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CYP21-catalyzed production of the long-term urinary metandienone metabolite 17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one: a contribution to the fight against doping

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Abstract

Anabolic-androgenic steroids are some of the most frequently misused drugs in human sports. Recently, a previously unknown urinary metabolite of metandienone, 17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one (20OH-NorMD), was discovered via LC-MS/MS and GC-MS. This metabolite was reported to be detected in urine samples up to 19 days after administration of metandienone. However, so far it was not possible to obtain purified reference material of this metabolite and to confirm its structure via NMR. Eleven recombinant strains of the fission yeast *Schizosaccharomyces pombe* that express different human hepatic or steroidogenic cytochrome P450 enzymes were screened for production of this metabolite in a whole-cell biotransformation reaction. 17,17-Dimethyl-18-norandrosta-1,4,13-trien-3-one, chemically derived from metandienone, was used as substrate for the bioconversion, because it could be converted to the final product in a single hydroxylation step. The obtained results demonstrate that CYP21 and to a lesser extent also CYP3A4 expressing strains can catalyze this steroid hydroxylation. Subsequent 5 l-scale fermentation resulted in the production and purification of 10 mg of metabolite and its unequivocal structure determination via NMR. The synthesis of this urinary metandienone metabolite via *S. pombe*-based whole-cell biotransformation now allows its use as a reference substance in doping control assays.

[a] These authors contributed equally to this work.

Keywords: anabolic steroids; CYP21; CYP3A4; doping; metabolite; metandienone; *Schizosaccharomyces pombe*; whole-cell biotransformation.

Introduction

Cytochromes P450 (P450s or CYPs) form a remarkably versatile enzyme superfamily and are involved in a variety of different reactions. These hemoproteins are spread throughout plants, animals and microorganisms where they participate in crucial physiological processes such as the biodegradation of xenobiotics during phase-I metabolism, steroidogenesis, bile acid synthesis or the metabolism (and sometimes even the activation) of a variety of carcinogenic substances. The reactions catalyzed by these enzymes include hydroxylation, N-, O- and S-dealkylation, sulfoxidation, epoxidation, deamination and N-oxide reduction. In view of their high substrate diversity it is not surprising that P450s have attracted the attention of different research fields such as biochemistry, pharmacology, physiology, organic chemistry and biotechnology (Bernhardt, 2005, 2006; Isin and Guengerich, 2007).

The human CYP superfamily comprises 57 members that are divided into 18 families and 43 subfamilies; among these, members of families 1–3 mediate 70–80% of all phase I-dependent metabolism of clinically used drugs and participate in the metabolism of a huge number of other xenobiotic chemicals (Guengerich, 2005; Ingelman-Sundberg et al., 2007). Depending on the subcellular localization of the respective P450 system, the electrons required for the activity of human P450s are provided in two different ways (Hannemann et al., 2007): mitochondrial P450s (which are mainly involved in steroidogenesis) obtain the two electrons needed for a hydroxylation reaction one by one via a short electron transfer chain consisting of the [2Fe-2S]-protein adrenodoxin and the FAD-containing NADPH-dependent adrenodoxin reductase, whereas microsomal CYPs (which play an important role in the degradation of a variety of different drugs, alkaloids and pesticides in the human liver) receive both electrons directly from the FAD- and FMN-containing cytochrome P450 reductase (CPR).

Owing to their significant contribution to the metabolism of anabolic steroids, P450s have also been the focus of antidoping laboratories. Previous investigations have shown that a variety of different P450s (including CYP3A4, the predominant liver P450) are capable of hydroxylating anabolic

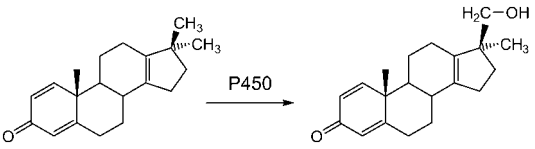

Figure 1 Reaction scheme for the CYP-dependent formation of 20OH-NorMD from 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one.

steroids at various positions. For example, CYP3A4 is mainly responsible for the 6β-hydroxylation of testosterone and of synthetic anabolic steroids such as metandienone (Schänzer, 1996; Rendic et al., 1999; Walsky and Obach, 2004). The latter steroid has been the focus of attention of antidoping researchers in the past number of years because several professional athletes were convicted of misusing this substance for doping purposes. Commonly used methods to detect the abuse of this anabolic steroid are based on the identification of different metandienone metabolites via LC-MS/MS and GC-MS analysis (Schänzer et al., 1991, 2006; Schänzer and Donike, 1993; Schänzer, 1996; Rendic et al., 1999). Among these metabolites is a recently identified urinary metabolite, 17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one (also referred to as '20OH-NorMD'), that allows the long-term detection of metandienone abuse up to 19 days after a single administration of 5 mg of the drug. This time span is significantly increased compared to the detection period of other metandienone metabolites reported detectable only between 4 and 6 days (Schänzer et al., 2006). Although the identification of 20OH-NorMD in urine samples by comparison with post-administration urines from controlled metandienone excretion studies is accepted by the WADA (World Anti-Doping Agency) as proof of metandienone abuse, it has not yet been possible to finally confirm its structure. Moreover, this metabolite was not available as purified reference substance because its chemical synthesis was not achieved. Therefore, it was the aim of this study to obtain 20OH-NorMD in sufficiently high quantities to allow both NMR structure identification and its use as reference material.

The *in vivo* formation of 20OH-NorMD from metandienone probably involves the participation of several enzymes that catalyze different reactions, and a biotechnological rebuilding of this pathway was considered to be very demanding. Therefore, 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one, a compound that is chemically derived from metandienone, was used as substrate because it can be conveniently synthesized and requires only a single hydroxylation reaction for its conversion into 20OH-NorMD (Figure 1). Moreover, it seems plausible to assume that this final reaction step is catalyzed by a P450 enzyme. Recombinant strains of the fission yeast *Schizosaccharomyces pombe* have been successfully used for the expression of a variety of different human CYPs as well as for the biotechnological production of P450 metabolites in whole-cell biotransformation assays (Bureik et al., 2002; Dragan et al., 2005, 2006b; Peters et al., 2007, 2009a,b; Zöllner et al., 2009). Therefore, a set of *S. pombe* strains that express different human hepatic or steroidogenic P450s was used to screen for the formation of 20OH-NorMD. Using P450 expressing fission yeast strains for screening purposes instead of microsome preparations enables a direct production of the metabolite of interest in the milligram to gram range and facilitates the establishment of an upscaling process.

Results

Cytochrome P450 screening assays

Eleven fission yeast strains that recombinantly express hepatic or steroidogenic CYPs (CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP3A7*2, CYP4Z1, CYP11B1, CYP11B2, CYP17 and CYP21, respectively) were tested for the bioconversion of chemically synthesized 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one to 20OH-NorMD (Figure 1). Recent studies indicate that coexpression of human CPR enhances the biotransformation activity of fission yeast strains that express human microsomal P450s compared with strains using only the endogenous CPR of *S. pombe* (Dragan et al., 2006b; Peters et al., 2007, 2009a,b). For the CYP17- and CYP21-dependent biotransformations we therefore used the CPR coexpressing strains CAD71 and CAD75 (Dragan et al., manuscript in preparation) instead of the previously described strains CAD8 and CAD18 (Dragan et al., 2006a,b) that solely express human CYP17 or CYP21, respectively. In addition to strains expressing the major liver P450s we also tested strain INA1 (Neunzig et al., submitted) which expresses CYP3A7*2, a polymorphic variant of the fetal enzyme CYP3A7 that displays improved activity towards some substrates (Rodriguez-Antona et al., 2005).

The performed screening assay showed that the CYP21 expressing *S. pombe* strain CAD75 was the most efficient one to catalyze the desired hydroxylation reaction (Table 1). The EI mass spectrum of trimethylsilylated (TMS) product of this biotransformation displayed the typical ion fragmentation pattern of 20OH-NorMD (Schänzer et al., 2006). In comparison to 20OH-NorMD (M^+=442) the analyzed substrate shows M^+=354. In addition, another hydroxylated metabolite was also produced by CYP21 in equal or even higher quantities than 20OH-NorMD. It also shows M^+=442 but in contrast to 20OH-NorMD no loss of 103 u (CH_2-O-TMS) is observed. The mass spectra of the TMS derivatives of the above-mentioned steroids are displayed in Figure 2. CYP3A4, which is reported to be the most abundant P450 in human liver, was also capable of producing the desired metabolite together with its 17-epimer 17α-hydroxymethyl-17β-methyl-18-norandrosta-1,4,13-triene-3-one (Table 1), which has been previously identified in urine samples (Schänzer et al., 2006; Parr et al., 2007). Moreover, CYP2C19 also metabolizes the substrate. However, the formed metabolite was not 20OH-NorMD and the amount of this CYP2C19 synthesized compound was too low to allow any further investigations. With all other tested P450 expressing fission yeast strains (i.e., CYP2B6, CYP2C9,

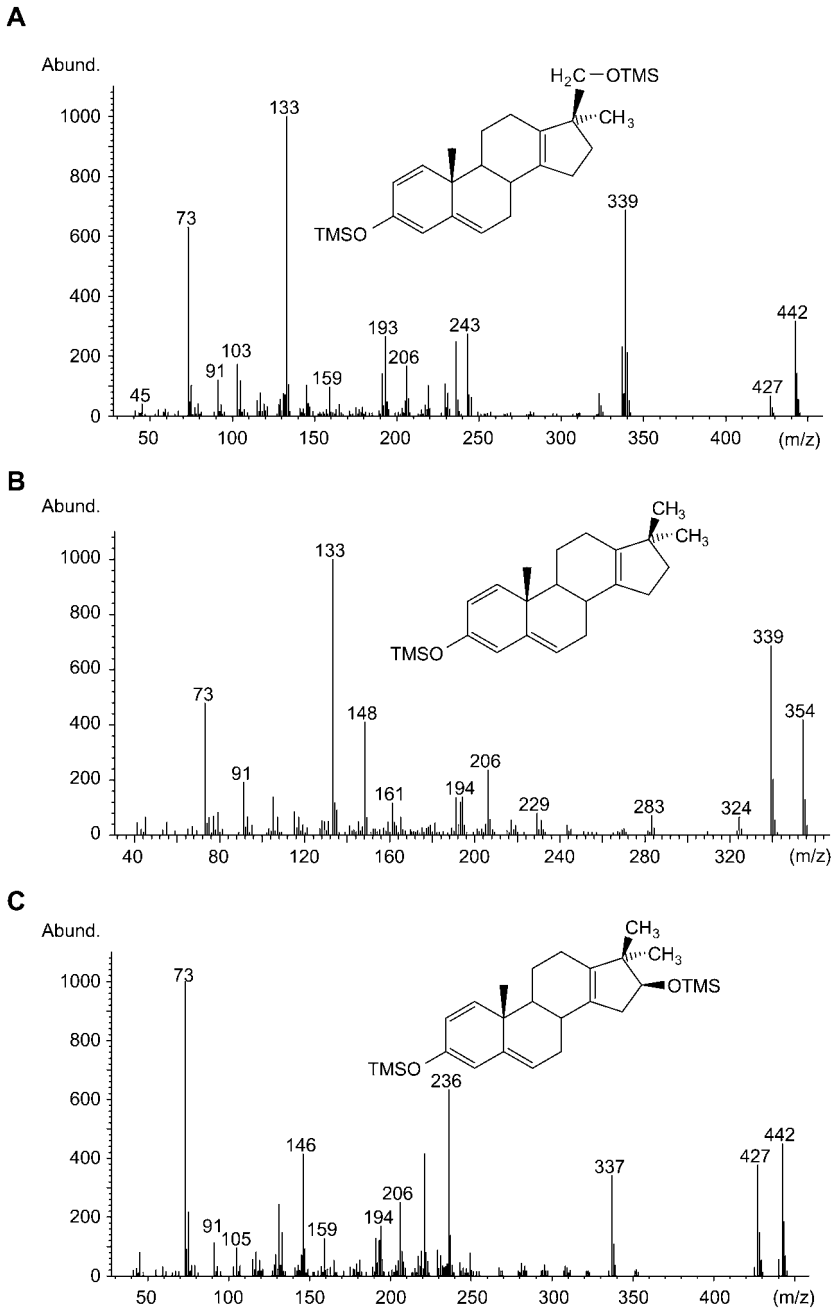

Figure 2 EI mass spectra of (A) trimethylsilylated 17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one, (B) trimethylsilylated 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one and (C) 16β-hydroxy-17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one.

CYP2D6, CYP3A7*2, CYP4Z1, CYP11B1, CYP11B2 and CYP17, respectively) no products of 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one were observed.

Large scale whole-cell biotransformation

For the production of larger amounts of 20OH-NorMD, a 5 l-scale fed-batch fermentation was performed using the CYP21 expressing strain CAD 75 (Figure 3). In this process, the glucose concentration was maintained at approximately 1 g/l during the initial growth phase of the cells (40 h) and the relative oxygen content within the fermentation broth steadily decreased from initially 100% to 20%. Preliminary small-scale experiments indicated that substrate concentrations between 175 and 225 μM lead to the highest product formation ratio, whereas higher amounts of substrate inhibit product formation (data not shown), an observation that has also been made with other P450s (Korzekwa et al., 1998; Lin et al., 2001). For the biotransformation the substrate was therefore added in two batches of 150 mg each (at an interval

Table 1 Results of the cytochrome P450 screening assays using recombinant *S. pombe* strains.

Cytochrome P450 expressed in *S. pombe*	Positive control reaction	Observed 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one metabolites
CYP2B6	Bupropion → hydroxybupropion	n.d.
CYP2C9	Tolbutamide → 4′-hydroxymethyltolbutamide	n.d.
CYP2C19	S-Mephenytoin → 4′-hydroxy-S-mephenytoin	Unidentified metabolite
CYP2D6	Dextromethorphan → dextrorphan	n.d.
CYP3A4	Testosterone → 6β-hydroxytestosterone	(A) 20OH-NorMD (B) 17α-Hydroxymethyl-17β-methyl-18-norandrosta-1,4,13-triene-3-one
CYP3A7*2	DHEA → 16α-hydroxy-DHEA	n.d.
CYP4Z1	Myristic acid → hydroxymyristic acids	n.d.
CYP11B2	Deoxycorticosterone → corticosterone	n.d.
CYP11B1	Deoxycortisol → cortisol	n.d.
CYP17	Progesterone → 17α-hydroxyprogesterone	n.d.
CYP21	17α-Hydroxyprogesterone → deoxycortisol	(A) 20OH-NorMD (B) 16β-Hydroxy-17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one

Only the CYP2C19, CYP3A4 and CYP21 expressing fission yeast strains were capable of metabolizing the substrate 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one. 20OH-NorMD was most efficiently produced by the human CYP21 expressing strain. All monitored standard reactions are indicated.
n.d.=no detectable product formation.

of 24 h) and the glucose concentration was increased, whereas the relative oxygen content remained approximately constant at around 20%. Because P450-catalyzed reactions require NADPH, which is predominantly formed in the pentose phosphate pathway, it is necessary that glucose is present in sufficiently high amounts in the fermentation culture. As shown in Figure 3, the biomass dry weight at the fermentation end was approximately 10 g/l. The product formation is expressed as relative areas of product obtained from total ion chromatograms after GC-MS analysis and was monitored at different time points as indicated in Figure 3. This approximate estimation of the product formation rate was necessary because an exact quantification of the product was not possible owing to the lack of reference material. Analysis of the relative peak areas of substrate and both major metabolites formed by the CYP21 expressing strain at the fermentation endpoint indicates that approximately 10% of the substrate was converted to the desired metabolite and 30% to the byproduct.

Product purification and NMR analysis

After extraction of the steroids from the fermentation broth using ethyl acetate and removal of remaining substrate, the

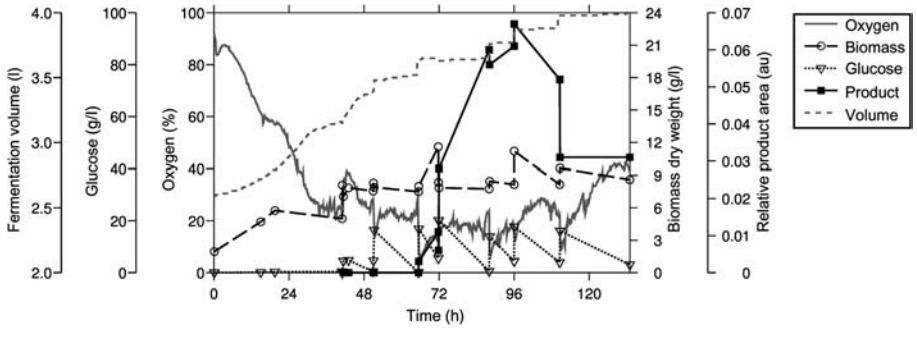

Figure 3 Whole-cell biotransformation of the CYP21 expressing *S. pombe* strain CAD75 in a 5 l stirred-tank fermenter.
Fermenter volume (dashed line), dissolved oxygen concentration (solid gray line), glucose concentration (dotted line with open triangles), biomass dry weight (dw; solid black line with open circles) and product formation (solid black line with filled boxes) were monitored throughout the fermentation process. Relative product peak areas were determined from total ion chromatograms obtained after GC-MS analysis and normalization against the internal standard metandienone.

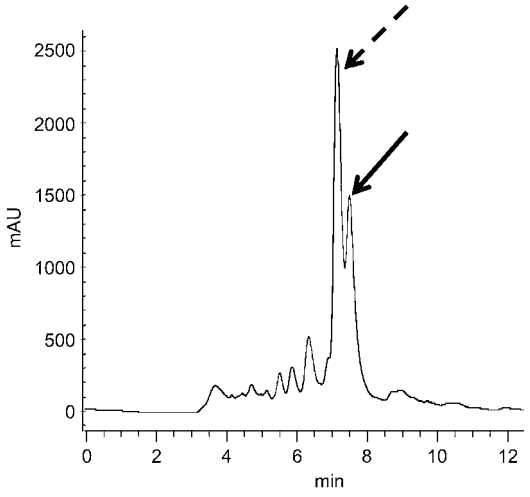

Figure 4 Chromatogram of the semi-preparative HPLC purification of 20OH-NorMD.
20OH-NorMD (solid black arrow) as well as 16β-hydroxy-17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one (dashed arrow) are separated from other byproducts and compounds generated by the CYP21 expressing *S. pombe* strain CAD75. Substrate remainder was previously removed as described in the materials and methods section. The product was isocratically eluted from the semi-preparative column [Nucleosil $N(CH_3)_2$, Macherey-Nagel] using *n*-hexane:2-propanol (95:5) as solvent at 5 ml/min.

final purification step was performed by semi-preparative HPLC. To identify the optimal purification conditions a variety of different HPLC materials and eluent compositions were tested [RP-C18, RP-C8 and $N(CH_3)_2$ column materials from Macherey and Nagel, Düren, Germany]. The best results were obtained using a $N(CH_3)_2$ column with *n*-hexane:isopropanol (95:5 v/v) as solvent. Under isocratic conditions the product was eluted after approximately 450 s, whereas the byproduct was eluted from the column after approximately 420 s (Figure 4). In this way, 10 mg of the product as well as 10 mg of the byproduct were isolated.

Subsequent NMR analysis [^{1}H NMR (600 MHz, $CDCl_3$) δ: 2.02/1.59 (m, 2H, H-16), 1.24 (s, 3H, H-19), 0.95 (s, 3H, H-20α), 3.51/3.37 (d, 2H, H-20β); ^{13}C NMR (150 MHz, $CDCl_3$) δ: 137.5 (C-13), 138.9 (C-14), 33.8 (C-16), 51.6 (C-17), 18.5 (C-19), 21.7 (C-20α), 68.9 (C-20β)] unequivocally confirmed the proposed structure of 20OH-NorMD as shown in Figure 1. Moreover, NMR analysis of the byproduct revealed that this CYP21 metabolite is β-hydroxylated at position C-16 [^{1}H NMR (600 MHz, $CDCl_3$) δ: 3.94 (dd, 1H, H-16), 1.16 (s, 3H, H-19), 0.99 (s, 3H, H-20α), 0.91 (s, 3H, H-20β); ^{13}C NMR (150 MHz, $CDCl_3$) δ: 140.7 (C-13), 131.5 (C-14), 80.3 (C-16), 47.9 (C-17), 18.5 (C-19), 24.6 (C-20α), 18.8 (C-20β)].

Discussion

Although the identification of the long-term urinary metandienone metabolite 20OH-NorMD in doping control samples without purified reference material has been accepted by the WADA as proof for metandienone abuse, until now, the athletes in question could challenge the results of the anti-doping laboratories resulting in prolonged trials. The results presented in this study demonstrate a successful combination of chemical methods (substrate synthesis) and biotechnological tools (whole-cell biotransformation) that allowed the synthesis of 20OH-NorMD which could not be accomplished by other means. The availability of 20OH-NorMD as reference compound will provide an incontestable proof for metandienone abuse even after 19 days of its intake helping anti-doping laboratories to identify athletes who have consumed the anabolic steroid metandienone, particularly during training periods. Because metandienone is usually not administrated during official competitions but during training periods the facilitated and unequivocal long-term detection of metandienone abuse will help to convict athletes who formerly had escaped conviction owing to the shorter detection periods of other metandienone metabolites. Thus, the availability of 20OH-NorMD as reference material is of great significance in the fight against doping.

As mentioned above, human liver CYPs clearly play an important role in the metabolism of anabolic steroids and, consequently, in the detection of steroid abuse (Schänzer, 1996; Rendic et al., 1999). But the activity of steroidogenic CYPs also needs to be considered, as is exemplified by the involvement of CYP19 in nandrolone biosynthesis (Reznik et al., 2001) and the influence of CYP17 polymorphisms on the testosterone/epitestosterone glucuronide ratio (Schulze et al., 2008). The results of the present study suggest that the CYP21-dependent metabolization of the metandienone metabolite 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one is added to this list. From an enzymatic point of view, it is very interesting to identify both a new substrate and new stereo-selective properties of human CYP21. To the best of our knowledge, the only activities that have so far been demonstrated for this enzyme are the 21-hydroxylations of progesterone and 17α-hydroxyprogesterone (Lorence et al., 1989; Wu et al., 1991). The CYP21-dependent steroid β-hydroxylations observed in this study indicate that the activity of this enzyme (like its closest human homolog CYP17) is not as strictly stereo- and regio-specific as has been thought previously. Thus, we might see other CYP21 metabolites of both natural and artificial steroids in the future.

The findings presented in this study naturally raise the question of whether CYP21 and CYP3A4 are actually both involved in the *in vivo* formation of 20OH-NorMD or not and, if so, to what extent each of them contributes to the production of this urinary metandienone metabolite over time. In this context, it is helpful to emphasize the fact that each of the two CYPs produces a second metabolite from 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one that is not formed by the other enzyme. Thus, the involvement of CYP3A4 seems to be very likely because the epimeric form of 20OH-NorMD (which was not observed in the CYP21-dependent biotransformations) has been previously identified in urine samples (Schänzer et al., 2006; Parr et al., 2007). In

contrast, the occurrence of the CYP21-specific 16β-hydroxy metabolite in urine samples of metandienone abusers still needs to be investigated. But even the participation of other human enzymes (CYPs or others) that were not included in this study cannot be ruled out. However, assessing these questions was not within the scope of the present study.

Other possible approaches to clarify the physiological role of CYP21 and CYP3A4 in the formation of 20OH-NorMD could either involve administration studies, e.g., including voluntary patients exhibiting a mild or non-classic CYP21 deficiency (congenital adrenal hyperplasia, CAH) (White and Speiser, 2000), the use of adequate animal models, the investigation of primary hepatic as well as adrenal cell cultures, or the examination of microsome preparations of the respective tissues.

The clinical manifestations of classic CAH are very severe ranging from patients suffering from extreme salt wasting, owing to the incapability of producing cortisol and aldosterone, the most important gluco- and mineralocorticoids, to a virilization of females caused by the excessive production of androgens (White and Speiser, 2000). The symptoms of the mildest from of CAH, non-classic CAH, are premature pubarche in children (Temeck et al., 1987), severe cystic acne (Marynick et al., 1983) and oligomenorrhea in young women (Rosenwaks et al., 1979). Only patients with a mild or non-classic CAH can be ethically suited for a metandienone administration study. However, owing to the mild clinical symptoms, patients with non-classical 21-hydroxylase deficiency very often remain undetected (White and Speiser, 2000).

Another possible approach to confirm the physiological participation of CYP21 in the formation of 20OH-NorMD might be the use of a mouse model. A naturally occurring mouse strain with a CYP21 deletion has been previously reported (Tajima et al., 1999), and a comparison of metandienone metabolization results obtained from this strain with those of wild type mice could help to clarify a possible role of CYP21. The use of primary human adrenal cells of healthy and CAH patients could also be used to assess this question. However, owing to their low proliferation rate primary human adrenocortical cells are difficult to obtain and to cultivate (Cardoso et al., 2009). Moreover, because extra-renal CYP21 activity has been reported before, e.g., in lymphocytes and heart tissue (Zhou et al., 1997; Kayes-Wandover and White, 2000; Forest, 2004), such experiments might not suffice to unequivocally clarify the role of CYP21 in the formation of 20OH-NorMD.

An investigation of the role of CYP3A4 could be conducted in similar ways as proposed for CYP21. For instance, administration studies on volunteers exhibiting a decreased CYP3A4 activity caused for example by a gene polymorphism (Pirmohamed and Park, 2003) or mutated CYP3A4 promoter regions (Ingelman-Sundberg et al., 2007) are an option. Moreover, the Cyp3A-knockout mouse model generated by van Herwaarden that lacks all 13 mouse CYP3A genes (van Herwaarden et al., 2007) seems to be a suited tool for such an investigation. In addition, primary HepG2 cell lines could be used to examine the role of CYP3A4 (Martinez-Jimenez et al., 2005). In these cell culture experiments, the use of known CYP3A4 inducers and inhibitors could help to elucidate the role of CYP3A4 in the formation of 20OH-NorMD.

If both CYP21 and CYP3A4 are found to be relevant for *in vivo* formation of 20OH-NorMD, it will be interesting to investigate to what extent each of them contributes to this reaction during the time span when the metabolite is detectable. As each of the fission yeast strains CAD67 and CAD75 strongly expresses the respective CYP gene under control of the strong endogenous *nmt1* promoter, the biotransformation rates observed in this study do not reflect the situation in the human body where the expression of the two genes is independently controlled by their native promoters in different tissues. Thus, in man the contribution of CYP3A4 to 20OH-NorMD formation might even be higher than that of CYP21.

In conclusion, by combining chemical and biotechnological methods it has been possible to produce and purify the long-term metandienone metabolite 20OH-NorMD in sufficient amounts to allow its structure confirmation by NMR. Two human P450s, CYP3A4 and CYP21, were identified as likely candidates for catalyzing the *in vivo* formation of this compound. Moreover, the techniques described in this paper will lead to the production of this important metabolite in sufficiently high quantities to allow anti-doping laboratories worldwide to use this urinary metabolite as reference substance.

Materials and methods

Chemicals

17α-Hydroxymethyl-17β-methyl-18-norandrosta-1,4,13-trien-3-one was synthesized as previously described (Parr et al., 2007). 17,17-Dimethyl-18-norandrosta-1,4,13-trien-3-one was chemically derived as follows: 1 g of metandienone was dissolved in 40 ml of a mixture of methanol: 1 N hydrochloric acid (1:1) and refluxed for 16 h. The product was extracted three times with 50 ml of n-pentane. All other steroids and reference substances used in this study were purchased from Sigma (Deisenhofen, Germany).

General techniques, media and strains

General DNA manipulation methods were performed according to standard techniques (Sambrook et al., 1989). Media and genetic methods for studying fission yeast were performed according to standard procedures (Moreno et al., 1991; Alfa et al., 1993). Unless indicated otherwise, strains were cultivated at 30°C in Edinburgh minimal medium (EMM) with 20 g/l glucose and supplements as required. The fermentation medium DM1 (1 l) was composed of 2.2 g Na_2HPO_4, 5 g NH_4Cl, 6 g KH_2PO_4, 22 ml salt stock solution (Moreno et al., 1991), 1 ml vitamin stock solution (Moreno et al., 1991) and 100 µl mineral stock solution (Moreno et al., 1991). In all constructs, expression of the human CYPs is regulated by the thiamine-repressible *nmt1* promoter (Maundrell, 1990) of fission yeast. Thiamine was used at a concentration of 5 µM throughout. All strains are listed in Table 2.

Table 2 Recombinant fission yeast strains expressing human cytochrome P450 systems.

Strain	Genotype	Expressed protein(s)	Standard substrate(s)	References
NCYC2036	*h⁻ ura4.dl18*	–	–	Losson and Lacroute, 1983
CAD62	*h⁻ ura4.dl18::pCAD1-CPR*	CPR	–	Dragan et al., submitted
AZ3	*h⁻ ura4.dl18::pCAD1-CPR/pREP1-CYP4Z1*	CPR, CYP4Z1	Myristic acid, lauric acid	Zöllner et al., 2009
CAD64	*h⁻ ura4.dl18::pCAD1-CPR/pREP1-CYP2D6*	CPR, CYP2D6	Dextromethorphan	Peters et al., 2009a
CAD65	*h⁻ ura4.dl18::pCAD1-CPR/pREP1-CYP2B6*	CPR, CYP2B6	Bupropion	Peters et al., 2009b
CAD66	*h⁻ ura4.dl18::pCAD1-CPR/pREP1-CYP2C19*	CPR, CYP2C19	Mephenytoin	Dragan et al., submitted
CAD67	*h⁻ ura4.dl18::pCAD1-CPR/pREP1-CYP3A4*	CPR, CYP3A4	Midazolam, testosterone	Dragan et al., submitted
CAD68	*h⁻ ura4.dl18::pCAD1-CPR/pREP1-CYP2C9*	CPR, CYP2C9	Tolbutamide	Dragan et al., submitted
CAD71	*h⁻ ura4.dl18::pCAD1-CPR/pNMT1-CYP17*	CPR, CYP17	Progesterone	Dragan et al., manuscript in preparation
CAD75	*h⁻ ura4.dl18::pCAD1-CPR/pNMT1-CYP21*	CPR, CYP21	Progesterone, 17α-OH-progesterone	Dragan et al., manuscript in preparation
INA1	*h⁻ ura4.dl18::pCAD1-CPR/pREP1-hCYP3A7*2*	CPR, CYP3A7*2	DHEA, testosterone	Neunzig et al., submitted
MB164	*h⁻ ura4.dl18::pINT5-CYP11B2*	CYP11B2	Deoxycorticosterone	Bureik et al., 2002
SZ1	*h⁻ ura4.dl18::pCAD1-CYP11B1*	CYP11B1	Deoxycortisol	Dragan et al., 2005

Cytochrome P450 screening biotransformation assays

Biotransformation assays with 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one using the recombinant fission yeast strains shown in Table 2 were started with a cell density of approximately 2×10^8 cells/ml and incubation was carried out in 96-well microtiter plates at a shaking velocity of 150 rpm for 24 h at 30°C. The substrate concentration was 250 μM. All steroids were extracted from the cell suspension twice with equal volumes of ethyl acetate and evaporated. Steroids were then derivatized to per-TMS products with an incubation at 60°C for at least 15 min in a glass test tube in 100 μl of a mixture of MSTFA, ammonium iodide and ethanethiol (100:0.2:0.3, v/w/v). GC-MS analyses were performed on an Agilent 6890/ 5973 instrument (Waldbronn, Germany) equipped with an HP Ultra1 GC column (length 17 m, i.d. 0.2 mm, film thickness 0.11 mm) as previously described (Schänzer et al., 2006).

The functionality of all P450 expressing fission yeast strains was confirmed using the known standard substrates (Table 1) at a concentration of 1 mM.

Fermentation conditions

Fed-batch cultivation was performed at 30°C in a 5 l stirred-tank fermenter (Biostat, Braun, Melsungen, Germany) at a stirrer speed of 400 rpm. The start volume of the culture was 2.6 l and the medium used for fermentation was DM1. The pH was continuously measured by a pH electrode (Easyferm Plus K8, Hamilton, NV, USA) and automatically kept constant at pH 5.5 by addition of 5 N NaOH. The fermenter was flushed with air at a flow rate of 3 l/min. The dissolved oxygen concentration was monitored continuously with a pO_2 measuring system (Oxyferm, Hamilton, NV, USA). The fermentation medium was inoculated with biomass from a 2 l EMM culture grown for 3 days in the absence of thiamine to stationary phase. After a biomass growth phase of 40 h, 150 mg of substrate dissolved in 3.0 ml ethanol was added. Another 150 mg of substrate was added after 65 h to yield an end concentration of approximately 200 μM. The total fermentation runtime was 132 h. During the first 40 h (biomass growth phase) feeding was carried out according to Jansen with minor modifications (Jansen et al., 2006). One liter of the used feed medium contained 1 g Na_2HPO_4, 20 g $(NH_4)_2SO_4$, 5 g KH_2PO_4, 4 g $MgSO_4$, 150 g glucose, 20 ml salt stock solution (Moreno et al., 1991), 1 ml vitamin stock solution (Moreno et al., 1991) and 100 μl mineral stock solution (Moreno et al., 1991). The glucose concentration was maintained at a value between 0.1 and 1 g/l. After 40 h, the glucose concentration was increased to a range between 2 and 8 g/l (biotransformation phase) by manually adding an adequate volume of an aqueous 60% glucose containing solution (w/v). Samples were frequently taken and analyzed for the determination of glucose concentration, biomass dry weight as well as product formation. Steroids were extracted from the fermentation broth with equal volumes of ethyl acetate and evaporated using a rotary evaporator (Büchi, Flawil, Switzerland).

Product purification

Purification of the product was accomplished in three steps. First, from the extraction residue the remaining substrate was removed with the n-pentane layer via liquid-liquid extraction from a solution in methanol:H_2O (4:1; v/v). Subsequently, the hydroxylated products were extracted with *n*-pentane from a mixture of methanol:aqueous KOH (1:9; v/v). Final purification was performed by HPLC using a semi-preparative column from Macherey-Nagel (Düren, Germany) [Nucleosil $N(CH_3)_2$, 10×250 mm].

The preparative HPLC system consisted of a Hewlett Packard (HP) 1050 series injector (Agilent Technologies, AT, Waldbronn,

Germany) equipped with a 1.0 ml loop, an AT 1200 series G1361A preparative pump, and an AT 1100 series diode array detector. The product was isocratically eluted from the column with a flow of 5 ml/min using *n*-hexane:2-propanol (95:5) as solvent. Fractions containing the desired metabolite were collected manually. The purity of the eluted products was analyzed via GC-MS as described previously.

NMR analysis

The nuclear magnetic resonance (NMR) analyses were performed in $CDCl_3$ (~4 mM solute) at 600 MHz (^{1}H NMR) and 150 MHz (^{13}C NMR) on a Bruker Avance II 600 instrument (Bruker, Rheinstetten, Germany) at 298 K. The spectra were recorded as ^{1}H; H,H COSY; APT; H,C HMQC; H,C HMBC and NOESY using tetramethylsilane as internal standard.

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