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Effects of Alternative Redox Partners and Oxidizing Agents on CYP154C8 Catalytic Activity and Product Distribution

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CYP154C8 catalyzes the hydroxylation of diverse steroids, as has previously been demonstrated, by using an NADH-dependent system including putidaredoxin and putidaredoxin reductase as redox partner proteins carrying electrons from NADH. In other reactions, CYP154C8 reconstituted with spinach ferredoxin and NADPH-dependent ferredoxin reductase displayed catalytic activity different from that of the NADH-dependent system. The NADPH-dependent system showed multistep oxidation of progesterone and other substrates including androstenedione, testosterone, and nandrolone. (Diacetoxyiodo)benzene was employed to generate compound I (FeO^{3+}), actively supporting the redox reactions catalyzed by CYP154C8. In addition to 16α-hydroxylation, progesterone and 11-oxoprogesterone also underwent hydroxylation at the 6β-position in reactions supported by (diacetoxyiodo)benzene. CYP154C8 was active in the presence of high concentrations (>10 mM) of H_2O_2, with optimum conversion surprisingly being achieved at ≈75 mM H_2O_2. More importantly, H_2O_2 tolerance by CYP154C8 was evident in the very low heme oxidation rate constant (*K*) even at high concentrations of H_2O_2. Our results demonstrate that alternative redox partners and oxidizing agents influence the catalytic efficiency and product distribution of a cytochrome P450 enzyme. More importantly, these choices affected the type and selectivity of reaction catalyzed by the P450 enzyme.

Introduction

Cytochrome P450s (CYPs) are widely distributed across all forms of life (archaea, prokarya, and eukarya).[1] Most CYPs catalyze oxidative reactions utilizing O_2 and two electrons supplied by NAD(P)H and transferred through redox partners such as flavin-containing reductases and iron–sulfur ferredoxins.[2] The mammalian CYPs are membrane-bound and reduced by NADPH–cytochrome P450 reductase (CPR), which is also a membrane-bound enzyme required for electron transfer from NADPH to CYPs in the endoplasmic reticulum. The FAD- and FMN-containing CPRs support CYPs of class II.[3] The class I redox system includes bacterial and mitochondrial iron–sulfur (Fe—S) containing proteins (ferredoxin/adrenodoxin, Fdx/Adx) and FAD reductase (ferredoxin/adrenodoxin reductase, Fdr/Adr). In addition, flavin-dependent proteins known as flavodoxins (flavin-mononucleotide-containing proteins) are present in some prokaryotic organisms and certain algae.[4] A distinct group is represented by the so-called self-sufficient CYPs, in which a CPR-like reductase domain is fused to a monooxygenase domain.[5–11] One well-known form is P450BM3 (CYP102A1, discovered in *Bacillus megaterium*), a natural fusion of fatty acid hydroxylase P450 to a soluble CPR.[5]

In addition to CYPs that are either linked to electron carrier proteins or require redox partner proteins to receive electrons from reducing equivalent, a few unusual CYPs (CYP170A1, CYP154A1, and CYP170A1) have been characterized with activity in the absence of redox partners.[12–14]

Only a few native CYPs have been found to display peroxygenase and peroxidase functions, catalyzing the peroxygenation of various substrates in the presence of H_2O_2 and other peroxy compounds.[15–20] By means of the "peroxide shunt" pathway, ferric CYP is directly transformed into a ferric hydroperoxo intermediate known as compound 0. The peroxide-shunt reaction is an attractive option for monooxygenation reactions through the action of CYP enzymes because these reactions are independent of redox partner proteins and, more importantly, the low cost of H_2O_2 is significant on industrial scales. Many efforts have been made to develop artificial H_2O_2-dependent CYPs in view of their benefits as practical biocatalysts.[21–23] In addition, the H_2O_2-dependent CYPs have been deployed to oxidize non-native substrates in the presence of

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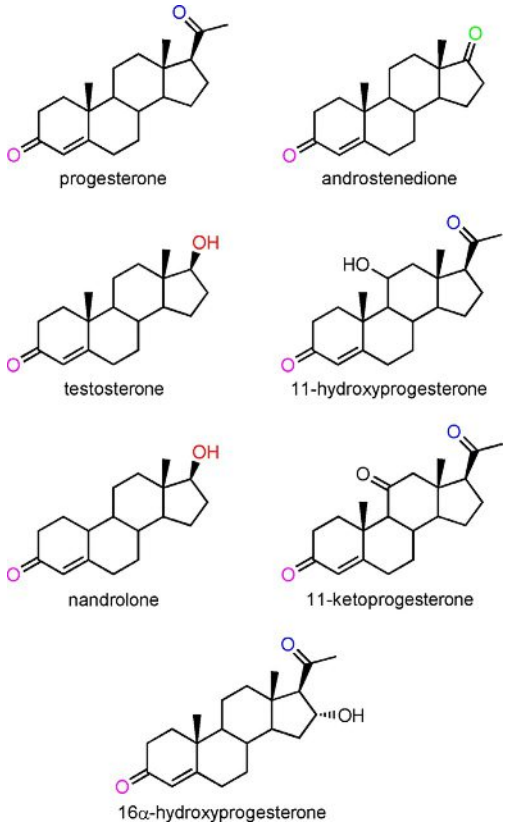
decoy molecules.[20,24,25] Further, single-oxygen donors (e.g., periodate and iodosobenzene) are also found to promote CYP-catalyzed monooxygenation reactions, resulting in a highly reactive ferryl heme π-cation radical known as compound I.[26–29] However, few CYPs are known to display such characteristics in the presence of specific chemical compounds. The characterization or synthetic application of CYPs has often been mediated through one or more surrogate redox partners either in an isolated form or after artificial fusion with the CYP complex, due to the difficulty in obtaining native redox partners.[30–34] It is generally believed that the choice of surrogate partners or their mode of action does not affect the type and selectivity of the reactions catalyzed by CYPs.[35] However, alternative redox partners might influence the catalytic efficiency and the product distribution.[29,35–40]

CYPs such as CYP106A1, CYP106A2, CYP109B1, CYP109E1, CYP154C3, CYP154C5, CYP260A1, and CYP260B1, originating from bacterial sources, are known to hydroxylate steroids.[32,33,41–45] CYP154C8 shows high similarity with CYP154C3 (74%) and CYP154C5 (66%), both of which are reported to hydroxylate steroids at C16α. CYP154C8 was previously characterized with an NADH-dependent system including putidaredoxin reductase (Pdr) and putidaredoxin (Pdx) as redox partner proteins as transferring electrons from NADH to CYP.[46] It was found to hydroxylate diverse steroids at different positions. Androstenedione, testosterone, and 11-oxoprogesterone were 16α-hydroxylated; two dihydroxylated products of progesterone were also formed, as the result of subsequent hydroxylation of 16α-hydroxyprogesterone in the presence of CYP154C8. The major product from corticosterone was 21-hydroxycorticosterone, and substrates with hydroxy or carbonyl groups at C11 and C21 showed a similar pattern of product formation to corticosterone.



Scheme 1. Steroids used as substrates for further characterization of CYP154C8.

In this work, in vitro reactions with steroids (Scheme 1) mediated by an NADPH-dependent redox partner system together with the oxidizing agents (diacetoxyiodo)benzene and H_2O_2 showed changes in the product distribution and catalytic activity of CYP154C8 in relation to an NADH-dependent system. However, other oxidizing agents including sodium periodate, sodium chlorite, and *tert*-butyl hydroperoxide failed to support CYP154C8-catalyzed in vitro reactions.

Results

Hydrogen peroxide tolerance of CYP154C8

Oxidative degradation of heme is a major challenge in H_2O_2-mediated CYP reactions. To determine the effect of H_2O_2 on CYP154C8 reaction behavior, the H_2O_2 tolerance was analyzed over the range of 0.2–100 mM H_2O_2. The decrease in the Soret absorbance in an oxidized form of CYP154C8 was monitored at different concentrations of H_2O_2 at intervals up to 30 min. The Soret absorbance at different intervals was plotted against time. The Soret peak intensity decreased over time during exposure to >1 mM H_2O_2 concentration (Figure 1). Surprisingly, the heme oxidation rate constant (K) was low even at higher concentrations of H_2O_2 (Table 1). These data demonstrate that CYP154C8 has high H_2O_2 tolerance even at high (>50 mM) H_2O_2 concentrations.

Table 1. Rates of heme oxidation (K) and absorbance amplitude (A) for heme Soret peak absorbance change at 417 nm of CYP154C8. Data were fitted to a one-phase exponential decay by use of GraphPad Prism 6.

H_2O_2 [mM]	K [min^{-1}]	A	H_2O_2 [mM]	K [min^{-1}]	A
0.2	n.d.	0.010	1	0.18 ± 0.040	0.016
3	0.09 ± 0.005	0.075	5	0.02 ± 0.020	0.092
20	0.12 ± 0.015	0.233	50	0.18 ± 0.012	0.235
75	0.51 ± 0.004	0.227	100	0.18 ± 0.050	0.223

n.d.: "not determined", due to small amplitudes of heme absorbance change.

Reaction behavior with NADPH and surrogate redox partners Fdx and Fdr

An in vitro approach involving spinach surrogate redox partners Fdx and Fdr, cofactor NADPH, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase for cofactor regeneration (as described in the Experimental Section) was highly preferred over the NADH system and oxidizing agents. The percentages of conversion for all substrates were found to be significantly

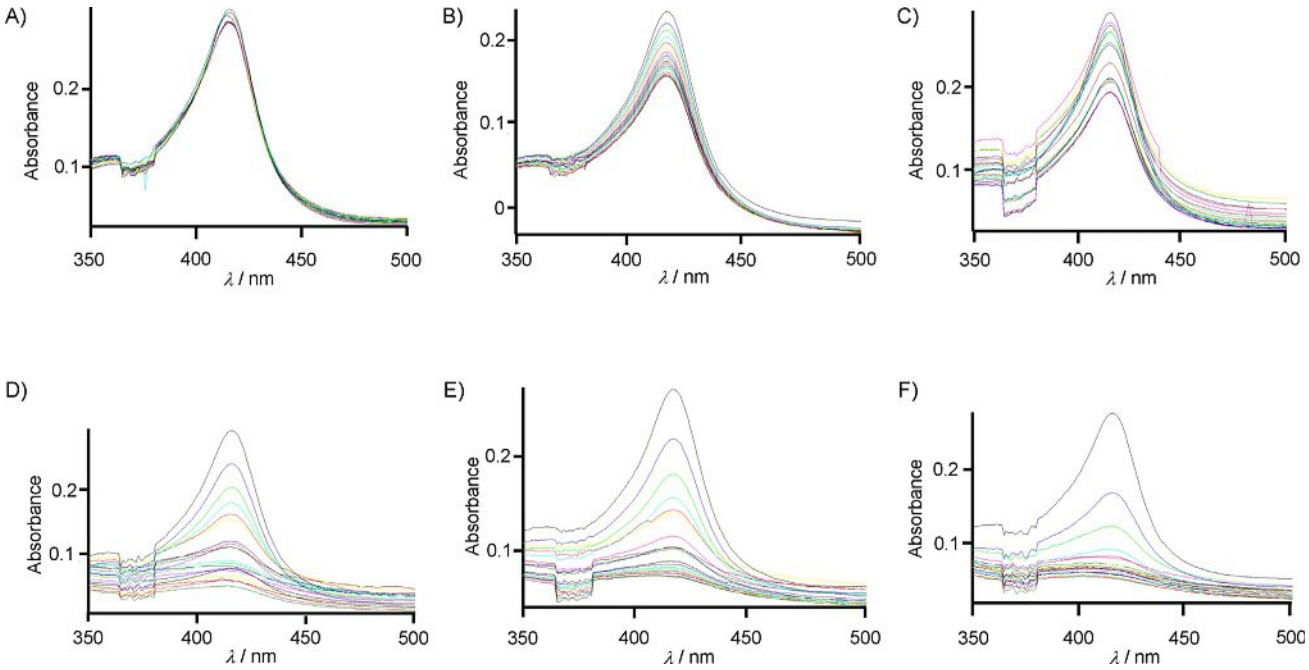


Figure 1. Tolerance of H_2O_2 by CYP154C8. The spectra show the absorbance over 30 min at intervals of 1.5 min at H_2O_2 concentrations of A) 1, B) 3, C) 5, D) 20, E) 50, and F) 100 mM.

increased with this system in relation to the NADH-dependent system (data not shown). Androstenedione and testosterone were completely converted into products. In addition to peaks corresponding to the 16α-hydroxylated products, both substrate reaction mixtures showed additional peaks in theirs HPLC chromatograms (Figure 2). LC-MS analysis of the reaction mixtures revealed both additional peaks to be dihydroxylated products of their corresponding substrates (Figure S1 in the Supporting Information). These dihydroxylated products must have resulted from subsequent hydroxylation of the 16α-hydroxylated products in the reaction mixtures. To confirm this, the 16α-hydroxylated products of androstenedione and testosterone were purified as described in the Experimental Section, and in vitro reactions with 16α-hydroxyandrostenedione and 16α-hydroxytestosterone—in place of androstenedione and testosterone, respectively—were carried out. HPLC (Figure S2) and LC-MS (data not shown) analysis confirmed that dihydroxylated products of androstenedione and testosterone were obtained as the result of hydroxylation of 16α-hydroxyandrostenedione and 16α-hydroxytestosterone, respectively.

The nandrolone reaction mixture also showed multiple peaks in its HPLC chromatograms (Figure S3), P4 being a major product whereas others reflected very low degrees of conversion. LC-MS analysis showed P1 to be a dihydroxylated product, and the remaining four products were each found to be hydroxylated at a single position (Figure S4).

Androstenedione, testosterone, and nandrolone were never converted into dihydroxylated products when CYP154C8 was supported by the NADH-dependent system.[46] 11-Hydroxyprogesterone showed a possible major peak due to 16α-hydroxylation and a very small additional product peak (Figure S5, inset I). Both peaks were identified as monohydroxylated products of 11-hydroxyprogesterone. However, 11-oxoprogesterone was hydroxylated at a single position (Figure S5, inset II). A comparative HPLC and LC-MS analysis with the previous standard showed hydroxylation at C16α.[46]

The progesterone reaction mixture supported by the NADPH-dependent system showed a product formation pattern similar to that obtained with the NADH-dependent system, based on HPLC analysis (Figure S6, inset I). In our previous report, progesterone in the NADH-dependent system was initially hydroxylated at C16α to form 16α-hydroxyprogesterone, which was then sequentially hydroxylated at two different positions to yield 6β,16α-dihydroxyprogesterone and 2α,16α-dihydroxyprogesterone.[46] The same 16α-hydroxyprogesterone and two dihydroxylated products (6β,16α-dihydroxyprogesterone and 2α,16α-dihydroxyprogesterone) were also found to have been formed with the NADPH-dependent system (Figure S6, inset II).

CYP154C8 reaction behavior with (diacetoxyiodo)benzene

When the reaction was carried out with the surrogate oxidant (diacetoxyiodo)benzene, in addition to the usual 16α-hydroxyprogesterone product (P1) from progesterone, another monohydroxylated product—P2, never observed with another system—was formed (Figure 3). NMR-based structural elucidation of the product P2 revealed it to be 6β-hydroxyprogesterone. Very low yields of the two dihydroxylated products were also detected in the same reaction mixture. Further, when 16α-hydroxyprogesterone was used as a substrate in place of progesterone and in the presence of (diacetoxyiodo)benzene, the reaction mixture showed the formation of two different dihydroxylated products with retention times precisely matching

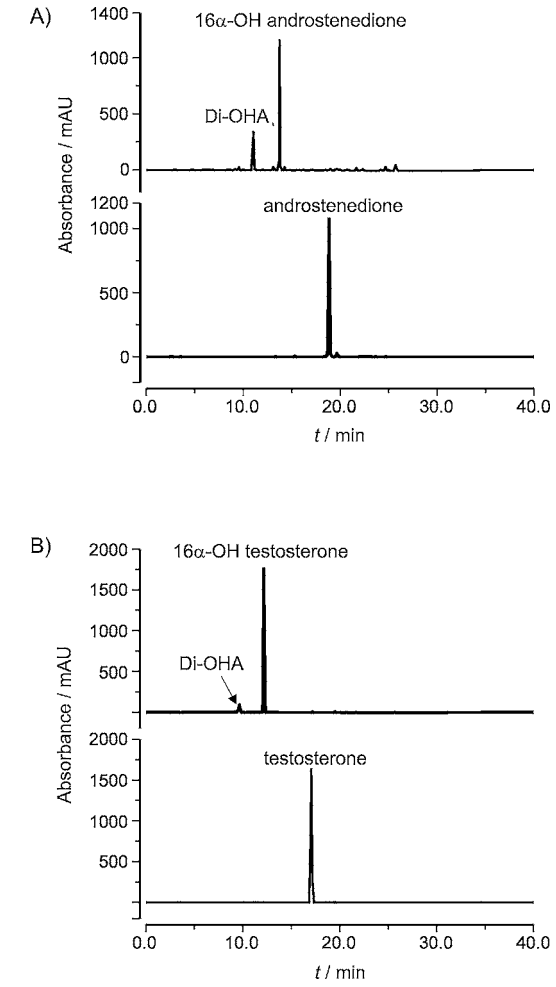


Figure 2. HPLC chromatograms of the reaction mixtures. A) Androstenedione, and B) testosterone supported by the NADPH-dependent system. The chromatograms for the reaction mixture and control system are shown above and below, respectively. Di-OHA and di-OHT denote dihydroxyandrostenedione and dihydroxytestosterone, respectively.

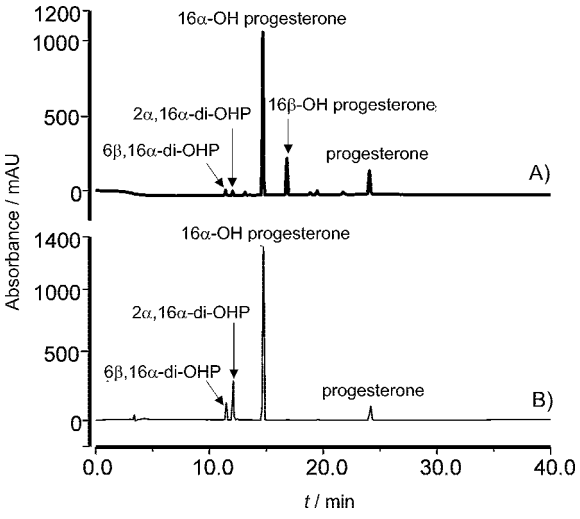


Figure 3. HPLC chromatograms of the progesterone reaction mixtures in the presence of A) (diacetoxyiodo)benzene, and B) NADPH. 2α,16α-Di-OHP and 6β,16α-di-OHP indicate 2α,16α-dihydroxyprogesterone and 6β,16α-dihydroxyprogesterone, respectively.

those of the two dihydroxylated products in the progesterone reaction mixture (Figure S7).

Similarly, HPLC analysis of 11-oxoprogesterone (Figure 4) and 11-hydroxyprogesterone (Figure S8) reaction mixtures also showed two monohydroxylated products in each case. LC-MS analysis (data not shown) revealed both peaks to be monohydroxylated products of the corresponding substrates. The

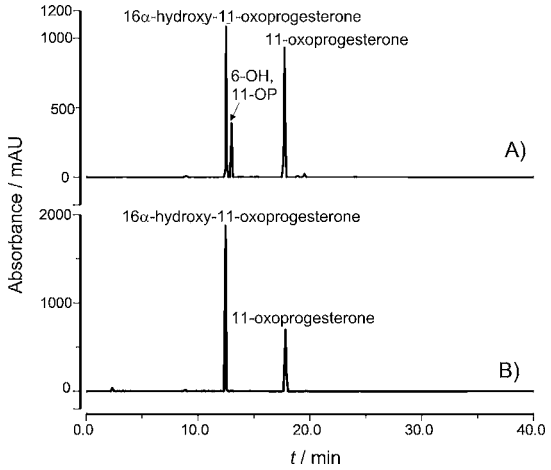


Figure 4. HPLC chromatogram of the 11-oxoprogesterone reaction mixture in the presence of A) (diacetoxyiodo)benzene, and B) NADPH. 6-OH and 11-OP indicate 6β-hydroxy and 11-oxoprogesterone, respectively.

structural elucidation of P2 from 11-oxoprogesterone by NMR showed hydroxylation at 6β, whereas another product (P1) was identified as 16α-hydroxy-11-oxoprogesterone, on the basis of comparison of HPLC retention times (Figure S9) and LC-MS (data not shown) with those of an authentic 16α-hydroxy-11-oxoprogesterone standard. (The authentic standard was previously characterized with CYP154C8 supported by an NADH-dependent system.[46]) A peak retention pattern similar to that of 11-hydroxyprogesterone was observed, thus suggesting that these products were also similarly modified.

Multiple peaks were observed in HPLC chromatograms of testosterone, nandrolone, and androstenedione reaction mixtures (Figure S10). LC-MS analysis showed at least four monohydroxylated products both for testosterone (Figure S11 A) and for nandrolone (Figure S11 B) as substrates, as well as three monohydroxylated products for androstenedione (Figure S11 C). In the cases both of androstenedione (Figure S12 A) and of testosterone (Figure S12 B) the major conversion was into the 16α-hydroxylated product, identified by HPLC and LC-MS (data not shown) and comparison with authentic standards.

All other additional products obtained through reactions catalyzed by CYP154C8 were found to be minor, being obtained with lower selectivity than the corresponding16α-hydroxylated products. Progesterone (≈83%) was the most favored substrate, whereas all other substrates showed at least 35% conversion yields in the presence of (diacetoxyiodo)benzene (Table 2).

Table 2. Conversion percentages of steroid substrates and selectivity of product formation supported by (diacetoxyiodo)benzene. The table also shows the retention times of substrates and their products.[a]

Substrates	Conversion [%]	Selectivity [%]	t_R [min]
progesterone	83.3	16α-OHP (70.0), 6β-OHP (25.0)	S (26.6), 16-OHP (14.6), 6-OHP (16.8)
androstenedione	36.2	16α-OHA (66.0), P1 (9.4), P2 (9.5), P3 (15.0)	S (18.7), 16-OHA (13.7), P1 (13.2), P2 (13.9), P3 (14.7)
testosterone	37.5	16α-OHT (76.0), P1 (3.0), P2 (17.0), P3 (4.0)	S (17.2), 16α-OHT (12.1), P1 (11.2), P2 (11.9), P3 (13.8)
nandrolone	68.6	P1 (13.5), P2 (31.5), P3 (50.4), P4 (4.3)	S (16.0), P1 (10.2), P2 (10.8), P3 (11.2), P4 (13.2)
11-hydroxyprogesterone	69.0	P1 (61.8), P2 (38.2)	S (15.6), P1 (10.7), P2 (11.0)
11-oxoprogesterone	50.3	P1 (75.0), P2 (25.0)	S (17.7), P1 (12.5), P2 (13.0)
16α-hydroxyprogesterone	14.0	P1 (9.5), P2 (4.5)	S (14.6), P1 (11.3), P2 (12.0)

[a] Products were quantified by correlating the peak areas of the products with the combined peak areas of products and substrates. S: substrate, 16-OHP: 16α-hydroxyprogesterone, 6-OHP: 6β-hydroxyprogesterone, 16-OHA: 16α-hydroxyandrostenedione, 16-OHT: 16α-hydroxyandrostenedione, P1–P4 represent the different products formed in the reactions with corresponding substrates.

Reaction behavior with H_2O_2

CYP154C8 was active in the presence of high concentrations (>10 mM) of H_2O_2. Optimum conversion of the substrates occurred at ≈75 mM H_2O_2. Surprisingly, 16α-hydroxyprogesterone was the most favored substrate for CYP154C8 in the presence of H_2O_2 (Figure 5). The two products 6β,16α-dihydroxyprogesterone and 2α,16α-dihydroxyprogesterone were predominantly observed, with overall conversion of ≈51%, the remaining substrates being obtained with very low conversion (Table S1).

Although the percentages of conversion were unacceptably low in the cases of 11-oxoprogesterone and 11-hydroxyprogesterone, each substrate reaction mixture showed two monohydroxylated product peaks by HPLC (Figure S13), further confirmed by LC-MS (data not shown). The retention times of the 11-oxoprogesterone products were similar to those of the previously identified 6β-hydroxy-11-oxoprogesterone and 16α-hydroxy-11-oxoprogesterone obtained in the reactions supported by H_2O_2. HPLC and LC-MS (data not shown) comparison of the progesterone (Figure S14A), androstenedione (Figure S14B), and testosterone (Figure S14C) reaction mixtures with the standards showed that the CYP154C8 in the presence of H_2O_2 was regio- and stereospecific for 16α-hydroxylation.

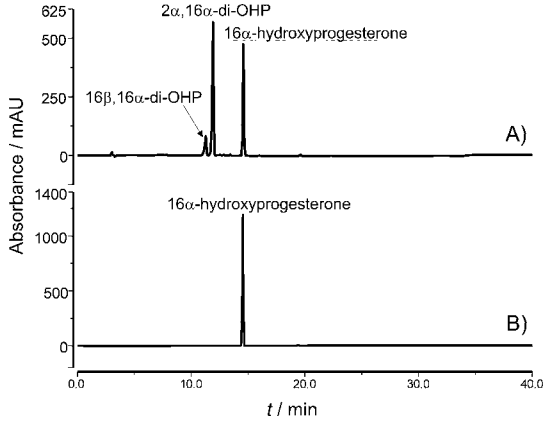


Figure 5. HPLC chromatograms of A) 16α-hydroxyprogesterone reaction mixture in the presence of H_2O_2, and B) 16α-hydroxyprogesterone standard. 2α,16α-Di-OHP and 6β,16α-di-OHP stand for 2α,16α-dihydroxyprogesterone and 6β,16α-dihydroxyprogesterone, respectively. The in vitro reaction was carried out in the presence of 3 μM CYP154C8, 0.5 mM substrate, and 75 mM H_2O_2 for 2 h at 30 °C.

Determination of catalytic efficiencies and kinetic parameters

Progesterone was found to be the most favored substrate for CYP154C8 supported by (diacetoxyiodo)benzene. In another reaction, 16α-hydroxyprogesterone—selected as a substrate for CYP154C8 supported by H_2O_2—yielded higher conversion than other steroid substrates. Hence, progesterone and 16α-hydroxyprogesterone were selected to determine the catalytic efficiency and kinetic parameters in the presence of (diacetoxyiodo)benzene and H_2O_2, respectively. The K_m and K_{cat} values for progesterone were estimated to be (75.94 ± 10.64) μM and (2.32 ± 0.10) min^{-1}, respectively (Figure 6A). Similarly, 16α-hydroxyprogesterone showed K_m and K_{cat} values of (134.50 ± 17.27) μM and (2.37 ± 0.13) min^{-1}, respectively (Figure 6B). In addition to the determination of the kinetic parameters, the time-dependent conversion of both substrates was demonstrated (Figure 7).

Discussion

In vitro reactions with an NADPH-dependent system have been used to achieve further characterization of CYP154C8. The catalytic efficiency of CYP154C8 was not only enhanced with the NADPH-dependent system in relation to the NADH-dependent system, but new products were also observed. The multistep oxidation of progesterone in the presence of CYP154C8 with the aid of an NADH-dependent system has been previously been reported.[46] The reactions supported by the NADPH system showed multistep oxidation of progesterone, androstenedione, testosterone, and nandrolone, indicating a preference of CYP154C8 for NADPH over NADH. Such product formation with the support of an NADPH system also showed the roles played by alternative redox partners and reducing equivalents with regard to the catalytic efficiency of CYP154C8 and its product distributions. In addition, it was also established that the appropriate choice of reducing equivalents and surrogate redox partners might play an important role with regard to catalytic efficiency and product distribu-

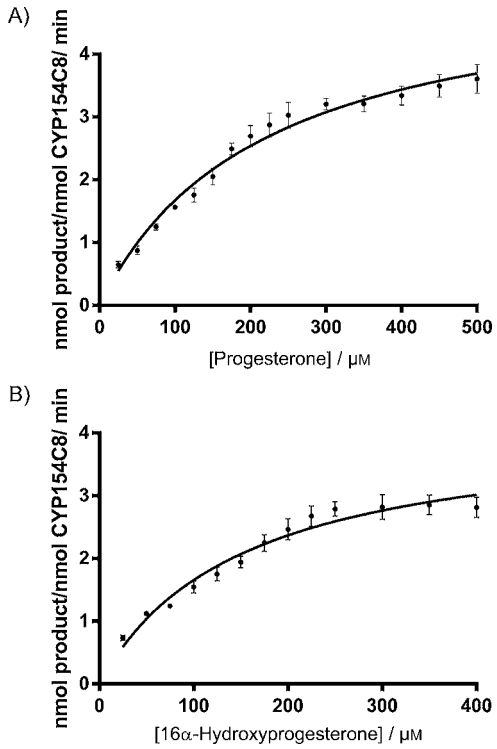


Figure 6. Hyperbolic fits for A) 16α-hydroxyprogesterone in the presence of (diacetoxyiodo)benzene, and B) 2α,16α-dihydroxyprogesterone and 6β,16α-dihydroxyprogesterone in the presence of H_2O_2.

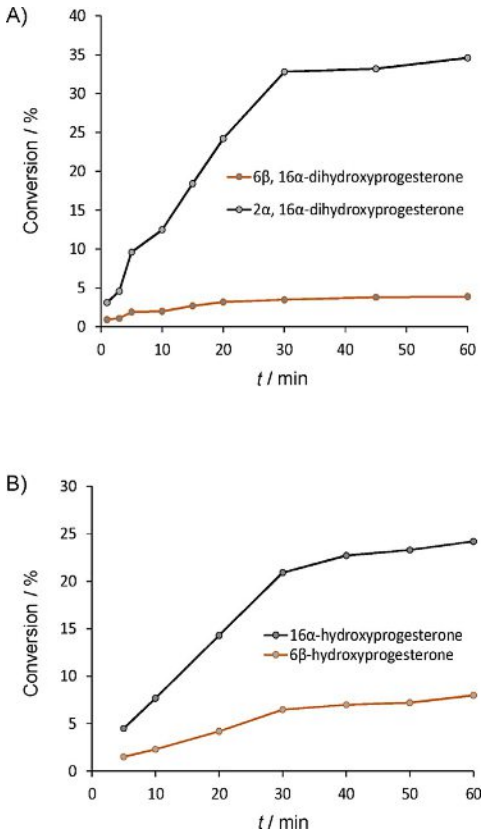
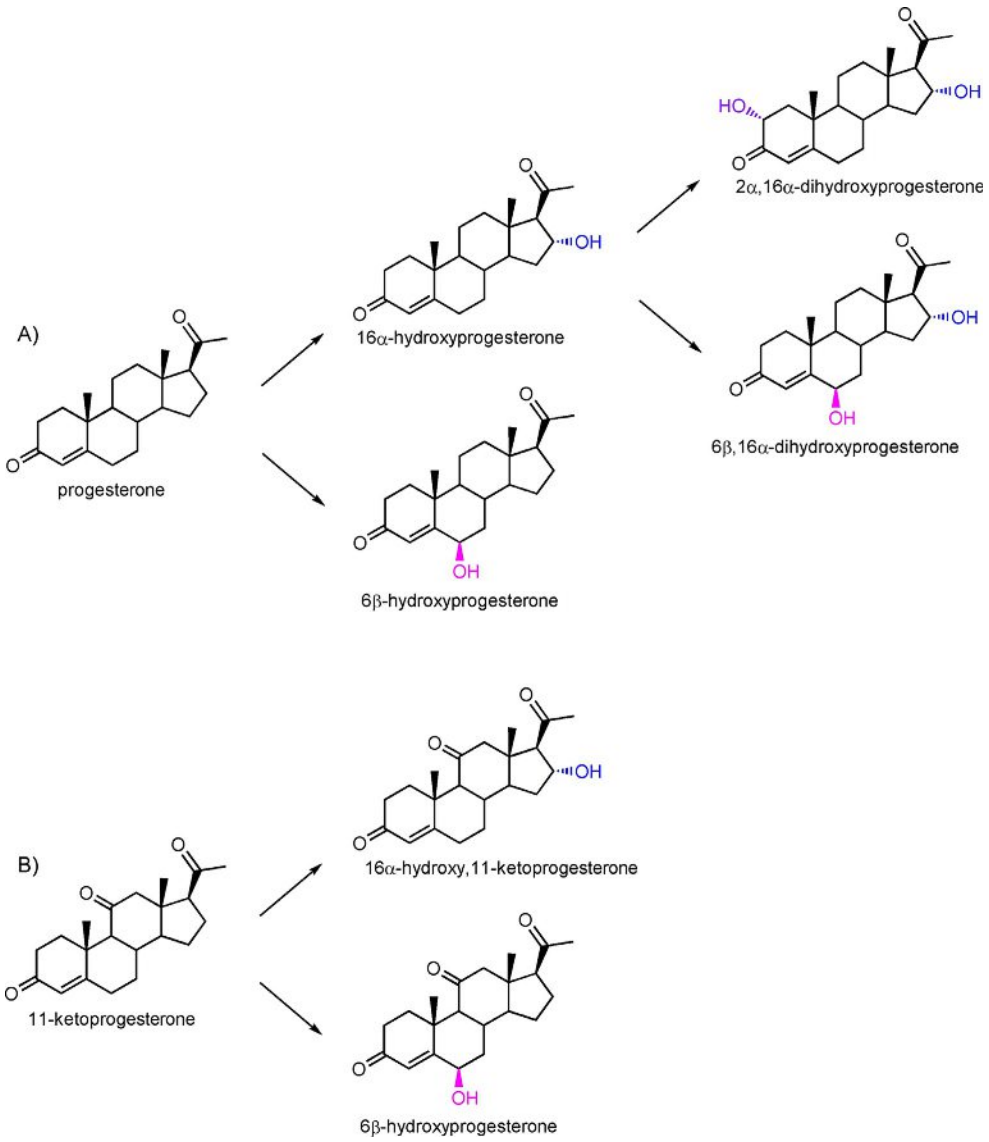


Figure 7. Time-dependent conversion of A) 16α-hydroxyprogesterone, and B) progesterone in the presence of H_2O_2 and (diacetoxyiodo)benzene, respectively. The reactions were carried out with 1 μM CYP154C8 and 0.5 mM substrate at 30 °C for different time intervals (1–60 min). The reactions involving 16α-hydroxyprogesterone and progesterone were initiated with 75 mM H_2O_2 and 2 mM (diacetoxyiodo)benzene, respectively.

tions for CYP enzymes generally.[29,35–40,48] The multistep oxidation of steroids in the presence of CYP154C8 was not previously observed for any bacterial source of CYPs, although cytochrome $P450_{BioI}$ (CYP107H1) and MycG from bacterial sources have been reported to catalyze the multistep oxidation of fatty acids and mycinamycin IV, respectively.[35,49] Some CYPs such as CYP11A1, CYP17A1, CYP19A1, and CYP24A1 from mammalian sources are widely known to catalyze the multistep oxidation of steroids.[50–53]

In addition to the formation of 16α-hydroxylated products of the substrates progesterone and 11-oxoprogesterone, that of 6β-hydroxylated products from both substrates with the support of the oxygen surrogate (diacetoxyiodo)benzene was unusual because such products had never been observed with the NAD(P)H-dependent system, although very low levels of conversion occurred in the presence of H_2O_2 (Scheme 2). However, is not clear whether such product formation occurs in the presence of only oxygen surrogates, as allosteric effects and interactions with redox partners affect the CYP activity and product distribution. Recently, it was reported that CYP17A1 yields different by-products in the presence of iodosobenzene and of NADPH-P450 reductase to form compound I.[29] The effect of cytochrome b5 on the catalytic activity and product formation of CYPs has been widely studied, and shown to induce the activity of specific CYPs.[54,55] In another experiment, MycG, a multifunctional P450 monooxygenase derived from a bacterial source (*Micromonospora griseorubida*) showed an altered type of catalysis in conjunction with an alternative surrogate redox partner, thus highlighting the roles played by variant redox partners in protein–protein interactions in the catalytic activity of CYP enzymes.[35] In addition to electron transfer, the complex of CYP101A1 with its natural redox partner protein Pdx demonstrates an important allosteric regulatory role of these redox partner proteins bound with CYPs.[56, 57] A few CYPs are known to display activity in the presence of (diacetoxyiodo)-benzene. Mammalian and bacterial CYPs such as CYP5A1, CYP17A1, CYP121, CYP101A1 ($P450_{cam}$), and CYP106A2 are known to show catalytic activity in the presence of iodosobenzene as a single-oxygen donor.[30,58–61] The mechanism of oxygenation supported by (diacetoxyiodo)benzene might be similar to that supported by iodosobenzene, probably mediated by two-electron transfer of a single oxygen atom from oxidant to ferric CYP to generate compound I, which is involved in substrate monooxygenation.[58] Hydroxylation of steroid substrates in the presence of CYP154C8 has usually occurred at the α-face of the D-ring. Interestingly, the hydroxylation occurred at the β-face in the cases of progesterone and 11-oxoprogesterone and regioselectivity switched from D-ring to B-ring. As reported previously, 16α-hydroxyprogesterone was sequentially hydroxylated at the 2α- and 6β-positions to yield the corresponding dihydroxylated products of progesterone.[46] CYP17A1, in contrast to its usual function, catalyzed hydroxylation at the 6β-position when 16α,17α-dihydroxyprogesterone



Scheme 2. CYP154C8-mediated hydroxylation of A) progesterone and B) 11-oxoprogesterone.

was used as a substrate; this was attributed to the presence of the two hydroxy groups at the 16α- and 17α-positions.[29] The switch in hydroxylation from D-ring to B-ring in the presence of CYP154C8 together with (diacetoxyiodo)benzene or H_2O_2 illustrates the effect of the oxidation system. Although both the NADPH- and the NADH-dependent systems efficiently supported the activity of CYP154C8, they never induced formation of 6β-hydroxylated product from the substrates progesterone and 11-oxoprogesterone or from any other substrates, thus suggesting the roles for redox partners in protein–protein interactions for modulating the specificity of CYPs. In addition, the functional groups, such as hydroxy or carbonyl groups, in the substrate can also influence the selectivity of hydroxylation, although such switching in the selectivity of different CYPs might not be consistent.[35,39] The factors underlying the B-ring hydroxylation in the presence of CYP154C8 supported by (diacetoxyiodo)benzene and H_2O_2 are unclear. However, the hydroxylation of progesterone at C16α might play a key role in the subsequent hydroxylation in the A-ring and B-ring to yield 2α,16α-dihydroxyprogesterone and 6β,16α-dihydroxyprogesterone, respectively.

The peroxide shunt pathway uses H_2O_2 (or the oxygen donors) to assist CYPs, and represents one of the most efficient ways of using these enzymes for industrial application. However, the oxidative degradation of heme by peroxide has been a major issue.[15,62] The activity of CYP154C8 in the presence of high concentrations of H_2O_2 is interesting and surprising. CYP154C8 displayed comparative tolerance to H_2O_2, although in vitro activity was only observed with >10 mM H_2O_2. More importantly, the heme oxidation rate constant (K) of CYP154C8 even at a high concentration (100 mM) of H_2O_2 was consistently low ($K>0.6\ min^{-1}$). Another widely studied CYP—CYP152L1—uses H_2O_2 in its catalysis of reactions, and recent studies investigating its hydrogen peroxide tolerance showed it to be greater than that of other CYPs (CYP121A1, P450 BM3, and CYP51B1).[63] Comparison of previously reported heme oxi-

dation rate constants (*K*) of CYP152L1 and CYP154C8 showed a higher tolerance of CYP154C8 for H_2O_2 over 30 min when the different CYPs were incubated for 1 h with different concentrations of H_2O_2. The activity of CYP154C8 in the presence of H_2O_2 with steroid substrates was low; in contrast, the elevated activity towards 16α-hydroxyprogesterone was unusual. 6β-Hydroxyprogesterone and 16α-hydroxyprogesterone are pharmaceutically important compounds: 6β-hydroxyprogesterone is used as an intermediate for the synthesis of anticancer compound 6β,14α-dihydroxyandrost-4-ene-3,17-dione, an inhibitor of the growth of breast cancer cells and of the 5α-reductase activity in male rats, thus representing a potential lead for the development of drugs against prostate cancer.[64–67]

Conclusions

In conclusion, CYP154C8 catalyzes the sequential oxidation of steroid substrates, with a preference for NADPH- over NADH-dependent systems. In addition, the use of alternative surrogate redox partners and reducing equivalents might alter the catalytic efficiency and product distribution. The unexpected changes in the product distribution patterns of steroid substrates observed with use of NADPH, (diacetoxyiodo)benzene, and H_2O_2 systems indicate altered roles for active oxygen species in P450-mediated oxidation reactions. The optimum activity of CYP154C8 in the presence of high concentrations of H_2O_2 is unusual and warrants further studies.

Experimental Section

Chemicals and reagents: All steroid substrates were purchased from Tokyo Chemical Industry Co., Ltd. (Republic of Korea). Isopropyl 1-thio-β-D-galactopyranoside (IPTG), 1,4-dithiothreitol (DTT), and kanamycin were obtained from Duchefa Biochemie (Republic of Korea). Ampicillin (Amp), chloramphenicol (Cm), α-aminolevulinic acid (ALA), nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH), hydrogen peroxide (H_2O_2), (diacetoxyiodo)benzene, cytochrome *c*, catalase, formate dehydrogenase, glucose 6-phosphate dehydrogenase, glucose 6-phosphate, spinach ferredoxin (Fdx), and spinach ferredoxin reductase (Fdr) were procured from Sigma–Aldrich. Restriction enzymes were obtained from Takara Clontech (Republic of Korea). T4 DNA ligase, DNA polymerase, and dNTPs were supplied by Takara Bio (Japan). All other chemicals were high-grade products obtained from commercially available sources.

Enzymatic in vitro assay with NADPH and its surrogate redox partners Fdx and Fdr: The heterologous expression and purification of CYP154C8 was performed as described previously.[46] Similarly, the surrogate redox partners Pdx and Pdr were expressed and purified as described elsewhere.[47] Reaction mixtures contained substrate (3 μM), CYP154C8 (3 μM), Fdx (6 μM), Fdr (0.1 U), glucose 6-phosphate (10 mM), glucose 6-phosphate dehydrogenase (1 U), catalase (100 $\mu g\,mL^{-1}$), $MgCl_2$ (1 mM), and NADPH (250 μM). All in vitro reaction mixtures in the enzymatic assay were incubated at 30 °C with shaking (200 rpm) for 2 h in potassium phosphate buffer (50 mM) containing equal concentrations of substrate (500 μM) and CYP enzyme (3 μM) in a final reaction volume of 0.5 mL. The reaction mixtures were extracted with ethyl acetate (2×500 μL) and dried under vacuum. The dried reaction mixture was dissolved in HPLC solvent acetonitrile (70%) and water (30%) for further analysis.

Enzymatic in vitro assay with surrogate oxidant: The reactions were initiated separately with the addition of H_2O_2 (biological hydroperoxide) and (diacetoxyiodo)benzene (exogenous oxidant). The in vitro activity of CYP154C8 was optimized by using different concentrations of H_2O_2 (ranging from 0.2–100 mM) and (diacetoxyiodo)benzene (1–5 mM). The data shown here were obtained from in vitro reactions that included 75 mM H_2O_2 or 2 mM (diacetoxyiodo)benzene, separately. Each reaction mixture (0.5 mL) contained steroid substrate (500 μM) and CYP154C8 (3 μM) in potassium phosphate buffer (pH 7.4). Reactions were triggered by the addition of H_2O_2 or (diacetoxyiodo)benzene, separately, at 30 °C and allowed to proceed for 2 h. The reaction mixtures were extracted as described previously.[46]

Hydrogen peroxide tolerance of CYP154C8: The hydrogen peroxide tolerance of CYP154C8 was monitored by UV/Vis spectroscopy with a Biochrome Libra S35PC spectrophotometer. Oxidative modification of CYP154C8 (3 μM) was carried out over the range of 0.2–100 mM concentrations of H_2O_2. After the addition of H_2O_2, the absorbance at a wavelength of 350–500 nm was recorded every 90 s for 30 min at room temperature. The Soret peak intensity (417 nm) of CYP154C8 was plotted against time, and the data were fitted by using one-phase decay in GraphPad Prism 6 software to calculate the rate constant for heme oxidation (*K*). Associated absorbance amplitudes (*A*) for heme were calculated as the differences between the highest and the lowest absorbances at the Soret peak.

Determination of catalytic efficiency and kinetic parameters: The time-dependent in vitro transformations of progesterone and 16α-hydroxyprogesterone were carried out by using (diacetoxyiodo)benzene (2 mM) and H_2O_2 (75 mM), respectively. Each reaction mixture (500 μL) contained CYP154C8 (1 μM) and substrate (500 μM). The reaction was performed at 30 °C with shaking (400 rpm) at intervals of 1–60 min. Each reaction mixture was extracted as described previously, and the conversion (%) of each product at different time intervals was calculated from the areas of the product peaks in the HPLC chromatogram. The product concentrations with the substrates progesterone and 16α-hydroxyprogesterone in the presence of (diacetoxyiodo)benzene (2 mM) and H_2O_2 (75 mM), respectively, were determined at different substrate concentrations ranging from 25–400 μM. Each reaction mixture was incubated and extracted as described elsewhere. With the assumption that the absorbance properties of products and substrate were the same, the products were quantified by correlating the peak area(s) of the product(s) in question with the combined peak area of product(s) and the substrate.

Product purification and characterization: Large-scale (300 mL) in vitro reactions were carried out to determine the structures of the products obtained in the presence of CYP154C8. Reactions were carried out separately in a 15 mL volume to determine the products obtained in the presence of (diacetoxyiodo)benzene (2 mM). All in vitro reactions were carried out in potassium phosphate buffer (pH 7.4, 50 mM) for 2 h in the presence of CYP154C8 (3 μM) and substrate (500 μM). The reaction mixture was extracted with ethyl acetate (300 mL), dried, and concentrated under reduced pressure, and the residue was dissolved in HPLC-grade methanol. The sample was filtered through a PTFE filter (pore size 0.45 μm) and subjected to preparative HPLC (Shimadzu) with a C_{18} column (Mightysil RP-18 GP, 150×4.6 mm, 5 μm, Kanto Chemical, Japan) for purification of the product peaks.

Analytical methods: The reaction mixture obtained after drying was used for analysis. The dried residue was dissolved in acetonitrile for analysis by ultra-high-performance liquid chromatography (UHPLC). The sample was injected into the UHPLC instrument and separated with use of a Mightysil reversed-phase C_{18} column (4.6× 250 mm, 5 μm). Water (A) and acetonitrile (B) were used as mobile phases for separation. The reaction mixture was analyzed by using a gradient system of B of 15% for 0–10 min, 50% for 10–20 min, 70% for 20–25 min, and 15% for 25–40 min at a flow rate of 1 mL min^{-1}. Substrates and their products were detected by UV-A at 242 and 245 nm. All reaction mixtures were analyzed by UHPLC quadrupole time-of-flight/ESI MS (SYNAPT G2-S/ACUITY, Waters) in positive-ion mode.

The purified hydroxylated products were dissolved in [D_6]DMSO and analyzed by NMR spectroscopy on a Unity INOVA spectrometer at 900 MHz (Varian). One-dimensional NMR spectra (^{1}H NMR and ^{13}C NMR) were obtained, with 2D NMR—heteronuclear multiple bond correlation, correlation spectroscopy, ROESY, and HSQC—used to delineate the structures where appropriate.

6β-Hydroxyprogesterone: ^{1}H NMR (900 MHz, [D_6]DMSO): δ = 5.67 (s, 1H), 5.13 (d, J = 2.9 Hz, 1H), 4.16 (q, J = 3.0 Hz, 1H), 2.59 (t, J = 9.2 Hz, 1H), 2.46 (ddd, J = 17.0, 15.0, 5.0 Hz, 1H), 2.21 (dt, J = 17.1, 3.5 Hz, 1H), 2.07 (s, 3H), 2.06–2.04 (m, 1H), 2.04–2.01 (m, 1H), 1.96 (ddd, J = 13.2, 5.1, 2.8 Hz, 1H), 1.86 (qd, J = 10.9, 3.3 Hz, 1H), 1.82 (dt, J = 13.6, 3.0 Hz, 1H), 1.65 (tt, J = 12.4, 3.8 Hz, 1H), 1.61 (dd, J = 14.1, 4.3 Hz, 1H), 1.60–1.58 (m, 0H), 1.58 (s, 1H), 1.46–1.38 (m, 2H), 1.29 (s, 3H), 1.20–1.16 (m, 1H), 1.16–1.13 (m, 2H), 0.91 (td, J = 11.3, 4.2 Hz, 1H), 0.58 ppm (s, 3H); ^{13}C NMR (226 MHz, [D_6]DMSO): δ = 209.08 (C20), 199.76 (C3), 169.40 (C5), 125.61 (C4), 71.46 (C6), 63.00 (C17), 55.66 (C14), 53.37 (C9), 43.85 (C13),39.11 (C7), 38.27 (C12), 38.02 (C10), 37.05 (C1), 34.33 (C2), 31.65 (C21), 29.82 (C8), 24.42 (C15), 22.71, 20.99 (C16), 19.39(C11), 13.50 ppm (C18).

6β-Hydroxy-11-oxoprogesterone: ^{1}H NMR (900 MHz, [D_6]DMSO): δ = 5.69 (s, 1H), 5.24 (d, J = 2.9 Hz, 1H), 4.18 (q, J = 2.7 Hz, 1H), 2.82 (t, J = 9.2 Hz, 1H), 2.68 (d, J = 12.4 Hz, 1H), 2.60 (ddd, J = 13.4, 5.0, 2.9 Hz, 1H), 2.51–2.48 (m, 1H), 2.46 (d, J = 12.4 Hz, 1H), 2.20 (qd, J = 11.3, 3.0 Hz, 1H), 2.17 (dt, J = 17.2, 3.4 Hz, 1H), 2.12 (d, J = 11.4 Hz, 1H), 2.09 (dt, J = 13.2, 9.7 Hz, 1H), 2.06 (s, 3H), 1.91 (dt, J = 13.5, 2.9 Hz, 1H), 1.87 (td, J = 11.7,7.2 Hz, 1H), 1.82–1.75 (m, 1H), 1.80–1.73 (m, 1H), 1.62 (td, J = 14.2, 4.3 Hz, 1H), 1.48 (s, 3H), 1.42 (ddd, J = 13.5, 11.8, 3.2 Hz, 1H),1.26 (qd, J = 11.6, 10.9, 5.3 Hz, 1H), 0.52 ppm (s, 2H); ^{13}C NMR (226 MHz, [D_6]DMSO): δ = 209.61 (C11), 208.57 (C20), 199.88 (C3), 167.94 (C5), 126.16 (C4), 70.99 (C6), 61.43 (C9), 61.33 (C17), 56.17 (C12), 53.49 (C14), 46.71 (C13), 39.32 (C7), 37.78 (C10), 35.79 (C1), 34.06 (C2), 31.43 (C21), 31.02 (C8), 23.93 (C15), 23.27 (C16), 18.92, 14.47 ppm (C18).

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Conflict of Interest

The authors declare no conflict of interest.

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