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Preparative-Scale Production of Testosterone Metabolites by Human Liver Cytochrome P450 Enzyme 3A4

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Abstract. Just like the drugs themselves, their metabolites have to be evaluated to succeed in a drug development and approval process. It is therefore essential to be able to predict drug metabolism and to synthesise sufficient metabolite quantities for further pharmacological testing. This study evaluates the possibility of using *in vitro* biotransformations to solve both these challenges in the case of testosterone as a representative component for steroids. The application of cells of *Pichia pastoris* with expressed membrane-associated human liver cytochrome P450 enzyme (P450) 3A4 in two cycles of a preparative-scale bioreactor experiment enabled the isolation of the common metabolites 6β-hydroxytestosterone and 6β-hydroxyandrostenedione on a 100 mg scale. Side-product formation caused by enzymes intrinsic to *P. pastoris* was reduced. In addition more polar testosterone metabolites formed by a P450 3A4-catalysed bioconversion, than the known mono-hydroxylated ones, are reported and 6-dehydro-15β-hydroxytestosterone as well as the di-hydroxylated steroids 6β,16β-dihydroxytestosterone, 6β,17β-dihydroxy-4-androstene-3,16-dione and 6β,12β-dihydroxyandrostenedione were isolated and verified by NMR analysis. Their respective biological significance remains to be investigated. Whole-cell P450 catalysts expressed in *P. pastoris* qualify as a tool for the preparative-scale synthesis of human metabolites. Biotransformation processes in combination with standard chemical procedures allow the isolation and characterisation even of minor drug metabolite products.

Keywords: human drug metabolites; cytochrome P450 3A4, Preparative-scale synthesis; steroids; whole cell biotransformation

Introduction

Poor pharmacokinetics or toxicity caused a major percentage of drugs to fail approval at a late stage of drug development processes,[1] although clinical trials are extremely costly and time-consuming.[2] Recently, the FDA acknowledged that individual drug metabolites might have a different pharmacological or chemical activity compared to the parent drug, and each must now be investigated separately to assess a drug's safety.[3] Consequently, an efficient, quick and authentic identification as well as preparative production of metabolites is highly important to the pharmaceutical industry.[4]

Traditional methods to elaborate a drug's metabolic profile include animal models,[5–7] liver microsomes[8,9] – vesicles of fragmented endoplasmic reticulum containing cytochrome P450 monooxygenases (P450s) – or computational predictions,[10,11] and there are also novel concepts emerging such as "organs-on-chips".[12] However, none of these approaches can provide sufficient metabolite quantities. While conventional methods of chemical synthesis could produce the required amounts of such materials, it is often difficult and time-demanding to functionalise structurally complex drugs with specific regioselectivity. For these reasons the potential of biocatalysis, operating in a synthetic late-stage fashion,[13] was investigated by many studies for the preparative-scale production of human metabolites. There are several examples of successful applications of recombinant human P450s,[14–16] and for some specific products also microbial P450s were deemed successful at providing typical human drug metabolites at such a scale.[17–25] However, in many

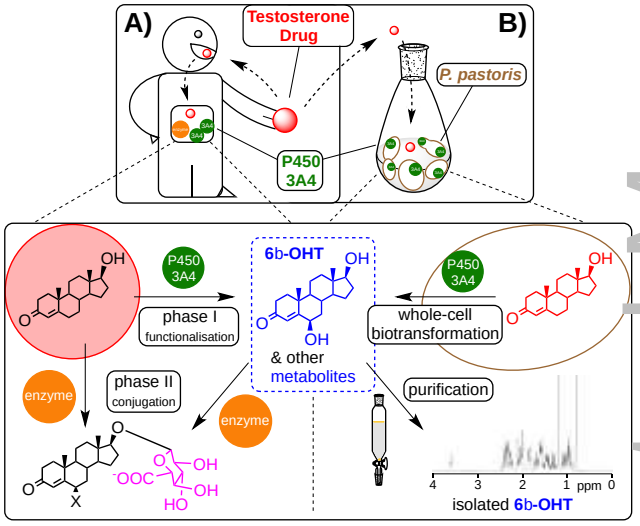
cases microbial P450s resulted in different product profiles and desired metabolites were only obtained after time intensive enzyme engineering.

The body's mechanism for clearance of chemicals like drugs, typically consists of two enzymatic phases with different significance.[26,27] In phase I the compound is made more polar *via* hydrolytic conversions or oxidative functionalisation, while phase II consists of a conjugation step attaching polar units like peptides, acids or sugar moieties to the newly installed or liberated functional group. More than 90% of the phase I enzymatic drug degradation reactions are caused by human liver P450s.[28] Among those, P450 3A4 is the key player in human xenobiotic clearance. It is the most abundant enzyme in this group,[29,30] and, due to its versatility, responsible for the degradation of more than 50% of approved drugs including testosterone (**Scheme 1A**).[28,31] Therefore, P450 3A4 in particular offers itself as a representative and meaningful model and tool for the study of enzymatic drug metabolism by P450 enzymes. The goal was to employ the hepatic function of this key enzyme for the preparative synthesis and identification of the respective metabolites to generate new data for future drug evaluations and models for drug metabolite predictions.

Liver P450 3A4 expressed in recombinant *E. coli* was described to hydroxylate testosterone (**1**) at four different positions, namely 6β, 2β, 1β and 15β in descending order of rates.[32] Thus, 6β-hydroxytestosterone (**2**) is typically used as the determinant of the overall testosterone bioconversion efficiency (**Scheme 2A**).[33] However, oxidation of **1** at positions 2α, 6α, 7α, 11β, 16α and 17 (forming the ketone androstenedione (**3**)) was also observed when using isolated human liver microsomes.[32,34] In fact, upon applying **1** to hepatic rat microsomes Pfeiffer and Metzler could detect at least 17 metabolites by HPLC analysis, some of which included di-hydroxylated derivatives of **1** and **3**.[35] Although rats are not a reliable model to predict drug metabolism in humans,[36,37] it was still hypothesised that the conversion of **1** in the human liver may produce a similarly high number of metabolic products. Many enzymes can produce metabolites; the related human liver P450 2D6 for example also converts steroids, albeit poorly.[38] Still, in that case a further presumption was that P450 3A4 itself could be mainly responsible for such a large metabolic product spectrum due to its prevalence in the testosterone metabolism and its extraordinary active site promiscuity,[39,40] cooperativity[41] and multiple substrate binding sites.[42,43]

In this study cells of *Komagataella phaffii (Pichia pastoris*) containing the main wild-type P450 3A4 at high expression level were used to simulate the human metabolism of testosterone and to synthesise its well-characterised metabolites such as 6β-hydroxytestosterone at a 100 mg scale (**Scheme 1B**).[44]

P. pastoris was the host organism of choice because of the observed advantages in a comparative expression study for human liver P450 2D6/CPR catalysts in different standard expression hosts. *P. pastoris* was identified to be superior to *Escherichia coli*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica*.[45] Furthermore, also compared to other yeasts convincing characteristics of *P. pastoris* are high cell density achievable in a cheap growth medium,[46] excellent capacity for native-like post-translational modifications of eukaryotic proteins,[47] tolerance for membrane protein production such as for P450 3A4,[48,49] and strongly regulated promoters that enable controlled growth on two individual carbon sources (glycerol/glucose and methanol).[50,51] These features allow a highly productive cell cultivation and efficient bioconversion under controlled conditions in a bioreactor.[46,49] First successful expression of active human P450 3A4 was reported in 2013.[52] Here we demonstrated the almost complete conversion of about one gram of testosterone in a 1.3 litre bioreactor system, validating the potential of this system for further up-scaling.



Scheme 1. Major routes of *in vivo* human drug metabolism are compared to an *in vitro* imitation approach. **A)** Administered testosterone drug (red) is metabolised in the liver by P450 3A4 (green) and conjugation enzymes (orange) in two phases to yield an *O*-glucuronide (pink) derivative for excretion. **B)** The same testosterone drug is added to cells of *P. pastoris* expressing recombinant P450 3A4 to simulate human phase I metabolism *in vitro*, and allow isolation of metabolites.

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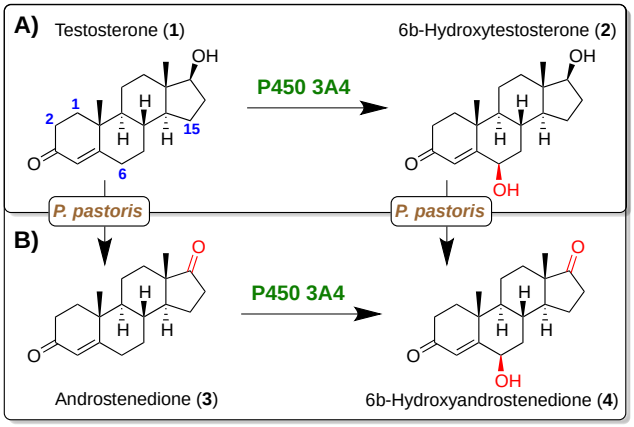

Scheme 2. Illustration of the major reactions observed in this study employing whole-cell catalysts. **A)** Cytochrome P450 3A4 catalysed the conversion of testosterone (**1**) to 6β-hydroxytestosterone (**2**); minor hydroxylation positions of **1** reported in the literature for this enzyme are indicated in blue. **B)** Competing redox enzymes intrinsic to *P. pastoris* can oxidise **1** to furnish 4-androstene-3,17-dione (**3**); Overexpressed P450 3A4 also accepted the latter as a substrate to yield the corresponding 6β-hydroxyandrostenedione **4**, confirming observations of previous studies.[31]

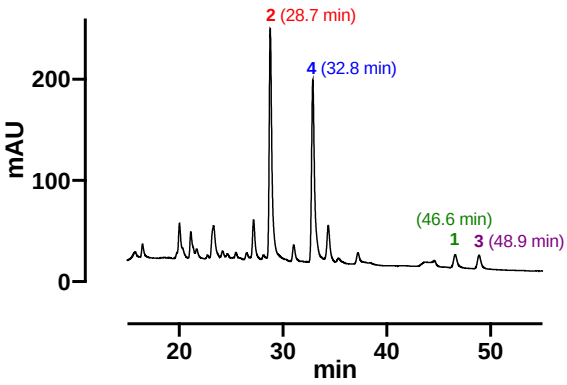

Figure 1. The HPLC analysis of metabolites of **1** obtained by the bioconversion of **1** with P450 3A4 (2 mM **1**, 1 mL total volume, cell concentration = 200 OD600, 225 rpm, 22h, 30 °C,).

Results and Discussion

The analysis of steroids is symptomatically challenging because of the small polarity changes induced upon functionalisation of the large hydrophobic scaffolds.[53] The development of a high-resolution separation method was therefore key to the successful analysis and isolation of steroidal metabolites, which is why the proven HPLC conditions of Pfeiffer and Metzler[35] were adapted. Commercial human P450 3A4/hCPR expressing *P. pastoris* cells (stored as frozen cells at -80 °C) were diluted to obtain standardised whole-cell catalyst stock solutions of a cell concentration of OD_{600} = 200, which was used for all experiments. **Figure 1** displays the metabolite profile obtained from a 1 mL-scale test experiment using 2.0 mM **1** after a 22 hours biotransformation. The high number of peaks with a wide R_f spread suggested the formation of various products with rather different polarity. Peaks eluting at 46.5, 28.7 and 48.8 min could be assigned to compounds **1**, **2** and **3**, respectively, with the aid of authentic reference materials. The peak of compound **4** was deduced from corresponding results shown in **Figure 3**. The other peaks around **2** and **4** could only partly be assigned during the course of this study.

Formation of ketones **3** and **4** occurred, although P450 3A4 is not known to oxidise **1** at position 17. In fact, this oxidation happened regardless of the presence of the expressed P450 as shown by the corresponding negative control experiment using wild-type *P. pastoris* cells, indicating the existence of an intrinsic oxidase or dehydrogenase in P. pastoris, competing with P450 3A4 for the substrate. After 24 hours more than 50% of **1** had been converted to **3** (**Figure 2**). This side reaction was noticed to be reversible by subjecting **3** as the sole substrate, which yielded an equilibrium between the two components as a consequence of the redox state of the host cells (**Figure S1**). The prevalence of low amounts of these additional oxidation products compared to the control reaction employing cells expressing no P450 3A4 indicated high efficiency of the expressed human P450 enzyme.

Furthermore, these results suggested the responsibility of a dehydrogenase for this reaction rather than an oxidase. The same effect had been noticed in the related yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* (*S. pombe*)[54] and the intrinsic glucose-6-phosphate dehydrogenase was suggested as a possible candidate for steroid interconversion in S. cerevisiae.[55] In *P. pastoris* related intrinsic alcohol dehydrogenases (ADHs) might be responsible for the oxidation of **1** to **3** over time. However, many dehydrogenases can be found in the organism's genome as dehydrogenases play a role in different biochemical pathways.[56] For example, three ADHs of *P. pastoris* that catalyse the reversible oxidation of alcohols with simultaneous reduction of the cofactor $NAD(P)^+$ to NAD(P)H had been described in more detail.[57] The identification of the one(s) specifically accounting for the observed steroid conversion remains to be investigated.

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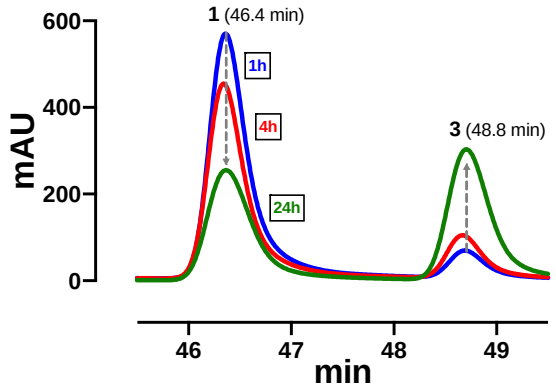

Figure 2. HPLC monitoring of a bioconversion of **1** using the empty vector control strain lacking P450 3A4 expression, which shows oxidation to **3** by *P. pastoris'* intrinsic enzymes (2.5 mM **1**, 30 mL, OD_{600} = 200, 130 rpm, 24h, 28 °C). After 24h, the concentration of ketone **3** exceeded that of substrate **1**.

Yet, due to efficient overexpression of the recombinant enzyme, the presented yeast model does not lead to an unrealistic metabolite profile for human testosterone metabolism in the liver as ketone formation also happens when using human liver microsomes as mentioned before.[34] However, the side-product formation negatively affected the preparative-scale metabolite synthesis, as the yield of individual components was reduced, and their subsequent separation became more complicated. Since **3** is likewise accepted by P450 3A4 as a substrate (**Scheme 2B**),[31] this leads to a much more complex product mixture, essentially composed of duplicate sets of complementary metabolites when all derivatives of **1** were produced in the equivalent ketone form.

Controlling the reaction progress thus became crucial to maximise product yield by anticipating the time, at which the rate of oxidation of **2** to **4** was higher than the rate of its formation, in order to stop the conversion beforehand. As indicated best in **Figure 3** (upper traces, 4.5 mM **1**), concentrations of **1** decreased over time while those of **3** increased, and likewise the peaks of the desired product **2** diminished, while those of **4** rose correspondingly. Because peaks of **2** at 8 hours reaction time were slightly lower than at 4 hours both for runs with 2.5 and 4.5 mM **1**, the optimum point in time had already passed in between. With 0.5 mM of **1**, one hour was sufficient to fully exhaust the substrate (data not shown). In comparison, a concentration of 4.5 mM **1** was too high for the biocatalyst to be fully used-up within 20 hours, indicating decay in enzyme activity over time or potential substrate/product inhibition at higher concentrations. Consequently, for subsequent biotransformations 2.5 mM of substrate **1** was chosen.

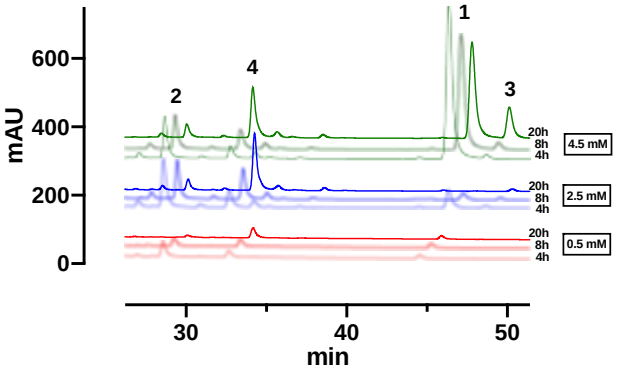

Figure 3. HPLC traces of the bioconversion of **1** at different concentrations (0.5, 2.5 and 4.5 mM shown in red, blue and green, respectively) by P450 3A4 in shake flasks (30 mL, 130 rpm, 24h, 28 °C, OD_{600} = 200). Samples of each biotransformation were taken at three different time points in between 4 and 20 hours.

Having established the optimal reaction time, it was attempted to further minimise the side-product formation by either changing the availability of the ADHs or their co-factors within the cells. The former can be achieved by addressing the ADH expression levels, which depend on the cell's metabolic state. Usually strains of *P. pastoris* are grown on glucose or glycerol as carbon source, and the recombinant expression of the desired protein is subsequently induced by the addition of methanol in Mut^S-type strains (Methanol utilisation Slow), only then activating the responsible, tightly regulated promoter.[58] A change in metabolism will also trigger or suppress the expression of ADHs.[59] Following methanol induction, cells were thus again exposed to either glucose or glycerol for 3 hours before the biotransformation. In another attempt to enhance the availability of reduced cofactors, methanol was added to the biotransformation not only to limit the availability of NAD(P)+, but also to supply a substrate competing with **1** for the ADH active site. In addition, high NAD(P)H concentrations supply sufficient electrons to P450 3A4 *via* the reductase, which often represents the rate-limiting step.[60] Because high methanol concentrations are rather lethal to *P. pastoris*,[61] three different concentrations from 0.5 to 3% were tested. The results of both these strategies are presented in **Figure 4**.

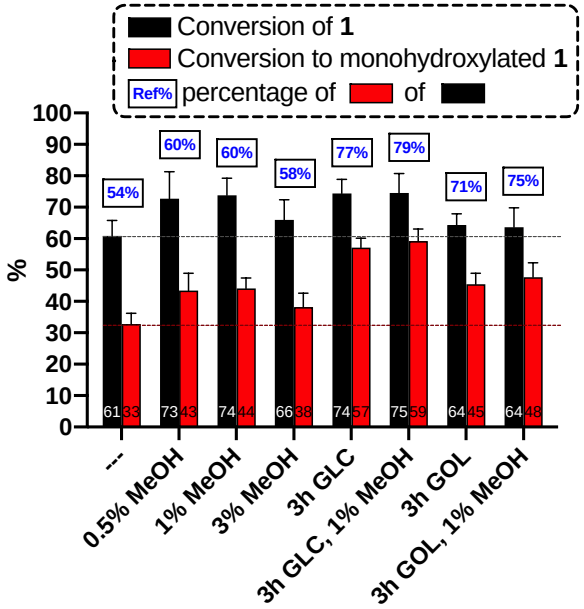

Figure 4. Conversion of **1** (black) to the respective fraction of mono-hydroxylated derivatives of **1** (red) are shown for biotransformations with cells in microwell plates under standard conditions (2.5 mM testosterone, 0.4 mL, 310 rpm, 17h, 28 °C, OD_{600} = 200) without (---) and with the addition of methanol (0.5, 1 and 3% MeOH), or with cells pre-treated by 3 hours cultivation in glycerol (3h GOL) or glucose (3h GLC) and a combination of cultivation and methanol addition (3h GOL, 1% MeOH; 3h GLC, 1% MeOH). For a comparison, the calculated Ref% indicates the percentage of formed conversion to mono-hydroxylated products relative to the total uptake of **1**.

All biotransformations were intentionally incubated for 17 hours, i.e. beyond the optimal reaction time, to intensify a potential reduction effect on the 17-ketone formation. "Ref%" was calculated for better quantification and facilitated comparison of the individual approaches. For the total conversion of **1** (black bars) all cumulated conversions caused by P450 3A4 were considered, while disregarding ADH involvement; thus **4** was included since hydroxylation must have preceded the oxidation, but not **3**. The total conversion data also acknowledge only three mono-hydroxylated derivatives of **1** leaving many extra peaks unaccounted (**Figure S2**). A significant difference could be observed across the different approaches relative to untreated cells. Addition of methanol alone increased the total conversion of **1** and the fraction of mono-hydroxylated products as seen by a slight increase in Ref%. Upon pre-incubation with glucose, the conversion increased to a similar level as for methanol addition, while glycerol pre-treatment had almost no effect. Interestingly though, less oxidation to ketones occurred in both cases as represented by significantly higher Ref% values suggesting that the ADHs present in *P. pastoris* cells that were tuned to carbon source metabolism cause less steroid oxidation. In combination with methanol addition both cases generally experienced a slight further boost. Therefore, it seems like both strategies for increasing product selectivity were successful individually as well as in combination, and suppression of ADH oxidation could enhance selectivity from 54% for untreated conditions to a peak value of 79% (glucose and methanol). A study demonstrated how the co-expression of the human 17β-hydroxysteroid dehydrogenase type 3 could further suppress the side-product formation in *P. pastoris*.[55] Another option would be the generation of knock-out strains lacking ADH genes *via* knock-out, eg. using recently established CRISPR/Cas9 technology.[62]

With reaction conditions optimised, a scale-up biotransformation (BT1) from a several 10 mL scale in 2.5 L cultivation flasks to 1.3 litres in a bioreactor under controlled conditions was performed next (**Figure 5**). Almost 1 gram of **1** was used with implementation of 1% methanol addition.

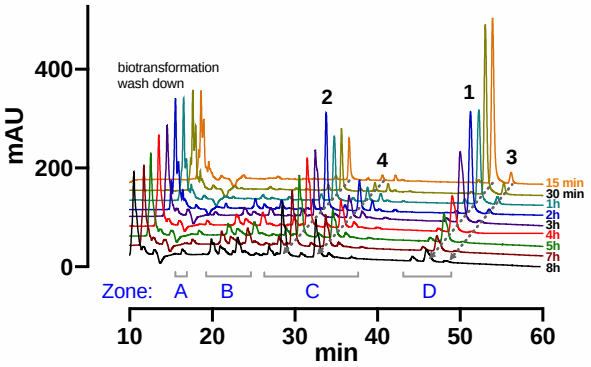

Figure 5. HPLC monitoring of the scale-up biotransformation (BT1) in a bioreactor under controlled conditions is shown (2.5 mM of **1** = 959 mg, 1.3 L, 400 rpm, 8h, 28 °C, OD_{600} = 200, pH 7.0, air flow = 5.0 L/min).

After 8 hours (black trace) a conversion of 85% and a mono-hydroxylation selectivity factor of 62% had been achieved, similar to the results in shake flasks (**Figure 3**), but significantly better than by 96-well plate cultivation (**Figure 4**). Enzyme stability and oxygen-transfer rates were considered to be some of the major limitations of P450s. Quite likely, the oxygen requirements of P450s had been met more accurately by the greater oxygen supply in the bioreactor.[63] A high cell density needs careful adaptation to match the enzyme's oxygen demand, and thus the full potential of a cell density at OD_{600} = 200 might not have been fully exploited.[64] On the other hand, the known rather poor stability of P450 3A4[30] may account for the observed shrinking in conversion rate from 21% (Ref% = 87%) during the first 15 minutes (15 min, orange) to 45% (81%) after one hour (1h, turquoise) and 73% (70%) after five hours (5h, green) of reaction time despite the application of whole-cell biocatalysts, which supports membrane-bound enzymes with the supply of cofactors and electrons.[60,65]

The recovered cells from the first batch were used for a second cycle of biotransformation (BT2) in the bioreactor under identical conditions in order to test the durability of the catalyst system and the amount of testosterone substrate that can be metabolised. In fact, within 9 hours (9h, blue) of the second cycle another 66% of 1 gram of the substrate was converted (**Figure 6**) with a Ref% of 58% that dramatically dropped afterwards. This is an extraordinary performance of a human P450 considering their poor stability and short lifetime usually described in literature.[66]

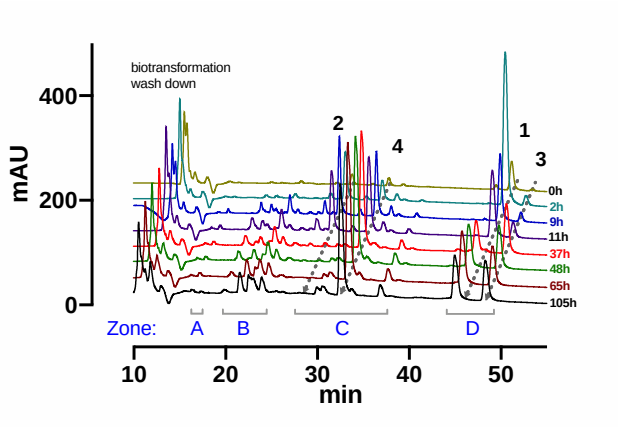

Figure 6. HPLC monitoring for the second cycle of the scale-up biotransformation (BT2) in a bioreactor under controlled conditions (2.5 mM of **1** = 959 mg, 1.3 L, 400 rpm, 8h, 28 °C, OD_{600} = 200, pH 7.0, air flow = 5.0 L/min) is shown.

Between 17 (not shown) and 37 hours the peaks of compounds **1** and **2** had vanished completely. Nevertheless, the biotransformation was continued for a total of 105 hours to ensure all derivatives of **1** had been dehydrogenated to their respective ketone equivalents. The reduced number of peaks simplified the HPLC analysis and revealed only those peaks belonging to derivatives of **3**. The controlled reactor conditions, efficient performance of the biocatalyst, large amount of substrate and frequent sampling provided a more comprehensive picture about the kinetics of substrate consumption and sequence of metabolite formation. Apparently, all metabolite peaks in **Figures 5 and 6** clustered into four distinct zones A to D according to their polarity and their elution times (marked in blue). Because zone D comprises the untreated, non-hydroxylated substrates **1** and **3** and zone C the mono-hydroxylated metabolites **2** and **4**, it seemed logical to assume di-hydroxylated metabolites in zone B and even tri-hydroxylated ones in zone A. The extent the regiochemistry of hydroxylation affects the polarity of the molecule decreases with rising degree of hydroxylation matching the smaller zone widths towards shorter elution times. Analysis of the same HPLC samples by mass detector indeed strongly supported such hypothesis (**Figure S3**). This experiment also indicated the presence of five, not just four mono-hydroxylated derivatives of **1**.

A zoomed-in perspective of zones A and B from **Figure 6** was not sufficient to analyse the different compounds in detail (**Figure S4**). Another HPLC method was developed specifically for compounds eluting in zones A and B and revealed a complex picture of more than ten different peaks as displayed in **Figure 7**.

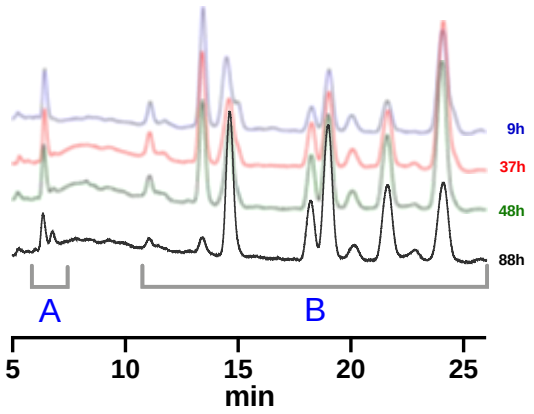

Figure 7. An illustration of the HPLC traces of zones A and B of BT2. A new HPLC method was used for better separation of the more polar metabolites.

For isolation and product characterisation, the extracted metabolite mixtures from runs BT1 and BT2 were separately pre-purified by manual column chromatography in order to simplify the product isolation by preparative HPLC. However, further HPLC purification was obsolete because clean fraction of **2** and **4** could be collected, which furnished 108 mg of **2** from the mixture of the first bioreactor cycle (yield = 11.3%, productivity[67] [g / g] using dry cell weight = 0.16%) and 87 mg of **4** from the second (9.1%, 0.13%). Higher masses were reported for human metabolites of other drugs,[68] but to the best of the author's knowledge the quantities isolated here represent the highest reported in literature for these particular metabolites in comparison to other preparative studies.[16,69,70] Additionally, a refinement or repetition of the purification procedure holds the potential of increasing the yield further.

From both mixtures of the two cycles of biotransformations executed in the bioreactor, two sets of two samples of about 10 mg each could also be isolated by column chromatography. They contained more polar compounds than **2** or **4** seemingly pure as determined by TLC analysis. NMR and HPLC analysis, however, clearly indicated the need for further purification, which was done by collecting the fractions manually using an analytical HPLC instrument as illustrated in **Figure S5**. At a

wavelength of 270 nm the metabolites had only about 10% absorbance enabling a more precise isolation of specific peaks than a preparative HPLC would have achieved (**Figures S6-S9**). Compounds 1β- (**5**) and 15β-hydroxytestosterone (**6**) could be isolated from BT1,[71,72] which confirmed the results published by Guengerich *et al*.[32] In addition, 6-dehydro-15β-hydroxytestosterone (**7**) could be uncovered as the minor component of a mixture with **6**. More polar compounds of BT1 were only present as mixtures and with quantities of less than 1 mg each, making an identification by NMR spectroscopy impossible. The same was true with many of the isolated fractions of samples of BT2. However, for the first time the NMR spectra of four isolated peaks confirmed the formation of the di-hydroxylated compounds 6β,16β-dihydroxytestosterone (**8**) 6β,17β-dihydroxy-4-androstene-3,16-dione (**9**) and 6β,12β-dihydroxyandrostenedione (**10**). Compounds **7**, **8** and **10** were also confirmed by high-resolution mass spectrometry, while the spectrum for **9** was inconclusive due to the presence of several other compounds. Their elution times and their structures are displayed in **Figure 8**. Compound **5** eluted between the peaks of **2** and **4**. Most likely, the peak eluting just after 4 is 2β-hydroxytestosterone, which was not isolated in this study. Consequently, the rate of formation of the mono-hydroxylated metabolites reported by Guengerich *et al*.[32] could not be confirmed here, but looks rather like 6β > 15β ≈ 2β > 1β.

Although the discovered metabolites **7** – **10** occupy a noteworthy area of the HPLC profile at least in this study, their existence remained unknown until now. Having found evidence for the presence of di-hydroxylated metabolites of P450 3A4, the peaks around those of **8** and **10** will likely be either of the same sort or of other oxidised species like **9** or **7**. Given the fact that many NMR spectra indicated the presence of compound mixtures, although some of these were derived from just one HPLC peak, there must be even a larger amount of individual di-hydroxylated compounds formed than represented by the number of peaks.

Remarkably, poly-hydroxylated testosterone products have barely been considered in combination with microsomal liver enzymes. Database searches yielded only few articles that suggested the presence of poly-hydroxylated testosterone metabolites since 1968.[35,73,74] Perhaps this might be due to the chronically bad stabilities and thus short lifetimes of recombinant human P450s causing a rapid decline in enzyme activity after a few hours in the most commonly used microbial host organisms.[45]

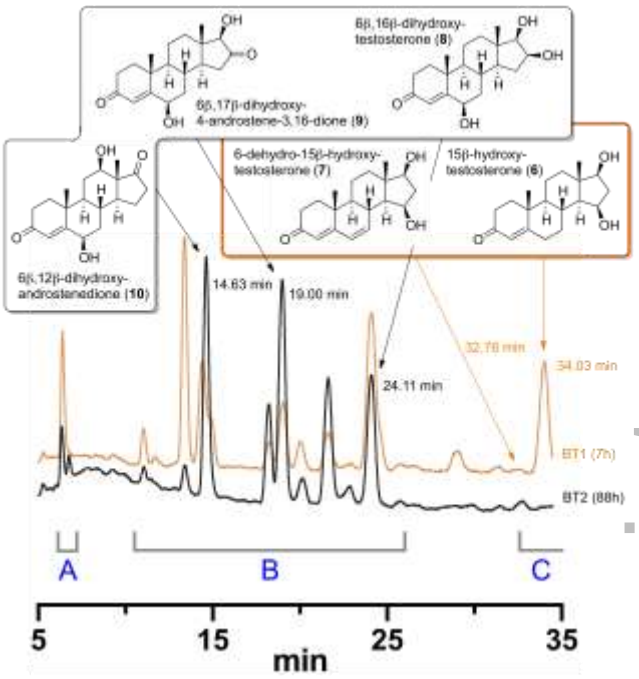

Figure 8. Display of the elution times of the newly discovered metabolites 6-dehydro-15β-hydroxytestosterone (**7**), 6β,16β-dihydroxytestosterone (**8**), 6β,17β-dihydroxy-4-androstene-3,16-dione (**9**) and 6β,12β-dihydroxyandrostenedione (**10**) relative to the already known 15β-hydroxytestosterone (**6**).

Providing more stable catalysts in combination with highly efficient (co-)expression levels of these enzymes could therefore generate a different metabolic profile. Here, high stability was achieved in form of whole-cell biocatalysts using robust yeast chassis, which also allows cost effective scale up and independence of NADPH addition or regeneration. Alternatively ancestral sequence reconstruction of the P450 3 family was also found to enhance stability of the enzyme itself without a loss in activity, albeit slightly changing its regioselectivity.[75]

Another reason might be that such profile is beyond the normal expectations. Not realising the complexity of the human liver P450s and their metabolic spectrum generated, which is still not completely understood nowadays,[31] many studies focused on the major metabolite **2**, which is accessed most easily and promises good results more quickly. Furthermore, the hydroxylation of **1** is not a prerequisite for entering phase II metabolism because the molecule already has an alcohol functional group attached (**Scheme 1**). The majority of phase I steps rather involve 17-oxidation, reductions of the A ring or the 3-position making consecutive hydroxylation steps less predictable.[76] Nevertheless, just like compound **5** was discovered in 2004 as a novel metabolite[32] and *in-vitro* experiments pointed towards some physiological potential,[77] the

existence of such poly-hydroxylated products might as well have pharmacological relevance, which is yet to be investigated.

Hydroxylated testosterone products may be already minor metabolites in the human liver,[76] though it is still desirable and necessary to identify and synthesise such minor metabolites in sufficient quantities.[78] Clearly, efficient biocatalysis enables access to them.[79] Owed to its high cell density, *P. pastoris* is frequently advertised by literature as excellently suitable for its application in large-scale bioreactor experiments[49,80–83]. Human P450 3A4 enzyme catalyst preparations based on *P. pastoris*[84–86] are commercially available from several companies, claiming also multiple cycles of biotransformation.[70] However, no peer-reviewed example of the bioreactor-scale application of human P450 enzymes produced by *P. pastoris* followed by several cycles of whole-cell biotransformations and description of experimental procedure for such scalable human P450-catalysed biotransformations had been published so far.

Reliable expression levels are difficult to obtain for membrane-bound proteins and hardly give representable information about the catalytic efficiency of an enzyme that is so dependent on the electron-transfer from the reductase to the heme domain.[60,87,88] The volumes used for cultivation and the subsequent biotransformation outcome provide better comparability: While in a bioreactor experiment using whole-cell *E. coli*[16] human P450 3A4 catalysts, Vail *et al.* needed more than 3 L of the *E. coli* culture to perform their conversion of **1** and to isolate 59 mg of **2** in 20.5% yield. In this study the reaction employed less than 0,5 L of the yeast culture broth to obtain 108 mg of the same metabolite (Table 1). In addition due to their size *P. pastoris* cells are easy to remove from the reaction broth. Yields of other metabolites than **2** were not discussed in the *E. coli* based biotransformation. Furthermore, confirmation of the identity of their product was only provided using LC-MS.

While P450 3A4 was also successfully expressed in other yeasts,[89,90] no preparative biotransformation with testosterone was performed with those catalysts. On the other hand, using *S. pombe* excellent human P450 expression and biocatalysis was exemplified by Drăgan *et al.*, but no direct comparison is possible as a different human P450 converting another drug was reported for their preparative synthesis.[68]

Table 1: Comparison of the cultivation and biotransformation parameters between this study and a previous publication of Vail *et al.*[16]

	Vail *et al.*[16]	**BT1, this study**
Cultivation Volume	10 L	5 L
Wet cell wt. produced	~ 300 g	~ 2000 g
Wet cell wt. used	100 g	200 g
Substrate **1** used	288 mg (25 mL, 40 mM)	959 mg (3.33 mL, 1 M)
Product **2** isolated	59 mg	108 mg
Yield of **2** (%)	20.5%	11.3%
Productivity[67]	*n.d. (no dry cell wt. data available)*	0.16%

Alternatively, *E. coli* expressing microbial monooxygenases was used to make some individual metabolites of testosterone with excellent yields.[21,79,91,92] Those enzymes are soluble, can be easily tuned *via* directed evolution, are often self-sufficient with high coupling efficiencies and turnover numbers, and have a more narrow substrate tolerance than the human liver P450s. However, often non-human metabolites are formed as main products and long lasting enzyme engineering was necessary to change the substrate and product selectivity, making it inconvenient human drug metabolites of new drug candidates.

Incubation of **2** with the empty plasmid cells as the negative control showed no conversion, other than the alcohol oxidation ruling out an influence of the yeast's ADHs. Therefore it seems like **2** and **4** are also acceptable substrates of P450 3A4 as already implied by the study of Pfeiffer and Metzler, who identified their di-hydroxylated metabolites by adding mono-hydroxylated ones to the liver microsomes.[35] The presence of a 6β-hydroxyl seems to change the enzyme's hydroxylation regioselectivity relative to **1** because positions 16 and 12 were observed as the second hydroxylation site. These results are in line with those by Guengerich *et al.*,[35] whose results suggested a major selectivity difference of P450 3A4 upon the small change in the substrate structure **1** to dihydrotestosterone with a reduced $\Delta^{4,5}$-bond. However, the here discovered α,β,γ,δ-unsaturated compound **7** was formed either after sequential or step-wise hydroxylation i.e. $\Delta^{6,7}$-elimination after the first or second hydroxylation at positions 6 and 15. This means that position 15 is either a conserved target of P450 3A4 with **2** as the substrate or the regioselectivity was not affected by the structural changes of double unsaturation. It seems also plausible that just like the 17β- and 16β-hydroxyl oxidation, the $\Delta^{4,5}$-bond reduction was caused by the ADHs, and compounds **7**, **9** and **10** are therefore no natural metabolites generated by P450 3A4 biocatalysis alone. But it seems reasonable to assume that the 17β-alcohol equivalent of compound **10** will be such metabolite. Interestingly, P450 3A4 seems to have a stereoselective preference for the β-face of the steroidal scaffold in agreement with the observations of Guengerich *et al*.[93] Not only all mono-, but also the newly identified di-hydroxylations of **1** occurred on the β-face.

Conclusion

Human liver enzyme P450 3A4 apparently diversifies the steroidal scaffold of **1** in a late-stage fashion to a larger extent than previously thought.[32] Not only four mono-hydroxylated derivatives at positions 1β, 2β, 6β and 15β are formed,[13] but also several di-hydroxylated and perhaps even at least one tri-hydroxylated metabolite. In this work so far the positions 16β and 12β could be clearly identified as accessible sites for a second hydroxylation by P450 3A4. However, the data presented suggest the presence of a vast number of additional poly-hydroxylated steroids opening up the opportunity for many interesting future discoveries with the help of an improved HPLC separation method combined with highly advanced analytical instrumentation used by Guengerich *et al.*[32]

This study displays how enzymatic metabolite production can balance the industrial production requirements of time, quantity and profile authenticity.[63] The constraints of efficiency, stability and scalability of recombinant human P450s often reported in literature[14,66] could be successfully bypassed using *P. pastoris*-based whole-cell biocatalysts at efficient expression levels. Such robust tool enabled the synthesis of new human metabolites at a preparative scale for the first time. The yeast's protein production features allow for easy transferability of the results to further scale-up strategies and potential industrial application[80] because the optimal conditions (pH, oxygen content, temperature, nutrient supply) for biomass growth, P450 enzyme expression and substrate conversion can differ dramatically among individual hosts and chemical reactions.[4,63] Hence, easy adaption to a bioreactor is just as important as strategies for optimisation strategies.[66] Moreover, the application of surprisingly simple standard chemical procedures for reaction work-up, product purification and analytical identification should make the implementation of this approach worthwhile for chemists and industry alike.

Experimental Section

All solvents and chemicals were purchased from Sigma-Aldrich/Merck (Steinheim/Darmstadt, Germany), VWR International (Fontenay-sous-Bois, France), Carl Roth GmbH (Karlsruhe, Germany) or Fisher Scientific (Loughborough, UK) in best available purity and were used as received without further purification. HPLC tubes were bought from Macherey-Nagel (Düren, Germany) and the corresponding caps and inserts from Bruckner Analysentechnik (Linz, Austria). In experiments (A) and (B) an Agilent Technologies 1100 Series HPLC was used, for experiments (C) and (D) an Agilent Technologies 1200 Series HPLC system coupled with a G1956B mass selective detector (MSD) and an Agilent Technologies 1100 Series HPLC system (D) were employed, respectively. The bioreactor Biostat C_{plus} from Sartorius BBI Systems was used for experiment (D). The cells of *P. pastoris* with expressed P450 3A4 were obtained from Bisy GmbH (Hofstaetten, Austria). OD measurements were executed with an Eppendorf BioPhotometer *plus*. They had been cultivated, then stored as frozen pellets at -80 °C. NMR spectra were recorded with a Varian/Agilent Inova 500 MHz NMR spectrometer equipped with an indirect detection probe 5 mm.

(A) Reaction tube biotransformation: HPLC profile analysis (Figure 1)

Test tubes with screw caps (20x150 mm) were used. A cell concentration OD_{600} of 200 was generated by resuspending cells in 100 mM phosphate buffer (pH 7.4). The biotransformation was started by adding 25 µL of 100 mM testosterone in DMSO to 975 µL of the cell solution and the reaction mixture was incubated at 28 °C and 225 rpm. The biotransformation was stopped after 22 hours by adding 1 mL of a 1:1 mixture (v/v) of acetonitrile/methanol. The resulting mixture was vortexed, centrifuged at top speed and the supernatant taken for HPLC analysis. Compounds were separated *via* a reverse-phase column Zorbax SB-C18 (21.2 mm i.d. x 25 cm). Water containing 0.1% acetic acid (A) and acetonitrile (B) were used for elution at 25 °C in the following ratios: 0 min: A/B 75/25; 50 min: A/B 0/100; 52 min: A/B 75/25; 60 min: A/B 75/25.

(B) 2.5 L shake flask biotransformations: Kinetic study (Figures 2 and 3)

Cells were resuspended in 30 mL of phosphate buffer until an OD_{600} of 200 was obtained and the broth was filled into a sterile 2.5 L shake flask, 0.5, 2.5 or 4.5 mM of 100 mM testosterone in DMSO or 2.5 mM of 100 mM androstenedione in DMSO was added. For the negative control, wild-type cells of P. pastoris were handled as the other samples with a 2.5 mM testosterone end concentration. The flasks were shaken at 130 rpm for 24 hours. Samples (1 mL) were taken after 4, 8, 20 and 24 hours, and treated as well as analysed as described above.

(C) 96-well plate biotransformations: Change in metabolism (Figure 4)

Cells were resuspended in 40 mL of 100 mM phosphate buffer (pH 7.4) until an OD_{600} of 200 was obtained and the broth was filled into a sterile 250 mL shake flask. Then 1030 µL of 20% glucose, 340 µL of 60% glycerol were added to obtain a final concentration of 0.5%. Control cells were left untreated. The cells were shaken for 3 hours at 140 rpm before they were used for biotransformation. The cell broth was added into a 96-deep-well-plate (390 µL each) and the reaction was started with the addition of 10 µL of 100 mM testosterone in DMSO. Methanol was added into the respective wells to obtain a final methanol concentration of 0.5, 1 or 3%. For each variation of the cell conditions, 14 repeats were tested. The plates were incubated at 28 °C at a speed of 320 rpm in a tilted orientation on the shaker to ensure maximal oxygen availability. The addition of 1 mL of a 1:1 mixture (v/v) of acetonitrile/methanol stopped the reaction. The plate was then centrifuged at 16,100 g for 10 min and the supernatant was transferred into 96-well GreinerV plates for HPLC analysis. Separation was carried out *via* a Kinetex C18 (100 Å; 50 x 4.6 mm; 2.6 µm) reverse-phase column. A positive electrospray ionisation mode was selected for the mass spectrometer. Water containing 0.1% acetic acid (A) and acetonitrile (B) were used for elution at 25 °C in the following ratios: 0 min: A/B 80/20; 1 min: A/B 80/20; 3 min: A/B 0/100; 5 min: A/B 0/100; 5.01 min: A/B 80/20; 6 min: A/B 80/20.

(D) Preparative-scale biotransformation in a bioreactor (Figures 5, 6, 7 and 8)

The obtained cells (ca. 200 g wet corresponding to 68 g dry cell weight) were resuspended in 100 mM phosphate buffer (pH 7.4) and filled up to 1.3 L to obtain an OD_{600} of 200. The cell broth was filled into the previously sterilised bioreactor, stirred at 400 rpm and kept at pH 7.4 with 1M solutions of potassium hydroxide and phosphorous acid.

The biotransformation was started with the addition of 13.3 mL of methanol and 3330 µL of a 1 M solution of testosterone (959 mg) in DMSO. Samples were taken regularly during the course of the reaction and simultaneously analysed using HPLC for end point determination. The biotransformation was stopped after 8 hours by centrifugation of the collected reaction broth and washing the cells twice with phosphate buffer. After the final centrifugation the cell pellet was suspended again in 1.3 L of phosphate buffer, filled into the same bioreactor and the second cycle of biotransformation was carried out analogously to the first one. Samples were taken with increasing time intervals and the biotransformation was stopped after 105 hours as before. Both aqueous reaction broths were worked up individually by liquid-liquid extraction washing the aqueous phase several times with ethyl acetate (3x 150 mL). The resulting organic layer was dried over $MgSO_4$, concentrated by rotary evaporation and loaded onto the column for chromatographic purification (50% EtOAc in hexane). 6β-hydroxytestosterone (108 mg, 11.3% yield, 0.16% productivity[67] [g / g] using dry cell weight for the catalyst) was isolated as a white crystalline solid, and 6β-hydroxyandrostenedione (87 mg, 9.1%, 0.13%) as an off-white solid. Mixtures of other mono- or dihydroxylated metabolites were also collected with masses up to 10 mg. These mixtures were further purified with the help of an Agilent Technologies 1100 Series HPLC system adapted for manual preparative collection. Separation was carried out *via* a reverse-phase Purospher Star RP-18e (5.0 µm; 250 x 4 mm) column at 35 °C and a flow rate of 1 mL/min, and water containing 0.1% acetic acid (A) and acetonitrile (B) were used as the eluents in a steadily increasing gradient. Then methanol (C) was mixed in to wash the column thoroughly. Mono-hydroxylated metabolites were purified using the following method with the ratios: 0 min: A/B 80/20; 31 min: A/B 75/25; 31.01 min: B/C: 60/40; 34.00 min: B/C 60/40; 34.01 min: A/B 80/20; 36 min: A/B 80/20. Di-hydroxylated metabolites were purified using the method with the ratios: 0 min: A/B 85/15; 33.50 min: A/B 80/20; 33.51 min: B/C: 60/40; 36.00 min: B/C 60/40; 36.01 min: A/B 85/15; 38 min: A/B 85/15. The aqueous HPLC solvents were removed under a stream of nitrogen. The compounds 1β- (**5**) and 15β-hydroxytestosterone (**6**), 6-dehydro-15β-hydroxytestosterone (**7**) and, 6β,16β-dihydroxytestosterone (**8**), 6β,17β-dihydroxy-4-androstene-3,16-dione (**9**) and 6β,12β-dihydroxyandrostenedione (**10**) could be identified. Compounds **5**, **6**, **7** and **8** were obtained as white/off-white solids, the appearance of **9** and **10** was hardly definable because of too little quantities. For the same reason other isolated compounds, which were also present as mixtures, could not be elucidated.

6β-hydroxytestosterone (**2**, $C_{19}H_{28}O_3$, white crystalline solid, 108 mg, 11%): ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.80 (1H, s, 4-H), 4.34 (1H, m, 6α-H), 3.65 (1H, dd, J = 10.3, 8.1 Hz, 17α-H), 2.50 (1H, ddd, J = 14.9, 4.5, 2.0 Hz, 2β-H), 2.40 (1H, ddd, J = 15.6, 4.3, 2.2, 2α-H), 2.02 (4H, m, 16α-H, 1α-H, 7β-H, 8-H), 1.88 (1H, ddd, J = 12.2, 4.1, 3.0 Hz, 12β-H), 1.70 (1H, dd, J = 14.2, 4.2 Hz, 1β-H), 1.61 (1H, m, 15α-H), 1.57 (1H, m, 11α-H), 1.49 (1H, m, 11β-H), 1.45 (1H, m, 16β-H), 1.40 (1H, m, 15β-H), 1.38 (3H, s, 19-CH_3), 1.21 (1H, m, 7α-H), 1.09 (1H, dd, J = 12.8, 4.3 Hz, 12α-H), 0.98 (1H, m, 14-H), 0.90 (1H, m, 9-H), 0.81 (3H, s, 18-CH_3). ^{13}C NMR (75 MHz, CDCl3): δ = 200.5 (C-3), 168.5 (C-5), 126.5 (C-4), 81.8 (C-17), 73.1 (C-6), 53.9 (C-9), 50.6 (C-14), 43.1 (C-13), 41.1 (C-10), 38.2 (C-7), 37.3 (C-1), 36.6 (C-12), 34.4 (C-2), 30.6 (C-16), 29.9 (C-8), 23.4 (C-15), 20.7 (C-11), 19.7 (C-19), 11.2 (C-18).

6β-hydroxyandrostenedione (**4**, $C_{19}H_{26}O_3$, off-white solid, 87 mg, 9%): ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.77 (1H, s, 4-H), 4.35 (1H, s, 6α-H), 2.49-2.36 (3H, m, 2α-H, 2β-H, 16β-H), 2.16-1.96 (5H, m, 1β-H, 7β-H, 8-H, 15α-H, 16α-H), 1.84 (1H, ddd, J = 12.4, 3.7, 2.7 Hz, 12β-H), 1.71-1.59 (3H, m, 1α-H, 11α-H, 15β-H), 1.51 (2H, ddd, J = 14.04, 13.3, 3.5 Hz, 11β-H), 1.36 (3H, s, 19-CH_3), 1.29-1.19 (3H, m, 7α-H, 12α-H, 14-H), 1.01-0.95 (1H, m, 9-H), 0.90 (3H, s, 18-CH_3). ^{13}C NMR (75 MHz, CDCl3): δ = 220.1 (C-17), 200.5 (C-3), 168.3 (C-5), 126.4 (C-4), 72.6 (C-6), 53.7 (C-9), 50.9 (C-14), 47.7 (C-13), 38.1 (C-10), 37.3 (C-7), 37.1 (C-1), 35.8 (C-12), 34.2 (C-2), 31.3 (C-16), 29.5 (C-8), 21.8 (C-15), 20.3 (C-11), 19.6 (C-19), 13.8 (C-18)

1β-hydroxytestosterone (**5**, $C_{19}H_{28}O_3$, white crystalline solid, 1 mg, 0.1%): ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.79 (1H, s, 4-H), 4.04 (1H, dd, J = 7.6, 7.0 Hz, 1α-H), 3.67 – 3.62 (1H, m, 17-H), 2.54 (2H, d, J = 7.8 Hz, 2α-, 2β-H), 2.49 (1H, dddd, J = 14.8, 13.8, 5.3, 1.3 Hz, 6β-H), 2.33 (1H, dddd, 14.2, 4.4, 2.6, 0.4 Hz, 6α-H), 2.11-2.03 (1H, m, 11α-H), 2.02-1.97 (1H, m, 16α-H), 1.91 – 1.85 (2H, m, 7β-, 12β-H), 1.69 – 1.57 (3H, m, 8-, 11β-, 15α-H), 1.49 – 1.41 (2H, m, 16β-H, 1β-OH), 1.33 – 1.29 (1H, m, 15β-H), 1.25 (3H, s, 19-H), 1.16 – 1.08 (2H, m, 9-, 12α-H), 1.06 – 0.94 (2H, m, 7α-, 14-H), 0.80 (3H, s, 18-H).

In the COSY spectrum the carbinol proton (δ 4.04 ppm) of interest was found to couple with protons in the region of 2.54 ppm corresponding to those of positions 2 or 6, and with a proton of the doublet at 1.46 ppm, which should correspond to the newly introduced hydroxyl group. No coupling to 8-H at δ 1.69 ppm was observed as previously described by Guengerich *et al.*[80,81] Additionally, the HSQC spectrum revealed the carbinol proton (δ 4.02 ppm) to be attached to the carbon at 74.0 (C-1) ppm. With the carbon shifts in hand, the HMBC spectrum confirmed the hydroxylation at C-1 due to the coupling of the 18-Hs to C-10, -9, -5 and the carbinol carbon -1. The NOESY spectrum indicated interactions between the carbinol proton and protons at 2.53 (2α- and 2β-H), 1.69 ppm (11β-H), 1.47 (1β-OH) and 1.11 (9-H). The correlation with 11β-H and the lack of it with the H-19 protons proved the hydroxylation having occurred at 1β.

15β-hydroxytestosterone (**6**, $C_{19}H_{28}O_3$, white solid, 1 mg, 0.1%): ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.75 (1H, s, 4-H), 4.24 – 4.20 (1H, m, 15α-H), 3.59 (1H, dd, J = 14.0, 8.7 Hz, 17α-H), 2.66 – 2.60 (1H, m, 16α-H), 2.47 (1H, ddd, J = 15.6, 15.3, 5.0 Hz, 6β-H), 2.43 (1H, dd, J = 14.1, 5.0 Hz, 2β-H), 2.37 – 2.28 (2H, m, 2α-, 6α-H), 2.13 – 2.08 (1H, m, 7β-H), 2.07 – 2.03 (1H, m, 1β-H), 2.00 (1H, ddd, J = 11.4 10.8, 2.95, 8-H), 1.87 – 1.83 (1H, m, 12β-H), 1.72 (1H, ddd, J = 14.2, 14.1, 4.3 Hz, 1α-H), 1.63 – 1.57 (2H, m, 11α-, 16β-H), 1.47 (1H, ddd, J = 13.2, 13.1, 3.9 Hz, 11β-H), 1.35 (1H, d, 15β-OH), 1.24 (3H, s, 19-H), 1.16 – 1.04 (2H, m, 7α-, 12α-H), 1.07 (3H, s, 18-H), 1.00 (1H, ddd, J = 12.3, 11.3, 4.0, Hz, 9-H), 0.85 (1H, dd, J = 11.3, 5.6 Hz, 14-H). ^{13}C NMR (75 MHz, CDCl3): δ = 199.8 (C-3), 171.3 (C-5), 124.0 (C-4), 81.2 (C-17), 69.2 (C-15), 55.3 (C-14), 54.4 (C-9), 42.4 (C-13), 38.9 (C-10), 38.0 (C-12), 35.9 (C-1), 34.0 (C-2), 32.8 (C-6), 31.6 (C-8), 31.2 (C-7), 20.7 (C-11), 17.5 (C-19), 13.9 (C-18).

6-dehydro-15β-hydroxytestosterone (**7**, $C_{19}H_{26}O_3$, white solid, 3 mg, 0.3%): ^{1}H NMR (500 MHz, $CDCl_3$): δ = 6.30 (1H, d, J = 9.86 Hz, 7-H), 6.16 (1H, dd, J = 9.86, 2.72 Hz, 6-H), 5.69 (1H, s, 3-H), 4.41 – 4.37 (1H, m, 15α-H), 3.65 – 3.60 (1H, m, 17-H), 2.70 – 2.66 (1H, m, 16α-H), 2.56 (1H, dd, J = 14.23, 5.41 Hz, 2β-H), 2.51 (1H, dd, J = 5.34, 1.53 Hz, 2α-H), 1.17 (3H, s, 19-C), 1.12 (3H, s, 18-H). HRMS (TOF-EI+) m/z: calcd. for $C_{19}H_{28}O_4$ 302.1882, found 302.1864.

This compound was isolated as the minor component in a mixture with 15β-hydroxytestosterone as reflected by HPLC, NMR and HRMS analysis. The peaks at 6.30 and 6.16 ppm implied the presence of another alkene group and the roof effect observed between two shifts indicates a strong second-order coupling effect. This was supported by their strong correlation in the NOESY spectrum. In the HMBC spectrum the 18-Hs at 1.13 ppm coupled with carbons at 80.9 (C-17), 52.8 (C-14), 43.8 (C-13) and 37.7 (C-12) ppm, and the 19-Hs at 1.19 ppm with carbons at 163.4 (C-5), 51.1 (C-9), 36.8 (C-10) and 32.6 (C-1) ppm ruling out the known α,β-unsaturated compound 1-dehydrotestosterone. The interaction of the proton at 6.16 ppm with the H-4 (5.69 ppm) in the NOESY spectrum

therefore revealed the alkene to be between positions 6 and 7. Here, a correlation between the other alkene proton (6.30 ppm) and the carbinol proton (4.39 ppm) also strongly pointed towards the hydroxylation to have occurred at position 15, on the β-face of the steroid due to the lack of a coupling to 18-H. Indeed, the carbinol proton (4.40 ppm) was attached to a carbon with a shift of 69.0 ppm in the HSQC spectrum, comparing well with the shifts of position 15 of 15β-hydroxytestosterone. Additionally, the carbinol proton (4.40 ppm) coupled to a proton at 2.69 ppm in the COSY spectrum, which in turn was found to correlate with 17-H (3.62 ppm), fitting well to 15α-H and 16α-H, respectively.

6β,16β-dihydroxytestosterone (**8**, $C_{19}H_{28}O_4$, off-white solid, 1 mg, 0.1%): ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.82 (1H, s, 4-H), 4.36 (1H, br, 6α-H), 4.22 – 4.17 (1H, m, 16α-H), 3.40 (1H, dd, J = 8.86, 7.98 Hz, 17-H), 1.40 (3H, s, 19-H), 0.94 (1H, ddd, J = 11.8, 11.2, 3.9 Hz, 9-H), 0.90 (3H, s, 18-H), 0.83 (1H, ddd, J = 13.2, 10.7, 7.1, 14-H). HRMS (TOF-EI+) m/z: calcd. for $C_{19}H_{28}O_4$ 320.1988, found 320.1975.

The first carbinol proton at 4.36 ppm was attached to a carbon at 73.0 ppm as determined by HSQC. Therefore it did not only have the same proton and carbon shifts as the 6α-H of **2**, but was also found to couple with protons of peaks at 2.04 (7β-H) and 1.23 (7α-H) ppm in the COSY spectrum. A slight coupling to a proton at 1.58 ppm was also visible in the COSY spectrum, which is likely the 6β-OH as it showed no other coupling and appeared as a broad singlet in the proton spectrum. In addition, the first carbinol proton (4.36 ppm) showed a clear correlation to 4-H in the HMBC and NOESY spectrum. The latter spectrum also revealed hydroxylation to have occurred at the β-face of testosterone by the lack of any correlation with the protons at position 19. The second carbinol proton at 4.19 ppm was attached to a carbon at 69.9 ppm as determined by HSQC. A strong correlation with 17-H in the COSY spectrum clearly indicated the other hydroxylation to have occurred at C-16. The presence of the same interaction in the NOESY spectrum in combination with the lack of a correlation with the C-18 methyl group suggested the hydroxyl group being in the equatorial position.

6β,17β-dihydroxy-4-androstene-3,16-dione (**9**, $C_{19}H_{26}O_4$, <1 mg, <0.1%): 5.85 (1H, s, 4-H), 4.40 (1H, br, 6α-H), 3.78 (1H, br, 17α-H), 2.62 (1H, d, J = 3.22 Hz, 17β-OH), 2.58 – 2.51 (1H, m, 2β-H), 2.45 – 2.36 (2H, m, 2α-, 15β-H), 1.62 (1H, br, 6β-OH), 1.42 (3H, s, 19-H), 1.15 – 1.10 (1H, m, 9-H), 0.81 (3H, s, 18-H)

The interaction between the first carbinol proton at 4.39 ppm and H-4 (5.96 ppm) in the NOESY spectrum pointed towards the hydroxylation to have occurred at position 6. In the HSQC spectrum the carbinol proton is attached to a carbon with a shift of 72.3 ppm fitting the usual 6α-proton shifts. It was also found to couple with protons at 1.98 (7α-H) and 1.36 (7β-H) ppm as well as to one proton at 1.62 ppm likely to be the 6β-OH in the COSY spectrum. The latter coupling is visible with the same shift and peak shape as in the proton spectra of 6β,16β-dihydroxytestosterone and 6β,12β-dihydroxyandrostenedione. In the HMBC spectrum a proton at 1.95 ppm was found to interact with a carbon at 215.0 ppm indicating the presence of a second ketone like in derivatives of androstenedione. However, the 18-Hs showed coupling to C-17 at 85.5 ppm as well as to other carbons in a range from 35 to 45 ppm. Consequently, the ketone had to be at a different position than 17. The 19-Hs coupled to C-5 at 158.7 ppm and to other carbons in a range from 30 to 53 ppm. The shifts of the A-ring in general were the same as for 6β-hydroxytestosterone. The peak shape of 9-H is quite distinct and could be identified easily in the proton spectrum. The proton interacted with carbons below 60 ppm. Therefore only positions 15 and 16 qualified having the ketone functional group attached. In the COSY 17α-H correlated with a proton at 2.58 ppm, however, quite likely this is 17β-OH as it appears as a high doublet signal in the ^{1}H spectrum integrating to only 0.6 protons. In contrast to the first carbinol proton H-6α of this compound or 17α-H of any other testosterone derivative determined here, no evidence for any other interaction of 17α-H was noticeable in the COSY spectrum. The same was observed in the NOESY spectrum, where 17α-H only coupled with two protons at 2.09 (12α-H) and 1.54 (14-H). The lack of coupling to neighbouring protons at position 16 strongly pointed towards their absence. In addition, C-17 (85.5 ppm) must have experienced a deshielding effect compared to its usual shift of about 81 ppm supporting the presence of a vicinal ketone functional group at position 16.

6β,12β-dihydroxyandrostenedione (**10**, $C_{19}H_{26}O_4$, <1 mg, <0.1%): ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.85 (1H, s, 4-H), 4.42 – 4.40 (1H, m, 6α-H), 3.80 (1H, dd, J = 11.5, 4.6 Hz, 12α-H), 3.50 – 3.48 (1H, m, 17-H), 1.63 (1H, br, 6β-OH), 1.42 (3H, s, 19-H), 1.08 (1H, ddd, J = 12.5, 11.5, 3.9 Hz, 9-H), 1.03 (3H, s, 18-H). HRMS (TOF-EI+) m/z: calcd. for $C_{19}H_{26}O_4$ 318.1831, found 318.1828.

In the proton spectrum two carbinol protons were found at 4.41 and 3.80 ppm, which are attached to carbons at 72.7 and 72.4 ppm, respectively. For this compound the HMBC spectrum was very informative. The coupling of the 18-Hs to a carbon at 222 ppm indicated that this compound had to be an androstenedione derivative with a ketone functional group at C-17. In addition to this interaction, the 18-Hs (1.05 ppm) couple with three other carbons at 48.7, 51.5 and 72.3 ppm corresponding to C-13, -14, and -12, respectively. Therefore, one of the hydroxylations occurred at C-12. The NOESY spectrum revealed that this carbinol proton at C-12 should be in the axial position because a correlations to 9-H (1.08 ppm), 14-H (1.27 ppm) and 11α-H (1.81 ppm) was visible. The other hydroxylation pattern (4.41, 72.7 ppm) compared well with the shifts of 6β-hydroxyandrostenedione. In the NOESY spectrum a strong interaction to the 4-H proton (5.85 ppm) was the most convincing. Two interactions with other protons at 2.15 and 1.29 ppm (7α-H and 7β-H), as well as one to a proton at 1.63 ppm in both the NOESY and COSY spectra left no room for doubt. The latter shift was assigned to the 6β-OH as it is a broad singlet in the proton spectrum with no other interactions in the COSY, just as for 6β,16β-dihydroxytestosterone and 6β,17β-dihydroxy-4-androstene-3,16-dione.

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Author contributions

N.D.F., M.S. and A.G. devised the study concept. N.D.F. conducted all experiments. M.S. and C.S. provided material. N.D.F. and M.S. developed methods. N.D.F. and H.W. performed data acquisition and analysis. C.S., D.S., U.S and A.G. jointly supervised. N.D.F. wrote the original manuscript. N.D.F. and A.G. reviewed and edited the manuscript. A.G. administrated the project. D.S., U.S. and A.G. acquired the funding. All authors critically reviewed and approved the manuscript.

References

[1] H. van de Waterbeemd, E. Gifford, *Nat. Rev. Drug Discov.* **2003**, *2*, 192–204.

[2] S. Piantadosi, *Clinical Trials: A Methodologic Perspective*, Wiley & Sons Inc., Hoboken, **2017**.

[3] FDA, *Guidance for Industry: Safety Testing of Drug Metabolites*, U.S. Department Of Health And Human Services, Food And Drug Administration, Center For Drug Evaluation And Research (CDER), **2016**.

[4] C. Martinez, S. Rupashinghe, *Curr. Top. Med. Chem.* **2013**, *13*, 1470–1490.

[5] D. S. Wishart, *Drugs R D* **2008**, *9*, 307–322.

[6] L. F. Shyur, N. S. Yang, *Curr. Opin. Chem. Biol.* **2008**, *12*, 66–71.

[7] O. Fiehn, D. Robertson, J. Griffin, M. van der Werf, B. Nikolau, N. Morrison, L. W. Sumner, R. Goodacre, N. W. Hardy, C. Taylor, J. Fostel, B. Kristal, R. Kaddurah-Daouk, P. Mendes, B. van Ommen, J. C. Lindon, S. A. Sansone, *Metabolomics* **2007**, *3*, 175–178.

[8] L. Di, E. H. Kerns, *Curr. Opin. Chem. Biol.* **2003**, *7*, 402–408.

[9] S. Asha, M. Vidyavathi, *Appl. Biochem. Biotechnol.* **2010**, *160*, 1699–1722.

[10] S. R. Kazmi, R. Jun, M. S. Yu, C. Jung, D. Na, *Comput. Biol. Med.* **2019**, *106*, 54–64.

[11] J. Kirchmair, A. H. Göller, D. Lang, J. Kunze, B. Testa, I. D. Wilson, R. C. Glen, G. Schneider, *Nat. Rev. Drug Discov.* **2015**, *14*, 387–404.

[12] E. W. Esch, A. Bahinski, D. Huh, *Nat. Rev. Drug Discov.* **2015**, *14*, 248–260.

[13] N. D. Fessner, *ChemCatChem* **2019**, *11*, 2226–2242.

[14] M. Winkler, M. Geier, S. P. Hanlon, B. Nidetzky, A. Glieder, *Angew. Chemie Int. Ed.* **2018**, *57*, 13406–13423.

[15] K. Schroer, M. Kittelmann, S. Lütz, *Biotechnol. Bioeng.* **2010**, *106*, 699–706.

[16] R. B. Vail, M. J. Homann, I. Hanna, A. Zaks, *J. Ind. Microbiol. Biotechnol.* **2005**, *32*, 67–74.

[17] J. M. Caswell, M. O'Neill, S. J. C. Taylor, T. S. Moody, *Curr. Opin. Chem. Biol.* **2013**, *17*, 271–275.

[18] H. M. Girvan, A. W. Munro, *Curr. Opin. Chem. Biol.* **2016**, *31*, 136–145.

[19] A. Worsch, F. K. Eggimann, M. Girhard, C. J. von Bühler, F. Tieves, R. Czaja, A. Vogel, C. Grumaz, K. Sohn, S. Lütz, M. Kittelmann, V. B. Urlacher, *Biotechnol. Bioeng.* **2018**, *115*, 2156–2166.

[20] D. Schmitz, S. Janocha, F. M. Kiss, R. Bernhardt, *Biochim. Biophys. Acta, Proteins Proteomics* **2018**, *1866*, 11–22.

[21] X. Ren, J. A. Yorke, E. Taylor, T. Zhang, W. Zhou, L. L. Wong, *Chem. - Eur. J.* **2015**, *21*, 15039–15047.

[22] G. Di Nardo, G. Gilardi, *Int. J. Mol. Sci.* **2012**, *13*, 15901–15924.

[23] M. Poraj-Kobielska, M. Kinne, R. Ullrich, K. Scheibner, G. Kayser, K. E. Hammel, M. Hofrichter, *Biochem. Pharmacol.* **2011**, *82*, 789–796.

[24] C. D. Murphy, *Biotechnol. Lett.* **2015**, *37*, 19–28.

[25] R. Weis, M. Winkler, M. Schittmayer, S. Kambourakis, M. Vink, J. David Rozzell, A. Glieder, *Adv. Synth. Catal.* **2009**, *351*, 2140–2146.

[26] O. Ghisalba, M. Kittelmann, in: *Mod. Biooxidation Enzym. React. Appl.* (Eds.: R. D. Schmid, V. Urlacher), Wiley-VCH, Weinheim, **2007**, pp. 211–232.

[27] T. Testa, in: *Pract. Med. Chem.* (Ed.: C. G. Wermuth), Elsevier Ltd, Amsterdam, **2008**, pp. 655–673.

[28] S. Rendic, F. P. Guengerich, *Chem. Res. Toxicol.* **2015**, *28*, 38–42.

[29] M. Miyazaki, K. Nakamura, Y. Fujita, F. P. Guengerich, R. Horiuchi, K. Yamamoto, *Drug Metab. Dispos.* **2008**, *36*, 2287–2291.

[30] P. Anzenbacher, E. Anzenbacherová, *Cell. Mol. Life Sci.* **2001**, *58*, 737–747.

[31] F. P. Guengerich, *Chem. Res. Toxicol.* **2017**, *30*, 2–12.

[32] J. A. Krauser, M. Voehler, L.-H. Tseng, A.-B. Schefer, M. Godejohann, F. P. Guengerich, *Eur. J. Biochem.* **2004**, *271*, 3962–3969.

[33] J. A. Krauser, F. P. Guengerich, *J. Biol. Chem.* **2005**, *280*, 19496–19506.

[34] H. C. Man, P. L. Skipper, J. S. Wishnok, S. R. Tannenbaum, *Drug Metab. Dispos.* **2005**, *33*, 714–718.

[35] E. Pfeiffer, M. Metzler, *Arch. Toxicol.* **2004**, *78*, 369–377.

[36] X. Cao, S. T. Gibbs, L. Fang, H. A. Miller, C. P. Landowski, H. C. Shin, H. Lennernas, Y. Zhong, G. L. Amidon, L. X. Yu, D. Sun, *Pharm. Res.* **2006**, *23*, 1675–1686.

[37] R. Zuber, E. Anzenbacherová, P. Anzenbacher, *J. Cell. Mol. Med.* **2002**, *6*, 189–198.

[38] M. Geier, A. Braun, P. Fladischer, P. Stepniak, F. Rudroff, C. Hametner, M. D. Mihovilovic, A. Glieder, *FEBS J.* **2013**, *280*, 3094–3108.

[39] M. Paloncýová, V. Navrátilová, K. Berka, A. Laio, M. Otyepka, *J Chem Theory Comput* **2016**, *12*, 2101–2109.

[40] M. Ekroos, T. Sjögren, *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 13682–13687.

[41] J. C. Hackett, *J. Biol. Chem.* **2018**, *293*, 4037–4046.

[42] Y. Kapelyukh, M. J. I. Paine, J. D. Maréchal, M. J. Sutcliffe, C. R. Wolf, G. C. K. Roberts, *Drug Metab. Dispos.* **2008**, *36*, 2136–2144.

[43] I. G. Denisov, P. J. Mak, Y. V. Grinkova, D. Bastien, G. Bérubé, S. G. Sligar, J. R. Kincaid, *J. Inorg. Biochem.* **2016**, *158*, 77–85.

[44] L. C. Wienkers, T. G. Heath, *Nat. Rev. Drug Discov.* **2005**, *4*, 825–833.

[45] M. Geier, A. Braun, A. Emmerstorfer, H. Pichler, A. Glieder, *Biotechnol. J.* **2012**, *7*, 1346–1358.

[46] G. P. L. Cereghino, J. L. Cereghino, C. Ilgen, J. M. Cregg, *Curr. Opin. Biotechnol.* **2002**, *13*, 329–332.

[47] V. Puxbaum, D. Mattanovich, B. Gasser, *Appl. Microbiol. Biotechnol.* **2015**, *99*, 2925–2938.

[48] A. Emmerstorfer, T. Wriessnegger, M. Hirz, H. Pichler, *Appl. Microbiol. Biotechnol.* **2014**, *98*, 7671–7698.

[49] B. Byrne, *Curr. Opin. Struct. Biol.* **2015**, *32*, 9–17.

[50] V. Juturu, J. C. Wu, *ChemBioChem* **2018**, *19*, 7–21.

[51] M. Ahmad, M. Hirz, H. Pichler, H. Schwab, *Appl. Microbiol. Biotechnol.* **2014**, *98*, 5301–5317.

[52] M. Geier, C. Schmid, A. Glieder, *Chim. Oggi* **2013**, *31*, 24–27.

[53] H. L. J. Makin, J. W. Honour, C. H. L. Shackleton, W. J. Griffith, in: *Steroid Anal.* (Eds.: H. L. J. Makin, D. B. Gower), Springer Science & Business Media, Dordrecht, **2010**, pp. 163–784.

[54] T. Pajič, M. Vitas, D. Žigon, A. Pavko, S. L. Kelly, R. Komel, *Yeast* **1999**, *15*, 639–645.

[55] M. Shao, X. Zhang, Z. Rao, M. Xu, T. Yang, H. Li, Z. Xu, S. Yang, *Green Chem.* **2016**, *18*, 1774–1784.

[56] R. A. Canuto, *Dehydrogenases*, InTech, London, **2012**.

[57] H. Zhang, Q. Li, L. Wang, Y. Chen, *Biochim. Biophys. Acta, Gen. Subj.* **2018**, *1862*, 1199–1208.

[58] F. W. Krainer, C. Dietzsch, T. Hajek, C. Herwig, O. Spadiut, A. Glieder, *Microb. Cell Fact.* **2012**, *22*, 1–14.

[59] T. Vogl, L. Sturmberger, T. Kickenweiz, R. Wasmayer, C. Schmid, A. M. Hatzl, M. A. Gerstmann, J. Pitzer, M. Wagner, G. G. Thallinger, et al., *ACS Synth. Biol.* **2016**, *5*, 172–186.

[60] E. O'Reilly, V. Köhler, S. L. Flitsch, N. J. Turner, *Chem. Commun.* **2011**, *47*, 2490–2501.

[61] W. Zhang, M. A. Bevins, B. A. Plantz, L. A. Smith, M. M. Meagher, *Biotechnol. Bioeng.* **2000**, *70*, 1–8.

[62] A. Weninger, A. M. Hatzl, C. Schmid, T. Vogl, A. Glieder, *J. Biotechnol.* **2016**, *235*, 139–149.

[63] M. T. Lundemo, J. M. Woodley, *Appl. Microbiol. Biotechnol.* **2015**, *99*, 2465–2483.

[64] W. A. Duetz, J. B. Van Beilen, B. Witholt, *Curr. Opin. Biotechnol.* **2001**, *12*, 419–425.

[65] R. A. Sheldon, D. Brady, *Chem. Commun.* **2018**, *54*, 6088–6104.

[66] V. B. Urlacher, M. Girhard, *Trends Biotechnol.* **2019**, *37*, 882–897.

[67] R. Kratzer, J. M. Woodley, B. Nidetzky, *Biotechnol. Adv.* **2015**, *33*, 1641–1652.

[68] C. A. Drăgan, F. T. Peters, P. Bour, A. E. Schwaninger, S. M. Schaan, I. Neunzig, M. Widjaja, J. Zapp, T. Kraemer, H. H. Maurer, M. Bureik, *Appl. Biochem. Biotechnol.* **2011**, *163*, 965–980.

[69] Y. Kanamori, K. Fujita, K. Nakayama, T. Kamataki, H. Kawai, *Drug Metab. Pharmacokinet.* **2003**, *18*, 42–47.

[70] M. Subramanian, S. Das, E. Martinez, D. . Callewaert, in *Rapid Identif. Prod. Metab. Using CypExpressTM 2D6, 3A4, 2C9*, 19th Annual Meeting Of The International Society For The Study Of Xenobiotics, San Francisco, **2014**.

[71] D. N. Kirk, H. C. Toms, C. Douglas, K. A. White, K. E. Smith, S. Latif, R. W. P. Hubbard, *J. Chem. Soc. Perkin Trans 2* **1990**, *2*, 1567–1594.

[72] I. Černý, J. Fajkoš, V. Pouzar, *Steroids* **1996**, *61*, 58–64.

[73] J. J. Sheets, R. W. Estabrook, *Biochemistry* **1985**, *24*, 6591–6597.

[74] B. P. Lisboa, J.-A. Gustafsson, J. Sjövall, *Eur. J. Biochem.* **1968**, *4*, 496–505.

[75] Y. Gumulya, J.-M. Baek, S.-J. Wun, R. E. S. Thomson, K. L. Harris, D. J. B. Hunter, J. B. Y. H. Behrendorff, J. Kulig, S. Zheng, X. Wu, J. E. Stok, J. J. De Voss, G. Schenk, U. Jurva, S. Andersson, E. M. Isin, M. Bodén, L. Guddat, E. M. J. Gillam, *Nat. Catal.* **2018**, *1*, 878–888.

[76] O. J. Pozo, J. Marcos, R. Ventura, A. Fabregat, J. Segura, *Anal. Bioanal. Chem.* **2010**, *398*, 1759–1770.

[77] C. Jo Corbin, S. M. Mapes, J. Marcos, C. H. Shackleton, D. Morrow, S. Safe, T. Wise, J. Joe Ford, A. J. Conley, *Endocrinology* **2004**, *145*, 2157–2164.

[78] D. J. B. Hunter, J. B. Y. H. Behrendorff, W. A. Johnston, P. Y. Hayes, W. Huang, B. Bonn, M. A. Hayes, J. J. De Voss, E. M. J. Gillam, *Metab. Eng.* **2011**, *13*, 682–693.

[79] S. Kille, F. E. Zilly, J. P. Acevedo, M. T. Reetz, *Nat. Chem.* **2011**, *3*, 738–743.

[80] V. Looser, B. Bruhlmann, F. Bumbak, C. Stenger, M. Costa, A. Camattari, D. Fotiadis, K. Kovar, *Biotechnol. Adv.* **2015**, *33*, 1177–1193.

[81] Z. Yang, Z. Zhang, *Biotechnol. Adv.* **2018**, *36*, 182–195.

[82] A. Schmideder, S. Hensler, M. Lang, A. Stratmann, U. Giesecke, D. Weuster-Botz, *Process Biochem.* **2016**, *51*, 177–184.

[83] C. W. Theron, J. Berrios, F. Delvigne, P. Fickers, *Appl. Microbiol. Biotechnol.* **2018**, *102*, 63–80.

[84] BioVision, "EZCyp," can be found under https://www.biovision.com/ezcyptm-active-human-cytochrome-p450-3a4.html, **2020**.

[85] S. Aldrich, "CYPExpress," can be found under https://www.sigmaaldrich.com/catalog/product/sigma/mtoxce3a4?lang=de&region=AT, **2020**.

[86] Bisy, "Biocatalysts," can be found under https://www.bisy.at, **2020**.

[87] Y. Farooq, G. C. K. Roberts, *Biochem. J.* **2010**, *432*, 485–493.

[88] V. R. Dodhia, C. Sassone, A. Fantuzzi, G. Di Nardo, S. J. Sadeghi, G. Gilardi, *Electrochem. Commun.* **2008**, *10*, 1744–1747.

[89] A. Braun, M. Geier, B. Bühler, A. Schmid, S. Mauersberger, A. Glieder, *Microb. Cell Fact.* **2012**, *11*, 106.

[90] B. Palabiyik, S. Karaer, N. Arda, S. Erturk Toker, G. Temizkan, S. Kelly, A. Topal Sarikaya, *Biologia (Bratisl).* **2008**, *63*, 450–454.

[91] H. Venkataraman, S. B. A. de Beer, L. A. H. van Bergen, N. van Essen, D. P. Geerke, N. P. E. Vermeulen, J. N. M. Commandeur, *ChemBioChem* **2012**, *13*, 520–523.

[92] D. Zehentgruber, F. Hannemann, S. Bleif, R. Bernhardt, S. Lütz, *ChemBioChem* **2010**, *11*, 713–721.

[93] Q. Cheng, C. D. Sohl, F. K. Yoshimoto, F. P. Guengerich, *J. Biol. Chem.* **2012**, *287*, 29554–29567.

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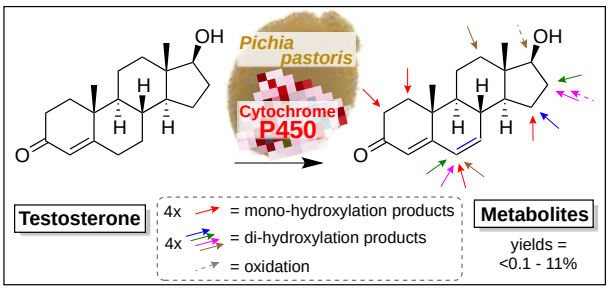
FULL PAPER

Preparative-Scale Production of Testosterone Metabolites by Human Liver Cytochrome P450 Enzyme 3A4



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