Diplomarbeit

Synthesis and Characterization of 17-Epistanozolol-\(N\)-Glucuronides for Anti-Doping Purposes

Durchgeführt am Institut für Angewandte Synthesechemie der Technischen Universität Wien

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Abstract:

Stanozolol is the most often used synthetic anabolic steroid as doping substance in professional sports. Therefore, the detection of metabolites of this molecule and comparison with synthetic reference materials is of high importance in anti-doping control. Especially, the synthesis of conjugated metabolites have risen in importance during the last years.

The aim of this thesis was the synthesis of urinary metabolites of the anabolic androgenic steroid stanozolol for anti-doping purposes and comparison with human metabolic molecules. Focus was put on the synthesis of the in position 17 epimerized metabolite of stanozolol and the $\text{N}$-glucuronides of this molecule. Synthesis of 17-epistanozolol was carried out starting from stanozolol via sulfation of the tertiary alcohol and hydrolysis under basic conditions. $\text{N}$-Glucuronides of this product were synthesized through modified Koenigs-Knorr glycosylation conditions with mercury salts for activation. The obtained synthetic metabolites were deprotected under basic conditions, fully characterized and compared with human urinary metabolic products.

Zusammenfassung

Stanozolol ist das am häufigsten als Dopingmittel genutzte synthetische anabole Steroid im professionellen Sport. Deshalb sind die Detektion von Metaboliten und der Vergleich mit synthetischen Referenzmaterialien von höchster Bedeutung in der Anti-Doping Kontrolle. Im Speziellen haben konjugierte Metaboliten in den letzten Jahren an Bedeutung gewonnen.

# Table of Contents

1. **Introduction** ............................................................................................................. 5  
   1.1. History of Doping ................................................................................................. 5  
   1.2. Definition and Prohibition of Doping ................................................................. 7  
   1.3. Modern Methods in Doping-Analysis ............................................................... 10  
   1.4. Metabolism of Steroids ....................................................................................... 12  
      1.4.1. Phase I Metabolism ....................................................................................... 12  
      1.4.2. Phase II Metabolism .................................................................................... 15  
   1.5. Methods for chemical Glycosylations ............................................................... 19  
      1.5.1. Koenigs-Knorr-Glycosylation ..................................................................... 19  
      1.5.2. Schmidt- and Yu-Glycosylations ................................................................. 20  
   1.6. Stanozolol ........................................................................................................... 21  
   1.7. Target Molecule and Task .................................................................................. 24  
   1.8. State of the Art .................................................................................................... 24  
      1.8.1. Synthesis of 17-Epistanozolol ..................................................................... 25  
      1.8.2. Synthesis of Glucuronides ......................................................................... 26  
2. **Results and Discussion** ......................................................................................... 27  
   2.1. Retrosynthetic Analysis ....................................................................................... 27  
   2.2. Synthesis of 17-Epistanozolol .......................................................................... 28  
      2.2.1. Approach A ................................................................................................... 29  
      2.2.2. Approach B ................................................................................................... 30  
      2.2.3. Approach C and D ....................................................................................... 31  
   2.3. Synthesis of Glycosyl-Donor-Molecules ............................................................ 36  
      2.3.1. Acetyl-Protection of Glucuronolactone ....................................................... 36  
      2.3.2. Koenigs-Knorr-Donor ................................................................................. 37  
      2.3.3. Schmidt-Donor ............................................................................................ 37  
   2.4. Synthesis of N-Glucuronides ........................................................................... 38  
      2.4.1. Koenigs-Knorr-Glycosylation ..................................................................... 39  
      2.4.2. Schmidt-Glycosylation ................................................................................. 41  
      2.4.3. Yu-Glycosylation ......................................................................................... 41  
      2.4.4. Deprotection of Glucuronides ................................................................... 42
3. Conclusion ............................................................................................................. 43
4. Experimental Part .................................................................................................. 45
  4.1. General ............................................................................................................. 45
  4.2. Synthetic Work ................................................................................................. 47
    4.2.1. 17α-Methyl-5α-androstano[3,2-c]pyrazol-17β-ol (2) and 17β-Methyl-5α-androstano[3,2-c]pyrazol-17α-ol (3) via Approach C ............................................................... 47
    4.2.2. 17α-Methyl-5α-androstano[3,2-c]pyrazol-17β-ol (2) and 17β-Methyl-5α-androstano[3,2-c]pyrazol-17α-ol (3) via Approach D ............................................................... 48
    4.2.3. 17β-Methyl-5α-androstano[3,2-c]pyrazol-17α-ol (3) and 17,17-Dimethyl-18-nor-5α-androstano[3,2-c]pyrazol-13-ene (13) ........................................................................ 50
    4.2.4. 1-Deoxy-1-(17α-hydroxy-17β-methyl-5α-androstano[3,2-c]pyraz-1-yl)-β-d-glucopyranuronic acid (9) .......................................................................................... 52
    4.2.5. 1-Deoxy-1-(17α-hydroxy-17β-methyl-5α-androstano[3,2-c]pyraz-2-yl)-β-d-glucopyranuronic acid (14) .................................................................................. 54
    4.2.6. 17β-Methyl-1’N-(4-methylphenyl-1-sulfonyl)-5α-androstano[3,2-c]pyrazol-17α-ol (16) and 17β-Methyl-2’N-(4-methylphenyl-1-sulfonyl)-5α-androstano[3,2-c]pyrazol-17α-ol (17) .................................................. 56
    4.2.7. 17-Methyl-5α-androstano[3,2-c]pyrazol-17-ene (20) and 17-Methyl-5α-androstano[3,2-c]pyrazol-16-ene (21) ........................................................................ 58
    4.2.8. Epoxidation of Products 20 and 21 (22 – 25) ................................................ 59
    4.2.9. Burgess-Elimination of Stanozolol (26 – 29) .............................................. 60
    4.2.10. Epoxidation of Products 26 – 29 (30 – 37) ................................................. 61
    4.2.11. Methyl (1,2,3,4-tetra-O-acetyl-β-d-glucopyranosid)-uronate (38) ............... 63
    4.2.12. Methyl (2,3,4-tri-O-acetyl-α-d-glucopyranosyl-1-bromide)-uronate (39) .......... 64
    4.2.13. Methyl (2,3,4-tri-O-acetyl-β-d-glucopyranosid)-uronate (40) ....................... 65
    4.2.14. Methyl [2,3,4-tri-O-acetyl-1-O-(trichloroacetimidoyl)-α-d-glucopyranosid]-uronate (41) ......................................................................................... 66
    4.2.15. 1’N-(Methyl 2,3,4-tri-O-acetyl-1-deoxy-β-d-glucuronosyluronate)-17β-methyl-5α-androstano[3,2-c]pyrazol-17α-ol (42) and 2’N-(Methyl 2,3,4-tri-O-acetyl-1-deoxy-β-d-glucuronosyluronate)-17β-methyl-5α-androstano[3,2-c]pyrazol-17α-ol (43) .......................... 67
    4.2.16. Methyl N-(triethylammoniumsulfonyl)carbamate (49) .............................. 70
5. References ............................................................................................................. 71
6. Appendix ................................................................................................................ 74
  6.1. Abbreviation .................................................................................................... 74
  6.2. Selected NMR-Spectra ..................................................................................... 78
1. Introduction

1.1. History of Doping

Doping implies the performance enhancement of athletes, goes against the rules and fair play in competitions and is known since ancient times.[1] The user of drugs usually wants to achieve a higher performance, especially to enhance strength with anabolics or to overcome fatigue using stimulants. In ancient times, it was typically an empiric knowledge of doping substances, which included to eat the organs of animals or even humans. Stimulants used during these ages were often plant based and utilized to overcome fatigue or to whitewash the pain of injuries.[2] It is known that the Berserkers of the Norse mythology took bufotein, which is a drug of the fly-agaric mushroom. Other folks like Andean Native Americans used alkaloids to push their performance by chewing or cooking tea out of coca leaves.[3]

With the upcoming modern medicine the establishment of performance enhancing drugs improved and thus they were used more frequently. In 1889, Brown-Sequard reported that self-injections of a mixture of testicular blood, semen and testicular extracts of animals refined his physical and mental energy, but after one month the effects worn off completely. This showed a reversibility in the proposed outcome, but experts considered these results as merely a Placebo-effect.[4]

As time goes by, the development of medical science increased and in 1912, the transplantation of testicular material of animals and humans has debuted. In 1935, the newly discovered testosterone replaced the old methods as it was more efficient and less invasive in use. In the following years, studies about sexual hormones confirmed the enhancement in physiological properties and the first “doping-case”, involving the horse Holloway, occurred in 1941. In the early 1950s, the excessive usage of testosterone preparations by bodybuilders emerged and it first appeared in the weight-lifting sport, because of the systematic utilization by the Soviets.[2]

In the field of sports the following users of anabolic steroids were top athletes of throwers in the mid-1960s, including world record holders and Olympic champions. Afterwards, athletes of other sports followed in the usage of steroids, since there was no ban during this period. The only debate was not about prohibition of anabolic steroids, but contained the effectiveness of
different substances. Aside from anabolic steroids, the utilization of blood doping, which means the reinfusion of oxygen-carrying red blood cells, was rising in endurance sports at the same time. After the Olympic games in 1980, the first urinary test for exogenous testosterone was developed and approximately twenty percent of the athletes were tested positive.[2]

Anabolic steroids were in the lead of all doping drugs in the 1980s, but the number of substances expanded dramatically during this time and different hormones, narcotics, stimulants, masking agents, diuretics and anabolics were in use. During the Olympic Games in Seoul in 1988, the first athlete was tested positive for stanozolol and afterwards, it was assumed that at least half of the sprint finalists were under the influence of anabolic steroids. The upcoming Olympic Games were branded for the excessive abuse of different doping agents, which led to the defamation of the Games in 1996, also known as the “Growth Hormone Games” and in 2000 known as “Dirty Games”.[4]

Rumors about nationally organized doping programs have been circulating for decades, but solid evidence came to light belated. First proof was the GDR, where since 1966 top ranked scientists did research work to enhance the performance of athletes in sports. Since the GDR was part of the Soviet Bloc, it was reasonable that this program has also been developed in the Soviet Union. Documents showed that the founding of a systematic doping program was planned since 1945. After the end of Communism in Europe, many scientists from GDR were hired for Chinese programs to found a scientific sports center. This led to the conclusion that systematic doping was happening in China as well, which was never proofed by documents, but is highly conceivable, since many positive doping tests of Chinese athletes occurred in the 1990s.[2] In 2014, the last systematic doping case appeared in media, in which Russia was accused by a documentary on the German television.[5] Afterwards, the WADA investigated on this case [6] and many Russian athletes were prohibited to start at the Olympic Games in Rio de Janeiro in 2016.[7]

Because of the uprising abuse of doping substances, the IOC founded a Medicinal Commission and created a prohibited list for substances and methods in 1967. One year afterwards, drug tests were introduced and the IOC declared the founding of the WADA at the World Conference on Doping in Sports in Lausanne (Switzerland) in 1999. The WADA was established to harmonize the Olympic Anti-Doping Code and implemented a single acceptable code for all stakeholders, which
included the creation of several international standards (IS). These ISs were developed for laboratories, testing, the prohibited list and therapeutic use exemptions.[8]

1.2. Definition and Prohibition of Doping

Since the establishment of WADA in 1999, it is an independent and internationally funded organization responsible for scientific research, education, development of anti-doping capacities and monitoring of the World Anti-Doping Code (WADC).[9] Therefore, the WADA is the most important instance for anti-doping internationally.

The World Anti-Doping Code WADC was first published in 2003, adapted in the years 2009 and 2013 and consists of rules, definitions and regulations on doping in sports. Doping is defined by WADA according to the WADC as the occurrence of one of the following points:[10]

- Presence of a Prohibited Substance or its Metabolites or Markers in an Athlete’s Sample
- Use or Attempted Use of a Prohibited Substance or Method by an Athlete
- Evading, Refusing or Failing to Submit to Sample Collection
- Whereabouts Failures
- Tampering or Attempted Tampering with any part of Doping Control
- Possession of a Prohibited Substance or Method
- Trafficking or Attempted Trafficking in any Prohibited Substance or Method
- Administration or Attempted Administration to any Athlete of any Prohibited Substance or Method
- Complicity
- Prohibited Association

WADA lists all prohibited substances and methods in a document on their website and actualizes this document every year. In the document, all prohibited substances and methods in- and out-of-competition as well as prohibited substances for selected sports are shown (see Table 1).[11]
### Table 1: Prohibited substances and methods in- and out-of-competition.[11]

<table>
<thead>
<tr>
<th>Prohibited substances (S) in- and out-of-competition (at all times)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S0</strong> Non-Approved Substances</td>
</tr>
<tr>
<td><strong>S1</strong> Anabolic Agents (AA)</td>
</tr>
</tbody>
</table>
| **S2** Peptide Hormones, Growth Factors, Related Substances and Mimetics | 1.1. dEPO, EPO, CERA, EPO-mimetics  
1.2. Cobalt, Daprodustat, Molidustat, Roxadustat, Vadadustat, Xenon (HIF activating agents)  
1.3. GATA inhibitors  
1.4. Luspatercept, Sotatercept (TGF-β signaling inhibitors)  
1.5. Asialo EPO, CEPO (Innate repair receptor agonists)  
2.1. Buserelin, gonadorelin (CG, LH and their releasing factor)  
2.2. Corticorelin (Corticotrophins and releasing factors)  
2.3. Growth Hormones  
3. Growth Factors |
| **S3** Beta-2 Agonists | Fenoterol, Formoterol, Higenamine, Indacaterol, Olodaterol, Procaterol, Reproterol, Salbutamol, Salmeterol, Terbutaline, Tretoquinol, Tulobuterol, Vilanterol, ... |
| **S4** Hormone and Metabolic Modulators | 1. Exemestane, Formestane, Letrozole (aromatase inhibitors)  
2. Bazedoxifene, Ospemifene, Raloxifene, Tamoxifen (SERMs)  
3. Clomifene, Cyclofenil (other anti-estrogenic substances)  
4. Follistatin, myostatin propeptide, Domagrozumab, Stamulumab (agents preventing activin receptor IIB activation)  
5. Activators of AMPK, insulins and insulin-mimetics, Meldonium, Trimetazidine (metabolic modulators) |
| **S5** Diuretics and Masking Agents | Desmopressin, Probenecid, plasma expanders, Acetazolamide, Amiloride, Bumetanide, Canrenone, Chlortalidone, Etacrynic acid, Furosemide, Indapamide, Metolazone, Spironolactone, Thiazides |
### Prohibited methods (M) in- and out-of-competition (at all times)

<table>
<thead>
<tr>
<th>M1</th>
<th>Manipulation of Blood and Blood Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>The Administration or reintroduction of any quantity of autologous, allogenic or heterologous blood, or red blood cell products of any origin.</td>
</tr>
<tr>
<td>2.</td>
<td>Artificially enhancing the uptake, transport or delivery of oxygen.</td>
</tr>
<tr>
<td>3.</td>
<td>Any form of intravascular manipulation of the blood or blood components by physical or chemical means.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M2</th>
<th>Chemical and Physical Manipulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Tampering, or attempting to tamper, to alter the integrity and validity of Samples collected during Doping Control.</td>
</tr>
<tr>
<td>2.</td>
<td>Intravenous infusions and/or injections of more than a total of 100 mL per 12 hour period except for those legitimately received in the course of hospital treatments, surgical procedures or clinical diagnostic investigations.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M3</th>
<th>Gene and Cell Doping</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>The use of nucleic acids or nucleic acid analogues that may alter genome sequences and/or alter gene expression.</td>
</tr>
<tr>
<td>2.</td>
<td>The use of normal or genetically modified cells.</td>
</tr>
</tbody>
</table>

### Prohibited substances (S) in-competition

<table>
<thead>
<tr>
<th>S6</th>
<th>Stimulants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrafinil, Benfluorex, Clobenzorex, Fencamine, Lisdexamfetamine, Mefenorex, Norfenfluramine, Phendimetrazine, Benzfetamine, Cathine, Dimetamfetamine, Ephedrine, Famprofazone, Heptaminol, Isomethptene, Levmetamfetamine, Meclofenoxate, Nikethamide, Octodrine,</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S7</th>
<th>Narcotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buprenorphine, Dextromoramide, Diamorphine, Fentanyl and its derivatives, Hydromorphone, Methadone, Morphine, Nicomorphine, Oxycodone, Oxymorphone, Pentazocine, Pethidine,</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S8</th>
<th>Cannabinoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>All natural and synthetic cannabinoids</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S9</th>
<th>Glucocorticoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betamethasone, Budesonide, Cortisone, Deflazacort, Dexamethasone, Fluticasone, Hydrocortisone, Methylprednisolone, Prednisolone, Prednisone, Triamcinolone</td>
<td></td>
</tr>
</tbody>
</table>

### Substances prohibited in particular sports (P)

<table>
<thead>
<tr>
<th>P1</th>
<th>Beta-Blocker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acebutolol, Alpenolol, Atenolol, Betaxolol, Bisoprolol, Bunolol, Carteolol, Carvedilol, Celiprolol, Esmolol, Labetalol, Metipranolol, Metoprolol, Nadolol, Oxprenolol, Pindolol, Propranolol, Sotalol, Timolol,</td>
<td></td>
</tr>
</tbody>
</table>
Every year, the WADA relates a detailed report about doping cases and the number of analyzed samples. The overall positive cases and the affiliations to the Prohibited List during the course of the last years are shown in Figure 1. The different substances and methods are referred to the used abbreviations in the Prohibited List (see Table 1). It is visible that the most often used category of the Prohibited List for Doping in the last few years is S1, the Anabolic Agents and therefore, it is the most important group for research workers.[12]

![Figure 1: Affiliation of positively analyzed doping cases to the categories used in the Prohibited List in the last years.][12]

1.3. Modern Methods in Doping-Analysis

Typically, WADA-correlated laboratories use urine- or blood-samples of athletes for doping-analysis, which were split in A- and B-samples. If the firstly analyzed A-samples indicate doping, B-samples will be checked as well. If both tests show doping abuse, the athlete will be punished according to the WADC.[10]

To analyze the collected samples, the accredited laboratories of the WADA have to use different methods according to the type of samples (urine or blood) and the category of substances or methods (see Figure 2).[13]
For analysis of hydrophobic substances, which are most of the used drugs and metabolites, the samples have to be extracted before carrying out measurements. Since most of the screened marker substances are conjugates (glucuronides, sulfates, see Chapter 1.4.2), the samples are hydrolyzed using suitable enzymes via required methods. Afterwards, the extracts are derivatized in some cases (for example anabolic agents via TMS-O-bonds for GC-MS-analysis) and used for the needed method.[14]

For anabolic androgenic steroids (AAS) the conjugates in urine samples are hydrolyzed via β-glucuronidase (for glucuronides) and/or arylsulfatase (for sulfates) in buffer-solution and extracted with organic solvents. Derivatization is carried out via MSTFA/TMSI-mixtures under heat, to derivatize every possible functionality (alcohol, ketone, amine, ...), to lower the boiling points of the substances and the samples were used for GC-analysis.[15] In the case of exogenous anabolic androgenic steroids (EAAS) GC-MS, GC-MS/MS for quantification or GC-IRMS for qualification can be used.[16] It is also possible to do HPLC-MS-analysis of conjugated metabolites via solid phase extraction, dilution and measurement without derivatization. Solid phase extraction is necessary, because of the high complexity of the used samples and derivatization is not required, since LC does not depend on the boiling points of metabolites.[17]
1.4. Metabolism of Steroids

Once the human body detects an exogenous substance, the organism tries to remove this molecule out of the bloodstream. For this purpose, the body transfers the substance into a form, in which it is better soluble in aqueous phase and this is called metabolism. Therefore, the organism harnesses different enzymes to generate functional groups, which enhance the water solubility of the molecules and removes the resulting metabolites via urine, sweat or excrement. Metabolism can be subdivided in two phases: Phase I (the interconversion of functional groups of the molecule) and Phase II (the conjugation of reactive head groups with more polar molecules).[18]

Both phases of the metabolism of steroids are described in the following chapters.

1.4.1. Phase I Metabolism

Phase I metabolism of steroids means the modification of functionalities of the steroidal molecule. The most common transformation possibilities are reductions, oxidations, hydrogenations and dehydrogenations.

For 3-keto-4-ene-steroids, the enzymes 5α- and 5β-reductase are of high importance, because of the hydrogenation of the C-4,5-bond, which reacts stereoselectively and yields the corresponding 5α- or 5β-steroid. Afterwards, the ketone is reduced by 3α- or 3β-hydroxysteroid dehydrogenase, forming either the 3α- or 3β-alcohol. Hydrogenation of 1,2-double bonds is carried out, if an alcohol in position 3 is available (see Scheme 1).[19]

![Scheme 1: Possible A-Ring-Metabolism of AAS.](image-url)
For 17β-hydroxy-17α-methylsteroids with hampered A-ring reduction possibilities, 6β-hydroxylation is carried out, but until now 6α-hydroxylation was not detected. Another option for B-ring metabolism is the dehydrogenation in 6,7-position, which was only observed in methandienone (see Scheme 2).[19]

![Scheme 2: Possible B-Ring-Metabolism of AAS.](image)

Metabolism of the C-ring is rarely observed, but hydroxylation in 12-position is possible and detected for some steroids, like stanozolol, with yet unknown stereochemistry in this position (see Scheme 3).[19]

![Scheme 3: Possible C-Ring-Metabolism of AAS.](image)

For 17β-hydroxysteroids, oxidation to 17-ketosteroids is a known metabolism, which is an equilibrium reaction, and the ketone can be reduced in some cases to form 17α-hydroxy-products. For 17-methyl-17-hydroxysteroids, hydroxylation in 16-position is possible and both stereomers were detected. Oxidation of 16-hydroxysteroids is observed in some cases as well. Epimerization of 17β-hydroxy-17α-methylsteroids exists, because of the conjugation with sulfates, which are not stable under aqueous conditions (see Chapter 1.4.2) (see Scheme 4).[19]
1.4.1.1. Cytochrome P450

Cytochrome P450 (CYP) is an enzyme with oxidative properties and one of the most common enzymes in the metabolism of steroids, since it converts apolar substances into more polar ones. It is a metalloenzyme, containing iron in its active site. Iron is linked to the protein by a heme-group and one side of the metal is blocked by the amino acid cysteine via a sulfide bond.[20] CYP is well-known for its occurrence in the metabolism and especially in the metabolism of steroids, where it transforms exogenous and endogenous steroids for their excretion. Therefore, the most common reaction the enzyme carries out, is the hydroxylation of C-H-bonds to modify the molecule with polar head groups (see Figure 3).[21]
As can be seen in Figure 3, the metal ion at the active site of the protein starts as Fe$^{3+}$ linked with water (A). The substrate (R-H) binds on the apolar parts of the protein and releases water (B). This step is followed by the reduction into Fe$^{2+}$ (C) and the oxidation of the iron ion by means of molecular oxygen in form of a diradical (D). Reduction and protonation of the oxygen radical yields to a peroxide structure (E'), which dehydrates to form an unstable oxide-complex (F). Since this complex is highly reactive, it oxidizes the substrate to form the corresponding alcohol and releases the product in the final step.[22]

1.4.2. Phase II Metabolism

Phase II metabolism defines the conjugation of polar head groups with more polar molecules to enhance the solubility of the substrate. In steroid metabolism, the most common groups are glucuronic acids and sulfates.[19]

3α-Hydroxysteroids are mainly conjugated with glucuronic acid, regardless of the configuration in 5-position. On the contrary, 3β-hydroxysteroids are mainly conjugated via sulfation and the sulfate is formed (see Scheme 5).[19]
Glucuronidation of secondary 17β-hydroxysteroids is well-known, but glucuronidation of tertiary 17β-hydroxy-17α-methylsteroids is not observed except for few cases. A similar picture emerges with sulfates, where secondary 17β-hydroxysteroids conjugated with sulfates are often observed (see Scheme 6). Sulfates of tertiary 17β-hydroxy-17α-methylsteroids are not detected, because of their instability in aqueous solution. They are epimerizing, eliminating or rearranging immediately as it is shown in Scheme 6.[19]
1.4.2.1. **UDP-Glucuronosyltransferase**

To link functionalities with glucuronic acid in the human body, the enzyme UDP-glucuronosyltransferase is used, which utilizes UDP-glucuronic acid as substrate, where UDP (uridine diphosphate) is bound via an α-linkage with glucuronic acid (see Figure 4).[23]

![Figure 4: UDP-glucuronic acid.](image)

Binding of UDP-glucuronic acid on UDP-glucuronosyltransferase leads to destabilization of the α-UDP-bond, which resulted in enhanced reactivity of the glucuronic acid. A nucleophile (the substrate) attacks the activated anomeric position of the glucuronic acid and the β-glucuronide is formed selectively, because of the blocked α-side of the molecule (see Scheme 7).[24]

![Scheme 7: Reaction of UDP-glucuronic acid with a nucleophile via UDP-glucuronosyltransferase.](image)
1.4.2.2. **Sulfotransferase**

Sulfotransferases are a part of enzymes, which conjugate molecules bearing nucleophilic functionalities with sulfate-groups via 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as sulfate-donor (see Figure 5).[19]

![Figure 5: 3´-Phosphoadenosine-5´-phosphosulfate.](image)

Usually, sulfotransferases can be separated in two classes in the case of steroids: estrogen sulfotransferases and hydroxysteroid sulfotransferases. Estrogens have an aromatic system at the A-ring and as the name predicts, estrogen sulfotransferases conjugate only phenolic hydroxyls and 16- or 17-hydroxyls of aromatic steroids. On the other hand, hydroxysteroid sulfotransferases have a wide substrate specificity and conjugate non phenolic hydroxyl-groups (see Scheme 8).[25]

![Scheme 8: Conjugation of a nucleophile via sulfotransferases.](scheme)

Sulfotransferases have a large variety in specificities, but the binding site of PAPS looks similar in all common enzymes. It consists of 5'-sulfophosphate-, 3'-phosphate- and PAPS-binding sites. In contrast to the PAPS-binding site, the substrate binding site is unspecific. It seems that the binding of PAPS activates the enzyme for reactivity with the substrate.[26]
1.5. Methods for chemical Glycosylations

1.5.1. Koenigs-Knorr-Glycosylation

The Koenigs-Knorr-glycosylation is one of the oldest known methods for chemical glycosylations and was first published in 1901.[27] Within this method, sugar bearing a bromide in the anomeric position and silver carbonate as activator are reacted with a corresponding nucleophile (alcohol or amine). Under these circumstances, silver ions are preferred, since the emerging silver halide precipitates during reaction and shifts the equilibrium to the product side (see Scheme 9). Later, it turned out that mercury salts are also functional as activators and show higher reactivity in some cases.[28]

![Scheme 9. Proposed Mechanism of Koenigs-Knorr-Glycosylations][28]

Since chemical glycosylations are forming mainly α-anomers by the influence of the anomeric effect and β-glycosides are privileged through the biological process, the neighboring group participation has to be used to obtain a higher ratio of β-anomers during the chemical processes. This can be achieved by usage of esters as protecting groups (PG).[28]
1.5.2. Schmidt- and Yu-Glycosylations

In 1987, Schmidt et al. [29] described a glycosylation method using trichloroimidates at the anomeric position as leaving group and Lewis acids for activation. In comparison to the Koenigs-Knorr-method, these glycosylation conditions are more advantageous as the leaving group is cleaved as trichloroacetamide, which makes the reaction irreversible and pushes the formation of product. Mechanistically, the Lewis acid activates the nitrogen of the imidate, which is cleaved and forms the positive charge at the anomeric position. The positively charged anomeric center can now be attacked by a nucleophile and forms the glycosidic bond (see Scheme 10).[28]

Scheme 10: Proposed Mechanism of Schmidt-Glycosylations.[28]

In 2008, Yu et al. [30] developed a new glycosylation method working via activation of an alkyne under gold catalysis and nucleophilic attack of the anomeric position. Similar to the Schmidt-glycosylation, this method activates sugars through an irreversible cleavage of the leaving group, which shifts the equilibrium of the reaction to the product side. Gold (I) activates the triple bond and triggers the formation of a lactone to give a positive charge in the anomeric position. The nucleophile can now attack the sugar forming a glycosidic bond and the remaining proton releases the gold catalyst (see Scheme 11).
1.6. Stanozolol

Stanozolol was first synthesized in 1959 by Clinton et al. [31] from oxymetholone (1) via condensation with hydrazine hydrate (see Scheme 12) [32] and was produced for its possible higher hormonal activity compared to the parent steroid and higher predicted anabolic/androgenic-effect-ratios.

Since its development, stanozolol is widely spread as an anabolic androgenic steroid for doping in sports. Until today, it is one of the most often used anabolic agents and the most often detected anabolic steroid in doping (see Figure 6).[12]
Detection of metabolites of stanozolol is similar to the description in Chapter 1.3 and is usually carried out via silylation reactions and GC-MS-analysis for unconjugated metabolites, which was first done by Massé et al. [33] in 1989, or direct HPLC-MS-analysis of conjugated metabolites.[34] Using these methods, Figure 7 shows the predicted possibilities for the metabolism of stanozolol (since too many metabolites are possible, only the reactive parts are marked with arrows).[35]

These metabolism pathways lead to the following summary of major metabolites:[14]
As it was discussed in Chapter 1.4, the consequence of sulfate hydrolysis is epimerization in 17-position and hydroxylation reactions were carried out in the human body through cytochrome P450 and oxygen as oxidizing agent.

Conjugation of metabolites with glucuronic acid or sulfate is generally possible with every hydroxyl-group and the N-positions of the pyrazole-ring, which leads to a plethora of potential phase II metabolites.[35] Therefore, some examples of phase II metabolites of stanozolol are shown in Figure 9.[36, 37]
1.7. Target Molecule and Task

Stanozolol is one of the mostly used anabolic agents and therefore, one of the most required substances in doping-control, which makes the necessity of reference metabolites for this molecule consistently high.[14] Since a few years ago, conjugated metabolites are of special interest, since the majority of tested marker substances in urine are in their conjugated form, either with glucuronic acid or sulfate.[35]

For that reason, the following metabolites were chosen as target molecules to further investigate the natural conjugation of 17-epistanozolol (3) and to have new long-term reference materials for doping-analysis (see Figure 10). Especially 17-epistanozolol-1’N-glucuronide (9) is of importance, since it is believed to be the naturally occurring conjugate of 17-epistanozolol (3).[38] 17-Epistanozolol-2’N-glucuronide (14) was chosen, because of scientific interest to compare these two molecules by means of their analytical data and to further investigate the natural occurrence of this substance.

![Figure 10: Target Molecules of this thesis. 9) 17-epistanozolol-1’N-glucuronide, 14) 17-epistanozolol-2’N-glucuronide.](image)

1.8. State of the Art

As mentioned before, Massé et al. [33] performed urinary studies on the metabolism of stanozolol in 1989 and detected 17-epistanozolol as possible new metabolite for this doping substance. The plausible metabolism pathway for the epimerization of 17β-hydroxy-17α-methylsteroids was also predicted in this case, because of the occurrence of corresponding eliminated and rearranged molecules. In accordance to the natural metabolism via conjugation of the 17β-hydroxyl group with sulfate, the first synthesis of 17-epistanozolol was carried out using this process in 1992.[39, 40]
Since only 2 – 10% of the detected metabolites were unconjugated, [33] the interest in conjugated metabolites of stanozolol is still on the uprise. In 2013, Schänzer et al. [37] performed studies on the conjugation of stanozolol metabolites and detected stanozolol-\(N\)-, stanozolol-\(O\)- and 17-epistanozolol-\(N\)-glucuronides as possible new marker substances in doping control. Synthesis of these substances using Koenigs-Knorr-conditions with silver carbonate showed easier availability of \(N\)-glucuronides, which makes them more valuable as potential reference materials in doping control. In 2015, Schänzer et al. [38] discovered the natural occurrence of stanozolol-1\(^{`}\)\(N\)- and stanozolol-2\(^{`}\)\(N\)-glucuronides in human urine, but only the 1\(^{`}\)\(N\)-glucuronide in the case of 17-epistanozolol was detected. This concluded in the 1\(^{`}\)\(N\)-glucuronide as the only naturally formed conjugate of 17-epistanozolol, therefore further examinations of different possible glucuronides have to be carried out.

1.8.1. Synthesis of 17-Epistanozolol

In 1992, Schänzer et al. [40] and Másse et al. [39] described methods to epimerize tertiary alcohols in position 17 of anabolic androgenic steroids separately by mimicking the biological process of the 17-epimerization. Therefore, both used a sulfation reagent to form the sulfate in 17 position, which is then hydrolyzed creating the 17-epimer as product. (see Scheme 13)
In the case of stanozolol (2), these simple and cheap methods lead to the formation of 17-epistanozolol (3) and the rearranged and eliminated product of stanozolol (13), which is formed via Wagner-Meerwein-rearrangement.

1.8.2. Synthesis of Glucuronides
Schänzer et al. [37] mentioned the synthesis of N-glucuronides of stanozolol using silver carbonate and Koenigs-Knorr-donor (39) for the glucuronidation reaction in 2013. Unfortunately, we were not able to reproduce the synthesis in our laboratory. In 2019, our group performed the synthesis of stanozolol-N-glucuronides (11, 12) with mercury salts and Koenigs-Knorr-donor (39), which yielded in a separable mixture of 1´N- and 2´N-glucuronide.[41] Comparison of the synthesized glucuronides with urinary metabolites showed mainly the formation of stanozolol-1´N-glucuronide (11) in the human body. Syntheses of 17-epistanozolol-N-glucuronides were not reported yet, but the 17-epistanozolol-1´N-glucuronide (9) is believed to be a secondary long-term metabolite of stanozolol with a detectability of nearly one month.[37]
2. Results and Discussion

2.1. Retrosynthetic Analysis

Scheme 14: Retrosynthetic Analysis of Target Molecule (9).

The first logical disconnection in target molecule 9 is between the anomeric carbon of glucuronic acid and the aromatic nitrogen of the pyrazole-ring. This leads to two molecules, 17-epistanozolol (3) and a fully protected form of glucuronic acid with a proper leaving group (LG) in the anomeric position depending on different glucuronidation-methods. The protecting groups (PG) have to be stable under the conditions of the glycosylations, easily introduced and cleaved without breaking the glycosidic bond. Acetyl was used as protecting group, because of its participating effect and easy accessibility. 17-Epistanozolol (3) can be synthesized from stanozolol (2) via inversion of the stereocenter in 17-position (see Chapter 2.2). The protected form of glucuronic acid can be obtained from glucuronolactone (15), which is commercially available (see Chapter 2.3).
2.2. Synthesis of 17-Epistanozolol

Scheme 15: Synthesis of 17-Epistanozolol (3). Approach A: red, Approach B: blue, Approach C: purple, Approach D: green, a) TosCl, 2,6-lutidine, DCM b) Ph₃P, DIAD, p-NBA, toluene, 80 °C c) POCI₃, pyridine, 50 °C d) m-CPBA, CHCl₃, pH=6.88 buffer, 0 °C e) LiAlH₄, THF, 60 °C f) SO₂-pyridine-complex, DMF, H₂O, K₂CO₃ g) burgess-reagent, toluene, 70 °C h) m-CPBA, CHCl₃, pH=6.88 buffer, 0 °C i) LiAlH₄, THF, 60 °C.
For the synthesis of 17-epistanozolol (3), different methods were explored, as depicted in Scheme 15: Approach A: sulfation of the tertiary alcohol and hydrolysis of the formed sulfate, Approach B: tosylation of the aromatic system and Mitsunobu-reaction of the tertiary alcohol, Approach C + D: dehydration of the tertiary alcohol, epoxidation of the formed alkenes and reduction of the obtained epoxides.

### 2.2.1. Approach A

![Scheme 16: Approach A for the Synthesis of 17-Epistanozolol (3).](image)

Epimerization was carried out according to literature, [40] using sulfur trioxide-pyridine-complex in DMF for sulfation and potassium carbonate in water for hydrolysis (see Scheme 16). This simple two-step, one-pot reaction yielded the desired product in 23% after purification. Formation of 17-epistanozolol (3) was confirmed by $^1$H-NMR-spectroscopy and comparison with literature (methyl-groups: 0.70, 0.75 and 1.20 ppm).[39] Eliminated fractions after column chromatography consist of substances 13, 20 and 21 in 49% yield with the rearranged molecule 13 as the major product in this reaction (13:20:21 = 10:1:1). This was confirmed through NMR-measurements and concise signals of the two methyl-groups in 17-position (0.97 ppm, 6H). Furthermore, the formation of a mixture of products 13, 20 and 21 was confirmed by TLC ($R_f = 0.35$ (ethyl acetate)) and MS-analysis ($m/z = 311$). Problems of this reaction are low yields (23%) and the low concentration during hydrolysis (1 mg/mL), which results in the practical work to be more challenging.
2.2.2. Approach B

Tosylation of stanozolol (2) was carried out according to literature with TosCl and 2,6-lutidine as base (see Scheme 17).[42] This reaction led in an overall yield of 99% to a mixture of products 16 and 17 in a ratio of 1:2. Both products were separated and purified by column chromatography, yielding 33% of 16 and 66% of 17. Tosylation took place on the pyrazole-ring exclusively, which was confirmed through $^1$H-NMR-experiments by the integrals of the aromatic signals and MS-analysis (m/z = 483). Initially, the tosylation was planned as an elimination-method of the tertiary alcohol, which was discarded due to the low reactivity of tertiary hydroxyl functionalities. This led to the conclusion that the tertiary alcohol is too sterically hindered for tosylation-reactions, but the N-tosylates are usable as stable protecting groups for other reactions.

Mitsunobu-reactions of substances 16 and 17 were carried out according to literature with DIAD, Ph$_3$P and $p$-NBA (see Scheme 17).[43] This reaction is well-known and widely used in synthetic chemistry, but unfortunately not suitable in this case, where the tertiary alcohol seems too sterically hindered. Also, the use of higher temperatures and higher amounts of reactants during reaction had no influence on the outcome of the experiments. Screenings with other azo-species, phosphines, or acids were not carried out, because of the working epimerization procedure via sulfation and hydrolysis (Approach A).
2.2.3. Approach C and D

2.2.3.1. Dehydration of Stanozolol

Since acidic conditions like phosphoric acid are inappropriate in this case due to the rearrangement problem, two different methods for the elimination of the tertiary alcohol were chosen. Phosphoryl chloride was used, because of its easy and cheap access and simple methodology, whereas burgess-reagent (49) was implemented for the simple synthesis of the reagent and its better exo:endo-ratio compared to phosphoryl chloride. An overview of the used reagents and methods for the dehydration reactions is depicted in Scheme 18 as well as the obtained products.
Synthesis of substances 20 and 21 was carried out according to literature with phosphoryl chloride and pyridine (see Scheme 18).[44] The reaction was constantly heated up to 50 °C to ensure stable conditions over the needed reaction time. After purification via column chromatography a yield of 49% as a mixture of products in a ratio of 20:21=54:46 (signals of double bonds: 20: 4.64 ppm, 21: 5.28 ppm) was obtained, which was confirmed by \(^1\)H-NMR-spectroscopy. Appearance of a dehydration reaction was also proven by MS-analysis (m/z = 311) and TLC (R<sub>f</sub> = 0.35 (ethyl acetate)). Separation of the dehydration products was not successful due to the similar polarity of the products and the strong influence of the pyrazole-ring on polarity.

Synthesis of substances 26 – 29 was carried out according to literature using burgess-reagent (49) in toluene (see Scheme 18).[45] Product formation was proven via NMR- and HPLC-MS-experiments, where the signals of the formed double bonds showed similar shifts like products 20 and 21 in \(^1\)H-NMR. Differences in polarity and mass compared to the POCl<sub>3</sub>-products verified reaction of the heterocyclic nitrogens of the aromatic system. This was confirmed through \(^1\)H-NMR, where a shift of 7.66 ppm for position 3′ proved reaction of the aromatic nitrogen, but no structural information of the rest was obtained. After analysis of MS-spectra and the fragmentation during these experiments as well as all NMR-spectra, the most likely reaction was the binding of burgess-reagent directly on the nitrogen, like it is shown in Scheme 18. Protection of the heterocyclic nitrogens was not carried out in this case, because it was believed that reductive conditions cleave this group as well (see Chapter 2.2.3.3). This reaction yielded to 86% of an inseparable mixture (26:27:28:29 = 2:1:4:2), which was confirmed using NMR (alkenes: 4.63, 5.27 ppm, methyl of Burgess-rest: 3.58, 3.61 ppm).
2.2.3.2. Epoxidation of Alkenes

Instead of direct oxymercuration, epoxidation and reduction were chosen, because of the directing effect of the methyl-group in position 13 of the steroid. This methyl-group is shielding the upper side (β) of the molecule, which enhances the ratio of α:β-epoxides in the case of epoxidations, but lower this ratio in the case of oxymercurations due to the differences in the mechanism. Epoxidations were carried out according to literature using m-CPBA as oxidizing agent in CHCl₃/buffer-solution (see Scheme 19).[46] Since N-oxide-formation could lead to certain issues, this mild epoxidation-method at 0 °C was used. Both reactions were yielding with 88% to an inseparable mixture of epoxides, which were subjected to the reduction steps as mixtures. To proof epoxide-formation and completion of the reaction, HPLC-MS-analysis resulted in conclusive signals (m/z = 327 for the products) swiftly, but was not successful in defining the ratios of the
formed products. To define the ratios of the different products, \(^1\)H-NMR-spectroscopy turned out to be successful.

Investigation of the NMR-spectra of the mixture of substances \(22 \rightarrow 25\) resulted in ratios of \(22:23 = 4:1\) and \(24:25 = 3:2\), which confirmed the major formation of \(\alpha\)-epoxides (22 and 24) and the influence of the methyl-group in position 13 on epoxide-formation.

The mixture of substances \(30 \rightarrow 37\) showed high complexity in \(^1\)H-NMR, which made assignment of spectra and differentiation of signals not feasible for this mixture of diastereomers. Since comparable ratios of diastereomers between the epoxides \(30 \rightarrow 37\) and \(22 \rightarrow 25\) are reasonable, an overall ratio of \(\alpha:\beta = 11:4\) (30 – 37) was assumed.

\[\text{2.2.3.3. Reduction of Epoxides} \]

\[\text{Scheme 20: Reduction of synthesized Epoxides (22-25, 30-37).}\]
Reductions were carried out according to literature using LiAlH₄ in THF (see Scheme 20).[47] Instead of TLC, the conversion was detected through HPLC-MS (starting material: m/z = 327, product: m/z = 329), since epoxides and products showed similarity in the Rf-values. The ratios between the products 2 and 3 were calculated using the integrals in ¹H NMR of the methyl groups at 0.76 and 0.88 ppm in the case of stanozolol (2) and 0.70 and 0.75 ppm in the case of 17-epistanozolol (3). The products were obtained as inseparable mixtures in 55% (Approach C) and 56% (Approach D) yields as depicted in Scheme 20. According to the mechanism of this reaction, the α-epoxides are yielding to 17-epistanozolol (3), which means that after reduction a better α:β-ratio of epoxides leads to a higher 3:2-ratio.

In the case of the reduction of substances 22 – 25, the ratio of 17-epistanozolol:stanozolol was 7:3 with 55% yield after workup and column chromatography. In the case of substances 30 – 37, the ratio of 17-epistanozolol:stanozolol was 11:4 with 56% yield after workup and purification. Since approach C and D required two more steps compared to approach A and resulted in similar overall yields and the separation of stanozolol (2) and 17-epistanozolol (3) was not possible by the methods available, the approaches C and D were discarded.
2.3. Synthesis of Glycosyl-Donor-Molecules

For the synthesis of glucuronides, it was necessary to have access to suitable glycosyl-donors depending on the method of glycosylation. Therefore, donor-molecules 39 and 41 were chosen, because of their simple synthesis and the used glucuronidation methods. An overview of the synthesized molecules and the required reaction conditions is shown in Scheme 21.

2.3.1. Acetyl-Protection of Glucuronolactone

Synthesis of substance 38 was carried out according to literature and is depicted in Scheme 22.[48, 49] Sodium methoxide, which was prepared by addition of sodium to methanol, worked to open the glucuronolactone (15) as methyl ester. Sodium instead of sodium methoxide was used due to its higher purity compared to the existing sodium methoxide in the laboratory. After evaporation of the reaction mixture, the crystallization in Et₂O was selected for purification, since the synthesis was accomplished in g-scale and preparative chromatography was inappropriate.
After workup, $^1$H-NMR exposed both isomers ($\alpha$: 6.37 ppm, 3.7 Hz, $\beta$: 5.75 ppm, 7.8 Hz) in a ratio of $\alpha$:$\beta = 1:1.2$ (45:55) in the crude mixture, but only the $\beta$-isomer (38) crystallized from the crude material by this method. Recrystallization attempts of the remaining residue were not successful, because of the good solubility of 38 in pyridine, which remained in the crude mixture after evaporation of the volatile components. This led to a yield of 34% of pure brownish crystals, containing $\beta$-isomer (38) exclusively.

2.3.2. Koenigs-Knorr-Donor


Synthesis of substance 39 was conducted according to literature and is shown in Scheme 23.[49] Crystallization done twice in ethanol led to 70% of pure $\alpha$-isomer, which was confirmed through $^1$H-NMR-spectroscopy ($J = 4.0$ Hz). Formation of only $\alpha$-anomer during this reaction is in consensus with literature.[13]

2.3.3. Schmidt-Donor

Scheme 24: Synthesis of Schmidt-Donor-Molecule (41).

Synthesis of substances 40 and 41 is shown in Scheme 24 and was carried out according to literature.[48] After chromatographic purification, the product 40 was obtained in 60% yield as mixture of anomers and was directly subjected to synthesis of substance 41.

Synthesis of molecule 41 provided only $\alpha$-anomer in 52% yield after purification, which was confirmed by $^1$H-NMR (6.64 ppm, 3.6 Hz) and is in consensus with literature.[48]
2.4. Synthesis of \( N \)-Glucuronides

Synthesis of the glucuronides 42 and 43 is depicted in Scheme 25. For glucuronidation-reactions a glycosyl-donor and an activator are required and proper reaction conditions were examined through small-scale testing reactions using different conditions. The conversion of starting material was determined via TLC and/or HPLC-MS and the formation of products was detected through \( ^1\)H-NMR-spectroscopy. An overview of the results of the screenings is presented in Table 2.

Table 2: Overview of the used Methods of Glucuronidation and the used Conditions.

<table>
<thead>
<tr>
<th>Activator</th>
<th>Conditions</th>
<th>Glycosyl-Donor</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Ph}_3\text{P} \text{AuNTf}_2 )</td>
<td>DCM, rt</td>
<td>46</td>
<td>No conversion</td>
</tr>
<tr>
<td>( \text{BF}_3\text{-OEt}_2 )</td>
<td>DCM, -20 °C to 0 °C</td>
<td>41</td>
<td>Mainly rearrangement (13)</td>
</tr>
<tr>
<td>TMSOTf</td>
<td>DCM, -70 °C</td>
<td>41</td>
<td>Rearrangement (13)</td>
</tr>
<tr>
<td>TMSOTf</td>
<td>DCM, -70 °C, DTBP</td>
<td>41</td>
<td>Rearrangement (13)</td>
</tr>
<tr>
<td>( \text{Hg(CN)}_2 ) \text{NO}_2\text{Me:PhH (1:1), 70 °C}</td>
<td>39</td>
<td>Mixture of orthoesters (44, 45) and products (42, 43)</td>
<td></td>
</tr>
<tr>
<td>( \text{Hg(CN)}_2, \text{HgBr}_2 )</td>
<td>( \text{NO}_2\text{Me:PhH (1:1), rt } \rightarrow \text{ 60 °C} )</td>
<td>39</td>
<td>Only orthoesters (44, 45)</td>
</tr>
<tr>
<td>( \text{Hg(CN)}_2, \text{HgBr}_2 )</td>
<td>( \text{NO}_2\text{Me:PhH (1:1), 70 °C} )</td>
<td>39</td>
<td>35% Yield of 42 and 43</td>
</tr>
</tbody>
</table>
Esters as protecting groups of the sugar are essential for this thesis, because of the necessity of β-products. These esters are troubling during glucuronidation-reactions, as can be seen in Table 2, since the formation of orthoesters (44 and 45, Figure 11) is a common problem and made optimization of the methods necessary.

![Figure 11: Orthoester products of 17-Epistanozolol.](image)

2.4.1. Koenigs-Knorr-Glycosylation

Since the glycosylation of stanozolol proposed by Schänzer et al. [38] turned out to be not functional in former studies of our laboratory, a method using mercury salts instead of silver salts was attempted, since a complex of pyrazole and mercury was expected in this case, which then activates the sugar bromide (39).[50] Glycosylation was carried out according to literature (see Scheme 26), [41] yielding to 35% of glucuronides (42: 10%, 43: 25%) and 40% recovery of 17-epistanozolol (3) after chromatographic separation and purification. The usage of column
chromatography with two different eluent systems led to the successful separation of the three major products (3, 42 and 43) in sufficient yields. To further enhance the purity of the glucuronide fractions, preparative HPLC-MS with RP-column can be advantageous (flow: 30 mL/min, eluent: MeOH + 0.1% FA 70 → 95%, H₂O + 0.1% FA 30 → 5%, m/z = 645).

To determine the structure of the obtained glucuronides, NMR-spectroscopy was chosen. The regiochemistry of the products was verified by the coupling of the anomeric proton (most shifted doublet, 1´N: 5.43 ppm, 2´N: 5.50 ppm) with a nearby carbon-atom (1´N: 138.5 ppm of carbon 3, 2´N: 126.0 ppm of carbon 3´) in HMBC-experiments. The stereochemistry of the anomeric position was proven by the coupling constants (1´N: 9.6 Hz, 2´N: 9.2 Hz) shown in ¹H-NMR, since β-glucuronides have coupling constants between 8 and 10 Hz.

As mentioned before, other methods for Koenigs-Knorr-glycosylation led to formation of orthoesters (44 and 45) as major products, which was confirmed by ¹H-NMR-spectroscopy (anomeric proton: 5.98 ppm, 4.8 Hz).

To clarify the occurrence of orthoesters in other methods using sugar bromide 39, the kinetics and thermodynamics as well as the equilibrium of this reaction have to be explained.[28] Basically, the orthoester can be considered as kinetic product and the glucuronide as a thermodynamic one, which is in consensus with the obtained data, since orthoester formation was observed at lower temperatures. The necessity to know the equilibrium can be perceived in experiments without HgBr₂, as this salt is the product of the reaction and shifts the reaction to the side of starting material. Absence of mercury bromide yields to unfavored orthoester, because the reaction cannot equilibrate and form the thermodynamic product.
2.4.2. Schmidt-Glycosylation

Schmidt-glucuronidation reactions were accomplished according to literature with BF$_3$·OEt$_3$ or TMSOTf as Lewis acids. [51, 52] Reactions were controlled through TLC and HPLC-MS and the formed products were analyzed using NMR-spectroscopy. Looking at the obtained MS-spectra during reaction, TMSOTf seems to be too acidic, since only the rearranged product 13 (m/z = 311) and the corresponding glucuronides (m/z = 627) were observed. Similarly, experiments with DTBP as additional base were yielding to rearrangement. Boron trifluoride as activator with lower acidity was also yielding to mainly rearranged substance 13, whereas conversion of the reaction is much lower compared to activation by TMSOTf (95% with TMSOTf, 30% with BF$_3$·OEt$_2$ according to HPLC-MS).

These experiments showed that Schmidt-glycosylation conditions are not practical in the case of androgens bearing tertiary alcohols in 17 position, because of the acidic reaction conditions, the possible elimination of the alcohol and the rearrangement problem. Since signals with m/z = 627 were found, it is reasonable that glycosylations of the heterocyclic nitrogens occurred, but there is no solid proof except of the mass available, since it was not possible to purify the products and observe proper NMR-spectra.

2.4.3. Yu-Glycosylation

Yu-Glycosylation was carried out according to literature by activation of 46 (see Figure 12) with Ph$_3$PAuNTf$_2$ as catalyst.[30] Unfortunately, no reaction took place due to different reasons. One reason is the possible complexation of gold at the pyrazole of 17-epistanozolol, which would stop the activation of sugar 46. Another eventuality is the different reactivity and nucleophilic property of aromatic nitrogen compared to alcoholic oxygen.
Cleavage of the protecting groups of the sugar was carried out according to literature through lithium hydroxide as base (see Scheme 27).[41] Since TLC of the products (9 and 14) was not possible due to the high polarity, the conversion of the reaction was controlled with HPLC-MS (starting material: m/z = 645, product: m/z = 505) and the consecutive cleavage of acetyl-groups was observed during the reaction. Since the experiments were conducted in HPLC-grade solvents, the mixtures were directly subjected to preparative HPLC (flow: 30 mL/min, m/z = 505, MeOH + 0.1% FA:H₂O + 0.1% FA = 70:30 → 85:15) after neutralization with Amberlyst-H⁺ and concentration of the solution in vacuo.

Product 9 was obtained in 61% yield after purification, whereas product 14 was yielded in 87% after chromatography. This difference can only be explained by the slightly lower purity of starting material 42, since the reactions were carried out under identical conditions. Both substances were gained as white crystalline solid with 95% purity according to ¹H-NMR-spectroscopy.
3. Conclusion

Scheme 28: Summary of the Synthesis of target molecules 9 and 14.
Synthesis of 17-epistanozolol (3) was accomplished via sulfation and hydrolysis of stanozolol (2) under basic conditions in a two-step, one-pot reaction in 23% yield. Unfortunately, this reaction was unselective and high amounts of byproduct 13 were formed (49% yield). Other routes through dehydration, epoxidation and reduction revealed to be higher in yielding (overall yields after 3 steps: POCl₃: 24%, Burgess: 56%), but the challenging separation of the obtained products made these routes unsuitable for the synthesis of 17-epistanozolol.

Synthesis of the protected N-glucuronides was carried out via Koenigs-Knorr-conditions using mercury salts for activation, which yielded with 35% to a mixture of glucuronides and 40% of starting material was recovered. After separation and purification 1’N-glucuronide (10% yield) and 2´N-glucuronide (25% yield) were obtained in sufficient purities and were subjected to the deprotection to form the final target molecules. Other experiments using Schmidt- or Yu-conditions for glucuronidation were not operable, because of the potential rearrangement during Schmidt-glycosylation and the conceivable complexation during Yu-glycosylation.

Deprotection of glucuronides under basic conditions through lithium hydroxide yielded with 61 – 87% to final target molecules (10 mg of 1’N-glucuronide, 25 mg of 2´N-glucuronide), which were handed over to Seibersdorf laboratories for analysis after characterization.

Synthesis of the target molecules was accomplished over 3 steps with an overall yield of 1% in the case of 9 and 5% in the case of 14. The required glycosylation-donor (39) was provided in 2 steps with an overall yield of 24%.

Both glucuronides proved to be the proposed metabolites, where 1´N-glucuronide is the naturally favored product and detectable for about one month in human urine after administration of stanozolol. In the future, this glucuronide can operate as practicable long-term reference material for anti-doping purposes.
4. Experimental Part

4.1. General

Chemicals and reagents:
All purchased reagents were used without further purification. HPLC-grade solvents (methanol, water, acetonitrile) were purchased from VWR. Sulfur trioxide-pyridine-complex and chlorosulfamylisocyanate were obtained from TCI, whereas MgSO$_4$ and DBU were purchased from FluoroChem. Dry dimethylformamide, dry dichloromethane and m-CPBA were purchased from Acros and deuterated solvents, like chloroform and methanol, from Eurisotop. All other chemicals were purchased from SigmaAldrich or obtained from the chemical database of TU Wien. Anhydrous tetrahydrofuran, methanol, diethyl ether and 1,4-dioxane were pre-dried using an Innovative Technologies PureSolv system.

Synthetic methods:
Reactions were carried out in flame-dried glass vessels under argon atmosphere using septa and Schlenk techniques, if not otherwise mentioned. All reactions were stirred magnetically by magnetic stirrers and magnetic bars. Chromatography was performed with glass columns filled with silica gel purchased from Merck (43 – 60 µm). Eluent mixtures were given in volumetric ratios.

Thin-layer chromatography:
TLC-analysis was performed using aluminum-backed silica plates (silica gel 60 F254, Merck). Compounds were stained by dipping in one of the following reagents: acidic phosphomolybdic acid/cerium sulfate solution (0.1 g Ce(SO$_4$)$_2$, 4.5 g phosphomolybdic acid, 100 mL H$_2$SO$_4$ (10%)), p-anise aldehyde/ethanol (20 mL p-anise aldehyde, 15 mL AcOH, 50 mL conc. H$_2$SO$_4$, 1 L EtOH) and basic potassium permanganate (2 g KMnO$_4$, 40 g K$_2$CO$_3$, 1 g NaOH, 320 mL H$_2$O). Subsequently, heating with a hot-air gun was carried out to stain the compounds.
High-pressure liquid chromatography (HPLC):

HPLC was carried out using an Autopurification system from Waters with AQUIDITY QDa detector and 2998 photodiode array detector. Separation was conducted using a XSELECT CSH C18 5 µm 4.6 × 150 mm column for analytical runs and a XSELECT CSH Prep. C18 5 µm OBD 30 × 150 mm column for preparative runs.

NMR-spectroscopy:

NMR-spectra were recorded on Bruker Avance 200, 400 or 600 at 200, 400 or 600 MHz. Chemical shifts are given in ppm and were referenced to the solvent peaks.[53] Coupling constants are given in Hz. Multiplets are given as abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, dd = doublet of doublets, m = multiplet. NMR files were processed using TopSpin 4.0.9 and MestreNova 14.1.1.

IR, melting point and specific rotation:

IR spectra were analyzed on a PerkinElmer Spectrum 65 using ATR-FT-IR-techniques and the processing was carried out with Spectragryph as program. Specific rotations ([α]D20) were detected by a MCP 500 polarimeter from Anton-Paar at 20 °C and 589 nm. Melting points were determined with a Leica Galen III Kofler hot-stage apparatus.

High resolution MS:

HRMS-analysis was carried out on an Agilent 6545 Q-TOF mass spectrometer equipped with an Agilent Dual AJS ESI Ion Source. The liquid chromatography system contained of an Agilent G7167B multi sampler, an Agilent G7120A binary pump with degasser and an Agilent G7116B oven. A precolumn from Phenomenex as stationary phase and 70% B (A: water + 0.1% formic acid, B: acetonitrile + 0.1% formic acid) isocratic for 1 min as mobile phase were used. The column temperature (30 °C), the flow rate (0.3 ml/min) and the injection volume (1 µl) were set as depicted in brackets. Nitrogen worked as drying gas (6 l/min, 250 °C) and steam gas (12 l/min, 200 °C) and the nebulizer pressure was adjusted with 60 psi. The capillary voltage (3.5 kV), fragmentor voltage (dual mode, 120 V, 180 V) and skimmer voltage (60 V) were fixed as described in brackets.
4.2. Synthetic Work

4.2.1. 17α-Methyl-5α-androstano[3,2-c]pyrazol-17β-ol (2) and 17β-Methyl-5α-androstano[3,2-c]pyrazol-17α-ol (3) via Approach C

Epoxidation products (22, 23, 24, 25, 16 mg, 0.049 mmol, 1 eq.) were dissolved in dry THF (1.5 mL) and one portion of LiAlH₄ (10 mg, 0.245 mmol, 5 eq.) was added to the solution. The grey suspension was heated up to 60 °C with an oil bath.

The reaction was cooled to rt after 6 d and quenched by slow addition of water (0.5 mL), until no reaction with the remaining LiAlH₄ took place. The mixture was diluted with Et₂O (5 mL) and dried over MgSO₄. The organic solvent was removed in vacuo, yielding to 21 mg of crude material.

Purification was carried out using column chromatography (3 g silica, LP:EA= 1:3, 2 mL fraction size) and the fractions containing products (5 – 17) were evaporated under reduced pressure, leading to 9 mg (55%) as a mixture of products 2 and 3.

Yield: 9 mg (55% yield, 2:3 = 3:7) of white crystalline solid.

TLC: Rᵣ = 0.27 (hexane:ethyl acetate + methanol = 1:1+1%).

¹H NMR (600 MHz, CDCl₃): δ = 0.70 (s, 3H) for 3 (2.1H), 0.75 (s, 3H) for 3 (2.3H), 0.76 (s, 3H) for 2 (1.1H), 0.80 – 0.86 (m, 1H), 0.88 (s, 3H) for 2 (1.2H), 0.89 – 0.95 (m, 1H), 0.97 – 1.04 (m, 1H) for 3 (0.8H), 1.13 – 1.19 (m, 1H), 1.20 (s, 3H) for 3 (2.5H), 1.23 (s, 3H) for 2 (1.3H), 1.24 – 1.50 (m, 6H), 1.52 – 1.77 (m, 6H), 1.79 – 1.90 (m, 1H), 2.09 – 2.15 (m, 1H), 2.22 – 2.33 (m, 1H), 2.54 – 2.67 (m, 2H), 7.28 (s, 1H) ppm.
4.2.2. 17α-Methyl-5α-androstano[3,2-c]pyrazol-17β-ol (2) and 17β-Methyl-5α-androstano[3,2-c]pyrazol-17α-ol (3) via Approach D

Epoxidation products (30, 31, 32, 33, 34, 35, 36, 37, 15 mg, 0.032 mmol, 1 eq.) were dissolved in dry THF (1.5 mL) under inert atmosphere and LiAlH$_4$ (6 mg, 0.162 mmol, 5 eq.) was added under stirring. The mixture was heated up to 60 °C through an oil bath. Water (0.4 mL) was added slowly to the solution after 5 d, until no reaction with remaining LiAlH$_4$ took place and the mixture was diluted with Et$_2$O (5 mL). The organic phase was dried over MgSO$_4$ and evaporated under reduced pressure, yielding to 18 mg of crude material.

Purification was conducted via column chromatography (2 g silica, DCM:MeOH = 20:1, 2 mL fraction size). Product containing fractions (3 – 7) were evaporated in vacuo, which gave 6 mg (56%) as a mixture of products (2 and 3).
Yield: 6 mg (56% yield, 2:3 = 4:11) as white crystals.

TLC: $R_f = 0.27$ (hexane:ethyl acetate + methanol = 1:1+1%).

$^1H$ NMR (600 MHz, CDCl$_3$): $\delta = 0.70$ (s, 3H) for 3 (2.2H), 0.75 (s, 3H) for 3 (2.3H), 0.76 (s, 3H) for 2 (1.0H), 0.80 – 0.86 (m, 1H), 0.88 (s, 3H) for 2 (1.1H), 0.89 – 0.95 (m, 1H), 0.97 – 1.04 (m, 1H) for 3 (0.9H), 1.13 – 1.19 (m, 1H), 1.20 (s, 3H) for 3 (2.6H), 1.23 (s, 3H) for 2 (1.2H), 1.24 – 1.50 (m, 6H), 1.52 – 1.77 (m, 6H), 1.79 – 1.90 (m, 1H), 2.09 – 2.15 (m, 1H), 2.22 – 2.33 (m, 1H), 2.54 – 2.67 (m, 2H), 7.28 (s, 1H) ppm.
4.2.3. 17β-Methyl-5α-androstano[3,2-c]pyrazol-17α-ol (3) and 17,17-Dimethyl-18-nor-5α-androstano[3,2-c]pyrazol-13-ene (13)

Stanozolol (2, 500 mg, 1.52 mmol, 1.0 eq.) was dissolved in DMF (10 mL) and SO₃-pyridine-complex (600 mg, 3.77 mmol, 2.5 eq.) was added to the solution subsequently. A solution of K₂CO₃ (3.33 g, 24.10 mmol, 16.0 eq.) in water (500 mL) was poured into the reaction after 2 h of reaction time.

After stirring for 3 d, the reaction was extracted with Et₂O (each 100 mL) several times, dried over MgSO₄ and evaporated under reduced pressure, yielding to 641 mg of crude material.

Purification was carried out using column chromatography (65 g silica, LP:EA = 2:5, 25 mL fraction size). Fractions containing products (13: 11 – 19, 231 mg and 3: 20 – 53, 117 mg) were collected and the solvent was removed *in vacuo*. Analytical data is in accordance with literature.[39, 40]
17β-Methyl-5α-androstano[3,2-c]pyrazol-17α-ol (3):

**Yield:** 117 mg (23% yield) of white crystals.

**TLC:** $R_f = 0.27$ (hexane:ethyl acetate + methanol = 1:1+1%).

**$^1$H NMR (600 MHz, CDCl$_3$):** $\delta = 0.70$ (s, 3H), 0.75 (s, 3H), 0.84 – 0.92 (m, 1H), 1.00 (qd, $J = 13.1$, 3.7 Hz, 1H), 1.11 – 1.19 (m, 1H), 1.20 (s, 3H), 1.25 (s, 1H), 1.32 – 1.53 (m, 5H), 1.54 – 1.79 (m, 6H), 1.87 (ddd, $J = 14.4$, 11.5, 3.0 Hz, 1H), 2.13 (d, $J = 15.1$ Hz, 1H), 2.22 – 2.33 (m, 1H), 2.54 – 2.67 (m, 2H), 7.28 (s, 1H) ppm.

**$^{13}$C NMR (151 MHz, CDCl$_3$):** $\delta = 11.7$, 16.0, 20.9, 22.8, 24.1, 26.4, 29.4, 30.1, 32.0, 34.9, 36.3, 36.6, 38.5, 42.5, 46.7, 50.0, 53.8, 82.3, 115.2, 132.3, 142.5 ppm.

**IR [cm$^{-1}$]:** 3166, 2924, 2852, 1732, 1446, 1377, 1086, 837, 737.

$[\alpha]_{D}^20$: +32.42 (c = 0.99, CHCl$_3$).

HRMS: (M+H)$^+$: Calc: 329.2588, found: 329.2589.

**M.p.:** 245 – 247 °C.


**Yield:** 231 mg (49% yield) of white crystals.

**TLC:** $R_f = 0.41$ (hexane:ethyl acetate + methanol = 1:1+1%).

**$^1$H NMR (600 MHz, CDCl$_3$):** $\delta = 0.71$ (s, 3H), 0.97 (s, 3H), 0.97 (s, 3H), 1.00 – 1.12 (m, 2H), 1.20 – 1.33 (m, 1H), 1.41 (qd, $J = 13.1$, 3.8 Hz, 1H), 1.56 – 1.69 (m, 4H), 1.81 – 1.98 (m, 3H), 1.98 – 2.11 (m, 3H), 2.15 (d, $J = 14.8$ Hz, 1H), 2.22 – 2.35 (m, 2H), 2.62 (dd, $J = 16.1$, 4.9 Hz, 1H), 2.71 (d, $J = 15.1$ Hz, 1H), 7.30 (s, 1H) ppm.

**$^{13}$C NMR (151 MHz, CDCl$_3$):** $\delta = 11.4$, 22.4, 22.9, 26.5, 26.6, 26.9, 29.9, 30.0, 31.1, 34.8, 36.6, 36.9, 39.7, 42.6, 45.5, 51.8, 114.8, 132.6, 136.0, 141.5, 142.7 ppm.

**IR [cm$^{-1}$]:** 3159, 2917, 2851, 1445, 1357, 963, 795, 735.

$[\alpha]_{D}^20$: +6.80 (c = 0.98, CHCl$_3$).

HRMS: (M+H)$^+$: Calc: 311.2482, found: 311.2489.
4.2.4. 1-Deoxy-1-(17\alpha-hydroxy-17\beta-methyl-5\alpha-androstano[3,2-c]pyraz-1′-yl)-\beta-D-glucopyranuronic acid (9)

Protected glucuronide (42, 23 mg, 0.036 mmol, 1 eq.) was dissolved in MeOH (HPLC-grade, 2 mL) and water (HPLC-grade, 0.5 mL) under stirring and two portions of LiOH (2 × 1.7 mg, 0.142 mmol, 4 eq.) were added to the reaction mixture over an interval of 15 min.

After 1 h, the reaction mixture was quenched with Amberlyst-H\(^+\) (18 mg) under vigorous stirring for 15 min, until the pH-value of the solution was slightly acidic. The mixture was filtered over silica (0.2 g) and washed twice with MeOH (2 × 0.5 mL). The resulting solution was concentrated \textit{in vacuo} and the obtained clear residue was subjected to prep. HPLC.

Purification was conducted via prep. HPLC (30 mL/min, MeOH:H\(_2\)O + 0.1% FA: 70:30 \(\rightarrow\) 85:15, m/z = 505). Product containing fractions were evaporated under reduced pressure to remove MeOH from the solution. The remaining mainly aqueous residue was frozen through liquid nitrogen and lyophilized. Product 9 was obtained as 10 mg (61%) of white crystalline solid.
Yield: 10 mg (61% yield) of white crystalline solid.

\[ ^1H \text{ NMR (600 MHz, CD}_3\text{OD):} \delta = 0.72 (s, 3H), 0.76 (s, 3H), 0.84 – 0.94 (m, 1H), 0.99 – 1.10 (m, 1H), 1.17 (s, 4H), 1.25 – 1.36 (m, 1H), 1.36 – 1.52 (m, 4H), 1.52 – 1.75 (m, 6H), 1.75 – 1.91 (m, 2H), 2.11 (d, \text{J}=15.1 \text{ Hz, 1H}), 2.30 (dd, \text{J}=15.6, 12.3 \text{ Hz, 1H}), 2.58 (d, \text{J}=15.1 \text{ Hz, 1H}), 2.67 (dd, \text{J}=17.0, 4.4 \text{ Hz, 1H}), 3.56 (t, \text{J}=9.0 \text{ Hz, 1H}), 3.64 (t, \text{J}=8.5 \text{ Hz, 1H}), 3.92 (d, \text{J}=9.4 \text{ Hz, 1H}), 4.07 (t, \text{J}=8.9 \text{ Hz, 1H}), 5.16 (d, \text{J}=8.7 \text{ Hz, 1H}), 7.30 (s, 1H) \text{ ppm.} \]

\[ ^{13}C \text{ NMR (151 MHz, CD}_3\text{OD):} \delta = 11.8, 16.4, 21.9, 22.4, 25.0, 26.6, 30.4, 31.3, 33.2, 36.1, 37.55, 37.59, 39.1, 43.6, 47.8, 51.0, 55.2, 72.9, 73.1, 78.4, 78.5, 87.0, 117.1, 140.0, 140.9, 174.2 \text{ ppm.} \]

IR [cm\(^{-1}\)]: 3250, 2916, 1594, 1386, 1079, 772.

\([\alpha]^{20}_D\): +8.89 (c = 0.63, MeOH).

HRMS: (M+H): Calc: 505.2908, found: 505.2917.

M.p.: >300 °C (decomposition).
4.2.5. 1-Deoxy-1-(17α-hydroxy-17β-methyl-5α-androstano[3,2-c]pyraz-2′-yl)-β-D-glucopyranuronic acid (14)

![Scheme 33]

To a solution of protected glucuronide (43, 22 mg, 0.036 mmol, 1 eq.) in MeOH (HPLC-grade, 2 mL) and water (HPLC-grade, 0.5 mL), two portions of LiOH (2 × 1.7 mg, 0.142 mmol, 4 eq.) were added to the reaction mixture over the course of 15 min.

After 1 h of reaction time, the reaction mixture was quenched with Amberlyst-H⁺ (18 mg) under vigorous stirring for 15 min, until the pH-value of the solution was slightly acidic. The mixture was filtered over silica (0.2 g) and washed with MeOH (2 × 0.5 mL). The resulting solution was concentrated under reduced pressure and the resulting clear residue was subjected to prep. HPLC.

Purification was carried out through prep. HPLC (30 mL/min, MeOH:H₂O + 0.1% FA: 70:30 → 85:15, m/z = 505). Product containing fractions were evaporated to remove MeOH from the solution. The remaining mainly aqueous solution was frozen by liquid nitrogen and lyophilized in vacuo. Product was obtained as 15 mg (87%) of white crystalline solid.
Yield: 15 mg (87% yield) of white crystalline solid.

$^1$H NMR (600 MHz, CD$_3$OD): $\delta = 0.72$ (s, 3H), 0.78 (s, 3H), 0.84 – 0.94 (m, 1H), 1.03 (qd, $J=13.0$, 3.7 Hz, 1H), 1.13 – 1.23 (m, 4H), 1.35 – 1.51 (m, 4H), 1.52 – 1.75 (m, 7H), 1.78 (dt, $J=9.1$, 2.8 Hz, 1H), 1.86 (ddd, $J=14.3$, 11.5, 2.5 Hz, 1H), 2.12 (d, $J=15.3$ Hz, 1H), 2.20 – 2.30 (m, 1H), 2.58 (dd, $J=16.5$, 5.0 Hz, 1H), 2.68 (d, $J=15.3$ Hz, 1H), 3.54 (t, $J=9.1$ Hz, 1H), 3.65 (t, $J=9.4$ Hz, 1H), 3.91 – 3.97 (m, 2H), 5.18 (d, $J=9.1$ Hz, 1H), 7.48 (s, 1H) ppm.

$^{13}$C NMR (151 MHz, CD$_3$OD) $\delta = 11.9, 16.3, 21.9, 22.4, 25.0, 28.5, 30.6, 31.3, 33.3, 35.8, 37.4, 37.6, 39.1, 43.9, 47.8, 51.0, 55.3, 72.9, 73.0, 78.3, 78.7, 82.9, 90.2, 117.5, 129.4, 150.6, 172.9$ ppm.

IR [cm$^{-1}$]: 3354, 2923, 2853, 1607, 1450, 1378, 1092, 1031.

$[\alpha]^2_0$: +14.57 (c = 0.95, MeOH).

HRMS: (M+Na)$^+$: Calc: 527.2726, found: 527.2726.

M.p.: $>300$ °C (decomposition).
4.2.6. 17β-Methyl-1′N-(4-methylphenyl-1-sulfonyl)-5α-androstano[3,2-c]pyrazol-17α-ol (16) and 17β-Methyl-2′N-(4-methylphenyl-1-sulfonyl)-5α-androstano[3,2-c]pyrazol-17α-ol (17)

Scheme 34

Stanozolol (2, 20 mg, 0.061 mmol, 1.0 eq.) was dissolved in DCM (2 mL) and 2,6-lutidine (27.5 µl, 0.237 mmol, 4.0 eq.) was added, followed by addition of TosCl (25 mg, 0.131 mmol, 2.1 eq.) at 0 °C. The reaction mixture was allowed to heat to rt slowly.

The mixture was diluted with DCM (3 mL) and quenched with sat. NaHCO₃-solution (1 mL) after 19 h of reaction time. The solution was extracted with DCM (4 × 1 mL), dried over MgSO₄ and the solvent was removed in vacuo, yielding to 63 mg of crude material.

Purification was conducted using column chromatography (5 g silica, LP:EA = 2:1, 2 mL fraction size). Fractions containing products (17: 16-22, 16: 23-26) were evaporated under reduced pressure to obtain the pure products 16 (10 mg, 33%) and 17 (20 mg, 66%) as white crystalline solids.
17β-Methyl-1′N-(4-methylphenyl-1-sulfonyl)-5α-androstano[3,2-c]pyrazol-17α-ol (16):

**Yield:** 10 mg (33% yield) of colorless glass.

**TLC:** $R_f = 0.37$ (hexane:ethyl acetate = 1:1).

**1H NMR (600 MHz, CDCl$_3$):** $\delta = 0.68$ (s, 3H), 0.74 – 0.81 (m, 1H), 0.86 (s, 3H), 0.87 – 0.95 (m, 1H), 1.12 – 1.20 (m, 1H), 1.21 (s, 3H), 1.23 – 1.33 (m, 3H), 1.33 – 1.46 (m, 2H), 1.46 – 1.55 (m, 1H), 1.55 – 1.62 (m, 3H), 1.65 (dq, $J = 13.4$, 3.5 Hz, 1H), 1.74 (dp, $J = 9.7$, 6.3, 5.5 Hz, 2H), 1.82 (td, $J = 13.3$, 12.3, 3.5 Hz, 1H), 2.02 (d, $J = 15.6$ Hz, 1H), 2.41 (s, 3H), 2.43 – 2.49 (m, 2H), 2.97 – 3.02 (m, 1H), 7.31 (d, $J = 8.0$ Hz, 2H), 7.45 (s, 1H), 7.82 – 7.86 (m, 2H) ppm.

**13C NMR (151 MHz, CDCl$_3$):** $\delta = 11.6$, 14.0, 20.9, 21.8, 23.4, 25.9, 27.9, 29.0, 31.5, 31.7, 35.0, 36.0, 36.6, 39.1, 42.2, 45.5, 50.6, 53.8, 81.8, 119.6, 127.8, 130.1, 135.4, 141.1, 144.4, 145.4 ppm.

**IR [cm$^{-1}$]:** 3423, 2923, 2853, 1377, 1175, 1091, 735, 671, 589.

[$\alpha$]$^D_{20}$: +23.77 (c = 0.59, CHCl$_3$).

**HRMS:** (M+Na)$^+$: Calc: 505.2495, found: 505.2503.

17β-Methyl-2′N-(4-methylphenyl-1-sulfonyl)-5α-androstano[3,2-c]pyrazol-17α-ol (17):

**Yield:** 20 mg (66% yield) of colorless glass.

**TLC:** $R_f = 0.46$ (hexane:ethyl acetate = 1:1).

**1H NMR (600 MHz, CDCl$_3$):** $\delta = 0.69$ (s, 3H), 0.78 (td, $J = 12.2$, 4.2 Hz, 1H), 0.85 (s, 4H), 1.13 – 1.20 (m, 1H), 1.21 (s, 3H), 1.22 – 1.34 (m, 3H), 1.34 – 1.43 (m, 2H), 1.43 – 1.64 (m, 5H), 1.68 – 1.76 (m, 2H), 1.81 (td, $J = 13.3$, 12.3, 3.4 Hz, 1H), 2.04 (d, $J = 15.8$ Hz, 1H), 2.24 (dd, $J = 17.4$, 12.4 Hz, 1H), 2.41 (s, 3H), 2.58 – 2.66 (m, 2H), 7.30 (d, $J = 8.0$ Hz, 2H), 7.73 (s, 1H), 7.85 (d, $J = 8.4$ Hz, 2H) ppm.

**13C NMR (151 MHz, CDCl$_3$):** $\delta = 11.7$, 14.0, 20.9, 21.8, 23.4, 25.9, 27.7, 29.3, 31.5, 31.7, 34.6, 36.2, 36.7, 39.1, 42.1, 45.5, 50.7, 53.8, 81.8, 119.8, 128.0, 128.3, 130.0, 134.8, 145.4, 155.7 ppm.

**IR [cm$^{-1}$]:** 3448, 2924, 2853, 1375, 1173, 1093, 1062, 670, 588, 542.

[$\alpha$]$^D_{20}$: +27.00 (c = 1.00, CHCl$_3$).

**HRMS:** (M+Na)$^+$: Calc: 505.2495, found: 505.2505.
4.2.7. 17-Methyl-5α-androstan[3,2-c]pyrazol-17-ene (20) and 17-Methyl-5α-androstan[3,2-c]pyrazol-16-ene (21)

Scheme 35

To a solution of stanozolol (2, 60 mg, 0.18 mmol, 1.0 eq.) in pyridine (1.5 mL), POCl₃ (0.13 mL, 1.40 mmol, 7.5 eq.) was added dropwise at rt and the reaction was heated up to 50 °C afterwards. After 2 h, the reaction was cooled to rt and poured into an ice/NaHCO₃-solution mixture (15 g: 7.5 mL), which was stirred for 15 min afterwards. The mixture was extracted with DCM (4 × 5 mL), dried over MgSO₄ and evaporated under reduced pressure, giving 85 mg of crude material. Purification was carried out via column chromatography (8 g silica, LP:EA=1:3, 2 mL fraction size). Fractions containing 19 and 20 (10 – 20) were collected and the solvent was removed in vacuo, yielding to 28 mg (49%) of a mixture of 19 and 20.

Yield: 28 mg (49% yield, 20:21 = 54:46) of colorless foam.

TLC: Rᵣ = 0.41 (hexane:ethyl acetate + methanol = 1:1+1%).

¹H NMR (600 MHz, CDCl₃): δ = 0.76 (s, 3H) for 20+21, 0.77 (s, 3H) for 21 (1.4H), 0.80 (s, 3H) for 20 (1.8H), 0.82 – 0.95 (m, 2H), 0.97 – 1.18 (m, 4H), 1.19 – 1.47 (m, 8H), 1.47 – 1.63 (m, 4H), 1.63 – 1.64 (m, 3H) for 21 (2.2H), 1.65 – 1.81 (m, 3H), 1.81 – 1.98 (m, 1H), 1.98 – 2.08 (m, 1H), 2.08 – 2.19 (m, 1H), 2.19 – 2.38 (m, 1H), 2.57 – 2.66 (m, 2H), 4.61 – 4.67 (m, 2H) for 20 (1H), 5.27 – 5.29 (m, 1H) for 21 (0.4H), 7.29 (s, 1H) ppm.

HRMS: (M+H)⁺: Calc: 311.2482, found: 311.2485.
4.2.8. Epoxidation of Products 20 and 21 (22 – 25)

Dehydrated products (20, 21, 16 mg, 0.0515 mmol, 1.0 eq.) were dissolved in CHCl₃ (0.5 mL) and phosphate-buffer (pH = 6.88, 0.3 mL) and cooled down to 0 °C through an ice bath. At this temperature, m-CPBA (70%, 19 mg, 0.0771 mmol, 1.5 eq.) was added to the mixture under vigorous stirring.

The reaction was quenched with sat. NaHCO₃-solution (1 mL) and sat. Na₂S₂O₃-solution (1 mL) after 3 h and extracted with DCM (3 × 5 mL). The organic phase was dried over MgSO₄ and evaporated under reduced pressure, which gave 34 mg of crude product.

Purification was conducted via column chromatography (3 g silica, LP:EA = 1:3, 2 mL fraction size). Product containing fractions (6 – 24) were collected and the solvent was removed in vacuo, yielding to 14 mg as a mixture of products (22 - 25).


TLC: Rf = 0.32 (hexane:ethyl acetate + methanol = 1:1+1%).

¹H NMR (400 MHz, CDCl₃): δ = 0.66 (s, 3H), 0.74 (s, 3H), 0.79 (s, 3H), 0.83 (s, 3H), 0.86 – 0.99 (m, 4H), 0.99 – 1.10 (m, 1H), 1.12 – 1.22 (m, 2H), 1.28 – 1.34 (m, 1H), 1.35 (s, 3H), 1.39 – 1.49 (m, 3H), 1.48 – 1.71 (m, 6H), 1.75 – 2.01 (m, 2H), 2.07 – 2.17 (m, 1H), 2.19 – 2.34 (m, 1H), 2.52 – 2.66 (m, 2H), 2.72 (dd, J=28.1, 4.5 Hz, 2H) for 22 (0.8H), 2.91 (d, J=5.1 Hz, 1H) for 25 (0.2H), 3.19 (s, 1H) for 24 (0.5H), 7.27 (s, 1H) ppm.

HRMS: (M+H)⁺: Calc: 327.2431, found: 327.2433.
4.2.9. Burgess-Elimination of Stanozolol (26 – 29)

Stanozolol (2, 100 mg, 0.305 mmol, 1 eq.) was suspended in dry toluene (5 mL), followed by addition of burgess-reagent (49, 140 mg, 0.588 mmol, 2 eq.). The mixture was heated up to 70 °C through an oil bath and stanozolol as well as burgess-reagent were dissolved at this temperature. After 2.5 h, the reaction was cooled to rt and diluted with EA (5 mL), followed by addition of water (5 mL). The mixture was extracted with EA (4 × 5 mL), dried over MgSO₄ and evaporated under reduced pressure, yielding to 159 mg of crude product.

The crude material was subjected to column chromatography (16 g silica, LP:EA = 1:7, 10 mL fraction size). Product containing fractions (5 – 17) were collected and the solvent was removed in vacuo, which yielded to 122 mg (87%) as a mixture of products (26:27:28:29 = 2:1:4:2).

Yield: 122 mg (87% yield, 26:27:28:29 = 2:1:4:2) of white crystalline solid.

TLC: 0.25 (ethyl acetate).

¹H NMR (600 MHz, CDCl₃): δ = 0.74 (s, 3H) for 27+29 (1.1H), 0.78 (s, 3H) for 26+28 (2.2H), 0.96 (s, 3H), 0.99 – 1.15 (m, 2H), 1.15 – 1.33 (m, 6H), 1.33 – 1.57 (m, 6H), 1.62 (s, 3H) for 27+29 (1.4H), 1.68 – 1.91 (m, 4H), 1.91 – 2.16 (m, 4H), 2.17 – 2.30 (m, 1H), 2.29 – 2.54 (m, 2H), 2.56 – 2.74 (m, 1H), 2.89 – 2.99 (m, 1H), 3.58 (s, 3H) for 26+27 (2.2H), 3.61 (s, 3H) for 28+29 (3.5H), 4.63 (d, J=9.0, 2H) for 26+28 (2H), 5.24 – 5.35 (m, 1H) for 27+29 (0.4H), 7.66 (s, 1H) ppm.

HRMS: (M+Na)^+: Calc: 470.2083, found: 470.2095.
4.2.10. Epoxidation of Products 26 – 29 (30 – 37)

Dehydrated products (26 - 29, 16 mg, 0.036 mmol, 1.0 eq.) were dissolved in CHCl₃ (0.5 mL) and phosphate-buffer (pH = 6.88, 0.3 mL) and were cooled down to 0 °C through an ice bath. At this temperature, m-CPBA (70%, 13 mg, 0.053 mmol, 1.5 eq.) was added to the mixture under vigorous stirring.

The reaction was quenched with sat. NaHCO₃-solution (1 mL) and sat. Na₂S₂O₃-solution (1 mL) after 3 h and extracted with DCM (3 × 5 mL). The organic phase was dried over MgSO₄ and evaporated under reduced pressure, which gave 32 mg of crude product.

Purification was conducted via column chromatography (3 g silica, EA, 2 mL fraction size). Product containing fractions (3 – 36) were collected and evaporated under reduced pressure, yielding to 14 mg as a mixture of products (30 - 37).
Yield: 14 mg (88% yield, 30, 31, 32, 33, 34, 35, 36 and 37) as white crystalline solid.

TLC: \( R_f = 0.17 \) (ethyl acetate).

\(^1\text{H NMR (400 MHz, CDCl}_3\)): \( \delta = 0.52 – 1.06 \) (m, 11H), 1.06 – 1.70 (m, 16H), 1.70 – 2.17 (m, 4H), 2.17 – 2.52 (m, 2H), 2.52 – 2.83 (m, 2H), 2.88 – 3.04 (m, 1H), 3.15 – 3.25 (m, 1H), 3.58 (s, 3H), 7.64 (s, 1H) ppm.

HRMS: (M+Na): Calc: 486.2033, found: 486.2043.
4.2.11. Methyl (1,2,3,4-tetra-O-acetyl-β-D-glucopyranosid)-uronate (38)

Glucuronolactone (15, 15 g, 85.2 mmol, 1.00 eq.) was suspended in anh. MeOH (150 mL) at 0 °C and Na (100 mg, 4.3 mmol, 0.05 eq.) was added to the solution subsequently. The ice bath was removed after 1 h and the yellow solution was stirred at rt for another 3.5 h. Acetic acid (0.3 mL, 5.2 mmol, 0.06 eq.) was added to the brown mixture and was evaporated to dryness under reduced pressure. The residue was taken up in pyridine (50 mL), followed by slow addition of acetic anhydride (45 mL, 476 mmol, 5.60 eq.) at 0 °C, which became a black solution immediately. The mixture was allowed to reach rt and the solution was concentrated in vacuo after 16 h.

The crude material was crystallized with Et₂O (100 mL), the crystals were filtered off and washed with small amounts of Et₂O (20 mL) and 1 M HCl (10 mL). The precipitate was dried under reduced pressure to obtain 10.76 g (34%) of pure β-anomer. Analytical data is in accordance with literature.[49]

**Yield:** 10.76 g (34% yield) of brownish powder (β-anomer).

**TLC:** Rᵋ = 0.42 (hexane:ethyl acetate = 1:1).

**¹H NMR (600 MHz, CDCl₃):** δ = 2.00 (s, 3H), 2.01 (s, 6H), 2.09 (s, 3H), 3.72 (s, 3H), 4.16 (d, J = 9.7 Hz, 1H), 5.12 (dd, J = 9.1, 7.8 Hz, 1H), 5.22 (t, J = 9.5 Hz, 1H), 5.29 (t, J = 9.2 Hz, 1H), 5.75 (d, J = 7.8 Hz, 1H) ppm.

**¹³C NMR (151 MHz, CDCl₃):** δ = 20.55, 20.62, 20.65, 20.9, 53.1, 69.0, 70.2, 71.9, 73.1, 91.4, 166.9, 168.9, 169.3, 169.5, 170.0 ppm.

[α]°D : +6.81 (c = 1.00, CHCl₃).

Scheme 39

Glucuronolactone (15, 15 g, 85.2 mmol, 1.00 eq.) was suspended in anh. MeOH (150 mL) at 0 °C and Na (100 mg, 4.3 mmol, 0.05 eq.) was added to the solution subsequently. The ice bath was removed after 1 h and the yellow solution was stirred at rt for another 3.5 h. Acetic acid (0.3 mL, 5.2 mmol, 0.06 eq.) was added to the brown mixture and was evaporated to dryness under reduced pressure. The residue was taken up in pyridine (50 mL), followed by slow addition of acetic anhydride (45 mL, 476 mmol, 5.60 eq.) at 0 °C, which became a black solution immediately. The mixture was allowed to reach rt and the solution was concentrated in vacuo after 16 h.

The crude material was crystallized with Et₂O (100 mL), the crystals were filtered off and washed with small amounts of Et₂O (20 mL) and 1 M HCl (10 mL). The precipitate was dried under reduced pressure to obtain 10.76 g (34%) of pure β-anomer. Analytical data is in accordance with literature.[49]

**Yield:** 10.76 g (34% yield) of brownish powder (β-anomer).

**TLC:** Rᵋ = 0.42 (hexane:ethyl acetate = 1:1).

**¹H NMR (600 MHz, CDCl₃):** δ = 2.00 (s, 3H), 2.01 (s, 6H), 2.09 (s, 3H), 3.72 (s, 3H), 4.16 (d, J = 9.7 Hz, 1H), 5.12 (dd, J = 9.1, 7.8 Hz, 1H), 5.22 (t, J = 9.5 Hz, 1H), 5.29 (t, J = 9.2 Hz, 1H), 5.75 (d, J = 7.8 Hz, 1H) ppm.

**¹³C NMR (151 MHz, CDCl₃):** δ = 20.55, 20.62, 20.65, 20.9, 53.1, 69.0, 70.2, 71.9, 73.1, 91.4, 166.9, 168.9, 169.3, 169.5, 170.0 ppm.

[α]°D : +6.81 (c = 1.00, CHCl₃).
4.2.12. Methyl (2,3,4-tri-O-acetyl-α-D-glucopyranosyl-1-bromide)-uronate (39)

Scheme 40

Substance 38 (2 g, 5.04 mmol, 1.0 eq.) was dissolved in HBr/AcOH (33%, 8 mL, 44.05 mmol, 8.7 eq.) at rt for 1 h and the reaction was stored at 4 °C overnight.

After 21 h the solvent was removed under reduced pressure, until a viscos brownish oil was obtained. The oil was taken up in chloroform (25 mL), which was washed with sat. NaHCO₃ (5 mL) and water (5 mL), dried over MgSO₄ and evaporated under reduced pressure (1.8 g).

The crude material was crystallized with anh. EtOH (6.5 mL) at -20 °C for 1.5 h and the precipitate was filtered off. The crystals were washed with cold EtOH (2 mL) and dried in vacuo (1.096 g). The mother liquor was evaporated under reduced pressure (0.535 g) and purified using the same procedure to obtain another 385 mg of product (39). Analytical data is in accordance with literature.[49]

Yield: 1.46 g (70% yield) of white crystals (α-anomer).

TLC: Rf = 0.55 (hexane:ethyl acetate = 1:1).

¹H NMR (600 MHz, CDCl₃): δ = 2.02 (s, 3H), 2.03 (s, 3H), 2.07 (s, 3H), 3.74 (s, 3H), 4.55 (d, J = 9.9 Hz, 1H), 4.83 (dd, J = 10.0, 4.1 Hz, 1H), 5.21 (dd, J = 10.3, 9.5 Hz, 1H), 5.58 (t, J = 9.8 Hz, 1H), 6.62 (d, J = 4.0 Hz, 1H) ppm.

¹³C NMR (151 MHz, CDCl₃): δ = 20.5, 20.7, 53.2, 68.6, 69.4, 70.4, 72.1, 85.5, 166.7, 169.5, 169.7, 169.74 ppm.

[α]₂⁰ᴰ: +114.83 (c = 1.24, CHCl₃).
4.2.13. Methyl (2,3,4-tri-O-acetyl-β-D-glucopyranosid)-uronate (40)

Substance 38 (0.5 g, 1.33 mmol, 1 eq.) was dissolved in DCM (20 mL), followed by the dropwise addition of benzylamine (0.3 mL, 2.75 mmol, 2 eq.) under stirring.

After 20 h the reaction was diluted with DCM (30 mL) and quenched with 1 M HCl (5 mL). The organic phase was washed with 1 M HCl (10 mL) and water (2 × 15 mL). The solution was dried over MgSO₄ and evaporated under reduced pressure (724 mg).

Purification was carried out via column chromatography (60 g silica, LP:EA = 2:1, 25 mL fraction size). Fractions containing product (52 – 72) were evaporated under reduced pressure (301 mg).

Analytical data is in accordance with literature.[48]

Yield: 301 mg (60% yield) of slightly yellow solid (α- and β-anomer).

TLC: Rf = 0.26 (hexane:ethyl acetate = 1:1).

1H NMR (600 MHz, CDCl₃): δ = 2.01 (s, 3H), 2.02 (s, 3H), 2.07 (s, 3H), 3.73 (s, 3H), 3.98 (s, 1H), 4.57 (d, J = 10.1 Hz, 1H), 4.88 (dd, J = 10.1, 3.6 Hz, 1H), 5.16 (dd, J = 10.0, 9.4 Hz, 1H), 5.53 (d, J = 3.4 Hz, 1H), 5.56 (t, J = 9.8 Hz, 1H) ppm.

13C NMR (151 MHz, CDCl₃): δ = 20.7, 20.8, 53.0, 68.1, 69.2, 69.6, 70.9, 90.3, 168.6, 169.8, 170.2, 170.3 ppm.

[α]D²⁰ : +70.56 (c = 0.98, CHCl₃).
4.2.14. Methyl \([2,3,4\text{-tri-}O\text{-acetyl-}1\text{-}O\text{-}(\text{trichloroacetimidoyl})\text{-}\alpha\text{-D-glucopyranosid}]\text{-uronate (41)}\)

Substance 40 (230 mg, 0.688 mmol, 1.0 eq.) was dissolved in anh. DCM (2 mL) in a Schlenk flask and cooled down to 0 °C. Trichloroacetonitrile (0.35 mL, 3.494 mmol, 5.1 eq.) was added slowly at this temperature and the mixture was stirred for 10 min. Subsequently, DBU (0.04 mL, 0.357 mmol, 0.5 eq.) was added dropwise under vigorous stirring and the ice bath was removed to allow the reaction to heat up to rt.

The reaction was quenched with sat. \(\text{NH}_4\text{Cl}\)-solution (2 mL) after 5 h and extracted with DCM (3 × 2 mL). The solution was washed with water (2 × 2 mL), dried over \(\text{MgSO}_4\) and evaporated under reduced pressure (513 mg).

Purification was carried out using column chromatography (50 g silica, LP:EA = 2:1, 25 mL fraction size). Fractions containing product (6 – 14) were collected and the solvent was removed \textit{in vacuo} (165 mg). Analytical data is in accordance with literature.[48]

**Yield:** 165 mg (52% yield) of white crystals (α-anomer).

**TLC:** \(R_f = 0.59\) (hexane:ethyl acetate = 1:1).

\(^1\text{H NMR (600 MHz, CDCl}_3\):} \(\delta = 2.01 \text{ (s, 3H), 2.04 (s, 3H), 2.04 (s, 3H), 3.74 (s, 3H), 4.49 (d, } J = 10.3\text{ Hz, 1H), 5.14 (dd, } J = 10.2, 3.6 \text{ Hz, 1H), 5.26 (t, } J = 9.5 \text{ Hz, 1H), 5.62 (t, } J = 9.9 \text{ Hz, 1H), 6.63 (d, } J = 3.3 \text{ Hz, 1H), 8.73 (s, 1H) ppm.}\)

\(^{13}\text{C NMR (151 MHz, CDCl}_3\):} \(\delta = 20.5, 20.6, 20.8, 53.2, 69.0, 69.2, 69.6, 70.6, 92.7, 160.7, 167.3, 169.6, 169.87, 169.91 \text{ ppm.}\)
4.2.15. 1´N-(Methyl 2,3,4-tri-O-acetyl-1-deoxy-β-D-glucuronosyluronate)-17β-methyl-5α-androstano[3,2-c]pyrazol-17α-ol (42) and 2´N-(Methyl 2,3,4-tri-O-acetyl-1-deoxy-β-D-glucuronosyluronate)-17β-methyl-5α-androstano[3,2-c]pyrazol-17α-ol (43)

Scheme 43

17-Epistanozolol (3, 117 mg, 0.356 mmol, 1.0 eq.) was suspended in a mixture of NO2Me/PhH (1:1, 5 mL) in a flame-dried Schlenk flask under argon. Hg(CN)2 (100 mg, 0.396 mmol, 1.1 eq.), HgBr2 (1 mg) and molecular sieves (3 Å, 10 mg) were added to the solution, followed by heating up to 40 °C and stirring for 15 min at this temperature. Substance 39 (181 mg, 0.463 mmol, 1.3 eq.) dissolved in the same solvent mixture (3 mL) was added to the suspension dropwise and the reaction was heated up to 70 °C immediately.

The reaction was cooled to rt after 4.5 h and the solvent was removed in vacuo at the Schlenk line. The residue was taken up with chloroform (30 mL) and washed with sat. KI-solution (3 × 10 mL) and water (10 mL). The organic phase was dried over MgSO4 and evaporated under reduced pressure to give 380 mg of crude material.

Separation was carried out using column chromatography (38 g silica, LP:EA = 2.5:1 → 1:1, 5 mL fraction size). Fractions containing 43 (77 – 104, 97 mg) and fractions containing a mixture of 3 and 42 (105 – 131, 104 mg) were collected separately and evaporated under reduced pressure. The mixture of 3 and 42 was subjected to column chromatography (10 g silica, LP:EA = 1:1, 2 mL fraction size) again. Fractions containing 42 (11 – 18, 39 mg) and the fractions containing 3 (19 – 43, 49 mg) were collected separately and evaporated under reduced pressure. The separated
products 42 and 43 were subjected to prep. HPLC-MS (30 mL/min, MeOH:H₂O + 0.1% FA: 70:30 → 95:5, m/z = 645). Fractions containing product were collected and MeOH was removed in vacuo. The mainly aqueous solutions were frozen through liquid nitrogen and lyophilized. Product 42 (23 mg, 10%) and 43 (58 mg, 25%) were obtained as white crystalline solids.

1’N-(Methyl 2,3,4-tri-O-acetyl-1-deoxy-β-D-glucuronosyluronate)-17β-methyl-5α-androstano[3,2-c]pyrazol-17α-ol (42):

Yield: 23 mg (10% yield) of white crystals.

TLC: Rf = 0.23 (hexane:ethyl acetate = 1:1).

1H NMR (600 MHz, CDCl₃): δ = 0.68 (s, 3H), 0.68 (s, 3H), 0.83 – 0.94 (m, 1H), 0.96 – 1.06 (m, 1H), 1.09 – 1.18 (m, 1H), 1.19 (s, 3H), 1.21 – 1.28 (m, 1H), 1.31 – 1.55 (m, 5H), 1.55 – 1.69 (m, 2H), 1.70 – 1.79 (m, 2H), 1.81 (s, 3H), 1.83 – 1.91 (m, 1H), 2.02 (s, 3H), 2.03 (s, 3H), 2.08 (s, 3H), 2.08 – 2.25 (m, 1H), 2.50 (d, J = 15.2 Hz, 1H), 2.60 (dd, J = 16.1, 5.0 Hz, 1H), 3.71 (s, 3H), 4.22 (d, J = 9.6 Hz, 1H), 5.36 (t, J = 9.5 Hz, 1H), 5.40 (t, J = 9.4 Hz, 1H), 5.43 (d, J = 9.2 Hz, 1H), 5.72 (t, J = 9.3 Hz, 1H), 7.30 (s, 1H) ppm.

13C NMR (151 MHz, CDCl₃): δ = 11.5, 16.0, 20.4, 20.6, 20.7, 22.8, 24.1, 26.0, 29.3, 30.0, 31.9, 35.0, 36.3, 36.5, 38.4, 42.0, 46.7, 49.9, 53.1, 53.6, 69.3, 70.0, 72.8, 75.0, 82.4, 84.6, 117.1, 138.5, 140.0, 166.78, 168.8, 169.5, 170.4 ppm.

IR [cm⁻¹]: 2921, 1746, 1444, 1372, 1217, 1098, 1038, 920.

[α]D²⁰: +8.49 (c = 1.02, CHCl₃).

HRMS: (M+Na)*: Calc: 667.3201, found: 667.3203.

M.p.: 171 – 173 °C.
2′N-(Methyl 2,3,4-tri-O-acetyl-1-deoxy-β-D-glucuronosyluronate)-17β-methyl-5α-androstano[3,2-c]pyrazol-17α-ol (43):

Yield: 58 mg (25% yield) of white crystals.

TLC: \( R_f = 0.35 \) (hexane:ethyl acetate = 1:1).

\(^1\)H-NMR (600 MHz, CDCl\(_3\)): \( \delta = 0.69 \) (s, 3H), 0.71 (s, 3H), 0.80 – 0.93 (m, 1H), 0.99 (qd, \( J = 13.1, 3.9 \) Hz, 1H), 1.07 – 1.19 (m, 1H), 1.20 (s, 3H), 1.22 – 1.28 (m, 1H), 1.28 – 1.50 (m, 5H), 1.50 – 1.63 (m, 2H), 1.63 – 1.69 (m, 3H), 1.69 – 1.78 (m, 2H), 1.82 – 1.93 (m, 4H), 2.04 (s, 6H), 2.06 (d, \( J = 15.4 \) Hz, 1H), 2.24 (dd, \( J = 16.7, 12.2 \) Hz, 1H), 2.55 – 2.68 (m, 2H), 3.71 (s, 3H), 4.22 (d, \( J = 9.9 \) Hz, 1H), 5.33 (t, \( J = 9.7 \) Hz, 1H), 5.40 (t, \( J = 9.3 \) Hz, 1H), 5.50 (d, \( J = 9.6 \) Hz, 1H), 5.56 (t, \( J = 9.4 \) Hz, 1H), 7.28 (s, 1H) ppm.

\(^{13}\)C-NMR (151 MHz, CDCl\(_3\)): \( \delta = 11.6, 16.0, 20.5, 20.6, 20.7, 20.8, 22.8, 24.1, 27.6, 29.5, 29.8, 30.0, 32.0, 34.8, 36.4, 38.5, 42.4, 46.7, 50.0, 53.1, 53.7, 69.4, 69.7, 72.9, 74.8, 82.3, 87.1, 118.1, 126.0, 150.4, 166.7, 169.2, 169.5, 170.1 \) ppm.

IR [cm\(^{-1}\)]: 3412, 2922, 2853, 1754, 1439, 1374, 1215, 1035, 734.

\([\alpha]_D^{20}\) : +4.01 (c = 1.00, CHCl\(_3\)).


M.p.: 201 – 204 °C.
4.2.16. Methyl \( N \)-\( \text{(triethylammoniumsulfonyl)} \)carbamate (49)

\[
\text{\textbf{Scheme 44}}
\]

Methyl (chlorosulfonyl)carbamate (48):

Chlorosulfamylisocyanate (47, 5 mL, 57.4 mmol, 1.1 eq.) was dissolved in toluene (15 mL) under stirring and methanol (HPLC-grade, 2.1 mL, 51.8 mmol, 1.0 eq.) was added dropwise over the course of 30 min. Afterwards, the reaction was stirred for one hour. The mixture was evaporated under reduced pressure and cooled down for crystallization. The crude product was recrystallized from toluene (10 mL) and precipitated at -20 °C. The crystals were filtered off, washed with toluene (2 mL) and dried \textit{in vacuo} to obtain 8.62 g (96%) of 48. Analytical data is in accordance with literature.[45]

\textbf{Yield:} 8.62 g (96% yield) of white crystalline solid.

\( ^1 \text{H NMR (200 MHz, CDCl}_3 \): \( \delta = 3.95 \text{ (s, 3H), 8.79 (s, 1H)} \) ppm.

Methyl \( N \)-\( \text{(triethylammoniumsulfonyl)} \)carbamate (49):

Triethylamine (16 mL, 114.7 mmol, 2.3 eq.) and benzene (50 mL) were combined in a flame-dried Schlenk flask under argon and the apparatus was fitted with a dropping funnel filled with substance 48 (8.6 g, 49.5 mmol, 1.0 eq.) dissolved in benzene (75 mL). The solution was added dropwise under stirring over the course of 2 h using a water bath for cooling the reaction. The precipitated ammonium salt was filtered off and washed with benzene (50 mL). The obtained mother liquor was evaporated and crystallized from toluene (20 mL) in the freezer. The crystals were filtered off, washed with toluene (10 mL) and dried \textit{in vacuo}, which gave a slightly pink powder (10.46 g) of substance 49. Analytical data is in accordance with literature.[45]

\textbf{Yield:} 10.46 g (89% yield) of slightly pink powder.

\( ^1 \text{H NMR (200 MHz, CDCl}_3 \): \( \delta = 1.40 \text{ (t, } J=7.3 \text{ Hz, 9H), 3.46 (q, } J=7.3 \text{ Hz, 6H), 3.68 (s, 3H)} \) ppm.
5. References


[47] V.A. Zolotsev, G.V. Ponomarev, M.O. Taratynova, et al., Conjugates of 17-substituted testosterone and epitestosterone with pyropheophorbide a differing in the length of linkers, Steroids 138((2018) 82-90.


6. Appendix

6.1. Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AA</td>
<td>Anabolic agent</td>
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<tr>
<td>AAS</td>
<td>Anabolic androgenic steroid</td>
</tr>
<tr>
<td>Ac</td>
<td>Acetyl</td>
</tr>
<tr>
<td>Ac₂O</td>
<td>Acetic anhydride</td>
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<td>AcOH</td>
<td>Acetic acid</td>
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<tr>
<td>AgBr</td>
<td>Silver bromide</td>
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<td>AMPK</td>
<td>Adenosine monophosphate-activated protein kinase</td>
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<td>Anhydrous</td>
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<td>Attached proton test</td>
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dEPO  Darbepoetin
DHCMT  Dehydrochloromethyltestosterone
DIAD  Diisopropyl azodicarboxylate
DMF  Dimethylformamide
DTBP  Di-2,6-tert.-butylpyridine
EA  Ethyl acetate
EAAS  Exogenous anabolic androgenic steroid
EPO  Erythropoietin
eq.  Equivalent
Et$_2$O  Diethyl ether
EtOH  Ethanol
FA  Formic acid
Fe  Iron
GC  Gas chromatography
GDR  German Democratic Republic
h  Hour
HBr  Hydrobromic acid
HCl  Hydrochloric acid
HgBr$_2$  Mercury-(II)-bromide
Hg(CN)$_2$  Mercury-(II)-cyanide
HIF  Hypoxia-inducible factor
HMBC  Heteronuclear multiple-bond correlation
H$_2$O  Water
HPLC  High-pressure liquid chromatography
HRMS  High-resolution mass spectroscopy
H$_2$SO$_4$  Sulfuric acid
HSQC  Heteronuclear single quantum coherence
Hz  Hertz
IOC  International Olympic Committee
IR  Infrared spectroscopy
IRM S  Isotope ratio mass spectrometry
IS  International standards
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>K₂CO₃</td>
<td>Potassium carbonate</td>
</tr>
<tr>
<td>KI</td>
<td>Potassium iodide</td>
</tr>
<tr>
<td>KMnO₄</td>
<td>Potassium permanganate</td>
</tr>
<tr>
<td>LA</td>
<td>Lewis acid</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LG</td>
<td>Leaving group</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>LiAlH₄</td>
<td>Lithium aluminum hydride</td>
</tr>
<tr>
<td>LiOH</td>
<td>Lithium hydroxide</td>
</tr>
<tr>
<td>LP</td>
<td>Light petroleum</td>
</tr>
<tr>
<td>m-CPBA</td>
<td><em>meta</em>-Chloroperoxybenzoic acid</td>
</tr>
<tr>
<td>Me</td>
<td>Methyl</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium sulfate</td>
</tr>
<tr>
<td>MHz</td>
<td>Mega Hertz</td>
</tr>
<tr>
<td>min.</td>
<td>Minute</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MSTFA</td>
<td>N-Methyl-N-(trimethylsilyl) trifluoroacetamide</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-charge ratio</td>
</tr>
<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NaOMe</td>
<td>Sodium methoxide</td>
</tr>
<tr>
<td>Na₂S₂O₃</td>
<td>Sodium thiosulfate</td>
</tr>
<tr>
<td>NEt₃</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>Ammonium chloride</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NO₂</td>
<td>Nitro</td>
</tr>
<tr>
<td>NO₂Me</td>
<td>Nitromethane</td>
</tr>
<tr>
<td>Nuc</td>
<td>Nucleophile</td>
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<tr>
<td>PAPS</td>
<td>3′-Phosphoadenosine-5′-phosphosulfate</td>
</tr>
<tr>
<td>PG</td>
<td>Protecting group</td>
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PhH  Benzene
Ph₃P  Triphenylphosphine
Ph₃PAuNTf₂  Triphenylphosphine gold(I) bis-(trifluoromethanesulfonyl)imidate
p-NBA  para-Nitrobenzoic acid
POCl₃  Phosphoryl chloride
Prep.  Preparative
Py  Pyridine
Rᵣ  Retention factor
RP  Reversed phase
rt  Room temperature
sat.  Saturated
SERM  Selective estrogen receptor modulator
SO₃  Sulfur trioxide
SO₃-py  Sulfur trioxide pyridine complex
TGF  Transforming growth factor
THF  Tetrahydrofuran
TLC  Thin layer chromatography
TMS  Trimethylsilyl
TMSI  Trimethylsilyl-iodide
TMSOTf  Trimethylsilyl-triflate
TosCl  para-Toluenesulfonyl chloride
UDP  Uridine diphosphate
WADA  World Anti-Doping Agency
WADC  World Anti-Doping Code
6.2. Selected NMR-Spectra

Figure 13: $^1$H-NMR of 17-Epistanozolol (3) at 600 MHz.

Figure 14: $^{13}$C-NMR of 17-Epistanozolol (3) at 151 MHz.
Figure 15: $^1$H-NMR of 17-Epistanozolol-1’N-Glucuronide (deprotected) (9) at 600 MHz.

Figure 16: $^{13}$C-NMR of 17-Epistanozolol-1’N-Glucuronide (deprotected) (9) at 151 MHz.
Figure 17: $^1$H-NMR of 17-Epistanozolol-2'N-Glucuronide (deprotected) (14) at 600 MHz.

Figure 18: $^{13}$C-NMR of 17-Epistanozolol-2'N-Glucuronide (deprotected) (14) at 151 MHz.
Figure 19: $^1$H-NMR of Koenigs-Knorr-Donor (39) at 600 MHz.

Figure 20: $^{13}$C-NMR of Koenigs-Knorr-Donor (39) at 151 MHz.
Figure 21: $^1$H-NMR of 17-Epistanozolol-1'N-Glucuronide (protected) (42) at 600 MHz.

Figure 22: $^{13}$C-NMR of 17-Epistanozolol-1'N-Glucuronide (protected) (42) at 151 MHz.
Figure 23: $^1$H-NMR of 17-Epistanozolol-2’N-Glucuronide (protected) (43) at 600 MHz.

Figure 24: $^{13}$C-NMR of 17-Epistanozolol-2’N-Glucuronide (protected) (43) at 151 MHz.
Curriculum vitae

Personal Data:

Georg Stadler
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Born 10th of January 1994 in Vienna, Austria

Educational Background:

Master Thesis: Synthesis and Characterization of 17-Epistanozolol-N-Glucuronides for Anti-Doping Purposes, under supervision of Prof. Peter Gärtner and Prof. Valentin Enev

03/2014 – 06/2018 Studies of Technical Chemistry – Bachelor curriculum, TU Wien
Bachelor Thesis: Synthesis of Dehydrochloromethyltestosterone-Metabolites as Reference Materials for Anti-Doping-Analysis, under supervision of Dr. Nicolas Kratena and Prof. Peter Gärtner

10/2013 – 03/2014 Military Service

09/2008 – 06/2013 Technical High School, HBLVA Rosensteingasse, Vienna
Acquiring the status of a trained Biochemical Technician
## Professional experience and Internships:

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<th>Date</th>
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<tr>
<td>10/2019 – 02/2021</td>
<td>Student Assistant/Tutor, Institute of Applied Synthetic Chemistry, TU Wien</td>
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<tr>
<td>06/2019</td>
<td>Internship with Dr. Christian Stanetty and Prof. Marko Mihovilovic, Institute of Applied Synthetic Chemistry, TU Wien</td>
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<td>Project/Title: <em>Building Blocks towards the Synthesis of a Labelled PI- Derivative</em></td>
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<td>02/2019 – 03/2019</td>
<td>Internship with Dipl.-Ing. Stefan Helfert and Prof. Robert Liska, Institute of Applied Synthetic Chemistry, TU Wien</td>
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<td>Project/Title: <em>Dependency of the thickness of polymer brushes with the concentration of monomers</em></td>
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<td>08/2016 – 09/2016</td>
<td>Internship with Dr. Nicolas Kratena and Prof. Peter Gärtner, Institute of Applied Synthetic Chemistry, TU Wien</td>
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<td>Project/Title: <em>Synthesis of Long-Term Metabolites of Oxandrolone</em></td>
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<td>07/2015</td>
<td>Internship, Baxalta Innovations GmbH, Vienna, GMP-Support</td>
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<tr>
<td>07/2012</td>
<td>Internship with Dr. Philipp Fruhmann and Prof. Johannes Fröhlich, Institute of Applied Synthetic Chemistry, TU Wien</td>
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<td>Project/Title: <em>Modeling of the Glycosylation of Deoxynivalenol and Deprotection with Pig Liver Esterase</em></td>
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<td>07/2011</td>
<td>Internship, LOBA GmbH &amp; Co. KG, Fischamend, Quality Control</td>
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<tr>
<td>07/2010</td>
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