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Lattemann et al.

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(54) 21-HYDROXYLATION OF STEROIDS

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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PCT Pub. Date: **May 6, 2016**

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(51) **Int. Cl.**
C12P 33/06 (2006.01)

(52) **U.S. Cl.**
CPC ***C12P 33/06*** (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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(Continued)

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(57) **ABSTRACT**

Generally, the present invention relates to the field of steroid hydroxylation. More specifically, the present invention relates to a method for the 21-hydroxylation of steroids in cells. It also relates to cells expressing a steroid 21-hydroxylating enzyme or steroid 21-hydroxylase, expression vectors comprising a nucleic acid encoding for a steroid 21-hydroxylase and a kit for carrying out the method for the 21-hydroxylation of steroids in cells.

14 Claims, 11 Drawing Sheets

Specification includes a Sequence Listing.

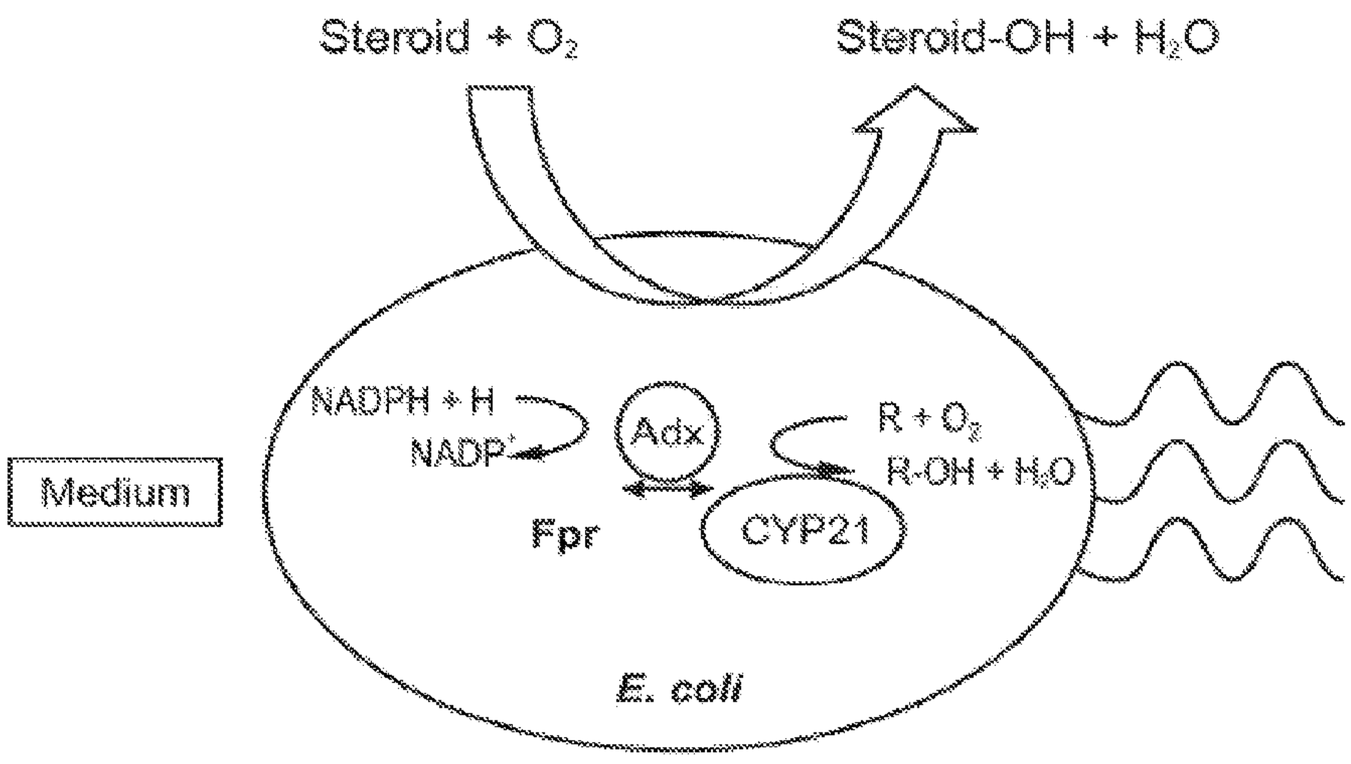

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FIG.1

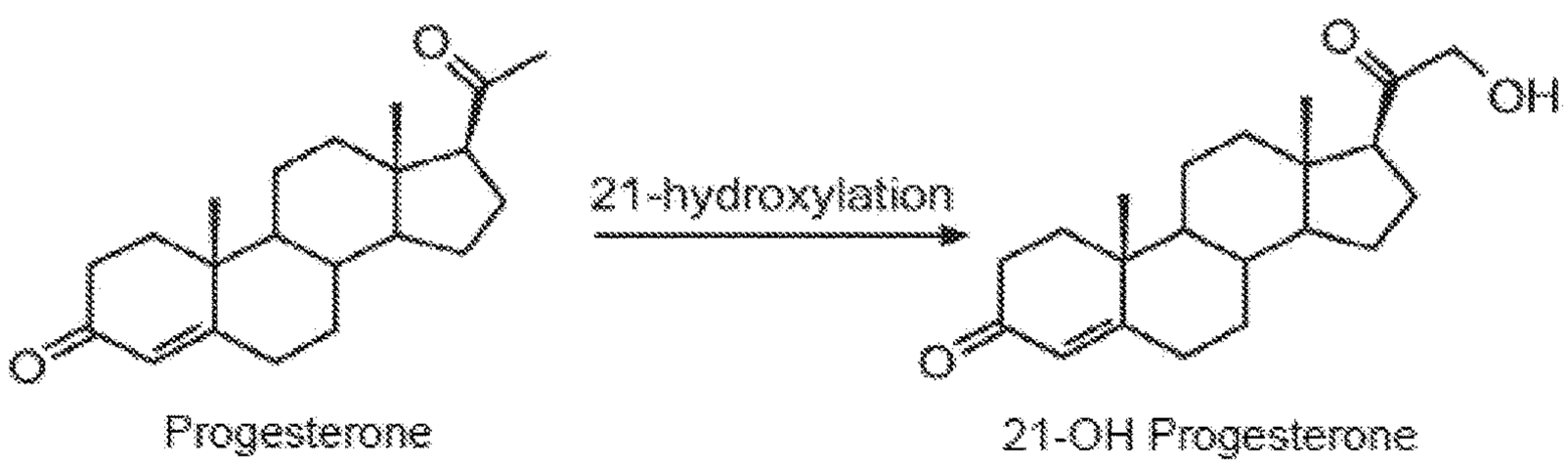


FIG.2

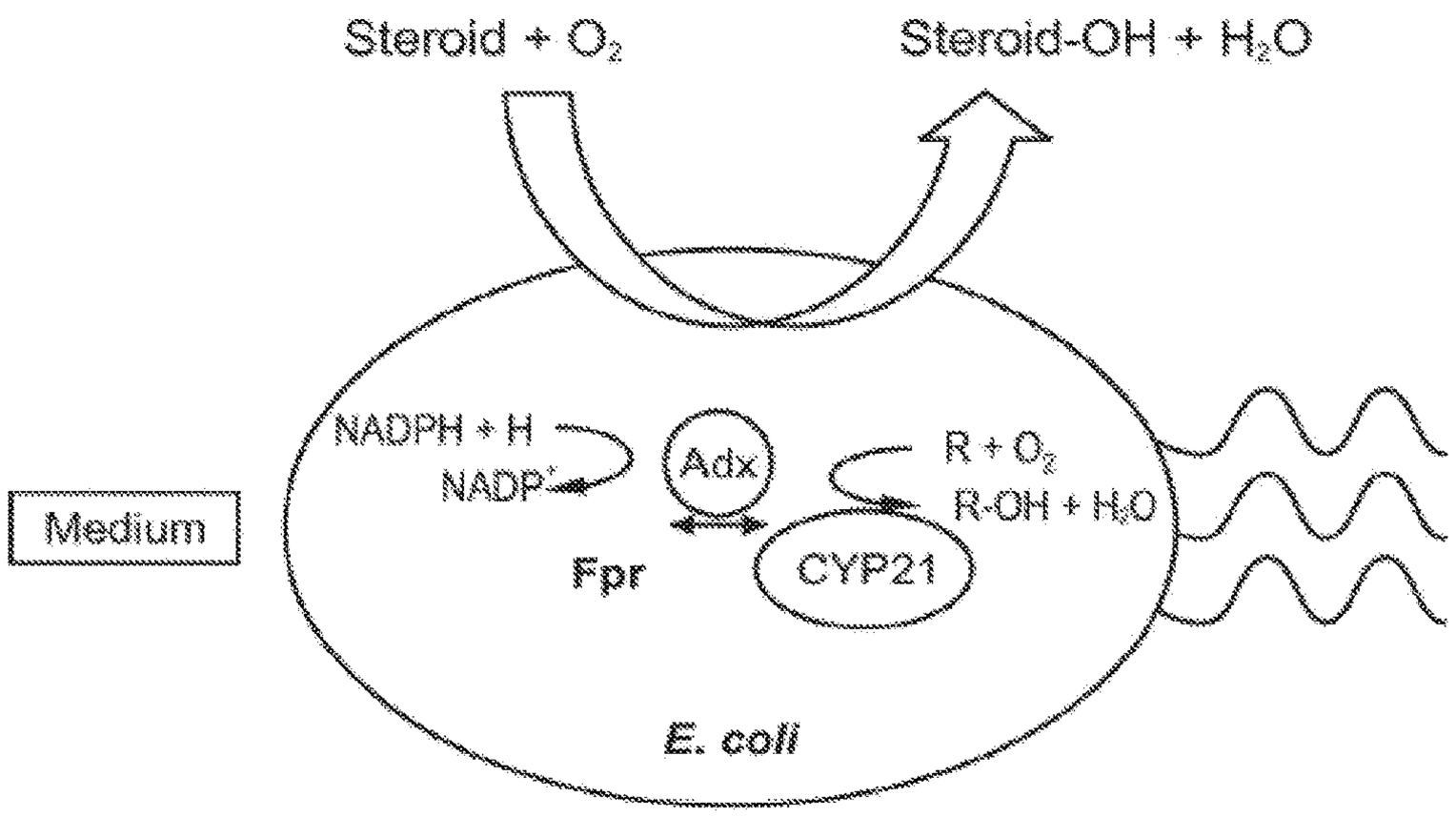
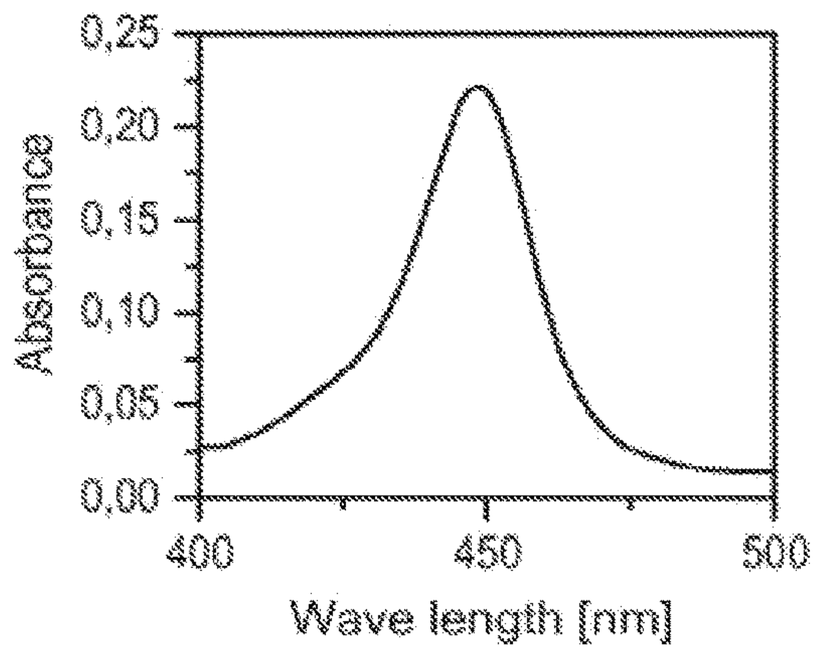

Steroid + O_2
Steroid-OH + H_2O
NADPH + H
NADP+
Adx
R + O_2
R-OH + H_2O
Fpr
CYP21
Medium
E. coli

FIG.3


0,25
0,20
0,15
0,10
0,05
0,00
Absorbance
400
450
500
Wave length [nm]

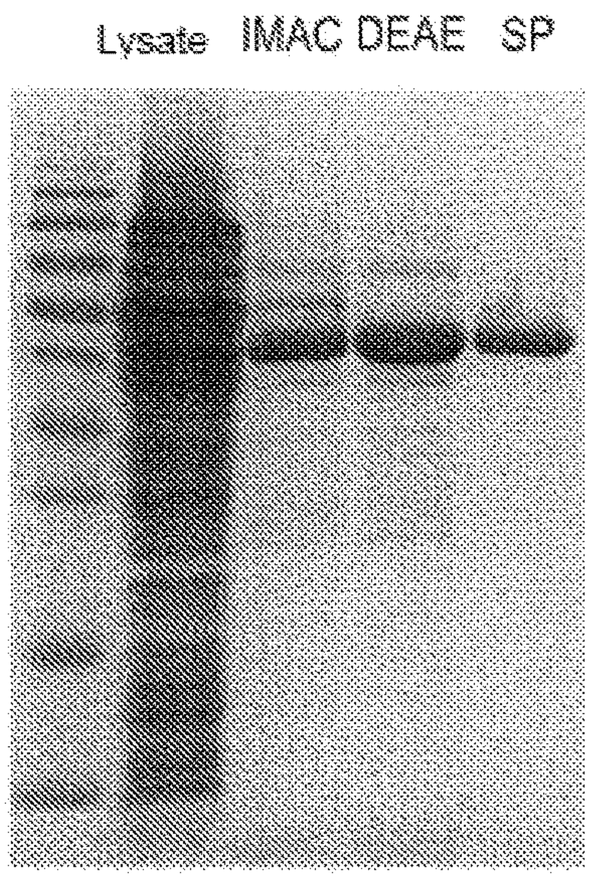

Lysate
IMAC
DEAE
SP

FIG.4

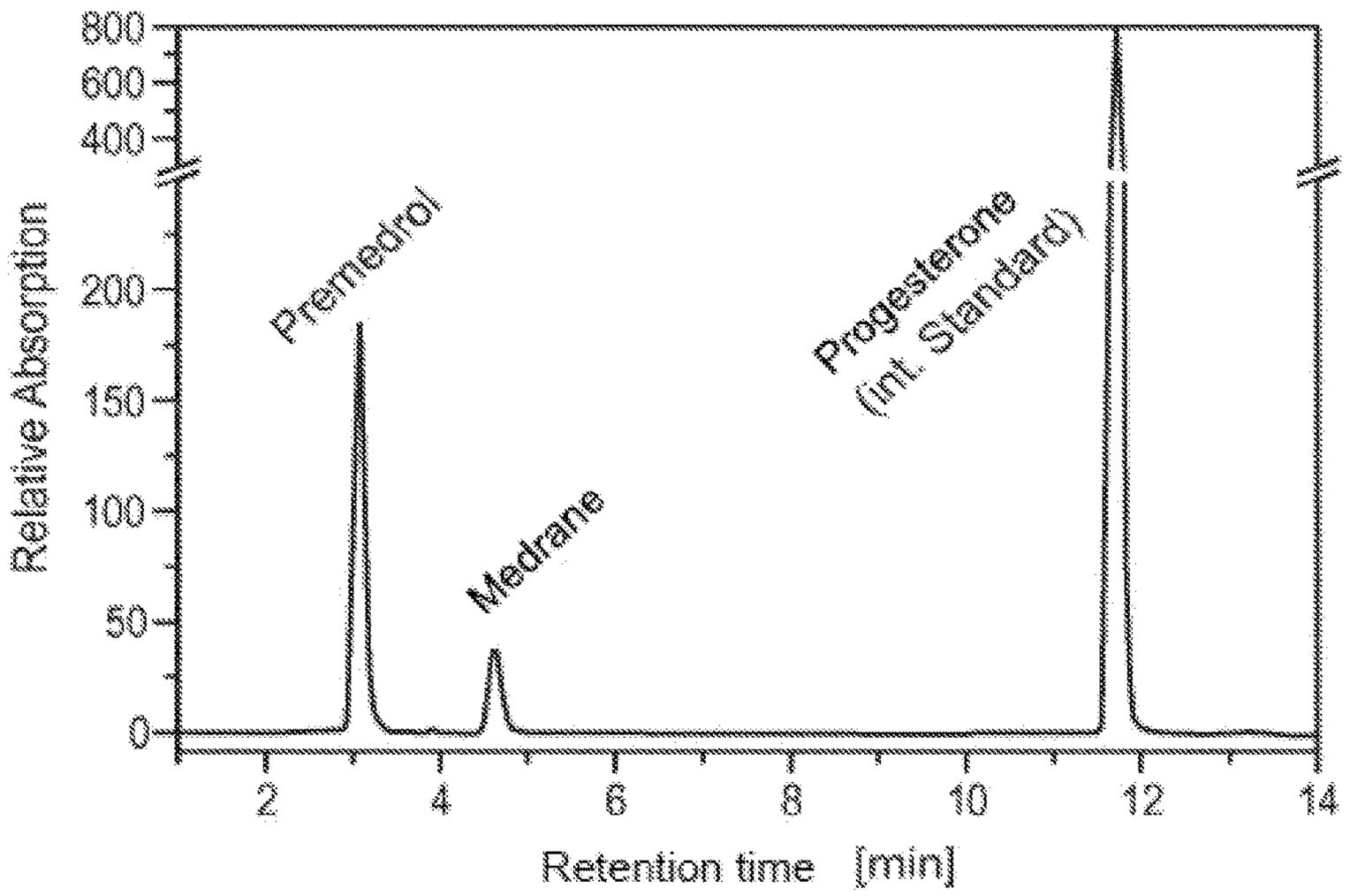

Relative Absorption
800
600
400
200
150
100
50
0
Premedrol
Medrane
Progesterone
(Int. Standard)
2
4
6
8
10
12
14
Retention time [min]

FIG.5A

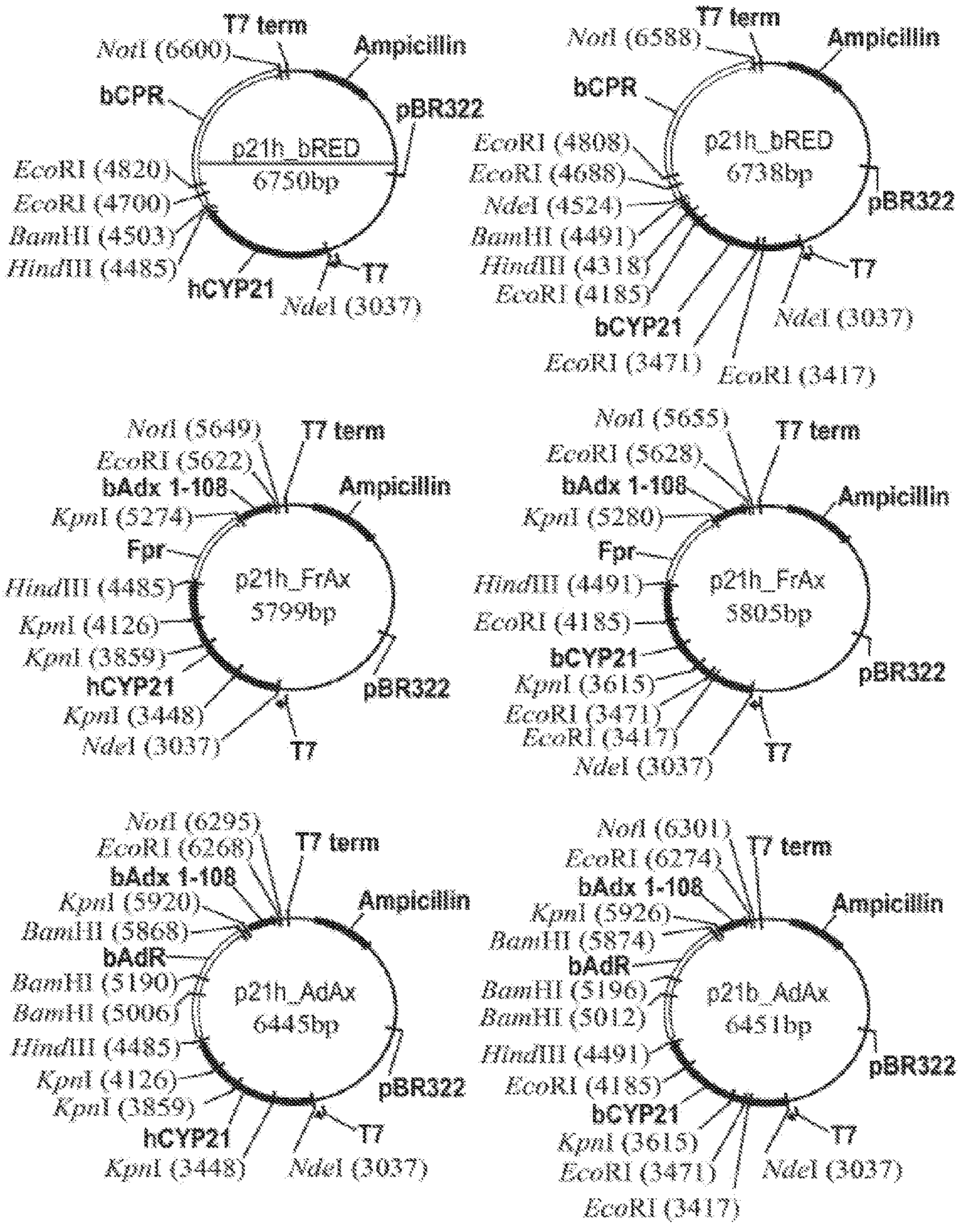
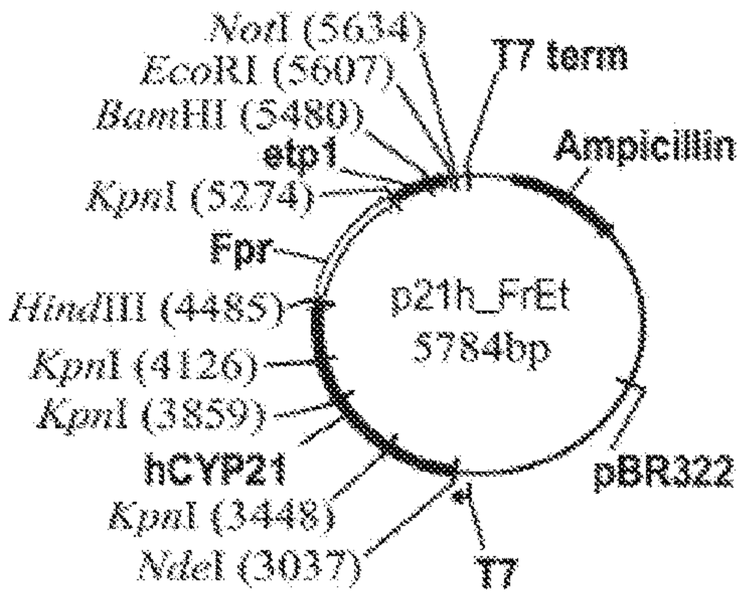
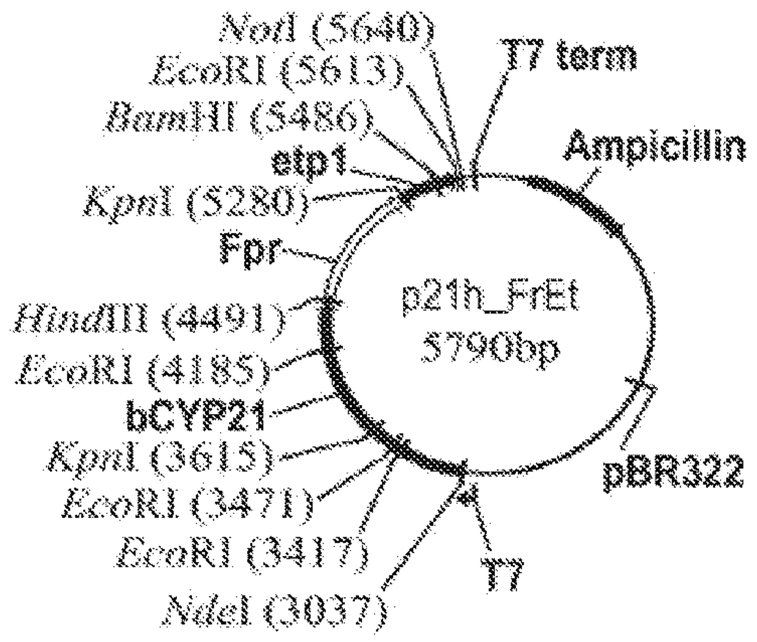
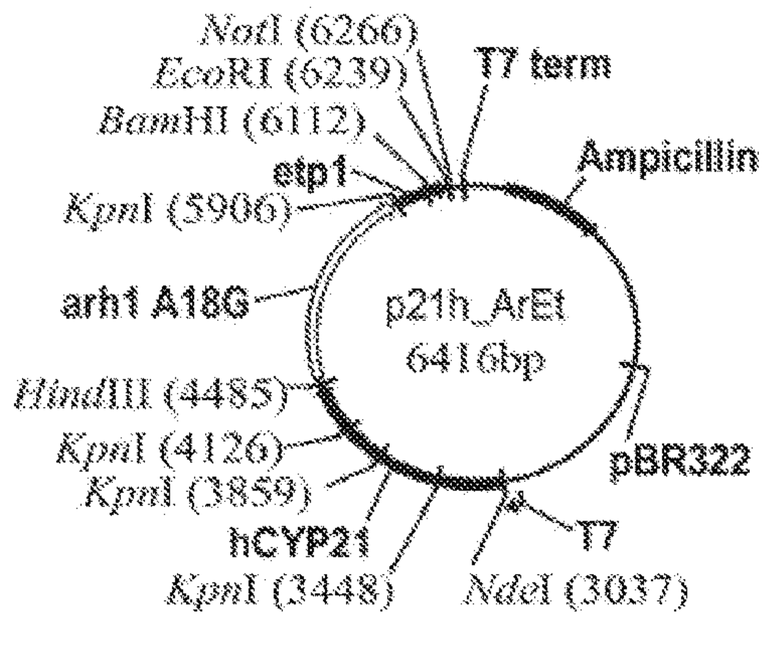
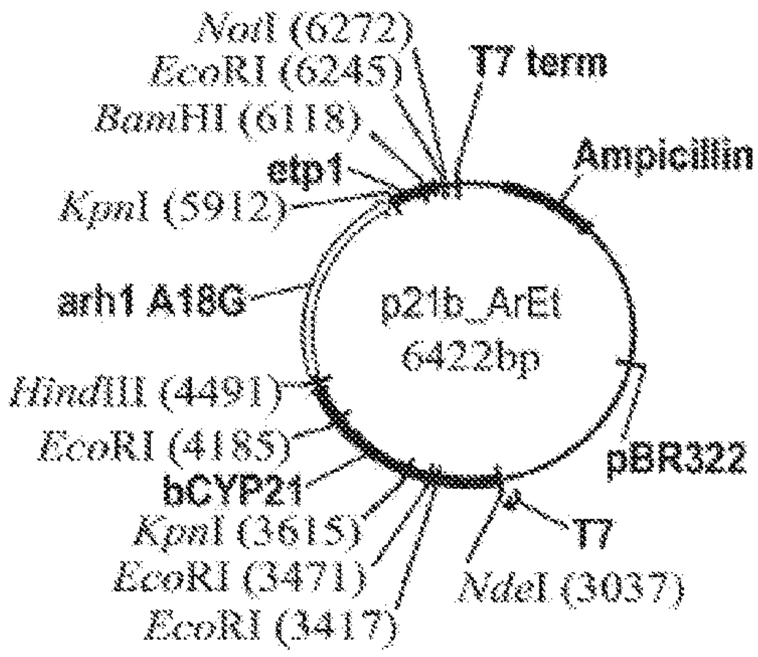

T7 term
NotI (6600)
Ampicillin
bCPR
pBR322
p21h_bRED
6750bp
EcoRI (4820)
EcoRI (4700)
BamHI (4503)
HindIII (4485)
hCYP21
T7
NdeI (3037)
T7 term
NotI (6588)
Ampicillin
bCPR
EcoRI (4808)
EcoRI (4688)
NdeI (4524)
BamHI (4491)
HindIII (4318)
EcoRI (4185)
bCYP21
EcoRI (3471)
p21h_bRED
6738bp
pBR322
T7
NdeI (3037)
EcoRI (3417)
NotI (5649)
T7 term
EcoRI (5622)
bAdx 1-108
Ampicillin
KpnI (5274)
Fpr
HindIII (4485)
KpnI (4126)
KpnI (3859)
hCYP21
KpnI (3448)
NdeI (3037)
p21h_FrAx
5799bp
pBR322
T7
NotI (5655)
T7 term
EcoRI (5628)
bAdx 1-108
Ampicillin
KpnI (5280)
Fpr
HindIII (4491)
EcoRI (4185)
bCYP21
KpnI (3615)
EcoRI (3471)
EcoRI (3417)
NdeI (3037)
p21h_FrAx
5805bp
pBR322
T7
NotI (6295)
EcoRI (6268)
T7 term
bAdx 1-108
Ampicillin
KpnI (5920)
BamHI (5868)
bAdR
BamHI (5190)
BamHI (5006)
HindIII (4485)
KpnI (4126)
KpnI (3859)
hCYP21
KpnI (3448)
NdeI (3037)
p21h_AdAx
6445bp
pBR322
T7
NotI (6301)
EcoRI (6274)
T7 term
bAdx 1-108
Ampicillin
KpnI (5926)
BamHI (5874)
bAdR
BamHI (5196)
BamHI (5012)
HindIII (4491)
EcoRI (4185)
bCYP21
KpnI (3615)
EcoRI (3471)
EcoRI (3417)
NdeI (3037)
p21b_AdAx
6451bp
pBR322
T7

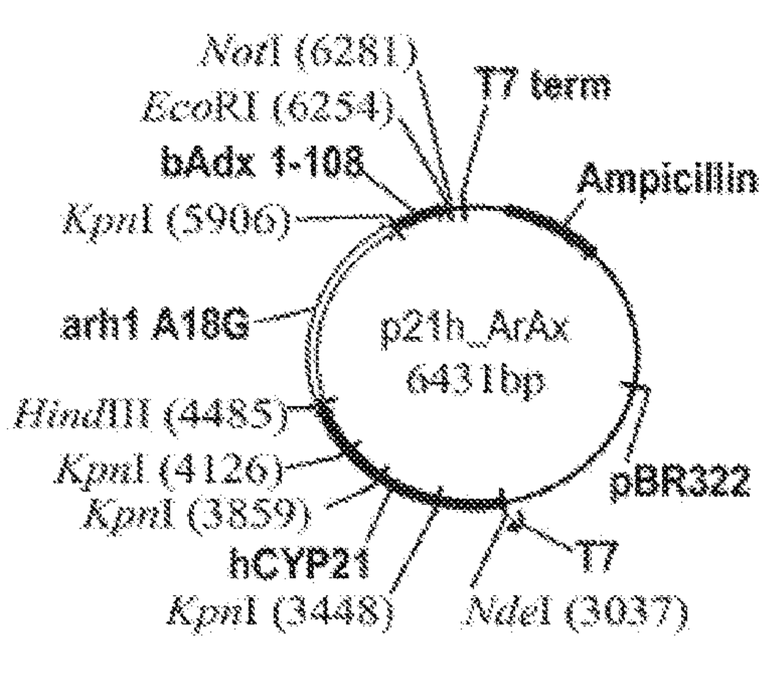
FIG.5B


NotI (5634)
EcoRI (5607)
BamHI (5480)
etp1
KpnI (5274)
Fpr
HindIII (4485)
KpnI (4126)
KpnI (3859)
hCYP21
KpnI (3448)
NdeI (3037)
T7
pBR322
Ampicillin
T7 term
p21h_FrEt
5784bp


NotI (5640)
EcoRI (5613)
BamHI (5486)
etp1
KpnI (5280)
Fpr
HindIII (4491)
EcoRI (4185)
bCYP21
KpnI (3615)
EcoRI (3471)
EcoRI (3417)
NdeI (3037)
T7
pBR322
Ampicillin
T7 term
p21h_FrEt
5790bp


NotI (6266)
EcoRI (6239)
BamHI (6112)
etp1
KpnI (5906)
arh1 A18G
HindIII (4485)
KpnI (4126)
KpnI (3859)
hCYP21
KpnI (3448)
NdeI (3037)
T7
pBR322
Ampicillin
T7 term
p21h_ArEt
6416bp


NotI (6272)
EcoRI (6245)
BamHI (6118)
etp1
KpnI (5912)
arh1 A18G
HindIII (4491)
EcoRI (4185)
bCYP21
KpnI (3615)
EcoRI (3471)
EcoRI (3417)
NdeI (3037)
T7
pBR322
Ampicillin
T7 term
p21b_ArEt
6422bp


NotI (6281)
EcoRI (6254)
bAdx 1-108
KpnI (5906)
arh1 A18G
HindIII (4485)
KpnI (4126)
KpnI (3859)
hCYP21
KpnI (3448)
NdeI (3037)
T7
pBR322
Ampicillin
T7 term
p21h_ArAx
6431bp

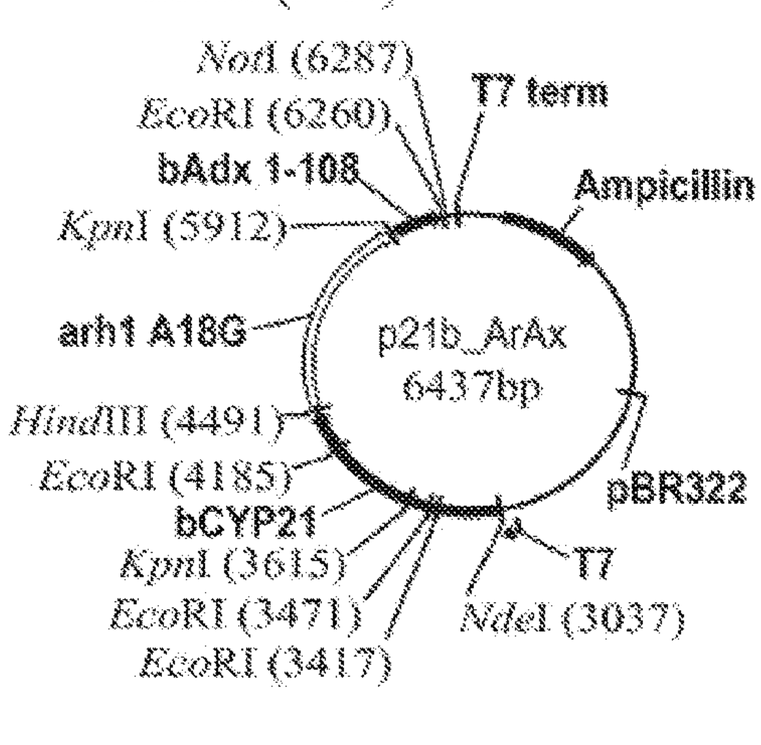

NotI (6287)
EcoRI (6260)
bAdx 1-108
KpnI (5912)
arh1 A18G
HindIII (4491)
EcoRI (4185)
bCYP21
KpnI (3615)
EcoRI (3471)
EcoRI (3417)
NdeI (3037)
T7
pBR322
Ampicillin
T7 term
p21b_ArAx
6437bp

FIG.6A

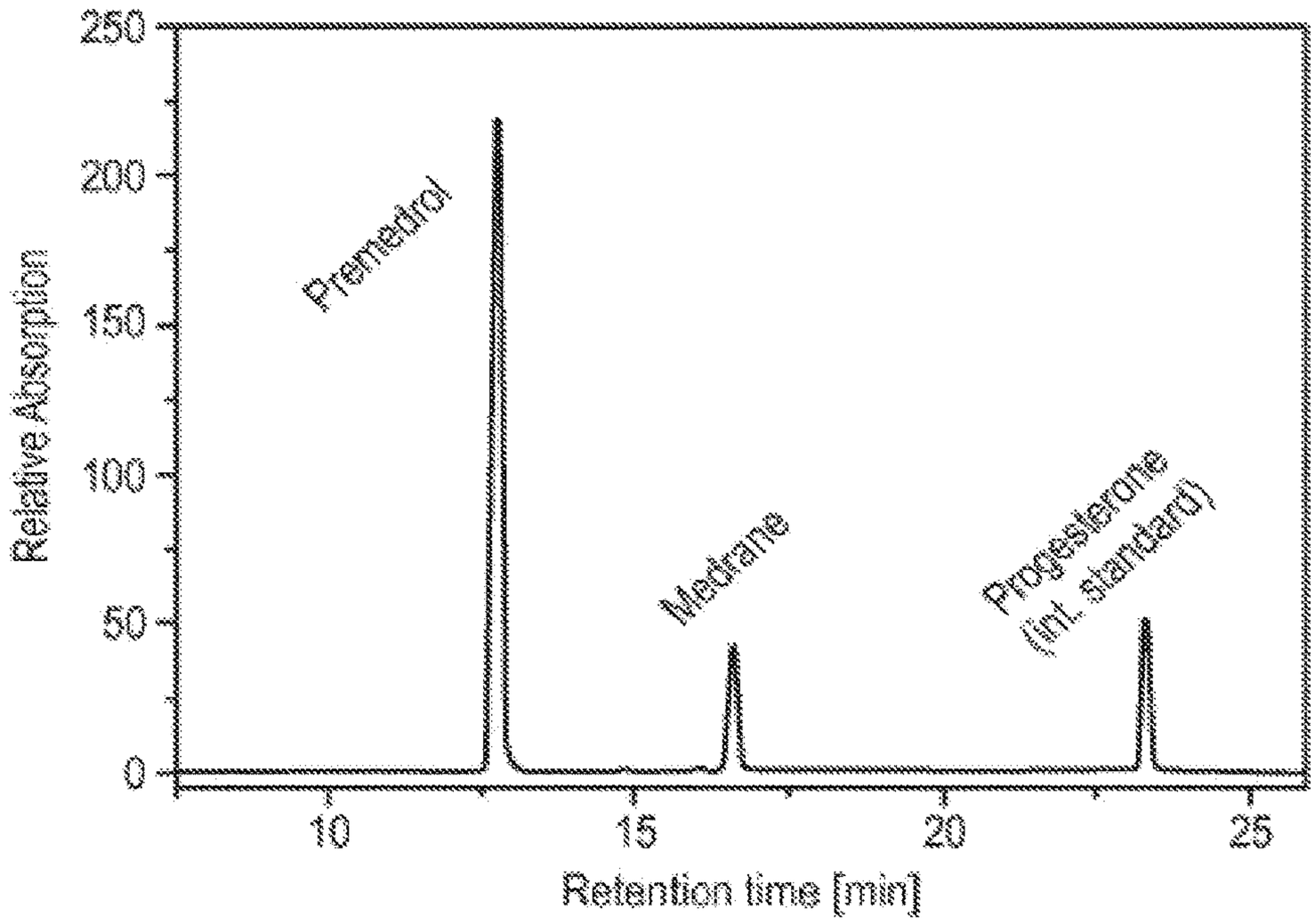

Relative Absorption
250
200
150
100
50
0
Pregnediol
Medrane
Progesterone
(int. standard)
10
15
20
25
Retention time [min]

FIG.6B

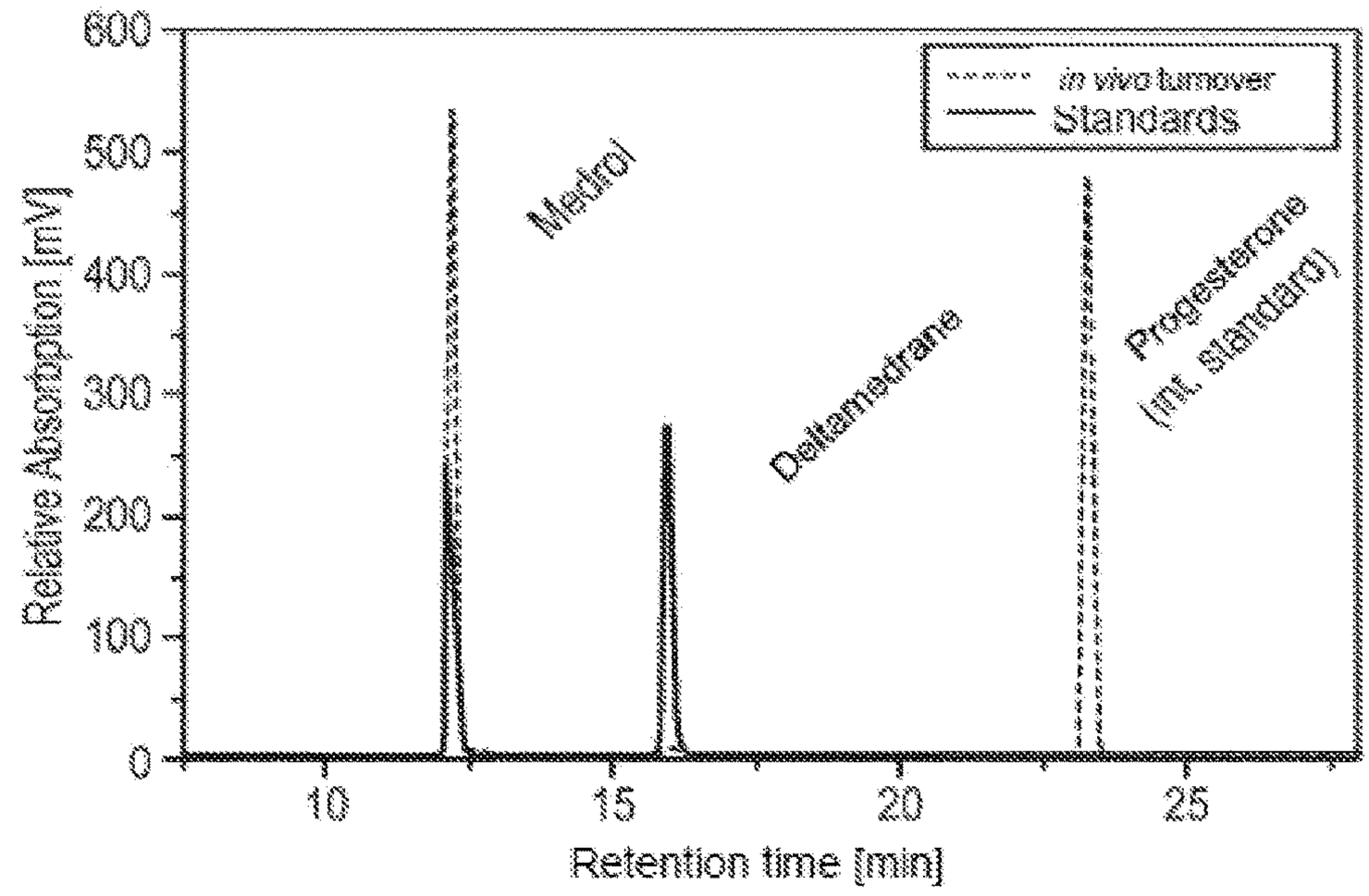

in vivo turnover
Standards
Relative Absorption [mV]
600
500
400
300
200
100
0
Medrol
Deltamedrane
Progesterone
(int. standard)
10
15
20
25
Retention time [min]

FIG.6C

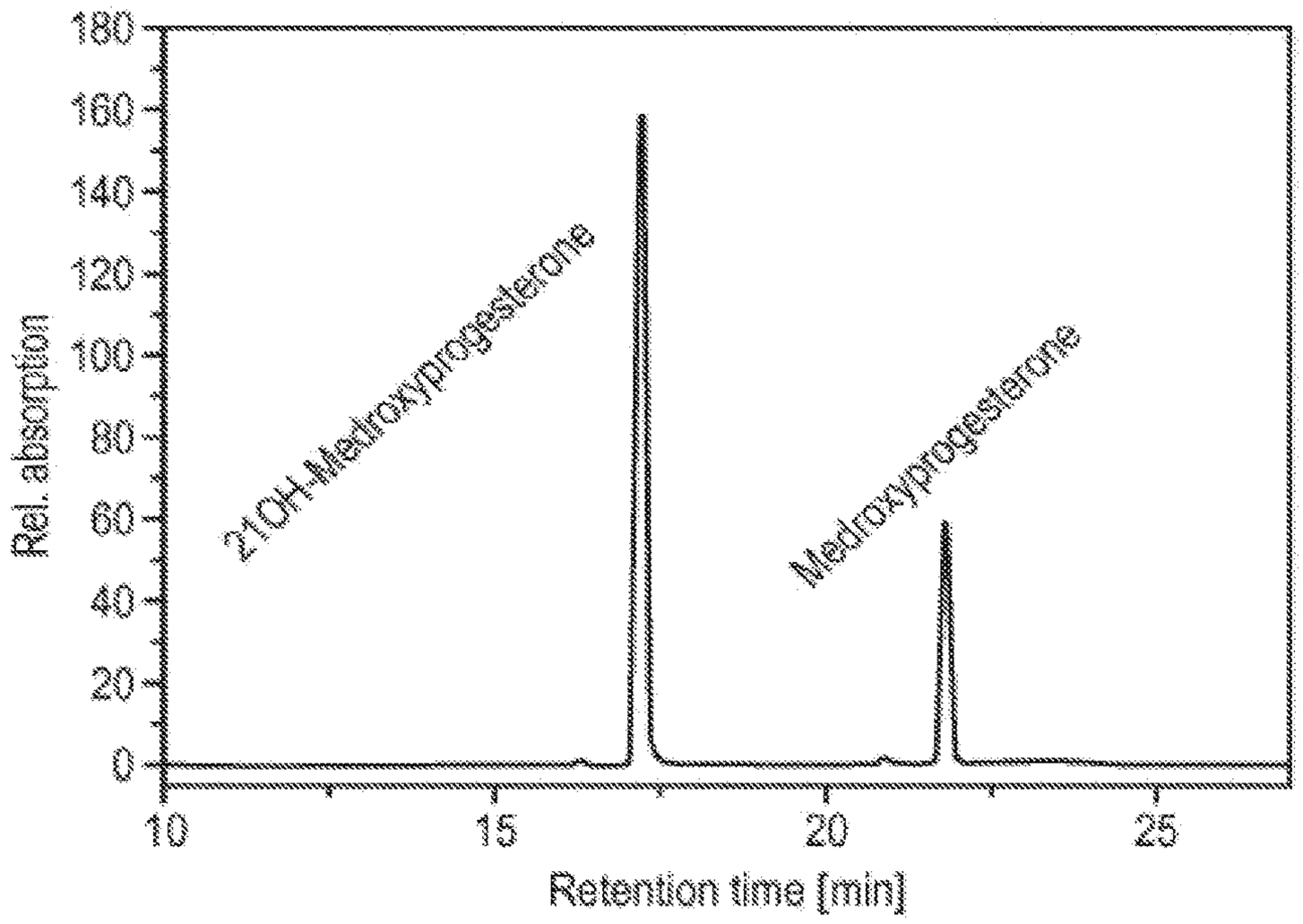

Rel. absorption
0
20
40
60
80
100
120
140
160
180
10
15
20
25
Retention time [min]
21OH-Medroxyprogesterone
Medroxyprogesterone

FIG.6D

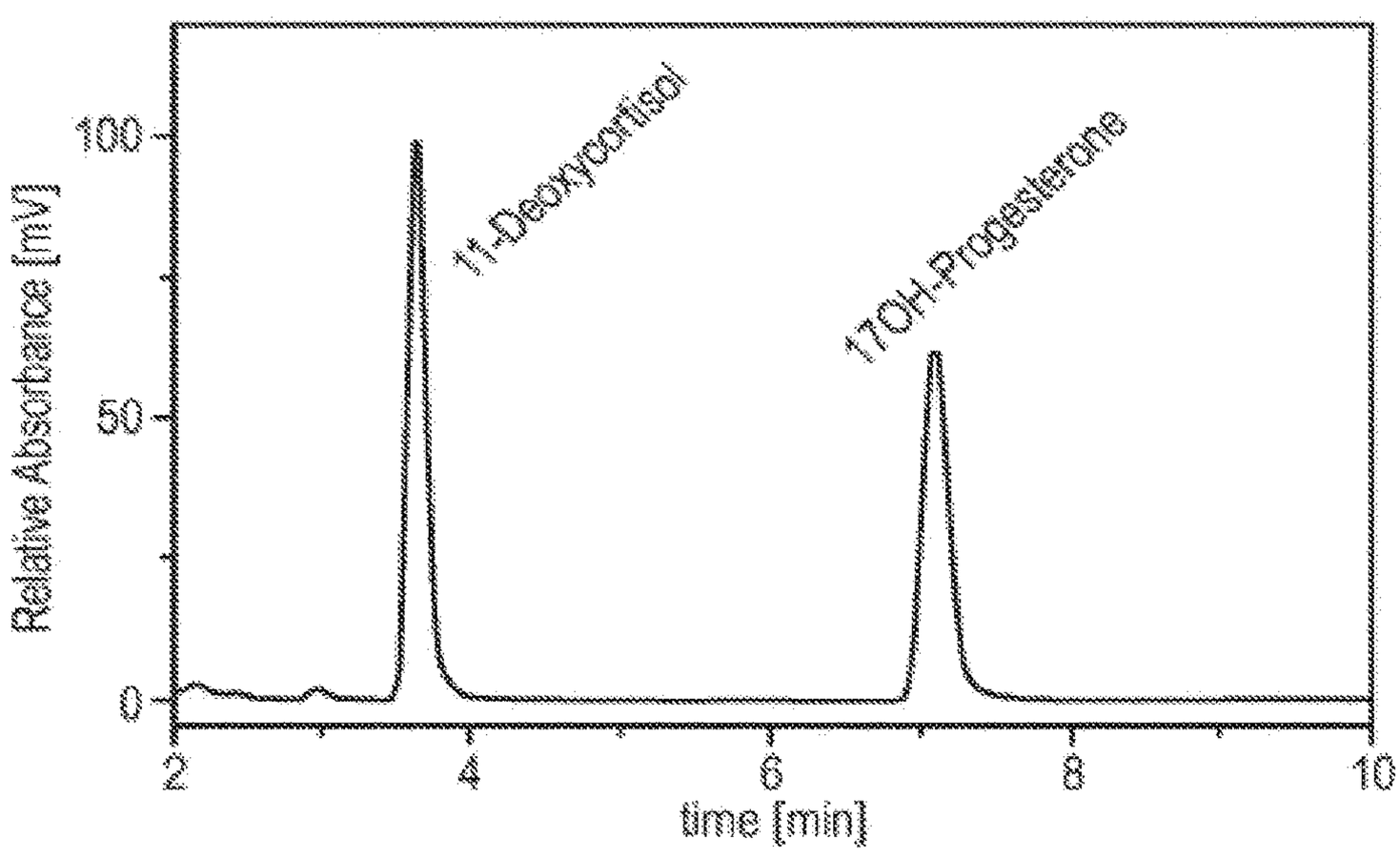

Relative Absorbance [mV]
0
50
100
2
4
6
8
10
time [min]
11-Deoxycortisol
17OH-Progesterone

FIG.7

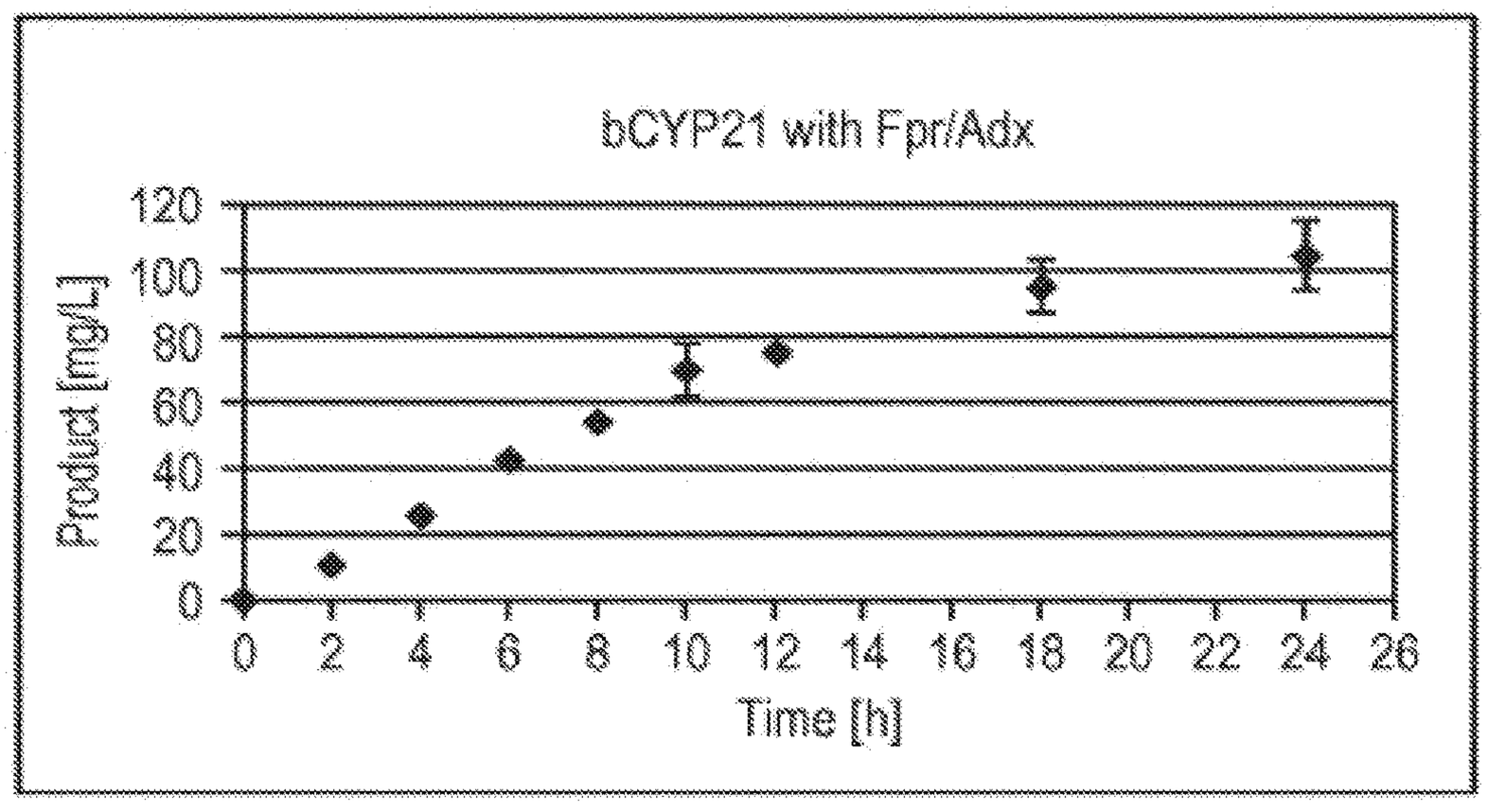

bCYP21 with Fpr/Adx
Product [mg/L]
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time [h]

FIG.8A

Wildtype human amino acid sequence:

MLLLGLLLLLPLLAGARLLWNWWKLRSLHLPPLAPGFLHLLQPDLPIYLLGLTQKFGPIYRLHL
GLQDVVVLNSKRTIEEAMVKKWADFAGRPEPLTYKLVSRNYPDLSLGDYSLLWKAHKKLTR
SALLLGIRDSMEPVVEQLTQEFCERMRAQPGTPVAIEEEFSLLTCSIICYLTFGDKIKDDNLM
PAYYKCIQEVLKTWSHWSIQIVDVIPFLRFFPNPGLRRLKQAIEKRDHIVEMQLRQHKESLVA
GQWRDMMDYMLQGVAQPSMEEGSGQLLEGHVHMAAVDLLIGGTETTANTLSWAVVFLLH
HPEIQQRLQEELDHELGPGASSSRVPYKDRARLPLLNATIAEVLRLRPVVPLALPHRTTRPS
SISGYDIPEGTVIIPNLQGAHLDETVWERPHEFWPDRFLEPGKNSRALAFGCGARVCLGEPL
ARLELFVVLTRLLQAFTLLPSGDALPSLQPLPHCSVILKMQPFQVRLQPRGMGAHSPGQSQ

Modified human amino acid sequence:

MAKKTSSKGKPPLAPGFLHLLQPDLPIYLLGLTQKFGPIYRLHLGLQDVVVLNSKRTIEEAMV
KKWADFAGRPEPLTYKLVSRNYPDLSLGDYSLLWKAHKKLTRSALLLGIRDSMEPVVEQLT
QEFCERMRAQPGTPVAIEEEFSLLTCSIICYLTFGDKIKDDNLMPAYYKCIQEVLKT WSHWSI
QIVDVIPFLRFFPNPGLRRLKQAIEKRDHIVEMQLRQHKESLVAGQWR DMMDYMLQGVAQP
SMEEGSGQLLEGHVHMAAVDLLIGGTETTANTLSWAVVFLLHHPEIQQRLQEELDHELGPG
ASSSRVPYKDRARLPLLNATIAEVLRLRPVVPLALPHRTTRPSSISGYDIPEGTVIIPNLQGAH
LDETVWERPHEFWPDRFLEPGKNSRALAFGCGARVCLGEPLARLELFVVLTRLLQAFTLLP
SGDALPSLQPLPHCSVILKMQPFQVRLQPRGMGAHSPGQSQ**HHHHHH**

FIG.8B

Wildtype bovine amino acid sequence:

*MVLAGLLLLLTLLAGAHLLWGRWKLRNLHL*PPLVPGFLHLLQPNLPIHLLSLTQKLGPVYRLR
LGLQEVVVLNSKRTIEEAMIRKWVDFAGRPQIPSYKLVSQRCQDISLGDYSLLWKAHKKLTR
SALLLGTRSSMEPWVDQLTQEFCERMRVQAGAPVTIQKEFSLLTCSIICYLTFGNKEDTLVH
AFHDCVQDLMKTWDHWSIQILDMVPFLRFFPNPGLWRLKQAIENRDHMVEKQLTRHKESM
VAGQWRDMTDYMLQGVGRQRVEEGPGQLLEGHVHMSVVDLFIGGTETTASTLSWAVAFL
LHHPEIQRRLQEELDRELGPGASCSRVTYKDRARLPLLNATIAEVLRLRPVVPLALPHRTTRP
SSIFGYDIPEGMVVIPNLQGAHLDETVWEQPHEFRPDRFLEPGANPSALAFGCGARVCLGE
SLARLELFVVLLRLLQAFTLLPPPVGALPSLQPDPYCGVNLKVQPFQVRLQPRGVEAGAWE
SASAQ

Modified bovine amino acid sequence:

MAKKTSSKGKPPLVPGFLHLLQPNLPIHLLSLTQKLGPVYRLRLGLQEVVVLNSKRTIEEAMI
RKWVDFAGRPQIPSYKLVSQRCQDISLGDYSLLWKAHKKLTRSALLLGTRSSMEPWVDQLT
QEFCERMRVQAGAPVTIQKEFSLLTCSIICYLTFGNKEDTLV HAFHDCVQDLMKTWDHWSIQ
ILDMVPFLRFFPNPGLWRLKQAIENRDHMVEKQLTRHKESMVAGQWRDMTDYMLQGVGR
QRVEEGPGQLLEGHVHMSVVDLFIGGTETTASTLSWAVAFLLHHPEIQRRLQEELDRELGP
GASCSRVTYKDRARLPLLNATIAEVLRLRPVVPLALPHRTTRPSSIFGYDIPEGMVVIPNLQG
AHLDETVWEQPHEFRPDRFLEPGANPSALAFGCGARVCLGESLARLELFVVLLRLLQAFTLL
PPPVGALPSLQPDPYCGVNLKVQPFQVRLQPRGVEAGAWESASAQ**HHHHHH**

21-HYDROXYLATION OF STEROIDS

This application is a national stage application under 35 U.S.C. § 371 of International Application No. PCT/EP2015/075096, filed Oct. 29, 2015, which claims the benefit of European Application No. EP 14306740.3, filed Oct. 30, 2014, the disclosures of which are explicitly incorporated herein in their entirety by reference.

Generally, the present invention relates to the field of steroid hydroxylation. More specifically, the present invention relates to a method for the 21-hydroxylation of steroids in cells. It also relates to cells expressing a steroid 21-hydroxylating enzyme or steroid 21-hydroxylase, expression vectors comprising a nucleic acid encoding for a steroid 21-hydroxylase and a kit for carrying out the method for the 21-hydroxylation of steroids in cells.

Synthetic glucocorticoids are descendent from the natural occurring stress hormone cortisol and play a crucial role in pharmaceutical industry because of their anti-inflammatory and immune suppressive effects. Moreover, synthetic molecules often act more effective than cortisol.

Currently, the synthesis of some pharmaceutically active steroids involves a 21-hydroxylation of their precursor (see FIG. **1** for an example), which consists of a long lasting chemical multistep synthesis. By means of synthetic chemistry this hydroxylation is also difficult, as the chemical oxidants are not selective to position 21. For this reason, other functional groups have to be protected to avoid their oxidation and to direct the hydroxylation reaction towards position 21. Furthermore, the synthesis is not environmentally friendly because of the use of reagents such as iodine. Therefore, a cheap and sustainable production of pharmaceutically active steroids is highly desirable to satisfy the high demand for these important drugs.

This problem has been addressed by the present inventors by the development of whole cell biotransformation of steroids in a one-step synthesis by the enzyme CYP21A2, which is a member of the protein family of the cytochrome P450 monooxygenases and which is able to perform a highly selective hydroxylation of steroids at the 21-position of the steroid scaffold (see FIG. **2** for a scheme of the process). CYP21A2 is a mammalian membrane anchored enzyme which is located in the endoplasmic reticulum and which plays a crucial role in the steroid hormone biosynthesis. The inventors have shown that the biocatalytic system of the invention is a promising candidate to replace the established chemical synthesis. In particular, they have shown that steroids could be modified within one single hydroxylation step, leading to the one desired product, which is saving time, is environmentally friendly (no by-products were observed) and facilitates downstream processing. Furthermore and advantageously, for the steroid production in whole cells according to the invention, enzymes do not have to be purified, remain stable in the host and the addition of costly redox equivalents like NADPH is not necessary, because the cell itself serves as a donor.

Before the present invention is described in detail below, it is to be understood that this invention is not limited to the particular methodology, protocols and reagents described herein as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art.

Preferably, the terms used herein are defined as described in "A multilingual glossary of biotechnological terms: (IUPAC Recommendations)", Leuenberger, H. G. W, Nagel, B. and Kölbl, H. eds. (1995), Helvetica Chimica Acta, CH-4010 Basel, Switzerland).

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including all patents, patent applications, scientific publications, manufacturer's specifications, instructions etc.), whether supra or infra, is hereby incorporated by reference in its entirety. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

In the following, the elements of the present invention will be described. These elements are listed with specific embodiments, however, it should be understood that they may be combined in any manner and in any number to create additional embodiments. The variously described examples and preferred embodiments should not be construed to limit the present invention to only the explicitly described embodiments. This description should be understood to support and encompass embodiments which combine the explicitly described embodiments with any number of the disclosed and/or preferred elements. Furthermore, any permutations and combinations of all described elements in this application should be considered disclosed by the description of the present application unless the context indicates otherwise.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", are to be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integer or step. As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural referents, unless the content clearly dictates otherwise.

In a first aspect, the present invention relates to a process for the hydroxylation of the carbon atom 21 of a steroid, comprising the steps of:

a) providing a cell expressing
   (i) a heterologous CYP21A2 protein or a functional variant thereof,
   (ii) at least one heterologous electron transfer system capable of transferring electrons to CYP21A2, and
   (iii) one or more chaperones facilitating folding of CYP21A2; and
b) adding the steroid to the cell.

A steroid is a type of organic compound that contains a characteristic arrangement of four cycloalkane rings that are joined to each other (shown below). The core of steroids is composed of seventeen carbon atoms bonded together that take the form of four fused rings: three cyclohexane rings (designated as rings A, B and C) and one cyclopentane ring (the D ring). The steroids vary by the functional groups attached to this four-ring core and by the oxidation state of the rings.

The hydroxylation of the carbon atom 21 of a steroid is the addition of an —OH group at position 21 as shown in the above-shown ABCD steroid ring system. The numbering of the carbon atoms is according to the IUPAC (International Union of Pure and Applied Chemistry)-approved ring lettering and atom numbering. The 21-hydroxylation of a steroid is shown in FIG. **1**.

In a particular embodiment of the process of the first aspect of the invention, the steroid is a 3-keto steroid. More particularly, the steroid is a non-natural steroid, i.e. a steroid

that is not produced and/or 21-hydroxylated in cells, especially human or bovine cells, which are not genetically altered.

In one embodiment, the steroid is selected from the group consisting of medrane, deltamedrane, progesterone, 17OH-progesterone, medroxyprogesterone, and 5-α-dihydro-progesterone. The 21-hydroxylation converts these particular steroids to premedrol, medrol, 11-deoxycorticosterone, 11-deoxycortisol, 21OH-medroxyprogesterone and 21OH-(5α-dihydroprogesterone), respectively. In one particular embodiment, in which the steroid is a non-natural steroid, the steroid is selected from the group consisting of medrane, deltamedrane, medroxyprogesterone, and 5-α-dihydro-progesterone.

The cell is in particular a cultured cell, cultured in any cell medium, e.g. in a growth medium, and is, in a particular embodiment, in a resting state. According to this embodiment, the cell is comprised in a buffer or medium capable of maintaining the cell rather than in a growth medium. The composition of the buffer depends on the particular cell and suitable buffers are well-known in the art. Depending on the cell-type, the cell is a suspension cell or an adherent cell. A suspension cell is a cell that may naturally live in suspension (i.e. without being attached to a surface), or a cell that has been modified to be able to survive in suspension cultures, for example to be grown to higher densities than adherent conditions would allow. An adherent cell is a cell that requires a surface, such as tissue culture plastic or micro-carrier, which may be coated with extracellular matrix (such as collagen and laminin) components to increase adhesion properties and provide other signals needed for growth and differentiation. In one embodiment, the adherent cell is a monolayer cell.

Generally, the cell may be a prokaryotic cell or a eukaryotic cell. A particular example of a prokarytic cell to be used in the process of the first aspect is an *E. coli* cell, e.g. of the *E. coli* strain C43(DE3) of the examples. A particular example of a eukaryotic cell is a yeast cell, e.g. a *S. cerevisae* cell or a *Schizosaccharomyces pombe* cell. However, the process of the first aspect is not limited to any particular cell type and any cell type may be used, in particular any cell type that can be grown and maintained in culture and that can be used as a recombinant expression system, like insect cells or mammalian cells in addition to the above-mentioned cells.

The term "heterologous" means that a protein is expressed in cell that does not normally (i.e. without human intervention) express that protein. CYP21A2, for example is an enzyme also called 21-hydroxylase, which is part of the cytochrome P450 family of enzymes. Cytochrome P450 enzymes are involved in many processes in the body, such as assisting with reactions that break down drugs and helping to produce cholesterol, certain hormones, and fats (lipids). The 21-hydroxylase enzyme is found in the adrenal glands, which are located on top of the kidneys and produce a variety of hormones that regulate many essential functions in the body. Therefore, with respect to heterologous expression, the CYP21A2 protein can be considered heterologous regarding any cell which is not an adrenal gland cell.

In particular, the term "heterologous" can also refer to the species a protein or gene is derived from in comparison to the cell in which it is expressed, in particular recombinantly expressed. A heterologous protein is then a protein that is derived from a different species than the cell it is expressed in, i.e. the cell of the process of the first aspect of the invention.

For example, the present inventors expressed mammalian, in particular human or bovine proteins in an *E. coli* cell, making these proteins heterologous with respect to the cell.

In a particular embodiment of the process of the first aspect of the invention, the CYP21A2 protein is of human or bovine origin. Human CYP21A2 (UniProt accession number P08686) has the sequence according to SEQ ID NO: 1 of the sequence listing. Bovine CYP21A2 (UniProt accession number P00191) has the sequence according to SEQ ID NO: 2 of the sequence listing. See also FIG. **8**. Homologous genes, however, do also exist in other mammalian species, such as *Canis lupus* (dog), *Macaca mulata* (resus monkey), *Rattus norvegicus* (rat), *Gallus gallus* (chicken), *Danio rerio* (zebra fish), *Mus musculus* (mouse), or *Pan troglodytes* (chimpanzee). Therefore, it is emphasized that any CYP21A2 protein can be used, in particular any mammalian CYP21A2 protein. A modified human or bovine CYP21A2 according to SEQ ID NO: 3 and SEQ ID NO: 4, respectively, can also be used. See also FIG. **8**.

The term "functional variant" is a protein variant that has at least 20% (e.g., at least: 20%; 30%; 40%; 50%; 60%; 70%; 80%; 90%; 95%; 98%; 99%; 99.5%; or 100% or even more) of the ability of the unaltered or CYP21A2 protein to 21-hydroxylate a steroid. This ability can be determined by the skilled person without undue burden using, for example, the methods shown in Examples 1 and 2 herein.

A "protein variant" is a protein that has an amino acid sequence that it at least 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the amino acid sequence of the CYP21A2 it is derived from, for example SEQ ID NO: 1 or 3 in case of human CYP21A2 or SEQ ID NO: 2 or 4 in case of bovine CYP21A2. The determination of percent identity between two sequences is accomplished using the mathematical algorithm of Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 90, 5873-5877, 1993. Such an algorithm is incorporated into the BLASTN and BLASTP programs of Altschul et al. (1990) J. Mol. Biol. 215, 403-410. To obtain gapped alignments for comparative purposes, Gapped BLAST is utilized as described in Altschul et al. (1997) Nucleic Acids Res. 25, 3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs are used.

Alternatively, a protein variant can also be defined as having up to 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 4, 3, 2, or 1 amino acid substitutions, in particular conservative amino acid substitutions. Conservative substitution tables are well known in the art (see for example Creighton (1984) Proteins. W. H. Freeman and Company). An overview of physical and chemical properties of amino acids is given in Table 1 below. In a particular embodiment, conservative substitutions are substitution made with amino acids have the same properties according to Table 1.

TABLE 1

Properties of naturally occuring proteins.

Charge properties/ hydrophobicity	Side group	Amino Acid
nonpolar hydrophobic	aliphatic	Ala, Ile, Leu, Val
	aliphatic, S-containing	Met
	aromatic	Phe, Trp
	imino	Pro

TABLE 1-continued

Properties of naturally occuring proteins.

Charge properties/ hydrophobicity	Side group	Amino Acid
polar uncharged	aliphatic	Gly
	amide	Asn, Gln
	aromatic	Tyr
	hydroxyl	Ser, Thr
	sulfhydryl	Cys
positively charged	basic	Arg, His, Lys
negatively charged	acidic	Asp, Gly

The term "variant" also includes protein fragments. A fragment of CYP21A2 has an N-terminal and/or C-terminal deletion of up to 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 4, 3, 2, or 1 amino acids in total. In a particular embodiment, the functional variant is a fragment of CYP21A2 lacking the hydrophobic anchor region, i.e. a truncation of 29 N-terminal amino acid residues (FIG. **8**).

In addition, the CYP21A2 protein may be modified, for example by N-terminal or C-terminal amino acid additions, such as tags or N-terminal modifications for improved bacterial expression. For example, the tag may be a C-terminal His-tag, e.g. 6× His-tag and the N-terminal modification an addition of ten amino acids from the N-terminus of CYP2C3 (see FIG. **8** and SEQ ID Nos 3 and 4).

An electron transfer system is a series of compounds that transfer electrons from electron donors to electron acceptors via redox reactions. The term "capable of transferring electrons to CYP21A2" thereby means that CYP21A2 is the electron acceptor of this series. The term "series" herein may include CYP21A2 and, thus, the electron transfer system may consist of only one protein in addition to CYP21A2. For the process of the first aspect of the invention, it is not crucial which members the electron transfer system consists of, as long as the system is capable of transferring electrons to CYP21A2. Suitable systems are well-known in the art. In a particular embodiment of the process of the first aspect of the invention, the at least one electron transfer system comprises (i) a CYP21A2 reductase, or (ii) a ferredoxin reductase, preferably an NADPH-dependent ferredoxin reductase (adrenodoxin reductase) and a ferredoxin. For example, the at least one electron transfer protein can be selected from the group consisting of an NADPH-dependent cytochrome P450 reductase (CPR, e.g. human or bovine), the combination of an adrenodoxin reductase (AdR, e.g. human or bovine) and an adrenodoxin (Adx4-108, e.g. human or bovine), the combination of a flavodoxin reductase (Fpr, e.g. from *E. coli*) and an adrenodoxin (Adx4-108, e.g. human or bovine), the combination of an adrenodoxin reductase homolog (Arh), e.g. *S. pombe* adrenodoxin reductase homolog (Arh1), and an adrenodoxin (Adx4-108, e.g. human or bovine), the combination of an adrenodoxin reductase homolog (Arh), e.g. *S. pombe* adrenodoxin reductase homolog (Arh1), and the ferredoxin domain of an electron transfer domain (etp^{fd}), e.g. the ferredoxin domain of the *S. pombe* electron transfer domain ($etp1^{fd}$), and the combination of a flavodoxin reductase (Fpr, e.g. from *E. coli*) and the ferredoxin domain of an electron transfer domain (etp^{fd}), e.g. the ferredoxin domain of the *S. pombe* electron transfer domain ($etp1^{fd}$).

In a particular embodiment, exogenous NADPH is not added to the cell. In this embodiment, NADPH is produced by the cell. In a specific embodiment, the NADPH-production in the cell may be increased by recombinant means, e.g. the heterologous expression of enzymes involved in NADPH production (e.g. one or more of glucose-6-phosphate dehydrogenase (G6PD), phosphogluconate dehydrogenase (PGD), malate dehydrogenase (MDH), and/or isocitrate dehydrogenase (ICDH)), and/or it may be increased by providing hormonal signals, i.e. adding hormones such as estradiol that enhance the level of endogenous NADPH production.

Similarly, the chaperone can be any chaperone as long as it is capable of facilitating folding of CYP21A2. The present inventors found that the process of the first aspect of the invention can rely only on endogenous chaperones, i.e. additional expression of suitable chaperones is not essential. However, an additional expression improves the production of functional CYP21A2 in the cell and can therefore be beneficial. Thus, in one embodiment of the process of the first aspect of the invention, the one or more chaperones are recombinantly expressed chaperones. In particular, the one or more chaperones may be heterologous chaperones. Exemplary chaperones are the *E. coli* chaperones GroEL and GroES or other chaperones like DnaK, DnaJ, GrpE, and ClpB as well as small heat shock proteins (sHSP) such as IbpA and IbpB (IbpAB).

Optionally, the same chaperone or one or more additional chaperone(s) to be expressed in the cell is/are capable of folding one or more of above electron transfer proteins.

Recombinant expression refers to the expression of a recombinant gene. Such a gene can be any gene introduced into the cell by methods of genetic engineering and is usually a heterologous gene and/or a gene regulated differently than a possible endogenous counterpart gene in terms of expression. In one embodiment, the recombinant expression is inducible.

In one embodiment of the process of the first aspect of the invention, the process further comprises adding one or more cell permeabilizing agents to the suspension of cells, for example after step b). Cell permeabilizing agents are reagents which increase the permeability of membranes. Examples are organic solvents, such as methanol, acetone or DMSO, detergents such as saponin, Triton X-100 or Tween-20, and EDTA. In particular, polymyxin B can be used as a cell permeabilizing agent. This agent proved to work particularly well in the process of the invention.

In another embodiment of the process of the first aspect of the invention, the process further comprises a step c) of extracting the 21-hydroxylated steroid from the cell and/or the supernatant of the cell (i.e. the buffer or medium the cell is comprised in), for example from a whole cell suspension. The extraction can be done with any solvents or extraction methods known in the art for extracting undissolved compounds. For example, a solvent such as 1-butanol, 2-butanone or chloroform may be used.

In a specific embodiment of the process of the first aspect of the invention, the expression of at least one tryptophanase gene is reduced or abolished in the cell. A tryptophanase or L-tryptophan indole-lyase (EC number 4.1.99.1.) is an enzyme catalyzing the reaction L-tryptophan+H2O=indole+pyruvate+NH3. Accordingly, the reduction or abolishment will lead to the decrease of indol production by the cell, which can improve the process, since indol is an inhibitor of CYP enzymes such as CYP21A2. As tryptophanase genes are generally (but not exclusively) found in prokaryotes, e.g. *E. coli*, this specific embodiment applies in particular to the embodiment of the process of the first aspect of the invention in which the cell is a prokaryotic cell. In one embodiment, the species of a cell expressing a tryptophansae gene is selected from the group consisting of *Aeromonas hydrophila, Bacillus* sp., *Bacteroides* sp., *Corynebacterium* sp.,

Enterobacter aerogenes, Enterobacter aerogenes SM-18, *Enterobacter* sp., *Erwinia* sp., *Escherichia aurescens, Escherichia coli, Fusobacterium necrophorum* subsp. *Funduliforme, Kluyvera* sp., *Micrococcus aerogenes, Morganella morganii, Paenibacillus alvei, Paracolobactrum coliforme, Paracolobactrum* sp., *Pasteurella* sp., *Photobacterium profundum, Porphyromonas gingivalis, Prevotella intermedia, Proteus vulgaris, Providencia rettgeri, Shigella alkalescens, Sphaerophorus* sp., *Symbiobacterium thermophilum, Vibrio* sp. and a mammalian species such as *Homo sapiens* and *Rattus norvegicus,*

In a further embodiment of the process of the first aspect of the invention, the cell further expresses a heterologous or recombinant gene encoding for an enzyme catalyzing a step in the heme biosynthesis pathway, in particular a heterologous hemA (glutamyl tRNA reductase) gene. An example for such cells is *E. coli*. This will advantageously reduce the need for feeding precursors for the synthesis of the CYP heme, such as the heme precursor δ-aminolevulinic acid, and reduce the costs for the biotransformation of steroids in such cells.

In a particular embodiment of the process of the first aspect of the invention, the nucleic acids encoding for (i) a heterologous CYP21A2 protein or a functional variant thereof, (ii) at least one heterologous electron transfer system capable of transferring electrons to CYP21A2, and optionally (iii) one or more chaperones facilitating folding of CYP21A2 are comprised in one or more expression cassette(s) which is/are integrated into the cell, in particular into its genome. The term "expression cassette" refers to a DNA fragment which comprises a gene operably linked to a regulatory sequence such as a promoter, necessary for gene expression. "Operably linked" refers to the linking of nucleotide regions encoding specific genetic information such that the nucleotide regions are contiguous, the functionality of the region is preserved and will perform relative to the other regions as part of a functional unit. The nucleic acids (i), (ii) and optionally (iii) may each be comprised in individual expression cassettes or in one or more multicistronic expression cassettes. As used herein, the term "multicistronic" means that multiple cistrons, namely, multiple nucleic acids or genes, are operably linked to the same regulatory sequence, e.g. a promoter.

In one embodiment, the nucleic acids encoding for (i) a heterologous CYP21A2 protein or a functional variant thereof, (ii) at least one heterologous electron transfer system capable of transferring electrons to CYP21A2, and optionally (iii) one or more chaperones facilitating folding of CYP21A2 are comprised in an expression vector comprised in the cell. An "expression vector" is a vehicle by means of which DNA fragments that contain nucleic acids encoding for a protein can be introduced into host cells where the nucleic acids can be expressed by the host cell. The nucleic acids (i), (ii) and optionally (iii) may each be comprised in individual expression vectors or in one or more multicistronic expression vectors.

Also, one or more of the nucleic acids (i), (ii) and optionally (iii) may be comprised in an expression cassette which is integrated into the cell genome, whereas the remaining nucleic acids (i), (ii) or optionally (iii) are comprised in an expression expression vector, both as set out above.

In a second aspect, the present invention relates to cell expressing

(i) a heterologous CYP21A2 protein or a functional variant thereof,

(ii) at least one heterologous electron transfer system capable of transferring electrons to CYP21A2, and

(iii) one or more chaperones facilitating folding of CYP21A2.

This cell as well as the cell of the process of the first aspect of the invention can also be described as a cell comprising one or more nucleic acids encoding for

(i) a heterologous CYP21A2 protein or a functional variant thereof,

(ii) at least one heterologous electron transfer system capable of transferring electrons to CYP21A2, and

(iii) one or more chaperones facilitating folding of CYP21A2.

In a particular embodiment, these nucleic acids are comprised in one or more expression cassettes.

The cell of the second aspect is in essence the cell used in the process of the first aspect of the invention and, therefore, further embodiments of the cell of the second aspect of the invention are as described above with respect to the process of the first aspect.

In a third aspect, the present invention relates to a multicistronic expression vector comprising (i) a nucleic acid encoding for a CYP21A2 protein or a functional variant thereof, (ii) one or more nucleic acids encoding for at least one heterologous electron transfer system capable of transferring electrons to CYP21A2, and optionally (iii) one or more nucleic acids encoding for chaperones facilitating folding of CYP21A2.

In a fourth aspect, the present invention relates to a kit comprising

a cell of the second aspect,

a multicistronic expression vector of the third aspect, or

(i) an expression vector comprising a nucleic acid encoding for a CYP21A2 protein or a functional variant thereof, (ii) one or more expression vectors comprising one or more nucleic acids encoding for at least one heterologous electron transfer system capable of transferring electrons to CYP21A2, and optionally (iii) one or more expression vectors comprising one or more nucleic acids encoding for chaperones facilitating folding of CYP21A2.

The term "kit" is used herein to mean a collection of all or some of the reagents, materials, and instructions necessary to carry out the process of the first aspect. This includes cell culture medium or buffer (both in dry or liquid form), one or more cell permeabilizing agents, solvents for extracting the steroid and, in particular, the steroid to be hydroxylated, in particular a 3-keto steroid, for example one or more of medrane, deltamedrane, progesterone, 17OH-progesterone, medroxyprogesterone, or 5α-dihydroprogesterone. Furthermore, it is envisaged that all reagents or materials described herein with relation to the process of the first aspect can be part of the kit of the fourth aspect.

In a particular embodiment, the process of the first aspect, the cell of the second aspect, the expression vector of the third aspect and the kit of the fourth aspect do not comprise a further step or component as applicable, especially a heterologous gene or protein, for steroid conversion or production which is not related to the 21-hydroxylation of steroids, particularly as described herein. Optionally, an exception of this may be downstream steps or components related to the further processing of the 21-hydroxylated steroid (such as the conversion from premedrol to medrol) or upstream steps or components related to the production of the steroid to be 21-hydroxylated (such as the production of medrane).

In the following figures and examples, some particular embodiments of the invention are described in more detail. Yet, no limitation of the invention is intended by the details of the particular embodiments. In contrast, the invention pertains to any embodiment which comprises details which are not explicitly mentioned in the embodiments herein, but which the skilled person finds without undue effort.

DESCRIPTION OF THE FIGURES

FIG. **1**: Hydroxylation of a steroid (here progesterone) at carbon atom 21.

FIG. **2**: Scheme of a whole cell biotransformation of a steroid by CYP21A2 and the needed electron transfer proteins in *E. coli*.

FIG. **3**: left: CO difference spectrum of purified bovine CYP21; right: SDS-PAGE of bCYP21 samples taken after indicated purification steps (IMAC/DEAE/SP).

FIG. **4**: HPLC chromatogram of the in vitro 21-hydroxylation of medrane to premedrol by human CYP21 and described electron transfer proteins, here AdR and Adx (system 2).

FIGS. **5**A-**5**B: Constructed vectors for whole cell biotransformation using human or bovine CYP21 with different electron transfer proteins.

FIGS. **6**A-D: HPLC chromatogram of the whole cell 21-hydroxylation of medrane to premedrol (A), delta-medrane to medrol (B), medroxyprogesterone to 21OH-medroxyprogesterone (C), and 17OH-progesterone to 11-deoxycortisol (D) by bovine CYP21 and described electron transfer proteins, here Fpr and Adx.

FIG. **7**: Time-dependent whole cell conversion of medrane to premedrol by bovine CYP21 and described electron transfer proteins, here Fpr and Adx.

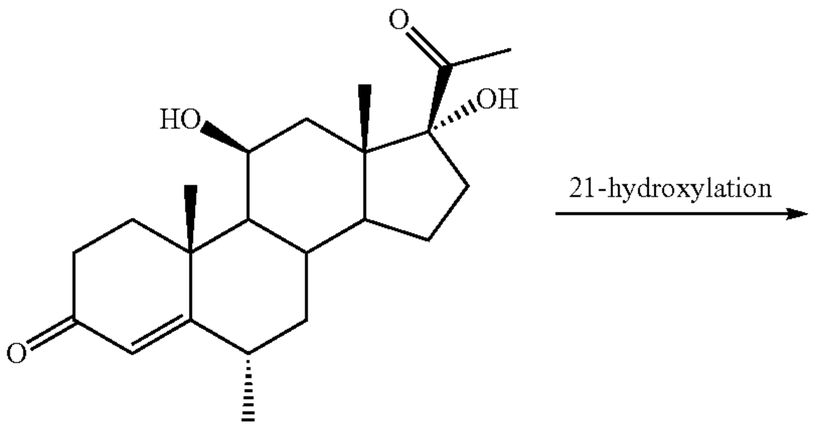
FIGS. **8**A-**8**B: Amino acid sequences of wildtype and modified human (A) and bovine (B) CYP21A2.

DESCRIPTION OF THE EXAMPLES

Example 1: In Vitro Hydroxylation

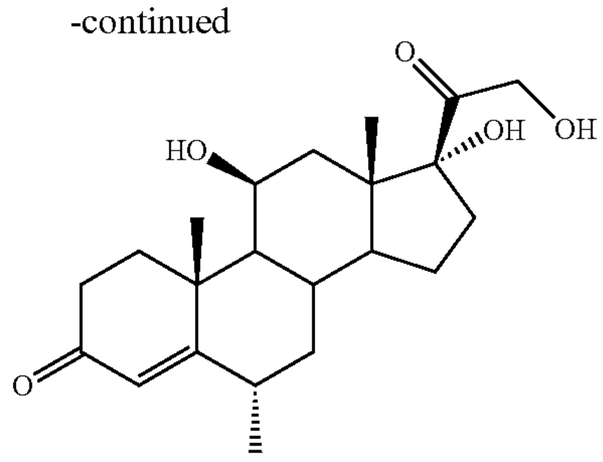
1.1 Expression/Purification of hCYP21/bCYP21

To show that both human and bovine CYP21 are able to hydroxylate steroids at position 21, in vitro studies with both enzymes were performed. As an exemplary 21-hydroxylation process, medrane was converted to premedrol:



Medrane

-continued



Premedrol
(Methylhydrocortison)

Premedrol (methylhydrocortisone) is a precursor of a highly effective pharmaceutical steroid medrol (methylprednisolone). Medrol is an important drug in therapy of autoimmune diseases, multiple sclerosis and in general for local and systematic treatment of inflammations.

Both enzymes were expressed in the *E. coli* strain C43 (DE3) by coexpression of the *E. coli* chaperones GroEL/GroES encoded in the vector pGro12. These chaperones ensure a correct protein folding which is important for an incorporation of the heme prosthetic group. In FIG. **3** an SDS-PAGE and a CO difference spectrum of purified bovine CYP21 are shown. As the CO difference spectrum shows, the enzyme was purified in an active form. To determine the binding of medrane to both isozymes the binding constants (K_D-values) were determined.

1.2 Expression of Electron Delivering Redox Partners

For an efficient substrate conversion, both isoforms require an electron transfer system which consists of two parts, the cytochrome P450 enzyme itself and one or two electron transfer proteins which are essential for a hydroxylation reaction. Without these transfer proteins, no reaction will take place. Electrons can be transferred to CYP21 for example by the six electron transfer systems listed in Table 2:

TABLE 2

Electron delivering proteins applied in CYP21-dependent substrate conversions and corresponding expression plasmids for whole-cell systems. hCYP21 or bCYP21 were combined in reconstituted systems or whole-cell systems with the indicated redox partners bCPR (bovine NADPH-dependent cytochrome P450 reductase), bAdR (bovine adrenodoxin reductase), $bAdx_{4\text{-}108}$ (bovine adrenodoxin), Fpr (*E. coli* flavodoxin reductase), Arh1 (*S. pombe* adrenodoxin reductase homolog), $etp1^{fd}$ (*S. pombe* electron transfer protein, ferredoxin domain).

	Protein combinations in reconstituted in vitro systems		Corresponding plasmids
	Reductase	Ferredoxin	in whole-cell systems
1	CPR		p21h_bRED/p21b_bRED
2	AdR	$Adx_{4\text{-}108}$	p21h_AdAx/p21b_AdAx
3	Fpr	$Adx_{4\text{-}108}$	p21h_FrAx/p21b_FrAx
4	Arh	$Adx_{4\text{-}108}$	p21h_ArAx/p21b_ArAx
5	Arh	$etp1^{fd}$	p21h_ArET/p21b_ArET
6	Fpr	$etp1^{fd}$	p21h_FrET/p21b_FrET

For in vitro studies and a verification of a substrate conversion, all redox partners were purified.

1.3 Reconstitution of Cytochrome P450 Systems In Vitro

In vitro substrate conversions with purified enzymes in a defined buffer and with an NADPH regeneration system revealed that both isoforms together with the here listed electron transfer proteins are able to convert medrane to

premedrol very efficiently. FIG. **4** shows the in vitro conversion of medrane by human CYP21 together with electron transfer system 2. This result indicates that steroids as exemplified by premedrol can be produced enzymatically by CYP21 together with a suitable redox system, e.g. as shown in Table 2.

Example 2: Whole-Cell Systems

In view of the successful in vitro conversion of steroids, a biotransformation in whole cells was developed.

Generally, in order to perform the hydroxylation in whole cells, the CYP21 as well as the necessary electron transfer proteins were expressed heterologously in *Escherichia coli* strain C43(DE3). For expression and following conversion, bi- or tricistronic vectors based on the plasmid pET17b were constructed, which carry the genes for the particular CYP21 and the particular redox system. FIG. **5** shows all constructed vectors. To facilitate correct protein folding, the *E. coli* chaperones GroEL and GroES were co-expressed on a second vector. The transformed *E. coli* cells were able to produce the CYP21 enzyme as well as the needed redox partners. After the protein expression, a substrate conversion took place which was started by the addition of the steroid to be hydroxylated (exemplified by medrane) as a substrate.

In particular, *E. coli* strain C43(DE3) was transformed with vector for whole cell biocatalysis (e.g. p21b_ArET) and the pGro12 which encodes the chaperones GroEL/ES. The culture comprised 200 mL TB medium (+antibiotics ampicillin and kanamycin) in a 2 L Erlenmeyer flask, inoculated with 2 mL seed culture, and was grown at 37° C. Expression was induced at OD 0.5 by addition of 1 mM IPTG, 1 mM δ-aminolevulinic acid, 4 mg/mL arabinose and maintained for 28 h at 27° C. For whole cell biotransformation, cells were harvested by centrifugation and washed with 50 mM potassium phosphate buffer (pH 7.4). Substrate conversion was started with the addition of 400 μM substrate with resting cells in 25 mL potassium phosphate buffer (50 mM) including 2% glycerol, 1 mM IPTG, 1 mM δ-aminolevulinic acid, 4 mg/mL arabinose in 300 mL buffled flasks for 24 h at a cell density of ca. 24 g/L (wet weight). Samples were taken after, e.g., 24 h and measurement was performed via RP-HPLC after steroid extraction with chloroform.

FIG. **6** shows that the steroid was converted to its 21-hydroxylated derivative and that the appearance of by-products was not observed, in contrast to a chemical synthesis. Time-dependent product formation was studied in whole cells with the six redox systems for each CYP21 isoform to determine not only an endpoint yield but also the velocity of the reaction which is of high interest regarding a biotechnological process (FIG. **7**).

Next to the medrane-to-premedrol conversion, both human and bovine CYP21 were able to hydroxylate all tested 3-ketosteroids which are not yet hydroxylated at position 21. In particular, the following steroid conversions could be shown:

- Medrane to premedrol (non-natural substrate)
- Deltamedrane to medrol (non-natural substrate)
- Progesterone to 11-deoxycorticosterone (natural substrate)
- 17OH-progesterone to 11-deoxycortisol (natural substrate)
- Medroxyprogesterone to 21OH-medroxyprogesterone (non-natural substrate)
- 5α-dihydroprogesterone to 21OH-(5α-dihydroprogesterone).

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 4

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<211> LENGTH: 495
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 1

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Arg Leu Leu Trp Asn Trp Trp Lys Leu Arg Ser Leu His Leu Pro Pro
            20                  25                  30

Leu Ala Pro Gly Phe Leu His Leu Leu Gln Pro Asp Leu Pro Ile Tyr
        35                  40                  45

Leu Leu Gly Leu Thr Gln Lys Phe Gly Pro Ile Tyr Arg Leu His Leu
    50                  55                  60

Gly Leu Gln Asp Val Val Val Leu Asn Ser Lys Arg Thr Ile Glu Glu
65                  70                  75                  80

Ala Met Val Lys Lys Trp Ala Asp Phe Ala Gly Arg Pro Glu Pro Leu
                85                  90                  95

Thr Tyr Lys Leu Val Ser Arg Asn Tyr Pro Asp Leu Ser Leu Gly Asp
            100                 105                 110

Tyr Ser Leu Leu Trp Lys Ala His Lys Lys Leu Thr Arg Ser Ala Leu
        115                 120                 125

Leu Leu Gly Ile Arg Asp Ser Met Glu Pro Val Val Glu Gln Leu Thr
    130                 135                 140
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Gln Glu Phe Cys Glu Arg Met Arg Ala Gln Pro Gly Thr Pro Val Ala
145                 150                 155                 160

Ile Glu Glu Glu Phe Ser Leu Leu Thr Cys Ser Ile Ile Cys Tyr Leu
                165                 170                 175

Thr Phe Gly Asp Lys Ile Lys Asp Asp Asn Leu Met Pro Ala Tyr Tyr
            180                 185                 190

Lys Cys Ile Gln Glu Val Leu Lys Thr Trp Ser His Trp Ser Ile Gln
        195                 200                 205

Ile Val Asp Val Ile Pro Phe Leu Arg Phe Phe Pro Asn Pro Gly Leu
    210                 215                 220

Arg Arg Leu Lys Gln Ala Ile Glu Lys Arg Asp His Ile Val Glu Met
225                 230                 235                 240

Gln Leu Arg Gln His Lys Glu Ser Leu Val Ala Gly Gln Trp Arg Asp
                245                 250                 255

Met Met Asp Tyr Met Leu Gln Gly Val Ala Gln Pro Ser Met Glu Glu
            260                 265                 270

Gly Ser Gly Gln Leu Leu Glu Gly His Val His Met Ala Ala Val Asp
        275                 280                 285

Leu Leu Ile Gly Gly Thr Glu Thr Thr Ala Asn Thr Leu Ser Trp Ala
    290                 295                 300

Val Val Phe Leu Leu His His Pro Glu Ile Gln Gln Arg Leu Gln Glu
305                 310                 315                 320

Glu Leu Asp His Glu Leu Gly Pro Gly Ala Ser Ser Ser Arg Val Pro
                325                 330                 335

Tyr Lys Asp Arg Ala Arg Leu Pro Leu Leu Asn Ala Thr Ile Ala Glu
            340                 345                 350

Val Leu Arg Leu Arg Pro Val Val Pro Leu Ala Leu Pro His Arg Thr
        355                 360                 365

Thr Arg Pro Ser Ser Ile Ser Gly Tyr Asp Ile Pro Glu Gly Thr Val
    370                 375                 380

Ile Ile Pro Asn Leu Gln Gly Ala His Leu Asp Glu Thr Val Trp Glu
385                 390                 395                 400

Arg Pro His Glu Phe Trp Pro Asp Arg Phe Leu Glu Pro Gly Lys Asn
                405                 410                 415

Ser Arg Ala Leu Ala Phe Gly Cys Gly Ala Arg Val Cys Leu Gly Glu
            420                 425                 430

Pro Leu Ala Arg Leu Glu Leu Phe Val Val Leu Thr Arg Leu Leu Gln
        435                 440                 445

Ala Phe Thr Leu Leu Pro Ser Gly Asp Ala Leu Pro Ser Leu Gln Pro
    450                 455                 460

Leu Pro His Cys Ser Val Ile Leu Lys Met Gln Pro Phe Gln Val Arg
465                 470                 475                 480

Leu Gln Pro Arg Gly Met Gly Ala His Ser Pro Gly Gln Ser Gln
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<210> SEQ ID NO 2
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<212> TYPE: PRT
<213> ORGANISM: Bos taurus

<400> SEQUENCE: 2

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His Leu Leu Trp Gly Arg Trp Lys Leu Arg Asn Leu His Leu Pro Pro
            20                  25                  30
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Leu Val Pro Gly Phe Leu His Leu Leu Gln Pro Asn Leu Pro Ile His
        35                  40                  45

Leu Leu Ser Leu Thr Gln Lys Leu Gly Pro Val Tyr Arg Leu Arg Leu
    50                  55                  60

Gly Leu Gln Glu Val Val Val Leu Asn Ser Lys Arg Thr Ile Glu Glu
65                  70                  75                  80

Ala Met Ile Arg Lys Trp Val Asp Phe Ala Gly Arg Pro Gln Ile Pro
                85                  90                  95

Ser Tyr Lys Leu Val Ser Gln Arg Cys Gln Asp Ile Ser Leu Gly Asp
            100                 105                 110

Tyr Ser Leu Leu Trp Lys Ala His Lys Lys Leu Thr Arg Ser Ala Leu
        115                 120                 125

Leu Leu Gly Thr Arg Ser Ser Met Glu Pro Trp Val Asp Gln Leu Thr
    130                 135                 140

Gln Glu Phe Cys Glu Arg Met Arg Val Gln Ala Gly Ala Pro Val Thr
145                 150                 155                 160

Ile Gln Lys Glu Phe Ser Leu Leu Thr Cys Ser Ile Ile Cys Tyr Leu
                165                 170                 175

Thr Phe Gly Asn Lys Glu Asp Thr Leu Val His Ala Phe His Asp Cys
            180                 185                 190

Val Gln Asp Leu Met Lys Thr Trp Asp His Trp Ser Ile Gln Ile Leu
        195                 200                 205

Asp Met Val Pro Phe Leu Arg Phe Phe Pro Asn Pro Gly Leu Trp Arg
    210                 215                 220

Leu Lys Gln Ala Ile Glu Asn Arg Asp His Met Val Glu Lys Gln Leu
225                 230                 235                 240

Thr Arg His Lys Glu Ser Met Val Ala Gly Gln Trp Arg Asp Met Thr
                245                 250                 255

Asp Tyr Met Leu Gln Gly Val Gly Arg Gln Arg Val Glu Glu Gly Pro
            260                 265                 270

Gly Gln Leu Leu Glu Gly His Val His Met Ser Val Val Asp Leu Phe
        275                 280                 285

Ile Gly Gly Thr Glu Thr Thr Ala Ser Thr Leu Ser Trp Ala Val Ala
    290                 295                 300

Phe Leu Leu His His Pro Glu Ile Gln Arg Arg Leu Gln Glu Glu Leu
305                 310                 315                 320

Asp Arg Glu Leu Gly Pro Gly Ala Ser Cys Ser Arg Val Thr Tyr Lys
                325                 330                 335

Asp Arg Ala Arg Leu Pro Leu Leu Asn Ala Thr Ile Ala Glu Val Leu
            340                 345                 350

Arg Leu Arg Pro Val Val Pro Leu Ala Leu Pro His Arg Thr Thr Arg
        355                 360                 365

Pro Ser Ser Ile Phe Gly Tyr Asp Ile Pro Glu Gly Met Val Val Ile
    370                 375                 380

Pro Asn Leu Gln Gly Ala His Leu Asp Glu Thr Val Trp Glu Gln Pro
385                 390                 395                 400

His Glu Phe Arg Pro Asp Arg Phe Leu Glu Pro Gly Ala Asn Pro Ser
                405                 410                 415

Ala Leu Ala Phe Gly Cys Gly Ala Arg Val Cys Leu Gly Glu Ser Leu
            420                 425                 430

Ala Arg Leu Glu Leu Phe Val Val Leu Leu Arg Leu Leu Gln Ala Phe
        435                 440                 445
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Thr Leu Leu Pro Pro Pro Val Gly Ala Leu Pro Ser Leu Gln Pro Asp
450 455 460

Pro Tyr Cys Gly Val Asn Leu Lys Val Gln Pro Phe Gln Val Arg Leu
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Gln Pro Arg Gly Val Glu Ala Gly Ala Trp Glu Ser Ala Ser Ala Gln
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<210> SEQ ID NO 3
<211> LENGTH: 481
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

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Phe Leu His Leu Leu Gln Pro Asp Leu Pro Ile Tyr Leu Leu Gly Leu
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Thr Gln Lys Phe Gly Pro Ile Tyr Arg Leu His Leu Gly Leu Gln Asp
35 40 45

Val Val Val Leu Asn Ser Lys Arg Thr Ile Glu Glu Ala Met Val Lys
50 55 60

Lys Trp Ala Asp Phe Ala Gly Arg Pro Glu Pro Leu Thr Tyr Lys Leu
65 70 75 80

Val Ser Arg Asn Tyr Pro Asp Leu Ser Leu Gly Asp Tyr Ser Leu Leu
85 90 95

Trp Lys Ala His Lys Lys Leu Thr Arg Ser Ala Leu Leu Leu Gly Ile
100 105 110

Arg Asp Ser Met Glu Pro Val Val Glu Gln Leu Thr Gln Glu Phe Cys
115 120 125

Glu Arg Met Arg Ala Gln Pro Gly Thr Pro Val Ala Ile Glu Glu Glu
130 135 140

Phe Ser Leu Leu Thr Cys Ser Ile Ile Cys Tyr Leu Thr Phe Gly Asp
145 150 155 160

Lys Ile Lys Asp Asp Asn Leu Met Pro Ala Tyr Tyr Lys Cys Ile Gln
165 170 175

Glu Val Leu Lys Thr Trp Ser His Trp Ser Ile Gln Ile Val Asp Val
180 185 190

Ile Pro Phe Leu Arg Phe Phe Pro Asn Pro Gly Leu Arg Arg Leu Lys
195 200 205

Gln Ala Ile Glu Lys Arg Asp His Ile Val Glu Met Gln Leu Arg Gln
210 215 220

His Lys Glu Ser Leu Val Ala Gly Gln Trp Arg Asp Met Met Asp Tyr
225 230 235 240

Met Leu Gln Gly Val Ala Gln Pro Ser Met Glu Glu Gly Ser Gly Gln
245 250 255

Leu Leu Glu Gly His Val His Met Ala Ala Val Asp Leu Leu Ile Gly
260 265 270

Gly Thr Glu Thr Thr Ala Asn Thr Leu Ser Trp Ala Val Val Phe Leu
275 280 285

Leu His His Pro Glu Ile Gln Gln Arg Leu Gln Glu Glu Leu Asp His
290 295 300

Glu Leu Gly Pro Gly Ala Ser Ser Ser Arg Val Pro Tyr Lys Asp Arg
305 310 315 320

Ala Arg Leu Pro Leu Leu Asn Ala Thr Ile Ala Glu Val Leu Arg Leu
325 330 335

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Arg Pro Val Val Pro Leu Ala Leu Pro His Arg Thr Thr Arg Pro Ser
340 345 350

Ser Ile Ser Gly Tyr Asp Ile Pro Glu Gly Thr Val Ile Ile Pro Asn
355 360 365

Leu Gln Gly Ala His Leu Asp Glu Thr Val Trp Glu Arg Pro His Glu
370 375 380

Phe Trp Pro Asp Arg Phe Leu Glu Pro Gly Lys Asn Ser Arg Ala Leu
385 390 395 400

Ala Phe Gly Cys Gly Ala Arg Val Cys Leu Gly Glu Pro Leu Ala Arg
405 410 415

Leu Glu Leu Phe Val Val Leu Thr Arg Leu Leu Gln Ala Phe Thr Leu
420 425 430

Leu Pro Ser Gly Asp Ala Leu Pro Ser Leu Gln Pro Leu Pro His Cys
435 440 445

Ser Val Ile Leu Lys Met Gln Pro Phe Gln Val Arg Leu Gln Pro Arg
450 455 460

Gly Met Gly Ala His Ser Pro Gly Gln Ser Gln His His His His His
465 470 475 480

His

<210> SEQ ID NO 4
<211> LENGTH: 482
<212> TYPE: PRT
<213> ORGANISM: bos taurus

<400> SEQUENCE: 4

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Phe Leu His Leu Leu Gln Pro Asn Leu Pro Ile His Leu Leu Ser Leu
20 25 30

Thr Gln Lys Leu Gly Pro Val Tyr Arg Leu Arg Leu Gly Leu Gln Glu
35 40 45

Val Val Val Leu Asn Ser Lys Arg Thr Ile Glu Glu Ala Met Ile Arg
50 55 60

Lys Trp Val Asp Phe Ala Gly Arg Pro Gln Ile Pro Ser Tyr Lys Leu
65 70 75 80

Val Ser Gln Arg Cys Gln Asp Ile Ser Leu Gly Asp Tyr Ser Leu Leu
85 90 95

Trp Lys Ala His Lys Lys Leu Thr Arg Ser Ala Leu Leu Leu Gly Thr
100 105 110

Arg Ser Ser Met Glu Pro Trp Val Asp Gln Leu Thr Gln Glu Phe Cys
115 120 125

Glu Arg Met Arg Val Gln Ala Gly Ala Pro Val Thr Ile Gln Lys Glu
130 135 140

Phe Ser Leu Leu Thr Cys Ser Ile Ile Cys Tyr Leu Thr Phe Gly Asn
145 150 155 160

Lys Glu Asp Thr Leu Val His Ala Phe His Asp Cys Val Gln Asp Leu
165 170 175

Met Lys Thr Trp Asp His Trp Ser Ile Gln Ile Leu Asp Met Val Pro
180 185 190

Phe Leu Arg Phe Phe Pro Asn Pro Gly Leu Trp Arg Leu Lys Gln Ala
195 200 205

Ile Glu Asn Arg Asp His Met Val Glu Lys Gln Leu Thr Arg His Lys
210 215 220

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Glu Ser Met Val Ala Gly Gln Trp Arg Asp Met Thr Asp Tyr Met Leu
225                 230                 235                 240

Gln Gly Val Gly Arg Gln Arg Val Glu Glu Gly Pro Gly Gln Leu Leu
                245                 250                 255

Glu Gly His Val His Met Ser Val Val Asp Leu Phe Ile Gly Gly Thr
            260                 265                 270

Glu Thr Thr Ala Ser Thr Leu Ser Trp Ala Val Ala Phe Leu Leu His
        275                 280                 285

His Pro Glu Ile Gln Arg Arg Leu Gln Glu Glu Leu Asp Arg Glu Leu
    290                 295                 300

Gly Pro Gly Ala Ser Cys Ser Arg Val Thr Tyr Lys Asp Arg Ala Arg
305                 310                 315                 320

Leu Pro Leu Leu Asn Ala Thr Ile Ala Glu Val Leu Arg Leu Arg Pro
                325                 330                 335

Val Val Pro Leu Ala Leu Pro His Arg Thr Thr Arg Pro Ser Ser Ile
            340                 345                 350

Phe Gly Tyr Asp Ile Pro Glu Gly Met Val Val Ile Pro Asn Leu Gln
        355                 360                 365

Gly Ala His Leu Asp Glu Thr Val Trp Glu Gln Pro His Glu Phe Arg
    370                 375                 380

Pro Asp Arg Phe Leu Glu Pro Gly Ala Asn Pro Ser Ala Leu Ala Phe
385                 390                 395                 400

Gly Cys Gly Ala Arg Val Cys Leu Gly Glu Ser Leu Ala Arg Leu Glu
                405                 410                 415

Leu Phe Val Val Leu Leu Arg Leu Leu Gln Ala Phe Thr Leu Leu Pro
            420                 425                 430

Pro Pro Val Gly Ala Leu Pro Ser Leu Gln Pro Asp Pro Tyr Cys Gly
        435                 440                 445

Val Asn Leu Lys Val Gln Pro Phe Gln Val Arg Leu Gln Pro Arg Gly
    450                 455                 460

Val Glu Ala Gly Ala Trp Glu Ser Ala Ser Ala Gln His His His His
465                 470                 475                 480

His His
```

The invention claimed is:

1. A process for the hydroxylation of the carbon atom 21 of a steroid, comprising the steps of:

(a) providing a cell expressing:

(i) a heterologous CYP21A2 protein or a functional variant thereof;

(ii) at least one heterologous electron transfer system capable of transferring electrons to CYP21A2; and

(iii) one or more chaperones facilitating folding of CYP21A2; and

(b) adding the steroid to the cell,

wherein the steroid is medrane or deltamedrane.

2. The process of claim **1**, further comprising a step (c) of extracting the 21-hydroxylated steroid from the supernatant of the cell.

3. The process of claim **1**, further comprising adding one or more cell permeabilizing agents to the cell after step (b).

4. The process of claim **1**, wherein the cell is a resting cell.

5. The process of claim **1**, wherein the cell is a prokaryotic cell or a eukaryotic cell.

6. The process of claim **1**, wherein the at least one heterologous electron transfer system comprises:

(a) a CYP21A2 reductase, and/or

(b) a ferredoxin reductase.

7. The process of claim **1**, wherein the one or more chaperones are recombinantly expressed chaperones.

8. The process of claim **1**, wherein the expression of at least one tryptophanase gene is reduced or abolished in the cell.

9. The process of claim **1**, wherein the cell further expresses a heterologous gene encoding for an enzyme catalyzing a step in the heme biosynthesis pathway.

10. The process of claim **1**, wherein the genes encoding for (i), (ii), and optionally (iii) are comprised in one or more expression cassettes which are integrated into the cell genome.

11. The process of claim **5**, wherein the cell is an *E. coli* cell.

12. The process of claim **5**, wherein the cell is a yeast cell.

13. The process of claim **9**, wherein the heterologous gene encoding for an enzyme catalyzing a step in the heme biosynthesis pathway is a hemA gene.

14. The process of claim **6**, wherein the at least one heterologous electron transfer system comprises an NADPH-dependent ferredoxin reductase and a ferredoxin.

* * * * *