TECHNISCHE UNIVERSITÄT WIEN

Dissertation

Synthesis of Glucuronidated Metabolites as Reference Substances for Doping Analysis

Ausgeführt zum Zwecke der Erlangung des akademischen Grades eines

Doktors der technischen Wissenschaften

unter der Leitung von:

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In conclusion, the efforts to search for a ‘better’ glycosylation reaction will not stop until non-specialists are able to make the glycosidic linkages in a manual way. The present review on a single glycosylation method with PTFAI donors could probably serve as ‘a single fallen leaf which tells of the oncoming autumn’ (a Chinese idiom 一叶知秋).

- Biao Yu

It´s better to have something than to have nothing.

– Peter Gärtner

Danksagung:

Zuerst möchte ich mich bei Prof. Peter Gärtner für die Betreuung dieser Arbeit bedanken. Seit meinem ersten Praktikum in seiner Forschungsgruppe sind nun fast neun Jahre vergangen, mehr als die Hälfte davon habe ich als Diplomand und Dissertand in seiner Gruppe verbracht. Ich hätte mich nicht besser entscheiden können! Danke für den immer geduldigen, aber hilfreichen Betreuungsstil und die Unterstützung in den für mich schwierigeren Zeiten.

Dr. Valentin Enev möchte ich für alle seine hilfreichen Anregungen, vorgetragen in seinem unverwechselbaren direkten Kommunikationsstil danken, sein oftmals lexikalisches Wissen war stets hilfreich beim Überwinden synthetischer Probleme.

Meinen StudentInnen Max Jöchl, Johanna Breinsperger und Lisa Ying danke ich für ihren Einsatz und ihre teils wichtigen Beiträge zu dieser Arbeit. An dieser Stelle sei auch die Mithilfe von Bagher Mohammadi erwähnt, der leider mit dem Jahr 2020 den denkbar schlechtesten Zeitpunkt für seinen Aufenthalt bei uns gewählt hat.

Meinem *Alter Ego* in Seibersdorf, Lorenz Göschl, danke ich für die gute Zusammenarbeit und die vielen unternommenen Fahrradkurierfahrten.

Meinen long-term-Laborkollegen David, Max, Nik und Philipp danke ich für die allzeit intellektuell stimulierende Umgebung im Labor, die vielen Stunden guter Unterhaltung und musikalischer Untermalung, und jetzt zum Schluss die Hilfe in den letzten zwei Wochen.

Ich möchte auch allen meinen TU-KollegInnen außerhalb der FGPG für die „good times“ danken, insbesondere aus der FGBSC, FGHF, und „Otech“. Das enge soziale Gefüge ist denke ich einer der Hauptgründe, warum sich die Zeit an der TU so „heimelig“ anfühlt, und ich werde es sicherlich vermissen.

Special shoutout auch an alle, die beteiligt waren mich während meiner Zeit an der TU zu ernähren: Die „Cooking Group“, Anna, „Jour Fixe“ – Robert und David, der mich unermüdlich mit Cola aus den Automaten versorgt hat.

Des Weiteren möchte ich in der Kategorie „hilfreiche Uni-Menschen“ noch besonders Sabine Stiedry und Florian Untersteiner hervorheben.

Auch außerhalb der Uni gibt es Leben: ich möchte all meinen FreundInnen danken, für all die gemeinsamen Unternehmungen und Erlebnisse in den vergangenen Jahren, die wichtig waren als ständiger Ausgleich zu meiner Arbeit:

Robert, Valle, Heci, Vroni, Nici, Resi, Max, Drasi, Fab, Dani, Niko & „Wienfreunde“, Manu, Felix & „Salzburgfreunde“, Miri, Motte, Michi, Gerry, Alina, Georg, Moelli *et. al.*

Zum Schluss gilt an dieser Stelle, dem vorläufigen Ende meines Bildungsweges, mein größter Dank noch meiner Mutter Ursula und meinem 2020 verstorbenen Vater Franz, die mich erst jahrelang gehegt und gepflegt und dann noch viele Jahre immer in meinem Werdegang unterstützt haben.

Abstract:

The goal of this work was the synthesis of different human primary and secondary metabolites as reference substances for doping analysis. The target compounds were mainly glucuronides of anabolic androgenic steroids (AAS) or glucuronides of their phase-I metabolites. The glucuronidation of the selective androgen receptor modulator (SARM) ostarine was also investigated. Namely, the synthesized metabolites include epiandrosterone glucuronide, metenolone glucuronide, epistanozolol glucuronide, trenbolone glucuronide and ostarine glucuronide.

The main effort throughout this work was spent on finding and synthesize suitable donor materials and reaction conditions for the glucuronidation of those target compounds that had either low nucleophilicity, were unstable under harsh reaction conditions, or combinations of both. A special focus was to find methods to prevent otherwise ubiquitous orthoester formation during the glucuronidation reaction. A silylated glucuronidation donor with distorted conformation was synthesized and studied by X-ray crystallography and DFT-calculations.

In cooperation with Seibersdorf Laboratories, the identities of two glucuronidated metabolites of dehydrochloromethyltestosterone were elucidated and efforts were made towards the synthesis of one of these compounds.

Kurzfassung:

Das Ziel dieser Arbeit war die Synthese verschiedener primärer und sekundärer menschlicher Metaboliten als Referenzsubstanzen für die Dopinganalyse. Die Zielverbindungen waren hauptsächlich Glucuronide von anabolen androgenen Steroiden (AAS) oder Glucuronide ihrer Phase-I-Metaboliten. Die Glucuronidierung des selektiven Androgenrezeptor-Modulators Ostarin wurde ebenfalls untersucht. Die synthetisierten Metaboliten umfassen Epiandrosteron-Glucuronid, Metenolon-Glucuronid, Epistanozolol-Glucuronid, Trenbolon-Glucuronid und Ostarin-Glucuronid.

Der Hauptaufwand in dieser Arbeit bestand darin, geeignete Donormaterialien und Reaktionsbedingungen für die Glucuronidierung jener Zielverbindungen zu finden und zu synthetisieren, die entweder eine geringe Nukleophilie aufwiesen, unter harschen Reaktionsbedingungen instabil waren oder Kombinationen aus beidem. Ein besonderer Schwerpunkt lag darauf, Methoden zur Verhinderung der sonst allgegenwärtigen Orthoesterbildung während der Glucuronidierungsreaktion zu finden. Ein silyliertes Donormolekül mit verzerrter Konformation wurde synthetisiert und mittels Röntgenkristallographie und DFT-Berechnungen untersucht.

In Zusammenarbeit mit den Seibersdorf Laboratories wurden die Identitäten zweier glucuronidierter Metaboliten von Dehydrochloromethyltestosteron aufgeklärt und es wurden Anstrengungen unternommen, um eine Synthese einer dieser Verbindungen zu ermöglichen.

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1 Introduction

1.1 Doping and Antidoping

1.1.1 History of Performance Enhancing Drugs

Ever since the advent of humanity, people have sought to improve their physical abilities, cognitive function and overall performance by the consumption of bioactive substances. The history of these substances spans thousands of years, from ancient civilizations to the present day.

From ancient civilizations, we know examples where they were employed for a variety of purposes:

Ancient Egyptians used various herbs and plants, such as the blue lotus, to enhance cognitive function and increase stamina [1]. The inhabitants of classical Greece consumed a variety of herbs and fungi, including hallucinogenic mushrooms [2], to improve their physical and mental performance in athletic competitions and warfare. Roman gladiators are mentioned as users of various blends of strength-increasing substances [3].

In medieval Europe and numerous other societies, alchemists and philosophers sought to create the "elixir of life" or "philosophers stone", meant to grant eternal youth and fitness [4].

In the 16th and 17th centuries caffeine was introduced to Europe in the form of coffee and tea. The mental stimulant supported the thinkers of enlightenment and the first natural scientists [5], and arguably continues to be important in this role to this day.

In the early 20th century, synthetic stimulants such as amphetamine and methamphetamine were first synthesized and extensively used by both sides in the second world war [6].

After the second world war, the era of synthetic anabolic androgenic steroids (AAS) began, (see chapter 1.2.1) and with it came the state-sponsored doping programs in the GDR and USSR, but also organized efforts in Western states [7].

Developments in the area of doping continue ceaselessly to this day. New techniques such as gene doping [8] and new substance classes such as selective androgen receptor modulators (SARMs) and recombinant proteins ensure that the fight against doping will stay an interesting scientific pursuit for years to come.

1.1.2 Timeline of Antidoping

The history of organized anti-doping efforts begins only in the 20th century [9]:

- 1908: Tom Hicks, a winner of the 1904 Olympics and competitor in the 1908 St. Louis marathon, is the first known victim of doping and dies after his use of a cognac-strychnine mixture during the race.
- 1928: After multiple incidents, the International Athletics Federation (IAF) is the first organization to ban doping
- 1960: Swedish cyclist Knut Jensen dies during the Rome Olympics, reportedly due to amphetamine use. This event highlights the need for stricter doping controls in sports.
- 1967: British cyclist Tom Simpson dies during the Tour de France, with amphetamines found in his body. Simpson's death prompts the International Olympic Committee (IOC) to take action against doping.
- 1971: The IOC establishes the "Medical Commission", responsible for creating and implementing anti-doping policies.
- 1972: The first IOC-accredited laboratory for doping control is established in Munich, Germany, during the Munich Summer Olympics.
- 1974: Anabolic Androgenic Steroids (AAS) are prohibited by the IOC
- 1983: The IOC introduces blood testing for doping control in addition to urine testing.
- 1999: Following the Festina cycling team scandal during the 1998 Tour de France, the World Anti-Doping Agency (WADA) is established to coordinate global efforts against doping in sports.
- 2004: WADA introduces the World Anti-Doping Code, a set of anti-doping rules and regulations that all Olympic sports organizations must adhere to.
- 2015: The Russian Anti-Doping Agency (RUSADA) is declared non-compliant with the World Anti-Doping Code, following revelations of widespread, state-sponsored doping in Russian sports.
- 2021: WADA introduces the new World Anti-Doping Code, which includes more stringent punishments for athletes and support personnel involved in doping, as well as increased emphasis on education and prevention programs [10].

1.2 Anabolic Androgenic Steroids (AAS)

Anabolic androgenic steroids (AAS) are a group of synthetic substances that mimic the effect of testosterone in the human body. They are commonly associated with the promotion of muscle growth and the development of male secondary sexual characteristics. Since their discovery, AAS have been utilized for various medical purposes, ranging from hormone replacement therapy to the treatment of wasting associated with cancer and AIDS. Soon after their initial development, the powerful effects of AAS were discovered by athletes and doping with AAS is widespread ever since.

1.2.1 History of AAS

The history of AAS can be traced back to the 1930s when scientists began to investigate the potential therapeutic applications of male hormones. In 1931, Adolf Butenandt and Kurt Tschernig successfully isolated the hormone androsterone, after a heroic campaign of distilling 17.000 l of male urine. This milestone inspired a lot of research interest, culminating in the first synthesis of testosterone, performed in 1935 by Leopold Ružička. Butenandt and Ružička shared the 1939 Nobel prize for chemistry [11].

Following the synthesis of testosterone, researchers quickly became interested in the potential benefits of this hormone. Early studies focused on the anabolic (tissue-building) effects of testosterone, exploring its potential use in treating a variety of medical conditions. These studies later led to the development of the first synthetic anabolic steroid, known as metandienone, in the early 1950s [12].

The end of World War II marked a turning point for anabolic steroid research. As the world became aware of the devastating effects of the war, particularly on prisoners of war (POWs), scientists sought ways to treat the severe malnutrition and muscle wasting that these individuals experienced. The intention was to help POWs regain their strength and health after their release.

In the late 1940s and early 1950s, several research projects were initiated to study the potential use of anabolic steroids for POW rehabilitation. These studies revealed that AAS could indeed promote muscle growth and overall recovery in individuals suffering from severe malnutrition. The research conducted during this period laid the groundwork for the eventual clinical use of AAS in a variety of medical conditions, including chronic diseases, hormonal imbalances, and recovery from major surgeries.

As the medical applications of AAS became more widely known, athletes and bodybuilders began to recognize the potential performance-enhancing effects of these substances. In the 1950s and 1960s, AAS-use began to spread among athletes, particularly in sports that required strength and power, such as weightlifting, football or athletics [13].

Bodybuilding, in particular, saw a surge in the popularity of AAS use that continues to this day. Competitive bodybuilders began to use steroids as a means to increase muscle mass, reduce body fat, and improve overall physical appearance. The use of AAS in bodybuilding quickly became widespread, with many athletes attributing their success to these substances [14].

The widespread use of AAS in sports led to growing concerns about the ethical and health implications of their use. Many sports organizations began to implement drug testing policies to discourage the use of performance-enhancing substances. The International Olympic Committee (IOC) introduced drug testing for the first time in 1972, with anabolic steroids added to the list of banned substances in 1976 [15].

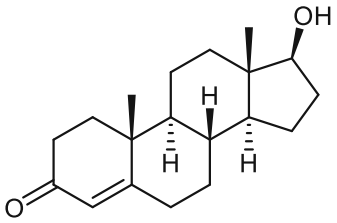
The dream of increased strength, muscle mass, and improved performance continued to drive the use of AAS among athletes and bodybuilders. The high-profile cases of athletes such as Lance Armstrong, Marion Jones, and Ben Johnson brought the issue of doping in sports to the forefront of public consciousness, sparking widespread debate about the role of AAS in sports and the lengths to which athletes would go to gain a competitive edge.

In recent times, the most significant case of doping was the Russian doping scandal. It came to light in 2015 when German television published a report detailing widespread state-sponsored doping in Russian sports. The investigation revealed that Russian officials, coaches, and athletes had been involved in a systematic doping program, aiming to enhance performance and secure victories in international competitions, including the Olympic Games.

Grigory Rodchenkov, who had been the director of the Moscow anti-doping laboratory, defected to the United States in 2016 and provided crucial evidence to investigators, including detailed accounts of the methods used to dope athletes and evade detection. His revelations led to a series of sanctions against Russian sports, including the banning of Russia from the 2016 Rio Olympics and the 2018 Pyeongchang Winter Olympics, as well as the suspension of the Russian anti-doping agency (RUSADA) [16].

1.2.2 Structure and Activity of AAS

Anabolic androgenic steroids derive their structure and activity from testosterone. They cause their different effects by binding to androgen receptors throughout the body. Their main effects can be categorized into anabolic and androgenic effects. Anabolic effects include increased nitrogen uptake and protein synthesis in muscle tissue, increased appetite and an increase in bone density. Androgenic effects include growth of hair on regions of the body other than the scalp and development of primary and secondary sexual characteristics after puberty [17].



Scheme 1: Structure of Testosterone

Each AAS has a characteristic anabolic-androgenic ratio (AR), that is measured with the "Hershberger-Assay" [18]: The value is derived from the differential weights the ventral prostate and levator ani muscle of male rats that had been castrated and then dosed with the AAS in question. Growth of the prostate is promoted by androgenic steroidal effects; growth of the levator ani muscle indicates anabolic effects. The ratio of growth of the two tissues is then normalized so that testosterone has AR of 1:1. The AR has been extensively used for marketing claims of steroids with almost exclusively anabolic properties, however, steroidal ARs in rats are not a reliable indicator for the effects of AAS in the human body. If AR was measured in human males, the ratios would typically be much less pronounced [19].

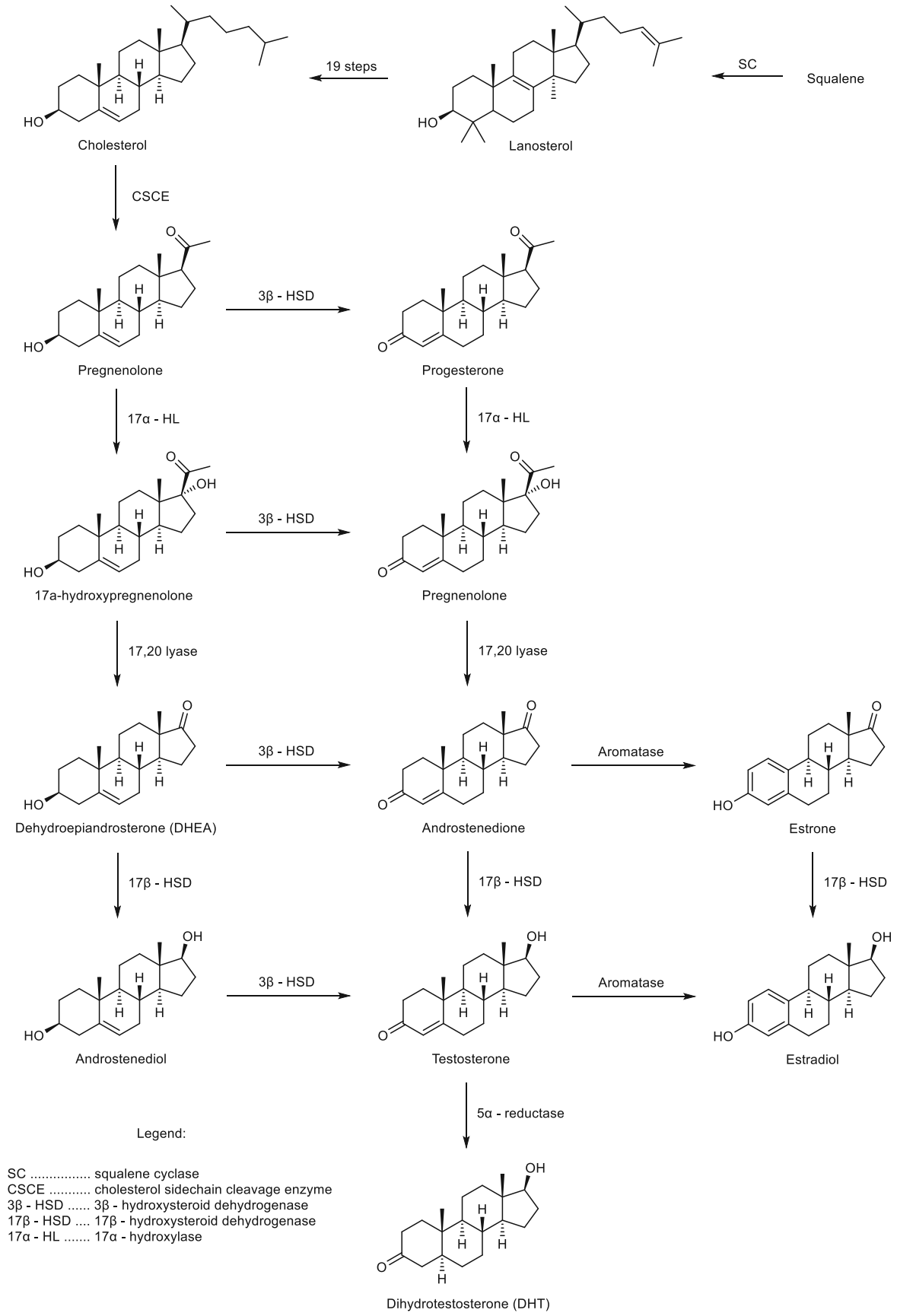
1.2.3 Biosynthesis of Testosterone

The biosynthesis of testosterone occurs primarily in the testicles in males and in the ovaries in females, with a smaller amount being produced in the adrenal glands of both sexes. On average, a healthy adult male will produce about 4-7 mg of testosterone per day while the number for females is considerably lower at 0.5 – 1.0 mg per day [20]. Testosterone production is regulated by the hypothalamic-pituitary-gonadal (HPG) axis, with luteinizing hormone (LH) stimulating testosterone synthesis in the gonads. Intake of exogenous AAS often results in the downregulation of LH production and therefore to unwanted side-effects related to testosterone and dihydrotestosterone deficiency, like shrinkage of the testicles, erectile dysfunction, and infertility [21].

Like for all steroids, the biosynthesis of Testosterone is a multi-step process, starting from squalene [22]:

1. Squalene is cyclized to Lanosterol and then converted to cholesterol by a 19-step sequence.
2. The cholesterol sidechain is oxidatively cleaved by cholesterol-side-chain-cleavage enzyme, giving pregnenolone.
3. Pregnenolone is oxidized at C17 to 17α-hydroxypregnenolone.
4. 17,20-lyase removes C20 in a retro-aldol reaction, dehydroepiandrosterone (DHEA) is formed.
5. DHEA is reduced at C17 to androstenediol.
6. Testosterone is formed by 3β-hydroxysteroid-dehydrogenase.

As can be seen in Scheme 2, this is only one of several routes that occur simultaneously.



Scheme 2: Biosynthesis and main metabolic pathways of testosterone

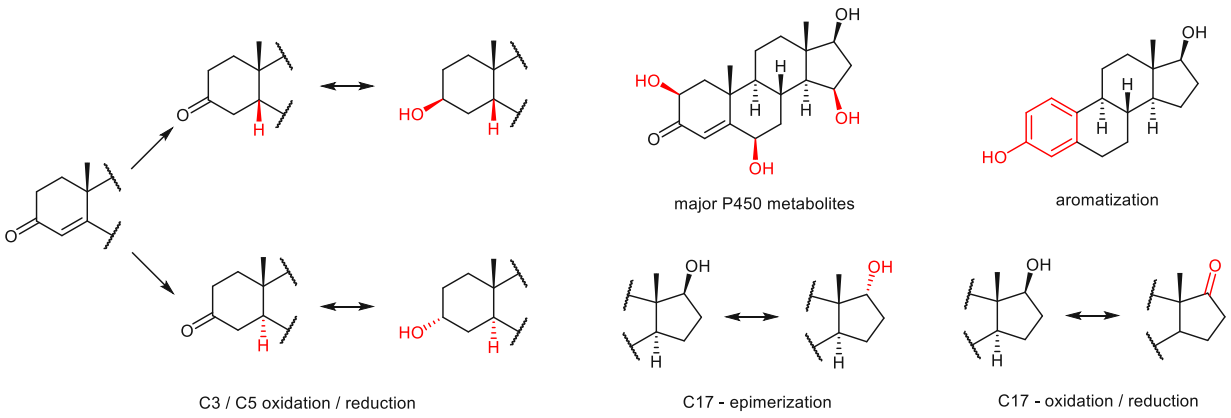
1.2.4 Metabolism of Testosterone and AAS

In general, the metabolism of AAS, so far as possible, follows the metabolic patterns of testosterone.

There are two main pathways of testosterone metabolism:

Reduction: Testosterone can be reduced by two different enzymes, 5α-reductase and 5β-reductase, to form dihydrotestosterone (DHT) and 5β-dihydrotestosterone (5β-DHT), respectively. Expression of 5α-reductase is concentrated in so-called "androgenic tissues" like certain hair follicles, sweat glands and the prostate, resulting in higher concentrations of DHT in these parts of the body [23].

Aromatization: Testosterone can be converted to estradiol, the primary female sex hormone, through a process called aromatization. This conversion is catalyzed by the enzyme aromatase which is mainly found in the ovaries, adipose tissue, and brain. Aromatization is important for maintaining a balance between androgens and estrogens in the body, particularly in females. It can lead to side-effects like gynecomastia when high doses of AAS are applied (if the AAS is a substrate for aromatase). Doping athletes can circumvent this problem by concurrent ingestion of aromatase-inhibitors and this class of compounds is therefore an interesting target for doping analysis [24][25].



Scheme 3:Phase-I metabolism of testosterone and other AAS

In addition to these two major pathways, testosterone and other AAS can undergo numerous other metabolic transformations. As soon as testosterone is hydrogenated to DHT, or in AAS without $\Delta^{4,5}$-double bond, reduction of the C3-ketone becomes possible [26]. If the previous (faster) metabolic pathways are blocked by structural features of AAS, or in small amounts in the case of testosterone, the promiscuous cytochrome-p450-enzymes of the liver can hydroxylate testosterone and AAS. The dominant product for testosterone is the 6β-hydroxylated metabolite, followed by the less important 2β- and 15β-hydroxy compounds [27][28]. Finally, oxidations and reductions at C17 can take place.

The phase-II metabolism of testosterone and AAS consists of conjugation with either glucuronic acid or sulfate. For glucuronidation, see chapter 1.4.3. While sulfation is an important metabolic pathway for

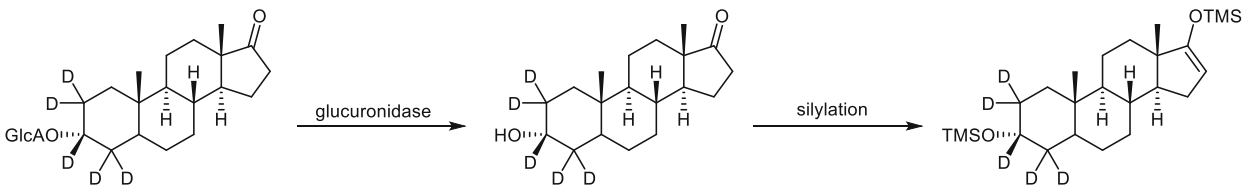
steroids and other substances in the human body, it is beyond the scope of this thesis and an extensive and interesting description of steroid sulfation can be found in Ref. [29].

1.2.5 Detection of AAS

Doping control of AAS is primarily performed on urine samples collected from athletes. Urine is advantageous for this purpose because it can be collected noninvasively and in larger volumes compared to blood. Furthermore, AAS metabolites are excreted from the body primarily via urine and AAS metabolites are therefore enriched in this medium [30][31].

In most cases, the analyte that will be detected in urine is not the parent AAS but a metabolite. Because many AAS are rapidly metabolized and excreted from the body, and many different metabolites can be formed from each parent steroid, elucidation of AAS metabolism is of paramount importance in the development of analytic methods for AAS-detection.

The most common method for detection of AAS is still gas chromatography coupled to mass spectroscopy (GC-MS), which has been in use for this purpose since the Los Angeles Olympic games in 1984 [32]. The routine protocol for AAS detection is described in WADA technical document "TD2021EAAS" [33]. In order to increase the volatility of the glucuronidated metabolites, enzymatic hydrolysis by *E. coli* glucuronidase is performed. The success of this step is validated by addition of isotopically labeled androsterone glucuronide (that is prepared by our research group, see chapter 1.2.6). This is followed by persilylation of free hydroxy- and oxo-moieties by use of a derivatization agent made of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), mercaptoethanol and ammonium iodide. Effectiveness of silylation is controlled by monitoring the ratio of mono- to disilylated androsterone reference material. In order to unambiguously identify doping with a specific AAS, a reference material of the metabolite in question is needed.



Scheme 4: Sample preparation steps for GC-MS, reactions illustrated on androsterone-d5 reference material

GC-based detection has the advantage of much higher ionization efficiency of steroids and metabolites compared to liquid chromatography, this was especially important in the early days of AAS analysis, when the used analytical instruments were much less sensitive than today. GC therefore allowed for more sensitive detection, but the GC-MS based approach also has several disadvantages.

1. The sequence of enzymatic hydrolysis – silylation that must be performed for every sample requires a lot of manual labor and is therefore also a source of human error [34].

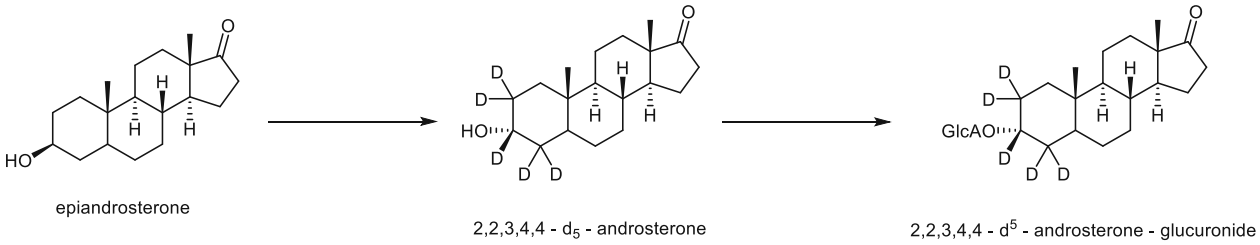
2. The efficiency of enzymatic hydrolysis can be reduced by external factors such as sample preparation or bacterial contamination, or inherent structural factors, as is the case with stanozolol glucuronides [35].
3. Some analytes are inherently not suitable for analysis by GC, as they are either not volatile enough even after silylation or thermally unstable.

The advances in instrumentation now allow for sufficiently sensitive detection of metabolites in LC-MS instruments [36]. The development of such methods has the potential to circumvent all the GC-specific problems, however the intact glucuronides are needed as reference substances for the introduction of such methods [37].

The sample preparation for the LC-MS methods typically just consists of purification of the sample by solid-phase-extraction (SPE). In the ideal case of very sensitive analytical instruments just dilution of the urine sample can suffice as sample preparation. Such methods are known as “dilute-and-shoot”- or “direct-injection”-methods and are highly sought-after due to their maximized operational simplicity [38][39][40].

1.2.6 Epiandrosterone

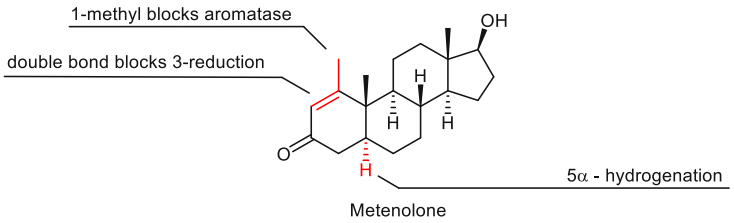
Epiandrosterone is a steroidal metabolite of dehydroepiandrosterone that has only low anabolic and androgenic potency by itself. As it is easy to access from epiandrosterone and relatively stable, the penta-deuterated version of androsterone - glucuronide is used as a reference standard to validate enzymatic hydrolysis steps during sample preparation for GC-MS doping analysis. A synthesis for this compound, starting from epiandrosterone, has previously been developed in our research group [41].



Scheme 5: Synthesis of d5 - androsterone - glucuronide

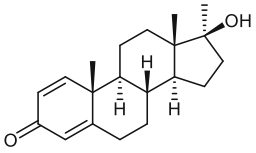
1.2.7 Metenolone

Metenolone was first synthesized in 1960 and is a 5α-steroid. It has a characteristic 1-methyl group and Δ-1,2 double bond. These structural features inhibit metabolism by 5α-reductase, 3α-hydroxysteroid dehydrogenase and aromatase, the primary metabolic pathways are therefore oxidation and reduction at C17 and subsequent glucuronidation [42][43].



Scheme 6: Structural features of metenolone

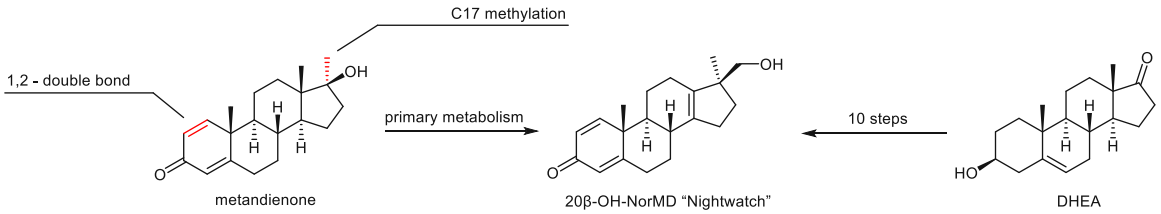
1.2.8 Metandienone



Scheme 7: Metandienone

In 1954 John Ziegler accompanied the US weightlifting team to Vienna for the World Weightlifting Championship [44][45]. During the event, he was told from a member of the Soviet delegation that their athletes were using testosterone to improve their performance. When Ziegler returned to the United States, he worked with CIBA company to develop "Dianabol" – the marketing term for Metandienone. While Dianabol was initially approved by the FDA and prescribed to burn victims and the elderly, Ziegler also continuously diverted the material for (at the time legal) use in the US weightlifting team.

Compared to testosterone, metandienone is C17 – methylated, allowing for oral bioavailability and possesses a $\Delta^{1,2}$ – double bond that increases the affinity to androgen receptors.



Scheme 8: Metandienon and its long-term metabolite

One important metandienone metabolite is the D-ring rearranged 20β-OH-NorMD "Nightwatch" metabolite, resulting from Wagner-Meerwein rearrangement and subsequent hydroxylation at C20. A chemical synthesis of this metabolite was previously developed in our research group and optimized as part of this dissertation [46].

1.2.9 Stanozolol

Stanozolol, also known under the brand name Winstrol, is an anabolic steroid derived from dihydrotestosterone (DHT). It was first developed by the American pharmaceutical company Winthrop Laboratories in 1962 [47].

This steroid gained widespread attention in the sports world during the 1988 Seoul Olympics when Canadian sprinter Ben Johnson tested positive for stanozolol after winning the 100-meter gold medal. Johnson's disqualification and subsequent ban from athletics brought the issue of doping and steroid use in sports to the forefront of public discussion [48].

In medical settings, stanozolol has been used to treat a variety of conditions such as hereditary angioedema, anemia, and certain types of breast cancer [49][50][51].

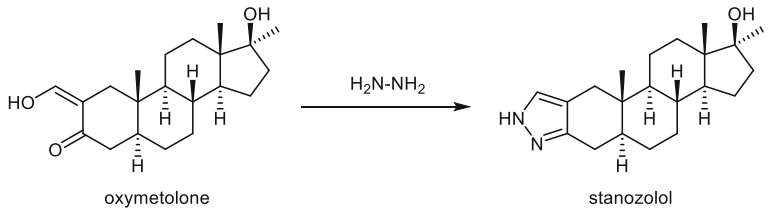
Even though it is relatively expensive, stanozolol is one of the most widespread AAS used in doping today (according to AAFs it is the most widespread, accounting for 15 % of all cases in the year 2021)

[52]. This can be attributed to its comparatively mild side-effect-profile and the reduced psychological barrier of oral AAS consumption compared to injections.

S1.1 Anabolic Agents	Occurrences	% within drug class
stanozolol	119	15%
drostanolone	94	12%
the GC/C/IRMS result is consistent with an exogenous origin	91	12%
metandienone	62	8%
19-norandrosterone (10 cases consistent with an exogenous origin)	62	8%
dehydrochloromethyltestosterone	53	7%
boldenone (15 cases consistent with an exogenous origin)	52	7%
oxandrolone	51	7%
trenbolone	41	5%
metenolone	41	5%
methasterone	23	3%
clostebol	19	2%
oxymetholone	17	2%
mesterolone	12	1.6%
methyltestosterone	6	0.8%
1-testosterone	5	0.7%
1-androsterone (3α-hydroxy-5α-androst-1-ene-17-one)	2	0.3%
testosterone propionate	2	0.3%
testosterone	2	0.3%
mestanolone	2	0.3%
1-androstenedione	2	0.3%
gestrinone	2	0.3%
methandriol	2	0.3%
fluoxymesterone	2	0.3%
oxabolone	1	0.1%
6a-hydroxy-androstenedione	1	0.1%
methyl-1-testosterone	1	0.1%
testosterone undecanoate	1	0.1%
methyldienolone	1	0.1%
TOTAL*	769	

Figure 1: Adverse analytical findings for AAS in the year 2021, stanozolol is the most detected AAS

Structurally, stanozolol is a derivate of dihydrotestosterone (DHT). It is synthesized by condensation of oxymetolone and hydrazine:



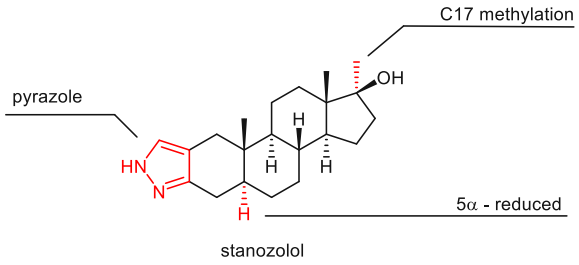
oxymetolone stanozolol

Scheme 9: Synthesis of Stanozolol

Its structural characteristics lead to the following effects [53]:

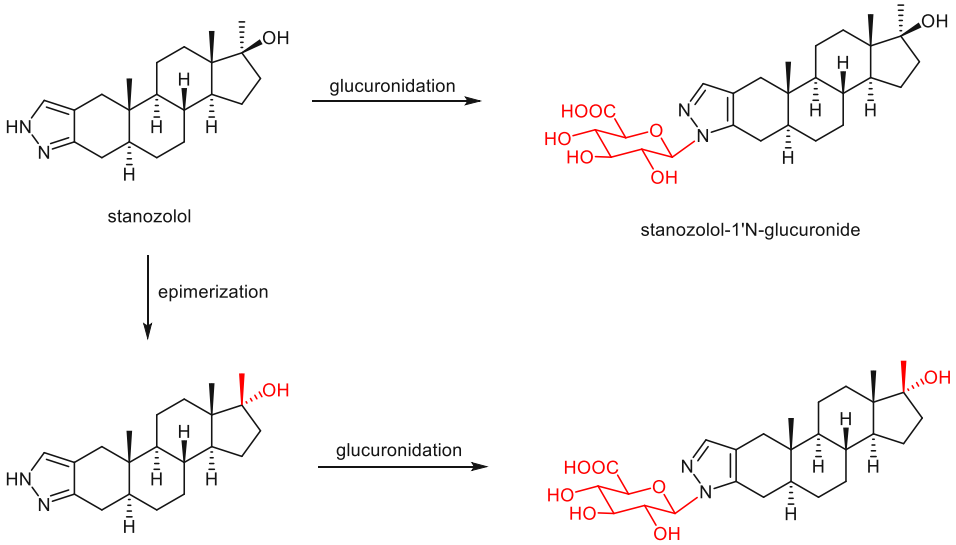
1. Stanozolol is a 5α-reduced steroid, and as such no substrate to 5α-reductase. Therefore, its metabolites cannot be enriched in "androgenic" tissues, improving the overall AR of stanozolol.
2. The characteristic pyrazole ring was introduced in the hope of further reduction of androgenic potential and inhibits action of aromatase on the A-ring, that would lead to estrogenic compounds.

3. The C-17 methylation inhibits (first-pass) liver metabolism at that position and therefore enables oral bioavailability. This kind of inhibition also leads to hepatotoxicity in case of excessive dosage.



Scheme 10: Structural characteristics of stanozolol

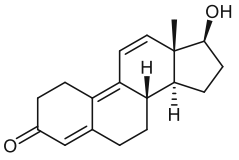
Because of its unique structural characteristics, the phase-I metabolism of stanozolol is limited to C17-epimerization and slow P450-hydroxylation at different sites. The pyrazole ring, however, is relatively nucleophilic and can be glucuronidated, forming stanozolol-1'*N*-glucuronide. Reference samples for this material have been synthesized by Thevi's and our group [54][55].



Scheme 11: Biosynthesis of epistanozolol-1'N-glucuronide

A metabolite that can be detected for longer periods than stanozolol-1'*N*-glucuronide (12 days) is 17-epistanozolol-1'*N*-glucuronide (28 days) [56]. The synthesis of this material is yet unpublished and was one of the projects that were worked on during this thesis.

1.2.10 Trenbolone



Scheme 12: Trenbolone

Trenbolone is a very powerful AAS with a very high anabolic ratio [57]. It was originally developed in the 1960s by the French company Roussel UCLAF (A predecessor of today's Sanofi) under the codename RU-2341 DOI [## US3453267A] and marketed under the brand "Parabolan" on the French market. Since the company voluntarily withdrew the product for human consumption in 1997, commercial usage of trenbolone continues as "finaplix®". This is a slow-release implant for cows, developed by Merck Animal Health which is widely used in the United States with the goal of increasing weight gain and feed efficiency in beef cattle [58].

The runoff from trenbolone feeding cattle farms is contaminated with significant amounts of the substance, which has been shown to skew sex ratios and lead to "Fish on Steroids" [59]

While the manufacturers have claimed that trenbolone is rapidly degraded by photoreactions in the environment, recent research has shown that trenbolone can be regenerated from the photodegradation products in the dark [60].

Even though medicinal human use of trenbolone was discontinued more than twenty years ago, trenbolone is still widely used in sports, especially in the bodybuilding community, where it is known as "tren". Bodybuilders (and amateurs) use trenbolone in doses of up to 1000 mg per week, which is five times the amount contained in the finaplix® implants for cattle. [59]

Trenbolone is known for its powerful side-effects, including increased aggression, insomnia, and significant effects on cardiovascular health. The typical user seems to accept these dangers in exchange for the reliable gains to strength, muscle mass and leanness that trenbolone offers.

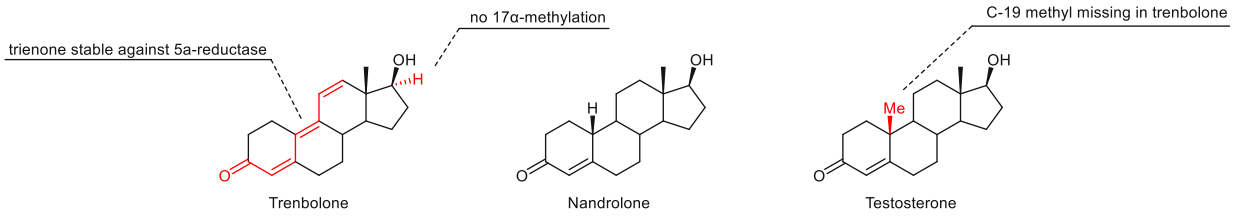
Trenbolone was part of the infamous "Dutchess"-Cocktail developed by Grigory Rodchenkov to enable Russian athletes to successfully avoid AAF during doping controls [61].

1.2.10.1 Structure, Activity and Metabolism

Trenbolone (estra-4,9,11-trien-17β-ol-3-one) is also be described as Δ-9,11-nandrolone, which is the 19-nor derivate of testosterone. Its structural features lead to the following effects [62]:

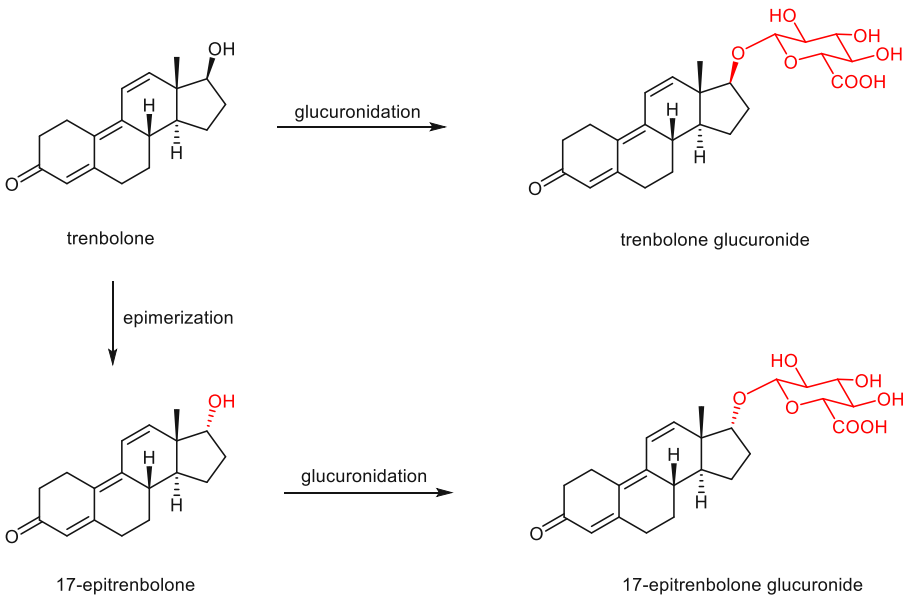
1. The absence of the 19-methyl prevents action of aromatase and therefore the conversion to estrogenic steroids.
2. The trienone - moiety hinders the action of 5α-reductase, preventing the formation of a more androgenic 4,5-dihydro derivate. The use of trenbolone as SARM has therefore been proposed [63].

3. Compared to other designer-steroids, the missing 17α-methylation precludes oral bioavailability, while reducing the risk of liver damage.



Scheme 13 Structural comparison of trenbolone with nandrolone and testosterone

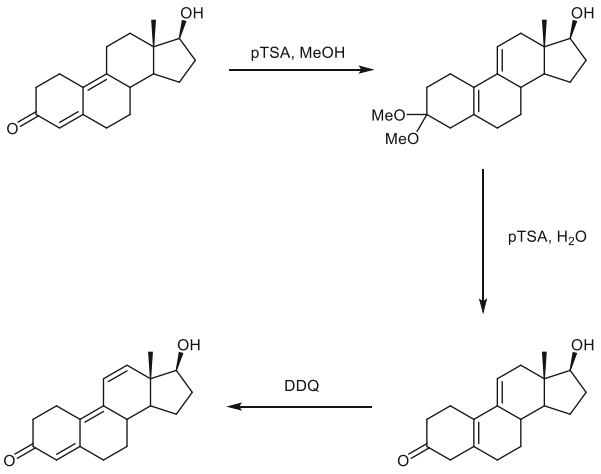
Because phase-I metabolism is extensively hindered by the structural features of trenbolone, similarly to the case of stanozolol, the molecule is directly glucuronidated without extensive prior phase-I modification. Again, detection of the 17-epi-glucuronide allows for a longer detection window (32 days) [64]. The syntheses of both trenbolone glucuronide and 17-epitrenbolone glucuronide were pursued over the course of this thesis.



Scheme 14: Synthesis of 17-epitrenbolone glucuronide

1.2.10.2 Synthesis of Trenbolone

One synthesis by Roussel UCLAF [65] starts from dienolone (RU-3118), which is transformed into the dimethyl acetal by pTSA in MeOH / trimethyl orthoformiate. In this process, the double bonds rearrange from 4,9 to 5(10), 9(11). When the acetal is removed by pTSA in water, the isomerization does not reverse and the formed compound can be transformed into trenbolone by action of DDQ.



Scheme 15: Synthesis of Trenbolone starting from dienolone

1.2.11 Dehydrochloromethyltestosterone (DHCMT)

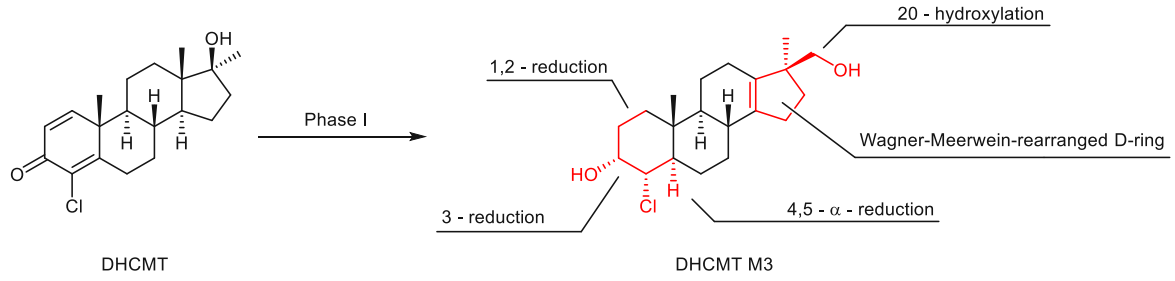
Dehydrochloromethyltestosterone (DHCMT), once marketed as Oral Turinabol or simply Turinabol, is an anabolic steroid developed in the early 1960s by the East German pharmaceutical company Jenapharm.

DHCMT became infamous during the 1970s and 1980s when it was revealed that East Germany had engaged in a state-sponsored doping program, using Oral Turinabol to enhance the performance of their athletes. The systematic administration of DHCMT to athletes, including minors, led to numerous gold medals but also resulted in significant long-term health problems for those involved. Today, the afflicted athletes are still fighting Jenapharm for compensation in the courts [66].

In recent years, DHCMT has experienced a resurgence in popularity among some athletes and bodybuilders seeking a relatively mild anabolic steroid with lower androgenic effects.

1.2.11.1 Structure, Activity and Metabolism

DHCMT is like all AAS a modified form of testosterone. Compared to testosterone, a methyl group was added at C17, a chlorine was added at C4 and a $\Delta^{1,2}$ – double bond was introduced. In previous investigations in our group, the structure of a prominent long-term metabolite "M3" of DHCMT was elucidated by synthesis of all eight possible A-ring isomers that corresponded to the observed mass of the metabolite [67][68]. The last compound of the series, the 3α-hydroxy-4α-chloro-5α-isomer was found to be the correct structure. *In vivo*, the metabolite is formed by a series of metabolic transformations that is depicted in Scheme 16:

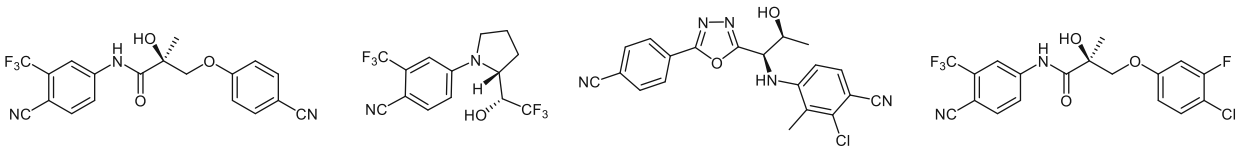


Scheme 16: Synthesis of phase-I long-term-metabolite DHCMT M3

Recent investigations on the phase-II metabolism of DHCMT-M3 were a part of the work conducted as part of this thesis and shall be discussed in chapter 2.5.

1.3 Selective Androgen Receptor Modulators (SARMs)

SARMs (Selective Androgen Receptor Modulators) are a class of nonsteroidal ligands binding to androgen receptors. The goal with their development was to find pharmaceuticals which can provide the anabolic effects of AAS, but without or reduced androgenic side effects. In order to achieve this, SARMs target androgen receptors in specific tissues such as muscle and bone, leading to increased muscle mass, bone density and strength, while not leading to virilization [63].



Scheme 17: Structures of selected SARMs

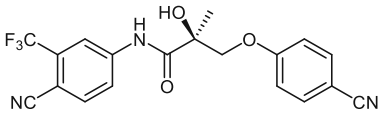
No SARM has made it through clinical trials, and their efficacy and long-term health effects remain under investigation. Nevertheless, they have been adopted by professional athletes, bodybuilders and fitness enthusiasts for their potential to enhance physical performance and appearance. SARMs were included in the WADA-list of prohibited substances in 2008 and the first AAF was reported when Russian cyclist Nikita Novikov delivered a out-of-competition urine sample that tested positive [69]. The number of AAF (Adverse Analytical Findings) involving SARMs has since been increasing every year [52].

S1.2 Other Anabolic Agents	Occurrences	% within drug class
clenbuterol	44	42%
SARMS enobosarm (ostarine)	26	25%
SARMS LGD-4033 (ligandrol)	22	21%
tibolone	6	6%
SARMS RAD140	3	3%
zilpaterol	2	2%
SARMS S-23	2	1.9%
ractopamine	1	0.9%
TOTAL*	106	

Figure 2: 2021 AAFs for SARMs and other nonsteroidal anabolic agents

1.3.1 Ostarine

In terms of clinical trials and also AAF, Ostarine (also called Enobarsam) is the most prominent SARM. The structure is based on the aryl-propionamide backbone common for many SARMs and it was initially developed by Merck and GTX, Inc. as MK-2866.



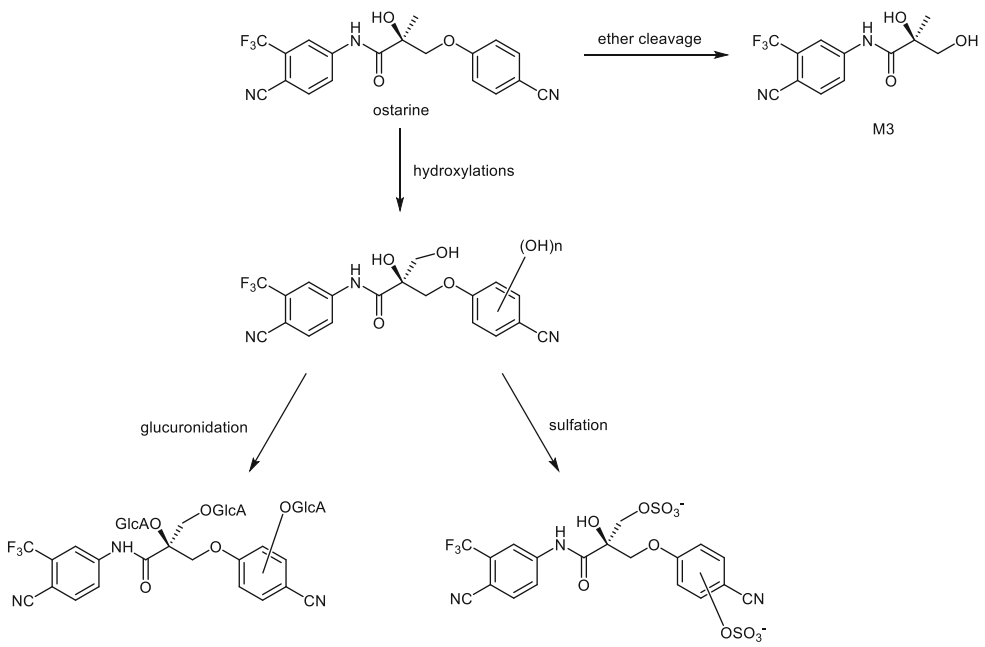
Scheme 18: Structure of ostarine

1.3.1.1 Structure, Activity and Metabolism

The propionamide backbone of Ostarine bears two aryl substituents: A 4-cyano-3-trifluoromethylaniline and 4-cyanophenol. Its SARM-like activity has been proven in both animal models and humans [70] [71].

As no SARM has been clinically approved, metabolic studies from the pharmaceutical industry are generally not available and had to be conducted by the anti-doping community. For Ostarine, Thevis *et. al.* published such a study in 2010 [72].

There are numerous metabolic pathways possible for ostarine, some of which are depicted in Scheme 19.



Scheme 19: Main metabolic pathways of ostarine

Primary metabolism includes (double-) hydroxylation at the phenol ether or methyl group. The phenol ether can also be cleaved off completely to give metabolite M3, which has previously been synthesized in our lab [73].

Secondary metabolism consists of glucuronidation of any free hydroxyl groups or sulfation of the primary or phenolic hydroxyls.

1.3.1.2 Detection

Many aryl propionamides can be detected in minute amounts because of their very high ionization efficiency in negative ion mode. In the case of Ostarine, application to volunteers of only 1 microgram of material resulted in Ostarine being detectable in their urine for up to nine days [74].

Because of this very high detectability, concerns about different forms of "contamination" have arisen. This is best illustrated by the famous case of Laurence Vincent Lapointe. In 2019, the canoe athlete failed a doping test for SARMs, but claimed to never have consumed any of those substances. A forensic investigation ensued and finally, she was cleared of any wrongdoing. The investigation had found that her boyfriend consumed supplements that were contaminated with andarine (another SARM) and traces of ostarine and the materials were apparently transmitted during intimate moments [75][76][77].

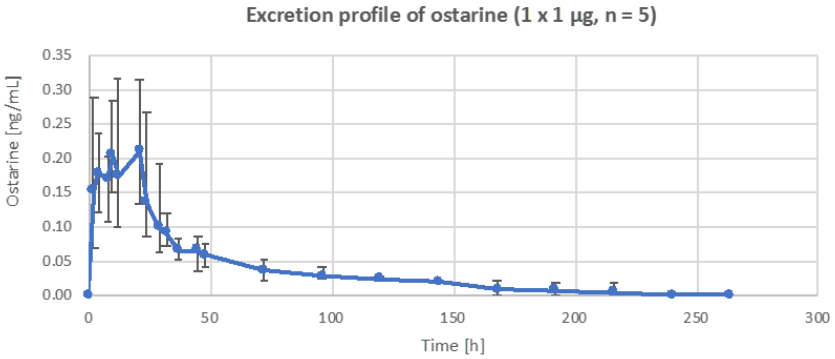


Figure 3: Excretion profile of ostarine after application of 1 µg

1.4 Biochemistry of Glucuronic Acid

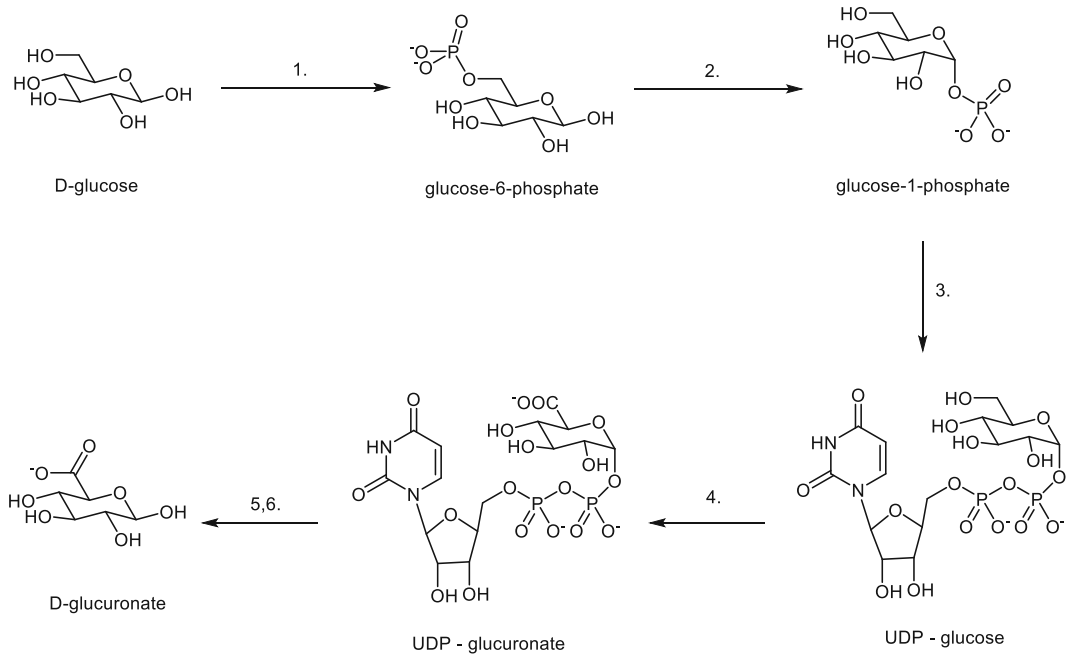
Glucuronic acid is an important carbohydrate in the human body, as it is not only essential for the detoxification of many endogenous and exogenous compounds in humans but is also very important in its role as constituent in glycosaminoglycans (GAGs).

1.4.1 Biosynthesis

Glucuronic acid is synthesized from glucose via the so-called uronic acid pathway. This series of enzymatic reactions happens as follows [78]:

1. D-Glucose is phosphorylated to glucose-6-phosphate (G6P), catalyzed by glucokinase or hexokinase.
2. G6P is isomerized to glucose-1-phosphate (G1P) by phosphoglucomutase.
3. G1P is conjugated with uridine triphosphate by UTP-G1P-uridylyltransferase, the products being UDP-glucose (UDPG) and diphosphate.
4. UDP-glucose is oxidized to UDP-glucuronate by UDPG dehydrogenase.
5. UDP-glucuronate can be cleaved by glucuronate-1-P uridylyl-transferase to reveal 1-P glucuronate.
6. 1-P glucuronate is hydrolyzed by glucuronokinase to free D-glucuronate

Both the biosyntheses of β-D-glucuronides and glycosaminoglycans do, however, use UDP-glucuronate as substrate and not the free glucuronate.

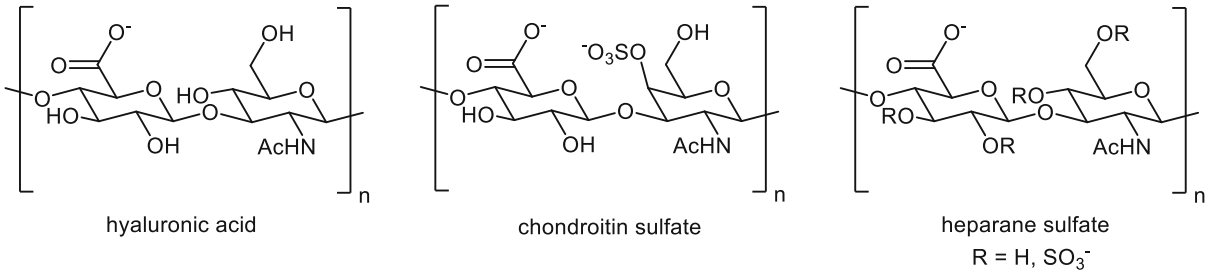


Scheme 20: Biosynthesis of UDP-glucuronate and free glucuronate

1.4.2 Glycosaminoglycans (GAGs)

Glycosaminoglycans [79] are long, unbranched polysaccharide chains made up of repeating disaccharide units. GAGs are a crucial part of the extracellular matrix, contributing to the structural integrity, hydration, and mechanical properties of tissues. They are involved in various biological processes, such as cell signaling, cell adhesion, and tissue repair.

The role of glucuronic acid in glycosaminoglycans lies in its contribution to the repeating disaccharide units. In most GAGs, glucuronic acid is combined with an amino sugar (either N-acetylglucosamine or N-acetylgalactosamine) to form the disaccharide unit. The glucuronic acid in the disaccharide structure provides a negative charge due to its carboxyl group, allowing GAGs to bind cations (such as sodium ions) and water molecules, which contribute to their physical properties.



Scheme 21: Schematic structures of the most important human glycosaminoglycans

One of the most common GAGs is hyaluronic acid, which is composed of glucuronic acid and N-acetylglucosamine. Hyaluronic acid is widespread in the body as part of connective tissues and plays an important role in hydration and lubrication in mechanically stressed parts of the body such as joints.

A further important GAG is chondroitin sulfate, made up of glucuronic acid and N-acetylgalactosamine which is an important structural component of cartilage and bone. It provides structural support, resistance to compression, and contributes to the mechanical properties of the extracellular matrix.

A third important GAG is heparan sulfate: made up of glucuronic acid and N-acetylglucosamine, it is a component of cell surfaces and plays a role in cell signaling, cell adhesion (which is important in cancer proliferation [80]) and the regulation of various growth factors and cytokines.

1.4.3 Glucuronidation

Glucuronidation is a major detoxification pathway that involves the conjugation of various endogenous and exogenous compounds with glucuronic acid. The enzyme responsible for this process is UDP-glucuronosyltransferase (UGT), which catalyzes the transfer of glucuronic acid from UDP-glucuronic acid to a substrate molecule. This reaction increases the water solubility and reduces the biological activity of the substrate, promoting its excretion via bile or urine.

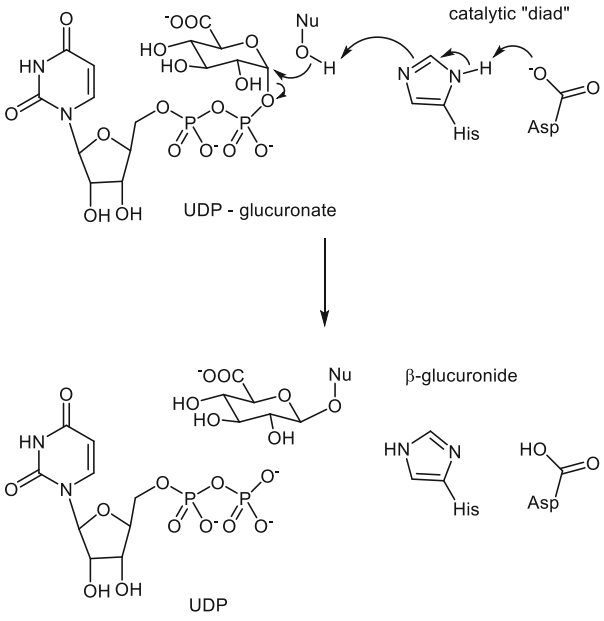
Endogenous substrates for glucuronidation include bilirubin, steroid hormones such as estrogens and androgens and lipids [81].

Exogenous substrates include many drugs, such as morphine derivates [82] or non-steroidal anti-inflammatory drugs (NSAIDs) such as acetaminophen or ibuprofen [83]. Because glucuronidation is an important pathway for the deactivation of these drugs, a lot of research into glucuronidation is conducted by pharmaceutical companies interested in studying the pharmacokinetics of their drug candidates. Another type of substrate for glucuronidation are environmental toxins and carcinogens such as mycotoxins.[84][85].

In humans, there are 22 UDP-glucuronosyltransferase (UGT) enzymes [86], each with specific substrate preferences and tissue distributions [87]. Genetic differences in UGT genes can result in altered enzyme activity, leading to interindividual differences in glucuronidation capacity. These genetic variations can affect drug metabolism, therapeutic response, and susceptibility to adverse effects or drug-drug interactions. Therefore, understanding an individual's genome could help in personalized medicine and drug therapy optimization. UGT activity is also very different between species, making it difficult to study glucuronidation in animal models.

Hereditary defects in glucuronidation can lead to pathological conditions such as Gilberts syndrome or Crigler-Najjar syndrome [88]. Similar symptoms can also be the result of liver damage caused by hepatotoxic substances, such as excessively high doses of some synthetic AAS or SARMs [89].

The mechanism of glucuronidation is a field of ongoing research interest [90]. As there are no crystal structures yet, firm conclusions about the shape of the catalytic cavity of UGTs can only be drawn from modeling approaches. Mutation-experiments and comparison with other glycosyl transferases allowed some mechanistic insight. One exemplary mechanism involves S_{N2}-Type displacement of UDP from the molecule. The nucleophile is activated by a “catalytic diad” of histidine and asparagine, before attacking at the α-configured anomeric center, yielding the β-glucuronide.

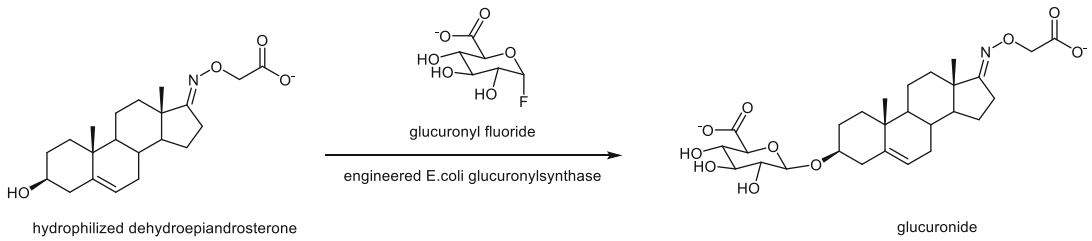


Scheme 22: Mechanism of action of UGT

1.4.4 Enzymatic Synthesis of AAS-Glucuronides

Enzymatic methods for the synthesis of AAS-glucuronides exist [91][92]. These rely either on recombinant expressed enzymes or extracts like rat-liver microsomes. While these methods are successful with some substrates, they share the common drawback of requiring expensive UDP-glucuronate (2k € / g) as donor material. A further limitation is the low aqueous solubility of steroids.

Approaches that use engineered enzymes that accept different glucuronic acid donors such as glucuronyl fluorides are very intriguing [93], however the enzymes involved in these procedures are not commercially available. A further disadvantage is the necessary substrate derivatization to ensure sufficient aqueous solubility. In conclusion, enzymatic synthesis of glucuronides is highly desirable, but, as of yet, seems not sufficiently developed in order to supplant synthetic approaches.



Scheme 23: Biochemical glucuronidation, using glycosyl fluoride as donor.

1.5 Chemical Glucuronidation

The stereoselective chemical glycosylation and glucuronidation of arbitrary substrates is an old problem that occupies synthetic chemists since the days of Emil Fischer [94]. The field has made great advances in the last 50 years and novel synthetic techniques have allowed rapid progress in carbohydrate chemistry, glycobiology and synthetic biology in the last few years [95][96][97].

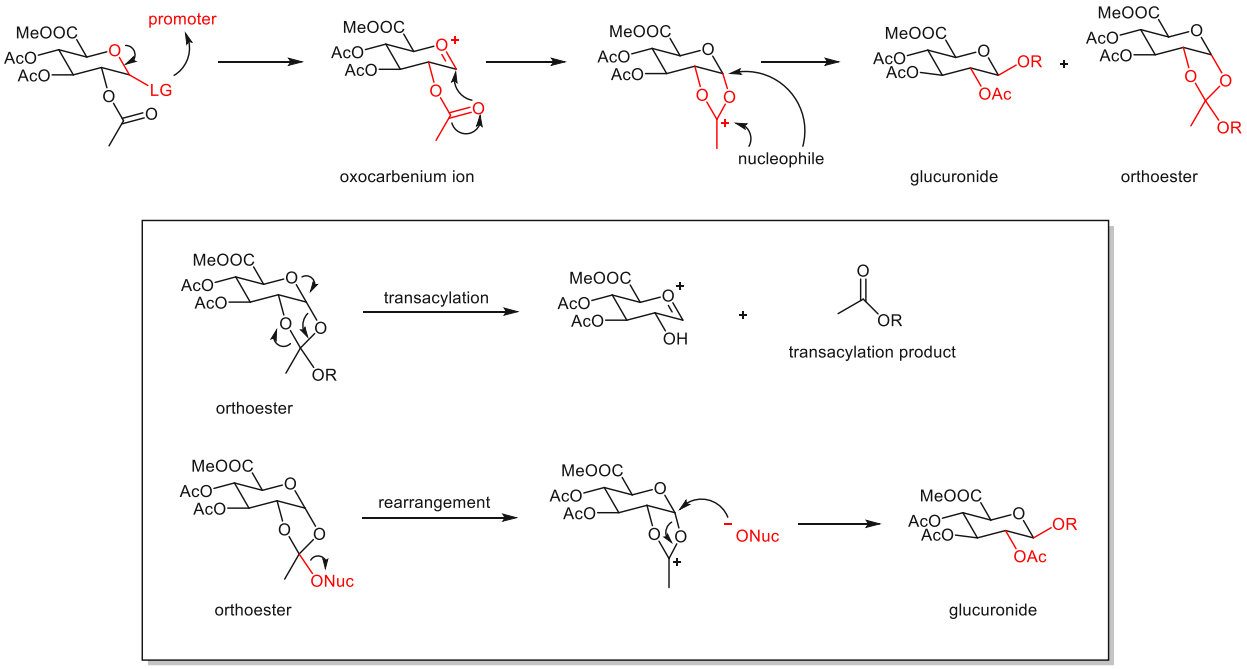
Even though a lot of progress has been made, there is still no general and dependable solution to the problem of glycosidic linkage creation.

Glucuronidation can be seen as a special case of glycosylation and is needed for the synthesis of mammalian secondary metabolites and a wide range of oligosaccharides [98]. It remains a particularly challenging task due to the comparably low reactivity and added functionality of glucuronidation donors [99].

If glucuronidation is not successful on a given substrate, a glycosylation – oxidation cascade can be attempted. As glycosyl donors are typically more reactive than their glucuronic counterparts, in some cases the reaction can be improved this way. The disadvantage of this method is that the resulting glycoside still has to be oxidized to the glucuronate. After deprotection, selective oxidation of the C6 –

primary hydroxyl is possible by use of TEMPO/NaOCl [100]. However, this approach can only be used if the stability of the substrate allows it [101].

Another problem for glycosylation reactions is stereoselectivity: The metabolic glucuronides of eukaryotic cells always feature β-glycosidic bonds. In order to achieve β-selective chemical glycosylation, traditionally the 1,2 – trans – effect (also known as anchimeric assistance) has been used [102]. This approach can lead to unwanted orthoester-formation. In most cases, the orthoester-formation is reversible and can be viewed as formation of a kinetic intermediate product in contrast to the glycosides as thermodynamical products. If the reaction conditions are too mild for equilibration to the thermodynamic product, the reaction can stop at the orthoester-stage and fail to give any of the desired glycoside. In the case of glucuronidation, this problem is exacerbated because the relatively low stability of glucuronic oxocarbenium ions means that the formed orthoesters are comparatively more stable than in the glycosyl case.



Scheme 24: Reaction pathways with 2O-acyl protecting groups

Several approaches have been described to circumvent the problematic orthoester formation, including the use of more bulky esters other than acetyl [103], the nitrophenylacetyl group [104], the 2O - picolyl group [105] or the use of 2O – carbonates [106]. The disadvantages of these approaches are the much more elaborate synthetic pathways needed to synthesize these donors and the need for additional orthogonal deprotection steps after the glycosylation.

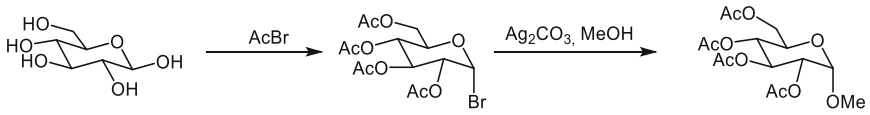
1.5.1 Königs-Knorr / Helferich

Königs-Knorr glycosylation was initially reported in 1901 by Wilhelm Königs and Eduard Knorr [107]. At the time, it represented an important advance in the methodology of glycosidic bond formation and it remains an important and frequently used method to this day.

In their report they detail how they, inspired by the "Acetochlorhydrose" by Colley, managed to transform glucose into analogous "Acetobromhydrose" by action of acetyl bromide. They observe:

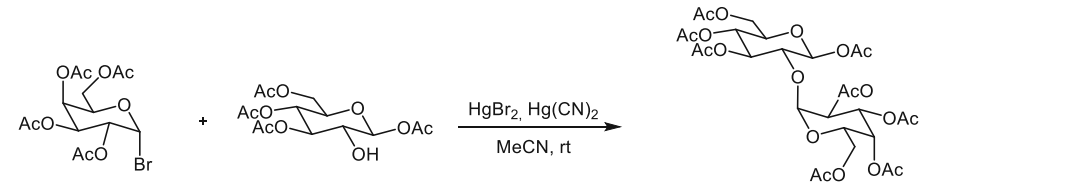
"Am bemerkenswertesten erscheint uns aber die Leichtigkeit, mit der das Brom ausgetauscht wird gegen Methoxyl oder Aethoxyl, wobei β-Alkylglucoside oder deren Acetylderivate entstehen. Schüttelt man die Lösung der Acetobromglucose in absolutem Methyl- oder Äthylalkohol bei gewöhnlicher Temperatur mit Silbercarbonat, so entwickelt sich reichlich Kohlensäure, und es bilden sich die bisher noch nicht bekannten, schön kristallisierenden Tetracetylverbindungen des β -Methyl- resp. β -Aethyl-Glucosids."

Translation: They report their synthesis of the β-methyl- and ethyl glycosides of tetraacetylglucose.



Scheme 25: The first reported glycoside synthesis using a bromo-donor and silver as promoter, reported by Königs and Knorr in their 1901 paper.

Helferich first reported on his method of glycosylation in 1962 [108]: Without giving much context, he introduces the use of $Hg(CN)_2$ / $HgBr_2$ as promotors for use with glycosyl bromides as donors. The advantage of his method is that these salts (in contrast to silver salts) are soluble in solvents like acetonitrile or hot nitromethane. Because the reactions can be conducted homogenously, the reactivity is generally better.



galactosyl α-bromide | 1,3,4,6-tetraacetyl glucose | octaacety-2-[α-D-galaktopyranosido]-α-D-glucopyranose

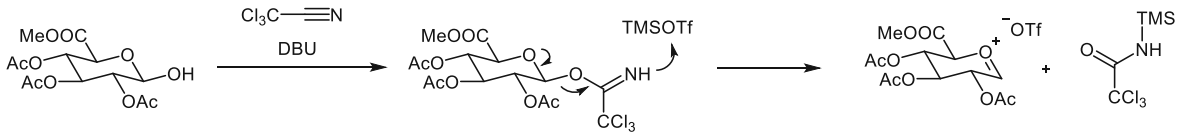
Scheme 26: One of the reactions described in Helferich's report

Both methods have in common the generation of stochiometric amounts of toxic heavy metal waste. Therefore, with the advent of greener methods like the Schmidt glycosylation, the relative importance of these methods has diminished somewhat.

1.5.2 Schmidt / Trichloroacetimidates (TCAIs)

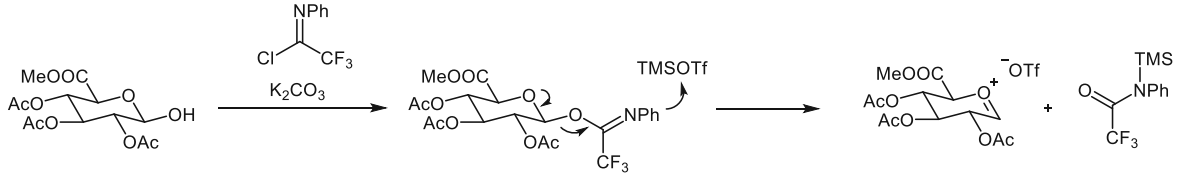
Since its initial report in 1986 [109], the Schmidt glycosylation or trichloroacetimidate (TCAI) method has found widespread application in carbohydrate chemistry [110].

It is based on the derivatization of lactol-form sugars with trichloroacetonitrile under basic conditions. The trichloroacetimidates that are formed in this manner are generally more stable than glycosyl halides but can be activated easily by catalytic amounts of (Lewis-) acids such as TMSOTf or $BF_3.Et_2O$. The activated group imidate leaves as almost inert trichloroacetamide.



Scheme 27: Synthesis and activation of trichloroacetimidate (TCAI) donors

One of the few drawbacks of the trichloroacetimidate method is that over time, if no good enough nucleophile is present in the reaction mixture, the formed oxocarbenium ion slowly reacts with trichloroacetamide, forming an inactive rearranged donor material. The Yu-group has developed a fix for this problem [111], their N-phenyltrifluoroacetimidate (TFAI) donors. Similarly to the TCAIs, these are formed from glycosyl lactol, base and 2,2,2-trifluoro-N-phenylacetimidoyl chloride. They behave similarly to the TCAIs but give improved yields on some substrates.

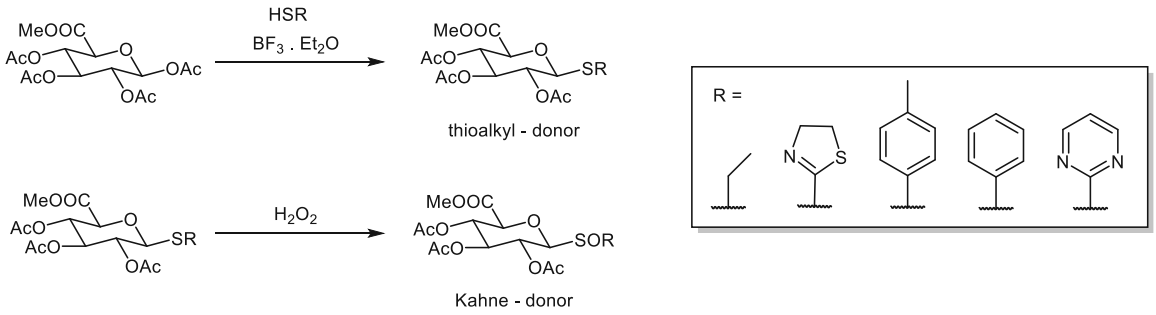


Scheme 28: Yu's TFAI donors

1.5.3 Thioalkyl / Kahne Methods

Due to their exceptional stability under a wide range of chemical conditions, thioalkyl donors and their derivates are today one of the main workhorses of carbohydrate chemistry [112]. They are easily synthesized due to the high nucleophilicity of their corresponding thiols and there are numerous choices for their activation, mostly combinations of catalytic acids with stoichiometric amounts of soft electrophiles such as TfOH / NIS.

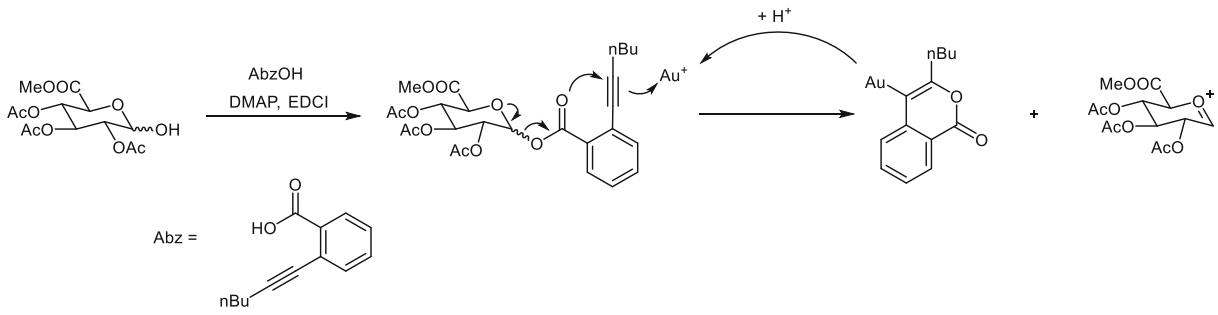
In 1989, Kahne reported the use of glycosyl sulfoxides for the glycosylation of sterically hindered substrates. His method works without the use of stoichiometric amounts of electrophile [113].



Scheme 29: Synthesis of thioalkyl- and sulfoxide-donors (Kahne)

1.5.4 Yu / *ortho* – Alkynylbenzoates

Yu and coworkers have reported an effective and selective glycosylation methodology based on *ortho*-alkynylbenzoate (Abz) - donors that are activated by catalytic amounts of gold[I]-triflates or -triflimides [114]. The group has since demonstrated the utility of their method on a diverse array of sensitive substrates, where standard glycosylation methodology like the Schmidt glycosylation failed [115]. The donor is typically synthesized by EDCI – coupling of glycosyl – lactol and o-alkynylbenzoic acid. After activation, a chromene is expelled from the molecule, and after protodeauration, the catalytic cycle can continue.

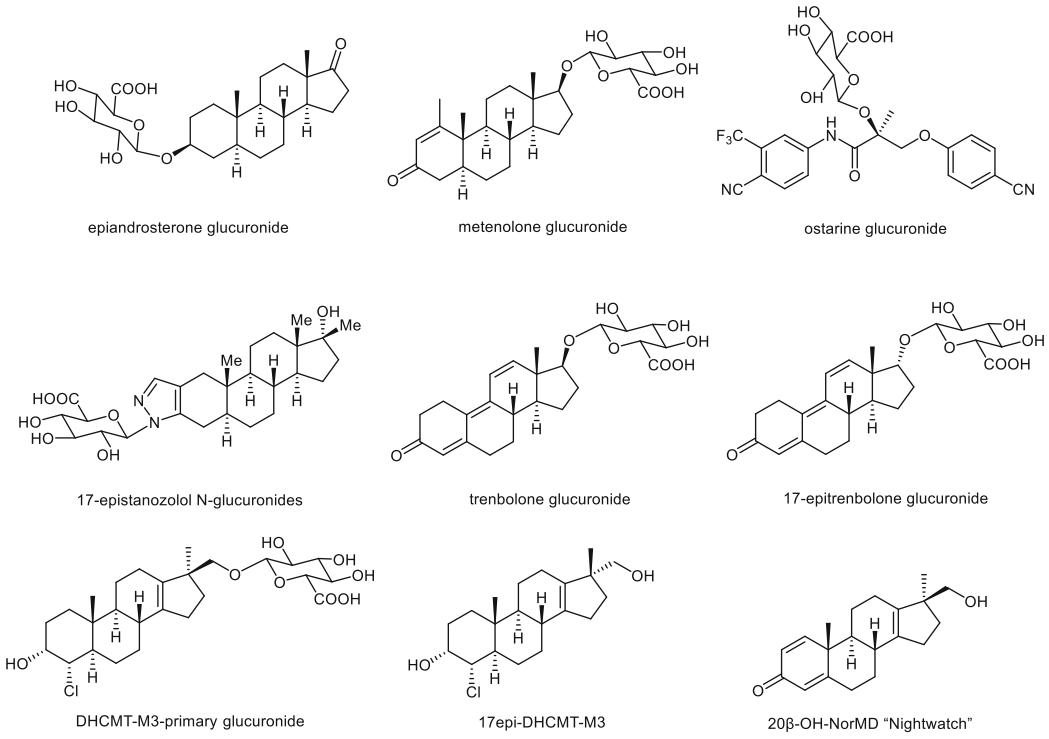


Scheme 30: Synthesis and activation of Yu's Abz-donors

2 Discussion and Results

The goal of this work was the synthesis of different, mostly glucuronidated, metabolites for the use as reference substances for doping analysis. Some of the target compounds were already previously known to literature and in these cases, the goal was to improve upon the published syntheses.

An overview of all the target compounds can be seen in Scheme 31:



Scheme 31: Overview of the targeted compounds

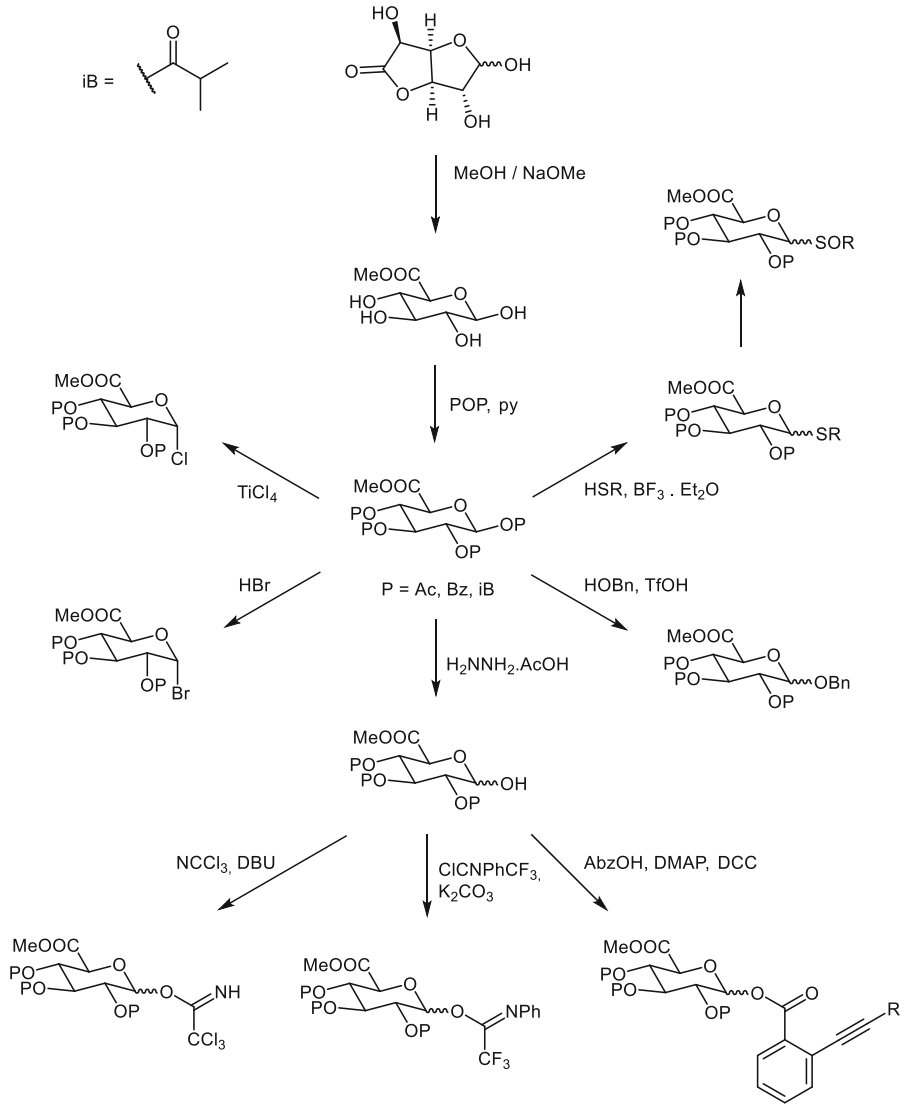
In addition to the synthesis of reference substances, another goal was the assignment and structural elucidation of the glucuronides of the known metabolite DHCMT-M3. To provide material for glucuronidation experiments, the synthesis of DHCMT-M3 was to be optimized. Over the course of this project, the need to synthesize 17epi-DHCMT-M3 arose, in order to further contribute to comprehensive knowledge of DHCMT metabolism.

2.1 Synthesis of Glucuronide Donors

2.1.1 Starting from Glucuronolactone

A diverse array of glucuronidation donors was synthesized during this thesis. Most syntheses started from D-glucuronolactone, which can be opened to the methyl ester by suspending it in dry methanol and adding a catalytic amount of base like NaOH, KOH, NaOMe or metallic Na. This reaction is complete as soon as everything is dissolved into a yellow or orange clear solution. After evaporation of methanol, there are two possibilities for further reaction. The first is esterification under basic conditions by dissolution in pyridine and addition of an acid chloride or anhydride. This yields a brown or black solution from which anomerically pure but colored crystals of β-acylated product can be obtained. The other route is acid-catalysed esterification by dissolution of the substrate in anhydride, followed by addition of a catalytic amount of strong acid like $HClO_4$ or HBr in acetic acid. In contrast to the base-catalysed variant, this approach yields colorless product and is operationally easier to perform in large scales. In our hands however, at times it let to partial relactonization, giving protected glucuronolactone that crystallizes together with the desired product.

With the tetraacylated compound in hand, the acyl group at the anomeric carbon was selectively cleaved and exchanged for various nucleophiles under acid catalysis. Treatment with HBr / AcOH gave the anomeric bromide, treatment with $TiCl_4$ the less reactive but more stable chloride [116]. Addition of HS-R catalyzed by $BF_3.Et_2O$ gave thioglucuronides, which were oxidized to the thiosulfone-donors introduced by Kahne. Another route was introduction of 1-O-benzyl catalyzed by TfOH or $BF_3.Et_2O$. This does not give a donor, but the benzyl acts as stable protecting group while the present acetyl groups could be removed and replaced with other protecting groups such as TBS.

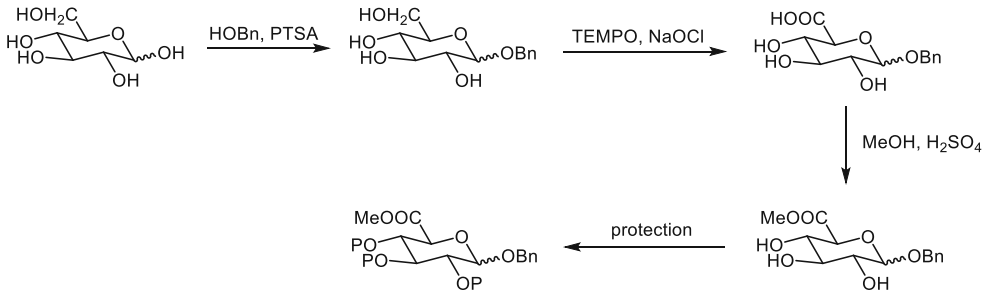


Scheme 32: Synthesis of glucuronyl-donors

An alternative route starting from the tetraacylated compound is selective removal of the anomeric protection to reveal the lactol. This was achieved by using hydrazinium acetate or benzylamine for nucleophilic removal of the anomeric ester. The resulting lactol was then elaborated by coupling with different electrophiles. Coupling with trichloroacetonitrile catalyzed by DBU gave the trichloroacetimidate (Schmidt) donor and coupling with N-phenyl trifluoroacetimidoyl chloride the improved version developed by Yu [111]. Coupling with o-alkynylbenzoic acids gave Abz-donors, also developed by Yu [115]. Finally, coupling with benzyl trichloroacetimidate opened an alternative route towards the 1-O-benzyl compounds.

2.1.2 Glucose-based Synthesis

Throughout the course of this thesis, the synthesis for most of the glucuronide donors was started from D-glucuronolactone. Even though glucuronolactone is cheap and readily available, glucose is still 100-times cheaper, and attempts were made to find an economic synthetic route towards glucuronides starting from glucose.



Scheme 33: Donor synthesis starting from glucose.

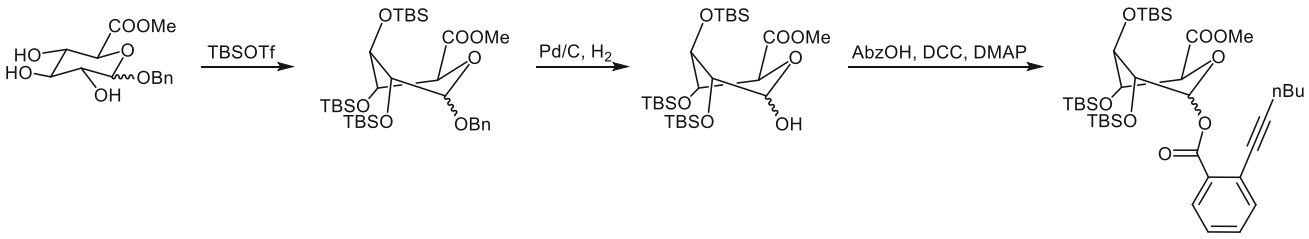
After some experimentation and review of the literature, it was found that glucose can be benzylated at the anomeric hydroxyl by heating in benzyl alcohol under acid catalysis [117]. The remaining benzyl alcohol could be removed by extraction with diethyl ether. The benzyl glycoside obtained this way could be oxidized to the uronic acid using catalytic TEMPO with cheap bleach as terminal oxidant [118]. The free acid could then be transformed into the methyl ester by heating in methanol under acid catalysis. While the benzyl glycoside is quite stable, formation of methyl glycoside in this step could explain the low observed yield of 25 %. The methyl ester could be purified by eluting over a plug of silica with pure ethyl acetate. The remaining free hydroxyls could then be protected by the protecting group of choice such as TBS or Fmoc [119][120]. These protecting groups were chosen because of their potential to effect β-selective glucuronidation. TBS was envisaged to achieve this via steric shielding of the α-face and Fmoc was chosen as a substitute for the Cbz-protection reported by Weber et. al. [106] (Hydrogenolysis, needed for Cbz-deprotection, was deemed incompatible with the substrate, trenbolone). The attempted synthesis of a triple-Fmoc protected donor failed and the triple-TBS protected donor shall be described in the next chapter.

While the route was able to provide large amounts of material, yields were very low (a typical run started with 200 g glucose) and the missing anomeric selectivity complicated characterization of the products and intermediates.

2.1.3 Synthesis of a potentially Super-Armed Glucuronidation Donor

In combining the ortho-alkynylbenzoate (Abz) leaving group with a super-armed silyl-protected glucuronic acid, we envisioned to create a very reactive glucuronidation donor without potential for orthoester formation. The β-selectivity was to be provided by steric shielding from the bulky TBS group at C-2. Additionally, it was known from studies by Bols and coworkers [120] that donor molecules can be forced into distorted conformations by persilylation with bulky silyl protecting groups like TBS. These distorted donor molecules were known to be comparatively reactive ("super-armed") and it was hoped this phenomenon would also apply to glucuronic acid derivates.

Starting from the 1O – benzylated product from the last chapter, persilylation was performed through extended action of TBSOTf in warm DCM. The benzyl group was cleaved by hydrogenolysis and the Abz-group was condensed with the resulting hemiacetal with DMAP / DCC.



Scheme 34: Synthesis of the TBS-Abz donor (conformation only confirmed for final compound)

This synthetic sequence led to an anomeric mixture of the envisaged donor materials, and serendipitously, the α-anomer could be crystallized. The crystal structure proved the molecule to be in a ring-flipped, axial-rich twist-boat-like conformation, at least in solid phase. In order to also gain information about the behaviour of the molecule in liquid phase, conformational analysis *via* the coupling data from ^{1}H-NMR was attempted, however, the results could not be interpreted. The failure of the NMR-study inspired investigation of the molecule by DFT-calculations. The calculations hinted at the existence of several populations of energetically similar conformational populations, with a structure closely resembling the X-ray structure having the highest weight. The different stable conformational states would equilibrate fast on the NMR-timescale and could explain the failure of the NMR-study (for details see Ref. [101]).

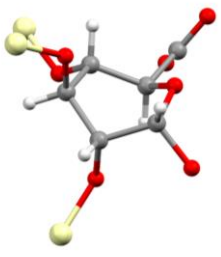
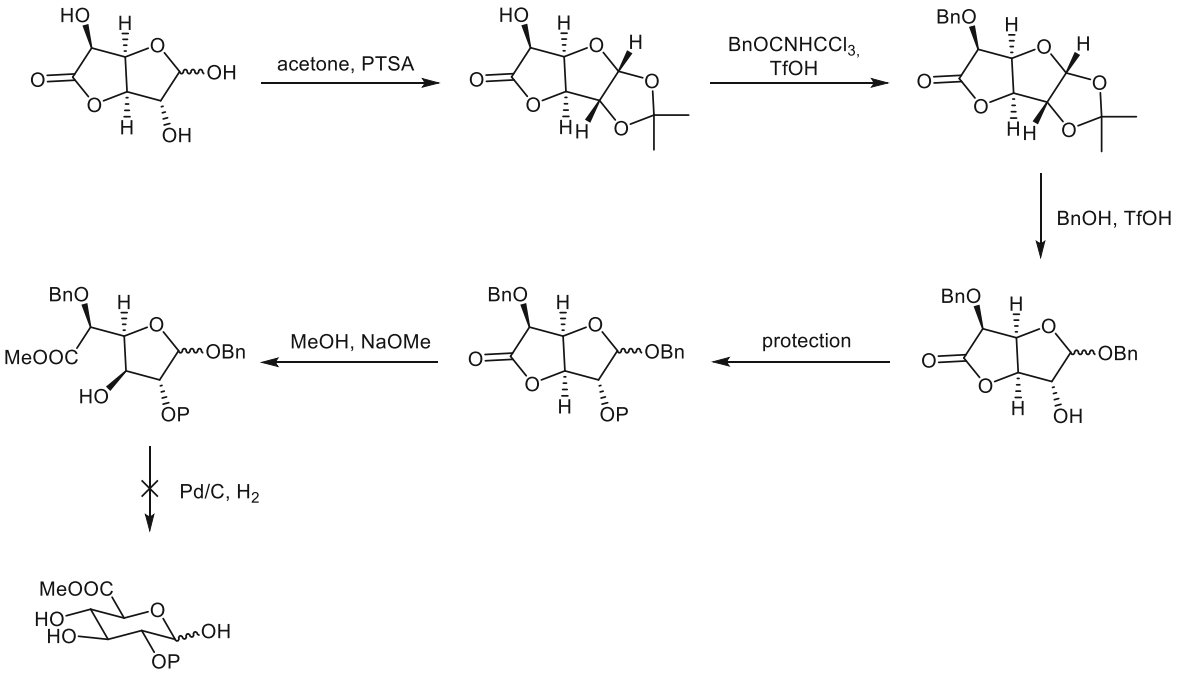


Figure 4: X-ray structure of the Abz-Donor, with the substituents curtailed for clarity.

2.1.4 Approach towards selective C2-protection

Frequently, orthoester formation was encountered as the sole product of attempted glucuronidations. A lot of literature exists about the topic of C2-protecting groups that do not form orthoester while still providing the 1,2-trans effect necessary for β-selectivity during glycosylation (see chapter 1.5).



Scheme 35: Approach towards selective C2-protection of glucuronides

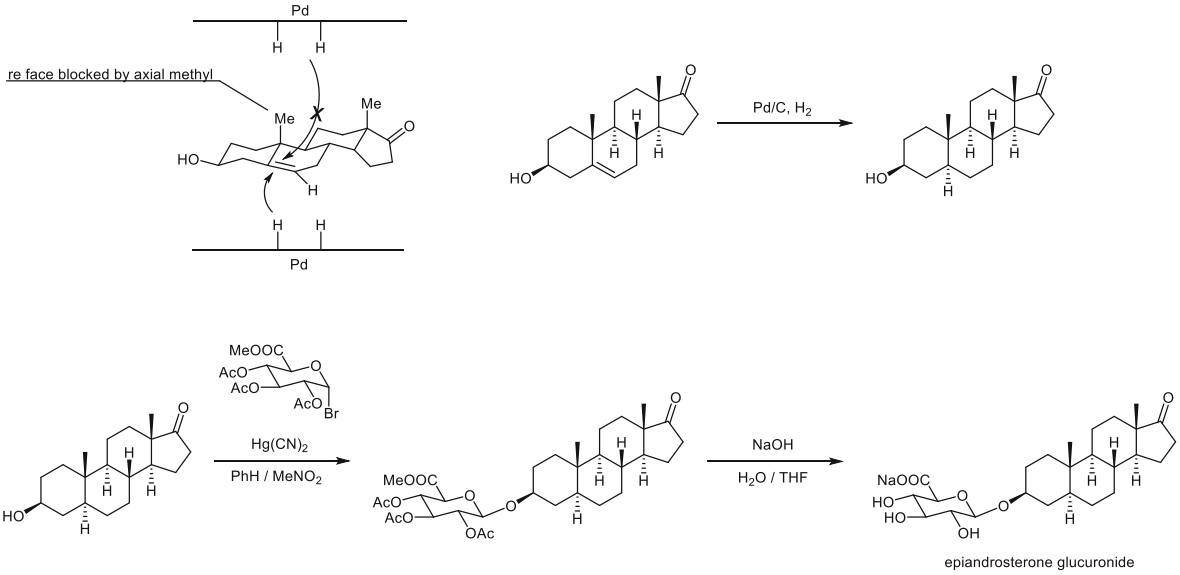
A synthetic route starting from glucuronolactone was envisaged that took advantage of the structure of the lactone and was aimed at rearrangement to the pyranose in a later step. It started by protecting the C1- and C2-hydroxyls as an acetonide. The C4-hydroxyl was then benzyl-protected via the benzyl trichloroacetimidate and the acetonide opened with benzyl alcohol / TfOH. This left the C2-hydroxyl unprotected, which was then protected by TIPS which was supposed to provide the 1,2-trans effect by means of steric hindrance. The lactone was then opened by MeOH / NaOMe and the product from this reaction subjected to hydrogenolysis of the benzyl protecting groups, allowing the molecule for rearrangement to the pyranose. Disappointingly, this reaction was unsuccessful, leading to a great number of different species populating the TLC plates, instead of elusive pyranose.

2.2 Synthesis of Known Compounds

2.2.1 Epiandrosterone Glucuronide

Epiandrosterone can be sourced commercially or can easily be obtained by catalytic hydrogenation of DHEA with palladium on charcoal [41]. Hydrogen is delivered exclusively from the si-face because the 10-β-methyl group blocks access to the palladium surface on the other side.

Schmidt and Königs-Knorr glycosylation did not deliver satisfactory results and finally the Helferich-method was chosen to deliver the glucuronic acid: Usage of the acetylated glycosyl bromide promoted by mercury cyanide in benzene / nitromethane at 110 °C delivered the product in 23 % yield.



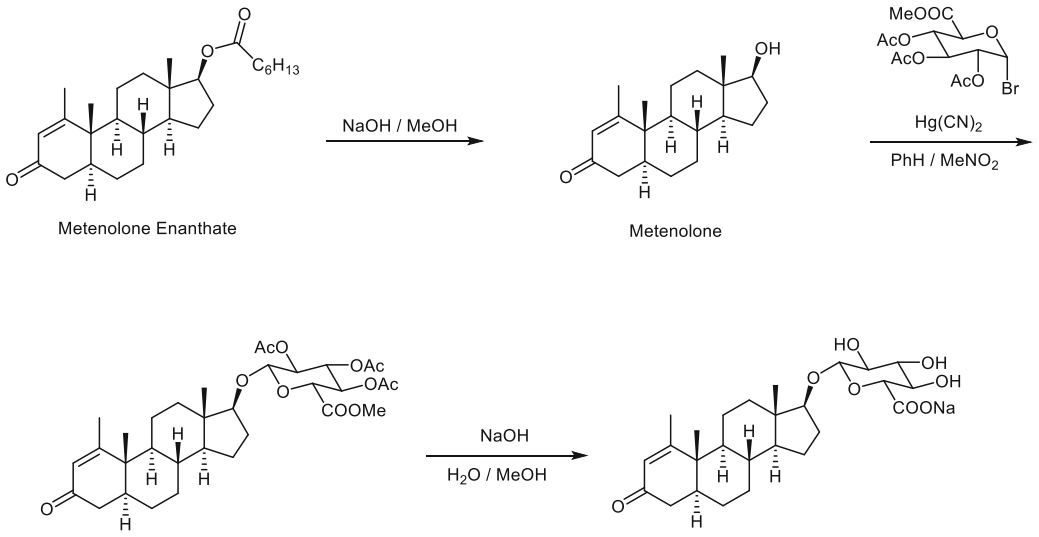
Scheme 36: Synthesis of epiandrosterone glucuronide

Deprotection was performed by sodium hydroxide in THF / water. After purification with RP-flash-chromatography, the product could be isolated in 65 % yield. After neutralization with 1.00 eq. of aq. sodium bicarbonate solution, the product was lyophilized and delivered to Seibersdorf Laboratories.

2.2.2 Metenolone Glucuronide

Metenolone was provided in form of injectable solutions of metenolone enanthate in “vegetable oil”. The oil and a multitude of other substances present could be removed by MPLC purification with pure petroleum ether as eluent. The purified enanthate ester was then hydrolysed to reveal pure Metenolone.

For glucuronidation, the same methodology as for Epiandrosterone glucuronide was applied [41]: Helferich glycosylation at 110 °C in nitromethane / benzene with glycosyl bromide as donor and promoted by mercury (II) cyanide delivered the 17-O-glucuronide in 31 % yield.



Scheme 37: Synthesis of metenolone glucuronide

The material was deprotected without issues with sodium hydroxide in methanol / water. However, in this solvent system, prolonged reaction times were necessary to cleave the methyl ester on the glucuronide. The glucuronide was purified on RP-HPLC, and the product neutralized with aq. $NaHCO_3$.

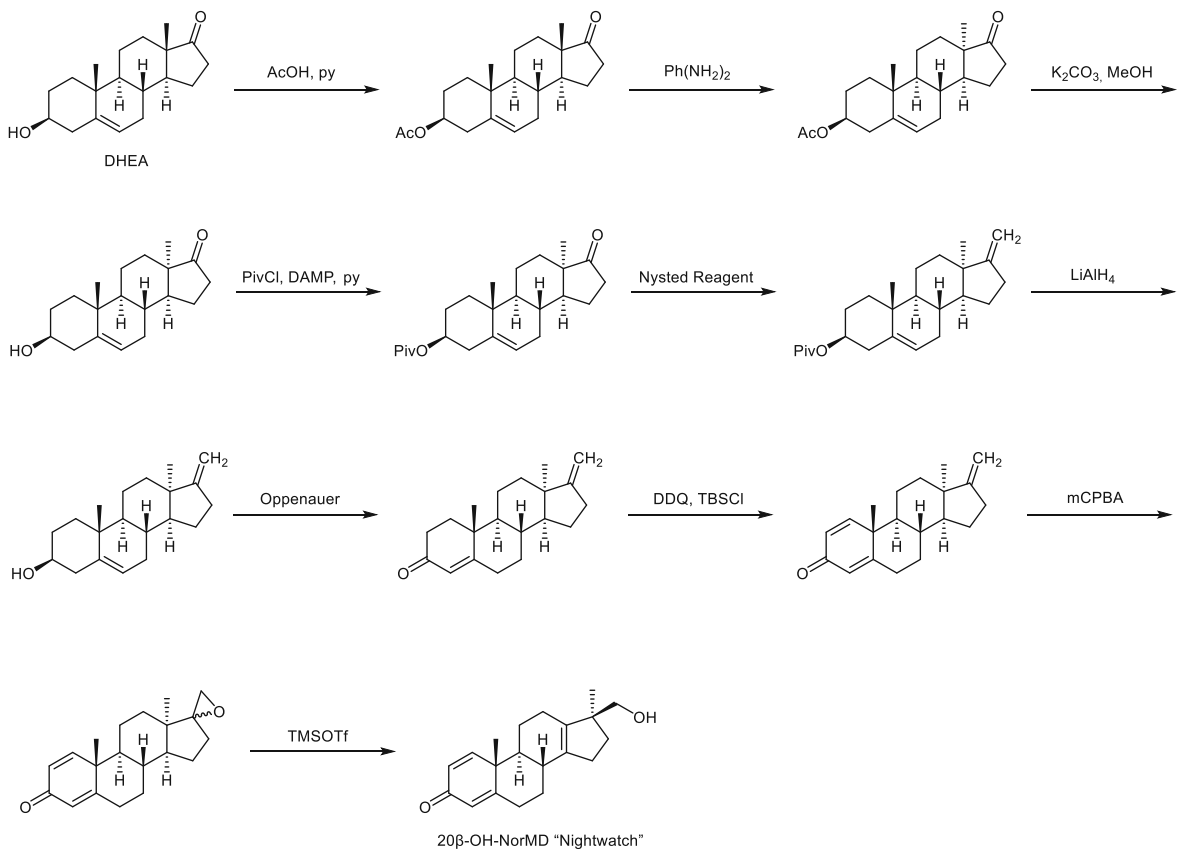
After lyophilization, the product was delivered to Seibersdorf Laboratories.

2.2.3 Metandienone Metabolite "Nightwatch"

The 10-step synthetic procedure for this synthesis was previously developed in our research group and the detailed experimental description can be found in the published report [46].

The synthesis started with acetylation and C13-epimerization of dehydroepiandrosterone (DHEA) [121]. The protecting group was changed for the more stable pivaloyl-group and the sterically shielded C17-carbonyl was methylenated with Nysted-reagent. Pivaloyl was quantitatively cleaved with $LiAlH_4$ and Oppenauer-oxidation was used to construct the testosterone-A-ring-motive. Dehydrogenation with DDQ / TBSCl was performed at this step to prevent Baeyer-Villiger-oxidation in the next step. Epoxidation gave two epimeric epoxides that were not separated. As opposed to the older pathway described in the reference, Wagner-Meerwein-rearrangement was performed by the newer TMSOTf-catalyzed method [68].

The final product was purified by HPLC and delivered to Seibersdorf Laboratories.

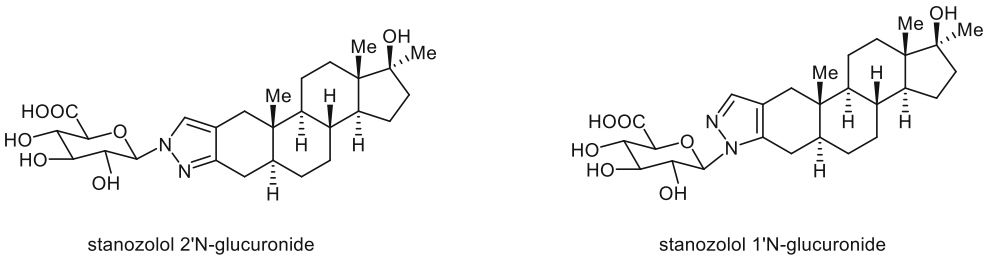


Scheme 38: Synthesis of 20β-OH-NorMD "Nightwatch" metabolite

2.3 17-Epistanozolol Glucuronides

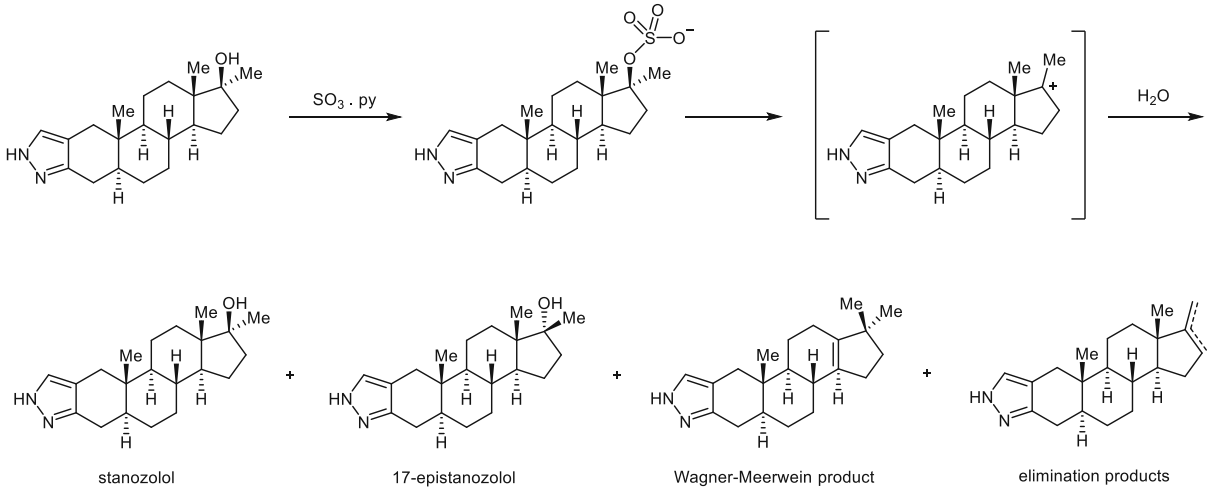
A more detailed description of this project and the experimental part are being prepared for publication as of the writing of this thesis and can also be found in the diploma thesis of Georg Stadler [122].

The 1'N- and 2'N-glucuronides of Stanozolol have been previously synthesized in the research group of Schänzer and our group [123][124]. The synthesized substances were found to correspond to known urinary metabolites of stanozolol. The structure of another known glucuronidated metabolite with a very long detectability window of up to thirty days was still unconfirmed at that point. This metabolite was theorized to be 17-epistanozolol-1'N-glucuronide.



Scheme 39: Stanozolol N-glucuronides

Epistanozolol was prepared according to a known procedure for 17-epimerization of steroids with tertiary hydroxyls at C17. The 17-sulfate is formed *via* utilization SO_3-pyridine complex. Elimination of the sulfate proceeds *via* the carbocation and in aqueous environment this leads to a separable mixture of 17-α/β-hydroxyl stanozolol, the Wagner-Meerwein-rearranged product and elimination products.

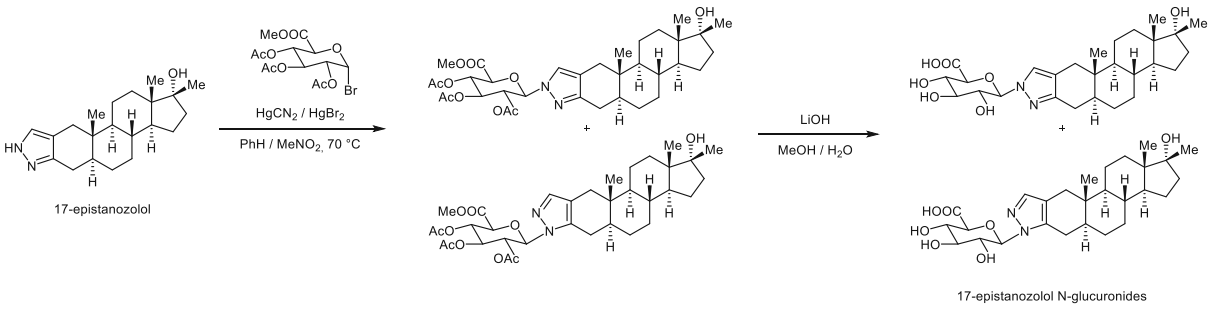


Scheme 40: Synthesis of epistanozolol

Epistanozolol could be separated from this mixture in 23 % yield.

While this method is neither very elegant nor very high yielding, it is operationally simple and no better alternatives were found. With the Epistanozolol in hand, different glucuronidation procedures were attempted, the same method that had been used previously for the synthesis of stanozolol glucuronides was found to be optimal.

As the tertiary hydroxyl group proved to be inert under most reaction conditions, protection was not deemed necessary.



Scheme 41: Synthesis of epistanozolol glucuronides

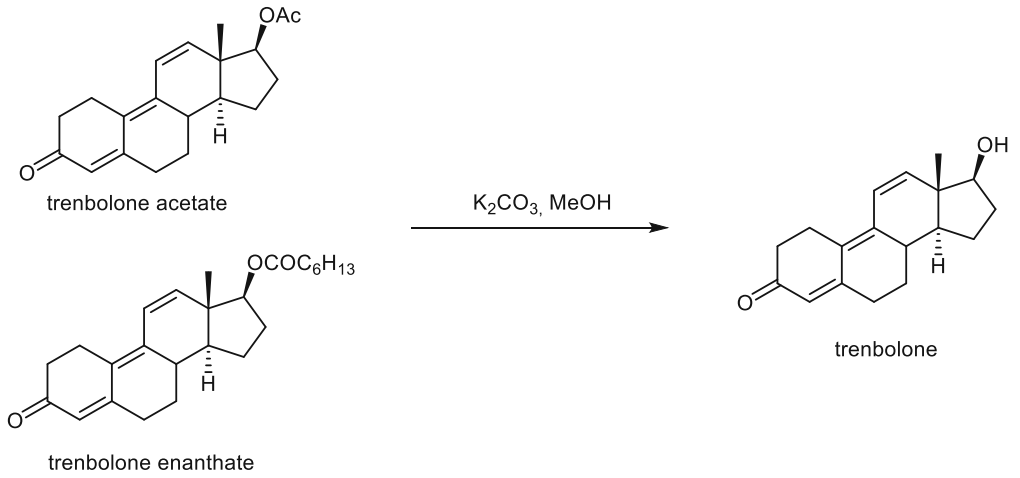
The glucuronidation was performed under Helferich conditions. The reaction was promoted by mercury (II) cyanide / bromide and the acetylated glucuronyl bromide was used as the donor material. The optimal temperature was found to be 70° C, with lower reaction temperatures producing predominantly orthoesters and higher reaction temperatures slightly reducing the yields. The two N-glucuronide regioisomers were formed in 10 % (1'N) and 25 % (2'N) yield and could be separated at this stage by NP - column chromatography followed by RP-HPLC separation.

The glucuronides were deprotected by use of lithium hydroxide in water / MeOH, purified by RP-HPLC, lyophilized, and sent to Seibersdorf Laboratories.

2.4 Trenbolone and Epitrenbolone Glucuronides

2.4.1 Trenbolone

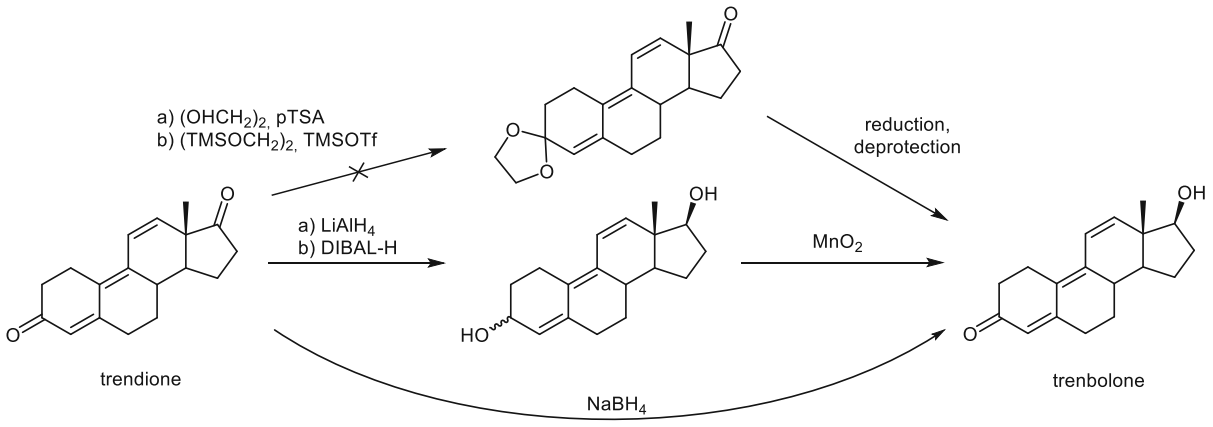
As with Metenolone, trenbolone was provided in form of injectable solutions of esters in "vegetable oil". The oil could be removed by MPLC the same way as in the case of metenolone (see chapter 2.2.2 and experimental part). In addition, trendione was provided in capsules. The trenbolone esters, including acetate, enanthate and mixtures thereof could be deprotected under basic conditions.



Scheme 42: Synthesis of trenbolone from its esters

The preparation of trenbolone starting from trendione was more complicated. It is known that reduction of C3-ketones in steroids is usually about 20-times faster than 17-reduction [125]. Therefore, protection of the C3-ketone as acetal was attempted. These reactions failed under standard and Noyori conditions [126].

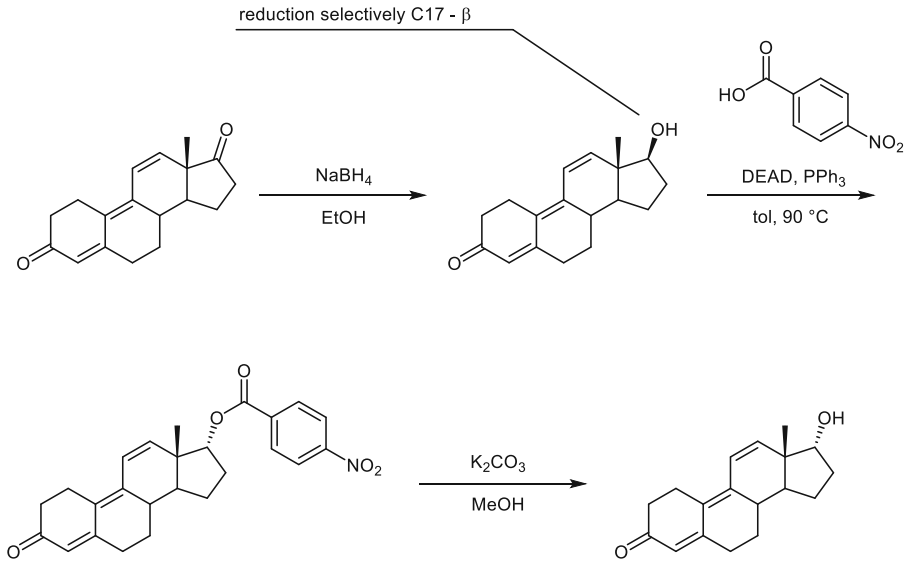
Double reduction to the diol was successful with both DIBAL-H and lithium aluminium hydride. Allylic oxidation with MnO_2 then gave Trenbolone but this reaction was very slow and low yielding. Finally, inspired by a method published for androstenedione [127], it was found that sodium borohydride in ethanol could selectively reduce the C17-ketone while leaving the trienone moiety untouched.



Scheme 43: Synthesis of trenbolone from trendione.

2.4.2 Synthesis of Epitrenbolone

As 17-α steroids are not accessible by reduction of 17-ketosteroids (like trendione), 17-epitrenbolone had to be synthesized by inversion of the sterically hindered 17-hydroxyl in trenbolone [129]. Lugar et. al. described an approach to this transformation using an enhanced Mitsunobu reaction [128]. Reactivity is increased by using relatively acidic *p*-nitrobenzoic acid, three equivalents of all reagents and 90 °C in toluene. This approach worked well in the case of trenbolone. Because the resulting trenbolone ester could not be separated from the hydrazide byproduct, the ester was directly cleaved after aqueous workup without further purification. Epitrenbolone was then separated from the hydrazide without trouble.



Scheme 44:Synthesis of epitrenbolone

2.4.3 (Epi-)Trenbolone Glucuronide

The first attempts at synthesizing trenbolone glucuronide were found to yield only low amounts of orthoester or no coupling products at all. The leaving groups used included the acetylated trichloroacetimidate (TCAI) for Schmidt glycosylation, the glycosyl thiophenolate / glycosyl sulfoxide for thio/Kahne-glycosylation [112][113] and the glycosyl bromide for Königs-Knorr / Helferich glycosylation [107][108]. When these approaches failed, the o-alkynylbenzoate (Abz) as reported by Yu was synthesized [111]. The reaction yielded only orthoester, but in markedly better yield than had been previously observed with the other approaches. Encouraged by this result, the isobutyryl-protected Abz-donor was synthesized, in the hope of suppressing orthoester formation. Inspiration for this was drawn from the synthesis of morphine-3,6-diglucuronide by Brown (2000) [103]. The more hindered pivalate group was also considered, but from the previous synthesis of epiandrosterone glucuronide in our group it was known to be very difficult to cleave ($LiAlH_4$ cannot be used) [41].

The isobutyryl-Abz donor led to very prolonged reaction time and after one week, exclusively orthoester was isolated once more (albeit in unprecedented 30 % yield).

Next, glucose-based Abz- and TCAI-donors were synthesized and tested. These reactions didn't meaningfully improve the reaction outcomes compared to their glucuronide counterparts and therefore no further work based on glucose was undertaken.

To avoid orthoester formation, TBS-protected Abz- and TCAI-donors were synthesized. Stereoselectivity for these donors was to be provided by shielding the α-face of the sugar with the steric bulk of the TBS-group. The TCAI-donor failed but the Abz-donor gave the glucuronide in 21 % yield and a pleasing β:α = 4:1 ratio. The anomers could be separated via RP-HPLC. Sadly, the molecule was largely decomposed during the extended exposure to TBAF that was needed to cleave the three sterically shielded TBS-groups.

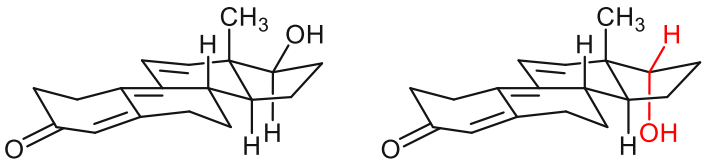
Many further protecting group strategies are published in literature (See chapter 1.5) that suppress orthoester formation while preserving β-selective glycosylation. Large scale synthesis of these donor materials, needed for the low-yielding glucuronidation of bad nucleophiles such as trenbolone seemed to be a daunting task with unclear payoff. Therefore, it was decided to first revisit the orthoester formed in previous attempts at glucuronidation and attempt the orthoester – glycoside rearrangement .

After TMSOTf, $BF_3.Et_2O$ and dilute TfOH turned out to be overly reactive and lead only to transacylated product, tetramethyl urea (TMU) – TfOH was chosen as rearrangement agent, as suggested by Banoub (1979) [130]. While still very low-yielding, this approach allowed the isolation of protected trenbolone glucuronide after normal-phase- followed by reversed-phase-chromatography. This result was slightly improved upon by generating TMU – TfOH *in situ* during the glucuronidation reaction, inspired by Hanessian (1977) [131]. The reaction utilizes acetoglucuronosyl bromide, activated by silver triflate. Triflic acid that is released over the course of the reaction is buffered by TMU and, over the course of four hours, rearranges the initially formed orthoester to the glucuronide. The small amounts of formed product were subjected to NP-column chromatography to remove the residual sugars and trenbolone acetate. This was followed by RP-MPLC or RP-HPLC, allowing separation from trenbolone. A yield of 4 % of purified glucuronide was obtained this way.

Deprotection was achieved without problems, using the conditions developed for ostarine glucuronide (see chapter 2.6): LiOH in THF / water rapidly cleaved the methyl ester, the reaction was then diluted with methanol to remove the acetyl groups. After HPLC-purification, the product was neutralized with sodium bicarbonate and lyophilized.

This final approach allowed for the synthesis of a total of 26 mg of trenbolone glucuronide sodium salt, a 3 % overall yield.

The synthesis of epitrenbolone suffers from the same problems as the synthesis of trenbolone, however, it seems to be even less nucleophilic. This is counterintuitive as on first glance, the 17-α hydroxyl seems less hindered as it is not shielded by the C-13 methyl.



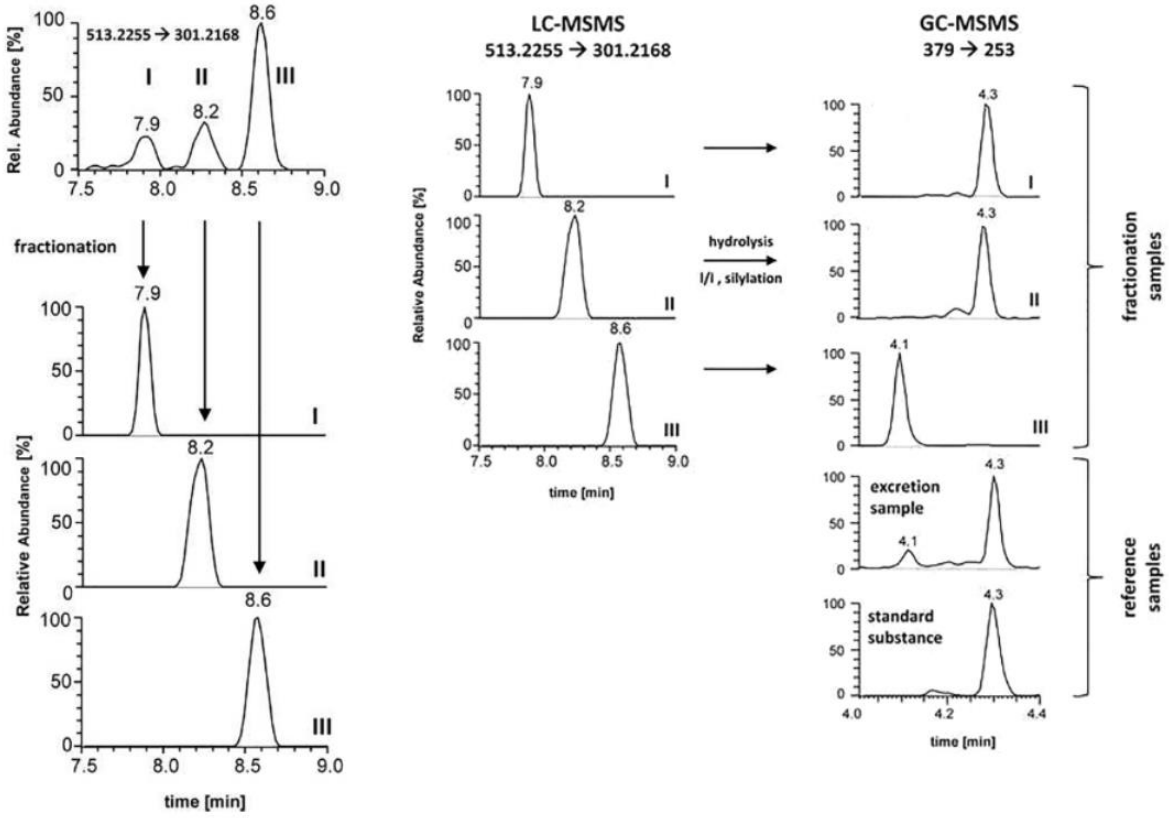
Scheme 45: Structural view of trenbolone vs epitrenbolone

No reaction conditions that allow the isolation of pure epitrenbolone glucuronide from the reaction mixture have been found as of the writing of this thesis. (However, its formation and the rearrangement of its orthoester have been observed by UPLC-MS).

2.5 DHCMT Glucuronides

2.5.1 Tritylation Experiments

A urine sample that had previously tested positive for DHCMT was made available for research purposes by WADA, with consent of the athlete. In this sample, three compound peaks (compound I, II and III) with m/z corresponding to the two possible glucuronides of DHCMT M3 metabolite could be identified [132]. Of these three, compounds I and II gave signals identical to M3 upon enzymatic hydrolysis, confirming they were the two possible glucuronides of M3. The third compound, upon enzymatic hydrolysis, yielded metabolite M15. This metabolite has the same molecular mass as M3 but its stereochemistry has not been assigned until now as no matching reference material is available. One likely option would be the C17-epimer of M3, as was mentioned by Sobolevsky in 2012 [133].

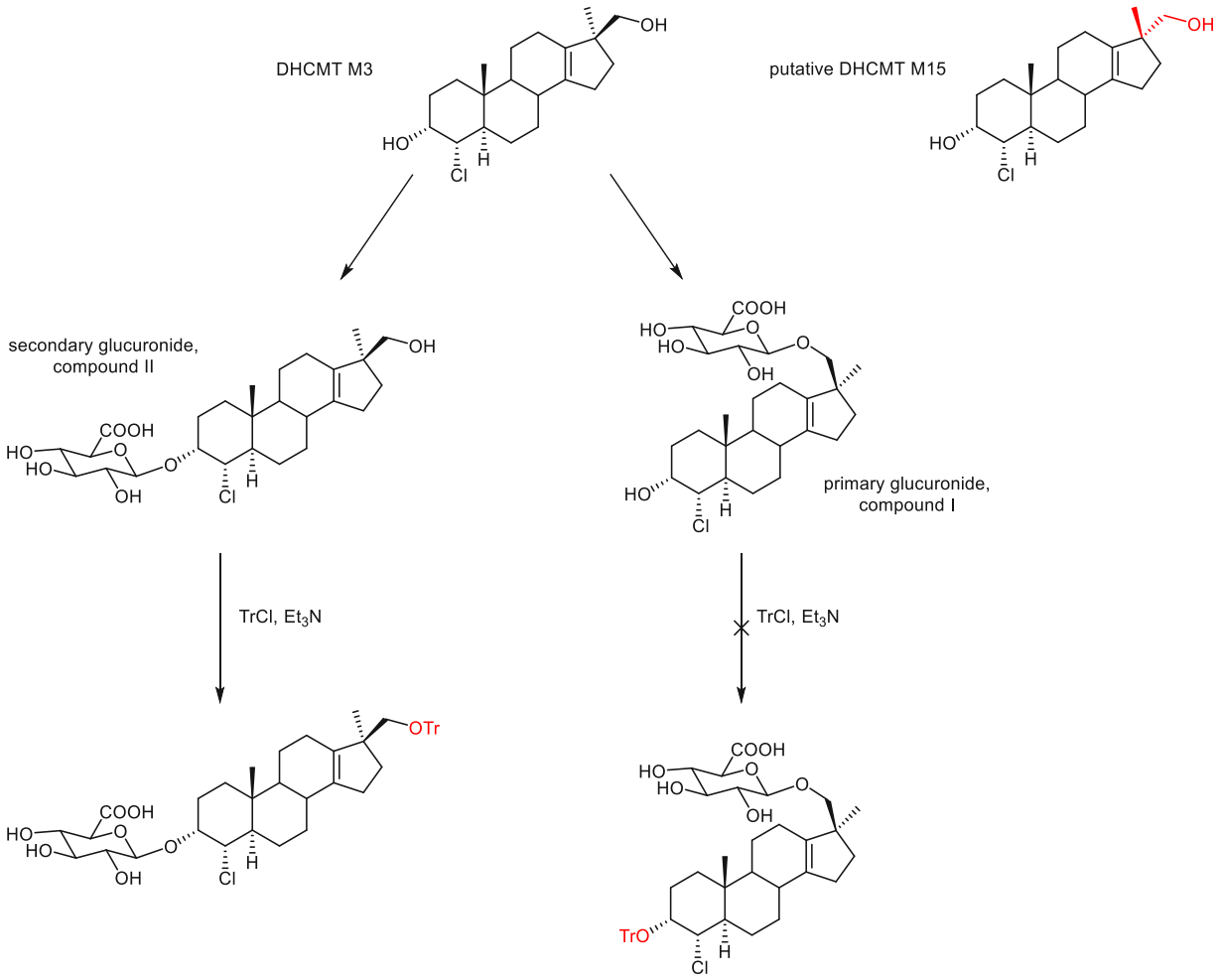


Scheme 46: Scan for m/z 513.2255 -> 301.2168 gave three compounds. After separation and enzymatic hydrolysis, compounds I and II were identical to M3 and

To distinguish the two M3 – glucuronides, a derivatization experiment was devised: As trityl chloride is known the react exclusively with primary alcohols in the presence of secondary alcohols [134], only the 3-OH glucuronide would react with trityl chloride. As the primary alcohol in the 17-hydroxymethyl glucuronide would be blocked, no reaction would be expected.

The glucuronide extract was fractionated on HPLC and the resulting residues separately incubated with a tritylating agent. The agent was prepared by dissolving TrCl in DMF to give a 1 N solution, followed

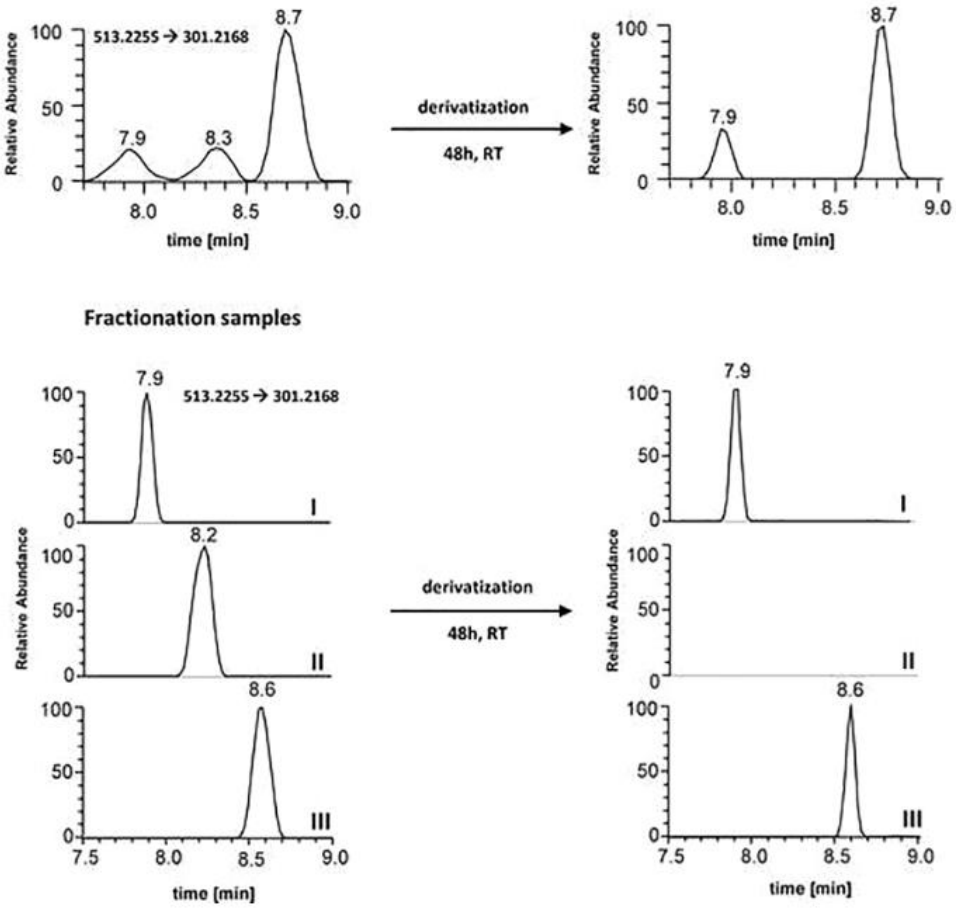
by addition of 1.3 eq Et_3N in respect to TrCl. The lyophilized glucuronide fractions were incubated with this solution at ambient temperature for 48 h. After quenching the reaction with sodium bicarbonate and evaporation of the solvent, the residue was redissolved in water and subjected to LC-HRMSMS analysis.



Scheme 47: Schematic depiction of the tritylation experiment

While compound I and III remained unharmed, the signal for compound II disappeared completely (see Scheme 48). This led to the conclusion that compound I must be the 17-hydroxymethyl glucuronide, while compound II must be the 3-hydroxy-glucuronide.

The success of the derivatization experiment was gratifying, however, the tritylated glucuronide could not be observed in LCMS. In order the further validate the result and confirm the identity of compound III, the synthesis of reference materials was necessary.



Scheme 48: Results from the tritylation experiments: compound II vanished while compounds I and III were unchanged

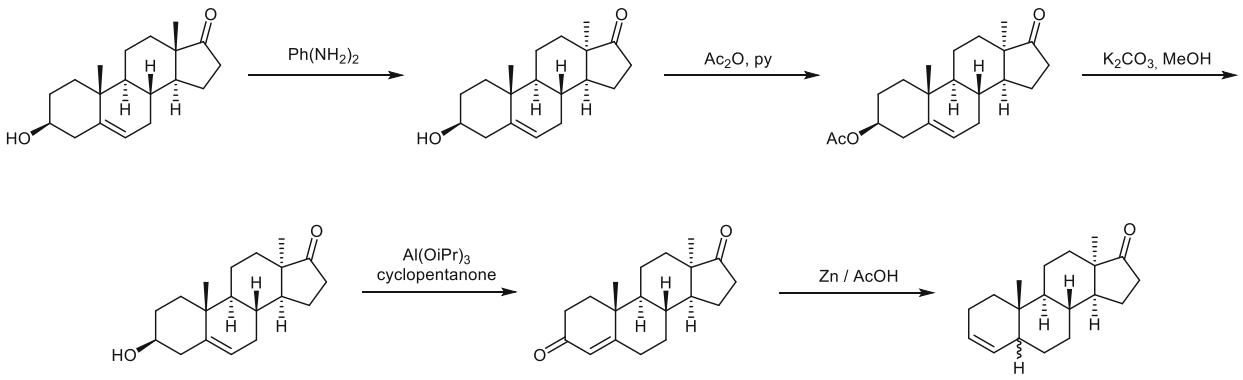
2.5.2 Synthesis of DHCMT M3 17-O-Glucuronide

DHCMT M3 17-O-Glucuronide was chosen as the target compound because the 3-hydroxyl group in M3 was known to be very sterically hindered and therefore didn't seem a promising target for glucuronidation. The synthesis of one glucuronide would be sufficient to unambiguously assign the identities of both compound I and compound II.

To be able to attempt the glucuronidation of DHCMT M3, resynthesis of the metabolite was necessary. The synthetic route that had been previously established in our group was planned towards the parallel synthesis of all metabolites M1 – M8 and could therefore be optimized towards the synthesis of M3 [68].

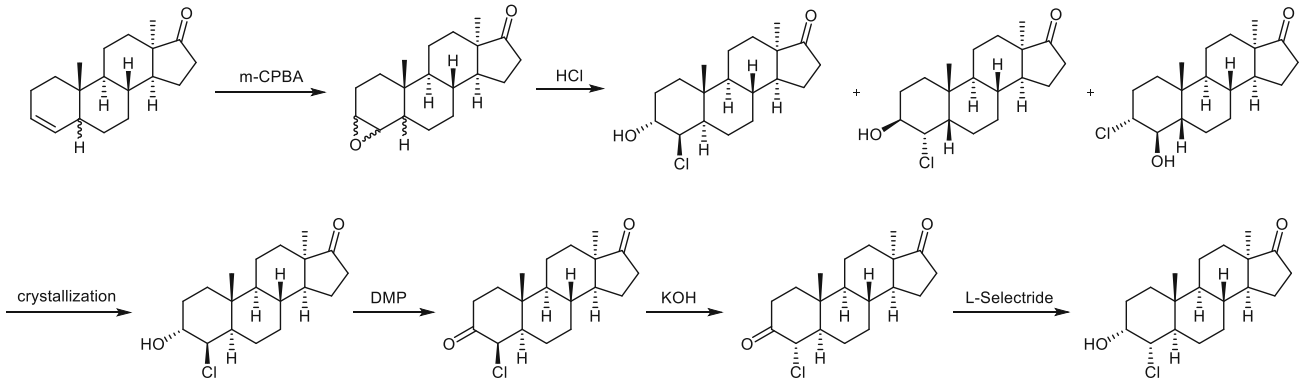
The synthesis started from dehydroepiandrosterone. The final Wagner-Meerwein-rearrangement for M3 must give the 17-β-hydroxymethyl. To achieve this, the stereochemistry of the C13-α-methyl has to be inverted. The method of choice for this transformation is epimerization by action of phenylenediamine in boiling acetic acid [121]. After 2 h, a 4:1 mixture of 13α:13β epimers could be isolated. During the transformation, partial acetylation took place at the 3-OH. To facilitate separation of the products,

acetylation is completed in acetic anhydride / pyridine before the epimers could be separated by crystallization from diisopropyl-ether. The remaining 13β-3O-acetate can be subjected to the epimerization conditions again or be recycled in subsequent synthetic campaigns. With enough of the 13α-epimer in hand, the A-ring could be elaborated after deacetylation. Oppenauer-oxidation achieves the dual goals of oxidizing the 3-OH while simultaneously causing isomerization of the Δ-5,6 double bond towards conjugation with the newly formed ketone. The replacement as oxidant of cyclohexanone with cyclopentanone resulted in simplified workup and reduced olfactory pollution of the lab space. The resulting enone-moiety was subjected to Clemmensen-reduction, giving the Δ-3,4 olefin. This reaction resulted in an inseparable epimeric mixture at C-5, resulting in the irrecoverable loss of half the material and a challenging purification step down the line. To circumvent this problem, a Birch-reduction-chlorination was attempted. Birch reduction of testosterone-like structures is known to give exclusively the desired 5-α-H product and moreover would have saved two steps [135]. After several attempts without any success, this idea had to be shelved.



Scheme 49: Synthetic scheme for the first part of M3 synthesis

The synthesis was continued with the 5-α/β-mixed Δ-3,4 olefin. Epoxidation with m-CPBA is stereospecific, depending on the geometry at C-5 and yields a mix of the Δ-3,4-β-epoxy-5β and Δ-3,4-α-epoxy-5α product. These were transformed to the corresponding chlorohydrins by action of conc. aq. HCl. The formation of three products could be observed: The Δ-3,4-β-epoxy-5β compound is opened to the 3β-hydroxy-4α-chloro-5β configuration and its regioisomer, the 3α-chloro-4β-hydroxy-5β compound. Starting from the Δ-3,4-α-epoxy-5α compound, only the desired 3α-hydroxy-4β-chloro-5α regioisomer is obtained. The resulting mixture can barely be separated by column chromatography. Serendipitously, it was found that the desired isomer has a strong propensity to crystallize and can be separated from the reaction mixture by this means almost completely.



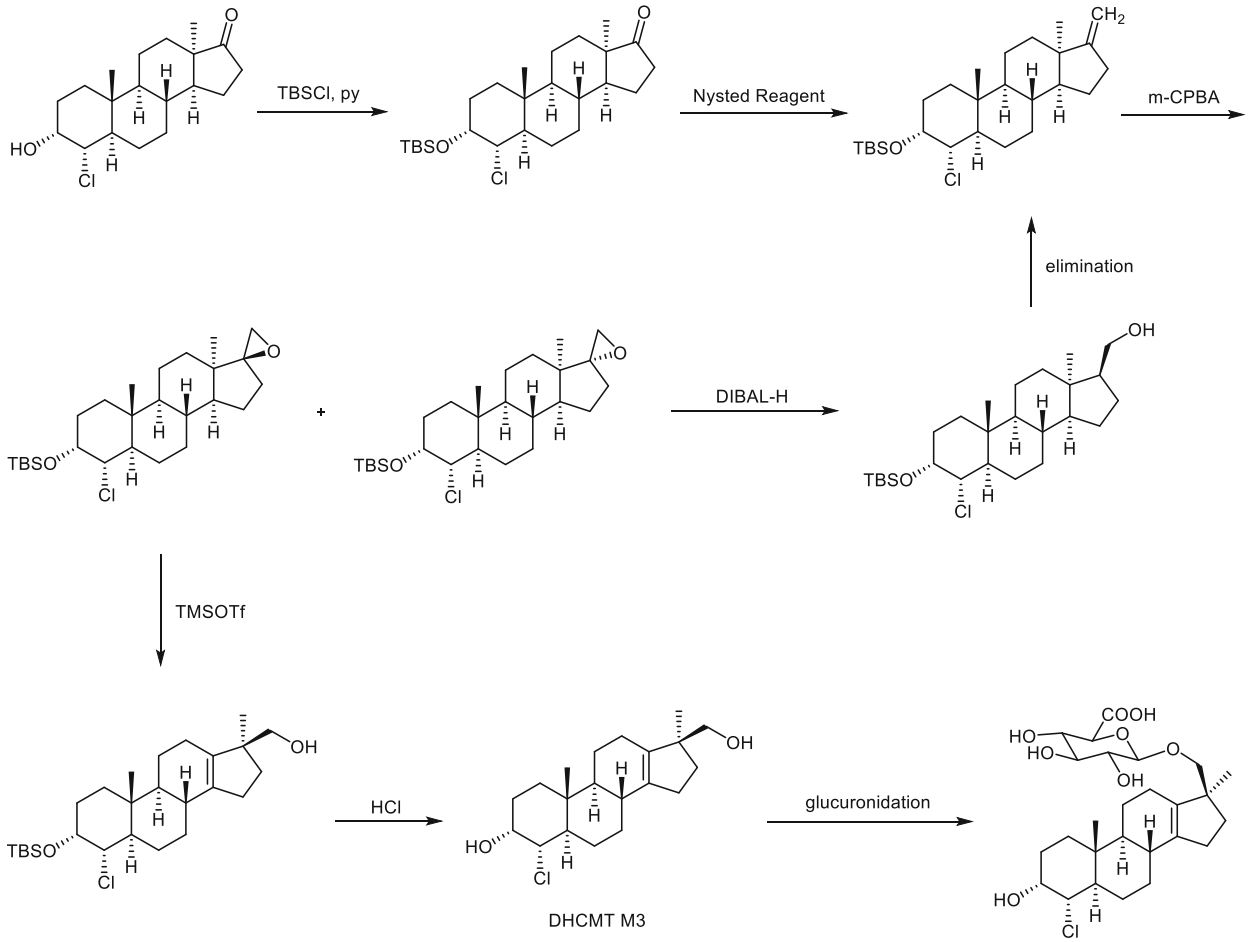
Scheme 50: Synthetic scheme for the second part of M3 synthesis, elaboration of the A-ring

Epimerization of the 4α-chloride was achieved by an oxidation – epimerization – reduction sequence. Oxidation with DMP was clean and uneventful but basic epimerization had to be stopped after 5 min. to prevent excessive degradation via Favorskii-rearrangement. Completion of the transformation can only subsequently be confirmed by ^{1}H-NMR. The original divergent route prescribed Meerwein-Ponndorf-Verley-reduction at this point, yielding a 1:1 mixture of 3-OH-epimers. This was successfully replaced by L-Selectride, yielding only the desired 3α-hydroxy-4α-chloro-5α product in good yields. This substance again shows a very strong propensity to crystallize.

In preparation for C-17 olefination, the free hydroxyl group was TBS-protected. Under standard silylation conditions (DMF, imidazole, TBSCl) this reaction took for four days to reach 80 % conversion. Attempts were made to improve upon this procedure, however, all variations, such as utilization of TBSOTf or stronger bases lead to negligible improvements in reactivity but coupled with lower yield. Complete deprotonation of the hydroxyl with LiHMDS at low temperatures before addition of TBSCl leads to faster reaction time with acceptable yields but finally, the original method was used for the bulk of the material. As the reaction was not complete even after four days, remaining starting material could be isolated and recycled for a next run or subsequent campaign.

After protection, the difficult C-17 olefination was performed. While this transformation is comparatively easy with a C13-β-methyl, the reaction is strongly hindered by the C13-α-methyl needed for the synthesis of M3. The existing method for this transformation was Nysted-olefination, yielding 29 % product and recovery of the remaining starting material. Attempts to improve upon this method via different olefination reagents such as Petasis- and Tebbe-reagent failed. The activity of the reaction could however be improved to 61 % conversion by using freshly prepared Nysted-reagent instead of the commercial suspension.

Subsequent epoxidation was slightly improved by changing the conditions to a biphasic mixture, somewhat improving the unfavorable diastereomeric ratio of products from 1:2 to 2:3. The epimeric epoxides were separated by chromatography. It was found that the undesired α-epoxide can be



Scheme 51: Synthetic scheme for the third part of M3 synthesis, elaboration of the D-ring

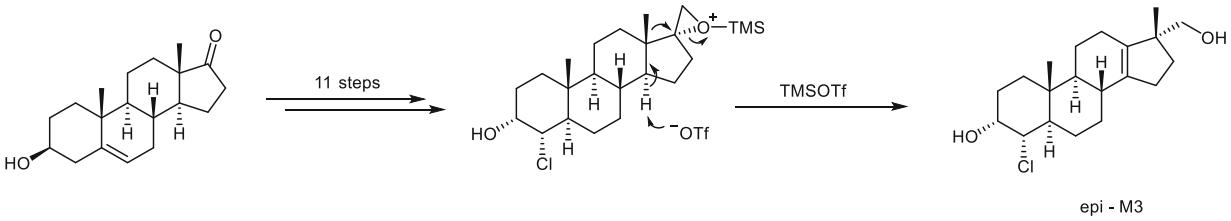
regioselectivity reduced to the primary alcohol with DIBAL-H, paving the way for recycling of the material by elimination.

The β-epoxide was Wagner-Meerwein-rearranged under TMSOTf catalysis, yielding 3O-TBS M3. Similar to the protection reaction, deprotection of the 3O-TBS was very slow and took 6 days to complete in 2 N aq. HCl. The reaction gave a mixture containing only a small amount of the desired material.

While only low milligram-amounts of M3 were made available through this campaign, synthesis of the glucuronide is performed with a primary alcohol as nucleophile and should therefore pose less of a problem than other glucuronidations in this thesis.

2.5.3 Synthesis of 17-epi-M3

As established previously, the most promising candidate for the identity of compound III is the primary glucuronide of Metabolite M15, ostensibly the C17-epimer of M3. The synthesis of this structure could be achieved by using the same route as is used in the synthesis of M3 but omitting the C-13 epimerization step in the beginning. This would lead to a final Wager-Meerwein rearrangement on the β-face of the D-ring and produce the desired compound.



Scheme 52: Wagner-Meerwein rearrangement to epi-M3

As the synthetic pathway to epi-M3 is substantially the same as for M3 for most reactions, it shall not be discussed in detail but only where reactivity departs significantly from the parent route:

1. The separation of the chlorohydrins by isomerization was just as successful as in the parent route.
2. The L-selectride-promoted reduction of the C3-ketone to the α-hydroxyl was significantly less regioselective. This was not unexpected as from previous experience, the C-17 ketone with adjacent C13-β-methyl was known to be much more reactive than the epimer.
3. Because of the unreactive nature of the C3-α-hydroxyl, and the therefore difficult protection and deprotection steps, it was decided to omit TBS-protection.
4. As expected, Nysted-olefination was much faster in this substrate because of the less hindered C17-ketone.

The synthesis of epi-M3 and characterization of the compounds on the pathway could not be completed due to lack of time before the end of the dissertation.

2.6 Ostarine Glucuronide

2.6.1 Ostarine

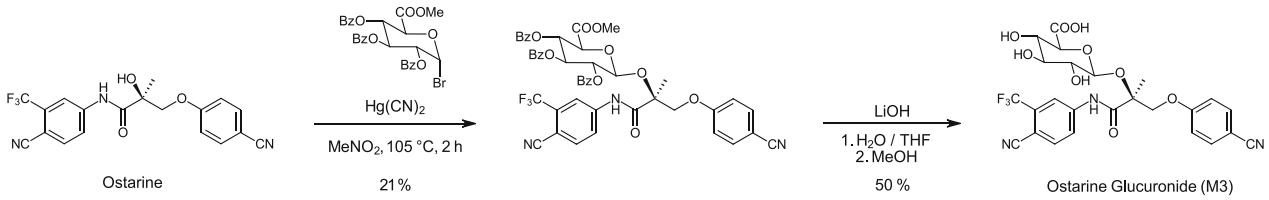
Ostarine was available in the form of capsules. The capsules were opened and the contained powder was extracted with dichloromethane. The powder was filtered off and ostarine could then be purified by column chromatography.

All reactions involving ostarine could easily be followed by TLC, as it has a characteristic violet fluorescence under the typical UV-lamp used for visualization of TLC plates. Reaction control *via* UPLC-MS was also aided by high UV-absorption and the very high ionization efficiency.

2.6.2 Ostarine Glucuronide

It was clear from the beginning that the hydroxyl group in ostarine would be very unreactive. In addition to it being a tertiary hydroxyl, the amide group in α-position withdraws electron density and therefore reduces electrophilicity. Even attempted acetylation of the hydroxyl group was difficult.

Many of the glucuronidation donors that had already been used for the trenbolone project were tried again for ostarine, but it was soon clear that harsher conditions would be needed to get any reaction at all. The Helferich method that had already been successful for the glucuronidation of androsterone and metenolone was employed and was met with some success. The key improvement was the change of acetyl- for benzoyl-protection on the glycosyl bromide. This allowed the reaction to proceed in up to 21 % yield for the glucuronidation.



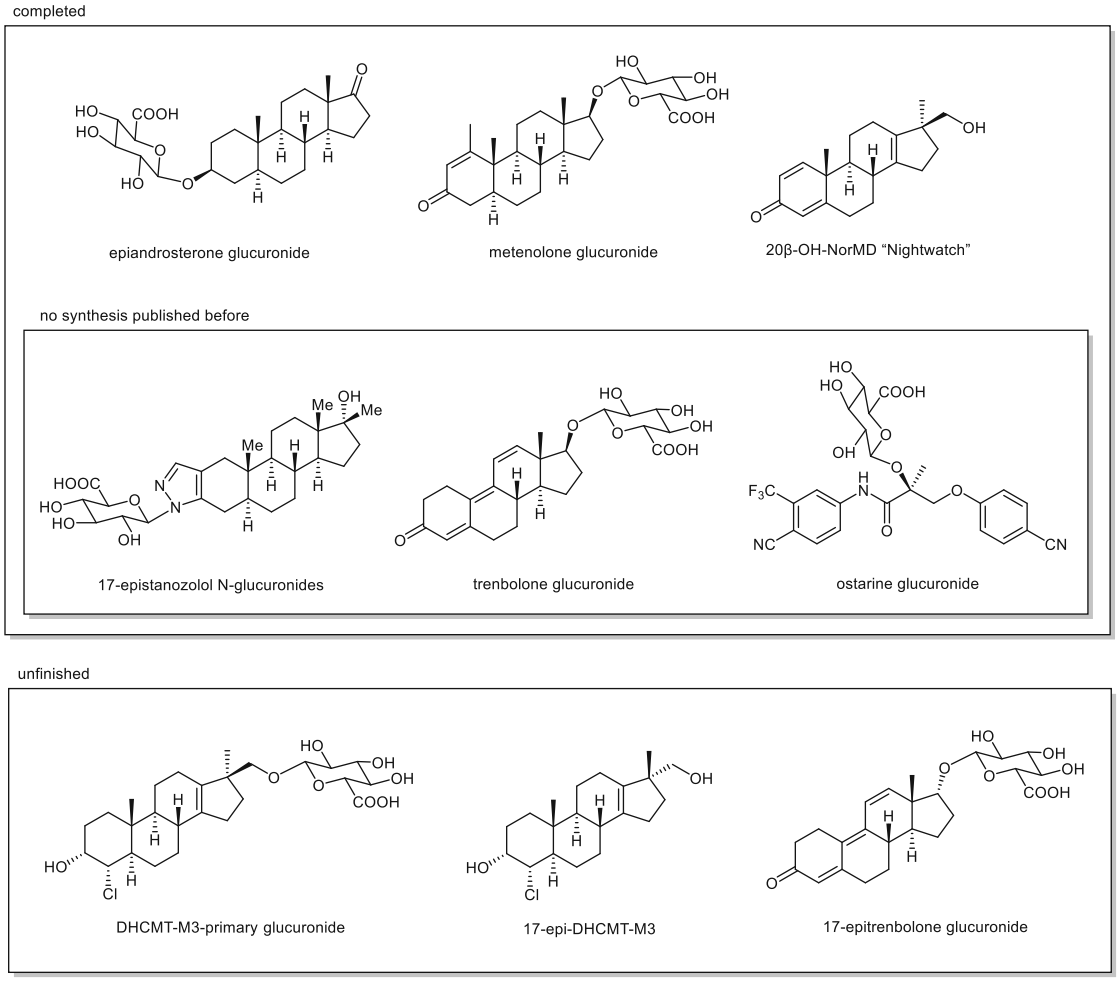
Scheme 53: Synthesis of ostarine glucuronide

During the long deprotection time required for the methyl ester with LiOH in MeOH / H_2O, significant amounts of elimination took place at the glucuronyl-C4. Deprotection was attempted in THF / H_2O. Under these conditions, the methyl ester was cleaved almost instantaneously, but the benzoyl groups stayed almost completely inert. When methanol was added to this reaction mixture, the benzoyl esters were cleaved after some time. From this experience, the final successful protocol was designed: The reaction is started in THF / H_2O at 0 °C and after 5 min, the reaction was diluted with MeOH to three times the initial volume and warmed up to ambient temperature. Usage of this protocol minimized the total reaction time to less than one hour and reduced the formation of elimination side-product.

The final product could be cleansed of the side-product by HPLC, but as the retention times were very similar and no baseline-separation could be achieved, some loss of the product had to be balanced with final purity.

3 Conclusion and Outlook

A variety of phase-I and phase-II metabolites were synthesized over the course of this thesis (including most of the initially targeted compounds shown in Scheme 31). These substances are now either in use as reference substances in routine doping analytics in WADA-accredited labs or will allow for the development of new analytic methods at Seibersdorf Laboratories.



Scheme 54: Overview over the results

While not all projects could be finished, the synthesis of doping-relevant metabolites will continue in our group and the experiences gained during this thesis will help with future endeavors in glucuronidation chemistry. In particular, the method developed for the synthesis of SARM-glucuronides seems applicable to many of the other aryl-propionamide-class SARMs.

The synthesis of trenbolone glucuronide was successfully performed, however, the low yields of the final method suggest that there is room for optimization in the future. An optimized method might also yield epitrenbolone glucuronide, which could not yet be successfully synthesized.

Contributions were made to the research into DHCMT – phase - II metabolism and the identities of two new glucuronides could be assigned by a trityl - derivatization experiment. To further validate the results from the derivatization experiments, the synthetic route towards DHCMT-M3 was optimized with the

goal of synthesizing one of the glucuronides. Furthermore, a campaign to synthesize 17epi-DHCMT-M3 was undertaken. These pursuits could not be finished due to time constraints but could potentially be finalized with comparatively little effort.

As “byproducts” of this thesis, several previously unknown glucuronidation donors were synthesized and characterized. This includes a distorted β-selective *ortho*-alkynylbenzoate glucuronidation donor, the conformations of which were studied via X-ray crystallography and a DFT-study.

4 Experimental Section

4.1 General Procedures

Most of the reagents and solvents were purchased from Merck & Co., TCI, ACROS Organics, Fluorochem or VWR and were used without further purification unless otherwise noted. Dry DCM, dioxane, Et_2O, MeOH, toluene and THF were provided by a PURESOLV® facility. Flash column chromatography, DCVC and normal phase MPLC separations were performed using silica gel 60 (Merck, 40-63 µm). Thin-layer chromatography was performed on Merck aluminium sheets precoated with silica gel (TLC Silica gel 60 F_{254}) and visualization was done by staining in either ceric ammonium molybdate in EtOH / H_2SO_4 or anisaldehyde in EtOH / H_2SO_4.

RP-MPLC was performed in MeCN / water or MeOH / water on a Büchi® "Pure" system with prepacked "Sfär C18 Duo" cartridges from Biotage®.

RP-HPLC separations were performed on an Autopurification system from Waters with an AQUITY QDa detector and a 2998 photoarray detector. Separation was conducted with a XSELECT CSH C18 5 µm 4.6 x 150 mm column for analytical runs and a XSELECT CSH Prep C18 5 µm OBD 30 x 150 mm column for preparative runs.

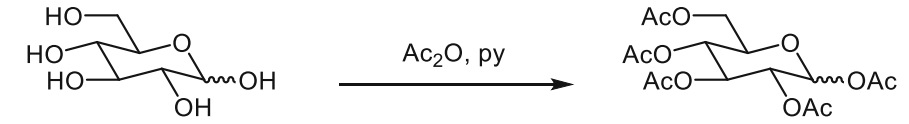
Specific Rotations were measured on an Anton Paar MCP 500 polarimeter at 20 °C and 589 nm.

^1H and ^{13}C NMR spectra were recorded on either Bruker Avance 200, Bruker Avance 400, or Bruker Avance III 600 instruments. Chemical shifts were reported in parts per million (ppm, δ) relative to Me_4Si (0 ppm) or $CDCl_3$ (7.26 ppm) or HDO (4.79 ppm) as the internal reference. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and bs = broad singlet), coupling constants in Hz.

For substances known to literature, spectral data can be found in the respective original papers.

4.2 Glucuronidation Donors

4.2.1 1,2,3,4,6-Penta-O-acetyl-D-glucopyranose



Molecular Weight: 180,16 Molecular Weight: 390,34

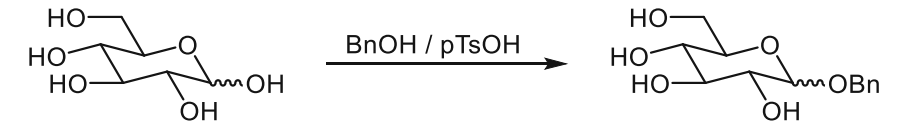
Glucose (10 g, 55 mmol) was dissolved in 50 mL dry pyridine and cooled to 0 °C. Acetic anhydride (50 mL, 555 mmol, 10 eq) was added dropwise over t4he course of 1 h and after completion, DMAP (670 mg, 0.1 eq) was added (exothermic, reaction warms to 40 °C).

After 5 h, the solution was poured into well stirred ice water and the water was extracted with 3 x EE and the organic phase evaporated. The residue was dried by coevaporation with toluene and then recrystallized from EtOH/H_2O (70° C). The crystals were washed with cold water and cold EtOH and then dried, yielding 20 g (92 %) pure 1,2,3,4,6-penta-O-acetyl-D-glucopyranoside.

Analytical data were in accordance with literature. [136]

^{1}H NMR (200 MHz, Chloroform-d) δ = 6.32 (d, J=3.6, 1H), 5.56 – 5.38 (m, 1H), 5.22 – 5.02 (m, 2H), 4.33 – 4.19 (m, 1H), 4.19 – 4.00 (m, 2H), 2.18 (s, 3H), 2.09 (s, 3H), 2.06 – 1.94 (m, 9H).

4.2.2 Benzyl α/ß-D-glucopyranoside



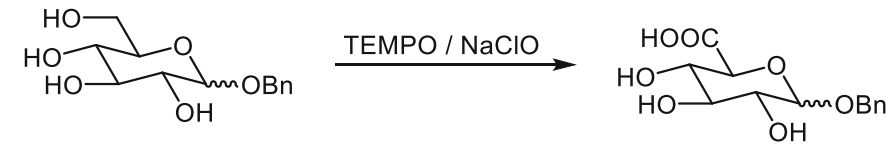
Molecular Weight: 180,16 Molecular Weight: 270,28

Glucose (100 g) was suspended in 200 mL BnOH and 11 g pTsOH were added. The mixture was heated to 83°C for 240 min. When everything had dissolved, UPLC-MS showed complete conversion and the white suspension had turned to a viscous yellow solution.

In order to remove benzyl alcohol, the mixture was cooled to ambient temperature and 300 mL water and 300 mL Et_2O were added. The phases were separated and the aqueous phase was extracted twice with 100 mL Et_2O. The water was evaporated and the residue coevaporated with toluene. The residue was crystallized from acetone to yield 1-O-benzyl glucose as a mixture of anomers (80 g, 59 %) which was used for the next step without further purification.

Spectra for β-anomer were in accordance with literature (supporting info, p43: [138])

4.2.3 Benzyl-α/ß-D-glucopyranosiduronic acid



Molecular Weight: 270,28

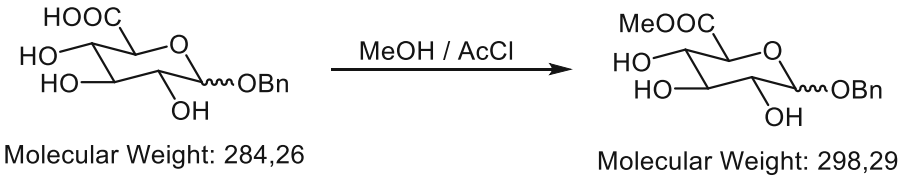
Molecular Weight: 284,26

In a 2000 mL flask, 1-O-benzyl-glucopyranose (23 g, 85 mmol) was dissolved in 40 mL water. Sodium bicarbonate (10 g, 110 mmol), TEMPO (130 mg, 0.01 eq) and KBr (530 mg, 0.10 eq) were added. The solution was cooled to 0 °C and NaOCl (130 mL, 3 eq, use min. 3 eq) was added dropwise (color turns yellow). After 4 h at 0 °C, the yellow color had vanished and HPLC-MS indicated complete conversion of the starting material (otherwise add more NaOCl). Na_2SO_3 (10 g, 0.5 eq) was added to quench residual hypochlorite and the pH was lowered to 3 with conc. H_2SO_4.

The water was evaporated and the residue redissolved in 250 mL methanol. The solution was filtered and used for the next step without further purification.

Spectra for β-anomer were in accordance with literature (supporting info, p44: [138])

4.2.4 Methyl (benzyl-α/ß-D-glucopyranosid)uronate I



Molecular Weight: 284,26 Molecular Weight: 298,29

The crude solution from the step before was introduced a round-flask cooled by an ice bath. Acetyl chloride (4 mL) was added dropwise until the solution was a bright yellow color. The mixture was warmed to ambient temperature and stirred for 3 h, after which UPLC confirmed complete consumption of starting material. The reaction was quenched with triethylamine (turns green) and the volatiles were evaporated.

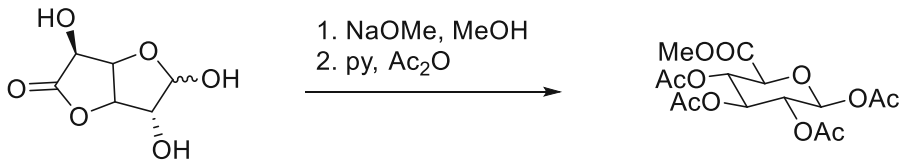
The product was purified by MPLC (200 g silica, 100 % EE or 200 g RP silica, gradient: 10 % - 60 % MeOH in H_2O) to yield the methyl ester (6.0 g, 24 % over two steps) as a mixture of anomers.

Spectra for β-anomer were in accordance with literature (supporting info, p44: [138])

R_f: 0.23 (100 % EA)

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.32 – 7.14 (m, 5H), 4.89 (d, J = 3.1 Hz, 1H,), 4.66 (d, J = 11.9 Hz, 1H), 4.48 (d, J = 3.3 Hz, 1H), 3.73 (d, J = 9.6 Hz, 2H), 3.70 (d, J = 1.0 Hz, 3H), 3.53 – 3.38 (m, 2H).

4.2.5 Methyl 1,2,3,4-tetraacetyl-β-D-glucopyranuroate



Molecular Weight: 176,12 Molecular Weight: 376,31

Glucuronolactone (17.6 g, 100 mmol) was suspended in 50 mL dry Methanol and 0.01 mL of 5 N NaOMe was added. The solution was stirred vigorously until everything had dissolved (1 h), giving a yellow solution. The solution was neutralized with acetic acid until a partial disoloration could be observed.

Methanol was evaporated and the resulting foam was redissolved in pyridine (40 mL, 500 mmol, 5 eq). When everything had dissolved (rotate in rotary evaporator bath), the solution was cooled to -10 °C and acetic anhydride (37,5 mL, 400 mmol, 4 eq) was added dropwise (dry DCM can be added to the mixture if stirring becomes difficult). The reaction mixture was warmed up to ambient temperature and stirring was continued overnight.

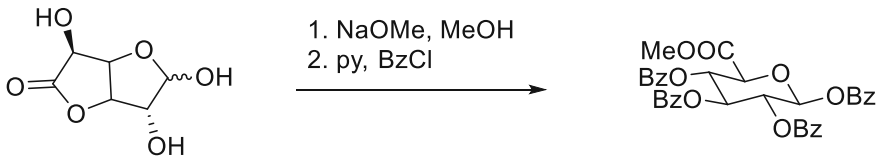
Before workup, the reaction mixture was diluted with 500 mL ethyl acetate. The dark organic solution was extracted with water, 1 N aq. HCl, sat. aq. $NaHCO_3$ and brine. The solvents were evaporated to yield the dark brown crude product. Pure β-anomer can be crystallized by redissolving in ethyl acetate and storing in the freezer overnight, giving off-white crystals (11.6 g, 31 %). The residue can be purified further by MPLC (20 % EA in PE) or crystallization from Et_2O to yield further product as a mixture of anomers (7.5 g).

Analytical data in accordance with literature [139], supporting info, S2.

R_f: 0.6 (50 % EE in hexanes)

^{1}H-NMR (400 MHz, Chloroform-d) δ = 5.77 (d, J=7.8, 1H), 5.36 – 5.19 (m, 2H), 5.15 (dd, J=8.9, 7.8, 1H), 4.18 (d, J=9.4, 1H), 3.75 (s, 3H), 2.12 (s, 3H), 2.04 (s, 6H).

4.2.6 Methyl 1,2,3,4-tetrabenzoyl-a/β-D-glucopyranuroate



Molecular Weight: 176,12

Molecular Weight: 624,60

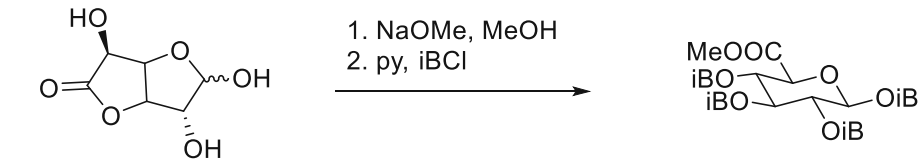
A 1000 mL round bottom flask was charged with glucorolactone (10.34 g, 58.7 mmol), which was then suspended in 150 mL Methanol. To the suspension 5 M NaOMe (0.1 mL, 0.6 mmol, 0.01 eq.) was added. The mixture was stirred for 15 minutes at room temperature. When everything had dissolved, yellow solution was concentrated to dryness. The residue was redissolved in pyridine (28 mL, 6 eq, dissolution slow, rotate flask on rotavap). The solution was cooled to 0 °C and benzoyl chloride (33 mL, 285 mmol, 5 eq.) was added dropwise. Dry DCM can be added to aid stirring. After stirring overnight, the reaction was diluted with 250 mL of DCM. The solution was washed with 1M HCl, saturated $NaHCO_3$ and dried over Na_2SO_4. The organic phase was evaporated.

The crude material was purified by filtering over silica gel (30 % EA in hexanes), and 38 g of brown resinuous product were obtained, which was pure enough to be used in the next step.

Spectral data were in accordance with literature [140]

R_f: 0.33 (33 % EA in hexanes)

4.2.7 Methyl 1,2,3,4-tetraisobutyryl-β-D-glucopyranuroate



Molecular Weight: 176,12 Molecular Weight: 488,53

Glucuronolactone (14.5 g, 82 mmol) was suspended in 100 mL dry Methanol and 0.05 mL of 5 N NaOMe were added. The solution was stirred vigorously until everything had dissolved (1 h), giving a yellow solution.

Methanol was evaporated and the resulting foam was redissolved in pyridine (45 mL, 570 mmol, 7 eq). When everything had dissolved (rotate in rotary evaporator bath), the solution was cooled to 0 °C and isobutyryl chloride (iBCl, 60 mL, 560 mmol, 6.8 eq) was added dropwise as a solution in 60 mL dry DCM. The addition was complete after 1 h, during which dry DCM was added as needed to facilitate stirring.

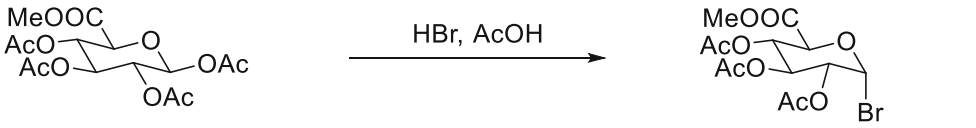
Before workup, the reaction mixture was diluted with 500 mL ethyl acetate. The dark organic solution was extracted with water, 1 N aq. HCl, sat. aq. $NaHCO_3$ and brine. The solution was dried and the solvents were evaporated to yield 37 g crude brown slurry. Pure β-anomer (13.7 g, 34 %) was obtained by trituration with light petrol. The mother liquor was concentrated and filtered over silica (500 g silica, 25 % EE in PE) to yield further 19 g of anomeric product mixture that was pure enough to be used for the next step.

Analytical data were in accordance to [103].

R_f: 0.81 (50 % EA in hexanes)

^{1}H-NMR (200 MHz, Chloroform-d) δ = 5.77 (d, J=8.0, 1H), 5.46 – 5.17 (m, 3H), 4.18 (d, J=9.8, 1H), 3.72 (s, 3H), 2.71 – 2.33 (m, 4H), 1.20 – 0.99 (m, 24H).

4.2.8 Methyl 2,3,4-triacetyl-1-bromo-1-deoxyglucopyranuroate



Molecular Weight: 376,31 Molecular Weight: 397,17

Glucuronic acid peracetate (3.4 g, 9.0 mmol) was suspended in 33 % HBr in glacial acetic acid (14 mL, 8.5 eq, 77 mmol), giving a brown suspension. After 1 h of stirring, all of the material had dissolved and stirring was continued at ambient temperature until TLC showed complete consumption of staring material (4 h).

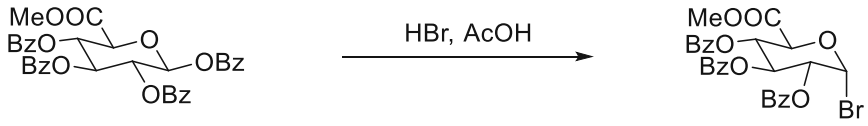
The reaction was diluted with 200 mL ethyl acetate and extracted with ice water, 2x sat. aq. $NaHCO_3$ and brine. The brwonish residue was recrystallized from EtOH and stored overnight in the fridge to yield the product as off-white crystals of pure α-anomer (2.6 g, 72 %).

Analytical Data in accordance with literature: [141] supp. p43

R_f: 0.7 (50 % EA in hexanes)

^{1}H-NMR (400 MHz, Chloroform-d) δ = 6.63 (d, J=4.1, 1H), 5.61 (d, J=9.8, 1H), 5.24 (dd, J=10.3, 9.5, 1H), 4.85 (dd, J=10.0, 4.1, 1H), 4.57 (dd, J=10.3, 0.7, 1H), 3.76 (s, 3H), 2.09 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H).

4.2.9 Methyl 2,3,4-tribenzoyl-1-bromo-1-deoxyglucopyranuroate



Molecular Weight: 624,60 Molecular Weight: 583,39

Glucuronic acid perbenzoate (4.7 g, 7.6 mmol) was dissolved in 10 mL dry DCM and 33 % HBr in glacial acetic acid (10 mL, 5.0 eq) was added dropwise, giving a brown suspension. After 5 h of stirring, TLC confirmed complete consumption of the starting material.

The reaction was diluted with 200 mL ethyl acetate and extracted with ice water, 2x sat. aq. $NaHCO_3$ and brine. The brownish residue was purified by MPLC (90 g silica, gradient: 10 % to 50 % EA in hexanes).

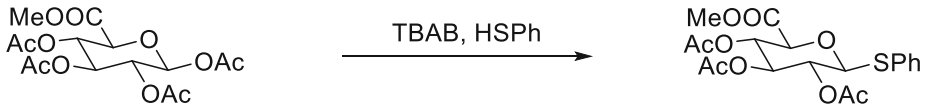
The product was obtained off-white resin of pure α-anomer (2.4 g, 54 %).

Analytical Data in accordance with literature [140].

R_f: 0.58 (33 % EA in hexanes)

^{1}H NMR: (400 MHz, CDCl3) δ 8.03 – 7.83 (m, 5H), 7.59 – 7.26 (m, 10H), 6.90 (d, J = 4.1 Hz, 1H), 6.28 (t, J = 9.8 Hz, 1H), 5.74 (dd, J = 10.2, 9.7 Hz, 1H), 5.35 (dd, J = 9.9, 4.1 Hz, 1H), 4.86 (dd, J = 10.2, 0.7 Hz, 1H), 3.69 (s, 3H).

4.2.10 Methyl 2,3,4-triacetyl-1-thiophenyl-1-deoxyglucopyranuroate



Molecular Weight: 376,31 Molecular Weight: 426,44

The substrate (500 mg, 1.3 mmol) and tetrabutyl ammoniumbromide (350 mg) were suspended in 10 mL EA and 10 mL 1 M K_2CO_3. Thiophenol (0.25 mL, 2.5 mmol, 1.85 eq) was added dropwise and the mixture was stirred vigorously at ambient temperature for 3 h.

After one additional hour, the reaction mixture was diluted with 70 mL EtOAc and washed with 1 M NaOH, sat. aq. $NaHCO_3$ and Brine. The solution was dried over sodium sulfate and the solvents were evaporated to give 700 mg of crude brown material.

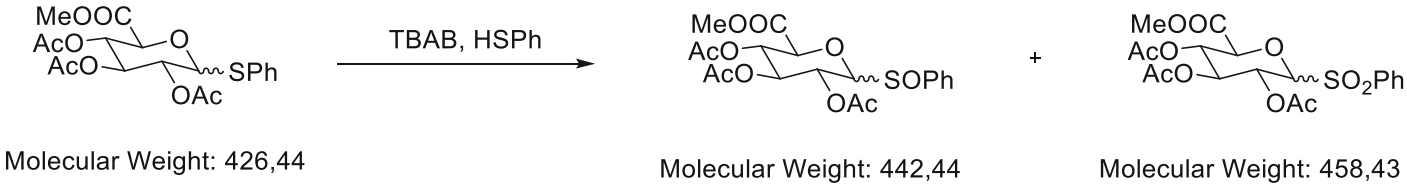
The material was purified by column chromatography (25 % EA in hexanes) to yield 340 mg (58 %) of an β-configured thioglucoside.

Spectral data of β-anomer were identical to reference: [142] supp. S14

R_f: 0.8 (50 % EE in hexanes)

^{1}H NMR (200 MHz, Chloroform-d) δ = 7.50 – 7.37 (m, 2H), 7.31 – 7.21 (m, 3H), 5.30 – 5.01 (m, 2H), 4.90 (dd, J=10.0, 8.7, 1H), 4.67 (d, J=10.0, 1H), 3.97 (d, J=9.4, 1H), 3.70 (s, 3H), 2.02 (s, 3H), 1.94 (d, J=2.8, 5H).

4.2.11 Methyl 2,3,4-triacetyl-1-phenylsulfinyl-1-deoxyglucopyranuroate



Molecular Weight: 426,44

Molecular Weight: 442,44

Molecular Weight: 458,43

The substrate (340 mg, 0.78 mmol) was dissolved in 3 mL DCM and cooled to – 70 °C. Solid *m*-CPBA (240 mg, 60 w% active, 0.84 mmol, 1.05 eq) was added in one batch. The reaction was warmed to ambient temperature over the course of two hours. TLC showed incomplete conversion but the reaction was worked up in order to avoid overoxidation.

Workup was performed by diluting with DCM, followed by quench of the reaction with sat. aq. $Na_2S_2O_3$. The organic phase was washed with sat. aq. $NaHCO_3$ and brine and dried over sodium sulfate. The solvent was evaporated to yield 320 mg of crude material.

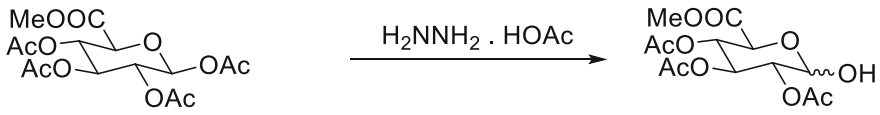
Purification was performed by column chromatography (20 g silica, 33 % EA in hexanes) and yielded 300 mg (85 %) of sulfoxoglycoside as a 3:1 mixture of diastereomers.

R_f = 0.29 (33 % EA in DCM)

^{1}H NMR (600 MHz, Chloroform-*d*) δ = 7.70 – 7.64 (m, 2H), 7.54 – 7.49 (m, 3H), 5.33 – 5.25 (m, 2H), 5.08 – 5.03 (m, 1H), 4.49 (d, *J*=8.8, 1H), 4.03 (d, *J*=9.8, 1H), 3.70 (s, 3H), 1.97 (s, 6H), 1.85 (s, 3H).

^{13}C-NMR (101 MHz, $CDCl_3$): δ 170.1, 169.0, 168.2, 165.3, 137.8, 130.8, 128.0, 124.7, 91.2, 74.9, 71.6, 67.5, 66.0, 51.9, 20.0, 19.5, 19.4.

4.2.12 Methyl 2,3,4-tri-O-acetyl-D-glucopyranuronate



Molecular Weight: 376,31 Molecular Weight: 334,28

Procedure derived from [143].

Glucuronic acid peracetate (2.0 g, 8.0 mmol) was dissolved in 5 mL DMF and heated to 55 °C. Hydrazinium acetate (750 mg, 1.5 eq) was added and the solution was stirred for 30 min.

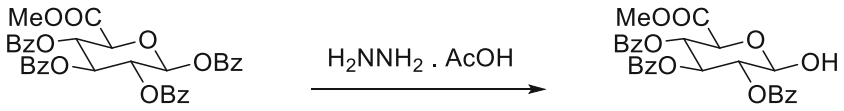
After TLC confirmed complete consumption of the starting material, the reaction was diluted with 100 mL EtOAc, washed three times with brine and then concentrated under reduced pressure. The product is immediately used for the next step without further purification.

Spectral data were in accordance with [144].

R_f: 0.5 (50 % EE in hexanes)

^{1}H-NMR (400 MHz, Chloroform-d) δ = 5.52 (m, 1H), 5.20 (m, 1H), 4.88 (m, 1H), 5.39–4.56 (m, 2H), 3.71 (m, 3H), 2.03 (m, 9H)

4.2.13 Methyl 2,3,4-tri-O-benzoyl-D-glucopyranuronate



Molecular Weight: 624,60 Molecular Weight: 520,49

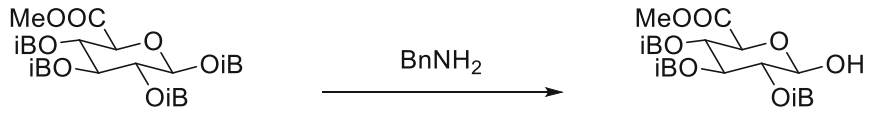
Glucuronic acid perabenzoate (3.0 g, 4.8 mmol) was dissolved in 10 mL DMF and heated to 55 °C. Hydrazinium acetate (580 mg, 1.3 eq) was added as a slurry and the solution was stirred for 60 min.

After TLC confirmed complete consumption of the starting material, the reaction was diluted with 100 mL EtOAc, washed three times with brine and then concentrated under reduced pressure. The product is immediately used for the next step without further purification.

Spectral data in accordance with: [145]

R_f: 0.45 (50 % EE in hexanes)

4.2.14 Methyl 2,3,4-tri-O-isobutyryl-D-glucopyranuronate



Molecular Weight: 488,53 Molecular Weight: 418,44

Glucuronic acid perisobutyrate (2.5 g, 5.0 mmol) was dissolved in 20 mL DCM and benzyl amine (1.4 mL, 12.5 mmol, 2.5 eq) was added. The mixture was stirred at ambient temperature overnight.

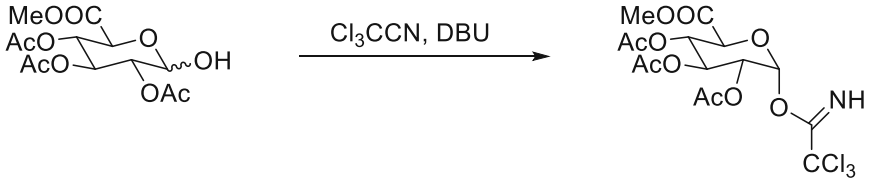
After TLC confirmed complete consumption of the starting material, the reaction was diluted with 100 mL EtOAc, washed with sat. aq. NH_4Cl, sat. aq. $NaHCO_3$ and brine, dried over sodium sulfate and evaporated. This procedure yielded 2.0 g of crude material, which contained the product as a α:β = 4:1 mixture. This material was used directly for the next step.

Analytical data were in accordance with: [104]

R_f: 0.65 (50 % EE in hexanes)

^{1}H-NMR (200 MHz, Chloroform-d) δ = 5.59 (t, J=9.8, 1H), 5.48 (d, J=3.6, 1H), 5.19 – 5.03 (m, 1H), 4.86 (dd, J=10.2, 3.6, 1H), 4.54 (d, J=10.2, 1H), 3.65 (d, J=4.0, 3H), 2.57 – 2.19 (m, 4H), 1.17 – 0.94 (m, 24H).

4.2.15 Methyl (2,3,4-tri-O-acetyl-D-glucopyranosyltrichloroacetimidate)uronate



Molecular Weight: 334,28 Molecular Weight: 478,66

Glycoside (500 mg, 1.5 µmol) was dissolved in 10 mL of dry DCM at -5 °C and trichloroacetonitrile (750 µL, 7.5 mmol, 5 eq) was added in one batch. DBU (0.07 mL, 0.5 mmol, 0.30 eq) was added, turning the colorless solution yellow. After warming to ambient temperature, the solution slowly got darker.

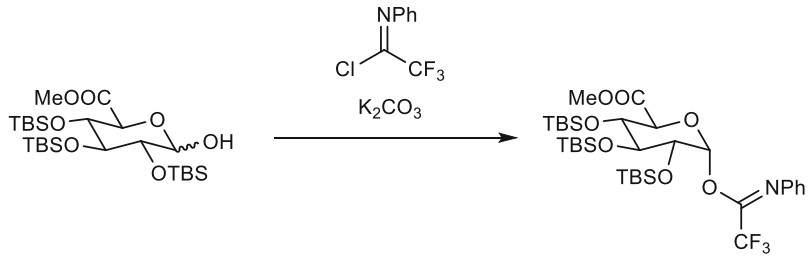
After 150 min reaction time, TLC showed complete conversion and the reaction mixture was concentrated to 3 mL and applied onto a silica gel column. Column chromatography was performed (25 g silica, 33% EA in PE) and yielded 470 mg (66 %) of pure α-trichloroacetimidate that crystallized slowly in the fridge.

Spectral Data identical to [144]

R_f: 0.8 (50 % EE in hexanes)

^{1}H-NMR (200 MHz, Chloroform-d) δ = 8.73 (s, 1H), 6.64 (d, J=3.6, 1H), 5.63 (t, J=9.8, 1H), 5.34 – 5.25 (m, 1H), 5.24 – 5.09 (m, 1H), 4.50 (d, J=10.2, 1H), 3.75 (s, 3H), 2.05 (s, 6H), 2.01 (s, 3H).

4.2.16 Methyl (2,3,4-tri-O-tert-butyltrimethylsilyl-D-glucopyranosyltrichloroacetimidate)uronate



Molecular Weight: 550,96

Molecular Weight: 722,08

The lactol substrate (300 mg, 0.54 mmol) was dissolved in 2 mL of dry acetone at 0 °C and 2,2,2-Trifluoro-N-phenylacetimidoyl chloride (95 µL, 0.6 mmol, 1.1 eq) was added in one batch. Dry potassium carbonate (150 mg, 1.1 mmol, 2 eq) was added. The suspension was stirred overnight, slowly turning a dark green color. When TLC showed complete consumption of the starting material, the reaction was evaporated. The residue was dissolved in ethyl acetate and water and washed with sat. aq. bicarbonate and brine. After drying over sodium sulfate, the organic phase was evaporated again.

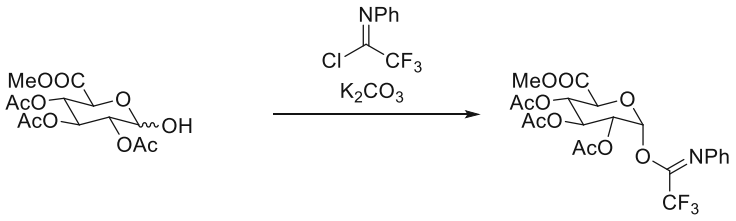
The product was purified by MPLC (40 g silica, 2 % -> 10 % EA in hexanes) and yielded 340 mg (86 %) of an 4:1 anomeric mixture. The anomers could not be assigned due to the spectral blurring caused by the slow rotation of the imine.

R_f: 0.4 (10 % EE in hexanes)

Major Anomer:

^{1}H-NMR (400 MHz, Chloroform-d) δ = 7.18 (dd, J=8.3, 7.4, 2H), 6.98 (dd, J=8.7, 7.2, 1H), 6.73 (d, J=7.7, 2H), 6.00 – 5.62 (bs, 1H), 4.21 (s, 1H), 3.82 (d, J=6.5, 1H), 3.71 (q, J=3.7, 2.6, 1H), 3.65 (d, J=4.4, 4H), 0.89 – 0.67 (m, 27H), -0.08 (d, J=13.9, 18H).

4.2.17 Methyl (2,3,4-tri-O-acetyl-D-glucopyranosyl(N-phenyl)trifluoroacetimidate)uronate



Molecular Weight: 334,28 Molecular Weight: 505,40

The hemiacetal (1.5 g, 4.5 mmol) was dissolved in 30 mL dry DCM and K_2CO_3 (1.9 g, 3 eq) and N-phenyl trifluoroacetimidoyl chloride (0.75 mL, 9.0 mmol, 2 eq) were added. The suspension was stirred at ambient temperature overnight after which TLC confirmed complete conversion of the starting material. The carbonate was filtered off over celite and the remaining mixture was concentrated. The residue was directly applied to column chromatography (75 g silica, 25 % EA in PE), which yielded the product (850 mg, 35 %) as a white foam.

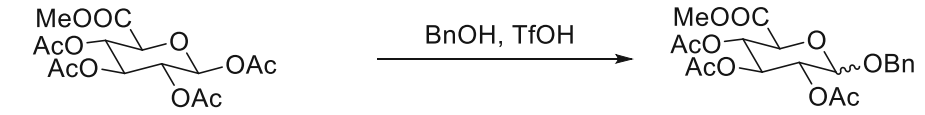
The anomers could not be assigned due to the spectral blurring caused by the slow rotation of the imine.

Spectral data in accordance with [146].

R_f: 0.8 (50 % EE in hexanes)

^{1}H-NMR (400 MHz, Chloroform-d) δ = 7.24 (t, J=6.0 Hz, 2H), 7.07 (d, J=6.6 Hz, 1H), 6.72 (d, J=7.4 Hz, 2H), 5.88 (s, 1H), 5.25-5.10 (m, 3H), 4.08 (d, J = 9.6 Hz, 1H), 3.69 (s, 3H), 1.99 (s, 9H).

4.2.18 Methyl (benzyl 2,3,4-tri-O-acetyl-α/ß-D-glucopyranosid)uronate



Molecular Weight: 376,31 Molecular Weight: 424,40

The substrate (8.6 g, 23 mmol) and 12.0 mL of benzyl alcohol (115 mmol, 5.00 eq) were dissolved in 120 mL dry DCM and 0.41 mL of triflic acid (4.6 mmol, 0.20 eq) were added dropwise at 0 °C. The mixture was stirred overnight.

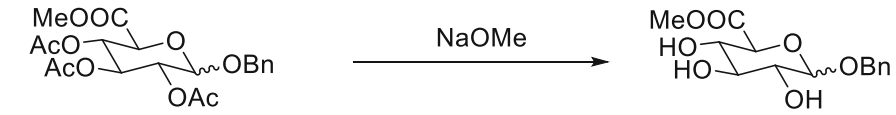
When TLC indicated complete consumption of starting material, the reaction was quenched with 20 mL of 1 M aq. K_2CO_3 and washed with water and brine. The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. After evaporation of the solvent, an amber oil was obtained. The oil contained residual benzyl alcohol and was used without further purification for the next step.

For analytical purposes, a small sample of the crude material was purified via column chromatography (20 g silica gel, petroleum ether/ethyl acetate = PE/EA = 5:1), obtaining pure β-anomer identical to prior reports [120].

R_f: 0.5 (50 % EE in hexanes)

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.35 (m, 5H), 5.59 – 5.55 (m, 1H), 5.26 – 5.14 (m, 2H), 5.13 (d, J = 7.3 Hz, 1H), 4.96 – 4.92 (m, 1H), 4.61 – 4.56 (m, 1H), 4.49 – 4.42 (m, 1H), 3.77 (s, 3H), 2.11 – 2.02 (m, 9H).

4.2.19 Methyl (benzyl-α/ß-D-glucopyranosid)uronate II



Molecular Weight: 424,40 Molecular Weight: 298,29

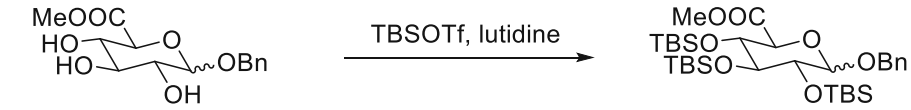
The substrate (16.3 g, 38.5 mmol, 1.00 eq) was dissolved in 300 mL MeOH and 0.77 mL of 5 M NaOMe solution (3.85 mmol, 0.1 eq) were added dropwise at 0 °C. After 1 h, TLC indicated complete deprotection and the solution was quenched with formic acid and evaporated. The resulting crude oil (15.3 g) was purified via flash column chromatography (150 g silica gel, PE/EA = 1:1, then EA/MeOH = 10:1), yielding 3.2 g (47 % over two steps) of a yellow foam.

Spectra for β-anomer were in accordance with literature (supporting info, p43: [138])

R_f: 0.23 (100 % EA)

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.32 – 7.14 (m, 5H), 4.89 (d, J = 3.1 Hz, 1H,), 4.66 (d, J = 11.9 Hz, 1H), 4.48 (d, J = 3.3 Hz, 1H), 3.73 (d, J = 9.6 Hz, 2H), 3.70 (d, J = 1.0 Hz, 3H), 3.53 – 3.38 (m, 2H).

4.2.20 Methyl (benzyl 2,3,4-tri-O-tert-butyldimethysilyl-α/ß-D-glucopyranosid)uronate



Molecular Weight: 298,29 Molecular Weight: 641,08

The substrate (1.02 g, 3.43 mmol, 1.00 eq) was dissolved in 40 mL dry DCM and 3.60 mL 2,6-lutidine (30.9 mmol, 9.00 eq) and 4.70 mL TBSOTf (5.44 g, 20.6 mmol, 6.00 eq) were added dropwise at 0 °C and then stirred for 1 h. The reaction mixture was heated to reflux and stirred for 6 h.

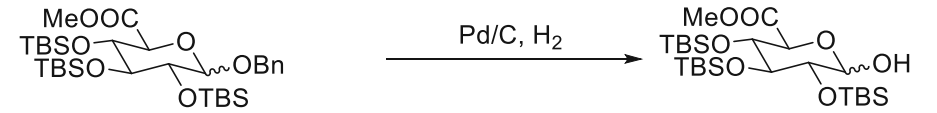
When TLC indicated complete conversion, the solution was diluted with 50 cm^3 DCM and washed with 1 M HCl, sat. aq. $NaHCO_3$ solution, water and brine. The organic layer was dried over Na_2SO_4 and the solvent removed under reduced pressure. The crude product (2.42 g) was purified *via* column chromatography (40 g silica gel, 10 % EE in hexanes), yielding 1.7 g (79 %) of a yellow oil.

Analytical Data in accordance with literature precedent [101].

R_f: 0.64 (10 % EA in hexanes)

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.18 – 7.13 (m, 5H), 4.90 (d, J = 6.1 Hz, 1H), 4.82 (d, J = 6.6 Hz, 1H), 4.64 (d, J = 12.3 Hz, 1H), 4.47 (d, J = 2.6 Hz, 1H), 4.29 (d, J = 1.2 Hz, 1H), 4.19 (dt, J_1 = 3.6 Hz, J_2 = 1.2 Hz, 1H), 3.62 (s, 1H), 3.60 (s, 3H), 0.76 (d, J = 4.00 Hz, 27H), 0.00 (m, 18H).

4.2.21 Methyl 2,3,4-tri-O-tert-butyldimethylsilyl-D-glucopyranuronate



Molecular Weight: 641,08 Molecular Weight: 550,96

The substrate (1.98 g, 2.96 mmol, 1.00 eq) was dissolved in 30 mL EtOH and Pd/C (0.59 g, 5 w % Pd) was added. The suspension was stirred under H_2 atmosphere overnight and filtered over a short plug of Celite. After evaporation of volatiles, 1.6 g of the crude residue was isolated. Purification was performed by column chromatography (100 g silica gel, 4 % EA in hexanes), yielding 1.45 g (85 %) of a colorless oil. An analytical sample of the β-anomer could be isolated from the anomeric mixture by repeated column chromatography (4 % EA in hexanes).

R_f: 0.42 (10 % EA in hexanes)

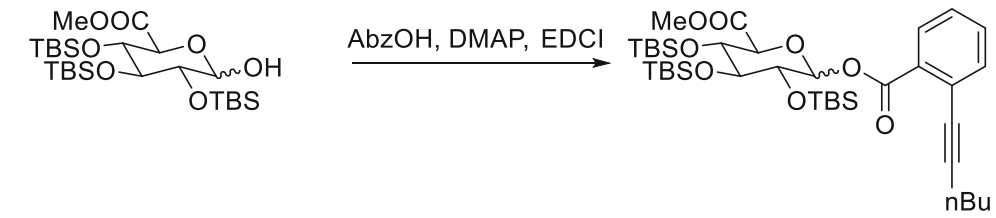
HRMS (ESI): m/z calcd. for $C_{25}H_{54}O_7Si_3$ ($[M + Na]^+$): 573.3075, found: 573.3097 (+5.0 ppm)

$[\alpha]_{20}D$ = + 16.8 ° (c = 1 g/100 mL, CH2Cl2)

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.38 (dd, J = 12.6, 3.0 Hz, 1H), 4.48 (dd, J = 6.3, 2.5 Hz, 1H), 4.17 – 4.07 (m, 1H), 3.92 – 3.84 (m, 1H), 3.75 (d, J = 6.8 Hz, 3H), 3.69 – 3.62 (m, 1H), 0.97 – 0.83 (m, 27H), 0.16 – 0.04 (m, 18H).

^{13}C-NMR (101 MHz, $CDCl_3$): δ 170.7, 89.2, 76.5, 73.5, 71.5, 71.3, 52.2, 26.2-25.7.

4.2.22 Methyl ((2-(butylethynyl)-benzoyl)-2,3,4-tri-O-tert-butyldimethylsilyl-α-D-glucopyranosid)uronate



Molecular Weight: 550,96

Molecular Weight: 735,19

The substrate (1.00 g, 2.66 mmol) was dissolved in 25 mL dry DCM and 1.11 cm³ Et3N (0.81 g, 7.97 mmol, 3 eq), DMAP (0.42 g, 3.45 mmol, 1.3 eq) and EDCI (0.66 g, 3.45 mmol, 3 eq) were added at ambient temperature and stirred for 10 minutes. 2-(1-Hexynyl)benzoic acid (0.64 g, 3.19 mmol, 1.2 eq) was added dropwise and the mixture was stirred overnight.

The reaction mixture was diluted with 100 mL DCM and washed with 1 M HCl, sat. aq. $NaHCO_3$ solution, water and brine. The organic layer was dried over Na_2SO_4 and volatiles were removed *in vacuo*.

The residue was purified by column chromatography (100 g silica gel, 4 % EA in hexanes). After purification, a mixture of anomers (452 mg, 23 %) was obtained and purified again via column chromatography (40 g silica gel, 4 % EA in hexanes). Separation of anomers was not possible, and 412 mg of a 3:2 anomeric mixture of α/β - anomers was obtained.

Crystals were grown by preparing a 2 % solution of the material in methanol, to which water was added until the solution became turbid. An oil separated after few minutes. Acetone was added dropwise under constant shaking until the solution became almost clear again and the oil redissolved. After storing this mixture at 4 °C overnight, colorless crystals of pure α-anomer could be harvested.

R_f = 0.29 (25 % EA in hexanes)

***α*–Anomer (J = 2.9 Hz):**

M.p.: 81.6 – 82.7 °C

HRMS (ESI): m/z calcd. for $C_{38}H_{67}O_6Si_3$ ([M + H]+): 735.4138, found: 735.4097 (-5.6 ppm)

$[\alpha]_{20}D$ = + 46,6 ° (c = 1 g/100 mL, CH_2Cl_2)

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.00 (dd, J = 8.0, 0.9 Hz, 1H), 7.50 (dd, J = 7.6, 1.5 Hz, 1H), 7.41 (td, J = 7.6, 1.4 Hz, 1H), 7.31 – 7.24 (m, 1H), 6.47 (d, J = 2.9 Hz, 1H), 4.69 (d, J = 5.8 Hz, 1H), 4.11 (ddd, J = 5.9, 2.0, 0.9 Hz, 1H), 4,07 (ddd, J = 4.0, 2.9, 0.9 Hz, 1H), 3.95 (dd, J = 4.2, 1.9 Hz, 1H), 3.76 (s, 3H), 2.48 (d, J = 7.2 Hz, 3H), 1.67 – 1.54 (m, 2H), 1.54 – 1.44 (m, 2H), 0.99 – 0.80 (m, 27H), 0.18 – -0.06 (m, 18H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 170.3, 164.2, 134.2, 131.7, 131.4, 130.7, 126.8, 125.2, 96.6, 91.6, 79.2, 76.5, 75.1, 73.4, 69.9, 52.1, 30.6, 26.0, 25.9, 25.8, 22.0, 19.5, 18.2, 18.1, 17.9, 13.7, -4.1, -4.3, -4.4, -4.5, -4.9, -5.0.

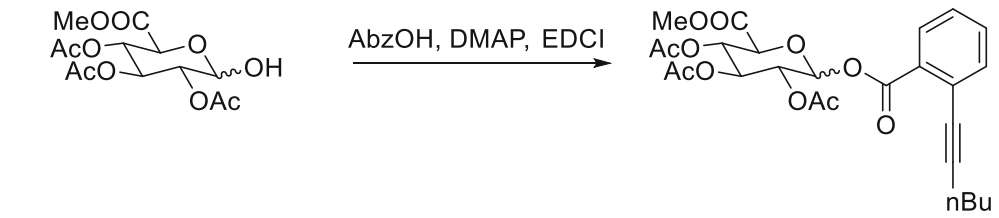
β–Anomer (J = 6.2 Hz) (analytical data from enriched mother liquor after crystallization):

HRMS (ESI): m/z calcd. for $C_{38}H_{67}O_6Si_3$ ([M + H]+): 735.4138, found: 735.4102 (-5.0 ppm)

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.06 (d, J = 8.0 Hz, 1H), 7.50 (d, J = 7.8 Hz, 1H), 7.40 (t, J = 7.7 Hz, 1H), 7.31 (t, J = 7.7 Hz, 1H), 6.25 (d, J = 6.2 Hz, 1H), 4.58 (d, J = 1.6 Hz, 1H), 4.36 (dd, J = 3.6, 1.6 Hz, 1H), 3.97 (d, J = 6.3 Hz, 1H), 3.84 (d, J = 3.5 Hz, 1H), 3.69 (s, 3H), 2.50 (t, J = 7.0 Hz, 2H), 1.66 – 1.45 (m, 6H), 1.00 – 0.84 (m, 27H), 0.17 – -0.05 (m, 18H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 169.9, 164.0, 134.4, 131.8, 130.9, 130.8, 126.7, 125.6, 96.9, 93.6, 79.3, 79.1, 77.4, 75.0, 72.6, 52.2, 30.7, 25.8, 25.7, 25.7, 22.0, 19.6, 18.0, 13.7, -4.4, -4.6, -4.6, -4.8, -4.9.

4.2.23 Methyl ((2-(butylethynyl)-benzoyl)-2,3,4-tri-acetyl-α/ß-D-glucopyranosid)uronate



Molecular Weight: 334,28

Molecular Weight: 518,52

The substrate (300 mg, 0.9 mmol) was dissolved in 6 mL dry DCM and DIPEA (0.38 mL, 2.7 mmol, 3 eq), DMAP (160 mg, 1.35 mmol, 1.5 eq) and EDCI (180 mg, 1.2 mmol, 1.3 eq) were added at ambient temperature and stirred for 10 minutes. 2-(1-Hexynyl)benzoic acid (AbzOH, 180 mg, 0.9 mmol, 1.0 eq) was added dropwise and the mixture was then stirred overnight at ambient temperature. When TLC showed complete consumption of the starting material, the reaction was diluted with DCM and extracted with water, 1 N HCl, sat. aq. $NaHCO_3$, and brine. The organic phase was dried over sodium sulfate and evaporated.

Purification was performed by column chromatography (20 g silica, 25 % EE in hexanes) and yielded 320 mg (57 %) of anomerically mixed product. Repeated column chromatography allowed separation of analytical amounts of pure anomers as yellow resins.

Anomers were assigned based on the coupling constant for the anomeric doublet.

***α*–Anomer (J = 3.7 Hz):**

R_f = 0.27 (33 % EA in hexanes)

HRMS (ESI): m/z calcd. for $C_{26}H_{31}O_{11}$ ([M + H]+): 519.1861, found: 519.1842 (-3.6 ppm)

^{1}H NMR (600 MHz, Chloroform-d) δ = 8.01 – 7.90 (m, 1H), 7.58 (d, J=7.8, 1H), 7.54 – 7.45 (m, 1H), 7.37 (d, J=7.7, 1H), 6.70 (d, J=3.7, 1H), 5.66 (t, J=9.9, 1H), 5.31 – 5.20 (m, 2H), 4.65 (d, J=10.2, 1H), 3.75 (s, 3H), 2.55 (q, J=7.3, 2H), 2.04 (s, 6H), 2.00 (s, 3H), 1.68 – 1.59 (m, 2H), 1.53 – 1.46 (m, 2H), 0.96 (t, J=7.3, 3H).

^{13}C NMR (151 MHz, $CDCl_3$) δ = 169.91, 169.71, 169.37, 167.33, 163.95, 135.28, 132.54, 130.89, 129.57, 127.40, 125.31, 97.36, 89.66, 79.79, 70.80, 69.35, 69.17, 69.16, 52.99, 30.75, 22.15, 20.69, 20.49, 20.47, 19.53, 13.68.

***β*–Anomer (J = 7.7 Hz):**

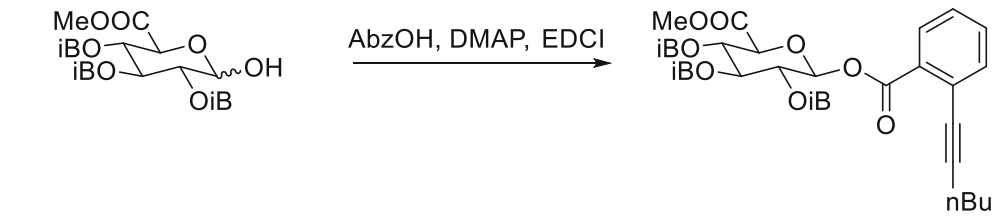
R_f = 0.29 (33 % EA in hexanes)

HRMS (ESI): m/z calcd. for $C_{26}H_{31}O_{11}$ ([M + H]+): 519.1861, found: 519.1838 (-4.3 ppm)

^{1}H NMR (600 MHz, Chloroform-*d*) δ = 7.99 – 7.88 (m, 1H), 7.53 (dd, *J*=7.9, 1.3, 1H), 7.47 (td, *J*=7.6, 1.4, 1H), 7.32 (td, *J*=7.7, 1.4, 1H), 6.01 (d, *J*=7.7, 1H), 5.41 – 5.35 (m, 1H), 5.35 – 5.29 (m, 2H), 4.27 (d, *J*=9.5, 1H), 3.70 (s, 3H), 2.50 (t, *J*=7.1, 2H), 2.05 (s, 6H), 2.00 (s, 3H), 1.68 – 1.55 (m, 2H), 1.55 – 1.44 (m, 2H), 0.96 (t, *J*=7.3, 3H).

^{13}C NMR (151 MHz, $CDCl_3$) δ = 169.93, 169.45, 169.30, 166.86, 163.13, 134.79, 132.72, 130.96, 128.89, 127.29, 126.06, 97.65, 91.82, 79.01, 73.15, 71.75, 70.06, 69.07, 52.97, 30.70, 22.12, 20.63, 20.56, 20.52, 19.56, 13.68.

4.2.24 Methyl ((2-(butylethynyl)-benzoyl)-2,3,4-tri-isobutyryl-ß-D-glucopyranosid)uronate



Molecular Weight: 418,44

Molecular Weight: 602,68

The substrate (1300 mg, 3.0 mmol) was dissolved in 20 mL dry DCM and DIPEA (1.0 mL, 9.0 mmol, 3 eq), DMAP (560 mg, 4.6 mmol, 1.5 eq) and EDCI (760 mg, 4.0 mmol, 1.3 eq) were added at ambient temperature and stirred for 10 minutes. 2-(1-Hexynyl)benzoic acid (AbzOH, 620 mg, 3.0 mmol, 1.0 eq) was added dropwise and the mixture was then stirred overnight at ambient temperature. When TLC showed complete consumption of the starting material, the reaction was diluted with DCM and extracted with water, 1 N HCl, sat. aq. $NaHCO_3$, and brine. The organic phase was dried over sodium sulfate and evaporated.

Purification was performed by column chromatography (60 g silica, 10 % EE in hexanes) and yielded 1100 mg (59 %) of predominantly (10:1) β-product.

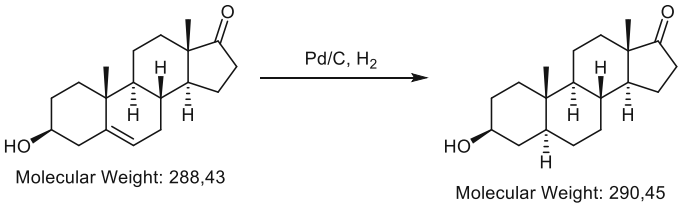
***β*–Anomer (J = 7.5 Hz):**

R_f = 0.79 (25 % EA in hexanes)

^{1}H NMR (200 MHz, Chloroform-*d*) δ = 7.86 (d, *J*=7.7, 1H), 7.55 – 7.30 (m, 3H), 7.30 – 7.22 (m, 1H), 5.96 (d, *J*=7.5, 1H), 5.48 – 5.09 (m, 3H), 4.21 (d, *J*=9.5, 1H), 3.65 (d, *J*=4.4, 3H), 2.61 – 2.24 (m, 2H), 1.63 – 0.72 (m, 28H).

4.3 Known Glucuronides

4.3.1 Epiandrosterone



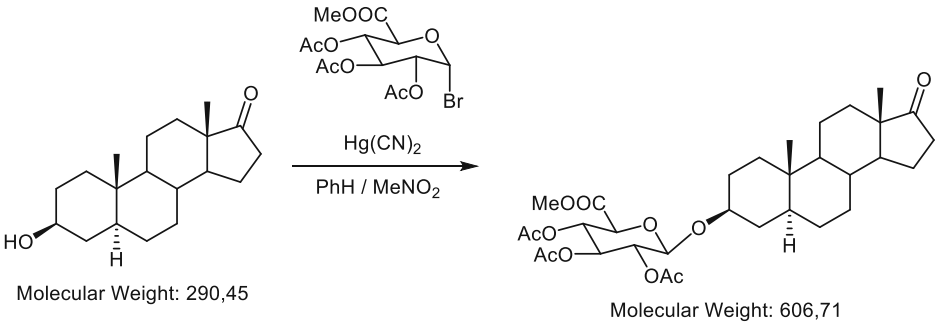
Dehydroepiandrosterone (DHEA, 1000 mg, 3.5 mmol) was dissolved in 20 mL EtOH and palladium on charcoal (37 mg, 10 w% Pd, 0.035 mmol Pd) was added. The suspension was stirred under hydrogen atmosphere overnight (the reaction can't be monitored by TLC).

Workup consists of filtration over celite followed by evaporation of the solvent. NMR confirmed complete conversion and no further purification was necessary. The product was isolated as colorless crystals (980 mg, 97 %).

R_f: 0.58 (33 % EE in hexane)

^{1}H NMR (400 MHz, Chloroform-*d*) δ = 3.67 – 3.52 (m, 1H), 2.44 (dd, *J*=19.2, 8.9, 1H), 2.13 – 1.99 (m, 1H), 1.99 – 1.87 (m, 1H), 1.87 – 1.77 (m, 2H), 1.77 – 1.05 (m, 12H), 1.05 – 0.92 (m, 2H), 0.86 (d, *J*=1.5, 3H), 0.84 (d, *J*=0.7, 3H), 0.76 – 0.64 (m, 1H).

4.3.2 Epiandrosterone Acetylglucuronide



Molecular Weight: 290,45

Molecular Weight: 606,71

Procedure derived from [41].

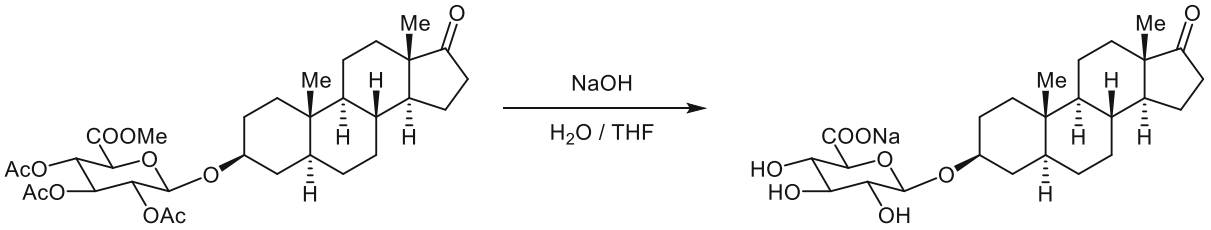
Epiandrosterone (220 mg, 0.76 mmol) was dissolved in 30 mL nitromethane and 30 mL dry benzene were added. The mixture was evaporated under reduced pressure to a residual volume of 20 mL and glycosyl bromide dissolved in little dry benzene (700 mg, 2.3 eq) and mercury cyanide (400 mg, 2 eq) were added. The suspension was heated to 110 °C for 1 h.

The mixture was cooled to ambient temperature and diluted with ethyl acetate. It was extracted with water, sat. aq. $NaHCO_3$ and brine (aqueous fractions contain mercury salts!). After drying over sodium sulfate, the solvents were evaporated and the crude mixture was purified by column chromatography (20 g silica, 25 % EA in hexanes) to yield 107 mg (23 %) of pure glucuronide.

R_f: 0.58 (33 % EE in hexane)

^{1}H NMR (400 MHz, Chloroform-*d*) δ = 5.31 – 5.17 (m, 2H), 4.95 (d, *J*=7.7, 1H), 4.65 (d, *J*=7.6, 1H), 4.02 (d, *J*=9.7, 1H), 3.75 (s, 3H), 3.66 – 3.51 (m, 1H), 2.51 – 2.36 (m, 1H), 2.15 – 2.07 (m, 2H), 2.07 – 1.97 (m, 9H), 1.97 – 1.42 (m, 12H), 1.37 – 1.16 (m, 6H), 1.14 – 1.02 (m, 1H), 1.02 – 0.89 (m, 2H), 0.85 (s, 3H), 0.81 (s, 3H), 0.72 – 0.59 (m, 1H).

4.3.3 Epiandrosterone Glucuronide Na-Salt



Molecular Weight: 606,71

Molecular Weight: 488,55

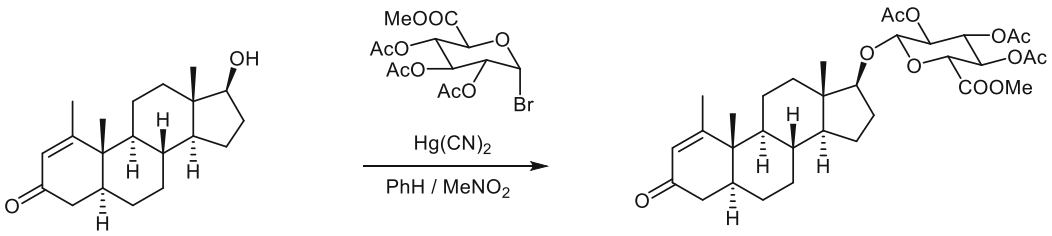
Epiandrosterone Glucuronide (107 mg, 0.18 mmol) was dissolved in 1 mL THF and 1 mL 1 N NaOH was added. The mixture was stirred for 2 h. After UPLC confirmed complete conversion, the solution was neutralised with an acidic ion exchange resin and filtered. The residue was washed with water.

The turbid solution was reduced to 5 mL and then purified by RP-MPLC (12 g C18-silica, 30% to 80% MeOH in water). The product was eluted at 50 % MeOH. The solvent was removed by lyophillization to give 55 mg (65 %) of pure glucuronate.

The substance was redissolved in water / MeOH and neutralized with 1 eq. $NaHCO_3$ and the lyophilized again.

Spectra were in accordance to [147].

4.3.4 Metenolone Acetylglucuronide



Molecular Weight: 302,46

Molecular Weight: 618,72

Metenolone (100 mg, 0.33 mmol) was dissolved in 15 mL nitromethane and 15 mL dry benzene were added. The mixture was evaporated under reduced pressure to a residual volume of 10 mL and bromosugar dissolved in little dry benzene (300 mg, 0.76 mmol, 2.3 eq) and mercury cyanide (200 mg, 0.66 mmol, 2 eq) were added. The suspension was heated to 100 °C for 2 h.

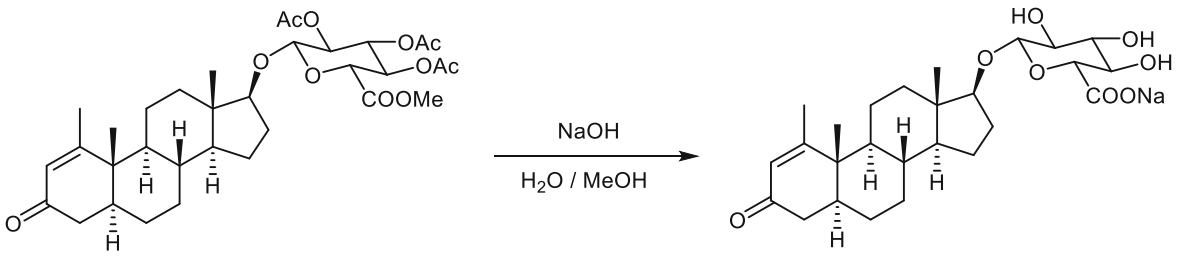
After 1 h, UPLC-MS indicated 80 % consumption of the starting material. After 3 h, the UPLC was almost unchanged and the reaction was stopped by cooling to ambient temperature.

Workup was performed by diluting the mixture with 50 mL diethyl ether and extraction with sat. aq. $NaHCO_3$, water and brine. The organic phase was dried with Na_2SO_4 and evaporated to give 600 mg crude yellow oil.

Purification was performed by RP-MPLC (40 g C18 Silica, 60% to 90% MeOH in water, product elutes at 85 %). The methanol was evaporated and the residual suspension was lyophylized to give 63 mg white solid residue (31 %).

Synthesis derived from ref. [40] and [148].

4.3.5 Metenolone Glucuronide Na-Salt



Molecular Weight: 618,72

Molecular Weight: 500,56

The substrate (40 mg, 0.06 mmol) was dissolved in 2 mL MeOH and 1 mL H_2O. Sodium hydroxide was added as a 1 N solution in water until the solubility limit for the substrate was reached (1 mL). The slightly cloudy solution was stirred for 1 h at ambient temperature, after which the solution was clear again and TLC and UPLC indicated complete consumption of the starting material.

The reaction was quenched by neutralization with sat. aq. ammonium chloride. Methanol was evaporated and the residual solution was purified by RP-MPLC (12 g C18 silica gel, gradient: 30% to 80% MeOH in water), yielding 21 mg (67 %) of purified glucuronide after lyophilization.

The substance was redissolved in water / MeOH and neutralized with 1 eq. $NaHCO_3$ and then lyophilized again.

Analytical data were in accordance with [148].

^{1}H NMR (600 MHz, Methanol-d4) δ = 5.78 (s, 1H), 4.51 (d, J=8.0, 1H), 3.86 (t, J=8.7, 1H), 3.76 – 3.62 (m, 1H), 3.57 – 3.43 (m, 2H), 3.39 – 3.23 (m, 1H), 2.59 – 2.41 (m, 1H), 2.27 – 1.85 (m, 10H), 1.77 – 1.49 (m, 7H), 1.49 – 1.41 (m, 2H), 1.41 – 1.29 (m, 3H), 1.29 – 1.15 (m, 2H), 1.09 (s, 4H), 0.88 (s, 5H).

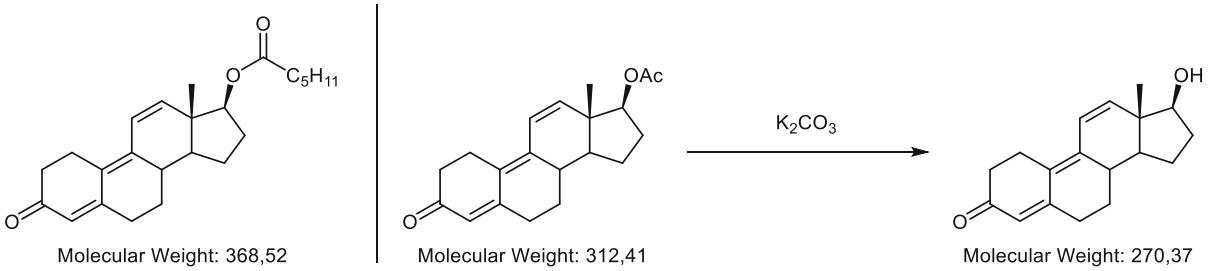
^{13}C NMR (151 MHz, D_2O) δ 204.80, 204.76, 180.39, 175.65, 171.03, 168.28, 126.96, 102.13, 88.55, 76.49, 75.62, 73.25, 71.95, 51.14, 49.68, 44.42, 44.35, 43.01, 42.99, 42.93, 42.70, 37.64, 37.58, 29.75, 28.01, 27.98, 27.75, 25.10, 24.91, 24.88, 22.84, 13.11, 13.09, 13.06, 11.53.

4.4 Trenbolone and Epitrenbolone Glucuronides

4.4.1 Trenbolone from Esters

When trenbolone esters were received as injectable solution in vegetable oil, they could be purified by MPLC: Up to 10 mL of solution were diluted with hexanes and applied onto a 90-g column. The oil was eluted with 1 % EA in hexanes, after which the esters can be eluted with 20 % EA in hexanes.

When trenbolone esters were received in pills, the contents can be extracted by opening the pills and stirring in DCM and subsequent filtration.



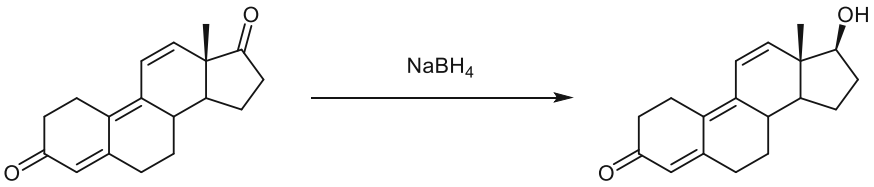
Trenbolone acetate (1 g, 3.2 mmol) was dissolved in 20 mL MeOH and potassium carbonate (880 mg, 6.4 mmol, 2 eq) was added. The suspension was stirred overnight at ambient temperature, after which TLC confirmed complete conversion. The solvent was evaporated and the residue taken up in dichloromethane/water. The phases were separated and the aqueous phase was washed once with DCM. The combined organic phases were reduced to 10 mL and this solution was purified by MPLC (90 g silica, gradient: 30 % - 80 % EA in hexanes), yielding 770 mg (89 %) of yellow crystalline material.

Trenbolone hexanoate (1 g, 2.7 mmol) was dissolved in 20 mL MeOH and potassium carbonate (780 mg, 5.4 mmol, 2 eq) was added. The suspension was heated to reflux for 4 h, after which TLC confirmed complete conversion. The solvent was evaporated and the residue taken up in dichloromethane/water. The phases were separated and the aqueous phase was washed once with DCM. The combined organic phases were reduced to 10 mL and this solution was purified by MPLC (90 g silica, gradient: 30 % - 80 % EA in hexanes), yielding 580 mg (80 %) of yellow crystalline material.

R_f: 0.5 (50 % EE in hexanes)

^{1}H-NMR (200 MHz, Chloroform-d) δ 6.56 – 6.34 (m, 2H), 5.78 (s, 1H), 3.89 (dd, J = 9.0, 6.8 Hz, 1H), 2.92 – 2.72 (m, 2H), 2.64 – 1.13 (m, 14H), 0.91 (s, 2H).

4.4.2 Trenbolone from Trendione



Molecular Weight: 268,36 Molecular Weight: 270,37

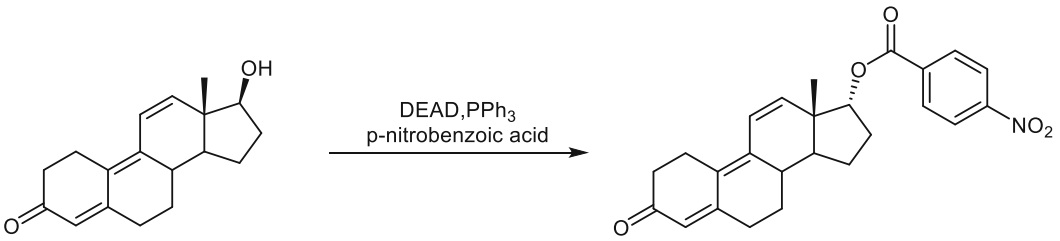
Trendione (190 mg, 0.71 mmol) and $NaBH_4$ (38 mg, 0.30 mmol, 1.4 eq) were cooled to -15 °C and suspended in 5 mL of dry MeOH. The colorless suspension was stirred for 4 h, after which TLC confirmed completion of the reaction.

The mixture was diluted with DCM and filtered over Celite. After evaporation of the solvent, purification was performed by column chromatography (80x silica, 30 % EA in hexanes) to give the product (117 mg, 61 %) as a yellow crystalline compound.

R_f: 0.5 (50 % EE in hexanes)

^{1}H-NMR (200 MHz, Chloroform-d) δ 6.56 – 6.34 (m, 2H), 5.78 (s, 1H), 3.89 (dd, J = 9.0, 6.8 Hz, 1H), 2.92 – 2.72 (m, 2H), 2.64 – 1.13 (m, 14H), 0.91 (s, 2H).

4.4.3 17-α Trenbolone *para*-nitrobenzoate



Molecular Weight: 270,37

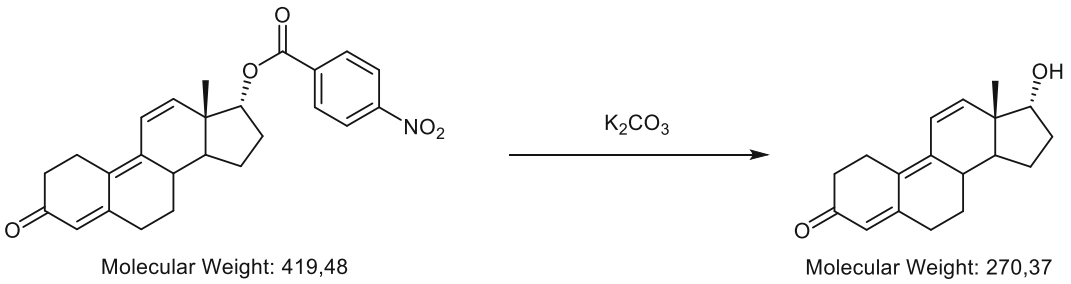
Molecular Weight: 419,48

Trenbolone (500 mg, 1.85 mmol), triphenyl phosphine (1200 mg, 4.6 mmol, 2.5 eq) and p-nitrobenzoic acid (770 mg, 4.6 mmol, 2.5 eq) were suspended in 10 mL dry toluene. DEAD (0,75 mL, 800 mg, 4.6 mmol, 2.5 eq) was added and the suspension was heated to 85 °C for 6 h or 70 °C overnight, turning it into an amber solution. After TLC confirmed complete conversion, the solvent was evaporated and the residue taken up in DCM. The solution was washed with water, bicarbonate and brine and the solvent was evaporated.

Purification was performed by MPLC (90 g silica, gradient: 10 % to 50 % EA in hexanes), to give a mixture (920 mg) of the product and reduced DEAD that could not be separated and was used directly for the next step.

R_f: 0.4 (33 % EE in hexanes)

4.4.4 17-α Trenbolone (Epitrenbolone)



Molecular Weight: 419,48 Molecular Weight: 270,37

The mixture from Mitsunobu reaction (920 mg) was dissolved in 20 mL MeOH and potassium carbonate (500 mg) was added. The suspension was stirred for 4 h at ambient temperature, after which TLC confirmed complete conversion. The solvent was evaporated and the residue taken up in dichloromethane/water. The phases were separated and the aqueous phase was washed once with DCM. The combined organic phases were reduced to 10 mL and this solution was purified by MPLC (45 g silica, gradient: 30 % - 80 % EA in hexanes), yielding 385 mg (77 % over two steps) of orange amorphous material.

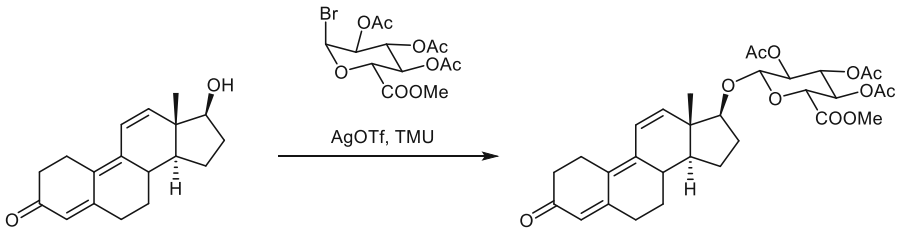
R_f: 0.5 (50 % EE in hexanes)

HRMS: Calcd. for $C_{18}H_{23}O_2$ (M+H): 271.1698, found: 271.1690 (-0.8 ppm)

^{1}H-NMR (400 MHz, Chloroform-d) δ 6.66 – 6.52 (m, 1H), 6.38 (d, J = 9.9 Hz, 1H), 5.79 (s, 1H), 3.98 (d, J = 5.8 Hz, 1H), 2.90 – 2.75 (m, 2H), 2.67 – 2.13 (m, 5H), 2.08 – 1.79 (m, 2H), 1.68 – 1.56 (m, 2H), 1.47 – 1.19 (m, 3H), 0.83 (s, 3H).

^{13}C NMR (151 MHz, Chloroform-d) δ 199.49, 156.73, 142.09, 141.02, 127.35, 125.74, 123.68, 78.21, 48.54, 46.21, 37.70, 36.68, 33.03, 31.60, 27.69, 24.38, 23.65, 18.57.

4.4.5 Trenbolone Acetyl-Glucuronide



Molecular Weight: 270,37 Molecular Weight: 586,63

Trenbolone (300 mg, 1.1 mmol) and the glucuronyl bromide (700 mg, 1.75 mmol, 1.6 eq) were dissolved in 2 mL dry DCM and tetramethyl urea (0.66 mL, 5.5 mmol, 5 eq) and 4 A molecular sieves were added. After stirring for 10 min, silver triflate (425 mg, 1.65 mg, 1.5 eq) was added and the suspension turned dark green immediately.

The reaction can be followed by UPLC: After 30 min, both product and orthoester are formed (orthoester elutes later). Stirring is continued until all the orthoester is rearranged to glucuronide (4 h). The reaction is worked up by eluting over a plug of silica with DCM.

Purification is performed by RP-MPLC (Gradient: 60 % - 99 % MeOH in water) followed by normal phase column chromatography (20 % EE in hexanes) to yield 25 mg (3 %) of glucuronide.

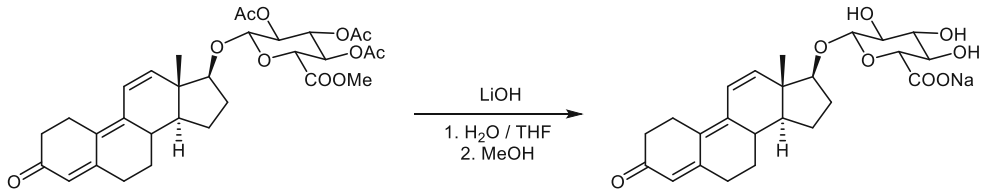
R_f: 0.45 (33 % EE in hexanes), Elutes at 90 % MeOH or 80 % MeCN in water.

HRMS: m/z calcd. for $C_{31}H_{38}O_{11}$ ([M + H]+): 587.2492 found: 587.2502 (+1.7 ppm)

^{1}H NMR (400 MHz, Chloroform-d) δ = 6.45 (d, J=9.8, 1H), 6.27 (d, J=9.8, 1H), 5.78 (s, 1H), 5.32 – 5.15 (m, 2H), 5.05 (dd, J=9.2, 7.9, 1H), 4.64 (d, J=7.9, 1H), 4.02 (d, J=9.5, 1H), 3.78 – 3.70 (m, 4H), 2.90 – 2.71 (m, 2H), 2.65 – 2.49 (m, 2H), 2.46 (t, J=7.3, 2H), 2.16 (s, 3H), 2.07 (s, 3H), 1.94 – 1.55 (m, 5H), 1.42 (d, J=11.5, 2H), 0.86 (s, 3H).

^{13}C NMR (151 MHz, Chloroform-d) δ 199.07, 170.21, 169.37, 169.08, 167.15, 156.17, 141.46, 141.41, 127.46, 123.97, 123.92, 101.75, 86.02, 77.35, 77.23, 77.03, 76.71, 72.58, 72.06, 71.35, 69.45, 52.90, 47.39, 45.44, 37.44, 36.68, 31.39, 30.93, 29.28, 28.69, 26.87, 24.38, 23.04, 20.71, 20.64, 20.51, 14.01.

4.4.6 Trenbolone Glucuronide Na-Salt



Molecular Weight: 586,63 Molecular Weight: 468,48

The substrate (35 mg, 0,04 mmol) was dissolved in 0.5 mL THF and water was added dropwise until cloudiness persisted. Lithium hydroxide monohydrate (9.5 mg, 5 eq) was added and after 10 min, UPLC indicated complete removal of the methyl ester. The reaction mixture was diluted with 2 mL MeOH and after 1 h, UPLC indicated complete removal of the acetyl groups. The reaction was quenched with sat. aq. NH_4Cl and diluted with water. The mixture was purified by RP-MPLC (20 to 80 % MeOH in water) and the collected product fractions lyophilized. After weighing, the product was redissolved in 50 % methanol in water and neutralized with 1 eq. $NaHCO_3$. After lyophilization, 25 mg (89 %) of product were obtained.

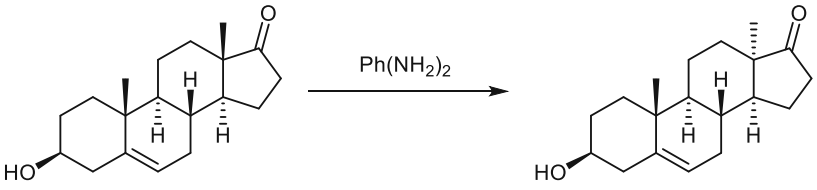
HRMS: m/z calcd. for $C_{24}H_{31}O_8$ ([M + H]+): 447.2013 found: 447.2009 (-1.0 ppm)

^{1}H-NMR (400 MHz, Chloroform-d) δ 6.66 – 6.52 (m, 1H), 6.38 (d, J = 9.9 Hz, 1H), 5.79 (s, 1H), 3.98 (d, J = 5.8 Hz, 1H), 2.90 – 2.75 (m, 2H), 2.67 – 2.13 (m, 5H), 2.08 – 1.79 (m, 2H), 1.68 – 1.56 (m, 2H), 1.47 – 1.19 (m, 3H), 0.83 (s, 3H).

^{13}C NMR (151 MHz, Chloroform-d) δ 199.49, 156.73, 142.09, 141.02, 127.35, 125.74, 123.68, 78.21, 48.54, 46.21, 37.70, 36.68, 33.03, 31.60, 27.69, 24.38, 23.65, 18.57.

4.5 Metabolite M3 Synthesis

4.5.1 3ß-Hydroxy-13α-methylandrost-5-en-17-one



Molecular Weight: 288,43 Molecular Weight: 288,43

Dehydroepiandrosterone (21.5 g, 79 mmol) was dissolved in 250 mL acetic acid and 13 g (1.6 eq, 126 mmol) of 1,2-diaminobenzene (o-phenylenediamine) were added. The solution was heated to reflux under exclusion of water and air and after 2 h, TLC showed no further changes, indication that equilibrium had been reached.

The solution was worked up by addition of 400 mL water and 100 mL brine. The dark brown suspension was extracted with 2 x 300 mL ethyl acetate. The organic phase was then washed with water, 1 N HCl, sat. aq. bicarbonate and brine. The organics were evaporated to give 25 g of crude isomeric mixture with 80 % of the starting material epimerized to the 13-α configuration and partial acetylation at the C-3 hydroxyl group.

The acetylation was completed by stirring with acetic anhydride (30 mL, 4 eq) and triethylamine (10 mL, 1 eq) overnight. The volatiles were evaporated and the residue taken up in ethyl acetate. The solution was extracted with water, sat. aq. bicarbonate and brine, dried over sodium sulphate and the volatiles were evaporated. The residue was crystallized from diisopropylether. The mother liquor was evaporated and the residue purified by MPLC (250 g silica, gradient: from 10 % to 30 % EE in hexanes)

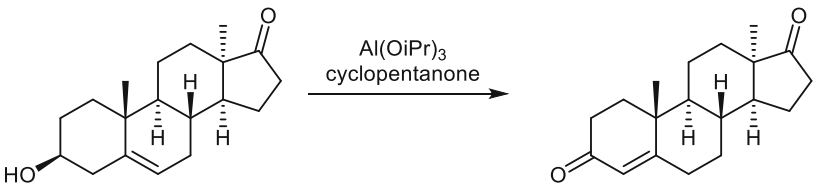
The combined acetylated products were dissolved in methanol and potassium carbonate (10 g) was added. The suspension was heated to reflux and after 1 h, TLC indicated complete deprotection. The solvent was evaporated and the residue taken up in ethyl acetate / water. The phases were separated and the organic phase washed with brine and dried over sodium sulphate. After concentration, the product was obtained as white solid (15 g, 70 %).

Spectra in accordance with the literature: [155]

R_f: 0.55 (50 % EA in hexane)

^{1}H NMR (400 MHz, Chloroform-d) δ = 5.37 (dt, J = 4.9, 2.2 Hz, 1H), 3.52 (m, 1H), 2.25 – 2.42 (m, 3H), 2.04 – 2.25 (m, 4H), 1.75 – 1.91 (m, 4H), 1.41 – 1.69 (m, 4H), 1.20 (td, J = 13.4, 3.7 Hz, 1H), 1.02 – 1.14 (m, 2H), 0.98 (s, 3H), 0.93 (dd, J = 11.9, 2.1 Hz, 1H),), 0.84 (s, 3H) 0.85 (m, 1H), 0.81 – 0.95 (m, 1H)

4.5.2 13α-Androst-4-en-3,17-dione



Molecular Weight: 288,43 Molecular Weight: 286,42

The substrate (10.2 g) was dissolved in 300 mL dry toluene and 50 mL cyclohexanone (or cyclopentanone for reduced smell) was added. The solution was heated to reflux under a Dean-Stark trap until no more water separated (30 min). Aluminium triisopropoxide (17.5 g, 2.5 eq) was added and the reaction continued at reflux temperature for 2.5 h.

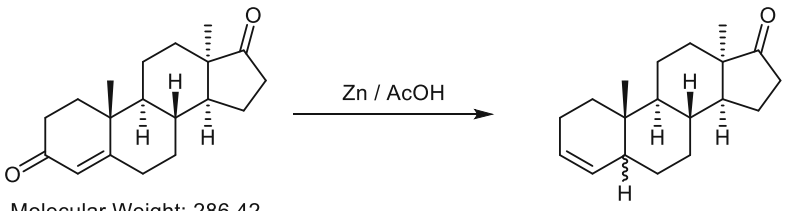
After the solution had cooled to ambient temperature, it was diluted to 500 mL and extracted with 1 N H_2SO_4 until no further change in turbidity could be observed. The solution was then extracted with water, sat. aq. $NaHCO_3$ and brine and dried over sodium sulphate. The solvent was evaporated (under high vacuum to remove the oxidant) and the residue purified by MPLC (300 g silica, gradient: 0 % to 50 % EA in hexanes), yielding 7.8 g (77 %) of crystalline product.

Spectra were in accordance with literature: [68]

R_f: 0.36 (20 % EA in hexane)

^{1}H NMR (400 MHz, CDCl3): δ = 5.7 (t, J = 0.7, 1H), 4.86 (t, J = 2.3, 1H), 4.71 (t, J = 2.3, 1H), 2.21–2.57 (m, 6H), 2.0–2.1 (m, 2H), 1.96 (dt, J = 13.5, 3.2, 1H), 1.8–1.92 (m, 1H), 1.52–1.75 (m, 3H), 1.44 (m, 1H), 1.35 (td, J = 19.73, 3.55, 1H), 1.13–1.27 (m, 2H), 1.05 (s, 3H), 0.97–1.03 (m, 1H), 0.94 (s, 3H), 0.91–0.97 (m, 1H).

4.5.3 13α,5ξ-Androst-3-en-17-one



Molecular Weight: 286,42

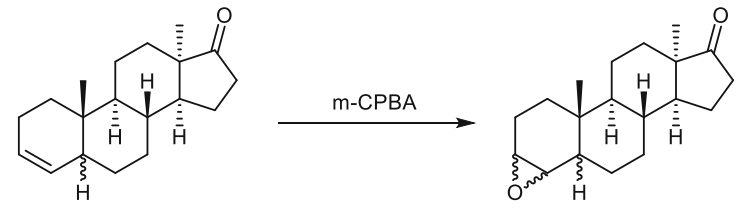
Molecular Weight: 272,43

The substrate (6.0 g, 21 mmol) was dissolved in 60 mL acetic acid and heated to 80 °C. Zinc dust (28 g, 20 eq) was added in portions over 15 minutes. After 1 h the reaction was finished according to TLC and the zinc residue was removed by filtration over celite. The zinc residue was 3 x washed with ethyl acetate and to combined organic liquids were washed with water, sat. aq. $NaHCO_3$ and brine and were dried over sodium sulphate. The volatiles were evaporated and the resulting residue (containing 5-α / 5-β product as 2:3 mixture) directly employed in the next step. If the product is not pure per TLC, it can be purified by MPLC (50x silica, 5 % EA in hexanes) to yield a colorless oil (5.3 g, 93 %)

Spectra were in accordance with literature: [68]

R_f: 0.8 (20 % EE in hexane)

4.5.4 3,4-Epoxy-13α-androst-17-one



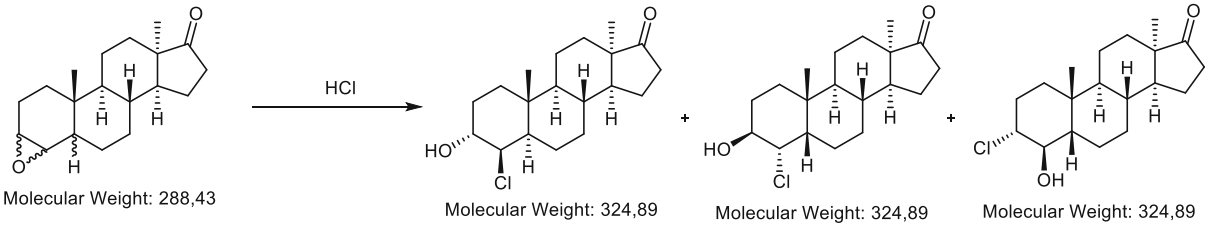
Molecular Weight: 272,43 Molecular Weight: 288,43

The substrate (5.3 g, 19.5 mmol) was dissolved in 200 mL DCM. A mixture of $KHCO_3$ (4 g, 2 eq) and 6.3 g m-CPBA (6.25 g 70%, 1.3 eq) was added. The suspension was stirred for 90 minutes until TLC confirmed complete conversion. The reaction was optionally quenched with $Na_2S_2O_3$ – solution and, after phase separation, extracted with sat. aq. $NaHCO_3$ and brine. After drying over sodium sulphate and evaporation of volatiles, the resulting crude mixture of epoxides (5.0 g) was used directly for the next step.

Spectra were in accordance with literature: [68]

R_f: 0.6 (20 % EE in hexane)

4.5.5 3α-Hydroxy-4ß-chloro-5α,13α-androstan-17-one



The substrate (5.0 g) was dissolved in 200 mL chloroform and 50 mL conc. HCl were added. The solution turned yellow and after 1 h, the phases were separated, and the organic phase washed with water, sat. aq. $NaHCO_3$ and brine. After evaporation of the volatiles (25 °C) the 3α-4β-5α product could be obtained by crystallization from hexane / acetone. The residue was purified by MPLC (180 g silica, EA in hexanes: 10 % to 50 %) and from the still impure fractions containing the product, a second crop of crystals could be obtained by evaporating and crystallizing again from hexane / acetone.

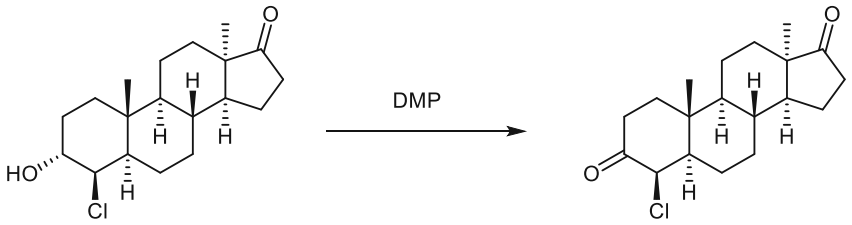
The combined yield of the harvested crystals was 2.05 g (33 % over two steps). The crystals should be handled quickly and stored in the freezer as they tend to degrade over time at ambient conditions.

Spectra were in accordance with literature: [68]

R_f: 0.28 (33 % EA in hexane)

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 4.09 (s, 1H), 3.91 (m, 1H), 2.28–2.38 (m, 1H), 1.97–2.21 (m, 5H), 1.78–1.87 (m, 2H), 1.63–1.77 (m, 2H), 1.40–1.61 (m, 5H), 1.23–1.35 (m, 2H), 0.98–1.17 (m, 2H), 0.94 (s, 3H), 0.88 (s, 3H), 0.64–0.73 (m, 2H).

4.5.6 4ß-Chloro-5α,13α-androstane-3,17-dione



Molecular Weight: 324,89 Molecular Weight: 322,87

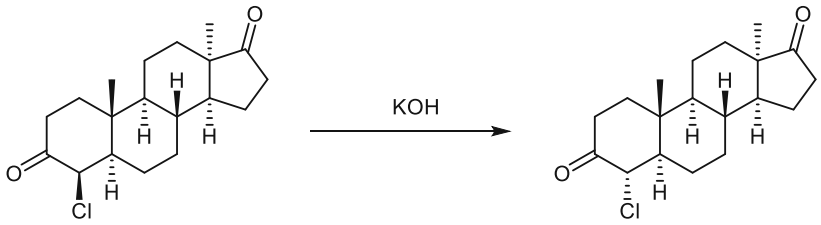
The substrate (2.05 g) was dissolved in 70 mL DCM and Dess-Martin-periodinane (3.6 g, 1.35 eq) was added. After 1 h, TLC indicated complete conversion and the reaction mixture was directly filtered over silica (50 g, 100 % DCM as eluent). The solvent was evaporated and the crude product (2.0 g) used directly for the next step.

Spectra were in accordance with literature: [68]

R_f: 0.35 (33 % EA in hexane)

^{1}H NMR (400 MHz, Chloroform-d) δ = 4.01 (m, 1H), 3.01 (td, J=14.7, 5.9, 1H), 2.43 – 2.23 (m, 2H), 2.23 – 2.07 (m, 5H), 1.91 – 1.74 (m, 3H), 1.57 – 1.29 (m, 4H), 1.18 (m, 1H), 1.12 – 1.10 (m, 3H), 1.10 – 0.99 (m, 2H), 0.98 (s, 3H), 0.88 – 0.62 (m, 2H).

4.5.7 4α-Chloro-5α,13α-androstane-3,17-dione



Molecular Weight: 322,87 Molecular Weight: 322,87

The substrate (2.0 g, 6.3 mmol) was dissolved in 200 mL MeOH and KOH (350 mg, 6.3 mmol, 1.0 eq, dissolved in 5 mL MeOH) was added. After 5 min, the solution was neutralized with 1 mL acetic acid. (If the reaction is left for longer, Favorskii-Rearrangement occurs!)

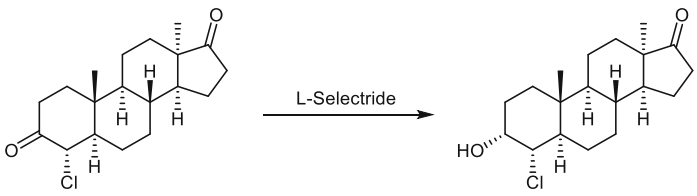
The volatiles were evaporated and the residue taken up in ethyl acetate / water. The phases were separated and the organic phase was washed with sat. aq. bicarbonate and brine. After drying over sodium sulphate, the volatiles were evaporated and the product was purified by MPLC (gradient EA in hexanes: 10 % to 50 %) to yield pure crystalline product (1.80 g, 90 %)

Spectra were in accordance with literature: [68]

R_f: 0.35 (33 % EA in hexane)

^{1}H NMR (400 MHz, Chloroform-d) δ = 4.01 (m, 1H), 3.01 (td, J=14.7, 5.9, 1H), 2.43 – 2.23 (m, 2H), 2.23 – 2.07 (m, 5H), 1.91 – 1.74 (m, 3H), 1.57 – 1.29 (m, 4H), 1.18 (m, 1H), 1.12 – 1.10 (m, 3H), 1.10 – 0.99 (m, 2H), 0.98 (s, 3H), 0.88 – 0.62 (m, 2H).

4.5.8 4α-Chloro-3α-hydroxy-5α,13α-androstan-17-one



Molecular Weight: 322,87 Molecular Weight: 324,89

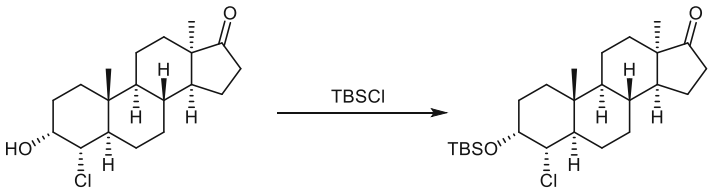
The substrate (1.39 g, 4.31 mmol) was dissolved in THF (low solubility) and cooled down to -70 °C. L-selectride (4.8 ml, 1 M, 4.7 mmol, 1.1 eq) was added dropwise and the reaction was stirred for 2 hours. When TLC showed complete consumption of starting material, the reaction was quenched by addition of sat. aq. NH_4Cl. The organic phase was diluted with ethyl acetate and washed with sat. aq. $NaHCO_3$ and brine and dried over Na_2SO_4. The volatiles were evaporated and purification was performed via MPLC (90 g silica, 25 % EA in hexanes) obtaining 1.0 g (72 %) of pure product.

Spectra were in accordance with literature: [68]

R_f: 0.36 (25 % EA in hexanes)

^{1}H-NMR: NMR (400 MHz, $CDCl_3$) δ 4.05 (dd, *J* = 11.9, 2.6 Hz, 1H), 4.02 – 3.96 (m, 1H), 2.40 – 2.28 (m, 2H), 2.22 – 2.09 (m, 1H), 2.13 – 2.00 (m, 1H), 1.98 – 1.83 (m, 2H), 1.82 (dd, *J* = 4.0, 1.6 Hz, 1H), 1.82 – 1.63 (m, 2H), 1.61 – 1.33 (m, 4H), 1.30 – 1.04 (m, 2H), 0.95 (s, 3H), 0.89 – 0.73 (m, 2H), 0.67 (s, 3H)

4.5.9 3α-(t-Butyldimethylsilyloxy)-4α-chloro-5α,13α-androstan-17-one



Molecular Weight: 324,89 Molecular Weight: 439,15

The substrate (500 mg, 1.5 mmol) was dissolved in 5 mL DMF and imidazole (280 mg, 4.1 mmol, 2.7 eq) and TBSCl (300 mg, 2.0 mmol, 1.3 eq) were added. The solution was stirred for 96 h, after which TLC showed about 75 % conversion. DMF was evaporated and the residue dissolved in 50 mL ethyl acetate. The organic phase was washed with 1 N HCl, bicarbonate and brine and the aqueous phases reextracted with ethyl acetate. The combined organic phases were dried over sodium sulfate and the solvent was evaporated.

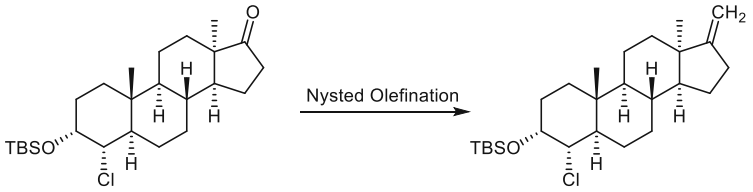
Purification was performed by MPLC (45 g silica, 5 – 20 % EA in hexanes), yielding 480 mg (71 %) of product and 95 mg (19 %) of starting material.

Spectra were in accordance with literature: [68]

R_f: 0.8 (25 % EA in hexanes)

^{1}H-NMR: NMR (400 MHz, $CDCl_3$) δ 4.05 (dd, *J* = 11.9, 2.6 Hz, 1H), 4.02 – 3.96 (m, 1H), 2.40 – 2.28 (m, 2H), 2.22 – 2.09 (m, 1H), 2.13 – 2.00 (m, 1H), 1.98 – 1.83 (m, 2H), 1.82 (dd, *J* = 4.0, 1.6 Hz, 1H), 1.82 – 1.63 (m, 2H), 1.61 – 1.33 (m, 4H), 1.30 – 1.04 (m, 2H), 0.95 (s, 3H), 0.89 – 0.73 (m, 2H), 0.67 (s, 3H)

4.5.10 3α-(t-Butyldimethylsilyloxy)-4α-chloro-17-methylene-5α,13α-androstane



Molecular Weight: 439,15 Molecular Weight: 437,18

Dibromomethane (0,64 mL, 9.1 mmol, 4.0 eq) was dissolved in 12 mL dry THF and zinc powder (4400 mg, 68 mmol, 30 eq) was added. The suspension was cooled to -10 °C and $TiCl_4$ (0.63 mL, 5.7 mmol, 2.5 eq) was added dropwise. The suspension was stirred at -10 °C for 2 h and then the substrate (1000 mg, 2.3 mmol) was added dropwise. The mixture was held at -10 °C for 1 h and then warmed up to ambient temperature. Stirring was continued overnight and the next day, TLC showed partial conversion.

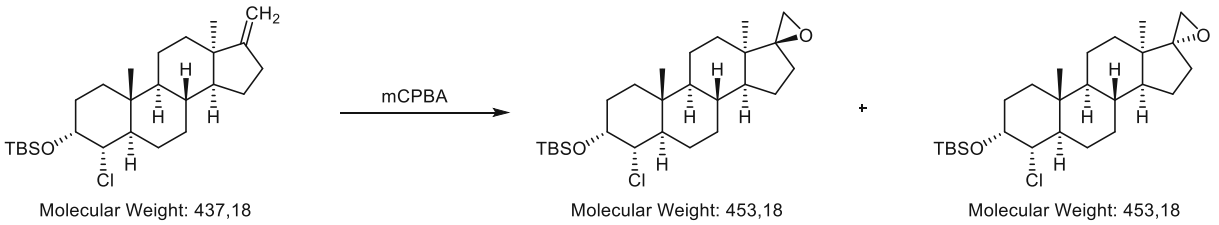
The reaction was quenched by dilution with 50 mL ethyl acetate and subsequent addition of 1 M HCl. The phases were separated and the organic phase washed with bicarbonate and brine. After drying over sodium sulphate, the volatiles were evaporated and purification was performed by MPLC (90 g silica, 0 – 20 % EA in hexanes). After evaporation of the eluents, 350 mg (35 %) of methylenated product and 280 mg (28 %) of starting material were isolated.

Spectra were in accordance with literature: [68]

R_f: 0.8 (20 % EA in hexanes)

^{1}H-NMR: (400 MHz, $CDCl_3$) δ 4.81 (d, *J* = 2.2 Hz, 1H), 4.68 (d, *J* = 2.6 Hz, 1H), 4.07 (dd, *J* = 11.9, 2.6 Hz, 1H), 4.00 (q, *J* = 2.9 Hz, 1H), 2.56 – 2.41 (m, 1H), 2.36 (m, 1H), 2.31 (d, *J* = 2.2 Hz, 1H), 2.04 – 1.87 (m, 3H), 1.86 – 1.63 (m, 3H), 1.63 – 1.48 (m, 2H), 1.48 – 1.16 (m, 5H), 1.16 – 0.95 (m, 2H), 0.93 (s, 2H), 0.82 (s, 3H), 0.70 (s, 3H).

4.5.11 (5α,13α)-3α-(t-Butyldimethylsilyloxy)-4α-chlorospiro[androstane-17-α/β-2′-oxirane]



Molecular Weight: 437,18 | Molecular Weight: 453,18 | Molecular Weight: 453,18

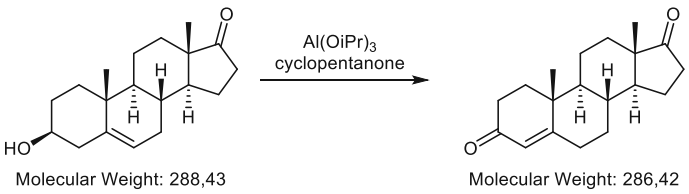
The substrate (350 mg, 0.81 mmol) was dissolved in 7 mL chloroform and 5 mL pH 7 phosphate buffer was added. Under vigorous stirring, mCPBA (250 mg, 70 % in benzoic acid, 1.0 mmol, 1.25 eq) was added. After 90 min, TLC showed complete conversion and the reaction mixture was diluted with DCM and water. The phases were separated and the organic phase was washed with sat. aq. $NaHCO_3$, water and brine. After drying over sodium sulfate, the volatiles were evaporated.

The mixture of epimeric epoxides and starting material was separated by column chromatography (30 g silica, 5 – 10% EA in hexanes). After evaporation of the solvents, 74 mg (21 %) of starting material, 123 mg (34 %) of 17-α epoxide and 102 mg (28 %) of 17-β epoxide were isolated.

Spectra were in accordance with literature: [68]

4.6 Metabolite Epi-M3 Synthesis

4.6.1 13ß-Androst-4-en-3,17-dione



Molecular Weight: 288,43 Molecular Weight: 286,42

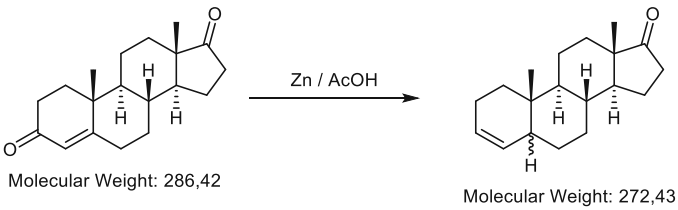
The substrate (10.2 g) was dissolved in 300 mL dry toluene and 50 mL cyclohexanone (or cyclopentanone for reduced smell) was added. The solution was heated to reflux under a Dean-Stark trap until no more water separated (30 min). Aluminium triisopropoxide (17.5 g, 2.5 eq) was added and the reaction continued at reflux temperature for 2.5 h.

After the solution had cooled to ambient temperature, it was diluted to 500 mL and extracted with 1 N H_2SO_4 until no further change in turbidity could be observed. The solution was then extracted with water, sat. aq. $NaHCO_3$ and brine and dried over sodium sulphate. The solvent was evaporated (under high vacuum to remove the oxidant) and the residue purified by MPLC (300 g silica, gradient: 0 % to 50 % EA in hexanes), yielding 7.8 g (77 %) of crystalline product.

Spectra were in accordance with Literature: [156]

R_f: 0.36 (20 % EA in hexane)

4.6.2 13ß,5ξ-Androst-3-en-17-one

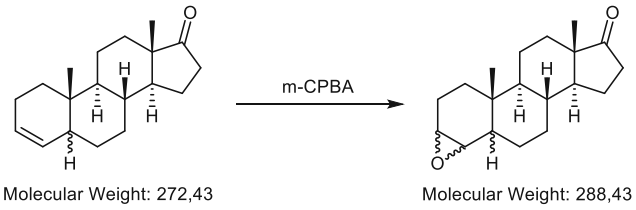


The substrate (4.2 g, 25 mmol) was dissolved in 80 mL acetic acid and heated to 110 °C. Zinc dust (41 g, 50 eq) was added in portions over 15 minutes. After 1 h the reaction was finished according to TLC and the zinc residue was removed by filtration over celite. The zinc residue was 3 x washed with ethyl acetate and to combined organic liquids were washed with water, sat. aq. $NaHCO_3$ and brine and were dried over sodium sulphate. The volatiles were evaporated and the resulting residue purified by MPLC (50x silica, 5 % EA in hexanes) to yield a colorless oil that crystallized upon standing (3.5 g, 88 %)

Spectra were in accordance with literature: [157]

R_f: 0.8 (20 % EE in hexane)

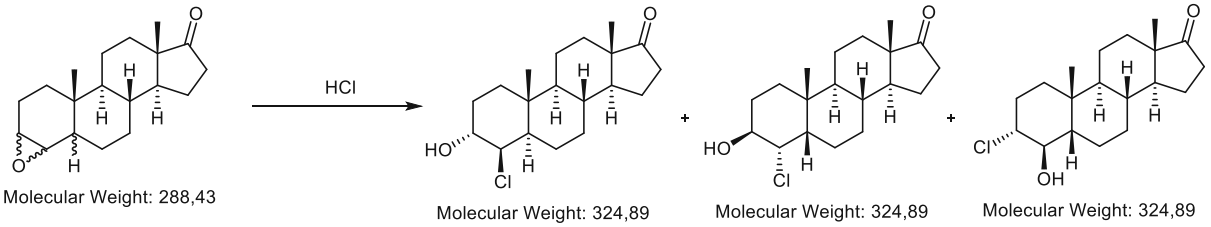
4.6.3 3,4-Epoxy-13ß-androst-17-one



The substrate (3.3 g, 12.3 mmol) was dissolved in 100 mL DCM. A mixture of $KHCO_3$ (2.5 g, 2 eq) and m-CPBA (70 %, 3.0 g, 1.1 eq) was added. The suspension was stirred for 120 minutes until TLC confirmed complete conversion. The reaction was quenched with $Na_2S_2O_3$ – solution and, after phase separation, extracted with sat. aq. $NaHCO_3$ and brine. After drying over sodium sulfate and evaporation of the volatiles, the resulting crude mixture of epoxides (3.4 g) was purified via MPLC (90 g silica, 0 to 40 % EA in hexanes). A purified mixture of epoxides was obtained (3.1 g, 89 % yield)

R_f: 0.6 (20 % EE in hexane)

4.6.4 3α-Hydroxy-4ß-chloro-5α,13ß-androstan-17-one



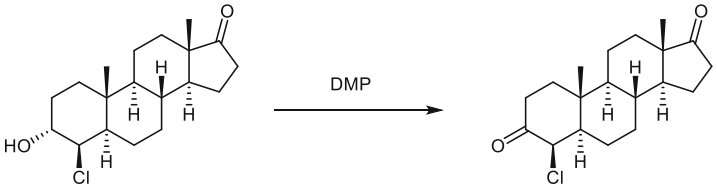
Molecular Weight: 288,43 | Molecular Weight: 324,89 | Molecular Weight: 324,89 | Molecular Weight: 324,89

The substrate (3.4 g) was dissolved in 70 mL chloroform and 25 mL conc. HCl were added. The solution turned yellow and after 2 h, the phases were separated, and the organic phase washed with water, sat. aq. $NaHCO_3$ and brine. After evaporation of the volatiles (25 °C) the 3α-4β-5α product could be obtained by crystallization from hexane / ethyl acetate. The residue was purified by MPLC (180 g silica, EA in hexanes: 10 % to 50 %) and from the still impure fractions containing the product, a second crop of crystals could be obtained by evaporating and crystallizing again from hexane / acetone.

The combined yield of the harvested crystals was 1.1 g (29 % over two steps). The crystals should be handled quickly and stored in the freezer as they tend to degrade over time at ambient conditions.

R_f: 0.51 (33 % EA in hexane)

4.6.5 4ß-Chloro-5α,13ß-androstane-3,17-dione



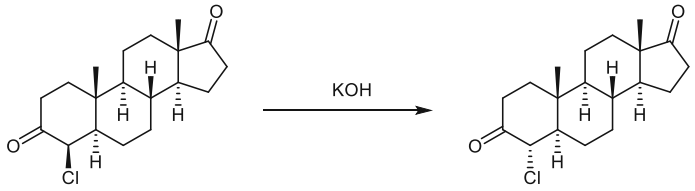
Molecular Weight: 324,89 | Molecular Weight: 322,87

The substrate (2.05 g) was dissolved in 70 mL DCM and Dess-Martin-periodinane (3.6 g, 1.35 eq) was added. After 1 h, TLC indicated complete conversion and the reaction mixture was directly filtered over silica (50 g, 100 % DCM as eluent). The solvent was evaporated and the crude product (2.0 g) used directly for the next step.

R_f: 0.68 (50 % EA in hexane)

^{1}H NMR (400 MHz, Chloroform-d) δ = 4.01 (m, 1H), 3.01 (td, J=14.7, 5.9, 1H), 2.43 – 2.23 (m, 2H), 2.23 – 2.07 (m, 5H), 1.91 – 1.74 (m, 3H), 1.57 – 1.29 (m, 4H), 1.18 (m, 1H), 1.12 – 1.10 (m, 3H), 1.10 – 0.99 (m, 2H), 0.98 (s, 3H), 0.88 – 0.62 (m, 2H).

4.6.6 4α-Chloro-5α,13β-androstane-3,17-dione



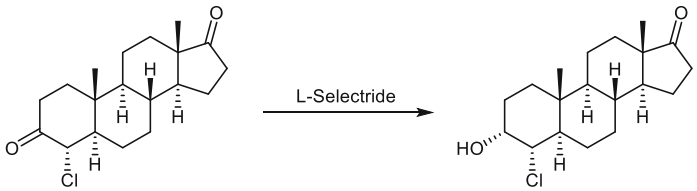
Molecular Weight: 322,87 Molecular Weight: 322,87

The substrate (0.75 g, 2.2 mmol) was dissolved in 70 mL MeOH and KOH (120 mg, 2.2 mmol, 1.0 eq, dissolved in 5 mL MeOH) was added. After 5 min, the solution was neutralized with sat. aq. NH_4Cl. (If the reaction is left for longer, Favorskii-Rearrangement occurs!)

The methanol was evaporated and the residue diluted with ethyl acetate. The phases were separated and the organic phase was washed with sat. aq. bicarbonate and brine. After drying over sodium sulfate, the volatiles were evaporated and the crude product (0.75 g) was of sufficient purity to be used directly in the next step.

R_f: 0.68 (50 % EA in hexane)

4.6.7 4α-Chloro-3α-hydroxy-5α,13β-androstan-17-one



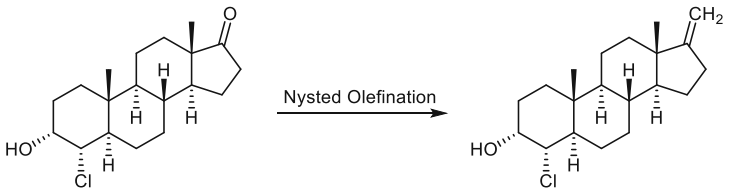
Molecular Weight: 322,87 Molecular Weight: 324,89

The substrate (0.7 g, 2.1 mmol) was dissolved in dry THF (12 mL, low solubility) and cooled down to -70 °C. L-selectride (3 ml, 1 M, 3 mmol, 1.4 eq) was added dropwise and the reaction was stirred for 2 hours. When TLC showed complete consumption of starting material, the reaction was quenched by addition of sat. aq. NH_4Cl. The organic phase was diluted with ethyl acetate and washed with sat. aq. $NaHCO_3$ and brine and dried over Na_2SO_4. The volatiles were evaporated and purification was performed via MPLC (90 g silica, 10 % to 50 % EA in hexanes) obtaining 215 mg (31 %) of pure product.

R_f: 0.4 (50 % EA in hexanes)

^{1}H-NMR: NMR (400 MHz, $CDCl_3$) δ 4.05 (dd, *J* = 11.9, 2.6 Hz, 1H), 4.02 – 3.96 (m, 1H), 2.40 – 2.28 (m, 2H), 2.22 – 2.09 (m, 1H), 2.13 – 2.00 (m, 1H), 1.98 – 1.83 (m, 2H), 1.82 (dd, *J* = 4.0, 1.6 Hz, 1H), 1.82 – 1.63 (m, 2H), 1.61 – 1.33 (m, 4H), 1.30 – 1.04 (m, 2H), 0.95 (s, 3H), 0.89 – 0.73 (m, 2H), 0.67 (s, 3H)

4.6.8 3α-hydroxy-4α-chloro-17-methylene-5α,13β-androstane



Molecular Weight: 324,89 Molecular Weight: 322,92

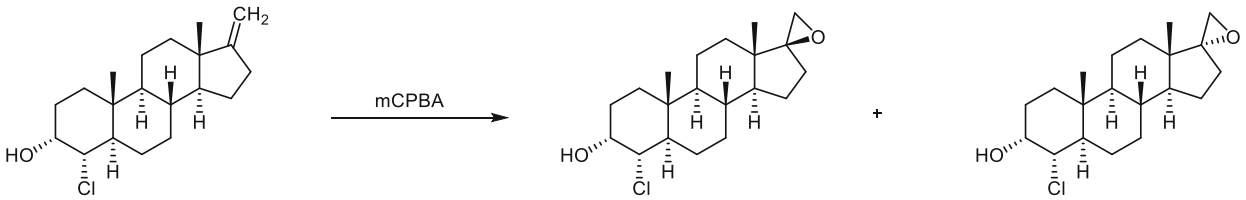
Dibromomethane (0,64 mL, 9.1 mmol, 4.0 eq) was dissolved in 12 mL dry THF and zinc powder (4400 mg, 68 mmol, 30 eq) was added. The suspension was cooled to -10 °C and $TiCl_4$ (0.63 mL, 5.7 mmol, 2.5 eq) was added dropwise. The suspension was stirred at -10 °C for 2 h and then the substrate (1000 mg, 2.3 mmol) was added dropwise. The mixture was held at -10 °C for 1 h and then warmed up to ambient temperature. Stirring was continued overnight and the next day, TLC showed partial conversion.

The reaction was quenched by dilution with 50 mL ethyl acetate and subsequent addition of 1 M HCl. The phases were separated and the organic phase washed with bicarbonate and brine. After drying over sodium sulphate, the volatiles were evaporated and purification was performed by MPLC (90 g silica, 0 – 20 % EA in hexanes). After evaporation of the eluents, 350 mg (35 %) of methylenated product and 280 mg (28 %) of starting material were isolated.

R_f: 0.8 (20 % EA in hexanes)

^{1}H-NMR: (400 MHz, $CDCl_3$) δ 4.81 (d, *J* = 2.2 Hz, 1H), 4.68 (d, *J* = 2.6 Hz, 1H), 4.07 (dd, *J* = 11.9, 2.6 Hz, 1H), 4.00 (q, *J* = 2.9 Hz, 1H), 2.56 – 2.41 (m, 1H), 2.36 (m, 1H), 2.31 (d, *J* = 2.2 Hz, 1H), 2.04 – 1.87 (m, 3H), 1.86 – 1.63 (m, 3H), 1.63 – 1.48 (m, 2H), 1.48 – 1.16 (m, 5H), 1.16 – 0.95 (m, 2H), 0.93 (s, 2H), 0.82 (s, 3H), 0.70 (s, 3H).

4.6.9 (5α,13β)-3α-hydroxy-4α-chlorospiro[androstane-17-α/β-2′-oxirane]



Molecular Weight: 322,92 | Molecular Weight: 338,92 | Molecular Weight: 338,92

The substrate (350 mg, 0.81 mmol) was dissolved in 7 mL chloroform and 5 mL pH 7 phosphate buffer was added. Under vigorous stirring, mCPBA (250 mg, 70 % in benzoic acid, 1.0 mmol, 1.25 eq) was added. After 90 min, TLC showed complete conversion and the reaction mixture was diluted with DCM and water. The phases were separated and the organic phase was washed with sat. aq. $NaHCO_3$, water and brine. After drying over sodium sulfate, the volatiles were evaporated.

The mixture of epimeric epoxides and starting material was separated by column chromatography (30 g silica, 5 – 10% EA in hexanes). After evaporation of the solvents, 74 mg (21 %) of starting material, 123 mg (34 %) of 17-α epoxide and 102 mg (28 %) of 17-β epoxide were isolated.

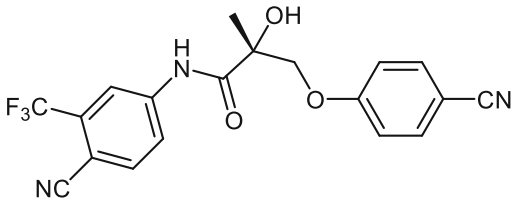
R_f: 0.8 (20 % EA in hexanes)

17-α epoxide

^{1}H-NMR: (400 MHz, $CDCl_3$) δ 4.81 (d, *J* = 2.2 Hz, 1H), 4.68 (d, *J* = 2.6 Hz, 1H), 4.07 (dd, *J* = 11.9, 2.6 Hz, 1H), 4.00 (q, *J* = 2.9 Hz, 1H), 2.56 – 2.41 (m, 1H), 2.36 (m, 1H), 2.31 (d, *J* = 2.2 Hz, 1H), 2.04 – 1.87 (m, 3H), 1.86 – 1.63 (m, 3H), 1.63 – 1.48 (m, 2H), 1.48 – 1.16 (m, 5H), 1.16 – 0.95 (m, 2H), 0.93 (s, 2H), 0.82 (s, 3H), 0.70 (s, 3H).

4.7 Ostarine Glucuronide

4.7.1 Ostarine



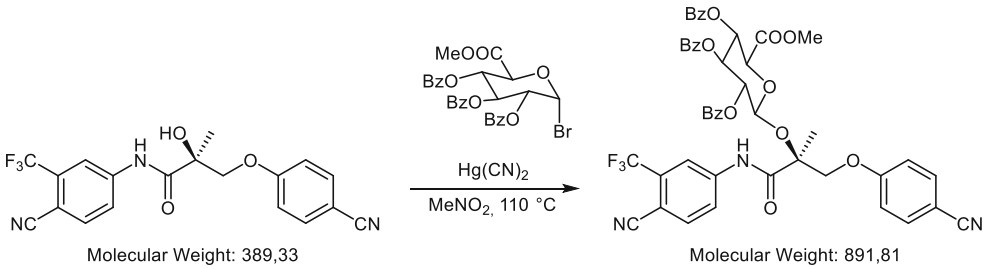
Molecular Weight: 389,33

Ostarine was received in Pills, which were opened and the contents extracted with DCM. The extract was purified with MPLC (45 g silica for 1 g extract, 50 % EE in hexanes) to yield ostarine as an amorphous , strongly UV-active yellow solid.

R_f: 0.3 (66 % EE in hexanes)

^{1}H-NMR (400 MHz, Chloroform-d) δ 9.11 (s, 1H), 8.11 (d, J = 2.2 Hz, 1H), 7.97 (dd, J = 8.5, 2.2 Hz, 1H), 7.82 (d, J = 8.5 Hz, 1H), 7.60 (d, J = 8.2 Hz, 2H), 6.97 (d, J = 8.2 Hz, 2H), 4.51 (d, J = 9.1 Hz, 1H), 4.07 (d, J = 9.1 Hz, 1H), 1.62 (s, 3H).

4.7.2 Ostarine Benzoyl-Glucuronide



Ostarine (299 mg, 0.768 mmol, 1 eq.), the glucuronyl donor (672 mg, 1.15 mmol, 1.5 eq.) and mercury [II] cyanide (310 mg, 1.23 mmol, 1.6 eq.) were dissolved / suspended in nitromethane/benzene 1:1 (10 mL : 10 mL). Liquid was evaporated until the volume of the solution had halved and it was topped up with benzene again. Liquid was again evaporated until the volume of the solution had halved again and the mixture was then heated to 110 °C. After 2 h, UPLC-MS showed that no further changes to the reaction mixture, which was then cooled down to ambient temperature.

The reaction was worked up by diluting with ethyl acetate and washing with sat. aq. bicarbonate and brine. The solution was then dried over sodium sulfate and evaporated.

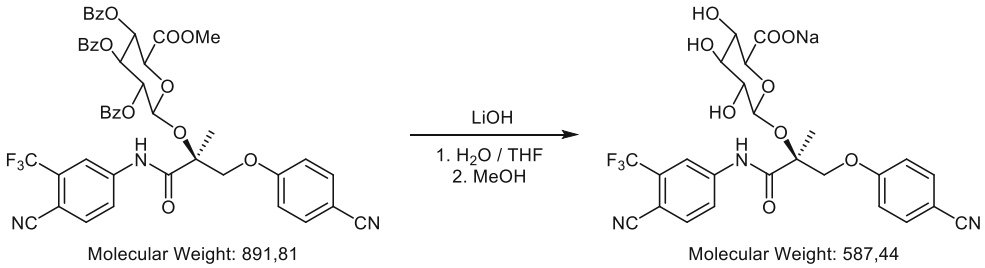
Purification was performed by column chromatography (LP/EA, linear gradient 10 % to 90 % EA). The pure product (173 mg, 25 % yield) was obtained as a yellowish viscous oil. Additionally, unconsumed ostarine can be recovered as a strongly UV-active yellow amorphous solid.

R_f: 0.65 (50 % EA in hexanes)

^{1}H NMR (600 MHz, Chloroform-d) δ = 9.52 (s, 1H), 8.36 (d, J=2.1, 1H), 8.19 (dd, J=8.5, 2.2, 1H), 7.95 – 7.86 (m, 6H), 7.84 (d, J=8.5, 1H), 7.59 (d, J=8.9, 4H), 7.42 – 7.37 (m, 4H), 7.33 (dd, J=8.4, 7.4, 2H), 6.97 (d, J=8.9, 2H), 6.07 (t, J=9.5, 1H), 5.68 – 5.59 (m, 2H), 5.26 (d, J=7.5, 1H), 4.47 (d, J=2.9, 2H), 4.22 (d, J=9.6, 1H), 3.39 (s, 3H), 1.56 (s, 3H).

^{13}C NMR (151 MHz, Chloroform-d) δ 168.7, 165.9, 165.4, 164.5, 164.1, 160.1, 140.8, 134.9, 133.3, 133.0, 132.7, 128.8, 128.7, 127.9, 127.6, 127.6, 127.5, 127.4, 127.3, 127.1, 120.9, 117.8, 114.3, 103.8, 95.0, 80.9, 76.2, 76.0, 75.8, 71.6, 71.5, 70.2, 69.9, 68.6, 51.8, 18.1, 0.0.

4.7.3 Ostarine Glucuronide – Sodium Salt



Molecular Weight: 891,81 | Molecular Weight: 587,44

The protected glucuronide (112 mg, 0.126 mmol, 1 eq.) was dissolved in THF and as much water was added as was permitted by solubility of the substrate. The solution was cooled to 0 °C and lithium hydroxide (26 mg, 0.628 mmol, 5 eq) was added. When the hydroxide had dissolved, THF was added until a homogenous solution was obtained. After 10 min, cleavage of the methyl ester was complete according to UPLC-MS and the solution was diluted with twice the volume of methanol (15 mL) and warmed to ambient temperature. After 40 min, UPLC-MS confirmed removal of the benzoyl groups and the reaction was quenched with sat. aq. ammonium chloride. The volatiles were evaporated to yield the crude product as a yellowish amorphous solid.

Purification was performed by preparative HPLC ($MeCN/H_2O$, linear gradient 50 % to 95 % MeCN). After lyophilization, the product was weighed and neutralized with 1 eq aq. 0.1 N $NaHCO_3$ to give, after another lyophilization, ostarine glucuronide sodium salt as a yellowish amorphous solid.

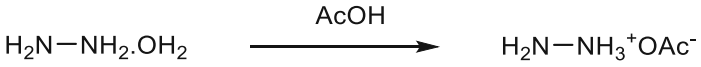
HRMS: m/z calcd. for $C_{25}H_{21}O_8N_3F_3$ ([M - H]-): 564.1235 found: 564.1207 (-3.1 ppm)

^{1}H NMR (600 MHz, DMSO-d6) δ = 8.34 (d, J=2.1, 1H), 8.22 – 8.00 (m, 2H), 7.71 (d, J=8.8, 2H), 7.11 (d, J=8.6, 2H), 4.70 (d, J=7.6, 1H), 4.38 – 4.23 (m, 2H), 3.35 (d, J=9.8, 1H), 3.24 (t, J=8.7, 1H), 3.18 (s, 0H), 3.16 – 3.09 (m, 15H), 1.61 (s, 3H).

^{13}C NMR (151 MHz, DMSO) δ 172.8, 172.5, 162.1, 143.2, 137.0, 134.6, 132.4, 132.2, 123.8, 122.9, 122.0, 119.5, 117.2, 117.2, 116.4, 116.2, 103.7, 102.8, 98.5, 82.1, 77.0, 74.8, 74.0, 72.6, 49.1, 18.5.

4.8 Various Reactions and Reagents

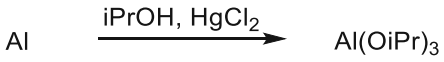
4.8.1 Hydrazinium Acetate



In a 500 mL round bottom flask, acetic acid (125 mL, 1.0 eq) was cooled to 17 °C (beginning to freeze). Hydrazine hydrate (200 mL, 1.2 eq) was added dropwise under constant stirring, keeping the temperature below 20 °C. After complete addition of hydrazine hydrate a viscous clear solution was obtained.

Water was evaporated overnight under vacuum, precipitating white crystals of Hydrazinium Acetate from the solution which were not further purified.

4.8.2 Aluminium Triisopropoxide

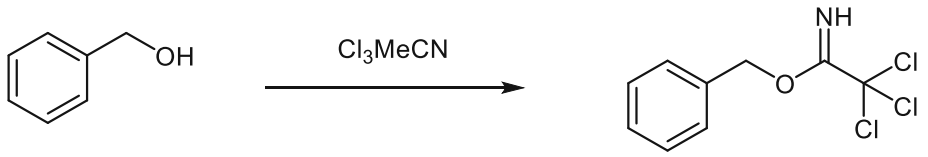


Aluminium foil (60 g, 2.2 mol) was cut into small pieces and suspended in 2 L dry isopropanol. The suspension was heated to reflux and mercury(II)chloride (600 mg, 0.001 eq) was added. After refluxing for two days, all of the aluminium foil had reacted and the remaining isopropanol was evaporated.

The product was purified by vacuum distillation (b.p.: 130 °C at 10 mbar). This distillation can be performed because resolidification of aluminium triisopropoxide is very delayed, it takes several days at ambient temperature. After distillation, 322 g (71 %) of product were obtained as white crystalline block.

Procedure from: [149]

4.8.3 Benzyl Trichloroacetimidate



Molecular Weight: 108,14 Molecular Weight: 252,52

[150]

Dry benzyl alcohol (100 mL) and trichloroacetonitrile (106 mL, 1.1 eq) were dissolved in 1000 mL n-hexane, cooled to 0 °C, and DBU (3 mL, 0,02 eq) was added. After a few seconds the solution became cloudy and stirring was continued. After 1 h, the cooling bath was removed.

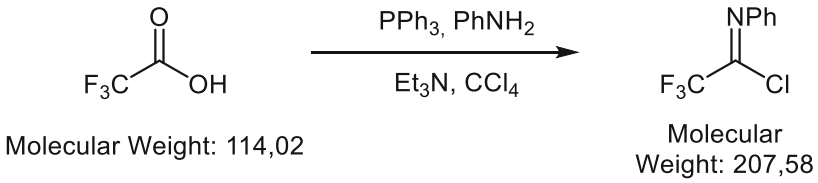
Workup was performed be extracting twice with sat. aq. NH_4Cl and the solution was dried over Na_2SO_4.

The solvent was evaporated to give 170 mL (95 %) benzyl trichloroacetimidate.

After prolonged storage, the product can be purified by vacuum distillation.

^{1}H NMR (400 MHz, Chloroform-*d*) δ = 8.41 (s, 1H), 7.59 – 7.30 (m, 5H), 5.36 (s, 2H).

4.8.4 N-phenyl trifluoracetimidoyl chloride



Molecular Weight: 114,02

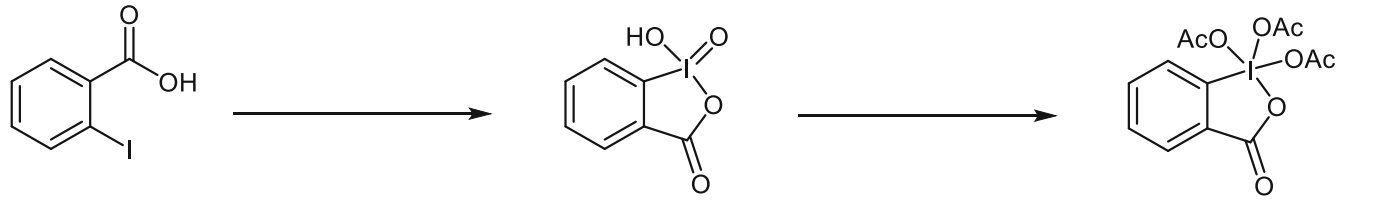
Molecular Weight: 207,58

Trifluoroacetic acid (3.4 mL, 44 mmol), triphenyl phosphine (34 g, 130 mmol, 3 eq), triethyl amine (7 mL, 51 mmol, 1.2 eq) and CCl_4 (20 mL, 5 eq) were mixed under ice cooling. After 5 min, aniline (4.6 mL, 51 mmol, 1.2 eq) dissolved in further 20 mL of CCl_4 was added to the reaction mixture dropwise. The mixture was heated to reflux for 3 h. The volatiles were evaporated and the residue was diluted with 50 mL n-hexane and filtered over celite. The hexane was evaporated and the resulting orange product used for the next step without further purification.

The product can be distilled at 100 °C at 10 mbar pressure.

Procedure derived from and analytical data in accordance to: [151]

4.8.5 Dess-Martin Periodinane



Molecular Weight: 248,02 Molecular Weight: 280,02 Molecular Weight: 424,14

Procedure from [152]

A 2-L, round-bottomed flask was charged with 80.0 g (0.48 mol) of potassium bromate and 750 mL of 2.0 M sulfuric acid. The resulting clear solution was heated to 65°C and 80.0 g of 2-iodobenzoic acid (0.32 mol) was added. The solution gradually turned red, and bromine vapors started to evolve (keep flask open so the vapors can escape), the product started to precipitate as a white solid. As the substrate tends to float on top of the solution, occasional manual stirring is necessary (a mechanical stirrer is advantageous). The reaction was heated at 65 °C for further 150 min. Cessation of bromine vapor formation indicates completion of the reaction.

The suspension was cooled in an ice-water bath and the product collected by vacuum filtration. The filter cake was washed with 500 mL cold water, 2 x 80 mL ethanol and then again water to give 80 g (90 %) of damp IBX. (don't dry completely, explosion hazard, see notes in reference)

A 1-L flask was charged with 80 g of moist IBX, 150 mL of dry acetic acid, and 300 mL of acetic anhydride. The mixture was heated to 85°C until all the solids had dissolved. Heating and stirring were discontinued and the reaction mixture was allowed to cool slowly to ambient temperature overnight. The resulting crystalline product was filtered off and washed with three 80-mL portions of dry ethyl ether. After drying in vacuum, 90 g (66% yield over 2 steps) of DMP were isolated.

^{1}H NMR (400 MHz, Chloroform-*d*) δ = 8.29 (d, J = 8.0, 2H), 8.09 (t, J = 8.1, 1 H), 7.91 (t, 1 H, J = 7.4), 2.32 (s, 3 H), 1.99 (s, 6 H)

4.8.6 Methyl *ortho*-Iodobenzoate

Molecular Weight: 248,02 Molecular Weight: 262,05

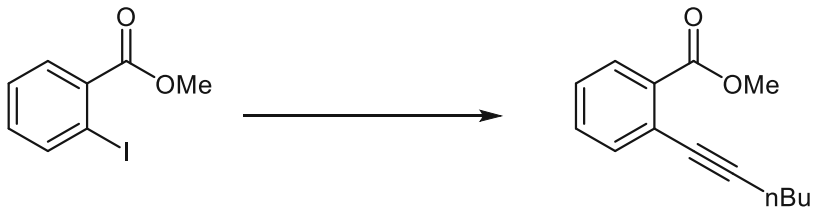
The substrate can be recrystallized from toluene / acetone

The acid (5g, 20 mmol, 1 eq) was dissolved in 150 mL absolute MeOH and 5 mL conc. H_2SO_4 were added to the brownish solution. The solution was heated to 50 °C and stirred overnight. When TLC indicated complete consumption of the starting material, the methanol was evaporated and the residue redissolved in toluene. The solution was washed with water and then with sat. aq. $NaHCO_3$ until no further gas formation was observed. After the solution was washed with brine, the solvent was evaporated to give 5.25 g (99 %) of product.

Analytical data in accordance with reference: [153]

^{1}H NMR (400 MHz, Chloroform-*d*) δ = 7.91 (d, *J*=8.0, 1H), 7.72 (d, *J*=7.8, 1H), 7.38 – 7.27 (m, 1H), 7.12 – 7.03 (m, 1H), 3.85 (s, 3H).

4.8.7 Methyl *ortho*-Hexynylbenzoate



Molecular Weight: 262,05 Molecular Weight: 216,28

The catalyst $Pd(PPh_3)Cl_2$ (130 mg, 0.01 eq) and CuI (80 mg, 0.03 eq) were suspended in 20 mL dry Et_3N and methyl orthoiodobenzoate (5 g, 2.8 mL, 1 eq) was added to the yellow suspension. The color changed to brown and stirring continued for 1 h. The flask was cooled to 0 °C and 1-hexyne (2.35 g, 3.27 mL, 1.5 eq) was added over the course of 10 min.

After stirring overnight, TLC confirmed complete conversion and the volatiles were evaporated. The residue was extracted 3x with hexanes + 1 % Et_2O and the resulting solution filtered over celite.

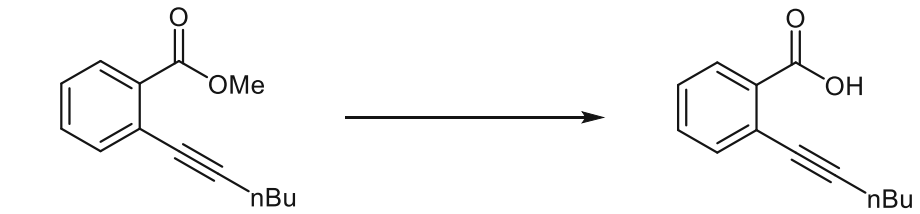
Purification was performed by MPLC (90 g Silica, PE:EA = 50:1), giving 3.71 g of pure product (90 %) as a yellow oil.

Analytical data identical to reference: [154]

R_f: 0.65 (25 % EE in hexanes)

^{1}H NMR (400 MHz, Chloroform-*d*) δ = 7.80 (dd, *J*=7.9, 1.5, 1H), 7.43 (dd, *J*=7.8, 1.5, 1H), 7.34 (td, *J*=7.7, 1.5, 1H), 7.23 (td, *J*=7.6, 1.4, 1H), 3.84 (s, 3H), 2.41 (t, *J*=7.0, 2H), 1.71 – 1.30 (m, 4H), 0.88 (t, *J*=7.3, 3H).

4.8.8 Ortho-Hexynylbenzoic Acid



Molecular Weight: 216,28

Molecular Weight: 202,25

The substrate (2.5 g, 11.5 mmol) was dissolved in 25 mL of EtOH and 10 mL H_2O (use maximal possible amount of water) and KOH (650 mg, 11.5 mmol, 1.0 eq) was added, deepening the yellow hue to amber. The solution was stirred at rt for 3 h.

After TLC showed full consumption of starting material, ethanol was evaporated, the remaining solution diluted to 100 mL water and washed with an equal amount Et_2O. The solution was carefully acidified with 1 M $KHSO_4$ to pH 3-4 (product crashes out of solution) and extracted twice with Et_2O. After drying over sodium sulphate, the solvent was evaporated to yield a 1.6 g (70 %) of yellow oil that solidifies in the freezer.

A small amount of methyl- and ethyl ester can be recovered from the initial organic extraction phase.

Analytical data identical to reference: [154]

R_f: 0.1 (25 % EE in hexanes)

^{1}H NMR (200 MHz, Chloroform-*d*) δ = 9.73 (s, 1H), 8.07 – 7.88 (m, 1H), 7.54 – 7.21 (m, 3H), 2.44 (t, *J* = 6.8 Hz, 2H), 1.69 – 1.27 (m, 4H), 0.89 (t, *J* = 7.1 Hz, 3H).

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5.1 Introduction

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5.3 Conclusion and Outlook

5.4 Experimental Section

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6 Abbreviations

6.1 General Abbreviations

AAF	Adverse Analytical Finding
AAS	Anabolic Androgenic Steroid
Abz	*ortho*-Alkynylbenzoate
Ac	Acyl
AIDS	Acquired Immunodeficiency Syndrome
Aq.	Aqueous
AR	Anabolic Ratio
Bn	Benzyl
n-BuLi	*n*-Butyl lithium
s-BuLi	*sec* – Butyl lithium
Bz	Benzoyl
CIBA	Chemische Industrie Basel
DCM	Dichloromethane
DCVC	Dry Column Vacuum Chromatography
DDQ	2,3-Dichloro-5,6-dicyano-1,4-benzoquinone
DHCMT	Dehydrochloromethyltestosterone
DIBAL-H	Diisobutyl Aluminium Hydride
DIPEA	Diisopropylethylamine “Hünig’s Base”
DOI	Digital Object Identifier
DMAP	N,N – Dimethyl 4-Aminopyridine
DMDO	Dimethyldioxirane
DMF	Dimethylformamide
DMP	Dess Martin Periodinane[14]
dr	Diastereomeric Ratio
Et	Ethyl
EE	Ethyl Acetate
EA	Ethyl Acetate
FDA	Food and Drug Administration
GAG	Glycosyminoglycan
GC-MS	Gas Chromatography – Mass Spectroscopy
GDR	German “Democratic” Republic

Glc	Glucuronyl
GlcA	Glucuronic Acid
HPG-axis	Hypothalamic-Pituitary-Gonadal-axis
HRMS	High Resolution Mass Spectroscopy
IAF	International Athletics Federation
IBX	2-Iodoxybenzoic acid
iBu	Isobutyryl-
IOC	International Olympic Committee
LC – MS	Liquid Chromatography – Mass Spectroscopy
LDA	Lithium diisopropyl amine
LH	Luteinizing Hormone
LiHMDS	Lithium Hexamethyldisilazane
2,6 lut	2,6-Lutidine
Me	Methyl
MPLC	Medium Pressure Liquid Chromatography
MSTFA	N-methyl-N-trimethylsilyl-trifluoroacetamide
NaHMDS	Sodium Hexamethyldisilazane
NMR	Nuclear Magnetic Resonance
NP-	Normal Phase (Chromatography)
PE	Petroleum Ether
Ph	Phenyl-
Piv	Pivaloyl-
POW	Prisoner of War
Py	Pyridine
RP-	Reversed Phase (Chromatography)
RUSADA	Russian Anti-Doping Agency
SARM	Selective Androgen Receptor Modulator
SPE	Solid-Phase Extraction
TBAF	Tetrabutylammonium Fluoride
TBS	*tert*-Butyldimethylsilyl-
TEMPO	(2,2,6,6-Tetramethylpiperidin-1-yl)oxyl
Tf	Trifluoromethanesulfonyl-
THF	Tetrahydrofuran
TIPS	Triisopropylsilyl-

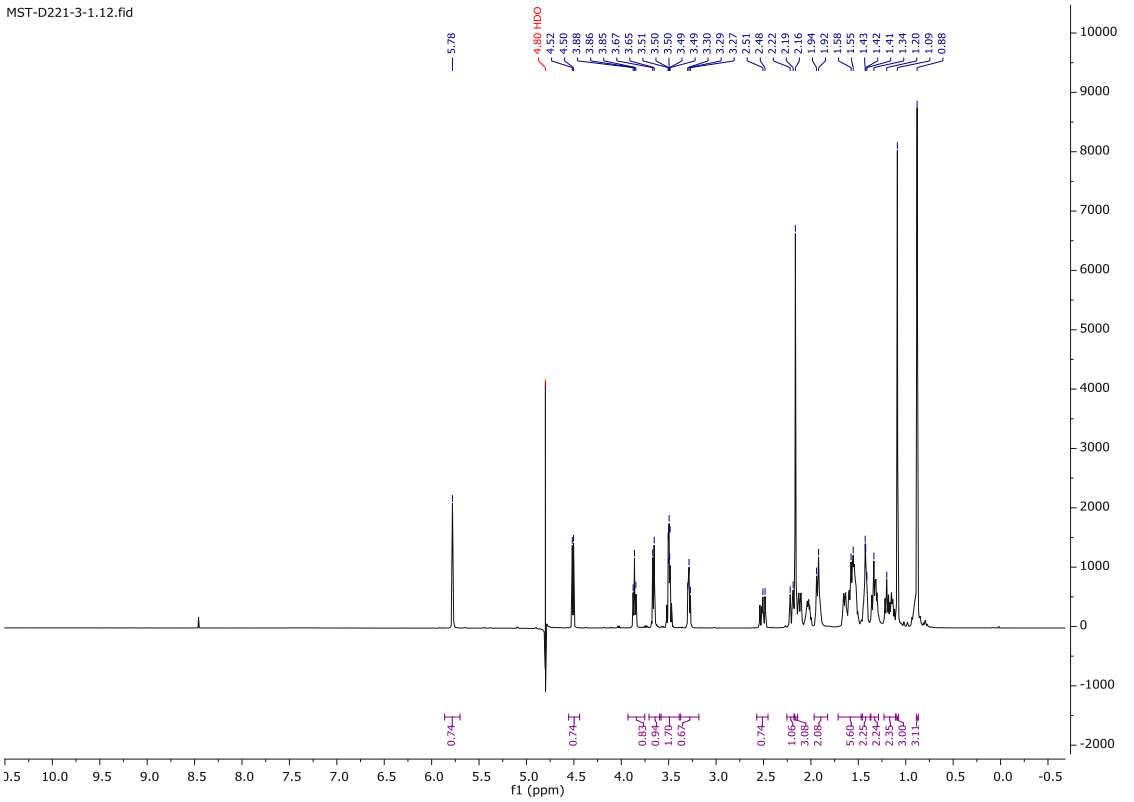
TLC	Thin Layer Chromatography
TMS	Trimethylsilyl-
TMSOTf	Trimethylsilyltrifluormethanesulfonate
Tol	Toluene
Tr	Trityl-
Ts	*para*-Toluenesulfone-
UDP	Uridine-diphosphate
UGT	Uridine 5'-diphospho-glucuronosyltransferase
USSR	Union of Soviet Socialist Republics
UV	Ultraviolet
WADA	World-Anti-Doping Agency

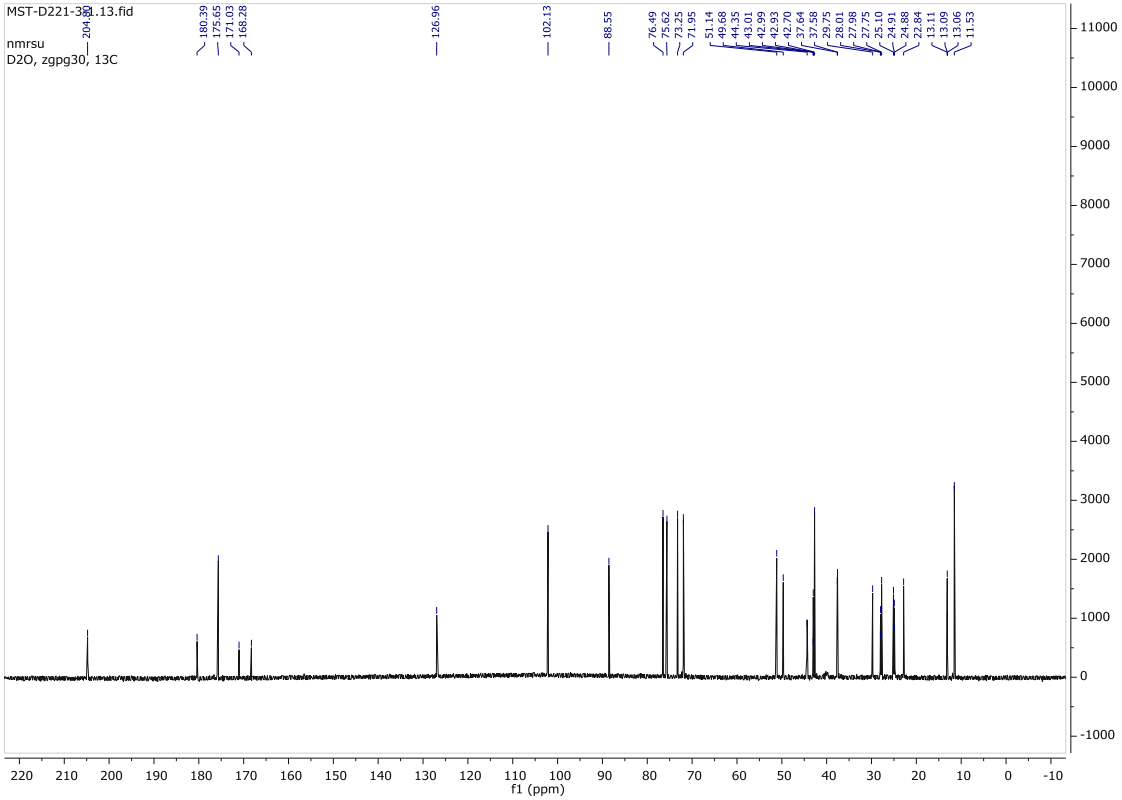
6.2 Steroids and SARMs

Trivial Name	Systematic Name
Andarine	(S)-3-(4-acetamidophenoxy)-2-hydroxy-2-methyl-N-(4-nitro-3-(trifluoromethyl)phenyl)propanamide
Androsterone	3α-hydroxy-5α-androstan-17-one
DHCMT	4-chlor-17β-hydroxy-17α-methylandrosta-1,4-dien-3-on
Dienolone	estra-4,9(10)-dien-17β-ol-3-one
Dihydrotestosterone (DHT)	17β-hydroxy-5α-androstan-3-one
Epiandrosterone	3β-hydroxy-5α-androstan-17-one
Epitrenbolone	estra-4,9,11-trien-17α-ol-3-one
Ligandrol	(S)-N-(4-cyano-3-(trifluoromethyl)phenyl)-3-(4-cyanophenoxy)-2-hydroxy-2-methylpropanamide
Metenolone	1-methyl-5α-androst-1-en-17β-ol-3-one
20β-OH-NorMD “Nightwatch”	17β-hydroxymethyl-17α-methyl-18-norandrost-1,4,13-trien-3-one
Ostarine	(R)-N-(4-cyano-3-(trifluoromethyl)phenyl)-3-(4-cyanophenoxy)-2-hydroxy-2-methylpropanamide
Oxymetholone	2-hydroxymethylene-17α-methyl-5α-androstan-17β-ol-3-one
Stanozolol	17α-methyl-2'H-5α-androst-2-eno[3,2-c]pyrazol-17β-ol
Trenbolone	estra-4,9,11-trien-17β-ol-3-one

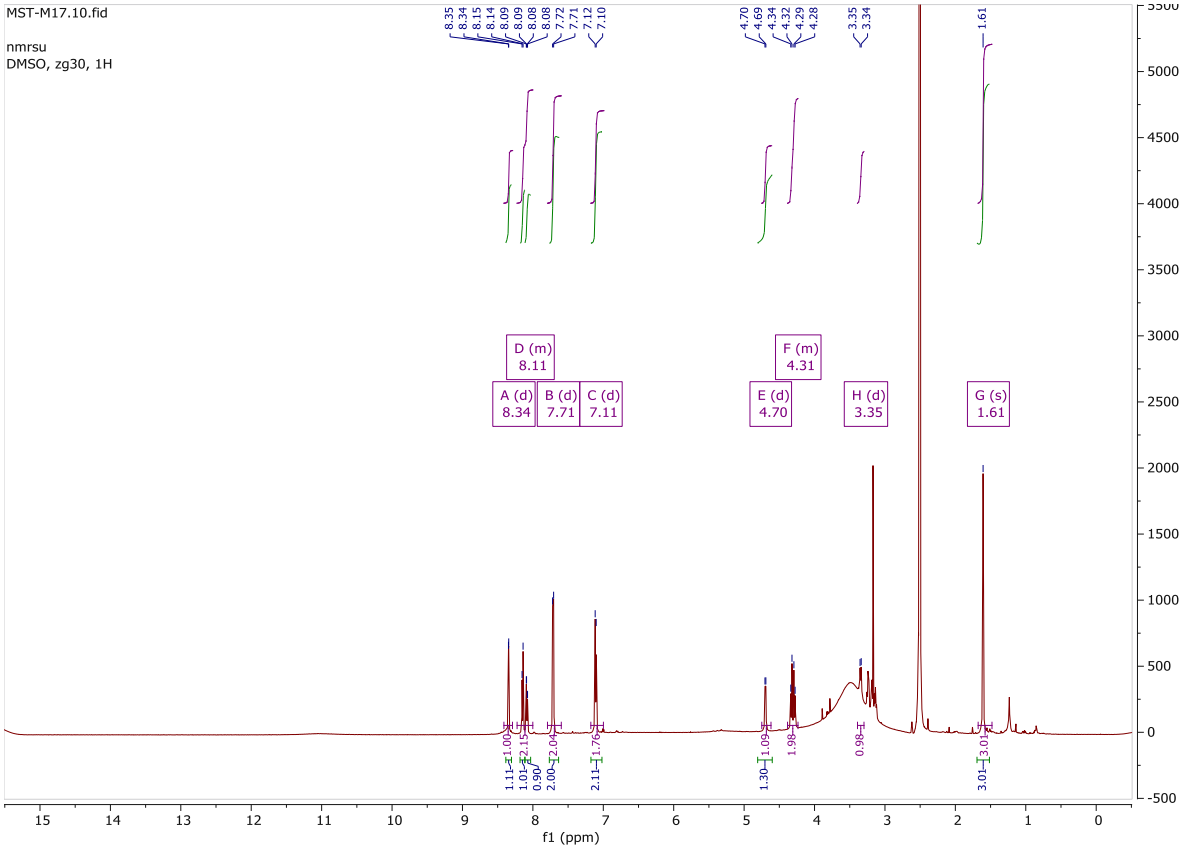
7 Selected Spectra

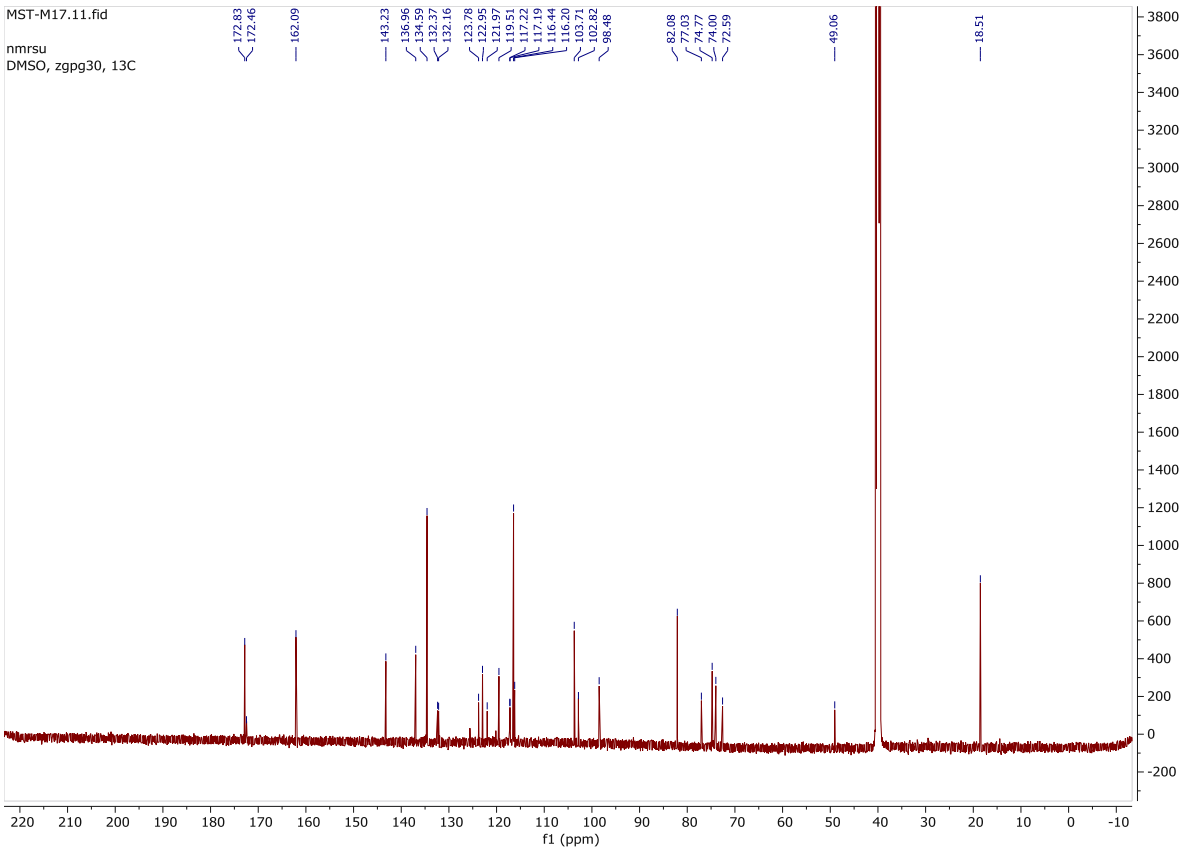
Metenolone Glucuronide-Na



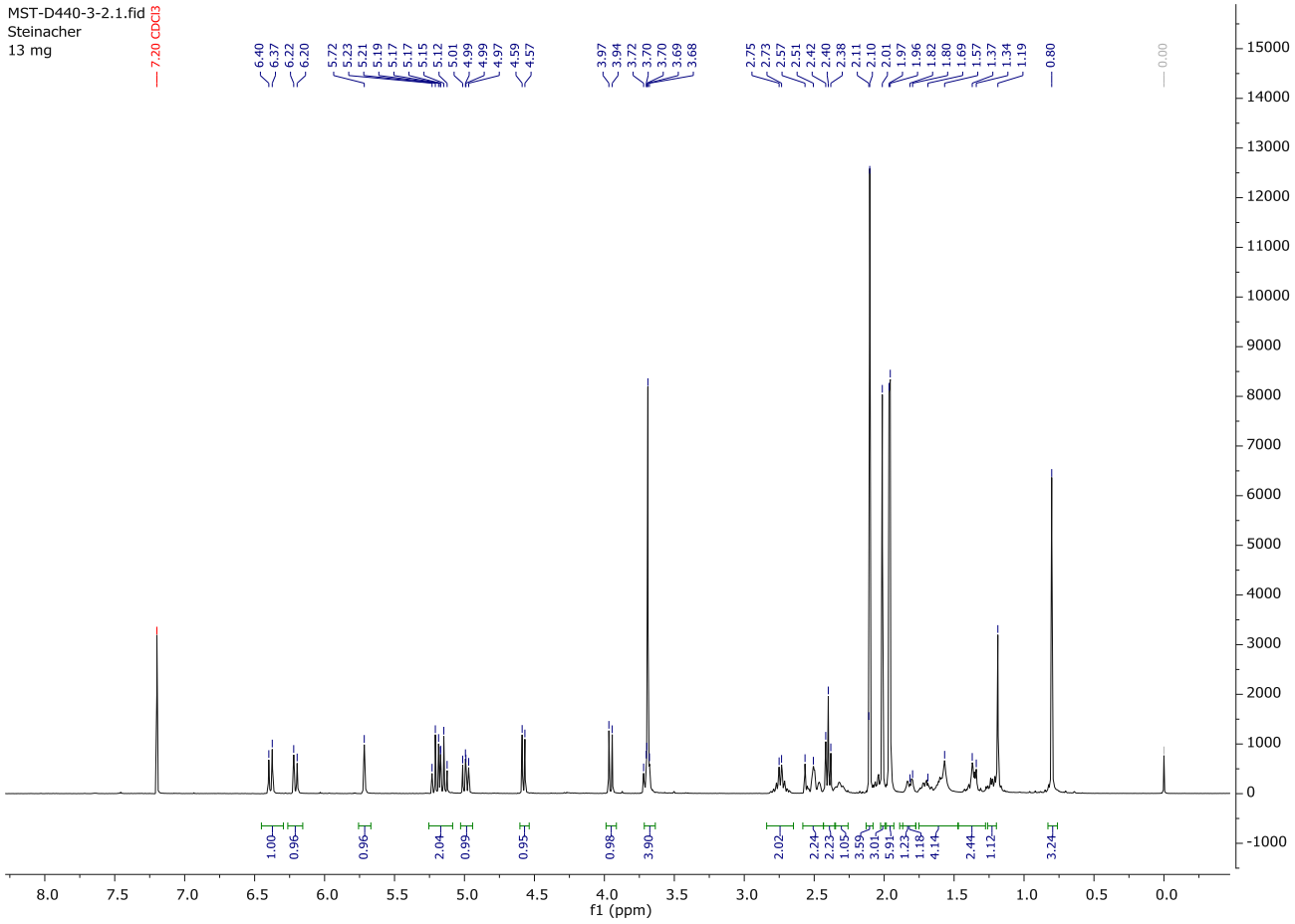


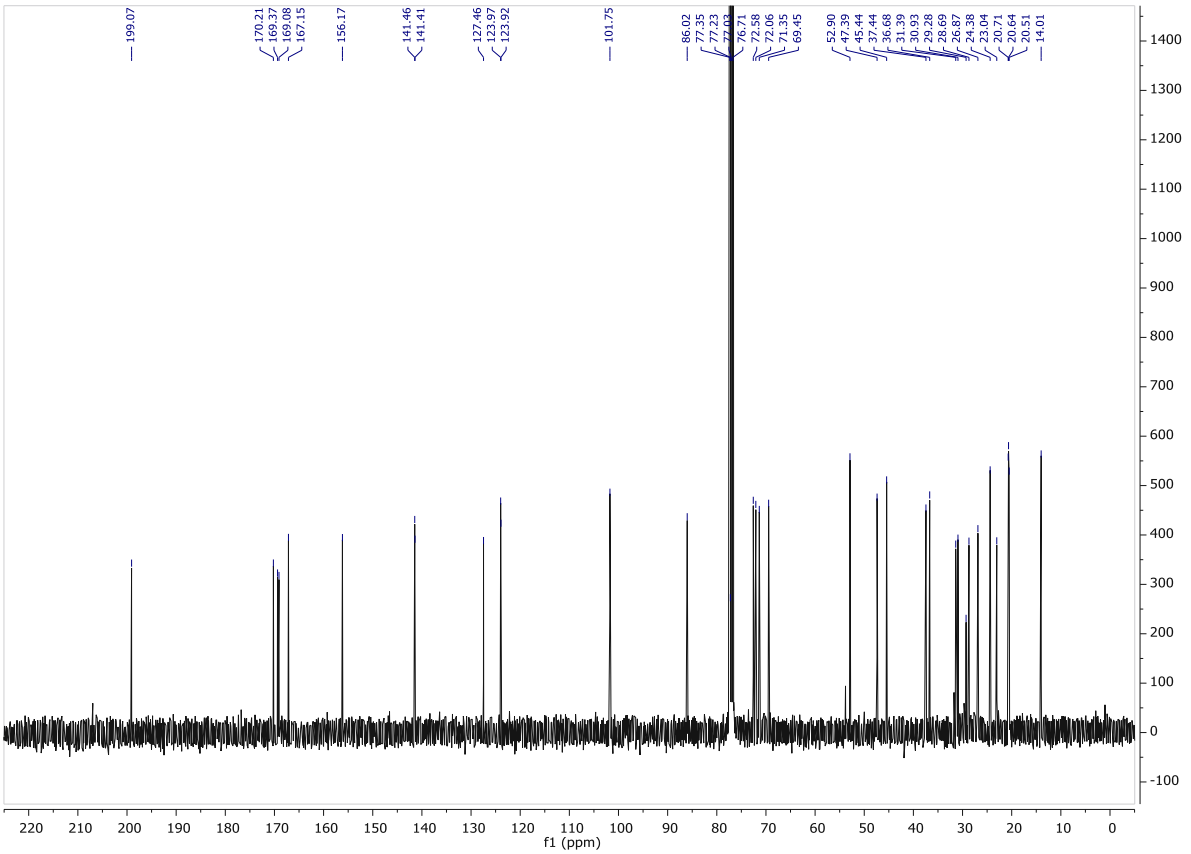
Ostarine Glucuronide-Na





Trenbolone Glucuronide (acetylated)





20-norMD - “Nightwatch”