Received Date : 20-Aug-2018

Revised Date : 26-Oct-2018

Accepted Date : 04-Nov-2018

Article type : Research Letter

Bacterial CYP154C8 catalyzes carbon-carbon bond cleavage in steroids

Bikash Dangi[1] and Tae-Jin Oh[1,2,3,*]

[1]*Department of Life Science and Biochemical Engineering, SunMoon University, 70 Sunmoon-ro 221, Tangjeong-myeon, Asan-si, Chungnam 31460, Republic of Korea*

[2]*Department of Pharmaceutical Engineering and Biotechnology, SunMoon University, 70 Sunmoon-ro 221, Tangjeong-myeon, Asan-si, Chungnam 31460, Republic of Korea*

[3]*Genome-based BioIT Convergence Institute, 70 Sunmoon-ro 221, Tangjeong-myeon, Asan-si, Chungnam 31460, Republic of Korea*

Author for correspondence: Prof. Tae-Jin Oh

Tel: +82(41)530-2677;

Fax: +82(41)530-2279;

E-mail: tjoh3782@sunmoon.ac.kr

Running title: Lyase activity of CYP54C8

Abstract

Here, we report the first bacterial cytochrome P450, CYP154C8, that catalyzes the C-C bond cleavage reaction of steroids. A major change in product distribution is observed with CYP154C8, when the reactions are supported by NADPH and spinach redox partners ferredoxin and ferredoxin reductase,

compared with previously reported reactions supported by NADH and redox partners containing putidaredoxin and putidaredoxin reductase. The NMR-based structural elucidation of reaction products reveals 21-hydroxyprednisone as the major product for prednisone, while the other product is identified as 1-dehydroadrenosterone obtained due to C-C bond cleavage. A similar pattern of product formation is observed with cortisone, hydrocortisone, and prednisone. The reaction catalyzed by CYP154C8 in the presence of oxygen surrogates also prominently shows the formation of C-C bond cleavage products.

Keywords: C-C bond cleavage; cytochrome P450; lyase; redox partner; steroid hydroxylase.

Introduction

Cytochrome P450 (CYP) enzymes are a superfamily of hemoproteins found throughout nature, well-known for catalyzing diverse reactions such as hydroxylation, epoxidation, dealkylation, deamination, dehydrogenation, dehydration, dehalogenations, demethylation, and carbon-carbon (C-C) bond cleavage (1,2). CYP enzymes play a crucial role in diverse chemical processes including drug metabolism, steroid biosynthesis, and detoxification of xenobiotics (3). Most of the CYP enzymes require redox partner proteins which can transfer two electrons from NADPH or NADH to reduce the oxygen atom from O_2 to water (4). In bacteria, CYP were initially considered to be mainly involved in the catabolism of the exogenous substrates as the source of energy; however, they were reported to play important role in secondary metabolism also. The substrates for the high numbers of CYPs in bacteria are unknown, yet they possess diverse roles in them (5).

C-C bond cleavage reactions are not common with CYP enzymes, especially from a bacterial source. Some of the well-known reactions that include C-C bond cleavage reactions are catalyzed by CYP51 (lanosterol 14α-demethylase) (6), CYP11 (cholesterol side chain cleavage) (7), CYP17 (C17α-C20 lyase) (8), and CYP19 (aromatase) (9). OleT (CY152L1), BS_β (cyp152A1), CYP51, and $P450_{Biol}$ (CYP107H1) are the only known CYPs isolated from a bacterial source that catalyze C-C bond cleavage reactions with fatty acids (10-13). Recently, two unspecific peroxygenases from mushrooms *Marasmius rotula* (MroUPO) and *Marasmius wettsteinii* (MweUPO) have been reported to catalyze the selective deacylation of corticosteroids (cortisone, 11-deoxycortisol, and prednisone) - via stepwise oxygenation and final C-C bond cleavage (14). Enzymatic cleavage of C-C bonds has been found to be a sequential oxidation reaction at the adjacent, which is usually catalyzed by a single CYP. These reactions generate unstable intermediates, which render the investigation of the mechanism involved and their characterization a difficult task (3,15). The hydroxylation reaction catalyzed by CYP uses Fe^{4+}–oxo intermediate, which is also known as compound I, although the participation of ferric peroxide (FeO_2^-) versus perferryl (FeO^{3+}, compound I) in the C-C cleavage reactions catalyzed with CYPs including 17A1, 19A1, and 51A1 has long been of interest and under debate (16-21). A recent study on OleT peroxide-dependent CYP, which is known to catalyze the C-C bond cleavage of fatty acids for the synthesis of alkenes demonstrated that CYP compound I species can promote such reactions that do not involve oxygen rebound (22,23).

The role of CYP-catalyzed C-C bond cleavage has attracted much attention due to its potential application in organic synthesis (4). This challenging reaction has currently gained considerable interest due to the broad arrays of applications, which include the production of anticorrosion agents, pharmaceutical compounds, pesticides, important chemicals with biofuel applications, bioremediation, and biodegradation. More importantly, despite the prevalence of lyase activity in the biological system, the mechanism behind it has not been understood clearly (24-27).

In the present work, we report the first bacterial CYP that catalyzes the C-C bond cleavage of steroid substrates such as prednisone, prednisolone, cortisone, and hydrocortisone (Scheme 1). To the best of our knowledge, until now, the C-C bond cleavage reaction with steroids as substrates has never been reported for any microbial CYP. The same enzyme was previously reported to synthesize mono-hydroxylated products of such substrates in a reaction reconstituted with NADH and the surrogate redox partners, putidaredoxin reductase (Pdr) and putidaredoxin (Pdx) (28). In this report, the reactions were supported by NADPH and spinach ferredoxin reductase (Fdr) and ferredoxin (Fdx) as the surrogate redox partners. In addition, we separately carried out the *in vitro* reaction with oxygen surrogates, diacetoxyiodobenzene (PIDA) and hydrogen peroxide (H_2O_2).

Materials and methods

Chemicals and reagents

Prednisone, cortisone, and hydrocortisone steroids substrates were purchased from Sigma Aldrich (Korea). Prednisolone and 17α-hydroxyprogesterone were purchased from TCI (Tokyo Chemical Industry co., Ltd). Isopropyl-1-thio-β-$_D$-galactopyranoside (IPTG) and 1,4-dithiothreitol (DTT) were obtained from Duchefa Biochemie (Korea). Ampicillin (Amp), α-aminolevulinic acid (ALA), nicotinamide adenine dinucleotide phosphate (NADPH), H_2O_2, PIDA, catalase, formate dehydrogenase, glucose-6-phosphate dehydrogenase, glucose-6-phosphate, spinach Fdx, and spinach Fdr were purchased from Sigma-Aldrich (Korea). Restriction enzymes were obtained from Takara Clontech (Korea). T4 DNA ligase, DNA polymerase, and dNTPs were from Takara Bio (Japan). High-performance liquid chromatography (HPLC) grade acetonitrile and water were purchased from Fisher Scientific (ACS, HPLC grade).

Determination of CYP154C8 and enzyme assay

Recombinant bacterial P450 CYP154C8 was expressed in *Escherichia coli* and purified as described previously (28). The enzyme concentration was determined based on the difference in CO-reduction absorbance spectra, which is the method widely used to determine the concentration of CYPs (29). *In vitro* enzymatic activity of CYP154C8 was assessed separately in the presence of NADPH, PIDA, and H_2O_2. The reaction supported by NADPH contained 6 μM Fdx and 0.1 U Fdr as redox partners from spinach, 10 mM glucose-6-phosphate and 1 U glucose-6-phosphate dehydrogenase for NADPH regeneration, 100 μg/ml catalase, 1 mM $MgCl_2$, and 250 μM NADPH. Another reaction was supported by 2 mM PIDA and 40 mM H_2O_2 as separate reactions. All the *in vitro* conversion reaction assays were carried out in potassium phosphate buffer (pH 7.4) containing 3 μM CYP154C8 and 0.5 mM substrate (steroids) in a final volume of 0.5 mL. The reactions were carried out in a shaking (500

rpm) reaction chamber at 30°C for 2 hr. The reactions mixtures were extracted twice with 0.5 mL ethyl acetate and dried for analytical purpose.

Product purification and analytical methods

The products, 21-hydroxyprednisone and 1-dehydroandrosterone were obtained by *in vitro* reaction of the substrate prednisone with PIDA and H_2O_2, respectively. The reactions were carried out in 10 ml volume, separately. Both the products were purified from a total reaction volume of 200 mL. All the conditions for the reactions were similar to enzyme assays method mentioned previously. The products were purified as previously reported (28). The reaction mixtures were initially analyzed with HPLC (Shimadzu). The samples were separated and run through Mightysil Reverse phase C_{18} GP column (4.6 × 250 mm, 5 µm, Kanto Chemical, Japan). The reaction mixtures were run with a gradient system as reported previously (28). Following the HPLC analysis, the samples were further analyzed with liquid chromatography-mass spectrometry (LC-MS) as described previously (28). The target peaks for identifying the position of the modification were purified with preparative HPLC with a C_{18} column (Mightysil RP-18 GP, 4.6 × 150 mm, 5 µm, Kanto Chemical, Japan) and prepared as reported previously (28).

Results

The *in vitro* reactions catalyzed by CYP154C8 were separately carried out in 3 different systems. The control reactions were performed separately without CYP enzyme supported by three different systems containing Fdx-Fdr-NADPH, H_2O_2, and PIDA. No product formation was observed with any of the supported systems. Previously, CYP154C8 was reported to hydroxylate prednisone, cortisone, hydrocortisone, and prednisolone at two different positions in a reaction supported by NADH and redox partners, Pdr and Pdx (28). However, in another CYP154C8 reaction supported by NADPH and the spinach redox partners, Fdr and Fdx, additional product peaks were noted in addition to 2 mono-hydroxylated products (observed in the NADH-supported reaction) (Fig. 1). LC-MS analysis of all the reactions with the four substrates (prednisone, cortisone, hydrocortisone, and prednisolone) showed 2 mono-hydroxylated products (P1 and P3), which were also observed with NADH system. Moreover, detection of at least 1 di-hydroxylated product (P4), a possible C-C bond cleavage productss (P6, prednisone, cortisone, and hydrocortisone; and P5, prednisolone), the hydroxylated product of cleavage product (P2), and the possible oxidation product of hydroxylated products (P5) was possible. The formation of C-C bond cleavage products (17-ketosteroids) in the reaction mixture was suspected based on the LC-MS analysis (Fig. 2) and peak retention pattern. Based on the similarity of all the 4 substrate reactions mixture, the reaction product of the substrate prednisone was characterized. The HPLC chromatogram (Fig. 1B) of prednisone reaction mixture supported by NADPH system showed 2 usual mono-hydroxylated products (P1 and P3) characterized by LC-MS and comparison of HPLC data (data not shown). The LC-MS analysis revealed P4 as a di-hydroxylated product ($[M+H]^+$ 391.1750), while P6 displayed an exact mass of m/z^+ $[M+H]^+$ 299.1634; and this mass exactly resembles with the mass of C-C bond cleavage product at C17 of prednisone. This observation was further confirmed by NMR (^1H, ^{13}C, and 2D NMR) based structural elucidation, which revealed the product as 1-dehydroadrenosterone formed due to C-C bond cleavage (Fig. 3A

and S1). Similarly, structural elucidation of another major mono-hydroxylated product peak (P1) by NMR showed the formation of 21-hydroxyprednisone as a result of hydroxylation at the C21 position of prednisone. The same NMR sample also showed another low concentration product, which was determined as dehydrated keto aldehyde form (Fig. 3B and S2). Such aldehyde bearing products have been observed in the geminal-diol form in the absence of water (14,30,31). The P2 was detected as a possible hydroxylated product of C-C bond cleavage product (P6) having an exact mass of $[M+H]^+$ 315.1591, which exactly resembles with the hydroxylated mass of 1-dehydroadrenosterone ($[M+H]^+$ 315.1591). Another product peak (P5) detected in traces showed the exact mass of $[M+H]^+$ 389.1958 indicating the possible oxidation like the product of di-hydroxylated prednisone (P4) obtained in the reactions. More than a single such possible oxidation like products from di-hydroxylated prednisone were detected in traces in LC-MS analysis, which were not clearly observed in HPLC chromatogram. The reaction with another substrate, hydrocortisone revealed a clear conversion (P5) of such product (oxidation like product of hydroxyhydrocortisone) formation with the exact mass of 377.1968; the calculated mass for oxidation product of hydroxyhydrocortisone is ($C_{21}H_{29}O_6^+$) $[M+H]^+$ 377.1953 (Fig. 1A). Another peak (P7) observed in hydrocortisone reaction had the major mass of $[M+H]^+$ 349.2009, which exactly resembles with the mass of a possible 11,17-dihydroxy-3-oxoandrost-4-ene-17-carboxylic acid ($[M+H]^+$ 349.2010). Such products (17-carboxylic acid of respective substrates) were also detected in traces in LC-MS (data not shown) with prednisone and cortisone, which had m/z^+ $[M+H]^+$ of 345.1697 (a possible 17-hydroxy-3,11-dioxo-1,4-diene-17-carboxylic acid) and 347.1847 (a possible, 17-hydroxy-3,11-dioxoandrost-4-ene-17-carboxylic acid), respectively. A similar product formation in traces has been reported by two unspecific peroxygenases characterized for lyase reactions employing similar substrates (14). CYP154C8 is known for the sequential oxidation of steroids. Henceforth, it can be stated that di-hydroxylated product (P3) of prednisone must have been obtained due to sequential hydroxylation of 21-hydroxyprednisone by CYP154C8. When 21-hydroxyprednisone was used instead of prednisone as the substrate, formation of further hydroxylated product (P1, m/z^+ $[M+H]^+$ 391.1752), possible oxidation product (P3) of di-hydroxylated product of prednisone (P1) with the mass of m/z^+ $[M+H]^+$ 389.1958, C-C cleavage product (P4, 1-dehydroadrenosterone), and a possible hydroxylated product of 1-dehydroadrenosterone (P2) was observed (Fig. S3). At least two possible oxidation products of di-hydroxylated prednisone were detected in LC-MS but at a very low conversion as observed with prednisone reaction mixture.

The time-dependent reactions of cortisone (Fig. S4) supported by NADPH system were carried out to investigate the effect of time on product formation. The reactions showed an increase in hydroxylated products with an increase in time. More importantly, increased the formation of 21-hydroxylated products (P1) with time was observed, which was further hydroxylated to give di-hydroxylated products (P3). However, with the increase in a time interval, the C-C bond cleavage product formation did not increase, although, a minor conversion of a possible hydroxylated product peak (P4) of C-C bond cleavage product was detected. Multiple other products with a very low conversion with the exact mass of possible oxidation-like product of hydroxylated products were also observed (data not shown).

CYP154C8 displayed catalytic function *in vitro* with H_2O_2 and PIDA, hence, the reaction was carried out in the presence of such oxygen surrogates. H_2O_2 supported reaction mixture of prednisone almost did not show the formation of 21-hydroxyprednisone, which was found to be the major product with NADPH system (Fig. 4A). While the formation of another mono-hydroxylated

product (P1, a possible 16α-hydroxyprednisone), product (P2) with the mass of $[M+H]^+$ 345.1697 (a possible 17-hydroxy-3,11-dioxo-1,4-diene-17-carboxylic acid), and possible oxidation like products (P3 and P4) of hydroxyprednisone and 1-dehydroadrenosterone (P5) was observed. The formation of P2 like products was prominently observed with other substrates with the H_2O_2 system. A similar pattern of product formation was observed with cortisone (Fig. 4A), hydrocortisone (Fig. 4B), and prednisolone (Fig. 4C).

PIDA supported reactions showed a major conversion of 21-hydroxyprednsione (P2), a minor conversion of a possible 16α-hydroxyprednisone (P1), and possible oxidation like product of 21-hydroxyprednisone with the exact mass of m/z^+ $[M+H]^+$ 373.1644 detected in traces in LC-MS analysis, which was not clearly observed in HPLC chromatogram (Fig. S5A). In addition, another product (P3) that had a low conversion with the mass of m/z^+ $[M+H]^+$ 345.1695 showing the formation of possible 17-hydroxy-3,11-dioxo-1,4-diene-17-carboxylic acid was identified and its formation was observed in the presence of other substrates but in traces (Fig. S5B-D). The fourth product (P4) was identified as the C-C bond cleavage product (1-dehydroadrenosterone) based on HPLC chromatogram and LC-MS (data not shown).

All the reactions supported by H_2O_2 and DIPA showed the formation of C-C lytic products which were confirmed by LC-MS analysis (data not shown). The C-C bond cleavage product of cortisone was identified as adrenosterone by means of HPLC comparison with the authentic standard (Fig. S6). Due to the low conversion of the remaining products, structural elucidation by NMR could not be achieved. It is hypothesized that the possible position of hydroxylation of another mono-hydroxylated product obtained in reactions might be at the C16 position because CYP154C8 is known to hydroxylate at the C16α position with steroids lacking C11 and C21 functional groups (hydroxyl or carbonyl). Based on our suggestion in a previous report (28), the P1 was found to be the 21-hydroxyproduct of prednisone and the structural elucidation reveals such type of products peaks (P1 of cortisone, hydrocortisone, and prednisolone) corresponding to 21-hydroxy (21-geminal diol) product of respective substrate. The other substrates, prednisolone, cortisone, and hydrocortisone also had a similar pattern of products formation with prednisone supported by NADPH, H_2O_2, and PIDA, separately.

The NADPH supported reaction showed much higher conversion by CYP154C8 compared to previously reported NADH system, revealing the effectiveness of spinach redox partner compared to Pdx and Pdr (Table S1). Assuming the absorbance properties of products and substrate to be similar, the products were quantified by correlating the peak area of the respective product(s) with the combined peak area of product(s) and the substrate. In contrast to NADH supported reactions, NADPH system displayed altered product profile where the 21-hydroxy product (further hydroxylated to form di-hydroxylated product) of the respective substrates was found to have major conversion (high selectivity). The mono-hydroxylated product (P3; a possible 16α-hydroxylation) had lower conversion (low selectivity). Hydrocortisone reaction mixture showed ~ 90% selectivity towards possible 21-hydroxycortisone. The NADH supported system previously reported by us showed almost similar conversion for both the products (21-hydroxy and a possible 16α-hydroxy product) with the substrates cortisone, prednisone, and prednisolone; whereas the selectivity for 21-hydroxyhydrocortisone was lower than the possible 16α-hydroxyhydrocortisone with the substrate hydrocortisone.

Discussion

CYP154C8 is the first bacterial CYP known to catalyze C-C bond cleavage reactions of the steroids with hydroxyacetyl and hydroxyl functionalities at C17. When CYP154C8 is reconstituted with NADH and the surrogate redox partners including Pdx and Pdr, detection of cleavage products is never achieved, while the formation of two mono-hydroxylated products remains apparent (28). However, NADPH and the surrogate redox partners, Fdx and Fdr from spinach were shown to affect the catalytic efficiency as well as product distribution by CYP154C8. In the present work, in addition to hydroxylated products, C-C bond cleavage product was detected with all the systems employed. Our results further support the contention that the choice of redox partner can alter the type and selectivity of the product formation by a CYP. More importantly, the alternative redox partner proteins can affect the complete characterization of a CYP. CYP154C8 has previously been reported to hydroxylate steroid substrates (progesterone, androstenedione, testosterone, and nandrolone) in a sequential manner. It has been shown that CYP154C8 catalyzes the sequential oxidation of steroid substrates including prednisone, cortisone, hydrocortisone, and cortisone when partnered with suitable and efficient redox partner proteins. The catalytic efficiency of CYP154C8 in the presence of NADPH and spinach redox partner proteins including Fdr and Fdx was higher compared to the reaction supported by NADH and redox partner containing Pdr and Pdx from *Pseudomonas putida*. This result reveals that the spinach Fdx and Fdr system is more effective as a redox donor for CYP154C8 compared to Pdx and Pdr redox system. However, there have been numerous reports on the effector role of alternative redox partner to the catalytic activity and product distribution of CYP enzymes (32-35). Moreover, we cannot deny the effector role of two different systems in varied product distribution catalyzed by CYP154C8. CYP154C8 displays higher selectivity for a 21-hydroxylated product with the substrates bearing functional group (hydroxyl or carbonyl) at C11 and C21 position, although this enzyme catalyzes regio- and stereo-specific hydroxylation at C16α of substrates lacking functional group from both the positions (28).

The exact mechanism of C-C bond cleavage products (17-ketosteroids) formation by CYP154C8 is not clear, although, the majority of the reactions catalyzed by CYPs involve sequential hydroxylation that generates the required functionality and promotes C-C bond cleavage (12,28,36-37). It is believed that a similar pattern of reaction might have been involved in C-C bond cleavage product formation catalyzed by CYP154C8 (Fig. 5). The formation of C-C bond cleavage product in a reaction where 21-hydroxyprednisone was used as a substrate instead of prednisone, revealed the involvement of such products (21-hydroxyprednisone) in C-C bond cleavage product formation. Recently, unspecific peroxygenase has been reported to catalyze C-C bond cleavage reactions of similar steroids in a multi-step manner. Both the peroxygenases catalyze hydroxylation at the C21 position of cortisone, prednisone, and 11-deoxycortisol substrates, and subsequently convert them into possible carboxylic acid that finally leads to C-C bond cleavage forming 17-ketosteroids (14). It is hypothesized that the reaction sequence for the formation of C-C bond cleavage product by CYP154C8 might be similar with these two unspecific peroxygenases. However, in contrast to the pattern of reaction products formation by peroxygenase, with the increase in the time of the reaction catalyzed by CYP154C8, no change in C-C bond cleavage product formation was observed, although sequential hydroxylation of such products (17-ketosteroids) was observed in traces; while increase in the formation of di-hydroxylated product from 21-hydroxyprednisone was observed with the increase in reaction time. This may indicate that CYP154C8 has a major function of hydroxylation; however, it is hypothesized that 21-hydroxylation of the steroid substrates with

hydroxyacetyl and hydroxyl functionalities at C17 may further proceed to C-C bond cleavage reactions, but with a low selectivity compared to di-hydroxylated product formation when the reaction is supported by NADPH system. The formation of possible oxidation such as hydroxylated products detected in traces was unclear. The NMR structural elucidation of prednisone mono-hydroxylated product (major) showed the hydroxylation at C21 position (21-geminal-diol), and the same sample showed prednisone 21-al formation but at a low concentration. Aldehyde formation was observed with geminal-diol under conditions of dehydration (18,30,31). The 21-aldehyde containing product in the reaction might have undergone further hydroxylation to form a possible oxidation like product. Similarly, the di-hydroxylated product of prednisone might have occurred in 21-aldehyde form and sequentially hydroxylated by CYP154C8 to form the respective oxidation like product of di-hydroxylated prednisone and similar substrates in the reaction mixture, since the di-hydroxylated product was obtained as a result of further hydroxylation of 21-hydroxyprednisone (Fig. S7). It is believed that the possibility of formation of oxidation products could be due to the presence of a double bond or formation of a keto or epoxide, as keto groups and epoxides can form from hydroxyl groups (38,39).

The formation of 17-hydroxy-3,11-dioxo-1,4-diene-17-carboxylic acid (17-carboxylic acid) like product with prednisone and similar products with respective substrates which were detected in traces with NADPH system but clearly observed with oxygen surrogates was also not clear. The possibility of the formation of such products might be due to the C-C bond cleavage between C20 and C21. Here, CYP154C8 might have been involved in the degradation of steroids with hydroxyacetyl and hydroxyl functionalities at C17 by cleaving the C-C bonds at different positions. Therefore, the formation of 17-hydroxy-3,11-dioxo-1,4-diene-17-carboxylic acid like product with prednisone and similar products formation with other substrates might be independent of the formation of 1-dehydroadrenosterone and similar products with respective substrates. Steroids are ubiquitous growth substrates for microorganisms, and bacterial hydroxylase plays an important role in the degradation of steroids (40). For example, CYP125A1, CYP142A1, and CYP124A1 play an essential role for the survival of *Mycobacterium tuberculosis* and spread of infection by the degradation of cholesterol by oxyfunctionalization at C27 to form terminal alcohol and subsequent oxidation to aldehyde and carboxylic acid (41-43). This reaction is found to be essential for the survival and virulence of *Mycobacterium tuberculosis.* The reaction leading towards the formation of di-hydroxyprednisone from 21-hydroxyprednisone was found to be much higher than the C-C bond cleavage product formation (1-dehydroadrenosterone) in an NADPH supported reaction. The involvement of di-hydroxyprednisone in C-C bond cleavage product formation is unclear but the pattern of product formation indicates that the hydroxylated products do not completely lead towards C-C bond cleavage. A widely studied mammalian CYP, CYP17A1 is known to catalyze 17α-hydroxylation of progesterone and pregnenolone where both the hydroxylated products are subsequently cleaved at C17-C20 bond to form androstenedione and dehydroepiandrosterone, respectively. Recently, the same enzyme was found to convert progesterone into 16α-hydroxyprogesterone as a minor product, and 16α-hydroxylation activity was observed with the substrates 17α-hydroxypregnenolone and 17α-hydroxyprogesterone. The products dehydroepiandrostenedione and androstenedione formed by lyase activity of CYP17A1 were also further hydroxylated at 16α-position (26,44-46). Similarly, zebrafish CYP17A1 and CYP17A2 at higher concentrations and under increased reaction time formed some additional products like 17α,21-

$(OH)_2$ progesterone, 16,17α-$(OH)_2$ progesterone, 6β,16,17α-$(OH)_3$ progesterone, 17α,21-$(OH)_2$ pregnenolone, and 16,17α-$(OH)_2$ pregnenolone (47).

CYP154C8 actively demonstrated the conversion of steroids in presence of oxygen surrogates H_2O_2 and PIDA. Both the oxygen surrogates supported reactions showed the distinct formation of C-C bond cleavage product. H_2O_2 is one of the efficient ways of using CYP for industrial application due to their cheap availability and not all CYP are capable of functioning in the presence of H_2O_2; however, oxidative degradation of heme by peroxide becomes a major issue (48). Surprisingly, CYP154C8 demonstrated its activity in the presence of high concentration of H_2O_2 where no products were observed below the concentration of 10 mM. The C-C bond cleavage product formation with the oxygen surrogate diacetoxyiodobenzene indicates that compound I (FeO^{3+}) is possibly responsible for C-C bond cleavage reactions catalyzed by CYP154C8. However, the participation of compound 0 ($Fe^{2+}O_2H$) in lyase reactions cannot be ignored because the reaction with H_2O_2 also showed the formation of lyase products. The role of compound I in the formation of lyase product with the oxygen surrogate has been the topic of interest and under debate. Recently, CYP17A1 with oxygen surrogate iodosylbenzene led to the formation of lyase product, thus providing the evidence that compound I reactions possibly participating in C-C bond cleavage reactions (26,47). In the reactions supported by H_2O_2 a very low conversion or no conversion of 21-hydroxylated products of the respective substrates was observed compared to PIDA, although both the systems clearly showed the formation of C-C bond cleavage product. The exact reason behind such a variable product distribution is unclear, but it is clearly evident that the two systems generate two different active oxygen species that possibly are involved in the oxidation of substrates. So, it is hypothesized that the reaction could be mechanistically distinct reactions initiated by two systems. In a reaction catalyzed by CYP2D6, differences in regioselectivity of oxidation products were observed when the reactions were separately supported by NADPH-P450 reductase, cumene hydroperoxide, and iodosobenzene due to the distinct chemical mechanism of the different system used (49). Similarly, in another reaction catalyzed by CYP2B1 and separately supported by P450/NADPH/O2- and P450/PhIO system, the observed mechanistic difference between two systems was most likely associated with the differences in the chemistries of the two systems, although active oxygen species in both the system was widely believed to be ferryl-oxo-species (50). Initially, it was believed that the compound I was the only key intermediate involved in catalytically active oxidant. However, subsequent experiments suggested the evidence of the presence of another oxygen species; hydroperoxy-iron species ($Fe^{III}O_2H$) which possibly functions as an active oxidant that can form altered product compared to compound I species (51,52).

In conclusion, CYP154C8 is a multifunctional enzyme that catalyzes the multi-step hydroxylation of diverse steroids, C-C bond cleavage of substrates bearing hydroxyl group at C17 and C21 positions, which are sequentially hydroxylated, and a possible oxidation of hydroxylated products (Fig. S8). The reaction carried out in the presence of NADPH system suggests that CYP154C8 catalyzes hydroxylation at the C21 position of all the substrates bearing hydroxyl or carbonyl group at C11 and C21 position. These reactions yielded 21-geminal-diol products of respective substrates. The 21-geminal-diol products were sequentially hydroxylated to yield di-hydroxylated products. It is hypothesized that the 21-geminal-diol, as well as the di-hydroxylated product of prednisone, might have further oxidized or aldehyde form of both the products could have further hydroxylated to form oxidized like products in traces. The 21-gem-diol was also involved in the formation of C-C bond cleavage product (1-dehydroadrenosterone), although the

exact reactions involved in the formation of such products is unclear. The cleavage product was further possibly hydroxylated at the C16α position.

Acknowledgments

This work was supported by the Korea Polar Research Institute (grant no. PE18210). We would like to thank Division of Magnetic Resonance, Korea Basic Science Institute, Ochang, Chungbuk, Korea for NMR analyses. In addition, the authors would like to sincerely thank Prof. Tokutaro Yamaguchi for his generous and considerable help regarding NMR analysis.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. McLean KJ, Leys D, Munro AW. (2015) Microbial cytochromes P450. In Cytochrome P450: structure, mechanism and biochemistry, 4th ed. Edited by Ortiz de Montellano PR. New York: Springer. pp. 261–408.
2. Munro AW, Girvan HM, McLean KJ. (2007) Variations on a (t)heme-novel mechanisms, redox partners and catalytic functions. Nat. Prod. Rep. 24:585–609.
3. Ortiz de Montellano PR. (2015) Substrate oxidation by cytochrome P450 enzymes in cytochrome P450: structure, mechanism, and biochemistry (Ortiz de Montellano, P. R., ed.). Springer International Publishing. pp. 111–176.
4. Xing Q, Lv H, Xia C, Li F. (2016) Iron-catalyzed aerobic oxidative cleavage of the C–C σ-bond using air as the oxidant: chemoselective synthesis of carbon chain-shortened aldehydes, ketones and 1,2-dicarbonyl compounds. Chem. Commun. 52:489–492.
5. Kelly SL, Kelly DE. (2013) Microbial cytochromes P450: biodiversity and biotechnology. Where do cytochromes P450 come from, what do they do and what can they do for us? Philos. Trans. R Soc. B Biol. Sci. 368:20120476.
6. Alexander K, Akhtar M, Boar RB, McGhie JF, Barton DH. (1972) Removal of the 32-carbon atom as formic acid in cholesterol biosynthesis. J. Chem. Soc. Chem. Commun. 383–385.
7. Burstein S, Middleditch BS, Gut M. (1975) Mass spectrometric study of the enzymic conversion of cholesterol to (22R)-22-hydroxycholesterol, (20R,22R)-20,22-dihydroxycholesterol, and pregnenolone, and of (22R)-22-hydroxycholesterol to the glycol and pregnenolone in bovine adrenocortical preparations. Mode of oxygen incorporation. J. Biol. Chem. 250:9028–9037.
8. Hosoda H, Fishman J. (1977) Unusually facile aromatization of 2β-hydroxy-19-oxo-4-androstene-3,17-dione to estrone. Implications in estrogen biosynthesis. J. Am. Chem. Soc. 96:7325–7329.
9. Arigoni D, Battaglia R, Akhtar M, Smith T. (1975) Stereospecificity of oxidation at C-19 in estrogen biosynthesis. J. Chem. Soc. Chem. Commun. 185–186.

10. Grant JL, Mitchell ME, Makris TM. (2016) Catalytic strategy for carbon−carbon bond scission by the cytochrome P450 OleT. Proc. Natl. Acad. Sci. U.S.A. 113:10049–10054.
11. Lepesheva GI, Waterman MR. (2007) Sterol 14α-demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms. Biochim. Biophys. Acta. 1770:467–477.
12. Cryle MJ, de Voss JJ. (2004) Carbon–carbon bond cleavage by cytochrome P450 (BioI) (CYP107H1). Chem. Commun. pp. 86–87.
13. Rude MA, Baron TS, Brubaker S, Alibhai M, Del Cardayre SB, Schirmer A. (2011) Terminal olefin (1-alkene) biosynthesis by a novel P450 fatty acid decarboxylase. Appl. Environ. Microbiol. 77:1718–1727
14. Ullrich R, Poraj-Kobielska M, Scholze S, Halbout C, Sandvoss M. (2018) Side chain removal from corticosteroids by unspecific peroxygenase. J. Inorg. Biochem. 183:84–93.
15. Guengerich FP, Yoshimoto FK. (2018) Formation and cleavage of C–C bonds by enzymatic oxidation–reduction reactions. Chem. Rev. 118:6573–6655.
16. Ortiz de Montellano PR. (2015) Substrate oxidation by cytochrome P450 enzymes in cytochrome P450: structure, mechanism, and biochemistry (Ortiz de Montellano, P. R., ed.). Springer International Publishing. pp. 111–176.
17. Mak PJ, Luthra A, Sligar SG, Kincaid JR. (2014) Resonance Raman spectroscopy of the oxygenated intermediates of human CYP19A1 implicates a compound I intermediate in the final lyase step. J. Am. Chem. Soc. 136:4825–4828.
18. Akhtar M, Calder MR, Corina DL, Wright JN. (1982) Mechanistic studies on C-19 demethylation in oestrogen biosynthesis. Biochem. J. 201:569–580.
19. Sen K, Hackett JC. (2012) Coupled electron transfer and proton hopping in the final step of CYP19-catalyzed androgen aromatization. Biochemistry 51:3039–3049.
20. Gregory MC, Denisov IG, Grinkova YV, Khatri Y, Sligar SG. (2013) Kinetic solvent isotope effect in human P450 CYP17A1-mediated androgen formation: evidence for a reactive peroxoanion intermediate. J. Am. Chem. Soc. 135:16245–16247.
21. Gregory M, Mak P, Sligar SG, Kincaid JR. (2013) Differential hydrogen bonding in human CYP17 dictates hydroxylation versus lyase chemistry. Angew. Chem. Int. Ed. Engl. 52:5342–5345.
22. Grant JL, Mitchell ME, Makris TM. (2016) Catalytic strategy for carbon−carbon bond scission by the cytochrome P450 OleT. Proc. Natl. Acad. Sci. U.S.A. 113:10049–10054.
23. Grant JL, Hsieh CH, Makris TM. (2015) Decarboxylation of fatty acids to terminal alkenes by cytochrome P450 compound I. J. Am. Chem. Soc. 137:4940–4943.
24. Matthews S, Tee KL, Rattray NJ, McLean KJ, Leys D, Parker DA, Blankley RT, Munro AW. (2017) Production of alkenes and novel secondary products by P450 OleTJE using novel H_2O_2-generating fusion protein systems. FEBS Lett. 591:737–750.
25. Singh A, Ward OP. (2004) Biodegradation and Bioremediation, Soil Biology, Vol. 2, Springer.
26. Yoshimoto FK, Gonzalez E, Auchus RJ, Guengerich FP. (2016) Mechanism of 17α,20-lyase and new hydroxylation reactions of human cytochrome P45017A1. 18O labeling and oxygen surrogate evidence for a role of a perferryl oxygen. J. Biol. Chem. 291:26587.
27. Allpress CJ, Grubel K, Fuller EZ, Arif AM, Berreau LM. (2013) Regioselective aliphatic carbon–carbon bond cleavage by a model system of relevance to iron-containing acireductone dioxygenase. J. Am. Chem. Soc. 135:659–668.

28. Dangi B, Kim KH, Kang SH, Oh TJ. (2018) Tracking down a new steroid-hydroxylating promiscuous cytochrome P450: CYP154C8 from *Streptomyces* sp. W2233-SM. ChemBioChem 19:1066–1077.
29. Omura T, Sato R. (1964) The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. 239:2370–2378.
30. Zhang F, Zhou J, Shi Y, Tavlarakis P, Karaisz K. (2016) Mechanism driven structural elucidation of forced degradation products from hydrocortisone in solution. J. Pharm. Biomed. Anal. 128:333–341.
31. Wang T, Shah YM, Matsubara T, Zhen Y, Tanabe T, Nagano T, Gonzalez FJ. (2010) Control of steroid 21-oic acid synthesis by peroxisome proliferator-activated receptor α and role of the hypothalamic-pituitary-adrenal Axis. J. Biol. Chem. 285:7670–7685.
32. Zhang W, Liu Y, Yan J, Cao S, Bai F, Yang Y, Huang S, Yao L, Anzai Y, Kato F, Podust LM, Sherman DH, Li S. (2014) New reactions and products resulting from alternative interactions between the P450 enzyme and redox partners. J. Am. Chem. Soc. 136:3640−3646.
33. Hiruma Y, Hass MA, Kikui Y, Liu WM, Olmez B, Skinner SP, Blok A, Kloosterman A, Koteishi H, Lohr F, Schwalbe H, Nojiri M, Ubbink M. (2013) The structure of the cytochrome p450cam-putidaredoxin complex determined by paramagnetic NMR spectroscopy and crystallography. J. Mol. Biol. 425:4353−4365.
34. Tripathi S, Li H, Poulos TL. (2013) Structural basis for effector control and redox partner recognition in cytochrome P450. Science 340:1227−1230.
35. Sagadin T, Riehm JL, Milhim M, Hutter MC, Bernhard R. (2018) Binding modes of CYP106A2 redox partners determine differences in progesterone hydroxylation product patterns. Commun. Biol. 1:99.
36. Cryle MJ, Schlichting I. (2008) Structural insights from a P450 carried protein complex reveal how specificity is achieved in the P450Biol ACP complx. Proc. Natl. Acad. Sci. U.S.A. 105:15696–15701.
37. Akhtar M, Alexander K, Boar RB, McGhie JF, Barton DH. (1978) Chemical and enzymatic studies on the characterization of intermediates during the removal of the 14alpha-methyl group in cholesterol biosynthesis. The use of 32-functionalized lanostane derivatives. Biochem. J. 169:449–463.
38. Khatri Y, Carius Y, Ringle M, Lancaster CR, Bernhardt R. (2016) Structural characterization of CYP260A1 from *Sorangium cellulosum* to investigate the 1α- hydroxylation of a mineralocorticoid. FEBS Lett. 590:4638–4648.
39. Chiang JY. (1998) Regulation of bile acid synthesis. Front. Biosci. 3:d176–193.
40. Bergstrand LH, Cardenas E, Holert J, Van Hamme JD, Mohn WW. (2016) Delineation of steroid-degrading microorganisms through comparative genomic analysis. MBio. 7:e00865-16.
41. Johnston JB, Ouellet H, Podust LM, Ortiz de Montellano PR. (2011) Structural control of cytochrome P450-catalyzed ω-hydroxylation. Arch. Biochem. Biophys. 507:86–94.
42. Johnston JB, Ouellet H, Ortiz de Montellano PR. (2010) Functional redundancy of steroid C26-monooxygenase activity in *Mycobacterium tuberculosis* revealed by biochemical and genetic analyses. J. Biol. Chem. 285:36352–36360.
43. Ouellet H, Johnston JB, de Montellano PR. (2011) Cholesterol catabolism as a therapeutic target in *Mycobacterium tuberculosis*. Trends Microbiol. 19:530–539.

44. Chung BC, Picado-Leonard J, Haniu M, Bienkowski M, Hall PF, Shively JE, Miller WL. (1987) Cytochrome P450c17 (steroid 17 alpha-hydroxylase/17,20 lyase): cloning of human adrenal and testis cDNAs indicates the same gene is expressed in both tissues. Proc. Natl. Acad. Sci. U.S.A. 84:407–411.
45. Yoshimoto FK, Auchus RJ. (2015) The diverse chemistry of cytochrome P450 17A1 (P450c17, CYP17A1). J. Steroid Biochem. Mol. Biol. 151:52–65.
46. Swart P, Swart AC, Waterman MR, Estabrook RW, Mason JI. (1993) Progesterone 16 alpha-hydroxylase activity is catalyzed by human cytochrome P450 17 alpha-hydroxylase. J. Clin. Endocrinol. Metab. 77:98–102.
47. Gonzalez E, Johnson KM, Pallan PS, Phan TTN, Zhang W, Lei L, Wawrzak Z, Yoshimoto FK, Egli M, Guengerich FP. (2018) Inherent steroid 17α,20-lyase activity in defunct cytochrome P450 17A enzymes. J. Biol. Chem. 293:541−566.
48. Munro AW, Girvan HM, Mason AE, Dunford AJ, McLean KJ. (2013) What makes a P450 tick? Trends Biochem. Sci. 38:140–150.
49. Hanna IH, Krauser JA, Cai H, Kim MS, Guengerich FP. (2001) Diversity in mechanisms of substrate oxidation by cytochrome P450 2D6. Lack of an allosteric role of NADPH-cytochrome P450 reductase in catalytic regioselectivity. J. Biol. Chem. 276:39553–39561.
50. Bhakta MN, Hollenberg PF, Wimalasena K. (2005). P_{450}/NADPH/O_2^- and P_{450}/PhIO-catalyzed N-dealkylations are mechanistically distinct. J. Am. Chem. Soc. 127:1376–1377.
51. Volz TJ, Rock DA, Jones JP. (2002) Evidence for two different active oxygen species in cytochrome P450 BM3 mediated sulfoxidation and N-dealkylation reactions. J. Am. Chem. Soc. 124:9724–9725.
52. Jin SX, Makris TM, Bryson TA, Sligar SG, Dawson JH. (2003) Epoxidation of olefins by hydroperoxo-ferric cytochrome P450. J. Am. Chem. Soc. 125:3406–3407.

Figure legends

Scheme 1. Steroid substrates used for *in vitro* reaction catalyzed by CYP54C8 in this study.

Figure 1. HPLC chromatogram of hydrocortisone (**A**), prednisone (**B**), cortisone (**C**), and prednisolone (**D**) reaction mixture catalyzed by CYP154C8 in an NADPH system. The HC, PD, CS, and PL indicates the substrate peak of hydrocortisone, prednisone, cortisone, and prednisolone, respectively. The P1 and P3 are mono-hydroxylated product peaks; P4 corresponds to the exact mass of di-hydroxylated product of respective substrates; P7 in inset A shows the exact mass of a possible 11,17-dihydroxy-3-oxoandrost-4-ene-17-carboxylic acid ($[M+H]^+$ 349.2010); P5 in inset A, B, and C shows the possible oxidation product peak of hydroxylated hydrocortisone. P5 in inset D and P6 in inset A, B, and C shows the peaks of C-C bond cleavage product.

Figure 2. LC-MS spectra and UV-absorbance of C-C bond cleavage product catalyzed by a CYP154C8 enzyme with the substrate hydrocortisone (**A**), cortisone (**B**), prednisone (**C**), and prednisolone (**D**).

Figure 3. ^{1}H NMR spectra and atom chemical shift of C-C bond cleavage product (1-dehydroadrenosterone) (**A**), major prednisone hydroxylated product (21-hydroxyprogesterone) (**B**), and its corresponding free aldehyde form obtained by the catalysis of CYP154C8. The supplementary figures S4 and S5 show the detailed two dimensional NMR spectra.

Figure 4. HPLC chromatogram of prednisone (**A**), prednisolone (**B**), hydrocortisone (**C**), and cortisone (**D**) reaction mixture catalyzed by CYP154C8 in an H_2O_2 system. Inset I and II show the HPLC chromatogram of the reaction performed in the presence and absence of CYP154C8, respectively. P1 shows the exact mass of mono-hydroxylated product of the respective substrates; P2 was identified as a C-C bond cleavage product of the respective substrate; P3 showed the exact mass resembling the 17-carboxylic acid of the respective substrate; P4 and P5 had an exact mass of possible oxidation product of the hydroxylated product.

Figure 5. Possible postulated reaction sequences involved in the formation of 1-dehydroadrenosterone (17-ketosteroid) from the substrate prednisone. **A**. The sequence might involve the initial hydroxylation at C21 position (**I**, prednisone 21-geminal-diol) followed by formation of carboxylic acid at C21 position (**II**, prednisone 21-oic acid) and finally, side chain removal to form 1-dehydroadrenosterone (**III**). **B**. Another possible reaction sequence in the formation of 1-dehydroadrenosterone (17-ketosteroid) product might have involved initial hydroxylation at C21 position (**I**) and finally would have undergone the C-C bond cleavage reaction to form 1-dehydroadrenosterone or prednisone may have directly undergone C-C cleavage and formed 1-dehydroadrenosterone (17-ketosteroid).

Scheme 1.

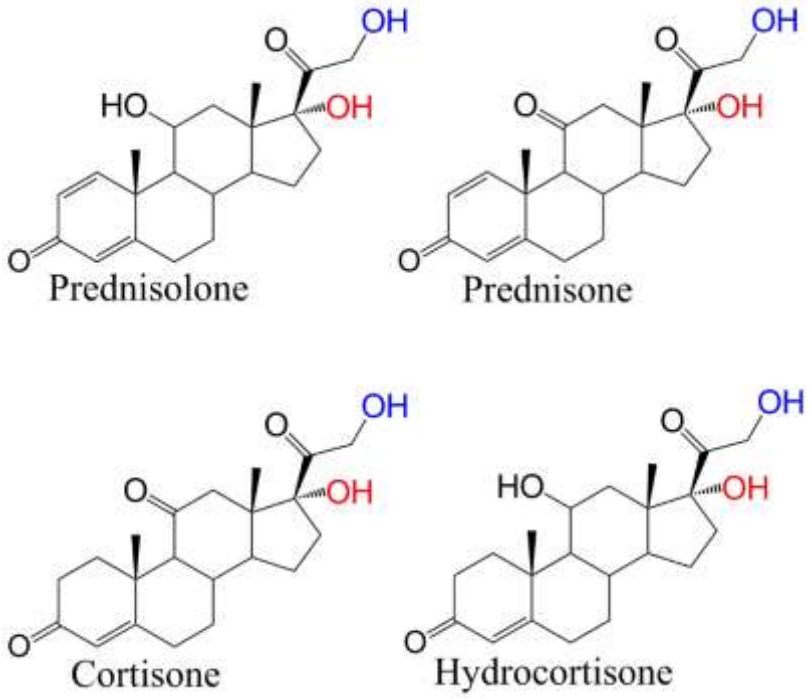


Fig. 1.

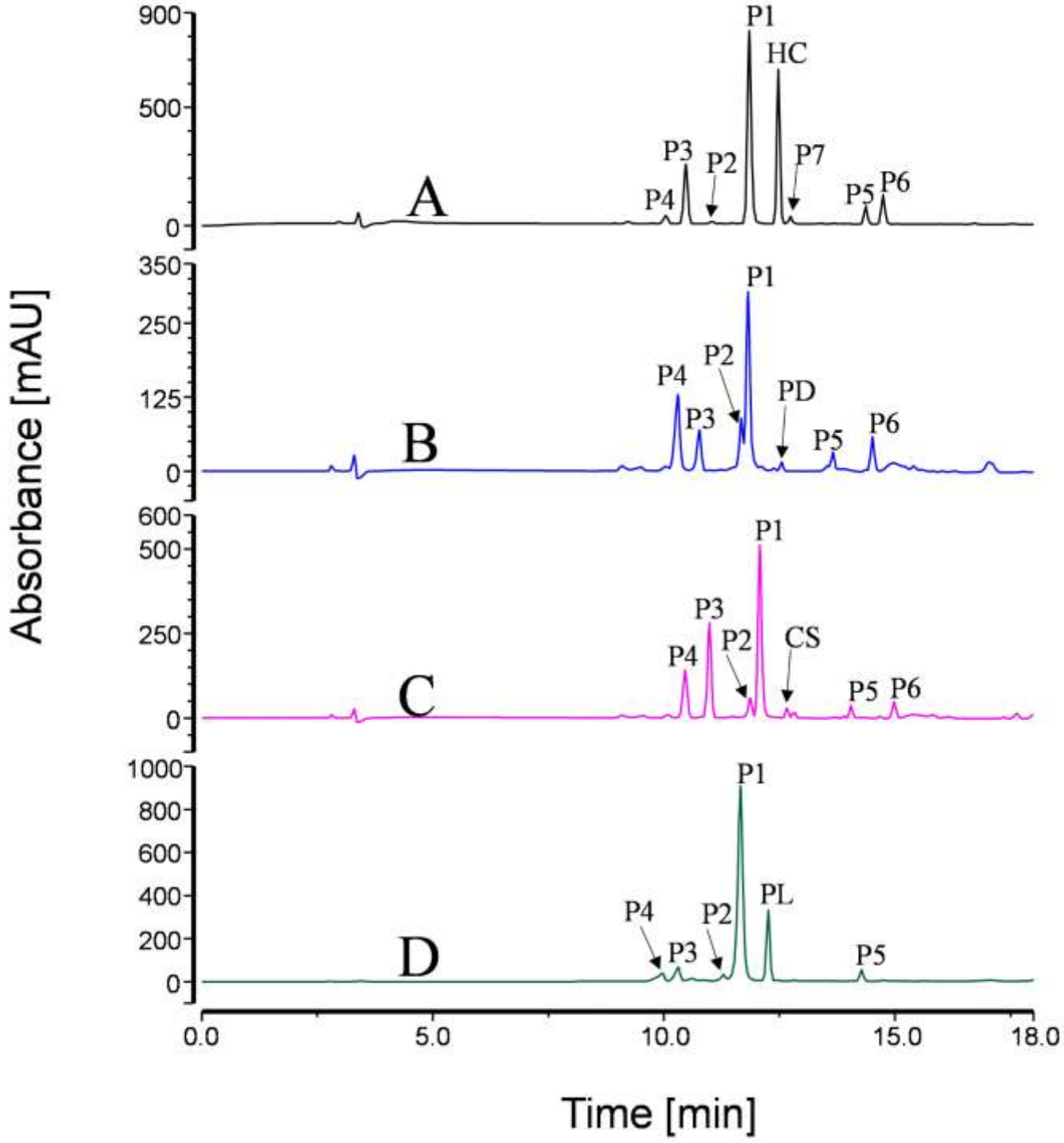


Fig. 2.

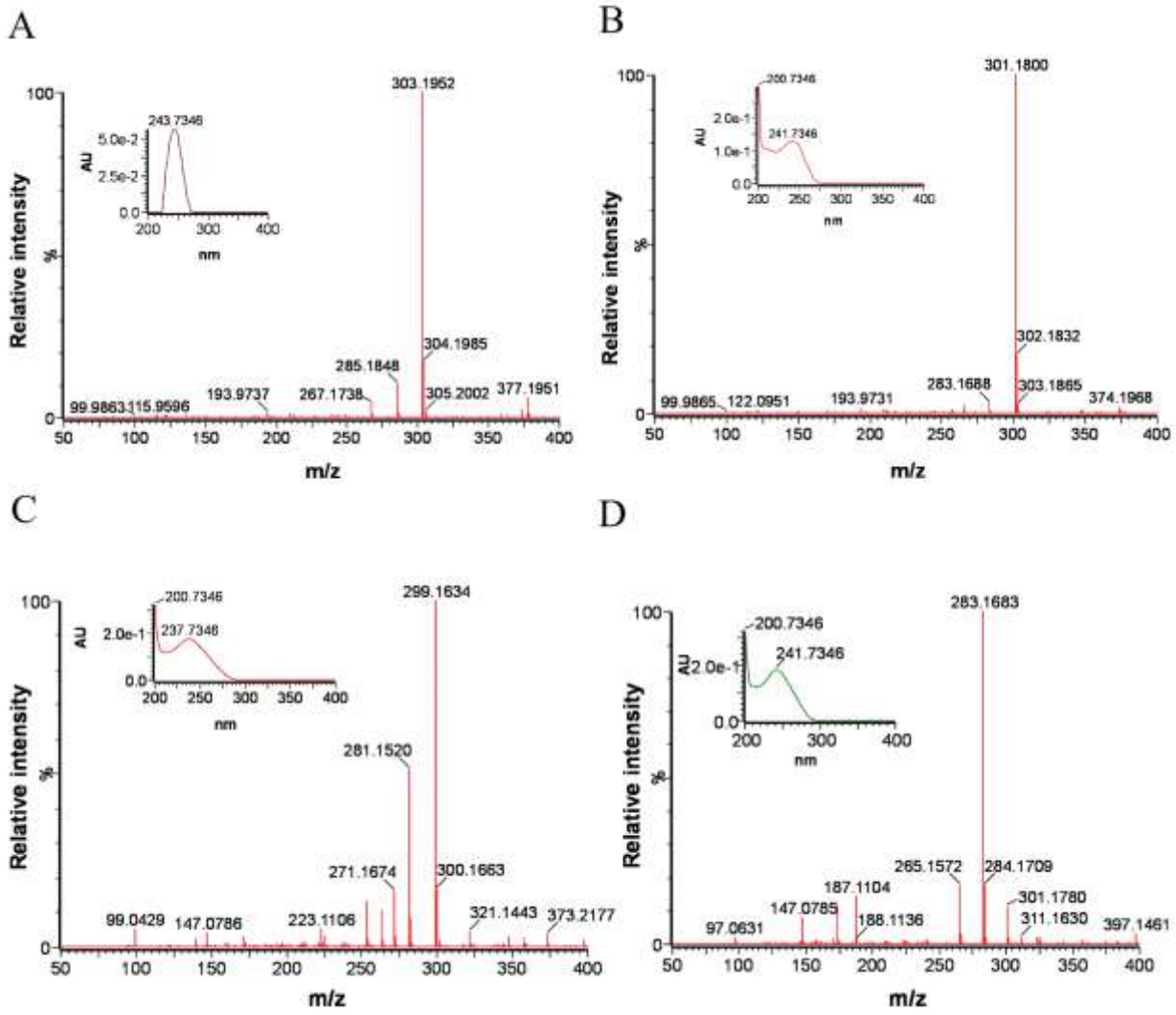
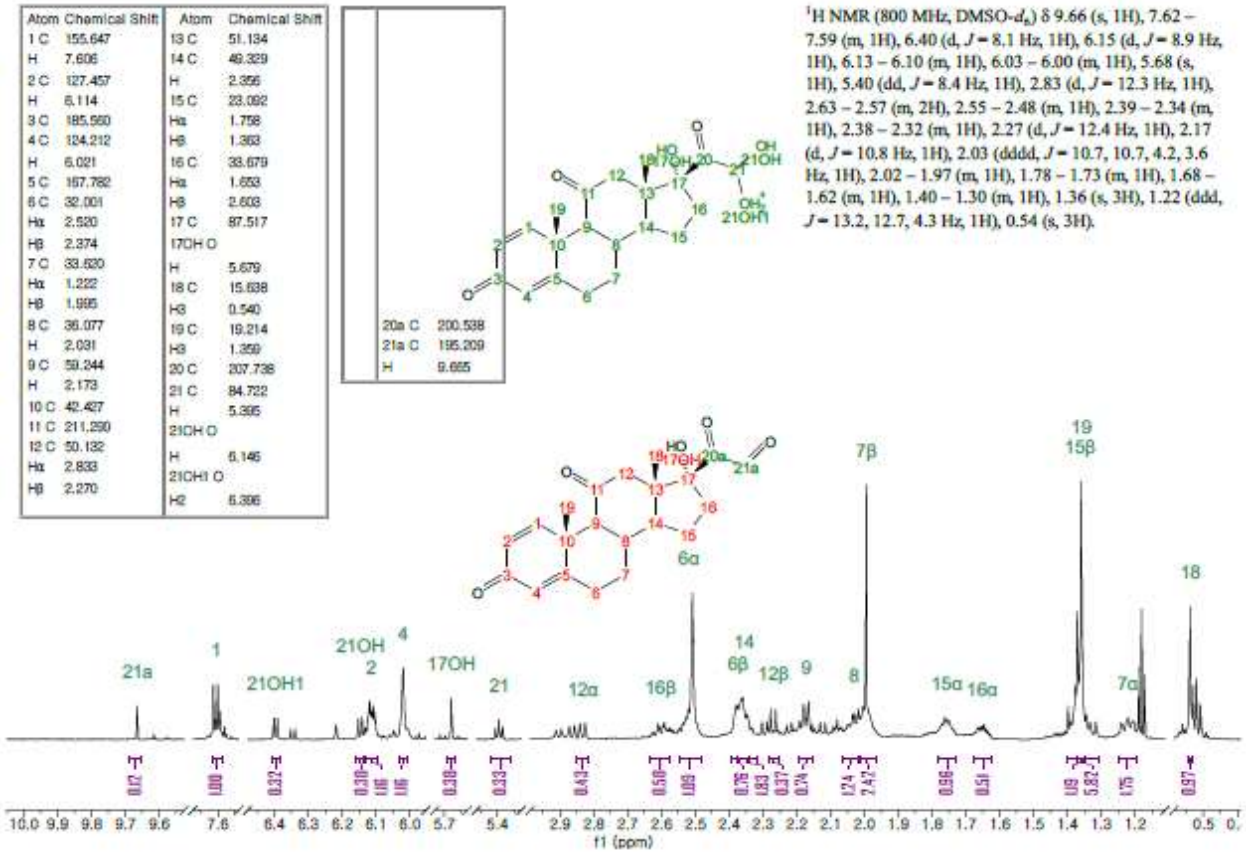


Fig. 3.

A.



B.

Atom	Chemical Shift	Atom	Chemical Shift
1 C	155.003	11 C	208.681
H	7.579	12 C	49.562
2 C	127.122	Hα	2.510
H	6.117	Hβ	2.146
3 C	185.057	13 C	50.010
4 C	123.823	14 C	47.711
H	6.040	H	2.009
5 C	167.208	15 C	21.140
6 C	31.297	Hα	1.994
Hα	2.404	Hβ	1.654
Hβ	2.534	16 C	35.683
7 C	31.731	Hα	2.520
Hα	1.204	Hβ	2.191
Hβ	2.120	17 C	216.827
8 C	35.150	18 C	14.486
H	2.205	H3	0.785
9 C	59.425	19 C	18.753
H	2.254	H3	1.389
10 C	41.970		

^{1}H NMR (900 MHz, DMSO-d_6) δ 7.58 (d, J = 10.3 Hz, 1H), 6.12 (dd, J = 10.3, 2.0 Hz, 1H), 6.04 (dd, J = 1.7, 1.7 Hz, 1H), 2.56 – 2.52 (m, 1H), 2.55 – 2.50 (m, 1H), 2.53 – 2.49 (m, 1H), 2.40 (ddd, J = 13.3, 4.2, 2.5 Hz, 1H), 2.25 (dd, J = 11.2, 0.9 Hz, 1H), 2.23 – 2.19 (m, 1H), 2.22 – 2.16 (m, 1H), 2.15 (d, J = 12.5 Hz, 1H), 2.12 (dddd, J = 12.5, 5.1, 3.5, 2.5 Hz, 1H), 2.04 – 1.97 (m, 1H), 2.01 – 1.97 (m, 1H), 1.68 – 1.63 (m, 1H), 1.39 (s, 3H), 1.20 (dddd, J = 13.5, 12.6, 11.8, 4.3 Hz, 1H), 0.79 (s, 3H).

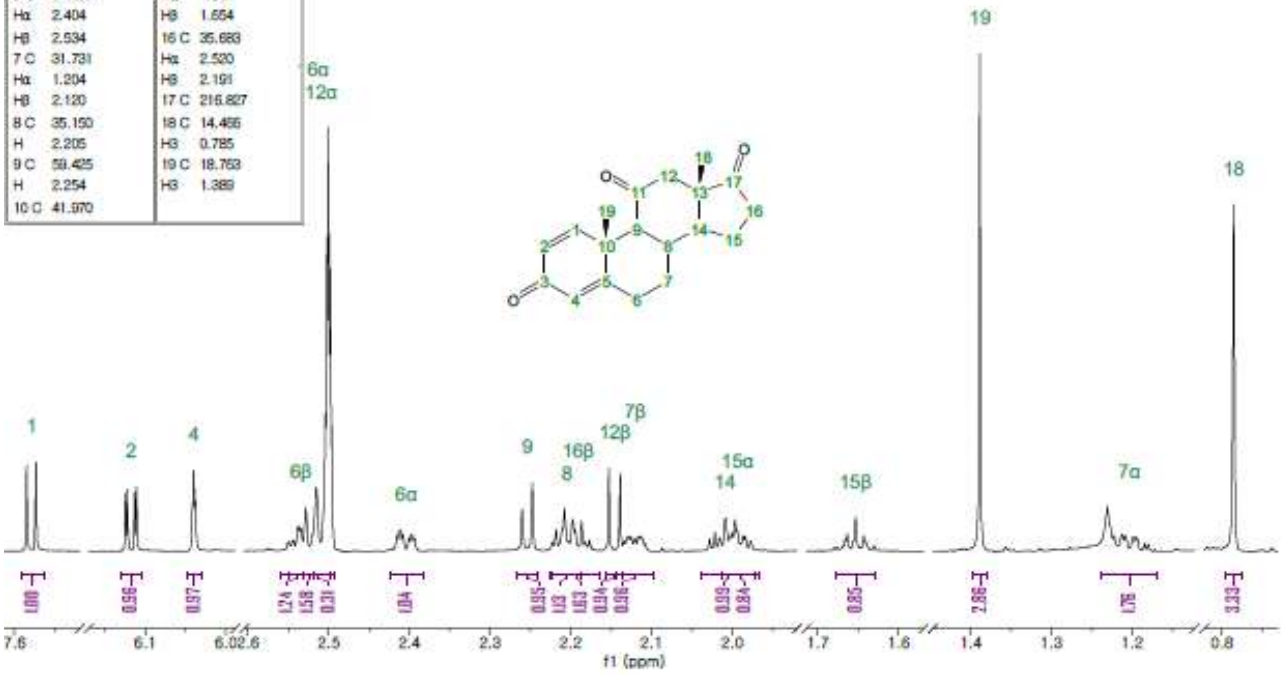


Fig. 4.

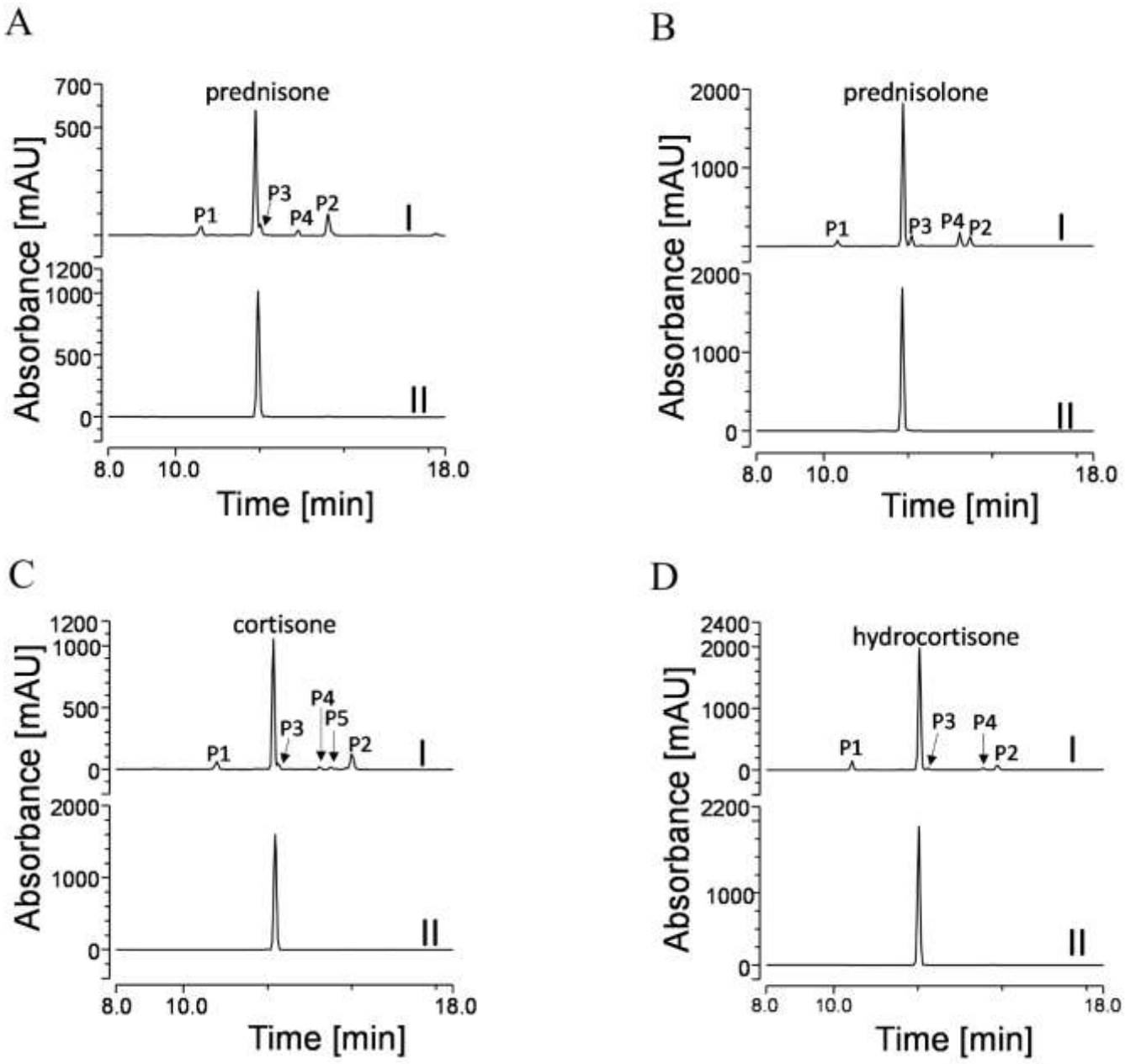


Fig. 5.