IN VITRO AND *IN VIVO* CHARACTERIZATION OF CYP11A1, CYP17A1 AND CHLORAMPHENICOL ACETYLTRANSFERASE - DEPENDENT STEROID CONVERSION

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DEDICATION

To the memory of my beloved parents, the greatest titles in the sacrifices, kindness and the reason for what I have become today

..... Allah have mercy on them

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…Praise be to Allah, Almighty for his invaluable help and guidance…

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THE SCIENTIFIC CONTRIBUTION IN THE MANUSCRIPTS FOR THE CUMULATIVE THESIS

The PhD work presented in this Thesis is formed from three publications. Two manuscripts were contributed by **Azzam Abdulsattar Mosa** as a first author and one publication as a co-author in refereed scientific journals.

3.1- A. Mosa, J. Neunzig, A. Gerber, J. Zapp, F. Hannemann, P. Pilak, R. Bernhardt
Journal of Steroid Biochemistry & Molecular Biology 150 (2015) 1–10.

The first author processed all data sets, performed all laboratory analysis and wrote the paper. The NMR analysis was carried out by Dr. Josef Zapp/ Institute of Pharmaceutical Biology, Saarland University. The CYP11A1 whole-cell system was developed in cooperation with Dr. Frank Hannemann, Adrian Gerber and Patrick Pilak/ Institute of Biochemistry, Saarland University. The evaluation and interpretation of the results as well as the writing of the manuscript were performed in close cooperation with Jens Neunzig and Adrian Gerber/ Institute of Biochemistry, Saarland University. The project was supervised by Prof. Dr. Rita Bernhardt.

3.2- Azzam Mosa, Michael C. Hutter, Josef Zapp, Rita Bernhardt and Frank Hannemann
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The first author was responsible for all laboratory work, except for the NMR analysis, which was carried out by Dr. Josef Zapp/ Institute of Pharmaceutical Biology, Saarland University, the docking experiments performed by Dr. Michael Hutter/ Center for Bioinformatics, Saarland University and the expression plasmid coding for a His6-tagged CATI was constructed by Dr. Frank Hannemann/ Institute of Biochemistry, Saarland University. The author also contributed to the writing the manuscript. The project was supervised by Prof. Dr. Rita Bernhardt and Dr. Frank Hannemann.

3.3- J. Neunzig, A. Sánchez-Guijo, **A. Mosa**, M.F. Hartmann, J. Geyer, S.A. Wudy, R. Bernhardt
Journal of Steroid Biochemistry & Molecular Biology 144 (2014) 324–333.

The author contributed in the methodological section reported in this publication especially the establishment of the expression and purification procedures for three proteins, CYP17A1, CPR and b_5 used in this study.

LIST OF ABBREVIATIONS

17-hydroxy-PregS	17-hydroxy-pregnenolone sulfate
AdR	Adrenodoxin reductase
Adx	Adrenodoxin
B	Corticosterone
b5	Cytochrome b_5
CAM	Chloramphenicol
CAM-1-A, CAM-3-A and CAM-1,3-A	Acetylated forms of chloramphenicol at position 1, 3 and 1,3
cAMP	Adenosine-3´,5´-cyclic monophosphate
camR	Chloramphenicol resistance gene
cat	Gene for CAT
CAT	Chloramphenicol acetyltransferase enzymes
CNS	Central nervous system
CO	Carbon monoxide
CPR	Cytochrome P450 reductase
CS	Cholesterol sulfate
CYP	Cytochrom P450
CYP11A1	Cytochrome P450 side chain cleavage enzyme
CYP11B1	Cytochrome P450 11β-hydroxylase
CYP11B2	Cytochrome P450 aldosterone synthase
CYP17	Cytochrome P450 17-hydroxylase/17,20 lyase
CYP19	Cytochrome P450 aromatase
CYP21	Cytochrome P450 21-hydroxylase
DHEA	Dehydroepiandrostenedione
DHEAS	Dehydroepiandrosterone sulfate
DMSO	Dimethylsulfoxide
DOC	11-deoxycorticosterone
DOCA	11-deoxycorticosterone-21-acetate
E. coli	*Escherichia coli*
ER	Endoplasmic reticulum
F	Cortisol (Hydrocortisone)
FAD	Flavine adenine dinucleotide
FMN	Flavine mononucleotide
FpR	Ferredoxin reductase
HSD	Hydroxysteroid dehydrogenase
k_{cat}	Catalytic rate constant
K_d	Dissociation constant
kDa	Kilodalton
K_m	Michaelis-Menten constant
KPP	Potassium phosphate buffer
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)

NMR	Nuclear magnetic resonance
P450	Cytochrome P450
P450scc	Cytochrome P450 side chain cleavage enzyme
PregS	Pregnenolone sulfate
RNA	Ribonucleic acid
RSS	11-deoxycortisol
SD	Standard division of the mean
StAR	Steroidogenic acute regulatory protein

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ABSTRACT

This Thesis provides an evidence for a direct stimulation of the CYP11A1-dependent side-chain cleavage activity by the sex hormones estrone and estradiol in a reconstituted *in vitro* system and demonstrates for the first time a novel activity for this enzyme to perform hydroxylation reactions on four steroid hormones 11-deoxycorticosterone (DOC), androstenedione, testosterone and dehydroepiandrosterone (DHEA). These new hydroxylated products might be useful to generate pharmaceutically interesting compounds.

Furthermore, another important steroid modification was performed in this study by the establishment of a novel and efficient *Escherichia coli* based biocatalyst using chloramphenicol acetyltransferase I (CATI) as a steroid acetyltransferase. The results clearly revealed the important role of bacterial CATI in a new strategy for 21-hydroxysteroid acetylation.

Moreover, it was investigated that pregnenolone sulfate (PregS) represents a starting material for a steroidogenic pathway of sulfonated steroids. The results in this work clearly proved the role of bovine CYP17 in the catalysis of PregS conversion to its corresponding hydroxylated product, 17-hydroxy-pregnenolone sulfate (17-hydroxy-PregS) and showed that cytochrome b_5 has no influence on 17,20-lyase activity of CYP17 dependent PregS conversion. For this, the expression and purification processes of three necessary proteins, bovine cytochrome P450 reductase (CPR), cytochrome b_5 and CYP17 were developed for the *in vitro* substrate characterizations.

ZUSAMMENFASSUNG

Die vorliegende Arbeit liefert Aufschluss über die direkte Stimulation der CYP11A1-katalysierten Seitenkettenspaltung von Cholesterol durch die Sexualhormone Estron und Estradiol in einem rekonstituierten *in vitro* System. Die Aktivität von CYP11A1 bei der Hydroxylierung der vier Steroidhormone 11-Deoxycorticosteron (DOC), Androstenedion, Testosteron und Dehydroepiandrosteron (DHEA) konnte zum ersten Mal gezeigt werden. Die hydroxylierten Produkte könnten Vorläufer für interessante Pharmazeutika sein.

Eine weitere wichtige Derivatisierungsreaktion von Steroiden wurde mit Hilfe der Chloramphenicolacetyltransferase I (CATI) etabliert. Dabei wirkt das Enzym in einem *Escherichia coli* basierten Ganzzellkatalysator als Steroidacetyltransferase. Die Ergebnisse zeigen, dass die bakterielle CATI eine zentrale Rolle bei einem neuen Ansatz zur Acetylierung von 21-Hydroxysteroiden einnimmt.

Darüber hinaus wurde Pregnenolonsulfat (PregS) als Ausgangsstoff der Biosynthese sulfonierter Steroide identifiziert. Die Ergebnisse dieser Arbeit zeigen, dass das bovine CYP17 die Hydroxylierung von PregS zu 17-Hydroxy-pregnenolonsulfat (17-hydroxy-PregS) katalysiert. Cytochrom b_5 hat keinen Einfluss auf die 17,20-Lyaseaktivität beim Umsatz von PregS durch CYP17. Für die Substratcharakterisierung *in vitro* wurde die Expression und Reinigung der notwendigen Proteine, bovine Cytochrom P450 Reduktase (CPR), Cytochrom b_5 und CYP17, etabliert.

1- GENERAL INTRODUCTION

1.1- CYTOCHROME P450

1.1.1- GENERAL FEATURES OF CYTOCHROME P450

The researchers, Garfinkel [1] and Klingenberg [2] first discovered cytochromes P450 (P450) during investigating steroid hormone metabolism in the liver microsomes from rats and pigs. The microsomal carbon monoxide-binding pigment with an absorption maximum at 450 nm was later demonstrated to contain an iron-protoporphyrin IX [3]. This iron (III) ion is equatorially bound to the four nitrogen atoms of the protoporphyrin ring. In contrast to most heme proteins, the fifth ligand is a thiolate anion of a cysteine, which characteristic leads to a specific maximum absorption at 450 nm in the reduced and CO-bound state [4].

Cytochromes P450 (EC 1.14.14.1; P450s) are one of most widely characterized and used monooxygenases with a molecular weight of approximately 50 kDa. More than 21,039 P450 genes have been found in all classes of living organisms (http://drnelson.uthsc.edu/P450.statsfile.html, August, 2013) [5-7]. They have been found in mammals, plants, archaea, fungi, bacteria, fish, yeasts, and insects. In mammals, they could not only be found in the liver but also in other organs, including lung, kidney, gastrointestinal tract, brain, gonads, skin and heart [8,9].

The most common reaction catalyzed by cytochromes P450 is the hydroxylation of C-H bond of organic compounds [5], however, P450s have become very versatile and developed a broad field of chemical activity to perform a number of difficult oxidative reactions [10]. This includes isomerizations, dehydratations, N-oxidation, N-, S-, and O-dealkylation, sulfoxidation, epoxidation of double bonds, peroxidation, deamination, desulfuration, dehalogenation and N-oxide reduction of numerous endogenous and exogenous compounds [11-13]. Furthermore, P450s are involved in the metabolism of a variety of xenobiotic chemical compounds, including drugs, alkaloids and carcinogens and other xenobiotics as well as the synthesis of important endogenous compounds like steroid hormones, bile acids or prostaglandins [14].

Noteworthy, the P450s are also of great interest in drug metabolism. They are often involved in the oxidation of drugs resulting in the generation of metabolites that are more easily excreted [15]. It was demonstrated that human genome encodes for 57 functional P450s and among these 57 P450s genes in human, 15 P450s are involved in the metabolism of clinically used drugs, 14 are primarily involved in the steroid metabolism, 6 are involved in vitamin metabolism and 9 P450s are involved in the metabolism of fatty acids and eicosanoids, whereas the remaining 13 human P450s are considered as orphans and their biological functions remain to be firmly established [16,17].

Due to the catalytic diversity and broad substrate range of P450s, they are attractive biocatalyst candidates for the production of fine chemicals, including pharmaceuticals [14,18]. In fact, the

remarkable ability of P450s to catalyze the hydroxylation of non-activated carbon atoms in a regio- and stereoselective manner makes P450 proteins very interesting for the production of these compounds, avoiding the use of protecting groups and several time-consuming chemical steps since the hydroxylation of non activated carbon bonds is often difficult to achieve by classical organic synthesis [19]. Therefore, researches focusing on steroid hydroxylation by P450s have attracted the interest of scientists for several decades. Nowadays, about 300 approved steroid drugs are known and are ranked among the most commercialized medical products representing the second largest category next to antibiotics [20]. The industrial applications of P450s comprise the synthesis of new anticancer drugs. Since these drugs are naturally only available in limited quantities from their biological sources, synthetic routes are required [14,21,22].

1.1.2- NOMENCLATURE OF CYTOCHROME P450

In order to assess the large number of currently known cytochrome P450s, these are not classified according to their function, but based on the sequence similarity of their amino acid chain. A regular classification of P450 enzymes according to their sequence similarity into families, subfamilies and finally into the individual species was established. Species belonging to the same family usually possess a sequence similarity of > 40% whereas members of the same subfamilies are > 55% identical. This classification resulted in a new nomenclature for all cytochrome P450 members in which CYP stands for cytochrome P450 followed by the number denoting the enzymes family. Further, a letter behind the family number designates the subfamily and an additional number represents the individual enzyme within the subfamily [23,24]. Accordingly, the cytochrome CYP11A1 (Figure 1), belongs to the family 11 and the subfamily A. The last number represents the individual enzyme.

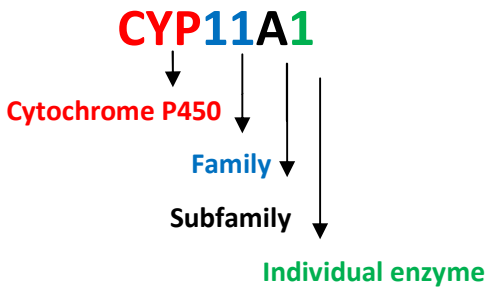


Figure 1: Nomenclature of the cytochromes P450 CYP11A1.

1.1.3- CYTOCHROME P450 CLASSIFICATION AND ELECTRON TRANSFER SYSTEM

P450s are oxidoreductases and in further classification they belong to external monooxygenases (Figure 2), in which the external electron donor is used to cleave the oxygen atoms [14]. In the reactions catalyzed by the cytochrome P450, an oxygen atom is transferred to the substrate, while the other one is reduced to water; they are, therefore, considered to be monooxygenases [25,26]. In

mammals, these monooxygenases can be divided into two broad classes based on their intracellular localization and requirement for redox partners, which provide electrons for the monooxygenase reaction: microsomal and mitochondrial P450s [9,27].

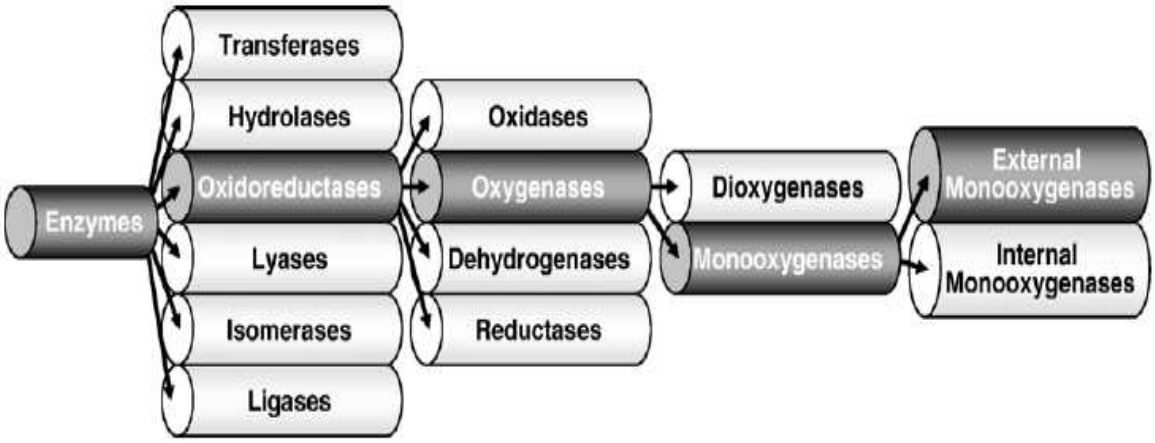


Figure 2: Classification of cytochrome P450 enzyme in groups. The members of this protein family belong to the dark gray sub-groups [26].

These cytochrome P450 classes obtain the required electrons from NADPH via two different electron transfer systems. In the mitochondrial P450s, which are arranged at the inner mitochondrial membrane, the high potential electron is transferred from NADPH to the flavoprotein, adrenodoxin reductase (AdR), and subsequently to a non heme iron- sulphur protein, adrenodoxin (Adx). Finally, Adx is able to reduce the cytochrome P450 (Figure 3A). In the microsomal system, the electrons are transferred from NADPH to the NADPH-cytochrome P450 reductase (CPR) and then to the microsomal cytochromes P450, which are abundantly present in the endoplasmic reticulum (ER) (Figure 3B) [26,28,29].

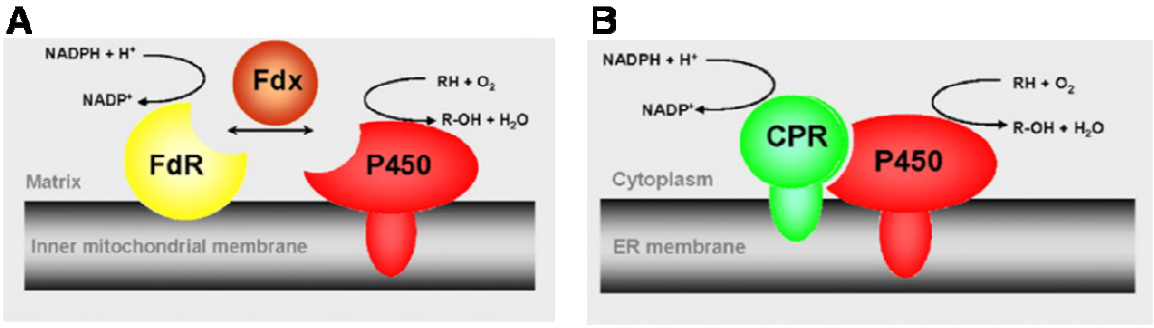
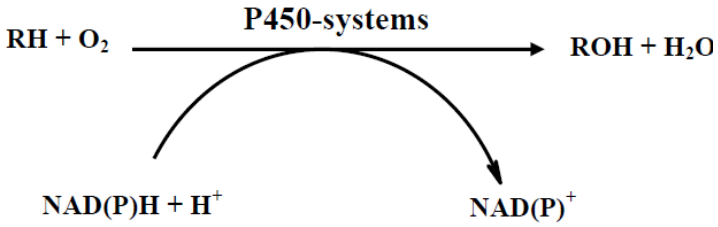


Figure 3: Schematic representation of the electron transfer system for P450s enzyme. A) class I, mitochondrial electron transfer system; B) class II, microsomal electron transfer system [26].

1.1.4- CYTOCHROME P450 CATALYZED REACTION AND CATALYTIC MECHANISM

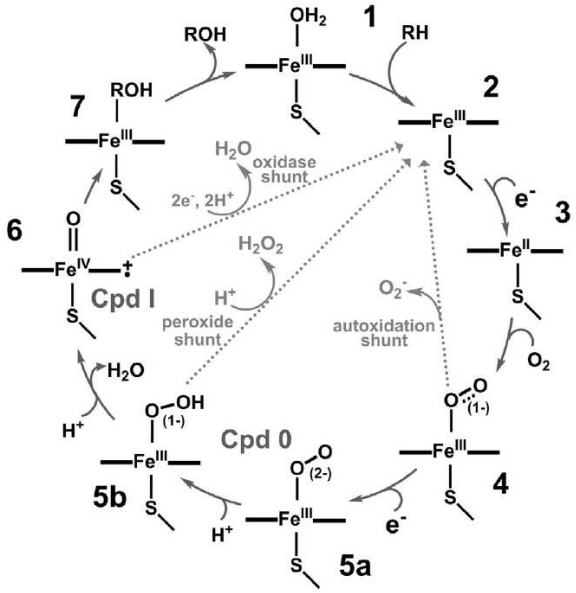
In general, P450 enzymes are proficient at highly regio-specific hydroxylation reactions. The usefulness of the P450s consists in the importance of their bioconversion activities of water insoluble chemicals into soluble ones by the introduction of single oxygen via a hydroxylation reaction, which is unique for P450 to insert hydroxyl groups into even non activated carbon bonds. Most P450s catalyze two-electron oxidation reactions of organic substrates. Dioxygen and the substrate (RH) bind at the heme active site and electrons from NADPH act to cleave the O-O bond leaving a high valent oxo-heme moiety that is capable of O-insertion into an otherwise inert C-H bond on the corresponding substrate. Thereby an oxygen molecule is transferred to the substrate and the other one is used to form a water molecule [14,30,31]. The reactions of all cytochrome P450s are similar (Scheme 1) and can be summarized as following:



Scheme 1: Reaction catalyzed by cytochrome P450. The RH represents a substrate and ROH the corresponding hydroxylated product.

The detailed catalytic cycle (Scheme 2) for substrate hydroxylation by P450s is described as follows:
In the absence of substrate, the ferric iron atom is six-coordinated, complexed with a water molecule. The binding of the substrate molecule to cytochrome P450 (1) partially or completely displaces the water molecule from the sixth coordination site of the heme iron and causes a shift from a hexa-coordinated low-spin complex to a penta-coordinated high spin ferric state (2). A single electron from the NADPH or NADH obtained from redox partners reduces the iron to the ferrous state (II), which allows subsequently the binding of oxygen to the ferrous iron. Binding of molecular oxygen to the ferrous protein (3) generates the ferrous oxygenated (oxy-ferrous) state (4), a key intermediate in P450 catalysis. The availability of a second electron to the oxy-ferrous intermediate from the redox partner yields the peroxo anion state (5a). Proton transfer to the distal oxygen atom orchestrated by the active site acid-alcohol pair and bound water molecules results in formation of the hydroperoxo state (5b). Each of these peroxo states (compound 0 (Cpd 0)) may undergo a non-productive release of peroxide, regenerating the ferric form of the enzyme (depicted as gray lines in the Scheme 2). Alternatively, a second protonation of the distal oxygen may occur, which reduces the O-O bond order resulting in cleavage and release of water molecule and the generation of a higher valent metal-oxo

species referred to as compound 1 (Cpd 1) (6), which then generates the hydroxylated substrate and the enzyme is further regenerated into its native ferric form [4,32-34].



Scheme 2: Proposed scheme for the mechanism action of cytochrome P450s [34].

1.2- STEROID HORMONES AND CYTOCHROMES P450

1.2.1- STEROIDOGENIC P450s

The biosynthesis of all steroid hormones starts from cholesterol in which two distinct groups of enzymes are involved: the cytochromes P450 and the hydroxysteroid dehydrogenases (HSD) [24]. The adrenal steroid hormones are produced in multi enzymatic steps in which different cytochromes P450 and hydroxysteroid dehydrogenases are involved. The P450s involved in the steroidogenic pathway include the following ones: CYP11A1 (side-chain cleavage enzyme), CYP17 (17-hydroxylase and 17,20-lyase), CYP21 (steroid 21-hydroxylase), CYP11B1 (steroid 11β-hydroxylase), CYP11B2 (aldosterone synthase) and CYP19 (aromatase) [9,11,35,36]. These P450s are expressed in steroidogenic tissues such as the adrenal gland (glucocorticoids, mineralocorticoids and androgens), testis, ovary, placenta (progestogens, androgens and oestrogens), and the skin (vitamin D metabolites). Further, they were also found in the brain [37-39].

1.2.2- STEROID HORMONE BIOSYNTHESIS

Steroid compounds are widespread in different living systems and represent an essential component for life and reproduction [40]. In mammals, steroids are mainly produced in the adrenals and gonads in a series of enzymatic steps in the mitochondria and the cytosol of steroidogenic tissues. They are known to play a key role in the management of human fertility, menopause, osteoporosis, and blood pressure regulation, also are of importance for cholesterol level regulation and cardiovascular and

neuroprotective functions. They are also involved in tissue differentiation, cell proliferation, regulation of signal transduction pathways and quorum sensing cell-to-cell communication processes [40-42].

The steroidogenesis is the process in which biologically active steroid hormones are synthesized from cholesterol. To initiate the production of any steroid hormone, the cholesterol must be transported from the outer to the inner mitochondrial membrane. This step is the rate-limiting step in steroidogenesis. The translocator protein and the steroidogenic acute regulatory protein (StAR) are the two major components of the mitochondrial cholesterol transport machinery [43]. These proteins deliver cholesterol to CYP11A1 in the inner mitochondrial membrane. The biosynthesis of all steroid hormones (Figure 4) is initiated by the mitochondrial steroid hydroxylase CYP11A1 with the side-chain cleavage of cholesterol yielding pregnenolone, the precursor of all other steroid hormones [44,45]. The formed pregnenolone is transformed into different steroid products through enzymatic reactions in a tissue-specific manner [27,43].

To ensure further steps in pregnenolone metabolism, the pregnenolone produced in the mitochondria moves into the cytosol where it serves as substrate for either CYP17 or 3β-HSD to produce either 17-hydroxy-pregnenolone or progesterone, respectively [46,47]. The formed 17-hydroxy-pregnenolone can then serve as substrate for the lyase activity of CYP17 to produce DHEA. DHEA and 17-hydroxy-pregnenolone also act as substrates for 3β-HSD. Pregnenolone, 17-hydroxy-pregnenolone and DHEA are all dehydrogenated at C3 by 3β-HSD, resulting in progesterone, 17-hydroxy-progesterone and androstenedione, respectively [48]. In a further step, progesterone can be hydroxylated at C17 by CYP17 to form 17-hydroxy-progesterone. In addition, the lyase activity of CYP17 can cleave the C17-C20 bond of 17-hydroxy-progesterone to yield androstenedione. Progesterone and 17-hydroxy-progesterone also act as substrates for CYP21, which catalyzes the formation of 11-deoxycorticosterone (DOC) and 11-deoxycortisol (RSS), respectively, by hydroxylation of these substrates at C21 position. These two intermediates move to the mitochondria where CYP11B1 catalyses 11-hydroxylation to yield cortisol (catalyzing the last step in the cortisol (hydrocortisone) biosynthesis). DOC serves as substrate for CYP11B2 (P450 aldo), which catalyses three sequential reactions: hydroxylation of positions 11 and 18 of the carbon skeleton of DOC and the oxidation step at position 18 hydroxyl group to yield the C18 aldehyde group of aldosterone [24,49]. Finally, the biosynthesis of estrogens is performed by CYP19 (P450arom), which catalyzes the conversion of androgens (androstenedione, testosterone and 16α-hydroxy-testosterone) to estrogens (estrone, 17β-estradiol and estriol, respectively) in a three-step reaction through the formation of 19-hydroxy and 19-aldehyde intermediates, followed by simultaneous elimination of the methyl group and aromatization of the A-ring [34].

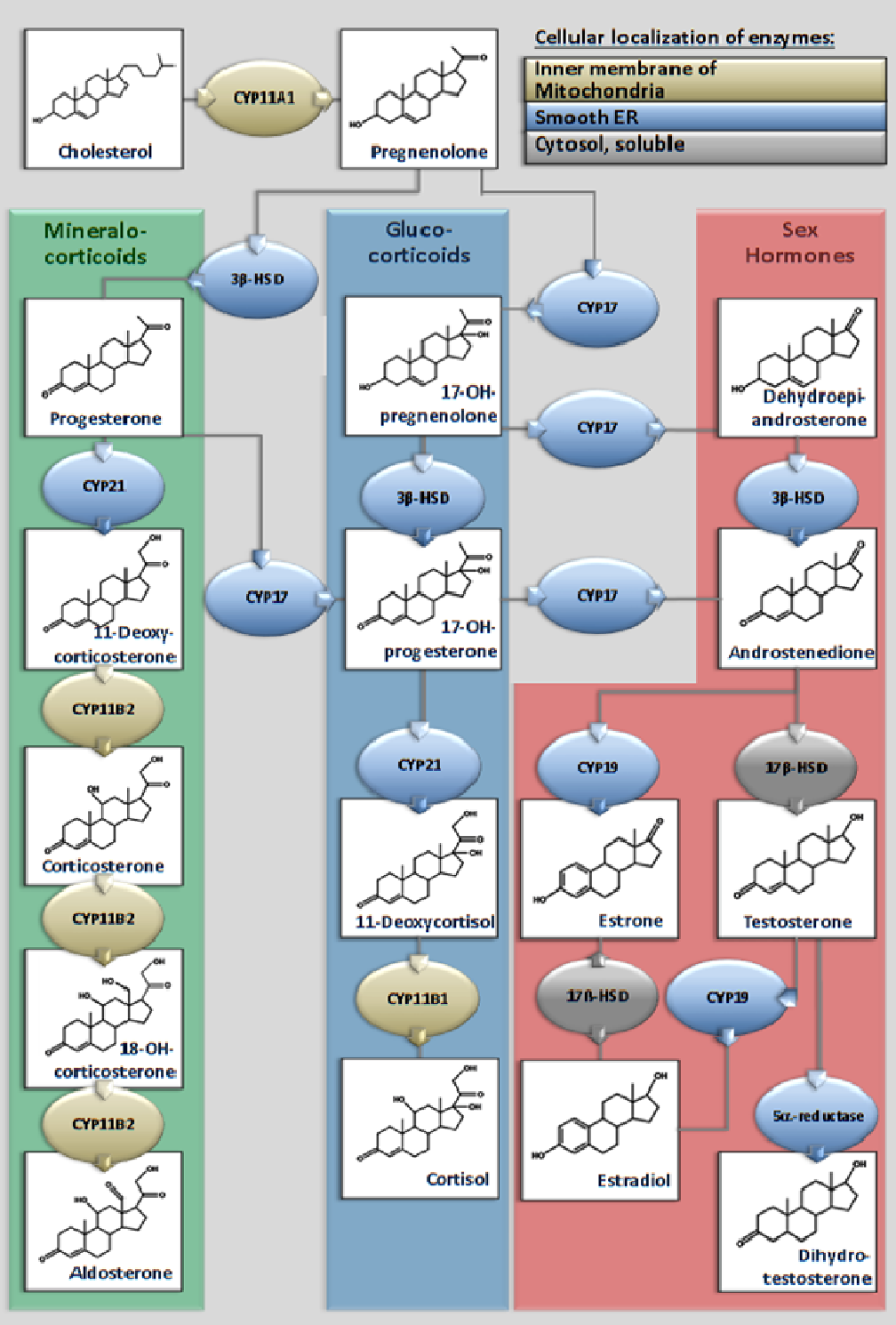


Figure 4: Biosynthesis of steroid hormones in adrenal glands and gonads. Schematic description of the different reactions taking place in the adrenal steroid hydroxylation system and all enzymes involved in the biosynthesis of the adrenal steroid hormones, corticosterone, cortisol, and aldosterone; and the gonadal steroid hormones, progesterone, estradiol, and testosterone [50].

The glucocorticoids, mineralocorticoids and adrenal androgens are produced in the adrenal cortex of the adrenal gland. Higher vertebrates possess two ovoid or bean-shaped adrenal glands situated above the kidney, embedded in fat tissue. The adrenal glands consist of three zones: medulla, cortex

and the capsule (Figure 5). In mammals, the structure of the adrenal cortex is complex and considered to be the largest section of the adrenal gland. In the adrenal cortex, three zones can be distinguished: the *zona glomerulosa*, the *zona fasciculata* and the *zona reticularis*. Each section contains different P450s, which together with two dehydrogenase enzymes are responsible for steroid hormone synthesis [9,36].

The distribution of different steroidogenic enzymes in the three zones of the adrenal cortex results in each zone's unique steroidogenic output. Within the adrenal cortex, CYP11A1 is expressed in all three zones of the adrenal cortex, while the outer *zona glomerulosa* consists of cells, which contain the CYP11B2 enzyme [49] and lack the CYP17 enzyme. These cells are, therefore, responsible for the synthesis of the major mineralocorticoid, aldosterone. The middle layer, *zona fasciculata* contains both the CYP17 and the CYP11B1 and is, therefore, responsible for the production of cortisol and corticosterone and trace amounts of the androgen precursors, (DHEA) [47,49,51]. The inner section of the adrenal gland is the *zona reticularis*, which is responsible for the production of trace amounts of glucocorticoids and the adrenal androgens DHEA, DHEA sulphate (DHEAS) and androstenedione due to the activity of CYP17 (17-hydroxylase/17,20-lyase) [9,47,51].

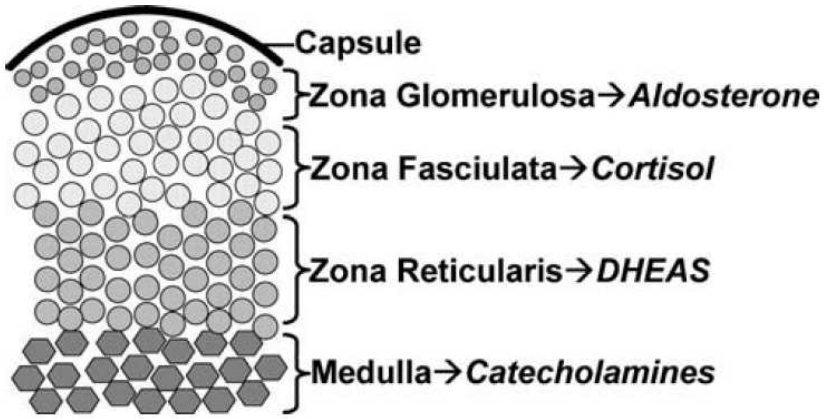


Figure 5: The three sections of the adrenal gland (zones) and correlated products. The outer *zona glomerulosa* is responsible for synthesis of the major mineralocorticoid, aldosterone. The middle *zona fasciculata* and inner *zona reticularis* are responsible for the synthesis of glucocorticoid and androgens, respectively [47].

1.3- CYTOCHROME P450 SIDE-CHAIN CLEAVAGE (CYP11A1)

1.3.1- GENERAL ASPECTS OF CYP11A1

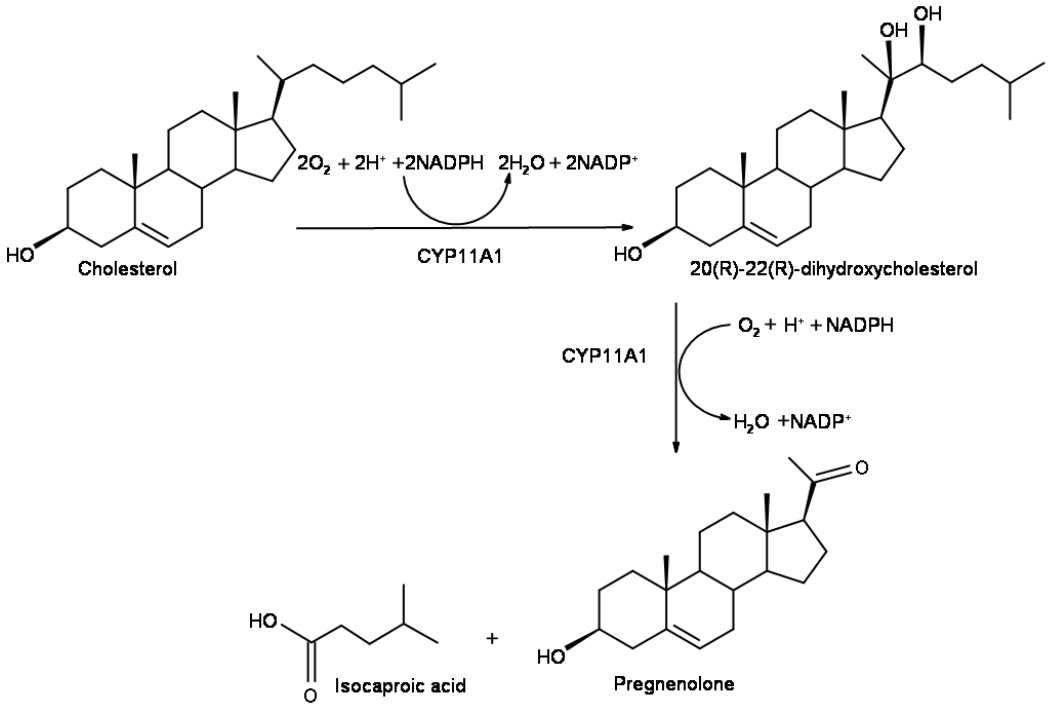
Cytochrome P450scc (CYP11A1), (EC 1.14.1.9), is a mitochondrial enzyme and the only known enzyme that cleaves the side-chain of cholesterol, yielding pregnenolone, the precursor of all steroid hormones. This is the first reaction in the process of steroidogenesis in all steroidogenic tissues

[52,53]. Among four enzymes in vertebrates (CYP7A1, CYP27A1, CYP46A1, and CYP11A1), CYP11A1 is the only one that initiates the biotransformation of cholesterol in different organs and thus plays an important role in the maintenance of cholesterol homeostasis. Furthermore, its deficiency leads to lipoid congenital adrenal hyperplasia, a lethal disease if untreated [54].

CYP11A1 is a membrane bound protein localized in the inner mitochondrial membrane of steroidogenic tissues such as the adrenal glands and gonads. CYP11A1 consists of 482 amino acids and has a molecular weight of about 54 kDa. It is expressed at all the major sites of steroidogenesis, namely the adrenal cortex, ovary, testis, and placenta under the control of pituitary hormones, through the cAMP-signaling pathway [9,24,55]. In the adrenal cortex, CYP11A1 is expressed in all three zones, the *zona fasciculata*, the *zona reticularis*, and the *zona glomerulosa*. In the ovary, CYP11A1 is expressed in the theca interna and the granulosa cells of ovulatory follicles, while in testis the only site of CYP11A1 expression are the Leydig cells. In addition, low expression levels of CYP11A1 have been detected in the central and peripheral nervous system, skin and heart. Several other tissues, such as brain, gastrointestinal tract and kidney, also express CYP11A1 [43,56].

1.3.2- REACTION CATALYZED BY CYP11A1

The conversion of cholesterol to pregnenolone by CYP11A1 is the first and rate-limiting enzymatic step in the biosynthesis of steroid hormones. Pregnenolone is formed via three sequential monooxygenation reactions. The details of the catalytic reactions in mitochondria involve two stereospecific sequential hydroxylations at position C22 and C20 (Scheme 3). First, cholesterol is hydroxlated at position C22 leading to the formation of 22R-hydroxy-cholesterol. This step is followed by the subsequent hydroxylation at position C20 with formation of 20R, 22R-dihydroxy-cholesterol, followed by the oxidative cleavage of the bond between C20 and C22 to yield the C21 steroid, pregnenolone and isocaproic acid [54,57].



Scheme 3: Pathway of pregnenolone formation according to Stryer et al. [58].

As shown in Scheme 3, the reaction requires three molecules of oxygen, three molecules of NADPH and the mitochondrial electron transfer system [54,59]. On the other hand, another catalytic reaction of CYP11A1 was noticed to perform modification of the side-chain for a wide spectrum of cholesterol analogues as well as the hydroxlytion reactions in the steroid skeleton (Table 1)

1.3.3- SUBSTRATE RANGE OF CYP11A1

Besides cholesterol, a wide range of steroids can also serve as substrates for CYP11A1, suggesting that this P450 possesses a wide spectrum of substrate specificity. In these steroid substrates, not only the analogues of cholesterol with modified side-chain are efficiently converted to pregnenolone, but also the hydroxylation occurs in the steroid skeleton. Some known substrates of CYP11A1 and their products are shown in Table 1.

Table 1: Well-known CYP11A1 substrates and their corresponding products.

Substrates	Products	References
20α-hydroxy-cholesterol	Pregnenolone	[60]
25-hydroxy-cholesterol	Pregnenolone	[61]
25-methyl-cholesterol	Pregnenolone	[62]
25,26-didehydro-cholesterol	Pregnenolone	[62]
20α,21-dihydroxy-cholesterol	21-hydroxy-pregnenolone	[63]
22(R)-hydroxy-cholesterol	Pregnenolone	[64]
22(R)-22, 23, 24, 25, 26-hydroxy-cholesterol	Pregnenolone	[62]
20(R),22(R)-20,22-dihydroxy-cholesterol	Pregnenolone	[62]
20(S)-20-hydroxy-cholesterol	Pregnenolone	[62]
20(S)-22-thia-cholesterol	(20(S),22(R)-22-thia-cholesterol S -oxide and (20(S),22(S)-22-thia-cholesterol S -oxide	[65]
(20S)-17α,20-dihydroxy-cholesterol	(20S,22R)-17α,20,22-trihydroxy-cholesterol and 17α-hydroxy-pregnenolone	[66]
Vitamins D2	20-hydroxy-vitamin D2; 17,20-dihydroxy-vitamin D2 and 17,20,24-trihydroxy-vitamin D2	[67,68]
20-hydroxy-vitamin D2	17,20,24-trihydroxy-vitamin D2	[67]
17,20-dihydroxy-vitamin D2	17,20,24-trihydroxy-vitamin D2	[67]
Vitamins D3	20-hydroxy-vitamin D3; 20,22-dihydroxy-vitamin D3; 20,23-dihydroxy-vitamin D3 and 17,20,23-trihydroxy-vitamin D3	[57,69,70]
20-hydroxy-vitamin D3	20,23-dihydroxy-vitamin D3	[70]
20,23-dihydroxy-vitamin D3	17,20,23-trihydroxy-vitamin D3	[70]
Pregn-5-en-3β-ol	Pregnenolone	[71]
Cholest-5-ene-3β, 20α-diol	Pregnenolone	[71]
Cholest-5-ene-3β, 24-diol	Pregnenolone	[71]
Cholest-5-ene-3β, 25-diol	Pregnenolone	[71]
Cholest-5-ene-3β, 26-diol	Pregnenolone	[71]
23,24-bisnor-5-cholen-3β-ol	Pregnenolone	[71]
5-cholen-3β-ol	Pregnenolone	[71]
26-norcholest-5-en-3β-ol	Pregnenolone	[71]
Desmosterol	Pregnenolone	[71]
7-dehydrocholesterol	7-dehydro-pregnenolone	[69,72]
Ergosterol	17α,24-dihydroxy-ergosterol; 20-	[56,73]

	hydroxy-22,23-epoxy-22,23-dihydro-ergosterol and 22-keto-23-hydroxy-22,23-dihydro-ergosterol	
Sitosterol	Pregnenolone	[62,71]
Campesterol	Pregnenolone	[62]
Cholesterol sulfate	Pregnenolone sulfate	[74,75]
11-deoxycorticosterone (DOC)	6β-hydroxy-deoxycorticosterone	[76]
Androstenedione	6β-hydroxy-androstenedione	[76]

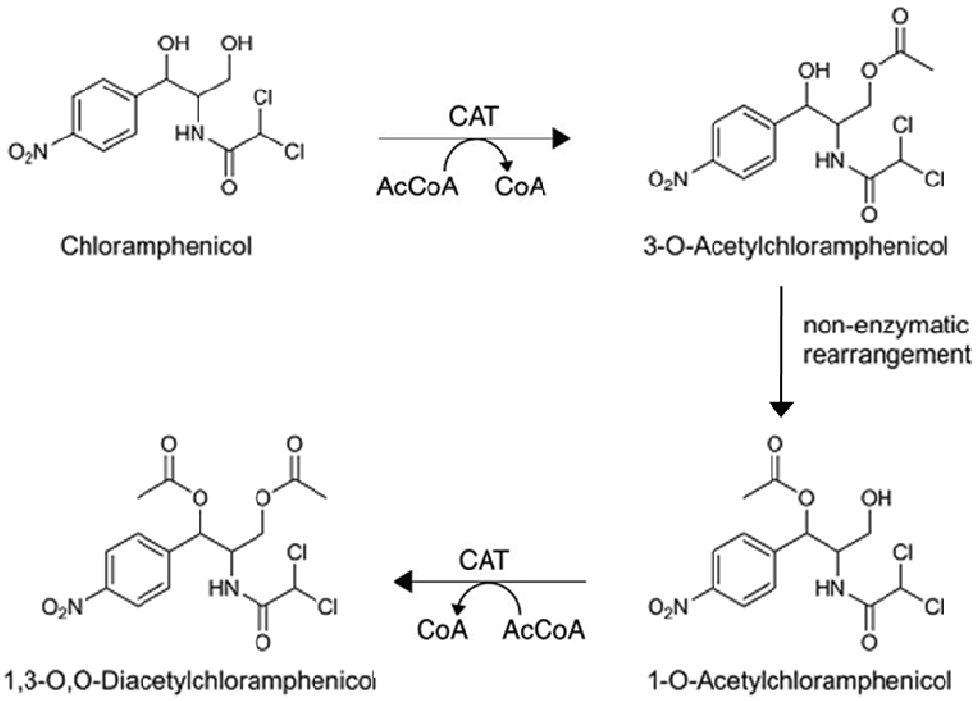
1.4- STEROID ACETYLATION

1.4.1- CHLORAMPHENICOL (CAM)

Chloramphenicol (CAM) was one of the first broad spectrum antibacterial drugs, isolated from the soil bacterium *Streptomyces venezuelae* in 1948 [77,78]. CAM represents the most successful first generation species of broad spectrum antibiotics; it is chemically 2, 2-dichloro-[1, 3-dihydroxy-1-(4-nitrophenyl) propan-2-yl] acetamide [79]. The relative simplicity of the CAM structure quickly led to economic routes of synthesis, and by 1950 the synthetic drug was in wide use clinically and had become accepted as a promising broad spectrum antibiotic [77,78,80]. CAM inhibits protein synthesis by interacting with the peptidyl transferase center of ribosomes, thereby blocking translation of the mRNA. The hydroxyl group of CAM is buried in the interface with the ribosome through direct hydrogen bonding, potassium ion-mediated electrostatic interactions as well as through van der Waals interactions with the RNA phosphosugar backbone [81,82].

1.4.2- MECHANISM OF ACTION OF CHLORAMPHENICOL ACETYLTRANSFERASE (CAT)

The bacterial resistance to chloramphenicol is normally conferred by the enzyme chloramphenicol acetyltransferase (CAT) encoded by *cat*. CAT [EC 2.3.1.28] is a cytoplasmic bacterial enzyme that detoxifies CAM by covalently attaching an acetyl group, derived from acetyl-CoA (acyl donor), to one of the hydroxyl groups of CAM to form the acetylated CAM and thus preventing the antibiotic from binding to a ribosome. CAT can acetylate chloramphenicol at both, one or two sites, and neither the mono- nor the diacetate forms have antibiotic activity and are thus destroying its ability to inhibit bacterial growth [83,84]. Previous studies have demonstrated that three reactions describe the fate of chloramphenicol in bacteria containing CAT. In the first reaction, the CAT enzyme acetylates CAM at the hydroxyl group in C3 position in the presence of acetyl CoA to produce chloramphenicol-3-acetate (3-O-acetyl-CAM). In the second reaction, this acetyl moiety is then shifted to the C1 hydroxyl group via a non-enzymatic rearrangement yielding chloramphenicol-1-acetate (1-O-acetyl-CAM). In the third step, 1-O-acetyl-CAM undergoes a new enzymatic acetylation at the C3 site in the presence of acetyl CoA producing chloramphenicol-1,3-diacetate (1,3-O,O-diacetyl-CAM) (Scheme 4) [85,86].



Scheme 4: Role of chloramphenicol acetyltransferase in chloramphenicol acetylation. Reactions (1) and (3) are both catalyzed by CAT, whereas reaction (2) is a non-enzymatic re-arrangement [86].

The mechanism of the acetylation of CAM involves the abstraction of a proton from the hydroxyl group in C3 position of the CAM molecule, whereby the imidazole side-chain of the catalytic histidine residue acts as general base. Loss of the proton from the hydroxyl group facilitates the subsequent nucleophilic attack of the anion at the carbonyl group of the acetyl-CoA thioester bond [87,88].

1.4.3- ACETYLATED STEROIDS

Steroid acetates represent important derivatives for pharmaceutical applications. Several steroid acetates have been used for the production of pharmaceuticals, which are administered in an acetate form [89,90], e.g: cortisol acetate to treat a variety of skin conditions and as anti inflammatory drug, or prednisolone acetate, which is used as anti-inflammatory agent for ophthalmic use.

In general, the acetylation reaction in the organic chemistry is the insertion of an acetyl group into a molecule. More specifically, the reaction replaces the hydrogen from an alcohol group by an acetyl group. Acetylation of alcohols is an important and one of the routinely utilized transformations in organic synthesis [91]. Concerning steroids, the acetylation has been carried out to produce a wide range of steroid compounds such as phytostanyl esters [92], esters of DHEA [93], aldosterone acetate [94,95], 17α-acetate [96], cortisol-21-acetate [97] and acetate of several steroid hormone metabolites [98], by using different acetylating agents. The acetylation of these compounds can be applied to isolate specific steroids from biological mixtures [99], to determine steroids in biological samples [98],

as well as to produce intermediates and products for pharmaceutical purposes [100-102], thus, confirming that many therapeutically useful steroids are administered as esters with the widespread distribution of the esterase activity in mammalian tissue ensuring the cellular availability of the free steroid [93]. For aforementioned purposes of steroid acetate, several synthetic methods have been described for the synthesis of these compounds. The application of biocatalysts and whole-cell biotransformation offer a good alternative for the production of steroid acetates, allowing mild and environmentally friendly reaction conditions and it is currently the preferred way to achieve selective acetylation of steroid compounds.

It is worth mentioning also that acetates of naturally occurring steroids have been detected as components of human blood. Cortisol-21-acetate is considered to be a regular blood constituent and the major portion is circulating endogenously. Corticosteroid acetates including corticosterone acetate, 11-dehydrocorticosterone acetate and other corticosteroid acetates also have been found to circulate in the human blood. The presence of acetylated steroids in circulating human peripheral blood is important for protein binding. It was demonstrated that acetylation of the C21-hydroxyl group of certain corticosteroids results in an increased interaction at carbon-3 with human albumin to facilitate the steroid transport [103,104]. Furthermore, the specific steroid binding albumin may protect steroids from degradation and thus avoids rapid fluctuations in active unbound steroid concentrations.

2- SCOPE AND OBJECTIVES OF THE THESIS

The first part of this Thesis focuses on the initial stage of the steroidogenic process, where CYP11A1 is involved. Among all steroidogenic P450s, CYP11A1 might represent a vital target for the regulation of steroidogenesis at the cellular level. Therefore, the main objective of this part was to provide a comprehensive overview regarding the interaction of steroid metabolites with CYP11A1 and in depth focusing on the *in vitro* characterization of the CYP11A1 activity in the presence of steroid intermediates. Hence, a recombinant expression system for CYP11A1 was developed as well as a purification procedure was established.

The *in vitro* conversion of cholesterol in the presence of some steroid metabolites was accompanied with the appearance of new product peaks in the HPLC chromatograms. Therefore, it was of special interest to determine whether CYP11A1 is also able to convert these steroids. For this reason, *in vitro* conversions were performed with these new steroids in absence of cholesterol.

Since the whole-cell system expressing P450 is one of the most important strategies of steroid functionalization, which is widely applied for steroid hydroxylations [105,106], an *Escherichia coli* based whole-cell system using tricistronic expression vectors coding for CYP11A1 and its electron transfer partners was established and sufficient amounts of the novel products were obtained for NMR spectroscopy analysis.

In the second part of this study, it was investigated whether a CYP11A1 whole-cell biocatalyst containing an expression vector (pACYC-FHH2) harboring a CAM resistance gene is able to perform the acetylation reaction at the hydroxyl group at C21 position of DOC. For this reason, we aimed to focus on the acetylation of steroids using the bacterial CATI to produce the acetylated steroids for pharmaceutical applications. In addition, the other objective of this study was to establish a novel and efficient *E. coli* based biocatalyst using chloramphenicol acetyltransferase I (CATI) for the regioselective acetylation of C21-hydroxyl groups in steroid compounds. In this study, a substrate screening for CATI was performed with 18 different biologically active steroids. Further, additional experiments were aimed to elucidate the optimization of the conversion assay. The study also aimed to prove that the acetylation activity of the whole-cell catalyst depends on CATI and that acetyl CoA is employed in that reaction as an acyl donor by performing the acetylation reactions of CAM as well as C21-hydroxysteroids with purified CATI. Therefore, an expression plasmid was constructed containing a gene coding for a His_6-tagged CATI and consequently, an *E. coli* expression system was used for the expression of CATI.

The third part of this study concentrates on the metabolism of sulfonated steroids, which are involved in steroidogenic pathway and might have a very important role in the production of biologically active

steroid hormones once they become de-sulfated. Therefore, it may contribute to the overall regulation of reproductive processes as well as affects steroid hormone biosynthesis on a molecular level [107,108]. Due to this reason, the third project aimed to illustrate the proposed steroidogenic pathway of sulfonated steroids using pregnenolone sulfate (PregS). The ability of PregS to serve as a substrate for CYP17 was assessed using the *in vitro* reaction by purified CYP17 and its electron transfer partner, CPR. Further, the effect of cytochrome b_5, which is known to enhance the CYP17 dependent lyase reaction, was determined. To achieve this aim, the expression systems and the purification procedures for recombinant proteins, CPR, b_5 and CYP17 were developed.

Since the uses of cytochrome P450s for steroid hydroxylation is restricted to whole-cell catalysis, the last part of the Thesis consisted in the establishment of CYP11A1 whole-cell system to improve their steroid biotransformation efficiency under desired process conditions. This included the improvement of a whole-cell system for CYP11A1, which has not been published so far. In this aspect, we investigated whether the bioconversion can be improved by avoiding all side effects of the medium components using the resting cells in potassium phosphate buffer to perform the biotransformation experiments. Moreover, the inhibitory effect of indole on the CYP11A1 activity on the *in vitro* and *in vivo* conversion was also estimated.

3- PUBLICATIONS RESULTING FROM THIS WORK

The results produced during the present work are published in the articles listed below.

3.1- MOSA ET AL., 2015 a

2β-AND 16β-HYDROXYLASE ACTIVITY OF CYP11A1 AND DIRECT STIMULATORY EFFECT OF ESTROGENS ON PREGNENOLONE FORMATION

A. Mosa, J. Neunzig, A. Gerber, J. Zapp, F. Hannemann, P. Pilak, R. Bernhardt

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2β- and 16β-hydroxylase activity of CYP11A1 and direct stimulatory effect of estrogens on pregnenolone formation

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ABSTRACT

The biosynthesis of steroid hormones in vertebrates is initiated by the cytochrome P450 CYP11A1, which performs the side-chain cleavage of cholesterol thereby producing pregnenolone. In this study, we report a direct stimulatory effect of the estrogens estradiol and estrone onto the pregnenolone formation in a reconstituted *in vitro* system consisting of purified CYP11A1 and its natural redox partners. We demonstrated the formation of new products from 11-deoxycorticosterone (DOC), androstenedione, testosterone and dehydroepiandrosterone (DHEA) during the *in vitro* reaction catalyzed by CYP11A1. In addition, we also established an *Escherichia coli*-based whole-cell biocatalytic system consisting of CYP11A1 and its redox partners to obtain sufficient yields of products for NMR-characterization. Our results indicate that CYP11A1, in addition to the previously described 6β-hydroxylase activity, possesses a 2β-hydroxylase activity towards DOC and androstenedione as well as a 16β-hydroxylase activity towards DHEA. We also showed that CYP11A1 is able to perform the 6β-hydroxylation of testosterone, a reaction that has been predominantly attributed to CYP3A4. Our results are the first evidence that sex hormones positively regulate the overall production of steroid hormones suggesting the need to reassess the role of CYP11A1 in steroid hormone biosynthesis and its substrate-dependent mechanistic properties.

1. Introduction

In mammals, steroid hormones exhibit many vital functions and are indispensable for normal development and reproduction. They are synthesized in steroidogenic tissues or organs, such as the adrenal glands or the gonads, in a series of reactions involving six different cytochromes P450 (CYPs) [1–3]. Steroid hormones are categorized into three main classes: the mineralocorticoids and glucocorticoids, regulating the salt and sugar homeostasis, respectively, as well as the sex hormones, which are necessary for the development of the secondary sexual characteristics and for reproduction [2,4]. Their availability is regulated by complicated mechanisms at different levels. The concentration of aldosterone, the main mineralocorticoid, is under the control of the renin–angiotensin–aldosterone system [5], whereas the release of cortisol, the main glucocorticoid, and the sex hormone supply are controlled by two similar mechanisms: the hypothalamic–pituitary–adrenal axis and the hypothalamic–pituitary–gonadal axis, respectively [6,7]. The compartmentalization of the production of steroid hormones in different organs or tissues represents an additional mechanism of regulation [8]. In order to interact with their corresponding receptors, steroid hormones need to be present in their free and unbound form. Therefore, the conjugation and deconjugation of steroids constitutes a further mechanism that controls the availability of free steroids on a cellular level [9].

The biosynthesis of steroid hormones starts with the side-chain cleavage of cholesterol producing pregnenolone. This is the first enzymatic and rate-limiting step and is catalyzed by the side-chain cleaving cytochrome P450 CYP11A1 [10,11], a mitochondrial cytochrome P450 found primarily in the steroidogenic cells of the adrenal cortex and the gonads as well as in the skin and brain [12]. The side chain cleavage of cholesterol by CYP11A1 is performed through three consecutive steps: two hydroxylations at C22 and C20, respectively, followed by the cleavage of the C20–C22 bond [2,10,13]. The resulting product, pregnenolone, is the precursor of all classes of steroid hormones: mineralocorticoids, glucocorticoids and sex hormones [14,15]. Therefore, CYP11A1 might represent a vital target for the regulation of steroidogenesis at the cellular level.

Babischkin et al. demonstrated an increased enzymatic activity of CYP11A1 in the presence of estrogens in the placenta of baboons

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0960-0760/

and postulated that the formation of pregnenolone by CYP11A1 might be regulated by estrogens [16].

For this reason, the aim of this study was to investigate whether the CYP11A1 activity is influenced by the presence of various steroid hormone metabolites employing a reconstituted *in vitro* system with purified CYP11A1 and its electron transfer partners to assess the effect of various steroids on the catalysis of CYP11A1 at a molecular level. HPLC analysis revealed a direct activation of CYP11A1 through the estrogens estradiol and estrone.

In addition, we identified new products from androstenedione, DOC, DHEA and testosterone. In order to elucidate the structure of these novel products, an *Escherichia coli* based whole-cells system was established to obtain sufficient amounts for NMR-characterization. These data revealed a novel hydroxylation pattern of CYP11A1-dependent steroid conversions at the positions 2β, 6β and 16β.

2. Materials

Cholesterol and other steroids were purchased from Sigma–Aldrich. All the reagents and solvents were of the highest purity.

3. Experimental

3.1. Expression of CYP11A1

E. coli strain C43(DE3) was used as a host for the expression of bovine CYP11A1, containing a C-terminal 6x-His tag. Cells were co-transformed with a CYP11A1-encoding pTrc99A plasmid [17,18] and the molecular chaperones GroEL/GroES-encoding plasmid pGro12 [19], which possess ampicillin and kanamycin resistance genes, respectively. Transformed cells were grown overnight at 37 °C under shaking at 180 rpm in 50 ml Terrific broth (TB) medium supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin. To prepare the main culture, 200 ml TB medium supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin were inoculated with 2 ml of the preculture and incubated in a 2 l Erlenmeyer baffled flask with 180 rpm shaking at 37 °C. When the culture reached an optical density of 0.8–1.0 at 600 nm, the induction of GroEL/GroES and the CYP11A1 synthesis was achieved by adding 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG), 1 mM delta-aminolevulinic acid (δ-ALA), 50 μg/ml ampicillin and 4 mg/ml arabinose. The culture was further incubated for 48 h at 28 °C and 95 rpm shaking and was harvested by centrifugation (4 °C, 4000 × *g* for 20 min) and the cell pellet was stored at −20 °C.

3.2. Purification of CYP11A1

The stored cell pellet was suspended in 50 ml lysis buffer (50 mM potassium phosphate buffer, pH 7.4, containing 20% (v/v) glycerol, 1.5% sodium cholate, 500 mM sodium acetate, 1.5% Tween-20, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1,4-dithiothreitol (DTT) and phenylmethanesulfonyl fluoride (PMSF). Cells were disrupted by sonication on ice (15 s pulse, 15 min, 13% amplitude) and the cell debris was removed by ultracentrifugation (4 °C, 70,500 × *g* for 30 min). The obtained supernatant was used for protein purification.

Approximately 50 ml of protein extract were applied to a Ni–NTA agarose column after equilibrating the column with equilibration buffer (50 mM potassium phosphate, pH 7.4, 500 mM sodium acetate, 20% glycerol, 1% sodium cholate, 1% Tween-20, 0.1 mM EDTA, DTT and PMSF). The column was washed with 50 ml washing buffer (50 mM potassium phosphate, pH 7.4, 500 mM sodium acetate, 20% glycerol, 1% sodium cholate, 1% Tween-20, 20 mM imidazole and 0.1 mM of EDTA, DTT and PMSF), followed by the same washing buffer containing 40 mM imidazole.

The bound CYP11A1 was eluted from the column by the same washing buffer containing 150 mM imidazole and was diluted with three volumes of dilution buffer (20% glycerol, 0.1 mM DTT, 0.1 mM EDTA and 1% sodium cholate). The dialyzed protein solution was applied to a SP-sepharose column equilibrated with 50 ml calibration buffer (20 mM potassium phosphate, pH 7.4, 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, 10 mM imidazole, 1% sodium cholate, and 0.1% Tween-20). The column was washed once again with 20 ml of equilibrium buffer followed by the second washing step with 30 ml of 40 mM phosphate buffer containing 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, 10 mM imidazole, 1% sodium cholate and 0.1% Tween-20. The column-bound protein was eluted with the 100–300 mM NaCl gradient of washing buffer. Finally, the protein was concentrated using a centrifugal filter (30 kDa) followed by washing with the dilution buffer (50 mM potassium phosphate, 20% glycerol, 1% sodium cholate, 0.05% Tween-20, 0.1 mM EDTA and 0.1 mM DTT). The protein was again concentrated in a centrifugal filter and stored at −80 °C until use.

3.3. Protein expression and purification of redox partners

Heterologous expression and purification of bovine adrenodoxin (Adx) and bovine adrenodoxin reductase were performed as described by Uhlmann et al. [20] and Sagara et al. [21], respectively.

3.4. Spectroscopic studies

UV–vis spectrum of CYP11A1 was measured at room temperature on a UV–vis scanning spectrophotometer (Shimadzu-2101PC). The concentration of expressed CYP11A1 was determined by reduced carbon-monoxide difference spectroscopy according to Omura and Sato [22], applying an extinction coefficient of 91 mM^{-1} cm^{-1} at 450 nm. The solution of CYP11A1 was treated with a few grains of reducing agent (sodium dithionite) and the baseline of the reduced spectrum was recorded between 400 and 500 nm. The sample was slowly bubbled with carbon monoxide for 20 s and the spectrum was recorded.

To determine the substrate binding spectra, difference spectroscopy was applied at room temperature using a Jasco V-630 spectrophotometer and two tandem cuvettes according to Schenkman [23]. Binding affinities for the different substrates of CYP11A1 were estimated by using 2 μM CYP11A1 in 800 μl 50 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.5% sodium cholate and 0.05% Tween-20. A baseline was recorded from 360 to 450 nm.

3.5. In vitro conversion of cholesterol by CYP11A1

The *in vitro* cholesterol conversion by CYP11A1 was performed as described before [24] with some modifications. In this study, the reaction mixture consisting of 1 μM CYP11A1, 20 μM adrenodoxin (Adx) and 0.5 μM adrenodoxin reductase was used in a final volume of 500 μl of 50 mM HEPES buffer (pH 7.4, 0.05% (v/v) Tween-20). In order to regenerate the NADPH, glucose-6-phosphate (5 mM), $MgCl_2$ (1 mM) and glucose-6-phosphate dehydrogenase (1U) were added to the reaction mixture. The substrate cholesterol was dissolved in 45% 2-hydroxypropyl-β-cyclodextrin prior to the addition to the reaction tube. The final concentration of cholesterol in the conversion buffer was 15 μM, the final cyclodextrin concentration was 0.337%. The enzymatic reaction was initiated by the addition of NADPH (500 μM) and incubated at 37 °C and 900 rpm in the Eppendorf thermomixer for 7 min. The assay was terminated by boiling the samples in a water bath for 5 min. In order to detect the cholesterol and pregnenolone product at 240 nm, a cholesterol oxidase reaction was performed during which cholesterol and

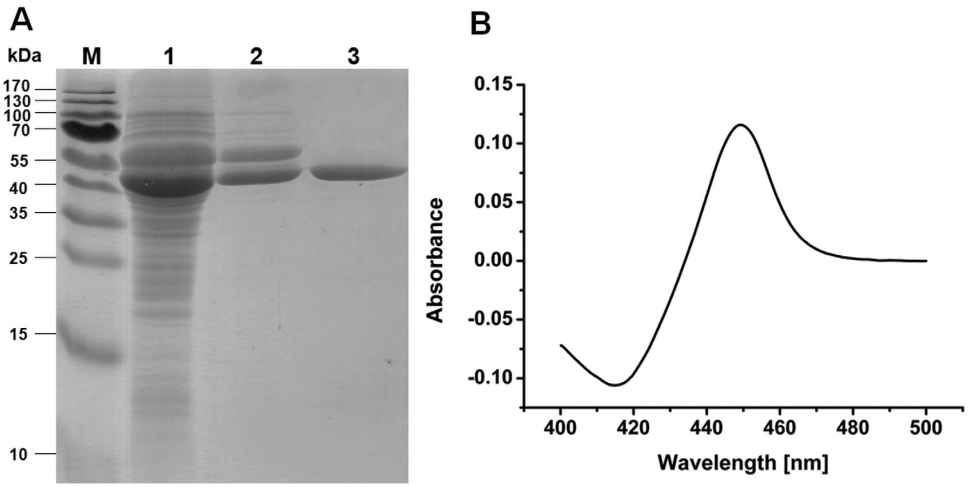

Fig. 1. (A) SDS-polyacrylamid gel electrophoresis. Lane M: protein marker (peqGOLD Protein–Marker IV); lane 1: cell lysate, lane 2: eluted protein after IMAC, lane 3: eluted protein after SP-sepharose chromatography, displaying pure CYP11A1. The cell lysate was mixed with an equal volume of SDS loading buffer and used for lane 1 while the samples of CYP11A1 after the two purification steps (lane 2 and 3) were analyzed by using 1 μg of purified protein in each well. (B) Dithionite reduced CO-difference spectrum of purified CYP11A1 exhibiting a characteristic peak at 450 nm.

pregnenolone are converted to cholestenone and progesterone, respectively. For this, the samples were incubated with the cholesterol oxidase at 37 °C for 40 min and 900 rpm [25]. The oxidase reaction was stopped and the steroids were extracted twice with ethyl acetate after adding either cortisol or DOC as an internal standard. The organic layer was pooled, evaporated and after complete drying the steroid extract was dissolved in 200 μl 20% acetonitrile prior to the RP-HPLC analysis. All conversions were performed for five times, and the product values represented the mean value of five separate conversions.

To quantify the formed pregnenolone by RP-HPLC, a calibration curve was prepared. The comparisons of the conversions with and without the influence of steroid metabolites were statistically analyzed using the *t*-test by comparing the pregnenolone concentration between the control group (performed without any metabolite) and the metabolite group (cholesterol with metabolites). The results were displayed as mean values with standard deviations (SD) and the statistical significance was assessed applying a *p*-value of <0.05.

The effect of fifteen different steroid metabolites which occur during mammalian steroid hormone biosynthesis was tested during the conversion of cholesterol by CYP11A1. These tested steroids were 11-deoxycorticosterone (DOC), testosterone, estradiol, estrone, estriol, aldosterone, cortisol, prednisolone, corticosterone, 11-deoxycortisol, androstenedione, dehydroepiandrosterone (DHEA), adrenosterone, 17α-hydroxy-progesterone and 17α-hydroxy-pregnenolone.

3.6. In vitro conversion of steroid metabolites by CYP11A1

In vitro conversions of 11-deoxycorticosterone (DOC), testosterone, androstenedione and dehydroepiandrosterone (DHEA) with CYP11A1 were performed using the same assay conditions as used for the cholesterol conversion, except that 300 μM of these steroids (stock solution dissolved in absolute ethanol, final ethanol concentration in the incubation 1%) were added to the CYP11A1 reaction mixture and incubated for 30 min. Since the oxidase reaction is not required for DOC, testosterone and androstenedione, the conversion was stopped by addition of an equal volume of ethyl acetate, while the samples containing DHEA were treated similar to the cholesterol conversions.

3.7. Kinetic analysis

The conversion assays were carried out to determine k_{cat} and K_m or $K_{0.5}$ values for the CYP11A1-catalyzed reactions, applying substrate concentration ranging from 0–60 μM for cholesterol in cyclodextrin, 0–800 μM for DOC in DMSO and from 0–1200 μM for androstenedione and DHEA in DMSO (the final DMSO concentrations were 1–1.2%), with fixed concentrations of CYP11A1 (1 μM). All other components and the assay conditions were similar as described above for the *in vitro* conversions. The quantified product represents the mean values of three experiments. The kinetic constants, k_{cat} and K_m or $K_{0.5}$ were determined by plotting the product (n mol product/nmol CYP11A1/min) against the corresponding substrate concentrations (μM). Michaelis–Menten or Hill equation (hyperbolic or sigmoidal fit, respectively) were used applying Origin 8.1G.

3.8. HPLC analysis

The extracted steroids were measured by high performance liquid chromatography (HPLC). Isocratic steroid separation was carried out on a Jasco HPLC system of the LC2000 series using a 4.0 mm × 125 mm Nucleodur C18ec reverse phase column (Macherey-Nagel) with a mobile phase consisting of 20% acetonitrile in water at the flow rate of 1 ml/min. The substrates were detected at 240 nm and the oven-temperature was 40 °C.

3.9. Whole cell biotransformation and purification of novel metabolites

Tricistronic expression vector PP04, based on pBR322 [26], coding for adrenodoxin (Adx), ferredoxin reductase (FpR) and

Table 1
Yields of purified bovine CYP11A1.

CYP11A1 yield (nmol/l)	Reference
40–60	[17]
50	[29]
250	[30]
400	[31]
640	[28]
712	This study

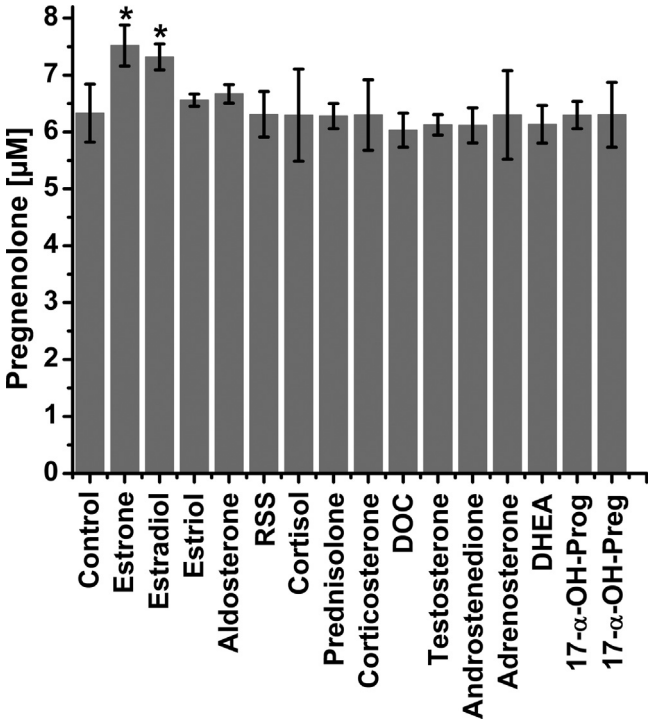

Fig. 2. Pregnenolone concentration (mean ± SD) after CYP11A1-dependent *in vitro* conversions using 15 μM cholesterol as a final substrate concentration in the presence of the same amount of steroid metabolites. * indicates significance ($p < 0.05$).

CYP11A1 were used in the present study for whole cell biotransformations. *E. coli* C43(DE3) cells were co-transformed with PP04 and the chaperone GroEL/GroES-encoding plasmid pGro12, possessing ampicillin and kanamycin resistance genes, respectively. Transformed cells were grown overnight in 50 ml Terrific broth (TB) medium supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin at 37 °C and shaking at 180 rpm.

For the preparative purification of the products, 200 ml TB medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin were inoculated (1:100) with the transformed cells and cultivated at 37 °C with rotary shaking at 180 rpm. After the optical density at 600 nm reached to 0.8–1.0, the culture was induced with 1 mM (IPTG), 1 mM (δ-ALA) and 4 mg/ml arabinose. The temperature and shaking speed were then reduced to 28 °C and 100 rpm, respectively. After incubation for 24 h, cells were harvested by centrifugation (4000 *g*) for 20 min at 4 °C and washed once with 200 ml 50 mM potassium phosphate buffer (KPP) (pH 7.4) in order to prevent side reactions and unspecific conversions of components of the complex medium [27]. After a second centrifugation, the cell pellets were resuspended in 200 ml KPP buffer supplemented with 100 mM glucose. The substrate was added to a final concentration of 300 μM and the culture was incubated for another 48 h at 30 °C and 140 rpm. Steroids were extracted twice with the same volume of ethyl acetate and the organic phase was evaporated using a rotary evaporator. Steroids were separated by reversed phase HPLC on a C18ec column (Nucleodur 250/8) (Macherey-Nagel) with a mobile phase consisting of 20% acetonitrile in water. The fraction containing the product was collected, evaporated and subjected to subsequent NMR spectroscopy analysis.

3.10. NMR analysis

The NMR spectra analysis of the hydroxylated products (6β-hydroxy-DOC, 2β-hydroxy-DOC, 6β-hydroxy-androstenedione, 2β-hydroxy-androstenedione, 6β-hydroxy-testosterone and 16β-hydroxy-DHEA) were recorded in $CDCl_3$ using a Bruker DRX 500 or a Bruker Avance 500 NMR spectrometer at 298 K. The chemical shifts were relative to $CHCl_3$ at δ 7.26 (^{1}H NMR) and $CDCl_3$ at δ 77.00 (^{13}C NMR), respectively, using the standard δ notation in parts per million. The 1D NMR (^{1}H and ^{13}C NMR, DEPT135) and the 2D NMR spectra (gs-HH-COSY, gs-NOESY, gs-HSQCED, and gs-HMBC) were recorded using the BRUKER pulse program library. All assignments were based on extensive NMR spectral evidence.

4. Results

4.1. Expression and purification of CYP11A1

CYP11A1 was successfully expressed in *E. coli* C43(DE3) cells, transformed with the CYP11A1-encoding pTrc99A and pGro12. The recombinant protein was purified from the cell lysate using immobilized-metal affinity chromatography (IMAC) followed by SP-sepharose chromatography. The purity of the eluted fractions was assessed by SDS-PAGE for each purification step (Fig. 1A). As shown in Fig. 1A, after purification by IMAC two bands could be seen in the gel (lane 2), one at approximately 54 kDa corresponding to the relative molecular mass of CYP11A1, and a second band corresponding to the expressed chaperone complex. The subsequent purification using SP-sepharose chromatography yielded a pure CYP11A1 protein, as displayed by the single band in lane 3 (Fig. 1A). The purified CYP11A1 showed the characteristic absorbance peak at 450 nm after CO binding by the dithionite reduced protein representing the stable Fe II(CO)-complex (Fig. 1B).

The yield of the purified protein calculated from the CO-difference spectrum was 712 ± 63 nmol/l *E. coli* cell culture, which represents the highest published value for this protein to date (Table 1) and is also in the range of our recent publication (640 nmol/l) [28]. The improved yield reflects the advantages of *E. coli* C43(DE3) strain as an expression host together with the co-expression of molecular chaperones.

4.2. Influence of steroid metabolites on the pregnenolone yield of CYP11A1-dependent in vitro reactions

To test the influence of steroid metabolites on the CYP11A1-catalyzed conversion of cholesterol, *in vitro* conversion assays were performed. The assay mixture consisted of CYP11A1 and its natural redox partners, which provided the required electrons necessary for the cytochrome P450 monooxygenases activity [1,32]. CYP11A1, wild type bovine adrenodoxin (Adx) and bovine adrenodoxin reductase were used in a molar ratio of 1:20:2. The activity of CYP11A1 with cholesterol as a substrate was successfully assessed using HPLC (Fig. S1, Supplemental data).

In order to test the influence of various intermediates of the steroid hormone biosynthesis on the CYP11A1-catalyzed cholesterol conversion, *in vitro* assays were performed in the presence of different steroid metabolites. Conversion rates were compared to

Table 2
Percentage of product obtained from the *in vitro* conversion of 300 μM steroid by CYP11A1 for 30 min. Each value represents the mean ± S.D of three replicates.

Steroids	Percentage of products (%)	
	Main product	Minor product
DOC	11.0 ± 0.6	3.3 ± 0.4
Androstenedione	18.0 ± 1.7	5.0 ± 0.4
DHEA	5.0 ± 0.9	2.0 ± 0.6
Testosterone	10.0 ± 0.3	–

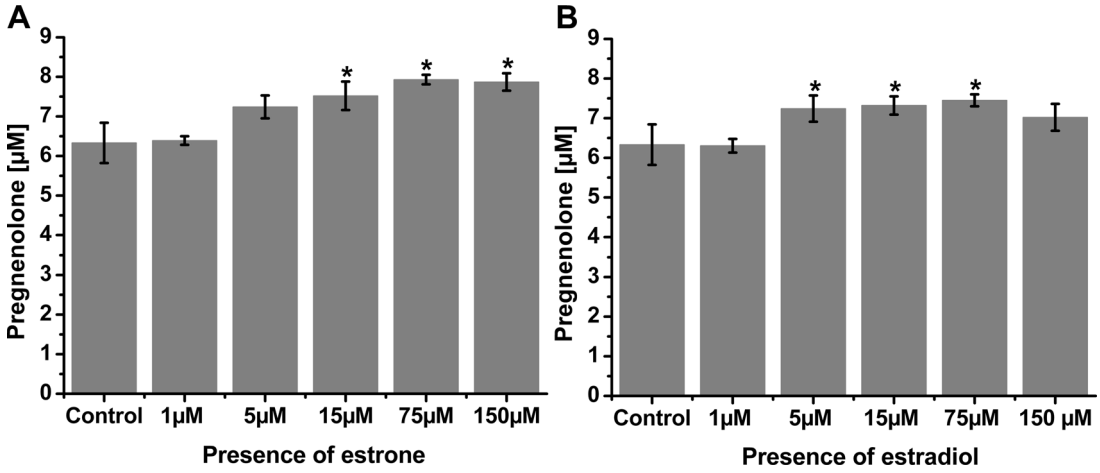

Fig. 3. Pregnenolone concentration (mean ± SD) after CYP11A1-dependent *in vitro* conversion of 15 μM cholesterol in the presence of the different concentrations of steroid metabolites. (A) Estrone, (B) Estradiol. * indicates significance ($p < 0.05$).

control reactions with 15 μM cholesterol as a sole substrate corresponding to the K_m value of cholesterol. The effects of equal amounts (15 μM) of 11-deoxycorticosterone (DOC), testosterone, estradiol, estrone, estriol, aldosterone, cortisol, prednisolone, corticosterone, 11-deoxycortisol, androstenedione, dehydroepiandrosterone (DHEA), adrenosterone, 17α-hydroxy-progesterone and 17α-hydroxy-pregnenolone on the conversion of cholesterol by CYP11A1 *in vitro* are shown in Fig. 2. In the presence of equal concentrations of estrone and estradiol, CYP11A1-mediated cholesterol metabolism was significantly increased ($p < 0.05$) compared with the control reactions. The presence of other steroids did not show any significant increase or decrease of the CYP11A1-dependent pregnenolone formation.

Because of the significant enhancement of the cholesterol conversion with 15 μM estrone and estradiol, we further investigated the influence of these steroids in more detail employing a broader concentration range of these steroids. The HPLC analysis (Fig. 3) showed a concentration-dependent increase of the CYP11A1-catalyzed cholesterol conversion in the presence of estrone and estradiol. Interestingly, amounts as low as 5 μM of estrone and estradiol already increased the CYP11A1-dependent cholesterol conversion, while very high concentrations of estradiol

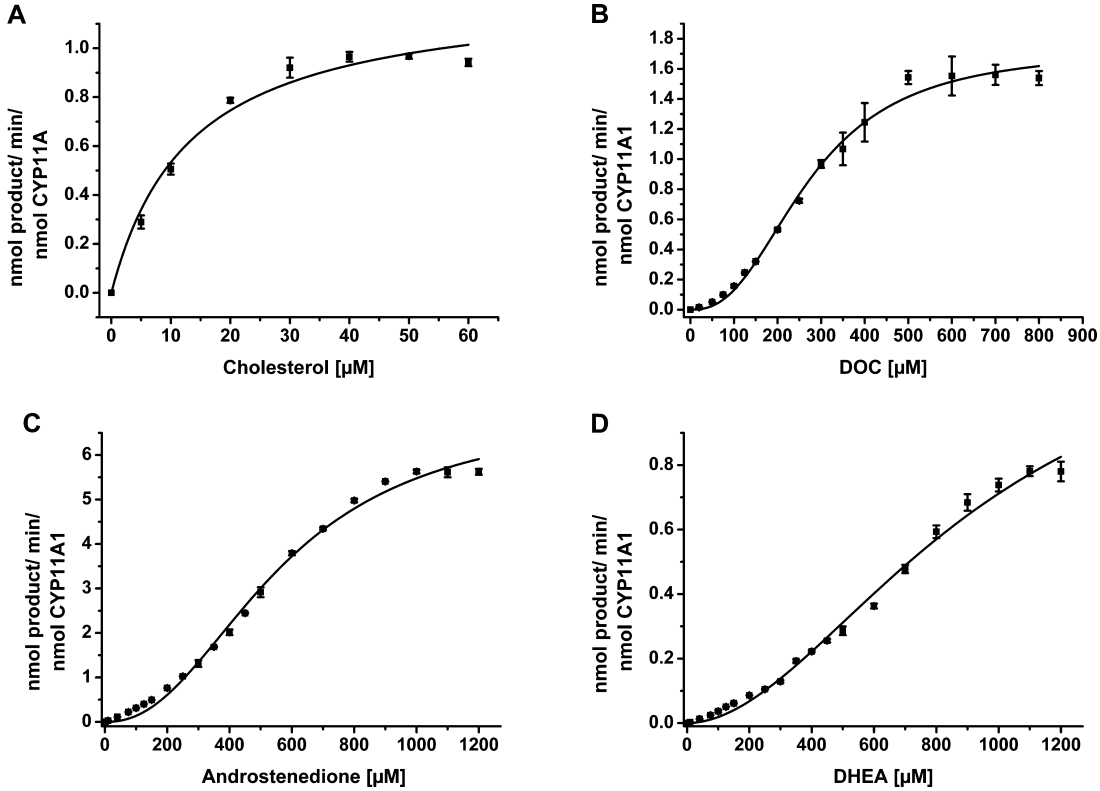

Fig. 4. Kinetic parameters for the conversion of cholesterol and other steroid substrates catalyzed by CYP11A1. (A) Cholesterol, (B) DOC, (C) Androstenedione, (D) DHEA. The product formation was determined by HPLC and each value is represented as mean ± SD of three separate measurements.

(150 μM) diminished the positive effect on pregnenolone formation exhibited by this steroid. Furthermore, 1 μM of both steroids had no significant effect on the product formation compared to control measurements.

4.3. Identification of new CYP11A1 substrates

Previous studies demonstrated that CYP11A1 is able to cleave the side chain of the cholesterol precursor 7-dehydrocholesterol [33], hydroxylate and epoxidate ergosterol [10], hydroxylate vitamin D2 [34,35] and vitamin D3 [31,33] as well as perform the 6β-hydroxylation of DOC and androstenedione [36].

During our studies, in some cases we observed the occurrence of new peaks in the HPLC chromatograms, although the tested steroid metabolites did not significantly affect the CYP11A1-dependent pregnenolone conversion. Therefore, we analyzed whether CYP11A1 is also able to accept these steroids as a substrate during *in vitro* conversions in the absence of cholesterol. Of the applied steroids, a successful *in vitro* conversion was achieved with DOC, androstenedione, testosterone and DHEA. The obtained data in Table 2 shows that the *in vitro* conversion of DOC, androstenedione and DHEA resulted in the formation of one major product (P1) and one minor product (P2), whereas testosterone conversion resulted in the formation of one major product (P2) and several minor products (see Fig. S2, Supplemental data).

4.4. Kinetic analysis

CYP11A1-dependent substrate conversion was examined using cholesterol, DOC, androstenedione or DHEA as substrates. As shown in Fig. 4A, the recombinant enzyme was able to convert cholesterol to pregnenolone with k_{cat} and K_m values of 1.33 ± 0.07 min^{-1} and 15.6 ± 2.4 μM, respectively, and a regression coefficient (R^2) of 0.99.

The kinetic parameters were also calculated for DOC, androstenedione and DHEA conversion utilizing the same reaction conditions as used for cholesterol, except that the steroids were dissolved in DMSO and the conversion time was extended from 7 min to 30 min. Since the conversions of DOC, androstenedione and DHEA gave one major and one minor product each, the catalytic activity was calculated for the total product amount.

The conversion of DOC showed two products with a k_{cat} of 2.0 ± 0.06 min^{-1} and $K_{0.5}$ value of 281 ± 12 μM ($R^2 = 0.99$, $n = 1.9 \pm 0.19$) (Fig. 4B). Likewise, CYP11A1 converted androstenedione into two products with a k_{cat} of 7 ± 0.4 min^{-1} and a $K_{0.5}$ value of 566 ± 33 μM ($R^2 = 0.99$, $n = 2.30 \pm 0.16$) (Fig. 4C). Similarly, with DHEA as a substrate, the k_{cat} and $K_{0.5}$ values were 1.34 ± 0.2 min^{-1} and 944 ± 151 μM ($R^2 = 0.99$, $n = 1.90 \pm 0.15$), respectively (Fig. 4D). Interestingly, cholesterol exhibited a hyperbolic fit, while the curve shapes of the kinetic measurements of CYP11A1 with DOC, androstenedione and DHEA revealed a sigmoidal nature. This is indicative for multiple binding sites. Whether these binding sites are specific as shown for CYP3A4 with testosterone [37,38] and progesterone [39], where two molecules of substrate enter into the active site, or unspecific as postulated for CYP17A1 with sulfonated pregnenolone [40], still has to be elucidated. The kinetic parameters were not determined for testosterone because of the formation of multiple products.

In a next step, we aimed to determine the binding constants (K_d) of the substrates to CYP11A1. The K_d of CYP11A1 for cholesterol was calculated to be 43 ± 3 μM (see Fig. S3, Supplemental data). However, none of the other substrates caused a spectral shift of CYP11A1, so that no K_d values could be calculated for DOC, androstenedione, DHEA and testosterone.

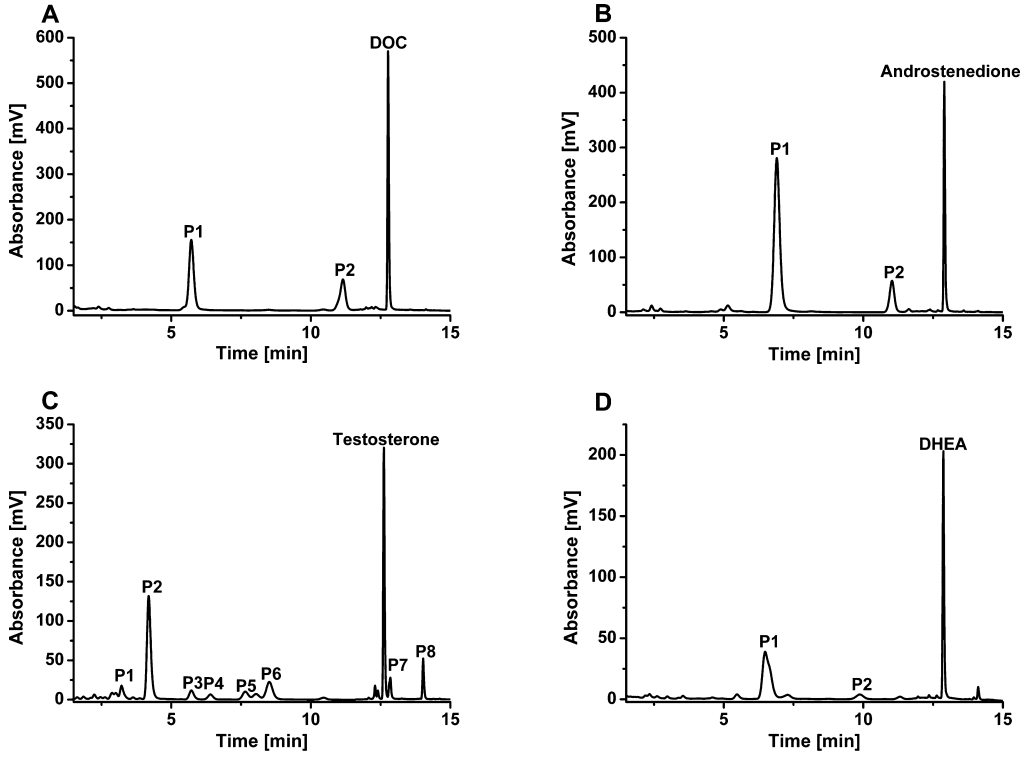

Fig. 5. HPLC chromatograms of the conversion of 300 μM steroid substrates in KPP buffer after 48 h using recombinant *E. coli* whole cell systems expressing CYP11A1. (A) 11-Deoxycorticosterone (DOC), (B) Androstenedione, (C) Testosterone, (D) DHEA.

4.5. Whole-cell biotransformation of new CYP11A1 substrates

Since the *in vitro* conversion of DOC, androstenedione, testosterone and DHEA by CYP11A1 resulted in the formation of novel products, we aimed to characterize their structures using NMR spectroscopy. In order to gain sufficient amounts of these steroids, an *E. coli* based whole-cell biocatalytic system was established. The conversions were performed in potassium phosphate buffer (KPP) to avoid possible side-product formations from components of the TB medium. Therefore, after 24 h of protein expression, the culture medium was shifted from TB to 50 mM KPP buffer (pH 7.4), containing 100 mM glucose. The respective substrates were added to a final concentration of 300 μM and the culture was incubated for another 48 h at 30 °C and 140 rpm. The samples of steroid bioconversions were then collected and extracted for HPLC analysis (Fig. 5). In addition, untransformed *E. coli* cells were used as a negative control for the steroid conversions (Fig. S4, Supplemental data).

As displayed in Fig. 5(A–D), the *in vivo* product patterns measured after the biotransformation are identical to those shown in the chromatograms obtained after the *in vitro* incubations (Fig. S2, Supplemental data). The *in vivo* conversion of DOC resulted in the formation 36 ± 1.6% of P1 product and 17 ± 1% P2 product. CYP11A1 forms two products with the yield of 60 ± 3% (P1) and 21 ± 2% (P2) from androstenedione. Furthermore, CYP11A1 was also able to convert DHEA into two products; the major product (P1) yield reached 20 ± 0.5% conversion. Moreover, several products were formed by incubation of testosterone and the bioconversion assay of testosterone produced 36 ± 2% P2 of testosterone as a major product.

4.6. NMR analysis

The structural identification of the obtained products was done by NMR spectroscopy as described in the method section. All products were purified in sufficient amounts, except for the minor products of testosterone and product 2 (P2) of DHEA. The amounts of the purified hydroxylated products were between 1 and 5 mg. The structural assignments of the products as obtained from the NMR data are shown in Fig. 6. The conversion of DOC and androstenedione resulted in the formation of two hydroxylated products; in both cases the 2β-hydroxy and 6β-hydroxy-derivatives were formed. The conversion of DHEA led to the production of 16β-hydroxy-DHEA as the main product, while testosterone conversion resulted in the formation of 6β-hydroxy-testosterone as a major product (P2).

The proton shifts of ring A and B in the 2β-hydroxylated steroids (DOC and androstenedione) were very close to each other

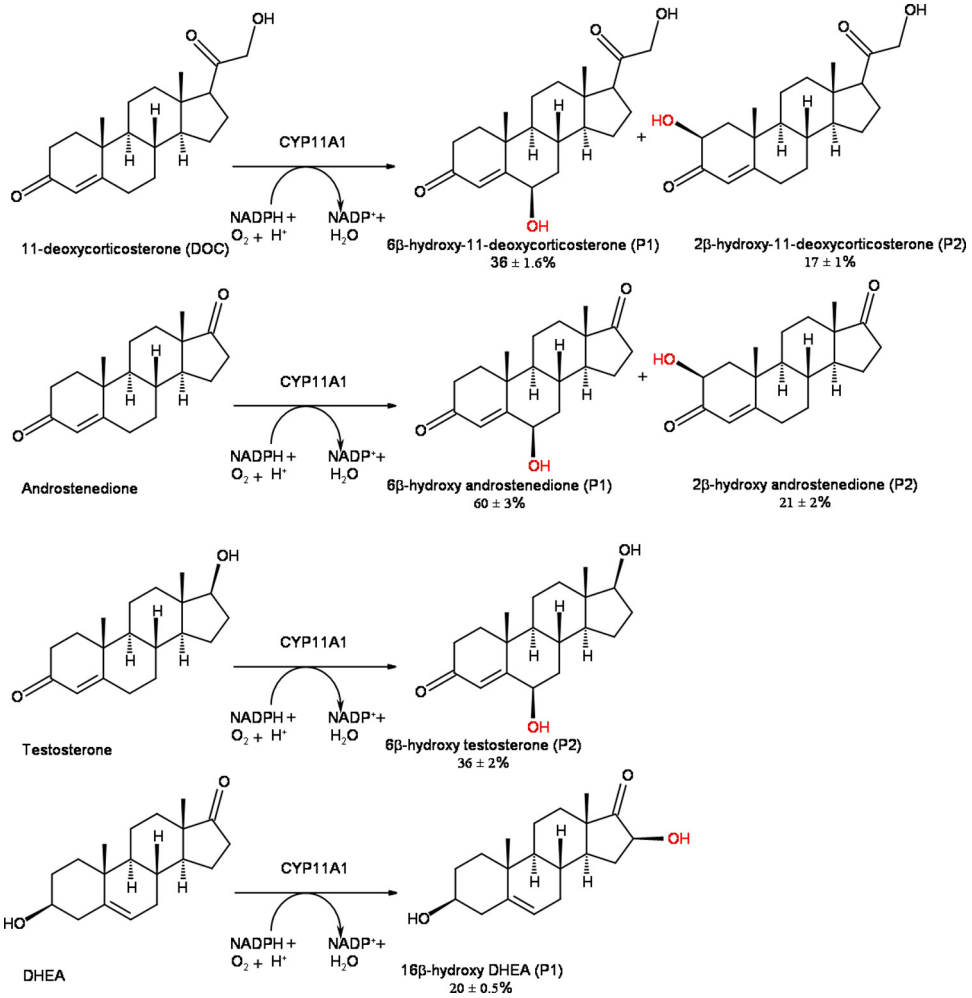

Fig. 6. The structures and product yields (in percent) of steroids converted by CYP11A1 according to the NMR data after 48 h conversion. Reactions contained 300 μM steroid and *E. coli* cells expressing CYP11A1 and its redox partners in KPP buffer. The obtained values are represented as mean ± SD of three separate experiments.

and to those of the known 2β-hydroxy-progesterone [41]. The same relation was found between the 6β-hydroxylated DOC and androstenedione and the known 6β-hydroxy-progesterone [41]. Matsuzaki et al. [42] reported selected ^{1}H NMR shifts for 6β-hydroxy-DOC, which are in good agreement to those of 6β-hydroxylated DOC as described. Jacobsen et al. [43] published the complete assignment of 2β-hydroxy-androstenedione measured in CD_3OD. Their data match perfectly with our additionally recorded data of 2β-hydroxy-androstenedione in methanol-d^4. ^{1}H NMR shifts of 6β-hydroxy-androstendione and 6β-hydroxy-testosterone could be found among others in the survey of high-field proton NMR spectra of steroid hormones by Kirk et al. [41]. They support our structures of 6β-hydroxy-androstendione and 6β-hydroxy-testosterone. Our data of 16β-hydroxy-DHEA showed a fair correlation with those of 16β-hydroxy-DHEA published in a mixture of C_6D_6/CD_3OD [44] and was refined by a re-measurement in the solvent mixture. The detailed NMR data for the formed products are shown in the Supplemental section.

5. Discussion

In this study, we investigated the influence of different steroid metabolites on the *in vitro* conversion of cholesterol to pregnenolone by CYP11A1. The functionality of the recombinant enzyme was analyzed by performing CYP11A1-dependent substrate conversions using cholesterol as a substrate (Fig. S1, Supplemental data). Because of the low solubility of cholesterol in aqueous solutions, the steroid was solubilized in 45% 2-hydroxypropyl-β-cyclodextrin prior to the addition into the conversion buffer. Cyclodextrins are commonly applied for the characterization of reaction kinetics with hydrophobic substrates [45]. Moreover, cyclodextrins are the solutes of choice for cholesterol and are mainly utilized for the conversion of cholesterol by CYP11A1 [46,47]. They, however, exhibit certain disadvantages: Tuckey et al. demonstrated the impact of cyclodextrins on the K_m value of CYP11A1 and cholesterol and found it to be dose-dependent [48]. Although this effect should be taken into consideration, in our study the final concentration of cyclodextrins was only 0.337%. Therefore, the CYP11A1 activity should not be considerably altered in the presence of the utilized cyclodextrin concentration.

In order to investigate whether steroid intermediates have an influence on the CYP11A1-dependent pregnenolone formation, *in vitro* conversion experiments were carried out using 15 μM cholesterol, which corresponds to the K_m-value of CYP11A1 and cholesterol [28]. At this substrate concentration the enzyme exhibits its half-maximal velocity, allowing the sensitive monitoring of activations or inhibitions.

Among all 15 tested steroid metabolites, only estrone and estradiol significantly affected the pregnenolone formation (Fig. 2), excluding an artificial effect from the displacement of cholesterol from the cyclodextrin molecules by these steroids, which would possibly affect the substrate conversion rate.

Determination of pregnenolone formation catalyzed by CYP11A1 in the presence of different concentrations of either estrone or estradiol revealed that the latter one is the more potent activator, exhibiting a stimulation of the CYP11A1 activity at a concentration as low as 5 μM (Fig. 3). This enhanced activity might be due to allosteric binding of these steroids to CYP11A1 resulting in conformational changes that lead to a more rapid sequential hydroxylation of the side-chain of cholesterol, an increase of the enzyme's affinity for the substrate or the redox proteins. However, the effect of estrone and estradiol on the CYP11A1 activity still needs to be elucidated at a molecular level in further investigations. We also observed that the presence of very high concentrations of estrone and estradiol (150 μM), resulted in less formation of pregnenolone by CYP11A1, suggesting that the structural changes of the enzyme become unfavorable for the substrate conversion or that higher concentrations of estrogens inhibit binding of cholesterol, the natural substrate.

It remains difficult to postulate a physiological relevance of the described activation of CYP11A1, as the concentrations of estrogens used were not in a physiological range. Moreover, concentrations and a possible enrichment of estrogens within cells are unknown, making it impossible to mimic physiological conditions in our reconstituted *in vitro* system.

Nevertheless, the impact of estrogens for the stimulation of the CYP11A1 activity is in agreement with a previous study analyzing the estradiol formation in human placental syncytiotrophoblasts, where a positive influence of estradiol on the pregnenolone formation has been shown [49]. In addition, an increased CYP11A1 activity and mRNA formation by estrogens in pregnant baboons was found, suggesting that pregnenolone formation is regulated by estrogens in the primate placenta [16]. In our study, we observed an increased pregnenolone formation in the presence of estrogens by ~20%. It is well known that the stimulation of pregnenolone synthesis is also induced by the expression of the steroidogenic acute regulator protein (StAR), which facilitates the transport of cholesterol to the inner mitochondrial membrane, representing the non-enzymatic rate-limiting step of the steroidogenesis. It has been shown that stimulation of the StAR expression in rat astrocyte cultures through dbcAMP caused an increase in the level of pregnenolone synthesis by ~50% [50], which is of the same order of magnitude as the estrogen stimulation observed in this study. Moreover, in the *preovulatory surge phase* of the menstrual cycle, concentrations of estrogens and luteinizing hormone (LH) are highly elevated. LH is involved in the hypothalamic–pituitary–gonadal (HPG)-axis, which regulates the production of androgens and estrogens. In the gonads, LH stimulates the expression of the steroidogenic acute regulator protein (StAR) and thus the delivery of cholesterol to the inner mitochondrial membrane, where CYP11A1 is located. In this context, the enhanced activity of CYP11A1 through estrogens might be a mechanism to save cell resources, as less additional CYP11A1 is required to metabolize the increased cholesterol concentrations.

Interestingly, during our *in vitro* assays, we observed a slight decrease in the level of pregnenolone formation in the presence of DOC, testosterone, androstenedione and DHEA, accompanied by the formation of additional products in the HPLC measurement; however they are not statistically significant. Therefore, we carried out *in vitro* conversions with these four potential substrates and, indeed, all of them were converted by CYP11A1, resulting into two products from DOC, androstenedione and DHEA, and multiple products from testosterone (Fig. S2, Supplemental data; see Table 2 for product ratios).

Since these new CYP11A1 substrates did not induce a type I spectral shift as opposed to cholesterol, these steroids might not bind in close proximity of the heme or do not displace the H_2O molecule from the sixth position of the heme iron. These findings are in agreement with the results of Simgen et al. [51], in which CYP106A2 from *Bacillus megaterium* showed the conversion of deoxycorticosterone without any spin shift of the heme iron. Likewise, Girhard et al. [52] also demonstrated the absence of spin-state shifts when testosterone or (+)-valencene were used for binding experiments with CYP109B1 from *Bacillus subtilis*, although these substances are converted by this enzyme.

Comparing the affinity of CYP11A1 towards cholesterol with the affinities for DOC, androstenedione and DHEA, the latter ones were remarkably lower as observed in the kinetic measurements. However, since the local concentration of the steroids in mitochondria under physiological and pathological conditions are not known and physiological effects of hydroxysteroids and hydroxycholesterol have been described on a picomolar to

nanomolar level [53], the obtained novel products might have an effect on physiological parameters.

In order to reveal the structures of the hydroxylated products of DOC, androstenedione, testosterone and DHEA, an *E. coli* based whole-cell system was established to produce sufficient amounts for NMR-analysis. Adequate yields of products 1 and 2 of DOC and androstenedione as well as of product 1 of DHEA and product 2 of testosterone could be obtained. In addition to the previously described 6β-hydroxylase activity of CYP11A1 towards DOC and androstenedione using the recombinant CYP11A1 enzyme [36,54], we identified a 2β-hydroxylation of both steroids. This suggests that these substrates enter the active site in a reverse orientation compared to cholesterol, suggesting that the previously described complexation of cholesterol at the entrance of the active site via hydrogen bonds is not necessary for these substrates [55]. Furthermore, testosterone and DHEA turned out to be new substrates for CYP11A1 producing 6β-hydroxy-testosterone and interestingly, 16β-hydroxy-DHEA, indicating that the presence of the hydroxy group at C3 influences the orientation of the steroid molecule in the active site of the enzyme.

In conclusion, the results from our *in vitro* experiments suggest that CYP11A1 might play a more vital role in the metabolism of steroids than previously assumed. For instance, the 6β-hydroxylation of testosterone has been ascribed mainly to CYP3A4 before [56–59]. In addition, 16β-hydroxy-DHEA serves as a substrate for human type I 3βHSD [60]. The resulting product, 16β-hydroxy-androstenedione might be a potential inhibitor of 17β-HSD. Furthermore, 16β-hydroxy-DHEA has been found to be a component of plasma and urine of adult humans. Plasma 16β-hydroxy-DHEA levels in normal subjects rise sharply during adolescence and then decline slowly throughout adult life. It is inferred that 16β-hydroxy-DHEA is secreted directly by the adrenal cortex and probably the gonads [61]. The functions of the other two new products remain to be elucidated, although 4β-hydroxy-androstenedione, with a structure closely similar to 2β-hydroxy-androstenedione, has been described as a suicide inhibitor of the aromatase CYP19A1 [62]. CYP19A1 is a target in anti-cancer therapy, since a correlation between estrogens and breast cancer is known and the development of specific inhibitors of CYP19A1 is of great importance. For this reason, 2β-hydroxy-androstenedione formation might be of particular interest and its potential ability to act as inhibitor of CYP19A1 should be investigated, since reasonable amounts of 2β-hydroxy-androstenedione can easily be produced using our whole-cell system.

In summary, we hereby report that the sex hormones estrone and estradiol directly stimulate the side-chain cleavage activity of CYP11A1 at a molecular level. In addition, it was shown that DOC, androstenedione, testosterone and DHEA are substrates of CYP11A1. After establishing an efficient whole-cell system for CYP11A1-dependent substrate conversion, we were able to identify novel 2β-hydroxylation activity towards DOC and androstenedione and 16β-hydroxylation of DHEA as well as 6β-hydroxylation of testosterone. Furthermore, we have demonstrated, for the first time, the ability of a CYP11A1 whole-cell biotransformation system to catalyze the hydroxylation reaction of these steroids. Our results imply that the role of CYP11A1 in steroid hormone biosynthesis might be broader than previously known, and that the new hydroxylated products of CYP11A1 might be useful to generate pharmaceutically interesting compounds.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2015.02.014.

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SUPPLEMENTAL DATA

2β- and 16β-hydroxylase activity of CYP11A1 and direct stimulatory effect of estrogens on pregnenolone formation

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NMR DATA

The NMR spectra analysis of the hydroxylated products (6β-hydroxy DOC, 2β-hydroxy DOC, 6β-hydroxy androstenedione, 2β-hydroxy androstenedione, 6β-hydroxy testosterone and 16β-hydroxy DHEA) were recorded in $CDCl_3$ with a Bruker DRX 500 or a Bruker Avance 500 NMR spectrometer at 298 K. The chemical shifts were relative to $CHCl_3$ at δ 7.26 (^{1}H NMR) and $CDCl_3$ at δ 77.00 (^{13}C NMR), respectively, using the standard δ notation in parts per million. The 1D NMR (^{1}H and ^{13}C NMR, DEPT135) and the 2D NMR spectra (gs-HH-COSY, gs-NOESY, gs-HSQCED, and gs-HMBC) were recorded using the BRUKER pulse program library. All assignments based on extensive NMR spectral evidence. The obtained NMR data for the formed products are as follows:

2β-hydroxy-11-deoxycorticosterone: ^{1}H NMR ($CDCl_3$, 500 MHz): *δ* 0.70 *s* (3xH-18), 1.07 *m* (H-7a), 1.18 *s* (3xH-19), 1.19 *m* (H-14), 1.33 *m* (H-15a), 1.39 *m* (H-12a), 1.44 *m* (H-9), 1.54 *m* (H-11a), 1.55 *m* (H-1a), 1.67 *m* (H-8), 1.73 *m* (H-15b), 1.77 *m* (H-16a), 1.80 *m* (H-11b), 1.97 *m* (H-12b), 1.99 *m* (H-7b), 2.22 *m* (H-16b), 2.26 *m* (H-6a), 2.46 *m* (H-1b), 2.47 *m* (H-17), 2.54 *m* (H-6b), 4.15 *dd* (8.5 and 5.5 Hz, H-2), 4.16 *d* (19.0 Hz, H-21a), 4.22 *d* (19.0 Hz, H-21b), 5.82 *d* (1.0 Hz, H-4). ^{13}C NMR ($CDCl_3$, 125 MHz): *δ* 13.66 (*CH_3*, C-18), 22.79 (2C, *CH_2* and *CH_3*, C-11 and C-19), 23.03 (*CH_2*, C-16), 24.45 (*CH_2*, C-15), 32.94 (*CH_2*, C-6), 34.85 (*CH_2*, C-7), 35.76 (*CH*, C-8), 38.33 (*CH_2*, C-12), 39.40 (*CH_2*, C-1), 41.32 (*C*, C-10), 45.11 (*C*, C-13), 48.89 (*CH*, C-9), 56.06 (*CH*, C-14), 58.94 (*CH*, C-17), 68.52 (*CH*, C-2), 69.38 (*CH_2*, C-21), 118.87 (*CH*, C-4), 174.51 (*C*, C-5), 199.57 (*C*, C-3), 210.03 (*C*, C-20).

6β-hydroxy-11-deoxycorticosterone: ^{1}H NMR ($CDCl_3$, 500 MHz): *δ* 0.72 *s* (3xH-18), 0.96 *ddd* (12.2, 10.5 and 4.2 Hz, H-9), 1.18 *ddd* (12.0, 11.0 and 7.0 Hz, H-14), 1.28 *m* (H-7a), 1.38 *s* (3xH-19), 1.39 *m* (H-12a), 1.40 *m* (H-15a), 1.51 *dddd* (3x13.0 and 4.0 Hz, H-11a), 1.62 *m* (H-11b), 1.72 *ddd* (2x14.5 and 4.5 Hz, H-1a), 1.78 *m* (H-15b), 1.79 *m* (H-16a), 1.97 *ddd* (13.0, 4.0 and 3.0 Hz, H-12b), 2.02 *m* (H-8), 2.03 *m* (H-7b), 2.05 *m* (H-1b), 2.25 *m* (H-16b), 2.40 *dddd* (17.0, 4.5, 2.7 and 1.0 Hz, H-2a), 2.47 *dd* (2x9.0 Hz, H-17), 2.51 *ddd* (17.0, 15.0 and 5.0 Hz, H-2b), 4.17 *d* (19.0 Hz, H-21a), 4.22 *d* (19.0 Hz, H-21b), 4.36 *dd* (2x3.0 Hz, H-6), 5.83 *d* (1.0

Hz, H-4). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 13.49 (*CH_3*, C-18), 19.52 (*CH_3*, C-19), 20.86 (*CH_2*, C-11), 22.91 (*CH_2*, C-16), 24.42 (*CH_2*, C-15), 29.37 (*CH*, C-8), 34.18 (*CH_2*, C-2), 37.10 (*CH_2*, C-1), 37.94 (*C*, C-10), 38.24 (2C, *2xCH_2*, C-7 and C-12), 44.75 (*C*, C-13), 53.38 (*CH*, C-9), 56.06 (*CH*, C-14), 59.09 (*CH*, C-17), 69.37 (*CH_2*, C-21), 72.99 (*CH*, C-6), 126.49 (*CH*, C-4), 167.68 (*C*, C-5), 200.13 (*C*, C-3), 210.07 (*C*, C-20).

2β-hydroxyandrost-4-ene-3,17-dione: ^{1}H NMR ($CDCl_3$, 500 MHz) : δ 0.92 *s* (3xH-18), 1.13 *dddd* (3x13.0 and 4.5 Hz, H-7a), 1.21 *s* (3xH-19), 1.33 *m* (H-14), 1.34 *m* (H-12a), 1.47 *ddd* (12.5, 10.0 and 3.5 Hz, H-9), 1.55 *m* (H-11a), 1.57 *m* (H-1a), 1.58 *m* (H-15a), 1.85 *m* (H-8), 1.89 *m* (2H, H-11b and H-12b), 1.94 *m* (H-15b), 2.11 *m* (2H, H-7b and H-16a), 2.32 *m* (H-6a), 2.48 *m* (2H, H-1b and H-16b), 2.57 *dddd* (2x13.0, 5.0 and 1.5 Hz, H-6b), 4.18 *dd* (8.5 and 5.5 Hz, H-2), 5.84 *d* (1.0 Hz, H-4). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 13.90 (*CH_3*, C-18), 21.76 (*CH_2*, C-15), 22.14 (*CH_2*, C-11), 22.76 (*CH_3*, C-19), 31.23 (*CH_2*, C-12) 32.72 (*CH_2*, C-6), 33.66 (*CH_2*, C-7), 35.37 (*CH*, C-8), 35.73 (*CH_2*, C-16), 39.40 (*CH_2*, C-1), 41.36 (*C*, C-10), 47.96 (*C*, C-13), 50.14 (*CH*, C-9), 50.79 (*CH*, C-14), 68.46 (*CH*, C-2), 119.02 (*CH*, C-4), 174.09 (*C*, C-5), 199.53 (*C*, C-3), 219.87 (*C*, C-17).

6β-hydroxyandrost-4-ene-3,17-dione: ^{1}H NMR ($CDCl_3$, 500 MHz) : δ 0.95 *s* (3xH-18), 0.98 *m* (H-9), 1.30 *m* (H-12a), 1.31 *m* (H-14), 1.33 *m* (H-7a), 1.41 *s* (3xH-19), 1.53 *m* (H-11a), 1.62 *m* (H-15a), 1.70 *m* (H-11b), 1.73 *m* (H-1a), 1.89 *ddd* (13.0, 4.0 and 2.8 Hz, H-12b), 2.00 *m* (H-15b), 2.06 *m* (H-1b), 2.10 *m* (H-16a), 2.15 *m* (H-7b), 2.19 *ddd* (2x11.3 and 3.0 Hz, H-8), 2.41 *m* (H-2a), 2.50 *m* (H-16b), 2.51 *ddd* (17.0, 15.0 and 5.0 Hz, H-2b), 4.41 *dd* (2x3.0 Hz, H-6), 5.84 *d* (1.0 Hz, H-4). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 13.76 (*CH_3*, C-18), 19.55 (*CH_3*, C-19), 20.25 (*CH_2*, C-11), 21.69 (*CH_2*, C-15), 29.39 (*CH*, C-8), 31.25 (*CH_2*, C-12), 34.16 (*CH_2*, C-2), 35.74 (*CH_2*, C-16), 37.07 (*CH_2*, C-1), 37.19 (*CH_2*, C-7), 38.10 (*C*, C-10), 47.61 (*C*, C-13), 50.89 (*CH*, C-14), 53.63 (*CH*, C-9), 72.82 (*CH*, C-6), 126.55 (*CH*, C-4), 167.61 (*C*, C-5), 200.11 (*C*, C-3), 220.44 (*C*, C-17).

3β,16β-dihydroxyandrost-5-ene-17-one: ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 0.89 *s* (3H, 3xH-18), 1.03 *m* (H-9), 1.04 *s* (3H, 3x-H-19), 1.10 *m* (H-1a), 1.26 *m* (H-12a), 1.27 m (H-14), 1.52 *m* (H-11a), 1.53 *m* (H-2a), 1.54 *m* (H-15a), 1.65 *m* (H-7a), 1.68 *m* (H-8), 1.69 *m* (H-11b), 1.85 *m* (H-12b), 1.86 *m* (H-1b), 1.87 *m* (H-2b), 2.13 *m*

(H-7b), 2.25 *m* (H-4a), 2.33 *m* (H-4b), 2.38 *m* (H-15b), 3.54 *m* (H-3), 3.96 *dd* (2x8.7 Hz, H-16), 5.39 *d* (5.3 Hz, H-6). ^{13}C-NMR (CDCl3, 125 MHz): δ 13.54 (*CH_3*, C-18), 19.41 (*CH_3*, C-19), 20.37 (*CH_2*, C-11), 30.79 (*CH_2*, C-15), 30.97 (*CH_2*, C-7), 31.45 (*CH_2*, C-12), 31.54 (*CH*, C-8), 31.58 (*CH_2*, C-2), 36.65 (*C*, C-10), 37.11 (*CH_2*, C-1), 42.18 (*CH_2*, C-4), 46.01 (*CH*, C-14), 47.54 (*C*, C-13), 50.47 (*CH*, C-9), 71.58 (*CH*, C-3), 75.34 (*CH*, C-16), 120.94 (*CH*, C-6), 141.01 (*C*, C-5), 221.14 (*C*, C-17).

6β-hydroxytestosterone: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.83 *s* (3xH-18), 0.91 *ddd* (12.0, 11.0 and 4.5 Hz, H-9), 0.98 *ddd* (12.0, 11.0 and 7.0 Hz, H-14), 1.10 *m* (H-12a), 1.21 *m* (H-7a), 1.36 *m* (H-15a), 1.39 *s* (3xH-19), 1.47 *m* (H-16a), 1.49 *m* (H-11a), 1.60 *m* (2H; H-11b and 15b), 1.70 *ddd* (2x14.3 and 4.5 Hz, H-1a), 1.88 *ddd* (13.0, 4.0 and 3.0 Hz, H-12b), 2.01 *m* (H-7b), 2.02 *m* (H-8), 2.05 *m* (H-1b), 2.09 *m* (H-16b), 2.40 *dddd* (17.0, 4.5, 2.7 and 1.0 Hz, H-2a), 2.53 *ddd* (17.0, 15.0 and 5.0 Hz, H-2b), 3.66 *dd* (2x8.5 Hz, H-17), 4.36 *dd* (2x3.0 Hz, H-6), 5.82 *d* (1.0 Hz, H-4). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 11.09 (*CH_3*, C-18), 19.56 (*CH_3*, C-19), 20.61 (*CH_2*, C-11), 23.30 (*CH_2*, C-15), 29.79 (*CH*, C-8), 30.31 (*CH_2*, C-16), 34.24 (*CH_2*, C-2), 36.42 (*CH_2*, C-12), 37.16 (*CH_2*, C-1), 38.04 (*CH_2*, C-7), 38.05 (*C*, C-10), 42.92 (*C*, C-13), 50.49 (*CH*, C-14), 53.74 (*CH*, C-9), 73.11 (*CH*, C-6), 81.70 (*CH*, C-17), 126.45 (*CH*, C-4), 168.05 (*C*, C-5), 200.23 (*C*, C-3).

Figure S1. *In vitro* conversion assay for the conversion of cholesterol by purified CYP11A1, using cortisol (F) as an internal standard (for the detection of cholesterol and pregnenolone at 240 nm, cholesterol and pregnenolone were converted to cholestenone and pregnenolone via an oxidase reaction using cholesterol-oxidase).

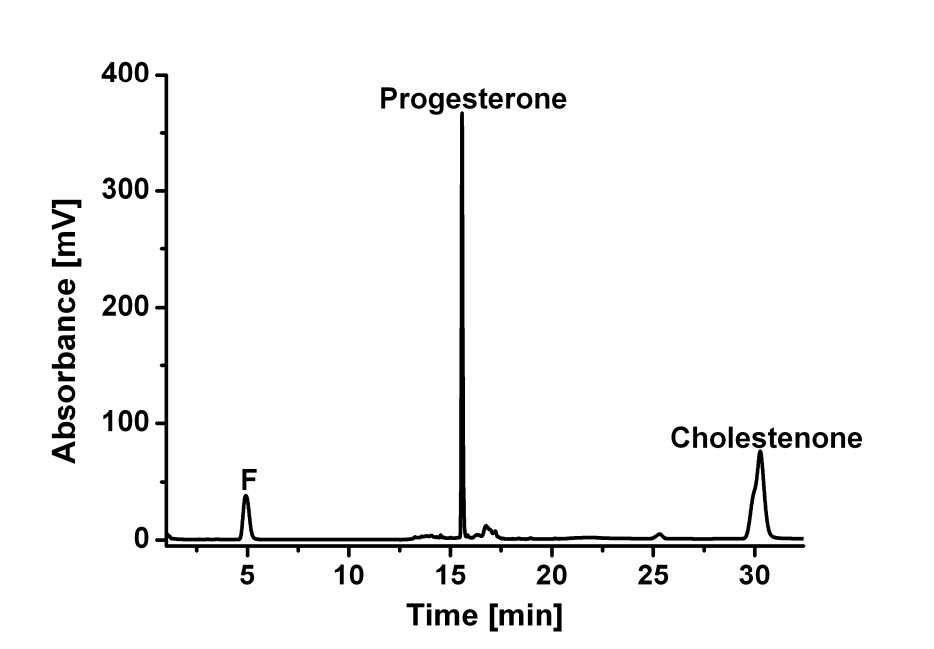


Figure S2. HPLC chromatograms of the conversion of 300 µM steroid substrates by CYP11A1 *in vitro* conversion after 30 min. (A) 11-Deoxycorticosterone (DOC), (B) Androstenedione, (C) Testosterone, (D) DHEA.

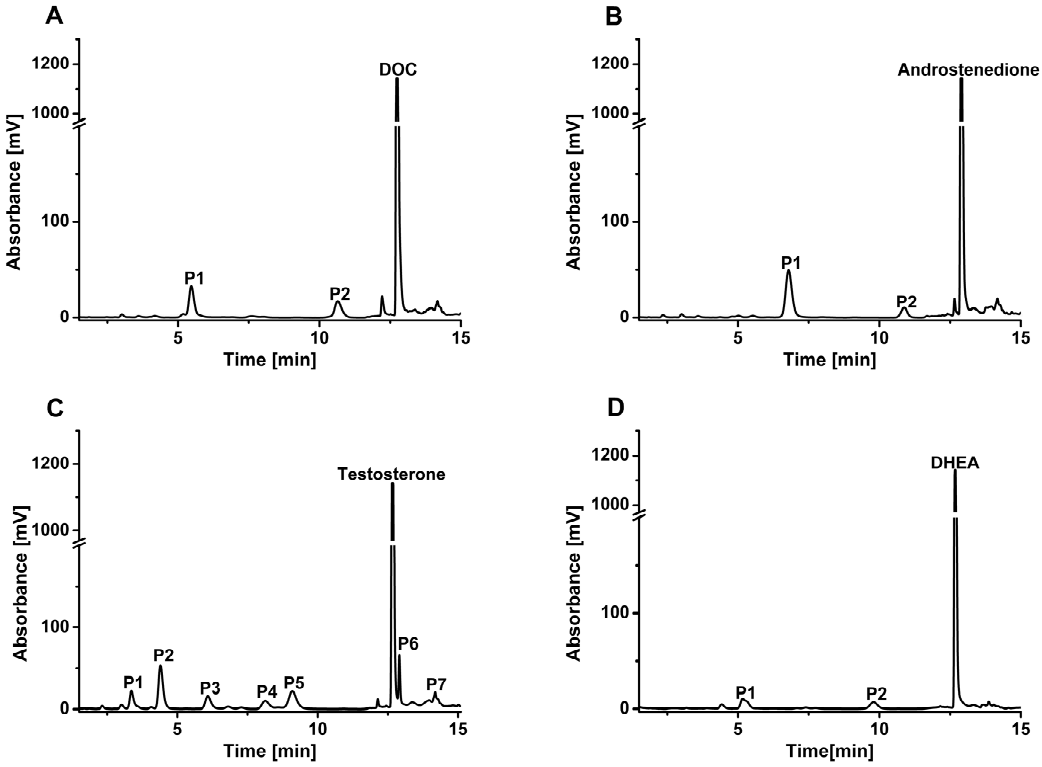


Figure S3. Determination of the spectral shift for the titration of different concentrations of cholesterol (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 26, 30, 34, 38, 42, 52, 62, 82, 102, 122, 142, 162, 182 µM) in 2-hydroxypropyl-β-cyclodextrin with 2 µM CYP11A1. The reaction was carried out using potassium phosphate buffer containing 20% glycerol, 1% sodium cholate and 0.05% Tween-20. The inset shows the spectral shift induced by cholesterol binding to CYP11A1.

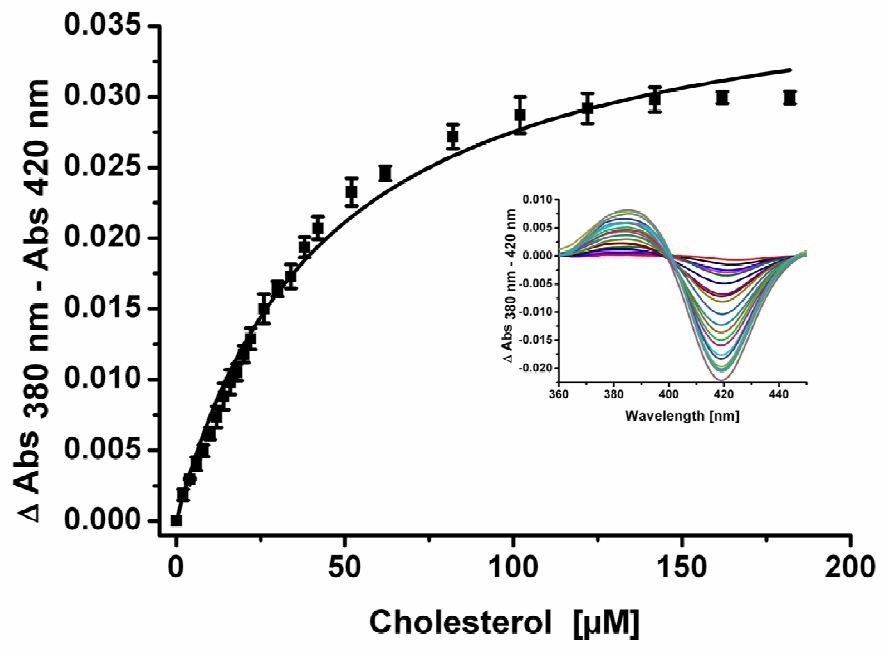
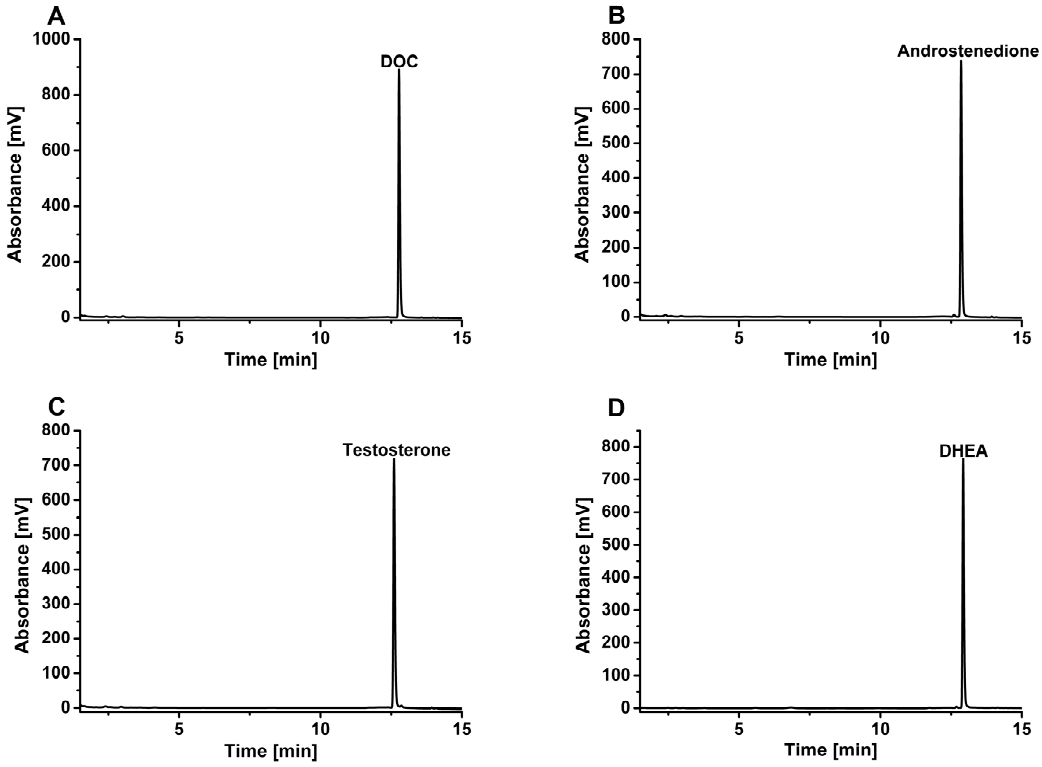


Figure S4. HPLC chromatograms for the negative control using *E. coli* C43(DE3) cells without plasmid in KPP buffer using 300 µM steroid substrates.(A) 11-Deoxycorticosterone (DOC), (B) Androstenedione, (C) Testosterone, (D) DHEA.



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REGIOSELECTIVE ACETYLATION OF C21 HYDROXYSTEROIDS BY THE BACTERIAL CHLORAMPHENICOL ACETYLTRANSFERASE I

Azzam Mosa, Michael C. Hutter, Josef Zapp, Rita Bernhardt and Frank Hannemann

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Regioselective Acetylation of C21 Hydroxysteroids by the bacterial Chloramphenicol Acetyltransferase I

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The chloramphenicol acetyltransferase I (CATI) detoxifies the antibiotic chloramphenicol and confers a corresponding resistance to bacteria. In this study we identified this enzyme as a steroid acetyltransferase and designed a novel and efficient *E. coli* based biocatalyst for the regioselective acetylation of C21 hydroxyl groups in steroids of pharmaceutical interest. The cells carried a recombinant *catI* gene controlled by a constitutive promoter. The capacity of the whole-cell system to modify different hydroxysteroids was investigated and NMR spectroscopy revealed that all substrates were selectively transformed into the respective 21-acetoxy derivatives. The biotransformation was optimized and the reaction mechanism is discussed on the basis of a computational substrate docking into the crystal structure of CATI.

Introduction

Chloramphenicol (CAM), a broad-spectrum antibiotic, was first isolated in 1947 from the soil bacterium *Streptomyces venezuelae*.[1] In general, CAM inhibits protein biosynthesis by binding to the 50S subunit of the bacterial ribosome. Recently solved crystal structures of the *Escherichia coli* ribosome's 50S subunit in complex with CAM revealed that CAM directly binds to the A-site of the 50S subunit and occupies the binding site for the aminoacyl moiety of the A-site tRNA.[2]

Resistance to CAM was recognized in some bacteria, being provided by a chloramphenicol acetyltransferase [EC 2.3.1.28], which covalently modifies CAM by transferring the acetyl group of acetyl-CoA to one of the hydroxyl groups of CAM, thus preventing the antibiotic from binding to the ribosome.[3] The mechanism of CAM acetylation (Scheme 1A) involves the abstraction of a proton from the hydroxyl group in C-3 position of the CAM molecule, whereby the imidazole side chain of the catalytic histidine residue in CATI acts as base. Loss of the proton from the hydroxyl group facilitates the subsequent nucleophilic attack of the anion at the carbonyl group of the acetyl-CoA thioester and the transfer of the acetyl group. [4]

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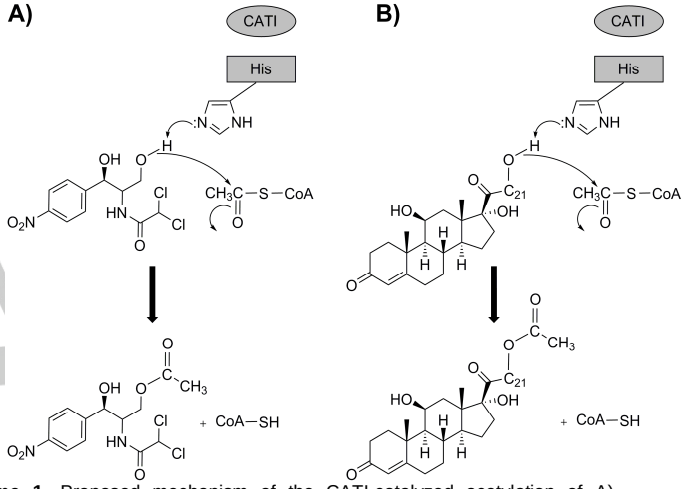


Scheme 1. Proposed mechanism of the CATI-catalyzed acetylation of A) chloramphenicol according to Day and Shaw [4a] , B) steroids, showing cortisol as a model substrate.

Various CAT enzymes have been isolated from bacterial strains, and in all cases the enzyme was identified as a homo-trimer, each subunit in the molecular weight range between 24 to 26 kD.[3a, 4a] CAT enzymes are divided into three classes: CATI, CATII, and CATIII, whereby the enzymes of all three classes catalyze the acetyl transfer to CAM yielding 3-O-acetyl-CAM.[4b, 5] Members of class I appear to be most prevalent and show a preference for binding various ligands. The substrate specificity of CATI includes perillyl alcohol (POH) in addition to CAM [6]. The enzyme also binds steroidal derivatives such as fusidic acid and bile salts, and has a high affinity for triphenylmethane dyes, such as crystal violet.[1a, 3b, 4b] Kinetic studies revealed that fusidic acid and bile salts bind to the active center of CATI, thus competing with CAM. This competitive inhibition could not be shown for other CAT types.[1a, 7] So far, neither the inhibitor fusidic acid nor other steroids have been reported to be acetylated in CATI reactions.

Acetylation of hydroxyl groups in steroids has been carried out chemically to produce a wide range of steroid compounds. Depending on the reaction conditions, most of the hydroxyl groups commonly found in steroids, except the highly hindered tertiary 17α- and secondary 11β-hydroxyls, can undergo an acetylation reaction with acetic anhydride-pyridine mixtures, for example.[8] However, the chemical acetylation of steroids has disadvantages. Chemical compounds and solvents required for the reaction, such as acid anhydrides or chlorides and pyridine, are highly polluting.[9] Moreover, choosing a suitable mixture and amount of acetylating agents is a demanding task for the stereo- and regioselective preparation of steroid acetates because of the differences in the reactivity of the targeted reactive groups in steroid molecules.[8, 10]

As an alternative to chemical reactions, hydrolases from different sources were shown to be effective biocatalysts for the mild and selective acetylation for a series of steroids. The applied hydrolases modified hydroxyl groups either on the A- or D-ring or on the side-chain of steroids. *P. cepacia* lipase, for example, performed the regio- and stereoselective acylation of primary and secondary hydroxyl groups in positions 22, 25 or 26 of steroid side chains,[11] while *C. viscosum* lipase reacts exclusively with OH groups in the C-3 position and *B. subtilis* protease shows a marked preference for the OH in the C-17 position.[12] However, the acetylation of C21 hydroxyl groups by isolated enzymes has not been documented so far, although there are indications that microorganisms can perform this reaction.[13]

We identified *E.coli* CATI as the first enzyme selectively performing this reaction, and developed a biotransformation in whole-cells, being prospectively competitive to chemical synthesis, and converted several synthetic and natural mineralo- and corticosteroids to their acetylated derivatives. Finally, we discuss potential pharmaceutical applications of the acetylated products.

Results

CATI-dependent whole-cell catalyst

E.coli CATI was expressed in *E. coli* JM109(DE3) cells, which harbored the plasmid pTG10 encoding the *catI* gene under the control of its own constitutive promoter (Figure 1A). These cells were used as whole-cell biocatalyst for exploring the steroid substrate spectrum as well as for analyzing the products of the CATI dependent reactions.

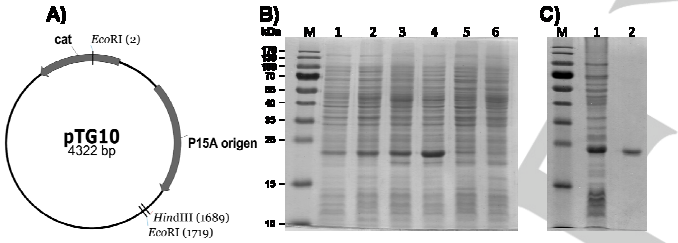


Figure 1. A) Plasmid map of pTG10 containing the *catI* gene, B) Coomassie-Brilliant Blue stained polyacrylamide gel after SDS-PAGE of *E. coli* JM109(DE3)pTG10 samples. M indicates the lane containing the molecular weight marker; lanes 1, 2, 3, 4, 5 and 6 represent samples of whole cell lysates collected at 3, 6, 12, 24, 48 and 72 h after culture inoculation, respectively, C) Polyacrylamid gel of samples taken during the purification of CATI (Lane M: protein marker; lane 1: whole cell lysate of an BL21(DE3)pET22b-CatI expression culture; lane 2: eluted protein after IMAC).

Steroid substrate spectrum of CATI

In order to explore the substrate spectrum of CATI, a substrate screening with resting cells of JM109(DE3)pTG10 in Tris-HCl buffer was performed utilizing 18 different biologically active steroids. The steroids were representatives of the major classes of steroids: androgens, estrogens, glucocorticoids, mineralocorticoids and progestagens. Particular consideration was given to the fact that the selected steroids contain available OH groups at positions 3, 11, 16, 17 and 21 in order to analyze the regioselectivity of the CATI catalyzed acetylation.

The HPLC analysis of reaction extracts showed that 6 steroids (11-deoxycorticosterone (DOC), cortisol (F), 11-deoxycortisol (S), aldosterone (ALDO), corticosterone (B) and prednisolone (PRL)) were new substrates of CATI (Figure 2). Each substrate was selectively converted to a product eluting at a higher retention time (13.48, 12.04, 12.95, 12.1, 13.0 and 12.33 min respectively). HPLC chromatograms of bioconversions performed with the other 12 steroids (cholesterol, pregnenolone, progesterone, testosterone, 17α-hydroxypregnenolone, 17α–hydroxyprogesterone, estrone, estriol, estradiol, adrenosterone, dehydroepiandrosterone and androstenedione) showed no apparent peak of a reaction product.

Whole-cell conversion of CAM

The activity of CATI toward chloramphenicol was analyzed at reaction conditions identical to that toward steroids. The HPLC separation of reaction extracts (Figure 2G) showed that chloramphenicol was converted to the expected acetylated products chloramphenicol-1-acetat (CAM-1-A), chloramphenicol-3-acetat (CAM-3-A), and chloramphenicol-1, 3-diacetate (CAM-1, 3-A)[1a], which was confirmed by comparing the retention time of the reference substances. The total amount of all three products was used to calculate a product concentration of 267 mg/L after 30 min conversion time (Fig.3 D, light gray bar).

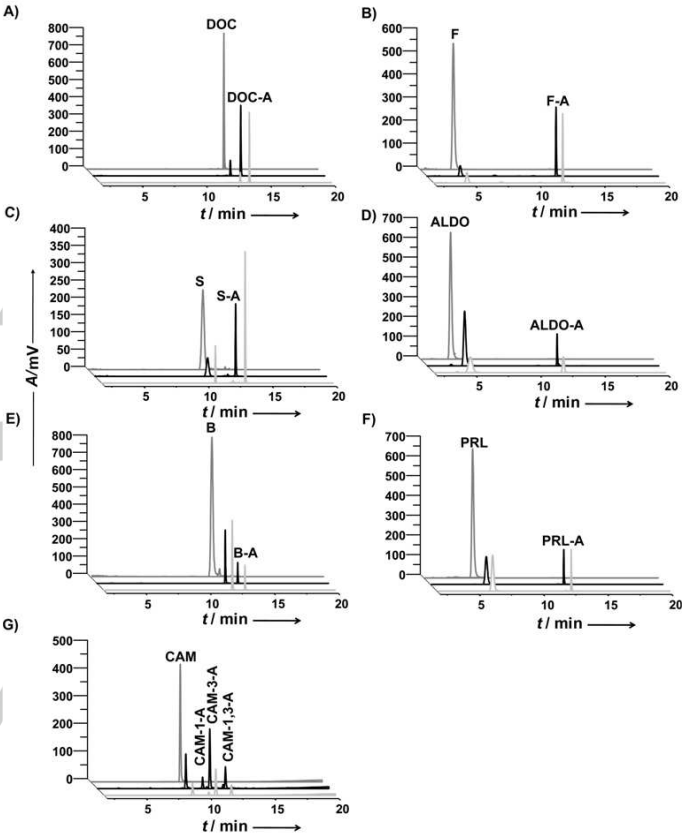


Figure 2. HPLC chromatograms of steroid and CAM conversions. The dark gray and black lines represent reaction extracts of JM109(DE3) (negative control) and JM109(DE3)pTG10 cultures, respectively. Light gray lines show chromatograms derived from conversions with purified CAT. The indicated substrates and corresponding products are A) DOC and DOC-A (11-deoxycorticosterone acetate), B) F and F-A (cortisol acetate), C) S and S-A (11-deoxycortisol acetate), D) ALDO and ALDO-A (aldosterone acetate), E) B and B-A (corticosterone acetate), F) PRL and PRL-A (prednisolone acetate) and G) CAM and CAM-1-A, CAM-3-A, and CAM-1,3-A.

Expression of CATI in JM109(DE3)pTG10

In order to characterize the reaction products of CATI by NMR spectroscopy, a sufficient amount of the steroids had to be synthesized and purified. Therefore the bioconversion of the steroids was optimized by studying the effect of various parameters. These parameters included in the first series of experiments the time-dependent analysis of the CATI synthesis.

The expression level of CATI was estimated by SDS-PAGE of samples taken at different time points from JM109(DE3)pTG10 cultures grown in TB medium, whereas the maximum of CATI expression was observed 24 h after inoculation. The amount of CATI decreased after 48 h, indicating that the protein was degraded after prolonged expression time (Figure 1B).

Effect of media and buffer composition on steroid conversion

In the second set of experiments, the effect of different media and buffers on the steroid conversion was investigated using DOC as representative. The substrate conversion was analyzed 24 h after feeding of 300 μM substrate. In case of bioconversions with growing cells, the product concentration was found to be higher when cells were cultivated in Enpresso tablet medium (50 ± 1.3 mg L^{-1}) than in TB medium (41 ± 3.5 mg L^{-1}), whereas the conversion performed with resting cells proved to be best in Tris-HCl (50 ± 1.4 mg L^{-1}) buffered cell suspensions compared with HEPES (42 ± 2.0 mg L^{-1}) or potassium phosphate buffer (43 ± 1.0 mg L^{-1}). The product concentration in Enpresso tablet medium and in Tris-HCl was similar but since the HPLC analysis of resting cell extracts showed a better separation of steroids and lower background, the following steroid conversions were performed using resting cells in Tris-HCl buffer.

In order to analyze the conversion time, cells were incubated in TB medium, suspended in 50 mM Tris–HCl buffer (pH 7.4), 300 μM DOC and 100 mM glucose. Samples of the bioconversion were collected 24, 48, and 72 h after substrate feeding and subjected to HPLC analysis. The HPLC analysis revealed that the reactions in Tris-HCl buffer resulted in a substrate conversion of 48 ± 2.2, 56 ± 1.9, and 58 ± 3.5 mg L^{-1}, respectively. However, the product concentration increased between 24 h and 48 h and remained on that level until 72 h conversion time. Thus, 48 h conversion time was selected for subsequent experiments.

Effect of glucose, substrate concentration and conversion time on steroid conversion

In the third set of optimization experiments, the glucose concentration and substrate concentration were studied and the conversion time was verified applying the newly obtained conditions. To estimate the effect of the glucose concentration on the steroid conversion, the conversion mixture in Tris-HCl buffer was supplemented with 1 mM DOC and different glucose concentrations (50 – 1000 mM) and incubated for 48 h. The best product concentration (301.3 ± 0.9 mg L^{-1}) was obtained in presence of 500 mM glucose (Figure 3A). Further increase of the glucose concentration to 600, 800, and 1000 mM in the conversion buffer decreased the product formation.

The influence of the substrate concentration was analyzed using cells suspended in Tris-HCl buffer and 500 mM glucose, after feeding with different DOC concentrations (0.3, 0.4, 1, 1.5, 2.0, 3.0, and 4.0 mM). The obtained data (Figure 3B) showed that the substrate was almost completely converted, if the suspensions contained 0.3 and 0.4 mM DOC, resulting in a product concentration of 95 ± 1.4 and 125 ± 3 mg L^{-1}, respectively. In the cultures, which contained 1 mM DOC the product concentration reached the maximum value of 292 ± 2 mg L^{-1}. We also observed that the product formation was lower in the presence of 1.5 and 2.0 mM substrate and further decreased at substrate concentrations of 3.0 and 4.0 mM.

The newly determined optimum conditions (24 h for protein expression, Tris-HCl buffer at pH 7.4, 500 mM glucose and 1 mM DOC) were applied to the whole-cell system and the optimum conversion time was reassessed. Samples were taken after 3, 6, 12, 24, 48 and 72 h conversion time and subjected to HPLC analysis. It was verified that the whole-cell system produces the maximum product concentration after 48 h conversion time (Figure 3C). In the next set of conversion experiments, all six steroid substrates identified in the substrate screening were added under optimum conditions obtained for DOC reactions. The bioconversions of DOC, F, S, ALDO, B and PRL resulted in a single reaction product. In each case, the concentration for the different steroid derivatives considerably varies between 89 ± 1.4 and 298 ± 4 mg L^{-1} for the substrates PRL and DOC, respectively (Figure 3D).

Since the DOC conversions shown in Figure 3C indicated that the catalytic activity of the system is changing with the time, further experiments were performed to characterize the initial rate of the CATI catalyzed reactions for all tested steroids at three different substrate concentrations. The product concentration increased in all cases in a linear manner in the analyzed time period of 6 h (Figure 4). The values of the initial catalytic activity of the whole-cell catalyst was, as expected, substrate concentration-dependent and reached values of 5.4 ± 0.1, 4.7 ± 0.05, 4.3 ± 0.08, 3.1 ± 0.01, 2.7 ± 0.05 and 2.7 ± 0.05 mg L^{-1} h^{-1} for the steroids DOC, F, S, ALDO, B and PRL, respectively, at a substrate concentration of 1 mM (Table 1).

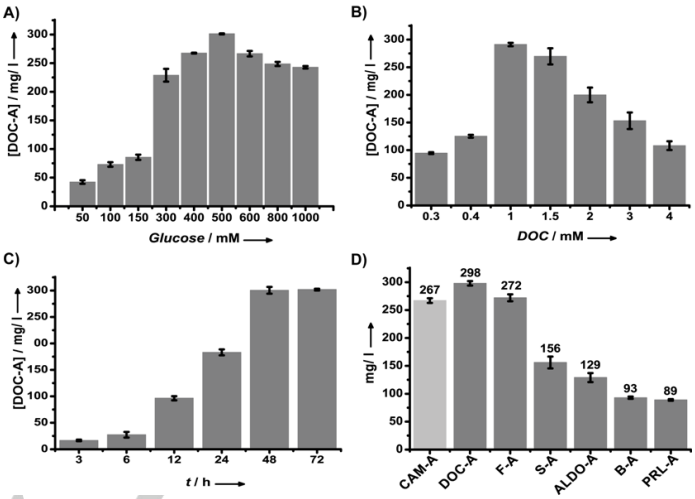


Figure 3. Product formation of whole-cell conversions using *E. coli* JM109(DE3)pTG10, A) in Tris-HCl buffer supplemented with 1 mM DOC and different concentrations of glucose after 48 h reaction time, B) in Tris-HCl buffer, 500 mM glucose and different concentrations of DOC after 48 h reaction time, C) in Tris-HCl buffer (50 mM, pH 7.4), 500 mM glucose and 1 mM DOC at different conversion times, and D) Product concentration after conversion of various steroids by the whole-cell biocatalyst in Tris-HCl buffer (50 mM, pH 7.4), 500 mM glucose, 1 mM steroid substrate after 48 h conversion time (dark gray bars) and 1 mM CAM after 30 min conversion time (light gray bar). The product concentration of CAM conversion represents the total amount of acetylated product. The standard deviation of each bar was calculated for three replicate experiments.

Table 1. Initial catalytic activity of CATI-catalyzed steroid bioconversions at different substrate concentrations. Values were calculated from a linear regression of the substrate concentration against the time shown in Figure 4.

Substrates	Catalytic activity in mg L^{-1} h^{-1}		
	0.2 mM	0.6 mM	1.0 mM
DOC	2.4 ± 0.06	3.2 ± 0.06	5.4 ± 0.1
F	2.0 ± 0.06	2.8 ± 0.06	4.7 ± 0.05
S	2.4 ± 0.03	3.2 ± 0.06	4.3 ± 0.08
ALDO	1.4 ± 0.02	2.1 ± 0.03	3.1 ± 0.01
B	1.2 ± 0.01	2.2 ± 0.04	2.7 ± 0.05
PRL	1.5 ± 0.03	2.3 ± 0.03	2.7 ± 0.05

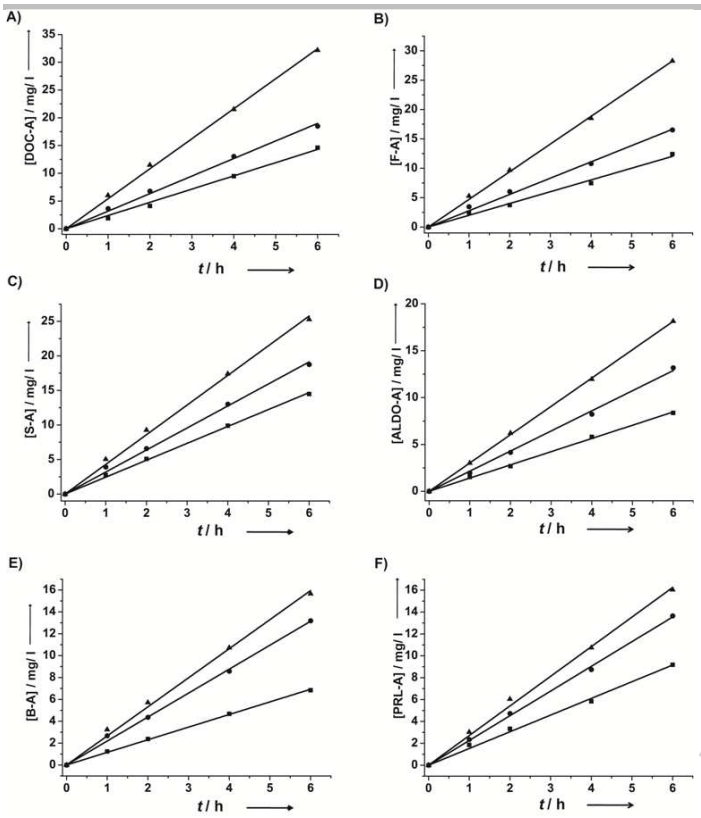
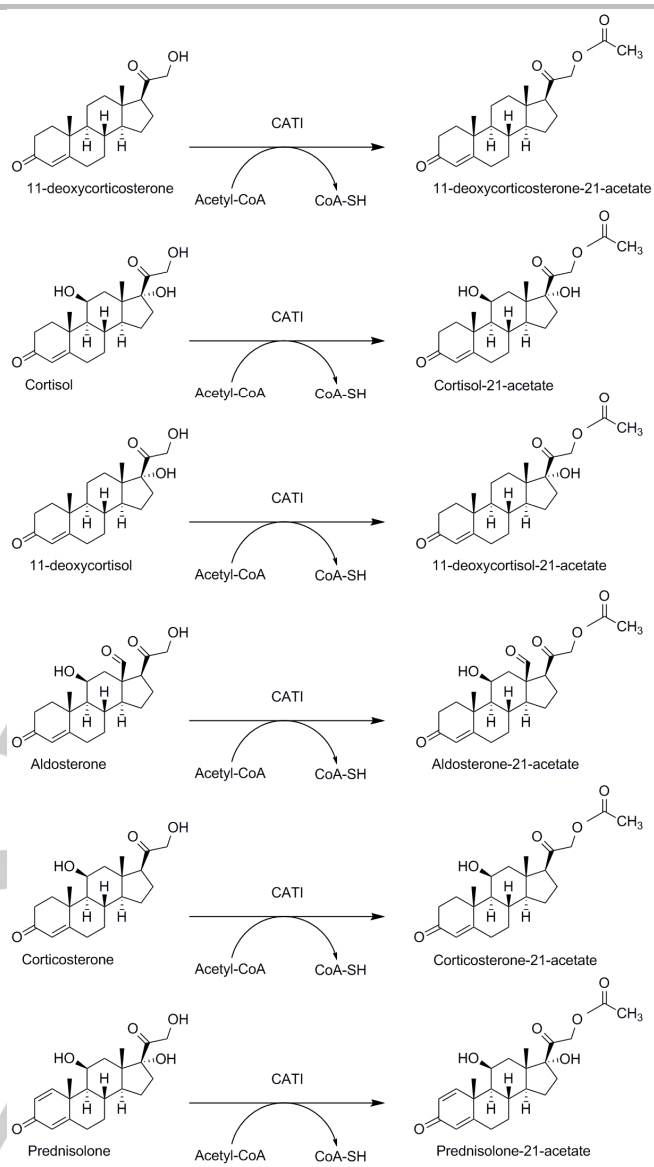


Figure 4. Product formation measured in the initial 6 h of the bioconversions. The slope of the linear regression lines represents the initial catalytic activity of the whole-cell catalyst for different steroids at the substrate concentrations 0.2 mM (■), 0.6 mM (●), and 1 mM (▲). A) DOC, B) F, C) S, D) ALDO, E) B and F) PRL.

A sufficient amount of DOC-A, F-A, S-A, ALDO-A, B-A, and PRL-A products was produced by whole-cell biotransformation in a culture volume of 200 mL followed by preparative purification yielding 20.4 mg, 20.2 mg, 17.3 mg, 14.6 mg, 13.3 mg, and 13.4 mg, respectively. A detailed H^1 and C^{13} NMR spectroscopy identified the products all being acetylated at the C21 OH function. The structures of substrates and products are summarized in Scheme 2.



Scheme 2. Reaction equations showing the CATI-catalyzed conversions of C21-OH steroids to the corresponding acetoxy steroids.

Activity determination of purified CATI

In order to show that the acetylation activity of the whole-cell catalyst is dependent on the *catI* gene and that acetyl CoA is employed in that reaction as an acyl donor, we constructed an expression plasmid containing a gene coding for a His_6-tagged CATI. The gene was successfully expressed in *E. coli* and the protein was purified to homogeneity by IMAC. A single polypeptide band on an SDS-PAGE gel stained with Coomassie blue was taken as indication of a pure protein (Figure 1C). Substrate conversions of CAM or steroids in absence of acetyl-CoA failed (data not shown), whereas conversions in presence of the co-substrate resulted in the same reaction products as in the whole-cell conversions (Figure 2), which clearly indicates that the acetylation of CAM as well as steroids is catalyzed by CATI and that the transfer of acetyl groups by CATI is conducted by acetyl-CoA in both cases.

Substrate docking

To obtain a deeper insight into the structural basis of the substrate binding to the active site of CATI and the selectivity of steroid acetylation, we performed molecular docking of the substrates and of fusidic acid as control.

Except for DOC and S all of the steroids used for docking (DOC, F, S, ALDO, B, and PRL) had their energetically most preferred binding pose showing hydrogen-bonding between their C21-OH group and N_ε of His193, as well as between their C11-OH group and the side chain of Tyr133 (Figure 5).

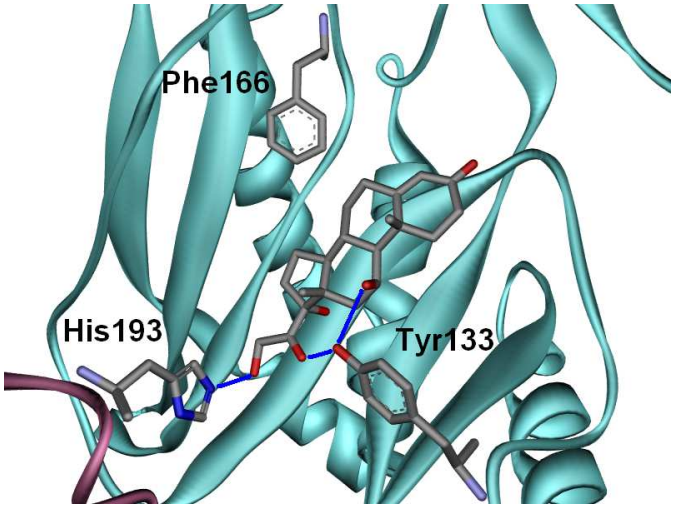


Figure 5. The energetically most preferred docking position found for cortisol (grey) shows hydrogen-bonding (blue lines) between the C21-OH group and His193, as well as between Tyr133 and the 11-OH group and the 20-carbonyl group. Phe166 is a close hydrophobic contact. Cyan: chain A, pink: chain B of 1Q23.pdb.

The obtained position for fusidic acid was found to be almost identical to that in the X-ray structure (1Q23.pdb, Figure 6). Since the conformations of the ring systems of fusidic acid given in the crystallographic data are almost planar, they deviate from the force field optimized geometry used for docking, whereas the overall orientation is preserved.

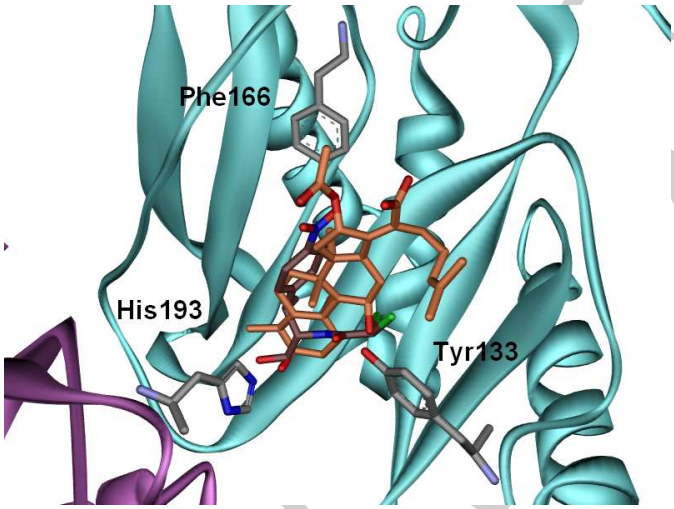


Figure 6. Overlay of fusidic acid (orange) and chloramphenicol (brown) as present in the crystallographic structures 1Q23.pdb and 3U9F.pdb, respectively. Nitrogen, oxygen and chlorine atoms are show in their respective CPK colors. Like for the steroid substrates, hydrogen-bonds to His193 and Tyr133 are realized, whereas the terminal sites of fusidic acid are solvent exposed. Compared to chloramphenicol, fusidic acid also occupies a much larger part of the binding pocket.

Discussion

Biocatalysts are applied for biotechnological conversions because of lower costs, less time consumption and less polluting processes compared to chemical reactions.[14] They are often used to convert the substrate in a one-step reaction to the desired product. Specifically in the steroid field, enzyme catalysis provides an alternative tool for the mild conversion of functional groups via regio- and stereoselective transformations of synthetic and related natural compounds.[15]

In our study, we identified the bacterial CATI as acetyltransferase for steroids and, for the first time, showed its ability to acetylate the 21-OH group of several steroids selectively *in vitro* and in a recombinant whole-cell biocatalyst. So far, only a few microorganisms including some fungal species[13b, 13c] and the bacterium *Clostridium sporogenes*,[13a] revealed the ability to acetylate the C21-hydroxyl function of steroids**.** Both, the enzymes conferring the acetylation activity and the corresponding genes are unknown, so far. The bacterium *C. sporogenes* has never been applied for whole-cell bioconversions of steroids because in addition to the acetylation activity the strain shows strong hydrolysis activity of steroid C21 acetates. Although different hydrolases were employed for the acetylation of steroids as described, [9b, 11-12, 15-16] however an enzyme capable of C21 hydroxyl acetylation has not been characterized.

The new whole-cell biocatalyst based on the *E. coli* enzyme CATI was successfully applied in our study to convert CAM and the steroids DOC, F, S, ALDO, B and PRL, respectively. The conditions for the bioconversion were optimized in shake flask cultures resulting in yields of 267 ± 4.2, 298 ± 4, 272 ± 6.2, 156 ± 10, 129 ± 8.1, 93 ± 2 and 89 ± 1.4 mg L^{-1}, which represent promising starting values for developing a bioconversion process in fermenter cultures and following industrial scale-up. Besides improving substrate concentration, expression and reaction time, the variation of the glucose concentration during the steroid conversion turned out to have a strong influence on the catalytic activity; because the substrate conversion was significantly improved in response to increased glucose concentration. The best product concentration was obtained after increasing the glucose concentration up to a critical value of 500 mM (Figure 3A). This effect is probably due to a higher concentration of the essential reaction cofactor acetyl-CoA. It was described before that glucose availability is linked to acetyl-CoA production in yeast and in *E. coli* and that the acetyl-CoA levels are consistently high when glucose concentration is abundant.[17] We noticed a decreased product formation upon increasing concentrations of glucose beyond the critical value of 500 mM (600, 800, and 1000 mM). This might be caused by a metabolic overflow leading to the acidification of the buffer solution during the maintenance of bacteria under aerobic conditions when the glucose uptake overrides a critical value.[18]

All reaction products obtained with the six steroid substrates were isolated and NMR analysis clearly demonstrated that only the 21-OH group was acetylated whereas other hydroxyl groups present in the converted steroids (11β-OH and 17α-OH), as well as other functional groups like the carbonyl groups at positions 3, 18, 20 and the double bonds at positions 1, 2 and 4, 5 were not modified. This can be attributed to the fact that position 21 has less steric constraints compared to OH-groups for example in positions 11 and 17, because it is separated from the steroid skeleton by a rotatable bond. In addition, due to the carbonyl group at the adjacent C20 position in the analyzed steroids, the 21-OH group becomes more acidic compared to OH-groups in other positions and can, thus, form an effective nucleophile for the subsequent attack at the carbonyl group of the acetyl-CoA more easily.

We suggest that the acetylation mechanism of steroids is similar to that proposed for CAM. This mechanism involves His193, which has been demonstrated to be one of the primary conserved catalytic residues to change its protonation pattern. The conformation of the protein ensures that the imidazole ring of His193 is aligned appropriately to deprotonate the 3-hydroxyl group of chloramphenicol, promoting a nucleophilic attack of acetyl-CoA to yield acetylated chloramphenicol and CoA-SH (Scheme 1A).[1b, 4a] In case of the acetylation of the C21 hydroxyl group of steroids the proposed reaction mechanism requires the His193 imidazole side chain to abstract a proton from the C21-

hydroxyl group of steroid compounds, thus facilitating the nucleophile formation. The subsequent attack at the acetyl-CoA-thioester bond would then produce acetoxy steroids and CoA-SH (Scheme 1B).

Additional support for a similar reaction mechanism for CAM and steroid substrates was provided in a study describing the crystal structure of CATI in complex with the steroidal antibacterial fusidic acid.[1b] In the CATI-fusidic acid complex, fusidic acid occupies the same binding site as CAM and its hydrophobic steroid ring system forms numerous hydrophobic contacts with active site residues, which explains the observed characteristic of fusidic acid as a competitive inhibitor of CATI (Figure 6). Also, the hydroxyl moiety of ring A of fusidic acid closely aligns with the 3-hydroxyl of CAM and forms a very strong hydrogen bond with the active site residue His 193. The side chains of Ala24 and Ala29 of CATI can accommodate the ring D and the 2-methylhex-2-ene tail of fusidic acid by providing a substrate binding cavity. The similarity in the steroid backbone of fusidic acid and C21-OH-steroids led us to assume that CATI binds both substrate types in a similar manner, and we decided to prove that hypothesis by molecular docking experiments.

The docking results corroborate the substrate binding and the proposed mechanism, showing the 21-OH group hydrogen-bonded to N_e of His193 (Figure 5). Where present in the steroids, also a hydrogen-bond between the 11-OH group and the side chain of Tyr133 is established. The same kind of interactions was also found between fusidic acid and the active site of CATI when fusidic acid was applied in a docking control, which is in excellent agreement with the X-ray crystallographic data. The absence of the 11-OH group in DOC and S is most likely the reason why we did not obtain corresponding docking positions for these two molecules, although they were found to be acetylated in position 21 as well. Since all residues were kept fixed during docking, we therefore assume that the side chain of Tyr133 undergoes a conformational change to form an alternative hydrogen-bond with another residue, when either DOC or S is bound to CATI.

Steroid acetates are used as pharmaceuticals and administered either orally, parenterally, or topically depending on the disease and the desired duration and effect of medication. The corticosteroid products of the new biocatalyst, DOC-A, F-A, S-A, and PRL-A respectively, are active pharmaceutical ingredients themselves or are important intermediates in pharmaceutical synthesis. DOC-A was used as an effective drug for treating adrenal insufficiency and Addison's disease in former days.[19] It has also been reported to regulate the salt appetite in brain of experimental animals,[20] and when it is administered together with sodium chloride to rats, the resulting DOC-A salt hypertensive rats represent an animal model of oxidative and inflammatory stress and can be applied to study hypertension and related diseases of the cardiovascular system.[21] S-A was not described as drug but it was used as substrate for the preparation of the effective corticosteroid drugs cortisol and cortisol-21-acetate. [14b, 22] F-A in turn was applied as an important intermediate for the synthesis of steroidal drugs such as triamcinolone acetonide[14b, 23] and, above all, it is known as drug to treat joint problems (e.g., arthritis, bursitis) and skin conditions (e.g. eczema, dermatitis, rash, and allergies). Less well-known is the application of F-A as drug in replacement therapies in adrenocortical insufficiency. PRL-A has, similar to F-A, a pronounced immune suppressive and also inflammatory and anti-allergic effect and is one of the most commonly used drugs for the treatment of corresponding diseases.

Conclusions

In our study, we discovered that CATI regioselectively acetylates the C21-OH group of steroids. A reaction mechanism similar to that for the natural substrate CAM was proposed based on molecular docking experiments. We established a whole-cell biotransformation using *E. coli* as host and demonstrated the biosynthesis of pharmaceutical important acetoxy steroids. The results lead us to expect that the new biocatalyst is competitive to chemical acetylation reactions and should be considered as an alternative approach for the industrial production of acetylated steroids.

Experimental Section

Chemicals

Steroids, solvents, media and other chemicals used were of analytical grade. Stock solutions of steroids were dissolved in dimethylsulfoxide (DMSO).

Bacterial strains and plasmids

E. coli strain JM109(DE3) was applied as host for whole-cell conversions. JM109(DE3) cells were transformed with the vector pTG10 (Figure 1A) [24] carrying the chloramphenicol acetyltransferase gene as a resistance marker resulting in the strain JM109(DE3)pTG10. *E. coli* strain BL21(DE3) containing the plasmid pET22b-CatI was used as host for the expression and purification of CATI.

Purification of CATI

A PCR fragment encoding a His_6-tagged CATI was amplified using plasmid pTG10 as template and inserted between the *Nde*I and *Xho*I sites of plasmid pET22b. *E. coli* strain BL21(DE3) was transformed with the resulting vector pET22b-CatI and cultures were grown to an optical density of 0.4 at 600 nm in 200 mL LB medium. The synthesis of the protein was induced by adding 1 mM isopropyl-β-D-1-thiogalactopyranoside. Cells were harvested after additional 3 h by centrifugation and suspended in potassium phosphate buffer (50 mM, pH 7.8) containing glycerol (10 %), sodium chloride (200 mM), β-mercaptoethanol (2 mM) and ethylenediaminetetraacetic acid (EDTA) (0.1 mM). Cells were disrupted by sonication on ice and the supernatant after centrifugation (4 °C, 70,500 *g* for 30 min) was used for protein purification by immobilized metal affinity chromatography (IMAC) on a Ni–NTA agarose column. CATI was eluted by potassium phosphate buffer (50 mM, pH 7.8) containing imidazole (250 mM), sodium chloride (200 mM) and glycerol (10 %). After buffer exchange to potassium phosphate (50 mM, pH 7.8), glycerol (10 %) and sodium chloride (200 mM) the protein was concentrated to ~10 mg mL^{-1}using centrifugal filter units and stored at -80 °C until use. The protein concentration was determined by the bicinchoninic acid assay using bovine serum albumin as a standard.

Activity determination of purified CATI

In order to determine the activity of purified CATI, substrate conversions were performed in a final volume of 1 mL Tris-HCl buffer (100 mM, pH 7.8) containing 0.2 mM of the co-substrate acetyl Coenzyme A and 200 µM of the substrate dissolved in DMSO. The reaction was initiated by the addition of 4 µM CATI, incubated at 37°C for 1h (steroids) or 5 min (CAM), and terminated by extracting the sample twice with an equal volume of ethyl acetate. The organic layer was isolated and completely evaporated prior to RP-HPLC analysis.

Steroid conversion with growing cells in TB medium

TB medium (30 mL) supplemented with chloramphenicol (25 µg mL^{-1}) was inoculated with a JM109(DE3)pTG10 seed culture (0.3 mL) and incubated in a 300 mL baffled flask at 37 °C with shaking at 180 rpm. When the optical density at 600 nm of the main culture reached a value between 0.4 - 0.6, the substrate (300 µM) was added and the cells were incubated for additional 24 h at the same temperature and shaking speed. The product formation was monitored by taking 1 mL samples 24 h after substrate feeding. In this study the following substrates were tested: cholesterol, pregnenolone, progesterone, testosterone, 17α-hydroxypregnenolone, 17α–hydroxyprogesterone, estrone, estriol, estradiol, cortisol, dehydroepiandrosterone, adrenosterone, 11-deoxycorticosterone, aldosterone, prednisolone, androstenedione, corticosterone and 11-deoxycortisol.

Steroid conversion with resting cells in buffer

JM109(DE3)pTG10 cultures were grown for 24 h at 37 °C with 180 rpm in 300 mL baffled flasks containing TB medium (30 mL) supplemented with chloramphenicol (25 µg mL^{-1}). Cells were then harvested by centrifugation

(20 min, 4000 *g*, 4 °C) and the pellet was washed once with 30 mL of 50 mM buffer (pH 7.4) (Tris-HCl, HEPES or potassium phosphate (KPP)) and centrifuged again for 20 min. After suspending the pellet in buffer (30 mL, 50 mM, pH 7.4) containing different concentrations of glucose and substrate, the suspensions were further incubated at 180 rpm and 37 °C.
Further, time and concentration depending experiments using resting cells in Tris-HCl buffer (50 mM, pH 7.4, 500 mM glucose) were performed to determine the initial catalytic activity. Substrate concentration studies were carried out using substrate concentrations of 0.2, 0.6 and 1.0 mM. In time course experiments, incubations were carried out for 0, 1, 2, 4, and 6 h.

Steroid conversion in an EnPresso^{TM} Tablet Cultivation set

In order to perform the steroid conversion in a medium based on EnPresso^{TM} tablets (BioSilta), the medium (50 mL) was prepared as described by the manufacturer and was inoculated with the seed culture of *E. coli* JM109(DE3) (2 mL) cells and incubated at 37 °C and 180 rpm. The substrate (300 µM) was added after 4 - 5 h simultaneously with the booster tablet, and the culture was further incubated at 30 °C and 180 rpm.

SDS-polyacrylamide gel electrophoresis

To determine the time period with the highest CATI expression level, cultures inoculated with a seed culture (0.3 mL) of *E. coli* JM109(DE3)pTG10 and grown at 37 °C and 180 rpm in TB medium (30 mL) supplemented with chloramphenicol (25 µg mL^{-1}). Samples were collected at different time points (3, 6, 12, 24, 48 and 72 h), cells were pelleted and suspended in SDS loading buffer and SDS-polyacrylamide gel electrophoresis was performed (Figure 1B).
To determine the purity of the CATI preparation, samples taken from an expression culture of BL21(DE3)pET22b-CatI and after the IMAC purification were separated by SDS-PAGE (Figure 1C).

Substrate extraction and analytic HPLC

Samples (1 mL) of the steroid or CAM conversions were extracted twice with ethyl acetate (1 mL). The organic phase was evaporated under vacuum. The residuum of steroid extracts was dissolved in a small amount of the mobile phase (20 % acetonitrile) for subsequent isocratic HPLC using a Jasco system of the 2000 series with a 4.0 mm x 125 mm Nucleodur C18ec RP column (Macherey-Nagel). The flow rate was 1 mL min^{-1} at 40 °C and steroids were detected at 240 nm. The residuum of CAM extracts was dissolved in 10 % ACN and analyzed using a gradient elution of acetonitrile 10-100 % containing 0.1 % trifluoroacetic acid from 0-18 min. The UV detection was monitored at 259 nm.

Preparative purification of steroids for NMR analysis

After optimizing the conditions for the whole cell biotransformation of steroids on an analytical scale, the procedure was applied for the preparative purification of the products for NMR spectroscopy. 200 mL of Terrific broth (TB) medium containing chloramphenicol (25 µg mL^{-1}) was inoculated with a JM109(DE3)pTG10 seed culture (2 mL) and cultivated for 24 h at 37 °C with rotary shaking at 180 rpm. Cells were harvested by centrifugation (4000 *g*, 20 min, 4 °C) and washed once with Tris–HCl buffer (200 mL, 50 mM, pH 7.4). After a second centrifugation cell pellets were suspended in Tris–HCl buffer (200 mL, 50 mM, pH 7.4) complemented with glucose (500 mM). The substrate (400 µM) was added and the culture was incubated for another 48 h at 37 °C and 180 rpm. Steroids were extracted twice using ethyl acetate (200 mL) and the organic layer was transferred to a 1 L round bottom flask for subsequent rotary evaporation. Steroids were separated by reversed phase HPLC on a C18ec column (Nucleodur 250/8, Macherey-Nagel) with a mobile phase consisting of 20% ACN. The fraction containing the product was collected and subjected to subsequent NMR analysis.

NMR analysis

The NMR spectra of 11-deoxycorticosterone-21-acetate (DOC-A), cortisol-21-acetate (F-A), 11-deoxycortisol-21-acetate (S-A), aldosterone-21-acetate (ALDO-A), corticosterone-21-acetate (B-A) and prednisolone-21-acetate (PRL-A) were recorded in $CDCl_3$ or CD_3OD with a Bruker DRX 500 or a Bruker Avance 500 NMR spectrometer at 300 K. The chemical shifts were relative to $CHCl_3$ at δ 7.26 or CH_3OD at δ 3.30 (^{1}H NMR) and $CDCl_3$ at δ 77.00 or CD_3OD at δ 49.00 (^{13}C NMR), respectively, using the standard δ notation in parts per million. The 1D NMR (^{1}H and ^{13}C NMR, DEPT135) and the 2D NMR spectra (gs-HH-COSY, gs-NOESY, gs-HSQCED, and gs-HMBC) were recorded using the BRUKER pulse program library. All assignments based on extensive NMR spectral evidence. The obtained NMR data for the steroid acetates are as follows:

11-Deoxycorticosterone-21-acetate: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.71 *s* (3xH-18), 0.98 *m* (H-9), 1.07 *m* (H-7a), 1.18 *ddd* (12.7, 11.0 and 7.0 Hz, H-14), 1.19 *s* (3xH-19), 1.33 *m* (H-15a), 1.39 *m* (H-12a), 1.48 *dddd* (3x13.0 and 4.0 Hz, H-11a), 1.58 *m* (H-8), 1.64 *m* (H-11b), 1.71 *m* (H-1a), 1.73 *m* (H-16a), 1.74 *m* (H-15b), 1.86 *m* (H-7b), 2.04 *ddd* (13.5, 5.0 and 3.2 Hz, H-1b), 2.09 *m* (H-12b), 2.17 *s* (3H, 21-OAc), 2.23 *m* (H-16b), 2.28 *m* (H-6a), 2.33 *m* (H-2a), 2.39 *m* (H-6b), 2.40 *ddd* (17.0, 15.0 and 5.0 Hz, H-2b), 2.51 *dd* (2x9.0 Hz, H-17), 4.52 *d* (17.0 Hz, H-21a), 4.72 *d* (17.0 Hz, H-21b), 5.74 *brs* (H-4). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 13.18 (CH_3, C-18), 19.50 (CH_3, C-19), 20.97 (CH_3, 21-OAc), 22.69 (CH_2, C-11), 22.83 (CH_2, C-16), 24.46 (CH_2, C-15), 31.87 (CH_2, C-7), 32.73 (CH_2, C-6), 33.93 (CH_2, C-2), 35.53 (*CH*, C-8), 35.70 (CH_2, C-1), 38.33 (CH_2, C-12), 38.55 (*C*, C-10), 44.65 (*C*, C-13), 53.56 (*CH*, C-9), 56.17 (*CH*, C-14), 59.07 (*CH*, C-17), 69.13 (CH_2, C-21), 123.97 (*CH*, C-4), 170.32 (*C*, C-5), 170.81 (*C*, 21-OAc), 199.48 (*C*, C-3), 203.62 (*C*, C-20).

Cortisol-21-acetate: ^{1}H NMR (CD_3OD, 500 MHz) : δ 0.90 *s* (3xH-18), 1.02 *dd* (11.2 and 3.5 Hz, H-9), 1.13 *m* (H-7a), 1.41 *m* (H-15a), 1.48 *s* (3xH-19), 1.49 *m* (H-16a), 1.79 *m* (-15b), 1.80 *m* (H-12a), 1.89 *ddd* (2x13.5 and 4.5 Hz, H-1a), 2.05 *m* (H-7b), 2.07 *m* (H-12b), 2.09 *m* (H-8), 2.14 *s* (3H, 21-OAc), 2.24 *ddd* (13.5 and 2x4.8 Hz, H-1b), 2.28 *m* (H-6a), 2.33 *ddd*(17.0 and 2x4.3 Hz, H-2a), 2.51 *ddd* (17.0, 13.7 and 5.0 Hz, H-2b), 2.57 *dddd* (2x14.5, 5.0 and 1.5 Hz, H-6b), 2.68 *ddd* (15.0, 11.5 and 3.0 Hz, H-16b), 4.42 *ddd* (3x3.3 Hz, H-11), 4.92 *d* (17.5 Hz, H-21a), 5.07 *d* (17.5 Hz, H-21b), 5.67 *d* (1.5 Hz, H-4). ^{13}C NMR(CD_3OD, 125 MHz): δ 17.39 (CH_3, C-18), 20.47 (CH_3, 21-OAc), 21.45 (CH_3, C-19), 24.60 (CH_2, C-15), 32.87 (*CH*, C-8), 33.30 (CH_2, C-6), 34.32 (CH_2, C-7), 34.62 (CH_2, C-2), 34.71 (CH_2, C-16), 35.87 (CH_2, C-1), 40.49 (CH_2, C-12), 40.73 (*C*, C-10), 48.59 (*C*, C-13), 53.45 (*CH*, C-14), 57.64 (*CH*, C-9), 68.74 (CH_2, C-11), 69.29 (CH_2, C-21), 90.60 (*C*, C-17), 122.50 (*CH*, C-4), 172.34 (*C*, 21-OAc) , 176.63 (*C*, C-5), 202.51 (*C*, C-3), 207.33 (*C*, C-20).

11-Deoxycortisol-21-acetate: ^{1}H NMR ($CDCl_3$, 500 MHz) : δ 0.73 *s* (3xH-18), 0.98 *ddd* (12.2, 10.5 and 4.0 Hz, H-9), 1.10 *dddd* (13.5, 12.5, 11.5 and 4.5 Hz, H-7a), 1.19 *s* (3xH-19), 1.37 *m* (H-15a), 1.46 *dddd* (3x13.5 and 4.5 Hz, H-11a), 1.52 *ddd* (15.0, 9.0 and 6.0 Hz, H-16a), 1.63 *m* (H-8), 1.65 *m* (H-12a), 1.66 *m* (H-11b), 1.71 *m* (H-1a), 1.73 *m* (1.79 *m* (H-12b), -15b), 1.87 *m* (H-7b), 2.04 *ddd* (13.5, 5.0 and 3.2 Hz, H-1b), 2.17 *s* (3H, 21-OAc), 2.29 *m* (H-6a), 2.36 *m* (H-2a), 2.41 *m* (H-6b), 2.43 *m* (H-2b), 2.74 *ddd* (15.0, 11.5 and 3.0 Hz, H-16b), 4.85 *d* (17.5 Hz, H-21a), 5.07 *d* (17.5 Hz, H-21b), 5.74 *d* (1.5 Hz, H-4). ^{13}C NMR ($CDCl_3$, 125 MHz): *δ* 14.77 (CH_3, C-18), 17.62 (CH_3, C-19), 20.78 (CH_3, 21-OAc), 20.86 (CH_2, C-11), 23.85 (CH_2, C-15), 30.27 (CH_2, C-12), 32.26 (CH_2, C-7), 33.02 (CH_2, C-6), 34.16 (CH_2, C-2), 35.08 (CH_2, C-16), 35.88 (*CH*, C-8), 35.95 (CH_2, C-1), 38.81 (*C*, C-10), 48.53 (*C*, C-13), 50.71 (*CH*, C-14), 53.54 (*CH*, C-9), 68.15 (CH_2, C-21), 90.22 (*C*, C-17), 124.19 (*CH*, C-4), 170.88 (*C*, 21-OAc), 171.22 (*C*, C-5), 194.83 (*C*, C-3), 205.36 (*CH*, C-20).

Aldosterone-21-acetate: According to literature,[25] this acetate exists in two tautomeric forms as (11-18) hemi-acetal and (18-20) hemi-ketal. In $CDCl_3$ the (18-20) hemi-ketal tautomer dominates as shown in the following data. ^{1}H NMR ($CDCl_3$, 500 MHz) : *δ* 1.07 *m* (H-9), 1.25 *m* (H-7a), 1.32 *s* (3xH-19), 1.46 *m* (H-15a), 1.48 *m* (H-12a), 1.49 *m* (1.64 *m* (H-16a), 1.72 *ddd* (2x 14.8 and 4.5 Hz, H-1a), 1.77 *m* (H-8), -15b), 2.04 *m* (H-7b), 2.13 *s* (3H, 21-OAc), 2.18 *m* (H-1b), 2.23 *m* (H-16b), 2.32 *m* (H-6a), 2.37 *m* (H-12b), 2.39 *ddd*(17.0 and 2x3.5 Hz, H-2a), 2.46 *m* (H-6b), 2.47 *brd* (10.0 Hz, H-17), 2.52 *ddd* (17.0, 15.0 and 5.0 Hz, H-2b), 4.02 *d* (11.3 Hz, H-21a), 4.19 *d* (11.3 Hz, H-21b), 4.86 *m* (H-11), 5.45 *brs*(H-18), 5.74 *brs* (H-4). ^{13}C NMR ($CDCl_3$, 125 MHz): *δ* 18.42 (CH_3, C-19), 20.79 (CH_3, 21-OAc), 25.77 (CH_2, C-16), 29.85 (CH_2, C-15), 31.95 (CH_2, C-7), 32.89 (CH_2, C-6), 33.63 (CH_2, C-2), 36.02 (CH_2, C-1), 38.17 (*CH*, C-8), 38.97 (*C*, C-10), 40.20 (CH_2,C-12), 47.85 (*CH*, C-17), 48.75 (*CH*, C-14), 57.16 (*CH*, C-9), 64.18 (*C*, C-13), 69.21 (CH_2, C-21), 81.61 (*C*, C-11), 104.88 (*C*, C-20), 106.72 (*CH*, C-18), 124.33 (*CH*, C-4), 169.79 (*C*, C-5), 170.80 (*C*, 21-OAc), 199.24 (*C*, C-3).

Corticosterone-21-acetate: ^{1}H NMR ($CDCl_3$, 500 MHz): *δ* 0.93 *s* (3xH-18), 0.98 *dd* (11.5 and 3.0 Hz, H-9), 1.07 *m* (H-7a), 1.12 *m* (1.39 *m* (H-15a), 1.43 *s* (3xH-19), 1.60 *m* (H-12a), -15b), 1.83 *m* (H-1a), 1.99 *m* (2H, H-7b and H-8), 2.15 *s* (3H, 21-OAc), 2.18 *m* (H-1b), 2.19 *m* (2H, H-12b and H-16b), 2.28 *m* (H-6a), 2.33 *m* (H-2a), 2.40 *m* (H-6b), 2.41 *m* (H-17),2.47 *m* (H-2b), 4.39 *m* (H-11), 4.48 *d* (17.0 Hz, H-21a), 4.71 *d* (17.0 Hz, H-21b), 5.67 *d* (1.5 Hz, H-4). ^{13}C NMR ($CDCl_3$, 125 MHz): *δ* 15.50 (CH_3, C-18), 20.48 (CH_3, 21-OAc), 20.92 (CH_3, C-19), 22.52 (CH_2, C-16), 24.43 (CH_2, C-15), 31.34 (*CH*, C-8), 32.56 (CH_2, C-7), 33.54 (CH_2, C-6), 33.80 (CH_2, C-2), 34.99.12 (CH_2, C-1), 39.25 (*C*, C-10), 43.76 (*C*, C-13), 47.91 (CH_2, C-12), 56.33 (*CH*, C-9), 57.64 (*CH*, C-14), 59.43 (*CH*, C-17), 67.99 (*CH*, C-11), 68.96 (CH_2, C-21), 59.43 (*CH*, C-17), 122.42 (*CH*, C-4),), 177.04 (*C*, C-5), 177.04 (*C*, 21-OAc), C-3, C-20 and 21-OOCCH_3 not detected).

Prednisolone-21-acetate: ^{1}H NMR ($CDCl_3$, 500 MHz) : δ 0.96 s (3H, H-18), 1.06 *d* (11.2 and 3.5 Hz, H-9), 1.11 *dddd*(3x13.0 and 4.5 Hz, H-7a), 1.43 *s* (3xH-19), 1.46 *m* (H-15a), 1.47 *m* (H-16a), 1.64 *m* (1.70 *m* (H-12a), -15b), 2.05 *m* (H-12b), 2.09 *m* (H-7b), 2.15 *m* (H-8), 2.16 *s* (3H, 21-OAc), 2.32 *ddd* (13.5, 5.0 and 2.0 Hz, H-6a), 2.55 *dddd*(2 x 13.5, 5.5 and 1.5 Hz, H-6b), 2.75 *dddd* (2 x 13.7 and 2x 3.5 Hz, H-16b), 4.47 *ddd* (3 x 3.5 Hz, H-11), 4.84 *d* (17.3 Hz, H-21a), 4.97 *d* (17.3 Hz, H-21b), 6.00 *dd* (2x1.8 Hz, H-4), 6.25 *dd*(10.0 and 1.8 Hz, H-2), 7.25 *d* (10.0 Hz, H-1). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 16.97 (CH_3, C-18), 20.54 (CH_3, 21-OAc), 21.08 (CH_3, C-19), 23.83 (CH_2, C-15), 31.24 (*CH*, C-8), 31.96 (CH_2, C-6), 34.00 (CH_2, C-7), 34.71 (CH_2, C-16), 39.63 (CH_2, C-12), 44.05 (*C*, C-10), 47.85 (*C*, C-13), 51.38 (*CH*, C-14), 55.32 (*CH*, C-9), 67.89 (CH_2, C-21), 70.16 (*CH*, C-11), 89.68 (*C*, C-17), 122.46 (*CH*, C-4), 127.88 (*CH*, C-2), 156.12 (*CH*, C-1), 169.92 (*C*, C-5), 170.79 (*C*, 21-OAc), 186.56 (*C*, C-3), 204.77 (*C*, C-20).

Computational Methods

The high resolution crystallographic structure of CATI from *E. coli* (pdb entry 1Q23.pdb, accession code P62577) contains a total of 12 chains. Here, only chain A and B were used, because fusidic acid and likewise chloramphenicol are bound in between the two monomeric units. Furthermore, all water molecules were removed. Compounds for docking were generated manually and energetically optimized applying the MM+ force field as implemented in HYPERCHEM. (HYPERCHEM, version 6.02, Hypercube Inc., Gainsville, FL, 1999). Gasteiger-Marsili charges were calculated with an in-house PERL script.[26] Further preparation steps for ligands and receptor were carried out by using the Windows version 1.5.6 of Autodock Tools.[27] His193 was modelled as being protonated only at N_δ. Flexible bonds of the compounds were assigned automatically and verified by manual inspection. To the protein part AMBER charges were assigned. A rectangular grid box (46 x 44 x 46 points) with a grid spacing of 0.375 Å was centered at 2.595, 13.265, -3.437 to comprise all potential amino acids that contribute to ligand binding. AUTODOCK (version 4.2) was applied for docking while keeping the protein part rigid.[28] For each of the ligands 200 docking runs were carried out applying the Lamarckian genetic algorithm using default parameter settings.

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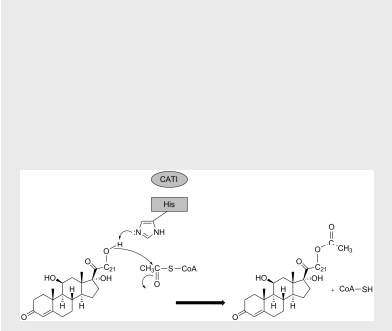
Entry for the Table of Contents (Please choose one layout)

Layout 1:

FULL PAPER

The bacterial chloramphenicol acetyltransferase I was identified as a steroid acetyltransferase. A novel and highly efficient *E. coli* based biocatalyst was designed for the regioselective acetylation of C21 hydroxyl groups in steroids of pharmaceutical interest.



Azzam Mosa, [a] Michael C.Hutter,[b] Josef Zapp, [c] Rita Bernhardt, [a] and Frank Hannemann[a]*

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A New and Regioselective Acetylation of C21 Hydroxysteroids by the bacterial Chloramphenicol Acetyltransferase I

3.3- NEUNZIG ET AL., 2014

A STEROIDOGENIC PATHWAY FOR SULFONATED STEROIDS: THE METABOLISM OF PREGNENOLONE SULFATE

J. Neunzig, A. Sánchez-Guijo, **A. Mosa**, M.F. Hartmann, J. Geyer, S.A. Wudy, R. Bernhardt

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A steroidogenic pathway for sulfonated steroids: The metabolism of pregnenolone sulfate

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ABSTRACT

In many tissues sulfonated steroids exceed the concentration of free steroids and recently they were also shown to fulfill important physiological functions. While it was previously demonstrated that cholesterol sulfate (CS) is converted by CYP11A1 to pregnenolone sulfate (PregS), further conversion of PregS has not been studied in detail. To investigate whether a steroidogenic pathway for sulfonated steroids exists similar to the one described for free steroids, we examined the interaction of PregS with CYP17A1 in a reconstituted *in-vitro* system. Difference spectroscopy revealed a K_d-value of 74.8 ± 4.2 μM for the CYP17A1–PregS complex, which is 2.5-fold higher compared to the CYP17A1–pregnenolone (Preg) complex. Mass spectrometry experiments proved for the first time that PregS is hydroxylated by CYP17A1 at position C17, identically to pregnenolone. A higher K_m- and a lower k_{cat}-value for CYP17A1 using PregS compared with Preg were observed, indicating a 40% reduced catalytic efficiency when using the sulfonated steroid. Furthermore, we analyzed whether the presence of cytochrome b_5 (b_5) has an influence on the CYP17A1 dependent conversion of PregS, as was demonstrated for Preg. Interestingly, with 17OH-PregS no scission of the 17,20-carbon–carbon bond occurs, when b_5 is added to the reconstituted *in-vitro* system, while b_5 promotes the formation of DHEA from 17OH-Preg. When using human SOAT-HEK293 cells expressing CYP17A1 and CPR, we could confirm that PregS is metabolized to 17OH-PregS, strengthening the potential physiological meaning of a pathway for sulfonated steroids.

1. Introduction

CYP11A1 initiates steroid hormone biosynthesis (Fig. 1) through a side-chain cleavage reaction on cholesterol yielding pregnenolone [1,2] which represents the precursor of all steroid hormones. In a series of reactions where six different cytochromes P450 (CYPs) and three hydroxysteroid dehydrogenases (HSD) participate, mineralocorticoids, glucocorticoids and sex hormones are formed.

In order to induce a biological response, steroid hormones interact with their corresponding receptor and thus e.g., regulation of blood pressure, provision of carbon hydrates or development of secondary sexual characteristics take place in mammalian organisms. Interestingly, sulfonated steroids or sulfated steroids [3] often circulate in mammals in considerably higher concentrations than unconjugated steroids [4–6]. Dehydroepiandrosterone (DHEA) for example, one of the most abundant steroid in humans, occurs to 99% in its sulfonated form [7], reaching concentrations of up to 10 μM in young adults [8]. Steroid sulfates are generated by sulfonation of free steroids by three different sulfotransferases (SULT1E1, SULT2B1, SULT2A1). These enzymes are widely distributed in mammalian organism, as NCBI EST profiles indicate. Sulfonation of unconjugated steroids seems to contribute to the modulation of the genomic action of steroids. For instance, SULT1E1 is highly active in cultured normal breast epithelial cells compared to tumor cell lines [9]. Since it is known that increased exposure of estrogens increases breast cancer development, sulfonation of estrogens might be a crucial mechanism to impede the danger of excessive estrogenic exposure [9]. In addition, alterations of steroid sulfonation have a severe impact on the development in mammals. DHEA is mainly produced in the adrenals and the gonads. In the gonads DHEA is further metabolized to sex hormones, but in the adrenals, due to the presence of SULT2A1, most of the DHEA is sulfonated. In case of

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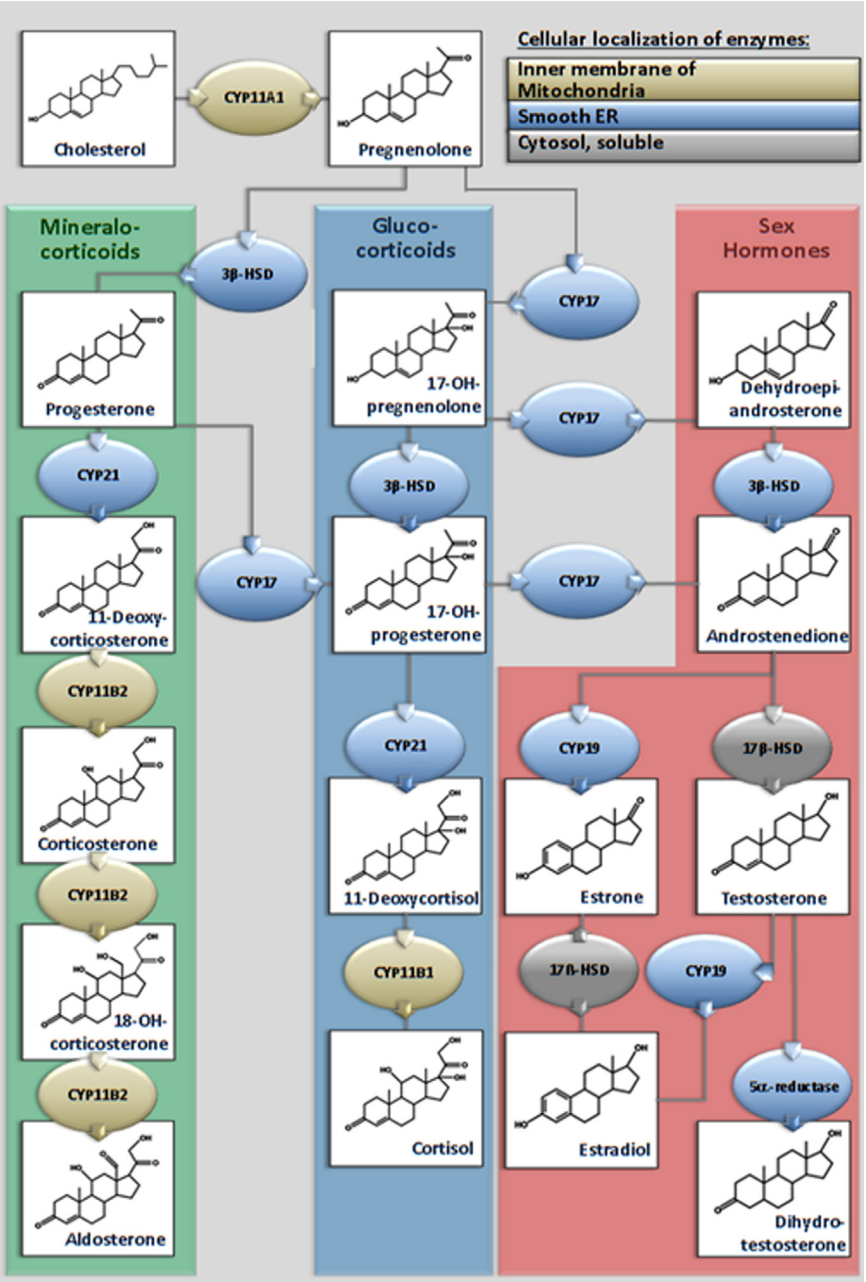

Fig. 1. Steroid hormone biosynthesis involving six cytochromes P450 and three hydroxysteroid dehydrogenases.

altered DHEA sulfonation, unconjugated DHEA accumulates, yielding to an excess of androgens and thus, virilization occurs in women [10].

On the other hand, excess of sulfonated steroids can lead to diseases, like the X-linked ichtiosis, which is caused by the accumulation of cholesterol sulfate in the epidermis [11].

Cell uptake of steroid sulfates depends on transporters of the SLC or SLCO family [12–14] as their hydrophilicity caused by the sulfate moiety hinders a passive passage. These transporters are expressed in many tissues, like liver, ovary, adrenal gland and hippocampus [15].

Sulfonated steroids have been regarded for a long time either as an end-product of xenobiotic metabolism designated for renal clearance [16] or as an inactive reservoir for unconjugated, active steroids [17]. Although the knowledge about the physiological role of steroid sulfates is still scarce, investigations about their biological function increased in the last decade. Recent studies showed that sulfonated steroid metabolites are involved in many different processes in mammalian organisms. Cholesterol sulfate (CS) possesses a stabilizing function in cell membranes; it is involved in the regulation of serine proteases and, moreover, in the differentiation of keratinocytes [18]. Recently, DHEAS was shown to induce a non-classical signaling pathway in spermatogenic cells [19]. Pregnenolone sulfate (PregS), on the other hand, represents a reservoir for pregnenolone (Preg), the precursor of mineralo-, and glucocorticoids, as well as sex hormones and it is described to act as neurosteroid modulating a big variety of ion channels, transporters, and enzymes [15]. For example, PregS was demonstrated to inhibit GABA-receptors [20], which play a crucial role in the neuronal network and to modulate *N*-methyl-D-aspartate (NMDA)-receptors. These receptors, which are essential for neuronal development and synapse formation, are heterodimers formed by several subunits (NR1, NR2A–D, NR3A–B). PregS modulates these receptors by enhancing the generation of

NR1/NR2A and NR1/NR2B receptors and inhibiting the formation of NR1/NR2C and NR1/NR2D receptors [21]. Further studies revealed that PregS promotes NMDA-receptor insertion in the cell surface [22] and thus, enhances the function of NMDA-receptors.

Moreover, PregS seems to play an essential role in reproduction as extremely increased concentrations during birth, pregnancy and parturition indicate [15].

It was demonstrated that PregS can be formed from CS through a side-chain cleavage reaction catalyzed by CYP11A1 [23] and seems to be metabolized to further sulfonated steroids as Korte et al. showed using tissue from human adrenals [24]. These findings inevitably lead to the question, whether a steroidogenic pathway for sulfonated steroids exists similar to the one described for free steroids. Trying to elucidate this question, we decided to investigate whether PregS serves as substrate for CYP17A1, a microsomal cytochrome P450 involved in the steroid hormone biosynthesis. CYP17A1 catalyzes the hydroxylation at position C17 of Preg or progesterone (Prog) yielding 17OH-Preg and 17OH-Prog, respectively, and subsequently catalyzes a 17,20-lyase reaction yielding dehydroepiandrosterone (DHEA) and androstenedione (andro) [25] (Fig. 2). In human and bovidae families (sheep, goat, bovine, bison), CYP17A1 exhibits lyase activity only toward delta5-steroids, like Preg and 17OH-Preg [26]. In these mammalian species CYP17A1 hydroxylates Prog at position C17 but 17OH-Prog is not further converted to androstenedione. Compared to the 17-hydroxylase activity, the 17,20-lyase reaction using 17OH-Preg as substrate is weak, but it is strongly enhanced in the presence of cytochrome b_5 (b_5) [27] or through phosphorylation of CYP17A1 at position 258 of its amino acid chain [28].

Here, we investigated the reaction of CYP17A1 with PregS as substrate in a reconstituted *in-vitro* system, consisting of recombinantly expressed and purified CYP17A1 and its electron transfer partner CPR, and were able to demonstrate for the first time its conversion to 17OH-PregS, but not to DHEAS.

2. Materials

Steroids were obtained from Sigma–Aldrich (Taufkirchen, Germany) or from C/D/N Isotopes Inc. (Quebec, Canada). 1,2-Dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), kanamycin sulfate, arabinose, magnesium chloride, sodium hydroxide, sulfur trioxide triethylamine, sulfatase from *Helix pomatia* Type H-1 and HPLC-grade acetonitrile were from Sigma–Aldrich (Taufkirchen, Germany). Yeast extract, technical was from Becton, Dickinson and Company (Heidelberg, Germany). Pepton, pancreatically digested and Na-acetate were from Merck (Darmstadt, Germany). LCMS grade water and ammonium hydroxide were purchased from Fluka (Taufkirchen, Germany). Methanol, pyridine and ethanol were obtained from Merck (Darmstadt, Germany). SepPak C18 (360 mg) columns were from Waters Corporation (Milford, MA, USA). DMEM/F12 cell culture media and fetal calf serum were from Gibco by Life Technologies (California, USA). Imidazole and ampicillin were from CarlRoth (Karlsruhe, Germany). NADPH was from Carbolution (Saarbrücken, Germany). Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Roche (Basel, Switzerland). Protino Ni-NTA was obtained from Macherey-Nagel (Düren, Germany). 17OH-PregS was synthesized in the Steroid Research & Mass Spectrometry Unit, Division of Pediatric Endocrinology & Diabetology, Center of Child and Adolescent Medicine, Justus-Liebig University. All other chemicals were of highest purity available.

3. Experimental

3.1. Construction of recombinant expression plasmids in E. coli

The plasmid pET-17b was utilized to express bovine CYP17A1, b_5 and CPR in *E. coli*. Each cDNA was cloned via the restriction sites NdeI and BamHI into the vector. The cDNAs of b_5 and CPR were obtained through amplification from a cDNA library of bovine liver (Zyagen, California, USA). CYP17A1 cDNA was kindly provided by Prof. M. Waterman (Vanderbilt University, Nashville, USA). The amino acid sequence of CYP17A1 is lacking its N-terminal hydrophobic anchor [29] and is extended at the C-terminus by a hexa-histidine-tag to facilitate purification. The CPR amino acid sequence is extended at the C-terminus by three glycines and six histidines and at the N-terminus has a lack of the first 27 amino acids [30]. Cytochrome b_5 is extended at the C-terminus by a hexa-histidine-tag. For human cell culture studies, the plasmid pVITRO1-neo-mcs (Invivogen) was used to express CYP17A1 and CPR. The cDNAs of CYP17A1 and CPR were cloned via the restriction sites AgeI and BsrGI (CYP17A1) and BspEI and AvrII (CPR) into the vector. Both cDNAs were obtained from a cDNA library of bovine adrenal (CYP17A1) and bovine liver (CPR).

3.2. Protein expression and purification

Bovine CYP17A1 was co-expressed with chaperones GroEL and GroES similar to CYP21 expression [31]. As host *E. coli* strain C43DE3 was used. The expression was performed in 2 L baffled flasks containing 400 ml TB medium supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin. The expression culture was inoculated from an overnight culture and grown at 37 °C and 120 rpm. Protein expression was induced at OD_{600} = 0.6 with 1 mM IPTG, 4 mg/ml arabinose, 1 mM δ-ALA and 50 μg/ml ampicillin. Afterwards, temperature was decreased to 26 °C and the cells incubated at 95 rpm for 48 h. Cells were harvested and sonicated as described elsewhere [32]. The purification was performed as described for CYP11B1 [33].

Bovine CPR was expressed in *E. coli* strain C43DE3 using similar conditions as described for bovine CYP17A1 with slight modifications: 2 L baffled flasks containing 300 ml TB medium were used and after induction of protein expression temperature was reduced to 30 °C and incubated at 100 rpm for 30 h. Cells were harvested and sonicated as described elsewhere [32]. CPR was purified via IMAC as described elsewhere [33] with 40 mM imidazole in the washing buffer and 200 mM imidazole in the elution buffer.

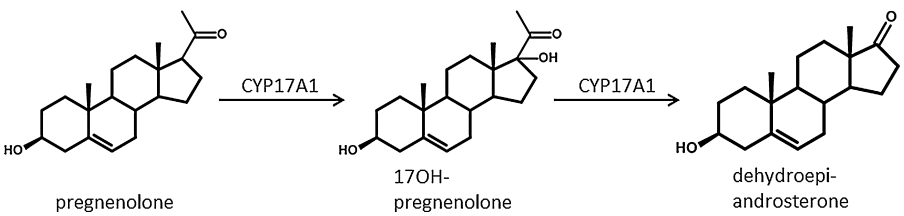

Fig. 2. Reaction catalyzed by CYP17A1. Pregnolone is hydroxylated at position C17 yielding 17OH-pregnenolone, and subsequently the 17,20-carbon–carbon bond scission takes place yielding DHEA.

Bovine b_5 was expressed according to Mulrooney et al. [34] using *E. coli* strain C43DE3. Purification was done as described for bovine CPR.

3.3. UV–vis spectroscopy

The protein concentration of the CPR was determined using a molar extinction coefficient $\varepsilon_{585} = 2.4\,mM^{-1}\,cm^{-1}$ [35]. Cytochrome b_5 concentration was calculated by using a difference extinction coefficient of $185\,mM^{-1}\,cm^{-1}$ for the absorbance change at 424–409 nm [34]. CYP17A1 concentration was calculated from a reduced carbon-monoxide difference spectroscopy according to Omura and Sato [36] with $\varepsilon_{448} = 91\,mM\,cm^{-1}$. Binding of Preg and PregS was investigated using difference spectroscopy, which was carried out in tandem cuvettes according to Schenkman [37]. Preg and PregS were dissolved in DMSO. The buffer utilized was composed of 50 mM HEPES (pH 7.4), 20% glycerol, and 100 μM dilauroyl phosphatidylcholin (DLPC). Difference spectra were recorded from 370 to 450 nm. To determine the dissociation constant (K_d), the values from three titrations were averaged and the resulting plots were fitted with hyperbolic regression.

3.4. Enzyme activity assay

In-vitro substrate conversion assays were done as described elsewhere [38] with slight modifications. The conversion buffer (50 mM HEPES, pH 7.4, 20% glycerol, 100 μM DLPC) contained 1 μM CYP17A1, 1 μM CPR, 1 mM $MgCl_2$, 5 mM glucose-6-phosphate, 1 U glucose-6-phosphate dehydrogenase for NADPH regeneration and varying concentrations of Preg or PregS as substrate. When substrate conversion assays were performed to investigate the influence of b_5, 4 μM of b_5 were added to the conversion buffer. After starting the reaction with 1 mM NADPH at 37 °C for 40, 50 and 60 min, the conversion was stopped in a boiling water bath for 5 min. Preg and the resulting product 17OH-Preg had to be converted to the corresponding 3-one-4-en form by cholesterol oxidase, to be detectable at 240 nm during HPLC analysis. Therefore, cholesterol oxidase was added to the boiled reaction mixture and incubated for 40 min at 37 °C according to Yamato [39]. Cortisol was added as internal standard. The reaction was stopped by addition of one reaction volume of ethylacetate. Extraction of steroids was performed twice with ethylacetate and the ethylacetate phase was evaporated. The steroids were resuspended in 20% acetonitrile for subsequent HPLC analysis. Steroids were separated on a Jasco reversed phase HPLC system LC2000 using a 4 mm × 125 mm Nucleodur C18 reversed phase column (Macherey-Nagel) with an acetonitrile/water gradient and a flow rate of 1 ml/min. Detection of the steroids was performed at 240 nm within 30 min at 40 °C. For analysis of sulfonated steroids mass spectrometry and liquid chromatography were utilized.

3.5. Substrate conversion in SOAT-HEK293 cells

For substrate conversion studies, 6-well plates were coated with poly-D-lysine for better attachment of the cells as described elsewhere [40]. 3×10^5 cells/well were plated and maintained in DMEM/F-12 (Invitrogen) medium with 10% fetal calf serum (Sigma), L-glutamine (4 mM), penicillin (100 units/ml) and tetracycline (1 μg/ml) (for SOAT expression). The cells were grown for 16 h at 37 °C and 5% CO_2 until they reached a confluency of 70%. Afterwards, they were transiently transfected with a plasmid containing the cDNAs of CYP17A1 and CPR with the Effectene Transfection Kit of Qiagen. After 5 h of incubation (37 °C and 5% CO_2), PregS (10 and 20 μM) was added and the cells were further incubated for 70 h, 37 °C and 5% CO_2. Subsequently, samples of 1 ml/well were collected and prepared for LC/MS-analysis.

3.6. Sample analysis by liquid chromatography-tandem mass spectroscopy (LC-MS/MS)

The studies were performed using a triple quadrupole mass spectrometer (TSQ, Quantum Ultra, Thermo Fisher Scientific, Dreieich, Germany) with electrospray ionization (ESI) in negative mode. Mass spectrometric parameters for sulfonated steroids were as described elsewhere [41]. For the in-house synthesized compound, 17OH-PregS, the collision energy and tube lens values were calculated, being similar to other sulfonated steroids. The selected quantifier transition was 411 → 97. The collision energy was 32 eV and the tube lens value was 180 V. A C18 reverse phase column was used for the chromatographic method (Hypersil Gold column 50 × 2.1 mm, 5 μm, Thermo Fisher Scientific, Dreieich, Germany), mounted within a HPLC system (Agilent 1200SL, Waldbronn, Germany). Different concentrations of pure water and pure methanol (MeOH) were applied to achieve the separation of the compounds (Table 1). The retention time for both the enzymatically obtained 17OH-PregS and for the synthetic 17OH-PregS were the same.

3.7. Sample preparation and calibrations

The samples were always diluted due to the high concentrations of the analytes, and of the presence of buffer components that may affect the ionization process. At least two dilutions had to be used for each sample, as the concentrations of PregS and 17OH-PregS after enzymatic conversion were very different (high concentrations of PregS when compared to 17OH-PregS concentrations). The dilutions were made in a mixture containing MeOH, water and ammonia to facilitate dissolution and to improve the ionization of the analytes in negative mode (90.00% MeOH, 9.95% H_2O and 0.05% NH_3). In case a precipitate was observed, the samples were centrifuged and the clear supernatant was analyzed. The dilution was usually performed in glass vials which were first vortexed and later shaked for 30 min to allow equilibration with the internal standards. The calibration points for the calibration curve were prepared adding the same amount of buffer and internal standards (IS). d6DHEAS was used as IS for 17OH-PregS and d4PregS for PregS. Calibrations showed values always higher than 0.99 for R^2. Data analysis was performed with Thermo Xcalibur 2.1 software (Thermo Fisher Scientific, Dreieich, Germany).

3.8. Determination of kinetic parameters

K_m and k_{cat}-values were determined by plotting the substrate conversion velocities versus the corresponding substrate concentrations and by using Michaelis–Menten kinetics (hyperbolic fit) for Preg or Hill kinetics (sigmoidal fit) for PregS utilizing the program OriginPro 8.6G.

Table 1
LC Table-flows and gradients.

Start	Seconds	Flow	% H_2O	% MeOH
0.00	60	0.45	70	30
1.00	60	0.50	55	45
2.00	20	0.50	40	60
2.33	80	0.50	5	95
3.67	60	0.50	40	60
4.67	30	0.50	55	45
5.17	30	0.45	70	30

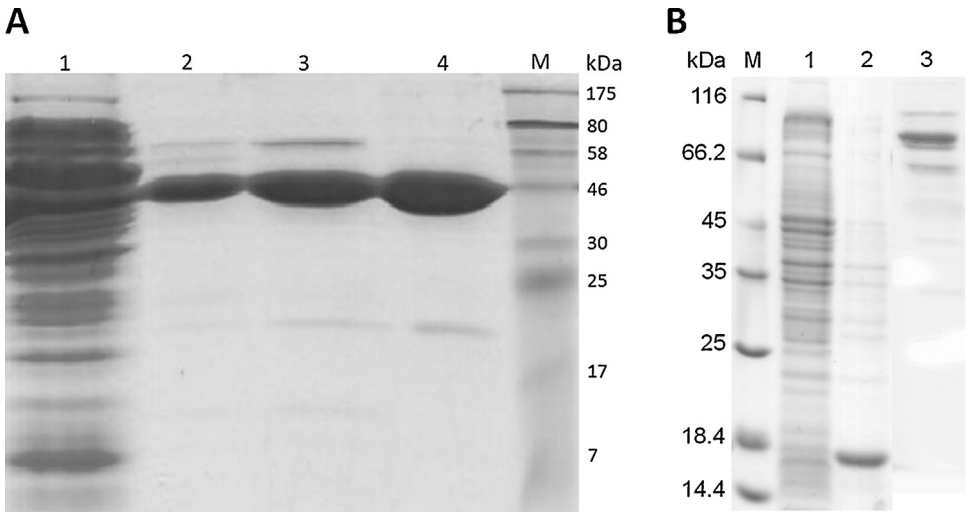

Fig. 3. SDS-polyacrylamide gel electrophoresis. (A) Lane 1: supernatant; lane 2: CYP17A1 after purification via IMAC; lane 3: CYP17A1 after purification via IMAC and DEAE-sepharose; lane 4: CYP17A1 after purification via IMAC, DEAE-sepharose and SP-sepharose; lane M: marker. (B) Lane M: marker; lane 1: supernatant; lane 2: b_5 after IMAC purification; lane 3: CPR after IMAC purification.

3.9. Synthesis and purification of 17OH-PregS

The method is based on Dusza et al. [42], with some modifications. Briefly, 1 mg of 17OH-Preg and 0.85 mg of triethylamine sulfur trioxide were mixed in 100 μl of anhydrous pyridine, in a 2 ml glass vial. The mixture was shaked at low speed during 3 h at room temperature, and consecutively evaporated. The residue was redissolved in 500 μl of ethanol. Then, 500 μl of NaOH 0.2 M in methanol were added, and a white precipitate was observed. After evaporation, the precipitate was washed twice with methanol.

Several HPLC-MS/MS analyses were performed to assess the purity of the reaction product. An aliquot of the solution was dissolved in a mixture of water:MeOH (1:1 v/v) and analyzed in positive full scan mode as previously described [41]. The product was free of 17OH-Preg, as no peak was present at 4.2 min, whereas a peak with m/z 297.2 at 0.75 min was observed. This mass-to-charge ratio value is identical to that of 17OH-Preg, and it is due to $(M - H_2O - H_2SO_4 + H)^+$.

The same chromatographic method was applied to ESI negative mode. The m/z value of the peak at 0.75 min was 411, molecular ion. A minority peak at 0.18 min was then found in negative mode. This peak corresponded to the disulfate fraction of 17OH-Preg, with m/z of 245, $(M - 2H)^{-2}$. Sulfonation of the hydroxyl group in position 3beta is favored over the sterically hindered 17alpha position.

Next, the mixture was purified with a solid phase extraction step. The precipitate was re-dissolved in a water:MeOH solution (70:30 v/v) and loaded onto a Sep-Pak column after activation. A gradient of MeOH was then applied (0–100%) and the fractions corresponding to 40–50% of MeOH were collected, discarding the rest. Those fractions were free of disulfate component, showing a clear single peak in the same chromatographic conditions described before.

The fractions collected after purification step were evaporated under nitrogen flow and weighed. A standard working solution was then prepared (250 μg/mL in MeOH).

In order to calculate the purity of the final solution, two aliquots, of 20 μl and 40 μl, were treated with 75 units of sulfatase at 37 °C during 6 h. The reaction products were then quantified using the aforementioned procedure described in Galuska et al. [41] for 17OH-Preg quantification. The analysis in negative mode proved that they were free of 17OH-PregS. The calculated purity was 90%.

4. Results and discussion

The levels of sulfonated steroids exceed in many species and tissues considerably the levels of the corresponding unconjugated steroids. In human, the plasma level of PregS varies significantly during life. The highest level is reached after birth with concentrations between 2–3 μM, depending on the gender, and afterwards rapidly decreases in the first year up to concentrations of about 30–50 nM [43]. During and after adrenarche, the PregS

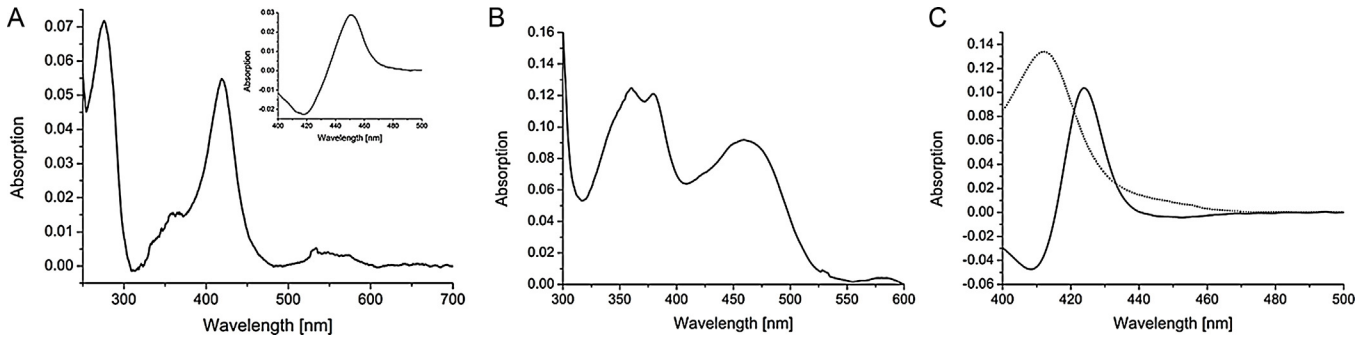

Fig. 4. Spectra of purified recombinant CYP17A1 (A), CPR (B) and b_5 (C). (A) Full spectrum of purified CYP17A1 recorded from 700 nm to 260 nm. The inset shows the CO-difference spectrum from 500 nm to 400 nm. (B) Spectrum of purified CPR recorded from 600 nm to 300 nm. (C) Spectrum of purified b_5 in its oxidized form (dotted line) and difference spectrum (reduced–oxidized) (solid line).

Table 2
Amount of heterologously expressed CPR, b_5 and CYP17A1.

	Yield of purified protein (mg/l)
CPR	34
b_5	22
CYP17A1	73

level in plasma again increases, achieving concentrations of 130–140 nM [43]. Although PregS represents a steroid hormone displaying high abundance in human and other mammalian organism [44] and exceeding its unconjugated form (0.9–6 nM [45]) by several folds, its influence on steroid hormone biosynthesis is poorly investigated. Moreover, it was already demonstrated that CS is converted to PregS through a CYP11A1 dependent reaction [23] leading necessarily to the issue whether also other sulfonated steroids are converted by enzymes involved in the steroid hormone biosynthesis, equally to free steroids. Korte et al. [24] suggested that CS is converted to PregS and afterwards to dehydroepiandrosterone sulfate (DHEAS), based on their observation that the fetal plasma contains very high levels of PregS and DHEAS. However, experiments to support this assumption were not performed in this early study. The conversion of PregS to sulfonated products was demonstrated by Jaffe et al. [46] and Lamont et al. [47] in the early 70 s, but, they did not identify the enzyme involved in this reaction. Therefore, it was the aim of our study to investigate, whether CYP17A1 is able to convert PregS.

4.1. Expression and purification of CPR, b_5 and CYP17A1

The three proteins necessary to perform *in-vitro* conversions of PregS were expressed in bacteria and purified from there.

For expression of the three proteins, *E. coli* C43DE3 were chosen as host, as this strain was described to display high expression yields for membrane-bound cytochrome P450's [33,38,48] and other membrane-bound proteins [49].

CPR and b_5 were purified by IMAC NTA, whereas CYP17A1 was purified at first by IMAC NTA followed by an anion exchange DEAE-sepharose and, in a last step, by an ion exchange SP-sepharose. The purity of the proteins was determined via SDS-gel electrophoresis (Fig. 3), in which a single band was obtained for CYP17A1 at 45 kDa and for b_5 at 17 kDa. CPR showed a main band at 70 kDa and two unknown bands located above and below the band corresponding to CPR. Further experiments, like the cytochrome c test (data not shown), spectral characterization (Fig. 4B) and substrate conversion experiments with CYP17A1 suggested an undisturbed catalytic function of CPR.

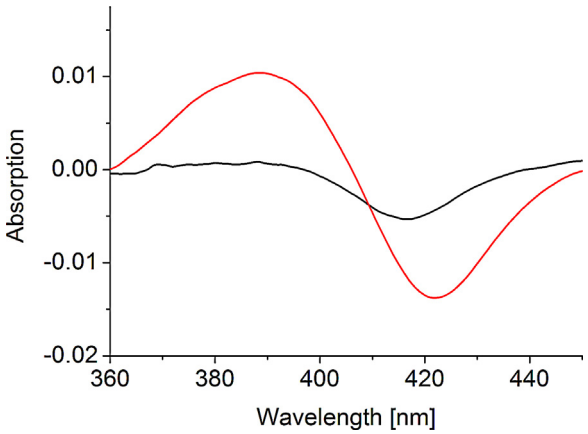

Fig. 6. Difference spectroscopy of CYP17A1 titrated with 150 μM PregS (red) and 150 μM 17OH-PregS (black). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The UV–vis spectrum categorizes the expressed CYP17A1 as a low-spin protein with the characteristic cytochrome P450 absorption spectrum: the Q bands at 569 nm (α-band) and 541 nm (β-band), the Soret band at 418 nm, the UV band at 360 nm and the protein-band at 278 nm (Fig. 4A). Moreover, the enzyme displays a typical CO-difference spectrum, with a major peak at 450 nm and a minor peak at 420 nm (Fig. 4A inset). Spectral analysis of CPR also reveals its typical absorption spectrum: the enzyme shows its major peaks at 360 nm, 380 nm, and at 453 nm, indicating its air-stable semiquinone (FMNH·, FAD) form [50] (Fig. 4B). Cytochrome b_5 exhibits a major peak at 412 nm in its oxidized state, as well as a major peak at 424 nm and a minor peak at 409 nm in the difference (reduced–oxidized) spectrum [34] (Fig. 4C).

The amounts of CPR, b_5 and CYP17A1, respectively, obtained after purification are listed in Table 2.

Using *E. coli* C43DE3 as host, the expression yield of b_5 is similar to literature data [51], that of CYP17A1 achieved 73 mg/l, which is a great improvement compared to published data [29]. The expression yield of bovine CPR described here for the first time was 34 mg/l.

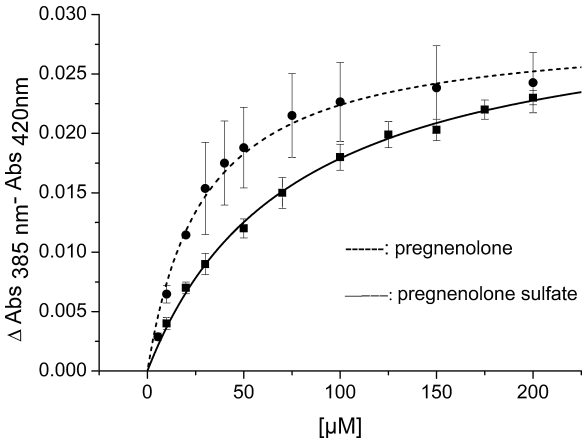

Fig. 5. Determination of substrate binding affinity to CYP17A1. The enzyme (1 μM) was titrated with increasing concentrations of Preg (dotted line) and PregS (solid line) dissolved in DMSO. The absorbance changes were plotted against the substrate concentration and fitted as described; R^2: 0.99; $n = 3$.

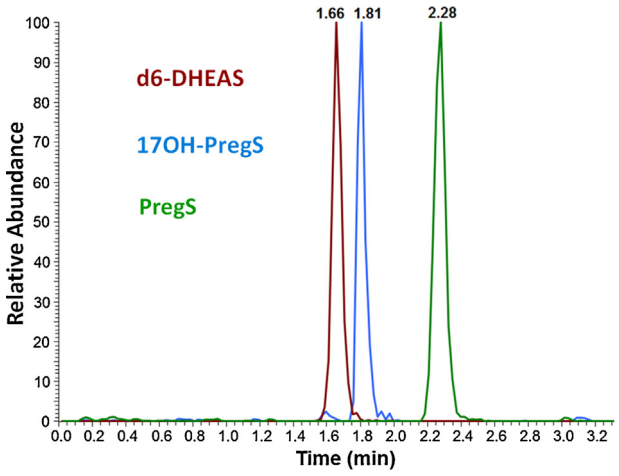

Fig. 7. Merged chromatogram of the analytes obtained from a conversion sample, as detected by ID-LC-MS/MS (peak at 1.66 min is d6-DHEAS, peak at 1.91 min is 17OH-PregS whereas peak at 2.28 min is PregS).

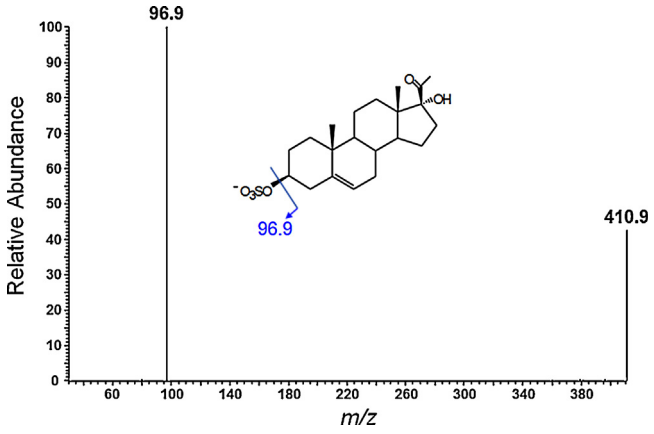

Fig. 8. Product-ion mass spectra of the molecular ion of 17OH-PregS (collision energy 32 eV). The predominant fragment ion at m/z 96.9 is due to the hydrogensulfate anion (HSO_4^-).

4.2. Determination of dissociation constants of CYP17A1 with pregnenolone and pregnenolone sulfate

In order to investigate the binding affinity of Preg and PregS with CYP17A1 in detail, we performed spectroscopic studies to determine dissociation constants (K_d). The experiments revealed a K_d-value for CYP17A1 and Preg of 28.9 ± 3.1 μM and for CYP17A1 and PregS of 74.8 ± 4.2 μM (Fig. 5), indicating a higher affinity of CYP17A1 toward the unconjugated pregnenolone.

Moreover, we aimed to compare the affinity of CYP17A1 toward PregS and its product 17OH-PregS. As the spectral change of CYP17A1 in the presence of 17OH-PregS was too low to determine the K_d-value, we compared the spectral change of CYP17A1 in the presence of 150 μM PregS and 150 μM 17OH-PregS (Fig. 6). In contrast to PregS, which induces a type I shift typical for substrates, 17OH-PregS does not, or only to a very low extend, influence the spectral properties of CYP17A1. This is an indication that 17OH-PregS might not be in a binding position suitable for further conversion.

4.3. Sample analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

In order to analyze the conversion of sulfonated steroids, we established a new LC-MS/MS method. This is the first time, to our knowledge, that 17OH-PregS has been quantified by LC-MS/MS, which represents the technique of choice to analyze sulfonated steroids [41]. In Fig. 7 a merged chromatogram for PregS, 17OH-PregS and DHEAS is displayed (detection by multiple reaction monitoring). All three steroid sulfates are baseline-resolved.

Fig. 8 shows the product-ion mass spectra of 17OH-PregS (molecular ion with m/z value of 411). This methodological development facilitates the analytical basis for the PregS metabolism in our reconstituted *in-vitro* system.

4.4. CYP17A1 dependent 17OH-pregnenolone and 17OH-pregnenolone sulfate conversion

Since we could clearly demonstrate that PregS efficiently interacts with CYP17A1, the next step was to evaluate whether the sulfonated steroid can be converted by CYP17A1. In mammalian steroidogenesis, CYP17A1 catalyzes the conversion of Preg to 17OH-Preg and DHEA. In our reconstituted *in-vitro* system nearly no lyase reaction and thus very little DHEA formation could be observed, because of the absence of b_5. Using PregS, we could demonstrate that it serves as a substrate for CYP17A1 and is converted to a considerable amount to 17OH-PregS. The kinetic parameters were determined to be $K_m = 30.00 \pm 3.31$ μM and $k_{cat} = 0.60 \pm 0.02$ (min^{-1}) for 17OH-Preg formation using Preg as substrate and $K_m = 36.72 \pm 3.22$ μM and $k_{cat} = 0.44 \pm 0.03$ (min^{-1}) for 17OH-PregS formation when PregS was utilized as substrate (Fig. 9). Comparing the curve shapes of the kinetics of CYP17A1 with Preg or with PregS a significant difference can be observed: in contrast to the hyperbolic shape of CYP17A1 with Preg (Fig. 9A), the kinetics of CYP17A1 with PregS display a sigmoidal form (Fig. 9B). When considering the surface of the CYP17A1 crystal structure [52], several positively charged regions that might act as potential interaction sites with the negatively charged sulfate moiety of PregS are found. This could lead to a decreased conversion rate at low substrate concentrations until these unspecific binding sites are saturated and could thus explain the sigmoidal character of the kinetics of CYP17A1 with PregS.

In Table 3, the kinetic parameters are summarized, revealing a catalytic efficiency k_{cat}/K_m for CYP17A1 toward Preg of 20.0 ($min^{-1}\,mM^{-1}$), which is two-fold higher compared to the catalytic efficiency toward PregS with $k_{cat}/K_m = 11.9$ ($min^{-1}\,mM^{-1}$).

Consequently, the nearly 2-fold increased catalytic efficiency of CYP17A1 converting Preg in comparison with PregS seems to be due to a higher affinity, as the K_d- and K_m-values indicate and to an higher k_{cat}-value. The K_d-value for CYP17A1 and PregS is about 2.5-fold higher compared with Preg (Fig. 5). Thus, PregS possesses less affinity to CYP17A1. By favoring Preg as a substrate, CYP17A1 might be able to compensate for the lower concentration

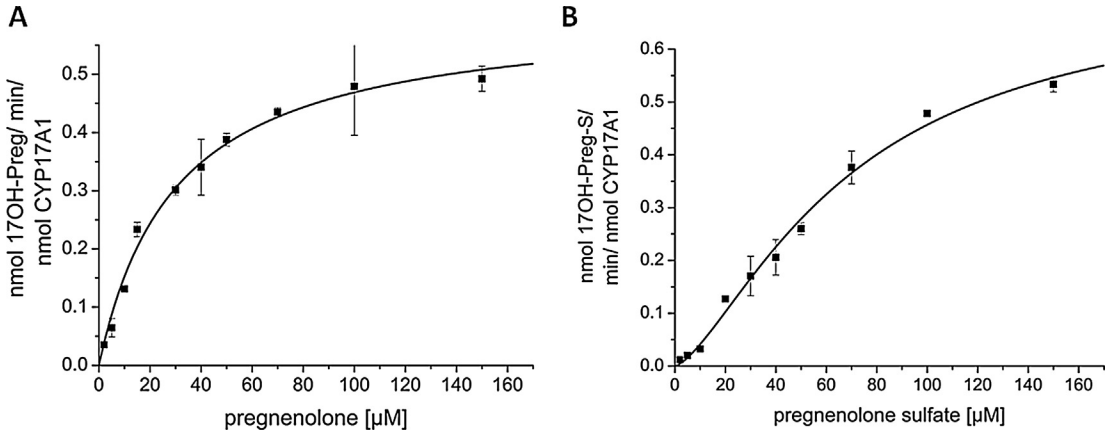

Fig. 9. (A) Kinetics of Preg to 17OH-Preg conversion catalyzed by CYP17A1 using CPR as redox partner; R^2: 0.99. (B) Kinetics of PregS to 17OH-PregS conversion catalyzed by CYP17A1 utilizing CPR as redox partner; R^2: 0.98.

Table 3
Kinetic parameters of CYP17A1 metabolizing Preg and PregS, respectively.

Pregnenolone			Pregnenolone sulfate		
K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
0.030 ± 0.003	0.60 ± 0.02	20.00	0.036 ± 0.003	0.44 ± 0.03	11.98

of Preg in the plasma. However, since concentrations of Preg and PregS in tissues expressing CYP17A1 are unknown, the physiological importance of this observation is not clear yet.

4.5. Effect of b_5 on CYP17A1 dependent substrate conversions

The first reaction step of CYP17A1 consists of a 17-hydroxylation of Preg and is followed by a 17,20-carbon–carbon bond scission yielding DHEA, as shown in Fig. 2. The augmentation by several folds of the lyase of CYP17A1 in the presence of b_5 is well reported [53,54]. It is postulated that the effect of b_5 on this second reaction step of CYP17A1 is based on an allosteric interaction of b_5 with CYP17A1, enhancing the alignment of the iron–oxygen complex onto the C20 rather than the C17 atom of the steroid, hence augmenting the lyase reaction [27]. Further studies are necessary to confirm this hypothesis. Very recently, Scott and co-workers [55] demonstrated that structural changes occur at the proximal surface of CYP17A1 depending on the substrate binding. In reverse, they claim that binding of b_5 to the proximal surface of CYP17A1 induces changes in the active site of CYP17A1, leading to an enhanced lyase activity.

For this reason, we investigated the effect of b_5 on CYP17A1 dependent conversion of Preg and PregS. In our reconstituted *in-vitro* system, the lyase reaction was nearly absent without addition of b_5. Using 30 μM Preg as substrate for CYP17A1, 0.08 ± 0.02 μM DHEA is formed. In contrast, in the presence of b_5 the CYP17A1 dependent conversion of Preg to DHEA is strongly increased, forming 1.02 ± 0.07 μM DHEA, thus augmenting the lyase activity about 10-fold (Fig. 10). The relatively low lyase activity is in accordance with previous studies of Barnes et al. [56] demonstrating that the formation of DHEA is time-depending and starts after accumulation of the intermediate 17OH-Preg. Studies using 17OH-Preg instead of Preg as substrate also show higher levels of DHEA formation [57].

To our surprise, the conversion of PregS was not influenced by addition of b_5. Whether or not b_5 was present, no scission of the 17,20-carbon–carbon bond took place and, consequently, no DHEAS was formed. It seems that the sulfate moiety hinders the alignment of the iron–oxygen complex onto the C20 atom of 17OH-PregS, possibly due to the increased size of the molecule and/or to the negative charge of the sulfate, which finally prevents the lyase reaction of CYP17A1 on 17OH-PregS. As discussed before and shown in Fig. 6, difference spectroscopy performed with CYP17A1 in the presence of 17OH-PregS showed that this metabolite might not to be capable to enter in the active site of CYP17A1, as nearly no spectral change of CYP17A1 was induced. This means that DHEAS seems to be produced solely by sulfonation of DHEA catalyzed by sulfotransferases, whereas PregS and 17OH-PregS can either be synthesized by sulfonation of unconjugated Preg and 17OH-Preg or by further conversion of CS or PregS.

4.6. CYP17A1 dependent substrate conversion in SOAT-HEK293 cells

To investigate whether the results concerning the conversion of PregS observed in our reconstituted *in-vitro* system may possess potential physiological meaning, we performed conversion experiments in SOAT-HEK293 cells. This cell line exhibits an integrative gene encoding for the human sodium-dependent organic anion transporter (SOAT), which was shown to display high specificity toward PregS [40]. SOAT-HEK293 cells were transiently transfected with a plasmid containing genes encoding for CYP17A1 and its electron transfer partner, CPR. LC-MS/MS analysis after three days of incubation with PregS could clearly confirm the formation of reasonable amounts of 17OH-PregS. As shown in Fig. 11, 2.30 ± 0.30 μM 17OH-PregS was synthesized when 20 μM PregS was used as substrate. Also in this case, no DHEAS could be detected, confirming our *in-vitro* results, where no 17,20-lyase activity could be attributed to CYP17A1 using PregS as substrate. In contrast, when using 20 μM Preg as substrate, 5.30 ± 0.35 μM 17OH-Preg, as well as 0.86 ± 0.2 μM DHEA are formed (Fig. 11).

The utilized cell line exhibits RNA expression of b_5 and MAPK14 as "The Human Protein Atlas" indicates. MAPK14 (p38alpha) is a kinase that phosphorylates CYP17A1 at position 258 of the amino acid chain [58]. This post-translational modification of CYP17A1 is described to enhance the lyase reaction of CYP17A1, similar to the effect of b_5 [28]. The presence of these two proteins (b_5, MAPK14) obviously supports the formation to 17OH-Preg and DHEA using Preg as substrate. However, the lyase reaction of CYP17A1 does not take place in these cells with PregS as substrate. In cell culture as well as in the reconstituted *in-vitro* system 17OH-PregS represents the end product of the CYP17A1 catalyzed reaction using PregS as substrate.

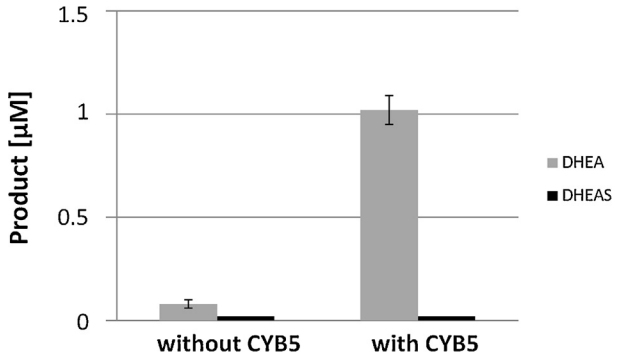

Fig. 10. Comparison of DHEA and DHEAS formation by CYP17A1 in absence and in the presence of b_5 using Preg and PregS, respectively, as substrate ($n = 5$).

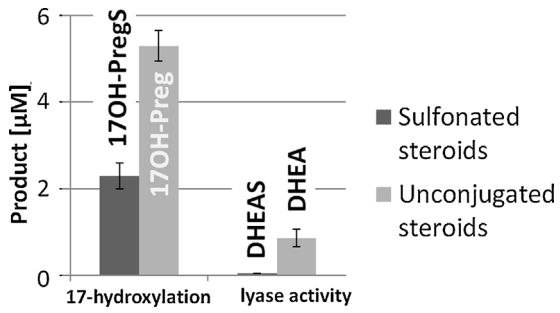

Fig. 11. Conversion of 20 μM PregS (black) and 20 μM Preg (grey) in SOAT-HEK293 cells. The product formation analyzed by LC-MS/MS (black) and HPLC (grey) is represented as mean ± standard deviation of five (black) and three (grey) individual experiments.

5. Summary

In this work we could clearly demonstrate that PregS serves as a substrate for CYP17A1, being converted to 17OH-PregS. Summarizing, we demonstrated for the first time that CYP17A1, which is involved in the steroid hormone biosynthesis, can convert sulfonated steroids in a similar manner as free steroids indicating a potential alternative steroidogenic pathway for sulfonated steroids. As already described, this pathway is initiated by the side-chain cleavage of CS by CYP11A1, yielding PregS [23]. Subsequently, PregS is metabolized in a CYP17A1 dependent hydroxylation reaction to 17OH-PregS, but not to DHEAS. At this point the steroidogenic pathway for sulfonated steroids differs from the one for free steroids yielding DHEA in large quantities. The k_{cat}- and K_m-values, as well as the K_d-value were determined for CYP17A1 with PregS. Moreover, we showed that b_5 does not enhance the 17,20-lyase activity of CYP17A1 when PregS was used as substrate, highlighting fundamental differences between the metabolism of free Preg and sulfonated Preg by CYP17A1. Utilizing a SOAT-HEK293 whole cell system, expressing SOAT, CYP17A1 and CPR, we confirmed the conversion of PregS into 17OH-PregS and the absence of the CYP17A1 dependent lyase reaction, indicating a potential physiological relevance of our findings.

Acknowledgement

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4- GENERAL DISCUSSION

The PhD topic is divided into the following parts:

4.1- STIMULATORY EFFECT OF ESTROGENS ON PREGNENOLONE FORMATION AND CHARACTERIZATION OF NOVEL 2β- AND 16β-HYDROXYLASE ACTIVITY OF CYP11A1

The goal of the first part of the work was to analyze the possible effects of steroid hormone intermediates on the activity of CYP11A1 in a reconstituted *in vitro* system. We reported a successful expression and purification of recombinant CYP11A1 in an *E. coli* cells in which different approaches have been performed. The expression of the protein was performed in *E. coli* C43(DE3) cells, which are particularly suitable for overproduction of membrane proteins [109] such as CYP11A1. The *E. coli* C43(DE3) cells are advisable for the efficient expression of other membrane bound P450s as well, as it was previously demonstrated for the expression of human CYP11B2 [110] and human CYP11B1 [111]. Further, lowering the temperature from 37 °C to 28 °C after the induction of the culture resulted in the increase of the amount of active protein, since a lower cell-divison rate improves the folding of the protein into an active form. Reduced temperature usually helps to control metabolic fluctuations and protein synthesis rates thereby avoiding the formation of insoluble protein aggregates typical of cells at higher growth rate [112].

To achieve high production of CYP11A1, the effect of media simultaneously with the co-expression with molecular chaperones GroEL/GroES on CYP11A1 expression was optimized. The results clearly showed that CYP11A1 expression can be dramatically enhanced by using Terrific broth (TB) medium and co-expressing the chaperones, which displays a significantly higher CYP11A1 yield. The reason for higher expression of CYP11A1 might be due to high nutrients in TB medium, compared to LB medium in which the cells were capable of multiplying, leading to an increase in the number of cells and to an increase in the amount of enzyme [113].

Concerning the expression of CYP11A1 with and without molecular chaperones GroEL/GroES, the co-expression with molecular chaperones resulted in a significantly higher expression level compared to the expression without chaperone (Figure 6A and B). As summarized in Figure 6B, using TB medium in absence of the molecular chaperone, the characteristic peak of cytochrome P450 around 450 nm was not significantly high; concluding that the co-expression of molecular chaperones was effective for the enhancement of CYP11A1 expression due to the great role of molecular chaperones in the correct folding of the protein [114]. These findings are in agreement with results described for human CYP19 [115], bovine CYP21 [116], human CYP11B1 [111], human CYP11B2 [110] as well as for bovine CYP17, cytochrome b_5 and CPR [50]. Noteworthy, previous investigations demonstrated that the co-

expression with chaperones leads to a tight association of P450s to *E. coli* membranes, which results in the high level expression of these enzymes [117].

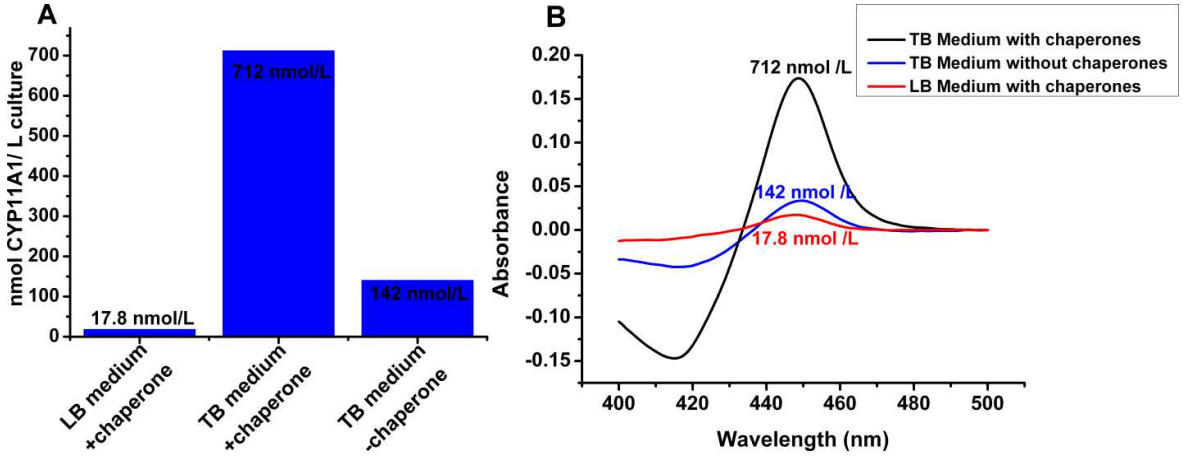

Figure 6: Effect of media and GroEL/GroES chaperones on CYP11A1 expression. A) The amount of purified CYP11A1 in nmol/ l culture, B) The reduced CO-difference spectrum of purified CYP11A1.

In order to investigate the influence of metabolites of steroid hormone biosynthesis on the CYP11A1 catalyzed pregnenolone formation, an *in vitro* assay was carried out in the presence of steroid metabolites similar to the concentration of cholesterol used. We found that only estrogens (estradiol and estrone) enhanced the enzyme activity and increased pregnenolone formation. Therefore, CYP11A1 might play a more vital role in the steroid hormones biosynthesis. However, the concentrations of estrone and estradiol used to investigate their influence on CYP11A1 are beyond physiological serum concentrations. This might also account for the CYP11A1 concentration utilized; although the amounts of steroids and active CYP11A1 in steroidogenic cells remain unclear. In contrast, other tested steroid metabolites besides estrone and estradiol did not significantly affect the CYP11A1 dependent pregnenolone conversion. More recently, a similar study by Neunzig and Bernhardt demonstrated that the CYP11A1 system might display a novel putative regulation of the steroid hormone biosynthesis on the cellular level. They showed that the elevation of dehydroepiandrosterone sulfate (DHEAS) concentrations increased the level of steroids designated for the production of sex hormones and this way might affect the steroid biosynthesis through an increased production of pregnenolone. As a result, the level of the end products of the steroid hormone biosynthesis might also increase [108].

Surprisingly, during the study of the effect of steroid metabolites on natural cholesterol conversion *in vitro*, we detected a slightly lower pregnenolone yield in the presence of DOC, androstendione, testosterone, and DHEA accompanied with additional products in the HPLC measurments (Figure 7). Therefore, *in vitro* conversions were performed with CYP11A1 using these steroids as the potential

substrates in absence of cholesterol. Successful *in vitro* conversion was achieved using DOC, androstenedione, testosterone and DHEA confirming that each of these steroids is converted by CYP11A1 as well as cholesterol.

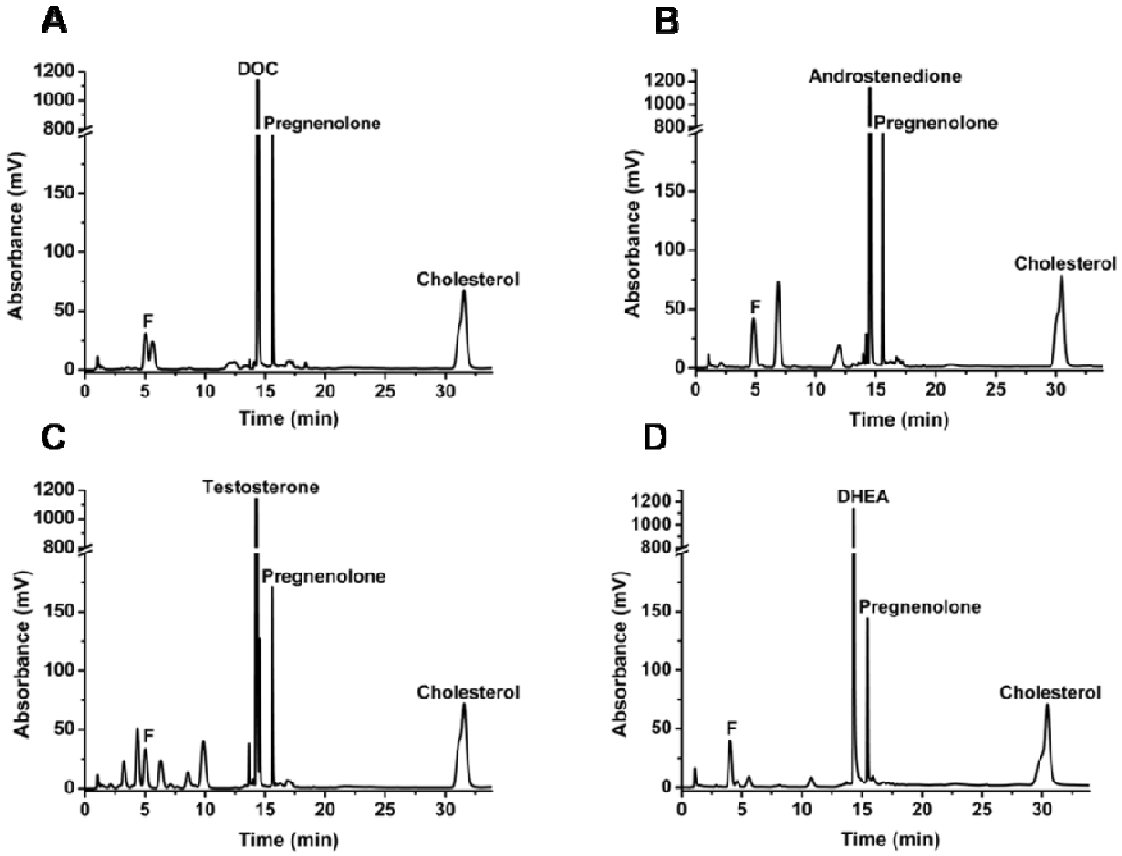

Figure 7: The *in vitro* conversion assay for cholesterol by purified CYP11A1 using cortisol (F) as an internal standard. A, B, C and D represent the cholesterol conversion in the presence of DOC, androstenedione, testosterone and DHEA respectively.

The whole-cell system with P450 enzymes as biocatalysts has the advantage of higher stability of P450 in a living organism and the regeneration of expensive cofactors by the host cell compared to purified enzymes [118]. In this study, a successful CYP11A1 whole-cell system was established for conversions of DOC, androstenedione, testosterone and DHEA. Our study demonstrated that the 6β-position is preferred by CYP11A1 in case of hydroxylation of DOC, androstenedione and testosterone. The 6β-hydroxylase reaction in the microsomes of the liver is the major route of P450 dependent oxidation in the metabolism of many steroids, including testosterone, androstenedione, and progesterone [119,120]. In contrast, the conversion of DOC to 6β-hydroxy-DOC by CYP11A1 provides further evidence for a possible physiological role of this reaction in the adrenal cortex [121]. Furthermore, there is increasing evidence that the 6β-hydroxylation represents a significant pathway for the excretion of steroids in the liver of rat, mouse, rabbit, dog, hamster, guinea pig and man [122]. It has

been demonstrated also that the metabolism of testosterone by rat liver microsomes produced several metabolites including 6β-hydroxy-testosterone. Our results indicated also, that CYP11A1 has, in addition to the 6β-hydroxylase activity, a 2β-hydroxylase activity towards DOC and androstenedione, as well as a 16β-hydroxylase activity towards DHEA. The 16β-hydroxy-DHEA is considered as a one of the normal components of plasma and urine of adult human. It is inferred that 16β-hydroxy-DHEA is secreted directly by the adrenal cortex and probably the gonads [123].

The functions of the other new products are not known and need to be investigated. The 2β-hydroxy-androstenedione for example is a putative inhibitor for the CYP19 enzyme, which is a target in anti-cancer therapy. Therefore the development of specific inhibitors of CYP19 is of great of interest. For this reason, performing more experiments concerning the *in vitro* characterization of CYP19 mediated substrate conversion in the presence of 2β-hydroxy-androstenedione will be particularly important for further investigations. For this purpose and in order to increase the amount of these products, further optimization steps of CYP11A1 will be essential for future studies like the engineering of more efficient CYP11A1 mutants via site-directed mutagenesis. Therefore, employing the homology modeling and substrate docking technology will be very helpful to identify the important amino acid residues for catalytic activity.

4.2- NOVEL AND EFFICIENT *E. coli* BASED BIOCATALYST FOR THE REGIOSELECTIVE ACETYLATION OF THE C21 HYDROXYL GROUP IN STEROIDS VIA BACTERIAL CHLORAMPHENICOL ACETYLTRANSFERASE I (CATI)

The key entrance for this part of the Thesis started during the establishment of the CYP11A1 whole-cell biocatalyst in which the system containing an expression vector (pACYC-FHH2) harboring a CAM resistance gene (camR) for the expression of CYP11A1 and pBar-Twin for the expression of Adx and AdR were used (Figure 8A and B). Interestingly, using this whole-cell system for the conversion of DOC resulted in the formation of unexpected acetylated DOC at the C21 position, which was not the expected product due to the action of P450s on steroid molecules.

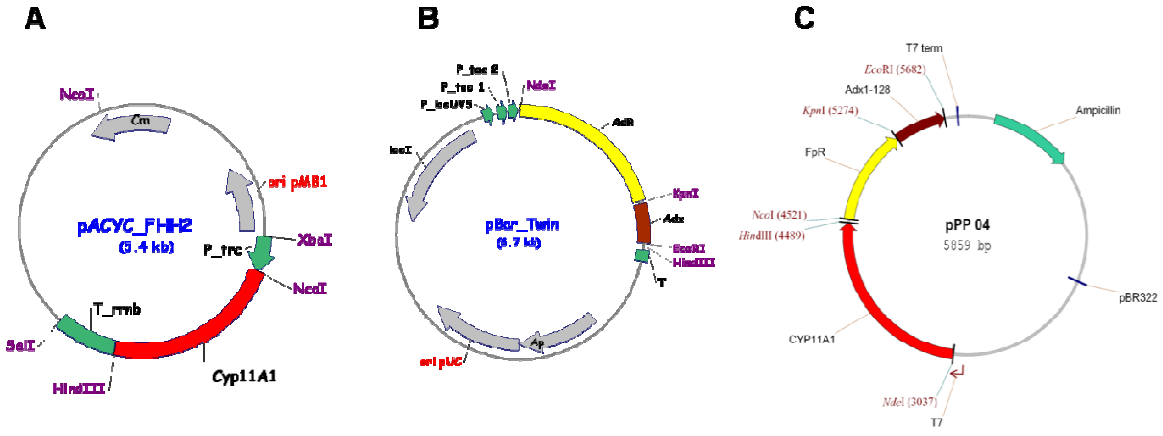

Figure 8: Plasmid maps of A) CYP11A1-containing plasmid (pACYC-FHH2), mediating CAM resistance gene, B) Twin Adx and AdR containing plasmid (pBar-Twin- Adx and AdR) with ampicillin as resistance marker, C) Tricistronic plasmid construct (pPP04) harbouring CYP11A1 gene and the redox partners, adrenodoxin (Adx) and ferredoxin reductase (FpR) with ampicillin as resistance gene.

The experiments proposed that using the expressing vector pACYC-FHH2 carrying the chloramphenicol acetyl transferase gene (*cat*) is responsible for the acetylation of the hydroxyl group of DOC. An *E. coli* whole-cell system using a tricistronic expression vector (pPP04) coding for adrenodoxin (Adx), ferredoxin reductase (FpR) and CYP11A1 used for recombinant CYP11A1 whole-cell biotransformation with ampicillin instead of chloramphenicol resistance marker (Figure 8C), in contrast, did not lead to steroid acetylation.

Taking this behavior of *cat* gene into account, it was of interest to analyze whether other biologically active steroids, which have structural similarities to DOC, are also capable of being acetylated by *cat*. This led us to the main idea behind further work to fully elucidate this characteristic of *cat* to produce acetylated steroids for biotechnological applications. As a consequence of the foregoing results, the expression vector pTG10 carrying the *cat* gene was used as a biocatalyst for steroid acetylation in

which six out of eighteen steroids: 11-deoxycorticosterone, cortisol, aldosterone, prednisolone, corticosterone and 11-deoxycortisol were identified as new substrates of CATI.

It is worth mentioning that the importance of steroid hormone acetylation is shown in the preparation of pharmaceuticals and intermediates for the synthesis of useful steroid acetate drugs, which play a significant role in the treatment of endometriosis and menopausal complaints [124,125], chronic infections [126] and as a popular anti-inflammatory agent [127]. The acetylation of steroids for these purposes were carried out previously by several synthetic routes, like using microorganisms, chemical approaches as well as hydrolases [92,113,127,128]. However, C21 steroids acetylation was not reported by hydrolases or other enzymes.

The application of biocatalysts is considered as an alternative approach for the industrial production of acetylated steroids. However, in this study we used the single plasmid harboring the single relevant gene as a tool for biocatalysis with a high selectivity. Furthermore, our biocatalyst system has not been identified so far as an acetylating agent for steroids and showed for the first time the ability of bacterial CATI as acetyltransferase to acetylate selectively the 21-hydroxl group of several steroids. On the other hand, it was clearly demonstrated that other hydroxyl groups as well as other functional groups (carbonyl and the double bond) were not modified**.** Therefore, this system seems to be an attractive approach with a new, easy and environmentally friendly technique for the production of acetylated steroids. It was demonstrated that biotransformation efficiency depends on the interaction between the whole-cell biocatalyst and bioprocess conditions [112]; therefore, the second approach of steroid acetylation by CATI focused on improving the whole-cell biocatalyst via optimization of conversion conditions to achieve a high yield of these steroids.

In terms of the development of a whole-cell system, it is of interest to mention that in preliminary optimization processes, *E. coli* strain JM109(DE3) harboring pTG10 plasmid was grown in TB medium containing DOC as substrates. The results showed that the conversion of DOC to its corresponding acetylated product, 11-deoxycorticosterone acetate (DOCA) is a time dependent conversion (Table 2).

Table 2: Amount of 11-deoxycorticosterone acetate (DOCA) ± SD, which was produced by *E. coli* JM109 (DE3) cells in TB medium after feeding of 300 µM DOC at different time points.

Time (hr)	DOCA ± SD (mg/L)
3	6.5 ± 0.9
6	24.80 ± 2.8
24	40.73 ± 1.5
48	-
72	-

The maximum product formation using TB medium was observed after 24 hr. Interestingly, after 48 and 72 hr there was no conversion observed (Figure 9). This might be because of the degradation of the protein, which demonstrates the highest protein expression after 24 hr incubation and a sharp decrease at 48 and 72 hr as shown by SDS-PAGE (Figure 1B, Publication 3.2). Additionally, using TB medium for the whole-cell conversion of steroid acetylation lead to a lower product yield compared with the other tested media and buffers. Another disadvantage of using TB medium is accompanied with the indole formation, which interferes with the product peak of some steroids and causes difficulties in the product separation and purification via HPLC. Therefore, subsequent experiments using resting cells were performed in buffer.

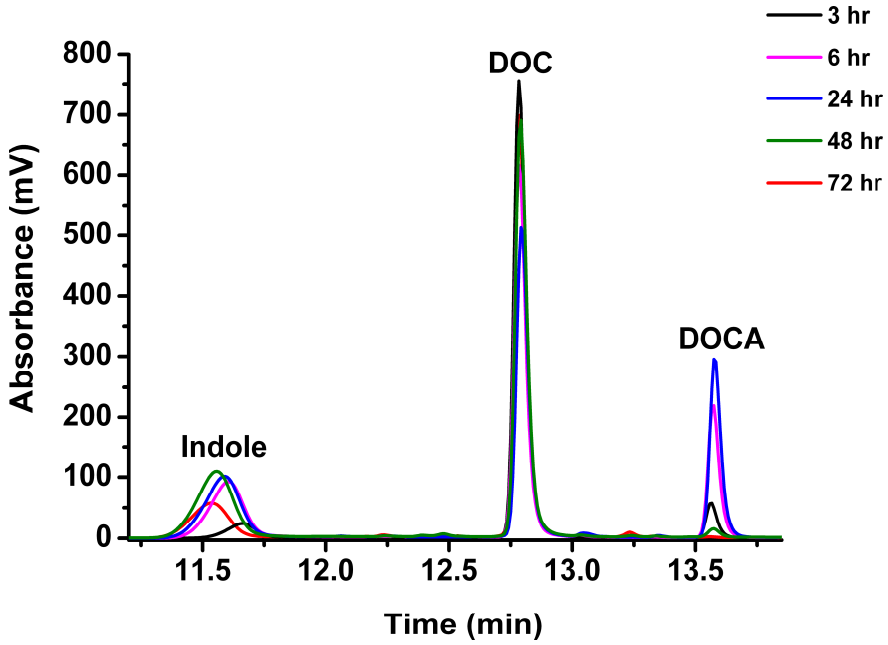

Figure 9: HPLC chromatograms for the 11-deoxycorticosterone acetate (DOCA) formation by the whole-cell conversions using *E. coli* JM109(DE3)pTG10 in TB medium at different conversion times.

Finally, the steroid acetylation by purified CATI was also performed. The *catI* gene was highly expressed in *E. coli* cells and the protein was successfully purified by IMAC. The purified protein was used for the *in vitro* conversions of 21-hydroxysteroids as well as CAM in the presence of acetyl CoA. The *in vitro* conversions of all of them were successfully achieved by purified CATI confirming that acetyl CoA is employed as an acyl donor in this conversion process.

4.3- INVESTIGATION OF STEROIDOGENIC PATHWAY FOR SULFONATED STEROIDS

The next part of this work is the investigation of the steroidogenic pathway for sulfonated steroids, studying the interaction of PregS with CYP17.

The special interest of the sufonated steroids stems from their function as prohormones, precursors to a great variety of steroids, both in the estrogenic and androgenic family. Previous studies have indicated that cholesterol sulfate (CS) is quantitatively the most important known sterol sulfate in human plasma [129]. It was also demonstrated, that the adrenal mitochondria of rats contains sulfonated cholesterol as an endogenous compound [130]. Further, cholesterol sulfate was metabolized efficiently by a mitochondrial side chain cleavage system producing PregS. However, further PregS conversion has not been studied in detail.

In this part of work, we could demonstrate that PregS serves as a substrate for CYP17 using the purified enzyme as well as in a SOATHEK293 cell culture system. We found that CYP17, which is involved in the steroid hormone biosynthesis, can convert PregS in a similar manner as free steroids indicating a potential alternative steroidogenic pathway.

Since our goal was to characterize the metabolism of PregS with CYP17 performing the *in vitro* conversion as well as the kinetic measurements, we established an efficient expression system for bovine CYP17, b_5 and CPR in the absence of detectable amounts of other proteins. It was demonstrated that some P450s have been poorly investigated because of the difficulties in obtaining sufficient quantities of purified protein. Therefore, the expression and purification procedures for the recombinant proteins were improved for a high yield and purity. All three proteins (CYP17, CPR and b_5) have been successfully expressed in bacteria, purified and used to perform the *in vitro* characterization.

As it is well known, the heterologously expressed P450 enzymes have the potential to be used in a variety of industrial applications. One big advantage of P450 expression in *E. coli* is the abundant generation of the corresponding proteins [45]. Common strategies for expression of P450s in *E. coli* include N-terminal sequence modification and truncation of the N-terminal hydrophobic region responsible for interaction of P450s with membranes and using lower temperatures (20–28 °C) to achieve the maximal yield of expression of the catalytically active protein [6]. For this reason, various approaches have been used during the expression and purification of bovine CYP17, b_5 and CPR proteins, including use of *E. coli* C43(DE3) host cells, as these cells are advisable for the over-expression of membrane-bound P450s and other membrane bound proteins using expression vector pET-17b, lowering the temperature during the expression and the expression in the presence of molecular chaperones, which is known to assist the correct folding of proteins. In addition, it was demonstrated that the truncation of the membrane anchor increases the solubility of P450s and

facilitates the expression of protein in *E. coli* [116]. Therefore, the N-terminal sequence modification has been performed utilizing the amino acid sequence of CYP17 lacking its N-terminal hydrophobic anchor. As well, the CPR amino acid sequence extended at the C-terminus by three glycines and lacking the first 27 amino acids at the N- terminus was used. It should also be noted that the protein purification procedures have been improved for all three proteins by extending the amino acid sequences at the C-terminus by a hexa-histidine-tag to facilitate the purification process by immobilized-metal affinity chromatography (IMAC) using Ni–NTA agarose column.

4.4- EFFECT OF INDOLE ON THE CYP11A1 MEDIATED BIOTRANSFORMATIONS

This part of the work involved the improvement of the whole-cell system expressing CYP11A1 and its electron transfer partners and investigation of the indole effect on *in vitro* and *in vivo* conversion reactions catalyzed by CYP11A1.

To perform the whole-cell conversion of the new CYP11A1 substrates (DOC, androstenedione, testosterone and DHEA), the complex medium Terrific Broth (TB) was chosen for cultivation. The whole-cell biotransformation of DOC, androstenedione, testosterone and DHEA by CYP11A1 carried out in TB medium did not lead to product formation for all tested substrates (Figure 10). It was suggested that there is a limiting factor in this process preventing the steroid conversion and we assumed that the indole production in TB medium might be responsible for the lack of steroid bioconversion. Furthermore, instead of the expected product peaks, presented in the *in vitro* conversion of the steroids, another peak was detected at ~12.2 minutes retention time, which was also seen in the control cultures (untransformed *E. coli* cells without plasmid). We expected this peak to belong to indole, which is produced by the tryptophanase from tryptophan as an endogenous metabolite in *E. coli* cells [131,132].

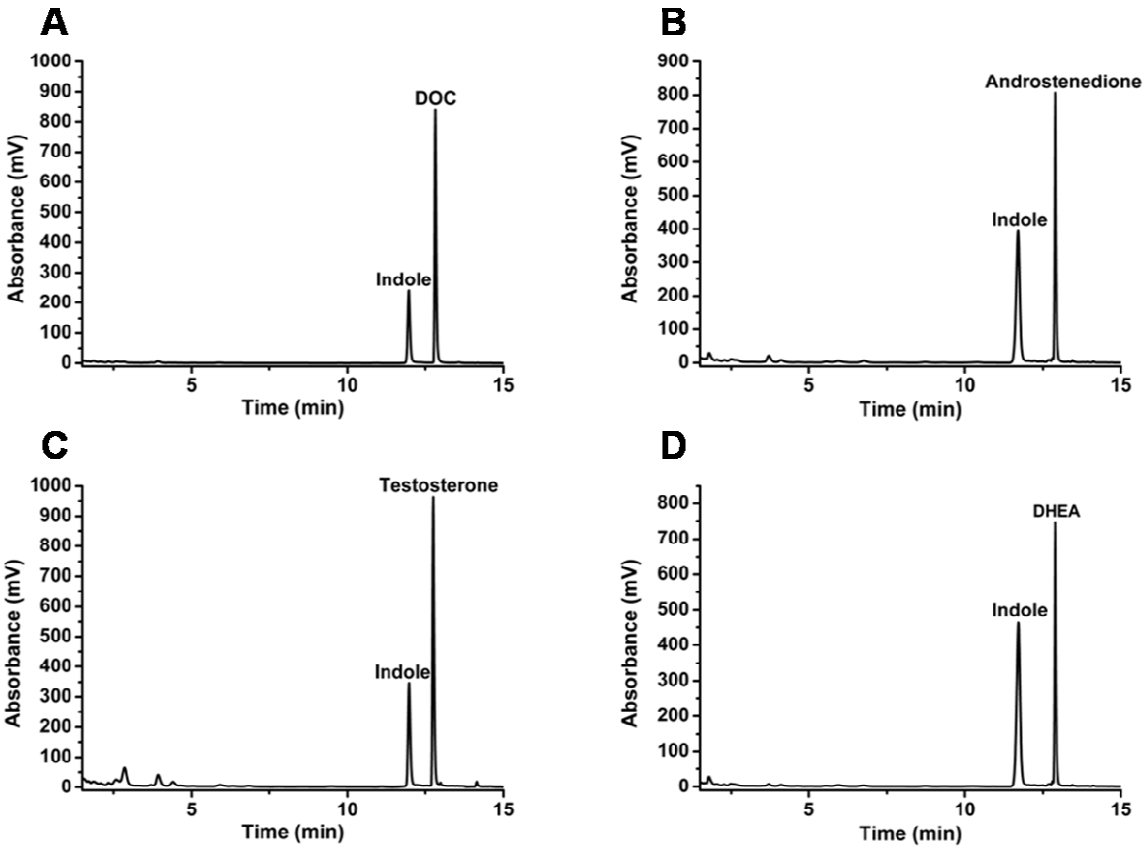

Figure 10: HPLC chromatograms of the whole-cell bioconversion of 300 µM of steroids using recombinant *E. coli* whole-cell system expressing CYP11A1 using TB medium after 24 hr from substrate adding. A) 11-deoxycorticosterone (DOC), B) Androstenedione, C) Testosterone, D) DHEA, with the indole peak present.

As this strong indole formation could possibly inhibit the activity of CYP11A1; this side product of TB medium was overcome by using resting cells in potassium phosphate buffer (KPP). Applying KPP buffer for the biotransformation experiments exhibited a removal of indole formation and improved the biotransformation of the respective steroids to corresponding hydroxylated products indicating that the indole formation has an inhibitory effect on the CYP11A1-dependent biotransformation. Furthermore, CYP11A1 system is well known to efficiently convert its natural substrate, cholesterol, to pregnenolone. An *E. coli* whole-cell system expressing CYP11A1 in TB medium failed also to perform the cholesterol conversion; however, successful *in vivo* formation of pregnenolone was achieved when the conversion performed with resting cells in buffer (Figure 11A and B).

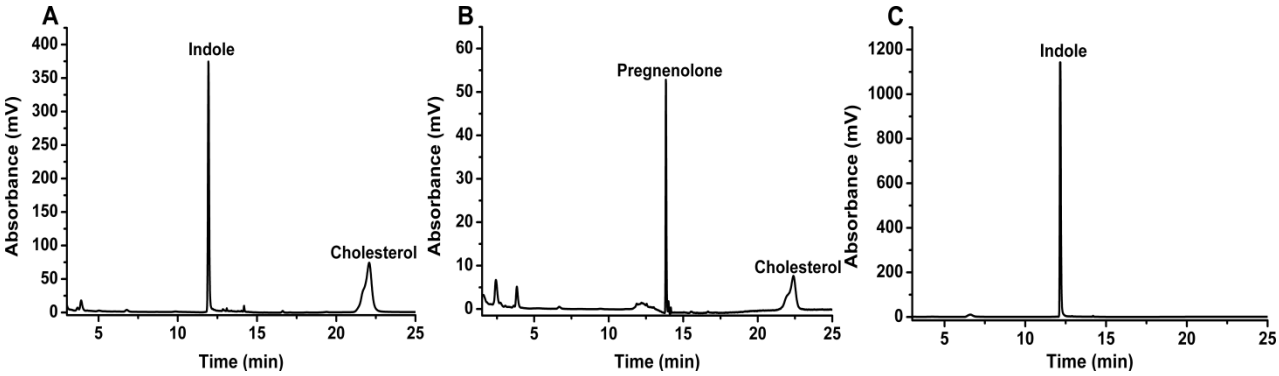

Figure 11: HPLC chromatograms of whole-cell conversion of cholesterol after 24 hr from substrate adding by using, A) Growing cells in TB medium, B) Resting cells in potassium phosphate buffer, C) HPLC chromatograme for the authentic indole injection.

To confirm this observation and to check whether indole has, indeed, a negative influence on the CYP11A1 activity, we performed a CYP11A1 whole-cell conversion of cholesterol in KPP buffer supplemented with different concentrations of indole. As well, the *in vitro* cholesterol conversion by purified CYP11A1 in the presence of various indole concentrations was conducted (Figure 12A and B).

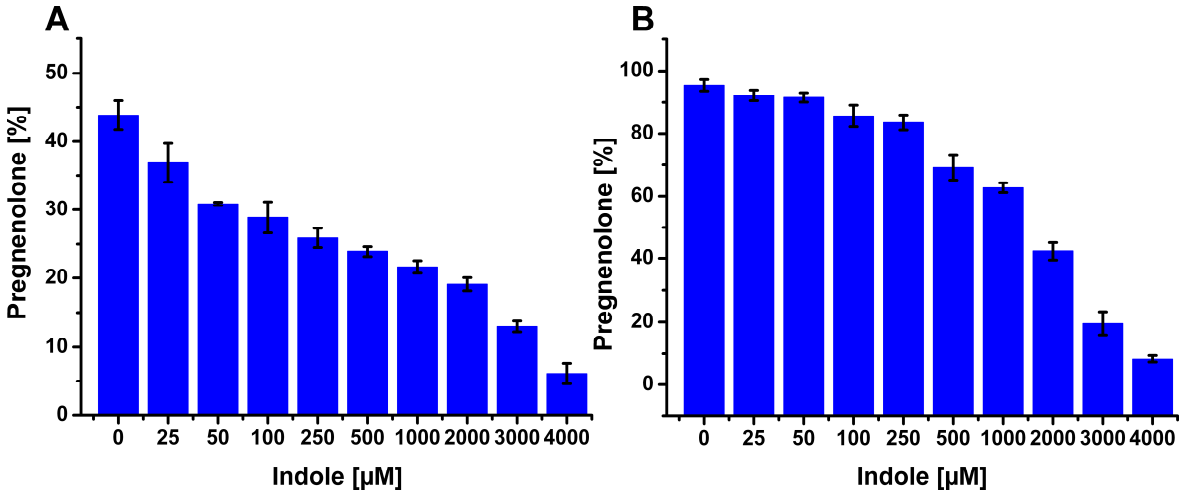

Figure 12: A) Pregnenolone formation (mean ± SD) after CYP11A1-dependent *in vitro* conversions using 15 µM cholesterol as final substrate concentration in the presence of different indole concentrations after 7 min. conversion time, B) Pregnenolone formation (mean ± SD) of whole-cell conversions in KPP buffer supplemented with 300 µM cholesterol, 100 mM glucose and different indole concentrations after 24 hr conversion time. The standard deviation of each bar was calculated for three replicate experiments. The indole dissolved in DMSO at different concentrations and equal volumes were used to achieve equal DMSO concentration in all measurements as well as with control.

As displayed in Figure 12B (first bar), it can clearly be seen that in the *in vivo* experiments performed with resting cells an almost complete substrate conversion took place when the suspensions

contained 300 µM cholesterol, resulting in a pregnenolone formation of 96 ± 1.8 %. Addition of indole, however, inhibited pregnenolone production. Summarizing, we demonstrated for the first time the ability of a CYP11A1 *E. coli* based whole-cell system to convert cholesterol, which has not been established so far.

For further understanding, it was important to study the indole effect with other steroids rather than cholesterol in order to exclude the substrate dependency. Therefore, *in vitro* and *in vivo* conversion assays were carried out using 300 µM androstenedione (as best substrate for CYP11A1 among all tested substrates in this study) in the presence of increasing concentrations of indole (25-4000 µM). Because CYP11A1 catalyzes the conversion of androstenedione to one major and one minor product, the percentage of product formation was calculated for the total amount of products (Figure 13A and B).

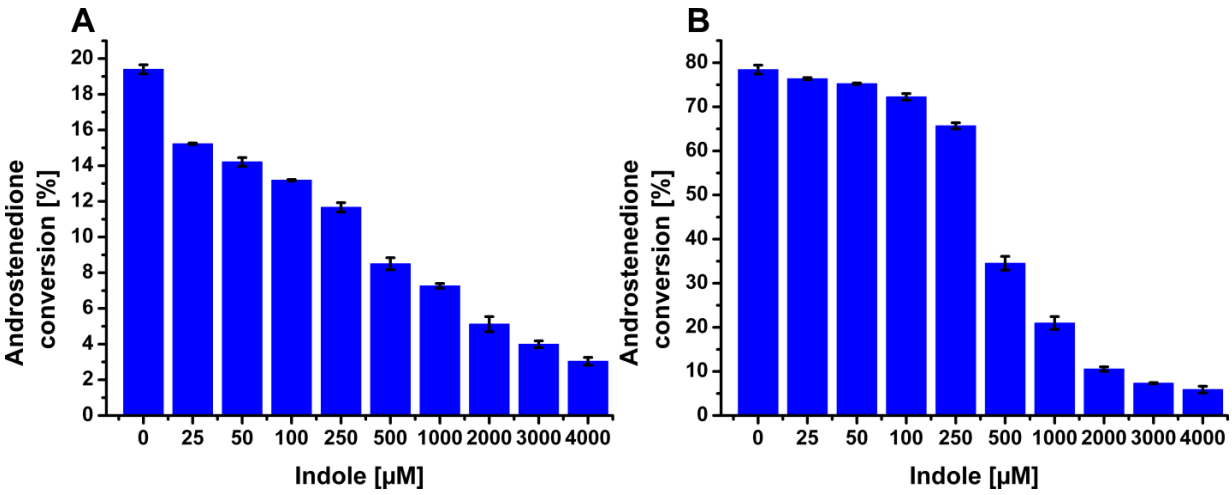

Figure 13: A) The effect of different indole concentrations on the conversion of androstenedione by CYP11A1 *in vitro*, which was carried out for 30 min at 37 °C employing the AdR–Adx redox system, B) The effect of different indole concentrations on the conversion of androstenedione by CYP11A1 using the whole-cell system after 48 hr conversion time using KPP buffer supplemented with 300 µM androstenedione and 100 mM glucose. The data expressed as a mean ± SD of three independent determinations.

The results shown in Figure 13 together with those obtained for the *in vitro* and *in vivo* conversions of cholesterol confirmed the hypothesis that the indole has a negative effect on the catalytic activity of CYP11A1. Furthermore, increasing the indole concentration, the inhibitory effect increased and the conversion of cholesterol and androstenedione diminished in both cases, confirming a concentration-dependent decrease of the CYP11A1-catalyzed conversions in the presence of indole. The reason might be that the indole blocks the active site of CYP11A1 and therefore it will negatively affect the interaction with the redox partners or the substrates. Furthermore, it was noticed that the cholesterol

conversion in the presence of higher concentration of indole (0.5-4 mM) was accompanied with formation of additional products (Figure 14), which need to be identified in further studies.

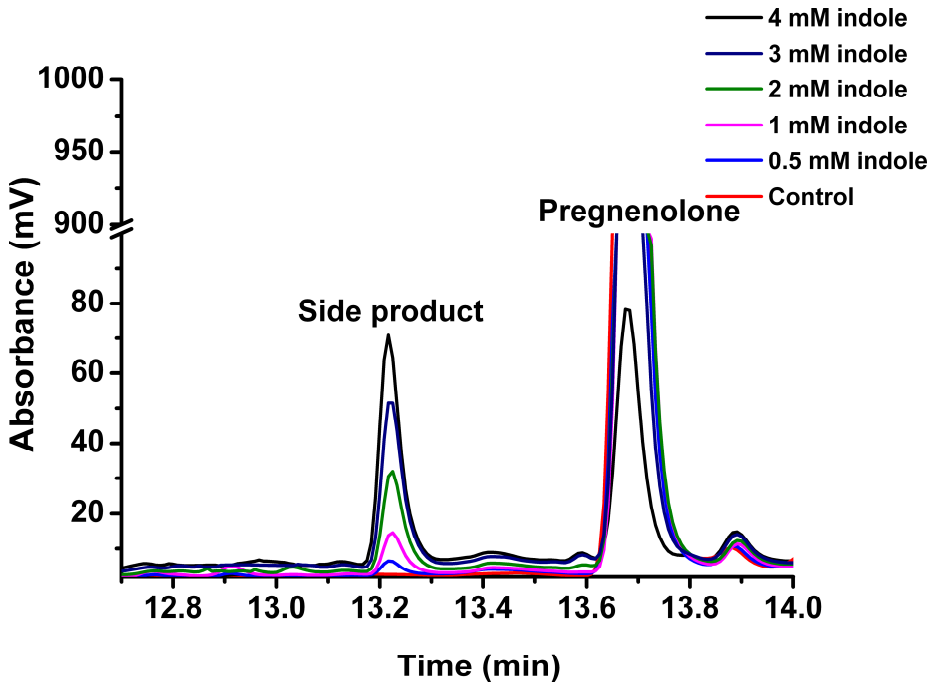

Figure 14: Side product formation during the *in vitro* cholesterol conversion by CYP11A1 in the presence of different indole concentrations (0.5-4 mM).

Our results on the inhibitory effect of indole are in agreement with a similar observation, which was recently reported for the *in vitro* conversion of myristic acid by CYP109B1 from *Bacillus subtilis* [133]. Furthermore, the negative influence of indole on the *in vivo* CYP11A1 conversion is consistent with the results of Ringle et al., which proved the inhibitory effect of indole on the *in vivo* conversion of 4-methyl-3-phenylcoumarin by a whole-cell system expressing CYP264A1 from *S. cellulosum Soce56* [134].

Taken together, the complex TB media containing a high amount of tryptophan is not recommended for CYP11A1-mediated biotransformation due to the inhibitory effect of indole. Thus, effective methods have to be considered for the design of CYP11A1 whole-cell experiments, like using resting cells in buffer or using M9 minimal medium supplemented with casamino acids (M9CA), which has only traces of tryptophan. Furthermore, it is also of special interest for future studies to use a homologous recombination method to prepare a tryptophanase knockout *E. coli* strain to overcome the inhibitory effect of indole.

5- CONCLUSIONS AND FUTURE PROSPECTS

The first step in the biosynthesis of steroid hormones occurs in the mitochondria, where cholesterol is converted to pregnenolone by CYP11A1. The main focus of this part of work was to characterize the influence of various metabolites of steroid hormones on the *in vitro* CYP11A1 mediated cholesterol conversion. The effect on this reaction is particularly interesting since a pro-hormone, pregnenolone, is the mandatory substrate for the synthesis of other steroid hormones.

We have reported that bovine CYP11A1 can be successfully expressed in *E. coli* in a high yield, purity and catalytically active form. Furthermore, we identified a novel function and new insights into the regulatory effects of estrogens, estradiol and estrone, on the CYP11A1 enzyme activity in a reconstituted *in vitro* system, which give an indication on the role of metabolites of steroid biosynthesis as activators of the CYP11A1-dependent cholesterol conversion. For this reason, it would be of great interest to use a cell culture model that expresses CYP11A1 (e.g. adrenocortical NCI-H295R cells) and see if estrogens regulate CYP11A1 in the same manner as in the *in vitro* experiments and if the same CYP11A1 product is formed. Moreover, further examinations of these estrogens as an activator of pregnenolone formation should be performed, especially the determination of the binding affinity and kinetic parameters for CYP11A1 with cholesterol in the presence of estradiol and estrone.

Since our *in vitro* experiments suggest the ability of purified CYP11A1 to convert DOC, androstenedione, testosterone and DHEA as new CYP11A1 substrates, it is of special interest also to see the influence of these steroids on CYP11A1 biochemically, focusing on the kinetics and the binding affinity measurements of CYP11A1 with its natural substrate, cholesterol under the influence of these steroid metabolites.

Moreover, the CYP11A1 whole-cell biocatalyst was successfully applied to convert these steroids to its hydroxylated form. The NMR identification of the formed products showed that the C2 and C6 positions were identified as sites of hydroxylation of DOC and androstenedione by CYP11A1 as well as C6 position in testosterone and C16 position in DHEA. These novel products of CYP11A1 might be important precursors in the synthesis of highly potent pharmaceutically interesting compounds. Thus, to produce these novel hydroxylated products in higher yield, it would be of interest to optimize the enzyme by the method of molecular evolution, thus, it is possible to generate CYP11A1 mutants to enhance the conversion of respective metabolites via random and site directed mutagenesis.

Further, in this work, the negative impact of indole, produced by the host microorganism *E. coli*, on CYP11A1 activity was demonstrated. This effect has been overcome by performing the biotransformation assays in buffer. It is, however, necessary to investigate a potential physiological role of this inhibition in future studies.

In the past years, several studies have demonstrated that the human gut is capable of *de novo* synthesis of glucocorticoids and sex steroids. This finding is based on the detection of steroidogenic enzyme expression such as CYP11A1, as well as the presence of bioactive steroids in both the rodent and human gut. The local production of immunoregulatory glucocorticoids contributes to intestinal homeostasis and has been linked to the pathophysiology of inflammatory bowel diseases. Intestinal epithelial cells also possess the ability to metabolize sex steroids, notably estrogen. This mechanism may impact colorectal cancer development. It was demonstrated, that commensal *E. coli* strains produce significant amounts of indole as a result of tryptophane degradation, leading to indole concentrations reaching from 250 to 1,100 mM in the human feces. Thus, it is likely that intestinal epithelial cells are continually exposed to high concentrations of indole.

Taking this into account, it is necessary to study the inhibitory effect of indole on CYP11A1 activity in more detail by determination of the inhibition constant K_i or IC_{50} value and the dissociation constant (K_d). Furthermore, these experiments should also be carried out with skatole, an important indole derivative in the mammalian digestive tract. As the inhibition of CYP11A1 would lead to overall decreased concentrations of glucocorticoids and sex hormones in the gut, an overly protein- or tryptohane-rich diet could have detrimental effects on steroid hormone homeostasis and potentially lead to chronic bowel diseases.

Concerning the acetylation of steroid hormones, it is well known, that the development of the whole-cell biocatalyst is of great interest for the synthesis of steroid hormone derivatives, which have several pharmaceutical and biotechnological applications. In this work, one of the successful applications of whole-cell biocatalysts has been proved to be a promising candidate for the acetylation of C21-hydroxysteroid compounds with a high regio- and stereoselectivity. The *E. coli* whole-cell biocatalyst expressing the bacterial chloramphenicol acetyltransferase I (CATI) was investigated for acetylation of C21-hydroxysteroid compounds. Six steroids: 11-deoxycorticosterone, cortisol, aldosterone, prednisolone, corticosterone and 11-deoxycortisol were identified as new substrates of CATI. Furthermore, the optimization of the experimental conditions displayed an increase in the activity of bacterial CATI enzyme to produce the desired acetylated products. For this, the *cat* gene expression was optimized for higher expression at 24 hr, utilizing Tris-HCl buffer, pH 7.4, containing 500 mM glucose as a carbon source and 1 mM substrate, in which the best condition to produce acetylated steroids by the resting cell was obtained after 48 hr. To accomplish this goal, and since the expression vectors pET22b or pET17b are known as high expression vectors for protein expression, it will be of great benefit to utilize these vectors carrying the *catI* gene for efficient synthesis of acetylated steroids in a higher yield for biotechnological applications. In addition, the catalytic activity of CATI should be studied in a wider range to involve other C21-hydroxysteroids and non steroidal

compounds, which might also have a pharmaceutical usage if they are converted to the acetylated form. Moreover, since the *in vitro* conversions of CAM as well as C21-hydroxysteroids were achieved with the purified CATI, it is possible to perform further experiments concerning the comparison of the kinetic parameters of CATI with CAM as well as steroid substrates.

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