TECHNISCHE UNIVERSITÄT WIEN

DISSERTATION

New Methods for the Synthesis of Glycosylated Natural Products with Application to Masked Mycotoxins

ausgeführt zum Zwecke der Erlangung des akademischen Grades einer Doktorin der technischen Wissenschaften

unter Leitung von

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Kurzfassung

Stoffwechselprodukte, die Kohlenhydrate enthalten, sind schon seit Langem bekannt, jedoch ist die Erforschung der genauen Funktion und Entstehung dieser Verbindungen oft noch sehr mangelhaft. Um die Rolle von Kohlenhydraten in biologischen Systemen, wie beispielsweise ihre Beteiligung an Krankheiten oder der Entgiftung von schädlichen Fremdstoffen, analysieren und feststellen zu können, werden reine und definierte Verbindungen in ausreichender Menge benötigt. Da es schwierig ist, homogene und chemisch definierte Kohlenhydrate aus biologischen Quellen zu erhalten, ist die chemische Synthese von Kohlenhydraten und Glykokonjugaten von großem Interesse. Daher wurden viele unterschiedliche Methoden zur Herstellung von Glykokonjugaten entwickelt, wobei unverändert an der Entwicklung neuer Strategien gearbeitet wird, mit dem Ziel allgemein anwendbarere Verfahren für die diastereoselektive Synthese von Glykosiden zu erhalten. Die bekanntesten und am häufigsten verwendeten Methoden für die chemische Glykosylierung sind die Königs-Knorr Methode, die Schmidt Glykosylierung und die Thioglykosid-Methode, welche Bromozucker, Glykosylacetamide bzw. Thioglykoside als Glykosylierungsreagenzien (Glykosyldonoren) verwenden. Nach wie vor ist eines der anspruchvollsten Probleme in der Synthese von Glykosiden die Notwendigkeit eine 1,2- *trans* oder 1,2-*cis* glykosidische Bindung diastereoselektiv zu bilden. Die Synthese von 1,2-*trans* Glykosiden unter Verwendung von Schutzgruppen, die einen Nachbargruppeneffekt ausüben, ist gut etabliert, jedoch gibt es nach wie vor Nachteile darunter Nebenreaktion wie die Orthoesterbildung und der Acyltransfer, und die limitierte Kompatibiltät von einigen Naturstoffen unter Reaktionsbedingungen, die üblicherweise zur Entschützung von den weit verbreitesten Schutzgruppen, wie zB. Ester-Funktionalitäten am O-2, verwendet werden. Daher war das Ziel dieser Arbeit die Entwicklung von neuen und vielseitig einsetzbaren Glykosylierungsstrategien, die für die 1,2-*trans* Glykosylierung von komplexen und labilen Naturstoffen angewendet werden können mit besonderem Schwerpunkt auf die Synthese von modifizierten Mykotoxinen.

Mykotoxine sind niedermolekulare sekündäre Metabolite von Schimmelpilzen. Sie treten häufig in befallenen Pflanzen wie Weizen, Mais, Gerste und Hafer auf und kontaminieren Getreide und Getreideprodukte weltweit. Da Mykotoxine akute als auch chronische Gesundheitsprobleme bei Menschen und Tieren verursachen können, stellen diese ein signifikantes Problem für die Lebens- und Futtermittelsicherheit dar. In Folge von Evaluierungen von internationalen Expertengremien über das Vorkommen, den Metabolismus und die Toxizität von Mykotoxinen, führten einige Länder Regulierungen ein. Jedoch sind Mykotoxine den lebenden Organismen fremd und werden daher wie andere Xenobiotika im Laufe von natürlichen Detoxifizierungsprozessen in Produkte, die sich strukturell von dem nativen Toxin unterscheiden, metabolisiert. Diese sogenannten modifizierten Mykotoxine werden in Routineanalysen nicht detektiert. Obwohl diese üblicherweise eine geringere Toxizität aufweisen als das ürsprüngliche Toxin, stellen sie trotzdem eine Gefahr für die Lebens- und Futtermittelsicherheit dar, da diese nach erfolgter Aufnahme hydrolysiert werden können, wodurch das eigentliche Toxin freigesetzt wird. Referenzsubstanzen werden benötigt für (i) die Entwicklung von routinemäßigen Analysenmethoden, die modifizierte Mykotoxine miteinbeziehen, (ii) die Bewertung des Vorkommens von modifizierten Mykotoxinen in Lebens- and Futtermitteln und für (iii) weiterführende biologische Untersuchungen. Daher wurden in dieser Arbeit verschiedene

Herstellungsmethoden für die Synthese von modifizerte Mykotoxine entwickelt und einige Mykotoxinkonjugate in ausreichender Menge hergestellt.

β-Glykoside von komplexen und basisch labilen Mykotoxinen können nicht über traditionelle Glykosylierungsmethoden hergestellt werden, daher wurden neuartige Methoden für die Synthese von Glykosiden entwickelt. Es wurden diastereoselektive, benzyl-geschützte Glykosyldonoren hergestellt, die die Synthese von β-Glykosiden und β-Glykosylestern ermöglichen, ohne dass vorhandene Estergruppen bei der finalen Entschützung gespalten werden. Weiters konnten diese erfolgreich für die regio- und diastereoselektive Synthese des Mykotoxinkonjugats Culmorin-11-*O*-β,D-glukosid eingesetzt werden. Jedoch stellen ungesättigte Naturstoffe mit Estergruppen unverändert ein Problem dar, da diese empfindlich gegenüber Hydrierungsreaktionen sind. Daher wurde eine weitere Glykosylierungsstrategie unter Verwendung von Schutzgruppen, welche unter milden reduktiven Bedingungen gespalten werden können, entwickelt. Diese wies zwar nur eine erhöhte β-Diastereoselektivität auf, konnte aber erfolgreich für die Synthese von T-2-Toxin-*O*-β,D-glukosid verwendet werden.

Weitere Phase II Metaboliten der Mykotoxine Deoxynivalenol und Zearalenon wurden hergestellt, indem optimierte Glykosylierungsmethoden verwendet wurden, um unter anderem die Bildung von Nebenprodukten zu verhindern. Unter Anwendung von [$^{13}C_6$]Glykosyldonoren konnten auch ^{13}C-isotopenmarkierte Mykotoxinkonjugate hergestellt werden, welche notwendig sind, um die exakte Quantifizierung von maskierten Mykotoxinen per LC-MS zu ermöglichen. Zusätzlich konnten mehrere Mykotoxinsulfate basierend auf der Entwicklung einer effizienten Methode zur chemischen Sulfatierung von Trichothecen-Mykotoxinen synthesiert werden. DON-3- und DOM-3-sulfat wurden als Referenzstandards verwendet, um die Bildung derartiger Mykotoxinsulfate in verschiedenen Geflügelarten nach dem Konsum von DON-kontaminierten Futter untersuchen zu können.

Zusammengefasst konnten neue Glykosylierungsmethoden entwickelt werden, die die Synthese von komplexen und labilen glykosylierten Naturstoffen ermöglichen. Diese und weitere Methoden konnten erfolgreich für die Synthese von β-Glykosiden und Mykotoxinkonjugaten im Allgemeinen eingesetzt werden.

Abstract

Carbohydrate-containing metabolites have been known for decades, but research devoted to the precise role of these compounds and their formation is often lacking. In order to be able to analyze and assess the roles of carbohydrates in biological systems, such as their precise involvement in diseases or in detoxification processes of harmful xenobiotics, there is a need for pure and defined target carbohydrates in reasonable quantities. As it is difficult to obtain homogeneous and chemically defined carbohydrates from biological sources, the chemical synthesis of carbohydrates and glycoconjugates is of high interest. Therefore, numerous glycosylation methods have been developed and the search for new glycosylation strategies is ongoing with the aim to find more generally applicable procedures for the stereoselective synthesis of glycosides. Most frequently used methods for chemical glycosylation include the Koenigs Knorr method, the Schmidt glycosylation and the thioglycoside method applying bromosugars, glycosyl acetimidates and thioglycosides, respectively, as glycosylation reagents (glycosyl donors). Still one of the most demanding problem in the synthesis of glycosides is the necessity to form either a 1,2-*trans* or 1,2-*cis* glycosidic bond with complete diastereoselectivity. The synthesis of 1,2-*trans* glycosides applying neighboring participating protective groups is well established, but there are still drawbacks including side reactions such as orthoester formation and acyl transfer, and limited compatibility of many natural products under reaction conditions usually applied for the removal of common participating groups, i.e. ester functionalities at O-2. Therefore, the main objective of this thesis was the development of new versatile glycosylation strategies that can be applied for 1,2-*trans* glycosylation of complex and labile natural products with emphasis on the synthesis of modified mycotoxins.

Mycotoxins are low-molecular-weight secondary metabolites of molds. They frequently occur on infected plants such as wheat, corn, barley and oats and thus contaminate grain and cereal products worldwide. As mycotoxins can cause acute and chronic health problems in human and animals, they pose a major concern for food and feed safety. As a result of evaluations of their occurrence, metabolism and toxicity by international expert bodies, many countries introduced regulation limits. However, mycotoxins are foreign for living organisms and therefore, similarly to other xenobiotics, metabolized in the course of natural detoxification processes into products structurally different from the native toxin. These so called modified mycotoxins fail to be recognized in routine analysis and are thus not regulated. Even though they are usually of lower toxicity than the parent toxin, they are a concern for food and feed safety as assimilated by food they might be hydrolyzed back to their precursor toxin during digestion, contributing to the overall toxicity. Reference standards are required for (i) the development of routine analytical methods that include modified mycotoxins, (ii) the evaluation of the occurrence of modified mycotoxins in food and feed, and (iii) further biological investigations. Hence, in this thesis various procedures for the synthesis of modified mycotoxins were developed and several mycotoxin conjugates were prepared in reasonable amounts.

β-Glycosides of complex and base labile mycotoxins cannot be prepared by traditional glycosylation methods, therefore new methods for the synthesis of glycosides were developed. Diastereoselective benzyl protected glucosyl donors were prepared that enabled the synthesis of β-glucosides and β-glucosyl esters without affecting ester groups during final deprotection. Furthermore, such a glucosyl donor could be applied for regio- and diastereoselective synthesis of the mycotoxin conjugate culmorin-11-*O*-β,D–glucoside. However, unsaturated natural products containing ester groups still pose a problem as these are sensible to hydrogenolysis. Therefore, a further glycosylation strategy applying protecting groups that can be cleaved under mild reductive conditions was developed. This

strategy demonstrated only an enhanced β-diastereoselectivity but could be applied for the synthesis of T-2-toxin-*O*-β,D-glucoside.

Further phase II metabolites of the mycotoxins deoxynivalenol and zearalenone were prepared by optimizing traditional glycosylation methods to avoid formation of byproducts and other side reactions. Applying [$^{13}C_6$]glycosyl donors, also various ^{13}C-isotope labeled mycotoxin conjugates were synthesized that are crucial to enable accurate quantification of masked mycotoxins by LC-MS. Additionally, based on the development of an efficient procedure for the sulfation of trichothecenes, various mycotoxin sulfates were prepared. These include DON-3- and DOM-3-sulfate that already served as reference standards to determine their formation in different poultry species after the consumption of DON contaminated feed.

In summary, novel glycosylation strategies were developed that enabled the synthesis of complex and labile glycosylated natural products. These and further methods have already been applied to the synthesis of β-glycosides and mycotoxin conjugates in general.

Aims and Structure of the Thesis

The main objective of this PhD thesis was the development of new versatile glycosylation strategies that can be applied for 1,2-*trans* glycosylation of complex and labile natural products to which traditional glycosylation methods are not amenable. Applying these newly developed methods reasonable amounts of β-glycosides and complex mycotoxin conjugates that are difficult to access otherwise should be prepared. Additionally, traditional glycosylation methods should be optimized to diminish or even eliminate side reactions and to develop efficient procedures for modified mycotoxins as reference materials for further studies. Furthermore a general applicable procedure for sulfation of various mycotoxins should be established.

This thesis is written as a cumulative work and consists of four parts (1) an introduction to the overall topic, (2) an overview of contributions, (3) the original works and (4) a short conclusion. Part three "Original Works" contains manuscript #1 to #8 (already published, submitted or as draft) and one technical note and is thematically divided into the following four subchapters:

3.1 Development of New Glycosylation Methods	(manuscript #1 to #3)
3.2 Synthesis of Trichothecene Metabolites	(manuscript #4 to #6)
3.3 Synthesis of Zearalenone Metabolites	(technical note and manuscript #7)
3.4 Synthesis of Zearalenone Metabolites	(manuscript #8)

In the appendix of the thesis the supporting informations can be found that contain most of the experimental details and data. The appendix also contains manuscript #9 and #10 as they were published before the start of the PhD thesis but are relevant to the topic of the thesis.

The applicant (J. Weber) is first author of five of these publications. These manuscripts were predominantly composed and written by J. Weber. Contributions of the applicant to the other manuscripts were manifold and essential for the completion of the respective research projects.

Chapter 1

Introduction

1.1 Synthesis of Glycosides- The Chemical Glycosylation

Importance of the Chemical Glycosylation

The chemical glycosylation is an important tool to offer access to glycosides, glycoconjugates and oligosaccharides that are indispensable for tackling problems in glycobiology. Glycoconjugates and glycosides are involved in a number of fundamental biological functions such as cell-cell recognition, cell-external agent interactions[1,2] and detoxification processes of harmful xenobiotics.[3,4] These functions can induce valuable biological events such as fertilization, cell growth and differentiation[5] as well as immune responses or damaging disease processes like viral or bacterial infections and cancer metastasis.[6] Oligosaccharides are found for instance in human breast milk as anti-infective agents that provide newborn with a mechanism for aborting infection processes[7] and the human blood groups are defined by cell surface glycans. Only little changes in the oligosaccharide structure are determining for the differentiation of the blood groups A, B and 0 (Fig. 1.1a). With increasing awareness of the numerous involvements of carbohydrates in biological events, interest into the science of glycobiology has emerged. In order to be able to fully explore and assess the roles of carbohydrates in biological systems, there is a need for pure and defined target carbohydrates in reasonable quantities. As it is difficult to obtain homogeneous, chemically defined and reasonable amounts of oligosaccharide, glycoconjugates and glycosides (Fig. 1.1b) from biological sources, advances of chemical synthesis are highly important. Therefore chemical glycosylation represents a significant method for providing material for biological, medicinal and pharmacological studies such as to elucidate biosynthetic pathways, to determine structural characteristics important for function, to assess the glycan function, to develop carbohydrate based vaccines[8,9] as well as non-natural glycosylated antibiotics.[10]

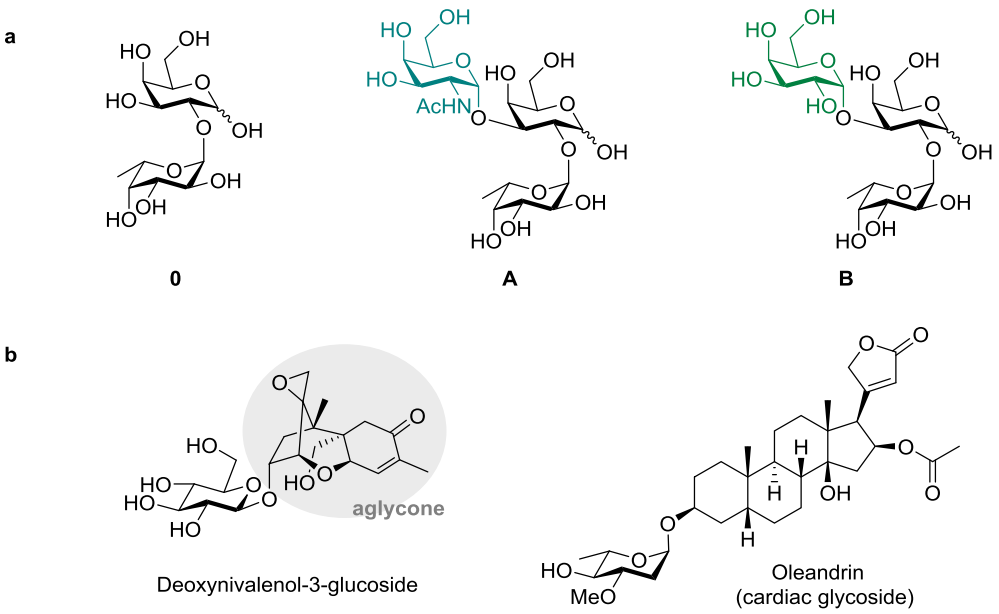


Figure 1.1 (a) structural basis of the ABO blood group antigens; (b) naturally occurring glycosides

Mechanism and types of glycosylation reactions

Since the first studies in the synthesis of glycosides by Michael[11] and Fischer,[12] followed by the pioneering work of Königs Knorr,[13] a very large number of glycosylation methods have been developed and still the search for new glycosylation strategies is ongoing with the aim to find a general applicable procedure for the stereoselective synthesis of glycosides.

The chemical synthesis of glycosides usually involves the preparation of an appropriate protected glycosyl donor, the glycosylation reaction and the final deprotection. The hydroxyl groups of the glycosyl donor are often protected with ester groups (eg. acetates, benzoates) or ether groups (eg. benzyl groups).[14] As leaving groups halides, acetimidates or alkylthios are most commonly installed at the anomeric center. Used in a glycosylation reaction, the prepared glycosyl donor is activated by a suitable promotor forming an oxocarbenium ion intermediate. This can then be attacked by a nucleophilic acceptor (aglycone), that generally contains only one free hydroxyl group, either at the α or β-face leading without a control of the stereoselectivity usually to a mixture of an α- and a β-glycoside (Fig. 1.2). After glycosylation typically deprotection follows and has to occur under conditions mild enough that do not affect the aglycone as well as the glycosidic bond.

Hence, requirements for an ideal glycosylation strategy are a simple preparation of the glycosyl donor, the use of only a catalytic amount of promotor, a complete stereoselective outcome, high yields and a possible scale-up. [15]

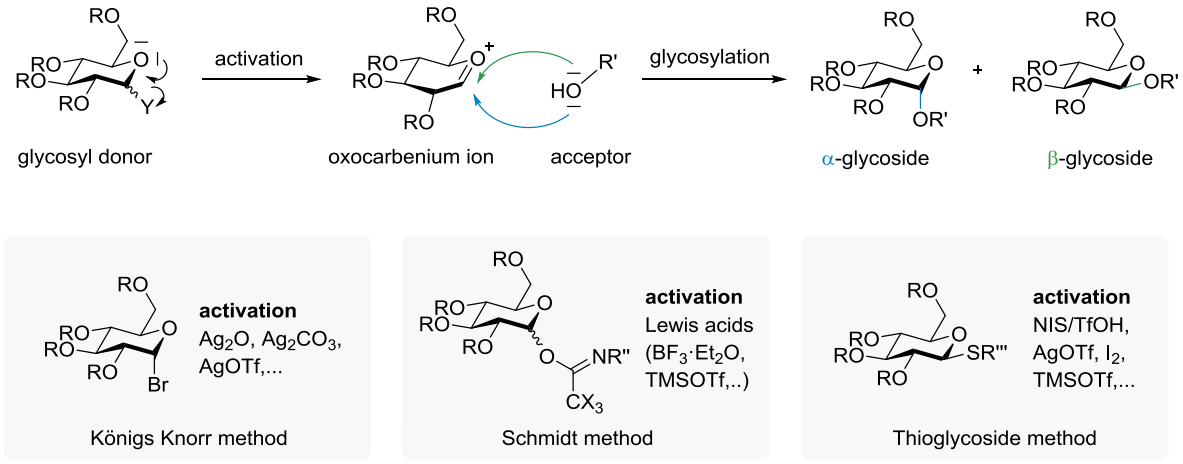


Figure 1.2 Most commonly applied glycosylation methods (shown in gluco-configuration); Y= leaving group, R''= H with X= Cl or R''= Ph with X= F, R'''= Et, Taz, Tol, Pym...

The best known and most common used glycosylation reactions so far, are the König Knorr-, the Schmidt- and the Thioglycoside method.[15]

The Königs Knorr glycosylation, was the first viable method developed for the controlled glycosidic bond formation in 1901,[13] and is still one of the most established methods for glycosylation. It uses glycosyl bromides as glycosyl donors that are easy to prepare or in some cases even commercially available. For activation of these donors an excess amount of silver(I) salts as promotor is required. A further development of this method that is especially suitable for the preparation of phenolic-O-glycosides, is the use of basic conditions, in particular phase transfer conditions, for coupling glycosyl bromides with acidic aglycons.[16,17]

The Schmidt method is a Lewis acid mediated glycosylation that is done under extremely mild glycosylation conditions. It requires only a catalytic amount of Lewis acid (in most cases TMSOTf or $BF_3{\cdot}Et_2O$) as promotor and uses glycosyl trichloroacetimidates as strong glycosyl donors. Therefore the Schmidt method is the most popular glycosylation type for the synthesis of complex glycosides. If the aglycone is very unreactive due to steric hindrance or poor nucleophilicity the glycosyl trichloroacetimidate can rearrange to the respective glycosyl trichloroacetamide (Fig. 1.3). To avoid this side reaction Yu and Tao developed N-phenyl trifluoroacetimidate (NPTFA) glucosyl donors as an alternative.[18]

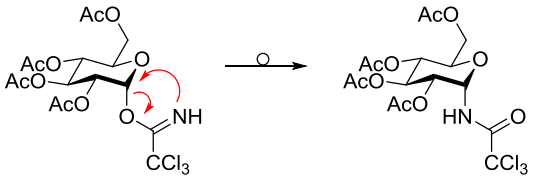


Figure 1.3 Possible rearrangement of glycosyl trichloroacetimidates

The Thioglycoside method has a wide applicability in synthetic carbohydrate chemistry as thioglycosides are in general easy to prepare, are shelf stable and are versatile in glycosylations. They can be activated by a wide variety of promotors such as NIS-TfOH, NIS-AgOTf, Ag_2OTf, TMSOTf, iodine and MeOTf but a stochiometric amount of promotor is required in all cases.[19] Thioglycosides also have a great stability under a wide range of conditions for protecting group manipulations. Thioether can thus act as temporary protecting group. In the synthesis of complex oligosaccharides, the feature of the tunable reactivity of thioglycosides and the fact that they can serve as glycosyl donor as well as glycosyl acceptor is often used.[15,20]

Regio-, stereoselectivity and side reactions of glucosylations

The control of the regio- and stereoselectivity of a glycosylation reaction is of high importance to avoid yield losses as well as laborious purifications of complex product mixtures. The problem of obtaining the proper regiochemistry can be solved by protection of all functional groups of the aglycone except the one to be glycosylated. For this purpose the application of orthogonal protection group strategies is often necessary. As the stereoselectivity of a glycosylation reaction is influenced by various factors including the protecting group pattern of the glycosyl donor and reaction conditions such as temperature, solvent, concentration and order of addition of reagents, the control of the stereoselectivity is still a challenge. However, in the case of glucosylations the synthesis of 1,2-*trans* glucosides can be in most cases reliably achieved by the use of a sterically demanding neighboring participating group at the 2-position.[21] Accordingly, non-neighboring participating groups are often used for the formation of 1,2-cis glucosides with the aim to harness the virtue of the anomeric effect. Nonetheless, this often leads only to a mixture of 1,2 cis- and 1,2 trans-glucosides (Fig.1.4).[15] Therefore the selective formation of α-glucosides is still a focus of ongoing research.

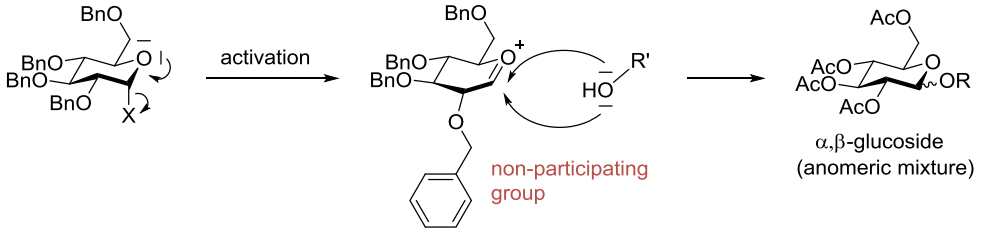


Figure 1.4 Glucosylation with a non-participating neighboring group

The use of neighboring participating groups at pos. 2 of glucosyl donors for the synthesis of β-glucosides is usually a reliable method; however it also has drawbacks owing to the sometimes occurring formation of by-products. These side reactions occur if the formed acetoxonium ion is not attacked at the anomeric center producing the desired β-glucoside but instead at the positively charged carbon between the two oxygens leading to an orthoester formation or an acyl transfer as illustrated in Fig. 1.5.[22,23] Prevention of these two side reactions can be achieved by the introduction of bulky protecting groups at position 2 of glucosyl donor such as ADMB[24] and AZMB.[25]

Therefore protecting groups play a key role in directing the stereochemistry and regiochemistry of glycosylation reactions. Furthermore they modulate the reactivity of glycosyl donors and acceptors.

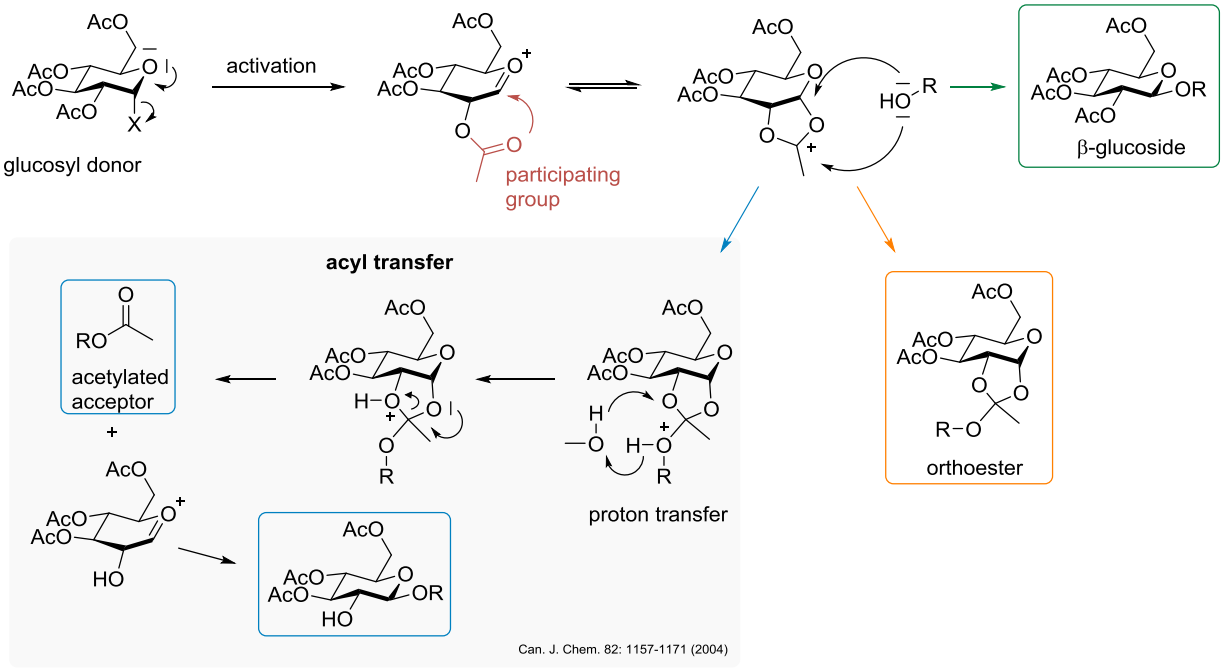


Figure 1.5 Neighboring group assisted glucosylation and its side reactions: the acyl transfer and the orthoester formation

1.2 Synthesis of Sulfate esters-The Chemical Sulfation

Sulfate esters are widespread in nature and are crucial for many biological functions. [27,28] There are sulfated carbohydrates, steroids, nucleosides and proteins. Furthermore sulfated compounds occur as phase II metabolites of plants and mammals in the course of detoxification processes of xenobiotics.[4,29] Therefore chemical sulfation is an important method to get access to sulfated biomolecules that can serve as reference material for further investigations.

Several methods for the synthetic preparation of alkyl and aryl sulfates were developed so far. The most applied technique is the use of commercially available sulfur trioxide amine or amide complexes ($SO_3{\cdot}NMe_3$, $SO_3{\cdot}Py$, $SO_3{\cdot}DMF$).[30] This sulfation procedure is very simple and efficient leading directly to the corresponding salt of the sulfate. Following often the counterion is exchanged by the use of an ion-exchange chromatography or resin (Fig. 1.6).

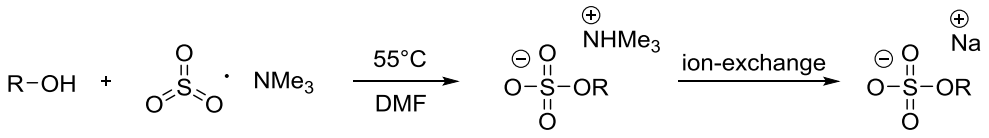


Figure 1.6 Sulfation using sulfur trioxide amine complexes

However, this method has its limitations in terms of chemical modifications following the introduction of the sulfate group, yield, regioselectivity and reproducibility. Sulfates are highly polar compounds that are acid sensitive and labile to elevated temperatures, therefore the sulfation step had to be originally carried out near or at the end of the synthesis to minimize purification and stability problems. To be able to introduce sulfate groups already at the beginning of syntheses, several protecting groups were investigated for sulfates in the last few decades. Taylor and co-workers introduced the 2,2,2-trichloroethyl (TCE) group for the sulfation of phenols which allowed an efficient preparation and good stability of protected aryl sulfates.[31] Cleavage of this protecting group is either achieved by catalytic transfer hydrogenation Pd/C, ammonium formate or under mild reductive conditions with Zn and ammonium formate leading to the ammonium salt of the sulfate (Fig. 1.7a). Since its introduction this protecting group found wide applicability and was used for the preparation of various sulfated compounds.[32–34] Furthermore sulfuryl imidazolium salt was described as reagent for incorporating trichloroethyl protected sulfate esters to carbohydrates (Fig. 1.7b).[35]

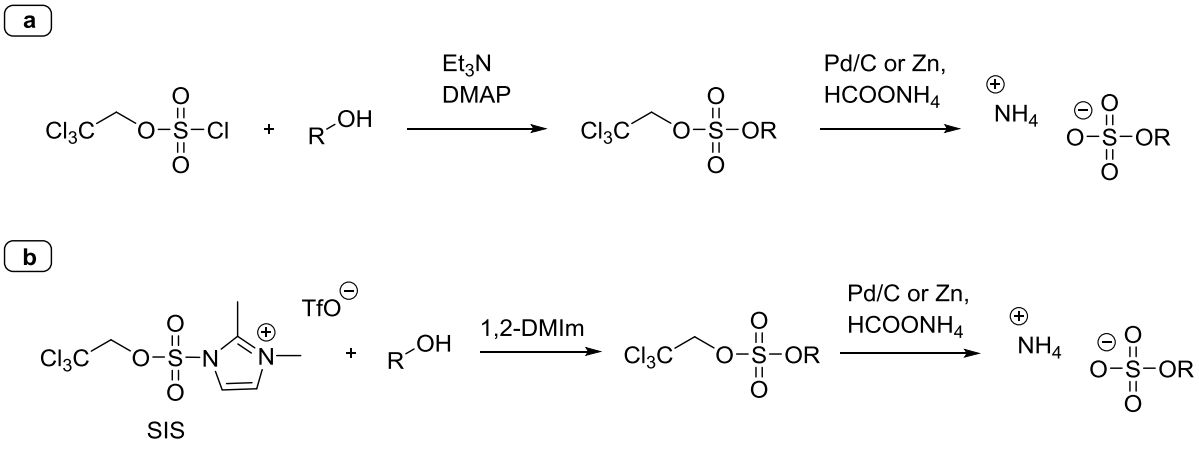


Figure 1.7 (a) Sulfation using 2,2,2 trichloroethyl as protecting group (b) Sulfation using a sulfuryl imidazolium salt

1.3 Mycotoxins

General Aspects

Mycotoxins are secondary metabolites of low molecular weight produced by various fungi, in particular molds, under certain environmental conditions. These compounds are toxic to human and animals even in low concentrations. However not all toxic compounds formed by fungi are called mycotoxins even though they all originate from fungi.[36] Molds that release mycotoxins into food and feed are phytopathogenic and saprophytic organisms. They infect plant commodities in the field during long cool moistly growing and respectively harvest seasons as well as during storage in warehouses. The production of mycotoxins by fungi depends on numerous environmental conditions including temperature, moisture, nutrient availability, growth substrate, competition with other microorganisms and the maturity of the fungal colony, hence the presence of fungal growth is no indication for the occurrence of mycotoxins.[37] The production of a certain mycotoxin is not limited to one species of fungi, mycotoxins may be produced by various species of fungi and a certain species of fungi can produce more than one mycotoxin.[38] Toxigenic fungis are commonly found in the outdoor environment.[39] The most common fungis, which produce mycotoxins that are found in food stuff, are *Aspergillus*, *Fusarium, Alternaria* and *Penicillium*.[40] The most important mycotoxins that are released into food and feed are aflatoxin B1 and M1, ochratoxin A and fumonisins, zearalenone (ZEN) and deoxynivalenol (DON).[41] Estimated over 1000 mycotoxins are described in literature so far, however this number will still rise in the next few decades as a consequence of advances in new analytical tools and the isolation of not yet known fungi.[42] Exposure to mycotoxins can cause acute intoxications including symptoms of severe illness as well as long term chronic or cumulative effects on health, including the induction of cancers and immune deficiency.[43] Classification of mycotoxins is very challenging due to their diverse chemical structures, biosynthetic origins, their various biological effects and their formation by a wide number of various fungal species. So far there is no satisfactory classification scheme established. Mycologists tend to classify mycotoxins according to the fungi that produce them eg. Fusarium toxins, Alternaria toxins…[44]

Mycotoxins of *Fusarium*

In the northern temperate regions several *Fusarium* species eg. *F. graminearum*, *F. culmorum* and *F. verticilliodes* are probably the most prevalent toxin-producing fungi. They colonize on maize, barley, oats, wheat and sorghum growing in temperate regions worldwide.[45] Devastating diseases of wheat and barley crops caused by the pathogens *F. graminearum* and *F. culmorum*, are named Fusarium head blight (Fig. 1.8) and Gibberella ear rot leading to severe yield losses and contaminations of the grain with predominantly the mycotoxins DON and ZEN.[45,46] As wheat and barley are responsible for approximately 80% of the European small grain production, a contamination of these crops with Fusarium toxins is a major health concern to human and animals.[47] The most common *Fusarium* toxins in cereal grains of northern Europe are fumonisins, zearalenone and trichothecenes such as T2-toxin, HT2-toxin, DON and NIV (Fig. 1.10).[43]

Figure 1.8 Fusarium head blight of wheat (© Mary Burrows, Montana State University, Bugwood.org)

Trichothecenes are a large family of related toxins mainly produced by fungi belonging to the genus Fusarium. Approximately, 200 trichothecenes have been isolated and characterized so far.[48] They can be classified according to their structure into four types A, B, C and D.[49] These compounds have in common that they all consist of a sesquiterpene skeleton with a C-12, C-13 epoxy ring (Fig. 1.9). Exposure to trichothecenes can cause acute or chronic intoxications appearing in vomiting, growth retardation, immunosuppression, neurotoxicity, haematotoxicity and animal feed refusal.[50,51] Trichothecenes of type A, such as T2-toxin and HT2-toxin as well as type B toxins including deoxynivalenol (DON) and nivalenol (NIV) are of the most concern due to their occurrence on edible crops and their highly toxic nature. In Nordic countries oats have been detected to be more severely contaminated by high DON and HT2 as well T2 concentrations compared to barley and wheat.[52]

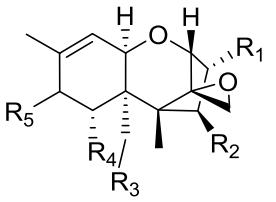


Figure 1.9 Core structure of trichothecenes

Zearalenone (ZEN) is a mycotoxin produced by several Fusarium species, mainly by *F. graminearum* and *F. culmorum* and is found in a variety of infected plants like maize, barley, oats, wheat and sorghum.[45] Maize is the most frequently contaminated commodity and levels of ZEN are found between 1 to 2900 µg/kg depending on climatic, harvest and storage conditions.[53] ZEN is a macrocyclic β-resorcyclic acid lactone containing a ring with two ketones and an aromatic ring substituted with two hydroxyl groups. As the structure of ZEN resembles natural estrogens, ZEN and its metabolites possess estrogenic activity in mammals, including pigs, cattle, and sheep.[54] Problems of the reproductive tract as well as impaired fertility and abnormal fetal development in farm animals can be caused by ZEN.[55] Furthermore, this mycotoxin can interfere with various enzymes involved in steroid metabolism, which was recently investigated.[56]

Culmorin belongs to the so called group of “emerging mycotoxins” as it is little investigated so far. Emerging mycotoxins frequently co-occur with other toxins and their toxicity or the effect of combinations with known toxins and their ecological roles are insufficiently studied so far.[57] CUL consists of a sesquiterpene diol core structure and is produced by several different Fusarium species. Its occurrence and contamination is correlated to the presence of DON. CUL is common in infected wheat, barley and oat.[58]

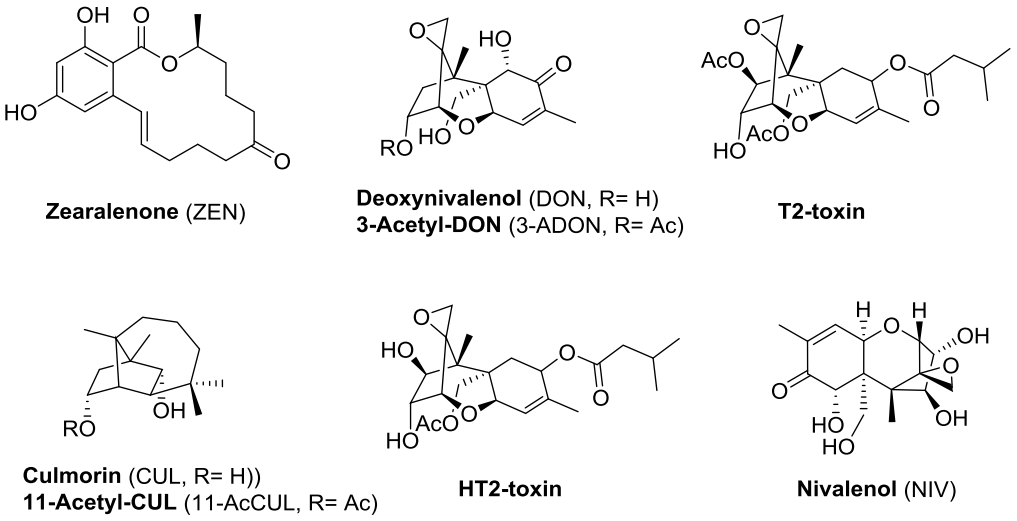


Figure 1.10 Structures of zearalenone, trichothecenes and culmorin as “emerging” mycotoxin

Limits and Regulations

Mycotoxins represent a major concern for food and feed safety as they can cause serious health problems and cannot be completely avoided. Therefore different international expert bodies, including those of the FAO/WHO and EFSA, assess their occurrence, exposure, metabolism and toxicity thoroughly.[59,60] As a result, regulatory limits for various mycotoxins were established in many countries to control their concentrations in food and feed and provisional maximum tolerable intakes (PMTDI) were set.[61] For instance for DON and its acetylated metabolites a PMTDI of 1 µg/kg[59] and for ZEN and its metabolites a PMTDI of 0.5 µg/kg were established.[62] Progress in the field of mycotoxin research such as the development of new analytical tools for the assessment of mycotoxins, the collection of further distribution data of mycotoxins in commodities and new investigations of toxicological effects is important for the validation of existing regulation limits as well as to spot out new mycotoxins that need to be regulated.

1.4 Modified Mycotoxins

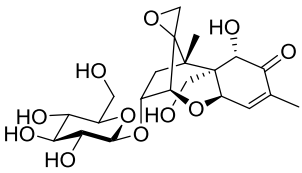
General Aspects

Mycotoxins are foreign for living organisms and therefore they are, similarly to other xenobiotics, metabolized in the course of natural detoxification processes into products structurally different from the native toxin. Plant and mammal metabolites of mycotoxins belong to the group of biologically modified mycotoxins.[63] Plant-generated mycotoxin metabolites are also called masked mycotoxins.[64] They are formed in the course of phase I and II metabolism. During phase I metabolism reactive functional groups are introduced via hydrolysis, oxidation and reduction reactions leading to metabolites that are not always decreased in toxicity. In some cases even an activated form or a product with equal toxicity to the native toxin is formed. During phase II metabolism conjugation reactions with hydrophilic moieties such as glucose, malonic acids, glutathiones, sulfates and amino acids take place leading to more water soluble and therefore less toxic metabolites. These modified mycotoxins of usually lower toxicity than the parent toxin pose a concern in food and feed safety as these metabolites assimilated by food might be hydrolyzed back to their precursor toxin during digestion, contributing to the overall toxicity.[65,66] Furthermore these altered forms of mycotoxins fail to be recognized in routine screenings, therefore their added contribution to the total mycotoxin content of a sample is missed and still not regulated. Hence, the chemical synthesis of mycotoxin metabolites and relevant isotope labelled analogues is of high importance for providing reference standards that are required for developing analytical methods including masked mycotoxins, for obtaining a more complete view of the occurrence of mycotoxin conjugates in food and feed and for carrying out studies on their potential toxicological effects in mammals.[67] Furthermore the availability of reference standards is important for the development of reliable biomarker methods for a proper exposure assessment of corresponding mycotoxins.[4,29]

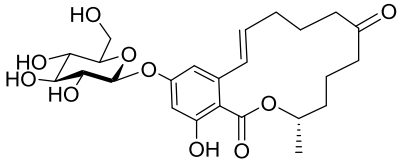
Masked mycotoxins

Plant generated mycotoxin metabolites, so called masked mycotoxins, have been identified so far for several mycotoxins including DON, NIV, ZEN, T2, HT2 and ochratoxin A (Fig. 1.11). The main strategy of plants for detoxification of xenobiotics is their conjugation to glucose. ZEN-14-glucoside and DON-3-glucoside have been found to occur in naturally infected cereals such as wheat, barley and maize.[64]

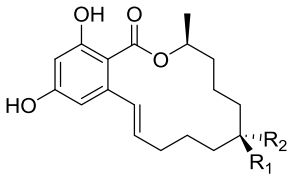
Furthermore the 3-O-glucosides of HT2 toxin and T2 toxin have been discovered in naturally contaminated wheat and oats.[43,68] With maize cell suspension cultures it was shown that ZEN is also modified to α and β-ZEL in the course of phase I metabolism, which can then be further conjugated with glucose leading to the respective glucosides (α-ZEL- and β-ZEL-glucoside).[69,70] ZEN-14-sulfate could be detected in different commodities such as biscuits, maize meal, crackers, wheat flour and whole-meal wheat bread.[71]



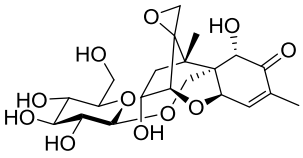
DON-3-glucoside



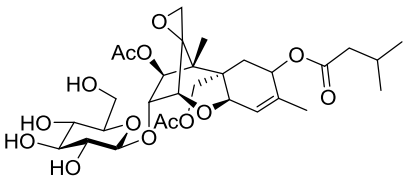
ZEN-14-glucoside



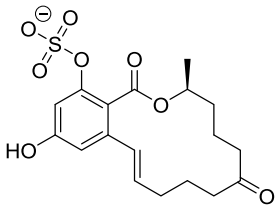
α-Zearalenol (α-ZEL, R_1 = H, R_2 = OH)
β-Zearalenol (β-ZEL, R_1 = OH, R_2 = H)



DON-15-glucoside



T2-glucoside

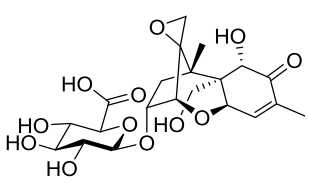


ZEN-14-sulfate

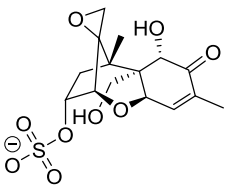
Figure 1.11 Structures of masked mycotoxins

Mammalian mycotoxin metabolites

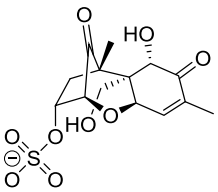
The mycotoxin metabolism of human and animals was studied so far for the mycotoxins ZEN and DON as well as for T2 toxin in animals. Glucuronides are the predominant products of phase II metabolism in mammals. DON-15-glucuronide could be identified as major product of phase II metabolism with minor contributions of DON-3- and DON-7-glucuronide in humans.[4] A minor metabolic pathway in humans is sulfation. Just recently, DON-3-sulfate was identified as a new human metabolite of DON.[29] Previously, DON-3-sulfate could also be discovered in different poultry species as well as the formation of deepoxy-deoxynivalenol(DOM)-3-sulfate (Fig. 1.12).[72] ZEN was shown to be metabolized to ZEN-14-glucuronide, α-ZEL and β-ZEL as well as their corresponding glucuronides.[4] Rapid deactylation of T2 to HT2 toxin could be observed in animals, furthermore deepoxidation and glucuronidation pose an important detoxification mechanism of animals.[47]



DON-3-glucuronide



DON-3-sulfate



DOM-3-sulfate

Figure 1.12 Modified mycotoxins produced by mammalian metabolism

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Chapter 2

Overview and Context of Contribution

Overview of Contributions

Chapter I-Development of New Glycosylation Methods

- Manuscript #1

  Weber, J.; Svatunek, D.; Tegl, G.; Krauter, S.; Hametner, C.; Kosma, P.; Fröhlich, J.; Mikula, H. **2-*O*-Benzyloxycarbonyl Protected Glucosyl Donors: A Revival Towards Diastereoselective Glycosylation**, *manuscript draft*.

- Manuscript #2

  Weber, J.; Krauter, S.; Schwarz, T.; Hametner, C.; Fröhlich, J.; Mikula, H. **(2-Benzyloxyphenyl)acetyl (BnPAc): A new relay protecting group for alcohols and especially carbohydrates**, *manuscript draft*.

- Manuscript #3

  Weber, J.; Schwarz, M.; Schiefer, A.; Hametner, C.; Schiessl, A.; Häubl, G.; Fröhlich, J.; Mikula, H. **(2-Nitrophenyl)acetyl-Protected Glucosyl Trichloroacetimidates as Novel Glucosyl donors for the Synthesis of Glycosylated Natural Products**, *manuscript draft*.

Chapter II-Synthesis of Trichothecene Metabolites

- Manuscript #4

  Weber, J.; Fruhmann, P.; Hametner, C.; Schiessl, A.; Häubl, G.; Fröhlich, J.; Mikula, H. **Synthesis of Isotope Labeled Deoxynivalenol-15-*O*-Glycosides**, *Eur. J. Org. Chem.* **2017**, doi:10.1002/ejoc.201700934.

- Manuscript #5

  Fruhmann, P.; Skrinjar, P.; **Weber, J.**; Mikula, H.; Warth, B.; Sulyok, M.; Krska, R.; Adam, G.; Rosenberg, E.; Hametner, C.; Fröhlich, J. **Sulfation of deoxynivalenol, its acetylated derivatives, and T2-toxin**, *Tetrahedron* **2014**, 70(34), 5260-5266.

- Manuscript #6

  Schwartz-Zimmermann, H.; Fruhmann, P.; Dänicke, S.; Wiesenberger, G.; Caha, S.; **Weber, J.**; Berthiller, F. **Metabolism of Deoxynivalenol and Deepoxy-Deoxynivalenol in Broiler Chickens, Pullets, Roosters and Turkeys**, *Toxins* ***2015***, 7(11), 4706-4729.

Chapter III-Synthesis of Zearalenone Metabolites

- Technical Note

  Weber, J.; Hametner, C.; Schiessl, A.; Häubl, G.; Fröhlich, J.; Mikula, H. **First Synthesis of Zearalenone-14-O-β,D-[$^{13}C_6$]glucoside**.

- Manuscript #7

  Mikula, H.; **Weber, J.**; Svatunek, D.; Skrinjar, P.; Adam, G.; Krska, R.; Hametner, C.; Fröhlich, J. **Synthesis of zearalenone-16-β,D-glucoside and zearalenone-16-sulfate: A tale of protecting resorcylic acid lactones for regiocontrolled conjugation**, *Beilstein J. Org. Chem.* **2014**, 10, 1129-1134.

Chapter IV-Synthesis of Culmorin Metabolites

- Manuscript #8

  Weber, J.; Vaclavikova, M.; Wiesenberger, G.; Haider,M.; Hametner, C.; Mikula, H.; Fröhlich, J.; Berthiller, F.; Adam, G.; Fruhmann, P.* **Chemical synthesis of culmorin metabolites and their biologic role in culmorin and acetyl-culmorin treated wheat cells**, *submitted manuscript*.

Appendix

- Manuscript #9

  Weber, J.; Mikula, H.; Fruhmann, P.; Hametner, C.; Varga, E.; Berthiller, F.; Krska, R.; Fröhlich, J. **Gentiobiosylation of β-Resorcylic Acid Esters and Lactones: First Synthesis and Characterization of Zearalenone-14-β,D-Gentiobioside**, *Synlett* **2013**, 14, 1830-1834.

- Manuscript #10

  Mikula, H.; Sohr, B.; Skrinjar, P.; **Weber, J.**; Hametner, C.; Berthiller, F.; Krska, R.; Adam, G.; Fröhlich, J. **Sulfation of β-resorcylic acid esters-first synthesis of zearalenone-14-sulfate**, *Tetrahedron Lett.* **2013**, 54, 3290-3293.

2.2 Context of Contributions

In this chapter the context of all manuscripts, paper drafts and one technical note will be outlined according to the following general aims.

- Development of new glycosylation methods
- Synthesis of trichothecene metabolites
- Synthesis of zearalenone metabolites
- Synthesis of culmorin metabolites

The next paragraphs will briefly describe the focus of the research, refer to theoretical fundamentals and reveal how these contribute to the main topic of this thesis. For detailed background information and respective literature see chapter 1 and 3.

Development of New Glycosylation Methods

In order to be able to analyze and assess the roles of carbohydrates in biological systems, such as their precise involvement in diseases or in detoxification processes of harmful xenobiotics, there is a need for pure and defined compounds in reasonable quantities. Therefore, the synthesis of carbohydrates and glycoconjugates is of high interest. One of the major challenges in the synthesis of glycosides is the necessity to form either a 1,2-*trans* or 1,2-*cis* glycosidic bond with complete diastereoselectivity. The synthesis of 1,2-*trans* glucosides applying neighboring participating protective groups is well established, but there are still drawbacks regarding the compatibility of many natural products under reaction conditions usually applied for the removal of the most common participating groups, i.e. ester functionalities at O-2 (Fig. 2.1).

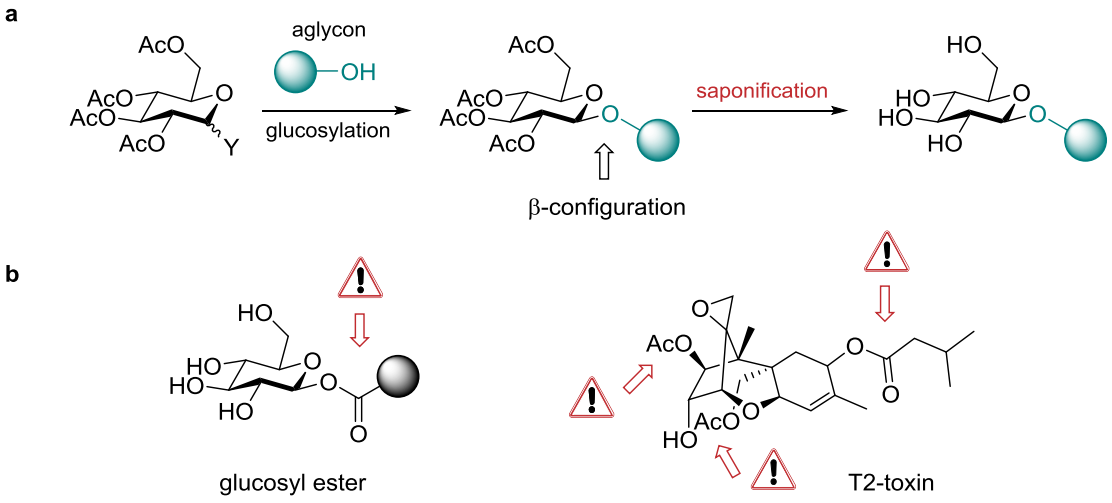

Figure 2.1 (a) General scheme for β-diastereoselective glucosylations applying acetyl-protected glucosyl donors (Y= leaving group), (b) general structure of glucosyl esters and T2 toxin indicating the labile ester groups that are not stable under saponification

Therefore, we aimed to develop glucosyl donors with high β-diastereoselectivity and a mild one-pot deprotection protocol compatible with base-sensitive natural products containing ester functionalities. Due to the excellent properties of thioglucosides, including versatile methods for selective activation and high stability, we considered different S-leaving groups (SEt, STaz, STol, SPym) for the development of efficient glucosyl donors. The introduction of a participating self-immolative linker between O-2 and the protective group led to the development of benzyloxycarbonyl (Cbz)-protected glucosyl donors that provide anchimeric assistance via the carbonyl group, whereas deprotection only depends on the benzyl moiety of the Cbz group, as cleavage leads to the formation of an unstable intermediate (Fig. 2.2).

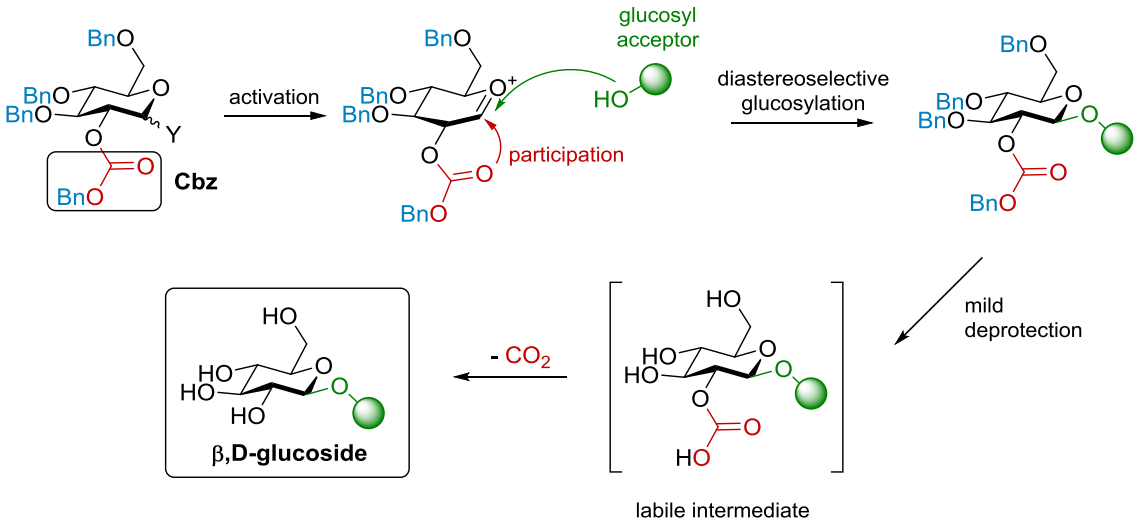

Figure 2.2 2-Benzyloxycarbonyl (Cbz) protected glucosyl donors for the 1,2-trans selective glycosylation of natural products (Y= SEt, SPym, STol, STaz or O(CNPh)CF_3)

Applying this concept, several 3,4,6-tri-*O*-benzyl-2-*O*-Cbz protected thioglucosides were prepared and tested in glycosylation reactions. Additionally, we were able to convert S-ethyl-thioglucosides to an N-phenyltrifluoroacetimidoyl glucosyl donor enabling Schmidt glycosylations. A mild one-pot deprotection procedure that does not affect ester groups was developed, also making these 2-O-Cbz glucosyl donors particularly suitable for the synthesis of glucosyl esters. The detailed synthesis of the various 2-*O*-Cbz glucosyl donors, optimized protocols for glycosylation and subsequent deprotection under mild reaction conditions, the orthogonality to ester groups and the application of this novel glycosylation method towards the synthesis of glycosylated natural products is shown and discussed in manuscript #1.

(2-Benzyloxyphenyl)acetyl (BnPAc) was developed as a new participating protecting group for hydroxyl groups. BnPAc can easily be introduced and cleaved applying a relay deprotection approach using catalytic hydrogenation in combination with proton sponge, conditions that do not affect ester groups. Furthermore, this protecting group can also be cleaved using conventional basic hydrolysis. Installed at position 2 of a thioglucosyl donor, BnPAc demonstrated an effective neighboring group participation in glucosylation reactions exclusively leading to the formation of β-glucosides (Fig. 2.3, manuscript #2).

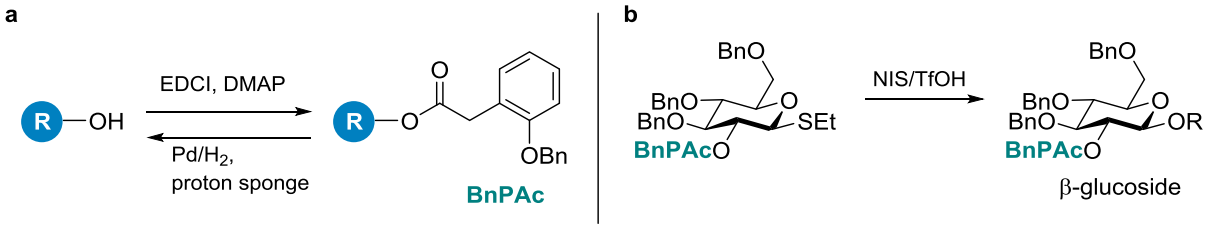

Figure 2.3 (a) Protection and deprotection conditions for the new hydroxyl protecting group (2-benzyloxyphenyl)acetyl (BnPAc) (b) BnPAc serves as effective neighboring participating group in gucosylations

2-*O*-BnPAc- and 2-*O*-Cbz-glucosyl donors represent new valuable tools for the synthesis of 1,2-*trans* glucosides of natural products containing ester groups. Moreover, these reagents are well suited for the synthesis of β-glucosyl esters. However, the synthesis of glycosylated natural products containing ester groups and double bonds (such as T2-toxin glucoside) is not feasible applying 2-O-BnPAc or 2-O-Cbz glucosyl donors (Fig. 2.4).

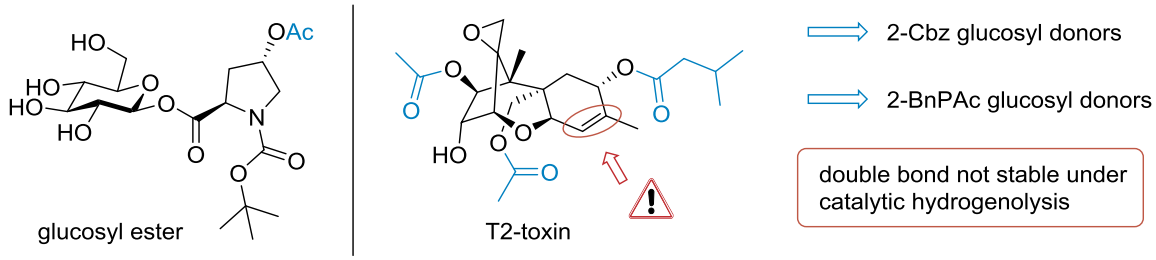

Figure 2.4 2-Cbz- and 2-BnPAc glucosyl donors are applicable for the glycosylation of base-sensitive natural products such as glycosyl esters but double bonds pose a problem

Hence, we became interested in the development of glucosyl donors that can be used for diastereoselective glucosylation of base-labile natural products that are also sensitive to hydrogenolysis such as T2-toxin (Fig. 2.5). In combination with a trichloroacetimidoyl leaving group, (2-nitrophenyl)acetyl (NPAc) groups were used as they can be cleaved under mild reductive conditions with zinc and ammonium chloride, conditions that are known to be orthogonal to ester groups. The detailed synthesis of the NPAc-protected Schmidt glucosyl donor, the optimization of the glycosylation reaction, the development of a one-pot procedure (glycosylation and subsequent deprotection) and the synthesis of T2-toxin glucoside is presented in manuscript #3.

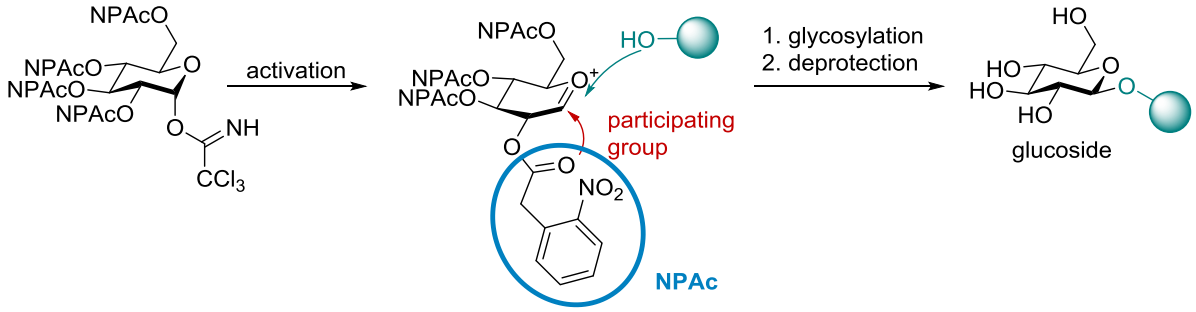

Figure 2.5 (2-Nitrophenyl)acetyl (NPAc) protected trichloroacetimidates as novel glucosyl donors for the glucosylation of base labile natural products that are also sensitive to hydrogenolysis

Synthesis of Trichothecene Metabolites

Trichothecene sulfates and glycosides can emerge after metabolization of the parent toxin in living plants, animals and humans. For structure confirmation, quantification and further biological investigations reference materials are required to be available in reasonable amounts. Therefore, we aimed to develop simple and efficient procedures for the preparation of various trichothecene glycosides and sulfates.

Deoxynivalenol, the most frequently detected trichothecene mycotoxin in cereal based food, consists of a trichothecene core structure and possesses three hydroxyl groups possible for conjugation that are quite different in reactivity (O-15 > O-3 >> O-7). Hence, for regioselective glycosylation of DON in position 15, 3-acetyldeoxynivalol (3-ADON) was used as starting material. Glycosylation applying Königs Knorr conditions failed, but we finally developed a reliable method for the synthesis of DON-15-glucoside by Schmidt glycosylation of 3-ADON followed by deprotection under mild reaction conditions avoiding the formation of undesired byproducts. This optimized procedure could then also be applied for the preparation of DON-15-gentiobioside after synthesizing an N-phenyltrifluoroacetimidoyl gentiobiosyl donor (according to manuscript #9). As isotope labeled standards are crucial for accurate quantification of modified mycotoxins by LC-MS, [$^{13}C_6$]glycosyl donors were synthesized that could be successfully used for the preparation of DON-15-O-β,D-[$^{13}C_6$]glucoside and DON-15-O-β,D-[$^{13}C_6$]gentiobioside, respectively (Fig. 2.6). The detailed optimization towards the efficient protocol for the preparation of DON-15-glycosides is described in manuscript #4.

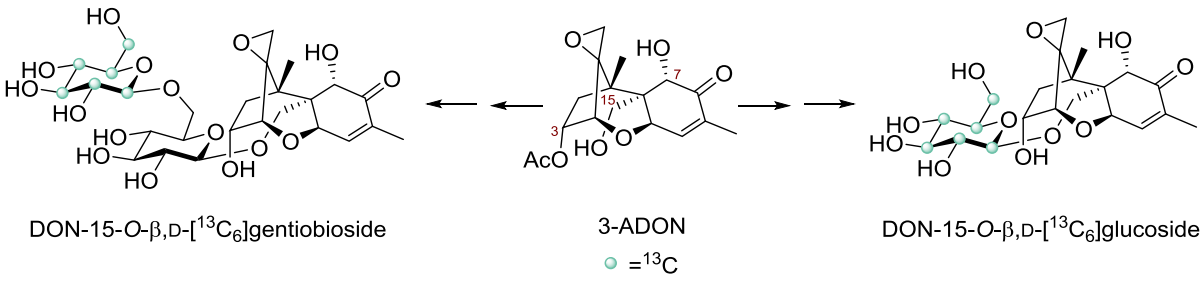

Figure 2.6 Successfully prepared ^{13}C isotope labelled DON-15-glycosides starting from 3-acetyldeoxynivalenol (3-ADON)

Sulfation of deoxynivalenol was achieved using an indirect and optimized method applying a sulfuryl imidazolium salt (Fig. 2.7). This procedure was applied to synthesize DON sulfates and acetylated analogs. Sulfation of DON with 2 equivalents of sulfuryl imidazolium salt yielded a mixture of 2,2,2-trichloroethyl (TCE)-protected DON-3,15-disulfate and DON-3-sulfate that could be separated by chromatography and deprotected to obtain the respective sulfates as ammonium salts. Even though O-15 is most reactive, sulfation at this position was only possible using 3-ADON as starting material followed by TCE-deprotection under reductive conditions and basic hydrolysis of the acetyl group. Furthermore, using the same sulfation procedure we have been able to prepare T2-toxin-3-sulfate (Manuscript #5).

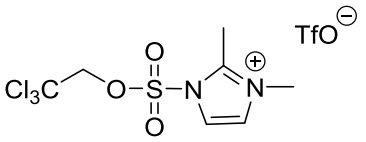

Figure 2.7 Sulfuryl imidazolium salt (SIS)

For an overview of all prepared DON conjugates and sulfates as described in manuscripts #4 and #5 see Fig. 2.8.

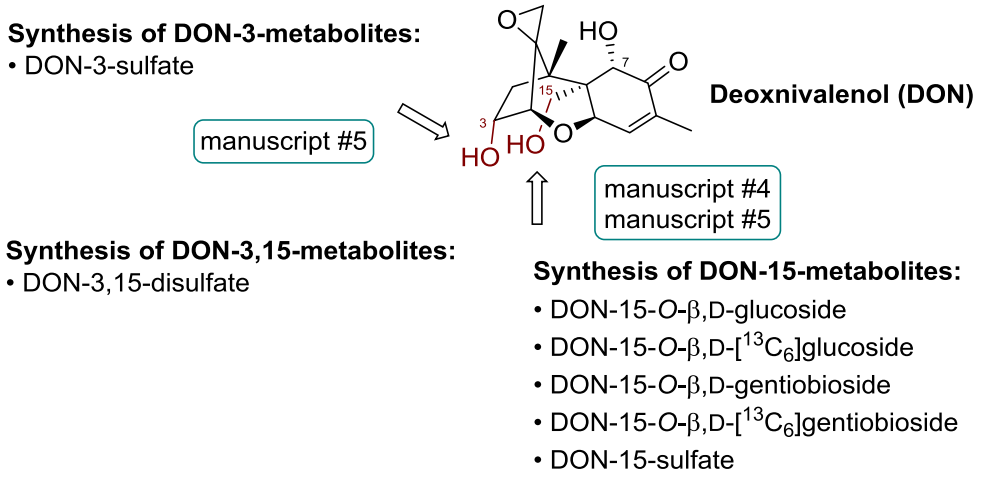

Figure 2.8 Overview of successfully performed syntheses of DON conjugates

Applying the optimized sulfation procedure of DON to de-epoxy-deoxynivalenol (DOM) the respective sulfates, DOM-3-sulfate and DOM-15-sulfate were prepared (Fig. 2.9). The synthesized DOM-3-sulfate and DON-3-sulfate (see manuscript #5) have been used for the development and validation of the first LC-MS/MS based method for the determination of these modified mycotoxins in excreta samples of chickens and turkeys. Furthermore, we have shown that DON-3-sulfate is the major DON-metabolite in different poultry species and that DOM-3-sulfate is formed after oral administration both in turkeys and chickens (manuscript #6).

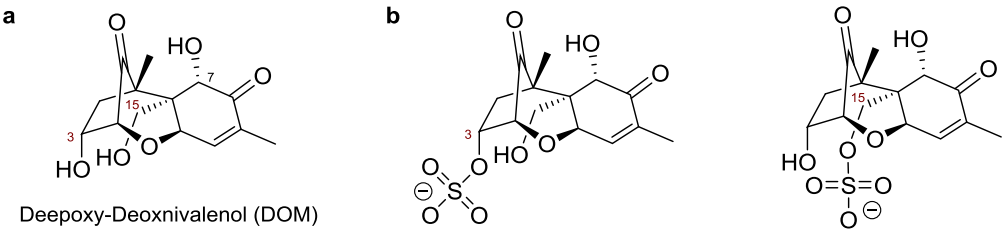

Figure 2.9 (a) Chemical structure of deepoxy-deoxynivalenol (DOM), (b) synthesized DOM-sulfates

Synthesis of Zearalenone Metabolites

Zearalenone is a macrocyclic β-resorcyclic acid lactone containing two phenolic OH groups of different reactivity due to hydrogen bonding of the OH group at position 16 with the carbonyl group (see Fig. 2.10). Therefore, glycosylation of ZEN at position 14 is strongly favored and prior protection of the hydroxyl group at position 16 is not required. ZEN-14-[$^{13}C_6$]glucoside was synthesized using acetobromo-α,D-[$^{13}C_6$]glucose in a phase transfer glycosylation and subsequent deprotection (technical note). A similar glycosylation approach was used for the gentiobiosylation of ZEN at position 14 to prepare ZEN-14-*O*-β,D-gentiobioside as first reference standard for late phase II metabolites (manuscript #9). An improved procedure for selective monosulfation of ZEN at position 14 and simultaneous preparation of mono- and disulfates could be developed after investigating chemical sulfation of ZEN (manuscript #10). Glycosylation and sulfation of ZEN at position 16 is more elaborate owing to the fact that conjugation has to occur at the less reactive site. Therefore,

different protective group strategies were tested finally leading to a regioselective TIPS protection of ZEN at position 14. This compound was conjugated at position 16 followed by deprotection to afford the respective ZEN-16-sulfate and ZEN-16-glucoside (manuscript #7). An overview of all prepared ZEN conjugates within this thesis is presented in Fig. 2.10.

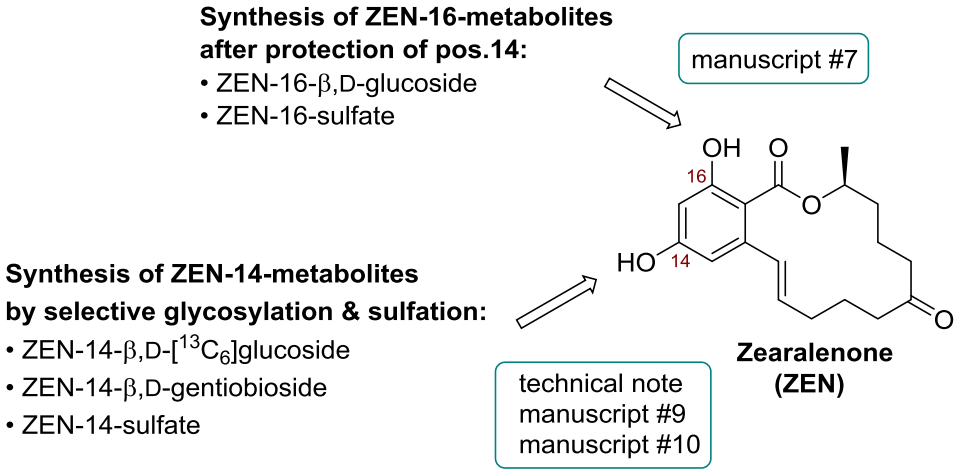

Figure 2.10 Overview of successfully prepared ZEN conjugates

Synthesis of Culmorin Metabolites

Culmorin (CUL) is a *Fusarium* metabolite that co-occurs with trichothecene mycotoxins and potentially influences their toxicity. The ecological role and fate of CUL in plants is still unknown. To provide reasonable amounts of reference standards for the investigation of the metabolism of CUL and 11-acetylculmorin in wheat suspension cultures, we aimed to develop selective methods for the synthesis of CUL sulfates and glucosides. Mono- and disulfation of CUL was achieved after development of an efficient procedure applying sulfur trioxide trimethylamine complex as sulfating reagent. To obtain CUL-8-sulfate, CUL was selectively acetylated at position 11 prior to sulfation. Diastereo- and regioselective glucosylation of culmorin to obtain CUL-8- and CUL-11-glucoside was achieved by (i) exploiting and (ii) preventing acyl transfer when using different glucosyl donors. The detailed synthesis of all CUL conjugates and their use as reference standards for investigating the role of CUL in plants is described in manuscript #8. An overview of all prepared CUL conjugates is presented in Fig. 2.11.

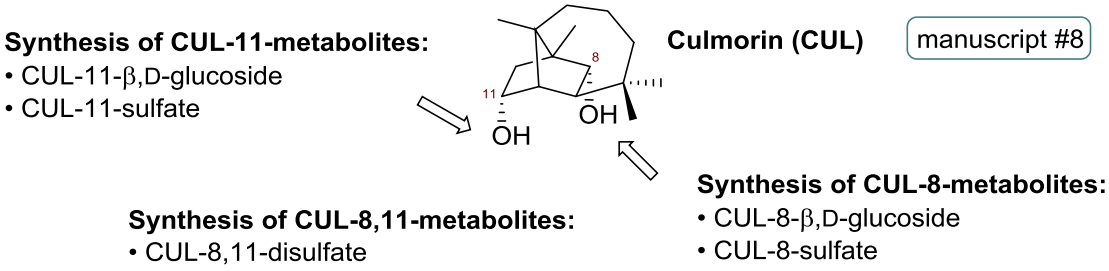

Figure 2.11 Overview of successfully performed syntheses of CUL conjugates

Chapter 3

Originial Works

3.1 Development of New Glycosylation Methods

Manuscript #1

Manuscript draft

Weber, J.; Svatunek, D.; Tegl, G.; Krauter, S.; Hametner, C.; Kosma, P.; Fröhlich, J.; Mikula, H. 2-*O*-Benzyloxycarbonyl Protected Glucosyl Donors: A Revival Towards Diastereoselective Glycosylation

2-*O*-Benzyloxycarbonyl Protected Glucosyl Donors: A Revival Towards Diastereoselective Glycosylation

Julia Weber,[a] Dennis Svatunek,[a]# Gregor Tegl,[b]# Simon Krauter,[b] Christian Hametner,[a] Paul Kosma,[b] Johannes Fröhlich,[a] and Hannes Mikula*[a]

Abstract: 2-O-Benzyloxycarbonyl protected glycosyl donors were developed for the β-diastereoselective glycosylation of structurally complex and/or labile natural products, particularly ones containing ester functionalities. The presented new thiodonor system could easily be prepared via two different routes and performed well in glycosylation reactions except with acceptors as small as methanol which formed mainly a cyclic carbonate as side product. The synthesized thiodonors could easily be transformed in the corresponding glycosyl imidates, which performed perfectly in glycosylations with just a catalytic amount of promotor. The prepared glycosides could be mildly deprotected via palladium-catalyzed hydrogenation in a one pot reaction without affecting ester groups. Hence, this new glycosylation strategy also showed to be perfectly suitable for the synthesis of glycosyl esters. As the benzyloxycarbonyl group can be easily exchanged with other alkyloxycarbonyl moieties, this new approach for 1,2- trans glycosylation shows a promising versatility with a wide applicability.

Carbohydrate-containing metabolites have been known for decades, but research devoted to the precise roles of these compounds and their formation is often lacking. Traditionally, carbohydrate ligands have been implicated in the control of drug pharmacokinetics,[1–3] but growing evidence has produced a change in this dogmatic view. Beside the structural and physicochemical influence of the sugar moiety, there also exist many examples in which removal of these critical ligands led to the formation of compounds with reduced or even no biological activity.[4–6] Furthermore, many organisms have evolved specific glycosylation mechanisms to detoxify harmful xenobiotics.[7–9] Carbohydrates are also involved in many fundamental biological events, in beneficial ones such as cell growth, immune responses, etc. as well as disease processes. In order to be able to analyze and assess the roles of carbohydrates in biological systems thoroughly, there is a need for pure and defined target carbohydrates in reasonable quantities. Therefore the synthesis of carbohydrates and glycoconjugates is becoming more and more of significant importance.[10,11] Many synthetic methods for the preparation of these natural products have been developed and applied for the synthesis of biologically active compounds, but the glycosylation reaction still remains a challenging synthetic transformation for which no general procedure can be applied so far.[12,13]

Still one of the most demanding problems in synthetic carbohydrate chemistry is the necessity to form either a 1,2-trans or 1,2-cis glycosidic bond with complete diastereoselectivity. The synthesis of 1,2-trans glycosides (e.g. β-glucosides) applying neighboring participating protective groups is well established,[14] but there are still drawbacks regarding the compatibility of many natural products and reaction conditions usually applied for the removal of the most common participating groups, i.e. ester functionalities at O-2. Furthermore the often emerging side reactions with acyl-protected glycosyl donor systems such as the orthoester formation[15,16] and the acyl transfer,[14,17] also show the need for the investigation of new glycosylation strategies. Hence, the development of novel methods for diastereoselective glycosylation of structurally complex and/or labile compounds is of high importance for carbohydrate chemistry.

Several methods are reported that might be applied to avoid such stability problems as well as the formation of common side products, including substituted phenylacetyl,[18,19] benzoyl[20–22] or benzyl ether[23] groups. The 2-O-picolinyl moiety, an arming participating group, developed by Smoot et al.[24] and an ester group reported by Crich et al.[25] are cleavable by palladium-catalyzed hydrogenation. All these protecting groups have in common that the classical neighboring participating group effect is used for stereocontrol of the glycosylation reaction. Recent studies also investigated new strategies to influence the diastereoselectivity of a glycosylation reaction by applying a linker effect,[26] remote protecting groups,[27] specific promotor-systems[13] or steric effects.[28] However, all these methods have a limited scope in terms of high ß-diastereoselectivity, reaction times, different glycosylation or deprotection methods as well as the ease of the donor preparation and a minimalist protecting group strategy.

In the course of ongoing research in the field of xenobiotic metabolism, we have become interested in the development of glucosyl donors which feature the qualities of forming only ß-glycosides, being highly reactive resulting therefore in good yields and having protecting groups which can be mildly deprotected in a one pot reaction compatible with base-sensitive natural products containing ester functionalities. The idea of introducing a participating immolative linker between O-2 and the protective group led to consideration of 2-O-

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Supporting information for this article is given via a link at the end of the document.

benzyloxycarbonyl protected glycosyl donors as reagents for diastereoselective glycosylation. The carbonyl group was considered to provide anchimeric assistance, whereas deprotection depends only on the benzyl substituent, as cleavage leads to the formation of an unstable intermediate (Figure 1). This approach could also easily be applied to the many different alkoxycarbonyl moieties which are available and commonly used as protective groups (e.g. Cbz, Alloc, Troc, Poc, Fmoc),[29,30] leading to a highly versatile strategy for 1,2-trans glycosylation. Similar investigations were already done by Gentil et al.[31] and Morère et al.,[32] but several drawbacks were observed including the presumed formation of a cyclic carbonate after donor activation. First successful results were reported by Ali et al.[33] by the use of methylsulfonylethoxycarbonyl (Msc) as carbonate protecting group at O-2 in a thiodonor system, efficient anchimeric assistance and good glycosylation yields could be observed. However, this donor system needs a two-step deprotection under mild basic conditions to cleave the Msc group.

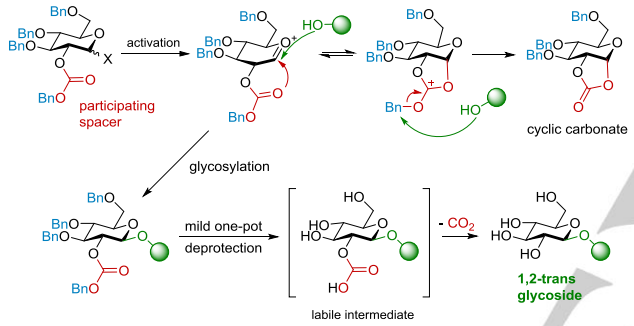


Figure 1. Participation of Benzyloxycarbonyl Groups as Strategy for 1,2-trans selective glycosylation

Herein we report the first results of applying the carbonate strategy to obtain 2-O-benzyloxycarbonyl protected thioglucosides as reagents for 1,2-trans glucosylation. Due to the excellent properties of thioglucosides, including versatile methods for selective activation and high stability,[12] we considered different S leaving groups (SEt, STol, STaz, SPym), for the development of efficient glucosyl donors. For this purpose selected benzyl protected 2-OH thioglucosides were prepared applying two different known strategies. Starting from the thioorthoester **1**[34], Lewis-acid mediated synthesis of the 2-O-acetyl protected intermediates **2-5** was applied followed by deprotection of the acetyl group to yield the corresponding 2-OH thioglucosides **6-9**. Applying DMDO (dimethyldioxirane) epoxidation of 3,4,6-tri-O-benzyl-D-glucal (**10**) and subsequent nucleophilic addition of the corresponding thiol directly led to **6**[35] and **7** (Figure 2).

Introduction of the benzyloxycarbonyl (Cbz) group was accomplished by reaction with benzyl chloroformate in the presence of TMEDA according to a general procedure developed by Adinolfi and coworkers (Figure 2).[36] Interestingly, the reaction did not lead to high conversion of the starting material even after addition of further reagents in combination with longer reaction times. Several other methods that are described and often used in the literature did not lead to desired product formation, including the use of pyridine,[37] triethylamine or DMAP in addition to Cbz-Cl in different solvents (THF, DMF).

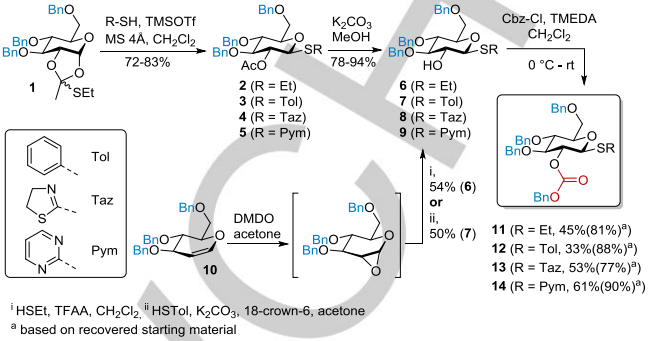


Figure 2. Synthesis of 2-O-Benzyloxycarbonyl Protected Thioglucosides

As thioglucosides can be easily converted in other glucosyl donors, we also prepared 2-O-benzyloxycarbonyl protected glucosyl imidates as donors, which have the advantage that they can be activated only by a catalytic amount of Lewis acid as promotor and have therefore a wide applicability.[13] Starting from thioglucoside **11**, the anomeric center was deprotected by addition of water and NIS to yield 1-hydroxysugar **15**.[38] The attempt to produce the trichloroacetimidate **16** by a DBU-catalyzed addition of the anomeric hydroxyl group to Cl_3CCN resulted mainly in the formation of cyclic carbonate **18** as side product. To reduce this problematic, N-phenyltrifluoroactimidate **17** was prepared by taking N-phenyltrifluoroacetimidoyl chloride as a strong electrophile combined with the weak base K_2CO_3. This resulted still in the formation of cyclic carbonate **18**, but the yield of the desired product could be doubled at least (Figure 3).

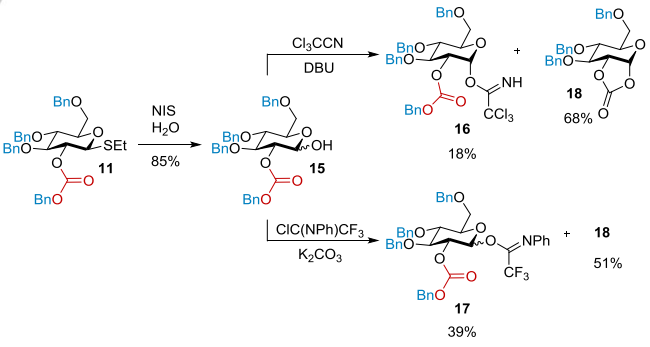
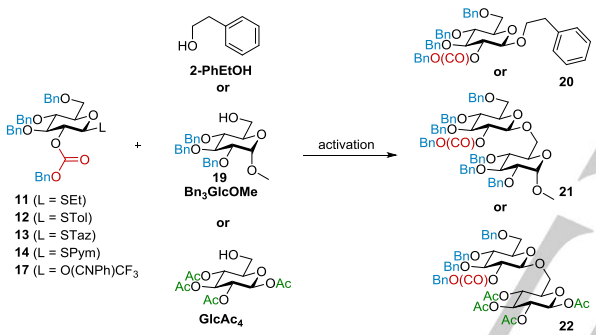


Figure 3. Synthesis of 2-O-Benzyloxycarbonyl Protected Glucosyl Imidates

First glycosylation experiments were done using the 2-O-benzyloxycarbonyl protected SEt glucosyl donor **11**. Different methods for activation were applied during the reaction with methanol and 2-phenylethanol as simple glucosyl acceptors. Interestingly, all glucosylations with methanol as acceptor formed no or only traces of product, leading mainly to the unwanted cyclic carbonate **18**.[39] While the glucosylations with

2-phenylethanol produced with different promotors 2-phenylethyl β,D-glucoside (**23**) in excellent yields. Only iodine turned out to be a bad promotor, leading only to low product yields and also formation of the undesired cyclic carbonate **18**. Encouraged by the results with 2-phenylethanol, further glucosylation reactions were performed applying Cbz-protected thioglucosyl donors **12-14** and 2-phenylethanol as simple acceptors as well as **19** and 1,2,3,4-tetraacetyl glucose ($GlcAc_4$) as typical carbohydrate acceptors (Table 1). In general, all glycosylations led to the corresponding products in high yields with complete ß-diastereoselectivity. Cbz-protected SEt and STol glucosyl donors **11** and **12** were best activated by NIS/TfOH, while the STaz- and SPym glucosyl donors **13** and **14** achieved with AgOTf as promotor the best results. The glycosyl donor **13** turned out to be the most labile donor during storage and shows noticeably the lowest yields in the glycosylation screening. Finally, the glucosyl imidate **17** was tested with 2-phenylethanol and **19** as acceptors, leading to perfect results in the shortest reaction time (2h). For activation only a catalytic amount of TMSOTf (0.1 eq.) was needed.

Table 1. Comparative Glucosylations of Glucosyl Donors **11**-**14** and **17**



entry	donor	acceptor (equ.)	activation	temp.	yield[a, b]
1	**11**	PhEtOH(1.5)	NIS/TfOH	-10°C	>99% (**20**)
2	**11**	PhEtOH(1.5)	I_2	rt	6 (22)*% (**20**)
3	**11**	$GlcAc_4$(1)	NIS/TfOH	-10°C	>99% (**22**)
4	**11**	$Bn_3GlcOMe$(1)	NIS/TfOH	-10°C	>99% (**21**)
5	**12**	PhEtOH(1.5)	NIS/TfOH	-10°C	98% (**20**)
6	**12**	$Bn_3GlcOMe$(1.5)	NIS/TfOH	-10°C	>99% (**21**)
7	**13**	PhEtOH (1.5)	AgOTf	rt	71% (**20**)
8	**13**	$Bn_3GlcOMe$(1.5)	AgOTf	rt	59% (**21**)
9	**14**	PhEtOH(1.5)	TMSOTf	-10°C	61% (**20**)
10	**14**	PhEtOH(1.5)	AgOTf	0°C	97% (**20**)
11	**14**	$Bn_3GlcOMe$(1)	AgOTf	0°C	98% (**21**)
12	**17**	PhEtOH(1.5)	TMSOTf	-10°C	94% (**20**)
13	**17**	$Bn_3GlcOMe$(1.5)	TMSOTf	-10°C	96% (**21**)

[a] non-isolated yield as determined by HPLC analysis (UV at 210 nm) after 3h reaction time [b] only β (as indicated by HPLC-analysis) *non-isolated yield after 24h

Deprotection of glucoside **20** and disaccharide **22** applying classic palladium-catalyzed hydrogenation was carried out under mild reaction conditions to obtain 2-phenylethyl β,D-glucoside (**23**) and 1,2,3,4-tetraacetyl gentiobioside (**24**) in good yields, showing that the deprotection conditions do not affect acetyl groups. This reveals the orthogonality to ester groups as well as the great capability of the presented approach. Selective deprotection of the acetyl groups of disaccharide **22** without causing the benzyl carbonate any harm could be achieved under very mild basic conditions to obtain **25** (Figure 4).

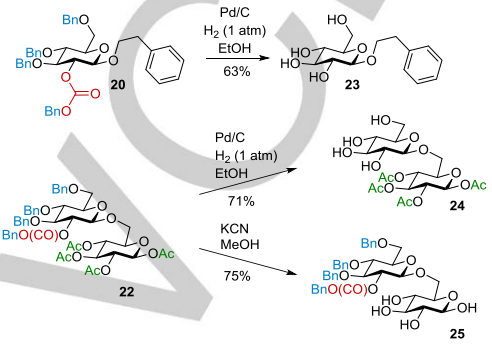


Figure 4. One-Pot Deprotection Applying Catalytic Hydrogenation and Demonstration of the Orthogonality to Ester Groups

Encouraged by the excellent yields of the glycosylation as well as deprotection reactions, we also focused on the stereocontrolled synthesis of the more labile class of compounds: the glycosyl esters. For improvement of the physicochemical properties of peptide pharmaceuticals, peptide-carbohydrate adducts play an important role.[40] To model the glycosyl ester bond formation between a peptide and a sugar molecule, we chose the amino acid trans-N-(tert-butoxycarbonyl)-4-acetoxy-L-proline (**26**) as representative for the derivatization site of the peptide. Glycosylation of **26** with glucosyl donor **11** formed the benzyl-protected glycosyl ester **27** with complete ß-diastereoselectivity. Deprotection was achieved under palladium-catalyzed hydrogenation to obtain glycosyl ester **28** in good yields without the observation of cleavage or migration of the acetyl group. Furthermore the β-glucosyl ester of acetylsalicyclic acid (**31**), a precursor of an aspirin prodrug,[41] could be synthesized from acetylsalicyclic acid (**29**) and glucosyl donor **11** under the same reaction conditions in perfect yields (Figure 5).

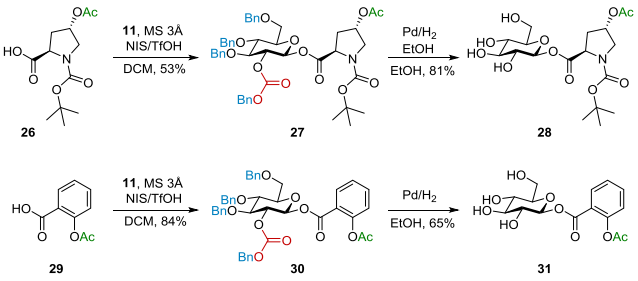


Figure 5. Synthesis of Glycosl Esters with 2-O-Benzyloxycarbonyl Protected Glucosyl Donors

In summary, the synthesis of several 3,4,6-tri-O-benzyl-2-O-Cbz protected thioglucosides and glucosyl imidates as novel efficient glucosyl donors is presented. First results of glucosylation studies are reported and simple deprotection by catalytic hydrogenation in a mild one-pot reaction is shown which affects no ester groups. Therefore the developed donor system is particularly suitable for the synthesis of glycosyl esters and base labile natural products. Orthoester formation and acyl transfer, the common side reactions of glycosylations, could be eliminated completely with this new approach. However, the formation of a cyclic carbonate in glycosylations with very small acceptors like methanol or with bad promotors emerged. The detailed mechanism of this side reaction should still be investigated more closely. The versatility of the general strategy of applying 2-O-alkyloxycarbonyl protection is shown to be a promising concept for the development of novel methods for 1,2 trans glycosylation.

Acknowledgements

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Keywords: 1,2-trans glycosylation • glycoconjugates• thioglucosides • anchimeric assistence • glycosyl ester

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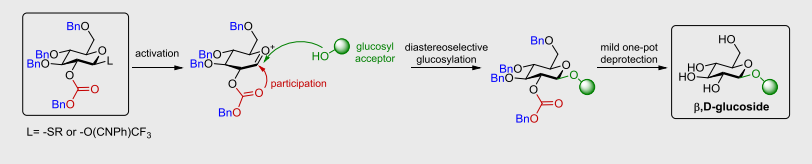

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*J. Weber, D. Svatunek, G. Tegl, S. Krauter, C. Hametner, P. Kosma, J. Fröhlich, and H. Mikula**

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2-*O*-Benzyloxycarbonyl Protected Glucosyl Donors: A Revival Towards Diastereoselective Glycosylation

Manuscript #2

Manuscript draft

Weber, J.; Krauter, S.; Schwarz, T.; Hametner, C.; Fröhlich, J.; Mikula, H. (2-Benzyloxyphenyl)acetyl (BnPAc): A new relay protecting group for alcohols and especially carbohydrates

(2-Benzyloxyphenyl)acetyl (BnPAc): A new relay protecting group for alcohols and in particular carbohydrates

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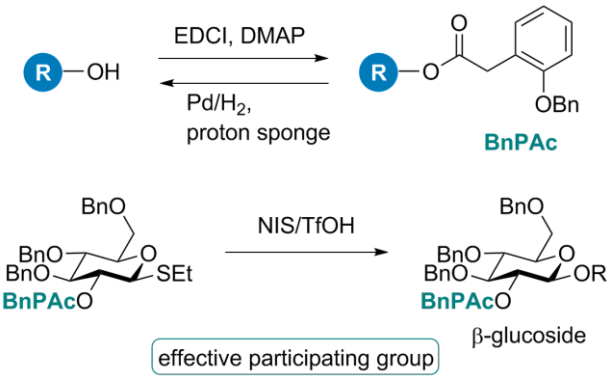

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Abstract The (2-benzyloxyphenyl)acetyl group was identified as a new protecting group for hydroxyl functions. Various alcohols could be easily protected with high yields and deprotection was achieved by a relay approach using Pd/H_2 in combination with proton sponge, conditions that are orthogonal to ester groups. The new protecting group is stable in glycosylation reactions demonstrating an effective neighboring group participation leading to the exclusive formation of 1,2-*trans* glycosides and glycosyl esters.

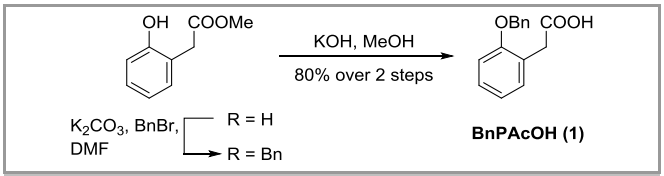
Key words protecting group, relay deprotection, neighboring participating group, alcohol, carbohydrate, glycoside

While simple molecules can often be prepared without protecting groups, they are crucial for the synthesis of complex and multifunctional organic compounds. Especially in the field of carbohydrate chemistry, the design of protecting groups and protecting group patterns for the differentiation of the multiple hydroxyl groups in regard to chemo- and regioselectivity plays an important role.[1,2] Routinely, in oligosaccharide synthesis there are only few persistent protecting groups (acetyl, benzyl or benzoyl groups) in use whereas a variety of temporary protecting groups depending on the structure of the target molecule and the type of the persistent protecting groups are applied.[3] The requirements for an ideal protecting group are an easy introduction with readily available, inexpensive reagents, a high stability during chemical transformations as well as purification steps and a selective deprotection under mild conditions without affecting other functional groups.[4] Even though there are already a vast number of protecting groups reported,[4,5] only few found really wide application. This is due to the fact, that usually they meet only partly the requirements of above. As a consequence, there is still a continuous need for the development of new protecting groups in carbohydrate chemistry.

Relay cleavage is a valuable tool for designing new protecting groups. In relay cleavage protecting groups have an auxiliary group that is stable under a wide range of conditions. Cleavage is initiated by a chemical transformation of this group leading to a readily cleavable form or even resulting in spontaneous deprotection under mild conditions. Although relay deprotection lengthens the cleavage process by an extra step of activation, this is compensated by the added measure of orthogonality of this kind of protecting group.[4] Based on this principle a variety of protecting groups have been already introduced so far, including among others AZMB,[6] AMPA,[7] APAc,[8] POMB,[9] CAMB,[10] NPAc,[11] PAC[12] and TMBPP.[13] Deprotection by hydrogenolysis within the class of relay protecting groups is represented only rarely, it was described for the PAC and its advancement, the TMBPP group. However, the TMBPP group, also an effective neighboring participating group in glycosylations, has still its failures in coupling reactions with bulky acceptors of low reactivity.[13]

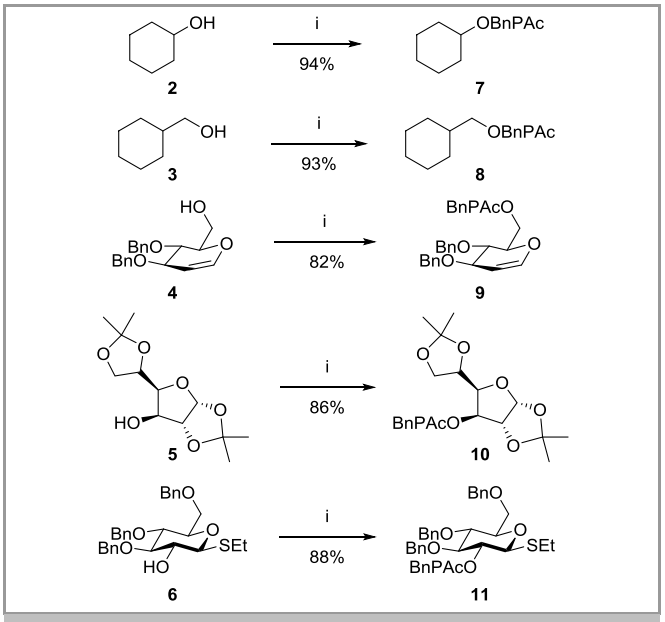
Herein, we introduce (2-benzyloxyphenyl)acetyl as a new protecting group for alcohols that can be cleaved orthogonal to ester or benzyl groups and is an effective participating group in glycosylations.

(2-Benzyloxyphenyl)acetic acid (BnPAcOH **1**, Scheme 1) was prepared starting from methyl 2-(2-hydroxyphenyl)acetate with benzyl bromide in the presence of potassium carbonate in dimethyl formamide, followed by basic hydrolysis of the methyl ester with potassium hydroxide in methanol.



Scheme 1 Synthesis of (2-benzyloxyphenyl)acetic acid

Introduction of the BnPAc group to different alcohols (**2**-**6**) was readily achieved by reaction with BnPAcOH (**1**) in the presence of EDCI and DMAP in dichloromethane.[14] In each case, the respective ester (**7**-**11**)[15–19] was obtained in good yields (88-94 %, Scheme 2).



Scheme 2 Introduction of the BnPAc-group on various alcohols. Reagents and conditions: i…BnPAcOH, EDCI, DMAP, DCM

Removal of the BnPAc group were accomplished either by a relay approach using catalytic hydrogenation in combination of proton sponge[20] or by conventional hydrolytic methods. [21] Compounds **7**, **8** and **10** were readily deprotected by using the relay cleavage resulting in the corresponding alcohol (**2**, **3** and **5**) and a lacton as side product.

Alternatively, compound **9** that is sensitive to hydrogenolysis, could be deprotected by basic hydrolysis with potassium carbonate in methanol to obtain **4**[22] in 90 % without affecting the double bond (Table 1).

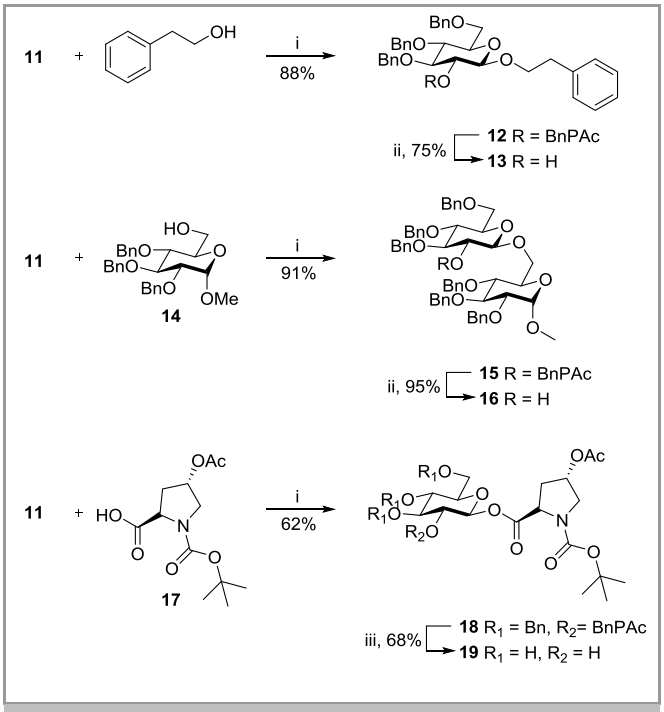
Table 1 Different deprotection conditions for the BnPAc-group

Entry	Substrate	Cleavage Conditions	Yield [%]
1	**7**	Pd/H_2+proton sponge	>99[a]
2	**8**	Pd/H_2+proton sponge	>99[a]
3	**10**	Pd/H_2+proton sponge	63
4	**9**	K_2CO_3 in MeOH	90

[a]non-isolated yield as determined by GC-MS

After having established a simple procedure for the introduction and two orthogonal ways for the removal of the BnPAc protecting group, we tested its compatibility in glycosylation reactions and explored its ability to perform neighboring group participation. Thioglycoside **11** was activiated with NIS and TfOH[23] and reacted with phenylethanol and glucosyl acceptor **14** leading exclusively to the β-glycosides **12**[24] and **15**[25] in yields of 88 and 91 %, respectively. Selective deprotection of the BnPAc group could be achieved in the presence of benzyl groups by basic hydrolysis to furnish glycosides **13**[26] and **16**.[27]

Encouraged by these results, we proceeded to perform a further glycosylation reaction with glucosyl donor **11** and the protected amino acid **17** as acceptor leading to the formation of the glycosyl ester **18**[28] with complete β-diastereoselectivity in good yields. Relay deprotection of **18** using palladium-catalyzed hydrogenolysis in combination with proton sponge enabled the simultaneous cleavage of the benzyl groups as well as the BnPAc group to obtain **19**[29] in 68 %. The relay deprotection method didn´t affect the acetyl group as well as the Boc group of **18**, demonstrating the orthogonality of the BnPAc group to ester groups (Scheme 3).



Scheme 3 Glycosylation reactions with 2-BnPAc-glucosyl donor **11**. Reagents and conditions: i…NIS/TfOH, CH_2Cl_2, ii…K_2CO_3 in MeOH, iii…Pd/H_2, proton sponge

In summary, the BnPAc ester is a stable protecting group that is easily introduced in almost quantitative yields and can be either cleaved by a relay approach using catalytic hydrogenation in combination with proton sponge or by basic hydrolysis. The BnPAc group can therefore either be orthogonal to ester groups or benzyl groups according to the applied deprotection protocol. In glycosylation reactions the BnPAc protecting group performed an effective neighboring participation with complete β-diastereoselectivity. Hence, we are confident that the BnPAc group will be a valuable tool in oligosaccharide synthesis and in the preparation of glycosyl esters.

Acknowledgment

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(14) **General Procedure A: Introduction of BnPAc**
Alcohol (0.5 mmol, 1 eq.), BnPAcOH (0.6 mmol, 1.2 eq.) and DMAP (0.05 mmol, 0.1 eq.) were dissolved in dry CH_2Cl_2 (3 mL) and cooled to 0 °C. After addition of EDCI (0.6 mmol, 1.2 eq.), the reaction mixture was stirred at r.t. until completion of the reaction. The reaction mixture was diluted with CH_2Cl_2 (10 mL) and washed with 1M HCl (2x 10 mL), saturated $NaHCO_3$ solution (2x 10 mL) and brine (10 mL). Aqueous phases were extracted with CH_2Cl_2 (5 mL) after each washing step. The combined organic layer was dried over Na_2SO_4 and concentrated. The crude product was purified by column chromatography (gradient elution hexanes:EE) to yield the desired product.

(15) **(2-Benzyloxyphenyl)acetic acid, cyclohexyl ester (7):** Starting from cyclohexanol (100 mg, 0.1 mmol) and following general procedure A BnPAc ester **7** was obtained as a white solid (306 mg, 94 %).
Analytical Data for 7
^{1}H NMR (200 MHz, $CDCl_3$) δ 7.44-7.10 (m, 7H), 6.85 (t, J = 7.4 Hz, 2H), 4.99 (s, 2H), 4.68 (tt, J = 8.6, 4.3 Hz, 1H), 3.58 (s, 2H), 1.81 - 0.98 (m, 10 H); ^{13}C NMR (50 MHz, CDCl3) δ 171.4 (s, 1C), 156.8 (s, 1C), 137.3 (s, 1C), 131.1 (d, 1C), 128.6 (d, 2C), 128.5 (d, 1C), 127.8 (d, 1C), 127.2 (d, 2C), 124.0 (s, 1C), 120.9 (d, 1C), 111.8 (d, 1C), 72.9 (d, 1C), 70.0 (t, 1C), 36.7 (t, 1C), 31.7 (t, 2C), 25.6 (t, 1C), 23.8 (t, 2C); HRMS calcd for $C_{21}H_{24}NaO_3^+$ $[M+Na]^+$ 347.1618, found 347.1617.

(16) **(2-Benzyloxyphenyl) acetic acid, cyclohexylmethyl ester (8):** Starting from cyclohexyl methanol (114 mg, 0.1 mmol) and following general procedure A BnPAc ester **8** was obtained as a white solid (315 mg, 93 %).
Analytical Data for 8
^{1}H NMR (200 MHz, CDCl3) δ 7.41-7.16 (m, 5H), 7.13 (d, J = 8.4 Hz, 2H), 6.85 (t, J = 7.4 Hz, 2H), 4.99 (s, 2H), 3.78 (d, J = 6.3 Hz, 2H), 3.60 (s, 2H), 1.72 - 0.64 (m, 11 H); ^{13}C NMR (50 MHz, CDCl3): δ = 172.1 (s, 1C), 156.8 (s, 1C), 137.3 (s, 1C), 131.2 (d, 1C), 128.6 (d, 3C), 127.9 (d, 1C), 127.1 (d, 2C), 123.8 (s, 1C), 120.9 (d, 1C), 111.8 (d, 1C), 70.0 (t, 1C), 69.9 (t, 1C), 37.2 (d, 1C), 36.3 (t, 1C), 29.7 (t, 2C), 26.5 (t, 1C), 25.8 (t, 2C); HRMS calcd for $C_{22}H_{26}NaO_3^+$ $[M+Na]^+$ 361.1774, found 361.1773.

(17) **(2-Benzyloxyphenyl) acetic acid, 3,4-di-O-benzyl glucuronal ester (9):** Starting from 3,4-di-O-benzyl glucuronal (200 mg, 0.61 mmol) and following general procedure A BnPAc ester **9** was obtained (276 mg, 82 %).
Analytical Data for 9
^{1}H NMR (400 MHz, CD_2Cl_2) δ 7.44-7.16 (m, 17H), 6.98-6.88 (m, 2H), 6.33 (dd, J = 6.2, 1.2 Hz, 1H), 5.05 (s, 2H), 4.91 (dd, J = 6.3, 2.7, 1H), 4.68 (, J = 11.1 Hz, 1H), 4.62 (d, J = 11.7 Hz, 1H), 4.52 (d, J = 11.7 Hz, 1H), 4.45 (d, J = 11.3 Hz, 1H), 4.42 (dd, *J* = 12.4, 2.4 Hz, 1H), 4.31 (dd, *J* = 12.1, 5.5 Hz, 1H), 4.17-4.13 (m, 1H), 4.06 (ddd, *J* = 8.3, 5.6, 2.6 Hz, 1H), 3.70 (s, 2H), 3.65 (dd, *J* = 8.2, 5.8 Hz, 1H); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 171.61 (s, 1C), 157.08 (s, 1C), 144.56 (d, 1C), 138.90 (s, 1C), 138.60 (s, 1C), 137.56 (s, 1C), 131.48 (d, 1C), 128.93 (d, 1C), 128.84 (d, 2C), 128.73 (d, 2C), 128.68 (d, 2C), 128.28 (d, 2C), 128.12 (d, 1C), 128.08 (d, 2C), 128.06 (d, 1C), 127.97 (d, 1C), 127.48 (d, 2C), 123.87 (s, 1C), 121.08 (d, 1C), 112.13 (d, 1C), 100.41 (d, 1C), 75.52 (d, 1C), 75.41 (d, 1C), 74.55 (d, 1C), 73.78 (t, 1C), 70.66 (t, 1C), 70.26 (t, 1C), 63.08 (t, 1C), 36.37 (t, 1C); HRMS calcd for $C_{35}H_{34}NaO_6^+$ $[M+Na]^+$ 573.22476, found 573.22444.

(18) **(2-Benzyloxyphenyl) acetic acid, 1,2,5,6-diisopropylidene glucosyl ester (10)**: Starting from diacetone-d-glucose (50 mg, 0.19 mmol) and following general procedure A BnPAc ester **10** was obtained (80 mg, 86 %).
Analytical Data for 10
^{1}H NMR (400 MHz, CD_2Cl_2) δ 7.46-7.37 (m, 4H), 7.36-7.31 (m, 1H), 7.3-7.25 (m, 1H), 7.21 (dd, J = 7.3, 1.4 Hz, 1H), 6.99-6.92 (m, 2H), 5.59 (d, J = 3.5 Hz, 1H), 5.11 (d, J = 3.1 Hz, 1H), 5.08 (s, 2H), 4.24 (d, J = 3.9 Hz, 1H), 4.16 (dd, J = 7.0, 3.1 Hz, 1H), 4.02 (dt, J = 7.0, 5.8 Hz, 1H), 3.85 (d, *J* = 5.84, 2H), 3.74 (d, *J* = 16.4 Hz, 1H), 3.65 (d, J = 16.4 Hz, 1H), 1.45 (s, 3H), 1.34 (s, 3H), 1.24 (s, 3H), 1.20 (s, 3H) ; ^{13}C NMR (100 MHz, CD_2Cl_2) δ 170.85 (s, 1C), 157.07 (s, 1C), 137.47 (s, 1C), 131.41 (d, 1C), 129.16 (d, 1C), 128.93 (d, 2C), 128.34 (d, 1C), 127.60 (d, 2C), 123.42 (s, 1C), 121.15 (d, 1C), 112.40 (s, 1C), 111.97 (d, 1C), 109.32 (s, 1C), 105.38 (d, 1C), 83.56 (d, 1C), 79.86 (d, 1C), 76.73 (d, 1C), 72.87 (d, 1C), 70.29 (t, 1C), 66.91 (t, 1C), 36.64 (t, 1C), 26.82 (q, 2C), 26.33 (q, 1C), 25.32 (q, 1C); HRMS calcd for $C_{27}H_{32}NaO_8^+$ $[M+Na]^+$ 507.19894, found 507.19900.

(19) **Ethyl 3,4,6-tri-O-benzyl-2-O-[(2-benzyloxyphenyl)acetyl]-1-thio-β,D-glucoside (11):** Starting from ethyl 3,4,6-tri-O-benzyl-1-thio-β,D-glucoside (4.4 g, 9 mmol) and following general procedure A glucosyl donor **11** was obtained as a white solid (5.2 g, 88 %).
Analytical Data for 11
^{1}H NMR (400 MHz, CD_2Cl_2) δ 7.50-7.09 (m, 22H), 6.95-6.85 (m, 2H), 5.04 (d, J = 12.5 Hz, 1H), 5.01 (d, J = 11.7 Hz, 1H), 5.01-4.97 (m, 1H), 4.77 (d, J = 10.9 Hz, 1H), 4.64 (d, J = 11.3 Hz, 1H), 4.59 (d, J = 12.1 Hz, 1H), 4.56 (d, J = 7.8 Hz, 2H), 4.53 (d, J = 9.0 Hz, 1H), 4.37 (d, J = 10.1 Hz, 1H), 3.78-3.61 (m, 6H), 3.51-3.43 (m, 1H), 2.64 (q, J = 2.6, 7.2 Hz, 2H), 1.20 (t, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 170.62 (s, 1C), 156.94 (s, 1C), 138.76 (s, 1C), 138.70 (s, 1C), 138.62 (s, 1C), 137.65 (s, 1C), 131.64 (d, 1C), 128.86 (d, 1C), 128.81 (d, 2C), 128.68 (d, 2C), 128.65 (d, 2C), 128.63 (d, 2C), 128.34 (d, 2C), 128.11 (d, 4C), 128.06 (d, 2C), 127.93 (d, 1C), 127.91 (d, 1C). 127.42 (d, 2C), 123.28 (s, 1C), 121.00 (d, 1C), 112.22 (d, 1C), 84.53 (d, 1C), 83.91 (d, 1C), 79.57 (d, 1C), 78.07 (d, 1C), 75.28 (t, 1C), 75.27 (t, 1C), 73.70 (t, 1C), 72.68 (d, 1C), 70.22 (t, 1C), 69.37 (t, 1C), 35.95 (t, 1C), 24.37 (t, 1C), 15.15 (q, 1C); HRMS calcd for $C_{44}H_{46}NaO_7S^+$ $[M+Na]^+$ 741.28565, found 741.28514.

(20) **General Procedure B1: Deprotection of BnPAc via catalytic hydrogenation**
To a solution of the respective BnPAc-protected compound (0.07 mmol, 1 eq.) in dry ethanol (1.5 mL) one small tip of a spatula of Pd/C was added under argon atmosphere. The argon balloon was changed for a H_2-balloon and the reaction mixture was stirred for 3-6 h at rt. The reaction mixture was filtered through a syringe filter. Proton sponge (1 eq.) was added to the filtrate and the reaction solution was heated up to 80 °C until completion of the reaction (4-8 h). The reaction mixture was concentrated and the residue was purified either by flash chromatography (gradient elution hexanes:EE) or by preparative-HPLC (H_2O:ACN) to yield the corresponding product.

(21) **General Procedure B2: Deprotection of BnPAc via basic hydrolysis**
To a solution of the respective BnPAc-protected compound in dry methanol K_2CO_3 (0.5 eq.) was added. The reaction mixture was stirred for 16 h at room temperature. In some cases, further addition of 0.5 eq. NaOMe was necessary for completion of the reaction. The reaction mixture was filtered, concentrated and the residue was purified by flash

chromatography (gradient elution hexanes:EE) or by preparative-HPLC (H_2O:ACN) to obtain the desired product.

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(23) **General Procedure C: Glycosylation with donor 11**
To a solution of the acceptor (680 µmol, 2.5 eq.) and glucosyl donor **11** (200 mg, 272 µmol, 1 eq.) in dry CH_2Cl_2 (5 mL) molecular sieve (3Å, 100 mg/mL) was added and the reaction mixture was stirred for 2 h at r.t.. After cooling to -10 °C, N-iodosuccinimide (133 mg, 544 µmol, 2 eq.), followed by trifluoromethanesulfonic acid (5 µl, 54 µmol, 0.2 eq.) was added and stirring was continued for 16 h. The reaction was quenched by addition of an aqueous saturated $NaHCO_3$ and Na_2SO_3 solution (1:1, 1 mL), then the reaction solution was diluted with CH_2Cl_2 (4 mL) and filtrated over Celite. The filtrate was washed with water (5 mL) and brine (3 mL), dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (hexanes/EtOAc gradient elution) to obtain the desired product.

(24) **2-Phenylethyl 3,4,6-tri-O-benzyl-2-O-[(2-benzyloxy-phenyl)acetyl]-β,D -glucoside (12):** General procedure C; starting from phenylethanol (83 mg, 0.68 mmol) and glucosyl donor **11** (200 mg, 0.27 mmol) **12** was obtained as a white solid (90 mg, 88 %).
Analytical Data for 12
^{1}H NMR (400 MHz, CD_2Cl_2) δ 7.43-7.11 (m, 27H), 6.92-6.81 (m, 2H), 5.01 (d, J = 12.9 Hz, 1H), 4.98 (d, J = 12.5 Hz, 1H), 4.94 (dd, J = 9.0, 8.2 Hz, 1H), 4.74 (d, J = 10.9 Hz, 1H), 4.61-4.45 (m, 5H), 4.35 (d, J = 8.2 Hz, 1H), 3.97 (dt, J = 9.9, 7.0 Hz, 1H), 3.75-3.68 (m, 2H), 3.67-3.54 (m, 5H), 3.47-3.40 (m, 1H), 2.78 (t, J = 7.2 Hz, 2H); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 170.46 (s, 1C), 156.85 (s, 1C), 139.11 (s, 1C), 138.75 (s, 1C), 138.73 (s, 1C), 138.64 (s, 1C), 137.61 (s, 1C), 131.42 (d, 1C), 129.33 (d, 2C), 128.83 (d, 2C), 128.78 (d, 1C), 128.67 (d, 2C), 128.65 (d, 3C), 128.59 (d, 2C), 128.32 (d, 2C), 128.14 (d, 2C), 128.10 (d, 2C). 128.09 (d, 2C), 128.04 (d, 1C), 127.91 (d, 1C), 127.87 (d, 1C), 127.42 (d, 2C), 126.55 (d, 1C), 123.46 (s, 1C), 121.04 (d, 1C), 112.22 (d, 1C), 101.33 (d, 1C), 83.07 (d, 1C), 78.16 (d, 1C), 75.38 (d, 1C), 75.22 (t, 1C), 75.03 (t, 1C), 73.90 (d, 1C), 73.71 (t, 1C), 70.67 (t, 1C), 70.22 (t, 1C), 69.23 (t, 1C), 36.40 (t, 1C), 35.82 (t, 1C); HRMS calcd for $C_{50}H_{50}NaO_8^+$ $[M+Na]^+$ 801.33979, found 801.33860.

(25) **Methyl 2,3,4,9,10,12-hexa-O-benzyl-8-O-[(2-benzyloxy-phenyl)acetyl]-α,D-gentiobioside (15):** General procedure C; starting from glucosyl donor **11** (60 mg, 0.08 mmol) and acceptor **14** (93 mg, 0.2 mmol) **15** (83 mg, 91 %) was obtained.
Analytical Data for 14
^{1}H NMR (400 MHz, CD_2Cl_2) δ 7.37-7.23 (m, 28H), 7.22-7.17 (m, 3H), 7.17-7.08 (m, 6H), 6.83-6.74 (m, 2H), 4.99 (dd, J = 8.6, 7.4 Hz, 1H), 4.96 (d, J = 11.3 Hz, 1H), 4.92 (d, J = 11.0 Hz, 1H), 4.86 (t, J = 12.3 Hz, 2H), 4.76 (d, J = 10.9 Hz, 1H), 4.72 (d, J = 10.9 Hz, 1H), 4.68 (d, J = 3.5Hz, 1H), 4.64 (d, J = 11.7 Hz, 1H), 4.61-4.55 (m, 3H), 4.54-4.48 (m, 3H), 4.45 (d, J = 11.3 Hz, 1H), 4.41 (d, J = 8.2 Hz, 1H), 4.01 (dd, J = 10.9, 1.6 Hz, 1H), 3.87 (t, J = 9.2 Hz, 1H), 3.78-3.55 (m, 8H), 3.50-3.39 (m, 3H), 3.32 (s, 3H); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 170.35 (s, 1C), 156.76 (s, 1C), 139.47 (s, 1C), 138.98 (s, 1C), 138.91 (s, 1C), 138.70 (s, 1C), 138.66 (s, 1C), 138.60 (s, 1C), 137.71 (s, 1C), 131.48 (d, 1C), 128.84 (d, 2C), 128.79 (d, 1C), 128.71 (d, 4C), 128.69 (d, 2C), 128.65 (d, 2C), 128.59 (d, 2C), 128.57 (d, 2C), 128.31 (d, 4C), 128.21 (d, 2C). 128.10 (d, 4C), 128.08 (d, 2C), 128.05 (d, 1C), 128.02 (d, 2C), 127.98 (d, 1C), 127.91 (d, 1C), 127.85 (d, 1C), 127.79 (d, 1C), 127.30 (d, 2C), 123.13 (s, 1C), 121.03 (d, 1C), 112.21 (d, 1C), 101.34 (d, 1C), 98.17 (d, 1C), 83.06 (d, 1C), 82.10 (d, 1C), 80.81 (d, 1C), 78.25 (d, 1C), 78.16 (d, 1C), 75.77 (t, 1C), 75.47 (d, 1C), 75.20 (t, 1C), 75.13 (t, 1C), 75.00 (t, 1C), 73.95 (d, 1C), 73.69 (t, 1C), 73.31 (t, 1C), 70.20 (d, 1C), 70.03 (t, 1C), 69.19 (t, 1C), 68.29 (t, 1C), 55.37 (q, 1C); HRMS calcd for $C_{70}H_{72}NaO_{13}^+$ $[M+Na]^+$ 1143.48651, found 1143.48627.

(26) **2-Phenylethyl 3,4,6-tri-O-benzyl-β,D-glucoside (13)**: Starting from compound **12** (51 mg, 0.07 mmol) and following general procedure B2 **13** was obtained as a white solid (27 mg, 75 %).
Analytical Data for 13
^{1}H NMR (400 MHz, CD_2Cl_2) δ 7.42-7.17 (m, 20H), 4.91 (d, J = 11.2 Hz, 1H), 4.84 (d, J = 6.6 Hz, 1H), 4.81 (d, J = 7.0 Hz, 1H), 4.57 (t, J = 11.7 Hz, 2H), 4..52 (d, J = 12.1 Hz, 1H), 4.27 (d, J = 7.6 Hz, 1H), 4.13 (dt, J = 9.6, 6.7 Hz, 1H), 3.79-3.68 (m, 3H), 3.60 (t, J = 9.2 Hz, 1H), 3.55 (t, J = 8.8 Hz, 1H), 3.49-3.43 (m, 2H), 2.96 (t, J = 7.0 Hz, 2H), 2.25 (d, J = 2.3 Hz, 1H); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 139.34 (s, 1C), 139.17 (s, 1C), 138.83 (s, 1C), 138.74 (s, 1C), 129.32 (d, 2C), 128.75 (d, 2C), 128.69 (d, 2C), 128.67 (d, 2C), 128.64 (d, 2C), 128.30 (d, 2C), 128.23 (d, 2C), 128.16 (d, 2C), 127.99 (d, 1C), 127.95 (d, 1C), 127.90 (d, 1C). 126.67 (d, 1C), 103.27 (d, 1C), 84.71 (d, 1C), 77.84 (d, 1C), 75.41 (d, 1C), 75.24 (t, 1C), 75.17 (t, 1C), 75.14 (d, 1C), 73.73 (t, 1C), 70.96 (t, 1C), 69.35 (t, 1C), 36.52 (t, 1C); HRMS calcd for $C_{35}H_{38}NaO_6^+$ $[M+Na]^+$ 577.25606, found 577.25570.

(27) **Methyl 2,3,4,9,10,12-hexa-O-benzyl-α,D-gentiobioside (16):** Starting from compound **15** (50 mg, 0.04 mmol) and following general procedure B2 **16** was obtained as a white solid (37 mg, 95 %).
Analytical Data for 16
^{1}H NMR (400 MHz, CD_2Cl_2) δ 7.44-7.25 (m, 28H), 7.23-7.18 (m, 2H), 4.96 (d, J = 10.9 Hz, 1H), 4.91 (d, J = 5.5 Hz, 1H), 4.88 (d, J = 5.5 Hz, 1H), 4.86-4.84 (m, 1H), 4.82 (t, J = 10.8 Hz, 2H), 4.76-4.70 (m, 2H), 4.67 (d, J = 5.1Hz, 1H), 4.64 (d, J = 4.3 Hz, 1H), 4.57 (t, J = 11.9 Hz, 2H), 4.52 (d, J = 12.1 Hz, 1H), 4.28 (d, J = 7.4 Hz, 1H), 4.11 (dd, J = 11.1, 2.0 Hz, 1H), 3.93 (t, J = 9.2, 1H), 3.80 (ddd, J = 10.1, 4.7, 1.9 Hz, 1H), 3.77-3.69 (m, 3H), 3.63-3.48 (m, 5H), 3.47-3.42 (m, 1H), 3.40 (s, 3H), 2.53 (bs, 1H); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 139.41 (s, 1C), 139.31 (s, 1C), 138.99 (s, 1C), 138.82 (s, 1C), 138.80 (s, 1C), 138.70 (s, 1C), 128.74 (d, 2C), 128.69 (d, 4C), 128.66 (d, 2C), 128.62 (d, 2C), 128.29 (d, 4C), 128.23 (d, 2C), 128.19 (d, 2C), 128.14 (d, 2C), 128.12 (d, 2C), 128.01 (d, 2C), 127.96 (d, 2C). 127.93 (d, 2C), 127.83 (d, 2C), 103.59 (d, 1C), 98.28 (d, 1C), 84.77 (d, 1C), 82.09 (d, 1C), 80.57 (d, 1C), 78.36 (d, 1C), 77.84 (d, 1C), 75.78 (t, 1C), 75.47 (d, 1C), 75.23 (t, 1C), 75.22 (t, 1C), 75.21 (t, 1C), 74.88 (d, 1C), 73.72 (t, 1C), 73.28 (t, 1C), 70.20 (d, 1C), 69.33 (t, 1C), 68.85 (t, 1C), 55.49 (q, 1C); HRMS calcd for $C_{55}H_{60}NaO_{11}^+$ $[M+Na]^+$ 919.40278, found 919.40238.

(28) ***trans*-N-(tert-butoxycarbonyl)-4-acetoxy-L-proline, 3,4,6-tri-O-benzyl-2-O-[(2-benzyloxyphenyl) acetyl]-β,D-glucosyl ester (18):** General procedure C; starting from glucosyl donor **11** (100 mg, 0.14 mmol) and acceptor **17** (57 mg, 0.21 mmol) **18** (80 mg, 62 %) was obtained.
Analytical Data for 18
^{1}H NMR (600 MHz, CD_2Cl_2) δ 7.43-7.08 (m, 22H), 6.92-6.82 (m, 2H), 5.68 (d, J = 8.2 Hz, 0.5H), 5.62 (d, J = 8.2 Hz, 0.5H), 5.22-5.16 (m, 0.5H), 5.12 (t, J = 8.8 Hz, 1H), 5.05-4.91 (m, 2.5H), 4.74 (dd, J = 11.2, 2.3 Hz, 1H), 4.62 (d, J = 11.2 Hz, 0.5H), 4.56 (d, J = 11.2 Hz, 0.5 H), 4.55-4.43 (m, 4H), 4.32 (t, J = 8.0 Hz, 0.5H), 4.26 (t, J = 8.4 Hz, 0.5 H), 3.87 (d, J = 16.0 Hz, 0.5H), 3.81 (d, J = 16.0 Hz, 0.5 H), 3.76-3.47 (m, 8H), 2.37-2.27 (m, 0.5H), 2.24-2.15 (m, 0.5H), 2.13-2.05 (m, 0.5H), 2.03 (s, 1.5H), 2.01 (s, 1.5H), 1.90-1.81 (m, 0.5H), 1.44 (s, 5H), 1.37 (s, 4H); ^{13}C NMR (150 MHz, CD_2Cl_2) δ 171.38 (s, 1C), 171.10 (s, 1C), 170.87 (s, 1C), 170.63 (s, 1C), 170.47 (s, 1C), 170.36 (s, 1C), 156.92 (s, 1C), 156.86 (s, 1C), 154.36 (s, 1C), 153.51 (s, 1C), 138.56 (s, 2C), 138.53 (s, 2C), 138.50 (s, 1C), 138.45 (s, 1C), 137.68 (s, 1C), 137.56 (s, 1C), 132.02 (d, 1C), 131.46 (d, 1C), 129.10 (d, 1C), 128.90 (d, 2C), 128.83 (d, 3C), 128.71 (d, 4C), 128.65 (d, 6C), 128.57 (d, 2C), 128.33 (d, 2C), 128.30 (d, 4C), 128.21 (d, 4C), 128.08 (d, 2C), 128.06 (d, 3C), 128.01 (d, 2C), 127.97 (d, 2C), 127.91 (d, 1C), 127.45 (d, 2C), 127.32 (d, 2C), 123.54 (s, 1C), 122.85 (s, 1C), 121.19 (d, 1C), 121.06 (d, 1C), 112.42 (d,

1C), 112.25 (d, 1C), 93.16 (d, 1C), 93.03 (d, 1C), 82.85 (d, 1C), 82.45 (d, 1C), 80.91 (s, 1C), 80.65 (s, 1C), 77.52 (d, 1C), 77.43 (d, 1C), 76.23 (d, 1C), 76.03 (d, 1C), 75.29 (t, 1C), 75.24 (t, 1C), 75.07 (t, 1C), 74.96 (t, 1C), 73.73 (t, 1C), 73.72 (t, 1C), 73.11 (d, 1C), 72.93 (d, 1C), 72.77 (d, 1C), 72.29 (d, 1C), 70.25 (t, 1C), 70.12 (t, 1C), 68.83 (t, 1C), 68.69 (t, 1C), 58.29 (d, 1C), 58.00 (d, 1C), 52.77 (t, 1C), 52.31 (t, 1C), 36.63 (t, 1C), 35.73 (t, 1C), 35.69 (t, 1C), 35.66 (t, 1C), 28.49 (q, 3C), 28.19 (q, 3C), 21.23 (q, 1C), 21.22 (q, 1C)[1]; HRMS calcd for $C_{54}H_{59}NaNO_{13}^+$ $[M+Na]^+$ 952.38786, found 952.38665.

(29) ***trans*-N-(tert-butoxycarbonyl)-4-acetoxy-L-proline, β,D-glucosyl ester (19):** Starting from compound **18** (35 mg, 0.04 mmol) and following general procedure B1 **19** was obtained as a colourless solid (11 mg, 68 %). Analytical data matched those reported in the literature.[30]

(30) Weber, J.; Svatunek, D.; Tegl, G.; Krauter, S.; Hametner, C.; Kosma, P.; Fröhlich, J.; Mikula, H. *manuscript in preparation* **2017**.

[1] There are two rotamers observable in the NMR spectra owing to the carbamate.

Manuscript #3

Manuscript draft

Weber, J.; Schwarz, M.; Schiefer, A.; Hametner, C.; Schiessl, A.; Häubl, G.; Fröhlich, J.; Mikula, H. (2-Nitrophenyl)acetyl-Protected Glucosyl Trichloroacetimidates as Novel Glucosyl donors for the Synthesis of Glycosylated Natural Products

(2-Nitrophenyl)acetyl-Protected Glucosyl Trichloroacetimidates as Novel Glucosyl Donors for the Synthesis of Glycosylated Natural Products

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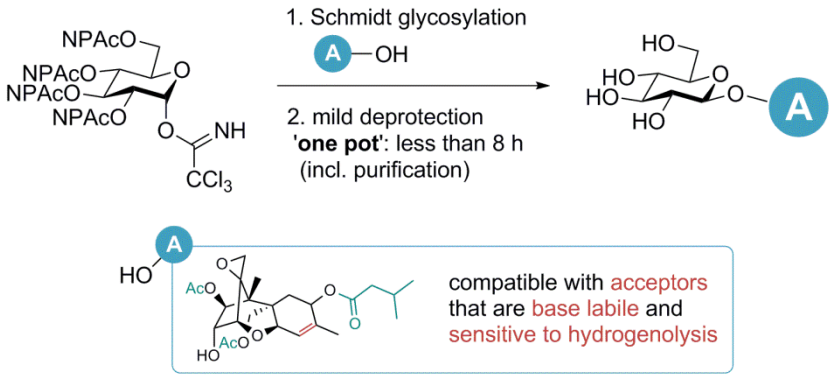

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Abstract The synthesis of (2-nitrophenyl)acetyl (NPAc)-protected glucosyl donors is described that were designed for the neighboring group assisted glycosylation of base-labile natural products that are also sensitive to hydrogenolysis. Glycosylation conditions were optimized with cyclohexyl methanol and (+)-menthol as simple acceptors and extended to a one-pot procedure for the synthesis of glycosides. Applying this procedure to T2-toxin, we were able to prepare T2-β-glucoside.

Key words carbohydrates, glycosylation, trichothecene, masked mycotoxin, glycoconjugate, (2-nitrophenyl)acetyl group, T2-toxin

Glycosides are widespread in nature, they are often found as secondary metabolites of plants or mammals formed due to xenobiotic metabolism.[1,2] Also various bioactive natural products are glycosylated in which the sugar part plays a pivotal role in the biological activity and recognition of cellular targets.[3,4] As the availability of glycosides from biological sources is usually scarce and glycobiologists are in need of significant amounts of these materials for biological, medicinal and pharmacological studies, the synthesis of carbohydrates and glycoconjugates is of high interest.[5]

Glycosylations of complex natural products still remain a challenging task as there is so far, no general procedure developed for the stereoselective synthesis of glycoconjugates.[6] The use of peracetylated glycosyl donors is the most commonly and reliable method in cases where there is a neighboring participating group effect required for the formation of ß-glucosides.[7,8] Peracetylated glucosyl donors are usually easy to prepare or even commercially available. However, if the aglycone is base-labile or has ester groups on its own, these donors fail as soon as it comes to deprotection of the acetyl groups. To circumvent this problem, several donor systems are reported that are perbenzylated and have a neighboring participating group at position 2 such as AZMB,[9] MSc,[10] picolinyl[11] and Cbz[12]. These donors work well for the diastereoselective glycosylation of base-sensitive acceptor molecules that are not sensitive towards hydrogenolysis. But as soon as there is a double bond in the molecule, these donors are usually inapplicable as the aglycone is then susceptible to be destroyed by the deprotection conditions. There are only very few glycosyl donor systems reported so far that might be applied for the glycosylation of base-labile natural products that are also sensitive to hydrogenolysis. For the synthesis of (-)-cassiode a fully PMB-protected sulfoxide glucosyl donor was used, which even presented surprisingly an enhanced stereoselectivity for β-glycosylation.[13] Even so, a fully PMB-protected thioglycosyl donor applied by Heuckendorff et al.[14] showed that the glycosylation selectivity is strongly dependent on the acceptor. A fully TBDMS-protected sulfoxide glucosyl donor could be applied for the synthesis of glycopeptides.[15] And a fully TIPS-protected thioethyl glucosyl donor was described by Okada et al.,[16] which showed good β-selectivities in glycosylation reactions with cyclohexyl methanol due to the restricted twist-boat conformation. However, by the use of more complex alcohols the stereoselective effect of this glucosyl donor decreases.[17] Hence a reliable glucosyl donor system for the diastereoselective glycosylation of complex and labile natural products is still missing. To the best of our knowledge, no donor system was reported so far that uses the classical neighboring group effect for the stereoselective glycosylation of base-labile natural products that contain at least one double bond.

Therefore, in the course of ongoing research in the field of phase II mycotoxin metabolites, we have become interested in the development of new glucosyl donors that can be used for the glucosylation of T2-toxin (**1**, Fig 1a) that bears acetyl groups and an isovaleryl group as well as a double bond in its structure. T2-toxin is a potent inhibitor of the eukaryotic protein synthesis and represents a contaminant of considerable concern to human and animal health.[18] Masked

T2-toxins, especially glycosides, can emerge after metabolization by living plants which fail to be recognized in conventional analyses.[19] As hydrolysis to the parent toxin can occur during digestion,[20,21] these masked toxins present a high risk for the underestimation of the total mycotoxin content in routine screenings. For the development of routine analyses that include masked mycotoxins[22] such as T2-glucoside (**2**) as well as for toxicity data and structure elucidation standards are urgently needed.

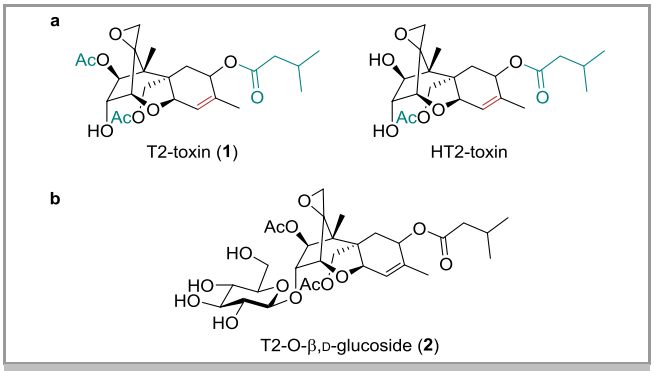


Figure 1 a) chemical structure of T2-toxin (**1**) and HT2-Toxin b) glycosylated structure of T2-toxin

Previously, glycosylation of T2-toxin was carried out under thioglycosylation conditions with the TIPS-protected thioethyl glucosyl donor developed by Okada et al.[16], leading to a glucoside mixture of β:α 5:1. The aim to improve this reaction in terms of reduction of the elaborate purification steps, versatility and stereoselective outcome of the glycosylation reaction led us to the development of a new glycosyl donor system with neighboring participating groups as protecting groups (Fig. 2). The new described (2-nitrophenyl)acetyl group for protection of hydroxy groups in 2010 by Daragics et al.[23] seemed for us the perfect choice as cleavage is possible under very mild reductive conditions and the introduction can be done with nitrophenylacetic acid in a Steglich esterification. Furthermore it was already shown that the (2-nitrophenyl)acetyl group is an effective participating neighboring group in glycosylations with thioglucosyl donors bearing a NPAc group at position 2, leading to the selective formation of 1,2 -*trans* glycosides.[23]

Herein we present the synthesis and application of a new glucosyl donor system that is suitable for the glycosylation of base-labile natural products that are not compatible with the hydrogenolytic removal of benzyl groups.

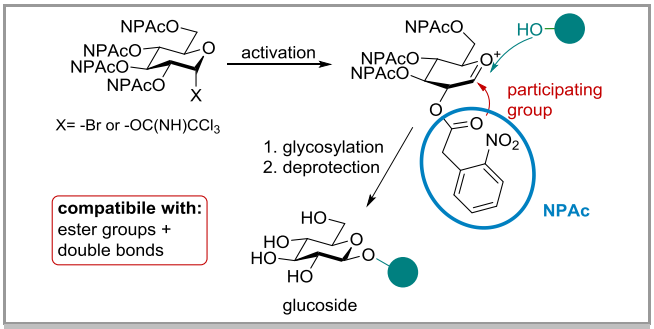
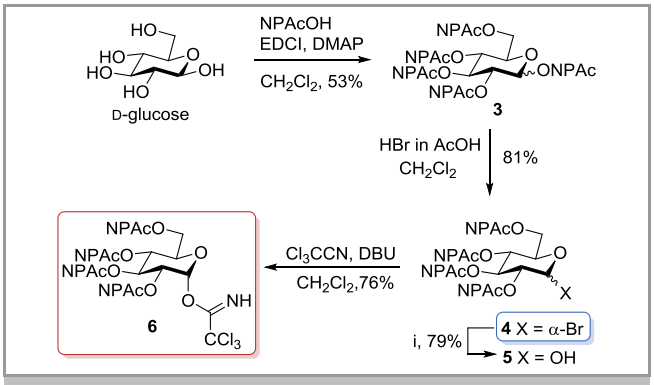


Figure 2 Principle of NPAc-protected glucosyl donors for glycosylation

Starting from D-glucose 1,2,3,4,6-NPAc-protected glucose **3** could be prepared only slightly modified after the protocol of Daragics et al.[23] in acceptable yields. According to already described procedures in literature, bromination of the anomeric center was done with HBr in acetic acid[5] to obtain glucosyl donor **4** that can be used in Königs Knorr glycosylations. Hydrolysis of the anomeric center of donor **4** with silver carbonate in acetone/water[24] yielded the OH-sugar **5** which was subsequently reacted with trichloroacetonitrile and 1,8-diazabicyclo[5,4,0]-undec-7-ene (DBU)[25] to obtain the trichloroacetimidate donor **6** for Schmidt glycosylations. Notably, first attempts to introduce the imidate group with potassium carbonate and trichloroacetonitrile[26] failed but changing the base to DBU, the reaction could be completed in 16h. The reported side reaction of Jacquinet et al. [27] that modifies NPAc groups in the presence of DBU and Cl_3CCN haven´t been observed in our case (Scheme 1).



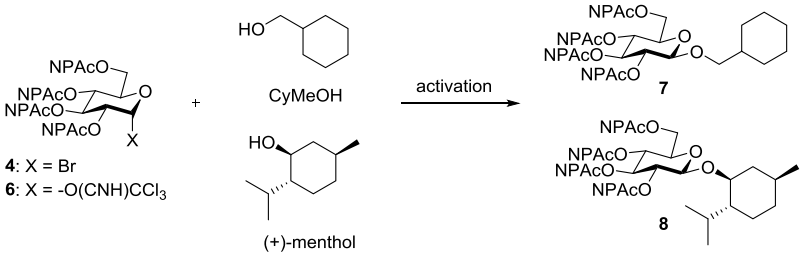
Scheme 1 Synthesis of 2,3,4,6-NPAc protected glucosyl donor **4** and **6**. [i...Ag_2CO_3, acetone/water]

Cyclohexyl methanol was used as model acceptor for primary alcohols to investigate glycosylation reactions with the synthesized glycosyl donors **4**[28] and **6**[29] under different conditions. Selected data of reaction optimization and screening is shown in Table 1. Königs Knorr glycosylations applying various silver salts, resulted in no conversion after 24h. Lewis-acid mediated glycosylations with donor **6** gave relatively good yields with TMSOTf as promotor whereas borontrifluoro diethyletherate was a bad activator leading only to very low product yields of **7**[30]. Encouraged by the result of donor **6** activated with TMSOTf, we also tested its applicability towards secondary alcohols with (+)-menthol as model substance, resulting also in good yields of product **8**[31].

Surprisingly, even though glycosyl donor **6** is equipped with a known neighboring participating group at C2, all glycosylation reactions led to a mixture of 1,2-cis and 1,2-trans glycosides, but anyway with an enhanced selectivity towards ß-glycosylation. We can only explain this by the number of NPAc-groups used for the protection of the hydroxyl groups of the glycosyl donor, which probably starts to influence the conformation of the sugar.

Deprotection of the NPAc- groups could be achieved under reductive conditions with zinc and ammonium chloride[23] by treatment with ultrasonic irradiation.

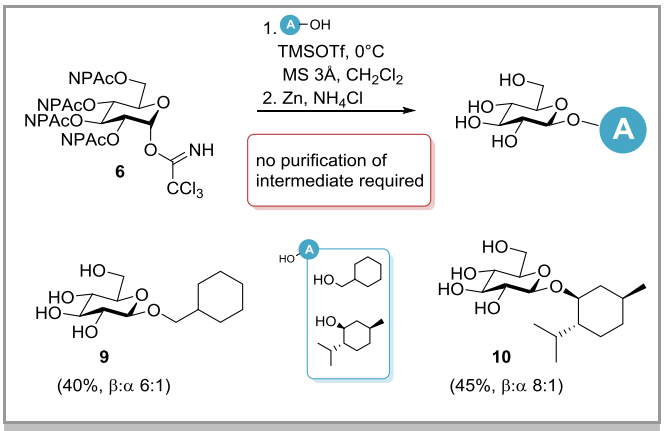
Table 1 Glycosylation of cyclohexyl methanol (CyMeOH) and (+)-menthol using glycosyl donors **4** and **6**



Entry	Donor (equiv)	Acceptor (equiv)	Activation (equiv)	Solvent	Yield (%)[a]
1	**5** (1.0)	CyMeOH (1.2)	Ag_2O (1.5)	ACN	0 (**8**)
2	**5** (1.0)	CyMeOH (1.2)	Ag_2CO_3 (1.5)	CH_2Cl_2	0 (**8**)
3	**7** (1.0)	CyMeOH (1.2)	$BF_3{\cdot}Et_2O$ (0.1)	CH_2Cl_2	5 (**8**)
4	**7** (1.2)	CyMeOH (1.0)	TMSOTf (0.1)	CH_2Cl_2	80 (**8**, β:α 6:1)
5	**7** (1.2)	(+)-menthol (1.0)	TMSOTf (0.1)	CH_2Cl_2	64 (**9**, β:α 8:1)

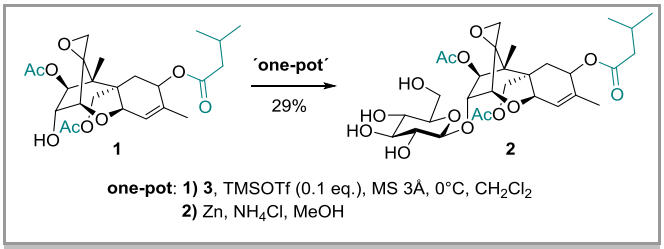
[a]Non-isolated yield determined by UV-HPLC (using standard addition)

As the protected glycosides **7** and **8** were obtained in a complex product mixture, purification of the intermediates was very laborious. To avoid this time-consuming and troublesome step, we developed a one-pot procedure[32] by first using cyclohexyl methanol and (+)-menthol as acceptors, leading to **9**[33] and **10**[34] in overall yields of 40 and 45%, respectively (Scheme 2).



Scheme 2 Optimized ´one-pot´ procedure for glycosylations with NPAc-protected glucosyl trichloroacetimidate **6**

Finally, we were able to synthesize T2-β,D-glucoside starting from T2 in an overall yield of 29%. Following the optimized Schmidt-glycosylation procedure the protected intermediate could be obtained in a crude mixture of isomers in a β:α ratio of 8:1 (determined by UV-HPLC). Subsequent deprotection under reductive conditions and purification via reversed-phase (C18) column chromatography gave exclusively T2-β,D-glucoside (**2**).[35]



Scheme 3 'One pot' synthesis of T2-β,D-glucoside (**2**)

In summary, we were able to develop a new glycosyl donor system that can be applied for the synthesis of glycosides consisting of a base labile aglycon that is also sensitive to hydrogenolysis. Reaction conditions for glycosylation could be optimized with simple model substances and finally simplified to a one-pot procedure avoiding the laborious purification step of the protected intermediates. With this optimized procedure, we could facilitate the synthesis of T2-β,D -glucoside and could enhance the selectivity of the glycosylation reaction towards the ß-isomer of the intermediate. Due to the ease and low-cost preparation of the glucosyl donor, we are convinced that this new donor system is anticipated to become a valuable tool in complex glycosylation reactions.

Acknowledgment

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(28) **General Procedure A: Königs Knorr Glycosylation**
To a solution of alcohol (0.01 mmol, 1 eq.) and glucosyl donor **4** (80 mg, 0.013 mmol, 1.2 eq.) in dry CH_2Cl_2 or MeCN (1 mL) molecular sieve (3Å, 0.1 g/ mL) was added. After stirring the reaction mixture at RT for 1h, promotor (0.017mmol, 1.5 eq.) was added and stirring was continued in the dark for 24h. A sample was taken and then measured on UV-HPLC. Standard addition was used for quantification.

(29) **General Procedure B: Schmidt Glycosylation.**
To a solution of alcohol (0.07 mmol, 1 eq.) and glucosyl donor **6** (80 mg, 0.08 mmol, 1.2 eq.) in dry CH_2Cl_2 (1 mL) molecular sieve (3Å, 0.1 g/ mL) was added, and the reaction mixture was stirred at RT for 1h. After cooling the reaction mixture to 0 °C, TMSOTf (1.6 µl, 9 µmol, 0.15 eq.) was added. At 0 °C the reaction mixture was stirred for 2 h, and quenched by the addition of Et_3N (0.15 equiv). The reaction mixture was filtered through Celite and concentrated. The crude product was purified by flash chromatography (gradient elution hexanes: EE) to yield the corresponding product.

(30) **Procedure for the Synthesis of 1-Methylcyclohexyl-2,3,4,6-tetra-*O*-(2-nitrophenyl)acetyl-β,d-glucopyranoside (7)**
General procedure A; starting from cyclohexyl methanol (12.3 mg, 0.11 mmol) and glucosyl donor **6** (127 mg, 0.13 mmol) **7** was obtained as a white solid (32 mg, 49%).
Analytical Data for 7
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21-8.04 (m, 4H), 7.68-7.42 (m, 10H), 7.41-7.29 (m, 2H), 5.33 (t, J = 9.6 Hz, 1H), 5.07 (t, J = 9.8 Hz, 1H), 4.99 (dd, J = 10.0, 7.7 Hz, 1H), 4.46 (d, J = 8.2 Hz, 1H), 4.38 (dd, J = 12.3, 1.8 Hz, 1H), 4.33-4.16 (m, 3H), 4.14-4.08 (m, 2H), 4.07-3.81 (m, 4H), 3.70 (ddd, J = 9.9, 5.5, 1.8 Hz, 1H), 3.65 (dd, J = 9.4, 5.8 Hz, 1H), 3.26 (dd, J = 9.6, 6.8 Hz, 1H), 1.80-1.60 (m, 4H), 1.31-1.15 (m, 4H), 1.00-0.78 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.81 (s, 1C), 169.73 (s, 1C), 169.34 (s, 1C), 168.77 (s, 1C), 148.84 (s, 1C), 148.79 (s, 1C), 148.65 (s, 1C), 148.52 (s, 1C), 134.49 (d, 1C), 134.21 (d, 1C), 134.12 (d, 1C), 133.99 (d, 1C), 133.85 (d, 1C), 133.80 (d, 1C), 133.71 (d, 1C), 133.68 (d, 1C), 130.16 (s, 1C), 129.82 (s, 1C), 129.71 (s, 1C), 129.65 (s, 1C), 128.85 (d, 1C), 128.80 (d, 1C), 128.75 (d, 1C), 128.64 (d, 1C), 125.41 (d, 1C), 125.31 (d, 2C), 125.26 (d, 1C), 101.05 (d, 1C), 75.76 (t, 1C), 72.66 (d, 1C), 72.13 (d, 1C), 72.00 (d, 1C), 68.95 (d, 1C), 62.88 (t, 1C), 39.76 (t, 2C), 39.62 (t, 2C), 37.86 (d, 1C), 29.88 (t, 1C), 29.70 (t, 1C), 26.69 (t, 1C), 25.96 (t, 1C), 25.91 (t, 1C); HRMS calcd for $C_{45}H_{44}N_4NaO_{18}^+$ $[M+Na]^+$ 951.2543, found 951.2536.

(31) **Procedure for the Synthesis of 1-(+)-Menthyl-2,3,4,6-tetra-*O*-(2-nitrophenyl)acetyl-β,d-glucopyranoside (8)**
General procedure A; starting from (+)-menthol (10.7 mg, 0.06 mmol) and glucosyl donor **6** (80 mg, 0.08 mmol) **8** was obtained as a slightly yellow solid (18 mg, 31 %).
Analytical Data for 8
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.18-8.05 (m, 4H), 7.65-7.40 (m, 10H), 7.35 (dd, J = 7.8, 1.6 Hz, 1H), 7.32 (dd, J = 7.4, 1.2 Hz, 1H), 5.36 (t, J = 9.6 Hz, 1H), 5.07 (t, J = 9.7 Hz, 1H), 5.03 (dd, J = 9.8, 7.8 Hz, 1H), 4.61 (d, J = 8.2 Hz, 1H), 4.44 (dd, J = 12.1, 1.9 Hz, 1H), 4.27 (d, J = 17.6 Hz, 1H), 4.22-4.03 (m, 6H), 3.92 (d, J = 17.2 Hz, 1H), 3.76 (d, J = 17.6 Hz, 1H), 3.74 (ddd, J = 10.1, 5.9, 2.0 Hz, 1H), 3.42 (td, J = 10.7, 4.3 Hz, 1H), 2.25-2.07 (m, 2H), 1.73-1.50 (m, 3H), 1.41-1.23 (m, 3H), 1.12-1.01 (m, 1H), 0.95 (d, J = 7.0 Hz, 3H), 0.88 (d, J = 6.6 Hz, 3H) 0.83 (d, J = 7.0 Hz, 3H) ; ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.89 (s, 1C), 169.73 (s, 1C), 169.42 (s, 1C), 168.74 (s, 1C), 148.87 (s, 1C), 148.83 (s, 1C), 148.68 (s, 1C), 148.57 (s, 1C), 134.49 (d, 1C), 134.22 (d, 1C), 134.05 (d, 1C), 133.92 (d, 1C), 133.86 (d, 1C), 133.68 (d, 1C), 133.67 (d, 1C), 133.61 (d, 1C), 130.15 (s, 1C), 129.80 (s, 1C), 129.70 (s, 1C), 129.59 (s, 1C), 128.94 (d, 1C), 128.74 (d, 1C), 128.69 (d, 1C), 128.66 (d, 1C), 125.51 (d, 1C), 125.31 (d, 1C), 125.26 (d, 1C), 125.19 (d, 1C), 101.82 (d, 1C), 82.31 (d, 1C), 72.88 (d, 1C), 72.50 (d, 1C), 71.82 (d, 1C), 69.13 (d, 1C), 63.13 (t, 1C), 48.27 (d, 1C), 43.07 (t, 1C), 39.66 (t, 1C), 39.65 (t, 1C), 39.56 (t, 1C), 39.47 (t, 1C), 34.23 (t, 1C), 31.77 (d, 1C), 25.08 (d, 1C), 22.86 (t, 1C), 22.40 (q, 1C), 21.48 (q, 1C), 16.23 (q, 1C); HRMS calcd for $C_{48}H_{50}N_4NaO_{18}^+$ $[M+Na]^+$ 993.3012, found 993.3012.

(32) **General Procedure C: One-pot Synthesis**
To a solution of alcohol (1 equiv) and glycosyldonor **6** (1.5 equiv) in dry CH_2Cl_2 (25 mL/mmol) molecular sieve (3 Å, 0.1 g/ mL) was added, and the reaction mixture was stirred at rt for 1 h. After cooling the reaction mixture to 0 °C, TMSOTf (0.15 equiv) was added and stirring of the reaction mixture was continued at 0 °C. After 2 h the reaction was quenched by the addition of Et_3N (0.15 equiv), filtered through Celite and concentrated. The crude product mixture was dissolved in MeOH (17 mL/mmol) and zinc dust (20 equiv.), followed by ammonium chloride (12 equ.) was added at rt. The reaction was treated with ultrasonic irradiation for 2-4h at 25 °C, then filtered over celite and concentrated under vacuum. Preparative HPLC (RP-C18, MeCN in H_2O, gradient elution) afforded the desired glycoside.

(33) **Procedure for the Synthesis of Methylcyclohexyl-β,d-glucopyranoside (9)**
General procedure C; starting from cyclohexyl methanol (14.6

mg, 0.13 mmol) and glucosyl donor **6** (150 mg, 0.15 mmol) **9** was obtained as a colourless solid (14 mg, 40%). Judged by NMR, a 6:1 (β:α) mixture of anomers was obtained.

Analytical Data for 9

^{1}H NMR (400 MHz, CD_3OD) δ 4.24 (d, J = 7.4 Hz, 1H), 3.87 (dd, J = 12.2, 1.9 Hz, 1H), 3.73 (dd, J = 9.4, 6.6 Hz, 1H), 3.70-3.65 (m, 1H), 3.39-3.23 (m, 4H), 3.18 (dd, J = 8.8, 7.9 Hz, 1H), 1.91-1.78 (m, 2H), 1.77-1.58 (m, 4H), 1.38-1.15 (m, 3H), 1.07-0.90 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 104.59 (d, 1C), 78.14 (d, 1C), 77.89 (d, 1C), 76.53 (t, 1C), 75.17 (d, 1C), 71.68 (d, 1C), 62.77 (t, 1C), 39.35 (d, 1C), 31.06 (t, 1C), 31.03 (t, 1C), 27.73 (t, 1C), 26.99 (t, 1C), 26.97 (t, 1C); HRMS calcd for $C_{14}H_{25}O_8^-$ [M+COOH]$^-$ 321.1555, found 321.1558.

(34) **Procedure for the Synthesis of (+)-Menthyl-β,d-glucopyranoside (10)**

General procedure C; starting from (+)-menthol (10.7 mg, 0.07 mmol) and glucosyl donor **6** (80 mg, 0.08 mmol) **10** was obtained as a white solid (10 mg, 45%). Judged by NMR, a 8:1 (β:α) mixture of anomers was obtained.

Analytical Data for 10

^{1}H NMR (400 MHz, CD_3OD) δ 4.32 (d, J = 7.8 Hz, 1H), 3.85 (dd, J = 11.9, 2.2 Hz, 1H), 3.67 (dd, J = 11.7, 5.1 Hz, 1H), 3.43 (td, J = 10.7, 4.3 Hz, 1H), 3.39-3.32 (m, 1H), 3.30-3.21 (m, 2H), 3.16 (t, J = 8.2 Hz, 1H), 2.45 (m, J = 13.9, 6.9, 2.4 Hz, 1H), 2.33-2.22 (m, 1H), 1.73-1.60 (m, 2H), 1.48-1.32 (m, 1H), 1.31-1.22 (m, 1H), 1.09-0.93 (m, 2H), 0.91 (d, J = 6.0 Hz, 3H), 0.90 (d, J = 7.6 Hz, 3H), 0.88-0.83 (m, 1H), 0.80 (d, J = 7.0 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 105.69 (d, 1C), 82.44 (d, 1C), 78.19 (d, 1C), 77.74 (d, 1C), 75.59 (d, 1C), 71.68 (d, 1C), 62.81 (t, 1C), 50.23 (d, 1C), 44.74 (t, 1C), 35.60 (t, 1C), 32.94 (d, 1C), 25.71 (d, 1C), 24.00 (t, 1C), 22.72 (q, 1C), 21.63 (q, 1C), 16.31 (q, 1C); HRMS calcd for $C_{16}H_{29}O_6^+$ [M-H]$^-$ 317.1970, found 317.1971.

(35) **Synthesis of T2-β,D-glucoside (2).**

General procedure C; starting from T2-toxin (30 mg, 0.06 mmol) and glucosyl donor **6** (94 mg, 0.096 mmol) T2-β,D -glucoside **(2)** was obtained as a white solid (11 mg, 29%).

Analytical Data for 2

^{1}H NMR (600 MHz, CD_3OH) δ 5.98 (d, J = 3.2 Hz, 1H), 5.78 (dt, J = 6.0; 1.0 Hz, 1H), 5.33 (d, J = 5.6 Hz, 1H), 4.48 (dd, J = 5.0, 3.0 Hz, 1H), 4.45 (d, J = 7.9 Hz, 1H), 4.39 (d, J = 6.7 Hz, 1H), 4.38 (d, J = 12.9 Hz, 1H), 4.09 (d, J = 12.7, 1H), 3.84 (dd, J = 12.0, 2.0 Hz, 1H), 3.72 (d, J = 5.0 Hz, 1H), 3.65 (dd, J = 12.1, 5.7 Hz, 1H), 3.35 (t, J = 9.0 Hz, 1H), 3.29 (t, J = 8.8 Hz, 1H), 3.25 (dd, J = 8.9, 7.5 Hz, 1H), 3.21 (ddd, J = 9.5, 5.7, 2.5 Hz, 1H), 3.04 (d, J = 3.8 Hz, 1H), 2.87 (d, J = 3.8 Hz, 1H), 2.38 (dd, J = 15.2, 5.9 Hz, 1H), 2.16 (dd, J = 7.0, 2.3 Hz, 2H), 2.09 (s, 3H), 2.07 (s, 3H), 2.06 - 2.03 (m, 1H), 1.94 (d, J = 15.3 Hz, 1H), 1.75 (s, 3H), 0.97 (d, J = 4.5 Hz, 3H), 0.96 (d, J = 4.4 Hz, 3H), 0.74 (s, 3H); ^{13}C NMR (150 MHz, CD_3OH) δ 173.97 (1C), 172.27 (1C), 172.19 (1C), 137.37 (1C), 125.07 (1C), 103.78 (1C), 83.84 (1C), 81.17 (1C), 80.48 (1C), 78.34 (1C), 78.06 (1C), 74.79 (1C), 71.43 (1C), 69.32 (1C), 68.49 (1C), 65.38 (1C), 65.26 (1C), 62.59 (1C), 50.07 (1C), 47.86 (1C), 44.50 (1C), 44.32 (1C), 28.76 (1C), 26.94 (1C), 22.77 (1C), 22.71 (1C), 21.21 (1C), 20.80 (1C), 20.44 (1C), 7.04 (1C); HRMS calcd for $C_{31}H_{45}O_{16}^-$ [M+COOH]$^-$ 673.2713, found 673.2716.

3.2 Synthesis of Trichothecene Metabolites

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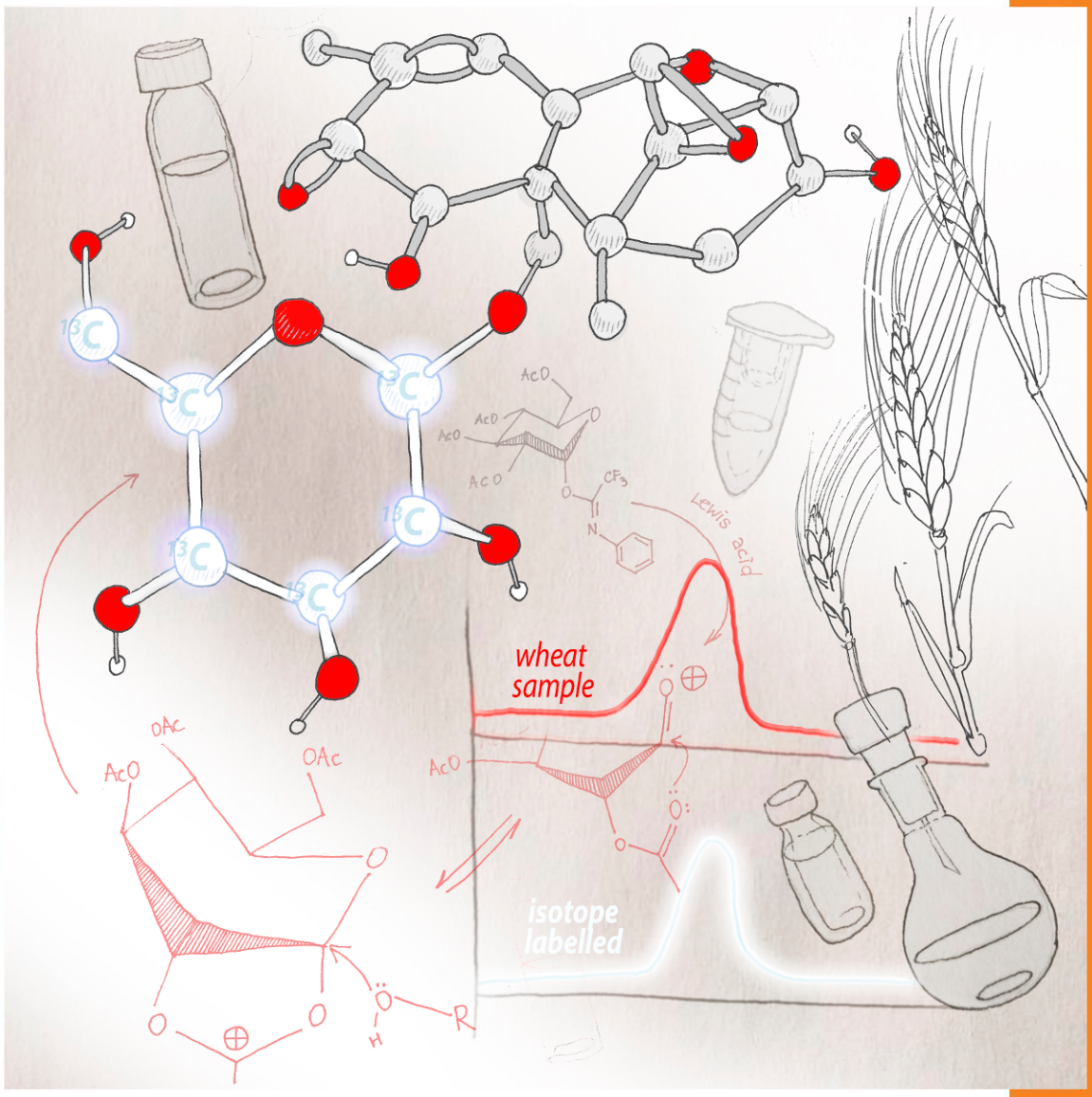

Front Cover

Weber et al.
Synthesis of Isotope Labeled Deoxynivalenol-15-*O*-Glycosides

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Full Paper

Glycosylation

Synthesis of Isotope-Labeled Deoxynivalenol-15-*O*-Glycosides

Julia Weber,[a] Philipp Fruhmann,[a,b] Christian Hametner,[a] Alois Schiessl,[c] Georg Häubl,[c] Johannes Fröhlich,[a] and Hannes Mikula*[a]

Abstract: A versatile and efficient protocol for the Schmidt glycosylation of 3-acetyldeoxynivalenol (3-ADON) and subsequent deprotection has been developed to gain access to deoxynivalenol-15-*O*-glycosides in reasonable amounts for biological analysis and further investigations. By using this protocol with [$^{13}C_6$]glycosyl donors, we were able to prepare isotope-labeled deoxynivalenol-15-*O*-glycosides, which are pivotal to enabling accurate quantification of masked mycotoxins by LC–MS.

Introduction

Deoxynivalenol (DON, **1**; Figure 1a) is a trichothecene mycotoxin produced by various *Fusarium* species, including *Fusarium graminearum* and *Fusarium culmorum*. These species mainly colonize wheat, barley, corn, and oats in temperate regions of Europe.[1] Infection of the crop by *Fusarium* fungi is favored during a prolonged cool, moist growing and harvest season.[2] DON is the most commonly detected trichothecene with the highest concentration in cereal-based foods. It is a potent protein-synthesis inhibitor, and has an acute toxicity, resulting in vomiting, nausea, abdominal pain, and diarrhea. Chronic exposure to low doses of DON can cause anorexia, growth retardation, immune dysregulation, and impaired reproduction and development.[3,4] To decrease the exposure to DON for humans and animals, the European Union has set maximum levels and guidance values, and started monitoring programs. In 2002, the Scientific Committee for Food (SCF) defined a provisional tolerable daily intake for DON and its acetylated derivatives of 1 μg/kg body weight.[2]

As *Fusarium* mycotoxins are plant pathogens, they are prone to metabolization by plants.[5] Infected plants are capable of biochemically modifying DON through xenobiotic metabolism. In phase I of this detoxification process, xenobiotics are oxidized or hydrolyzed; in phase II, conjugates are formed by glycosylation, sulfation, addition of glutathione, etc. The most significant conjugate of DON is DON-3-*O*-β-D-glucoside (Figure 1b). Until recently, this metabolite was the only DON conjugate that had been shown to occur in naturally infected cereals (e.g., wheat).[5] Schmeitzl et al. recently reported on the formation of

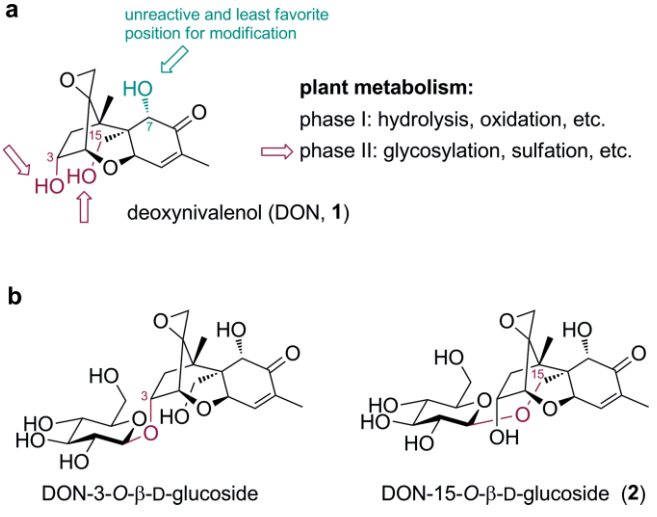

Figure 1. (a) Structure of deoxynivalenol (DON, **1**), and reaction sites for metabolic modification; (b) naturally occurring DON glucosides.

DON-15-*O*-β-D-glucoside (**2**; Figure 1b) in wheat.[6] These altered forms, often called masked mycotoxins, escape routine detection, but can release the parent toxin during food processing or digestion.[7] Therefore, the presence of such metabolites can cause an underestimation of the potential toxicity of a particular sample, and thus represent a risk for food and feed safety.[8]

Awareness of these DON metabolites is increasing, but reference compounds for structure elucidation, analytics, and toxicity testing are still scarce.[8,9] For example, **2** could only be tentatively identified by Schmeitzl et al. by mass spectrometric analysis, and so a method for accurate quantification could not be achieved.[6] For the development of improved and accurate analytical methods and toxicological risk assessment, sufficient quantities of these compounds are required in high purity. Furthermore, isotope-labeled conjugates are crucial for accurate quantification of mycotoxin metabolites by LC–MS.[6,10]

Previously described glycosylation reactions of DON were carried out under Königs–Knorr conditions ($CdCO_3$ in toluene

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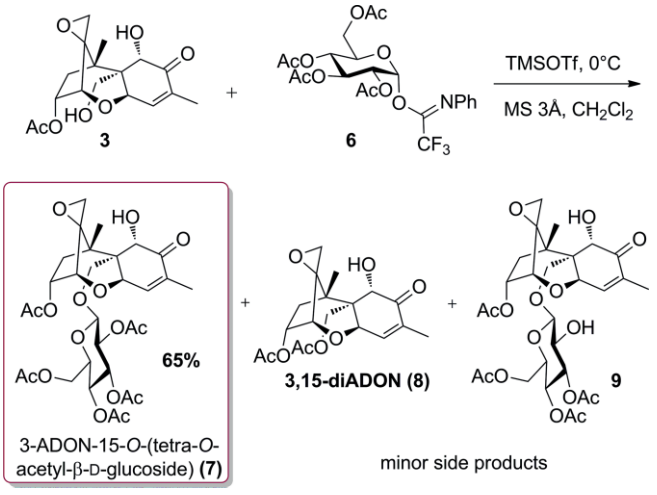
under reflux) in 38 % yield.[11] Recent advances have been made in the preparation of DON-3-*O*-β-D-glucoside by an enzymatic approach. Michlmayr et al. described a DON-conjugating UDP-glucosyltransferase from rice that produces DON-3-*O*-β-D-glucoside in 69 % under optimized conditions.[12] The synthesis of ^{13}C-labeled DON-3-*O*-β-D-[$^{13}C_6$]glucoside was accomplished by Königs–Knorr glycosylation (Ag_2CO_3 in dichloromethane) by Habler et al. very recently. However, the authors reported several drawbacks of their method: (i) an excess of isotope-labeled glucosyl donor; (ii) a very long reaction time (5 d); and (iii) failed scale-up of the reaction.[10]

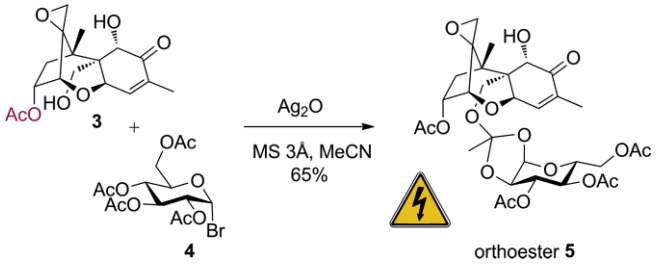
In this paper, we present the development of an efficient general procedure for the preparation of DON-15-*O*-glycosides, and the synthesis of isotope-labeled analogs using a $^{13}C_6$-labeled *N*-phenyltrifluoroacetimidate glucosyl donor.



Scheme 2. Schmidt glucosylation of 3-ADON.

Results and Discussion

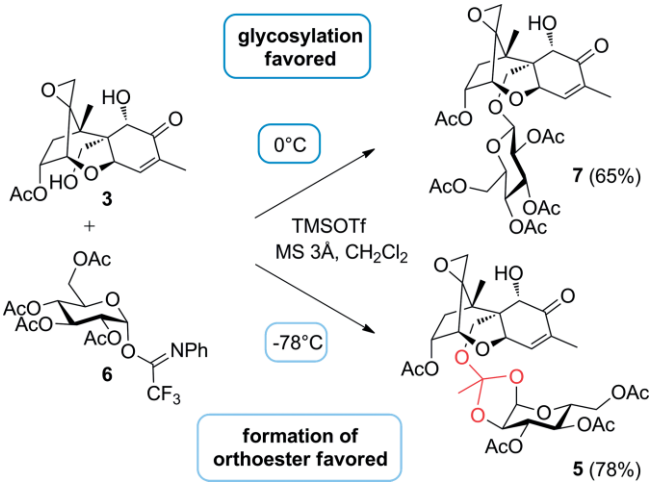
For glycosylation at the 15-position, 3-acetyldeoxynivalenol (3-ADON, **3**)[13,14] was used as the starting material; the second free OH group at the 7-position is known to be biochemically and chemically inert.[5,15,16] Bearing in mind the previously reported procedures for the glycosylation of DON, we tested Königs–Knorr conditions [acetobromo-α-D-glucose (**4**), Ag_2O, MeCN] to synthesize DON-15-*O*-β-D-glucoside, but we observed exclusive formation of the undesired orthoester **5** (Scheme 1).



Scheme 1. Königs–Knorr glucosylation of 3-ADON leading to undesired orthoester formation.

In a second approach, we used Schmidt glucosylation using *N*-phenyltrifluoroacetimidate donor **6**[17] at 0 °C, which successfully yielded acetyl-protected 3-ADON glucoside **7** in good yield (67 %) with only two minor side products **8** and **9** (Scheme 2).

To avoid the undesired formation of 3,15-diacetyl-DON (3,15-diADON, **8**), the reaction temperature was lowered to –78 °C. This fully prevented acyl transfer, but none of the desired glucoside was formed. Instead, orthoester **5** was isolated in excellent yield (Scheme 3). As rarely described in the literature,[18] temperature-dependent orthoester vs. glucoside formation was observed. This observation confirms the results of DFT calculations carried out by Berces et al., which showed that acyl transfer is a related but separate reaction from orthoester formation. To prevent acyl transfer without leading to orthoester formation, the steric bulk of the acyl residue at O-2 needs to be increased.[19] Thus, to improve the outcome of the glycosylation reaction, a more complex glycosyl donor would have to be synthesized. Because the aim of this study was to develop a simple procedure starting from commercially available peracetylated glucose, we did not consider any further modification of the glycosyl donor. In principle, orthoesters can often be converted into the glucosides under acidic conditions, but in such reactions acyl transfer can again emerge as an undesired side reaction.[20,21]



Scheme 3. Temperature-dependent formation of orthoester vs. glycosylation.

The removal of acyl groups is usually carried out by saponification (basic hydrolysis) or base-catalyzed transesterification (Zemplén conditions).[22–25] The use of sodium methoxide in methanol for the deacetylation of **7** led to the formation of a mixture of the desired DON-15-*O*-β-D-glucoside (**2**) and iso-DON-15-*O*-β-D-glucoside (**10**) in a ratio of 1:0.6, which was confirmed by NMR spectroscopy.[26] Preparative separation of the two isomers by column chromatography was not possible in our hands, but the isomers can be analytically distinguished based on their different UV-absorption maxima (**2**: 228 nm, **10**: 280 nm; Figure 2).

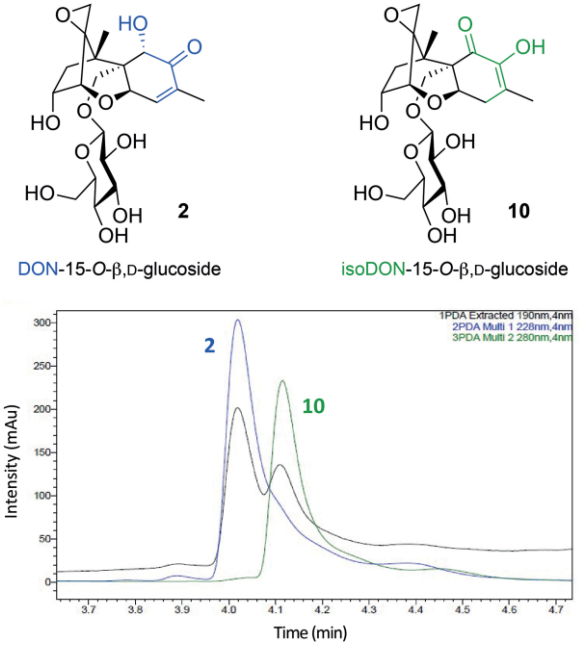
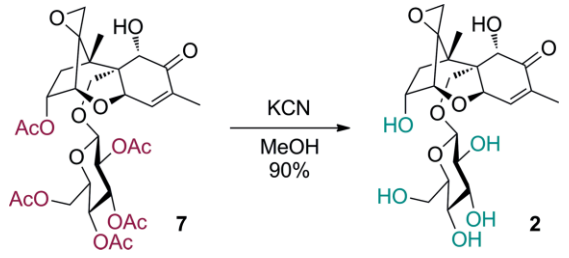


Figure 2. HPLC analysis of an inseparable mixture of DON-15-*O*-β-D-glucoside (**2**) and isoDON-15-*O*-β-D-glucoside (**10**).

We tested several base-catalyzed and saponification methods such as K_2CO_3 in MeOH,[27] KOH in THF/H_2O,[23] and $Mg(OMe)_2$ in MeOH,[28] but these all led to the formation of inseparable mixtures of **2** and **10**. Finally, the selective deprotection of **7** was successfully carried out by a very mild procedure using potassium cyanide in MeOH[29] to give DON-15-*O*-β-D-glucoside (**2**) in excellent yield (Scheme 4).

Having developed an optimized procedure, we applied it to the synthesis of other DON-15-*O*-glycosides. Gentiobiosyl donor **11**[30] was treated with **3**, and then deprotection of the intermediate **12** gave DON-15-β-gentiobioside (**13**; Scheme 5). Even though a lower yield of 32 % was achieved in the glycosylation step (compared to 65 % for the synthesis of **7**) due to a more complex product mixture, and thus more difficult separation, the final product was obtained in an overall yield of 30 %.

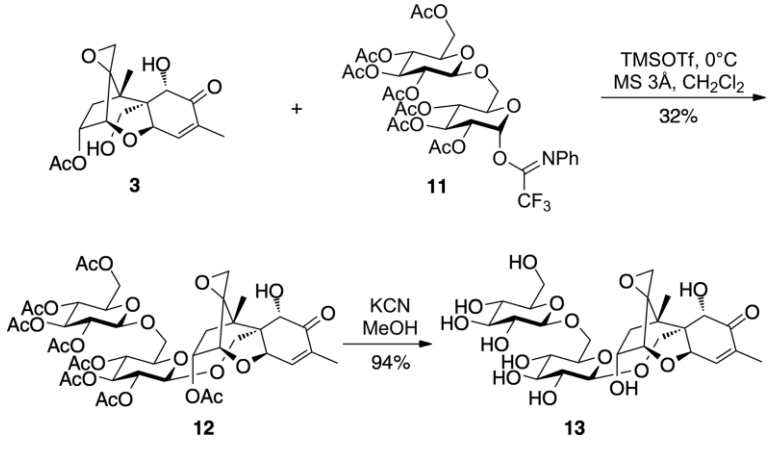


Scheme 4. Deprotection of **7** to give DON-15-*O*-β-D-glucoside (**2**).

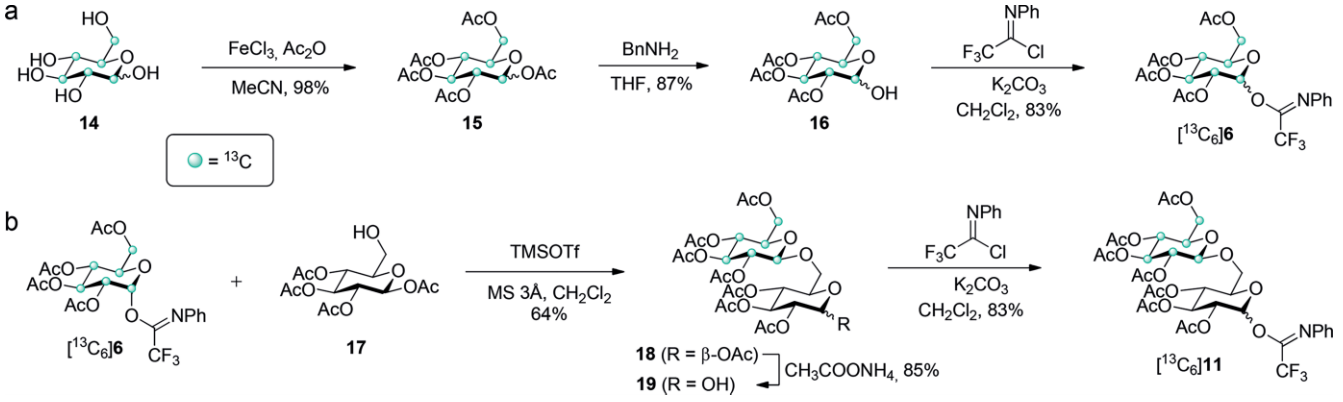
As the availability of isotope-labeled compounds for use as internal standards is essential for the development of robust and accurate analytical methods, we prepared ^{13}C-labeled glycosyl donors to enable the preparation of isotope-labeled DON-15-*O*-glycosides. Compared to using [$^{13}C_{15}$]DON,[31] the use of [$^{13}C_6$]glycosyl donors makes the synthesis of isotope-labeled DON glycosides significantly easier and cheaper. The resulting partially isotope-labeled DON-15-*O*-[$^{13}C_6$]glycoside can be used as a standard for reliable quantification in LC–MS analysis.

The $^{13}C_6$-labeled glucosyl donor [$^{13}C_6$]**6** was synthesized starting from [$^{13}C_6$]glucose (**14**). Acetylation with Ac_2O and a catalytic amount of $FeCl_3$ under ultrasonic irradiation gave **15**.[32] Anomeric deprotection with benzylamine[33] yielded OH-sugar **16**, which was subsequently treated with *N*-phenyltrifluoroacetimidoyl chloride[34] to give [$^{13}C_6$]glucosyl donor [$^{13}C_6$]**6** in high yield (Scheme 6a). [$^{13}C_6$]Gentiobiosyl donor [$^{13}C_6$]**11** was synthesized by preparing [$^{13}C_6$]gentiobiose octaacetate (**18**) by Schmidt glycosylation of 1,2,3,4-tetra-*O*-acetyl-β-D-glucose (**17**) with [$^{13}C_6$]glucosyl donor [$^{13}C_6$]**6**. Anomeric deprotection of **17** with ammonium acetate[35] gave OH-sugar **19**, and then introduction of an *N*-phenyltrifluoroacetimidate leaving group gave $^{13}C_6$-labeled gentiobiosyl donor [$^{13}C_6$]**11** in good overall yield (Scheme 6b).

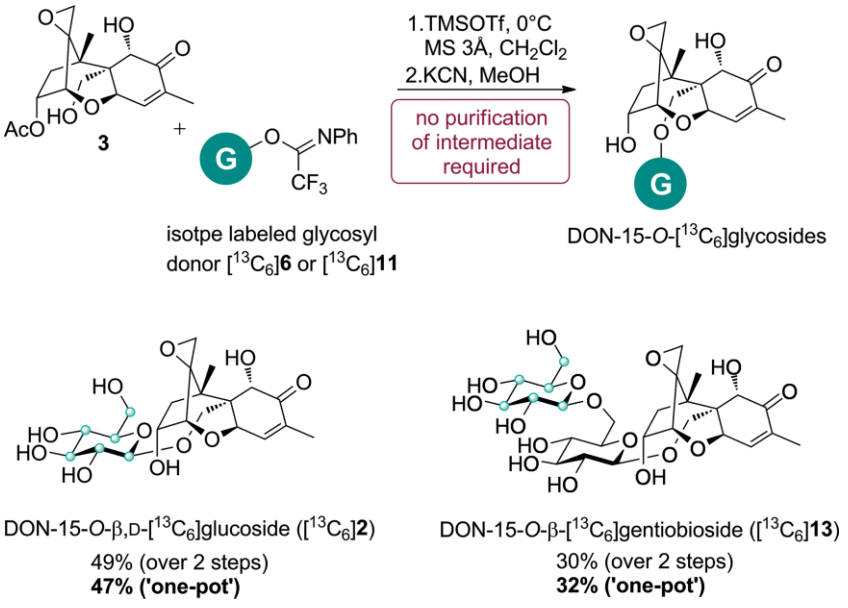
Following our developed procedure, Schmidt glycosylation of **3** using $^{13}C_6$-labeled glycosyl donors [$^{13}C_6$]**6** and [$^{13}C_6$]**11** and subsequent deprotection gave DON-15-*O*-β-D-[$^{13}C_6$]glucoside ([$^{13}C_6$]**2**) and DON-15-*O*-β-[$^{13}C_6$]gentiobioside ([$^{13}C_6$]**13**), respectively. Furthermore, to avoid laborious purification of the inter-



Scheme 5. Synthesis of DON-15-*O*-β-gentiobioside (**13**).



Scheme 6. Synthesis of isotope-labeled glycosyl donors [$^{13}C_6$]**6** and [$^{13}C_6$]**11** for Schmidt glycosylation starting from [$^{13}C_6$]glucose (**14**).



Scheme 7. Synthesis of isotope-labeled DON-15-*O*-[$^{13}C_6$]glycosides.

mediate acetyl-protected 3-ADON glycosides, a "one-pot" procedure was developed. Using this fast and efficient procedure, we were able to obtain both products ([$^{13}C_6$]**2** and [$^{13}C_6$]**13**) in sufficient quantities (up to 40 mg per batch; only limited by the amount of **3**) in less than 8 h (including final purification by preparative HPLC; Scheme 7).

Conclusions

We have developed an efficient method for the synthesis of DON-15-*O*-glycosides by Schmidt glycosylation of 3-ADON (**3**) followed by deprotection under mild reaction conditions, avoiding the formation of undesired by-products. This procedure was successfully used for the preparation of isotope-labeled [$^{13}C_6$]glycosides. The method was further optimized and simplified by the development of a "one-pot" protocol, avoiding the troublesome and time-consuming purification of the intermediate protected glycosides. We are convinced that this method will find applications in the synthesis of further (isotope-labeled) DON-15-*O*-glycosides in reasonable amounts for biological analysis and ongoing investigations in the field of masked and conjugated mycotoxins. Starting from 15-*O*-acetyl-DON instead of 3-ADON (**3**), this method might also be useful for the synthesis of DON-3-*O*-glycosides. The glycosylation step might require further optimization (bearing in mind the lower reactivity of the secondary 3-OH compared to the primary 15-OH) but the optimized reaction conditions for deprotection, avoiding the formation of isoDON derivatives, could be used for the synthesis of any DON conjugate.

Experimental Section

General Remarks: All reactions were carried out under an argon atmosphere. 3-Acetyldeoxynivalenol (3-ADON; **3**) was obtained from Romer Labs (Tulln, Austria). All other chemicals were purchased from ABCR (Germany), Sigma–Aldrich (Germany), or Carbosynth (UK). Anhydrous solvents (dichloromethane, tetrahydrofuran, methanol, and diethyl ether) were dried using a PureSolv system (inert technology, Amesbury, MA, USA). Molecular sieves (3 Å) were activated under vacuum at 200 °C before use. The progress of reactions was monitored by thin-layer chromatography (TLC) on silica

gel 60 F_{254}; plates were visualized either by using UV light or by heat staining using cerium(IV) ammonium molybdate in ethanol/sulfuric acid. LC–ESI-MS/MS was carried out with an HCT ion-trap mass spectrometer (Bruker, Germany) in full-scan mode. Chromatographic separation was carried out with a 1200 series HPLC system (Agilent Technologies, Germany) using a Luna RP-C18 column (3.0 × 150 mm, 3 µm particle size, Phenomenex, Germany). Preparative column chromatography was carried out on silica gel 60 (Merck, 40–63 µm) using a Sepacore™ Flash System or a Grace Reveleris Prep Purification System (Büchi, Switzerland). Preparative HPLC separation was carried out with a Grace Reveleris Prep system (Büchi, Switzerland) using a Luna Prep C18(2) column (10 × 250 mm, 10 µm, Phenomenex, Germany). NMR spectra were recorded with an Avance IIIHD 600 MHz spectrometer equipped with a Prodigy BBO cryoprobe (Bruker, Germany), or with an Avance DRX-400 MHz spectrometer (Bruker, Germany) at 20 °C. Data were recorded and evaluated using TOPSPIN 3.5 (Bruker Biospin, Germany). Chemical shifts are given in ppm relative to tetramethylsilane; spectra were calibrated using residual solvent signals.

General Procedure A: Preparation of *O*-Glycosyl *N*-Phenyltrifluoroacetimidates: The 1-hydroxy sugar (1 equiv.) was dissolved in CH_2Cl_2 (9 mL/mmol), and K_2CO_3 (2 equiv.) was added, followed by *N*-phenyl-2,2,2-trifluoroacetimidoyl chloride (2 equiv.). The reaction mixture was stirred at room temperature for 24 h, then it was filtered and concentrated. The residue was purified by flash chromatography (gradient elution, hexanes/EtOAc) to give the corresponding trifluoroacetimidate.

General Procedure B: Glycosylation of 3-ADON (3): 3-ADON (**3**; 1 equiv.) and glycosyl donor (1.5 equiv.) were dissolved in dry CH_2Cl_2 (8 mL/mmol), and molecular sieves (3 Å; 0.1 g/mL) were added. The reaction mixture was stirred at room temp. for 30 min. The reaction mixture was then cooled to 0 °C, and TMSOTf (0.1 equiv.) was added. The reaction mixture was stirred at 0 °C for 2 h, and then it was quenched by the addition of Et_3N (0.15 equiv.). The reaction mixture was filtered through Celite and concentrated. The crude product was purified by flash chromatography (MeOH in CH_2Cl_2, gradient elution) and preparative HPLC (RP-C18, MeCN in H_2O, gradient elution).

General Procedure C: Deprotection of Acetyl-Protected 3-ADON-15-*O*-Glycosides: The acetyl-protected glycoside (1 equiv.) was dissolved in MeOH (14 mL/µmol), and KCN (0.5 equiv.) was added at 0 °C. The reaction mixture was slowly warmed to room temperature and stirred for 2–4 h. Water (1 mL) was then added, and the MeOH was evaporated under reduced pressure. The residue was purified by preparative HPLC (RP-C18, MeCN in H_2O, gradient elution) to give the desired glycoside.

General Procedure D: "One-Pot" Procedure: 3-ADON (**3**; 1 equiv.) and glycosyl donor (1.5 equiv.) were dissolved in dry CH_2Cl_2 (8 mL/mmol), and molecular sieves (3 Å; 0.1 g/mL) were added. The reaction mixture was stirred at room temp. for 30 min. The reaction mixture was then cooled to 0 °C, and TMSOTf (0.1 equiv.) was added. The reaction mixture was stirred at 0 °C for 2 h, and then it was quenched by the addition of Et_3N (0.15 equiv.). The reaction mixture was filtered through Celite and concentrated.

The crude product mixture was dissolved in MeOH (14 mL/µmol), and KCN (0.5 equiv.) was added at 0 °C. The reaction mixture was slowly warmed to room temperature and stirred for 2–4 h. Water (1 mL) was added, and the MeOH was evaporated. The residue was purified by HPLC (RP-C18, MeCN in H_2O, gradient elution) to give the desired glycoside.

3-ADON-15-*O*-(tetra-*O*-acetyl-β-D-glucoside) (7): General procedure B; starting from 3-ADON (**3**; 40 mg, 0.12 mmol) and donor **6**[17] (92 mg, 0.18 mmol), **7** was obtained (54 mg, 68 %) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.54 (dq, *J* = 5.8, 1.5 Hz, 1 H), 5.20 (dt, *J* = 11.3, 4.5 Hz, 1 H), 5.12 (t, *J* = 9.4 Hz, 1 H), 5.04 (t, *J* = 9.6 Hz, 1 H), 4.84 (dd, *J* = 9.4, 7.8 Hz, 1 H), 4.78 (d, *J* = 2.0 Hz, 1 H), 4.72 (d, *J* = 5.4 Hz, 1 H), 4.29 (d, *J* = 8.2 Hz, 1 H), 4.27 (dd, *J* = 12.4, 5.0 Hz, 1 H), 4.11 (dd, *J* = 12.3, 2.5 Hz, 1 H), 4.03 (d, *J* = 10.2 Hz, 1 H), 3.88 (d, *J* = 4.3 Hz, 1 H), 3.67 (d, *J* = 1.9 Hz, 1 H), 3.66–3.60 (m, 1 H), 3.48 (d, *J* = 10.1 Hz, 1 H), 3.15 (d, *J* = 4.5 Hz, 1 H), 3.10 (d, *J* = 4.3 Hz, 1 H), 2.42 (d, *J* = 15.2, 4.3 Hz, 1 H), 2.17 (s, 3 H), 2.11 (dd, *J* = 15.0, 11.2 Hz, 1 H), 2.09 (s, 3 H), 2.08 (s, 3 H), 2.01 (s, 3 H), 1.98 (s, 3 H), 1.85 (s, 3 H), 1.09 (s, 3 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 199.5 (s, 1 C), 170.7 (s, 1 C), 170.6 (s, 1 C), 170.3 (s, 1 C), 169.5 (s, 1 C), 169.4 (s, 1 C), 138.7 (d, 1 C), 135.8 (s, 1 C), 100.6 (d, 1 C), 79.2 (d, 1 C), 73.9 (d, 1 C), 72.9 (d, 1 C), 72.1 (d, 1 C), 71.2 (d, 1 C), 71.1 (d, 1 C), 69.7 (t, 1 C), 68.5 (d, 1 C), 68.1 (d, 1 C), 65.1 (s, 1 C), 62.0 (t, 1 C), 51.5 (s, 1 C), 47.6 (t, 1 C), 45.9 (s, 1 C), 40.6 (t, 1 C), 21.1 (q, 1 C), 20.9 (q, 1 C), 20.8 (q, 1 C), 20.7 (q, 2 C), 15.3 (q, 1 C), 13.8 (q, 1 C) ppm. HRMS: calcd. for $C_{31}H_{40}O_{16}^+$ [M + Na]$^+$ 691.2209; found 691.2206.

DON-15-*O*-β-D-glucoside (2): General procedure C; starting from **7** (27.8 mg, 42 µmol), **2** was obtained (18.7 mg, 98 %) as a white solid. ^{1}H NMR (600 MHz, [D_4]methanol): δ = 6.50 (dq, *J* = 5.9, 1.5 Hz, 1 H), 4.98 (d, *J* = 6.2 Hz, 1 H), 4.85 (s, 1 H), 4.37 (dt, *J* = 11.2, 4.4 Hz, 1 H), 4.18 (d, *J* = 10.5 Hz, 1 H), 4.03 (d, *J* = 7.9 Hz, 1 H), 3.84 (dd, *J* = 11.9, 1.9 Hz, 1 H), 3.63 (dd, *J* = 11.7, 5.6 Hz, 1 H), 3.57 (d, *J* = 10.6 Hz, 1 H), 3.53 (d, *J* = 4.7 Hz, 1 H), 3.30–3.27 (m, 1 H), 3.24–3.18 (m, 2 H), 3.10 (d, *J* = 4.4 Hz, 1 H), 3.07 (d, *J* = 4.4 Hz, 1 H), 3.04 (dd, *J* = 9.2, 7.8 Hz, 1 H), 2.44 (dd, *J* = 14.8, 4.3 Hz, 1 H), 1.96 (dd, *J* = 14.7, 11.2 Hz, 1 H), 1.83 (s, 3 H), 1.11 (s, 3 H) ppm. ^{13}C NMR (150 MHz, [D_4]methanol): δ = 202.5 (s, 1 C), 140.3 (d, 1 C), 136.9 (s, 1 C), 104.8 (d, 1 C), 82.2 (d, 1 C), 78.0 (d, 1 C), 77.8 (d, 1 C), 75.7 (d, 1 C), 75.2 (d, 1 C), 71.5 (d, 2 C), 69.8 (t, 1 C), 69.7 (d, 1 C), 66.7 (s, 1 C), 62.8 (t, 1 C), 53.3 (s, 1 C), 48.1 (t, 1 C), 47.3 (s, 1 C), 44.5 (t, 1 C), 15.3 (q, 1 C), 14.8 (q, 1 C) ppm. HRMS: calcd. for $C_{21}H_{30}O_{11}^+$ [M + Na]$^+$ 481.1681; found 481.1685.

3-ADON-15-(hepta-*O*-acetyl-β-gentiobioside) (12): General procedure B; starting from 3-ADON (**3**; 50 mg, 148 µmol) and donor **11**[30] (180 mg, 223 µmol), **12** was obtained (46 mg, 32 %) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.55 (dq, *J* = 6.0, 1.4 Hz, 1 H), 5.25–5.19 (m, 1 H), 5.16 (t, *J* = 9.4 Hz, 1 H), 5.12 (t, *J* = 9.4 Hz, 1 H), 5.06 (t, *J* = 9.6 Hz, 1 H), 4.96 (dd, *J* = 9.3; 8.2 Hz, 1 H), 4.90–4.82 (m, 3 H), 4.76 (d, *J* = 5.9 Hz, 1 H), 4.63 (d, *J* = 8.2 Hz, 1 H), 4.28–4.22 (m, 2 H), 4.19 (d, *J* = 10.1 Hz, 1 H), 4.11 (dd, *J* = 12.5, 2.4 Hz, 1 H), 3.94 (d, *J* = 1.96 Hz, 1 H), 3.89 (d, *J* = 4.2 Hz, 1 H), 3.79 (dd, *J* = 11.7, 1.9 Hz, 1 H), 3.68 (dd, *J* = 11.9, 7.2 Hz, 1 H), 3.64–3.57 (m, 2 H), 3.34 (d, *J* = 9.8 Hz, 1 H), 3.20 (d, *J* = 4.3 Hz, 1 H), 3.11 (d, *J* = 4.0 Hz, 1 H), 2.60 (dd, *J* = 15.3, 4.3 Hz, 1 H), 2.17 (s, 3 H), 2.09 (s, 3 H), 2.08 (s, 3 H), 2.02 (s, 3 H), 2.01 (s, 6 H), 2.00 (s, 3 H), 1.98 (s, 3 H), 1.85 (s, 3 H), 1.61 (s, 1 H), 1.09 (s, 3 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 199.6 (s, 1 C), 170.7 (s, 1 C), 170.5 (s, 1 C), 170.4 (s, 1 C), 170.3 (s, 1 C), 169.6 (s, 1 C), 169.5 (s, 1 C), 169.4 (s, 1 C), 169.2 (s, 1 C), 138.3 (d, 1 C), 136.0 (s, 1 C), 100.8 (d, 1 C), 100.6 (d, 1 C), 79.4 (d, 1 C), 74.4 (d, 1 C), 74.3 (d, 1 C), 73.1 (d, 1 C), 72.7 (d, 1 C), 72.1 (d, 1 C), 71.5 (d, 1 C), 71.3 (d, 1 C), 71.2 (d, 1 C), 69.4 (d, 1 C), 68.9 (d, 1 C), 68.4 (d, 1 C), 68.3 (t, 1 C), 67.5 (t, 1 C), 65.3 (s, 1 C), 61.9 (t, 1 C), 51.5 (s, 1 C), 47.7 (t, 1 C), 45.9 (s, 1 C), 40.7 (t, 1 C), 21.1 (q, 1 C), 20.9 (q, 2 C), 20.8 (q, 1 C), 20.7 (q, 4 C), 15.3 (q, 1 C), 13.8 (q, 1 C) ppm. HRMS: calcd. for $C_{43}H_{56}O_{24}^+$ [M + Na]$^+$ 979.3054; found 979.3060.

DON-15-*O*-β-gentiobioside (13): General procedure C; starting from **12** (37.7 mg, 39.4 µmol), **13** was obtained (23 mg, 94 %) as a white solid. ^{1}H NMR (600 MHz, [D_4]methanol): δ = 6.62 (dq, *J* = 6.0,

1.6 Hz, 1 H), 4.98 (d, *J* = 4.98 Hz, 1 H), 4.84 (s, 1 H), 4.39–4.34 (m, 2 H), 4.15 (d, *J* = 10.6 Hz, 1 H), 4.12 (dd, *J* = 11.6, 1.9 Hz, 1 H), 4.03 (d, *J* = 7.9 Hz, 1 H), 3.86 (dd, *J* = 11.9, 2.2 Hz, 1 H), 3.74 (dd, *J* = 11.9, 6.3 Hz, 1 H), 3.66 (dd, *J* = 11.8, 5.5 Hz, 1 H), 3.57 (d, *J* = 10.6 Hz, 1 H), 3.53 (d, *J* = 4.4 Hz, 1 H), 3.42–3.38 (m, 1 H), 3.37 (t, *J* = 8.9, 1.9 Hz, 1 H), 3.32–3.23 (m, 4 H), 3.20 (dd, *J* = 9.0, 8.0 Hz, 1 H), 3.10 (d, *J* = 4.4 Hz, 1 H), 3.07–3.03 (m, 2 H), 2.45 (dd, *J* = 14.7, 4.4 Hz, 1 H), 1.96 (dd, *J* = 14.6, 11.2 Hz, 1 H), 1.83 (s, 3 H), 1.12 (s, 3 H) ppm. ^{13}C NMR (150 MHz, [D$_4$]methanol): δ = 202.4 (s, 1 C), 140.2 (d, 1 C), 136.8 (s, 1 C), 104.9 (d, 1 C), 104.5 (d, 1 C), 82.2 (d, 1 C), 78.0 (d, 1 C), 77.9 (d, 1 C), 77.7 (d, 1 C), 77.2 (d, 1 C), 75.7 (d, 1 C), 75.2 (d, 1 C), 75.0 (d, 1 C), 71.6 (d, 1 C), 71.5 (d, 1 C), 71.4 (d, 1 C), 69.8 (t, 1 C), 69.7 (d, 1 C), 69.7 (t, 1 C), 66.8 (s, 1 C), 62.7 (t, 1 C), 53.3 (s, 1 C), 48.1 (t, 1 C), 47.3 (s, 1 C), 44.5 (t, 1 C), 15.5 (q, 1 C), 14.9 (q, 1 C) ppm. HRMS: calcd. for $C_{27}H_{40}O_{16}^+$ [M + Na]$^+$ 643.2209; found 643.2221.

[$^{13}C_6$]Glucose pentaacetate (15): [$^{13}C_6$]Glucose (**14**; 1 g, 5.37 mmol, 1 equiv.) was suspended in acetonitrile (3 mL), and the mixture was cooled to 0 °C. $FeCl_3$ (87 mg, 0.54 mmol, 0.1 equiv.) was added, followed by the dropwise addition of acetic anhydride (2.8 mL, 29.6 mmol, 5.5 equiv.). The reaction mixture was warmed to room temperature and treated with ultrasonic irradiation for 45 min. The mixture was then diluted with CH_2Cl_2 (10 mL) and washed with water. The organic layer was dried with Na_2SO_4 and concentrated. The crude product was filtered through a pad of silica gel, eluting with hexanes/EtOAc (1:1) to give **15** (2.1 g, 98 %) as a white solid. Analytical data matched those reported in the literature.[36]

2,3,4,6-Tetra-*O*-acetyl-D-[$^{13}C_6$]glucopyranose (16): [$^{13}C_6$]Glucose pentaacetate (**15**; 2.1 g, 5.3 mmol, 1 equiv.) was dissolved in dry THF (40 mL), and benzylamine (625 mg, 5.8 mmol, 1.1 equiv.) was added. The reaction mixture was stirred at room temperature for 24 h, then it was concentrated, and the residue was dissolved in CH_2Cl_2 (40 mL). The solution was washed with HCl (1 N aq.; 25 mL) and water (25 mL). The organic layer was dried with Na_2SO_4 and concentrated. The residue was purified by flash chromatography (hexanes/EtOAc, 1:1; 90 g of silica gel) to give compound **16** (1.64 g, 87 %) as a yellowish oil that slowly crystallized on storage. Analytical data matched those reported in the literature.[36]

2,3,4,6-Tetra-*O*-acetyl-α-D-[$^{13}C_6$]glucopyranosyl-1-(*N*-phenyl)-2,2,2-trifluoroacetimidate ([$^{13}C_6$]6): General procedure A; starting from **16** (1.54 g, 4.4 mmol), [$^{13}C_6$]**6** was obtained (1.9 g, 83 %) as a colorless oil that slowly crystallized on storage in the freezer. ^{13}C-decoupled ^{1}H NMR spectroscopic data matched those reported in the literature.[17] HRMS: calcd. for $C_{16}(^{13}C)_6H_{24}NO_{10}$ [M + Na]$^+$ 548.1446; found 548.1462.

3-ADON-15-*O*-(tetra-*O*-acetyl-β-D-[$^{13}C_6$]glucoside) ([$^{13}C_6$]7): General procedure B; starting from 3-ADON (**3**; 80 mg, 0.24 mmol) and donor [$^{13}C_6$]**6** (189 mg, 0.36 mmol), [$^{13}C_6$]**7** was obtained (85 mg, 53 %) as a white solid. ^{13}C-decoupled ^{1}H NMR spectroscopic data matched those of compound **7**. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 199.5 (s, 1 C), 170.7 (s, 1 C), 170.6 (s, 1 C), 170.3 (s, 1 C), 169.5 (s, 1 C), 169.4 (s, 1 C), 138.7 (d, 1 C), 135.8 (s, 1 C), 100.9–100.1 (m, 1 C), 79.2 (d, 1 C), 73.9 (d, 1 C), 73.5–70.5 (m, 4 C), 69.7 (t, 1 C), 68.8–67.8 (m, 2 C), 65.1 (s, 1 C), 62.4–61.5 (m, 1 C), 51.5 (q, 1 C), 47.6 (t, 1 C), 45.9 (s, 1 C), 40.6 (t, 1 C), 21.1 (q, 1 C), 20.9 (q, 1 C), 20.8 (q, 1 C), 20.7 (q, 2 C), 15.3 (q, 1 C), 13.8 (q, 1 C) ppm. HRMS: calcd. for $C_{25}(^{13}C)_6H_{40}O_{16}$ [M + Na]$^+$ 697.2410; found 697.2430.

DON-15-*O*-β-D-[$^{13}C_6$]glucoside ([$^{13}C_6$]2): General procedure C; starting from [$^{13}C_6$]**7** (62 mg, 92 µmol), [$^{13}C_6$]**2** was obtained (40 mg, 93 %) as a white solid. ^{13}C-decoupled ^{1}H NMR spectroscopic data matched those of compound **2**. ^{13}C NMR (150 MHz, [D$_4$]methanol): δ = 202.5 (s, 1 C), 140.3 (d, 1 C), 136.9 (s, 1 C), 104.0–102.9 (m, 1 C), 82.2 (d, 1 C), 77.3–75.7 (m, 2 C), 75.7 (d, 1 C), 75.6–74.7 (m, 1 C), 71.9–71.1 (m, 2 C), 69.8 (t, 1 C), 69.7 (d, 1 C), 66.7 (s, 1 C), 63.1–62.4 (m, 1 C), 53.3 (s, 1 C), 48.1 (t, 1 C), 47.3 (s, 1 C), 44.5 (t, 1 C), 15.3 (q, 1 C), 14.8 (q, 1 C) ppm. HRMS: calcd. for $C_{15}(^{13}C)_6H_{30}O_{11}^+$ [M + Na]$^+$ 487.1882; found 487.1884.

[$^{13}C_6$]Gentiobiose Octaacetate (18): 1,2,3,4-Tetra-*O*-acetyl-β-D-glucose (**17**; 80 mg, 0.23 mmol, 1 equiv.) and [$^{13}C_6$]glucosyl donor [$^{13}C_6$]**6** (144 mg, 0.27 mmol, 1.2 equiv.) were dissolved in dry CH_2Cl_2 (3 mL). Molecular sieves (3 Å; 300 mg) were added, and the mixture was stirred at room temp. under argon for 1 h. The mixture was then cooled to –60 °C, and TMSOTf (68 mg, 0.03 mmol, 0.15 equiv.) was added. The reaction mixture was slowly warmed to room temp. and stirred for 1 h. The reaction was then quenched by the addition of Et_3N, and the mixture was filtered through Celite and concentrated. The crude product was purified by column chromatography (hexanes/EtOAc, 5:1 to 1:1) to give **18** (100 mg, 64 %) as a white solid. ^{13}C-decoupled ^{1}H NMR spectroscopic data matched those reported in the literature.[37] HRMS: calcd. for $C_{22}(^{13}C)_6H_{38}O_{19}^+$ [M + Na]$^+$ 707.2101; found 707.2117.

[$^{13}C_6$]Gentiobiose Heptaacetate (19): [$^{13}C_6$]Gentiobiose octaacetate (**18**; 138 mg, 0.20 mmol, 1 equiv.) was dissolved in dry DMF (1 mL), and ammonium acetate (31 mg, 0.40 mmol, 2 equiv.) was added. The reaction mixture was stirred for 16 h at room temp., then it was concentrated. The residue was purified by column chromatography to give **19** (109 mg, 85 %) as a white solid. ^{13}C-decoupled ^{1}H NMR spectroscopic data matched those reported in the literature.[38] HRMS: calcd. for $C_{20}(^{13}C)_6H_{36}O_{18}^+$ [M + Na]$^+$ 665.1996; found 665.2007.

1-*N*-Phenyl-2,2,2-trifluoroacetimidoyl-[$^{13}C_6$]gentiobiose Heptaacetate ([$^{13}C_6$]11): General procedure A; starting from **19** (67 mg, 0.10 mmol), [$^{13}C_6$]**11** was obtained (70 mg, 83 %) as a colourless viscous syrup that crystallized in the freezer; ^{13}C-decoupled ^{1}H NMR spectroscopic data matched those of nonlabeled **11**, as reported in the literature.[30] HRMS: calcd. for $C_{28}(^{13}C)_6H_{40}F_3NO_{18}^+$ [M + Na]$^+$ 836.2291; found 836.2283.

DON-15-*O*-β-[$^{13}C_6$]gentiobioside ([$^{13}C_6$]13): General procedure D ("one-pot"); starting from 3-ADON (**3**; 38 mg, 113 µmol) and donor [$^{13}C_6$]**11** (138 mg, 170 µmol), [$^{13}C_6$]**13** was obtained (23 mg, 32 %) as a white solid. ^{13}C-decoupled ^{1}H NMR spectroscopic data matched those of nonlabeled **13**. ^{13}C NMR (150 MHz, [D$_4$]methanol): δ = 202.4 (s, 1 C), 140.2 (d, 1 C), 136.8 (s, 1 C), 105.3–104.5 (m, 1 C), 104.5 (d, 1 C), 82.2 (d, 1 C), 78.5–77.5 (m, 3 C), 77.2 (d, 1 C), 75.7 (d, 1 C), 75.5–74.7 (m, 2 C), 72.1–70.9 (m, 3 C), 69.8 (t, 1 C), 69.7 (d, 1 C), 69.7 (t, 1 C), 66.8 (s, 1 C), 63.23–61.9 (m, 1 C), 53.3 (s, 1 C), 48.1 (t, 1 C), 47.3 (s, 1 C), 44.5 (t, 1 C), 15.5 (q, 1 C), 14.9 (q, 1 C) ppm. HRMS: calcd. for $C_{21}(^{13}C)_6H_{40}O_{16}^+$ [M + H]$^+$ 627.2590; found 627.2591.

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Keywords: Glycosylation · Glycosides · Orthoesters · Natural products · Isotopic labeling

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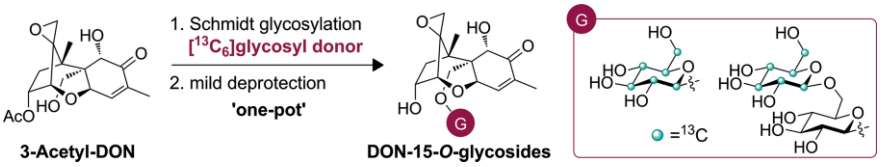
Glycosylation



An efficient and fast procedure for the synthesis of 15-*O*-glycosides of deoxynivalenol (DON) was developed, allowing access to isotope-labeled compounds. By using this method, DON-15-*O*-β-D-[$^{13}C_6$]glucoside and DON-15-*O*-β-[$^{13}C_6$]gentiobioside were prepared in reasonable amounts for characterization and use as reference materials for further investigations.

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Sulfation of deoxynivalenol, its acetylated derivatives, and T2-toxin☆

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ABSTRACT

The synthesis of several sulfates of trichothecene mycotoxins is presented. Deoxynivalenol (DON) and its acetylated derivatives were synthesized from 3-acetyldeoxynivalenol (3ADON) and used as substrate for sulfation in order to reach a series of five different DON-based sulfates as well as T2-toxin-3-sulfate. These substances are suspected to be formed during phase-II metabolism in plants and humans. The sulfation was performed using a sulfuryl imidazolium salt, which was synthesized prior to use. All protected intermediates and final products were characterized via NMR and will serve as reference materials for further investigations in the fields of toxicology and bioanalytics of mycotoxins.

1. Introduction

Deoxynivalenol (DON), its acetylated derivatives, which occur as 3-acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON), and 3,15-diacetyldeoxynivalenol (3,15-diADON) as well as T2-toxin (Fig. 1) are very common and widespread trichothecene mycotoxins. They are predominantly produced by different *Fusarium* species and can contaminate food and feed. They are known to act as a protein biosynthesis inhibitor,[1,2] neurotoxin, immunosuppressive or nephrotoxin[3] and can cause acute and chronic symptoms[4] after uptake. Based on this knowledge, regulatory limits regarding the toxin concentration for food and feed were established to minimize the daily uptake.

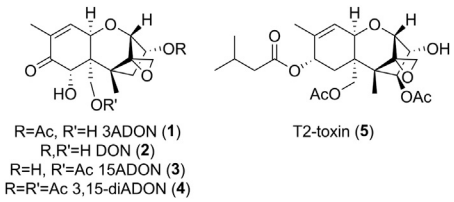

Fig. 1. Structures of DON, its derivatives, and T2-toxin.

Although these limits cover the toxins themselves, there is still a lack of information regarding the occurrence and toxicity of their conjugated forms. DON, for example, can undergo glycosylation during late phase-II metabolism in the plant to end up as DON-3-β-D-*O*-glucoside, which is classified as masked mycotoxin.[5] A similar conjugation leads to the corresponding T2-toxin glucoside, which was recently discovered.[6] These masking mechanisms can also lead to di- and triglycosides, which are difficult to investigate due to a lack of authentic reference standards. The main concern regarding these masked forms is the fact that the occurring conjugates can be cleaved in the stomach after uptake, whereby releasing the parent toxin. In addition to the stomach, this cleavage could also occur within the process of malting,[7] leading to an increase of free DON. Besides the formation of glycosides, other masking processes like sulfation or thia-michael addition can take place. Zearalenone is another prominent mycotoxin produced by *Fusarium* species and its metabolite zearalenone-14-sulfate[8] was previously synthesized[9] in order to serve as reference material for contamination and

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toxicity studies. Additionally, the synthesis of different *Alternaria* toxin sulfates[10] was recently published showing the emerging research interest within the field of mycotoxin sulfur conjugates. Besides the occurrence of different masked mycotoxins in plants, many parent toxins also undergo phase-II metabolism in living organisms, leading mainly to their corresponding glucuronides[11] but also to their sulfates.[12,13] Besides the occurring glucuronides, which are accessible[14] and were already used for the successful development of a biomarker method[15–17] for human deoxynivalenol exposure estimation, little is known about possible occurring trichothecene sulfates. Considering that sulfate conjugates are described for different substance classes like steroids,[18] pollutants,[19] and drugs,[20,21] we assume that there might be even more trichothecene-derived sulfates occurring during metabolism.

The objective of this work was the development of a reliable synthetic strategy for the sulfation of trichothecenes to access all possible DON-sulfates incorporating their acetylated derivatives and T2-toxin-3-sulfate. Since the reactivity of the three hydroxyl groups in deoxynivalenol is somewhat different in the order 15>3>>7 (Fig. 2), we expected all kinds of 3- and 15-sulfates to occur. However, we were interested in evaluating the possibility to obtain also some C7-sulfates.

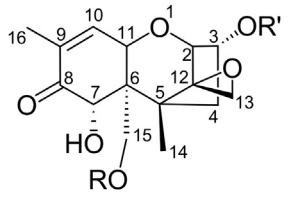


Fig. 2. Structural numbering of trichothecenes.

3ADON was isolated and crystallized[22] from an extract of *Fusarium graminearum* and was directly used after comparison with a 3ADON standard. Deprotection and the following acetylation of DON[23] led us to the four desired structures, which we wanted to utilize for sulfation (Scheme 1) to access a series of different sulfates.

reproducibility, and regioselectivity, several newer protective groups for the sulfation of organic molecules are described in literature. Besides the fact that we already made good experiences in our group regarding the separation of intermediates and yields by the use of 2,2,2-trichloroethyl (TCE) protective groups[25] within chemical sulfation and glycosylation reactions, the use of this protective group also offers the possibility of mild deprotection conditions. The cleavage could therefore be done via catalytic transfer hydrogenation (Pd/C) or via different mild reductive methods including ammonium formate or Zn/ammonium formate.

In addition, a very mild protocol using sulfuryl imidazolium salts was recently employed in the synthesis of trichloroethyl-protected sulfates on carbohydrates and offered a good possibility of the sulfation of the fragile trichothecene scaffold.

2. Results and discussion

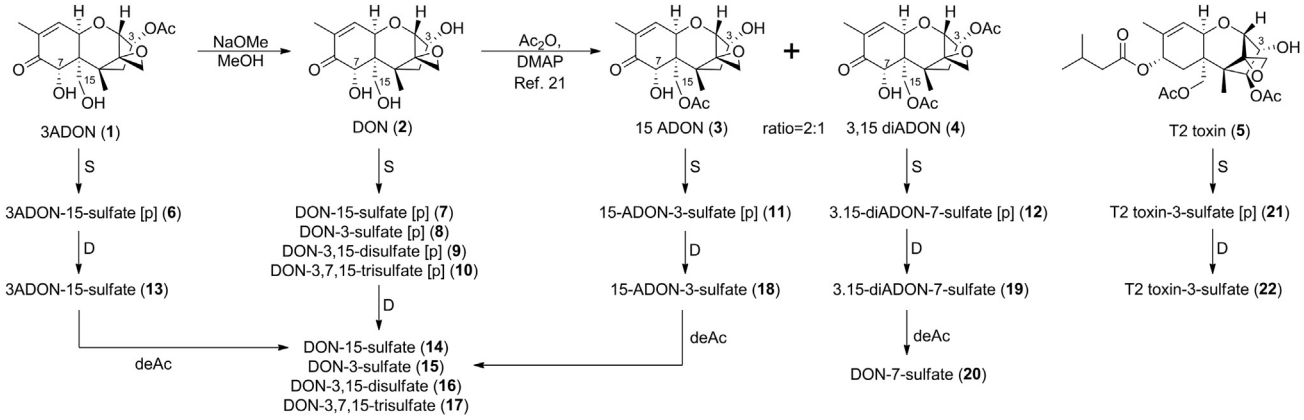
After synthesis of the proper sulfuryl imidazolium salt[26] (Scheme 2a, **26**), the method evaluation was done by the use of a simple mimic[27] (Scheme 2b, **27**), which was selected because of an incorporated primary alcohol group, a labile acetic ester group (like T2-toxin), and its UV activity.

The reaction of **26** and **27** to **28** proceeds smoothly if freshly sublimed dimethylimidazole is used. The deprotection toward **29** was done in an ultrasonic bath at room temperature, which shortened the reaction time to 20 min. This is a crucial achievement since trichothecenes often own labile ester groups, which are rapidly cleaved in different solvents. The deprotection toward **30** was done via NaOMe and was carried out as a proof of concept regarding the deprotection of acetylated sulfates.

The reactions toward the different acetylated DON derivates (Scheme 3) were carried out as described in literature[23] and led to all desired products.

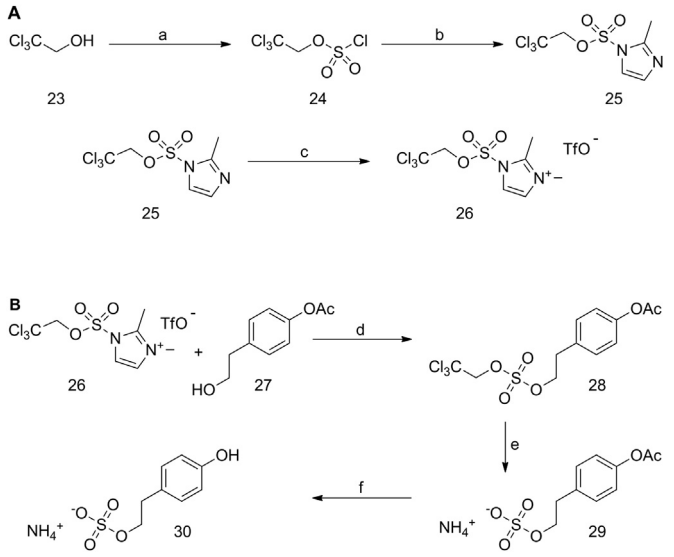
The sulfation of **1** yielded (as expected) only one product, which was identified as the protected 3ADON-15-sulfate, and sulfation of **3** yielded only the protected 15ADON-3-sulfate. Both substances were successfully deprotected to the free sulfates as ammonium salt. In case of **4** no reaction was observed at all, even with 4 equiv of **26** and prolonged reaction time (Scheme 4).

In case of **2** we isolated protected DON-3,15-disulfate surprisingly together with DON-3-sulfate instead of the expected 15-

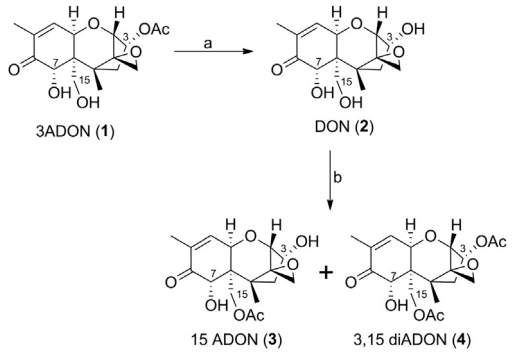


Scheme 1. Synthetic strategies toward all possible sulfate conjugates. S=sulfation, D=deprotection, deAc=deacetylation.

Regarding sulfation itself, numerous synthetic methods[24] can be found within the literature mainly based on the application of commercially available sulfur trioxide complexes. Since these methods are limited concerning possible chemical modifications after installation of the sulfate group as well as yield, sulfate (Scheme 5). Additionally, these two products were the only ones, which were isolated. In general, we were expecting position 7 to be somewhat unreactive due to steric hindrance and poor nucleophilicity. Nevertheless, since acetylation resulted in a 2:1 mixture of the 15- to the 3-product, we also expected a similar



Scheme 2. Synthesis of the protected sulfate donor (A, **26**) and method evaluation with a simple mimic (B, **27**). Conditions: (a) pyridine, SO_2Cl_2, −80 °C to rt, 24 h, 97%, (b) 2-methyl imidazole, 2 h, 99%, (c) Et_2O, 0 °C, MeOTf, 6 h, 94%, (d), 1,2-dimethylimidazole, 0 °C, DCM, 24 h, 82%, (e) MeOH, $HCOONH_4$, Zn, 20 min, 81%, (f) MeOH, NaOMe, 1 h, 42%.



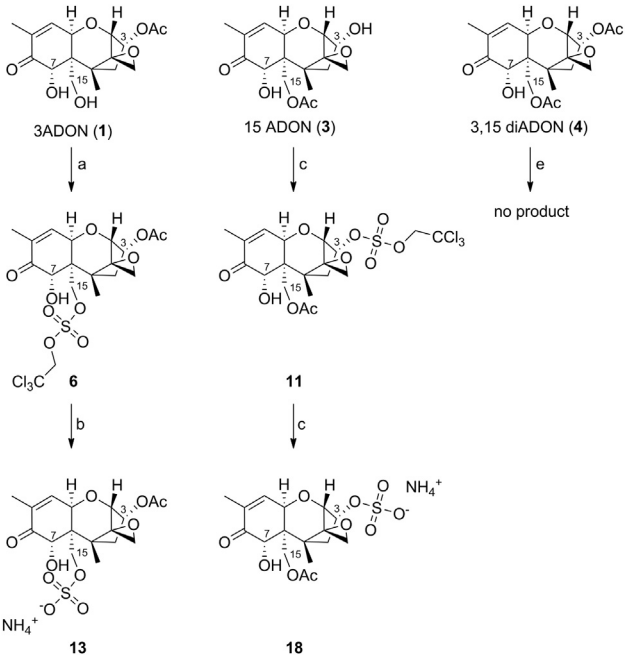
Scheme 3. Synthesis of different deoxynivalenol derivatives. Conditions: (a) NaOMe, MeOH, 2 h, 94%, (b) Ac_2O, DMAP, 18 h, 47% for **3**, 23% for **4**, sum=70%.

regioselectivity and ratio of the sulfation reaction. The reaction itself has not undergone any decomposition (observed via TLC) during workup, so we could also exclude the theory of degradation from the disulfate species toward the monosulfate. Deprotection of both substances yielded DON-3-sulfate as ammonium salt and DON-3,15-disulfate as the corresponding diammonium salt.

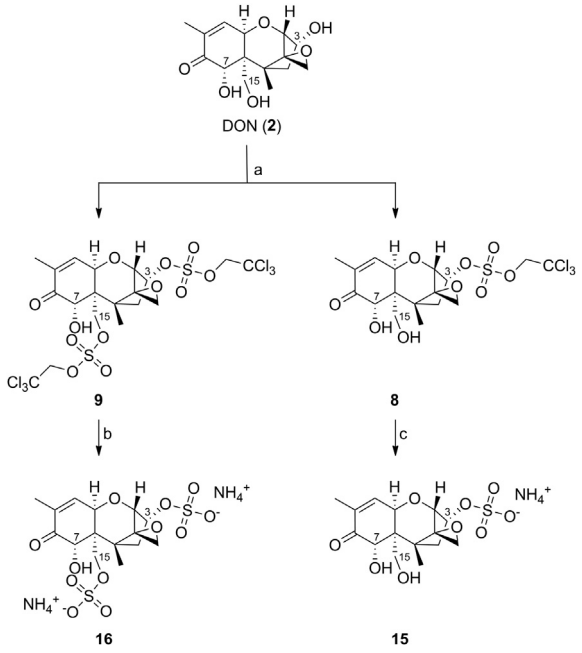
Position 7 was not sulfated during any reaction. Of the remaining five theoretically possible sulfates, only DON-15-sulfate was not isolated after direct sulfation (Scheme 1). For this reason we used deacetylation of 3ADON-15-sulfate (Scheme 6) to complete the set of sulfates.

Having proven that the method is well working for trichothecenes, we aimed also for the sulfation of T2-toxin, which was carried out in a similar way, whereby leading to the corresponding 3-sulfate as ammonium salt (Scheme 7).

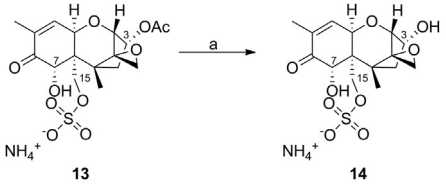
Finally, all isolated protected intermediates as well as all ammonium sulfates were characterized via NMR, and ^1H shifts were assigned using several NMR references[28,29] for DON and its derivatives. For the assignment of the ^1H shifts of T2-toxin-3-sulfate, COSY and HMBC spectra were recorded. We tried to purify all intermediates very quickly over short columns (10–15 g silica), since we have encountered deacetylation of all types of trichothecenes during column chromatography (silica as well as RP). Therefore, we ended up with traces of 1,2-dimethylimidazole within the



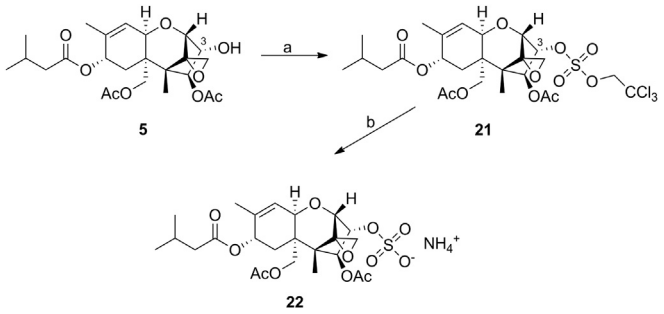
Scheme 4. Synthesis of different acetyldeoxynivalenol-derived sulfates. Conditions: (a) DCM, 2.5 equiv 1,2-dimethylimidazole, 1.25+0.75 equiv 26, 0 °C, 18 h, 52%, (b) MeOH, 9 equiv $HCOONH_4$, 3 equiv Zn, 20 min, 72%, (c) DCM, 2.7 equiv 1,2-dimethylimidazole, 1.35 equiv 26, 0 °C, 18 h, 34%, (d) MeOH, 9 equiv $HCOONH_4$, 3 equiv Zn, 20 min, 89%.



Scheme 5. Synthesis of DON-3-sulfate and DON-3,15-disulfate as their ammonium salts. Conditions: (a) DCM, 4 equiv 1,2-dimethylimidazole, 2 equiv 26, 0 °C, 18 h, 24% for **9**, 30% for **8**, (b) MeOH, 18 equiv $HCOONH_4$, 6 equiv Zn, 2 h, 54%, (c) MeOH, 9 equiv $HCOONH_4$, 3 equiv Zn, 30 min, 98%.



Scheme 6. Synthesis of DON-15-sulfate as ammonium salt. Conditions: (a) MeOH, NaOMe, 2 h, 69%.



Scheme 7. Synthesis of T2-toxin-3-sulfate as ammonium salt. Conditions: (a) DCM, 4 equiv 1,2-dimethylimidazole, 2 equiv 26, 0 °C, 18 h, 49%, (b) MeOH, 9 equiv $HCOONH_4$, 3 equiv Zn, 1 h, 29%.

protected intermediates. After the deprotection step, column chromatography was done with very polar solvents (DCM/MeOH/NH_4OH=10:4:1 or 10:2.5:0.5), which led to $HCOONH_4$ impurities in our products. Because of this we usually made a second column chromatography, followed by lyophilization, and continued drying for several days to remove remaining $HCOONH_4$. All NMR spectra and ^1H chemical shift assignments can be found in the Supplementary data.

3. Conclusion

Considering the proven unreactivity of the C7 position to chemical sulfation, we have synthesized, isolated, and characterized all possible DON-sulfates including its acetylated derivatives. In case of T2-toxin we were also able to synthesize the desired T2-toxin-3-sulfate. Therefore, we have proven that the utilized method is well working for trichothecenes and provides a good way to access the class of trichothecene sulfates via sulfation of the parent toxins including their acetylated derivatives. Separation of the protected intermediates was done using column chromatography, followed by fast deprotection by the use of Zn/$HCOONH_4$ within an ultrasonic bath. All products and intermediates were characterized by ^1H and ^{13}C NMR, and all ^1H chemical shifts were assigned to the substances. The gathered ^1H NMR information will serve as a valuable reference for naturally isolated material. Finally, all standards will be used for identification and quantification of their occurrence and formation within human and plant metabolism.

4. Experimental section

4.1. General remarks

CAUTION: All used toxins are strong protein biosynthesis inhibitors and can cause a series of acute and chronic symptoms. Therefore, we strongly recommend considering their toxicity within all reactions!

All reactions were carried out under an argon atmosphere and the progress of all reactions was monitored using thin-layer chromatography (TLC) over silica gel $60F_{254}$ (Merck, Germany). All chromatograms were visualized by heat staining using ceric ammonium molybdate/Hanessian's stain[30] in ethanol/sulfuric acid. Chromatographic separation was done on silica gel 60 (40–63 μm) using a SepacoreTM Flash System (Büchi, Switzerland) or glass columns. All samples were measured via LC–ESI-MS/MS and LC–APCI-MS/MS and in a negative ionization mode. These measurements were performed on an HCT ion trap mass spectrometer (Bruker, Germany). A TLC–MS interface (Camag, Germany) was used for ESI-MS analysis after TLC. ^1H and ^{13}C spectra were recorded upon using a Bruker DPX-200 spectrometer as well as an Avance DRX-400 MHz spectrometer (both Bruker, Germany). Data were recorded and evaluated using TOPSPIN 1.3 (Bruker Biospin). All chemical shifts are given in parts per million relative to tetramethylsilane. The calibration was done using residual solvent signals.[31] Multiplicities are abbreviated as s (singlet), d (doublet), t (triplet), q (quartet), and br (broad signal). 3-ADON was obtained from BOKU, Dept. for Agrarbiotechnology (IFA-Tulln) as crude fermentation extract, purified via column chromatography, and used after the ^1H NMR purity check. All other chemicals were purchased from ABCR (Germany) and Sigma–Aldrich (Austria/Germany).

4.2. 2,2,2-Trichloroethyl 2-(4-acetoxyphenyl)ethylsulfate (28)

Compound **27** (121.8 mg, 0.68 mmol, 1.0 equiv) was dissolved in 3 mL DCM and a solution of 1,2-dimethylimidazole (194.9 mg, 2.03 mmol, 3.0 equiv) in 1 mL DCM was added at 0 °C. Then, **26** (464.0 mg, 1.01 mmol, 1.5 equiv) was added in one portion and the reaction was allowed to warm to room temperature over night. After TLC indicated full conversion the reaction was directly purified via column chromatography (hexane/EtOAc=1:1) to yield **28** (215.8 mg, 82%) as white solid. ^1H NMR (200 MHz, $CDCl_3$) δ 7.28 (s, 4H), 4.82 (s, 2H), 4.27 (t, J=6.8 Hz, 2H), 2.95 (t, J=6.8 Hz, 2H), 2.02 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.0 (s, 1C), 148.9 (s, 1C), 138.1 (s, 1C), 130.6 (d, 2C), 121.2 (d, 2C), 92.5 (s, 1C), 80.5 (t, 1C), 64.5 (t, 1C), 34.5 (t, 1C), 21.0 (q, 1C). HRMS m/z calcd for $C_{12}H_{14}O_6SCl_3^+$ $[M+H]^+$, 390.9571, found 390.9578.

4.3. 2-(4-Acetoxyphenyl)ethylsulfate, ammonium salt (29)

Compound **28** (190.0 mg, 0.49 mmol, 1.0 equiv) was dissolved in 5 mL MeOH. Ammonium formate (276.5 mg, 4.39 mmol, 9.0 equiv) and Zn dust (95.6 mg, 1.46 mmol, 3.0 equiv) were added and the reaction was placed in an ultrasonic bath. After 20 min, TLC revealed complete conversion of the starting material and the reaction mixture was filtered through Celite and concentrated to 1 mL. Direct purification of this solution using column chromatography (DCM/MeOH/NH_4OH=10:2.5:0.5) yielded **29** (109.5 mg, 81%) as white solid. ^1H NMR (200 MHz, methanol-d_4) δ=7.22 (br, 4H), 4.93 (br, NH_4^+, H_2O), 4.23 (t, J=6.9 Hz, 2H), 2.91 (t, J=6.9 Hz, 2H), 2.00 (s, 3H); ^{13}C NMR (50 MHz, methanol-d_4) δ 172.9 (s, 1C), 152.5 (s, 1C), 136.0 (s, 1C), 130.6 (d, 2C), 122.5 (d, 2C), 66.2 (t, 1C), 35.3 (t, 1C), 20.8 (q, 1C). HRMS m/z calcd for $C_{10}H_{11}O_6S^-$ $[M-NH_4]^-$, 259.0282, found 259.0278.

4.4. 2-(4-Hydroxyphenyl)ethylsulfate, ammonium salt (30)

Compound **29** (69.0 mg, 0.25 mmol, 1.00 equiv) was dissolved in 2 mL dry MeOH and NaOMe (14.1 mg, 0.26 mmol, 1.05 equiv) was added. Since no reaction occurred after 30 min we added the same amount of NaOMe again. After stirring for 30 min, TLC indicated full conversion and the reaction was directly purified via column chromatography (DCM/MeOH/NH_4OH=10:2.5:0.5) to yield **9** (28.7 mg, 42%) as white solid. ^1H NMR (200 MHz, methanol-d_4) δ=7.21 (s, 4H), 4.90 (br, NH_4^+, H_2O), 3.73 (t, J=6.9 Hz, 2H), 2.80 (t, J=6.9 Hz, 2H); ^{13}C NMR (50 MHz, methanol-d_4) δ 152.3 (s, 1C), 137.0 (s, 1C), 130.6 (d, 2C), 122.5 (d, 2C), 64.2 (t, 1C), 39.5 (t, 1C). HRMS m/z calcd for $C_8H_9O_5S^-$ $[M-NH_4^+]$, 217.0176, found 217.0174.

4.5. 15-ADON (3) and 3,15-diADON (4)

3-ADON (**1**) (95.6 mg, 0.28 mmol, 1.0 equiv) was dissolved in 5 mL methanol, followed by the addition of NaOMe (13.7 mg, 0.25 mmol, 0.9 equiv). After 2 h, TLC showed full conversion of the **1**. The reaction was concentrated to 1 mL and directly purified by the use of column chromatography ($CHCl_3$/MeOH=9:1), which yielded deoxynivalenol (**2**, 79.0 mg, 94%) as white solid. The reaction product was proven to be identical to an authentic sample by

TLC and, thus, was directly used for acetylation. For this purpose, **2** (79.0 mg, 0.27 mmol) was dissolved in 50 mL dry dichloromethane. Pyridine (1 mL) and 4-DMAP (app. 10 mg) were added, followed by the dropwise addition of acetic anhydride (27.2 mg, 0.27 mmol). The reaction was stirred over night, treated with 20 mL 2 N HCl, and extracted with dichloromethane. After drying with Na_2SO_4, filtration, and evaporation of the solvent, the remaining residue was subjected to column chromatography ($CHCl_3$/MeOH=95:5) to yield **3** (42.0 mg, 47%) and **4** (23.5 mg, 23%) as white solid. Total yield=70%, 93% conversion. 15-ADON (**3**): ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.61 (dq, J=5.7, 1.6 Hz, 1H), 4.89 (d, J=5.7 Hz, 1H), 4.83 (d, J=1.6 Hz, 1H), 4.52 (dt, J=10.2, 4.7 Hz, 1H), 4.24 (s, 2H), 3.78 (d, J=1.8 Hz, 1H), 3.63 (d, J=4.5 Hz, 1H), 3.13 (d, J=4.3 Hz, 1H), 3.08 (d, J=4.3 Hz, 1H), 2.22 (dd, J=14.8, 4.7 Hz, 1H), 2.08 (dd, J=14.7, 10.4 Hz, 1H), 1.88 (s, 3H), 1.87 (s, 3H), 1.07 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ=199.6 (s), 170.3 (s), 138.8 (d), 135.6 (s), 80.7 (d), 73.5 (d), 70.1 (d), 68.9 (s), 65.5 (s), 62.2 (t), 51.4 (s), 47.4 (t), 46.3 (s), 43.3 (t), 20.7 (q), 15.4 (q), 13.8 (q). 3,15-diADON (**4**): ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.56 (dq, J=5.8, 1.4 Hz, 1H), 5.20 (dt, J=10.9, 4.6 Hz, 1H), 4.80 (d, J=2.0 Hz, 1H), 4.69 (d, J=5.8 Hz, 1H), 4.27 (d, J=12.1 Hz, 1H), 4.20 (d, J=12.1 Hz, 1H), 3.89 (d, J=4.3 Hz, 1H), 3.80 (d, J=2.0 Hz, 1H), 3.14 (d, J=4.3 Hz, 1H), 3.09 (d, J=4.3 Hz, 1H), 2.31 (dd, J=15.2, 4.8 Hz, 1H), 2.15 (dd, J=15.2, 10.9 Hz, 1H), 2.12 (s, 3H), 1.88 (s, 3H), 1.87 (s, 3H), 1.08 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 199.3 (s), 170.3 (s), 170.2 (s), 138.4 (d), 135.6 (s), 78.9 (d), 73.4 (d), 71.1 (d), 70.1 (d), 64.9 (s), 62.1 (t), 51.5 (s), 47.4 (t), 45.8 (s), 40.3 (t), 21.0 (q), 20.6 (q), 15.3 (q), 13.6 (q).

4.6. 2,2,2-Trichloroethyl 3-acetyl-DON-15-sulfate (6)

Compound **1** (45.1 mg, 133 μmol, 1.0 equiv) was dissolved in 2.5 mL of DCM and cooled to 0 °C and 1,2-dimethylimidazole (32.0 mg, 333 μmol, 2.5 equiv) in 1 mL DCM was added to the reaction. Then, **26** (76.2 mg, 167 μmol, 1.25 equiv) was added and the reaction was allowed to reach room temperature over night. Since TLC showed remaining starting material after 18 h, the reaction was cooled again to 0 °C and another 0.75 equiv of **26** (45.7 mg, 100 μmol) were added. After another 48 h, TLC showed still starting material, but also the formation of substantial amounts of product. The reaction was directly used for column chromatography ($CHCl_3$/MeOH=95:5), yielding **6** (37.8 mg, 52%) as white solid. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.66 (dq, J=5.9, 1.4 Hz, 1H), 5.24 (ddd, J=9.5, 6.0, 4.5 Hz, 1H), 4.89 (d, J=1.4 Hz, 1H), 4.80 (d, J=5.9 Hz, 1H), 4.64 (d, J=10.8 Hz, 1H), 4.57 (d, J=10.8 Hz, 1H), 4.56 (d, J=10.6 Hz, 1H), 4.43 (d, J=10.6 Hz, 1H), 3.95 (d, J=4.5 Hz, 1H), 3.84 (d, J=1.4 Hz, 1H), 3.16 (d, J=4.1 Hz, 1H), 3.13 (d, J=4.1 Hz, 1H), 2.10–2.36 (m, 2H), 2.16 (s, 3H), 1.91 (br, 3H), 1.11 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 198.8 (s, 1C), 170.2 (s, 1C), 138.7 (d, 1C), 136.1 (s, 1C), 92.4 (s, 1C), 79.7 (t, 1C), 78.8 (d, 1C), 73.0 (d, 1C), 71.6 (t, 1C), 70.7 (d, 1C), 69.1 (d, 1C), 64.6 (s, 1C), 51.3 (s, 1C), 47.4 (t, 1C), 45.8 (s, 1C), 40.4 (t, 1C), 20.9 (q, 1C), 15.2 (q, 1C), 13.5 (q, 1C); HRMS m/z calcd for $C_{19}H_{24}O_{10}SCl_3^+$ $[M+H]^+$, 549.0150, found 549.0160.

4.7. 2,2,2-Trichloroethyl 15-acetyl-DON-3-sulfate (11)

Compound **3** (76.5 mg, 226 μmol, 1.0 equiv) was dissolved in 3 mL of DCM and cooled to 0 °C and 1,2-dimethylimidazole (58.7 mg, 610 μmol, 2.7 equiv) in 1 mL DCM was added to reaction. Then **26** (139.7 mg, 305 μmol, 1.35 equiv) was added and the reaction was allowed to reach room temperature over night. TLC showed substantial amounts of product and the reaction was directly used for column chromatography (DCM/MeOH=95:5) yielding **11** (42.6 mg, 34%) as white solid. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.60 (dq, J=5.8, 1.5 Hz, 1H), 5.31 (dt, J=11.1, 4.3 Hz, 1H), 4.81 (s, 1H), 4.79 (s, 2H), 4.70 (d, J=5.8 Hz, 1H), 4.27 (d, J=12.1 Hz, 1H), 4.18 (d, J=12.1 Hz, 1H), 4.00 (d, J=4.3 Hz, 1H), 3.71 (s, 1H), 3.21 (d, J=4.1 Hz, 1H), 3.15 (d, J=4.1 Hz, 1H), 2.64 (dd, J=15.7, 4.3 Hz, 1H), 2.29 (dd, J=15.7, 11.1 Hz, 1H), 1.93 (s, 3H), 1.91 (br, 3H), 1.12 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 198.9 (s, 1C), 170.2 (s, 1C), 137.9 (d, 1C), 136.1 (s, 1C), 92.7 (s, 1C), 80.4 (d, 1C), 80.0 (t, 1C), 78.9 (d, 1C), 73.5 (d, 1C), 70.1 (d, 1C), 64.5 (s, 1C), 62.0 (t, 1C), 51.2 (s, 1C), 47.4 (t, 1C), 46.1 (s, 1C), 40.2 (t, 1C), 20.8 (q, 1C), 15.4 (q, 1C), 13.7 (q, 1C); HRMS m/z calcd for $C_{19}H_{24}O_{10}SCl_3^+$ $[M+H]^+$, 549.0150, found 549.0146.

4.8. 2,2,2-Trichloroethyl DON-3-sulfate (8) and bis(2,2,2-trichloroethyl) DON-3,15-disulfate (9)

Compound **2** (40.6 mg, 137 μmol, 1.0 equiv) was dissolved in 5 mL of DCM and cooled to 0 °C and 1,2-dimethylimidazole (52.7 mg, 548 μmol, 4.0 equiv) in 1 mL DCM was added to the reaction. Then, **26** (125.4 mg, 274 μmol, 2.0 equiv) was added and the reaction was allowed to reach room temperature over night. TLC after 24 h showed nearly full conversion of the starting material and the reaction was directly used for column chromatography (DCM/MeOH gradient from 100:0 → 95:5), yielding **8** (22.4 mg, 30%) and **9** (23.9 mg, 24%) as white solid. ^{1}H NMR of **8** (200 MHz, $CDCl_3$) δ 6.62 (dq, J=5.9, 1.4 Hz, 1H), 5.31 (dt, J=11.2, 4.4 Hz, 1H), 4.81 (s, 1H), 4.78 (s, 2H), 4.74 (d, J=5.9 Hz, 1H), 3.99 (d, J=4.5 Hz, 1H), 3.86 (d, J=11.5 Hz, 1H), 3.77 (br, 1H), 3.76 (d, J=11.5 Hz, 1H), 3.22 (d, J=4.1 Hz, 1H), 3.14 (d, J=4.1 Hz, 1H), 2.80 (dd, J=15.7, 4.3 Hz, 1H), 2.25 (dd, J=15.7, 11.2 Hz, 1H), 1.91 (br, 3H), 1.71 (br, 1H), 1.17 (s, 3H); ^{13}C NMR of **8** (50 MHz, $CDCl_3$) δ 199.6 (s, 1C), 138.0 (d, 1C), 136.3 (s, 1C), 92.7 (s, 1C, $-CCl_3$ tiny signal!), 80.8 (d, 1C), 79.9 (t, 1C), 79.1 (d, 1C), 74.5 (d, 1C), 70.1 (d, 1C), 64.8 (s, 1C), 62.0 (t, 1C), 51.8 (s, 1C), 47.7 (t, 1C), 46.1 (s, 1C), 40.3 (t, 1C), 15.4 (q, 1C), 14.1 (q, 1C); HRMS m/z calcd for $C_{17}H_{20}O_9SCl_3^-$ $[M-H^+]^-$, 504.9899, found 504.9878. ^{1}H NMR of **9** (200 MHz, $CDCl_3$) δ 6.69 (dq, J=5.9, 1.5 Hz, 1H), 5.32 (dt, J=11.1, 4.4 Hz, 1H), 4.88 (s, 1H), 4.81 (dt, J=5.7, 1.5 Hz, 1H), 4.79 (s, 2H), 4.66 (d, J=11.0 Hz, 1H), 4.60 (d, J=11.0 Hz, 1H), 4.48 (s, 2H), 4.04 (d, J=4.4 Hz, 1H), 3.83 (br, 1H), 3.21 (d, J=4.1 Hz, 1H), 3.17 (d, J=4.1 Hz, 1H), 2.57 (dd, J=15.7, 4.2 Hz, 1H), 2.33 (dd, J=15.7, 11.1 Hz, 1H), 1.93 (br, 3H), 1.15 (s, 3H); ^{13}C NMR of **9** (50 MHz, $CDCl_3$) δ 198.5 (s, 1C), 138.2 (d, 1C), 136.6 (s, 1C), 2×80.0 (d, 1C/t, 1C), 79.9 (t, 1C), 78.9 (d, 1C), 73.3 (d, 1C), 71.5 (t, 1C), 69.2 (d, 1C), 64.3 (s, 1C), 51.3 (s, 1C), 47.7 (t, 1C), 46.2 (s, 1C), 40.3 (t, 1C), 15.3 (q, 1C), 13.6 (q, 1C), 2× CCl_3 between 92 and 93 ppm are missing, but the corresponding CH_2 groups are located at 80.0 and 79.9; HRMS m/z calcd for $C_{17}H_{20}O_{12}S_2Cl_3^-$ $[M-TCE]^-$, 584.9467, found 584.9505.

4.9. General deprotection procedure

The protected intermediate was dissolved in MeOH (1 mL/10 μmol starting material). $HCOONH_4$ (3 equiv) as well as Zn dust (9 equiv) were added and the reaction was placed in an ultrasonic bath at room temperature. The reaction was followed via TLC until substantial amounts of products were formed (20–90 min). After filtration through Celite the remaining residue was subjected to column chromatography to end up with the corresponding sulfates as ammonium salts. For all acetylated DON derivatives and T2-toxin, DCM/MeOH/NH_4OH=10:2.5:0.5 was used for purification. In case of DON-3- and 15-sulfate and 3,15-disulfate, a mixture of DCM/MeOH/NH_4OH=10:4:1 was used. Since all products contained accompanying $HCOOHNH_4$, we tried to purify some products via a second and third column chromatography as well as via lyophilization. Nevertheless, we obtained all desired products as a white misty veil.

4.10. 3-Acetyl-DON-15-sulfate, ammonium salt (13)

Following the general deprotection procedure, **6** (34.0 mg, 62 μmol) was converted into **13** (19.3 mg, 72%). ^{1}H NMR (200 MHz, methanol-d_4) δ 6.63 (dq, J=6.1, 1.4 Hz, 1H), 5.11 (dt, J=11.3, 4.4 Hz,

1H), 4.92 (d, *J*=6.1 Hz, 1H), 4.89 (s, 1H), 4,87 (br, NH_4^+, H_2O), 4.27 (d, *J*=11.0 Hz, 1H), 3.94 (d, *J*=11.1 Hz, 1H), 3.85 (d, *J*=4.5 Hz, 1H), 3.16 (d, *J*=4.3 Hz, 1H), 3.12 (d, *J*=4.3 Hz, 1H), 2.79 (dd, *J*=15.3, 4.3 Hz, 1H), 2.08 (dd, *J*=15.3, 11.3 Hz, 1H), 2.13 (s, 3H), 1.85 (br, 3H), 1.18 (s, 3H); ^{13}C NMR (50 MHz, methanol-d_4) δ 201.0 (s, 1C), 172.5 (s, 1C), 139.4 (d, 1C), 137.1 (s, 1C), 80.5 (d, 1C), 75.8 (d, 1C), 72.7 (d, 1C), 70.7 (d, 1C), 67.1 (t, 1C), 66.3 (s, 1C), 52.3 (s, 1C), 48.4 (t, 1C), 46.8 (s, 1C), 41.7 (t, 1C), 20.8 (q, 1C), 15.3 (q, 1C), 14.4 (q, 1C); HRMS *m*/*z* calcd for $C_{17}H_{21}O_{10}S^-$ $[M-NH_4^+]^-$, 417.0861, found 417.0834.

4.11. 15-Acetyl-DON-3-sulfate, ammonium salt (18)

Following the general deprotection procedure, **11** (13.1 mg, 24 μmol) was converted into **18** (9.2 mg, 89%). ^{1}H NMR (400 MHz, methanol-d_4) δ 6.65 (dq, *J*=5.9, 1.5 Hz, 1H), 4.70–5.10 (m, NH_4^+, C3–H, C7–H, C11–H, H_2O), 4.30 (d, *J*=12.1 Hz, 1H), 4.23 (d, *J*=12.1 Hz, 1H), 3.82 (d, *J*=4.5 Hz, 1H), 3.12 (s, 2H), 2.63 (dd, *J*=15.3, 4.3 Hz, 1H), 2.11 (dd, *J*=15.3, 11.2 Hz, 1H), 1.90 (s, 3H), 1.85 (br, 3H), 1.11 (s, 3H); ^{13}C NMR (100 MHz, methanol-d_4) δ 201.1 (s, 1C), 171.9 (s, 1C), 139.7 (d, 1C), 137.0 (s, 1C), 81.2 (d, 1C), 75.2 (d, 1C), 75.0 (d, 1C), 71.3 (d, 1C), 65.9 (s, 1C), 63.3 (t, 1C), 52.6 (s, 1C), 48.2 (t, 1C), 47.0 (s, 1C), 42.5 (t, 1C), 20.6 (q, 1C), 15.4 (q, 1C), 14.4 (q, 1C); HRMS *m*/*z* calcd for $C_{17}H_{21}O_{10}S^-$ $[M-NH_4^+]^-$, 417.0861, found 417.0824.

4.12. DON-3-sulfate, ammonium salt (15)

Following the general deprotection procedure, **8** (18.8 mg, 37 μmol) was converted into **15** (14.3 mg, 98%). ^{1}H NMR (400 MHz, methanol-d_4) δ 6.61 (dq, *J*=6.1, 1.4 Hz, 1H), 4.75–4.95 (m, NH_4^+, C3–H, C11–H, H_2O), 4.79 (s, 1H), 3.80 (d, *J*=4.4 Hz, 1H), 3.78 (d, *J*=12.3 Hz, 1H), 3.68 (d, *J*=12.3 Hz, 1H), 3.12 (d, *J*=4.5 Hz, 1H), 3.09 (d, *J*=4.5 Hz, 1H), 2.75 (dd, *J*=15.2, 4.4 Hz, 1H), 2.06 (dd, *J*=15.2, 11.4 Hz, 1H), 1.83 (br, 3H), 1.12 (s, 3H); ^{13}C NMR (100 MHz, methanol-d_4) δ 201.7 (s, 1C), 139.4 (d, 1C), 137.0 (s, 1C), 81.2 (d, 1C), 75.8 (d, 1C), 75.4 (d, 1C), 71.6 (d, 1C), 66.3 (s, 1C), 61.8 (t, 1C), 53.6 (s, 1C), 48.2 (t, 1C), 46.7 (s, 1C), 42.5 (t, 1C), 15.4 (q, 1C), 14.6 (q, 1C); HRMS *m*/*z* calcd for $C_{15}H_{19}O_9S^-$ $[M-NH_4^+]^-$, 375.0755, found 375.0741.

4.13. DON-15-sulfate, ammonium salt (14)

Compound **13** (12.0 mg, 28 μmol, 1.0 equiv) was dissolved in 5 mL MeOH and NaOMe (3.0 mg, 55 mmol, 2.0 equiv) was added. After stirring for 2 h, TLC indicated full conversion of the starting material and the reaction was subjected to column chromatography (DCM/MeOH/NH_4OH=10:4:1), yielding **14** (7.5 mg, 69%) as white solid. ^{1}H NMR (200 MHz, methanol-d_4) δ 6.65 (dq, *J*=6.1, 1.4 Hz, 1H), 5.04 (d, *J*=6.1 Hz, 1H), 4.85–4.95 (m, NH_4^+, C7–H, H_2O), 4.76 (dt, *J*=11.1, 4.5 Hz, 1H), 4.25 (d, *J*=11.0 Hz, 1H), 3.96 (d, *J*=11.0 Hz, 1H), 3.55 (d, *J*=4.5 Hz, 1H), 3.12 (d, *J*=4.5 Hz, 1H), 3.06 (d, *J*=4.5 Hz, 1H), 2.57 (dd, *J*=14.8, 4.4 Hz, 1H), 1.99 (dd, *J*=14.8, 11.1 Hz, 1H), 1.84 (br, 3H), 1.14 (s, 3H); ^{13}C NMR (50 MHz, methanol-d_4) δ 201.1 (s, 1C), 139.9 (d, 1C), 136.9 (s, 1C), 82.3 (d, 1C), 75.9 (d, 1C), 70.7 (d, 1C), 69.6 (d, 1C), 67.2 (s, 1C), 66.7 (t, 1C), 52.5 (s, 1C), 48.2 (t, 1C), 47.5 (s, 1C), 45.0 (t, 1C), 15.3 (q, 1C), 14.4 (q, 1C); HRMS *m*/*z* calcd for $C_{15}H_{19}O_9S^-$ $[M-NH_4^+]^-$, 375.0755, found 375.0746.

4.14. DON-3,15-disulfate, diammonium salt (16)

Following the general deprotection procedure, twice the amount of $HCOONH_4$ (6 equiv) and Zn dust (18 equiv) were used to convert **9** (20.0 mg, 28 μmol) into **16** (7.4 mg, 54%). ^{1}H NMR (400 MHz, methanol-d_4) δ 6.67 (dq, *J*=6.0, 1.5 Hz, 1H), 4.80–5.00 (m, NH_4^+, C3–H, C7–H, C11–H, H_2O), 4.24 (d, *J*=10.9 Hz, 1H), 3.98 (d, *J*=10.9 Hz, 1H), 3.84 (d, *J*=4.5 Hz, 1H), 3.15 (d, *J*=4.3 Hz, 1H), 3.10 (d, *J*=4.3 Hz, 1H), 2.97 (dd, *J*=15.3, 4.5 Hz, 1H), 2.10 (dd, *J*=15.3, 11.4 Hz, 1H), 1.85 (br, 3H), 1.16 (s, 3H); ^{13}C NMR (100 MHz, methanol-d_4) δ 201.3 (s, 1C), 139.6 (d, 1C), 137.2 (s, 1C), 81.3 (d, 1C), 75.8 (d, 1C), 75.7 (d, 1C), 70.5 (d, 1C), 67.4 (s, 1C), 66.1 (t, 1C), 52.4 (s, 1C), 48.4 (t, 1C), 47.1 (s, 1C), 42.4 (t, 1C), 15.3 (q, 1C), 14.5 (q, 1C); HRMS *m*/*z* calcd for $C_{15}H_{20}O_{12}S_2^-$ $[M-2\times NH_4^+ + H^+]^-$, 455.0323, found 455.0300.

4.15. 2,2,2-Trichloroethyl T2-toxin-3-sulfate (21)

Compound **5** (37.4 mg, 80 μmol, 1.0 equiv) was dissolved in 3 mL of DCM, cooled to 0 °C, and 1,2-dimethylimidazole (30.8 mg, 321 mmol, 4.0 equiv) in 1 mL DCM was added to the reaction. Then, **26** (73.4 mg, 160 mmol, 2.00 equiv) was added and the reaction was allowed to reach room temperature over night. After 18 h, TLC showed substantial amounts of product and the reaction was directly used for column chromatography (DCM/MeOH=95:5), yielding **21** (26.4 mg, 49%) as white solid. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.16 (d, *J*=3.1 Hz, 1H), 5.77 (dt, *J*=5.7, 1.4 Hz, 1H), 5.28 (d, *J*=5.5 Hz, 1H), 5.10 (dd, *J*=4.9, 3.1 Hz, 1H), 4.80 (s, 2H), 4.31 (d, *J*=12.7 Hz, 1H), 4.25 (d, *J*=5.7 Hz, 1H), 4.10 (d, *J*=12.7 Hz, 1H), 3.96 (d, *J*=4.9 Hz, 1H), 3.09 (d, *J*=3.9 Hz, 1H), 2.85 (d, *J*=3.9 Hz, 1H), 2.35 (dd, *J*=15.2, 6.0 Hz, 1H), 2.11 (s, 3H), 2.09 (s, 3H), 2.00–2.25 (m, 3H), 1.80 (d, *J*=16.6 Hz, 1H), 1.76 (s, 3H), 0.96 (d, *J*=6.3 Hz, 3H), 0.95 (d, *J*=6.3 Hz, 3H), 0.74 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.8 (s, 1C), 170.4 (s, 1C), 170.2 (s, 1C), 137.1 (s, 1C), 123.0 (d, 1C), 92.6 (s, 1C), 87.1 (d, 1C), 80.1 (t, 1C), 78.4 (d, 1C), 77.4 (d, 1C), 67.7 (d, 1C), 67.5 (d, 1C), 64.6 (t, 1C), 63.8 (s, 1C), 48.7 and 47.5 (1t, 1s, 2× 1C), 43.7 (t, 1C), 43.1 (s, 1C), 28.4 (t, 1C), 25.9 (d, 1C), 22.6 (q, 1C), 22.5 (q, 1C), 21.2 (q, 1C), 20.8 (q, 1C), 20.4 (q, 1C), 6.5 (q, 1C); HRMS *m*/*z* calcd for $C_{24}H_{33}O_{12}S^-$ [M–TCE-group]$^-$, 545.1698, found 545.1679.

4.16. T2-toxin-3-sulfate, ammonium salt (22)

Following the general deprotection procedure, **21** (20.8 mg, 31 μmol) was converted into **22** (5.0 mg, 29%). ^{1}H NMR (400 MHz, methanol-d_4) δ 6.03 (d, *J*=3.1 Hz, 1H), 5.78 (dt, *J*=5.5 Hz, 1H), 5.33 (d, *J*=5.5 Hz, 1H), 4.80–4.94 (m, NH_4^+, C3–H, H_2O), 4.33 (d, *J*=12.1 Hz, 1H), 4.32 (d, *J*=5.5 Hz, 1H), 4.16 (d, *J*=12.1 Hz, 1H), 3.79 (d, *J*=5.1 Hz, 1H), 3.04 (d, *J*=3.9 Hz, 1H), 2.87 (d, *J*=3.9 Hz, 1H), 2.38 (dd, *J*=15.3, 5.5 Hz, 1H), 2.13–2.18 (m, 2H), 2.07 (s, 3H), 2.06 (s, 3H), 2.00–2.12 (m, 1H), 1.92 (d, *J*=15.3 Hz, 1H), 1.74 (s, 3H), 0.97 (d, *J*=6.7 Hz, 3H), 0.96 (d, *J*=6.7 Hz, 3H), 0.72 (s, 3H); ^{13}C NMR (100 MHz, methanol-d_4) δ 174.0 (s, 1C), 172.3 (s, 1C), 172.2 (s, 1C), 137.2 (s, 1C), 125.1 (d, 1C), 82.3 (d, 1C), 81.5 (d, 1C), 79.7 (d, 1C), 69.4 (d, 1C), 68.5 (d, 1C), 65.8 (t, 1C), 65.0 (s, 1C), 50.1 and 47.8 (1t, 1s, 2× 1C), 44.5 (t, 1C), 44.3 (s, 1C), 28.8 (t, 1C), 26.9 (d, 1C), 22.8 (q, 1C), 22.7 (q, 1C), 21.3 (q, 1C), 20.7 (q, 1C), 20.4 (q, 1C), 6.9 (q, 1C); HRMS *m*/*z* calcd for $C_{24}H_{33}O_{12}S^-$ $[M-NH_4^+]^-$, 545.1698, found 545.1682.

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Supplementary data

NMR spectra of all protected and isolated sulfates as well as tables for the ^{1}H chemical shifts of all substances. Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tet.2014.05.064. These data include MOL files and InChiKeys of the most important compounds described in this article.

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Article

Metabolism of Deoxynivalenol and Deepoxy-Deoxynivalenol in Broiler Chickens, Pullets, Roosters and Turkeys

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Abstract: Recently, deoxynivalenol-3-sulfate (DON-3-sulfate) was proposed as a major DON metabolite in poultry. In the present work, the first LC-MS/MS based method for determination of DON-3-sulfate, deepoxy-DON-3-sulfate (DOM-3-sulfate), DON, DOM, DON sulfonates 1, 2, 3, and DOM sulfonate 2 in excreta samples of chickens and turkeys was developed and validated. To this end, DOM-3-sulfate was chemically synthesized and characterized by NMR and LC-HR-MS/MS measurements. Application of the method to excreta and chyme samples of four feeding trials with turkeys, chickens, pullets, and roosters confirmed DON-3-sulfate as the major DON metabolite in all poultry species studied. Analogously to DON-3-sulfate, DOM-3-sulfate was formed after oral administration of DOM both in turkeys and in chickens. In addition, pullets and roosters metabolized DON into DOM-3-sulfate. *In vitro* transcription/translation assays revealed DOM-3-sulfate to be

2000 times less toxic on the ribosome than DON. Biological recoveries of DON and DOM orally administered to broiler chickens, turkeys, and pullets were 74%–106% (chickens), 51%–72% (roosters), and 131%–151% (pullets). In pullets, DON-3-sulfate concentrations increased from jejunum chyme samples to excreta samples by a factor of 60. This result, put into context with earlier studies, indicates fast and efficient absorption of DON between crop and jejunum, conversion to DON-3-sulfate in intestinal mucosa, liver, and possibly kidney, and rapid elimination into excreta via bile and urine.

Keywords: mycotoxins; biomarkers; HPLC-MS/MS; poultry; deoxynivalenol-3-sulfate; deoxynivalenol sulfonates

1. Introduction

After being discovered in 1972 [1], the Fusarium mycotoxin deoxynivalenol (DON) is one of the most common contaminants of cereal-based food and feed. Since its discovery, a lot of research has been carried out on its occurrence [2,3], toxicity [4], and reduction [5,6] as well as on its metabolization by plants [7], humans [8], and animals [9]. Nevertheless, the current knowledge, particularly on metabolization by different animal species, is far from complete.

One metabolization type common to most studied animal species is de-epoxidation. Formation of deepoxy-DON (DOM) is predominantly achieved by the gut microbiota. Its extent varies greatly both between species and also between individuals of one species [9,10]. The second metabolization pathway of DON observed in most of the investigated animal species is glucuronidation. Glucuronidation is carried out by endogenous UDP-glucuronosyltransferases in liver, and possibly also in intestinal microsomes [11]. In general, the extent of glucuronidation and the regiospecificity of the reaction are species-dependent with additional great individual differences [11–13].

Both de-epoxidation and glucuronidation have been studied as metabolization pathways of DON for many years. In humans and pigs, de-epoxidation is of minor importance [14–18], whereas glucuronidation is a major metabolization step [8,14,18–20]. In ruminants [21,22] and in rats [23], both de-epoxidation and glucuronidation of DON and of the formed DOM are significant metabolic processes. In addition, very recently two independent research groups discovered sulfonation as the main metabolization pathway of DON in rats [24,25]. Poultry, however, shows only minor metabolization of DON by glucuronidation, de-epoxidation, and sulfonation [26]. In contrast, sulfation was recently discovered as a major metabolization pathway, with DON-3α-sulfate as the predominant metabolic product of DON in chickens and turkeys [25,26]. According to Wan and co-workers who used radioisotope counting radio-HPLC for quantitation, DON-3-sulfate in excreta of chickens accounted for 89% ± 7% of the administered dose of DON (2.5 mg/kg body weight (b.w.)) [25]. Very recently, the toxicokinetic behavior of DON after oral application to turkeys and broiler chickens was investigated, and the peak areas of DON-3-sulfate and DON in plasma were compared [26]. Five minutes after administration of DON, the DON-3-sulfate to DON ratio in plasma was already 38 for turkeys and almost 2000 for broiler chickens. Thirty minutes after treatment, ratios increased to 141 and to nearly 10,000

for turkeys and broilers, respectively. These data indicate rapid absorption as well as very rapid and extensive metabolization of DON to DON-3-sulfate in both investigated avian species.

Although DOM is a minor metabolite in poultry [27,28], de-epoxidation of DON-3-sulfate or rapid sulfation of DOM may still be significant processes. We therefore hypothesized that DOM-3-sulfate might equally be a natural DON metabolite in poultry. The first aim of our work was therefore to synthesize and characterize DOM-3-sulfate and DOM-15-sulfate and to investigate their toxicity on ribosomes, the molecular targets of trichothecenes. The second aim was to develop and validate the first LC-MS/MS-based method for simultaneous quantitation of DON, DOM, DON-, and DOM-sulfate as well as DON- and DOM-sulfonates (DONS and DOMS) in excreta of different poultry species. The third objective of the work was to prove the hypothesis of formation of DON-3-sulfate and DOM-3-sulfate, as well as of DONS and DOMS in poultry consuming feed contaminated with DON and/or DOM. To this end, excreta and, in one occasion, chyme samples from four previous feeding trials with turkeys, chickens, pullets [28], and roosters [29,30] were re-analyzed. In three of these trials, feed consumption was recorded and the biological recoveries of orally administered DON and DOM could be assessed. In addition, the effect of species (turkey and chicken) and concomitant infection with *Ascaridia galli* on DON metabolization could be investigated. The results of this work will contribute to the understanding of DON metabolization in poultry**.**

2. Results

2.1. Synthesis, Purification, and Characterization of DOM-3-Sulfate and DOM-15-Sulfate

Similarly to production of DON-sulfates [31], synthesis of DOM-sulfates is a two-step reaction. In the first step, three protected intermediates were produced: 2,2,2-trichloroethyl-DOM-3-sulfate (71.0 mg, 29%), 2,2,2-trichloroethyl-DOM-15-sulfate (14.0 mg, 6%) and, as by-product, bis (2,2,2-trichloroethyl) DOM-3,15-disulfate (30.0 mg, 9%). These substances were recovered as white solid with an overall yield of 44%. NMR data of the protected intermediates are given in the electronic supplementary material; NMR spectra are shown in Figure S1. Deprotection of the intermediates and column chromatography with ammonium hydroxide as a mobile phase additive yielded 36.7 mg (67% of the protected intermediate) of DOM-3-sulfate, 5.3 mg (49%) of DOM-15-sulfate (both as ammonium salt), and 9.2 mg (45%) of the by-product DOM-3,15-disulfate (as diammonium salt). By using LC-HR-MS, the following molecular masses were obtained. DOM-3-sulfate: 360.0880 g/mol (exact molecular mass: 360.0879 g/mol); DOM-15-sulfate: 360.0879 g/mol (exact molecular mass: 360.0879 g/mol); DOM-3,15-disulfate: 440.0448 g/mol (exact molecular mass: 440.0447 g/mol). Results of NMR measurements are summarized in the electronic supplementary material; NMR and LC-HR-MS/MS spectra are given in Figures S2 and S3. The structures of DON- and DOM-sulfates are provided in Figure 1.

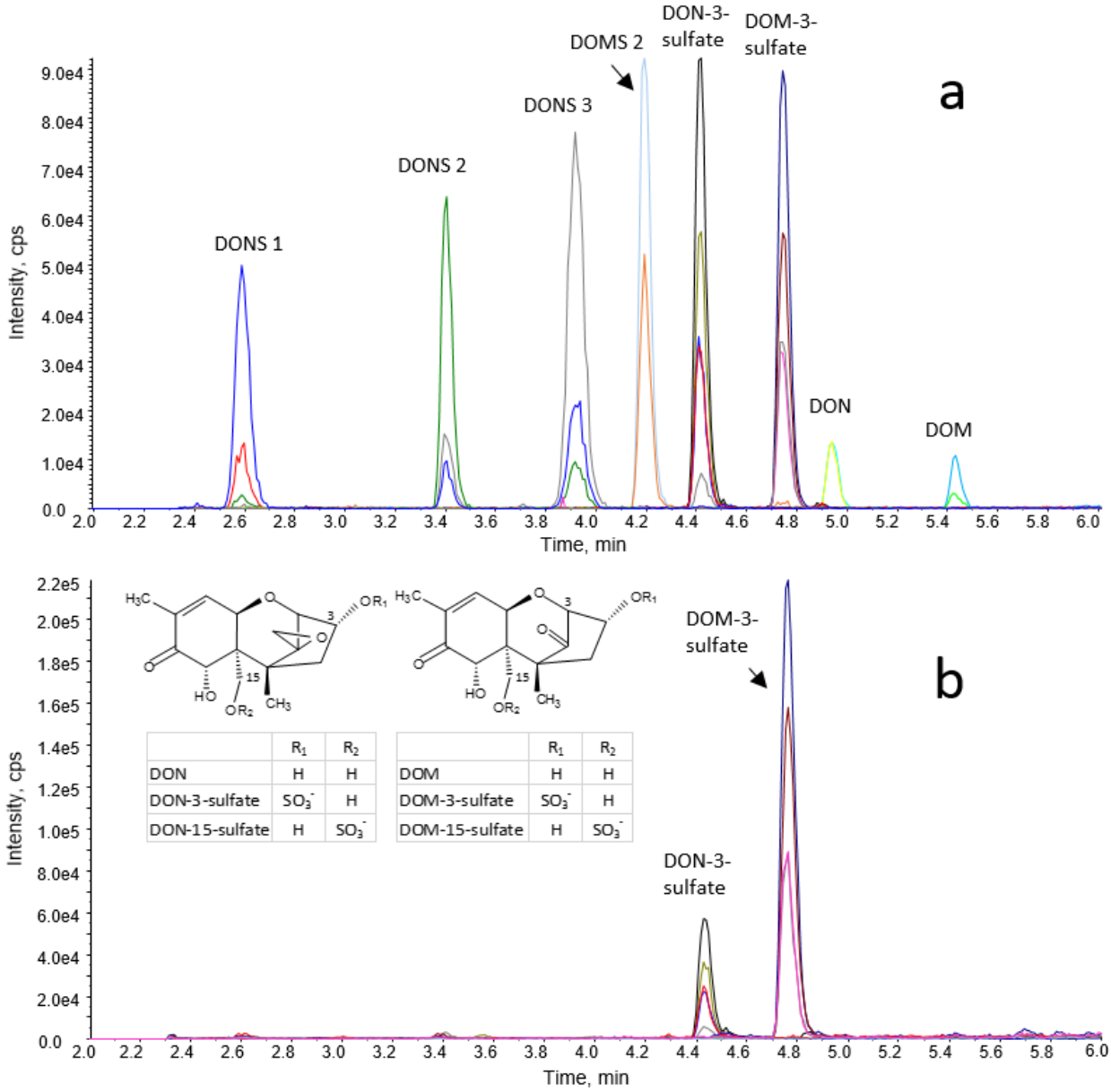

Figure 1. LC-MS/MS chromatogram of (**a**) a standard solution (30 ng/mL of DONS 1, DONS 2, DONS 3, DOMS 2, DON-3-sulfate, DOM-3-sulfate, DON, and DOM) and (**b**) of an excreta extract of a turkey fed a DOM-contaminated diet (1.6 mg/kg DOM and 0.2 mg/kg DON in feed).

2.2. Method Validation

For LC-MS/MS analysis, two LC gradient methods were developed, a long method capable of separating the 3/15 isomers of DON- and DOM-sulfate and a short method where the 3/15 isomers of DON- and DOM-sulfate are only partially separated. Chromatograms of a standard solution and of a turkey extract measured by the short method are shown in Figure 1, and a standard chromatogram recorded by the long method is depicted in Figure S4.

Prior to method validation, between four and six randomly selected samples of each matrix (excreta of turkeys, chickens, pullets, roosters, and chyme of pullets from both the DON- and DOM group, if available) were extracted and measured by the long gradient method. Due to presence of DON-3-sulfate

and in part DOM-3-sulfate, but absence of the 15-sulfate isomer in all investigated samples, the long gradient method was validated only with respect to matrix effects, whereas the short gradient method was selected for full validation and later on for measurement of all samples.

Apparent recoveries (R_{AS}), recoveries of extraction (R_{ES}), and matrix effects (SSE) for the short routine method are summarized in Table 1. Signal suppression and enhancement (SSE) of the long gradient method are given in Table S1 in the Supplementary Material. Recoveries of extraction ranged between 87% and 96% for all analytes. Hence, apparent recoveries were mainly determined by mass spectrometric SSE. Matrix effects for DON and DOM were between 80% and 108% in all sample extracts, resulting in apparent recoveries between 71% and 107%. Matrix effects for DON-3-sulfate, DOM-3-sulfate, as well as for DON- and DOM sulfonates were mostly between 100% and 120% with the exception of all analytes in chyme sample extracts and DON- and DOM sulfonates in excreta extracts of chickens. Accordingly, R_{AS}s mostly ranged between 90% and 110%. However, matrix enhancement by approximately 150% was observed for DON-3-sulfate, DOM-3-sulfate, and DONS 1 in jejunum and ileum of pullets. Exceptionally large matrix enhancement occurred for DONS 2, DONS 3, and DOMS 2 in chyme sample extracts and additionally for DONS 3 in excreta extracts of chickens. One reason is the lower dilution factor for chyme samples compared to excreta extracts, which was required because of lower analyte concentrations in jejunum and ileum compared to excreta. Additionally, DON sulfonates and DON-3-sulfate exhibit severe matrix enhancement in cereal sample extracts [32,33], and chyme samples still contain greater proportions of undigested cereal matrix. As indicated by these data, quantitation of DON- and DOM- sulfates and sulfonates in chyme and excreta samples of poultry requires determination of matrix effects in appropriately diluted blank extracts and correction by the respective R_{AS}.

Table 1. Validation of the short gradient method. R_A: apparent recovery, R_E: recovery of extraction, SSE: matrix effects.

		Dilution of Extract (v + v)	Average ± Standard Deviation (n = 3, Values in %)							
			DON-3-sulfate	DOM-3-sulfate	DON	DOM	DONS 1	DONS 2	DONS 3	DOMS 2
R_E	Excreta of turkeys		90 ± 1	91 ± 1	91 ± 1	89 ± 1	90 ± 2	90 ± 3	91 ± 3	90 ± 2
	Excreta of chickens		86 ± 5	88 ± 3	93 ± 4	87 ± 3	91 ± 3	96 ± 3	87 ± 7	90 ± 3
SSE	Excreta of turkeys	1 + 2	102 ± 1	99 ± 0	96 ± 2	80 ± 2	103 ± 2	122 ± 3	105 ± 1	107 ± 1
	Excreta of chickens	1 + 2	117 ± 2	112 ± 3	117 ± 2	97 ± 1	121 ± 1	138 ± 1	208 ± 6	131 ± 1
	Jejunum of pullets	1 + 1	153 ± 1	150 ± 1	93 ± 1	107 ± 1	149 ± 2	229 ± 14	334 ± 25	242 ± 20
	Ileum of pullets	1 + 1	149 ± 7	138 ± 1	99 ± 1	107 ± 1	177 ± 2	292 ± 9	365 ± 9	256 ± 6
	Excreta of pullets	1 + 5	100 ± 1	110 ± 1	101 ± 0	101 ± 0	121 ± 3	115 ± 1	110 ± 2	115 ± 0
	Excreta of roosters	1 + 9	93 ± 0	110 ± 2	101 ± 1	108 ± 1	133 ± 2	116 ± 2	116 ± 0	107 ± 1
R_A	Excreta of turkeys	1 + 2	92 ± 3	90 ± 2	87 ± 3	71 ± 3	92 ± 2	111 ± 1	96 ± 1	96 ± 2
	Excreta of chickens	1 + 2	98 ± 6	98 ± 6	107 ± 6	84 ± 4	110 ± 4	132 ± 7	178 ± 15	118 ± 2
	Jejunum of pullets	1 + 1	135 ± 2	134 ± 3	86 ± 2	94 ± 1	135 ± 1	213 ± 1	297 ± 5	218 ± 1
	Ileum of pullets	1 + 1	131 ± 1	124 ± 1	91 ± 1	94 ± 1	160 ± 2	272 ± 13	325 ± 22	230 ± 18
	Excreta of pullets	1 + 5	88 ± 6	98 ± 1	93 ± 1	89 ± 1	110 ± 2	107 ± 8	98 ± 8	104 ± 5
	Excreta of roosters	1 + 9	82 ± 1	98 ± 1	93 ± 0	95 ± 0	120 ± 3	108 ± 1	103 ± 2	96 ± 0

Limits of detection (LODs) and quantitation (LOQs) are given in Table 2. In pure standard solution, LODs ranged between 0.3 (sulfates and sulfonates of the series 2 and 3) and 1.5 ng/mL (DON, DOM),

LOQs between 1.0 and 4.5 ng/mL. LODs and LOQs in lyophilized samples depended on the dilution factor of the extract. In chyme samples, LODs were between 40 (sulfates and sulfonates of the series 2) and 200 (DOM) ng/g, LOQs between 120 and 600 ng/g. Highest LODs (up to 1000 ng/g for DOM) and LOQs (up to 3000 ng/g) were obtained for excreta of roosters, the extract of which was diluted 1 + 9 (v + v) before measurement.

Table 2. Limits of detection (LODs) in pure standard solution and in freeze-dried chyme and excreta samples of poultry. Limits of quantitation (LOQs) were by the factor of 3.3 higher.

Matrix	DON-3-sulfate	DOM-3-sulfate	DON	DOM	DONS 1	DONS 2	DONS 3	DOMS 2
Pure standard solution (ng/mL)	0.3	0.3	1.5	1.5	0.6	0.3	0.3	0.3
Excreta of turkeys (ng/g)	59	98	196	300	119	59	68	59
Excreta of chickens (ng/g)	51	98	196	400	119	59	68	69
Jejunum of pullets (ng/g)	40	40	130	200	79	40	46	40
Ileum of pullets (ng/g)	40	40	130	200	79	40	46	40
Excreta of pullets (ng/g)	102	147	391	800	237	119	137	138
Excreta of roosters (ng/g)	198	245	652	1000	396	198	228	198

Calibration functions in neat standard solution were linear from the LOQ up to at least 674 ng/mL for all analytes. Matrix-matched calibration functions were linear in the investigated calibration ranges (up to 674 ng/mL for excreta extracts of turkeys and chickens; up to 600 ng/mL for chyme and excreta extracts of pullets and excreta extracts of roosters) with the following exceptions: calibration curves of DONS 2, DONS 3, and DOMS 2 were linear up to 300 ng/mL in chyme extracts, and calibration functions of DONS 3 were linear up to 337 ng/mL in excreta extracts of chickens.

The relative standard deviation (RSD) of triplicate work-up and measurement of blank turkey and chicken excreta samples spiked at 4 concentration levels on one day was below 10% for each substance at each concentration level (mostly between 2% and 5%). The inter-day RSD of work-up and analysis of one freeze-dried excreta sample of one turkey of the DOM group containing 834 ng/g DON-3-sulfate and 3420 ng/g DOM-3-sulfate was 11% and 9% for DON-3-sulfate and DOM-3-sulfate, respectively (n = 10). The inter-day RSD of sample preparation and measurement of one broiler excreta sample containing 7720 ng/g DON-3-sulfate that was worked-up and analysed on 10 different days was 5%.

2.3. Analysis of Samples

Excreta and chyme samples of four different feeding trials with different poultry species were carried out in the past in order to study the metabolization of DON and DOM, to assess biological recoveries, and to investigate the effects of DON on health and performance in poultry. These samples were re-analysed using the newly developed LC-MS/MS method, which included DON- and DOM-3-sulfate as well as DON- and DOM sulfonate metabolites. An overview of these four feeding trials is given in Table 5.

Turkey and chicken trial: The two feeding trials with turkeys and chickens were carried out in order to study the metabolization of DON and DOM in poultry and in order to assess the biological recoveries of these two mycotoxins after oral administration at concentrations relevant in practice. For that,

the mycotoxin content in poultry feed (between 1.5 and 1.7 mg/kg) was kept well below the guidance value of 5 mg/kg in complementary and complete feeding stuffs. As birds excrete a mixture of white pasty urine and feces via the cloaca, only the combined excreta samples were collected.

Both in turkeys and in chickens, DON-3-sulfate was the major DON metabolite, followed by DON. Analogous to that, DOM-3-sulfate turned out to be the major metabolite of orally administered DOM. DOM itself could not be detected in any of the excreta samples of the turkey and the chicken trial, nor could any of the DON- or DOM sulfonates. The average DON equivalent concentrations of the main DON- and DOM metabolites in excreta of turkeys and chickens ($n = 4$ for each species) at the individual sampling times are given in Figure 2. In lyophilized excreta of the individual turkeys of the DON group, maximum DON equivalent concentrations of DON-3-sulfate ranged from 8.8 to 16 μg/g and were obtained between 2 h (mean concentration 9.6 μg/g) and 4 h (mean concentration 8.8 μg/g) after provision of feed. Maximum DON equivalent concentrations of DOM-3-sulfate in excreta of turkeys of the DOM group were measured in excreta collected 2 h after start of feeding and ranged between 5.3 and 18 μg/g (average 13 μg/g). Due to the presence of 0.3 mg/kg DON in feed of the DOM group, DON-3-sulfate was also excreted by turkeys of the DOM group. The excretion pattern was similar to that of DOM-3-sulfate, with a maximum mean value of 2.5 μg/g in DON equivalents. A chromatogram of a turkey excreta extract of the DOM group is given in Figure 1.

In freeze-dried excreta of chickens, average DON equivalent concentrations of DON-3-sulfate in the DON group and of DOM-3-sulfate in the DOM group were 2.8 and 3.0 μg/g, respectively, 3 h after provision of feed. At the later sampling points (6, 9, 12 and 24 h after start of feeding), average DON equivalent concentrations of DON-3-sulfate increased to between 4.8 and 5.8 μg/g in the DON group and mean DON equivalent concentrations of DOM-3-sulfate were between 4.0 and 4.8 μg/g in the DOM group. The average DON equivalent concentration of DON-3-sulfate in excreta of the control group was between 0.8 and 1.3 μg/g.

The feed consumption by turkeys was not significantly different for the three feeding groups (DON, DOM, control) and ranged from 110 to 380 g per turkey on the day of the feeding trial. Likewise, feed consumption in chickens was similar across the three feeding groups with average values between 105 and 115 g per chicken and day. In Table 3, the average amounts of DON-3-sulfate, DOM-3-sulfate and DON excreted by turkeys and chickens between 2 h and 24 h after the start of feeding are given. Comparison of DON equivalent amounts of total excreted metabolites and ingested toxins yielded biological recoveries in turkeys between 34% and 71% (52% ± 16%, average ± standard deviation, $n = 4$) in the DON group, between 33% and 76% (51% ± 18%) in the DOM group, and between 68% and 75% (72% ± 3%) in the control group. In chickens, biological recoveries ranged between 69% and 95% (80% ± 13%) in the DON group, between 68 and 78% (74% ± 4%) in the DOM group, and between 93% and 126% (106% ± 14%) in the control group.

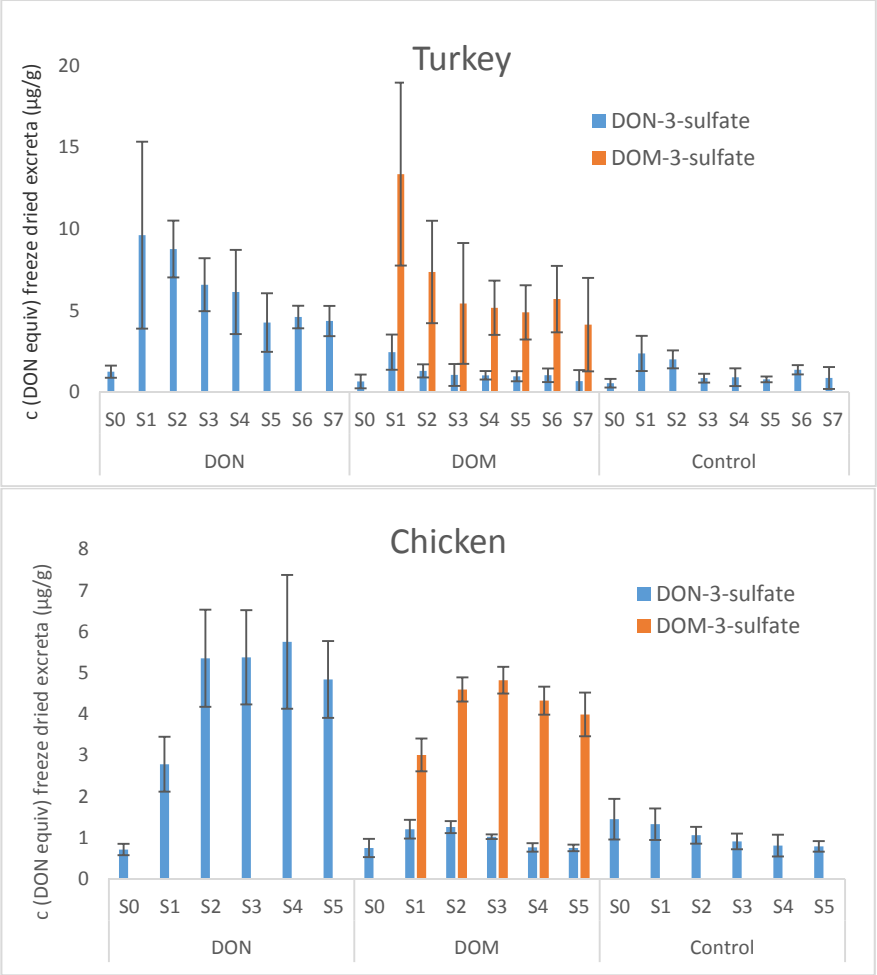

Figure 2. Average DON equivalent concentrations ± standard deviation (n = 4) of DON-3-sulfate and DOM-3-sulfate in excreta of turkeys and chickens at different sampling times (turkeys: 0, 2, 4, 6, 8, 11, 15 and 24 h after start of feeding; chickens: 0, 3, 6, 9, 12 and 24 h after start of feeding).

Table 3. Amounts of DON- and DOM metabolites excreted by turkeys and chickens between 2 h and 24 h after start of feeding (average ± natural standard deviation, n = 4, values in µg DON equivalents).

	Group	DON-3-sulfate	DOM-3-sulfate	DON	Sum Excreted	Consumed *	Biological Recovery (%)
Turkey	DON	144 ± 40	0.7 ± 0.8	5.2 ± 1.2	150 ± 42	304 ± 96	52 ± 16
	DOM	27 ± 13	149 ± 67	1.0 ± 1.4	177 ± 80	359 ± 159	51 ± 18
	Control	29 ± 7	0 ± 0	0 ± 0	29 ± 7	40 ± 10	72 ± 3
Chicken	DON	450 ± 112	0 ± 0	17 ± 12	476 ± 118	591 ± 77	80 ± 13
	DOM	72 ± 5	321 ± 30	1 ± 3	397 ± 33	536 ± 17	74 ± 4
	Control	71 ± 15	0 ± 0	0 ± 0	71 ± 15	67 ± 5	106 ± 14

* The average amounts of DON and DOM consumed were calculated by multiplying the concentration in feed by the amount of feed consumed by the individual animals between start and end of feeding.

Pullet trial: The original aim of the feeding trial with pullets was to investigate interactions between *Ascaridia galli* infection of pullets and DON contamination of feed [28]. As DON-3-sulfate and

DOM-3-sulfate had not been suspected as major DON metabolites in poultry at that time, biological recoveries had been poor (≤5%). Therefore, the excreta samples obtained in this animal experiment were re-analysed by the newly developed LC-MS/MS method including DON- and DOM-sulfate and sulfonate metabolites. As contents of ileum and jejunum were also collected, the metabolite pattern across the pullets' GI tract could be studied as well.

The average concentrations of DON metabolites in chyme and excreta samples are given in Table 4. Again, DON-3-sulfate was the main DON metabolite. However, contrary to chicken of the feeding trial described above, DOM-3-sulfate was also present in ileum and excreta samples of pullets. In addition, DON sulfonate metabolites of the series 1, 2 and 3 could be detected and quantified in jejunum and ileum samples. In general, both the metabolite concentrations and the metabolite pattern changed across the GI tract. In jejunum, DON-3-sulfate concentrations in the DON groups with and without *A. galli* infection were on average 3.5 times lower than in ileum and 60 times lower than in excreta samples. DOM-3-sulfate concentrations also increased from jejunum to excreta, reaching about 12%–15% of the DON-3-sulfate concentration in the latter matrix. DON sulfonate 2 (DONS 2) and DON sulfonate 3 (DONS 3) could be quantified in jejunum of the DON groups. In ileum, the concentration of DONS 2 remained similar, but DONS 3 could not be detected, most likely due to its instability under alkaline conditions [24,34]. However, traces of DONS 1 were observed in ileum, which might result from decomposition of DONS 3. In excreta, limits of quantitation were higher because of measurement of more diluted samples, so that only traces of DONS 2 were detected.

Table 4. Average concentrations of DON metabolites in chyme and excreta samples of pullets receiving 65 g feed containing 4.4 mg/kg DON (corresponding to 286 µg DON) per day. CON: negative control, -: no *A. galli* infection, +: *A. galli* infection, n.d.: not detected, tr: traces.

Matrix	Group	*n*	c (Average ± std dev, µg/g Freeze Dried Sample in DON Equivalents)					
			DON-3-Sulfate	DOM-3-Sulfate	DON	DONS 1	DONS 2	DONS 3
Jejunum	CON -	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	CON +	3	tr *	n.d.	n.d.	n.d.	n.d.	n.d.
	DON -	3	0.34 ± 0.04	n.d.	tr	n.d.	0.20 ± 0.05	0.08 ± 0.02
	DON +	3	0.35 ± 0.04	n.d.	tr	n.d.	0.17 ± 0.00	0.12 ± 0.04
Ileum	CON -	2	tr	n.d.	n.d.	n.d.	n.d.	n.d.
	CON +	3	tr	n.d.	n.d.	n.d.	n.d.	n.d.
	DON - **	2	1.26 ± 0.53	tr	tr	tr	0.10 ± 0.01	n.d.
	DON +	3	1.09 ± 0.11	1.00 ± 1.38	tr	tr	0.20 ± 0.12	n.d.
Excreta	CON -	9	1.83 ± 0.34 [a]	tr	n.d.	n.d.	n.d.	n.d.
	CON +	9	2.00 ± 1.00 [a]	tr	n.d.	n.d.	n.d.	n.d.
	DON -	9	22.9 ± 0.8 [b]	3.47 ± 0.14 [a]	tr	n.d.	tr	n.d.
	DON +	9	20.3 ± 1.8 [c]	2.49 ± 1.02 [b]	tr	n.d.	tr	n.d.

*: tr in one sample of three. **: for groups with $n = 2$, the value is given as average ± (max-min)/2. Statistical analysis (excreta): mean values with different superscripts within one column are significantly different.

Biological recoveries reached values of 151% ± 5% in the DON group without *A. galli* infection and 131% ± 11% in the DON group infected by *A. galli* (see Table S2 in the Supplementary Material).

Interestingly, statistical analysis revealed significant differences both between the concentrations and between the excreted amounts of DON-3-sulfate and DOM-3-sulfate in excreta of pullets of the DON groups with and without infection by *A. galli*. Excreta of pullets not infected by *A. galli* showed significantly greater concentrations of both sulfate metabolites than excreta of pullets infected with worms. Likewise, pullets not infected by *A. galli* excreted significantly greater amounts of DON-3-sulfate and of DOM-3-sulfate than pullets infected with *A. galli*.

Roosters: Excreta samples of roosters administered feed contaminated with 11 mg/kg DON [29] were analysed in order to study the metabolite pattern in roosters. Average DON-3-sulfate concentrations were 29 ± 3 μg/g freeze-dried excreta in DON equivalents. Surprisingly, mean DOM-3-sulfate concentrations amounted to 15 ± 3 μg/g in DON equivalents, yielding a DON-3-sulfate to DOM-3-sulfate ratio of 1.9 to 1. DONS 2 occurred at 0.8 ± 0.1 μg/g in DON equivalents, whereas only traces of DON were detected. Other compounds were below the respective LODs in rooster excrement extracts (see Table 2).

2.4. Toxicity assessment of DOM-3-Sulfate and DOM-15-Sulfate

The toxicity of DON, DON-3-sulfate, and DON-15-sulfate on the ribosome had recently been assessed in *in vitro* transcription/translation assays with wheat germ extract [31]. In short, DON-3-sulfate did not show any toxicity in the investigated concentration range, whereas DON-15-sulfate was *ca*. 44 times less toxic than DON. To compare the toxicities of DON, DOM, DOM-3-sulfate, and DOM-15-sulfate, *in vitro* transcription/translation assays were performed with mammalian ribosomes (Figure 3). While 50% reduction of *in vitro* translation by rabbit reticulocyte lysate was obtained at 0.6 μM DON, the IC_{50} value of DOM was 410 μM. DOM-3-sulfate and DOM-15-sulfate did not significantly inhibit *in vitro* translation at concentrations up to 100 μM. The translation efficiency was still 81% and 75% at 500 μM DOM-3-sulfate and DOM-15-sulfate, respectively. Inhibition of translation was not significantly different for DOM-3-sulfate and DOM-15-sulfate at substrate concentrations up to 250 μM. At a concentration of 500 μM, differences in translation were minor, but significant. Based on IC_{20} values for DON (0.25 μM), DOM (122 μM), DOM-3-sulfate (490 μM), and DOM-15-sulfate (420 μM), DOM-3-sulfate is less toxic than DOM by a factor of four and less toxic than DON by almost a factor of 2000. Similarly, DOM-15-sulfate shows lower toxicity on the ribosome than DOM by a factor of 3.4 and lower toxicity than DON by a factor of nearly 1700. Statistical analysis revealed significantly greater inhibition of translation by DOM compared to DOM-3-sulfate and DOM-15-sulfate from 250 μM onwards. These results confirm that de-epoxidation of DON leads to a less toxic metabolite and that sulfation of trichothecenes is, similar to glucosylation in plants, a detoxification mechanism.

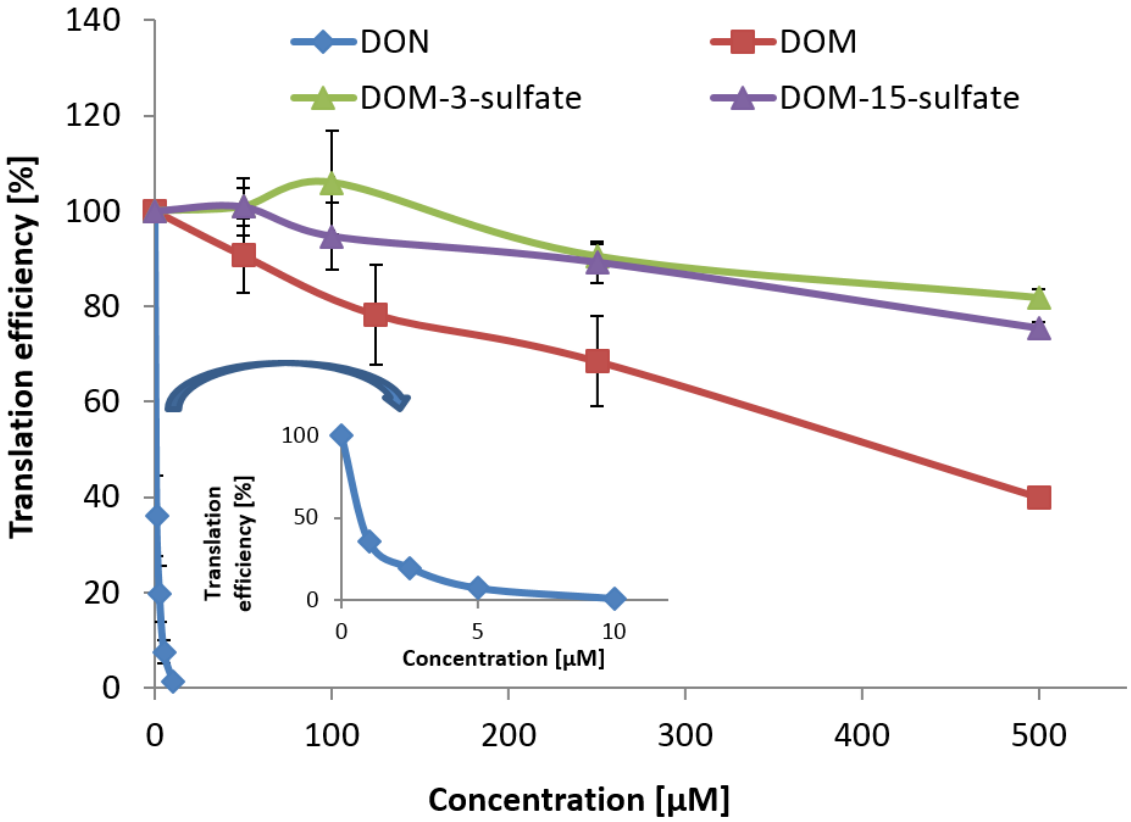

Figure 3. Comparative toxicity of DON, DOM, DOM-3-sulfate, and DOM-15-sulfate on the ribosome, determined by an *in vitro* transcription/translation assay with rabbit reticulocyte lysate. Error bars denote the analytical standard deviation of three independent determinations.

3. Discussion

Concluding from our results and those published in the most recent literature [25,26], DON-3-sulfate and DOM-3-sulfate are the missing link to quantitative biological recoveries of DON in poultry. Similar to previous studies reporting percentages of DON in excreta as being between 2% and 6% of total ingested DON [28,35], excreted DON was 2% and 3% of ingested DON in our turkey and chicken trial and approximately 4% in the pullet experiment. DOM was rarely detected in excreta samples in the literature. Whereas Wan *et al.* and Awad and co-workers could not detect DOM in excreta extracts [25,35], Dänicke *et al.*, applying immunoaffinity clean-up as sample preparation, determined 0.5 µg DON/kg b.w./day for pullets consuming *ca.* 300 µg DON/kg b.w./day, corresponding to approximately 0.2% of ingested DON [28]. Re-analyzing these pullet excreta samples, we could not detect DOM, most likely due to higher LODs due to greater dilution factors (see below). Similarly, DOM could not be detected in any of the excreta samples of our turkey and chicken experiments. One reason might be lack of microbes capable of de-epoxidizing DON. Another reason is that our methods were not designed to detect trace concentrations of DOM, but to allow co-determination of DON- and DOM-sulfates, DONS, DOMS, DON and DOM. Separation of DONS and DOMS requires formic acid as a mobile phase additive [24]. However, use of formic acid resulted in fivefold higher LODs for DON and DOM compared to acetic acid. Still, this compromise had to be made for proper separation of DON sulfonates. LOQ values for DON and DOM in a pure standard solution (5 ng/mL) were higher than those

obtained by Devreese *et al.* (0.1 and 0.5 ng/mL for DON and DOM), but similar to those obtained in our previous work on DON- and DOM sulfonates (2 ng/mL on 5500 QTrap, 5 ng/mL on 4000 QTrap). LOQ values in lyophilized pullet feces obtained by Dänicke and co-workers were 0.8 and 1.6 ng/g [28], and as such far better than ours. The reason for this great difference is that Dänicke *et al.* used multi-step sample preparation including defatting and immunoaffinity clean-up. In our work, on the contrary, great dilution factors were chosen on purpose due to the high concentrations of DON-3-sulfate and in order to minimize matrix effects.

As DOM and DOM-sulfates are much less toxic than DON in an *in vitro* translation assay using animal ribosomes, and the main mode of action of all trichothecenes is the suppression of translation, these metabolites can be considered as detoxification products of DON. In the following, the question about location of formation, absorption and elimination of DON-3-sulfate and DOM-3-sulfate will be tackled by putting our findings and recent literature results in context with older studies on DON metabolization in poultry. For this purpose, it is important to note that poultry excrete white pasty urine into the cloaca where it is mixed with solid excrements from the GI tract. Hence, administration of native DON to poultry and collection of excrements does not give information about the origin of excreted DON-3-sulfate and DOM-3-sulfate. Another peculiarity of poultry is that, in addition to the hepatic portal system, a renal portal system exists. Both systems drain the intestine via the *Vena mesenterica cranialis* and *V. mesenterica caudalis*, respectively, and might contribute to renal and/or hepatic metabolism and first pass elimination as suggested by Rotter *et al.* [36].

As early as in 1986, Prelusky and co-workers administered a single oral dose of ^{14}C-labeled DON to white Leghorn hens by crop intubation [37]. They observed low levels of radioactivity in plasma and reported the total body burden (excluding bile, GI tract and excreta) of radioactively-labeled DON and its metabolites to be less than 2%. The highest accumulation of radioactivity was determined in bile 6 h after treatment. Within 24 h, 79% of radioactivity was excreted. These results suggested low systemic absorption of DON and/or its metabolites and significant biliary excretion.

Two years later, Lun *et al.* [38], administering native DON to laying domestic chickens, reported that DON disappeared from the GI tract between the crop and the jejunum. However, only low levels of DON were detected in portal vein blood. Considering little decrease of DON when incubated with gastric fluid or juice from the small intestine [38], they set up the hypothesis of rapid post-absorptive modification of DON by enterocytes and hepatic elimination into bile. In the same study, *in vitro* incubation of native DON with contents of colon and caecae resulted in the reduction of the DON concentration by factors of 3, 9 and 20 after 6, 12 and 24 h of incubation, respectively.

One year later, Lun and co-workers administered tritium labeled DON by crop intubation to colostomized white Leghorn hens [39]. Interestingly, 68% of the administered radioactivity was excreted within 24 h into urine, whereas only 6% was eliminated via feces. Radioactivity in systemic blood accounted for only 7% of the administered radioactivity. Radioactivity along the GI tract of intact crop intubated hens decreased rapidly after the crop, but increased again in the large intestine six hours after administration, which is most likely due to retrograde movement of urine from cloaca into colon. However, contrary to observations by Prelusky and co-workers [37], biliary excretion of radioactivity was <1%. These results confirmed the hypothesis set up by the same working group in 1988 that ingested DON is rapidly absorbed between crop and upper jejunum and metabolized shortly after absorption [38].

Yet, extensive excretion of the altered DON via bile was not confirmed as most of the radioactivity was excreted into urine.

Results obtained in more recent studies where native or radiolabeled DON was administered to poultry mostly confirmed and complemented the earlier published data. Yunus *et al.* [27] recovered only 0.04% of total DON fed to broilers 1 h after oral treatment in plasma in the form of DON and DOM. Likewise, Dänicke and co-workers determined only trace levels of DON in plasma of pullets fed with poultry feed containing 4.4 mg/kg DON. However, two recent studies reported an oral bioavailability of 20% for DON administered to broiler chickens [40], and 21% for DON fed to turkeys [26]. Higher values than previously reported might be due to different ways of calculating the oral bioavailability. In accordance with earlier studies, rapid absorption, fast elimination, and extensive biotransformation were reported [26,40]. Comparison of oral and IV application of DON and inclusion of DON-3-sulfate in the analytical method for semiquantitative determination yielded precious information on the toxicokinetic behavior of DON and on the location of sulfation in turkeys and broiler chickens [26]. Already 5 min after oral administration, DON and DON-3-sulfate were detected in plasma. Maximum values of DON were reached 10 min post dosing, and maximum values of DON-3-sulfate between 20 and 30 min after application. Only 5 min after IV administration of DON, the average peak area ratio of DON-3-sulfate to DON was 10 in turkeys and 243 in broiler chickens. This finding points to very rapid sulfation, with the liver, extrahepatic tissues, and the intestinal mucosa being potential metabolism sites. In addition, average peak area ratios of DON-3-sulfate to DON were much greater after oral, rather than after IV administration in both avian species. This observation strongly substantiates the hypothesis by Lun *et al.* [38,39] that sulfation is already performed in enterocytes of the intestinal mucosa.

Our current turkey and chicken trial revealed that metabolization of DON and DOM was very similar in both avian species. Excretion kinetics could not be established because animals had *ad libitum* access to DON and DOM contaminated feed during the day. Despite large inter-individual differences between the turkeys of one feeding group, maximum metabolite concentrations in excreta between 2 and 4 h after the start of feeding and maximum feed intake in the morning suggest fast transit times in turkeys. In chickens, excreta were sampled 3 and 6 h after provision of DON or DOM contaminated feed, so that transit times can only roughly be estimated. Still, maximum concentrations obtained at the latest 6 h after start of feeding corroborate fast excretion of DON- and DOM metabolites also in chickens.

Low concentrations of DON-3-sulfate and DON in chyme samples (content of jejunum and ileum) of pullets support the hypothesis of rapid and extensive absorption of DON from the small intestine [39]. The origin of DON-3-sulfate in jejunum and ileum might be biliary excretion of DON-3-sulfate formed in enterocytes or in the liver. As chyme samples were collected 3 h after administration of DON, biliary excretion might not have arrived at its maximum which was reported to occur *ca.* 6 h after dosing [37].

Presence of DOM-3-sulfate in excreta of pullets and roosters might be due to formation of DOM by intestinal microbes, absorption of DOM from the GI tract, sulfation in the intestinal mucosa, liver and/or kidney, and excretion into the cloaca via urine or back into the GI tract via bile. The latter could also explain low concentrations of DOM-3-sulfate in ileum. An additional hypothesis is antiperistaltic retrograde movement of DON-3-sulfate from the cloaca to the microbial rich caecae and microbe-mediated formation of DOM-3-sulfate from DON-3-sulfate. A third possibility might be sulfation in DOM by intestinal microbes. Absence of DOM-3-sulfate in excreta samples of our turkey and chicken trial might be due to differences in the intestinal microflora, specifically due to lack of microbes capable of

de-epoxidizing DON. Although infection with *A. galli* resulted in significantly lower formation of DON-3-sulfate and DOM-3-sulfate in pullets, the formation pattern of DON metabolites was unaltered by worm infection.

Concluding from the DON sulfonate pattern across the GI tract of pullets, DON sulfonates are formed early in the digestion process. The mechanism of DON sulfonate formation is currently unknown. Some experiments aiming at elucidating the mechanism of DON-, DOM-, and DON-3-glucoside (D3G) sulfonate formation in rats had been carried out lately [24], but were inconclusive. However, because the major part of the sulfonates was recovered in feces of DON treated rats and the formation pattern shifted to DOM sulfonate on the second day after treatment, authors speculated that DON-, DOM-, and D3G sulfonates might be formed in a Michael addition with reactive inorganic sulfur species possibly present in intestinal chyme rather than by liver enzymes.

Biological recoveries of DON in the current study are much higher than those previously published for native DON administered to chickens, which were ≤5% [28]. However, they compare nicely to values published for experiments with radiolabeled DON. Wan *et al.* [25], using radioisotope counting radio-HPLC, reported biological recoveries of 81%, 89% and 95% in the time span of 24, 48 and 72 h after oral administration of a single dose of radiolabeled DON. Likewise, Prelusky *et al.* [37] recovered 79%, 92% and 99% of radiolabeled DON by 24, 48 and 72 h after single bolus administration to white Leghorn hens. The finding that excretion is not complete 24 h after treatment explains incomplete biological recoveries in our feeding trials with turkeys and chickens. Lower biological recoveries in turkeys than in chickens hint at delayed excretion in turkeys compared to chickens. Recoveries greater than 100% in the pullet trial might be due to presence of DON-3-glucoside (D3G) in the Fusarium contaminated diet used in the animal experiment. As D3G was not determined in the administered feed, its presence and subsequent *in vivo* metabolization to DON- and DOM-3-sulfate cannot be excluded.

To summarize, our findings and data from the recent literature substantiate, complement, and extend results and hypotheses from earlier published articles on the absorption, metabolism, and excretion of DON in poultry. DON is, to a great extent, rapidly absorbed from the GI tract between crop and jejunum. After absorption, DON is extensively metabolized to DON-3-sulfate in the intestinal mucosa, the liver, and possibly in the kidney. DON-3-sulfate is rapidly and completely eliminated into bile by the liver and/or into urine by the kidney. DOM-3-sulfate is an important natural DON metabolite in pullets and roosters and might originate from sulfation of microbe-formed DOM by intestinal mucosa, liver, and/or kidney or from microbe mediated deepoxidation of urinary DON-3-sulfate transported into caecae by retrograde movement.

4. Experimental Section

4.1. Reagents

Methanol (MeOH, LC gradient grade) and formic acid (98%–100%, p.a.) were purchased from Merck (Darmstadt, Germany). Formic acid (LC-MS gradient grade) for LC-MS/MS was obtained from Sigma Aldrich (Vienna, Austria). Reagents for synthesis of DOM-sulfates were purchased from ABCR (Karlsruhe, Germany) and Sigma Aldrich (Vienna, Austria). In all experiments, ultra-pure water (delivered by a Purelab Ultra system (ELGA LabWater, Celle, Germany)) was used.

4.2. Synthesis, Purification and Characterization of DOM-3-Sulfate and DOM-15-Sulfate

DOM as the starting material for synthesis of DOM-3-sulfate and DOM-15-sulfate was produced from DON as described earlier [24] and purified by preparative chromatography. The preparative HPLC system and column were the same as described in [34]. Mobile phase A consisted of methanol/water/formic acid (10/89.9/0.1, v/v/v), mobile phase B of methanol/formic acid (99.9/0.1, v/v). Gradient elution started at 0% B from 0 to 1 min and continued with linear increase to 100% B from 1 to 6 min. After 1 min at 100% B the starting conditions were re-established from 7 to 7.1 min and the column was re-equilibrated at 0% B until 10 min. The injection volume was 700 µL, the flow rate 16 mL/min and DOM was detected by measurement of UV absorption at 220 nm. The collection window of DOM was 5.66–6.03 min.

The synthesis of DOM-3-sulfate and DOM-15-sulfate was carried out as described for DON [31] with minor modifications. In short, it included synthesis of protected intermediates (4.2.1) and subsequent deprotection (4.2.2) followed by purification by column chromatography. The progress of all reactions was monitored by thin layer chromatography (TLC) using silica gel 60 F_{254} TLC plates (Merck, Germany). All chromatograms were visualized using ceric ammonium molybdate/Hanessian's stain in EtOH/sulphuric acid. Column chromatography was performed on silica gel 60 (40–63 µm) using a Sepacore™ Flash System (Büchi, Switzerland) or small glass columns.

4.2.1. Synthesis of the Protected Intermediates

DOM (140.0 mg, 0.50 mmol, 1.0 equivalents (eq.)) was dissolved in 20 mL of dry dichloromethane (DCM), cooled to 0 °C, and 1,2-dimethylimidazole (144.0 mg, 1.50 mmol, 3.0 eq.) was added to the mixture. Finally, 2,3-dimethyl-1-((2,2,2-trichloroethoxy)sulfonyl)-1H-imidazolium trifluoromethanesulfonate (342.8 mg, 0.75 mmol, 1.5 eq.) was added and the reaction was allowed to reach room temperature overnight. TLC after 1 day showed nearly full conversion of the starting material. Hence, the reaction was directly subjected to column chromatography (hexane/ethyl acetate 3/1, v/v) upon which three protected intermediates (2,2,2-trichloroethyl-DOM-3-sulfate (71.0 mg, 29%), 2,2,2-trichloroethyl-DOM-15-sulfate (14 mg, 6%) and bis(2,2,2-trichloroethyl) DOM-3,15-disulfate (30.0 mg, 9%)) were obtained.

4.2.2. Deprotection of Protected Intermediates

Deprotection of the protected intermediates was carried out as described in [31]. The starting material was dissolved in MeOH (1 mL/10 µmol). Ammonium formate (3 eq. for the monosulfates, 6 eq. for the disulfate) as well as Zn dust (9 and 18 eq., respectively) were added, and the reaction mixture was placed in an ultrasonic bath at room temperature. The progress of the reaction was monitored by TLC until substantial amounts of products were formed (20 to 120 min). After filtration through celite, the remaining residue was subjected to column chromatography to end up with the corresponding sulfates as ammonium salts. For this purpose, a mobile phase of DCM/MeOH/NH_4OH (10/4/1, v/v/v) was used. The final products were dissolved in water, lyophilized, and finally obtained as a white powder.

4.2.3. NMR Spectroscopy

^{1}H and ^{13}C spectra were recorded on a Bruker Avance DRX-400 MHz and a Bruker Avance III 600 MHz spectrometer (Bruker, Germany). Data were recorded and evaluated using TOPSPIN 1.3 and TOPSPIN 3.2 (Bruker Topspin). All chemical shifts are given in ppm relative to tetramethylsilane. The calibration was done using residual solvent signals. Multiplicities are abbreviated as s (singlet), d (doublet), t (triplet), q (quartet) and b (broad signal). Deuterated solvents were purchased from Eurisotop (Gif sur Yvette Cedex, Paris, France).

4.2.4. LC-HR-MS/MS

LC-HR-MS(/MS) spectra were recorded on a 6550 iFunnel Q-TOF instrument coupled to a 1290 Infinity UHPLC system (both Agilent Technologies, Waldbronn, Germany). Chromatographic separation was carried out on a Zorbax Eclipse Plus C18 Rapid Resolution High Definition column (2.1 × 150 mm, 1.8 µm particle size, Agilent, Waldbronn, Germany) at a flow rate of 0.25 mL/min using gradient elution (0 min: 15% B, 6 min: 100% B, 7 min: 100% B, 7.1 min: 15% B, 9 min: 15% B). Mobile phase A was water/formic acid (99.9/0.1, v/v), mobile phase B MeOH/formic acid (99.9/0.1, v/v). Compounds were ionized by electrospray ionization in the negative mode and measured first in full scan and then in targeted MS/MS mode at a collision energy of 30 eV (both in the range from *m/z* 40-1000). Electrospray ionization was carried out at a gas temperature of 140 °C, drying gas flow of 14 L/min, nebulizer pressure of 35 psig, sheath gas temperature of 350 °C, and sheath gas flow of 11 L/min. The capillary voltage was 4500 V, the nozzle voltage 300 V. Data acquisition was achieved in the 2 GHz extended dynamic range mode.

4.3. Standards and Standard Solutions

Solid DON (purity >95%) as well as standard solutions of DON and DOM (both 50 mg/L in acetonitrile) were supplied by Romer Labs GmbH (Tulln, Austria). DON-3-sulfate and DON-15-sulfate (both 95% purity) were synthesized according to [31]. DOM, DOM-3-sulfate, and DOM-15-sulfate were produced as described above (4.2). DON sulfonates 1, 2, 3 and DOM sulfonate 2 were produced as described in [24].

A mixed standard solution containing 300 mg/L of DON-3-sulfate, DOM-3-sulfate, DON, DOM, DONS 1, DONS 2, DONS 3, and DOMS 2 was prepared in methanol/water/formic acid (20/79.9/0.1, v/v/v). This solution and several dilutions thereof were used for spiking experiments and the establishment of pure solvent and matrix-matched calibration functions.

4.4. Sample Preparation Methods for Determination of DON, DOM, and Their Sulfates in Excreta and GI Samples of Poultry

Extraction of DON, DOM and their sulfate- and sulfonate metabolites from excreta samples was carried out according to a procedure previously optimized for the extraction of DON- and DOM sulfonates from rat feces [24]. A 300 mg aliquot of homogenized lyophilized excreta or GI content sample was consecutively extracted with 4, 3 and 3 mL of methanol/water/formic acid (49.5/49.5/1, v/v/v) by shaking for 30, 20 and 10 min in 15 mL polypropylene tubes. Prior to LC-MS/MS analysis,

aliquots of the pooled extracts were diluted with water (turkeys, chickens: 1 + 2; pullets: jejunum and ileum: 1 + 1, excreta: 1 + 5; roosters: 1 + 9) and centrifuged at 14,000 × *g*.

4.5. LC-MS/MS Analysis of DON, DOM, Their Sulfates and Their Sulfonates in Excreta of Poultry

LC-MS/MS analyses were carried out on an Agilent 1290 series UHPLC system coupled to a 6500 QTrap mass spectrometer equipped with an IonDrive Turbo V® source (Sciex, Foster City, CA, USA). Chromatographic separation was achieved on a Kinetex Biphenyl column (150 × 3 mm, 2.6 µm) protected by a SecurityGuard ULTRA pre-column of the same stationary phase (both Phenomenex, Aschaffenburg, Germany) at 30 °C and at a flow rate of 0.4 mL/min. Mobile phase A consisted of water/formic acid, mobile phase B of methanol/formic acid (both 99.9/0.1, v/v). Two different gradient methods were used, a short one for routine measurements, and a long one for separation of the 3/15 isomers of DON- and DOM-sulfate. The gradient of the short routine method was: 0.0–0.5 min: 10% B, 6.0 min: 90% B, 6.1–7.5 min: 100% B, 7.6–10.0 min: 10% B. Time segments of the gradient of the long method were: 0.0–0.5 min: 10% B, 4.5 min: 40% B, 8.0 min: 45% B, 8.5–10.9 min: 100% B, 11.0–13.5 min: 10% B. The injection volume was 3 µL and the LC eluent was diverted to the MS between 2.3 and 6.0 min (8.0 min for the long method).

Tandem mass spectrometric detection was performed in negative selected reaction monitoring (SRM) mode after electrospray ionization. The following ion source settings were used: Temperature 400 °C, ion spray voltage -4500 V, curtain gas 35 psi, ion source gas 1 80 psi, ion source gas 2 90 psi, collision gas (N_2) high. SRM parameters (declustering potential, collision energy, collision cell exit potential) were optimized for the individual analytes by software-controlled compound optimization and are listed in Supplementary Table S3. Analyst® software version 1.6.2 (Sciex) was used for instrument control and data evaluation.

4.6. Method Validation

Validation of the short routine method included determination of the apparent recovery (R_A), recovery of extraction (R_E), and mass spectrometric matrix effects (SSE) for excreta of turkeys and chickens as well as assessment of SSE for extracts of excreta and GI content samples of pullets, and excreta extracts of roosters. In addition, intra- and inter-day repeatability of sample work-up and analysis, limits of detection and quantitation, and linear range of calibration functions were evaluated. The long gradient method was validated with respect to SSE in excreta extracts of turkeys and chickens.

For assessment of R_A, R_E, and SSE in excreta samples of turkeys and chickens, 300 mg aliquots of freeze dried excreta collected before administration of toxins were spiked with 60 µL aliquots of spiking solutions containing between 0.5 and 300 mg/L of DON-3-sulfate, DOM-3-sulfate, DON, DOM, DONS 1, DONS 2, DONS 3, and DOMS 2, resulting in 0.1 to 60 mg/kg of these analytes in the freeze dried sample aliquots. Spiking was performed at seven concentration levels (0.1/0.3/1/3/10/30/60 mg/kg) in triplicate. One hour after spiking, 21 spiked and three unspiked samples were worked-up and diluted as described above, and measured in the same run as matrix-matched and pure solvent calibration functions. Pooled diluted extracts of unspiked samples were used to prepare matrix-matched calibration functions as described in detail in [24]. Both matrix-matched and pure solvent calibration functions were established at seven concentration levels (1.1/3.4/11/34/112/337/674 ng/mL), which corresponded to the theoretical

analyte concentrations in measurement solutions of samples spiked prior to work-up in the case of 100% apparent recovery. R_A, R_E, and SSE were calculated by comparing the slopes of the standard addition curve (k_{SA}), matrix-matched calibration curve (k_{MM}), and pure solvent calibration curve (k_{SOL}) as described in [41]. The following equations were used: $R_A = k_{SA}/k_{SOL} \times 100$; $R_E = k_{SA}/k_{MM} \times 100$; $SSE = k_{MM}/k_{SOL} \times 100$. Matrix-matched calibration functions were established between 3 and 600 ng/mL in diluted extracts of chyme and excreta samples of the negative control group of pullets and in excreta sample extracts of roosters, and used for calculation of SSE in these matrices. Apparent recoveries in the same matrices were estimated by multiplying the SSE of each individual analyte by the average R_E of the same analyte determined in excreta of turkeys and chickens (see above).

The intra-day repeatability was determined by triplicate work-up and measurement of blank turkey and chicken excreta samples spiked at four concentration levels (1, 3, 10, 30 ng/g) on one day. The inter-day repeatability was assessed by work-up and analysis of two randomly selected excreta samples (one of turkey, one of chicken) on each day sample preparation and analysis was carried out.

Limits of detection (LOD, signal to noise ratio (S/N) 3/1) and quantitation (LOQ, S/N 10/1) were determined in pure solvent standard solutions and in matrix-matched standard solutions. LODs and LOQs in freeze-dried samples were calculated by dividing LODs and LOQs in matrix-matched solutions by the recovery of extraction and by multiplying by the dilution factor. The linear range in matrix-matched calibration curves defined the upper end of the working range.

4.7. Design of the Feeding Trials

In total, excreta samples from four different feeding trials with poultry were analyzed. The parameters of the individual feeding trials are summarized in Table 5. Turkeys, chickens, and pullets of the negative control groups were fed with basal poultry feed naturally contaminated with 0.2–0.3 mg/kg DON. Turkeys and chickens of the DON group received basal poultry feed enriched with DON from culture material to a concentration of 1.5–1.7 mg/kg. Similarly, turkeys and chickens of the DOM group were fed with basal poultry feed artificially contaminated with the equimolar concentration of DOM. Pullets were exposed to 4.4 mg/kg DON in feed. As this feed was prepared from wheat contaminated with different Fusarium toxins [28], it also contained traces of 3-acetyl-DON (0.13 mg/kg) and 15-acetyl-DON (0.03 mg/kg). Roosters received diet containing 11 mg/kg DON.

All feeding trials and animal experiments were conducted following the European Guidelines for the Care and Use of Animals for Research Purpose [42]. The feeding trials with turkeys and chickens lasted for one day during which animals had unlimited access to feed and water until feed was removed in the evening. Animals were housed under a light/dark cycle of 18/6 h. Feed consumption of the individual animals was recorded. Excreta samples were collected at regular intervals during the day and in the morning of the following day. After the experiment, animals received basal poultry feed for two weeks before they were reintegrated into the meat production process. In the animal experiment with pullets, pullets received 65 g feed/day in two equal portions for two weeks and excreta samples were collected twice a day in the second week [28]. Pooled excreta samples were used for determination of biological recoveries. On the last day of the trial, pullets were slaughtered 3 h after the last feeding and chyme samples (contents of jejunum and ileum) were taken. Roosters were fed the DON contaminated feed at amounts of 90 g/day and excreta were collected for seven days [30]. Samples taken on different days

were pooled for each animal prior to analysis. All excreta and chyme samples were stored frozen until lyophilization. Freeze-dried samples were stored at −20 °C.

Table 5. Overview of four DON/DOM feeding trials with poultry.

	Turkeys	**Chickens**	**Pullets**	**Roosters**
Reference	-	-	[28]	[29,30]
Animals	Hybrid Converter	Ross 308	Lohmann LSL	New Hampshire hybrids
Age	11 weeks	5 weeks	12 weeks	adult
No. of animals/group	4	4	9	8
c (DON) in feed (DON group) (mg/kg)	1.5	1.7	4.4	11
c (DON, DOM) in feed (DOM group) (mg/kg)	DON: 0.3 DOM: 1.4	DON: 0.2 DOM: 1.6	-	-
c (DON) in feed negative control group (mg/kg)	0.3	0.2	0.2	No NC
Feeding	Ad libitum access from 7:00 to 22:00	Ad libitum access from 7:00 to 19:00	65 g feed/day (2 equal portions)	90 g feed /day
Duration	1 day	1 day	2 weeks	9 days *
Sampling times	Every 2 h until 15:00; 18:00; 22:00; 7:00 next day	Every 3 h until 19 h; 7:00 next day	Excreta: morning and afternoon for 1 week; chyme: 3 h after administration	Morning and afternoon for 1 week
Samples taken	Excreta	Excreta	Excreta, content of jejunum and ileum	Excreta

* The duration of the animal experiment was 10 months, but the period of restricted feeding lasted for nine days.

4.8. Analysis of Samples and Data Evaluation

Excreta and GI content samples were worked-up in duplicate, each pooled and diluted extract was measured once. Analyte concentrations in freeze-dried samples were determined on the basis of pure solvent calibration functions (peak area *versus* analyte concentration) established routinely between 1 and 300 ng/mL under consideration of the apparent recoveries. In case the concentrations determined by duplicate sample work-up and analysis differed by more than 20%, work-up and analysis was repeated in duplicate and the new values were taken for further data processing.

For determination of the biological recoveries of ingested DON and DOM in the trials with turkeys, chickens, and pullets, DON equivalent amounts of all DON- and DOM metabolites quantified in excreta were calculated and divided by the DON equivalent amounts of DON or DOM ingested in the corresponding time period (one day for turkeys and chickens, one week for pullets). Statistical evaluation of differences in metabolite concentrations in excreta samples of pullets with and without *A. galli* infection was performed by one-way analysis of variance in MS Excel 2013.

4.9. Toxicity Assessment of DOM-3-Sulfate and DOM-15-Sulfate

To determine the *in vitro* toxicities of DON, DOM, DOM-3-sulfate, and DOM-15-sulfate, an *in vitro* transcription/translation assay with rabbit reticulocyte lysate (Promega, Madison, WI, USA) was employed. Transcription/translation reactions were carried out according to the manufacturer's instructions as described in [33] for wheat germ extract with two modifications. Firstly, animal ribosomes (rabbit reticulocyte lysate) were used instead of wheat ribosomes. Secondly, the reactions were stopped after 20 min instead of 30 min. All substances tested were dissolved in water prior to use. The concentrations of DON in the assay were between 0 and 10 μM, while the concentration range of DOM and DOM-sulfates was 0–500 μM. For each compound, three independent assays were performed on three different days using the same batch of the reticulocyte lysate. For determining the translation efficiency at different inhibitor concentrations, one single test reaction was performed for each inhibitor at each concentration level. In addition, a control reaction was carried out at each concentration level where water was used instead of the test substance. The luciferase activity determined for the control reaction was set to 100% (uninhibited reaction) and the readouts from the other reactions were related to this control. Statistical evaluation was performed with IBM SPSS Statistics software. Comparison of mean values was performed with a two-sided *T*-test with Welch correction (equal variances not assumed). Results were considered significant at $p < 0.05$.

5. Conclusions

An LC-MS/MS based method for quantitative determination of DON-3-sulfate, DOM-3-sulfate, DON, DOM, DON sulfonates 1, 2, 3, and DOM sulfonate 2 in excreta and chyme samples of poultry was developed and validated. Application of the method to excreta and chyme samples from four different feeding trials with turkeys, broiler chickens, pullets, and roosters confirmed DON-3-sulfate as major DON metabolite in all investigated poultry species. Orally administered DOM was equally extensively metabolized to its 3-sulfate metabolite. In addition, DOM-3-sulfate was shown to be an important natural DON metabolite in pullets and roosters, where it amounted to *ca.* 12% and 33%, respectively, of all the detected metabolites. Interestingly, pullets not infected with *A. galli* excreted significantly greater concentrations and greater amounts of DON-3-sulfate and DOM-3-sulfate than pullets infected with worms. DON sulfonates of the series 1, 2 and 3 were detected in chyme samples of broilers, albeit only at trace levels. Biological recoveries of orally administered DON in the form of DON-3-sulfate and DOM-3-sulfate in chickens were close to 100%, supporting the hypothesis of nearly quantitative conversion of DON to its 3-sulfate metabolites in chickens. Although turkeys showed very similar metabolization of DON and DOM as chickens, biological recoveries of orally administered DON and DOM were only between 50% and 70%, suggesting faster excretion of DON and DOM metabolites in chickens than in turkeys.

Similar to DON-3-sulfate, DOM-3-sulfate was much less toxic than DON on the ribosome. Although *in vitro* transcription/translation assays cannot predict all aspects of *in vivo* toxicity, greatly reduced toxicity on the ribosome, the molecular target of trichothecenes, and low susceptibility of poultry to DON indicates that sulfation serves as detoxification mechanism for DON in poultry. Concluding from literature reports and our current study, locations of formation of DON-3-sulfate are most likely the

intestinal mucosa, liver, and possibly the kidney. Elimination into excreta is probably achieved via bile and via urine. DOM-3-sulfate might also be formed by microbial conversion of DON-3-sulfate after retrograde movement of urinary DON-3-sulfate into the microbial rich caecae. In order to confirm this hypothesis, more complex animal experiments with separate collection of blood (hepatic and renal portal vein blood, systemic blood), bile, urine, contents of jejunum, ileum, caecae, and cloaca samples are required which demands highly sophisticated methods of sampling.

Supplementary Materials

Supplementary materials can be accessed at: http://www.mdpi.com/2072-6651/7/11/4706/s1.

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Author Contributions

P.F. and J.W. synthesized the DOM-sulfates. P.F. carried out NMR analysis as well as NMR data evaluation and wrote one part of the manuscript. S.D. conceived and designed the feeding trials with pullets and roosters and provided valuable information for the discussion part of the manuscript. G.W. performed and evaluated the toxicity tests. H.E.S.-Z. and S.C. performed the lab analyses. H.E.S.-Z. analyzed the data, contributed to study design and wrote the manuscript. F.B. contributed to study design and data analysis. F.B., P.F., S.D., G.W. and J.W. carefully revised the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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3.3 Synthesis of Zearalenone Metabolites

Technical Note

Weber, J.; Hametner, C.; Schiessl, A.; Häubl, G.; Fröhlich, J.; Mikula, H. First Synthesis of Zearalenone-14-O-β,d-[$^{13}C_6$]glucoside

First Synthesis of Zearalenone-14-*O*-β,D-[$^{13}C_6$]glucoside

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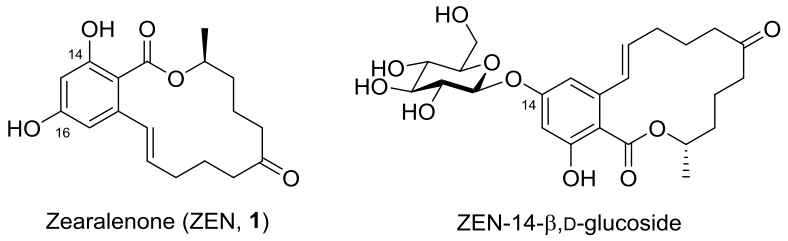
Zearalenone (ZEN **1**, Fig. 1) is a mycotoxin produced by several Fusarium species mainly by *F. graminearum* and *F. culmorum*. It is found in a variety of infected plants like maize, barley, oats, wheat and sorghum.[1] Maize is the most frequently contaminated commodity and levels of ZEN are found between 1 to 2900 µg/kg depending on climatic, harvest and storage conditions.[2] As the structure of ZEN resembles natural estrogens, it is able to bind to the estrogenic receptor causing hyperestrogenism, severe productive and infertility problems in mammals.[3] Therefore, the amount of ZEN in certain foods for human consumption was regulated with 11 other mycotoxins by the European Commission in 2006.[4] However, ZEN is prone to be metabolized by plants leading to so called "masked mycotoxins" that fail to be detected in conventional analysis. One major emerging metabolite of ZEN is ZEN-14-glucoside; it was shown by Schneweis et al. that in wheat samples the relative proportion of ZEN-14-glucoside to free ZEN are on average 27%.[5] ZEN-14-glucoside is according to toxicological studies less toxic than ZEN itself but can be hydrolyzed back to the parent toxin during digestion or food processing.[6] Hence, missing ZEN-14-glucoside in standard approaches presents a serious health risk for human and animals as it can contribute to the base contamination of ZEN in food samples.

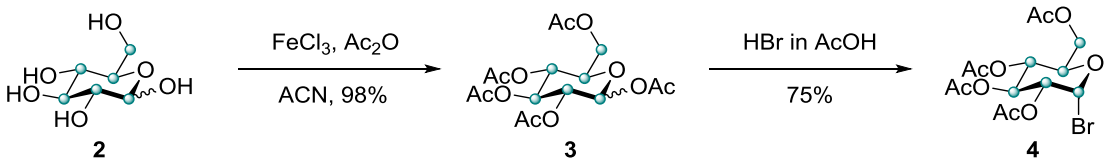


Zearalenone (ZEN, **1**) ZEN-14-β,D-glucoside

Figure 1 Chemical structure of zearalenone (ZEN, 1) and a frequent co-contaminant of ZEN in nature

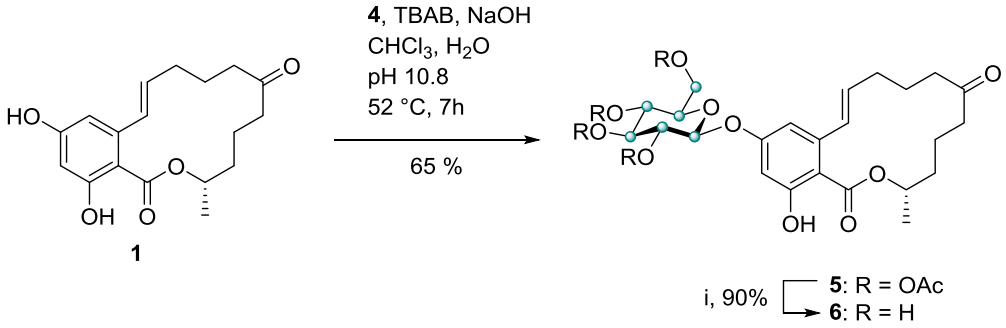
In order to be able to develop routine screenings including masked mycotoxins, reference standards are needed. Zearalenone-14-*O*-β,D-glucoside could already be successfully synthesized via a phase transfer glycosylation by Grabley et al.[7] However, for accurate quantification of mycotoxin metabolites by LC-MS isotope labeled standards are crucial. Therefore, we decided to develop a simple and cheap procedure for the synthesis of Zearalenone-14-*O*-β,D-[$^{13}C_6$]glucoside.

Acetylation of [$^{13}C_6$]glucose (**2**) with acetic anhydride in the presence of dry iron(III) chloride in acetonitrile[8] formed [$^{13}C_6$]glucose pentaacetate (**3**) in 98%. Subsequently **3** was treated with HBr in acetic acid[9] leading to acetobromo-α,D-[$^{13}C_6$]glucose that could be applied as glucosyl donor in the following conjugation step (Scheme 1).



Scheme 1 Synthesis of ^{13}C-labeled glycosyl donor **4**

ZEN was successfully glycosylated in a phase-transfer reaction[7] with glucosyl donor **4** to yield acetyl-protected ZEN-14-O-β,D-[$^{13}C_6$]glucoside **5** in 65%. Deprotection of **5** was achieved after a protocol developed by Mikula et al.[10] with potassium hydroxide in THF-H_2O to obtain ZEN-14-O-β,D-[$^{13}C_6$]glucoside (**6**) in excellent yields (Scheme 2).



Scheme 2 Synthesis of ZEN-14-O-β,D-[$^{13}C_6$]glucoside [(i) KOH, THF-H_2O (4:1)]

In summary, we could synthesize ZEN-14-O-β,D-[$^{13}C_6$]glucoside in an overall yield of 59% that can now be used for the accurate quantification of ZEN-14-glucoside in natural samples by LC-MS.

Experimental

[$^{13}C_6$] Glucose pentaacetate (3)

To a suspension of [$^{13}C_6$]-glucose (1 g, 5.37 mmol, 1 eq.) in acetonitrile (3 mL) cooled to 0 °C was added $FeCl_3$ (87 mg, 0.54 mmol, 0.1 eq.) followed by dropwise addition of acetic anhydride (2.8 mL, 29.6 mmol, 5.5 eq.). The reaction mixture was warmed to room temperature and treated with ultrasonic irradiation for 45 min. The reaction mixture was diluted with CH_2Cl_2 (10 mL) and washed with water. The organic layer was dried over Na_2SO_4 and concentrated. The crude product was filtered through a pad of silica gel eluting with hexanes/EtOAc (1:1) to afford the title compound (2.1 g, 98%) as a white solid. Analytical data matched those reported in literature.[11]

Acetobromo-α,D-[$^{13}C_6$]glucose (4)

To a hydrogen bromide solution (33 wt-% in acetic acid, 1.8 mL) cooled to 0 °C was added in small portions [$^{13}C_6$]glucose pentaacetate (1 g, 2.5 mmol, 1 eq.). The reaction was allowed to warm to room temperature and stirred for 1 h. The reaction mixture was quenched by the addition of ice water (100 mL) and diluted with CH_2Cl_2 (2 x 100 mL). The organic layer was separated, washed with saturated aqueous $NaHCO_3$ solution, dried over Na_2SO_4 and concentrated. The residue was purified by crystallization from Et_2O/hexanes to give **2** as a white solid (790 mg, 75%). Analytical data matched those reported in literature.[12]

Zearalenone-14-*O*-(tetra-*O*-acetyl-β,D-[$^{13}C_6$]glucoside) (5)

To a solution of ZEN (100 mg, 0.31 mmol, 1 eq.), acetobromo-α,D-[$^{13}C_6$]glucose (367 mg, 0.88 mmol, 2.8 eq.) and tetrabutylammonium bromide (TBAB) (105 mg, 0.33 mmol, 1.04 eq.) in chloroform (30 mL) was added borate buffer (pH 11.0, 30 mL). The reaction mixture was heated to 52°C and the pH was kept constant at 10.8 by addition of 0.1N NaOH for 7 hours. The organic phase was separated, dried over Na_2SO_4 and concentrated. The crude product was purified by flash chromatography (hexanes:EtOAc 6:1 to 1:1, 70 g silica gel) to afford the title compound (145 mg, 65%). ^{13}C decoupled ^{1}H-NMR matched those reported in the literature[7]; ESI-MS calcd for $C_{26}(^{13}C)_6H_{40}NaO_{14}^+$ $[M+Na]^+$ 677.2, found 677.1.

Zearalenone-14-O-β,D-[$^{13}C_6$]glucoside (6)

To a solution of Zearalenone-14-*O*-(tetra-*O*-acetyl-β,D-[$^{13}C_6$]glucoside) (145 mg, 0.2 mmol, 1 eq.) in THF/H_2O (4:1, 11 mL) was added KOH (125 mg, 2.2 mmol, 10 eq.). The reaction mixture was stirred at room temperature for 2 h. The pH was adjusted to 6.8 by dropwise addition of 0.1 N HCl. The solution was immediately extracted with EtOAc (3 x 20 ml). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. Flash chromatography (MeOH in DCM, gradient elution, 0% to 10%, 20 g silica gel) afforded the title compound as a white solid (97 mg, 90%). ^{1}H NMR (600 MHz, MeOD): δ 6.96 (d, J = 15.5 Hz, 1H), 6.69 (d, J = 2.3 Hz, 1H), 6.53 (d, J = 2.3 Hz, 1H), 5.84 (ddd, J = 15.1, 10.3, 4.7 Hz, 1H), 5.08 – 5.01 (m, 1H), 4.98 (d, J = 7.0 Hz, 1H), 3.91 (d, J = 11.7 Hz, 1H), 3.68 (dd, J = 12.3, 6.2 Hz, 1H), 3.50 – 3.42 (m, 3H), 3.39-3.33 (m, 1H), 2.80 (ddd, J = 18.8, 11.4, 2.3, 1H), 2.66 – 2.59 (m, 1H), 2.36 -2.27 (m, 2H), 2.23-2.11 (m, 2H), 2.07-1.98 (m, 1H), 1.83-1.70 (m, 2H), 1.69 – 1.55 (m, 3H), 1.38 (d, J = 6.2 Hz, 3H); ^{13}C NMR (150 MHz, MeOD): δ 213.8 (s, 1C), 171.3 (s, 1C), 165.2 (s, 1C), 162.9 (s, 1C), 144.1 (s, 1C), 134.1 (d, 1C), 133.7 (d, 1C), 109.5 (d, 1C), 106.2 (s, 1C), 104.1 (d, 1C), 101.9-100.6 (m, 1C), 78.9-77.4 (m, 2C), 75.2-74.2 (m, 2C), 71.9-70.9 (m, 1C), 63.0-62.0 (m, 1C), 43.9 (t, 1C), 37.6 (t, 1C), 35.9 (t, 1C), 32.2 (t, 1C), 23.2 (t, 1C), 22.0 (t, 1C), 20.9 (q, 1C); ESI-MS calcd for $C_{18}(^{13}C)_6H_{32}NaO_{10}^+$ $[M+Na]^+$ 509.1, found 509.1.

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Synthesis of zearalenone-16-β,D-glucoside and zearalenone-16-sulfate: A tale of protecting resorcylic acid lactones for regiocontrolled conjugation, 1129-1134

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Synthesis of zearalenone-16-β,D-glucoside and zearalenone-16-sulfate: A tale of protecting resorcylic acid lactones for regiocontrolled conjugation

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Abstract

The development of a reliable procedure for the synthesis of the 16-glucoside and 16-sulfate of the resorcylic acid lactone (RAL) type compound zearalenone is presented. Different protective group strategies were considered and applied to enable the preparation of glucosides and sulfates that are difficult to access up to now. Acetyl and *p*-methoxybenzyl protection led to undesired results and were shown to be inappropriate. Finally, triisopropylsilyl-protected zearalenone was successfully used as intermediate for the first synthesis of the corresponding mycotoxin glucoside and sulfate that are highly valuable as reference materials for further studies in the emerging field of masked mycotoxins. Furthermore, high stability was observed for aryl sulfates prepared as tetrabutylammonium salts. Overall, these findings should be applicable for the synthesis of similar RAL type and natural product conjugates.

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Introduction

Resorcylic acid lactones (RALs, Figure 1), a compound class of benzannulated macrolides, are pharmacologically active secondary metabolites produced by a variety of different fungal species [1]. Zearalenone (ZEN, **1**) is a well-known RAL type mycotoxin for which maximum tolerated levels in food and feed were enacted and recommended, respectively, in Europe [2,3]. ZEN is produced by several plant pathogenic *Fusarium* species, including *Fusarium graminearum* and *Fusarium culmorum*. These species, which are the most frequently occurring toxin-producing fungi of the northern temperate zone, are commonly found in cereals and crops throughout the world [4,5]. Significant levels of ZEN are prevalently found in grains such as maize, wheat, and rice [6]. It is known that ZEN can cause problems of the reproductive tract (e.g., impaired fertility) in animals [7]. Physiological studies revealed binding of ZEN to recombinant human estrogen receptors [8] and have furthermore shown a ZEN-induced stimulation of the growth of human breast cancer cells [9].

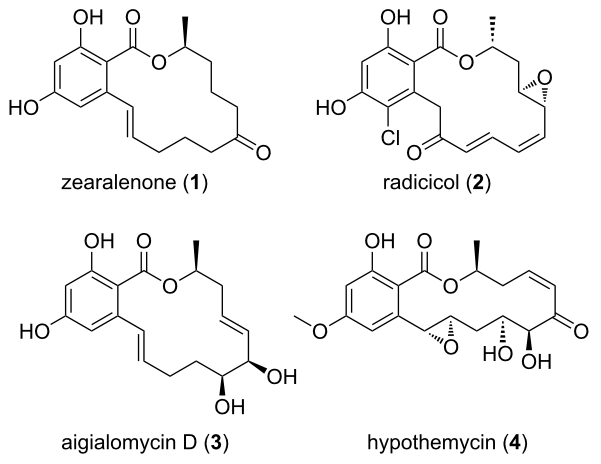

Figure 1: Structure of selected RAL type fungal secondary metabolites.

Additionally, masked mycotoxins, especially altered derivatives formed through conjugation to sugar moieties or sulfate, emerge after metabolization by living plants. Due to changed chemical structures and properties compared to the parent mycotoxins, these conjugates can usually not be detected applying standard analytical techniques [10,11]. Responsible biochemical transformations are catalyzed usually by enzymes within detoxification processes [12]. Schneweis et al. reported the occurrence of ZEN-14-β,D-glucoside (**5**, Figure 2A) in wheat [13] and the first chemical synthesis of this compound applying phase transfer glycosylation has been reported by Grabley et al. [14]. ZEN-14-sulfate (**6**, Figure 2A) was first isolated from *F. graminearum*-inoculated rice [15] and both, the glucoside **5** and the sulfate **6**, were identified as ZEN metabolites in *Arabidopsis thaliana* [16]. These conjugates are easily hydrolyzed back to the parental mycotoxin during digestion of contaminated grain, and should therefore be considered as masked mycotoxins [12]. Recently it has been shown that ZEN treated barley, wheat and *Brachypodium distachyon* cells produce both the ZEN-14 and the ZEN-16-glucoside, with up to 18-fold higher levels of ZEN-16-glucoside than ZEN-14-glucoside in barley roots [17]. We therefore intended to develop a synthetic method for regiocontrolled conjugation of ZEN. Basically, the RAL type moiety of ZEN contains two possible sites for glycosylation/sulfation, but due to the higher reactivity of the phenol group at position 14, reactions at this site are strongly favored compared to conjugation at C16–OH [18-20]. Although natural products containing a RAL type moiety conjugated at the phenol group in ortho position to the carboxyl group (2'-OH) were already detected and identified [21-25], to the best of our knowledge there are no reliable synthetic procedures and strategies towards this class of compounds described in the literature so far. The synthesis of the natural glucoside delphoside by Saeed was performed using methyl ether protection at O-6 of the isocoumarin core structure during glucosylation and rather harsh unfavorable demethylation with boron tribromide in the last step [26]. Without structure verification and characterization ZEN-16-β,D-glucoside (**7**, Figure 2B) was tentatively identified as a byproduct of the Königs–Knorr glucosylation of ZEN for preparation of ZEN-14-glucoside [27]. In the course of ongoing research in the emerging field of masked mycotoxins, we were able to prepare

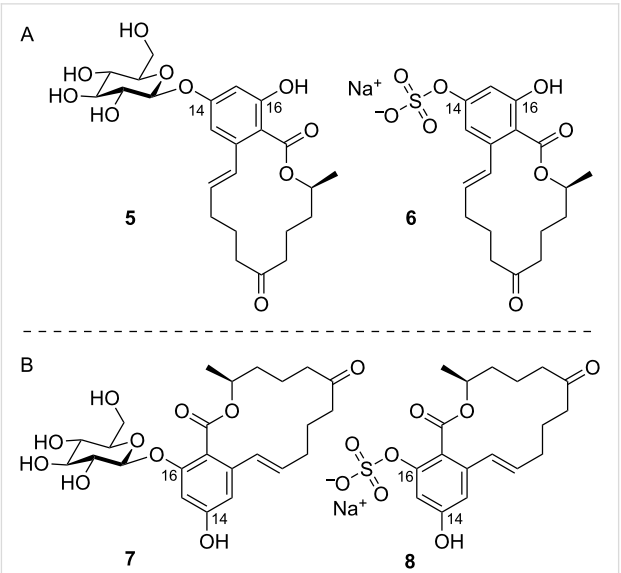

Figure 2: Structures of zearalenone conjugates: (A) ZEN-14-β,D-glucoside (**5**) and ZEN-14-sulfate (**6**), (B) ZEN-16-β,D-glucoside (**7**) and ZEN-16-sulfate (**8**). Sulfates shown as sodium salts.

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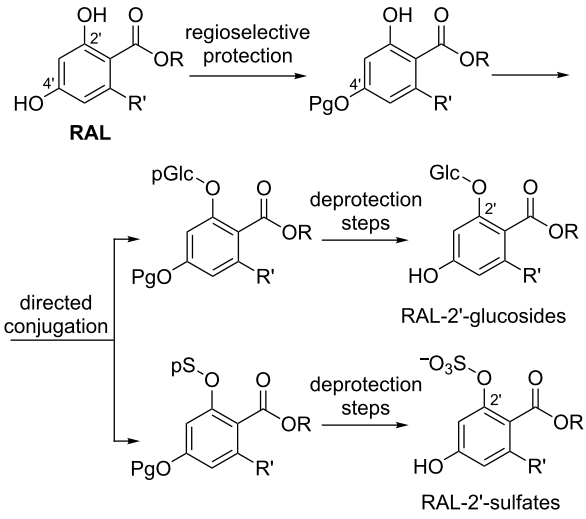
ZEN-16-β,D-glucoside (**7**) and ZEN-16-sulfate (**8**, Figure 2B) in reasonable amounts after the development of reliable procedures that should be generally applicable to resorcylic acid lactones. Additionally the first chemical synthesis of the ZEN-derivative 14-O-acetylzearalenone (14-AcZEN, produced by some Fusarium strains) [28] is reported.

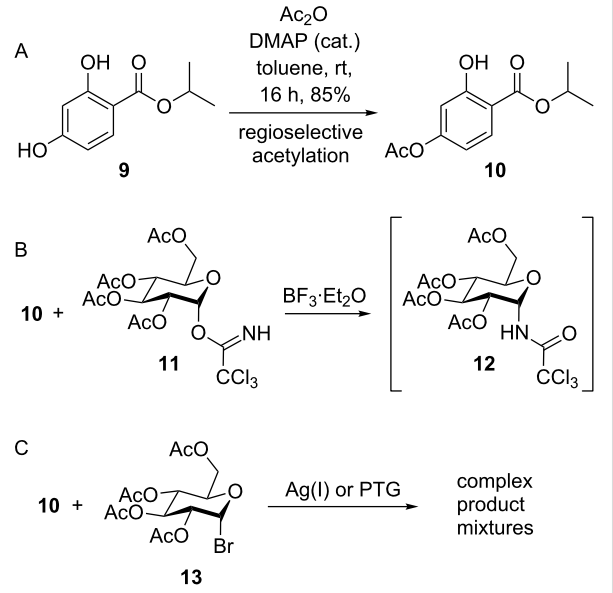
Results and Discussion

The general strategy for regiocontrolled conjugation at position 2' of resorcylic acid esters and lactones is shown in Scheme 1. Regioselective protection of the more nucleophilic 4'-phenol and subsequent glucosylation or sulfation should lead to the desired products.



Scheme 1: General strategy for the synthesis of RAL-2'-conjugation (Pg: protective group, pGlc: protected glucose moiety, pS: protected sulfate, Glc: β,D-glucoside).

For the development of a reliable protective group strategy and subsequent reaction optimization, 2,4-dihydroxybenzoic acid isopropyl ester (**9**) [29] was used as a RAL mimic. For protection of the 4-OH group we first considered an acetyl group that could be regioselectively introduced by reaction of **9** with acetic anhydride and catalytic amounts of 4-(dimethylamino)pyridine (DMAP) to obtain the acetylated RAL mimic **10** (Scheme 2A). Different methods for glycosylation were investigated using acetyl-protected glucosyl donors since diastereoselective β-conjugation, which is needed for the preparation of glucosides formed during phase II metabolism, is commonly achieved applying the participation of acyl groups at O-2 of the glycosyl donor (anchimeric effect) [30]. Lewis acid-mediated glucosylation using the trichloroacetimidate donor **11** according to the procedure of Saeed [26] did not lead to the desired product, which can be explained by the weak nucleophilicity of the 2-OH group of the acceptor **10**. This assumption was supported by detection of the glucosyl acetamide **12**, which is known to be formed by rearrangement of **11** when activated in the presence of a weak acceptor (Scheme 2B) [31-34]. Königs–Knorr glucosylation, which in general is most frequently used for the glucosylation of phenols, using commercially available bromo sugar **13** activated by silver(I) salts or under phase transfer conditions led to complex product mixtures (Scheme 2C).



Scheme 2: (A) Regioselective acetylation of resorcylic acid ester **9**. (B) Lewis acid mediated glucosylation; no conversion of **10**, partially rearrangement of **11** to glucosyl acetamide **12**. (C) Königs–Knorr glucosylation; Ag(I): Ag_2CO_3 or Ag_2O, CH_2Cl_2 or MeCN; PTG: phase transfer glucosylation, NaOH, tetrabutylammonium bromide, pH 10–11, $CHCl_3/H_2O$.

Nevertheless, the procedure for selective acetylation of resorcylic acid esters and lactones was applied for the first synthesis of 14-O-acetylzearalenone (**14**) (Scheme 3).

To avoid undesired cleavage of the acetyl group during glucosylation of **10**, *p*-methoxybenzyl (PMB) protection was applied instead, since the PMB group was considered to be inert to the reactions conditions of the Königs–Knorr procedure, thus forcing conjugation at position 2. Regioselective *p*-methoxybenzylation of **9** was achieved by reaction with PMB-Cl and Cs_2CO_3 in dry DMF after optimization in terms of base and solvent type (Scheme 4A). Königs–Knorr glucosylation of the PMB-protected mimic **15** afforded **16**, which was deprotected using 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) for oxidative PMB cleavage and subsequent ester saponification to yield the desired glucoside **17** in an overall yield of 30% (Scheme 4B).

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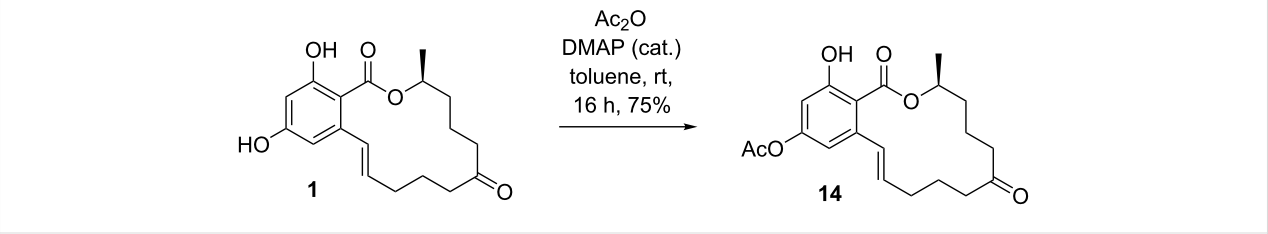
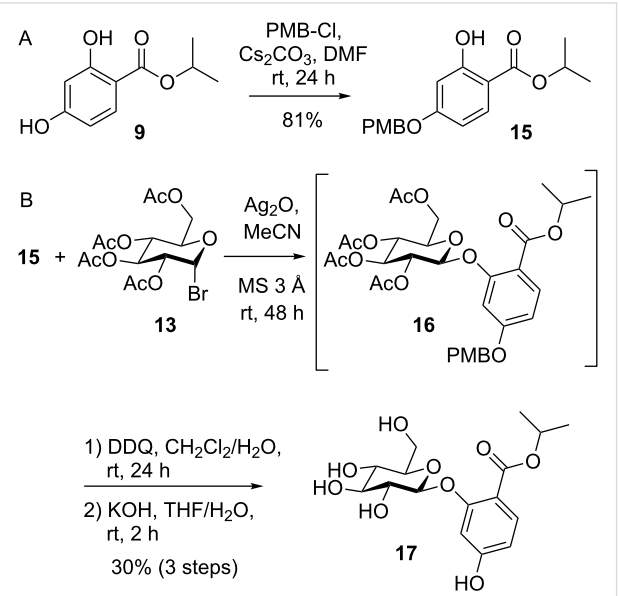
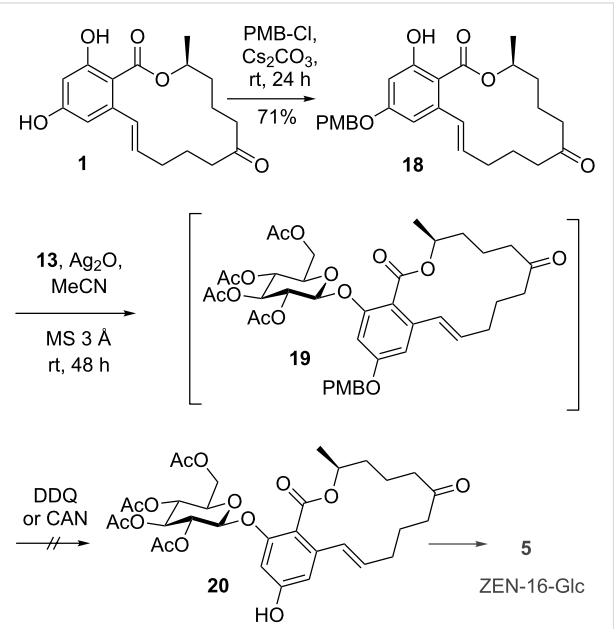


Scheme 3: Regioselective acetylation of ZEN (**1**) affording 14-O-acetylzearalenone (**14**).



Scheme 4: (A) Regioselective p-methoxybenzylation of **9**. (B) Synthesis of the ZEN-16-Glc mimic **17**.



Scheme 5: Regioselective protection of ZEN (**1**) and failed PMB cleavage of the glucosylated intermediate **19**.

Applying this procedure to ZEN (**1**) afforded the glucosylated intermediate **19** after Königs–Knorr glucosylation of 14-PMB-ZEN (**18**), but subsequent deprotection using DDQ did not afford the desired product. Also an alternative procedure for oxidative PMB cleavage using cerium ammonium nitrate (CAN) did not lead to the formation of the deprotected compound **20** (Scheme 5). Beside unreacted **19**, LC–MS/MS analysis showed the formation of a product with an *m*/*z* value 16 amu higher than calculated for **19** indicating oxidation of this intermediate but no deprotection.

Since the DDQ-promoted cleavage of phenolic PMB ethers can be complicated by overoxidation, especially with electron-rich phenolic compounds [35], we assume a significant effect of the conjugated olefinic double bond at C-6’ of the resorcylic acid moiety being responsible for the observed different behavior of ZEN (**1**) and the ZEN mimic **9**.

After this second setback the protective group strategy was changed again within a third approach. Considering steric hindrance and methods for selective deprotection under relatively mild conditions led us to the use of triisopropylsilyl (TIPS) protection for regiocontrolled glucosylation of resorcylic acid esters and lactones. Regioselective silylation of **9** and ZEN (**1**) was readily achieved affording compounds **21** and **22** (14-TIPS-ZEN), respectively, in nearly quantitative yields by reaction with TIPS-Cl and imidazole in dry CH_2Cl_2. Applying this strategy we were finally able to accomplish the synthesis of the ZEN mimic glucoside **17** (Scheme 6A) as well as of the target compound ZEN-16-β,D-glucoside (**7**) as shown in Scheme 6B. Reasonable yields of 41% (**17**) and 34% (**7**), respectively, were obtained applying an optimized purification protocol.

Additionally, TIPS protection was applied for the synthesis of ZEN-16-sulfate (**8**) using a procedure that was successfully applied for the synthesis of ZEN-14-sulfate (**6**) as described recently [20]. Reaction of **22** with the 2,2,2-trichloroethyl (TCE) protected sulfuryl imidazolium salt **23** [36,37] gave the

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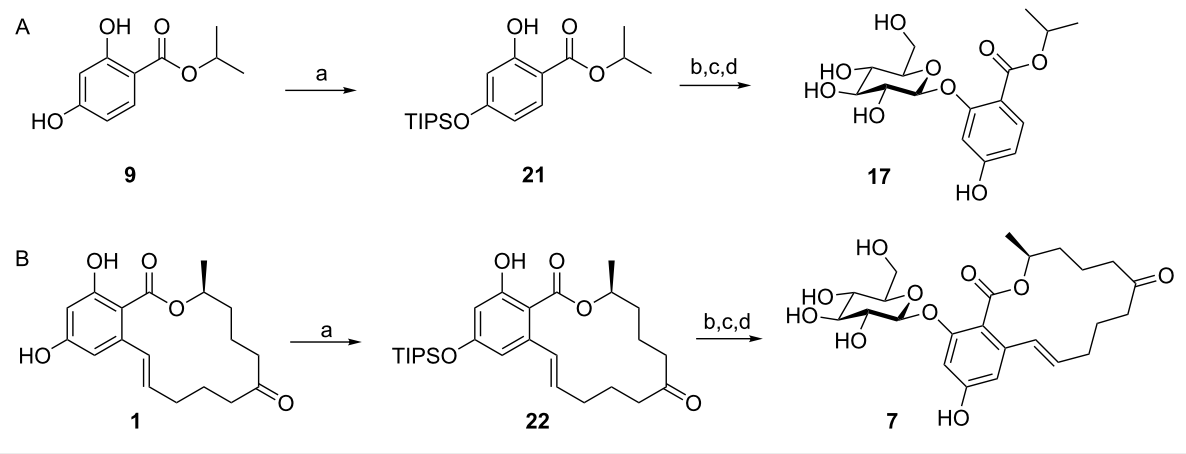
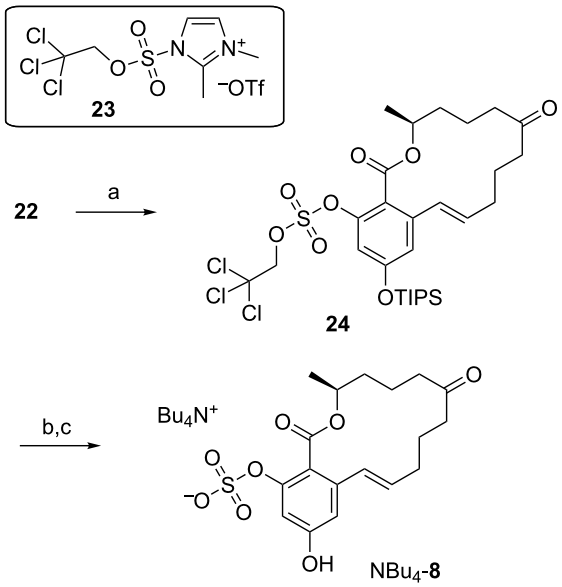


Scheme 6: Synthesis of (A) ZEN mimic glucoside **17** and (B) ZEN-16-β,D-glucoside (**7**); a: TIPS-Cl, imidazole, 16 h, rt, >99% for **21** and **22**; b: **13**, Ag_2O, MeCN, 96 h, rt; c: TBAF, AcOH, THF, 24 h; d: KOH, THF/H_2O, 4 h, rt, 41% for **17**, 34% for **7** (3 steps).

protected sulfate **24**. TIPS cleavage and subsequent TCE deprotection using Zn/ammonium formate ($HCOONH_4$) yielded the desired product. Interestingly, when using the crude intermediate after TIPS deprotection without purification directly in the second step, we obtained the tetrabutylammonium salt of ZEN-16-sulfate (NBu_4-**8**) in good yield (65% over 2 steps) as shown in Scheme 7. The stability of this compound was significantly increased compared to the corresponding sodium salt, which is of great importance in terms of preparation of reference material and long term stability of appropriate standard solutions for further investigations.



Scheme 7: Chemical sulfation using the 2,2,2-trichloroethyl (TCE)-protected sulfuryl imidazolium salt **23** yielding ZEN-16-sulfate (**8**) as tetrabutylammonium salt; a: **23**, 1,2-dimethylimidazole, CH_2Cl_2, 12 h, rt, 91%, b: TBAF, AcOH, THF, 3 h, −10 °C, c: Zn, $HCOONH_4$, MeOH, 16 h, rt, 65% (2 steps).

Conclusion

In summary, different methods for the regioselective protection of resorcylic acid esters and lactones were investigated for subsequent regiocontrolled glucosylation and sulfation. Whereas acetyl and *p*-methoxybenzyl protection led to undesired products, TIPS-protected RALs were successfully used as intermediates for the preparation of corresponding glucosides and sulfates applying the Königs–Knorr glucosylation and chemical sulfation using TCE-protected sulfuryl imidazolium salt **23**, respectively. These methods were used for the first chemical synthesis of the ZEN-16-conjugates **7** and **8** in reasonable amounts for ongoing research and further investigations in the field of conjugated/masked mycotoxins.

Supporting Information

Supporting Information File 1

Experimental details (including remarks and general procedures), characterization data, copies of NMR spectra of new compounds, 2D NMR spectra of glucoside **7**.
[http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-10-112-S1.pdf]

Acknowledgements

The Theodor Körner fund (Vienna, Austria), the Austrian Federal Ministry of Economy, Family and Youth as well as the National Foundation for Research, Technology and Development are gratefully acknowledged for financial support. GA and RK were supported by the Austrian Science Fund (FWF) special research project SFB Fusarium (F3706 and F3708).

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3.4 Synthesis of Culmorin Metabolites

Manuscript #8

Submitted manuscript

Weber, J.; Vaclavikova, M.; Wiesenberger, G.; Haider,M.; Hametner, C.; Mikula, H.; Fröhlich, J.; Berthiller, F.; Adam, G.; Fruhmann, P.* Chemical synthesis of culmorin metabolites and their biologic role in culmorin and acetyl-culmorin treated wheat cells

Organic & Biomolecular Chemistry

ARTICLE

Chemical synthesis of culmorin metabolites and their biologic role in culmorin and acetyl-culmorin treated wheat cells

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The Fusarium metabolite culmorin is receiving increased attention as "emerging mycotoxin". It is co-occurring with trichothecene mycotoxins and potentially influences their toxicity. Its ecological role and fate in plants is unknown. We synthesized sulfated and glucosylated culmorin conjugates as potential metabolites, which are expected to be formed *in planta*. Therefore, an efficient procedure for the synthesis of culmorin sulfates was developed. Diastereo- and regioselective glucosylation of culmorin was achieved by exploiting or preventing unexpected acyl transfer when using different glucosyl donors. Treatment of a wheat suspension culture with culmorin revealed an *in planta* conversion of culmorin into culmorin-8-glucoside and culmorin acetate, but no sulfates or cumorin-11-glucoside were found. Treatment of wheat cells with the fungal metabolite 11-acetylculmorin revealed its rapid deacetylation, but also showed formation of 11-acetylculmorin-8-glucoside. These results show that plants are capable to extensively metabolize culmorin.

Introduction

Mycotoxins are widespread contaminants in food and feed. In depth knowledge about their toxicity, metabolism and occurrence is of great importance.[1] Beside a number of well investigated mycotoxins such as deoxynivalenol, several compounds get increasing attention as so called "emerging mycotoxins". They frequently co-occur with other toxins and their toxicity, ecological role and the effect of combinations with known toxins are insufficiently investigated so far.[2]

Culmorin (CUL, **1**, Fig. 1a) is considered as "emerging mycotoxin" although it was isolated already in 1937, followed by structural characterization in 1967.[3, 4] It contains a sesquiterpene diol core structure and is produced by several different *Fusarium* species, such as *F. culmorum* (name giving), *F. graminearum, F. crookwellense* and *F. venenatum*. Moreover it is produced by a recently identified basal species of the *F. graminearum* species complex, *Fusarium praegraminearum*, indicating that CUL production is an ancestral trait. In general, CUL is little investigated and might be produced also by other *Fusarium* species. Outside of the genus *Fusarium* it has been identified in the marine ascomycete *Leptosphaeria oreamaris*.[5-9] Besides CUL, various *Fusarium* species have been shown to produce related compounds including hydroxyculmorins, culmorone and hydroxyculmorone (Fig. 1).[7, 10, 11]

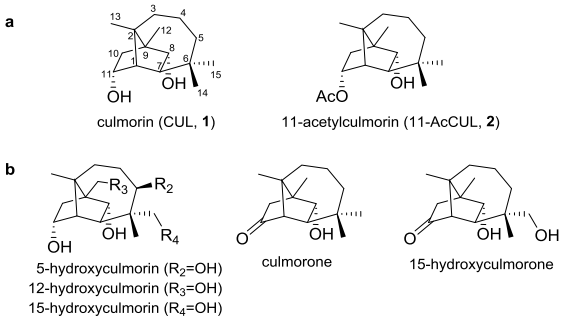

Figure 1 (a) Chemical structure of culmorin (CUL, **1**) and 11-acetylculmorin (11-AcCUL, **2**); (b) naturally occurring related compounds of CUL (**1**)

The natural occurrence and contamination levels of CUL are usually strongly related with the occurrence and levels of deoxynivalenol (DON). Typically CUL was found to occur in concentrations about 3-fold higher than those of DON in naturally contaminated samples.[12, 13] Although different parameters may influence the ratio of CUL and its metabolites, there are several examples for their occurrence in different regions of the world. In a recent study, CUL was detected in nearly all wheat, barley and oat samples from Norway with a median concentration of 100 (wheat), 292 (barley) and 2000 µg/kg (oats).[13] A study with feed and feed raw material samples mainly from Austria, Denmark and Hungary led to similar results, where 63 % of all samples contained CUL in a median concentration of 195 µg/kg.[14] Frequent occurrence of CUL has been shown in a recent study reporting a median concentration

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

of approximately 100 µg/kg in cereals, nuts and their processed products from Cameroon.[15]

There are only a limited number of studies describing the toxicological relevance of CUL. The compound has been shown to possess antifungal and phytotoxic properties.[2, 7, 11] Previous studies about the toxicity of CUL include tests in baby hamster kidney cells (toxic at 20 µg/mL), a chick embryotic screening test (CHEST, LD_{50} around 70 µg CUL/egg) and the estimation of the LD_{50} (i.p.) for mice with a range of 250 to 1000 µg/kg BW[7, 8, 16]. In all these trials only high amounts of CUL where used and the obtained values therefore suggest a low toxicity to animal cells. The metabolism of CUL in humans or animals is unknown. Up to date, *in vivo* studies were only carried out in swine and two insect species.[17, 18] Caterpillars of corn earworm (*Heliothis zea*) and armyworm (*Spodoptera frugiperda*) reared on a CUL containing diet (25 mg/kg, 7 days) showed unaltered weight gain and mortality rate. A similar study with growing piglets (2 mg/kg diet, 21 days) also showed no negative impact. In these two studies additionally the combined effect of DON and CUL was investigated. Interestingly, the combination of DON (25 mg/kg diet) and CUL (10 mg/kg diet) significantly increased the mortality and decreased the weight gain of corn earworms.[17] Although the evidence for a synergistic effect is still very limited, an enzyme involved in the biosynthesis of CUL has been proposed to be a target for mycotoxin reduction.[19] The role of CUL in plant pathogen interaction is unknown.

The biosynthesis of CUL has been elucidated in *F. graminearum*. A terpenoid synthase encoded by *CLM1* produces longiborneol that is subsequently hydroxylated at C11 by a cytochrome P450 encoded by *CLM2*.[20, 21] CUL biosynthesis is induced *in planta* during infection of several crop species and is co-regulated with DON biosynthesis[22, 23] indicating a possible role of CUL as a Fusarium virulence factor. In general, plants may have the ability to at least partly inactivate and counteract fungal virulence factors using various mechanisms.[24] Data regarding CUL metabolites formed by plants and their biological significance are lacking. Since the most prominent and important metabolites are usually the glycosylated forms of the parent toxin, it seems obvious to speculate that also CUL-glucosides (CUL-Glc) might be formed.[25] In addition, the occurrence of sulfated derivatives similar to other mycotoxins like DON, ZEN or AOH/AME seems possible (Fig. 2).[26-29]

CUL is a longifolane sesquiterpene with a tricyclo-[6.3.0.0] undecane skeleton and the core structure appears in several other natural products such as longifolane or longiborneol. Several strategies for the synthesis of CUL and its core structure have been reported.[30-33] Acetylation of CUL affording 8-acetyl- and 11-acetylculmorin (11-AcCUL **2**, Fig 1a) was described just recently by Bahadoor *et al.*[20] Besides, no further synthetic modifications or derivatizations have been described.

We got interested in potential metabolites of CUL and thus set out to synthesize glucosides and sulfates, which could be used to develop analytical methods to allow investigating the natural occurrence of these conjugates.

Results and discussion

Synthesis of culmorin metabolites

To obtain sufficient amounts of CUL for synthetic studies, it was necessary to produce it on our own. For this purpose, *tri1* knockout strains derived from *Fusarium graminearum* strains PH-1 and WG-9 were selected[34] and a purification protocol was developed to obtain CUL (885 + 120 mg) and 11-AcCUL (42 mg). The identity and purity of both compounds was confirmed by NMR spectroscopy.[10, 20]

For the synthesis of the desired CUL sulfates, we used a procedure originally developed for the sulfation of carbohydrates.[34] In a first attempt CUL was reacted with SO_3-trimethylamine (NEt_3) complex in N,N-dimethylformamide (DMF) at 55°C showing unexpected exclusive formation of CUL-11-sulfate as trimethylamine salt. To obtain sodium salt **3,** which is required for biological investigations, the purified product was submitted to a cation exchange resin. Using the same procedure with a 50-fold excess of SO_3-NEt_3, CUL was sulfated at position O-8 and O-11 affording CUL disulfate (**5**). The reactivity difference of the two hydroxyl groups is noteworthy and made it apparent that the hydroxyl group of CUL at position 11 needs to be protected to be able to prepare CUL-8-sulfate (**4**). Hence, 11-AcCUL (**2**) that was previously isolated from the Fusarium culture material was used as starting material and sulfated in a similar approach. Deprotection of the acetyl group was finally accomplished under basic conditions[35] yielding directly the sodium salt of the sulfate **4**. To make 11-AcCUL (**2**) easily accessible via a synthetic route, we screened for a

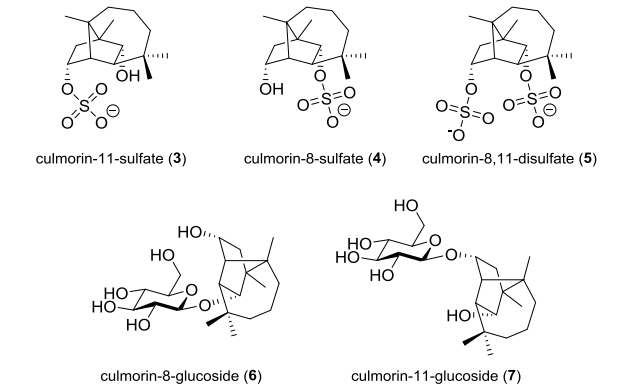
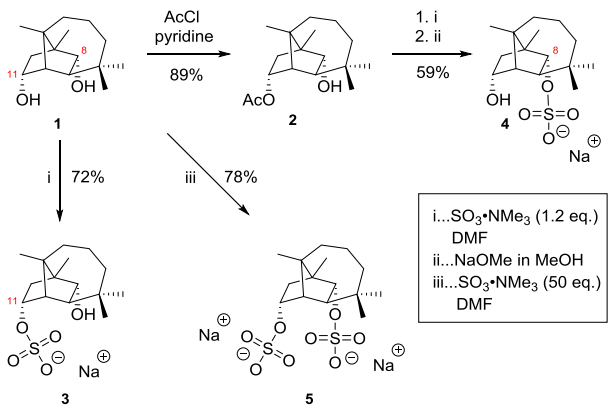


Figure 2 Target molecules for the *in planta* investigation: culmorin sulfates (**3, 4**), the corresponding disulfate (**5**) and both possible glucosides (**6, 7**)



Scheme 1 Synthesis of culmorin sulfates **3**, **4** and culmorin disulfate **5**

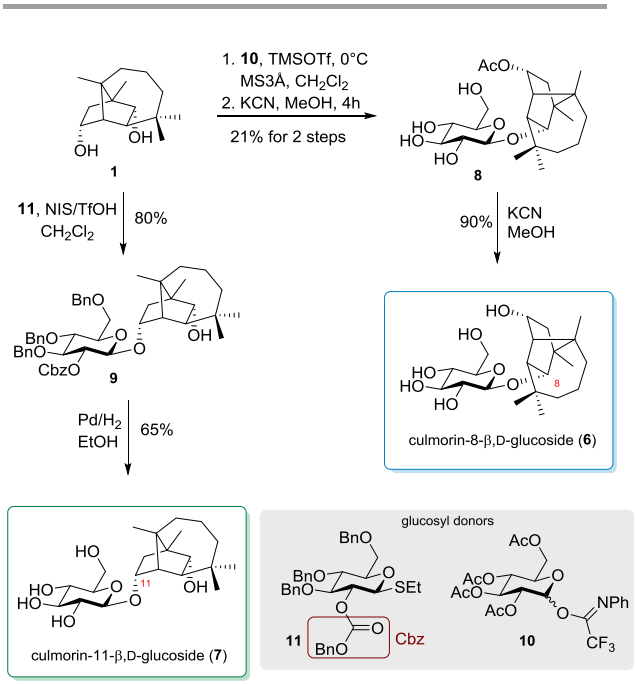
regioselective acetylation method and were successful by treating CUL with acetyl chloride in pyridine (Scheme 1).

Glucosylation of CUL was done applying Lewis acid mediated glycosylation using N-phenyltrifluoroacetimidoyl (NPTFA) glucosyl donor **10**[35, 36] and a catalytic amount of TMSOTf (0.1 eq.). Unexpectedly, glycosylation of CUL didn´t take place like the sulfation of CUL on the hydroxyl group at pos. 11 but on pos. 8 and formed 11-AcCUL-8-β,D-tetra-*O*-acetylglucoside as intermediate. We assume, this is caused by glucosylation after an initial acyl transfer, a known side reaction of glycosylations[37, 38]. Selective deprotection of the sugar moiety was achieved using sodium cyanide [39] yielding 11-AcCUL-8-glucoside (11-AcCUL-8-Glc, **8**). Further deprotection by using a fourfold excess of sodium cyanide afforded CUL-8-glucoside (CUL-8-Glc, **6**).

For the preparation of CUL-11-glucoside (CUL-11-Glc, **7**) we aimed to use glucosyl donors as just recently developed in our group.[40] These 2-O-benzyloxycarbonyl (Cbz) protected donors can be applied for diastereoselective glucosylation without the need for acetyl or any acyl groups in general. Hence, side reactions such as orthoester formation and acyl transfer are prevented. Activation of 2-Cbz glycosyl donor **11** with N-iodosuccinimide and trifluoromethanesulfonic acid selectively formed benzyl protected CUL-11-glucoside **9**. Subsequent deprotection by palladium-catalyzed hydrogenation[41] afforded CUL-11-glucoside **7** in a yield of 90 % (Scheme 2).

Metabolism of culmorin (1) and 11-acetyl-culmorin (9) in a wheat suspension culture

To investigate the metabolic fate of CUL *in planta*, a wheat suspension culture was treated with either CUL or 11-AcCUL (each 100 mg/L) and the respective metabolites were analyzed after 1 day and 1 week. At the end point the supernatant was removed, and mixed 1:1 with methanol to stop further reactions. The cell pellet was washed twice with 50% methanol to remove adsorbed compounds, and the combined wash solution was brought up to the 2-fold volume of the initial culture. The cell pellet was disrupted by sonication and also brought up in the twofold culture volume with 50% methanol final concentration (see supporting information).



Scheme 2 Regioselective glycosylation of culmorin (**1**) to yield culmorin-8-β,D-glucoside (**6**) via a Schmidt-glycosylation and culmorin-11-β,D-glucoside (**7**) with the 2-benzyloxycarbonyl (2-Cbz)-donor **11**.

In case of 11-AcCUL only 1 day incubation was investigated, because of the expected rapid deacetylation by the cells. The medium without cells was used as a control showing no background signals for CUL or any of its metabolites. The values obtained for CUL dissolved in cell free medium after 1 week were about 30% higher than the theoretical value of 50,000 µg/L (due to 1:1 dilution with methanol). This can only be partially explained by evaporation of the 1.5 mL culture medium, but most likely by considerable matrix effects, which may be different and even more significant in case of the cell culture and highly depending on the age of the cells. Therefore our results are only semiquantitative. Yet, in the medium without cells CUL seemed to be stable for a week, while only about 37% of the 11-AcCUL added to the medium without cells were measured in the spiked medium after 1 day. Nevertheless, no CUL was found, excluding non-enzymatic hydrolysis in the medium, and also pointing to matrix effects.

Treatment of wheat cells with CUL revealed a clear reduction of the remaining CUL concentration in the medium (supernatant after spinning down the cells). While about 94% of CUL were detected after day 1, only 7% were observed after 1 week in one replicate, and in the other two replicates CUL was already below the detection limit. Also the wash solution revealed interesting results. Assuming that in the worst case 50 µl medium may be retained in the 250 mg cell pellet about 3% remaining CUL could be explained in the absence of any metabolization. Interestingly, in the wash solution on both the 1-day and the 7-day time points about 11% of the CUL input was recovered, indicating considerable reversible binding of CUL to cells, presumably due to adsorption to cell wall structures preventing further metabolism.

CUL was metabolized in wheat cells to CUL-8-glucoside, and unexpectedly to 11-AcCUL, but no CUL-sulfates were detected (Table 1 & 2). Within 7 days the level of CUL-8-glucoside in the supernatant increased from an average value of 95 µg/L to 355 µg/L. CUL-8-glucoside could also be detected with an increasing concentration in the wash solution. CUL has two hydroxyl groups, so theoretically two different glucosides are possible. Yet, only one was found in the plant extract and this had the same retention time and fragmentation pattern as the synthesized CUL-8-Glc. The importance of CUL-11-Glc therefore seems to be negligible from a plant physiology point of view.

Table 1 Average concentrations of CuI-8-Glc in CUL treated wheat suspension culture extracts at two time points (mean of 3 repetitions).

	day	[CUL-8-Glc] µg/L
Culture supernatant	1	95.0
Culture supernatant	7	355.0
Cell wash solution	1	17.1
Cell wash solution	7	98.1
Cell extract	1	108.0
Cell extract	7	86.1

Interestingly, we also observed formation of 11-AcCUL when treating wheat cells with CUL suggesting the presence of wheat enzymes capable of acetylating CUL. Yet, the concentrations were lower than observed for the glucosides (day 1) and surprisingly 11-AcCUL was not detected in the cell extract.

Table 2 Concentrations of the formed 11-AcCUL in the supernatant.

	day	11-Ac-CUL [µg/L]			average
		# 1	# 2	# 3	[µg/L]
supernatant	1	11.8	11.9	10.2	11.3
supernatant	7	7.4	< LOD	< LOD	-

As the acetylation is performed by intracellular transferases depending on acetyl-CoA, the product AcCUL seemingly is rapidly pumped out of the cell.

We also performed a feeding assay with 11-AcCUL at 100 mg/L. By normalization to the 11-AcCUL concentration found after 1 day in the medium without cells (which should in first approximation correct for matrix effects), only one of three replicates showed 0.2% remaining 11-AcCUL in the supernatant, whereas the concentration was below the detection limit in case of the two other replicates. 11-AcCUL was obviously rapidly deacetylated – indicated by a maximum level of 47,300 µg/L CUL in the supernatant. In addition, CUL-8-Glc (32.5 µg/L found in the supernatant), formed by glucosylation of CUL, and 11-AcCUL-8-Glc (483 µg/L) were detected indicating that 11-AcCUL can enter the cells followed by glucosylation and excretion of 11-AcCUL-8-Glc.

Although we observed acetylation of CUL, no acetylated CUL-glucoside was found in any CUL treated sample. Hence, we assume that intracellular levels of AcCUL sufficient for glycosylation are only reached in 11-AcCUL treated cells, while in case of intracellular formation of 11-AcCUL it seems to be excreted more rapidly than glycosylated.

Conclusions

We were able to develop selective methods for the synthesis of CUL sulfates and glucosides (Supplementary figures NMR and MS spectra). Different glucosyl donors could be applied to exploit acetyl transfer to obtain CUL-8-Glc and CUL-11-Glc without the need for laborious separation of two regioisomers. Considering the potential of the combination of the already described NPTFA-donor **10** and our newly developed donor **11**, we aim to use this system on other mycotoxins in order to selectively access masked mycotoxins. The in depth evaluation of this donor and its use within the synthesis of other masked mycotoxins and metabolites is already in progress.

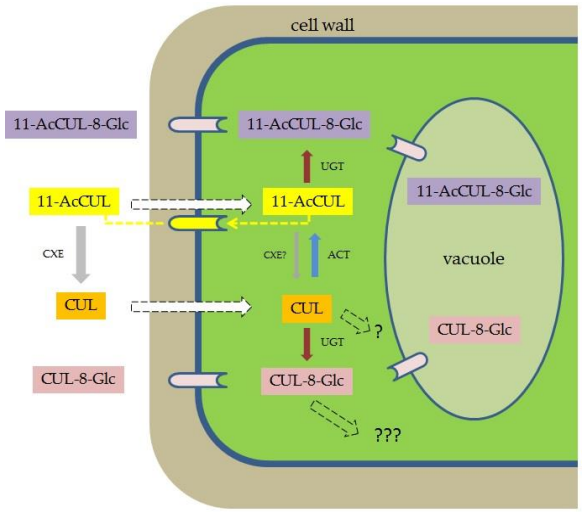


Figure 3 In CUL treated wheat cells CUL disappears and CUL-8-Glc is formed by cytosolic UDP-glucosyltransferase (UGT), but this can only account for a small fraction of the missing CUL. CUL is therefore either also metabolized into other unknown ways or CUL-8-Glc is further metabolized. A minor fraction of added CUL is also converted to 11-AcCUL by plant acetyltransferase (ACT) and translocated.When cells are treated with 11-AcCUL most of it is rapidly deacetylated to CUL by carboxylesterase (CXE). 11-AcCUL entering the cell (presumably by diffusion - dashed white arrow) is also directly glycosylated, and potentially could in part deacetylated by intracellular CXEs. Both glucosides are presumably concentrated in the vacuole but also translocated across the plasma membrane (blue line) to the apoplast, where they could be incorporated into cell wall material.

Using the synthesized standards as calibrants we analyzed the culture media (supernatant), wash solution, and the extracts of the cells. Calibrants in neat solvents were used, rendering the results semiquantitative by not considering matrix effects. A validation of the analytical method would be necessary to evaluate the natural occurrence of CUL metabolites in cereals. A schematic representation of our results is shown in Fig. 3.

In contrast to a similar study treating wheat suspension culture cells with DON, no sulfate conjugates were identified *in planta*.[28] While sulfation seems to be of minor relevance in plants, it is a prominent detoxification process of xenobiotica in poultry and other animal species.[42] Recently the presence of DON-sulfate in human urine has been described for the first time.[43] Data on the metabolic fate of CUL in humans and livestock are lacking. In an upcoming study, we intend to use the prepared CUL sulfates as analytical standards to assess the metabolization of CUL in poultry.

11-AcCUL is a fungal metabolite and potentially a biosynthetic precursor of CUL similar to DON, which is formed by deacetylation of the initial metabolites 3- and 15-acetyl-DON. Acetylated CUL may have higher membrane permeability, and thus reach targets in plants more easily. The wheat cells can rapidly deacetylate 11-AcCUL, seemingly by extracellular esterase. If high concentrations of 11-AcCUL are applied externally, the compound enters the cell and is converted to 11-AcCUL-8-Glc by cytosolic glucosyltransferases.

Likewise when high concentrations of CUL are applied, CUL-8-Glc is formed intracellularly.

Assuming that in the original culture only about 250 µl (250 mg wet weight of cells on average) of the total 1500 µl culture volume corresponds to intracellular space, the actual intracellular glucoside concentration is about 6x higher than what was measured in the extract. Consequently, the intracellular concentration of CUL-Glc (6 x 108 µg/L) is about 6.8 fold higher than the glucoside concentration found in the medium (average 95 µg/L). Although this result is only semiquantitative it clearly indicates that at least a part of the CUL-Glc formed inside the plant is concentrated (presumably in vacuoles) but partly also translocated to the apoplast and released to the medium in case of the suspension culture.

Interestingly, a small portion of the added CUL was converted into 11-AcCUL and found in the medium, but not in cells. The observed ability of the plant to acetylate mycotoxins is in line with recent findings in studies of the fate of T-2 and HT-2 in various cereals, wherein formation of acetylated derivatives has been reported.[44, 45] In case of trichothecenes acetylating the C3-OH is clearly a detoxification reaction.[46] It is unknown which plant enzymes are responsible for this reaction, but CUL might be a competing substrate, modulating the toxicity of trichothecenes. In depth studies are required to investigate whether a synergism exists between CUL and trichothecenes in plants.

On a molecular basis only a minor part of CUL can be accounted for the formation of the identified metabolites. Beside other possible metabolization routes, CUL might be rapidly metabolized mainly into CUL-Glc and then further to still uncharacterized metabolites and conjugates. The low recovery is comparable to previously reported results for the mycotoxin zearalenone. Formation of several di-glucosides and malonylglucosides was reported as well as a large portion that eventually ends up as "insoluble residue".[47, 48] Further studies with stable isotope or radiolabeled CUL will help to better understand its fate *in planta* and its ecological role. Since the toxicity of CUL itself is low, the newly described masked mycotoxins are probably of minor toxicological relevance, but give insights on how the plant cope with the suspected DON synergist.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest

Supplementary materials

Synthetic procedures, NMR data of all compounds and details about the plant experiments are available in the supporting information.

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Chapter 4

Conclusion

In summary, three novel glycosyl donor systems could be developed that enable the synthesis of glycosyl esters and the preparation of complex and labile glycosylated natural products containing ester functionalities. Two of these systems demonstrated high yields and complete β-diastereoselectivity in glucosylations but were not applicable for the glycosylation of natural products containing double bonds. Therefore the NPAc-protected glucosyl trichloroacetimidate was developed as third glycosyl donor system that finally enabled the glycosylation of base labile natural products that are also sensitive to hydrogenolysis. Unfortunately, this glycosyl donor system even though it contains a neighboring participating group at C-2, presented only an enhanced selectivity towards β-glycosylation (Fig. 1). However, it could be successfully applied in a one-pot procedure for the preparation of T2-O-β,D- glucoside, a phase II metabolite of the mycotoxin T2.

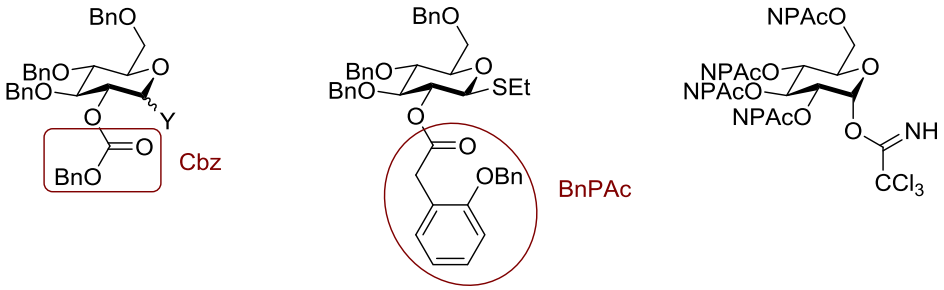

Figure 1 Novel glucosyl donors developed for the glycosylation of complex and labile natural products [Y= SEt, STaz, STol, SPym, $O(CNPh)CF_3$]

Further trichothecene phase II metabolites could be synthesized after the development of a reliable and efficient method for the synthesis of DON-15-glycosides by Schmidt-glycosylation of 3-ADON with an acetyl protected Schmidt glycosyl donor followed by deprotection under mild reaction conditions. The common side reactions of acetyl protected glycosyl donors, namely the formation of orthoesters and the acyl transfer, could be largely eliminated. Applying this protocol in combination with [$^{13}C_6$]glycosyl donors isotope labeled DON-15-glycosides could be synthesized, which are crucial to enable accurate quantification of masked mycotoxins by LC-MS.

Additionally, based on the development of an efficient sulfation procedure for trichothecenes, various mycotoxin sulfates could be synthesized including DON-3- and DOM-3-sulfate that were already applied by our collaboration partners from the University of Natural Resources and Life Sciences, Vienna (BOKU) as reference materials to determine the formation of these modified mycotoxins in different poultry species.

Selective glycosylation and sulfation procedures could be developed for the mycotoxins zearalenone and culmorin to prepare their respective glycosides and sulfates. Simply for conjugation of ZEN at the less reactive site a suitable protecting group strategy had to be established first. The developed 2-O-Cbz-glycosyl donors for the glycosylation of base labile natural products could be successfully applied for the synthesis of culmorin-11-glucoside revealing a further advantage of these type of glycosyl donors of not undergoing acyl transfer (Fig. 2).

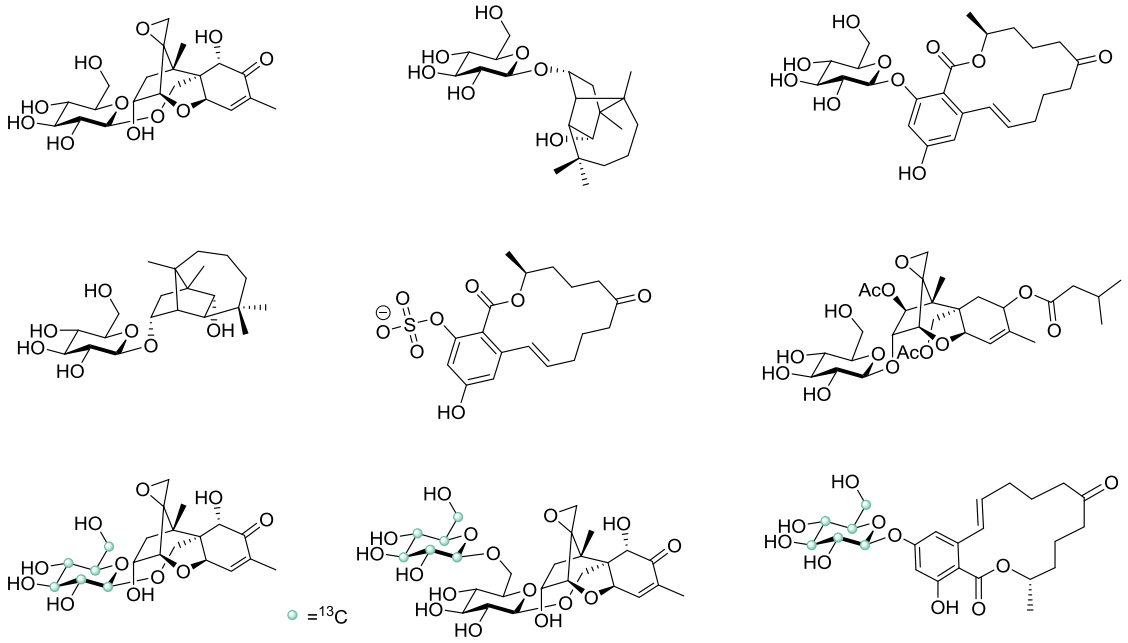

Figure 2 Various modified mycotoxins synthesized within this PhD thesis

Chapter 5

Statement of Contribution

3.1-Development of New Glycosylation Methods

Manuscript #1

Preliminary work such as the synthesis of the various glycosyl donors and first glycosylation experiments were performed by D. Svatunek, S. Krauter and G. Tegl within their bachelor and diploma thesis, respectively. The applicant carried out the large glycosylation screening, demonstrated the applicability of the developed method by synthesizing various glucosides and glucosyl ester, developed a mild deprotection protocol and presented its orthogonality to ester groups. P. Kosma contributed in terms of NMR measurements and supported the project with his comprehensive expertise. The manuscript was composed and written by H. Mikula and the applicant. All authors contributed to the manuscript discussion and correction.

Manuscript #2

The main synthetic work within this manuscript was performed by the applicant. S. Krauter and T. Schwarz contributed to the synthesis of the protecting group and the glucosyl donor within their bachelor thesis. The manuscript was predominantly composed and written by the applicant. All authors contributed to the manuscript discussion and correction.

Manuscript #3

The research subject was initiated by H. Mikula and the applicant. Preliminary studies were performed by M. Schwarz within his bachelor thesis and A. Schiefer contributed to the synthesis of the glucosyl donor during an internship. All other parts of the synthetic work were conducted by the applicant. G. Häubl and A. Schiessl provided the mycotoxin. The manuscript was predominantly composed and written by the applicant. All authors contributed to the manuscript discussion and correction.

3.2-Synthesis of Trichothecene Metabolites

Manuscript #4

The research subject was initiated and independently performed by the applicant. All the synthetic work was carried out by the applicant. ^{13}C decoupled and correlated NMR spectra were recorded by C. Hametner. G. Häubl and A. Schiessl provided the mycotoxin. The manuscript was composed and written by the applicant with a revision by H. Mikula. All authors contributed to the manuscript discussion and correction.

Manuscript #5

The research subject of manuscript #5 was initiated and to a great extent performed by P. Fruhmann. The applicant assisted in the improvement of the sulfation procedure and contributed to the purification of some DON sulfates. The manuscript was written by P. Fruhmann. The applicant contributed to manuscript revision and discussion.

Manuscript #6

This collaborative project was initiated by H. Schwartz-Zimmermann. P. Fruhmann and the applicant performed the synthesis of the DOM-sulfates and contributed to the manuscript discussion and correction.

3.3-Synthesis of Zearalenone Metabolites

Technical Note

All the synthetic work was performed by the applicant. A. Schiessl and G. Häubl provided the mycotoxin. The ^{13}C decoupled NMR spectra were recorded by C. Hametner. The technical note was written by the applicant.

Manuscript #7

The applicant was already involved in this project before starting with the PhD thesis within the scope of an internship. Further improvement and optimization, especially of the synthesis of Zearelenone-16-glucoside was done in the course of the PhD thesis of the applicant. The manuscript was written by H. Mikula. The applicant contributed to the manuscript revision and discussion.

3.4-Synthesis of Culmorin Metabolites

Manuscript #8

The research subject was initiated by P. Fruhmann and the applicant. All the synthetic work was performed by the applicant. M. Vaclavikova performed the biological studies. The manuscript was predominantly written by P. Fruhmann and the applicant. All authors contributed to the manuscript discussion and correction.

Chapter 6

Appendix

Manuscript #9

Reprinted from Synlett (2013), 14
Weber, J.; Mikula, H.; Fruhmann, P.; Hametner, C.; Varga, E.; Berthiller, F.; Krska, R.; Fröhlich, J. Gentiobiosylation of β-Resorcylic Acid Esters and Lactones: First Synthesis and Characterization of Zearalenone-14-β,d-Gentiobioside, 1830-1834.

Gentiobiosylation of β-Resorcylic Acid Esters and Lactones: First Synthesis and Characterization of Zearalenone-14-β,D-Gentiobioside

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Abstract: The development of an optimized protocol for the gentiobiosylation of β-resorcylic acid esters and lactones (β-RAL) is presented. Different gentiobiosyl donors were prepared and used for regioselective and diastereoselective glycosylation affording a reliable synthetic strategy towards this class of natural product glycosides. The improved procedure was finally used for the preparation of the masked *Fusarium* mycotoxin zearalenone-14-β,D-gentiobioside.

Key words: carbohydrates, glycosides, glycosylation, phenols, natural products

Zearalenone (ZEN, **1**, Figure 1) is a mycotoxin produced by several plant pathogenic *Fusarium* species, including *Fusarium graminearum*, *Fusarium culmorum*, and *Fusarium cerealis*. This mycotoxin is common in maize, but also barley, oats, wheat, and rice are susceptible to contamination with ZEN.[1] Fusarium species are probably the most prevalent toxin-producing fungi of the northern temperate regions and are commonly found in cereals grown worldwide.[2] ZEN and its metabolites possess estrogenic activity in mammals, including pigs, cattle, and sheep.[3] Problems of the reproductive tract as well as impaired fertility and abnormal fetal development in farm animals can be caused by ZEN.[4] Furthermore, this mycotoxin can interfere with various enzymes involved in steroid metabolism, which was recently investigated.[5] Therefore ZEN is of significant importance from an agricultural, economic, and health perspective.[6]

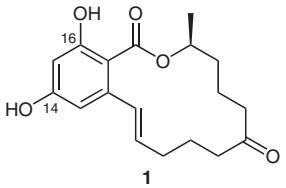

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Figure 1 Structure of zearalenone (ZEN, **1**)

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Conjugated mycotoxins, especially glycosides, can emerge after metabolization by living plants. The occurrence of ZEN-14-β,D-glucoside (**2**, Figure 2) in wheat was shown by Schneweis et al.[7] Furthermore ZEN-14-β,D-gentiobioside (formerly described as ZEN-14-diglucoside) was shown to be one of the major (late) phase II metabolites of **1** formed in the model plant *Arabidopsis thaliana* (Figure 2).[8] Awareness of such altered forms, often called masked mycotoxins, is increasing, but reliable analytical methods, standards as well as structural, occurrence, and toxicity data are still scarce.[9]

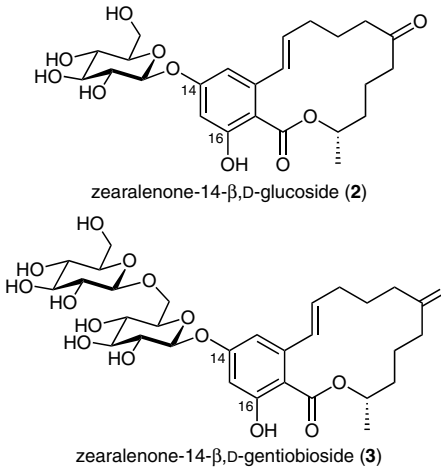


Figure 2 Structures of glycosylated ZEN metabolites (masked mycotoxins)

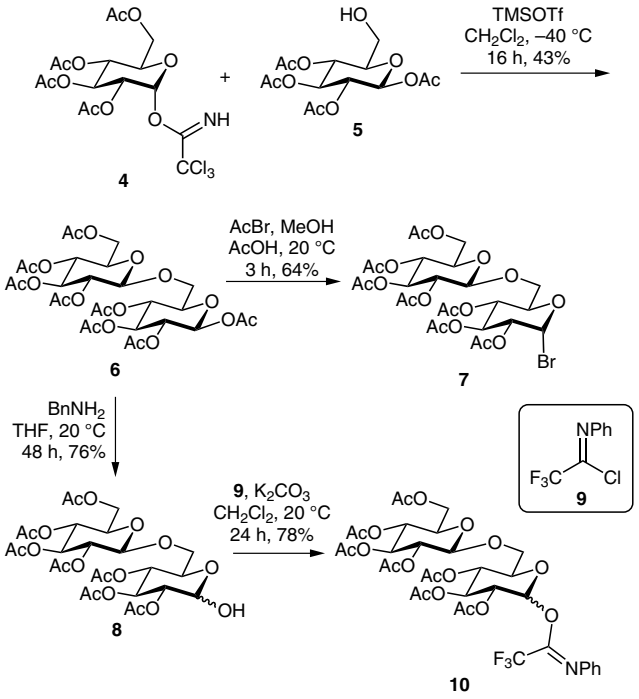
Gentiobiosylation has been shown to be an important metabolic pathway and several biologically active and relevant compounds have been described.[10] Additionally, further mycotoxin gentiobiosides have been identified as natural contaminants of corn and are also reported to be present in beer samples.[11]

In the course of ongoing research in the emerging field of masked and conjugated mycotoxins we focused on the synthesis of ZEN-14-β,D-gentiobioside (**3**) for structure confirmation and an accurate differentiation between **3** and the isomeric metabolite ZEN-14,16-di(β,D-glucoside).[12]

The first synthesis of a ZEN-glycoside, ZEN-14-β,D-glucoside (**2**) was achieved applying a Königs–Knorr procedure under phase-transfer conditions,[13] but the analogous reaction to produce ZEN-14-β,D-glucuronide was described to be unsuccessful under a variety of coupling conditions.[14] We were able to develop a fast and reproducible procedure for the chemical synthesis of ZEN-14-β,D-glucuronide, which was published very recently.[15]

Gentiobiosylation of phenols is often achieved by stepwise glucosylation and application of protective-group strategies.[16] Although similar approaches were developed for direct phenol glycosylation using a disaccharidic donor,[17] to the best of our knowledge there is no procedure reported for direct gentiobiosylation of phenols. Shimoda and coworkers applied cultured cells of *Eucalyptus perriniana* as biocatalysts for the synthesis of a mixture of glucosides and gentiobiosides of the isoflavonoids genistein and glycitein.[18]

Considering already described procedures for glucosylation of β-resorcylic acid esters and lactones (β-RAL), different disaccharidic donors were prepared for direct β-gentiobiosylation. Since acetyl protective groups were shown to be applicable for diastereoselective glycosylation of β-RAL, β-gentiobiose octaacetate (**6**) was selected as key intermediate. A reliable procedure[19] for β-glucosylation of 1,2,3,4-tetra-*O*-acetyl-β,D-glucoside (**5**)[20] using the trichloroacetimidate donor **4**[21] was applied to obtain larger amounts of **6**.[22] Gentiobiosyl bromide **7** was prepared according to Hunsen et al.,[23] and anomeric deprotection of **6** by reaction with benzyl amine[24] yielded the OH-sugar **8**,[25] which was subsequently reacted with *N*-phenyl-2,2,2-trifluoroacetimidoyl chloride (**9**)[26] to obtain the acetimidate gentiobiosyl donor **10**[27] (Scheme 1).



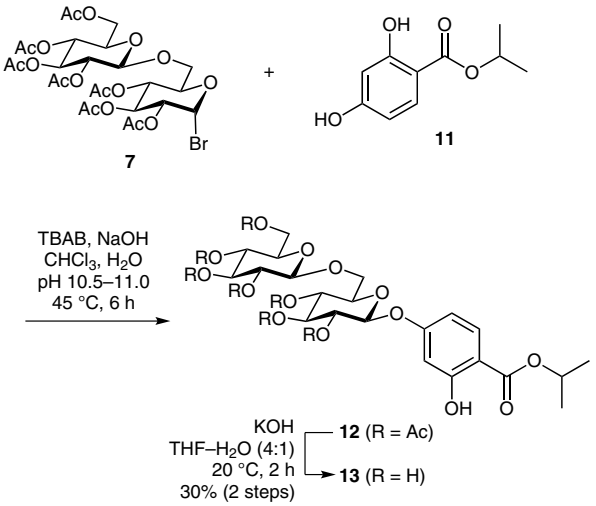
Scheme 1 Synthesis of gentiobiosyl donors **7** and **10**

2,4-Dihydroxybenzoic acid isopropyl ester (**11**)[28] was used as ZEN mimic to investigate the gentiobiosylation of resorcylic acid esters applying **7** and **10** as glycosyl donors under different reaction conditions. Selected data of reaction optimization and screening is shown in Table 1. Königs–Knorr glycosylation applying silver(I) salts for activation[29] was observed to result in nearly quantitative conversion after 48 hours. The highest yield (as determined by ^{1}H NMR after standard addition to the crude product mixture) was obtained when using Ag_2CO_3 for activation of the gentiobiosyl donor **7**.

Lewis acid mediated glycosylation[30] applying the acetimidate donor **10** gave only poor conversion or even complete rearrangement of **10** into the corresponding *N*-glycosyl acetamide (as indicated by NMR spectroscopy).

Phase-transfer glycosylation (Table 1, entry 7) was carried out applying an optimized procedure[31] according to the described approach for ZEN glucosylation.[13] The obtained results in terms of conversion and yield were quite comparable to that observed applying classic Königs–Knorr conditions.

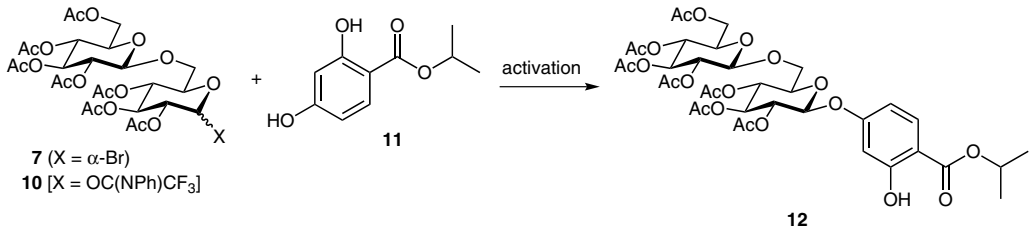
In all cases **12** was obtained within a complex product mixture mainly containing carbohydrate byproducts, but best results were achieved using phase-transfer glycosylation. Most of the impurities were removed by silica gel filtration, and the crude product was used without further purification in the deprotection step. Basic hydrolysis was achieved by reaction with an excess of KOH in THF–H_2O (4:1),[32] and the deacetylated product **13**[33] was finally isolated by reversed-phase (C18) column chromatography (Scheme 2).



Scheme 2 Synthesis of gentiobioside **13**

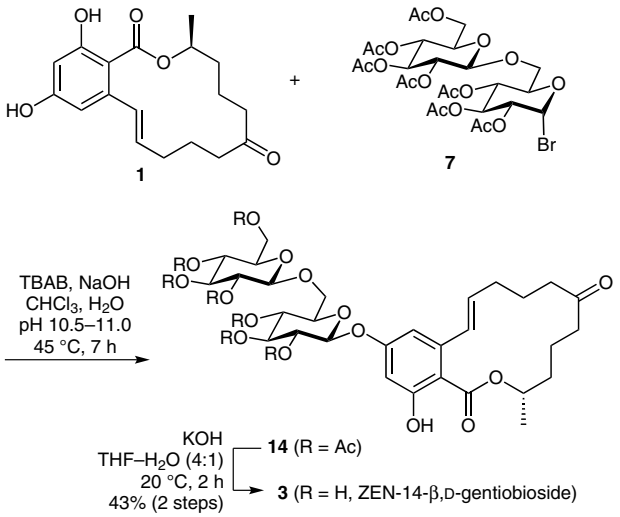
Finally, we were able to prepare ZEN-14-β,D-gentiobioside (**3**)[34] starting from ZEN (**1**) in an overall yield of 43% following general procedures for glycosylation and subsequent basic hydrolysis of the acetylated intermediate **14** according to the synthesis of the mimic gentiobioside **13** (Scheme 3).

Table 1 Gentiobiosylation of ZEN Mimic **11** Using Glycosyl Donors **7** and **10**



Entry	Donor (equiv)	Activation (equiv)	Solvent	Conversion of **11** (%)[a]	Yield (%)[b]
1	**7** (2.5)	Ag_2O (1.5)	MeCN	100	54
2	**7** (2.5)	Ag_2O (1.5)	CH_2Cl_2	90	52
3	**7** (2.5)	Ag_2CO_3 (1.5)	CH_2Cl_2	95	83
4	**10** (1.5)	TMSOTf (0.1)	CH_2Cl_2	13	9
5	**10** (2.5)	TMSOTf (0.02)	1,4-dioxane–toluene	0[c]	0[c]
6	**10** (1.5)	$BF_3{\cdot}OEt_2$ (0.1)	CH_2Cl_2	24	18
7	**7** (2.8)	TBAB (1.0), NaOH	$CHCl_3$–H_2O	92	52

[a] As determined by ^{1}H NMR.
[b] Nonisolated yield, determined by ^{1}H NMR spectroscopy (after standard addition of **11**).
[c] Rearrangement of the acetimidate **10** to the corresponding *N*-glycosyl acetamide was observed, as indicated by NMR spectroscopy.



Scheme 3 Gentiobiosylation of **1** and preparation of the target compound ZEN-14-β,D-gentiobioside (**3**)

In summary we were able to develop a reliable procedure for the gentiobiosylation of β-RAL, which in general should be applicable for many phenolic compounds. The first synthesis of a mycotoxin gentiobioside was accomplished yielding **3** in reasonable amounts for further studies.

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Supporting Information for this article is available online at http://www.thieme-connect.com/ejournals/toc/synlett. General experimental details and full characterization data (MS and NMR spectra) of **3** are included.

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(19) **Procedure for the Lewis Acid Mediated Synthesis of β-Gentiobiose Octaacetate (6)**
1,2,3,4-Tetra-*O*-acetyl-β,D-glucose (**4**, 3.8 g, 11 mmol) and trichloroacetimidoyl donor **5** (5.8 g, 11.8 mmol) were dissolved in dry CH_2Cl_2 (100 mL). MS 3 Å (10 g) was added, and the mixture was stirred at r.t. under argon for 1 h. After cooling to –40 °C, TMSOTf (0.2 mL, 1.1 mmol) was added, and the reaction mixture was stirred at –40 °C for 16 h. The reaction was quenched by addition of Et_3N, filtered through Celite, and concentrated. The crude product was purified by column chromatography (hexanes–EtOAc, 5:1 to 1:1) to obtain **6** (3.1 g, 43%) as a white solid. Analytical data matched those reported in the literature.[22]

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(24) **Procedure for Anomeric Deprotection of 6**
To a solution of **6** (1.31 g, 1.9 mmol) in dry THF (45 mL) was added benzylamine (0.23 mL, 2.1 mmol), and the reaction mixture was stirred at r.t. for 48 h. The solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2 (100 mL), washed with 1 M HCl (2 × 100 mL) and H_2O (100 mL). The organic layer was dried over Na_2SO_4 and concentrated. Column chromatography (hexanes–EtOAc, 1:1 to 1:3) afforded the desired product **8** (0.91 g, 76%) as a white solid. Analytical data matched those reported in the literature.[25]

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(27) **Procedure for the Synthesis of Gentiobiosyl Acetimidate Donor 10**
To a solution of gentiobiose heptaacetate (**8**, 0.8 g, 1.3 mmol) in dry CH_2Cl_2 (20 mL) was added K_2CO_3 (0.36 g, 2.6 mmol) and *N*-phenyl-2,2,2-trifluoroacetimidoyl chloride (**9**, 0.4 mL, 2.6 mmol). The reaction mixture was stirred at r.t. and under argon for 24 h. The solvent was removed on a rotary evaporator, and the residue was purified by column chromatography (hexanes–EtOAc, 3:1) to yield **10** (0.82 g, 78%) as a white solid.
Analytical Data for 10
^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.39–7.22 (m, 2 H), 7.18–7.06 (m, 1 H), 6.86 (d, *J* = 7.6 Hz, 2 H), 5.77 (br s, 1 H), 5.25–5.15 (m, 2 H), 5.11 (d, *J* = 10.3 Hz, 1 H), 5.05–4.92 (m, 3 H), 4.56 (d, *J* = 7.8 Hz, 1 H), 4.25 (dd, *J* = 12.3, 4.7 Hz, 1 H), 4.12 (dd, *J* = 12.3, 2.3 Hz, 1 H), 3.90 (d, *J* = 9.4 Hz, 1 H), 3.76–3.56 (m, 3 H), 2.08 (s, 3 H), 2.06 (s, 3 H), 2.03 (s, 3 H), 2.02 (s, 3 H), 2.01 (s, 3 H), 2.00 (s, 3 H), 1.95 (s, 3 H). ^{13}C NMR (50 MHz, $CDCl_3$): δ = 170.8 (s, 1 C), 170.3 (s, 1 C), 170.27 (s, 1 C), 169.6 (s, 1 C), 169.52 (s, 1 C), 169.49 (s, 1 C), 169.1 (s, 1 C), 142.9 (s, 1 C), 128.8 (d, 2 C), 124.6 (d, 1 C), 119.2 (d, 2 C), 100.5 (d, 1 C), 94.3 (d, 1 C), 74.1 (d, 1 C), 72.6 (d, 1 C), 72.4 (d, 1 C), 71.9 (d, 1 C), 70.8 (d, 1 C), 70.2 (d, 1 C), 68.3 (d, 1 C), 68.2 (d, 1 C), 67.3 (t, 1 C), 61.7 (t, 1 C), 20.9 (q, 1 C), 20.7 (q, 4 C), 20.6 (q, 1 C), 20.5 (q, 1 C). HRMS (ESI$^+$): *m/z* calcd for $C_{34}H_{40}F_3NNaO_{18}^+$ $[M + Na]^+$: 830.2090; found: 830.2099.

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(29) **General Procedure A: Silver(I)-Activated Königs–Knorr Glycosylation Using Gentiobiosyl Bromide 7**
To a solution of the glycosyl acceptor (1 equiv) and gentiobiosyl bromide **7** (2.5 equiv) in CH_2Cl_2 or MeCN (5 mL/mmol) MS 3 Å (0.1 g/mL) was added, and the reaction mixture was stirred at r.t. under argon for 1 h. After addition of silver(I) salt (1.5 equiv) stirring was continued in the dark for an additional period of 48 h. Filtration through Celite and concentration under reduced pressure afforded the crude product mixture.

(30) **General Procedure B: Lewis Acid Mediated Glycosylation Using Acetimidate 10**
To a solution of the glycosyl acceptor (1 equiv) and gentiobiosyl bromide **7** (1.5–2.5 equiv) in CH_2Cl_2 or dioxane–toluene (5 mL/mmol) MS 3 Å (0.1 g/mL) was added, and the reaction mixture was stirred at r.t. under argon for 1 h. After cooling to –10 °C, TMSOTf or $BF_3{\cdot}OEt_2$ (0.02–0.1 equiv) was added, and stirring was continued at –10 °C for an additional period of 16 h. The reaction mixture was filtered through Celite, washed with sat. aq $NaHCO_3$, dried over Na_2SO_4, and concentrated under reduced pressure to afford the crude product mixture.

(31) **General Procedure C: Phase-Transfer Glycosylation Using Gentiobiosyl Bromide 7**
Glycosyl acceptor (1 equiv), **7** (2.8 equiv), and TBAB (1 equiv) were dissolved in $CHCl_3$ (80 mL/mmol). Borate buffer (80 mL/mmol) was added, and the reaction mixture was warmed to 45 °C. The pH was kept between 10.5 and 11.0 by dropwise addition 0.1 M aq NaOH. After 6 h the organic layer was separated, dried over Na_2SO_4, and concentrated to afford the crude product mixture.

(32) **General Procedure D: Basic Hydrolysis of Crude Acetyl-Protected Gentiobiosides**
Crude acetyl-protected gentiobioside (**12** or **14**) was filtered over silica gel (hexanes–EtOAc = 3:1 to 1:2) to remove most of the carbohydrate impurities. Appropriate fractions were pooled and concentrated. The residue was dissolved in THF–H_2O (4:1, 50 mL/mmol), KOH (10 equiv) was added, and the reaction mixture was stirred for 2 h at r.t. After addition of 0.1 N HCl (pH 6.8), the solution was diluted with H_2O and immediately extracted with EtOAc. The combined organic layer was dried over Na_2SO_4 and concentrated. RP-C18 column chromatography (MeCN–H_2O gradient elution, used for **13**) or preparative RP-C18-HPLC (MeCN–H_2O gradient elution, used for **3**) yielded the desired gentiobioside.

(33) **Synthesis of Gentiobioside 13**
Starting from ZEN mimic **11** (11.7 mg, 0.06 mmol) and following general procedures C and D, gentiobioside **13** was obtained as a white solid (9.3 mg, 30%).
Analytical Data for 13
^{1}H NMR (400 MHz, MeOD): δ = 7.77 (d, *J* = 8.8 Hz, 1 H), 6.71 (d, *J* = 1.8 Hz, 1 H), 6.64 (dd, *J* = 8.8, 1.8 Hz, 1 H), 5.26 (sept, *J* = 6.2 Hz, 1 H), 4.98 (d, *J* = 6.9 Hz, 1 H), 4.36 (d, *J* = 7.5 Hz, 1 H), 4.17 (d, *J* = 11.3 Hz, 1 H), 3.91–3.83 (m, 1 H), 3.88–3.78 (m, 2 H), 3.81–3.74 (m, 1 H), 3.67 (dd, *J* = 11.3, 5.4 Hz, 1 H), 3.50–3.44 (m, 2 H), 3.44–3.38 (m, 1 H), 3.36–3.30 (m, 1 H), 3.28–3.20 (m, 2 H), 1.38 (d, *J* = 6.2 Hz, 6 H). ^{13}C NMR (100 MHz, MeOD): δ = 170.7 (s, 1 C), 164.6 (s, 1 C), 164.4 (s, 1 C), 132.4 (d, 1 C), 109.6 (d, 1 C), 108.4 (s, 1 C), 104.94 (d, 1 C), 104.88 (d, 1 C), 101.32 (d, 1 C), 78.0 (d, 1 C), 77.9 (d, 1 C), 77.8 (d, 1 C), 77.3 (d, 1 C), 75.1 (d, 1 C), 74.7 (d, 1 C), 71.6 (d, 1 C), 71.4 (d, 1 C), 70.2 (d, 1 C), 70.1 (t, 1 C), 62.7 (t, 1 C), 22.1 (q, 2 C). HRMS (ESI$^-$): *m/z* calcd for $C_{22}H_{31}O_{14}^-$ [M – H]$^-$: 519.1719; found: 519.1707.

(34) **Synthesis of ZEN-14-β,D-gentiobioside (3)**
Starting from ZEN (**1**, 33.4 mg, 0.105 mmol) and following general procedures C and D, ZEN-14-β,D-gentiobioside (**3**) was obtained as a white solid (29 mg, 43%).
Analytical Data for 3
^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.43 (br s, 1 H), 6.65 (d, *J* = 1.5 Hz, 1 H), 6.53 (d, *J* = 1.5 Hz, 1 H), 6.40 (d, *J* = 15.4 Hz, 1 H), 6.01 (dt, *J* = 15.4, 7.1, 1 H), 5.36 (d, *J* = 4.3 Hz, 1 H), 5.18 (d, *J* = 3.2 Hz, 1 H), 5.11–5.04 (m, 1 H), 4.98–4.86 (m, 2 H), 4.90 (d, *J* = 7.8 Hz, 1 H), 4.53 (br s, 1 H), 4.19 (d, *J* = 7.8 Hz, 1 H), 3.98 (d, *J* = 10.2 Hz, 1 H), 3.66 (d, *J* = 12.1 Hz, 1 H), 3.63–3.55 (m, 2 H), 3.47–3.41 (m, 1 H), 3.30–3.18 (m, 3 H), 3.16–3.09 (m, 1 H), 3.08–3.03 (m, 2 H), 2.98 (t, *J* = 8.1 Hz, 1 H), 2.39–2.32 (m, 1 H), 2.32–2.26 (m, 2 H), 2.25–2.16 (m, 1 H), 2.07–1.95 (m, 1 H), 1.83–1.71 (m, 1 H), 1.70–1.59 (m, 3 H), 1.58–1.45 (m, 2 H), 1.27 (d, *J* = 6.1 Hz, 6 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 211.2 (s, 1 C), 168.7 (s, 1 C), 160.0 (s, 1 C), 158.5 (s, 1 C), 138.4 (s, 1 C), 133.3 (d, 1 C), 129.8 (d, 1 C), 112.8 (s, 1 C), 105.2 (d, 1 C), 103.9 (d, 1 C), 103.1 (d, 1 C), 100.6 (d, 1 C), 77.3 (d, 1 C), 77.1 (d, 1 C), 76.8 (d, 1 C), 74.0 (d, 1 C), 73.6 (d, 1 C), 71.9 (d, 1 C), 70.4 (d, 1 C), 69.9 (d, 1 C), 68.9 (t, 1 C), 61.4 (t, 1 C), 43.5 (t, 1 C), 37.1 (t, 1 C), 34.8 (t, 1 C), 31.4 (t, 1 C), 21.4 (t, 2 C), 20.3 (q, 2 C). HRMS (ESI$^-$): *m/z* calcd for $C_{30}H_{41}O_{15}^-$ [M – H]$^-$: 641.2451; found: 641.2455.

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Sulfation of β-resorcylic acid esters—first synthesis of zearalenone-14-sulfate

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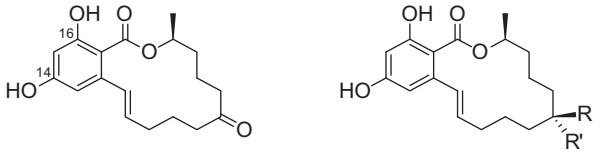
ABSTRACT

The chemical sulfation of β-resorcylic acid esters was investigated by applying state of the art procedures for the synthesis and deprotection of 2,2,2-trichloroethyl protected sulfates as appropriate intermediates. The selectivity of monosulfation was studied and reaction optimization was performed considering the effect of the solvent, different bases as well as the sulfation reagent itself. Finally the obtained protocols were applied for the first synthesis of zearalenone-14-sulfate (ammonium salt), an important conjugated (masked) mycotoxin, as reference material for further investigations in the field of bioanalytics as well as toxicology.

Zearalenone (ZEN, **1**, Fig. 1) is a mycotoxin produced by several plant pathogenic *Fusarium* species, including *Fusarium graminearum*, *Fusarium culmorum*, and *Fusarium cerealis*. This mycotoxin is common in maize, but also barley, oats, wheat, and rice are susceptible to contamination with ZEN.[1] *Fusarium* species are probably the most prevalent toxin-producing fungi of the northern temperate regions and are commonly found in cereals grown in America, Europe, and Asia, but also in Africa.[2,3] ZEN and its phase I metabolites possess estrogenic activity in mammals, including pigs, cattle, and sheep.[4] Problems of the reproductive tract as well as impaired fertility and abnormal fetal development in farm animals can be caused by ZEN.[5] Furthermore ZEN and its metabolites α-zearalenol (α-ZEL, **2a**) and β-ZEL (**2b**, Fig. 1) can interfere with various enzymes involved in steroid metabolism, which was recently investigated.[6–8] Therefore ZEN is of significant importance from an agricultural, economic, and health perspective.[9]

Additionally, conjugated mycotoxins, for example, glucosides and sulfates can emerge after metabolization by living plants.[10] The occurrence of ZEN-14-β,D-glucoside (**3**, Fig. 2) in wheat was shown by Schneweis et al.[11] and preparation of this conjugate to obtain reasonable amounts of reference material for further investigations has been first reported by Grabley et al. by applying an optimized Königs–Knorr procedure under phase transfer conditions.[12] Basically there are two sites for glycosylation present in



Zearalenone (**1**)

α-Zearalenol (**2a**): R = H, R' = OH
β-Zearalenol (**2b**): R = OH, R' = H

Figure 1. Structures of zearalenone (ZEN) and its main phase I metabolites α-zearalenol (α-ZEL) and β-ZEL.

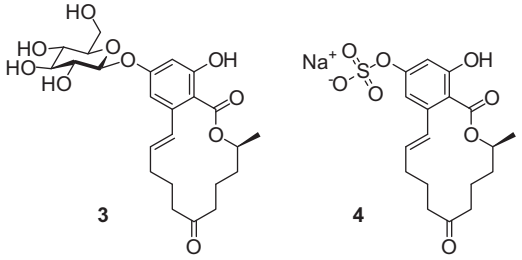


Figure 2. Structures of conjugated zearalenone (ZEN) metabolites: ZEN-14-β,D-glucoside (**3**) and ZEN-14-sulfate, sodium salt (**4**).

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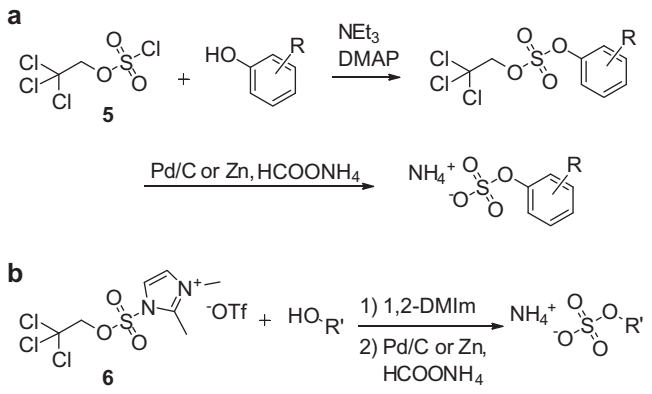
ZEN, but conjugation in position 14 (ring system numbering following the scheme proposed by Metzler[13]) is strongly favored compared to position 16.[14] ZEN-14-sulfate (formerly known as ZEN-4-sulfate) was first isolated by Plasencia and Mirocha from rice inoculated with *F. graminearum*.[15] El-Sharkawy et al. reported the conversion of ZEN into ZEN-14-sulfate (**4**, Fig. 2) by various microorganisms[16] and Berthiller et al. identified **4** as phase II metabolite of ZEN in the model plant *Arabidopsis thaliana*.[17] The conjugate most likely retains biological properties of the mycotoxin, since the sulfate moiety is easily cleaved under acidic conditions and in rats.[16] Therefore, ZEN-14-sulfate was included in analytical methods for the determination of free and masked mycotoxins during the last years.[18–21] Vendl et al. identified **4** as the most abundant analyte in 84 cereal based food products by LC–MS/MS measurements.[22]

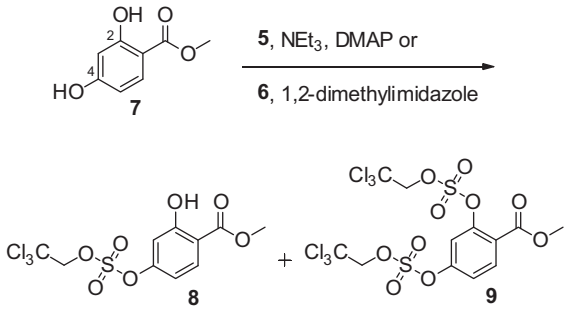
Nevertheless, reference material of **4** is still produced by time-consuming *F. graminearum* inoculation of rice. Due to its low stability the obtained sulfate is furthermore not suitable for long term storage.

Although alkyl and aryl sulfates as well as resorcylic acid lactones (cyclic 2,4-dihydroxybenzoates) such as ZEN are widespread in biological systems,[23–28] to the best of our knowledge there is still no procedure reported for the synthesis of sulfated resorcylic acid lactones or esters, which may be formed during plant or human metabolism (phase II detoxification). In general several synthetic methods were developed for the preparation of aryl sulfates, mainly by applying commercially available sulfur trioxide amine and amide complexes.[29] Since these methods are limited in terms of chemical modifications following installation of the sulfate group as well as regioselectivity, yield, and reproducibility, protecting groups for chemical sulfation were investigated during the last decade. Simpson and Widlanski introduced neopentyl and isobutyl chlorosulfates for chemical sulfation of phenols,[30] whereas the 2,2,2-trichloroethyl (TCE) group was used by Taylor and co-workers.[31] Application of the TCE group allows for efficient preparation (applying sulfuryl chloride **5** as reagent) and good stability of protected aryl sulfates. Catalytic transfer hydrogenation using Pd/C, ammonium formate ($HCOONH_4$) as well as cleavage by reaction with Zn/$HCOONH_4$ was reported for the deprotection of the TCE group under mild reductive conditions yielding arylsulfate ammonium salts (Scheme 1a). Additionally sulfuryl imidazolium salt **6** was introduced as reagent for incorporating trichloroethyl protected sulfate esters to carbohydrates (Scheme 1b).[32]

Considering different methods for the synthesis of aryl sulfates, we have started carrying out the chemical sulfation of β-resorcylic acid methyl ester (**7**) as a mimic for ZEN[14,33] by applying several



Scheme 1. (a) Synthesis of aryl sulfates by applying chlorosulfuric acid 2,2,2-trichloroethyl ester (**5**),[31] (b) sulfuryl imidazolium salt **6** for the preparation of alkyl sulfates (e.g. carbohydrates),[32] DMAP = 4-(dimethylamino)pyridine, and 1,2-DMIm = 1,2-dimethylimidazole.



Scheme 2. Chemical sulfation of β-resorcylic acid methyl ester (**7**), by applying sulfuryl chloride **5** or imidazolium triflate **6** as reagent.

sulfur trioxide complexes ($SO_3{\cdot}NMe_3$, $SO_3{\cdot}$Pyr, and $SO_3{\cdot}$DMF) all leading to complex product mixtures or low conversion of **7** as indicated by HPLC. Therefore, chlorosulfuric acid 2,2,2-trichloroethyl ester (**5**)[31] was used as the sulfation reagent for further investigations. In a first attempt reaction of **7** and **5** (1.2 equiv) in the presence of 4-(dimethylamino)pyridine (DMAP, 1.0 equiv) and NEt_3 (1.2 equiv), according to the original procedure of Taylor and co-workers[31] led to a conversion of 68% yielding the desired monosulfated product **8** (44%), but also the disulfate **9** (24%, Scheme 2). In contrast to glycosylation,[14,12] acetylation[34], silylation[35], and benzylation[36] no significant selectivity was observed for monosulfation in position 4 of compound **7** by applying this procedure. Since we have reasoned this outcome with the higher reactivity of sulfuryl chlorides compared to glycosyl donors as well as common acylation, silylation, and benzylation reagents, an optimization study was performed considering the effect of the solvent, different bases as well as the sulfation reagent itself (Table 1).

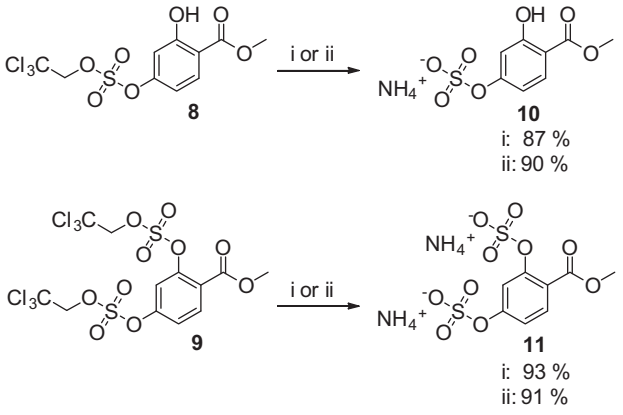
Starting by changing the solvent from tetrahydrofuran (THF) to dichloromethane (DCM) the selectivity of monosulfation by reaction of **7** with sulfuryl chloride **5** was increased to a **8**:**9** ratio of 3.5:1 using 0.5 M equiv of DMAP (Table 1, entry 5), whereas the application of pyridine instead of DMAP basically led to no (DCM) or only poor (THF) conversion of compound **7**. Interestingly sulfation of the monosulfate **8** (to form the disulfate **9**) was observed to occur faster than the sulfation of the ZEN mimic **7** when using an excess of DMAP instead of NEt_3/DMAP (entry 6). Basically higher selectivity was observed in DCM compared to reactions carried out in THF. Enhanced selectivity was achieved by applying imidazolium salt **6** for sulfation of **7**. This reaction was studied in terms of varying the amount of the sulfating reagent starting from 1.05 to 2.0 M equiv leading to **8**:**9** ratios between 8:1 and 1:3. In particular using 1.05 equiv of **6** and 1.5 equiv of 1,2-dimethylimidazole (1,2-DMIm), sulfation at 20 °C afforded 80% of the monosulfated product **8** (entry 7). Applying these conditions at −10 °C (entry 11) or using exactly one equivalent of the reagent (entry 12) did not lead to a significant improvement in terms of selectivity and conversion. Therefore conditions according to entry 7 were applied for the sulfation of **7** affording compounds **8** and **9** in reasonable isolated yields (75% and 10%, respectively). These TCE protected sulfates were readily separated and purified by silica gel chromatography revealing an important advantage of this strategy compared to common methods directly leading to nonprotected sulfate salts.

Deprotection of TCE protected sulfates was carried out by applying both strategies known from the literature. Catalytic transfer hydrogenation (Pd/C, $HCOONH_4$) as well as cleavage by reaction with zinc/ammonium formate ($HCOONH_4$) yielded the desired monosulfate **10** as well as the disulfate **11** as ammonium salts in excellent yields after purification by simple filtration over a pad of silica gel eluting with DCM/MeOH/NH_4OH (10:4:1) (Scheme 3). Both compounds were observed to be stable in solid form as well

Table 1
Optimization study on the sulfation of β-resorcylic acid methyl ester (**7**) via Scheme 1

Entry	Reagent (equiv)	Conditions[a]	**8** (%)[b]	**9** (%)[b]	Unreacted **7** (%)[b]
1	**5** (1.20)	NEt_3 (1.2 equiv), DMAP (1.0 equiv), THF, 20 °C	44	24	32
2	**5** (1.20)	NEt_3 (1.2 equiv), DMAP (1.0 equiv), DCM, 20 °C	67	20	13
3	**5** (1.20)	NEt_3 (1.2 equiv), Pyridine (1.0 equiv), THF, 20 °C	4	0	96
4	**5** (1.20)	NEt_3 (1.2 equiv), Pyridine (1.0 equiv), DCM, 20 °C	No conversion		100
5	**5** (1.20)	NEt_3 (1.2 equiv), DMAP (0.5 equiv), DCM, 20 °C	73	21	6
6	**5** (1.20)	DMAP (2.0 equiv), DCM, 20 °C	17	48	35
7	**6** (1.05)	1,2-DMIm (1.5 equiv), DCM, 20 °C	80	11	9
8	**6** (1.25)	1,2-DMIm (1.5 equiv), DCM, 20 °C	71	24	5
9	**6** (1.50)	1,2-DMIm (1.5 equiv), DCM, 20 °C	52	47	1
10	**6** (2.00)	1,2-DMIm (1.5 equiv), DCM, 20 °C	23	77	0
11	**6** (1.05)	1,2-DMIm (1.5 equiv), DCM, -10 °C	64	18	18
12	**6** (1.00)	1,2-DMIm (1.5 equiv), DCM, 20 °C	80	8	12

[a] Addition of reagent **5** or **6** at −10 °C to the reaction mixture and stirring over-night at the indicated temperature (DMAP = 4-(dimethylamino)pyridine, 1,2-DMIm = 1,2-dimethylimidazole).
[b] Determined by ^1H NMR after aqueous work-up (integration of H-6 signals; see Supplementary data for detailed information).



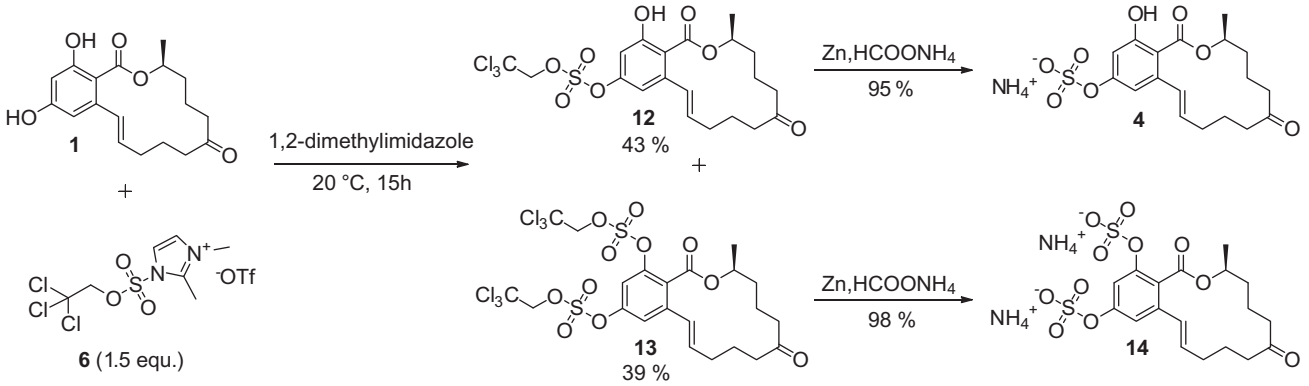
Scheme 3. Deprotection of TCE sulfates **8** and **9** ((i) Pd/C, $HCOONH_4$; (ii) Zn, $HCOONH_4$).

as dissolved in methanol at −20 °C and even room temperature (RT) for several weeks and days, respectively. This outcome was quite surprising, since these compounds were originally suspected to be unstable in protic solvents. An NMR sample of the disulfate **11** (in MeOH) showed slight degradation to the monosulfate **10** after several days at RT indicating lower stability of the sulfate moiety in position 2 compared to the 4-sulfate.

Based on these results and by applying the obtained optimized procedures, ZEN-14-sulfate (**4**) as well as ZEN-14,16-disulfate (**14**) were prepared in high overall yields. Due to the unexpected noticeable stability of compound **11** we also got interested in the preparation of disulfated ZEN (**14**) which is why reaction conditions according to entry 9 (see Table 1) were applied yielding both protected sulfates, **12** and **13** in an approximate ratio of 1:1. To avoid undesired hydrogenation of the conjugated double bond present in ZEN, Zn/$HCOONH_4$ was applied for reductive cleavage of TCE groups (Scheme 4).

The protected ZEN sulfates **12** and **13** were separated and purified by silica gel chromatography. Similar to compounds **10** and **11**, both ZEN sulfates, **4** and **14** were obtained as ammonium salts in pure form after short filtration over a pad of silica gel eluting with DCM/MeOH/NH_4OH (10:4:1). The obtained ZEN-14-sulfate was identical (NMR, ESI-MS) to natural occurring material that was previously isolated and characterized.[15,37]

In conclusion, the chemical sulfation of β-resorcylic acid esters by applying TCE protection was investigated leading to improved procedures for selective monosulfation as well as for simultaneous preparation of mono- and disulfates. Sulfuryl imidazolium salt **6**, which can be easily prepared in large scale starting from 2,2,2-trichloroethanol,[32] was shown to be an appropriate reagent for controlled synthesis of aryl sulfates and reductive conditions were applied for efficient deprotection of the TCE group after simple purification of the intermediates by silica gel chromatography. These protocols were used for the first chemical synthesis of zearalenone-14-sulfate (**4**). This fast and efficient procedure can easily be reproduced in other labs to obtain the masked/conjugated mycotoxin **4** in reasonable amounts and in short time for further investigations, for example, as reference material for bioanalytical studies or for toxicological testing. Additionally, the stability of ammonium salts of these sulfates was reported to be adequate for long term storage in solid or dissolved form.



Scheme 4. Preparation of ZEN-14-sulfate (**4**) and ZEN-14,16-disulfate (**14**) using sulfuryl imidazolium salt **6** and an optimized procedure for the sulfation of ZEN. Reductive conditions (Zn, $HCOONH_4$) were applied for the cleavage of the 2,2,2-trichloroethyl group.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2013.04.059.

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Supporting Information

Manuscript #1

Supporting Information

2-*O*-Benzyloxycarbonyl Protected Glucosyl Donors: A Revival Towards Diastereoselective Glycosylation

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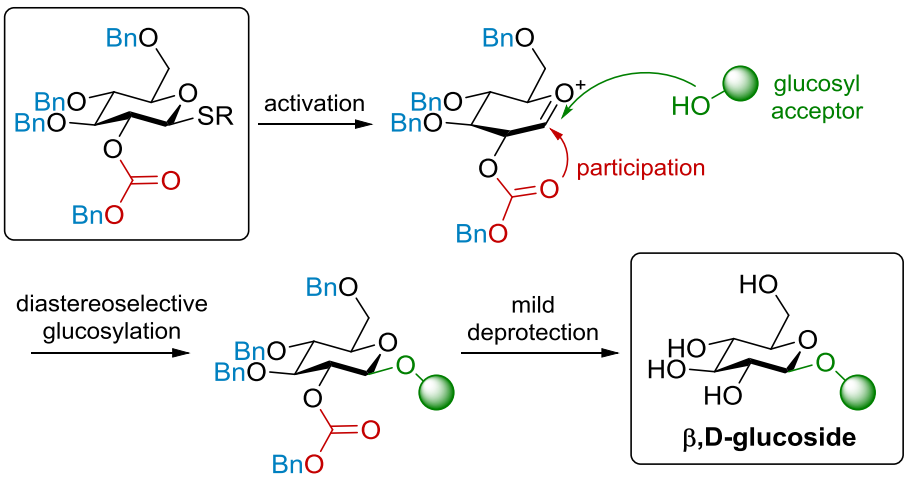

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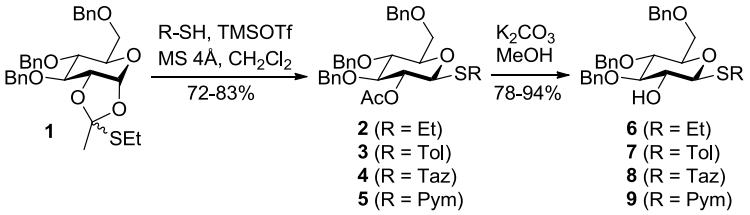
1) General Remarks

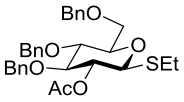
All reactions were performed under an argon atmosphere. Anhydrous solvents like dichloromethane, tetrahydrofuran, methanol and diethyl ether were dried using a PURESOLV facility of it-innovative technology. Molecular sieve 3Å was activated under vacuum at 200 °C before use. Thin-layer chromatography (TLC) was performed over silica gel 60 F254 (Merck). The chromatograms were visualized either by UV irradiation (254 or 366 nm) or by heat staining with ceric ammonium molybdate in ethanol/sulfuric acid. A TLC-MS interface (Camag, Germany) was used for ESI-MS analysis after TLC. Chromatographic separation was done on a 3000 series HPLC-UV system (Dionex UltiMate 3000, Thermo Scientific) using a Chiralpak IB column (Cellulose tris-(3,5-dimethylphenylcarbamate) immobolised on 5 µm silica-gel, 4.6x250mm, Chiral Technologies Europe) and a n-heptane/iPrOH gradient elution (flow rate: 1 mL/min, 0-4 min: 4% iPrOH, 4-25 min: 4 to 20% iPrOH linear gradient, 25-30 min: 20% iPrOH, 30-30.1 min: 20 to 4% iPrOH linear gradient, 30.1-35 min: 4% iPrOH). Preparative column chromatography was performed using a Büchi Sepacore Flash System (2 x Büchi Pump Module C-605, Büchi Pump Manager C-615, Büchi UV Photometer C-635, Büchi Fraction Collector C-660) or a Grace Reveleris Prep Purification System using silica gel 60 (40-63 µm) as obtained from Merck and distilled or redistilled solvents. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX 200-MHz, an Avance DRX-400 MHz or an Avance IIIHD 600-MHz spectrometer equipped with a Prodigy BBO cryo probe (Bruker, Germany). Data were recorded and evaluated using TOPSPIN 3.5 (Bruker Biospin). Chemical shifts are reported in ppm (δ) relative to tetramethylsilane and calibrated using solvent residual peaks. Multiplicities are abbreviated as s (singlet), d (doublet), t (triplet), q (quartet), b (broad signal). All chemicals were purchased either from ABCR (Germany) or Sigma-Aldrich (Austria/Germany). HR-MS analysis was carried out from methanol solutions (concentration: 10 ppm) by using an HTC PAL system autosampler (CTC Analytics AG, Zwingen, Switzerland), an Agilent 1100/1200 HPLC with binary pumps, degasser and column thermostat (Agilent Technologies, Waldbronn, Germany) and Agilent 6230 AJS ESI–TOF mass spectrometer (Agilent Technologies, Palo Alto, United States). 3,4,6-Tri-O-benzyl-1,2-O-(1-ethylthioethylidene)-α,D-glucopyranosre (**1**)[1], dimethyldioxirane (DMDO)[2] and methyl 2,3,4-tri-O-benzyl-1-O-β,D-glucopyranoside (**19**)[3–5] were synthesized according to published procedures.

2) Experimental Procedures

a. Synthesis of 2-OH Thioglucosides 6-9 Applying the Orthoester Strategy



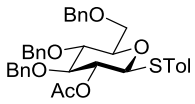
Ethyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-1-thio-β,D-glucoside (2)



To a solution of the thioorthoester **1** (16 g, 29.8 mmol) in dry CH_2Cl_2 (150 mL) molecular sieve (4Å, 4 g) was added and the suspension was stirred at room temperature for 1 h. After cooling to 0 °C, TMSOTf (0.27 mL, 1.5 mmol) was added and stirring was continued at room temperature for 4 h. The reaction was quenched by addition of NEt_3 (4 mL) and the mixture was filtrated over Celite and concentrated under reduced pressure. The residue was purified by filtration over silica (hexanes/EtOAc gradient elution) to obtain **2** as a reddish highly viscous oil (13.3 g, 83 %); R_f 0.45 (hexanes/EtOAc = 5/1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.41-7.26 (m, 15H), 5.15-5.05 (m, 1H), 4.88 (d, *J* = 11.1 Hz, 1H), 4.86 (d, *J* = 10.5 Hz, 1H), 4.76 (d, *J* = 11.2 Hz, 1H), 4.68 (d, *J* = 11.9 Hz, 1H), 4.64 (d, *J* = 10.9 Hz, 1H), 4.62 (d, *J* = 12.2 Hz, 1H), 4.43 (d, *J* = 10.0 Hz, 1H), 3.86-3.68 (m, 4H), 3.63-3.48 (m, 1H), 2.88-2.65 (m, 2H), 2.03 (s, 3H), 1.32 (t, *J* = 7.5 Hz, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 169.3 (s, 1C), 138.0 (s, 1C), 138.0 (s, 1C), 137.8 (s, 1C), 128.2 (d, 1C), 128.15 (d, 1C), 128.0 (d, 1C), 127.85 (d, 1C), 127.7 (d, 1C), 127.5 (d, 1C), 84.2 (d, 1C), 83.4 (d, 1C), 79.4 (d, 1C), 77.6 (d, 1C), 75.1 (t, 1C), 75.0 (t, 1C), 73.3 (t, 1C), 71.5 (d, 1C), 68.6 (t, 1C), 23.6 (t, 1C), 20.8 (q, 1C), 14.8 (q, 1C); NMR data matched that reported.[1]

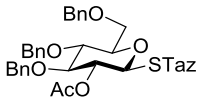
General procedure for the preparation of 2-OAc thioglucosides 3-5. To a solution of the thioorthoester **1** (2.68 g, 5 mmol) in dry CH_2Cl_2 (80 mL) molecular sieve (3Å, 2 g) was added, followed by the thiol (R-SH) (40 mmol). After stirring at room temperature for 30 min and subsequent cooling to 0 °C, TMSOTf (0.28 g, 1.25 mmol) was added and the reaction mixture was stirred at room temperature for 12 h. The reaction was quenched by addition of NEt_3 (0.8 mL) and the mixture was filtrated over Celite, washed with aq. NaOH (1 %) and water. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (hexanes/EtOAc gradient elution + 0.1 % NEt_3) to obtain the desired product.

p-Tolyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-1-thio-β,D-glucoside (3)



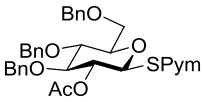
white solid (2.40 g, 80 %); R_f 0.64 (hexanes/EtOAc = 3/1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.53 (d, *J* = 8.2 Hz, 2H), 7.49-7.27 (m, 15H), 7.14 (d, *J* = 8.2 Hz, 2H), 5.15-5.04 (m, 1H), 4.92 (d, *J* = 11.6 Hz, 1H), 4.90 (d, *J* = 10.8 Hz, 1H), 4.78 (d, *J* =11.6 Hz, 1H), 4.69 (d, *J* = 10.6 Hz, 1H), 4.66 (d, *J* = 10.10 Hz, 1H), 3.97-3.83 (m, 2H), 3.82-3.74 (m, 2H), 3.69-3.57 (m, 1H), 2.41 (s, 3H), 2.12 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 169.5 (s, 1C), 138.3 (s, 1C), 138.1 (s, 2C), 137.9 (s, 1C), 133.2 (d, 2C), 129.6 (d, 2C), 128.5 (d, 4C), 128.4 (d, 2C), 128.0 (d, 2C), 127.9 (d, 3C), 127.8 (d, 1C), 127.7 (d, 2C), 127.6 (d, 1C), 86.1 (d, 1C), 84.4 (d, 1C), 84.2 (d, 1C), 79.4 (d, 1C), 77.8 (d, 1C), 75.3 (t, 1C), 75.1 (t, 1C), 73.5 (t, 1C), 71.8 (d, 1C), 68.9 (t, 1C), 21.1 (q, 1C), 21.0 (q, 1C); NMR data matched that reported.[6]

1,3-Thiazolin-2-yl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-1-thio-β,D-glucoside (4)



white solid (2.13 g, 72 %); R_f 0.40 (hexanes/EtOAc = 3/2); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.41-7.13 (m, 15), 5.34 (d, *J* = 10.4 Hz, 1H), 5.20-5.07 (m, 1H), 4.84-4.50 (m, 6H), 4.29-4.08 (m, 2H), 3.88-3.67 (m, 4H), 3.67-3.56 (m, 1H), 3.35 (t, *J* = 8.1 Hz, 2H), 1.97 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 169.6 (s, 1C), 163.6 (s, 1C), 138.1 (s, 2C), 137.9 (s, 1C), 128.5 (d, 2C), 128.4 (d, 2C), 128.3 (d, 2C), 128.0 (d, 2C), 127.9 (d, 3C), 127.8 (d, 3C), 127.6 (d, 1C), 85.3 (d, 1C), 83.8 (d, 1C), 83.3 (d, 1C), 79.7 (d, 1C), 77.7 (d, 1C), 75.3 (t, 1C), 75.1 (t, 1C), 73.4 (t, 1C), 71.4 (d, 1C), 68.4 (t, 1C), 64.2 (t, 1C), 35.1 (t, 1C), 20.9 (q, 1C); NMR data matched that reported.[7]

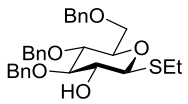
2-Pyrimidyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-1-thio-β,D-glucoside (5):



yellowish solid (2.12 g, 72 %); R_f 0.44 (hexanes/EtOAc = 3/2); ^{1}H NMR (200 MHz, $(CD_3)_2CO$) δ 8.61 (d, *J* = 4.9 Hz, 2H), 7.36-7.24 (m, 15H), 7.21 (t, *J* = 4.9 Hz, 1H), 5.78 (d, *J* = 10.7 Hz, 1H), 5.12 (dd, *J* = 10.6, 9.0 Hz, 1H), 4.88 (d, *J* = 11.4 Hz, 1H), 4.86 (d, *J* = 10.9 Hz, 1H), 4.77 (d, *J* = 11.4, 1H), 4.68 (d, *J* = 10.9 Hz, 1H), 4.57 (d, *J* = 12.1 Hz, 1H), 4.49 (d, *J* = 12.1 Hz, 1H), 4.00-3.89 (m, 1H), 3.82-3.69 (m, 4H), 1.96 (s, 3H); ^{13}C NMR (50 MHz, $(CD_3)_2CO$) δ 170.4 (s, 1C), 170.1 (s, 1C), 158.7 (d, 2C), 139.6 (s, 1C), 139.53 (s, 1C), 139.47 (s, 1C), 129.13 (d, 2C), 129.11 (d, 2C), 129.0 (d, 2C), 128.8 (d, 2C), 128.6 (d, 2C), 128.5 (d, 2C), 128.42 (d, 1C), 128.40 (d, 1C), 128.2 (d, 1C), 118.8 (d, 1C), 85.2 (d, 1C), 82.7 (d, 1C), 80.4 (d, 1C), 78.9 (d, 1C), 75.8 (t, 1C), 75.5 (t, 1C), 73.6 (t, 1C), 71.9 (d, 1C), 69.7 (t, 1C), 21.0 (q, 1C); HRMS calcd for $C_{33}H_{34}N_2NaO_6S^+$ $[M+Na]^+$ 609.2030, found 609.2042.

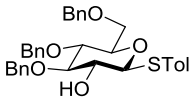
General procedure for de-acetylation of 2-OAc thioglucosides yielding 6-9. To a solution/suspension of the 2-OAc thioglucoside (1 mmol) in dry MeOH (5 mL) K_2CO_3 (28 mg, 0.2 mmol) was added and the reaction mixture was stirred at room temperature until the starting material had completely dissolved (up to 72 h). The reaction mixture was quenched by addition of acidic cation exchange resin (Amberlite® IR120H), filtrated and concentrated under reduced pressure. The residue was purified by column chromatography (hexanes/EtOAc gradient elution + 0.1 % NEt_3) to obtain the desired product.

Ethyl 3,4,6-tri-*O*-benzyl-1-thio-β,D-glucoside (6)



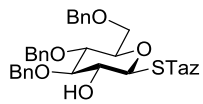
white solid (420 mg, 85 %); R_f 0.21 (hexanes/EtOAc = 4/1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.31 – 7.16 (m, 13H), 7.11 – 7.08 (m, 2H), 4.86 (d, *J* = 11.3 Hz, 1H), 4.77 (d, *J* = 11.3 Hz, 1H), 4.76 (d, *J* = 12.1 Hz, 1H), 4.58 – 4.42 (m, 3H), 4.22 (d, *J* = 9.1 Hz, 1H), 3.67 (dd, *J* = 1.8, 10.9 Hz, 1H), 3.61 (dd, *J* = 4.5, 10.9 Hz, 1H), 3.56 - 3.39 (m, 4H), 2.70 – 2.60 (m, 2H), 1.24 (t, *J* = 4.0 Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 138.7 (s, 1C), 138.3 (s, 1C), 138.1 (s, 1C), 128.6 (d, 2C), 128.5 (d, 2C), 128.4 (d, 2C), 128.1 (d, 2C), 128.0 (d, 2C), 127.9 (d, 1C), 127.8 (d, 3C), 127.7 (d, 1C), 86.2 (d, 1C), 86.1 (d, 1C), 79.5 (d, 1C), 77.5 (d, 1C), 75.3 (t, 1C), 75.2 (t, 1C), 73.5 (t, 1C), 73,4 (d, 1C), 69.1 (t, 1C), 24.4 (t, 1C), 15.5 (q, 1C); NMR data matched that reported.[8]

p-Tolyl 3,4,6-tri-*O*-benzyl-1-thio-β,D-glucoside (7)



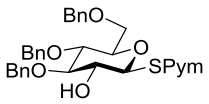
white solid (523 mg, 94 %); R_f 0.49 (hexanes/EtOAc = 4/1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.50 (d, *J* = 8.2 Hz, 2H), 7.38 – 7.19 (m, 15H), 7.07 (d, *J* = 7.8 Hz, 2H), 4.97 – 4.79 (m, 3H), 4.67 – 4.50 (m, 3H), 4.45 (d, *J* = 9.4 Hz, 1H), 3.81 – 3.75 (m, 2H), 3.64 – 3.40 (m, 4H), 2.33 (s, 3H), 1.97 (bs, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 138.6 (s, 1C), 138.6 (s, 1C), 138.5 (s, 1C), 138.2 (s, 1C), 133.8 (d, 2C), 129.9 (d, 2C), 128.60 (d, 2C), 128.56 (d, 2C), 128.5 (d, 2C), 128.1 (d, 2C), 128.08 (d, 2C), 127.9 (d, 2C), 127.8 (d, 2C), 127.7 (d, 1C), 127.6 (s, 1C), 88.2 (d, 1C), 86.0 (d, 1C), 79.6 (d, 1C), 77.5 (d, 1C), 75.5 (t, 1C), 75.2 (t, 1C), 73.6 (t, 1C), 72.6 (d, 1C), 69.1 (t, 1C), 21.3 (q, 1C); NMR data matched that reported.[6]

1,3-Thiazolin-2-yl 3,4,6-tri-*O*-benzyl-1-thio-β,D-glucoside (8):



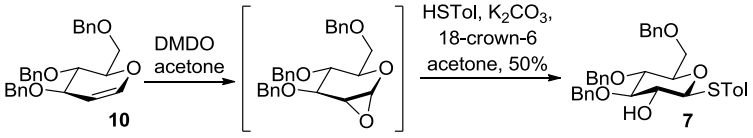
white solid (430 mg, 78 %); R_f 0.27 (hexanes/EtOAc = 3/2); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.31-7.02 (m, 15H), 5.08 (d, *J* = 9.2 Hz, 1H), 4.90 (d, *J* = 11.2 Hz, 1H), 4.75 (d, *J* = 11.3 Hz, 1H), 4.74 (d, *J* = 10.9 Hz, 1H), 4.53 (d, *J* = 12.1 Hz, 1H), 4.46 (d, *J* = 10.9 Hz, 1H), 4.42 (d, *J* = 12.1 Hz, 1H), 4.13-4.01 (m, 2H), 3.72-3.44 (m, 6H), 3.22 (t, *J* = 8.1 Hz, 2H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 164.5 (s, 1C), 137.6 (s, 1C), 137.1 (s, 1C), 137.0 (s, 1C), 127.4 (d, 2C), 127.33 (d, 2C), 127.30 (d, 2C), 126.94 (d, 2C), 126.87 (d, 2C), 126.8 (d, 2C), 126.7 (d, 2C), 126 (d, 1C), 85.5 (d, 1C), 84.5 (d, 1C), 78.7 (d, 1C), 75.9 (d, 1C), 74.4 (t, 1C), 74.0 (t, 1C), 73.3 (d, 1C), 72.4 (t, 1C), 67.6 (t, 1C), 62.8 (t, 1C), 34.3 (t, 1C); NMR data matched that reported.[7]

2-Pyrimidyl 3,4,6-tri-*O*-benzyl-1-thio-β,D-glucoside (9)



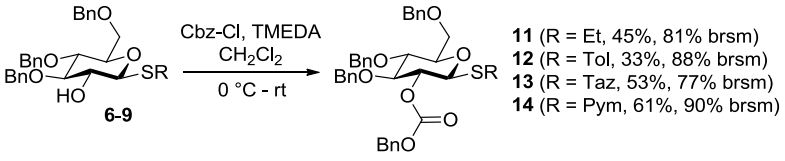
yellowish solid (479 mg, 88 %); R_f 0.21 (hexanes/EtOAc = 3/2); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.51 (d, *J* = 4.9 Hz, 2H), 7.43-7.41 (m, 2H), 7.36-7.26 (m, 11H), 7.24-7.22 (m, 2H), 6.96 (t, *J* = 5.0 Hz, 1H), 5.65 (d, *J* = 9.8 Hz, 1H), 5.04 (d, *J* = 11.4 Hz, 1H), 4.93 (d, *J* = 11.4 Hz, 1H), 4.90 (d, *J* = 10.8 Hz, 1H), 4.63 (d, *J* = 12.2 Hz, 1H), 4.62 (d, *J* = 10.8 Hz, 1H), 4.53 (d, *J* = 12.2 Hz, 1H), 3.82-3.72 (m, 6H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.3 (s, 1C), 157.6 (d, 2C), 138.7 (s, 1C), 138.3 (s, 1C), 138.2 (s, 1C), 128.5 (d, 2C), 128.4 (d, 2C), 128.3 (d, 2C), 128.02 (d, 2C), 127.96 (d, 2C), 127.9 (d, 2C), 127.7 (d, 2C), 127.6 (d, 1C), 117.5 (d, 1C), 86.6 (d, 1C), 84.6 (d, 1C), 79.6 (d, 1C), 77.4 (d, 1C), 75.4 (t, 1C), 75.0 (t, 1C), 73.4 (t, 1C), 73.3 (d, 1C), 68.8 (t, 1C); HRMS calcd for $C_{31}H_{32}N_2NaO_5S^+$ $[M+Na]^+$ 567.1924, found 567.1909.

b. p-Tolyl 3,4,6-tri-O-benzyl-1-thio-β,D-glucoside (7) via DMDO Epoxidation of 10



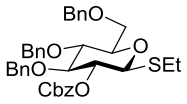
3,4,6-Tri-*O*-benzyl-D-glucal (**10**, 1.25 g, 3 mmol) was reacted with DMDO (78.7 mL, 0.046 M in acetone) at 0 °C for 30 min. The solvent was evaporated and the residue was dissolved in dry acetone (100 mL). After addition of p-thiocresol (1.86 g, 15 mmol), K_2CO_3 (4.15 g, 30 mmol) and 18-crown-6 (80 mg, 0.3 mmol), the reaction mixture was heated to reflux for 2 h, subsequently filtrated and evaporated. The residue was purified by column chromatography (hexanes/EtOAc gradient elution) to yield **7** as a white solid (0.84 g, 50 %); R_f 0.49 (hexanes/EtOAc = 4/1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.50 (d, *J* = 8.2 Hz, 2H), 7.38 – 7.19 (m, 15H), 7.07 (d, *J* = 7.8 Hz, 2H), 4.97 – 4.79 (m, 3H), 4.67 – 4.50 (m, 3H), 4.45 (d, *J* = 9.4 Hz, 1H), 3.81 – 3.75 (m, 2H), 3.64 – 3.40 (m, 4H), 2.33 (s, 3H), 1.97 (bs, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 138.6 (s, 1C), 138.6 (s, 1C), 138.5 (s, 1C), 138.2 (s, 1C), 133.8 (d, 2C), 129.9 (d, 2C), 128.60 (d, 2C), 128.56 (d, 2C), 128.5 (d, 2C), 128.1 (d, 2C), 128.08 (d, 2C), 127.9 (d, 2C), 127.8 (d, 2C), 127.7 (d, 1C), 127.6 (s, 1C), 88.2 (d, 1C), 86.0 (d, 1C), 79.6 (d, 1C), 77.5 (d, 1C), 75.5 (t, 1C), 75.2 (t, 1C), 73.6 (t, 1C), 72.6 (d, 1C), 69.1 (t, 1C), 21.3 (q, 1C); NMR data matched that reported.[6]

c. Cbz-Introduction



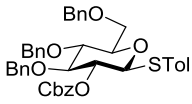
General procedure. To a solution of the 2-OH thioglucoside (0.5 mmol) in dry CH_2Cl_2 (5 mL), cooled to 0 °C, TMEDA (58 mg, 0.5 mmol) was added, followed by Cbz-Cl (127 mg, 0.75 mmol). The reaction mixture was stirred for 48 h, poured into water (20 mL) and extracted with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (hexanes/EtOAc gradient elution) to yield the desired Cbz-protected thioglucoside.

Ethyl 3,4,6-tri-*O*-benzyl-2-*O*-benzyloxycarbonyl-1-thio-β,D-glucoside (11)



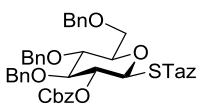
white solid (420 mg, 45 %); R_f 0.42 (hexanes/EtOAc = 6/1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.30-7.27 (m, 2H), 7.26-7.23 (m, 6H), 7.22-7.17 (m, 8H),7.15-7.12 (m, 2H), 7.11-7.08 (m, 2H), 5.11 (s, 2H), 4.79-4.75 (m, 1H), 4.72 (d, *J* = 10.9 Hz, 1H), 4.69 (d, *J* = 11.0 Hz, 1H), 4.62 (d, *J* = 11.0 Hz, 1H), 4.53 (d, *J* = 12.2 Hz, 1H), 4.50 (d, *J* = 10.9 Hz, 1H), 4.47 (d, *J* = 12.1 Hz, 1H), 4.34 (d, *J* = 10.0 Hz, 1H), 3.68 (dd, *J* = 11.0, 2.1 Hz, 1H), 3.66-3.59 (m, 3H), 3.42 (ddd, *J* = 9.2, 4.4, 1.8 Hz, 1H), 2.70-2.60 (m, 2H), 1.19 (t, *J* = 7.5 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 154.4 (s, 1C), 138.2 (s, 1C), 138.0 (s, 1C), 137.9 (s, 1C), 135.1 (s, 1C), 128.6 (d, 1C), 128.6 (d, 1C), 128.4 (d, 3C), 128.37 (d, 3C), 128.3 (d, 2C), 128.0 (d, 2C), 127.9 (d, 2C), 127.8 (d, 2C), 127.71 (d, 2C), 127.69 (d, 1C), 127.6 (d, 1C), 84.3 (d, 1C), 83.4 (d, 1C), 79.5 (d, 1C), 77.7 (d, 1C), 76.3 (d, 1C), 75.4 (t, 1C), 75.1 (t, 1C), 73.5 (t, 1C), 70.0 (t, 1C), 68.8 (t, 1C), 23.8 (t, 1C), 14.9 (q, 1C); HRMS calcd for $C_{37}H_{40}NaO_7S^+$ $[M+Na]^+$ 651.2387, found 651.2402.

p-Tolyl 3,4,6-tri-*O*-benzyl-2-*O*-benzyloxycarbonyl-1-thio-β,D-glucoside (12)



white solid (523 mg, 33 %); R_f 0.47 (hexanes/EtOAc = 6/1); ^{1}H NMR (400 MHz, CD_2Cl_2) δ 7.43-7.34 (m, 11H), 7.33-7.25 (m, 7H), 7.24-7.17 (m, 4H), 7.07 (d, *J* = 8.2 Hz, 2H), 5.23 (d, *J* = 12.1 Hz, 1H), 5.16 (d, *J* = 12.1, 1H), 4.78 (t, *J* = 11.1 Hz, 2H), 4.74 (dd, *J* = 9.9, 8.8 Hz, 1H), 4.66 (d, *J* = 11.3 Hz, 1H), 4.62 (d, *J* = 10 Hz, 1H), 4.61-4.50 (m, 3H), 3.77 (dd, *J* = 10.9, 2.0 Hz, 1H), 3.750-3.69 (m, 2H), 3.66 (t, *J* = 9.2 Hz, 1H), 3.55-3.45 (m, 1H), 2.32 (s, 3H); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 154.7 (s, 1C), 138.7 (s, 1C), 138.6 (s, 1C), 138.52 (s, 1C), 138.50 (s, 1C), 135.8 (s, 1C), 133.3 (d, 2C), 130.0 (d, 2C), 129.2 (s, 1C), 129.0 (d, 1C), 128.9 (d, 1C), 128.69 (d, 4C), 128.67 (d, 2C), 128.6 (d, 2C), 128.4 (d, 2C), 128.2 (d, 2C), 128.13 (d, 1C), 128.10 (d, 3C), 128.0 (d, 1C), 127.9 (d, 1C), 86.5 (d, 1C), 84.6 (d, 1C), 79.6 (d, 1C), 77.9 (d, 1C), 76.6 (d, 1C), 75.7 (t, 1C), 75.3 (t, 1C), 73.7 (t, 1C), 70.3 (t, 1C), 69.3 (t, 1C), 21.2 (q, 1C); HRMS calcd for $C_{42}H_{42}NaO_7S^+$ $[M+Na]^+$ 713.2543, found 713.2570.

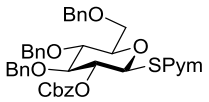
1,3-Thiazolin-2-yl 3,4,6-tri-*O*-benzyl-2-*O*-benzyloxycarbonyl-1-thio-β,D-glucoside (13)



white solid (430 mg, 53 %); R_f 0.48 (hexanes/EtOAc = 3/2); ^{1}H NMR (200 MHz, $(CD_3)_2CO$) δ 7.43-7.23 (m, 20H), 5.56 (d, *J* = 10.6 Hz, 1H), 5.23 (s, 2H), 4.90-4.80 (m, 3H), 4.72 (d, *J* = 11.3 Hz, 1H), 4.68 (d, *J* = 10.6 Hz, 1H), 4.62 (d, *J* = 11.8 Hz, 1H), 4.56 (d, *J* = 11.8 Hz, 1H), 4.24-4.12 (m, 2H), 3.94 (t, *J* = 8.9 Hz, 1H), 3.84-3.68 (m, 4H), 3.44 (t, *J* = 8.2 Hz, 2H); ^{13}C NMR (50 MHz, $(CD_3)_2CO$) δ 162.3 (s, 1C), 155.3 (s, 1C), 139.5 (s, 1C), 139.4 (s, 1C), 139.3 (s,

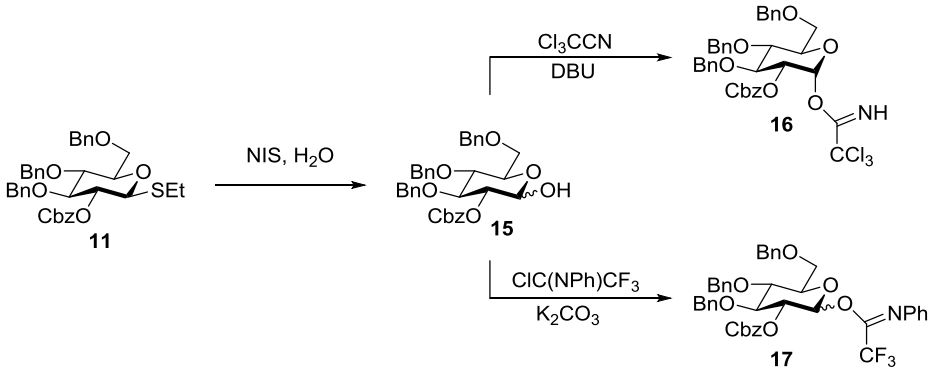
1C), 136.5 (s, 1C), 129.4 (d, 2C), 129.3 (d, 1C), 129.1 (d, 6C), 129.0 (d, 2C), 128.7 (d, 2C), 128.5 (d, 4C), 128.4 (d, 1C), 128.3 (d, 1C), 128.2 (d, 1C), 84.8 (d, 1C), 83.6 (d, 1C), 80.3 (d, 1C), 78.4 (d, 1C), 76.8 (d, 1C), 75.9 (t, 1C), 75.5 (t, 1C), 73.7 (t, 1C), 70.5 (t, 1C), 69.5 (t, 1C), 65.1 (t, 1C), 35.7 (t, 1C); HRMS calcd for $C_{38}H_{39}NNaO_7S_2^+$ $[M+Na]^+$ 708.2060, found 708.2078.

2-Pyrimidyl 3,4,6-tri-*O*-benzyl-2-*O*-benzyloxycarbonyl-1-thio-β,D-glucoside (14)

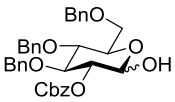


yellowish solid (479 mg, 61 %); R_f 0.66 (hexanes/EtOAc = 3/2); ^{1}H NMR (400 MHz, $(CD_3)_2CO$) δ 8.48 (d, *J* = 4.8 Hz, 2H), 7.24-7.10 (m, 20H), 7.08 (t, *J* = 5.4 Hz, 1H), 5.70 (d, *J* = 10.7 Hz, 1H), 5.08 (d, *J* = 12.2 Hz, 1H), 5.04 (d, *J* = 12.2 Hz, 1H), 4.81 (dd, *J* = 10.3, 9.3 Hz, 1H), 4.72 (d, *J* = 10.7 Hz, 1H), 4.70 (d, *J* = 11.0 Hz, 1H), 4.59 (d, *J* = 10.9 Hz, 1H), 4.54 (d, *J* = 11.1 Hz, 1H), 4.42 (d, *J* = 11.9 Hz, 1H), 4.36 (d, *J* = 12.1 Hz, 1H), 3.84 (t, *J* = 8.3 Hz, 1H), 3.66-3.56 (m, 4H); ^{13}C NMR (100 MHz, $(CD_3)_2CO$) δ 169.4 (s, 1C), 157.9 (d, 2C), 154.5 (s, 1C), 138.6 (d, 1C), 138.5 (d, 1C), 138.4 (d, 1C), 135.6 (d, 1C), 128.5 (d, 2C), 128.3 (d, 1C), 128.2 (d, 4C), 128.15 (d, 2C), 128.1 (d, 2C), 127.9 (d, 2C), 127.7 (d, 2C), 127.6 (d, 2C), 127.54 (d, 1C), 127.49 (d, 1C), 127.3 (d, 1C), 118.0 (d, 1C), 84.2 (d, 1C), 81.6 (d, 1C), 79.5 (d, 1C), 77.8 (d, 1C), 75.6 (d, 1C), 75.0 (t, 1C), 74.6 (t, 1C), 72.7 (t, 1C), 69.6 (t, 1C), 68.8 (t, 1C); HRMS calcd for $C_{39}H_{38}N_2NaO_7S^+$ $[M+Na]^+$ 701.2292, found 701.2306.

d. Synthesis of O-Glucosyl Imidates



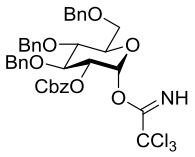
3,4,6-Tri-*O*-benzyl-2-*O*-benzyloxycarbonyl-glucose (15)



To a solution of glucosyl donor **11** (300 mg, 0.477 mmol, 1 eq.) in ACN:H_2O (9:1, 4 mL), NIS was added. The reaction mixture was stirred for 5 min at rt, quenched with an aqueous saturated solution of $Na_2S_2O_3$, diluted with CH_2Cl_2, and washed with $Na_2S_2O_3$-solution and brine. The organic phases were combined, dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography (hexanes/ EtOAc, gradient elution) to obtain the desired product **15** as a mixture of α,β-isomers (~4:1 as judged by NMR) in good yield (235 mg, 85 %).[9] ^{1}H NMR (400 MHz, CD_2Cl_2) δ 7.41- 7.32 (m, 9H), 7.32- 7.25 (m, 8H), 7.24-7.18 (m, 3H), 5.41 (d, *J* = 3.5 Hz, 1H), 5.18 (d, J = 12.1 Hz, 1H), 5.14 (d, J = 12.1 Hz, 1H), 4.86- 4.78 (m, 1H), 4.77- 4.73 (m, 2H), 4.73- 4.70 (m, 1H),

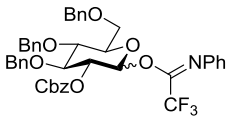
4.56 (d, *J* = 11.3 Hz, 1H), 4.52 (q, *J* = 23.9, 11.9 Hz, 2H), 4.09- 3.99 (m, 2H), 3.74- 3.66 (m, 2H), 3.62 (dd, *J* = 9.76, 8.96 Hz, 1H); ^{13}C NMR (100 MHz, CD_2Cl_2): **α-(15)**: δ 154.93 (s, 1C), 138.86 (s, 1C), 138.67 (s, 1C), 138.46 (s, 1C), 135.66 (s, 1C), 128.96 (d, 1C), 128.89 (d, 1C), 128.72 (d, 2C), 128.67 (d, 2C), 128.63 (d, 2C), 128.59 (d, 2C), 128.28 (d, 2C), 128.27 (d, 2C), 128.21 (d, 3C), 128.04 (d, 2C), 127.93 (d, 1C), 90.74 (d, 1C), 79.98 (d, 1C), 78.36 (d, 1C), 77.51 (d, 1C), 75.77 (t, 1C), 75.29 (t, 1C), 73.62 (t, 1C), 70.70 (d, 1C), 70.21 (t, 1C), 69.21 (t, 1C); **β-(15)**: δ 155.52 (s, 1C), 138.57 (s, 1C), 138.46 (s, 1C), 138.37 (s, 1C), 135.60 (s, 1C), 128.96 (d, 1C), 128.89 (d, 1C), 128.72 (d, 2C), 128.67 (d, 2C), 128.63 (d, 2C), 128.59 (d, 2C), 128.28 (d, 2C), 128.27 (d, 2C), 128.21 (d, 3C), 128.04 (d, 2C), 127.93 (d, 1C), 95.61 (d, 1C), 82.79 (d, 1C), 79.76 (d, 1C), 78.07 (d, 1C), 75.64 (t, 1C), 75.29 (t, 1C), 73.76 (t, 1C), 70.70 (d, 1C), 70.41 (t, 1C), 69.09 (t, 1C) ; HRMS calcd for $C_{35}H_{36}NaO_8^+$ $[M+Na]^+$ 607.2302, found 607.2301.

3,4,6-Tri-*O*-benzyl-2-*O*-benzyloxycarbonyl-α,D-glucopyranosyl trichloroacetimidate (16)



To a solution of compound **15** (994 mg, 1.7 mmol, 1 eq.) in CH_2Cl_2 (25 mL), trichloroacetonitrile (736 mg, 5.1 mmol, 3 eq.), followed by DBU (39 mg, 255 µmol, 0.1 eq.) was added. The reaction mixture was stirred at room temperature for 2 h and then concentrated. The residue was purified by column chromatography (hexanes/ EtOAc, gradient elution) to afford **16** as a colourless viscous liquid (220 mg, 18 %).[10] ^{1}H NMR (200 MHz, $(CD_3)_2CO$) δ 9.31 (s, 1H), 7.44-7.19 (m, 20H), 6.62 (d, J = 3.5 Hz, 1H), 5.20 (s, 2H), 4.96–4.84 (m, 2H), 4.56 (d, J = 4.5 Hz, 2H), 4.76-4.61 (m, 1H), 4.60-4.48 (m, 2H), 4.19-3.99 (m, 2H), 3.92-3.65 (m, 3H); ^{13}C NMR (50 MHz, $(CD_3)_2CO$) δ 160.72 (s, 1C), 155.31 (s, 1C), 139.42 (s, 1C), 139.34 (s, 1C), 139.28 (s, 1C), 136.53 (s, 1C), 129.40 (d, 2C), 129.28 (d, 1C), 129.11 (d, 4C), 129.08 (d, 3C), 129.06 (d, 3C), 128.84 (d, 2C), 128.65 (d, 1C), 128.56 (d, 1C), 128.47 (d, 1C), 128.37 (d, 1C), 128.30 (d, 1C), 94.24 (d, 1C), 80.46 (d, 1C), 77.94 (d, 1C), 77.06 (d, 1C), 75.95 (t, 1C), 75.76 (t, 1C), 74.55 (d, 1C), 73.74 (t, 1C), 70.50 (t, 1C), 69.16 (t, 1C); ESI-MS calcd for $C_{37}H_{36}Cl_3NNaO_8^+$ $[M+Na]^+$ 750.1, found 750.1.

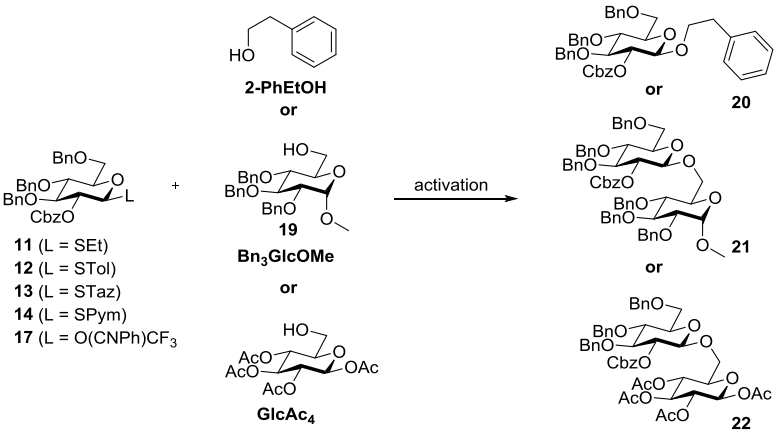
3,4,6-Tri-*O*-benzyl-2-*O*-benzyloxycarbonyl-β,D-glucopyranosyl-1-(N-phenyl)-2,2,2-trifluoroacetimidate (17)



To a solution of compound **15** (170 mg, 291 µmol, 1 eq.) in CH_2Cl_2 (3 mL), K_2CO_3 (88 mg, 581 µmol, 2 eq.) and N-phenyltrifluoroacetimidoyl chloride[11] (121 mg, 581 µmol, 2 eq.) was added. The reaction mixture was stirred at room temperature for 16 h, filtrated over Celite and concentrated. The residue was purified by column chromatography (hexanes/ EtOAc, gradient elution) to afford **17** as a colourless viscous liquid (86 mg, 39 %).[10] ^{1}H NMR (600 MHz, CD_2Cl_2): δ 7.42-7.26 (m, 18H), 7.26-7.19 (m, 4H), 7.15- 7.09 (m, 1H), 6.86-6.77 (m, 2H), 6.10-5.52 (m, 1H), 5.18 (q, J = 23.4; 12.0 Hz, 2H), 5.05 - 4.96 (m, 1H), 4.81 (t, J = 11.8 Hz, 2H), 4.69 (d, J = 11.4 Hz, 1H), 4.60 (d, J = 11.8 Hz, 1H), 4.59 (d, J = 10.9 Hz, 1H), 4.54 (d, J = 11.8 Hz, 1H), 3.81 (t, J = 9.3 Hz, 1H), 3.78- 3.68 (m, 3H), 3.66-3.39 (m, 1H); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 154.52 (s, 1C), 143.59 (s, 1C), 138.44 (s, 1C), 138.35 (s, 1C), 138.34 (s, 1C), 135.50 (s, 1C), 129.16 (d, 2C), 129.00 (d, 3C), 128.73 (d, 6C), 128.67 (d, 2C), 128.37 (d, 2C), 128.25 (d, 2C), 128.20 (d, 3C), 128.14 (d, 2C), 128.05 (d, 2C),

124.81 (d, 1C), 119.59 (d, 1C), 82.61 (d, 1C), 77.52 (d, 1C), 76.77 (d, 1C), 76.26 (d, 1C), 75.65 (t, 1C), 75.38 (t, 1C), 73.69 (t, 1C), 70.55 (t, 1C), 68.46 (t, 1C); HRMS calcd for $C_{43}H_{40}F_3NNaO_8^+$ $[M+Na]^+$ 778.2598, found 778.2607.

e. Glycosylations



General Procedure for Glucosylation Reactions with Thioglucosyldonors in small scale

To a solution of the glucosyl donor (0.05 mmol) and the acceptor (0.05 or 0.075 mmol) in dry CH_2Cl_2 (1 mL) molecular sieve (3Å, 100 mg) was added and the reaction mixture was stirred for 14 h at room temperature. After cooling to the appropriate temperature, activator (see Table S1) was added and stirring was continued in the dark for 24 h. A sample of the reaction mixture (100 µl) was taken after 3 h and a second one after 24 h in single cases, and diluted with 1.9 mL CH_2Cl_2. To quench the reaction, the reaction solution was washed with 1 mL of aqueous saturated $NaHCO_3$ or Na_2SO_3 solution, followed by 0.5 mL water. The organic phase was separated, dried over Na_2SO_4 and concentrated. The residue was diluted in 3 mL acetonitrile, 1 mL was taken and filtered through a syringe filter. This sample was then analyzed by HPLC-UV using isolated material as reference material (external and internal calibration).

General Procedure for Glucosylation Reactions with N-phenyltrifluoroacetimidoyl glucosyldonor

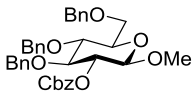
To a solution of the glucosyl donor (0.03 mmol) and the acceptor (0.05-0.06 mmol) in dry CH_2Cl_2 (2 mL) molecular sieve (3Å, 100 mg) was added and the reaction mixture was stirred for 2 h at room temperature. After cooling to the appropriate temperature, activator (see Table S1) was added and stirring was continued for 2 h. The reaction was quenched by addition of NEt_3. A sample of the reaction mixture (100 µl) was taken; this was diluted with 0.9 mL ACN and filtered through a syringe filter. This sample was

then analyzed by HPLC-UV using isolated material as reference material (external and internal calibration).

Table S1. Glycosylation Methods

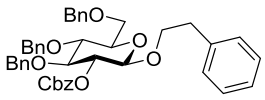
Procedure	Activator	Temperature	Donor
A	NIS (2 eq.) TfOH (0.2 eq.)	-10 °C	SEt, STol
B	I_2 (2 eq.)	rt	SEt
C	TMSOTf (2 eq.)	-10 °C	SPym
D	AgOTf (2 eq.)	0 °C resp. rt	SPym, STaz
E	TMSOTf (0.1 eq.)	-10 °C	O(CNPh)CF_3

Methyl 3,4,6-tri-*O*-benzyl-2-*O*-benzyloxycarbonyl-β,D-glucoside



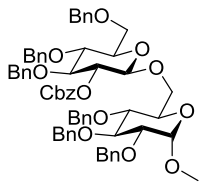
To a solution of glucosyldonor **11** (81.7 mg, 0.13 mmol, 1 eq.) and methanol (8.3 mg, 0.26 mmol, 2 eq.) in dry CH_2Cl_2 (2.5 mL) molecular sieve 3Å (125 mg) was added and the reaction mixture was stirred overnight at room temperature. After cooling to -10 °C, NIS (58.5 mg, 0.3 mmol, 2 eq.) and TfOH (3.9 mg, 0.03 mmol, 0.2 eq.) was added and stirring was continued for 4 h at -10 °C. The reaction was quenched by addition of Et_3N, the reaction solution was diluted with CH_2Cl_2 and filtrated over Celite. The filtrate was washed with water and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (hexanes/EtOAc gradient elution) to obtain the title compound (15 mg, 19 %) as a colourless solid.; R_f 0.40 (hexanes/EtOAc = 4/1); ^{1}H NMR (600 MHz, CD_2Cl_2) δ 7.40-7.28 (m, 16H), 7.26-7.23 (m, 4H), 5.19 (d, *J* = 12.1 Hz, 1H), 5.17 (d, *J* = 12.1 Hz, 1H), 4.84 (d, *J* = 11.1 Hz, 1H), 4.79 (d, *J* = 11.1 Hz, 1H), 4.75-4.72 (m, 1H), 4.69 (d, *J* = 11.3 Hz, 1H), 4.63 (d, *J* = 12.1 Hz, 1H), 4.62 (d, *J* = 10.9 Hz, 1H), 4.57 (d, *J* = 11.8 Hz, 1H), 4.37 (d, *J* = 8.0 Hz, 1H), 3.79 (dd, *J* = 11.1, 2.3 Hz, 1H), 3.76 (dd, *J* = 11.1, 4.4 Hz, 1H), 3.75-3.70 (m, 2H), 3.51 (s, 3H), 3.52-3.49 (m, 1H); ^{13}C NMR (150 MHz, CD_2Cl_2) δ 154.5 (s, 1C), 138.3 (s, 1C), 138.24 (s, 1C), 138.22 (s, 1C), 135.4 (s, 1C), 128.6 (d, 1C), 128.5 (d, 1C), 128.33 (d, 3C), 128.28 (d, 2C), 128.2 (d, 1C), 128.1 (d, 2C), 127.9 (d, 2C), 127.79 (d, 2C), 127.75 (d, 2C), 127.73 (d, 2C), 127.62 (d, 1C), 127.59 (d, 1C), 101.6 (d, 1C), 82.8 (d, 1C), 77.8 (d, 1C), 77.6 (d, 1C), 75.2 (t, 1C), 75.1 (d, 1C), 74.9 (t, 1C), 73.4 (t, 1C), 69.8 (t, 1C), 68.7 (t, 1C), 56.8 (q, 1C). HRMS calcd for $C_{36}H_{38}NaO_8^+$ $[M+Na]^+$ 621.2459, found 621.2462.

2-Phenylethyl 3,4,6-tri-*O*-benzyl-2-*O*-benzyloxycarbonyl-β,D-glucoside (20)



To a solution of glucosyldonor **14** (81.5 mg, 0.12 mmol, 1 eq.) and phenylethanol (29.3 mg, 0.24 mmol, 2 eq.) in dry CH_2Cl_2 (2.5 mL) molecular sieve 3Å (250 mg) was added and the reaction mixture was stirred overnight at room temperature. After cooling to -10°C, TMSOTf (43 µl, 0.24 mmol, 2 eq.) was added and stirring was continued for 16 h at -10 °C. Analysis by TLC indicated remaining starting material, thus additional phenylethanol (1 eq.) and TMSOTf (2 eq.) was added. After 2 h the reaction mixture was slowly warmed to room temperature and stirred for 16 h. The reaction was quenched by addition of Et_3N, the reaction solution was diluted with CH_2Cl_2 and filtrated over Celite. The filtrate was washed with water and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (hexanes/EtOAc gradient elution) to obtain the title compound **20** (35 mg, 43 %) as a colourless solid.; R_f 0.39 (hexanes/EtOAc = 4/1); ^{1}H NMR (400 MHz, $(CD_3)_2CO$) δ 7.44-7.14 (m, 25H), 5.21 (d, *J* = 12.3 Hz, 1H), 5.16 (d, *J* = 12.3 Hz, 1H), 4.83 (d, *J* = 11.1 Hz, 1H), 4.78 (d, *J* = 11.4 Hz, 1H), 4.73 (t, *J* = 8.7 Hz, 1H), 4.67 (d, *J* = 7.9 Hz, 1H), 4.62 (d, *J* = 7.6 Hz, 1H), 4.63-4.53 (m, 3H), 4.07-3.98 (m, 1H), 3.83-3.57 (m, 6H), 2.87-2.80 (m, 2H); ^{13}C NMR (100 MHz, $(CD_3)_2CO$) δ 155.3 (s, 1C), 139.8 (s, 1C), 139.6 (s, 1C), 139.4 (s, 2C), 136.8 (s, 1C), 129.9 (d, 2C), 129.4 (d, 2C), 129.2 (d, 2C), 129.1 (d, 4C), 129.0 (d, 4C), 128.7 (d, 2C), 128.5 (d, 2C), 128.4 (d, 2C), 128.3 (d, 2C), 128.2 (d, 2C), 126.9 (d, 1C), 101.3 (d, 1C), 83.7 (d, 1C), 78.9 (d, 1C), 78.5 (d, 1C), 75.8 (d, 1C), 75.6 (t, 1C), 75.4 (t, 1C), 73.7 (t, 1C), 70.9 (t, 1C), 70.2 (t, 1C), 69.7 (t, 1C), 36.8 (t, 1C). HRMS calcd for $C_{43}H_{44}NaO_8^+$ $[M+Na]^+$ 711.2928, found 711.2932.

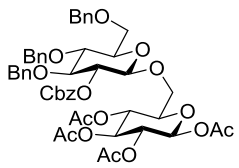
Methyl 2,3,4,9,10,12-hexa-*O*-benzyl-8-*O*-benzyloxycarbonyl-α,D-gentiobioside (21)



To a solution of glucosyl donor **11** (200 mg, 0.32 mmol, 1 eq.) and the glucosyl acceptor **19** (369 mg, 0.8 mmol, 2.5 eq.) in dry CH_2Cl_2 (6 mL) molecular sieve 3Å (300 mg) was added and the reaction mixture was stirred for 2 h at room temperature. After cooling to -10 °C, NIS (143 mg, 0.64 mmol, 2 eq.) and TfOH (10 mg, 0.06 mmol, 0.2 eq.) was added and stirring was continued for 14 h. The reaction was quenched by addition of Et_3N, the mixture was diluted with CH_2Cl_2 and filtrated over Celite. The filtrate was washed with water and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (hexanes/EtOAc gradient elution) to obtain the desired product **21** (314 mg, 87 %) in good yields; R_f 0.64 (hexanes/ EtOAc = 2/1); ^{1}H NMR (600 MHz, CD_2Cl_2) δ 7.41-7.31 (m, 11H), 7.30-7.22 (m, 16H), 7.22-7.17 (m, 8H), 5.14 (d, *J* = 12.1 Hz, 1H), 4.96 (t, *J* = 11.0 Hz, 2H), 4.82-4.76 (m, 4H), 4.76-4.71 (m, 3H), 4.66 (d, *J* = 4.9 Hz, 1H), 4.64 (d, *J* = 4.3 Hz, 1H), 4.61 (d, J = 12.0 Hz, 1H), 4.57 (d, J = 10.9 Hz, 1H), 4.54 (d, J = 2.2 Hz, 1H), 4.52 (d, J = 3.2 Hz, 1H), 4.43 (d, J = 8.0 Hz, 1H), 4.07 (dd, J = 10.5, 1.5 Hz, 1H), 3.89 (t, J = 9.3 Hz, 1H), 3.77-3.70 (m, 4H), 3.70-3.64 (m, 2H), 3.55 (dd, J = 9.6, 3.5 Hz, 1H), 3.49-3.42 (m, 2H), 3.35 (s, 3H); ^{13}C NMR (150 MHz, CD_2Cl_2) δ 154.80 (s, 1C), 139.53 (s, 1C), 139.00 (s, 1C), 138.87 (s, 1C), 138.62 (s, 1C), 138.53 (s, 1C), 138.52 (s, 1C), 135.46 (s, 1C), 128.91 (d, 2C), 128.82 (d, 1C), 128.70 (d, 6C), 128.66 (d, 2C), 128.62 (d, 2C), 128.60 (d, 3C), 128.32 (d, 2C), 128.25

(d, 4C), 128.17 (d, 2C), 128.12 (d, 3C), 128.01 (d, 1C), 128.05 (d, 2C), 128.01 (d, 2C), 127.95 (d, 1C), 127.88 (d, 1C), 127.77 (d, 1C), 101.15 (d, 1C), 98.24 (d, 1C), 83.12 (d, 1C), 82.10 (d, 1C), 80.69 (d, 1C), 78.11 (d, 1C), 78.06 (d, 1C), 77.91 (d, 1C), 75.76 (t, 1C), 75.60 (t, 1C), 75.41 (d, 1C), 75.28 (t, 1C), 75.12 (t, 1C), 73.68 (t, 1C), 73.27 (t, 1C), 70.27 (t, 1C), 70.07 (d, 1C), 69.00 (t, 1C), 68.37 (t, 1C), 55.37 (q, 1C); HRMS calcd for $C_{63}H_{66}NaO_{13}^+$ $[M+Na]^+$ 1053.4396, found 1053.4392.

1,2,3,4-Tetra-*O*-acetyl-9,10,12-tri-*O*-benzyl-8-*O*-benzyloxycarbonyl gentiobioside (22)



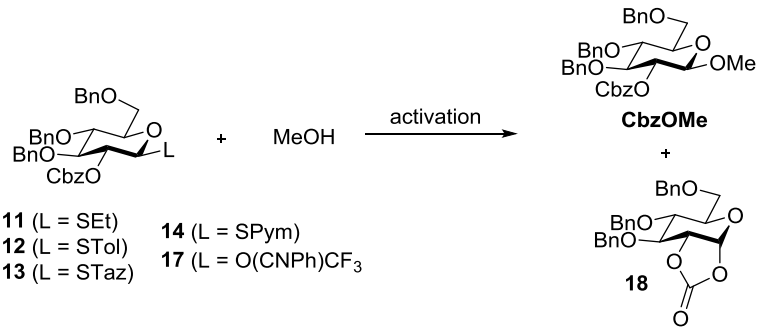
To a solution of glucosyldonor **11** (200 mg, 0.32 mmol) and the acceptor 1,2,3,4-tetraacetylglucose (166 mg, 0.48 mmol) in dry CH_2Cl_2 (6 mL) molecular sieve 3Å (600 mg) was added and the reaction mixture was stirred for 2h at room temperature. After cooling to -10 °C, NIS (143 mg, 0.64 mmol) and TfOH (10 mg, 0.06 mmol) was added and stirring was continued for 14 h at -10 °C. The reaction was quenched by addition of an aqueous saturated $NaHCO_3$ and Na_2SO_3 solution (1:1), the reaction solution was diluted with CH_2Cl_2 and filtrated over Celite. The filtrate was washed with water and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (hexanes/EtOAc gradient elution) to obtain the desired product **22** (225 mg, 77 %) in good yields; R_f 0.29 (hexanes/ EtOAc = 2/1); ^{1}H NMR (600 MHz, CD_2Cl_2) δ 7.43-7.32 (m, 9H), 7.31-7.23 (m, 7H), 7.22-7.17 (m, 4H), 5.71 (d, *J* = 8.2 Hz, 1H), 5.28-5.14 (m, 3H), 5.12-5.02 (m, 2H), 4.82-4.86 (m, 3H), 4.67-4.51 (m, 4H), 4.45 (d, J = 7.8 Hz, 1H), 3.95 (dd, *J* = 11.3, 2.3 Hz, 1H), 3.82-3.75 (m, 1H), 3.75-3.71 (m, 2H), 3.70-3.65 (m, 2H), 3.59 (dd, J = 11.1, 4.9 Hz, 1H), 3.49-3.41 (m, 1H), 2.07 (s, 3H), 2.02 (s, 3H), 2.00 (s, 3H), 1.97 (s, 3H); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 170.31 (s, 1C), 169.71 (s, 1C), 169.52 (s, 1C), 169.28 (s, 1C), 154.73 (s, 1C), 138.69 (s, 1C), 138.59 (s, 1C), 138.57 (s, 1C), 136.03 (s, 1C), 128.94 (d, 2C), 128.82 (d, 1C), 128.73 (d, 2C), 128.70 (d, 2C), 128.68 (d, 4C), 128.35 (d, 2C), 128.22 (d, 2C), 128.19 (d, 2C), 128.12 (d, 1C), 128.02 (d, 1C), 127.97 (d, 1C), 100.96 (d, 1C), 92.11 (d, 1C), 83.12 (d, 1C), 78.06 (d, 1C), 77.59 (d, 1C), 75.59 (t, 1C), 75.56 (d, 1C), 75.28 (t, 1C), 74.25 (d, 1C), 73.78 (t, 1C), 73.25 (d, 1C), 70.71 (d, 1C), 70.27 (t, 1C), 69.02 (t, 1C), 68.67 (d, 1C), 67.57 (t, 1C), 21.03 (q, 1C), 20.79 (q, 2C), 20.76 (q, 1C); HRMS calcd for $C_{49}H_{54}NaO_{17}^+$ $[M+Na]^+$ 937.3254, found: 937.3251

f. Methanol as Acceptor in the Glycosylation-Screening

To a solution of the glucosyl donor (0.03 mmol, 1 eq.) and MeOH (see Table S2) in dry CH_2Cl_2 (1 mL) molecular sieve (3Å, 100 mg) was added and the reaction mixture was stirred for 14 h at room temperature. After cooling to the appropriate temperature, activator (see Table S2) was added and stirring was continued in the dark for 24 h. A sample of the reaction mixture (100 µl) was taken after 3 h and diluted with 1.9 mL CH_2Cl_2. To quench the reaction, the reaction solution was washed with 1 mL of aqueous saturated $NaHCO_3$ or Na_2SO_3 solution, followed by 0.5 mL water. The organic phase was

separated, dried over Na_2SO_4 and concentrated. The residue was diluted in 3 mL acetonitrile, 1 mL was taken and filtered through a syringe filter. This sample was then analyzed by HPLC-UV using isolated material as reference material (external and internal calibration).

Table S2. Glycosylation screening with MeOH as scceptor



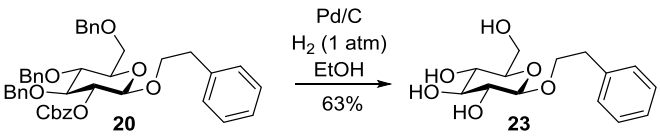
entry	donor	MeOH	activation	temp.	yield[a] (**CbzOMe**)	yield[a] (**18**)	glucosyl donor
1	11	0 eq.	NIS/TfOH	-10°C	0%	93%	7%
2	11	2 eq.	NIS/TfOH	-10°C	0%	96%	4%
3	11	15 eq.	NIS/TfOH	-10°C	78%	19%	3%
4	12	2 eq.	NIS/TfOH	-10°C	0%	85%	15%
5	13	2 eq.	AgOTf	rt	2%	28%	70%
6	14	2 eq.	AgOTf	0°C	0%	31*%	69%
7	17	2 eq.	TMSOTf	-10°C	0%	19%	81%

[a] non-isolated yield as determined by HPLC-UV analysis (UV at 210nm) after 3h reaction time
*non-isolated yield after 24h

Chromatographic separation was done on a 3000 series HPLC-UV system (Dionex UltiMate 3000, Thermo Scientific) using a Chiralpak IB column (Cellulose tris-(3,5-dimethylphenylcarbamate) immobolised on 5 µm silica-gel, 4.6x250mm, Chiral Technologies Europe) and a n-heptane/iPrOH gradient elution (flow rate: 1 mL/min, 0-4 min: 4% iPrOH, 4-25 min: 4 to 25% iPrOH linear gradient, 25-30 min: 25% iPrOH, 30-30.1 min: 25 to 4% iPrOH linear gradient, 30.1-35 min: 4% iPrOH).

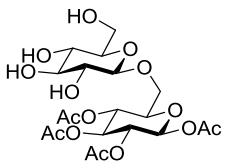
g. Deprotection

2-Phenylethyl-β,D-glucoside (23)



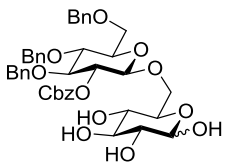
To a suspension of compound **20** (20 mg, 0.03 mmol) in dry ethanol (1 mL) one small tip of a spatula of Pd/C was added under argon atmosphere. The argon balloon was changed for a H_2-balloon and the reaction mixture was stirred for 4 h at rt. The reaction mixture was filtered through a syringe filter and the filtrate was concentrated. The residue was dissolved in water and purified via preparative-HPLC to yield **23** as a white solid (5 mg, 63 %). The obtained material was identical with reference material of **23** previously prepared using a known procedure for Königs-Knorr glucosylation of 2-phenylethanol.[13]

1,2,3,4-Tetra-O-acetylgentiobioside (24)



To a suspension of the disaccharide **22** (50 mg, 0.05 mmol) in dry ethanol (3 mL) two small tips of a spatula of Pd/C was added under argon atmosphere. The argon balloon was changed for a H_2-balloon and the reaction mixture was stirred for 1 h at rt. The reaction mixture was filtered through a syringe filter and the filtrate was concentrated. The residue was dissolved in water and purified via preparative-HPLC to yield **24** as a white solid (20 mg, 71 %). ^{1}H NMR (600 MHz, CD_2Cl_2) δ 5.81 (d, J = 8.5 Hz, 1H), 5.34 (t, J = 9.7 Hz, 1H), 5.16 (t, *J* = 9.5 Hz, 1H), 5.06 (dd, J = 9.5, 8.4 Hz, 1H), 4.25 (d, J = 7.9 Hz, 1H), 4.03-3.98 (m, 2H), 3.86 (dd, J = 11.9, 2.2, 1H), 3.69-3.64 (m, 2H), 3.34 (t, *J* = 8.9 Hz, 1H), 3.28 (t, J = 9.1 Hz, 1H), 3.26-3.22 (m, 1H), 3.20 (dd, J = 9.1, 7.9 Hz, 1H), 2.08 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 1.98 (s, 3H); ^{13}C NMR (150 MHz, CD_2Cl_2) δ 171.61 (s, 2C), 170.99 (s, 1C), 170.55 (s, 1C), 104.45 (d, 1C), 92.97 (d, 1C), 77.98 (d, 1C), 77.81 (d, 1C), 75.01 (d, 1C), 74.91 (d, 1C), 74.28 (d, 1C), 71.77 (d, 1C), 71.45 (d, 1C), 69.87 (d, 1C), 68.63 (t, 1C), 62.65 (t, 1C), 20.70 (q, 1C), 20.61 (q, 1C), 20.54 (q, 1C), 20.44 (q, 1C); HRMS calcd. for $C_{20}H_{30}NaO_{15}^+$ $[M+Na]^+$ 533.1477, found: 533.1482.

9,10,12-Tri-*O*-benzyl-8-*O*-(benzyloxycarbonyl)gentiobioside (25)

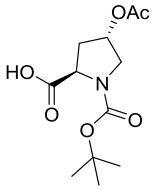


To a suspension of the disaccharide **22** (80 mg, 0.09 mmol) in dry methanol (20 mL) KCN (3 mg, 0.05 mmol) was added at 0 °C. The reaction mixture was slowly warmed to room temperature and stirring was continued for 4 h. Water was added and the reaction mixture was concentrated to a third of its volume. The residue was diluted with ACN:H_2O 1:1 and purified via preparative HPLC to obtain compound **25**, as an anomeric mixture in good yields (50 mg, 75 %).[14] ^{1}H NMR (600 MHz, MeOD) δ 7.38-7.34 (m, 4H), 7.33-7.29 (m, 5H), 7.28-

7.24 (m, 4H), 7.23-7.20 (m, 3H), 7.17-7.13 (m, 4H), 5.21 (dd, J = 12.5, 5.7 Hz, 1H), 5.12 (dd, J = 12.0, 5.0 Hz, 1H), 5.07 (d, *J* = 3.5 Hz, 0.5H), 4.75-4.68 (m, 2.5H), 4.67-4.65 (m, 1H), 4.64-4.57 (m, 2.5H), 4.52 (t, J = 11.6 Hz, 2H), 4.44 (d, J = 7.6 Hz, 0.5H), 4.11 (dd, J = 11.4, 2.1 Hz, 0.5H), 4.06 (dd, J = 11.2, 2.3 Hz, 0.5H), 3.92 (ddd, J = 10.1, 5.2, 2.0 Hz, 0.5H), 3.78 (dd, J = 11.2, 5.0 Hz, 0.5H), 3.76-3.66 (m, 4H), 3.61 (td, J = 9.4, 2.3 Hz, 1H), 3.55-3.50 (m, 1H), 3.44-3.39 (m, 0.5H), 3.36-3.32 (m, 1.5H), 3.24 (dd, J = 9.5, 8.9 Hz, 0.5H), 3.13 (dd, J = 9.2, 7.8 Hz, 0.5H); ^{13}C NMR (150 MHz, MeOD) δ 156.09 (s, 1C), 156.08 (s, 1C), 139.5 (s, 4C), 139.4 (s, 1C), 139.3 (s, 1C), 137.0 (s, 2C), 129.7 (d, 2C), 129.6 (d, 2C), 129.53 (d, 2C), 129.49 (d, 2C), 129.46 (d, 2C), 129.45 (d, 2C), 129.41 (d, 2C), 129.35 (d, 2C), 129.33 (d, 4C), 129.15 (d, 4C), 129.0 (d, 3C), 128.84 (d, 3C), 128.83 (d, 2C), 128.80 (d, 2C), 129.78 (d, 2C), 128.76 (d, 2C), 128.68 (d, 2C), 102.32 (d, 1C), 102.31 (d, 1C), 98.13 (d, 1C), 93.95 (d, 1C), 84.0 (d, 1C), 83.9 (d, 1C), 79.11 (d, 2C), 79.08 (d, 2C), 78.0 (d, 1C), 77.2 (d, 1C), 76.2 (d, 1C), 76.17 (t, 2C), 76.10 (d, 1C), 76.03 (d, 1C), 75.9 (t, 2C), 74.8 (d, 1C), 74.4 (t, 2C), 73.8 (d, 1C), 72.07 (d, 1C), 71.85 (d, 1C), 71.81 (d, 1C), 70.9 (t, 2C), 70.2 (t, 1C), 70.1 (t, 1C), 69.65 (t, 2C), HRMS calcd for $C_{41}H_{46}NaO_{13}^+$ $[M+Na]^+$ 769.2831, found: 769.2829

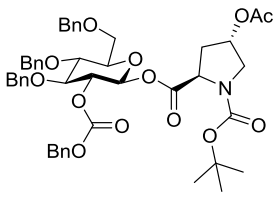
h. Application to Glycosyl Esters

trans-N-(tert-butoxycarbonyl)-4-acetoxy-L-proline (26)



The title compound was synthesized according to the procedure described by Wong.[15] white solid (588 mg, 99 %); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.41-5.16 (m, 1H), 4.48 (t, J = 7.7 Hz, 0.5H), 4.36 (t, J = 7.9 Hz, 0.5H), 3.83-3.44 (m, 2H), 2.58-2.24 (m, 2H), 2.06 (s, 3H), 1.46 (s, 4.5H), 1.42 s (4.5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.28 (s, 1C), 175.65 (s, 1C), 170.62 (s, 1C), 170.56 (s, 1C), 155.73 (s, 1C), 153.73 (s, 1C), 81.83 (s, 1C), 81.19 (s, 1C), 72.40 (d, 1C), 71.98 (d, 1C), 57.86 (d, 1C), 57.77 (d, 1C), 52.48 (t, 1C), 52.09 (t, 1C), 36.61 (t, 1C), 34.93 (t, 1C), 28.46 (q, 3C), 28.33 (q, 3C), 21.15 (q, 2C);[1] ESI-MS calcd for $C_{12}H_{19}NNaO_6^+$ $[M+Na]^+$ 296.1, found 296.0.

trans-N-(*tert*-butoxycarbonyl)-4-acetoxy-L-proline, 3,4,6-tri-*O*-benzyl-2-*O*-benzyloxycarbonyl-β,D-glucosyl ester (27)

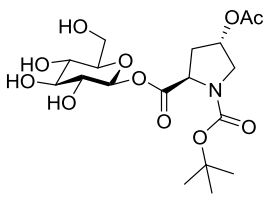


To a solution of glucosyl donor **11** (100 mg, 0.16 mmol) and trans-N-(tert-butoxycarbonyl)-4-acetoxy-L-proline (65 mg, 0.24 mmol) in dry CH_2Cl_2 (3 mL) molecular sieve 3Å (150 mg) was added and the reaction mixture was stirred for 2h at room temperature. After cooling to -10°C, NIS (72 mg, 0.32 mmol) and TfOH (5 mg, 0.03 mmol) was added and stirring was continued for 2 h at -10 °C. The reaction was quenched by addition of an aqueous saturated $NaHCO_3$ and Na_2SO_3 solution (1:1), the

[1] There are two rotamers observable in the NMR spectra owing to the carbamate.

reaction solution was diluted with CH_2Cl_2 and filtrated over Celite. The filtrate was washed with water and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (hexanes/EtOAc gradient elution) to obtain the desired product **27** (71 mg, 53 %) in good yields; Rf 0.45 (hexanes/ EtOAc = 2/1)); ^{1}H NMR (400 MHz, CD_2Cl_2) δ 7.41-7.15 (m, 20H), 5.71 (d, J = 8.2 Hz, 0.4H), 5.65 (d, J = 8.2 Hz, 0.6H), 5.24-5.09 (m, 3H), 4.95-4.84 (m, 1H), 4.83-4.73 (m, 2H), 4.66 (d, J = 10.9 Hz, 1H), 4.62-4.43 (m, 3H), 4.37-4.28 (m, 1H), 3.82-3.68 (m, 4H), 3.66-3.49 (m, 3H), 2.39-2.27 (m, 1H), 2.16-1.96 (m, 1H), 2.04 (s, 3H), 1.43 (s, 3H), 1.36 (s, 6H); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 171.19 (s, 1C), 171.10 (s, 1C), 170.62 (s, 1C), 170.52 (s, 1C), 154.68 (s, 2C), 154.38 (s, 1C), 153.55 (s, 1C), 138.52 (s, 1C), 138.48 (s, 1C), 138.43 (s, 4C), 135.80 (s, 1C), 135.52 (s, 1C), 129.09 (d, 2C), 129.04 (d, 2C), 128.94 (d, 2C), 128.79 (d, 2C), 128.73 (d, 10C), 128.49 (d, 2C), 128.35 (d, 4C), 128.30 (d, 2C), 128.26 (d, 2C), 128.23 (d, 4C), 128.21 (d, 4C), 128.11 (d, 2C), 128.06 (d, 2C), 92.80 (d, 2C), 82.98 (d, 1C), 82.74 (d, 1C), 80.97 (s, 1C), 80.64 (s, 1C), 77.60 (d, 1C), 77.55 (d, 1C), 76.83 (d, 1C), 76.65 (d, 1C), 76.35 (d, 1C), 76.25 (d, 1C), 75.71 (t, 2C), 75.35 (t, 2C), 73.79 (t, 1C), 73.76 (t, 1C), 73.07 (d, 1C), 72.18 (d, 1C), 70.46 (t, 1C), 70.41 (t, 1C), 68.65 (t, 1C), 68.57 (t, 1C), 58.23 (d, 1C), 57.98 (d, 1C), 52.76 (t, 1C), 52.39 (t, 1C), 36.75 (t, 1C), 35.74 (t, 1C), 28.46 (q, 2C), 28.18 (q, 4C), 21.23 (q, 2C)[2]; ESI-MS calcd for $C_{47}H_{53}NNaO_{13}^+$ $[M+Na]^+$ 862.3, found 862.1.

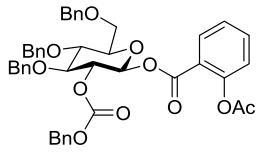
***trans*-N-(*tert*-butoxycarbonyl)-4-acetoxy-L-proline, ß,D-glucosyl ester (28)**



To a suspension of compound **27** (71 mg, 0.085 mmol) in dry ethanol (1.5 mL) was added two small tip of a spatula of Pd/C under argon atmosphere. The argon balloon was changed for a H_2-balloon and the reaction mixture was stirred for 16 h at rt. The reaction mixture was filtered through a syringe filter and the filtrate was concentrated. The residue was dissolved in a mixture of acetonitrile-water and purified via preparative-HPLC to yield **28** as a white solid (30 mg, 81 %). 1H NMR (600 MHz, MeOD) δ 5.50 (d, J = 8.2 Hz, 0.4H), 5.48 (d, J = 8.2 Hz, 0.6H), 5.29-5.22 (m, 1H), 4.45 (q, J = 15.4, 7.8 Hz, 1H), 3.86-3.78 (m, 1H), 3.72-3.62 (m, 2H), 3.61-3.55 (m, 1H), 3.43 (t, J = 8.8 Hz, 1H), 3.40-3.32 (m, 3H), 2.51-2.42 (m, 1H), 2.37-2.27 (m, 1H), 2.05 (s, 3H), 1.46 (s, 3H), 1.43 (s, 6H); ^{13}C NMR (150 MHz, MeOD) δ 172.66 (s, 1C), 172.48 (s, 1C), 172.12 (s, 1C), 172.11 (s, 1C), 156.05 (s, 1C), 155.62 (s, 1C), 96.44 (d, 1C), 96.39 (d, 1C), 82.47 (s, 1C), 82.21 (s, 1C), 78.95 (d, 1C), 78.83 (d, 1C), 77.90 (d, 1C), 77.72 (d, 1C), 74.15 (d, 1C), 74.01 (d, 1C), 73.81 (d, 1C), 73.46 (d, 1C), 71.06 (d, 1C), 71.03 (d, 1C), 62.38 (t, 1C), 62.29 (t, 1C), 59.04 (d, 1C), 58.82 (d, 1C), 53.43 (t, 1C), 53.05 (t, 1C), 37.02 (t, 1C), 36.22 (t, 1C), 28.61 (q, 3C), 28.51 (q, 3C), 20.88 (q, 2C)[2]; ESI-MS calcd for $C_{18}H_{29}NNaO_{11}^+$ $[M+Na]^+$ 458.2, found 458.1.

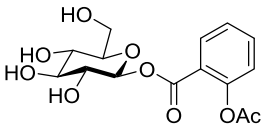
[2] There are two rotamers observable in the NMR spectra owing to the carbamate.

Acetylsalicylic acid, 3,4,6-tri-*O*-benzyl-2-*O*-benzyloxycarbonyl-β,D-glucosyl ester (30)



To a solution of glucosyl donor **11** (100 mg, 0.16 mmol) and acetylsalicylic acid (43 mg, 0.24 mmol) in dry CH_2Cl_2 (3 mL) molecular sieve 3Å (150 mg) was added and the reaction mixture was stirred for 2 h at room temperature. After cooling to -10°C, NIS (72 mg, 0.32 mmol) and TfOH (5 mg, 0.03 mmol) was added and stirring was continued for 2 h at -10 °C. The reaction was quenched by addition of an aqueous saturated $NaHCO_3$ and Na_2SO_3 solution (1:1), the reaction solution was diluted with CH_2Cl_2 and filtrated over Celite. The filtrate was washed with water and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (hexanes/EtOAc gradient elution) to obtain the desired product **30** (100 mg, 84 %) in good yields; Rf 0.67 (hexanes/ EtOAc = 2/1); ^{1}H NMR (400 MHz, CD2Cl2) δ 8.01 (dd, J = 8.0, 1.8 Hz, 1H), 7.64 (td, J = 7.8, 1.6 Hz, 1H), 7.37-7.20 (m, 21H), 7.15 (dd, J = 8.2, 0.8 Hz, 1H), 5.84 (d, J = 8.2 Hz, 1H), 5.09 (s, 2H), 5.06-4.99 (m, 1H), 4.82 (t, J = 10.7 Hz, 2H), 4.71 (d, J = 10.9 Hz, 1H), 4.62 (d, J = 10.9 Hz, 1H), 4.58 (d, J = 12.1 Hz, 1H), 4.51 (d, J = 12.1 Hz, 1H), 3.83 (dd, J = 6.8, 2.5 Hz, 2H), 3.77 (d, J = 2.8 Hz, 2H), 3.69 (dt, J = 9.8, 2.8 Hz, 1H), 2.3 (s, 3H); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 169.85 (s, 1C), 162.55 (s, 1C), 154.75 (s, 1C), 151.88 (s, 1C), 138.49 (s, 1C), 138.45 (s, 2C), 135.55 (s, 1C), 135.08 (d, 1C), 132.38 (d, 1C), 128.89 (d, 2C), 128.83 (d, 1C), 128.73 (d, 6C), 128.38 (d, 4C), 128.27 (d, 4C), 128.19 (d, 1C), 128.13 (d, 1C), 128.04 (d, 1C), 126.58 (d, 1C), 124.44 (d, 1C), 122.29 (s, 1C), 92.76 (d, 1C), 82.90 (d, 1C), 77.63 (d, 1C), 76.83 (d, 1C), 76.36 (d, 1C), 75.76 (t. 1C), 75.40 (t, 1C), 73.82 (t, 1C), 70.38 (t, 1C), 68.64 (t, 1C), 21.14 (q, 1C); ESI-MS calcd for $C_{44}H_{42}NaO_{11}^+$ $[M+Na]^+$ 769.3, found 769.3.

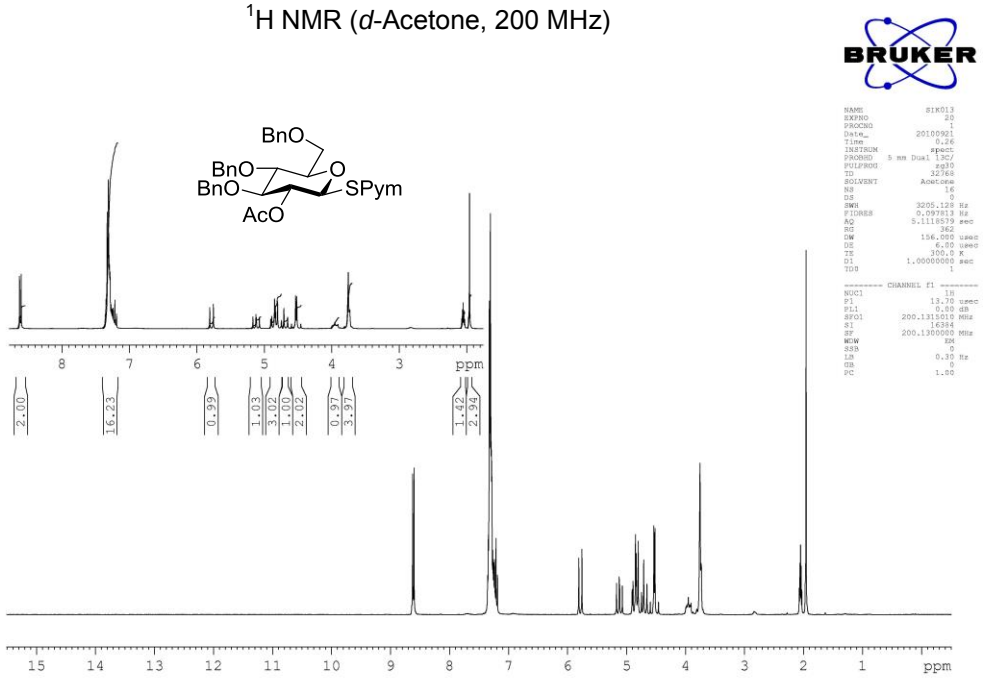
Acetylsalicylic acid, ß,D-glucosyl ester (31)



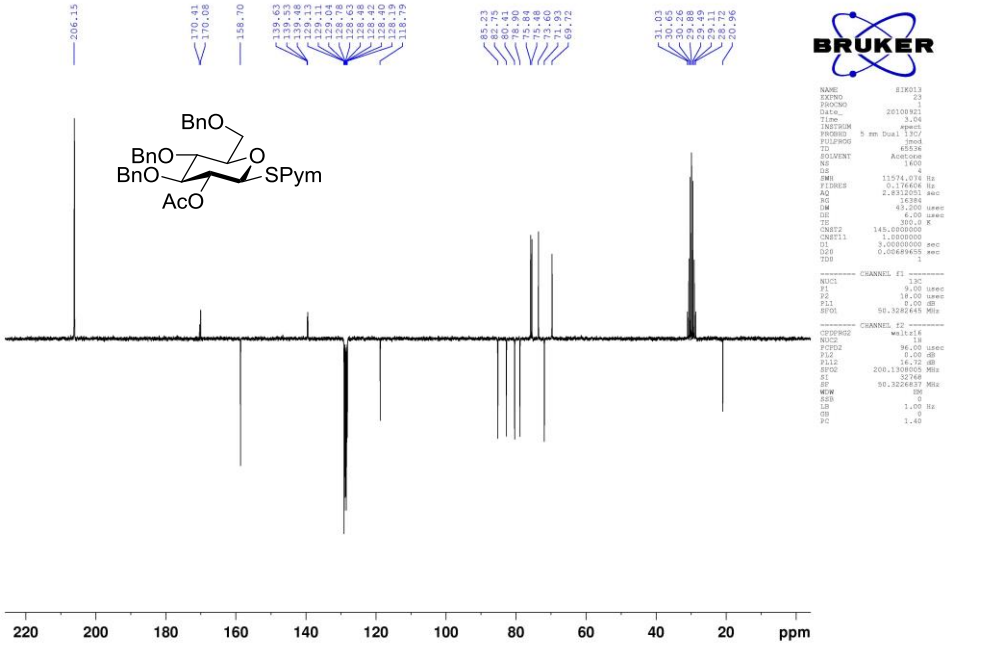
To a suspension of compound **30** (98 mg, 0.13 mmol) in dry ethanol (1.5 mL) three small tip of a spatula of Pd/C was added under argon atmosphere. The argon balloon was changed for a H_2-balloon and the reaction mixture was stirred for 16 h at rt. The reaction mixture was filtered through a syringe filter and the filtrate was concentrated. The residue was dissolved in acetonitrile-water and purified via preparative-HPLC to yield **31** as a white solid (29 mg, 65 %). ^{1}H NMR (600 MHz, MeOD) δ 8.11 (dd, J = 7.9, 1.7 Hz, 1H), 7.66 (td, J = 7.8, 1.5 Hz, 1H), 7.39 (td, J = 7.6, 1.1 Hz, 1H), 7.18 (dd, J = 8.2, 0.9 Hz, 1H), 5.69 (d, J = 7.9 Hz, 1H), 3.86 (dd, J = 12.2, 2.1 Hz, 1H), 3.71 (dd, J = 12.3, 5.0 Hz, 1H), 3.51-3.45 (m, 2H), 3.44-3.42 (m, 1H), 3.42-3.38 (m, 1H), 2.32 (s, 3H); ^{13}C NMR (150 MHz, MeOD) δ 171.45 (s, 1C), 164.40 (s, 1C), 152.45 (s, 1C), 135.65 (d, 1C), 132.88 (d, 1C), 127.22 (d, 1C), 125.15 (d, 1C), 123.93 (s, 1C), 96.12 (d, 1C), 78.97 (d, 1C), 78.06 (d, 1C), 73.99 (d, 1C), 71.01 (d, 1C), 62.28 (t, 1C), 21.00 (q, 1C); ESI-MS calcd for $C_{15}H_{18}NaO_{19}^+$ $[M+Na]^+$ 365.1, found 365.0.

3) NMR Spectra

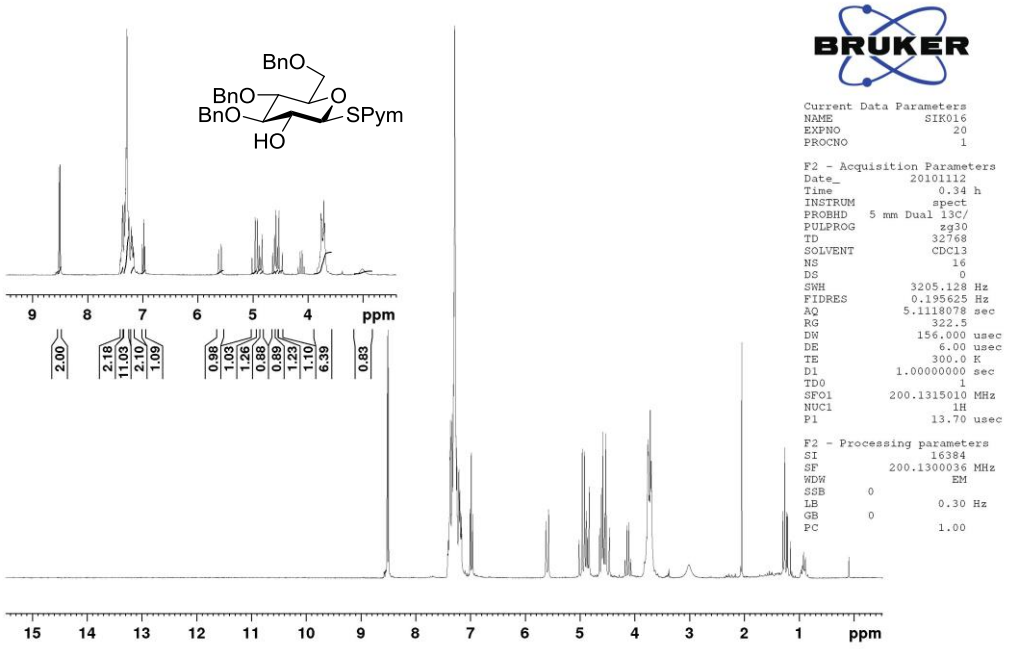
^{1}H NMR (*d*-Acetone, 200 MHz)



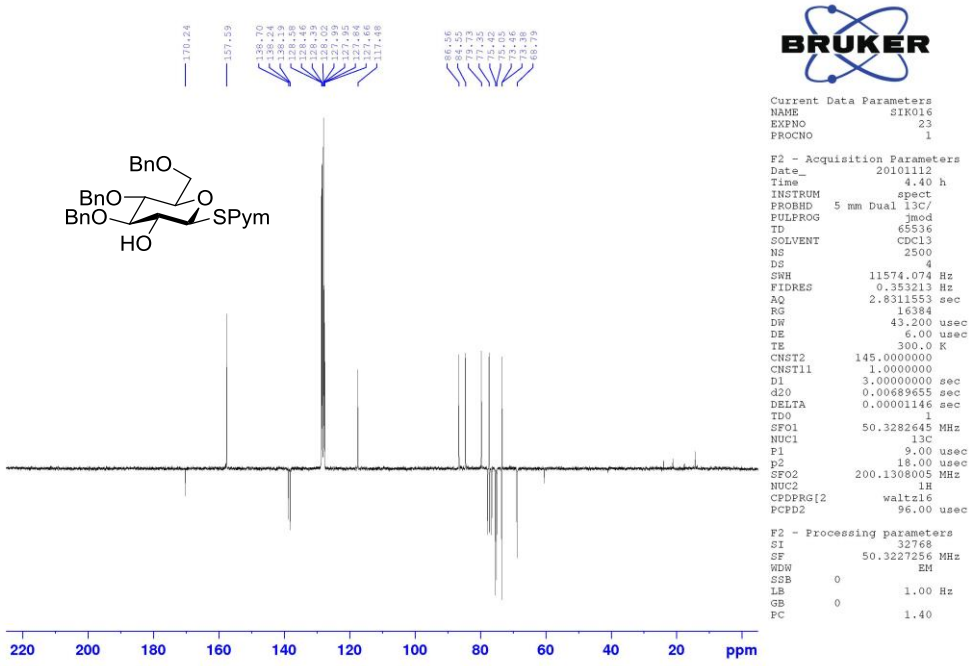
^{13}C NMR (*d*-Acetone, 50 MHz)



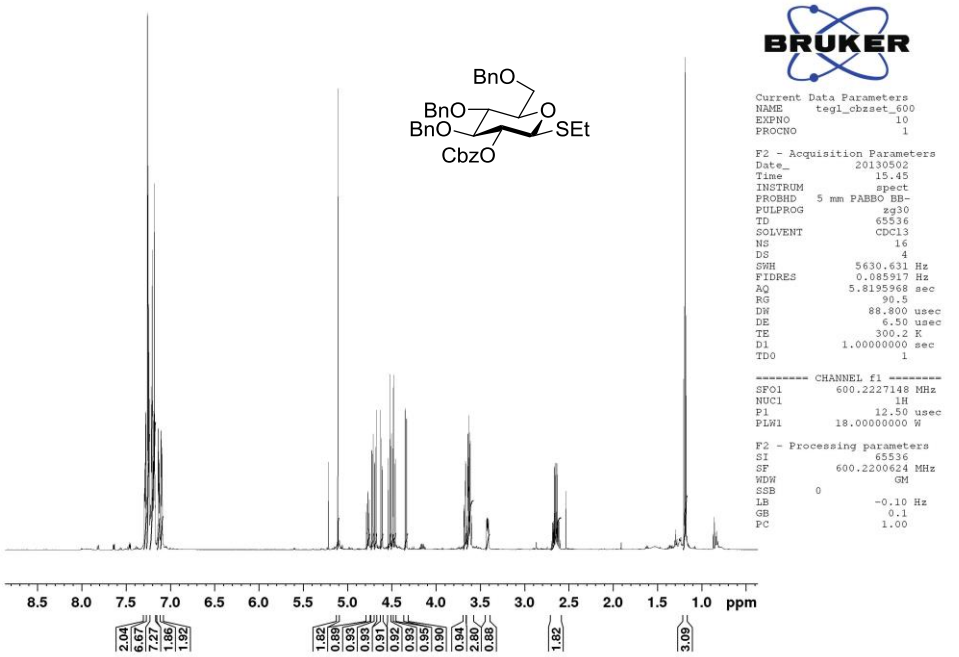
^{1}H NMR (*d*-Chloroform, 200 MHz)



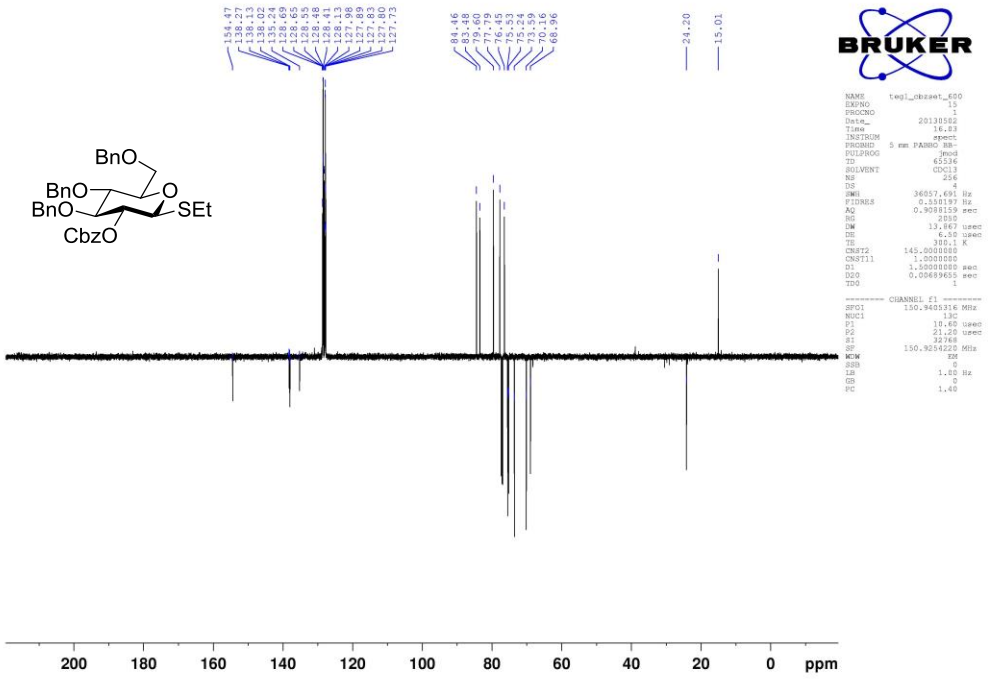
^{13}C NMR (*d*-Chloroform, 50 MHz)



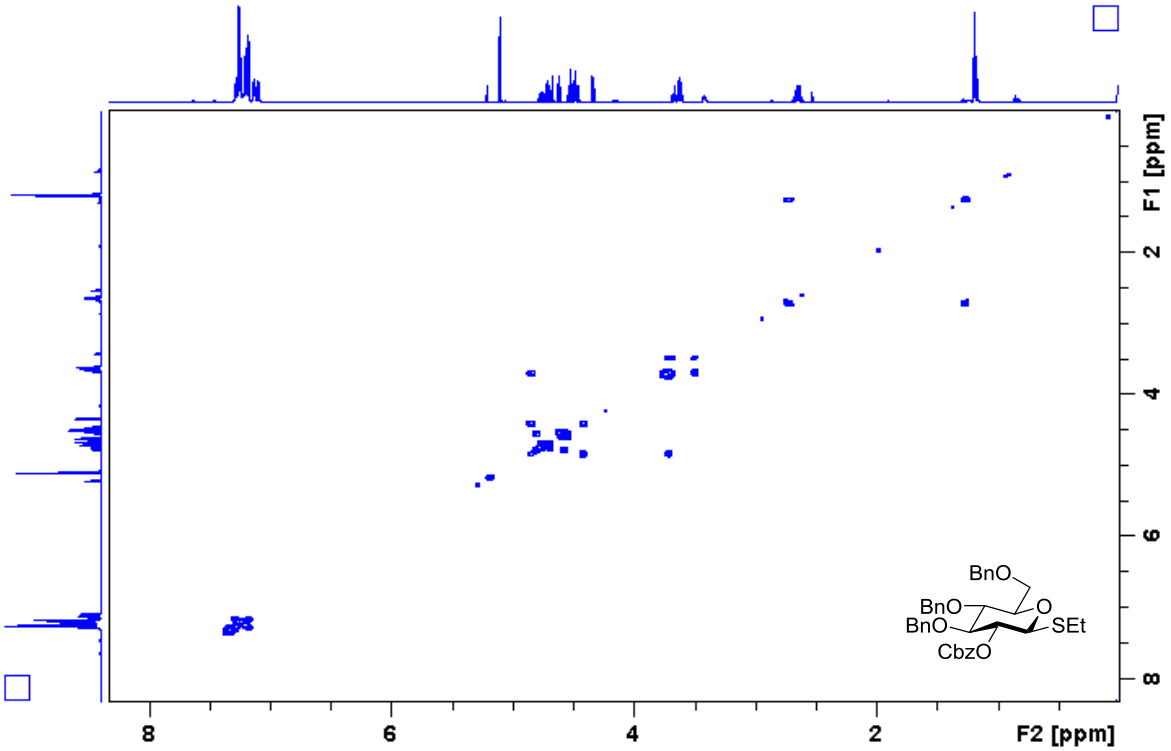
^{1}H NMR (*d*-Chloroform, 600 MHz)



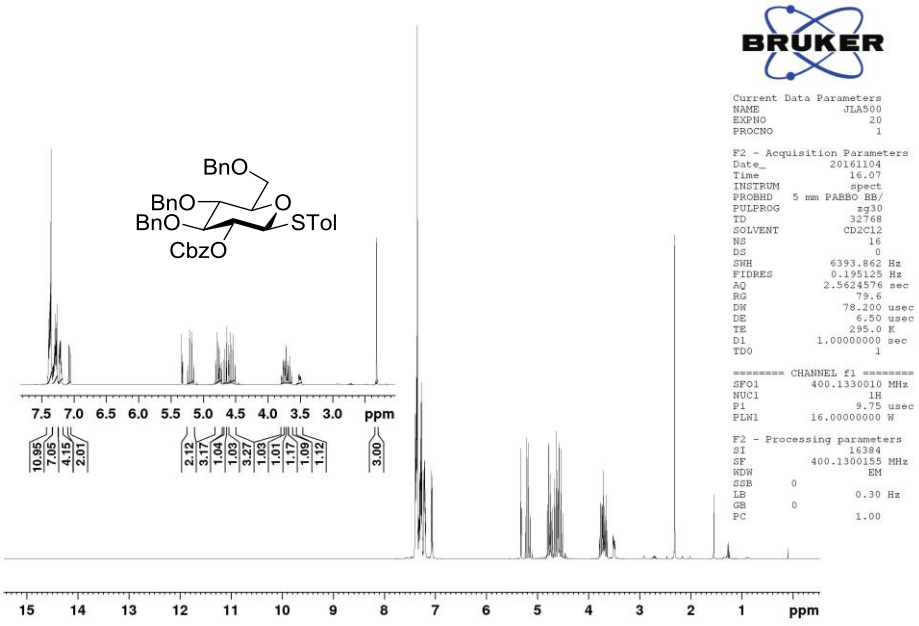
^{13}C NMR (*d*-Chloroform, 150 MHz)



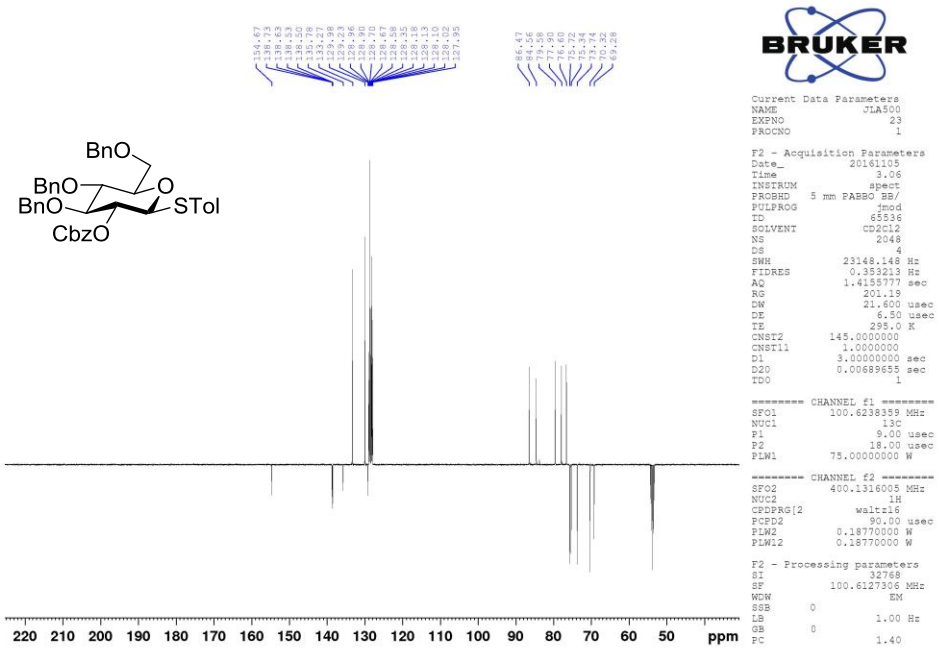
H,H-COSY (*d*-Chloroform, 600 MHz)



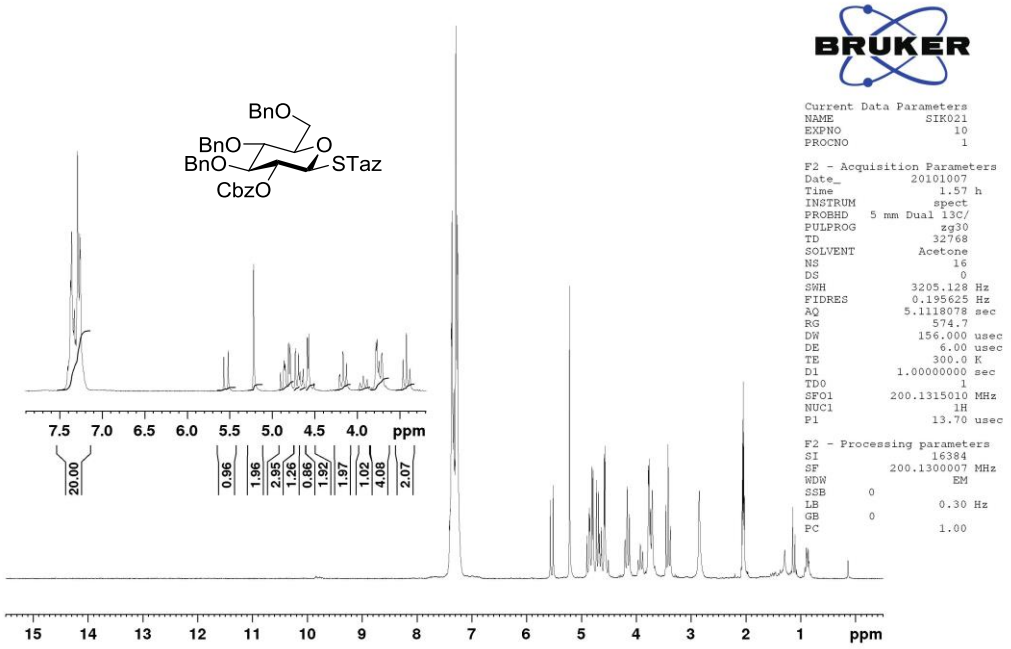
^{1}H NMR (*d*-Methylen chloride, 400 MHz)



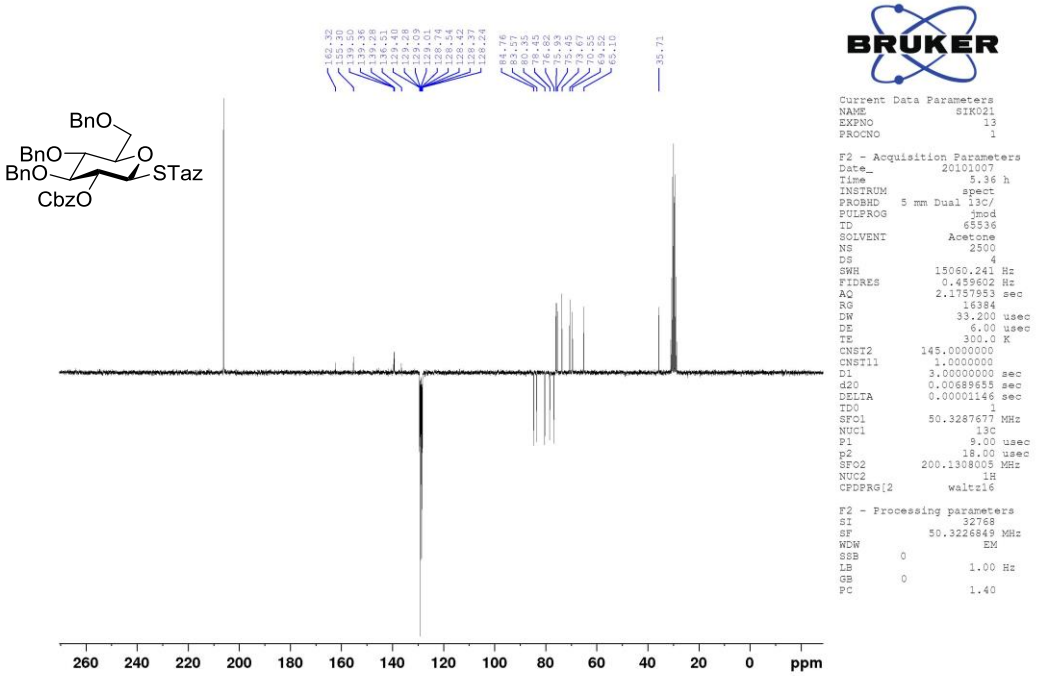
^{13}C NMR (*d*-Methylen chloride, 100 MHz)



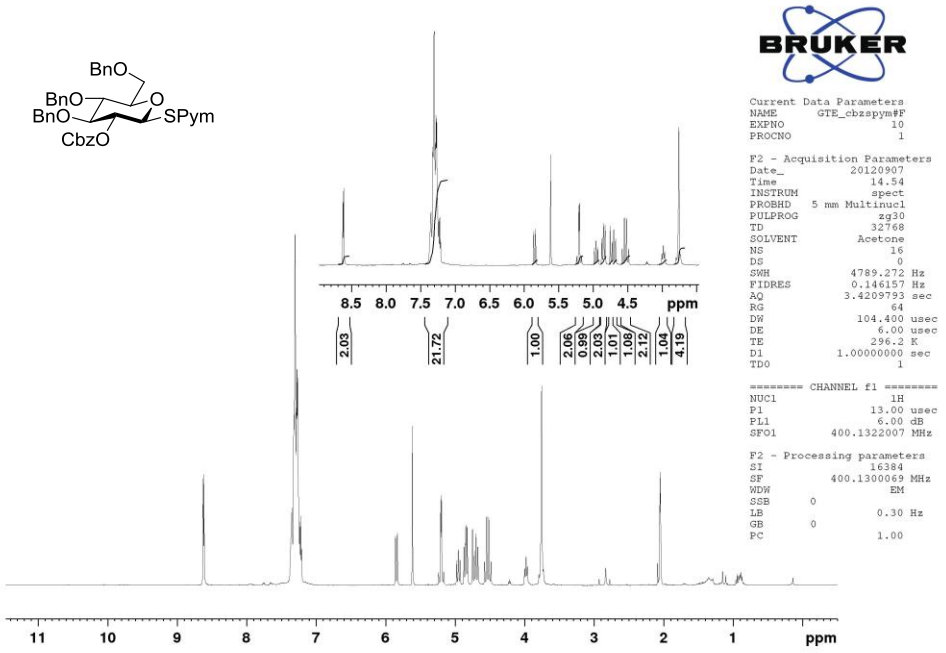
^{1}H NMR (*d*-Acetone, 200 MHz)



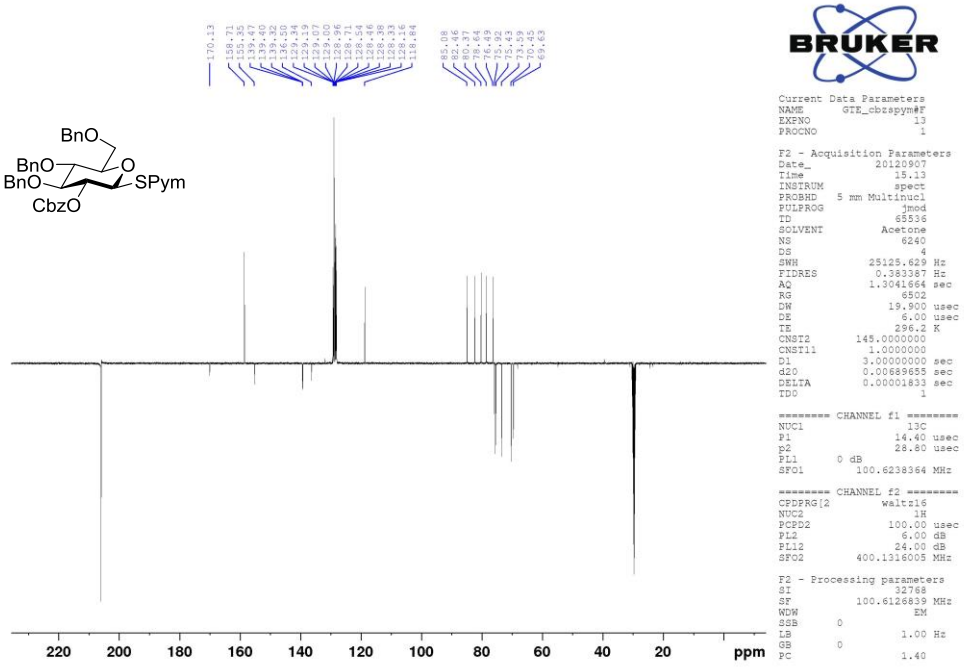
^{13}C NMR (*d*-Acetone, 50 MHz)



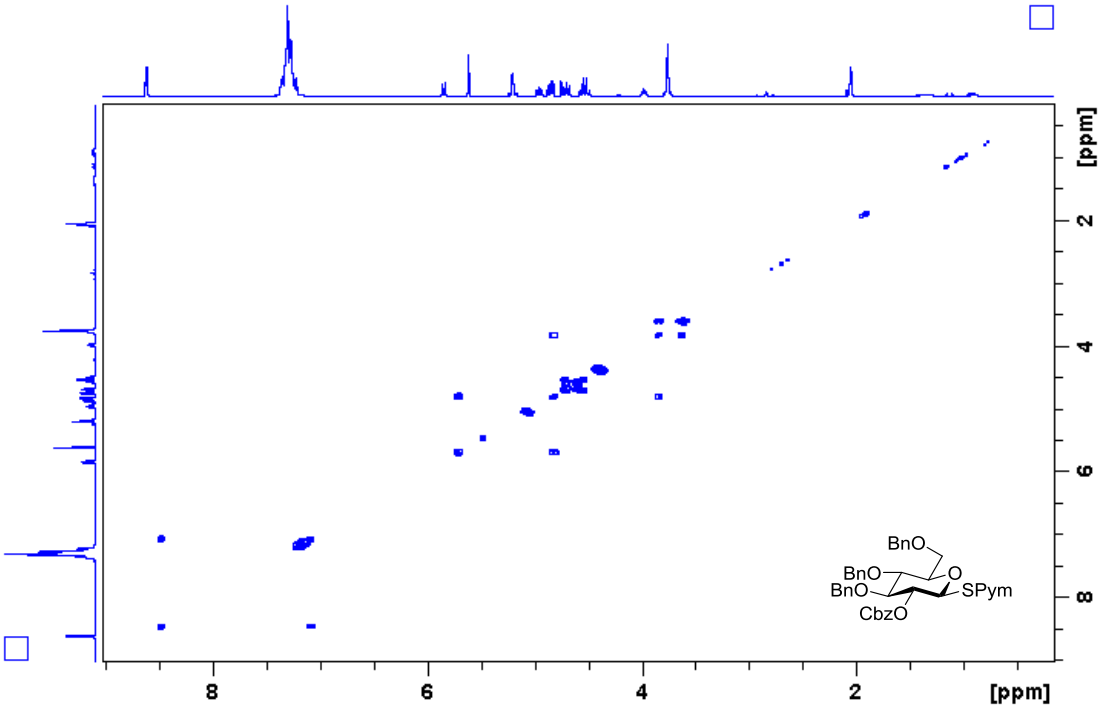
^{1}H NMR (*d*-Acetone, 400 MHz)



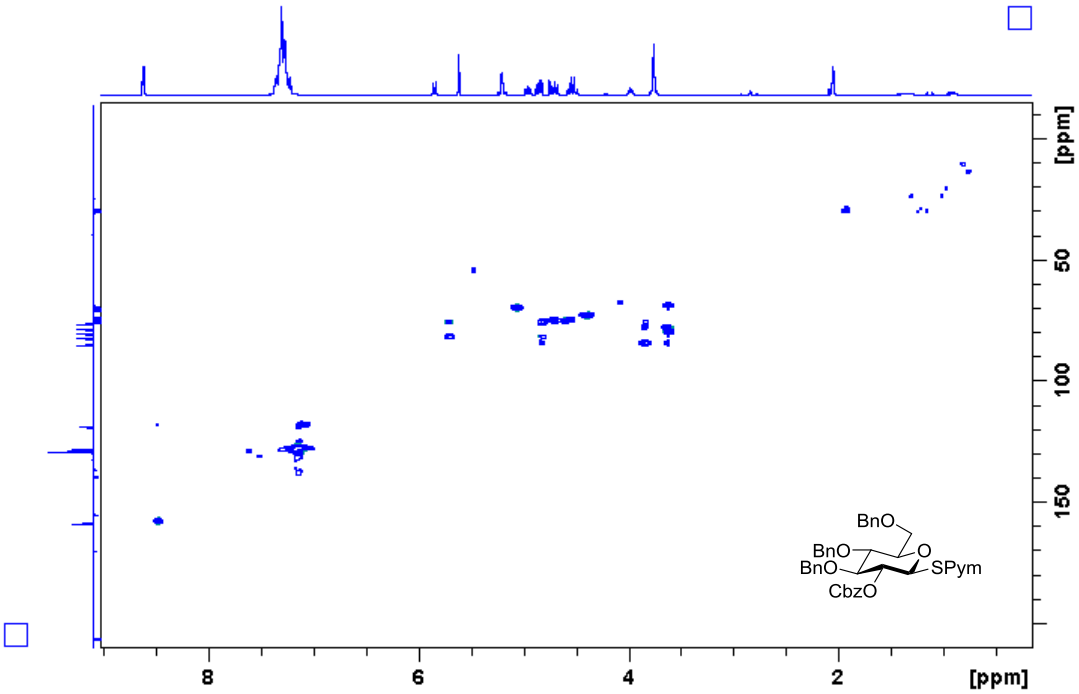
^{13}C NMR (*d*-Acetone, 100 MHz)



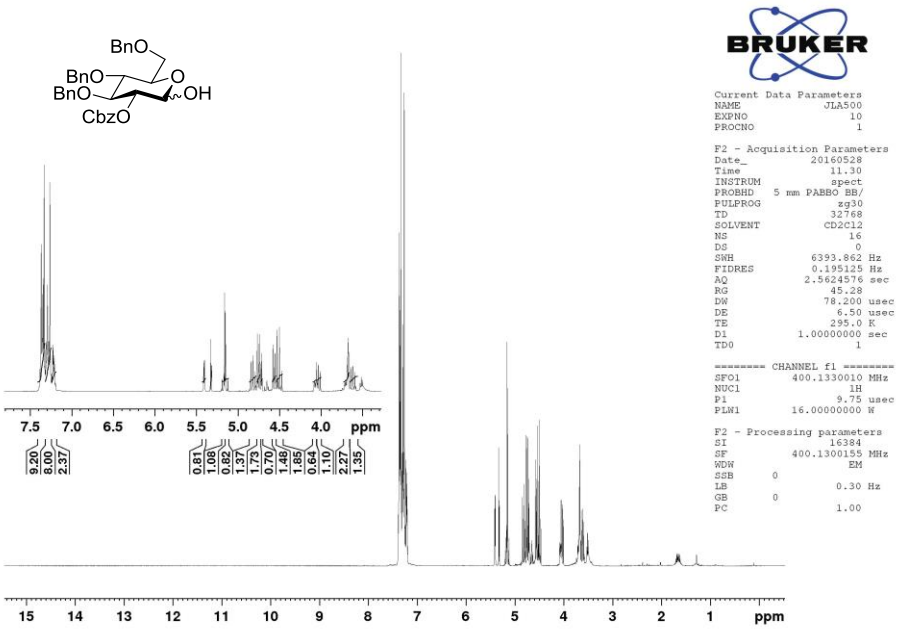
H,H-COSY (*d*-Acetone, 400 MHz)



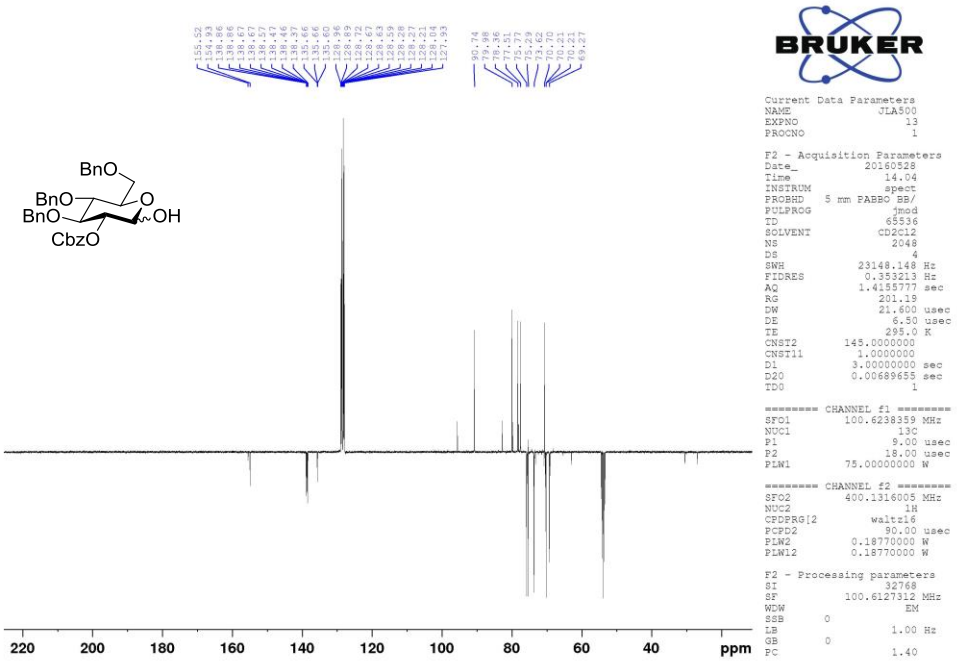
HSQC (*d*-Acetone, 400 MHz)



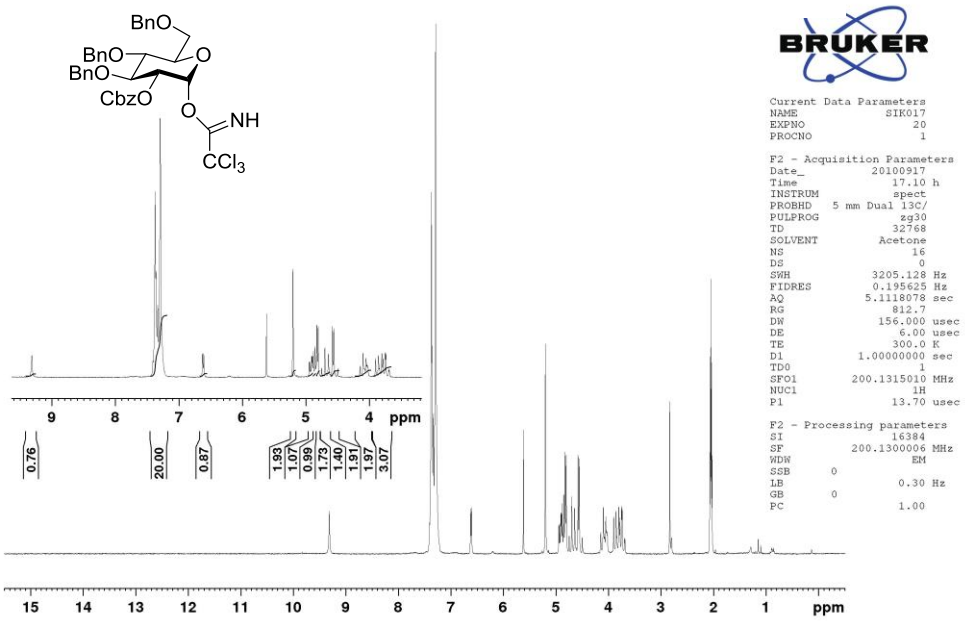
^{1}H NMR (*d*-Methylene chloride, 400 MHz)



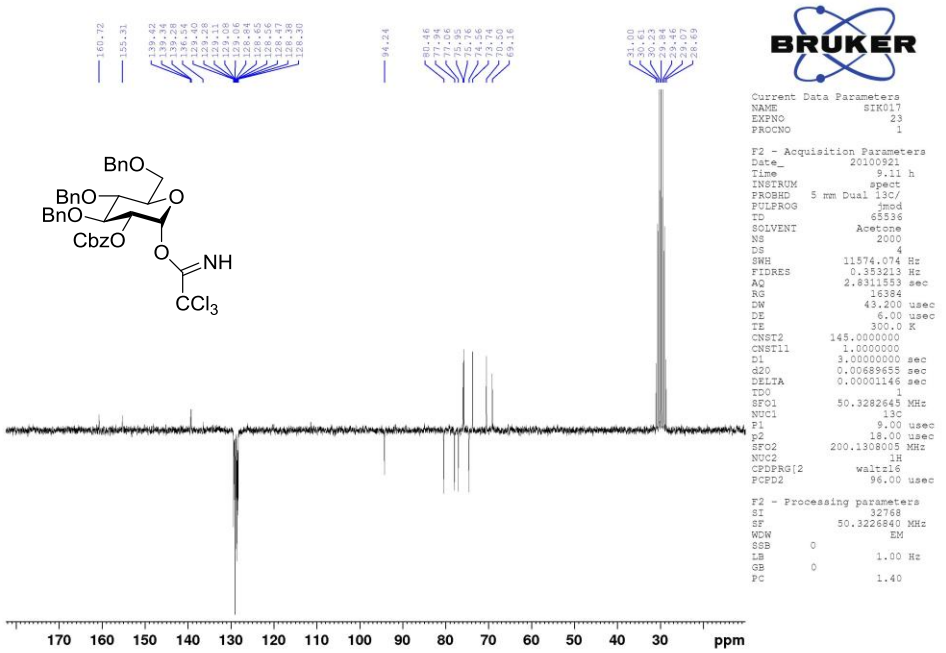
^{13}C NMR (*d*-Methylene chloride, 100 MHz)



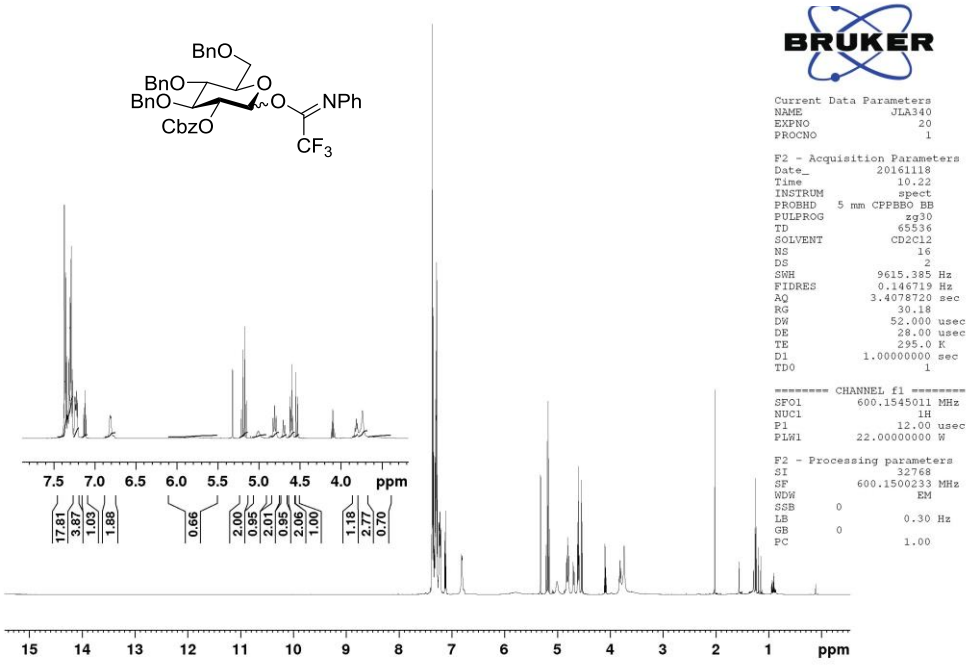
^{1}H NMR (*d*-acetone, 200 MHz)



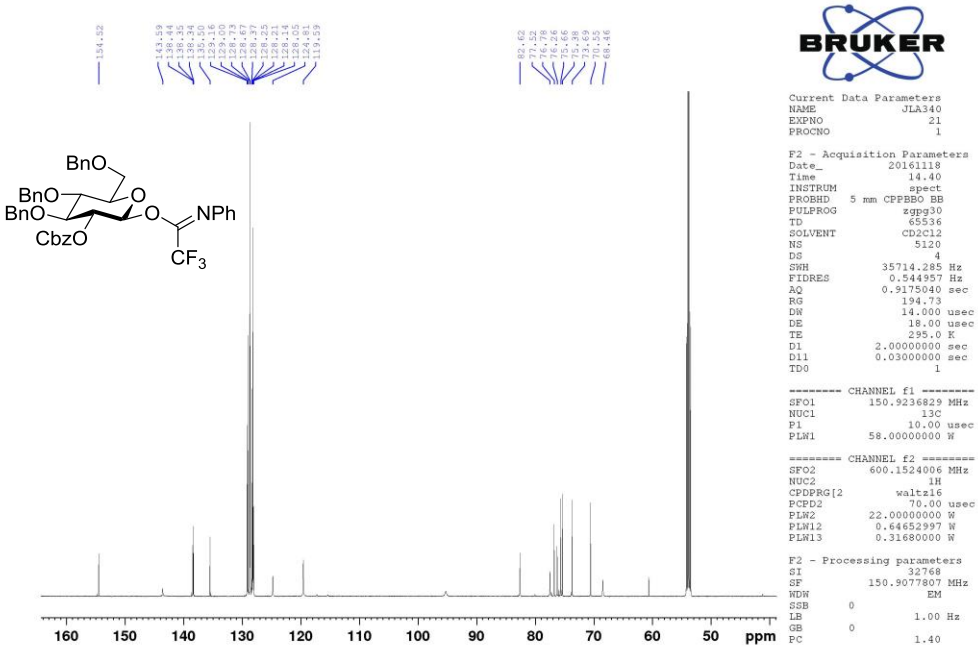
^{13}C NMR (*d*-acetone, 50 MHz)



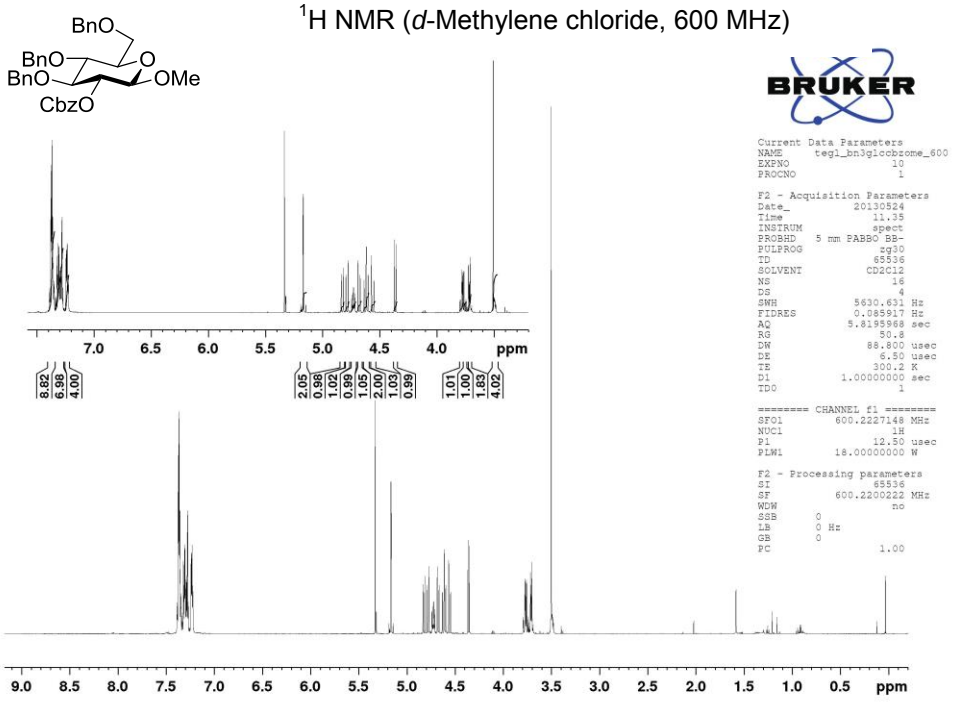
^{1}H NMR (*d*-Methylene chloride, 600 MHz)



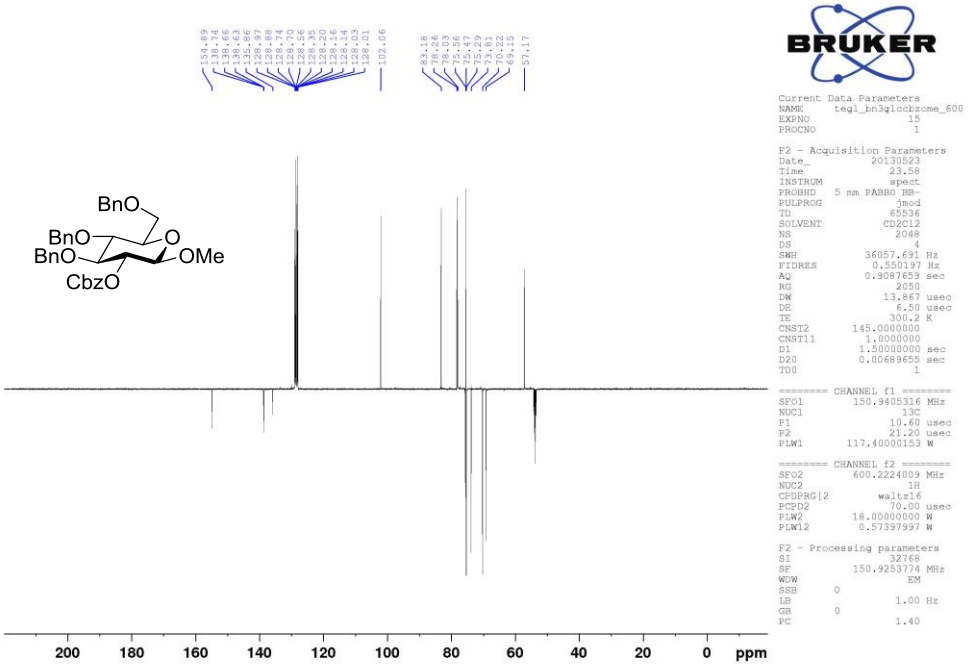
^{13}C NMR (*d*-Methylene chloride, 150 MHz)



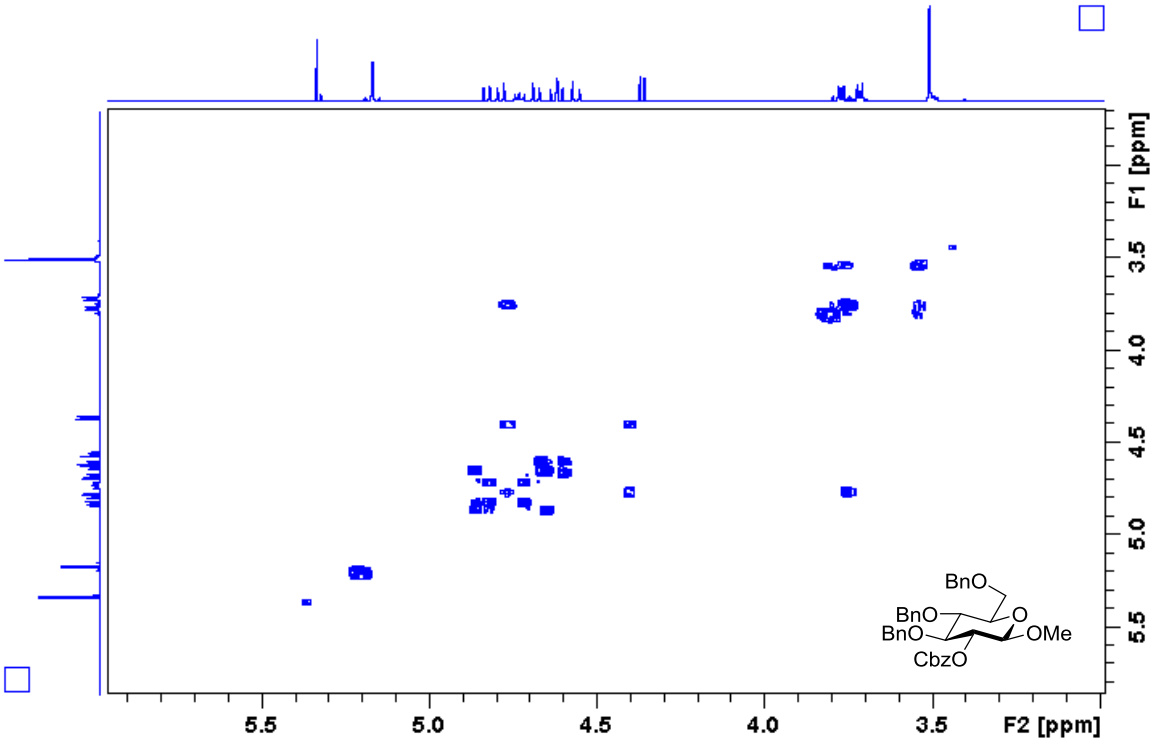
^{1}H NMR (*d*-Methylene chloride, 600 MHz)



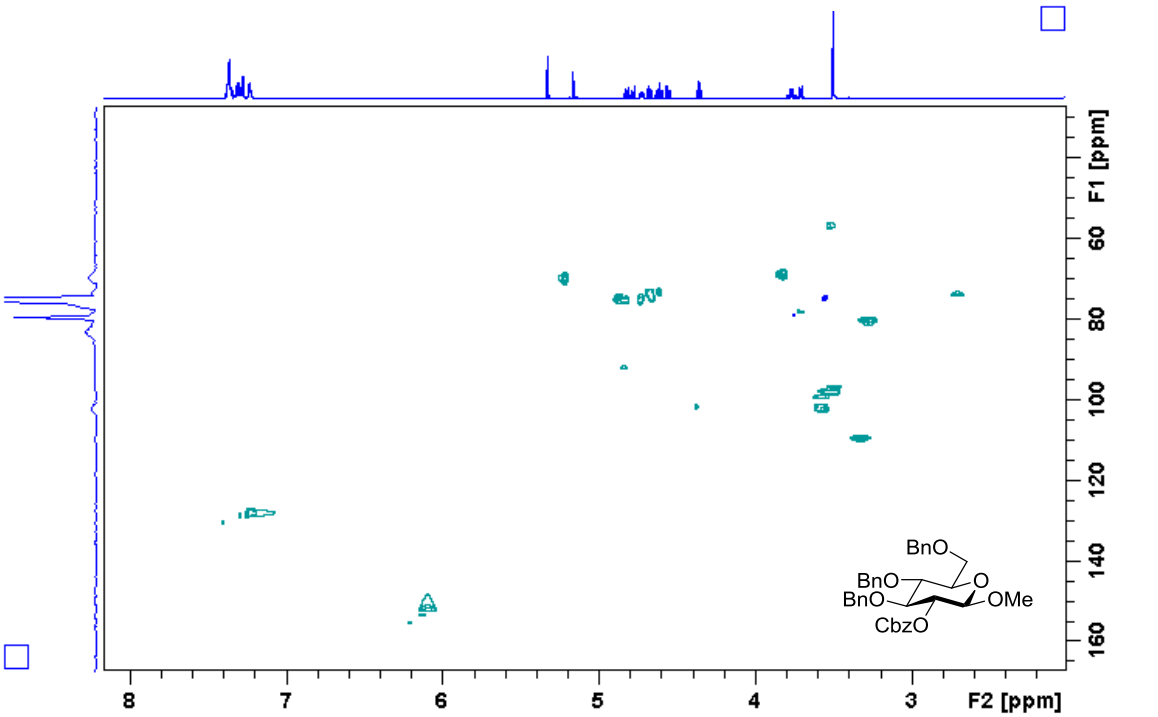
^{13}C NMR (*d*-Methylene chloride, 150 MHz)

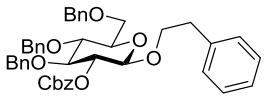


H,H COSY (*d*-Methylene chloride, 600 MHz)

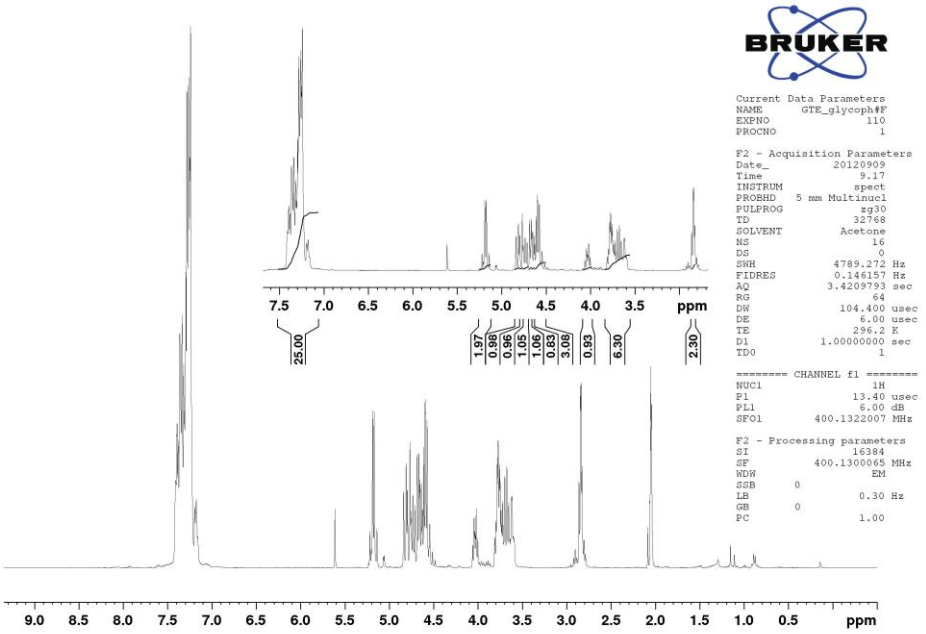


HSQC (*d*-Methylene chloride, 600 MHz)

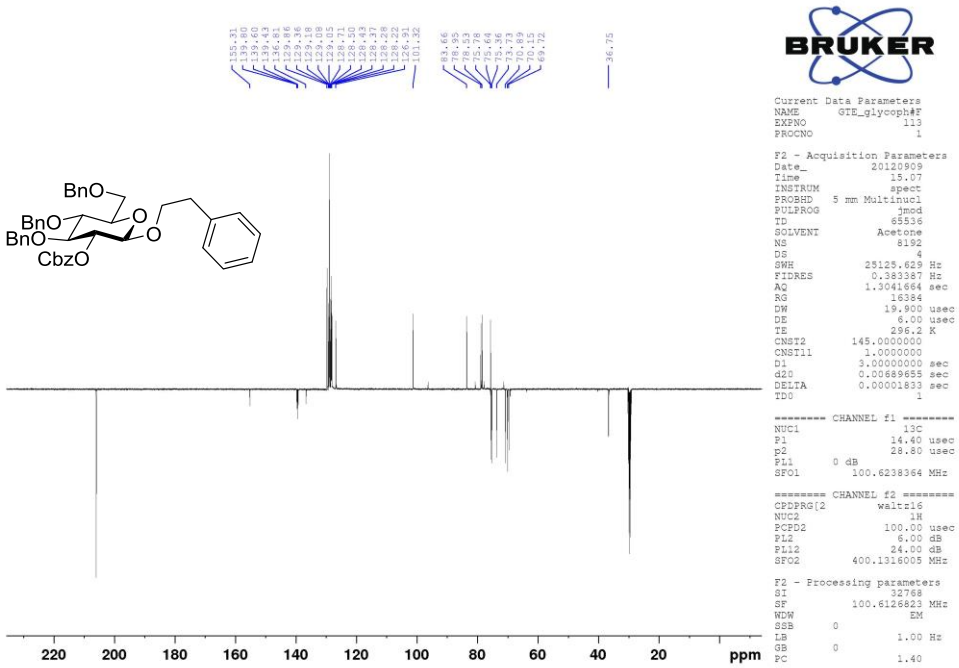




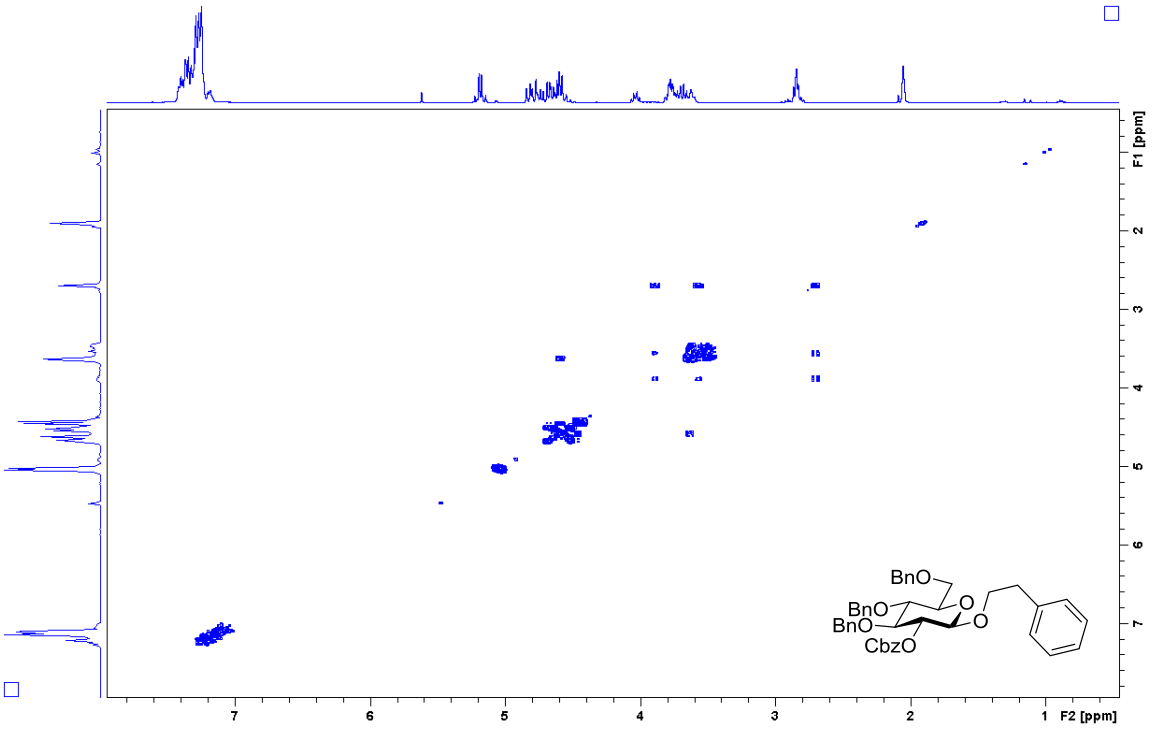
^{1}H NMR (*d*-Acetone, 400 MHz)



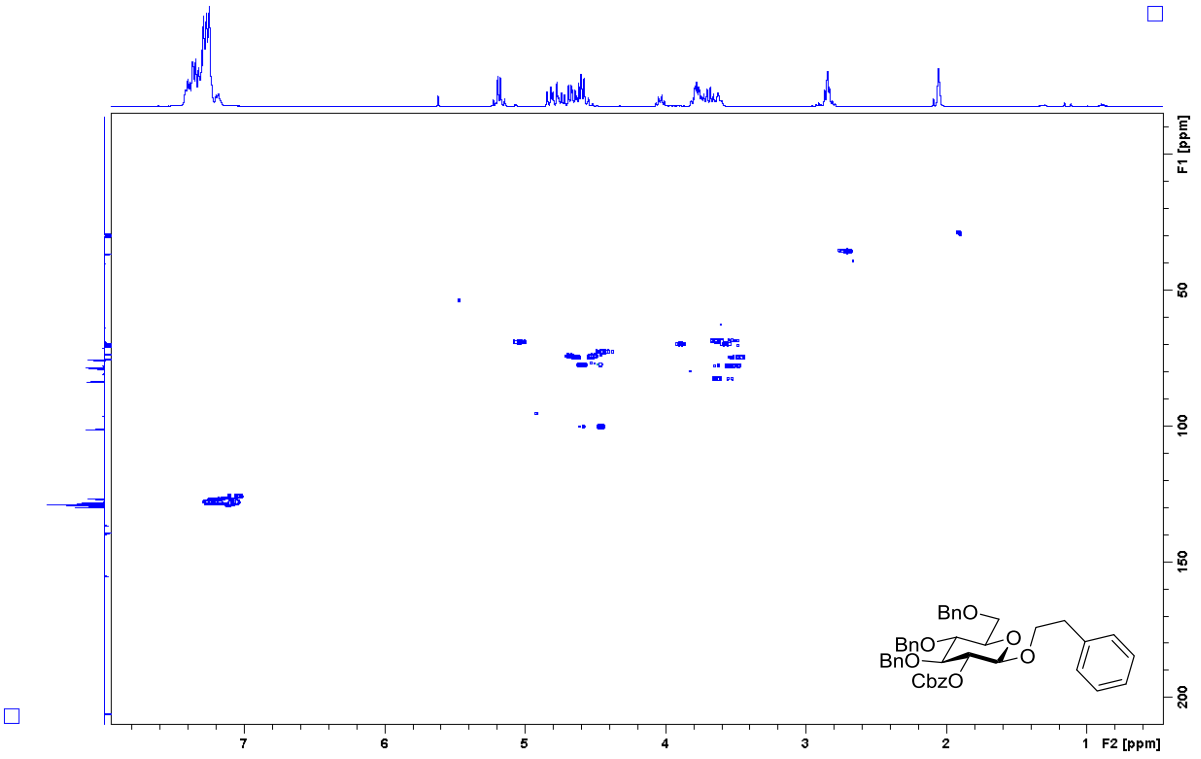
^{13}C NMR (*d*-Acetone, 100 MHz)



H,H COSY (*d*-Acetone, 400 MHz)



HSQC (*d*-Acetone, 400 MHz)



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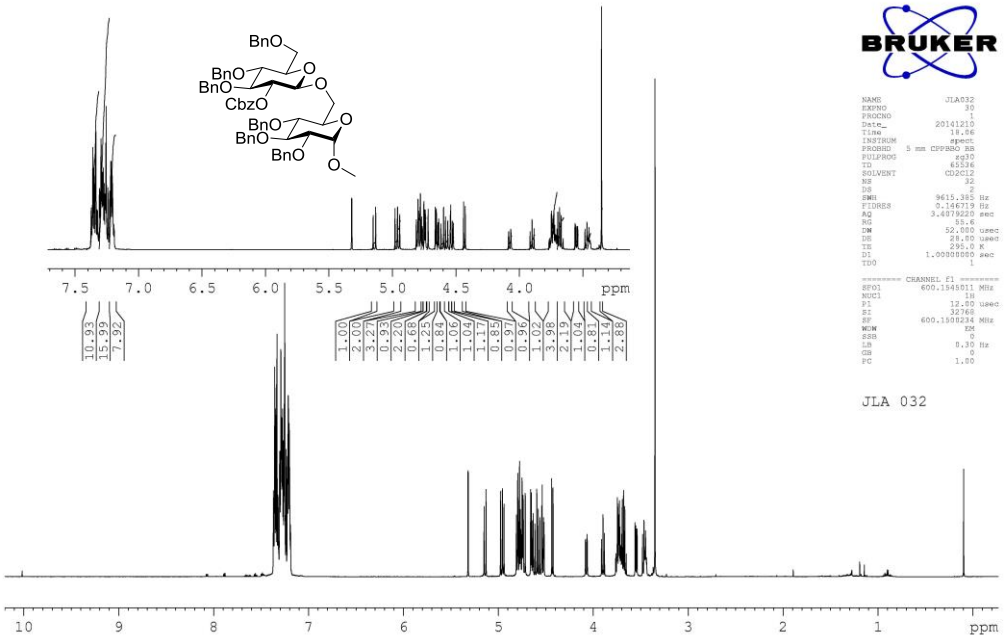

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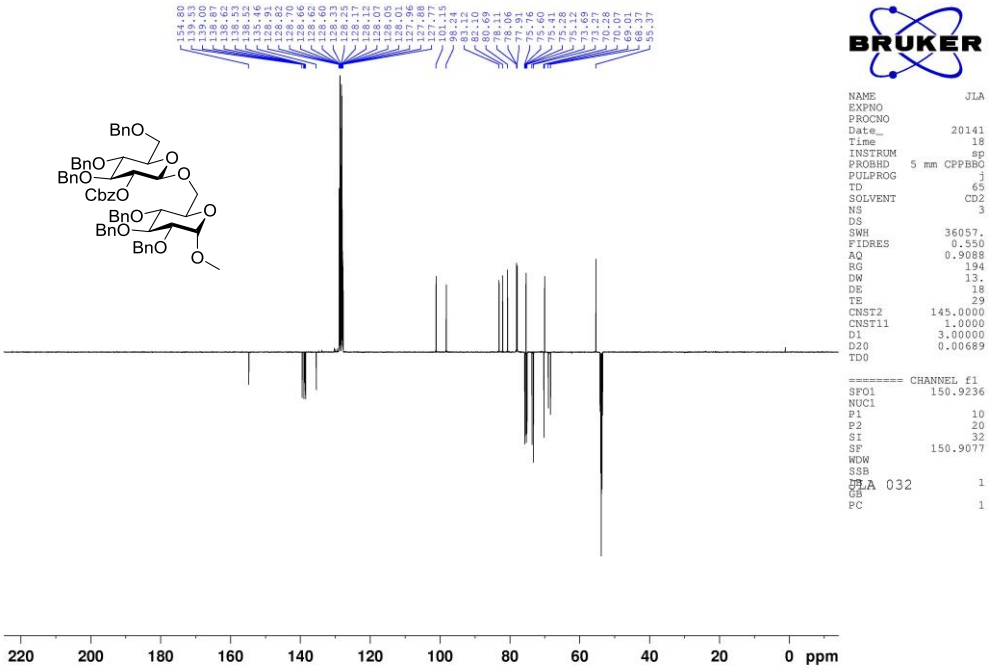

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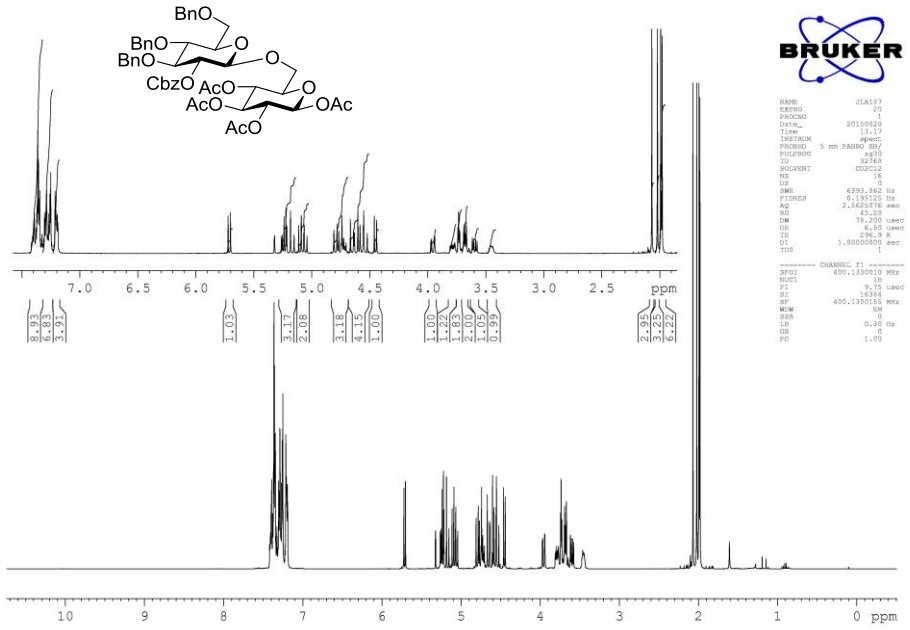

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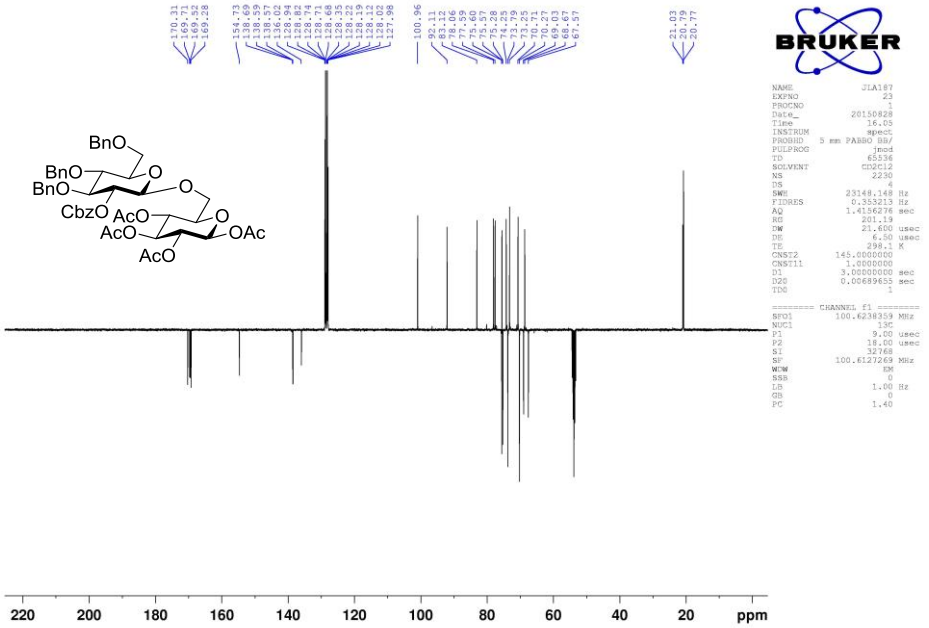

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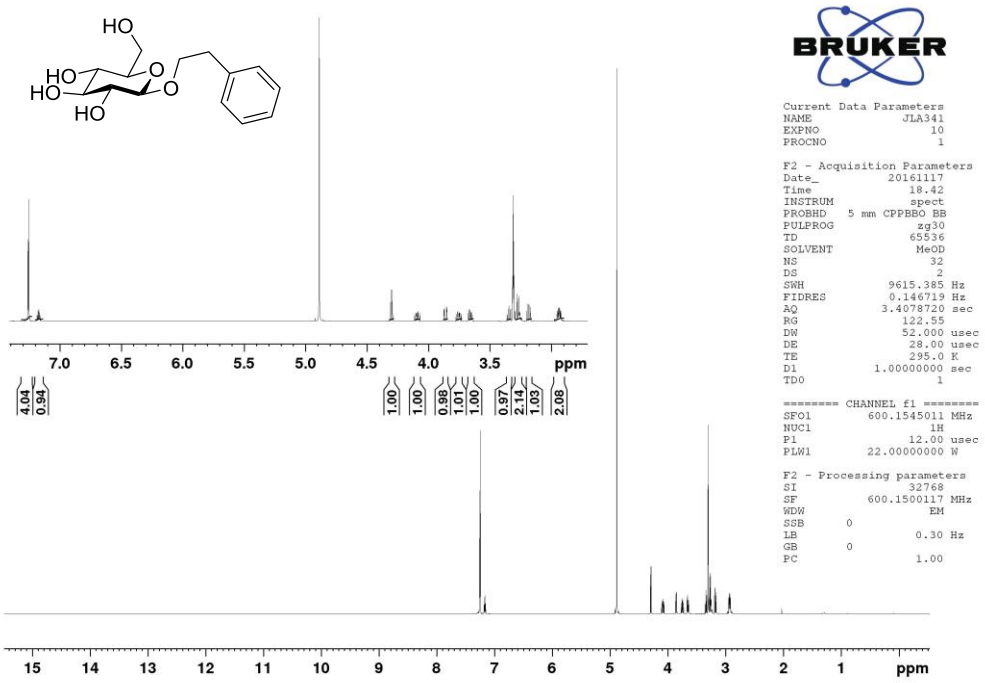

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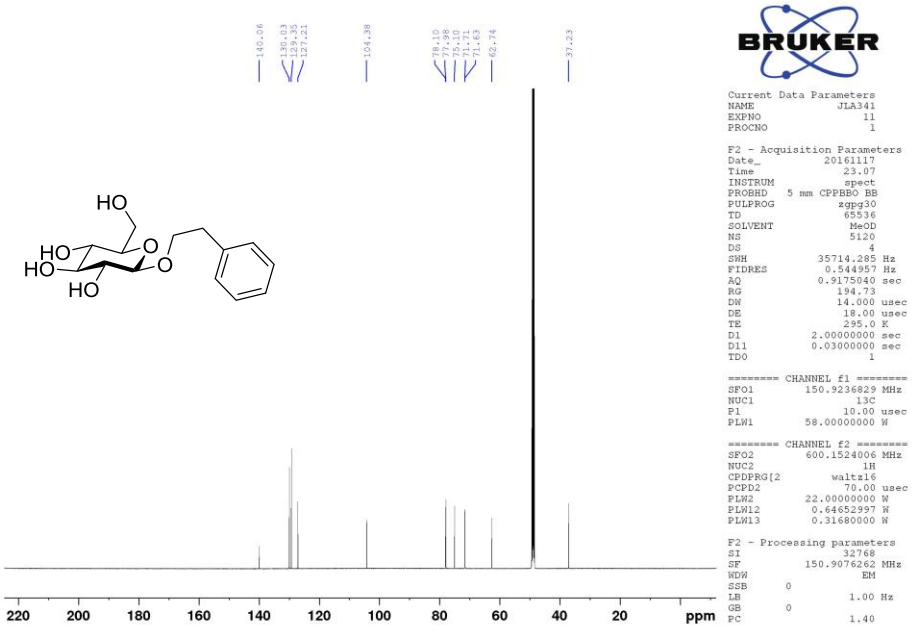

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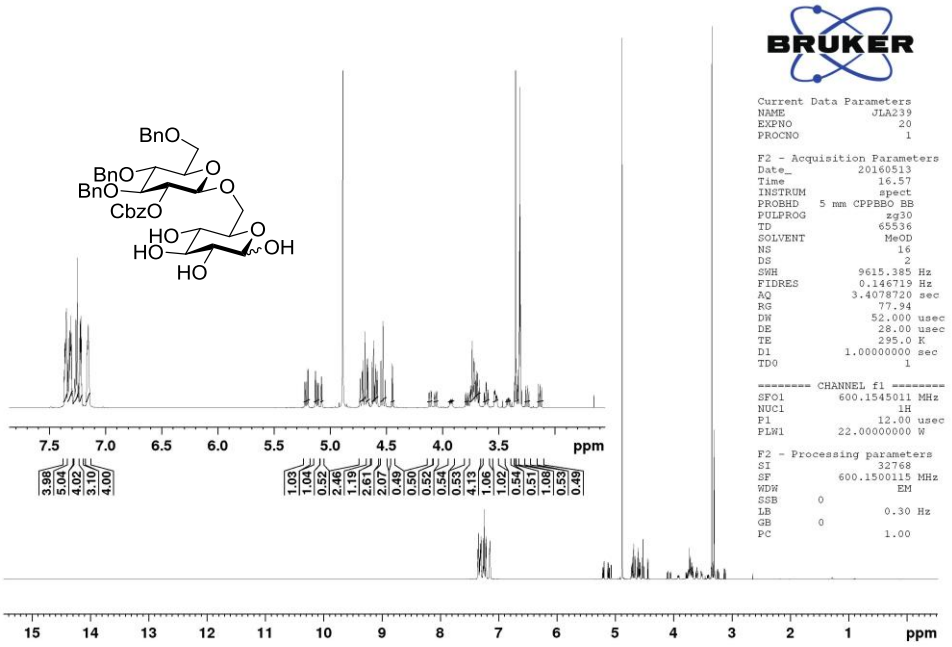

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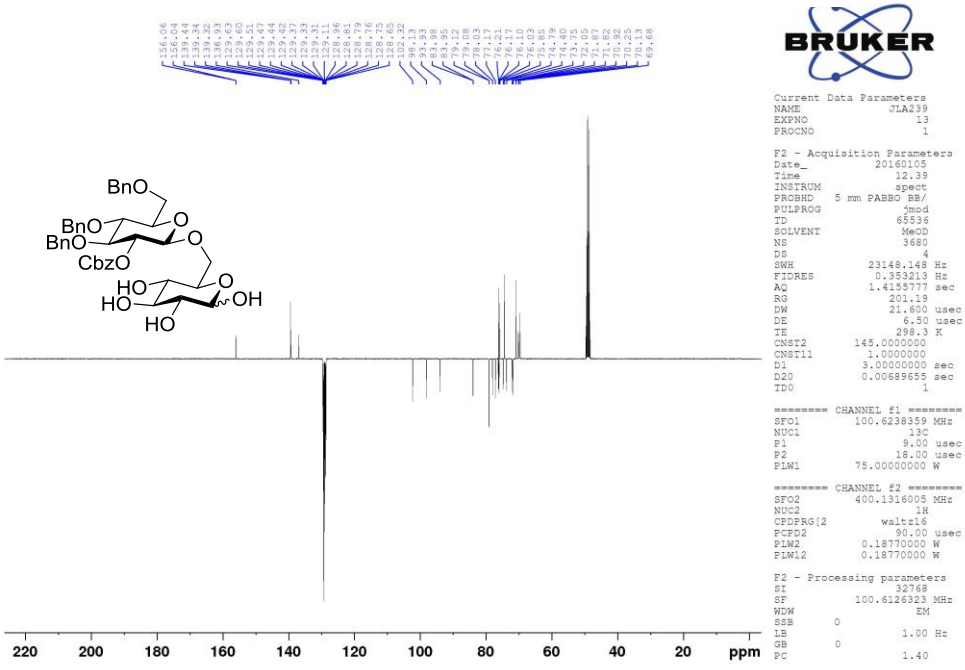

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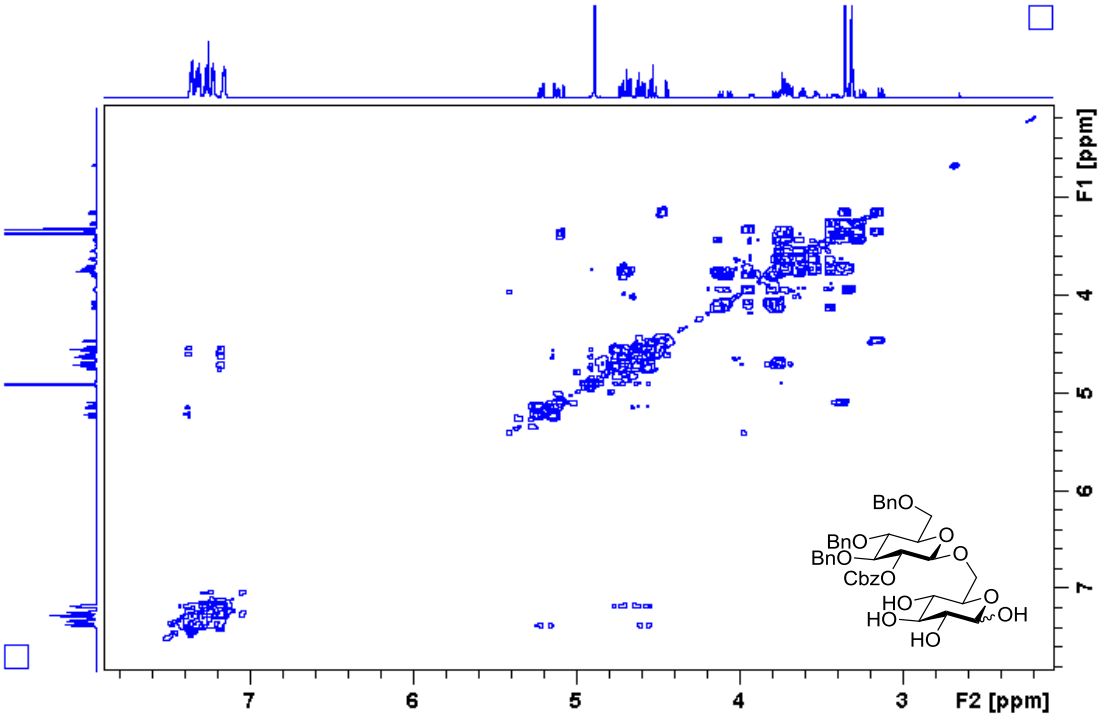

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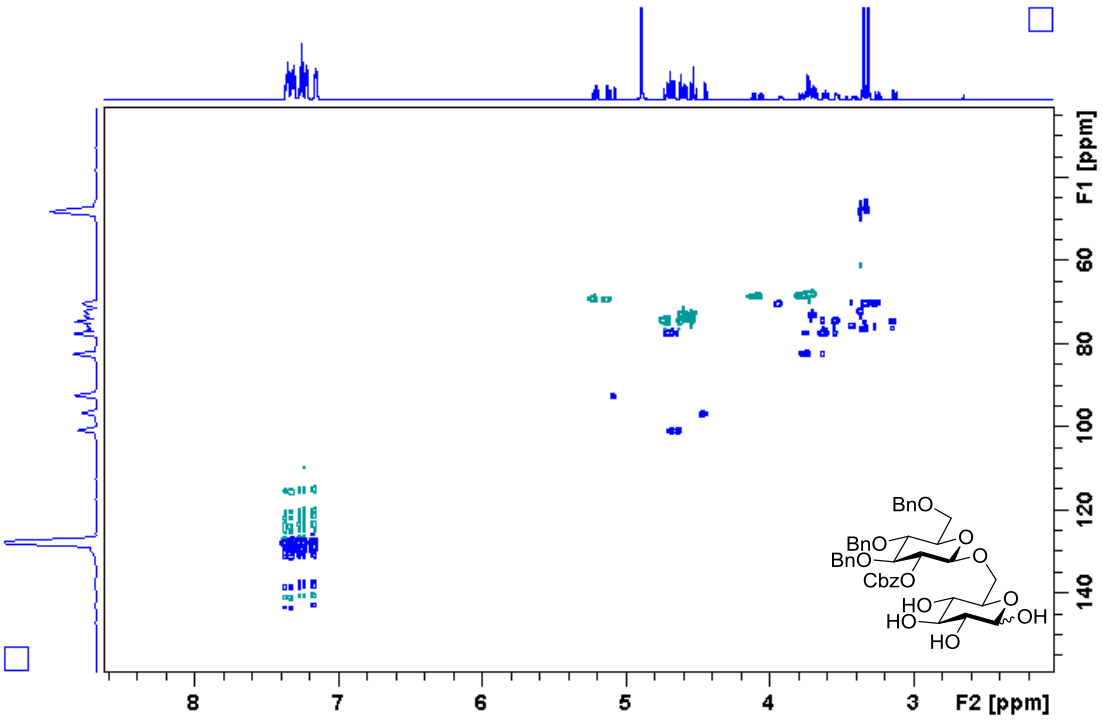

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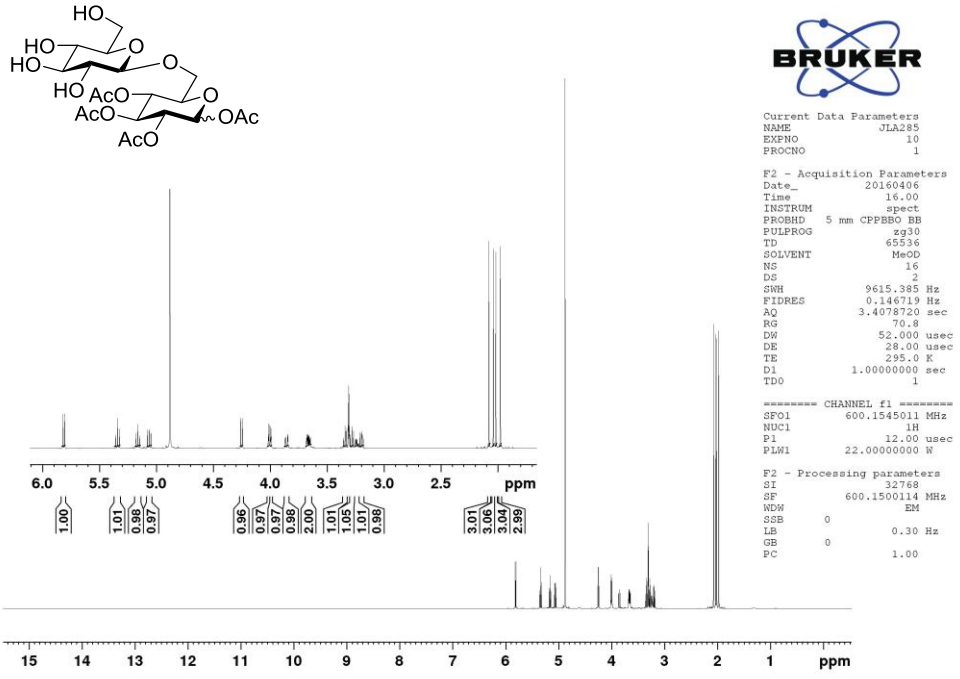

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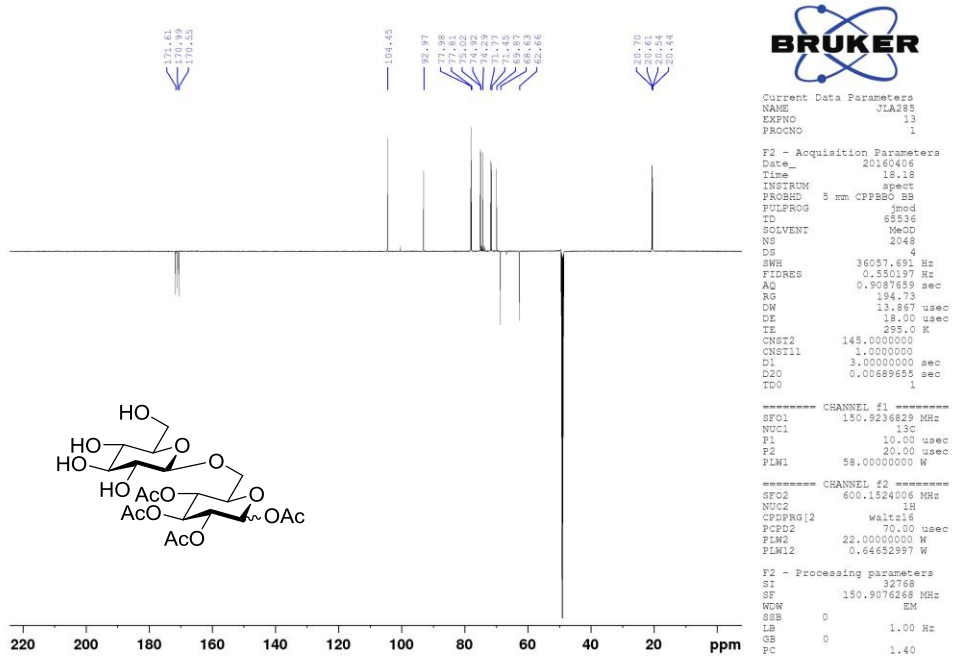

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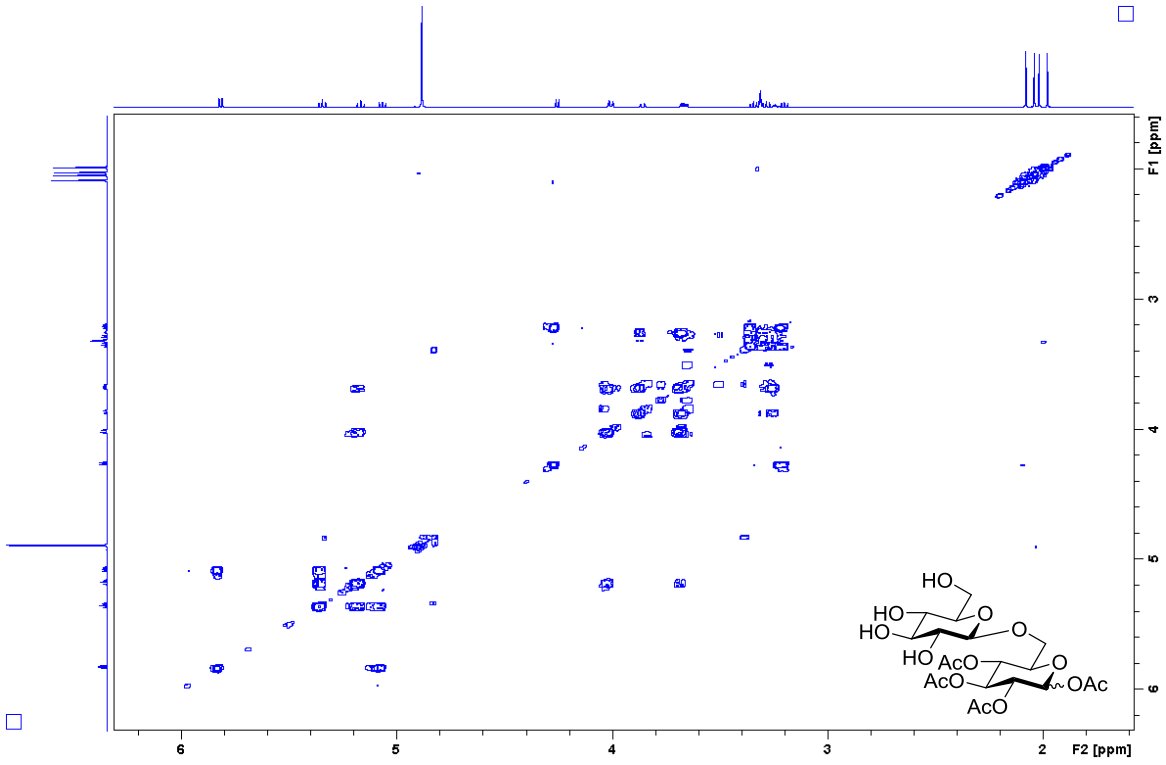
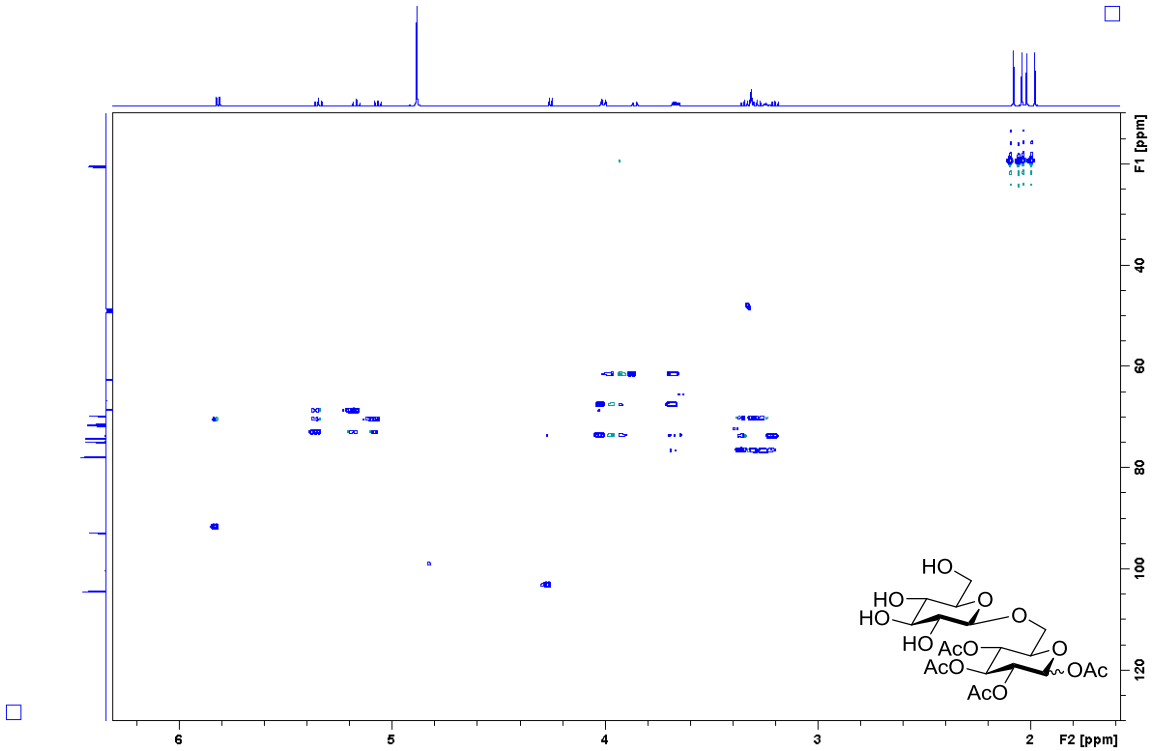
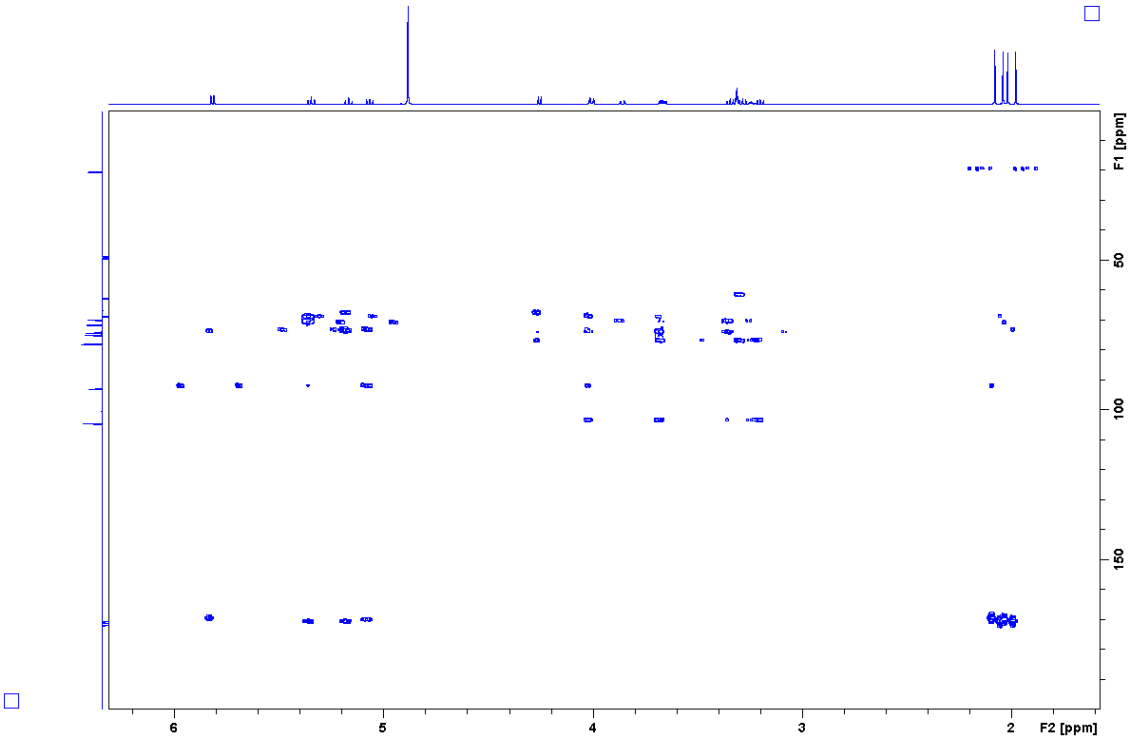


HSQC (*d*-MeOH, 600 MHz)



HMBC (*d*-MeOH, 600 MHz)



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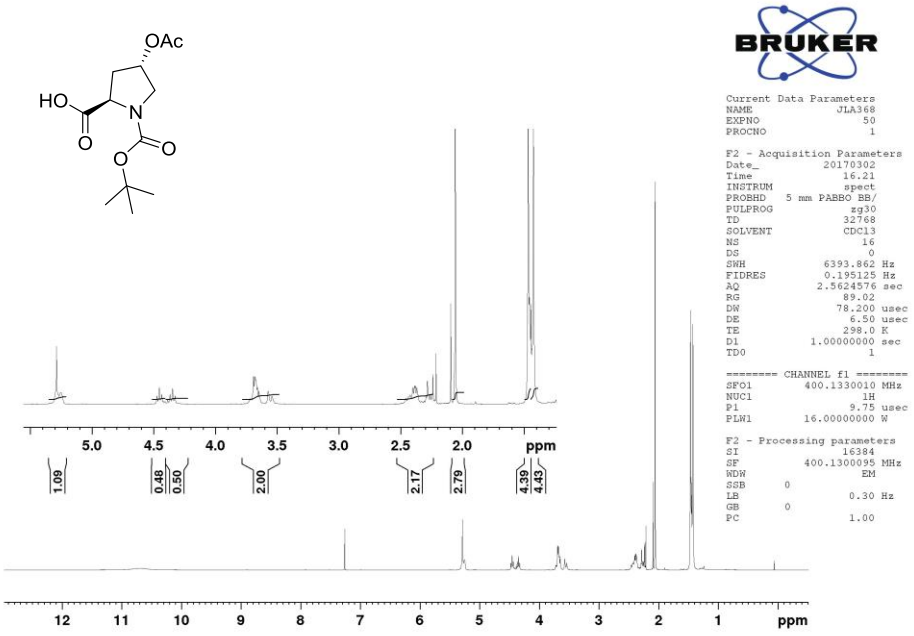

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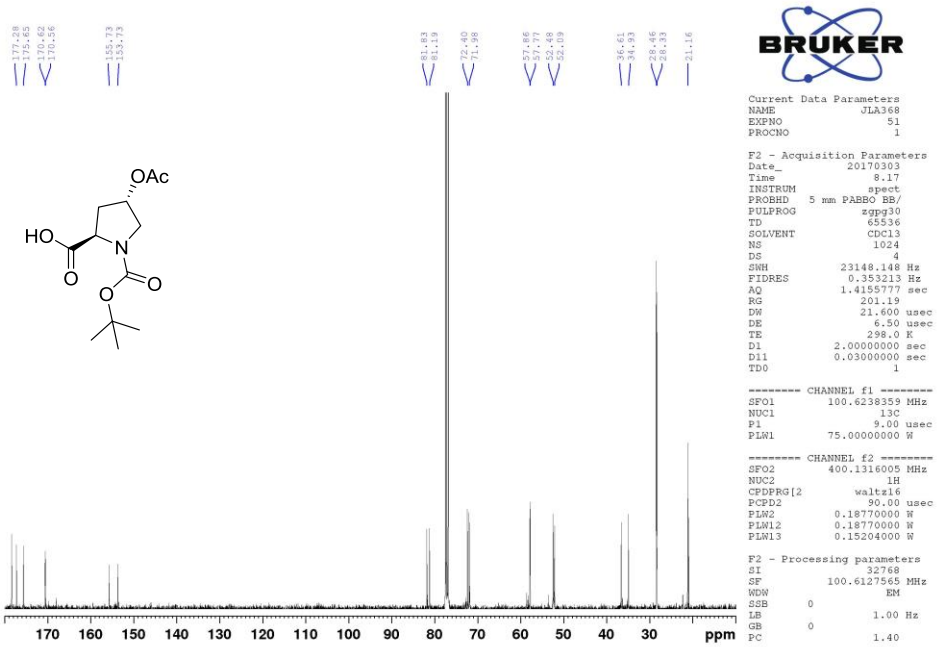

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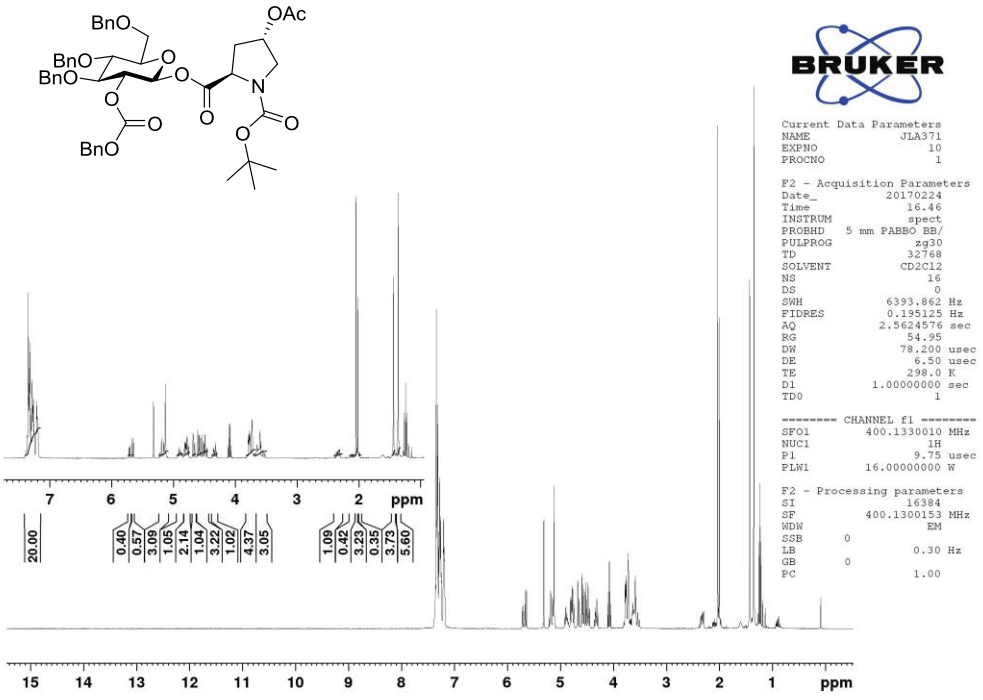
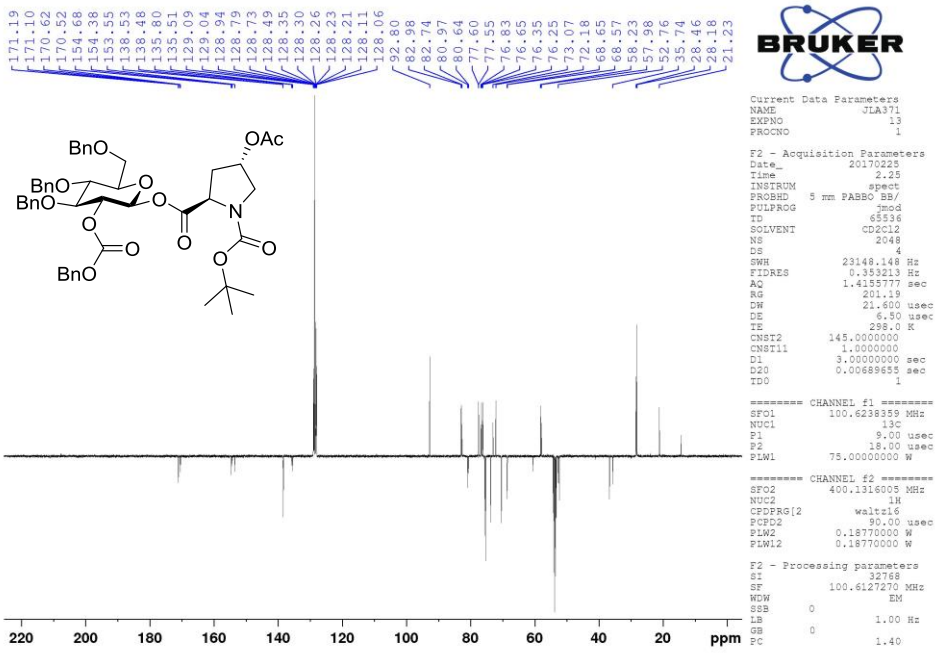
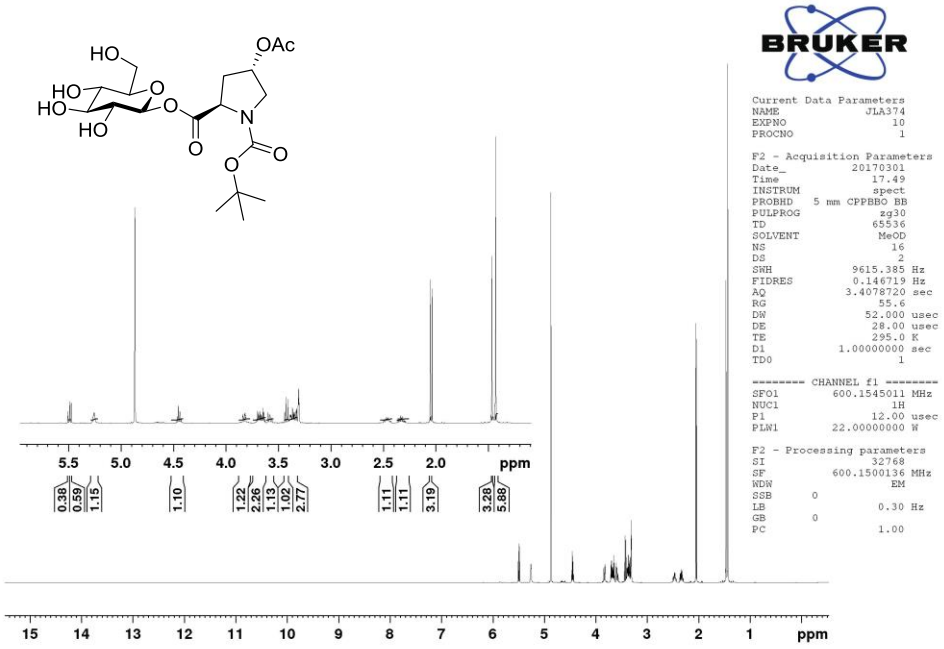


^{13}C NMR (*d*-Dichloromethane, 100 MHz)



^{1}H NMR (*d*-Methanol, 600 MHz)



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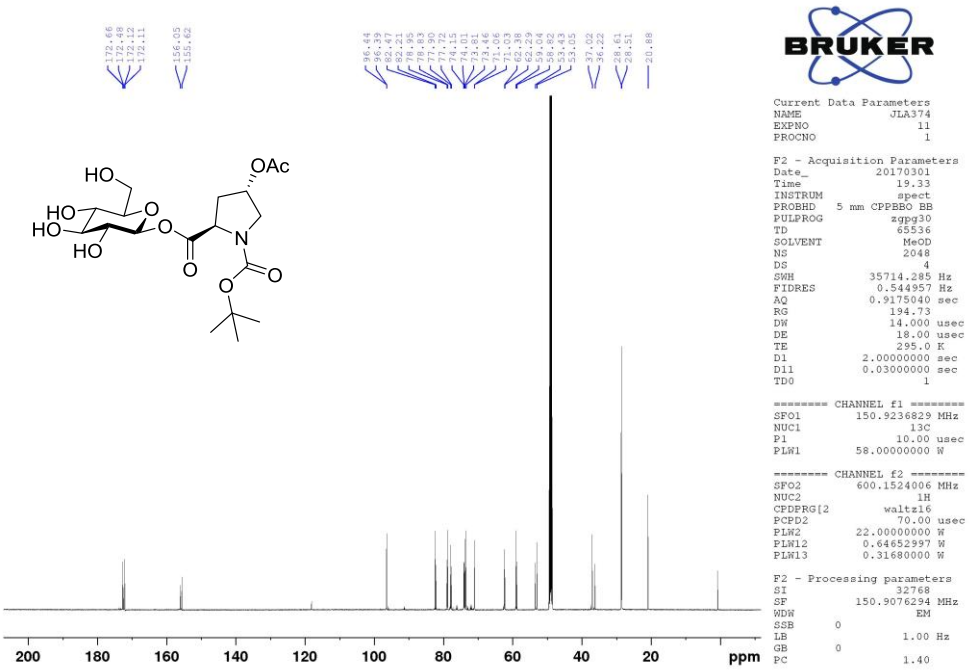


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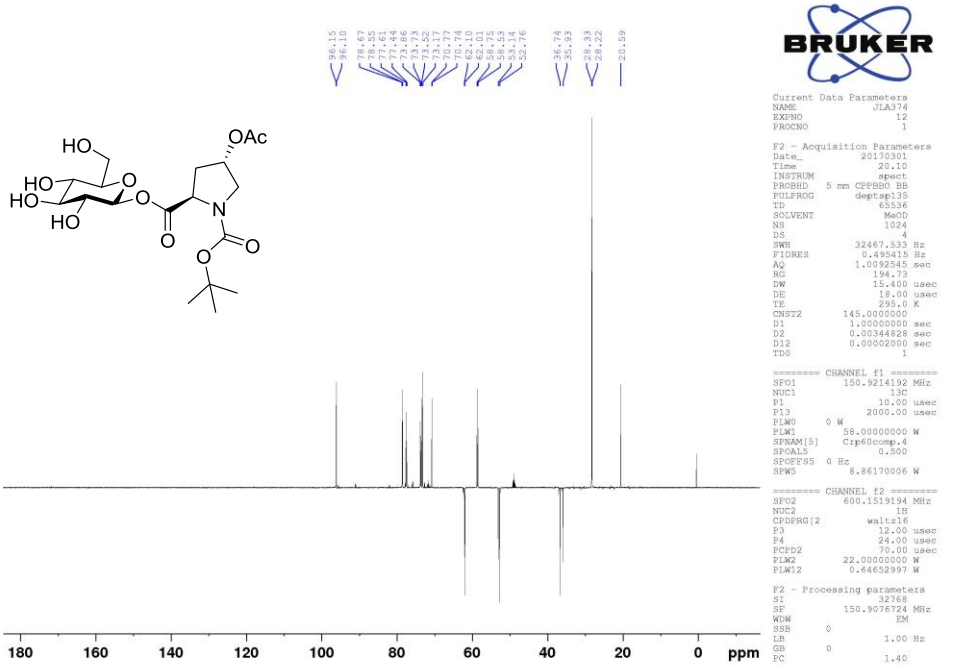


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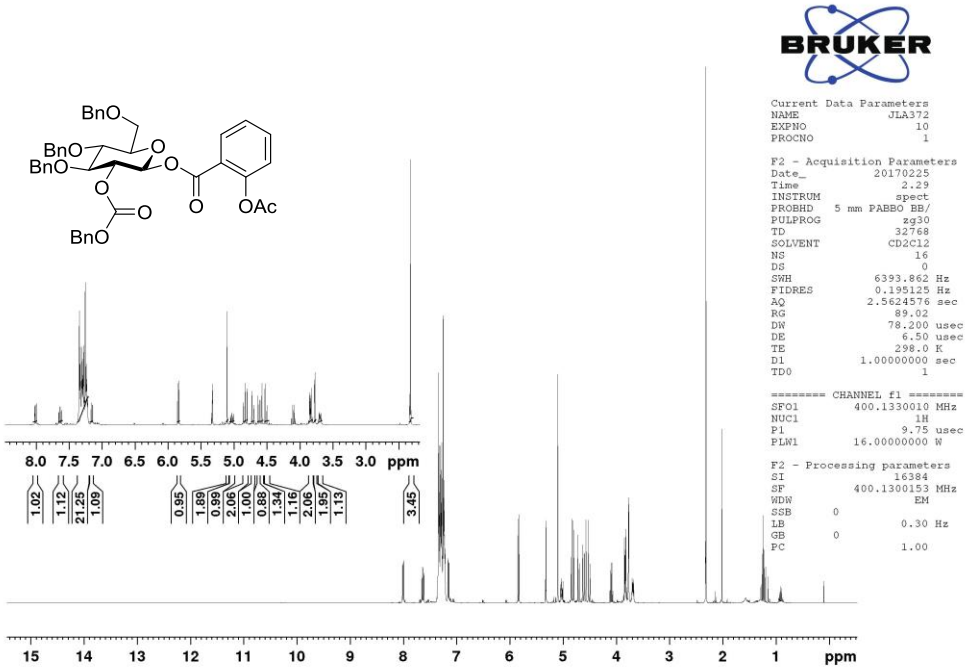
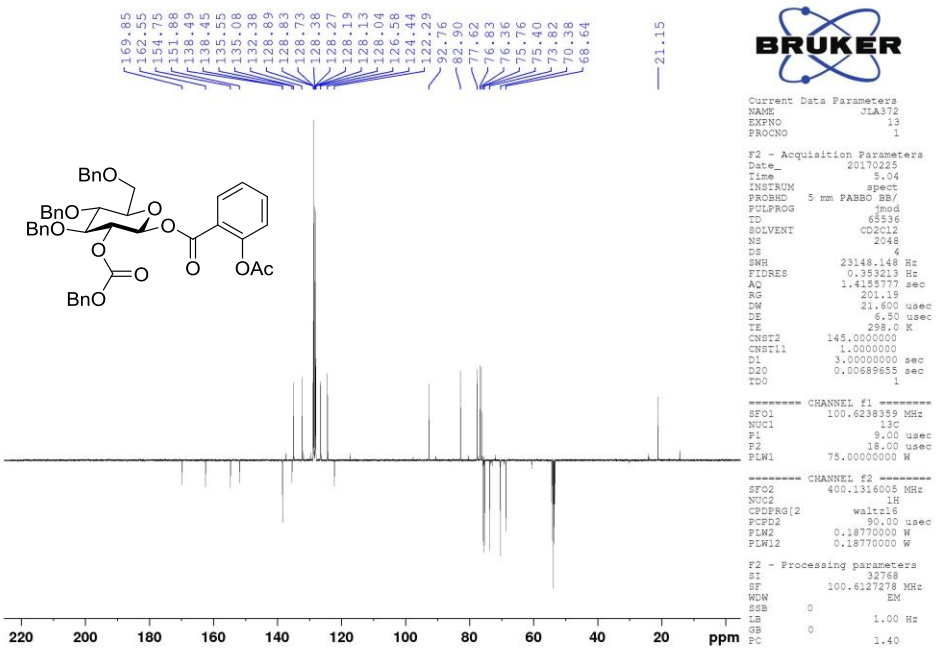
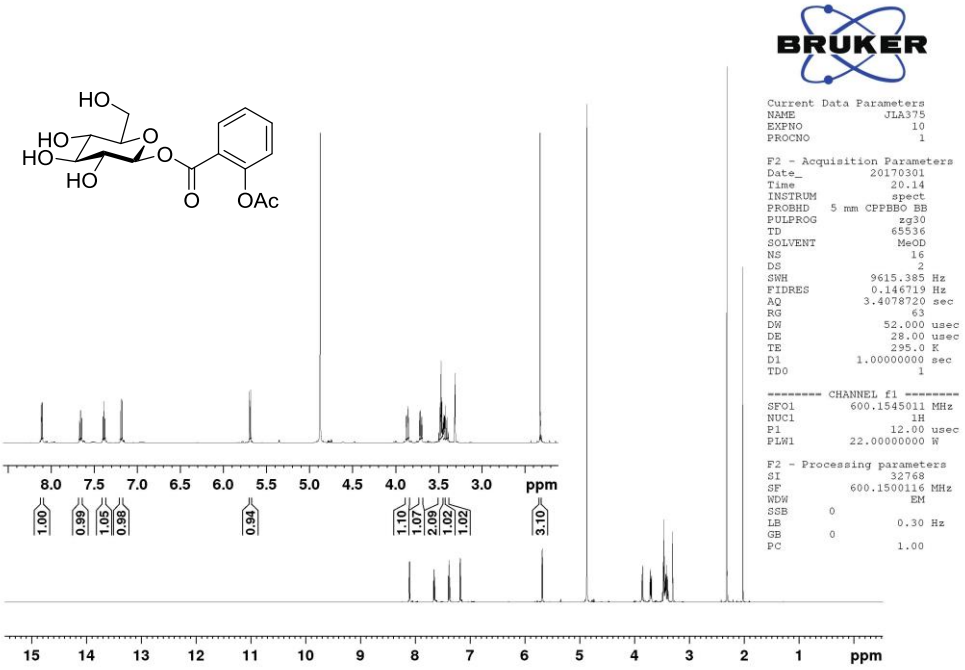
^{1}H NMR (*d*-Dichloromethane, 400 MHz)



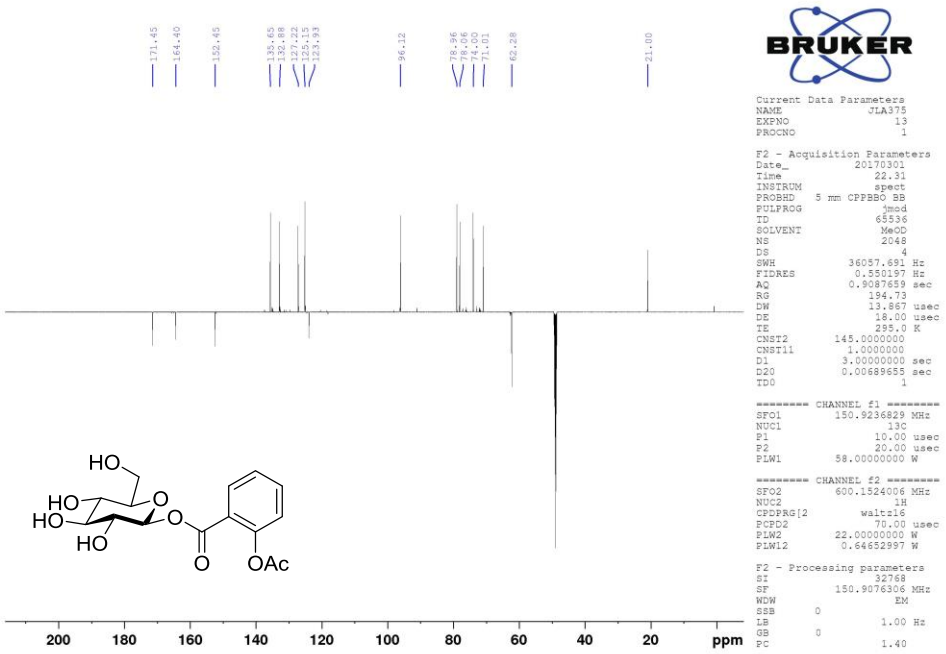
^{13}C NMR (*d*-Dichloromethane, 100 MHz)



^{1}H NMR (*d*-Methanol, 600 MHz)



^{13}C NMR (*d*-Methanol, 150 MHz)



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Supporting Information

Manuscript #2

Supplementary Data

(2-Benzyloxyphenyl)acetyl ester as a new relay protecting group for alcohols and in particular carbohydrates

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Vienna University of Technology

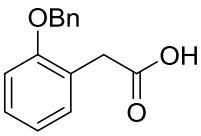
Content

1) General remarks

All reactions were performed under argon atmosphere. Anhydrous solvents like dichloromethane, tetrahydrofuran, methanol and diethyl ether were dried using a PURESOLV facility of it-innovative technology. Molecular sieve 3 Å was activated under vacuum at 200°C before use. The progress of the reactions was monitored by thin layer chromatography (TLC) over silica gel 60 F_{254}. Visualization of the spots was achieved either by UV irradiation (254 or 366 nm) or by heat staining with ceric ammonium molybdate in ethanol/sulfuric acid. LC-ESI-MS/MS was performed on an HCT ion trap mass spectrometer (Bruker, Germany) in full scan mode. Chromatographic separation was done on a 1200 series HPLC system (Agilent Technologies, Germany) using a Luna RP-C18 column (3.0 x 150 mm, 3 µm particle size, Phenomenex, Germany) and application of pure substances was achieved using a TLC-MS interface (Camag, Germany). Preparative column chromatography was performed on silica gel 60 (Merck, 40-63 µm) using a Büchi Sepacore™ Flash System or a Grace Reveleris Prep Purification System. Preparative HPLC separation was done on a Grace Reveleris Prep system using a Luna Prep C18(2), 10 µm, 250x10 mm column (Phenomenex). NMR spectra were recorded on a Bruker Avance IIIHD 600Mhz spectrometer equipped with a Prodigy BBO cryo probe or on a Bruker Avance DRX-400 MHz spectrometer at 20°C. Data were recorded and evaluated using TOPSPIN 3.5 (Bruker Biospin). All chemical shifts are given in ppm relative to tetramethylsilane. The calibration was done using residual solvent signals. Multiplicities are abbreviated as s (singlet), d (doublet), t (triplet), q (quartet), b (broad signal). All chemicals were purchased either from ABCR (Germany) or Sigma-Aldrich (Austria/Germany). HR-MS analysis was carried out from acetonitrile solutions (concentration: 10 ppm) either by using an HTC PAL system autosampler (CTC Analytics AG, Zwingen, Switzerland), an Agilent 1100/1200 HPLC with binary pumps, degasser and column thermostat (Agilent Technologies, Waldbronn, Germany) and Agilent 6230 AJS ESI–TOF mass spectrometer (Agilent Technologies, Palo Alto, United States) or by using a HTC PAL Autosampler, Thermo Ulitmate 3000 HPLC and a Thermo Q Exactive Focus mass spectrometer.

2) Experimental Procedures

2-(2-(Benzyloxy)phenyl)acetic acid (1)

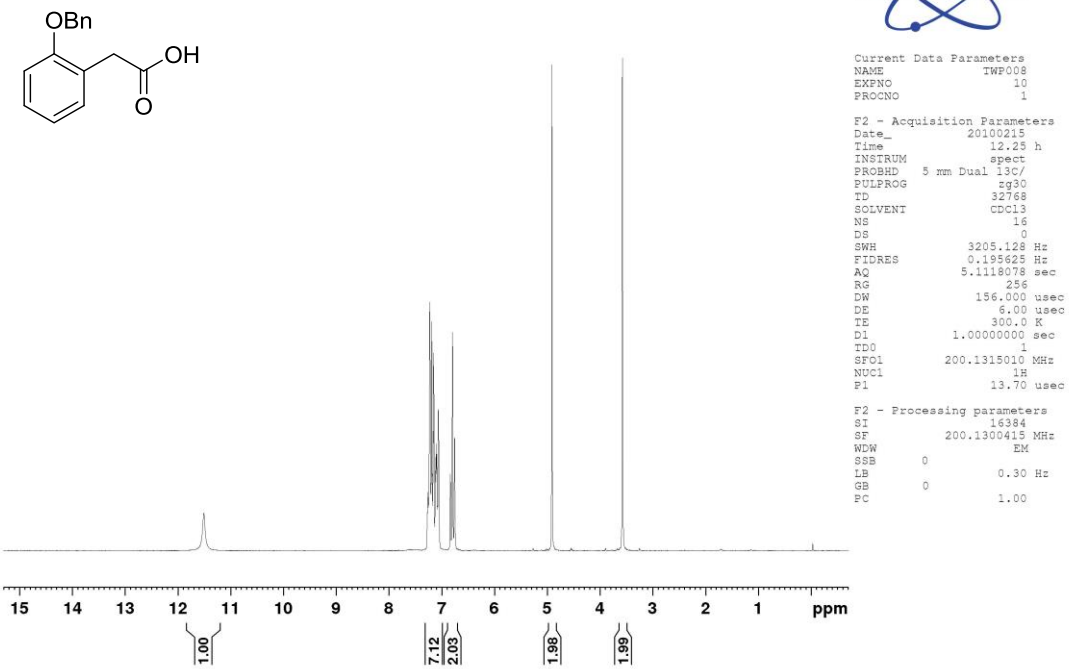


To a solution of methyl 2-(2-hydroxyphenyl)acetate (3.32 g, 20 mmol, 1 eq.) in DMF (50 mL) K_2CO_3 (8.3 g, 60 mmol, 3 eq.) and benzyl bromide (4.3 g, 25 mmol, 1.25 eq.) was added. After stirring for 16 h, the reaction mixture was filtered, diluted with Et_2O (200 mL) and washed with a saturated solution of NH_4Cl (2x100 mL) and water (1x100 mL). The aqueous phases were reextracted with Et_2O (100 mL), the organic phases combined, dried over Na_2SO_4 and concentrated. The crude product (2-(2-(benzyloxy)phenyl)acetic acid methyl ester) was dissolved in THF (80 mL) and KOH (4.48 g, 80 mmol, 4 eq.) dissolved in water (20 mL) was added. The reaction mixture was stirred at 0 °C for 30 min, then methanol (60 mL) was added and stirring was continued for 1h. To the reaction mixture water (200 mL) was added; this solution was then extracted with CH_2Cl_2 (3x150 mL) and the combined organic phases were reextracted with water (2x150 mL). The aqueous phases were acidified to a pH of 2 (leading to a partially precipitation of the acid) and extracted with Et_2O (2x150 mL). The combined organic phases were dried over Na_2SO_4 and concentrated to yield compound **1** (3.88 g, 80 %) as a white solid; ^{1}H NMR (200 MHz, $CDCl_3$): δ = 11.51 (s, 1H), 7.31 - 7.01 (m, 7H), 6.79 (t, J = 8.0 Hz, 2H), 4.91 (s, 2H), 3.57 (s, 2H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 178.6 (s, 1C), 156.7 (s, 1C), 137.0 (s, 1C), 131.2 (d, 1C), 128.9 (d, 1C), 128.6 (d, 2C), 127.9 (d, 1C), 127.1 (d, 2C), 122.9 (s, 1C), 121.0 (d, 1C), 112.0 (d, 1C), 70.1 (t, 1C), 36.2 (t, 1C)

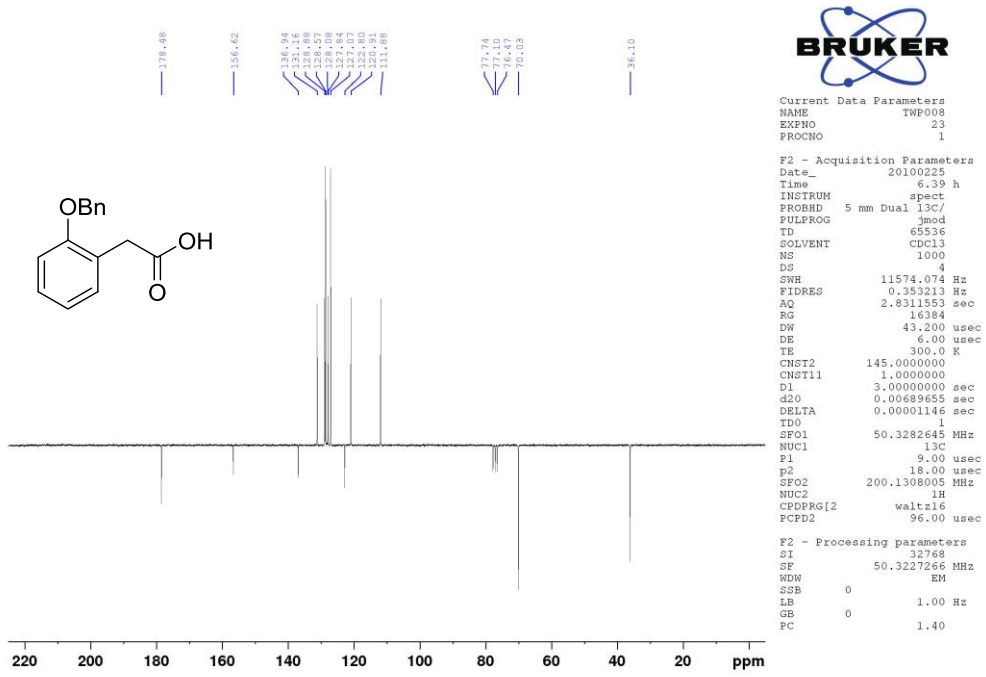
3) Copies of NMR spectra

a. (2-Benzyloxyphenyl)acetic acid (1)

^{1}H NMR (*d*-Chloroform, 200 MHz)

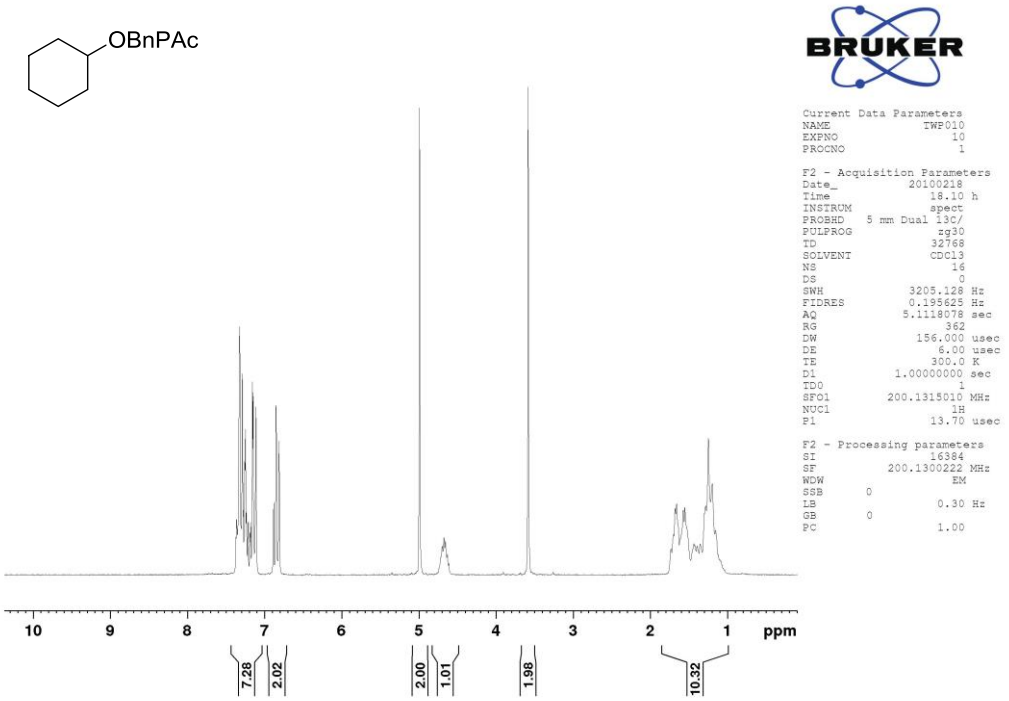


^{13}C NMR (*d*-Chloroform, 50 MHz)

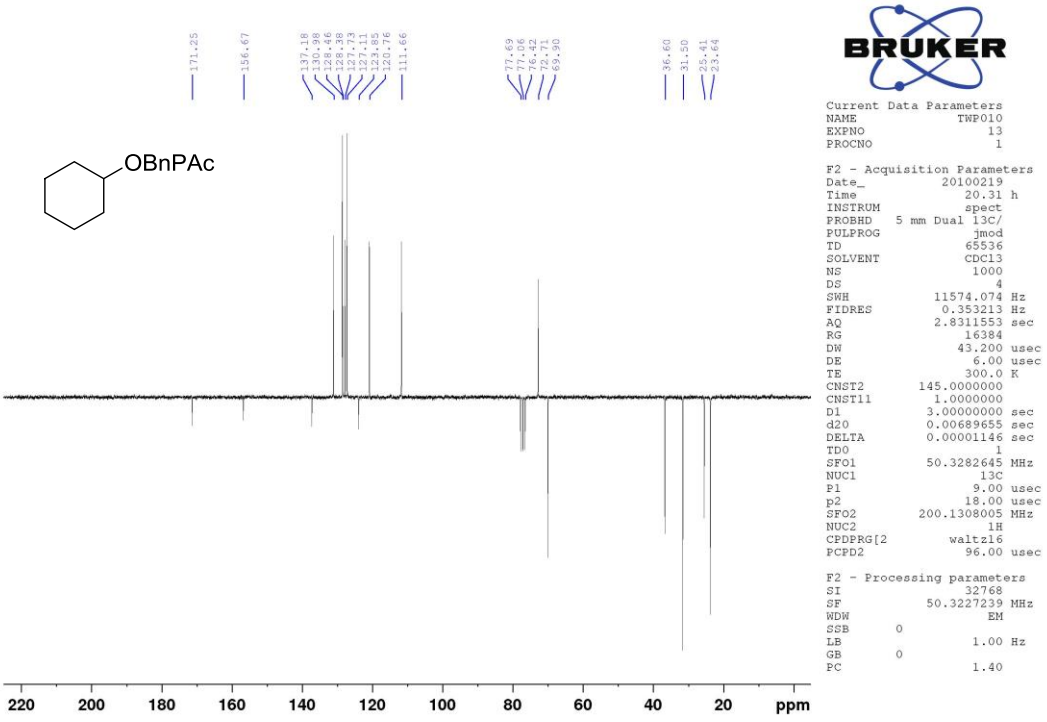


b. (2-Benzyloxyphenyl)acetic acid, cyclohexyl ester (7)

^{1}H NMR (*d*-Chloroform, 200 MHz)

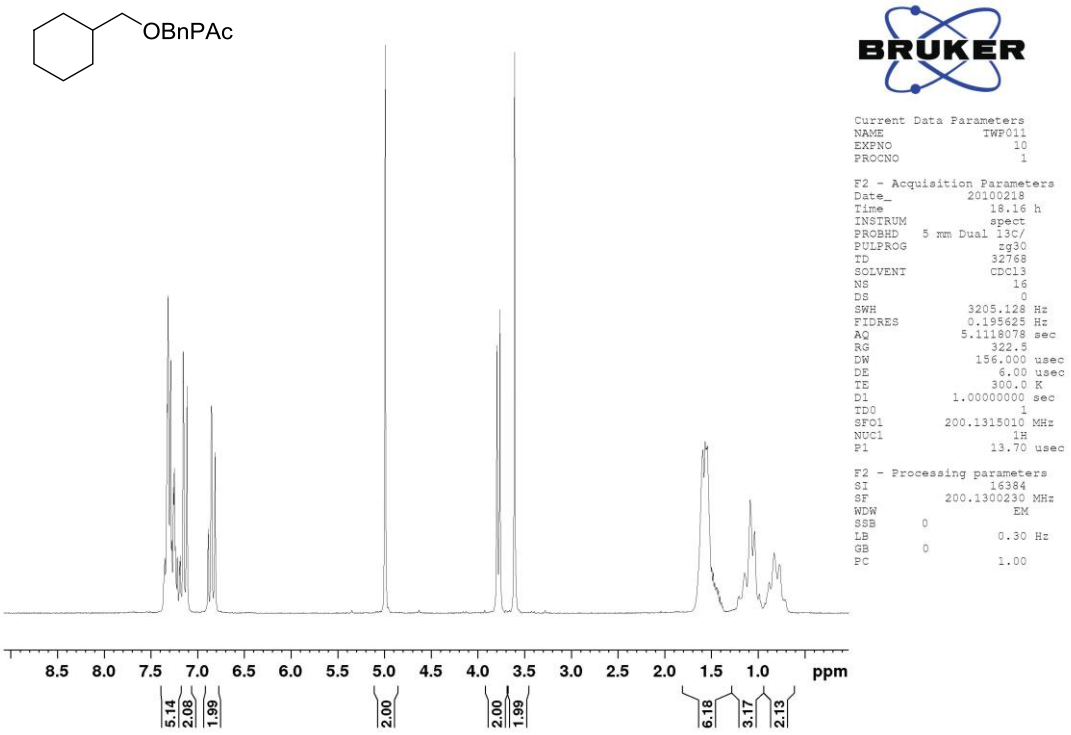


^{13}C NMR (*d*-Chloroform, 50 MHz)

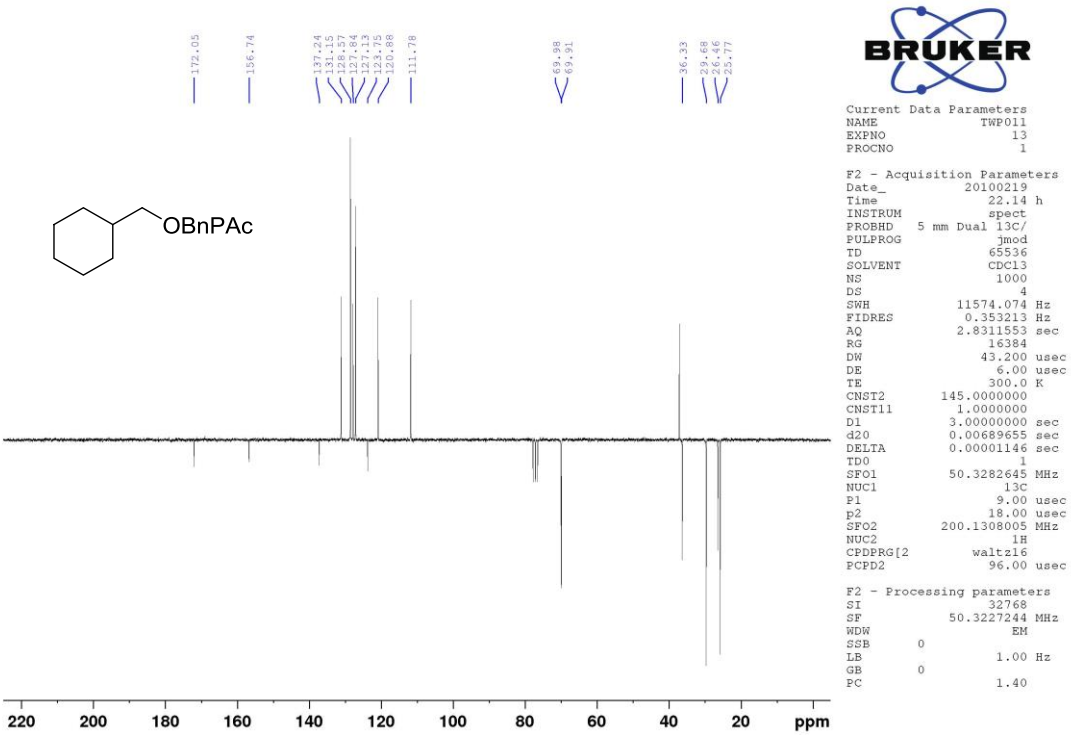


c. (2-Benzyloxyphenyl) acetic acid, cyclohexylmethyl ester (8)

^{1}H NMR (*d*-Chloroform, 200 MHz)

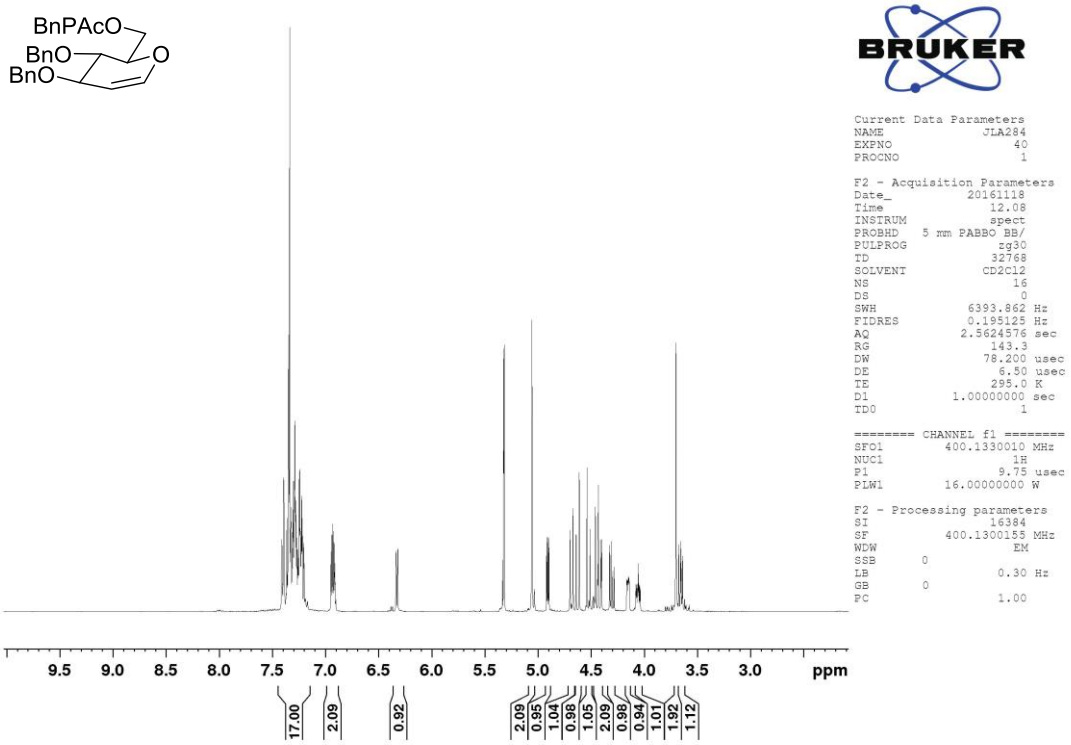


^{13}C NMR (*d*-Chloroform, 50 MHz)

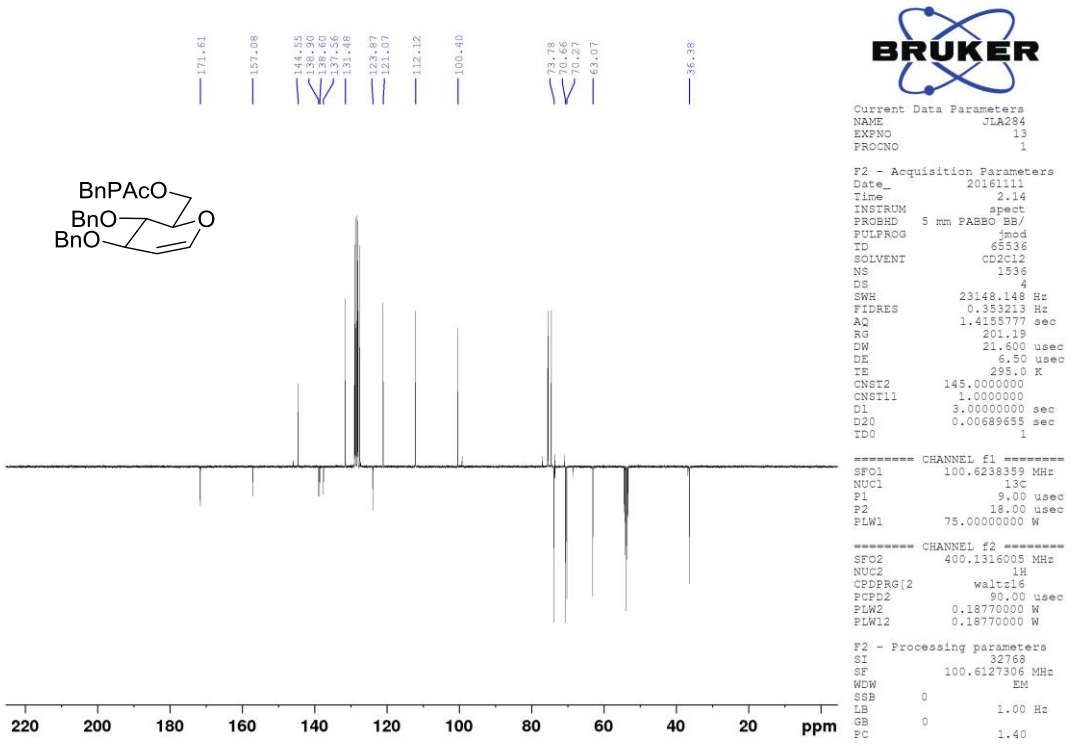


d. (2-Benzyloxyphenyl) acetic acid, 3,4-di-O-benzyl glucuronal ester (9)

^{1}H NMR (*d*-Dichloromethane, 400 MHz)

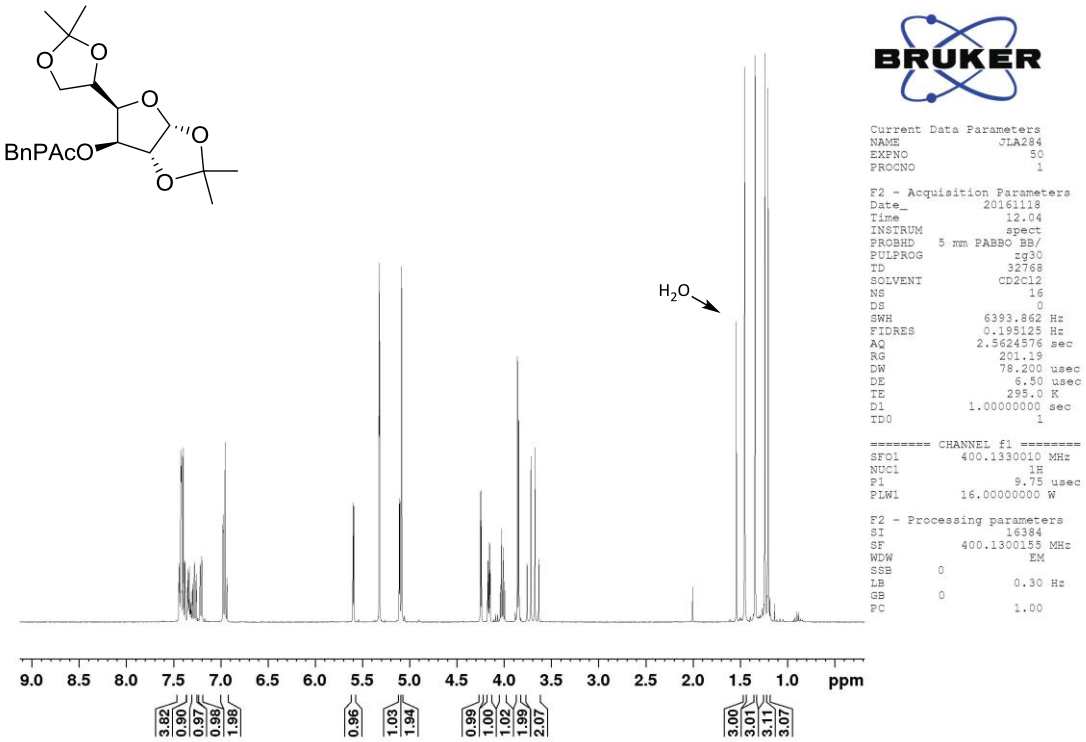


^{13}C NMR (*d*-Dichloromethane, 100 MHz)

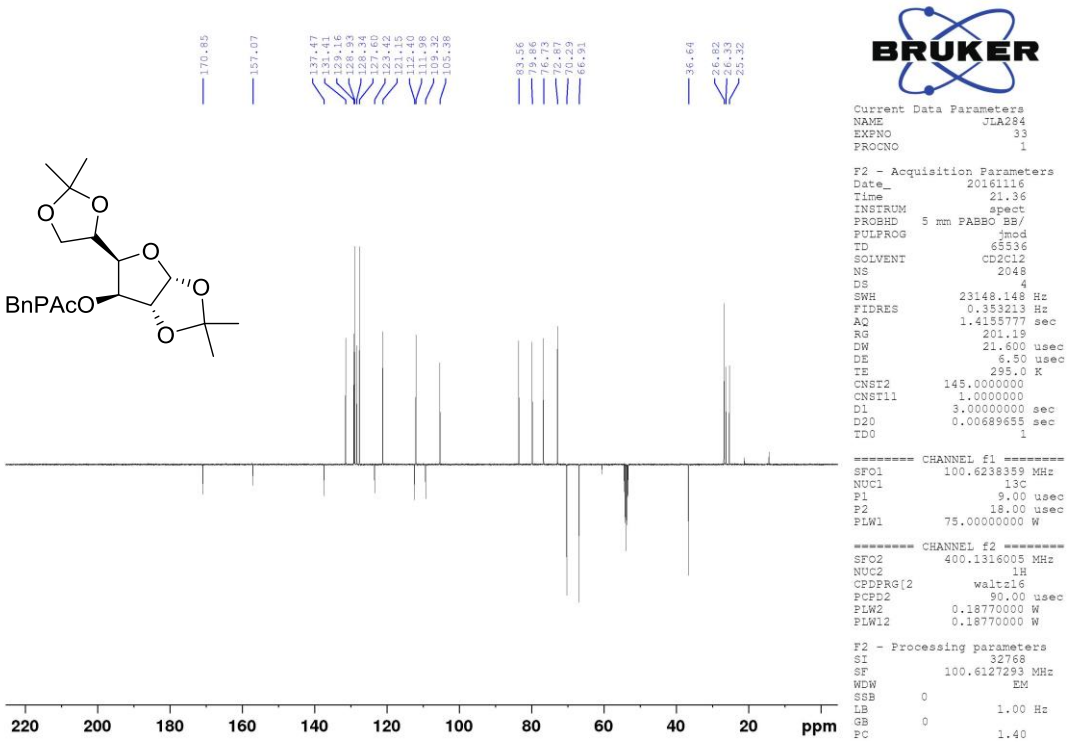


e. (2-Benzyloxyphenyl) acetic acid, 1,2,5,6-diisopropylidene glucosyl ester (10)

^{1}H NMR (*d*-Dichloromethane, 400 MHz)

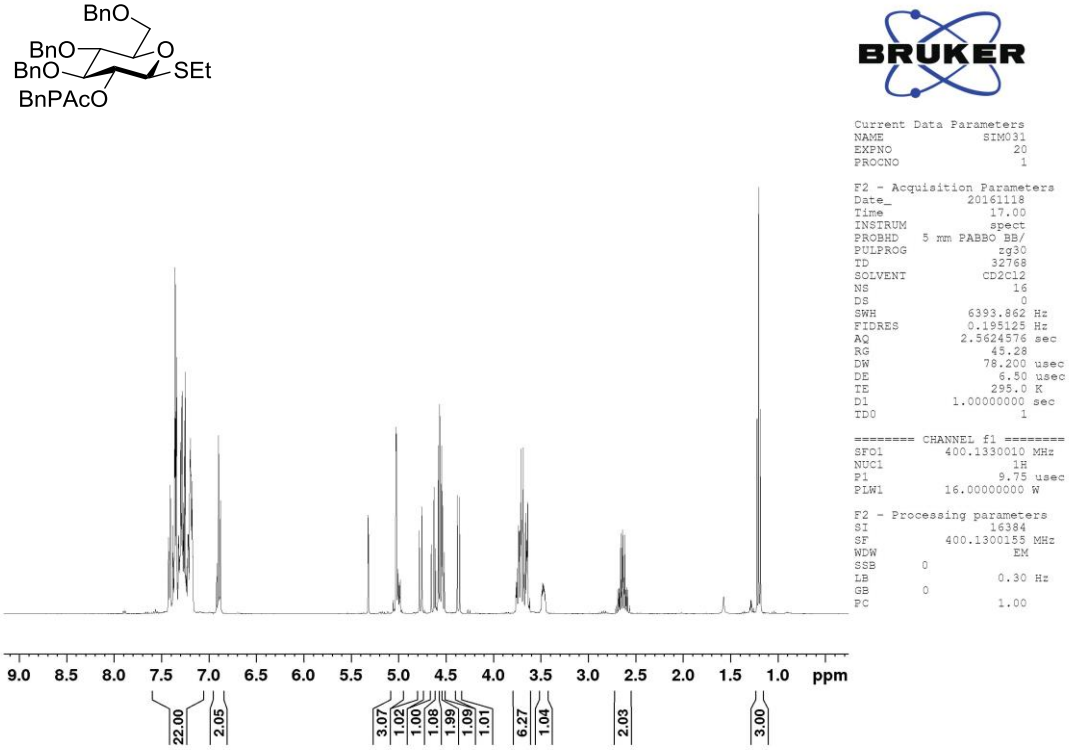


^{13}C NMR (*d*-Dichloromethane, 100 MHz)

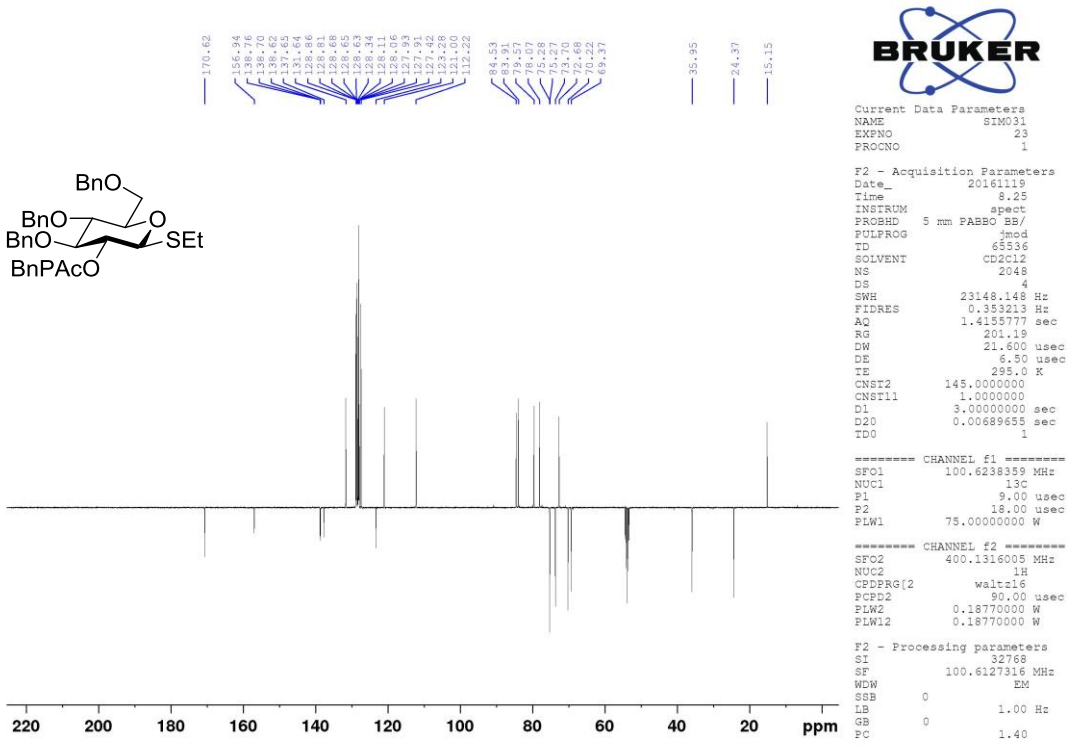


f. Ethyl 3,4,6-tri-O-benzyl-2-O-[(2-benzyloxyphenyl)acetyl]-1-thio-β,D-glucoside (11)

^{1}H NMR (*d*-Dichloromethane, 400 MHz)

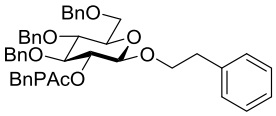


^{13}C NMR (*d*-Dichloromethane, 400 MHz)



g. 2-Phenylethyl 3,4,6-tri-O-benzyl-2-O-[(2-benzyloxyphenyl)acetyl]-β,D-glucoside (12)

^{1}H NMR (*d*-Dichloromethane, 400 MHz)



BRUKER

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PROCNO                     1

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PULPROG               zg30
TD                   32768
SOLVENT           CD2Cl2
NS                        16
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FIDRES               0.195125 Hz
AQ                 2.5624576 sec
RG                    201.19
DW                    78.200 usec
DE                     6.50 usec
TE                   298.0 K
D1                 1.00000000 sec
TD0                        1

======== CHANNEL f1 ========
SFO1           400.1330010 MHz
NUC1                      1H
P1                     9.75 usec
PLW1           16.00000000 W

F2 - Processing parameters
SI                     16384
SF              400.1300152 MHz
WDW                       EM
SSB          0
LB                     0.30 Hz
GB           0
PC                     1.00
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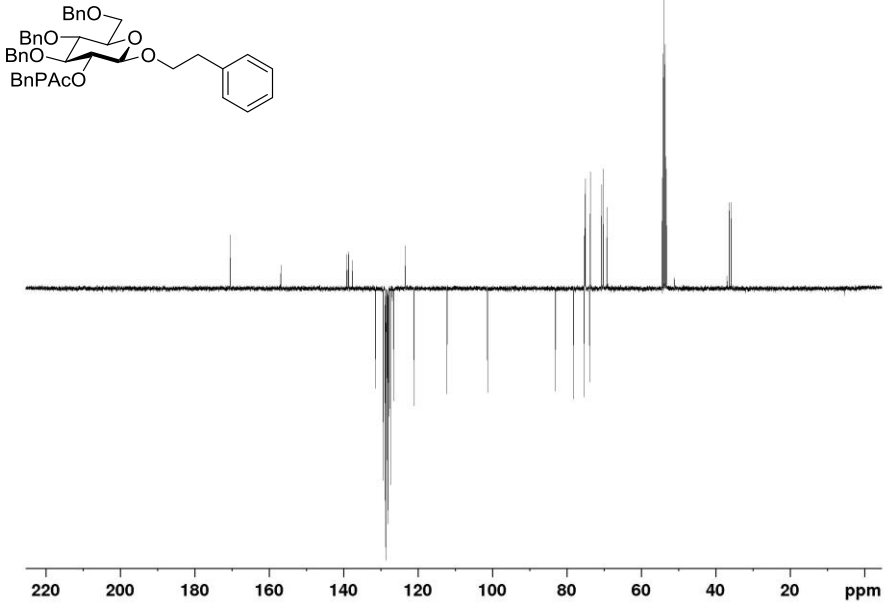
^{13}C NMR (*d*-Dichloromethane, 100 MHz)



BRUKER

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EXPNO                     13
PROCNO                     1

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PULPROG              jmod
TD                   65536
SOLVENT           CD2Cl2
NS                      2048
DS                         4
SWH               23148.148 Hz
FIDRES               0.353213 Hz
AQ                 1.4155777 sec
RG                    201.19
DW                    21.600 usec
DE                     6.50 usec
TE                   295.0 K
CNST2             145.0000000
CNST11              1.0000000
D1                 3.00000000 sec
D20                0.00689655 sec
TD0                        1

======== CHANNEL f1 ========
SFO1           100.6238359 MHz
NUC1                      13C
P1                     9.00 usec
P2                    18.00 usec
PLW1           75.00000000 W

======== CHANNEL f2 ========
SFO2           400.1316005 MHz
NUC2                      1H
CPDPRG[2             waltz16
PCPD2                 90.00 usec
PLW2           0.18770000 W
PLW12          0.18770000 W

F2 - Processing parameters
SI                     32768
SF              100.6127297 MHz
WDW                       EM
SSB          0
LB                     1.00 Hz
GB           0
PC                     1.40
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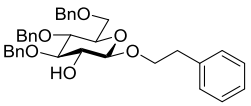
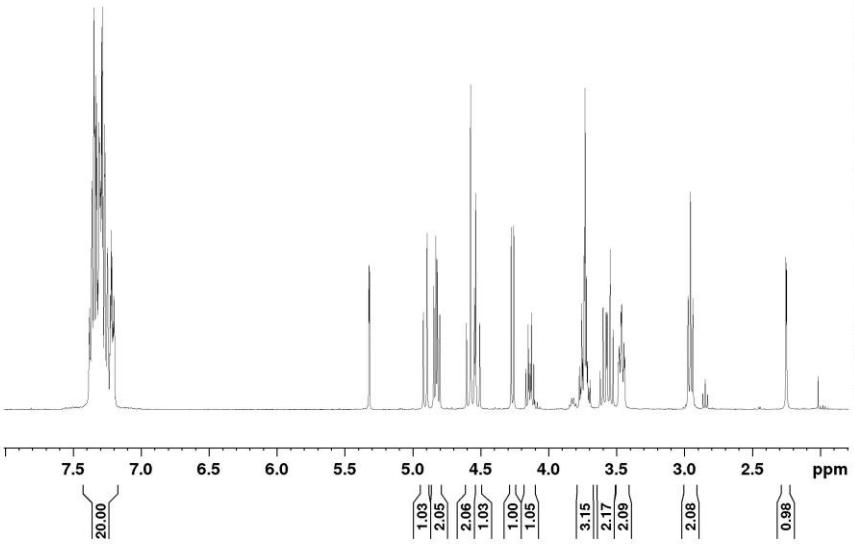
h. 2-Phenylethyl 3,4,6-tri-O-benzyl-β,D-glucoside (13)

^{1}H NMR (*d*-Dichloromethane, 400 MHz)



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EXPNO                     10
PROCNO                     1

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Date_              20161213
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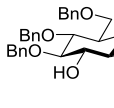
PULPROG                zg30
TD                    32768
SOLVENT            CD2Cl2
NS                        16
DS                         0
SWH                6393.862 Hz
FIDRES            0.195125 Hz
AQ                2.5624576 sec
RG                     79.6
DW                   78.200 usec
DE                    6.50 usec
TE                   295.0 K
D1                1.00000000 sec
TD0                        1

======== CHANNEL f1 ========
SFO1             400.1330010 MHz
NUC1                       1H
P1                       9.75 usec
PLW1            16.00000000 W

F2 - Processing parameters
SI                        16384
SF               400.1300154 MHz
WDW                       EM
SSB          0
LB                    0.30 Hz
GB           0
PC                     1.00
```

^{13}C NMR (*d*-Dichloromethane, 100 MHz)

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PROCNO                     1

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PULPROG               jmod
TD                    65536
SOLVENT            CD2Cl2
NS                      2048
DS                         4
SWH               23148.148 Hz
FIDRES            0.353213 Hz
AQ                1.4155777 sec
RG                    201.19
DW                   21.600 usec
DE                    6.50 usec
TE                   295.0 K
CNST2            145.0000000
CNST11             1.0000000
D1                3.00000000 sec
D20               0.00689655 sec
TD0                        1

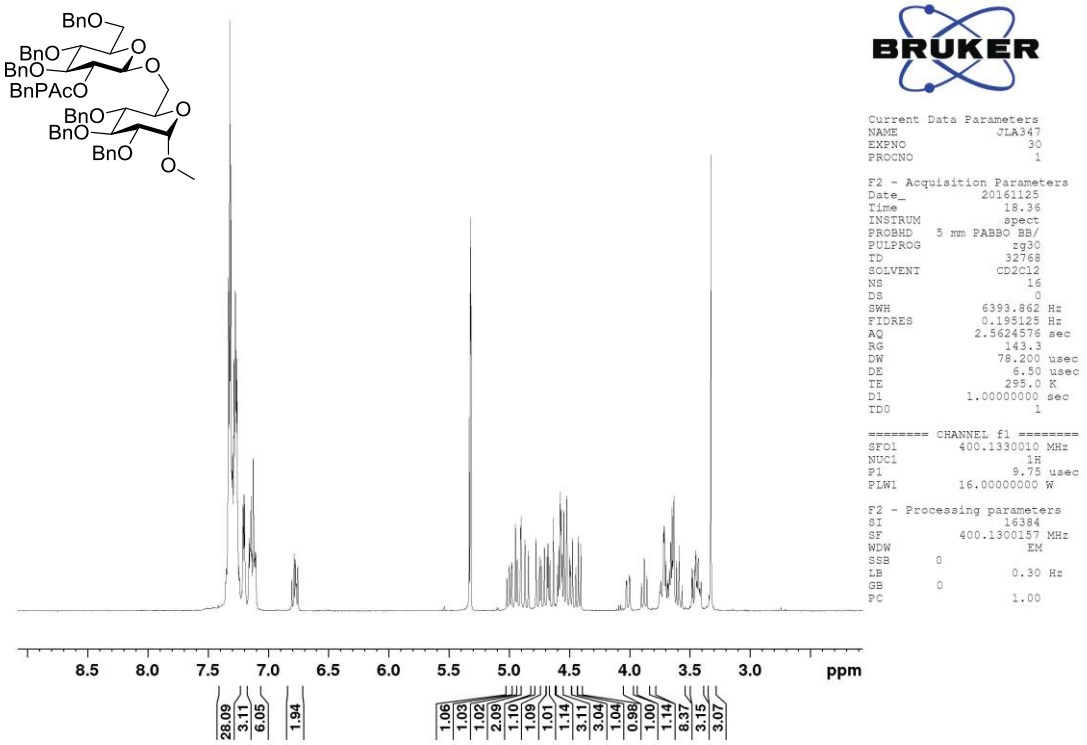
======== CHANNEL f1 ========
SFO1             100.6238359 MHz
NUC1                      13C
P1                       9.00 usec
P2                      18.00 usec
PLW1            75.00000000 W

======== CHANNEL f2 ========
SFO2             400.1316005 MHz
NUC2                       1H
CPDPRG[2              waltz16
PCPD2                   90.00 usec
PLW2             0.18770000 W
PLW12            0.18770000 W

F2 - Processing parameters
SI                        32768
SF               100.6127300 MHz
WDW                       EM
SSB          0
LB                    1.00 Hz
GB           0
PC                     1.40
```

i. Methyl 2,3,4,9,10,12-hexa-*O*-benzyl-8-*O*-[(2-benzyloxyphenyl)acetyl]-α,D-gentiobioside (15)

^{1}H NMR (*d*-Dichloromethane, 400 MHz)



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EXPNO                     30
PROCNO                     1

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PULPROG               zg30
TD                   32768
SOLVENT           CD2Cl2
NS                       16
DS                        0
SWH               6393.862 Hz
FIDRES            0.195125 Hz
AQ                2.5624576 sec
RG                    143.3
DW                   78.200 usec
DE                    6.50 usec
TE                  295.0 K
D1                1.00000000 sec
TD0                        1

======== CHANNEL f1 ========
SFO1            400.1330010 MHz
NUC1                      1H
P1                     9.75 usec
PLW1           16.00000000 W

F2 - Processing parameters
SI                       16384
SF              400.1300157 MHz
WDW                        EM
SSB          0
LB                     0.30 Hz
GB           0
PC                     1.00
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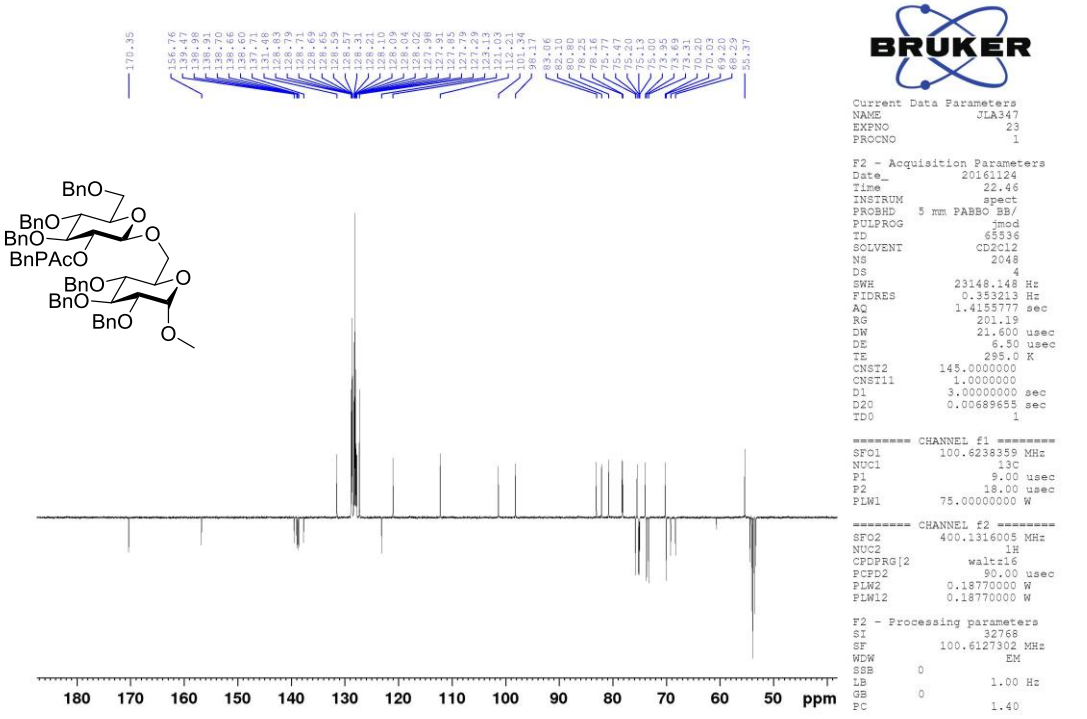
^{13}C NMR (*d*-Dichloromethane, 100 MHz)



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PULPROG               jmod
TD                   65536
SOLVENT           CD2Cl2
NS                     2048
DS                        4
SWH              23148.148 Hz
FIDRES            0.353213 Hz
AQ                1.4155777 sec
RG                   201.19
DW                   21.600 usec
DE                    6.50 usec
TE                  295.0 K
CNST2           145.0000000
CNST11            1.0000000
D1                3.00000000 sec
D20               0.00689655 sec
TD0                        1

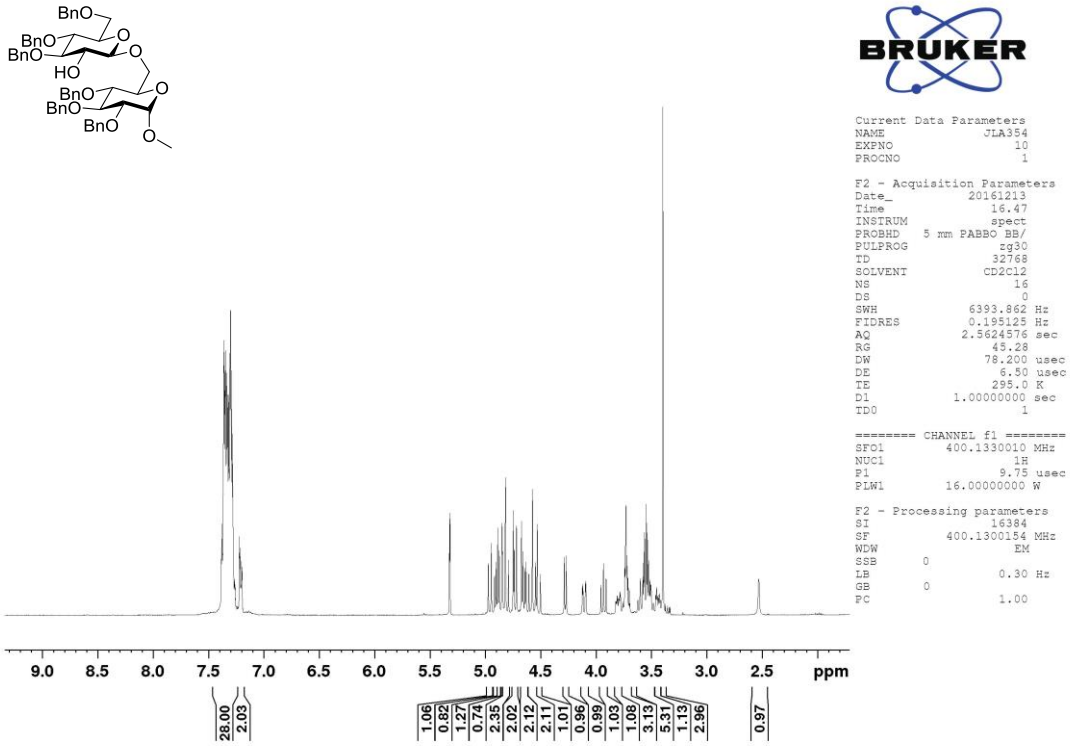
======== CHANNEL f1 ========
SFO1            100.6238359 MHz
NUC1                      13C
P1                     9.00 usec
P2                    18.00 usec
PLW1           75.00000000 W

======== CHANNEL f2 ========
SFO2            400.1316005 MHz
NUC2                      1H
CPDPRG[2              waltz16
PCPD2                 90.00 usec
PLW2           0.18770000 W
PLW12          0.18770000 W

F2 - Processing parameters
SI                       32768
SF              100.6127302 MHz
WDW                        EM
SSB          0
LB                     1.00 Hz
GB           0
PC                     1.40
```

j. Methyl 2,3,4,9,10,12-hexa-*O*-benzyl-α,D-gentiobioside (16)

^{1}H NMR (*d*-Dichloromethane, 400 MHz)



BRUKER

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NAME                  JLA354
EXPNO                     10
PROCNO                     1

F2 - Acquisition Parameters
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Time                  16.47
INSTRUM               spect
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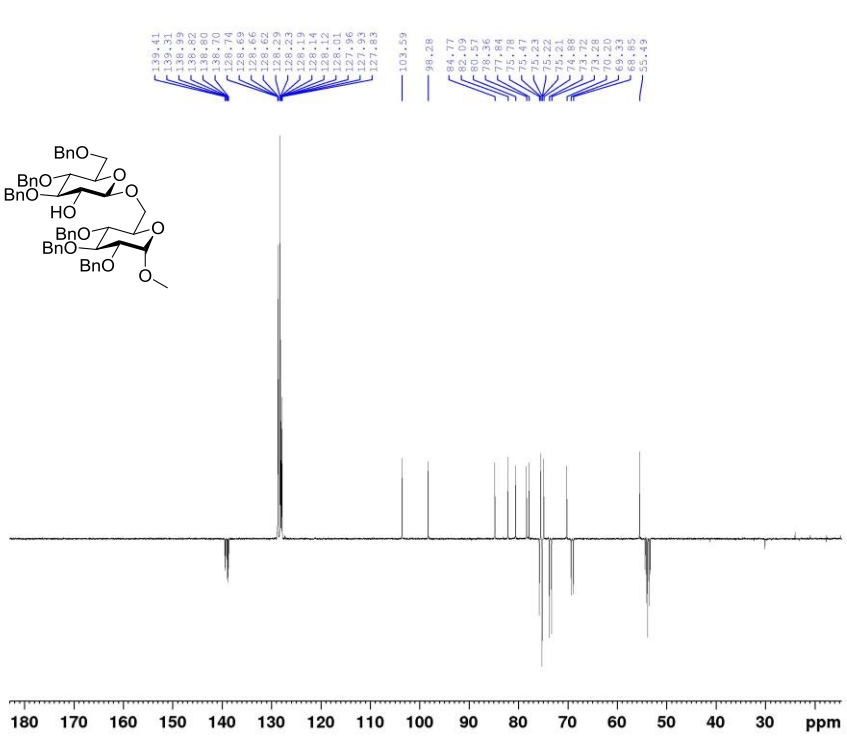
PULPROG                zg30
TD                    32768
SOLVENT            CD2Cl2
NS                        16
DS                         0
SWH                6393.862 Hz
FIDRES            0.195125 Hz
AQ                2.5624576 sec
RG                    45.28
DW                  78.200 usec
DE                    6.50 usec
TE                   295.0 K
D1                1.00000000 sec
TD0                        1

======== CHANNEL f1 ========
SFO1             400.1330010 MHz
NUC1                       1H
P1                      9.75 usec
PLW1            16.00000000 W

F2 - Processing parameters
SI                        16384
SF               400.1300154 MHz
WDW                       EM
SSB                        0
LB                      0.30 Hz
GB                         0
PC                      1.00
```

^{13}C NMR (*d*-Dichloromethane, 100 MHz)



BRUKER

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PROCNO                     1

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PULPROG               zgmod
TD                    65536
SOLVENT            CD2Cl2
NS                      2048
DS                         4
SWH               23148.148 Hz
FIDRES            0.353213 Hz
AQ                1.4155777 sec
RG                    201.18
DW                  21.600 usec
DE                    6.50 usec
TE                   295.0 K
CNST2            145.0000000
CNST11             1.0000000
D1                3.00000000 sec
D20               0.00689655 sec
TD0                        1

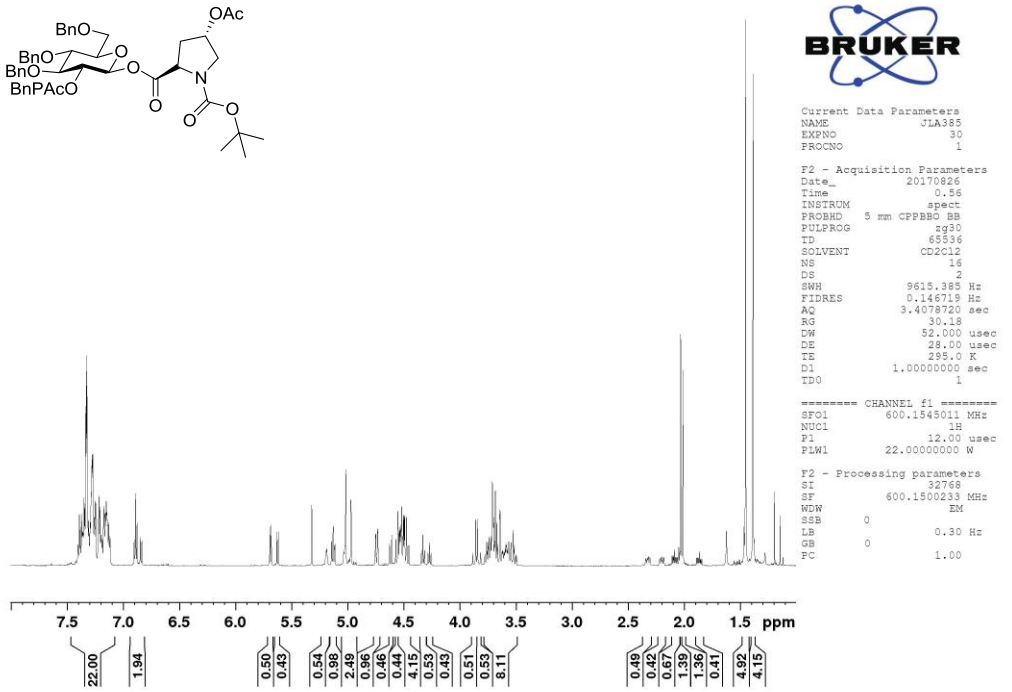
======== CHANNEL f1 ========
SFO1             100.6238359 MHz
NUC1                      13C
P1                      9.00 usec
P2                     18.00 usec
PLW1            75.00000000 W

======== CHANNEL f2 ========
SFO2             400.1316005 MHz
NUC2                       1H
CPDPRG[2              waltz16
PCPD2                  90.00 usec
PLW2             0.18770000 W
PLW12            0.18770000 W

F2 - Processing parameters
SI                        32768
SF               100.6127304 MHz
WDW                       EM
SSB                        0
LB                      1.00 Hz
GB                         0
PC                      1.40
```

k. *trans*-N-(tert-butoxycarbonyl)-4-acetoxy-L-proline, 3,4,6-tri-*O*-benzyl-2-*O*-[(2-benzyloxyphenyl) acetyl]-β,D-glucosyl ester (18)

^{1}H NMR (*d*-Dichloromethane, 600 MHz)



BRUKER

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NAME                  JLA385
EXPNO                     30
PROCNO                     1

F2 - Acquisition Parameters
Date_              20170826
Time                   0.56
INSTRUM               spect
PROBHD   5 mm CPPBBO BB
PULPROG                zg30
TD                    65536
SOLVENT            CD2Cl2
NS                        16
DS                         2
SWH                9615.385 Hz
FIDRES             0.146719 Hz
AQ                 3.4078720 sec
RG                     30.18
DW                    52.000 usec
DE                    28.00 usec
TE                    295.0 K
D1                 1.00000000 sec
TD0                        1

======== CHANNEL f1 ========
SFO1           600.1545011 MHz
NUC1                      1H
P1                    12.00 usec
PLW1           22.00000000 W

F2 - Processing parameters
SI                     32768
SF             600.1500233 MHz
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SSB                    0
LB                     0.30 Hz
GB                     0
PC                     1.00
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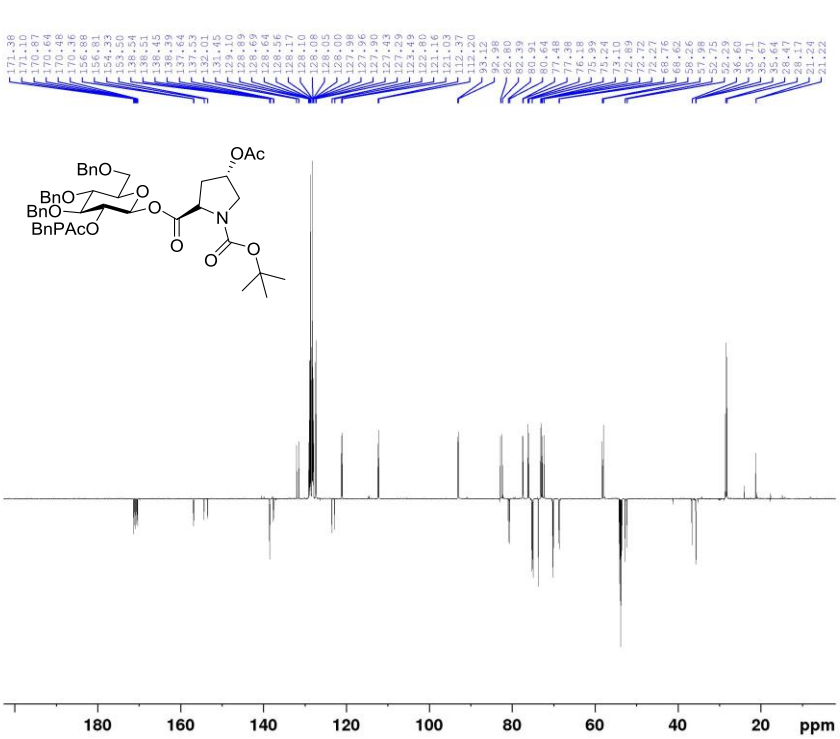
^{13}C NMR (*d*-dichloromethane, 150 MHz)



BRUKER

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EXPNO                     33
PROCNO                     1

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Time                   5.29
INSTRUM               spect
PROBHD   5 mm CPPBBO BB
PULPROG             zgpg30
TD                    65536
SOLVENT            CD2Cl2
NS                      4096
DS                         4
SWH               36057.691 Hz
FIDRES             0.550197 Hz
AQ                 0.9087659 sec
RG                    194.73
DW                    13.867 usec
DE                    18.00 usec
TE                    295.0 K
CNST2             145.0000000
CNST11              1.0000000
D1                 3.00000000 sec
D20                0.00689655 sec
TD0                        1

======== CHANNEL f1 ========
SFO1           150.9236829 MHz
NUC1                      13C
P1                    10.00 usec
P2                    20.00 usec
PLW1           58.00000000 W

======== CHANNEL f2 ========
SFO2           600.1524006 MHz
NUC2                      1H
CPDPRG[2             waltz16
PCPD2                 70.00 usec
PLW2           22.00000000 W
PLW12           0.64652997 W

F2 - Processing parameters
SI                     32768
SF             150.9077810 MHz
WDW                       EM
SSB                    0
LB                     1.00 Hz
GB                     0
PC                     1.40
```

Supporting Information

Manuscript #3

Supplementary Data

(2-Nitrophenyl)acetyl-Protected Glucosyl Trichloroacetimidates as Novel Glucosyl Donors for the Synthesis of Glycosylated Natural Products

Julia Weber, Markus Schwarz, Andrea Schiefer, Christian Hametner, Alois Schiessl, Georg Häubl, Johannes Fröhlich, Hannes Mikula*

* hannes.mikula@tuwien.ac.at, Institute of Applied Synthetic Chemistry, Vienna University of Technology

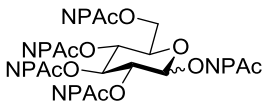
Content

1) General remarks

All reactions were performed under an argon atmosphere. Anhydrous solvents like dichloromethane, tetrahydrofuran, methanol and diethyl ether were dried using a PURESOLV facility of it-innovative technology. Molecular sieve 3 Å was activated under vacuum at 200 °C before use. The progress of the reactions was monitored by thin layer chromatography (TLC) over silica gel 60 F_{254}. Visualization of the spots was achieved either by UV irradiation (254 or 366 nm) or by heat staining with ceric ammonium molybdate in ethanol/sulfuric acid. LC-ESI-MS/MS was performed on an HCT ion trap mass spectrometer (Bruker, Germany) in full scan mode. Chromatographic separation was done on a 1200 series HPLC system (Agilent Technologies, Germany) using a Luna RP-C18 column (3.0 x 150 mm, 3 µm particle size, Phenomenex, Germany) and application of pure substances was achieved using a TLC-MS interface (Camag, Germany). Preparative column chromatography was performed on silica gel 60 (Merck, 40-63 µm) using a Büchi Sepacore™ Flash System or a Grace Reveleris Prep Purification System. Preparative HPLC separation was done on a Grace Reveleris Prep system using a Luna Prep C18(2), 10 µm, 250x10 mm column (Phenomenex). NMR spectra were recorded on a Bruker Avance IIIHD 600Mhz spectrometer equipped with a Prodigy BBO cryo probe or on a Bruker Avance DRX-400 MHz spectrometer at 20°C. Data were recorded and evaluated using TOPSPIN 3.5 (Bruker Biospin). All chemical shifts are given in ppm relative to tetramethylsilane. The calibration was done using residual solvent signals. Multiplicities are abbreviated as s (singlet), d (doublet), t (triplet), q (quartet), b (broad signal). T2-toxin was obtained from Romer Labs (Tulln) and all other chemicals were purchased from ABCR (Germany) or Sigma-Aldrich (Austria/Germany). HR-MS analysis was carried out from acetonitrile solutions (concentration: 10 ppm) by using an HTC PAL system autosampler (CTC Analytics AG, Zwingen, Switzerland), an Agilent 1100/1200 HPLC with binary pumps, degasser and column thermostat (Agilent Technologies, Waldbronn, Germany) and Agilent 6230 AJS ESI–TOF mass spectrometer (Agilent Technologies, Palo Alto, United States).

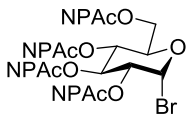
2) Experimental Procedures

1,2,3,4,6-Penta-*O*-(2-nitrophenyl)acetyl-β,D-glucopyranoside (3)



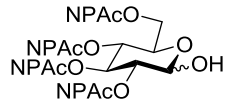
To a solution of glucose (1.0 g, 5.6 mmol, 1 eq.) and 2-nitrophenylacetic acid (6.1 g, 33.6 mmol, 6 eq.) in dry dichloromethane (50 mL) (4-dimethylamino)pyridine (1.1 g, 5.6 mmol, 1 eq.) followed by EDCI (6.4 g, 5.6 mmol, 6 eq.) was added. The reaction mixture was stirred at room temperature for 16 hours, washed with 1N HCl (2x50 mL) and saturated $NaHCO_3$-solution (2x50 mL), dried over Na_2SO_4, filtered and concentrated. The crude product was purified by flash chromatography (DCM:EtOAc, gradient elution 100:1 to 20:1) to obtain the title compound as a white foam (3.0 g, 53 %). Judged by NMR, a 4:1 (α:β) mixture of anomers was obtained; ^{1}H NMR (400 MHz, $CDCl_3$): **α-(4)**: δ 8.20-8.11 (m, 4H), 8.09 (dd, J = 8.2, 1.2 Hz, 1H), 7.70-7.57 (m, 5H), 7.56-7.46 (m, 8H), 7.43 (td, J = 7.8, 1.3, 1H), 7.34 (dd, J = 7.6, 1.3 Hz, 1H), 6.37 (d, J = 3.5 Hz, 1H), 5.59 (t, J = 9.9 Hz, 1H), 5.14 (t, J = 9.9 Hz, 1H), 5.08 (dd, J = 10.1, 3.9 Hz, 1H), 4.39 (dd, J = 12.3, 1.4 Hz, 1H), 4.32-4.09 (m, 9H), 4.08-3.93 (m, 3H); **β-(4)**: δ 8.20-8.11 (m, 4H), 8.09 (dd, J = 8.2, 1.2 Hz, 1H), 7.70-7.57 (m, 5H), 7.56-7.46 (m, 8H), 7.43 (td, J = 7.8, 1.3, 1H), 7.34 (dd, J = 7.6, 1.3 Hz, 1H), 5.79 (d, J = 8.2 Hz, 1H), 5.43 (t, J = 9.7 Hz, 1H), 5.22 (dd, J = 9.7, 8.2 Hz, 1H), 5.14 (t, J = 9.7 Hz, 1H), 4.36 (dd, J = 12.5, 4.6 Hz, 1H), 4.32-4.09 (m, 9H), 4.08-3.93 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): **α-(4)**: δ 169.81 (s, 2C), 169.62 (s, 1C), 169.29 (s, 1C), 168.50 (s, 1C), 148.75 (s, 1C), 148.57 (s, 1C), 148.56 (s, 1C), 148.52 (s, 1C), 148.49 (s, 1C), 134.42 (d, 1C), 134.32 (d, 1C), 134.25 (d, 1C), 134.15 (d, 1C), 134.12 (d, 3C), 133.91 (d, 1C), 133.76 (d, 1C), 133.73 (d, 1C), 130.11 (s, 1C), 129.88 (s, 1C), 129.80 (s, 1C), 129.59 (s, 1C), 129.52 (s, 1C), 129.07 (d, 1C), 128.89 (d, 1C), 128.87 (d, 1C), 128.77 (d, 1C), 128.65 (d, 1C), 125.54 (d, 1C), 125.35 (d, 2C), 125.31 (d, 1C), 125.25 (d, 1C), 89.29 (d, 1C), 70.18 (d, 1C), 70.10 (d, 1C), 69.84 (d, 1C), 68.11 (d, 1C), 62.48 (t, 1C), 39.84 (t, 1C), 39.74 (t, 1C), 39.69 (t, 1C), 39.55 (t, 1C), 39.50 (t, 1C); **β-(4)**: δ 169.69 (s, 1C), 169.66 (s, 1C), 169.24 (s, 1C), 169.18 (s, 1C), 168.86 (s, 1C), 148.56 (s, 1C), 148.52 (s, 1C), 148.49 (s, 1C), 148.46 (s, 1C), 148.42 (s, 1C), 134.56 (d, 1C), 134.45 (d, 1C), 134.25 (d, 1C), 134.15 (d, 1C), 134.12 (d, 3C), 133.82 (d, 1C), 133.76 (d, 1C), 133.73 (d, 1C), 130.06 (s, 1C), 129.88 (s, 1C), 129.83 (s, 1C), 129.55 (s, 1C), 129.46 (s, 1C), 129.00 (d, 1C), 128.89 (d, 1C), 128.87 (d, 1C), 128.80 (d, 1C), 128.61 (d, 1C), 125.42 (d, 1C), 125.35 (d, 2C), 125.31 (d, 1C), 125.25 (d, 1C), 91.97 (d, 1C), 72.86 (d, 1C), 72.37 (d, 1C), 70.51 (d, 1C), 68.32 (d, 1C), 62.15 (t, 1C), 39.62 (t, 1C), 39.55 (t, 1C), 33.91 (t, 1C), 25.67 (t, 1C), 24.99 (t, 1C); HRMS calcd for $C_{46}H_{37}N_5NaO_{21}^+$ $[M+Na]^+$ 1018.1873, found 1018.1875.

1-Bromo-1-deoxy-2,3,4,6-tetra-*O*-(2-nitrophenyl)acetyl-α,D-glucopyranose (4)



To a solution of compound **3** (4.0 g, 4.02 mmol, 1 eq.) in dry dichloromethane (3 mL) hydrogen bromide in acetic acid (30 % (wt), 5 mL)) was added at 0 °C. The reaction was warmed to room temperature and stirred for 4 h. The reaction mixture was quenched by the addition of ice water (30 mL), diluted with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$ solution. The organic layer was dried over Na_2SO_4 and the solvents were removed under reduced pressure to give the desired product **4** as a yellow foam (2.9 g, 81%); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.19-8.14 (m, 3H), 8.09 (dd, J = 8.2, 1.2 Hz, 1H), 7.67-7.57 (m, 4H), 7.53-7.47 (m, 5H), 7.46-7.42 (m, 2H), 7.31 (dd, J = 7.7, 0.9 Hz, 1H), 6.51 (d, J = 4.1 Hz, 1H), 5.66 (t, J = 9.7 Hz, 1H), 5.21 (t, J = 9.8 Hz, 1H), 4.96 (dd, J = 9.8, 3.9 Hz, 1H), 4.38 dd (J = 13.1, 4.2 Hz, 1H), 4.33-4.29 (m, 2H), 4.27 (d, J = 17.0 Hz, 1H), 4.22-4.14 (m, 3H), 4.08 (d, J = 17.0 Hz, 1H), 4.03 (d, J = 17.0 Hz, 1H), 3.97 (d, J = 5.3 Hz, 1H), 3.95 (d, J = 5.6 Hz, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 146.35 (s, 1C), 146.20 (s, 1C), 146.08 (s, 1C), 146.82 (s, 1C), 125.40 (s, 1C), 125.15 (s, 3C), 111.07 (d, 1C), 110.88 (d, 2C), 110.84 (d, 2C), 110.80 (d, 1C), 110.39 (d, 1C), 110.38 (d, 1C), 106.73 (s, 1C), 106.28 (s, 1C), 106.17 (s, 1C), 106.13 (s, 1C), 105.72 (d, 1C), 105.64 (d, 1C), 105.54 (d, 1C), 105.40 (d, 1C), 102.16 (d, 1C), 102.07 (d, 1C), 102.03 (d, 1C), 102.02 (d, 1C), 63.26 (d, 1C), 49.02 (d, 2C), 47.56 (d, 1C), 46.99 (d, 1C), 38.28 (t, 1C), 16.45 (t, 3C), 16.24 (t, 1C); ESI-MS calcd for $C_{38}H_{32}BrN_4O_{17}^+$ $[M+H]^+$ 895.1, found 895.1.

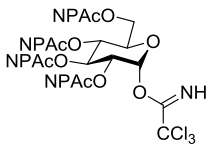
2,3,4,6-Tetra-*O*-(2-nitrophenyl)acetyl-D-glucopyranose (5)



To a solution of compound **4** (1.45 g, 1.67 mmol, 1 eq.) in acetone (9 mL) water (30 µl) and Ag_2CO_3 (0.52 g, 1.84 mmol, 1.1 eq.) was added. After stirring at room temperature for 20 h with exclusion of UV-light, the reaction mixture was filtered over celite and the filtrate was concentrated under reduced pressure. For purification the residue was dissolved in DCM and filtered over a short pad of silica gel (hexanes:EtOAc 1:1) to obtain a 4:1 (α:β) mixture of anomers of **5** (1.1 g, 79 %) in good yields.; ^{1}H NMR (400 MHz, $CDCl_3$): **α-(6)**: δ 8.21-8.08 (m, 4H), 7.68-7.54 (m, 5H), 7.53-7.41 (m, 6H), 7.36-7.30 (m, 1H), 5.65 (t, J = 9.9 Hz, 1H), 5.43 (d, J = 3.5 Hz, 1H), 5.03 (t, J = 9.8 Hz, 1H), 4.94 (dd, J = 10.1, 3.5 Hz, 1H), 4.35-4.26 (m, 2H), 4.25-4.18 (m, 2H), 4.18-4.11 (m, 3H), 4.10-4.00 (m, 3H), 3.95 (d, J = 17.6 Hz, 1H), 3.37 (bs, 1H); **β-(6)**: δ 8.21-8.08 (m, 4H), 7.68-7.54 (m, 5H), 7.53-7.41 (m, 6H), 7.36-7.30 (m, 1H), 5.39 (t, J = 9.9 Hz, 1H), 5.08 (t, J = 9.9 Hz, 1H), 4.97 (dd, J = 9.8, 8.2 Hz, 1H), 4.77 (d, J = 7.8 Hz, 1H), 4.35-4.26 (m, 2H), 4.25-4.18 (m, 2H), 4.18-4.11 (m, 3H), 4.10-4.00 (m, 3H), 3.95 (d, J = 17.6 Hz, 1H), 3.37 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): **α-(6)**: δ 169.88 (s, 1C), 169.67 (s, 1C), 169.57 (s, 1C), 169.43 (s, 1C), 148.52 (s, 4C), 134.37 (d, 1C), 134.18 (d, 1C), 134.10 (d, 4C), 133.95 (d, 1C), 133.78 (d, 1C), 130.23 (s, 1C), 129.98 (s, 2C), 129.67 (s, 1C), 128.89 (d, 1C), 128.86 (d, 1C), 128.75 (d, 2C), 125.46 (d, 1C), 125.44 (d, 1C),

125.35 (d, 1C), 125.28 (d, 1C), 90.14 (d, 1C), 71.78 (d, 1C), 70.07 (d, 1C), 69.05 (d, 1C), 67.24 (d, 1C), 62.91 (t, 1C), 39.95 (t, 1C), 39.82 (t, 2C), 39.77 (t, 1C); **β-(6)**: 170.09 (s, 1C), 169.80 (s, 1C), 169.57 (s, 1C), 169.35 (s, 1C), 148.61 (s, 1C), 148.52 (s, 2C), 148.49 (s, 1C), 134.47 (d, 1C), 134.23 (d, 1C), 134.10 (d, 4C), 133.95 (d, 1C), 133.78 (d, 1C), 130.12 (s, 1C), 129.92 (s, 1C), 129.72 (s, 1C), 129.63 (s, 1C), 128.94 (d, 1C), 128.86 (d, 1C), 128.79 (d, 1C), 128.75 (d, 1C), 125.46 (d, 1C), 125.44 (d, 1C), 125.31 (d, 1C), 125.28 (d, 1C), 95.49 (d, 1C), 73.77 (d, 1C), 72.21 (d, 1C), 72.19 (d, 1C), 68.87 (d, 1C), 62.59 (t, 1C), 39.90 (t, 1C), 39.82 (t, 1C), 39.71 (t, 1C), 39.64 (t, 1C); HRMS calcd for $C_{38}H_{32}N_4NaO_{18}^+$ $[M+Na]^+$ 855.1603, found 855.1604.

2,3,4,6-Tetra-*O*-(2-nitrophenyl)acetyl-α,D-glucopyranosyl trichloroacetimidate (6)

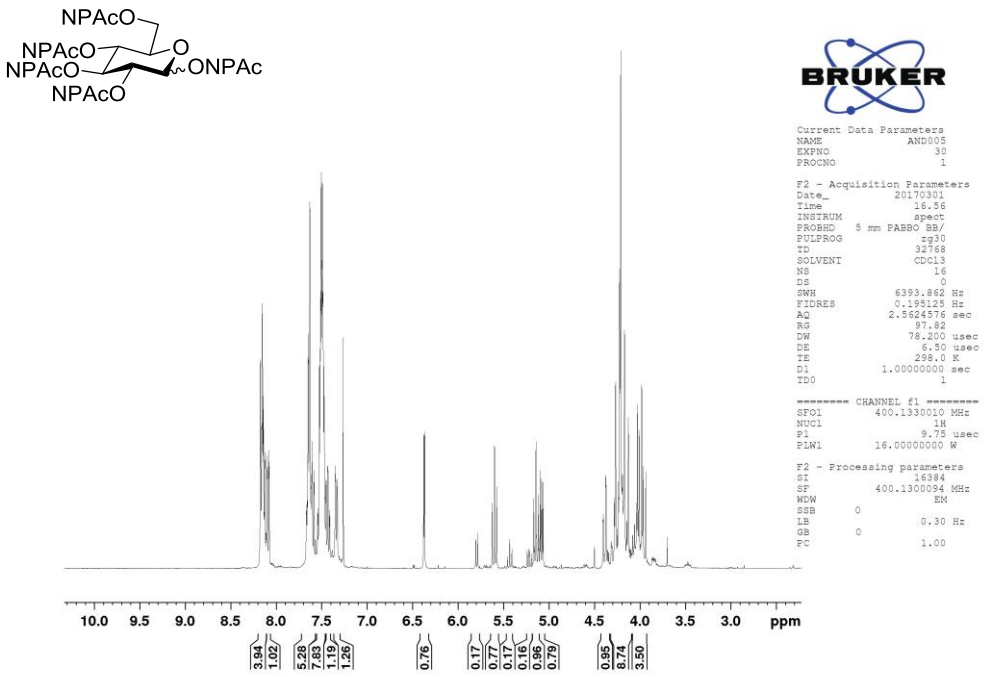


To a solution of compound **5** (1.2 g, 1.4 mmol, 1 eq.) in dry CH_2Cl_2, trichloroacetonitrile (43 µl, 4.3 mmol, 3 eq.) was slowly added at 0 °C, followed by DBU (21 µl, 0.14 mmol, 0.1 eq.). The reaction mixture was slowly warmed to room temperature and stirring was continued for 16 h. The solution was concentrated under vacuum and the crude product was purified by flash chromatography (gradient elution hexanes:EE) to obtain the title compound as a lightly yellow foam (1.07 g, 76 %); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.70 (s, 1H), 8.17 (dd, J = 8.3, 1.1 Hz, 1H), 8.15 (dd, J = 8.0, 1.2 Hz, 1H), 8.12 (dd, J = 8.2, 1.2 Hz, 1H), 8.01 (dd, J = 8.2, 1.3 Hz, 1H), 7.69-7.56 (m, 4H), 7.54-7.41 (m, 7H), 7.31 (dd, J = 7.6, 1.2, 1H), 6.52 (d, J = 3.8 Hz, 1H), 5.71 (t, J = 9.8 Hz, 1H), 5.25 (t, J = 9.9 Hz, 1H), 5.22 (dd, J = 10.1, 3.7 Hz, 1H), 4.35 (dd, J = 12.6, 3.8 Hz, 1H), 4.31 (dd, J = 12.6, 1.7 Hz, 1H), 4.24 (ddd; J = 10.5, 3.9, 2.1, 1H), 4.22 (d, J = 6.5 Hz, 1H), 4.20-4.14 (m, 3H), 4.09 (d, J = 16.9 Hz, 1H), 3.99 (d, J = 14.9 Hz, 1H), 3.96 (d, J = 14.1 Hz, 1H), 3.91 (d, J = 17.3 Hz, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.76 (s, 1C), 169.69 (s, 1C), 169.50 (s, 1C), 168.24 (s, 1C), 160.61 (s, 1C), 148.76 (s, 1C), 148.56 (s, 1C), 148.48 (s, 1C), 148.44 (s, 1C), 143.43 (d, 1C), 134.21 (d, 2C), 134.10 (d, 1C), 134.08 (d, 1C), 133.69 (d, 2C), 133.65 (d, 1C), 130.14 (s, 1C), 129.64 (s, 1C), 129.57 (s, 1C), 129.47 (s, 1C), 128.97 (d, 1C), 128.90 (d, 1C), 128.83 (d, 1C), 128.66 (d, 1C), 125.44 (d, 1C), 125.35 (d, 1C), 125.31 (d, 2C), 92.96 (d, 1C), 90.80 (s, 1C), 70.33 (d, 1C), 70.27 (d, 1C), 69.93 (d, 1C), 67.80 (d, 1C), 62.10 (t, 1C), 39.76 (t, 1C), 39.69 (t, 1C), 39.52 (t, 1C), 39.49 (t, 1C); HRMS calcd for $C_{40}H_{32}Cl_3N_5NaO_{18}^+$ $[M+Na]^+$ 998.0700, found 998.0702.

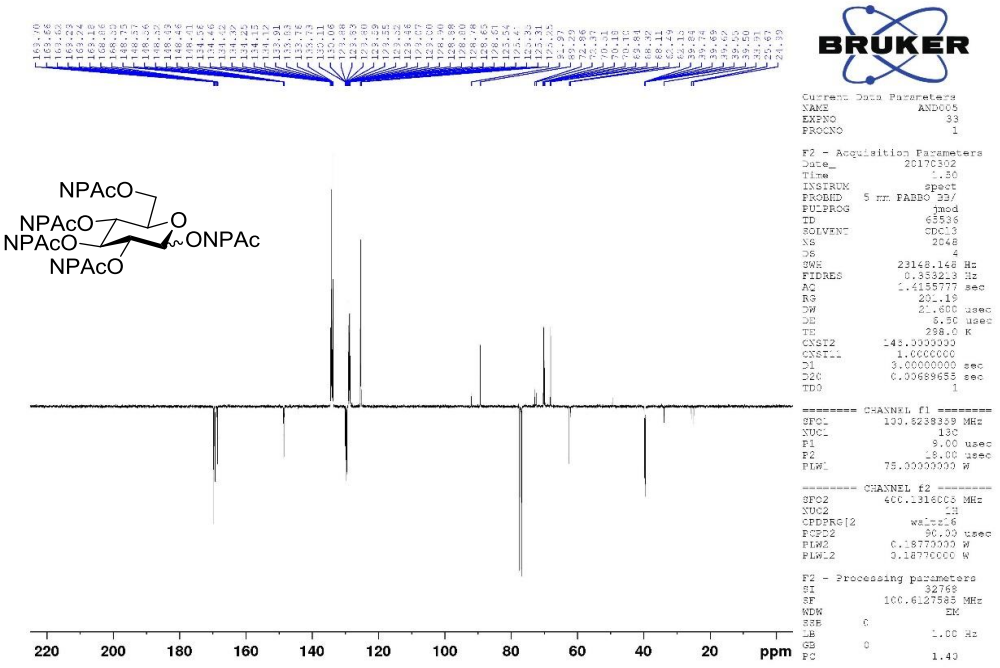
3) Copies of NMR spectra

a. 1,2,3,4,6-Penta-O-(2-nitrophenyl)acetyl-β,d-glucopyranoside (3)

^{1}H NMR (*d*-Chloroform, 400 MHz)

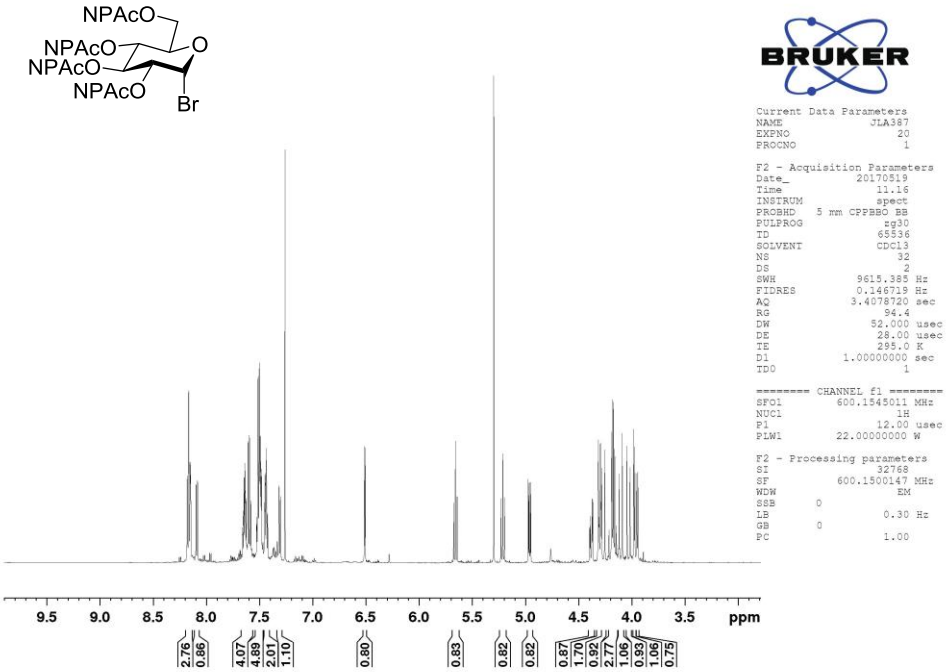


^{13}C NMR (*d*-Chloroform, 100 MHz)

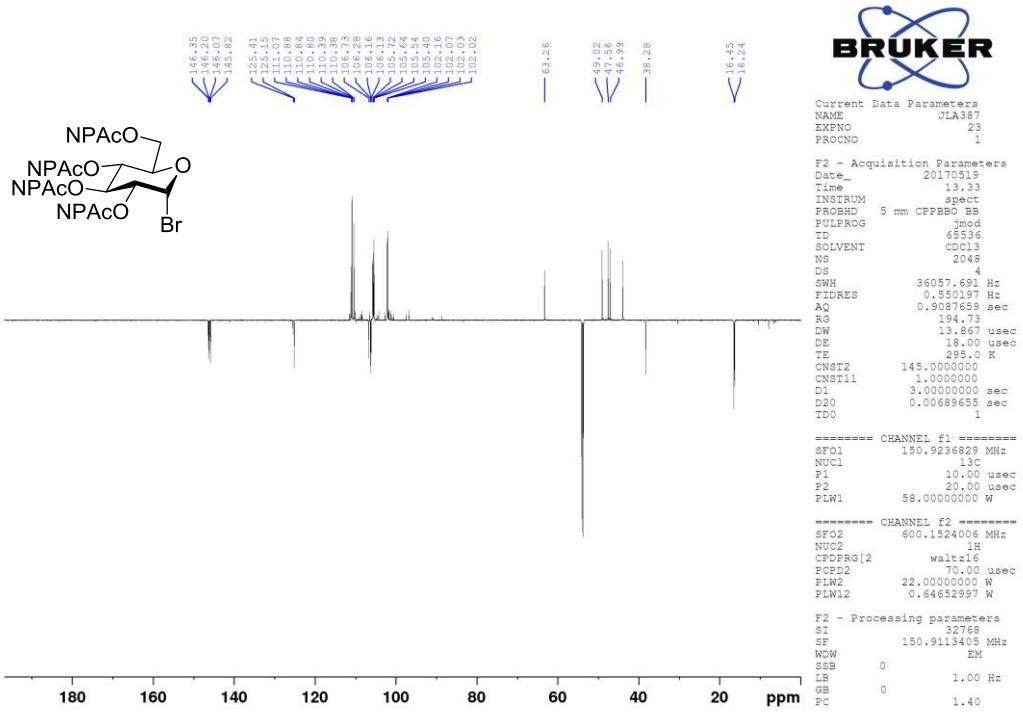


b. 1-Bromo-1-deoxy-2,3,4,6-tetra-*O*-(2-nitrophenyl)acetyl-α,d-glucopyranose (4)

^{1}H NMR (*d*-Chloroform, 600 MHz)

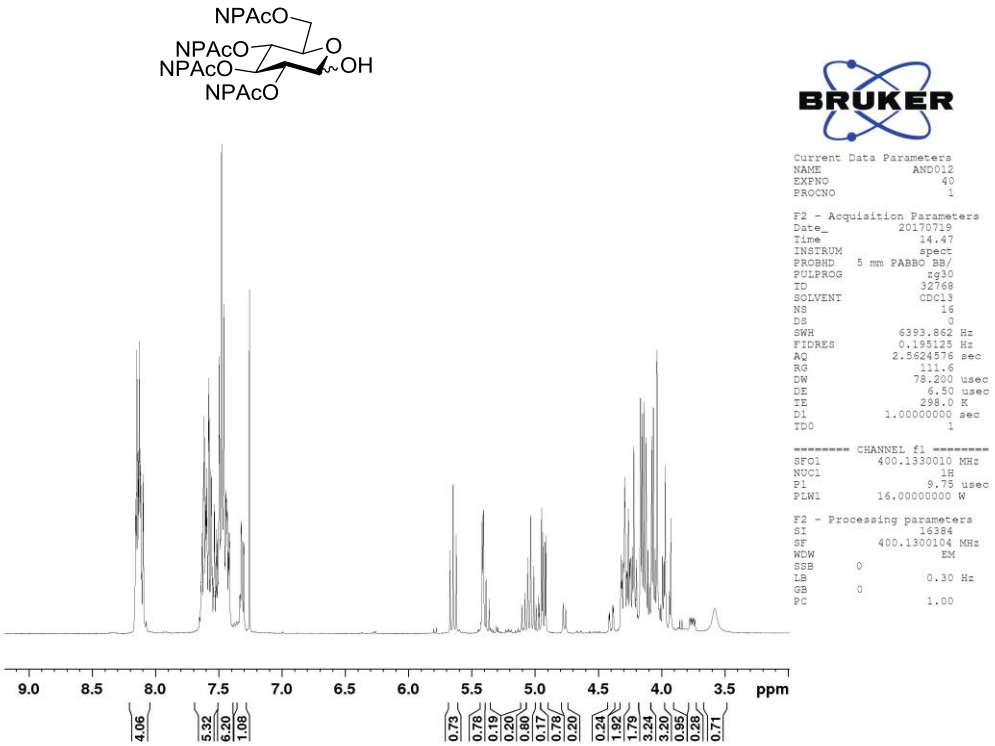


^{13}C NMR (*d*-Chloroform, 150 MHz)

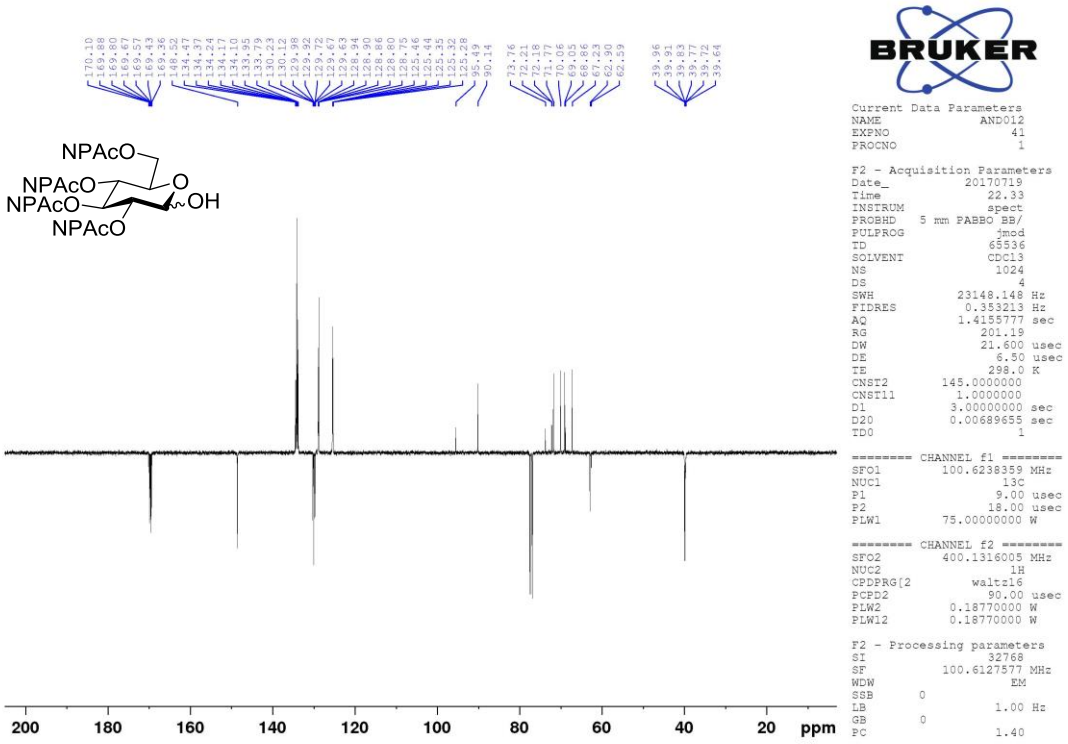


c. 2,3,4,6-Tetra-*O*-(2-nitrophenyl)acetyl-d-glucopyranose (5)

^{1}H NMR (*d*-Chloroform, 400 MHz)

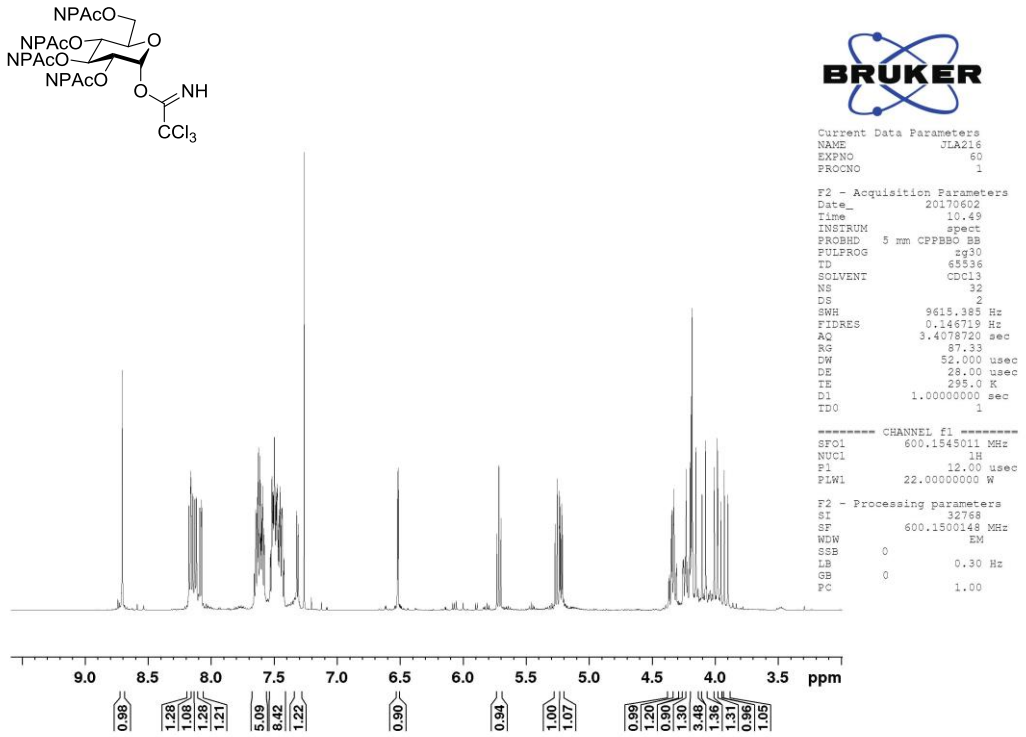


^{13}C NMR (*d*-Chloroform, 100 MHz)

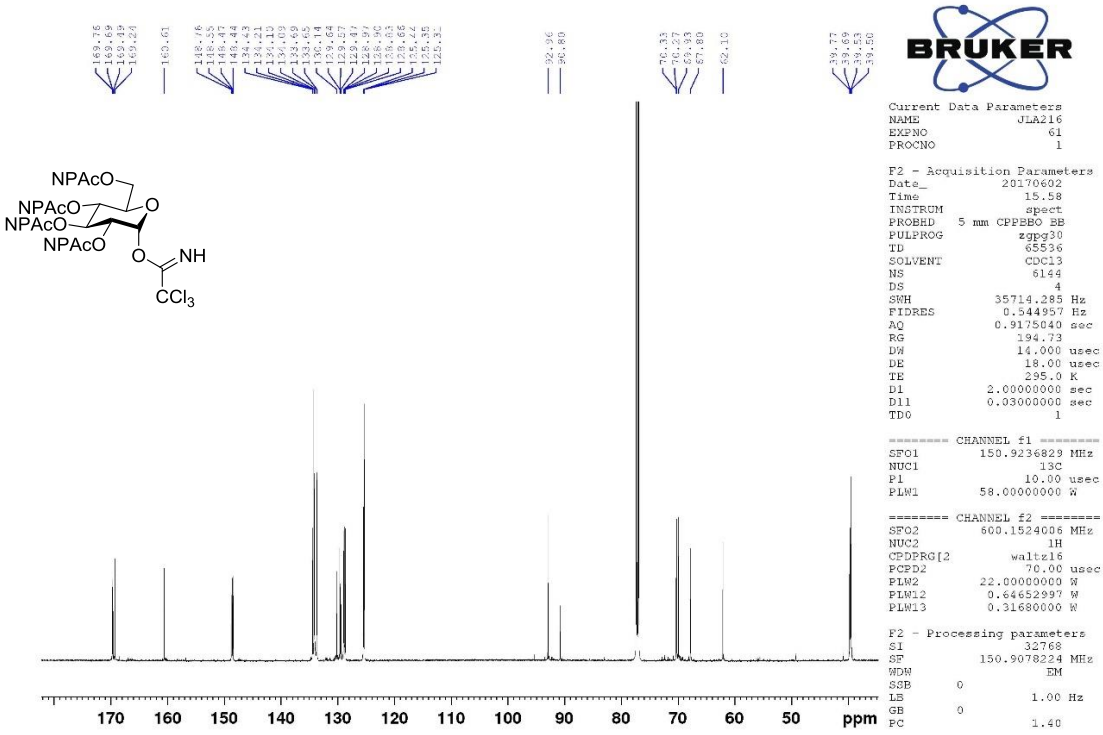


d. 2,3,4,6-Tetra-*O*-(2-nitrophenyl)acetyl-α,d-glucopyranosyl trichloroacetimidate (6)

^{1}H NMR (*d*-Chloroform, 600 MHz)

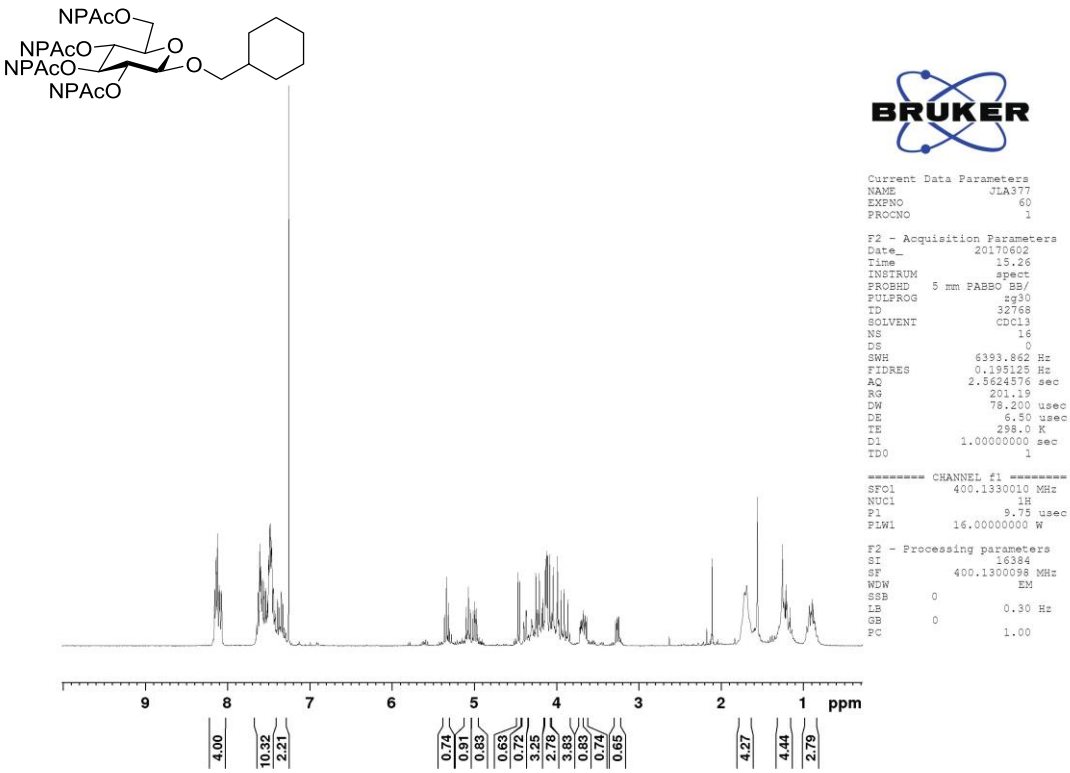


^{13}C NMR (*d*-Chloroform, 150 MHz)

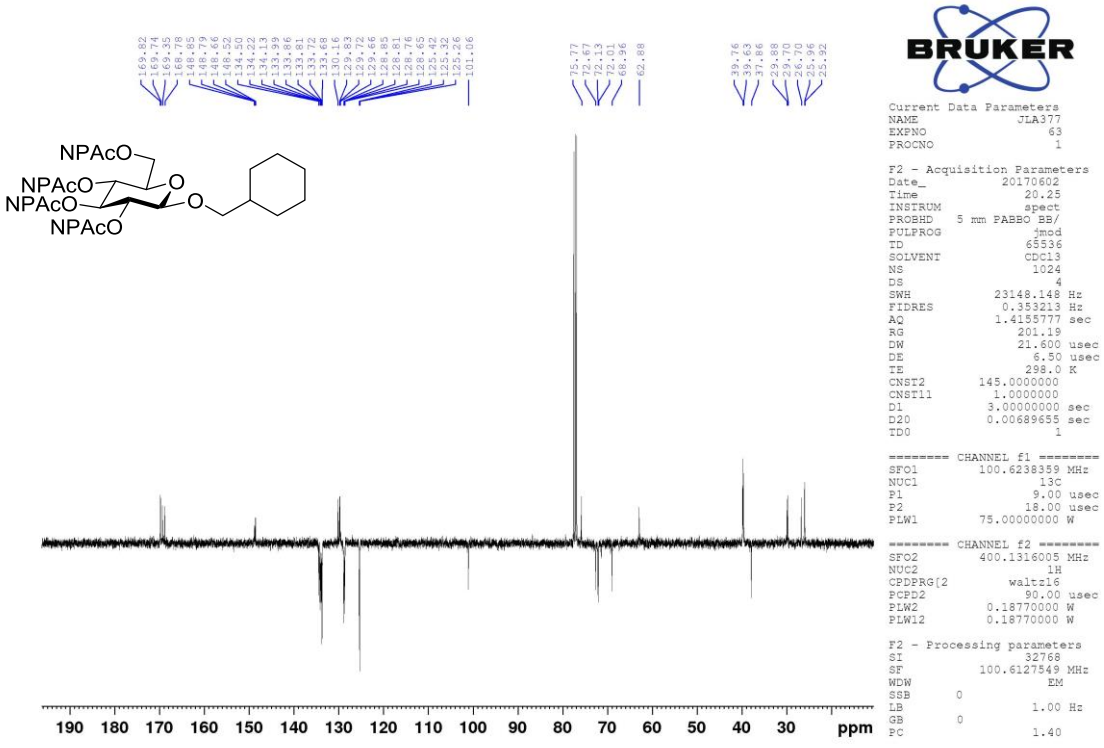


e. 1-Methylcyclohexyl-2,3,4,6-tetra-*O*-(2-nitrophenyl)acetyl-β,d-glucopyranoside (7)

^{1}H NMR (*d*-Chloroform, 400 MHz)

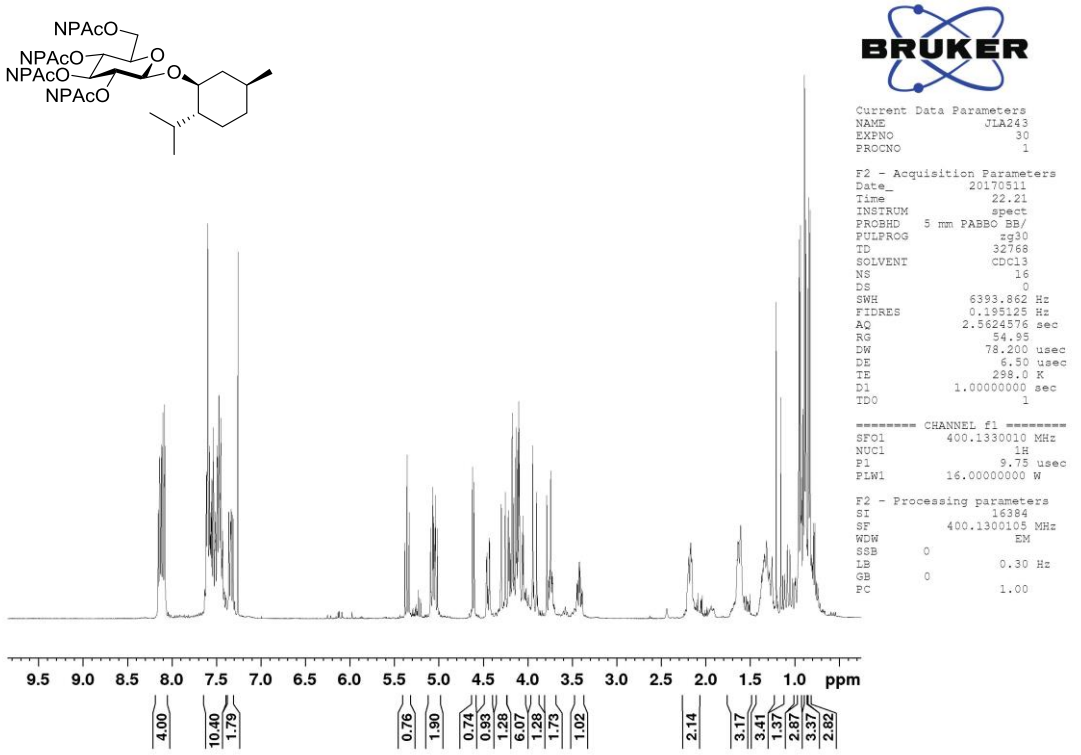


^{13}C NMR (*d*-Chloroform, 100 MHz)

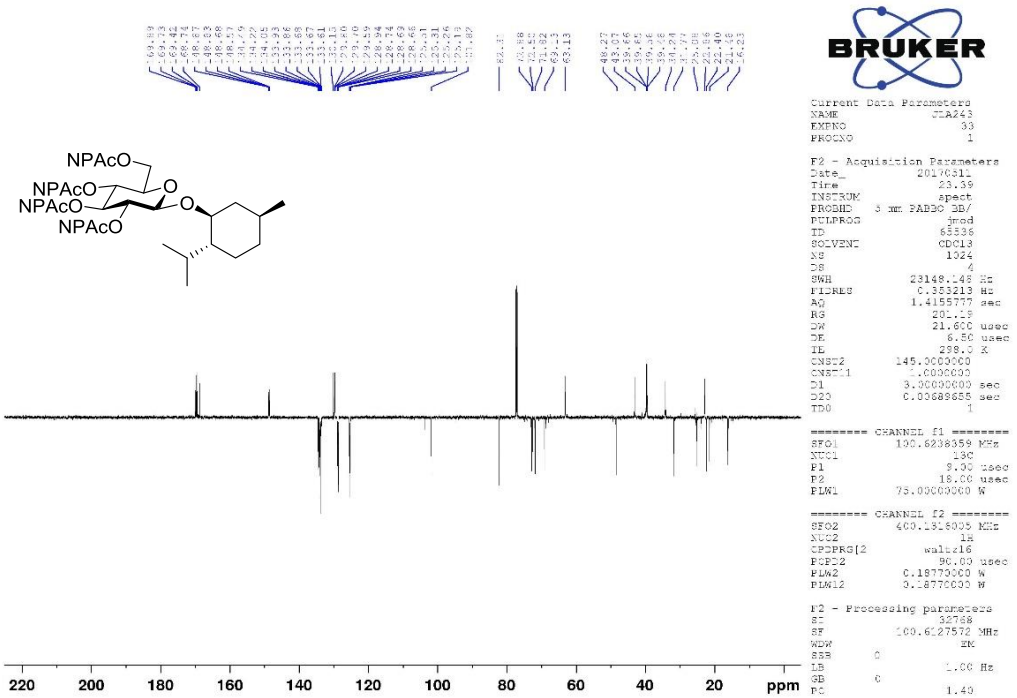


f. 1-(+)-Menthyl-2,3,4,6-tetra-*O*-(2-nitrophenyl)acetyl-β,d-glucopyranoside (8)

^{1}H NMR (*d*-Chloroform, 400 MHz)

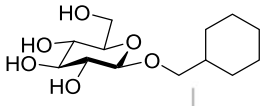


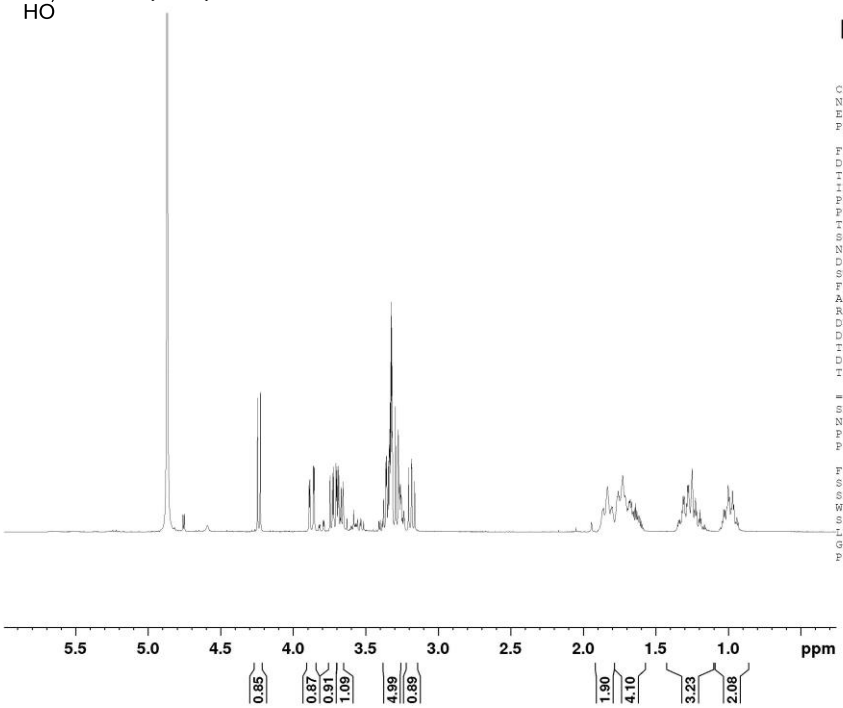
^{13}C NMR (*d*-Chloroform, 100 MHz)



g. Methylcyclohexyl-β,D-glucopyranoside (9)

^{1}H NMR (*d*-Methanol, 400 MHz)



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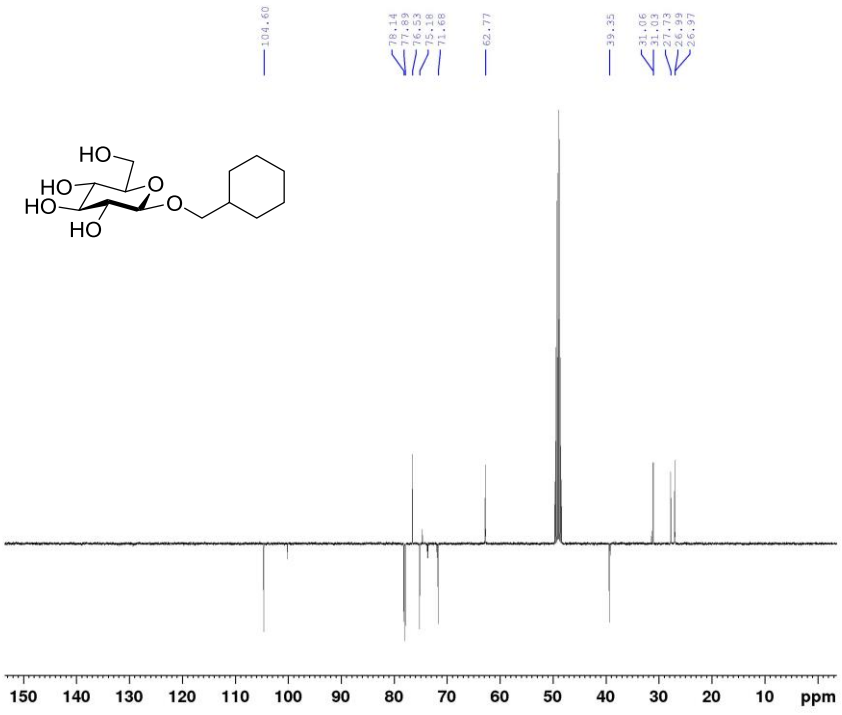
^{13}C NMR (*d*-Methanol, 100 MHz)

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PROCNO                     1

F2 - Acquisition Parameters
Date_               20170626
Time                   21.39
INSTRUM                spect
PROBHD   5 mm PABBO BB/
PULPROG                jmod
TD                    65536
SOLVENT                MeOD
NS                      1024
DS                         4
SWH               23148.148 Hz
FIDRES            0.353213 Hz
AQ                1.4155777 sec
RG                    201.19
DW                  21.600 usec
DE                    6.50 usec
TE                   298.0 K
CNST2            145.0000000
CNST11             1.0000000
D1                 3.00000000 sec
D20                0.00689655 sec
TD0                       1

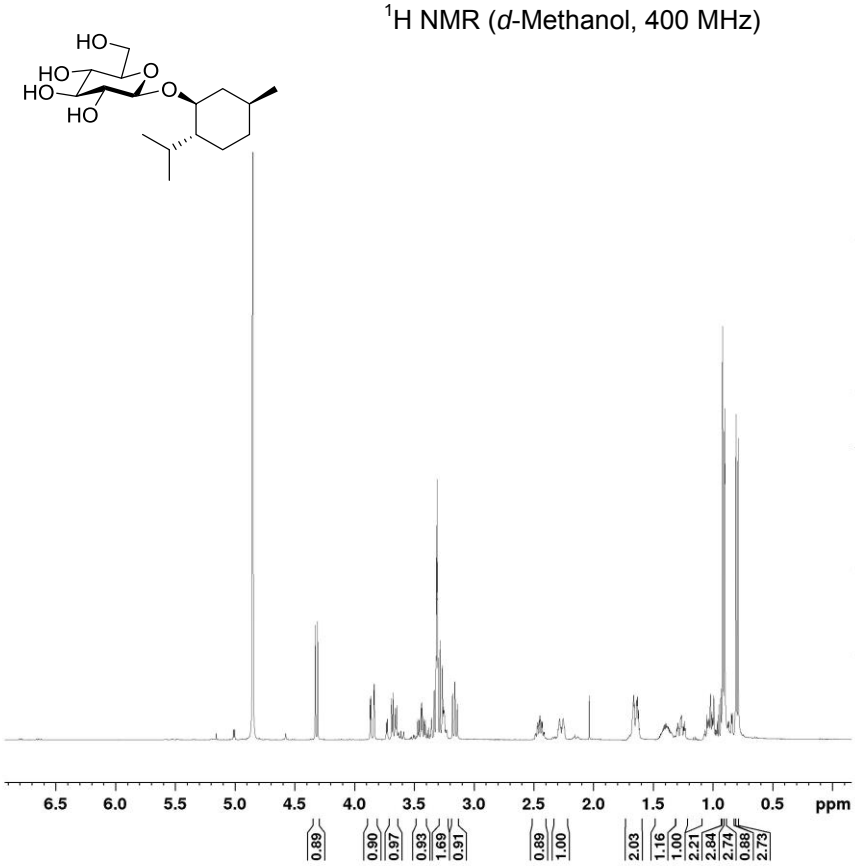
======== CHANNEL f1 ========
SFO1            100.6238359 MHz
NUC1                      13C
P1                      9.00 usec
P2                     18.00 usec
PLW1           75.00000000 W

======== CHANNEL f2 ========
SFO2            400.1316005 MHz
NUC2                      1H
CPDPRG[2             waltz16
PCPD2                 90.00 usec
PLW2           0.18770000 W
PLW12          0.18770000 W

F2 - Processing parameters
SI                    32768
SF              100.6126281 MHz
WDW                      EM
SSB                       0
LB                     1.00 Hz
GB                        0
PC                     1.40
```

h. (+)-Menthyl-β,d-glucopyranoside (10)

^{1}H NMR (*d*-Methanol, 400 MHz)



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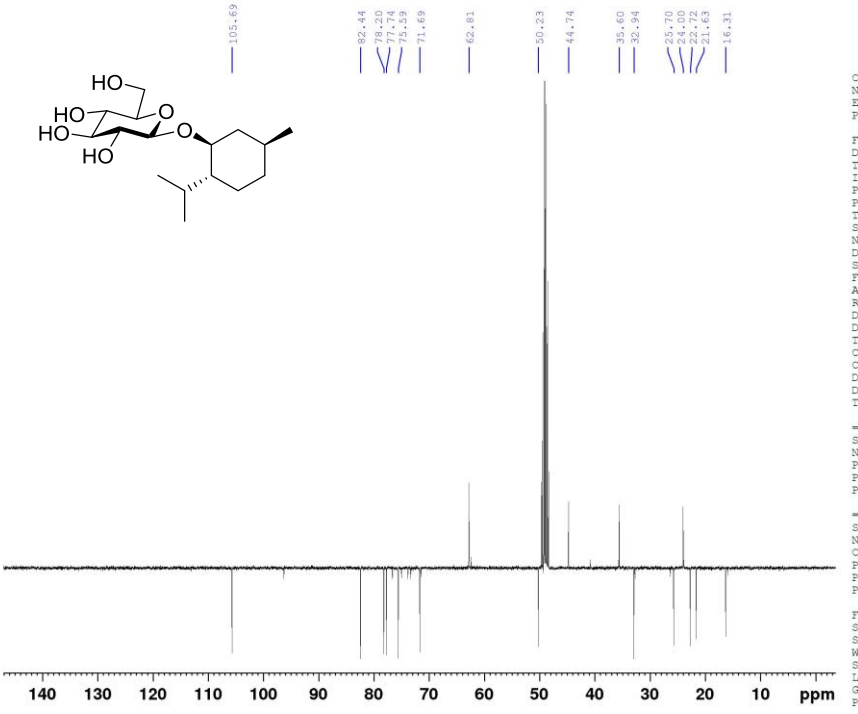
```
Current Data Parameters
NAME                  JLA392
EXPNO                     10
PROCNO                     1

F2 - Acquisition Parameters
Date_              20170626
Time                  14.13
INSTRUM               spect
PROBHD    5 mm PABBO BB/
PULPROG                zg30
TD                    32768
SOLVENT                MeOD
NS                       16
DS                        0
SWH                6393.862 Hz
FIDRES            0.195125 Hz
AQ                2.5624576 sec
RG                    127.26
DW                  78.200 usec
DE                    6.50 usec
TE                   298.0 K
D1              1.00000000 sec
TD0                       1

======== CHANNEL f1 ========
SFO1           400.1330010 MHz
NUC1                     1H
P1                    9.75 usec
PLW1           16.00000000 W

F2 - Processing parameters
SI                    16384
SF             400.1300077 MHz
WDW                     EM
SSB          0
LB                    0.30 Hz
GB           0
PC                    1.00
```

^{13}C NMR (*d*-Methanol, 100 MHz)



BRUKER

```
Current Data Parameters
NAME                  JLA392
EXPNO                     13
PROCNO                     1

F2 - Acquisition Parameters
Date_              20170626
Time                  20.20
INSTRUM               spect
PROBHD    5 mm PABBO BB/
PULPROG                jmod
TD                    65536
SOLVENT                MeOD
NS                     1024
DS                        4
SWH               23148.148 Hz
FIDRES            0.353213 Hz
AQ                1.4155777 sec
RG                    201.19
DW                  21.600 usec
DE                    6.50 usec
TE                   298.0 K
CNST2          145.0000000
CNST11           1.0000000
D1              3.00000000 sec
D20             0.00689655 sec
TD0                       1

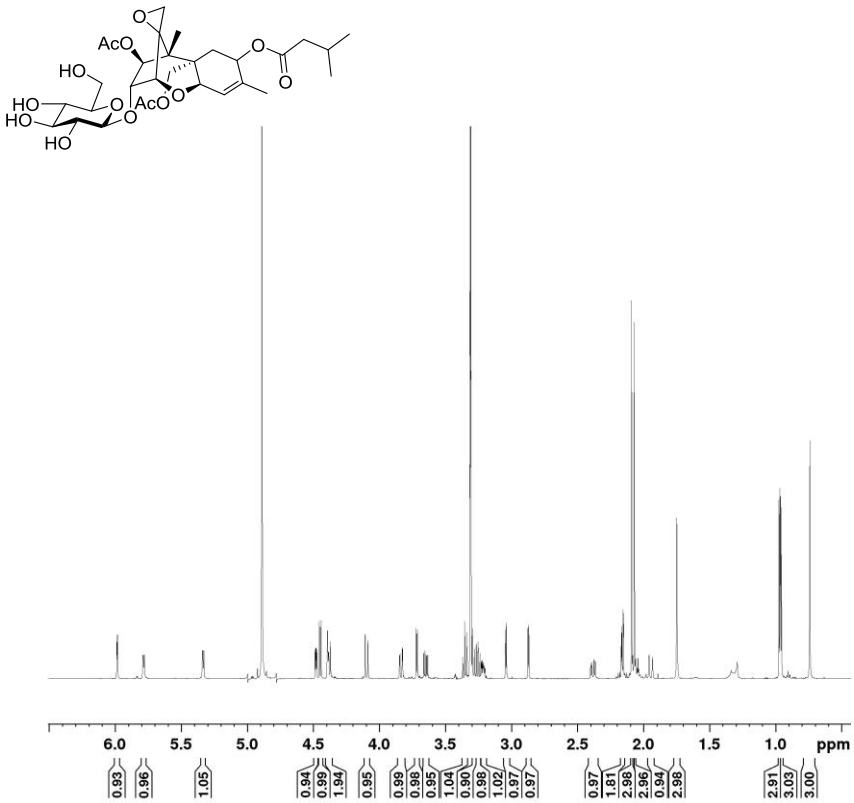
======== CHANNEL f1 ========
SFO1           100.6238359 MHz
NUC1                    13C
P1                    9.00 usec
P2                   18.00 usec
PLW1           75.00000000 W

======== CHANNEL f2 ========
SFO2           400.1316005 MHz
NUC2                     1H
CPDPRG[2           waltz16
PCPD2                90.00 usec
PLW2           0.18770000 W
PLW12          0.18770000 W

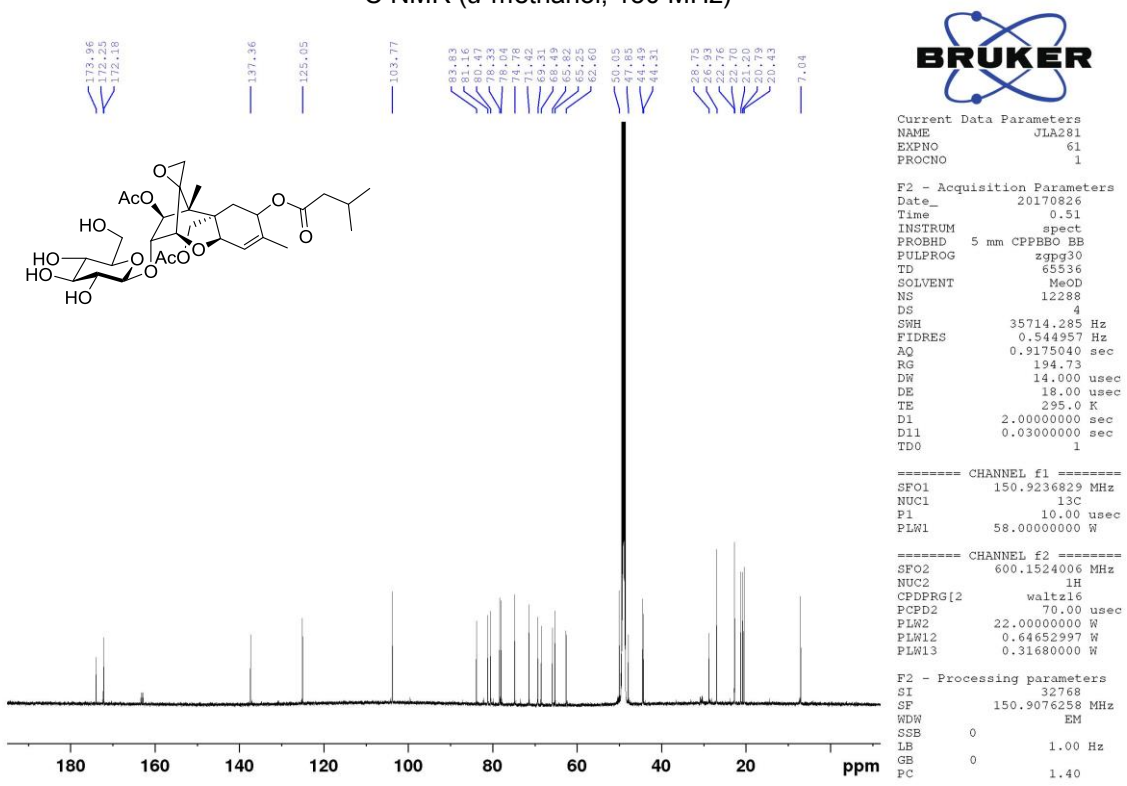
F2 - Processing parameters
SI                    32768
SF             100.6126280 MHz
WDW                     EM
SSB          0
LB                    1.00 Hz
GB           0
PC                    1.40
```

i. T2-β,D-glucoside (2)

^{1}H NMR (*d*-methanol, 600 MHz)



^{13}C NMR (*d*-methanol, 150 MHz)



Supporting Information

Manuscript #4

Eur. J. Org. Chem. **2017** · ISSN 1099–0690

https://doi.org/10.1002/ejoc.201700934

SUPPORTING INFORMATION

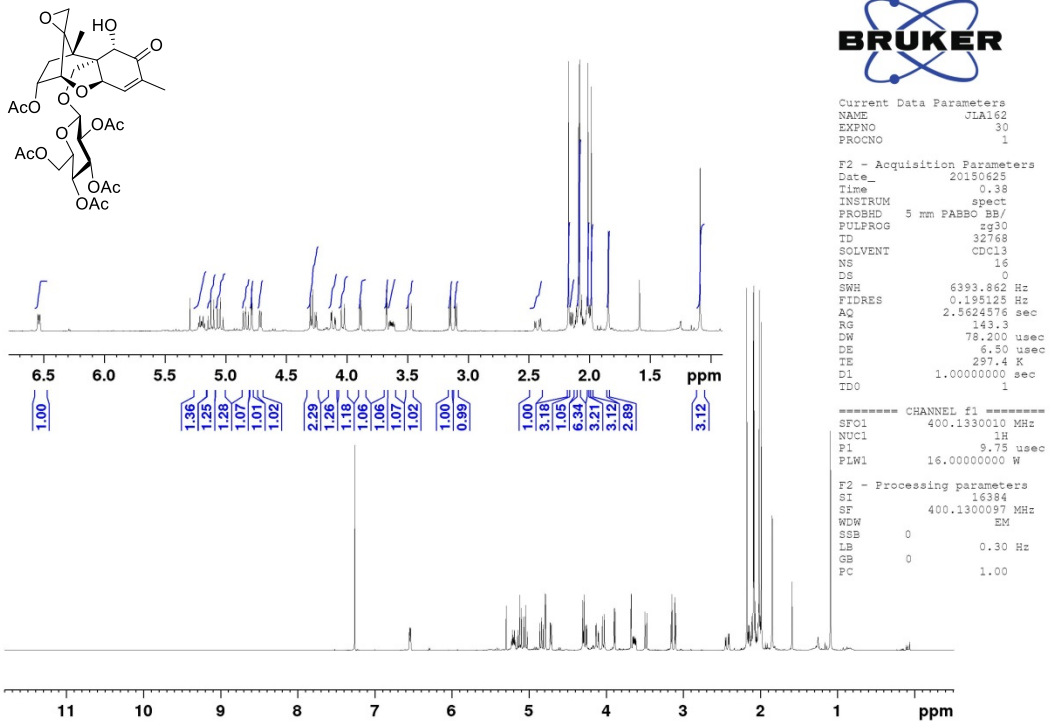
Title: Synthesis of Isotope-Labeled Deoxynivalenol-15-*O*-Glycosides

Author(s): Julia Weber, Philipp Fruhmann, Christian Hametner, Alois Schiessl, Georg Häubl, Johannes Fröhlich, Hannes Mikula*

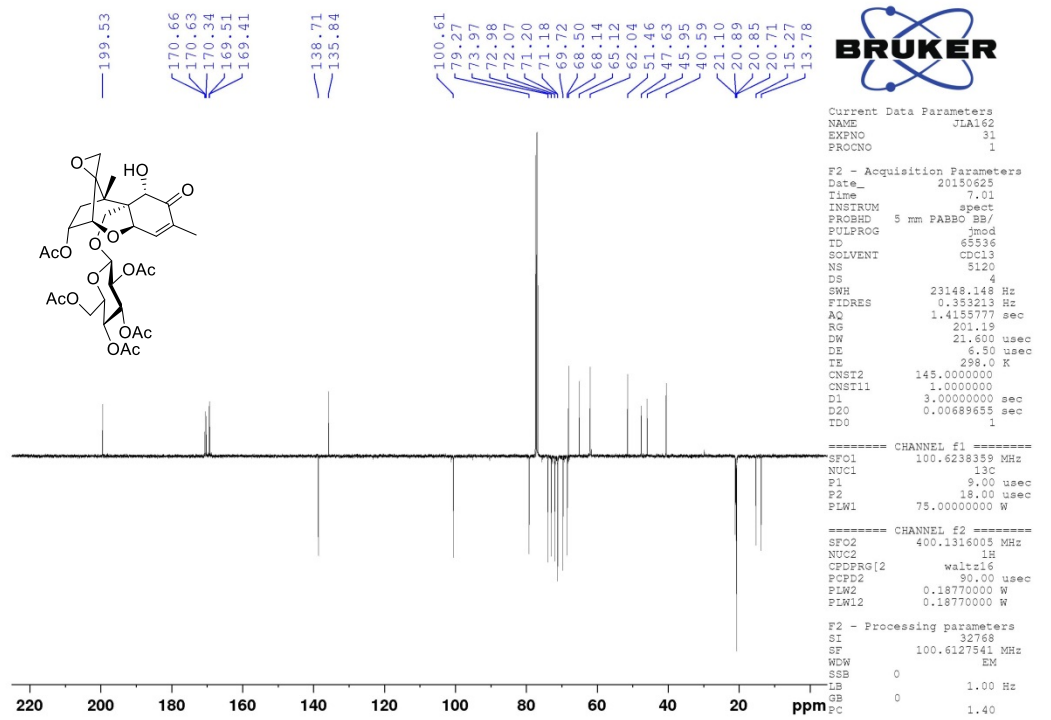
NMR spectra and LCMS

a) 3-Acetyldeoxynivalenol-15-*O*-(tetra-*O*-acetyl-ß,D-glucopyranoside) (7)

^{1}H NMR ($CDCl_3$, 400 MHz)

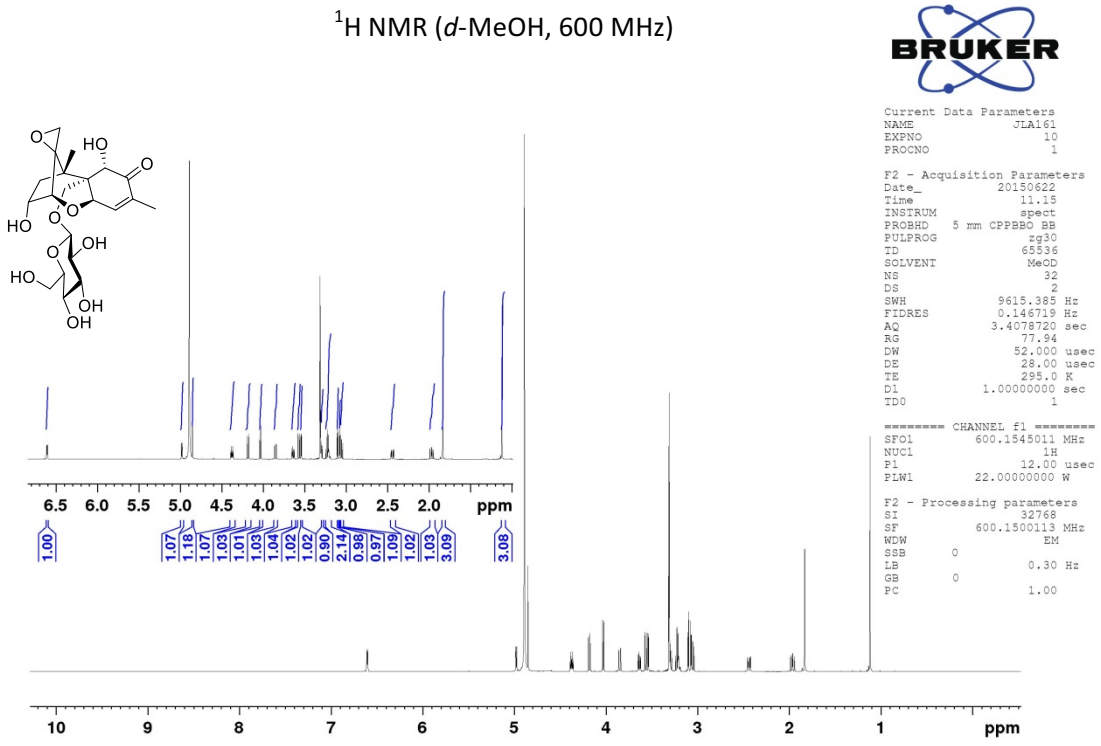


^{13}C NMR (*d*-Chloroform, 100 MHz)

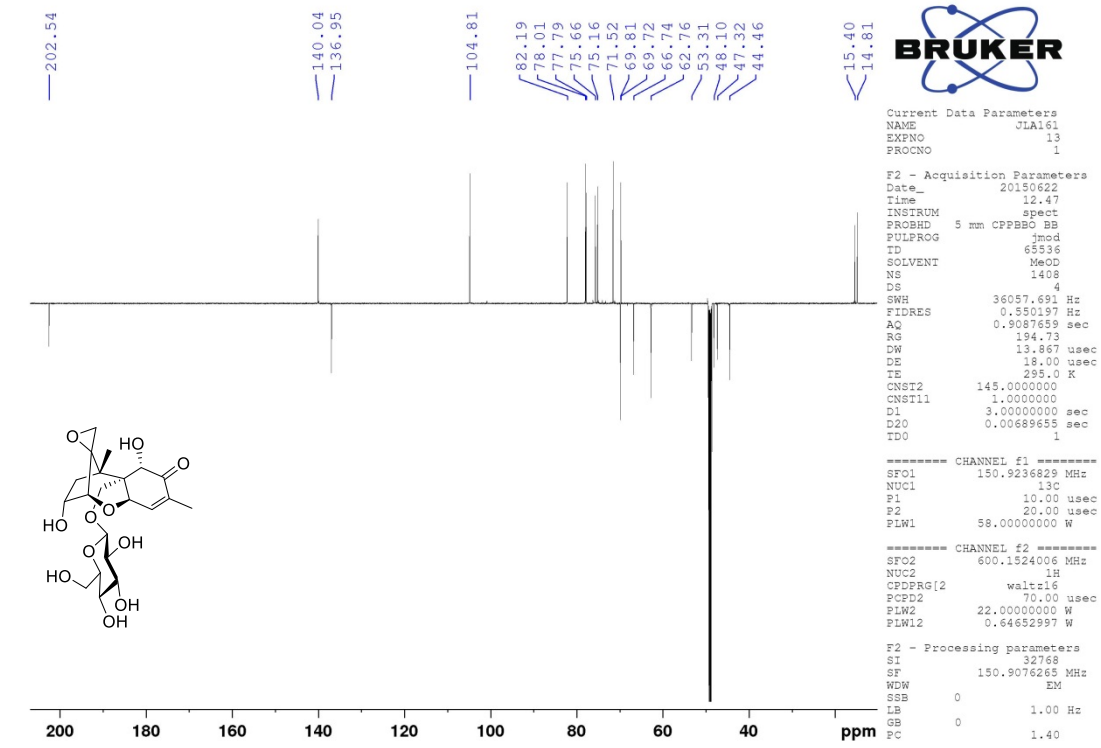


b) Deoxynivalenol-15-*O*-ß,D-glucopyranoside (2)

^{1}H NMR (*d*-MeOH, 600 MHz)

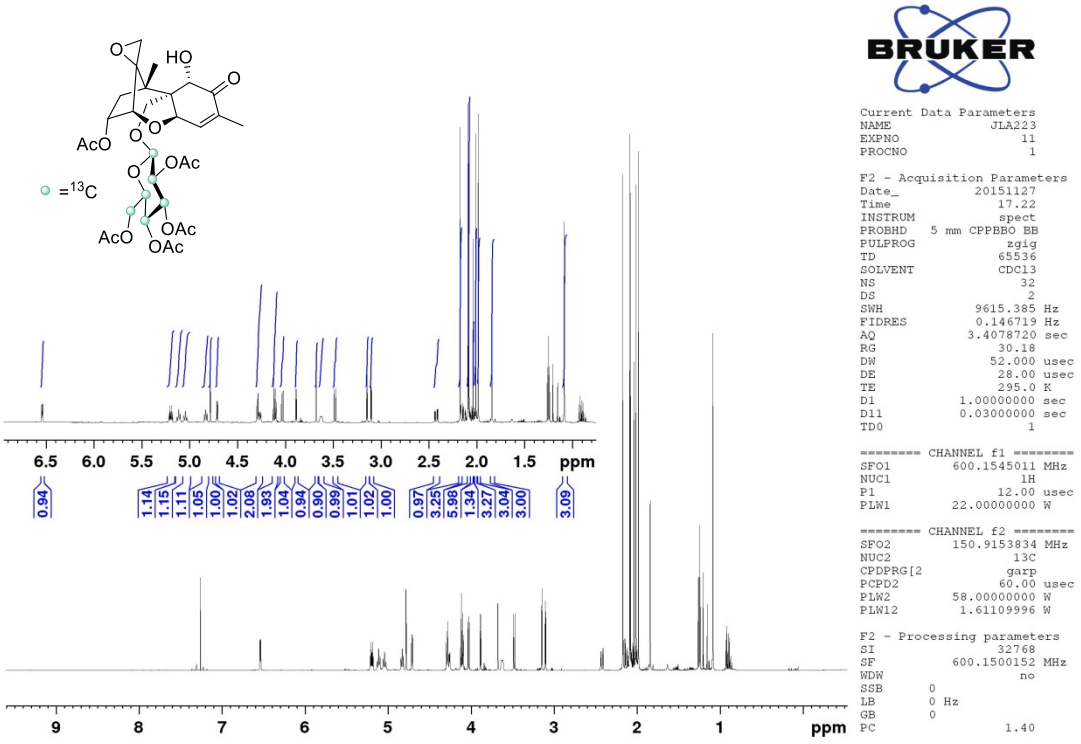


^{13}C NMR (*d*-MeOH, 150 MHz)



c) 3-Acetyldeoxynivalenol-15-*O*-(tetra-*O*-acetyl-ß,D-[$^{13}C_6$]glucopyranoside) ([$^{13}C_6$]7)

^{1}H NMR ($CDCl_3$, 600 MHz, ^{13}C-decoupled)



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```
Current Data Parameters
NAME                  JLA223
EXPNO                     11
PROCNO                     1

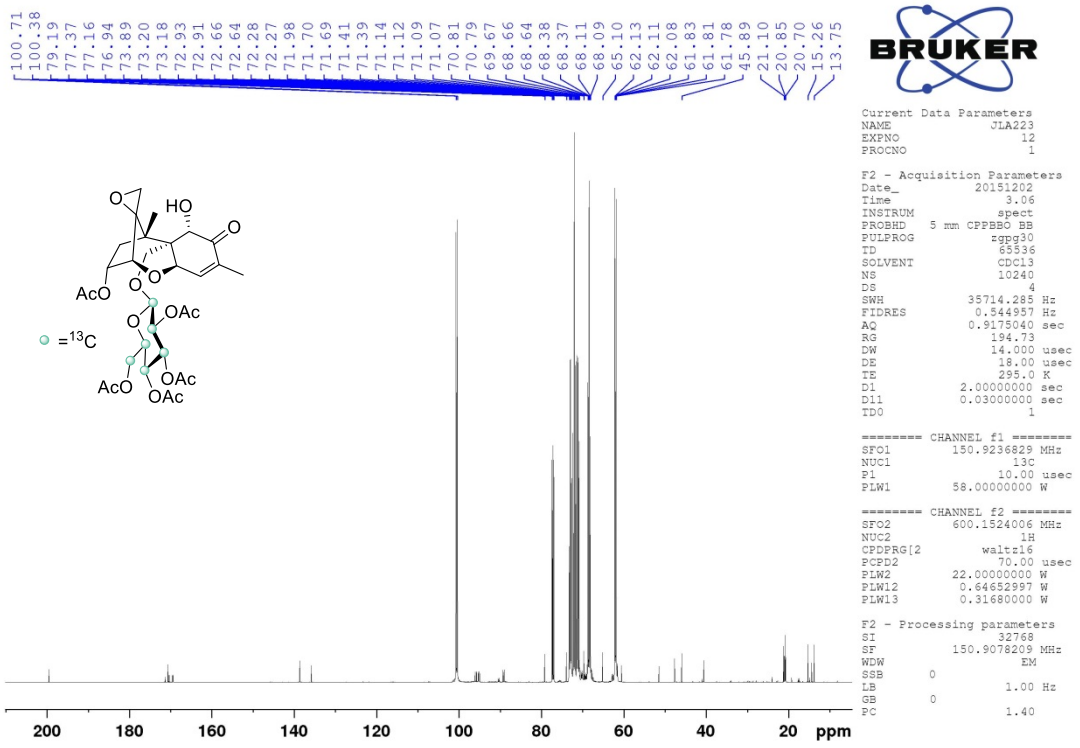
F2 - Acquisition Parameters
Date_              20151127
Time                  17.22
INSTRUM                spect
PROBHD    5 mm CPPBBO BB
PULPROG                zgig
TD                    65536
SOLVENT               CDCl3
NS                       32
DS                        2
SWH                9615.385 Hz
FIDRES            0.146719 Hz
AQ                3.4078720 sec
RG                    30.18
DW                   52.000 usec
DE                   28.00 usec
TE                   295.0 K
D1                1.00000000 sec
D11               0.03000000 sec
TD0                        1

======== CHANNEL f1 ========
SFO1           600.1545011 MHz
NUC1                     1H
P1                    12.00 usec
PLW1           22.00000000 W

======== CHANNEL f2 ========
SFO2           150.9153834 MHz
NUC2                    13C
CPDPRG[2               garp
PCPD2                 60.00 usec
PLW2           58.00000000 W
PLW12           1.61109996 W

F2 - Processing parameters
SI                       32768
SF             600.1500152 MHz
WDW                         no
SSB          0
LB           0 Hz
GB           0
PC                      1.40
```

^{13}C NMR ($CDCl_3$, 150 MHz)



BRUKER

```
Current Data Parameters
NAME                  JLA223
EXPNO                     12
PROCNO                     1

F2 - Acquisition Parameters
Date_              20151202
Time                   3.06
INSTRUM                spect
PROBHD    5 mm CPPBBO BB
PULPROG             zgpg30
TD                    65536
SOLVENT               CDCl3
NS                    10240
DS                        4
SWH               35714.285 Hz
FIDRES            0.544957 Hz
AQ                0.9175040 sec
RG                   194.73
DW                   14.000 usec
DE                   18.00 usec
TE                   295.0 K
D1                2.00000000 sec
D11               0.03000000 sec
TD0                        1

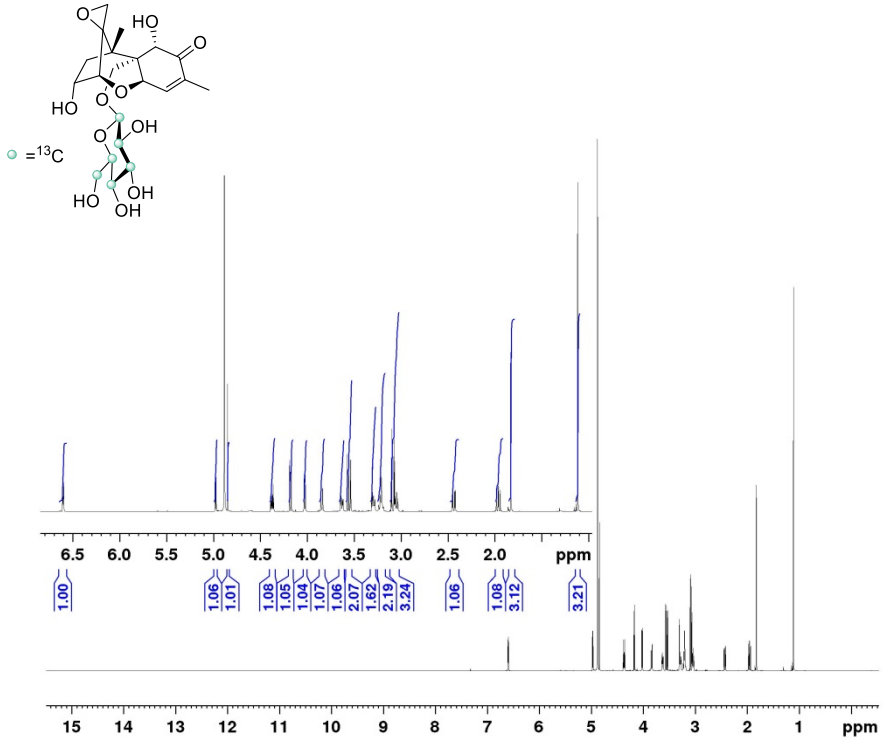
======== CHANNEL f1 ========
SFO1           150.9236829 MHz
NUC1                    13C
P1                    10.00 usec
PLW1           58.00000000 W

======== CHANNEL f2 ========
SFO2           600.1524006 MHz
NUC2                     1H
CPDPRG[2           waltz16
PCPD2                 70.00 usec
PLW2           22.00000000 W
PLW12           0.64652997 W
PLW13           0.31680000 W

F2 - Processing parameters
SI                       32768
SF             150.9078209 MHz
WDW                         EM
SSB          0
LB                    1.00 Hz
GB           0
PC                      1.40
```

d) Deoxynivalenol-15-*O*-ß,D-[$^{13}C_6$]glucopyranoside ([$^{13}C_6$]2)

^{1}H NMR (*d*-MeOH, 600 MHz, ^{13}C-decoupled)




```
Current Data Parameters
NAME                  JLA237
EXPNO                     10
PROCNO                     1

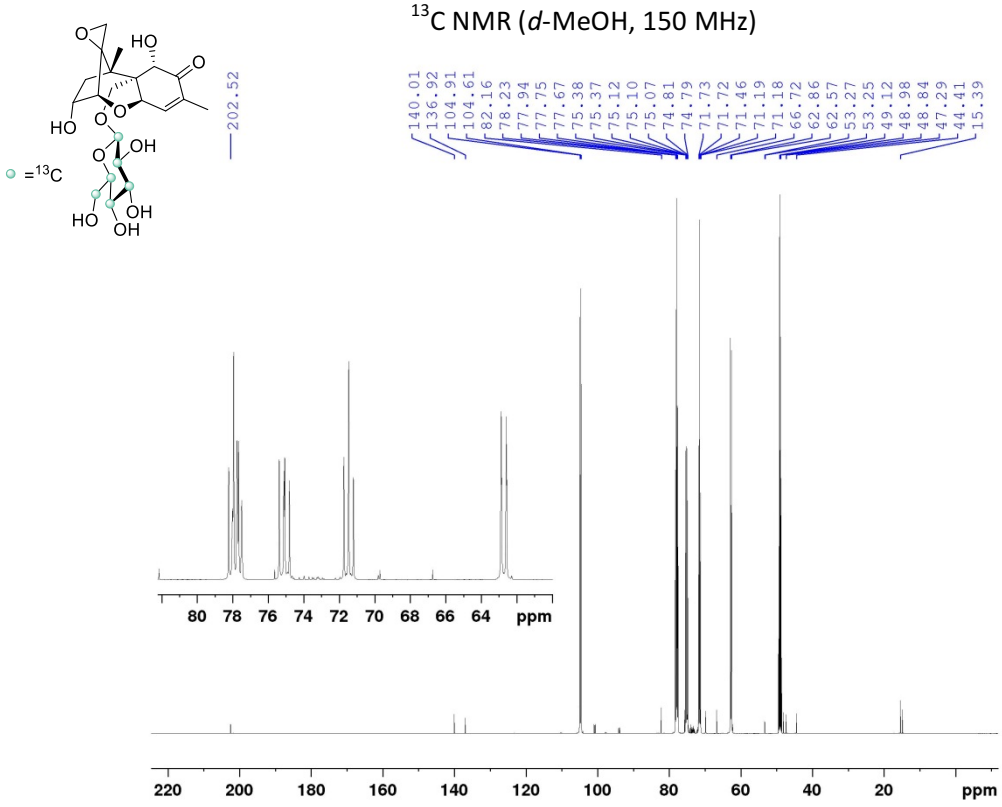
F2 - Acquisition Parameters
Date_              20151211
Time                  14.27
INSTRUM               spect
PROBHD    5 mm CPPBBO BB
PULPROG               zgig
TD                   65536
SOLVENT               MeOD
NS                      32
DS                       2
SWH                9615.385 Hz
FIDRES             0.146719 Hz
AQ                 3.4078720 sec
RG                    30.18
DW                   52.000 usec
DE                   28.00 usec
TE                   295.0 K
D1                1.00000000 sec
D11               0.03000000 sec
TD0                        1

======== CHANNEL f1 ========
SFO1             600.1545011 MHz
NUC1                      1H
P1                     12.00 usec
PLW1             22.00000000 W

======== CHANNEL f2 ========
SFO2             150.9153834 MHz
NUC2                     13C
CPDPRG[2                garp
PCPD2                 60.00 usec
PLW2             58.00000000 W
PLW12             1.61109996 W

F2 - Processing parameters
SI                       32768
SF               600.1500124 MHz
WDW                         no
SSB          0
LB           0 Hz
GB           0
PC                       1.40
```

^{13}C NMR (*d*-MeOH, 150 MHz)




```
Current Data Parameters
NAME                  JLA237
EXPNO                     11
PROCNO                     1

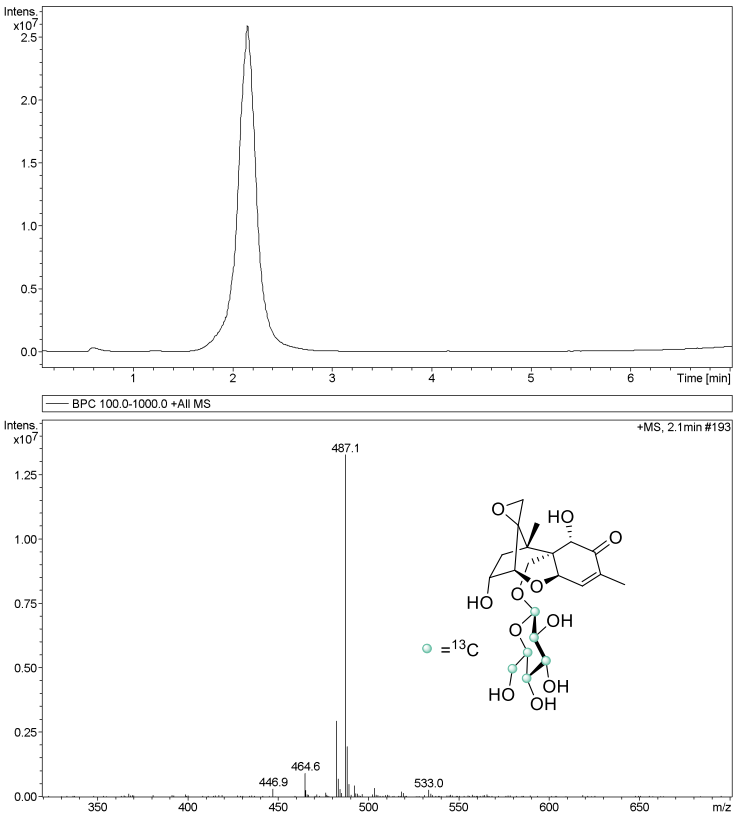
F2 - Acquisition Parameters
Date_              20151211
Time                  23.33
INSTRUM               spect
PROBHD    5 mm CPPBBO BB
PULPROG             zgpg30
TD                   65536
SOLVENT               MeOD
NS                     8192
DS                        4
SWH               35714.285 Hz
FIDRES             0.544957 Hz
AQ                 0.9175040 sec
RG                   194.73
DW                   14.000 usec
DE                   18.00 usec
TE                   295.0 K
D1                2.00000000 sec
D11               0.03000000 sec
TD0                        1

======== CHANNEL f1 ========
SFO1             150.9236829 MHz
NUC1                     13C
P1                     10.00 usec
PLW1             58.00000000 W

======== CHANNEL f2 ========
SFO2             600.1524006 MHz
NUC2                      1H
CPDPRG[2          waltz16
PCPD2                 70.00 usec
PLW2             22.00000000 W
PLW12             0.64652997 W
PLW13             0.31680000 W

F2 - Processing parameters
SI                       32768
SF               150.9076304 MHz
WDW                         EM
SSB          0
LB                    1.00 Hz
GB           0
PC                       1.40
```

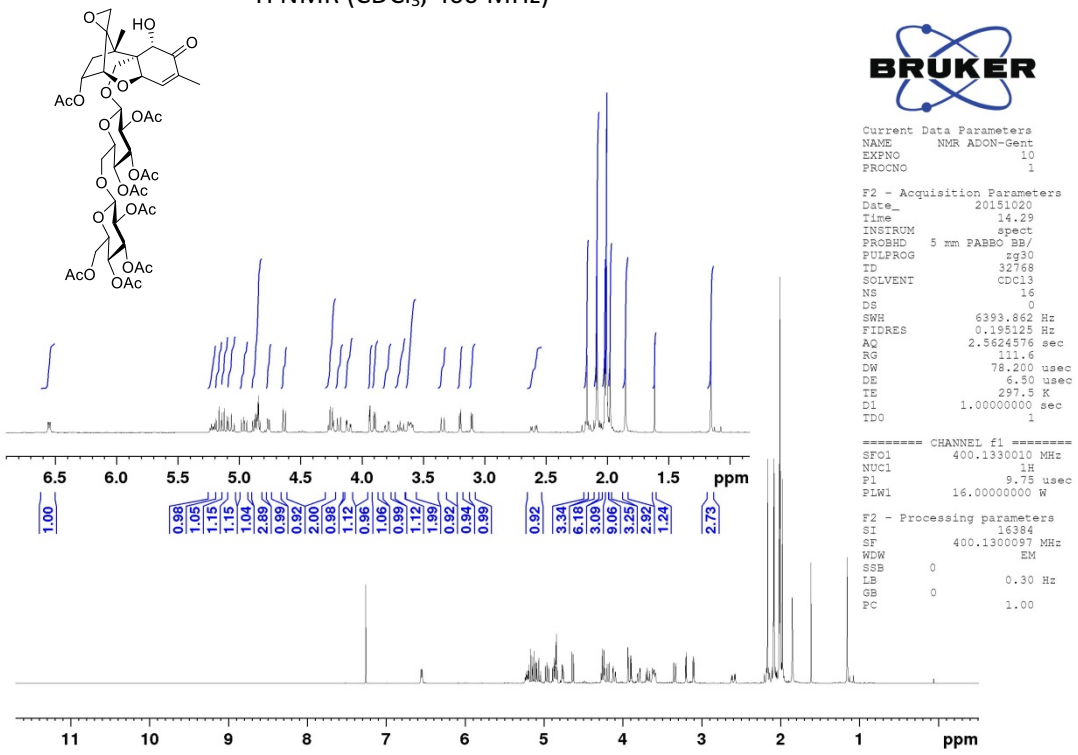
LCMS



Gradient:
Eluent A: ACN+0.1% formic acid;
Eluent B: H_2O+0.1% formic acid;
After an initial hold time at 5% B for 0.6 min, B was linearly increased to 60% within the next 5.0 min and hold at 60% B for 0.4 min.

e) 3-Acetyldeoxynivalenol-15-*O*-ß-hepta-O-acetylgentiobioside (12)

^{1}H NMR ($CDCl_3$, 400 MHz)



BRUKER

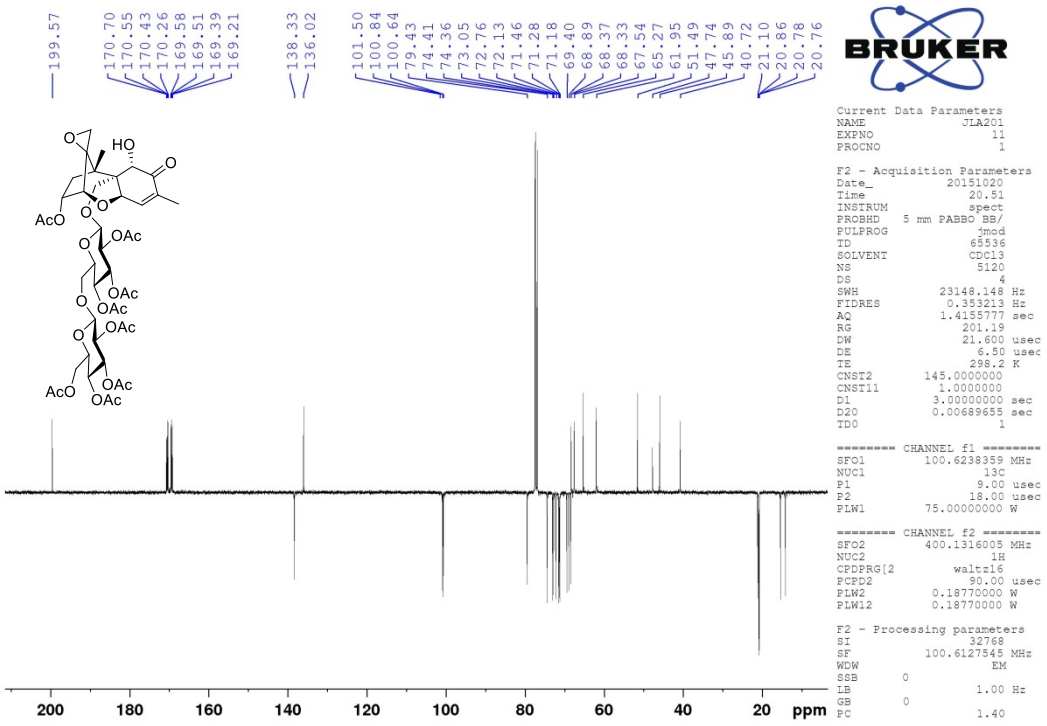
```
Current Data Parameters
NAME       NMR ADON-Gent
EXPNO                 10
PROCNO                 1

F2 - Acquisition Parameters
Date_           20151020
Time               14.29
INSTRUM            spect
PROBHD   5 mm PABBO BB/
PULPROG            zg30
TD                32768
SOLVENT           CDCl3
NS                   16
DS                    0
SWH           6393.862 Hz
FIDRES        0.195125 Hz
AQ            2.5624576 sec
RG                111.6
DW               78.200 usec
DE                6.50 usec
TE                297.5 K
D1           1.00000000 sec
TD0                    1

======== CHANNEL f1 ========
SFO1         400.1330010 MHz
NUC1                   1H
P1                  9.75 usec
PLW1        16.00000000 W

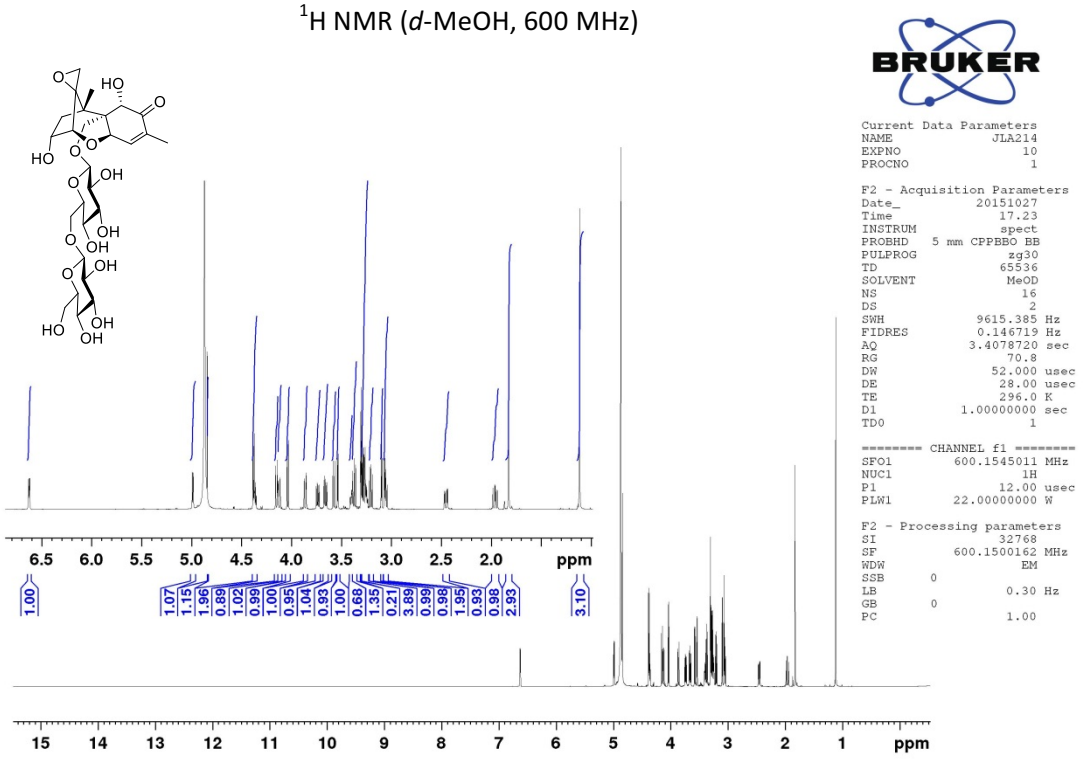
F2 - Processing parameters
SI                 16384
SF           400.1300097 MHz
WDW                   EM
SSB       0
LB                  0.30 Hz
GB        0
PC                  1.00
```

^{13}C NMR ($CDCl_3$, 100 MHz)

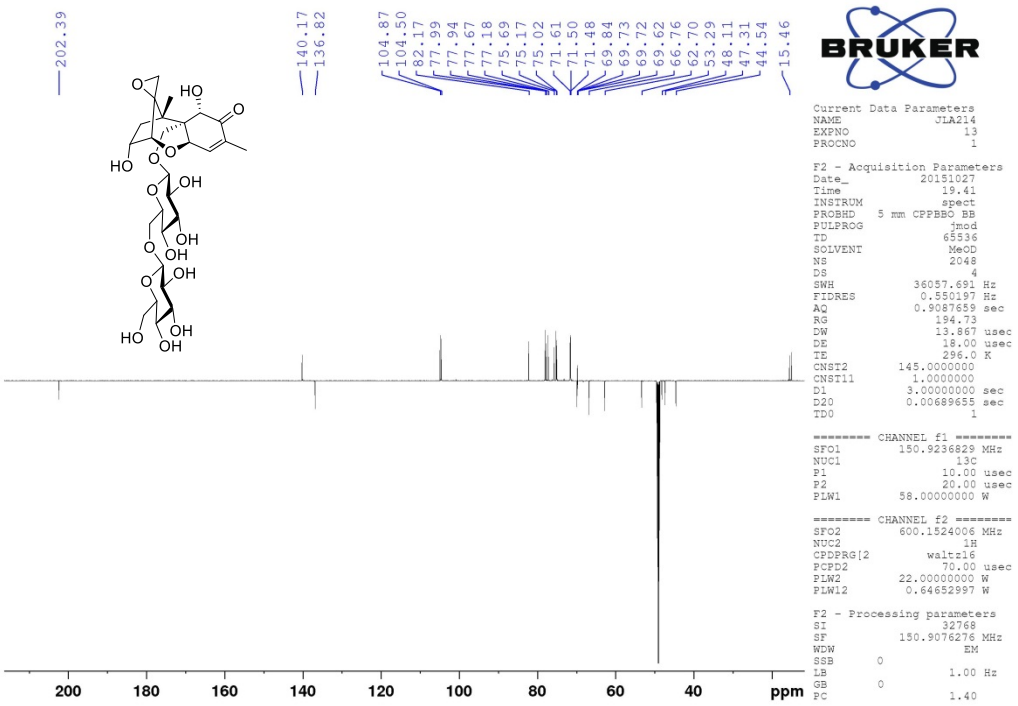


f) Deoxynivalenol-15-*O*-ß-gentiobioside (13)

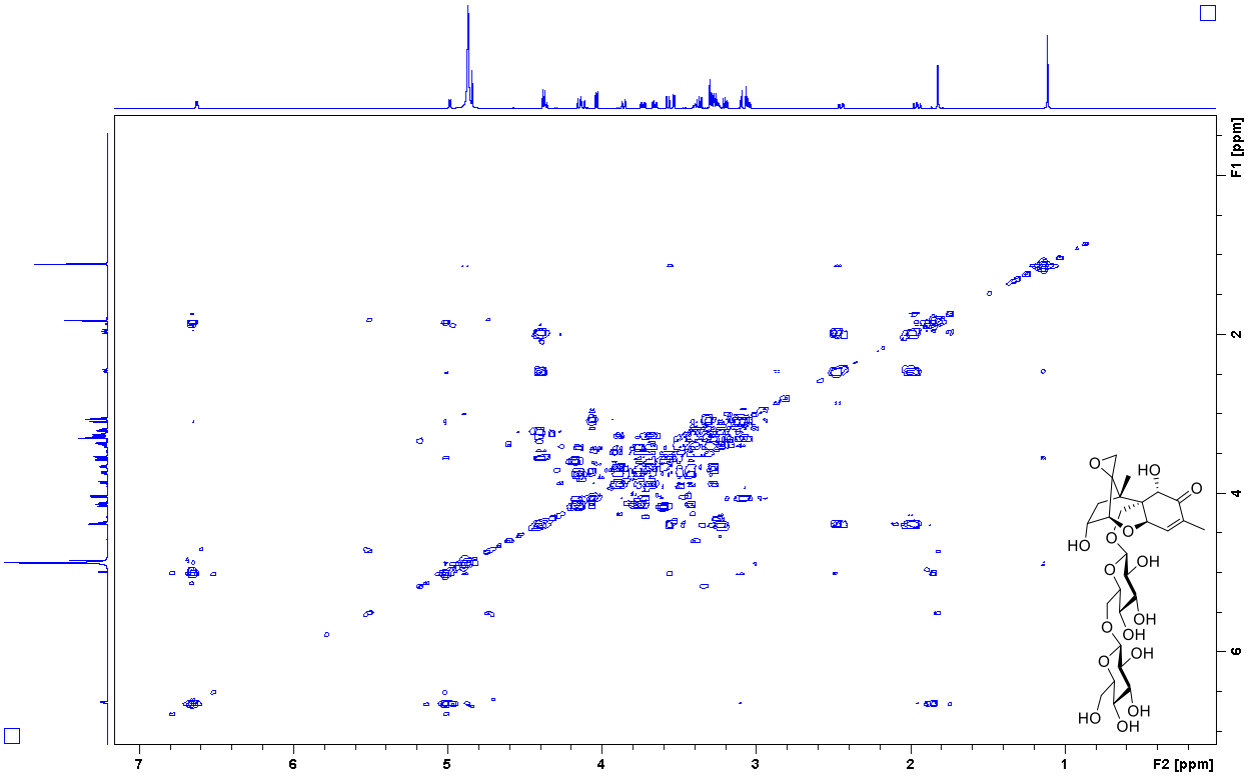
^{1}H NMR (*d*-MeOH, 600 MHz)



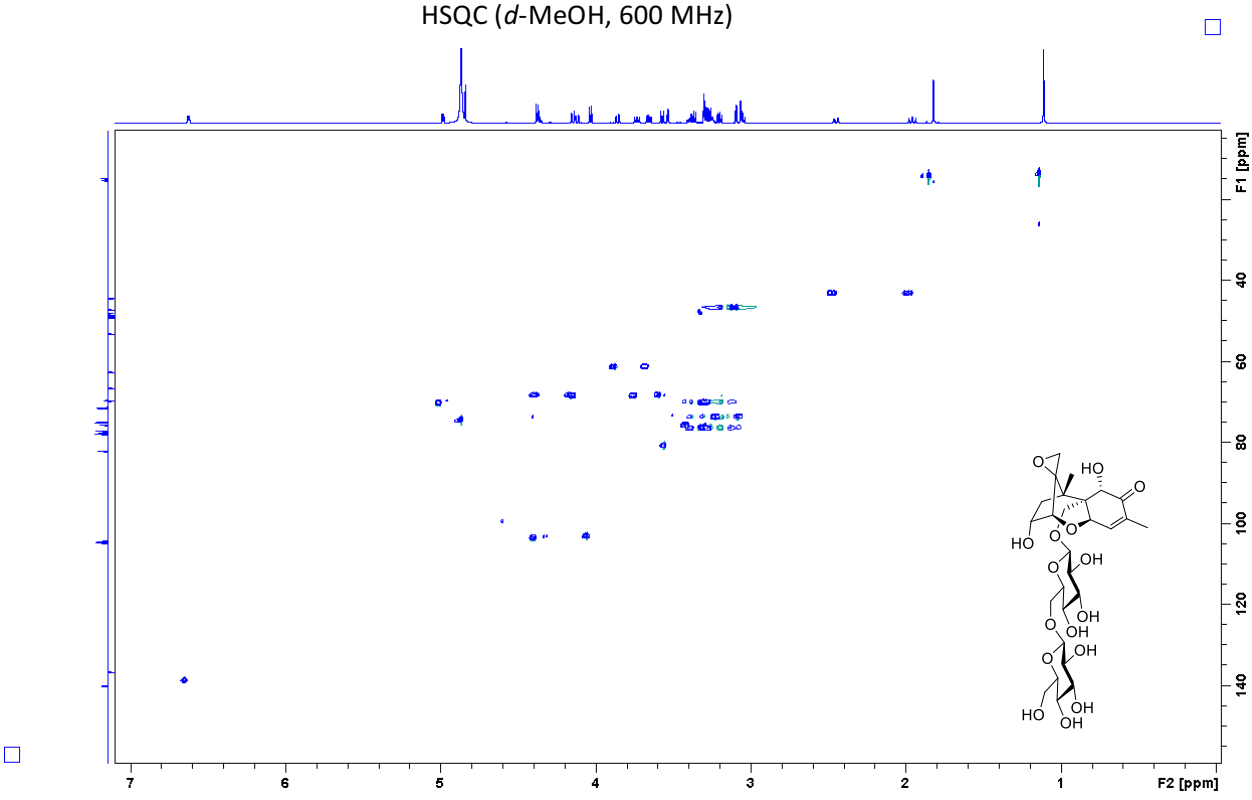
^{13}C NMR (*d*-MeOH, 150 MHz)



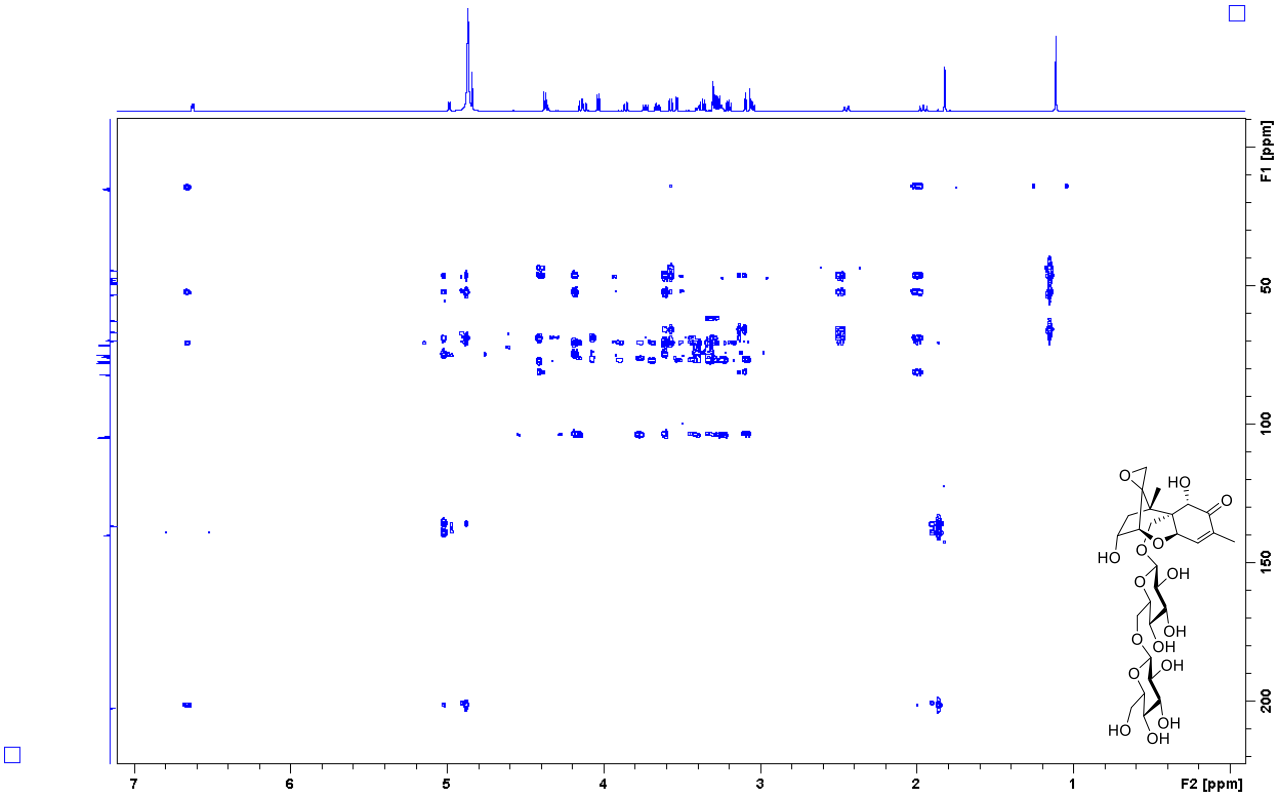
H,H-COSY (*d*-MeOH, 600 MHz)



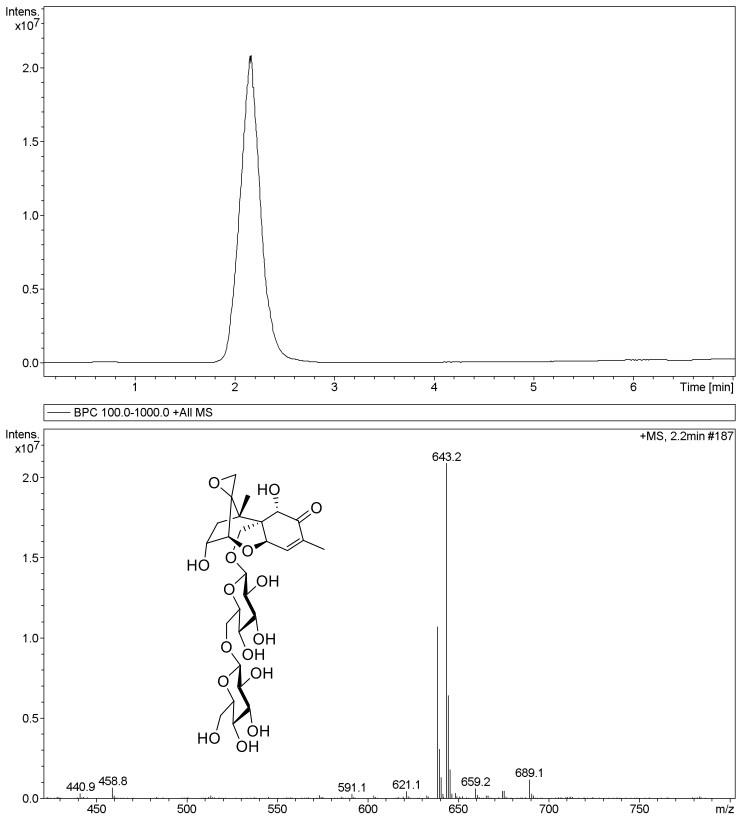
HSQC (*d*-MeOH, 600 MHz)



HMBC (*d*-MeOH, 600 MHz)

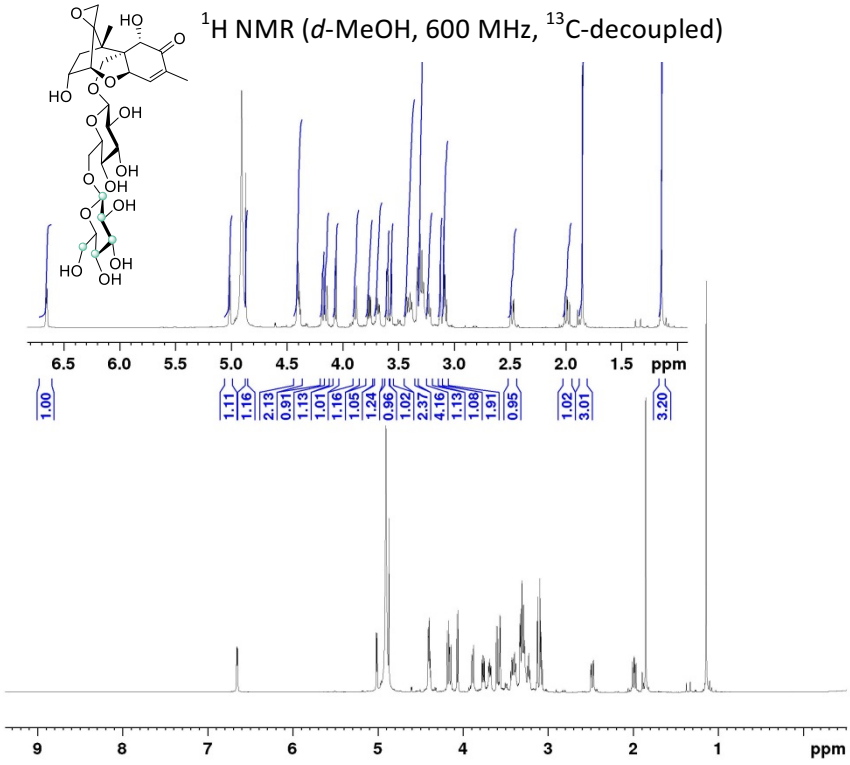


LCMS



Gradient:
Eluent A: ACN+0.1% formic acid;
Eluent B: H_2O+0.1% formic acid;
After an initial hold time at 5% B for 0.6 min, B was linearly increased to 60% within the next 5.0 min and hold at 60% B for 0.4 min.

g) Deoxynivalenol-15-*O*-ß-[$^{13}C_6$]gentiobioside ([$^{13}C_6$]13)

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Current Data Parameters
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EXPNO                     14
PROCNO                     1

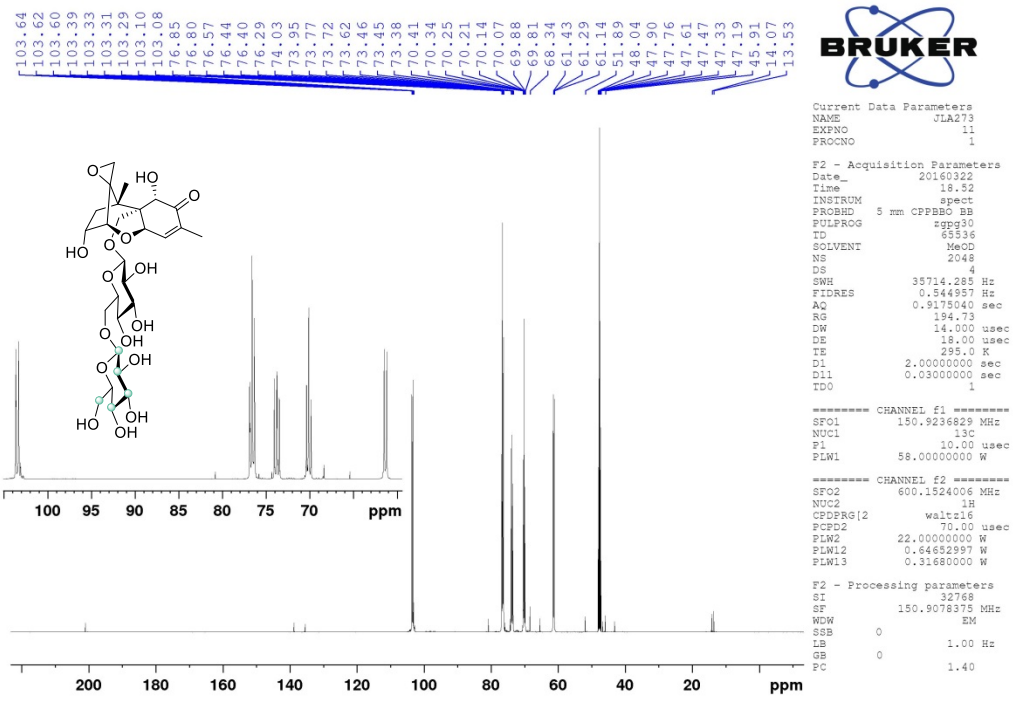
F2 - Acquisition Parameters
Date_              20160322
Time                  12.07
INSTRUM               spect
PROBHD   5 mm CPPBBO BB
PULPROG                zgig
TD                    65536
SOLVENT                MeOD
NS                       16
DS                        2
SWH                9615.385 Hz
FIDRES             0.146719 Hz
AQ                3.4078720 sec
RG                     30.18
DW                   52.000 usec
DE                   28.00 usec
TE                   295.0 K
D1              1.00000000 sec
D11             0.03000000 sec
TD0                        1

======== CHANNEL f1 ========
SFO1          600.1545011 MHz
NUC1                      1H
P1                    12.00 usec
PLW1          22.00000000 W

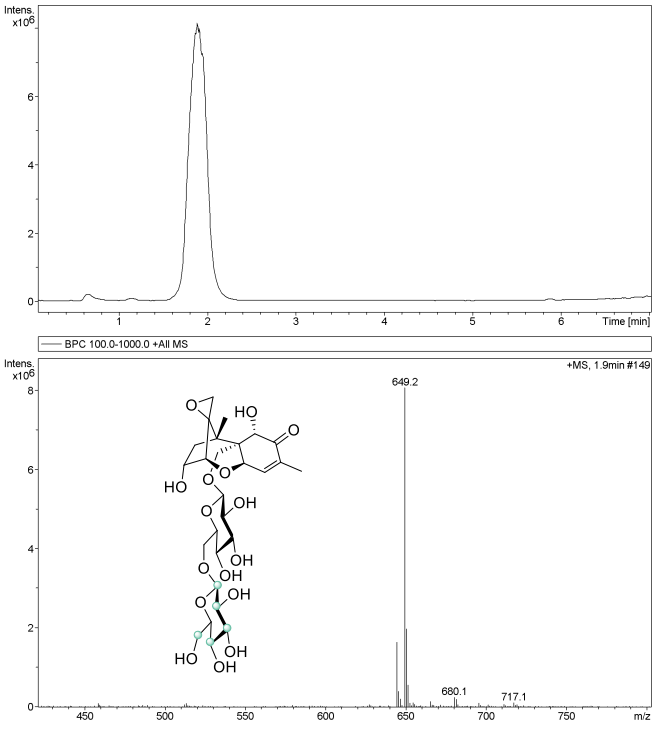
======== CHANNEL f2 ========
SFO2          150.9153834 MHz
NUC2                     13C
CPDPRG[2               garp
PCPD2                 60.00 usec
PLW2          58.00000000 W
PLW12          1.61109996 W

F2 - Processing parameters
SI                       32768
SF            600.1500000 MHz
WDW                         EM
SSB       0
LB                     1.00 Hz
GB        0
PC                     1.40
```

^{13}C NMR (*d*-MeOH, 150 MHz)

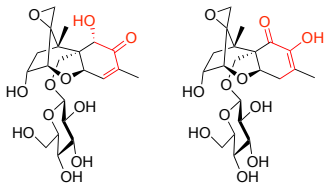


LCMS



Gradient:
Eluent A: ACN+0.1% formic acid;
Eluent B: H_2O+0.1% formic acid;
After an initial hold time at 5% B for 0.6 min, B was linearly increased to 60% within the next 5.0 min and hold at 60% B for 0.4 min.

h) Mixture of deoxynivalenol-15-*O*-ß,D-glucoside (2) and iso-deoxynivalenol-15-*O*-ß,D-glucoside (10)

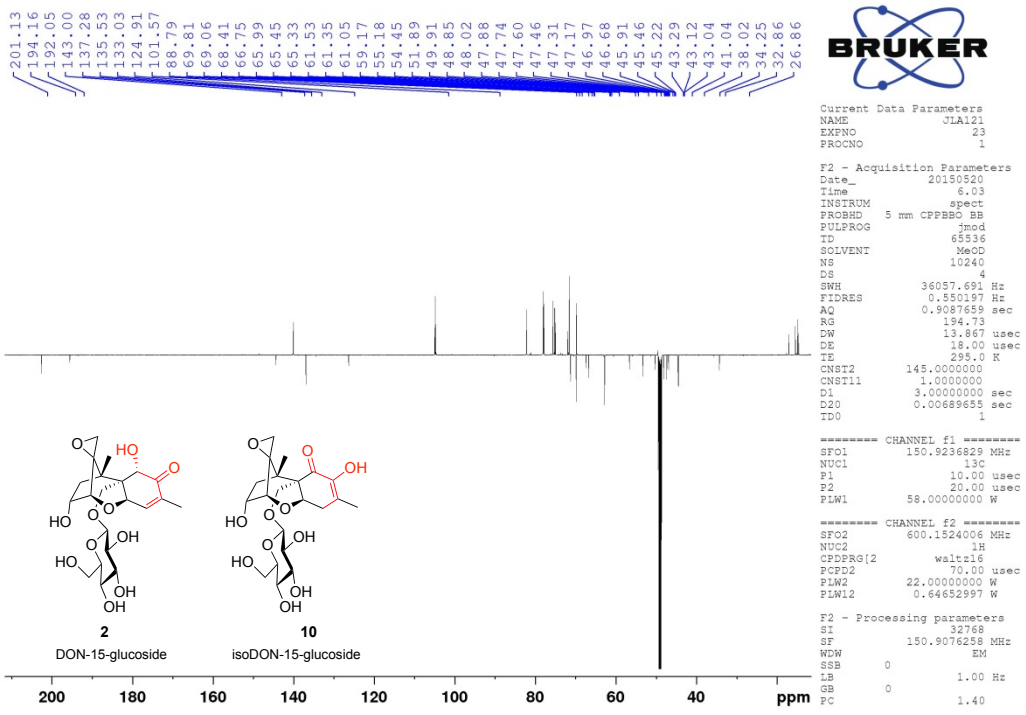


2 DON-15-glucoside

10 isoDON-15-glucoside

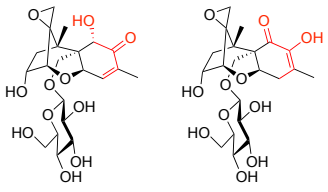
^{1}H NMR (*d*-MeOH, 600 MHz)

^{13}C NMR (*d*-MeOH, 150 MHz)



2 DON-15-glucoside

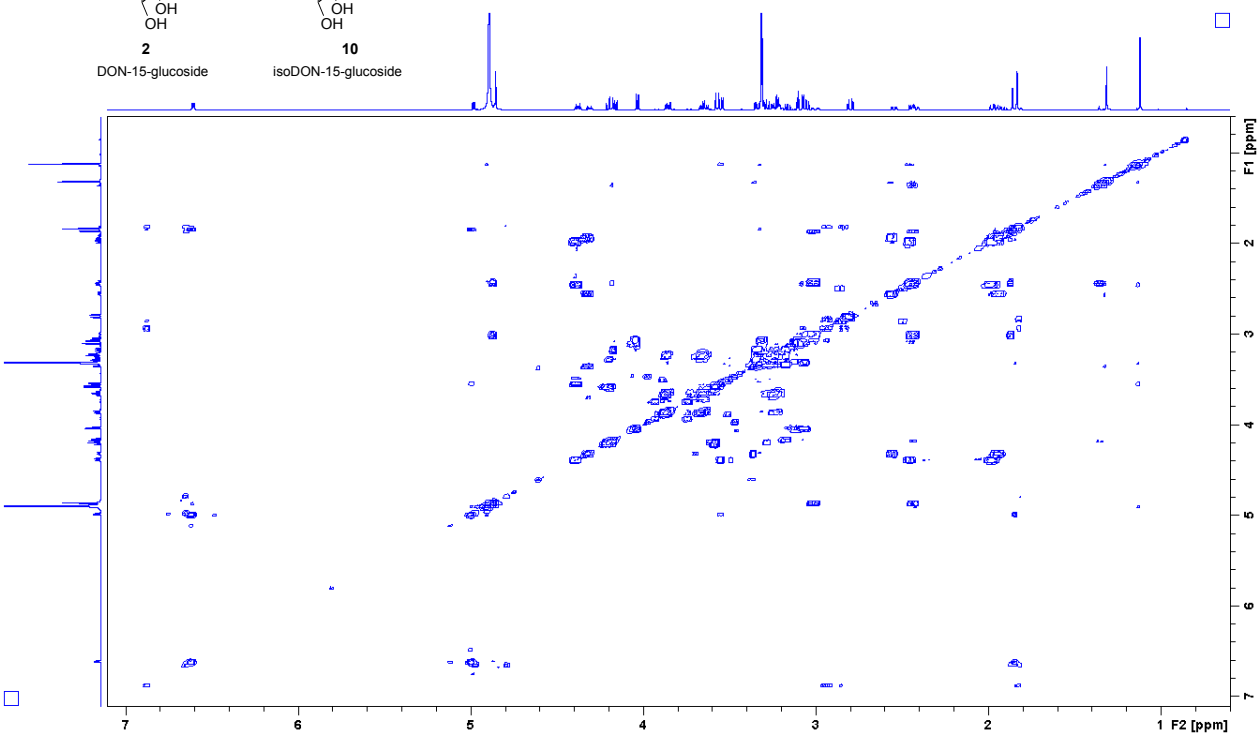
10 isoDON-15-glucoside



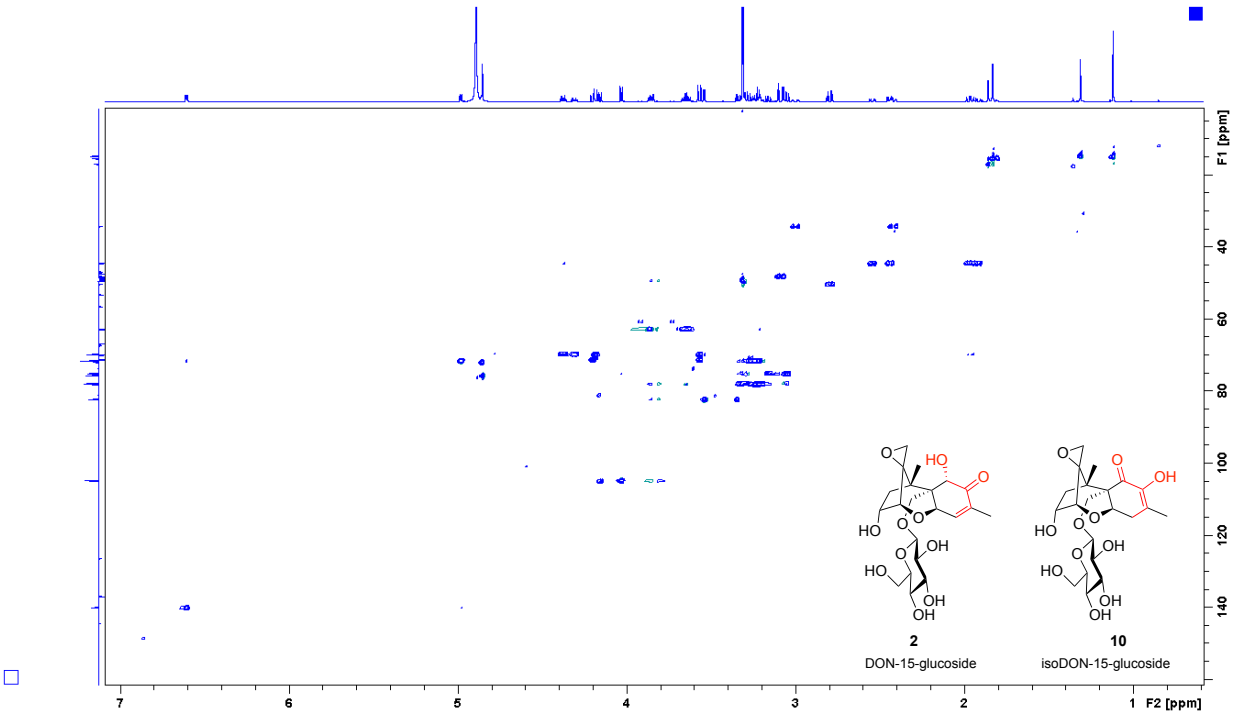
2
DON-15-glucoside

10
isoDON-15-glucoside

H,H-COSY (*d*-MeOH, 600 MHz)

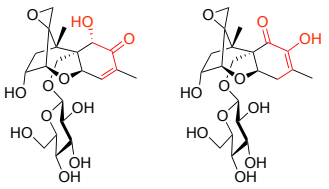


HSQC (*d*-MeOH, 600 MHz)



2
DON-15-glucoside

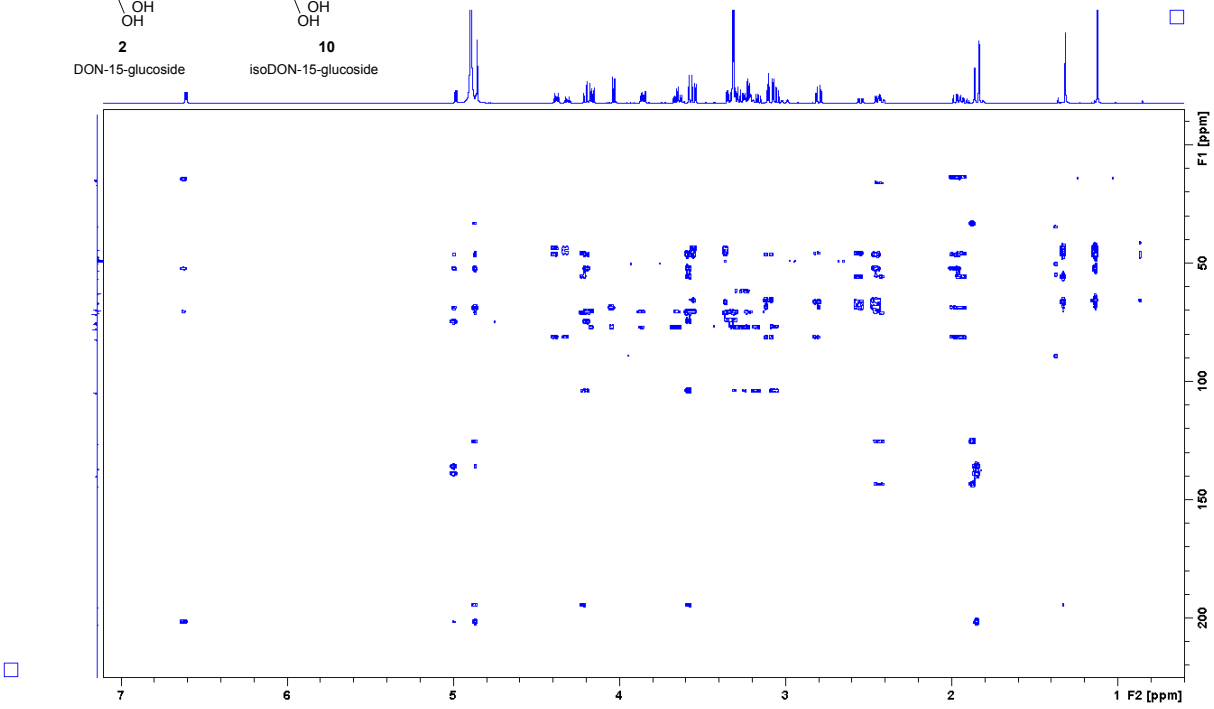
10
isoDON-15-glucoside



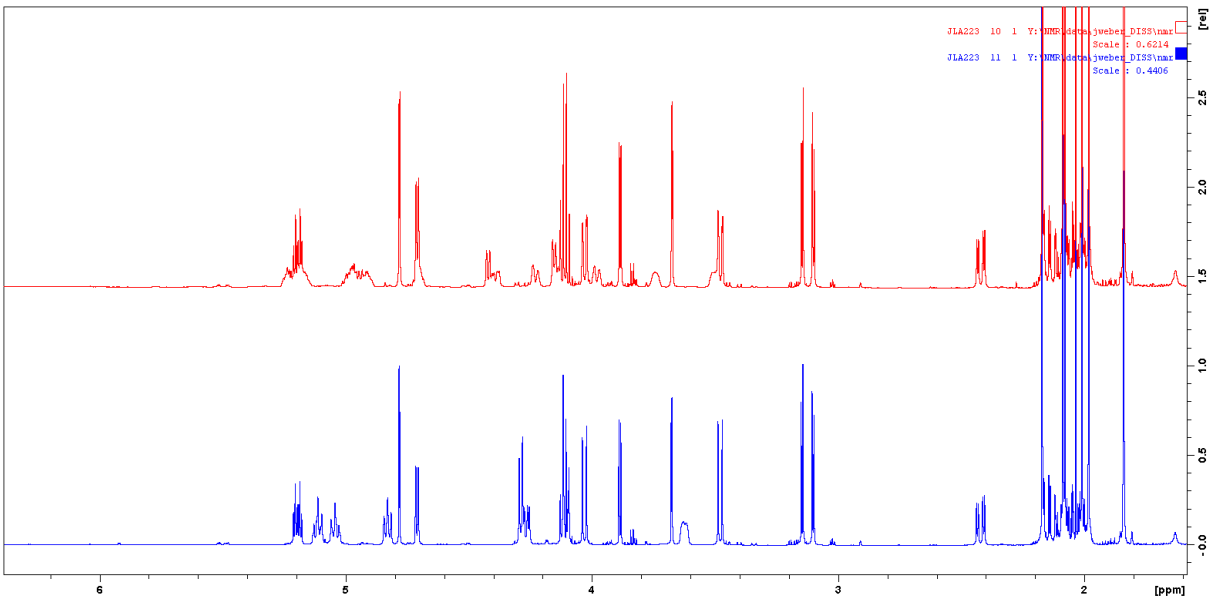
2
DON-15-glucoside

10
isoDON-15-glucoside

HMBC (*d*-MeOH, 600 MHz)



Comparison of spectra of [$^{13}C_6$]**7** recorded in the typical and decoupled mode:



Supporting Information

Manuscript #5

Supplementary Information

Sulfation of deoxynivalenol, its acetylated derivatives and T2-toxin

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Konrad Lorenz Str. 20, A-3430 Tulln, Austria
[c] *University of Natural Resources and Life Sciences, Vienna (BOKU), Dept. of Applied Genetics and Cell Biology, Konrad Lorenz Str. 20, A-3430 Tulln, Austria*
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Table of content

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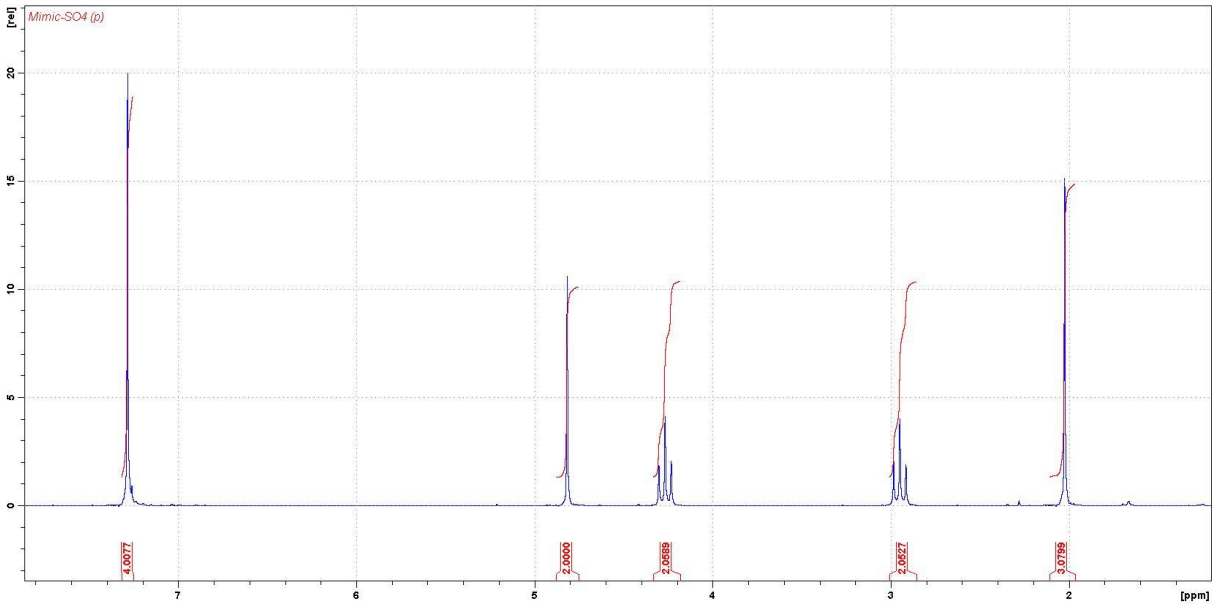
Tel.: +43-1-58801-163737; fax: +43-1-58801-15499

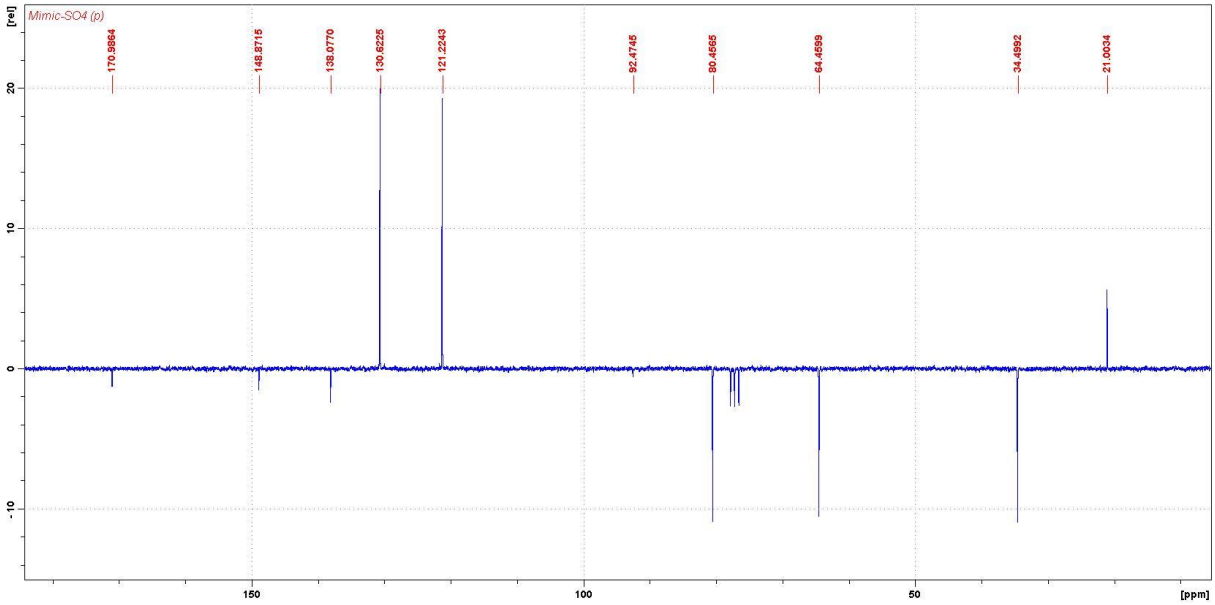
Name / Position	2	3	4 α	4 β	7 α	7 β	8 β	10	11	13 a	13 b	14	15 a	15 b	16	3 Ac/OH	7 Ac/OH	15 Ac/OH	3 TCE	15 TCE
DON (2) in CDCl3	3.63 d [4.5]	4.53 bt	2.23 dd [14.8; 4.6]	2.08 dd [14.9; 10.6]	x	4.84 bs	x	6.62 dq [5.9; 1.4]	4.81 d [5.7]	3.08 d [4.3]	3.17 d [4.3]	1.1 s	3.74 d [11.5]	3.89 d [11.5]	1.9 bs	2.37 d [2.4]	3.86 bs, 1H	1.75 bs, 1H	x	x
DON (2) in MeOD	3.53 d [4.5]	4.37 dt [11.0; 4.5]	2.39 dd [14.6; 4.5]	1.95 dd [14.6; 11.0]	x	4.72 s	x	6.50 dq [5.9; 1.5]	4.93 d [5.7]	3.05 d [4.3]	3.10 d [4.3]	1.1 s	3.73 s, 2H		2 bs	x	x	x	x	x
3 ADON (1) in CDCl3	3.91 d [4.7]	5.23 dt [11.0; 4.5]	2.38 dd [15.1; 4.5]	2.17 dd [15.1; 11.2]	x	4.84 d [1.8]	x	6.61 dq [5.8; 1.5]	4.70 d [5.9]	3.12 d [4.3]	3.18 d [4.3]	1.2 S	3.72 - 3.88 m, 3H		1.90 bs	2.14 s, 3H	3.72 - 3.88 m, 3H	1.67 bt, 1H	x	x
15 ADON (3) in CDCl3	3.60 d [4.5]	4.49 dt [9.8; 4.9]	2.20 dd [14.8; 4.8]	2.06 dd [14.8; 10.5]	x	4.81 bs	x	6.59 dq [5.9; 1.4]	4.88 d [5.7]	3.06 d [4.3]	3.11 d [4.3]	1.1 s	4.22 bs	4.22 bs	1.9 bs	2.92 bs, 1H	3.8 bs, 1H	1.86 bs	x	x
3,15 diADON (4) in CDCl3	3.89 d [4.5]	5.19 dt [10.9; 4.6]	2.30 dd [15.2; 4.8]	2.14 dd [15.2; 10.9]	x	4.79 d [2.0]	x	6.55 dq [5.7; 1.4]	4.68 d [5.7]	3.08 d [4.3]	3.13 d [4.3]	1.1 s	4.19 d [12.1]	4.27 d [12.1]	1.9 bs	2.11 s, 3H	3.79 d [2.0], 1H	1.86 bs	x	x
T2 toxin (5) in CDCl3	3.64 d [4.9]	4.18 dd [4.9; 2.9]	5.34 d [2.9]	x	1.9-2.2 m	2.38 dd [15.1; 5.4]	5.3 d [5.5]	5.78 dt [5.9; 1.2]	4.35 d [6.3]	2.79 d [3.9]	3.05 d [3.9]	0.8 s	4.06 d [12.7]	4.30 d [12.7]	1.7 bs	2.00 - 2.20 2xs, 2x3H, 6H	x	2.00 - 2.20 2xs, 2x3H, 6H	x	x
3 ADON-15-sulfate [p] (6) in CDCl3	3.95 d [4.5]	5.24 ddd [9.5; 6.0; 4.5]	2.10 - 2.36 m, 2 H	2.10 - 2.36 m, 2 H	x	4.89 d [1.4]	x	6.66 dq [5.9; 1.4]	4.8 d [5.9]	3.13 d [4.1]	3.16 d [4.1]	1.1 s	4.43 d [10.6]	4.56 d [10.6]	1.9 bs	2.16 s, 3H	3.84 d [1.4], 1H	x	x	4.64; 4.57 2 x d [10.8]
15 ADON-3-sulfate [p] (11) in CDCl3	4.00 d [4.3]	5.31 dt [11.1; 4.3]	2.64 dd [15.7; 4.3]	2.29 dd [15.7; 11.1]	x	4.81 s	x	6.60 dq [5.8; 1.5]	4.70 d [5.8]	3.15 d [4.1]	3.21 d [4.1]	1.1 s	4.18 d [12.1]	4.27 d [12.1]	1.9 bs	x	3.71 s, 1H	1.93 s	4.79 s, 2H	x
DON-3,15-disulfate [pp] (9) in CDCl3	4.04 d [4.4]	5.32 dt [11.1; 4.4]	2.57 dd [15.7; 4.2]	2.33 dd [15.7; 11.1]	x	4.88 bs	x	6.69 dq [5.9; 1.5]	4.81 dt [5.7; 1.5]	3.17 d [4.1]	3.21 d [4.1]	1.2 s	4.60 d [11.0]	4.66 d [11.0]	1.9 bs	x	3.83 bs, 1H	x	4.79 s, 2H	4.48 s, 2H
DON-3-sulfate [p] (15) in CDCl3	3.99 [4.5]	5.31 dt [11.2; 4.4]	2.80 dd [15.7; 4.3]	2.25 dd [15.7; 11.2]	x	4.81 s	x	6.62 dq [5.9; 1.4]	4.74 d [5.9]	3.14 d [4.1]	3.22 d [4.1]	1.2 s	3.76 d [11.5]	3.86 d [11.5]	1.9 bs	x	3.77 bs, 1H	1.71 bs, 1H	x	4.78 s, 2H
T2 toxin-3-sulfate [p] (21) in CDCl3	3.96 d [4.9]	5.10 dd [4.9; 3.1]	6.16 d [3.1]	x	1.80 d [16.6]	2.35 dd [15.2; 6.0]	5.3 d [5.5]	5.77 dt [5.7; 1.4]	4.25 d [5.7]	2.85 d [3.9]	3.09 d [3.9]	0.7 s	4.1 d [12.7]	4.31 d [12.7]	1.8 bs	2.00 - 2.20 2xs, 2x3H, 6H	x	2.00 - 2.20 2xs, 2x3H, 6H	4.80 s, 2H	x
3 ADON-15-sulfate (13) in MeOD	3.85 d [4.5]	5.11 dt [11.3; 4.4]	2.79 dd [15.3; 4.3]	2.08 dd [15.3; 11.3]	x	4.89 s	x	6.63 dq [6.1; 1.4]	4.92 d [6.1]	3.12 d [4.3]	3.16 d [4.3]	1.2 s	3.94 d [11.0]	4.27 d [11.0]	1.9 bs	2.13 s, 3H	x	x	x	x
15 ADON-3-sulfate (18) in MeOD	3.82 d [4.5]	4.70-5.10 m [+NH4, H2O]	2.63 dd [15.3; 4.3]	2.11 dd [15.3; 11.2]	x	4.70-5.10 m [+NH4, H2O]	x	6.65 dq [5.9; 1.5]	4.70-5.10 m [+NH4, H2O]	3.12 s	3.12 s	1.1 s	4.23 d [12.1]	4.30 d [12.1]	1.9 bs	x	x	1.90 s, 3H	x	x
DON-3-sulfate (15) in MeOD	3.80 d [4.4]	4.90 dt [11.2; 4.5]	2.75 dd [15.2; 4.4]	2.06 dd [15.2; 11.4]	x	4.79 s	x	6.61 dq [6.1; 1.4]	4.86 d [5.8]	3.09 d [4.5]	3.12 d [4.5]	1.1 s	3.68 d [12.3]	3.78 d [12.3]	1.8 bs	x	x	x	x	x
DON-15-sulfate (14) in MeOD	3.55 d [4.5]	4.76 dt [11.1; 4.45]	2.57 dd [14.8; 4.4]	1.99 dd [14.8; 11.1]	x	4.85-4.95 m [+NH4, H2O]	x	6.65 dq [6.1; 1.4]	5.04 d [6.1]	3.06 d [4.5]	3.12 d [4.5]	1.1 s	3.96 d [11.0]	4.25 d [11.0]	1.8 bs	x	x	x	x	x
DON-3,15-disulfate (16) in MeOD	3.84 d [4.5]	4.80 - 5.00 m [+NH4, H2O]	2.97 dd [15.3; 4.5]	2.10 dd [15.3; 11.4]	x	4.80-5.00 m [+NH4, H2O]	x	6.67 dq [6.0; 1.5]	4.80-5.00 m [+NH4, H2O]	3.10 d [4.3]	3.15 d [4.3]	1.2 s	4.24 d [10.9]	3.98 d [10.9]	1.9 bs	x	x	x	x	x
T2 toxin-3-sulfate (22) in MeOD	3.79 d [5.1]	5.10 dd [4.9; 3.1]	6.03 d [3.1]	x	1.92 d [16.6]	2.38 dd [15.3; 5.9]	5.3 d [5.5]	5.78 d [5.5]	4.32 d [5.5]	2.87 d [3.9]	3.04 d [3.9]	0.7 s	4.16 d [12.1]	4.33 d [12.1]	1.7 bs	2.00 - 2.20 2xs, 2x3H, 6H	x	2.00 - 2.20 2xs, 2x3H, 6H	x	x

28.) 2,2,2-Trichloroethyl 2-(4-acetoxyphenyl)ethylsulfate

^{1}H NMR (200 MHz, $CDCl_3$) δ 7.28 (s, 4H), 4.82 (s, 2H), 4.27 (t, *J*=6.8 Hz, 2H), 2.95 (t, *J*=6.8 Hz, 2H), 2.02 (s, 3H)

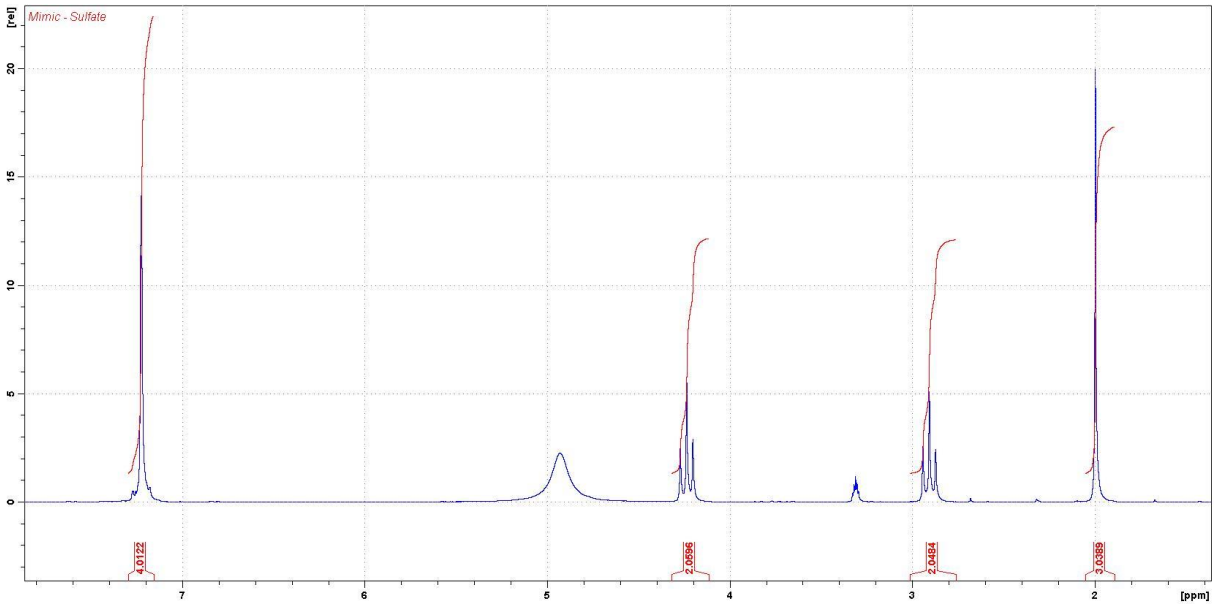


^{13}C NMR (50 MHz, $CDCl_3$) δ 171.0 (s, 1C), 148.9 (s, 1C), 138.1 (s, 1C), 130.6 (d, 2C), 121.2 (d, 2C), 92.5 (s, 1C), 80.5 (t, 1C), 64.5 (t, 1C), 34.5 (t, 1C), 21.0 (q, 1C)

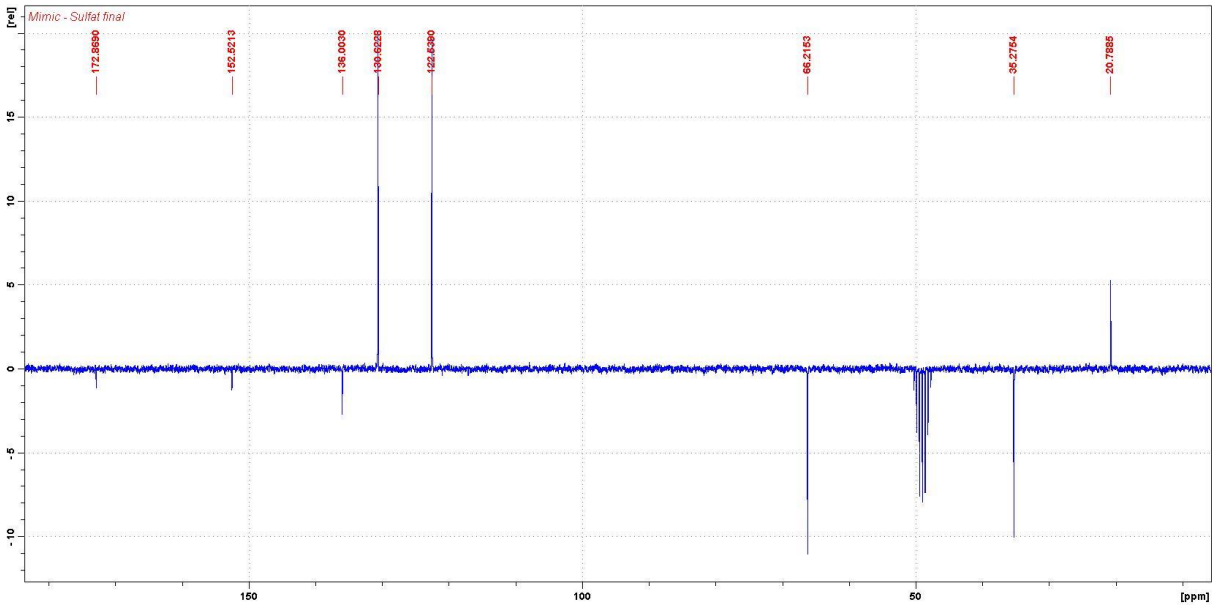


29.) 2-(4-Acetoxyphenyl)ethylsulfate, ammonium salt

^{1}H NMR (200 MHz, methanol-d_4) δ = 7.22 (b, 4H), 4.93 (b, NH_4^+, H_2O), 4.23 (t, *J*=6.9, 2H), 2.91 (t, *J*=6.9 Hz, 2H), 2.00 (s, 3H);

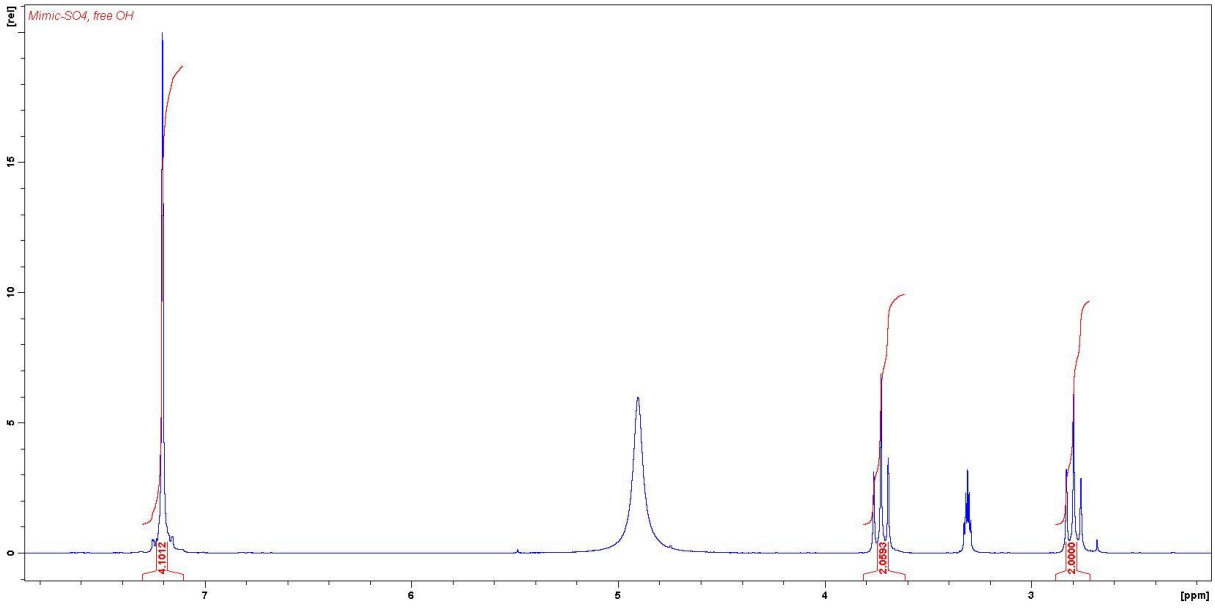


^{13}C NMR (50 MHz, methanol-d_4) δ 172.9 (s, 1C), 152.5 (s, 1C), 136.0 (s, 1C), 130.6 (d, 2C), 122.5 (d, 2C), 66.2 (t, 1C), 35.3 (t, 1C), 20.8 (q, 1C)

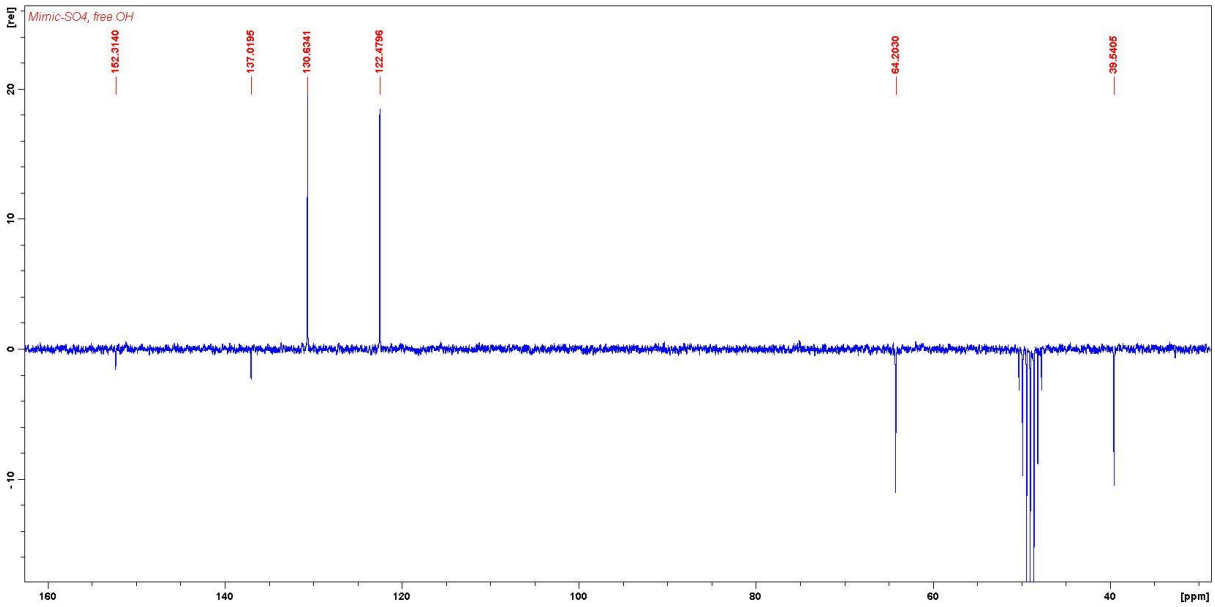


30.) 2-(4-Hydroxyphenyl)ethylsulfate, ammonium salt

^{1}H NMR (200 MHz, methanol-d_4) δ = 7.21 (s, 4H), 4.90 (b, NH_4^+, H_2O), 3.73 (t, *J*=6.9, 2H), 2.80 (t, *J*=6.9 Hz, 2H)

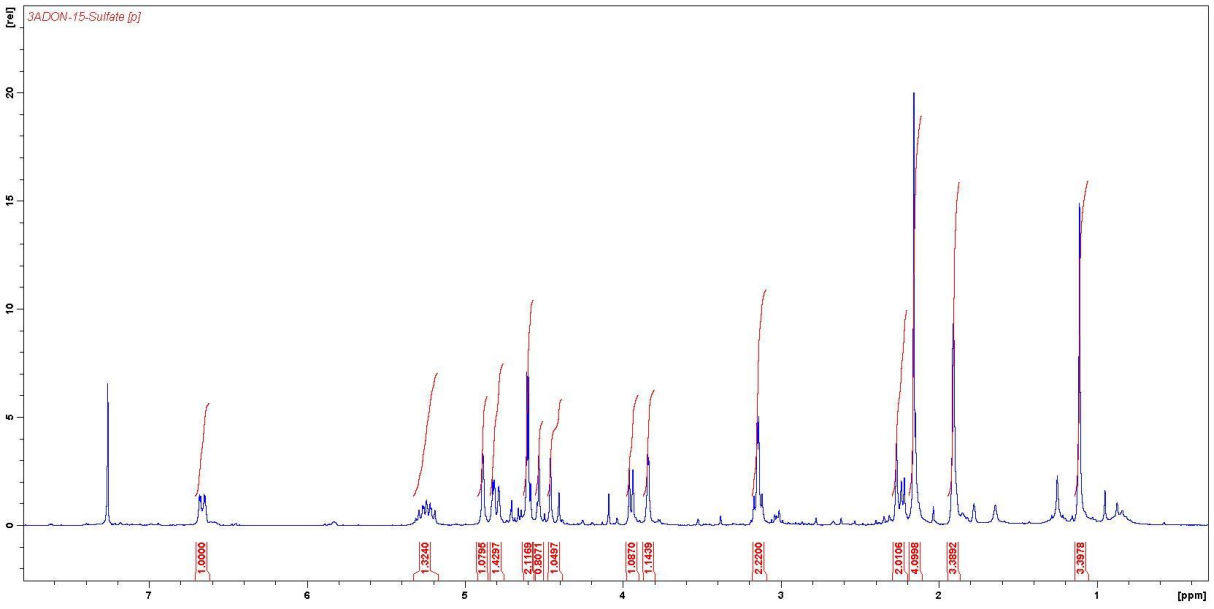


^{13}C NMR (50 MHz, methanol-d_4) δ 152.3 (s, 1C), 137.0 (s, 1C), 130.6 (d, 2C), 122.5 (d, 2C), 64.2 (t, 1C), 39.5 (t, 1C)

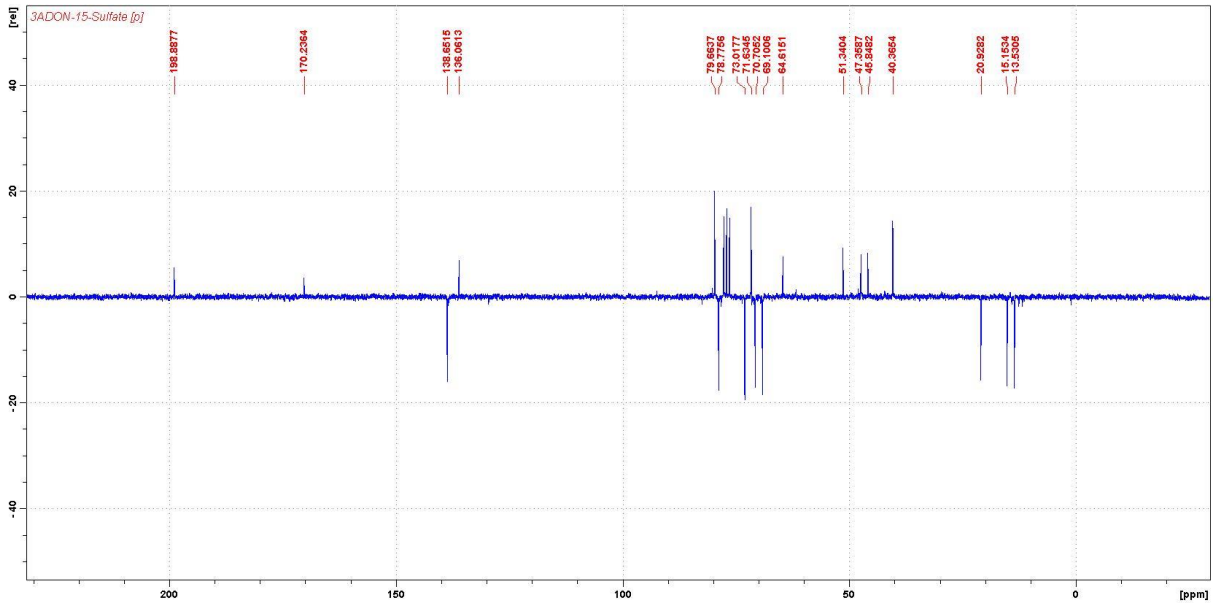


6.) 2,2,2-Trichloroethyl 3-acetyl-DON-15-sulfate

^{1}H NMR (200 MHz, $CDCl_3$) δ 6.66 (dq, *J*=5.9, 1.4 Hz, 1H), 5.24 (ddd, *J*=9.5, 6.0, 4.5 Hz, 1H), 4.89 (d, *J*=1.4 Hz, 1H), 4.80 (d, *J*=5.9 Hz, 1H), 4.64 (d, *J*=10.8 Hz, 1H), 4.57 (d, *J*=10.8 Hz, 1H), 4.56 (d, *J*=10.6 Hz, 1H), 4.43 (d, *J*=10.6 Hz, 1H), 3.95 (d, *J*=4.5 Hz, 1H), 3.84 (d, *J*=1.4 Hz, 1H), 3.16 (d, *J*=4.1 Hz, 1H), 3.13 (d, *J*=4.1 Hz, 1H), 2.10-2.36 (m, 2H), 2.16 (s, 3H), 1.91 (b, 3H), 1.11 (s, 3H)

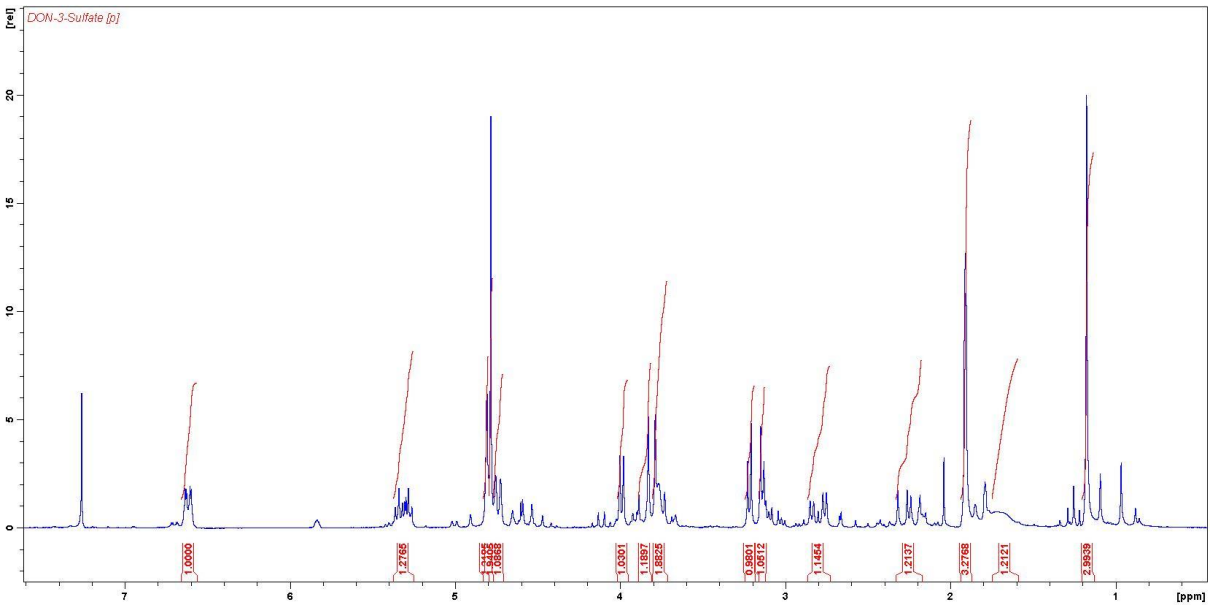


^{13}C-NMR (50 MHz, $CDCl_3$) δ 198.8 (s, 1C), 170.2 (s, 1C), 138.7 (d, 1C), 136.1 (s, 1C), 92.4 (s, 1C, -CCl_3 tiny signal!), 79.7 (t, 1C), 78.8 (d, 1C), 73.0 (d, 1C), 71.6 (t, 1C), 70.7 (d, 1C), 69.1 (d, 1C), 64.6 (s, 1C), 51.3 (s, 1C), 47.4 (t, 1C), 45.8 (s, 1C), 40.4 (t, 1C), 20.9 (q, 1C), 15.2 (q, 1C), 13.5 (q, 1C)

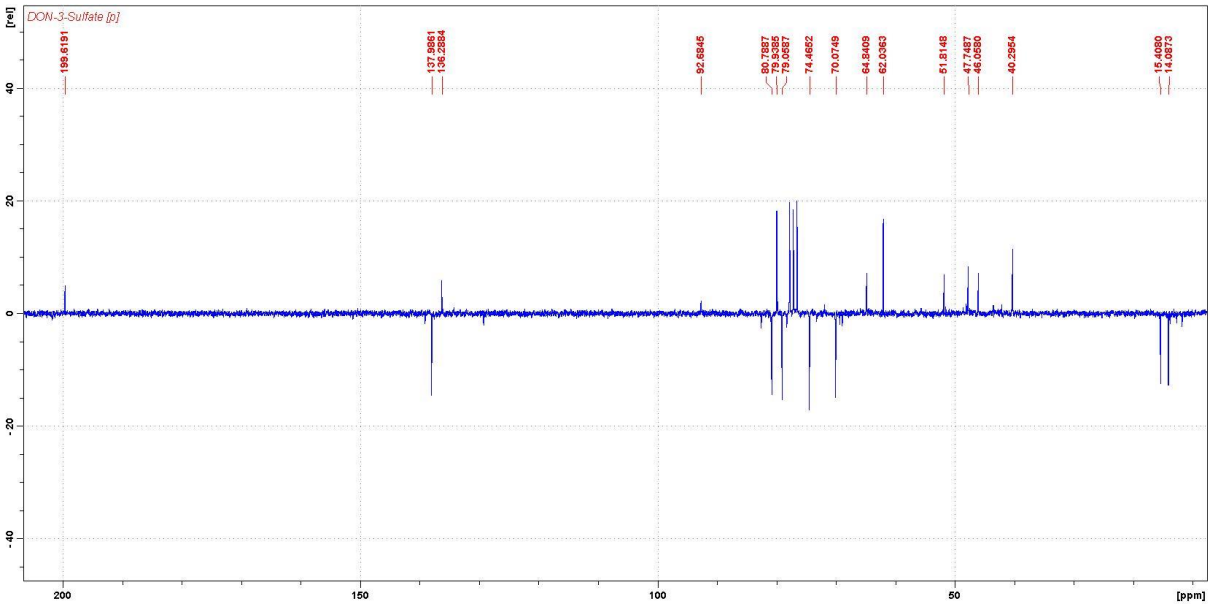


8.) 2,2,2-Trichloroethyl DON-3-sulfate

^{1}H NMR (200 MHz, $CDCl_3$) δ 6.62 (dq, *J*=5.9, 1.4 Hz, 1H), 5.31 (dt, *J*=11.2, 4.4, 1H), 4.81 (s, 1H), 4.78 (s, 2H), 4.74 (d, *J*=5.9 Hz, 1H), 3.99 (d, *J*=4.5 Hz, 1H), 3.86 (d, *J*=11.5 Hz, 1H), 3.77 (b, 1H), 3.76 (d, *J*=11.5 Hz, 1H), 3.22 (d, *J*=4.1 Hz, 1H), 3.14 (d, *J*=4.1 Hz, 1H), 2.80 (dd, *J*=15.7, 4.3 Hz, 1H), 2.25 (dd, *J*=15.7, 11.2 Hz, 1H), 1.91 (b, 3H), 1.71 (b, 1H), 1.17 (s, 3H)

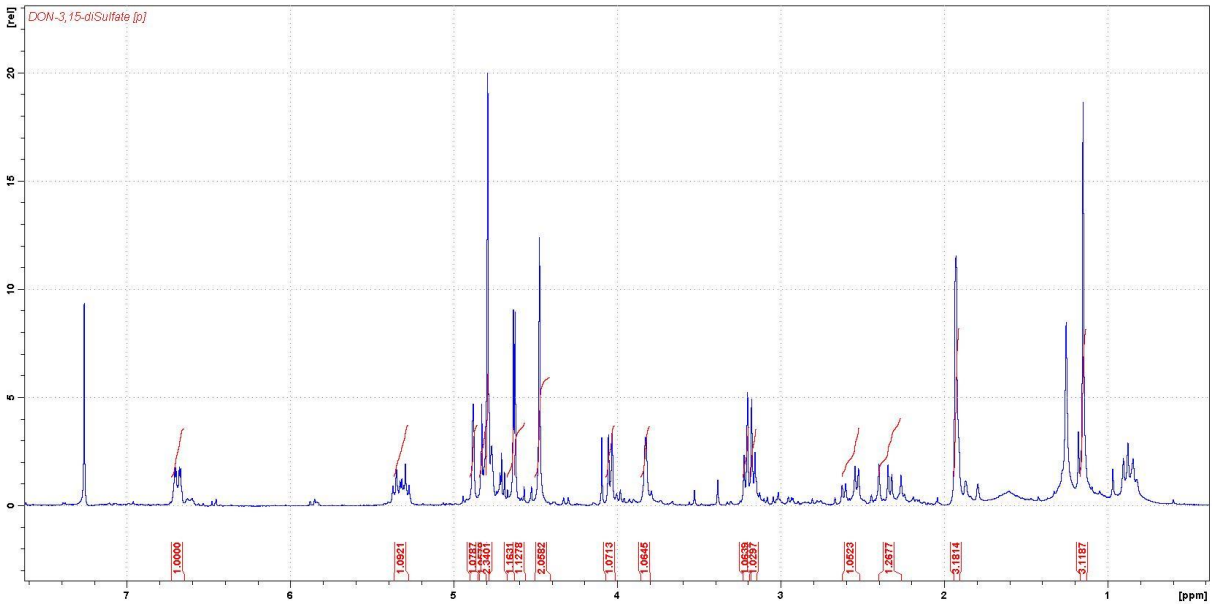


^{13}C-NMR (50 MHz, $CDCl_3$) δ 199.6 (s, 1C), 138.0 (d, 1C), 136.3 (s, 1C), 92.7 (s, 1C, -CCl_3 tiny signal!), 80.8 (d, 1C), 79.9 (t, 1C), 79.1 (d, 1C), 74.5 (d, 1C), 70.1 (d, 1C), 64.8 (s, 1C), 62.0 (t, 1C), 51.8 (s, 1C), 47.7 (t, 1C), 46.1 (s, 1C), 40.3 (t, 1C), 15.4 (q, 1C), 14.1 (q, 1C)

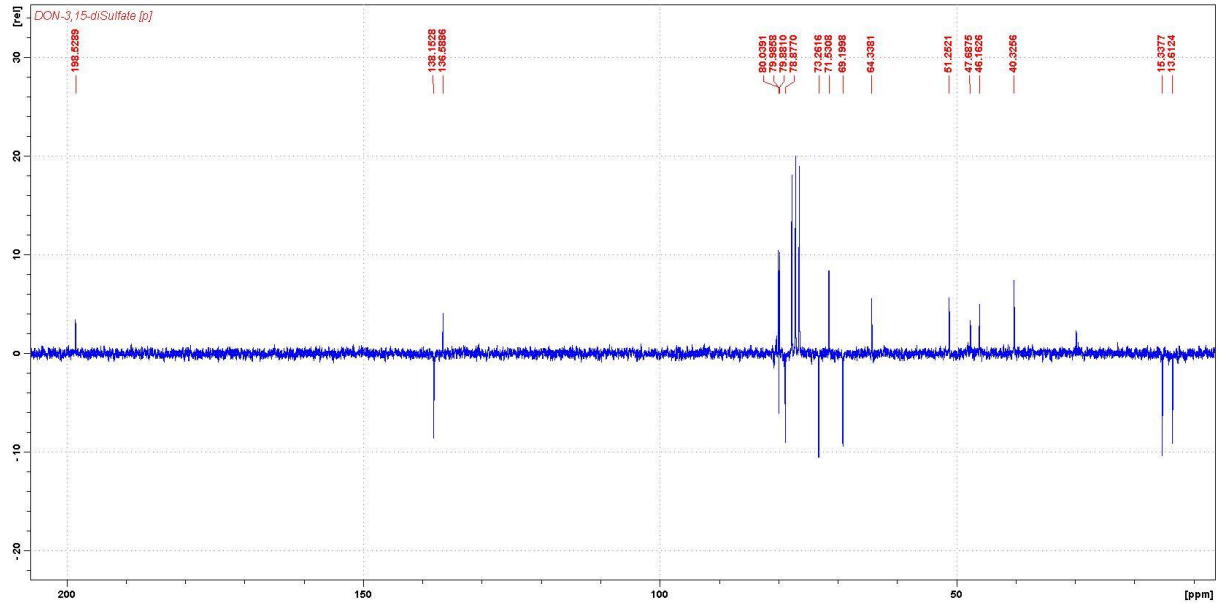


9.) bis(2,2,2-Trichloroethyl) DON-3,15-disulfate

^{1}H NMR (200 MHz, $CDCl_3$) δ 6.69 (dq, *J*=5.9, 1.5 Hz, 1H), 5.32 (dt, *J*=11.1, 4.4, 1H), 4.88 (s, 1H), 4.81 (dt, *J*=5.7, 1.5 Hz, 1H), 4.79 (s, 2H), 4.66 (d, *J*=11.0 Hz, 1H), 4.60 (d, *J*=11.0 Hz, 1H), 4.48 (s, 2H), 4.04 (d, *J*=4.4 Hz, 1H), 3.83 (b, 1H), 3.21 (d, *J*=4.1 Hz, 1H), 3.17 (d, *J*=4.1 Hz, 1H), 2.57 (dd, *J*=15.7, 4.2 Hz, 1H), 2.33 (dd, *J*=15.7, 11.1 Hz, 1H), 1.93 (b, 3H), 1.15 (s, 3H)

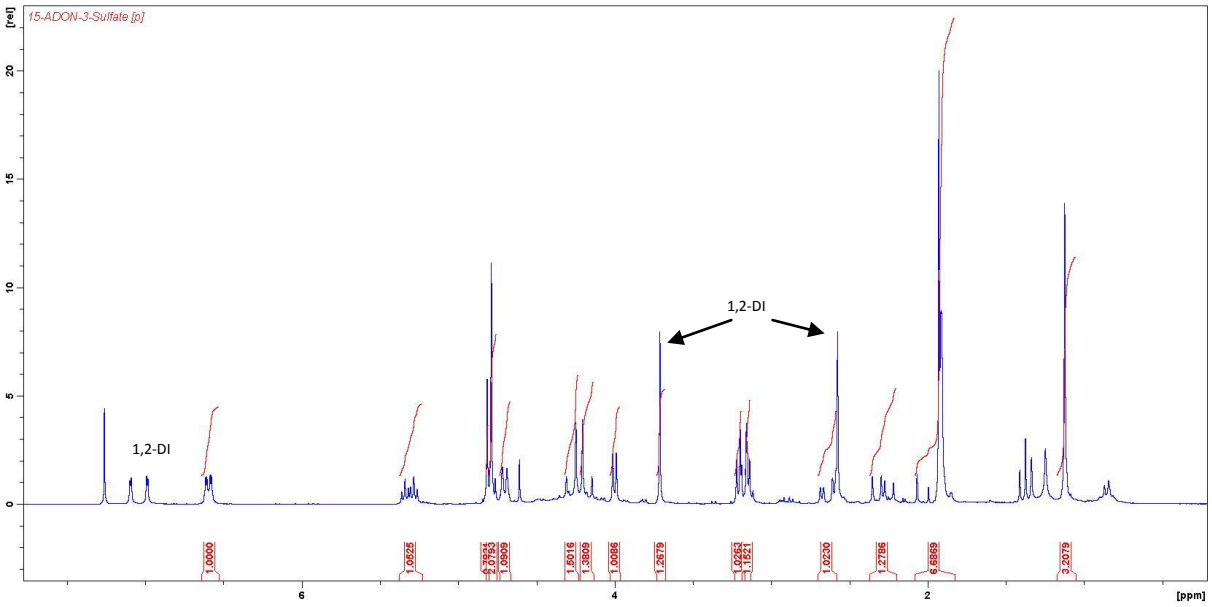


^{13}C-NMR (50 MHz, $CDCl_3$) δ 198.5 (s, 1C), 138.2 (d, 1C), 136.6 (s, 1C), 2 x 80.0 (d, 1C/t, 1C), 79.9 (t, 1C), 78.9 (d, 1C), 73.3 (d, 1C), 71.5 (t, 1C), 69.2 (d, 1C), 64.3 (s, 1C), 51.3 (s, 1C), 47.7 (t, 1C), 46.2 (s, 1C), 40.3 (t, 1C), 15.3 (q, 1C), 13.6 (q, 1C), 2 x CCl_3 between 92 and 93 ppm are missing, but the corresponding CH_2 groups are located at 80.0 and 79.9

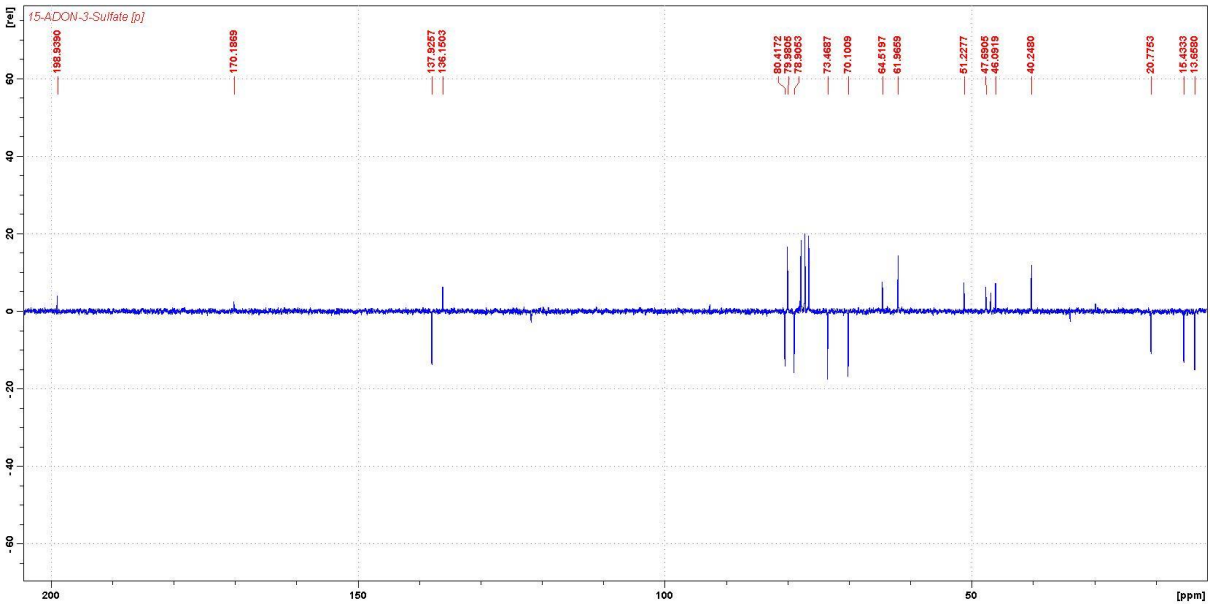


11.) 2,2,2-Trichloroethyl 15-acetyl-DON-3-sulfate

^{1}H NMR (200 MHz, $CDCl_3$) δ 6.60 (dq, *J*=5.8, 1.5 Hz, 1H), 5.31 (dt, *J*=11.1, 4.3, 1H), 4.81 (s, 1H), 4.79 (s, 2H), 4.70 (d, *J*=5.8 Hz, 1H), 4.27 (d, *J*=12.1 Hz, 1H), 4.18 (d, *J*=12.1 Hz, 1H), 4.00 (d, *J*=4.3 Hz, 1H), 3.71 (s, 1H), 3.21 (d, *J*=4.1 Hz, 1H), 3.15 (d, *J*=4.1 Hz, 1H), 2.64 (dd, *J*=15.7, 4.3 Hz, 1H), 2.29 (dd, *J*=15.7, 11.1 Hz, 1H), 1.93 (s, 3H), 1.91 (b, 3H), 1.12 (s, 3H); 1,2-DI = traces of 1,2-dimethylimidazole

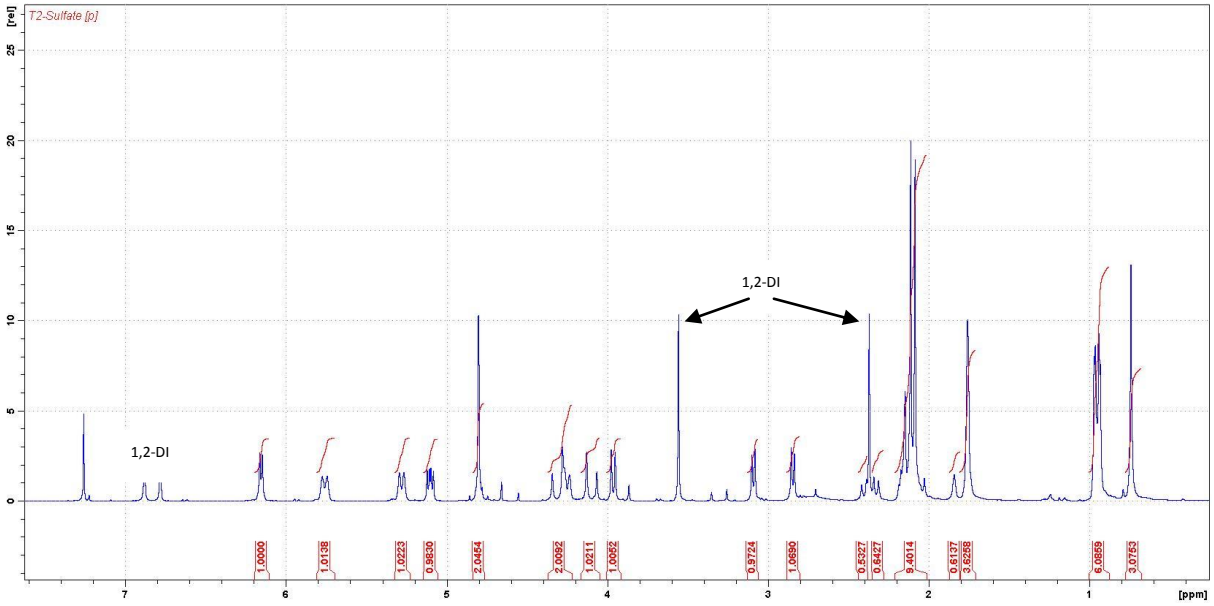


^{13}C-NMR (50 MHz, $CDCl_3$) δ 198.9 (s, 1C), 170.2 (s, 1C), 137.9 (d, 1C), 136.1 (s, 1C), 92.7 (s, 1C, -CCl_3 tiny signal!), 80.4 (d, 1C), 80.0 (t, 1C), 78.9 (d, 1C), 73.5 (d, 1C), 70.1 (d, 1C), 64.5 (s, 1C), 62.0 (t, 1C), 51.2 (s, 1C), 47.4 (t, 1C), 46.1 (s, 1C), 40.2 (t, 1C), 20.8 (q, 1C), 15.4 (q, 1C), 13.7 (q, 1C)

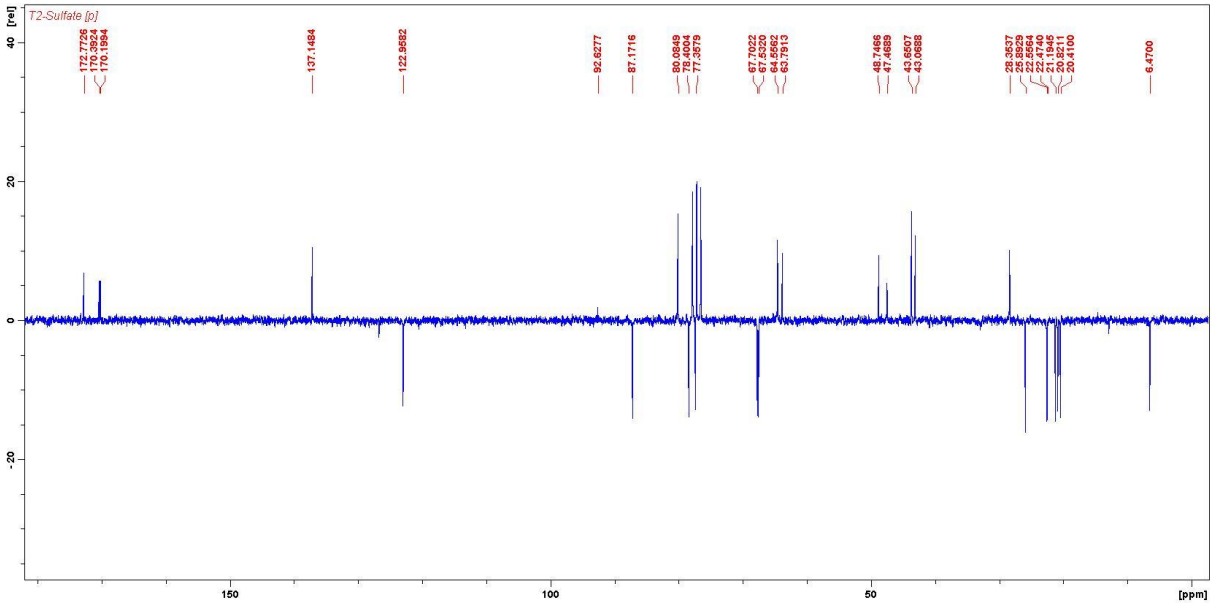


21.) 2,2,2-Trichloroethyl T2-toxin-3-sulfate

^{1}H NMR (200 MHz, $CDCl_3$) δ 6.16 (d, *J*=3.1 Hz, 1H), 5.77 (dt, *J*=5.7, 1.4 Hz, 1H), 5.28 (d, *J*=5.5 Hz, 1H), 5.10 (dd, *J*=4.9, 3.1 Hz, 1H), 4.80 (s, 2H), 4.31 (d, *J*=12.7 Hz, 1H), 4.25 (d, *J*=5.7 Hz, 1H), 4.10 (d, *J*=12.7 Hz, 1H), 3.96 (d, *J*=4.9 Hz, 1H), 3.09 (d, *J*=3.9 Hz, 1H), 2.85 (d, *J*=3.9 Hz, 1H), 2.35 (dd, *J*=15.2, 6.0 Hz, 1H), 2.11 (s, 3H), 2.09 (s, 3H), 2.00-2.25 (m, 3H), 1.80 (d, *J*=16.6 Hz, 1H), 1.76 (s, 3H), 0.96 (d, *J*=6.3 Hz, 3H), 0.95 (d, *J*=6.3 Hz, 3H), 0.74 (s, 3H); 1,2-DI = traces of 1,2-dimethylimidazole

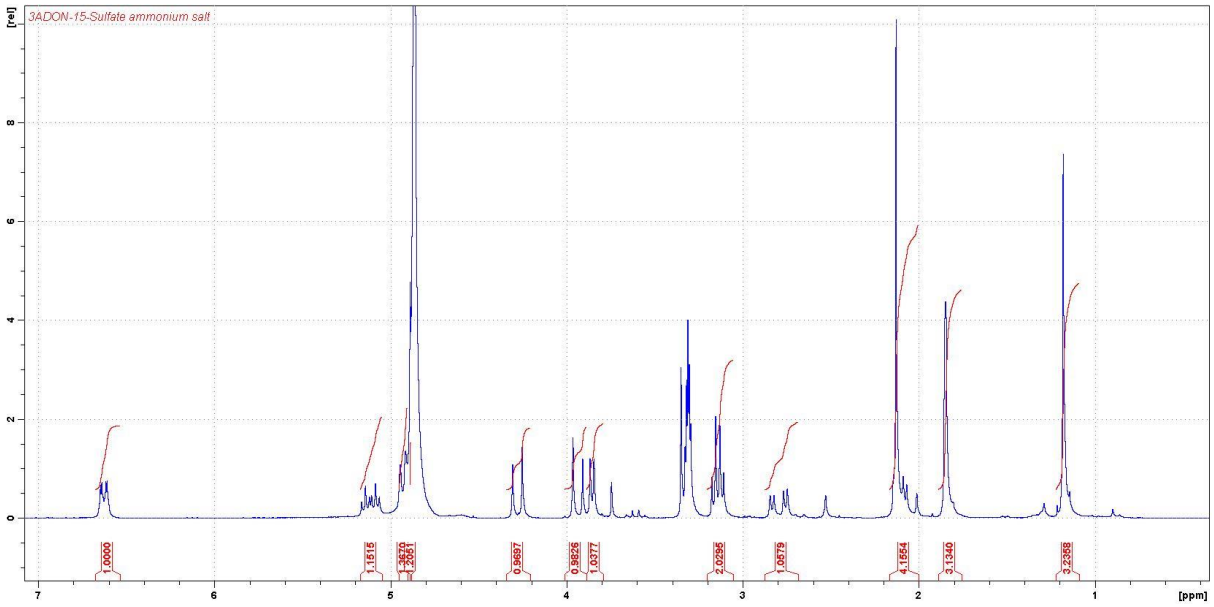


^{13}C NMR (50 MHz, $CDCl_3$) δ 172.8 (s, 1C), 170.4 (s, 1C), 170.2 (s,1C), 137.1 (s, 1C), 123.0 (d, 1C), 92.6 (s, 1C), 87.1 (d, 1C), 80.1 (t, 1C), 78.4 (d, 1C), 77.4 (d, 1C), 67.7 (d, 1C), 67.5 (d, 1C), 64.6 (t, 1C), 63.8 (s, 1C), 48.7 & 47.5 (1t, 1s, 2x1C), 43.7 (t, 1C), 43.1 (s, 1C), 28.4 (t, 1C), 25.9 (d, 1C), 22.6 (q, 1C), 22.5 (q, 1C), 21.2 (q, 1C), 20.8 (q, 1C), 20.4 (q, 1C), 6.5 (q, 1C)

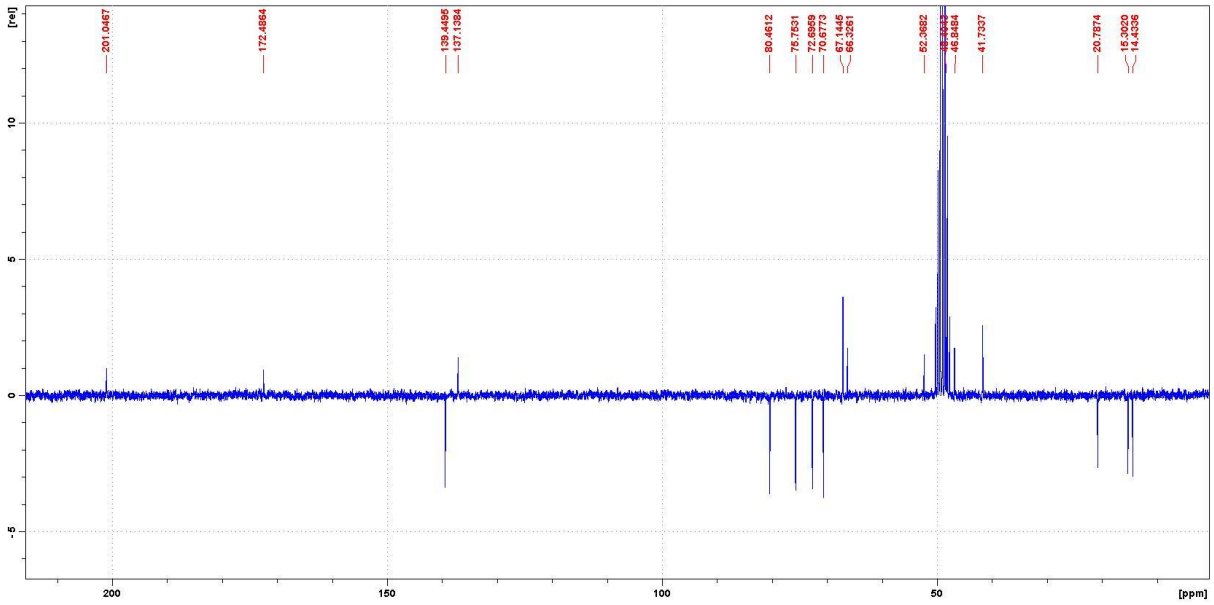


13.) 3-Acetyl-DON-15-sulfate, ammonium salt

^{1}H NMR (200 MHz, methanol-d_4) δ 6.63 (dq, *J*=6.1, 1.4 Hz, 1H), 5.11 (dt, *J*=11.3, 4.4 Hz, 1H), 4.92 (d, *J*=6.1 Hz, 1H), 4.89 (s, 1H), 4,87 (b, NH_4^+, H_2O) 4.27 (d, *J*=11.0 Hz, 1H), 3.94 (d, *J*=11.1 Hz, 1H), 3.85 (d, *J*=4.5 Hz, 1H), 3.16 (d, *J*=4.3 Hz, 1H), 3.12 (d, *J*=4.3 Hz, 1H), 2.79 (dd, *J*=15.3, 4.3 Hz, 1H), 2.08 (dd, *J*=15.3, 11.3 Hz, 1H), 2.13 (s, 3H), 1.85 (b, 3H), 1.18 (s, 3H)

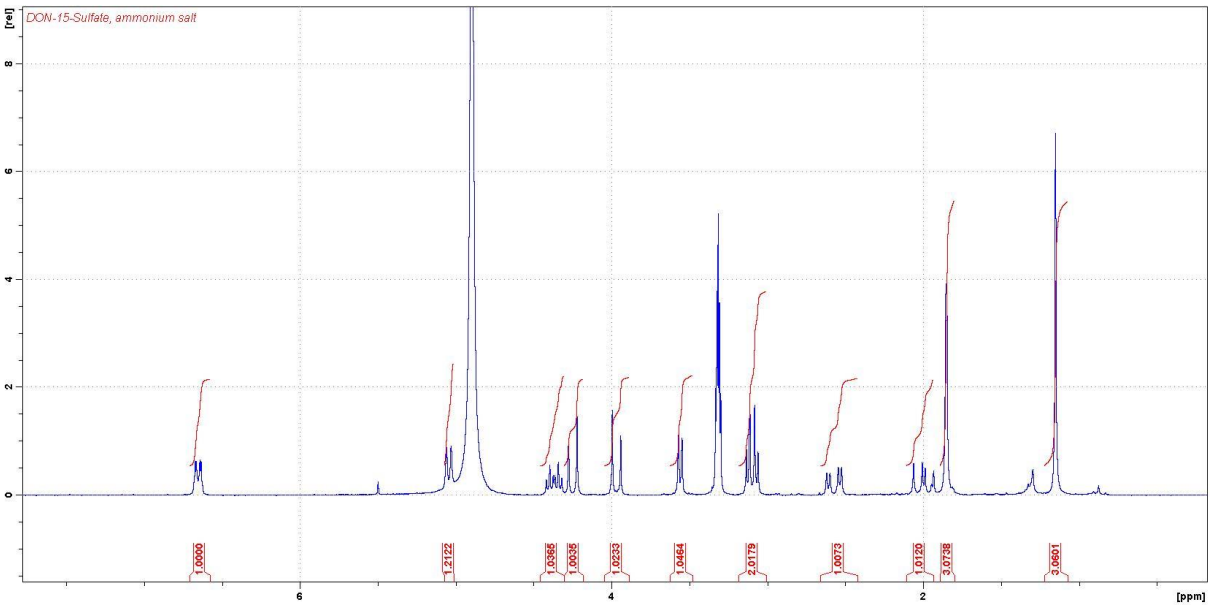


^{13}C NMR (50 MHz, methanol-d_4) δ 201.0 (s, 1C), 172.5 (s, 1C), 139.4 (d, 1C), 137.1 (s, 1C), 80.5 (d, 1C), 75.8 (d, 1C), 72.7 (d, 1C), 70.7 (d, 1C), 67.1 (t, 1C), 66.3 (s, 1C), 52.3 (s, 1C), 48.4 (t, 1C), 46.8 (s, 1C), 41.7 (t, 1C), 20.8 (q, 1C), 15.3 (q, 1C), 14.4 (q, 1C)

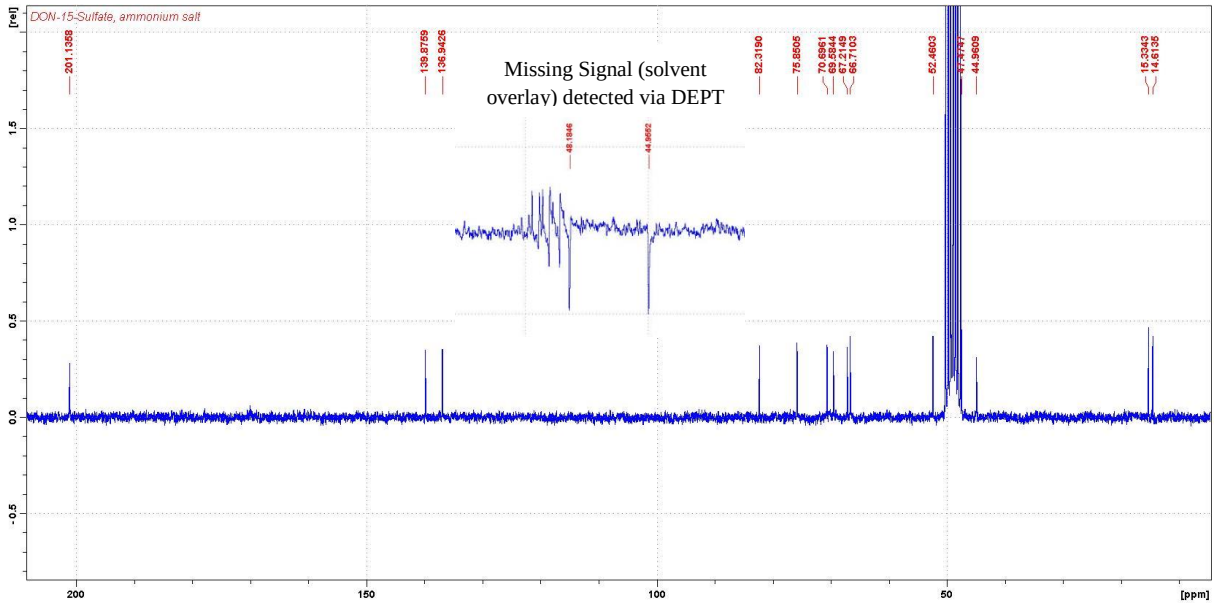


14.) DON-15-sulfate, ammonium salt

^{1}H NMR (200 MHz, methanol-d_4) δ 6.65 (dq, *J*=6.1, 1.4 Hz, 1H), 5.04 (d, *J*=6.1 Hz, 1H), 4.85-4.95 (m, NH_4^+, C7-H, H_2O), 4.76 (dt, *J*=11.1, 4.5, 1H), 4.25 (d, *J*=11.0 Hz, 1H), 3.96 (d, *J*=11.0 Hz, 1H), 3.55 (d, *J*=4.5 Hz, 1H), 3.12 (d, *J*=4.5 Hz, 1H), 3.06 (d, *J*=4.5 Hz, 1H), 2.57 (dd, *J*=14.8, 4.4 Hz, 1H), 1.99 (dd, *J*=14.8, 11.1 Hz, 1H), 1.84 (b, 3H), 1.14 (s, 3H)

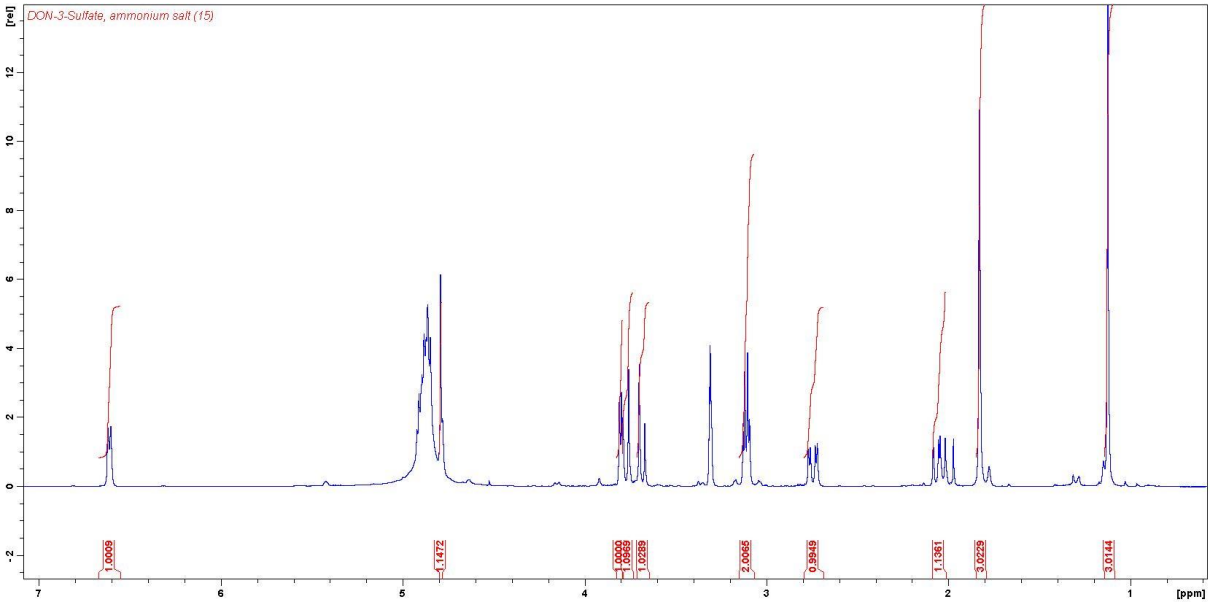


^{13}C NMR (50 MHz, methanol-d_4) δ 201.1 (s, 1C), 139.9 (d, 1C), 136.9 (s, 1C), 82.3 (d, 1C), 75.9 (d, 1C), 70.7 (d, 1C), 69.6 (d, 1C), 67.2 (s, 1C), 66.7 (t, 1C), 52.5 (s, 1C), 48.2 (t, 1C), 47.5 (s, 1C), 45.0 (t, 1C), 15.3 (q, 1C), 14.4 (q, 1C)

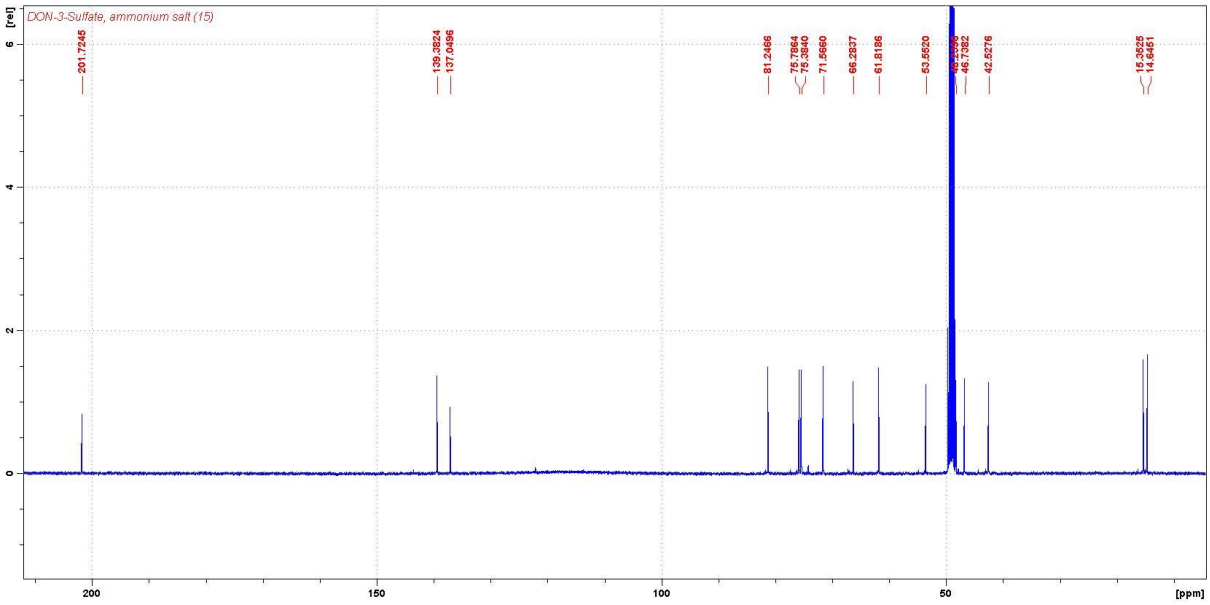


15.) DON-3-sulfate, ammonium salt

^{1}H NMR (400 MHz, methanol-d_4) δ 6.61 (dq, *J*=6.1, 1.4 Hz, 1H), 4.75-4.95 (m, NH_4^+, C3-H, C11-H, H_2O), 4.79 (s, 1H), 3.80 (d, *J*=4.4 Hz, 1H), 3.78 (d, *J*=12.3 Hz, 1H), 3.68 (d, *J*=12.3 Hz, 1H), 3.12 (d, *J*=4.5 Hz, 1H), 3.09 (d, *J*=4.5 Hz, 1H), 2.75 (dd, *J*=15.2, 4.4 Hz, 1H), 2.06 (dd, *J*=15.2, 11.4 Hz, 1H), 1.83 (b, 3H), 1.12 (s, 3H)

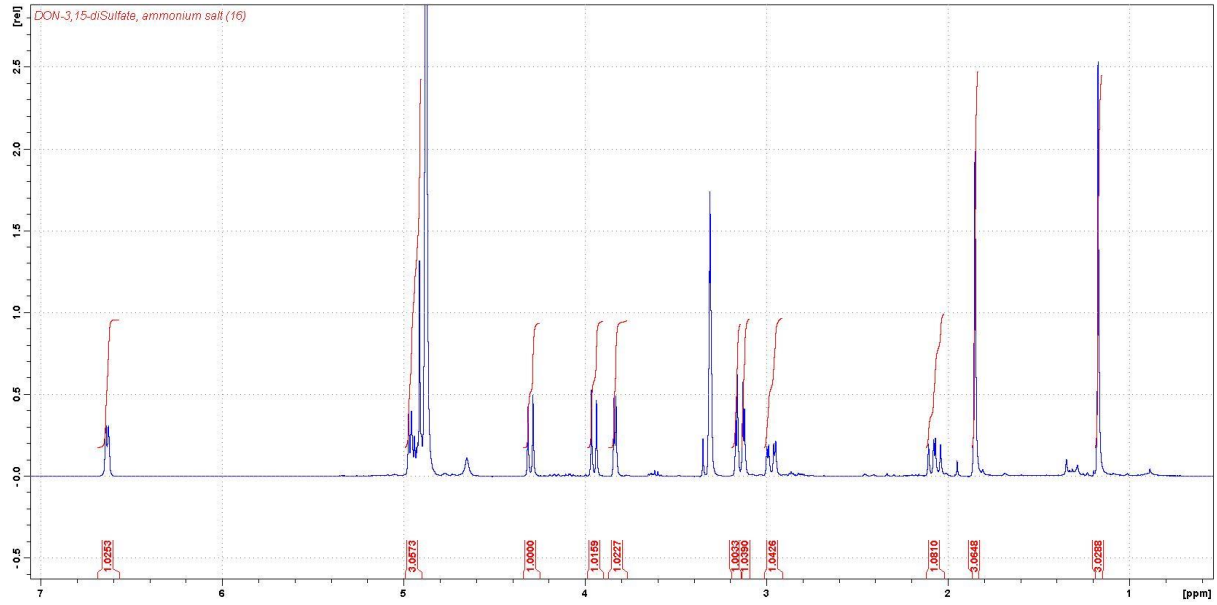


^{13}C NMR (100 MHz, methanol-d_4) δ 201.7 (s, 1C), 139.4 (d, 1C), 137.0 (s, 1C), 81.2 (d, 1C), 75.8 (d, 1C), 75.4 (d, 1C), 71.6 (d, 1C), 66.3 (s, 1C), 61.8 (t, 1C), 53.6 (s, 1C), 48.2 (t, 1C), 46.7 (s, 1C), 42.5 (t, 1C), 15.4 (q, 1C), 14.6 (q, 1C)

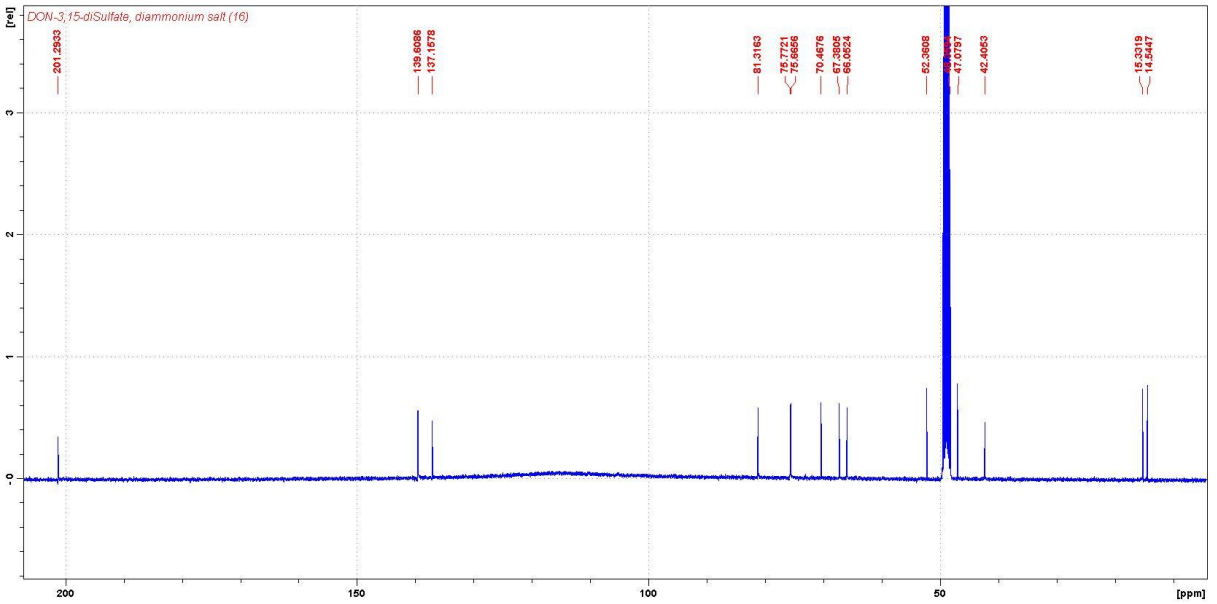


16.) DON-3,15-disulfate, diammonium salt

^{1}H NMR (400 MHz, methanol-d_4) δ 6.67 (dq, *J*=6.0, 1.5 Hz, 1H), 4.80-5.00 (m, NH_4^+, C3-H, C7-H, C11-H, H_2O), 4.24 (d, *J*=10.9 Hz, 1H), 3.98 (d, *J*=10.9 Hz, 1H), 3.84 (d, *J*=4.5 Hz, 1H), 3.15 (d, *J*=4.3 Hz, 1H), 3.10 (d, *J*=4.3 Hz, 1H), 2.97 (dd, *J*=15.3, 4.5 Hz, 1H), 2.10 (dd, *J*=15.3, 11.4 Hz, 1H), 1.85 (b, 3H), 1.16 (s, 3H)

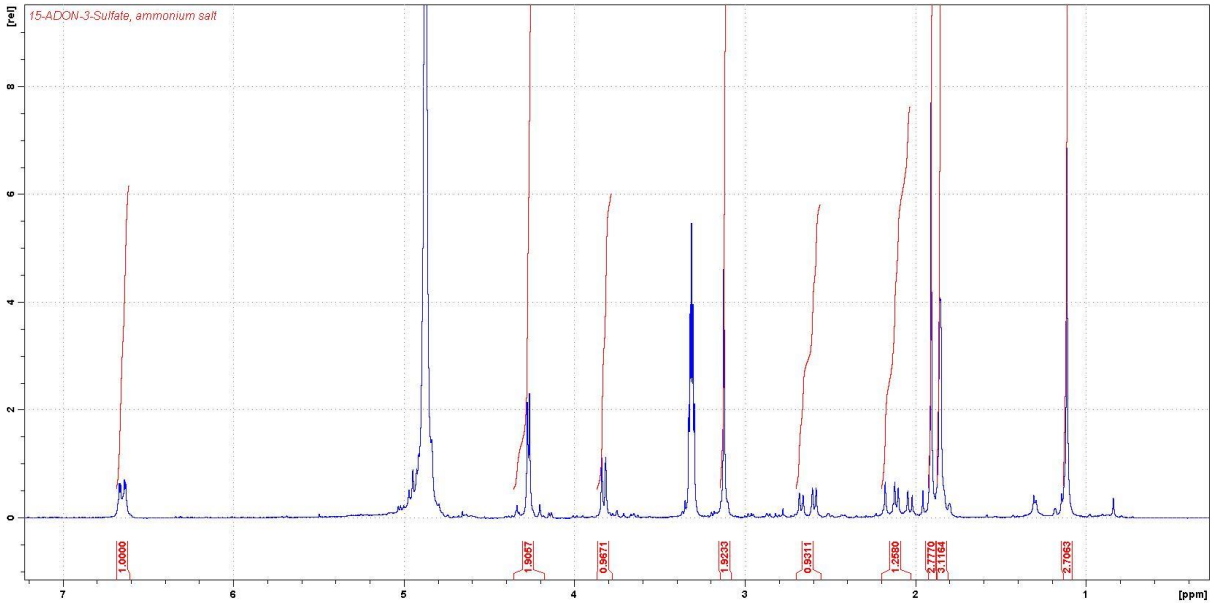


^{13}C NMR (100 MHz, methanol-d_4) δ 201.3 (s, 1C), 139.6 (d, 1C), 137.2 (s, 1C), 81.3 (d, 1C), 75.8 (d, 1C), 75.7 (d, 1C), 70.5 (d, 1C), 67.4 (s, 1C), 66.1 (t, 1C), 52.4 (s, 1C), 48.4 (t, 1C), 47.1 (s, 1C), 42.4 (t, 1C), 15.3 (q, 1C), 14.5 (q, 1C)

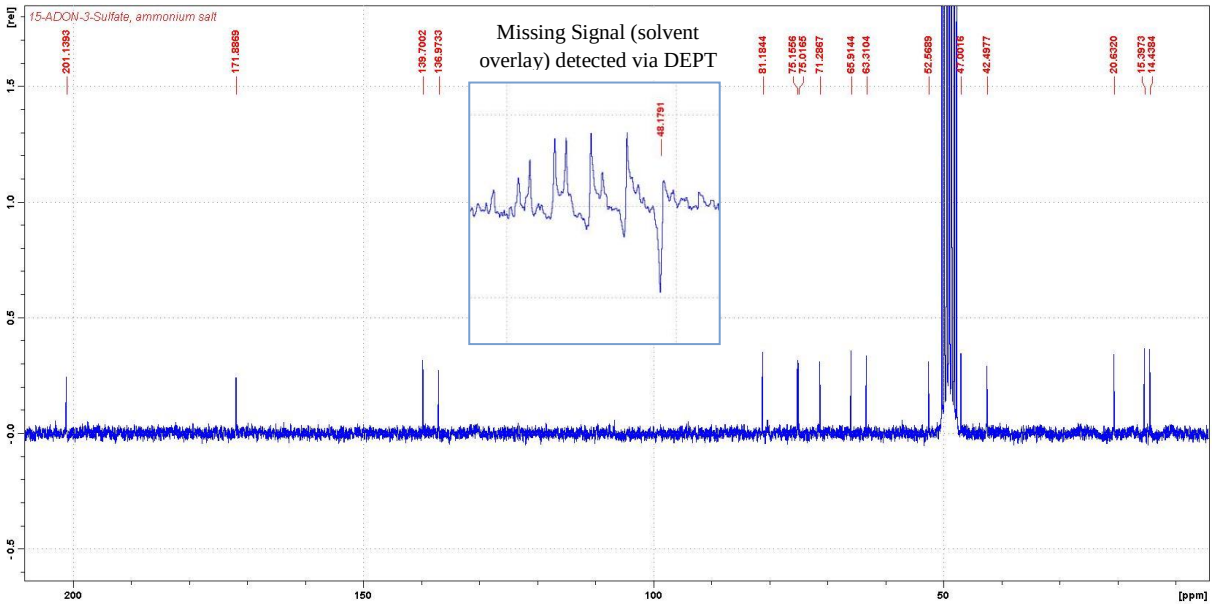


18.) 15-Acetyl-DON-3-sulfate, ammonium salt

^{1}H NMR (400 MHz, methanol-d_4) δ 6.65 (dq, *J*=5.9, 1.5 Hz, 1H), 4.70-5.10 (m, NH_4^+, C3-H, C7-H, C11-H, H_2O), 4.30 (d, *J*=12.1 Hz, 1H), 4.23 (d, *J*=12.1 Hz, 1H), 3.82 (d, *J*=4.5 Hz, 1H), 3.12 (s, 2H), 2.63 (dd, *J*=15.3, 4.3 Hz, 1H), 2.11 (dd, *J*=15.3, 11.2 Hz, 1H), 1.90 (s, 3H), 1.85 (b, 3H), 1.11 (s, 3H)

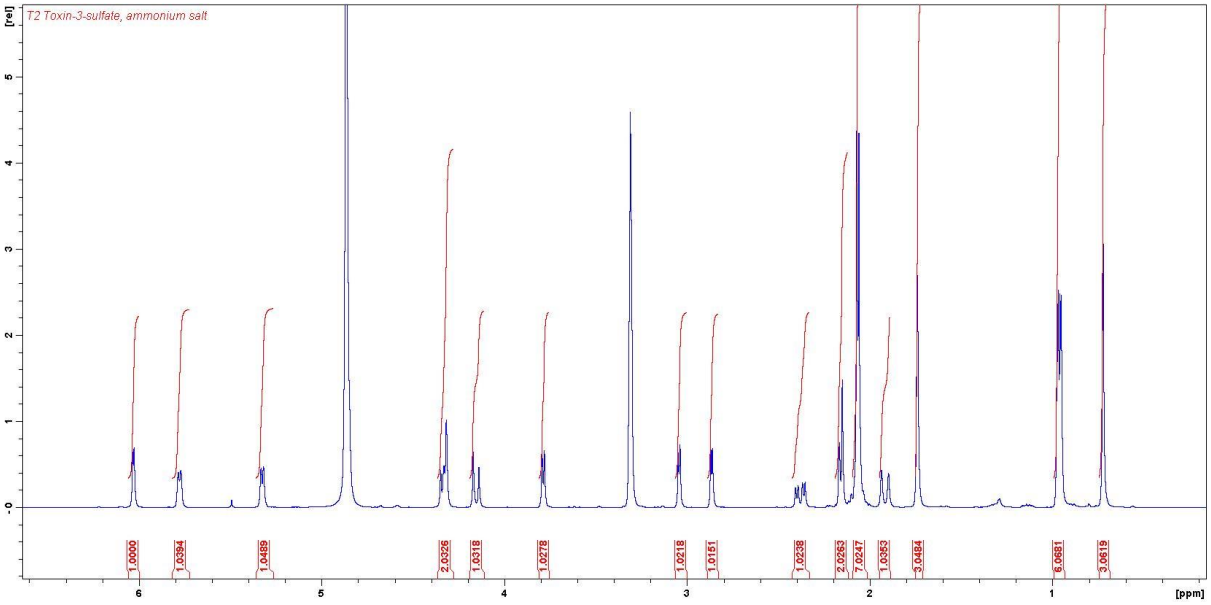


^{13}C-NMR (100 MHz, methanol-d_4) δ 201.1 (s, 1C), 171.9 (s, 1C), 139.7 (d, 1C), 137.0 (s, 1C), 81.2 (d, 1C), 75.2 (d, 1C), 75.0 (d, 1C), 71.3 (d, 1C), 65.9 (s, 1C), 63.3 (t, 1C), 52.6 (s, 1C), 48.2 (t, 1C), 47.0 (s, 1C), 42.5 (t, 1C), 20.6 (q, 1C), 15.4 (q, 1C), 14.4 (q, 1C)

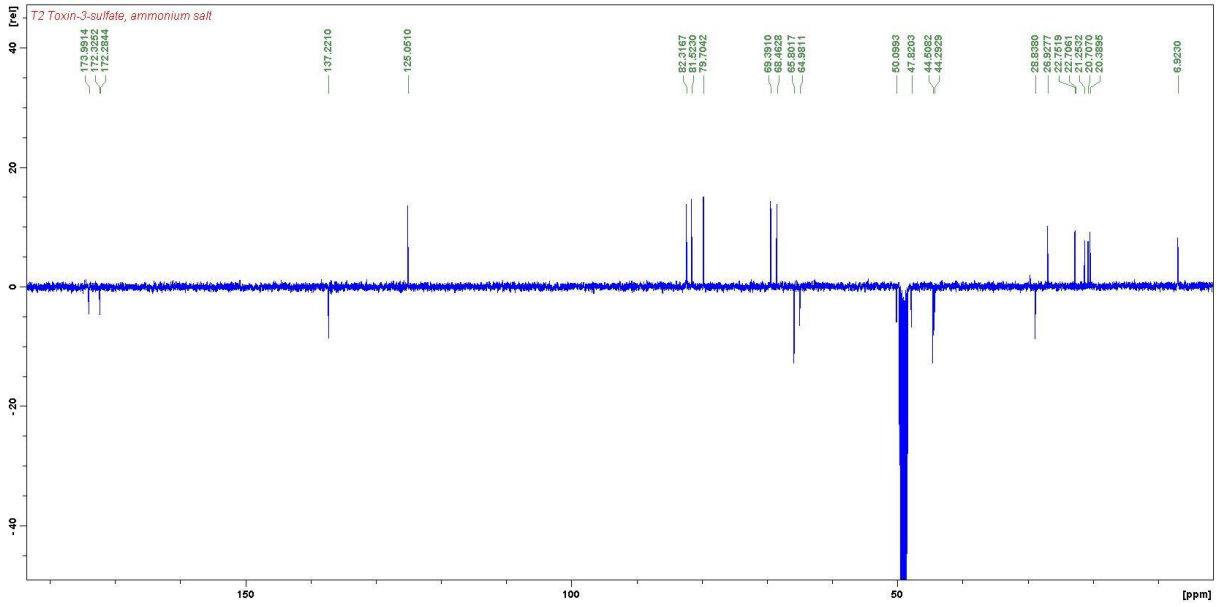


22.) T2-toxin-3-sulfate, ammonium salt

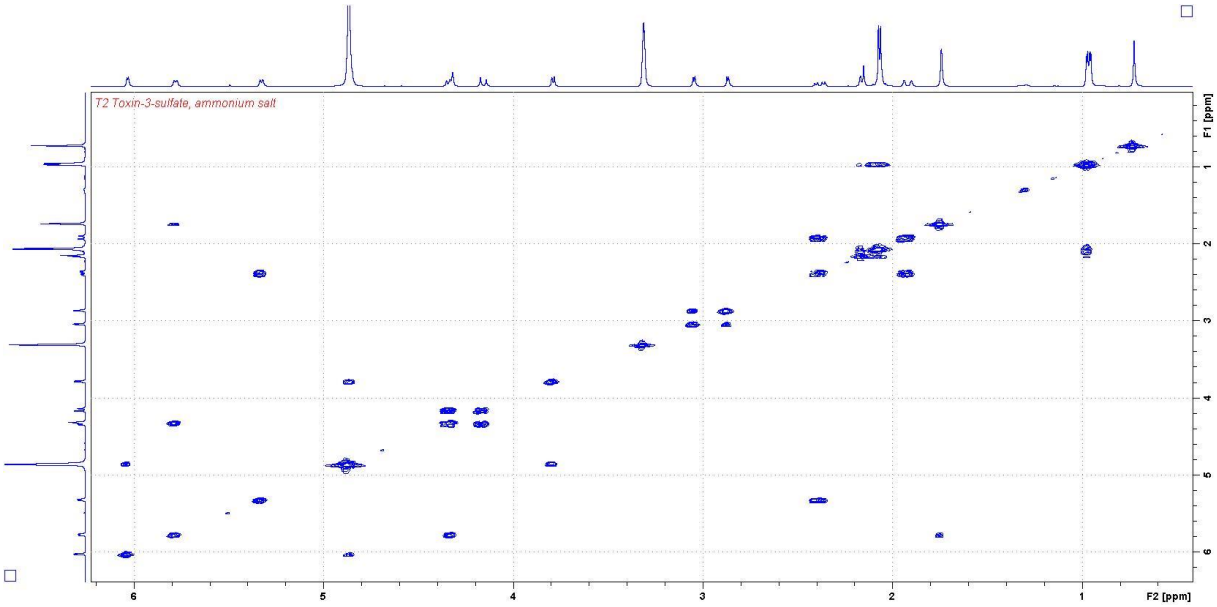
^{1}H NMR (400 MHz, methanol-d_4) δ 6.03 (d, *J*=3.1 Hz, 1H), 5.78 (dt, *J*=5.5 1H), 5.33 (d, *J*=5.5 Hz, 1H), 4.80-4.94 (m, NH_4^+, C3-H, H_2O), 4.33 (d, *J*=12.1, 1H), 4.32 (d, *J*=5.5 Hz, 1H), 4.16 (d, *J*=12.1 Hz, 1H), 3.79 (d, *J*=5.1 Hz, 1H), 3.04 (d, *J*=3.9 Hz, 1H), 2.87 (d, *J*=3.9 Hz, 1H), 2.38 (dd, *J*=15.3, 5.5 Hz, 1H), 2.13-2.18 (m, 2H), 2.07 (s, 3H), 2.06 (s, 3H), 2.00-2.12 (m, 1H), 1.92 (d, *J*=15.3 Hz, 1H), 1.74 (s, 3H), 0.97 (d, *J*=6.7 Hz, 3H), 0.96 (d, *J*=6.7 Hz, 3H), 0.72 (s, 3H)



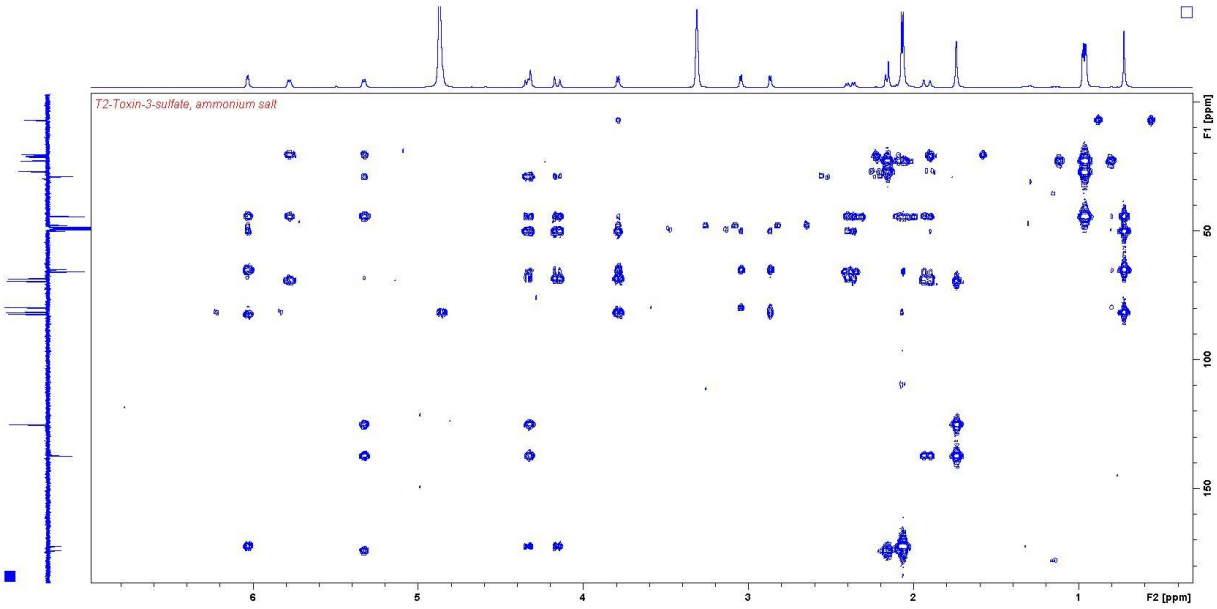
^{13}C NMR (100 MHz, methanol-d_4) δ 174.0 (s, 1C), 172.3 (s, 1C), 172.2 (s, 1C), 137.2 (s, 1C), 125.1 (d, 1C), 82.3 (d, 1C) 81.5 (d, 1C), 79.7 (d, 1C), 69.4 (d, 1C), 68.5 (d, 1C), 65.8 (t, 1C), 65.0 (s, 1C), 50.1 & 47.8 (1t, 1s, 2x1C), 44.5 (t, 1C), 44.3 (s, 1C), 28.8 (t, 1C), 26.9 (d, 1C), 22.8 (q, 1C), 22.7 (q, 1C), 21.3 (q, 1C), 20.7 (q, 1C), 20.4 (q, 1C), 6.9 (q, 1C)



22.) T2-toxin-3-sulfate, ammonium salt - COSY



22.) T2-toxin-3-sulfate, ammonium salt – HMBC



Manuscript #6

Reprinted from Toxins (2015), 7 (11)
Schwartz-Zimmermann, H.; Fruhmann, P.; Dänicke, S.; Wiesenberger, G.; Caha, S.; Weber, J.; Berthiller, F. Metabolism of Deoxynivalenol and Deepoxy-Deoxynivalenol in Broiler Chickens, Pullets, Roosters and Turkeys, 4706-4729

Supplementary Information

NMR and LC-HR-MS Data of Protected Intermediates:

NMR Data of 2,2,2-Trichloroethyl-DOM-3-Sulfate:

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.57 (dq, *J* = 5.8, 1.6 Hz, 1H), 5.31 (s, 1H), 5.18 (s, 1H), 5.07 (dt, *J* = 11.9, 4.6 Hz, 1H), 4.82 (d, *J* = 10.9 Hz, 1H), 4.79 (d, *J* = 10.9 Hz, 1H), 4.68 (d, *J* = 5.9 Hz, 1H), 4.66 (s, 1H), 4.57 (d, *J* = 4.3 Hz, 1H), 3.80 (s, *2*H), 3.75 (s, 1H), 2.75 (dd, *J* = 15.6, 4.3 Hz, 1H), 2.09 (dd, *J* = 15.6, 11.6 Hz, 1H), 1.96 (br, 1H) 1.88 (s, 3H), 1.46 (s, 3H);
^{13}C-NMR (100 MHz, $CDCl_3$) δ 200.3 (s, 1C), 149.7 (s, 1C), 138.2 (d, 1C), 136.1 (s, 1C), 110 (t, 1C), 92.8 (s, 1C), 81.0 (d, 1C), 79.9 (t, 1C), 79.1 (d, 1C), 74.4 (d, 1C), 70.1 (d, 1C), 62.0 (t, 1C), 52.3 (s, 1C), 48.6 (s, 1C), 40.8 (t, 1C), 19.6 (q, 1C), 15.5 (q, 1C); LC-HR-MS *m/z* calcd for $C_{17}H_{20}O_8SCl_3^-$ $[M - H^+]^-$, 488.9950, found 488.9948.

NMR data of 2,2,2-Trichloroethyl-DOM-15-Sulfate:

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.67 (dq, *J* = 5.9, 1.6 Hz, 1H), 5.26 (s, 1H), 5.10 (s, 1H), 4.94 (d, *J* = 5.9 Hz, 1H), 4.72 (s, 1H), 4.62 (d, *J* = 10.1 Hz, 1H), 4.61 (d *J* = 10.9 Hz, 1H), 4.56 (d, *J* = 10.9 Hz; 1H), 4.45 (d, *J* = 10.5 Hz, 1H), 4.30 (dt, *J* = 10.7, 4.2 Hz, 1H), 4.26 (d, *J* = 4.3 Hz, 1H), 3.78 (s, 1H), 2.20 (dd, *J* = 15.0, 4.1 Hz, 1H), 1.94–2.06 (m, 2H), 1.89 (s, 3H), 1.37 (s, 3H);
^{13}C-NMR (100 MHz, $CDCl_3$) δ 199.9 (s, 1C), 151.1 (s, 1C), 139.1 (d, 1C), 136.0 (s, 1C), 109.8 (t, 1C), 92.6 (s, 1C), 81.2 (d, 1C), 79.8 (t, 1C), 73.1 (d, 1C), 72.2 (t, 1C), 69.5 (d, 1C), 69.3 (d, 1C), 51.8 (s, 1C), 49.0 (s, 1C), 44.3 (t, 1C), 19.4 (q, 1C), 15.5 (q, 1C); LC-HR-MS *m/z* calcd for $C_{17}H_{20}O_8SCl_3^-$ $[M - H^+]^-$, 488.9950, found 488.9942.

NMR Data of Bis (2,2,2-Trichloroethyl) DOM-3,15-Disulfate:

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.65 (dq, *J* = 5.6, 1.6 Hz, 1H), 5.39 (s, 1H), 5.24 (s, 1H), 5.09 (dt, *J* = 11.5, 4.4 Hz, 1H), 4.81 (s, 2H), 4.77 (d, *J* = 5.9 Hz, 1H), 4.70 (s, 1H), 4.61–4.66 (m, *2*H), 4.59 (d, *J* = 10.5 Hz, 1H), 4.54 (d, *J* = 10.5 Hz, 1H), 4.47 (d, *J* = 10.5 Hz, 1H), 3.76 (br, 1H), 2.58 (dd, *J* = 16.0, 4.3 Hz, 1H), 2.18 (dd, *J* = 15.6, 11.3 Hz, 1H), 1.91 (s, 3H), 1.43 (s, 3H);
^{13}C-NMR (100 MHz, $CDCl_3$) δ 199.3 (s, 1C), 148.3 (s, 1C), 138.3 (d, 1C), 136.4 (s, 1C), 112.0 (t, 1C), 92.7 (s, 1C), 92.5 (s, 1C), 80.2 (t, 1C), 80.0 (t, 1C), 79.8 (d, 1C), 78.9 (d, 1C), 73.0 (d, 1C), 71.8 (t, 1C), 69.3 (d, 1C), 51.6 (s, 1C), 48.4 (s, 1C), 40.9 (t, 1C), 19.1 (q, 1C), 15.4 (q, 1C); LC-HR-MS *m/z* calcd for $C_{19}H_{21}O_{11}S_2Cl_6^-$ $[M - H^+]^-$, 698.8662, found 698.8681.

NMR and LC-HR-MS Data of DOM-Sulfates:

NMR Data of DOM-3-Sulfate, Ammonium Salt:

^{1}H NMR (400 MHz, methanol-d_4) δ 6.59 (dq, *J* = 5.9, 1.6 Hz, 1H), 5.21 (s, 1H), 5.04 (s, 1H), 4.94–4.80 (m, 7H), 4.68 (dt, *J* = 11.3, 4.5 Hz, 1H), 4.60 (s, 1H), 4.41 (d, *J* = 4.3 Hz, 1H), 3.76 (d, *J* = 12.1 Hz, 1H), 3.72 (d, *J* = 12.1 Hz, 1H), 2.73 (dd, *J* = 15.0, 4.5 Hz, 1H), 1.93 (dd, *J* = 15.0, 11.5 Hz, 1H), 1.81 (q, *J* = 0.8 Hz, 3H), 1.42 (s, 3H); ^{13}C NMR (100 MHz, methanol-d_4) δ 202.3 (s, 1C), 153.9 (s, 1C), 139.6 (d, 1C), 136.9 (s, 1C), 108.3 (t, 1C), 81.5 (d, 1C), 75.7 (d, 1C), 75.6 (d, 1C), 71.7

(d, 1C), 61.7 (t, 1C), 54.1 (s, 1C), 49.5 (s, 1C), 43.3 (t, 1C), 20.1 (q, 1C), 15.4 (q, 1C); LC-HR-MS: *m/z* calculated for $C_{15}H_{19}O_8S^-$ $[M - H^+]^-$: 359.0806, *m/z* measured: 359.0807.

NMR Data of DOM-15-Sulfate, Ammonium Salt:

^{1}H NMR (400 MHz, methanol-*d*$_4$) δ 6.62 (dq, *J* = 6.0, 1.5 Hz, 1H), 5.16 (s, 1H), 5.02 (d, *J* = 6.2 Hz, 1H), 5.01 (s, 1H), 4.95–4.80 (s, NH_4^+, 2 x CH, H_2O), 4.70 (s, 1H), 4.20 (d, *J* = 10.5 Hz, 1H), 4.16 (s, 1H), 4.14 (dt, *J* = 15.2, 4.5 Hz, 1H), 4.01 (d, 10.9 Hz, 1H), 2.56 (dd, *J* = 14.6, 4.1 Hz, 1H), 1.88 (dd, *J* = 15.0, 10.7 Hz, 1H), 1.83 (s, 3H), 1.43 (s, 3H); ^{13}C NMR (100 MHz, methanol-*d*$_4$) δ 201.7 (s, 1C), 154.7 (s, 1C), 140.1 (d, 1C), 136.9 (s, 1C), 107.8 (t, 1C), 82.8 (d, 1C), 76.0 (d, 1C), 70.9 (d, 1C), 70.1 (d, 1C), 67.1 (t, 1C), 52.8 (s, 1C), 50.2 (s, 1C), 45.7 (t, 1C), 20.0 (q, 1C), 15.4 (q, 1C); LC-HR-MS: *m/z* calculated for $C_{15}H_{19}O_8S^-$ $[M - H^+]^-$: 359.0806, *m/z* measured: 359.0806.

NMR Data of DOM-3,15-Disulfate, Diammonium Salt:

^{1}H NMR (600 MHz, methanol-*d*$_4$) δ 6.64 (dq, *J* = 5.7, 1.5 Hz, 1H), 5.23 (s, 1H), 5.07 (s, 1H), 5.05 – 4.80 (m, 2 x NH_4^+ + 1H + H_2O), 4.72–4.60 (m, 3H), 4.45 (d, *J* = 4.4 Hz, 1H), 4.23 (d, *J* = 10.9 Hz, 1H), 4.03 (d, *J* = 10.9 Hz, 1H), 2.81 (dd, *J* = 15.3, 4.4 Hz, 1H), 1.99 (dd, *J* = 15.3, 11.4 Hz, 1H), 1.83 (s, 3H), 1.46 (s, 3H); ^{13}C NMR (150 MHz, methanol-*d*$_4$) δ 201.6 (s, 1C), 153.6 (s, 1C), 139.9 (d, 1C), 137.1 (s, 1C), 108.6 (t, 1C), 81.5 (d, 1C), 75.7 (d, 1C), 75.5 (d, 1C), 71.0 (d, 1C), 67.1 (t, 1C), 52.9 (s, 1C), 49.1 (s, 1C), 43.4 (t, 1C), 19.9 (q, 1C), 15.5 (q, 1C); LC-HR-MS: *m/z* calculated for $C_{15}H_{19}O_{11}S_2^-$ $[M - H^+]^-$: 439.0374, *m/z* measured: 439.0375.

Table S1. Matrix effects of the long gradient method. Dilution of extracts: 1 + 2, v + v.

	Average ± standard deviation (*n* = 3)									
	DON-3-sulfate	**DON-15-sulfate**	**DOM-3-sulfate**	**DOM-15-Sulfate**	**DON**	**DOM**	**DONS 1**	**DONS 2**	**DONS 3**	**DOMS 2**
Excreta of turkey	87 ± 3	95 ± 1	89 ± 5	103 ± 2	89 ± 1	90 ± 1	89 ± 4	105 ± 5	101 ± 5	108 ± 0
Excreta of broiler (1)	105 ± 2	135 ± 0	104 ± 1	114 ± 2	107 ± 2	107 ± 2	108 ± 1	119 ± 4	188 ± 4	117 ± 1

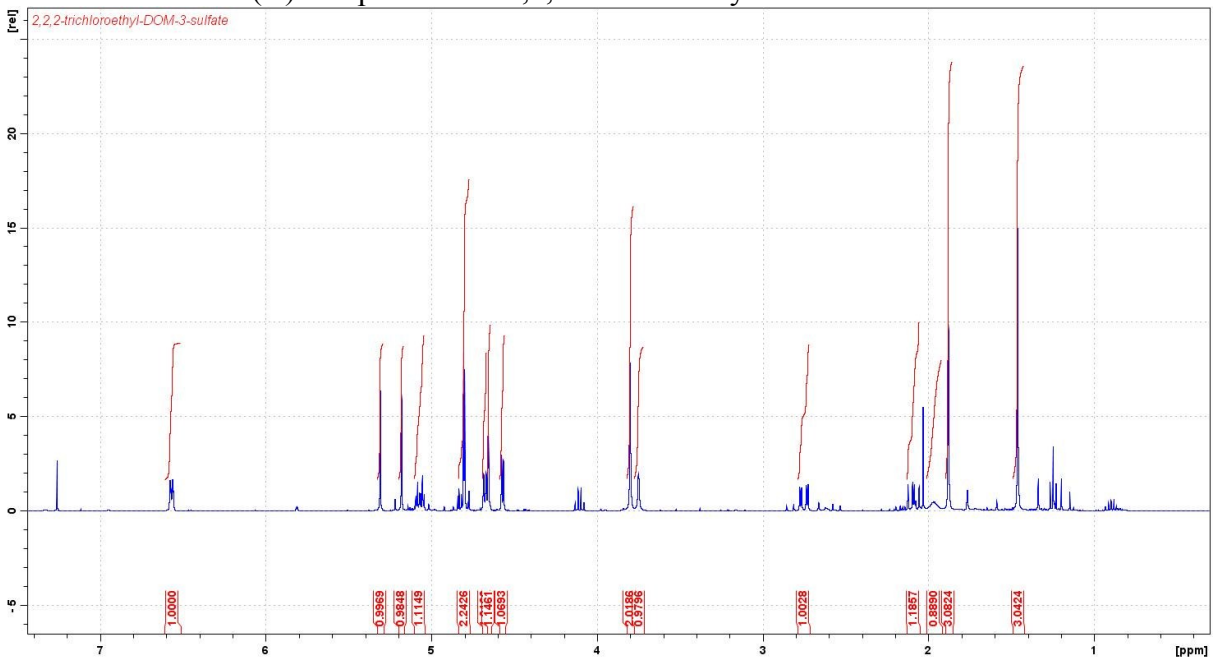
Table S2. Calculation of biological recoveries in the pullet experiment. -: no *A. galli* infection, +: *A.galli* infection.

	Amount of Lyophilized Excreta (g)	**µg Excreted Over 7 Days (in DON Equivalents)**						
		DON-3-sulfate	**DOM-3-sulfate**	**DON**	**DONS 2**	**SUM**	**µg DON Ingested**	**Biological Recovery (%)**
DON -	109 ± 3	2496 ± 104	379 ± 20	84 ± 3	16 ± 1	2975 ± 121	1973 ± 23	151 ± 5
DON +	110 ± 9	2227 ± 182	272 ± 113	85 ± 7	14 ± 5	2598 ± 214	1981 ± 0	131 ± 11

Table S3. Optimized SRM parameters.

Analyte	Retention Time (min)		Precursor Ion	Ion Spezies	DP	Product Ions	CE	Relative Intensity
	Short Method	Long Method	(m/z)		(V)	(quant/qual) (m/z)	(eV)	(qual/quant)
DONS 1	2.60	2.76	377.1	$[M-H]^-$	−130	80.0/331.0	−98/−52	0.25
DONS 2	3.40	3.81	377.1	$[M-H]^-$	−105	81.0/347.0	−68/−36	0.21
DONS 3	3.92	4.66	377.1	$[M-H]^-$	−125	347.0/80.0	−36/−98	0.29
DOMS 2	4.19	5.10	361.1	$[M-H]^-$	−25	81.0/249.0	−65/−30	0.51
DON-3-sulfate	4.43	5.58	375.1	$[M-H]^-$	−125	344.9/247.0	−36/−38	0.58
DON-15-sulfate	4.38	5.46	375.1	$[M-H]^-$	−110	97.0/229.0	−38/−42	0.20
DOM-3-sulfate	4.74	6.18	359.1	$[M-H]^-$	−125	97.0/329.0	−38/−34	0.65
DOM-15-sulfate	4.80	6.28	359.1	$[M-H]^-$	−125	97.0/80.0	−28/−118	0.26
DON	4.95	6.43	341.1	$[M+HCO_2]^-$	−25	265.1/45.0	−14/−38	1.01
DOM	5.44	7.50	325.1	$[M+HCO_2]^-$	−25	45.0/249.1	−40/−17	0.32

(**A**) ^{1}H spectrum of 2,2,2-trichloroethyl-DOM-3-sulfate



(**B**) ^{13}C spectrum of 2,2,2-trichloroethyl-DOM-3-sulfate

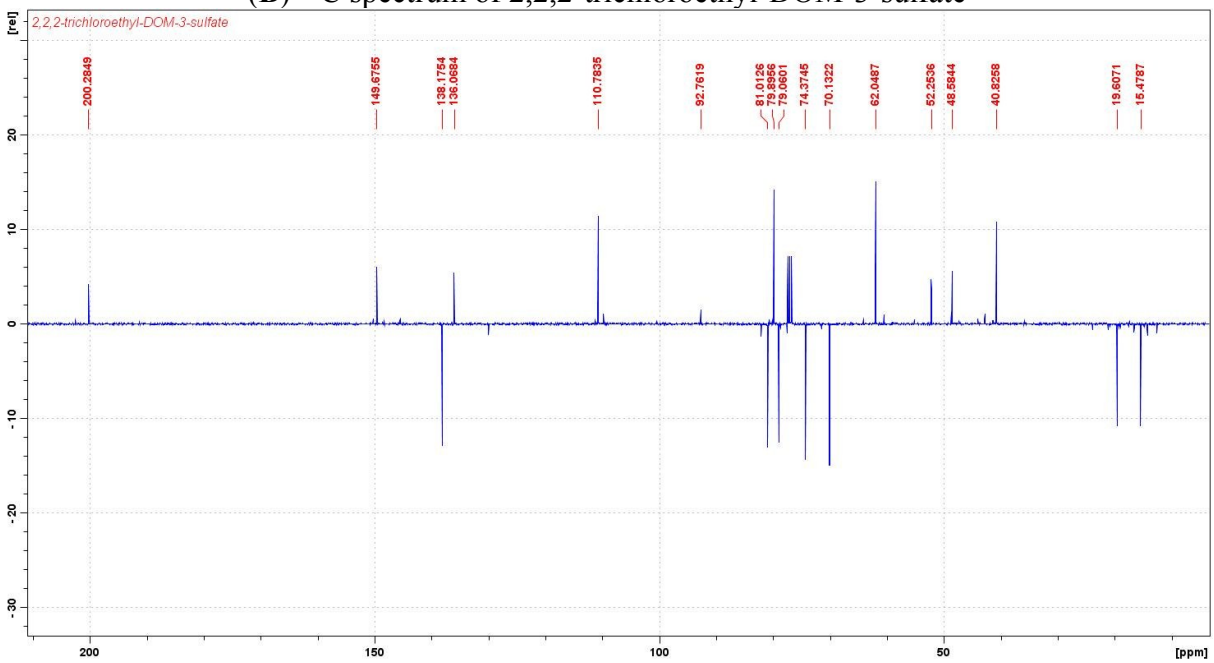
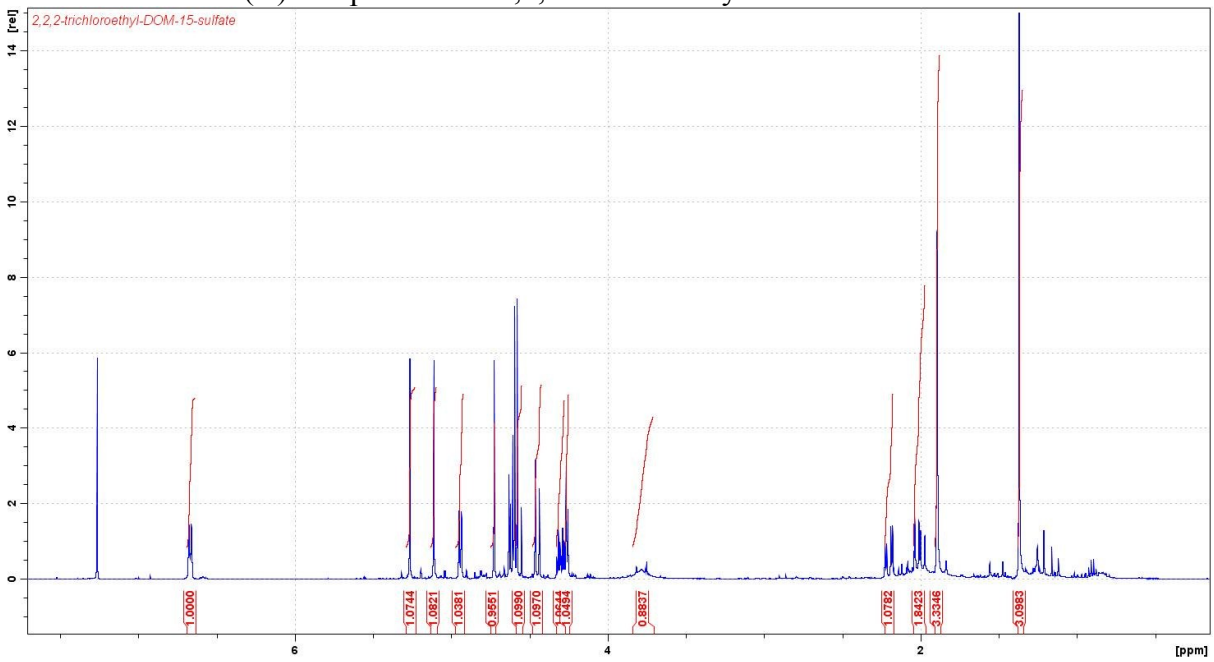


Figure S1. *Cont.*

(**C**) ^{1}H spectrum of 2,2,2-trichloroethyl-DOM-15-sulfate



(**D**) ^{13}C spectrum of 2,2,2-trichloroethyl-DOM-15-sulfate

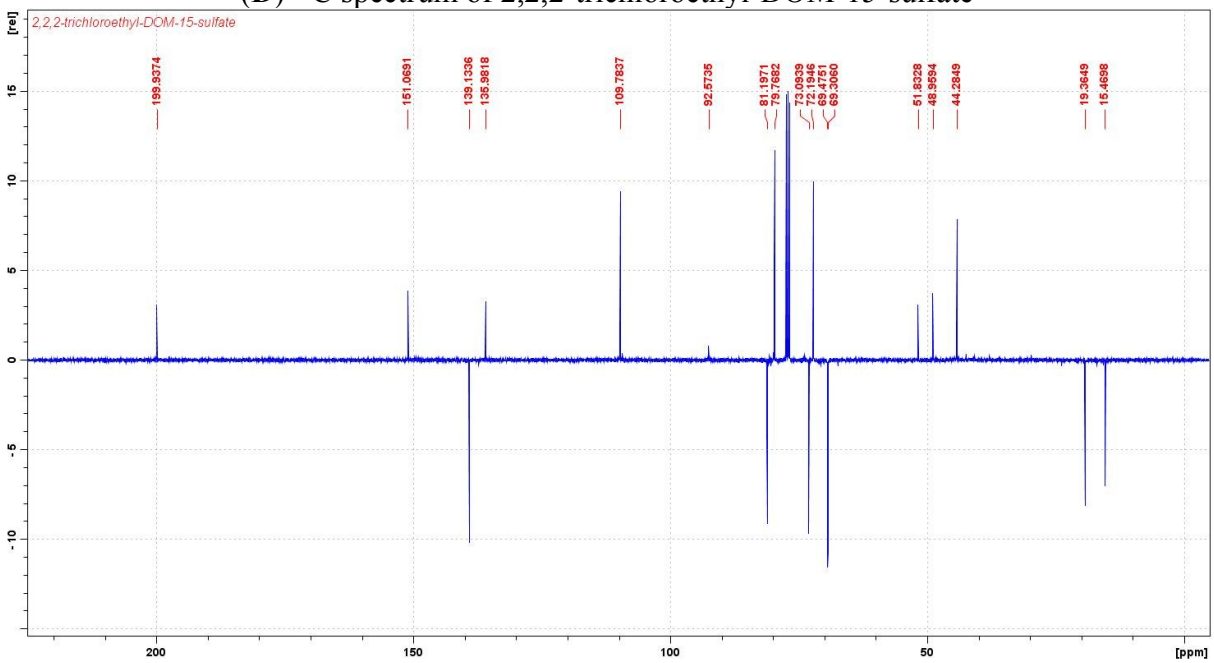
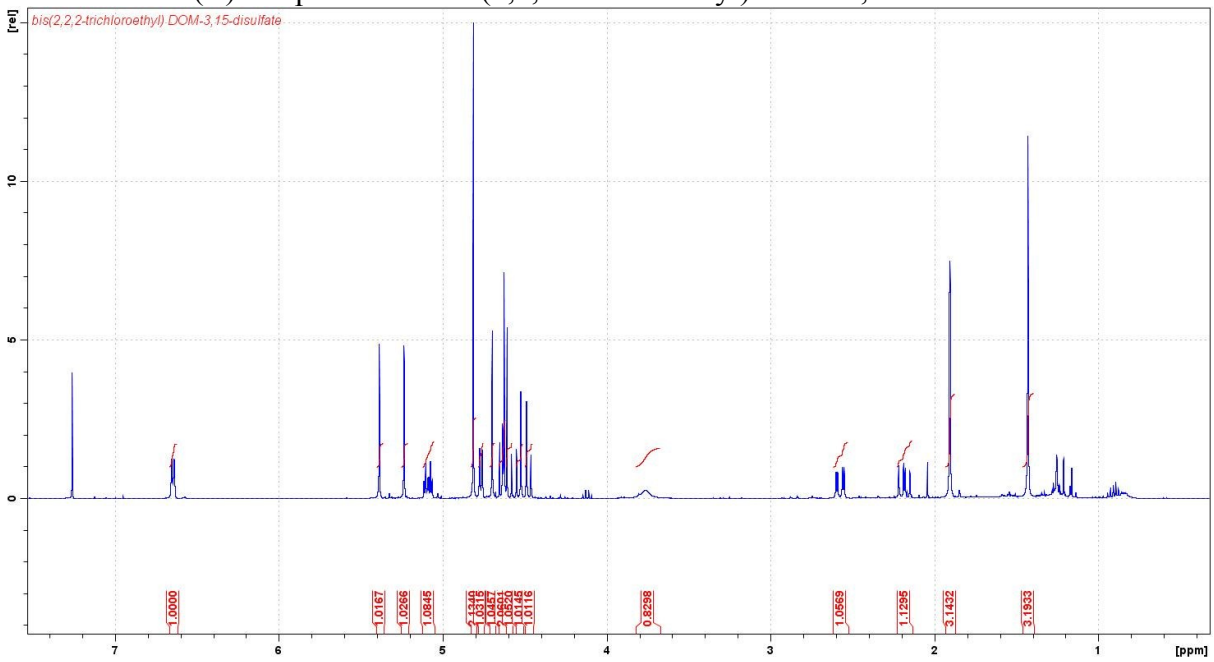


Figure S1. *Cont.*

(**E**) ^{1}H spectrum of bis(2,2,2-trichloroethyl) DOM-3,15-disulfate



(**F**) ^{13}C spectrum of bis(2,2,2-trichloroethyl) DOM-3,15-disulfate

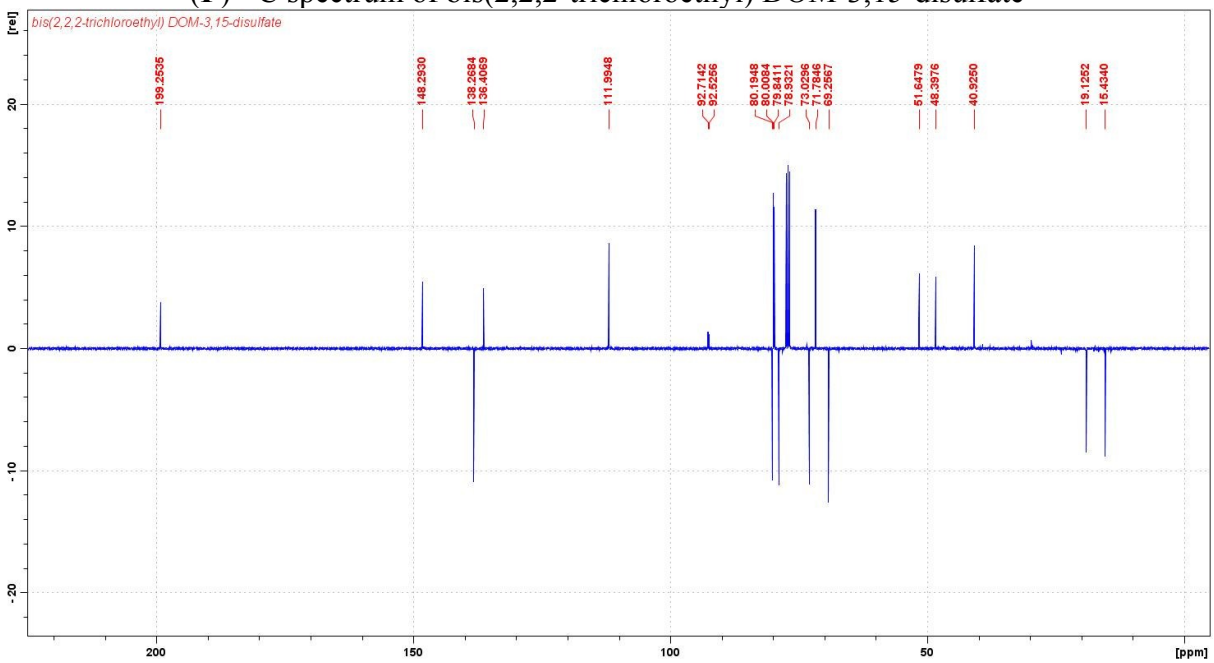
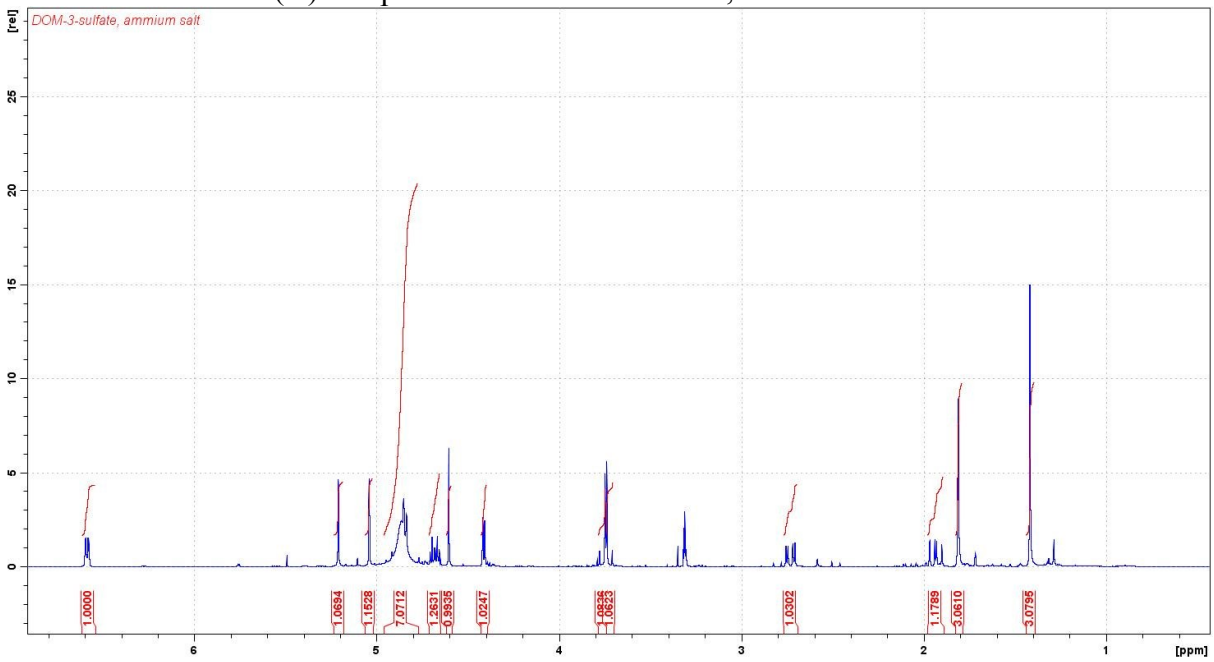


Figure S1. NMR spectra of protected intermediates.

(**A**) ^{1}H spectrum of DOM-3-sulfate, ammonium salt



(**B**) ^{13}C spectrum of DOM-3-sulfate, ammonium salt

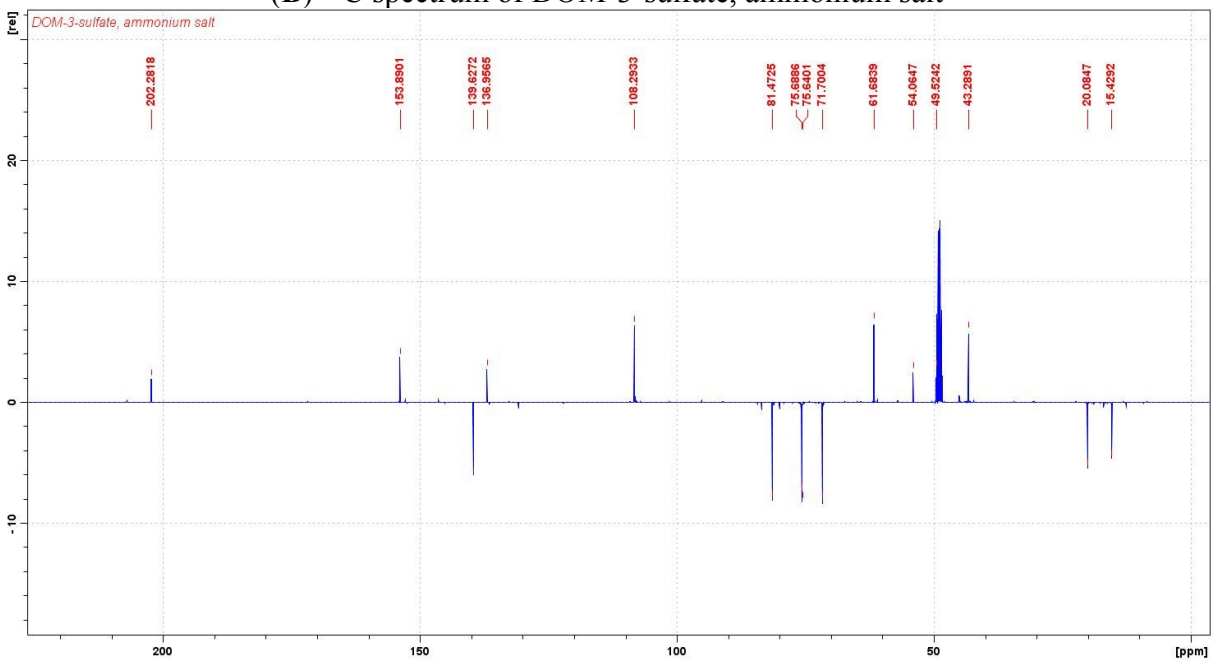
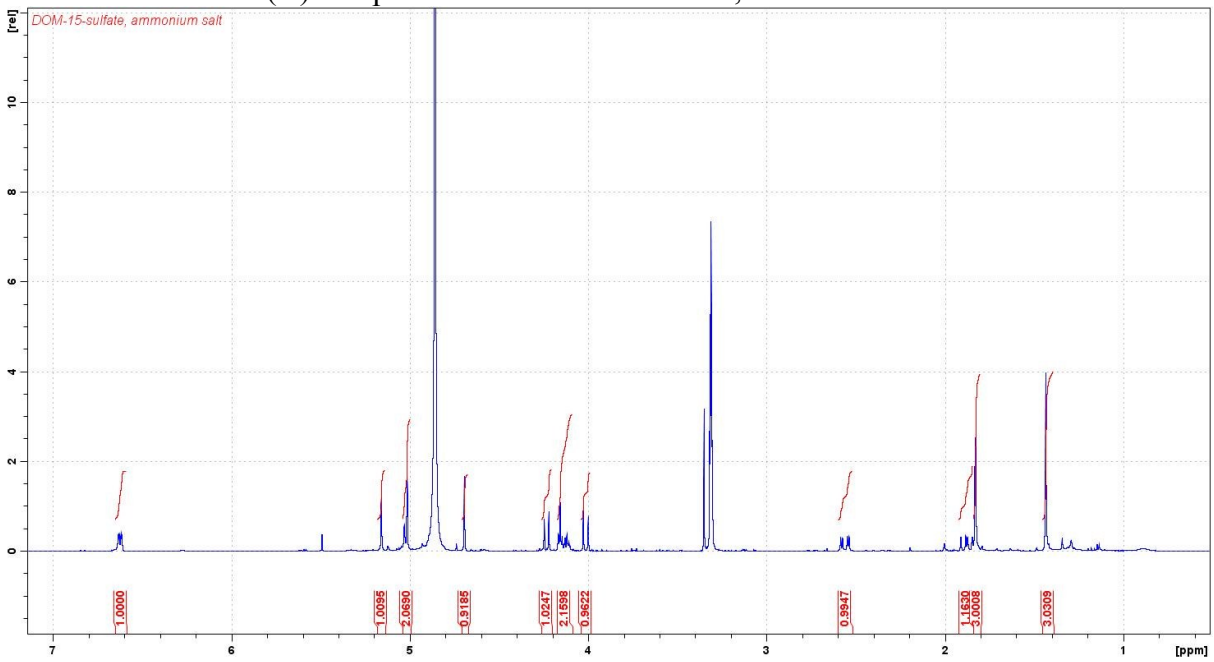


Figure S2. *Cont.*

(**C**) ^{1}H spectrum of DOM-15-sulfate, ammonium salt



(**D**) ^{13}C spectrum of DOM-15-sulfate, ammonium salt

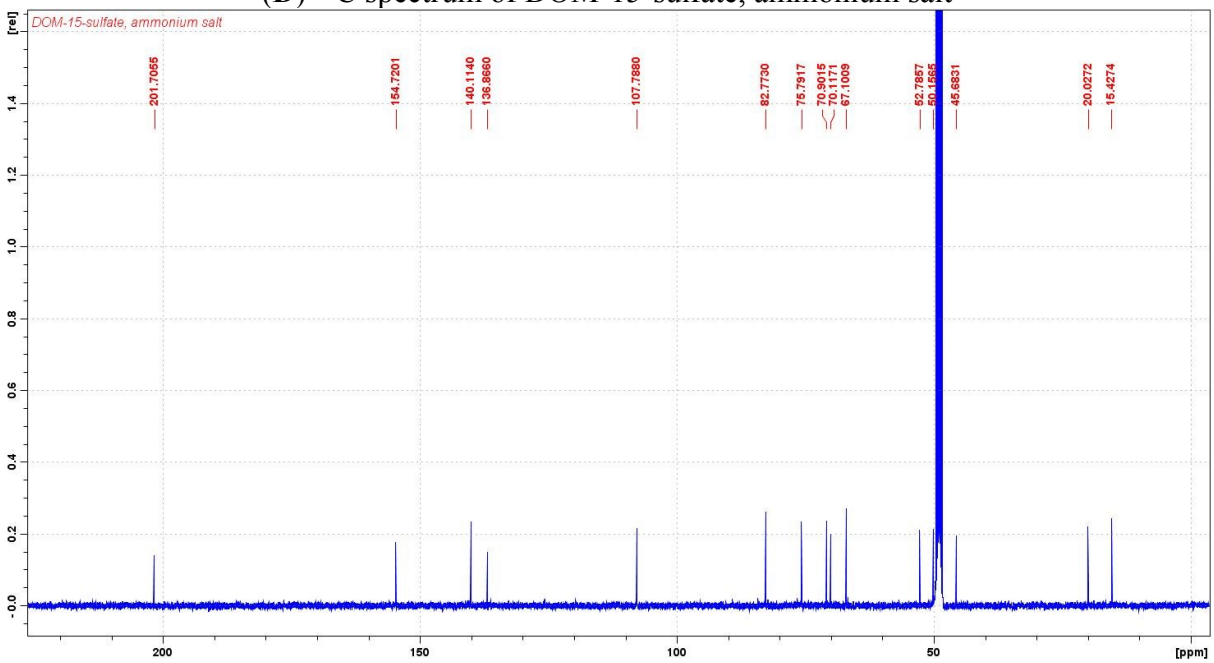
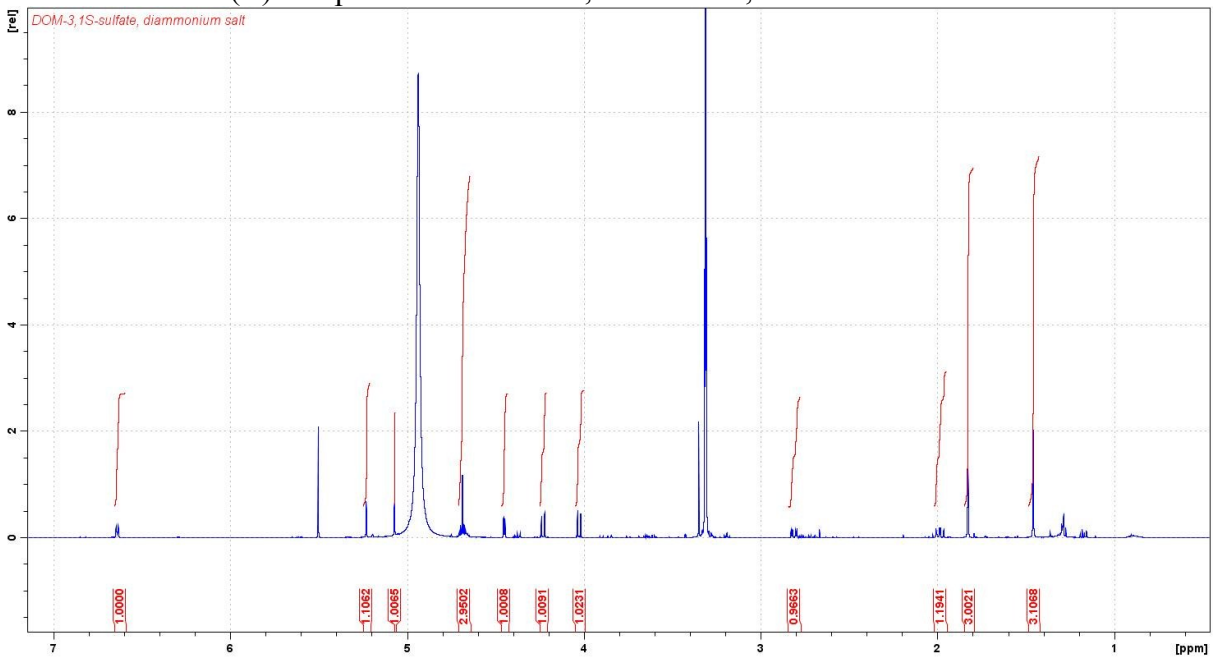


Figure S2. *Cont.*

(**E**) ^{1}H spectrum of DOM-3,15-disulfate, diammonium salt



(**F**) ^{13}C spectrum of DOM-3,15-disulfate, diammonium salt

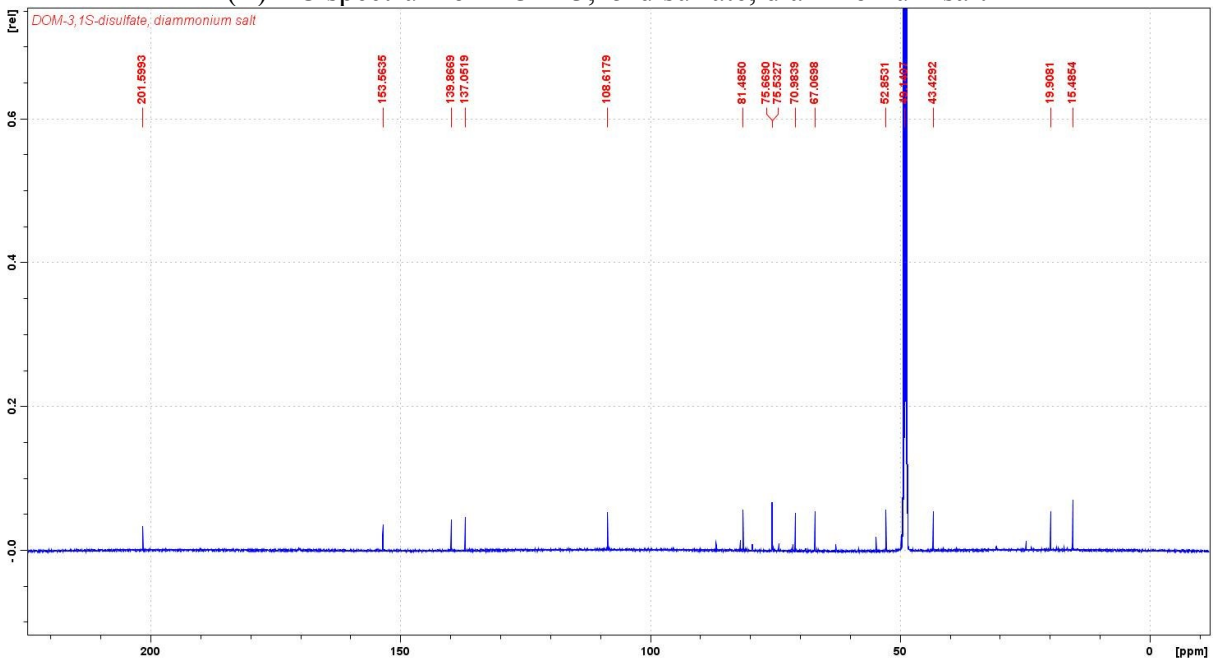


Figure S2. NMR Spectra of DOM-sulfates (ammonium salts).

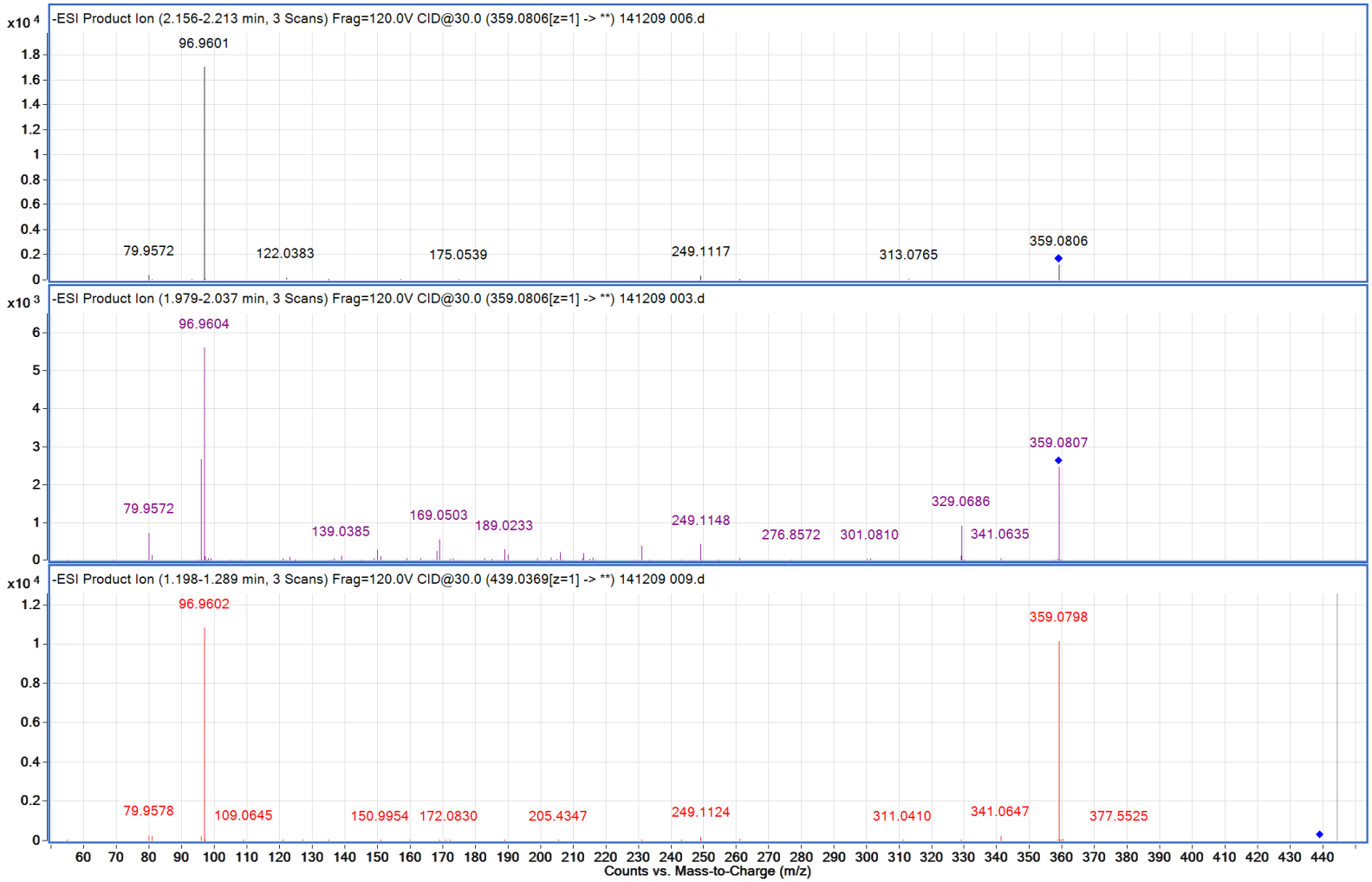


Figure S3. HR-MS/MS spectra of DOM-3-sulfate, DOM-15-sulfate and DOM-3,15-disulfate (CE 30 eV).

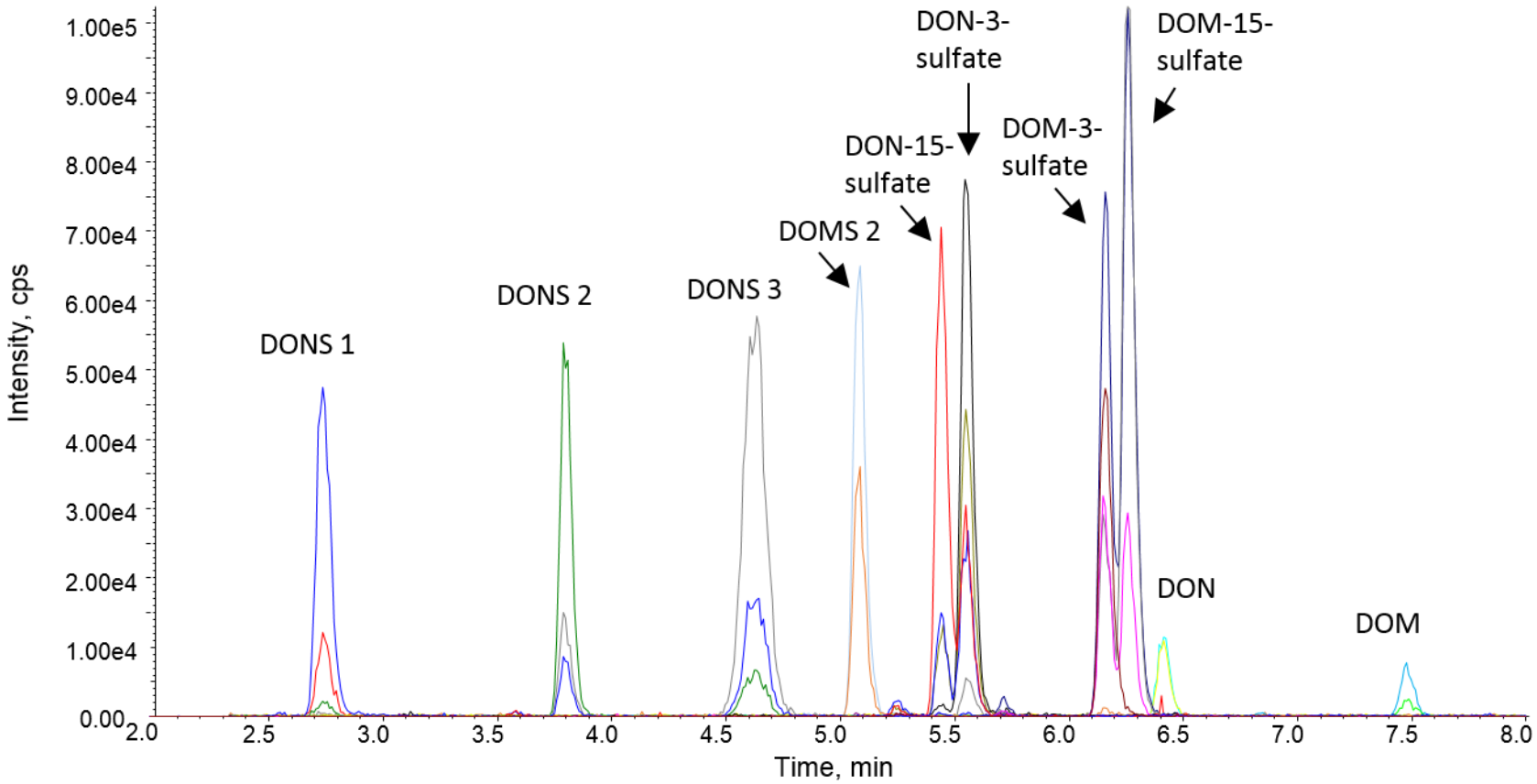


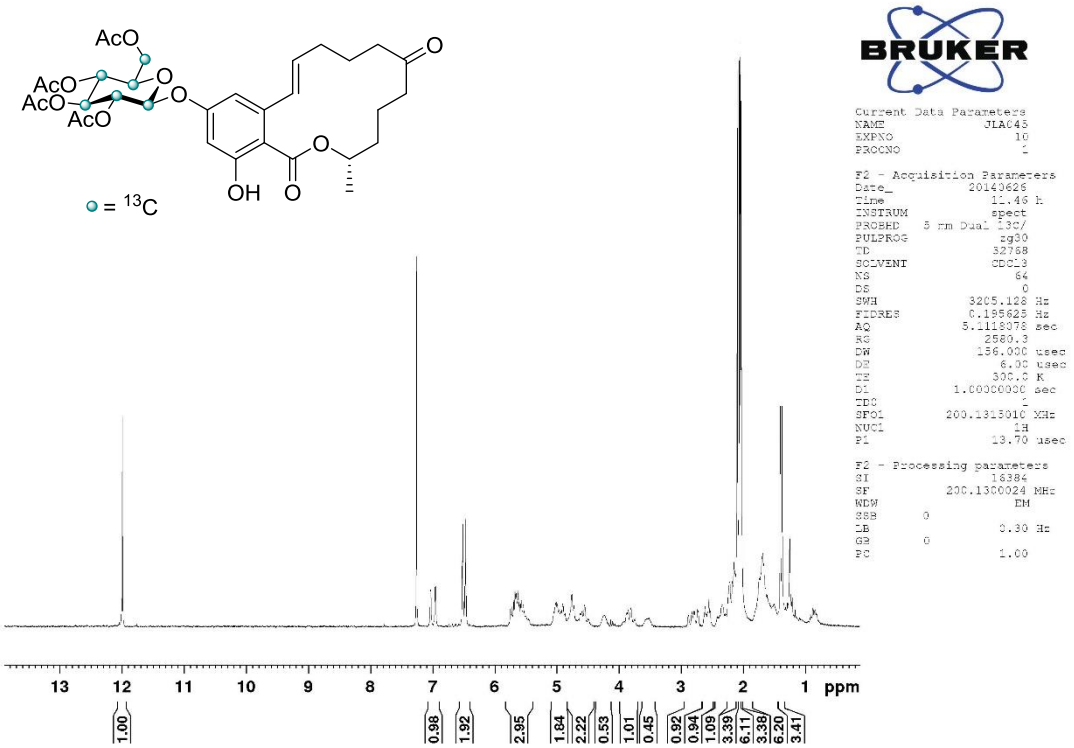
Figure S4. LC-MS/MS chromatogram of a standard solution containing 30 ng/mL of all analytes (long gradient method).

Supporting Information

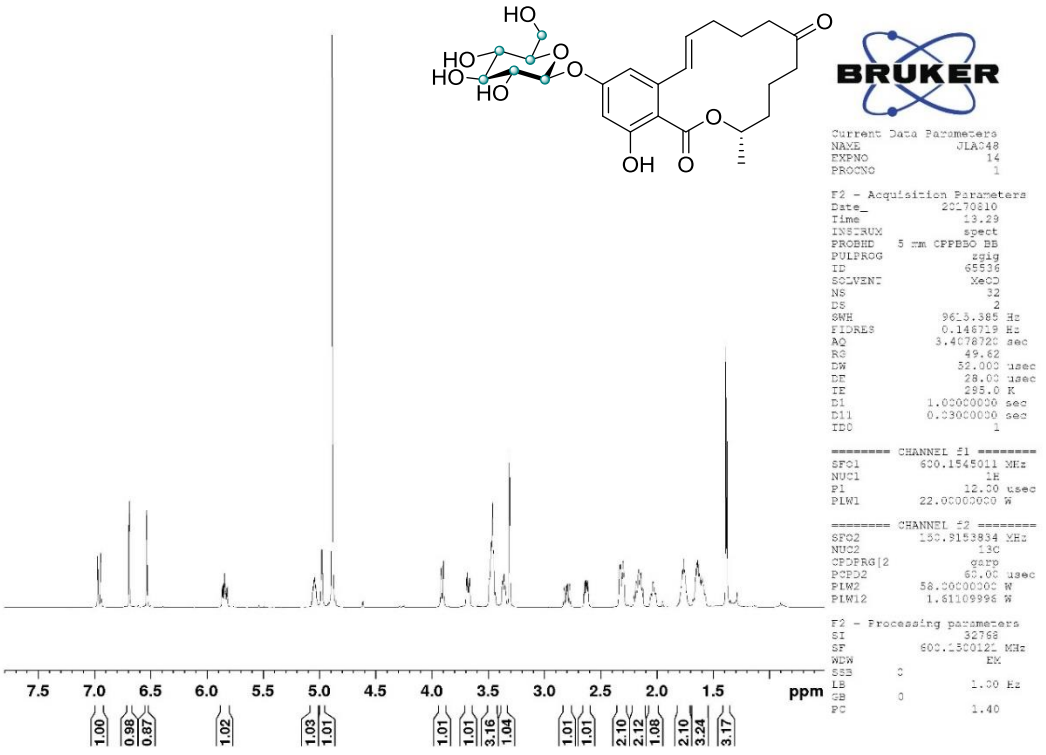
Technical Note

NMR Spectra and LC-MS

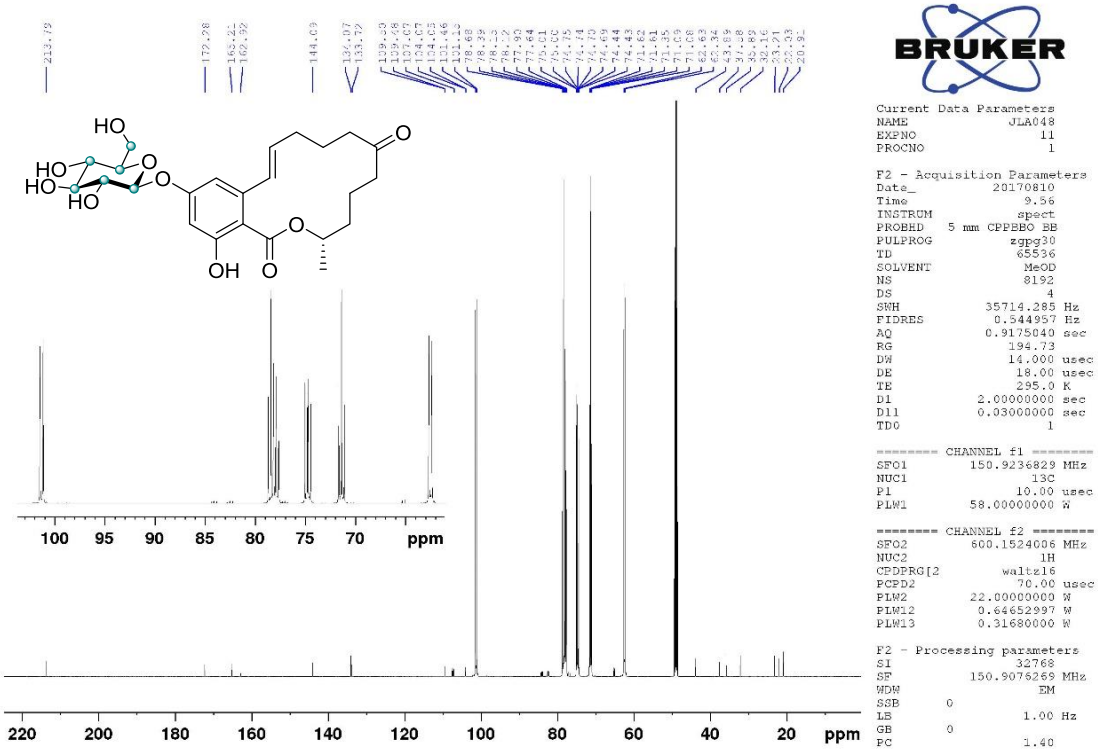
^{1}H NMR (*d*-chloroform, 200 MHz)



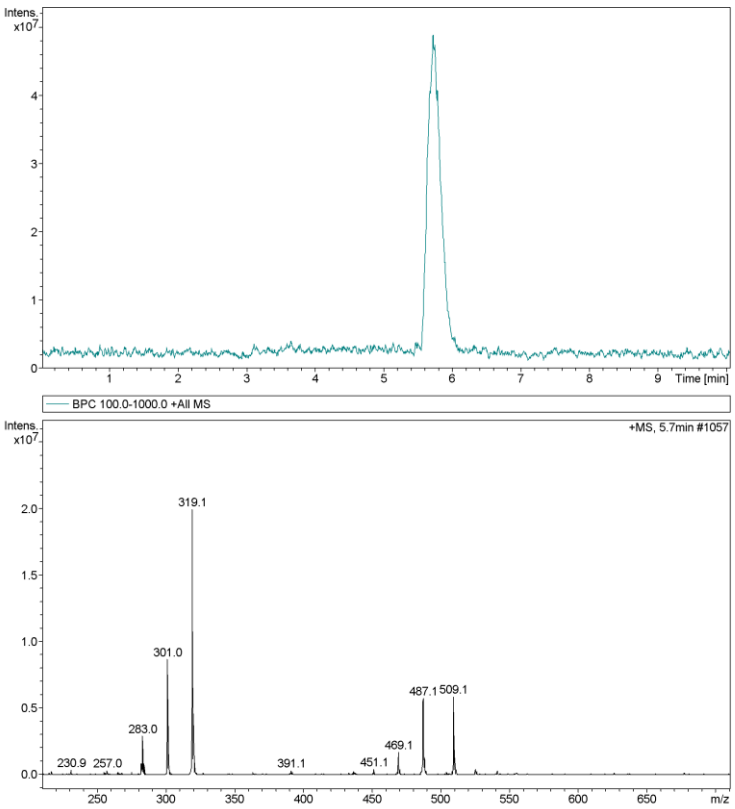
^{1}H NMR (*d*-methanol, 600 MHz, ^{13}C-decoupled)



^{13}C NMR (*d*-methanol, 150 MHz)



LCMS



LC-MS analysis using a Luna RP-C18 column (4.6 x 250 mm, 5 µm particle size, Phenomenex, Germany) gave a retention time of 5.7 min, if conditioned with 40 % acetonitrile in water at a flow rate of 1mL/min and holding this value for 5 min followed by a 5 min ramp reaching 65 % acetonitrile in water. During the whole analysis the eluent contained 0.1 % acetic acid.

Supporting Information

Manuscript #7

Supporting Information

for

Synthesis of zearalenone-16-β,D-glucoside and zearalenone-16-sulfate: A tale of protecting resorcylic acid lactones for regiocontrolled conjugation

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Experimental details (including remarks and general procedures), characterization data, copies of NMR spectra of new compounds, 2D NMR spectra of glucoside 7.

Content

1. Experimental

General remarks

All reactions were performed under an argon atmosphere. The progress of reactions was monitored by thin-layer chromatography (TLC) over silica gel 60 F254 (Merck). The chromatograms were visualized by irradiation with ultraviolet light or by heat staining with ceric ammonium molybdate in ethanol/sulfuric acid. LC–ESI–MS/MS was performed on an HCT ion trap mass spectrometer (Bruker, Germany) in full scan mode. Chromatographic separation was done on a 1200 series HPLC system (Agilent Technologies, Germany) using a Luna RP-C18 column (3.0 × 150 mm, 3 µm particle size, Phenomenex, Germany) and application of pure substances was achieved using a TLC–MS interface (Camag, Germany). Preparative column chromatography was performed on silica gel 60 (Merck, 40-63 µm) or RP-C18 silica gel (Merck, 40–63 µm) using a Büchi Sepacore™ Flash System. NMR spectra were recorded on a Bruker DPX-200 MHz or Avance DRX-400 MHz spectrometer. Data were recorded and evaluated using TOPSPIN 1.3 (Bruker Biospin). All chemical shifts are given in ppm relative to tetramethylsilane. The calibration was done using residual solvent signals. Multiplicities are abbreviated as s (singlet), d (doublet), t (triplet), q (quartet), b (broad signal). Zearalenone was obtained from Fermentek (Israel) and all other chemicals were purchased from ABCR (Germany) or Sigma-Aldrich (Austria/Germany).

General procedure A: regioselective acetylation of RAL type compounds

To a solution of the RAL type compound (1.0 mmol) and DMAP (1.2 mg, 10 µmol) in dry toluene (5 mL) at 0 °C was slowly added a solution of Ac_2O (110 mg, 1.1 mmol) in dry toluene (1 mL). The reaction mixture was gradually warmed to room temperature and stirred for further 16 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (hexanes/EtOAc gradient elution) to yield the desired 4'-O-acetylated product.

4-Acetoxy-2-hydroxybenzoic acid isopropyl ester (10)

Following general procedure A, **10** was obtained as a white solid (203 mg, 85%); R_f 0.4 (hexanes/EtOAc, 9/1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 11.07 (s, 1H, 2-OH), 7.84 (d, J = 8.8 Hz, 1H, H-6), 6.72 (d, J = 2.3 Hz, 1H, H-3), 6.62 (dd, J = 8.8, 2.3 Hz, 1H, H-5), 5.26 (sept., J = 6.2 Hz, 1H, COOCH), 2.27 (s, 3H, CH_3COO), 1.36 (d, J = 6.2 Hz, 6H, $(CH_3)_2CH$); ^{13}C NMR (50 MHz, $CDCl_3$) δ 169.2 (s), 168.5 (s), 162.9 (s), 156.1 (s), 131.0 (d), 112.9 (d), 110.7 (s), 110.4 (d), 69.3 (d), 21.8 (q), 21.1 (q); HRMS *m/z* calcd for $C_{12}H_{14}NaO_5^+$ $[M+Na]^+$ 261.0733, found 261.0750.

14-*O*-Acetylzearalenone (14)

Following general procedure A (reduced scale), **14** was obtained starting from ZEN (**1**, 32 mg, 0.1 mmol) as a white solid (27 mg, 75%); R_f 0.4 (hexanes/EtOAc, 2/1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 11.88 (s, 1H, 16-OH), 7.00 (d, J = 15.6 Hz, 1H, H-12), 6.67-6.62 (m, 2H, H-13, H-15), 5.71 (ddd, J = 15.4, 10.1, 4.3 Hz, 1H, H-11), 5.11-4.93 (m, 1H, H-3), 2.81 (ddd, J = 18.7, 12.1, 2.5 Hz, 1H, H_a-8), 2.59 (m, 1H, H_a-6), 2.37-2.05 (m, 5H, H_a-5, H_b-6, H_b-8, H_{ab}-10), 2.29 (s, 3H, CH_3COO), 1.85-1.54 (m, 5H, H_{ab}-4, H_b-5, H_{ab}-9), 1.39 (d, J = 6.1 Hz, 3H, CH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ 211.0 (s, C-7), 171.1 (s, CH_3COO), 168.8 (s, C-1), 164.6 (s, C-14), 154.9 (s, C-16), 143.4 (s, C-12a), 133.5 (d, C-11), 132.6 (d, C-12), 113.7 (d, C-13), 109.6 (d, C-15), 108.3 (s, C-16a), 74.1 (d, C-3), 43.0 (t, C-6), 36.7 (t, C-8), 34.7 (t, C-4), 31.1 (t, C-10), 22.2 (t, C-5), 21.2 (q, CH_3COO), 21.0 (t, C-9), 20.8 (q, CH_3); NMR data matched that reported previously by Munoz et al.[1] HRMS *m/z* calcd for $C_{20}H_{24}NaO_6^+$ $[M+Na]^+$ 383.1465, found 383.1466.

General procedure B: regioselective *p*-methoxybenzylation of RAL type compounds

To a solution of the RAL type compound (1 mmol) and Cs_2CO_3 (433 mg, 1.5 mmol) in dry DMF (5 mL) was slowly added a solution of *p*-methoxybenzyl chloride (PMB-Cl, 172 mg, 1.1 mmol) in dry DMF (1 mL). The reaction mixture was stirred at room temperature for 24 h, diluted with water (20 mL) and extracted with EtOAc (3 × 15 mL). The combined organic layer was washed with brine,

[1] Munoz, L.; Castro, J.L.; Cardelle, M.; Castedo, L.; Riguera, R. *Phytochemistry* **1989**, *28*, 83-85.

dried over Na_2SO_4 and concentrated. Column chromatography (hexane/EtOAc gradient elution) afforded the desired 4'-O-PMB protected compound.

2-Hydroxy-4-((*p*-methoxybenzyl)oxy)benzoic acid isopropyl ester (15)

Following general procedure B, **15** was obtained as a white solid (256 mg, 81%); R_f 0.8 (hexanes/EtOAc, 3/1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 11.07 (s, 1H, 2-OH), 7.66 (d, J = 8.5 Hz, 1H, H-6), 7.26 (d, J = 8.5 Hz, 2H, PMB), 6.84 (d, J = 8.5 Hz, 2H, PMB), 6.46-6.36 (m, 2H, H-3, H-5), 5.17 (sept., J = 6.5 Hz, 1H, COOCH), 4.92 (s, 2H, OCH_2), 3.74 (s, 3H, OCH_3), 1.29 (d, J = 6.4 Hz, 6H, $(CH_3)_2CH$); ^{13}C NMR (50 MHz, $CDCl_3$) δ 169.6 (s), 164.6 (s), 163.8 (s), 159.6 (s), 131.2 (d), 129.3 (d), 128.1 (s), 114.1 (d), 108.0 (d), 106.1 (s), 101.6 (d), 69.9 (t), 68.7 (q), 55.3 (q), 21.9 (q); HRMS *m/z* calcd for $C_{18}H_{20}NaO_5^+$ $[M+Na]^+$ 339.1203, found 339.1195.

14-*O*-(*p*-Methoxybenzyl)zearalenone (18)

Following general procedure B (reduced scale), **18** was obtained starting from ZEN (**1**, 32 mg, 0.1 mmol) as a white solid (31 mg, 71%); R_f 0.6 (hexanes/EtOAc, 2/1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 12.01 (s, 1H, 16-OH), 7.27 (d, J = 8.6 Hz, 2H, PMB), 6.94 (d, J = 14.9 Hz, 1H, H-12), 6.84 (d, J = 8.8 Hz, 2H, PMB), 6.46 (d, J = 2.6 Hz, 1H, H-13), 6.39 (d, J = 2.6 Hz, 1H, H-15), 5.60 (ddd, J = 15.5, 10.3, 4.0 Hz, 1H, H-11), 5.03-4.83 (m, 3H, H-3, OCH_2), 3.74 (s, 3H, OCH_3), 2.77 (ddd, J = 18.2, 12.2, 2.9 Hz, 1H, H_a-8), 2.60-2.45 (m, 1H, H_a-6), 2.37-1.96 (m, 5H, H_a-5, H_b-6, H_b-8, H_{ab}-10), 1.78-1.45 (m, 5H, H_{ab}-4, H_b-5, H_{ab}-9), 1.31 (d, J = 6.1 Hz, 3H, CH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ 211.0 (s, C-7), 171.4 (s, C-1), 165.6 (s, C-14), 163.2 (s, C-16), 159.6 (s, Ar-OCH_3), 143.3 (s, C-12a), 133.3 (d, C-11), 132.4 (d, C-12), 129.3 (d, PMB), 128.1 (s, PMB), 114.0 (d, PMB), 108.9 (d, C-13), 103.7 (s, C-16a), 100.8 (d, C-15), 73.4 (d, C-3), 69.9 (t, OCH_2), 55.4 (q, OCH_3), 42.9 (t, C-6), 36.6 (t, C-8), 34.7 (t, C-4), 31.0 (t, C-10), 22.3 (t, C-5), 21.0 (t, C-9), 20.9 (q, CH_3); HRMS *m/z* calcd for $C_{26}H_{30}NaO_6^+$ $[M+Na]^+$ 461.1935, found 461.1957.

General procedure C: regioselective TIPS-protection of RAL type compounds

To a solution of the RAL type compound (1 mmol) and imidazole (156 mg, 2.3 mmol) in dry CH_2Cl_2 (10 mL) was slowly added TIPS-Cl (216 mg, 1.1 mmol). The reaction mixture was stirred at room temperature for 16 h, diluted with CH_2Cl_2 (20 mL), washed with brine, dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography (hexanes/EtOAc gradient elution).

2-Hydroxy-4-(triisopropylsilyloxy)benzoic acid isopropyl ester (21)

Following general procedure C, **21** was obtained as a colorless viscous oil (350 mg, 99%); R_f 0.9 (hexanes/EtOAc, 12/1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 11.0 (s, 1H, 2-OH), 7.70 (d, J = 8.7 Hz, 1H, H-6), 6.44 (d, J = 2.3 Hz, 1H, H-3), 6.39 (dd, J = 8.6, 2.3 Hz, 1H, H-5), 5.24 (sept., J = 6.2 Hz, 1H, COOCH), 1.35 (d, J = 6.3 Hz, 6H, (CH_3)$_2$CH), 1.31-1.16 (m, 3H, SiCH), 1.12-1.04 (m, 18H, SiCH(CH_3)$_2$); ^{13}C NMR (50 MHz, $CDCl_3$) δ 169.6 (s), 163.5 (s), 162.5 (s), 131.2 (d), 112.1 (d), 107.5 (d), 106.6 (s), 68.6 (d), 21.9 (q), 17.8 (q), 12.9 (d); HRMS *m/z* calcd for $C_{19}H_{32}NaO_4Si^+$ $[M+Na]^+$ 375.1962, found 375.1960.

14-*O*-(Triisopropylsilyl)zearalenone (22)

Following general procedure C (reduced scale), **22** was obtained starting from ZEN (**1**, 100 mg, 0.31 mmol) as a white solid (145 mg, 99%); R_f 0.8 (hexanes/EtOAc, 2/1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 11.85 (s, 1H, 16-OH), 6.90 (d, J = 15.2 Hz, 1H, H-12), 6.30 (d, J = 2.5 Hz, 1H, H-13), 6.25 (d, J = 2.5 Hz, 1H, H-15), 5.51 (ddd, J = 15.3, 10.2, 3.8 Hz, 1H, H-11), 4.97-4.79 (m, 1H, H-3), 2.75 (ddd, J = 18.4, 12.2, 3.0 Hz, 1H, H_a-8), 2.55-2.41 (m, 1H, H_a-6), 2.34-1.93 (m, 5H, H_a-5, H_b-6, H_b-8, H_{ab}-10), 1.76-1.35 (m, 5H, H_{ab}-4, H_b-5, H_{ab}-9), 1.26 (d, J = 6.1 Hz, 3H, CH_3), 1.21-1.09 (m, 3H, SiCH), 1.02-0.93 (m, 18H, SiCH(CH_3)$_2$); 13C NMR (50 MHz, $CDCl_3$) δ 211.1 (s, C-7), 171.4 (s, C-1), 165.3 (s, C-16), 161.2 (s, C-14), 143.4 (s, C-12a), 133.4 (d, C-11), 132.1 (d, C-12), 113.0 (s, C-13), 106.7 (d, C-15), 104.1 (d, C-16a), 73.3 (d, C-3), 43.0 (t, C-6), 36.7 (t, C-8), 34.8 (t, C-6), 31.1 (t,

C-10), 22.3 (t, C-5), 21.0 (t, C-9), 20.9 (q, CH_3), 17.9 (q, SiCH(CH_3)$_2$), 12.7 (d, SiCH); HRMS *m/z* calcd for $C_{27}H_{42}NaO_5Si^+$ $[M+Na]^+$ 497.6294, found 497.6299.

General procedure D: Königs–Knorr glucosylation

To a solution of the glucosyl acceptor (0.5 mmol) and bromoacetoglucose **13** (617 mg, 1.5 mmol) in dry MeCN (10 mL) was added molecular sieve 3 Å (1 g) and the resulting suspension was stirred at room temperature for 1 h. After addition of Ag_2O (174 mg, 0.75 mmol), the reaction mixture was stirred in the dark for 48 h. Since TLC analysis indicated remaining glucosyl acceptor, **13** (411 mg, 1 mmol) and Ag_2O (116 mg, 0.5 mmol) were added and stirring was continued in the dark for 24 h. The reaction mixture was diluted with CH_2Cl_2, filtered through Celite and concentrated under reduced pressure. Silica gel filtration (hexane/EtOAc gradient elution) was performed to remove most the carbohydrate byproducts. Since we were not able to fully purify the crude glucosylated products, these compounds were directly subjected to deprotection within subsequent steps.

5-Hydroxy-2-(isopropoxycarbonyl)phenyl-β,D-glucoside (17)

Following general procedure D, crude **16** was obtained starting from the PMB protected ZEN mimic **15** (95 mg, 0.3 mmol). This material was dissolved in CH_2Cl_2/H_2O (10/1, 6 mL) and cooled to 0 °C. After addition of DDQ (102 mg, 0.45 mmol) the reaction mixture was gradually warmed to room temperature. TLC/MS analysis indicated full conversion after stirring for 24 h. The reaction mixture was diluted with CH_2Cl_2 (20 mL), washed with satd. aq. $NaHCO_3$, dried over Na_2SO_4 and concentrated. The crude product was dissolved in THF/H_2O (4/1, 7 mL) and KOH (168 mg, 3 mmol) was added. After stirring for 2 h at room temperature, the solution was diluted with water (15 mL) and acidified to pH 6.5 by slow addition of 0.2 M aq. HCl followed by extraction with EtOAc (4 × 15 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (MPLC, RP-C18, MeCN/H_2O, gradient elution) to yield the desired product **17** as a white solid (32 mg, 30%); R_f 0.5 (CH_2Cl_2/MeOH, 5/1); ^{1}H NMR

(200 MHz, CD_3OD) δ 7.68 (d, J = 8.8 Hz, 1H, H-6), 6.80 (d, J = 2.3 Hz, 1H, H-3), 6.52 (dd, J = 8.8, 2.3 Hz, 1H, H-5), 5.16 (sept., J = 6.3 Hz, 1H, COOCH), 4.82 (d, J = 7.2 Hz, 1H, H-1'), 3.94 (dd, J = 11.8, 1.5 Hz, 1H, H_a-6'), 3.80-3.68 (m, 1H, H_b-6'), 3.56-3.39 (m, 4H, H-2', H-3', H-4', H-5'), 1.34 (d, J = 6.3 Hz, 6H, $(CH_3)_2CH$); ^{13}C NMR (50 MHz, CD_3OD) δ 167.3 (s), 164.4 (s), 161.4 (s), 134.1 (d), 113.2 (s), 111.0 (d), 105.9 (d), 104.3 (d), 78.5 (d), 77.5 (d), 74.9 (d), 71.2 (d), 69.4 (d), 62.5 (t), 22.2 (q), 22.1 (q); HRMS *m/z* calcd for $C_{16}H_{22}NaO_9^+$ $[M+Na]^+$ 381.1156, found 381.1145.

Zearalenone-16-β,D-glucoside (7)

Following general procedure D, crude protected ZEN-16-glucoside was obtained starting from **22** (47 mg, 0.1 mmol). This material was dissolved in dry THF (2 mL) and the resulting solution was cooled to −10 °C. After addition of AcOH (6 mg, 0.1 mmol) and TBAF (0.1 mL, 0.1 mmol, 1 M in THF), the reaction was stirred at −10 °C for 3 h. TLC/MS analysis indicated full conversion of the starting material to the desired product. The solvent was evaporated and the residue was dissolved in THF/H_2O (4/1, 6 mL). KOH (56 mg, 1 mmol) was added and the reaction mixture was stirred at room temperature for 2 h. The solution was diluted with water (15 mL) and acidified to pH 6.5 by slow addition of 0.2 M aq. HCl followed by extraction with EtOAc (4 × 15 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (MPLC, RP-C18, MeCN/H_2O, gradient elution) to yield the desired product **7** as a white solid (16 mg, 34%, 3 steps); R_f 0.4 (CH_2Cl_2/MeOH, 6/1); ^1H and ^{13}C NMR data matched that reported[2] (for additional 2D NMR spectra see Figures S1, S2 and S3); HRMS *m/z* calcd for $C_{24}H_{32}NaO_{10}^+$ $[M+Na]^+$ 503.1888, found 503.1905.

16-*O*-((2,2,2,-Trichloroethoxy)sulfonyl)-14-*O*-(triisopropylsilyl)zearalenone (24)

To a solution of **22** (47.5 mg, 0.1 mmol) in dry CH_2Cl_2 (2 mL) cooled to 0 °C was added 1,2-dimethylimidazole (28 mg, 0.5 mmol) and **23** (184 mg, 0.4 mmol). The reaction mixture was warmed

[2] Kovalsky Paris, M. P.; Schweiger, W.; Hametner, C.; Stückler, R.; Muehlbauer, G. J.; Varga, E.; Krska, R.; Berthiller, F.; Adam, G. *J. Agric. Food Chem*. **2014**, *62*, 1181-1189.

to room temperature and stirred overnight. After dilution with CH_2Cl_2 (10 mL), the solution was washed with water, dried over Na_2SO_4 and concentrated. Column chromatography over silica gel (hexanes/EtOAc gradient elution) gave the desired product **24** as a colorless viscous oil (62 mg, 91%); R_f 0.4 (hexanes/EtOAc, 5/1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.96 (d, J = 2.2 Hz, 1H, H-13), 6.92 (d, J = 2.2 Hz, 1H, H-15), 6.45 (d, J = 15.6 Hz, 1H, H-12), 5.93 (ddd, J = 15.5, 9.5, 4.7 Hz, 1H, H-11), 5.42-5.25 (m, 1H, H-3), 4.92 (d, J = 11.0 Hz, 1H, CH_{2a}), 4.84 (d, J = 11.0 Hz, 1H, CH_{2b}), 2.65 (ddd, J = 17.6, 10.9, 3.2 Hz, 1H, H_a-8), 2.45-2.18 (m, 4H, H_{ab}-6, H_b-8, H_a-10), 2.15-1.93 (m, 2H, H_a-5, H_b-10), 1.80-1.51 (m, 5H, H_{ab}-4, H_b-5, H_{ab}-9), 1.37 (d, J = 6.3 Hz, 3H, CH_3), 1.31-1.18 (m, 3H, SiCH), 1.14-1.05 (m, 18H, SiCH(CH_3)$_2$); ^{13}C NMR (50 MHz, $CDCl_3$) δ 211.1 (s, C-7), 165.2 (s, C-1), 158.0 (s, C-16), 147.8 (s, C-14), 138.7 (s, C-12a), 134.8 (d, C-11), 128.4 (d, C-12), 118.6 (s, C-16a), 116.1 (d, C-13), 110.8 (d, C-15), 92.4 (s, CCl_3), 80.6 (t, OCH_2), 72.2 (d, C-3), 43.7 (t, C-6), 37.6 (t, C-8), 35.1 (t, C-4), 31.4 (t, C-10), 21.5 (t, C-5), 21.2 (t, C-9), 19.8 (q, CH_3), 17.9 (q, SiCH(*C*H$_3$)$_2$), 12.60 (d, SiCH); HRMS *m/z* calcd for $C_{29}H_{43}Cl_3NaO_8SSi^+$ $[M+Na]^+$ 707.1406, found 707.1433.

Zearalenone-16-sulfate, tetrabutylammonium salt (NBu_4-8)

To a solution of the **24** (55 mg, 0.08 mmol) in dry THF (1 mL), cooled to −10 °C, was added AcOH (5 mg, 0.083 mmol) and TBAF (83 µL, 0.083 mmol, 1 M in THF). The reaction mixture was stirred at −10 °C for 3 h and subsequently concentrated under reduced pressure. The residue was dissolved in dry MeOH (1 mL) and after addition of $HCOONH_4$ (44 mg, 0.7 mmol) and zinc dust (16 mg, 0.25 mmol), the reaction mixture was stirred for 16 h at room temperature. Filtration through Celite and evaporation of the solvent afforded the crude product, which was purified by column chromatography over silica gel (CH_2Cl_2/MeOH/NH_4OH = 10/4/1). **8** was obtained as tetrabutylammonium salt starting from **24** as a white solid (33 mg, 65%); R_f 0.5 (CH_2Cl_2/MeOH/NH_4OH, 20/5/1); ^{1}H NMR (400 MHz, CD_3OD) δ 7.01 (d, J = 1.9 Hz, 1H, Ar-H), 6.75 (d, J = 1.9 Hz, 1H, Ar-H), 6.39 (d, J = 15.7 Hz, 1H, H-12), 6.01 (ddd, J = 15.7, 10.0, 4.3 Hz,

1H, H-11), 5.33-5.24 (m, 1H, H-3), 3.26-3.19 (m, 8H, N^+CH_2), 2.72 (ddd, *J* = 17.1, 11.1, 3.1 Hz, 1H, H_a-8), 2.50 (ddd, *J* = 13.2, 8.5, 4.5 Hz, 1H, H_a-6), 2.37-2.21 (m, 3H, H_b-6, H_b-8, H_a-10), 2.10-1.97 (m, 2H, H_a-5, H_b-10), 1.85-1.70 (m, 2H, H_a-4, H_a-9), 1.69-1.54 (m, 11H, $N^+CH_2CH_2$, H_b-4, H_b-5, H_b-9), 1.47-1.35 (m, 11H, $N^+CH_2CH_2CH_2$, CH_3), 1.02 (t, *J* = 7.4 Hz, 12H, $N^+CH_2CH_2CH_2CH_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 212.7 (s, C-7), 167.6 (s, C-1), 159.0 (s, C-14), 150.8 (s, C-16), 136.7 (s, C-12a), 132.7 (d, C-11), 128.7 (d, C-12), 117.8 (s, C-16a), 107.5 (d, C-13), 106.8 (d, C-15), 71.2 (d, C-3), 58.05 (q_Nt_H, $^2J_{CN}$ = 2.1 Hz, N^+CH_2, 4C), 43.4 (t, C-6), 37.1 (t, C-8), 34.8 (t, C-4), 31.0 (t, C-10), 23.4 (t, $N^+CH_2CH_2$), 21.2 (t, C-5), 21.0 (t, C-9), 19.3 (t, $N^+CH_2CH_2CH_2$), 18.9 (q, CH_3), 12.6 (q, $N^+CH_2CH_2CH_2CH_3$); HRMS *m/z* calcd for $C_{18}H_{21}O_8S^-$ $[M\text{-}NBu_4]^-$ 397.0963, found 397.0961.

2. NMR spectra

Figure S1: COSY spectrum of **7**

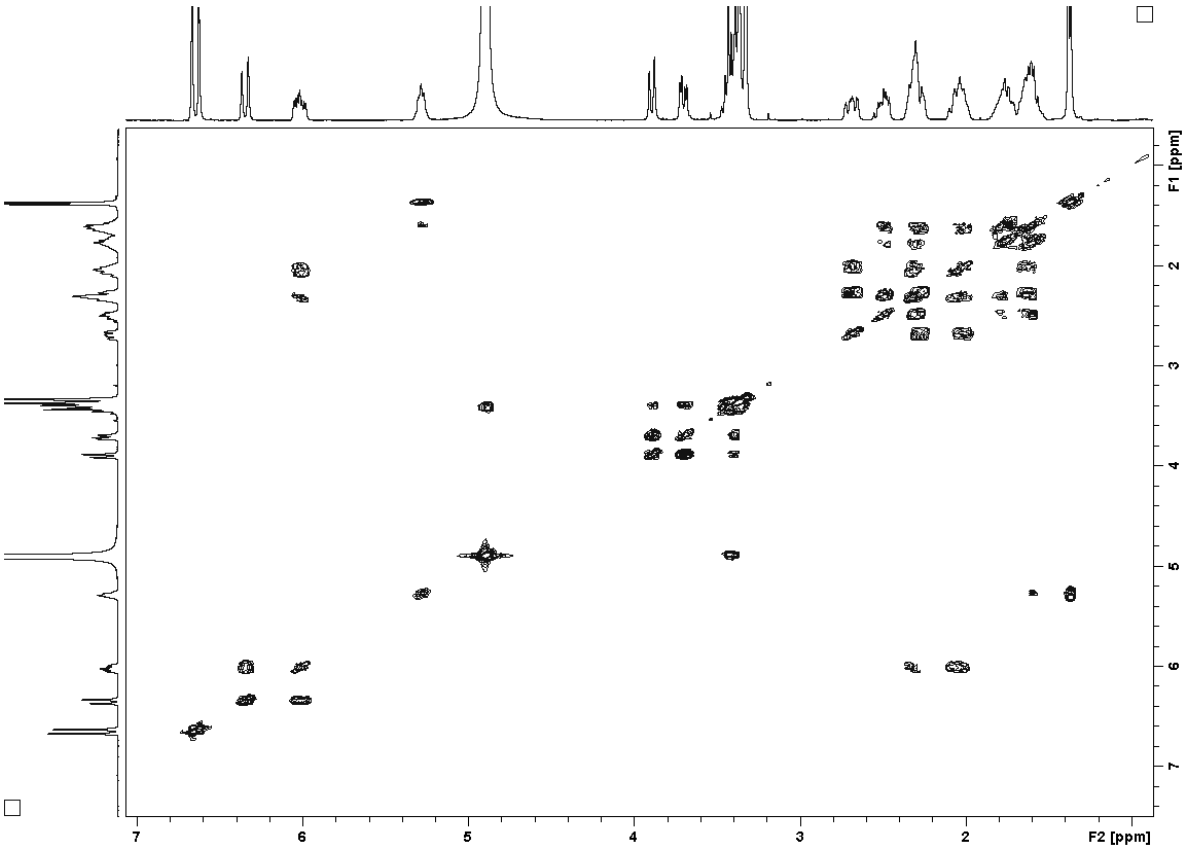


Figure S2: HSQC spectrum of **7**

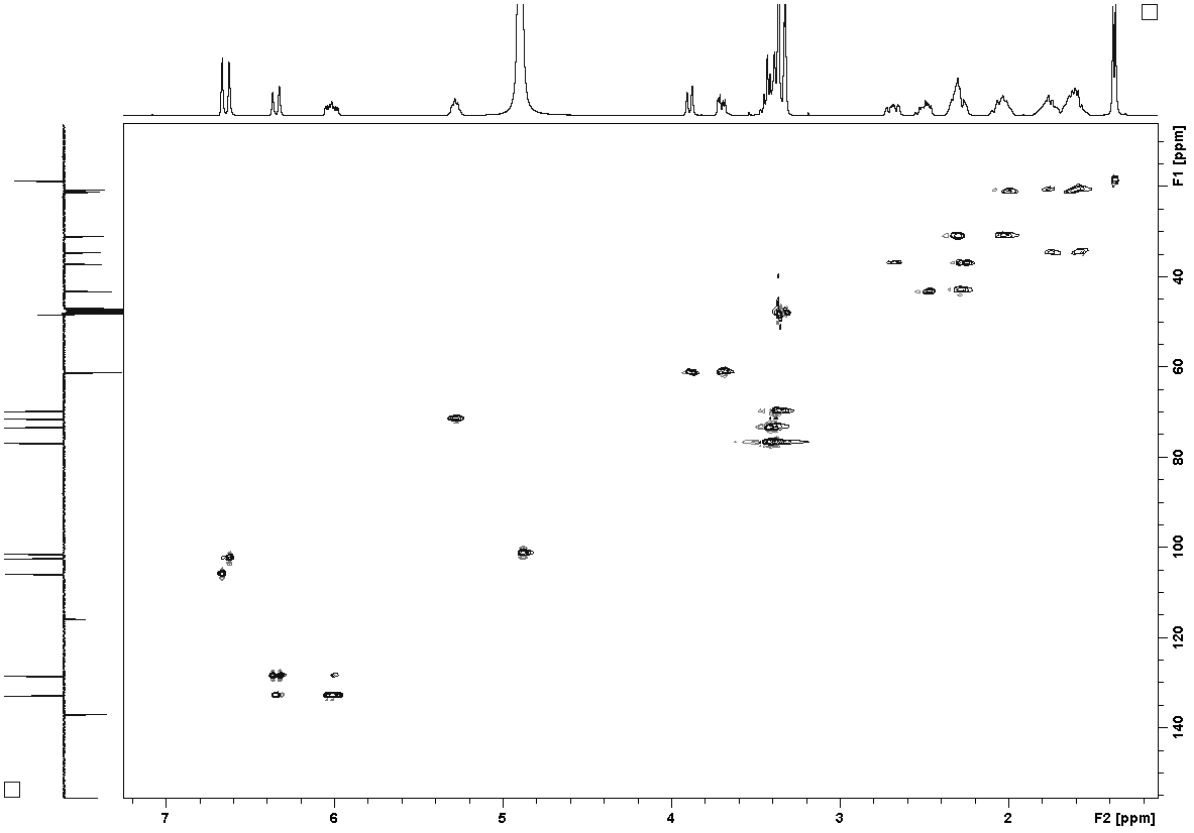


Figure S3: HMBC spectrum of **7**

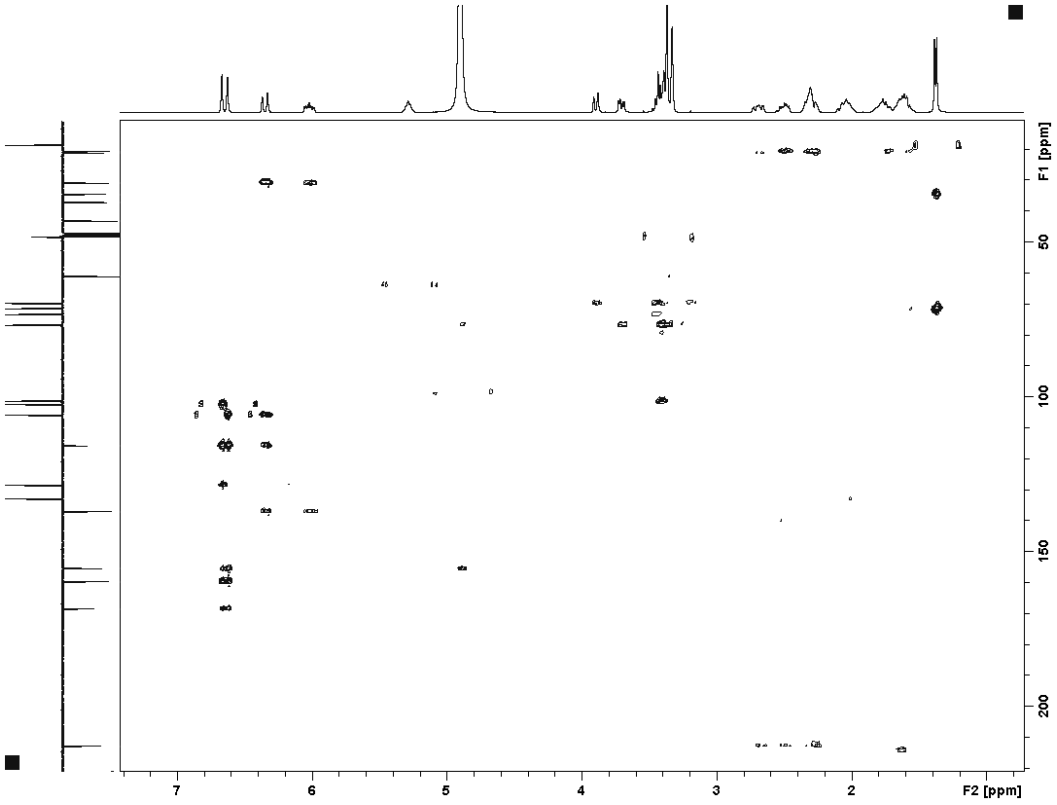


Figure S4: ^{1}H NMR spectrum of **8**

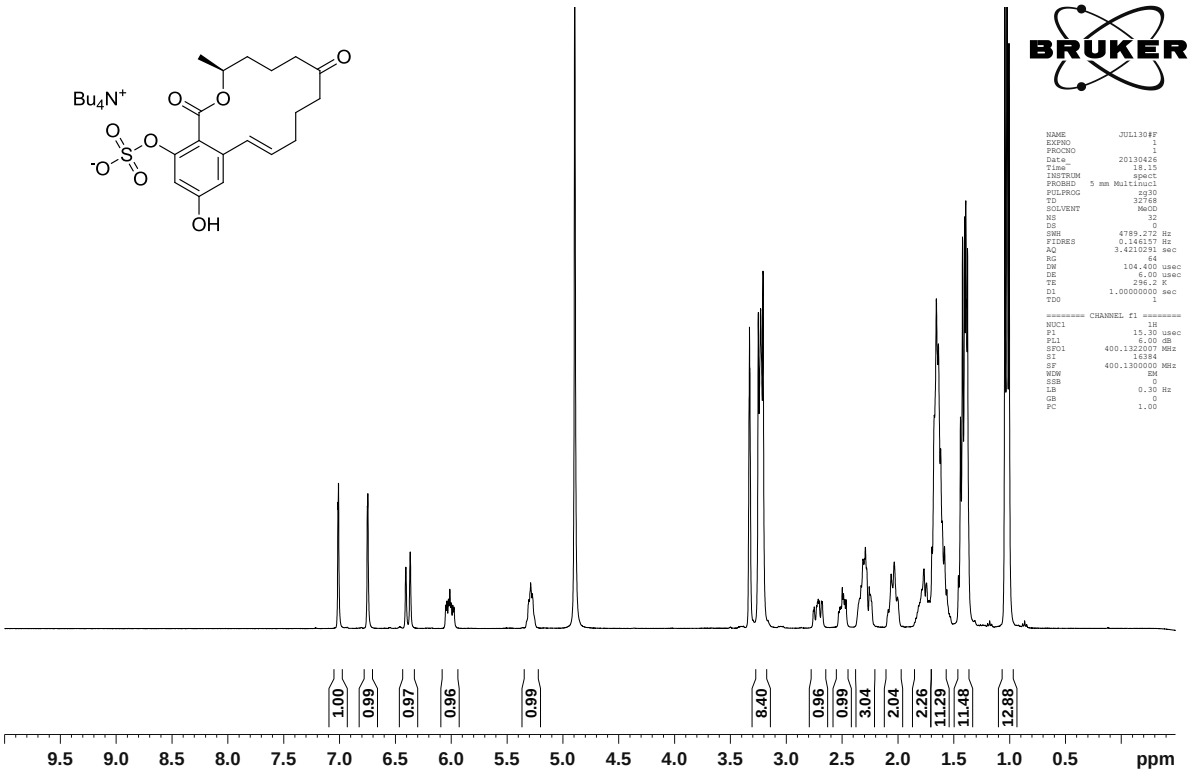


Figure S5: ^{13}C NMR spectrum of **8**

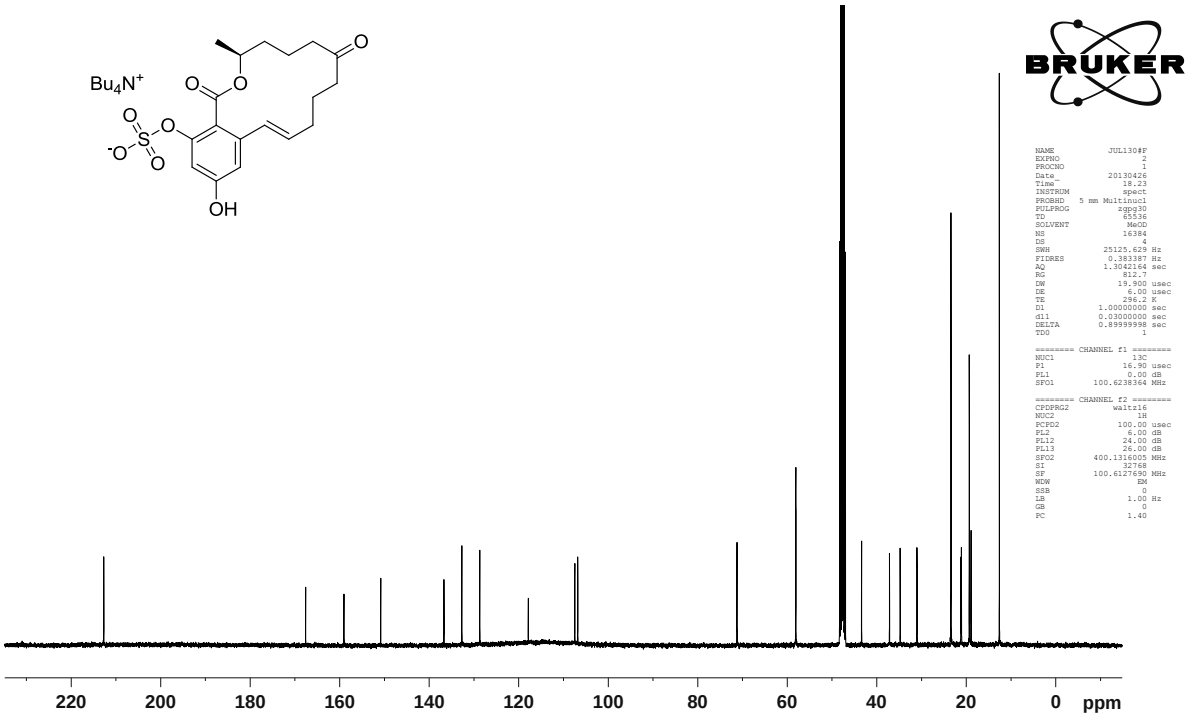


Figure S6: ^{1}H NMR spectrum of **10**

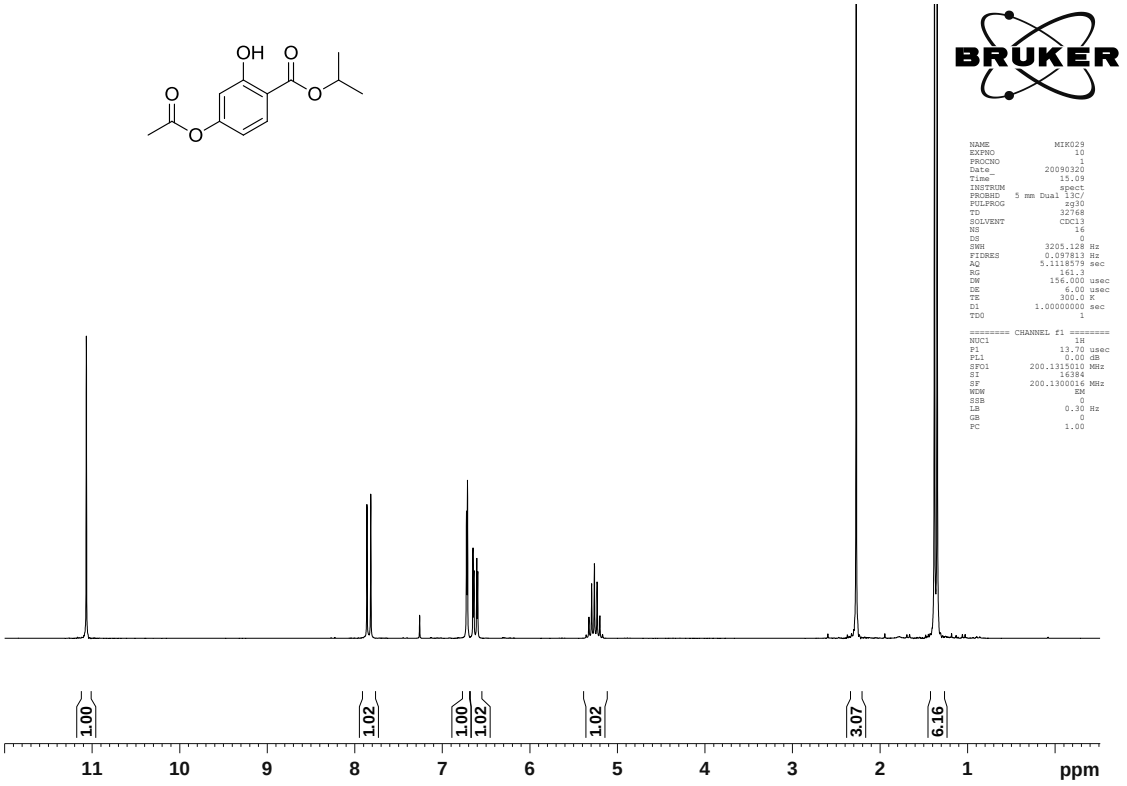


Figure S7: ^{13}C NMR spectrum of **10**

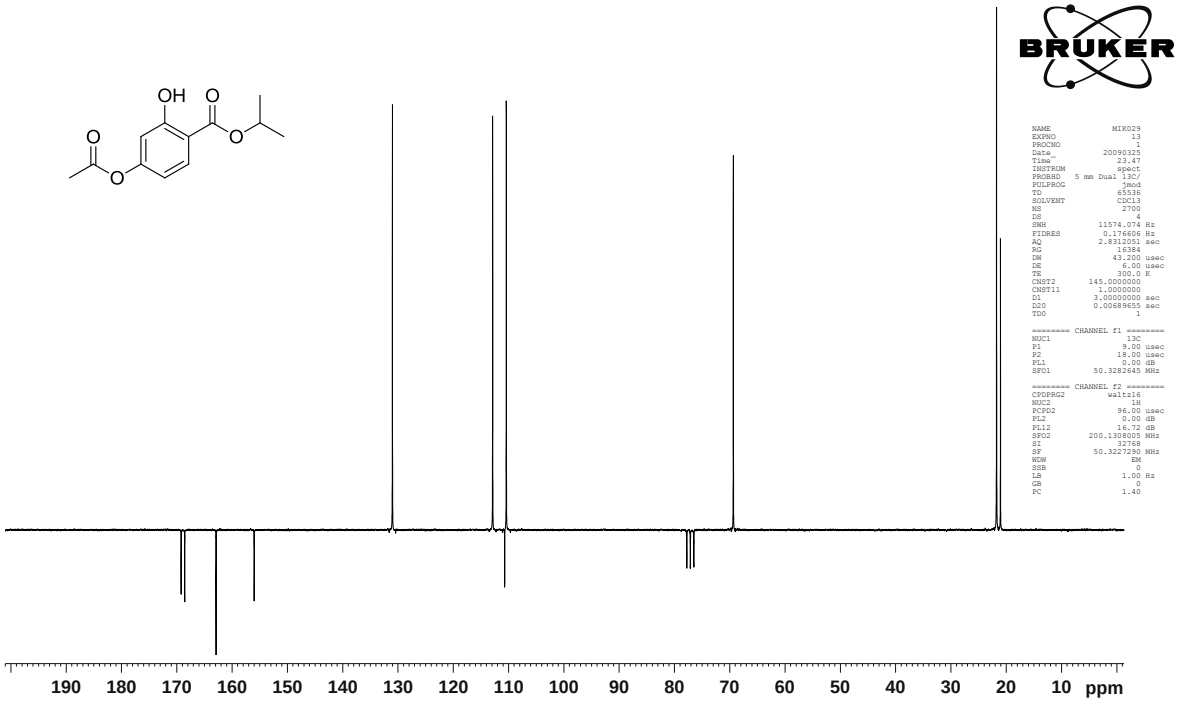


Figure S8: ^{1}H NMR spectrum of **14**

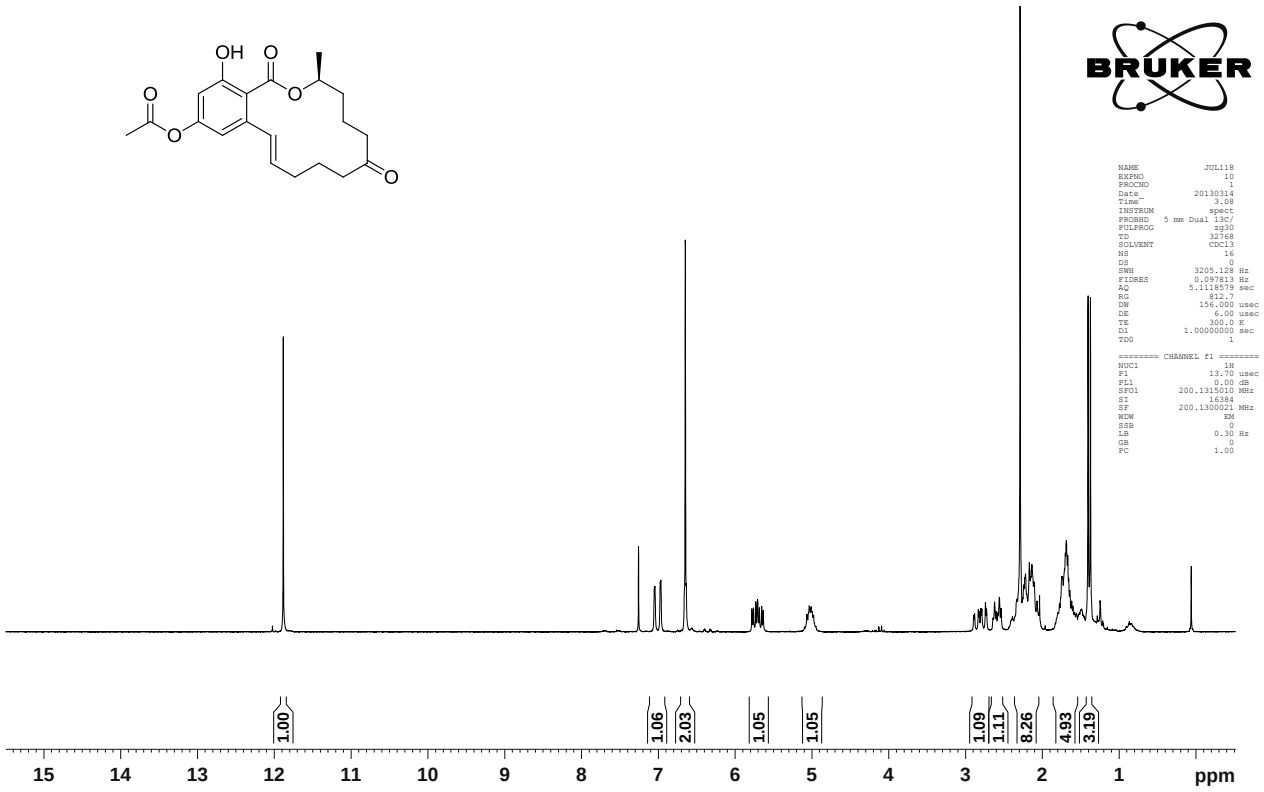


Figure S9: ^{13}C NMR spectrum of **14**

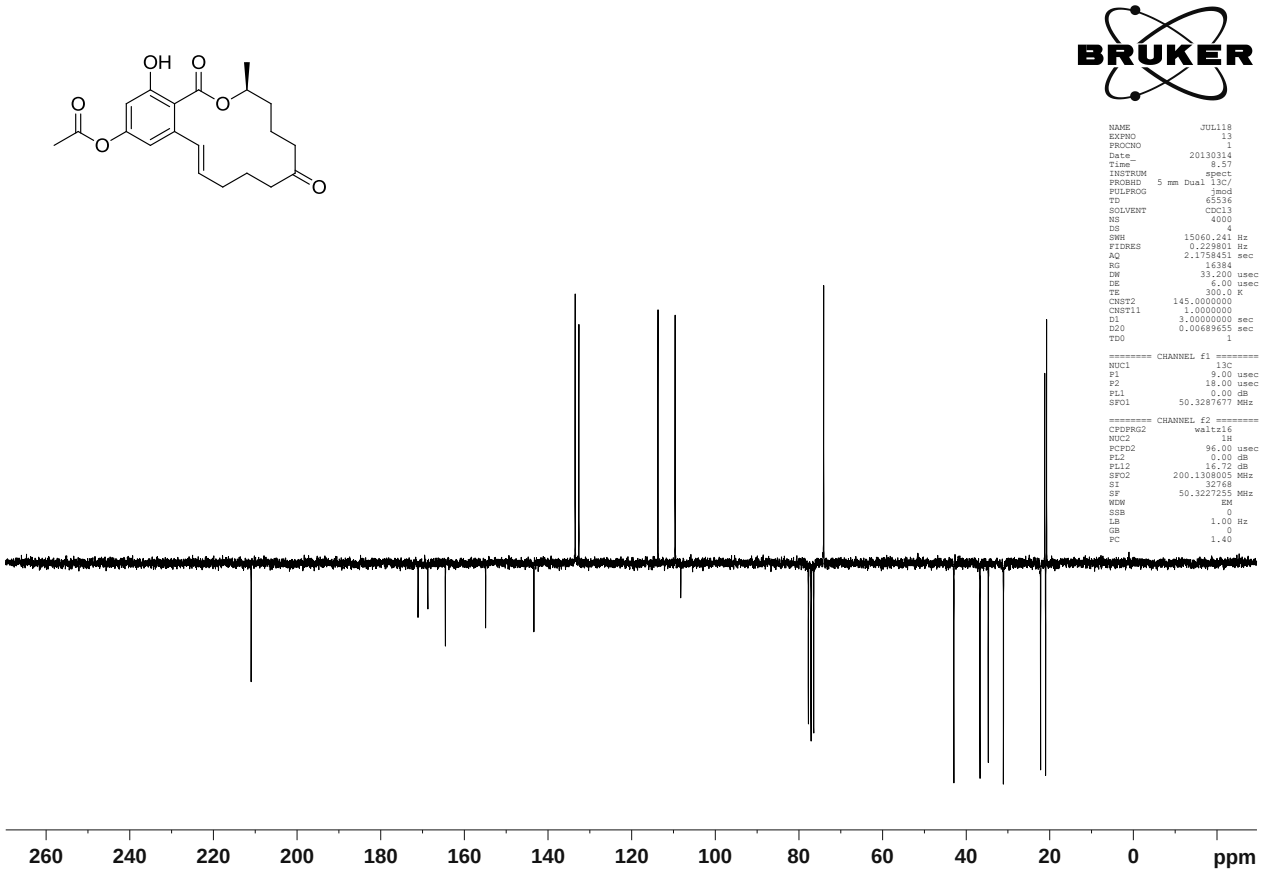


Figure S10: ^{1}H NMR spectrum of **15**

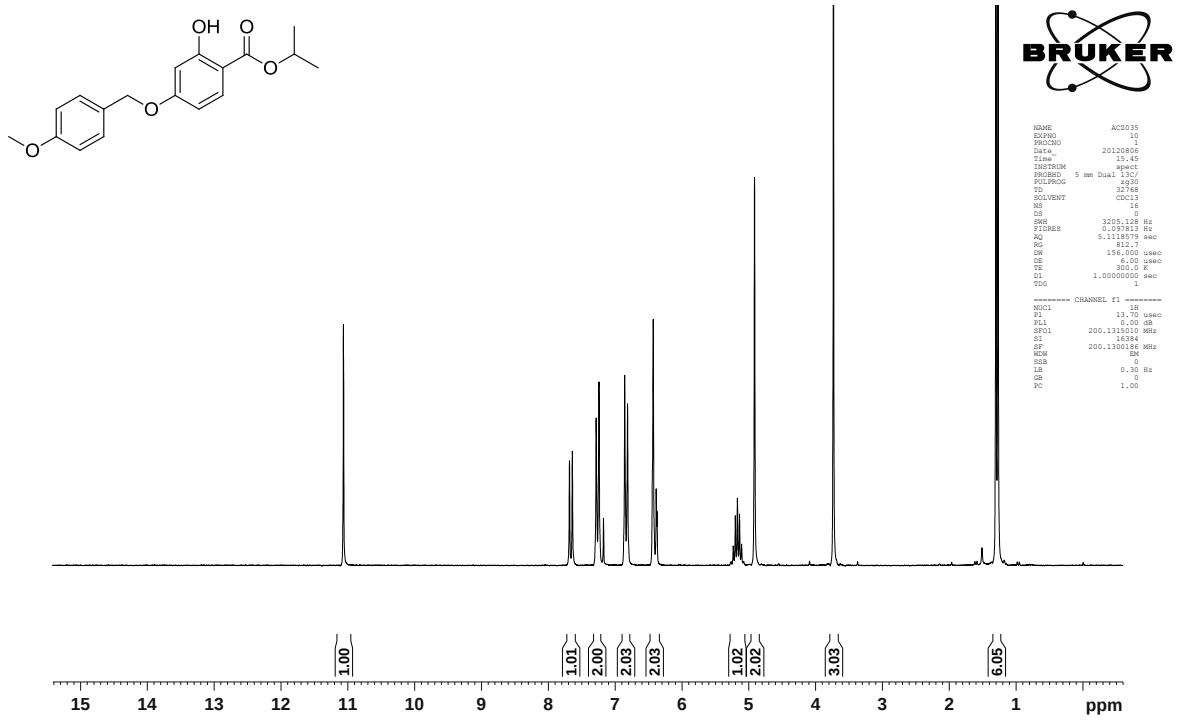


Figure S11: ^{13}C NMR spectrum of **15**

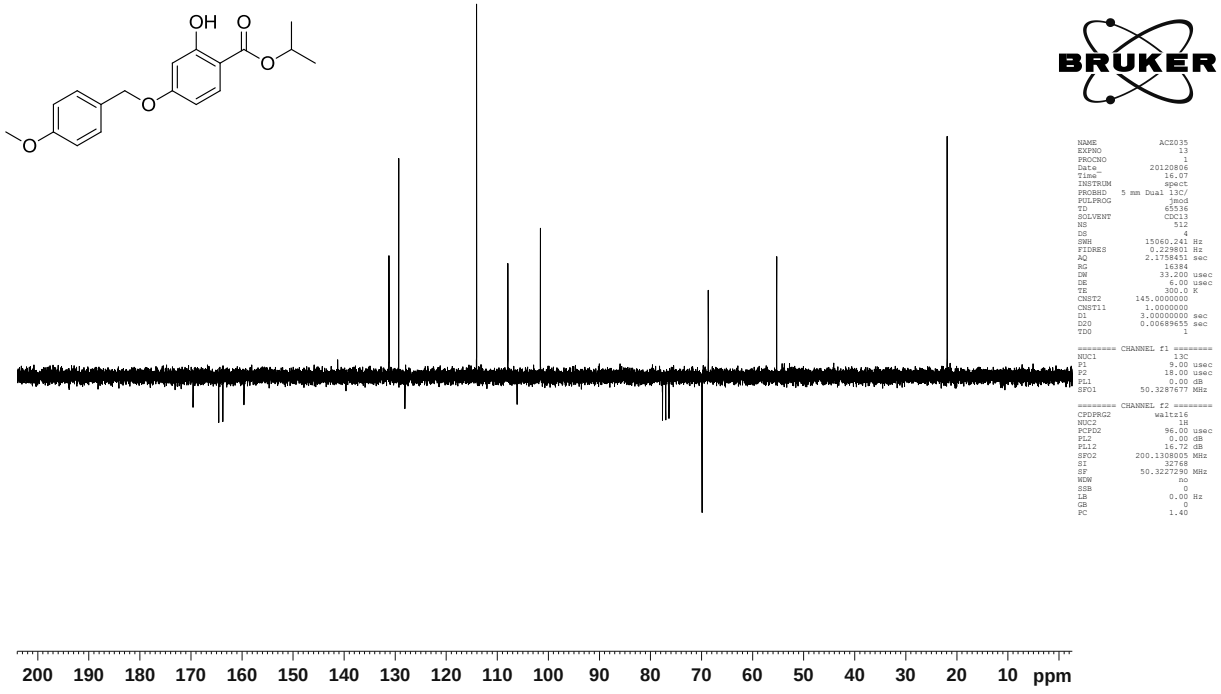


Figure S12: ^{1}H NMR spectrum of **17**

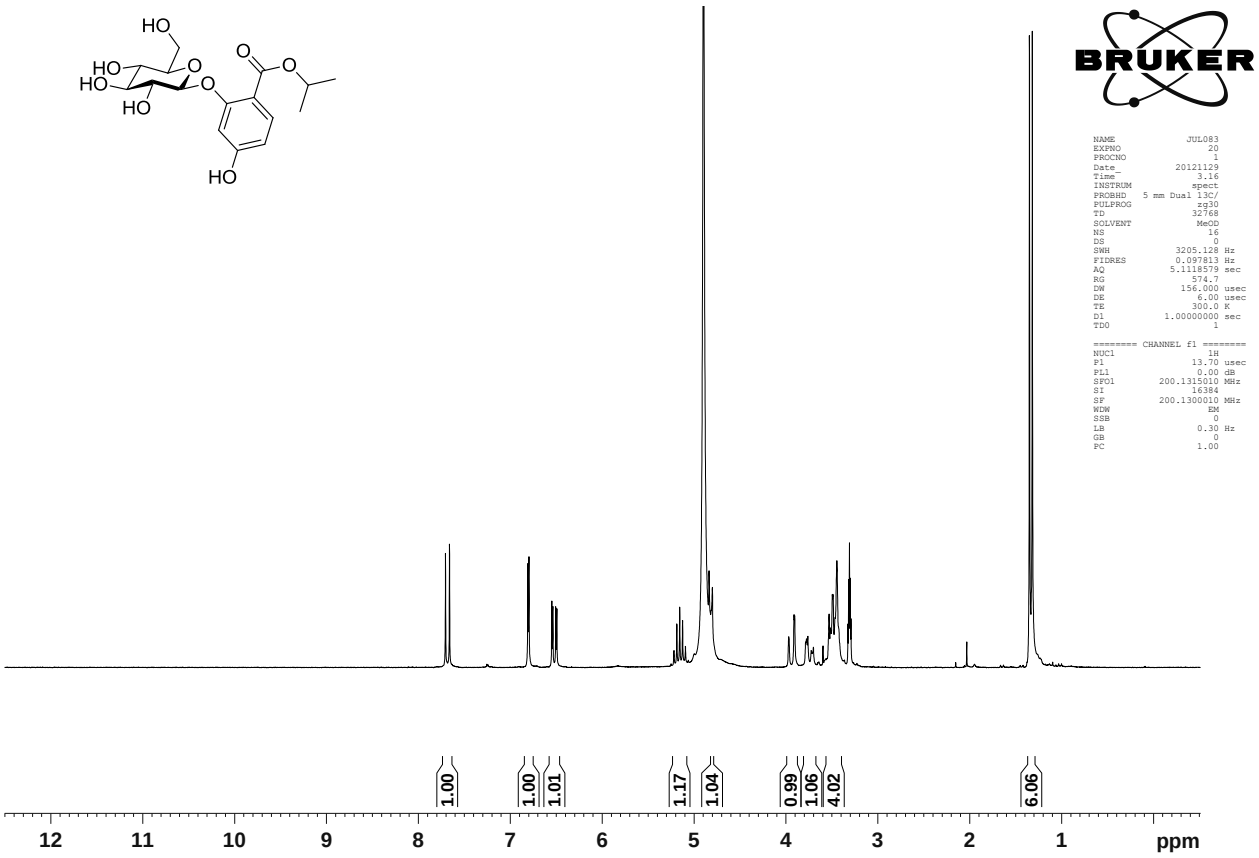


Figure S13: ^{13}C NMR spectrum of **17**

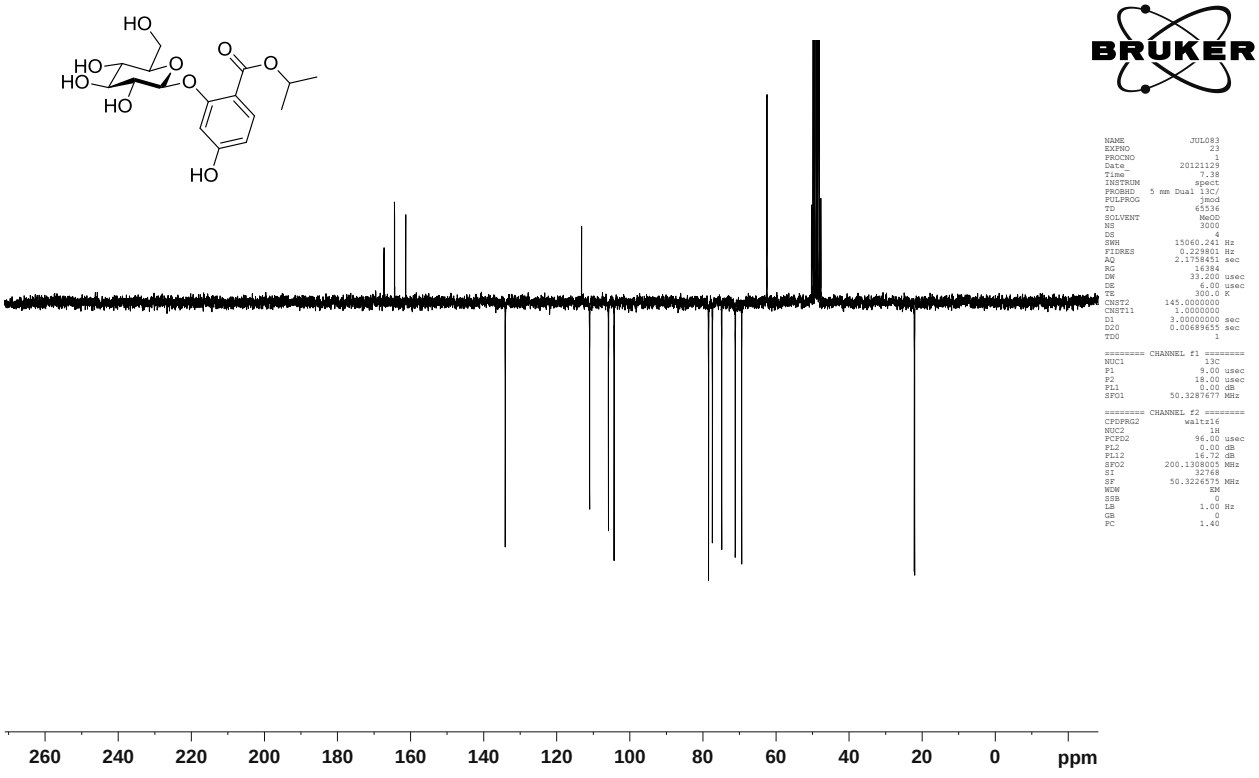


Figure S14: ^{1}H NMR spectrum **of 18**

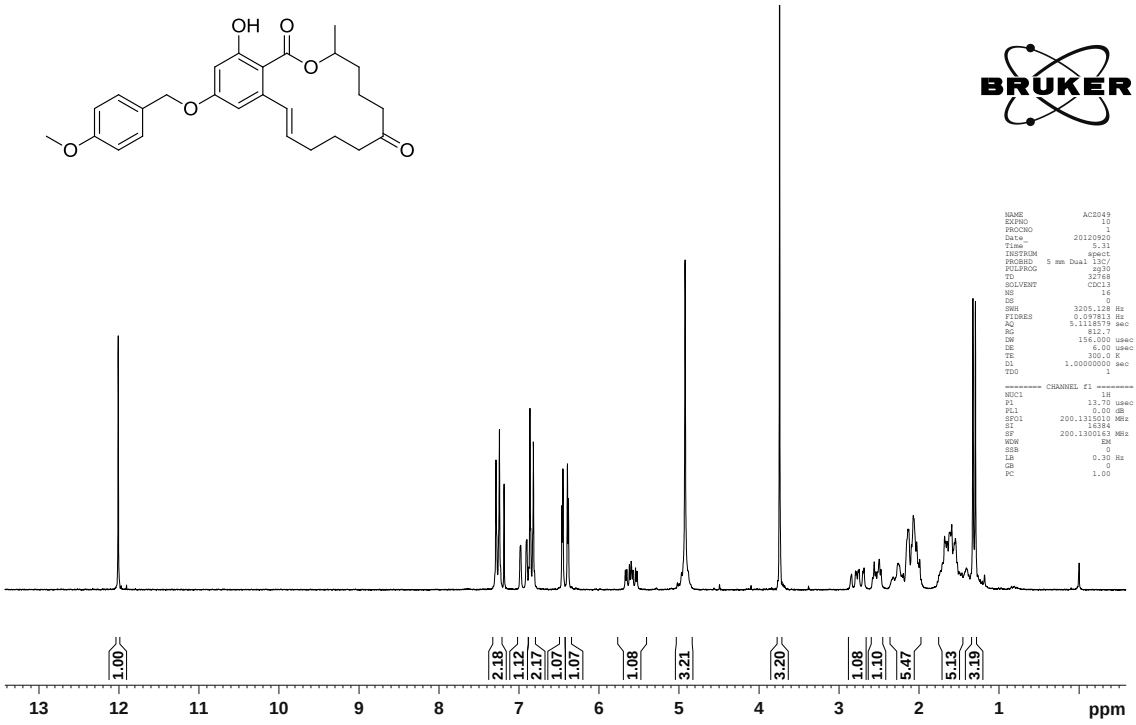


Figure S15: ^{13}C NMR spectrum of **18**

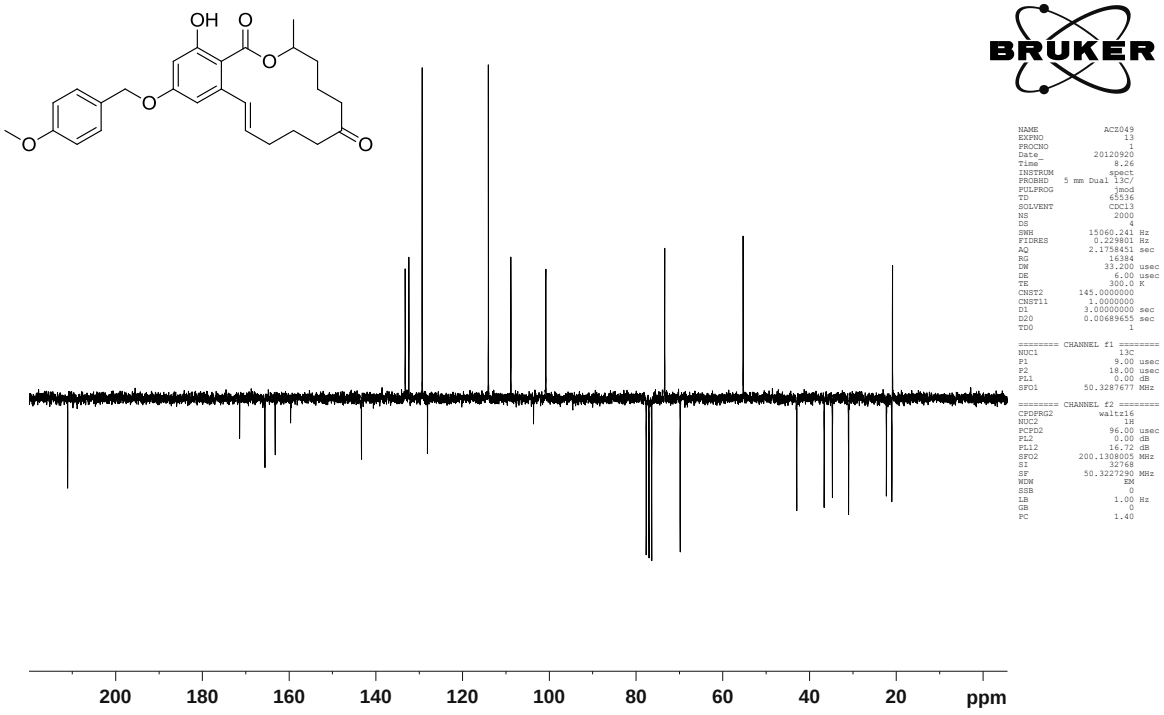


Figure S16: ^{1}H NMR spectrum of **21**

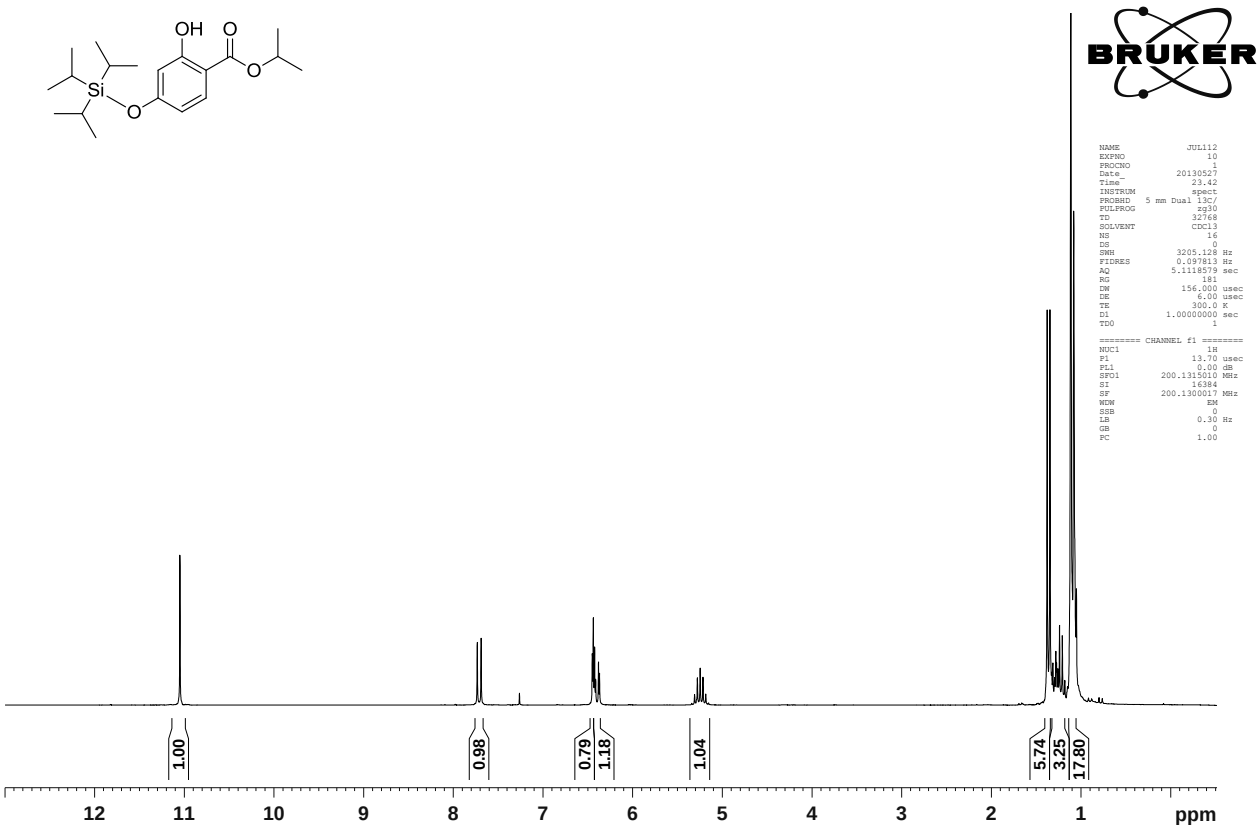


Figure S17: ^{13}C NMR spectrum of **21**

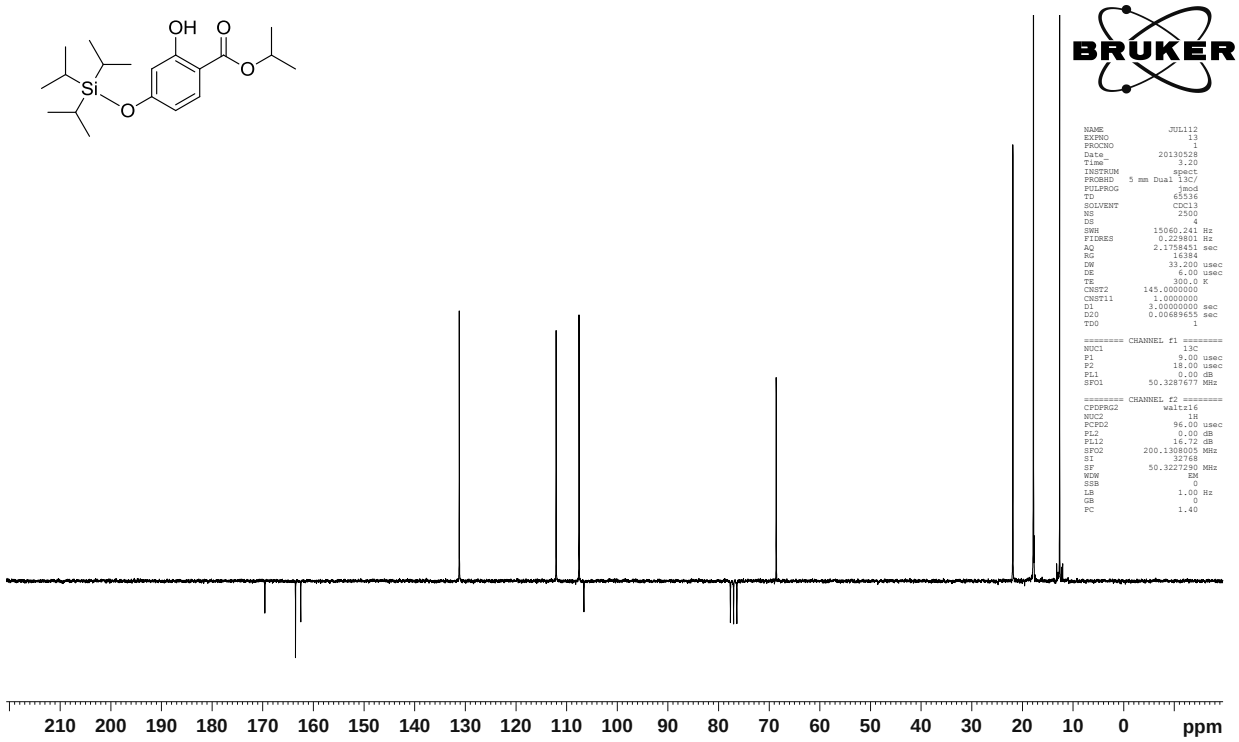


Figure S18: ^{1}H NMR spectrum of **22**

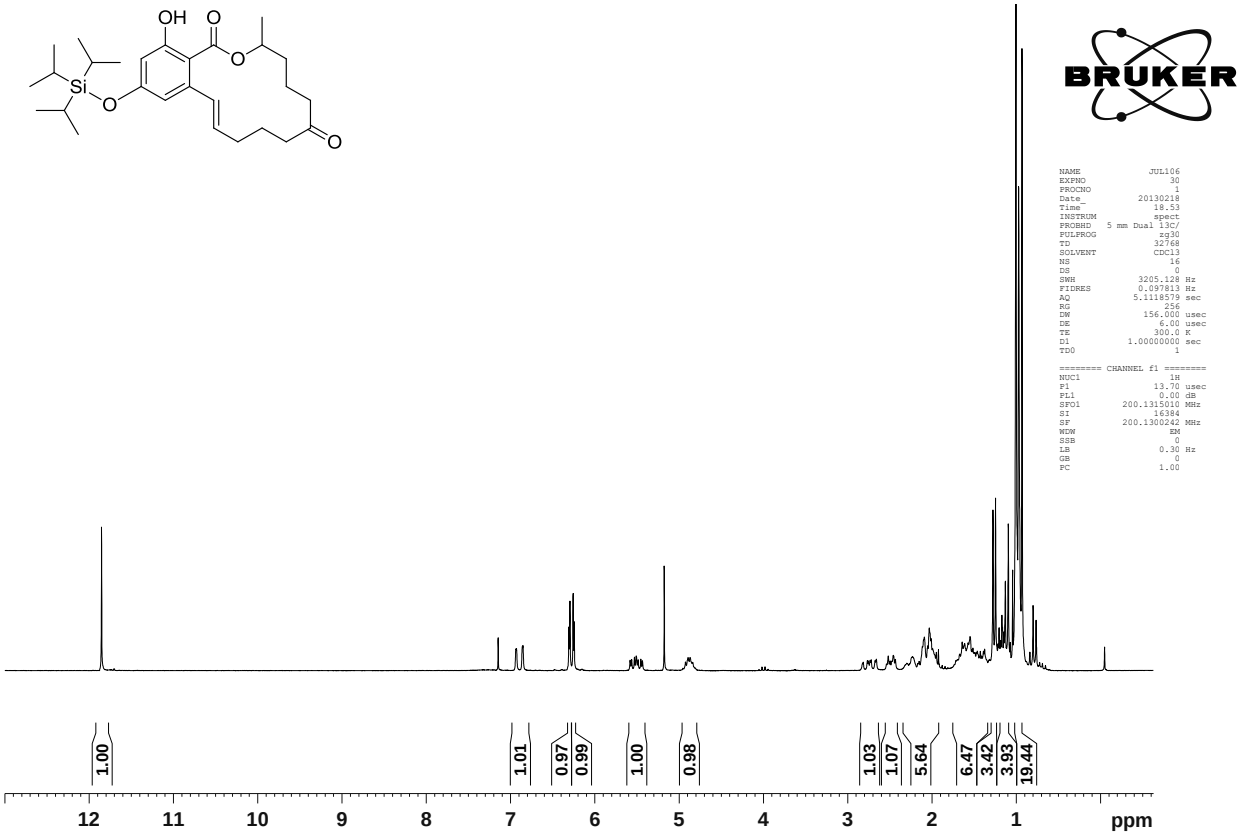


Figure S19: ^{13}C NMR spectrum of **22**

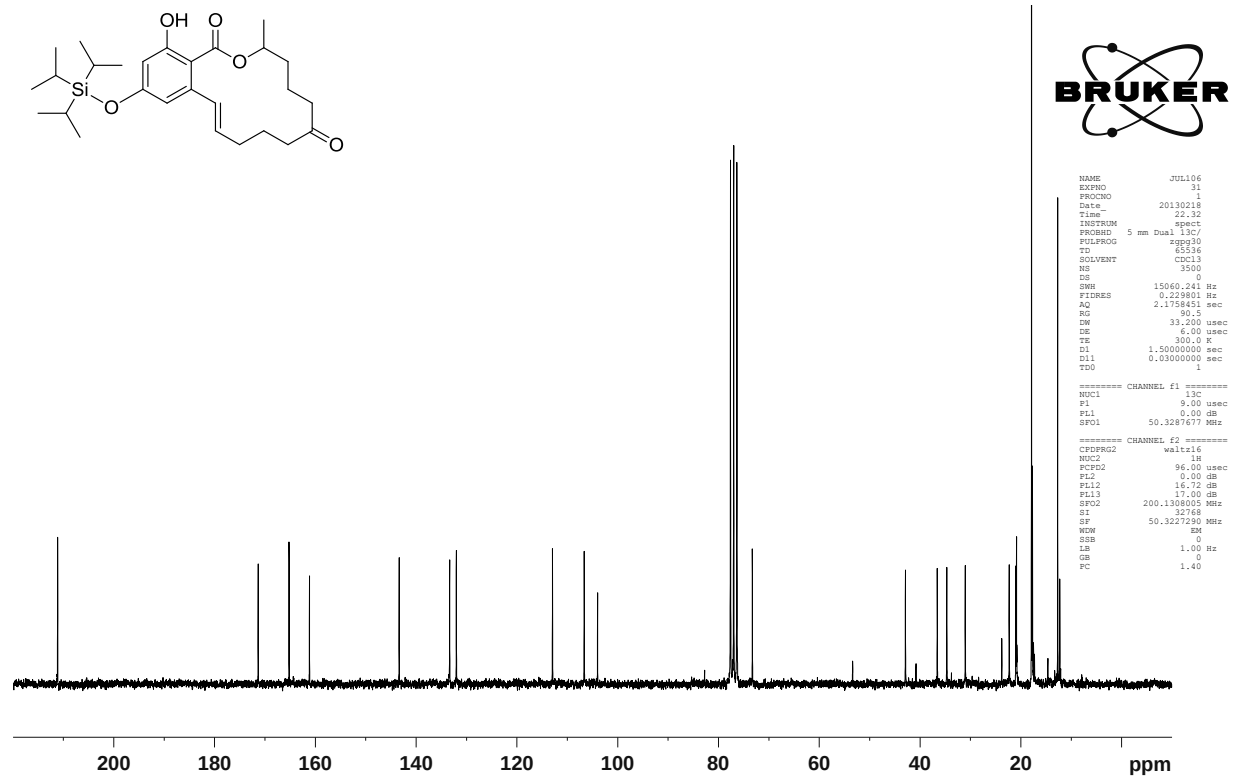


Figure S20: ^{1}H NMR spectrum of **24**

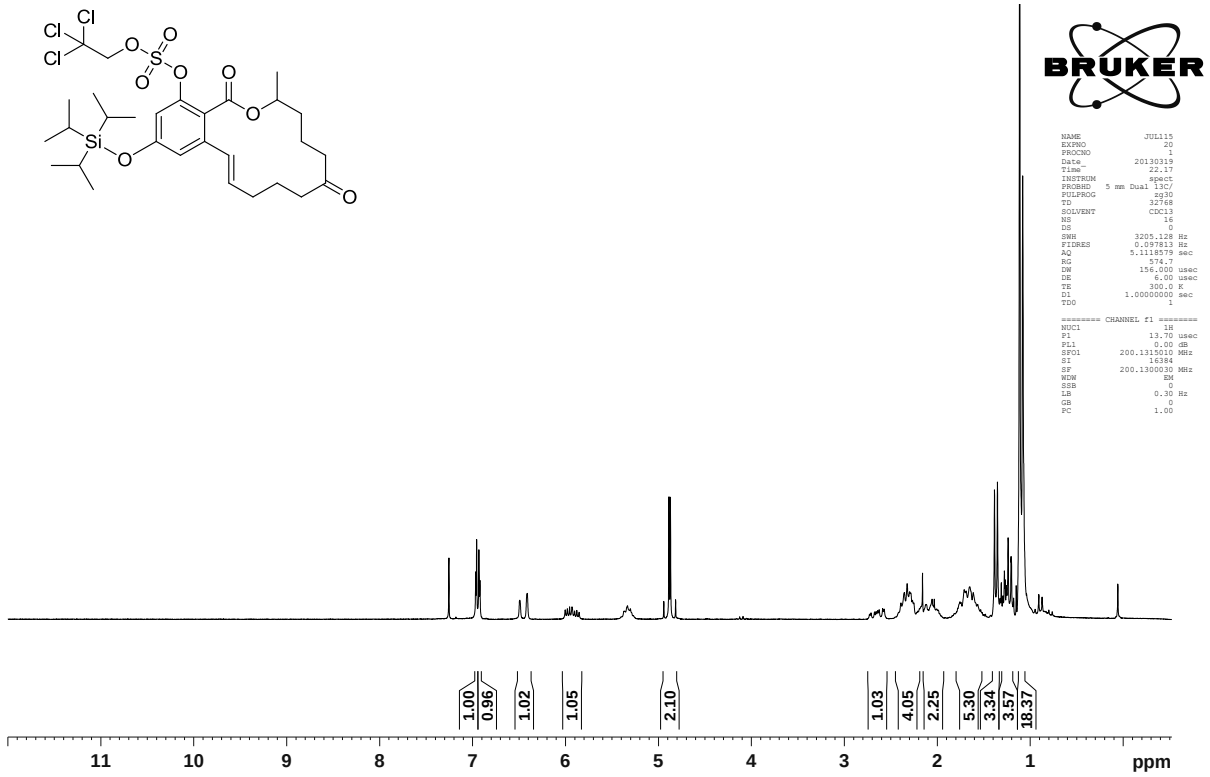
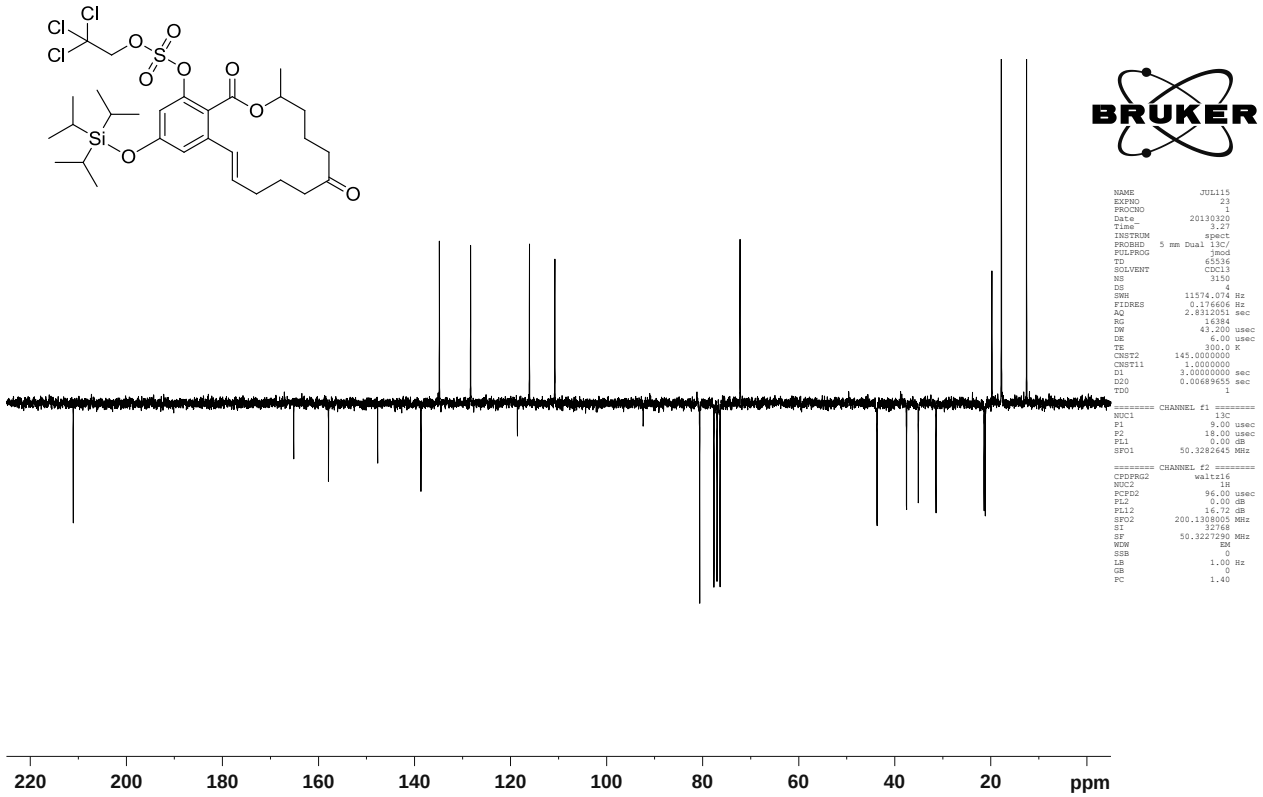


Figure S21: ^{13}C NMR spectrum of **24**



Supporting Information

Manuscript #8

Chemical Synthesis of Culmorin Metabolites and their biologic role in Culmorin and Acetyl-Culmorin Treated Wheat Cells

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Electronic Supplementary Information (ESI)

Content

1) Experimental

a) General remarks

All reactions were performed under argon atmosphere. The progress of the reactions was monitored by thin layer chromatography (TLC) over silica gel 60 F_{254}. All chromatograms were visualized either by UV irradiation (254 or 366 nm) or by heat staining with ceric ammonium molybdate (Hanessian's stain) in ethanol/sulfuric acid.[1] All samples were measured via LC-ESI-MS/MS in negative or positive ionization mode. These measurements were performed on an HCT ion trap mass spectrometer (Bruker, Germany). A TLC-MS interface (Camag, Germany) was used for ESI-MS analysis after TLC. Preparative column chromatography was performed on silica gel 60 (Merck, 40-63 µm) using a Büchi Sepacore™ Flash System or a Grace Reveleris Prep Purification System. Preparative HPLC separation was done on a Grace Reveleris Prep system using a Luna Prep C18(2), 10 µm, 250x10 mm column (Phenomenex). NMR spectra were recorded on a Bruker Avance IIIHD 600Mhz spectrometer equipped with a Prodigy BBO cryo probe or on a Bruker Avance DRX-400 MHz spectrometer at 20°C. Data were recorded and evaluated using TOPSPIN 3.5 (Bruker Biospin). All chemical shifts are given in ppm relative to tetramethylsilane. The calibration was done using residual solvent signals.[2] Multiplicities are abbreviated as s (singlet), d (doublet), t (triplet), q (quartet), b (broad signal). If not stated otherwise, all chemicals were purchased from Sigma Aldrich (Austria/Germany) or ABCR (Germany). Dry solvents were obtained from an in-house PURESOLV facility of it-innovative technology (USA). Molecular sieve 3 Å was activated under vacuum at 200°C before use. HR-MS analysis was carried out from acetonitrile/water solutions (concentration: 10 ppm) by using an HTC PAL system auto-sampler (CTC Analytics AG, Zwingen, Switzerland), an Agilent 1100/1200 HPLC with binary pumps, degasser and column thermostat (Agilent Technologies, Waldbronn, Germany) and Agilent 6230 AJS ESI–TOF mass spectrometer (Agilent Technologies, Palo Alto, United States). In addition to this, also a Thermo Ulitmate 3000 HPLC and Thermo Q Exactive Focus mass spectrometer equipped with the same auto-sampler was used for four samples.

b) Production and Purification of Culmorin

In order to obtain culmorin, two different ways of production, on autoclaved rice and in liquid still cultures were investigated. Two *tri1* knockout strains (IAPT24 (*Fusarium graminearum* PH-1 *tri1*Δ::*hph*) and IAWT2 (*Fusarium graminearum* WG-9 *tri1*Δ::*hph)*[3] selected for purification of the trichothecene calonectrin produced considerable amounts of culmorin and allowed simultaneous purification of culmorin as a by-product.

***Rice cultures using strain* IAPT24**

For the rice culture medium 10 g of long-grained rice and 10 mL Millipore water were filled into 200-mL baby jars. After incubation for one hour at room temperature the jars were autoclaved for 60 minutes at 121 °C. Strain IAPT24 was sporulated in mung bean medium (10 g mung beans were added to 450 mL boiling water and cooked for 20 min. After removal of the mung beans, the extract was filtrated through a folded filter, filled up to 1 L and autoclaved for 20 min at 121°C. Conidia were separated

from mycelia by filtration through a glass-wool filter and sedimented overnight at 4 °C. After removal of the medium spores were re-suspended in water and counted in a Fuchs-Rosenthal chamber.).

36 rice cultures were inoculated with 10^5 spores of IAPT24/jar and incubated at 20°C in the dark for one, two or three weeks, respectively. At each time point 12 glasses were transferred to -20°C and were kept frozen unti further use. For extraction all cultures were thawed at room temperature for about one hour, 40 mL ethylacetate (EtOAc) were added to each tube, the rice cakes were broken up with a spatula and subsequently homogenized using an Ultra Turrax at maximum speed. After addition of 60 mL EtOAc the suspensions were shaken at 20°C with 180 rpm for 1 hour. After removal of small samples for analysis all samples were pooled and worked up together as follows: they were centrifuged in 550-g portions with 4000 g at room temperature. The supernatants were collected and pooled. Each rice pellet was extracted once more with 150 mL EtOAc and treated as described above. Finally, all supernatants were pooled and concentrated to a yellow viscous solution of about 500 mL. This remaining crude extract was used for the purification and crystallization step.

***Liquid still cultures using strain* IAWT2**

Strain IAWT2 was sporulated as described above. 36 Magenta boxes containing modified 2-stage medium (2-SM: 3 g/L KH_2PO_4, 0,2 g/L $MgSO_4 \cdot 7\ H_2O$, 5 g/L NaCl, 1 g/L $(NH_4)_2HPO_4$, 40 g/L sucrose, 10 g/L glycerol; 10 mg/L citric acid, 10 mg/L $ZnSO_4 \cdot 6\ H_2O$, 2 mg/L $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$, 0.5 mg/L $CuSO_4 \cdot 5\ H_2O$, 0.1 mg/L $MnSO_4$, 0.1 mg/L H_3BO_4, 0.1 mg/L $Na_2MoO_4 \cdot 2\ H_2O$; 25 mL/container) were inoculated with 10^5 conidia/jar and incubated as described for the rice cultures. 12 cultures per time point were frozen after one, two or three weeks, respectively. Small aliquots of three samples/time point were used for analysis, the rest and the remaining samples were pooled and the media was concentrated *in vacuo* to a yellow viscous solution of approx. 500 mL which was used for the purification and crystallization step.

Purification and crystallization of culmorin

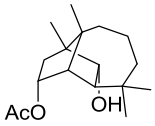
The crude culmorin extract was used for silica gel filtration in order to obtain a pre-concentrated suspension. For this reason, a large and porous suction filter was equipped with filter paper and about 350 g of silica gel 60 (0.015 – 0.040 mm). The crude extract was diluted with a small amount of EtOAc to lower the viscosity and placed on the silica gel layer. EtOAc was subsequently flushed through the material and fractions of around 500 mL were collected. Thin layer chromatography (TLC) in either pure EtOAc or dichloromethane:methanol (95:5) was used to localize CUL in the fractions. The confirmation of the occurrence of CUL was done via the molecular weight using a Camag TLC-MS machine. All CUL enriched fractions were pooled together and concentrated again in vacuo to app. 500 mL of a yellow viscous liquid. This step was repeated two times in order to remove as much by-products as possible and to finally obtain 500 mL of a slightly yellow and still viscous solution. This solution was treated with a small amount of active charcoal followed by column chromatography (DCM:MeOH = 95:5; 400 g silica gel 60) in order to obtain a nearly colourless solution with only minor by-products (according to TLC and TLC-MS). This step was again repeated two times in order to get a colourless solution of nearly pure CUL. This fraction was concentrated in vacuo to a minimal amount

of sticky resin followed by the addition of small amounts of EtOAc (~ 5 mL per portion). This addition was continued till everything of the residue was dissolved and once again a solution was obtained. During this step, colourless crystals precipitated and even more were formed upon standing over night in the fridge. These crystals were filtered, dried under vacuum and confirmed as culmorin via NMR spectroscopy and the available reference literature.[4] Based on this purification procedure, ~120 mg of CUL were obtained from the rice culture with strain IAPT24 and 885 mg of CUL were obtained with the liquid cultures from strain IAWT2. In addition to culmorin, small amounts of 11-AcCul were also present in the media and 42 mg were isolated from a side fraction using several times straight phase chromatography (DCM:MeOH = 98:2, R_f value of 11-AcCul = 0.125). The NMR data of the isolated compound is identical to the synthesized substance.

Culmorin (**1**): ^{1}H NMR (600 MHz, $CDCl_3$): δ = 4.35 (ddd, J = 7.4, 6.5, 4.5 Hz, 1H), 3.82 (d, J = 5.3 Hz, 1H), 2.17 (bs, 2H), 1.90 (d, J = 4.6 Hz, 1H), 1.74 (d, J = 5.1 Hz, 1H), 1.65 (d, J = 6.4 Hz, 2H), 1.48 – 1.26 (m, 6H), 1.00 (s, 3H), 0.91 (s, 3H), 0.86 (s, 3H), 0.81 (s, 3H); ^{13}C NMR (150 MHz, MeOD): δ = 79.7 (d, 1C), 71.2 (d, 1C), 52.5 (s, 1C), 52.1 (d, 1C), 51.3 (s, 1C), 49.7 (d, 1C), 41.4 (t, 1C), 36.3 (t, 1C), 36.0 (t, 1C), 33.0 (s, 1C), 29.4 (q, 1C), 28.9 (q, 1C), 22.7 (t, 1C), 22.2 (q, 1C), 13.3 (q, 1C)

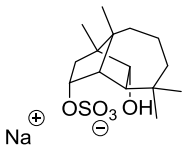
c) Synthetic procedures for culmorin sulfates

11-Acetylculmorin (2)



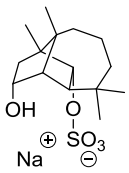
To a solution of culmorin (20 mg, 84 µmol, 1 eq.) in pyridine (2 mL) was added acetyl chloride (6.5 µl, 92 µmol, 1.1 eq.) at -5 °C. After stirring the reaction mixture for 16 h at -5 °C, the reaction was concentrated under reduced pressure and the residue was purified by column chromatography (hexanes: EtOAc 9-17 %) yielding the target compound (21 mg, 89 %); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 5.06 (dt, J = 10.1, 4.3 Hz, 1H), 3.86 (dd, J = 5.2, 1.9 Hz, 1H), 2.18 (d, J = 4.2 Hz, 1H), 2.05 (s, 3H), 1.85 (dd, J = 14.1, 4.4 Hz, 1H), 1.69 (ddd, J = 13.9, 10.2, 2.1 Hz, 1H), 1.58 (d, J = 5.3 Hz, 1H), 1.47 – 1.38 (m, 3H), 1.38 – 1.29 (m, 3H), 0.98 (s, 3H), 0.92 (s, 3H), 0.85 (s, 3H), 0.83 (s, 3H); ^{13}C NMR (150 MHz, MeOD): δ = 171.3 (s, 1C), 79.2 (d, 1C), 74.8 (d, 1C), 53.2 (d, 1C), 52.1 (s, 1C), 51.0 (s, 1C), 47.5 (d, 1C), 41.1 (t, 1C), 35.8 (t, 1C), 33.2 (t, 1C), 32.9 (s, 1C), 29.3 (q, 1C), 29.0 (q, 1C), 22.7 (t, 1C), 22.3 (q, 1C), 21.5 (q, 1C), 13.1 (q, 1C); HRMS calcd for $C_{17}H_{28}NaO_3^+$ $[M+Na]^+$ 303.1931, found 303.1930.

Culmorin-11-sulfate, sodium salt (3)



Culmorin (30 mg, 0.13 mmol, 1 eq.) and SO_3•Me_3N (21 mg, 0.15 mmol, 1.2 eq.) was stirred in DMF (1 mL) at 55 °C for 16 h. The solvent was evaporated under high vacuum and the residue was purified by preparative HPLC (water-MeCN 5 to 30 %). To obtain the sodium salt, the product was dissolved in water and passed through a small column packed with sodium ion exchange resin (Amberlite IRC7481 Chelating Resin) to give **3** as a white solid (31 mg, 72 %). ^{1}H NMR (600 MHz, MeOD): δ = 4.85 (dt, J = 10.2, 4.3 Hz, 1H), 3.78 (dd, J = 5.3, 1.8 Hz, 1H), 2.23 (d, J = 4.4 Hz, 1H), 2.02 (dd, J = 14.1, 4.1 Hz, 1H), 1.85 (d, J = 5.6 Hz, 1H), 1.66 (ddd, J = 14.1, 10.3, 2.1 Hz, 1H), 1.55 – 1.44 (m, 3H), 1.42 – 1.35 (m, 2H), 1.35 – 1.29 (m, 1H), 0.98 (s, 3H), 0.95 (s, 3H), 0.93 (s, 3H), 0.81 (s, 3H); ^{13}C NMR (150 MHz, MeOD): δ = 79.8 (d, 1C), 79.5 (d, 1C), 53.1 (s, 1C), 52.7 (d, 1C), 51.4 (s, 1C), 49.4 (d, 1C), 42.5 (t, 1C), 36.9 (t, 1C), 34.9 (t, 1C), 33.9 (s, 1C), 29.8 (q, 1C), 29.1 (q, 1C), 23.7 (t, 1C), 22.7 (q, 1C), 13.2 (q, 1C); HRMS calcd for $C_{15}H_{26}O_5S^-$ $[M-H]^-$ 317.1428, found 317.1426.

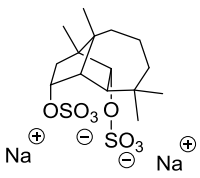
Culmorin-8-sulfate, sodium salt (4)



11-Acetylculmorin (21 mg, 0.08 mmol, 1 eq.) and SO_3•Me_3N (12.5 mg, 0.09 mmol, 1.2 eq.) was stirred in DMF (1.5 mL) at 55 °C for 16 h. The solvent was evaporated under high vacuum. For basic hydrolysis, the crude product was dissolved in MeOH (2 mL), followed by the addition of NaOMe in MeOH (45 µl, 15 wt%, 2 eq.) at 0 °C. The reaction mixture was slowly warmed to room temperature. After stirring for 4 h, the reaction was concentrated under reduced pressure, the residue was dissolved in water and purified via preparative HPLC (water-MeCN 5 to 30 %) to obtain compound **4** (15 mg, 59 %); ^{1}H NMR (600

MHz, MeOD): δ = 4.75 (dd, J = 4.8, 2.2 Hz, 1H), 4.29 (dt, J = 10.1, 4.3 Hz, 1H), 2.13 (d, J = 4.4 Hz, 1H), 1.91 (d, J = 1.9 Hz, 1H), 1.86 (dd, J = 13.8, 4.1 Hz, 1H), 1.60 (ddd, J = 13.5, 9.9, 2.1 Hz, 1H), 1.57 – 1.44 (m, 4H), 1.41 – 1.30 (m, 2H), 1.10 (s, 3H), 0.99 (s, 3H), 0.92 (s, 3H), 0.88 (s, 3H); ^{13}C NMR (150 MHz, MeOD): δ = 89.3 (d, 1C), 71.6 (d, 1C), 53.7 (s, 1C), 52.3 (s, 1C), 50.7 (d, 1C), 50.4 (d, 1C), 42.4 (t, 1C), 37.3 (t, 1C), 37.2 (t, 1C), 33.9 (s, 1C), 29.8 (q, 1C), 29.6 (q, 1C), 23.7 (t, 1C), 22.6 (q, 1C), 14.2 (q, 1C); HRMS calcd for $C_{15}H_{26}O_5S^-$ $[M-H]^-$ 317.1428, found 317.1429.

Culmorin-8,11-disulfate, sodium salt (5)



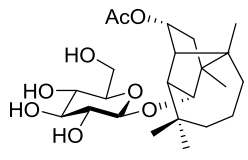
Culmorin (20 mg, 0.084 mmol, 1 eq.) and SO_3•Me_3N (585 mg, 4.2 mmol, 50 eq.) was stirred in DMF (700 µL) at 55 °C for 16 h. The solvent was evaporated under high vacuum, the residue was redissolved in MeOH and filtered to remove the excess of the sulfate complex. The filtrate was concentrated under vacuum and purified by preparative HPLC (water-MeCN 5 to 30 %). To obtain the sodium salt, the product was dissolved in water and passed through a small column packed with sodium ion exchange resin (Amberlite IRC7481 Chelating Resin) to obtain compound **5** as a white solid (32 mg, 87 %); ^{1}H NMR (600 MHz, MeOD): δ = 4.86 (dt, J = 10.2, 4.3 Hz, 1H), 4.75 (dd, J = 5.0, 2.0 Hz, 1H), 2.26 (d, J = 4.7 Hz, 1H), 2.16 (dd, J = 13.9, 4.3 Hz, 1H), 2.11 (d, J = 5.0 Hz, 1H), 1.71 (ddd, J = 14.1, 10.2, 2.2 Hz, 1H), 1.61 – 1.43 (m, 4H), 1.41 – 1.33 (m, 2H), 1.09 (s, 3H), 1.01 (s, 3H), 0.95 (s, 3H), 0.92 (s, 3H); ^{13}C NMR (150 MHz, MeOD): δ = 88.8 (d, 1C), 79.3 (d, 1C), 53.3 (s, 1C), 51.9 (s, 1C), 51.3 (d, 1C), 49.1 (d, 1C), 42.4 (t, 1C), 37.1 (t, 1C), 35.6 (t, 1C), 34.1 (s, 1C), 29.8 (q, 1C), 29.5 (q, 1C), 23.6 (t, 1C), 22.6 (q, 1C), 14.1 (q, 1C); HRMS calcd for $C_{15}H_{24}O_8S_{22}^-$ $[^M/_2]^-$ 198.0454, found 198.0452

Preparative HPLC procedures for all culmorin sulfates

Preparative HPLC separation was done on a Grace Reveleris Prep system using a Luna Prep C18(2), 10 µm, 250x10 mm column (Phenomenex). As eluents were used water (A) and MeCN (B) and the flow rate was 10 mL/min. After an initial hold time at 5 % B for 4 min, the proportion of B was linearly increased to 40 % B within the next 10 min, it was hold at 40 % B for 1 min and increased to 90 % within another 1 min.

d) Synthetic procedures for culmorin glucosides

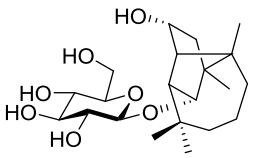
11-Acetylculmorin-8-glucoside (8)



To a solution of culmorin (100 mg, 420 µmol, 1 eq.) and 2,3,4,6-tetra-O-acetyl-α,D-glucopyranosyl-1-(N-phenyl)-2,2,2-trifluoroacetimidate (327 mg, 629 µmol, 1.5 eq.) in dry CH_2Cl_2 (3 mL), molecular sieve (3 Å, 300 mg) was added and the reaction mixture was stirred at room temperature for 30 min.

After cooling the reaction mixture to 0 °C, TMSOTf (7.6 µL, 420 µmol, 1 eq.) was added. At 0 °C the reaction mixture was stirred for 1 h, and quenched by the addition of Et_3N (0.15 eq.). The reaction mixture was filtered through celite and concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 and purified by flash chromatography (gradient elution hexane:EtOAc) to obtain 11-acetylculmorin-8-tetra-O-acetyl-glucoside with traces of impurities (80 mg, 31 %). This crude fraction (80 mg, 0.13 mmol, 1 eq.) was dissolved in dry MeOH and potassium cyanide (4.3 mg, 0.06 mmol, 0.5 eq.). After stirring for 4 h at room temperature, the reaction mixture was reduced to a volume of 1 mL and purified by preparative HPLC yielding compound **8** (38 mg, 66 %). Preparative HPLC separation was done on a Grace Reveleris Prep system using a Kinetex XB-C18, 5 µm, 100x30 mm column (Phenomenex). As eluents were used water (A) and MeCN (B) and the flow rate was 50 mL/min. After an initial hold time at 10 % B for 2 min, the proportion of B was linearly increased to 68 % B within the next 11 min, and within another 1 min to 90 % B; ^{1}H NMR (600 MHz, MeOD): δ = 5.03 (dt, J = 10.2, 4.3 Hz, 1H), 4.32 (d, J = 7.6 Hz, 1H), 3.94 (dd, J = 4.5, 1.9 Hz, 1H), 3.84 (dd, J = 11.8, 2.5 Hz, 1H), 3.69 (dd, J = 11.7, 5.3 Hz, 1H), 3.36-3.32 (m, 2H), 3.25-3.21 (m, 1H), 3.21- 3.17 (m, 1H), 2.19 (d, J = 4.4 Hz, 1H), 2.08 (dd, J = 14.2, 4.1 Hz, 1H), 2.03 (s, 3H), 1.91 (d, J = 5.0 Hz, 1H), 1.65 (ddd, J = 13.9, 10.2, 2.0 Hz, 1H), 1.59 – 1.45 (m, 3H), 1.41 – 1.31 (m, 3H), 1.03 (s, 3H), 0.98 (s, 3H), 0.92 (s, 3H), 0.87 (s, 3H); ^{13}C NMR (150 MHz, MeOD): δ = 173.3 (s, 1C), 104.7 (d, 1C), 100.0 (d, 1C), 78.3 (d, 1C), 77.5 (d, 1C), 76.8 (d, 1C), 75.4 (d, 1C), 71.6 (d, 1C), 62.7 (t, 1C), 53.4 (s, 1C), 52.8 (d, 1C), 52.4 (s, 1C), 48.6 (d, 1C), 42.2 (t, 1C), 37.0 (t, 1C), 34.9 (t, 1C), 33.7 (s, 1C), 30.3 (q, 1C), 30.0 (q, 1C), 23.6 (t, 1C), 22.7 (q, 1C), 21.2 (q, 1C), 14.1 (q, 1C); HRMS calcd for $C_{23}H_{38}O_8^+$ $[M+Na]^+$ 465.2459, found 465.2469.

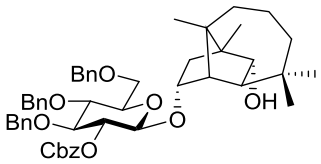
Culmorin-8-glucoside (6)



To a solution of 11-acetylculmorin-8-glucoside (18 mg, 40 µmol, 1 eq.) in MeOH (10 mL) was added potassium cyanide (10 mg, 160 µmol, 4 eq.). The reaction mixture was stirred at room temperature till LC-MS measurements showed full conversion, concentrated under reduced pressure to a volume of 1 mL and purified by preparative HPLC to obtain compound **6** as a white solid (13 mg, 80 %). The separation was done on a Grace Reveleris Prep system using a Luna Prep C18(2), 10 µm, 250x21.2 mm column (Phenomenex). As eluents were used water (A) and MeCN (B) and the flow rate was 20 mL/min. After an initial hold time at 10 % B for 2 min, the proportion of B was linearly increased to 63 % B within the next 11 min, it was hold at 63 % B for 2 min and increased to 90 % within another 5 min; ^{1}H NMR (600 MHz, MeOD): δ = 4.38 (d, J = 7.6 Hz, 1H), 4.31 (dt, J = 10.1, 4.2 Hz, 1H), 3.92 (dd, J = 4.4, 1.5 Hz, 1H), 3.85 (dd, J = 11.7, 2.4 Hz, 1H), 3.68 (dd, J = 11.7, 5.6 Hz, 1H), 3.38-3.28 (m, 2H), 3.27-3.22 (m, 1H), 3.17 (dd, J = 9.3, 7.8 Hz, 1H), 2.04 (d, J = 4.1 Hz, 1H), 1.91 (d, J = 4.7 Hz, 1H), 1.85 (dd, J = 13.7, 4.0, 1H), 1.56 (ddd, J = 13.7, 10.4, 1.8 Hz, 1H), 1.53 – 1.43 (m, 3H), 1.41 – 1.28 (m, 3H), 1.03 (s, 3H), 0.93 (s, 3H), 0.90 (s, 3H), 0.88 (s, 3H); ^{13}C NMR (150 MHz, MeOD): δ = 103.6 (d, 1C), 89.5 (d, 1C), 77.7 (d, 1C), 77.6 (d, 1C), 75.4 (d, 1C), 71.9 (d, 1C), 71.8 (d, 1C), 62.9 (t, 1C), 53.7 (s, 1C), 52.5 (s, 1C), 52.3 (d, 1C), 51.3 (d, 1C), 42.5 (t, 1C), 37.6 (t, 1C), 37.5 (t, 1C), 33.5 (s,

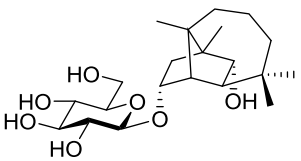
1C), 30.9 (q, 1C), 30.1 (q, 1C), 23.6 (t, 1C), 22.5 (q, 1C), 14.1 (q, 1C); HRMS calcd for $C_{21}H_{36}O_7^+$ $[M+Na]^+$ 423.2354, found 423.2359.

Culmorin-11-(3,4,6-tri-O-benzyl-2-O-benzyloxycarbonyl)-β,D-glucoside (9)



To a solution of glucosyl donor **11** (44 mg, 70 µmol, 1 eq.) and CUL (25 mg, 105 µmol, 1.5 eq.) in dry CH_2Cl_2 (1.5 mL) was added molecular sieve (3 Å, 75 mg) and the reaction was stirred for 2 h at room temperature. After cooling to -10 °C, N-iodosuccinimide (32 mg, 140 µmol, 2 eq.) and TfOH (2 mg, 14 µmol, 0.2 eq.) was added and stirring was continued at -10 °C for 3 h. The reaction was quenched by addition of an aqueous saturated $NaHCO_3$ and Na_2SO_3 solution (1:1), the reaction solution was diluted with CH_2Cl_2 and filtrated over celite. The filtrate was washed with water and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (hexanes/EtOAc gradient elution) to obtain the desired product **9** (45 mg, 80 %) in good yields; ^{1}H NMR (400 MHz, CD_2Cl_2): δ = 7.47-7.13 (m, 20H), 5.20 (d, J = 12.1 Hz, 1H), 5.12 (d, J = 12.1 Hz, 1H),4.79 (t, J = 10.5 Hz, 1H), 4.75-4.69 (m, 2H), 4.66 (d, J = 11.3 Hz, 1H), 4.60-4.46 (m, 3H), 4.43 (d, J = 7.8 Hz, 1H), 4.28 (dt, J = 9.4, 4.3 Hz, 1H), 3.78-3.62 (m, 5H), 3.48-3.41 (m, 1H), 2.08 (d, J = 4.7 Hz, 1H), 1.75 (d, J = 5.0 Hz, 1H), 1.56-1.48 (m, 2H), 1.46 – 1.41 (m, 3H), 1.38 – 1.32 (m, 3H), 0.98 (s, 3H), 0.93 (s, 3H), 0.86 (s, 3H), 0.81 (s, 3H); ^{13}C NMR (100 MHz, CD_2Cl_2): δ = 154.8 (s, 1C), 138.72 (s, 1C), 138.67 (s, 1C), 138.6 (s, 1C), 135.9 (s, 1C), 128.9 (d, 2C), 128.8 (d, 1C), 128.72 (d, 2C), 128.70 (d, 2C), 128.69 (d, 2C), 128.6 (d, 2C), 128.4 (d, 2C), 128.21 (d, 2C), 128.18 (d, 2C), 128.1 (d, 1C), 127.9 (d, 2C), 100.3 (d, 1C), 83.0 (d, 1C), 79.5 (d, 1C), 78.5 (d, 1C), 78.3 (d, 1C), 78.2 (d, 1C), 75.4 (t, 1C), 75.31 (t, 1C), 75.27 (d, 1C), 73.7 (t, 1C), 70.3 (t, 1C), 69.3 (t, 1C), 53.0 (d, 1C), 52.3 (s, 1C), 51.1 (s, 1C), 49.0 (d, 1C), 41.7 (t, 1C), 36.1 (t, 1C), 33.6 (t, 1C), 33.3 (s, 1C), 29.7 (q, 1C), 28.7 (q, 1C), 22.9 (t, 1C), 22.4 (q, 1C), 13.3 (q, 1C) ; HRMS calcd for $C_{50}H_{60}NaO_9^+$ $[M+Na]^+$ 827.4130, found 827.4122.

Culmorin-11-β,D-glucoside (7)



To a suspension of the protected Culmorin-11-glucoside **9** (42 mg, 52 µmol, 1 eq.) in dry ethanol (2 mL) was added two small tips of a spatula of Pd/C under argon atmosphere. The argon balloon was changed for a H_2-balloon and the reaction mixture was stirred for 4 h at rt. The reaction mixture was filtered through a syringe filter and the filtrate was concentrated. The residue was dissolved in a mixture of acetonitrile/water (1:2) and purified via preparative-HPLC to yield **7** as a white solid (13 mg, 65 %). The separation was done on a Grace Reveleris Prep system using a Luna Prep C18(2), 10 µm, 250x21.2 mm column (Phenomenex). As eluents were used water (A) and MeCN (B) and the flow rate was 18 mL/min. After an initial hold time at 15 % B for 2 min, the proportion of B was linearly increased to 60 % B within the next 12 min, it was hold at 60 % B for 3 min and increased to 95 % within another 1 min; ^{1}H NMR (600 MHz, MeOD): δ = 4.39 (dt, J = 9.5, 4.2 Hz, 1H), 4.26 (d, J = 7.6 Hz, 1H), 3.87 (dd, J = 11.9, 2.5 Hz, 1H), 3.80 (dd, J = 5.3, 1.7 Hz, 1H), 3.66 (dd, J = 11.9, 5.4 Hz,

1H), 3.35 (t, J = 9.1 Hz, 2H), 3.28 (dd, J = 9.6, 8.6 Hz, 1H), 3.24 (ddd, J = 9.6, 5.6, 2.2 Hz, 1H), 3.19 (dd, J = 9.2, 7.8 Hz, 1H), 2.07 (d, J = 4.4 Hz, 1H), 1.93 (d, J = 5.3 Hz, 1H), 1.79 (dd, J = 13.4, 3.9, 1H), 1.59 (ddd, J = 13.4, 9.6, 1.9 Hz, 1H), 1.53 – 1.43 (m, 3H), 1.40 – 1.36 (m, 2H), 1.34 – 1.26 (m, 1H), 0.98 (s, 3H), 0.92 (s, 3H), 0.88 (s, 3H), 0.81 (s, 3H); ^{13}C NMR (150 MHz, MeOD): δ = 103.5 (d, 1C), 80.0 (d, 1C), 78.7 (d, 1C), 77.90 (d, 1C), 77.87 (d, 1C), 75.2 (d, 1C), 71.7 (d, 1C), 62.8 (t, 1C), 53.1 (s, 1C), 52.0 (d, 1C), 51.8 (s, 1C), 49.9 (d, 1C), 42.6 (t, 1C), 37.0 (t, 1C), 34.8 (t, 1C), 34.0 (s, 1C), 29.8 (q, 1C), 29.2 (q, 1C), 23.6 (t, 1C), 22.7 (q, 1C), 13.5 (q, 1C); HRMS calcd for $C_{21}H_{36}NaO_7^+$ $[M+Na]^+$ 423.2353, found 423.2353.

e) Plant cell experiments

The wheat suspension culture (PC-998, DSMZ, Braunschweig Germany) was maintained in B5 medium and rediluted 1:1 every week as previously described.[5] For the experiment 14 mL round bottom vented tubes (allowing gas exchange) were used (Greiner Bio-One, Kremsmünster, Austria; #191161). The tara of the empty tubes was determined, then 30 µL of a 5,000 mg/L CUL or 11-AcCUL stock solution in DMSO was added and combined with 1.47 mL of the suspension culture (1 day after passage to new medium). The resulting final concentration of the toxin was therefore 100 mg/L (2% final solvent concentration) and the wet weight of the cells per tube was about 250 mg. The tubes were placed upright on a rotary shaker (100 rpm, 20°C) for the duration of the experiment. At the end point the content of the tube was split into three parts (supernatant, wash solution, and pellet extract), which each was brought up to the doubled volume of the initial culture (3 mL) and having a final concentration of 50% methanol. Experiments with CUL were performed in triplicates and with AcCUL in duplicates.

Supernatant: The culture medium was removed by pushing a 1000 µL pipette tip down to the bottom of the 14 mL tube, to avoid aspiration of cells. The medium was completely removed in two steps and transferred to an Eppendorf tube, which was then centrifuged (2 min 5000 rpm) to pellet the small amounts of aspirated fine suspension cells. The cleared supernatant was mixed 1:1 with methanol and transferred into the glass vials for LC-MS culmorin measurement, or further diluted 10-times up to 1000-times with mixture of methanol:water (1:1), depending on analytes concentration.

Washing solution: To remove residual culture medium from the cells and to remove unspecifically adsorbed toxin 750 µL 10% MeOH was added and after gently mixing by hand (to avoid lysing cells) the supernatant was removed by again pushing the tip to the bottom of the tube (beneath the cells). The solution was transferred to an Eppendorf tube. Then another 750 µL aliquot of 10% methanol was added to the cells and the supernatant again removed and combined with the first aliquot. After a short mild centrifugation step (2 min 5000 rpm) 500 µL of the supernatant were combined 1:1 with 90% methanol to give the wash sample.

Pellet extract: the weight of the tube with the remaining cell pellet was determined and the wet weight calculated. To break the cells the tubes were frozen at -80 and thawed by adding 2x 750 µL 100% methanol prewarmed to 37 °C. The suspension was then sonified with a Branson Digital Sonifier (W-250-D, VWR International, Vienna) using a standard resonator with ½'' diameter and the settings: 2x 15 sec, 20% Amplitude. (The resonator was extensively rinsed with methanol between samples.) The resulting suspension was cleared by centrifugation (5 min, 11,000 rpm) and diluted 1:1 with distilled water to also yield a 50% methanol solution.

f) HPLC-MS/MS analysis of culmorin and its metabolites

Semiquantitative analysis of culmorin and its metabolites was performed by high performance liquid chromatography hyphenated to tandem mass spectrometry (HPLC-MS/MS). Since an appropriate separation of all metabolites is rather complicated due to the identical molecular mass and similar structure (e.g. CUL-8-sulfate and CUL-11-sulfate), two different methods were used for analysis of all compounds of interest. Analyses were performed using Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA) equipped with electrospray ionization (ESI).

In the first method, mass spectrometer was operated in positive ionization mode (ESI+) and covered the analysis of CUL and 11-AcCUL. Chromatographic separation of analytes was carried out using Gemini C18 (Phenomenex) HPLC column (150 x 4.6 mm; 5 µm) operated at 25°C, mobile phase consisted of water:methanol (80:20, v/v) (eluent A) and methanol:water (97:3, v/v) (eluent B), both containing 5 mM ammonium acetate. Time of analysis was 10 min with constant flow rate of 800 µL/min and injection volume of 10 µL. Gradient elution started at 80% of B, was held for 1 min and afterwards increased to 100% B within 5 min and held for another 2 min. Column was equilibrated at initial conditions for 2 min.

Second method involved analysis of CUL-8-sulfate, CUL-11-sulfate, CUL-8,11-disulfate, 11-Ac-CUL-8-glucoside and CUL-8-glucoside. The application of Kinetex Biphenyl UHPLC column (150 x 4.6 mm; 2.6 µm) enabled partial separation of both culmorin sulfates. Column operated at 25°C and the run time of analysis was also 10 min. Mobile phase was running at 300 µL/min and consisted of H_2O with 0.1% acetic acid (eluent A) and methanol with 0.1% acetic acid (eluent B). Gradient started at 3% B, was held for 1 min, then increased up to 90% B within 2 min, and consequently up to 100% B within another 3 min. At 100% B the gradient was held for 2 min and finally decreased down to initial conditions of mobile phase, at which stayed for 2 min.

The following source settings were used: temperature, 550°C; ion spray voltage, 4 kV (positive mode) and 4 kV (negative mode); curtain gas, 30 lb/in^2; source gas one and two, both 50 lb/in^2; and collision gas (nitrogen) set to high. For quantitation, two selected reaction-monitoring transitions per compound

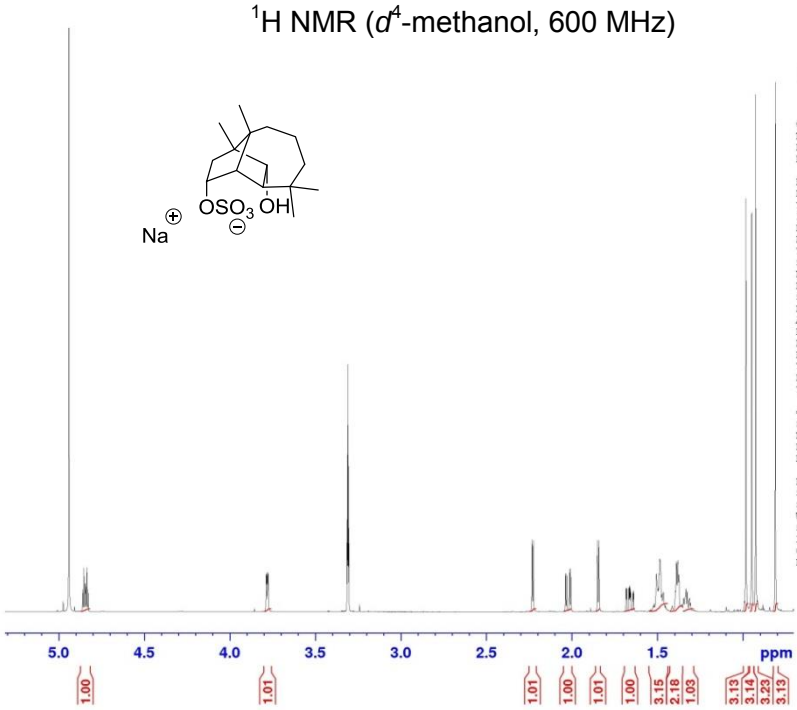
were acquired with a dwell time of 25 ms. Retention times and MS/MS parameters of all analytes are provided in the following table.

Analyte	Tr (min)	Q1	Q3	Declustering potential (V)	Collision energy (V)
CUL	4.25	256.2	221.3	41	17
		256.2	203.2	41	21
11-AcCUL	4.85	298.3	203.1	41	17
		298.3	161.1	41	33
CUL-8-glucoside	5.74	459.3	399.1	-70	-24
		459.3	58.9	-70	-48
11-AcCUL-8-glucoside	6.09	501.3	441.0	-75	-24
		501.3	399.1	-75	-34
CUL-8-sulfate	5.50	317.1	96.8	-145	-56
		317.1	79.9	-145	-116
CUL-11-sulfate	5.62	317.1	96.9	-140	-60
		317.1	79.9	-140	-116
CUL-disulfate	5.11	198.1	79.9	-70	-44
		198.1	96.9	-70	-36

2) Copies of NMR spectra

Culmorin-11-sulfate, sodium salt (3)

^{1}H NMR (*d*^{4}-methanol, 600 MHz)



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DS                        2
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RG                    87.33
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DE                   28.00 usec
TE                   295.0 K
D1                1.00000000 sec
TD0                        1

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F2 - Processing parameters
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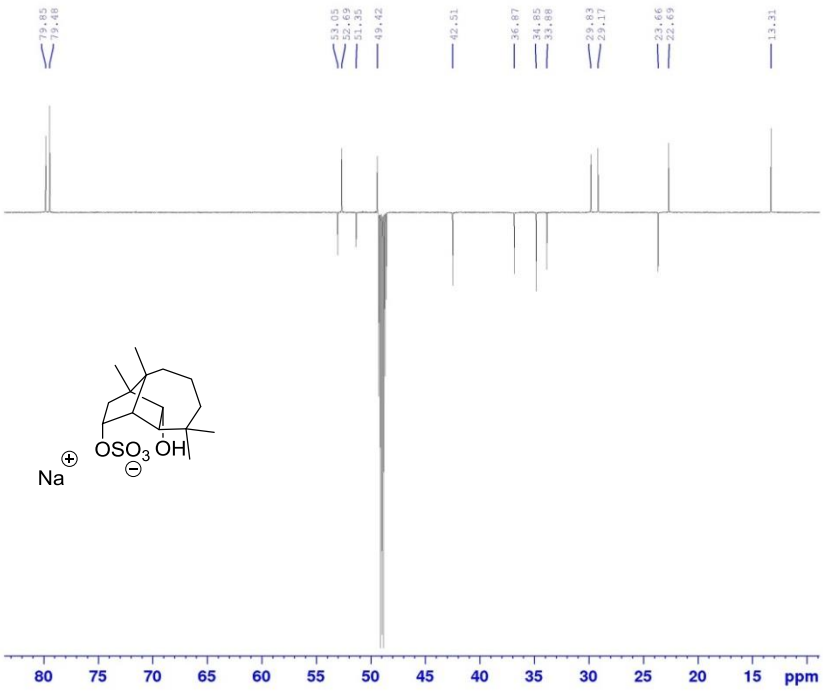
^{13}C NMR (*d*^{4}-methanol, 150 MHz)



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SWH               36057.691 Hz
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AQ                 0.9087659 sec
RG                    194.73
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CNST2             145.0000000
CNST11              1.0000000
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TD0                        1

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F2 - Processing parameters
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WDW                         EM
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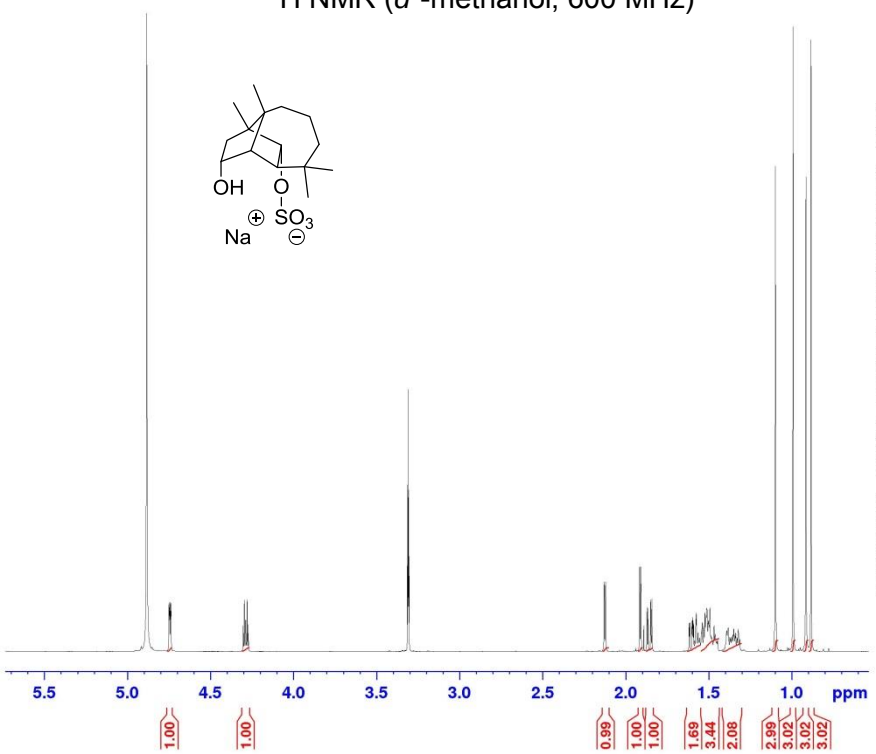
Culmorin-8-sulfate, sodium salt (4)

^{1}H NMR (d^{4}-methanol, 600 MHz)

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NS                        16
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SWH                 9615.385 Hz
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AQ                  3.4078720 sec
RG                     77.94
DW                    52.000 usec
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TE                    295.0 K
D1                 1.00000000 sec
TD0                        1

======== CHANNEL f1 ========
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NUC1                      1H
P1                     12.00 usec
PLW1           22.00000000 W

F2 - Processing parameters
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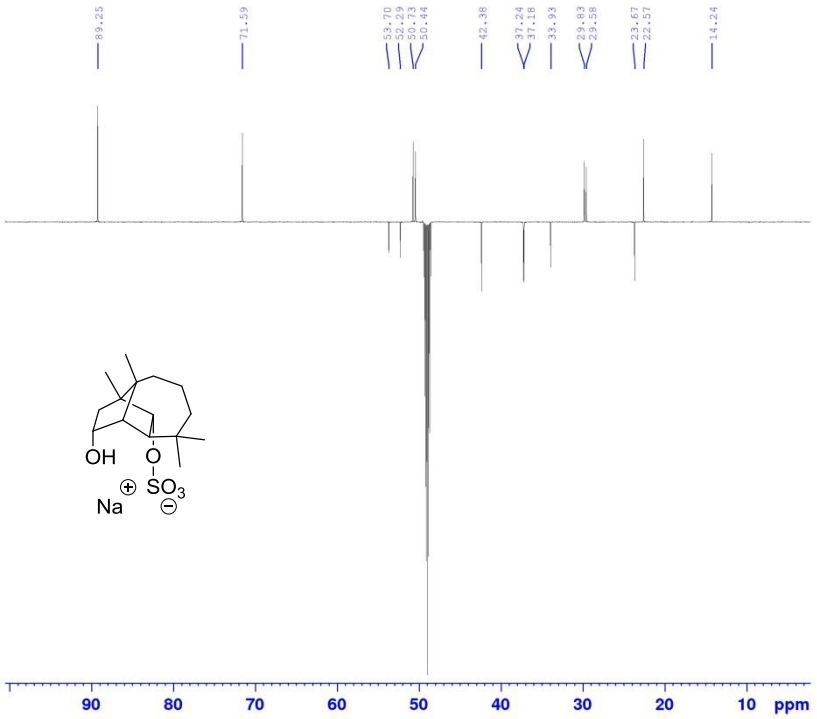
^{13}C NMR (d^{4}-methanol, 150 MHz)

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FIDRES              0.550197 Hz
AQ                  0.9087659 sec
RG                     194.73
DW                    13.867 usec
DE                    18.00 usec
TE                    295.0 K
CNST2             145.0000000
CNST11              1.0000000
D1                  3.00000000 sec
D20                 0.00689655 sec
TD0                        1

======== CHANNEL f1 ========
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NUC1                      13C
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P2                     20.00 usec
PLW1           58.00000000 W

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F2 - Processing parameters
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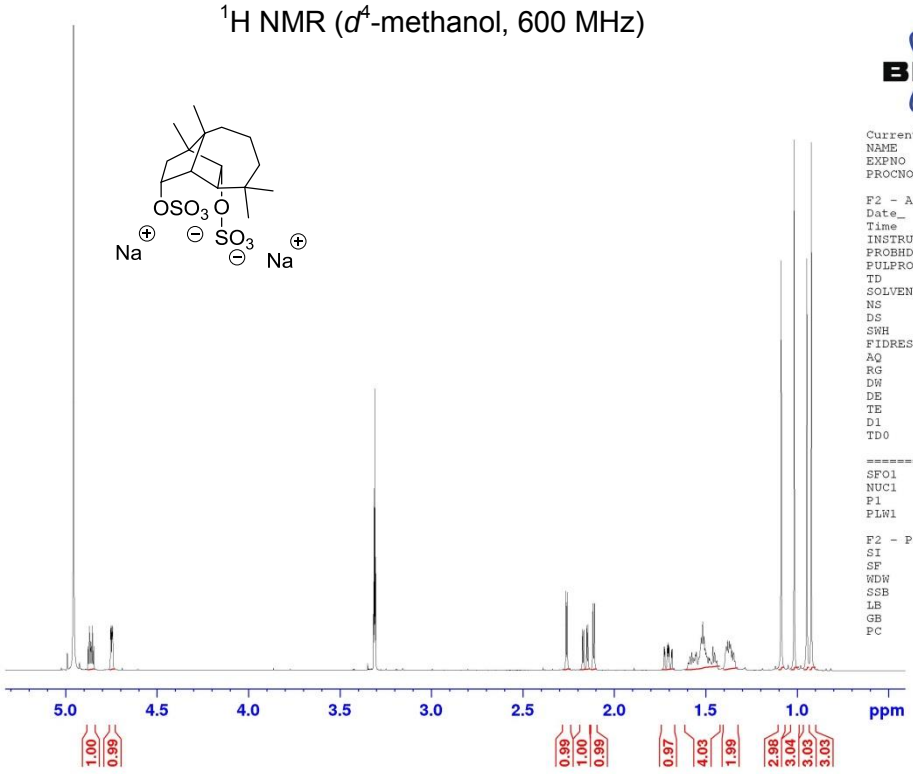
Culmorin-8,11-disulfate, sodium salt (5)

^{1}H NMR (d^{4}-methanol, 600 MHz)

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Time                  14.07
INSTRUM                spect
PROBHD    5 mm CPPBBO BB
PULPROG                zg30
TD                    65536
SOLVENT                MeOD
NS                        16
DS                         2
SWH                9615.385 Hz
FIDRES            0.146719 Hz
AQ                3.4078720 sec
RG                    77.94
DW                   52.000 usec
DE                   28.00 usec
TE                   295.0 K
D1                1.00000000 sec
TD0                        1

======== CHANNEL f1 ========
SFO1             600.1545011 MHz
NUC1                       1H
P1                      12.00 usec
PLW1            22.00000000 W

F2 - Processing parameters
SI                        32768
SF               600.1500116 MHz
WDW                       EM
SSB          0
LB                      0.30 Hz
GB           0
PC                      1.00
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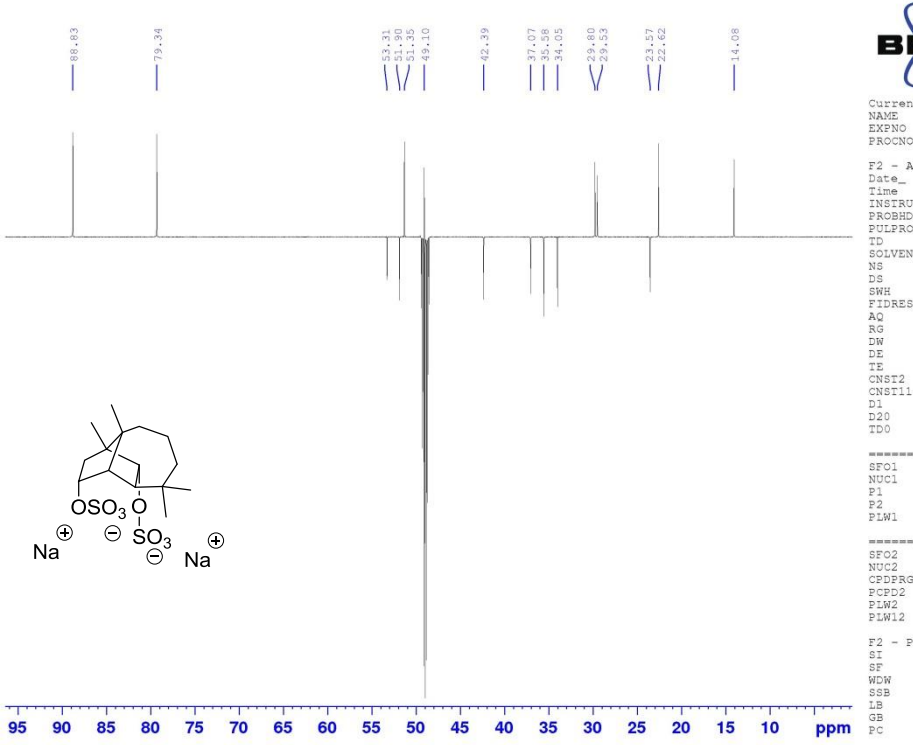
^{13}C NMR (d^{4}-methanol, 150 MHz)

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Current Data Parameters
NAME                  JLA301
EXPNO                     23
PROCNO                     1

F2 - Acquisition Parameters
Date_              20160905
Time                  21.15
INSTRUM                spect
PROBHD    5 mm CPPBBO BB
PULPROG                jmod
TD                    65536
SOLVENT                MeOD
NS                      3072
DS                         4
SWH               36057.691 Hz
FIDRES            0.550197 Hz
AQ                0.9087659 sec
RG                    194.73
DW                   13.867 usec
DE                   18.00 usec
TE                   295.0 K
CNST2            145.0000000
CNST11             1.0000000
D1                 3.00000000 sec
D20                0.00689655 sec
TD0                        1

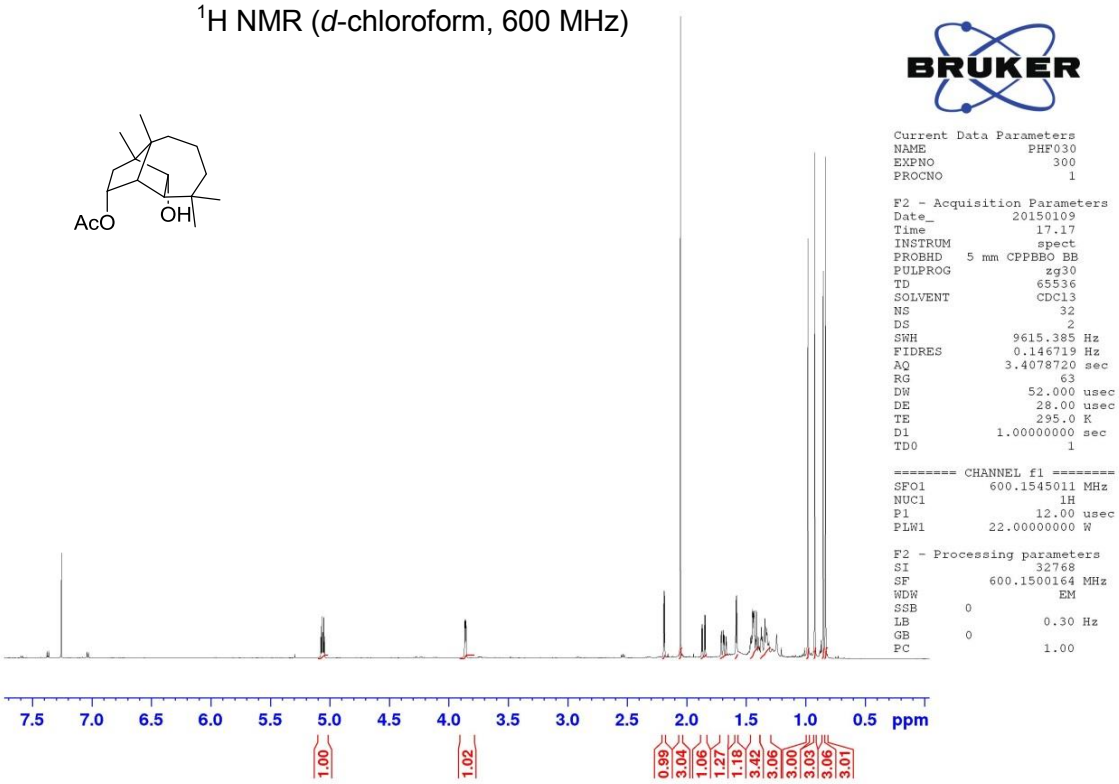
======== CHANNEL f1 ========
SFO1             150.9236829 MHz
NUC1                      13C
P1                      10.00 usec
P2                      20.00 usec
PLW1            58.00000000 W

======== CHANNEL f2 ========
SFO2             600.1524006 MHz
NUC2                       1H
CPDPRG[2              waltz16
PCPD2                   70.00 usec
PLW2            22.00000000 W
PLW12            0.64652997 W

F2 - Processing parameters
SI                        32768
SF               150.9076293 MHz
WDW                       EM
SSB          0
LB                      1.00 Hz
GB           0
PC                      1.40
```

11-Acetylculmorin (2)

^{1}H NMR (*d*-chloroform, 600 MHz)

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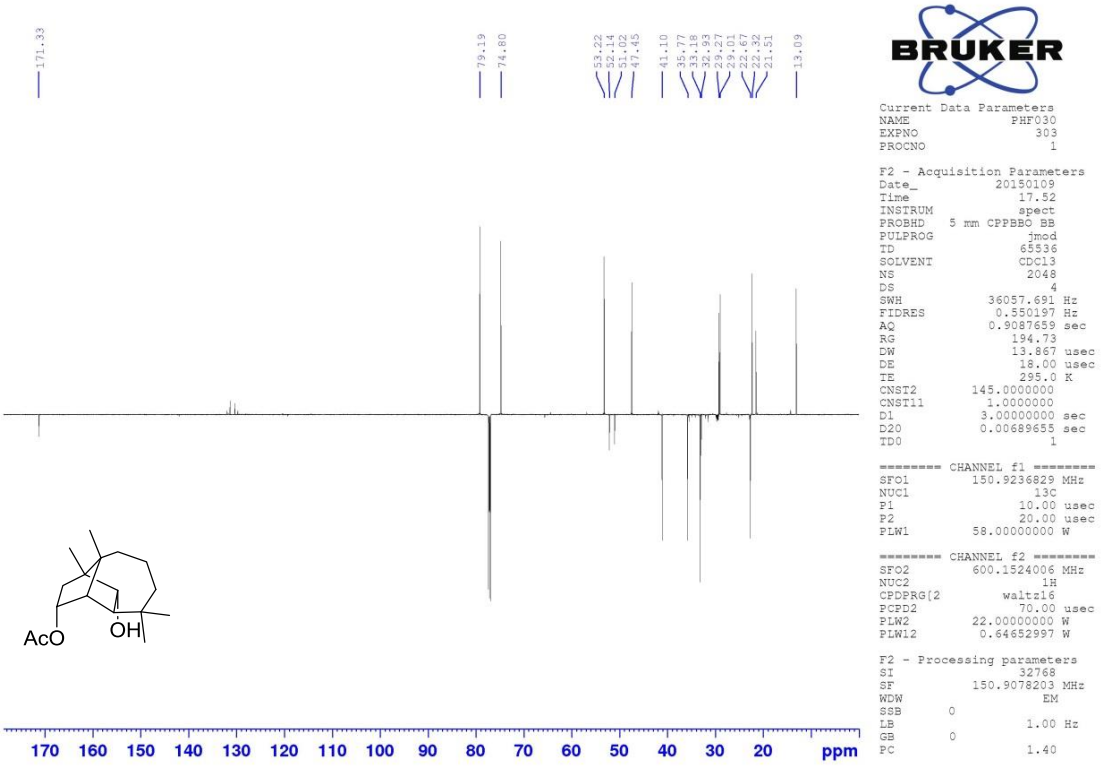
Current Data Parameters
NAME                  PHF030
EXPNO                    300
PROCNO                     1

F2 - Acquisition Parameters
Date_              20150109
Time                 17.17
INSTRUM              spect
PROBHD   5 mm CPPBBO BB
PULPROG              zg30
TD                  65536
SOLVENT             CDCl3
NS                     32
DS                      2
SWH               9615.385 Hz
FIDRES            0.146719 Hz
AQ                3.4078720 sec
RG                     63
DW                  52.000 usec
DE                  28.00 usec
TE                  295.0 K
D1              1.00000000 sec
TD0                     1

======== CHANNEL f1 ========
SFO1            600.1545011 MHz
NUC1                   1H
P1                  12.00 usec
PLW1           22.00000000 W

F2 - Processing parameters
SI                     32768
SF              600.1500164 MHz
WDW                    EM
SSB          0
LB                  0.30 Hz
GB           0
PC                  1.00
```

^{13}C NMR (*d*-chloroform, 150 MHz)

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Current Data Parameters
NAME                  PHF030
EXPNO                    303
PROCNO                     1

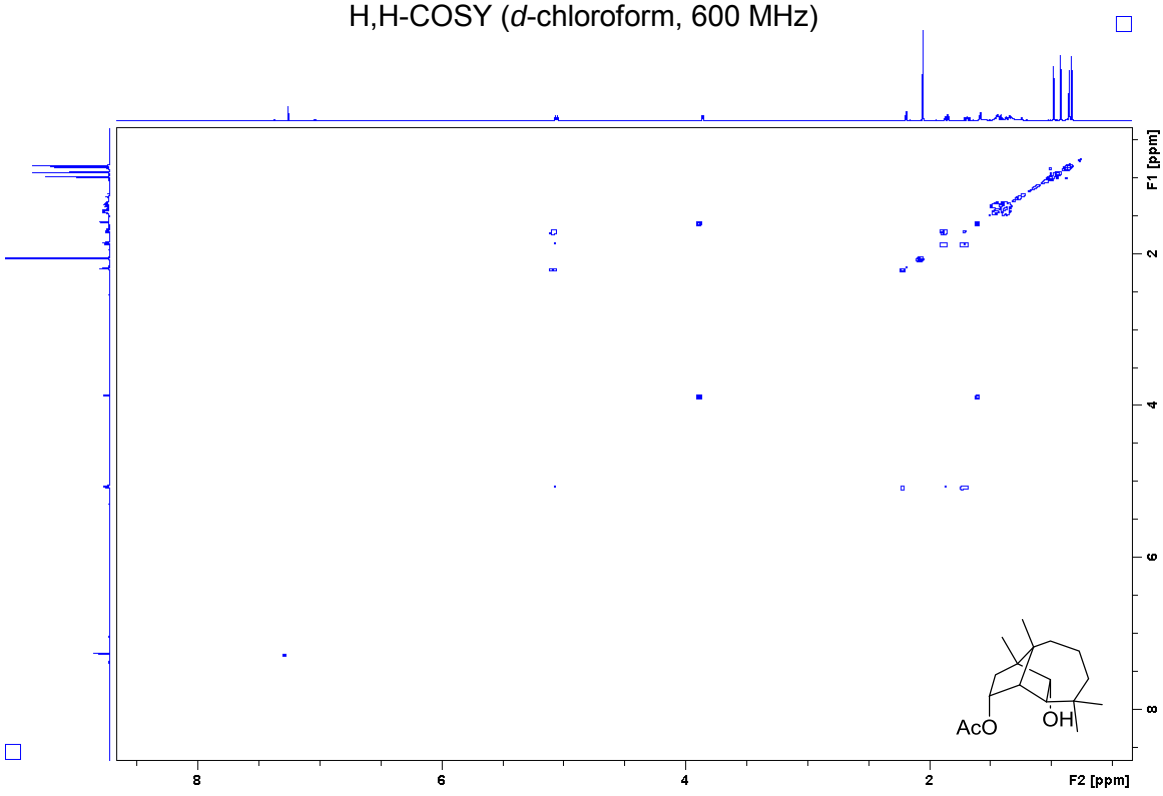
F2 - Acquisition Parameters
Date_              20150109
Time                 17.52
INSTRUM              spect
PROBHD   5 mm CPPBBO BB
PULPROG             jmod
TD                  65536
SOLVENT             CDCl3
NS                    2048
DS                       4
SWH              36057.691 Hz
FIDRES            0.550197 Hz
AQ                0.9087659 sec
RG                   194.73
DW                  13.867 usec
DE                  18.00 usec
TE                  295.0 K
CNST2           145.0000000
CNST11            1.0000000
D1              3.00000000 sec
D20             0.00689655 sec
TD0                     1

======== CHANNEL f1 ========
SFO1            150.9236829 MHz
NUC1                   13C
P1                  10.00 usec
P2                  20.00 usec
PLW1           58.00000000 W

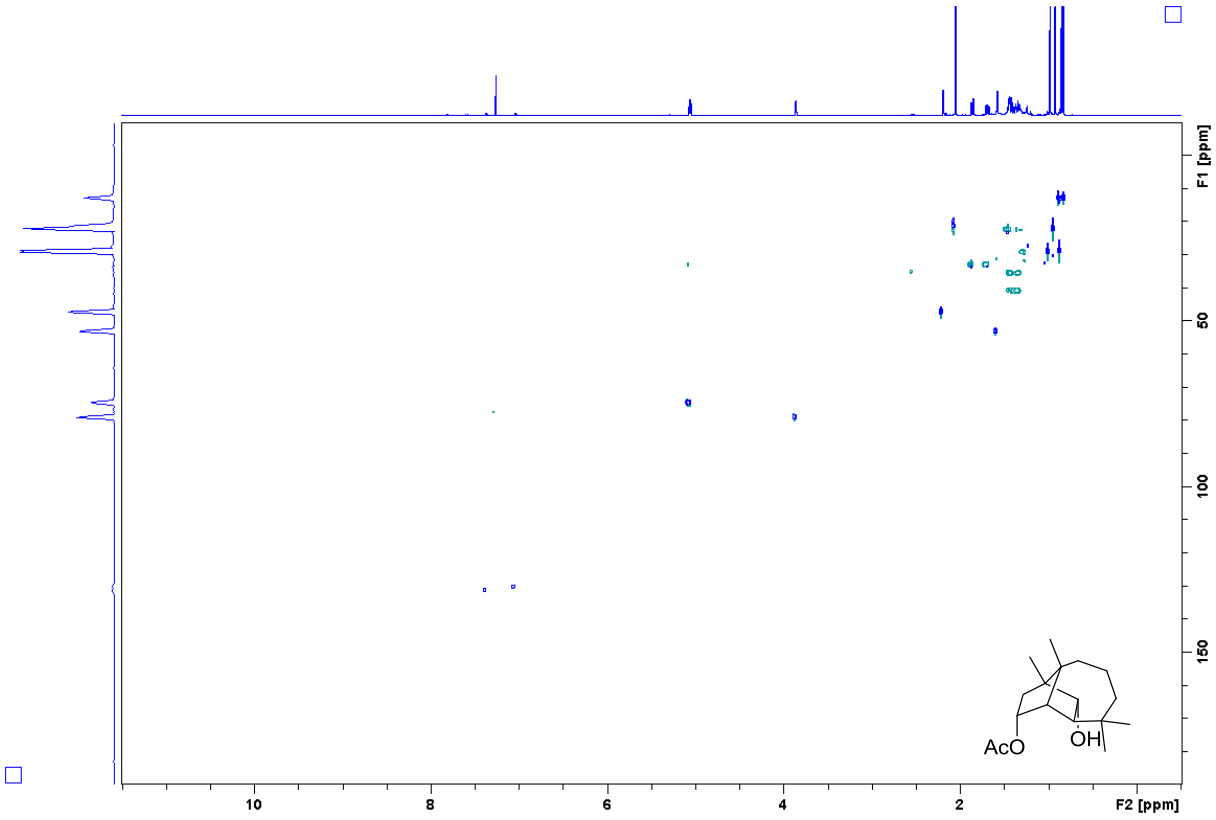
======== CHANNEL f2 ========
SFO2            600.1524006 MHz
NUC2                   1H
CPDPRG[2            waltz16
PCPD2               70.00 usec
PLW2           22.00000000 W
PLW12           0.64652997 W

F2 - Processing parameters
SI                     32768
SF              150.9078203 MHz
WDW                    EM
SSB          0
LB                  1.00 Hz
GB           0
PC                  1.40
```

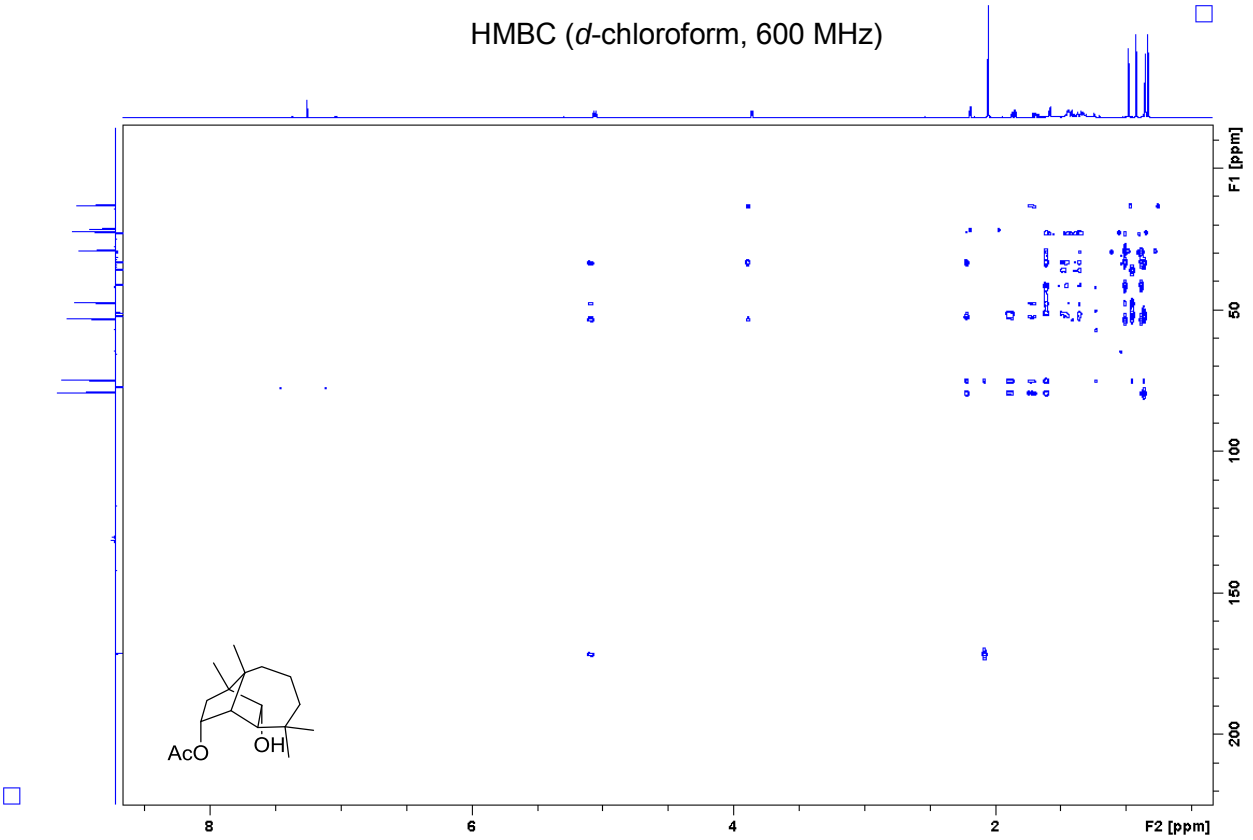
H,H-COSY (*d*-chloroform, 600 MHz)



HSQC (*d*-chloroform, 600 MHz)

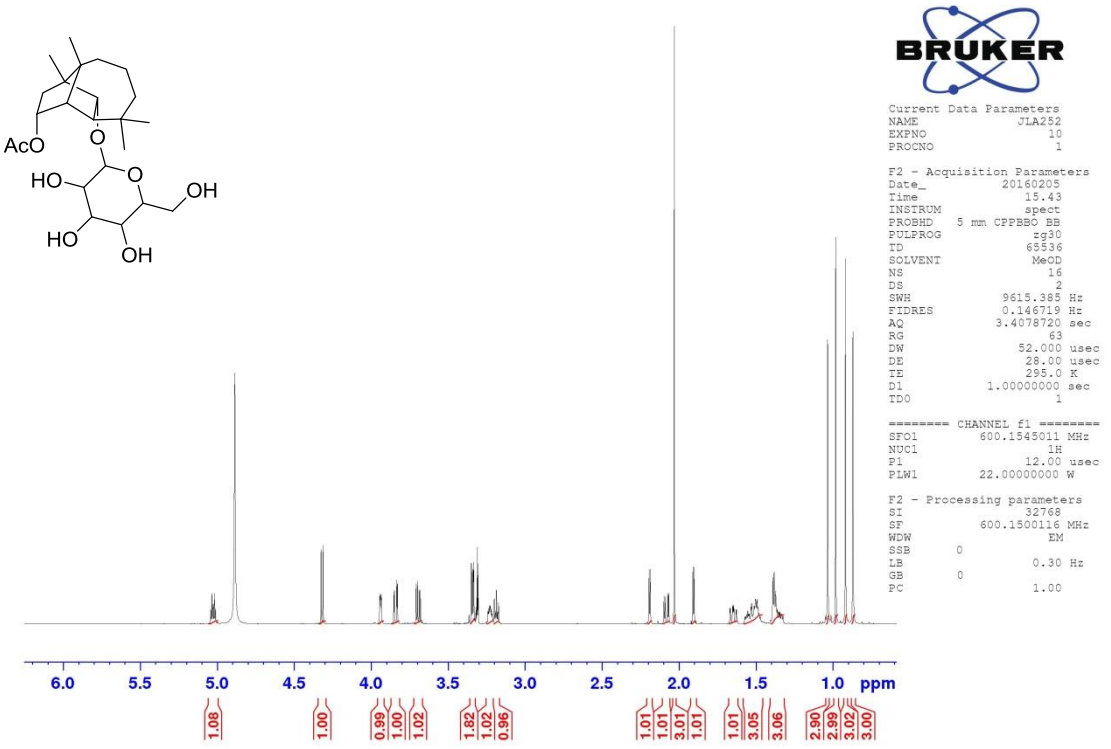


HMBC (*d*-chloroform, 600 MHz)

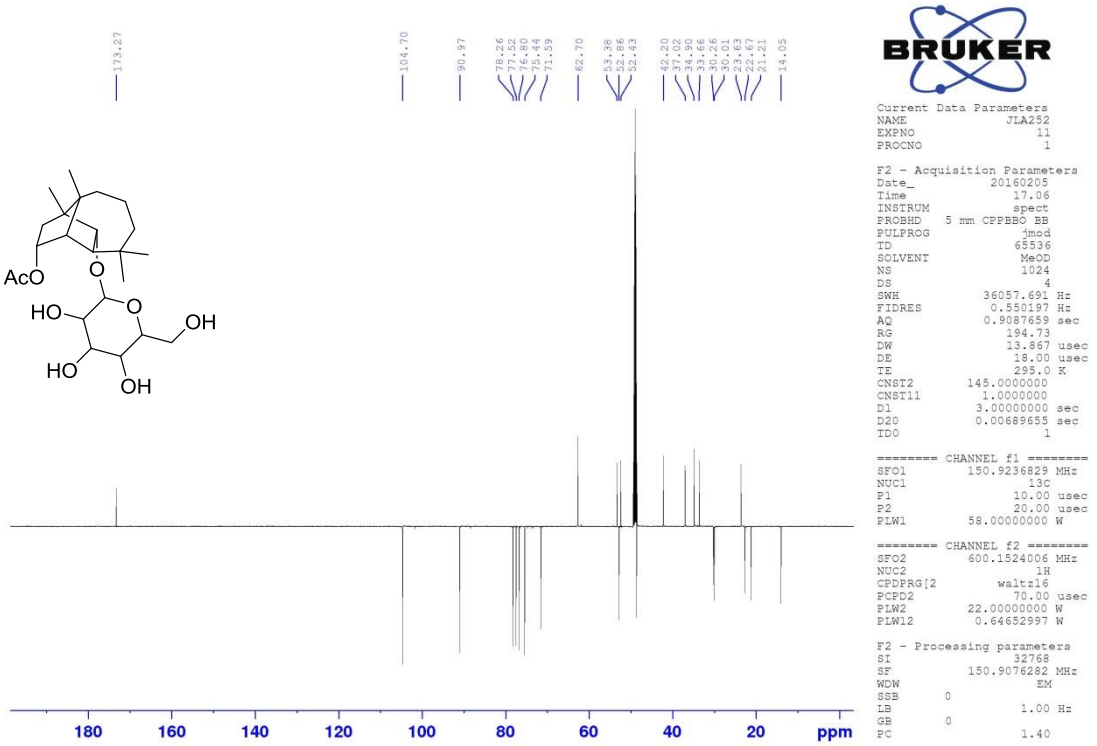


11-Acetylculmorin-8-β,D-glucoside (8)

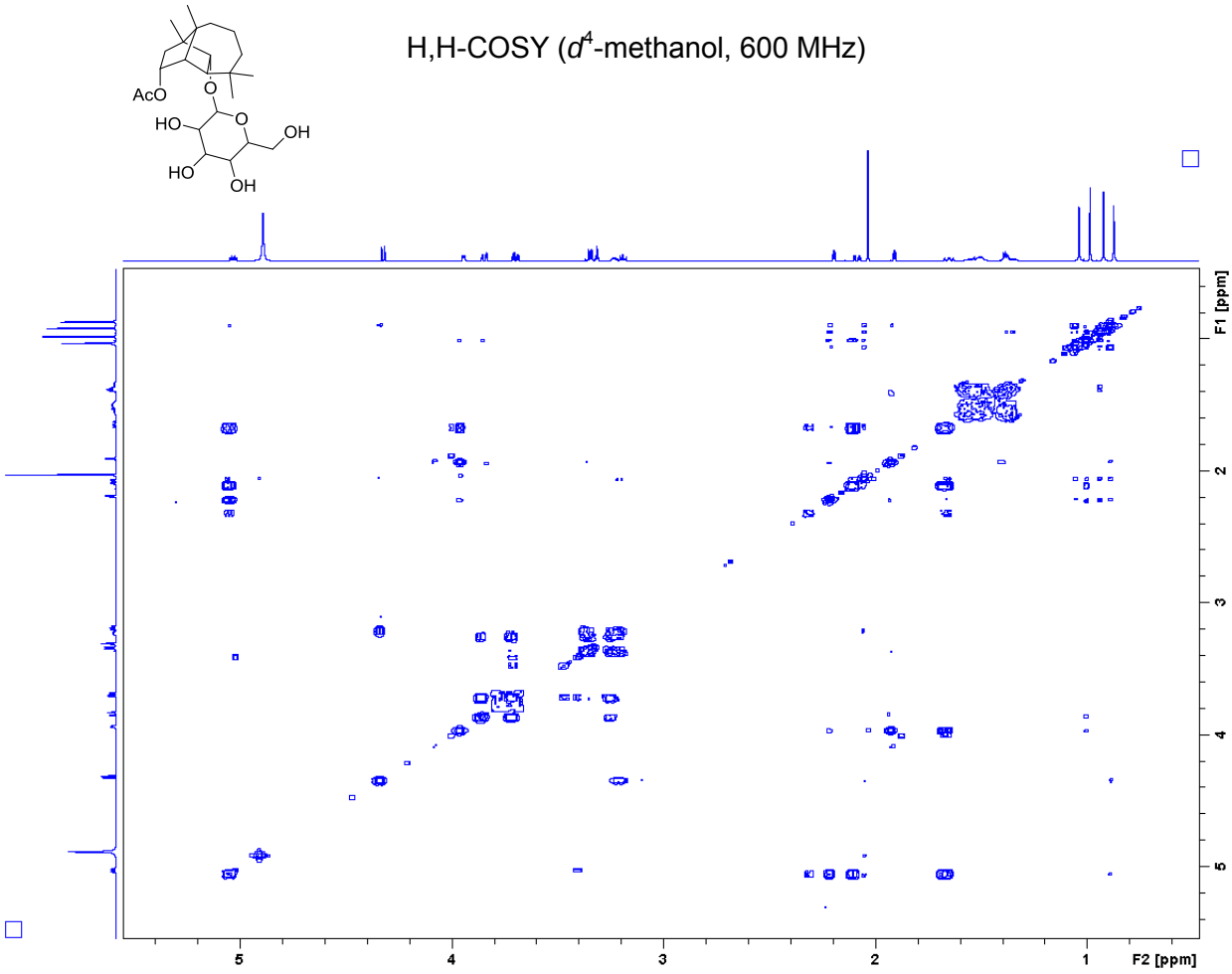
^{1}H NMR (*d*^{4}-methanol, 600 MHz)



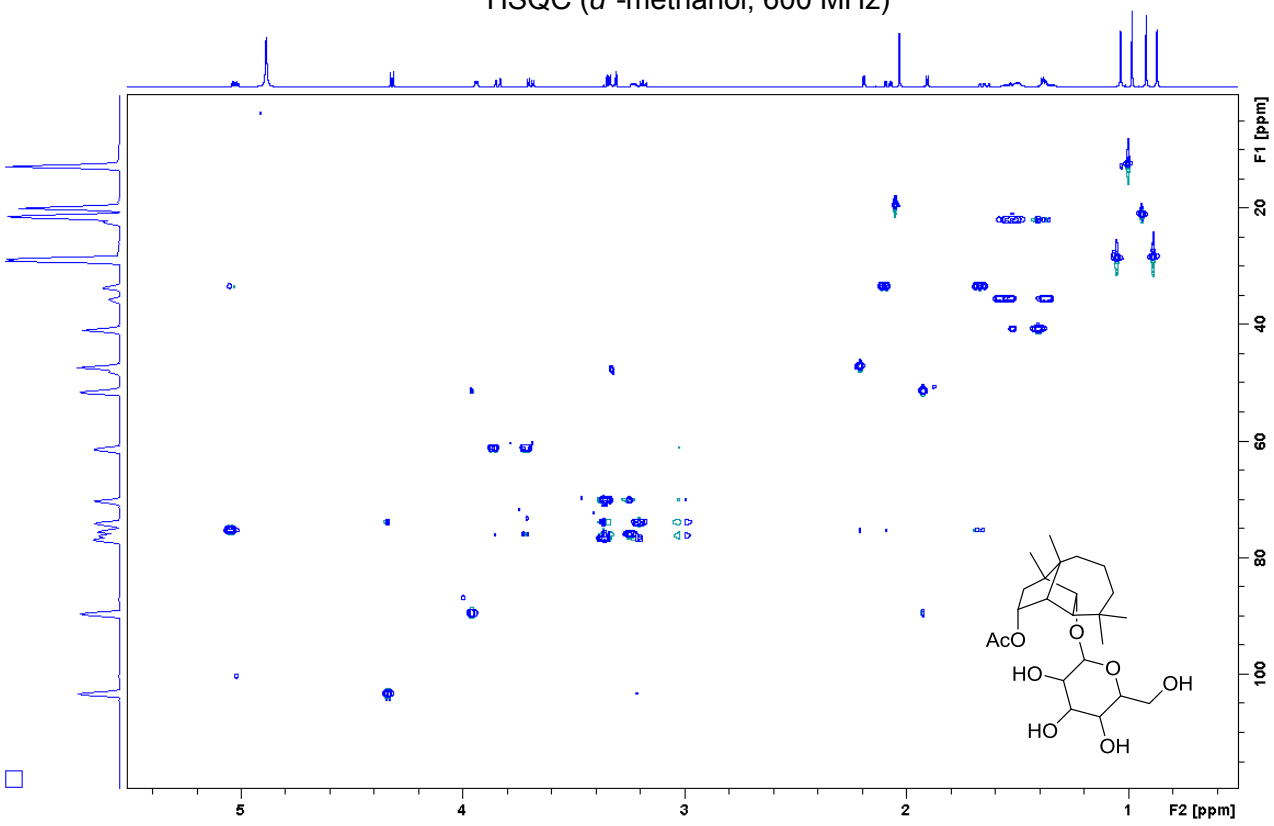
^{13}C NMR (d^4-methanol, 150 MHz)



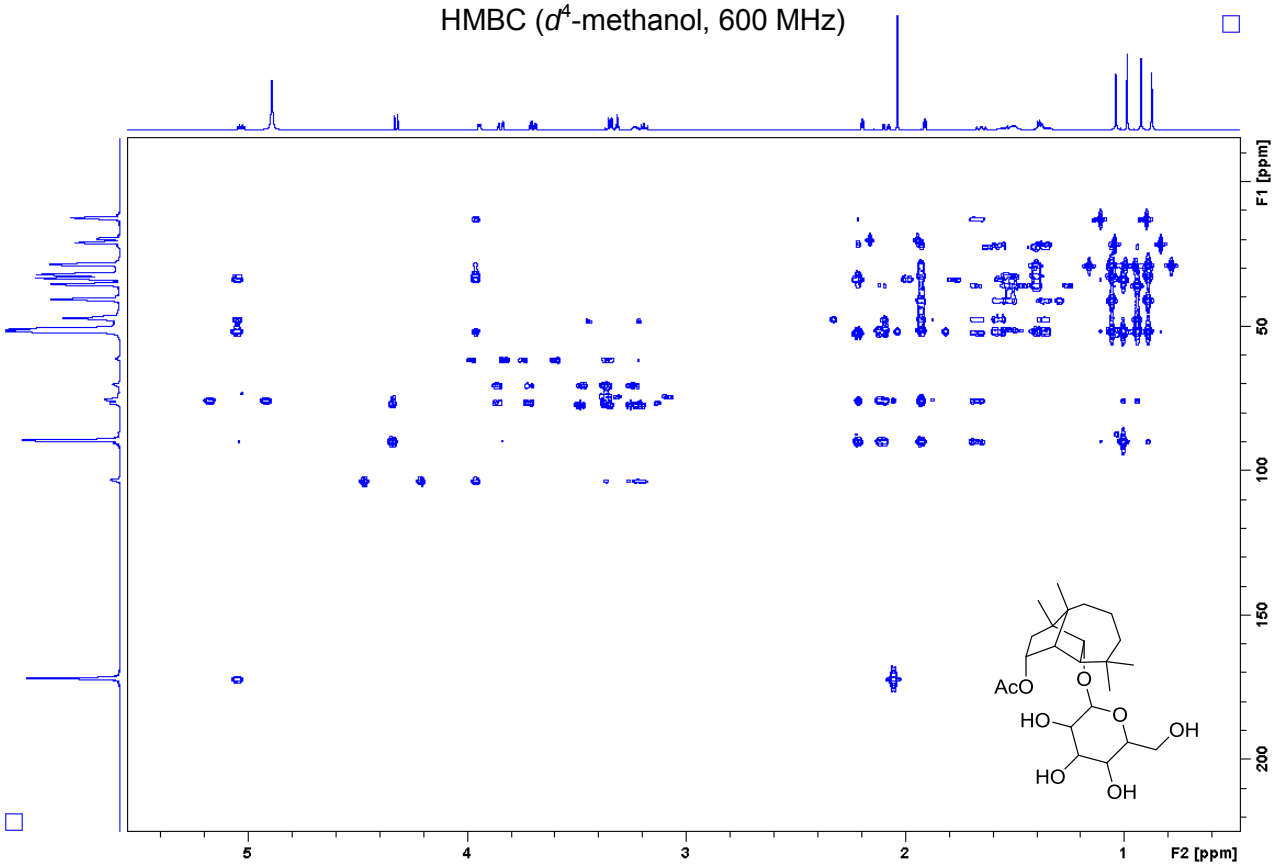
H,H-COSY (d^4-methanol, 600 MHz)



HSQC (d^4-methanol, 600 MHz)

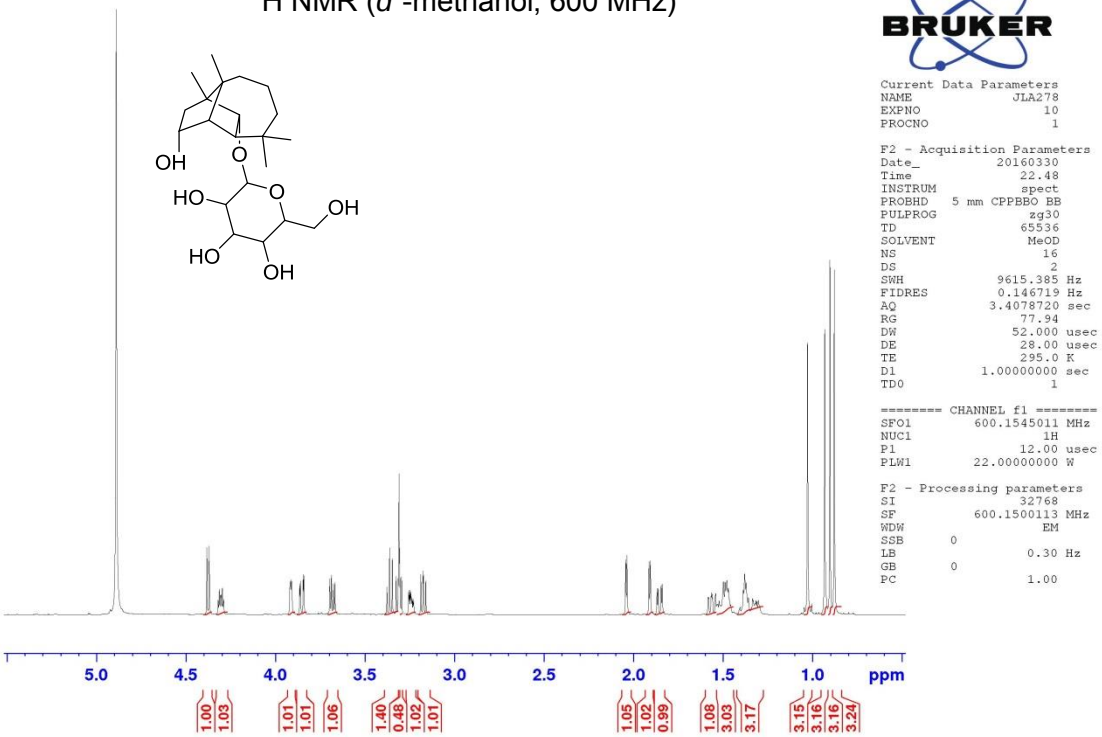


HMBC (d^4-methanol, 600 MHz)



Culmorin-8-β,D-glucoside (6)

^{1}H NMR (d^{4}-methanol, 600 MHz)

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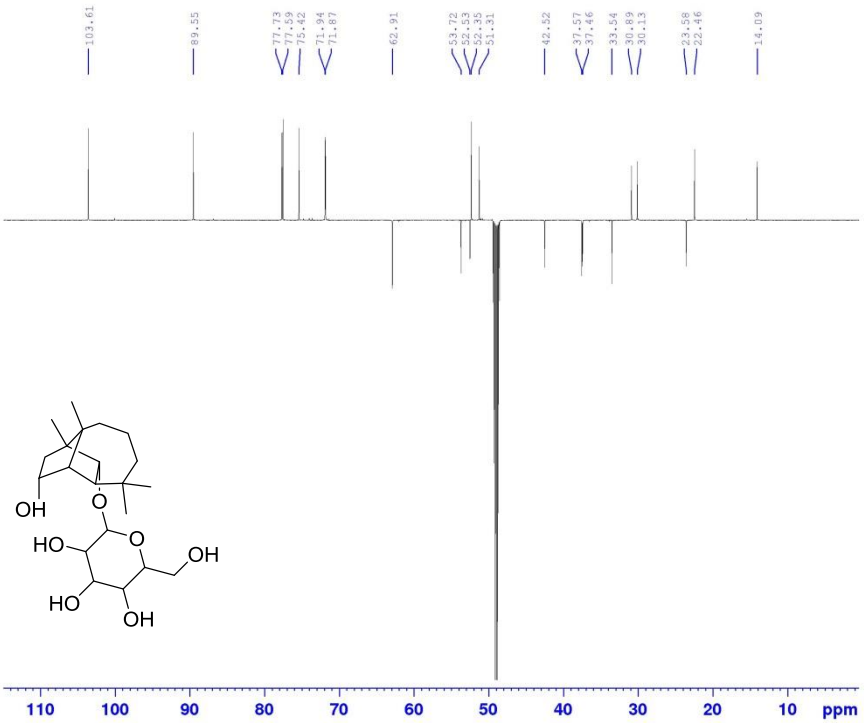
Current Data Parameters
NAME                  JLA278
EXPNO                     10
PROCNO                     1

F2 - Acquisition Parameters
Date_              20160330
Time                  22.48
INSTRUM                spect
PROBHD   5 mm CPPBBO BB
PULPROG                zg30
TD                    65536
SOLVENT                MeOD
NS                       16
DS                        2
SWH               9615.385 Hz
FIDRES            0.146719 Hz
AQ                3.4078720 sec
RG                    77.94
DW                  52.000 usec
DE                  28.00 usec
TE                  295.0 K
D1                1.00000000 sec
TD0                        1

======== CHANNEL f1 ========
SFO1            600.1545011 MHz
NUC1                      1H
P1                     12.00 usec
PLW1           22.00000000 W

F2 - Processing parameters
SI                        32768
SF              600.1500113 MHz
WDW                        EM
SSB          0
LB                     0.30 Hz
GB           0
PC                     1.00
```

^{13}C NMR (d^{4}-methanol, 150 MHz)

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Current Data Parameters
NAME                  JLA278
EXPNO                     13
PROCNO                     1

F2 - Acquisition Parameters
Date_              20160331
Time                   4.27
INSTRUM                spect
PROBHD   5 mm CPPBBO BB
PULPROG               jmod
TD                    65536
SOLVENT                MeOD
NS                      5120
DS                         4
SWH              36057.691 Hz
FIDRES            0.550197 Hz
AQ                0.9087659 sec
RG                   194.73
DW                  13.867 usec
DE                   18.00 usec
TE                   295.0 K
CNST2           145.0000000
CNST11            1.0000000
D1                3.00000000 sec
D20               0.00689655 sec
TD0                        1

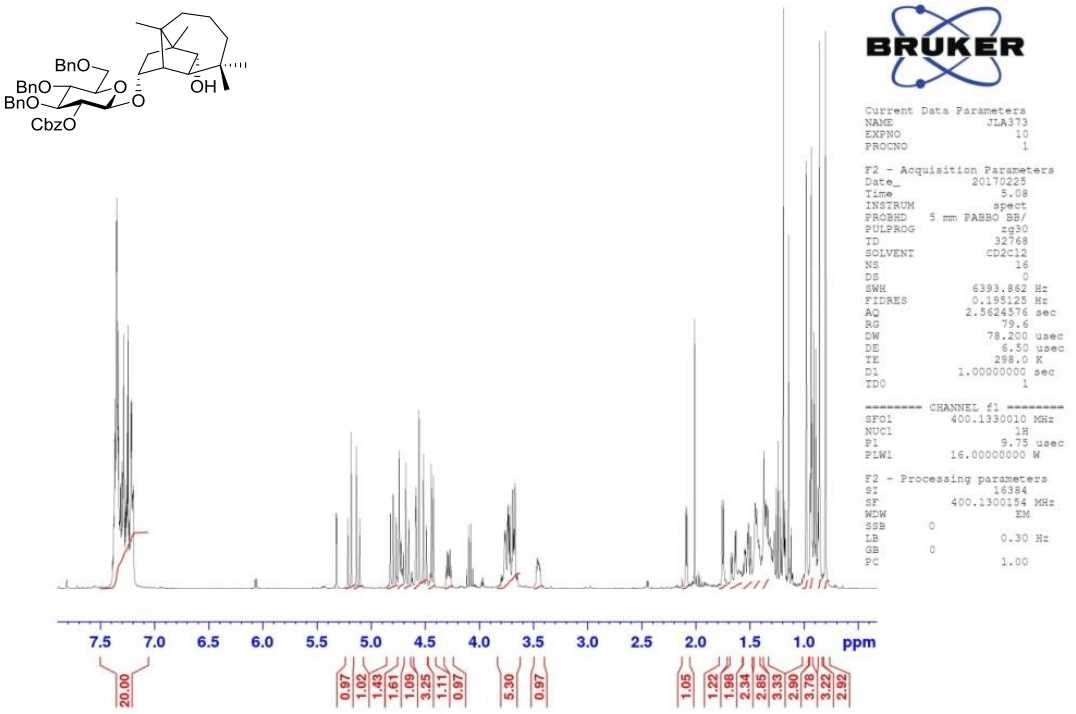
======== CHANNEL f1 ========
SFO1            150.9236829 MHz
NUC1                      13C
P1                     10.00 usec
P2                     20.00 usec
PLW1            58.00000000 W

======== CHANNEL f2 ========
SFO2            600.1524006 MHz
NUC2                      1H
CPDPRG[2             waltz16
PCPD2                  70.00 usec
PLW2            22.00000000 W
PLW12            0.64652997 W

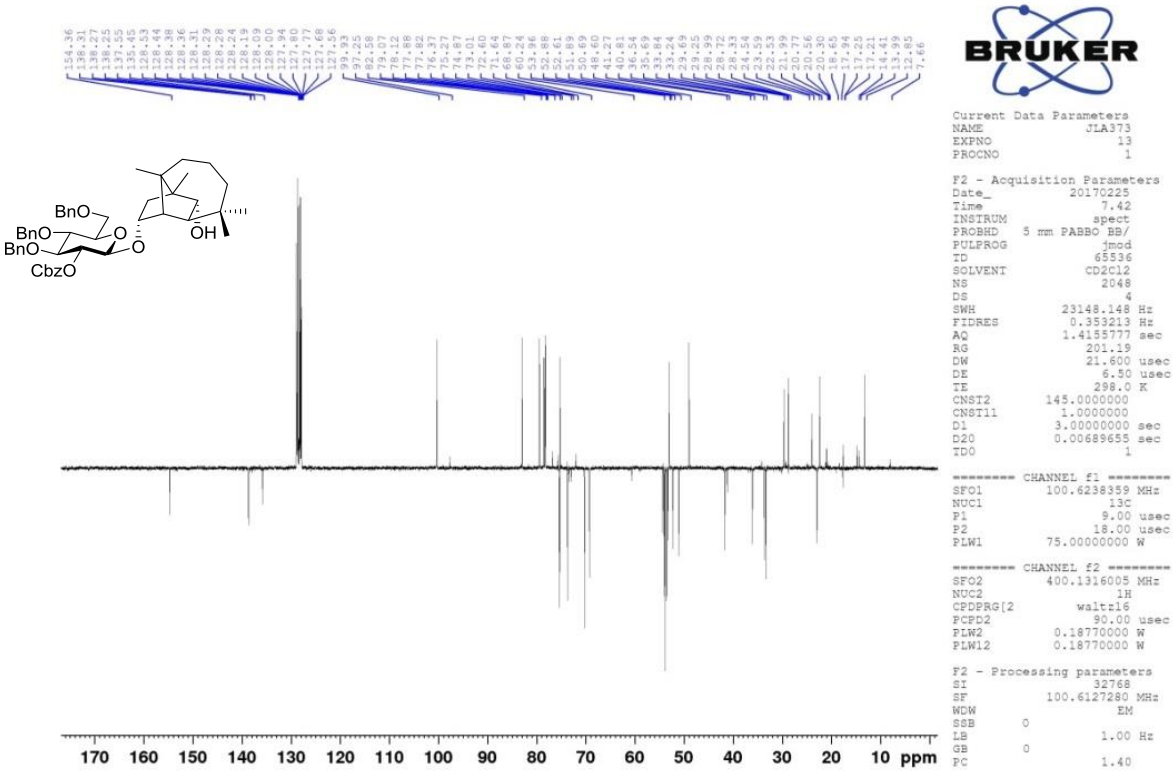
F2 - Processing parameters
SI                        32768
SF              150.9076273 MHz
WDW                        EM
SSB          0
LB                     1.00 Hz
GB           0
PC                     1.40
```

Culmorin-11-(3,4,6-tri-O-benzyl-2-O-benzyloxycarbonyl)-β,D-glucoside (9)

^{1}H NMR (d^{2}-methylene chloride, 400 MHz)

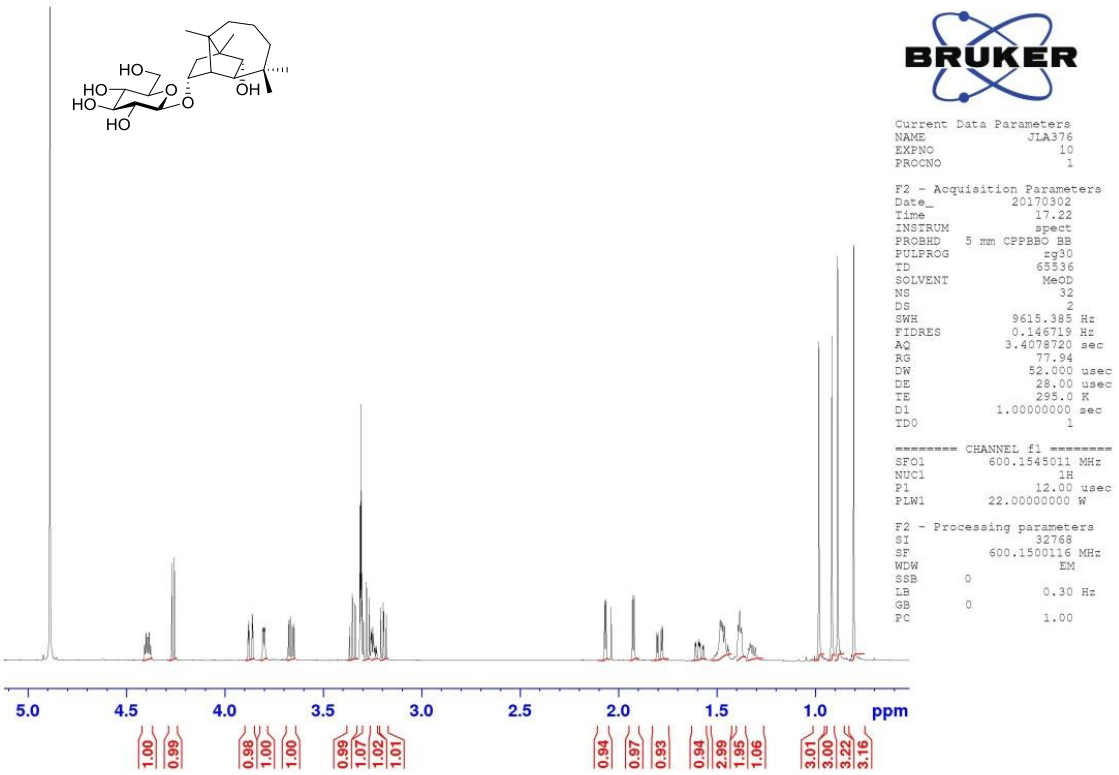


^{13}C NMR (d^{2}-methylene chloride, 100 MHz)

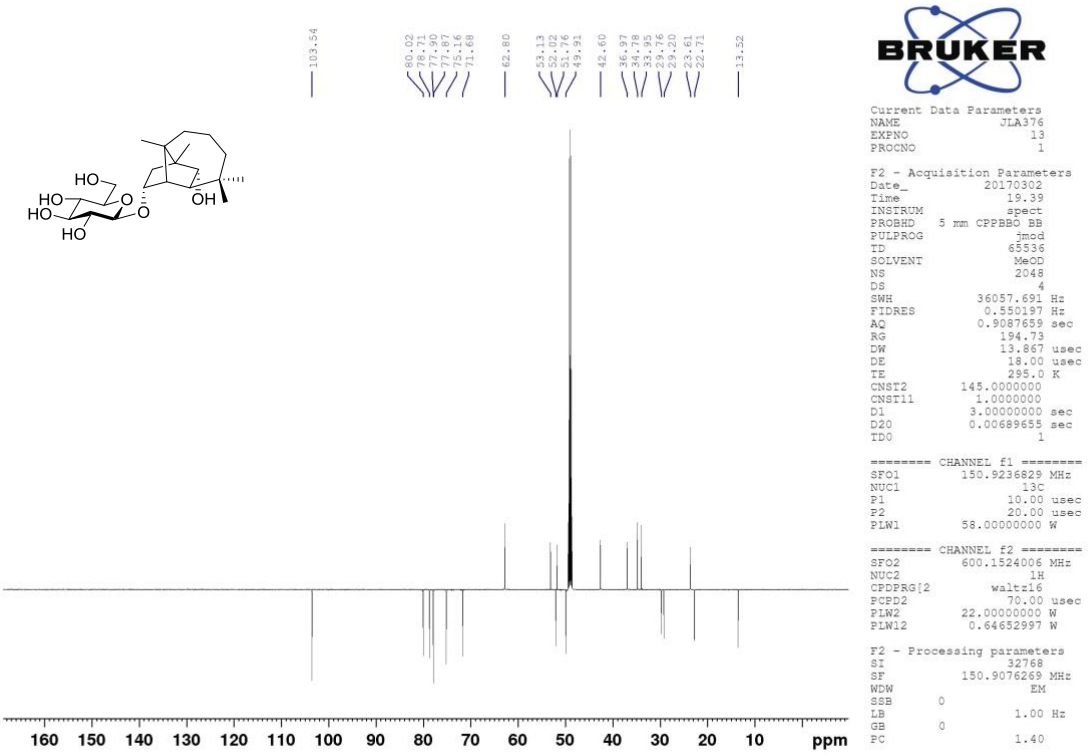


Culmorin-11-β,D-glucoside (7)

^{1}H NMR (*d*^{4}-methanol, 600 MHz)



^{13}C NMR (*d*^{4}-methanol, 150 MHz)



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3. E. Varga, G. Wiesenberger, C. Hametner, T. J. Ward, Y. Dong, D. Schofbeck, S. McCormick, K. Broz, R. Stuckler, R. Schuhmacher, R. Krska, H. C. Kistler, F. Berthiller and G. Adam, *Environmental microbiology*, 2015, **17**, 2588-2600.
4. G. C. Kasitu, J. W. ApSimon, B. A. Blackwell, D. A. Fielder, R. Greenhalgh and J. D. Miller, *Canadian Journal of Chemistry*, 1992, **70**, 1308-1316.
5. C. Schmeitzl, B. Warth, P. Fruhmann, H. Michlmayr, A. Malachová, F. Berthiller, R. Schuhmacher, R. Krska and G. Adam, *Toxins*, 2015, **7**, 3112.

Chapter 7

Curriculum Vitae

Curriculum Vitae

Julia Weber
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Address: Bandgasse 27, 1070 Vienna, Austria
Email: julia.weber@tuwien.ac.at

EDUCATION

2005	High School Exit Exam (Matura), BRG Rahlgasse, Vienna, Austria
Since October 2005	Studies on Chemistry at TU Wien, Vienna, Austria
April, 2012	**Bachelor of Science**, Technical Chemistry, TU Wien
June, 2013	**Master of Science**, Chemistry-Synthesis, TU Wien
	Diploma Thesis: Synthesis of (late) Phase II Mycotoxin Metabolites focused on the *Fusarium* Mycotoxin Zearalenone Advisor: Prof. Johannes Fröhlich
Since February 2014	**PhD studies**, TU Wien
	PhD Thesis: New Methods for the Synthesis of Glycosylated Natural Products with Application to Masked Trichothecene Mycotoxins Supervision: Prof. Johannes Fröhlich & Dr. Hannes Mikula

PUBLICATIONS

J. Weber, H. Mikula, P. Fruhmann, C. Hametner, E. Varga, F. Berthiller, R. Krska, J. Fröhlich: "*Gentiobiosylation of β-Resorcylic Acid Esters and Lactones: First Synthesis and Characterization of Zearalenone-14-β,D-Gentiobioside*"; Synlett, **24** (2013), 1830-1834.

H. Mikula, J. Weber, S. Lexmüller, G. Bichl, H. Schwartz, E Varga, F. Berthiller, C. Hametner, R. Krska, J. Fröhlich: "*Simultaneous preparation of α/β-zearalenol glucosides and glucuronides*"; Carbohydrate Research, **373** (2013), 59-63.

H. Mikula, B. Sohr, P. Skrinjar, J. Weber, C. Hametner, F. Berthiller, R. Krska, Gerhard A., J. Fröhlich: "*Sulfation of β-resorcylic acid esters-first synthesis of zearalenone-14-sulfate*"; Tetrahedron Letters, **54** (2013), 3290-3293.

H. Mikula, J. Weber, D. Svatunek, P. Skrinjar, G. Adam, R. Krska, C. Hametner, J. Fröhlich: "*Synthesis of Zearalenone-16-β,D-Glucoside and Zearalenone-16-Sulfate: A Tale of Protecting Resorcylic Acid Lactones for Regiocontrolled Conjugation*"; Beilstein Journal of Organic Chemistry, **10** (2014), 1129-34.

P. Fruhmann, P. Skrinjar, J. Weber, H. Mikula, B. Warth, M. Sulyok, R. Krska, G. Adam, E. Rosenberg, C. Hametner, J. Fröhlich: "*Sulfation of Deoxynivalenol, its acetylated derivatives, and T2-toxin*"; Tetrahedron, **70** (2014), 5260-66.

H. Schwartz-Zimmermann, P. Fruhmann, S. Dänicke, G. Wiesenberger, S. Caha, J. Weber, F. Berthiller: "*Metabolism of Deoxynivalenol and Deepoxy-Deoxynivalenol in Broiler Chickens, Pullets, Roosters and Turkeys*"; Toxins, **7** (2015), 4076-29.

J. Weber, P. Fruhmann, C. Hametner, A. Schiessl, G. Häubl, J. Fröhlich, H. Mikula: "*Synthesis of isotope labeled deoxynivalenol-15-β,D-glycosides*"; Eur. J. Org. Chem. 2017, *accepted manuscript*.

CONFERENCE PROCEEDINGS

2012 J. Weber, P. Fruhmann, H. Mikula, C. Hametner, J. Fröhlich: "*Towards the Synthesis of Late Phase II Metabolites of Mycotoxins*"; 34th Mycotoxin Workshop, Braunschweig, Germany

2013 J. Weber, H. Mikula, P. Fruhmann, C. Hametner, E. Varga, F. Berthiller, R. Krska, J. Fröhlich: "*Synthesis of Zearalenon-14-β,D-gentiobioside*"; 35th Mycotoxin Workshop, Ghent, Belgium

2015 J. Weber, P. Fruhmann, C. Hametner, J. Fröhlich, H. Mikula, A. Schiessl, G. Häubl: "*Synthesis of Deoxynivalenol-15-β,D-glucoside*"; 37th Mycotoxin Workshop, Bratislava, Slovakia

2015 J. Weber, H. Mikula, P. Fruhmann, C. Hametner, J. Fröhlich: "*Mycotoxin-Conjugates as Reference Materials for Environmental Monitoring*"; Vienna young Scientist Symposium, TU Vienna

2016 J. Weber, P. Fruhmann, C. Hametner, A. Schiessl, G. Häubl, J. Fröhlich, H. Mikula: "*Optimized Synthesis of Deoxynivalenol-15-β,D-glycosides*"; 38th Mycotoxin Workshop, Berlin, Germany

2017 J. Weber, D. Svatunek, G. Tegl, S. Krauter, C. Hametner, P. Kosma, J. Fröhlich, H. Mikula: "*2-O-Benzyloxycarbonyl Protected Thioglucosides: A Revival Towards Diastereoselective Glycosylation*"; 19th European Carbohydrate Symposium, Barcelona, Spain

PROFESSIONAL EXPERIENCE

Since Sept. 2013 **Research Assistant** ('*Projektassistentin'*), TU Wien

TEACHING/MENTORING EXPERIENCE

2013 **Student Assistant**
TU Wien, Institute of Applied Synthetic Chemistry; Main activities and responsibilities: Instructor in the organic chemistry laboratory course

Since 2014 **University Assistant**
TU Wien, Institute of Applied Synthetic Chemistry; Main activities and responsibilities: Supervision of laboratory courses and Bachelor's theses

PROFESSIONAL TRAINING

September, 2012	Summer school „**1D- and 2D-NMR Spectroscopy in Liquids**“, Austria
September, 2014	Summer school „**Chromatography and Coupling to Mass Spectrometry**“, Austria

GRANTS, FELLOWSHIPS & AWARDS

October, 2012	**Scholarship for Scientific Work**, Austrian Federal Ministry for Science and Research, Vienna
2014	**GÖCH Prize** for best diploma theses
2015	**VSS prize** for the best Scientific Speed Talk

REFERENCES

Prof. Johannes Fröhlich
Vice rector for Research, Vienna University of Technology, Vienna, Austria

Dr. Hannes Mikula
Postdoc, Vienna University of Technology, Vienna, Austria

Prof. Rudolf Krska
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