Optimizing the Process of Introducing New Metabolites of Anabolic-Androgenic Steroids – Method Evaluation, Synthesis, Verification

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"The great tragedy of science – the slaying of a beautiful hypothesis by an ugly fact."

Thomas Henry Huxley

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I Abbreviations

AAF	Adverse Analytical Finding
AAS	Anabolic-Androgenic Steroid
ADRV	Anti-Doping Rule Violation
ASOIF	Association of Summer Olympic International Federations
CYP	Cytochrome P450
DHCMT	Dehydrochloromethyltestosterone
GC	Gas Chromatography
GC-EI-MS	Gas Chromatography coupled by Electron Ionization to Single Quadrupole-Mass Spectrometry
GC-EI-QQQ-MS	Gas Chromatography coupled by Electron Ionization to Triple Quadrupole-Mass Spectrometry
GC-EI-QTOF-MS	Gas Chromatography coupled by Electron Ionization to Quadrupole Time-of-Flight-Mass Spectrometry
IOC	International Olympic Committee
LC-ESI-QTOF-MS	High-Performance Liquid Chromatography coupled by Electrospray Ionization to Quadrupole Time-of-Flight-Mass Spectrometry
LC-ESI-MS	High-Performance Liquid Chromatography coupled by Electrospray Ionization to Mass Spectrometry
MD	Metandienone
MT	17α-Methyltestosterone
NMR	Nuclear Magnetic Resonance Spectroscopy
WADA	World Anti-Doping Agency

1 Introduction

The use of drugs to increase physical and mental abilities in sports and everyday life is recorded since mankind exists and is still a part of it till today [1]. Although this kind of performance improvement was never socially accepted outside of sports and proscribed or punished in sports, it could never be stopped entirely. The Olympic motto "citius, altius, fortius" ("faster, higher, stronger") [2] spurs the athletes, but some of them, driven by it, use illegal methods, harming the spirit of the sport as well as their health. Therefore, it is crucial to convict as many doping athletes as possible to make the sport fairer. "Doping" is defined as the use of forbidden substances or methods; it will be explained in detail later (2.1.1).

As an independent organization, the World Anti-Doping Agency (WADA) oversees an enormous system that tries to catch doping athletes. To leave no doubt about the potential findings in the analysis of samples, it is indispensable that the process of doping conviction itself is transparent and reliable. Therefore, doping control samples are collected from athletes in and out of competition and analyzed with methods that detect the substance itself and already known metabolites and markers. The flawless identification of those substances is indispensable to avoid any doubts about a positive sample. Hence, completely characterized reference material is one of many key points in anti-doping analysis to identify the metabolites beyond doubt. The need for reference material was the origin of this thesis and accompanied the whole work.

The work focuses on substances from the still widely used group of anabolic agents defined by WADA [3]. Most of these substances are extensively metabolized, and after a brief period, none of the original compounds is excreted. To catch the athletes even after a long time after misusing substances, the detection window of the substances can be enlarged by research on so-called long-term metabolites. Although there are already methods to detect the abuse of the prohibited substances and studies on their metabolism and the resulting metabolites, it is essential to further improve the methods by lowering the detection limit of the known substances by increasing the selectivity and sensitivity, to find new metabolites, and to confirm the structures of postulated metabolites. The discovery and verification of new metabolic products will help understand human metabolism, find the unfair playing athletes, and increase public health by keeping athletes from taking prohibited substances.

The synthesis of the mentioned long-term metabolites is often a complex and time-consuming process. Still, it helped in the past to find the athletes guilty of doping after introducing new metabolites to the methods in anti-doping laboratories. Nevertheless, it is indispensable that the synthesized compounds are proven as metabolites from the investigated substance. This is feasible with an administration study including blank samples as negative control. Combining the above mentioned is one of the best ways of improving anti-doping analysis.

The aim of this work is to further elucidate steroid metabolism and contribute a small part to the worldwide fight against doping. Therefore, this work demonstrates the establishment of potential new long-term metabolites. The biosynthesis process was evaluated by elucidating the structural requirements of enzymes using endogenous and exogenous compounds. New metabolites of prohibited substances have been produced. Finally, the metabolite formation out of the administered drug was proven by a controlled administration study.

2 Theoretical Background

2.1 Doping in Sports

2.1.1 Aims and Prohibited List

As an independent authority in the worldwide fight for a doping-free sporting environment, the WADA formulates its mission as "lead[ing] a collaborative worldwide movement for doping-free sport" [4]. It defines doping as "the occurrence of one or more of the anti-doping rule violations" [5]. Amongst others, this is specified as "Presence of a Prohibited Substance or its Metabolites or Markers in an Athlete's Sample," "Possession of a Prohibited Substance or a Prohibited Method by an Athlete or Athlete Support Person," or "Use or Attempted Use by an Athlete of a […] Prohibited Method" [5].

Therefore, a list of prohibited substances and methods is published by WADA every year to define which substances and methods are forbidden to be used in sports [3]. This goes back to the first attempts of the International Olympic Committee (IOC) introducing such a catalog [6]. The so-called "prohibited list" classifies the substances in groups related to their pharmacological effects and their "time of prohibition." Some substances are prohibited at all times (S0-S5, M1-M3), some in the competition only (S6-S9), and others just in particular sports (P1). The different groups are displayed in **Figure 1**.

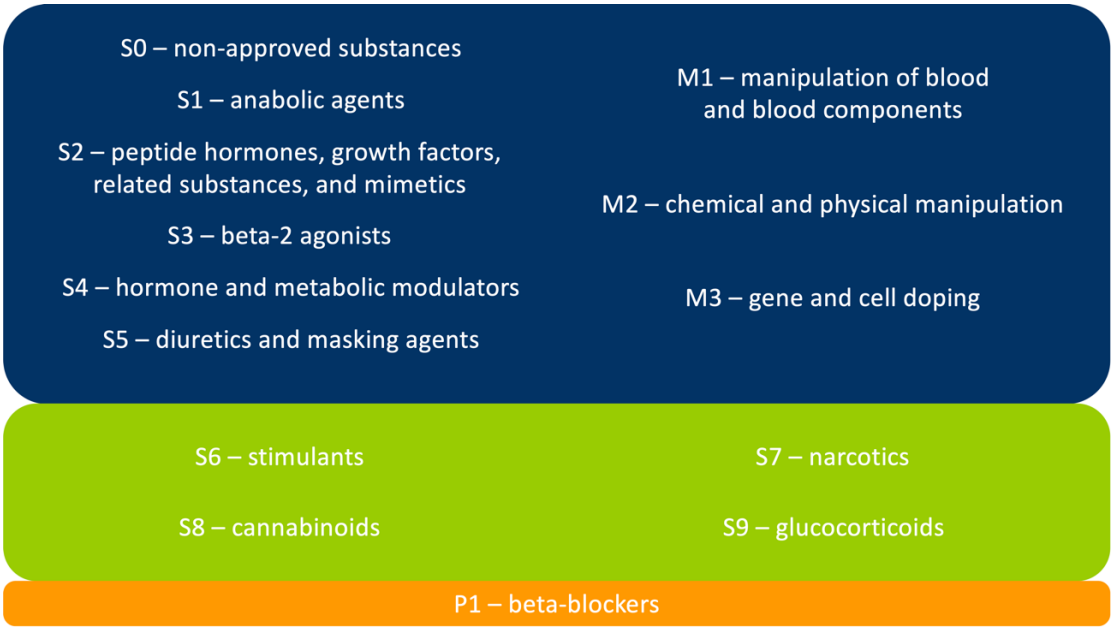


Figure 1: *Groups of the Prohibited List 2021 of WADA [3]; blue: prohibited at all times; green: prohibited in competition only; orange: prohibited in particular sports*

2.1.2 Doping Control System

To achieve its goals of a doping-free sport and to unveil the prohibited use of performance-enhancing substances by athletes, WADA keeps a complex and highly regulated system of sample collection and analysis. The process is summarized for urine analysis in the following section but taking blood samples is also possible. First, the sample is collected (at least 90 mL), and the specific gravity is measured since highly diluted samples could result in false-negative results. Subsequently, the sample is divided into two containers, the A sample, and the B sample, and both are sealed. The two containers are made anonymous and transported to one of 27 WADA accredited laboratories for doping control analysis [7]. The A sample is directly used for analysis, while the B sample is stored for potential later use. For the first analysis, an aliquot of the A sample is analyzed using various methods to capture as many analytes as possible, e.g., gas chromatography coupled by electron ionization to triple quadrupole-mass spectrometry (GC-EI-QQQ-MS) and high-performance liquid chromatography coupled to electrospray ionization-orbitrap-mass spectrometry. If there are findings or indications of potential prohibited substances or metabolites, a confirmation analysis is performed. If this analysis also detects forbidden substances, this is called an adverse analytical finding (AAF). In case of any negative finding, may it be during the screening analysis or confirmation analysis, the sample is declared negative. In case of a positive confirmation analysis, the athlete or a representative, e.g., a manager or trainer, has the right to claim a (supervised) analysis of the B sample. The analysis procedure for this sample is the same as described before. If the athlete waives a further analysis or if the B sample is also tested positive, the AAF is confirmed [8-10].

An AAF is not synonymous with an anti-doping rule violation (ADRV). Instead, an AAF indicates a prohibited substance or method in a specific sample of an athlete. If an AAF occurs, the following legal steps are required to declare an ADRV [11]. In addition, an ADRV may result in various consequences, such as reprimand or a period of ineligibility [12].

2.2 Steroids

2.2.1 General Information

Steroids are an important class of molecules in the human body. Besides their function as part of cell membranes or digestion, they play a substantial role in human signaling processes; therefore, they form their own class of hormones [13]. The effect of steroids is mediated through several pathways. The classical way is via agonism at the respective intracellular receptor, which then migrates into the nucleus and regulates the control of transcription or the interaction with regulatory sections of the DNA [14]. Another way of action is happening faster because of non-genomic effects, e.g., by interaction with G protein-coupled receptors [15].

Steroid hormones can be divided into two major classes: corticosteroids and sex steroids. Subsequently, both classes are further subdivided. The subclasses of corticosteroids are glucocorticoids and mineralocorticoids, and sex steroids can be parted into androgens, progestogens, and estrogens [13].

Every of the mentioned classes of steroid hormones has a specific lipophilic backbone: Most of the androgens share the structure of androstane, progestogens, mineralo- and glucocorticoids go back to pregnane, and estrogens have an estrane structure. All five classes derive from cholesterol (cholest-5-en-3β-ol) with a cholestane backbone and are shown in **Figure 2**.

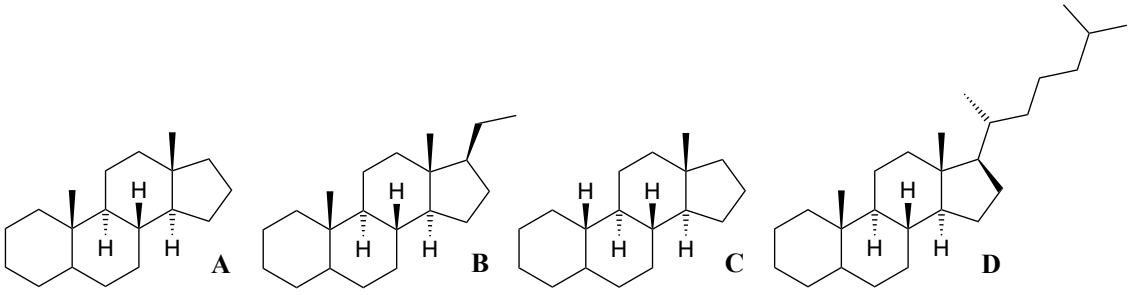


Figure 2: *Backbones of steroid hormones: androstane (A), pregnane (B), estrane (C), and cholestane (D)*

All backbones have in common that they consist of four rings: three cyclohexane rings (A, B, C) and one cyclopentane ring (D). The stereo-specific orientation of all substituents is named related to the methyl group between rings A and B (C-10): if they are heading in the same direction (cis configured), they are named beta; if the substituents stand contrarily (trans), it is called alpha. This is continued even if the substituent at position 10 is missing, e.g., estrogens (double bond 5(10)). The numbering of carbons and the naming of rings in steroids are displayed in **Figure 3** [16].

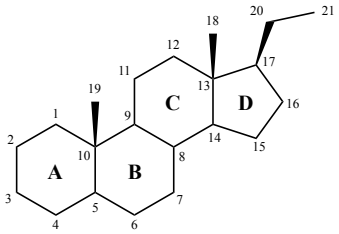


Figure 3: *The method of counting the carbon atoms and naming the rings using the example of pregnane*

2.2.2 Steroidogenesis

The formation of the different classes of steroids originates from cholesterol (1). A simplified overview of steroidogenesis is given in **Figure 4** [13, 16, 17].

In a first step, pregnenolone (3β-hydroxy-pregn-5-en-20-one, 2) as a progestogen is formed out of cholesterol, catalyzed by cytochrome P450 11A1 (CYP11A1). Subsequent oxidation in position 3 via 3β-hydroxysteroid dehydrogenase results in progesterone (pregn-4-ene-3,20-dione, 4). The hydroxylation in position 17 by CYP17A1 is leading to their 17α-hydroxy derivatives (3β,17α-dihydroxy-pregn-5-en-20-one, 3; 17α-hydroxy-pregn-4-ene-3,20-dione, 5). A subsequent CYP21A2-catalyzed hydroxylation of steroids 3 and 5 in position 21 leads to deoxycorticosterone (21-hydroxy-pregn-4-ene-3,20-dione, 11), a mineralocorticoid, or 11-deoxycortisol (17α,21-dihydroxy-pregn-4-ene-3,20-dione, 12), a glucocorticoid. Further reactions result in the generation of more mineralo- or glucocorticoids.

Androgens (dehydroepiandrosterone, 3β-hydroxy-androst-5-en-17-one, 6; androstenedione, androst-4-ene-3,17-dione, 8) are formed out of progestogens by CYP17A2-catalyzed cleavage of the pregnane-sidechain (carbons 20, 21). The reduction of the 17-oxo group to a hydroxy group gives androstenediol (androst-5-ene-3β,17β-diol, 7) and testosterone (17β-hydroxy-androst-4-en-3-one, 9) and is catalyzed by 17β-hydroxysteroid dehydrogenase [18].

Estrogens are formed out of androstenedione (8) and testosterone. This reaction is catalyzed by CYP19A1 (aromatase) [19].

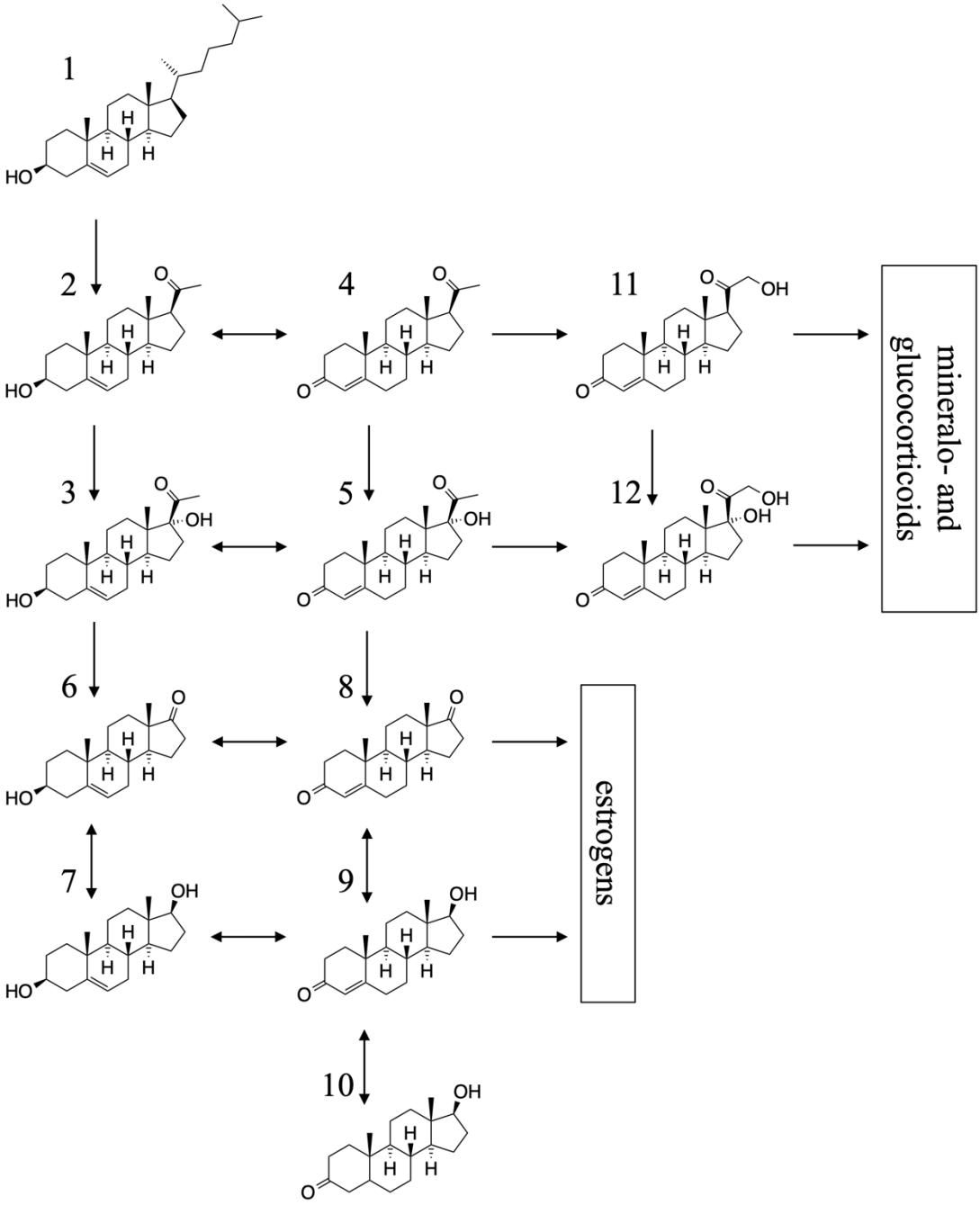


Figure 4: *Simplified human steroidogenesis; 1: cholesterol, 2: pregnenolone, 3: 17α-hydroxypregnenolone, 4: progesterone, 5: 17α-hydroxyprogesterone, 6: dehydroepiandrosterone, 7: androstenediol, 8: androstenedione, 9: testosterone, 10: dihydrotestosterone, 11: deoxycorticosterone, 12: 11-deoxycortisol; 2-5: progestogens, 6-10: androgens, 11: mineralocorticoid, 12: glucocorticoid*

2.3 Anabolic-Androgenic Steroids

2.3.1 Effects of Androgens

Anabolic-Androgenic Steroids (AAS) are a class of substances related to the male sex hormone testosterone.

Testosterone itself is mainly produced by Leydig cells in the testicles in males, while females produce small amounts in the ovaries and adrenal glands [20, 21]. The effect of testosterone is mediated by affecting the androgen receptor. The potency of its metabolite 5α-dihydrotestosterone (17β-hydroxy-androstan-3-one), produced at the effector organ by 5α-reductase (SRD5A1-3), is much higher. Therefore, the characteristic anabolic effects like muscle growth and increase of strength and androgenic effects as preservation of libido or sexual maturation are mainly caused by 5α-dihydrotestosterone [22, 23].

2.3.2 Modifications of AAS

Steroids with the effects as mentioned above are on the one hand endogenous, which means that the human body itself produces them, e.g., testosterone, 5α-dihydrotestosterone, and androstenedione, on the other hand also exogenous, that are synthesized chemically or occur in plants, e.g., euphol or phytoestrogens, and do not occur naturally in humans [24]. This group is specified as AAS.

Synthetic exogenous steroids are modified compared to testosterone in that they favor either the androgenic or the anabolic way, depending on their potential application. To evaluate those properties, an assay was introduced known as Hershberger assay nowadays [25]. Castrated male rats are exposed to the investigated compound over a distinct period, either by oral or subcutaneous administration. After euthanatizing the animals, the androgen-dependent tissues (depending on the assay: ventral prostate, seminal vesicle, levator ani-bulbocavernosus muscle, paired Cowper's glands, glans penis) are weighted in comparison to a control group. The ratio of the masses of the observed tissues of actively treated rats versus the control group gives information about the androgenic or anabolic effect of the tested substance [25-27]. Due to the broad variability of structural modifications of AAS, some of them have bigger anabolic than androgenic activity and vice versa [28].

Research on AAS aims most of the time to increase the anabolic effects while decreasing the androgenic ones and preventing undesired side effects. The structural-

activity relationship of modifications in AAS is complex, although some of them are proven. A brief overview of potential modifications is given in **Figure 5**.

As depicted, some of the modifications tend to hamper the aromatization of the AAS. This should minimize one of the most prominent side effects of AAS in males like gynecomastia, infertility, or erectile dysfunction [29, 30]. The aromatic system is formed by two hydroxylations at C-19 and the following dehydration to an aldehyde. Subsequently, the enolization of the 3-oxo group takes place while the 19-methyl group leaves the molecule as formic acid [31, 32]. This is one theory, among others. The introduction of a hydroxy group in position 2 is also discussed [33]. A 3-hydroxy group in an aromatic A-ring can interact with the estrogen receptor and hence causes the above-mentioned side effects. Even though some of the steroids are not affected by the aromatase, there is often still an estrogenic effect because of a small amount aromatized anyway. These metabolites can bind the estrogen receptor like estradiol (estr-1,3,5(10)-triene-3,17β-diol), the estrogen formed out of testosterone. For example, metandienone (MD, 17β-hydroxy-17α-methyl-androsta-1,4-dien-3-one) is metabolized to 17α-methylestradiol (17α-methyl-estra-1,3,5(10)-triene-3,17β-diol). Even it has a lower affinity to the estrogen receptor, the inactivation process is very slow [34, 35]. Thus, MD is provoking estrogenic side effects [36, 37].

Another often observed adverse reaction is the hepatoxicity of 17α-alkylated drugs, e.g., MD or dehydrochloromethyltestosterone (DHCMT, 4-chloro-17β-hydroxy-17α-methyl-androsta-1,4-dien-3-one). The methylation in position 17 slows down the

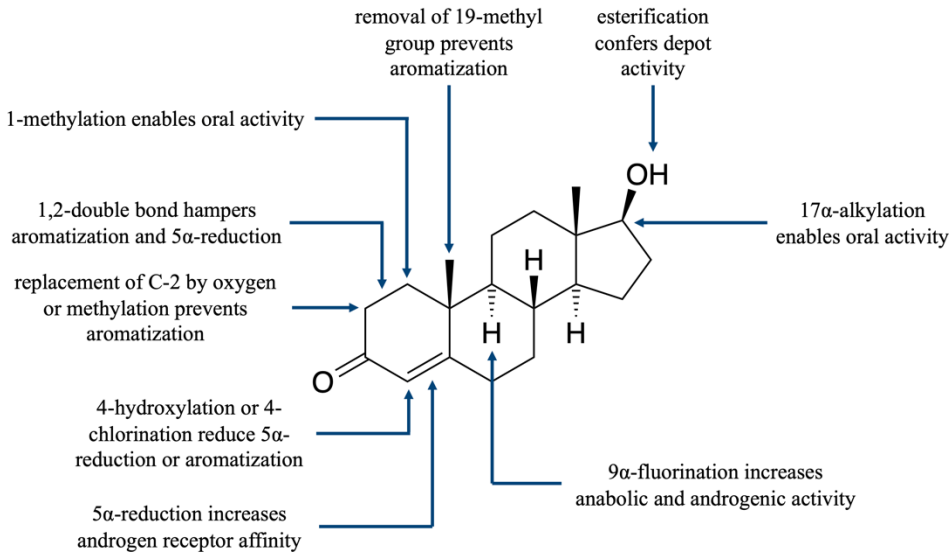


Figure 5: *Overview of potential modifications of AAS including the pharmacological effect; adapted from [28, 38-40]*

extensive first-pass effect taking place in the liver. Therefore, the oxidation of the 17β-hydroxy group is hindered. The subsequent glucuronidation of the hydroxy group is possible and leads to increased hepatotoxicity properties of the metabolite due to the structural similarities to bile acids [35, 41, 42].

2.3.3 Usage of AAS

Androsterone (3α-hydroxy-5α-androstan-17-one) was the first androgen isolated. It was obtained in 1931 out of 15,000 L urine from men. In 1935, testosterone could be crystallized out of 100 kg of bull testes. The structure determination of these and the following androgens mark the beginning of AAS development [43].

The first designed exogenous AAS indications were the treatment of hypogonadism in men with methyltestosterone (17β-hydroxy-17α-methyl-androst-4-en-3-one, MT) in 1937 [44]. Later marketed steroids were used to treat wasting conditions after surgeries or severe burns, against anemia, psychoses, or depressions [45]. Today, some AAS, e.g., testosterone or oxandrolone are still approved and used as treatment for cachexia in terminal HIV-infections, hypogonadism, or anemia [46-48].

It is reported that during the late 1940s and 1950s, athletes began to use testosterone and synthetic steroids to increase their muscle mass and strength. In the following time, many athletes of the Soviet Union and the German Democratic Republic used AAS as performance-enhancing drugs. Although the IOC banned the use of testosterone and its derivatives in 1974, the then illicit use of AAS continued [45, 49].

Today, most of the AAS are not marketed as approved drugs anymore but are produced by underground laboratories and sold via the internet. Due to the lack of control of these products, the quality of the preparations is volatile. Therefore, the consumers expose themselves to a high and incalculable risk of severe health problems [50-52].

Though the use of AAS is forbidden, the group of “Anabolic Agents” of WADA’s prohibited list not only represents a constant high number of AAFs but since 2003 also the highest proportion among all other groups. An overview of findings by WADA accredited laboratories is given in **Figure 6** [53-69].

The use of AAS concentrates on a few sports. In a comparison of the AAF of AAS in disciplines of ASOIF (Association of Summer Olympic International Federations) of the years between 2017 to 2019, it is noticeable that four of the five fields with the highest number of AAFs are unchanged over the years (athletics – long distance 3000 m or greater, cycling – road, rugby – fifteens, weightlifting). This is illustrated in **Figure 7**.

This is in accord with specific studies done in weightlifting and may indicate that these particular sports have a severe problem with AAS [70].

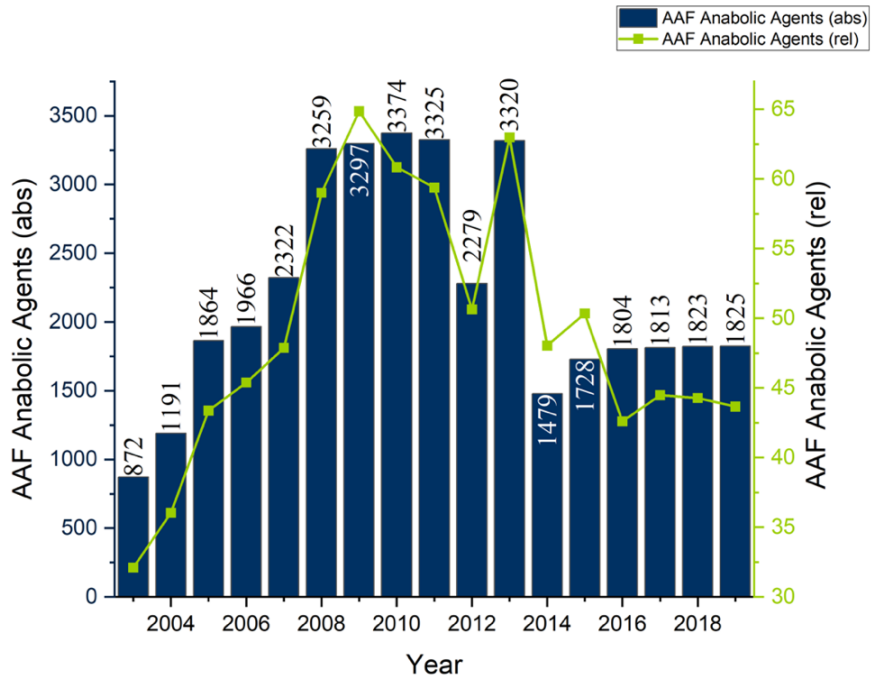


Figure 6: *AAFs of AAS between 2003 and 2019 by WADA accredited laboratories [53-69]; blue: total AAFs of AAS, green: AAFs of AAS in relation to the total amount of AAFs of all groups*

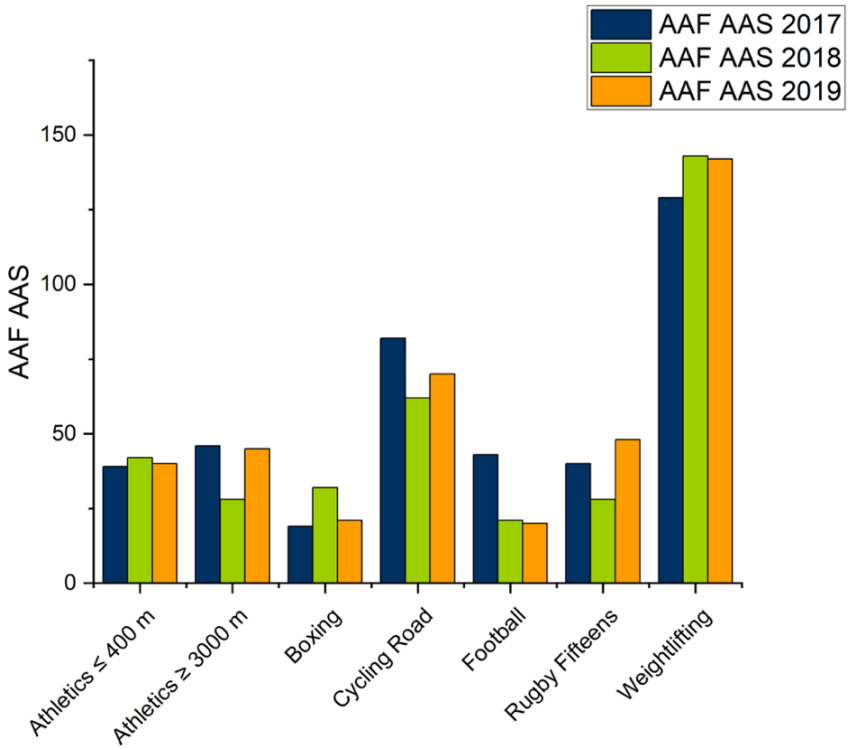


Figure 7: *Disciplines of ASOIF sports with the highest number of AAFs in the group of AAS between 2017 to 2019 [67-69]; a discipline is displayed if it was one of the five disciplines with the highest number of AAFs in the group of AAS in any of the years 2017 to 2019 regardless its number in other years*

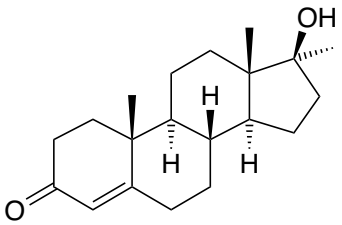
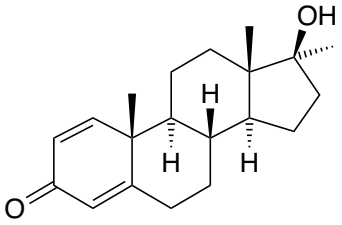
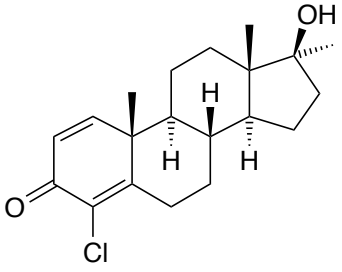
Unfortunately, the misuse of AAS is not only a problem in competitive sports but also amateur sports. The estimated number of unreported cases in the latter case is probably even higher. This seems reasonable, not at least because an exogenous application of supraphysiological doses of testosterone affects the muscle size and strength in normal men even without training [71].

An analysis from 2007 calculated the total amount of AAS worldwide at 700 t, based on the quantity of seized substances. This is enough to supply 15,000,000 people with these harmful substances [72]. Furthermore, based on the global trend of supply chains and the increasingly easy way of ordering even illegal products from the internet, it is supposable that this number raised in the meantime [72].

2.3.4 Focus on Special Steroids

This work pays special attention to the following orally active AAS: MT, MD, and DHCMT. The structures are given in **Table 1**.

Table 1: *Structures, names, and modifications of 17α-methyltestosterone, metandienone, and dehydrochloromethyltestosterone*

Name	Structure	Modifications compared to testosterone
17α-Methyltestosterone		17α-methylation
Metandienone		17α-methylation, 1,2-double bond
Dehydrochloro-methyltestosterone		17α-methylation, 1,2-double bond, 4-chloro

The number of AAFs of these compounds has been consistently high over the last years, although the substances have been off the market for over two decades. An overview of AAF from 2003 to 2019 is displayed in **Figure 8** [53-69].

The increase of AAFs of MD in 2006 and the following years is most likely caused by introducing a new long-term metabolite by the anti-doping laboratory in Cologne [73]. The peak of DHCMT in 2013 results from the publication of Sobolevsky *et al.* in 2012 [74]. The proposed new structures of potential long-term metabolites based on the findings of Schänzer *et al.*, which extended the detection window of MD from seven to 19 days and led to an enormous increase of AAF [73, 75]. This structural element was also found in metabolites of DHCMT (Parr *et al.*) [76].

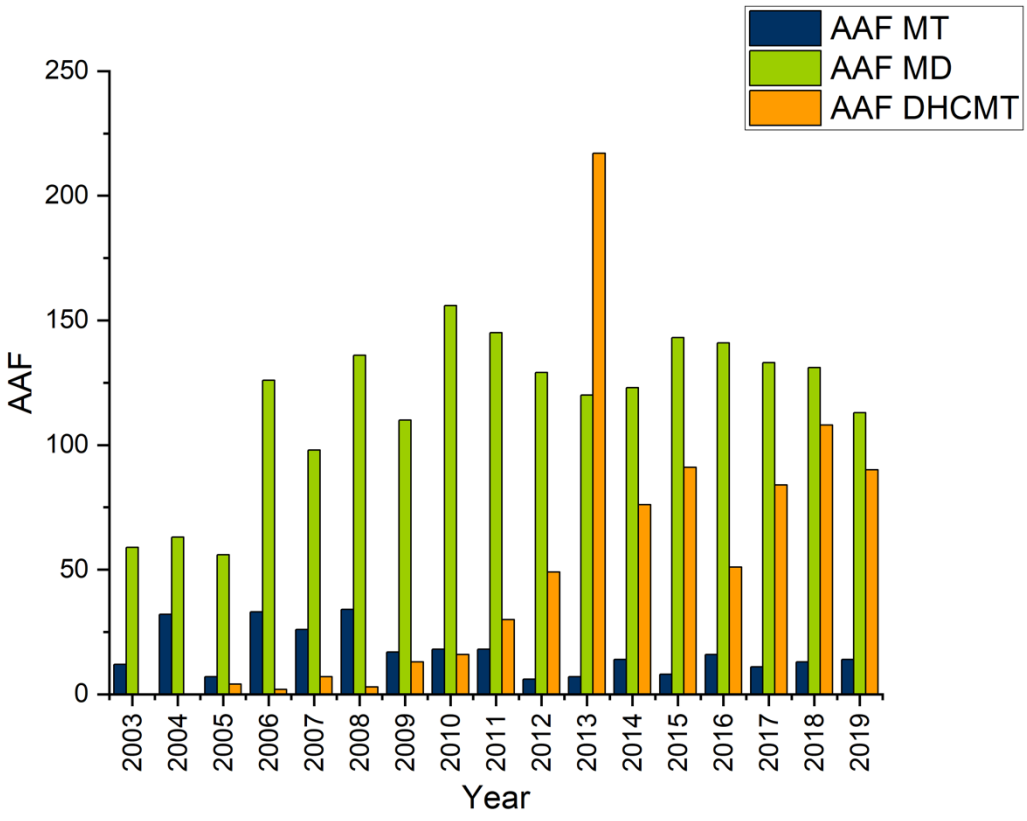


Figure 8: *AAFs of 17-methyltestosterone, metandienone, and dehydrochloromethyltestosterone between 2003 and 2019 [53-69]*

2.3.5 Metabolism of AAS

AAS are lipophilic substances that are hardly soluble in water [77]. They are transported through the body by binding to transport proteins. This complex is not excreted due to renal reabsorption mechanisms. This also applies to the small amount of free hormone [13]. Thus, AAS are often extensively metabolized by various enzymes as part of biotransformation [19]. Based on these modifications, the body can eliminate the drug via urine or other hydrophilic systems like sweat [78].

Because of that, methods in anti-doping analysis are not only targeting the prohibited compound itself (also called "parent compound") but also its different metabolites. Moreover, many of these metabolites are excreted over a more extended period than the parent compound, allowing to prove the misuse even after none of the original substance is traceable anymore.

The discovery of new metabolites can help convict athletes of doping even years after taking the sample. The storage of samples for up to ten years was implemented by the IOC for the Summer Olympics in Athens in 2004. The first reanalysis of samples from the Olympic Games took place in 2009, six months after the Beijing Games. Substantial discoveries of new long-term metabolites in AAS group were provided by the anti-doping laboratory in Cologne in 2006 or by the anti-doping laboratory in Moscow in 2012 [73, 74]. These findings resulted in an increased number of AAFs over the following years [57, 63, 79].

AAS can be metabolized in various positions and ways. For example, phase I metabolism includes reductions, oxidations (hydroxylation as a particular case of oxidation processes), and hydrolysis [80].

Hydroxylation reactions can nearly take place in every part of the steroid backbone. They have been reported for positions 1, 2, 4, 6, 7, 11, 12, 15, 16, 18, 19, and 20 [31, 81-84].

Oxidations of hydroxy groups and the introduction of new double bonds are also common reactions. Reductions of double bonds and oxo groups are also part of the phase I metabolism of AAS, as already described in 2.2.2 [81, 83].

The epimerization of position 17 is well known for 17α-methyl steroids, e.g., bolasterone (7α,17α-dimethyl-17β-hydroxy-androst-4-en-3-one) or MD. First, the 17β-hydroxy group is sulfonated, followed by the loss of the newly formed sulfate as anion. The resulting carbocation is then attacked by water to create either the 17α-hydroxy-

17β-methyl structure, the original 17β-hydroxy-17α-methyl element, or different side products [81, 85]. A relatively newly discovered kind of metabolization is the rearrangement of the D-ring in 17α-methyl steroids, e.g., in MD or DHCMT. The first part is similar to the epimerization. Still, instead of the water attack, the methyl group in position 13 (which is C-18) is transferred to the sp^2-hybridized and positively charged C-17 (Wagner-Meerwein rearrangement) followed mainly by elimination of H-14 to form the C-13(14) double bond. After this introduction of the double bond, the formed 18-nor derivative is hydroxylated in position 20 by CYP21A2 [86, 87]. The formation of the 17-epimer and the rearranged D-ring metabolite in the case of MD are displayed in **Figure 9**. Other pathways have been described for MD involving different enzymes (CYP3A4, CYP11B2), e.g., the hydroxylation of the methyl group in position 18 and subsequently the Wagner-Meerwein-rearrangement [84]. The positions of potential metabolic phase I reactions for MD are shown in **Figure 10**.

The processes of phase II metabolism include the conjugation with glucuronic acid or sulfates of the parent compound or the compounds formed after phase I metabolization. Like this, even more polar substances are formed. These conjugates can then be excreted easily into the urine [20].

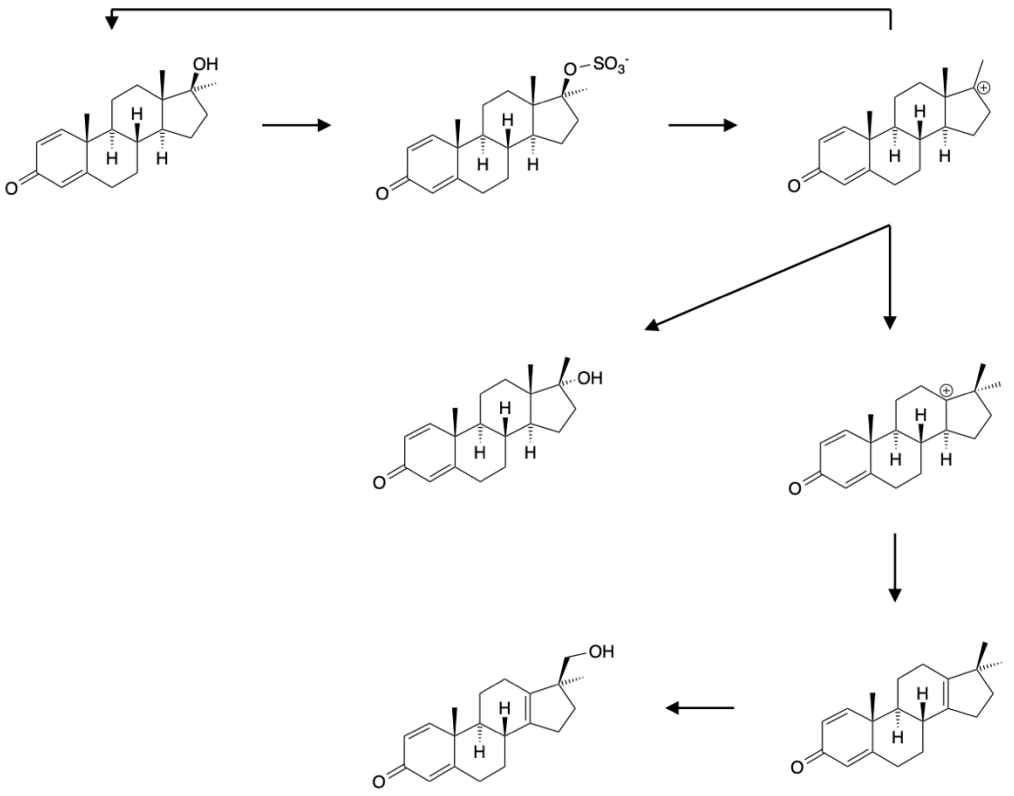


Figure 9: *Proposed formation of 17-epimer and rearranged D-ring; adapted from [81] and [86]*

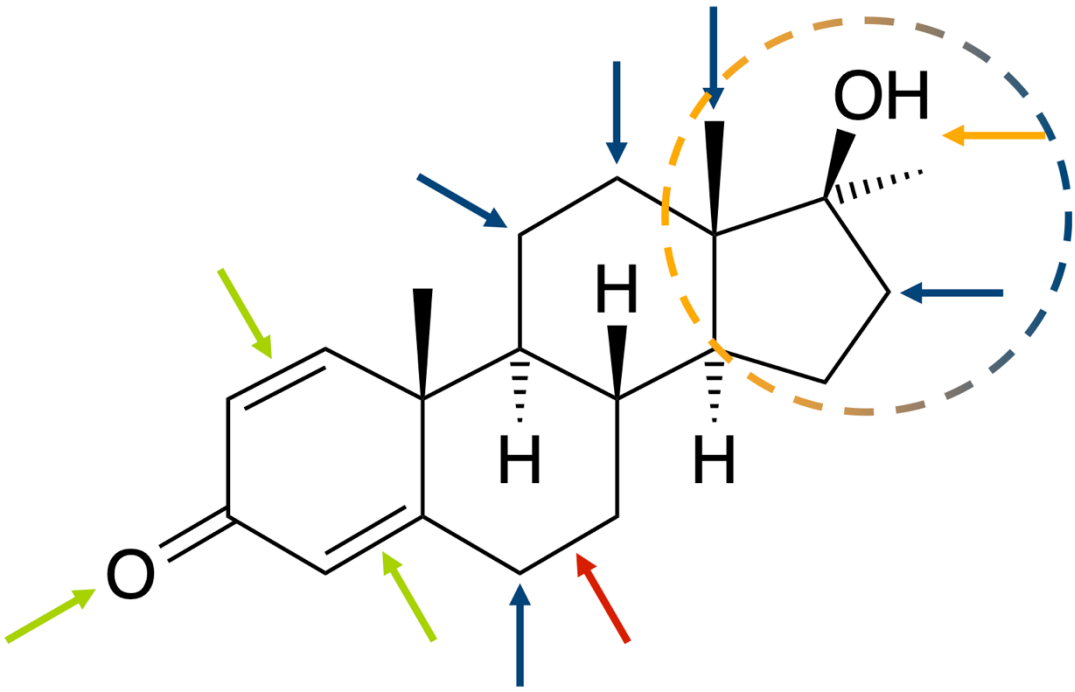


Figure 10: *Described positions of metabolization reactions for metandienone; red: introduction of a double bond, green: reductions of double bonds or oxo groups, blue: hydroxylations, orange: epimerization or rearrangements [81, 84, 85, 88-92]*

2.4 Cytochrome P450

Cytochrome P450 (CYP enzymes, CYPs) are enzymes that play a crucial role in the human metabolism of endogenous and exogenous substances.

In humans, 57 different CYP enzymes are known to be expressed [93]. They contain a single heme molecule and can be divided into three distinct classes depending on their location: in the membrane of the endoplasmic reticulum, mitochondria, or cytosol [17, 94, 95].

CYPs act as monooxygenases by catalyzing reactions following the equation:

$$R\text{-}H + NADPH + H^+ + O_2 \rightarrow R\text{-}OH + NADP^+ + H_2O \text{ [96]}$$

Most of the described reactions in steroidogenesis (2.2.2) are catalyzed by CYP enzymes, which explains the severe symptoms of people with a deficiency or excess of these enzymes, e.g., patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency or isolated 17,20-lyase deficiency.

About ten hepatic CYPs are responsible for the oxidative metabolism of xenobiotics; seven of them account for 90 % of all drugs (CYP3A4, CYP3A5, CYP3A7, CYP2B6, CYP2C9, CYP2C19, CYP2D6) [80, 97]. Therefore, they are an integral part of human metabolism, whether endogenous or exogenous substances.

Some cytochrome P450 enzymes are subject to polymorphism. This can change the activity of the enzyme and therefore affect the whole metabolic process. Well-known representatives are 1A2, 2D6, 2C9, and 2C19 [98]. CYP2D6 polymorphism, for example, may result in people with non-functioning (poor metabolizer), semi-functioning (intermediate metabolizer), functioning (extensive metabolizer) or over-functioning enzymes (ultra-rapid metabolizer). This also plays a role in pharmacotherapy and clinical therapy [99, 100].

CYP enzymes can also be induced or inhibited in their activity by food ingredients and other drugs [101]. This may result in higher or lower concentrations of the administered drug and, therefore, causing severe side effects or no effect at all [102]. Through specific nutrition, these effects can be forced or prevented.

3 Manuscripts

3.1 Manuscript I: Corticosteroid Biosynthesis Revisited: No Direct Hydroxylation of Pregnenolone by Steroid 21-Hydroxylase

Steffen Loke, Anna Stoll, David Machalz, Francesco Botrè, Gerhard Wolber, Matthias Bureik, Maria Kristina Parr

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Abstract: Cytochrome P450s (CYPs) are an essential family of enzymes in the human body. They play a crucial role in metabolism, especially in human steroid biosynthesis. Reactions catalyzed by these enzymes are highly stereo- and regio-specific. Lack or severe malfunctions of CYPs can cause severe diseases and even shorten life. Hence, investigations on metabolic reactions and structural requirements of substrates are crucial to gain further knowledge on the relevance of different enzymes in the human body functionalities and origin of diseases. One key enzyme in the biosynthesis of gluco- and mineralocorticoids is CYP21A2, also known as steroid 21-hydroxylase. To investigate the steric and regional requirements of substrates for this enzyme, we performed whole-cell biotransformation assays with the fission yeast *Schizosaccharomyces pombe* recombinantly expressing CYP21A2. The progestogens progesterone, pregnenolone, and their 17α-derivatives were used as substrates. After incubation, samples were analyzed using gas chromatography coupled to mass spectrometry. For progesterone and 17α-progesterone, their corresponding 21-hydroxylated metabolites desoxycortone and cortodoxone were detected, while after incubation of pregnenolone and 17α-pregnenolone, no hydroxylated product was observed. Findings were confirmed with authentic reference material. Molecular docking experiments agree with these results and suggest that interaction between the 3-oxo group and arginine-234 of the enzyme is a strict requirement. The presented results demonstrate that the presence of an oxo-group in position 3 of the steroid is indispensable, where a 3-hydroxy group prevents hydroxylation in position C-21 by CYP21A2. This knowledge may be transferred to other CYP21A2 substrates and hence help to gain essential insights into steroid metabolism.

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ORIGINAL RESEARCH
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Corticosteroid Biosynthesis Revisited: No Direct Hydroxylation of Pregnenolone by Steroid 21-Hydroxylase

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Cytochrome P450s (CYPs) are an essential family of enzymes in the human body. They play a crucial role in metabolism, especially in human steroid biosynthesis. Reactions catalyzed by these enzymes are highly stereo- and regio-specific. Lack or severe malfunctions of CYPs can cause severe diseases and even shorten life. Hence, investigations on metabolic reactions and structural requirements of substrates are crucial to gain further knowledge on the relevance of different enzymes in the human body functions and the origin of diseases. One key enzyme in the biosynthesis of gluco- and mineralocorticoids is CYP21A2, also known as steroid 21-hydroxylase. To investigate the steric and regional requirements of substrates for this enzyme, we performed whole-cell biotransformation assays using a strain of fission yeast *Schizosaccharomyces pombe* recombinantly expressing CYP21A2. The progestogens progesterone, pregnenolone, and their 17α-hydroxy-derivatives were used as substrates. After incubation, samples were analyzed using gas chromatography coupled to mass spectrometry. For progesterone and 17α-hydroxyprogesterone, their corresponding 21-hydroxylated metabolites 11-deoxycorticosterone and 11-deoxycortisol were detected, while after incubation of pregnenolone and 17α-hydroxypregnenolone, no hydroxylated product was observed. Findings were confirmed with authentic reference material. Molecular docking experiments agree with these results and suggest that interaction between the 3-oxo group and arginine-234 of the enzyme is a strict requirement. The presented results demonstrate once more that the presence of an oxo-group in position 3 of the steroid is indispensable, while a 3-hydroxy group prevents hydroxylation in position C-21 by CYP21A2. This knowledge may be transferred to other CYP21A2 substrates and hence help to gain essential insights into steroid metabolism.

Keywords: corticosteroid, cytochrome P450, CYP21A2, GC-MS, fission yeast (*Schizosaccharomyces pombe*), molecular docking, steroid biosynthesis

INTRODUCTION

Cytochrome P450 enzymes (CYPs) are an important class of enzymes in the human body. Acting mainly as hydroxylases, they are responsible for a wide variety of metabolic reactions. CYPs form a remarkably versatile enzyme superfamily that has been found in all domains of life and already includes more than 370,000 named members (1). In humans, there are 57 isoforms that can be subdivided by sequence homology into 18 families and 43 subfamilies. However, they may also be categorized according to their subcellular localization, their electron transport chains, or their biological functions (2, 3). On the one hand, substances like xenobiotic drugs can be excreted easier after CYP catalyzed hydroxylation; on the other hand, they also play an essential role in the synthesis of many endogenous compounds (4). Six of these enzymes are reported to fulfill critical physiological functions in steroid hormone biosynthesis, namely CYP11A1 (cytochrome P450 scc, side-chain cleavage enzyme), CYP17A1 (cytochrome P450 17α, steroid 17α-hydroxylase and 17, 20-lyase), CYP21A2 (cytochrome P450 c21, steroid 21-hydroxylase), CYP11B1 (cytochrome P450 11β, steroid 11β-hydroxylase), CYP11B2 (cytochrome P450 aldo, aldosterone synthase) and CYP19A1 (cytochrome P450 aro, aromatase) (5).

Steroidogenic CYPs display high substrate specificity, which only allows a small group of substances with a defined structure to be transformed. This regio- and stereospecific oxy functionalization is of high biological relevance, and especially CYPs involved in steroid biosynthesis have been considered to be unable to metabolize xenobiotics. In the last decade, this hypothesis, however, was demonstrated to be not correct (6–10). Still, strict structural requirements for substrates have been demonstrated. As recently reported, substrates of CYP21A2 require a 3-oxo functionality at the steroid A-ring to be successfully hydroxylated at the side chain attached to the D-ring (7).

CYP21A2 is an important enzyme in the biotransformation of progestogens into corticosteroids. The enzyme converts the endogenous progestogens progesterone (pregn-4-ene-3,20-dione, PRO) and 17α-hydroxyprogesterone (17α-hydroxypregn-4-ene-3,20-dione, 17αOH-progesterone, 17PRO) to 11-deoxycorticosterone (21-hydroxypregn-4-ene-3,20-dione, DOC) and 11-deoxycortisol (17α,21-dihydroxypregn-4-ene-3,20-dione, RSS), respectively, by hydroxylation at the C-21 position (11). Therefore, it plays an essential role in the biosynthesis of both gluco- and mineralocorticoids. Hence, humans with reduced activity of CYP21A2 may suffer from congenital adrenal hyperplasia (CAH), which is characterized by decreased production of cortisol (12). Due to feedback mechanisms of the hypothalamic-pituitary-adrenal axis, increased concentrations of progestogens, but also of androgens resulting from alternative metabolic pathways, are observed in these patients (13).

Based on recent findings on substrate selectivity of human CYP21A2, this work gives a deeper insight into the endogenous compounds PRO, 17PRO, pregnenolone (PRE), and 17α-hydroxypregnenolone (17PRE). These substances have been reported as substrates of the enzyme in the KEGG database and by Cathro et al. (14). The data presented in this work help to extrapolate findings to other potential substrates of CYP21A2 and may thus help to identify new metabolites or inhibitors of the enzyme.

MATERIALS AND METHODS

Chemicals and Reagents

The substrates PRO, PRE, 17PRO, and 17PRE as well as the analytical references DOC and RSS, were obtained from Steraloids Inc. (Newport, Rhode Island, USA). *N*-Methyl-*N*-(trimethylsilyl)trifluoro acetamide (MSTFA) was purchased from Chemische Fabrik Karl Bucher GmbH (Waldstetten, Germany). All other chemicals and solvents were purchased from AppliChem GmbH (Darmstadt, Germany), Merck (Darmstadt, Germany), Karl Roth (Karlsruhe, Germany), Sigma Aldrich (Steinheim, Germany), Thermo Fischer (Karlsruhe, Germany), or VWR International (Darmstadt, Germany).

GC-MS Analyses

Gas chromatographic-mass spectrometric (GC–MS) analysis of the samples was performed on an Agilent 7890A gas chromatographic system coupled to an Agilent 5975 C inert mass selective detector equipped with an Agilent HP1 column (17 m, 0.2 mm id, 0.11 µm film thickness). The following parameters were used for the analysis of products: carrier gas: helium, oven program: 183 °C, +3 °C/min to 232 °C (rate 1), +40 °C/min to 310 °C (rate 2), hold for 2 min, injection volume: 2 µL, split 16:1, injection temperature: 300 °C, electron ionization (EI): 70 eV, full scan mode from m/z 40 to m/z 1000. Prior to GC–MS analysis the dried residues were pertrimethylsilylated (TMS derivatives) by reaction with 100 µL TMIS reagent (MSTFA/ammonium iodide/ethanethiol, 1000:2:3, v:w:v) at 75 °C for 20 min.

Steroid 21-Hydroxylation Assay

All experiments were conducted using the recombinant fission yeast strain CAD75. As reported earlier, it co-expresses the recombinant human steroid 21-hydroxylase together with human cytochrome P450 reductase (CPR) (genotype: *h- ura4-D.18 leu1:: pCAD1-hCPR/pNMT1-CYP21A2*) (15). Whole-cell biotransformation assays were performed as described before (7). In brief, cells were cultured for three days at 30 °C on plates containing 5 µM thiamin. Pre- and main cultures were prepared in Edinburgh Minimal Medium (EMM) (8). Substrates, dissolved in ethanol (PRO, PRE, 17PRO) or methanol/dimethyl sulfoxide (1 + 1 v/v) (17PRE), were added in a concentration of 500 µM to cell suspension of CAD75 fission yeast in EMM and incubated for 72 h at 30 °C under mild agitation. The cofactor NADPH (needed for the P450-dependent bioconversion) is provided by the fission yeast cells itself in sufficient quantity (16). After centrifugation and separation from the cell pellet, the

supernatant was extracted with ethyl acetate. The organic phase was dried, and product analysis was performed by GC-MS after trimethylsilyl-derivatization, as described in the section *GC-MS Analyses*.

Molecular Modeling

Molecular docking experiments were performed using GOLD software [Genetic Optimisation for Ligand Docking, The Cambridge Crystallographic Data Centre, UK (17, 18) v.5.2] with 10 genetic algorithm (GA) runs. 3D starting geometries for substrates PRE, 17PRO, and 17PRE were obtained with CORINA [3D Structure Generator CORINA Classic, Molecular Networks GmbH, Nuremberg, Germany (19)]. The X-ray structure of CYP21A2 co-crystallized with PRO [PDB: 4Y8W (20)] served as protein conformation. The binding pocket was defined by a sphere of 12 Å radius with the C-8 atom of PRO as its center. GOLD was executed with standard settings, besides setting search efficiency to 200% and enabling the 'Generate diverse solutions' option. Key binding site residue Arg234 was marginally rotated during its energy minimization conducted in the MOE software package (Molecular Operating Environment 2019.01; Chemical Computing Group ULC, Montreal, Canada) to allow for optimal hydrogen bonding to 17PRE. This Arg234 orientation was kept for all other compounds. Subsequently, energy minimization of and 3D pharmacophore modeling for the obtained poses was conducted in LigandScout (21, 22) v.4.2. using the MMFF94 force field (23).

RESULTS

Steroid 21-Hydroxylation Assay

The substrates PRO, PRE, 17PRO, and 17PRE, were incubated with steroid 21-hydroxylase in a whole-cell biotransformation assay. After work-up, the GC-MS analysis of the incubation

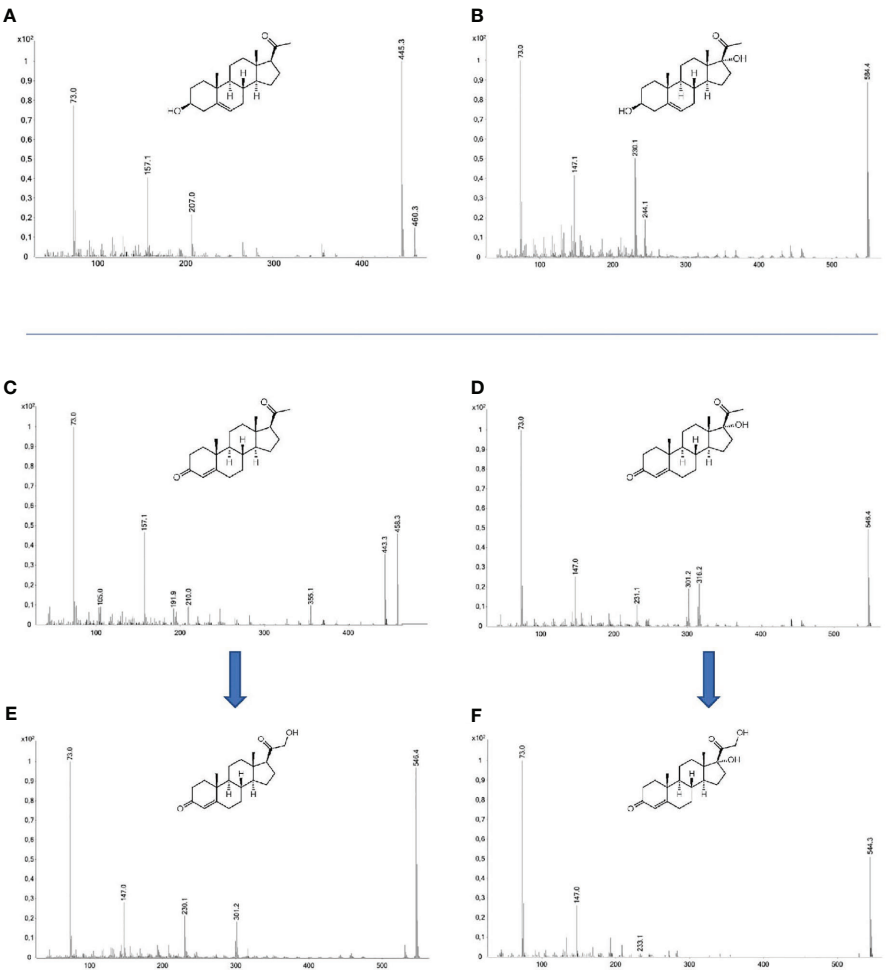

FIGURE 1 | Mass spectra (GC-EI-MS) of the TMS derivatives of pregnenolone **(A)**, 17α-hydroxypregnenolone **(B)**, progesterone **(C)**, 17α-hydroxyprogesterone **(D)**, 11-deoxycorticosterone **(E)**, 11-deoxycortisol **(F)**; abscissa: m/z, ordinate. intensity normalized to the highest peak.

broth showed characteristic peaks for the 21-hydroxylated products of PRO and 17PRO. The product of PRO showed peaks at m/z 546 and a loss of 245, the product of 17PRO showed an abundant peak at m/z 544 and a subsequent loss of 311. The corresponding mass spectra of the TMS derivatives are presented in **Figures 1E, F**. Additionally, peaks of the remaining substrate were present in the chromatograms as well (mass spectra in **Figures 1C, D**). Product structures were deduced from the mass spectra from fragmentation analysis and later confirmed by comparison with an authentic reference material of DOC and RSS analyzed by GC-MS in parallel. Similar retention times and characteristic fragment ions were obtained for both, reference and products of hydroxylation.

In contrast, no hydroxylated substrate was detectable after incubation of PRE or 17PRE. Mass spectra of substrates are displayed in **Figures 1A, B**. Comparison of the chromatograms revealed no product formation for these two substrates. The chromatograms of the incubated substances in comparison with the authentic reference material are displayed in **Figure 2**. As reported earlier, multiple peaks may be detected for one analyte due to the formation of derivatization isomers (24).

Modeling Binding of 17PRO, PRE and 17PRE to Steroid 21-Hydroxylase

In order to rationalize the observed differences in the 21-hydroxylation activity of CYP21A2, docking experiments for

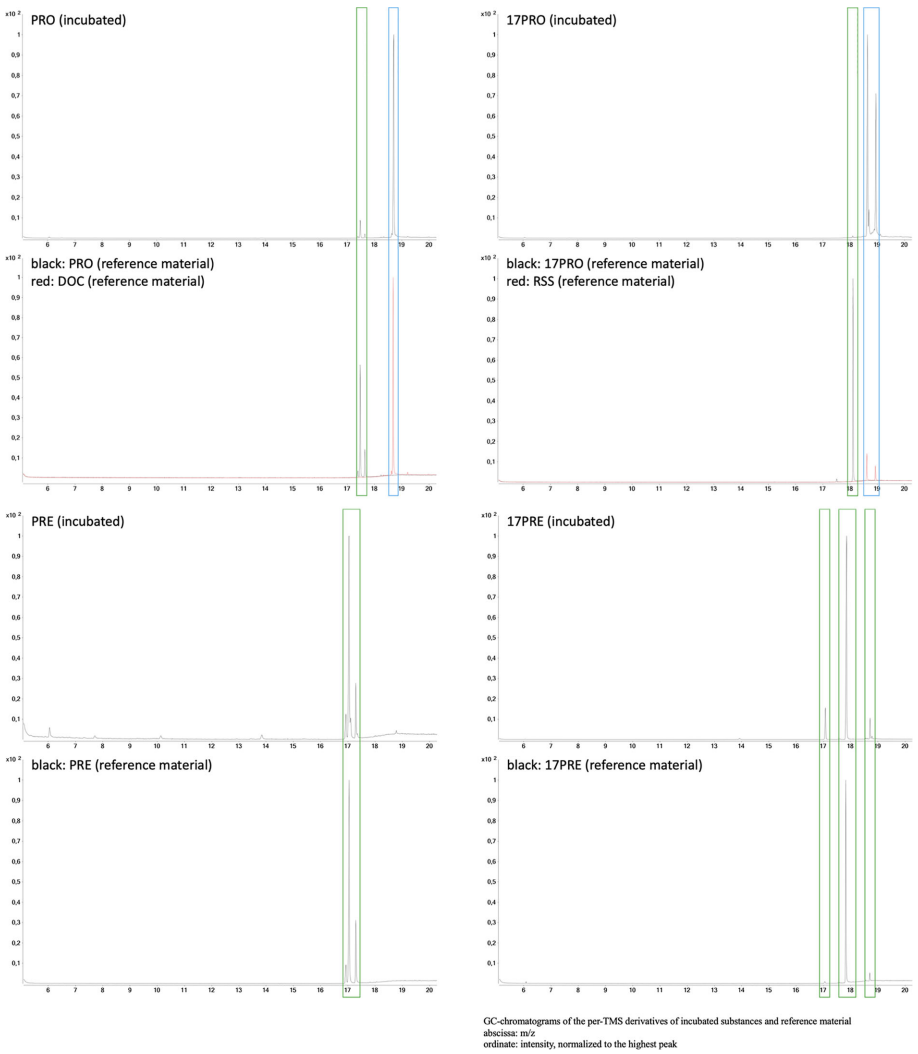

FIGURE 2 | Upper two rows: Chromatograms (GC-EI-MS) of the TMS derivatives of progesterone (PRO), 17-hydroxyprogesterone (17PRO), and their hydroxylated products after incubation in comparison to the authentic reference material (due to the formation of derivatization isomers, multiple peaks are obtained for one analyte). Lower two rows: Chromatograms (GC-EI-MS) of the TMS derivatives of pregnenolone (PRE) and 17-hydroxypregnenolone (17PRE) after incubation in comparison to the authentic reference material (due to the formation of derivatization isomers, multiple peaks are obtained for one analyte).

PRE, 17PRO and 17PRE were performed based on the X-ray structure of CYP21A2 co-crystallized with PRO [PDB: 4Y8W (20), **Figure 3**]. The catalytically competent binding mode of PRO present in the X-ray structure places the C-21 atom 4 Å away from the heme iron. The hydrogen bond of Arg234 and the oxo group at C-3 of the substrate is critical to catalytically competent binding (7) (**Figure 3A**). According to the pharmacophore model the substrate methyl groups of PRO and 17PRO form hydrophobic contacts to the active site of CYP21A2. The key hydrogen bond to Arg234 is also present in the pharmacophore model of the most plausible docking pose of 17PRO, which is highly similar to the orientation of PRO (**Figure 3B**). The C-21 atoms of PRO and 17PRO are in a highly similar position (4 Å away from the heme) and hence the 17PRO pose is deemed catalytically competent as well.

In contrast, the most plausible docking poses of PRE and 17PRE corresponding to catalytically competent orientation do not involve hydrogen bonding to key residue Arg234 (**Figures 3C, D**), because the hydroxy groups at C3 represent only very weak hydrogen bond acceptor properties. We suggest that these orientations and productive binding of PRE or 17PRE to steroid 21-hydroxylase (CYP21A2) does not occur *in vivo*. Hence, we further suggest that conversion of PRE and 17PRE to 21-hydroxypregnenolone (T) and 17, 21-dihydroxypregnenolone (R), respectively, is highly unlikely.

DISCUSSION

As expected, PRO and 17PRO are substrates for 21-hydroxylation catalyzed by human CYP21A2 (**Figure 4A**). The resulting products were confirmed as DOC and RSS by GC-MS comparison with authentic reference material [confidence level 1, according to Schymanski et al. (25)]. GC-MS analysis showed a characteristic loss of 245 for DOC corresponding to a fragment of the hydroxylated D-ring (**Figure 1E**). For RSS, no peak for the molecular ion (m/z 634) could be detected (**Figure 1F**). However, a signal with m/z 544 was observed, which is identified as $[M\text{-}90]^+$ (a loss of TMSOH), and a subsequent loss of 311, resulting in a fragment with m/z 233, which is caused by B/C-ring fragmentation.

The rates of successful hydroxylation in the assays were calculated by comparison of the areas in the GC-chromatograms. After 72 h PRO was hydroxylated to 92%, 17PRO was hydroxylated to over 99%. In a previous publication the product space-time yields for the conversion of

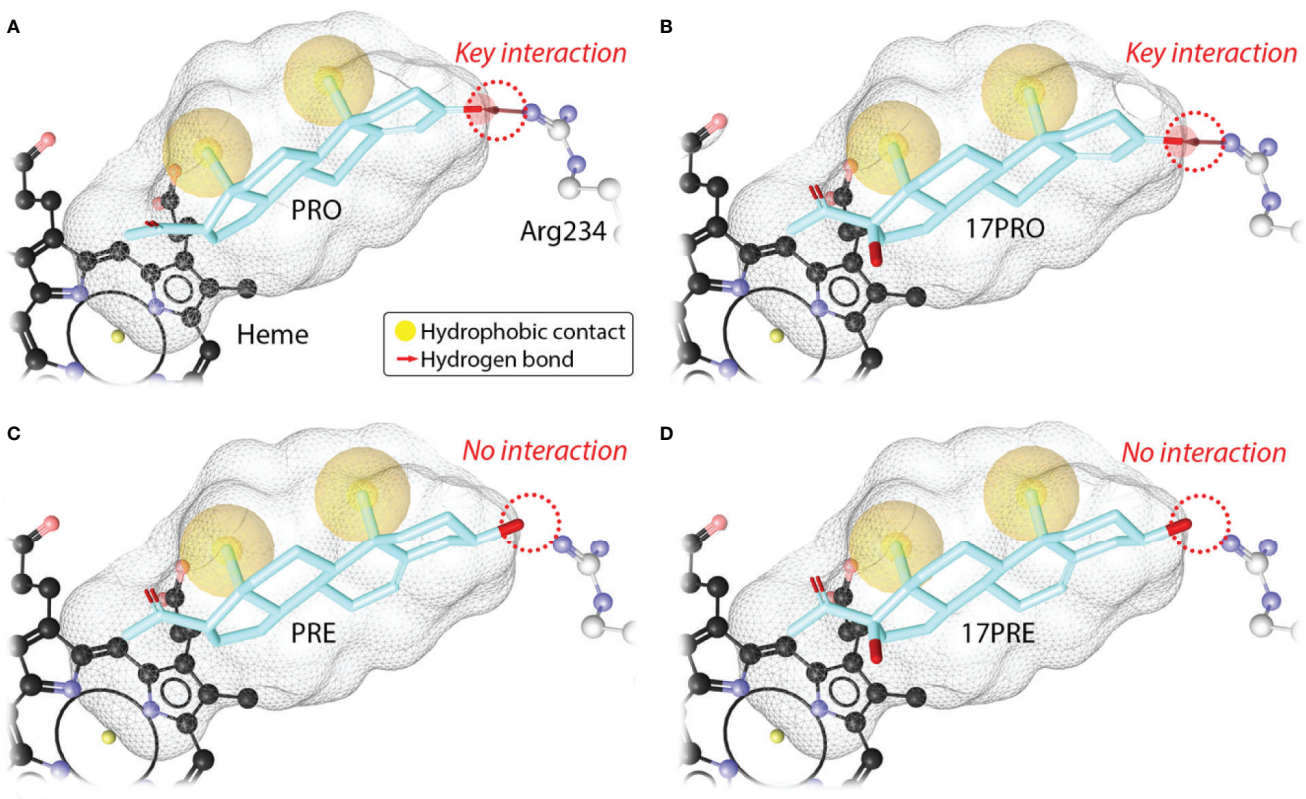

FIGURE 3 | Substrate binding to the active site of CYP21A2. Progesterone binding mode from X-ray structure (PDB: 4Y8W) (**A**, PRO). Suggested binding modes of 17α-hydroxyprogesterone (**B**, 17PRO), pregnenolone (**C**, PRE) and 17α-hydroxypregnenolone (**D**, 17PRE) obtained from docking. Active substrates **(A, B)** form the key interaction to Arg234. Inactive substrates **(C, D)** do not show this interaction, which makes productive binding highly unlikely.

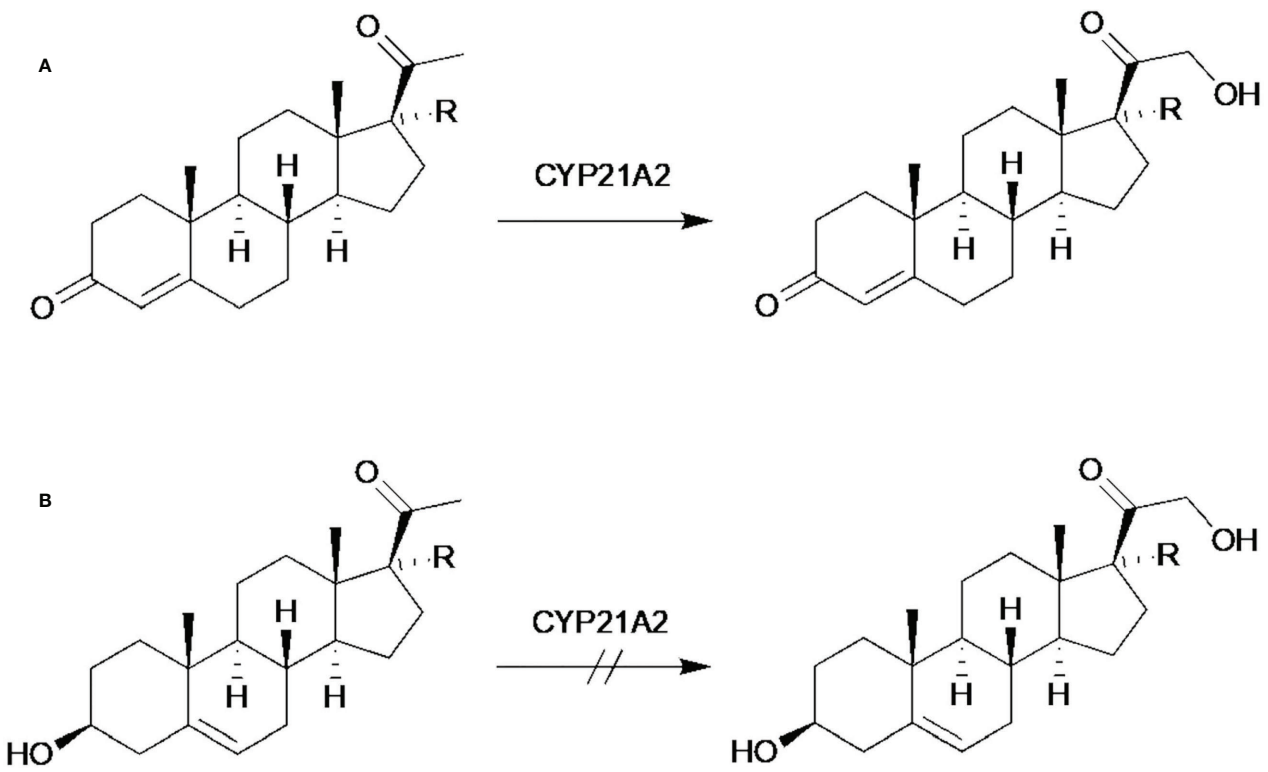

FIGURE 4 | A-ring structure dependent hydroxylation of progestogens **(A)** successful hydroxylation of 3-keto steroids (R = H = progesterone; R = OH = 17α-hydroxyprogesterone), **(B)** no hydroxylation of 3-hydroxy steroids (R = H = pregnenolone; R = OH = 17α-hydroxypregnenolone).

PRO (1.6 ± 0.5 µmol/g/day) and 17PRO (2.1 ± 0.7 µmol/g/day) by whole-cell biotransformation with strain CAD75 were reported (15). The absence of any signal for potential products of hydroxylation in case of PRE and 17PRE substantiates the hypothesis of no hydroxylation in the latter cases.

These results are in line with common knowledge of steroid biosynthesis, where the progestogens PRO and 17PRO are converted to corticoids (5) by 21-hydroxylation. Similarly, Lattemann et al. (26) reported the successful 21-hydroxylation of 3-keto steroids in whole-cell biotransformation utilizing human CYP21A2 recombinantly expressed in *E. coli*. They disclosed the successful conversion of natural and non-natural steroid substrates, all showing a 3-oxo group. As demonstrated in earlier experiments, the A-ring composition of CYP21A2-substrates plays a crucial role in hydroxylation. It was demonstrated that the 3-oxo group is a strict prerequisite for CYP21A2 substrates (7). In line with these findings, the incubation of endogenous 3-hydroxy steroids PRE and 17PRE did not lead to their 21-hydroxylated analogs (**Figure 4B**). No signals corresponding to hydroxylated metabolites of PRE or 17PRE were detected following their incubation with CYP21A2. Molecular docking experiments supported the structure requirements. Interaction of the carbonyl group at C-3 of the substrate to the side-chain Arg234 of the enzyme is indispensable.

In contrast, earlier reports from literature postulate the 21-hydroxylation of PRE based on *in vitro* experiments utilizing adrenal microsomes (27). Human adrenal microsomes yielded R after incubation of PRE in their assay. Already reported in the 1960s, PRE is transformed into PRO prior to 21-hydroxylation (28). Significant amounts of T *in vivo* are reported in newborn infants (day 0-3 after delivery) or adult males after corticotrophin stimulation, while generally only small amounts of T were detected in adult humans (29, 30). Cathro et al. thus postulated the direct 21-hydroxylation as a subsidiary pathway in humans (30). Furthermore, Cathro et al. (14) postulated the direct 21-hydroxylation of PRE, while 17PRE is reported to undergo side-chain cleavage to yield dehydroepiandrosterone (DHEA). However, in 1980 Kaufmann et al. hypothesized that alternative pathways of corticosteroid biosynthesis exist. As postulated, T, and R are most likely generated *via* 21-hydroxy- or 17,21-dihydroxydesmosterol involving adrenocorticotropin (ACTH) rather than direct hydroxylation of PRE or 17PRE (31). According to our results we consider it likely that also the 21-hydroxylation of desmosterol is not catalyzed by steroid 21-hydroxylase in humans (**Figure 5**). The presented results hence help to better understand corticoid formation and may thereby provide important basic knowledge for the treatment of diseases that are linked to enzyme deficiencies involved in corticoid biosynthesis.

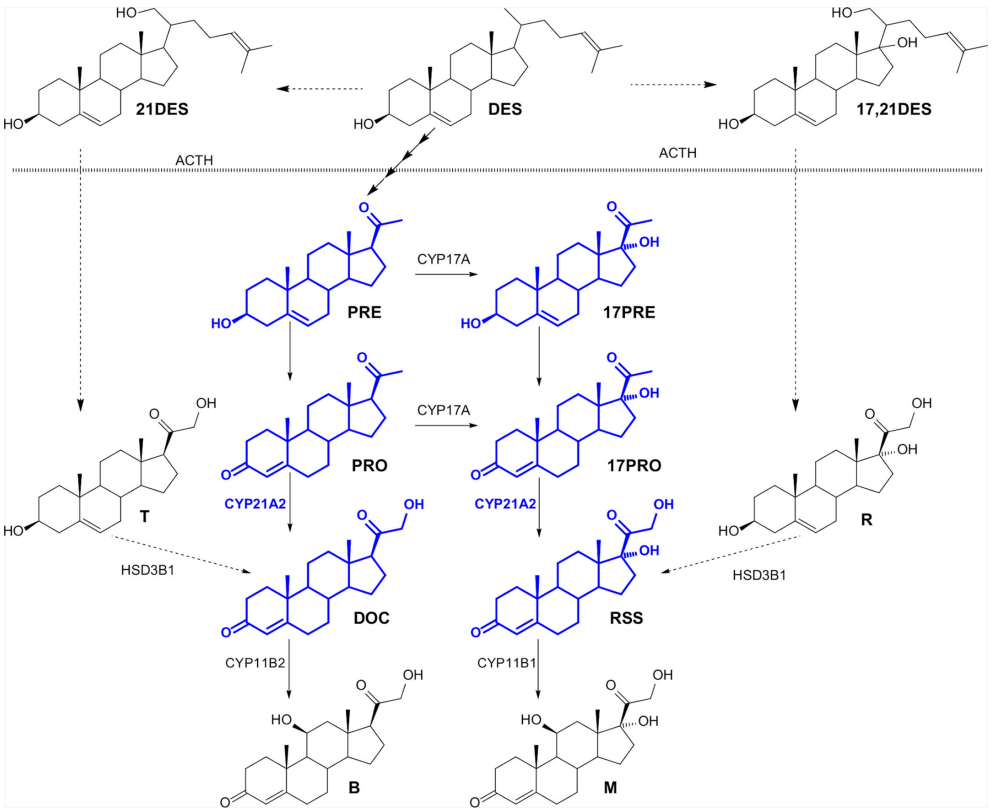

FIGURE 5 | Hypothesized corticosteroid biosynthesis, adapted from Kaufmann et al. (31) steroid precursor desmosterol (DES) is converted *via* different pathways to corticosterone (B) and cortisol (M) involving 21-hydroxydesmosterol (21DES), 17,21-dihydroxydesmosterol (17,21DES), pregnenolone (PRE), 17-hydroxypregnenolone (17PRE), progesterone (PRO), 17-hydroxyprogesterone (17PRO), 11-deoxycorticosterone (DOC), 21-hydroxypregnenolone (T), 11-deoxycortisol (RSS) and 17,21-dihydroxypregnenolone (R).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

Conceptualization, MB and MP. *In vitro* methodology and data analysis, SL, AS, MB, and MP. *In silico* methodology and analysis, DM and GW. Resources, GW, MB, and MP. Data curation, SL, AS, and DM. Writing—original draft preparation, SL and MP. Writing—review and editing, AS, DM, GW, FB, and MB. Visualization, SL, AS, and DM. Supervision, GW and MP. Project administration, MP. Funding acquisition, FB, MB, and MP. All authors have read and agreed to the published version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.2 Manuscript II: Fine-mapping of the substrate specificity of human steroid 21-hydroxylase (CYP21A2)

Anna Stoll, Steffen Loke, Jan Felix Joseph, David Machalz, Xavier de la Torre, Francesco Botrè, Gerhard Wolber, Matthias Bureik, Maria Kristina Parr

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Abstract: Cytochrome P450 enzymes (CYPs) are capable of catalyzing regio- and stereo-specific oxy functionalization reactions, which otherwise are major challenges in organic chemistry. In order to make the best possible use of these biocatalysts it is imperative to understand their specificities. Human CYP21A2 (steroid 21-hydroxylase) acts on the side-chain attached to C-17 in ring D of a steroid substrate, but the configuration of ring A also plays a prominent role in substrate cognition. Here, we comprehensively investigated this relationship using sixteen 17,17-dimethyl-18-nor-13-ene steroids with different arrangements of hydroxy-, oxo-, fluoro- and chloro-groups and in the presence or absence of double bonds (Δ1 and/or Δ4) and heteroatoms in ring A. The results show that presence of a 3-oxo group is a strict requirement for a CYP21A2 substrate, while the other configurations tested were all tolerated. This was also confirmed by control experiments using endogenous steroids. While progesterone and 17-hydroxyprogesterone were hydroxylated at C-21, (17-hydroxy-) pregnenolone did not react. Molecular docking experiments indicate that the interaction of the carbonyl group at C-3 to the side-chain Arg234 of the enzyme is indispensable.

3.3 Manuscript III: New Insights into the Metabolism of Methyltestosterone and Metandienone: Detection of Novel A-ring Reduced Metabolites

Steffen Loke, Lingyu Liu, Maxi Wenzel, Heike Scheffler, Michele Iannone, Xavier de la Torre, Nils Schlörer, Francesco Botrè, Annekathrin Martina Keiler, Matthias Bureik, Maria Kristina Parr

Molecules 2021, 26(5), 1354 https://doi.org/10.3390/molecules26051354

Abstract: Metandienone and methyltestosterone are orally active anabolic-androgenic steroids with a 17α-methyl structure that are prohibited in sports but are frequently detected in anti-doping analysis. Following the previously reported detection of long-term metabolites with a 17ξ- hydroxymethyl-17ξ-methyl-18-nor-5ξ-androst-13-en-3ξ-ol structure in the chlorinated metandienone analog dehydrochloromethyltestosterone ("oral turinabol"), in this study we investigated the formation of similar metabolites of metandienone and 17α-methyltestosterone with a rearranged D-ring and a fully reduced A-ring. Using a semi-targeted approach including the synthesis of reference compounds, two diastereomeric substances, viz. 17α-hydroxymethyl-17β- methyl-18-nor-5β-androst-13-en-3α-ol and its 5α-analog, were identified following an administration of methyltestosterone. In post-administration urines of metandienone, only the 5β-metabolite was detected. Additionally, 3α,5β-tetrahydro-epi-methyltestosterone was identified in the urines of both administrations besides the classical metabolites included in the screening procedures. Besides their applicability for anti-doping analysis, the results provide new insights into the metabolism of 17α-methyl steroids with respect to the order of reductions in the A-ring, the participation of different enzymes, and alterations to the D-ring.

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New Insights into the Metabolism of Methyltestosterone and Metandienone: Detection of Novel A-ring Reduced Metabolites

Steffen Loke [1], Lingyu Liu [1], Maxi Wenzel [1], Heike Scheffler [1], Michele Iannone [2], Xavier de la Torre [2], Nils Schlörer [3], Francesco Botrè [2,4], Annekathrin Martina Keiler [5,6], Matthias Bureik [7], and Maria Kristina Parr [1,*]

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Abstract: Metandienone and methyltestosterone are orally active anabolic-androgenic steroids with a 17α-methyl structure that are prohibited in sports but are frequently detected in anti-doping analysis. Following the previously reported detection of long-term metabolites with a 17ξ-hydroxymethyl-17ξ-methyl-18-nor-5ξ-androst-13-en-3ξ-ol structure in the chlorinated metandienone analog dehydrochloromethyltestosterone ("oral turinabol"), in this study we investigated the formation of similar metabolites of metandienone and 17α-methyltestosterone with a rearranged D-ring and a fully reduced A-ring. Using a semi-targeted approach including the synthesis of reference compounds, two diastereomeric substances, viz. 17α-hydroxymethyl-17β-methyl-18-nor-5β-androst-13-en-3α-ol and its 5α-analog, were identified following an administration of methyltestosterone. In post-administration urines of metandienone, only the 5β-metabolite was detected. Additionally, 3α,5β-tetrahydro-epi-methyltestosterone was identified in the urines of both administrations besides the classical metabolites included in the screening procedures. Besides their applicability for anti-doping analysis, the results provide new insights into the metabolism of 17α-methyl steroids with respect to the order of reductions in the A-ring, the participation of different enzymes, and alterations to the D-ring.

Keywords: 17α-methyl steroids, long-term metabolites, gas chromatography-mass spectrometry, 17-hydroxymethyl-17-methyl-18-nor, D-ring alteration, doping control, metabolism

1. Introduction

Metandienone (17β-hydroxy-17α-methyl-androsta-1,4-dien-3-one, MD, **12**; list of steroids available in supplement S1) and methyltestosterone (17β-hydroxy-17α-methylandrost-4-en-3-one, MT, **18**) are anabolic-androgenic steroids. They were introduced to the market in 1960 (MD) [1] and 1939 (MT) [2] as orally active anabolic androgenic steroids. Although there is no approved drug available anymore, they are still widely marketed and misused as performance-enhancing drugs in sports, even though

they are prohibited in and outside of competition by the World Anti-Doping Agency [3]. Many so-called "adverse analytical findings" (AAFs) in doping control have been reported, and their numbers in the last 17 years are displayed in Figure 1 Over the last few years, metandienone and methyltestosterone represent 10% and 1% of all AAFs in the class of anabolic agents. In 2018, only five other substances out of all prohibited compound classes were identified more frequently than metandienone (n = 131), with clenbuterol giving the highest number (n = 320).

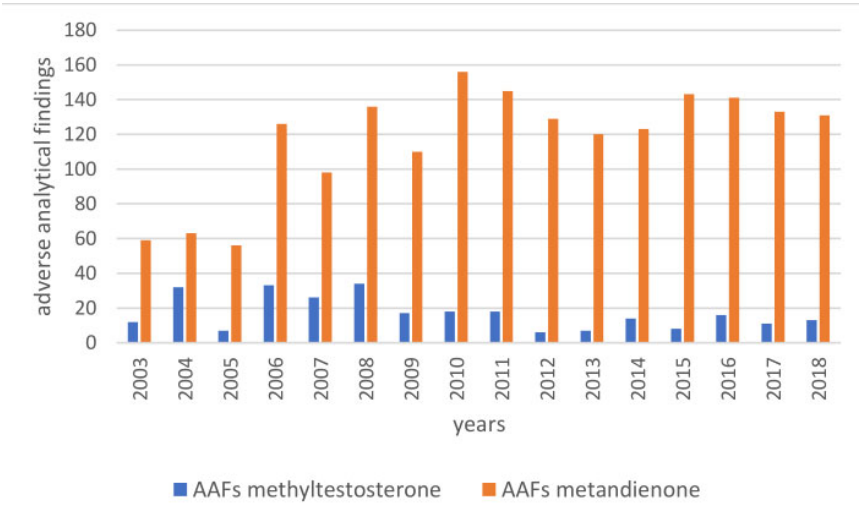

Figure 1. Adverse analytical findings of methyltestosterone and metandienone between 2003 and 2018, according to [4].

Due to the 17α-methyl group, the steroids become orally active, because it prevents the first-pass metabolism by hindering the oxidation of the 17β-hydroxy group sterically, while the introduction of a double bond in position 1 was intended to avoid aromatization and reduced the activity of A-ring-reducing enzymes [5–7].

Many metabolites related to the intake of metandienone are reported in the literature, and a number of these have been known for decades. These are generated by both phase I and phase II drug metabolizing enzymes. Phase I reactions include the introduction of a double bond at position 6, the reduction of double bonds in the A-ring, the reduction of the 3-oxo group, hydroxylations in positions 6, 11, 12, 16, or 18, epimerization in position 17, and rearrangement of the D-ring [8–15]. With respect to phase II reactions, both glucuronidation and sulfonation have been reported [16,17]. In recent years, new investigations on long-term metabolites of MD (**12**) identified further metabolites with 17β-hydroxymethyl-17α-methyl-13-ene structure [18–21]. The known metabolites are shown in Figure 2. Anti-doping laboratories mostly target the parent compound (**12**), 6-OH-metandienone (**13**), epi-metandienone (**14**), epi-metendiol (**15**), nor-epi-metendiol (**16**), and 20βOH-nor-metandienone (**17**) [18,22,23].

The intake of methyltestosterone leads to several metabolites, which derive from hydroxylations in positions 2, 4, 6, 11, or 20, reduction of the 4,5-double bond, reduction of the 3-oxo group, oxidation yielding a 1,2- or a 6,7-double bond, epimerization in position 17, and rearrangement of the D-ring [24–28]. Subsequent phase II reactions are also leading to both glucuronides and sulfates [26,29]. The structures of metabolites of MT (**18**) are shown in Figure 3. Laboratories mainly screen for the parent compound itself (**18**) and two reduced derivatives (3α5α-THMT, **19**; 3α5β-THMT, **20**). The metabolites of both substances are frequently monitored by gas chromatography-mass spectrometry after hydrolysis of the glycosidic bond of glucuronides as aglycons [22,23].

For other steroids with a similar structure, such as dehydrochloromethyltestosterone, there is a metabolite described with a fully reduced A-ring and a rearranged D-ring [30], which was synthesized in 2018 [31,32]. This metabolite led to an extended detection time of the intake for this substance and thereby increased the number of adverse analytical findings.

As the chemical structures of metandienone (**12**) and methyltestosterone (**18**) are similar to dehydrochloromethyltestosterone, it is conceivable that intake of these substances results in metabolites with a related structure. The discovery of such new metabolites may help in extending the time of detection after the intake of metandienone (**12**) or methyltestosterone (**18**), which would be a considerable contribution to the fight against doping, as cheating may be traced back over a longer period. Additionally, such findings may help to further elucidate the metabolism of synthetic steroids and therefore improve the understanding of human biotransformation.

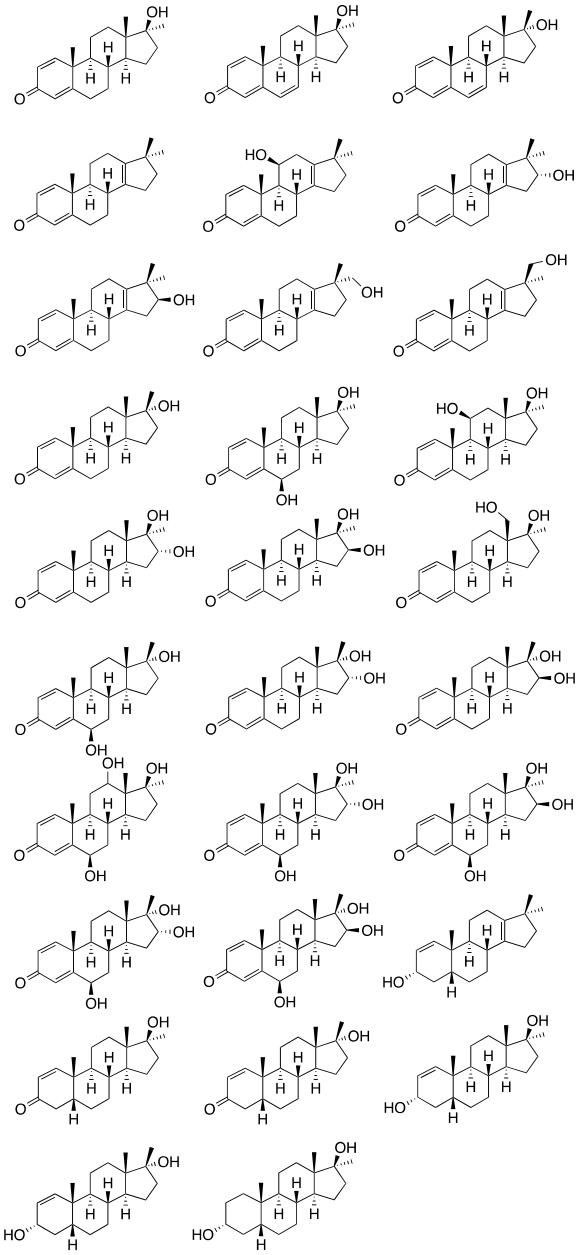

Figure 2. Chemical structures of phase I metabolites of metandienone reported in the literature.

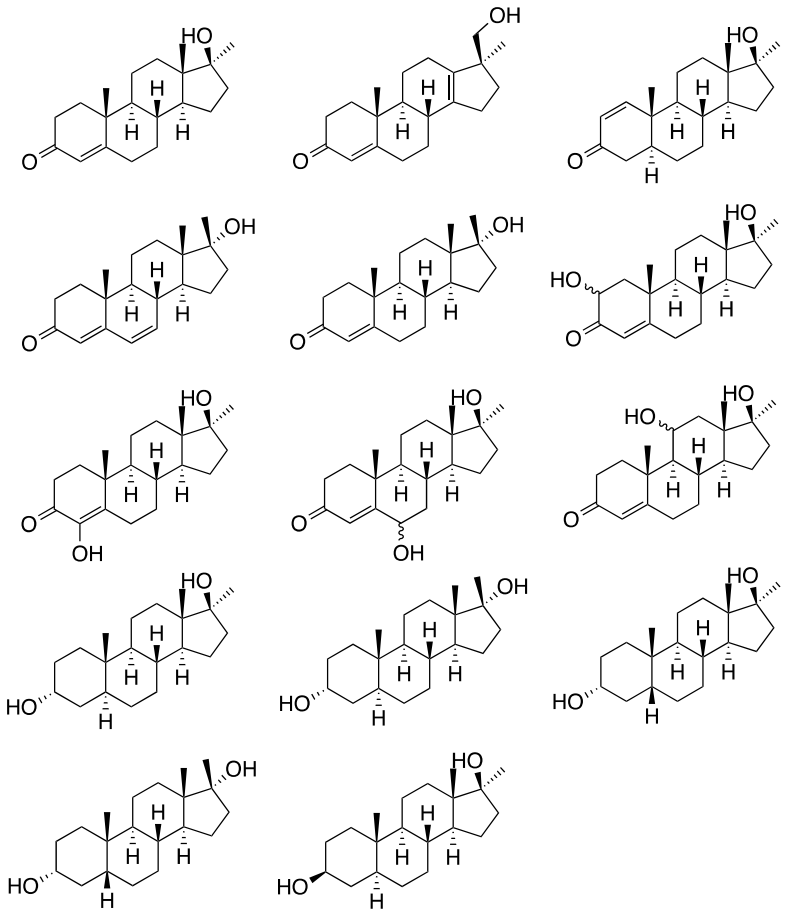

Figure 3. Chemical structures of phase I metabolites of methyltestosterone reported in the literature.

2. Results

2.1. Synthesis and Characterization of Reference Steroids

2.1.1. 17-Hydroxymethyl-17-methyl-18-nor-13-enes

Different diastereomeric 17α-hydroxymethyl-17β-methyl-18-nor-5ξ-androst-13-en-3ξ-ols were synthesized using 3-hydroxyandostan-17-ones as starting material by modifying the D-ring. The method was adapted from Kratena et al. [33] but started with regularly C13β-CH_3 configured androstanes in contrast to the *ent*-configurated (C13α-CH_3) androstanes used by Kratena et al. As the first step of synthesis, attachment of an additional carbon-atom at C17 was achieved using Nysted reagent. The epoxidation of the newly introduced 17(20) double bond and subsequent acid catalyzed ring-opening was accompanied by the stereoselective Wagner–Meerwein rearrangement, resulting in 17α-hydroxymethyl-17β-methyl-18-nor-5ξ-androst-13-en-3ξ-ols as the major product, while the 17β-hydroxymethyl-17α-methyl analogs were obtained as minor side products. The reaction scheme is displayed in Figure 4. The preceding synthesis of etiocholanolone (**5**) is described in the supplementary material (S2). The other educt androsterone (**5a**) was obtained from commercial sources.

As is common in diastereomers, all yielded very similar mass spectra. As an example, the spectrum of 17α-hydroxymethyl-17β-methyl-18-nor-5β-androst-13-en-3α-ol (**8**) is displayed in Figure 5. Using electron ionization at low energy (15 eV, low energy electron ionization, LEI) the molecular ion, which was literally invisible at regular ionization energy (viz. 70 eV), was detected at the accurate mass *m*/*z* 448.3162. The retention time of the bis-trimethylsilyl (TMS) derivatives of the diastereomers are given in Table 1. Further

structure confirmation was achieved by 1D and 2D-NMR analysis. Assignments are provided in Table 2.

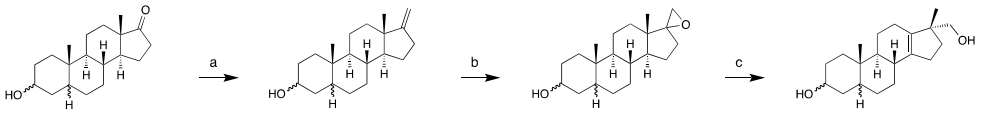

Figure 4. Reaction scheme for 17α-hydroxymethyl-17β-methyl-18-nor-5ξ-androst-13-en-3ξ-ol steroids.

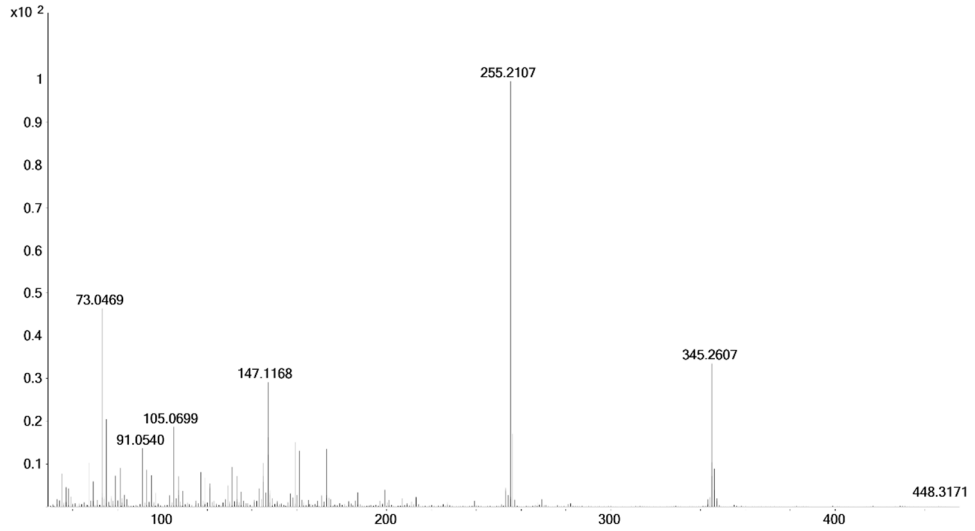

Figure 5. Mass spectrum (GC-EI-QTOF-MS, 70 eV) of 17α-hydroxymethyl-17β-methyl-18-nor-5β-androst-13-en-3α-ol (**8**), bis- trimethylsilyl) (TMS (x-axis: *m*/*z*; y-axis: relative abundance).

Table 1. Retention times (GC-QQQ-MS), molecular ions ($M^{\bullet+}$) in low electron ionization (LEI, 20 eV), and mass difference to exact mass (m/z_{ther} 448.3187, $C_{26}H_{48}O_2Si_2^{+\bullet}$) of diasteromeric 17$\alpha$-hydroxymethyl-17$\beta$-methyl-18-nor-5$\xi$-androst-13-en-3$\xi$-ols as per-TMS derivatives.

No.	Stereochemical Assignment	RT [min]	Molecular Ion (LEI)	$\Delta m/z$ [ppm]
8	3α, 5β, 17α-CH_2OH	9.80	448.3162	−5.6
8a	3α, 5α, 17α-CH_2OH	10.13	448.3164	−5.1

Table 2. ^1H and ^{13}C NMR spectral data of 17α-hydroxymethyl-17β-methyl-18-nor-5β-androst-13-en-3α-ol (8) and 17α-hydroxymethyl-17β-methyl-18-nor-5α-androst-13-en-3α-ol (8a). Multiplicity of signals indicated as singlet (s), doublet (d)

	17α-hydroxymethyl-17β-methyl-18-nor-5β-androst-13-en-3α-ol (8)		**17α-hydroxymethyl-17β-methyl-18-nor-5α-androst-13-en-3α-ol (8a)**	
	δ_C	δ_H	δ_C	δ_H
1	35.18	α: 1.92 β: 1.06	31.93	α: 1.35 β: 1.58
2	30.64	α: 1.37 β: 1.72	28.93	α: 1.66 β: 1.75
3	71.75	β: 3.66	66.43	β: 4.08
4	36.59	α: 1.75 β: 1.56	35.70	α: 1.41 β: 1.53
5	41.75	β: 1.47	39.03	α: 1.60
6	27.63	α: 1.35 β: 1.93	28.86	α: 1.25 β: 1.30

7	26.08	α: 1.72 β: 1.21	31.47	α: 1.04 β: 1.95
8	37.41	β: 2.14	36.97	β: 2.10
9	38.44	α: 1.67	52.03	α: 1.01
10	34.67	-	36.13	-
11	22.43	α: 1.79 β: 1.14	22.14	α: 1.90 β: 1.16
12	22.65	α: 1.83 β: 2.02	22.59	α: 1.80 β: 2.01
13	135.94	-	135.85	-
14	141.76	-	141.81	-
15	30.60	α: 2.32 β: 2.12	30.61	α: 2.33 β: 2.11
16	34.20	α: 1.58 β: 1.97	34.13	α: 1.97 β: 1.58
17	51.66	-	51.54	-
19	22.93	0.93 (s)	10.61	0.78 (s)
20βCH_3	21.72	1.00 (s)	21.75	0.99 (s)
20αCH_2OH	68.97	3.34 (d) 3.44 (d)	68.99	3.31 (d) 3.42 (d)

2.1.2. 17β-Methyl-5β-androstane-3α,17α-diol (**11**)

Additionally, the diastereomeric 17β-methyl-5ξ-androstane-3ξ,17α-diols were synthesized using epi-methyltestosterone (17α-hydroxy-17β-methyl-androst-4-en-3-one, **9**) as educt. After reduction of the 4,5-double bond and the 3-oxo group, the four fully reduced products **(11**, **11a**, **11b**, **11c)** were obtained as shown in Figure 6. In parallel, reduction of the 3-oxo group in epi-mestanolone (17α-hydroxy-17β-methyl-5α-androstan-3-one, **10a**) yielded the two products **11a** and **11c**. Assignment of the stereochemistry was based on the known stereoselectivity of the reductions, the comparison of the two reactions, and the elution order of the bis-TMS derivatives in GC-MS [24,34]. As a major product 3α,5β-epi-tetrahydromethyltestosterone (**11**) was obtained (Figure 7). The mass spectrum of its bis-TMS derivative is displayed in Figure 8. In LEI the molecular ion was detected at *m*/*z* 450.3352 (accurate mass), confirming the elemental composition $C_{26}H_{50}O_2Si_2^{+\bullet}$ (exact mass *m*/*z* 450.3344, difference Δ*m*/*z* = 1.78 ppm).

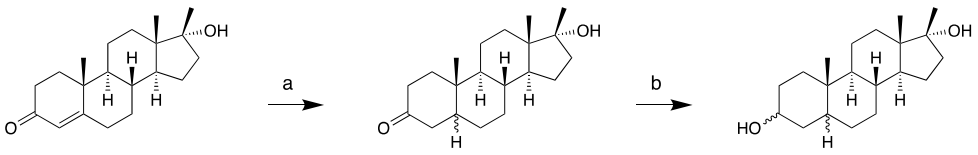

Figure 6. Synthesis route for 17β-methyl-5β-androstane-3α,17α-diol (**11**).

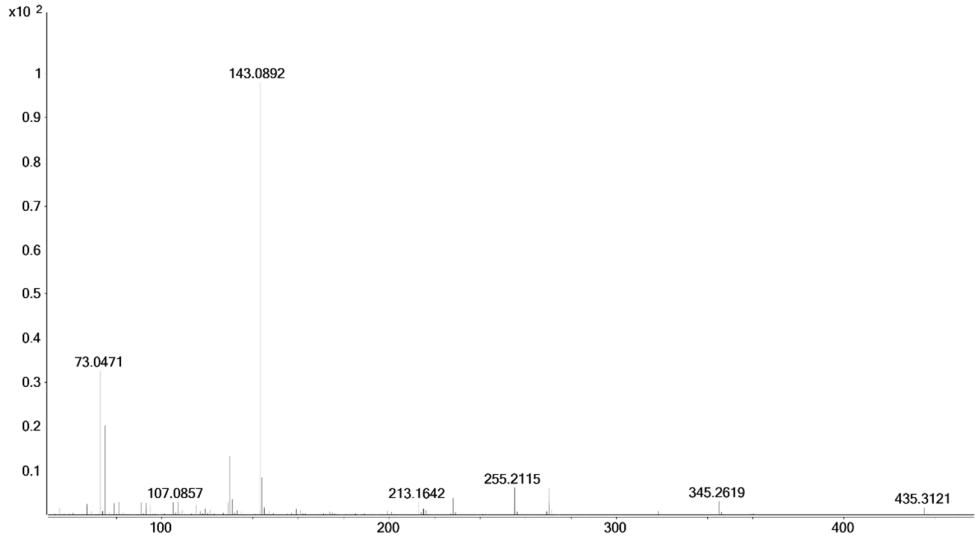

Figure 7. Mass spectrum (GC-EI-QTOF-MS, 70 eV) of 17β-methyl-5β-androstane-3α,17α-diol (**11**), bis TMS (x-axis: *m*/*z*; y-axis: relative abundance).

2.2. Post-Administration Urines

Urine samples from the administration trials were analyzed with a GC-QTOF-MS and GC-QQQ-MS after per-TMS derivatization.

The common metabolites of MT (**18**) and MD (**12**) were detected by comparison of retention time and quantifier and qualifier transitions, as reported in Table 3. Corresponding chromatograms are available as supplemental material (S3).

Table 3. Retention times (GC-QQQ-MS) and ion transitions of currently targeted metabolites in anti-doping analysis.

Compound (Parent Compound)	RT [min]	Ion Transitions (*m*/*z*) & Collision Energies
17β-methyl-5β-androst-1-ene-3α,17α-diol (**15**)	9.87	358.0 → 301.0 (10 eV) 358.0 → 169.0 (30 eV) 358.0 → 196.0 (10 eV) 358.0 → 194.0 (10 eV) 216.0 → 159.0 (5 eV) 268.0 → 211.0 (10 eV) 216.0 → 187.0 (5 eV)
6β,17β-dihydroxy-17α-methyl-androsta-1,4-dien-3-one (**13**)	16.19	517.5 → 229.0 (5 eV) 517.5 → 297.0 (5 eV) 517.5 → 205.0 (30 eV) 517.5 → 429.4 (5 eV)
17α-hydroxy-17β-methyl-androsta-1,4-dien-3-one (**14**)	13.77	444.4 → 206.0 (10 eV) 444.4 → 191.0 (30 eV) 339.0 → 270.0 (20 eV) 444.4 → 283.0 (30 eV)
17,17-dimethyl-18-nor-5β-androsta-1,13-dien-3α-ol (**16**)	6.19	253.0 → 185.0 (20 eV) 253.0 → 197.0 (20 eV) 253.0 → 105.0 (30 eV) 216.0 → 131.0 (20 eV) 216.0 → 145.0 (20 eV)

17β-hydroxymethyl-17α-methyl-18-nor-androsta-1,4,13-trien-3-one (**17**)	13.84	236.0 → 133.0 (5 eV) 339.0 → 193.0 (20 eV) 442.4 → 243.0 (15 eV) 442.4 → 133.0 (15 eV) 339.0 → 133.0 (20 eV) 339.0 → 243.0 (20 eV)
17α-methyl-5β-androstane-3α,17β-diol (**20**)	13.36	228.0 → 174.0 (5 eV) 270.0 → 157.0 (30 eV) 270.0 → 171.0 (30 eV) 270.0 → 199.0 (30 eV)
17α-methyl-5α-androstane-3α,17β-diol (**19**)	13.22	318.0 → 199.0 (10 eV) 318.0 → 187.0 (10 eV) 318.0 → 182.0 (10 eV) 450.4 → 365.0 (10 eV) 450.4 → 261.0 (10 eV)

Monitoring of the ion transitions *m*/*z* 345.3 → 255.0, *m*/*z* 345.3 → 173.0, and *m*/*z* 345.3 → 159.0, selected for the 17ξ-hydroxymethyl-17ξ-methyl-18-nor-5ξ-androst-13-en-3ξ-ol isomers, resulted in the detection of two signals at $RT_{metabolite\ A}$ = 9.56 min and $RT_{metabolite\ B}$ = 9.80 min in a case of metandienone (**12**). In the post-administration (p.a.) samples of MT (**18**), three signals were detected—one in addition to the two mentioned above ($RT_{metabolite\ A}$ = 9.56 min, $RT_{metabolite\ B}$ = 9.80 min, and $RT_{metabolite\ C}$ = 10.13 min). The comparison with the synthesized reference compounds assigned the metabolites common for MD (**12**) and MT (**18**) to 17α-hydroxymethyl-17β-methyl-18-nor-5β-androst-13-en-3α-ol (**8**) and 3α,5β-epi-tetrahydromethyltestosterone (**11**). The additional metabolite in MT administration was assigned to 17α-hydroxymethyl-17β-methyl-18-nor-5α-androst-13-en-3α-ol (**8a**).

The 3α,5β-epi-tetrahydromethyltestosterone was identified as the first peak in positive urine samples of metandienone and methyltestosterone at 9.56 min (Figure 8).

Another substance with a slightly different structure as compound **11**, namely 3α,5α-epi-tetrahydromethyltestosterone (**11a**), has almost the same retention time as compound **8**. However, **8** does not show the transition *m*/*z* 450 → 345 because of its structure ($M^{\bullet+}$ as TMS-derivative: *m*/*z* 448). As this transition is present in the urine sample, the 3α5α-epi-tetrahydromethyltestosterone (**11a**) can be excluded as the metabolite at 9.80 min (Figure 9). Another closely eluting metandienone metabolite, 17β-methyl-5β-androst-1-ene-3α,17α-diol (15, RT_{15} = 9.87 min, $M^{+\bullet}$ = 448), was mainly separated and identified by the selective ion transitions given in Table 3.

Two more diastereomers with 17α-methyl-17β-hydroxy configurations (17α-methyl-5α-androstane-3α,17β-diol (**19**) and 17α-methyl-5β-androstane-3α,17β-diol (**20**)) were commercially available and used for retention time comparison and urinary metabolite identification.

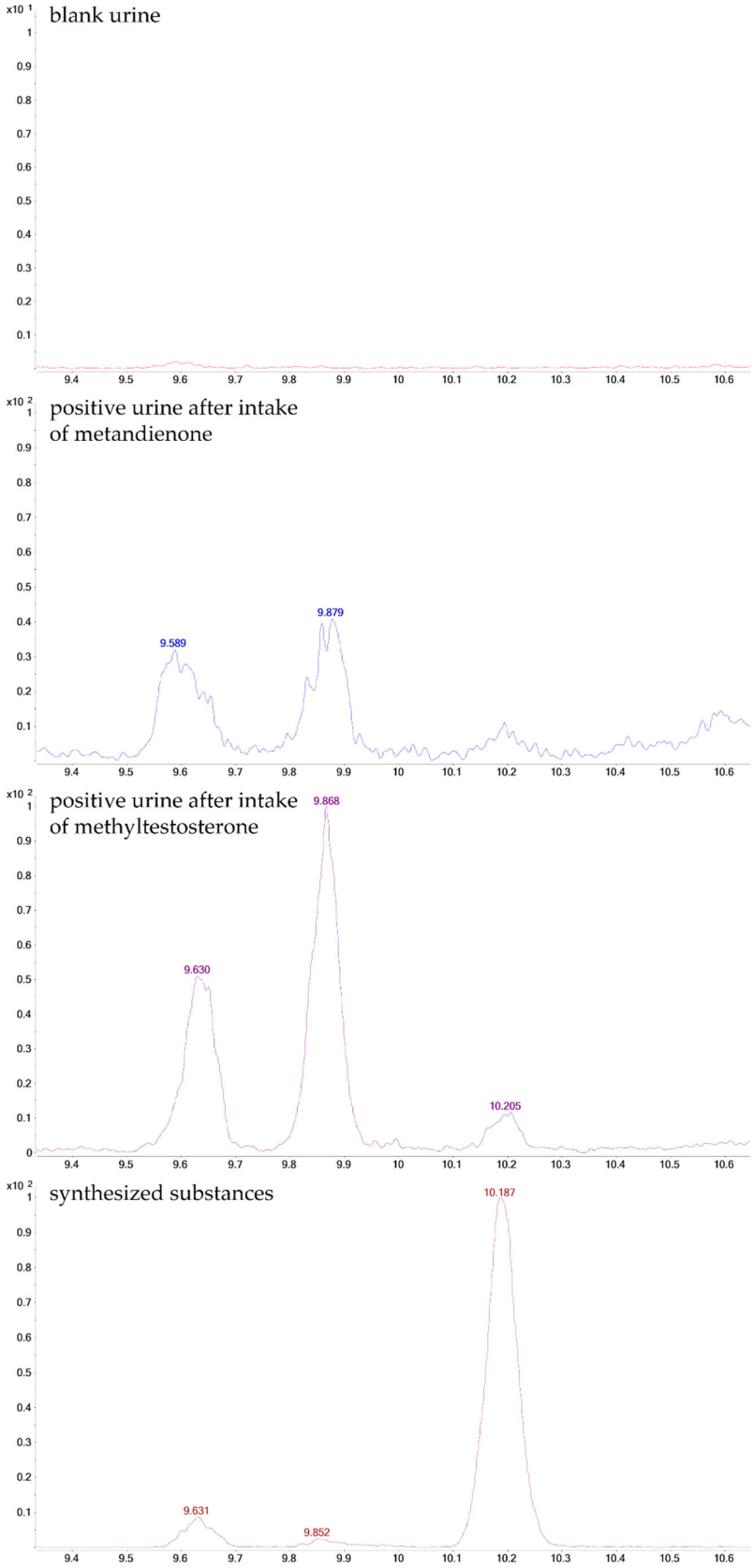

Figure 8. GC-QQQ-MS chromatograms (MRM, *m*/*z* 345 → 173).

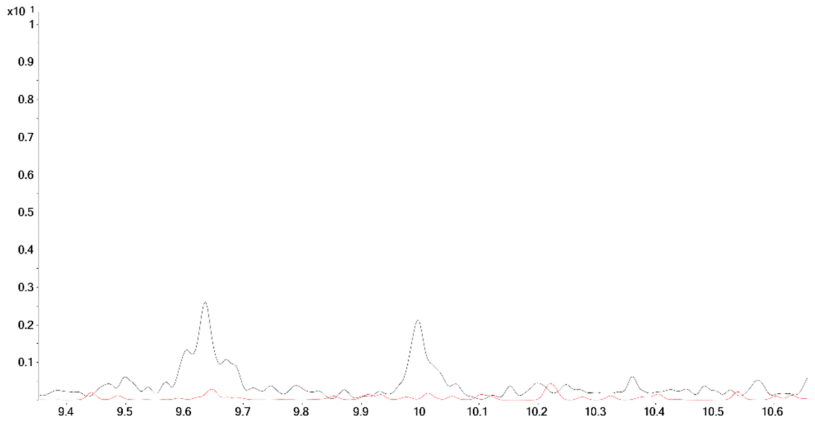

Figure 9. Chromatograms (MRM, *m*/*z* 450 → 345); black: positive urine sample after intake of methyltestosterone; red: synthesized substances.

3. Discussion

3.1. Chemical Syntheses and Characterization of Reference Material

The described syntheses starting from etiocholanolone (**5**) or androsterone (**5a**) led to androstane derivatives with a fully reduced A-ring (**8**: 3α-hydroxy-5β-; 8a: 3-hydroxy-5α-) and a rearranged D-ring (17α-hydroxymethyl-17β-methyl-18-nor-13-ene). As expected from the reactions, stereochemistry at C3 and C5 was retained unchanged. Due to the commonly known remaining stereochemistry of the 13β-methyl group during the Wagner–Meerwein rearrangement, the 17α-hydroxymethyl-17β-methyl products were the major products as expected. In GC-EI-MS, using common ionization energy of 70 eV, literally no molecular ions were obtained, due to extensive fragmentation. As the dominant fragment, $[M\text{-}CH_2\text{-}OTMS]^+$ (accurate mass *m*/*z* 345.2607, exact mass *m*/*z* 345.2608, Δ*m*/*z* = −0.29 ppm) was found. The loss of 103 Da is considered characteristic for the TMS derivatized 17α-hydroxymethyl-17β-methyl-18-nor-13-ene steroids [18,35]. The base peak with an accurate mass *m*/*z* 255.2107 (exact mass *m*/*z* 255.2107, Δ*m*/*z* = 0.00 ppm) corresponds to an additional loss of TMSOH. This transition was selected as target in the GC-QQQ-MS method. As qualifiers the transitions to *m*/*z* 159 ($C_{12}H_{15}^+$, accurate mass *m*/*z* 159.1168, exact mass *m*/*z* 159.1168, Δm/z = 0.00 ppm) and *m*/*z* 173 ($C_{13}H_{17}^+$, accurate mass *m*/*z* 173.1324, exact mass *m*/*z* 173.1325, Δ*m*/*z* = −0.56 ppm) are monitored.

NMR data confirmed the structure assignments. In 17α-hydroxymethyl-17β-methyl-18-nor-5β-androst-13-en-3α-ol (**8**) stereochemistry at C5 was assigned by the downfield shifted C19 (δ_{C19} = 22.93 ppm) signal. C19 shifts δ_{C19} > 22 ppm are known to be characteristic for 5β-androstanes [36]. Configuration at C3 was deduced from the multiplicity of H3 (δ_{H3} = 3.66 ppm, dddd, J = 11/11/5/5 Hz). The diaxial coupling with H-4_{ax} and H-2_{ax} substantiated the axial orientation of H3(β), thus confirming 3α-hydroxy configuration. The NMR data for the residues attached to C17 ($\delta_{C20\text{-}CH3}$ = 21.72 ppm, $\delta_{H20\text{-}CH3}$ = 1.00 ppm and $\delta_{C20\text{-}CH2OH}$ = 68.97 ppm, $\delta_{H20\text{-}CH2OH}$ = 3.34 ppm and 3.44 ppm) together with NOESY experiments confirmed the 17α-hydroxymethyl-17β-methyl assignment.

In case of 17α-hydroxymethyl-17β-methyl-18-nor-5α-androst-13-en-3α-ol (**8a**), stereochemistry at C5 was assigned by the upfield shifted C19 (δ_{C19} = 10.61 ppm) signal. C19 shifts δ_{C19} < 17 ppm are known to be characteristic in 5α-androstanes [36]. Configuration at C3 was deduced from the multiplicity of H3 (δ_{H3} = 4.08 ppm, dddd, *J* = 3/3/3/3 Hz) representing coupling constants of H-3_{eq} with H-2_{eq}, H-2_{ax}, H-4_{eq} and H-4_{ax}. This substantiated the orientation of H3β, thus confirming 3α-hydroxy configuration. Further confirmation was achieved by selective NOE experiments (irradiation of H19, δ_{H19} = 0.78 ppm). The NMR data for the residues attached to C17 ($\delta_{C20\text{-}CH3}$ = 21.7 ppm, $\delta_{H20\text{-}CH3}$ = 0.97 ppm and $\delta_{C20\text{-}CH2OH}$ = 21.7 ppm, $\delta_{H20\text{-}CH2OH}$ = 0.97 ppm) together with NOESY experiments confirmed the 17α-hydroxymethyl-17β-methyl assignment.

In comparison to the administered drug, the product of the last synthesis (17β-methyl-5β-androstane-3α,17α-diol, **11**) has a different stereochemistry at C17. Starting from epi-methyltestosterone (17α-hydroxy-17β-methylandrost-4-en-3-one, **9**), the first reduction using hydrogen gas and palladium on charcoal as catalyst leads to the 5β-product (**10**) with huge excess [37]. The subsequent reduction of the 3-oxo group of 5β-dihydro-epi-methyltestosterone (**10**) with sodium borohydride mainly results in the 3α-isomer (88:12, 3α-OH:3β-OH according to Schänzer et al. [24]; **11**). Stereochemistry at C17 is retained during these reactions and thus assigned to 17α-hydroxy-17β-methyl. Due to the collision energy of 70 eV, nearly no molecular ion is found, whereas prominent fragments occur. The two dominant fragments of the above mentioned 17α-hydroxymethyl-17β-methyl steroids, *m*/*z* 345 and *m*/*z* 255, are present in the spectrum of epi-tetrahydromethyltestosterone as well. The signal at *m*/*z* 345 is caused by $[M\text{-}CH_3\text{-}HOTMS]^+$ (accurate mass *m*/*z* 345.2619, exact mass *m*/*z* 345.2608, Δ*m*/*z* = 3.19 ppm), that at *m*/*z* 255 by another loss of TMSOH (accurate mass *m*/*z* 255.2115, exact mass *m*/*z* 255.2107, Δ*m*/*z* = 3.13 ppm), and the base peak at *m*/*z* 143 by a characteristic D-ring fragment of 17-methyl steroids (accurate mass *m*/*z* 143.0892, exact mass *m*/*z* 143.0887, Δ*m*/*z* = 3.49 ppm).

3.2. Urinary Metabolites

As is common in several doping control laboratories, glucuronidated metabolites are enzymatically cleaved and determined as their aglycons together with their analogs that are excreted as unconjugated compounds. Due to the low abundance of some of the target analytes, GC-QQQ-MS in MRM mode is considered as a better-suited technique for metabolite detection after optimization of the ion transitions. As described in the literature [8,24,38], GC-QQQ-MS analysis detected 17α-methyl-5β-androstane-3α,17β-diol (**20**, MT M1) following the administration of both steroids, MD (**12**) and MT (**18**), in all samples. Its 3α,5α-analog (**19**, MT M2) was detected following the administration of MT (**18**), while in MD (**12**) p.a. samples, only very minor corresponding signals were detectable in the 48 h urine and remained unconfirmed due to the low signal-to noise ratio of the qualifier transitions. According to earlier studies, these two metabolites are considered as longest detectable by GC-QQQ-MS after MT (**18**) administration in GC-MS [38].

Exclusively after MD (**12**) administration, the parent compound (**12**), epimetendiol (**15**, M1: EMD), 6-hydroxymetandienone (**13**, M2: 6OH-MD), epimetandienone (**14**, M3: EpiMD), normetendiol (17,17-dimethyl-18-nor-5β-androsta-1,13-dien-3α-ol, **16**, M4: NorEMD), and the long-term metabolite 17β-hydroxymethyl-17α-methyl-18-nor-androsta-1,4,13-trien-3-one (**17**, M6: 20βOH-NorMD) were detected, which is in agreement with earlier findings [8,12,13,18].

In addition to the commonly monitored metabolites, the two synthesized 17-hydroxymethyl-17-methyl steroids were found in p.a. urines of methyltestosterone (17α-hydroxymethyl-17β-methyl-18-nor-5α-androst-13-en-3α-ol, **8a**, 17α-hydroxymethyl-17β-methyl-18-nor-5β-androst-13-en-3α-ol, **8**) by GC-MS comparison.

Aberrantly, only 17α-hydroxymethyl-17β-methyl-18-nor-5β-androst-13-en-3α-ol (**8**) was confirmed in the p.a. urines of metandienone (**12**). The stereochemistry at C17 is the opposite of the currently monitored long-term metabolite of MD and also to 17β-hydroxymethyl-17α-methyl-18-nor-androsta-4,13-dien-3-one, which was detected earlier after administration of MT [38]. They are also different from the majority of metabolites of analogous 17-methyl steroids [30,31,38–42]. Only less abundant 17α-hydroxymethyl-17β-methyl metabolites of metandienone, methyl-1-testosterone (17β-hydroxy-17α-methyl-5α-androst-1-en-3-one) and oxandrolone [13,41], as well as the recently identified 4-chloro-17α-hydroxymethyl-17β-methyl-18-nor-androsta-4,13-dien-3β-ol (named "M4" by Sobolevsky in 2012) as metabolite of 4-chlorometandienone (dehydrochloromethyltestosterone, active component in Oral Turinabol) [33,43] have a similar stereochemistry at C17.

Interestingly, the structure of the long-term metabolite of 4-chlorometandienone with modified D-ring structure and a fully reduced A-ring (Sobolevsky's "M3") was assigned to 4α-chloro-17β-hydroxymethyl-17α-methyl-18-nor-5α-androst-13-en-3α-ol by Forsdahl

et al. [31]. The metabolites proposed for MT and MD as described above show an inverse stereochemistry at the D-ring in comparison to these assignments.

Additionally, the product of the last synthesis (17β-methyl-5β-androstane-3α,17α-diol, **11**) has an inverse D-ring at C17 in comparison to the parent compounds and the fully A-ring reduced metabolites, 17α-methyl-5β-androstane-3α,17β-diol (**20**, MT M1) and 17α-methyl-5α-androstane-3α,17β-diol (**19**, MT M2). The latter are formed through reduction of the 1,2- and 4,5-double bond and the 3-oxo group. The 17-epimer was found in the urines after the intake of both mentioned anabolic-androgenic steroids. In the case of MT administration, the metabolite **11** was also described earlier, but found with shorter detection times than the 17α-methyl analogs **19** and **20** [38]. After the intake of MD, this was also found earlier, but with a problem in separation of the four diastereomers [15].

The epimerization of position 17 is a common reaction of 17α-methyl steroids and was first described in 1971 [44]. In humans, it is generated through sulfonation of the tertiary 17-hydroxy group and its subsequent hydrolysis [9]. Besides 17-epimerization, the sulfate may also undergo an elimination of sulfuric acid and concomitant Wagner–Meerwein rearrangement, leading to 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one. This may undergo A-ring reduction, leading to the metandienone metabolite normetendiol (17,17-dimethyl-18-nor-5β-androsta-1,13-dien-3α-ol, **16**) [11].

Generation of 17α-hydroxymethyl-17β-methyl-18-norandrosta-1,4,13-trien-3-one is generated from the intermediate 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one by CYP3A4 catalyzed hydroxylation [20], while CYP21A1-catalyzed hydroxylation leads to the formation of a 17β-hydroxymethyl-17α-methyl analog [20].

The stereoselectivity of the A-ring reduction is dependent on the parent compound. For metandienone, there is only very limited generation of metabolites with a 5α-structure. This is is likely due to the 1,2-double bond, which inhibits the activity of 5α-reductase [45]. In contrast, methyltestosterone is metabolized to 5α- and 5β-isomers. This substantiates our hypothesis of metabolite generation due to the A-ring structure with a double bond in position 4 and its already saturated positions 1 and 2 in methyltestosterone, while MD (**12**) has an unsaturated A-ring (i.e., 3-oxo-1,4-diene). Thus, it is reasonable that the 17α-hydroxymethyl-17β-methyl-18-nor-5α-androst-13-en-3α-ol-derivative (**8a**) is only detectable in p.a. samples of methyltestosterone (**18**), while the 5β-analog (**8**) is observed after MT or MD administration. This supports our concept of the order of reductions: if the 1,2-double bond was reduced before the 4,5-double bond, there would have also been 5α-metabolites in p.a. urines of metandienone [8].

Thus, the order of the following two reductions of metandienone (1,2-double bond, 3-oxo group) is not yet confirmed, but it seems to be more likely that the formation of the 3-hydroxy group takes place before the hydrogenation of the 1,2-double bond, because there are known metabolites of metandienone with a 3-hydroxy-1-ene structure but not with a 3-oxo group in a fully reduced A-ring. Both potential ways represent the last step of the proposed formation of the metabolites **8** and **11**. They are displayed in Figure 10. The other reactions of the metabolism of both investigated compounds are displayed in Figure 11.

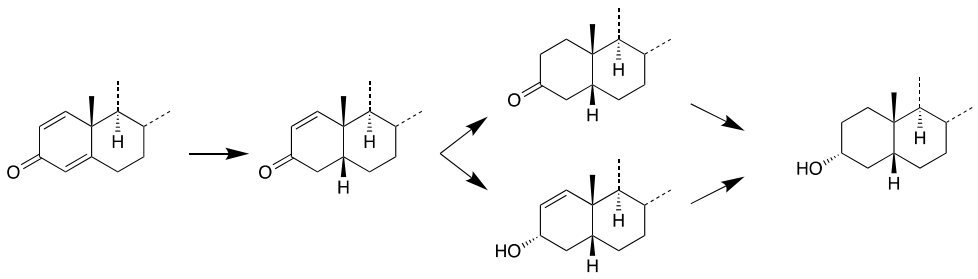

Figure 10. Potential ways of A-ring reduction.

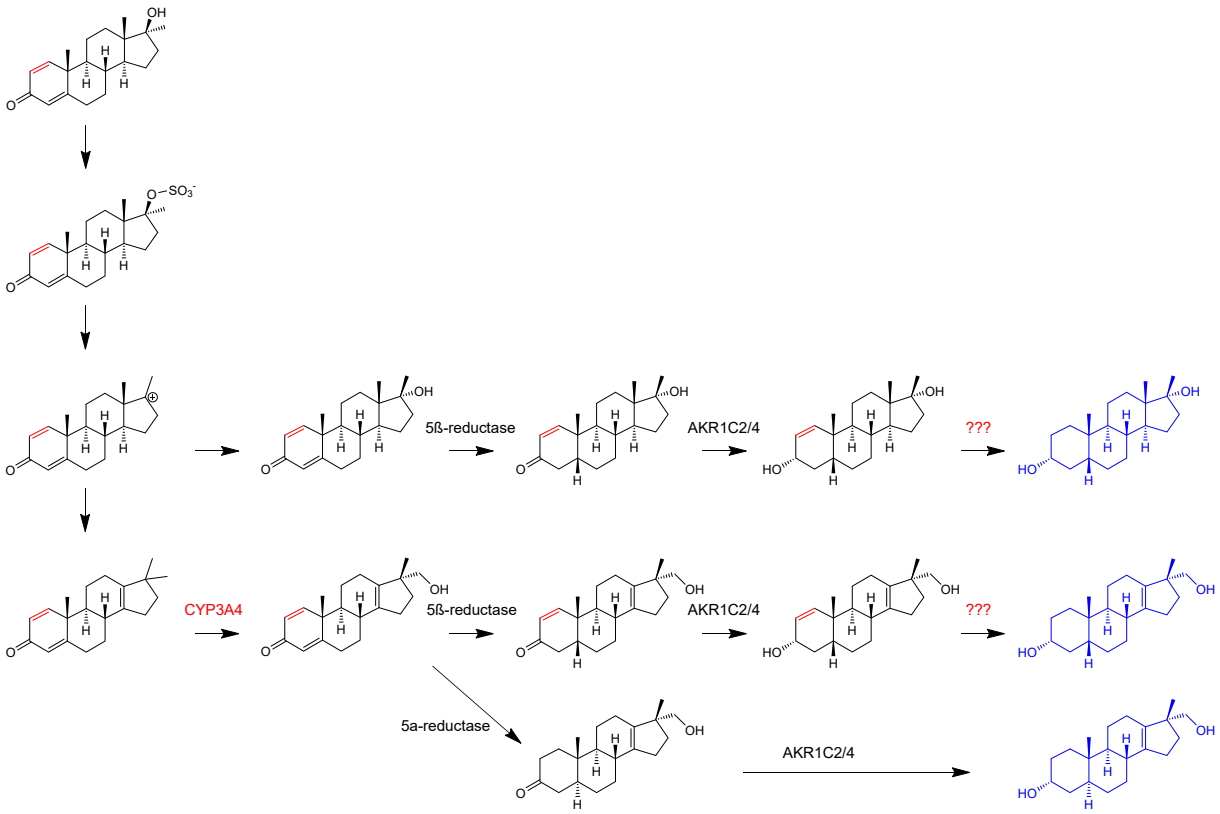

Figure 11. Proposed metabolism of methyltestosterone (black, **18**) and metandienone (red, **12**) to the found metabolites 17α-hydroxymethyl-17β-methyl-18-nor-5β-androst-13-en-3α-ol (**8**), 17α-hydroxymethyl-17β-methyl-18-nor-5α-androst-13-en-3α-ol (**8a**), and 17β-methyl-5β-androstane-3α,17α-diol (**11**) except last step of metandienone. The question marks represent reactions whose enzymes have not been elucidated yet.

Based on preliminary data, the mentioned substances are detected for at least 48 h after the intake of parent compounds. Excretion studies with a higher number of volunteers and prolonged sample collection will be performed in the near future to evaluate the detection windows of the new metabolites.

In addition to that, a potential next step will be the investigation of the substrate specificity of 5α-reductase towards 1,2-ene steroids by means of molecular modelling to elucidate structural requirements for generation of 5α-metabolites of androgenic steroids.

The detection and structure identification of the above-mentioned substances in the urine samples help to gain further insights into human metabolism of metandienone and 17α-methyltestosterone. Due to the similarity of other anabolic androgenic steroids to the investigated compounds, it is probable that other metabolites with related structures may be found in further 17α-methyl steroids. Finally, the results may support the fight against doping by introducing new analytes for screening in anti-doping analysis.

4. Materials and Methods

4.1. Instrumentation

4.1.1. GC-MS/MS

The gas chromatographic-tandem mass spectrometric analysis was performed on an Agilent 7890A gas chromatographic system coupled to an Agilent 7000 GC/MS triple quadrupole mass spectrometer (Agilent Technologies, Milano, Italy). The following conditions for the analysis of the intermediates and products were applied: Agilent HP1 column (17 m, 0.20 mm, 0.11 µm), carrier gas: helium, oven program: 188 °C, hold for 2.5 min, +3 °C/min to 211 °C, hold for 2.0 min, +10 °C/min to 238 °C, +40 °C to 320 °C, hold for

3.2 min, injection volume: 2 µL, split: 20:1, injection temperature: 280 °C, electron ionization (EI): 70 eV, transitions: *m*/*z* 345 → 255 (5 eV), *m*/*z* 345 → 173 (20 eV), *m*/*z* 345 → 159 (10 eV). Prior to injection, samples were treated with 50 µL of trimethyliodosilane (TMIS) reagent (*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA)/ethanethiol/ammonium iodide, 1000:6:4, *v*:*v*:*w*) at 75 °C for 20 min before analysis to generate the per-TMS derivatives.

4.1.2. GC-QTOF-MS

High resolution accurate mass analyses were performed on an Agilent GC-QToF 7890B/7250 (Agilent Technologies, Milano, Italy), equipped with an Agilent HP1 column (17 m, 0.20 mm; 0.11 µm) with helium as carrier gas. Injection was performed in split mode with a 1:10 ratio at 280 °C. The oven program had the following heating rates: 188 °C hold for 2.5 min, 3 °C/min to 211 °C and hold for 2 min, 10 °C/min to 238 °C, 40 °C/min to 320 °C, and hold for 3.2 min. The coupled QToF was operated in full scan with an ionization energy of 70 eV. Aberrantly, in LEI an ionization energy of 15 eV was applied. Ions were detected from *m*/*z* 50 to 750.

4.1.3. HPLC Purification

The purification of the synthesized reference steroids was performed by semi-preparative HPLC using an Agilent 1260 Infinity Quaternary HPLC system coupled to an Agilent Infinity 1260 diode array detector (Agilent Technologies GmbH, Waldbronn, Germany). Chromatographic separation was achieved on a Hypersil ODS C18 column (pore size: 120 A°, 250 mm length, 10 mm ID, 5 µm particle size, Thermo Scientific, Schwerte, Germany). Isocratic elution was accomplished at a flow rate of 3 mL/min using acetonitrile:water (7:3, *v*:*v*) as the mobile phase. The UV signal was monitored at 194 nm.

4.1.4. Nuclear Magnetic Resonance

The nuclear magnetic resonance (NMR) analyses were performed at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR) at 296 K on a Bruker (Rheinstetten, Germany) Avance III instrument equipped with a nitrogen-cooled 5 mm inverse TCI cryoprobe with actively shielded z-gradient coil. Chemical shifts are reported in δ values (ppm) relative to tetramethylsilane. Solutions of about 5 mg of each compound in deuterated dimethylsulfoxide (d_6-DMSO) were used for conducting ^{1}H; H,H COSY; ^{13}C; edited HSQC; HMBC, selective NOE and NOESY experiments. Two-dimensional experiments were recorded in non-uniform sampling (NUS) mode.

4.2. Chemicals and Reagents

Androst-4-ene-3,17-dione (**1**) was purchased from TCI (Tokyo, Japan), androsterone (**5a**), and $TiCl_4$ from Acros Organics (Fair Lawn, New Jersey, USA), 17α-methyltestosterone (**18**), palladium on charcoal, nysted reagent, K-Selectride and meta-chloroperoxybenzoic acid from Aldrich (Steinheim, Germany). 17β-Methyltestosterone (epi-MT, **9**) was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). 17α-methyl-5β-androstane-3α,17β-diol (**20**) and 17α-methyl-5α-androstane-3α,17β-diol (**19**) were purchased from the National Measurement Institute (North Ryde, Australia). Benzene was delivered from Thermo Fisher (Karlsruhe, Germany), hexane, ethyl acetate, methanol, dichloromethane, diethyl ether, hydrochloric acid, sodium bicarbonate, and potassium carbonate from Fisher Scientific (Loughborough, United Kingdom). MSTFA was obtained from from Chemische Fabrik Karl Bucher GmbH (Waldstetten, Germany). THF, monosodium phosphate, sodium borohydride, and p-toluenesulfonic acid were purchased from Merck (Darmstadt, Germany), while TBME, potassium hydroxide, and sodium carbonate were bought from Carl Roth (Darmstadt, Germany). Hydrogen gas was provided by Air Liquide (Düsseldorf, Germany) and β-glucuronidase from Roche Diagnostics (Mannheim, Germany). All other chemicals were purchased from VWR (Darmstadt, Germany).

4.3. Synthesis of Reference Steroids

4.3.1. Diastereomeric 17-hydroxymethyl-17-methyl-18-nor-5-androst-13-en-3-ols

17-Methylene-5ξ-androstan-3ξ-ol (**6**, **6a**)

A flask was flushed with argon gas and held under an argon atmosphere. After cooling to 0 °C, Nysted reagent (20 %) was diluted with absolute tetrahydrofurane (THF abs.), and titanium tetrachloride was added dropwise. After 15 min of stirring, the mixture was brought to room temperature. The precursor steroid (**5**, or **5a**) was dissolved in 10 mL THF (abs.) and was added dropwise to the mixture. The reaction was held under these conditions overnight. After cooling down the mixture to 0 °C, aqueous hydrochloric acid (2 M) and ice-cold water were added, and it was extracted four times with diethyl ether. The organic phases were combined, washed with sodium hydrogen carbonate and brine, dried over sodium sulfate, and evaporated to dryness. Detailed amounts of reactants and solvents are available in the supplements (S4).

Spiro[5 ξ-androstan-17,2′-oxirane]-3ξ-ol (**7**, **7a**)

The crude substance was dissolved in dichloromethane, and potassium hydrogen carbonate and meta-chloroperoxybenzoic acid were added. The solution was stirred for 3 h at ambient temperature. Afterwards, the mixture was poured into water and extracted three times with dichloromethane. The organic phases were washed with brine and then dried over sodium sulfate. Further details are disclosed as supplemental information.

17α-Hydroxymethyl-17β-methyl-18-nor-5β-androst-13-en-3α-ol (**8**)

The intermediate product **7** was dissolved in 5 mL methanol plus 5 mL aqueous hydrochloric acid (1 M). The solution was stirred overnight. Then, 10 mL water were added, and the mixture was extracted three times with 20 mL t-butyl methyl ether. The organic phases were combined and dried over sodium sulfate. The product was purified by column chromatography (silica gel 60, 300 mm × 30 mm, particle size 40–63 µm), using hexane/ethyl acetate (3:2, *v:v*) followed by HPLC fractionation. The finally purified product (**8**) was obtained in a total amount of 16 mg (yield: 1.51 %, purity >98%).

17α-Hydroxymethyl-17β-methyl-18-nor-5α-androst-13-en-3α-ol (**8a**)

The intermediate product **7a** was dissolved in 20 mL of methanol and 20 mL of aqueous hydrochloric acid (1 M). The solution was stirred overnight. Then, 30 mL of water was added, and the mixture was extracted three times with 50 mL t-butyl methyl ether. The organic phases were combined and dried over sodium sulfate. The product was purified by column chromatography (silica gel 60, 300 mm × 30 mm, particle size 40–63 µm), using hexane/ethyl acetate (3:2, *v:v*) followed by HPLC fractionation. A total amount of 263 mg (yield: 57.1 %, purity >98%) of the final product (**8a**) was obtained.

4.3.2. Epi-Tetrahydromethyltestosterones

17β-Methyl-5β-androstane-3α,17α-diol (**11**)

A mixture of 450 µL methanol and 50 µL potassium hydroxide solution (5 M) was prepared, and 100 µg epi-methyltestosterone (**9**) was dissolved. A spatula tip of palladium on charcoal was added, and hydrogen gas flushed through the solution for 5 min. After adding 2 mL of water, the mixture was extracted three times with 3 mL of hexane and evaporated to give the product **10**. The residue was dissolved in methanol/water (9:1, *v:v*) and a spatula tip of sodium borohydride was added. The solution was stirred for one hour at room temperature. After adding ammonium chloride to stop the reaction, potassium hydroxide solution (1 M) was added to yield alkaline solution. Then, the solution was extracted three times with dichloromethane and evaporated to give the product **11**.

17β-Methyl-5α-androstane-3α,17α-diol (**11a**)

A spatula tip of epi-mestanolon (**10a**) was dissolved in 2 mL of absolute THF, 80 µL of K-Selectride was added and the mixture was stirred for 1 h at ambient temperature. Afterwards, 100 µL of aqueous hydrochloric acid (1 M) was added until there was no formation of bubbles anymore. Then, 150 µL of potassium hydroxide solution (1 M) was added and the mixture was extracted three times with 5 mL of hexane. The hexane-phase was evaporated to give the product **11a**.

4.4. Human Administration Trial

Urine samples out of the stock of the anti-doping laboratory in Rome were available for analysis. Samples collected before and after an oral administration of either MD or MT were used for evaluation of the excretion of the hypothized metabolites. The excretion study with MT was carried out by a healthy male volunteer (Caucasian, 50 years old, 80 kg and normal body mass index). A single oral dose of 10 mg of MT (Metadren®, Novartis, Basel, Switzerland) was administered. For investigation of MD metabolism, a single oral dose of 5 mg MD (Dianabol®, Ciba-Geigy, Basel, Switzerland) was administered to a healthy male volunteer (Caucasian, 45 years old, 82 kg and normal body mass index).

4.5. Urine Sample Preparation

An aliquot of 6 mL urine was used for the following analysis. As internal standard methyltestosterone (50 µL of a solution of 100 µg/mL) was added. After the addition of 750 µL of phosphate buffer (0.8 M) and 50 µL β-glucuronidase, the mixture was incubated at 55 °C for 60 min. Afterwards, 500 µL of carbonate buffer (20 %) was added and the mixture was extracted with 10 mL of TBME. After evaporation, 50 µL of TMIS reagent was added to the sample and the mixture was treated at 75 °C for 20 min before analysis to generate the per-TMS derivatives.

Supplementary Materials: The following are available online: Supplement S1: Table of steroids, Supplement S2: Preceding synthesis of etiocholanolone, Supplement S3: Chromatograms of urine samples. Supplement S4: Detailed amounts of reactants and solvents in the synthesis of 17-hydroxymethyl-17-methyl-18-nor-5-androst-13-en-3-ols.

Author Contributions: Conceptualization, M.K.P., S.L., A.M.K., and X.d.l.T.; methodology, M.K.P., S.L. and X.d.l.T.; investigation, S.L., L.L., H.S., M.W., M.I., X.d.l.T. and N.S.; resources, M.K.P., N.S., A.M.K., M.B. and F.B.; data curation, M.K.P., S.L., M.I., X.d.l.T. and N.S.; writing—original draft preparation, S.L.; writing—review and editing, M.K.P.; visualization, M.K.P., S.L., M.I., X.d.l.T. and N.S.; supervision, M.K.P., M.B., and F.B.; project administration, M.K.P.; funding acquisition, M.K.P., A.M.K., M.B., and F.B. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Raw data are stored at the authors.

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Sample Availability: Samples of the compounds are kept amongst the authors.

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3.4 Manuscript IV: Long-Term Detection of Dehydrochloromethyltestosterone: Insights from Controlled Administration in Humans

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Abstract: Dehydrochloromethyltestosterone (DHCMT) is an anabolic-androgenic steroid that was developed by Jenapharm in the 1960s and was marketed as Oral Turinabol®. It is prohibited in sports at all times; nevertheless, there are several findings by anti-doping laboratories every year. New long-term metabolites have been proposed in 2011/12, which resulted in adverse analytical findings in retests of the Olympic games of 2008 and 2012. However, no controlled administration trial monitoring these long-term metabolites was reported until now. In this study, DHCMT (5 mg, p.o.) was administered to five healthy male volunteers and their urine samples were collected for a total of 60 days. The unconjugated and the glucuronidated fraction were analyzed separately by gas chromatography coupled to tandem mass spectrometry. The formation of the described long-term metabolites was verified, and their excretion monitored in detail.

Due to interindividual differences there were several varieties in the excretion profiles among the volunteers. The metabolite M3, which has a fully reduced A-ring and modified D-ring structure, was identified by comparison with reference material as 4α-chloro-17β-hydroxymethyl-17α-methyl-18-nor-5α-androstan-13-en-3α-ol. It was found to be suitable as long-term marker for the intake of DHCMT in four of the volunteers. In one of the volunteers, it was detectable for 45 days after single oral dose administration. However, in two of the volunteers M5 (already published as long-term metabolite in the 1990s) showed longer detection windows. In one volunteer M3 was undetectable but another metabolite, M2, was found as the longest detectable metabolite.

The last sample clearly identified as positive was collected between 9.9 and 44.9 days. Furthermore, the metabolite epiM4 (partially reduced A-ring and a modified D-ring structure which is epimerized in position 17 compared to M3) was identified in the urine

of all volunteers with the help of chemically synthesized reference as 4-chloro-17α-hydroxymethyl- 17β-methyl-18-nor-androsta-4,13-dien-3β-ol. It may serve as additional confirmatory metabolite.

It is highly recommended to screen for all known metabolites in both fractions, glucuronidated and unconjugated, to improve identification of cheating athletes. This study also offers some deeper insights into the metabolism of DHCMT and of 17α-methyl steroids in general.

Controlled Administration of Dehydrochloromethyltestosterone in Humans: Urinary Excretion and Long-Term Detection of Metabolites for Anti-Doping Purpose

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Competing interests

All authors declare no conflict of interest.

Abstract

Dehydrochloromethyltestosterone (DHCMT) is an anabolic-androgenic steroid that was developed by Jenapharm in the 1960s and was marketed as Oral Turinabol®. It is prohibited in sports at all times; nevertheless, there are several findings by anti-doping laboratories every year. New long-term metabolites have been proposed in 2011/12, which resulted in adverse analytical findings in retests of the Olympic games of 2008 and 2012. However, no controlled administration trial monitoring these long-term metabolites was reported until now. In this study, DHCMT (5 mg, p.o.) was administered to five healthy male volunteers and their urine samples were collected for a total of 60 days. The unconjugated and the glucuronidated fraction were analyzed separately by gas chromatography coupled to tandem mass spectrometry. The formation of the described long-term metabolites was verified, and their excretion monitored in detail.

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It is highly recommended to screen for all known metabolites in both fractions, glucuronidated and unconjugated, to improve identification of cheating athletes. This study also offers some deeper insights into the metabolism of DHCMT and of 17α-methyl steroids in general.

Keywords

Oral-Turinabol, administration study, gas chromatography-tandem mass spectrometry, long-term metabolites, metabolism

Introduction

Dehydrochloromethyltestosterone (4-chloro-17β-hydroxy-17α-methyl-androsta-1,4-dien-3-one, DHCMT) is an anabolic-androgenic steroid which was introduced into the market as "Oral-Turinabol" by East German pharmaceutical company Jenapharm in the 1960s [1, 2]. It is a derivative of testosterone with enhanced anabolic properties which is orally bioavailable due to its alkylation at C-17 [3, 4]. According to Zafferoni *et al.* the chlorination at C-4 does not affect the anabolic or androgenic activity of DHCMT, which is contrary to the activity of progestogens or corticoids after substitution in this position [5]. However, the chloro-atom in position 4 hampers the reduction of the 4,5-double bond by 5α-reductase [6, 7]. 4-chlorination as well as the 1,2-double bond prevent the aromatization of the A-ring [4, 6]. Even if the androgenic activity is lower than that of testosterone, muscle tightness, acne, functional liver damage, and amenorrhea in women are reported as side effects [1, 8].

DHCMT was primarily developed to support recovery after major surgeries. However, shortly after its introduction on the market, it was misused in sports as performance-enhancing drug in one of the biggest systematic doping programs, until the German Democratic Republic's collapse [1]. "Oral-Turinabol" was sold in two dosages, 1 and 5 mg, with recommendations for daily intake up to 50 mg [1]. It is explicitly mentioned as anabolic androgenic steroid (AAS) in class S1 in the list of prohibited substances published by the World Anti-Doping Agency (WADA) every year [9]. Even if discontinued as approved drug, the substance is still misused in sports until today. DHCMT gained new importance in doping control in the last 15 years with the number of its adverse analytical findings steadily increasing (Figure 1) through illegal marketing of steroid hormones mainly as so called "supplements" sold over the internet [10, 11]. Unfortunately, the control of these products is nearly impossible [12]. The WADA-accredited laboratories reported 108 adverse analytical findings for DHCMT in 2018 [13]. This fact is a clear indicator of the continuing relevance of research on substances even if they are on the market for a long time.

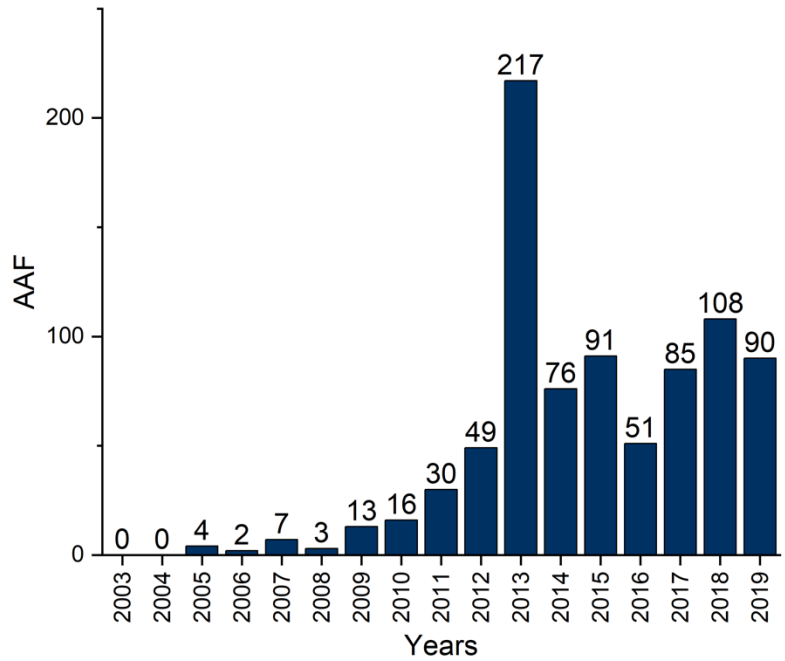

Figure 1: Adverse analytical findings of DHCMT between 2003 and 2019, according to [13]

Anabolic-androgenic steroids are extensively modified in the body by phase I and phase II metabolism. These reactions result in more polar substances facilitating the excretion via the urine [14, 15].
17β-Hydroxymethyl-17α-methyl-18-norandrost-13-ene metabolites of 17α-methyl steroids were first discovered in 2006, with the identification of a long-term metabolite of metandienone [16-18]. Subsequent research revealed analogous metabolites for other 17α-methyl steroids with capabilities for long-term detection, especially in case of oxandrolone and DHCMT [19-27].
As intermediates 17,17-dimethyl-18-norandrost-13-ene compounds are postulated. The subsequent hydroxylation at 17β-CH_3 is catalyzed by steroid 21-hydroxylase (CYP21A2) [19, 27-30]. Continuing investigations on A-ring reduced long-term metabolites like 20ξOH-nortetrahydrochloromethyltestosterone ("M3") by Sobolovsky and Rodchenkov led to several adverse analytical findings in retests of samples of the 2008 and 2012 Olympic Games [13, 31]. The underlying method of the research by Sobolovsky and Rodchenkov was based on the analysis of pooled urines from doping control samples already tested positive for DHCMT metabolites. As a controlled administration study was not yet reported, Kopylov *et al.* challenged these results of Sobolevsky [32]. In recent investigations the metabolite named "M3" in Sobolevsky's publication was assigned to 4α-chloro-17β-hydroxymethyl-17α-methyl-18-nor-5α-androst-13-en-3α-ol by comparison with synthesized reference material by Kratena *et al.* [33]. Sobolevsky's "M4" was analogously assigned to 4-chloro-17α-hydroxymethyl-17β-methyl-18-norandrosta-4,13-dien-3β-ol by our group in 2019 [26] and confirmed by Kratena *et al.* [25].
The pathways of DHCMT metabolism are not fully uncovered yet, but due to the work of Liu *et al.* and Stoll *et al.* [27, 29] some enzymes and metabolic processes came to the fore.

The trial reported in this manuscript aimed to monitor metabolite excretion after the intake of DHCMT based on a controlled administration trial in five male volunteers. Information about the long-term metabolism of DHCMT may help to further improve the fight against doping, support anti-doping laboratories with information on best target analytes and their detection windows. Furthermore, knowledge on biotransformation of 17β-hydroxy-17α-methyl-androstan-3-one steroids in general is increased.

Materials and Methods

Chemicals and Reagents

Methanol (HPLC-grade), t-butyl methyl ether (TBME, HPLC-grade), ethyl acetate, K_2CO_3, and NaH_2PO_4 were purchased from Merck KGaA (Darmstadt, Germany). $KHCO_3$ was provided by Honeywell Fluka (Charlotte, USA). Na_2HPO_4 was bought from Carlo Erba (Val de Reuil, France). β-Glucuronidase was purchased from Roche Diagnostics (Mannheim, Germany), β-Glucuronidase from Helix Pomatia (G0876) and sulfatase from Helix Pomatia (S9626) were provided by Sigma Aldrich (Milano, Italy). Hydrochloric acid and acetic acid were provided by Fisher Scientific (Loughborough, United Kingdom). 17α-methyltestosterone was delivered by Steraloids (Newport, R.I., USA). Etiocholanolone-D_5, androsterone-D_4-glucuronide, and 4-chloro-6β,17β-dihydroxy-17α-methyl-androst-1,4-dien-3-one are coming from the National Measurement Institute (West Lindfield, New South Wales, Australia). N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was bought from Chemische Fabrik Karl Bucher GmbH (Waldstetten, Germany). Dehydrochloromethyltestosterone (DHCMT) was obtained from Cohnchem Scientific Co., Ltd (Derby, UK). 4α-Chloro-17β-hydroxymethyl-17α-methyl-18-nor-5α-androst-13-en-3α-ol was obtained from the Austrian Anti-Doping Laboratory, provided by the World Association of Anti-Doping Scientists (WAADS). All other chemicals were purchased from Sigma-Aldrich (Munich, Germany).

GC-QQQ-MS analysis

The gas chromatographic-mass spectrometric analysis of urine samples was performed on an Agilent 7890A gas chromatographic system coupled to an Agilent 7000 GC/MS triple quadrupol (QQQ) mass spectrometer with the following parameters for the analysis of the intermediates and products as reported by Martinez-Brito et al. [34]: Agilent HP1 (17 m, 0.20 mm, 0.11 µm), carrier gas: helium, oven program: 188 °C, hold for 2.5 min, +3 °C/min to 211 °C, hold for 2.0 min, +10 °C/min to 238 °C, +40 °C to 320 °C, hold for 3.2 min, injection volume: 2 µL, split: 20:1, injection temperature: 280 °C, electron ionization (EI): 70 eV, transitions, collision energy and retention time for each substance are displayed in Table 1.

Table 1: Overview of screened compounds including the retention time, the transitions and the collision energy used for analysis

Substance	Retention time [min]	Transition	CE [eV]
DHCMT	16.40	478.4 → 240	5
		478.4 → 225	5
		240 → 225	5
		240 → 189	5
		225 → 189	5
M1	16.68	315 → 227	10
		227 → 163	5
		227 → 93	5
M2	14.50	377 → 251	15
		377 → 185	22
		287 → 251	15
		287 → 185	12
M3	15.25	381 → 343	15
		381 → 253	15
		379 → 343	7
		379 → 253	13
		379 → 147	15
epiM3	14.40	381 → 343	15
		381 → 253	15
		379 → 343	7
		379 → 253	10
		379 → 147	15
M4	14.30	379 → 149	15
		377 → 149	13
		287 → 125	22
epiM4	15.10	379 → 149	15
		377 → 149	13
		287 → 125	22
M5	16.60	658.6 → 244	15
		658.6 → 244	17

Every sample was treated with 50 µL of TMIS reagent (MSTFA / ammonium iodide / ethanethiol, 1000:2:3, v:w:v) at 75 °C for 20 min prior to injection to generate the per-TMS derivatives.

GC-QTOF-MS analysis

High resolution accurate mass analyses were performed on an Agilent GC-quadrupol time-of-flight (QToF) 7890B/7250 (Agilent Technologies, Milano, Italy), equipped with an Agilent HP1 column (17 m, 0.20 mm, 0.11 µm) with helium as carrier gas as reported earlier [35, 36]. Injection was performed in split mode with a 1:10 ratio at 280 °C. The oven program had the following heating rates: 188 °C hold for 2.5 min, 3 °C/min to 211 °C and hold for 2 min, 10 °C/min to 238 °C, 40 °C/min to 320 °C and hold for 3.2 min. The hyphenated QToF was

operated in full scan with an ionization energy of 70 eV. Aberrantly, in LEI an ionization energy of 15 eV. Ions were detected from *m/z* 50 to 750.

GC-MS analysis

The gas chromatographic-mass spectrometric analysis of the extended steroid profile samples was performed on an Agilent 6890N gas chromatographic system coupled to an Agilent 5973N GC/MSD with the following parameters for the analysis of the intermediates and products: Agilent HP5 (17 m, 0.20 mm, 0.11 µm), carrier gas: helium, oven program: 60 °C, hold for 0.5 min, +60 °C/min to 210 °C, +2.5 °C/min to 270 °C, +30 °C to 320 °C, hold for 2.5 min, injection volume: 2 µL, split: 20:1, injection temperature: 280 °C, electron ionization (EI): 70 eV

Nuclear Magnetic Resonance

The nuclear magnetic resonance (NMR) analyses were performed at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR) at 296 K on a Bruker (Rheinstetten, Germany) Avance III 500 instrument equipped with a nitrogen-cooled 5 mm inverse probe head (Prodigy TCI) with actively shielded z-gradient coil. Chemical shifts are reported in δ values (ppm) relative to tetramethylsilane. Solutions of about 5 mg of each compound in deuterated dimethylsulfoxid (d_6-DMSO) were used for conducting ^{1}H; H,H COSY; ^{13}C{^{1}H} APT; H,C HMQC; H,C HMBC and H,H NOESY experiments. 2D experiments were acquired applying non-uniform sampling (NUS, [37]).

Synthesis of Reference Substances

4-Chloro-17α-hydroxymethyl-17β-methyl-18-norandrosta-4,13-dien-3β-ol and three of its diastereomers were synthesized as described in the supplemental information. After purification analytical characterization was performed using GC-QTOF-MS and NMR analyses.

Preparative Liquid Chromatography

The preliminary purification runs were performed on ISOLERA ONE (Biotage AB, Uppsala, Sweden) instrument equipped with UV-Vis detector (detection wavelength 254 nm with 15 mAU as threshold). The column used was a BIOTAGE SNAP (Biotage AB, Uppsala, Sweden) Ultra (10 g, 25 µm). The flow used was 12 mL/min with an isocratic elution with solvent composition of hexane:EtOAc (60:40, v:v).

HPLC Separation

The HPLC runs were performed on Agilent 1260 instrument equipped with C18 (250 mm x 10 mm, particle size 5 µm) THERMO electron corporation (Waltham, Massachusetts, USA) column. The flow was 2.5 mL, the maximum flow gradient was 1 mL/min. An isocratic run with solvent composition MeOH:H_2O (70:30, v:v) was performed. The injection volume was 0.5 mL. The selected wavelength for the UV detection were 250 nm, 254 nm and 210 nm.

Human Administration Trial

An administration study with a single oral intake of 5 mg DHCMT was performed in five healthy male volunteers (age: 30 - 67 years). The study was approved by the ethics committee of the School of Pharmaceutical Science and Technology, Tianjin University. The study was

performed following the recommendations of the Helsinki declaration and written informed consent was obtained from the participants. The anthropometric data and further characteristics of the volunteers are shown in Table 2.
Blank urines were collected within 24 hours before the administration of DHCMT. During the first 72 hours after intake, every urine was collected. Afterwards, every first urine in the morning was collected until day 60 post administration. The urines were directly frozen after every collection and kept at -24 °C until analysis. Specific gravity for each sample was determined with two drops of urine on a Mettler Toledo RE50 refractometer prior to analysis. The correction factor was calculated by (1,020-1,000)/(SG-1,000). SG is the specific gravity of the sample and 1,020 is the value to which the specific gravity of urine is normalized.

Table 2: Characteristics of the volunteers

Volunteer	Age [years]	Weight [kg]	Height [cm]	Race	Additional medication
1	30	101	188	Caucasian	75 µg thyroxine/day
2	34	85	162	Caucasian	---
3	31	80	178	Caucasian	"Pure Encapsulation Magnesium Energy"
4	67	72	178	Caucasian	---
5	29	64	172	Caucasian	---

Sample Preparation for Metabolite Analysis

Samples were analyzed using the method adapted from the Anti-Doping Laboratory Rome for DHCMT metabolite confirmation. Briefly, an aliquot of 6 mL was used for the following analysis. After the addition of an internal standard (50 µL solution of methyltestosterone [100 µg/mL]), the urine was extracted with 10 mL of TBME. The organic layer was separated and evaporated to dryness to result in the fraction of unconjugated metabolites (free fraction).
For the extraction of the glucuronide fraction, the aqueous layer was used. The internal standard (50 µL solution of methyltestosterone [100 µg/mL]) and a standard containing deuterated glucuronides (50 µL solution of a mixture of androsterone-D_4-glucuronide, etiocholanolone-D_5; 24 µg/mL) were added. After the addition of 750 µL of phosphate buffer (0.8 M; 71.2 g Na_2HPO_4, 55.0 g NaH_2PO_4, ad 1000 mL H_2O) and 50 µL β-glucuronidase, the mixture was incubated at 55 °C for 60 min. Afterward, 500 µL of carbonate/bicarbonate buffer (20 %; 200 g K_2CO_3, 200 g $KHCO_3$, ad 1000 mL H_2O) was added and the mixture was extracted with 10 mL of TBME. The ether layer was evaporated to dryness. Analyses were performed by GC-QQQ-MS and GC-QTOF-MS.
A standard containing 90 µL of a solution of DHCMT, metabolite M1, and metabolite M3 (1 µg/mL each), and 9 µL epiM4 (100 µg/mL), and 50 µL methyltestosterone (100 µg/mL) was evaporated to dryness. This mixture was measured together with every batch of samples. The chromatogram of this mixture is displayed in Figure 2.

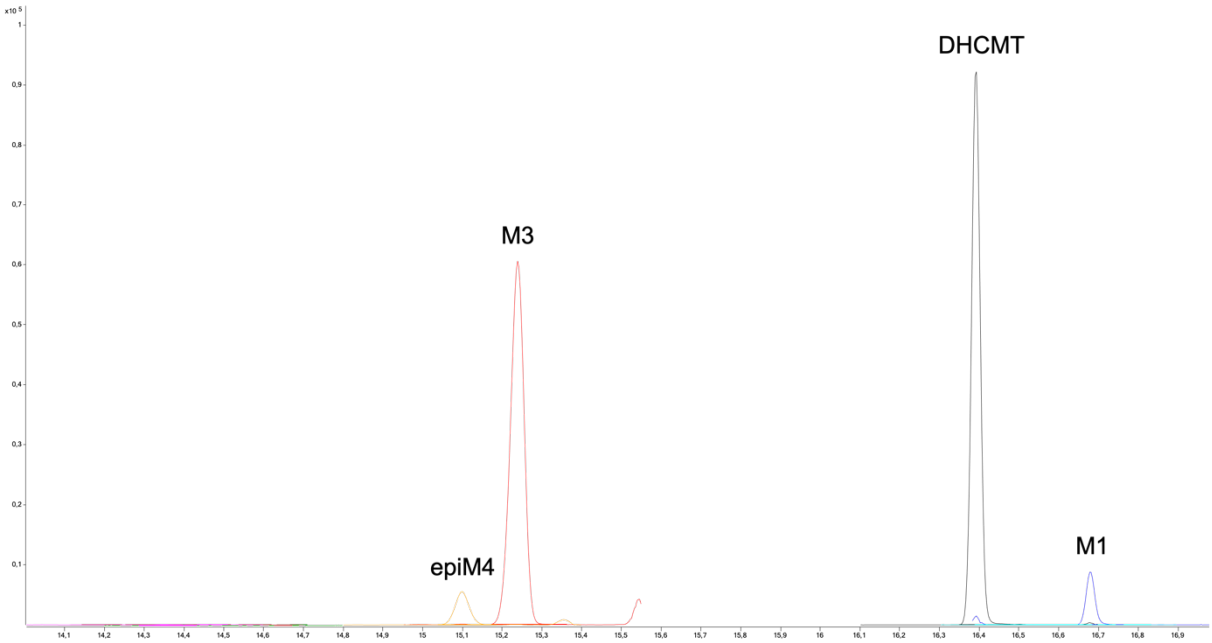

Figure 2: GC-QQQ-MS chromatogram of the standard mixture; yellow: epiM4, red: M3, black: parent compound, blue: M1

Extended Steroid Profile

Solid-phase extraction was performed using Waters Sep-Pak Classic C_{18} cartridges (WAT051910, Waters Corporation, Massachusetts, USA). The cartridge was conditioned with 2 x 2 mL of each methanol and water, loaded with 2 mL of blank urine, washed with 2 x 2 mL of water, and eluted with 2 x 2 mL of methanol. The combined eluates were evaporated to dryness. After adding β-glucuronidase and sulfatase from *Helix pomatia* in acetate buffer, the mixture was incubated at 55 °C for 3 h. Afterwards, 500 µL of carbonate buffer was added, and the mixture was extracted with 5 mL TBME, and an internal standard was added (50 µL of a mixture of 5α-androstane-3α,17α-diol, stigmasterol, cholesterol butyrate, androsterone-D_4, etiocholanolone-D_5-sulfate, cortisol-D4; concentration: 5 µg/mL each). The ether layer was evaporated to dryness. The derivatization was performed in three steps: after the addition of 100 µL of N-methylhydroxylamine hydrochloride in pyridine (2 %), the sample was heated to 55 °C for 1 hour, the solvent was evaporated afterwards. Following the addition of 50 µL 1-(trimethylsilyl)imidazole, the sample was heated with a microwave oven, extracted with cyclohexane, and evaporated to dryness; finally, 50 µL MSTFA were added, and the sample was heated to 75 °C for 10 minutes. Analysis of the derivatized residues was performed by GC-MS.

Results

Metabolite Detection

The analysis mainly focused on metabolites Sobolevsky *et al.* proposed in 2012 [23] as target analytes. Analysis was performed separately for the unconjugated and for the glucuronidated metabolites. Structure assignments of the aglycons extracted in the different fractions (with or without deglucurinidation) are based on the comparison with authentic reference material in case of M1, M3 and epiM4 as described below.

The success of the deglucuronidation was evaluated by comparison of two substances in the added standard: etiocholanolone-[d_5] and androsterone-[d_4]-glucuronide. The mean value of deglucuronidation in all volunteers was 59.0 %.

Most of the compounds were only excreted as glucuronides (M2, M3, M4, epiM4) and thus detectable in the extract after deconjugation (glucuronide fraction). The other substances (DHCMT, M1, M5) were found in both fractions (unconjugated and glucuronide fraction). The metabolite epiM3 could not be confirmed in any sample but urines of volunteer 5 gave some samples with signals different to the blank urine between 1.7 and 7.0 days. Figure 3 gives an overview of the found metabolites in comparison to a blank sample.

Aberrantly from the other volunteers, volunteer 4 displayed a completely different excretion profile. Neither M3 nor M4 were detected in his samples. The last sample with a positive metabolite finding was collected after 18 days. The metabolite detected was M2.

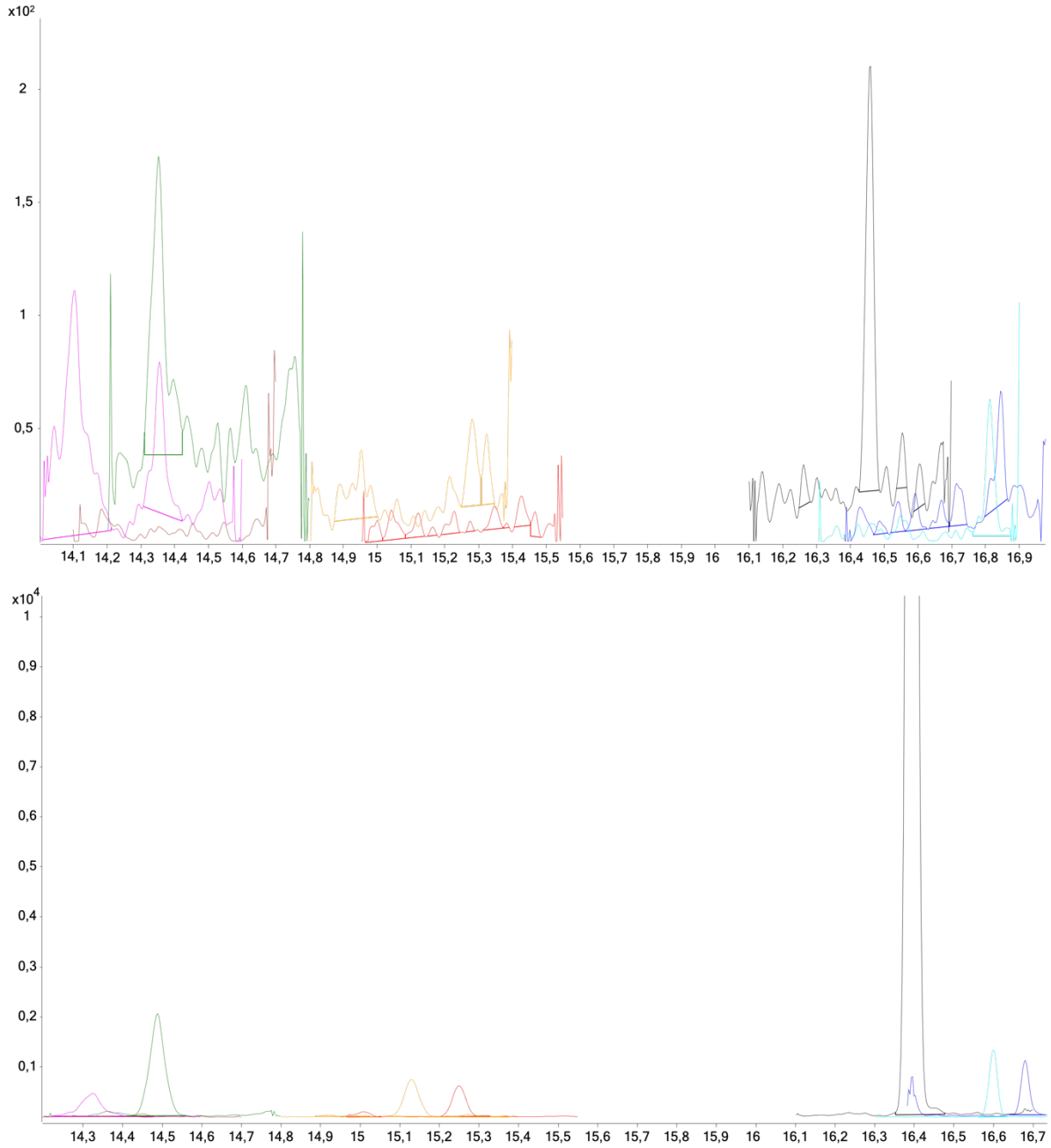

Figure 3: upper picture: GC-QQQ-MS chromatogram of a blank sample of volunteer 1; lower picture: GC-QQQ-MS chromatogram of a post-administration sample of volunteer 1 (25.3 h after intake); magenta: M4, green: M2, yellow: epiM4, red: M3, black: parent compound, turquoise: M5, blue: M1

Synthesis of 4-Chloro-17α-hydroxymethyl-17β-methyl-18-nor-androsta-4,13-dien-3β-ol

For proper structure confirmation of M4/epiM4, different diastereomers of 4-chloro-17α-hydroxymethyl-17β-methyl-18-nor-androsta-4,13-dien-3β-ol were synthesized starting from 4-chloro-androst-4-ene-3,17-dione. The synthesis route is described in the supplement.

After the regioselective reduction of the 3-oxo group, the protection of the 3-hydroxy group followed and the intermediate was transformed using a method adapted from Kratena *et al.* [25]. A new carbon-atom at position 17 was introduced. After epoxidation of the 17(20)-double bond, the epoxide was opened under acidic conditions which went along with a Wagner-Meerwein rearrangement mainly resulting in 17α-hydroxymethyl-17β-methyl derivatives. This is called epiM4 following Sobolevsky's nomenclature that 17α-

hydroxymethyl-17β-methyl derivatives are called “epi” whereas the 17β-hydroxymethyl-17α-methyl metabolites are described as, e.g., “M4”. The final step was the acid catalyzed deprotection of the 3-hydroxy group. Four diastereomeric products were obtained. The detailed steps are described in the supplement. The retention times and relative abundances are displayed in Table 3. As expected, their mass spectra are very similar. As an example, the spectrum of the main product, 4-chloro-17α-hydroxymethyl-17β-methyl-18-nor-androstane-4,13-dien-3β-ol, is displayed in Figure 4.

The obtained main products had the 4-chloro-17α-hydroxymethyl-17β-methyl-18-nor-androstane-4,13-dien-3ξ-ol structure with the 3β-ol as major product. The 4-chloro-17β-hydroxymethyl-17α-methyl-18-nor-androstane-4,13-dien-3ξ-ol structures appeared as minor byproducts.

Table 3: Retention times and relative abundances of the 4-chloro-17ξ-hydroxymethyl-17ξ-methy-18-norl-androstane-4,13-dien-3ξ-ol isomers

Tentative Isomer	Retention Time [min]	Relative Abundance [%]
3α17α	13.56	36.9
3α17β	14.29	3.2
3β17α	15.06	100
3β17β	15.32	21.3

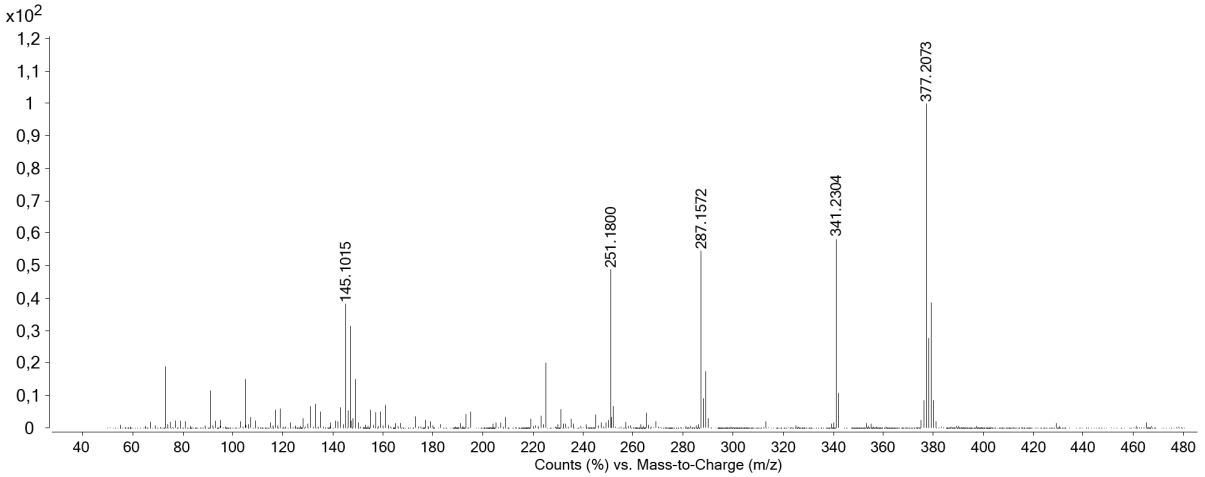

Figure 4: Mass spectrum (GC-EI-QTOF-MS, 70 eV) of 4-chloro-17α-hydroxymethyl-17β-methyl-18-nor-androstane-4,13-dien-3β-ol (bis-trimethylsilyl)

After purification of the major diastereomer by liquid chromatography, structure confirmation by NMR was achieved. All signals were assigned from shifts and correlations of 1D and 2D measurements:
^{1}H NMR (500 MHz, DMSO-d_6): δ = 5.11 (d, ^{3}J = 6.7 Hz, 1H, C-3-OH); 4.31 (t, ^{3}J = 5.5 Hz, 1H, C-17-CH_2-OH); 3.92 (ddm, ^{3}J = 13.3/6.7 Hz, 1H, H-3a); 3.14 (d, ^{3}J = 5.5 Hz, 2H, C-17-CH_2-OH); 2.88 (ddd, ^{3}J = 13.8/3.7/2.5 Hz, 1H, H-6a); 2.19 (m, 1H, H-15a) ; 2.14 (m, 1H, H-8); 2.04 (m, 1H, H-15b); 2.00 (m, 1H, H-6b); 1.95 (m, 1H, H-12b); 1.94 (m, 1H, H-7b); 1.91 (m, 1H, H-2a); 1.86 (m, 1H, H-16a); 1.80 (m, 1H, H-12a); 1.78 (m, 1H, H-11a); 1.74 (m, 1H, H-1b); 1.56 (m, 1H, H-2b); 1.36 (m, 1H, H-16b); 1.32 (m, 1H, H-1a); 1.12 (m, 1H, H-11b); 1.01 (s, 3H, C-10-CH_3); 1.00 (m, 1H, H-9); 0.90 (s, 3H, C-17-CH_3); 0.84 (m, 1H, H-7a).

^{13}C NMR (125 MHz, DMSO-d_6): δ = 141.4 (s, C-5), 138.8 (s, C-13), 137.4 (s, C-14), 130.4 (s, C-4), 68.7 (d, C-3), 67.9 (t, C-17-$\underline{CH_2}$-OH), 51.8 (d, C-9), 51.4 (s, C-17), 40.3 (s, C-10), 36.6 (d, C-8), 34.2 (t, C-16), 33.5 (t, C-1), 31.2 (t, C-7), 29.9 (q, C-15), 29.6 (t, C-2), 27.4 (t, C-6), 23.0 (t, C-12), 22.9 (t, C-11), 22.1 (q, C-17-$\underline{CH_3}$), 19.1 (q, C-10-$\underline{CH_3}$).

Metabolite Structure Assignment

The targeted metabolites are different in their structures. Tentative structures have been proposed by Sobolevsky *et al.* [23] and Schänzer *et al.* [38].

The first metabolite, M1, is a monohydroxylated derivative of the parent compound. Its structure was confirmed as 6β-hydroxy-DHCMT by comparison of retention time and ion transitions with commercial reference material.

The metabolite M5 is also hydroxylated in position 6 but also functionalized in position 16 (16-oxo) and reduced in positions 3 (3-hydroxy) and 4(5) (no double bond). Its structure was proposed as 3α,6β,17β-trihydroxy-17α-methyl-4ξ-chloro-5β-androst-1-en-16-one by Schänzer *et al.* [38] based on MS fragmentations. To the best of our knowledge, no reference material for confirmation is available until now.

The other screened metabolites (M2, M3/epiM3 and M4/epiM4) have a rearranged D-ring which goes together with a shift of the 18-methyl group, concomitant with formation of a 13(14) double bond and hydroxylation at 17-CH_3. As proposed by Sobolevsky *et al.* [23] the metabolites M2, M3/epiM3 and M4/epiM4 share the reduction in position 3 (3-hydroxy). The metabolites M3/epiM3 have a fully reduced A-ring. The structure of M3 was further elucidated by comparison with the authentic reference material of 4α-chloro-17β-hydroxymethyl-17α-methyl-18-nor-5α-androst-13-en-3α-ol, with matching retention time and ion transitions according to WADA criteria [39]. Supported by elution order epiM3 is supposed to be the 17-epimer of M3 [19, 28]. Metabolites M2 and M4/epiM4 still contain one double bond in the A-ring. While metabolite M2 is assigned to the 1-ene isomer, metabolites M4/epiM4 are supposedly reduced in position 1(2). Comparison with the inhouse synthesized reference confirmed epiM4 as 4-chloro-17α-hydroxymethyl-17β-methyl-18-nor-androstane-4,13-dien-3β-ol, while M4 is assigned to 4-chloro-17β-hydroxymethyl-17α-methyl-18-nor-androstane-4,13-dien-3α-ol with matching retention times and ion transitions in GC-QQQ-MS.

Excretion Profiles of Metabolites

The excretion period is profoundly different depending on the fraction, the volunteer, or the compound itself. The given signal area was adjusted by the area of the internal standard and the measured specific gravity. Figure 5 displays the excretion kinetics of DHCMT from the glucuronide fraction.

The parent compound (DHCMT) was detected in the unconjugated fraction up to 72 hours after the oral intake. In the glucuronide fraction it was found for a longer time (up to 6.9 days, volunteer 2). The maximum concentration was detected in the first few hours (midtime of sampling up to 10 hours post administration). Volunteers 1 and 5 had a distinct second peak in the excretion of the unconjugated DHCMT as well as of the conjugated substance (Figure 5). The above-mentioned fact of two peaks in the excretion profile is visible throughout all metabolites and volunteers, but it is remarkable only in volunteers 1 and 5.

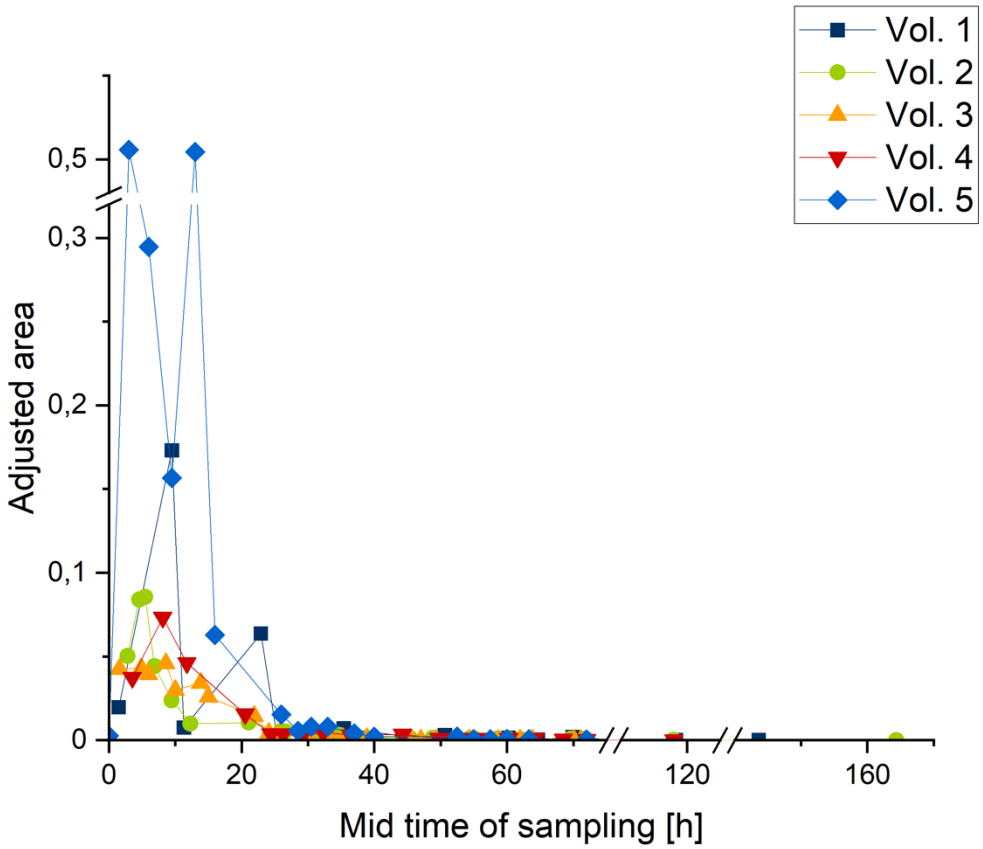

Figure 5: Elimination profile of parent compound DHCMT (detected in glucuronide fraction); peak area adjusted by internal standard and specific gravity

The total amounts over all positive samples are displayed in Figure 6. The relative amount of DHCMT varies from 1.013 % (volunteer 4) and 7.127 % (volunteer 1) for the glucuronidated and between 0.009 % (volunteer 3) and 0.037 % (volunteer 1) for the unconjugated substance of the administered dose of 5 mg.

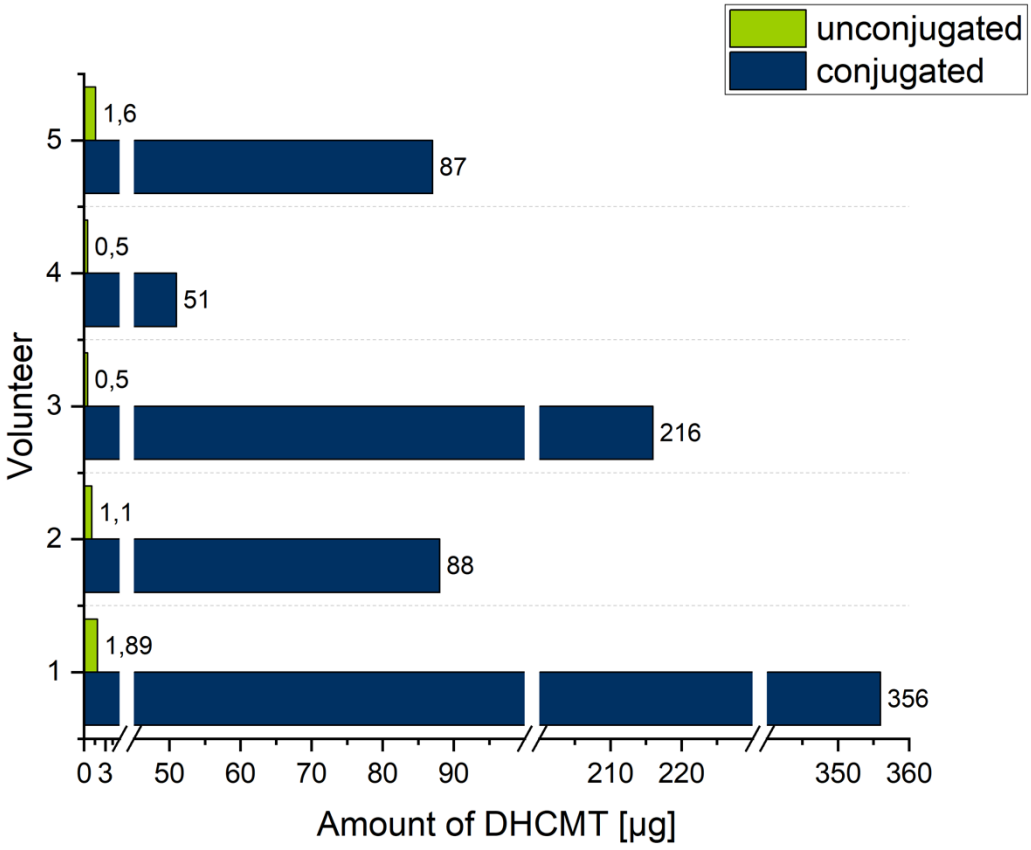

Figure 6: Total amount of excreted DHCMT as conjugated and unconjugated substance in µg

As mentioned above, three of the targeted compounds are found in both fractions. Besides the parent compound, the detection window of two metabolites, M1 and M5, was significantly longer in the unconjugated fraction than in the glucuronide fraction: M1 between 11 h (volunteer 5; glucuronide fraction: 26 h, free fraction: 37 h) and 48.2 h (volunteer 4; glucuronide fraction: 44,3 h, free fraction: 92,5 h), M5 between 44.0 h (volunteer 5; glucuronide fraction: 194 h, free fraction: 240 h) and 144.1 h (volunteer 1; glucuronide fraction: 213.1 h, free fraction: 357.2 h) longer. The detection times of all volunteers for both metabolites in the free and glucuronide fraction are displayed in Figure 7.

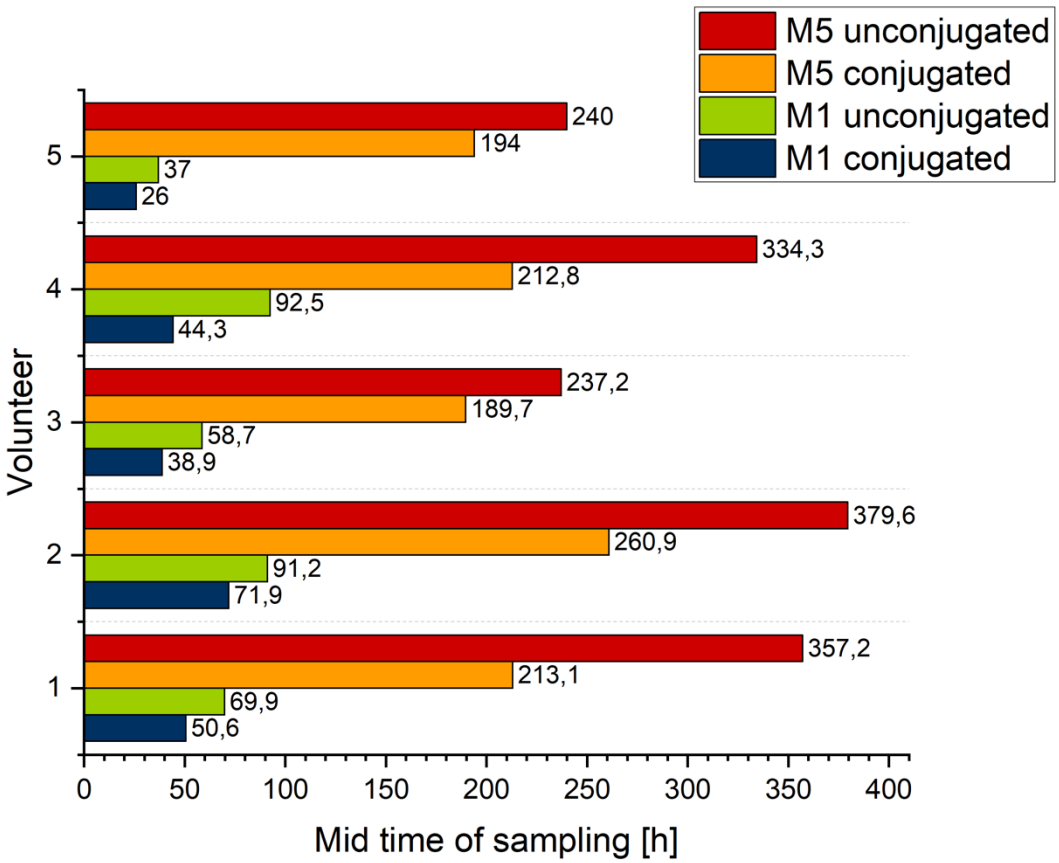

Figure 7: Comparison of the excretion times of metabolites M1 and M5 in the free vs. the glucuronide fraction (all volunteers)

The longest detectable metabolite of every volunteer (separately for unconjugated and glucuronide fraction) for each volunteer is displayed in Figure 8. The metabolite, which is excreted for the longest time, is different between the volunteers. Volunteer 1 excreted M3 for 44.9 days and volunteer 5 for 19.0 days in the glucuronide fraction. In contrast, volunteers 2 and 3 excreted the unconjugated M5 over 15.8 days and 9.9 days whereas M3 was only detectable for 7.8 and 4.9 days (Figure 9). It is noticeable that the later samples of volunteers 1, 2, 3, and 5 had signals different to the blank urine for the metabolite M3 (vol. 1: 58.9 days; vol. 2: 58.8 days; vol. 3: 56.9 days; vol. 5: 57.1 days; Figure 10).
The elimination profiles of the other metabolites (M1, M2, M4, epiM4, M5) are displayed in the supplement.
Excretion times after intake are displayed in Figure 11 and Figure 12.

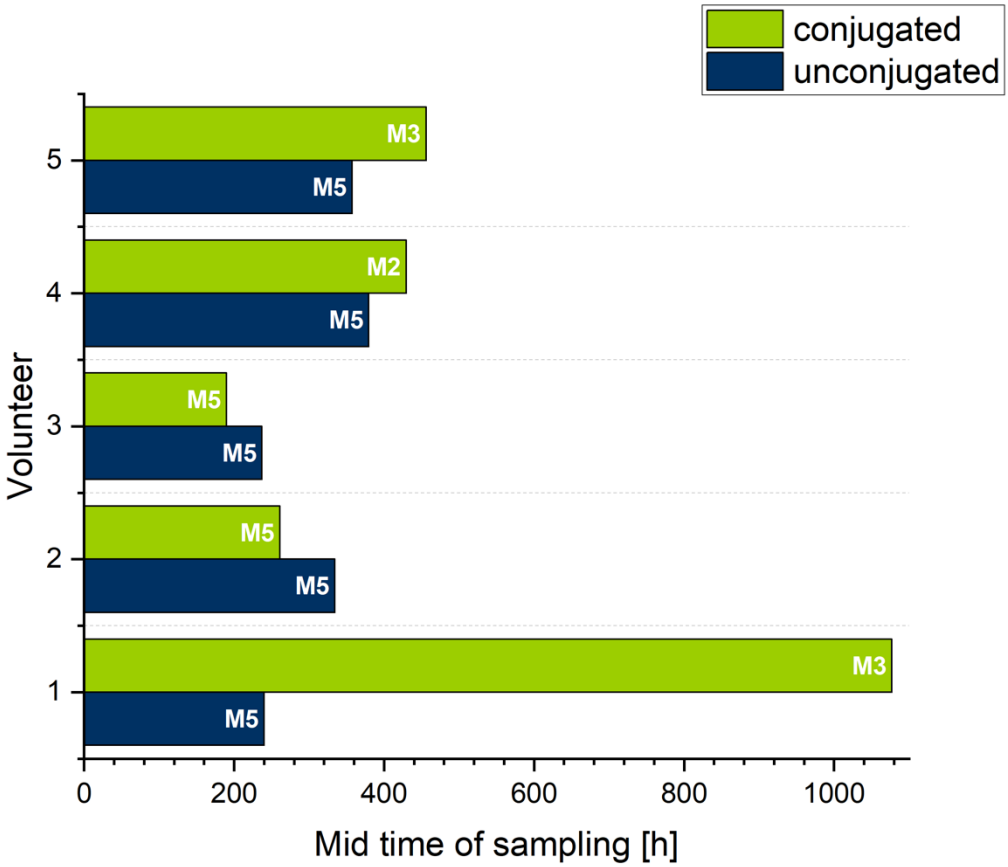

Figure 8: Comparison of the longest detectable metabolites in the free vs. the glucuronide fraction (all volunteers)

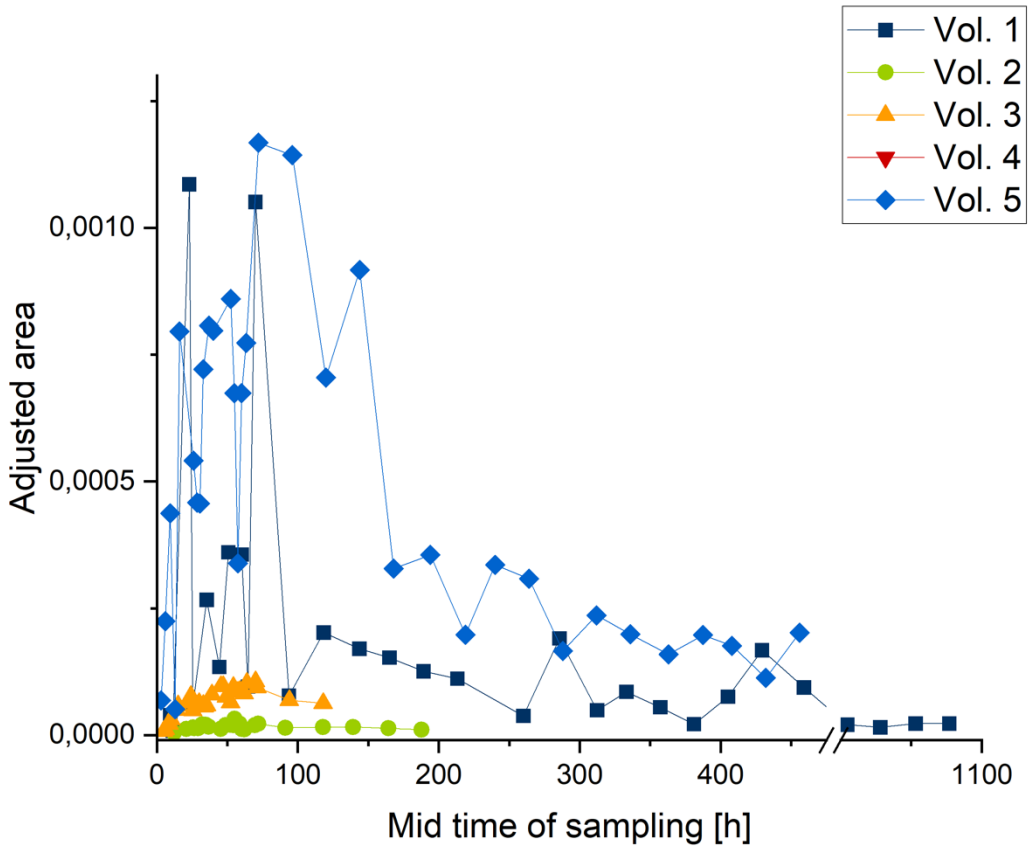

Figure 9: Elimination profile of metabolite M3 (detected in glucuronide fraction); peak area adjusted by internal standard and specific gravity

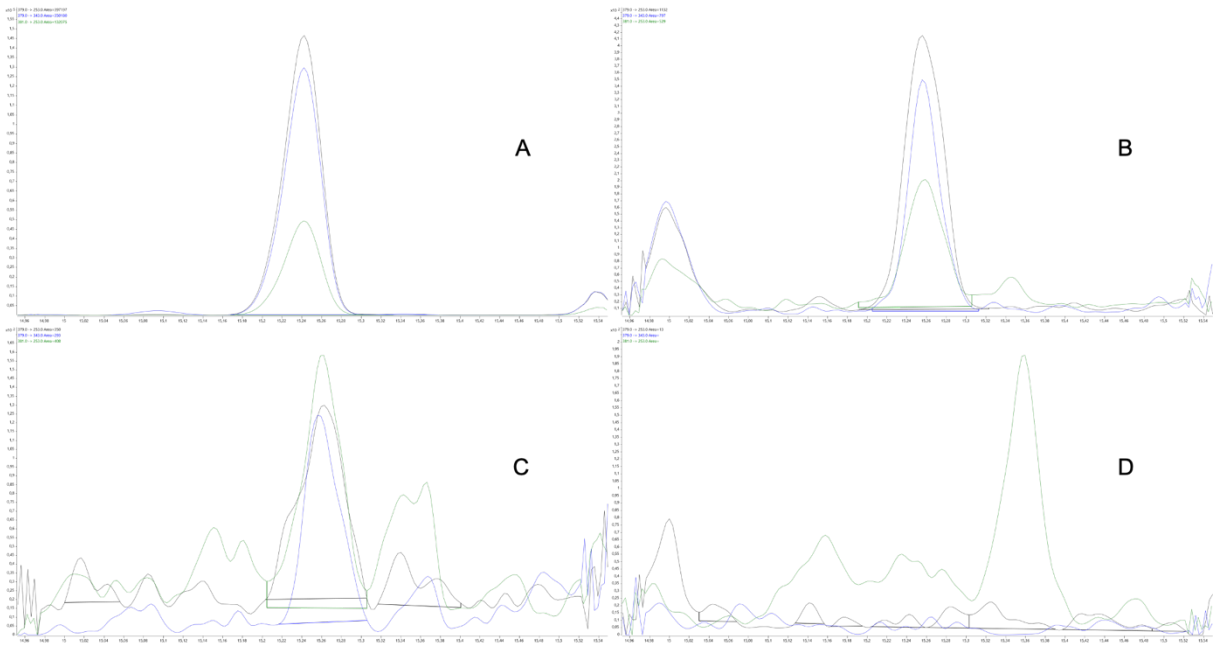

Figure 10: chromatograms (GC-QQQ-MS, 379→253, 379→343, 381→253) of volunteer 4: A standard M3 (1.8 ng/µL); B 24.1 h after intake; C 885.5 h after intake; D blank urine

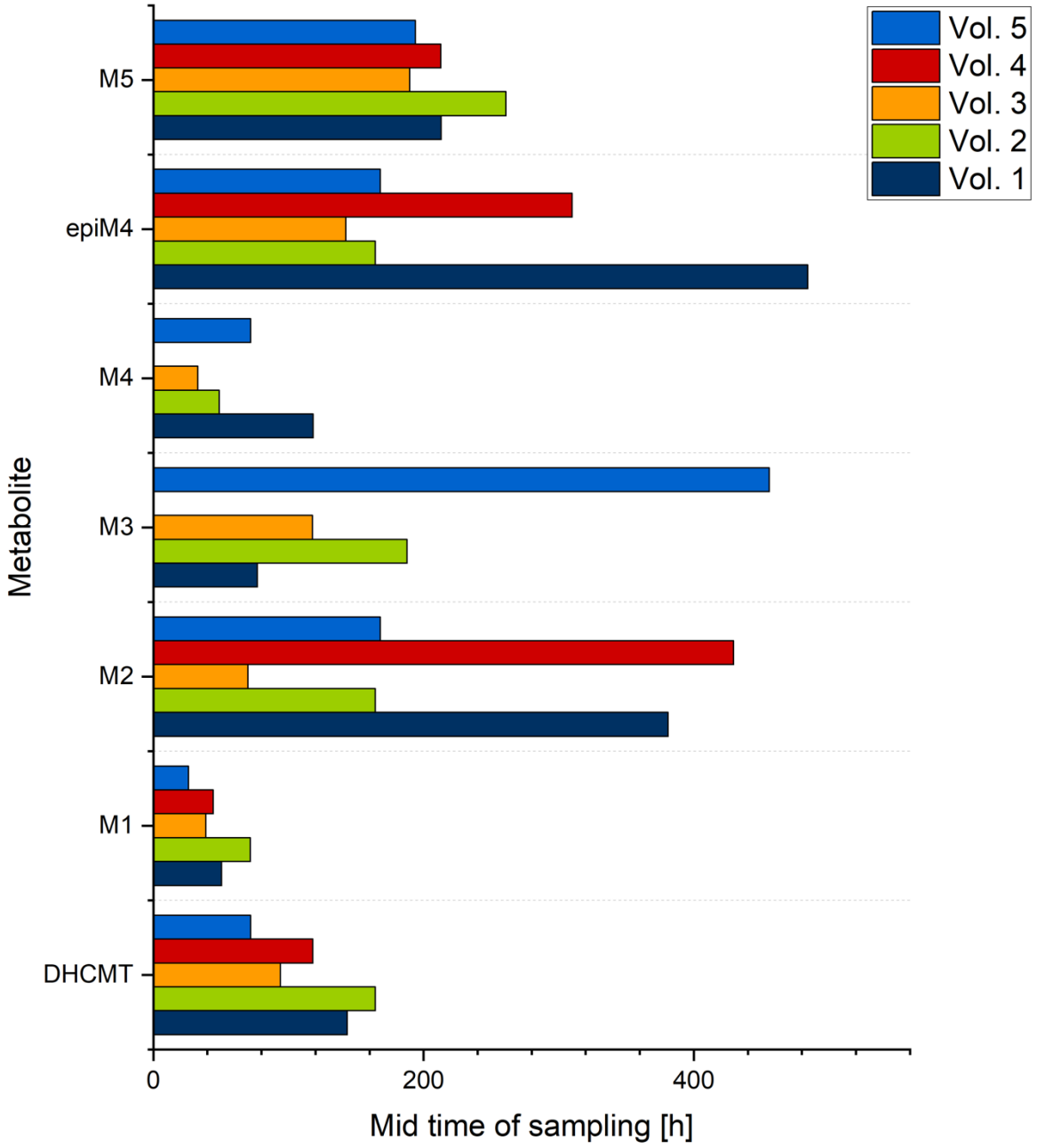

Figure 11: Last positive sample in the glucuronide fraction (time after intake in hours)

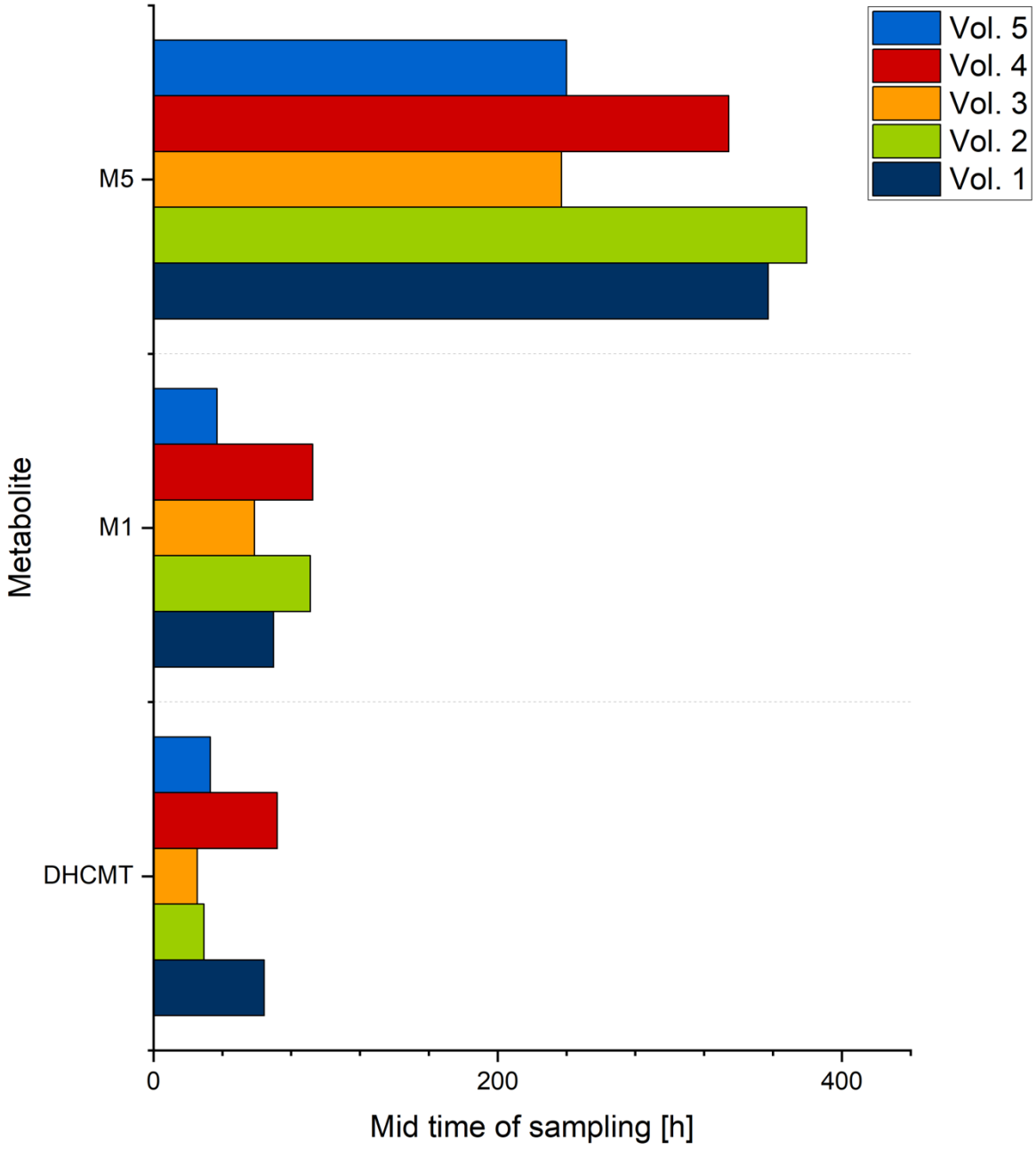

Figure 12: Last positive sample in the free fraction (time after intake in hours)

Evaluation of Activities of Crucial Enzymes of Steroid Metabolism

For the calculation of the activity of the steroid 21-hydroxylase (CYP21A2), the areas of 3α5β-tetrahydrocortisone, 3α5β-tetrahydrocortisol, 3α5α-tetrahydrocortisol, pregnanetriol, and 17-hydroxypregnanolone in the blank samples were compared, as described by Krone *et al.* [40]. For evaluation of the activity of the 3-oxo-5α-steroid 4-dehydrogenase 2 (SRD5A2), the areas of etiocholanolone, androsterone, 3α5β-tetrahydrocorticosterone, 3α5α-tetrahydrocorticosterone, 3α5β-tetrahydrocortisol, and 3α5α-tetrahydrocortisol were used. The ratios are displayed in Table 4 and Table 5.

Table 4: Diagnostic ratios for diagnosis of inborn errors of steroidogenesis and steroid metabolism (steroid 21-hydroxylase) as used by Krone et al. [40]; 17HP: 17-OH-pregnanolone; THE: Tetrahydrocortisone; THF: Tetrahydrocortisol; 5α-THF: 5α-Tetrahydrocortisol; PT: Pregnanetriol

Volunteer	Ratio 1 17HP / (THE + THF + 5αTHF)	Ratio 2 PT / (THE + THF + 5αTHF)
1	0.041	0.175
2	0.020	0.920
3	0.022	0.020
4	0.010	0.031
5	0.016	0.036

Table 5: Diagnostic ratios for diagnosis of inborn errors of steroidogenesis and steroid metabolism (3-oxo-5α-steroid 4-dehydrogenase 2) as used by Krone et al. [40]; Et: Etiocholanolone; An: Androsterone; THB: Tetrahydrocorticosterone; 5α-THB: 5α-Tetrahydrocorticosterone; THF: Tetrahydrocortisol; 5α-THF: 5α-Tetrahydrocortisol

Volunteer	Ratio 1 Et / An	Ratio 2 THB / 5αTHB	Ratio 3 THF / 5αTHF
1	0.362	1.000	0.424
2	1.314	1.609	0.824
3	0.955	1.001	1.348
4	1.851	1.001	1.495
5	0.508	1.058	0.655

The displayed values for each volunteer are representing the ratios of the peak areas of the investigated compounds. For example, the ratio 1 of the 3-oxo-5α-steroid 4-dehydrogenase 2 is formed out of the areas of etiocholanolone and androsterone. When testosterone is metabolized, the first step is the reduction of the 4,5-double bond, giving the 5α- and the 5β-isomer. The following steps resulting in the above-mentioned compounds are catalyzed by the same enzymes for both ways (3α-HSD, 17β-HSD). Therefore, the ratios are giving an overview of the capacity of the 5α-reductase. A value above 1 means that the 5β-derivative is favored, and this is interpreted in a deficiency of the 5α-way.

Discussion

All urine samples were analyzed by GC-QQQ-MS to meet the ultra-trace levels of metabolites. Due to the trace amounts of metabolites excreted in the urines, full direct identification was impossible, even after purification. Thus, GC-MS comparison with authentic reference material allowed for identification of DHCMT, 6-hydroxy-DHCMT and M3, resulting in level 1 confidence [41].

For confirmation of the tentative structures of M4/epiM4 no reference material was available. Thus, inhouse synthesis was required. Four diastereomeric androstane derivatives with a partially reduced A-ring (3ξ-hydroxy-4-ene) and a rearranged D-ring (17ξ-hydroxymethyl-17ξ-methyl-18-nor-13-ene) were obtained. As reported by Schänzer *et al.* [42] the reduction of 4-chloro-androst-4-ene-3,17-dione using K-selectride mainly results in the 3β-hydroxy isomer (ratio α/β ≈ 1/4). Due to the stereoselectivity of Wagner-Meerwein rearrangement the majority of C-17 substitution is hypothesized to yield 17α-hydroxymethyl-17β-methyl products.

After purification of the main product accurate mass spectrometry (GC-QTOF-MS) and NMR confirmed its structure as 4-chloro-17α-hydroxymethyl-17β-methyl-18-nor-androsta-4,13-dien-3β-ol.

Due to the extensive fragmentation caused by the high ionization energy of 70 eV no molecular ion was obtained. The dominant fragment $[M\text{-}CH_2\text{-}OTMS]^+$ (accurate mass *m/z* 377.2073, exact mass *m/z* 377.2067, Δ*m/z* = 1.59 ppm) was found. The loss of 103 Da is considered characteristic for per-TMS derivatives of 17-hydroxymethyl-17-methyl-18-nor-13-ene steroids. The fragment of *m/z* 287.1572 corresponds to another loss of TMSOH (exact mass *m/z* 287.1567, Δ*m/z* = 1.74 ppm). The fragments *m/z* 341.2304 and *m/z* 251.1800 are formed out of the above-mentioned fragments by loss of hydrochloric acid (exact mass *m/z* 341.2301, Δ*m/z* = 0.88 ppm; exact mass *m/z* 251.1800, Δ*m/z* = 0.00 ppm).

NMR data confirmed the structure proposal. Correlation analysis and multiplicity were used to assign 3-hydroxy as β-oriented. The related H-3α (3.92 ppm, ddm) showed a coupling with H-2β with ^3J = 13.3 Hz, which may be explained by the pseudoaxial orientation. NOE of H-3α with H-2α (1.91 ppm, m) and H-1α (1.32 ppm, m) confirmed this assignment. The most shielded signal of C-17-CH_3 detected at 0.90 ppm pinpoints towards β-orientation of the methyl group, concomitant with α-orientation of the 17-hydroxymethyl group. This assignment is also confirmed by correlation signals in NOESY. For confirmation of the substituent pattern at positions C-3 and C-4, predictions of the ^{13}C chemical shifts for 4-chloro-17α-hydroxymethyl-17β-methyl-18-nor-androsta-4,13-dien-3β-ol and 3β-chloro-17α-hydroxymethyl-17β-methyl-18-nor-androsta-4,13-dien-4-ol were first calculated using the HOSE code in nmrshiftdb [43]: The observed and predicted shifts of C-3 are in good agreement for the expected OH group and C-4 for the expected chloro substituent. Utilization of high resolution $^{13}C\{^1H\}$ NMR yielded two signals for C-4 (130.47 ppm, 130.46 ppm). The second signal represents the carbon atom coupled to ^{37}Cl isotope. No split signal was observed for C-3 (neither in 1D nor in HSQC), thus, confirming 3-hydroxy-4-chloro substitution [44, 45].

The side products were identified assigned based on the elution order (see Figure 13). As reported earlier [46], TMS derivatives of 3β-hydroxyandrost-4-enes elute later than their 3α-analogs. Similarly, 17α-hydroxymethyl-17β-methyl show shorter retention times than their 17β-hydroxymethyl-17α-methyl analogs [28]. Thus, the latest eluting isomer is assigned to 4-chloro-17β-hydroxymethyl-17α-methyl-18-nor-androsta-4,13-dien-3β-ol, the first eluting isomer is assigned to 4-chloro-17α-hydroxymethyl-17β-methyl-18-nor-androsta-4,13-dien-3α-ol. These assignments are also in line with the relative abundances of the isomers.

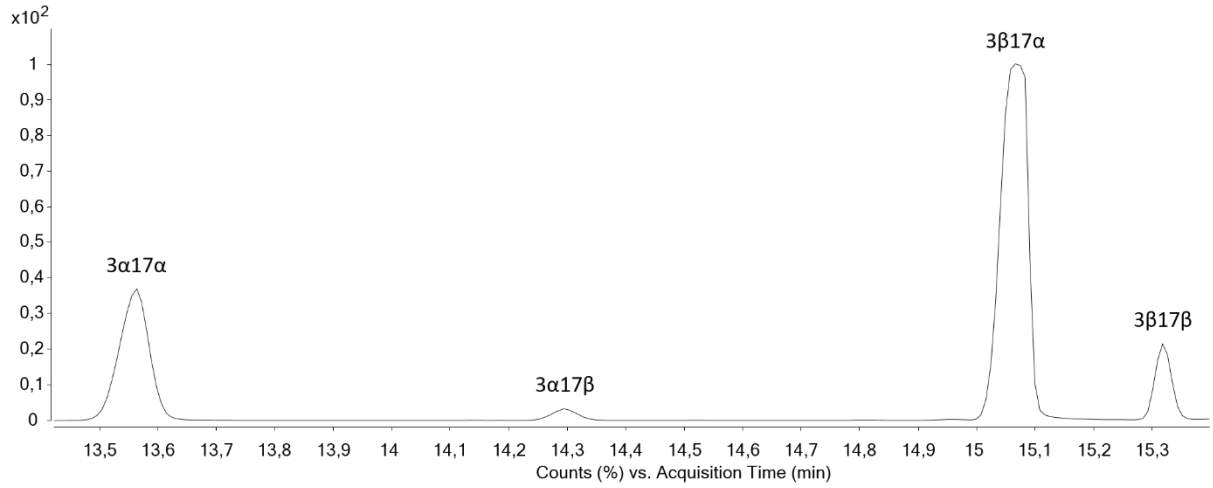

Figure 13: Elution order of the given isomers after synthesis (supposed stereochemistry)

GC-MS comparison of the synthesized references with the results obtained for the urine samples identified M4 and epiM4 as metabolites. Remarkably, that our structure assignments are challenging the hypotheses of Sobolevsky *et al.* in 2012 [23]. The DHCMT metabolite at the retention time of 15.1 min was assigned by Sobolevsky as 4-chloro-17β-hydroxymethyl-17α-methyl-18-nor-androstane-4,13-dien-3α-ol (3α17β). However, the isomer with this retention time is the main product from our synthesis (3β17α). For consistency in metabolite nomenclature, we call this metabolite epiM4, due to its 17α-hydroxymethyl structure. This data was confirmed by Kratena *et al.* [25]. Sobolevsky found an isomeric structure at a retention time of 14.3 min, which he called epiM4. He proposed a structure which is epimerized in position 17 (3α17α). The isomer assigned from our synthetic approach fitting this retention time has a configuration of 3α17β. Again, to be consistent in metabolite nomenclature we use "M4" for this 17β-hydroxymethyl metabolite. As reported [19, 28], the 17α-hydroxymethyl derivatives elute before their 17β-hydroxymethyl isomers. This is just the case if the rest of the molecule is identical. The observed structures in the study have a different A-ring conformation so this correlation does not apply. An overview of the isomer assignments is given in Table 6.

However, based on well-known principles of phase II metabolism, it is likely that the 3-hydroxy group of the metabolites has an α-conformation as all examined substances are excreted as glucuronides. In contrast, steroids with a 3β-hydroxy group are mainly excreted as sulfates [15]. The subsequent investigation could focus on the sulfate fraction of the samples to verify this assumption. The glucuronidation of the hydroxy group at position 17 or 20 is unlikely because it is sterically hindered [15, 47]. Nevertheless, a glucuronidation of a 3β-hydroxy group or of the hydroxy groups in positions 17 and 20 might be possible.

Table 6: Nomenclature of isomeric metabolites M4 and epiM4 in comparison to Sobolevsky's nomenclature [23] including retention times

Retention Time [min]	Nomenclature in this publication	Confirmed Structure (short form)	Nomenclature Sobolevsky 2012	Hypothesized Structure (short form)
15.1	M4	4-chloro-17β-hydroxymethyl-17α-methyl-18-nor-	epiM4	4-chloro-17β-hydroxymethyl-17α-methyl-18-nor-

		androsta-4,13-dien-3α-ol (3α17β)		androstane-4,13-dien-3α-ol (3α17β)
14.3	epiM4	4-chloro-17α-hydroxymethyl-17β-methyl-18-nor-androsta-4,13-dien-3β-ol (3β17α)	M4	4-chloro-17α-hydroxymethyl-17β-methyl-18-nor-androsta-4,13-dien-3α-ol (3α17α)

The data reported here clearly show that it is not to monitor one single long-term metabolite, but to ideally screen for the entirety of all metabolites. This fact becomes apparent when considering the maximum detection time after intake of the substance: Two of the five volunteers excreted the metabolite M3 for the longest time (volunteers 1 and 5), in the samples of two other volunteers (volunteers 2 and 3), metabolite M5 was found for a longer time than any other. The last volunteer (volunteer 4) excreted metabolite M2 as longest detectable substance, while M3 could not be confirmed in any of these samples at all. Therefore, it is indispensable to track any use of DHCMT by screening for all these metabolites. Another crucial point is the combined examination of free and conjugated metabolites. Six of the eight targeted compounds are excreted over a longer period in the glucuronide fraction but for the mentioned metabolites M1 and M5 it is the other way round. The detection of M1 and M5 in the free fraction may be explained by fact that these metabolites are polar enough for being excreted directly after phase I metabolism without the need for conjugation to glucuronic acid. This is plausible due to the additional hydroxy group in position 6 (M1, M5) or the extra oxo group (M5). The calculated log P values are confirming this: log P (DHCMT) = 3.25; log P (M1) = 2.16; log P (M5) = 1.77. In a former investigation by Schänzer *et al.* [38] the metabolite M5 was found in the free fraction up to 9 days after intake of 40 mg of DHCMT. This goes in line with the findings from our study.
The common practice in anti-doping analysis is the cleavage of the glucuronides and analysis of the combined free and glucuronide fraction. This is suggested to be a reasonable way to target all metabolites in one analytical run. Furthermore, especially relevant for DHCMT, M1, and M5, this procedure increases the concentration in the final extract, which may further prolong their detectability.

Sobolevsky *et al.* [23] estimated the excretion time of the metabolite M3 to 40-50 days. In volunteer 1, we indeed found the proposed long-time marker up to 45 days. However, due to the samples that had different signals than the blank urine but were not clearly identified as positive (e.g., lack of single transitions, different ratios of transitions to each other), there is a chance that this window can be further extended by starting from a greater volume of urine (> 6 mL).

For many drugs or xenobiotic substances in general, the body's distribution is displayed by the Bateman function (Figure 14**Fehler! Verweisquelle konnte nicht gefunden werden.**). We assumed idealized one-compartment body model with first-order absorption and elimination as a basis [48]. A first-order kinetics is characterized by the fact that an even proportion of the substance is absorbed or eliminated per time unit [49, 50]. Based on the data from our analyses, especially the two or more cases of increasing concentrations of metabolites after a period of decreasing concentrations (cf. excretion of parent compound of volunteers 1 and 5),

it is highly unlikely that the kinetics of DHCMT, i.e., absorption, distribution, metabolism, excretion, follow a first-order kinetics. Therefore, it cannot be easily described by the Bateman function. Another fact that hampers a smooth excretion curve is the potential effect of enterohepatic cycling. This is well described for estrogens but not investigated for androgens [51]. Another compounding factor could be the different time interval of each days' urine collection: The volunteers did not orient themselves toward the collection process, so on every day there is a variable period between the last urine in the evening that was discarded and the saved one in the morning: this and the minimal concentration of the analytes exacerbate the identification of a defined excretion order.

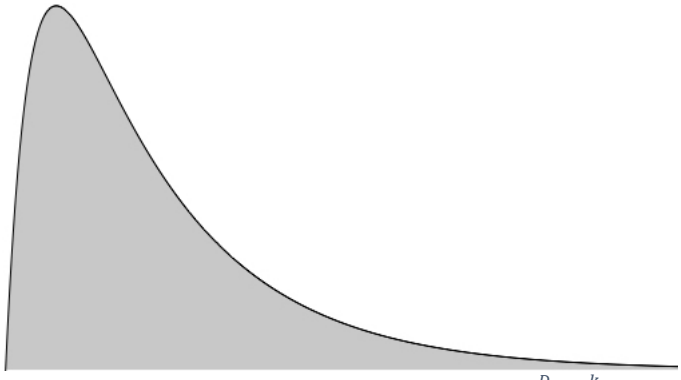

Figure 14: Bateman function; general form: $c(t) = f * \frac{D}{V} * \frac{k_a}{k_a - k_e} * (e^{-k_e * t} - e^{-k_a * t})$

Based on our findings and the common metabolism of anabolic-androgenic steroids, we propose the following mechanisms in the formation of the metabolites investigated in this study.

M1: CYP3A4 hydroxylates testosterone in position 6 [52, 53]. Due to the structural similarity, Rendic *et al.* demonstrated that 6β-hydroxylation also occurred *in vitro* after incubation of DHCMT with recombinant CYP3A4 while CYP2C9 or CYP2B6 did not catalyze this reaction in DHCMT. The stereoselectivity of the catalyzed reaction is explained by the electronic effects of 3-keto-4-ene steroids through the stabilization of the substrate-radical intermediate formed during the reaction [54].

M2-M4: The formation of the altered D-ring structure (17β-hydroxymethyl,17α-methyl-18-norandrost-13-ene) starts with the sulfonation of the 17β-hydroxy group, followed by elimination of sulfuric acid, and a Wagner-Meerwein rearrangement [55]. The hydroxylation of the 17β-methyl group follows catalyzed by CYP21A2 [19]. This results in the intermediate generation of 4-chloro-17β-hydroxymethyl-17α-methyl-18-norandrost-1,4,13-trien-3-one [27]. For generation of the A-ring reduced metabolites, these steps are required to happen first because the hydroxylation of the 17β-methyl group only takes place in the presence of a 3-oxo function. The hydroxylation by CYP21A2 will not take place in the case of 3-hydroxy steroids [29]. An alternative pathway towards this intermediate may be the hydroxylation of C-18 by CYP11B2, followed by a Wagner-Meerwein rearrangement, which was demonstrated for metandienone [28]. Investigations on structure requirements for these reactions should be carried out in the future.

M2: As described by Schänzer, the initial and rate-limiting step in the reduction of the A-ring is the reduction of the 4,5-double bond, directly followed by the reduction of the 3-oxo group. The first step is likely catalyzed by the 5β-reductase as the chlorine atom in position 4 as well as the double-bond in position 1 hamper 5α-reductase (no 5α-metabolite reported for 3-oxo-1,4-diene steroids except M3). The second step is most likely catalyzed by the 3α-hydroxysteroid dehydrogenase. No 3β-formation in a 5β-steroid was ever reported in humans

and 3^{α}-steroids are reported to be mainly excreted as glucuronides, which is also the case for M2 [15].

M3: The structure assignment by Forsdahl *et al.* is 3α-hydroxy-4α-chloro-5α. It is remarkable that the A/B-ring connection is *trans* (5α), while the other reduced metabolites of DHCMT show likely 5β-orientation. Recent data of our group elucidating the structure of new metabolites of metandienone and methyltestosterone including a proposed order of reductions in the A-ring [56] suggest that the double bond in position 1 is reduced before the one in position 4 (cf. supposed formation of M2). Subsequently, the reduction of the 4,5-double bond and of the 3-oxo group take place. Nevertheless, the reduction of the 1,2-double bond is described for metandienone in the presence of a 3-hydroxy group [57]. Massé *et al.* considered a different order in A-ring reduction of metandienone to be possible (first reduction of the 1,2-double bond, then of the 4,5-double bond / 3-oxo group). Unfortunately, no proof of this assumption is published until now [58].

M4: For M4, the next steps are the reductions of the 3-oxo group by 3α-hydroxysteroid dehydrogenase (3α-HSD) and of the 1,2-double bond.

M5: The hydroxylation in position 6 is described above for M1. The introduction of the oxo-function in position 16 can be a product of 16-hydroxylation by CYP3A4, followed by oxidation. 16-Hydroxylation is well-known in the metabolism of estrogens [59] but also reported for testosterone [53]. The enzyme responsible for the following step, the oxidation of the 16-hydroxy group, remains unclear for now, whereas the steps afterward (reduction 4,5-double bond, reduction 3-oxo group) are catalyzed as described above.

The formation of epiM3 and epiM4 is not elucidated yet. For the case of epiM3 no sample was clearly marked positive.

In case of the metabolites M2, M3, M4, and M5, the formation is hypothesized. Further experiments for confirmation are desired in the future. An overview of the postulated metabolism of DHCMT is displayed Figure 15.

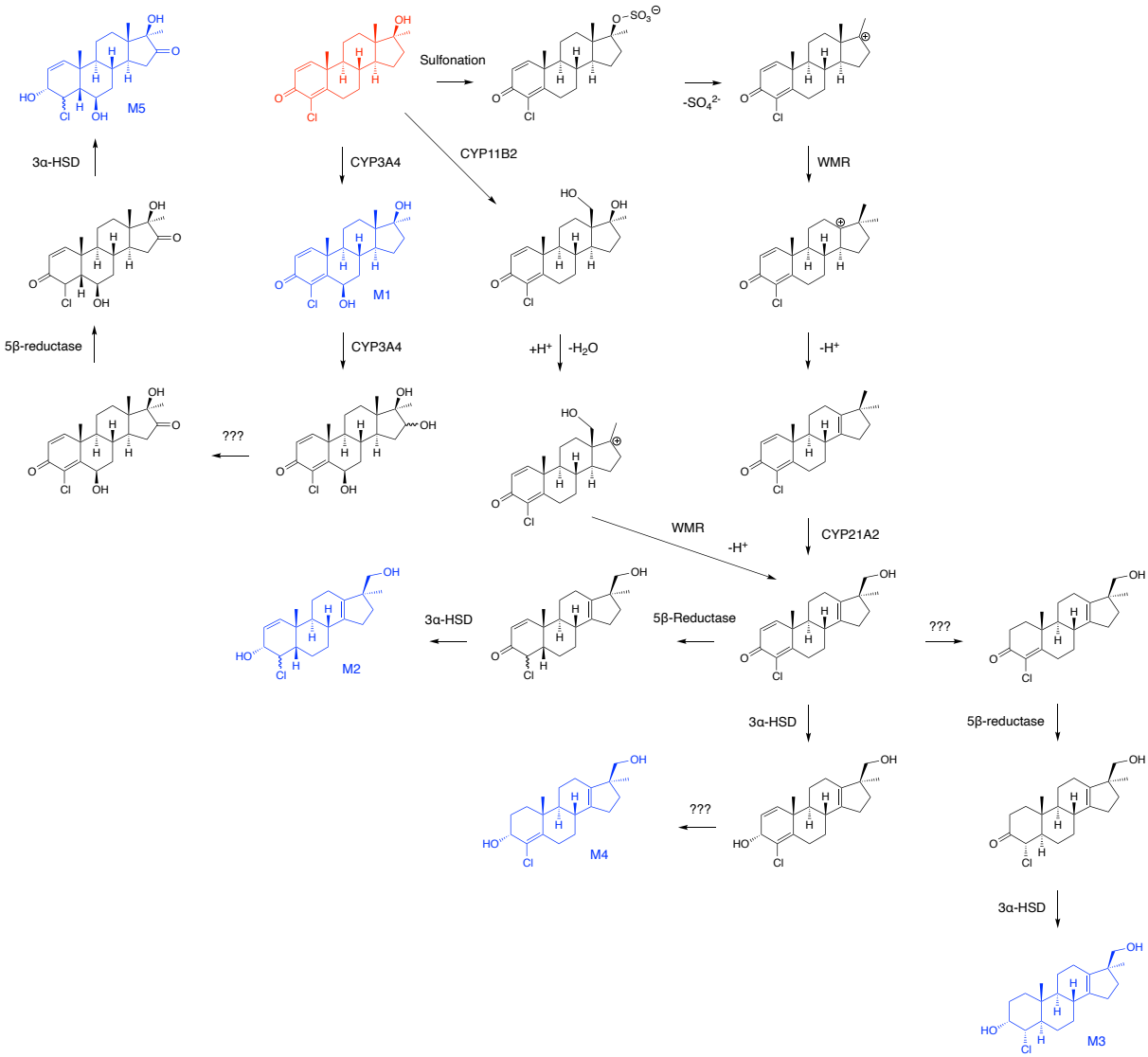

Figure 15: Postulated metabolism of DHCMT including the formation of metabolites M1-M5; question marks indicate enzymes that have not been elucidated yet; red = parent compound; blue = targeted metabolites

The differences in the excretion of the long-time markers M3 and M4 between the younger volunteers (vol. 1, 2, 3, and 5) and the older one (vol. 4) may be explained by the proposed metabolic pathways leading to these metabolites. As M2, M3, and M4 supposably share their D-ring structure, it is assumed that the difference in pathways of formation happens later. From the data of the extended steroid profiles all volunteers show a sufficient activity of CYP21A2. It remains unclear why volunteer 4 excretes epiM4 and M2 but not M3/M4. Therefore, a deficiency in the enzyme that reduces the 1,2-double bond is not plausible. The metabolite epiM3 was not clearly identified in any sample. This could have been caused by the fact that the other ways of metabolite formation are highly favored in comparison to epiM3. Other possibilities are a different location of the involved enzymes or a kinetic problem. Due to the fact that the metabolism of 17-epi metabolites of DHCMT is not elucidated yet, these occurrences cannot be explained for now.

The activity of 5α-reductase and its impact on the formation of different metabolites is hard to evaluate, because the finally excreted amount of metabolite is caused by the complex metabolism. Additionally, the structures of the metabolites M2, epiM3 and M5 are not confirmed yet due to the lack of reference material. Nevertheless, the values can provide some interesting information regarding the structure of the metabolites. Volunteer 4 excretes

the metabolite M2 longer than any other volunteer. At the same time, he has the lowest 5α-reductase activity. This could provide an indication of the structure of this metabolite and favor the 5β-structure. If the volunteers 2 and 3 had higher 5α-reductase activity, perhaps they would produce more M3 and thus excrete it longer. Because of the almost independent excretion of metabolite M5 of the 5α-reductase activity, this indicates that the structure assignment of Sobolevsky was right (5β).

Finally, the various differences in metabolite formation in combination with the excreted amount of parent compound underline the fact that anabolic-androgenic steroids are extensively metabolized and that the location and point in time of metabolism are mostly unknown.

Conclusion

The controlled administration study confirmed that the analytes proposed by Sobolevsky *et al.* can be used to trace back the intake of DHCMT. Our findings support Sobolevsky's hypothesis of a long-term metabolite with a reduced A-ring and an 18-nor-13-ene-17α-methyl-17β-hydroxymethyl structure. These findings support the current practice of routine doping analysis by targeting these metabolites.

In routine anti-doping control, an aliquot of 2 mL urine is prepared prior to analysis combining the unconjugated and glucuronidated metabolites. In this study 6 mL of urine have been used to enlarge the detection time of the metabolites. Separate analysis of the phase-II metabolite fractions gave further insights into the metabolism. The results show that some metabolites can be identified clearly until a certain point of time but are likely excreted even longer. Still, higher amounts of urine, such as 20 mL, might be used to possibly further extend the detection window of this metabolite. Due to the difficulties in handling such a large amount of sample volume, this procedure may not be suitable for routine analysis but could be interesting in confirmatory testing or in future studies of metabolism.

In this study, all volunteers belong to the Caucasian ethnicity. Nevertheless, the excretion profiles of the different volunteers are considerably different. Therefore, it is indispensable to monitor all metabolites irrespective of the expectation of the longest detectable metabolite.

To verify the proposed metabolite structures, it is mandatory to synthesize reference material to achieve the highest confidence level [41]. A level 1 confirmation of the structures of M2 and M5 would be highly desirable.

A potential next experiment will be the inclusion of volunteers from other ethnic groups to investigate potential different metabolism pathways of non-Caucasian test persons. The determination of characteristics (polymorphism, dysfunctionality, et cetera) of the future volunteers' enzymes like UDP-glucuronosyltransferases or cytochromes P450 would be helpful. Additionally, blood samples and tissue could be collected during the next study to calculate the volume of distribution and to uncover potential drug and metabolite depots. This may help in understanding the distribution of the substances.

To elucidate the remaining 'blind spots' in DHCMT metabolism, further experiments with potential intermediate metabolites and purified enzymes are mandatory. Parallel investigations on the substrate selectivity using molecular modeling techniques may explain and substantiate the experimental data.

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Synthesis of 4-Chloro-17α-hydroxymethyl-17β-methyl-18-nor-androsta-4,13-dien-3β-ol

A 0.05 M solution of 4-chloro-androst-4-ene-3,17-dione in dry Et_2O under argon atmosphere has been treated with 1.1 eq of K-selectride (1 M) by dropwise addition to the stirring solution. After 1.5 h the reaction mixture was diluted with water and extracted three times with Et_2O. The combined organic phases were washed with brine and dried with Na_2SO_4. Evaporation at reduced pressure gave the crude compound as a yellow solid.

The reduced substance was dissolved in dry DMF (0.1 M) then 2.5 eq of imidazole and 1.5 eq of TBSCl have been added at room temperature. After 48 h of stirring the solvent was evaporated. The crude solid has been solubilized in EtOAc/H_2O and transferred in a separatory funnel. The obtained organic phases have been washed with brine and dried with Na_2SO_4. After evaporation the product was obtained as a yellow oil.

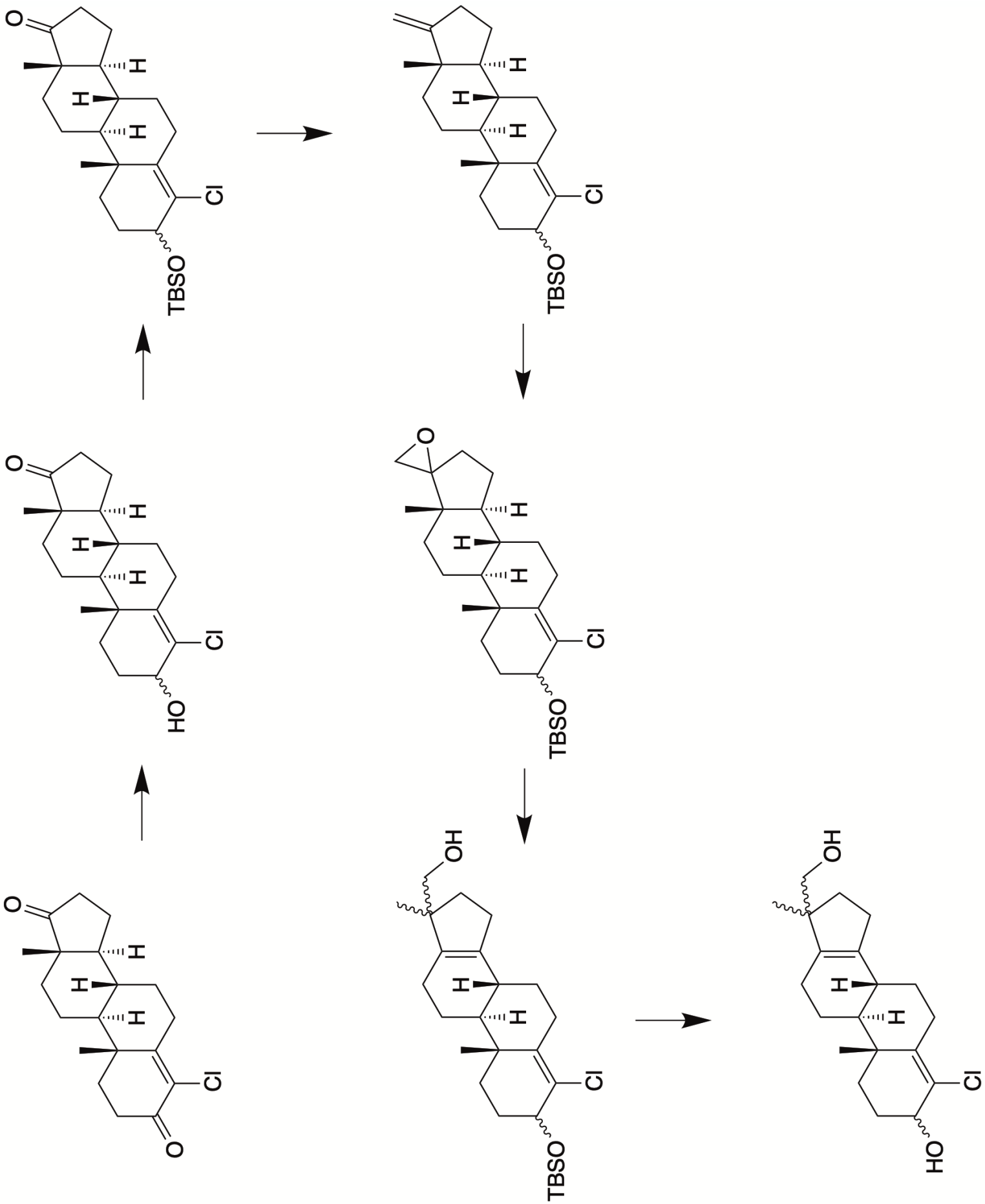
A solution of Nysted reagent (20 % wt., 5 eq) diluted in half volume of dry THF was cooled to 0 °C and subsequently treated with 2.2 eq of $TiCl_4$. After stirring for 15 minutes at 0 °C the solution was allowed to warm to room temperature. Next, a solution of starting material dissolved in dry THF was added dropwise to the reaction flask to give a violet mixture. After overnight reaction at room temperature the reaction was quenched with HCl_{aq} (1 M) at 0 °C. Following dilution with water and the mixture was extracted three times with Et_2O. The combined organic phases were washed with brine and dried over Na_2SO_4. Evaporation at reduced pressure gave the crude compound as a yellow solid.

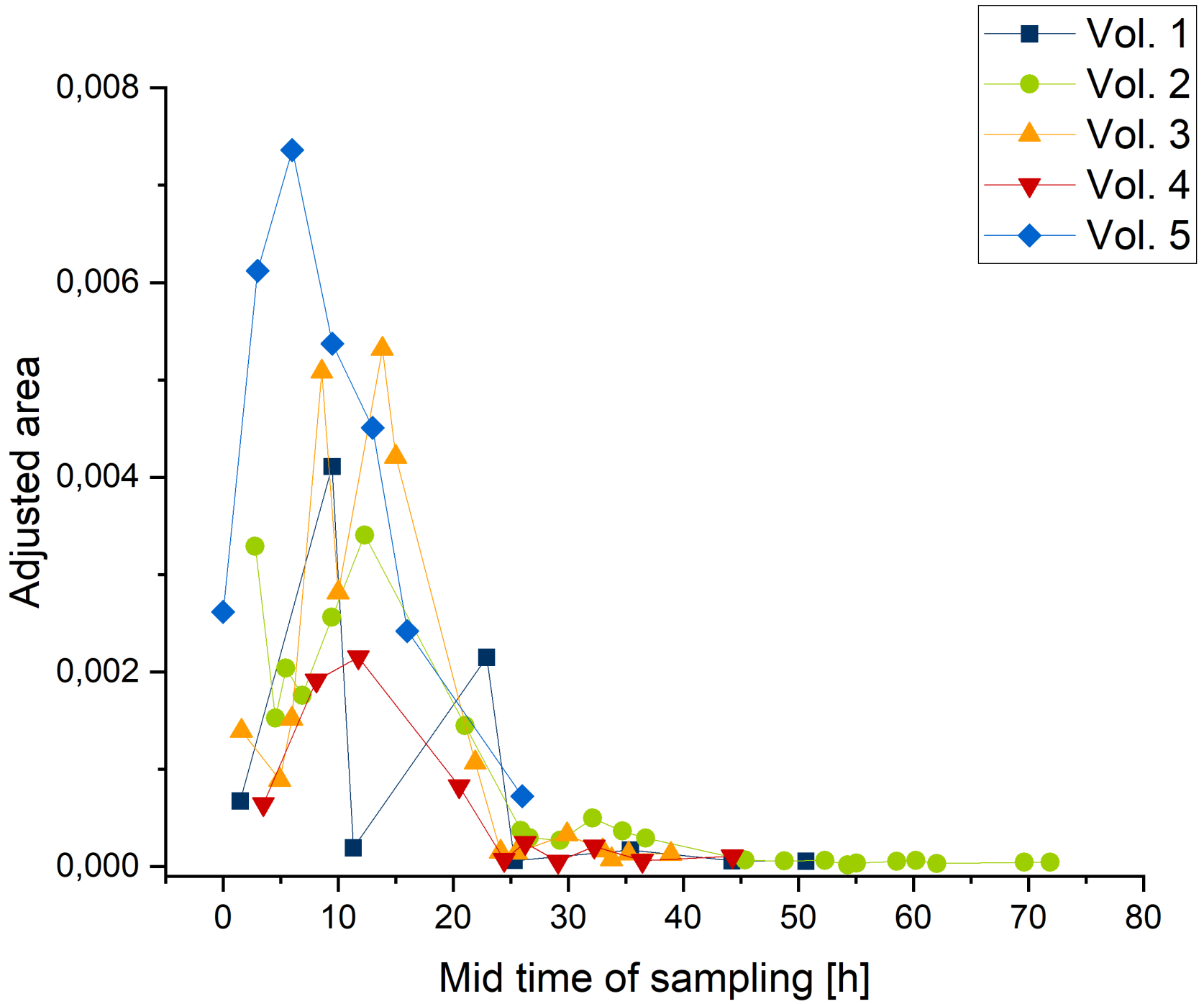
To a 0.1 M solution of the olefin in DCM, 3 eq of $KHCO_3$ and 1.2 eq of m-CPBA have been added at room temperature. After 3 hours, the solution was diluted with water and extracted with DCM in a separatory funnel. The combined organic phases were washed with brine and dried with Na_2SO_4, then evaporated to give the crude compound as a yellow solid.

To a solution 0.04 M of DCM under argon atmosphere at -78 °C, 2.2 eq of 2,6-lutidine and 2 eq of TMSOTf have been added dropwise. After five minutes the compound has been solubilized in DCM and subsequently added dropwise to the reagent's solution at -78 °C. After one hour the reaction mixture has been quenched by the addition of HCl_{aq} 2 M (10 eq) and let stir for 30 minutes at room temperature. Then, the solution was diluted with water and extracted with DCM in a separatory funnel. The combined organic phases were washed with brine and dried with Na_2SO_4, then evaporated to give yellow mixture.

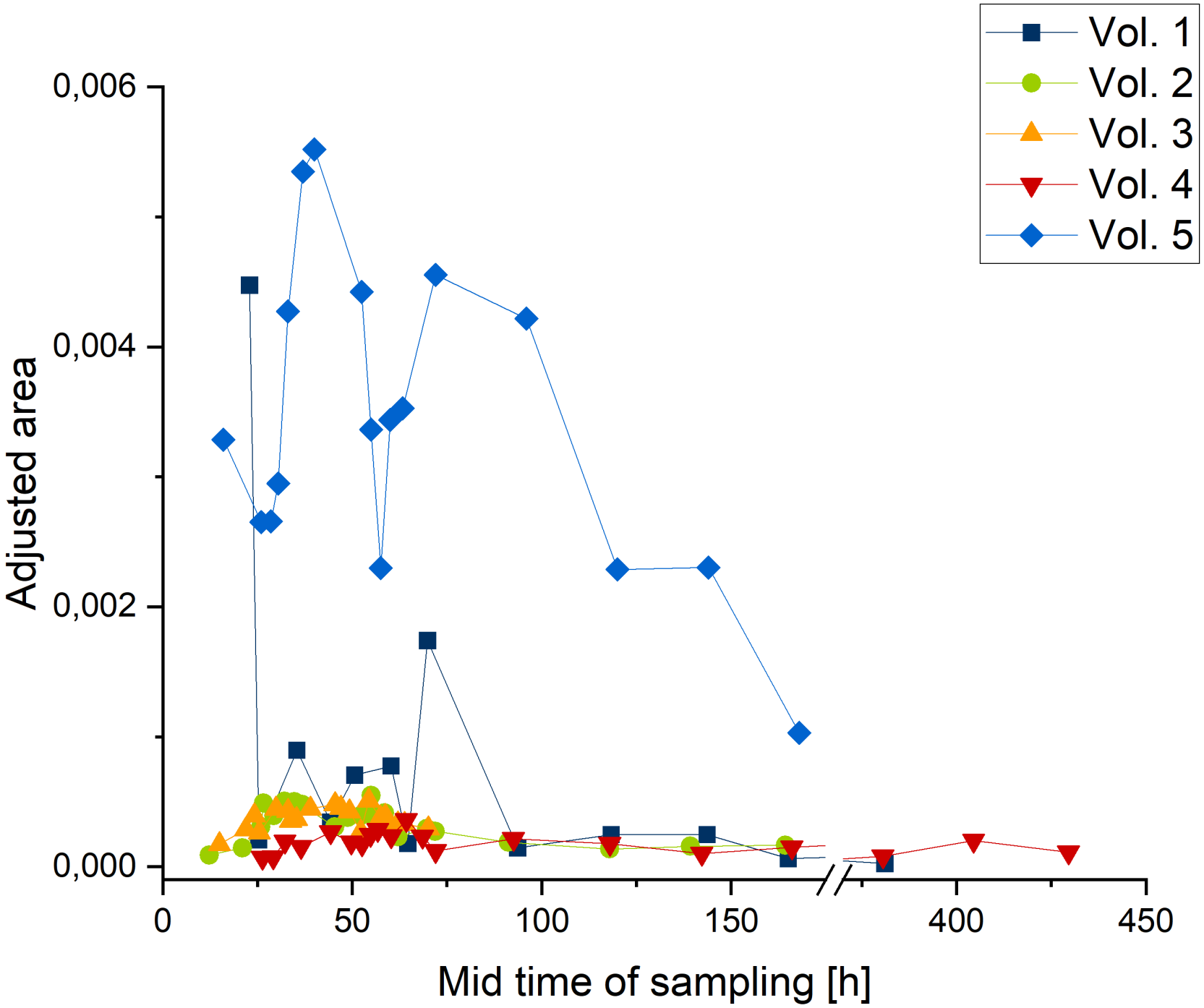
To a solution 0.04 M of epoxide in MeOH, 5 eq of aqueous HCl have been added dropwise. After 1 hour the Wagner-Meerwein rearrangement and deprotection was already accomplished. The mixture was let react overnight at room temperature. Then it has been quenched by addition of water and diluted in TBME and extracted in a separatory funnel. The combined organic phases were washed with brine and dried with Na_2SO_4 then evaporated to give the crude final product as a yellow oil.

Supplement S2
Synthesis of epiM4 isomers

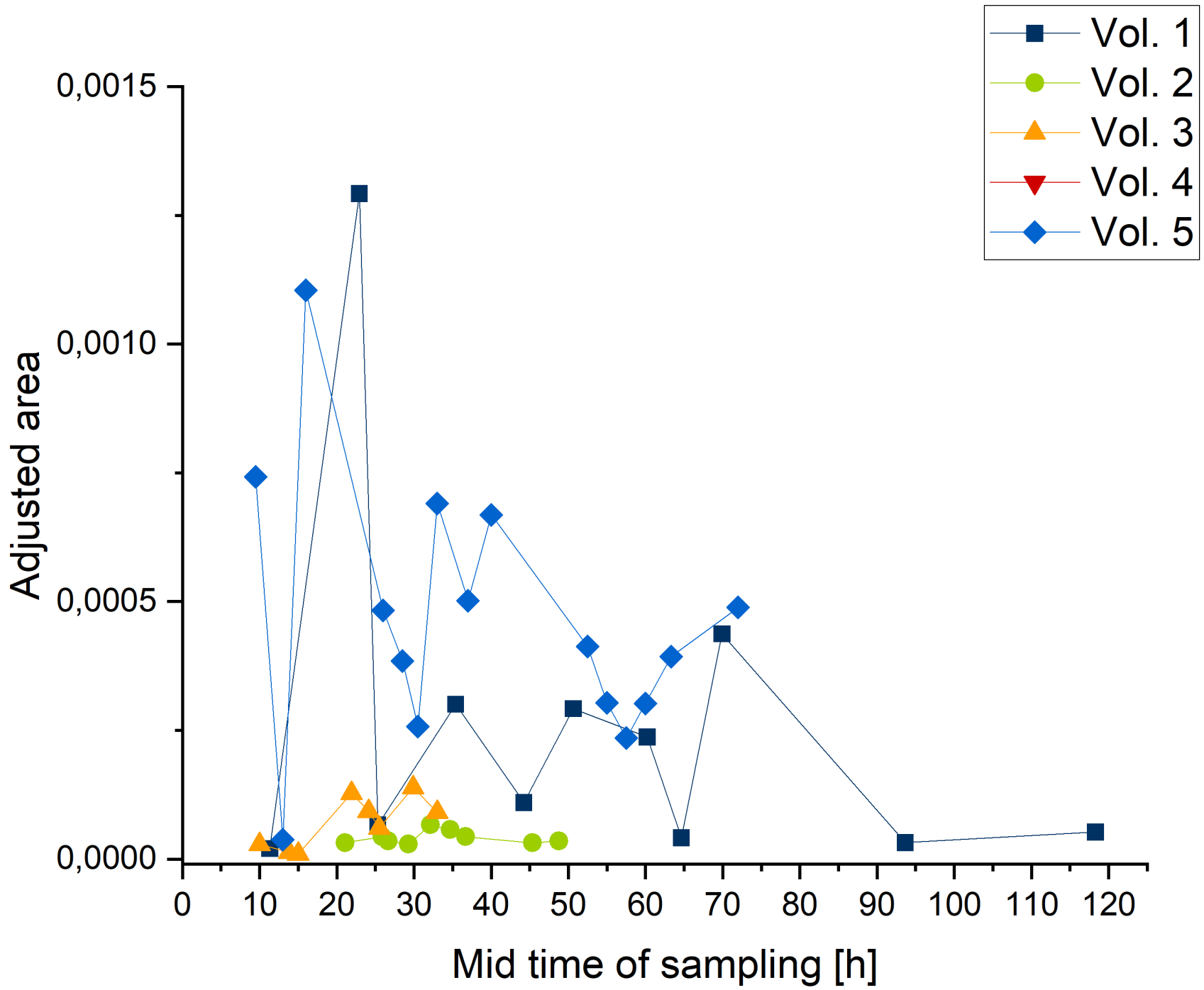




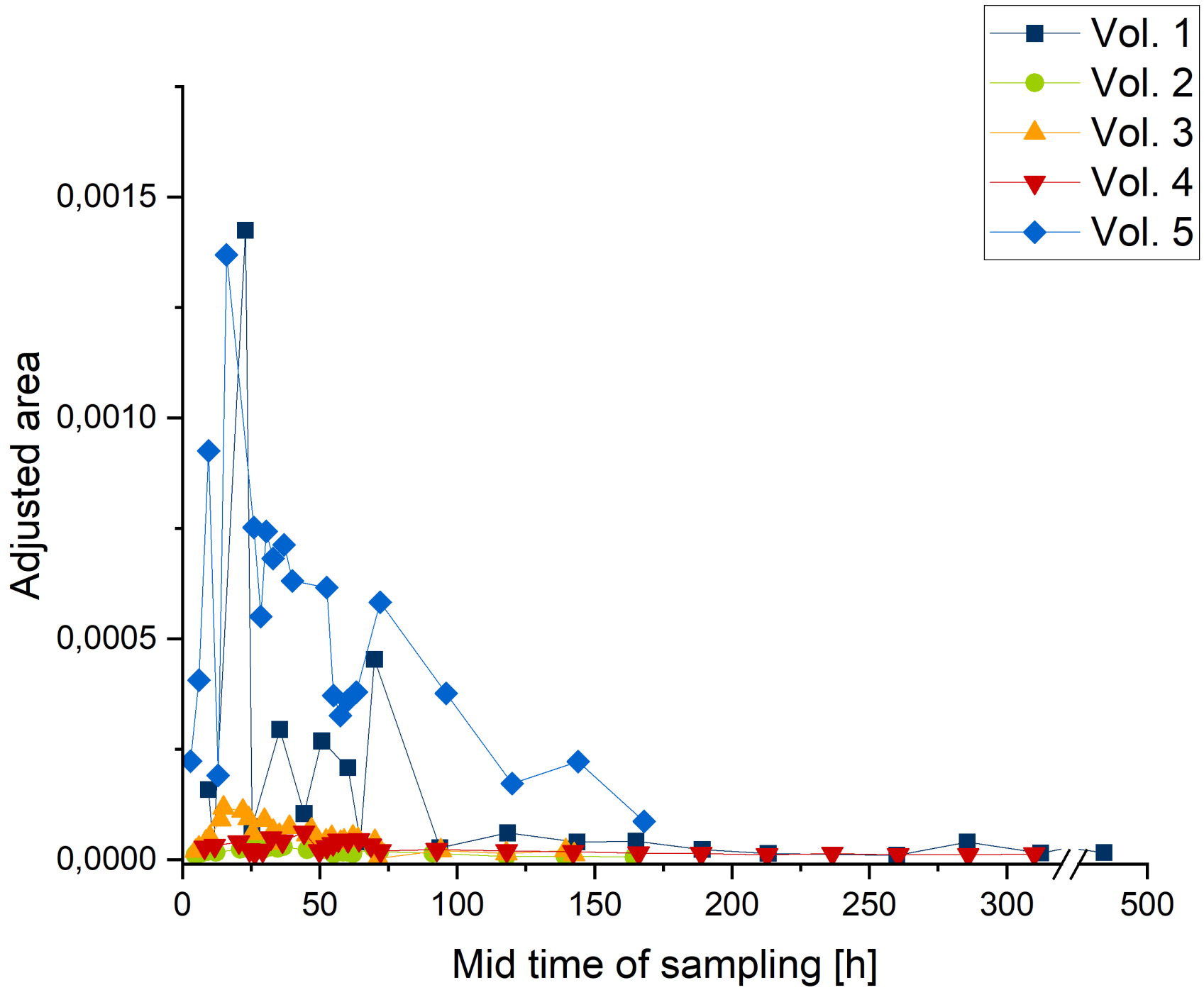
Supplement S3
Excretion profile M1 (glucuronide)



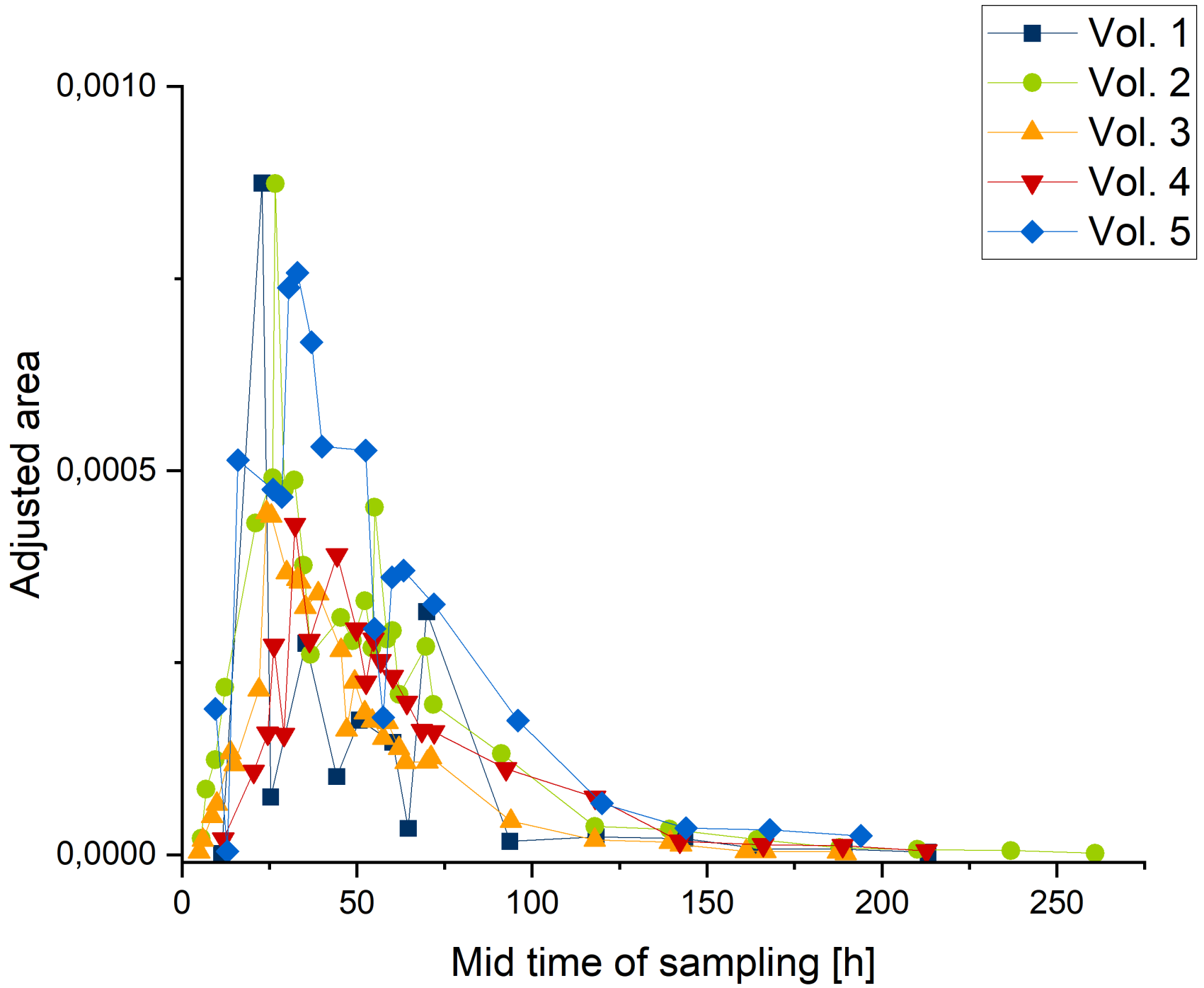
Supplement S4
Excretion profile M2 (glucuronide)



Supplement S5
Excretion profile M4 (glucuronide)



Supplement S6
Excretion profile epiM4 (glucuronide



Supplement S7
Excretion profile M5 (glucuronide)

4 Declaration of Own Contribution

The following list displays the author's contribution to the publications listed under "Manuscripts".

Manuscript I

- *In-vitro* methodology in cooperation with co-authors
- Data curation in cooperation with co-authors
- Preparation of manuscript in cooperation with co-authors
- Visualization

Manuscript II

- Synthesis of compounds used as substrates for subsequent biotransformation
- Execution and evaluation of biotransformation in cooperation with co-authors
- Data curation in cooperation with co-authors
- Preparation of manuscript in cooperation with co-authors

Manuscript III

- Conceptualization in cooperation with co-authors
- Methodology in cooperation with co-authors
- Investigation in cooperation with co-authors
- Data curation in cooperation with co-authors
- Preparation of manuscript

Manuscript IV

- Conceptualization in cooperation with co-authors
- Methodology
- Investigation
- Data curation
- Preparation of manuscript

5 Discussion and Outlook

This work provides deep insights into the substrate specificity of CYP21A2, the metabolism of the AAS MD, MT, and DHCMT, and steroid metabolism in general. Therefore, the results obtained are essential for the research in anti-doping analysis, biochemistry, and endocrinology. This list is not terminatory and may be continued by areas focusing on general metabolism, disease markers and the synthesis of reference material.

The findings from the publications as mentioned above shall be discussed concerning their benefits and limitations in anti-doping analysis and steroid metabolism, as well as their potential continuation in the future.

During the last years, WADA's anti-doping analysis practice and performed tests were criticized [103]. The work of Kopylov will be used as the basis for the following discussion [104].

As shown before, a first step in identifying a doping athlete is an AAF. Specific requirements for chromatographic retention time and mass spectrometric mass shift are claimed by WADA for the analyte identification. The analyte in the sample is compared to a spiked positive control, a reference collection, or reference material [105]. A reference collection is defined as a collection of samples, for example, coming from a controlled administration study, which can be used to determine the identity of an unknown substance [10]. Although reference material is not always needed according to WADA, it remains the best way to confirm an AAF. Therefore, it is desired to make reference material available to the scientific community [106]. This does not only conciliate the critics but makes anti-doping analysis more transparent and evident.

The synthesis of the desired compounds often implicates various difficulties due to the reaction's regio- or stereo-specific requirements. Hence, not only chemical but also biotechnological methods are increasingly used. Based on former investigations of Zöllner *et al.* [87] and Liu *et al.* [86] using CYP21A2 for the synthesis of a MD and a DHCMT long-term metabolite, the idea of this thesis was to transfer the method to other 17α-methyl steroids to potentially generate more metabolites with a 17β-hydroxymethyl-17α-methyl-18-nor-13-ene structure which could then function as reference standards for potential long-term markers as well. Therefore, it was indispensable to understand how the enzyme is working and which requirements must be met to use it reasonably. As

described in manuscript I, the endogenous substrates of CYP21A2, progesterone, and 17α-hydroxyprogesterone, were tested together with their A-ring reduced derivatives pregnenolone and 17α-hydroxypregnenolone in a whole-cell biotransformation assay using a strain of the recombinant fission yeast *Schizosaccharomyces pombe* (strain CAD75). The incubation yielded the successful hydroxylation of progesterone, giving 11-deoxycorticosterone, and 17α-hydroxyprogesterone, giving 11-deoxycortisol. In contrast pregnenolone and its 17α-hydroxy derivative remained unchanged. A subsequent molecular modeling experiment highlighted the need for the presence of a 3-oxo group. Hence, substances with a 3-hydroxy group could not be hydroxylated in position 21.

To find more metabolites of AAS with the previously described 17β-hydroxymethyl-17α-methyl-18-nor-13-ene structure, 16 substances were incubated with CYP21A2 in the same manner as described for manuscript I. The substances had a 17,17-dimethyl-18-nor-13-ene structure in common and various modifications in rings A-C. The substances with a 3-oxo group were hydroxylated at C-20. In contrast, the potential substrates with a hydroxy group or chlorine at position 3 did not undergo the hydroxylation reaction. Modifications at other positions than C-3, e.g., fluorine at C-9, a hydroxy group at C-11, or substitution of C-2 by an oxygen atom, did not affect the hydroxylation (manuscript II).

On the one hand, these results provide an insight into the substrate specificity of CYP21A2, which will allow future syntheses to be designed with the aid of this enzyme. Stereoselective reactions may be performed easier compared to chemical synthesis. Furthermore, manuscript II elucidates the metabolism of steroids, focusing on CYP21A2. This is helpful to understand how metabolites are formed in the human body and which intermediate products are involved. The results have also falsified some ideas of steroidogenesis in which the hydroxylation of pregnenolone was supposed to be catalyzed by steroid 21-hydroxylase [107].

The knowledge about the substrate specificity of enzymes in general and CYP21A2, in particular, is essential not only for the synthesis of reference material but also for the evaluation of the metabolic profile of athletes. If one enzyme usually plays a key role in the formation of metabolites but is deficient in one person, it becomes apparent that screening for metabolites in anti-doping analysis should not only focus on one category of excreted substances, even if they are known as long-term markers. The analytical method should also investigate various structures to catch athletes with a different excretion profile. It is more likely to convict the athletes of the use of AAS if the targeted metabolites are as heterogenic as possible.

Although the investigated exogenous substances MT, MD, and DHCMT have been used in men and women for at least 50 years or, in the case of the endogenous compounds progesterone, pregnenolone, and their 17α-hydroxy derivatives, have been isolated in the first half of the 20th century, the metabolism has not been fully elucidated yet. This fact opens the floodgates to people who question the results and, therefore, try to find a way to avoid a disciplinary measure. Therefore, it is crucial to investigate the metabolism itself. Additionally, the stereochemical properties of the formed metabolites should be elucidated as well.

Sobolevsky *et al.* made the assumption based on the prior mentioned literature that the structure of the mainly excreted long-term metabolite of DHCMT was 17β-hydroxymethyl-17α-methyl-18-nor-13-ene as Schänzer *et al.* described this for MD (besides an additionally fully reduced A-ring, a 3-hydroxy-4-ene or a 3-hydroxy-1-ene structure) [73, 74]. The stereochemical orientation of position 17 was confirmed by comparison with the synthesis of the M3 (4α-chloro-17β-hydroxymethyl-17α-methyl-18-nor-5α-androst-13-ene-3α-ol) in 2018 [108, 109]. The M4 was also successfully synthesized and confirmed as 4-chloro-17α-hydroxymethyl-17β-methyl-18-nor-androst-4,13-dien-3β-ol by our group and later verified by Kratena *et al.* [110-112]. The noticeable fact is the stereochemistry in position 17: While M3 goes in line with the assumption of Sobolevsky *et al.,* the mainly excreted metabolite in the case of M4 is the one with a 17α-hydroxymethyl element which is opposite to the postulated structures. Following this, the synthesis of all potential isomers of M3 (containing 17α-hydroxymethyl-17β-methyl) is absolutely necessary to identify the structure of epiM3 and elucidate if there is also a switch like in M4.

Based on these findings, the synthesis of other potential metabolites of 17α-methylated steroids with the epimerized structure in position 17 and a fully reduced A-ring was performed. As mentioned above, the substances MT and MD can be considered as derivatives of DHCMT. Because of the missing chlorine, the number of potential isomers is reduced. In a full chemical approach, metabolites with a 17α-hydroxymethyl-17β-methyl-18-nor-13-ene and a 17α-hydroxy-17β-methyl structure had been synthesized out of androsterone derivatives. The produced metabolites were detectable in post-administration urine for up to 48 hours (manuscript III).

Additionally, the findings raise questions for other steroids: The found metabolites from MD have a 5β-backbone, while in MT, also a 5α-structure occurs. This is in line with earlier insights with substances having a 1,4-diene-3-oxo structure: If the 1,2-double

bond is present during the reduction of the 4,5-double bond, no 5α-product is detectable [113]. The DHCMT metabolite M3 has an A-ring structure with only α-orientated substituents, even though the parent compound contains a 1,2-double bond and chlorine in position 4 [81]. As mentioned in manuscript III, this is only possible if the double bond in position 1 is reduced before the hydration of the one in position 4 so that a 5α-reduction can take place. Therefore, it is absolutely indispensable to elucidate the involved enzymes and intermediates, e.g., by performing molecular docking experiments with 5α-reductase and 1,4-dien-3-oxo steroids. This may help to better understand the metabolism and facilitate the transfer of the results to other steroids.

Although the metabolites of MT have been found in previously positive tested urine samples, the lack of a controlled administration study is a point Kopylov mentioned that needs to be considered [104]. He noted this in the case of DHCMT, but it needs to be pointed out in any case of newly proposed metabolites.

The excretion study described in manuscript IV was necessary to prove that the intake of DHCMT leads to the found metabolites of Sobolevsky *et al.*, who pooled urine of athletes from "risky sports" followed by fractionation. It is not evident that the found results came directly from the intake of DHCMT. Therefore, blank urines were collected from each volunteer and used as negative control samples in the executed study. The results give a deep insight into the excretion and metabolism of DHCMT. Based on a specific selection of volunteers over various age groups, the different metabolite patterns were connected to the individual properties. Over 60 years after introducing DHCMT to the market, an administration study focusing on long-term metabolites was finally published. On the one hand, it increased the understanding of DHCMT-metabolism. On the other hand, this verified Sobolevsky's assumptions of the existence of DHCMT metabolites as well as the proposed excretion times (assumption for M3: 40-50 days; last positive sample: 4.9-44,9 days).

Additionally, the urines collected for the excretion study were analyzed by high-performance liquid chromatography coupled by electrospray ionization to quadrupole time-of-flight-mass spectrometry (LC-ESI-QTOF-MS). This technique was used because it enables the identification of intact phase II metabolites. Due to potential incomplete hydrolysis of the conjugates, e.g., by inhibitors of β-glucuronidase in the urine matrix or the stability of sulfate conjugates, only hydrolyzable phase I metabolites can be detected in gas chromatography (GC) [114]. Therefore, one aliquot of 2 mL urine was hydrolyzed in the same way as described in manuscript IV (glucuronide fraction); another aliquot of

2 mL urine was not treated (free fraction). Both were concentrated 1:10 by solid-phase extraction before analysis. Additionally, the sulfates of the metabolites proposed by Fernández-Álvarez *et al.* [115] and the metabolites of the described GC method, including their potential glucuronides and sulfates, have been added.

The results were profoundly different from the ones acquired with GC-EI-QQQ-MS. Using LC-ESI-QTOF-MS (full scan, MS1), there were only findings of substances with an unsaturated A-ring (1,4-dien-3-one): parent compound (free and glucuronidated), 6-hydroxy-DHCMT (free and conjugated), and 4-chloro-17,17-dimethyl-androsta-1,4,13-trien-3-one (free). Due to the ionization technique, completely saturated steroids are hardly ionized, making them impossible to detect in combination with their small urinary concentrations. The longest detectable compound was the unconjugated parent compound in volunteer 4 for up to 36.4 hours. With the GC method, it was detected in samples of the same volunteer up to 72.0 hours after administration. All conjugated metabolites were glucuronides, and no sulfate-conjugate was found. Based on the results acquired with GC-EI-QQQ-MS and because glucuronidation is one of the most prominent routes of detoxification of xenobiotics from the human body [116], the presence of glucuronides was expected. Therefore, all metabolites were searched for in parallel to the unconjugated substance, also as glucuronide. Unfortunately, this was not successful. Thus, and based on the data presented in manuscript IV, the analysis should be further performed by GC-EI-QQQ-MS after deglucuronidation, unless a potential stable long-term marker is found, which is detectable with liquid chromatography coupled by electrospray ionization to mass spectrometry (LC-ESI-MS).

The structures of MD metabolites mentioned in manuscript III, a substance containing the 17α-hydroxymethyl element and a fully reduced A-ring, could be promising regarding the excretion time. In the case of MT and MD, simple hydroxylated derivatives without rearrangement of the D-ring are not suitable as long-term metabolites [82, 117]. In contrast, for the newly synthesized compounds, this needs to be evaluated. Hence, administration studies of MD and MT would not only verify the existence of the metabolites as Kopylov [104] requested but also identify the detection window.

WADA's aim of enabling sport in a doping-free environment could be achieved by the improvement of anti-doping analysis by further developed analytic devices, or by the introduction of new metabolites. At the same time, the enlargement of detection times could keep some athletes from taking the substances and therefore improve public health simultaneously.

Many AAS have never been marketed legally or have been released during the middle of the last century. This means that the requirements of the regulatory authorities were not as strict as nowadays. Therefore, data of kinetic properties or metabolism were not published until today. However, since the anti-doping community needs such data, accredited laboratories or associated research groups perform studies, like the one shown in manuscript IV. Moreover, the realization of a study is difficult due to various regulations. Among other things, a positive ethical vote is required for the administration of an unapproved drug. Therefore, there is often a single-digit number of volunteers participating in those administration studies. Thus, the value of the received results can be challenged by critics [103, 118]. Besides the mentioned criticism and the request for bigger groups of volunteers (including placebo groups), which would help to simulate the actual situation in laboratories and to increase the statistical power, a bigger heterogeneity of volunteers would be desirable. This includes people from different ethnic groups or with different compositions of enzymes, e.g., poor metabolizers. An ascertainment of genetic varieties in parallel to other factors could provide helpful information, e.g., malfunction or excessive function of enzymes. This goes in line with the demand for clinical trials including non-Caucasian people because they may respond different to the administered drugs [119].

In the future, other steroids should be investigated concerning potential metabolites with the mentioned structure (17ξ-hydroxymethyl-17ξ-methyl-18-nor-13-ene). Even if the 17β-isomer was not found in previous studies [76], the potential diastereomer might play a role in the metabolism, e.g., stanozolol (17α-methyl-pyrazolo[4',3':2,3]-5α-androstan-17β-ol). Hence, synthesis needs to be performed. Because 17α-hydroxymethyl derivatives are probably not formed via CYP21A2, the substrate specificity of the currently unknown enzyme could be highly different [84]. As it was claimed for the A-ring reduction, it is essential to elucidate the formation of this particular D-ring rearrangement. Regardless of structure, the discovery of new metabolites, in addition to the revealed metabolism, would likely lead to an increased number of AAFs [70].

Apart from anti-doping analysis, the results also provide significant findings regarding the metabolism of steroids in general. In case of a deficiency of CYP21A2, the classical pathway of steroidogenesis described above (2.2.2) is hindered. Nevertheless, amounts of 21-hydroxypregnenolone (3β,21-dihydroxy-pregn-5-en-20-one) that are then metabolized to 11-deoxycorticosterone (21-hydroxy-pregn-4-ene-3,20-dione) are

found [120]. According to the findings in manuscript I, the formation of 21-hydroxypregnenolone must follow an alternative pathway. Kaufmann *et al.* postulated a way including 21-hydroxydesmosterol (cholesta-5,24-diene-3β,21-diol) that could not be refuted [121]. This knowledge may help to understand the biosynthesis of corticosteroids better and, therefore, probably find more suitable markers for the detection of enzyme-linked diseases.

The elucidation of the metabolism is also important for androgens, which include MT, MD, and DHCMT. The general metabolism of androgens is well known and described [122], nevertheless, the gained knowledge gives deep insights into the specificity of the involved enzymes and may help to bring other enzymes and reactions into focus. For example, the postulated metabolism of MD and its order of reduction steps shown in manuscript III might help design new structure elements for 5α-reductase inhibitors that are widely used in drug therapy of benign prostatic hyperplasia. In addition, many therapeutic used glucocorticoids have a 1,4-dien-3-oxo structure in common. The obtained results may also contribute elucidating the metabolism of these drugs.

Some of the metabolic processes may only occur in certain groups. Additionally, the potential differences of transgender people are not investigated yet. This leads back to the field of anti-doping research, when the first transgender athlete will soon compete in the Olympics [123].

6 Conclusion

The presented thesis concentrates on the metabolism of three AAS in the human body and the synthesis of potential new long-term metabolites. It demonstrates an effective way of introducing new markers to anti-doping analysis and finally confirming the proposed structures.

First, the substrate specificity of a human CYP (21A2) was elucidated to use its stereoselective hydroxylation capacity for the targeted synthesis of new metabolites of several AAS. Second, new metabolites of MD and MT were synthesized chemically, characterized, and detected in urine samples. Third, a controlled administration study with DHCMT was performed to reveal the metabolism of this doping substance and provide kinetic data of the metabolic products. Different synthesis methods were used depending on the structure: either a full chemical or a combined chemical and biotechnological approach.

Several analysis techniques were used to provide sufficient information during the particular project steps. To verify intermediate products in the synthesis, gas chromatography coupled by electron ionization to single quadrupole-mass spectrometry (GC-EI-MS) was used. NMR experiments together with GC-EI-MS and gas chromatography coupled by electron ionization to quadrupole time-of-flight-mass spectrometry (GC-EI-QTOF-MS) were used for the structure elucidation of the synthesized reference material. Post administration samples were analyzed by GC-EI-QQQ-MS and LC-ESI-QTOF-MS.

The results of this thesis show the ideal way of providing reference material to the anti-doping community or every other research field that is dealing with the metabolism of endogenous and exogenous substances in case that the concentrations are too low for other means of comprehensive identification. In a first step, the synthesis of reference material must be planned, and the used compounds, e.g., enzymes or reagents, must be evaluated for their potential use. Subsequently, the synthesis has to be performed, and the product needs to be fully characterized so that there is no doubt of its structure. Finally, an administration of the parent compound and subsequent analysis of the excreted metabolites confirm the previous work.

In summary, this work shows how new ideas about metabolism should be dealt with. It helps to dispel the doubts that have existed for years about postulated results. At the

same time, it shows that steroid metabolism still has various blind spots, whose elucidation must be the goal of further research. Although anti-doping was the all-dominant topic, the gained results are also relevant for fields in physiology and pharmacology.

7 Zusammenfassung

Die vorliegende Arbeit konzentriert sich auf den Metabolismus von drei AAS im menschlichen Körper und die Synthese von potentiellen neuen Langzeitmetaboliten. Es wird ein effektiver Weg aufgezeigt, um neue Marker in die Anti-Doping-Analyse einzuführen und schließlich die vorgeschlagenen Strukturen zu bestätigen.

Zuerst wurde die Substratspezifität eines menschlichen CYP (21A2) aufgeklärt, um dessen stereoselektive Hydroxylierungskapazität für die gezielte Synthese neuer Metaboliten mehrerer AAS zu nutzen. Folgend wurden neue Metaboliten von MD und MT chemisch synthetisiert, mittels Kernspinresonanzspektroskopie (NMR) charakterisiert und in Urinproben nachgewiesen. Um den Metabolismus dieser Dopingsubstanz aufzudecken und kinetische Daten der Metabolite zu erhalten, erfolgte letztlich eine kontrollierte Ausscheidungsstudie mit DHCMT. Je nach Struktur wurden unterschiedliche Synthesemethoden verwendet: entweder ein vollchemischer oder ein kombinierter chemisch-biotechnologischer Ansatz.

Um in den einzelnen Projektschritten ausreichend Informationen zu erhalten, wurden verschiedene Analysetechniken eingesetzt. Um die Zwischenprodukte in der Synthese zu verifizieren, wurde Gaschromatographie gekoppelt mit Elektronenionisations-Single-Quadrupol-Massenspektrometrie (GC-EI-MS) verwendet. Für die Strukturaufklärung des synthetisierten Referenzmaterials wurden NMR-Experimente zusammen mit GC-MS und Gaschromatographie gekoppelt an Elektronenionisations-Quadrupol-Flugzeit-Massenspektrometrie (GC-EI-QTOF-MS) verwendet. Die Proben der Ausscheidungsstudie wurden mittels GC-EI-QQQ-MS und LC-ESI-QTOF-MS analysiert.

Die Ergebnisse dieser Arbeit zeigen den idealen Weg, der Anti-Doping-Gemeinschaft oder anderen Forschungsbereichen, die sich mit dem Metabolismus von endogenen und exogenen Substanzen beschäftigen, Referenzmaterial zur Verfügung zu stellen, falls die Konzentrationen zu gering sind, um sie mit anderen Mitteln umfassend charakterisieren zu können. In einem ersten Schritt muss die Synthese von Referenzmaterial geplant und die verwendeten Verbindungen, z.B. Enzyme oder Reagenzien, auf ihre mögliche Verwendung hin evaluiert werden. Anschließend muss die Synthese durchgeführt und das Produkt vollständig charakterisiert werden, so dass kein Zweifel an seiner Struktur

besteht. Schließlich bestätigen eine Verabreichung der Ausgangsverbindung und die anschließende Analyse der ausgeschiedenen Metaboliten die bisherige Arbeit.

Zusammenfassend zeigt diese Arbeit, wie mit neuen Ideen zum Metabolismus umgegangen werden sollte. Sie hilft, die seit Jahren bestehenden Zweifel an postulierten Ergebnissen auszuräumen. Gleichzeitig zeigt sie, dass der Steroidstoffwechsel noch verschiedene blinde Flecken hat, deren Aufklärung das Ziel weiterer Forschung sein muss. Obwohl Anti-Doping das alles beherrschende Thema war, sind die gewonnenen Ergebnisse auch für Bereiche der Physiologie und Pharmakologie relevant.

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9 List of Figures

10 List of Tables

11 Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

12 List of Publications

12.1 Publications in scientific peer reviewed journals

Loke S, Stoll A, Machalz D, Botrè F, Wolber G, Bureik M, Parr MK. Corticosteroid Biosynthesis Revisited: No Direct Hydroxylation of Pregnenolone by Steroid 21-Hydroxylase. *Frontiers in Endocrinology* 12 (2021) 633785
https://doi.org/10.3389/fendo.2021.633785

Stoll A, Loke S, Joseph JF, Machalz D, de la Torre X, Botrè F, Wolber G, Bureik M, Parr MK. Fine-mapping of the substrate specificity of human steroid 21-hydroxylase (CYP21A2). *Journal of Steroid Biochemistry and Molecular Biology* 194 (2019) 105446
https://doi.org/10.1016/j.jsbmb.2019.105446

Loke S, Liu L, Wenzel M, Scheffler H, Iannone M, de la Torre X, Schlörer N, Botrè F, Keiler AM, Bureik M, Parr MK. New Insights into the Metabolism of Methyltestosterone and Metandienone: Detection of Novel A-Ring Reduced Metabolites. *Molecules* 26 (2021) 1354
https://doi.org/10.3390/molecules26051354

Loke S, de la Torre X, Iannone M, La Piana G, Schlörer N, Botrè F, Bureik M, Parr MK. Controlled Administration of Dehydrochloromethyltestosterone in Humans: Urinary Excretion and Long-Term Detection of Metabolites for Anti-Doping Purpose. *Journal of Steroid Biochemistry and Molecular Biology* [in revision]
DOI

12.2 Poster Presentations in national and international conferences

Parr MK, Stoll A, Gorczyca D, Krzemiński M, Loke S, Joseph JF, de la Torre X, Botrè F. Towards the synthesis of long-term metabolite of Oral-Turinabol for revelation of doping abuse at Beijing and London Olympics. 24th Conference on Isoprenoids Book of Abstracts, The Polish Chemical Society (2018) 104

Loke S, Stoll A, Parr MK. Controlled administration trial of Oral-Turinabol - metabolite confirmation and elimination profiles with special respect to long-term metabolites. DPhG Conference 2018 “Shaping future pharmaceutical research“, Book of Abstracts (2018) Poster P28

Loke S, Stoll A, Joseph J, Machalz D, de la Torre X, Botrè F, Wolber G, Bureik M, Parr M. Corticosteroid Biosynthesis Revisited: Substrate Specificity of Steroid-21-Hydroxylase. Experimental Biology FASEB 2020 (2020) Poster D173

12.3 Oral presentations in national and international conferences

The speaker is emphasized by underlining.

Parr MK, La Piana G, Stoll A, Joseph JF, Loke S, Schloerer N, de la Torre X, Botrè F. Tracing back drug misuse – proper metabolite identification requires synthesis. The 57th Annual Meeting of the International Association of Forensic Toxicologists, Book of Abstracts (2019) SP1

13 Independence Declaration

I hereby affirm that I have completed the presented cumulative dissertation independently and without unauthorized assistance. No aids other than these listed in the text were used in the writing of the dissertation.

A doctoral procedure has never been completed at any other university or applied to another department.

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Steffen Loke