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DIPLOMARBEIT

Synthesis of two oxandrolone long-term metabolite glucuronides

ausgeführt am

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unter der Anleitung von

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ABSTRACT

Anabolic androgenic steroids are still the most prominent substance class in use as illicit doping agents. In anti-doping analysis the detection of steroid abuse can be quite challenging as steroids are extensively metabolised by the human body. In search for suitable marker molecules steroid long-term metabolites are of increasing interest due to their prolonged detection window. Their respective conjugated phase II metabolites, mostly glucuronides and sulphates, give an additional advantage over the phase I metabolites with regards to sample preparation. They can be analysed directly from urine without the need for prior sample preparation involving enzymatic hydrolysis. The aim of this work is to synthesise the β-D-glucuronides of two C17-epimeric oxandrolone long-term metabolites and to verify their presence in human urine via LC-HRMS measurements by comparing the synthesised reference material to authentic urine samples obtained from excretion studies. With an analytical technique adapted for the direct injection of urine the synthesised oxandrolone long-term metabolite glucuronides can serve as reference material in the confirmation of oxandrolone abuse.

DEUTSCHE KURZFASSUNG

Anabole androgene Steroide bilden noch immer die Substanzgruppe, die am häufigsten als illegale Dopingmittel in Verwendung ist. In der Anti-Doping Analytik kann die Detektion von Steroidmissbrauch herausfordernd sein, weil Steroide sehr vielfältig im menschlichen Körper metabolisiert werden. Auf der Suche nach geeigneten Marker-Molekülen sind die Langzeitmetabolite der Steroide von großer Bedeutung wegen ihres verlängerten Detektionsfensters. Hier haben besonders die konjugierten Phase II Metabolite, vor allem Glucuronide und Sulfate, in der Probenvorbereitung einen Vorteil gegenüber den Phase I Metaboliten. Sie können durch Direktmessung von Urinproben analysiert werden ohne vorhergehende Probenvorbereitung mit enzymatischer Hydrolyse. Das Ziel dieser Arbeit ist die Synthese der β-D-Glucuronide von zwei C17-epimeren Oxandrolon Langzeitmetaboliten und die Bestätigung ihres Vorkommens im menschlichen Urin. Dazu werden LC-HRMS Messungen des synthetisierten Referenzmaterials verglichen mit authentischen Urinproben aus Ausscheidungsversuchen. Mit einer adaptierten analytischen Methode, die geeignet ist für die direkte Injektion von Urin, können die synthetisierten Oxandrolon Langzeitmetabolit-Glucuronide als Referenzmaterial in der Bestätigung von Oxandrolon Missbrauch verwendet werden.

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1 GENERAL PART

1.1 OXANDROLONE – A DOPING AGENT

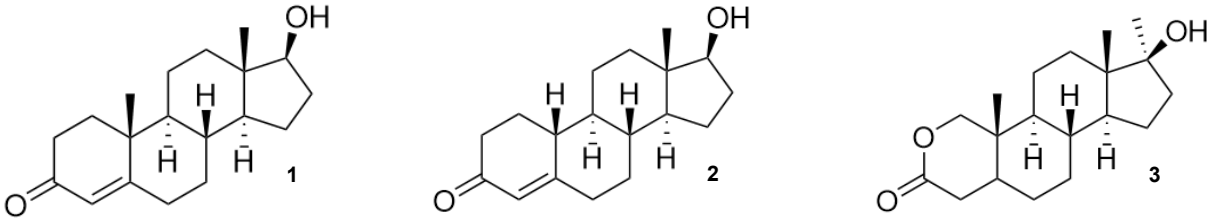
Anabolic androgenic steroids (AAS) are derivatives of the primary male sexual hormone testosterone that have a history of illicit use as doping agents since the 1950s because of their skeletal muscle building properties.[1,2] In 1935 testosterone was isolated from a natural source (testis of bulls) by David et al.[3] and independently synthesised by Butenandt and Hanisch[4] and Ruzicka et al.[5] leading to extended synthetic efforts to produce derivatives of this sexual hormone and separating the anabolic effect from the less favourable androgenic.[2] Nandrolone, the 19-nor testosterone, which also occurs endogenously, was the first testosterone derivative where this separation was found to be successful enough for it to be introduced into practice and administered to weightlifters.[6] It was also found that the oral application of testosterone showed no effect as it is rapidly metabolised in the liver (first pass effect).[7] Therefore, the efforts to synthesise testosterone derivatives aims at installing a 17α-methyl group and A-ring modifications to slow the metabolic rate and increase anabolic properties.[1]

In 1962 oxandrolone (17β-hydroxy-17α-methyl-2-oxaandrostan-3-one) was first synthesised by Pappo et al.[8] as part of their efforts to synthesise 2-oxa derivatives of testosterone [Figure 1]. Oxandrolone was found to be more active as anabolic agent with decreased androgenic properties than the corresponding 3-ketone without the lactone functionality in the A-ring.[8] As oxandrolone is also a 17-methyl derivative of testosterone it can be administered orally without undergoing first pass metabolism ensuring a prolonged biological activity.[1] In 1964 it was launched on the US market under the name of Anavar® and prescribed in the treatment of amyotrophia and microsomia, amongst others symptoms of Turner syndrome.[9]

In 1974 the International Olympic Committee (IOC) banned the use of anabolic androgenic steroids in sports and since 1976 analytical testing of human urine by gas chromatography coupled with mass spectrometry has been implemented for the detection of anabolic androgenic steroid abuse.[10]

In their role to coordinate international anti-doping efforts the World Anti-Doping Agency (WADA) publishes the Prohibited List as part of the World Anti-Doping Code where oxandrolone is listed among 43 other exogenous anabolic androgenic steroids that are prohibited at all times (in and out of competition).[11,12] In 2017 according to the anti-doping testing figures report[13] anabolic androgenic steroids made up 44 % of adverse analytical findings (i.e. positive test results) of all WADA accredited laboratories [Figure 2]. In order to prolongate the detection window of anabolic

androgenic steroids their respective urinary phase I and phase II metabolites are of increasing interest.[14]



testosterone nandrolone oxandrolone

Figure 1: testosterone and nandrolone, two endogenous anabolic androgenic steroids and oxandrolone, an exogenous anabolic androgenic steroid

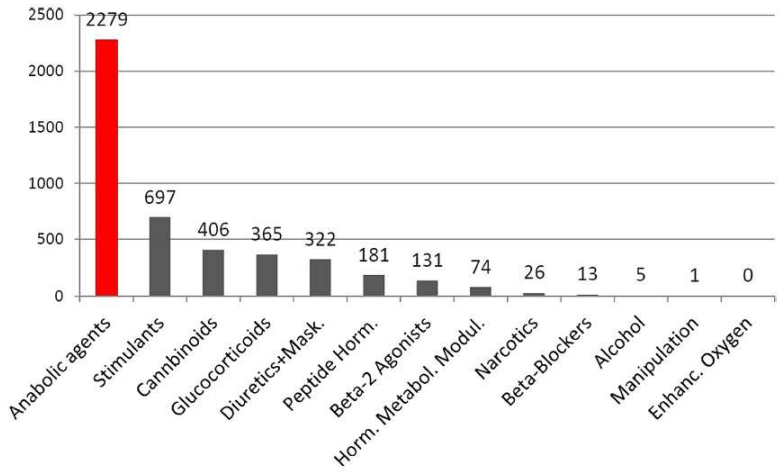


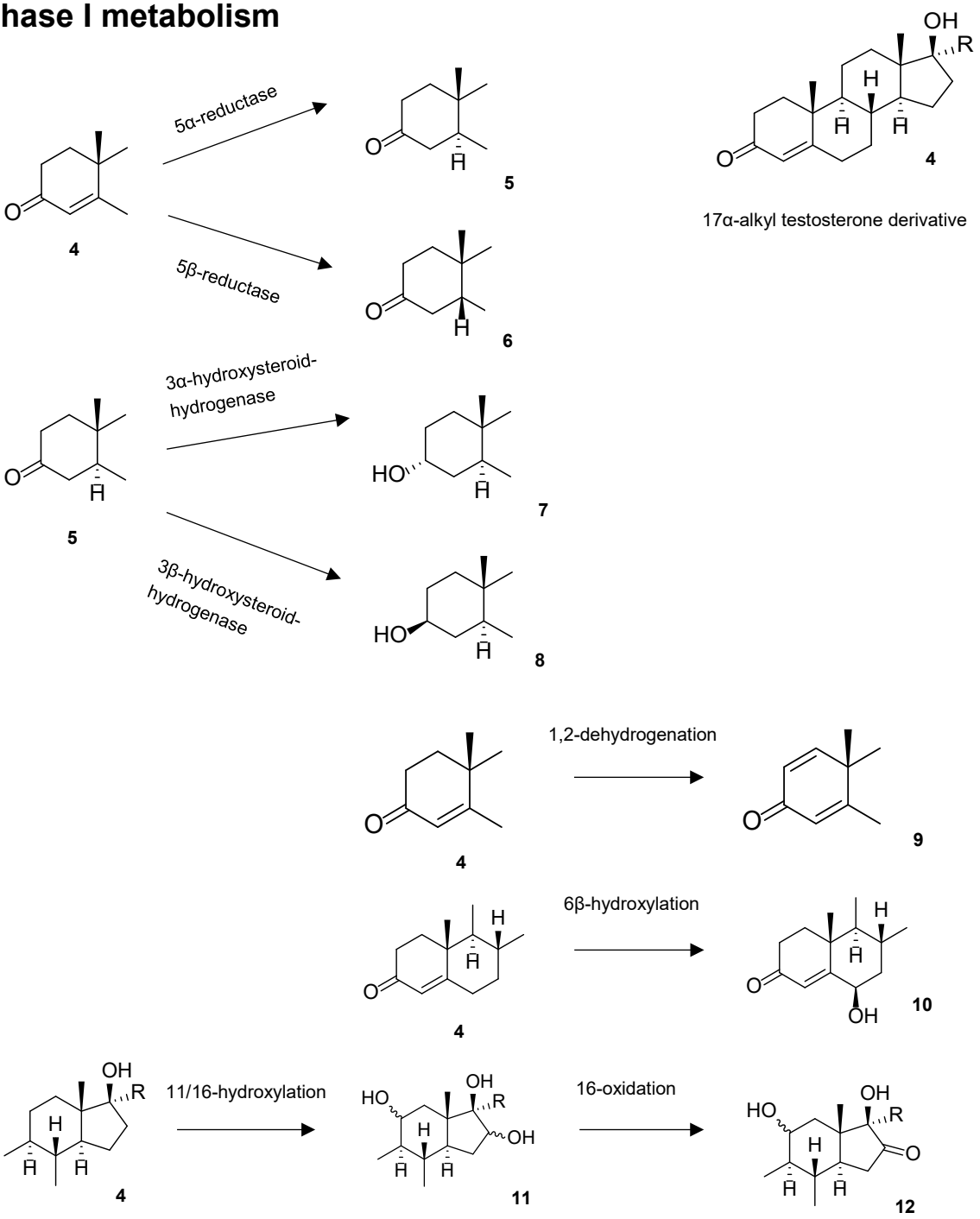
Figure 2: anti-doping testing figures report; anabolic agents make up 44% of adverse analytical findings.[14]

1.2 THE METABOLISM OF OXANDROLONE

General metabolism of AAS and determination of new metabolites

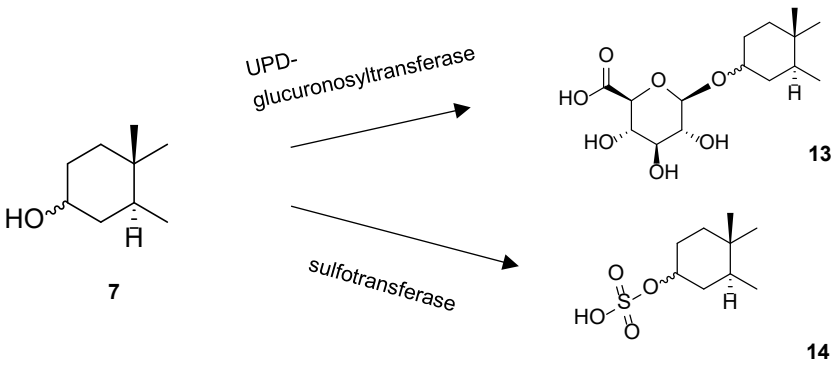
Anabolic androgenic steroids undergo heavy metabolism in the human body.[15] The metabolic pathway for testosterone was thoroughly studied in animal models and human clinical studies and serves as a model for the study of the metabolism of synthetic anabolic androgenic steroids.[15] The first phase (phase I metabolism) includes enzymatically catalysed reduction, hydroxylation and oxidation reactions that can occur on all four rings of the steroid molecule generally leading to the inactivation of the drug [Scheme 1].[15] Metabolic modification of the A- and D-ring are more common than of the B- and C-ring.[15] The second phase (phase II metabolism) involves enzymatic conjugation reactions with glucuronic acid and sulphates leading to compounds that are more polar than the parent drug and more readily excreted via urine [Scheme 2].[15]

phase I metabolism



Scheme 1: selected reactions occurring in phase I metabolism of a model 17α-alkyl-testosterone derivative[15]

phase II metabolism

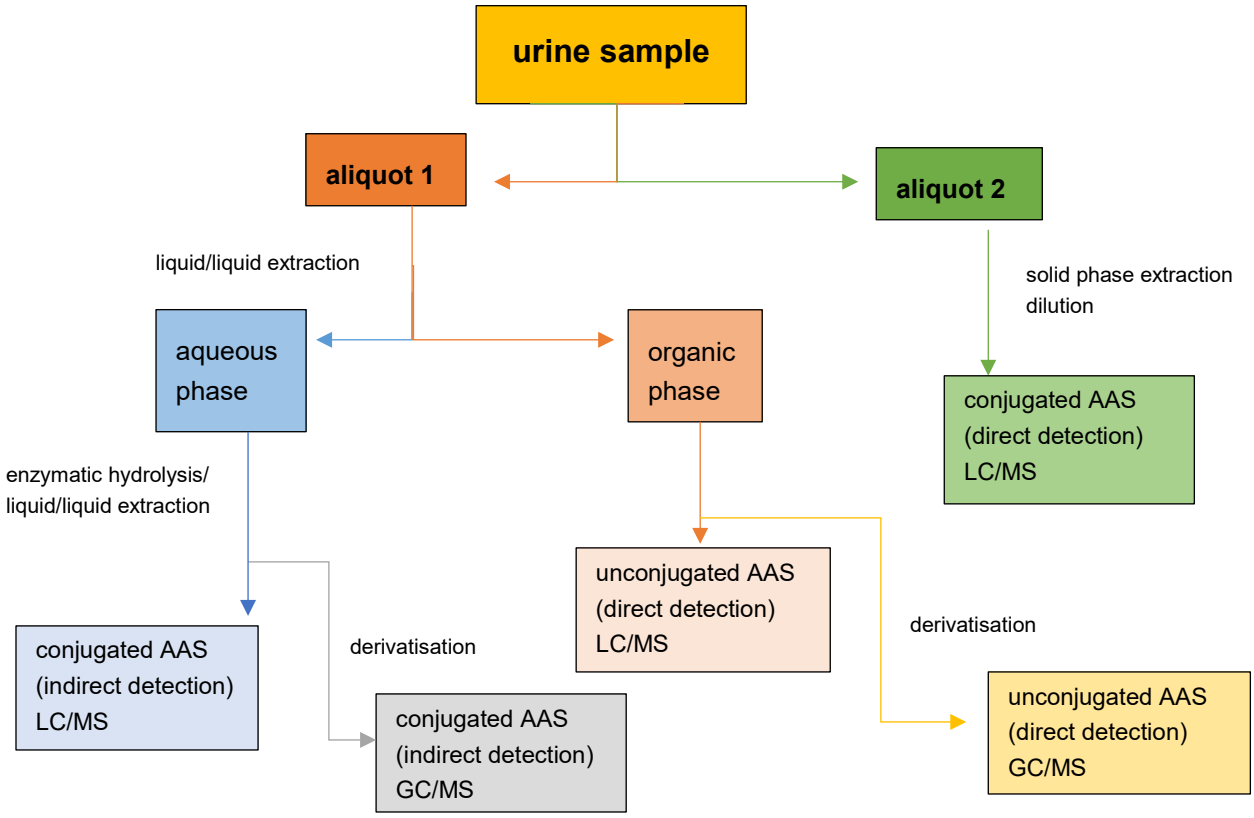


Scheme 2: conjugation reactions with glucuronic acid and sulphate as most common phase II metabolic reactions[15]

Anti-doping laboratories are required to not only monitor the parent compound but also identify and detect the respective metabolites.[15,16] The trend is finding metabolites that are excreted in urine for longer times after administration (long-term metabolites).[15,16] The general strategy for finding and determining new metabolites of anabolic androgenic steroids includes the following steps: comprehensively analysing positive urine samples (e.g. post administration urine or routine doping control samples) by GC-MS(/MS) and LC-MS(/MS) [Scheme 3], postulating a structure for the new metabolite with information from mass spectra and synthesising all possible stereoisomers of the postulated metabolite as there is no stereochemical information available from mass spectra.[16,17] Comparing the synthesised material with authentic urine samples verifies the presence of the metabolite. There the retention time and the relative abundance of the fragment ions in MS/MS experiments have to be identical.[16,17]

The behaviour of AAS in GC-MS analysis employing electron ionisation (EI) including the most common fragmentation pathways has been thoroughly studied in the past 50 years.[15,16] Nevertheless GC-MS reaches its limitations with polar compounds as the free hydroxy groups have to be converted into their respective silyl ethers to ensure volatility.[16] LC-MS methods have therefore become increasingly important in the detection of phase I and phase II metabolites.[18] The major disadvantage of this method is the poor ionisation efficiency of AAS in electro spray ionisation (ESI).[16] The ionic sulphate group and the polar glucuronic acid group of conjugated metabolites improve ionisation via ESI and coupled with high resolution mass spectrometers ensure enough sensitivity for the detection of phase II metabolites.[19] Another

advantage of the detection of conjugated metabolites is the possibility to measure a urine sample directly without the need for prior enzymatic hydrolysis, extraction and derivatisation procedures that were necessary for GC-MS measurements.[20] Hydrolysis involves the incubation of urine samples with ß-glucuronidase from *Escherichia coli* at elevated temperatures (50 °C).[21] Other urine components and bacteria can inhibit the enzymatic hydrolysis or lead to degradation of the analytes giving inaccurate results.[22] The possibility to analyse phase II metabolites directly from urine saves a time consuming and expensive sample preparation.



Scheme 3: comprehensive analysis of urine samples via GC/LC-MS[16]

Phase I metabolism of oxandrolone

In 1989 Massé et al. published their GC-MS investigations on the excretion of oxandrolone in urine.[23,24] Oxandrolone is mainly excreted in unconjugated form as the parent drug and as the respective 17-epimer (17-epioxandrolone).[23,24] Schaenzer et al. found that 1.9 % of orally administered oxandrolone is excreted as 17-epioxandrolone.[1]

The first 17-epimer of an anabolic steroid found in human urine was 17-epimetandienone with elucidation of the structure and synthesis of the reference compound by MacDonald et al.[25] The formation of 17-epimetandienone is suggested to go via the 17β-sulphate conjugate, a metabolic pathway first described by Edlund

et al.[26] They found that the hydrolysis of metandienone-17-sulphate gave 17-epimetandienone.[26] In their LC-MS investigations of horse urine it was found that the 17β-sulphate conjugate was the source of 17-epimetandienone, but up to this point it is still not evident that this sulphate is also formed in the human body or if the epimer arises from a different conjugate.[26]

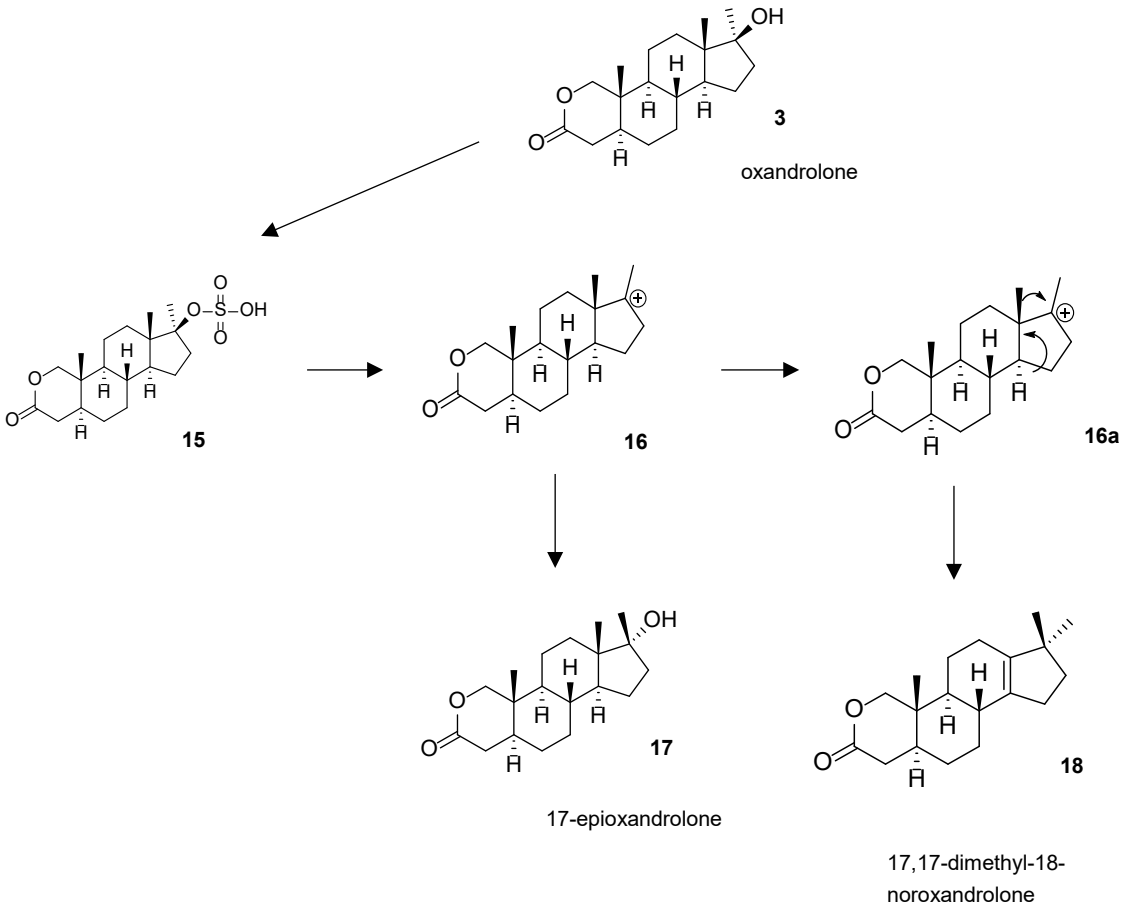
The formation of 17-epioxandrolone involves the decomposition of the sulphate in the urinary aqueous phase leading to a carbocation intermediate that further undergoes hydroxylation to give 17-epioxandrolone and rearrangement to give 17,17-dimethyl-18-noroxandrolone [Scheme 4].[1,15]

The detection window for oxandrolone and 17-epioxandrolone is reported to be around 3 days after a single oral dose of 5 mg of oxandrolone.[27] The detection window for 17,17-dimethyl-18-noroxandrolone is reportedly twice as long (6 days), still there is a need for finding and determining possible long-term metabolites to prolong detection periods and increase the effectiveness of anti-doping analysis.[27]

In 2006 the mass spectrometric detection of a novel metandienone metabolite (Nightwatch metabolite) with a 17-hydroxymethyl-17-methyl-18-norandrost-13-ene moiety in the D-ring gave rise to a significant increase of adverse analytical findings for metandienone.[28] Following this discovery other 17-methyl steroids were investigated for a similar metabolic modification of the D-ring. For oxandrolone it was found by Parr et al. that steroid **19** could be detected in traces after oxandrolone application [Scheme 5]. The formation is proposed to go via hydroxylation of 17,17-dimethyl-18-noroxandrolone by cytochrome P450 enzymes (CYP21, CYP3A4) or by hydroxylation of the 18-methyl group and subsequent Wagner-Meerwein rearrangement [Scheme 5].[29]

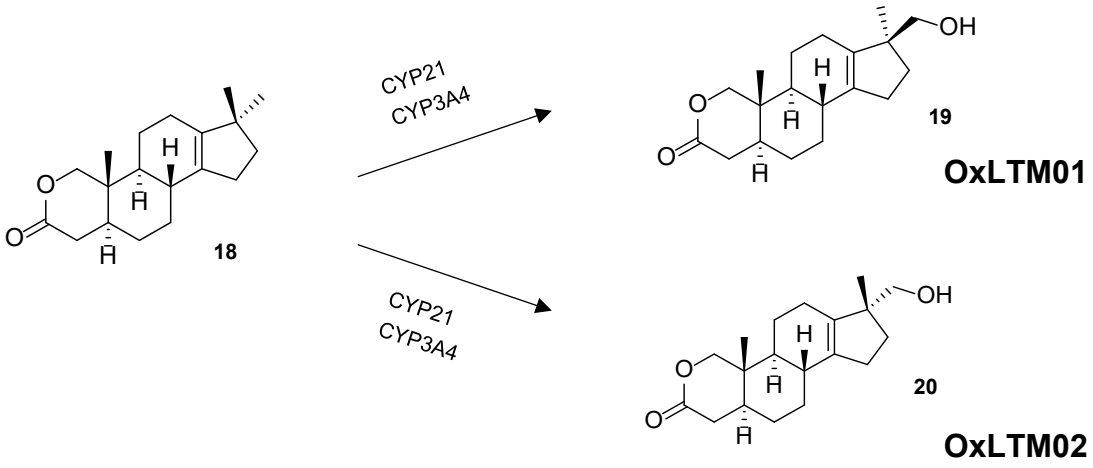
Guddat et al. synthesised 17β-hydroxymethyl-17α-methyl-18-nor-2-oxa-5α-androsta-13-en-3-one (OxLTM01) and its epimer 17α-hydroxymethyl-17β-methyl-18-nor-2-oxa-5α-androsta-13-en-3-one (OxLTM02) using a fungal in vitro system (*Cunninghamella elegans)*.[27] They characterised these newly found oxandrolone metabolites and used them as reference material in the analysis of urine samples.[27] Both epimers are found to be metabolites of oxandrolone formed by the human body.[27] After a single oral dose of 5 mg oxandrolone it was possible to detect OxLTM02 up to 15 days after administration, OxLTM01 was still found after 18 days.[27] Kratena et al. published the first fully synthetic way to obtain these two metabolites in 2017.[30]

formation of 17-epioxandrolone and 17,17-dimethyl-18-noroxandrolone



Scheme 4: phase I metabolism of oxandrolone. Formation of 17-epioxandrolone and 17,17-dimethyl-18-noroxandrolone[15,29]

formation of OxLTM01 and OxLTM02 via hydroxylation of 17,17-dimethyl-18-noroxandrolone



Scheme 5: phase I metabolism of oxandrolone. Formation of two oxandrolone long-term metabolites via enzymatic hydroxylation of 17,17-dimethyl-18-noroxandrolone [29]

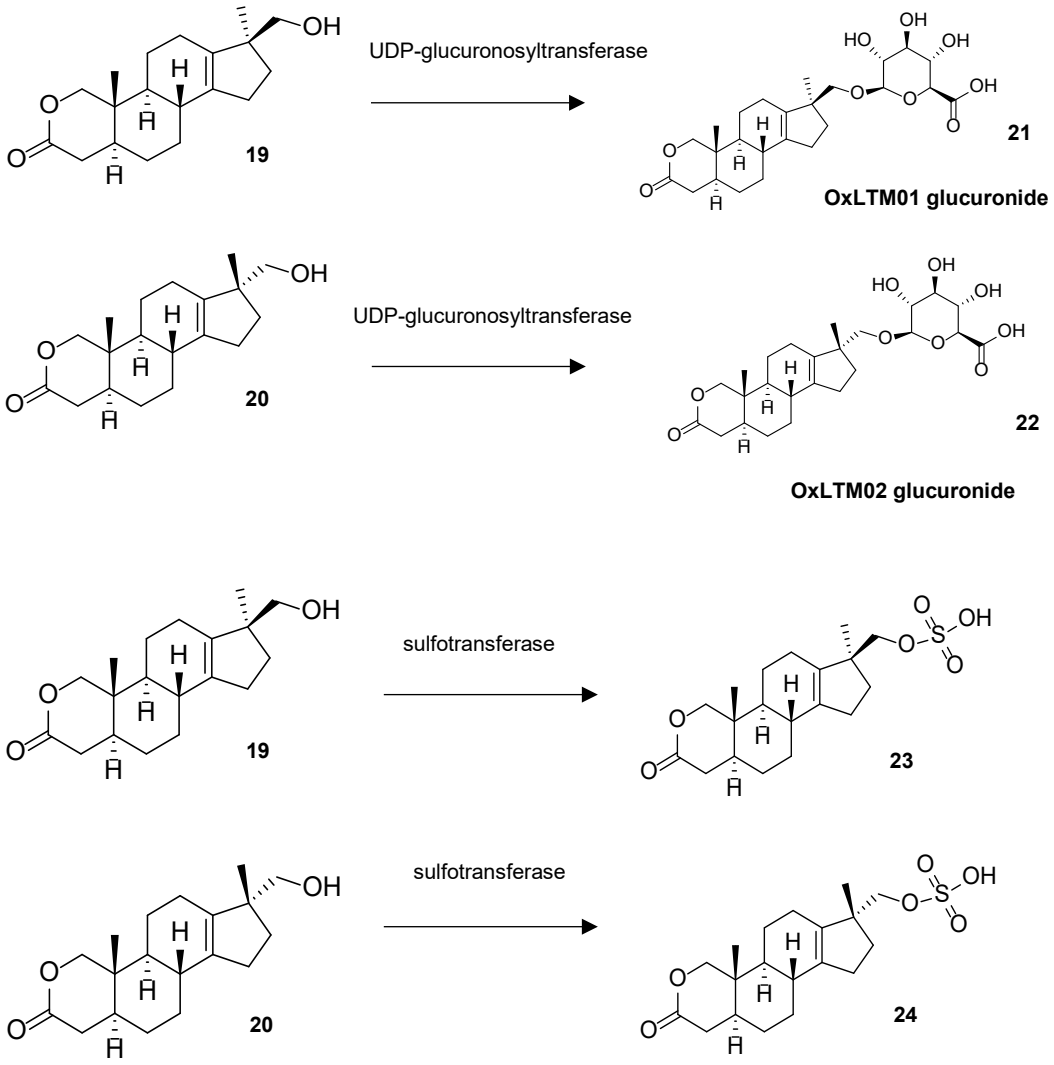
Phase II metabolism of oxandrolone

Conjugates of oxandrolone and 17-epioxandrolone with glucuronic acid have not been reported. The tertiary hydroxy group was believed to be too sterically hindered for the formation of glucuronides.[15] When Parr et al. discovered the oxandrolone long-term metabolites (OxLTM01 and OxLTM02) they reported that these metabolites occurred in the conjugate fraction of the urine.[29] To detect OxLTM01 and OxLTM02 in unconjugated form urine samples have been subjected to hydrolysis by β-glucuronidase.[29] As this enzymatic hydrolysis gave unconjugated OxLTM01 and OxLTM02 it can be assumed that these metabolites occur as β-glucuronides.[29] Up to this point no analytical data and no reference material exists for these OxLTM-β-D-glucuronides [Scheme 6].

Rzeppa et al. reported the detection via HPLC-MS/MS of the oxandrolone long-term metabolites OxLTM01 and OxLTM02 as sulphate conjugates.[31] The conjugated metabolites were isolated from urine by preparative HPLC and subjected to solvolysis with sulphuric acid in methanol and incubation at 55 °C for 2 hours.[31] Only the 17β-epimer (OxLTM01) was isolated in relevant amounts. Subsequent analysis of the unconjugated metabolite confirmed the presence of OxLTM01.[31] Up to this point there is no reference material available for these sulphate conjugates [Scheme 6].[31]

The aim of this work was therefore to synthesise the β-D-glucuronides of OxLTM01 and OxLTM02, to compare this synthetic reference material to authentic urine

samples and to confirm the presence of OxLTM01 and OxLTM02 as glucuronic acid conjugates in human urine.



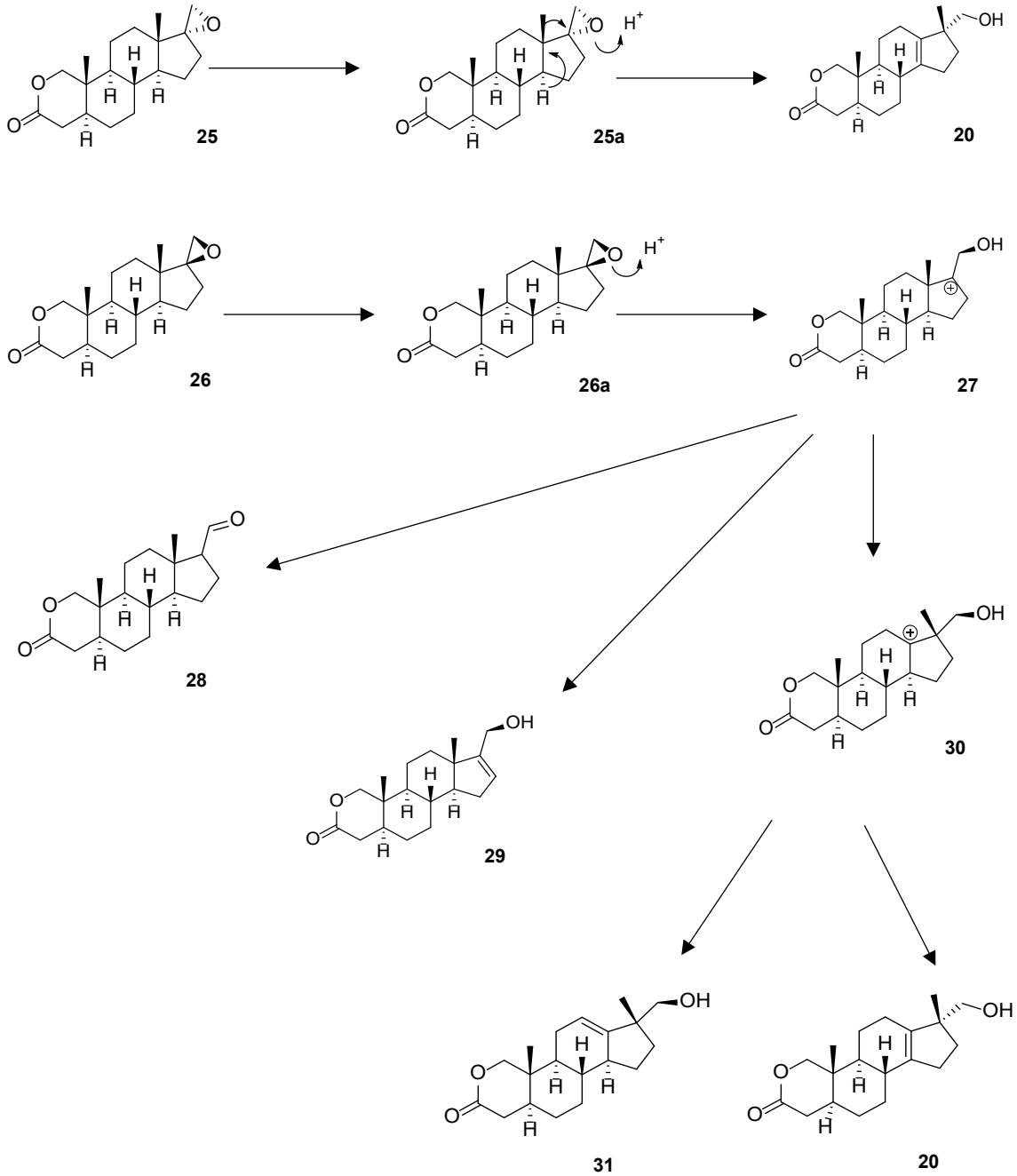
Scheme 6: phase II metabolism of oxandrolone. Conjugation reactions with glucuronic acid and sulphate[29,31]

1.3 SYNTHESIS OF OXANDROLONE LONG-TERM METABOLITES

In the first fully synthetic approach to synthesise OxLTM01 and OxLTM02 Kratena et al.[30] employed acid catalysed Wagner-Meerwein rearrangement as key step to introduce the 17-hydroxymethyl-17-methyl-18-norandrost-13-ene moiety in the D-ring.[30] As precursor a spiro-epoxide was used that was opened during rearrangement by the migrating methyl group.[30] This approach was found in retrosynthetic considerations based on the proposed mechanism for the formation of these 17-hydroxymethyl metabolites in the human body [Scheme 4].[29,30]

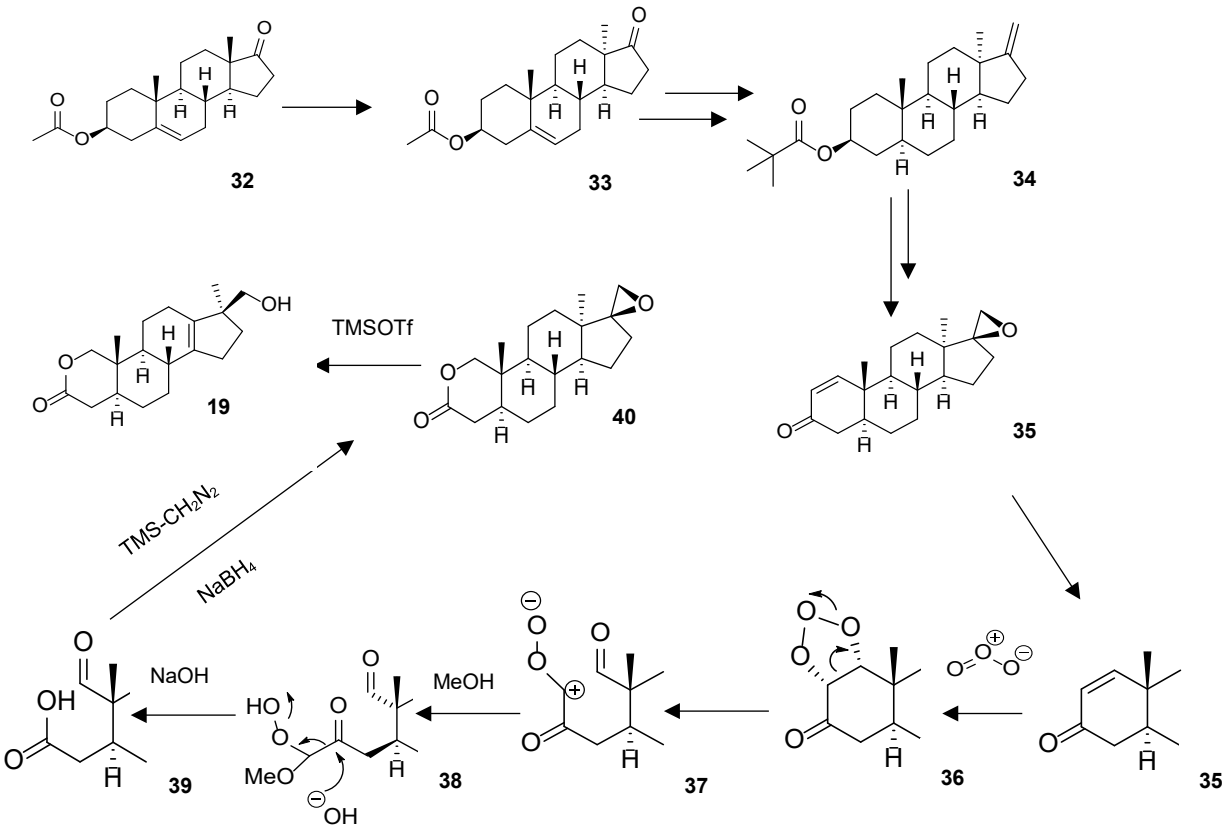
The synthesis of OxLTM02 (17α-epimer) started from oxandrolone where the 18-methyl group already is in the right configuration (β) for the stereospecific Wagner-Meerwein rearrangement and opening of the respective α-epoxide **25**.[30] The

epoxide ring opens under acidic activation and the methyl group migrates in a concerted rearrangement with higher selectivity than in the other case where both the methyl group and the epoxide are on the same face of the ring.[30] Then epoxide **26** is opened under acidic catalysis leading to the formation of a carbocation that can react in a number of ways to give isomeric products [Scheme 7].[30]



Scheme 7: mechanism of acid catalysed Wagner-Meerwein rearrangement with different reaction products depending of the configuration of the epoxide and the 18-methyl group[30]

Consequently, for the formation of OxLTM01 (17β-epimer) the 18-methyl group had to be epimerised to α-configuration to give the desired product in the Wagner-Meerwein rearrangement.[30] Therefore, the synthesis started from dehydroepiandrosterone acetate (DHEA acetate) where a literature known epimerisation procedure was applied.[32] Olefination using the Nysted reagent gave the first key intermediate **34** with an exocyclic double bond needed for the installation of the epoxide.[33] Oxidation and installation of an α,β-unsaturated ketone in the A-ring gave a second key intermediate. Epoxide **35** was subjected to an ozonolysis procedure used in the industrial synthesis of oxandrolone.[34] The ozonolysis mechanism of α,β-unsaturated carbonyl compounds in protic solvents was studied by Yamamoto et al. and suggested to go via a carbonyl oxide **37** (formed after the decomposition of the primary ozonide **36**) which is attacked by methanol to give the hydroperoxide **38** [Scheme 8].[35] This decomposes to methyl formate and the respective seco-acid by adding aqueous sodium hydroxide.[36] Formation of the methyl ester and lactonisation under reductive conditions gave the third key intermediate **40**. The Wagner-Meerwein rearrangement could be conducted under mild Lewis acid catalysis (TMSOTf).[30]



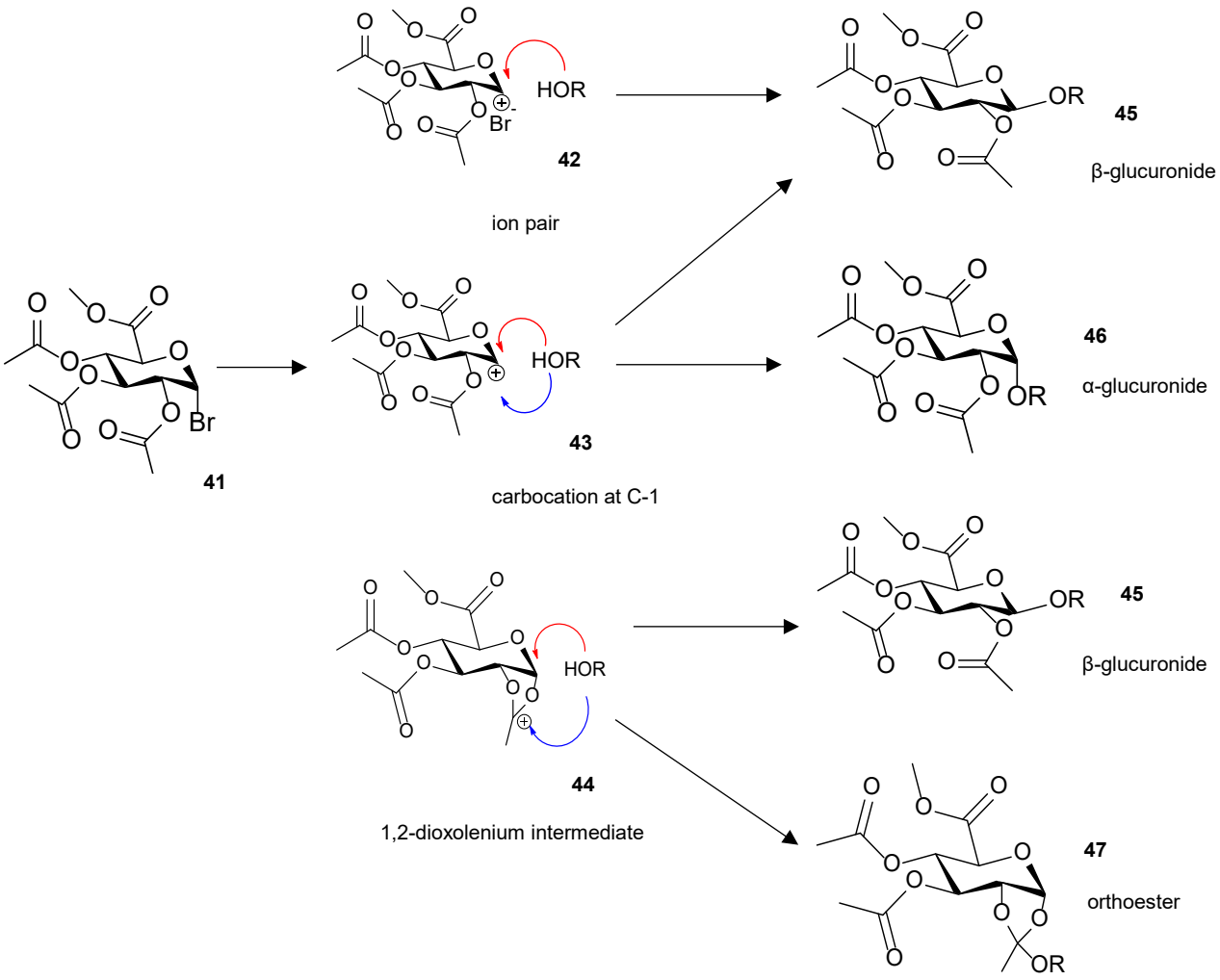
Scheme 8: synthetic route to the formation of OxLTM01 starting from DHEA acetate (with key intermediates and ozonolysis mechanism)[30,35,36]

1.4 SYNTHESIS OF GLYCOSIDES

Koenigs-Knorr glycosylation

In a classic glycosylation procedure, the Koenigs-Knorr method, an aglycon with a free hydroxy group is reacted with a glycosyl donor that is substituted in the anomeric position by a halide.[37] In the glycosylation reaction the halide leaving group on the anomeric carbon has to be activated by heavy metal salts to enable the formation of a new glycosidic bond.[37] To ensure the selective formation of a glycosidic bond to the anomeric carbon the other hydroxy groups of the sugar donor are protected as mostly esters, ethers and acetals.[37]

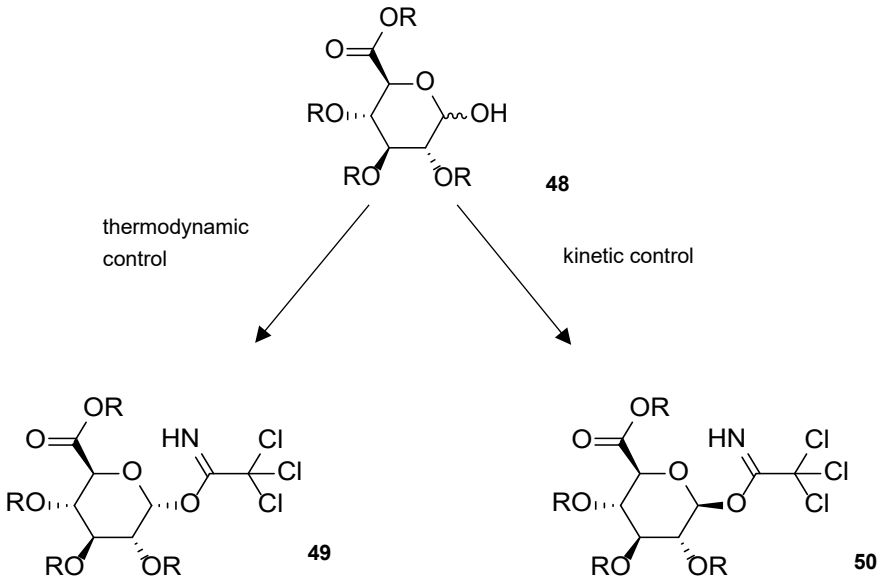
In the synthesis of steroid glycosides, the β-glycosides are generally of more interest than the α-anomers as most naturally occurring steroid glycosides display β-configuration.[29] For the stereoselective formation of a β-glycosidic bond the anchimeric effect of neighbouring acyl groups has been intensely studied.[38,39] Reactions of 1,2-cis-glucopyranosyl halides especially with a C-2 O-acetyl protecting group have proven to selectively occur with inversion of the configuration at C-1 while ether protecting groups that do not show an anchimeric effect gave a mixture of α- and β-glycosides.[38,39] Isbell et al.[38] postulated a S_N2 like mechanism and formation of a tight ion pair **42** as reason for this stereoselectivity where the anion shields the α-side from a nucleophilic attack [Scheme 9]. Hough et al.[40] suggest a S_N1 like mechanism where after the cleavage of the halide leaving group the carbocation **43** is formed. This can be either attacked by the aglycon from both sides to give a glycoside product or stabilised by the formation of a 1,2-dioxolenium ion **44** with contribution from the carboxylic ester protecting group [Scheme 9].[40] The cation is not only stabilised but the reacting nucleophile has to attack from the opposite side leading to the formation of a β-glycoside in case it attacks the C-1 carbon.[40] Within this reaction mechanism the possibility of the formation of an orthoester **47** is also possible [Scheme 9].[39] Wallace reported the formation of such orthoesters as intermediates in glycoside formation.[39] The orthoester appeared to be unstable when $Hg(CN)_2$ was used as activating species due to the formation of HBr and/or HCN that catalysed the reaction of the orthoester intermediate with the nucleophile to give the β-glycoside.[39]



Scheme 9: possible products of a Koenigs Knorr glycosylation reaction depending on the protecting groups and reaction conditions[38,39,40]

Schmidt glycosylation

In 1980 Schmidt introduced a novel glycosyl donor, an O-glycosyl trichloroacetimidate that arises from the reaction of a 1-OH sugar (with a variety of protecting groups attached to the other hydroxy groups) with trichloroacetonitrile under base catalysis.[41] Schmidt et al. studied a variety of bases and reaction conditions to selectively synthesise the thermodynamically more stable α-anomer **49** and the β-anomer **50** that arise from kinetic control of the reaction [Scheme 10].[42] The formation of the kinetic product is postulated to be a result of the unfavourable free orbital interactions of the β-configuration that lead to increased nucleophilicity of the anomeric oxygen.[42]



Scheme 10: synthesis of two different Schmidt donors depending on reaction control[42]

In a glycosylation reaction the activated sugar donor is reacted with an aglycon under Lewis acid catalysis.[43] Schmidt suggests two different ways to control the reaction depending on the reactivity of the donor and the acceptor molecules.[43] In the first method the catalyst is added to a mixture of donor and acceptor leading to the formation of an activated donor molecule that can react with the acceptor.[43] For this reaction to be successful the donor and acceptor have to have similar reactivities.[43] If the donor is much more reactive than the acceptor it will decompose after the addition of the catalyst before reacting with the acceptor.[43] Therefore Schmidt suggested an “inverse“ addition method where the catalyst is added to a solution of the acceptor forming a cluster to which the donor is added and activated in the vicinity of the acceptor.[43]

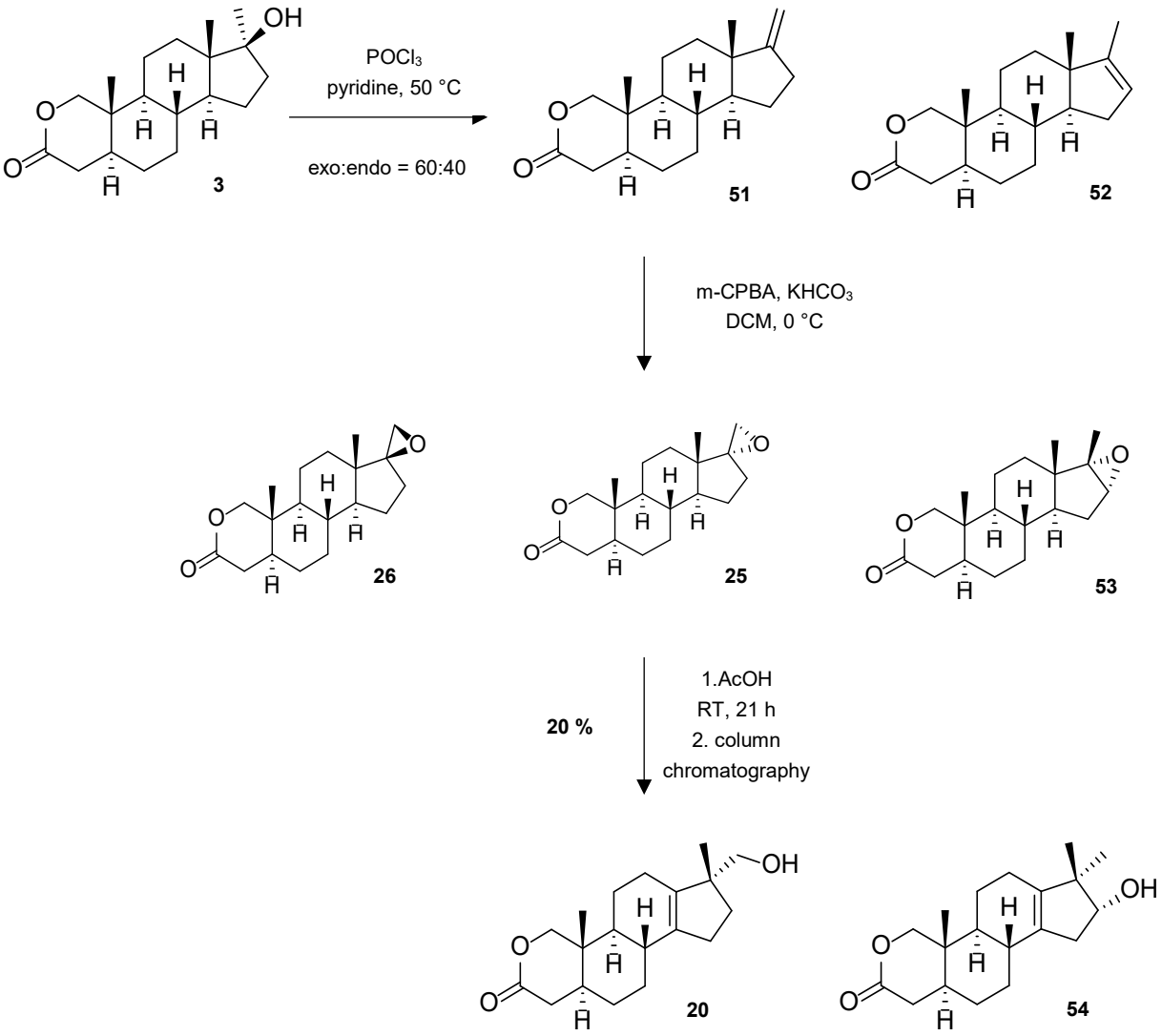
Depending on the solvent and the catalyst the stereoselectivity of the glycosylation can be influenced.[43] Schmidt found that with $BF_3{\cdot}Et_2O$ as catalyst in an apolar solvent like dichloromethane the reaction proceeds via a S_N2 type mechanism with a tight ion pair favouring the formation of the glycoside with the inverse configuration in the anomeric centre.[43] With TMSOTf the reaction follows more a S_N1 type mechanism with a carbocation intermediate that can be either stabilised by a neighbouring acyl group or give rise to the glycoside with retention of the configuration in the anomeric centre.[43] Orthoester formation is found to be reduced due to the acidic reaction conditions.[44] However, especially for acetyl protecting groups transacetylation where an acetyl protecting group is transferred to the aglycon is reported.[45] This side reaction is found to be less prominent with sterically more hindered protecting groups like pivaloyl or isobutyryl groups.[46]

2 SPECIAL PART

2.1 SYNTHESIS OF OXANDROLONE LONG-TERM METABOLITES

Synthesis of OxLTM02

The synthesis of OxLTM02 followed a previously published procedure [Scheme 11].[30]



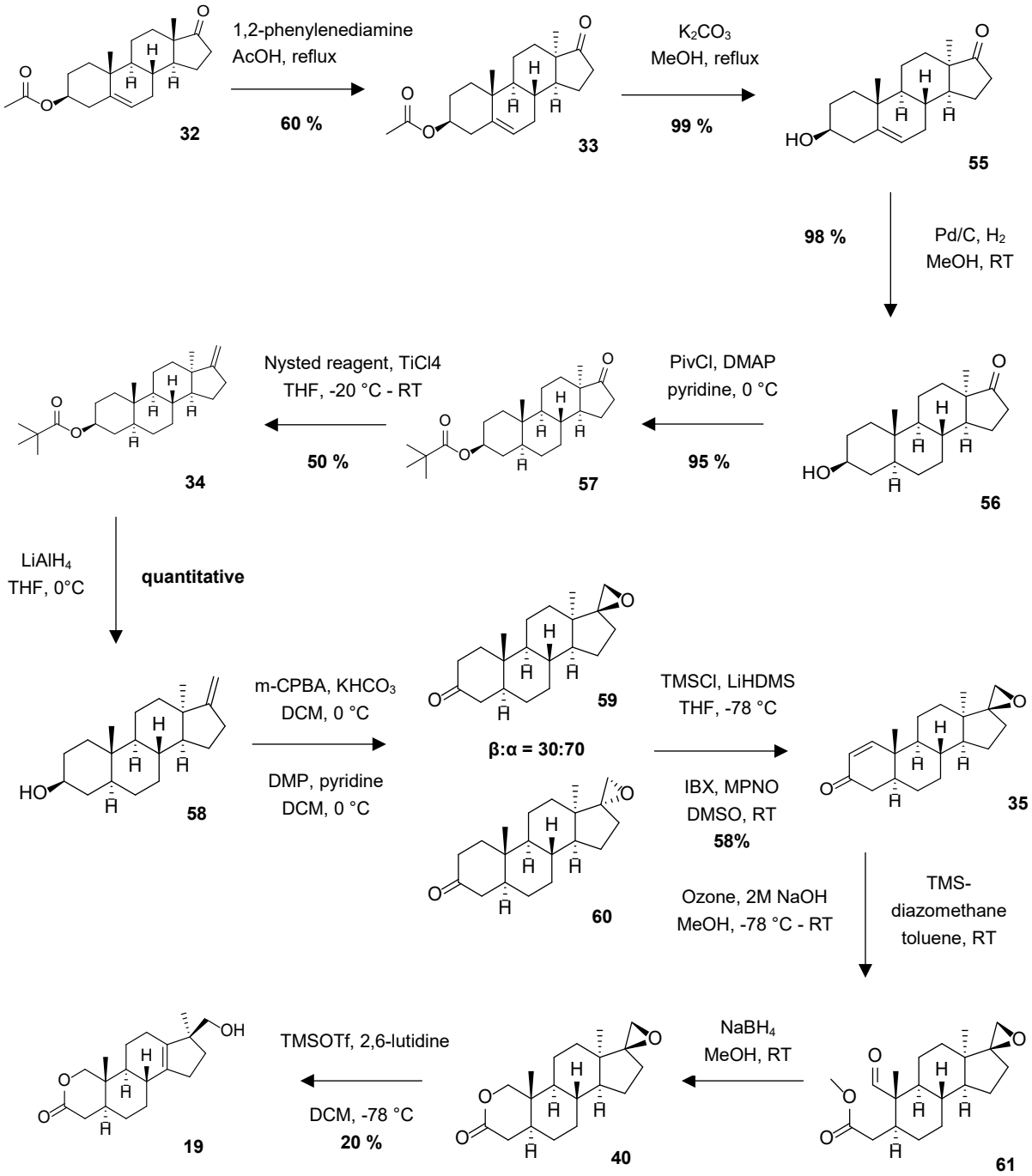
Scheme 11: synthetic route to OxLTM02 starting from oxandrolone[30]

Oxandrolone was isolated from tablets and in the first step dehydrated with $POCl_3$ in pyridine to give an approximately 60:40 mixture of compounds **51** and **52** (estimated from ^{1}H-NMR). Martin sulfurane was used as another dehydrating agent resulting in a slightly better ratio in favour of the wanted exocyclic isomer (exo:endo = 70:30 from ^{1}H-NMR). As these isomers were not separable by column chromatography the mixture was subjected to an epoxidation procedure using meta-

chloroperoxybenzoic acid and potassium carbonate.[47] The resulting mixture of epoxides was subjected to acid catalysed Wagner-Meerwein rearrangement. With TMSOTf and 2,6-lutidine, conditions that were successful in previous synthesis of 17-hydroxymethyl steroids[47], 60 % of the starting material decomposed and consequently the rearrangement was carried out in acetic acid as previously outlined.[30] OxLTM02 was synthesised in 20 % overall yield.

Synthesis of OxLTM01

The synthesis of OxLTM01 followed previously published procedures [Scheme 12].[30,48]



Scheme 12:synthetic route to OxLTM01 starting from dehydroepiandrosterone acetate[30,48]

To meet the stereochemical requirements of the Wagner-Meerwein rearrangement the 18-methyl group had to be epimerised for the synthesis of OxLTM01. As there are not many literature known procedures to get to a 13α-steroid, this synthesis sequence started from dehydroepiandrosterone acetate where a known epimerisation procedure was applied.[32] The reaction is suggested to go via an ion-radical mechanism and to reach equilibrium at 85 % formed epimer **33**.[32] Depending on the strategy to separate the epimer from the starting material varying yields of

product could be isolated. Column chromatography and recrystallisation from n-hexane gave 60 % yield while recrystallisation from diisopropyl ether gave only 40 % yield.

After cleavage of the acetate protecting group with potassium carbonate in refluxing methanol hydrogenation on palladium activated charcoal in hydrogen atmosphere was carried out to selectively give compound **56**.

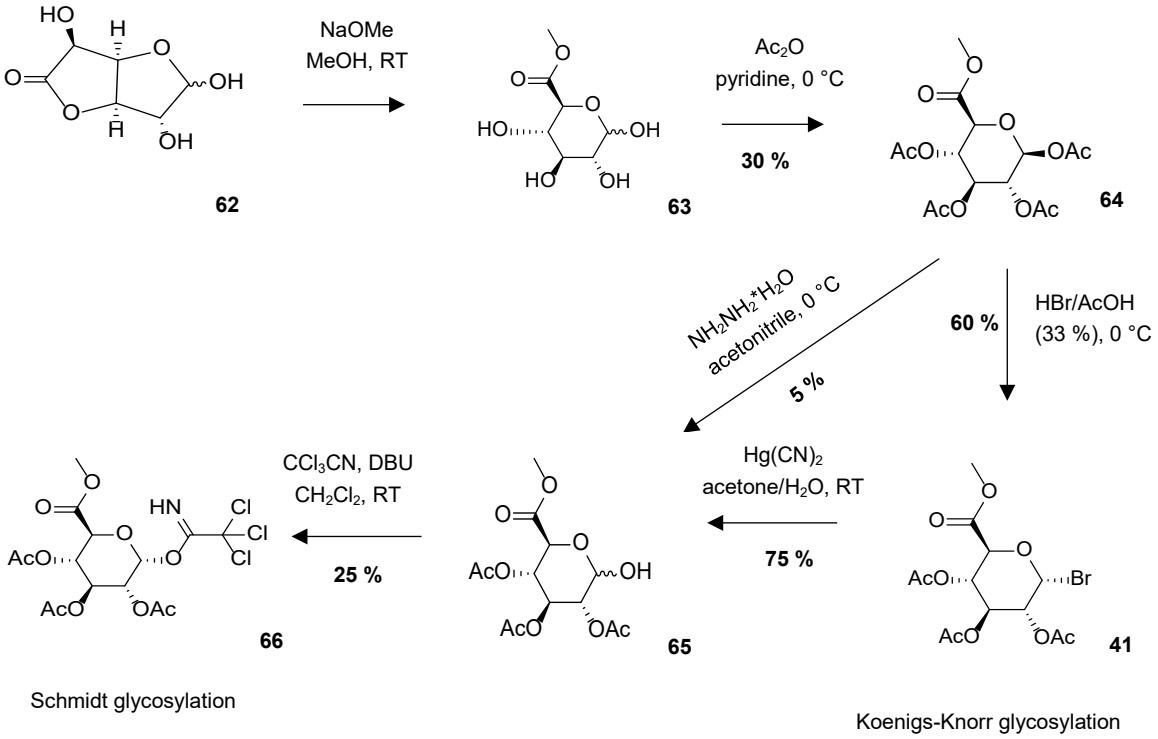
Installation of a pivaloyl protecting group was necessary before the methylenation with the Nysted reagent. In previous work it was outlined that other olefination reagents (Peterson, Tebbe) were unsuccessful, because of the sterical hindered 17-carbonyl group arising from the cis-configuration of the C and D ring.[48] The Nysted reagent was prepared in situ in tetrahydrofuran using zinc powder (10 µm), catalytic amounts of $PbCl_2$, and $TiCl_4$ in dibromomethane. The reaction gave mainly the desired olefin **34** and the product was isolated in 50 % yield.

Cleavage of the pivaloyl protecting group under reducing conditions and subsequent epoxidation of the exocyclic double bond and oxidation of the C-3 hydroxy group with Dess Martin periodinane gave a 3:1 mixture of epoxides **59** and **60**. The β-epoxide is the stereoisomer with the right configuration for the Wagner-Meerwein rearrangement and was therefore used for the following reactions. Compound **35** was prepared from the 2,3-TMS-enolate that was treated with IBX and 4-methoxypyridine-N-oxide in 58 % yield. The subsequent ozonolysis followed an industrial procedure for the synthesis of oxandrolone.[34] To neutralise the added sodium hydroxide an equimolar amount of acetic acid was added and pH was controlled not to drop below 7 because of the instability of the epoxide moiety under acidic conditions. The reaction mixture was extensively extracted with dichloromethane and the solvent carefully removed in vacuo. ^{1}H-NMR of the crude seco-acid showed that the epoxide was still intact and the crude product was immediately converted to the corresponding methyl ester **61** with trimethylsilyl diazomethane. A side product was formed during the reaction that, with knowledge from previous synthesis, could be assigned to the over oxidised dimethyl ester.[30] This mixture was not purified but subjected to the last two steps of this synthesis sequence due to the instability of the epoxide. Under reducing conditions ($NaBH_4$) lactone formation was achieved and in the last step, the Wagner-Meerwein rearrangement, TMSOTf and 2,6-lutidine lead to the formation of the wanted oxandrolone long-term metabolite. After purification of the crude product OxLTM01 could be isolated in 20 % yield over the last 4 steps.

2.2 SYNTHESIS OF OXLTM GLUCURONIDES

Synthesis of suitable sugar donors for glucuronidation of OxLTM

Considering previously acquired knowledge on suitable donors for glucuronidation reactions on steroids (section 1.4) the literature known Schmidt glycosyl donor **66** seemed the most useful for the trichloroacetimidate method.[45,46,49,50] For applying Koenigs-Knorr conditions sugar bromide **41** was a suitable donor to start the effort to synthesise the OxLTM glucuronides.[22,51]



Scheme 13: synthetic route to sugar donors for Koenigs-Knorr and Schmidt glycosylation reactions starting from D-glucuronic acid γ-lactone[49,51,52,53]

Compound **63** can be easily obtained from the commercially available D–glucuronic acid γ-lactone via a base catalysed lactone opening in methanol. Installation of the acetate protecting groups and synthesis of sugar **64** was achieved by reacting methyl D-glucopyranuronate with acetic anhydride in pyridine.[51]

Using acetate protecting groups has the advantage of easy introduction and mild cleaving conditions over other ester groups. As the oxandrolone long-term metabolites still carry the lactone functionality in the A-ring only mild basic conditions can be applied in order to avoid lactone opening. Small scale screening experiments involving stirring OxLTM02 in methanolic potassium hydroxide at 0 °C lead to partial opening of the lactone. By adding an ion exchange resin (Amberlyst® 15 hydrogen

form) neutralisation and re-cyclisation of the six membered lactone ring could be achieved.

As the oxandrolone long-term metabolites are reported to occur as their respective β-glucuronides[29] a β-selectivity in the glycosylation reactions is wanted. As previously outlined (section 1.4) acetate protecting groups are known to favour β-configuration in glycosylation reactions because of the anchimeric effect.[38,39] This mechanism requires α-configuration in the anomeric centre.[38,39]

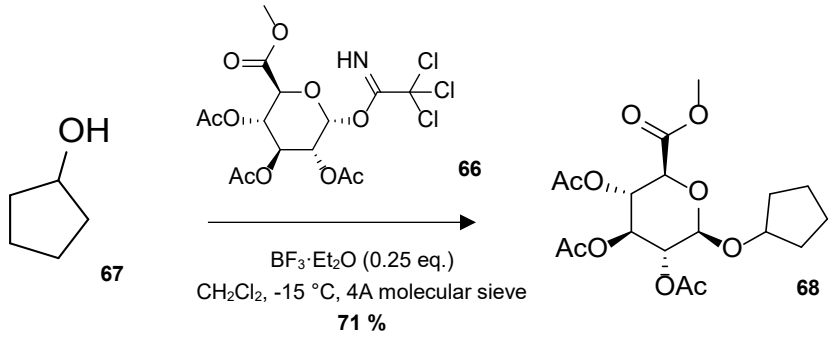
Compound **64** proved to be a useful intermediate in the synthesis of the two sugar donors of interest. Stirring in HBr (33% in acetic acid)[51] gave selectively sugar **41** after recrystallisation in pure form in 60% yield.

The acetate group on the anomeric carbon could also be selectively cleaved by hydrazine hydrate[52] giving compound **65** but only in 5 % yield. As sugar bromide **41** was available, this compound was subjected to hydrolysis with $Hg(CN)_2$ activation giving 75 % yield of the desired product.[53]

From this 1-OH sugar Schmidt glycosyl donor **66** could be selectively prepared by thermodynamically controlling the reaction with trichloroacetonitrile.[42] In previous work 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) was used as base catalyst and the reaction was stirred for 24 hours.[49] Applying these reaction conditions gave sugar **66** in 25 % yield.

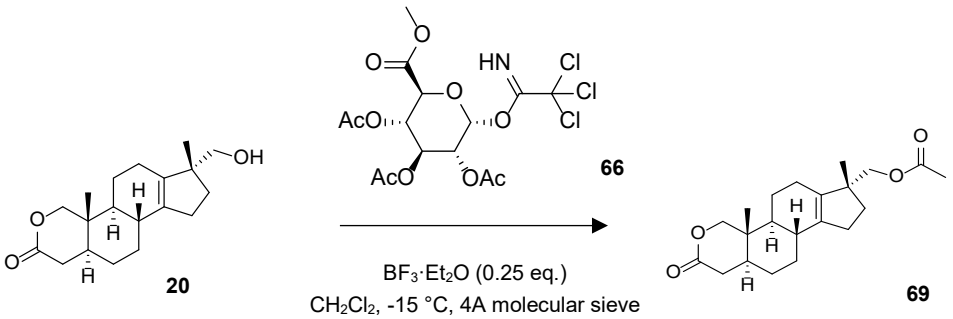
Synthesis of OxLTM glucuronides via Schmidt glycosylation

The Schmidt glycosylation conditions applied previously in the synthesis of steroid glucuronides[46,50] were used in a test reaction with cyclopentanol [Scheme 14]. The alcohol and the sugar donor molecule were dissolved in anhydrous dichloromethane and $BF_3 \cdot Et_2O$ was added in substoichiometric amounts at -15 °C. The reaction finished while warming to room temperature. After aqueous workup and purification by column chromatography glycoside **68** was isolated in pure form in 71 % yield.



Scheme 14: Schmidt glycosylation test reaction with cyclopentanol

Similar conditions were used for OxLTM02 as aglycon [Scheme 15]. The reaction yielded the transacetylation product and no detectable amount of the wanted glucuronide was formed. Instead the protons of the hydroxymethyl group coupled to a carbon with a shift of 171 ppm in ^{13}C-NMR which would fit to an acetate group. Comparison with NMR spectra of OxLTM02 acetate reference material (synthesised from OxLTM02 with acetic anhydride in pyridine) confirmed the formation of the transacetylated product **69**.



Scheme 15: attempted Schmidt glycosylation with OxLTM02 as aglycon resulted in the formation of the transacetylated compound

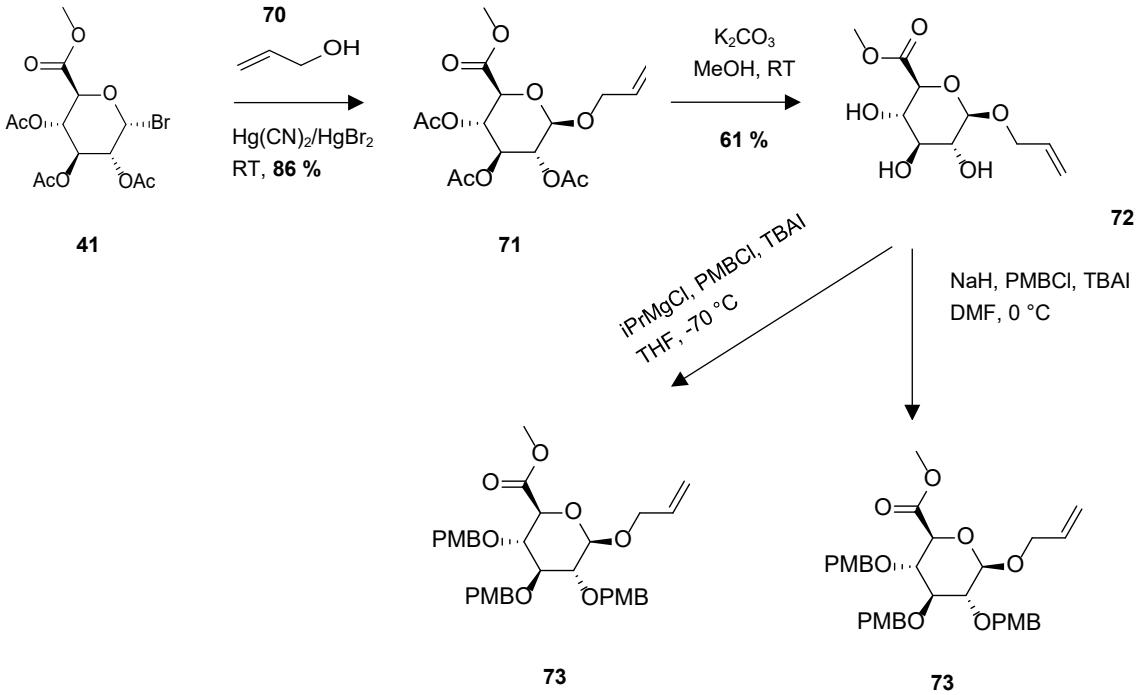
As this transacetylation is known to occur when sugar donors with acetate protecting groups are used[46,50], the inverse addition procedure was employed which was suggested by Schmidt to be helpful when reactivity of the sugar donor exceeds that of the acceptor molecule.[43] When monitoring the reaction by TLC and UPLC-MS it was found that the sugar donor reacts within minutes upon addition of the Lewis acid catalyst while most of the starting material remains unreacted. Harding et al. also found in their efforts to synthesise various steroid glucuronides that the inverse addition procedure gave by trend better yields of the glucuronides over the transacetylation products.[46]

Therefore, OxLTM02 was stirred with of $BF_3 \cdot Et_2O$ and the Schmidt sugar donor **66** was added at -15 °C. The reaction outcome did not differ from that of the previously applied order of addition. The transacetylated product **69** was formed again with no detectable amount of glucuronide. Variation of amount of catalyst (0.1, 0.25 and 0.5 equivalents $BF_3 \cdot Et_2O$) and of sugar donor (1, 1.2, 1.5 equivalents) did not result in product formation.

A different activator (TMSOTf) was also tested. Both in the normal and inverse addition procedure it resulted mostly in decomposition of the steroid starting material.

The installation of sterically more demanding ester protecting groups like pivaloyl where this transacetylation is reportedly suppressed[46] is not possible due to the lactone functionality of the oxandrolone long-term metabolites. The cleaving conditions (refluxing in base) are not compatible with this steroid.

An attempt was made to install ether protecting groups where transacetylation is not possible. As the oxandrolone long-term metabolites carry a double bond, benzyl ethers that are cleaved by palladium catalysed hydrogenation could not be installed. Instead it was attempted to install para-methoxybenzyl groups that could be cleaved by DDQ or under Birch conditions [Scheme 16].



Scheme 16: attempted synthetic route to install PMB protecting groups on the sugar donor

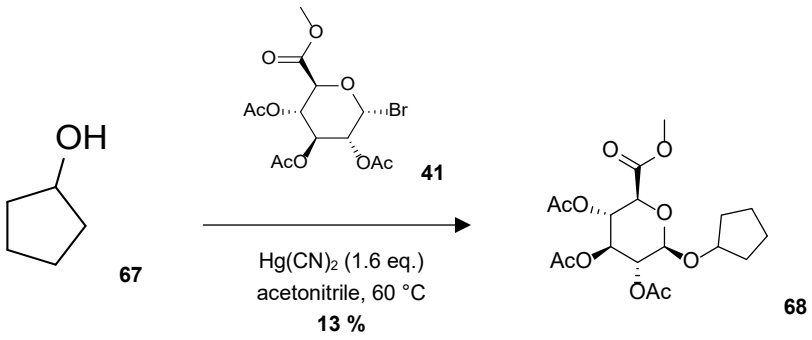
The synthesis followed a protocol applied for the installation of benzyl ether protecting groups.[54] Sugar **71** was obtained from Koenigs-Knorr glucuronidation of allylic alcohol with sugar bromide **41** using a mixed mercury activator $(Hg(CN)_2$ and

$HgBr_2$). The cleavage of the acetate protecting groups under basic conditions gave compound **72**. The three hydroxy groups were attempted to be converted to the corresponding para-methoxybenzyl ethers with para-methoxybenzyl chloride and sodium hydride [Scheme 16].[55] These conditions did not result in product formation. Also, deprotonation of the hydroxy groups with isopropyl magnesium chloride and subsequent reaction with para-methoxybenzyl chloride was unsuccessful.

Synthesis of OxLTM glucuronides via Koenigs-Knorr glycosylation

Mercury salts $Hg(CN)_2$ and $HgBr_2$ were chosen as activating species for the attempt to synthesis the OxLTM glucuronides under Koenigs Knorr conditions. They have already proven to be successful in the glucuronidation of simple alcohols like allylic alcohol where the alcohol also functioned as the solvent. A mixed activator ($Hg(CN)_2$ and $HgBr_2$) was used and reaction with sugar bromide **41** gave the desired product **71** in 86 % yield after 16 hours.

Conditions that were successful in a previous synthesis of androsterone-β-D-glucuronide were tested on cyclopentanol [Scheme 17].[22] The reaction yielded 13 % glycoside **68** after 5 h.



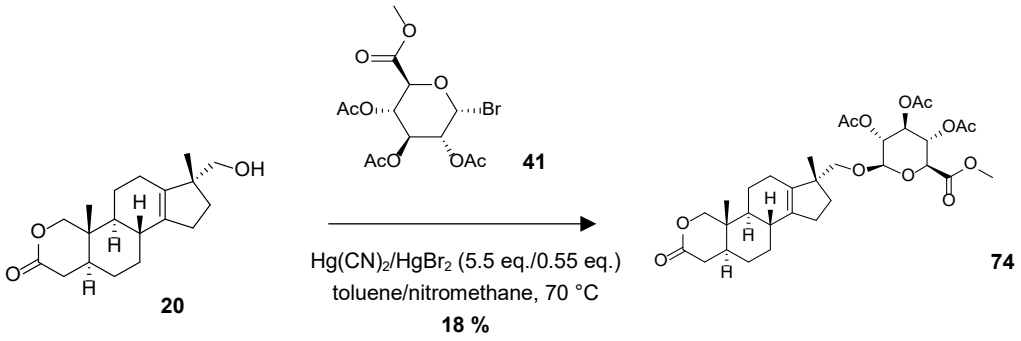
Scheme 17: Koenigs-Knorr glycosylation test reaction with cyclopentanol

In a reaction of OxLTM02 in dry acetonitrile with sugar bromide **41** and $Hg(CN)_2$ at 60 °C traces of glucuronide product could be isolated after 48 h. At lower temperatures (40 °C) no reaction occurred.

Different solvent systems were screened for their applicability in Koenigs-Knorr glycosylation (benzene, benzene/nitromethane (3:1), toluene, toluene/nitromethane (3:1), toluene/nitromethane (1:1) and toluene/acetonitrile (1:1)). The conditions were based on a glucuronidation attempt on analytical scale for the 17β-hydroxymethyl metandienone long-term metabolite (Nightwatch).[56] The reaction (1 mg OxLTM02 scale) was stirred at 75 °C for 60 hours and in all cases product formation was observed via UPLC-MS (Nexera, C18 column). Where benzene and

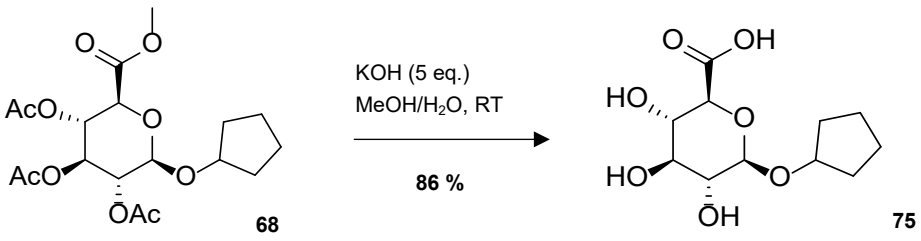
benzene/nitromethane were used as solvent starting material was still present. With $AgCO_3$ as activator product formation was not observed.

Toluene/nitromethane (3:1) was then chosen as solvent for the reaction on a preparative scale (30 mg OxLTM02). The β-glycoside was selectively formed. The anomeric proton occurred as duplet with a coupling constant of 7.5 Hertz and showed long-range coupling to the protons of the hydroxymethyl group in 2D-NMR experiments.



Scheme 18: Koenigs-Knorr glycosylation reaction with OxLTM02 as aglycone resulted in product formation

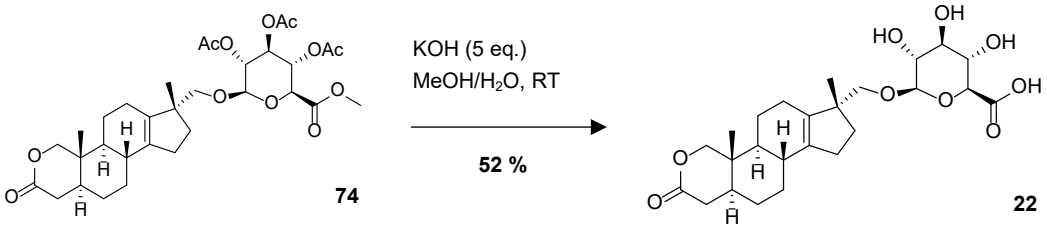
For cleavage of the acetate groups potassium hydroxide in methanol was tested on the lactone moiety of the steroid and it was found that the opened lactone ring re-cyclises under acidification with Amberlyst® ion exchange resin. These cleavage conditions were tested on the previously synthesised glycoside **68** to see if the acidification has an impact on the glycosidic bond [Scheme 19]. With 10 % water added the methyl ester could be hydrolysed as well and glucuronide **75** could be isolated with the glycosidic bond intact.



Scheme 19: cleavage conditions tested on methyl O^1-cyclopentyl-O^2,O^3,O^4-triacetyl-D-β-glucopyranuronate

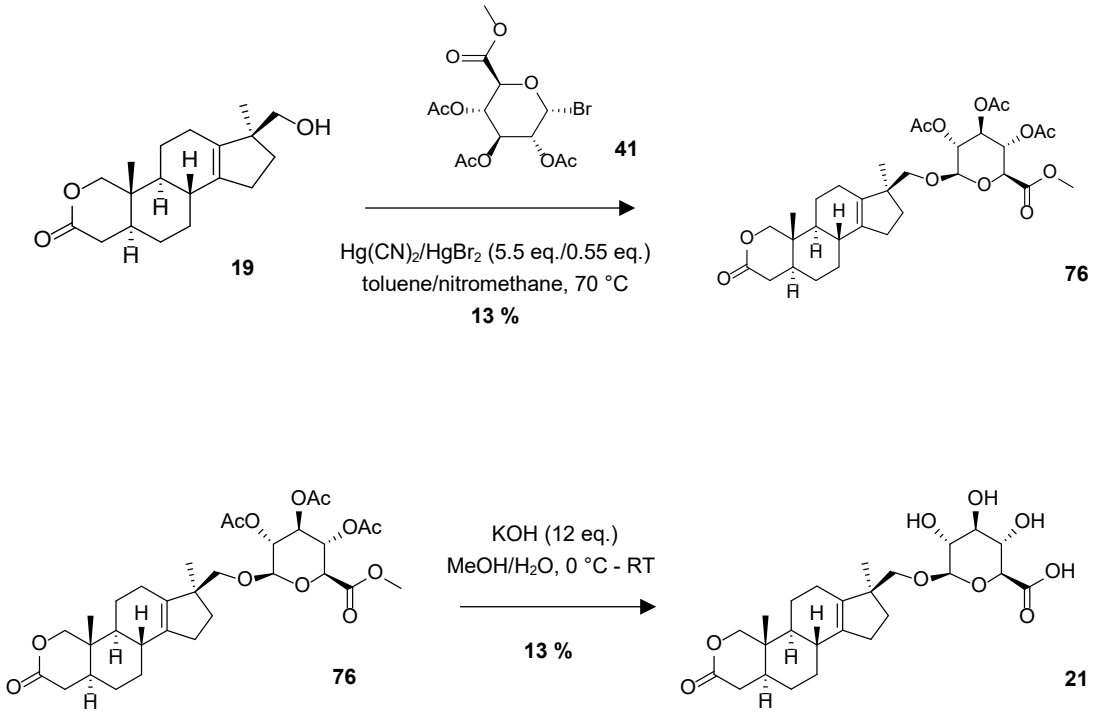
Glycoside **74** was stirred in methanol (with 10 % water) with 5 equivalents of potassium hydroxide for 2 hours at room temperature. After acidification with

Amberlyst® ion exchange resin and purification on a Waters preparative HPLC system OxLTM02 glucuronide **22** could be isolated in 52 % yield [Scheme 20].



Scheme 20: cleavage of the acetate protecting groups resulted in formation of the OxLTM02 glucuronide

Similar conditions were applied in the synthesis of the OxLTM01 glucuronide. The Koenigs-Knorr glucuronidation with mercury salt activation gave the respective glycoside **76** with acetate protecting groups in 13 % yield. Cleavage under basic conditions and purification on a Waters preparative HPLC system gave OxLTM01 glucuronide **21** in pure form in 13 % yield [Scheme 21].



Scheme 21: Koenigs-Knorr glycosylation resulted in the formation of OxLTM01 glucuronide

2.3 DETECTION OF OXLTM GLUCURONIDES IN HUMAN URINE

Conjugated metabolites can be measured directly from urine via LC-MS without the need for prior enzymatic hydrolysis or derivatisation (cf. section 1.2). The polar carboxy and hydroxy groups of the glucuronic acid also enhance the ion efficiency in electro spray ionisation and therefore increase sensitivity of the measurement.

As direct injection does not involve a preparation step where unwanted matrix components are removed and the analytes are concentrated, a high resolving mass spectrometer (Thermo Vanquish QExactive) coupled to a high performance liquid chromatography system was chosen over a routine triple quadrupole mass spectrometer due to its higher selectivity and sensitivity. As for the separation via liquid chromatography, a washing step was included before eluting the analytes on the analytical column. For this purpose a 10 mm phenylhexyl precolumn was used to trap the analytes during the initial washing step.

The ESI ionisation of OxLTM glucuronides was studied and revealed advantages over the detection of OxLTM in unconjugated form. For the unconjugated steroids the protonated molecular ion is not observed but a fragment with m/z = 275 that occurs after the cleavage of formaldehyde [Figure 3]. This leads to further uncharacteristic fragmentation in MS/MS experiments that lowers selectivity and enhances matrix interferences.

For the OxLTM glucuronides the protonated molecular ion is observed and fragmentation in MS/MS experiments with argon as collision gas at 30 eV gives two characteristic fragments: m/z = 287 after the cleavage of glucuronic acid and again the m/z = 275 fragment where the hydroxymethyl group is cleaved as well [Figure 3]. The mass spectra for the two compounds show a difference in the relative abundance of these two fragments with m/z = 287 being the most abundant fragment for the 17α-isomer OxLTM02 glucuronide and m/z = 275 for the 17β-isomer OxLTM01 glucuronide [annex Figure 30 and 31].

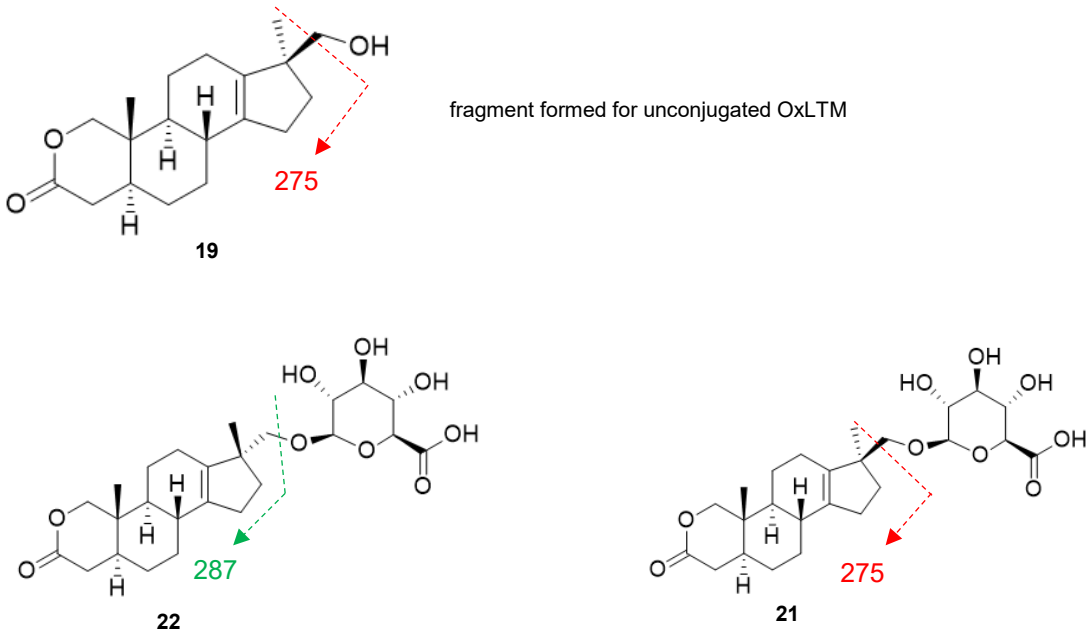
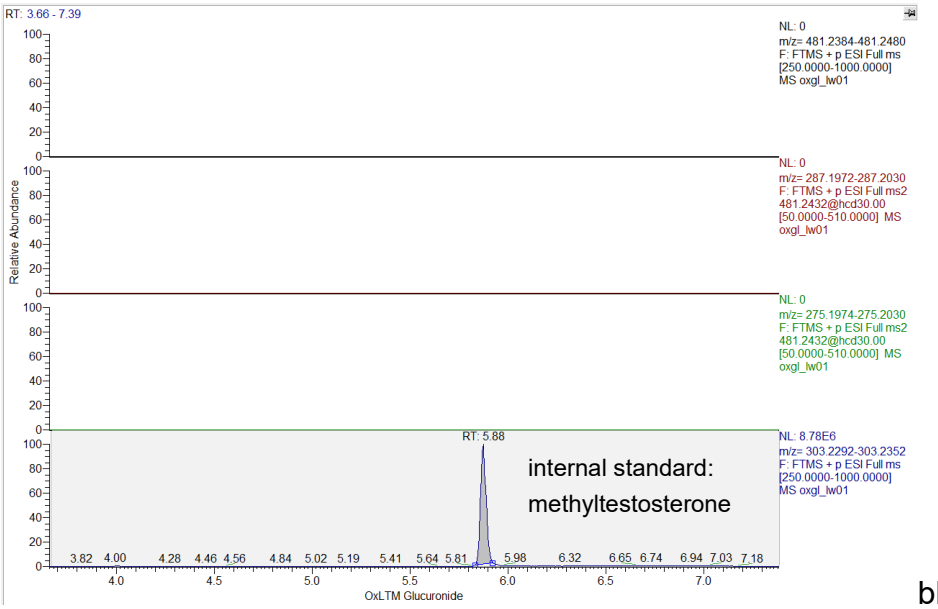


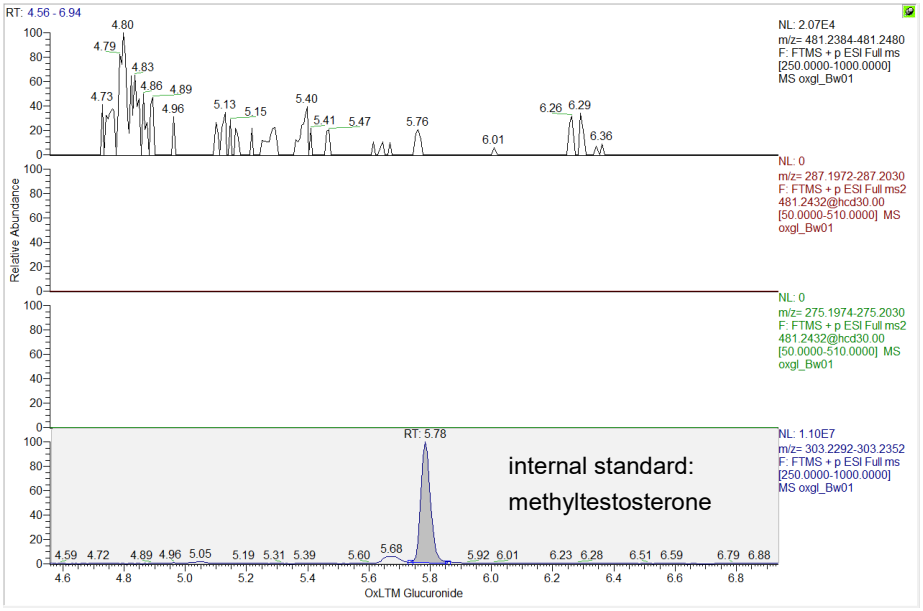
Figure 3: ESI-MS/MS behaviour of OxLTM and OxLTM glucuronides

The chromatograms of the blank samples (water and blank urine) show that there are no interfering background and matrix signals and show the selectivity of the chosen method and mass transitions [Figures 4 and 5].



blank (water)

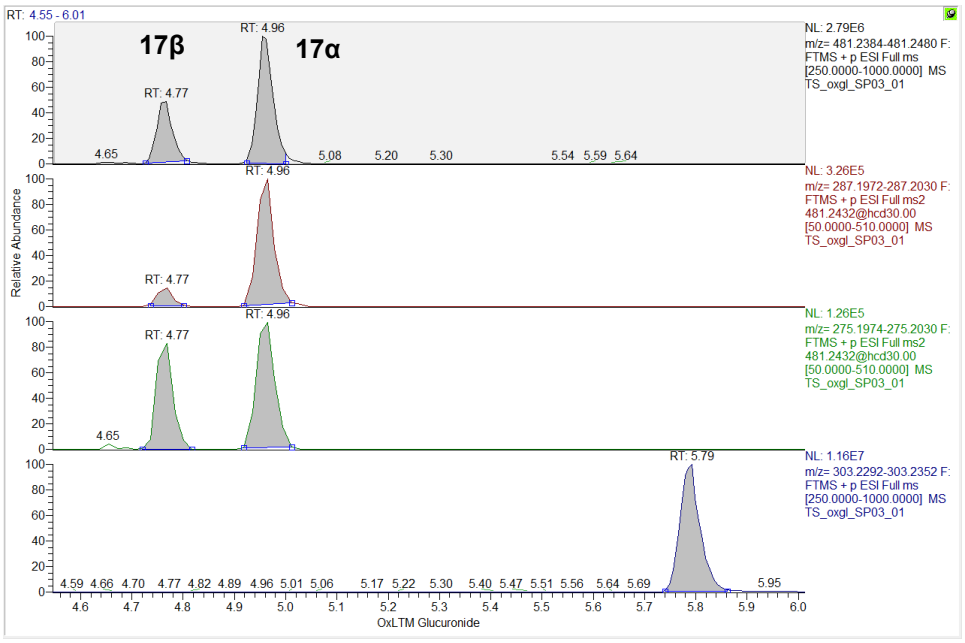
Figure 4: chromatograms of the water blank sample (from top: selected ion chromatogram for OxLTM glucuronides, mass transition to fragment with m/z = 287, mass transition to fragment with m/z = 275, selected ion chromatogram for internal standard methyltestosterone



blank (urine)

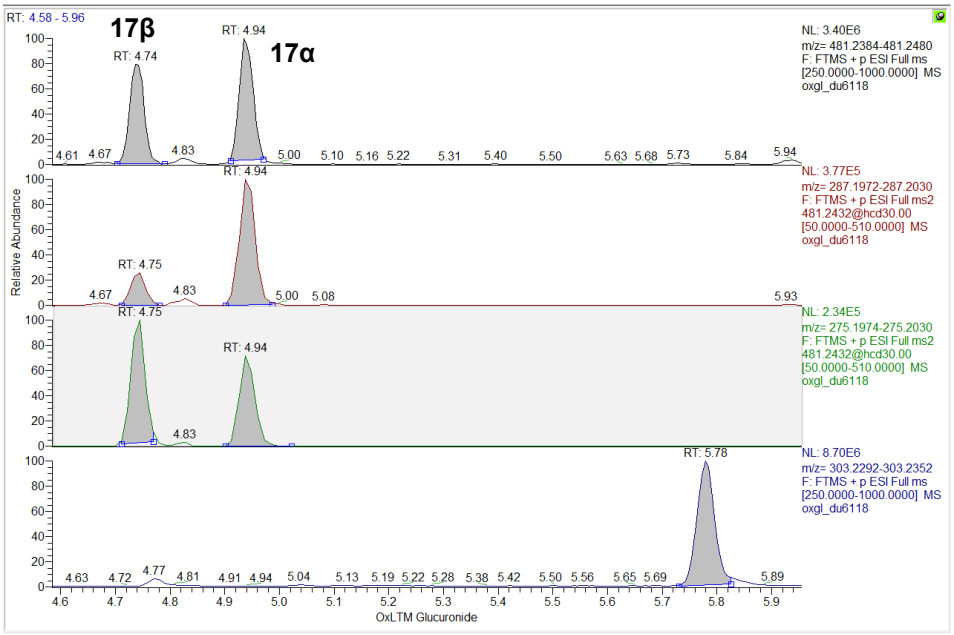
Figure 5: chromatograms of the urine blank sample (from top: selected ion chromatogram for OxLTM glucuronides, mass transition to fragment with m/z = 287, mass transition to fragment with m/z = 275, selected ion chromatogram for internal standard methyltestosterone

Comparing authentic urine samples from excretion studies and positive routine samples to blank urine spiked with OxLTM glucuronides (quality control samples) confirms their presence in human urine after oxandrolone application. The retention time and the ion ratio of the two selected mass transitions in the urine sample fit those of the quality control sample [Figures 6 and 7].



reference

Figure 6: chromatograms of the quality control sample (from top: selected ion chromatogram for OxLTM glucuronides, mass transition to fragment with m/z = 287, mass transition to fragment with m/z = 275, selected ion chromatogram for internal standard methyltestosterone



urine sample

Figure 7: chromatograms of the authentic urine sample (from top: selected ion chromatogram for OxLTM glucuronides, mass transition to fragment with m/z = 287, mass transition to fragment with m/z = 275, selected ion chromatogram for internal standard methyltestosterone

The retention time for the 17β-isomer is 4.77 minutes under the applied measurement conditions (cf. section 4.10) and 4.96 min for the 17α-isomer. In the urine sample the retention time difference is 0.01 minutes after correction with the internal standard which is within the WADA minimum criteria for substance identification that allows a deviation of 1 %.[57] The ion ratio calculated from the relative intensities of the fragment ions is 0.46 (287/275) for the 17β-glucuronide and 0.39 (275/287) for the 17α-glucuronide. In the urine sample the ion ratio is 0.42 for the 17β-glucuronide and 0.43 for the 17α-glucuronide which is also within the WADA acceptance criteria that allow a deviation of 20 %.[57] Therefore, the presence of both oxandrolone long-term metabolite glucuronides in urine after oxandrolone application is confirmed.

From the measurement results of an excretion study where the excretion of oxandrolone is monitored over 5 days it can be said that the OxLTM glucuronides start to occur 12 hours after application and are still detectable after 5 days.

3 CONCLUSION

The synthesis of the β-D-glucuronides of two C17-epimeric oxandrolone long-term metabolites was achieved by employing Koenigs-Knorr glycosylation conditions with mercury salt activation. The synthesised material was characterised by NMR spectroscopy and high-resolution mass spectrometry. Comparison with authentic urine samples from excretion studies revealed the presence of these oxandrolone long-term metabolite glucuronides in human urine after oxandrolone application. The synthesised glucuronides can serve as reference material in the confirmation of oxandrolone abuse in anti-doping analysis.

4 EXPERIMENTAL PART

4.1 CHEMICALS AND MATERIALS

In Table 1 the chemicals and materials in use are listed:

chloroform, dichloromethane, ethyl acetate, diethyl ether, petrol ether, hexane, potassium bicarbonate, silica gel 0- 63 µm 60 A, silica gel 60 F_{254} (TLC), sodium borohydride, dimethyl sulfoxide, toluene, Amberlyst® 15 (hydrogen form), phenylenediamine, Celite 545, lithium aluminium hydride, mercury(II) cyanide, lead(II) chloride, acetic anhydride	Merck
dichloromethane (p.A.), dichloromethane (extra dry), acetic acid, toluene (extra dry), nitromethane, formic acid, dimethylformamide (extra dry), acetonitrile (extra dry), meta-chloroperoxybenzoic acid, trimethylsilyl trifluoromethanesulfonate, hydrogen bromide (33 wt% solution in glacial acetic acid), acetone, sodium hydroxide, potassium hydroxide	Acros Organics
methanol for HPLC, water for HPLC, tetrahydrofuran, sodium thiosulphate, hydrochloric acid, ammonium chloride	VWR
pyridine	Alfa Aesar
ethanol (abs.)	Chem Lab
silver(II) carbonate	ChemPur
phosphoryl chloride, trimethylacetyl chloride, zinc, Martin sulfurane, titanium(IV) tetrachloride, trimethylsilyl chloride, palladium on carbon	Aldrich

(10 wt% loading), trimethylsilyl diazomethane (2M in diethyl ether), boron trifluoride etherate, hydrazine hydrate, lithium bis(trimethylsilyl)amide, 2,6-lutidine, para-methoxybenzyl chloride, D-glucuronic acid γ-lactone, dibromomethane, Dess Martin periodinane, sodium hydride (60 % in mineral oil dispersion), allyl alcohol, isopropyl magnesium chloride (2M in THF), 1,8-diazabicyclo(5.4.0)undec-7-ene, trichloroacetonitrile, tetrabutylammonium iodide	
sodium bicarbonate, magnesium sulphate, diisopropyl ether	Sigma
potassium carbonate, mercury(II) bromide	Fluka
dehydroepiandrosterone acetate, 4-(dimethylamino)-pyridine	FluoroChem
tetrahydrofuran (abs.), diethyl ether (abs.)	Innovative Technologies PureSolv System

Table 1: list of chemicals and materials in use

4.2 INSTRUMENTATION

NMR spectra were recorded on a Bruker AC400 and Bruker AC600. IR spectra were recorded on a Perkin Elmer Spectrum 65. TLC analysis was performed with precoated aluminium-backed plates (Silica gel 60 F254, Merck). Compounds were visualized by submerging in an acidic phosphomolybdic acid/cerium sulfate solution or an acidic vanillin solution and heating. Melting points were determined with a Kofler hot-stage apparatus. LC-MS measurements were carried out on a Nexera UHPLC system by Shimadzu using a XSelect CSH C18 2.5 µm column XP (3x50 mm). Preparative HPLC was carried out on a Waters prep 150 LC system using a XSelect CSH perpC18 5 µm OBD column (30x150 mm). High resolution high accuracy mass measurements were carried out on a Thermo Vanquish QExactive Hybride Quadrupole Orbitrap mass spectrometer coupled to a Thermo Survey LC system.

4.2 SYNTHESIS OF OXLTM02

The synthesis of OxLTM02 followed a previously published procedure.[30]

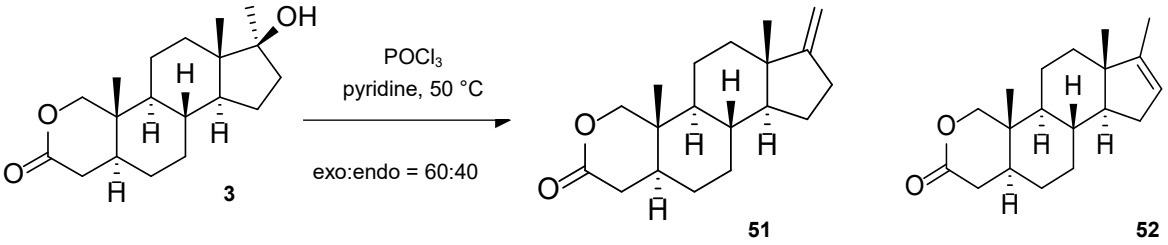
Isolation of oxandrolone from tablets

40 tablets (Magnus Pharmaceuticals) à 10 mg oxandrolone and 48 tablets (General European Pharmaceuticals) à 10 mg oxandrolone were grounded and the powder

suspended four times with 50 mL $CHCl_3$ (200 mL in total) in a glass sinter funnel. The filtrate was a slightly opaque solution and evaporation of the solvent *in vacuo* gave 900 mg of pure oxandrolone as a white solid. Spectral data (^{1}H-NMR) was in accordance with previously published data.[1]

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 4.25 – 4.22 (1H, d, J = 10.75), 3.94 – 3.91 (1H, d, J = 10.75), 2.55 – 2.49 (1H, dd, J = 6.02, 18.88), 2.27 – 2.19 (1H, dd, J = 12.94, 18.66), 1.86 – 1.55 (5H, m), 1.54 – 1.23 (10H, m), 1.22 – 1.16 (5H, m; therein 3H, s, C20-methyl group), 1.01 (3H, s), 0.87 (3H, s).

Synthesis of 17-methylene-2-oxaandrost-13-en-3-on (51) with $POCl_3$



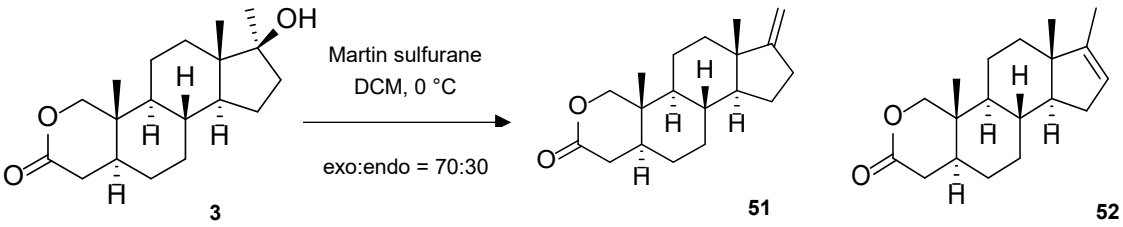
Scheme 22: dehydration of oxandrolone with $POCl_3$

$POCl_3$ (2.1 mL, 10 eq.) was added to a solution of oxandrolone (700 mg, 2.3 mmol) in dry pyridine (20 mL) at room temperature. The solution was stirred at 50 °C oil bath for 90 minutes. The resulting dark red solution was slowly poured onto 100 g ice and 50 mL water resulting in a brown suspension that was transferred to a separation funnel and extracted with dichloromethane. The combined organic phases were washed with saturated sodium bicarbonate solution and dried over magnesium sulphate. Evaporation of the solvent *in vacuo* gave 760 mg of a light-yellow solid that was used without further purification. From ^{1}H-NMR the ratio of the two formed isomers was estimated to give exo: endo = 60:40. Spectral data (^{1}H-NMR) was in accordance with previously published data.[30]

^{1}H-NMR data are given for the exo-isomer:

^{1}H-NMR (400 MHz, CD_3Cl): δ = 4.65 – 4.62 (2H, d, J = 11.52), 4.26 – 4.23 (1H, d, J = 10.72), 3.95– 3.92 (1H, d, J = 10.72), 2.55 – 2.49 (1H, dd, J = 6, 18.8), 2.27 – 2.19 (1H, m), 2.12 – 0.8 (16H, m), 1.00 (3H, s), 0.79 (3H, s)

Synthesis of 17-methylene-2-oxaandrost-13-en-3-on (51) with Martin sulfurane



Scheme 23: dehydration of oxandrolone with Martin sulfurane dehydrating agent

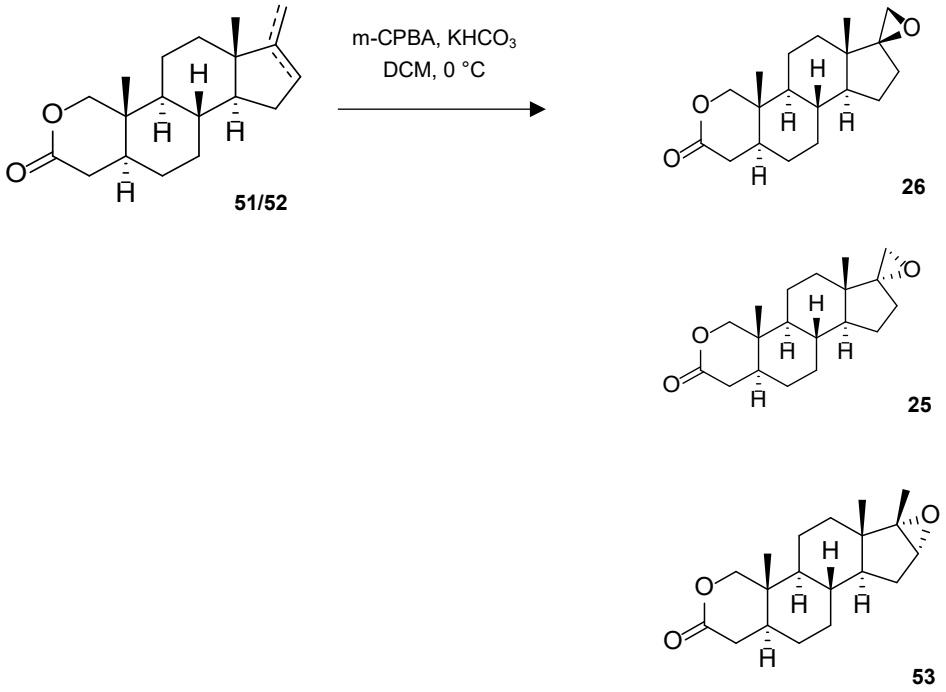
The experimental procedure to dehydrate oxandrolone with Martin sulfurane was based on a previously published procedure.[58]

A Schlenk flask was charged with oxandrolone (200 mg, 6.5 mmol) in dry dichloromethane (10 mL) and cooled to 0 °C. Martin sulfurane (770 mg, 1.7 eq.) was added portion wise. The mixture was stirred at the same temperature for 45 minutes and then the reaction was quenched with saturated NH_4Cl solution. The mixture was separated in a separation funnel and the aqueous layer extracted with dichloromethane. The combined organic phases were dried over magnesium sulphate and the solvent evaporated *in vacuo* to give 760 mg of a yellow oil. The crude product was purified by column chromatography (15 g silica, PE:EA = 5:1 → 3:1) to yield 135 mg of a mixture of compounds **51** and **52** (approximately 70:30 from ^{1}H-NMR). Spectral data (^{1}H-NMR) was in accordance with previously published data.[30]

^{1}H-NMR data are given for the exo-isomer (**51**):

^{1}H-NMR (400 MHz, CD_3Cl): δ = 4.65 – 4.62 (2H, d, J = 11.52), 4.26 – 4.23 (1H, d, J = 10.72), 3.95– 3.92 (1H, d, J = 10.72), 2.55 – 2.49 (1H, dd, J = 6, 18.8), 2.27 – 2.19 (1H, m), 2.12 – 0.8 (16H, m), 1.00 (3H, s), 0.79 (3H, s)

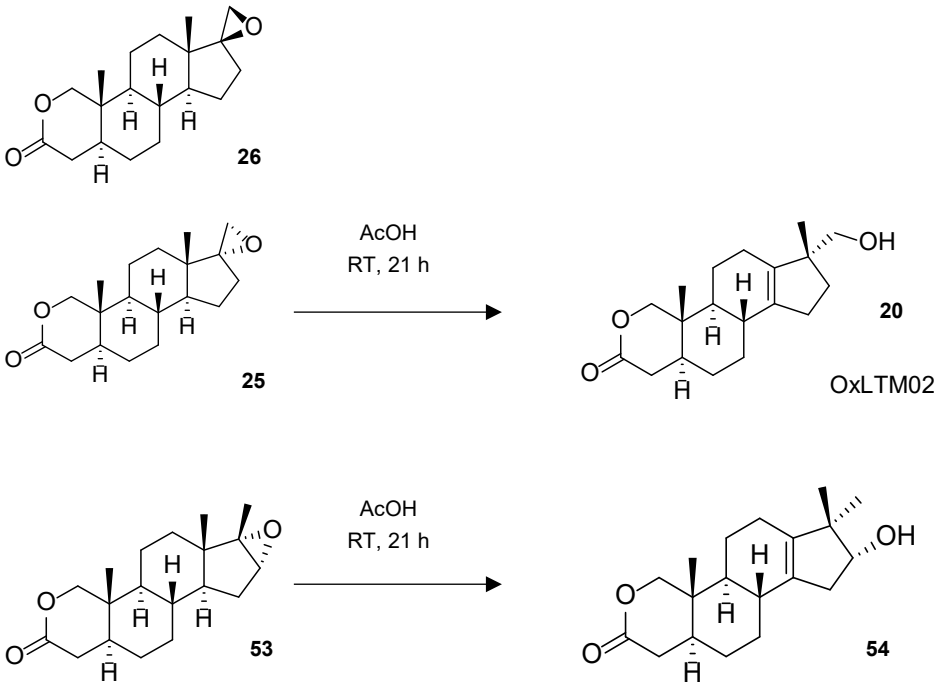
Synthesis of (5α,13β)-spiro(2-oxaandrost-17,2'oxirane)-3-one (25)



Scheme 24: epoxidation with m-CPBA

Potassium bicarbonate (979 mg, 3.1 eq.) was added to a solution of compounds **51** and **52** (895 mg, 3.1 mmol) in dichloromethane (20 mL) at room temperature. The solution was cooled to 0 °C and m-CPBA (70 % pure, 1.05 g, 1.4 eq.) was added as solid in small portions. The reaction mixture was allowed to reach room temperature over the course of 1.5 hours and stirred for further 2 hours at that temperature. The reaction was quenched by the addition of saturated sodium thiosulfate and sodium bicarbonate solutions. The mixture was extracted with dichloromethane, the combined organic phases washed with brine and dried over magnesium sulphate. Evaporation of the solvent *in vacuo* yielded 960 mg of a light yellow solid. The mixture of epoxides was not separated and used for the next step without further purification.[30]

Synthesis of 17α-hydroxymethyl-17β-methyl-18-nor-2-oxaandrost-13-en-3-one (20) – Wagner-Meerwein rearrangement with acetic acid

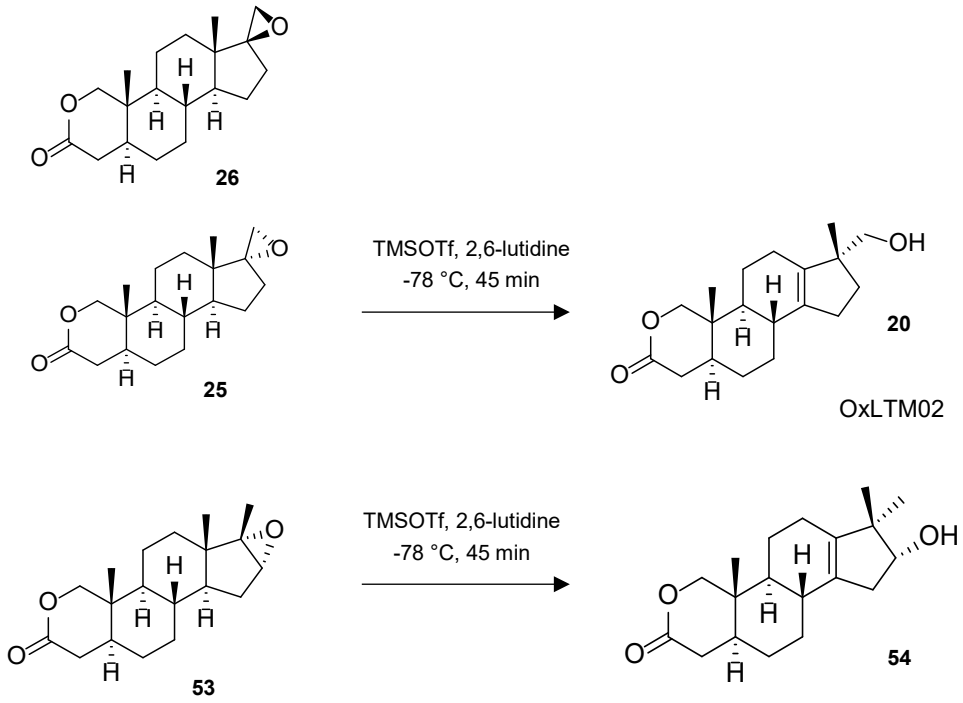


Scheme 25: Wagner-Meerwein rearrangement with acetic acid

The crude mixture of epoxides (960 mg) was dissolved in acetic acid (15 mL) and stirred at room temperature for 21 hours. 50 mL of water were added and the solution was neutralized by adding potassium bicarbonate portion wise until pH reached approximately 7. The mixture was extracted with dichloromethane, the combined organic phases washed with brine and dried over magnesium sulphate. Evaporation of the solvent *in vacuo* gave 780 mg of a yellow oil. The crude product was purified by column chromatography (90 g Silica, PE:EA = 3:1 → 1:2) to give 186 mg of OxLTM02 (**20**) (20 % overall yield) in pure form as a white waxy solid. The spectral data (^{1}H-NMR, ^{13}C-NMR) were in accordance with previously published data.[30]

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 4.32 – 4.29 (1H, d, J = 10.71), 3.97 – 3.95 (1H, d, J = 10.71), 3.42 - 3.30 (2H, dd, J = 10.44, 35.14), 2.57 – 2.51 (1H, dd, J = 5.85, 18.74), 2.36 – 2.17 (2H, m), 2.1 – 1.4 (11H, m), 1.4 - 1.32 (2H, m), 1.2 - 0 99 (2H, m), 0.97 (6H, s). ^{13}C-NMR (100 MHz, $CDCl_3$): 170.57, 140.17, 136.65, 81.13, 69.13, 51.76, 47.49, 40.41, 36.26, 34.86, 34.12, 33.89, 30.74, 30.58, 27.67, 23.04, 22.34, 21.84, 10.03

Synthesis of 17α-hydroxymethyl-17β-methyl-18-nor-2-oxaandrost-13-en-3-one (20) – Wagner-Meerwein rearrangement with TMSOTf



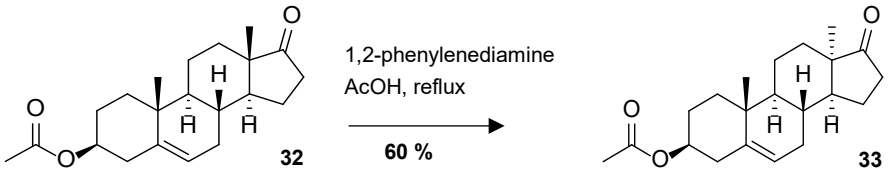
Scheme 26: Wagner-Meerwein rearrangement with TMSOTf

A flame dried Schlenk flask was charged with dry dichloromethane (3 mL) and cooled to -78 °C. TMSOTf (0.1 mL, 2.5 eq.) and 2,6-lutidine (0.1 mL, 3 eq.) were added at the same temperature. After 10 minutes a solution of epoxides (10 mg, 0.033 mmol) in dry dichloromethane (0.3 mL) was added. The reaction was stirred at -78 °C for 45 minutes and then quenched by the addition of 0.5 mL 2M HCl. The mixture was extracted with dichloromethane and washed with saturated sodium bicarbonate solution. Evaporation of the solvents *in vacuo* gave 20 mg of the crude product as yellow oil. The crude product was purified by column chromatography (1 g silica, PE:EA = 2:1 → 1:1) to give 4 mg of a mixed fraction of OxLTM02 (**20**) and the isomeric rearrangement product (**54**).

4.3 SYNTHESIS OF OXLTM01

The synthesis of OxLTM01 followed previously published procedures.[30,47,48]

Synthesis of 3β-hydroxy-13α-androst-5-en-17-one acetate (33)



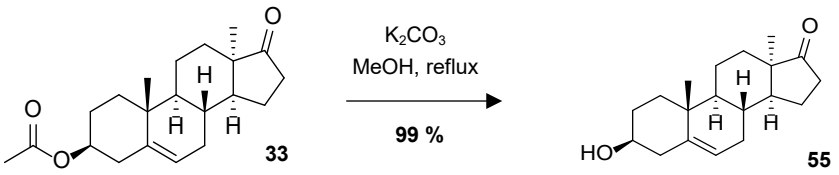
Scheme 27: epimerisation of dehydroepiandrosterone acetate

1,2-phenylendiamine (5.81 g, 1.6 eq.) was added to a solution of dehydroepiandrosterone acetate (10.85 g, 33 mmol) in acetic acid (130 mL). The light brown solution was heated to reflux upon which all solid residues dissolved completely. After 2 hours the solution turned dark green and the reaction mixture was refluxed overnight. Upon cooling the colour of the solution changed again from dark green to light brown. 150 mL deionised water were added and a white precipitate formed. The reaction mixture was extracted with 200 mL of ethyl acetate. The combined organic phases were washed with water and saturated sodium bicarbonate solution and dried over magnesium sulphate. Evaporation of the solvent *in vacuo* gave 10.5 g of a dark orange oil. The crude product was attempted to be purified by column chromatography (180 g silica, PE:Et_2O = 5:1 → 2:1), but no sufficient separation between dehydroepiandrosterone acetate and its 13α-epimer was achieved. 1 g of recovered starting material was obtained as well as 7.7 g of a mixed fraction which was recrystallised from n-hexane to give 6.5 g of compound **33** (60 % yield) in pure form as white crystals. Spectral data (^{1}H-NMR) was in accordance with previously published data.[30,48]

^{1}H-NMR (400 MHz, CD_3Cl): δ = 5.4 (1H, m), 4.64 – 4.56 (1H, m), 2.42 – 2.07 (6H, m), 2.03 (3H, s), 1.91 – 1.75 (3H, m), 1.68 – 1.52 (6H, m), 1.25 – 1.04 (4H, m), 0.98 (3H, s), 0.85 (3H, s)

The reaction was repeated with 9.9 g of dehydroepiandrosterone acetate where the crude oil was directly recrystallised from diisopropyl ether which yielded 4.3 g of compound **33** as an orange solid (43 % yield). Spectral data (^{1}H-NMR) was in accordance with previously published data.[30,48]

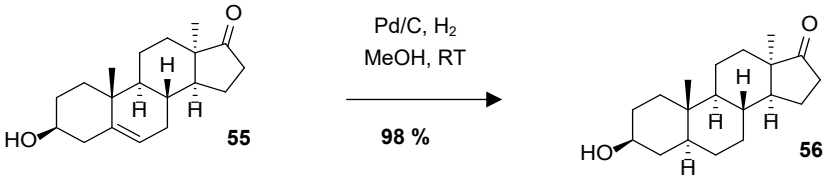
Synthesis of 3β-hydroxy-13α-androst-5-en-17-one (55)



Scheme 28: acetate cleavage

Compound **33** (10.8 g, 33 mmol) and K_2CO_3 (12.65 g, 2.8 eq.) were dissolved in 200 mL methanol under reflux and stirred for 1 hour. Approximately 100 mL methanol were removed *in vacuo* and the solution was diluted with water and extracted with dichloromethane. The combined organic extracts were washed with brine and dried over magnesium sulphate. Evaporation of the solvent *in vacuo* yielded 9.4 g of compound **55** (99 % yield) as white crystalline solid.[48]

Synthesis of 3β-hydroxy-13α,5α-androstan-17-one (56)

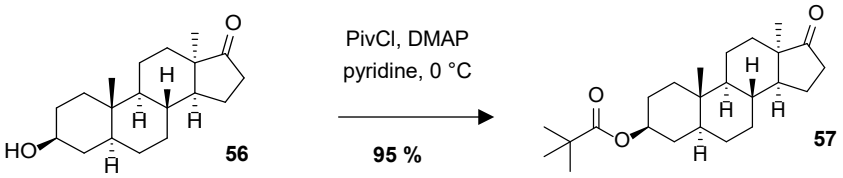


Scheme 29: hydrogenation of 3β-hydroxy-13α-androst-5-en-17-one

Compound **55** (5 g, 17mmol) was weighed into a 1000 mL three necked flask equipped with a stopper and two stopcocks. The flask was flushed with argon and 400 mL methanol were added. The solution was stirred at room temperature until the starting material dissolved completely. Palladium on carbon (938 mg, 5 mol% Pd, 10 wt%) was added under argon atmosphere at room temperature. The atmosphere was changed to hydrogen atmosphere by three times evacuating and flushing the flask with hydrogen. After stirring under hydrogen atmosphere for 68 hours the reaction was complete. The flask was flushed with argon and the reaction mixture filtered over Celite 545 and washed with ethyl acetate. Evaporation of the solvent *in vacuo* gave 4.9 g of a white solid (98 % yield). Spectral data (^{1}H-NMR) was in accordance with previously published data.[48]

^{1}H-NMR (400 MHz, CD_3Cl): δ = 3.59 (1H, m), 2.38 – 2.31 (1H, m), 2.22 – 2.12 (2H, m), 2.08 – 1.99 (3H, m), 1.86 – 1.74 (4H., m), 1.60 – 1.49 (4H, m), 1.40 – 1.11 (7H, m), 0.96 (3H, s), 0.95 – 0.67 (2H, m), 0.65 (3H, s)

Synthesis of 3β-hydroxy-13α,5α-androstan-17-one pivalate (57)

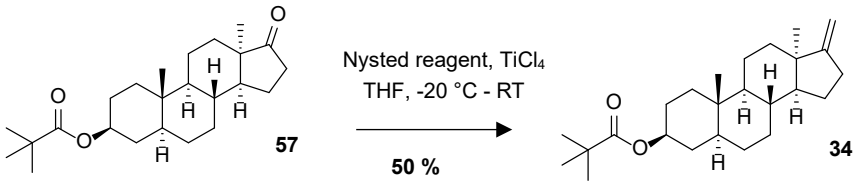


Scheme 30: pivaloyl protection

A solution of compound **56** (2.5 g, 10 mmol) and a catalytic amount of 4-dimethylamino in dry pyridine (20 mL) was cooled to 0 °C. Pivaloyl chloride (1.27 mL, 1.2 eq.) was added via syringe. The reaction was allowed to reach room temperature over night and was finished after 13 hours. Pyridine was removed *in vacuo* giving a white residue which was dissolved in dichloromethane and saturated sodium bicarbonate solution. The mixture was separated and extracted with dichloromethane. Evaporation of the solvent *in vacuo* yielded 3.05 g of product as white solid (95 % yield).[48]

^{1}H-NMR (400 MHz, CD_3Cl): δ = 4.69 – 4.61 (1H, m), 2.37 – 2.30 (1H, dd, J = 9.08, 19.21), 2.21 – 2.11 (2H, m), 2.07 – 1.99 (2H, m), 1.86 – 1.75 (3H, m), 1.57 (3H, s), 1.53 – 1.43 (3H, m), 1.36 – 1.21 (3H, m), 1.16 (9H, s), 1.04 – 0.98 (2H, m), 0.97 (3H, s), 0.93 – 0.70 (3H, m), 0.67 (3H, s)

Synthesis of 17-methylene-13α,5α–androstan-3β-ol pivalate (34)



Scheme 31: Nysted olefination

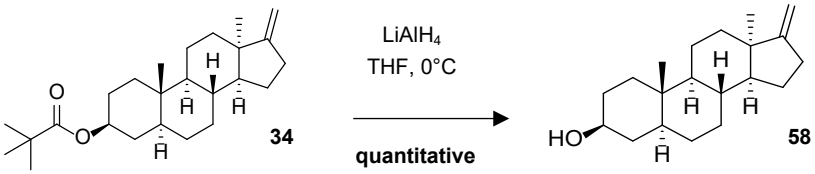
A flamed dried 250 mL three necked flask was charged with zinc (7.8 g, 15eq.), dry THF (70 mL), catalytic amounts of $PbCl_2$ and CH_2Br_2 (3.34 mL, 6 eq.) in that order. The reaction was cooled to -20°C and $TiCl_4$ (2.6 mL, 3 eq.) was added dropwise under argon over the course of 30 minutes. The mixture was stirred for 1.5 hours while slowly warming to room temperature. At 0 °C a solution of ketone **57** (3 g, 8 mmol) in dry THF (15 mL) was added dropwise to the solution. The reaction was taken out of the cooling bath and stirred overnight. Upon warming to room temperature it turned dark brown. After 16 hours the reaction was quenched by pouring the mixture on 300 g ice and 200 mL 2M HCl. The mixture was extracted with

diethyl ether. The combined organic phases were washed with saturated sodium bicarbonate solution, washed with brine and dried over magnesium sulphate. Evaporation of the solvent *in vacuo* gave 3.3 g of a white oily solid.

The crude product was purified by flash chromatography (30 g silica, PE:DCM = 2:1) giving 1.5 g of olefin **34** (50% yield) as white solid. Spectral data (^{1}H-NMR) was in accordance with previously published data.[30]

^{1}H-NMR (400 MHz, CD_3Cl): δ = 4.81 (1H, s), 4.68 (1H, s), 4.65 – 4.61 (1H, m), 2.51 – 2.31 (2H, m), 1.92 – 1.88 (2H, m), 1.79 – 1.76 (2H, m), 1.57 – 1.49 (5H, m), 1.48 – 1.21 (6H, m), 1.16 (9H, s), 1.06 – 0.97 (2H, m), 0.94 (3H, s), 0.88 – 0.74 (3H, m), 0.70 (3H, s)

Synthesis of 17-methylene-13α,5α–androstan-3β-ol (58)

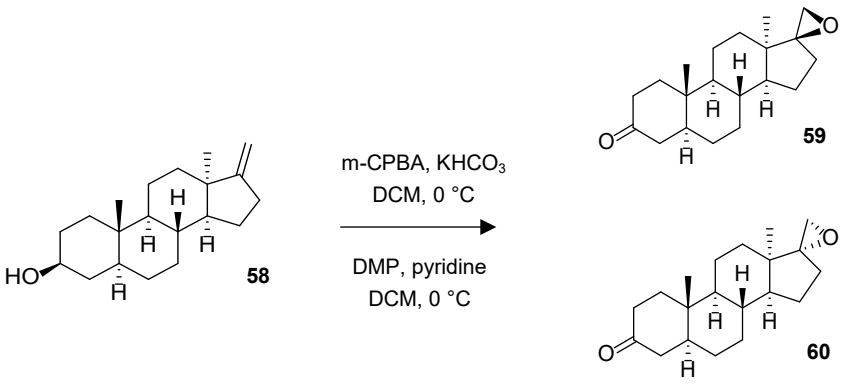


Scheme 32: pivaloyl cleavage

A solution of olefin **34** (2.2 g, 6 mmol) in dry THF (20 mL) was cooled to 0 °C. $LiAlH_4$ (560 mg, 2.5 eq.) was added portion wise to the solution. After 15 minutes the reaction was quenched by the slow addition of 2 mL H_2O. 50 mL diethyl ether were added and the mixture was stirred for 5 minutes and dried over magnesium sulphate. Salts and insoluble residues were filtered off and washed with diethyl ether. Evaporation of the solvent *in vacuo* yielded 2.23 g of alcohol **58** which was used without further purification.[30]

^{1}H-NMR (400 MHz, CD_3Cl): δ = 4.81 (1H, s), 4.68 (1H, s), 3.63 – 3.55 (1H, m), 2.50 – 2.30 (2H, m), 1.93 – 1.74 (2H, m), 1.58 – 1.55 (2H, m), 1.53 – 1.47 (5H, m), 1.47 – 0.97 (8H, m), 0.95 – 0.92 (1H, m), 0.91 (3H, s), 0.89 – 0.69 (2H, m), 0.67 (3H, s), 0.64 – 0.58 (1H, m)

Synthesis of (5α,13α)-spiro(androst-17β,2'-oxirane)-3-one (59)



Scheme 33: epoxidation with m-CPBA and oxidation with Dess Martin periodinane

$KHCO_3$ (2.3 g, 3 eq.) was added to a solution of crude alcohol **58** (2.2 g, 5 mmol) in dichloromethane (50 mL) at room temperature. The reaction was cooled to 0 °C and m-CPBA (70% pure, 2.35 g, 1.25 eq.) was added as solid. After 30 minutes the reaction was taken out of the cooling bath and stirred at room temperature for 2 hours. The reaction was quenched with saturated sodium thiosulphate and sodium bicarbonate solutions and extracted three times with dichloromethane. Evaporation of the solvent *in vacuo* gave 2 g of a mixture of isomers (α:β epoxide = 70:30) which was used without further purification.

The mixture of epoxides (2 g, 6.6 mmol) was dissolved in dichloromethane (50 mL) and pyridine (2 mL). Dess Martin periodinane (2.95 g, 1.06 eq.) was added as solid at 0 °C and the solution was stirred for 2 hours while warming to room temperature. The reaction was quenched by the addition of saturated sodium thiosulphate and sodium bicarbonate solutions. The mixture was extracted with dichloromethane and diethyl ether. Evaporation of the solvent *in vacuo* gave 3.43 g of crude product.

This mixture was purified by column chromatography (100 g silica, PE:EA = 8:1) giving 370 mg β-epoxide (minor diastereomer, 16% yield) and 740 mg α-epoxide (major diastereomer, 32% yield) in pure form. Spectral data (^{1}H-NMR) was in accordance with previously published data.[30]

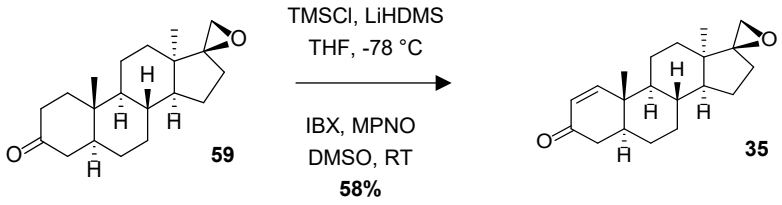
For the minor stereoisomer:

^{1}H-NMR (400 MHz, CD_3Cl): δ = 2.71 – 2.70 (1H, d, J = 4.57), 2.62 – 2.61 (1H, d, J = 4.57), 2.43 – 1.96 (7H, m), 1.89 – 1.71 (3H, m), 1.57 – 1.41 (1H, m), 1.38 – 0.99 (10H, m), 0.95 (3H, s), 0.93 (3H, s), 0.74 – 0.68 (1H, m)

For the major stereoisomer:

^{1}H-NMR (400 MHz, CD_3Cl): δ = 2.83 – 2.82 (1H, d, J = 5.08), 2.77 – 2.76 (1H, d, J = 5.08), 2.42 – 2.22 (3H, m), 2.13 – 1.85 (7H, m), 1.60 – 1.50 (1H, m), 1.38 – 1.02 (10H, m), 0.99 (3H, s), 0.93 (3H, s), 0.78 – 0.72 (1H, m)

Synthesis of (5α,13α)-spiro(androst-17β,2'-oxirane)-1-en-3-one (35)



Scheme 34: oxidation with IBX and 4-methoxypyridine-N-oxide

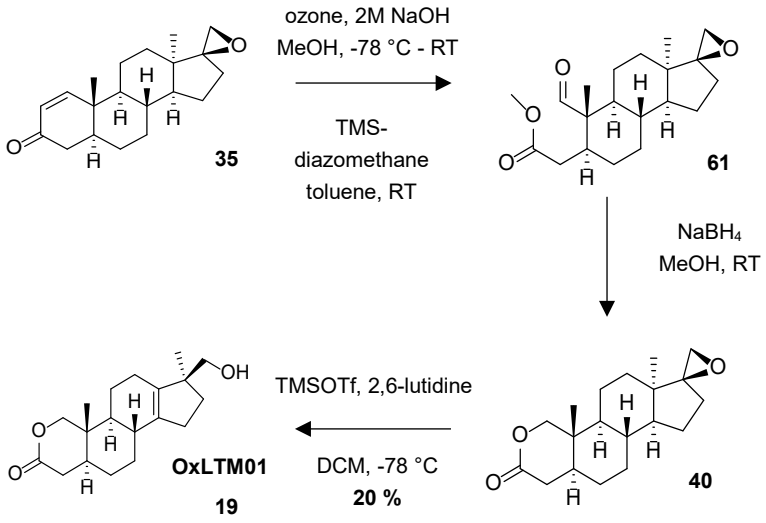
A flame dried 50 mL schlenk flask was charged with dry THF (4mL) and cooled to -78 °C. LiHMDS (1.3 mL 1M solution in THF, 2 eq.) was added as well as freshly distilled TMSCl (0.13 mL, 1.5 eq.) at that temperature. The reaction was stirred for 5 minutes and a solution of epoxide **59** (minor diastereomer, 200 mg, 0.66 mmol) in dry THF (3 mL) was added. The reaction was stirred for 2 hours and slowly warmed to room temperature. After 2 hours the reaction was taken out of the cooling bath and quenched by the addition of 12 mL saturated sodium bicarbonate solution. The reaction was stirred for 5 minutes and 100 mL toluene were added. The mixture was transferred to a separation funnel and shaken until the organic phase became clear. The organic phase was washed with brine and dried over magnesium sulphate. Evaporation of the solvent *in vacuo* gave 290 mg of a yellow oil. The silyl ether was used without further purification for the next step which was carried out immediately afterwards.

IBX (303 mg, 1.4 eq.) and 4-methoxypyridine-N-oxide (135 mg, 1.4 eq.) were dissolved in DMSO (1.2 mL) and stirred at room temperature until complete dissolution took place (15 min). A solution of freshly prepared silyl ether in DMSO/DCM (1 mL, 50:50) was added dropwise at room temperature. After 30 min the reaction was quenched by the addition of saturated sodium bicarbonate and sodium thiosulphate solutions. The mixture was extracted with dichloromethane to give 540 mg of crude product as a yellow oil.

The crude product was purified by column chromatography (25 g silica, PE:EA = 10:1 → 5:1) to give 115 mg epoxide **35** (58 % yield) in pure form as white needles. Spectral data (^{1}H-NMR) was in accordance with previously published data.[30]

^{1}H-NMR (400 MHz, CD_2Cl_2): δ = 7.13 – 7.11 (1H, d, J = 10.18), 5.80 – 5.78 (1H, d, J = 10.18), 2.67 – 2.65 (1H, d, J = 4.70), 2.59 – 2.58 (1H, d, J = 4.70), 2.39 – 2.31 (1H, m), 2.19 – 1.62 (8H, m), 1.45 – 1.11 (9H, m), 0.93 (6H, s)

Synthesis of 17β-hydroxymethyl-17α-methyl-18-nor-2-oxaandrost-13-en-3-one (19)



Scheme 35: ozonolysis, methyl ester formation, reductive lactone formation and Wagner-Meerwein rearrangement with TMSOTf

A solution of epoxide **35** (61.5 mg, 0.2 mmol) in methanol (20 mL, HPLC grade) and dichloromethane (2 mL) was cooled to -78 °C. Ozone (100 l/h, 0.4 A) was bubbled through the solution until it turned light blue (1 min). The reaction was stirred for 2.5 hours during which the temperature reached -40 °C. The reaction was quickly warmed to -25 °C and a 2.19 M NaOH solution (1.87 mL, 20 eq.) was added. The reaction was stirred for an hour during which it reached room temperature. After stirring for another hour at the same temperature acetic acid (0.23 mL, 20 eq.) was added dropwise until pH reached 7. The mixture was extracted with dichloromethane and washed with water. The organic phase was dried over magnesium sulphate and evaporation of the solvent *in vacuo* (water bath 35 °C) gave 125 mg crude seco-acid which was immediately turned into the corresponding methyl ester.

Trimethylsilyl diazomethane (0.25 mL) was added to a solution of seco-acid in toluene/methanol (5 mL, 20:1) at room temperature until the yellow colour of the solution persisted. The reaction was stirred for 15 minutes after which the solvents were removed *in vacuo* to give 73 mg crude methyl ester. Spectral data (^{1}H-NMR) showed that the epoxide was still intact.

Due to the lability of the epoxide moiety the lactonization and rearrangement reactions were carried out immediately afterwards without isolation of the pure intermediates.

$NaBH_4$ (32 mg, 4 eq.) was added as solid to a solution of crude methyl ester in methanol (5 mL). The reaction was stirred for 1 hour at room temperature. The

reaction was quenched with acetone (1 mL) and H_2O (2 mL). The mixture was extracted with dichloromethane to give 46 mg crude lactone.

A flame dried Schlenk flask was charged with dry dichloromethane (3 mL) and cooled to -75 °C. 2,6-lutidine (0.035 mL, 2 eq.) and TMSOTf (0.046 mL, 1.7 eq.) were added as well as a solution of crude lactone in dichloromethane (0.7 mL). The reaction was stirred at -75 °C for 30 minutes and then quenched with methanol (2 mL) and 2M HCl (0.2 mL). The mixture was extracted with dichloromethane and washed with saturated sodium bicarbonate. Evaporation of the solvent *in vacuo* gave 55 mg of crude product.

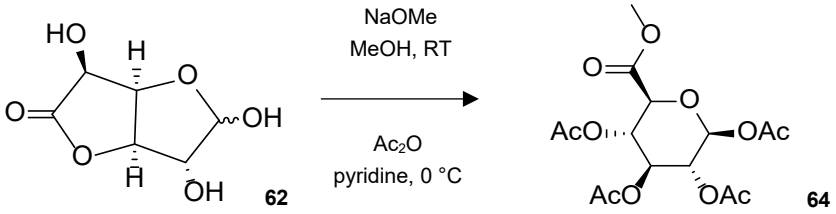
The crude product was purified by column chromatography (10 g silica, DCM:MeOH = 1000:1 → 100:1) to give 12 mg of OxLTM01 (**19**) (20 % yield over 4 steps) as white waxy solid. Spectral data (^{1}H-NMR, ^{13}C-NMR) were in accordance with previously published data.[30]

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 4.32 – 4.29 (1H, d, J = 10.71), 3.97 – 3.95 (1H, d, J = 10.71), 3.48 - 3.31 (2H, dd, J = 10.67, 60.32), 2.57 – 2.51 (1H, dd, J = 5.98, 18.74), 2.35 – 2.08 (4H, m), 2.02 – 1.83 (4H, m), 1.78 - 1.67 (1H, m), 1.66 – 1.49 (3H, m), 1.43 - 0.99 (5H, m), 0.966 (3H, s), 0.951 (3H, s). ^{13}C-NMR (100 MHz, $CDCl_3$): 170.60, 139.87, 136.97, 81.17, 69.01, 51.79, 47.34, 40.44, 35.94, 34.83, 33.93, 33.86, 30.64, 30.33, 27.68, 22.96, 22.24, 21.87, 9.97

4.4 SYNTHESIS OF METHYL 1-BROMO-1-DESOXY-O^2,O^3,O^4-TRIACETYL-D-alpha-GLUCOPYRANURONATE

The synthesis of methyl 1-bromo-1-desoxy-O^2,O^3,O^4-triacetyl-D-α-glucopyranuronate followed a previously published procedure.[51]

Synthesis of methyl O^1,O^2,O^3,O^4-tetraacetyl-D-glucopyranuronate (64)

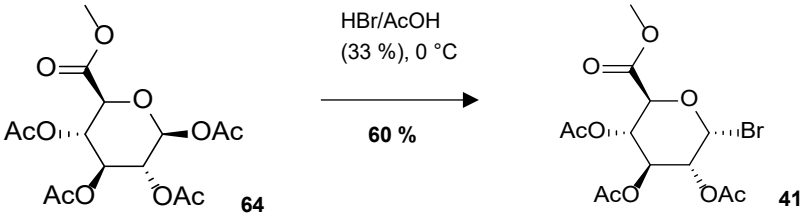


Scheme 36: based catalyzed opening of D-glucuronic acid γ-lactone and peracetylation

A flame dried 250 mL round bottom flask was charged with methanol (100 mL) and sodium methoxide (0.18 g, 0.03 eq.). D-glucuronic acid γ-lactone (20 g, 113 mmol) was added at room temperature and the mixture was stirred until complete dissolution took place (2 hours). Methanol was removed *in vacuo* to give an orange syrup which was dissolved in pyridine (50 mL). The solution was cooled to 0°C and acetic anhydride (70 mL) were added dropwise over the course of one hour. The reaction was stirred for 18 hours while warming to room temperature. The dark brown solution was filtered over a glass sinter funnel to give a white filter cake which was washed with diethyl ether. Drying *in vacuo* gave 13 g of methyl O^1,O^2,O^3,O^4-tetraacetyl-D-glucopyranuronate (30 % yield) as white solid. Analytical data were in accordance with previously published data.[22,51]

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 5.77 – 5.75 (1H, d, J = 7.75), 5.33 – 5.22 (2H, m), 5.16- 5.12 (1H, m), 4.19 – 4.16 (1H, d, J = 9.42), 3.74 (3H, s), 2.11 (3H, s), 2.04 (3H, s), 2.03 (3H, s). IR [cm^{-1}]: 2955, 1753, 1443, 1371, 1205, 1144, 1088, 1039, 981, 907, 779, 736, 693, 606, 567, 527, 488. $[\alpha]^{20}_D$ = +9.891 (c 0.37, dichloromethane). m.p.: 170 – 174 °C

Synthesis of methyl 1-bromo-1-desoxy-O^2,O^3,O^4-triacetyl-D-α-glucopyranuronate (41)



Scheme 37: C1 bromination with HBr/AcOH

Sugar **64** (1.03 g, 2.7 mmol) was dissolved in 33 % HBr in acetic acid (4 mL) and stirred at room temperature until complete dissolution took place (30 minutes). The solution was stored in the refrigerator for 24 hours. The solvents were removed *in vacuo* to give a yellow oil as residue which was taken up in chloroform and extracted with cold saturated sodium bicarbonate solution and water. After removal of the solvent the remaining slightly pink syrup was dissolved in anhydrous ethanol (3 mL) and stored at -20 °C. After 5 hours the formed crystals were filtered off and dried *in vacuo* to give 650 mg sugar **41** (60 % yield) as a white crystalline solid. Analytical data were in accordance with previously published data.[22,51,59]

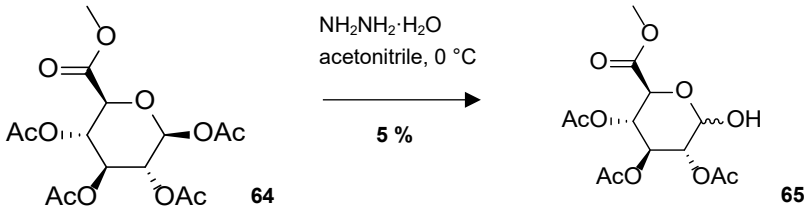
^{1}H-NMR (400 MHz, $CDCl_3$): δ = 6.64 – 6.63 (1H, d, J = 4.13), 5.63 – 5.58 (1H, dd, J = 9.7, 9.7), 5.26- 5.21 (1H, dd, J = 9.7, 9.7), 4.87 – 4.83 (1H, dd, J = 4.09, 10.07), 4.59 – 4.56 (1H, d, J = 10.31), 3.76 (3H, s), 2.09 (3H, s), 2.05 (3H, s), 2.04 (3H, s). ^{13}C-NMR (100 MHz, $CDCl_3$): 169.78, 169.75, 169.56, 166.78, 85.47, 72.16, 70.46, 69.41, 68.60, 53.24, 20.71, 20.56. IR [cm^{-1}]: 1745, 1616, 1435, 1377, 1333, 1205, 1110, 1044, 1006, 961, 897, 777, 750, 705, 673, 642, 600, 556, 506. $[\alpha]^{20}_{D}$ = +173.195 (c 0.178, dichloromethane). m.p.: 80 – 82 °C

4.5 SYNTHESIS OF METHYL O^2,O^3,O^4-TRIACETYL-O^1-(TRICHLOROACETIMIDOYL)-D-alpha-GLUCOPYRANURONATE

The synthesis of methyl O^2,O^3,O^4-triacetyl-O^1-(trichloroacetimidoyl)-D-α-glucopyranuronate followed previously published procedures.[49,52,53]

Synthesis of methyl 1-hydroxy-O^2,O^3,O^4-triacetyl-D-glucopyranuronate (65) with hydrazine hydrate

The synthesis of methyl 1-hydroxy-O^2,O^3,O^4-triacetyl-D-glucopyranuronate followed a previously published procedure.[52]



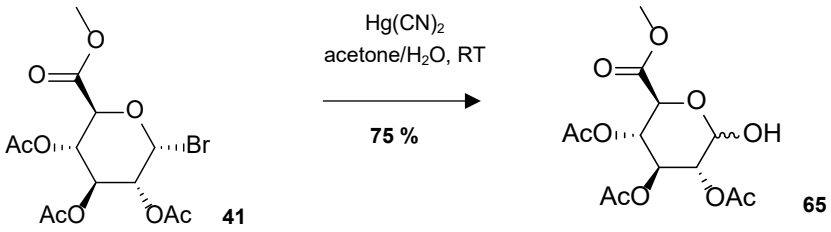
Scheme 38: C1 acetate cleavage with hydrazine hydrate

A solution of sugar **64** (600 mg, 1.3 mmol) in acetonitrile (6 mL) was cooled to 0 °C. Hydrazine hydrate (0.08 mL, 1.2 eq.) was added via syringe at the same temperature. The reaction was stirred for 22 hours while slowly warming to room temperature. A small amount of ion exchange resin Amberlyst® 15 was added and the reaction stirred for 15 minutes at room temperature. The resin was filtered off and washed with dichloromethane. Removal of the solvent in vacuo gave a yellow syrup

which was purified by column chromatography (10 g silica, PE:EA = 1:1) to give sugar **65** as a white solid. Spectral data (^{1}H-NMR) was in accordance with previously published data.[60]

Synthesis of methyl 1-hydroxy-O^2,O^3,O^4-triacetyl-D-glucopyranuronate (65) via hydrolysis of the sugar bromide

The synthesis of methyl 1-hydroxy-O^2,O^3,O^4-triacetyl-D-glucopyranuronate followed a previously published procedure.[53]



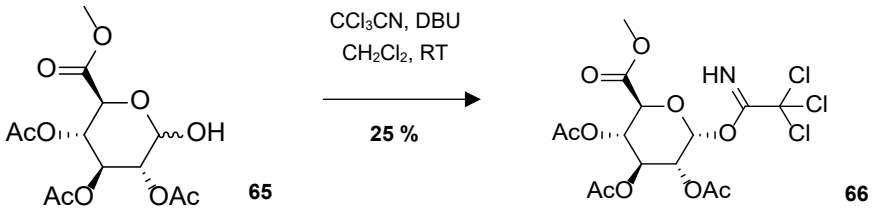
Scheme 39: hydrolysis of the sugar bromide

Sugar **41** (550 mg, 1.4 mmol) was dissolved in acetone (3 mL) and H_2O (0.6 mL). $Hg(CN)_2$ (350 mg, 1 eq.) was added at room temperature and the reaction was stirred overnight. The mixture was extracted with dichloromethane and washed with water. The crude product was purified by column chromatography (3 g silica, PE:EA = 3:1) to give 350 mg sugar **65** (75 % yield) as white solid. Spectral data (^{1}H-NMR) were in accordance to previously published data.[60]

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 5.60 – 5.55 (1H, m), 5.21 – 5.16 (1H, dd, J = 9.72, 9.72), 4.93 – 4.89 (1H, dd, J = 3.64, 10.26), 4.60 – 4.57 (1H, d, J = 10.01), 3.75 (3H, s), 3.36 – 3.35 (1H, d, J = 3.41), 2.08 (3H, s), 2.04 (3H, s), 2.03 (3H, s)

Synthesis of methyl O^2,O^3,O^4-triacetyl-O^1-(trichloroacetimidoyl)-D-α-glucopyranuronate (66)

The synthesis of methyl O^2,O^3,O^4-triacetyl-O^1-(trichloroacetimidoyl)-D-α-glucopyranuronate followed a previously published procedure.[49]



Scheme 40: synthesis of Schmidt sugar donor with trichloroacetonitrile and DBU

A flame dried Schlenk flask was charged with a solution of sugar **65** (100 mg, 0.3 mmol) in dry dichloromethane (1 mL). Trichloroacetonitrile (0.15 mL, 5 eq.) was added at room temperature as well as 0.1 mL of a DBU stock solution (0.4 mL DBU

in 10 mL dry dichloromethane, 0.1 eq.) resulting in a dark red reaction mixture. The reaction was stirred for 24 hours at room temperature. Solvents were removed *in vacuo* to give a dark brown oil which was purified by flash chromatography (3 g silica, PE:EA = 3:1) to give 35 mg sugar **66** (25 % yield) as white crystalline solid. Analytical data were in accordance with previously published data.[61]

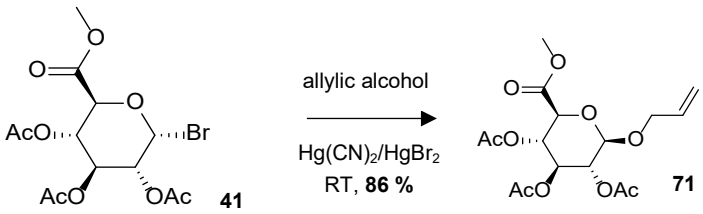
^{1}H-NMR (400 MHz, $CDCl_3$): δ = 8.73 (1H, s), 6.63 – 6.62 (1H, d, J = 3.55), 5.64 – 5.59 (1H, dd, J = 9.76, 9.76), 5.28 – 5.23 (1H, dd, J = 10.01, 10.01), 5.16 – 5.12 (1H, dd, J = 3.77, 10,19), 3.74 (3H, s), 2.04 (6H, s), 2.00 (3H, s). ^{13}C-NMR (100 MHz, $CDCl_3$): 169.90, 169.85, 169.60, 167.26, 160.69, 92.72, 70.59, 69.57, 69.20, 69.05, 53.17, 20.79, 20.61, 20.52. IR [cm^{-1}]: 3313, 2960, 1740, 1686, 1437, 1373, 1279, 1214, 1152, 1032, 975, 935, 833, 790, 679, 638, 600, 543, 508, 476. $[\alpha]^{20}_{D}$ = +83.741 (c 0.235, dichloromethane). m.p.: 86 – 89 °C

4.6 SYNTHESIS OF METHYL O^2,O^3,O^4-TRI-PARA-METHOXYBENZYL-O^1-(TRICHLOROACETIMIDOYL)-D-alpha-GLUCOPYRANURONATE

The attempt to synthesise methyl O^2,O^3,O^4-tri-para-methoxybenzyl-O^1-(trichloroacetimidoyl)-D-α-glucopyranuronate followed previously published procedures.[54,55]

Synthesis of methyl O^1-allyl-O^2,O^3,O^4-triacetyl-β-D-glucopyranuronate (71)

The synthesis of methyl O^1-allyl-O^2,O^3,O^4-triacetyl-β-D-glucopyranuronate followed a previously published procedure.[54]



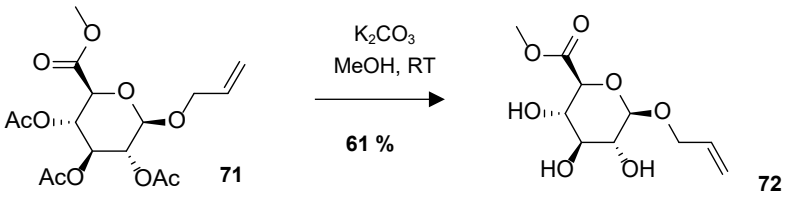
Scheme 41: synthesis of methyl O^1-allyl-O^2,O^3,O^4-triacetyl-β-D-glucopyranuronate

A flame dried schlenk flask was charged with sugar **41** (1.04 g, 2.6 mmol) and freshly distilled cold allyl alcohol (5 mL) under argon atmosphere. $Hg(CN)_2$ (339 mg, 0.5 eq.) and $HgBr_2$ (47 mg, 0.05 eq.) were added at room temperature and the reaction was stirred for 16 hours. A white precipitate formed. The solvent was removed *in vacuo* and the residue was taken up in chloroform:heptane (1:1) and washed with cold H_2O and with 1M potassium iodide solution. Drying of the organic phase with magnesium sulphate and removal of the solvent *in vacuo* gave 840 mg (86 % yield) of sugar **71** as white amorphous solid. Spectral data (^{1}H-NMR) was in accordance with previously published data.[54,60]

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 5.88 – 5.78 (1H, m), 5.30 – 5.19 (4H, m), 5.06 – 5.02 (1H, m), 4.62 – 4.60 (1H, d, J = 7.33), 4.38 – 4.34 (1H, dd, J = 4.78, 13.27), 4.12 – 4.07 (1H, m), 4.04 – 4.02 (1H, m), 3.76 (3H, s), 2.05 (3H, s), 2.02 (6H, s)

Synthesis of methyl O^1-allyl-β-D-glucopyranuronate (72)

The synthesis of methyl O^1-allyl-β-D-glucopyranuronate followed a previously published procedure.[54]



Scheme 42: acetate cleavage

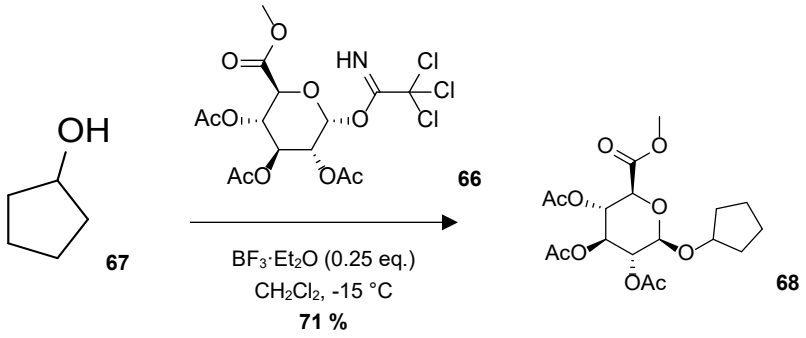
Potassium carbonate (1.36 g, 5 eq.) was added to a solution of sugar **71** (740 mg, 1.98 mmol) in methanol (30 mL) at room temperature. The solution turned yellow and was stirred for 10 minutes at room temperature. The solution was neutralised by dropwise adding acetic acid until pH reached 7. The solvent was removed *in vacuo* and the crude yellow oil was purified by column chromatography (5 g silica, EA:MeOH = 12:1) giving 300 mg sugar **72** (61 % yield) as yellow oil. Spectral data (^{1}H-NMR) was in accordance with previously published data.[54]

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 5.99 – 5.89 (1H, m), 5.36 – 5.31 (1H, dd, J = 1.34, 17.20), 5.26 – 5.23 (1H, d, J = 10.50), 4.41 – 4.39 (1H, d, J = 7.74), 4.18 – 4.11 (2 H, m), 3.88 – 3.85 (1H, m), 3.84 (3H, s), 3.81 – 3.77 (1H, dd, J = 8.7, 8.7), 3.65 – 3.61 (1H, dd, J = 9.01, 9.01), 3.51 – 3.47 (1H, dd, J = 7.71, 7.71)

4.7 SYNTHESIS OF O^1-CYCLOPENTYL-D-beta-GLUCOPYRANURONIC ACID

Synthesis of methyl O^1-cyclopentyl-O^2,O^3,O^4-triacetyl-D-β-glucopyranuronate (68) via Schmidt glucuronidation

The glucuronidation reaction using Schmidt conditions followed previously published procedures.[46,50]



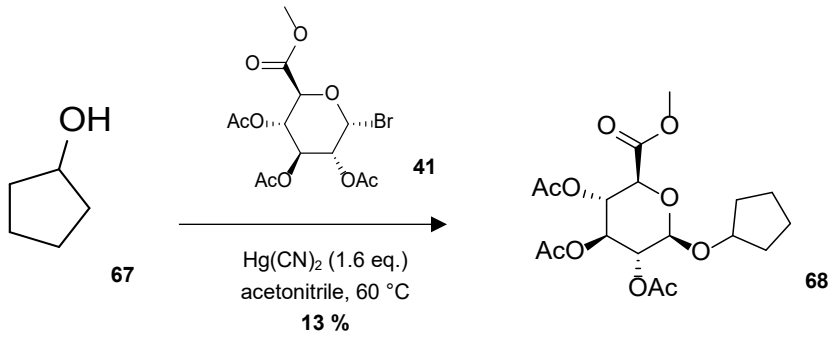
Scheme 43: Schmidt glucuronidation of cyclopentanol

A flame dried Schlenk flask was charged with molecular sieve 4A and a solution of cyclopentanol (0.17 mL, 9 eq.) in dry dichloromethane (2 mL) under argon atmosphere. After stirring for 2.5 hours at room temperature a solution of sugar **66** (100 mg, 0.21 mmol) in dry dichloromethane (2 mL) was added and the reaction stirred for another 50 minutes at room temperature before cooling to -15 °C. At the same temperature 1 mL $BF_3{\cdot}Et_2O$ stock solution (0.25 eq., 0.07 mL freshly distilled $BF_3{\cdot}Et_2O$ in 10 dry dichloromethane) was added dropwise over the course of 10 minutes. The reaction was slowly warmed to room temperature and after one hour it was quenched by the addition of saturated sodium bicarbonate solution. The mixture was extracted with dichloromethane and ethyl acetate. The combined organic phases were dried over magnesium sulphate and evaporation of the solvents *in vacuo* gave the crude product as colourless oil. The crude product was flashed over 1 g silica (PE:EA = 3:1) to give 60 mg pure glycoside **68** (71% yield) as white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 5.27 – 5.17 (2H, m), 4.96 – 4.91 (1H, dd, J = 7.88, 7.88), 4.57 – 4.55 (1H, d, J = 7.77), 4.31 – 4.26 (1H, m), 4.03 – 4.00 (1H, d, J = 9.39), 3.75 (3H, s), 2.02 (3H, s), 2.00 (6H, s), 1.80 – 1.45 (8H, m). ^{13}C-NMR (100 MHz, $CDCl_3$): 170.38, 169.54, 169.33, 167.51, 99.58, 81.70, 72.77, 72.29, 71.53, 69.63, 52.99, 33.21, 32.13, 23.47, 23.24, 20.77, 20.72, 20.65. IR [cm^{-1}]: 3372, 3320, 3243, 3185, 2960, 2925, 2853, 1746, 1693, 1617, 1441, 1370, 1210, 1165, 1109, 1070, 1036, 909, 831, 748, 649, 618, 550, 490. $[\alpha]^{20}_D$ = -20.802 (c 0.61, dichloromethane). m.p.: 70 – 73 °C. HR-MS: [M+Na]: calcd: 425.1418, found: 425.1437.

Synthesis of methyl O^1-cyclopentyl-O^2,O^3,O^4-triacetyl-D-β-glucopyranuronate (68) via Koenigs-Knorr glucuronidation

The glucuronidation reaction using Koenigs-Knorr conditions followed a previously published procedure.[22]

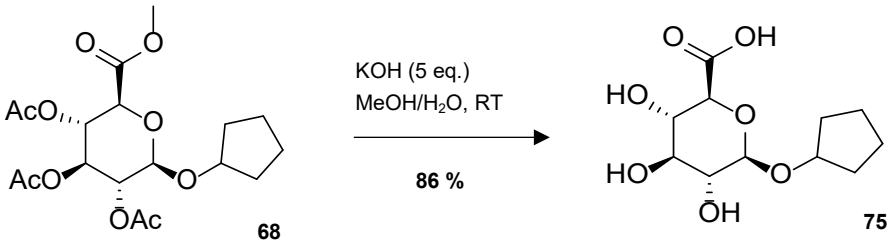


Scheme 44: Koenigs-Knorr glucuronidation of cyclopentanol

A flame dried Schlenk flask was charged with dry acetonitrile (5 mL) and 1 mL cyclopentanol stock solution (0.52 mL in 10 mL acetonitrile, 50 mg, 0.58 mmol) under argon atmosphere. Sugar **41** (267 mg, 1.15 eq.) was added at room temperature as well as $Hg(CN)_2$ (240 mg, 1.6 eq.). The reaction was stirred at 60 °C oil bath for 5 hours. The reaction mixture was diluted with diethyl ether and washed with saturated sodium bicarbonate solution and brine. Evaporation of the solvent in vacuo gave the crude product as yellow oil which was purified by column chromatography (10 g silica, PE:EA = 5:1 → 1:1, and a second time with DCM:EA = 10:1) to give 30 mg of glycoside **68** (13% yield) as white solid. Spectral data (^{1}H, ^{13}C) were in accordance with the product from the Schmidt glucuronidation.

Synthesis of O^1-cyclopentyl-D-β-glucopyranuronic acid (75)

Cleavage of the acetate groups followed previously published procedures.[62,63]



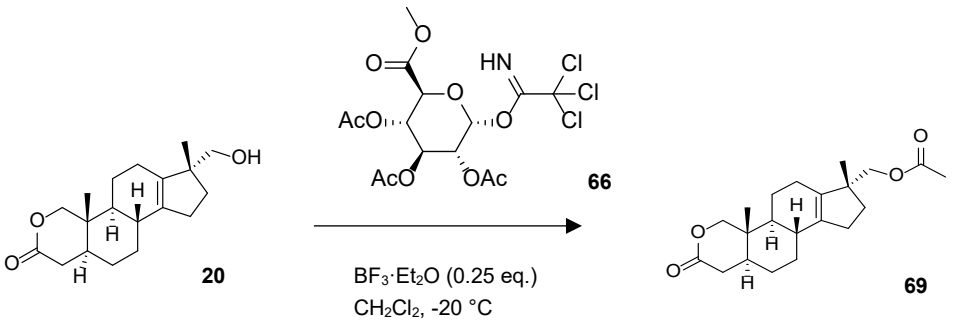
Scheme 45: acetate cleavage

Potassium hydroxide (15 mg, 5 eq.) was added to a solution of glycoside 68 (30 mg, 0.075 mmol) in acetone (1 mL) and H_2O (0.04 mL) at room temperature. The reaction was stirred at that temperature for 2 hours. 0.3 mL H_2O were added as well as ion exchange resin Amberlyst® 15 until pH reached 6. Filtration of the resin and removal of the solvents *in vacuo* gave a yellow oil which was washed with dichloromethane and diethyl ether. 17 mg of glucuronide **75** (86 % yield) were obtained as yellow oil.

^{1}H-NMR (400 MHz, CD_3OD): δ = 4.48 – 4.41 (1H, m), 4.32 – 4.30 (1H, d, J = 7.83), 3.56 – 3.54 (1H, d, J = 9.40), 3.45 – 3.37 (2H, m), 3.19 – 3.15 (1H, m), 1.84 – 1.68 (6H, m), 1.58 – 1.48 (2H, m). ^{13}C-NMR (100 MHz, CD_3OD): 176.97, 102.76, 81.66, 77.88, 76.34, 74.94, 73.74, 34.12, 32.85, 24.47, 24.31. IR [cm^{-1}]: 2950, 2873, 1582, 1410, 1363, 1311, 1079, 1025, 913, 864, 825, 750, 646, 598, 542. $[\alpha]^{20}_D$ = -29.219 (c 0.295, methanol). flowing point: 119 - 121°C. HR-MS: [M+Na]: calcd: 285.0945, found: 285.0937.

4.8 SYNTHESIS OF OXLTM02 GLUCURONIDE

$BF_3{\cdot}Et_2O$ activation: normal procedure



Scheme 46: attempted Schmidt glucuronidation of OxLTM02 with BF_3 activation

A flame dried Schlenk flask was charged with molecular sieve 4A and a solution of OxLTM02 (21 mg, 0.065 mmol) in dry dichloromethane (1 mL) under argon atmosphere. The solution was stirred for 1 hour at room temperature. A solution of sugar **66** (30 mg, 1.eq.) in dry dichloromethane (1 mL) was added at the same temperature and the solution was stirred for another hour. The solution was cooled to -20 °C and 0.5 mL of a $BF_3{\cdot}Et_2O$ stock solution (0.25 eq., 0.04 mL freshly distilled $BF_3{\cdot}Et_2O$ in 10 mL dichloromethane) was added dropwise. The reaction was allowed to reach room temperature over the course of 2 hours and then the reaction was quenched by the addition of saturated sodium bicarbonate solution. The aqueous phase was extracted with dichloromethane and ethyl acetate. The combined organic phases were washed with brine and dried over magnesium sulphate. Evaporation of the solvents *in vacuo* gave the crude product as yellow oil which was purified by column chromatography (3 g silica, PE:EA = 5:1 and a second time with 1 g silica, DCM:EA = 5:1) to give 3 mg of a white solid which turned out to be OxLTM02 acetate (**69**).

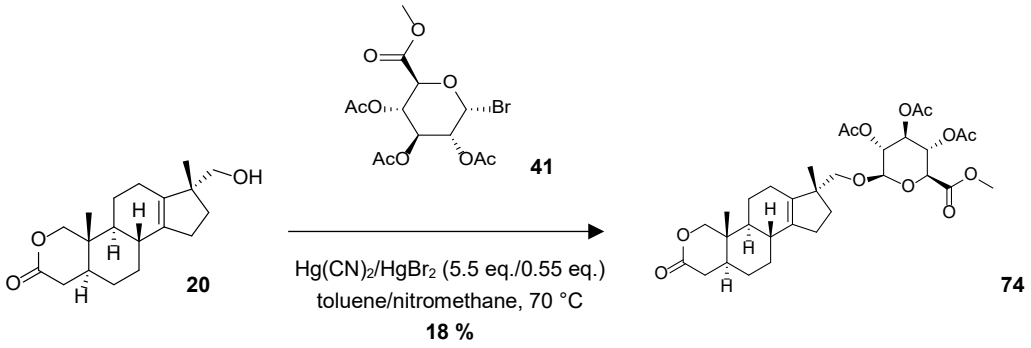
$BF_3{\cdot}Et_2O$ activation: inverse procedure

A flame dried Schlenk flask was charged with molecular sieve 4A and a solution of OxLTM02 (21 mg, 0.065 mmol) in dry dichloromethane (1 mL) under argon atmosphere. The solution was stirred for 0.5 hour at room temperature and 0.5 mL of a $BF_3{\cdot}Et_2O$ stock solution (0.25 eq., 0.04 mL freshly distilled $BF_3{\cdot}Et_2O$ in 10 mL dichloromethane) was added dropwise at the same temperature. The reaction was stirred for further 1.5 hours at room temperature and then cooled to -20°C. A solution of sugar **66** (30 mg, 1.eq.) in dry dichloromethane (1 mL) was added dropwise at the same temperature. The reaction reached room temperature over the course of

2 hours and was then quenched by the addition of saturated sodium bicarbonate solution. The aqueous phase was extracted with dichloromethane and ethyl acetate. The combined organic phases were washed with brine and dried over magnesium sulphate. Evaporation of the solvents *in vacuo* gave the crude product as yellow oil which was purified by column chromatography (3g silica, PE:EA = 3:1 and a second time with 1g silica, DCM:EA = 5:1) to give 1 mg of a white solid which turned out to be OxLTM02 acetate.

Synthesis of 17α-(methyl O^2,O^3,O^4-triacetyl-β-D-glucuronosyl-1-oxy)-17β-methyl-18-nor-2-oxaandrost-13-en-3-one (74) via Koenigs-Knorr glucuronidation

The synthesis of the glucuronide of OxLTM02 via Koenigs-Knorr glucuronidation relied on previously published procedures.[22,56,64]



Scheme 47: synthesis of OxLTM02 glucuronide via Koenigs-Knorr glucuronidation with mercury salt activation

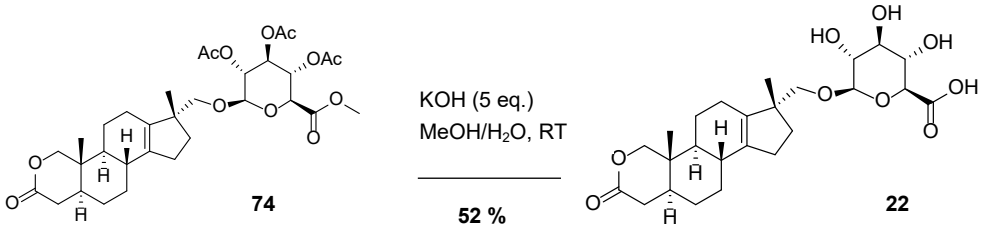
A flame dried screw-cap 7 mL vial was charged with a solution of OxLTM02 (30 mg, 0.098 mmol) in toluene (2 mL) and nitromethane (1.5 mL). Sugar **41** (194 mg, 5 eq.), $Hg(CN)_2$ (137 mg, 5.5 eq.) and $HgBr_2$ (19.4 mg, 0.55 eq.) were added at room temperature. The reaction was stirred for 10 hours at 70 °C. The mixture was diluted with diethyl ether and the insoluble mercury salts were filtered off. The solution was washed with saturated sodium bicarbonate solution and the organic phase was dried over magnesium sulphate. Evaporation of the solvents *in vacuo* gave the crude product as yellow oil which was purified by column chromatography (5 g silica, DCM:EA = 5:1). The product fractions (29 mg) were combined, dissolved in 1 mL methanol and separated on a C_{18} column of a Waters preparative HPLC system using a mass detector. The solvents used were water (+0.1 % formic acid) and methanol (+0.1 % formic acid). The gradient started at 80% methanol with a constant flow rate of 30 mL/min, reached 98% methanol over the course of 10 minutes and was followed by 1 minute of equilibration with the starting conditions. The product

fractions were combined, methanol evaporated in vacuo (water bath at 40 °C) and water removed by lyophilisation. 11 mg of glycoside **74** (18 % yield) were obtained in pure form as white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 5.26 – 5.19 (2H, m), 5.02 – 4.98 (1H, m), 4.52 – 4.49 (1H, d, J = 7.53), 4.30 – 4.28 (1H, d, J = 10.75), 4.02 – 3.97 (2H, m), 3.75 (3H, s), 3.74 – 3.71 (1H, d, J = 8.95), 3.14 – 3.12 (1H, d, J = 8.95), 2.56 – 2.50 (1H, dd, J = 5.80, 18.74), 2.26 – 2.18 (2H, m), 2.10 – 2.05 (1H, m), 2.02 (3H, s), 2.01 (6H, s), 1.96 – 1.89 (2H, m), 1.87 – 1.79 (2H, m), 1.78 – 1.68 (1H, m), 1.64 – 1.42 (4H, m), 1.35 - 1.18 (2H, m), 1.10 – 1.00 (2H, m), 0.95 (6H, s). ^{13}C-NMR (100 MHz, $CDCl_3$): 170.73, 170.32, 169.52, 169.14, 167.48, 138.37, 137.41, 101.41, 81.17, 76.98, 72.65, 72.28, 71.43, 69.65, 53.00, 50.13, 47.14, 40.33, 36.03, 34.81, 34.59, 33.90, 30.25(2C), 27.74, 23.06, 22.53, 21.86, 20.78 (2C), 20.66, 9.95. IR [cm^{-1}]: 2928, 2867, 1738, 1438, 1375, 1286, 1218, 1168, 1089, 1030, 908, 859, 796, 599, 556, 526, 494. $[\alpha]^{20}_{D}$ = -24.881 (c 0.89, dichloromethane). m.p.: 140 – 143 °C. HR-MS: [M+Na]: calcd: 643.2725, found 643.2749. [M+H]: calcd: 621.2906, found: 621.2910

Synthesis of 17α-(β-D-glucuronosyl-1-oxy)-17β-methyl-18-nor-2-oxaandrost-13-en-3-one (22)

Cleavage of the acetate protecting groups in presence of a lactone moiety relied on previously published procedures.[62,63]



Scheme 48: acetate cleavage and synthesis of OxLTM02 glucuronide

A solution of glycoside **74** (10 mg, 0.016 mmol) in methanol (1 mL) was cooled to 0°C. 0.5 mL of a potassium hydroxide stock solution was added (40 mg KOH dissolved in 3 mL water and 2 mL methanol, 5eq.) and the reaction was stirred at the same temperature for 20 minutes and then taken out of the cooling bath. The reaction was stirred for another 2 hours at room temperature. Amberlyst® 15 ion exchange resin was added until pH reached 6. The resin was filtered off over a short pat of silica and washed with methanol. Evaporation of the solvents *in vacuo* gave the crude product as colourless oil which was dissolved in 0.5 mL methanol and purified on a Waters preparative HPLC system with mass detection. The solvents used were water (+0.1 % formic acid) and methanol (+0.1 % formic acid). The gradient started at 85% methanol with a constant flow rate of 30 mL/min, reached 98% methanol over the course of 8 minutes and was followed by 1 minute of equilibration with the starting conditions. The product fractions were

combined, methanol evaporated *in vacuo* (water bath at 40 °C) and water removed by lyophilisation. 4 mg pure OxLTM02 glucuronide (**22**) (52 % yield) were obtained as white solid.

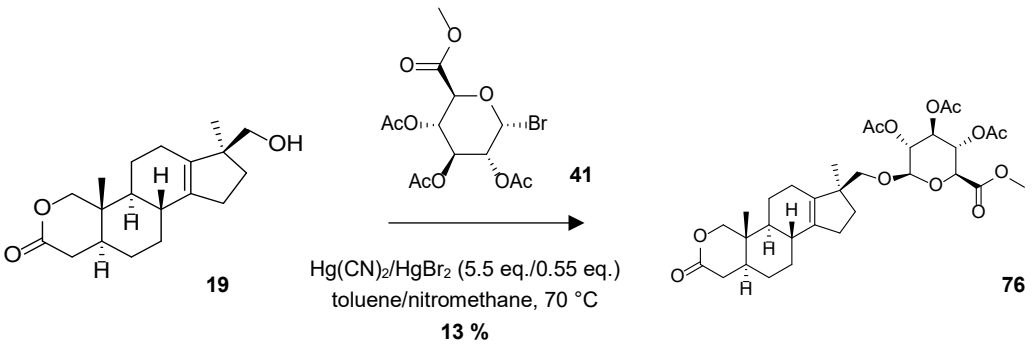
^{1}H-NMR (400 MHz, CD_3OD): δ = 8.15 (1H, s), 4.33 – 4.31 (1H, d, J = 10.63), 4.26 – 4.24 (1H, d, J = 7.76), 4.09 – 4.06 (1H, d, J = 10.78), 3.71 – 3.68 (2H, m), 3.53 – 3.48 (1H, dd, J = 9.13, 9.13), 3.38 – 3.34 (1H, dd, J = 9.13, 9.13), 3.27 (1H, m), 3.23 – 3.19 (1H, dd, J = 8.69, 8.69), 2.54 – 2.48 (1H, dd, J = 5.76, 18.85), 2.36 – 2.19 (2H, m), 2.14 – 1.91 (6H, m), 1.87 – 1.75 (1H, m), 1.70 – 1.61 (1H, m), 1.56 – 1.44 (2H, m), 1.35 – 1.23 (1H, m), 1.20 – 1.03 (3H, m), 1.02 (3H, s), 0.96 (3H, s).

^{1}H-NMR (600 MHz, CD_3OD): δ = 4.33 – 4.31 (1H, d, J = 10.63), 4.23 – 4.22 (1H, d, J = 7.69), 4.08 – 4.06 (1H, d, J = 10.73), 3.81 – 3.79 (1H, d, J = 9.16), 3.59 – 3.54 (1H, m), 3.49 – 3.43 (1H, m), 3.39 – 3.35 (1H, m), 3.25 – 3.19 (2H, m), 2.53 – 2.49 (1H, dd, J = 5.88, 18.80), 2.35 – 2.28 (1H, m), 2.27 – 2.17 (1H, m), 2.14 – 2.05 (2H, m), 2.04 – 1.92 (5H, m), 1.85 – 1.77 (1H, m), 1.69 – 1.58 (2H, m), 1.55 – 1.44 (2H, m), 1.18 – 1.06 (2H, m), 1.03 (3H, s), 0.96 (3H, s). ^{13}C-NMR (151 MHz, CD_3OD): 173.81 (2C), 139.62, 138.95, 105.03, 82.56, 78.01, 77.42, 75.06, 73.71, 51.36, 48.22, 47.82, 41.14, 37.31, 35.77, 35.52, 34.54, 31.46, 30.94, 28.55, 24.14, 23.63, 22.26, 9.97. IR [cm^{-1}]: 2924, 2860, 1716, 1446, 1410, 1372, 1211, 1030, 917, 880, 799, 677, 612, 561, 529, 492, 462. $[\alpha]^{20}_D$ = -18.999 (c 0.25, methanol). decomposition: 121 - 125°C. HR-MS: [M+H]: calcd: 481.2432, found: 481.2448.

4.9 SYNTHESIS OF OXLTM01 GLUCURONIDE

Synthesis of 17β-(methyl O^2,O^3,O^4-triacetyl-β-D-glucuronosyl-1-oxy)-17α-methyl-18-nor-2-oxaandrost-13-en-3-one (76) via Koenigs-Knorr glucuronidation

The synthesis of the glucuronide of OxLTM01 via Koenigs-Knorr glucuronidation relied on previously published procedures.[22,56,64]



Scheme 49: synthesis of OxLTM01 glucuronide via Koenigs-Knorr glucuronidation with mercury salt activation

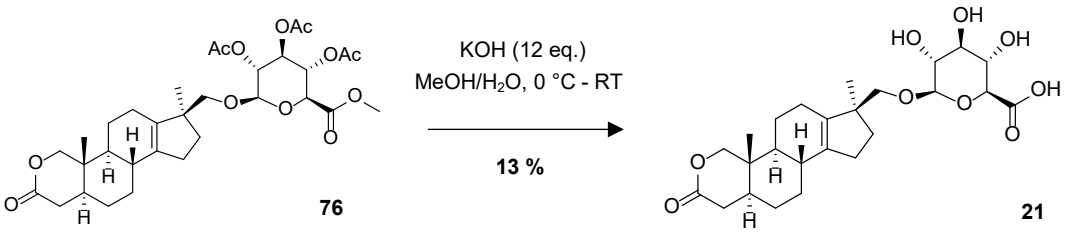
A flame dried 7 mL screw-cap vial was charged with a solution of OxLTM01 (15 mg, 0.05 mmol) in toluene (2 mL) and nitromethane (0.5 mL). Sugar **41** (97 mg, 5 eq.), $Hg(CN)_2$ (68 mg, 5.5 eq.) and $HgBr_2$ (9 mg, 0.55 eq.) were added at room temperature. The reaction was stirred for 4 hours at 70 °C and monitored via UPLC-MS. The mixture was diluted with diethyl ether and the insoluble mercury salts were filtered off. The solution was washed with saturated sodium bicarbonate solution and the organic phase was dried over magnesium sulphate. Evaporation of the solvents *in vacuo* gave the crude product as yellow oil which was dissolved in 1.5 mL methanol and separated on a C_{18} column of a Waters preparative HPLC system using a mass detector. The solvents used were water (+0.1 % formic acid) and methanol (+0.1 % formic acid). The gradient started at 80% methanol with a constant flow rate of 30 mL/min, reached 98% methanol over the course of 10 minutes and was followed by 1 minute of equilibration with the starting conditions. The product fractions were combined, methanol evaporated in vacuo (water bath at 40 °C) and water removed by lyophilisation. 4 mg of glycoside **76** (13 % yield) were obtained in pure form as white solid.

^{1}H-NMR (400 MHz, CD_2Cl_2): δ = 5.25 – 5.21 (1H, dd, J = 9.45, 9.45), 5.15 – 5.11 (1H, dd, J = 9.63, 9.63), 4.97 – 4.93 (1H, dd, J = 8.72), 4.51 – 4.49 (1H, d, J = 7.85), 4.29 – 4.26 (1H, d, J = 10.70), 4.03 – 4.01 (1H, d, J = 9.76), 3.95 – 3.93 (1H, d, J = 10.70), 3.73 – 3.71 (4H, m; therein 3H, s, methyl ester), 3.23 – 3.20 (1H, d, J = 9.22), 2.50 – 2.44 (1H, dd, J = 5.83, 18.65), 2.28 – 2.14 (2H, m), 2.13 – 2.04 (2H, m), 2.01 (3H, s), 1.99 (6H, s), 1.95 – 1.78 (4H, m), 1.77 – 1.66 (2H, m), 1.65 – 1.11 (4H, m), 1.09 – 0.99 (2H, m), 0.94 (6H, s)

^{1}H-NMR (600 MHz, CD_2Cl_2): δ = 5.25 – 5.21 (1H, dd, J = 9.67, 9.67), 5.14 – 5.11 (1H, dd, J = 9.77, 9.77), 4.96 – 4.93 (1H, dd, J = 7.66, 9.46), 4.50 - 4.49 (1H, d, J = 7.78), 4.28 – 4.26 (1H, d, J = 10.70), 4.03 - 4.01 (1H, d, J = 9.88), 3.95 – 3.93 (1H, d, J = 10.66), 3.72 – 3.70 (4H, m, therein 3H, s methyl ester), 3.22 – 3.20 (1H, d, J = 9.21), 2.48 – 2.44 (1H, dd, J = 5.80, 18.58), 2.28 – 2.15 (2H, m), 2.11 – 2.04 (2H, m), 2.01 (3H, s), 1.99 (3H, s), 1.98 (3H, s), 1.97 – 1.91 (2H, m), 1.90 – 1.79 (2H, m), 1.75 – 1.67 (1H, m), 1.63 – 1.57 (2H, m), 1.51 – 1.44 (3H, m), 1.07 – 0.99 (2H, m), 0.95 (3H, s), 0.94 (3H, s). ^{13}C-NMR (151 MHz, CD_2Cl_2): 170.47, 170.26, 169.74, 169.28, 167.75, 138.59, 137.96, 101.66, 81.36, 76.88, 72.81, 72.16, 71.42, 69.93, 53.11, 50.31, 47.39, 40.61, 36.12, 34.99, 34.75, 34.12, 30.59, 30.21, 27.86, 23.17, 22.83, 21.91, 20.89, 20.85, 20.69, 9.98. IR [cm^{-1}]: 2924, 2854, 1749, 1611 1438, 1368, 1214, 1097, 1036, 971, 888, 796, 772, 714, 657, 600, 562, 527, 494. $[\alpha]^{20}_D$ = -10.172 (c 0.29, dichloromethane), decomposition: 145 °C. HR-MS: [M+H]: calcd: 621.2911, found: 621.2899

Synthesis of 17β-(β-D-glucuronosyl-1-oxy)-17α-methyl-18-nor-2-oxaandrost-13-en-3-one (21)

Cleavage of the acetate protecting groups in presence of a lactone moiety relied on previously published procedures.[62,63]



Scheme 50: acetate cleavage and synthesis of OxLTM01 glucuronide

A solution of glycoside **76** (11 mg, 0.018 mmol) in methanol (0.5 mL) was cooled to 0 °C. 1 mL of a potassium hydroxide stock solution (12 eq. KOH, 60 mg KOH dissolved in 3 mL water and 2 mL methanol) was added and the reaction was slowly warmed to room temperature under stirring. After 2.5 hours Amberlyst® 15 ion exchange resin was added until pH reached 6 and stirred until lactone re-cyclisation was observed via UPLC-MS. The resin was filtered off over a short pat of silica and washed with methanol. Evaporation of the solvents *in vacuo* gave the crude product as colourless oil which was dissolved in 0.5 mL methanol and purified on a Waters preparative HPLC system with mass detection. The solvents used were water (+0.1 % formic acid) and methanol (+0.1 % formic acid). The gradient started at 85% methanol with a constant flow rate of 30 mL/min, reached 98% methanol over the course of 8 minutes and was followed by 1 minute of equilibration with the starting conditions. The product fractions were combined, methanol evaporated in vacuo (water bath at 40 °C) and water removed by lyophilisation. 1.1 mg pure OxLTM01 glucuronide (**21**) (13 % yield) were obtained as white solid.

^{1}H-NMR (600 MHz, CD_3OD): δ = 4.35 – 4.33 (1H, d, J = 10.79), 4.23 – 4.22 (1H, d, J = 7.55), 4.06 – 4.04 (1H, d, J = 10.79), 3.79 – 3.78 (1H, d, J = 9.71), 3.66 – 3.64 (1H, d, J = 9.63), 3.49 – 3.46 (1H, dd, J = 9.25, 9.25), 3.37 - 3.36 (1H, dd, J = 9.25, 9.25), 3.29 (1H, m), 3.22 – 3.20 (1H, dd, J = 8.51, 8.51), 2.53 – 2.49 (1H, dd, J = 5.84, 18.75), 2.30 – 2.22 (2H, m), 2.15 – 2.04 (3H, m), 1.99 – 1.97 (1H, m), 1.92 – 1.87 (1H, m), 1.84 – 1.78 (1H, m), 1.66 – 1.64 (1H, m), 1.55 – 1.47 (2H. m), 1.41 – 1.29 (3H, m), 1.12 – 1.04 (2H, m), 0.99 (3H, s), 0.97 (3H, s). ^{13}C-NMR (151 MHz, CD_3OD): 173.77, 139.61, 139.18, 105.04, 82.55, 77.78, 76.72, 76.49, 74.88, 73.46, 51.37, 41.15, 37.11, 35.79, 35.36, 34.50, 31.49, 30.89, 28.54, 23.81, 23.53, 22.48, 10.04

IR [cm^{-1}]: 2924, 2864, 2645, 2323, 2286, 2230, 2162, 2075, 2050, 1980, 1715, 1446, 1409, 1384, 1247, 1210, 1034, 918, 801, 667, 609, 570, 539, 456. $[\alpha]^{20}_{D}$ = -45.635 (c 0.06, methanol), decomposition: 125 - 128 °C. HR-MS: [M+H]: calcd: 481.2432, found: 481.2431

4.10 DETECTION OF OXLTM GLUCURONIDES IN HUMAN URINE

Sample preparation

0.5 mL urine was diluted with 0.5 mL of an internal standard solution (methyltestosterone in MQ water with 0.1% formic acid added). The quality control samples (MQ and blank urine) were spiked with the synthesised reference materials OxLTM02 glucuronide and OxLTM01 glucuronide.

Urine samples were taken from an excretion study following the application of a single oral dose of 5 mg oxandrolone [Table 2].

Sample Code	Date	Volume (mL)	pH	Density (g/mL)	oxandrolone concentration (ng/mL)
Oxa00	2000-08-02 15:10:00	278	5.00	1.008	0
Oxa01	2000-08-02 17:30:00	237	5.00	1.020	15.63
Oxa02	2000-08-02 20:00:00	208	5.50	1.020	144.23
Oxa06	2000-08-03 2:00:00	297	5.50	1.005	86.51
Oxa07	2000-08-03 7:00:00	285	5.80	1.015	283.85
Oxa08	2000-08-03 9:40:00	153	5.80	1.023	291.77
Oxa09	2000-08-03 14:00:00	269	7.00	1.022	75.58
Oxa10	2000-08-03 17:20:00	299	7.10	1.020	13.09
Oxa11	2000-08-04 13:00:00	251	7.10	1.027	9.74
Oxa12	2000-08-07 10:00:00	301	6.50	1.028	0

Table 2: urine samples taken after the oral application of 5 mg of oxandrolone

LC-HRMS conditions

LC-HRMS measurements were carried out on a Thermo Survey LC system coupled to a Thermo Vanquish QExactive mass spectrometer. Separation was carried out on a Phenomenex Kinetex (2.6µ Evo C18, 100 A, 100 x 2.1 mm) column equipped with a Thermo Accucore Phenylhexyl precolumn (2.6 µm, 10 x 2.1 mm). The solvents used were H_2O + 0.2% formic acid (biosolve) and acetonitrile (biosolve). The gradient started with a constant flow rate of 0.4 mL/min at 10 % acetonitrile, reached 65 % after 6 minutes and 100 % after 6.2 min. An isocratic elution of 1.3 min followed. Equilibration with the starting conditions took 2.5 minutes. The optimal injection volume was found to be 50 µL.

The samples were measured in Full-MS mode in a mass range from 250 to 1000 m/z applying electrospray ionisation in positive ion mode. The resolution was set to 35,000. The masses selected for MS/MS experiments were $[M+H]^+$: 307.22677 for oxandrolone and $[M+H]^+$: 481.24321 for the OxLTM glucuronides. Argon was used as collision gas and the collision energy for the fragmentation of oxandrolone was set to 20 eV and to 30 eV for the OxLTM glucuronides.

The mass transitions found for oxandrolone (and 17-epioxandrolone respectively) were 307.2261 → 289.2156 and 307.2261 → 271.2051. The most prominent fragments for the OxLTM glucuronides were found to be 481.2430 → 287.2001 and 481.2430 → 275.2002.

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6 ANNEX

6.1 NMR SPECTRA

OxLTM02 glucuronide with protecting groups

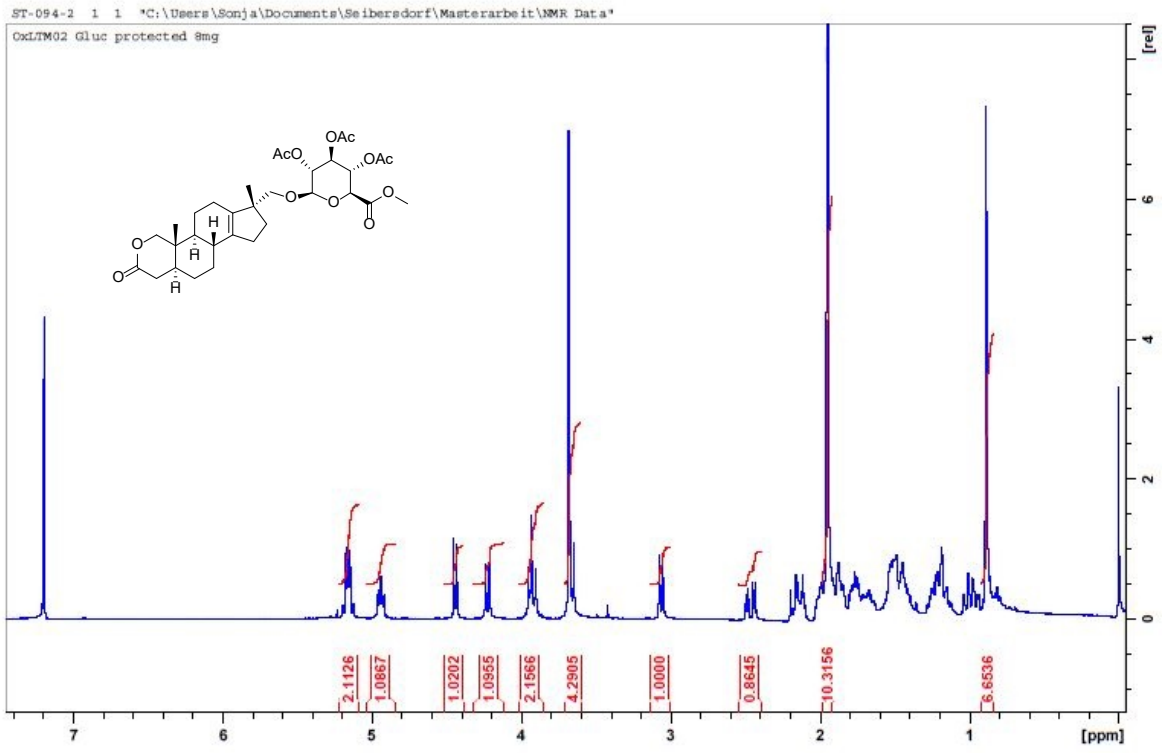

Figure 8: ^{1}H-NMR spectrum: OxLTM02 glucuronide with protecting groups

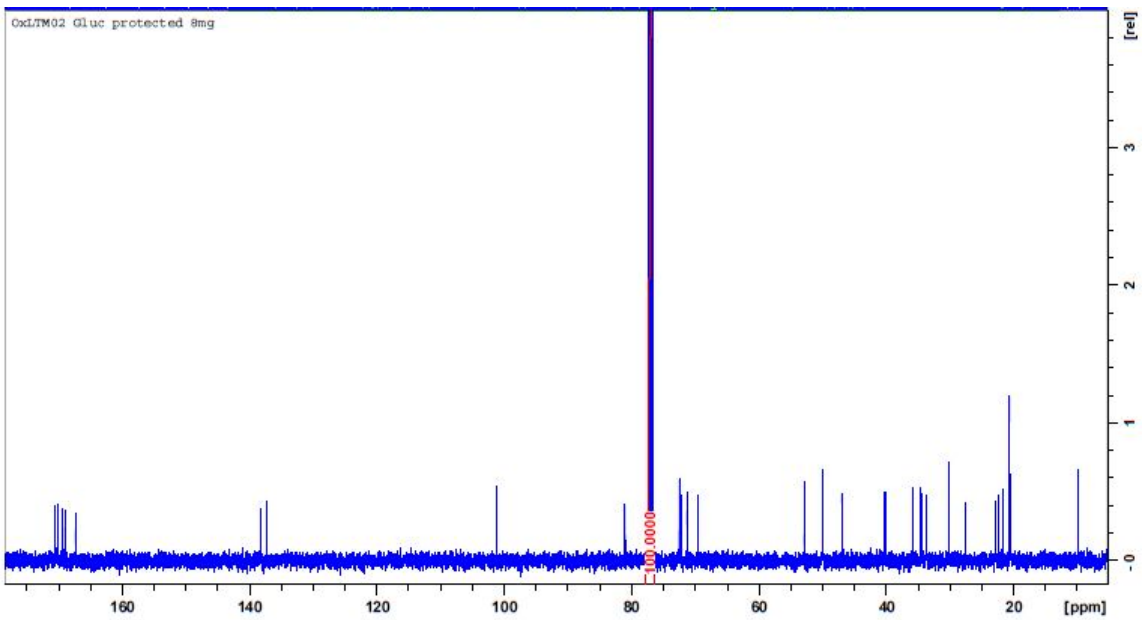

Figure 9: ^{13}C-NMR spectrum: OxLTM02 glucuronide with protecting groups

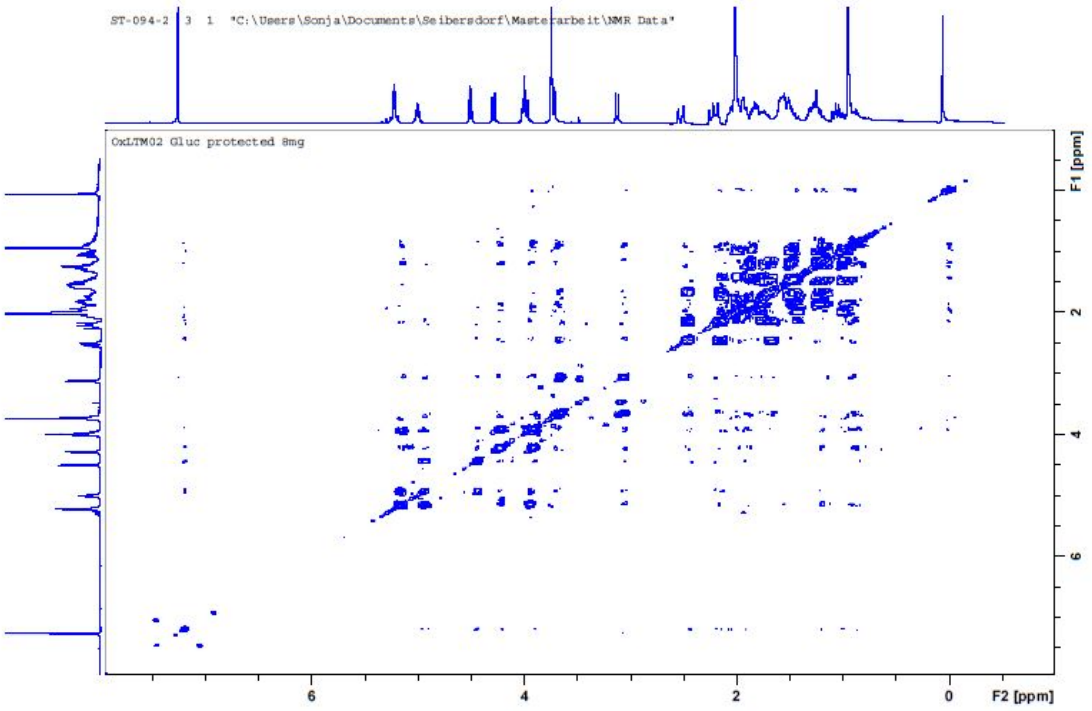

Figure 10: COSY spectrum: OxLTM02 glucuronide with protecting groups

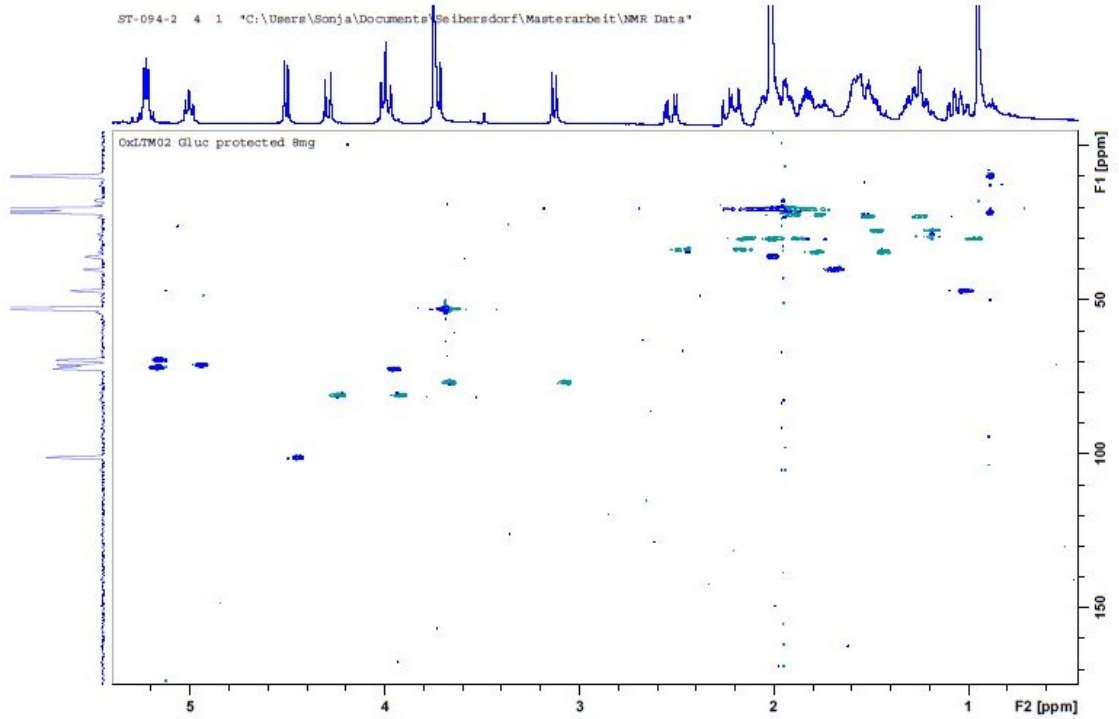

Figure 11: HSQC spectrum: OxLTM02 glucuronide with protecting groups

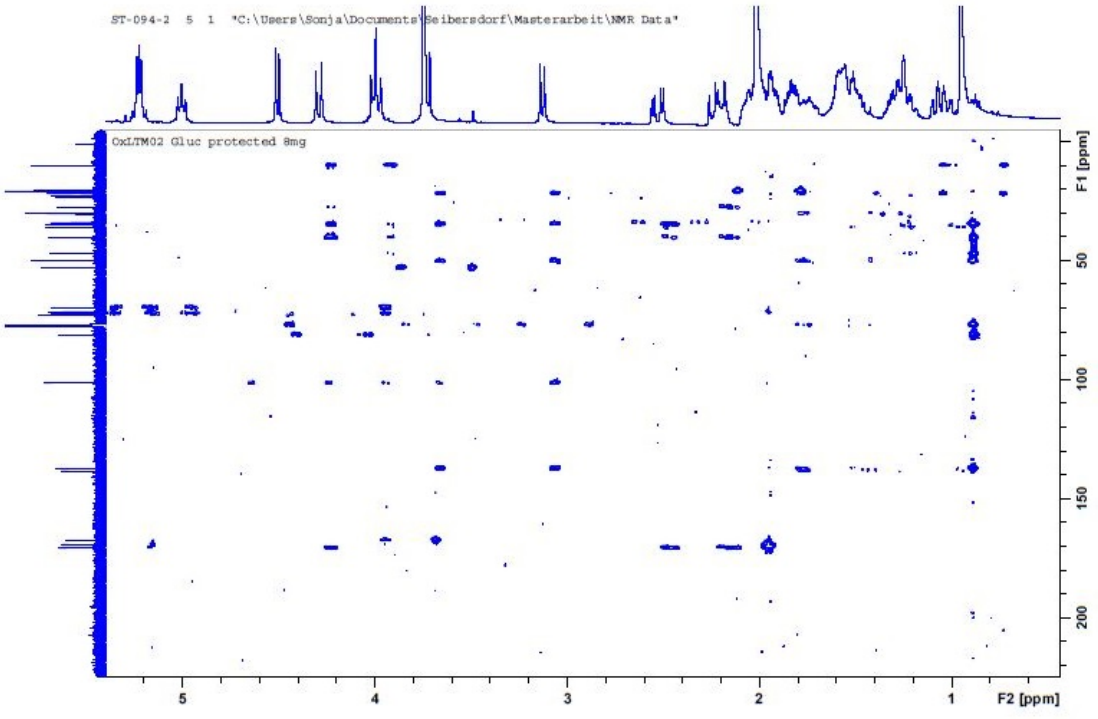

Figure 12: HMBC spectrum: OxLTM02 glucuronide with protecting groups

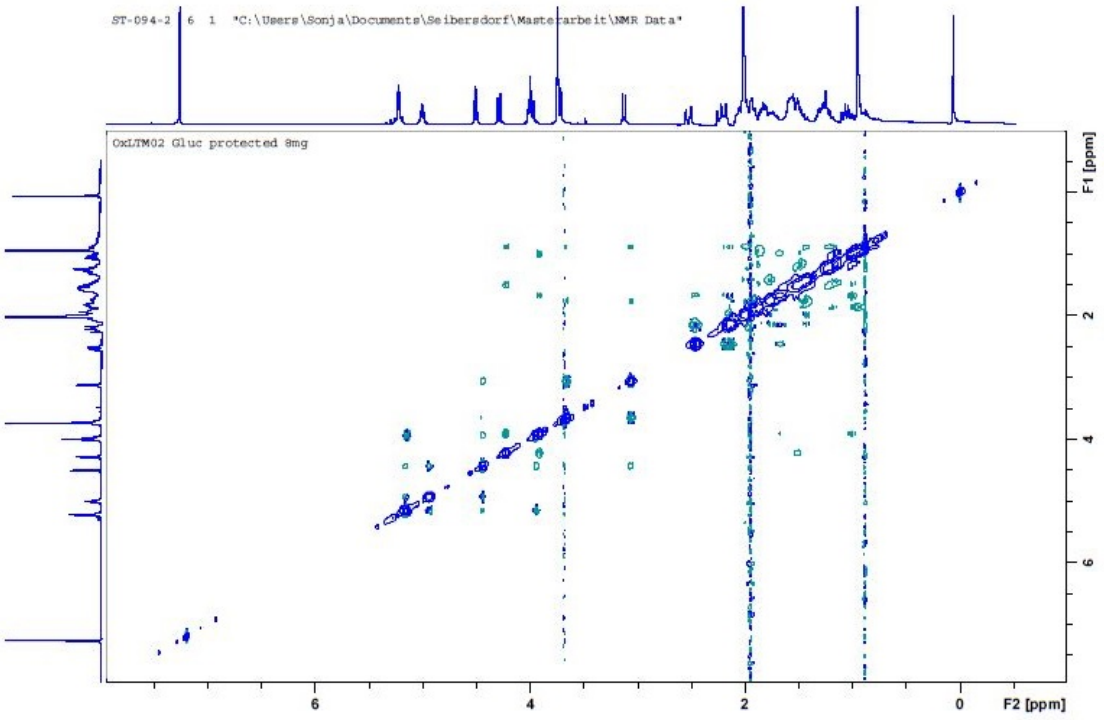

Figure 13: NOESY spectrum: OxLTM02 glucuronide with protecting groups

OxLTM02 glucuronide

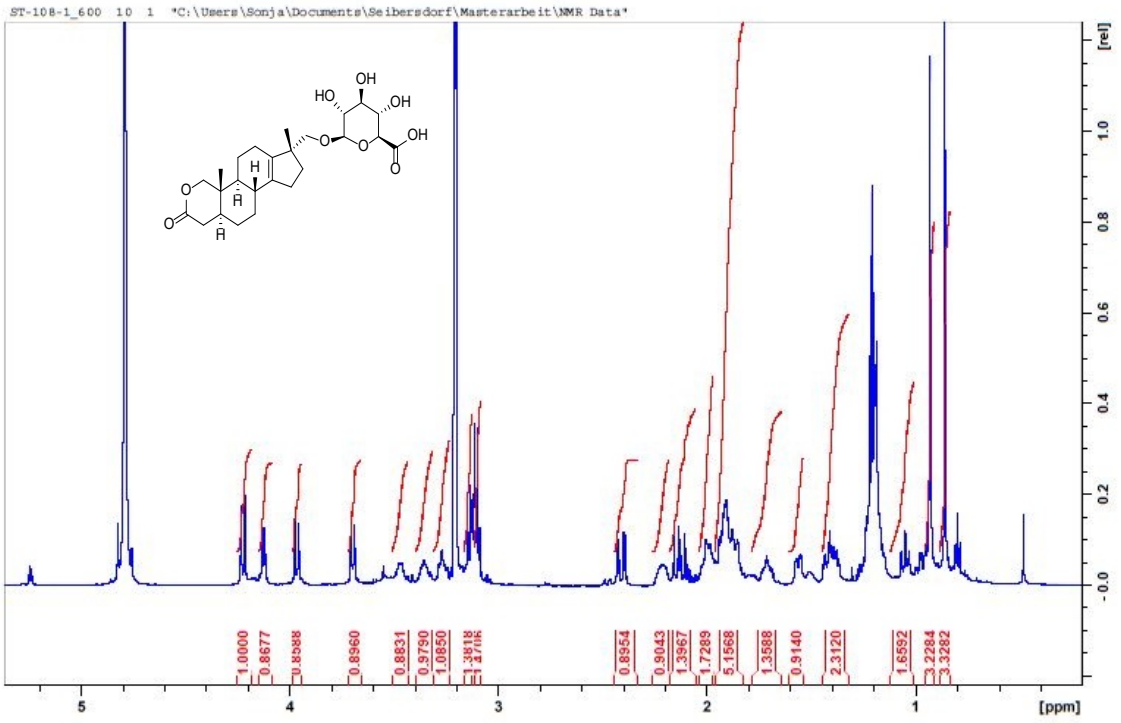

Figure 14: [1]H-NMR spectrum: OxLTM02 glucuronide

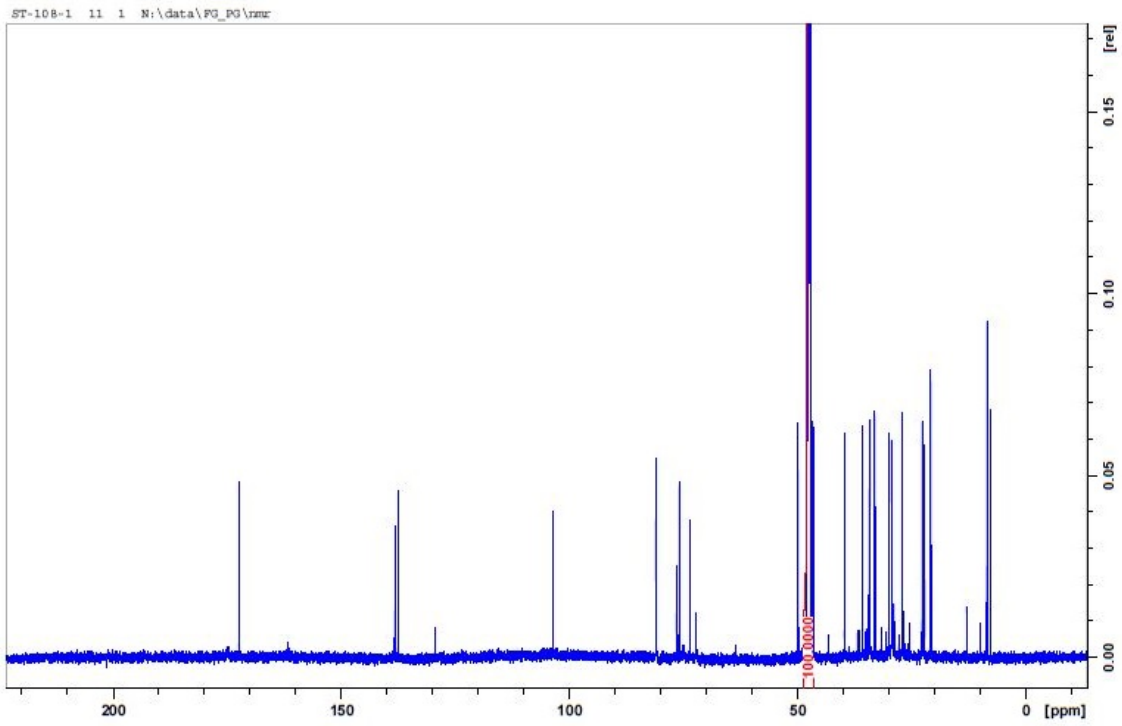

Figure 15: [13]C-NMR spectrum: OxLTM02 glucuronide

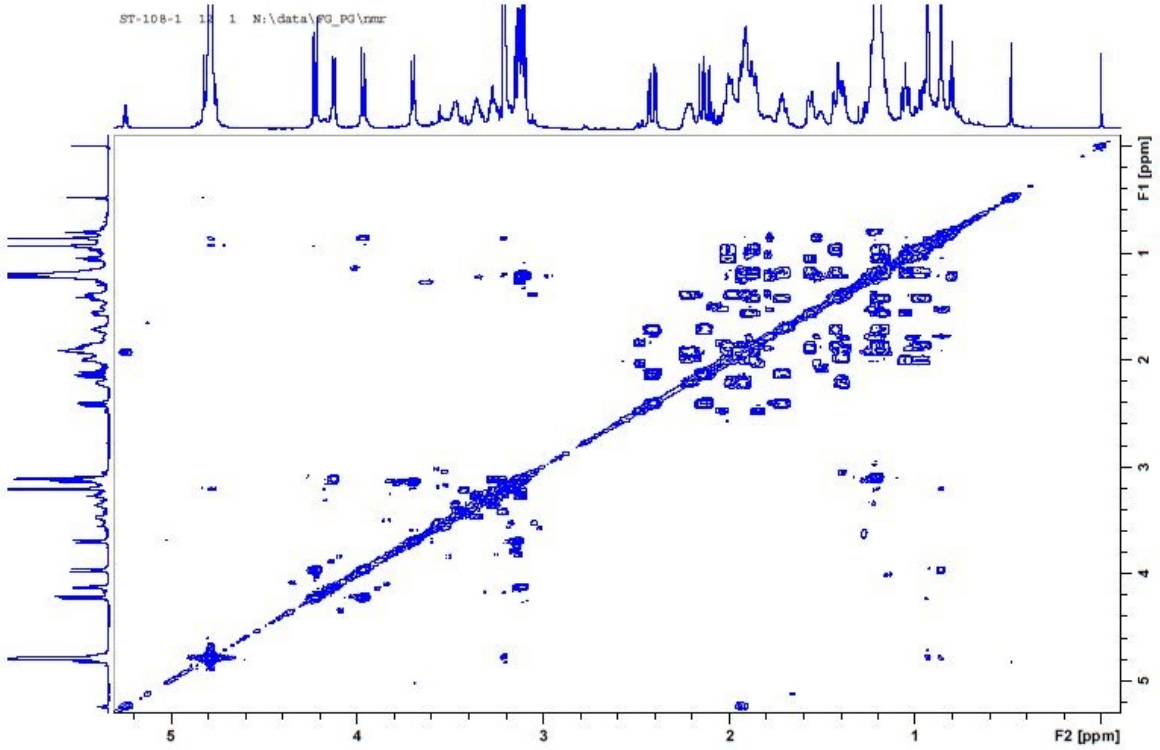

Figure 16: COSY spectrum: OxLTM02 glucuronide

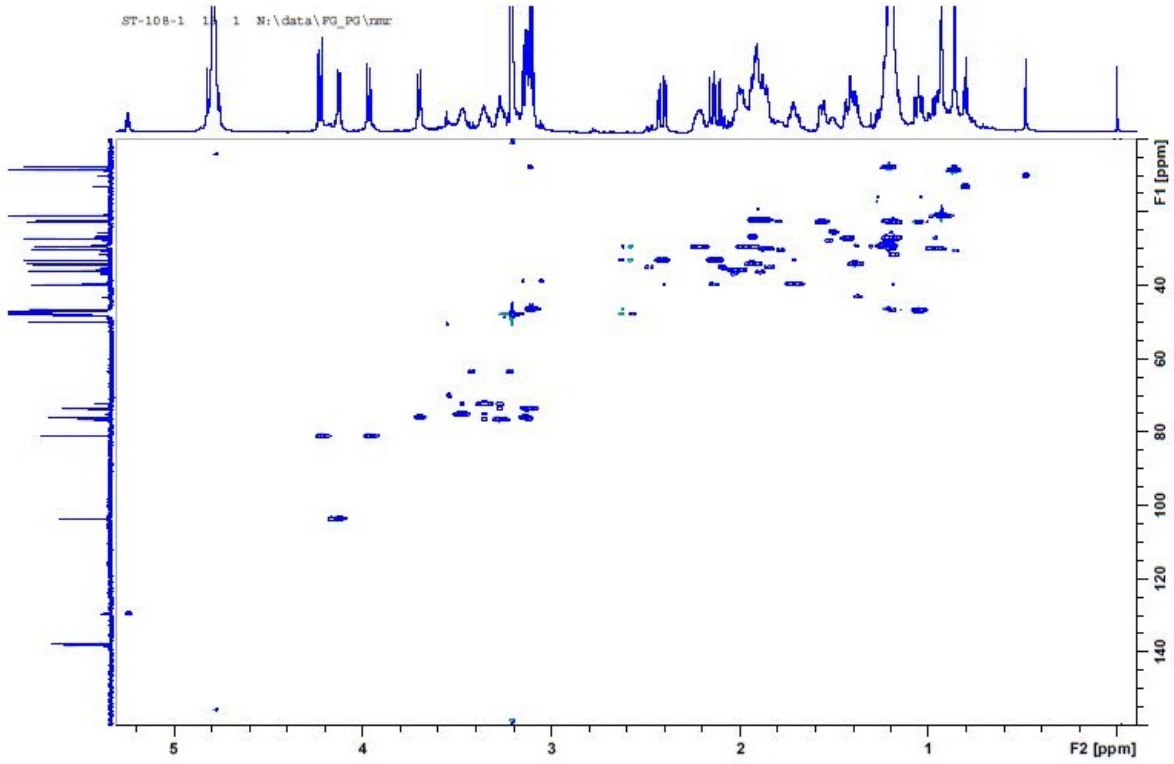

Figure 17: HSQC spectrum: OxLTM02 glucuronide

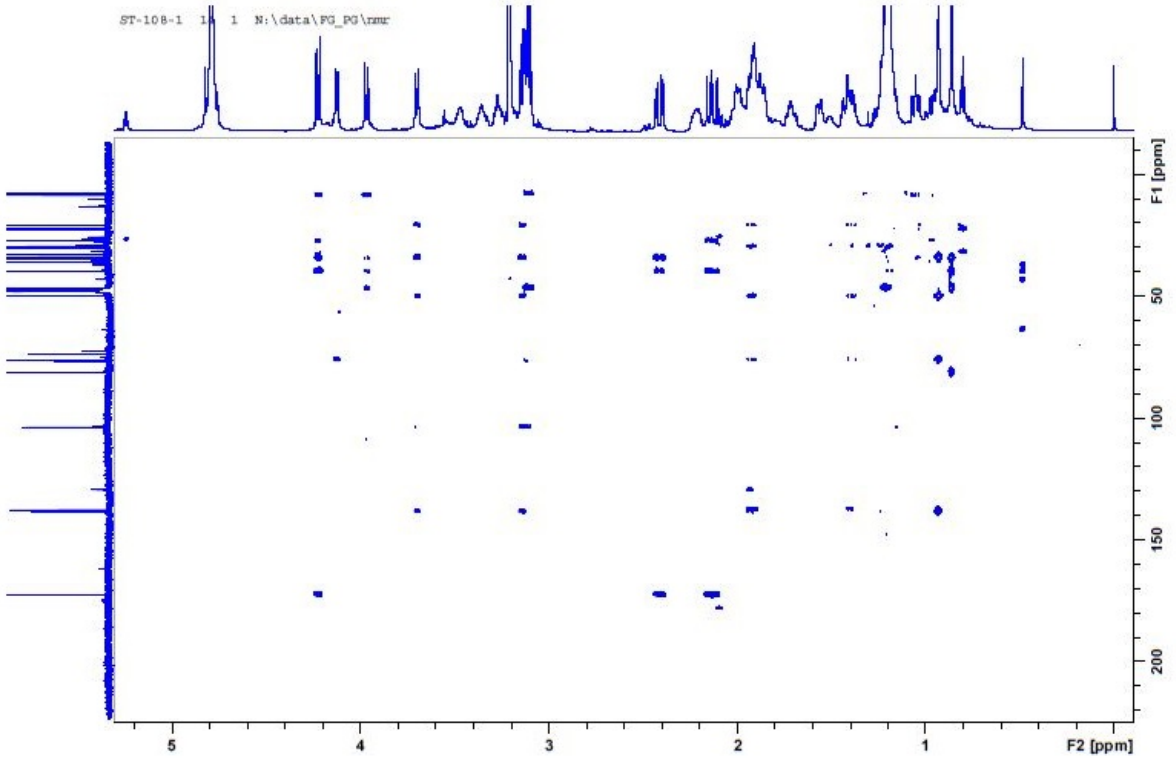

Figure 18: HMBC spectrum: OxLTM02 glucuronide

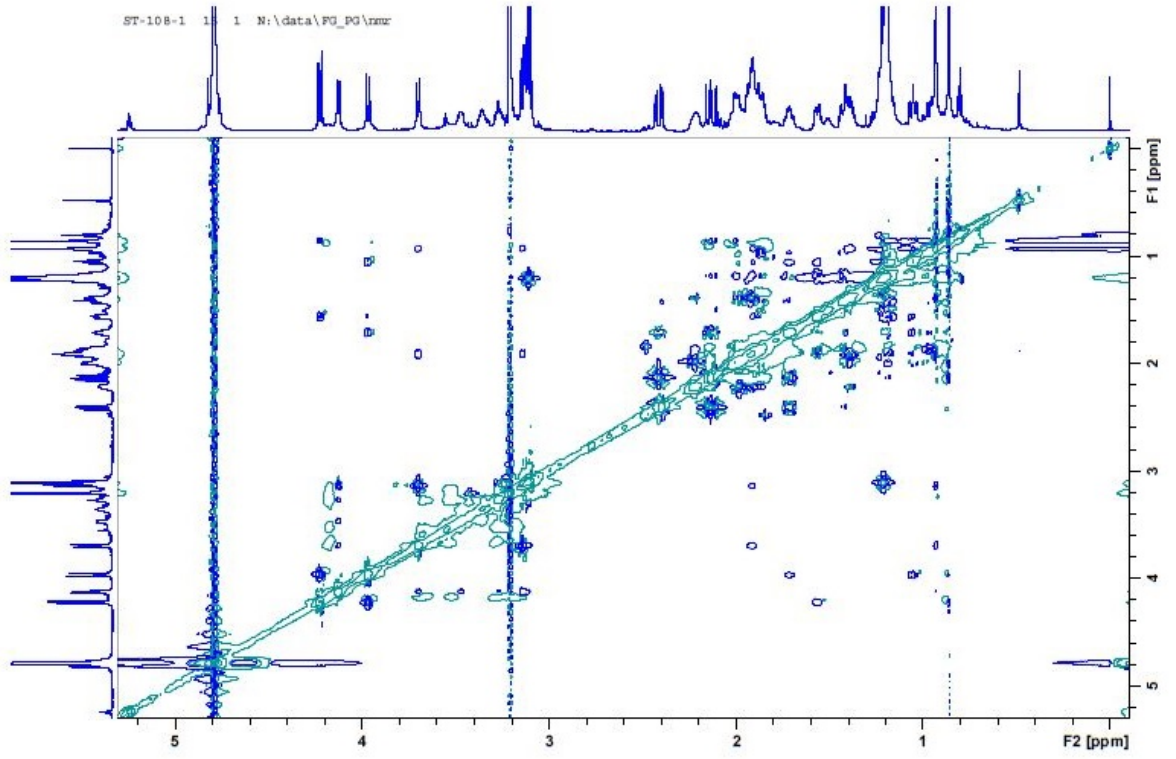

Figure 19: NOESY spectrum: OxLTM02 glucuronide

OxLTM01 glucuronide with protecting groups

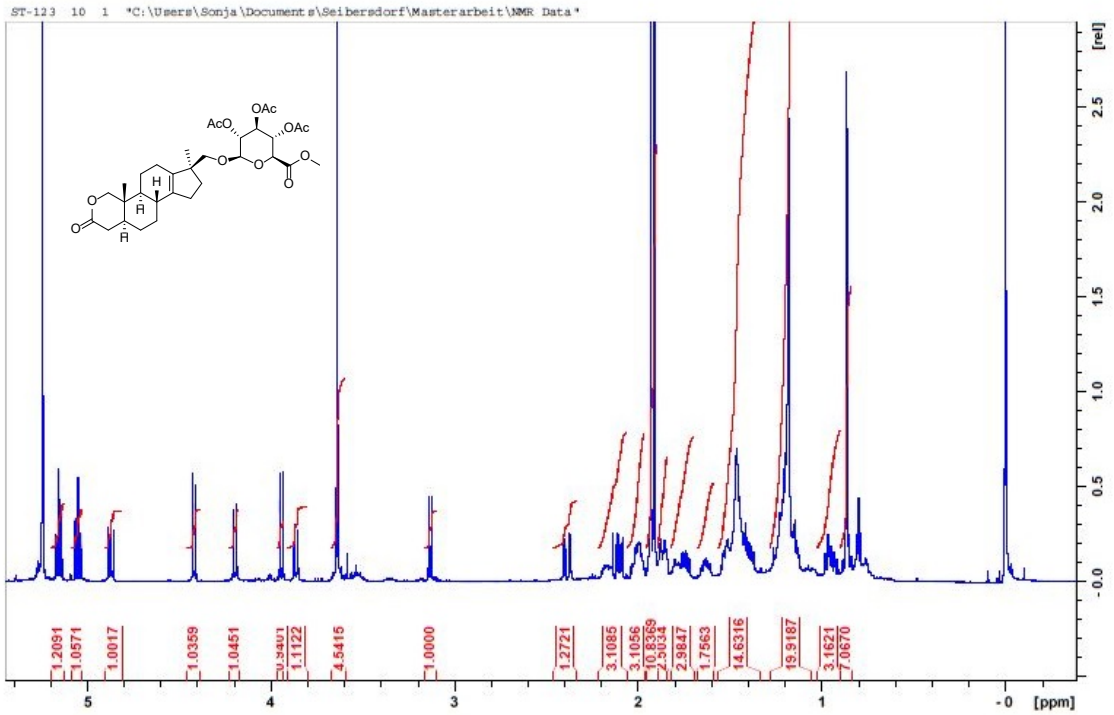

Figure 20: [1]H-NMR spectrum: OxLTM01 glucuronide with protecting groups

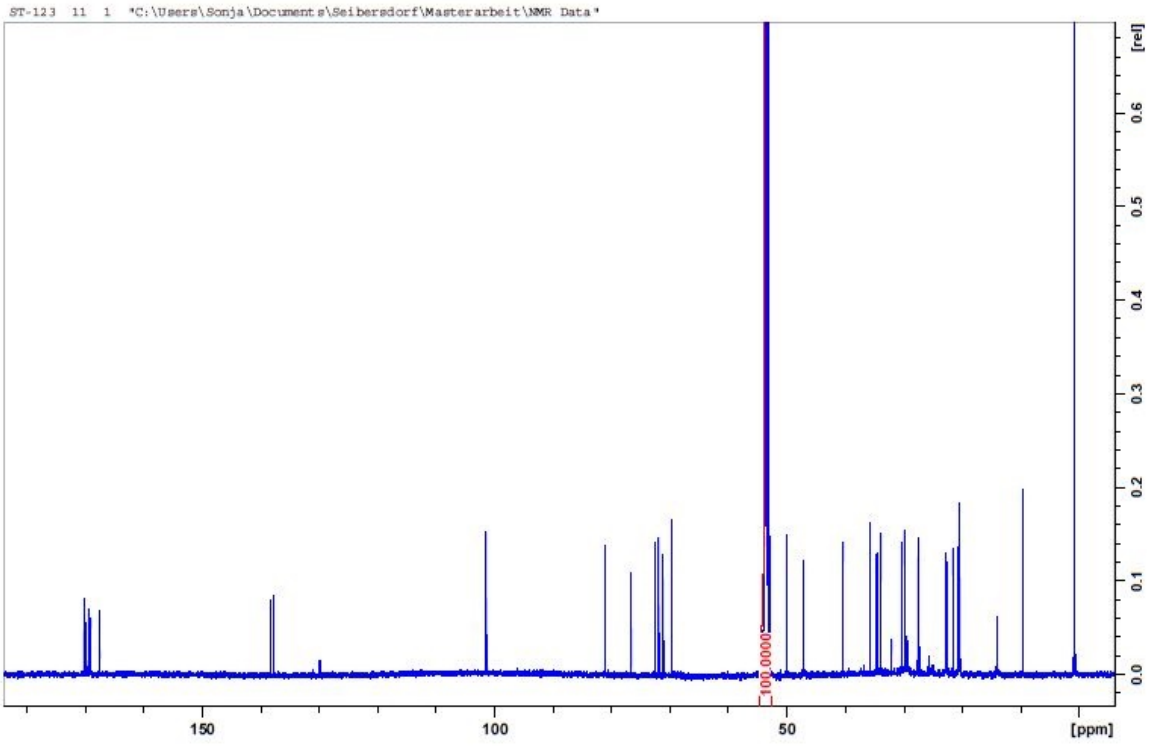

Figure 21: [13]C-NMR spectrum: OxLTM01 glucuronide with protecting groups

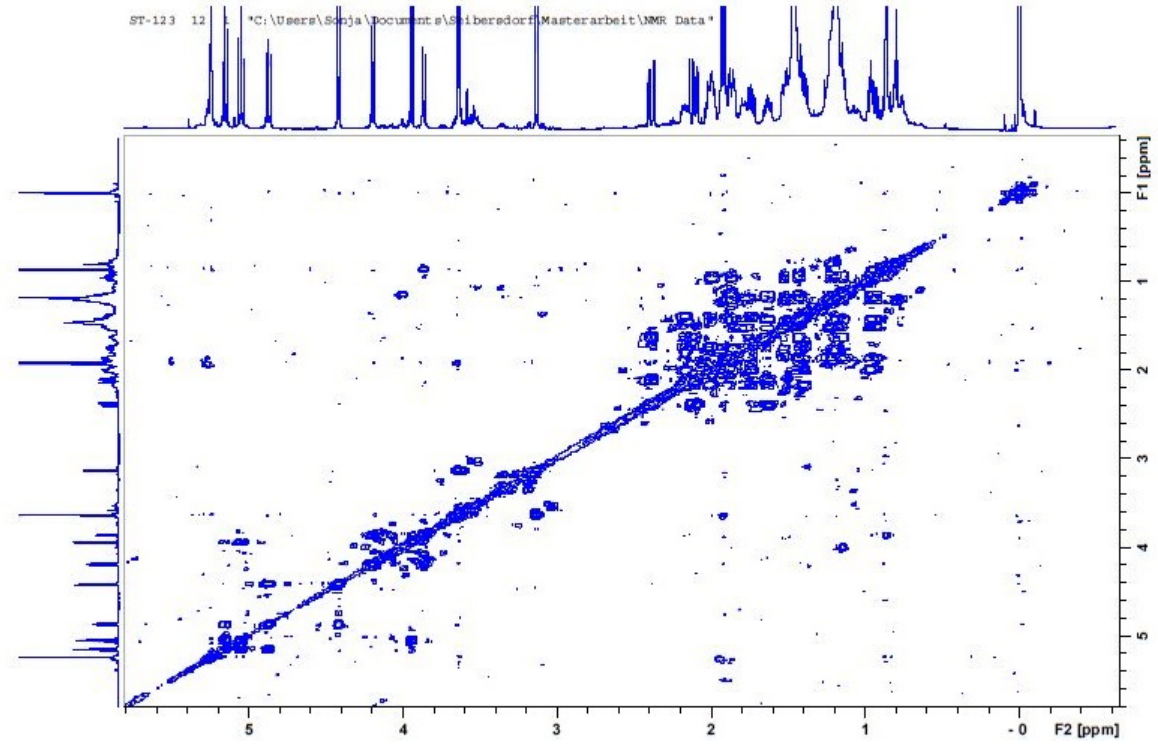

Figure 22: COSY spectrum: OxLTM01 glucuronide with protecting groups

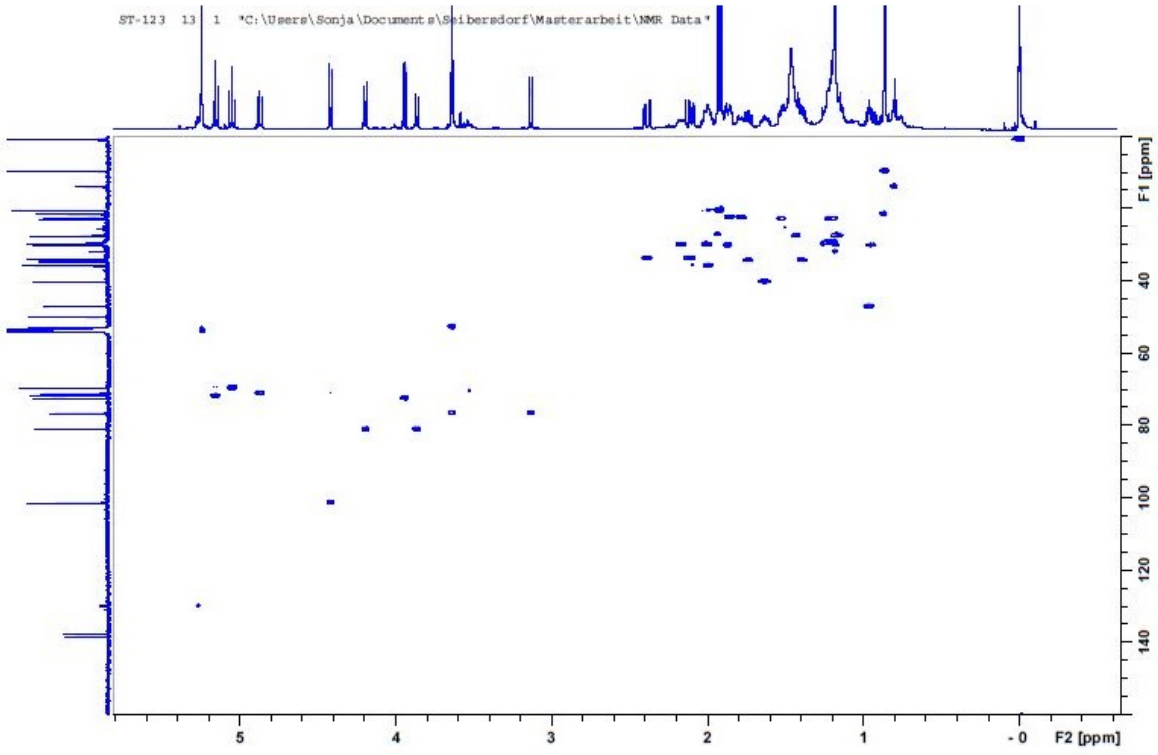

Figure 23: HSQC spectrum: OxLTM01 glucuronide with protecting groups

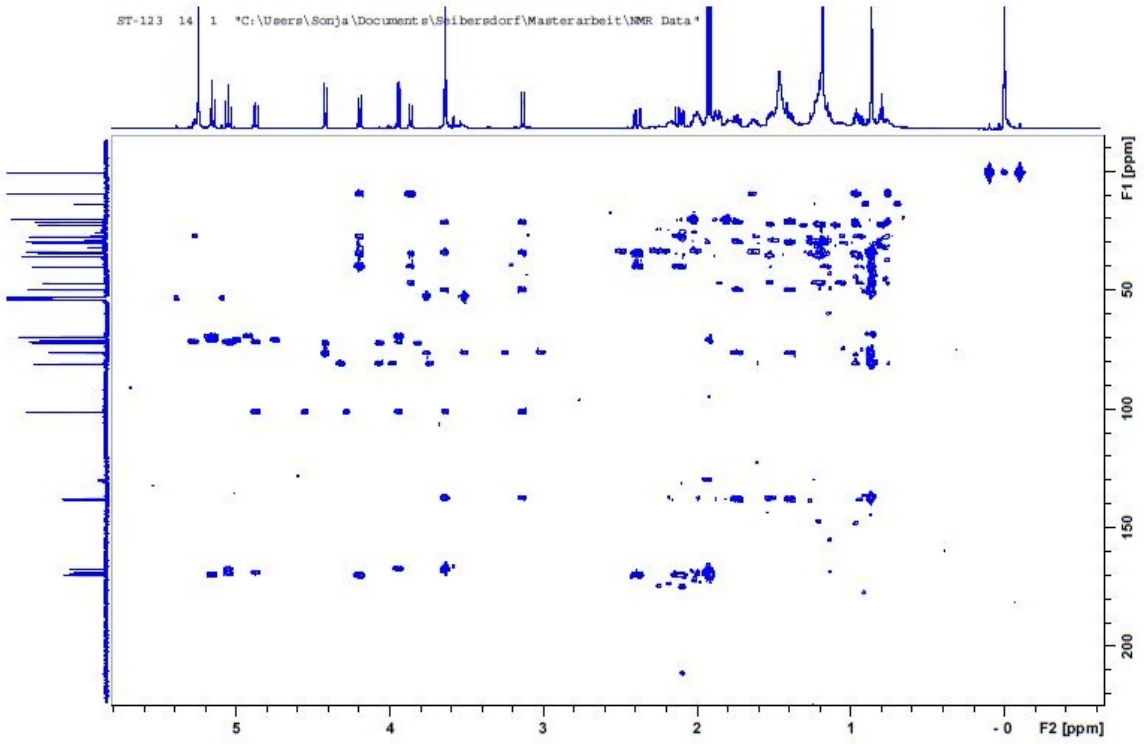

Figure 24: HMBC spectrum: OxLTM01 glucuronide with protecting groups

OxLTM01 glucuronide

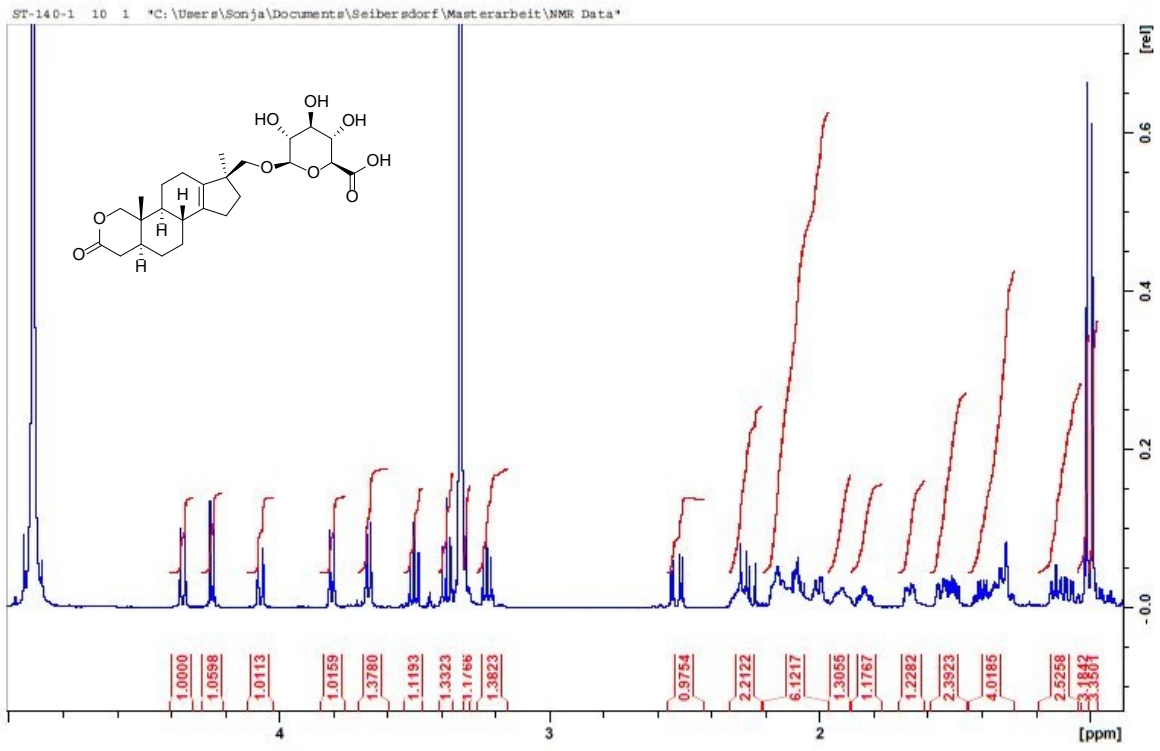

Figure 25: ^{1}H-NMR spectrum: OxLTM01 glucuronide

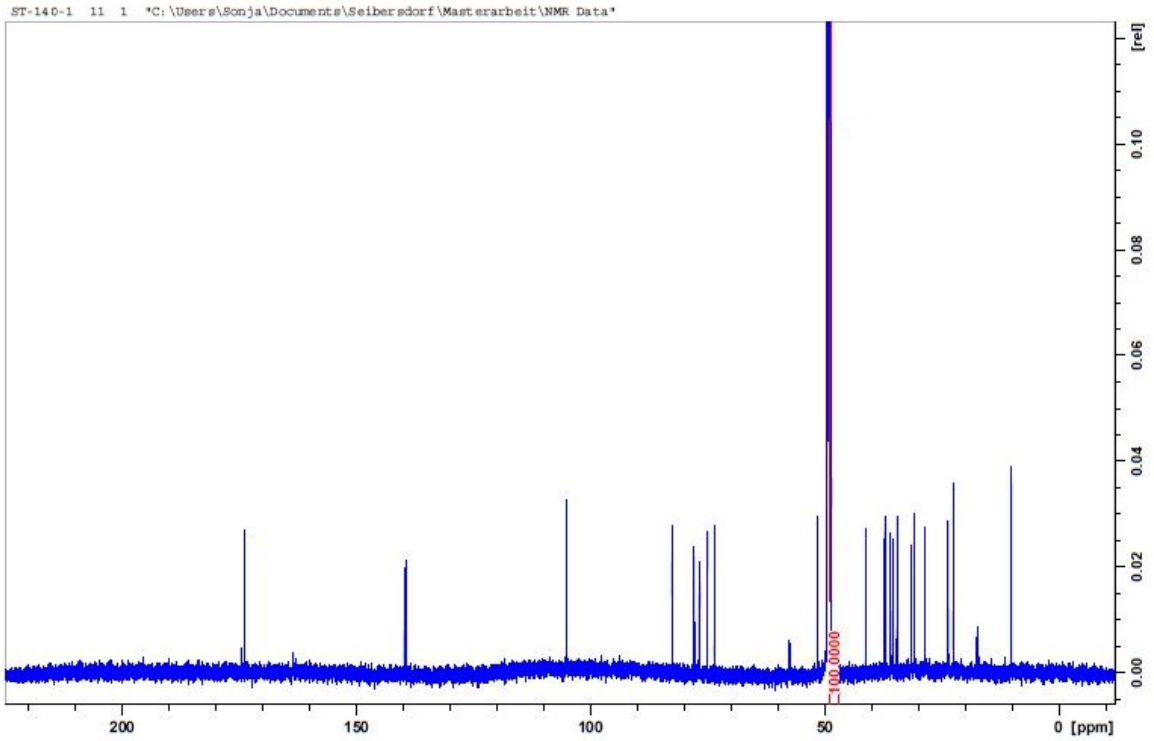

Figure 26: ^{13}C-NMR spectrum: OxLTM01 glucuronide

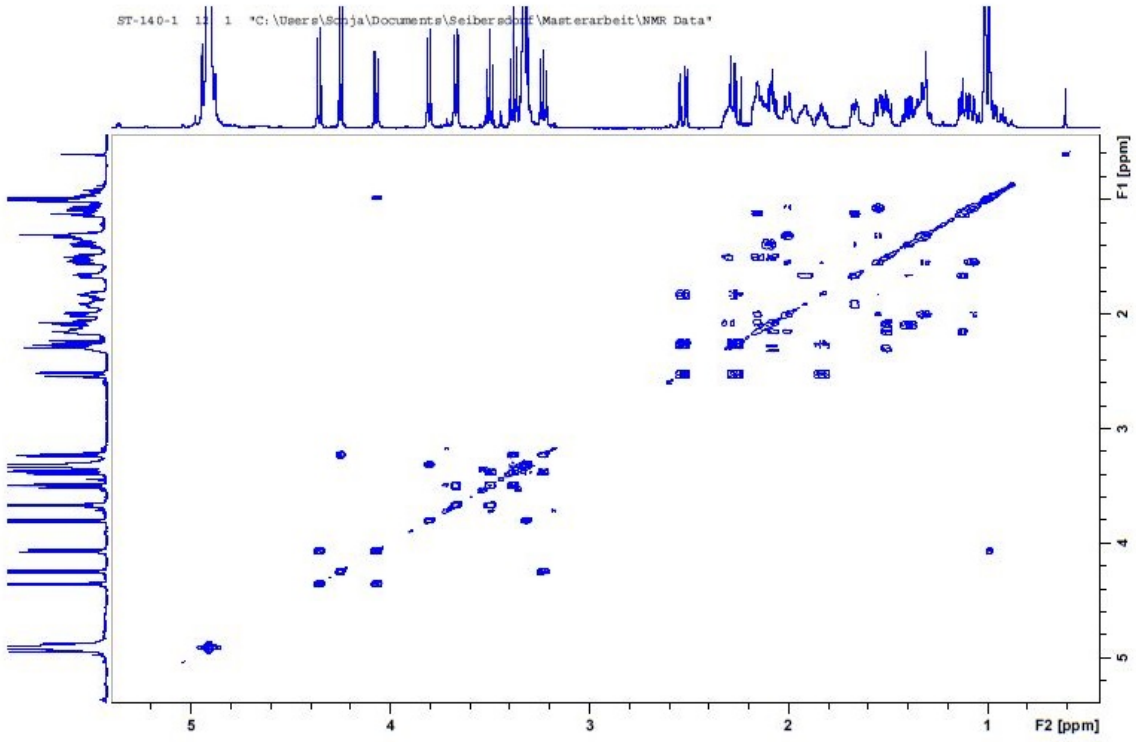

Figure 27: COSY spectrum: OxLTM01 glucuronide

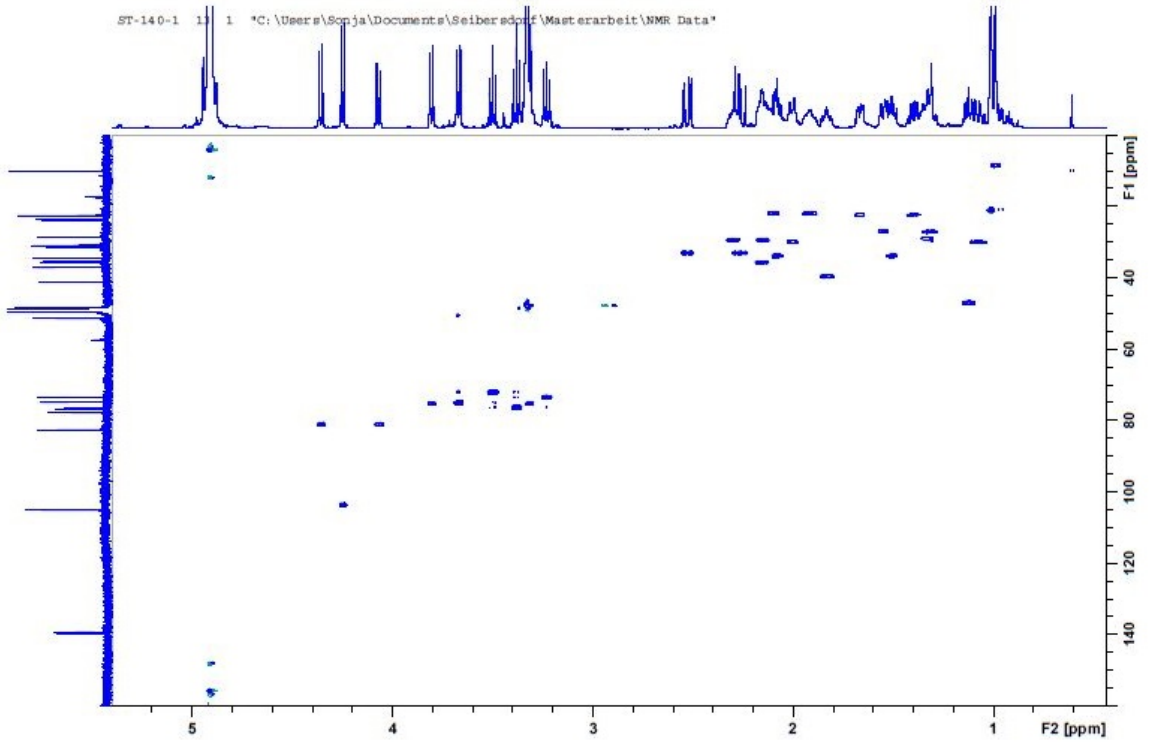

Figure 28: HSQC spectrum: OxLTM01 glucuronide

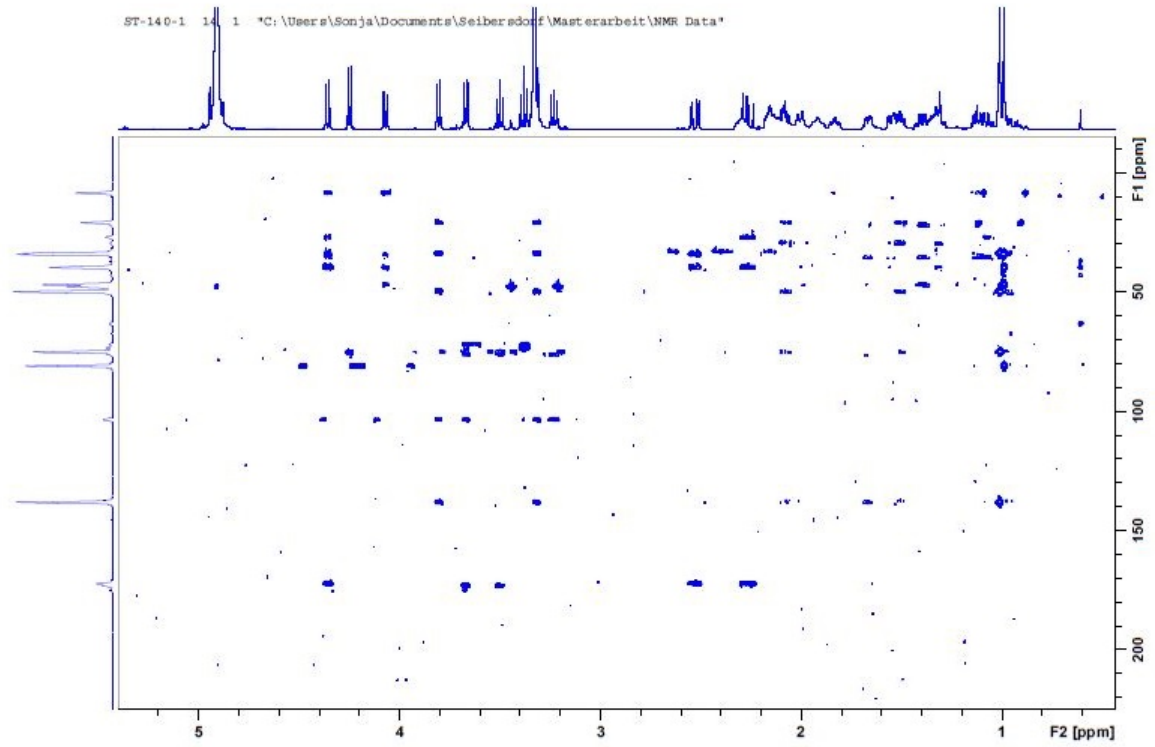

Figure 29: HMBC spectrum: OxLTM01 glucuronide

6.2 MASS SPECTRA

OxLTM02 glucuronide: High resolution MS/MS spectrum

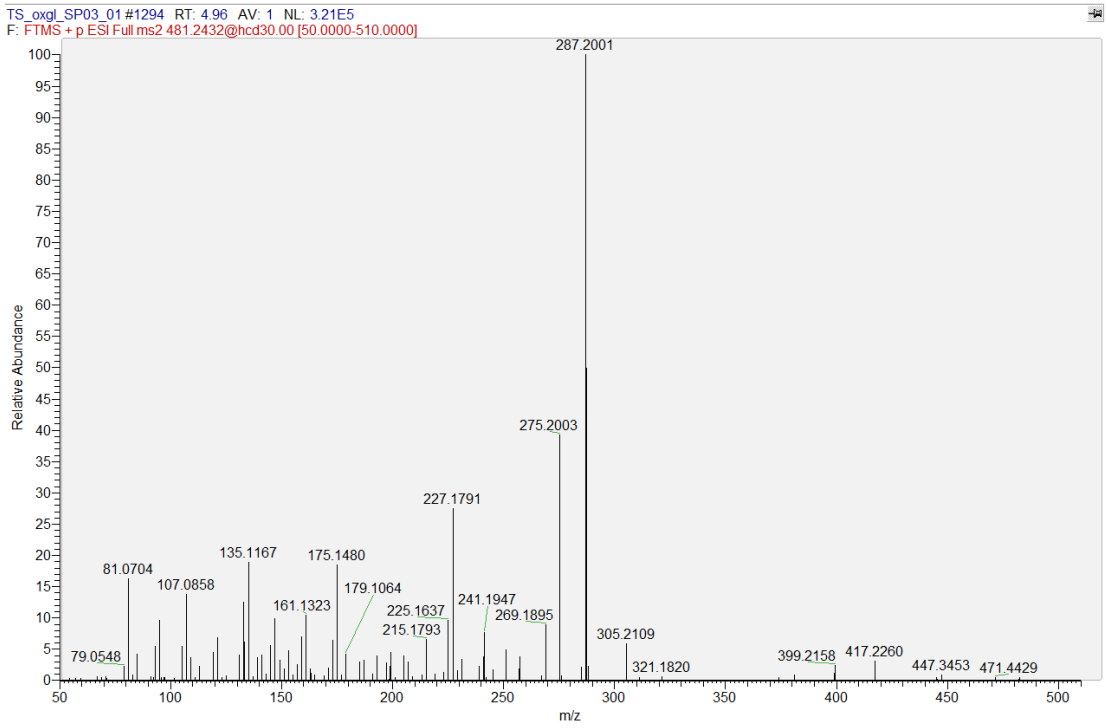

Figure 30: high resolution MS/MS spectrum recorded at 30 eV collision energy

OxLTM01 glucuronide: High resolution MS/MS spectrum

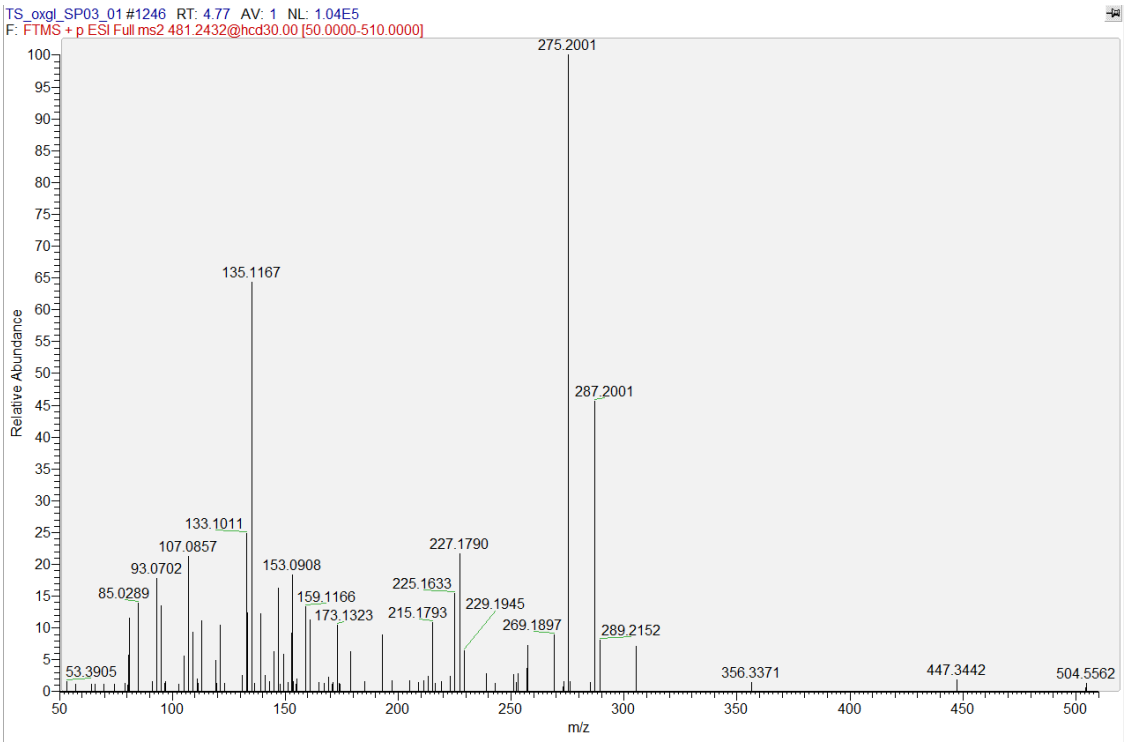

Figure 31: high resolution MS/MS spectrum recorded at 30 eV collision energy