

P450 BM3 MONOOXYGENASE VARIANTS FOR C19-HYDROXYLATION OF STEROIDS**FIELD OF THE DISCLOSURE**

The present invention relates to novel recombinant *Bacillus megaterium* cytochrome P450-monooxygenase (P450-BM3) variants for the C19 hydroxylation of steroids and derivatives thereof or for improved BM3 protein expression. In particular, the present invention also relates to methods and processes using P450-BM3 variants for the production of estrone and estradiol. The invention further relates to nucleotide sequences, constructs and vectors for the expression of these P450-BM3 variants.

BACKGROUND

10 The steroid hormones estrone and estradiol and their derivatives are used as medications, for instance in menopausal hormone therapy, contraception and oncology or are important intermediates for the synthesis of further steroid products. Estrone and estradiol can be generated in several steps starting from phytosterols. The different degradation products of phytosterols contain a methyl group in position C19 of the steroid ring systems which needs to be removed in order to synthesize estrone and estradiol and their derivatives under aromatization of the A ring of the steroid ring system. Inhoffen describes this aromatization by thermolysis for the synthesis of estradiol (Inhoffen 1947) and Hershberg *et al* describe this aromatization by thermolysis for the synthesis of estrone (Hershberg 1950). However, yields are not optimal and various side products are formed. Templeton *et al.* and Numazawa *et al.* describe this aromatization through steroid products which have been oxidized in position C19 (F. Templeton 1997), (Mitsuteru Numazawa 2009). Therefore, processes hydroxylating the methyl group in position C19 and thus allowing for further derivatization and eventually removal of the substituent in position 19 can pave the way for novel and improved syntheses of estrone and estradiol and their derivatives.

25 Enzymes as catalysts are typically characterized by an excellent regio- and/or stereoselectivity for the natural substrates but may show low thermal and solvent stabilities. Due to a narrow substrate spectrum, enzymes are often very specialized compared to chemical catalyst, which limits their broad applicability. It is therefore a challenging endeavor to find or build an enzyme which catalyzes a specific reaction with commercially exploitable yield and sufficient purity.

30 Cytochrome P450 monooxygenases (P450s) comprise a large group of heme enzymes that are ubiquitous in the natural world. Cytochrome P450 BM3 (P450-BM3, BM3), obtained from *Bacillus megaterium* has previously been described to catalyze the NADPH-dependent hydroxylation of long-chain fatty acids, alcohols, and amides, as well as the epoxidation of unsaturated fatty acids (see e.g. (Narhi 1986) and (Capdevila 1996)). For P450-BM3 the

reductase (65 kDa) and monooxygenase (55 kDa) domains of the enzyme are fused and produced as a catalytically self-sufficient 120 kDa enzyme. In consequence, P450-BM3 enzymes exhibit the highest rate of catalysis amongst P450 monooxygenases due to the efficient electron transfer between the fused reductase and heme domains, see e.g., (Noble MA 1999);
5 and (Munro 1996).

BM3 variants have been described for various biotransformation processes. Wild-type and mutant P450 BM3 were applied as biocatalysts in the production of chemicals, including pharmaceuticals (Hazel M Girvan 2016). For example, (WO200107630A1) discloses processes for the microbiological oxidation of different organic substrates, such as N-heterocyclic aromatic
10 compounds, particularly methods for the preparation of indigo and indian ruby using special cytochrome P450-monooxygenases with altered substrate specificity. (EP1196603A1) claims a P450 BM3 variant with an altered profile in enzymatic hydroxylation of aliphatic carboxylic acids, owing to site-specific mutagenesis of its substrate-binding region, wherein F87 is replaced by Val, Ala or Leu, and L188 is replaced by Asn, Gln, Arg, Lys, Ala, Gly, Ser or Trp, and optionally
15 at least one of amino acid positions 26, 47, 72, 74 and 354 is altered. (WO2016007623A1) discloses cytochrome P450 BM3 variants to obtain improved activity with regard to substrates selected from nifedipine, propranolol, verapamil and diclofenac. (EP1131440A2) discloses a process for oxidising a substrate which is an acyclic or cyclic terpene selected from monoterpenes, sesquiterpenes and diterpenes, or a cycloalkene; or a substituted derivative
20 thereof, which process comprises oxidising said compound with a mutant P450 BM3 enzyme, the mutant comprising the substitution of an amino acid in the active site by an amino acid with a less polar side-chain.

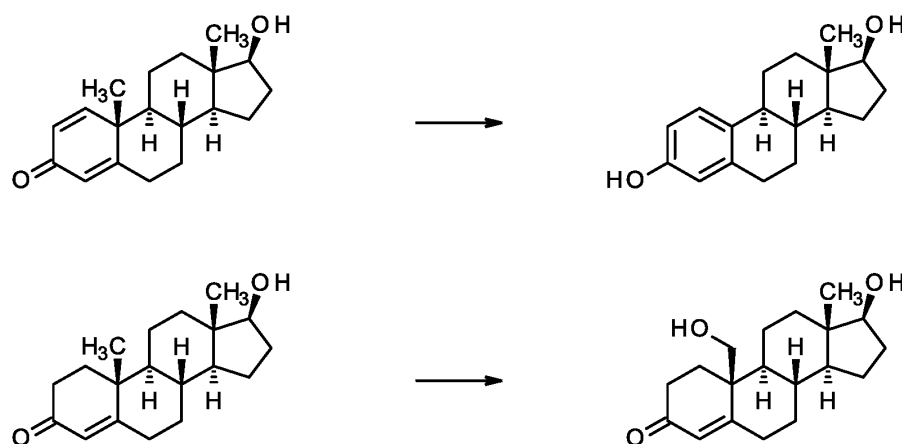
None of the mentioned documents discloses the specific structure of the BM3 variants according to the current invention. Furthermore, none of the above indicated documents relates to the C19
25 hydroxylation of steroid derivatives, e.g. for the production of estradiol or estrone. Instead it has been described that BM3 wildtype (WT) does not accept testosterone nor steroids in general as a substrate.

(Kille Sabrina 2011), (Kille 2010) and (Acevedo-Rocha 2018) have described a library of P450 BM3 variants and the use of directed evolution of P450 BM3. Acevedo-Rocha *et al.* have
30 reported the directed evolution of P450 BM3 for the selective hydroxylation of testosterone. With the resulting mutants, however, relevant amounts of hydroxylation of testosterone were obtained only at positions 16 α / β . These findings discouraged further developments of BM3 variants for C19 hydroxylation, because there was a prejudice to obtain only trace amounts at the position C19 due to preferential hydroxylation at other positions (Acevedo-Rocha 2018).

35 According to the current invention it was nevertheless surprisingly found that specific novel variants of bacterial cytochrome P450 BM3 monooxygenases (CYPs) can be used to catalyze

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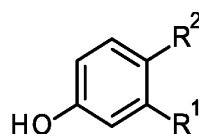
the efficient synthesis of estrone and estradiol and their derivatives through C19-hydroxylation, also in an efficient and commercially exploitable way. In particular, it was surprisingly found that C19-hydroxylation of substrates testosterone, delta-1-testosterone and androsta-1,4-dien-3,17-dione can be achieved using mutants of bacterial cytochrome P450 BM3 monooxygenases as biocatalysts.



These uses can be further supported by incorporating mutations into the BM3 variants which improve the protein expression of BM3.

SUMMARY

According to a first aspect of the invention there is provided a Cytochrome P450 BM3 monooxygenase (BM3) variant for catalyzing the C19 hydroxylation of a steroid or steroid derivative. According to a first embodiment of the first aspect, the BM3 variant comprises the mutation F87A and at least one and preferably two further mutations selected from (i) a mutation at position V78, preferably V78F, V78Y, V78M, V78I or V78L, (ii) a mutation at position A82, preferably A82E, A82Q or A82P. According to a second aspect of the current invention, there is provided a nucleic acid encoding for a Cytochrome P450 BM3 monooxygenase variant as described herein. According to a third aspect of the current invention, there is provided a host cell for the production of a Cytochrome P450 BM3 monooxygenase variant. In a preferred embodiment, the host cell comprises a nucleic acid encoding for a BM3 variant according to any of the aspects described herein. According to a fourth aspect of the current invention, there is provided the use of a Cytochrome P450 BM3 monooxygenase (BM3) variant for the production of a compound according to formula I, wherein R1 and R2 form a six-membered ring as part of a steroid.

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According to a fifth aspect of the current invention, there is provided the use of a BM3 variant for the C19-hydroxylation of a steroid or steroid derivative. According to a sixth aspect of the current invention, there is provided a process for C19-hydroxylation of a steroid or derivative thereof comprising (i) (a) culturing a recombinant Cytochrome P450 BM3 monooxygenase (BM3) variant producing microorganism in a culture medium, in the presence of an exogenous or intermediately formed substrate; or (b) incubating a substrate-containing reaction medium with a Cytochrome P450 BM3 monooxygenase; and (ii) isolating the oxidation product formed, or a secondary product thereof, from the medium; said process being further characterized in that said BM3 variant is a BM3 variant as described herein. According to a seventh aspect there is provided a method for obtaining optimized BM3 variants for the C19 hydroxylation of steroids, said method comprising (i) (a) culturing a recombinant microorganism expressing a BM3 variant (test variant) in a culture medium, in the presence of an exogenous or intermediately formed steroid or steroid derivative; or (b) incubating a steroid or steroid derivative-containing reaction medium with a BM3 variant (test variant); and (ii) comparing the obtained product yield and/or selectivity for the C19 hydroxylation product formed by the test variant or a secondary product thereof with the respective value obtained for a parent variant of the test variant capable of catalyzing the C19 hydroxylation of a steroid, and (iii) selecting the test variant as optimized for the C19 hydroxylation of steroids, if the test variant has an improved product yield/and or selectivity compared to the parent variant. According to a further aspect, there are provided BM3 variants characterized by an improved BM3 protein expression relative to BM3 wildtype (WT), wherein the BM3 variant comprise specific mutations.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 a shows the estradiol yield obtained in biotransformation reactions for BM3-268 daughter variants. The estradiol yield (from 50 mg/L Δ 1-testosterone as substrate) is given as ratio to the parent type. In comparison with the parent type, estradiol yields could be further improved by introducing mutations at positions V26, L29, R47, V48, T49, R50, Y51, E64, N70, S72, A74, L75, F77, L78, F81, G85, L86, T88, S89, W90, M118, E143, T146, S176, M177, V178, L181, A184, M185, L188, K224, R255, F261, L262, I263, A264, H266, E267, T268, V314, A321, W325, T327, P329, A330, T365, P392, F393, A399, I401, G402, G415, L437, T438.

Figure 1 b shows the selectivity factor for estradiol obtained in biotransformation reactions for BM3-268 daughter variants. The selectivity factor (from 50 mg/L Δ 1-testosterone as substrate)

is given as ratio to the parent type. In comparison with the parent type, selectivity factors could be further improved by introducing mutations at positions V26, L29, R47, V48, T49, R50, Y51, E64, N70, S72, A74, L75, F77, L78, F81, G85, L86, T88, S89, W90, M118, E143, T146, S176, M177, V178, L181, A184, M185, L188, K224, R255, F261, L262, I263, A264, H266, E267, T268, V314, A321, W325, T327, P329, A330, T365, P392, F393, A399, I401, G402, G415, L437, T438.

Figure 1 c shows Δ 1-testosterone to estradiol conversion obtained in biotransformation reactions (50 mg/L Δ 1-testosterone as substrate) with BM3-268 (SEQ ID No. 5) variants, wherein the positions L78, E82 and A87 have been altered. Estradiol was obtained with BM3-268 and variants L78[I, L, M, V] of BM3-268.

Figure 2 a, b show the estradiol product yield (a) and selectivity factor (b), respectively, obtained in biotransformation reactions with BM3-268 (SEQ ID No. 5) variants comprising the depicted double mutations. Both estradiol yield (from 50 mg/L Δ 1-testosterone as substrate) and selectivity factor are given as ratio to the parent type. Variants having an improved yield or selectivity factor compared to BM3-268 are depicted in grey. In comparison with the BM3-268 parent variant, product yields could be further improved by introducing the following mutations: (S72C, A74[C, I, L, V]), (S72D, A74C), (S72G, A74[C, F, H, I, L, S, V, Y]), (S72H, A74C), (S72N, A74[C, I, V]), (L75I, F81[C, I, L, S, V]), (L75V, F81[C, L, V, Y]), (I263H, A264G), (A74[C, R, V], L75V). In comparison with the BM3-268 parent variant, selectivity factors could be further improved by introducing the following mutations: (S72C, A74[C, I, L, V, Y]), (S72D, A74[C, F]), (S72G, A74[C, F, H, I, L, S, V, Y]), (S72H, A74[C, G, S, Y]), (S72N, A74[C, I, N, V]), (S72Y, A74V), (L75H, F81S), (L75I, F81[C, G, H, I, L, S, V, Y]), (L75V, F81[C, H, I, L, V, Y]), (I263H, A264G), (A74G, L75C), (A74[C, R, V], L75V).

Figure 3 a, b show the estradiol product yield (a) and selectivity factor (b), respectively, obtained in biotransformation reactions with variants of BM3-268; M177Y, A184Y (SEQ ID No. 15) and further mutations as depicted. Both, estradiol yield and selectivity factor (from 100 mg/L Δ 1-testosterone as substrate) are given as ratio to the parent type. Variants of SEQ ID No. 15 comprising an additional mutation at position L20, V26, L29, R47, T49, L78, T146, L150, F173, F205, M212, H266, E267, G271, L272, W325, T327, A330, F331, E352, and/or T438 were surprisingly found to have a further improved product yield compared with the parent variant according to SEQ ID No. 15. Variants of SEQ ID No. 15 comprising an additional mutation at position L20, V26, L29, R47, T49, A74, L78, F81, E82, L86, T88, T146, L150, F205, M212, I259, F261, E267, L272, W325, T327, A330, F331, E352, M354, L356, and/or T438 were surprisingly found to have an improved selectivity compared with the parent variant according to SEQ ID No. 15.

Figure 3 c shows Δ 1-testosterone to estradiol conversion obtained in biotransformation reactions with 100 mg/L Δ 1-testosterone as substrate, using BM3-268; M177Y, A184Y (SEQ ID

No. 15) and comprising mutation(s) at position L78, E82 or A87 as specified. Estradiol was obtained with the variant having the SEQ ID No. 15. Furthermore, estradiol was obtained with SEQ ID No. 15 derived variants comprising at least mutation(s) L78[L, F, M, Y] or E82[E, P, Q], or combinations thereof.

5 **Figure 4 a, b, c, d, e, f** show product yield and selectivity factor for estradiol obtained in biotransformation reactions with BM3-268; M177Y, A184Y (SEQ ID No. 15) variants (Var) having up to six mutations. 100 mg/L Δ 1-testosterone were used as substrate. Each variant was compared to BM3-268 (SEQ ID No. 5) and improved variant BM3-268; M177Y, A184Y (PT) (SEQ ID No. 15), respectively. Furthermore, the last two columns show whether a variant (having
10 the depicted set of mutations) was improved in yield or selectivity (SF) compared to the best analyzed variant (best) from a set of variants comprising the same subset of mutations but at least one mutation less.

Figure 5 a and b show estradiol product yield and selectivity factor (SF) obtained in biotransformation reactions with BM3-268 variants having up to seven mutations. 50 mg/L Δ 1-
15 testosterone were used as substrate. Each variant was compared to BM3-268 (SEQ ID No. 5) with regard to product yield and selectivity factor (columns 1 and 2). Furthermore, columns 3 and 4 show whether a variant (having the depicted set of mutations) was improved in yield or selectivity (SF) compared to the best analyzed variant (best) from a set of variants comprising the same subset of mutations but at least one mutation less.

20 **Figures 6 a and b** show product yield (a) and selectivity factor (b), respectively, for estrone obtained in biotransformation reactions with BM3-268; M177Y, A184Y (SEQ ID No. 15) variants having the depicted mutations. Estrone yield/selectivity factor from 100 mg/L ADD as substrate is given as ratio to the parent type (SEQ ID No. 15). In comparison with the parent type, estrone yields could be further improved by introducing mutations at positions L20, V26, R47, T49, Y51,
25 A74, L78, F81, E82, T146, L150, F205, M212, I259, G271, L272, W325, T327, A330, E352, M354, T436 and/or T438. Selectivity factors could be further improved in comparison to the parent type by introducing mutations at positions L20, V26, T49, A74, L78, F81, T146, L150, F173, F205, M212, I259, F261, I263, H266, E267, G271, L272, W325, T327, E352, M354, L356 and/or T438.

30 **Figure 6 c** shows ADD to estrone conversion obtained in biotransformation reactions with 100 mg/L ADD as substrate using BM3-268; M177Y, A184Y (SEQ ID No. 15) variants having a mutation at position L78, E82 or A87. Estrone was obtained with the variant having SEQ ID No. 15 and variants L78[M], E82[P] of SEQ ID No. 15.

Figure 7 a, b, c, d, e, f show product yield and selectivity factor for estrone obtained in
35 biotransformation reactions with BM3-268; M177Y, A184Y (SEQ ID No. 15) variants (Var) having

up to six mutations. 100 mg/L ADD were used as substrate. Each variant was compared to BM3-268 (SEQ ID No. 5) and improved variant BM3-268; M177Y, A184Y (PT) (SEQ ID No. 15). Furthermore, the last two columns show whether a variant (having the depicted set of mutations) was improved in yield or selectivity (SF) compared to the best analyzed variant (best) from a set of variants comprising the same subset of mutations but at least one mutation less.

Figure 8 shows product yield and selectivity (SF) for estrone obtained in biotransformation reactions with BM3-268 variants having multiple mutations. 100 mg/L ADD were used as substrate. Each variant was compared to BM3-268 (SEQ ID No. 5) with regard to product yield and selectivity (columns 1 and 2). Furthermore, columns 3 and 4 show whether a variant (having the depicted set of mutations) was improved in yield or selectivity (SF) compared to the best analyzed variant (best) from a set of variants comprising the same subset of mutations but at least one mutation less.

Figures 9 and 10 show product titer (A) and target product ratio (B), respectively, for estrone obtained in biotransformation reactions with BM3-268; S72G, V78L, A82E, F87A, M177Y, V178P, L181Y, A184Y, L188F (SEQ ID No. 117) variants having the depicted mutations. Estrone titer/selectivity factor from 2000 mg/L ADD as substrate is given as ratio to the parent type (SEQ ID No. 117). In comparison with the parent type, estrone titers could be further improved by introducing mutations at positions M5, A44, S53, Q73, K76, R79, D80, H92, G114, E140, P172, F173, I174, T175, S176, R179, D182, E183, N186, K187, Q189, R190, A225, G227, H236, G240, I258, T269, S270, V299, E337, Q397, A399, Q403, Q404 and/or K440. Selectivity could be further improved in comparison to the parent type by introducing mutations at positions M5, A44, G46, S53, Q73, K76, R79, H92, G114, E140, P172, F173, I174, T175, S176, R179, D182, E183, N186, K187, Q189, R190, A225, H236, G240, I258, T269, S270, V299, E337, A399, Q403, Q404, E409 and/or K440.

Figure 11 shows product titer and the target product ratio (TPR) for estrone obtained in biotransformation reactions with BM3-268; S72G, V78L, A82E, F87A, M177Y, V178P, L181Y, A184Y, L188F (SEQ ID No. 117) variants having multiple mutations. 2000 mg/L ADD were used as substrate. Columns 1 and 2 show whether a variant (having the depicted set of mutations) was improved in product titer or selectivity compared to the best analyzed variant from a set of variants comprising the same subset of depicted mutations but at least one mutation less.

BRIEF DESCRIPTION OF THE SEQUENCE IDs

The Sequence Listing associated with this application is filed in electronic format and hereby incorporated by reference into the specification in its entirety.

SEQ ID NO.	SUBJECT	MUTATIONS	TYPE
1	BM3 WT		PRT

2	BM3-254	V78Y, A82E, F87A	PRT
3	BM3-261	V78M, A82E, F87A	PRT
4	BM3-263	V78I, A82E, F87A	PRT
5	BM3-268	V78L, A82E, F87A	PRT
6	BM3 variant	V78Y, A82P, F87A	PRT
7	BM3 variant	V78M, A82P, F87A	PRT
8	BM3 variant	V78I, A82P, F87A	PRT
9	BM3 variant	V78L, A82P, F87A	PRT
10	BM3 variant	V78Y, A82Q, F87A	PRT
11	BM3 variant	V78M, A82Q, F87A	PRT
12	BM3 variant	V78I, A82Q, F87A	PRT
13	BM3 variant	V78L, A82Q, F87A	PRT
14	BM3 variant	V78F, A82E, F87A, M177Y, A184Y	PRT
15	BM3 variant	V78L, A82E, F87A, M177Y, A184Y	PRT
16	BM3 variant	V78I, A82E, F87A, M177Y, A184Y	PRT
17	BM3 variant	V78M, A82E, F87A, M177Y, A184Y	PRT
18	BM3 variant	V78Y, A82E, F87A, M177Y, A184Y	PRT
19	BM3 variant	V78F, A82P, F87A, M177Y, A184Y	PRT
20	BM3 variant	V78L, A82P, F87A, M177Y, A184Y	PRT
21	BM3 variant	V78I, A82P, F87A, M177Y, A184Y	PRT
22	BM3 variant	V78M, A82P, F87A, M177Y, A184Y	PRT
23	BM3 variant	V78Y, A82P, F87A, M177Y, A184Y	PRT
24	BM3 variant	V78F, A82Q, F87A, M177Y, A184Y	PRT
25	BM3 variant	V78L, A82Q, F87A, M177Y, A184Y	PRT
26	BM3 variant	V78I, A82Q, F87A, M177Y, A184Y	PRT
27	BM3 variant	V78M, A82Q, F87A, M177Y, A184Y	PRT
28	BM3 variant	V78Y, A82Q, F87A, M177Y, A184Y	PRT
29	BM3 variant	V78F, A82E, F87A, M177Y	PRT
30	BM3 variant	V78L, A82E, F87A, M177Y	PRT
31	BM3 variant	V78I, A82E, F87A, M177Y	PRT
32	BM3 variant	V78M, A82E, F87A, M177Y	PRT
33	BM3 variant	V78Y, A82E, F87A, M177Y	PRT
34	BM3 variant	V78F, A82P, F87A, M177Y	PRT
35	BM3 variant	V78L, A82P, F87A, M177Y	PRT
36	BM3 variant	V78I, A82P, F87A, M177Y	PRT
37	BM3 variant	V78M, A82P, F87A, M177Y	PRT
38	BM3 variant	V78Y, A82P, F87A, M177Y	PRT
39	BM3 variant	V78F, A82Q, F87A, M177Y	PRT
40	BM3 variant	V78L, A82Q, F87A, M177Y	PRT
41	BM3 variant	V78I, A82Q, F87A, M177Y	PRT
42	BM3 variant	V78M, A82Q, F87A, M177Y	PRT
43	BM3 variant	V78Y, A82Q, F87A, M177Y	PRT
44	BM3 variant	V78F, A82E, F87A, A184Y	PRT
45	BM3 variant	V78L, A82E, F87A, A184Y	PRT
46	BM3 variant	V78I, A82E, F87A, A184Y	PRT
47	BM3 variant	V78M, A82E, F87A, A184Y	PRT
48	BM3 variant	V78Y, A82E, F87A, A184Y	PRT
49	BM3 variant	V78F, A82P, F87A, A184Y	PRT
50	BM3 variant	V78L, A82P, F87A, A184Y	PRT

51	BM3 variant	V78I, A82P, F87A, A184Y	PRT
52	BM3 variant	V78M, A82P, F87A, A184Y	PRT
53	BM3 variant	V78Y, A82P, F87A, A184Y	PRT
54	BM3 variant	V78F, A82Q, F87A, A184Y	PRT
55	BM3 variant	V78L, A82Q, F87A, A184Y	PRT
56	BM3 variant	V78I, A82Q, F87A, A184Y	PRT
57	BM3 variant	V78M, A82Q, F87A, A184Y	PRT
58	BM3 variant	V78Y, A82Q, F87A, A184Y	PRT
59	WT		NUC
60	BM3-254	V78Y, A82E, F87A	NUC
61	BM3-261	V78M, A82E, F87A	NUC
62	BM3-263	V78I, A82E, F87A	NUC
63	BM3-268	V78L, A82E, F87A	NUC
64	BM3 variant	V78Y, A82P, F87A	NUC
65	BM3 variant	V78M, A82P, F87A	NUC
66	BM3 variant	V78I, A82P, F87A	NUC
67	BM3 variant	V78L, A82P, F87A	NUC
68	BM3 variant	V78Y, A82Q, F87A	NUC
69	BM3 variant	V78M, A82Q, F87A	NUC
70	BM3 variant	V78I, A82Q, F87A	NUC
71	BM3 variant	V78L, A82Q, F87A	NUC
72	BM3 variant	V78F, A82E, F87A, M177Y, A184Y	NUC
73	BM3 variant	V78L, A82E, F87A, M177Y, A184Y	NUC
74	BM3 variant	V78I, A82E, F87A, M177Y, A184Y	NUC
75	BM3 variant	V78M, A82E, F87A, M177Y, A184Y	NUC
76	BM3 variant	V78Y, A82E, F87A, M177Y, A184Y	NUC
77	BM3 variant	V78F, A82P, F87A, M177Y, A184Y	NUC
78	BM3 variant	V78L, A82P, F87A, M177Y, A184Y	NUC
79	BM3 variant	V78I, A82P, F87A, M177Y, A184Y	NUC
80	BM3 variant	V78M, A82P, F87A, M177Y, A184Y	NUC
81	BM3 variant	V78Y, A82P, F87A, M177Y, A184Y	NUC
82	BM3 variant	V78F, A82Q, F87A, M177Y, A184Y	NUC
83	BM3 variant	V78L, A82Q, F87A, M177Y, A184Y	NUC
84	BM3 variant	V78I, A82Q, F87A, M177Y, A184Y	NUC
85	BM3 variant	V78M, A82Q, F87A, M177Y, A184Y	NUC
86	BM3 variant	V78Y, A82Q, F87A, M177Y, A184Y	NUC
87	BM3 variant	V78F, A82E, F87A, M177Y	NUC
88	BM3 variant	V78L, A82E, F87A, M177Y	NUC
89	BM3 variant	V78I, A82E, F87A, M177Y	NUC
90	BM3 variant	V78M, A82E, F87A, M177Y	NUC
91	BM3 variant	V78Y, A82E, F87A, M177Y	NUC
92	BM3 variant	V78F, A82P, F87A, M177Y	NUC
93	BM3 variant	V78L, A82P, F87A, M177Y	NUC
94	BM3 variant	V78I, A82P, F87A, M177Y	NUC
95	BM3 variant	V78M, A82P, F87A, M177Y	NUC
96	BM3 variant	V78Y, A82P, F87A, M177Y	NUC
97	BM3 variant	V78F, A82Q, F87A, M177Y	NUC
98	BM3 variant	V78L, A82Q, F87A, M177Y	NUC
99	BM3 variant	V78I, A82Q, F87A, M177Y	NUC

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100	BM3 variant	V78M, A82Q, F87A, M177Y	NUC
101	BM3 variant	V78Y, A82Q, F87A, M177Y	NUC
102	BM3 variant	V78F, A82E, F87A, A184Y	NUC
103	BM3 variant	V78L, A82E, F87A, A184Y	NUC
104	BM3 variant	V78I, A82E, F87A, A184Y	NUC
105	BM3 variant	V78M, A82E, F87A, A184Y	NUC
106	BM3 variant	V78Y, A82E, F87A, A184Y	NUC
107	BM3 variant	V78F, A82P, F87A, A184Y	NUC
108	BM3 variant	V78L, A82P, F87A, A184Y	NUC
109	BM3 variant	V78I, A82P, F87A, A184Y	NUC
110	BM3 variant	V78M, A82P, F87A, A184Y	NUC
111	BM3 variant	V78Y, A82P, F87A, A184Y	NUC
112	BM3 variant	V78F, A82Q, F87A, A184Y	NUC
113	BM3 variant	V78L, A82Q, F87A, A184Y	NUC
114	BM3 variant	V78I, A82Q, F87A, A184Y	NUC
115	BM3 variant	V78M, A82Q, F87A, A184Y	NUC
116	BM3 variant	V78Y, A82Q, F87A, A184Y	NUC
117	BM3 variant	S72G, V78L, A82E, F87A, M177Y, V178P, L181Y, A184Y, L188F	PRT
118	BM3 variant	S72G, V78L, A82E, F87A, T146F, M177Y, V178W, L181Y, A184Y	PRT
119	BM3 variant	S72G, V78L, A82E, F87A, T146F, M177Y, V178W, L181Y, A184Y, G457S, I458G, A477N	PRT
120	BM3 variant	S72G, V78L, A82E, F87A, M177Y, V178P, L181Y, A184Y, L188F	NUC
121	BM3 variant	S72G, V78L, A82E, F87A, T146F, M177Y, V178W, L181Y, A184Y	NUC
122	BM3 variant	S72G, V78L, A82E, F87A, T146F, M177Y, V178W, L181Y, A184Y, G457S, I458G, A477N	NUC
123	Insert	GKHIIQGPMYDVSGTAPVNVN	PRT
124	Insert	LRASVSAIQTTKQIYDDQSLVNV	PRT
125	Insert	AASKPEFIKPEDVSAEVVEKEYQVQL	PRT
126	Insert	AKKPLNTEGVMKSRN	PRT
127	Insert	SAAAATPAVRTVPQYKYAAGVRNPQQHLNAQPQV TMQQPAVHVQGGQEPL	PRT
128	Insert	TVHFESFGATNTNARENTPF	PRT
129	Insert	ENPGVTQLNRLAAHPPFAS	PRT
130	Insert	VKMVRVQAIEKNRYRA	PRT
131	Insert	VREALLRRQRAFRELPG	PRT
132	Insert	IRPRAIGGSKPRVA	PRT
133	Insert	DETYVPKEFNAETF	PRT
134	Insert	WQYTAATATPTIGHV	PRT
135	Insert	ARKLLSPEVAND	PRT
136	Insert	VQTPGHANQSMMPQFIMPANNQFSAQ	PRT
137	Insert	GGGGSGGGSGGGSGGGGS	PRT
138	Insert	GGGGSGGGSGGGSGGGGS	PRT
139	Insert	APAPAPAPAPAPAP	PRT
140	Insert	PSTEQSAKKVRKKAEN	PRT
141	Insert	PSPSTEQSKVRKKAEN	PRT
142	Insert	PSPSTEQSAKKVR	PRT

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143	Insert	TKLPEAQQRVGGCFLNLMQMKTLYLTICANHPSA VNVL	PRT
144	Insert	GNVPELPDTTEHSRTDL	PRT
145	Insert	TARKGKGAFcNGQKLQV	PRT
146	Insert	PSDNVDAQLYNGFFSDAD	PRT
147	Insert	AMSLYLAGEISLLILDEPT	PRT
148	Insert	GCAWYELMPAETTVRLRA	PRT
149	Insert	LSLEVAEEIARLEAEV	PRT
150	Insert	ASSYTAPQPGLG	PRT
151	Insert	IPVPTRDLEKPF	PRT
152	Insert	VIGYMPIPVGVA	PRT
153	Insert	RGRWMARLARM	PRT
154	Insert	FLMKLINRPPII	PRT
155	Insert	LSPDEAP	PRT
156	Insert	VEPPDL	PRT
157	Insert	FITPII	PRT
158	Insert	LRKMRTGKP	PRT
159	Insert	SNSTMKKHGR	PRT
160	Insert	YLSITFL	PRT
161	Insert	AAAEKNVPLY	PRT
162	Insert	SEFEDPLV	PRT
163	Insert	KRSPW	PRT
164	Insert	NASCT	PRT
165	Insert	IQRKK	PRT
166	Insert	EDSGF	PRT
167	Insert	FVGVL	PRT
168	Insert	GGGGSGGGGS	PRT
169	Insert	GGGGS	PRT
170	Insert	PAPAP	PRT
171	Insert	PSPSTEVRKKAEN	PRT
172	Insert	PSPSTKKAEN	PRT
173	Insert	PEN	PRT

DETAILED DESCRIPTION

DEFINITIONS

Unless otherwise defined, all scientific and technical terms used in the description, figures and claims have their ordinary meaning as commonly understood by one of ordinary skill in the art.

- 5 All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will prevail. If two or more documents incorporated by reference include conflicting and/or inconsistent disclosure with respect to each other, then the document having the later effective date shall control. The materials, methods, and examples are illustrative only and not
- 10 intended to be limiting. Unless stated otherwise, the following terms used in this document, including the description and claims, have the definitions given below.

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The terms "**comprising**", "**including**", "**containing**", "**having**" etc. shall be read expansively or open-ended and without limitation. Singular forms such as "**a**", "**an**" or "**the**" include plural references unless the context clearly indicates otherwise. Unless otherwise indicated, the term "**at least**" preceding a series of elements is to be understood to refer to every element in the series. The terms "**at least one**" and "**at least one of**" include for example, one, two, three, four, five, six, seven, eight, nine, ten or more elements.

It is furthermore understood that slight variations above and below a stated range can be used to achieve substantially the same results as a value within the range. Also, unless indicated otherwise, the disclosure of ranges is intended as a continuous range including every value between the minimum and maximum values.

Where protein or amino acid sequences are provided throughout the application it is also understood by the skilled person that single or multiple amino acids may be exchanged by amino acids with similar properties to achieve substantially the same effect, i.e. an equivalent result. The skilled person furthermore knows that a defined protein or amino acid sequence may be encoded by various nucleic acid sequences. For a given amino acid sequence as defined herein, each of the countable nucleic acid sequences encoding the specific amino acid sequence shall be deemed to be disclosed herein. Where nucleic acid sequences are provided throughout the application it is furthermore understood that silent mutations may be introduced.

The terms "**peptide**", "**polypeptide**", and "**protein**" are used interchangeably herein, and refer to a compound which comprises at least two amino acid residues covalently linked by at least one peptide bond. No limitation is placed on the maximum number of amino acids that can comprise a peptide's sequence. A "peptide" may comprise without limitation modified amino acids, non naturally-occurring amino acids and/or D amino acids. Unless otherwise indicated, a particular peptide sequence also encompasses variants wherein at least one amino acid has been replaced by an amino acid which is characterized by similar structural properties. A "peptide" may be a natural peptide, a recombinant peptide, a synthetic peptide, or a combination thereof. A "peptide" may be, for example, a biologically active fragment, an oligopeptide, a homodimer, a heterodimer, a peptide variant, a modified peptide, a peptide derivative, a peptide analog, a fusion protein, among others.

The term "**amino acid**" or "**amino acid residue**" ("**aa**") as used herein typically refers to a naturally-occurring amino acid but may also refer to a non naturally-occurring amino acid. The term typically refers to an L-amino acid but may also encompass a D-amino acid. An amino acid may or may not be modified as described elsewhere herein. The one letter code is used herein to refer to the respective amino acid. As used herein, a "**charged amino acid**" is an amino acid which is negatively charged or positively charged. "**Negatively charged amino acids**" are aspartic acid (D) and glutamic acid (E). "**Positively charged amino acids**" are arginine (R)

lysine (K) and histidine (H). “**Polar amino acids**” are all amino acids that form hydrogen bonds as donors or acceptors. These are all charged amino acids and asparagine (N), glutamine (Q), serine (S), threonine (T), tyrosine (Y) and cysteine (C). “**Polar uncharged amino acids**” are asparagine (N), glutamine (Q), serine (S), threonine (T), tyrosine (Y) and cysteine (C).

- 5 “**Amphiphatic amino acids**” are tryptophan (W), tyrosine (Y) and methionine (M). “**Aromatic amino acids**” are phenylalanine (F), tyrosine (Y), and tryptophan (W). “**Hydrophobic amino acids**” are glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), proline (P), phenylalanine (F), methionine (M) and cysteine. “**Small amino acids**” are glycine (G), alanine (A), serine (S), proline (P), threonine (T), aspartic acid (D) and asparagine (N).
- 10 Two amino acids are “**characterized by similar structural properties**” if (a) both are charged amino acids, preferably both are negatively charged amino or both are positively charged amino acids, (b) both are polar amino acids, (c) both are polar uncharged amino acids, (d) both are amphiphatic amino acids, (e) both are aromatic amino acids, (f) both are hydrophobic amino acids, or (g) both are small amino acids. As understood by the skilled person, where an amino
- 15 acid sequence is provided throughout the disclosure, amino acids characterized by similar structural properties may be exchanged with each other to achieve substantially the same or an equivalent result.

- The terms “**cytochrome P450 BM3 monooxygenase**”, “**cytochrome P450-BM3**”, “**P450-BM3**” (also **BM3**, **BM-3**) refer to the cytochrome P450 enzyme obtained from *Bacillus megaterium* that
- 20 catalyzes the hydroxylation of long-chain fatty acids, alcohols, and amides, as well as the epoxidation of unsaturated fatty acids. Without being bound by theory, P450-BM3 is naturally expressed by *Bacillus megaterium* strains ATCC 14581, DSM 32, JCM 2506, NBRC 15308, NCIMB 9376, NCTC 10342, VKM B-512. Alternative names comprise “*Bifunctional cytochrome P450/NADPH--P450 reductase*”. The P450-BM3 protein is encoded by the gene *cyp102A1*.
 - 25 Sequence(s) are accessible via UniProt Identifier P14779 (CPXB_BACMB). Different isoforms and variants may exist for the different strains and are all comprised by the term. Where a specific mutation can be exchanged without changing the described catalytic properties of the initial sequence, it is clear that the sequence having such a functionally silent mutation is equivalent with regard to the initial sequence. In addition, the protein may furthermore be subject
 - 30 to various modifications, e.g, synthetic or naturally occurring modifications.

- The gene encoding the cytochrome P450-monooxygenases (P450-BM3) from *Bacillus megaterium* was originally amplified from genomic DNA by PCR and subsequently cloned using restriction endonucleases *NcoI* and *SacI* into the polylinker sequence of plasmid pETM11, resulting in pETM11-BM3 (Kille 2010). This procedure adds a hexa-histidine tag and an 18 amino
- 35 acid linker sequence to the N-terminus of the resulting protein, which is however not essential for function. Introducing the *NcoI* site at the 5' end of the gene sequence resulted in alteration of

the second codon of the P450-BM3 gene and to an amino acid exchange in the resulting protein (T1A).

5 An **amino acid position** is typically given by specifying the number of the respective position behind the amino acid of the wildtype using the one letter code (e.g. V78 where the wildtype has a valine at position 78). A list of comma separated letters in squared brackets specifies the **mutation or amino acid exchange**. For example, where at position 78 of the wildtype variant the valine is replaced by any one of isoleucine, leucine, or methionine, or is not mutated at all, this is abbreviated as V78[I, L, M, V].

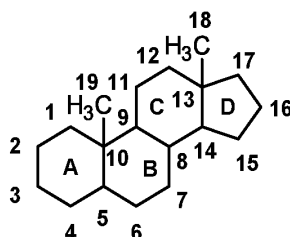
10 Throughout this application **counting of amino acid positions** of the P450-BM3 amino acid sequence starts with the first amino acid that appears in the protein sequence underlying the crystal structure of a P450-BM3 variant (PDB ID: 2UWH) that was used for designing protein libraries. This is also common practice in P450-BM3-related literature, see e.g. (Kille 2010), (Kille Sabrina 2011), (Acevedo-Rocha 2018). The N-terminal methionine is ignored such that counting starts with threonine 1, which was converted to an alanine in the cloning process of pETM11-
15 BM3 and which is codon 28 according to the SEQ IDs listed herein.

The term “**P450-BM3 variant**” refers to a **P450-BM3** comprising at least one mutation relative to the wildtype sequence. In particular, the term refers to proteins having at least 60 %, 70 %, 80 % or 90 % sequence identity with the P450-BM3 wildtype (SEQ ID No. 1). Preferably, P450-BM3 variants are functional, i.e. catalytically active with regard to at least one substrate. Where
20 mutations of a variant are specified, these mutations are restrictive, i.e. a BM3-268; M177Y, A184Y (SEQ ID No. 15) variant having up to six mutations is required to have the mutations M177Y and A184Y.

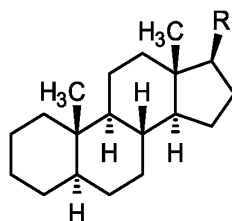
A “**daughter variant**” of a “**parent variant**” is a variant having the sequence of its parent variant and at least one further mutation. A daughter variant can have multiple parent variants.

25 According to the IUPAC definition “**steroids**” are naturally occurring compounds and synthetic analogues, which are based on the cyclopenta[a]phenanthrene carbon skeleton, and can be partially or completely hydrogenated. Usually but not necessarily, there are methyl groups at C-10 and C-13, and often but not necessarily there is an alkyl group at C-17. By extension, one or more bond scissions, ring expansions and/or ring contractions of the skeleton may or may not
30 have occurred (IUPAC kein Datum). Throughout this application, steroids are numbered and rings are lettered as in the following formula:

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When the rings of a steroid are denoted as projections onto the plane of the paper, the formula is normally to be oriented as in the following formula:



- 5 An atom or group attached to a ring depicted as in this orientation is termed “**alpha**” if it lies below the plane of the paper or “**beta**” if it lies above the plane of the paper. Examples for steroids include androgens, estrogens, and progestogens, corticosteroids, glucocorticoids, mineralocorticoids, cholesterol, estradiol, testosterone, dexamethasone, lanosterol, progesterone, medrogestone, β -sitosterol.
- 10 The term **steroid derivative** refers to molecules derived from a steroid, i.e. molecules comprising a cyclopenta[a]phenanthrene carbon skeleton.

The term “**C19 hydroxylation**” refers to the introduction of a hydroxyl group (-OH) into a steroid or steroid derivative at the position C19.

- 15 The “**selectivity**” of a biotransformation process or enzyme can be determined based on the selectivity factor or target product ratio. Other methods to determine selectivity are known to the skilled person. In case of doubt the selectivity factor as defined herein shall control.

- 20 The “**selectivity factor**” of a reaction is a measure for the selectivity of the reaction and is calculated by dividing the measured product concentration by the consumed substrate concentration (which is the difference between the initial substrate concentration and the measured substrate concentration after the biotransformation process). Product and substrate concentration can be measured as known in the art and as described in the examples. Usual methods comprise HPLC analyses using substrate and product standards as references and for quantification. If not specified otherwise, the selectivity factor for the C19 hydroxylation of at least one steroid or derivative thereof is calculated based on the steroid or derivative thereof (for the consumed substrate concentration) and the C19 hydroxylated steroid or derivative thereof (for
- 25

the product concentration). Where the C19 hydroxylated steroid is further converted into a secondary product, selectivity factor may also be calculated with regard to the commercially relevant product.

The “**target product ratio**” (TPR) of a reaction is calculated as integrated area (in HPLC chromatogram) for the desired product divided by the sum of all integrated areas for products. The product may be the C19 hydroxylated steroid. Where the C19 hydroxylated steroid is further converted into a secondary product, target product ratio may also be calculated with regard to the commercially relevant product. For example, with regard to the conversion from ADD to the product estrone, the area under the curve for estrone was divided by the area under the curve for all other products (i.e. not for the educt ADD). In case of doubt, reference is made to the various examples described throughout the disclosure.

The “**product yield**” is calculated by dividing the measured product concentration by the initial substrate concentration in the experiment. The product yield for a given P450-BM3 variant is therefore specific for a given substrate and a given product. For example, the substrate may be a steroid and the product may be the C19 hydroxylated steroid. Where the C19 hydroxylated steroid is further converted into a secondary product, product yield may also be calculated with regard to the commercially relevant product.

The “**product titer**” is provided in mg/L and is an alternative measure to describe the obtainable yields for a biotransformation product. If not specified elsewhere herein, product titer is determined under standard conditions as described in Example 2.

The term “**nucleic acid**” refers to deoxyribonucleotides or ribonucleotides and polymers thereof composed of monomers (nucleotides) containing a sugar, phosphate and a base that is either a purine or pyrimidine. For example and without limitation nucleic acids may occur in single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogs of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzner et al., (1991); Ohtsuka et al., (1985); Rossolini et al., (1994)).

“**Sequence identity**”, “**percent identity**” or “**percent (%) sequence identity**” describes how similar a query sequence is to a target sequence, more precisely how many characters in each sequence are identical after alignment. Sequence identity can be calculated using BLAST (basic

local alignment search tool, NCBI), which performs comparisons between pairs of sequences, searching for regions of local similarity. Suitable alignment methods are known in the art, e.g. Needleman-Wunsch algorithm for global-global alignment, using BLOSUM62 matrix, with gap opening penalty of 11 and a gap extension penalty of 1. Afterwards, the pairs of aligned identical
5 residues can be counted and then divided by the total length of the alignment (including gaps, internal as well as external) to arrive at the percent identity value.

A "**host cell**" is a cell that is used in to receive, maintain, reproduce and amplify a vector. A host cell can also be used to express the polypeptide encoded by the vector. The nucleic acid contained in the vector is replicated when the host cell divides, thereby amplifying the nucleic
10 acids.

The term "**expression system**" refers to any protein production system known in the art and suitable for production of P450-BM3 variants according to the current invention. Suitable expression systems are well known in the art and example systems comprise *E. coli* strains such as DH5alpha, BL21 (DE3), or Rosetta (DE3), but also various other bacterial and non bacterial
15 systems. Suitable expression systems include gram positive bacteria, such as *Bacillus* or *Rhodococcus*, such as *Bacillus megaterium* or *Bacillus subtilis*.

The term "**vector**", as used herein, is known in the art and refers to a nucleic acid molecule capable of propagating a nucleic acid molecule to which it is linked. The term further comprises plasmids (non-viral) and viral vectors.

20 EMBODIMENTS

According to a **first aspect** of the invention there is provided a Cytochrome P450 BM3 monooxygenase (BM3) variant for catalyzing the C19 hydroxylation of a steroid or steroid derivative.

According to a **first embodiment** of the first aspect, the BM3 variant comprises the mutation
25 F87A and at least one and preferably two further mutations selected from

- (i) a mutation at position V78, preferably V78F, V78Y, V78M, V78I or V78L, and
- (ii) a mutation at position A82, preferably A82E, A82Q or A82P.

These BM3 may or may not comprise further mutations.

For example the mutation according to (i) can be V78F and the mutation according to (ii) can be
30 A82E, A82Q or A82P. For example the mutation according to (i) can be V78Y and the mutation according to (ii) can be A82E, A82Q or A82P. For example the mutation according to (i) can be V78M and the mutation according to (ii) can be A82E, A82Q or A82P. For example the mutation according to (i) can be V78I and the mutation according to (ii) can be A82E, A82Q or A82P. For

example the mutation according to (i) can be V78L and the mutation according to (ii) can be A82E, A82Q or A82P. For example the mutation according to (ii) can be A82E and the mutation according to (i) can be V78F, V78Y, V78M, V78I or V78L. For example the mutation according to (ii) can be A82Q and the mutation according to (i) can be V78F, V78Y, V78M, V78I or V78L.

- 5 For example the mutation according to (ii) can be A82P and the mutation according to (i) can be V78F, V78Y, V78M, V78I or V78L.

The optional further mutations may comprise a mutation at position M177 and/or A184. For example the mutations at position M177 and/or A184 can be M177Y and/or A184Y.

- 10 Generation of enzyme variants according to the current invention may be performed as known in the art. For example, mutations can be introduced into the nucleic acid sequences which encode the BM3 wildtype or BM3 variants by any means appropriate for replacing nucleotides in nucleic acid sequences. A useful method for preparing a mutated nucleic acid sequence according to the invention and the corresponding protein comprises carrying out site-directed mutagenesis on codons encoding one or more amino acids which are selected in advance,
- 15 thereby changing the selected codons in a way that they encode for different amino acids. The methods for obtaining these site-directed mutations are well known to the skilled person and widely described in the literature (in particular: (McPherson 1991)). Various kits are commercially available, for example the QUIKCHANGE™ lightening mutagenesis kit from Qiagen or Stratagene.

- 20 According to a second **embodiment** of the first aspect, there are provided variants which may or may not be variants according to the first embodiment. The P450-BM3 variants according to the second embodiment comprise

(i) at least the mutations

- a) V78Y, A82E and F87A (BM3-254, SEQ ID No. 2),
- 25 b) V78M, A82E and F87A (BM3-261, SEQ ID No. 3),
- c) V78I, A82E and F87A (BM3-263, SEQ ID No. 4),
- d) V78L, A82E and F87A (BM3-268, SEQ ID No. 5).
- e) V78Y, A82P and F87A (SEQ ID No. 6),
- f) V78M, A82P and F87A (SEQ ID No. 7),
- 30 g) V78I, A82P and F87A (SEQ ID No. 8),
- h) V78L, A82P and F87A (SEQ ID No. 9),
- i) V78Y, A82Q and F87A (SEQ ID No. 10),
- j) V78M, A82Q and F87A (SEQ ID No. 11),

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k) V78I, A82Q and F87A (SEQ ID No. 12), or

l) V78L, A82Q and F87A (SEQ ID No. 13),

and at least one, two, three, four, five, six, seven, eight, nine or ten further mutation(s),

and/or

5 (ii) at least the mutations

a) V78F, A82E, F87A, M177Y and A184Y (SEQ ID No. 14),

b) V78L, A82E, F87A, M177Y and A184Y (SEQ ID No. 15),

c) V78I, A82E, F87A, M177Y and A184Y (SEQ ID No. 16),

d) V78M, A82E, F87A, M177Y and A184Y (SEQ ID No. 17),

10 e) V78Y, A82E, F87A, M177Y and A184Y (SEQ ID No. 18),

f) V78F, A82P, F87A, M177Y and A184Y (SEQ ID No. 19),

g) V78L, A82P, F87A, M177Y and A184Y (SEQ ID No. 20),

h) V78I, A82P, F87A, M177Y and A184Y (SEQ ID No. 21),

i) V78M, A82P, F87A, M177Y and A184Y (SEQ ID No. 22),

15 j) V78Y, A82P, F87A, M177Y and A184Y (SEQ ID No. 23),

k) V78F, A82Q, F87A, M177Y and A184Y (SEQ ID No. 24),

l) V78L, A82Q, F87A, M177Y and A184Y (SEQ ID No. 25),

m) V78I, A82Q, F87A, M177Y and A184Y (SEQ ID No. 26),

n) V78M, A82Q, F87A, M177Y and A184Y (SEQ ID No. 27),

20 o) V78Y, A82Q, F87A, M177Y and A184Y (SEQ ID No. 28),

p) V78L, A82E, F87A, S72G, M177Y, V178P, L181Y, A184Y and L188F (SEQ ID No. 117),

q) V78L, A82E, F87A, S72G, T146F, M177Y, V178W, L181Y and A184Y (SEQ ID No. 118), or

25 r) V78L, A82E, F87A, S72G, T146F, M177Y, V178W, L181Y, A184Y, G457S, I458G and A477N (SEQ ID No. 119).

In some highly preferred of these second embodiments, the P450-BM3 variant comprises S72G.

In some highly preferred of these second embodiments, the P450-BM3 variant comprises

T146F. In some highly preferred of these second embodiments, the P450-BM3 variant

30 comprises M177Y. In some highly preferred of these second embodiments, the P450-BM3

- variant comprises V178W. In some highly preferred of these second embodiments, the P450-BM3 variant comprises V178P. In some highly preferred of these second embodiments, the P450-BM3 variant comprises L181Y. In some highly preferred of these second embodiments, the P450-BM3 variant comprises A184Y. In some highly preferred of these second
- 5 embodiments, the P450-BM3 variant comprises L188F. In some highly preferred of these second embodiments, the P450-BM3 variant comprises G457S. In some highly preferred of these second embodiments, the P450-BM3 variant comprises I458G. In some highly preferred of these second embodiments, the P450-BM3 variant comprises A477N. Except where obviously incompatible with each other, these highly preferred embodiments can be combined.
- 10 The BM3 variants according to the second embodiment may or may not comprise further mutation(s).

Some preferred of the variants according to the second embodiment of the first aspect comprise at least one further mutation, preferably at least one, two, three, four, five, six, seven, eighth, nine, ten, eleven, twelve, thirteen, fourteen, fifteen or more further mutations.

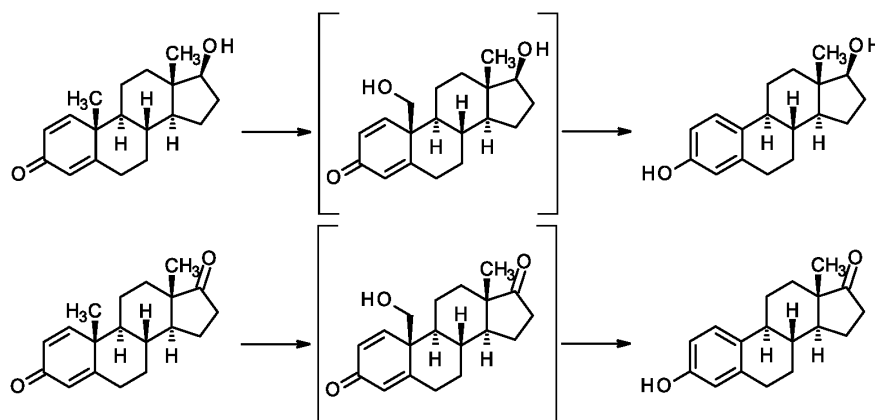
- 15 While the P450-BM3 wild type as well as hundreds of tested variants from literature or generated *de novo* were not able to catalyze the C19 hydroxylation of steroids or steroid derivatives, the variants according to the first aspect were surprisingly found to be suitable catalysts for the efficient C19 hydroxylation of steroids or derivatives thereof.

- Without being bound by theory, the described variants according to the aspect at hand, in
- 20 particular the variants according to SEQ ID No. 2 to 28 and 117 to 119 are technically related, such that they induce a tertiary protein structure which enables not only the binding of steroids but also their efficient C19 hydroxylation, without supporting side reactions, such as other oxidation reactions, e.g. as mentioned herein.

- In brief, the P450-BM3 variants according to the first aspect recognize steroids as substrates, in
- 25 particular steroids comprising a 1,4-dien-3-one-A-ring, or steroids comprising a 4-en-3-one-A-ring. Furthermore, the P450-BM3 variants according to the first aspect can catalyze the C19 hydroxylation of these steroids.

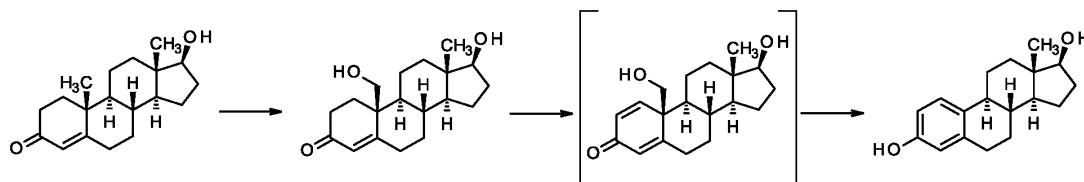
- Without being bound by theory, for steroids with a **1,4-dien-3-one-A-ring** such as (17 β)-17-hydroxyandrosta-1,4-dien-3-one (synonyms: 1-dehydrotestosterone, Δ 1-testosterone, boldenone) or androsta-1,4-dien-3,17-dione (synonym: ADD) a C19-hydroxylation is believed to
- 30 lead to an instable intermediate undergoing immediate aromatization and formation of estradiol or estrone and their derivatives.

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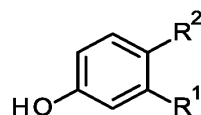
Example 8, Table E2 show the obtained screening yields of estradiol based on delta-1-testosterone as a substrate for P450-BM3 variants BM3-254 (SEQ ID No. 2), BM3-261 (SEQ ID No. 3), BM3-263 (SEQ ID No. 4) and BM3-268 (SEQ ID No. 5).

- 5 Without being bound by theory, for steroids with a **4-en-3-one-A-ring** like (17β)-17-hydroxyandrost-4-en-3-one (synonym: testosterone) the product of C19-hydroxylation is typically stable and can be isolated. These kind of products can then by further functionalization under dehydrogenation form the same instable intermediate and subsequently under aromatization estradiol and estradiol derivatives (as shown for similar estrone derivatives by (F. Templeton 1997).
- 10



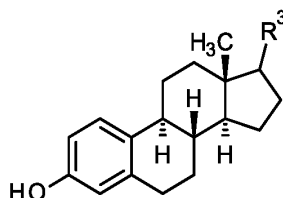
Example 9, Table E5 show the obtained screening yields for C19 hydroxylated products based on testosterone as a substrate for P450-BM3 variants BM3-254 (SEQ ID No. 2), BM3-261 (SEQ ID No. 3), BM3-263 (SEQ ID No. 4) and BM3-268 (SEQ ID No. 5).

- 15 In consequence, the described P450-BM3 variants are suitable catalysts for the C19-hydroxylation of steroids, thereby enabling production of compounds of formula (I)



(I),

wherein R^1 and R^2 form a six-membered ring as part of a steroid. In particular, the described P450-BM3 variants according to the first aspect are suitable catalysts for the C19-hydroxylation of steroids, thereby enabling production of compounds of formula (II)



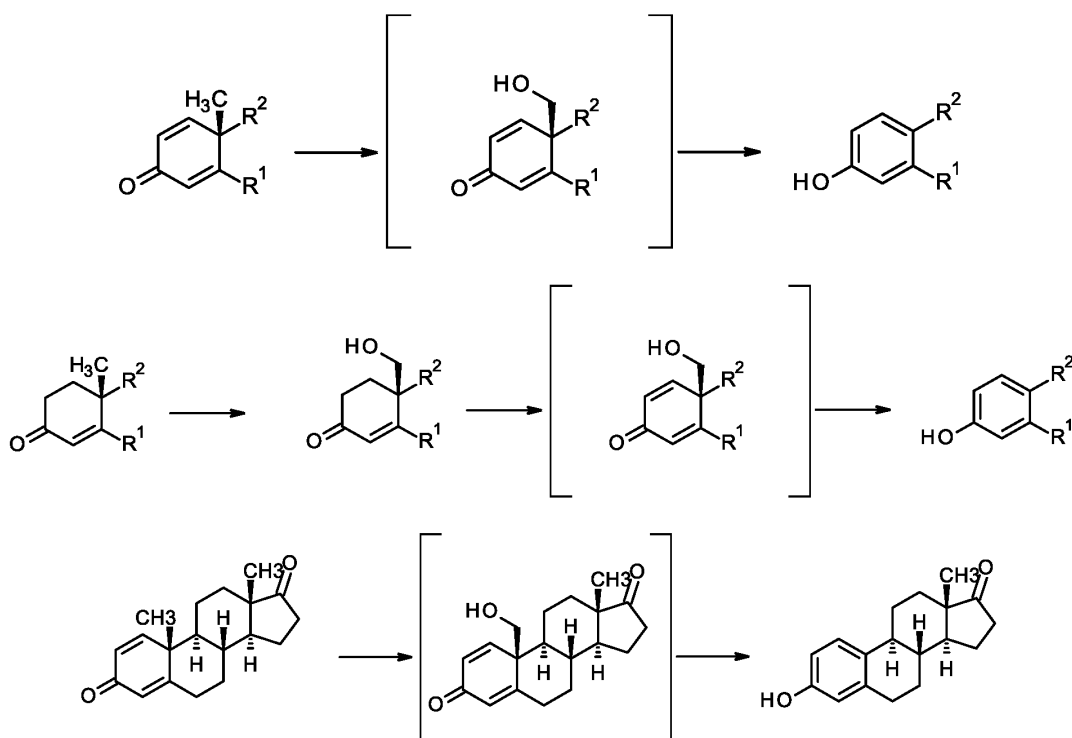
5

(II),

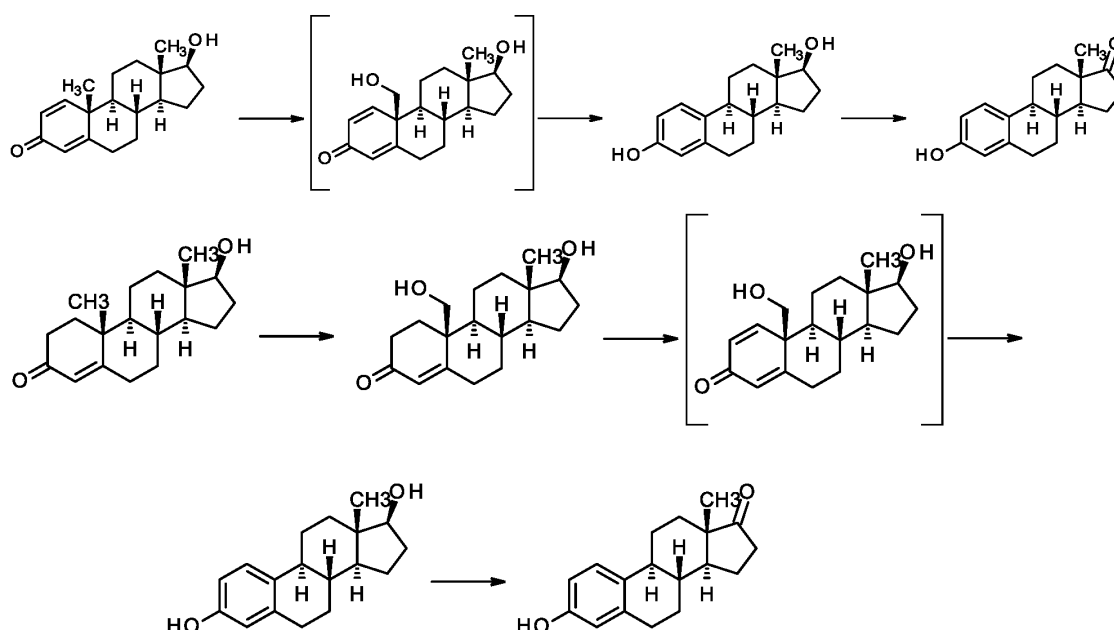
wherein R^3 is a beta-hydroxy group (-OH) or an oxo group (=O).

According to some **highly preferred embodiments** the P450-BM3 variants according to the first aspect catalyze the C19-hydroxylation of steroids for the production of compounds of formula (I) wherein the formula (I) is preferably formula (II).

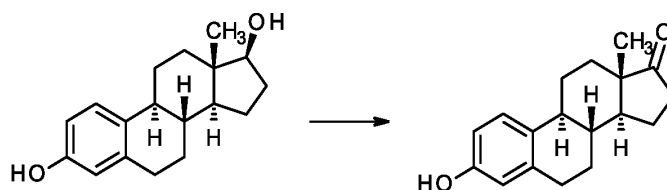
- 10 Without being bound by theory, by using the P450-BM3 variants as catalysts, the compounds of formula (I) and (II) can thus be synthesized in the following ways:



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Estradiol and estradiol derivatives can be oxidized to estrone and estrone derivatives, e.g. as described by (Kawahara R 2012).



- 5 According to a **third embodiment of the first aspect**, there are provided variants which are characterized by a product yield for at least one C19 hydroxylated steroid or derivative thereof or a secondary product thereof which is higher than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 92, 92, 93, 94 or 95 %.

For example, said product yield for the at least one C19 hydroxylated steroid or derivative thereof, or a secondary product thereof can be > 5 %, preferably > 8 %, >10 %, or > 12 %, even more preferably > 15%, >20 %, >25 %, > 30 %, >35 %, > 40 %, > 45 %, or > 50 %, most preferably > 55 %, > 60 %, > 65 %, > 70 % or > 75 %. For example, said product yield can be the product yield for the product estradiol or estrone, or derivatives thereof. For example, said steroid or steroid derivative can be a steroid or steroid derivative comprising a 1,4-dien-3-one-A-ring or a 4-en-3-one-A-ring, such as d1-testosterone or ADD.

The third embodiment according to the first aspect can be and is suggested to be combined with each embodiment or example according to the first aspect and in particular the first, second, fourth, fifth or sixth embodiment of the first aspect.

For example there is provided a BM3 variant comprising the mutation F87A and at least one and
5 preferably two further mutations selected from

- (i) a mutation at position V78, preferably V78F, V78Y, V78M, V78I or V78L, and
- (ii) a mutation at position A82, preferably A82E, A82Q or A82P,

and optionally comprising further mutations, e.g. as specified elsewhere herein, said BM3 variant having a product yield for the at least one C19 hydroxylated steroid or derivative thereof or a
10 secondary product thereof > 5 %, preferably > 8 %, >10 %, or > 12 %, even more preferably > 15%, >20 %, >25 %, > 30 %, >35 %, > 40 %, > 45 %, or > 50 %, most preferably > 55 %, > 60 %, > 65 %, > 70 % or > 75 %.

For example, the BM3 variant may comprise the mutations M177Y and/or A184Y.

For example there is provided a BM3 variant comprising

15 (i) at least the mutations

- a) V78Y, A82E and F87A (BM3-254, SEQ ID No. 2),
- b) V78M, A82E and F87A (BM3-261, SEQ ID No. 3),
- c) V78I, A82E and F87A (BM3-263, SEQ ID No. 4),
- d) V78L, A82E and F87A (BM3-268, SEQ ID No. 5).

20 e) V78Y, A82P and F87A (SEQ ID No. 6),

f) V78M, A82P and F87A (SEQ ID No. 7),

g) V78I, A82P and F87A (SEQ ID No. 8),

h) V78L, A82P and F87A (SEQ ID No. 9),

i) V78Y, A82Q and F87A (SEQ ID No. 10),

25 j) V78M, A82Q and F87A (SEQ ID No. 11),

k) V78I, A82Q and F87A (SEQ ID No. 12), or

l) V78L, A82Q and F87A (SEQ ID No. 13),

and at least one, two, three, four, five, six, seven, eight, nine or ten further mutation(s),

and/or

30 (ii) at least the mutations

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- a) V78F, A82E, F87A, M177Y and A184Y (SEQ ID No. 14),
 b) V78L, A82E, F87A, M177Y and A184Y (SEQ ID No. 15),
 c) V78I, A82E, F87A, M177Y and A184Y (SEQ ID No. 16),
 d) V78M, A82E, F87A, M177Y and A184Y (SEQ ID No. 17),
 5 e) V78Y, A82E, F87A, M177Y and A184Y (SEQ ID No. 18),
 f) V78F, A82P, F87A, M177Y and A184Y (SEQ ID No. 19),
 g) V78L, A82P, F87A, M177Y and A184Y (SEQ ID No. 20),
 h) V78I, A82P, F87A, M177Y and A184Y (SEQ ID No. 21),
 i) V78M, A82P, F87A, M177Y and A184Y (SEQ ID No. 22),
 10 j) V78Y, A82P, F87A, M177Y and A184Y (SEQ ID No. 23),
 k) V78F, A82Q, F87A, M177Y and A184Y (SEQ ID No. 24),
 l) V78L, A82Q, F87A, M177Y and A184Y (SEQ ID No. 25),
 m) V78I, A82Q, F87A, M177Y and A184Y (SEQ ID No. 26),
 n) V78M, A82Q, F87A, M177Y and A184Y (SEQ ID No. 27),
 15 o) V78Y, A82Q, F87A, M177Y and A184Y (SEQ ID No. 28),
 p) V78L, A82E, F87A, S72G, M177Y, V178P, L181Y, A184Y and L188F (SEQ ID No. 117),
 q) V78L, A82E, F87A, S72G, T146F, M177Y, V178W, L181Y and A184Y (SEQ ID No. 118), or
 20 r) V78L, A82E, F87A, S72G, T146F, M177Y, V178W, L181Y, A184Y, G457S, I458G and A477N (SEQ ID No. 119),

said BM3 variant having a product yield for at least one C19 hydroxylated steroid or derivative thereof or a secondary product thereof > 5 %, preferably > 8 %, >10 %, or > 12 %, even more preferably > 15%, >20 %, >25 %, > 30 %, >35 %, > 40 %, > 45 %, or > 50 %, most preferably > 25 55 %, > 60 %, > 65 %, > 70 % or > 75 %.

Table A1 shows estradiol product yields for d1-testosterone to estradiol conversion from 50 mg/L substrate for variants derived from the parent type V78L, A82E, F87A (SEQ ID No. 5) having further mutations as depicted.

Mutation	Yield Estradiol [%]	Mutation	Yield Estradiol [%]	Mutation	Yield Estradiol [%]	Mutation	Yield Estradiol [%]
A74I	52.8	V178M	10.9	L181I	7.7	M177G	6.0

A74V	42.2	A184Q	10.8	A399Q	7.7	M118K	6.0
S72G, A74V	40.2	A321G	10.7	R50D	7.7	L29Y	6.0
F77A	38.3	P392Q	10.5	M177A	7.6	A399L	6.0
A184G	37.4	A399E	10.5	S72C, A74C	7.6	M177I	6.0
M177Y	36.5	M177F	10.4	S89V	7.6	T49H	6.0
V178P	36.3	T365I	10.3	R50S	7.5	R47C	6.0
A184Y	34.2	L75V, F81L	10.3	V371A	7.5	I401V	5.9
A74T	33.3	F77P	10.2	E143I	7.5	T146A	5.9
A184R	31.5	V314P	10.2	T49Q	7.5	S176L	5.9
R50S, A74V	30.7	S72G, A74Y	10.2	T365G	7.5	S72G, A74F	5.9
S72G, A74I	30.2	S89E	10.1	F77M	7.5	K224W	5.9
A74C	29.2	M118E	10.0	T146D	7.5	E267T	5.9
M185V	29.1	T146R	10.0	R47H	7.4	M185D	5.8
L188K	27.0	V26I	9.9	R255K	7.4	Y51L	5.8
A330R	26.3	E64Q	9.9	E143V	7.4	M118S	5.8
K224P	26.0	W90Y	9.7	V314F	7.3	M118T	5.8
V48H	25.9	L29T	9.6	A330F	7.3	A399G	5.8
V26N	25.9	T327V	9.6	Y51W	7.3	A330C	5.8
A184E	24.9	F81W	9.6	M118A	7.3	K224Y	5.8
F77V	24.8	H266N	9.6	T49D	7.3	S89G	5.7
A184W	24.7	A184F	9.6	E267D	7.2	S72N, A74I	5.7
A74M	24.2	V48S	9.5	F261Y	7.2	S72C, A74L	5.7
F77S	24.1	M177T	9.5	T365L	7.2	E143Q	5.7
A74L	23.8	G85A	9.5	W90D	7.2	R47D	5.7
V26H	23.5	M118Y	9.5	L29M	7.2	A264V	5.6
F77L	23.0	L437I	9.5	L75I	7.2	R47M	5.6
A184D	22.3	R50Y	9.4	V26A	7.2	N70R	5.6
M177V	21.9	M118W	9.4	T49W	7.2	I401M	5.6
F77T	20.5	A74R, L75V	9.3	R50P	7.2	F393W	5.6
T146F	20.3	E64V	9.3	L29V	7.2	A74D, F81V	5.6
V178W	20.0	A321N	9.2	R47T	7.1	G415D	5.6
L188R	19.7	M118L	9.2	S72N, A74C	7.1	L75I, F81I	5.6
A74F	19.7	L188A	9.2	V48Y	7.1	T49G	5.6
F77K	19.7	T365W	9.2	T146C	7.1	S89N	5.5
L188N	19.5	T49C	9.2	E143M	7.1	E64A	5.5
T49R	19.4	I263L	9.1	V48F	7.0	E143G	5.5
S89T	19.4	T146M	9.1	R255P	7.0	A330N	5.5
L188W	19.1	T49A	9.1	R50I	7.0	H266E	5.5
A74W	19.0	V48T	9.0	H266A	7.0	R50F	5.5
V178Y	19.0	M118P	8.9	M118V	7.0	F77G	5.4
A74Y	18.9	V314E	8.9	L29A	7.0	R47E	5.4

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M177C	18.8	M177L	8.8	E143K	7.0	R255A	5.4
A184K	18.4	F77Y	8.8	E64Y	6.9	A74V, L75V	5.4
A184N	18.2	R50G	8.8	L29I	6.9	L78M	5.4
H266S	18.1	R47L	8.8	F77D	6.9	T146N	5.3
S89C	18.1	R50N	8.7	T49F	6.8	F261V	5.3
L188F	17.9	F77H	8.7	S89I	6.8	K224L	5.3
V178Q	17.6	E143F	8.7	F81Y	6.8	I401A	5.3
S176P	17.5	F261I	8.7	V48R	6.8	R47W	5.3
F77E	17.2	V178R	8.7	R255N	6.8	T365D	5.3
L188I	17.0	A184L	8.7	V178N	6.8	N70F	5.2
S72W	16.9	A74R	8.6	S72N, A74V	6.8	N70C	5.2
S72G, A74C	16.9	T49L	8.6	E267R	6.7	S176N	5.2
A399V	16.8	P392G	8.6	E143P	6.7	L86V	5.2
L181Y	16.4	F77N	8.6	L75V	6.7	E143N	5.2
S176G	15.8	F261M	8.6	R50C	6.6	V48M	5.2
L181P	15.8	S89Q	8.6	T438V	6.6	K224M	5.1
S176M	15.4	T365Q	8.6	V48L	6.6	A321T	5.1
G415A	15.4	E143C	8.6	R47A	6.6	A321E	5.1
F77W	15.2	F77I	8.5	E143A	6.6	E64L	5.1
T49V	15.2	M177H	8.5	A330T	6.5	G85S	5.1
M118G	15.2	V26M	8.5	V48I	6.5	T146V	5.1
A330K	14.9	V178S	8.5	L75V, F81Y	6.5	L75I, F81L	5.1
K224H	14.9	V314A	8.5	L86M	6.5	M177E	5.1
S72G, A74L	14.8	I263K	8.5	S176K	6.5	M118N	5.1
A184H	14.7	V178L	8.5	S72H, A74C	6.5	I401T	5.0
M177Q	14.6	T49N	8.4	A321I	6.5		
A330W	14.5	A330V	8.4	L29Q	6.4		
L188H	14.2	E143D	8.3	T365K	6.4		
A399I	14.0	F77R	8.3	S176H	6.4		
V178D	14.0	S176F	8.3	M177D	6.4		
T327L	13.7	A399N	8.2	S89L	6.4		
A74H	13.6	G415T	8.2	E344K	6.3		
G415S	13.5	R47Q	8.2	K224C	6.3		
T365V	13.4	E64G	8.1	F81S	6.3		
S176C	13.1	V26K	8.1	L29H	6.3		
V314H	13.0	R50M	8.1	F261C	6.3		
T365Y	12.8	A74P	8.1	W325F	6.3		
S72G, A74S	12.8	S176D	8.1	F393M	6.3		
T365P	12.7	L29C	8.1	E143Y	6.3		
A74S	12.6	A321V	8.1	A321D	6.3		
F77C	12.6	R50V	8.0	K224I	6.2		

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L188M	12.5	E143R	8.0	S72G, A74H	6.2
R50Q	12.5	V314Q	8.0	T365F	6.2
R255I	12.4	E64H	8.0	V178T	6.2
V178I	12.4	P392R	8.0	M118H	6.2
V314S	12.2	L188S	8.0	V26G	6.2
A399M	12.0	R47K	8.0	F261A	6.2
A74C, L75V	12.0	I263N	7.9	E344G	6.2
L188D	11.8	V178K	7.9	S176Q	6.2
T49P	11.6	V26L	7.9	K224E	6.1
P392V	11.6	F81L	7.8	A74N	6.1
L188E	11.5	S72C, A74V	7.8	M177N	6.1
T49I	11.4	R50L	7.8	V178A	6.1
A399C	11.4	V371P	7.8	L75I, F81V	6.1
M118F	11.3	V48W	7.8	R255L	6.1
E143W	11.1	V48D	7.8	S176E	6.1
L29F	10.9	L262I	7.7	A330D	6.1

Table A2 shows estradiol product yields for $\Delta 1$ -testosterone to estradiol conversion from 100 mg/L substrate for variants derived from the parent type V78L, A82E, F87A, M177Y, A184Y (SEQ ID No. 15) having further mutations as depicted.

Mutation	Yield Estradiol [%]	Mutation	Yield Estradiol [%]	Mutation	Yield Estradiol [%]	Mutation	Yield Estradiol [%]
L150W	65.2	L20R	39.4	F205R	44.0	L20C	33.3
L78M	64.0	T149P	39.3	A330W	43.8	T146V, A191T	33.2
L20W	59.5	F205K	38.8	F205D	43.4	L20Y	32.3
M212F	52.2	W325Y	38.6	F205D, D208N	43.2	L20M	31.4
L20F	52.2	P9S	38.3	L150Y	43.1	T49A	31.3
A330Y	50.1	V26Q	37.0	T327M	42.3	T146A, L272W	30.8
M212Y	48.9	R47S	36.4	L20N	41.9	T49L	30.7
E267D	48.3	L272V	36.1	K210E	41.7	L150F	30.2
L20P	47.5	S89T	35.5	T146A, L150A	41.7	T146L	28.5
M212W	47.1	F205H	35.1	R47W	41.5	L20D	28.2
F205Y	46.8	W325F	34.4	F205E	41.4	L20S	28.1
F205G	46.4	E93G	34.2	F77V	41.2	L20I	27.7
F205W	45.0	T49P	34.0	L20T	40.7	L20V	26.6
T146A, F205G	44.2	E352N	33.5	F205T	39.7	T49I	25.9

Table A3 shows product yield for estradiol obtained in biotransformation reactions with BM3-

268; M177Y, A184Y (SEQ ID No. 15) variants having up to six mutations. 100 mg/L Δ 1-testosterone were used as substrate.

Mutations	Yield Estradio I [%]	Mutations	Yield Estradio I [%]
S72G,A74V,T146F,V178W,L181Y	75.9	T146F,V178W,L188F	49.1
S72G,A74T,T146F,V178W,L181Y,L188R	72.8	A74T,T146F,V178P,L181Y	47.9
S72G,A74V,T146F,V178W,L188R	72.3	S72G,V178W	47.7
S72G,A74T,T146F,V178W,L188R	72.2	V178P	47.0
S72G,A74V,T146F,L181Y,L188R	72.1	T146F,V178W,L188K	46.1
S72G,T146F,V178W,L188F	65.9	S72G,V178W,L181Y	45.8
S72G,A74V,T146F,L181Y	65.8	S72G,A74T,L181Y,L188F	45.1
S72G,V178P,L181Y	64.8	S72G,V178W,L188F	45.0
A74T,T146F,L181Y,L188R	63.9	S72G,L188F	44.9
S72G,A74I,V178P,L181Y	62.6	S72G,A74T,T146F,V178W,L188F	44.7
S72G,A74V,T146F,V178W	61.2	S72G,A74V,T146F,V178P,L181Y,L188R	44.5
A74I,T146F,V178W	61.0	T146F,V178W	43.7
S72G,T146F,V178W,L188R	61.0	S72G	43.6
S72G,A74I,T146F,L181Y,L188K	60.7	S72G,A74T,T146F,L188R	43.1
S72G,T146F,V178W,L181Y,L188F	59.0	S72G,T146F,V178W	42.9
T146F,V178W,L188R	58.9	S72G,A74V,T146F,V178P,L181Y,L188K	41.9
A74V,V178P,L181Y,L188K	58.4	S72G,V178W,L188R	39.9
T146F,L181Y,L188F	58.1	S72G,T146F,L188F	38.8
S72G,V178P,L181Y,L188F	57.6	S72G,A74I,T146F,L181Y,L188F	35.0
V178P,L181Y	56.9	S72G,T146F	34.7
S72G,V178P,L181Y,L188K	56.0	A74V,V178P,L181Y,L188F	34.4
S72G,A74V,T146F,V178P,L181Y	56.0	S72G,A74T,L181Y,L188K	33.7
S72G,V178P,L181Y,L188R	55.8	S72G,A74I,T146F,L181Y,L188R	29.9
S72G,T146F,V178W,L181Y	55.4	A74T,T146F,L188K	29.8
S72G,T146F,V178W,L188K	55.4	S72G,A74V,L188R	29.5
T146F,L181Y	55.3	S72G,A74T,T146F,V178P,L181Y	29.2
S72G,A74T,T146F,L181Y,L188K	54.8	S72G,V178W,L188K	29.1
S72G,A74V,V178P,L181Y,L188K	54.1	S72G,A74I,T146F,L181Y	28.8
S72G,T146F,V178W,L181Y,L188K	53.9	S72G,T146F,V178P,L188R	28.3
A74T,T146F,L181Y,L188K	53.9	S72G,A74T,V178P,L188F	28.2
T146F,V178W,L181Y,L188R	52.6	V178W	28.2
A74V,V178P,L181Y,L188R	52.1	S72G,T146F,L181Y,L188R	26.6
V178P,L181Y,L188R	52.0	A74T,T146F,V178P,L188R	26.2
A74T,T146F,V178W	51.3		

Table A4 shows estrone product yields for ADD to estrone conversion from 100 mg/L substrate for variants derived from the parent type BM3-268; M177Y, A184Y (SEQ ID No. 15) having further mutations as depicted.

Mutation(s)	Yield Estrone	Mutation(s)	Yield Estrone	Mutation(s)	Yield Estrone
F205Y	24.5	F205K	8.4	L272W	6.3
M212Y	18.9	L150G	8.1	L272F	6.3
L150Y	18.4	F205P	8.0	T146A, L272H	6.1
M212F	17.2	E352I	7.9	E352N	5.8
F205H	15.8	L20F	7.9	T438V	5.8
F205R	15.6	M212T	7.6	E93G	5.7
L20W	15.4	E352V	7.5	T49Q	5.7
F205G	15.2	F81D	7.4	F205S	5.6
L78M	14.0	L20P	7.4	D363G, T438C	5.5
F205W	13.2	F173Y, I174F	6.8	W325Y	5.4
M212W	13.0	T49I	6.7	M212H	5.4
K210E	11.6	T49R	6.7	T438C	5.3
L20Y	11.3	L272Y	6.7	L150W	5.3
T146F	10.7	L20R	6.7	F205I	5.1
L150F	10.5	F205N	6.6	F205V	5.1
S89T	10.0	E352M	6.6	F81Y	5.1
T146A, F205G	9.7	R203C	6.5		
F205D, D208N	9.5	F205D	6.4		

- 5 **Table A5** shows the product yield for estrone obtained in biotransformation reactions with SEQ ID No. 15 derived variants having up to six mutations. 100 mg/L ADD were used as substrate.

Mutation(s)	Yield Estrone [%]	Mutation(s)	Yield Estrone [%]	Mutation(s)	Yield Estrone [%]
S72G, T146F, V178W, L181Y	66.6	S72G, V178P, L181Y, L188R	14.8	S72G, V178W, L188R	7.9
S72G, T146F, V178W, L181Y, L188F	58.4	S72G, V178W, L181Y	14.7	S72G, T146F, V178P, L188F	7.6
S72G, T146F, V178W	55.2	S72G, A74T, T146F, V178P	14.6	S72G, A74I, T146F, L181Y, L188R	7.6
S72G, T146F, V178W, L188F	53.1	S72G, A74V, V178W, L181Y	14.6	S72G, A74V, T146F, V178P, L188R	7.5
S72G, V178P, L181Y	38.1	S72G, A74V, V178P, L188R	14.1	S72G, A74I, T146F, V178P, L188K	7.5
S72G, T146F, L181Y, L188R	37.4	S72G, A74I, T146F, V178P, L181Y	14.0	S72G, T146F, V178P, L181Y, L188F	7.2
S72G, T146F, L181Y, L188F	37.3	S72G, T146F	13.6	A74T, T146F, V178P, L188K	7.0

S72G,A74V,T146F,V178W,L181Y	36.3	S72G,T146F,V178W,L188K	13.5	S72G,A74I,T146F,V178P,L181Y,L188F	6.9
S72G,T146F,V178W,L188R	33.5	S72G,V178P,L188R	13.2	S72G,A74V,T146F,L181Y,L188K	6.9
S72G,A74T,T146F,V178W,L181Y,L188R	30.0	S72G,A74V,L181Y	13.1	S72G,A74I,T146F,V178P,L188F	6.9
S72G,A74V,T146F,L181Y	29.4	S72G,A74V,T146F,V178P,L181Y,L188R	13.0	S72G,L188K	6.8
S72G,T146F,V178P,L181Y	29.0	T146F,L181Y,L188F	12.9	S72G,A74V,V178P,L181Y,L188R	6.6
S72G,A74T,V178P,L181Y	27.6	S72G	12.7	S72G,T146F,L188K	6.6
S72G,A74T,T146F,V178W,L181Y	26.5	S72G,A74T,T146F,V178P,L181Y	12.3	S72G,A74I,T146F,L181Y	6.5
S72G,V178P,L181Y,L188F	25.2	S72G,L188F	11.9	S72G,A74V,T146F,V178W,L188R	6.3
T146F,L181Y	25.2	S72G,A74I,V178P,L181Y	11.8	T146F,V178W,L188R	6.2
S72G,A74V,T146F,L181Y,L188F	24.1	S72G,A74T,V178P,L188F	11.7	A74I,T146F,L181Y	6.2
S72G,A74V,V178P,L181Y,L188F	23.1	A74T,T146F,V178P,L181Y	11.5	A74T,T146F,V178W	6.2
S72G,A74T,T146F,V178W,L188F	23.0	S72G,A74V,T146F,V178W	11.3	S72G,A74V,V178P	6.1
S72G,A74T,T146F,V178W,L181Y,L188K	22.7	S72G,T146F,V178P	11.3	T146F,V178W,L188F	6.0
S72G,T146F,L181Y,L188K	21.4	S72G,V178P	11.1	V178P	6.0
S72G,A74V,T146F,V178P,L181Y	20.8	A74I,T146F,V178P	11.0	S72G,A74V,T146F,L188F	5.9
S72G,A74I,T146F,V178W,L181Y	20.7	S72G,V178W,L188F	10.8	S72G,A74I,T146F,V178P,L188R	5.9
S72G,A74V,T146F,L181Y,L188R	20.6	T146F	10.7	S72G,L188R	5.9
S72G,A74T,V178P,L181Y,L188K	20.3	S72G,A74V,T146F,V178P,L188F	10.3	S72G,V178W,L188K	5.8
T146F,V178W,L181Y,L188R	19.6	S72G,V178P,L181Y,L188K	10.0	S72G,A74I,T146F,V178P	5.8
S72G,L181Y,L188R	18.8	S72G,A74V,T146F,V178W,L181Y,L188R	10.0	S72G,A74T,T146F,V178P,L188R	5.7
S72G,T146F,V178W,L181Y,L188K	18.8	V178P,L181Y	9.9	S72G,T146F,L188R	5.6
A74T,T146F,V178W,L181Y	18.3	A74T,T146F,L181Y,L188K	9.3	S72G,T146F,L181Y	5.6
S72G,A74T,T146F,L181Y,L188K	18.0	S72G,A74V,V178P,L188F	9.3	T146F,L181Y,L188K	5.4
S72G,L181Y,L188K	18.0	S72G,V178W,L181Y,L188R	9.0	A74I,T146F,V178P,L188R	5.3

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S72G,T146F,V178P,L181Y,L188R	17.4	S72G,T146F,V178P,L188R	8.9	S72G,L181Y	5.3
S72G,A74V,T146F,V178P,L181Y,L188K	16.8	A74T,T146F,V178P	8.8	S72G,A74I,T146F,L181Y,L188F	5.3
S72G,V178P,L188F	16.5	S72G,A74T,T146F,V178P,L188F	8.7	S72G,A74T,T146F,V178W,L181Y,L188F	5.2
A74I,T146F,V178W,L188R	15.9	S72G,T146F,L188F	8.6	S72G,A74I,T146F,V178P,L181Y,L188R	5.2
S72G,V178P,L188K	15.3	T146F,V178P,L181Y,L188R	8.5	A74V,T146F,L181Y,L188R	5.1
T146F,V178W,L188K	15.3	T146F,V178W	8.4	T146F,V178P	5.1
S72G,A74T,T146F,V178W,L188R	14.9	S72G,T146F,V178P,L181Y,L188K	8.2		

Table A6 shows estrone product titers for ADD to estrone conversion from 2000 mg/L substrate for variants derived from the parent type BM3-268; M177Y, A184Y; S72G, V178P, L181Y, L188F (SEQ ID No. 117) having further mutations as depicted.

Mutation	Estrone titer (mg/L)	Mutation	Estrone titer (mg/L)	Mutation	Estrone titer (mg/L)	Mutation	Estrone titer (mg/L)
R179L	303.62	P172A	95.82	N186E	73.51	H236D	60.79
K440C	210.34	D182I	95.57	Q404F	73.20	A44T	60.30
Q403R	180.62	H236A	95.57	K76C	72.91	A225M	60.06
R179A	178.01	R190S	94.64	G227N	72.90	H92Y	59.91
F173W	169.97	Q73E	93.66	H236P	72.87	G114L	59.70
G240K	169.04	T175N	93.23	R190C	71.96	E140L	59.57
R179V	152.25	R179H	93.07	A44Q	71.48	Q189I	59.51
R190V	148.61	N186Y	92.47	N186Q	71.19	A225S	59.38
K440T	148.43	N186F	91.72	E140D	70.70	D80L	59.34
R179M	147.50	I258C	90.90	G114N	70.12	M5E	59.30
K440N	145.90	I174T	90.35	R190W	69.96	A225N	58.19
R79K	141.47	T269V	89.15	N186D	69.25	Q189N	57.96
D182V	141.05	K76E	88.93	D182M	67.40	M5P	57.88
I258M	139.83	Q73L	88.56	K76N	67.21	Q73S	57.80
T175M	136.11	G240S	86.69	S270A	66.64	D80C	57.66
Q403K	134.74	N186K	85.42	H236M	66.60	D182A	56.31
K187N	127.66	T175Q	82.76	V299L	66.47	Q397R	55.83
H236K	125.70	R190G	82.37	S270D	66.38	D80S	55.82
D182C	125.68	N186H	82.29	H236R	66.18	I258L	55.62
G240R	124.47	G240H	81.19	E337V	65.96	N186V	55.58
A399R	122.05	D182E	81.05	E140K	65.62	K187A	55.20
H236S	121.43	G240N	80.92	S176M	64.58	D80W	54.87
R190M	121.39	N186A	79.82	Q73Y	64.36	G240C	54.73
K76I	119.26	N186L	79.45	Q189V	64.26	E140M	54.62

V299W	119.23	H236C	79.42	E140A	64.05	G114K	54.56
H236I	118.01	T175L	79.26	I174V	63.70	K187R	54.55
R190N	114.74	Q73T	79.25	D182N	63.37	K440M	53.99
A225T	114.35	Q73F	78.92	Q189K	63.12	E183D	53.53
T175G	114.34	K76F	78.76	T175R	63.01	D80M	53.46
N186M	104.02	D182W	78.45	G114W	62.88	E337L	53.35
N186T	103.98	D80T	77.88	G240A	62.81	M5C	53.31
T175F	103.16	Q73W	77.22	S53C	62.05	D182H	52.88
K76V	100.58	S176D	76.43	K76H	61.81	A225Q	52.37
Q404Y	100.03	A225C	75.67	G114C	61.50	I258V	51.53
T175H	98.82	P172D	75.44	H236F	61.26	D80N	48.87
T175Y	98.67	R179P	74.92	Q189E	61.00	G227R	46.02
K187G	98.01	A225Y	74.80	H92F	60.99		
R190K	96.19	P172G	74.43	E140T	60.90		

- According a **fourth embodiment of the first aspect**, there are provided variants which are characterized by a selectivity factor for the C19 hydroxylation of at least one steroid or derivative thereof, which is higher than 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.11, 0.12, 0.13, 0.14, 0.15, 0.16, 0.17, 0.18, 0.19, 0.2, 0.21, 0.22, 0.23, 0.24, 0.25, 0.26, 0.27, 0.28, 0.29, 0.3, 0.31, 0.32, 0.33, 0.34, 0.35, 0.36, 0.37, 0.38, 0.39, 0.4, 0.41, 0.42, 0.43, 0.44, 0.45, 0.46, 0.47, 0.48, 0.49, 0.5, 0.51, 0.52, 0.53, 0.54, 0.55, 0.56, 0.57, 0.58, 0.59, 0.6, 0.61, 0.62, 0.63, 0.64, 0.65, 0.66, 0.67, 0.68, 0.69, 0.7, 0.71, 0.72, 0.73, 0.74, 0.75, 0.76, 0.77, 0.78, 0.79, 0.8, 0.81, 0.82, 0.83, 0.84, 0.85, 0.86, 0.87, 0.88, 0.89, 0.9, 0.91, 0.92, 0.93, 0.94, 0.95, 0.96, 0.97, 0.98 or 0.99.
- For example, said selectivity factor for the C19 hydroxylation of a steroid or steroid derivative can be > 0.1, preferably > 0.2 or > 0.3, even more preferably > 0.4 or > 0.5, most preferably > 0.5, > 0.6, > 0.7, > 0.8 or > 0.9. For example, said steroid or steroid derivative can be a steroid or steroid derivative comprising a 1,4-dien-3-one-A-ring or a 4-en-3-one-A-ring, such as d1-testosterone or ADD.
- The fourth embodiment according to the first aspect can be and is suggested to be combined with each embodiment according to the first aspect and in particular with the first, second, third, fifth, or sixth embodiment of the first aspect.

For example there is provided a BM3 variant comprising the mutation F87A and at least one and preferably two further mutations selected from

- (i) a mutation at position V78, preferably V78F, V78Y, V78M, V78I or V78L, and
- (ii) a mutation at position A82, preferably A82E, A82Q or A82P,

and optionally comprising further mutations, e.g. as described elsewhere herein, said BM3 variant having a selectivity factor for the C19 hydroxylation of at least one steroid or steroid

derivative > 0.1, preferably > 0.2 or > 0.3, even more preferably > 0.4 or > 0.5, most preferably > 0.5, > 0.6, > 0.7, > 0.8 or > 0.9. Preferably, the BM3 variant is furthermore characterized by a product yield for the C19 hydroxylation of a steroid or steroid derivative > 5 %, more preferably > 8 %, > 10 %, or > 12 %, even more preferably > 15 %, > 20 %, > 25 %, > 30 %, > 35 %, > 40 %, 5 > 45 %, or > 50 %, most preferably > 55 %, > 60 %, > 65 %, > 70 % or > 75 %.

Preferably, the BM3 variant comprises the mutations M177Y and/or A184Y.

For example there is provided a BM3 variant comprising

(i) at least the mutations

- a) V78Y, A82E and F87A (BM3-254, SEQ ID No. 2),
- 10 b) V78M, A82E and F87A (BM3-261, SEQ ID No. 3),
- c) V78I, A82E and F87A (BM3-263, SEQ ID No. 4),
- d) V78L, A82E and F87A (BM3-268, SEQ ID No. 5).
- e) V78Y, A82P and F87A (SEQ ID No. 6),
- f) V78M, A82P and F87A (SEQ ID No. 7),
- 15 g) V78I, A82P and F87A (SEQ ID No. 8),
- h) V78L, A82P and F87A (SEQ ID No. 9),
- i) V78Y, A82Q and F87A (SEQ ID No. 10),
- j) V78M, A82Q and F87A (SEQ ID No. 11),
- k) V78I, A82Q and F87A (SEQ ID No. 12), or
- 20 l) V78L, A82Q and F87A (SEQ ID No. 13),

and at least one, two, three, four, five, six, seven, eight, nine or ten further mutation(s),
and/or

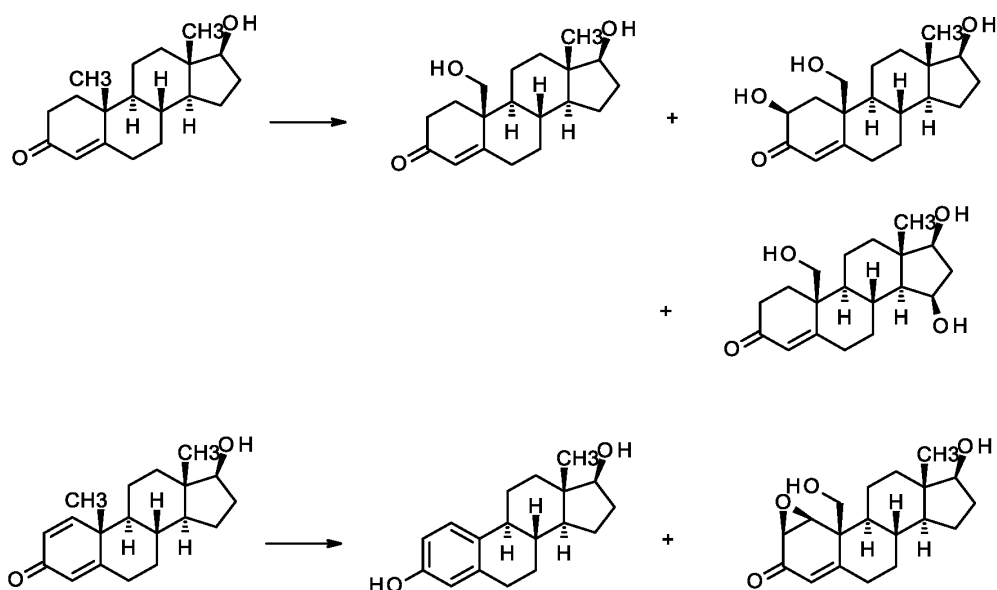
(ii) at least the mutations

- a) V78F, A82E, F87A, M177Y and A184Y (SEQ ID No. 14),
- 25 b) V78L, A82E, F87A, M177Y and A184Y (SEQ ID No. 15),
- c) V78I, A82E, F87A, M177Y and A184Y (SEQ ID No. 16),
- d) V78M, A82E, F87A, M177Y and A184Y (SEQ ID No. 17),
- e) V78Y, A82E, F87A, M177Y and A184Y (SEQ ID No. 18),
- f) V78F, A82P, F87A, M177Y and A184Y (SEQ ID No. 19),
- 30 g) V78L, A82P, F87A, M177Y and A184Y (SEQ ID No. 20),

- h) V78I, A82P, F87A, M177Y and A184Y (SEQ ID No. 21),
- i) V78M, A82P, F87A, M177Y and A184Y (SEQ ID No. 22),
- j) V78Y, A82P, F87A, M177Y and A184Y (SEQ ID No. 23),
- k) V78F, A82Q, F87A, M177Y and A184Y (SEQ ID No. 24),
- 5 l) V78L, A82Q, F87A, M177Y and A184Y (SEQ ID No. 25),
- m) V78I, A82Q, F87A, M177Y and A184Y (SEQ ID No. 26),
- n) V78M, A82Q, F87A, M177Y and A184Y (SEQ ID No. 27),
- o) V78Y, A82Q, F87A, M177Y and A184Y (SEQ ID No. 28),
- p) V78L, A82E, F87A, S72G, M177Y, V178P, L181Y, A184Y and L188F (SEQ ID
- 10 No. 117),
- q) V78L, A82E, F87A, S72G, T146F, M177Y, V178W, L181Y and A184Y (SEQ ID
- No. 118), or
- r) V78L, A82E, F87A, S72G, T146F, M177Y, V178W, L181Y, A184Y, G457S,
- I458G and A477N (SEQ ID No. 119),
- 15 said BM3 variant having a selectivity factor for the C19 hydroxylation of a steroid or steroid derivative > 0.1, preferably > 0.2 or > 0.3, even more preferably > 0.4 or > 0.5, most preferably > 0.5, > 0.6, > 0.7, > 0.8 or > 0.9. Preferably, the BM3 variant is furthermore characterized by a product yield for the C19 hydroxylation of a steroid or steroid derivative > 5 %, more preferably > 8 %, > 10 %, or > 12 %, even more preferably > 15 %, > 20 %, > 25 %, > 30 %, > 35 %, > 40 %, > 45 %, or > 50 %, most preferably > 55 %, > 60 %, > 65 %, > 70 %, or > 75 %.
- 20

The P450-BM3 parent variants, in particular the variants according to SEQ ID No. 2 to 5 and variants derived thereof catalyze not only C19 hydroxylation but also further oxidative reactions. Significant generation of products with C19-hydroxylation and an additional oxidation in other positions of the steroid molecule was observed. With testosterone as substrate (2beta)-2,19-dihydroxytestosterone and (15beta)-15,19-dihydroxytestosterone were isolated, with delta-1-

25 testosterone as substrate (1beta,2beta)-1,2-epoxy-19-hydroxytestosterone was isolated.

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Thus, for example the P450-BM3 variants may catalyze formation of (6β)-OH, (15β)-OH or (1β,2β)-1,2-epoxy derivatives either instead of or in combination with C19 hydroxylation (see Examples 8, 9, Tables E3, E5). The formation of these side products leads to an impaired selectivity factor. However, for the listed BM3 parent variants it was surprisingly found that even where side reactions occur, the resulting C19 hydroxylation (overoxidation) products may still be relevant for the commercial steroid synthesis. Compared with their respective parent variant the variants according to the fourth embodiment of the first aspect were superior with regard to selectivity.

- 10 **Table B1** shows estradiol selectivity factors for d1-testosterone to estradiol conversion from 50 mg/L substrate for variants derived from the parent type BM3-268 (SEQ ID No. 5) having further mutations as depicted.

Mutation	SF (Estradiol)	Mutation	SF (Estradiol)	Mutation	SF (Estradiol)	Mutation	SF (Estradiol)
V26E	0.58	L188H	0.14	M177N	0.09	V26G	0.07
A74I	0.53	I263L	0.14	H266V	0.09	L86M	0.07
G85L	0.48	V178D	0.14	E64V	0.09	T146C	0.07
A74V	0.42	T146M	0.14	M118N	0.09	E143M	0.07
I263C	0.42	S72C, A74I	0.14	A321N	0.09	V48F	0.07
V178P	0.42	T327L	0.14	M118L	0.09	R50I	0.07
S72G, A74V	0.40	L29Q	0.14	E143R	0.09	A321D	0.07
F77A	0.38	S72N, A74I	0.14	L75I, F81G	0.09	R255L	0.07
A184G	0.37	G415S	0.13	L188A	0.09	M118V	0.07
M177Y	0.37	S89Q	0.13	T365W	0.09	L29A	0.07

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A330V	0.36	T365V	0.13	T49C	0.09	T365K	0.07
A184Y	0.34	L75V, F81L	0.13	E143D	0.09	E64Y	0.07
A74T	0.33	S176C	0.13	V178K	0.09	L29I	0.07
H266S	0.32	L75I, F81L	0.13	V48T	0.09	L181H	0.07
T146F	0.31	G402A	0.13	V178R	0.09	T49F	0.07
A184R	0.31	V314H	0.13	M118P	0.09	V48R	0.07
A184F	0.31	T365Y	0.13	R50E	0.09	V178N	0.07
R50S, A74V	0.31	M177T	0.13	G415T	0.09	S72D, A74C	0.07
S72G, A74I	0.30	T365P	0.13	M177L	0.09	E267R	0.07
A74C	0.29	L188D	0.13	S72N, A74V	0.09	K224I	0.07
M185V	0.29	L262Y	0.13	R50G	0.09	S176Q	0.07
A330R	0.28	P392V	0.13	V48W	0.09	L75V	0.07
M177K	0.28	A74S	0.13	R47L	0.09	L75I, F81V	0.07
L188K	0.28	F77C	0.13	R50N	0.09	V48L	0.07
I263N	0.27	L188M	0.13	F81Y	0.09	R47A	0.07
A184W	0.27	I263H, A264G	0.13	F77H	0.09	E143A	0.07
A74M	0.27	R50Q	0.12	E143F	0.09	L78M	0.07
S89L	0.27	R255I	0.12	S176F	0.09	A330T	0.07
K224P	0.26	S72G, A74Y	0.12	L75I, F81Y	0.09	V48I	0.07
V48H	0.26	V178I	0.12	F261I	0.09	S176K	0.07
V26N	0.26	W90D	0.12	F77D	0.09		
A399I	0.26	L75I, F81S	0.12	A184L	0.09		
A74L	0.26	P392G	0.12	F77N	0.09		
A184E	0.25	V314S	0.12	F261M	0.09		
F77V	0.25	V178M	0.12	E143C	0.09		
F77S	0.24	A74R	0.12	F77I	0.09		
V178W	0.24	M177F	0.12	V26M	0.08		
V26H	0.24	A74G, L75C	0.12	V178S	0.08		
L181P	0.23	L29S	0.12	V314A	0.08		
F77L	0.23	L75I, F81H	0.12	R47K	0.08		
F77W	0.23	I263K	0.12	T49N	0.08		
A184D	0.22	A399M	0.12	R50P	0.08		
S72W	0.22	V178L	0.12	L75I, F81C	0.08		
M177V	0.22	F261V	0.12	F77R	0.08		
V178Y	0.22	S72Y, A74V	0.12	L29H	0.08		
A74H	0.21	A399C	0.12	T49W	0.08		
L181V	0.21	S72C, A74L	0.12	V48D	0.08		
A74C, L75V	0.21	L188E	0.11	A399N	0.08		
S176P	0.21	G415D	0.11	R47Q	0.08		
F77T	0.21	T49I	0.11	E64G	0.08		
S89E	0.20	M118F	0.11	V26K	0.08		
M177R	0.20	L75V, F81C	0.11	R50M	0.08		

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T49R	0.20	T88C	0.11	A321V	0.08
A74Y	0.20	E143W	0.11	R50V	0.08
A74W	0.20	V314P	0.11	V314Q	0.08
M177C	0.20	F81W	0.11	E64H	0.08
S72C, A74V	0.20	L181I	0.11	P392R	0.08
L188R	0.20	S89V	0.11	M185L	0.08
A74F	0.20	T438V	0.11	L188S	0.08
F77K	0.20	S176D	0.11	V26L	0.08
A74R, L75V	0.19	L86V	0.11	F81L	0.08
F261D	0.19	L29F	0.11	L29Y	0.08
L188N	0.19	I401T	0.11	R50L	0.08
S89T	0.19	A184Q	0.11	V371P	0.08
L188W	0.19	A321G	0.11	E267D	0.08
M177H	0.19	M118Y	0.11	L262W	0.08
T49P	0.19	W90Y	0.11	A399Q	0.08
F81S	0.19	L262I	0.11	M177A	0.08
A184K	0.18	T146R	0.11	E143G	0.08
A184N	0.18	L29C	0.11	V371A	0.08
M177Q	0.18	P392Q	0.10	M177P	0.08
L75H, F81S	0.18	A399E	0.10	S72N, A74C	0.08
S89C	0.18	H266E	0.10	R50S	0.08
S89M	0.18	S72C, A74Y	0.10	S176E	0.08
L188F	0.18	T49A	0.10	E143I	0.08
L181Y	0.18	S72C, A74C	0.10	T49Q	0.08
V178Q	0.18	L75V, F81Y	0.10	T365G	0.07
F77E	0.18	T365I	0.10	F77M	0.07
F261C	0.17	F77P	0.10	R50C	0.07
L188I	0.17	R50Y	0.10	T146D	0.07
F261S	0.17	S72G, A74F	0.10	R47H	0.07
S72G, A74C	0.17	M118E	0.10	R255K	0.07
A399V	0.17	S72H, A74C	0.10	E143V	0.07
A330K	0.17	A74P	0.10	R255P	0.07
S72G, A74H	0.17	V26I	0.10	V48Y	0.07
F261A	0.16	E64Q	0.10	V314F	0.07
S176M	0.16	L75V, F81I	0.10	A330F	0.07
A74N	0.16	V314E	0.10	Y51W	0.07
S176G	0.16	T146P	0.10	M118A	0.07
T365Q	0.16	T49L	0.10	E143P	0.07
S89N	0.16	M177E	0.10	T49D	0.07
G415A	0.15	L29T	0.10	E143K	0.07
A264V	0.15	T327V	0.10	R255N	0.07
S89I	0.15	H266N	0.10	H266A	0.07

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T49V	0.15	V48S	0.10	F261Y	0.07
M118G	0.15	R50D	0.10	T365L	0.07
K224H	0.15	G85A	0.09	L29M	0.07
S72G, A74L	0.15	L437I	0.09	L75I	0.07
A184H	0.15	F77Y	0.09	V26A	0.07
A330W	0.15	T146S	0.09	L29V	0.07
S72G, A74S	0.14	M118W	0.09	R47T	0.07

Table B2 shows estradiol selectivity factors for $\Delta 1$ -testosterone to estradiol conversion from 100 mg/L substrate for variants derived from the parent type BM3-268; M177Y, A184Y (SEQ ID No. 15) having further mutations as depicted.

Mutation	SF Estradiol	Mutation	SF Estradiol	Mutation	SF Estradiol
L150W	0.65	T49I	0.51	F205Y	0.47
L78M	0.64	F205G	0.51	K210E	0.47
W325Y	0.61	A330W	0.51	L20I	0.47
L20W	0.60	L20N	0.50	F205R	0.46
E93G	0.59	F205H	0.50	T49A	0.46
T149P	0.59	F205D, D208N	0.50	L20D	0.46
V26Q	0.57	L150Y	0.50	L20V	0.46
W325F	0.56	E267D	0.50	L20S	0.46
M212F	0.55	L20C	0.49	L20M	0.45
S89T	0.55	T146L	0.49	T146A, F205G	0.45
M212W	0.54	M212Y	0.49	R47W	0.45
L20T	0.54	F205T	0.49	T146A, L272W	0.45
L150F	0.53	L20R	0.49	F205W	0.45
A330Y	0.53	F77V	0.48	R47S	0.45
L20F	0.53	F205E	0.48	T327M	0.43
T49P	0.52	T49L	0.48	F205K	0.42
L20P	0.52	T146V, A191T	0.47	T146A, L150A	0.42
L272V	0.52	F205D	0.47	E352N	0.38
L20Y	0.51	P9S	0.47		

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Table B3 shows selectivity factors for estradiol obtained in biotransformation reactions from 100 mg/L $\Delta 1$ -testosterone as substrate with BM3-268; M177Y, A184Y (SEQ ID No. 15) variants having up to six mutations.

Mutations	SF Estradiol	Mutations	SF Estradiol
S72G,A74I,V178P,L181Y	0.83	S72G,V178P,L181Y,L188F	0.61
S72G,A74V,V178P,L181Y,L188K	0.83	S72G,V178W,L188R	0.61
A74V,V178P,L181Y,L188R	0.83	T146F,L181Y	0.6
S72G,A74V,T146F,V178P,L181Y,L188R	0.82	T146F,V178W,L188R	0.59
A74V,V178P,L181Y,L188K	0.8	S72G,T146F,V178W	0.59

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S72G,A74V,T146F,V178P,L181Y,L188K	0.8	T146F,L181Y,L188F	0.58
S72G,A74V,T146F,V178P,L181Y	0.79	A74T,T146F,L181Y,L188K	0.58
S72G,A74I,T146F,L181Y,L188F	0.77	A74T,T146F,V178W	0.58
S72G,A74V,T146F,V178W,L181Y	0.76	V178P	0.58
S72G,A74T,T146F,V178W,L188R	0.76	S72G,A74T,T146F,L188R	0.58
S72G,A74V,T146F,L181Y,L188R	0.75	S72G,V178P,L181Y,L188K	0.57
S72G,A74V,T146F,L181Y	0.75	S72G,V178W,L188F	0.57
A74V,V178P,L181Y,L188F	0.74	S72G,V178P,L181Y,L188R	0.56
S72G,A74T,T146F,V178W,L181Y,L188R	0.73	S72G,T146F,V178W,L181Y,L188K	0.56
S72G,A74V,T146F,V178W,L188R	0.72	S72G,T146F,V178W,L188K	0.55
A74T,T146F,V178P,L181Y	0.72	T146F,V178W,L181Y,L188R	0.55
A74I,T146F,V178W	0.68	V178P,L181Y,L188R	0.55
S72G,A74T,T146F,L181Y,L188K	0.68	T146F,V178W,L188F	0.55
S72G,A74T,L181Y,L188F	0.68	S72G,A74V,L188R	0.55
S72G,A74T,T146F,V178P,L181Y	0.68	S72G,A74T,V178P,L188F	0.55
S72G,A74T,T146F,V178W,L188F	0.67	S72G,T146F,V178P,L188R	0.54
S72G,T146F,V178W,L188F	0.66	S72G,V178W,L188K	0.53
S72G,A74I,T146F,L181Y,L188K	0.66	V178W	0.53
S72G,A74I,T146F,L181Y,L188R	0.66	S72G,V178W	0.52
S72G,V178P,L181Y	0.65	S72G,T146F,L188F	0.52
A74T,T146F,L181Y,L188R	0.65	S72G,T146F,L181Y,L188R	0.52
V178P,L181Y	0.65	A74T,T146F,V178P,L188R	0.51
S72G,V178W,L181Y	0.65	T146F,V178W	0.49
S72G,T146F,V178W,L181Y,L188F	0.63	S72G	0.47
S72G,T146F,V178W,L181Y	0.63	A74T,T146F,L188K	0.47
S72G,A74T,L181Y,L188K	0.63	T146F,V178W,L188K	0.46
S72G,A74I,T146F,L181Y	0.63	S72G,L188F	0.46
S72G,A74V,T146F,V178W	0.62	S72G,T146F	0.46

Table B4 shows estrone selectivity factors for ADD to estrone conversion from 100 mg/L substrate for variants derived from the parent type BM3-268; M177Y, A184Y (SEQ ID No. 15) having further mutations as depicted.

Mutation(s)	SF Estrone	Mutation(s)	SF Estrone	Mutation(s)	SF Estrone
I259M	0.75	T146A, F205G	0.29	L20R	0.23
L150W	0.72	F205E	0.29	F205W	0.23
L272C	0.65	F205S	0.29	V26S	0.23
F205D	0.45	M354Q	0.29	E352A	0.23
F205N	0.44	W325Y	0.29	E352I	0.23
R203C	0.44	F205I	0.28	M212F	0.22
F205V	0.42	F205G	0.28	M212T	0.22
E93G	0.41	M212H	0.28	L272W	0.21
F173Y, I174F	0.40	T438V	0.28	E352M	0.20
F205T	0.39	T438C	0.27	L150F	0.20
M212Y	0.37	M354W	0.27	T49Q	0.20
F205P	0.36	T49R	0.27	L272Y	0.20

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L78M	0.36	T327M	0.27	L20F	0.20
F205Y	0.34	F81Y	0.27	T146F	0.20
F205D, D208N	0.34	F205R	0.26	L150G	0.19
M212W	0.34	S89T	0.26	K210E	0.19
T146L	0.32	I259D	0.26	T49I	0.18
D363G, T438C	0.31	L150Y	0.25	F81D	0.18
F205K	0.31	L20Y	0.24	L20P	0.17
L20W	0.30	T438I	0.24	L272F	0.16
F205C	0.30	F205H	0.24	E352N	0.15
T146A, L272H	0.30	E352V	0.24		

Table B5 shows the selectivity factor for estrone obtained in biotransformation reactions from 100 mg/L ADD with BM3-268; M177Y, A184Y (SEQ ID No. 15) derived variants having up to six mutations.

Mutation(s)	SF Estrone	Mutation(s)	SF Estrone	Mutation(s)	SF Estrone
S72G,A74V,V178P,L181Y,L188F	0.94	S72G,A74T,L188R	0.54	S72G,A74I,T146F,V178P,L188F	0.37
S72G,A74V,L181Y,L188F	0.88	A74V,V178P,L181Y,L188F	0.54	T146F,V178W,L188F	0.37
S72G,A74V,T146F,V178P,L181Y,L188K	0.85	S72G,L181Y,L188R	0.53	S72G,L181Y,L188F	0.37
S72G,A74T,V178P,L181Y	0.84	S72G,A74T,T146F,V178P,L181Y	0.53	A74I,T146F,V178P	0.36
S72G,A74V,T146F,V178P,L181Y,L188R	0.83	S72G,T146F,L181Y,L188R	0.52	S72G,A74I,T146F,L181Y	0.36
S72G,A74V,T146F,L181Y,L188K	0.83	S72G,A74V,T146F,L181Y,L188R	0.52	S72G,L181Y	0.36
S72G,A74V,T146F,V178W,L181Y,L188K	0.82	A74T,T146F,V178P,L181Y	0.51	S72G,A74T,T146F,V178W,L181Y,L188F	0.36
S72G,A74V,V178W,L181Y	0.81	S72G,V178P,L188F	0.50	S72G,A74V,T146F,V178P	0.36
S72G,A74V,V178W,L181Y,L188F	0.79	A74T,T146F,V178W,L188R	0.50	S72G,A74T,T146F,V178P,L188F	0.35
S72G,A74T,T146F,V178W,L181Y	0.78	S72G,L181Y,L188K	0.49	S72G,A74V,V178P,L188R	0.34
S72G,A74I,T146F,V178W,L181Y	0.78	S72G,A74I,V178P,L181Y	0.49	S72G,T146F,V178P,L181Y,L188F	0.34
S72G,A74V,T146F,V178W,L181Y,L188R	0.78	S72G,A74V,V178P,L188F	0.49	V178P,L181Y	0.33
S72G,A74I,T146F,V178P,L181Y,L188R	0.77	S72G,T146F,L188F	0.49	S72G,V178W,L181Y,L188F	0.33
S72G,A74V,T146F,L181Y,L188F	0.76	S72G,V178P,L181Y,L188F	0.48	S72G,A74I,T146F,V178P	0.32
S72G,V178W,L181Y	0.75	S72G,A74T,T146F,V178P	0.48	S72G,T146F,L188R	0.32
S72G,V178W,L181Y,L188R	0.73	S72G,V178W,L188R	0.48	T146F,V178P	0.32
S72G,A74V,T146F,V178P,L181Y	0.71	S72G,A74T,T146F,V178P,L188R	0.48	S72G,A74T,T146F,L181Y	0.32
S72G,A74I,T146F,V178W,L181Y,L188K	0.71	S72G,T146F,V178W,L181Y,L188K	0.47	T146F,V178W,L181Y,L188R	0.31

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S72G,T146F,V178W,L181Y	0.69	S72G,A74I,T146F,V178P,L181Y	0.47	S72G,V178P,L188K	0.31
S72G,A74T,T146F,V178W,L181Y,L188K	0.69	S72G,T146F,V178P,L181Y,L188K	0.47	S72G,L188F	0.31
A74I,T146F,V178W,L188R	0.69	S72G,T146F,V178P,L181Y	0.45	S72G,V178P,L181Y,L188K	0.30
S72G,A74T,V178P,L181Y,L188K	0.68	S72G,T146F,L188K	0.44	S72G,A74T,V178P,L188F	0.29
S72G,T146F,V178W,L181Y,L188F	0.67	S72G,A74T,T146F,L181Y,L188K	0.43	S72G,T146F,V178P,L188R	0.29
S72G,A74V,T146F,V178P,L188F	0.66	S72G,A74V,T146F,V178W,L188R	0.43	S72G,A74V,V178P	0.29
S72G,A74V,T146F,L188F	0.66	S72G,V178P,L188R	0.42	V178P	0.29
S72G,A74T,T146F,V178W,L188R	0.65	T146F,L181Y,L188F	0.42	S72G,A74I,T146F,V178P,L188R	0.29
S72G,A74V,L181Y	0.65	T146F,L181Y	0.41	S72G,V178W	0.29
S72G,T146F,V178W,L188F	0.64	S72G,T146F,V178W,L188K	0.41	A74I,T146F,L181Y,L188K	0.29
S72G,A74V,T146F,V178W,L181Y	0.64	A74T,T146F,V178P	0.41	A74T,T146F,L181Y,L188K	0.28
S72G,A74T,L181Y,L188R	0.64	S72G,A74V,T146F,V178P,L188R	0.41	S72G,A74V,V178P,L181Y,L188R	0.28
S72G,T146F,V178W,L188R	0.63	T146F,L181Y,L188K	0.41	S72G,L188R	0.27
A74T,T146F,V178W,L181Y	0.63	S72G,T146F	0.40	S72G,A74V,T146F,V178P,L188K	0.27
S72G,A74I,T146F,L181Y,L188F	0.63	S72G	0.40	S72G,A74V,L188R	0.26
S72G,A74V,T146F,L181Y	0.62	S72G,A74I,T146F,V178P,L188K	0.40	S72G,T146F,V178P,L188K	0.26
S72G,A74T,T146F,V178W,L181Y,L188R	0.61	S72G,A74I,T146F,V178P,L181Y,L188F	0.40	A74I,T146F,V178W,L188K	0.26
S72G,T146F,L181Y,L188K	0.61	S72G,T146F,L181Y	0.40	T146F,V178P,L181Y,L188R	0.25
A74I,T146F,L181Y	0.61	A74I,T146F,V178P,L188R	0.40	T146F,V178W	0.25
S72G,T146F,V178W	0.59	A74V,T146F,L181Y,L188R	0.40	A74T,T146F,V178W	0.25
S72G,T146F,L181Y,L188F	0.59	S72G,A74V,T146F,V178W	0.39	S72G,L188K	0.24
S72G,T146F,V178P,L188F	0.56	S72G,V178P	0.39	S72G,A74V,L188K	0.24
S72G,V178W,L188K	0.56	S72G,A74I,T146F,L181Y,L188R	0.39	T146F,V178W,L188R	0.23
S72G,T146F,V178P	0.55	S72G,V178P,L181Y	0.38	T146F,V178W,L188K	0.21
S72G,V178W,L188F	0.55	S72G,V178P,L181Y,L188R	0.38	T146F	0.20
S72G,A74T,T146F,V178P,L188K	0.55	A74T,T146F,V178P,L188K	0.38		
S72G,A74T,T146F,V178W,L188F	0.54	S72G,T146F,V178P,L181Y,L188R	0.37		

Table B6 shows the target product ratio (TPR) for ADD to estrone conversion from 2000 mg/L substrate for variants derived from the parent type BM3-268; M177Y, A184Y; S72G, V178P, L181Y, L188F (SEQ ID No. 117) having further mutations as depicted.

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Mutation	TPR Estrone (%)	Mutation	TPR Estrone (%)	Mutation	TPR Estrone (%)	Mutation	TPR Estrone (%)
R79K	76.80	D182W	58.98	G240H	54.09	T175R	51.15
R179L	74.43	K76E	58.51	T269A	54.01	Q404F	51.10
I174V	70.26	N186L	58.42	E140K	54.01	Q189V	51.07
K187N	67.17	T175Q	58.42	H236G	54.01	V299L	51.03
R179M	65.99	A44Q	58.41	H236P	53.97	H92Y	50.93
K440C	65.82	T175M	58.25	A225S	53.86	R190W	50.61
F173W	65.23	H236C	58.23	G114C	53.81	S270D	50.56
D182A	64.50	T269V	58.21	S176D	53.74	E140T	50.47
N186K	64.40	N186Y	58.09	E409N	53.71	Q189I	50.31
D182I	64.27	V299W	58.05	R190S	53.68	G114K	50.30
K76V	63.28	R190K	57.79	T175G	53.63	S53C	50.24
D182H	62.78	K187G	57.79	G114L	53.61	A225V	50.06
R179A	62.59	G240A	57.78	A225G	53.39	N186E	50.02
D182E	62.29	G240R	57.66	M5P	53.22	H236F	50.01
D182V	62.26	H236I	57.50	N186V	53.02	Q189K	49.82
D182C	62.21	A399R	57.44	E140D	52.94	A225N	49.79
K76I	62.18	Q73F	57.39	K76H	52.86	K76C	49.74
G240S	61.85	N186A	57.23	G240C	52.86	Q73A	49.62
I258L	61.68	K440T	57.21	K187A	52.78	H236R	49.59
R190V	61.58	P172G	56.97	R179V	52.77	A225Y	49.58
Q73L	61.26	A225T	56.85	R190G	52.74	G240N	49.45
N186M	61.23	G114N	56.33	Q73W	52.67	T175H	49.43
Q403R	61.19	N186F	55.94	R190C	52.45	G46T	49.27
T175F	61.08	H236K	55.94	S270G	52.40	I258V	49.20
T175Y	61.06	D182M	55.78	T175N	52.37	H236A	49.15
H236Y	61.01	Q73T	55.69	G240M	52.11	M5C	49.13
K440N	61.00	G240Q	55.69	I174T	52.08	K76L	49.12
H236D	60.98	E140A	55.42	S270A	51.99	E183T	49.08
Q403K	60.90	K76N	55.22	A225Q	51.90	A44G	49.03
R190M	60.87	H236S	55.17	R179P	51.77	E337L	48.74
R190N	59.98	Q73E	54.76	Q73S	51.75	K187R	48.64
K76F	59.97	R179H	54.69	N186Q	51.68	M5E	48.63
P172A	59.90	I258M	54.67	E140M	51.65	R79E	48.59
N186T	59.83	G114W	54.67	M5Q	51.59	G240V	48.49
N186H	59.68	D182N	54.47	N186D	51.43		
T175L	59.57	P172D	54.40	H92F	51.29		
G240K	59.46	Q404Y	54.31	A225C	51.22		

Provided are variants according to a **fifth embodiment** according to the first aspect, wherein said P450-BM3 variant comprises further mutation(s) at at least one, two, three, four, five, six, seven, eight, nine, ten or more position(s). In particular, the variants according to the fifth

embodiment can be variants according to the first or second embodiment of the first aspect, such as variants derived from variants according to SEQ ID No. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 117, 118 or 119.

Preferrably, said position(s) are selected from (A184), (A191), (A221), (A264), (A321), (A328),
5 (A33), (A330), (A399), (A74), (D168), (D208), (D222), (D363), (E13), (E143), (E267), (E352),
(E64), (E82), (E93), (F173), (F205), (F261), (F331), (F393), (F77), (F81), (G240), (G271),
(G402), (G415), (G570), (G677), (G85), (H266), (H659), (I153), (I174), (I258), (I259), (I263),
(I401), (K210), (K224), (L150), (L181), (L188), (L20), (L262), (L272), (L29), (L324), (L333),
(L356), (L437), (L75), (L78), (L86), (M118), (M177), (M185), (M212), (M354), (N70), (P243),
10 (P326), (P329), (P392), (P9), (R147), (R161), (R190), (R203), (R255), (R323), (R47), (R50),
(S164), (S176), (S54), (S72), (S89), (T146), (T149), (T260), (T268), (T269), (T327), (T365),
(T436), (T438), (T49), (T88), (V178), (V26), (V299), (V314), (V371), (V48), (W325), (W367),
(W90), (Y51), (A225), (A44), (D182), (D80), (E140), (E183), (E337), (E409), (G114), (G227),
(G46), (H236), (H92), (K187), (K440), (K76), (M5), (N186), (P172), (Q189), (Q397), (Q403),
15 (Q404), (Q73), (R179), (R79), (S270), (S53) and (T175).

Preferably said BM3 variant has an improved selectivity and/or product yield for C19 hydroxylation compared with its corresponding parent BM3 variant. Preferably, the BM3 variant comprises the mutations M177Y and/or A184Y.

It was surprisingly found that targeting the(se) position(s) and introducing a "suitable" amino acid
20 exchange could further improve either product yield (cf. Tables A1, A2, A3, A4, A5, and A6) or
selectivity (cf. Tables B1, B2, B3, B4, B5 and B6) of the C19 hydroxylation, thereby further
improving the efficiency of the C19 hydroxylation process. Evaluation which amino acid is
"suitable" for a position to obtain the improvement is easily feasible where the targeting position
is disclosed herein. To evaluate which of the 20 amino acids is "suitable" for the given position,
25 one simply has to mutate the given position by introducing the amino acid as known in the art
(see also Example 1) and evaluate the performance of the obtained variant for C19
hydroxylation, e.g in comparison with the parent variant and/or in comparison with the remaining
18 amino acids.

There is provided a **first preferred subset** comprising variants according to the **fifth**
30 **embodiment**, wherein the P450-BM3 variants have an improved product yield or product titer
for C19 hydroxylation in comparison with the parent variant. Comprised within this subset are
variants wherein said position(s) are selected from (A184), (A191), (A221), (A264), (A321),
(A33), (A330), (A399), (A74), (D208), (D222), (D363), (E13), (E143), (E267), (E352), (E64),
(E82), (E93), (F173), (F205), (F261), (F331), (F393), (F77), (F81), (G240), (G271), (G402),
35 (G415), (G677), (G85), (H266), (H659), (I153), (I174), (I258), (I259), (I263), (I401), (K210),
(K224), (L150), (L181), (L188), (L20), (L262), (L272), (L29), (L324), (L356), (L437), (L75), (L78),

(L86), (M118), (M177), (M185), (M212), (M354), (N70), (P243), (P329), (P392), (P9), (R147), (R161), (R190), (R203), (R255), (R47), (R50), (S176), (S54), (S72), (S89), (T146), (T149), (T260), (T268), (T269), (T327), (T365), (T436), (T438), (T49), (T88), (V178), (V26), (V314), (V371), (V48), (W325), (W367), (W90), (Y51), (A225), (A44), (D182), (D80), (E140), (E183),
5 (E337), (G114), (G227), (H236), (H92), (K187), (K440), (K76), (M5), (N186), (P172), (Q189), (Q397), (Q403), (Q404), (Q73), (R179), (R79), (S270), (S53), (T175) and (V299).

There is provided a **second preferred subset** comprising variants according to the **fifth embodiment**, wherein the P450-BM3 variants have an improved selectivity (as determined by SF or TPR) for C19 hydroxylation in comparison with the parent variant (see figure 1b).
10 Comprised within this subset are variants wherein said position(s) are selected from (A184), (A191), (A221), (A264), (A321), (A328), (A33), (A330), (A399), (A74), (D168), (D208), (D222), (D363), (E143), (E267), (E352), (E64), (E82), (E93), (F173), (F205), (F261), (F331), (F393), (F77), (F81), (G240), (G271), (G402), (G415), (G570), (G677), (G85), (H266), (H659), (I153), (I174), (I258), (I259), (I263), (I401), (K210), (K224), (L150), (L181), (L188), (L20), (L262), (L272),
15 (L29), (L324), (L333), (L356), (L437), (L75), (L78), (L86), (M118), (M177), (M185), (M212), (M354), (N70), (P243), (P326), (P329), (P392), (P9), (R147), (R161), (R190), (R203), (R255), (R323), (R47), (R50), (S164), (S176), (S54), (S72), (S89), (T146), (T149), (T260), (T268), (T269), (T327), (T365), (T436), (T438), (T49), (T88), (V178), (V26), (V299), (V314), (V48), (W325), (W367), (W90), (Y51), (A225), (A44), (D182), (E140), (E183), (E337), (E409), (G114),
20 (G46), (H236), (H92), (K187), (K440), (K76), (M5), (N186), (P172), (Q189), (Q403), (Q404), (Q73), (R179), (R79), (S270), (S53) and (T175).

There is provided a **third preferred subset** comprising variants according to the **fifth embodiment**, wherein the P450-BM3 variants have an improved selectivity (as determined by SF or TPR) and an improved product yield or product titer for C19 hydroxylation in comparison
25 with the parent variant. Comprised within this subset are variants wherein said position(s) are selected from (A184), (A191), (A221), (A264), (A321), (A33), (A330), (A399), (A74), (D208), (D222), (D363), (E13), (E143), (E267), (E352), (E64), (E82), (E93), (F173), (F205), (F261), (F331), (F393), (F77), (F81), (G240), (G271), (G402), (G677), (G85), (H266), (H659), (I153), (I174), (I258), (I259), (I263), (I401), (K210), (K224), (L150), (L181), (L188), (L20), (L262), (L272),
30 (L29), (L324), (L356), (L437), (L75), (L78), (L86), (M118), (M177), (M185), (M212), (M354), (N70), (P243), (P329), (P392), (P9), (R147), (R161), (R190), (R203), (R255), (R47), (R50), (S176), (S54), (S72), (S89), (T146), (T149), (T268), (T269), (T327), (T365), (T436), (T438), (T49), (T88), (V178), (V26), (V314), (V48), (W325), (W367), (W90), (Y51), (A225), (A44), (D182), (E140), (E337), (G114), (H236), (H92), (K187), (K440), (K76), (M5), (N186), (P172),
35 (Q189), (Q403), (Q404), (Q73), (R179), (R79), (S270), (S53), (T175) and (V299).

The fifth embodiment and each of the subsets provided can be and is suggested to be combined with all previous embodiments, as understood by the skilled person, and may or may not be an embodiment according to the **second embodiment** of the first aspect.

5 Provided are variants according to a **sixth embodiment** according to the first aspect, wherein said P450-BM3 variant comprise at least one, two, three, four, five, six, seven, eight, nine, ten or more further mutations. In particular the variants according to the sixth embodiment can be variants according to the first or second embodiment of the first aspect, such as variants derived from variants according to SEQ ID No. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 117, 118 or 119. The sixth embodiment can be and is suggested to be combined with all previous embodiments, and may or may not be an embodiment according to the fifth embodiment.

Preferrably, said further mutation(s) according to the sixth embodiment of the first aspect are (A184D) or (A184D, L188K) or (A184E) or (A184F) or (A184G) or (A184H) or (A184I) or (A184K) or (A184L) or (A184N) or (A184Q) or (A184R) or (A184W) or (A184W, L188F) or (A184Y) or 15 (A184Y, L188K) or (A184Y, R50S) or (A221S) or (A264V) or (A321C) or (A321D) or (A321E) or (A321G) or (A321I) or (A321N) or (A321T) or (A321V) or (A328P) or (A330C) or (A330D) or (A330E) or (A330F) or (A330G) or (A330H) or (A330I) or (A330K) or (A330L) or (A330M) or (A330N) or (A330Q) or (A330R) or (A330S) or (A330T) or (A330T, E352A) or (A330T, F331V) or (A330V) or (A330W) or (A330Y) or (A330Y, W367C) or (A33V) or (A399C) or (A399E) or 20 (A399G) or (A399I) or (A399L) or (A399M) or (A399N) or (A399Q) or (A399R) or (A399S) or (A399T) or (A399V) or (A74C) or (A74C, L75V) or (A74D) or (A74D, F81V) or (A74E) or (A74F) or (A74G) or (A74G, L75C) or (A74H) or (A74I) or (A74I, A184W, L188F) or (A74I, F77A, L181Y, A184W) or (A74I, L181Y, L188K) or (A74I, L181Y, L188R) or (A74I, L188F) or (A74I, L188K) or (A74I, L188R) or (A74I, M177Y, A184R, L188F) or (A74I, M177Y, V178P, A184G) or (A74I, 25 T146F) or (A74I, T146F, L181Y) or (A74I, T146F, L181Y, L188K) or (A74I, T146F, L181Y, L188R) or (A74I, T146F, L188F) or (A74I, T146F, L188K) or (A74I, T146F, V178P) or (A74I, T146F, V178P, L181Y, L188F) or (A74I, T146F, V178P, L181Y, L188K) or (A74I, T146F, V178P, L188K) or (A74I, T146F, V178P, L188R) or (A74I, T146F, V178W) or (A74I, T146F, V178W, L181Y, L188F) or (A74I, T146F, V178W, L181Y, L188R) or (A74I, T146F, V178W, L188F) or 30 (A74I, T146F, V178W, L188K) or (A74I, T146F, V178W, L188R) or (A74I, V178P) or (A74I, V178P, L181Y) or (A74I, V178P, L181Y, L188R) or (A74I, V178P, L188F) or (A74I, V178P, L188K) or (A74I, V178P, L188R) or (A74I, V178W, L188F) or (A74I, V178W, L188K) or (A74I, V178W, L188R) or (A74K) or (A74L) or (A74M) or (A74N) or (A74P) or (A74Q) or (A74R) or (A74R, L75V) or (A74S) or (A74T) or (A74T, A184D) or (A74T, F77A, L181Y, A184D, L188K) or 35 (A74T, F77A, V178W, A184Y) or (A74T, F77L, L181Y, A184D, L188K) or (A74T, L181Y) or (A74T, L181Y, A184D, L188K) or (A74T, L181Y, A184Y, L188K) or (A74T, L181Y, L188K) or

(A74T, L181Y, L188R) or (A74T, L188K) or (A74T, M177Y, L181Y, A184D, L188K) or (A74T, T146F, L181Y, A184D, L188K) or (A74T, T146F, L181Y, L188K) or (A74T, T146F, L181Y, L188R) or (A74T, T146F, L188F) or (A74T, T146F, L188K) or (A74T, T146F, L188R) or (A74T, T146F, V178P) or (A74T, T146F, V178P, L181Y) or (A74T, T146F, V178P, L188K) or (A74T, T146F, V178P, L188R) or (A74T, T146F, V178W) or (A74T, T146F, V178W, L181Y) or (A74T, T146F, V178W, L188K) or (A74T, T146F, V178W, L188R) or (A74T, V178P) or (A74T, V178P, L188F) or (A74T, V178P, L188K) or (A74T, V178P, L188R) or (A74T, V178W, L181Y, L188K) or (A74T, V178W, L188K) or (A74T, V178W, L188R) or (A74V) or (A74V, F77A, A184N, L188F) or (A74V, L181Y) or (A74V, L181Y, A184D, L188K) or (A74V, L181Y, A184W, L188F) or (A74V, L181Y, L188F) or (A74V, L188F) or (A74V, L188K) or (A74V, L75V) or (A74V, T146F, L181Y, L188K) or (A74V, T146F, L181Y, L188R) or (A74V, T146F, L188K) or (A74V, T146F, L188R) or (A74V, T146F, V178W) or (A74V, T146F, V178W, L181Y, L188F) or (A74V, V178P, A184W, L188K) or (A74V, V178P, L181Y, L188F) or (A74V, V178P, L181Y, L188K) or (A74V, V178P, L181Y, L188R) or (A74V, V178P, L188F) or (A74V, V178W, L188K) or (A74W) or (A74Y) or (D168Y, F173D) or (D363G, T438C) or (E13D, R47L) or (E143A) or (E143C) or (E143D) or (E143F) or (E143G) or (E143I) or (E143K) or (E143L) or (E143M) or (E143N) or (E143P) or (E143Q) or (E143R) or (E143S) or (E143V) or (E143W) or (E143Y) or (E267A) or (E267C) or (E267D) or (E267G) or (E267K) or (E267P) or (E267R) or (E267S) or (E267T) or (E267Y) or (E352A) or (E352D) or (E352F) or (E352G) or (E352I) or (E352L) or (E352M) or (E352N) or (E352P) or (E352R) or (E352S) or (E352T) or (E352V) or (E352W) or (E352Y) or (E64A) or (E64G) or (E64H) or (E64I) or (E64K) or (E64L) or (E64M) or (E64N) or (E64Q) or (E64V) or (E64W) or (E64Y) or (E82P) or (E93G) or (F173C) or (F173C, F205G) or (F173D) or (F173I) or (F173K) or (F173L) or (F173M) or (F173N) or (F173P) or (F173Q) or (F173R) or (F173S) or (F173S, F205P) or (F173V) or (F173W) or (F173Y) or (F173Y, I174F) or (F205A) or (F205C) or (F205D) or (F205D, D208N) or (F205E) or (F205G) or (F205H) or (F205I) or (F205K) or (F205L) or (F205M) or (F205N) or (F205P) or (F205R) or (F205S) or (F205T) or (F205V) or (F205W) or (F205Y) or (F261A) or (F261C) or (F261D) or (F261G) or (F261I) or (F261L) or (F261M) or (F261N) or (F261Q) or (F261S) or (F261T) or (F261V) or (F261W) or (F261Y) or (F331C) or (F331H) or (F331I) or (F331L) or (F331M) or (F331N) or (F331P) or (F331T) or (F331V) or (F331W) or (F331Y) or (F393M) or (F393W) or (F77A) or (F77A, A184W) or (F77A, L181P, A184Y, L188K) or (F77A, M177Y) or (F77C) or (F77D) or (F77E) or (F77G) or (F77H) or (F77I) or (F77K) or (F77L) or (F77M) or (F77N) or (F77P) or (F77R) or (F77S) or (F77T) or (F77V) or (F77V, L86M) or (F77W) or (F77Y) or (F81A) or (F81C) or (F81D) or (F81I) or (F81L) or (F81P) or (F81R) or (F81S) or (F81T) or (F81V) or (F81W) or (F81Y) or (G271A) or (G271C) or (G271D) or (G271E) or (G271F) or (G271H) or (G271K) or (G271L) or (G271M) or (G271N) or (G271P) or (G271Q) or (G271R) or (G271S) or (G271T) or (G271V) or (G271W) or (G271Y) or (G402A) or (G415A) or (G415D) or (G415S) or (G415T) or (G415V) or (G85A) or (G85L) or

(G85S) or (H266A) or (H266C) or (H266D) or (H266E) or (H266F) or (H266G) or (H266I) or (H266K) or (H266M) or (H266N) or (H266P) or (H266Q) or (H266R) or (H266S) or (H266T) or (H266V) or (H266W) or (H266Y) or (I153F, G271L) or (I153L) or (I153L, F173Y) or (I259A) or (I259C) or (I259D) or (I259F) or (I259G) or (I259H) or (I259K) or (I259L) or (I259M) or (I259N) or (I259Q) or (I259S) or (I259T) or (I259V) or (I259W) or (I259Y) or (I263A) or (I263C) or (I263E) or (I263F) or (I263G) or (I263H, A264G) or (I263K) or (I263L) or (I263M) or (I263N) or (I263Q) or (I263S) or (I263T) or (I263V) or (I263Y) or (I401A) or (I401L) or (I401M) or (I401T) or (I401V) or (K210E) or (K210T, G271V) or (K224C) or (K224E) or (K224F) or (K224H) or (K224I) or (K224L) or (K224M) or (K224P) or (K224Q) or (K224W) or (K224Y) or (L150A) or (L150C) or (L150D) or (L150E) or (L150F) or (L150G) or (L150H) or (L150I) or (L150K) or (L150M) or (L150N) or (L150Q) or (L150R) or (L150R, F205R) or (L150S) or (L150S, F173L) or (L150T) or (L150V) or (L150W) or (L150Y) or (L181H) or (L181I) or (L181M) or (L181P) or (L181V) or (L181Y) or (L181Y, A184D) or (L181Y, L188F) or (L181Y, L188K) or (L181Y, L188K, H659R) or (L188A) or (L188D) or (L188E) or (L188F) or (L188H) or (L188I) or (L188K) or (L188M) or (L188N) or (L188Q) or (L188R) or (L188S) or (L188W) or (L20C) or (L20D) or (L20E) or (L20F) or (L20G) or (L20G, R47L) or (L20I) or (L20M) or (L20N) or (L20P) or (L20R) or (L20S) or (L20T) or (L20V) or (L20W) or (L20Y) or (L262I) or (L262V) or (L262W) or (L262Y) or (L272A) or (L272C) or (L272E) or (L272F) or (L272G) or (L272I) or (L272K) or (L272M) or (L272N) or (L272Q) or (L272R) or (L272S) or (L272T) or (L272V) or (L272W) or (L272Y) or (L29A) or (L29C) or (L29D) or (L29F) or (L29H) or (L29I) or (L29M) or (L29M, R47G) or (L29P) or (L29Q) or (L29S) or (L29T) or (L29V) or (L29W) or (L29Y) or (L324F) or (L356C) or (L356F) or (L356H) or (L356I) or (L356M) or (L356N) or (L356Q) or (L356S) or (L356T) or (L356V) or (L356W) or (L437I) or (L437M) or (L75H, F81S) or (L75I) or (L75I, F81C) or (L75I, F81G) or (L75I, F81H) or (L75I, F81I) or (L75I, F81L) or (L75I, F81S) or (L75I, F81V) or (L75I, F81Y) or (L75V) or (L75V, F81C) or (L75V, F81H) or (L75V, F81I) or (L75V, F81L) or (L75V, F81V) or (L75V, F81Y) or (L78F) or (L78I) or (L78M) or (L78V) or (L78Y) or (L86A, S89T) or (L86I) or (L86I, S89T) or (L86M) or (L86M, S89T) or (L86N, S89T) or (L86V) or (L86V, S89T) or (M118A) or (M118E) or (M118F) or (M118G) or (M118H) or (M118I) or (M118K) or (M118L) or (M118N) or (M118P) or (M118Q) or (M118S) or (M118T) or (M118V) or (M118W) or (M118Y) or (M177A) or (M177C) or (M177C, V178Y) or (M177D) or (M177E) or (M177F) or (M177G) or (M177H) or (M177I) or (M177K) or (M177L) or (M177N) or (M177P) or (M177Q) or (M177R) or (M177T) or (M177V) or (M177Y) or (M177Y, A184W) or (M177Y, A184Y) or (M177Y, M185V) or (M177Y, R50S) or (M177Y, V178P) or (M177Y, V178P, A184Y, L188F) or (M177Y, V178W) or (M177Y, V178W, A184Y) or (M177Y, V178W, A184Y, L188F) or (M185C) or (M185D) or (M185E) or (M185G) or (M185H) or (M185K) or (M185L) or (M185N) or (M185Q) or (M185R) or (M185V) or (M185Y) or (M212A) or (M212C) or (M212C, I259L) or (M212D) or (M212E) or (M212F) or (M212G) or (M212H) or (M212K) or (M212L) or (M212L, I259F) or (M212P) or (M212Q) or (M212R) or (M212S) or (M212T) or

(M212V) or (M212W) or (M212Y) or (M354A) or (M354C) or (M354D) or (M354E) or (M354G) or (M354I) or (M354K) or (M354K, D363Y) or (M354L) or (M354N) or (M354Q) or (M354R) or (M354S) or (M354T) or (M354V) or (M354W) or (M354Y) or (N70A) or (N70C) or (N70F) or (N70G) or (N70H) or (N70K) or (N70R) or (N70W) or (N70Y) or (P243T, H266L) or (P326C) or
5 (P326D) or (P326G) or (P326N) or (P326S) or (P326T) or (P329A) or (P329C) or (P329G) or (P329K) or (P329S) or (P329T) or (P392C) or (P392G) or (P392Q) or (P392R) or (P392V) or (P9S) or (R147C, H266G) or (R161C, G271D) or (R190L, F261V) or (R203C) or (R255A) or (R255F) or (R255G) or (R255I) or (R255K) or (R255L) or (R255M) or (R255N) or (R255P) or (R255T) or (R255W) or (R323C) or (R47A) or (R47C) or (R47D) or (R47E) or (R47F) or (R47G)
10 or (R47H) or (R47H, A74W) or (R47I) or (R47K) or (R47L) or (R47M) or (R47N) or (R47P) or (R47Q) or (R47S) or (R47T) or (R47V) or (R47W) or (R50A) or (R50C) or (R50D) or (R50E) or (R50F) or (R50G) or (R50I) or (R50L) or (R50M) or (R50N) or (R50P) or (R50Q) or (R50S) or (R50S, A184W, L188F) or (R50S, A74I, A184W, L188F) or (R50S, A74M, A184Y, L188K) or (R50S, A74T, L181Y, A184D, L188K, G240R) or (R50S, A74V) or (R50S, M177Y, V178P, A184Y) or (R50S, M177Y, V178W, A184W) or (R50S, M177Y, V178W, A184Y) or (R50S, M177Y, V178W, A184Y, A330R) or (R50S, M177Y, V178W, A184Y, A74V, G677D) or (R50S, M177Y, V178W, A184Y, H266S) or (R50S, M177Y, V178W, A184Y, L181P) or (R50S, M177Y, V178W, A184Y, L181Y) or (R50S, M177Y, V178W, A184Y, L188K) or (R50S, M177Y, V178W, A184Y, L188R) or (R50S, M177Y, V178W, A184Y, T146F) or (R50S, M177Y, V178W, A184Y, V26N) or (R50S, M177Y, V178W, L181Y, A184Y) or (R50S, S72G, A74V) or (R50S, S72G, A74V, M177Y, A184Y) or (R50S, S72G, A74V, M177Y, V178P, A184Y) or (R50S, S72G, A74V, M177Y, V178W, A184Y) or (R50S, S72G, A74V, M177Y, V178W, A184Y, L188R) or (R50S, T146F, M177Y, V178W, A184Y) or (R50V) or (R50Y) or (S164N, F261L) or (S176A, A184Y, L188K) or (S176C) or (S176D) or (S176E) or (S176F) or (S176G) or (S176H) or (S176K) or
25 (S176L) or (S176M) or (S176N) or (S176P) or (S176Q) or (S176R) or (S72C, A74C) or (S72C, A74I) or (S72C, A74L) or (S72C, A74V) or (S72C, A74Y) or (S72D, A74C) or (S72D, A74F) or (S72G) or (S72G, A74C) or (S72G, A74F) or (S72G, A74H) or (S72G, A74I) or (S72G, A74I, A184W, L188F) or (S72G, A74I, F77A, M177Y) or (S72G, A74I, F77L) or (S72G, A74I, F77L, M177Y) or (S72G, A74I, L181Y) or (S72G, A74I, L181Y, L188F) or (S72G, A74I, L181Y, L188K) or (S72G, A74I, L181Y, L188R) or (S72G, A74I, L188F) or (S72G, A74I, L188K) or (S72G, A74I, L188R) or (S72G, A74I, T146F) or (S72G, A74I, T146F, L181Y) or (S72G, A74I, T146F, L181Y, L188F) or (S72G, A74I, T146F, L181Y, L188K) or (S72G, A74I, T146F, L181Y, L188R) or (S72G, A74I, T146F, L188F) or (S72G, A74I, T146F, L188K) or (S72G, A74I, T146F, L188R) or (S72G, A74I, T146F, V178P) or (S72G, A74I, T146F, V178P, L181Y) or (S72G, A74I, T146F, V178P, L181Y, L188F) or (S72G, A74I, T146F, V178P, L181Y, L188K) or (S72G, A74I, T146F, V178P, L181Y, L188R) or (S72G, A74I, T146F, V178P, L188F) or (S72G, A74I, T146F, V178P, L188K) or (S72G, A74I, T146F, V178W) or (S72G, A74I,

T146F, V178W, L181Y) or (S72G, A74I, T146F, V178W, L181Y, L188F) or (S72G, A74I, T146F, V178W, L181Y, L188K) or (S72G, A74I, T146F, V178W, L181Y, L188R) or (S72G, A74I, T146F, V178W, L188F) or (S72G, A74I, T146F, V178W, L188K) or (S72G, A74I, T146F, V178W, L188R) or (S72G, A74I, V178P) or (S72G, A74I, V178P, L181Y) or (S72G, A74I, V178P, L181Y, L188K) or (S72G, A74I, V178P, L181Y, L188R) or (S72G, A74I, V178P, L188F) or (S72G, A74I, V178P, L188K) or (S72G, A74I, V178P, L188R) or (S72G, A74I, V178W) or (S72G, A74I, V178W, L181Y, L188F) or (S72G, A74I, V178W, L181Y, L188K) or (S72G, A74I, V178W, L181Y, L188R) or (S72G, A74I, V178W, L188F) or (S72G, A74I, V178W, L188K) or (S72G, A74I, V178W, L188R) or (S72G, A74L) or (S72G, A74M, A184Y, L188K) or (S72G, A74S) or (S72G, A74T) or (S72G, A74T, L181Y, A184D, L188K) or (S72G, A74T, L181Y, L188F) or (S72G, A74T, L181Y, L188K) or (S72G, A74T, L181Y, L188R) or (S72G, A74T, L188F) or (S72G, A74T, L188K) or (S72G, A74T, L188R) or (S72G, A74T, T146F) or (S72G, A74T, T146F, L181Y) or (S72G, A74T, T146F, L181Y, L188K) or (S72G, A74T, T146F, L181Y, L188R) or (S72G, A74T, T146F, L188F) or (S72G, A74T, T146F, L188K) or (S72G, A74T, T146F, L188R) or (S72G, A74T, T146F, V178P) or (S72G, A74T, T146F, V178P, L181Y) or (S72G, A74T, T146F, V178P, L188F) or (S72G, A74T, T146F, V178P, L188K) or (S72G, A74T, T146F, V178P, L188R) or (S72G, A74T, T146F, V178W) or (S72G, A74T, T146F, V178W, L181Y) or (S72G, A74T, T146F, V178W, L181Y, L188F) or (S72G, A74T, T146F, V178W, L181Y, L188K) or (S72G, A74T, T146F, V178W, L181Y, L188R) or (S72G, A74T, T146F, V178W, L188F) or (S72G, A74T, T146F, V178W, L188R) or (S72G, A74T, T146F, V178W, L188F) or (S72G, A74T, T146F, V178W, L188K) or (S72G, A74T, T146F, V178W, L188R) or (S72G, A74T, T146F, V178W, L188F) or (S72G, A74T, T146F, V178W, L188K) or (S72G, A74T, T146F, V178W, L188R) or (S72G, A74V) or (S72G, A74V, A184W) or (S72G, A74V, A221V) or (S72G, A74V, F77A) or (S72G, A74V, F77A, M177Y) or (S72G, A74V, F77L, M177Y) or (S72G, A74V, F77S) or (S72G, A74V, L181Y) or (S72G, A74V, L181Y, L188F) or (S72G, A74V, L181Y, L188K) or (S72G, A74V, L188F) or (S72G, A74V, L188K) or (S72G, A74V, L188R) or (S72G, A74V, T146F) or (S72G, A74V, T146F, L181Y) or (S72G, A74V, T146F, L181Y, L188F) or (S72G, A74V, T146F, L181Y, L188K) or (S72G, A74V, T146F, L181Y, L188R) or (S72G, A74V, T146F, L188F) or (S72G, A74V, T146F, L188K) or (S72G, A74V, T146F, L188R) or (S72G, A74V, T146F, V178P) or (S72G, A74V, T146F, V178P, L181Y) or (S72G, A74V, T146F, V178P, L181Y, L188F) or (S72G, A74V, T146F, V178P, L181Y, L188K) or (S72G, A74V, T146F, V178P, L181Y, L188R) or (S72G, A74V, T146F, V178P, L188F) or (S72G, A74V, T146F, V178P, L188K) or (S72G, A74V, T146F, V178P, L188R) or (S72G, A74V, T146F, V178W) or (S72G, A74V, T146F, V178W, L181Y) or (S72G, A74V, T146F, V178W, L181Y, L188K) or (S72G, A74V, T146F, V178W, L181Y, L188R) or (S72G,

A74V, T146F, V178W, L188F) or (S72G, A74V, T146F, V178W, L188K) or (S72G, A74V, T146F, V178W, L188R) or (S72G, A74V, V178P) or (S72G, A74V, V178P, L181Y) or (S72G, A74V, V178P, L181Y, L188F) or (S72G, A74V, V178P, L181Y, L188K) or (S72G, A74V, V178P, L181Y, L188R) or (S72G, A74V, V178P, L188F) or (S72G, A74V, V178P, L188K) or (S72G, A74V, V178P, L188R) or (S72G, A74V, V178W) or (S72G, A74V, V178W, L181Y) or (S72G, A74V, V178W, L181Y, L188F) or (S72G, A74V, V178W, L181Y, L188K) or (S72G, A74V, V178W, L181Y, L188R) or (S72G, A74V, V178W, L188F) or (S72G, A74V, V178W, L188K) or (S72G, A74V, V178W, L188R) or (S72G, A74Y) or (S72G, L181Y) or (S72G, L181Y, L188F) or (S72G, L181Y, L188K) or (S72G, L181Y, L188R) or (S72G, L188F) or (S72G, L188K) or (S72G, L188R) or (S72G, M177Y, V178W) or (S72G, M177Y, V178W, L188K) or (S72G, T146F) or (S72G, T146F, L181Y) or (S72G, T146F, L181Y, L188F) or (S72G, T146F, L181Y, L188K) or (S72G, T146F, L181Y, L188R) or (S72G, T146F, L188F) or (S72G, T146F, L188K) or (S72G, T146F, L188R) or (S72G, T146F, V178P) or (S72G, T146F, V178P, L181Y) or (S72G, T146F, V178P, L181Y, L188F) or (S72G, T146F, V178P, L181Y, L188K) or (S72G, T146F, V178P, L181Y, L188R) or (S72G, T146F, V178P, L188F) or (S72G, T146F, V178P, L188K) or (S72G, T146F, V178P, L188R) or (S72G, T146F, V178W) or (S72G, T146F, V178W, L181Y) or (S72G, T146F, V178W, L181Y, L188F) or (S72G, T146F, V178W, L181Y, L188K) or (S72G, T146F, V178W, L181Y, L188R) or (S72G, T146F, V178W, L188F) or (S72G, T146F, V178W, L188K) or (S72G, T146F, V178W, L188R) or (S72G, V178P) or (S72G, V178P, L181Y) or (S72G, V178P, L181Y, L188F) or (S72G, V178P, L181Y, L188K) or (S72G, V178P, L181Y, L188R) or (S72G, V178P, L188F) or (S72G, V178P, L188K) or (S72G, V178P, L188R) or (S72G, V178W) or (S72G, V178W, L181Y) or (S72G, V178W, L181Y, L188F) or (S72G, V178W, L181Y, L188K) or (S72G, V178W, L181Y, L188R) or (S72G, V178W, L188F) or (S72G, V178W, L188K) or (S72G, V178W, L188R) or (S72H, A74C) or (S72H, A74G) or (S72H, A74S) or (S72H, A74Y) or (S72N, A74C) or (S72N, A74I) or (S72N, A74N) or (S72N, A74V) or (S72T) or (S72W) or (S72W, A74I, A184W, L188F) or (S72W, A74V) or (S72W, L333R) or (S72W, W90Y, V299G) or (S72Y, A74V) or (S89C) or (S89E) or (S89G) or (S89I) or (S89L) or (S89M) or (S89N) or (S89Q) or (S89R) or (S89T) or (S89V) or (T146A) or (T146A, D222Y, I263M) or (T146A, F173D) or (T146A, F173V) or (T146A, F205G) or (T146A, F261C) or (T146A, F261S) or (T146A, F261V) or (T146A, G271R) or (T146A, G271S) or (T146A, G271T) or (T146A, H266T) or (T146A, H266V) or (T146A, I258T, I259S) or (T146A, I259H) or (T146A, I263F) or (T146A, I263L) or (T146A, I263N) or (T146A, I263V) or (T146A, L150A) or (T146A, L150G) or (T146A, L150K) or (T146A, L150R, F173I) or (T146A, L272H) or (T146A, L272R) or (T146A, L272S) or (T146A, L272W) or (T146A, M212A) or (T146C) or (T146D) or (T146E) or (T146F) or (T146F, L181Y) or (T146F, L181Y, L188F) or (T146F, L181Y, L188K) or (T146F, L181Y, L188R) or (T146F, V178P) or (T146F, V178P, L181Y, L188R) or (T146F, V178P, L188K) or (T146F, V178P, L188R) or (T146F, V178W) or (T146F, V178W, L181Y, L188R) or (T146F, V178W, L188F) or (T146F, V178W, L188K) or (T146F, V178W,

L188R) or (T146G) or (T146H) or (T146L) or (T146M) or (T146N) or (T146P) or (T146R) or
 (T146S) or (T146V) or (T146V, A191T) or (T146Y) or (T149P) or (T260A) or (T260S) or (T260W)
 or (T268A) or (T268S) or (T269K) or (T327A) or (T327A, A330W) or (T327C) or (T327D) or
 (T327E) or (T327G) or (T327I) or (T327L) or (T327M) or (T327N) or (T327P) or (T327Q) or
 5 (T327S) or (T327V) or (T365A) or (T365C) or (T365D) or (T365F) or (T365G) or (T365H) or
 (T365I) or (T365K) or (T365L) or (T365N) or (T365P) or (T365Q) or (T365V) or (T365W) or
 (T365Y) or (T436A) or (T436C) or (T436D) or (T436E) or (T436F) or (T436G) or (T436H) or
 (T436I) or (T436K) or (T436L) or (T436M) or (T436N) or (T436P) or (T436Q) or (T436R) or
 (T436S) or (T436V) or (T436W) or (T436Y) or (T438A) or (T438C) or (T438G) or (T438I) or
 10 (T438L) or (T438S) or (T438V) or (T49A) or (T49C) or (T49D) or (T49E) or (T49F) or (T49G) or
 (T49H) or (T49I) or (T49K) or (T49L) or (T49L, S54N) or (T49M) or (T49N) or (T49P) or (T49Q)
 or (T49R) or (T49R, R50C) or (T49R, R50S, M177Y, V178W, A184Y) or (T49S) or (T49V) or
 (T49W) or (T49Y) or (T88A) or (T88C) or (T88S) or (T88V) or (V178A) or (V178D) or (V178F) or
 (V178H) or (V178I) or (V178K) or (V178L) or (V178M) or (V178N) or (V178P) or (V178P, L181Y)
 15 or (V178P, L181Y, L188R) or (V178P, L188F) or (V178P, L188K) or (V178P, L188R) or (V178Q)
 or (V178R) or (V178S) or (V178T) or (V178W) or (V178W, A184Y) or (V178W, L181Y, L188K)
 or (V178W, L181Y, L188R) or (V178W, L188R) or (V178W, R50S) or (V178Y) or (V26A) or
 (V26C) or (V26E) or (V26F) or (V26G) or (V26G, R47S) or (V26H) or (V26I) or (V26K) or (V26L)
 or (V26M) or (V26N) or (V26N, R50S, M177Y, V178W, A184Y) or (V26Q) or (V26R) or (V26S)
 20 or (V26T) or (V26W) or (V26Y) or (V314A) or (V314E) or (V314F) or (V314H) or (V314P) or
 (V314Q) or (V314S) or (V314Y) or (V371P) or (V48A) or (V48D) or (V48E) or (V48F) or (V48G)
 or (V48H) or (V48I) or (V48L) or (V48M) or (V48P) or (V48Q) or (V48R) or (V48S) or (V48T) or
 (V48W) or (V48Y) or (W325A) or (W325C) or (W325D) or (W325E) or (W325F) or (W325G) or
 (W325I) or (W325K) or (W325L) or (W325M) or (W325Q) or (W325R) or (W325S) or (W325T)
 25 or (W325V) or (W325Y) or (W90D) or (W90Y) or (W90Y, V299G, G570D) or (Y51A) or (Y51C)
 or (Y51E) or (Y51F) or (Y51G) or (Y51H) or (Y51I) or (Y51L) or (Y51M) or (Y51N) or (Y51P) or
 (Y51Q) or (Y51S) or (Y51S, F77V) or (Y51T) or (Y51V) or (Y51W) or (A225C) or (A225G) or
 (A225M) or (A225N) or (A225Q) or (A225S) or (A225T) or (A225V) or (A225Y) or (A44G) or
 (A44Q) or (A44T) or (D182A) or (D182C) or (D182E) or (D182H) or (D182I) or (D182M) or
 30 (D182N) or (D182V) or (D182W) or (D80C) or (D80L) or (D80M) or (D80N) or (D80S) or (D80T)
 or (D80W) or (E140A) or (E140D) or (E140K) or (E140L) or (E140M) or (E140T) or (E183D) or
 (E183T) or (E337L) or (E337V) or (E409N) or (G114C) or (G114K) or (G114L) or (G114N) or
 (G114W) or (G227N) or (G227R) or (G240A) or (G240C) or (G240H) or (G240K) or (G240M) or
 (G240N) or (G240Q) or (G240R) or (G240S) or (G240V) or (G46T) or (H236A) or (H236C) or
 35 (H236D) or (H236F) or (H236G) or (H236I) or (H236K) or (H236M) or (H236P) or (H236R) or
 (H236S) or (H236Y) or (H92F) or (H92Y) or (I174T) or (I174V) or (I258C) or (I258L) or (I258M)
 or (I258V) or (K187A) or (K187G) or (K187N) or (K187R) or (K440C) or (K440M) or (K440N) or

(K440T) or (K76C) or (K76E) or (K76F) or (K76H) or (K76I) or (K76L) or (K76N) or (K76V) or (M5C) or (M5E) or (M5P) or (M5Q) or (N186A) or (N186D) or (N186E) or (N186F) or (N186H) or (N186K) or (N186L) or (N186M) or (N186Q) or (N186T) or (N186V) or (N186Y) or (P172A) or (P172D) or (P172G) or (Q189E) or (Q189I) or (Q189K) or (Q189N) or (Q189V) or (Q397R) or
 5 (Q403K) or (Q403R) or (Q404F) or (Q404Y) or (Q73A) or (Q73E) or (Q73F) or (Q73L) or (Q73S) or (Q73T) or (Q73W) or (Q73Y) or (R179A) or (R179H) or (R179L) or (R179M) or (R179P) or (R179V) or (R190C) or (R190G) or (R190K) or (R190M) or (R190N) or (R190S) or (R190V) or (R190W) or (R79E) or (R79K) or (S270A) or (S270D) or (S270G) or (S53C) or (T175F) or (T175G) or (T175H) or (T175L) or (T175M) or (T175N) or (T175Q) or (T175R) or (T175Y) or
 10 (T269A) or (T269V) or (V299L) or (V299W) or (A225V, H236K, G240V, T269V) or (D80I, D182H, A225V, G240V, T269V) or (F173W, R179L, R190V, A225V) or (F173W, R179L, R190V, K440C) or (F173W, R179L, T269V, K440C) or (F173W, R190V, H236K, T269V) or (H236K, G240V, T269V, K440C) or (K76I, F173W, R179L, D182I, R190V, K440C) or (Q73W, D80I, N186V, G240V) or (R179L, A225S) or (R179L, A225V, G240V, K440C) or (R179L, R190V) or (R190V,
 15 A225V, T269V, K440C) or (T175M, H236D) or (T175M, R190V).

The variants according to the **sixth embodiment** were surprisingly found to show an improved product yield or titer or selectivity of the C19 hydroxylation, thereby further improving efficiency of the C19 hydroxylation process in comparison with the parent variant.

There is provided a **first preferred subset** comprising variants according to the **sixth**
 20 **embodiment**, wherein the P450-BM3 variants have an improved product yield or product titer for C19 hydroxylation in comparison with the parent variant. Comprised within this subset are variants comprising at least the mutation(s) (A184D) or (A184D, L188K) or (A184E) or (A184F) or (A184G) or (A184H) or (A184I) or (A184K) or (A184L) or (A184N) or (A184Q) or (A184R) or (A184W) or (A184W, L188F) or (A184Y) or (A184Y, L188K) or (A184Y, R50S) or (A221S) or
 25 (A264V) or (A321C) or (A321D) or (A321E) or (A321G) or (A321I) or (A321N) or (A321T) or (A321V) or (A330C) or (A330D) or (A330E) or (A330F) or (A330G) or (A330H) or (A330I) or (A330K) or (A330L) or (A330M) or (A330N) or (A330Q) or (A330R) or (A330S) or (A330T) or (A330T, E352A) or (A330V) or (A330W) or (A330Y) or (A330Y, W367C) or (A33V) or (A399C) or (A399E) or (A399G) or (A399I) or (A399L) or (A399M) or (A399N) or (A399Q) or (A399R) or
 30 (A399S) or (A399T) or (A399V) or (A74C) or (A74C, L75V) or (A74D) or (A74D, F81V) or (A74E) or (A74F) or (A74H) or (A74I) or (A74I, A184W, L188F) or (A74I, F77A, L181Y, A184W) or (A74I, L181Y, L188R) or (A74I, L188F) or (A74I, L188K) or (A74I, L188R) or (A74I, M177Y, A184R, L188F) or (A74I, M177Y, V178P, A184G) or (A74I, T146F) or (A74I, T146F, L181Y) or (A74I, T146F, L181Y, L188K) or (A74I, T146F, L181Y, L188R) or (A74I, T146F, L188F) or (A74I,
 35 T146F, L188K) or (A74I, T146F, V178P) or (A74I, T146F, V178P, L181Y, L188F) or (A74I, T146F, V178P, L181Y, L188K) or (A74I, T146F, V178P, L188K) or (A74I, T146F, V178P,

L188R) or (A74I, T146F, V178W) or (A74I, T146F, V178W, L181Y, L188F) or (A74I, T146F, V178W, L181Y, L188R) or (A74I, T146F, V178W, L188F) or (A74I, T146F, V178W, L188K) or (A74I, T146F, V178W, L188R) or (A74I, V178P) or (A74I, V178P, L181Y) or (A74I, V178P, L181Y, L188R) or (A74I, V178P, L188F) or (A74I, V178P, L188K) or (A74I, V178P, L188R) or
5 (A74I, V178W, L188F) or (A74I, V178W, L188R) or (A74K) or (A74L) or (A74M) or (A74N) or (A74P) or (A74Q) or (A74R) or (A74R, L75V) or (A74S) or (A74T) or (A74T, A184D) or (A74T, F77A, L181Y, A184D, L188K) or (A74T, F77A, V178W, A184Y) or (A74T, F77L, L181Y, A184D, L188K) or (A74T, L181Y) or (A74T, L181Y, A184D, L188K) or (A74T, L181Y, A184Y, L188K) or (A74T, L181Y, L188K) or (A74T, L181Y, L188R) or (A74T, L188K) or (A74T, M177Y, L181Y, A184D, L188K) or (A74T, T146F, L181Y, A184D, L188K) or (A74T, T146F, L181Y, L188K) or (A74T, T146F, L181Y, L188R) or (A74T, T146F, L188F) or (A74T, T146F, L188K) or (A74T, T146F, L188R) or (A74T, T146F, V178P) or (A74T, T146F, V178P, L181Y) or (A74T, T146F, V178P, L188K) or (A74T, T146F, V178P, L188R) or (A74T, T146F, V178W) or (A74T, T146F, V178W, L181Y) or (A74T, T146F, V178W, L181Y, L188K) or (A74T, T146F, V178W, L188R) or
10 (A74T, V178P) or (A74T, V178P, L188F) or (A74T, V178P, L188K) or (A74T, V178P, L188R) or (A74T, V178W, L181Y, L188K) or (A74T, V178W, L188K) or (A74T, V178W, L188R) or (A74V) or (A74V, F77A, A184N, L188F) or (A74V, L181Y) or (A74V, L181Y, A184D, L188K) or (A74V, L181Y, A184W, L188F) or (A74V, L181Y, L188F) or (A74V, L188F) or (A74V, L188K) or (A74V, L75V) or (A74V, T146F, L181Y, L188K) or (A74V, T146F, L181Y, L188R) or (A74V, T146F, L188K) or (A74V, T146F, L188R) or (A74V, T146F, V178W, L181Y, L188F) or (A74V, V178P, A184W, L188K) or (A74V, V178P, L181Y, L188F) or (A74V, V178P, L181Y, L188K) or (A74V, V178P, L181Y, L188R) or (A74V, V178P, L188F) or (A74V, V178W, L188K) or (A74W) or (A74Y) or (D363G, T438C) or (E13D, R47L) or (E143A) or (E143C) or (E143D) or (E143F) or (E143G) or (E143I) or (E143K) or (E143L) or (E143M) or (E143N) or (E143P) or (E143Q) or
15 (E143R) or (E143S) or (E143V) or (E143W) or (E143Y) or (E267D) or (E267R) or (E267T) or (E352A) or (E352D) or (E352F) or (E352G) or (E352I) or (E352L) or (E352M) or (E352N) or (E352P) or (E352R) or (E352S) or (E352T) or (E352V) or (E352W) or (E352Y) or (E64A) or (E64G) or (E64H) or (E64K) or (E64L) or (E64M) or (E64N) or (E64Q) or (E64V) or (E64Y) or (E82P) or (E93G) or (F173C) or (F173C, F205G) or (F173D) or (F173I) or (F173K) or (F173L) or (F173M) or (F173N) or (F173P) or (F173Q) or (F173R) or (F173S) or (F173S, F205P) or (F173V) or (F173W) or (F173Y) or (F173Y, I174F) or (F205A) or (F205C) or (F205D) or (F205D, D208N) or (F205E) or (F205G) or (F205H) or (F205I) or (F205K) or (F205L) or (F205M) or (F205N) or (F205P) or (F205R) or (F205S) or (F205T) or (F205V) or (F205W) or (F205Y) or (F261A) or (F261C) or (F261D) or (F261G) or (F261I) or (F261L) or (F261M) or (F261Q) or
20 (F261S) or (F261T) or (F261V) or (F261Y) or (F331H) or (F331I) or (F331L) or (F331M) or (F331V) or (F331W) or (F331Y) or (F393M) or (F393W) or (F77A) or (F77A, A184W) or (F77A, L181P, A184Y, L188K) or (F77A, M177Y) or (F77C) or (F77D) or (F77E) or (F77G) or (F77H) or

(F77I) or (F77K) or (F77L) or (F77M) or (F77N) or (F77P) or (F77R) or (F77S) or (F77T) or (F77V) or (F77V, L86M) or (F77W) or (F77Y) or (F81D) or (F81I) or (F81L) or (F81R) or (F81S) or (F81V) or (F81W) or (F81Y) or (G271A) or (G271C) or (G271D) or (G271E) or (G271F) or (G271H) or (G271K) or (G271L) or (G271M) or (G271N) or (G271P) or (G271Q) or (G271S) or (G271T) or (G271V) or (G271W) or (G271Y) or (G402A) or (G415A) or (G415D) or (G415S) or (G415T) or (G415V) or (G85A) or (G85L) or (G85S) or (H266A) or (H266C) or (H266E) or (H266F) or (H266I) or (H266K) or (H266M) or (H266N) or (H266Q) or (H266S) or (H266V) or (H266W) or (H266Y) or (I153F, G271L) or (I153L) or (I153L, F173Y) or (I259A) or (I259C) or (I259D) or (I259F) or (I259H) or (I259K) or (I259L) or (I259M) or (I259N) or (I259Q) or (I259S) or (I259T) or (I259V) or (I263A) or (I263C) or (I263E) or (I263F) or (I263H, A264G) or (I263K) or (I263L) or (I263M) or (I263N) or (I263Q) or (I263S) or (I263V) or (I263Y) or (I401A) or (I401M) or (I401T) or (I401V) or (K210E) or (K210T, G271V) or (K224C) or (K224E) or (K224H) or (K224I) or (K224L) or (K224M) or (K224P) or (K224Q) or (K224W) or (K224Y) or (L150A) or (L150C) or (L150D) or (L150E) or (L150F) or (L150G) or (L150H) or (L150I) or (L150K) or (L150M) or (L150N) or (L150Q) or (L150R) or (L150R, F205R) or (L150S) or (L150S, F173L) or (L150T) or (L150V) or (L150W) or (L150Y) or (L181H) or (L181I) or (L181M) or (L181P) or (L181V) or (L181Y) or (L181Y, A184D) or (L181Y, L188F) or (L181Y, L188K) or (L181Y, L188K, H659R) or (L188A) or (L188D) or (L188E) or (L188F) or (L188H) or (L188I) or (L188K) or (L188M) or (L188N) or (L188Q) or (L188R) or (L188S) or (L188W) or (L20C) or (L20D) or (L20E) or (L20F) or (L20G) or (L20G, R47L) or (L20I) or (L20M) or (L20N) or (L20P) or (L20R) or (L20S) or (L20T) or (L20V) or (L20W) or (L20Y) or (L262I) or (L262V) or (L262W) or (L272A) or (L272C) or (L272E) or (L272F) or (L272G) or (L272I) or (L272K) or (L272M) or (L272N) or (L272Q) or (L272S) or (L272T) or (L272V) or (L272W) or (L272Y) or (L29A) or (L29C) or (L29D) or (L29F) or (L29H) or (L29I) or (L29M) or (L29M, R47G) or (L29Q) or (L29S) or (L29T) or (L29V) or (L29W) or (L29Y) or (L324F) or (L356C) or (L356F) or (L356I) or (L356M) or (L356V) or (L437I) or (L437M) or (L75I) or (L75I, F81C) or (L75I, F81I) or (L75I, F81L) or (L75I, F81S) or (L75I, F81V) or (L75V) or (L75V, F81C) or (L75V, F81L) or (L75V, F81V) or (L75V, F81Y) or (L78F) or (L78I) or (L78M) or (L78V) or (L86I) or (L86I, S89T) or (L86M) or (L86M, S89T) or (L86V) or (M118A) or (M118E) or (M118F) or (M118G) or (M118H) or (M118I) or (M118K) or (M118L) or (M118N) or (M118P) or (M118Q) or (M118S) or (M118T) or (M118V) or (M118W) or (M118Y) or (M177A) or (M177C) or (M177C, V178Y) or (M177D) or (M177E) or (M177F) or (M177G) or (M177H) or (M177I) or (M177L) or (M177N) or (M177P) or (M177Q) or (M177R) or (M177T) or (M177V) or (M177Y) or (M177Y, A184W) or (M177Y, A184Y) or (M177Y, M185V) or (M177Y, R50S) or (M177Y, V178P) or (M177Y, V178P, A184Y, L188F) or (M177Y, V178W) or (M177Y, V178W, A184Y) or (M177Y, V178W, A184Y, L188F) or (M185C) or (M185D) or (M185E) or (M185G) or (M185H) or (M185K) or (M185L) or (M185N) or (M185Q) or (M185V) or (M212A) or (M212C) or (M212D) or (M212E) or (M212F) or (M212G) or (M212H) or (M212K) or (M212L) or (M212L,

I259F) or (M212Q) or (M212R) or (M212S) or (M212T) or (M212V) or (M212W) or (M212Y) or (M354A) or (M354C) or (M354D) or (M354E) or (M354G) or (M354I) or (M354K) or (M354K, D363Y) or (M354L) or (M354N) or (M354Q) or (M354R) or (M354S) or (M354T) or (M354V) or (M354W) or (M354Y) or (N70A) or (N70C) or (N70F) or (N70G) or (N70H) or (N70K) or (N70R) or (N70Y) or (P243T, H266L) or (P329A) or (P392C) or (P392G) or (P392Q) or (P392R) or (P392V) or (P9S) or (R147C, H266G) or (R161C, G271D) or (R190L, F261V) or (R203C) or (R255A) or (R255G) or (R255I) or (R255K) or (R255L) or (R255M) or (R255N) or (R255P) or (R255T) or (R255W) or (R47A) or (R47C) or (R47D) or (R47E) or (R47F) or (R47G) or (R47H) or (R47H, A74W) or (R47I) or (R47K) or (R47L) or (R47M) or (R47N) or (R47P) or (R47Q) or (R47S) or (R47T) or (R47V) or (R47W) or (R50A) or (R50C) or (R50D) or (R50E) or (R50F) or (R50G) or (R50I) or (R50L) or (R50M) or (R50N) or (R50P) or (R50Q) or (R50S) or (R50S, A184W, L188F) or (R50S, A74I, A184W, L188F) or (R50S, A74M, A184Y, L188K) or (R50S, A74T, L181Y, A184D, L188K, G240R) or (R50S, A74V) or (R50S, M177Y, V178P, A184Y) or (R50S, M177Y, V178W, A184W) or (R50S, M177Y, V178W, A184Y) or (R50S, M177Y, V178W, A184Y, A330R) or (R50S, M177Y, V178W, A184Y, A74V, G677D) or (R50S, M177Y, V178W, A184Y, L181P) or (R50S, M177Y, V178W, A184Y, L181Y) or (R50S, M177Y, V178W, A184Y, L188K) or (R50S, M177Y, V178W, A184Y, L188R) or (R50S, M177Y, V178W, A184Y, T146F) or (R50S, M177Y, V178W, A184Y, V26N) or (R50S, M177Y, V178W, L181Y, A184Y) or (R50S, S72G, A74V) or (R50S, S72G, A74V, M177Y, A184Y) or (R50S, S72G, A74V, M177Y, V178P, A184Y) or (R50S, S72G, A74V, M177Y, V178W, A184Y) or (R50S, S72G, A74V, M177Y, V178W, A184Y, L188R) or (R50S, T146F, M177Y, V178W, A184Y) or (R50V) or (R50Y) or (S176A, A184Y, L188K) or (S176C) or (S176D) or (S176E) or (S176F) or (S176G) or (S176H) or (S176K) or (S176L) or (S176M) or (S176N) or (S176P) or (S176Q) or (S72C, A74C) or (S72C, A74I) or (S72C, A74L) or (S72C, A74V) or (S72D, A74C) or (S72G) or (S72G, A74C) or (S72G, A74F) or (S72G, A74H) or (S72G, A74I) or (S72G, A74I, A184W, L188F) or (S72G, A74I, F77A, M177Y) or (S72G, A74I, F77L) or (S72G, A74I, F77L, M177Y) or (S72G, A74I, L181Y) or (S72G, A74I, L181Y, L188F) or (S72G, A74I, L181Y, L188K) or (S72G, A74I, L181Y, L188R) or (S72G, A74I, L188F) or (S72G, A74I, L188K) or (S72G, A74I, L188R) or (S72G, A74I, T146F) or (S72G, A74I, T146F, L181Y) or (S72G, A74I, T146F, L181Y, L188F) or (S72G, A74I, T146F, L181Y, L188R) or (S72G, A74I, T146F, L181Y, L188F) or (S72G, A74I, T146F, L181Y, L188R) or (S72G, A74I, T146F, V178P) or (S72G, A74I, T146F, V178P, L181Y) or (S72G, A74I, T146F, V178P, L181Y, L188F) or (S72G, A74I, T146F, V178P, L181Y, L188K) or (S72G, A74I, T146F, V178P, L181Y, L188R) or (S72G, A74I, T146F, V178P, L188F) or (S72G, A74I, T146F, V178P, L188K) or (S72G, A74I, T146F, V178P, L188R) or (S72G, A74I, T146F, V178W, L181Y, L188F) or (S72G, A74I, T146F, V178W, L181Y, L188K) or (S72G, A74I, T146F, V178W, L181Y, L188R) or (S72G, A74I, T146F, V178W, L188F) or (S72G, A74I, T146F, V178W, L188K) or

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(S72G, A74I, T146F, V178W, L188R) or (S72G, A74I, V178P) or (S72G, A74I, V178P, L181Y)
or (S72G, A74I, V178P, L181Y, L188R) or (S72G, A74I, V178P, L188F) or (S72G, A74I, V178P,
L188K) or (S72G, A74I, V178P, L188R) or (S72G, A74I, V178W) or (S72G, A74I, V178W,
L181Y, L188F) or (S72G, A74I, V178W, L181Y, L188K) or (S72G, A74I, V178W, L181Y, L188R)

5 or (S72G, A74I, V178W, L188F) or (S72G, A74I, V178W, L188R) or (S72G, A74L) or (S72G,
A74M, A184Y, L188K) or (S72G, A74S) or (S72G, A74T) or (S72G, A74T, L181Y, A184D,
L188K) or (S72G, A74T, L181Y, L188F) or (S72G, A74T, L181Y, L188K) or (S72G, A74T
L181Y, L188R) or (S72G, A74T, L188F) or (S72G, A74T, L188K) or (S72G, A74T, L188R) or
(S72G, A74T, T146F) or (S72G, A74T, T146F, L181Y) or (S72G, A74T, T146F, L181Y, L188K)

10 or (S72G, A74T, T146F, L181Y, L188R) or (S72G, A74T, T146F, L188F) or (S72G, A74T,
T146F, L188K) or (S72G, A74T, T146F, L188R) or (S72G, A74T, T146F, V178P) or (S72G,
A74T, T146F, V178P, L181Y) or (S72G, A74T, T146F, V178P, L188F) or (S72G, A74T, T146F,
V178P, L188K) or (S72G, A74T, T146F, V178P, L188R) or (S72G, A74T, T146F, V178W) or
(S72G, A74T, T146F, V178W, L181Y) or (S72G, A74T, T146F, V178W, L181Y, L188F) or

15 (S72G, A74T, T146F, V178W, L181Y, L188K) or (S72G, A74T, T146F, V178W, L181Y, L188R)
or (S72G, A74T, T146F, V178W, L188F) or (S72G, A74T, T146F, V178W, L188K) or (S72G,
A74T, T146F, V178W, L188R) or (S72G, A74T, V178P) or (S72G, A74T, V178P, L181Y) or
(S72G, A74T, V178P, L181Y, L188F) or (S72G, A74T, V178P, L181Y, L188K) or (S72G, A74T,
V178P, L188F) or (S72G, A74T, V178P, L188K) or (S72G, A74T, V178P, L188R) or (S72G,

20 A74T, V178W) or (S72G, A74T, V178W, L181Y, L188F) or (S72G, A74T, V178W, L181Y,
L188K) or (S72G, A74T, V178W, L188F) or (S72G, A74T, V178W, L188K) or (S72G, A74T,
V178W, L188R) or (S72G, A74V) or (S72G, A74V, A184W) or (S72G, A74V, A221V) or (S72G,
A74V, F77A) or (S72G, A74V, F77A, M177Y) or (S72G, A74V, F77L, M177Y) or (S72G, A74V,
F77S) or (S72G, A74V, L181Y) or (S72G, A74V, L181Y, L188F) or (S72G, A74V, L181Y, L188K)

25 or (S72G, A74V, L188F) or (S72G, A74V, L188K) or (S72G, A74V, L188R) or (S72G, A74V,
T146F) or (S72G, A74V, T146F, L181Y) or (S72G, A74V, T146F, L181Y, L188F) or (S72G,
A74V, T146F, L181Y, L188K) or (S72G, A74V, T146F, L181Y, L188R) or (S72G, A74V, T146F,
L188F) or (S72G, A74V, T146F, L188K) or (S72G, A74V, T146F, L188R) or (S72G, A74V,
T146F, V178P) or (S72G, A74V, T146F, V178P, L181Y) or (S72G, A74V, T146F, V178P,

30 L181Y, L188F) or (S72G, A74V, T146F, V178P, L181Y, L188K) or (S72G, A74V, T146F, V178P,
L181Y, L188R) or (S72G, A74V, T146F, V178P, L188F) or (S72G, A74V, T146F, V178P,
L188K) or (S72G, A74V, T146F, V178P, L188R) or (S72G, A74V, T146F, V178W) or (S72G,
A74V, T146F, V178W, L181Y) or (S72G, A74V, T146F, V178W, L181Y, L188K) or (S72G,
A74V, T146F, V178W, L181Y, L188R) or (S72G, A74V, T146F, V178W, L188F) or (S72G,

35 A74V, T146F, V178W, L188K) or (S72G, A74V, T146F, V178W, L188R) or (S72G, A74V,
V178P) or (S72G, A74V, V178P, L181Y) or (S72G, A74V, V178P, L181Y, L188F) or (S72G,
A74V, V178P, L181Y, L188K) or (S72G, A74V, V178P, L181Y, L188R) or (S72G, A74V, V178P,

L188F) or (S72G, A74V, V178P, L188K) or (S72G, A74V, V178P, L188R) or (S72G, A74V, V178W) or (S72G, A74V, V178W, L181Y) or (S72G, A74V, V178W, L181Y, L188F) or (S72G, A74V, V178W, L181Y, L188K) or (S72G, A74V, V178W, L181Y, L188R) or (S72G, A74V, V178W, L188F) or (S72G, A74V, V178W, L188K) or (S72G, A74V, V178W, L188R) or (S72G, A74Y) or (S72G, L181Y) or (S72G, L181Y, L188F) or (S72G, L181Y, L188K) or (S72G, L181Y, L188R) or (S72G, L188F) or (S72G, L188K) or (S72G, L188R) or (S72G, M177Y, V178W) or (S72G, M177Y, V178W, L188K) or (S72G, T146F) or (S72G, T146F, L181Y) or (S72G, T146F, L181Y, L188F) or (S72G, T146F, L181Y, L188K) or (S72G, T146F, L181Y, L188R) or (S72G, T146F, L188F) or (S72G, T146F, L188K) or (S72G, T146F, L188R) or (S72G, T146F, V178P) or (S72G, T146F, V178P, L181Y) or (S72G, T146F, V178P, L181Y, L188F) or (S72G, T146F, V178P, L181Y, L188R) or (S72G, T146F, V178P, L188F) or (S72G, T146F, V178P, L188K) or (S72G, T146F, V178P, L188R) or (S72G, T146F, V178W) or (S72G, T146F, V178W, L181Y) or (S72G, T146F, V178W, L181Y, L188F) or (S72G, T146F, V178W, L181Y, L188K) or (S72G, T146F, V178W, L188F) or (S72G, T146F, V178W, L188K) or (S72G, T146F, V178W, L188R) or (S72G, V178P) or (S72G, V178P, L181Y) or (S72G, V178P, L181Y, L188F) or (S72G, V178P, L181Y, L188K) or (S72G, V178P, L181Y, L188R) or (S72G, V178P, L188F) or (S72G, V178P, L188K) or (S72G, V178P, L188R) or (S72G, V178W) or (S72G, V178W, L181Y) or (S72G, V178W, L181Y, L188F) or (S72G, V178W, L181Y, L188K) or (S72G, V178W, L181Y, L188R) or (S72G, V178W, L188F) or (S72G, V178W, L188K) or (S72G, V178W, L188R) or (S72H, A74C) or (S72N, A74C) or (S72N, A74I) or (S72N, A74V) or (S72W) or (S72W, A74V) or (S89C) or (S89E) or (S89G) or (S89I) or (S89L) or (S89N) or (S89Q) or (S89R) or (S89T) or (S89V) or (T146A) or (T146A, D222Y, I263M) or (T146A, F173D) or (T146A, F173V) or (T146A, F205G) or (T146A, F261C) or (T146A, F261S) or (T146A, F261V) or (T146A, G271S) or (T146A, G271T) or (T146A, H266V) or (T146A, I258T, I259S) or (T146A, I259H) or (T146A, I263F) or (T146A, I263L) or (T146A, I263N) or (T146A, I263V) or (T146A, L150A) or (T146A, L150G) or (T146A, L150K) or (T146A, L150R, F173I) or (T146A, L272H) or (T146A, L272S) or (T146A, L272W) or (T146A, M212A) or (T146C) or (T146D) or (T146E) or (T146F) or (T146F, L181Y) or (T146F, L181Y, L188F) or (T146F, L181Y, L188K) or (T146F, L188K) or (T146F, L188R) or (T146F, V178P) or (T146F, V178P, L181Y, L188R) or (T146F, V178P, L188K) or (T146F, V178P, L188R) or (T146F, V178W) or (T146F, V178W, L181Y, L188R) or (T146F, V178W, L188F) or (T146F, V178W, L188K) or (T146F, V178W, L188R) or (T146G) or (T146H) or (T146L) or (T146M) or (T146N) or (T146P) or (T146R) or (T146S) or (T146V) or (T146V, A191T) or (T146Y) or (T149P) or (T260W) or (T268A) or (T268S) or (T269K) or (T327A) or (T327A, A330W) or (T327C) or (T327D) or (T327E) or (T327I) or (T327L) or (T327M) or (T327N) or (T327P) or (T327Q) or (T327S) or (T327V) or (T365C) or (T365D) or (T365F) or (T365G) or (T365H) or (T365I) or (T365K) or (T365L) or (T365N) or (T365P) or (T365Q) or (T365V) or (T365W) or (T365Y) or (T436A) or (T436C) or (T436D) or (T436E) or

(T436F) or (T436G) or (T436H) or (T436I) or (T436K) or (T436L) or (T436M) or (T436N) or (T436P) or (T436Q) or (T436R) or (T436S) or (T436V) or (T436W) or (T436Y) or (T438A) or (T438C) or (T438I) or (T438S) or (T438V) or (T49A) or (T49C) or (T49D) or (T49E) or (T49F) or (T49G) or (T49H) or (T49I) or (T49K) or (T49L) or (T49L, S54N) or (T49M) or (T49N) or (T49P) or (T49Q) or (T49R) or (T49R, R50C) or (T49R, R50S, M177Y, V178W, A184Y) or (T49S) or (T49V) or (T49W) or (T49Y) or (T88C) or (T88S) or (T88V) or (V178A) or (V178D) or (V178H) or (V178I) or (V178K) or (V178L) or (V178M) or (V178N) or (V178P) or (V178P, L181Y) or (V178P, L181Y, L188R) or (V178P, L188F) or (V178P, L188K) or (V178P, L188R) or (V178Q) or (V178R) or (V178S) or (V178T) or (V178W) or (V178W, A184Y) or (V178W, L181Y, L188K) or (V178W, L181Y, L188R) or (V178W, L188R) or (V178W, R50S) or (V178Y) or (V26A) or (V26C) or (V26F) or (V26G) or (V26H) or (V26I) or (V26K) or (V26L) or (V26M) or (V26N) or (V26N, R50S, M177Y, V178W, A184Y) or (V26Q) or (V26S) or (V26T) or (V26W) or (V26Y) or (V314A) or (V314E) or (V314F) or (V314H) or (V314P) or (V314Q) or (V314S) or (V314Y) or (V371P) or (V48A) or (V48D) or (V48E) or (V48F) or (V48G) or (V48H) or (V48I) or (V48L) or (V48M) or (V48P) or (V48Q) or (V48R) or (V48S) or (V48T) or (V48W) or (V48Y) or (W325A) or (W325C) or (W325D) or (W325E) or (W325F) or (W325G) or (W325I) or (W325K) or (W325L) or (W325M) or (W325Q) or (W325S) or (W325T) or (W325V) or (W325Y) or (W90Y) or (Y51A) or (Y51C) or (Y51F) or (Y51H) or (Y51I) or (Y51L) or (Y51M) or (Y51P) or (Y51S, F77V) or (Y51T) or (Y51V) or (Y51W) or (A225C) or (A225M) or (A225N) or (A225Q) or (A225S) or (A225T) or (A225Y) or (A44Q) or (A44T) or (D182A) or (D182C) or (D182E) or (D182H) or (D182I) or (D182M) or (D182N) or (D182V) or (D182W) or (D80C) or (D80L) or (D80M) or (D80N) or (D80S) or (D80T) or (D80W) or (E140A) or (E140D) or (E140K) or (E140L) or (E140M) or (E140T) or (E183D) or (E337L) or (E337V) or (G114C) or (G114K) or (G114L) or (G114N) or (G114W) or (G227N) or (G227R) or (G240A) or (G240C) or (G240H) or (G240K) or (G240N) or (G240R) or (G240S) or (H236A) or (H236C) or (H236D) or (H236F) or (H236I) or (H236K) or (H236M) or (H236P) or (H236R) or (H236S) or (H92F) or (H92Y) or (I174T) or (I174V) or (I258C) or (I258L) or (I258M) or (I258V) or (K187A) or (K187G) or (K187N) or (K187R) or (K440C) or (K440M) or (K440N) or (K440T) or (K76C) or (K76E) or (K76F) or (K76H) or (K76I) or (K76N) or (K76V) or (M5C) or (M5E) or (M5P) or (N186A) or (N186D) or (N186E) or (N186F) or (N186H) or (N186K) or (N186L) or (N186M) or (N186Q) or (N186T) or (N186V) or (N186Y) or (P172A) or (P172D) or (P172G) or (Q189E) or (Q189I) or (Q189K) or (Q189N) or (Q189V) or (Q397R) or (Q403K) or (Q403R) or (Q404F) or (Q404Y) or (Q73E) or (Q73F) or (Q73L) or (Q73S) or (Q73T) or (Q73W) or (Q73Y) or (R179A) or (R179H) or (R179L) or (R179M) or (R179P) or (R179V) or (R190C) or (R190G) or (R190K) or (R190M) or (R190N) or (R190S) or (R190V) or (R190W) or (R79K) or (S270A) or (S270D) or (S53C) or (T175F) or (T175G) or (T175H) or (T175L) or (T175M) or (T175N) or (T175Q) or (T175R) or (T175Y) or (T269V) or (V299L) or (V299W) or (A225V, H236K, G240V, T269V) or (D80I, D182H, A225V, G240V, T269V) or (F173W, R179L,

T269V, K440C) or (F173W, R190V, H236K, T269V) or (H236K, G240V, T269V, K440C) or (Q73W, D80I, N186V, G240V) or (R179L, A225S) or (T175M, H236D) or (T175M, R190V).

There is provided a **second preferred subset** comprising variants according to the **sixth embodiment**, wherein the P450-BM3 variants have an improved selectivity (as determined by

5 SF or TPR) for C19 hydroxylation in comparison with the parent variant (see figure 1b). Comprised within this subset are variants comprising at least the mutation(s) (A184D) or (A184D, L188K) or (A184E) or (A184F) or (A184G) or (A184H) or (A184I) or (A184K) or (A184L) or (A184N) or (A184Q) or (A184R) or (A184W) or (A184W, L188F) or (A184Y) or (A184Y, L188K) or (A184Y, R50S) or (A221S) or (A264V) or (A321C) or (A321D) or (A321E) or (A321G) or

10 (A321I) or (A321N) or (A321T) or (A321V) or (A328P) or (A330C) or (A330D) or (A330E) or (A330F) or (A330G) or (A330H) or (A330I) or (A330K) or (A330L) or (A330M) or (A330N) or (A330Q) or (A330R) or (A330S) or (A330T) or (A330T, E352A) or (A330T, F331V) or (A330V) or (A330W) or (A330Y) or (A330Y, W367C) or (A33V) or (A399C) or (A399E) or (A399G) or (A399I) or (A399L) or (A399M) or (A399N) or (A399Q) or (A399R) or (A399S) or (A399T) or

15 (A399V) or (A74C) or (A74C, L75V) or (A74D) or (A74D, F81V) or (A74E) or (A74F) or (A74G) or (A74G, L75C) or (A74H) or (A74I) or (A74I, A184W, L188F) or (A74I, F77A, L181Y, A184W) or (A74I, L181Y, L188K) or (A74I, L181Y, L188R) or (A74I, L188F) or (A74I, L188K) or (A74I, L188R) or (A74I, M177Y, A184R, L188F) or (A74I, M177Y, V178P, A184G) or (A74I, T146F) or (A74I, T146F, L181Y) or (A74I, T146F, L181Y, L188K) or (A74I, T146F, L181Y, L188R) or (A74I,

20 T146F, L188F) or (A74I, T146F, L188K) or (A74I, T146F, V178P) or (A74I, T146F, V178P, L181Y, L188F) or (A74I, T146F, V178P, L181Y, L188K) or (A74I, T146F, V178P, L188K) or (A74I, T146F, V178P, L188R) or (A74I, T146F, V178W) or (A74I, T146F, V178W, L181Y, L188F) or (A74I, T146F, V178W, L181Y, L188R) or (A74I, T146F, V178W, L188F) or (A74I, T146F, V178W, L188K) or (A74I, T146F, V178W, L188R) or (A74I, V178P) or (A74I, V178P,

25 L181Y) or (A74I, V178P, L181Y, L188R) or (A74I, V178P, L188F) or (A74I, V178P, L188K) or (A74I, V178P, L188R) or (A74I, V178W, L188F) or (A74I, V178W, L188K) or (A74I, V178W, L188R) or (A74K) or (A74L) or (A74M) or (A74N) or (A74P) or (A74Q) or (A74R) or (A74R, L75V) or (A74S) or (A74T) or (A74T, A184D) or (A74T, F77A, L181Y, A184D, L188K) or (A74T, F77A, V178W, A184Y) or (A74T, F77L, L181Y, A184D, L188K) or (A74T, L181Y) or (A74T, L181Y,

30 A184D, L188K) or (A74T, L181Y, A184Y, L188K) or (A74T, L181Y, L188K) or (A74T, L181Y, L188R) or (A74T, L188K) or (A74T, M177Y, L181Y, A184D, L188K) or (A74T, T146F, L181Y, A184D, L188K) or (A74T, T146F, L181Y, L188K) or (A74T, T146F, L181Y, L188R) or (A74T, T146F, L188F) or (A74T, T146F, L188K) or (A74T, T146F, L188R) or (A74T, T146F, V178P) or (A74T, T146F, V178P, L181Y) or (A74T, T146F, V178P, L188K) or (A74T, T146F, V178P,

35 L188R) or (A74T, T146F, V178W) or (A74T, T146F, V178W, L181Y) or (A74T, T146F, V178W, L181Y, L188K) or (A74T, T146F, V178W, L188R) or (A74T, V178P) or (A74T, V178P, L188F) or (A74T, V178P, L188K) or (A74T, V178P, L188R) or (A74T, V178W, L181Y, L188K) or (A74T,

V178W, L188K) or (A74T, V178W, L188R) or (A74V) or (A74V, F77A, A184N, L188F) or (A74V, L181Y) or (A74V, L181Y, A184D, L188K) or (A74V, L181Y, A184W, L188F) or (A74V, L181Y, L188F) or (A74V, L188F) or (A74V, L188K) or (A74V, L75V) or (A74V, T146F, L181Y, L188K) or (A74V, T146F, L181Y, L188R) or (A74V, T146F, L188K) or (A74V, T146F, L188R) or (A74V, T146F, V178W) or (A74V, T146F, V178W, L181Y, L188F) or (A74V, V178P, A184W, L188K) or (A74V, V178P, L181Y, L188F) or (A74V, V178P, L181Y, L188K) or (A74V, V178P, L181Y, L188R) or (A74V, V178P, L188F) or (A74V, V178W, L188K) or (A74W) or (A74Y) or (D168Y, F173D) or (D363G, T438C) or (E13D, R47L) or (E143A) or (E143C) or (E143D) or (E143F) or (E143G) or (E143I) or (E143K) or (E143L) or (E143M) or (E143N) or (E143P) or (E143Q) or (E143R) or (E143S) or (E143V) or (E143W) or (E143Y) or (E267A) or (E267C) or (E267D) or (E267G) or (E267K) or (E267P) or (E267R) or (E267S) or (E267T) or (E267Y) or (E352A) or (E352D) or (E352F) or (E352G) or (E352I) or (E352L) or (E352M) or (E352N) or (E352P) or (E352R) or (E352S) or (E352T) or (E352V) or (E352W) or (E352Y) or (E64A) or (E64G) or (E64H) or (E64I) or (E64K) or (E64L) or (E64M) or (E64N) or (E64Q) or (E64V) or (E64W) or (E64Y) or (E82P) or (E93G) or (F173C) or (F173C, F205G) or (F173D) or (F173I) or (F173K) or (F173L) or (F173M) or (F173N) or (F173P) or (F173Q) or (F173R) or (F173S) or (F173S, F205P) or (F173V) or (F173W) or (F173Y) or (F173Y, I174F) or (F205A) or (F205C) or (F205D) or (F205D, D208N) or (F205E) or (F205G) or (F205H) or (F205I) or (F205K) or (F205L) or (F205M) or (F205N) or (F205P) or (F205R) or (F205S) or (F205T) or (F205V) or (F205W) or (F205Y) or (F261A) or (F261C) or (F261D) or (F261G) or (F261I) or (F261L) or (F261M) or (F261N) or (F261Q) or (F261S) or (F261T) or (F261V) or (F261W) or (F261Y) or (F331C) or (F331H) or (F331I) or (F331L) or (F331M) or (F331N) or (F331P) or (F331T) or (F331V) or (F331W) or (F331Y) or (F393M) or (F393W) or (F77A) or (F77A, A184W) or (F77A, L181P, A184Y, L188K) or (F77A, M177Y) or (F77C) or (F77D) or (F77E) or (F77G) or (F77H) or (F77I) or (F77K) or (F77L) or (F77M) or (F77N) or (F77P) or (F77R) or (F77S) or (F77T) or (F77V) or (F77V, L86M) or (F77W) or (F77Y) or (F81A) or (F81C) or (F81D) or (F81I) or (F81L) or (F81P) or (F81R) or (F81S) or (F81T) or (F81V) or (F81W) or (F81Y) or (G271A) or (G271C) or (G271D) or (G271E) or (G271F) or (G271H) or (G271K) or (G271L) or (G271M) or (G271N) or (G271P) or (G271Q) or (G271R) or (G271S) or (G271T) or (G271V) or (G271W) or (G271Y) or (G402A) or (G415A) or (G415D) or (G415S) or (G415T) or (G415V) or (G85A) or (G85L) or (G85S) or (H266A) or (H266C) or (H266D) or (H266E) or (H266F) or (H266G) or (H266I) or (H266K) or (H266M) or (H266N) or (H266P) or (H266Q) or (H266R) or (H266S) or (H266T) or (H266V) or (H266W) or (H266Y) or (I153F, G271L) or (I153L) or (I153L, F173Y) or (I259A) or (I259C) or (I259D) or (I259F) or (I259G) or (I259H) or (I259K) or (I259L) or (I259M) or (I259N) or (I259Q) or (I259S) or (I259T) or (I259V) or (I259W) or (I259Y) or (I263A) or (I263C) or (I263E) or (I263F) or (I263G) or (I263H, A264G) or (I263K) or (I263L) or (I263M) or (I263N) or (I263Q) or (I263S) or (I263T) or (I263V) or (I263Y) or (I401A) or (I401L) or (I401M) or (I401T) or (I401V) or (K210E) or (K210T,

G271V) or (K224C) or (K224E) or (K224F) or (K224H) or (K224I) or (K224L) or (K224M) or (K224P) or (K224Q) or (K224W) or (K224Y) or (L150A) or (L150C) or (L150D) or (L150E) or (L150F) or (L150G) or (L150H) or (L150I) or (L150K) or (L150M) or (L150N) or (L150Q) or (L150R) or (L150R, F205R) or (L150S) or (L150S, F173L) or (L150T) or (L150V) or (L150W) or (L150Y) or (L181H) or (L181I) or (L181M) or (L181P) or (L181V) or (L181Y) or (L181Y, A184D) or (L181Y, L188F) or (L181Y, L188K) or (L181Y, L188K, H659R) or (L188A) or (L188D) or (L188E) or (L188F) or (L188H) or (L188I) or (L188K) or (L188M) or (L188N) or (L188Q) or (L188R) or (L188S) or (L188W) or (L20C) or (L20D) or (L20E) or (L20F) or (L20G) or (L20G, R47L) or (L20I) or (L20M) or (L20N) or (L20P) or (L20R) or (L20S) or (L20T) or (L20V) or (L20W) or (L20Y) or (L262I) or (L262V) or (L262W) or (L262Y) or (L272A) or (L272C) or (L272E) or (L272F) or (L272G) or (L272I) or (L272K) or (L272M) or (L272N) or (L272Q) or (L272R) or (L272S) or (L272T) or (L272V) or (L272W) or (L272Y) or (L29A) or (L29C) or (L29D) or (L29F) or (L29H) or (L29I) or (L29M) or (L29M, R47G) or (L29P) or (L29Q) or (L29S) or (L29T) or (L29V) or (L29W) or (L29Y) or (L324F) or (L356C) or (L356F) or (L356H) or (L356I) or (L356M) or (L356N) or (L356Q) or (L356S) or (L356T) or (L356V) or (L356W) or (L437I) or (L437M) or (L75H, F81S) or (L75I) or (L75I, F81C) or (L75I, F81G) or (L75I, F81H) or (L75I, F81I) or (L75I, F81L) or (L75I, F81S) or (L75I, F81V) or (L75I, F81Y) or (L75V) or (L75V, F81C) or (L75V, F81H) or (L75V, F81I) or (L75V, F81L) or (L75V, F81V) or (L75V, F81Y) or (L78F) or (L78I) or (L78M) or (L78V) or (L78Y) or (L86A, S89T) or (L86I) or (L86I, S89T) or (L86M) or (L86M, S89T) or (L86N, S89T) or (L86V) or (L86V, S89T) or (M118A) or (M118E) or (M118F) or (M118G) or (M118H) or (M118I) or (M118K) or (M118L) or (M118N) or (M118P) or (M118Q) or (M118S) or (M118T) or (M118V) or (M118W) or (M118Y) or (M177A) or (M177C) or (M177C, V178Y) or (M177D) or (M177E) or (M177F) or (M177H) or (M177I) or (M177K) or (M177L) or (M177N) or (M177P) or (M177Q) or (M177R) or (M177T) or (M177V) or (M177Y) or (M177Y, A184W) or (M177Y, A184Y) or (M177Y, M185V) or (M177Y, R50S) or (M177Y, V178P) or (M177Y, V178P, A184Y, L188F) or (M177Y, V178W) or (M177Y, V178W, A184Y) or (M177Y, V178W, A184Y, L188F) or (M185C) or (M185D) or (M185E) or (M185G) or (M185H) or (M185K) or (M185L) or (M185N) or (M185Q) or (M185R) or (M185V) or (M185Y) or (M212A) or (M212C) or (M212C, I259L) or (M212D) or (M212E) or (M212F) or (M212G) or (M212H) or (M212K) or (M212L) or (M212L, I259F) or (M212P) or (M212Q) or (M212R) or (M212S) or (M212T) or (M212V) or (M212W) or (M212Y) or (M354A) or (M354C) or (M354D) or (M354E) or (M354G) or (M354I) or (M354K) or (M354K, D363Y) or (M354L) or (M354N) or (M354Q) or (M354R) or (M354S) or (M354T) or (M354V) or (M354W) or (M354Y) or (N70A) or (N70C) or (N70F) or (N70G) or (N70H) or (N70K) or (N70R) or (N70W) or (N70Y) or (P243T, H266L) or (P326C) or (P326D) or (P326G) or (P326N) or (P326S) or (P326T) or (P329A) or (P329C) or (P329G) or (P329K) or (P329S) or (P329T) or (P392C) or (P392G) or (P392Q) or (P392R) or (P392V) or (P9S) or (R147C, H266G) or (R161C, G271D) or (R190L, F261V) or (R203C) or (R255A) or (R255F) or (R255G) or (R255I)

or (R255K) or (R255L) or (R255M) or (R255N) or (R255P) or (R255T) or (R255W) or (R323C)
 or (R47A) or (R47C) or (R47D) or (R47E) or (R47F) or (R47G) or (R47H) or (R47H, A74W) or
 (R47I) or (R47K) or (R47L) or (R47M) or (R47N) or (R47P) or (R47Q) or (R47S) or (R47T) or
 (R47V) or (R47W) or (R50A) or (R50C) or (R50D) or (R50E) or (R50F) or (R50G) or (R50I) or
 5 (R50L) or (R50M) or (R50N) or (R50P) or (R50Q) or (R50S) or (R50S, A184W, L188F) or (R50S,
 A74I, A184W, L188F) or (R50S, A74M, A184Y, L188K) or (R50S, A74T, L181Y, A184D, L188K,
 G240R) or (R50S, A74V) or (R50S, M177Y, V178P, A184Y) or (R50S, M177Y, V178W, A184W)
 or (R50S, M177Y, V178W, A184Y) or (R50S, M177Y, V178W, A184Y, A330R) or (R50S,
 M177Y, V178W, A184Y, A74V, G677D) or (R50S, M177Y, V178W, A184Y, H266S) or (R50S,
 10 M177Y, V178W, A184Y, L181P) or (R50S, M177Y, V178W, A184Y, L181Y) or (R50S, M177Y,
 V178W, A184Y, L188K) or (R50S, M177Y, V178W, A184Y, L188R) or (R50S, M177Y, V178W,
 A184Y, T146F) or (R50S, M177Y, V178W, A184Y, V26N) or (R50S, M177Y, V178W, L181Y,
 A184Y) or (R50S, S72G, A74V) or (R50S, S72G, A74V, M177Y, A184Y) or (R50S, S72G, A74V,
 M177Y, V178P, A184Y) or (R50S, S72G, A74V, M177Y, V178W, A184Y) or (R50S, S72G,
 15 A74V, M177Y, V178W, A184Y, L188R) or (R50S, T146F, M177Y, V178W, A184Y) or (R50V) or
 (R50Y) or (S164N, F261L) or (S176A, A184Y, L188K) or (S176C) or (S176D) or (S176E) or
 (S176F) or (S176G) or (S176H) or (S176K) or (S176L) or (S176M) or (S176N) or (S176P) or
 (S176Q) or (S176R) or (S72C, A74C) or (S72C, A74I) or (S72C, A74L) or (S72C, A74V) or
 (S72C, A74Y) or (S72D, A74C) or (S72D, A74F) or (S72G) or (S72G, A74C) or (S72G, A74F)
 20 or (S72G, A74H) or (S72G, A74I) or (S72G, A74I, A184W, L188F) or (S72G, A74I, F77A,
 M177Y) or (S72G, A74I, F77L) or (S72G, A74I, F77L, M177Y) or (S72G, A74I, L181Y) or (S72G,
 A74I, L181Y, L188F) or (S72G, A74I, L181Y, L188K) or (S72G, A74I, L181Y, L188R) or (S72G,
 A74I, L188F) or (S72G, A74I, L188K) or (S72G, A74I, L188R) or (S72G, A74I, T146F) or (S72G,
 A74I, T146F, L181Y) or (S72G, A74I, T146F, L181Y, L188F) or (S72G, A74I, T146F, L181Y,
 25 L188K) or (S72G, A74I, T146F, L181Y, L188R) or (S72G, A74I, T146F, L188F) or (S72G, A74I,
 T146F, L188K) or (S72G, A74I, T146F, L188R) or (S72G, A74I, T146F, V178P) or (S72G, A74I,
 T146F, V178P, L181Y) or (S72G, A74I, T146F, V178P, L181Y, L188F) or (S72G, A74I, T146F,
 V178P, L181Y, L188K) or (S72G, A74I, T146F, V178P, L181Y, L188R) or (S72G, A74I, T146F,
 V178P, L188F) or (S72G, A74I, T146F, V178P, L188K) or (S72G, A74I, T146F, V178P, L188R)
 30 or (S72G, A74I, T146F, V178W) or (S72G, A74I, T146F, V178W, L181Y) or (S72G, A74I, T146F,
 V178W, L181Y, L188F) or (S72G, A74I, T146F, V178W, L181Y, L188K) or (S72G, A74I, T146F,
 V178W, L181Y, L188R) or (S72G, A74I, T146F, V178W, L188F) or (S72G, A74I, T146F,
 V178W, L188K) or (S72G, A74I, T146F, V178W, L188R) or (S72G, A74I, V178P) or (S72G,
 A74I, V178P, L181Y) or (S72G, A74I, V178P, L181Y, L188K) or (S72G, A74I, V178P, L181Y,
 35 L188R) or (S72G, A74I, V178P, L188F) or (S72G, A74I, V178P, L188K) or (S72G, A74I, V178P,
 L188R) or (S72G, A74I, V178W) or (S72G, A74I, V178W, L181Y, L188F) or (S72G, A74I,
 V178W, L181Y, L188R) or (S72G, A74I, V178W, L188F) or (S72G, A74I, V178W, L188K) or

(S72G, A74I, V178W, L188R) or (S72G, A74L) or (S72G, A74M, A184Y, L188K) or (S72G, A74S) or (S72G, A74T) or (S72G, A74T, L181Y, A184D, L188K) or (S72G, A74T, L181Y, L188F) or (S72G, A74T, L181Y, L188K) or (S72G, A74T, L181Y, L188R) or (S72G, A74T, L188F) or (S72G, A74T, L188K) or (S72G, A74T, L188R) or (S72G, A74T, T146F) or (S72G, A74T, T146F, L181Y) or (S72G, A74T, T146F, L181Y, L188K) or (S72G, A74T, T146F, L181Y, L188R) or (S72G, A74T, T146F, L188F) or (S72G, A74T, T146F, L188K) or (S72G, A74T, T146F, L188R) or (S72G, A74T, T146F, V178P) or (S72G, A74T, T146F, V178P, L181Y) or (S72G, A74T, T146F, V178P, L188F) or (S72G, A74T, T146F, V178P, L188K) or (S72G, A74T, T146F, V178P, L188R) or (S72G, A74T, T146F, V178W) or (S72G, A74T, T146F, V178W, L181Y) or (S72G, A74T, T146F, V178W, L181Y, L188F) or (S72G, A74T, T146F, V178W, L181Y, L188K) or (S72G, A74T, T146F, V178W, L181Y, L188R) or (S72G, A74T, T146F, V178W, L188F) or (S72G, A74T, T146F, V178W, L188K) or (S72G, A74T, T146F, V178W, L188R) or (S72G, A74T, V178P) or (S72G, A74T, V178P, L181Y) or (S72G, A74T, V178P, L181Y, L188F) or (S72G, A74T, V178P, L181Y, L188K) or (S72G, A74T, V178P, L188F) or (S72G, A74T, V178P, L188K) or (S72G, A74T, V178P, L188R) or (S72G, A74T, V178W) or (S72G, A74T, V178W, L181Y, L188F) or (S72G, A74T, V178W, L181Y, L188K) or (S72G, A74T, V178W, L188F) or (S72G, A74V) or (S72G, A74V, A184W) or (S72G, A74V, A221V) or (S72G, A74V, F77A) or (S72G, A74V, F77A, M177Y) or (S72G, A74V, F77L, M177Y) or (S72G, A74V, F77S) or (S72G, A74V, L181Y) or (S72G, A74V, L181Y, L188F) or (S72G, A74V, L181Y, L188K) or (S72G, A74V, L188F) or (S72G, A74V, L188K) or (S72G, A74V, L188R) or (S72G, A74V, T146F) or (S72G, A74V, T146F, L181Y) or (S72G, A74V, T146F, L181Y, L188F) or (S72G, A74V, T146F, L181Y, L188K) or (S72G, A74V, T146F, L181Y, L188R) or (S72G, A74V, T146F, L188F) or (S72G, A74V, T146F, L188K) or (S72G, A74V, T146F, L188R) or (S72G, A74V, T146F, V178P) or (S72G, A74V, T146F, V178P, L181Y) or (S72G, A74V, T146F, V178P, L181Y, L188F) or (S72G, A74V, T146F, V178P, L181Y, L188K) or (S72G, A74V, T146F, V178P, L181Y, L188R) or (S72G, A74V, T146F, V178P, L188F) or (S72G, A74V, T146F, V178P, L188K) or (S72G, A74V, T146F, V178P, L188R) or (S72G, A74V, T146F, V178W) or (S72G, A74V, T146F, V178W, L181Y) or (S72G, A74V, T146F, V178W, L181Y, L188K) or (S72G, A74V, T146F, V178W, L181Y, L188R) or (S72G, A74V, T146F, V178W, L188F) or (S72G, A74V, T146F, V178W, L188K) or (S72G, A74V, T146F, V178W, L188R) or (S72G, A74V, V178P) or (S72G, A74V, V178P, L181Y) or (S72G, A74V, V178P, L181Y, L188F) or (S72G, A74V, V178P, L181Y, L188K) or (S72G, A74V, V178P, L181Y, L188R) or (S72G, A74V, V178P, L188F) or (S72G, A74V, V178P, L188K) or (S72G, A74V, V178P, L188R) or (S72G, A74V, V178W) or (S72G, A74V, V178W, L181Y) or (S72G, A74V, V178W, L181Y, L188F) or (S72G, A74V, V178W, L181Y, L188K) or (S72G, A74V, V178W, L181Y, L188R) or (S72G, A74V, V178W, L188F) or (S72G, A74V, V178W, L188K) or (S72G, A74V, V178W, L188R) or (S72G, A74Y) or (S72G, L181Y) or (S72G, L181Y, L188F) or

(S72G, L181Y, L188K) or (S72G, L181Y, L188R) or (S72G, L188F) or (S72G, L188K) or (S72G, L188R) or (S72G, M177Y, V178W) or (S72G, M177Y, V178W, L188K) or (S72G, T146F) or (S72G, T146F, L181Y) or (S72G, T146F, L181Y, L188F) or (S72G, T146F, L181Y, L188K) or (S72G, T146F, L181Y, L188R) or (S72G, T146F, L188F) or (S72G, T146F, L188K) or (S72G, T146F, L188R) or (S72G, T146F, V178P) or (S72G, T146F, V178P, L181Y) or (S72G, T146F, V178P, L181Y, L188F) or (S72G, T146F, V178P, L181Y, L188K) or (S72G, T146F, V178P, L181Y, L188R) or (S72G, T146F, V178P, L188F) or (S72G, T146F, V178P, L188K) or (S72G, T146F, V178P, L188R) or (S72G, T146F, V178W) or (S72G, T146F, V178W, L181Y) or (S72G, T146F, V178W, L181Y, L188F) or (S72G, T146F, V178W, L181Y, L188K) or (S72G, T146F, V178W, L181Y, L188R) or (S72G, T146F, V178W, L188F) or (S72G, T146F, V178W, L188K) or (S72H, A74C) or (S72H, A74G) or (S72H, A74S) or (S72H, A74Y) or (S72N, A74C) or (S72N, A74I) or (S72N, A74N) or (S72N, A74V) or (S72T) or (S72W) or (S72W, A74I, A184W, L188F) or (S72W, A74V) or (S72W, L333R) or (S72W, W90Y, V299G) or (S72Y, A74V) or (S89C) or (S89E) or (S89G) or (S89I) or (S89L) or (S89M) or (S89N) or (S89Q) or (S89R) or (S89T) or (S89V) or (T146A) or (T146A, D222Y, I263M) or (T146A, F173D) or (T146A, F173V) or (T146A, F205G) or (T146A, F261C) or (T146A, F261S) or (T146A, F261V) or (T146A, G271R) or (T146A, G271S) or (T146A, G271T) or (T146A, H266T) or (T146A, H266V) or (T146A, I258T, I259S) or (T146A, I259H) or (T146A, I263F) or (T146A, I263L) or (T146A, I263N) or (T146A, I263V) or (T146A, L150A) or (T146A, L150G) or (T146A, L150K) or (T146A, L150R, F173I) or (T146A, L272H) or (T146A, L272R) or (T146A, L272S) or (T146A, L272W) or (T146A, M212A) or (T146C) or (T146D) or (T146E) or (T146F) or (T146F, L181Y) or (T146F, L181Y, L188F) or (T146F, L181Y, L188K) or (T146F, L188K) or (T146F, L188R) or (T146F, V178P) or (T146F, V178P, L181Y, L188R) or (T146F, V178P, L188K) or (T146F, V178P, L188R) or (T146F, V178W) or (T146F, V178W, L181Y, L188R) or (T146F, V178W, L188F) or (T146F, V178W, L188K) or (T146F, V178W, L188R) or (T146G) or (T146H) or (T146L) or (T146M) or (T146N) or (T146P) or (T146R) or (T146S) or (T146V) or (T146V, A191T) or (T146Y) or (T149P) or (T260A) or (T260S) or (T268A) or (T268S) or (T269K) or (T327A) or (T327A, A330W) or (T327C) or (T327D) or (T327E) or (T327G) or (T327I) or (T327L) or (T327M) or (T327N) or (T327P) or (T327Q) or (T327S) or (T327V) or (T365A) or (T365C) or (T365D) or (T365F) or (T365G) or (T365H) or (T365I) or (T365K) or (T365L) or (T365N) or (T365P) or (T365Q) or (T365V) or (T365W) or (T365Y) or (T436A) or (T436C) or (T436D) or (T436E) or (T436F) or (T436G) or (T436H) or (T436I) or (T436K) or (T436L) or (T436M) or (T436N) or (T436P) or (T436Q) or (T436R) or (T436S) or

(T436V) or (T436W) or (T436Y) or (T438A) or (T438C) or (T438G) or (T438I) or (T438L) or (T438S) or (T438V) or (T49A) or (T49C) or (T49D) or (T49E) or (T49F) or (T49G) or (T49H) or (T49I) or (T49K) or (T49L) or (T49L, S54N) or (T49M) or (T49N) or (T49P) or (T49Q) or (T49R) or (T49R, R50C) or (T49R, R50S, M177Y, V178W, A184Y) or (T49S) or (T49V) or (T49W) or (T49Y) or (T88A) or (T88C) or (T88S) or (T88V) or (V178D) or (V178F) or (V178H) or (V178I) or (V178K) or (V178L) or (V178M) or (V178N) or (V178P) or (V178P, L181Y) or (V178P, L181Y, L188R) or (V178P, L188F) or (V178P, L188K) or (V178P, L188R) or (V178Q) or (V178R) or (V178S) or (V178T) or (V178W) or (V178W, A184Y) or (V178W, L181Y, L188K) or (V178W, L181Y, L188R) or (V178W, L188R) or (V178W, R50S) or (V178Y) or (V26A) or (V26C) or (V26E) or (V26F) or (V26G) or (V26G, R47S) or (V26H) or (V26I) or (V26K) or (V26L) or (V26M) or (V26N) or (V26N, R50S, M177Y, V178W, A184Y) or (V26Q) or (V26R) or (V26S) or (V26T) or (V26W) or (V26Y) or (V314A) or (V314E) or (V314F) or (V314H) or (V314P) or (V314Q) or (V314S) or (V314Y) or (V48A) or (V48D) or (V48E) or (V48F) or (V48G) or (V48H) or (V48I) or (V48L) or (V48M) or (V48P) or (V48Q) or (V48R) or (V48S) or (V48T) or (V48W) or (V48Y) or (W325A) or (W325C) or (W325D) or (W325E) or (W325F) or (W325G) or (W325I) or (W325K) or (W325L) or (W325M) or (W325Q) or (W325R) or (W325S) or (W325T) or (W325V) or (W325Y) or (W90D) or (W90Y) or (W90Y, V299G, G570D) or (Y51A) or (Y51C) or (Y51E) or (Y51F) or (Y51G) or (Y51H) or (Y51I) or (Y51L) or (Y51M) or (Y51N) or (Y51P) or (Y51Q) or (Y51S) or (Y51S, F77V) or (Y51T) or (Y51V) or (Y51W) or (A225C) or (A225G) or (A225N) or (A225Q) or (A225S) or (A225T) or (A225V) or (A225Y) or (A44G) or (A44Q) or (D182A) or (D182C) or (D182E) or (D182H) or (D182I) or (D182M) or (D182N) or (D182V) or (D182W) or (E140A) or (E140D) or (E140K) or (E140M) or (E140T) or (E183T) or (E337L) or (E409N) or (G114C) or (G114K) or (G114L) or (G114N) or (G114W) or (G240A) or (G240C) or (G240H) or (G240K) or (G240M) or (G240N) or (G240Q) or (G240R) or (G240S) or (G240V) or (G46T) or (H236A) or (H236C) or (H236D) or (H236F) or (H236G) or (H236I) or (H236K) or (H236P) or (H236R) or (H236S) or (H236Y) or (H92F) or (H92Y) or (I174T) or (I174V) or (I258L) or (I258M) or (I258V) or (K187A) or (K187G) or (K187N) or (K187R) or (K440C) or (K440N) or (K440T) or (K76C) or (K76E) or (K76F) or (K76H) or (K76I) or (K76L) or (K76N) or (K76V) or (M5C) or (M5E) or (M5P) or (M5Q) or (N186A) or (N186D) or (N186E) or (N186F) or (N186H) or (N186K) or (N186L) or (N186M) or (N186Q) or (N186T) or (N186V) or (N186Y) or (P172A) or (P172D) or (P172G) or (Q189I) or (Q189K) or (Q189V) or (Q403K) or (Q403R) or (Q404F) or (Q404Y) or (Q73A) or (Q73E) or (Q73F) or (Q73L) or (Q73S) or (Q73T) or (Q73W) or (R179A) or (R179H) or (R179L) or (R179M) or (R179P) or (R179V) or (R190C) or (R190G) or (R190K) or (R190M) or (R190N) or (R190S) or (R190V) or (R190W) or (R79E) or (R79K) or (S270A) or (S270D) or (S270G) or (S53C) or (T175F) or (T175G) or (T175H) or (T175L) or (T175M) or (T175N) or (T175Q) or (T175R) or (T175Y) or (T269A) or (T269V) or (V299L) or (V299W) or (F173W, R179L, R190V, A225V) or (F173W, R179L, R190V, K440C) or (F173W, R179L, T269V, K440C) or (H236K,

G240V, T269V, K440C) or (K76I, F173W, R179L, D182I, R190V, K440C) or (R179L, A225V, G240V, K440C) or (R179L, R190V) or (R190V, A225V, T269V, K440C).

There is provided a **third preferred subset** comprising variants according to the **sixth embodiment**, wherein the P450-BM3 variants have an improved product yield or product titer and an improved selectivity (as determined by SF or TPR) for C19 hydroxylation in comparison with the parent variant. Comprised within this subset are variants comprising at least the mutation(s) (A184D) or (A184D, L188K) or (A184E) or (A184F) or (A184G) or (A184H) or (A184I) or (A184K) or (A184L) or (A184N) or (A184Q) or (A184R) or (A184W) or (A184W, L188F) or (A184Y) or (A184Y, L188K) or (A184Y, R50S) or (A221S) or (A264V) or (A321C) or (A321D) or (A321E) or (A321G) or (A321I) or (A321N) or (A321T) or (A321V) or (A330C) or (A330D) or (A330E) or (A330F) or (A330G) or (A330H) or (A330I) or (A330K) or (A330L) or (A330M) or (A330N) or (A330Q) or (A330R) or (A330S) or (A330T) or (A330T, E352A) or (A330V) or (A330W) or (A330Y) or (A330Y, W367C) or (A33V) or (A399C) or (A399E) or (A399G) or (A399I) or (A399L) or (A399M) or (A399N) or (A399Q) or (A399R) or (A399S) or (A399T) or (A399V) or (A74C) or (A74C, L75V) or (A74D) or (A74D, F81V) or (A74E) or (A74F) or (A74H) or (A74I) or (A74I, A184W, L188F) or (A74I, F77A, L181Y, A184W) or (A74I, L181Y, L188R) or (A74I, L188F) or (A74I, L188K) or (A74I, L188R) or (A74I, M177Y, A184R, L188F) or (A74I, M177Y, V178P, A184G) or (A74I, T146F) or (A74I, T146F, L181Y) or (A74I, T146F, L181Y, L188K) or (A74I, T146F, L181Y, L188R) or (A74I, T146F, L188F) or (A74I, T146F, L188K) or (A74I, T146F, V178P) or (A74I, T146F, V178P, L181Y, L188F) or (A74I, T146F, V178P, L181Y, L188K) or (A74I, T146F, V178P, L188R) or (A74I, T146F, V178W) or (A74I, T146F, V178W, L181Y, L188F) or (A74I, T146F, V178W, L181Y, L188R) or (A74I, T146F, V178W, L188F) or (A74I, T146F, V178W, L188K) or (A74I, T146F, V178W, L188R) or (A74I, V178P) or (A74I, V178P, L181Y) or (A74I, V178P, L181Y, L188R) or (A74I, V178P, L188F) or (A74I, V178P, L188K) or (A74I, V178P, L188R) or (A74I, V178W, L188F) or (A74I, V178W, L188R) or (A74K) or (A74L) or (A74M) or (A74N) or (A74P) or (A74Q) or (A74R) or (A74R, L75V) or (A74S) or (A74T) or (A74T, A184D) or (A74T, F77A, L181Y, A184D, L188K) or (A74T, F77A, V178W, A184Y) or (A74T, F77L, L181Y, A184D, L188K) or (A74T, L181Y) or (A74T, L181Y, A184D, L188K) or (A74T, L181Y, A184Y, L188K) or (A74T, L181Y, L188K) or (A74T, L181Y, L188R) or (A74T, L188K) or (A74T, M177Y, L181Y, A184D, L188K) or (A74T, T146F, L181Y, A184D, L188K) or (A74T, T146F, L181Y, L188K) or (A74T, T146F, L181Y, L188R) or (A74T, T146F, L188F) or (A74T, T146F, L188K) or (A74T, T146F, L188R) or (A74T, T146F, V178P) or (A74T, T146F, V178P, L181Y) or (A74T, T146F, V178P, L188K) or (A74T, T146F, V178P, L188R) or (A74T, T146F, V178W) or (A74T, T146F, V178W, L181Y) or (A74T, T146F, V178W, L181Y, L188K) or (A74T, T146F, V178W, L188R) or (A74T, V178P) or (A74T, V178P, L188F) or (A74T, V178P, L188K) or (A74T, V178P, L188R) or (A74T, V178W, L181Y, L188K) or (A74T, V178W, L188K) or (A74T, V178W, L188R) or (A74V) or (A74V, F77A, A184N, L188F) or (A74V,

L181Y) or (A74V, L181Y, A184D, L188K) or (A74V, L181Y, A184W, L188F) or (A74V, L181Y, L188F) or (A74V, L188F) or (A74V, L188K) or (A74V, L75V) or (A74V, T146F, L181Y, L188K) or (A74V, T146F, L181Y, L188R) or (A74V, T146F, L188K) or (A74V, T146F, L188R) or (A74V, T146F, V178W, L181Y, L188F) or (A74V, V178P, A184W, L188K) or (A74V, V178P, L181Y, L188F) or (A74V, V178P, L181Y, L188K) or (A74V, V178P, L181Y, L188R) or (A74V, V178P, L188F) or (A74V, V178W, L188K) or (A74W) or (A74Y) or (D363G, T438C) or (E13D, R47L) or (E143A) or (E143C) or (E143D) or (E143F) or (E143G) or (E143I) or (E143K) or (E143L) or (E143M) or (E143N) or (E143P) or (E143Q) or (E143R) or (E143S) or (E143V) or (E143W) or (E143Y) or (E267D) or (E267R) or (E267T) or (E352A) or (E352D) or (E352F) or (E352G) or (E352I) or (E352L) or (E352M) or (E352N) or (E352P) or (E352R) or (E352S) or (E352T) or (E352V) or (E352W) or (E352Y) or (E64A) or (E64G) or (E64H) or (E64K) or (E64L) or (E64M) or (E64N) or (E64Q) or (E64V) or (E64Y) or (E82P) or (E93G) or (F173C) or (F173C, F205G) or (F173D) or (F173I) or (F173K) or (F173L) or (F173M) or (F173N) or (F173P) or (F173Q) or (F173R) or (F173S) or (F173S, F205P) or (F173V) or (F173W) or (F173Y) or (F173Y, I174F) or (F205A) or (F205C) or (F205D) or (F205D, D208N) or (F205E) or (F205G) or (F205H) or (F205I) or (F205K) or (F205L) or (F205M) or (F205N) or (F205P) or (F205R) or (F205S) or (F205T) or (F205V) or (F205W) or (F205Y) or (F261A) or (F261C) or (F261D) or (F261G) or (F261I) or (F261L) or (F261M) or (F261Q) or (F261S) or (F261T) or (F261V) or (F261Y) or (F331H) or (F331I) or (F331L) or (F331M) or (F331V) or (F331W) or (F331Y) or (F393M) or (F393W) or (F77A) or (F77A, A184W) or (F77A, L181P, A184Y, L188K) or (F77A, M177Y) or (F77C) or (F77D) or (F77E) or (F77G) or (F77H) or (F77I) or (F77K) or (F77L) or (F77M) or (F77N) or (F77P) or (F77R) or (F77S) or (F77T) or (F77V) or (F77V, L86M) or (F77W) or (F77Y) or (F81D) or (F81I) or (F81L) or (F81R) or (F81S) or (F81V) or (F81W) or (F81Y) or (G271A) or (G271C) or (G271D) or (G271E) or (G271F) or (G271H) or (G271K) or (G271L) or (G271M) or (G271N) or (G271P) or (G271Q) or (G271S) or (G271T) or (G271V) or (G271W) or (G271Y) or (G402A) or (G415A) or (G415D) or (G415S) or (G415T) or (G415V) or (G85A) or (G85L) or (G85S) or (H266A) or (H266C) or (H266E) or (H266F) or (H266I) or (H266K) or (H266M) or (H266N) or (H266Q) or (H266S) or (H266V) or (H266W) or (H266Y) or (I153F, G271L) or (I153L) or (I153L, F173Y) or (I259A) or (I259C) or (I259D) or (I259F) or (I259H) or (I259K) or (I259L) or (I259M) or (I259N) or (I259Q) or (I259S) or (I259T) or (I259V) or (I263A) or (I263C) or (I263E) or (I263F) or (I263H, A264G) or (I263K) or (I263L) or (I263M) or (I263N) or (I263Q) or (I263S) or (I263V) or (I263Y) or (I401A) or (I401M) or (I401T) or (I401V) or (K210E) or (K210T, G271V) or (K224C) or (K224E) or (K224H) or (K224I) or (K224L) or (K224M) or (K224P) or (K224Q) or (K224W) or (K224Y) or (L150A) or (L150C) or (L150D) or (L150E) or (L150F) or (L150G) or (L150H) or (L150I) or (L150K) or (L150M) or (L150N) or (L150Q) or (L150R) or (L150R, F205R) or (L150S) or (L150S, F173L) or (L150T) or (L150V) or (L150W) or (L150Y) or (L181H) or (L181I) or (L181M) or (L181P) or (L181V) or (L181Y) or (L181Y, A184D) or (L181Y, L188F) or (L181Y,

L188K) or (L181Y, L188K, H659R) or (L188A) or (L188D) or (L188E) or (L188F) or (L188H) or
 (L188I) or (L188K) or (L188M) or (L188N) or (L188Q) or (L188R) or (L188S) or (L188W) or
 (L20C) or (L20D) or (L20E) or (L20F) or (L20G) or (L20G, R47L) or (L20I) or (L20M) or (L20N)
 or (L20P) or (L20R) or (L20S) or (L20T) or (L20V) or (L20W) or (L20Y) or (L262I) or (L262V) or
 5 (L262W) or (L272A) or (L272C) or (L272E) or (L272F) or (L272G) or (L272I) or (L272K) or
 (L272M) or (L272N) or (L272Q) or (L272S) or (L272T) or (L272V) or (L272W) or (L272Y) or
 (L29A) or (L29C) or (L29D) or (L29F) or (L29H) or (L29I) or (L29M) or (L29M, R47G) or (L29Q)
 or (L29S) or (L29T) or (L29V) or (L29W) or (L29Y) or (L324F) or (L356C) or (L356F) or (L356I)
 or (L356M) or (L356V) or (L437I) or (L437M) or (L75I) or (L75I, F81C) or (L75I, F81I) or (L75I,
 10 F81L) or (L75I, F81S) or (L75I, F81V) or (L75V) or (L75V, F81C) or (L75V, F81L) or (L75V,
 F81V) or (L75V, F81Y) or (L78F) or (L78I) or (L78M) or (L78V) or (L86I) or (L86I, S89T) or
 (L86M) or (L86M, S89T) or (L86V) or (M118A) or (M118E) or (M118F) or (M118G) or (M118H)
 or (M118I) or (M118K) or (M118L) or (M118N) or (M118P) or (M118Q) or (M118S) or (M118T)
 or (M118V) or (M118W) or (M118Y) or (M177A) or (M177C) or (M177C, V178Y) or (M177D) or
 15 (M177E) or (M177F) or (M177H) or (M177I) or (M177L) or (M177N) or (M177P) or (M177Q) or
 (M177R) or (M177T) or (M177V) or (M177Y) or (M177Y, A184W) or (M177Y, A184Y) or (M177Y,
 M185V) or (M177Y, R50S) or (M177Y, V178P) or (M177Y, V178P, A184Y, L188F) or (M177Y,
 V178W) or (M177Y, V178W, A184Y) or (M177Y, V178W, A184Y, L188F) or (M185C) or
 (M185D) or (M185E) or (M185G) or (M185H) or (M185K) or (M185L) or (M185N) or (M185Q) or
 20 (M185V) or (M212A) or (M212C) or (M212D) or (M212E) or (M212F) or (M212G) or (M212H) or
 (M212K) or (M212L) or (M212L, I259F) or (M212Q) or (M212R) or (M212S) or (M212T) or
 (M212V) or (M212W) or (M212Y) or (M354A) or (M354C) or (M354D) or (M354E) or (M354G)
 or (M354I) or (M354K) or (M354K, D363Y) or (M354L) or (M354N) or (M354Q) or (M354R) or
 (M354S) or (M354T) or (M354V) or (M354W) or (M354Y) or (N70A) or (N70C) or (N70F) or
 25 (N70G) or (N70H) or (N70K) or (N70R) or (N70Y) or (P243T, H266L) or (P329A) or (P392C) or
 (P392G) or (P392Q) or (P392R) or (P392V) or (P9S) or (R147C, H266G) or (R161C, G271D) or
 (R190L, F261V) or (R203C) or (R255A) or (R255G) or (R255I) or (R255K) or (R255L) or
 (R255M) or (R255N) or (R255P) or (R255T) or (R255W) or (R47A) or (R47C) or (R47D) or
 (R47E) or (R47F) or (R47G) or (R47H) or (R47H, A74W) or (R47I) or (R47K) or (R47L) or (R47M)
 30 or (R47N) or (R47P) or (R47Q) or (R47S) or (R47T) or (R47V) or (R47W) or (R50A) or (R50C)
 or (R50D) or (R50E) or (R50F) or (R50G) or (R50I) or (R50L) or (R50M) or (R50N) or (R50P) or
 (R50Q) or (R50S) or (R50S, A184W, L188F) or (R50S, A74I, A184W, L188F) or (R50S, A74M,
 A184Y, L188K) or (R50S, A74T, L181Y, A184D, L188K, G240R) or (R50S, A74V) or (R50S,
 M177Y, V178P, A184Y) or (R50S, M177Y, V178W, A184W) or (R50S, M177Y, V178W, A184Y)
 35 or (R50S, M177Y, V178W, A184Y, A330R) or (R50S, M177Y, V178W, A184Y, A74V, G677D)
 or (R50S, M177Y, V178W, A184Y, L181P) or (R50S, M177Y, V178W, A184Y, L181Y) or (R50S,
 M177Y, V178W, A184Y, L188K) or (R50S, M177Y, V178W, A184Y, L188R) or (R50S, M177Y,

V178W, A184Y, T146F) or (R50S, M177Y, V178W, A184Y, V26N) or (R50S, M177Y, V178W, L181Y, A184Y) or (R50S, S72G, A74V) or (R50S, S72G, A74V, M177Y, A184Y) or (R50S, S72G, A74V, M177Y, V178P, A184Y) or (R50S, S72G, A74V, M177Y, V178W, A184Y) or (R50S, S72G, A74V, M177Y, V178W, A184Y, L188R) or (R50S, T146F, M177Y, V178W, A184Y) or (R50V) or (R50Y) or (S176A, A184Y, L188K) or (S176C) or (S176D) or (S176E) or (S176F) or (S176G) or (S176H) or (S176K) or (S176L) or (S176M) or (S176N) or (S176P) or (S176Q) or (S72C, A74C) or (S72C, A74I) or (S72C, A74L) or (S72C, A74V) or (S72D, A74C) or (S72G) or (S72G, A74C) or (S72G, A74F) or (S72G, A74H) or (S72G, A74I) or (S72G, A74I, A184W, L188F) or (S72G, A74I, F77A, M177Y) or (S72G, A74I, F77L) or (S72G, A74I, F77L, M177Y) or (S72G, A74I, L181Y) or (S72G, A74I, L181Y, L188F) or (S72G, A74I, L181Y, L188K) or (S72G, A74I, L181Y, L188R) or (S72G, A74I, L188F) or (S72G, A74I, L188K) or (S72G, A74I, L188R) or (S72G, A74I, T146F) or (S72G, A74I, T146F, L181Y) or (S72G, A74I, T146F, L181Y, L188F) or (S72G, A74I, T146F, L181Y, L188K) or (S72G, A74I, T146F, L181Y, L188R) or (S72G, A74I, T146F, L188F) or (S72G, A74I, T146F, L188K) or (S72G, A74I, T146F, V178P) or (S72G, A74I, T146F, V178P, L181Y) or (S72G, A74I, T146F, V178P, L181Y, L188F) or (S72G, A74I, T146F, V178P, L181Y, L188K) or (S72G, A74I, T146F, V178P, L181Y, L188R) or (S72G, A74I, T146F, V178P, L188F) or (S72G, A74I, T146F, V178P, L188K) or (S72G, A74I, T146F, V178P, L188R) or (S72G, A74I, T146F, V178W) or (S72G, A74I, T146F, V178W, L181Y) or (S72G, A74I, T146F, V178W, L181Y, L188F) or (S72G, A74I, T146F, V178W, L181Y, L188K) or (S72G, A74I, T146F, V178W, L181Y, L188R) or (S72G, A74I, T146F, V178W, L188F) or (S72G, A74I, T146F, V178W, L188K) or (S72G, A74I, T146F, V178W, L188R) or (S72G, A74I, V178P) or (S72G, A74I, V178P, L181Y) or (S72G, A74I, V178P, L181Y, L188R) or (S72G, A74I, V178P, L188F) or (S72G, A74I, V178P, L188K) or (S72G, A74I, V178P, L188R) or (S72G, A74I, V178W) or (S72G, A74I, V178W, L181Y, L188F) or (S72G, A74I, V178W, L181Y, L188R) or (S72G, A74L) or (S72G, A74M, A184Y, L188K) or (S72G, A74S) or (S72G, A74T) or (S72G, A74T, L181Y, A184D, L188K) or (S72G, A74T, L181Y, L188F) or (S72G, A74T, L181Y, L188K) or (S72G, A74T, L181Y, L188R) or (S72G, A74T, L188F) or (S72G, A74T, L188K) or (S72G, A74T, L188R) or (S72G, A74T, T146F) or (S72G, A74T, T146F, L181Y) or (S72G, A74T, T146F, L181Y, L188K) or (S72G, A74T, T146F, L181Y, L188R) or (S72G, A74T, T146F, L188F) or (S72G, A74T, T146F, L188K) or (S72G, A74T, T146F, V178P) or (S72G, A74T, T146F, V178P, L181Y) or (S72G, A74T, T146F, V178P, L188F) or (S72G, A74T, T146F, V178P, L188K) or (S72G, A74T, T146F, V178P, L188R) or (S72G, A74T, T146F, V178W) or (S72G, A74T, T146F, V178W, L181Y) or (S72G, A74T, T146F, V178W, L181Y, L188F) or (S72G, A74T, T146F, V178W, L181Y, L188K) or (S72G, A74T, T146F, V178W, L181Y, L188R) or (S72G, A74T, T146F, V178W, L188F) or (S72G, A74T, T146F, V178W, L188K) or (S72G, A74T, T146F, V178W, L188R) or (S72G, A74T, V178P) or (S72G, A74T, V178P, L181Y) or

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(S72G, A74T, V178P, L181Y, L188F) or (S72G, A74T, V178P, L181Y, L188K) or (S72G, A74T, V178P, L188F) or (S72G, A74T, V178P, L188K) or (S72G, A74T, V178P, L188R) or (S72G, A74T, V178W) or (S72G, A74T, V178W, L181Y, L188F) or (S72G, A74T, V178W, L181Y, L188K) or (S72G, A74T, V178W, L188F) or (S72G, A74T, V178W, L188K) or (S72G, A74T, V178W, L188R) or (S72G, A74V) or (S72G, A74V, A184W) or (S72G, A74V, A221V) or (S72G, A74V, F77A) or (S72G, A74V, F77A, M177Y) or (S72G, A74V, F77L, M177Y) or (S72G, A74V, F77S) or (S72G, A74V, L181Y) or (S72G, A74V, L181Y, L188F) or (S72G, A74V, L181Y, L188K) or (S72G, A74V, L188F) or (S72G, A74V, L188K) or (S72G, A74V, L188R) or (S72G, A74V, T146F) or (S72G, A74V, T146F, L181Y) or (S72G, A74V, T146F, L181Y, L188F) or (S72G, A74V, T146F, L181Y, L188K) or (S72G, A74V, T146F, L181Y, L188R) or (S72G, A74V, T146F, L181Y, L188R) or (S72G, A74V, T146F, V178P) or (S72G, A74V, T146F, V178P, L181Y) or (S72G, A74V, T146F, V178P, L181Y, L188F) or (S72G, A74V, T146F, V178P, L181Y, L188K) or (S72G, A74V, T146F, V178P, L181Y, L188R) or (S72G, A74V, T146F, V178P, L188F) or (S72G, A74V, T146F, V178P, L188K) or (S72G, A74V, T146F, V178P, L188R) or (S72G, A74V, T146F, V178W) or (S72G, A74V, T146F, V178W, L181Y) or (S72G, A74V, T146F, V178W, L181Y, L188K) or (S72G, A74V, T146F, V178W, L181Y, L188R) or (S72G, A74V, T146F, V178W, L188F) or (S72G, A74V, T146F, V178W, L188K) or (S72G, A74V, T146F, V178W, L188R) or (S72G, A74V, V178P) or (S72G, A74V, V178P, L181Y) or (S72G, A74V, V178P, L181Y, L188F) or (S72G, A74V, V178P, L181Y, L188K) or (S72G, A74V, V178P, L181Y, L188R) or (S72G, A74V, V178P, L188F) or (S72G, A74V, V178P, L188K) or (S72G, A74V, V178P, L188R) or (S72G, A74V, V178W) or (S72G, A74V, V178W, L181Y) or (S72G, A74V, V178W, L181Y, L188F) or (S72G, A74V, V178W, L181Y, L188K) or (S72G, A74V, V178W, L181Y, L188R) or (S72G, A74V, V178W, L188F) or (S72G, A74V, V178W, L188K) or (S72G, A74V, V178W, L188R) or (S72G, A74Y) or (S72G, L181Y) or (S72G, L181Y, L188F) or (S72G, L181Y, L188K) or (S72G, L181Y, L188R) or (S72G, L188F) or (S72G, L188K) or (S72G, L188R) or (S72G, M177Y, V178W) or (S72G, M177Y, V178W, L188K) or (S72G, T146F) or (S72G, T146F, L181Y) or (S72G, T146F, L181Y, L188F) or (S72G, T146F, L181Y, L188K) or (S72G, T146F, L181Y, L188R) or (S72G, T146F, L188F) or (S72G, T146F, L188K) or (S72G, T146F, L188R) or (S72G, T146F, V178P) or (S72G, T146F, V178P, L181Y) or (S72G, T146F, V178P, L181Y, L188F) or (S72G, T146F, V178P, L181Y, L188K) or (S72G, T146F, V178P, L181Y, L188R) or (S72G, T146F, V178P, L188F) or (S72G, T146F, V178P, L188K) or (S72G, T146F, V178P, L188R) or (S72G, T146F, V178W) or (S72G, T146F, V178W, L181Y) or (S72G, T146F, V178W, L181Y, L188F) or (S72G, T146F, V178W, L181Y, L188K) or (S72G, T146F, V178W, L188F) or (S72G, T146F, V178W, L188K) or (S72G, T146F, V178W, L188R) or (S72G, V178P) or (S72G, V178P, L181Y) or (S72G, V178P, L181Y, L188F) or (S72G, V178P, L181Y, L188K) or (S72G, V178P, L181Y, L188R) or (S72G, V178P, L188F) or (S72G, V178P, L188K) or (S72G, V178P, L188R) or (S72G,

V178W) or (S72G, V178W, L181Y) or (S72G, V178W, L181Y, L188F) or (S72G, V178W, L181Y, L188K) or (S72G, V178W, L181Y, L188R) or (S72G, V178W, L188F) or (S72G, V178W, L188K) or (S72G, V178W, L188R) or (S72H, A74C) or (S72N, A74C) or (S72N, A74I) or (S72N, A74V) or (S72W) or (S72W, A74V) or (S89C) or (S89E) or (S89G) or (S89I) or (S89L) or (S89N) or (S89Q) or (S89R) or (S89T) or (S89V) or (T146A) or (T146A, D222Y, I263M) or (T146A, F173D) or (T146A, F173V) or (T146A, F205G) or (T146A, F261C) or (T146A, F261S) or (T146A, F261V) or (T146A, G271S) or (T146A, G271T) or (T146A, H266V) or (T146A, I258T, I259S) or (T146A, I259H) or (T146A, I263F) or (T146A, I263L) or (T146A, I263N) or (T146A, I263V) or (T146A, L150A) or (T146A, L150G) or (T146A, L150K) or (T146A, L150R, F173I) or (T146A, L272H) or (T146A, L272S) or (T146A, L272W) or (T146A, M212A) or (T146C) or (T146D) or (T146E) or (T146F) or (T146F, L181Y) or (T146F, L181Y, L188F) or (T146F, L181Y, L188K) or (T146F, L188K) or (T146F, L188R) or (T146F, V178P) or (T146F, V178P, L181Y, L188R) or (T146F, V178P, L188K) or (T146F, V178P, L188R) or (T146F, V178W) or (T146F, V178W, L181Y, L188R) or (T146F, V178W, L188F) or (T146F, V178W, L188K) or (T146F, V178W, L188R) or (T146G) or (T146H) or (T146L) or (T146M) or (T146N) or (T146P) or (T146R) or (T146S) or (T146V) or (T146V, A191T) or (T146Y) or (T149P) or (T268A) or (T268S) or (T269K) or (T327A) or (T327A, A330W) or (T327C) or (T327D) or (T327E) or (T327I) or (T327L) or (T327M) or (T327N) or (T327P) or (T327Q) or (T327S) or (T327V) or (T365C) or (T365D) or (T365F) or (T365G) or (T365H) or (T365I) or (T365K) or (T365L) or (T365N) or (T365P) or (T365Q) or (T365V) or (T365W) or (T365Y) or (T436A) or (T436C) or (T436D) or (T436E) or (T436F) or (T436G) or (T436H) or (T436I) or (T436K) or (T436L) or (T436M) or (T436N) or (T436P) or (T436Q) or (T436R) or (T436S) or (T436V) or (T436W) or (T436Y) or (T438A) or (T438C) or (T438I) or (T438S) or (T438V) or (T49A) or (T49C) or (T49D) or (T49E) or (T49F) or (T49G) or (T49H) or (T49I) or (T49K) or (T49L) or (T49L, S54N) or (T49M) or (T49N) or (T49P) or (T49Q) or (T49R) or (T49R, R50C) or (T49R, R50S, M177Y, V178W, A184Y) or (T49S) or (T49V) or (T49W) or (T49Y) or (T88C) or (T88S) or (T88V) or (V178D) or (V178H) or (V178I) or (V178K) or (V178L) or (V178M) or (V178N) or (V178P) or (V178P, L181Y) or (V178P, L181Y, L188R) or (V178P, L188F) or (V178P, L188K) or (V178P, L188R) or (V178Q) or (V178R) or (V178S) or (V178T) or (V178W) or (V178W, A184Y) or (V178W, L181Y, L188K) or (V178W, L181Y, L188R) or (V178W, L188R) or (V178W, R50S) or (V178Y) or (V26A) or (V26C) or (V26F) or (V26G) or (V26H) or (V26I) or (V26K) or (V26L) or (V26M) or (V26N) or (V26N, R50S, M177Y, V178W, A184Y) or (V26Q) or (V26S) or (V26T) or (V26W) or (V26Y) or (V314A) or (V314E) or (V314F) or (V314H) or (V314P) or (V314Q) or (V314S) or (V314Y) or (V48A) or (V48D) or (V48E) or (V48F) or (V48G) or (V48H) or (V48I) or (V48L) or (V48M) or (V48P) or (V48Q) or (V48R) or (V48S) or (V48T) or (V48W) or (V48Y) or (W325A) or (W325C) or (W325D) or (W325E) or (W325F) or (W325G) or (W325I) or (W325K) or (W325L) or (W325M) or (W325Q) or (W325S) or (W325T) or (W325V) or (W325Y) or (W90Y) or (Y51A) or (Y51C) or (Y51F) or (Y51H) or

(Y51I) or (Y51L) or (Y51M) or (Y51P) or (Y51S, F77V) or (Y51T) or (Y51V) or (Y51W) or (A225C) or (A225N) or (A225Q) or (A225S) or (A225T) or (A225Y) or (A44Q) or (D182A) or (D182C) or (D182E) or (D182H) or (D182I) or (D182M) or (D182N) or (D182V) or (D182W) or (E140A) or (E140D) or (E140K) or (E140M) or (E140T) or (E337L) or (G114C) or (G114K) or (G114L) or (G114N) or (G114W) or (G240A) or (G240C) or (G240H) or (G240K) or (G240N) or (G240R) or (G240S) or (H236A) or (H236C) or (H236D) or (H236F) or (H236I) or (H236K) or (H236P) or (H236R) or (H236S) or (H92F) or (H92Y) or (I174T) or (I174V) or (I258L) or (I258M) or (I258V) or (K187A) or (K187G) or (K187N) or (K187R) or (K440C) or (K440N) or (K440T) or (K76C) or (K76E) or (K76F) or (K76H) or (K76I) or (K76N) or (K76V) or (M5C) or (M5E) or (M5P) or (N186A) or (N186D) or (N186E) or (N186F) or (N186H) or (N186K) or (N186L) or (N186M) or (N186Q) or (N186T) or (N186V) or (N186Y) or (P172A) or (P172D) or (P172G) or (Q189I) or (Q189K) or (Q189V) or (Q403K) or (Q403R) or (Q404F) or (Q404Y) or (Q73E) or (Q73F) or (Q73L) or (Q73S) or (Q73T) or (Q73W) or (R179A) or (R179H) or (R179L) or (R179M) or (R179P) or (R179V) or (R190C) or (R190G) or (R190K) or (R190M) or (R190N) or (R190S) or (R190V) or (R190W) or (R79K) or (S270A) or (S270D) or (S53C) or (T175F) or (T175G) or (T175H) or (T175L) or (T175M) or (T175N) or (T175Q) or (T175R) or (T175Y) or (T269V) or (V299L) or (V299W) or (F173W, R179L, T269V, K440C) or (H236K, G240V, T269V, K440C).

According to **one embodiment** there is provided a BM3 variant wherein said variant has an improved selectivity and/or product yield or product titer for C19 hydroxylation compared with at least one BM3 variant according to SEQ ID No. 2 to 28, or 117 to 119, preferably with at least one BM3 variant selected from BM3-254, BM3-261, BM3-263, or BM3-268. This embodiment can be and is suggested to be combined with all previous embodiments, in particular with the first, second, third, fourth, fifth and/or sixth embodiment or the described subsets.

According to a highly preferred embodiment of the current invention, there is provided a BM3 variant for catalyzing the C19 hydroxylation of a steroid or steroid derivative, wherein an amino acid sequence (insert) has been introduced between amino acid positions 458 and 477, and wherein the insert has at least 90 %, 95 %, 99 % or 100 % sequence identity with a sequence according to any of SEQ ID No. 123 to 173. Most preferably this embodiment is a BM3 variant according to any of the previously described embodiments of the first aspect.

It was surprisingly found that BM3 variants for C19 hydroxylation of steroids comprising these amino acid sequence inserts had improved product titer or TPR, for example if the insert was introduced into a BM3 variant with Seq ID No. 118 or 119.

According to a **separate aspect 1B** there is provided a BM3 variant characterized by an improved BM3 protein expression relative to BM3 wildtype (WT), wherein the BM3 variant comprises mutations at at least one, two, three, four, five, six, seven, eight, nine, ten or all amino acid positions of (C62), (S106), (Q110), (A117), (Q128), (T152), (G157), (G227), (P243), (H285),

(Q288), (A295), (N319), (G368), (V371), (K391), (N395) and (H408). These BM3 variants may also be in accordance with some embodiments of the first aspect.

In particular provided herein is a BM3 variant characterized by an improved BM3 protein expression relative to BM3 wildtype (WT), wherein the BM3 variant comprises at least one, two, 5 three, four, five, six, seven, eight, nine, ten or all mutations of (C62L), (S106A), (Q110E), (A117P), (Q128E), (T152I), (G157L), (G227P), (P243E), (H285E), (Q288E), (A295D), (N319E), (G368E), (V371P), (K391L), (N395F) and (H408L).

In some more specific embodiments, there is provided the BM3 variant according to the previous 10 embodiments, wherein the BM3 variant comprises the mutations (S106A, Q128E) or (S106A, H285E, Q288E) or (S106A, Q128E, H285E) or (C62L, A117P, G157L, N319E) or (C62L, G227P, N319E, K391L) or (Q110E, Q128E, Q288E, N319E) or (Q128E, H285E, Q288E, N319E) or (C62L, S106A, A117P, A295D, V371P) or (C62L, S106A, A117P, G227P, P243E) or (C62L, S106A, Q128E, G227P, K391L) or (S106A, Q110E, G227P, H285E, K391L) or (C62L, S106A, Q128E, G227P, Q288E, K391L) or (S106A, G227P, H285E, V371P, K391L, H408L) or (S106A, 15 Q110E, G227P, N319E, K391L, N395F) or (C62L, S106A, Q128E, T152I, G227P, P243E, H408L) or (S106A, Q110E, Q128E, G227P, Q288E, N319E, K391L) or (C62L, S106A, Q128E, G157L, P243E, A295D, G368E, K391L) or (S106A, A117P, Q128E, T152I, G227P, N319E, V371P, N395F).

As shown in Table E7E and Example 7E, introduction of these mutations resulted in an improved 20 protein expression of the BM3 protein. Protein expression can be measured as known in the art, e.g. by use of SDS PAGE, Western Blot, Mass Spectrometry or any other suitable method. These mutations and BM3 variants can thus be used to solve the same problem, i.e. to improve the C19 hydroxylation.

As understood by the skilled person, the BM3 embodiments for the improved protein production 25 can be combined and are suggested to be combined with each of the previously described embodiments, which are suitable to improve selectivity or yield for C19 hydroxylation.

In addition, the BM3 embodiments for the improved protein production are however not restricted to C19 hydroxylation and can also be used more broadly, i.e. to improve the protein production for any BM3 protein, independent of its suitability for steroid hydroxylation.

30 According to a **second aspect** of the current invention, there is provided a nucleic acid encoding for a Cytochrome P450 BM3 monooxygenase variant as described herein.

A defined protein or amino acid sequence may be encoded by various nucleic acid sequences. Where an amino acid sequence has been defined herein, each of the nucleic acids encoding this amino acid sequence shall also be deemed disclosed herein. It is therefore well understood

that the nucleic acid sequence as provided e.g. in SEQ ID No. 59 for the wildtype sequence has to be read in an exemplary fashion.

Preferably, the nucleic acid encodes for a variant according to the first aspect. Even more preferably, the nucleic acid encodes for a variant according to the first aspect, first embodiment or the nucleic acid encodes for a variant according to the first aspect, second embodiment, e.g. variants according to SEQ ID No. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 117, 118 or 119 or functional variants derived thereof. Furthermore, the nucleic acid can be a nucleic acid encoding for a variant according to the third, fourth, fifth, or sixth embodiment of the first aspect.

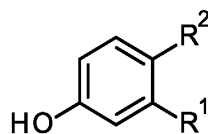
10 Nucleic acids according to the second aspect can be generated *de novo* as known in the art or can be generated by mutating available BM3 nucleic acid sequences. The nucleic acid encoding the P450-BM3 variant according to the current invention can then be introduced into a suitable expression system or host cell for expression of the BM3 variant. Sequences of the nucleic acids can be verified as known in the art. Exemplary methods for sequence verification are disclosed
15 for example in (Green and Sambrook 2012).

According to a **third aspect** of the current invention, there is provided a host cell for the production of a Cytochrome P450 BM3 monooxygenase variant. In a preferred embodiment, the host cell comprises a nucleic acid encoding for a BM3 variant according to any of the aspects described herein.

20 Suitable expression systems are well known in the art and example systems comprise *E. coli* strains such as DH5alpha, BL21 (DE3), or Rosetta (DE3), but also various other bacterial and non bacterial systems. In some preferred embodiments, the host cell is a procaryotic cell (e.g. an *E. coli* cell) or a eukaryotic cell (e.g. a CHO cell). In a preferred embodiment, the host cell is an *E. coli* cell, e.g. DH5alpha, BL21 (DE3), or Rosetta (DE3). In another preferred embodiment,
25 the host cell is from *E. coli* strain W3110 (NC_007779.1), JM101 or MG1655 (NC_000913.3). Introduction of nucleic acids encoding for a BM3 variant, e.g. according to the second aspect, into the host cell may occur as known in the art, i.e. using suitable vectors or transfer systems. For example, where bacterial expression systems such as DH5alpha, BL21 (DE3), or Rosetta (DE3) are used, suitable vectors are known in the art, such as pSE420 (Invitrogen), pET21a
30 (EMD Biosciences), pET22b (EMD Millipore), pLys (EMD Biosciences) or pETM11 (EMBL Vector Collection, Germany). For a detailed description see e.g. section 4.2 "*Cloning of cyp102A1 and pETM11-BM3 construction*" of Supporting Information of (Acevedo-Rocha 2018) and the examples provided herein.

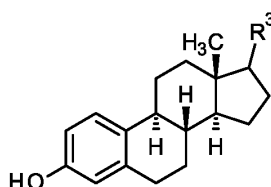
-76-

According to a **fourth aspect** of the current invention, there is provided the use of a Cytochrome P450 BM3 monooxygenase (BM3) variant for the production of a compound according to formula I, wherein R¹ and R² form a six-membered ring as part of a steroid.



(I)

In a preferred embodiment according to the fourth aspect, formula I is formula (II), wherein R³ is a beta-hydroxy group (-OH) or an oxo group (=O).



(II),

As described also elsewhere herein, it was surprisingly found, that BM3 variants can be used for the production of a compound according to formula I and II in a commercially relevant setup, i.e. for an industrial application. To this end, a BM3 variant suitable for catalyzing C19 hydroxylation is incubated with a suitable substrate, e.g. as described in examples 2, 8, 10, 13, 17, 20, 21, 24, 25.

In some highly preferred embodiments according to the fourth aspect, the substrate for the BM3 variant is a steroid or steroid derivative.

According to a **fifth aspect** of the current invention, there is provided the use of a BM3 variant for the C19-hydroxylation of a steroid or steroid derivative.

As described also elsewhere herein, it was surprisingly found, that BM3 variants can be used for the C19-hydroxylation of a steroid or steroid derivative in a commercially relevant setup, i.e. for an industrial application. To this end, a BM3 variant suitable for catalyzing C19 hydroxylation is incubated with a suitable substrate, e.g. as described in examples 2, 8, 10, 13, 17, 20, 21, 24, 25.

According to some highly preferred embodiments of the **fourth or fifth aspect**, the BM3 variant is a variant as described herein. Preferably, the BM3 variant can be a variant according to the first aspect. Even more preferably, the BM3 variant can be a variant according to the first aspect, first embodiment or the BM3 variant can be a variant according to the first aspect, second embodiment, e.g. a variant according to SEQ ID No. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15,

16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 117, 118 or 119, or a variant derived from any of these. Furthermore, the BM3 variant can be a variant according to the third, fourth, fifth, or sixth embodiment of the first aspect.

In some embodiments according to the **fourth or fifth aspect**, the BM3 variant is purified prior
5 to incubation with the substrate using a purification technique known in the art, such as but not restricted to immobilized metal affinity chromatography, ion exchange chromatography or size exclusion chromatography. In some other embodiments according to the fourth aspect, the BM3 variant remains in a cell suspension when incubated with the substrate, e.g. no separation of BM3 variant and expression system occurs prior to the biotransformation, e.g. whole-cell
10 biotransformation.

According to some preferred embodiments of the **fourth or fifth aspect** said steroid or steroid derivatives comprise a 1,4-dien-3-one-A-ring or a 4-en-3-one-A-ring. According to some even more preferred embodiments according to the **fourth or fifth aspect** said steroid or steroid derivative is androsta-1,4-dien-3,17-dione (ADD) or (17beta)-17-hydroxyandrosta-1,4-dien-3-
15 one (delta1 testosterone).

In some embodiments of the **fourth or fifth aspect** the substrate steroid or steroid derivative is added to the biotransformation reaction as a purified compound. In some embodiments the substrate steroid or steroid derivative is dissolved in a suitable solvent and can be added in solution to the biotransformation mixture. In other embodiments the substrate steroid or steroid
20 derivative is formed in the course of a reaction preceding the C19 hydroxylation. The reaction preceding the C19 hydroxylation reaction may be another biotransformation reaction or a chemical reaction, e.g. a reduction of delta1 testosterone to testosterone or an oxidation of delta1 testosterone to ADD.

In some embodiments of the fourth or fifth aspect the formed C19 hydroxylated steroid or steroid
25 derivative is extracted from the biotransformation reaction mixture using a method known in the art, e.g. with 4-methyl-2-pentanone (see example 10). In some other embodiments according to the fourth or fifth aspect the formed C19 hydroxylated steroid or steroid derivative is converted into a secondary product. This conversion may occur e.g. by means of biotransformation or chemical reaction. A secondary product may be for example estrone or a derivative thereof.

30 Extraction of the C19 hydroxylated steroid or steroid derivative or a secondary product thereof may occur as known in the art, e.g. by stirring the reaction mixture with 4-methyl-2-pentanone, separation of the organic phase and concentration by evaporation of the organic solvents. Further purification of the C19 hydroxylated steroid or steroid derivative or a secondary product thereof may be performed using chromatographic techniques, e.g. flash chromatography using
35 silica gel or preparative HPLC, or crystallization.

Analysis of the production of the C19 hydroxylated steroid, steroid derivative or secondary product can occur as known in the art, e.g. by using MS- or NMR-methods.

Suitable biotransformation conditions are described in the examples provided herein. However, it is routine for the skilled person to optimize the reaction conditions for a BM3 variant to obtain optimal yields for the reaction product. For example, incubation may occur in a KH₂PO₄/K₂HPO₄ buffer comprising an aqueous EDTA solution in a pH range of 6.6 to 7.4. Suitable incubation times may be for example between 12 and 48 hours.

According to a **sixth aspect** of the current invention, there is provided a process for C19-hydroxylation of a steroid or derivative thereof comprising

- 10 (i) (a) culturing a recombinant Cytochrome P450 BM3 monooxygenase (BM3) variant producing microorganism in a culture medium, in the presence of an exogenous or intermediately formed substrate; or
(b) incubating a substrate-containing reaction medium with a Cytochrome P450 BM3 monooxygenase; and
- 15 (ii) isolating the oxidation product formed or a secondary product thereof from the medium;

said process being further characterized in that said BM3 variant is a BM3 variant as described herein.

- 20 The recombinant BM3 variant producing microorganism is a microorganism comprising a nucleic acid encoding a BM3 variant. The recombinant BM3 variant producing microorganism can be derived from any suitable expression system. Preferably, the recombinant BM3 variant producing microorganism is a microorganism according to the third aspect. For example, the BM3 variant may be a variant according to the first aspect.

- 25 In some embodiments of the sixth aspect, the process comprises culturing the recombinant BM3 variant producing microorganism in a culture medium. This can occur as known in the art, e.g. as described in the examples. However, the skilled person is aware of a variety of setups for the cultivation of microorganisms. Suitable culture media are described in the art for the respective expression system and can be easily adapted.

- 30 In some embodiments of the sixth aspect, the BM3 variant is purified prior to incubation with the substrate using a purification technique known in the art, such as immobilized metal affinity chromatography, ion exchange chromatography or size exclusion chromatography. In some other embodiments of the sixth aspect, the BM3 variant remains in a cell suspension when incubated with the substrate, e.g. no separation of BM3 variant and expression system occurs prior to the biotransformation, e.g. whole-cell biotransformation.

In some embodiments, the process comprises incubating a substrate-containing reaction medium with a BM3 variant (e.g. purified or crude mixture with cells of the expression system). Suitable media and conditions are described in the examples provided herein. However, it is routine for the skilled person to optimize the reaction conditions for a BM3 variant to optimize yields for the reaction product.

For example, the substrate can be an exogenous substrate. Such an exogenous substrate is added to the biotransformation reaction, e.g. as a purified compound, solution or mixture. The substrate can also be an intermediately formed substrate, i.e. the substrate can be formed in the course of a reaction preceding the C19 hydroxylation. The reaction preceding the C19 hydroxylation reaction may be another biotransformation reaction or a chemical reaction.

Suitable substrates are steroids or steroid derivatives, in particular those comprising a 1,4-dien-3-one-A-ring or a 4-en-3-one-A-ring. For example ADD or d1-testosterone are suitable substrates for the described BM3 variants. In some preferred embodiments according to the sixth aspect, said steroid or derivative thereof comprises a 1,4-dien-3-one-A-ring and a 19-methyl group. In some preferred embodiments according to the sixth aspect, said steroid or derivative thereof comprises a 4-en-3-one-A-ring and a 19-methyl group.

Following the biotransformation, the C19 hydroxylation product can be isolated from the reaction medium as described in the art. In the alternative, a secondary product may be isolated from the reaction medium. The isolation may be performed for example by chromatographic techniques such as preparative or analytical HPLC, or by extraction. Extraction of the C19 hydroxylated steroid or steroid derivative or a secondary product thereof may occur as known in the art, e.g. with 4-methyl-2-pentanone (see example 10). Further purification of the C19 hydroxylated steroid or steroid derivative or a secondary product thereof may be performed using chromatographic techniques, e.g. flash chromatography using silica gel or preparative HPLC, or crystallization.

Analysis of the production of the C19 hydroxylated steroid, steroid derivative or secondary product can occur as known in the art, e.g. by MS- or NMR-methods.

According to a **seventh aspect** there is provided a method for obtaining optimized BM3 variants for the C19 hydroxylation of steroids, said method comprising

- (i) (a) culturing a recombinant microorganism expressing a BM3 variant (test variant) in a culture medium, in the presence of an exogenous or intermediately formed steroid or steroid derivative; or
(b) incubating a steroid or steroid derivative-containing reaction medium with a BM3 variant (test variant); and

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- (ii) Comparing the obtained product yield or titer and/or selectivity for the C19 hydroxylation product formed by the test variant or a secondary product thereof with the respective value obtained for a parent variant of the test variant capable of catalyzing the C19 hydroxylation of a steroid, and
- 5 (iii) Selecting the test variant as optimized for the C19 hydroxylation of steroids, if the test variant has an improved product yield or titer and/or selectivity compared to the parent variant.

The description provided for the process according to the fifth and sixth aspect applies *mutatis mutandis*, in particular with regard to step (i) a and b.

- 10 In a preferred embodiment, the steroid or steroid derivative comprises an 1,4-dien-3-one-A-ring or a 4-en-3-one-A-ring. In a highly preferred embodiment the steroid or steroid derivative is ADD or d1-testosterone. In some preferred embodiments of the seventh aspect, the steroid or derivative thereof comprises a 1,4-dien-3-one-A-ring and a 19-methyl group. In some preferred embodiments of the seventh aspect, said steroid or derivative thereof comprises a 4-en-3-one-
- 15 A-ring and a 19-methyl group.

- Product yield and selectivity (factor) are calculated as disclosed elsewhere herein. The product yield can be the product yield for the C19 hydroxylated steroid or steroid derivative, or a product derived thereof. Preferably the product yield is the product yield for estradiol or estrone. The selectivity factor can be the selectivity factor for the C19 hydroxylated steroid or steroid
- 20 derivative.

Preferably, the daughter variant has the sequence of its parent variant and at least one further mutation. Preferably, the parent variant is a variant according to the first aspect.

EXAMPLES

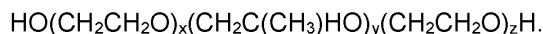
- If not stated otherwise, culture media were prepared in demineralized water and sterilized at 121
- 25 °C for 20 minutes or sterile filtered. All solutions were prepared with MilliQ water and sterile filtered, unless otherwise described. All preparations were performed under sterile conditions.

Oxford trace element solution contained FeCl₃ x 6 H₂O (27 g/L), ZnCl₂ (1.31 g/L), CoCl₂ x 6 H₂O (2.87 g/L), CuCl₂ x 2 H₂O (1.27 g/L), Boric acid (0.5 g/L), CaCl₂ x 2 H₂O (1.32 g/L), Na₂MoO₄ x 2 H₂O (2.35 g/L), Hydrochloric acid (37%) (100 mL/L).

- 30 List of abbreviations: Δ...deletion from amino acid; DMSO...Dimethyl sulfoxide; DMF...Dimethylformamide; EDTA...Ethylenediaminetetraacetate; × g...times gravity; IPTG...isopropyl β-D-thiogalactopyranoside; LB...Luria-Bertani broth; OD₅₅₀...optical density at 550 nm; rpm...revolutions per minute;

SOC Outgrowth Medium...#B9020S-from BioLabs: 2 % Vegetable Peptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM Glucose.

Pluronic® PE 8100... Pluronic PE types are low-foaming, nonionic surfactants. They are block copolymers in which the central polypropylene glycol group is flanked by two polyethylene glycol groups. PE 8100 conforms to the following structural formula:



PE 8100 is a polypropylene glycol block copolymer with a molar mass of 2300 g/mol and 10 % polypropylene glycol in the molecule.

Example 1

10 Cloning of P450 BM3 (BM3) variants

Nucleotide sequences encoding P450-BM3 variants as described herein can be synthesized as known in the art, e.g. as offered by respective service providers such as Eurofins Genomics GmbH (Eurofins Genomics GmbH, Anzinger Str. 7a, 85560 Ebersberg, Germany). In brief, nucleic acid sequences encoding P450-BM3 WT (e.g. SEQ ID No. 59) or other P450-BM3 variants as described herein were cloned into an expression vector based on the vector pETM-11 (EMBL Vector Collection, Germany) or another suitable vector. For a detailed description see section 4.2 "*Cloning of cyp102A1 and pETM11-BM3 construction*" of Supporting Information of (Acevedo-Rocha 2018). Genetic elements were introduced into the modified pETM-11 vector by means of commonly known methods. For expression of BM3 variants, the expression vector was introduced into *Escherichia coli* strain BL21 Star™ (DE3) (Invitrogen™, Lifetechnologies) cells, *E. coli* BL 21 (DE3) cells or *E. coli* BL 21 Gold (DE3) ΔdkgA :: FRT T7 gdh (the construction is described in (Agudo R 2012)) cells.

Generation of enzyme variants

Nucleotide substitutions (replacements) were introduced into the nucleic acid parent sequences, e.g. to obtain a T88 exchange into other amino acids on SEQ ID No. 5 as parent. Several Molecular Biological methods can be used to achieve these replacements. A useful method for preparing a mutated nucleic acid according to the invention and the corresponding protein comprises carrying out site-directed mutagenesis on codons encoding one or more amino acids, which are selected in advance. The methods for obtaining these site-directed mutations are well known to the skilled person and widely described in the literature (in particular: Directed Mutagenesis: A Practical Approach, 1991, Edited by M.J. McPHERSON, IRL PRESS), or are methods for which it is possible to employ commercial kits (for example the QUIKCHANGE™ lightening mutagenesis kit from Qiagen or Stratagene). After site-directed mutagenesis, nucleic acids were transformed into the *Escherichia coli* strain BL21 Star™ (DE3) (Invitrogen™, Lifetechnologies). Replacements within the BM3 gene were generated by first introducing Bsal-

sites into a vector backbone with harboring SEQ ID No. 122, yielding an entry vector, and subsequent ligation of insert sequences from a library with different length and properties in-frame into the BM3 gene.

Transformed cells were tested in appropriate biotransformation reactions, in order to determine product yield and selectivity. Appropriate biotransformation reactions are described below, see e.g. Example 2. Sequence verification was performed as known in the art.

Glycerol stocks of the *E. coli* cultures transformed with the respective expression plasmids were prepared by adding one volume of 40% glycerol solution to one volume of *E. coli* culture.

For isolation of single bacterial colonies, appropriate dilutions of *E. coli* cultures were plated onto LB-Agar plates containing suitable concentrations of kanamycin and incubated at 37°C until single colonied were obtained.

Example 2

Analysis of P450-BM3 variants for estradiol or estrone production

Cultivation

For screening purposes *E. coli* strain BL21 Star™ (DE3) (Invitrogen™, Lifetechnologies) was used as host for expression plasmids (Example 1). For pre-cultures sterilized 2.5-mL 96-well deep-well plates (850301, HJ-Bioanalytik, Erkelenz, Germany) were filled with 490 µL LB-medium (LB Broth Miller, Fisher Bioreagents, BP1426-500) supplemented with kanamycin (Sigma-Aldrich, 50 µg/mL), inoculated with 10 µL of glycerol stocks of the respective variants (that were prepared in 96-well microtiter plates) or alternatively 500 µL LB-medium were inoculated with cell material from Agar plate colonies. Pre-cultures were incubated for 17 hours at 37°C and 250 rpm in a climo shaker ISF1-X (Kühner AG, Birsfelden, Switzerland).

Medium for expression cultures contained Tryptone (12 g/L), Yeast extract (24 g/L), Meat Peptone, tryptically digested (2 g/L), KH₂PO₄ (2.2 g/L), K₂HPO₄ (9.4 g/L), glycerol (0.4% [v/v]), Riboflavin (0.001 g/L), Thiamine hydrochloride (0.337 g/L) and Oxford trace element solution (250 µL/L). Tryptone, Yeast extract and Meat peptone were prepared as 50 g/L stock solutions, and K-PO₄ was prepared as a 50x stock solution. These stock solutions were sterilized at 121 °C for 20 minutes. Riboflavin and Thiamine hydrochloride were freshly prepared as 0.06 g/L and 10 g/L stock solutions and sterile-filtered, respectively. The medium was prepared from the stock solutions and Kanamycin (Kanamycin solution, K0254, Sigma-Aldrich, St. Louis, MO, USA) was added for a final concentration of 50 mg/L.

For expression cultures sterilized 2.5-mL 96-well deep-well plates (850301, HJ-Bioanalytik, Erkelenz, Germany) were filled with 440 µL expression medium supplemented with kanamycin (50 mg/L); expression cultures were inoculated with 50 µL of pre-cultures. The expression culture plates were incubated at 37°C and 250 rpm in a climo shaker. After 4 h incubation 10 µL

of 30 mM IPTG diluted in expression medium (supplemented with 50 mg/L kanamycin) was added per well to induce enzyme expression (resulting in a final concentration of 0.6 mM IPTG). The expression culture plates were subsequently incubated for 20 hours at 27°C in a shaking incubator.

- 5 Cells were harvested by centrifugation of the expression cultures for 15 minutes at 4°C and 2500 × g. The culture supernatant was discarded, and the remaining cell pellets were resuspended in 495 µL of P450 assay buffer (100 mM KPO4-buffer at pH 7.4, 50 g/L glucose, 35 g/L glycerol, 1 mM EDTA) or 250 µL of 2x P450 assay buffer (200 mM KPO4-buffer at pH 7.0, 100 g/L glucose, 70 g/L glycerol, 2 mM EDTA). The deep-well plates were stored at -80°C until use.

10 Biotransformation

- The deep-well plates containing the cell suspensions were thawed at room temperature. The biotransformation reaction was started (i) by addition of 5 µL of Δ1-testosterone or androsta-1,4-diene-3,17-dione (ADD) stock solution (at 5 mg/mL or 10 mg/mL in *N,N*-Dimethylformamide) to cells resuspended in 495 µL of P450 assay buffer, or (ii) by addition of 250 µL of androsta-1,4-diene-3,17-dione (ADD) stock (at 4000 mg/mL) to 250 µL cell suspension in 2x P450 assay buffer, and incubation occurred for 22 h at 27°C in a shaking incubator. Reactions were stopped by adding 500 µL of Stop solution (containing Acetonitrile, Methanol and Dimethylsulfoxide in a 1:1:1 ratio), and for extraction of steroids incubated on a shaker for 30 min at 20°C. Afterwards the plates were centrifuged for 15 min at 4°C and 2500× g. From each well 120 µL of the supernatant was transferred to a 96-well filter plate (Corning, 3504) that was subsequently centrifuged for 5 min at 4°C and 650× g. The filtered samples were stored at -20°C or directly submitted to HPLC analysis.

Analytical HPLC method

Analytical HPLC was performed with the following setup:

- 25 Instrument: Agilent Technologies 1290 Infinity; Column: Ascentis Express C18, 50 x 2.1mm, 2.7 µm; Eluent A: Water (+0.025% or 0.005% Trifluoroacetic acid); Eluent B: Acetonitrile; Flow 1.5 mL/min; Temperature: 45 °C; injection volume: 4 µL, detection at 280 nm. Gradient: 0 - 0.18 min 5% solvent B, 0.18 - 0.4 min 5 - 10% solvent B, 0.4 – 4.6 min 10 – 27% solvent B, 4.6 – 7.0 min 27 – 95% solvent B, 7.01 – 7.5 min 5% solvent B.
- 30 Androsta-1,4-diene-3,17-dione (ADD), Δ1-testosterone, estradiol and estrone (dissolved in *N,N*-Dimethylformamide) were used as reference substances and as a standard for quantification. Appropriate dilutions were prepared covering the range up to the maximum concentration used in the biotransformation.
- Observed retention times were 4.01 min for Δ1-testosterone, 4.22 min for ADD, 4.37 min for estradiol and 4.86 min for estrone. The concentration of the respective compounds in samples was calculated by comparing the obtained peak areas with those of the standards.

Example 3

Conversion of Δ 1-testosterone to estradiol using BM3 variants based on parent type BM3-268 (Seq ID No. 5)

5 Variants of BM3-268 (Seq ID No. 5) with mutations in one or two positions were prepared as described in Example 1 and screened for Δ 1-testosterone to estradiol conversion. Cultivation, biotransformation and HPLC analysis was performed as described in Example 2. Glycerol stocks were used as inoculum for cultivation, and the initial substrate (Δ 1-testosterone) concentration was 50 mg/L.

10 Results for estradiol product yield and the selectivity factor for estradiol are shown in Figures 1 a, b and 2 a, b. For BM3-268 derived variants comprising mutations at position L78, E82 or A87 results for Δ 1-testosterone to estradiol conversion are presented in Figure 1 c.

Example 4

Conversion of Δ 1-testosterone to estradiol using BM3 variants based on BM3-268; M177Y, A184Y (SEQ ID No. 15)

15 Variants of the BM3 derivative BM3-268; M177Y, A184Y (Seq ID No. 15) with further mutations in up to six positions (Example 1) were screened for Δ 1-testosterone to estradiol conversion. Cultivation, biotransformation and HPLC analysis was performed as described in Example 2. Glycerol stocks were used as inoculum for cultivation, and the initial Δ 1-testosterone concentration used in the experiment was 100 mg/L.

20 Resulting estradiol product yields and estradiol selectivity factors are shown in Figure 3 a, b and Figure 4 a, b, c, d, e, f. For Seq ID No. 15 derived variants comprising mutations at position L78, E82 or A87 results for Δ 1-testosterone to estradiol conversion are presented in Figure 3 c.

Example 5

25 Conversion of Δ 1-testosterone to estradiol using BM3-268 (Seq ID No. 5) variants with up to seven mutations.

30 Variants of BM3-268 (Seq ID No. 5) with up to seven mutations in different positions (Example 1) were screened for Δ 1-testosterone to estradiol conversion. Cultivation, biotransformation and HPLC analysis was performed as described in Example 2. Agar plate colonies were used as inoculum for cultivation, and the initial Δ 1-testosterone concentration used in the experiment was 50 mg/L. Results for estradiol yield and the selectivity factor for estradiol are shown in Figure 5 a and b.

Example 6

Conversion of androsta-1,4-diene-3,17-dione (ADD) to estrone using BM3 variants based on BM3-268; M177Y, A184Y (SEQ ID No. 15)

5 Variants of the BM3-268; M177Y, A184Y (Seq ID No. 15) with further mutations in up to six positions (Example 1) were screened for androsta-1,4-diene-3,17-dione (ADD) to estrone conversion. Cultivation, biotransformation and HPLC analysis was performed as described in Example 2. Glycerol stocks were used as inoculum for cultivation, and the initial ADD concentration used in the experiment was 100 mg/L.

10 Results for estrone yield and selectivity factor for estrone are shown in Figure 6 a, b and Figure 7 a, b, c, d, e, f. For Seq ID No. 15 derived variants comprising mutations at position L78, E82 or A87 results for ADD to estrone conversion are presented in Figure 6 c.

Example 7

Conversion of androsta-1,4-diene-3,17-dione (ADD) to estrone using BM3-268 (Seq ID No. 5) variants with up to six mutations.

15 Variants of BM3-268 (Seq ID No. 5) with up to six mutations in different positions (Example 1) were screened for androsta-1,4-diene-3,17-dione (ADD) to estrone conversion. Cultivation, biotransformation and HPLC analysis was performed as described in Example 2. Agar plate colonies were used as inoculum for cultivation, and the initial ADD concentration used in the experiment was 100 mg/L. Results for estrone yield and the selectivity factor for estrone are shown in Figure 8.

20 **Example 7A**

Conversion of androsta-1,4-diene-3,17-dione (ADD) to estrone using BM3 variants based on BM3-268; S72G, V78L, A82E, F87A, M177Y, V178P, L181Y, A184Y, L188F (SEQ ID No. 117)

25 Variants of the BM3-268; S72G, V78L, A82E, F87A, M177Y, V178P, L181Y, A184Y, L188F (SEQ ID No. 117) with further mutations in one or two positions (Example 1) were screened for androsta-1,4-diene-3,17-dione (ADD) to estrone conversion. Cultivation, biotransformation and HPLC analysis was performed as described in Example 2. Glycerol stocks were used as inoculum for cultivation, and the initial ADD concentration used in the experiment was 2000 mg/L.

30 Results for obtained estrone titers and target product ratio for estrone are shown in Figure 9 and Figure 10.

Example 7B

Conversion of androsta-1,4-diene-3,17-dione (ADD) to estrone using BM3-268; S72G, V78L, A82E, F87A, M177Y, V178P, L181Y, A184Y, L188F (SEQ ID No. 117) variants with up to five mutations.

5 Variants of BM3-268; S72G, V78L, A82E, F87A, M177Y, V178P, L181Y, A184Y, L188F (SEQ ID No. 117) with up to five mutations in different positions (Example 1) were screened for androsta-1,4-diene-3,17-dione (ADD) to estrone conversion. Cultivation, biotransformation and HPLC analysis was performed as described in Example 2. Agar plate colonies were used as inoculum for cultivation, and the initial ADD concentration used in the experiment was 2000 mg/L. Results for estrone titer and the target product ratio for estrone are shown in Figure 11.

10 **Example 7C**

Conversion of androsta-1,4-diene-3,17-dione (ADD) to estrone using BM3-268; S72G, V78L, A82E, F87A, T146F, M177Y, V178W, L181Y, A184Y (SEQ ID No. 118) variants with three mutations.

15 Variant G457S, I458G, A477N of BM3-268; S72G, V78L, A82E, F87A, T146F, M177Y, V178W, L181Y, A184Y (SEQ ID No. 118) (see Example 1) was screened for androsta-1,4-diene-3,17-dione (ADD) to estrone conversion. Cultivation, biotransformation and HPLC analysis was performed as described in Example 2. Glycerol stocks were used as inoculum for cultivation, and the initial ADD concentration used in the experiment was 2000 mg/L. Results for the obtained estrone titer and target product ratio (TPR) are listed in Table E7C.

20 **Table E7C:** Obtained estrone titers and target product ratio (TPR) with BM3 variants Seq ID No. 118 and 119.

BM3-268 variant	SEQ ID No.	Estrone titer (mean) (mg/L)	Estrone titer (standard deviation) (mg/L)	TPR Estrone (mean)	TPR Estrone (standard deviation)
S72G, T146F, M177Y, V178W, L181Y, A184Y	118	209.22	21.26	0.732	0.169
S72G, T146F, M177Y, V178W, L181Y, A184Y, G457S, I458G, A477N	119	253.32	26.78	0.784	0.146

Example 7D

25 Conversion of androsta-1,4-diene-3,17-dione (ADD) to estrone using BM3-268; S72G, T146F, M177Y, V178W, L181Y, A184Y, G457S, I458G, A477N (SEQ ID No. 119) with exchanges of the amino acid sequence between positions 458 and 477 by inserts with varying length and properties.

Variants of BM3-268; S72G, T146F, M177Y, V178W, L181Y, A184Y, G457S, I458G, A477N (SEQ ID No. 119) with amino acid sequence exchanges between positions 458 and 477

(Example 1) were screened for androsta-1,4-diene-3,17-dione (ADD) to estrone conversion. Cultivation, biotransformation and HPLC analysis was performed as described in Example 2. Glycerol stocks were used as inoculum for cultivation, and the initial ADD concentration used in the experiment was 2000 mg/L.

- 5 Results for the obtained estrone titers and target product ratios (TPR) are listed in Table E7D. Only those variants are listed where - relative to the parent type - the insert ("linker") improved (i) product yield as determined by titer and/or (ii) selectivity as determined by TPR.

Table E7D: Obtained estrone titer and target product ratio (TPR) with BM3 variants with amino acid sequence exchanges between positions 458 and 477 in BM3-268; S72G, T146F, M177Y, V178W, L181Y, A184Y, G457S, I458G, A477N (SEQ ID No. 119)

Linker/insert Variant	Estron-Titer (mg/L)	Estrone titer ratio relative to SEQ ID No. 119	TPR Estrone	TPR (variant to SEQ ID No. 119)
GKHIIQGPMYDVSGTAPVNVN	265.68	1.05	0.737	1.016
LRASVSAIQQTTKQIYDDQSLVNV	282.72	1.12	0.718	0.989
AASKPEFIKPEDVSAEVVEKEYQVQL	254.76	1.01	0.722	0.996
AKKPLNTEGVMKSRN	254.20	1.01	0.734	1.012
SAAAATPAVRTVPQYKYAAGVRNPQQHL	296.28	1.17	0.745	1.028
NAQPQVTMQQPAVHVQGGQEPL	265.56	1.05	0.951	1.000
TVHFESFGATNTNARENTPF	260.11	1.03	0.714	0.985
ENPGVTQLNRLAAHPPFAS	377.77	1.49	0.968	1.018
VKMVRVQAIEKNRYRA	327.64	1.29	0.958	1.008
VREALLRRQRAFRELPG	260.09	1.03	0.723	0.997
IRPRAIGGSKPRVA	256.58	1.02	0.751	1.036
DETYVPKEFNAETF	257.69	1.02	0.814	1.122
WQYTAATATPTIGHV	260.90	1.03	0.750	1.034
ARKLLSPEVAND	300.62	1.19	0.802	1.106
VQTPGHANQSMMPQFIMPANNQFSAQ	277.31	1.10	0.839	1.157
GGGGSGGGGSGGGGSGGGGS	272.08	1.08	0.769	1.060
APAPAPAPAPAPAPAP	253.89	1.01	0.724	0.999
PSTEQSAKKVRKKAEN	346.70	1.37	0.964	1.014
PSPSTEQSKVRKKAEN	290.90	1.15	0.965	1.015
PSPSTEQSAKKVR	259.95	1.03	0.829	1.143
TKLPEAQQRVGGCFLNLMPQMKTLYLTYC	135.79	0.54	0.975	1.025
ANHPSAVNVL	232.63	0.92	0.741	1.022
GNVPELPDTTEHSRTDL	237.15	0.94	0.961	1.011
TARKGKGAFCCNGQKLQV	225.89	0.89	0.961	1.010
PSDNVDAQLYNGFFSDAD	94.19	0.37	1.000	1.052
AMSLYLAGEISLLILDEPT	239.61	0.95	0.795	1.096
GCAWYELMPAETTVRLRA	250.97	1.00	0.740	1.021
LSLEVAEEIARLEAEV				

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ASSYTAPQPGLG	169.78	0.67	1.000	1.052
IPVPTRDLEKPF	189.87	0.75	0.981	1.032
VIGYMPIPVGVA	233.26	0.92	0.756	1.042
RGRVMARLARM	153.66	0.61	1.000	1.052
FLMKLINRPII	162.51	0.64	0.992	1.044
LSPDEAP	207.67	0.82	1.000	1.052
VEPPDL	193.74	0.76	1.000	1.052
FITPII	191.08	0.75	0.960	1.010
LRKMRTGKP	188.21	0.74	1.000	1.052
SNSTMKKHGR	244.12	0.96	0.808	1.114
YLSITFL	112.07	0.44	1.000	1.052
AAAEKNVPLY	216.16	0.85	0.977	1.028
SEFEDPLV	199.76	0.79	0.964	1.014
KRSPW	198.09	0.78	1.000	1.052
NASCT	176.89	0.70	0.955	1.005
IQRKK	197.47	0.78	1.000	1.052
EDSGF	222.51	0.88	0.969	1.019
FVGVL	188.23	0.74	1.000	1.052
GGGGSGGGGS	241.84	0.95	0.804	1.109
GGGGGS	205.94	0.81	0.966	1.016
PAPAP	128.37	0.51	0.952	1.001
PSPSTEVRKKAEN	249.06	0.99	0.748	1.031
PSPSTKKAEN	212.52	0.84	0.994	1.046
PEN	207.56	0.82	1.000	1.052

Example 7E

BM3 protein level of BM3 variants based on the wild type (SEQ ID No. 1) with up to eight amino acid exchanges.

- 5 Variants of wild type BM3 (SEQ ID No. 1) with up to eight mutations (Example 1) in different positions were screened for BM3 protein level. Cultivation of cells was carried out as described in Example 2. Cellular protein levels were analyzed on PAGE gel (NuPAGE™ 4 to 12%, Bis-Tris, 1.0 mm, Mini Protein Gel, 15-well; Thermo Fisher Scientific). Expression cultures were adjusted to an OD_{600 nm} of 0.5, and one volume of a 2-fold blend of NuPAGE® LDS Sample Buffer
- 10 (4X) and NuPAGE® Sample Reducing Agent (10X) (both Thermo Fisher Scientific) were added. Samples were denatured for 5 minutes at 99° C in a Thermocycler. Subsequently, 10 µL of denatured samples were loaded on the gel, and electrophoresis was carried out at for 60 minutes at 180 Volts in NuPAGE® MOPS SDS Running Buffer (Thermo Fisher Scientific). After electrophoresis the gels were stained using SimplyBlue™ SafeStain (Thermo Fisher Scientific),
- 15 and a digital image was taken. Quantification of protein bands was carried out using the software package ImageJ (ImageJ version 1.53e, National Institutes of Health, Bethesda, MD, USA). The gel image was converted to 8-bit grey scale and a background subtraction was performed using

the setting 'light background' at a rolling ball radius of 50. Areas in the gel profile were calculated for each lane for the BM3 protein peak and for the sum of all peaks (total protein). Based on the area values of BM3 protein and total protein the ratio of BM3 to total protein was calculated for each sample, and those values were compared to the wild type control samples ((area BM3/ area total protein for variant)/ (area BM3/ area total protein for wild type)). The results for the BM3 protein level relative to the BM3 wild type are listed in **Table E7E**.

Table E7E: BM3 protein levels of BM3 variants with up to eight mutations relative to the wild type (SEQ ID No. 1)

Mutations	BM3 area/ total area	BM3 area/ total area Variant-to-WT
C62L, A117P, G157L, N319E	0.498	1.255
C62L, G227P, N319E, K391L	0.399	1.048
C62L, S106A, A117P, A295D, V371P	0.400	1.053
C62L, S106A, A117P, G227P, P243E	0.448	1.129
C62L, S106A, Q128E, G157L, P243E, A295D, G368E, K391L	0.431	1.133
C62L, S106A, Q128E, G227P, K391L	0.400	1.052
C62L, S106A, Q128E, G227P, Q288E, K391L	0.407	1.070
C62L, S106A, Q128E, T152I, G227P, P243E, H408L	0.498	1.310
Q110E, Q128E, Q288E, N319E	0.415	1.046
Q128E, H285E, Q288E, N319E	0.401	1.011
S106A, A117P, Q128E, T152I, G227P, N319E, V371P, N395F	0.390	1.025
S106A, G227P, H285E, V371P, K391L, H408L	0.415	1.045
S106A, H285E, Q288E	0.539	1.358
S106A, Q110E, G227P, H285E, K391L	0.421	1.108
S106A, Q110E, G227P, N319E, K391L, N395F	0.583	1.469
S106A, Q110E, Q128E, G227P, Q288E, N319E, K391L	0.424	1.069
S106A, Q128E	0.400	1.008
S106A, Q128E, H285E	0.523	1.317

Example 8

- 10 Method for screening P450-BM3 variants for the C19 hydroxylation of (17 β)-17-hydroxyandrosta-1,4-dien-3-one (delta-1-testosterone)

Media

Tryptone (10 g/L), sodium chloride (10 g/L) and yeast extract (5 g/L) in demineralized water. The medium was sterilized at 121 °C for 20 minutes. Afterwards kanamycin (50 mg/L) was added.

- 15 Preculture

Each well of a 96 deep well plate contained 0.25 ml of the medium which was inoculated with the *E. coli* strains (2 μ L) containing the P450-BM3 variants from glycerol stocks. The plate was shaken at 37 °C and 800 rpm for 17 hours.

Main culture

Each well of a 96 deep well plate contained 1.5 ml of the medium which was inoculated with 50 μ L from the preculture. The cultures were shaken at 37 °C and 800 rpm for 3 hours. Then the temperature was decreased to 27 °C within 1 hour and IPTG (1 mM) in demineralized water was added to start the protein expression. After 4 hours of expression, the plates were harvested by centrifugation, the supernatant was discarded, the pellets were resuspended in 500 μ L reaction buffer (KH₂PO₄ (4 g/L), K₂HPO₄ (12.3 g/L), glycerol (4% (v/v)), glucose (5% (w/v)), EDTA (0.5 mM)), frozen in liquid nitrogen and stored at –80 °C.

Biotransformation

The plates were thawed and the reaction was started by addition of 5 μ L of steroid stock solution of (17 β)-17-hydroxyandrost-1,4-dien-3-one (12.5 μ g dissolved in 5 μ L of DMF) and plates were shaken for 22 h at 700 rpm and 27 °C while covered with lids. The plates were frozen in liquid nitrogen and stored at –80 °C. The thawed samples were prepared for HPLC screening by adding 500 μ L mixture of acetonitrile / methanol / DMSO 1:1:1 (HPLC grade). The plates were shaken and centrifuged and 200 μ L of the supernatant was transferred to an analytics plate. The plate was covered with a silicone sealing mat and submitted to HPLC analysis.

HPLC AnalysisAnalytical chiral HPLC method:

Instrument: Waters Acquity UPLCMS SingleQuad; Column: Thermo Accucore C30 2.6 μ m, 50x2.1 mm; Eluent A: water + 0.05 vol % formic acid, eluent B: acetonitrile + 0.05 vol % formic acid; Gradient: 0.0-2.0 min 5-30 % B, 2.0-5.0 min 85 % B; injection volume 4 μ L; flow 0.9 mL/min; temperature: 45 °C; DAD scan: 248-280 nm.

Table E1: HPLC retention times

Substance	Retention Time
Delta-1-testosterone	2.40
Estradiol (Example 10)	2.55
(15 β -OH)-delta-1-testosterone (Example 12)	1.39
(1 α ,2 α -epoxy)-testosterone (Example 11)	2.46
(1 α ,2 α -epoxy-15 β -OH)-testosterone (Example 15)	1.35
(6 β -OH)-delta-1-testosterone (Example 16)	1.50
(1 α ,2 α -epoxy-19-OH)-testosterone (Example 14)	1.56
(15 β -OH)-estradiol (Example 13)	1.69

Results

After testing hundreds of P450-BM3 variants, only few mutants allowed for the C19-hydroxylation of delta-1-testosterone leading to estradiol as final product:

Table E2: Estradiol product yield in % for some P450 BM3 variants.

	mutations	substrate	product	Screening yield (%)
BM3 254	V78Y, A82E, F87A	delta-1-testosterone	estradiol	1.9
BM3 261	V78M, A82E, F87A	delta-1-testosterone	estradiol	6
BM3 263	V78I, A82E, F87A	delta-1-testosterone	estradiol	4.8
BM3 268	V78L, A82E, F87A	delta-1-testosterone	estradiol	6.6

In a subsequent screening experiment of four BM3 variants the following screening yields were found for the following (side) products:

Table E3: Products formed with delta-1-testosterone as substrate for some P450 BM3 variants.

Variant	Substrate	Screening yield (%)					
		Example 10	Example 12	Example 11	Example 15	Example 16	Example 14
BM3 254	delta-1-testosterone	2.5	10.3	8.6	3.4	2.2	-
BM3 261	delta-1-testosterone	6.3	15	13.2	9.3	0.4	10.1
BM3 263	delta-1-testosterone	3	21.6	1.6	11.8	1.5	6.5
BM3 268	delta-1-testosterone	6.7	26.3	5.4	7.8	4.1	10.3

5 **Example 9**

Method for screening P450-BM3 variants for the C19 hydroxylation of (17beta)-17-hydroxyandrost-4-en-3-one (testosterone) as substrate

Media, preculture and main culture were prepared as described for delta-1-testosterone (Example 8).

10 **HPLC Analysis**

Analytical chiral HPLC method:

Instrument: Waters Acquity UPLCMS SingleQuad; Column: Ascentis Express C18 2.7 µm, 50x2.1 mm; Eluent A: water + 0.05 vol % formic acid, eluent B: acetonitrile + 0.05 vol % formic acid; Gradient: 0.0-3.0 min 5-30 % B, 3.0-5.0 95 % B, 5.0-5.5 5 % B; injection volume 4 µL; flow 0.9 mL/min; temperature: 45 °C; DAD scan: 244 nm.

Table E4: HPLC retention times

Substance	Retention Time
Testosterone	3.45
(19-OH)-testosterone (Example 17)	2.19
(2beta,19-di-OH)-testosterone (Example 20)	1.63

(15beta, 19-di-OH)-testosterone (Example 18)	1.23
(2beta, 15beta-di-OH)-testosterone (Example 19)	1.17
(15beta-OH)-testosterone	1.95
(2beta-OH)-testosterone	2.92

Results

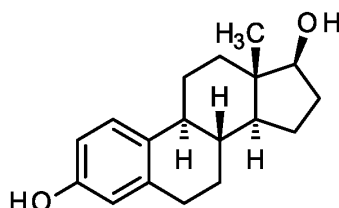
After testing hundreds of P450-BM3 variants, only few variants allowed for the C19-hydroxylation of testosterone:

Table E5: Products formed with testosterone as substrate for some P450 BM3 variants.

Variant	Substrate	Screening yield (%)					
		Example 17	Example 20	Example 18	Example 19	(2beta-OH)-testosterone	(15beta-OH)-testosterone
BM3 254	testosterone	8	4	-	-	29	5
BM3 261	testosterone	-	23	-	13	31	17
BM3 263	testosterone	-	11	4	5	35	16
BM3 268	testosterone	-	39	5	7	9	20

5 Example 10

(17beta)-estra-1(10),2,4-triene-3,17-diol



Expression system

Expression system *E. coli* BL 21(DE3) pETM11 with BM3-268 was provided by M. T. Reetz and is described in (Kille Sabrina 2011) (DOI:10.1038/NCHEM.1113). The respective protein and gene sequence such as pETM11-BM3-268 is given in SEQ ID No. 5 and 63, respectively.

Cultivation

Preculture medium comprised tryptone (10 g/L), sodium chloride (10 g/L) and yeast extract (5 g/L) in demineralized water. The medium was sterilized before kanamycin (50 mg/L) was added. One preculture (100 mL) was inoculated with the strain *E. coli* BL 21 (DE3) pETM11-BM3-268 (SEQ ID No.5) (50 µL) containing the desired plasmids and was shaken at 37 °C and 165 rpm for 16 hours. This preculture (100 mL) was used to inoculate one 5 L steel fermenter. The cultivation medium was prepared in the fermenter. Tryptone (20 g/L), sodium chloride (20 g/L), yeast extract (10 g/L) and Pluronic® PE 8100 (0.5 mL) were dissolved in demineralized water (4.0 L) and sterilized for 30 minutes at 121 °C in the fermenter. Afterwards kanamycin (0.25 g)

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in demineralized water (10 mL) was added. After the inoculation of the fermenter (16 hours old preculture), the inoculated culture was stirred at 500 rpm at 37 °C with an aeration rate of 2.5 L/min and an oxygen partial pressure of 30 % regulated by the stirring rate of up to 1500 rpm. After 3 hours an aqueous glucose solution (25 %, 40 g/h) was added. After 4 hours and 25 minutes an OD550 of 16.16 was reached, the temperature was decreased to 28 °C within 10 minutes and IPTG (1.19 g) in demineralized water (50 mL) was added to start the protein expression. After additional 8 hours and 35 minutes the cells (124.6 g) were harvested by centrifugation, suspended in buffer (125 mL; KH₂PO₄ (4 g/L), K₂HPO₄ (12.3 g/L), glycerol (4% (v/v)), glucose (5% (w/v)), EDTA (0.5 mM)), frozen in liquid nitrogen and stored at -80 °C until further use in aliquots of 4 x 45 mL, 1 x 35 mL und 10 x 1 mL.

Biotransformation

To a 5 L steel fermenter which contained KH₂PO₄ (15 g), K₂HPO₄ (46.15 g), Pluronic® PE 8100 (0.5 mL), demineralized water (3.8 L), aqueous glucose solution (50 %, 375 mL) and aqueous EDTA solution (0.5 M, 3.75 mL) (17beta)-17-hydroxyandrosta-1,4-dien-3-one (500 mg, 1.75 mmol) dissolved in DMF (10 mL) was added and the mixture was stirred at 140 rpm at 27 °C with an aeration rate of 2.0 L/min. Cells in buffer (215 mL) were added and the biotransformation was stirred at an oxygen partial pressure of 50 % regulated by the stirring rate of up to 800 rpm. The pH value was maintained at pH 7.4 by addition of an aqueous solution of sodium hydroxide (16 %). After 26 hours and 5 minutes the biotransformation was extracted with 4-methyl-2-pentanone. The organic layer was concentrated to give a crude oil (4.98 g) which was slurried with methanol, filtered and concentrated to give an oil (4 g). The crude product was further purified by flash chromatography using silica gel (dichloromethane/acetone gradient) and by preparative HPLC to give the title compound (4.3 mg, 0.8 % yield).

Preparative chiral HPLC method

Instrument: Waters Autopurificationsystem; Column: Waters XBrigde C18 5 µ 100x30 mm; Eluent A: water + 0.2 vol-% aqueous ammonia (32 %), Eluent B: acetonitrile; gradient: 0.00-0.50 min 17 % B (40-70 mL/min), 0.51-5.50 min 34-44 % B (70 mL/min), DAD scan: 210-400 nm.

Analytical chiral HPLC method

Instrument: Waters Acquity UPLCMS SingleQuad; Column: Acquity UPLC BEH C18 1.7 µm, 50x2.1 mm; eluent A: water + 0.2 vol % aqueous ammonia (32 %), eluent B: acetonitrile; gradient: 0-1.6 min 1-99 % B, 1.6-2.0 min 99 % B; flow 0.8 mL/min; temperature: 60 °C; DAD scan: 210-400 nm.

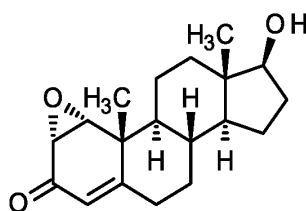
LC-MS: R_t = 1.01 min; MS (ESI^{neg}): m/z = 271 [M-H]⁻

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¹H-NMR (400 MHz, CHLOROFORM-d) δ [ppm]: 0.000 (1.83), 0.710 (4.29), 1.102 (0.18), 1.113 (0.30), 1.132 (0.31), 1.141 (0.29), 1.160 (0.32), 1.185 (0.85), 1.213 (0.54), 1.224 (0.54), 1.243 (0.60), 1.255 (0.59), 1.273 (0.57), 1.286 (0.46), 1.303 (0.52), 1.317 (0.44), 1.329 (0.32), 1.348 (0.47), 1.355 (0.45), 1.375 (0.54), 1.398 (0.55), 1.407 (0.56), 1.429 (0.57), 1.438 (0.51), 1.460 (0.41), 1.471 (0.41), 1.509 (0.63), 1.590 (0.24), 1.599 (0.21), 1.616 (0.31), 1.621 (0.29), 1.633 (0.23), 1.640 (0.26), 1.775 (0.23), 1.783 (0.23), 1.790 (0.24), 1.807 (0.22), 1.814 (0.24), 1.821 (0.20), 1.851 (0.23), 1.859 (0.34), 1.867 (0.24), 1.882 (0.22), 1.890 (0.31), 1.898 (0.20), 2.030 (0.22), 2.043 (0.24), 2.053 (0.23), 2.063 (0.26), 2.077 (0.30), 2.100 (0.32), 2.211 (0.25), 2.218 (0.25), 2.244 (0.24), 2.252 (0.22), 2.554 (16.00), 2.736 (0.53), 2.749 (0.54), 2.776 (0.22), 3.642 (0.29), 3.664 (0.50), 3.685 (0.27), 6.498 (0.70), 6.546 (0.36), 6.552 (0.31), 6.567 (0.39), 6.574 (0.34), 7.076 (0.58), 7.097 (0.53).

Example 11

(1 α ,2 α ,17 β)-17-hydroxy-1,2-epoxyandrost-4-en-3-one



For the preparation of the title compound see preparation of Example 10. The crude product was further purified by flash chromatography using silica gel (dichloromethane/acetone gradient) and by preparative HPLC to give the title compound (13 mg).

Preparative chiral HPLC method

Instrument: Waters Autopurification system; Column: Waters XBrigde C18 5 μ 100x30 mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0.00–0.50 min 16 % B (25-70 mL/min), 0.51–5.50 min 33-48 % B (70 mL/min), DAD scan: 210-400 nm.

Analytical chiral HPLC method

Instrument: Waters Acquity UPLCMS SingleQuad; Column: Acquity UPLC BEH C18 1.7 μ m, 50x2.1 mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0-1.6 min 1-99 % B, 1.6-2.0 min 99 % B; Flow: 0.8 mL/min; Temperature: 60 °C; DAD scan: 210-400 nm.

Analytical Chiral HPLC: R_t = 2.04 min.

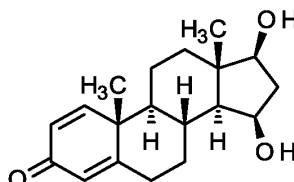
¹H-NMR (600MHz, Pyridine-d₅): δ [ppm]: 0.77 - 0.91 (m, 2H), 0.98 - 1.05 (m, 1H), 0.99 (s, 3H), 1.11 (td, 1H), 1.23 - 1.32 (m, 1H), 1.34 (s, 3H), 1.43 - 1.53 (m, 2H), 1.55 - 1.67 (m, 2H), 1.73 -

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1.81 (m, 2H), 2.01 - 2.14 (m, 3H), 2.20 (td, 1H), 3.63 (dd, 1H), 3.74 (d, 1H), 3.89 (td, 1H), 5.91 - 5.93 (m, 1H), 6.18 (d, 1H).

Example 12

(15 β ,17 β)-15,17-dihydroxyandrosta-1,4-dien-3-one



5

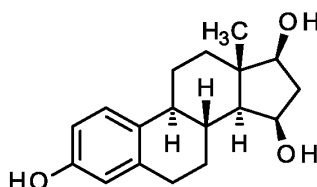
For the preparation of the title compound see preparation of Example 10. The crude product was further purified by flash chromatography using silica gel (dichloromethane/acetone gradient) and by preparative HPLC to give the title compound (78 mg).

Analytical HPLC: R_t = 0.74 min; MS (ESIpos): m/z = 303 $[M+H]^+$

- 10 $^1\text{H-NMR}$ (600 MHz, CDCl_3) δ [ppm]: 0.82 (dd, 1H), 1.05 - 1.16 (m, 3H), 1.11 (s, 3H), 1.28 (s, 3H), 1.58 - 1.65 (m, 1H), 1.71 (qd, 1H), 1.75 - 1.80 (m, 1H), 1.86 (dt, 1H), 2.09 (qd, 1H), 2.20 - 2.29 (m, 1H), 2.37 - 2.42 (m, 1H), 2.55 (tdd, 1H), 2.63 (ddd, 1H), 3.58 (t, 1H), 4.21 (ddd, 1H), 6.09 (s, 1H), 6.24 (dd, 1H), 7.08 (d, 1H).

Example 13

- 15 (15 β ,17 β)-estra-1(10),2,4-triene-3,15,17-triol

**Cultivation**

- 20 Medium of the preculture with tryptone (16 g/L), sodium chloride (10 g/L) and yeast extract (10 g/L) in demineralized water was adjusted to pH 7.3 with sodium hydroxide solution (16 % in water), and sterilized at 121 °C for 20 minutes. Afterwards kanamycin (50 mg/L) was added.

- One preculture (100 mL each) was inoculated with the *Escherichia coli* strain BL 21 Gold (DE3) $\Delta\text{dkgA} :: \text{FRT T7 gdh}$ (50 μL) expressing BM3-268; F77L and was shaken at 37 °C and 165 rpm for 16 hours. These precultures (100 mL) were used to inoculate one 10 L steel fermenter. The cultivation media was prepared in the fermenters. Tryptone (12 g/L), yeast extract (24 g/L),
25 predigested beef extract (2 g/L), KH_2PO_4 (2.2 g/L), K_2HPO_4 (9.4 g/L) and glycerol (87 %, 4.6

g/L) were dissolved in demineralized water (9.2 L) and sterilized for 20 minutes at 121 °C in the fermenter. Afterwards kanamycin (0.5 g) in water (20 mL), riboflavin (10 mg) in water (20 mL), thiamine hydrochloride (3.37 g) in water (10 mL) and Oxford trace metal solution (2.5 mL) were added. After the inoculation with the 17 hours old preculture, the inoculated culture was stirred at 315 rpm at 37 °C with an aeration rate of 3.3 L/min partial pressure at pH 6.6 which was regulated by addition of aqueous sodium hydroxide solution (16 %) or aqueous phosphoric acid solution (16 %). After 2 hours an OD550 of 0.92 was reached, the temperature was decreased to 27 °C within 15 minutes and IPTG (2.38 g) in demineralized water (40 mL) and aminolevulinic acid (838 mg) in demineralized water (40 mL) were added to start the protein expression. After 12 hours the aqueous phosphoric acid solution (16 %) was substituted for an aqueous glucose solution (50 %) for pH regulation. After additional 24 hours the cells were harvested by centrifugation (96.86 g), suspended in buffer (97 mL; KH₂PO₄ (4 g/L), K₂HPO₄ (12.3 g/L), glycerol (4% (v/v)), glucose (5% (w/v)), EDTA (0.5 mM)), frozen in liquid nitrogen and stored at -80 °C until further use in aliquots of 2 x 40 mL, 1 x 80 mL und 5 x 1 mL.

15 Biotransformation 1

To one 1 L Biostat Q fermenter which contained KH₂PO₄ (4 g), K₂HPO₄ (12.3 g), Pluronic® PE 8100 (0.1 mL), demineralized water (930 mL), aqueous glucose solution (50 %, 20 mL) and aqueous EDTA solution (0.5 M, 1 mL) (17beta)-17-hydroxyandrosta-1,4-dien-3-one (50 mg, 174.5 µmol) dissolved in DMF (4 mL) was added and the mixture was stirred at 300 rpm at 27 °C with an aeration rate of 0.5 L/min. The pH value was maintained at pH 7.4. Cells in buffer (20 mL) were added and the biotransformation was maintained at an oxygen partial pressure of 50 % by varying the stirring rate to up to 1200 rpm. After 24 hours and 40 minutes the biotransformation was frozen and stored at -20 °C.

Biotransformation 2

25 To one 1 L Biostat Q fermenter which contained KH₂PO₄ (4 g), K₂HPO₄ (12.3 g), Pluronic® PE 8100 (0.1 mL), demineralized water (930 mL), aqueous glucose solution (50 %, 20 mL) and aqueous EDTA solution (0.5 M, 1 mL) (17beta)-17-hydroxyandrosta-1,4-dien-3-one (50 mg, 174,5 µmol) dissolved in DMF (4 mL) was added and the mixture was stirred at 300 rpm at 27 °C with an aeration rate of 0.5 L/min. The pH value was maintained at pH 7.4. Cells in buffer (40 mL) were added and the biotransformation was maintained at an oxygen partial pressure of 50 % by varying the stirring rate to up to 1200 rpm. After 24 hours and 40 minutes the biotransformation was harvested, frozen and stored at -20 °C.

Biotransformation 3 + 4

To two 1 L Biostat Q fermenter which contained KH₂PO₄ (4 g), K₂HPO₄ (12.3 g), Pluronic® PE 8100 (0.1 mL), demineralized water (930 mL), aqueous glucose solution (50 %, 20 mL) and

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aqueous EDTA solution (0.5 M, 1 mL) (17 β)-17-hydroxyandrosta-1,4-dien-3-one (50 mg, 174.5 μ mol) dissolved in DMF (4 mL) was added and the mixture was stirred at 300 rpm at 27 °C with an aeration rate of 0.5 L/min. The pH value was maintained at pH 7.4. Cells in buffer (40 mL) were added and the biotransformation was maintained at an oxygen partial pressure of 50 % by varying the stirring rate to up to 1200 rpm. After 5 hours and 20 minutes the culture was harvested, frozen and stored at -20 °C.

The harvested four biotransformations were thawed, combined and extracted with 4-methyl-2-pentanone.

The organic phases were concentrated to give a crude oil (1.69 g) which was slurried with methanol and was filtered and concentrated to give an oil (1.04 g). The crude product was further purified by flash chromatography using silica gel (dichloromethane/ethanol gradient) and by preparative HPLC to give the title compound (13.0 mg).

Preparative chiral HPLC method

Instrument: Waters Autopurificationsystem; Column: Waters XBrigde C18 5 μ 50x50 mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0.00–0.50 min 5 % B (50-100 mL/min), 0.51–8.00 min 5 - 35 % B (100 mL/min), DAD scan: 210-400 nm.

Analytical chiral HPLC method

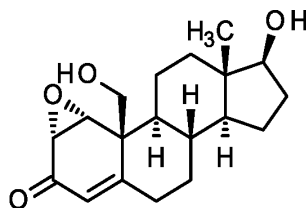
Instrument: Waters Acquity UPLCMS SingleQuad; Column: Acquity UPLC BEH C18 1.7 μ m, 50x2.1mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0-1.6 min 1-99 % B, 1.6-2.0 min 99 % B; Flow 0.8 mL/min; Temperature: 60 °C; DAD scan: 210-400 nm.

Analytical Chiral HPLC: R_t = 0.78 min.

$^1\text{H-NMR}$ (600MHz, Pyridine- d_5): δ [ppm]: 1.08 (dd, 1H), 1.37 (td, 1H), 1.49 (qd, 1H), 1.61 (s, 3H), 1.65 - 1.74 (m, 1H), 2.17 - 2.24 (m, 2H), 2.28 (ddd, 1H), 2.32 - 2.39 (m, 2H), 2.49 - 2.56 (m, 1H), 2.81 - 2.89 (m, 2H), 2.94 - 3.01 (m, 1H), 3.96 (td, 1H), 4.55 (s, 1H), 5.85 (d, 1H), 6.25 (d, 1H), 7.02 (d, 1H), 7.12 (dd, 1H), 7.38 (d, 1H), 11.16 (s, 1H).

Example 14

(1 α ,2 α ,17 β)-17,19-dihydroxy-1,2-epoxyandrosta-4-en-3-one



For the preparation of the title compound see Example 13. The crude product (1.04 g) was further purified by flash chromatography using silica gel (dichloromethane/ethanol gradient) and by preparative HPLC to give the title compound (7.4 mg).

Preparative chiral HPLC method

- 5 Instrument: Waters Autopurificationsystem; Column: Waters XBrigde C18 5 μ 50x50 mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0.00–0.50 min 5 % B (50-100 mL/min), 0.51–8.00 min 5 - 35 % B (100 mL/min), DAD scan: 210-400 nm.

Analytical chiral HPLC method

- Instrument: Waters Acquity UPLCMS SingleQuad; Column: Acquity UPLC BEH C18 1.7 μ m,
10 50x2.1mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0-1.6 min 1-99 % B, 1.6-2.0 min 99 % B; Flow 0.8 mL/min; Temperature: 60 °C; DAD scan: 210-400 nm.

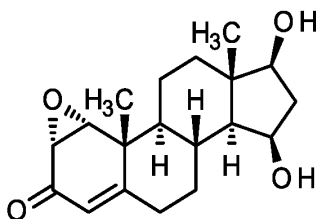
Analytical Chiral HPLC: R_t = 0.80 min.

- ¹H-NMR (600 MHz, CDCl₃) δ [ppm]: -0.006 (0.17), 0.000 (4.06), 0.005 (0.19), 0.772 (0.44), 0.816
15 (16.00), 0.880 (0.19), 0.969 (0.54), 0.981 (0.62), 0.987 (0.73), 0.989 (0.80), 0.999 (0.79), 1.001 (0.80), 1.007 (0.70), 1.015 (0.41), 1.020 (0.77), 1.036 (0.77), 1.044 (0.88), 1.057 (0.93), 1.064 (0.94), 1.069 (1.08), 1.076 (1.00), 1.086 (0.47), 1.090 (0.59), 1.096 (0.51), 1.253 (0.81), 1.260 (0.82), 1.266 (0.73), 1.278 (0.76), 1.282 (1.02), 1.287 (0.83), 1.299 (0.81), 1.305 (0.86), 1.313 (0.39), 1.324 (0.90), 1.334 (0.94), 1.344 (1.03), 1.354 (1.03), 1.365 (0.45), 1.374 (0.43), 1.432
20 (0.59), 1.437 (0.64), 1.445 (0.72), 1.451 (0.96), 1.454 (0.88), 1.457 (0.79), 1.460 (0.87), 1.465 (0.68), 1.468 (0.78), 1.471 (0.71), 1.474 (0.96), 1.480 (0.52), 1.488 (0.46), 1.494 (0.47), 1.506 (0.43), 1.512 (0.49), 1.528 (0.85), 1.534 (1.04), 1.550 (0.85), 1.556 (0.84), 1.572 (0.55), 1.574 (0.61), 1.579 (0.75), 1.586 (0.60), 1.590 (0.81), 1.595 (0.92), 1.602 (0.83), 1.607 (1.01), 1.611 (0.91), 1.616 (0.83), 1.623 (1.01), 1.628 (1.22), 1.640 (1.51), 1.761 (0.34), 1.767 (0.37), 1.779
25 (0.68), 1.785 (0.77), 1.799 (0.70), 1.806 (0.68), 1.816 (0.29), 1.823 (0.30), 1.862 (0.36), 1.868 (0.80), 1.873 (1.01), 1.882 (1.30), 1.884 (1.47), 1.889 (1.08), 1.898 (1.30), 1.905 (1.12), 1.910 (0.58), 1.992 (0.36), 2.000 (0.71), 2.004 (0.72), 2.014 (0.49), 2.022 (0.68), 2.026 (0.67), 2.033 (0.35), 2.052 (0.38), 2.062 (0.43), 2.067 (0.72), 2.074 (0.56), 2.077 (0.75), 2.083 (0.62), 2.090 (0.70), 2.092 (0.54), 2.100 (0.62), 2.105 (0.40), 2.115 (0.33), 2.312 (0.50), 2.319 (0.68), 2.324
30 (0.56), 2.334 (0.85), 2.339 (1.05), 2.346 (0.78), 2.372 (0.43), 2.396 (0.84), 2.403 (0.73), 2.405 (0.74), 2.418 (0.98), 2.426 (0.94), 2.439 (0.42), 2.441 (0.42), 2.447 (0.39), 3.358 (2.18), 3.361 (2.22), 3.364 (2.35), 3.367 (2.14), 3.633 (0.88), 3.647 (1.53), 3.662 (0.85), 3.944 (2.87), 3.950 (2.82), 4.173 (0.59), 4.263 (2.71), 4.281 (1.88), 5.900 (2.52), 7.267 (12.12).

Example 15

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(1 α ,2 α ,15 β ,17 β)-15,17-dihydroxy-1,2-epoxyandrost-4-en-3-one



For the preparation of the title compound see Example 13. The crude product (1.04 g) was further purified by flash chromatography using silica gel (dichloromethane/ethanol gradient) and by preparative HPLC to give the title compound (2.8 mg).

Preparative chiral HPLC method

Instrument: Waters Autopurificationsystem; Column: Waters XBrigde C18 5 μ 50x50 mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0.00–0.50 min 5 % B (50-100 mL/min), 0.51–8.00 min 5 - 35 % B (100 mL/min), DAD scan: 210-400 nm.

10 Analytical chiral HPLC method

Instrument: Waters Acquity UPLCMS SingleQuad; Column: Acquity UPLC BEH C18 1.7 μ m, 50x2.1mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0-1.6 min 1-99 % B, 1.6-2.0 min 99 % B; Flow 0.8 mL/min; Temperature: 60 °C; DAD scan: 210-400 nm.

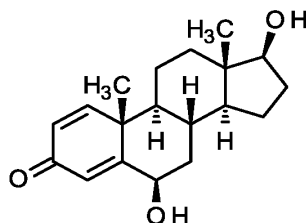
15 Analytical Chiral HPLC: R_t = 0.73 min.

$^1\text{H-NMR}$ (600 MHz, CHLOROFORM- d) δ [ppm]: -0.006 (0.48), 0.000 (11.79), 0.005 (0.44), 0.825 (1.12), 0.835 (1.15), 0.844 (1.18), 0.854 (1.23), 0.857 (1.22), 0.881 (0.35), 1.070 (0.62), 1.076 (0.66), 1.091 (1.49), 1.104 (13.48), 1.112 (1.31), 1.119 (1.11), 1.134 (0.39), 1.141 (0.37), 1.227 (0.58), 1.234 (0.66), 1.254 (2.09), 1.266 (0.93), 1.272 (0.78), 1.398 (0.70), 1.440 (15.22), 1.457 (1.29), 1.513 (0.45), 1.546 (0.44), 1.571 (2.00), 1.576 (2.63), 1.590 (16.00), 1.610 (1.91), 1.614 (2.15), 1.619 (1.27), 1.635 (0.91), 1.641 (0.91), 1.657 (0.40), 1.663 (0.37), 1.786 (0.38), 1.792 (0.80), 1.797 (0.85), 1.804 (0.43), 1.808 (0.34), 1.815 (0.61), 1.820 (0.62), 1.872 (0.61), 1.877 (0.96), 1.883 (0.58), 1.893 (0.64), 1.898 (0.85), 1.905 (0.51), 2.108 (0.34), 2.114 (0.32), 2.126 (0.65), 2.133 (0.70), 2.145 (0.62), 2.152 (0.65), 2.215 (0.47), 2.219 (0.62), 2.226 (0.54), 2.236 (0.60), 2.240 (0.78), 2.247 (0.68), 2.262 (0.38), 2.269 (0.60), 2.274 (0.52), 2.283 (0.45), 2.291 (0.57), 2.294 (0.51), 2.405 (0.51), 2.414 (0.47), 2.428 (0.86), 2.435 (0.79), 2.449 (0.41), 2.457 (0.36), 2.603 (0.67), 2.616 (0.88), 2.617 (0.84), 2.627 (0.79), 2.630 (0.83), 2.640 (0.83), 2.642 (0.82), 2.654 (0.65), 3.385 (1.56), 3.388 (1.69), 3.391 (1.66), 3.395 (1.55), 3.571 (0.58), 3.585 (1.12), 3.600 (0.56), 3.678 (2.43), 3.684 (2.34), 4.182 (0.51), 4.191 (0.87), 4.199 (0.51), 5.746 (2.23).

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Example 16

(6 β ,17 β)-6,17-dihydroxyandrost-1,4-dien-3-one



For the preparation of the title compound see Example 13. The crude product (1.04 g) was further purified by flash chromatography using silica gel (dichloromethane/ethanol gradient) and by preparative HPLC to give the title compound (2.4 mg).

Preparative chiral HPLC method

Instrument: Waters Autopurificationsystem; Column: Waters XBrigde C18 5 μ 50x50 mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0.00–0.50 min 5 % B (50–100 mL/min), 0.51–8.00 min 5 - 35 % B (100 mL/min), DAD scan: 210–400 nm.

Analytical chiral HPLC method

Instrument: Waters Acquity UPLCMS SingleQuad; Column: Acquity UPLC BEH C18 1.7 μ m, 50x2.1mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0–1.6 min 1–99 % B, 1.6–2.0 min 99 % B; Flow 0.8 mL/min; Temperature: 60 °C; DAD scan: 210–400 nm.

Analytical Chiral HPLC: R_t = 0.75 min.

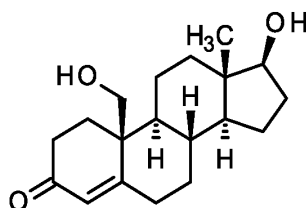
¹H-NMR (600 MHz, CDCl₃) δ [ppm]: 0.000 (7.05), 0.850 (0.34), 0.857 (10.48), 0.868 (0.34), 0.960 (0.40), 0.972 (0.39), 0.979 (0.55), 0.990 (0.55), 0.998 (0.48), 1.010 (0.46), 1.056 (0.37), 1.065 (0.43), 1.075 (0.61), 1.082 (0.65), 1.089 (0.52), 1.094 (0.57), 1.096 (0.56), 1.103 (1.02), 1.110 (0.72), 1.118 (0.75), 1.131 (0.41), 1.139 (0.42), 1.215 (0.47), 1.220 (0.44), 1.235 (0.62), 1.238 (0.70), 1.241 (0.73), 1.243 (0.71), 1.253 (1.01), 1.258 (1.09), 1.264 (0.79), 1.377 (0.64), 1.387 (0.69), 1.398 (0.76), 1.407 (0.80), 1.418 (0.40), 1.427 (0.45), 1.440 (1.03), 1.456 (12.77), 1.464 (0.72), 1.470 (0.66), 1.473 (0.63), 1.476 (0.51), 1.478 (0.58), 1.487 (0.52), 1.490 (0.47), 1.492 (0.64), 1.499 (0.39), 1.506 (0.37), 1.512 (0.39), 1.562 (0.37), 1.571 (0.42), 1.602 (1.00), 1.608 (1.08), 1.614 (1.25), 1.619 (1.41), 1.623 (1.48), 1.628 (1.35), 1.631 (1.37), 1.635 (1.48), 1.638 (1.35), 1.644 (1.17), 1.651 (1.05), 1.656 (0.96), 1.705 (0.40), 1.712 (0.39), 1.727 (0.56), 1.734 (0.56), 1.748 (0.66), 1.754 (0.70), 1.768 (0.66), 1.773 (0.86), 1.777 (0.87), 1.780 (0.57), 1.785 (0.56), 1.792 (0.36), 1.795 (0.39), 1.799 (0.37), 1.883 (0.57), 1.889 (0.73), 1.895 (0.53), 1.905 (0.56), 1.910 (0.66), 1.916 (0.46), 2.052 (0.49), 2.057 (0.81), 2.062 (0.63), 2.069 (0.34),

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2.074 (0.85), 2.081 (1.05), 2.085 (1.41), 2.090 (0.63), 2.093 (0.33), 2.097 (0.54), 2.102 (0.65), 2.107 (0.74), 2.112 (0.39), 2.122 (0.72), 2.127 (0.45), 3.646 (0.92), 3.660 (1.30), 3.674 (0.85), 4.543 (0.81), 4.548 (1.50), 4.553 (0.85), 6.162 (2.12), 6.165 (2.42), 6.210 (1.34), 6.213 (1.21), 6.226 (1.36), 6.229 (1.27), 7.052 (1.95), 7.069 (1.96), 7.264 (16.00).

5 Example 17

(17 β)-17,19-dihydroxyandrost-4-en-3-one



Preparation 1

Expression system

- 10 The strain *E. coli* BL 21 (DE3) pETM11-BM3-254 was provided by M. T. Reetz and is described in (Kille Sabrina 2011) (DOI:10.1038/NCHEM.1113). The protein and gene sequence are listed under SEQ ID No. 2 and 60, respectively.

Cultivation

- Medium of the preculture: tryptone (10 g/L), sodium chloride (10 g/L) and yeast extract (5 g/L)
15 in demineralized water. The media was sterilized at 121 °C for 20 minutes. Afterwards kanamycin (50 mg/L) was added.

- One preculture (100 mL) was inoculated with the strain *E. coli* BL 21 (DE3) pETM11-BM3-254 (50 μ L) containing the desired plasmids and was shaken at 37 °C and 165 rpm for 16 hours. This preculture (10 mL) was used to inoculate one 1 L Biostat Q fermenter. The cultivation
20 medium was prepared in the fermenter. Tryptone (20 g/L), sodium chloride (20 g/L), yeast extract (10 g/L) and Pluronic® PE 8100 (0.1 mL) were dissolved in demineralized water (1.0 L) and sterilized for 30 minutes at 121 °C in the fermenter. Afterwards kanamycin (50 mg) in demineralized water (5 mL) was added. After the inoculation of the fermenter the inoculated culture was stirred at 500 rpm at 37 °C with an aeration rate of 0.4 L/min and an oxygen partial
25 pressure of 30 % was maintained by the stirring at a rate of up to 1500 rpm. After 3 hours an aqueous glucose solution (50%, 4 g/h) was added. After 4 hours and 50 minutes an OD550 of 16.1 was reached, the temperature was decreased to 28 °C within 5 minutes and IPTG (238 mg) in demineralized water (10 mL) was added to start the protein expression. After additional 9 hours the cells (39.1 g) were harvested by centrifugation, suspended in buffer (39.1 mL; KH₂PO₄

(4 g/L), K₂HPO₄ (12.3 g/L), glycerol (4 % (v/v)), glucose (5 % (w/v)), EDTA (0.5 mM)), frozen in liquid nitrogen and stored at –80 °C until further use in aliquots of 1 x 75 mL, 3 x 1 mL.

Biotransformation

To two 1 L Biostat Q fermenter each containing KH₂PO₄ (3 g), K₂HPO₄ (9.23 g), Pluronic® PE 8100 (0.1 mL), demineralized water (0.75 L), aqueous glucose solution (50 %, 15 mL) and aqueous EDTA solution (0.5 M, 0.75 mL) (17beta)-17-hydroxyandrost-4-en-3-one (75.0 mg, 260 µmol) dissolved in DMF (5 mL) was added and the mixture was stirred at 300 rpm at 27 °C with an aeration rate of 0.5 L/min. Cells in buffer (each 37 mL) were added and the biotransformation was maintained at an oxygen partial pressure of 50 % by varying the stirring rate to up to 800 rpm. The pH value was maintained at pH7.4. After 26 hours aqueous glucose solution was added (50 %, 60 mL). After 102.5 hours the biotransformation was extracted with 4-methyl-2-pentanone. The organic layer was concentrated to give an oil (1.09 g) which was dissolved in methanol and was concentrated to give an oil (0.91 g). The crude product was further purified by flash chromatography using silica gel (dichloromethane/ methanol gradient) and by preparative HPLC to give the title compound (4.0 mg, 2,52 % yield).

Preparative chiral HPLC method:

Instrument: Labomatic HD5000, Labocord-5000; Gilson GX-241, Labcol Vario 4000, Column: Chiralpak IA 5 µ 250x30 mm; Eluent A: Acetonitrile + 0.1 Vol-% Diethylamine (99 %); Eluent B: Ethanol; Isocratic: 90 % A + 10 % B; Flow 50.0 mL/min; UV 254 nm.

Analytical chiral HPLC method:

Instrument: Agilent HPLC 1260; Column: Chiralpak IA 3 µ 100x4.6 mm; Eluent A: Acetonitrile + 0.1 Vol-% Diethylamine (99 %); Eluent B: Ethanol; Isocratic: 90 % A + 10 % B; Flow 1.4 mL/min; Temperature: 25 °C; DAD 254 nm.

¹H-NMR (600 MHz, CHLOROFORM-d) δ [ppm]: 0.79 (s, 3H), 0.92 - 0.99 (m, 1H), 1.02 - 1.09 (m, 3H), 1.31 (qd, 2H), 2.05 - 2.13 (m, 1H), 2.32 - 2.44 (m, 4H), 2.75 (ddd, 1H), 3.65 (t, 1H), 3.92 (d, 1H), 4.08 (dd, 1H), 5.96 (s, 1H).

Preparation 2

Expression system

In a second preparation the title compound was synthesized with the strain *E. coli* BL 21 Gold (DE3) ΔdkgA :: FRT T7 gdh and plasmid pETM11-BM3-254. The construction of the *E. coli* BL 21 Gold (DE3) ΔdkgA :: FRT T7 gdh strain is described in (Agudo R 2012). The strain was rendered competent and transformed with pETM11-BM3-254.

Preparation of competent cells & transformation

Required solutions: TFB1 buffer containing KCH₃COO (2.95 g/L), KCl (7.46 g/L), CaCl₂ (1.11 g/L), glycerol (130 mL). TFB2 buffer containing KCl (0.75 g/L), CaCl₂ (11.1 g/L), MOPS buffer (2.1 g/L), glycerol (130 mL). The buffers were autoclaved. A solution of MgCl₂·6H₂O (95.2 g/L) was prepared including sterile filtration.

- 5 A culture (5 mL) of Luria-Bertani broth medium (10 g/L bacto tryptone, 5 g/L bacto yeast extract, 10 g/L sodium chloride) was inoculated with a glycerol culture (2 µl) of the strain *E. coli* BL 21 Gold (DE3) Δ dkgA :: FRT T7 gdh. This culture was shaken at 37 °C and 165 rpm overnight. The overnight culture (1 mL) was used to inoculate a culture (100 mL) of Luria-Bertani broth medium. The culture was incubated at 37 °C and 165 rpm until an OD₆₀₀ of 0.4-0.5 was reached, whereupon the culture was subjected to centrifugation at 4 °C and 4000 rpm for 15 minutes. All following substances were pre-cooled in ice. The supernatant of the culture was discarded and the cell pellet was resuspended in TFB1 buffer (30 mL) and 3.2 mL MgCl₂ solution (3.2 mL) and incubated on ice for 15 min. The mixture was subjected to centrifugation at 4 °C and 4000 rpm for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in TFB2 buffer (4 mL) and was incubated for 15 min on ice. The cells were aliquoted à 50 µL, were snap frozen in liquid nitrogen and stored at -80 °C.

- For transformation 50 µL of the competent cells were thawed on ice for 15 min, 1-2 µL of plasmid (pETM11-BM3-254) were added, and the mixture was incubated on ice for 30 min. A heat shock was carried out for 45 sec at 42 °C, followed by incubation on ice for 5 min. After the addition of SOC Outgrowth Medium (350 µL), the mixture was incubated for 1 h at 37 °C and 1000 rpm shaking. The cells (100 µL) were placed on Luria-Bertani broth agar plates with kanamycin (50 mg/L) and incubated overnight at 37 °C. Single colonies were picked and sequenced.

Cultivation of *E. coli* BL 21 Gold (DE3) Δ dkgA :: FRT T7 gdh pETM11-BM3-254 and protein expression:

- 25 Media of the preculture: tryptone (10 g/L), sodium chloride (10 g/L) and yeast extract (5 g/L) in demineralized water. The media was sterilized at 121 °C for 20 minutes. Afterwards kanamycin (50 mg/L) was added.

Cell cultivation 1

- One preculture (100 mL) was inoculated with the strain *E. coli* BL 21 Gold (DE3) Δ dkgA :: FRT T7 gdh pETM11-BM3-254 (50 µL) containing the desired plasmid and was shaken at 37 °C and 165 rpm for 16 hours. This preculture (10 mL) was used to inoculate one 1 L Biostat Q fermenter. The cultivation media was prepared in the fermenters. Tryptone (20 g/L), sodium chloride (20 g/L), yeast extract (10 g/L) and Pluronic® PE 8100 (0.1 mL) were dissolved in demineralized water (1.0 L) and sterilized for 30 minutes at 121 °C in the fermenter. Afterwards kanamycin (50 mg) in demineralized water (5 mL) and Oxford trace metal solution (0.25 mL) was added. After

the inoculation of the fermenter, the inoculated culture was stirred at 500 rpm at 37 °C with an aeration rate of 0.4 L/min and an oxygen partial pressure of 30 % maintained by the stirring up to 1500 rpm. After 3 hours an aqueous glucose solution was added (25 %, 8 g/h). After 5 hours and 30 minutes OD550 of 15.72 was reached, the temperature was decreased to 28 °C within 10 minutes and IPTG (238 mg) in demineralized water (10 mL) was added to start the protein expression. After additional 9 hours and 50 minutes the cells (34.88 g) were harvested by centrifugation, suspended in buffer (34.88 mL; KH₂PO₄ (4 g/L), K₂HPO₄ (12.3 g/L), glycerol (4% (v/v)), glucose (5% (w/v)), EDTA (0.5 mM)), frozen in liquid nitrogen and stored at –80 °C until further use in aliquots of 2 x 25 mL, 3 x 1 mL and 1 x 13 mL.

10 Cell cultivation 2

One preculture (100 mL) was inoculated with the strain *E. coli* BL 21 Gold (DE3) Δ dkgA :: FRT T7 gdh pETM11-BM3-254 (50 μ L) containing the desired plasmid and was shaken at 37 °C and 165 rpm for 16 hours. This preculture (10 mL) was used to inoculate one 1 L Biostat Q fermenter. The cultivation media was prepared in the fermenters. Tryptone (20 g/L), sodium chloride (20 g/L), yeast extract (10 g/L) and Pluronic® PE 8100 (0.1 mL) was dissolved in demineralized water (4.0 L) and sterilized for 30 minutes at 121 °C in the fermenter. Afterwards kanamycin (50 mg) in demineralized water (5 mL) and Oxford trace metal solution (0.25 mL) was added. After inoculation of the fermenter, the inoculated culture was stirred at 500 rpm at 37 °C with an aeration rate of 0.4 L/min, and an oxygen partial pressure of 30 % was maintained by the stirring up to 1500 rpm. After 3 hours an aqueous glucose solution (25%, 8 g/h) was added. After 5 hours and 5 minutes an OD550 of 16.72 was reached, the temperature was decreased to 28 °C within 5 minutes and IPTG (238 mg) in demineralized water (10 mL) and aminolevulinic acid (42 mg) in demineralized water (5 mL) were added to start the protein expression. After additional 9 hours and 10 minutes the cells (38 g) were harvested by centrifugation, suspended in buffer (38 g; KH₂PO₄ (4 g/L), K₂HPO₄ (12.3 g/L), glycerol (4% (v/v)), glucose (5% (w/v)), EDTA (0.5 mM)), frozen in liquid nitrogen and stored at –80 °C until further use in aliquots of 2 x 25 mL, 3 x 1 mL and 1 x 19 mL.

Cell cultivation 3

One preculture (100 mL) was inoculated with the strain *E. coli* BL 21 Gold (DE3) Δ dkgA :: FRT T7 gdh pETM11-BM3-254 (50 μ L) containing the desired plasmid and was shaken at 37 °C and 165 rpm for 16 hours. This preculture (100 mL) was used to inoculate one 10 L steel fermenter. The cultivation media was prepared in the fermenter. Tryptone (20 g/L), sodium chloride (20 g/L), yeast extract (10 g/L) and Pluronic® PE 8100 (0.1 mL) were dissolved in demineralized water (8.8 L) and sterilized for 30 minutes at 121 °C in the fermenter. Afterwards kanamycin (500 mg) in demineralized water (10 mL) and Oxford trace metal solution (2.5 mL) was added. After inoculation of the fermenter (16 hours old preculture), the inoculated culture was stirred at

400 rpm at 37 °C with an aeration rate of 4.0 L/min and an oxygen partial pressure of 30 % maintained by stirring up to 1200 rpm. After 3 hours an aqueous glucose solution (25%, 80 g/h) was added. After 5 hours and 20 minutes an OD550 of 16.69 was reached, the temperature was decreased to 28 °C within 5 minutes and IPTG (2.38 g) in demineralized water (20 mL) and aminolevulinic acid (420 mg) in demineralized water (20 mL) was added to start the protein expression. After additional 9 hours and 25 minutes the cells (298.42g) were harvested by centrifugation, suspended in buffer (298.42 g; KH₂PO₄ (4 g/L), K₂HPO₄ (12.3 g/L), glycerol (4% (v/v)), glucose (5% (w/v)), EDTA (0.5 mM)), frozen in liquid nitrogen and stored at -80 °C until further use in aliquots of 7 x 25 mL, 3 x 1 mL and 7 x 50 mL.

10 Biotransformation 1

To one 1 L Biostat Q fermenter which contained KH₂PO₄ (4 g), K₂HPO₄ (12.3 g), Pluronic® PE 8100 (0.1 mL), demineralized water (930 mL), aqueous glucose solution (50 %, 20 mL) and aqueous EDTA solution (0.5 M, 1 mL) (17beta)-17-hydroxyandrost-4-en-3-one (100 mg, 347 µmol) dissolved in DMF (5 mL) was added and the mixture was stirred at 300 rpm at 27 °C with an aeration rate of 0.5 L/min. The pH value was maintained at pH 7.4. Cells in buffer (50 mL of cell cultivation 1) were added, and the biotransformation was maintained at an oxygen partial pressure of 50 % by varying the stirring rate to up to 800 rpm. After 26.5 hours the biotransformation was extracted with 4-methyl-2-pentanone.

Biotransformation 2

20 To one 1 L Biostat Q fermenter which contained KH₂PO₄ (4 g), K₂HPO₄ (12.3 g), Pluronic® PE 8100 (0.1 mL), demineralized water (930 mL), aqueous glucose solution (50 %, 20 mL) and aqueous EDTA solution (0.5 M, 1 mL) (17beta)-17-hydroxyandrost-4-en-3-one (100 mg, 347 µmol) dissolved in DMF (5 mL) was added and the mixture was stirred at 300 rpm at 27 °C with an aeration rate of 0.5 L/min. The pH value was maintained at pH 7.4. Cells in buffer (50 mL of cell cultivation 2) were added, and the biotransformation was maintained at an oxygen partial pressure of 50 % by varying the stirring rate to up to 800 rpm. After 26.5 hours the biotransformation was extracted with 4-methyl-2-pentanone.

Biotransformation 3

To one 1 L Biostat Q fermenter which contained KH₂PO₄ (4 g), K₂HPO₄ (12.3 g), Pluronic® PE 8100 (0.1 mL), demineralized water (930 mL), aqueous glucose solution (50 %, 20 mL) and aqueous EDTA solution (0.5 M, 1 mL) (17beta)-17-hydroxyandrost-4-en-3-one (100 mg, 347 µmol) dissolved in DMF (5 mL) was added and the mixture was stirred at 300 rpm at 27 °C with an aeration rate of 0.5 L/min. The pH value was maintained at pH 7.4. Cells in buffer (50 mL of cell cultivation 3) were added, and the biotransformation was maintained at an oxygen partial pressure of 50 % by varying the stirring rate to up to 1200 rpm. After 4 hours additional cells in

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buffer (25 mL of cell cultivation 3) were added. After 26 hours and 25 minutes the biotransformation was extracted with 4-methyl-2-pentanone.

Biotransformation 4 and 5

To two 1 L Biostat Q fermenter each containing KH₂PO₄ (4 g), K₂HPO₄ (12.3 g), Pluronic® PE 8100 (0.1 mL), demineralized water (930 mL), aqueous glucose solution (50 %, 20 mL) and aqueous EDTA solution (0.5 M, 1 mL) (17beta)-17-hydroxyandrost-4-en-3-one (25 mg, 87 µmol) dissolved in DMF (2.5 mL) was added and the mixture was stirred at 300 rpm at 27 °C with an aeration rate of 0.5 L/min. The pH value was maintained at pH 7.4. Cells in buffer (50 mL of cell cultivation 3) were added, and the biotransformation was maintained at an oxygen partial pressure of 50 % by varying the stirring rate to up to 800 rpm. After 26 hours and 25 minutes the biotransformation was extracted with 4-methyl-2-pentanone.

The organic phases of the five biotransformations were combined and concentrated to give an oil (4.45 g) which was slurried with methanol and was filtered and concentrated to give an oil (1.23 g). The crude product was further purified by flash chromatography using silica gel (dichloromethane/ethanol gradient) and by preparative HPLC to give the title compound (13.0 mg).

Analytical HPLC method:

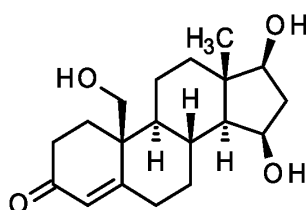
Instrument: Waters Acquity UPLCMS SingleQuad; Column: Acquity UPLC BEH C18 1.7 µm, 50x2.1 mm; Eluent A: Water + 0.1 Vol-% formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0-1.6 min 1-99 % B, 1.6-2.0 min 99 % B; Flow 0.8 mL/min; Temperature: 60 °C; DAD scan: 210-400 nm.

Preparative HPLC method:

Instrument: Waters Autopurificationsystem; Column: Waters XBrigde C18 5 µ 150x50 mm; Eluent A: Water + 0.1 Vol-% formic acid (99 %), Eluent B: Acetonitril; Gradient: 0.00–0.50 min 9 % B (40-100 mL/min), 0.51-8.50 min 19-41 % B (100 mL/min), DAD scan: 210-400 nm.

Example 18

(15beta,17beta)-15,17,19-trihydroxyandrost-4-en-3-one



For the preparation of the title compound see second preparation of Example 17. The crude product (1.23 g) was further purified by flash chromatography using silica gel (dichloromethane/ethanol gradient) and by preparative HPLC to give the title compound (2.2 mg).

5 Preparative chiral HPLC method:

Instrument: Waters Autopurificationsystem; Column: Waters XBrigde C18 5 μ 150x50 mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0.00–0.50 min 5 % B (40-100 mL/min), 0.51–8.50 min 5-30 % B (100 mL/min), DAD scan: 210-400 nm.

Analytical chiral HPLC method:

- 10 Instrument: Waters Acquity UPLCMS SingleQuad; Column: Acquity UPLC BEH C18 1.7 μ m, 50x2.1 mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0-1.6 min 1-99 % B, 1.6-2.0 min 99 % B; Flow: 0.8 mL/min; Temperature: 60 °C; DAD scan: 210-400 nm.

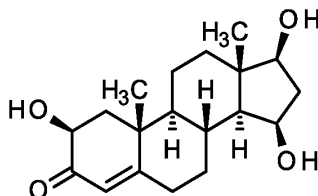
Analytical Chiral HPLC: R_t = 0.63 min.

- 15 $^1\text{H-NMR}$ (600MHz, DMSO- d_6) δ [ppm]: 0.61 (dd, 1H), 0.82 - 0.97 (m, 6H), 1.37 - 1.47 (m, 2H), 1.51 - 1.57 (m, 1H), 1.61 (td, 1H), 1.64 - 1.68 (m, 1H), 1.89 - 1.97 (m, 1H), 2.07 - 2.17 (m, 2H), 2.22 - 2.32 (m, 3H), 2.37 - 2.45 (m, 2H), 2.58 - 2.67 (m, 2H), 3.26 - 3.32 (m, 1H), 3.73 (dd, 1H), 3.85 (dd, 1H), 3.91 - 3.98 (m, 1H), 4.35 (d, 1H), 4.46 (d, 1H), 4.72 (t, 1H), 5.74 (s, 1H).

- $^1\text{H-NMR}$ (600 MHz, DMSO- d_6) δ [ppm]: -0.006 (0.85), 0.005 (0.77), 0.593 (1.06), 0.603 (1.08),
20 0.612 (1.10), 0.621 (1.08), 0.832 (0.48), 0.847 (0.83), 0.853 (0.89), 0.869 (0.54), 0.875 (0.56), 0.889 (16.00), 0.900 (0.68), 0.907 (0.64), 0.924 (1.06), 0.943 (1.10), 0.957 (0.56), 0.964 (0.54), 1.383 (0.68), 1.387 (0.70), 1.398 (0.85), 1.402 (0.95), 1.406 (0.83), 1.410 (0.77), 1.421 (1.12), 1.425 (1.31), 1.440 (0.68), 1.446 (0.66), 1.529 (0.77), 1.535 (0.83), 1.552 (0.58), 1.557 (0.54), 1.588 (0.46), 1.602 (0.83), 1.611 (0.85), 1.625 (0.48), 1.634 (0.46), 1.651 (1.02), 1.656 (0.60),
25 1.666 (0.64), 1.671 (0.95), 1.918 (0.73), 1.924 (0.79), 1.937 (0.73), 1.942 (0.75), 2.076 (0.44), 2.087 (0.60), 2.092 (0.79), 2.099 (0.56), 2.113 (0.73), 2.120 (1.37), 2.126 (1.14), 2.134 (0.54), 2.141 (0.58), 2.145 (0.64), 2.230 (0.77), 2.234 (0.56), 2.248 (0.79), 2.255 (1.31), 2.270 (1.35), 2.275 (0.93), 2.280 (1.24), 2.283 (1.22), 2.294 (1.43), 2.306 (0.77), 2.382 (0.81), 2.385 (1.16), 2.388 (1.64), 2.391 (1.31), 2.405 (0.75), 2.411 (0.70), 2.519 (3.59), 2.522 (3.63), 2.525 (3.05),
30 2.600 (0.58), 2.610 (1.08), 2.613 (1.04), 2.616 (1.33), 2.619 (1.02), 2.624 (0.79), 2.628 (0.75), 2.633 (0.70), 2.637 (0.62), 2.652 (0.62), 2.661 (0.48), 3.265 (0.54), 3.273 (0.58), 3.279 (1.14), 3.287 (1.16), 3.293 (0.58), 3.302 (0.56), 3.713 (0.99), 3.723 (1.04), 3.732 (1.31), 3.741 (1.26), 3.837 (1.02), 3.846 (1.08), 3.855 (0.81), 3.863 (0.77), 3.943 (0.87), 3.949 (0.87), 4.348 (2.88), 4.355 (2.82), 4.454 (2.47), 4.462 (2.38), 4.711 (1.18), 4.719 (2.47), 4.728 (1.16), 5.740 (3.34),
35 6.552 (0.56), 8.318 (1.16).

Example 19

(2beta,15beta,17beta)-2,15,17-trihydroxyandrost-4-en-3-one



For the preparation of the title compound see second preparation of Example 17. The crude product (1.23 g) was further purified by flash chromatography using silica gel (dichloromethane/ethanol gradient) and by preparative HPLC to give the title compound (1 mg).

Preparative chiral HPLC method:

Instrument: Waters Autopurificationsystem; Column: Waters XBrigde C18 5 μ 150x50 mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0.00–0.50 min 5 % B (40-100 mL/min), 0.51–8.50 min 5-30 % B (100 mL/min), DAD scan: 210-400 nm.

Analytical chiral HPLC method:

Instrument: Waters Acquity UPLCMS SingleQuad; Column: Acquity UPLC BEH C18 1.7 μ m, 50x2.1 mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0-1.6 min 1-99 % B, 1.6-2.0 min 99 % B; Flow: 0.8 mL/min; Temperature: 60 °C; DAD scan: 210-400 nm.

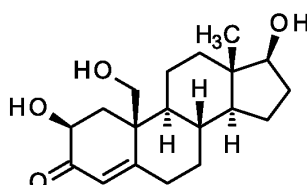
Analytical Chiral HPLC: $R_t = 0.64$ min.

¹H-NMR (400 MHz, CHLOROFORM-d) δ [ppm]: 0.774 (1.91), 0.788 (1.77), 0.802 (1.30), 0.816 (1.29), 0.997 (0.71), 1.010 (14.33), 1.027 (0.71), 1.031 (0.70), 1.040 (0.91), 1.051 (1.46), 1.061 (0.86), 1.072 (0.57), 1.082 (0.82), 1.092 (0.42), 1.129 (0.40), 1.136 (0.42), 1.148 (0.61), 1.160 (16.00), 1.170 (0.89), 1.174 (0.86), 1.184 (3.29), 1.191 (1.47), 1.210 (0.65), 1.349 (0.55), 1.358 (0.57), 1.381 (0.95), 1.389 (0.89), 1.407 (0.92), 1.415 (0.98), 1.434 (0.69), 1.439 (0.56), 1.443 (0.76), 1.456 (0.60), 1.464 (2.08), 1.474 (1.47), 1.498 (3.80), 1.507 (1.42), 1.517 (1.52), 1.523 (1.55), 1.533 (2.86), 1.538 (1.94), 1.554 (1.66), 1.559 (1.68), 1.640 (0.47), 1.708 (0.49), 1.716 (0.89), 1.724 (0.89), 1.733 (0.51), 1.740 (0.42), 1.748 (0.63), 1.757 (0.62), 1.779 (0.74), 1.788 (1.01), 1.796 (0.61), 1.811 (0.61), 1.819 (0.93), 1.828 (0.54), 1.943 (0.50), 1.979 (0.98), 2.026 (1.06), 2.036 (0.61), 2.057 (0.70), 2.063 (0.57), 2.106 (0.64), 2.191 (0.71), 2.198 (1.06), 2.207 (1.84), 2.215 (0.62), 2.227 (1.79), 2.234 (1.28), 2.240 (0.81), 2.400 (1.05), 2.414 (1.07), 2.434 (0.99), 2.448 (0.98), 2.512 (0.43), 2.516 (0.45), 2.521 (0.76), 2.532 (0.66), 2.535 (0.71), 2.540 (1.23), 2.543 (1.28), 2.558 (0.89), 2.562 (1.02), 2.568 (0.44), 2.577 (1.01), 2.580 (1.05), 2.598 (0.68), 3.504 (1.26), 3.526 (2.58), 3.547 (1.18), 4.093 (1.59), 4.101 (0.86), 4.107 (1.71), 4.114

(1.31), 4.119 (0.79), 4.127 (1.90), 4.133 (0.80), 4.142 (1.13), 5.234 (4.88), 5.755 (3.02), 5.757 (2.97).

Example 20

(2 β ,17 β)-2,17,19-trihydroxyandrost-4-en-3-one



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Media of the preculture: tryptone (10 g/L), sodium chloride (10 g/L) and yeast extract (5 g/L) in demineralized water. The media was sterilized at 121 °C for 20 minutes. Afterwards kanamycin (50 mg/L) was added.

Cell cultivation 1

- 10 One preculture (100 mL) was inoculated with the strain *E. coli* BL 21 Gold (DE3) Δ dkgA :: FRT T7 gdh pETM11-BM3-254 (50 μ L) containing the desired plasmids and was shaken at 37 °C and 165 rpm for 16 hours. This preculture (10 mL) was used to inoculate one 1 L Biostat Q fermenter. The cultivation media was prepared in the fermenter. Tryptone (20 g/L), sodium chloride (20 g/L), yeast extract (10 g/L) and Pluronic® PE 8100 (0.5 mL) were dissolved in demineralized
- 15 water (1.0 L) and sterilized for 30 minutes at 121 °C in the fermenter. Afterwards kanamycin (50 mg) in demineralized water (5 mL) was added. After the inoculation of the fermenter, the inoculated culture was stirred at 500 rpm at 37 °C with an aeration rate of 0.4 L/min and an oxygen partial pressure of 30 % was maintained by stirring of up to 1500 rpm. After 3 hours an aqueous glucose solution (25 %, 8 g/h) was added. After 5 hours and 30 minutes an OD550 of
- 20 16.2 was reached, the temperature was decreased to 28 °C within 15 minutes and IPTG (0.24 g) in demineralized water (10 mL) was added to start the protein expression. After additional 10 hours and 5 minutes the cells were harvested by centrifugation (38.8 g), suspended in buffer (38.8 mL; KH₂PO₄ (4 g/L), K₂HPO₄ (12.3 g/L), glycerol (4% (v/v)), glucose (5% (w/v)), EDTA (0.5 mM)), frozen in liquid nitrogen and stored at –80 °C until further use in aliquots of 3 x 1 mL
- 25 and 7 x 10 mL.

Cell cultivation 2

- One preculture (100 mL) was inoculated with the strain *E. coli* BL 21 Gold (DE3) Δ dkgA :: FRT T7 gdh pETM11-BM3-254 (50 μ L) containing the desired plasmids and was shaken at 37 °C and 165 rpm for 16 hours. This preculture (10 mL) was used to inoculate one 1 L Biostat Q fermenter.
- 30 The cultivation media was prepared in the fermenter. Tryptone (20 g/L), sodium chloride (20

g/L), yeast extract (10 g/L) and Pluronic® PE 8100 (0.5 mL) were dissolved in demineralized water (1.0 L) and sterilized for 30 minutes at 121 °C in the fermenter. Afterwards kanamycin (50 mg) in demineralized water (5 mL) was added. After the inoculation of the fermenter, the inoculated culture was stirred at 500 rpm at 37 °C with an aeration rate of 0.4 L/min and an oxygen partial pressure of 30 % was maintained by stirring of up to 1500 rpm. After 3 hours an aqueous glucose solution (25 %, 8 g/h) was added. After 5 hours an OD550 of 16.6 was reached, the temperature was decreased to 28 °C within 15 minutes and IPTG (0.24 g) in demineralized water (10 mL) was added to start the protein expression. After additional 9 hours and 15 minutes the cells were harvested by centrifugation (42 g), suspended in buffer (42 mL; KH₂PO₄ (4 g/L), K₂HPO₄ (12.3 g/L), glycerol (4% (v/v)), glucose (5% (w/v)), EDTA (0.5 mM)), frozen in liquid nitrogen and stored at –80 °C until further use in aliquots of 3 x 1 mL and 7 x 10 mL.

Cell cultivation 3

One preculture (100 mL) was inoculated with the strain *E. coli* BL 21 Gold (DE3) ΔdkgA :: FRT T7 gdh pETM11-BM3-254 (50 μL) containing the desired plasmids and was shaken at 37 °C and 165 rpm for 16 hours. This preculture (10 mL) was used to inoculate one 1 L Biostat Q fermenter. The cultivation media was prepared in the fermenter. Tryptone (20 g/L), sodium chloride (20 g/L), yeast extract (10 g/L) and Pluronic® PE 8100 (0.5 mL) were dissolved in demineralized water (1.0 L) and sterilized for 30 minutes at 121 °C in the fermenter. Afterwards kanamycin (50 mg) in demineralized water (5 mL) was added. After the inoculation of the fermenter, the inoculated culture was stirred at 500 rpm at 37 °C with an aeration rate of 0.4 L/min and an oxygen partial pressure of 30 % was maintained by stirring of up to 1500 rpm. After 3 hours an aqueous glucose solution (25 %, 8 g/h) was added. After 5 hours and 5 minutes an OD550 of 16.72 was reached, the temperature was decreased to 28 °C within 15 minutes and IPTG (0.238 g) in demineralized water (10 mL) and 5-aminolevulinic acid hydrochloride (0.042 g) in demineralized water (5 mL) was added to start the protein expression. After additional 9 hours and 40 minutes the cells were harvested by centrifugation (43 g), suspended in buffer (43 mL; KH₂PO₄ (4 g/L), K₂HPO₄ (12.3 g/L), glycerol (4% (v/v)), glucose (5% (w/v)), EDTA (0.5 mM)), frozen in liquid nitrogen and stored at –80 °C until further use in aliquots of 3 x 1 mL and 5 x 15 mL.

Biotransformation 1

To a 1 L Biostat Q fermenter which contained KH₂PO₄ (4 g), K₂HPO₄ (12.3 g), Pluronic® PE 8100 (0.1 mL), demineralized water (0.93 L), aqueous glucose solution (50 %, 20 mL) and aqueous EDTA solution (0.5 M, 1 mL) (17β)-17-hydroxyandrost-4-en-3-one (100 mg, 0.35 mmol) dissolved in DMF (5 mL) was added, and the mixture was stirred at 300 rpm at 27 °C with an aeration rate of 0.5 L/min. Cells in buffer (50 mL of preculture 2) were added, and the biotransformation was maintained at an oxygen partial pressure of 50 % by varying the stirring

rate to up to 800 rpm. The pH value was maintained at pH 7.4 by addition of an aqueous solution of sodium hydroxide (16 %). After 4 hours, cells (20 mL) in buffer were added again. After 28 hours and 10 minutes the biotransformation was stopped.

Biotransformation 2

- 5 To a 1 L Biostat Q fermenter which contained KH₂PO₄ (4 g), K₂HPO₄ (12.3 g), Pluronic® PE 8100 (0.1 mL), demineralized water (0.93 L), aqueous glucose solution (50 %, 20 mL) and aqueous EDTA solution (0.5 M, 1 mL) (17beta)-17-hydroxyandrosta-4-en-3-one (100 mg, 0.35 mmol) dissolved in DMF (5 mL) was added and the mixture was stirred at 300 rpm at 27 °C with an aeration rate of 0.5 L/min. Cells in buffer (50 mL of cell cultivation 3) were added, and the
- 10 biotransformation was maintained at an oxygen partial pressure of 50 % by varying the stirring rate to up to 800 rpm. The pH value was maintained at pH 7.4 by addition of an aqueous solution of sodium hydroxide (16 %). After 27 hours and 45 minutes the biotransformation was stopped.

Biotransformation 3

- To a 1 L Biostat Q fermenter which contained KH₂PO₄ (4 g), K₂HPO₄ (12.3 g), Pluronic® PE
- 15 8100 (0.1 mL), demineralized water (0.93 L), aqueous glucose solution (50 %, 20 mL) and aqueous EDTA solution (0.5 M, 1 mL). (17beta)-17-hydroxyandrosta-4-en-3-one (4 x 25 mg, 0.35 mmol) dissolved in DMF (4 x 2.5 mL) was added in intervals after 0 h, 2 h, 4 h and 6 h and the mixture was stirred at 300 rpm at 27 °C with an aeration rate of 0.5 L/min. Cells in buffer (50 mL of cell cultivation 1) were added at 0 h, and the biotransformation was maintained at an
- 20 oxygen partial pressure of 50 % by varying the stirring rate between 800 and 1200 rpm. The pH value was maintained at pH 7.4 by addition of an aqueous solution of sodium hydroxide (16 %). After 28 hours the biotransformation.

- The three biotransformations were combined and extracted with 4-methyl-2-pentanone. The organic layer was concentrated to give an oil (2.2 g) which was slurried with methanol and was
- 25 filtered and concentrated to give an oil (1.06 g). The crude product was further purified by flash chromatography using silica gel (dichloromethane/methanol gradient) and by preparative HPLC to give the title compound (7.8 mg, 2 % yield).

Preparative chiral HPLC method:

- Instrument: Waters Autopurificationsystem; Column: Waters XBrigde C18 5 µ 100x30 mm;
- 30 Eluent A: water + 0.1 vol-% aqueous ammonia (99 %), Eluent B: acetonitrile; gradient: 0.00-0.50 min 10 % B (40-70 mL/min), 0.51-5.50 min 10-40 % B (70 mL/min), DAD scan: 210-400 nm.

Analytical chiral HPLC method:

Instrument: Waters Acquity UPLCMS SingleQuad; Column: Acquity UPLC BEH C18 1.7 µm, 50x2.1 mm; eluent A: water + 0.1 vol % aqueous ammonia (99 %), eluent B: acetonitrile;

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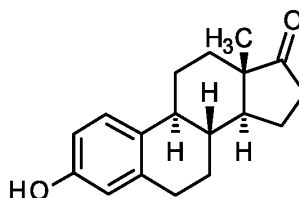
gradient: 0-1.6 min 1-99 % B, 1.6-2.0 min 99 % B; flow 0.8 mL/min; temperature: 60 °C; DAD scan: 210-400 nm.

Analytical Chiral HPLC: R_t = 0.73 min.

$^1\text{H-NMR}$ (600 MHz, CDCl_3) δ [ppm]: 0.79 (s, 3H), 0.95 - 1.11 (m, 3H), 1.24 - 1.36 (m, 3H), 1.38 - 1.50 (m, 3H), 1.57 - 1.63 (m, 1H), 1.69 - 1.77 (m, 1H), 1.81 (dq, 1H), 1.87 (dt, 1H), 1.95 - 2.02 (m, 1H), 2.03 - 2.14 (m, 1H), 2.20 - 2.27 (m, 2H), 2.30 (s, 1H), 2.35 - 2.41 (m, 1H), 2.44 - 2.54 (m, 1H), 3.60 - 3.73 (m, 3H), 4.09 - 4.17 (m, 2H).

Example 21

3-hydroxyestra-1(10),2,4-trien-17-one



Expression system

E. coli BL21 Star™ (DE3) pETM11-BM3-268; S72G, A74V, F77A

Cultivation

Media of the preculture: tryptone (16 g/L), sodium chloride (10 g/L) and yeast extract (10 g/L) in demineralized water. The pH value was maintained at pH 7.3. The medium was sterilized at 121 °C for 20 minutes. Afterwards kanamycin (50 mg/L) was added.

One preculture (100 mL) was inoculated with the strain *E. coli* BL21 Star™ (DE3) pETM11-BM3-268; S72G, A74V, F77A (50 μL) containing the desired plasmids and was shaken at 37 °C and 165 rpm for 17 hours. This preculture (100 mL) was used to inoculate one 10 L steel fermenter.

The cultivation media were prepared in the fermenter. Tryptone (12 g/L), yeast extract (24 g/L), predigested beef extract (2 g/L), KH_2PO_4 (2.2 g/L), K_2HPO_4 (9.4 g/L) and glycerol (87%, 4.6 g/L) were dissolved in demineralized water (9.2 L) and sterilized for 20 minutes at 121 °C in the fermenter. Afterwards kanamycin (0.5 g) in water (20 mL), riboflavin (10 mg) in water (20 mL), thiamine hydrochloride (3.37 g) in water (10 mL) and Oxford trace metal solution (2.5 mL) were added. After the inoculation of the fermenter, the culture was stirred at 315 rpm at 37 °C with an aeration rate of 3.3 L/min at pH 6.6 which was regulated by addition of aqueous sodium hydroxide solution (16%) or aqueous phosphoric acid solution (16%). After 2.55 hours, an OD550 of 0.94 was reached, the temperature was decreased to 27 °C within 10 minutes and IPTG (1.43 g) in water (40 mL) were added to start the protein expression. After 9.15 hours the

aqueous phosphoric acid solution (16%) was substituted for an aqueous glucose solution (50%) for pH regulation. After additional 23.5 hours an OD550 of 23.92 was reached, the cells were harvested by centrifugation (215.6 g), suspended in buffer (215.6 mL; KH₂PO₄ (4 g/L), K₂HPO₄ (12.3 g/L), glycerol (4% (v/v)), glucose (5% (w/v)), EDTA (0.5 mM)), frozen in liquid nitrogen and stored at -80 °C until further use in aliquots of 1 x 150 mL, 1 x 130 mL, 2 x 40 mL und 5 x 1 mL.

Biotransformation

To a 10 L steel fermenter which contained KH₂PO₄ (40 g), K₂HPO₄ (123 g), Pluronic® PE 8100 (1 mL), demineralized water (9.0 L), aqueous glucose solution (50 %, 200 mL) and aqueous EDTA solution (0.5 M, 10 mL) androsta-1,4-diene-3,17-dione (500 mg, 1.75 mmol) dissolved in DMF (10 mL) was added and the mixture was stirred at 315 rpm at 27 °C with an aeration rate of 3.3 L/min. Cells in buffer (400 mL) were added, and the biotransformation was maintained at an oxygen partial pressure of 50 % by varying the stirring rate to up to 1200 rpm. The pH value was maintained at pH 7.4 by addition of an aqueous solution of sodium hydroxide (16 %). After 26 hours the biotransformation was extracted with 4-methyl-2-pentanone. The organic layer was concentrated to give an oil (2.37 g) which was slurried with methanol and was filtered and concentrated to give an oil (2.05 g). The crude product was further purified by flash chromatography using silica gel (dichloromethane/methanol gradient) and twice by preparative HPLC to give the title compound (14 mg).

1. Preparative HPLC method

Instrument: Waters Autopurificationsystem; Column: Waters XBrigde C18 5µ 50x50 mm; Eluent A: Water + 0.2 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0.00–0.50 min 23 % B (50-100 mL/min), 0.51–13.50 min 23 - 43 % B (100 mL/min), DAD scan: 210-400 nm.

1. Analytical HPLC method

Instrument: Waters Acquity UPLCMS SingleQuad; Column: Acquity UPLC BEH C18 1.7 µm, 50x2.1mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0-2.6 min 1-99 % B, 2.6-3.0 min 99 % B; Flow 0.8 mL/min; Temperature: 60 °C; DAD scan: 210-400 nm.

Analytical HPLC: R_t = 1.10 min.

2. Preparative HPLC method

Instrument: Sepiatec: Prep SFC100; Column: Chiralpak IG 5µ 250x30 mm; eluent A: CO₂; eluent B: methanol; isocratic: 20 % B; flow: 100 mL/min; temperature: 40 °C; BPR: 150 bar; UV: 280 nm.

2. Analytical HPLC method

Instrument: Agilent: 1260, Aurora SFC-Modul; Column: Chiralpak IG 5 μ 100x4.6 mm; eluent A: CO₂; eluent B: methanol; isocratic: 20 % B; flow: 4 mL/min; temperature: 37.5 °C; BPR: 100 bar; UV: 280 nm.

Analytical HPLC: R_t = 3.66 min.

- 5 ¹H-NMR (400 MHz, DMSO-d₆) δ [ppm]: 0.820 (16.00), 1.293 (0.48), 1.322 (0.95), 1.329 (0.75), 1.348 (2.38), 1.371 (1.82), 1.401 (0.24), 1.450 (0.87), 1.461 (0.83), 1.476 (1.50), 1.492 (0.75), 1.523 (0.55), 1.529 (0.44), 1.545 (0.36), 1.552 (0.59), 1.575 (0.36), 1.583 (0.32), 1.726 (0.99), 1.733 (0.91), 1.748 (0.79), 1.754 (0.67), 1.882 (0.51), 1.890 (0.55), 1.896 (0.59), 1.915 (0.75), 1.921 (0.83), 1.935 (0.63), 1.943 (0.67), 1.964 (0.48), 1.976 (0.44), 2.011 (0.59), 2.033 (0.91),
- 10 2.058 (0.83), 2.071 (0.20), 2.080 (1.19), 2.102 (0.51), 2.136 (0.55), 2.284 (0.40), 2.301 (0.71), 2.314 (0.71), 2.326 (0.51), 2.331 (0.24), 2.394 (0.95), 2.414 (1.07), 2.440 (0.75), 2.461 (0.95), 2.518 (0.71), 2.522 (0.48), 2.539 (0.44), 2.664 (0.20), 2.669 (0.24), 2.673 (0.20), 2.710 (0.20), 2.735 (1.35), 2.747 (1.43), 2.775 (0.51), 3.298 (0.20), 6.444 (2.06), 6.450 (2.42), 6.496 (1.35), 6.502 (1.07), 6.517 (1.39), 6.523 (1.15), 7.036 (1.82), 7.056 (1.66), 9.033 (4.63).
- 15 ¹H-NMR (400 MHz, DMSO-d₆) δ [ppm]: 0.82 (s, 3H), 1.22 - 1.41 (m, 3H), 1.42 - 1.61 (m, 3H), 1.69 - 1.79 (m, 1H), 1.86 - 1.99 (m, 2H), 2.00 - 2.19 (m, 2H), 2.25 - 2.35 (m, 1H), 2.43 (dd, 1H), 2.68 - 2.83 (m, 2H), 6.45 (d, 1H), 6.51 (dd, 1H), 7.05 (d, 1H), 9.03 (s, 1H).

For another preparation of the title compound see Example 25. The crude product was further purified by flash chromatography using silica gel (dichloromethane/methanol gradient) and by

20 preparative HPLC to give the title compound (91 mg).

Furthermore the title compound was synthesized using androsta-1,4-diene-3,17-dione (ADD) as a substrate by the following procedures with the screening yields mentioned below.

BM3 Variant	Biotransformation variant	Substrate concentration (mg/L)	OD550	pH	Screening yield (%) Example 21 after 23 h
A	2	400	35	7.0	61
A	4	800	44	6.6	58
A	3	1500	210	6.6	55
B	1	400	26	6.6	29
C	1	400	36	6.6	28
C	1	400	28	6.6	61*
D	2	400	35	7.0	7
D	2	400	32	7.0	32
E	1	400	34	6.6	32
F	1	400	30	6.6	48
G	1	400	36	6.6	66
H	1	400	29	6.6	46
I	1	400	34	6.6	60

*after 47 h cultivation

Expression systems

A: *E. coli* BL21 Star™ (DE3) pETM11-BM3-268; M177Y, A184Y (Prot Seq ID No. 15; DNA Seq ID No. 73) + S72G, V178P, L181Y, L188F

5 B: *E. coli* BL21 Star™ (DE3) pETM11-BM3-268; M177Y, A184Y (Prot Seq ID No. 15; DNA Seq ID No. 73) + S72G, T146F, V178W, L181Y

C: *E. coli* BL21 Star™ (DE3) pETM11-BM3-268; S72G, M177Y, V178P, L181Y, A184Y, L188F (Prot Seq ID No. 117; DNA Seq ID No. 120) + R179L

D: *E. coli* BL21 Star™ (DE3) pETM11-BM3-268; S72G, M177Y, V178P, L181Y, A184Y, L188F (Prot Seq ID No. 117; DNA Seq ID No. 120)

10 E: *E. coli* BL21 Star™ (DE3) pETM11-BM3-268; S72G, T146F, M177Y, V178W, L181Y, A184Y, G457S, I458G, A477N (Prot Seq ID No. 119; DNA-Seq ID No. 122)

F: *E. coli* BL21 Star™ (DE3) pETM11-BM3-268; S72G, T146F, M177Y, V178W, L181Y, A184Y, G457S, I458G, A477N (Prot Seq ID No. 119; DNA-Seq ID No. 122) + insert with SEQ ID No. 137

15 G: *E. coli* BL21 Star™ (DE3) pETM11-BM3-268; S72G, T146F, M177Y, V178W, L181Y, A184Y, G457S, I458G, A477N (Prot Seq ID No. 119; DNA-Seq ID No. 122) + insert with SEQ ID No. 126

20 H: *E. coli* BL21 Star™ (DE3) pETM11-BM3-268; S72G, T146F, M177Y, V178W, L181Y, A184Y, G457S, I458G, A477N (Prot Seq ID No. 119; DNA-Seq ID No. 122) + insert with Seq ID No. 139

I: *E. coli* BL21 Star™ (DE3) pETM11-BM3-268; S72G, T146F, M177Y, V178W, L181Y, A184Y, G457S, I458G, A477N (Prot Seq ID No. 119; DNA-Seq ID No. 122) + insert with SEQ ID No. 145

25 Media of the preculture: tryptone (16 g/L), sodium chloride (10 g/L) and yeast extract (10 g/L) in demineralized water. The pH value was maintained at pH 7.3. The medium was sterilized at 121 °C for 20 minutes. Afterwards kanamycin (50 mg/L) was added.

30 One preculture (100 mL) was inoculated with the strain (50 µL) containing the desired plasmids and was shaken at 37 °C and 165 rpm for 17 hours. This preculture (100 mL) was used to inoculate one 10 L steel fermenter. The cultivation media were prepared in the fermenter. Tryptone (12 g/L), yeast extract (24 g/L), predigested beef extract (2 g/L), KH₂PO₄ (2.2 g/L), K₂HPO₄ (9.4 g/L) and glycerol (87%, 4.6 g/L) were dissolved in demineralized water (9.2 L) and sterilized for 20 minutes at 121 °C in the fermenter. Afterwards kanamycin (0.5 g) in water (20 mL), riboflavin (10 mg) in water (20 mL), thiamine hydrochloride (3.37 g) in water (10 mL)

and Oxford trace metal solution (2.5 mL) were added. After the inoculation of the fermenter, the culture was stirred at 315 rpm at 37 °C with an aeration rate of 3.3 L/min at pH 6.6 which was regulated by addition of aqueous sodium hydroxide solution (16%) or aqueous phosphoric acid solution (16%). After an OD550 of 0.9-1.0 was reached, the temperature was decreased to 27 °C within 10 minutes and IPTG (1.43 g) in water (40 mL) was added to start the protein expression. The aqueous phosphoric acid solution (16%) was substituted for an aqueous glucose solution (50%) for pH regulation after the time described in the table below. After the culturing time mentioned in the table below the cells were harvested by centrifugation and resuspended in buffer, 1 g cell pellet in 1 mL buffer (KH₂PO₄ (4 g/L), K₂HPO₄ (12.3 g/L), glycerol (4% (v/v)), glucose (5% (w/v)), EDTA (0.5 mM)), frozen in liquid nitrogen and stored at –80 °C until further use in aliquots of 80 mL.

Variant	culturing time (h)	time of phosphoric acid Glucose switch (h)
A	24	12.3
A	48	12.0
A	48	12.0
B	31	23.5
C	24	14.3
C	48	14.0
D	24	14.5
D	31	24.5
E	48	9.3
F	48	11.3
G	48	9.8
H	48	14.0
I	48	9.5

Biotransformation 1

To a 1 L glass vessel fermenter which contained KH₂PO₄ (9.54 g), K₂HPO₄ (5.19 g), 0.1 mL of Pluronic® PE 8100, demineralized water (0.9 L), aqueous glucose solution (50%, 20 mL) and aqueous EDTA solution (0.5 mM, 1 mL), androsta-1,4-diene-3,17-dione (400 mg, 1.41 mmol/L) dissolved in DMF (8 mL) was added and the mixture was stirred at 300 rpm at 27 °C with an aeration rate of 0.5 L/min. Cells in buffer (80 mL) were added, and the biotransformation was maintained at a dissolved oxygen partial pressure of 50% by varying the stirring rate up to 2000 rpm. After Glucose concentration dropped below 5 g/L an aqueous glucose solution (50%) feeding started. The pH value was maintained at pH 6.6 by addition of an aqueous solution of sodium hydroxide (16 %). After 0 h, 1 h, 2 h, 3 h, 5 h, 7 h, 23 h and 47 h an analytical sample (2 mL) was taken and the titer for Example 21 was measured with the methods mentioned below.

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Variant	Start Glucose feeding (h)	total quantity Glucose (g)
B	3	68.5
C	3	66.7
C	7	25.1
E	5	51.7
F	5	70.6
G	5	70.9
H	1	61.2
I	7	63.9

Biotransformation 2

To a 1 L glass vessel fermenter which contained KH₂PO₄ (6.8 g), K₂HPO₄ (8.7 g), 0.1 mL of Pluronic® PE 8100, demineralized water (0.9 L), aqueous glucose solution (50%, 20 mL) and aqueous EDTA solution (0.5 mM, 1 mL), androsta-1,4-diene-3,17-dione (400 mg, 1.41 mmol/L) dissolved in DMF (8 mL) was added and the mixture was stirred at 300 rpm at 27 °C with an aeration rate of 0.5 L/min. Cells in buffer (80 mL) were added, and the biotransformation was maintained at a dissolved oxygen partial pressure of 50% by varying the stirring rate up to 2000 rpm. After the glucose concentration dropped below 5 g/L an aqueous glucose solution (50%) feeding started. The pH value was maintained at pH 7.0 by addition of an aqueous solution of sodium hydroxide (16 %). After 0 h, 1 h, 2 h, 3 h, 5h, 7 h and 23 h an analytical sample (2 mL) was taken and the titer for Example 21 was measured with methods mentioned below.

Variant	Start Glucose feeding (h)	total quantity Glucose (g)
A	7	no data
D	5	79
D	5	66.3

Biotransformation 3

To a 1 L glass vessel fermenter which contained KH₂PO₄ (9.54 g), K₂HPO₄ (5.19 g), 0.1 mL of Pluronic® PE 8100, demineralized water (0.5 L), aqueous glucose solution (50%, 20 mL), 20 mL of DMF and aqueous EDTA solution (0.5 mM, 1 mL), androsta-1,4-diene-3,17-dione (1500 mg, 5.27 mmol/L) as aqueous suspension was added (15.4 mL of an 125 g/L stock solution; 78 %) and the mixture was stirred at 500 rpm at 27 °C with an aeration rate of 0.5 L/min. Cells in buffer (400 mL) were added, and the biotransformation was maintained at an dissolved oxygen partial pressure of 50 % by varying the stirring rate up to 2000 rpm and addition of pure oxygen to aeration within the range of 0.4 L/min and reducing air aeration by the same volume per minute to keep aeration rate of 0.5 L/min. After 3 hours an aqueous glucose solution (50 %) feeding started. The amount of glucose measured should not drop below 5 g/L. The total amount of added glucose was 97.9 g. The pH value was maintained at pH 6.6 by addition of an aqueous

solution of sodium hydroxide (16 %). After 0 h, 1 h, 2 h, 3 h, 5 h, 7 h and 23 h an analytical sample (2 mL) was taken and the titer for Example 21 was measured with methods mentioned below.

Biotransformation 4

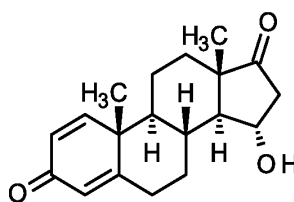
- 5 To a 1 L glass vessel fermenter which contained KH₂PO₄ (9.54 g), K₂HPO₄ (5.19 g), 0.1 mL of Pluronic® PE 8100, demineralized water (0.9 L), aqueous glucose solution (50%, 20 mL) and aqueous EDTA solution (0.5mM, 1 mL), androsta-1,4-diene-3,17-dione (800 mg, 2.81 mmol/L) dissolved in DMF (16 mL) was added and the mixture was stirred at 300 rpm at 27 °C with an aeration rate of 0.5 L/min. Cells in buffer (80 mL) were added, and the biotransformation was
- 10 maintained at a dissolved oxygen partial pressure of 50 % by varying the stirring rate up to 2000 rpm. After 23 hours an aqueous glucose solution (50 %) feeding started. The amount of glucose measured should not drop below 5 g/L. The total amount of added glucose was 67.5 g. The pH value was maintained at pH 6.6 by addition of an aqueous solution of sodium hydroxide (16 %). After 0 h, 1 h, 2 h, 3 h, 5 h, 7 h and 23 h an analytical sample (2 mL) was taken and the titer for
- 15 Example 21 was measured with methods mentioned below.

Analytical HPLC method

- Instrument: Waters Acquity UPLC (I-Class, H-Class) or Thermo Scientific ISQ EM SingleQuad;
Column: Ascentis Express C18 2.7 µm, 150x2.1 mm; Eluent A: Water + 0.05 % Formic acid (99%); Eluent B: Acetonitrile + 0.05 % Formic acid; Gradient: 0-9 min 5-30 % B, 9-15 min 30-
20 95 % B; Flow 0.7mL/min; Temperature: 45 °C; DAD: 243 nm + 280 nm.

Example 22

15α-hydroxyandrosta-1,4-diene-3,17-dione



- For the preparation of the title compound see Example 21. The crude product was further purified
- 25 by flash chromatography using silica gel (dichloromethane/methanol gradient) and twice by preparative HPLC to give the title compound (14 mg).

1. Preparative HPLC method

Instrument: Waters Autopurificationsystem; Column: Waters XBrigde C18 5 μ 50x50 mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0.00–0.50 min 17 % B (50-100 mL/min), 0.51–8.50 min 17 - 37 % B (100 mL/min), DAD scan: 210-400 nm.

1. Analytical HPLC method

- 5 Instrument: Waters Acquity UPLCMS SingleQuad; Column: Acquity UPLC BEH C18 1.7 μ m, 50x2.1mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0-2.6 min 1-99 % B, 2.6-3.0 min 99 % B; Flow 0.8 mL/min; Temperature: 60 °C; DAD scan: 210-400 nm.

Analytical HPLC: R_t = 0.82 min.

10 2. Preparative HPLC method

Instrument: PrepCon Labomatic HPLC; Column: YMC Cellulose SC 5 μ , 250x30 mm; eluent A: hexane + 0.1 vol % diethylamine; eluent B: 2-propanol; isocratic: 70 % A + 30 % B; flow: 50 mL/min; temperature: 25 °C; UV: 254 nm.

2. Analytical HPLC method

- 15 Instrument: Waters Alliance 2695; Column: YMC Cellulose SC 3 μ , 100x4.6 mm; eluent A: hexane + 0.1 vol % diethylamine; eluent B: 2-propanol; isocratic: 70 % A + 30 % B; flow: 1.4 mL/min; temperature: 25 °C; UV: 254 nm.

Analytical HPLC: R_t = 9.28 min.

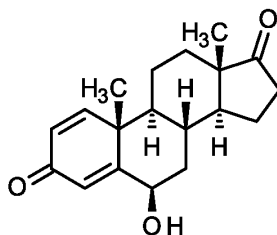
- ¹H-NMR (600 MHz, CDCl₃) δ [ppm]: 0.000 (2.45), 0.736 (0.49), 0.839 (0.64), 0.848 (0.52), 0.856 (0.53), 0.862 (0.46), 0.869 (0.88), 0.881 (1.19), 0.892 (0.62), 1.071 (0.16), 1.139 (0.57), 1.146 (0.65), 1.157 (0.67), 1.159 (0.78), 1.163 (0.80), 1.166 (0.69), 1.177 (0.65), 1.184 (0.62), 1.189 (0.46), 1.197 (0.55), 1.210 (0.78), 1.217 (0.88), 1.233 (1.06), 1.239 (1.17), 1.254 (5.41), 1.262 (3.03), 1.270 (14.17), 1.281 (2.20), 1.288 (1.62), 1.297 (1.04), 1.306 (16.00), 1.333 (0.30), 1.348 (0.42), 1.356 (0.21), 1.376 (0.22), 1.399 (0.16), 1.412 (0.17), 1.601 (0.17), 1.642 (0.51), 1.700 (0.19), 1.724 (0.23), 1.731 (0.33), 1.747 (0.53), 1.753 (0.50), 1.768 (0.70), 1.774 (0.62), 1.789 (0.32), 1.796 (0.33), 1.840 (0.70), 1.844 (1.02), 1.851 (1.63), 1.858 (0.95), 1.861 (0.97), 1.866 (1.73), 1.868 (1.14), 1.873 (1.06), 1.877 (0.57), 1.884 (0.28), 2.163 (0.24), 2.169 (0.28), 2.181 (0.55), 2.187 (0.64), 2.201 (0.54), 2.206 (0.60), 2.219 (0.21), 2.225 (0.26), 2.267 (0.30), 2.271 (0.41), 2.276 (0.50), 2.280 (0.46), 2.288 (0.41), 2.292 (0.42), 2.297 (0.46), 2.301 (0.44), 2.307 (0.28), 2.434 (0.41), 2.438 (0.48), 2.441 (0.51), 2.445 (0.42), 2.456 (0.59), 2.461 (0.67), 2.463 (0.68), 2.468 (0.52), 2.503 (0.69), 2.513 (0.64), 2.535 (1.79), 2.545 (1.79), 2.567 (0.41), 2.569 (0.53), 2.575 (2.58), 2.590 (0.64), 2.592 (0.66), 2.598 (0.63), 2.600 (0.64), 2.607 (0.88), 2.612 (0.34), 2.614 (0.31), 2.621 (0.27), 2.623 (0.37), 4.576 (0.66), 4.585 (1.06), 4.593 (0.65), 6.107

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(1.41), 6.109 (2.45), 6.112 (1.50), 6.245 (1.70), 6.248 (1.64), 6.262 (1.64), 6.266 (1.59), 7.060 (2.35), 7.078 (2.17), 7.267 (8.94).

Example 23

6 β -hydroxyandrosta-1,4-diene-3,17-dione



5

For the preparation of the title compound see Example 21. The crude product was further purified by flash chromatography using silica gel (dichloromethane/methanol gradient) and twice by preparative HPLC to give the title compound (2 mg).

1. Preparative HPLC method

- 10 Instrument: Waters Autopurificationsystem; Column: Waters XBrigde C18 5 μ 50x50 mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0.00–0.50 min 17 % B (50-100 mL/min), 0.51–8.50 min 17 - 37 % B (100 mL/min), DAD scan: 210-400 nm.

1. Analytical HPLC method

- 15 Instrument: Waters Acquity UPLCMS SingleQuad; Column: Acquity UPLC BEH C18 1.7 μ m, 50x2.1mm; Eluent A: Water + 0.1 Vol-% Formic acid (99 %), Eluent B: Acetonitrile; Gradient: 0-2.6 min 1-99 % B, 2.6-3.0 min 99 % B; Flow 0.8 mL/min; Temperature: 60 °C; DAD scan: 210-400 nm.

Analytical HPLC: R_t = 0.82 min.

2. Preparative HPLC method

- 20 Instrument: PrepCon Labomatic HPLC; Column: YMC Cellulose SC 5 μ , 250x30 mm; eluent A: hexane + 0.1 vol % diethylamine; eluent B: 2-propanol; isocratic: 70 % A + 30 % B; flow: 50 mL/min; temperature: 25 °C; UV: 254 nm.

2. Analytical HPLC method

- 25 Instrument: Waters Alliance 2695; Column: YMC Cellulose SC 3 μ , 100x4.6 mm; eluent A: hexane + 0.1 vol % diethylamine; eluent B: 2-propanol; isocratic: 70 % A + 30 % B; flow: 1.4 mL/min; temperature: 25 °C; UV: 254 nm.

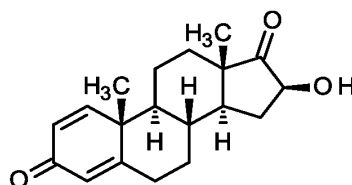
Analytical HPLC: R_t = 6.58 min.

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¹H-NMR (400 MHz, DMSO-d₆) δ [ppm]: 0.697 (1.62), 0.717 (0.16), 0.833 (0.65), 0.851 (1.06), 0.879 (16.00), 0.986 (0.57), 0.996 (0.57), 1.014 (0.89), 1.023 (0.97), 1.042 (0.73), 1.052 (0.65), 1.083 (0.32), 1.147 (0.65), 1.164 (1.06), 1.172 (0.89), 1.184 (1.38), 1.195 (1.87), 1.232 (5.28), 1.256 (1.38), 1.272 (1.06), 1.289 (1.38), 1.293 (1.46), 1.300 (1.22), 1.317 (0.97), 1.330 (0.97), 1.347 (0.65), 1.351 (0.65), 1.373 (16.00), 1.472 (0.41), 1.495 (0.81), 1.502 (0.65), 1.517 (0.57), 1.526 (1.14), 1.534 (0.57), 1.548 (0.73), 1.557 (0.65), 1.579 (0.32), 1.598 (0.32), 1.609 (0.41), 1.631 (0.65), 1.641 (0.81), 1.668 (2.60), 1.692 (0.89), 1.698 (1.22), 1.708 (0.65), 1.784 (0.73), 1.795 (0.81), 1.805 (0.57), 1.822 (0.65), 1.833 (0.81), 1.847 (0.65), 1.853 (0.73), 1.867 (0.73), 1.883 (0.57), 1.898 (0.49), 1.959 (0.81), 1.967 (0.81), 1.975 (1.22), 1.981 (1.71), 2.006 (1.46), 2.017 (0.73), 2.028 (1.38), 2.051 (0.49), 2.080 (0.32), 2.089 (0.32), 2.109 (0.73), 2.116 (0.65), 2.138 (0.73), 2.165 (0.32), 2.173 (0.24), 2.331 (0.97), 2.336 (0.49), 2.380 (1.14), 2.401 (1.14), 2.428 (1.30), 2.450 (1.79), 2.518 (3.65), 2.522 (2.44), 2.539 (0.49), 2.673 (0.89), 2.678 (0.41), 4.398 (1.71), 4.405 (1.71), 5.331 (2.27), 5.336 (2.19), 6.054 (3.49), 6.058 (4.30), 6.084 (2.60), 6.089 (1.79), 6.109 (2.36), 6.114 (1.95), 7.168 (3.17), 7.193 (3.09).

15 **Example 24**

16β-hydroxyandrosta-1,4-diene-3,17-dione

Expression system

E. coli BL21 StarTM (DE3) pETM11-BM3-268; F77A, M177Y

20 **Cultivation**

Media of the preculture: tryptone (10 g/L), sodium chloride (10 g/L) and yeast extract (5 g/L) in demineralized water. The media was sterilized at 121 °C for 20 minutes. Afterwards kanamycin (50 mg/L) was added.

One preculture (100 mL) was inoculated with the strain *E. coli* BL21 StarTM (DE3) pETM11-BM3-268; F77A, M177Y (50 μL) containing the desired plasmids and was shaken at 37 °C and 165 rpm for 17 hours. This preculture (100 mL) was used to inoculate one 10 L steel fermenter. The cultivation media were prepared in the fermenter. Tryptone (12 g/L), yeast extract (24 g/L), predigested beef extract (2 g/L), KH₂PO₄ (2.2 g/L), K₂HPO₄ (9.4 g/L) and glycerol (87%, 4.6 g/L) were dissolved in demineralized water (9.2 L) and sterilized for 20 minutes at 121 °C in the fermenter. Afterwards kanamycin (0.5 g) in water (20 mL), riboflavin (10 mg) in water (20 mL),

thiamine hydrochloride (3.37 g) in water (10 mL) and Oxford trace metal solution (2.5 mL) were added. After the inoculation of the fermenter, the culture was stirred at 315 rpm at 37 °C with an aeration rate of 3.3 L/min at pH 6.6 which was regulated by addition of aqueous sodium hydroxide solution (16%) or aqueous phosphoric acid solution (16%). After 3.15 hours, an
5 OD550 of 1.0 was reached, the temperature was decreased to 27 °C within 10 minutes and IPTG (1.43 g) in water (40 mL) were added to start the protein expression. After 8 hours the aqueous phosphoric acid solution (16%) was substituted for an aqueous glucose solution (50%) for pH regulation. After additional 24 hours an OD550 of 25.2 was reached, the cells were harvested by centrifugation (284.2 g), suspended in buffer (284.2 mL; KH₂PO₄ (4 g/L), K₂HPO₄
10 (12.3 g/L), glycerol (4% (v/v)), glucose (5% (w/v)), EDTA (0.5 mM)), frozen in liquid nitrogen and stored at -80 °C until further use in aliquots of 2 x 150 mL, 1 x 140 mL, 2 x 40 mL und 5 x 1 mL.

Biotransformation

To a 10 L steel fermenter which contained KH₂PO₄ (40 g), K₂HPO₄ (123 g), Pluronic® PE 8100 (1 mL), demineralized water (9.0 L), aqueous glucose solution (50 %, 200 mL) and aqueous
15 EDTA solution (0.5 M, 10 mL) androsta-1,4-diene-3,17-dione (500 mg, 1.75 mmol) dissolved in DMF (10 mL) was added and the mixture was stirred at 315 rpm at 27 °C with an aeration rate of 3.3 L/min. Cells in buffer (400 mL) were added, and the biotransformation was maintained at an oxygen partial pressure of 50 % by varying the stirring rate to up to 1200 rpm. The pH value was maintained at pH7.4 by addition of an aqueous solution of sodium hydroxide (16 %). After
20 26 hours the biotransformation was extracted with 4-methyl-2-pentanone. The organic layer was concentrated to give an oil (1.71 g) which was slurried with methanol and was filtered and concentrated to give an oil (1.55 g). The crude product was further purified by flash chromatography using silica gel (dichloromethane/methanol gradient) and by preparative HPLC to give the title compound (11 mg).

25 Preparative HPLC method

Instrument: Sepiatec: Prep SFC100; Column: Reprosil Chiral NR 8 μ 250x30 mm; eluent A: CO₂; eluent B: 2-propanol; isocratic: 25 % B; flow: 100 mL/min; temperature: 40 °C; BPR: 150 bar; UV: 254 nm.

Analytical HPLC method

30 Instrument: Agilent: 1260, Aurora SFC-Modul; Column: Reprosil Chiral NR 5 μ 100x4.6 mm; eluent A: CO₂; eluent B: 2-propanol; isocratic: 25 % B; flow: 4 mL/min; temperature: 37.5 °C; BPR: 100 bar; UV: 254 nm.

Analytical HPLC: R_t = 3.41 min.

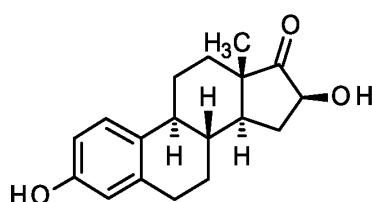
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¹H-NMR (600 MHz, CDCl₃) δ [ppm]: 0.000 (2.08), 1.032 (13.48), 1.117 (0.25), 1.124 (0.30), 1.129 (0.47), 1.137 (0.99), 1.147 (0.90), 1.150 (0.65), 1.154 (0.70), 1.157 (0.93), 1.168 (0.97), 1.175 (0.53), 1.180 (0.33), 1.188 (0.30), 1.222 (0.50), 1.230 (0.53), 1.237 (0.38), 1.239 (0.59), 1.244 (0.71), 1.248 (0.70), 1.252 (0.83), 1.256 (0.66), 1.262 (0.78), 1.272 (16.00), 1.322 (0.38), 1.330 (0.43), 1.337 (0.26), 1.344 (0.81), 1.351 (0.86), 1.366 (0.46), 1.373 (0.45), 1.558 (0.71), 1.573 (0.73), 1.578 (0.78), 1.580 (0.67), 1.593 (0.82), 1.596 (0.70), 1.600 (0.71), 1.616 (0.71), 1.723 (0.35), 1.730 (0.38), 1.745 (0.66), 1.752 (0.68), 1.767 (0.71), 1.774 (0.75), 1.788 (0.35), 1.796 (0.36), 1.850 (0.36), 1.854 (0.71), 1.857 (0.73), 1.861 (0.91), 1.869 (0.48), 1.872 (0.82), 1.879 (0.99), 1.884 (0.55), 1.892 (0.77), 1.897 (0.59), 1.909 (0.25), 1.916 (0.27), 1.928 (0.59), 1.933 (0.66), 1.935 (0.65), 1.940 (0.51), 1.950 (0.57), 1.954 (0.62), 1.957 (0.60), 1.961 (0.46), 2.073 (0.30), 2.077 (0.40), 2.081 (0.47), 2.083 (0.44), 2.085 (0.44), 2.092 (0.39), 2.099 (0.40), 2.102 (0.44), 2.104 (0.43), 2.113 (0.27), 2.380 (0.61), 2.388 (0.62), 2.393 (0.63), 2.400 (0.80), 2.408 (0.60), 2.413 (0.59), 2.422 (0.84), 2.426 (0.51), 2.430 (0.43), 2.441 (0.67), 2.446 (0.73), 2.448 (0.72), 2.453 (0.59), 2.504 (0.41), 2.507 (0.45), 2.512 (0.45), 2.515 (0.44), 2.526 (0.65), 2.529 (0.66), 2.535 (0.65), 2.538 (0.61), 2.549 (0.29), 2.552 (0.31), 2.557 (0.27), 2.560 (0.25), 2.623 (5.61), 3.948 (1.17), 3.962 (1.59), 3.977 (1.10), 6.101 (1.45), 6.103 (2.45), 6.107 (1.46), 6.241 (1.54), 6.244 (1.48), 6.258 (1.70), 6.262 (1.62), 7.031 (2.46), 7.048 (2.39), 7.266 (8.11).

¹H-NMR (600 MHz, CDCl₃) δ [ppm]: 1.03 (s, 3H), 1.11 - 1.20 (m, 2H), 1.21 - 1.28 (m, 1H), 1.27 (s, 3H), 1.31 - 1.38 (m, 1H), 1.59 (ddd, 1H), 1.76 (qd, 1H), 1.84 - 1.97 (m, 3H), 2.06 - 2.12 (m, 1H), 2.37 - 2.46 (m, 2H), 2.50 - 2.57 (m, 1H), 2.78 (s, 1H), 3.96 (t, 1H), 6.10 (t, 1H), 6.25 (dd, 1H), 7.04 (d, 1H).

Example 25

3,16β-dihydroxyestra-1(10),2,4-trien-17-one

25 Cultivation

Media of the preculture: tryptone (16 g/L), sodium chloride (10 g/L) and yeast extract (10 g/L) in demineralized water. The pH value was maintained at pH 7.3. The medium was sterilized at 121 °C for 20 minutes. Afterwards kanamycin (50 mg/L) was added.

Cell cultivation 1: One preculture (100 mL) was inoculated with the strain *E. coli* BL21 Star™ (DE3) pETM11-BM3-268; M177Y, A184Y, S72G, V178P, L181Y, L188F (50 μL) containing the

desired plasmids and was shaken at 37 °C and 165 rpm for 17 hours. This preculture (100 mL) was used to inoculate one 10 L steel fermenter. The cultivation media was prepared in the fermenter. Tryptone (12 g/L), yeast extract (24 g/L), predigested beef extract (2 g/L), KH₂PO₄ (2.2 g/L), K₂HPO₄ (9.4 g/L) and glycerol (87%, 4.6 g/L) were dissolved in demineralized water (9.2 L) and sterilized for 20 minutes at 121 °C in the fermenter. Afterwards kanamycin (0.5 g) in water (20 mL), riboflavin (10 mg) in water (20 mL), thiamine hydrochloride (3.37 g) in water (10 mL) and Oxford trace metal solution (2.5 mL) were added. After the inoculation of the fermenter, the culture was stirred at 315 rpm at 37 °C with an aeration rate of 3.3 L/min at pH 6.6 which was regulated by addition of aqueous sodium hydroxide solution (16%) or aqueous phosphoric acid solution (16%). After 4 hours, an OD₅₅₀ of 0.7 was reached, the temperature was decreased to 27 °C within 10 minutes and IPTG (1.43 g) in water (40 mL) were added to start the protein expression. After 9.50 hours the aqueous phosphoric acid solution (16%) was substituted for an aqueous glucose solution (50%) for pH regulation. After additional 24 hours an OD₅₅₀ of 19.6 was reached, the cells were harvested by centrifugation (194.4 g), suspended in buffer (194.4 mL; KH₂PO₄ (4 g/L), K₂HPO₄ (12.3 g/L), glycerol (4% (v/v)), glucose (5% (w/v)), EDTA (0.5 mM)), frozen in liquid nitrogen and stored at –80 °C until further use in aliquots of 1 x 150 mL, 1 x 100 mL, 2 x 40 mL und 5 x 1 mL.

Cell cultivation 2: One preculture (100 mL) was inoculated with the strain *E. coli* BL21 Star™ (DE3) pETM11-BM3-268; M177Y, A184Y, S72G, V178P, L181Y, L188F (50 µL) containing the desired plasmids and was shaken at 37 °C and 165 rpm for 17 hours. This preculture (100 mL) was used to inoculate one 10 L steel fermenter. The cultivation media was prepared in the fermenter. Tryptone (12 g/L), yeast extract (24 g/L), predigested beef extract (2 g/L), KH₂PO₄ (2.2 g/L), K₂HPO₄ (9.4 g/L) and glycerol (87%, 4.6 g/L) were dissolved in demineralized water (9.2 L) and sterilized for 20 minutes at 121 °C in the fermenter. Afterwards kanamycin (0.5 g) in water (20 mL), riboflavin (10 mg) in water (20 mL), thiamine hydrochloride (3.37 g) in water (10 mL) and Oxford trace metal solution (2.5 mL) were added. After the inoculation of the fermenter, the culture was stirred at 315 rpm at 37 °C with an aeration rate of 3.3 L/min at pH 6.6 which was regulated by addition of aqueous sodium hydroxide solution (16%) or aqueous phosphoric acid solution (16%). After 3.55 hours, an OD₅₅₀ of 0.69 was reached, the temperature was decreased to 27 °C within 10 minutes and IPTG (1.43 g) in water (40 mL) were added to start the protein expression. After 9 hours the aqueous phosphoric acid solution (16%) was substituted for an aqueous glucose solution (50%) for pH regulation. After additional 24 hours an OD₅₅₀ of 27.75 was reached, the cells were harvested by centrifugation (231 g), suspended in buffer (231 mL; KH₂PO₄ (4 g/L), K₂HPO₄ (12.3 g/L), glycerol (4% (v/v)), glucose (5% (w/v)), EDTA (0.5 mM)), frozen in liquid nitrogen and stored at –80 °C until further use in aliquots of 2 x 150 mL, 1 x 50 mL, 2 x 40 mL und 5 x 1 mL.

Biotransformation

To a 10 L steel fermenter which contained KH₂PO₄ (40 g), K₂HPO₄ (123 g), Pluronic® PE 8100 (1 mL), demineralized water (9.0 L), aqueous glucose solution (50 %, 200 mL) and aqueous EDTA solution (0.5 M, 10 mL) androsta-1,4-diene-3,17-dione (500 mg, 1.75 mmol) dissolved in DMF (10 mL) was added and the mixture was stirred at 315 rpm at 27 °C with an aeration rate of 3.3 L/min. Cells in buffer (330 mL of cell cultivation 1 and 150 mL of cell cultivation 2) were added, and the biotransformation was maintained at an oxygen partial pressure of 50 % by varying the stirring rate to up to 1200 rpm. The pH value was maintained at pH 7.4 by addition of an aqueous solution of sodium hydroxide (16 %). After 4 hours the biotransformation was extracted with 4-methyl-2-pentanone. The organic layer was concentrated to give an oil (5.65 g) which was dissolved in methanol, and was concentrated to give an oil (3.73 g).

The crude product was further purified by flash chromatography using silica gel (dichloromethane/ethanol gradient) and by preparative HPLC to give the title compound (54 mg).

Preparative HPLC method

Instrument: Waters Autopurificationsystem; Column: XBrigde C18 5 µ, 100x30 mm; eluent A: water + 0.1 vol % formic acid; eluent B: acetonitrile; gradient: 0.0-0.5 min 23 % B (35-70 mL/min), 0.5-5.5 min 23-58 % B; flow: 70 mL/min; temperature: 25 °C; DAD scan: 210-400 nm.

Analytical HPLC method

Instrument: Waters Acquity UPLCMS SingleQuad; Column: Acquity UPLC BEH C18 1.7 µ, 50x2.1 mm; eluent A: water + 0.1 vol % formic acid; eluent B: acetonitrile; gradient: 0-2.6 min 1-99 % B, 2.6-3.0 min 99 % B; flow: 0.8 mL/min; temperature: 60 °C; DAD scan: 210-400 nm.

Analytical HPLC: R_t = 0.84 min.

¹H-NMR (600 MHz, Pyridin-d₅) δ [ppm]: 1.04 (s, 3H), 1.28 - 1.41 (m, 2H), 1.45 - 1.62 (m, 3H), 1.75 (ddd, 1H), 1.86 - 1.93 (m, 1H), 1.98 - 2.08 (m, 1H), 2.21 - 2.27 (m, 1H), 2.28 - 2.36 (m, 1H), 2.45 - 2.53 (m, 1H), 2.80 - 2.92 (m, 2H), 4.30 (t, 1H), 7.03 (d, 1H), 7.11 (dd, 1H), 7.28 (d, 1H), 7.91 (s, 1H), 11.24 (s, 1H).

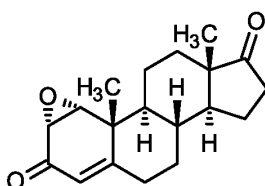
¹H-NMR (600 MHz, Pyridin-d₅) δ [ppm]: -0.002 (1.01), 0.000 (2.61), 0.965 (0.35), 1.038 (16.00), 1.292 (0.33), 1.303 (0.42), 1.313 (0.94), 1.323 (1.00), 1.333 (1.12), 1.344 (1.18), 1.353 (0.90), 1.355 (0.90), 1.365 (1.11), 1.370 (1.07), 1.374 (1.06), 1.378 (0.87), 1.388 (0.84), 1.396 (0.76), 1.461 (0.25), 1.467 (0.28), 1.483 (0.83), 1.489 (0.69), 1.504 (1.07), 1.508 (1.19), 1.524 (2.72), 1.539 (1.21), 1.545 (1.23), 1.553 (0.73), 1.557 (0.76), 1.561 (0.70), 1.571 (1.25), 1.574 (1.22), 1.590 (1.08), 1.593 (1.04), 1.608 (0.40), 1.612 (0.36), 1.722 (0.73), 1.737 (0.96), 1.741 (1.08), 1.744 (0.91), 1.757 (1.08), 1.759 (0.97), 1.764 (0.84), 1.779 (0.67), 1.875 (0.85), 1.881 (0.87), 1.886 (0.98), 1.890 (0.83), 1.896 (0.93), 1.901 (0.84), 1.907 (0.81), 2.007 (0.29), 2.026 (1.65),

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2.031 (1.03), 2.042 (1.15), 2.046 (1.18), 2.051 (0.70), 2.224 (0.72), 2.241 (1.12), 2.253 (0.50),
2.260 (0.54), 2.302 (1.04), 2.313 (0.84), 2.317 (0.78), 2.324 (1.11), 2.329 (1.01), 2.474 (0.74),
2.482 (0.89), 2.487 (0.99), 2.494 (1.19), 2.502 (0.94), 2.506 (0.82), 2.515 (0.68), 2.813 (0.44),
2.823 (0.52), 2.841 (1.32), 2.849 (1.37), 2.858 (0.95), 2.869 (0.89), 2.879 (0.83), 2.888 (0.90),
5 2.907 (0.31), 2.917 (0.27), 3.615 (0.54), 4.287 (0.83), 4.301 (1.48), 4.314 (0.78), 5.038 (1.80),
7.025 (2.83), 7.029 (2.71), 7.099 (1.47), 7.103 (1.36), 7.113 (1.74), 7.117 (1.46), 7.212 (11.96),
7.270 (2.43), 7.284 (2.07), 7.580 (5.57), 7.908 (0.88), 8.729 (10.46), 11.240 (0.77).

Example 26

1 α ,2 α -1,2-epoxyandrost-4-ene-3,17-dione



10

For the preparation of the title compound see Example 25. The crude product was further purified by flash chromatography using silica gel (dichloromethane/ethanol gradient) and by preparative HPLC to give the title compound (5.8 mg).

Preparative HPLC method

15 Instrument: Waters Autopurificationsystem; Column: XBrigde C18 5 μ , 100x30 mm; eluent A: water + 0.1 vol % formic acid; eluent B: acetonitrile; gradient: 0.0-0.5 min 23 % B (35-70 mL/min), 0.5-5.5 min 23-58 % B; flow: 70 mL/min; temperature: 25 °C; DAD scan: 210-400 nm.

Analytical HPLC method

Instrument: Waters Acquity UPLCMS SingleQuad; Column: Acquity UPLC BEH C18 1.7 μ ,
20 50x2.1 mm; eluent A: water + 0.1 vol % formic acid; eluent B: acetonitrile; gradient: 0-2.6 min 1-99 % B, 2.6-3.0 min 99 % B; flow: 0.8 mL/min; temperature: 60 °C; DAD scan: 210-400 nm.

Analytical HPLC: R_t = 1.06 min.

$^1\text{H-NMR}$ (600 MHz, CHLOROFORM- d) δ [ppm]: 0.95 (s, 3H), 1.13 (qd, 1H), 1.23 - 1.36 (m, 4H),
1.42 (s, 3H), 1.54 - 1.68 (m, 2H), 1.84 - 1.94 (m, 2H), 1.88 - 1.98 (m, 1H), 2.05 - 2.15 (m, 2H),
25 2.22 - 2.31 (m, 1H), 2.36 - 2.43 (m, 1H), 2.48 (dd, 1H), 3.40 (dd, 1H), 3.67 (d, 1H), 5.76 (s, 1H).

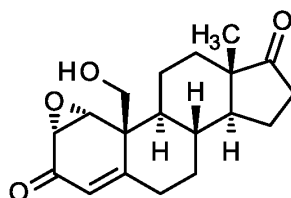
$^1\text{H-NMR}$ (600 MHz, CHLOROFORM- d) δ [ppm]: 0.000 (1.78), 0.880 (0.44), 0.935 (0.39), 0.949
(14.78), 0.988 (1.17), 1.093 (0.36), 1.100 (0.42), 1.114 (0.85), 1.121 (0.85), 1.136 (0.89), 1.143
(0.90), 1.157 (0.45), 1.164 (0.41), 1.238 (0.73), 1.244 (0.81), 1.255 (2.21), 1.279 (1.39), 1.288
(1.42), 1.303 (1.60), 1.309 (1.87), 1.318 (0.94), 1.327 (1.16), 1.419 (16.00), 1.545 (0.66), 1.560

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(1.16), 1.565 (1.06), 1.576 (0.92), 1.581 (1.72), 1.587 (1.04), 1.596 (1.54), 1.602 (1.28), 1.610 (1.26), 1.617 (1.71), 1.633 (1.26), 1.638 (1.20), 1.654 (0.67), 1.662 (0.58), 1.849 (0.39), 1.856 (0.43), 1.868 (1.11), 1.875 (1.61), 1.880 (1.14), 1.887 (1.23), 1.895 (1.53), 1.900 (2.16), 1.918 (0.88), 1.925 (1.35), 1.936 (0.62), 1.940 (0.70), 1.950 (0.73), 1.956 (0.56), 1.961 (0.61), 1.971 (0.54), 2.013 (0.39), 2.072 (0.90), 2.086 (1.84), 2.093 (0.67), 2.103 (1.67), 2.119 (1.48), 2.134 (0.65), 2.245 (0.61), 2.252 (0.74), 2.257 (0.60), 2.267 (0.85), 2.271 (0.99), 2.278 (0.76), 2.291 (0.41), 2.371 (0.67), 2.380 (0.75), 2.393 (1.15), 2.402 (1.07), 2.414 (0.57), 2.423 (0.47), 2.454 (1.10), 2.468 (1.06), 2.486 (0.96), 2.501 (0.92), 2.625 (0.45), 3.393 (1.58), 3.396 (1.81), 3.398 (1.89), 3.402 (1.65), 3.662 (2.62), 3.668 (2.55), 5.756 (2.73), 7.264 (14.93).

10 **Example 27**

19-hydroxy-1 α ,2 α -1,2-epoxyandrost-4-ene-3,17-dione



For the preparation of the title compound see Example 25. The crude product was further purified by flash chromatography using silica gel (dichloromethane/ethanol gradient) and twice by preparative HPLC to give the title compound (3.7 mg).

1. Preparative HPLC method

Instrument: Waters Autopurificationsystem; Column: XBrigde C18 5 μ , 100x30 mm; eluent A: water + 0.1 vol % formic acid; eluent B: acetonitrile; gradient: 0.0-0.5 min 23 % B (35-70 mL/min), 0.5-5.5 min 23-58 % B; flow: 70 mL/min; temperature: 25 °C; DAD scan: 210-400 nm.

20 **1. Analytical HPLC method**

Instrument: Waters Acquity UPLCMS SingleQuad; Column: Acquity UPLC BEH C18 1.7 μ , 50x2.1 mm; eluent A: water + 0.1 vol % formic acid; eluent B: acetonitrile; gradient: 0-2.6 min 1-99 % B, 2.6-3.0 min 99 % B; flow: 0.8 mL/min; temperature: 60 °C; DAD scan: 210-400 nm.

2. Preparative HPLC method

25 Instrument: Waters Autopurificationsystem; Column: XBrigde C18 5 μ , 100x30 mm; eluent A: water + 0.1 vol % formic acid; eluent B: acetonitrile; gradient: 0.0-0.5 min 20 % B (35-70 mL/min), 0.5-5.5 min 20-30 % B; flow: 70 mL/min; temperature: 25 °C; DAD scan: 210-400 nm.

2. Analytical HPLC method

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Instrument: Waters Acquity UPLCMS SingleQuad; Column: Acquity UPLC BEH C18 1.7 μ , 50x2.1 mm; eluent A: water + 0.1 vol % formic acid; eluent B: acetonitrile; gradient: 0-2.6 min 1-99 % B, 2.6-3.0 min 99 % B; flow: 0.8 mL/min; temperature: 60 °C; DAD scan: 210-400 nm.

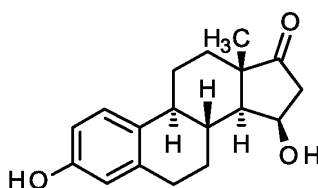
Analytical HPLC: R_t = 0.82 min.

5 $^1\text{H-NMR}$ (600 MHz, CHLOROFORM-d) δ [ppm]: 0.95 (s, 3H), 1.12 - 1.21 (m, 1H), 1.23 - 1.37 (m, 3H), 1.54 - 1.65 (m, 2H), 1.84 - 2.01 (m, 4H), 2.06 - 2.18 (m, 2H), 2.33 - 2.41 (m, 2H), 2.43 - 2.52 (m, 2H), 3.38 (dd, 1H), 3.95 (d, 1H), 4.16 (t, 1H), 4.29 (d, 1H), 5.92 (s, 1H).

$^1\text{H-NMR}$ (600 MHz, CDCl_3) δ [ppm]: 0.881 (0.18), 0.904 (0.57), 0.927 (0.28), 0.948 (16.00), 1.136 (0.33), 1.143 (0.36), 1.157 (0.87), 1.164 (0.89), 1.179 (0.96), 1.186 (0.96), 1.200 (0.48),
10 1.208 (0.57), 1.240 (0.68), 1.247 (0.73), 1.263 (1.38), 1.269 (1.30), 1.287 (1.12), 1.291 (0.93), 1.297 (0.82), 1.308 (0.99), 1.315 (0.97), 1.318 (0.96), 1.326 (1.21), 1.331 (0.88), 1.336 (0.89), 1.347 (1.18), 1.364 (0.77), 1.369 (0.76), 1.414 (0.40), 1.545 (0.55), 1.552 (0.60), 1.568 (1.36), 1.574 (1.53), 1.590 (2.54), 1.597 (2.84), 1.606 (4.35), 1.621 (2.17), 1.627 (1.63), 1.642 (0.73), 1.881 (0.80), 1.887 (1.28), 1.893 (0.88), 1.904 (0.80), 1.909 (1.20), 1.915 (0.87), 1.926 (0.50),
15 1.937 (1.32), 1.945 (1.51), 1.952 (1.50), 1.958 (2.52), 1.963 (2.30), 1.970 (1.32), 1.981 (1.80), 2.076 (0.82), 2.090 (1.33), 2.108 (1.19), 2.115 (0.59), 2.123 (2.28), 2.138 (1.12), 2.143 (0.92), 2.148 (0.85), 2.367 (1.13), 2.373 (1.49), 2.388 (1.21), 2.394 (1.41), 2.399 (0.97), 2.435 (0.78), 2.443 (0.82), 2.458 (1.29), 2.464 (2.12), 2.478 (1.61), 2.487 (0.59), 2.495 (1.14), 2.510 (1.01), 2.623 (0.54), 3.377 (1.81), 3.380 (2.08), 3.383 (2.14), 3.387 (1.91), 3.942 (2.83), 3.949 (2.78),
20 4.146 (0.55), 4.163 (1.05), 4.179 (0.71), 4.278 (2.45), 4.297 (1.82), 5.920 (3.16), 7.266 (11.03).

Example 28

3,15beta-dihydroxyestra-1(10),2,4-trien-17-one



For the preparation of the title compound see Example 25. The crude product was further purified
25 by flash chromatography using silica gel (dichloromethane/ethanol gradient) and by preparative HPLC to give the title compound (0.8 mg).

Preparative HPLC method

Instrument: Waters Autopurificationsystem; Column: XBrigde C18 5 μ , 100x30 mm; eluent A: water + 0.1 vol % formic acid; eluent B: acetonitrile; gradient: 0.0-0.5 min 25 % B (35-70 mL/min),
30 0.5-5.5 min 25-35 % B; flow: 70 mL/min; temperature: 25 °C; DAD scan: 210-400 nm.

Analytical HPLC method

Instrument: Waters Acquity UPLCMS SingleQuad; Column: Acquity UPLC BEH C18 1.7 μ , 50x2.1 mm; eluent A: water + 0.1 vol % formic acid; eluent B: acetonitrile; gradient: 0-2.6 min 1-99 % B, 2.6-3.0 min 99 % B; flow: 0.8 mL/min; temperature: 60 °C; DAD scan: 210-400 nm.

5 Analytical HPLC: R_t = 0.85 min.

$^1\text{H-NMR}$ (600 MHz, Pyr) δ [ppm]: 0.000 (1.30), 1.267 (0.19), 1.452 (0.59), 1.458 (0.76), 1.469 (0.83), 1.477 (0.91), 1.492 (4.48), 1.530 (0.23), 1.551 (0.43), 1.574 (0.61), 1.596 (0.42), 1.618 (0.31), 2.018 (0.47), 2.037 (0.49), 2.137 (0.40), 2.155 (0.41), 2.172 (0.16), 2.301 (0.26), 2.318 (0.42), 2.336 (0.26), 2.352 (0.45), 2.379 (0.51), 2.496 (0.25), 2.666 (0.30), 2.676 (0.32), 2.697 (0.48), 2.707 (0.47), 2.816 (0.89), 2.833 (0.26), 2.848 (0.98), 2.859 (0.55), 2.879 (0.32), 2.887 (0.31), 2.898 (0.31), 4.782 (0.56), 4.989 (1.05), 6.502 (0.65), 7.002 (1.01), 7.106 (0.53), 7.118 (0.60), 7.211 (16.00), 7.313 (0.74), 7.327 (0.66), 7.578 (7.55), 8.731 (12.51), 11.201 (0.27).

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