Supporting Information

Simplification of Corticosteroids Biosynthetic Pathway by Engineering P450BM3

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Table of Contents

Materials and methods

Chemicals

Standards of Progesterone, corticosterone, 17α-hydroxy-PG, 21-hydroxy-PG, 16β-hydroxy-PG, hydrocortisone, and 11α-hydrocortisone (Analytical Reagent) were purchased from Macklin Reagent (Shanghai, China). Nicotinamide adenine dinucleotide phosphate ($NADP^+$) and Glucose dehydrogenase (GDH) were both purchased from Glpbio (Tianjing, China) and Macklin Reagent (Shanghai, China), respectively. NaCl, K_2HPO_4, KH_2PO_4, tryptone, Glucose, yeast extract, glycerol, Isopropyl *β*-D-1-thiogalactopyranoside, and 5-aminolevulinic acid hydrochloride (Analytical Reagent) were all purchased from Sangon (Shanghai, China).

Gene synthesis and Plasmid construction

Sangon Biotech (Shanghai, China) synthesized codon optimized P450 genes (Table S1) and primers (Table S2). *E. coli* JM109 and *E. coli* BL21 (DE3) were purchased from TransGen Biotech (Beijing, China) and used as hosts for gene cloning and protein expression, respectively. The synthetic *P450BM3* gene was inserted at the *Bam*HI/*Hin*dIII sites of pET28a-1 to form pET28a-P450BM3. The synthetic *CYP5311B2* and *CPR* genes was inserted at the Multiple Cloning Site I (MCSI) and MSCII of pETDuet-1 by Gibson tech, respectively, and finally formed pETDuet-CYP5311B2-CPR. The synthetic *gdh* genes was inserted at the MCSI of pACYCDuet-1 to form pACYCDuet-gdh by Gibson tech. MultiF Seamless Assembly Mix, used for seamless cloning, was purchased from ABclonal technology (Wuhan, China). Genes of VD1721-BM3 and FA11a-BM3 was respectively inserted at the MCSI and MCSII of pETDuet-1 to form pETDuet-VD1721BM3-FA11aBM3. Genes of VD1721-BM3 and CYP5311B2-RBS_CPR was respectively inserted at the MCSI and MCSII of pETDuet-

1 to form pETDuet-VD1721BM3-CYP5311B2-CPR. *Nco*I/*Not*I and *Mfe*I/*Xho*I were selected as the cleavage sites for genes inserting into MCSI and MSCII, respectively.

Construction of P450BM3 variants library

P450BM3 mutation site introduction achieved through overlapping PCR strategy. All primers used for mutating residues of P450BM3 were listed in Table S3. Single point mutation construction method refers to our previous study.[1] The introduction of saturated mutated residues was achieved through degenerate base NNK.

In this study, S72/L437/A330/A74 of P450BM3 were saturation mutagenesis by using Gibson assembly strategy.[2] First, we designed primers F1 and R1 to PCR amplify the gene sequence between S72 and A330. By incorporating NNK codon substitutions into the primers, we targeted the original sequences of S72, A74, and A330 to achieve saturation mutagenesis. Subsequently, we designed primers F2 and R2 to amplify the gene sequence between A330 and L437. By integrating the NNK codon into the primer design, we targeted the original sequence of L437 to generate saturation mutants. Finally, primers F3 and R3 were designed to circularize the other segments of the target plasmid. The three fragments were digested with DpnI (Vazyme, Nanjing-China) then assembled to achieve saturation mutagenesis at four positions. Above three sequences assembly system ratio reference the instructions for using 2× MultiF Seamless Assembly Mix (https://abclonal.com.cn/catalog/RK21020).[3] The assembled products were transformed into *E. coli* BL21(DE3) and used for subsequent P450 variants expression.

P450 mutation library screening

The P450 mutant library was transformed into *E. coli* BL21(DE3). The transformed

products were spread on LB solid plates containing antibiotics and incubated at 37°C for 12 hours. Subsequently, we picked individual colonies from the plate and inoculated them into a 96 deep well plate, with each well containing 700μL of LB medium. The cultures were then incubated at 37°C for 12 hours. We transferred 100μL of the bacterial suspension into 500μL of TB medium, and then added IPTG to a final concentration of 0.2mM. The cultures were then incubated at 25°C for 24 hours. Moreover, we also added 100μL of the bacterial suspension into another 96 deep-well plate for subsequent sequencing of P450 variants.

After the induction of P450 expression, the cell pellets were harvested and washed with 500μL 100mM Kpi (pH=8.0) by centrifugation at 4°C and 4000 rpm for 15 min. The 96-well plate was stored at -80°C until further use. When P450 needed to initiate the catalytic reaction, the 96-well plate was thawed at room temperature (RT). The entire cells were resuspended with 600μL of 100 mM KPi buffer (pH=8.0) containing 100 mM glucose, 10% glycerol, 1mM $NADP^+$, and 1U/mL GDH. The reaction mixture was immediately frozen in liquid nitrogen. Thaw the 96-well plate at RT for 5 min, then immersed in warm water (25°C) for about 30-35 minutes. The reaction was initiated by adding 5μL of substrate (100 mM, DMF dissolution) and was allowed to proceed for 24 hours at 25°C with shaking at 220rpm. After the reaction was completed, added 500μL of ethyl acetate and vortex vigorously, followed by centrifugation (30 min, 4000rpm, 20°C). After centrifugation, collect 300μL of the organic phase, dry at 60°C, and reconstitute in 200μL of acetonitrile. Filter through an organic membrane before HPLC and GC-MS analysis. Finally, effective P450BM3 variants were identified, and the original bacterial cultures stored at -80°C would be retrieved for further expansion. Subsequently, the mutated sequences were determined in Sangon (Shanghai, China).

The composition of bacterial culture medium was as follows: Luria-Bertani

medium (LB) containing: 10g tryptone, 5g yeast extract, 10g NaCl, and deionized water to reach 1000mL. Terrific Broth (TB) containing: 24g yeast extract, 12g tryptone, 9.4g K_2HPO_4, 2.2g KH_2PO_4, 4mL glycerol, and deionized water to reach 1000mL. 100mM Kpi (pH=8.0) contained 55.9g K_2HPO_4 and 4.1g KH_2PO_4 were diluted to 1000mL with water.

P450 variants purification and kinetic parameters analysis

All processes of protein purification were carried out on ice. Extraction and purification of P450 enzymes were conducted following the published method.[4, 5] *E. coli* BL21(DE3) with P450 variants were collected and resuspended for ultrasonic crushing in a 10mL 100mM Kpi with 10mM imidazole. The *ProteinSafe*™ Protease Inhibitor Cocktail (Transgen, Beijing) consisted of AEBSF, aprotinin, bestatin, E-64, leupeptin and pepstatin A, with a final concentration of 0.1mM was added to the suspension bacteria before ultrasonic crushing. The conditions of the ultrasonic breaking of *E. coli* were: 6mm horn; power 300W; working time 3s; interval time 3s, for 12min, in an ice bath. The crude enzymes were collected by collecting centrifugation at 12000*rpm, 4°C for 30min. The enzymes with his-tag were purified by ProteinIso® Ni IDA resin (Transgen, China). Equilibrium solution was comprised of 300mM NaCl, 50mM NaH_2PO_4, 10mM imidazole. Five mL resin was added to the 6mL Ni-NTA Chromatography Column. The pure enzymes were eluted with 2mL protein eluent (200mM imidazole, pH=7.0). Moreover, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were performed as described previously with 10% polyacrylamide gels.[4-6] Protein concentration was measured using the UV method using Nanodrop Ultra-Micro Reflectometer (Implen Gmbh, Munich, Germany).

Kinetic parameters were determined by measuring NADPH consumption at 340 nm using a UV–visible 2600 spectrophotometer (UNICO®, Shanghai, China). The method was referenced from Li et.al's work.[7] The catalytic system of P450BM3 or its variants was contained with 100 mM glucose, 1 mM NADPH, and 200 mg/L P450 variants. The catalytic system of CYP5311B2-CPR was contained with 100 mM glucose, 1 mM NADPH, and 200 mg/L *Ao*CYP5311B2, and 200 mg/L *Ao*CPR. P450s kinetic analysis was carried out in different substrate concentrations (0.0625, 0.125, 0.25, 0.5, 0.75, 1, 1.5, and 2 mM) at 25 °C, pH 8.0 for 20 min. The kinetic parameters of five P450 variants were shown in Table S1.

Whole-cell catalytic synthesis of 11α-hydrocortisone and hydrocortisone

Whole-cell catalytic **system1** for 11α-hydrocortisone production were constructed by co-introducing pETDuet-VD1721BM3-FA11aBM3 and pACYCDuet-gdh into *E. coli* BL21(DE3). Whole-cell catalytic **system2** for hydrocortisone production were constructed by co-introducing pETDuet-VD1721BM3-CYP5311B2-CPR and pACYCDuet-gdh into *E. coli* BL21(DE3). The conditions for inducing expression and catalytic system refer to "P450 Mutation Library Screening". The difference was that the bacterial solution was inoculated with 50mL TB medium at a rate of 1%, and after OD_{600} grows to 0.6-0.8, IPTG with a final concentration of 0.25mM was added. Subsequently, collecting the *E. coli* and resuspend them with kPi solution (pH=8.0) into OD_{600}=20, and added a final concentration of 1mM of $NADP^+$ in 50 mL centrifuge tube. After rapid freezing with liquid nitrogen, put centrifuge tube stand at RT for 5 minutes and place it in a thermostat water bath at 25 ℃. 1g/L PG was added to the system to initiate the reaction and catalyze for 12 hours at 25℃, 220 rpm. Take 100uL of fermentation products per hour for HPLC analysis. After reaction, the products of

whole-cell catalytic systems were extracted with an equal volume of ethyl acetate and dried with Na_2SO_4. Subsequently, the solvent was removed with a rotary evaporator. Samples were purified using a SHIMADZU LC-20AR semi-preparative chromatography system using a Shim-pack GIST C18 column (10 mm × 250 mm, 5 μm, SHIMADZU, Japan) at 254 nm, 30°C. The elution program consisted of acetonitrile and water (50:50) at a flow rate of 5 mL/min and elution for 40 minutes. The organic solvent was then evaporated to obtain the purified samples.

Molecular docking and molecular dynamics (MD) simulation

The structure of $P450_{BM3}$ from *Bacillus megaterium* ATCC 14581 was obtained from PDB website (pdb ID: 1FAG). The structure of CYP5311B2 from *Absidia orchidis* was obtained by using AphaFold2. Moreover, the structures of all P450BM3 variants were re-built by using 1FAG as template in Swiss Model. The models of progesterone and cortexolone were obtained by using Chem 3D (19.0). Docking of substrates and P450 was carried out by AutoDock Vina. The method of molecular docking was referred to our previous study.[8] The docking box is selected within a range of 7Å above the heme center. The conformation with the lowest affinity (kcal/mol) is generally selected as the optimal docking result.

The coordinates of five ligands were optimized in Gaussian 09 package with B3LYP theory level and 6-31G* basis set.[9] The parameters of ligands for molecular dynamics were carried out by the Antechamber tool in the General Amber Force Field form, and charges were fitted by the RESP method.[10, 11] The topology of protein was described by Amber 99SB-ILDN all-atoms force field.[12] After energy minimization, NVT ensemble and NPT ensemble equilibrium, 100ns molecular dynamics simulation were applied for each protein-ligand group with GROMACS 2021.5 package with

periodic boundary conditions.[13-15] The Nose-Hoover thermostat coupling methods were used to maintain the system temperature at 298K.[16] The cut-off switching function for non-bonded van der Waals interaction started at 1.2 nm and reached zero at 1.35 nm. Long-range electrostatic interactions were calculated by Particle mesh Ewald summation.[17] And the cut-off distance was set at 1.2 nm to separate the direct and reciprocal space. The linear constraint solver algorithm was applied to describe the bond length constraint.[18] Protein-ligands complex was dissolved in a box full of simple point charge water molecules with sodium and chloride ions as counterions.[19] Simulations were carried out with a time step of 2 fs, and data were saved every 4 ps. Snapshots of simulation results were visualized by VMD software.[20] Calculate the distance between atoms by grabbing atoms from the index file and by using "gmx distance" command to calculate the distance variation between 0-100ns separately. Using gmx_mmPBSA toolkit to disassemble and calculate the P450's residues Free-energy in 80-100ns, taking the average value as the final result.

HPLC, GC-MS, and NMR analysis for the separated and purified products

Progesterone, cortexolone, and their hydroxylation product were measured by HPLC analysis. The column was C18 (Agilent Poroshell 120 EC-C18, 1.9μm, 2.1 × 50 mm, 699675-902, USJSA03892, B19249). Mobile phase A: 0.5% trifluoroacetic acid aqueous solution, B: acetonitrile. The liquid phase conditions were flowing rate: 1mL/min, column temperature: 30 °C, dual wavelength: 241nm and 254nm. Gradient elution conditions: 0min→15min: 5% A and 95% B to 80% A and 20% B. 15min→ 20min: 80% A and 20% B. 20min→30min: 80% A and 20% B to 5% A and 95% B. The testing time for each sample was 30 minutes.

Gas chromatography-mass spectrometry (GC-MS) analyses were carried out with

a Shimadzu TQ8050 NX instrument equipped with a flame ionization detector (FID) and an AI1310 autosampler using HP-5 MS column (length 30m, internal diameter 0.25mm, film thickness 0.25mm) with helium as carrier gas at a flow rate of 1mL/min. The injector was held at 300°C and the FID temperature was 280°C. The oven temperature was held at 220°C for 1min, then raised at 20°C/min to 300°C and held at this temperature for 5 min.

The purified samples were dissolved in $CDCl_3$ or DMSO-d_6 and subsequently characterized by nuclear magnetic resonance (NMR). The purified samples (20 mg/mL) were dissolved in $CDCl_3$ and subsequently characterized by nuclear magnetic resonance (NMR) spectra with a Bruker Avance III 600 MHz nuclear magnetic resonance spectrometer (Bruker BioSpin, Karlsruhe, Germany). NMR spectra were recorded on 500 MHz for 1H and 126 MHz for 13C in $CDCl_3$. MestReNova software (version 14.0) was used to analyze and process data.

NMR data

17α-hydroxy-PG (a):

^{1}H NMR (500 MHz, DMSO-d_6) δ 5.63 (s, 1H), 5.24 (s, 1H), 2.62 – 2.31 (m, 2H), 2.27 – 2.13 (m, 2H), 2.01 – 1.91 (m, 2H), 2.09 (s, 3H), 1.87 – 1.26 (m, 10H), 1.14 (s, 3H), 1.06 – 0.76 (m, 2H), 0.53 (s, 3H).

^{13}C NMR (126 MHz, DMSO-d_6) δ 210.48, 197.97, 170.85, 123.22, 89.15, 53.01, 49.88, 46.23, 38.17, 35.17, 35.03, 33.63, 32.25, 32.06, 31.87, 30.32, 26.81, 23.18, 20.32, 16.92, 14.57.

21-hydroxy-PG (b):

^{1}H NMR (500 MHz, DMSO-d_6) δ 5.63 (s, 1H), 5.09 – 4.67 (m, 1H), 4.16 – 3.79 (m, 2H), 3.34 (s, 1H), 2.70 – 2.32 (m, 4H), 2.20 (m, 2H), 2.00 (m, 2H), 1.91 – 1.71 (m, 2H),

1.73 – 1.45 (m, 3H), 1.35 (m, 2H), 1.25 – 1.18 (m, 1H), 1.14 (s, 3H), 1.06 – 0.84 (m, 2H), 0.58 (s, 3H).

^{13}C NMR (126 MHz, DMSO-d_6) δ 210.44, 198.04, 170.84, 123.22, 68.73, 57.56, 55.38, 53.01, 43.72, 38.17, 37.74, 35.13, 34.89, 33.63, 31.96, 31.63, 24.10, 22.39, 20.55, 16.88, 13.25.

17α,21-dihydroxy-PG (c):

^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.73 (brs, 1H), 4.67 (d, *J* = 19.9 Hz, 1H), 4.30 (d, *J* = 19.9 Hz, 1H), 2.68 (ddd, *J* = 14.8, 11.5, 3.0 Hz, 1H), 2.47 – 2.32 (m, 3H), 2.32 – 2.23 (m, 1H), 2.03 (ddd, *J* = 13.4, 5.0, 3.2 Hz, 1H), 1.92 – 1.79 (m, 2H), 1.78 – 1.54 (m, 5H), 1.48 – 1.32 (m, 3H), 1.18 (s, 3H), 1.10 (qd, *J* = 12.5, 4.4 Hz, 1H), 0.97 (ddd, *J* = 12.0, 10.5, 4.1 Hz, 1H), 0.70 (s, 3H).

^{13}C NMR (151 MHz, Chloroform-*d*) δ 212.46, 199.70, 171.00, 124.12, 89.15, 67.60, 53.43, 50.44, 48.76, 38.70, 35.84, 35.75, 34.68, 34.05, 32.90, 32.14, 30.23, 23.88, 20.68, 17.53, 15.18.

16β-hydroxy-PG (d):

^{1}H NMR (500 MHz, DMSO-d_6) δ 5.65 (s, 1H), 4.24 (s, 1H), 2.62 – 1.33 (m, 21H), 1.25 (s, 3H), 0.81 (s, 3H).

^{13}C NMR (126 MHz, DMSO-d_6) δ 197.96, 170.58, 125.10, 75.61, 46.75, 44.21, 43.71, 36.71, 35.36, 33.82, 31.27, 28.07, 26.89, 25.69, 23.73, 21.19, 19.43, 12.52.

Hydrocortisone (e):

^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.69 (d, *J* = 1.7 Hz, 1H), 4.66 (d, *J* = 19.8 Hz, 1H), 4.48 (q, *J* = 3.3 Hz, 1H), 4.31 (d, *J* = 19.8 Hz, 1H), 2.72 (m, 1H), 2.51 (m, 1H), 2.45 (m, 1H), 2.36 (dt, *J* = 16.6, 4.4 Hz, 1H), 2.25 (ddd, *J* = 14.3, 4.6, 2.0 Hz, 1H), 2.18 (dt, *J* = 13.3, 4.6 Hz, 1H), 2.08 (td, *J* = 10.9, 5.4 Hz, 1H), 2.05 – 1.98 (m, 2H), 1.93 – 1.80 (m, 2H), 1.71 (m, 1H), 1.61 – 1.49 (m, 2H), 1.48 (t, *J* = 5.9 Hz, 1H), 1.44 (s, 3H),

1.13 (qd, *J* = 12.3, 4.5 Hz, 1H), 1.03 (dd, *J* = 11.2, 3.4 Hz, 1H), 0.96 (s, 3H), 0.90 – 0.77 (m, 1H).

^{13}C NMR (151 MHz, Chloroform-*d*) δ 212.22, 199.54, 171.84, 122.62, 88.84, 68.46, 67.59, 56.13, 51.90, 48.10, 39.93, 39.33, 35.19, 34.43, 33.94, 32.86, 32.15, 31.57, 23.91, 21.18, 17.77.

11α-Hydrocortisone (f):

^{1}H NMR (500 MHz, DMSO-d_6) δ 5.61 (d, *J* = 1.4 Hz, 1H), 5.33 (s, 1H), 4.74 (t, *J* = 6.0 Hz, 1H), 4.50 (dd, *J* = 19.2, 6.5 Hz, 1H), 4.35 (d, *J* = 7.0 Hz, 1H), 4.11 (dd, *J* = 19.2, 5.7 Hz, 1H), 3.78 (tdd, *J* = 11.0, 6.9, 4.8 Hz, 1H), 2.64 (dt, *J* = 13.9, 4.5 Hz, 1H), 2.58 – 2.53 (m, 1H), 2.42 – 2.30 (m, 2H), 2.23 (dt, *J* = 14.0, 3.3 Hz, 1H), 2.12 (dt, *J* = 16.5, 4.1 Hz, 1H), 1.90 (td, *J* = 14.0, 4.3 Hz, 1H), 1.84 – 1.72 (m, 2H), 1.66 – 1.41 (m, 2H), 1.24 (s, 3H), 1.18 (m, 1H), 1.00 (m, 2H), 0.55 (s, 3H).

^{13}C NMR (126 MHz, DMSO-d_6) δ 211.83, 206.95, 198.76, 171.71, 123.58, 88.19, 67.66, 66.03, 58.24, 49.55, 47.30, 41.82, 37.06, 34.34, 33.89, 33.31, 32.97, 31.74, 23.24, 17.94, 15.80.

Tables

Table S1. Analyzing enzyme kinetic parameters of VD16-BM3, VD17-BM3, VD21-BM3, VD1721-BM3, FA11a-BM3, and CYP5311B2-CPR for catalyzing steroids.

P450	Sub.	k_{cat} (S^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} S^{-1})
VD16-BM3	**1**	171.45 ± 8.1	0.52 ± 0.08	329.7
VD17-BM3	**1**	273 ± 15	0.72 ± 0.1	379
VD21-BM3	**1**	245 ± 10.5	0.65 ± 0.2	377
VD1721-BM3	**1**	224 ± 15	0.88 ± 0.05	254
FA11a-BM3	**2**	264 ± 10	1.1 ± 0.2	240
CYP5311B2-CPR	**2**	288.5 ± 6.6	0.8 ± 0.1	360

Reaction conditions: 0-1 mM substrate concentrations, 1 mM NADPH, 10 mM glucose, 2 units of GDH, and 200 mg/L enzyme in 200 μL of KPi buffer (pH 8.0) for 5 min at 220 rpm and 30 °C. Notes: progesterone (**1**), cortexolone (**2**).

Table S2. Strains and plasmids used in this study.

Name	Relative characteristics	Reference
Strain		
E. coli JM109	*endA1 hsdR*17 [$r^{-}m^{+}$] *supE*44 *thi-1 recA1 gyrA* [NalR] *relA relA1* Δ[*lacZYA-argF*] *U169 deoR* [Ø80Δ (*LacZ*) M15]	TransGen
E. coli BL21 (DE3)	Chemically Competent Cell, F-, *ompT*, *hsdS*($r^{-}m^{+}$), *gal*, *dcm*(DE3)	TransGen
Plasmid		
pET28a-1	Kan^{r}, lacZ, T7 promoter	Sangon
pET28a-P450BM3	P450BM3 (*Bacillus megaterium* ATCC 14581) expression plasmid	This study
pET28a-VDBM3	P450BM3-F87A/P25A/P329A/E435D (VD-BM3) expression plasmid	This study
pET28a-VD16-BM3	VD-BM3-S72H/L437A expression plasmid	This study
pET28a-VD17-BM3	VD-BM3-S72Q/L437G expression plasmid	This study
pET28a-VD21-BM3	VD-BM3-S72Q/L437G/A330Y/A74G expression plasmid	This study
pET28a-VDSP-BM3	VD-BM3-S72Q/L437G/A330Y/A74P (VDSP-BM3) expression plasmid	This study
pET28a-VD1721-BM3	VD-BM3- S72Q/L437G/A330Y/A74P/D433G/K435G expression plasmid	This study
pET28a-FA11a-BM3	P450BM3-F87A-P25F-A330W-S72W-L437G-L439G expression plasmid	This study
pETDuet-1	Amp^{r}, lacZ, T7 promoter	Sangon

Name	Relative characteristics	Reference
pETDuet-VD1721BM3-FA11aBM3	Co-expression plasmid of VD1721-BM3 and FA11a-BM3 enzymes	This study
pETDuet-VD1721BM3-CYP5311B2-CPR	Co-expression plasmid of VD1721-BM3 and CYP5311B2 enzymes	This study
pACYCDuet-1	Cm^r, lacI, T7 promoter	Sangon
PACYCDuet-gdh	GDH (*Bacillus Subtilis*) expression plasmid	This study

Table S3. Primers used for plasmid construction in this study.

Primer	Sequence (5′-3′)
P450BM3-F	GATATACCATGACAATAAAAGAAATGCCACAACCCAAGACGTTC GGTGAAC
P450BM3-R	GCAAGCTTTTAGACGTCCTTAGCATAACGGCCTTTTTCTTCTAAT TGCTGC
Vec28a-F	AGGACGTCTAAAAGCTTGCGGCCGCACTCGAGCACCACCACCAC CAC
Vec28a-R	CCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCAT GACAAT
CYP5311B2-F	gatataccATGTTGACTGAATATATTCATCATTTTATTAATAATTTTG ATCAAAAG
CYP5311B2-R	cttaagcaTTATTTTCTTGGAACAATTTTGAATCTACCCATTGGAATT C
VecDuet1-F	GAAAATAAtgcttaagtcgaacagaaagtaatcgtattgtacacggc
VecDuet1-R	CAGTCAACATggtatatctccttattaaagttaaacaaaattatttctacaggggaa
CPR-F	gatataccATGGATTTGCCAACAGCTACTGATATTAATGAAAAACCA AAATTATC
CPR-R	ctaggttaaTTAAGCCCAAACATCTTCAACATATCTATTAGATTTTCTC
VecDuet2-F	GGCAAATCCATggtatatctccttcttatacttaactaatatactaagatggggaattg
VecDuet2-R	GGGCTTAAttaacctaggctgctgccaccgctgagcaataactag
GDH-F	CTTTAATAAGGAGATATACCATGTATAAGGATCTGGAGGGCAAG G
GDH-R	CTTTCTGTTCGACTTAAGCATTAGCCGCGGCCGGCCTGGAA
Vec3-GDH-F	TGCTTAAGTCGAACAGAAAGTAATCGTATTGTACACGGCCGCAT
Vec3-GDH-R	GGGACATCTTTATTAAAACAAATTGAAATTATTCCTCTATATGG
1-F	CTTAAGTTCGTGCGCGACTTTNNKGGTGACGGTCTGGCGACCTC GTGGACCCATGAG

Primer	Sequence (5′-3′)
1-R	AGTCGCGCACGAACTTAAGAGCTTGCGACAGGTTCTTGTCGAAG CGGCTTTC
2-F	CTGATTGCAGGCCACGAAACCACCTCTGGCTTGCTGAGCTTTGCT CTGTACTTC
2-R	TTCGTGGCCTGCAATCAGAAANNKAATAATTTGATATCTGATGT TTTCGTCGTCTAGC
3-F	GTATGCAAAGGAGGACACCGTTCTCGGTGGTGAATATCCGTTGG AGAAGGGCGACG
3-R	GGTGTCCTCCTTTGCATACAGCGAGAANNKAGGNNKGGTCGGCC ACAGACGCAGCGC

Table S4. Primers used for P450 variants construction in this study.

Primer	Sequence (5′-3′)
F87A-F	ACGGTCTGGCGACCTCGTGGACCCATGAGAAAAATTGGAAAAAGGCCCAC
F87A-R	CCACGAGGTCGCCAGACCGTCACCCGCAAAGTCGCGCACGAACTTAAGAG
F87V-F	ACGGTCTGGTGACCTCGTGGACCCATGAGAAAAATTGGAAAAAGGCCCAC
F87V-R	CCACGAGGTCACCAGACCGTCACCCGCAAAGTCGCGCAC
F87G-F	ACGGTCTGGGTACCTCGTGGACCCATGAGAAAAATTGGAAAAAGGCCCAC
F87G-R	CCACGAGGTACCCAGACCGTCACCCGCAAAGTCGCGCAC
P25A-F	CCGACAAAGCGATTCAGACGCTGATGAAAATCGCGGACGAGCTGG
P25A-R	CGTCTGAATCGCTTTGTCGGTATTCAGGAGCGGAAGGTTCTTCAGTTCACCGAAC
P25V-F	CCGACAAAGTGATTCAGACGCTGATGAAAATCGCGGACGAGCTGG
P25V-R	CGTCTGAATCACTTTGTCGGTATTCAGGAGCGGAAGGTTCTTCAGTTCACCGAAC
P25G-F	CCGACAAAGGTATTCAGACGCTGATGAAAATCGCGGACGAGCTGG
P25G-R	CGTCTGAATACCTTTGTCGGTATTCAGGAGCGGAAGGTTCTTCAGTTCACCGAAC
P329A-F	GACCGCGGCGGCTTTCTCGCTGTATGCAAAGGAGGACACCGTTCTCGGTG
P329A-R	GCGAGAAAGCCGCCCGCGGTCGGCCACAGACGCAGCGCTTCATACAGTACC
P329V-F	GACCGCGGTGGCTTTCTCGCTGTATGCAAAGGAGGACACCGTTCTCGG
P329V-R	GCGAGAAAGCCACCGCGGTCGGCCACAGACGCAGCGCTTCATACAGT

Primer	Sequence (5′-3′)
P329G-F	GACCGCGGGTGCTTTCTCGCTGTATGCAAAGGAGGACACCGTTCTCGGTG
P329G-R	GCGAGAAAGCACCCGCGGTCGGCCACAGACGCAGCGCTTCATACAGTACC
E435A-F	ATATCAAGGCGACCCTGACCTTGAAGCCGGAGGGGTTCGTTGTTAAAG
E435A-R	GGTCAGGGTCGCCTTGATATCTAATTCGTAGTTAGTGTGATCTTCGAAATC
E435V-F	ATATCAAGGTGACCCTGACCTTGAAGCCGGAGGGGTTCGTTGTTAAAG
E435V-R	GGTCAGGGTCACCTTGATATCTAATTCGTAGTTAGTGTGATCTTCGAAATC
E435G-F	ATATCAAGGGTACCCTGACCTTGAAGCCGGAGGGGTTCGTTGTTAAAG
E435G-R	GGTCAGGGTACCCTTGATATCTAATTCGTAGTTAGTGTGATCTTCGAAATC
E435D-F	ATATCAAGGATACCCTGACCTTGAAGCCGGAGGGGTTCGTTGTTAAAG
E435D-R	GGTCAGGGTATCCTTGATATCTAATTCGTAGTTAGTGTGATCTTCGAAATC
MuS72-F	GAACCTGNNKCAAGCTCTTAAGTTCGTGCGCGACTTTGCGGGTGACGG
MuS72-R	CTTAAGAGCTTGCGACAGGTTCTTGTCGAAGCGGCTTTCATCGCAGGCCTC
MuL437-F	AGGAAACCNNKACCTTGAAGCCGGAGGGGTTCGTTGTTAAAGCGAAG
MuL437-R	CTTCAAGGTCAGGGTTTCCTTGATATCTAATTCGTAGTTAGTGTGATCTTCGAAATC
MuA330-F	CGACCGCGCCTNNKTTCTCGCTGTATGCAAAGGAGGACACCGTTCTC
MuA330-R	AGAAAGCAGGCGCGGTCGGCCACAGACGCAGCGCTTCATACAGTACCATACC

Primer	Sequence (5′-3′)
MuA330-F	CGACCGCGCCTNNKTTCTCGCTGTATGCAAAGGAGGACACCGTTCTC
MuA330-R	AGAAAGCAGGCGCGGTCGGCCACAGACGCAGCGCTTCATACAGTACC ATACC
MuA74-F	GAACCTGTCGCAANNKCTTAAGTTCGTGCGCGACTTTGCGGGTGACGG TC
MuA74-R	CTTAAGAGCTTGCGACAGGTTCTTGTCGAAGCGGCTTTCATCGCAGGC CTC
R66G-F	GATGAAAGCGGTTTCGACAAGAACCTGTCGCAAGCTCTTAAGTTCGTG
R66G-R	TTGTCGAAGACCCTTTCATCGCAGGCCTCTTTGATCAGGCGTTGGCTG
I259G-F	ATCAAATTGGTACGTTTCTGATTGCAGGCCACGAAACCACCTCTGGCTT G
I259G-R	TCAGAAACGTACCAATTTGATATCTGATGTTTTCGTCGTCTAGCGGTTC ACC
F261G-F	AAATTATTACGGGTCTGATTGCAGGCCACGAAACCACCTCTGGCTTGC
F261G-R	CAATCAGACCCGTAATAATTTGATATCTGATGTTTTCGTCGTCTAGCGG TTC
E267G-F	TGCAGGCCACGGTACCACCTCTGGCTTGCTGAGCTTTGCTCTGTAC
E267G-R	AAGCCAGAGGTGGTACCGTGGCCTGCAATCAGAAACGTAATAATTTGA TATCTG
V317G-F	ACGTTGGTATGGGTCTGAACGAAGCGCTGCGTCTGTGGCCGACCGCGC CTG
V317G-R	TTCAGACCCATACCAACGTACTTCAATTGTTTCACGTGTTTGTAAGATG GC
L324G-F	GCGCTGCGTGGTTGGCCGACCGCGCCTGCTTTCTCGCTGTATGCAAAG
L324G-R	GTCGGCCAACCACGCAGCGCTTCGTTCAGTACCATACCAACGTACTTC AATTG

Primer	Sequence (5′-3′)
T324G-F	TGTGGCCGGGTGCGCCTTGGTTCTCGCTGTATGCAAAGGAGGACACCG TTCTC
T324G-R	CCAAGGCGCACCCGGCCACAGACGCAGCGCTTCGTTCAGTACCATAC
E348G-F	TCCGTTGGGTAAGGGCGACGAGCTTATGGTGCTGATCCCGCAACTGCA CC
E348G-R	GTCGCCCTTACCCAACGGATATTCACCACCGAGAACGGTGTCCTCCTTT GC
A399G-F	GGCCAGCGTGGTTGCATTGGTCAGCAGTTTGCGTTGCACGAAGCGACT
A399G-R	GACCAATGCAACCACGCTGGCCATTCCCGAACGGTTTGAATGCATGC
A406G-F	TCAGCAGTTTGGTTTGCACGAAGCGACTCTGGTTCTGGGCATGATGCT G
A406G-R	CTTCGTGCAAACCAAACTGCTGACCAATGCACGCACGCTGGCCATTC
Y429G-F	CACTAACGGTGAATTAGATATCAAGGAAACCCTGACCTTGAAGCCGGA G
Y429G-R	CTAATTCACCGTTAGTGTGATCTTCGAAATCAAAATGTTTCAGCATCAT G
D432G-F	ACTACGAATTAGGTATCAAGGAAACCCTGACCTTGAAGCCGGAGGGGT TC
D432G-R	GTTTCCTTGATACCTAATTCGTAGTTAGTGTGATCTTCGAAATCAAAAT G
K434G-F	AGATATCGGTGAAACCCTGACCTTGAAGCCGGAGGGGTTCGTTG
K434G-R	GGGTTTCACCGATATCTAATTCGTAGTTAGTGTGATCTTCGAAATC
E435G-F	GATATCAAGGGTACCCTGACCTTGAAGCCGGAGGGGTTCG
E435G-R	TCAGGGTACCCTTGATATCTAATTCGTAGTTAGTGTGATCTTCGAAATC
L439G-F	CCCTGACCGGTAAGCCGGAGGGGTTCGTTGTTAAAGCGAAGAGC
L439G-R	TCCGGCTTACCGGTCAGGGTTTCCTTGATATCTAATTCGTAGTTAGTGT G

Primer	Sequence (5′-3′)
P25F-F	CCGACAAATTCATTCAGACGCTGATGAAAATCGCGGACGAGCTGG
P25F-R	CGTCTGAATGAATTTGTCGGTATTCAGGAGCGGAAGGTTCTTCAGTTC ACCGAAC
P329G-F	GACCGCGTCCGCTTTCTCGCTGTATGCAAAGGAGGACACCGTTCTCGG TG
P329G-R	GCGAGAAAGCGAACGCGGTCGGCCACAGACGCAGCGCTTCATACAGT ACC
F174A-F	AACCTCACCCGGGTATCACCAGCATGGTGCGCGCGCTGGACGAAGCTA TG
F174A-R	GCTGGTGATACCCGGGTGAGGTTGGTCACGATAAAAGGAGTTGAATCT GTAG
R179K-F	CAGCATGGTGAAAGCGCTGGACGAAGCTATGAATAAACTGCAGCG
R179K-R	GTCCAGCGCTTTCACCATGCTGGTGATGAACGGGTGAGGTTGG
W325S-F	CGCTGCGTCTGTCGCCGACCGCGCCTGCTTTCTCGCTGTATGCAAAGGA GG
W325S-R	GCGGTCGGCGACAGACGCAGCGCTTCGTTCAGTACCATACCAACGTAC TTC
F331A-F	CGACCGCGCCTGCTGCGTCGCTGTATGCAAAGGAGGACACCGTTCTCG G
F331A-R	ACAGCGACGCAGCAGGCGCGGTCGGCCACAGACGCAGCGCTTCGTTC AGTACCATAC
D433G-F	AACTACGAATTAGGGATCAAGGAAACCCTGACCTTGAAGCCGGAGGG G
D433G-R	TTTCCTTGATCCCTAATTCGTAGTTAGTGTGATCTTCGAAATCAAAATG TTTC

Table S5. Codon optimized wild-type P450BM3 gene sequence.

Sequence (5′-3′)
ATGACAATAAAAGAAATGCCACAACCCAAGACGTTCGGTGAACTGAAGAACCTTC
CGCTCCTGAATACCGACAAACCGATTCAGACGCTGATGAAAATCGCGGACGAGCT
GGGCGAAATCTTTAAATTCGAAGCTCCGGGTCGCGTGACCCGCTACCTGAGCAGCC
AACGCCTGATCAAAGAGGCCTGCGATGAAAGCCGCTTCGACAAGAACCTGTCGCA
AGCTCTTAAGTTCGTGCGCGACTTTGCGGGTGACGGTCTGGCGACCTCGTGGACCC
ATGAGAAAAATTGGAAAAAGGCCCACAATATTCTGTTACCGTCCTTTAGTCAACAA
GCTATGAAAGGCTATCATGCAATGATGGTGGACATCGCCGTGCAGCTGATTCAAAA
ATGGGAACGTCTGAACACCGACGAACACATTGAAGTTCCGGAGGATATGACCCGTT
TGACCTTGGACACCATCGGCCTTTGCGGTTTTAACTACAGATTCAACTCCTTTTATC
GTGACCAACCTCACCCGTTCATCACCAGCATGGTGCGCGCGCTGGACGAAGCTATG
AATAAACTGCAGCGCGCCAACCCGGATGACCCGGCATATGATGAGAACAAACGTC
AGTTCCAGGAGGACATCAAGGTGATGAACGATCTGGTCGACAAAATTATCGCAGA
TCGTAAGGCGAGCGGCGAGCAAAGTGATGATCTGTTGACCCATATGTTGAACGGCA
AAGATCCAGAAACCGGTGAACCGCTAGACGACGAAAACATCAGATATCAAATTAT
TACGTTTCTGATTGCAGGCCACGAAACCACCTCTGGCTTGCTGAGCTTTGCTCTGTA
CTTCTTAGTCAAGAACCCGCATGTTTTGCAAAAGGCTGCGGAGGAGGCCGCGCGTG
TGCTCGTGGATCCCGTGCCATCTTACAAACAAGTGAAACAATTGAAGTACGTTGGT
ATGGTACTGAACGAAGCGCTGCGTCTGTGGCCGACCGCGCCTTGGTTCTCGCTGTA
TGCAAAGGAGGACACCGTTCTCGGTGGTGAATATCCGTTGGAGAAGGGCGACGAG
CTTATGGTGCTGATCCCGCAACTGCACCGTGATAAGACCATCTGGGGTGATGATGT
GGAGGAGTTCCGCCCTGAGCGCTTTGAAAACCCGAGCGCCATCCCGCAGCATGCAT
TCAAACCGTTCGGGAATGGCCAGCGTGCGTGCATTGGTCAGCAGTTTGCGTTGCAC
GAAGCGACTCTGGTTCTGGGCATGATGCTGAAACATTTTGATTTCGAAGATCACAC
TAACTACGAATTAGATATCAAGGAAACCCTGACCTTGAAGCCGGAGGGGTTCGTTG
TTAAAGCGAAGAGCAAGCAAATTCCGCTGGGTGGTATCCCGTCACCGAGCCGTGA
ACAGAGCGCGAAAAAAGAGCGAAAAACCGTTGAGAACGCACACAACACTCCGCTG

TTGGTACTGTACGGCTCCAACATGGGTACTGCTGAAGGTACAGCCCGTGATCTGGC

CGACATCGCCATGAGCAAAGGCTTCGCGCCGCAGGTTGCGACGCTGGACAGCCAT

GCAGGCAACCTTCCGCGTGAAGGTGCGGTTCTGATTGTTACCGCGAGCTACAATGG

CCACCCGCCGGACAACGCAAAAGAGTTCGTTGACTGGCTGGATCAGGCTTCGGCTG

ATGAGGTGAAAGGCGTGCGTTACAGCGTGTTTGGCTGCGGTGACAAGAACTGGGC

AACGACCTACCAAAAGGTGCCGGCCTTCATCGATGAGACGTTCGCGGCAAAGGGC

GCTGAGAACATCGCGGAACGTGGTGAGGCGGACGCATCTGACGATTTTGAAGGTA

CATACGAAGAATGGCGTGAGCACATGTGGAGCGATCTGGCTGCGTATTTCAATCTG

GACATTGAGAACAGCGAAGAAAATGCCTCGACCCTGTCCCTGCAATTCGTTGACAG

CGCAGCGGATATGCCGCTGGCGAAGATGCATCGTGCGTTTTCCGCGAATGTGGTCG

CGTCCAAAGAATTGCAAAAACCGGGTAGCGCGCGTTCCACCCGTCATCTGGAGATT

GAACTGCCAAAAGAGGCCTCTTACCAGGAGGGCGACCACTTAGGTGTTATTCCGCG

CAATTATGAAGGCATCGTTAATCGTGTTGCAACTCGCTTTGGTCTGGATGCAAGCC

AGCAGATCAGGTTAGAGGCGGAGGAGGAGAAGCTGGCGCATCTGCCGCTTGGCAA

AACGGTTTCCGTCGAAGAGCTCCTCCAGTATGTTGAGTTGCAGGATCCGGTTACTC

GCACCCAGTTGCGTGCGATGGCGGCTAAGACCGTGTGTCCGCCGCACAAAGTTGAG

CTGGAGGTTCTGTTGGAAAAGCAGGCGTACAAGGAGCAGGTGCTGGCAAAGCGCC

TGACGATGCTTGAGTTGCTAGAGAAATATCCGGCGTGCGAAATGGAATTTTCTGAG

TTCATCGCTCTCCTCCCGTCTATGAGACCACGTTACTATTCGATCAGCAGCTCCCCG

CGTGTTGACGAAAAGCAGGCGTCTATTACCGTTAGTGTTGTGTCCGGTGAGGCGTG

GTCAGGCTACGGCGAATACAAGGGTATCGCTAGCAACTACCTGGCGAATCTGCAA

GAGGGCGACACGATTACCTGTTTTGTTAGCACCCCGCAGAGCGGCTTCACCTTGCC

GAAAGGACCGGAAACTCCGCTGATCATGGTCGGTCCAGGCACGGGTGTGGCGCCTT

TTCGTGGTTTCGTGCAAGCGCGGAAACAACTGAAAGAGCAGGGTCAGAGCTTAGG

TGAAGCACACTTGTACTTCGGCTGCCGTTCGCCGCACGAGGATTATTTGTACCAGA

AAGAGCTAGAGAACGCGCAGAATGAGGGTATAATTACCCTGCACACCGCGTTCAG

CCGCGTACCAAATCAGCCGAAAACCTACGTGCAACACGTCATGGAACAAGACGGT

AAGAAACTGATTGAATTACTGGATCAGGGTGCGCACTTTTATATTTGTGGTGATGG

TAGCCAGATGGCACCGGATGTCGAGGCCACCCTGATGAAGAGCTATGCTGAGGTG

CATCAGGTCTCCGAAGCAGACGCACGTCTGTGGCTGCAGCAATTAGAAGAAAAAG

GCCGTTATGCTAAGGACGTCTAA

Table S6. Codon optimized GDH gene sequence.

Sequence (5′-3′)
ATGTATAAGGATCTGGAGGGCAAGGTGGTCGTGATCACCGGCTCCAGCACGGGCCT
GGGCAAGTCCATGGCCATCCGCTTCGCCACCGAGAAGGCCAAGGTGGTCGTGAACT
ATCGCAGCAAGGAGGACGAGGCCAACTCCGTCCTGGAGGAAATCAAGAAAGTGGG
TGGCGAAGCCATCGCCGTGAAGGGCGATGTCACCGTGGAGTCCGATGTGATCAACC
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GATCGACACCAACCTGACCGGCGCCTTCCTGGGCTCCCGCGAGGCCATCAAGTATT
TCGTCGAGAACGACATCAAGGGCACCGTCATCAACATGAGCTCCGTCCATGAGAA
GATCCCGTGGCCGCTGTTCGTCCATTATGCCGCGAGCAAGGGTGGCATGAAGCTGA
TGACCCGCACCCTGGCCCTGGAATATGCCCCGAAGGGCATCCGCGTCAACAATATC
GGCCCGGGCGCCATCAACACCCCGATCAACGCCGAAAAGTTCGCCGACCCGGAGC
AGCGCGCCGACGTCGAGAGCATGATCCCGATGGGCTATATCGGCGAGCCGGAGGA
AATCGCCGCGGTCGCCGCGTGGCTGGCCAGCTCCGAAGCCAGCTATGTGACCGGCA
TCACGCTGTTCGCCGACGGTGGCATGACGCTGTATCCGAGCTTCCAGGCCGGCCGC
GGCTAA

Table S7. Codon optimized CYP5311B2 gene sequence.

Sequence (5′-3′)
ATGTTGACTGAATATATTCATCATTTTATTAATAATTTTGATCAAAAGAAAACTATG
GATCAATTACAAACTATGGTTTCTTCAAAAGAAGGTATGATTGGTTTAGCAACAGC
TGCCGTATTAATGTCTGGTGCTGCTGTTTATAAATCTACTAGAATTGAAAGAGGTTG
TCCTCAAGTTCCTAATCAATCATATTTTATGGGTTCTACTAAAGAATATAGAAATAA
TCCAGCTGCTTTTATTGAAAAATGGGAAAAAGAATTAGGTCCAGTTTATGGTGCAT
ATTTGTTTGGTCAATATACTACAGTTGTTTCTGGTCCACAAGTTAGAGAAGTATTTT
TAAATGATGATTTTGATTTTATTGCAGGTATTGATAGAGACTTTGATACTAACTTAT
TAAGTAATGGTGGTGACTTAAGAGACTTACCAGTTCATAAATTTGCAGGTTCTATT
AAGAAAAATTTGTCTCCTAAATTGCCTTTTTATACATCTAGAGTTATTGAACATTTG
AAAATTGGTTTAAAAGAATTTTGTGGAGTAGTACCAGATGAAGGTAAAGAATTTGA
TCATGTTTATCCATTAGTTCAACATATGGTTGCTAAAGCTTCAGCTTCTGTTTTTGTT
GGTCCTGAATTAGCTAAAAATGAACAATTGATTGATTCTTTTAAAAACATGGTTTTA
GAAGTTGGTTCAGAATTAGCTCCTAAACCTTATTTGGAATTTTTCCCAAATTTGATG
AGATTGAGAATGTGGTTTATTGGTAAAACTAGTCAAAAAGTTAAAAGACATAGAG
ATCAATTAAGAGCTGCCTTAGCACCTCAAGTTGAATATAGATTGAAAGCTATGAAA
GAAAATGATAGTAATTGGGATAGACCAAATGACTTTTTACAAGATATTTTAGAAAG
TGGAGATATACCAGCTCATGTTGATGTAACAGATCATTGTTGTGATTGGATGACTC
AAATTATTTTTGCTGCACTACATACTACTTCAGAAAATGGTACTTTGAGTTTTTATA
GATTATTAGATAATCCAAAAGTTTTAGAAGATTTGTTAGAAGAACAAAATCAAGTT
TTAGAAGATGCAGGATATGATTCTAGTGTTGGACCTGAAGTTTTTACTAGGGAAAT
TTTGAATAAGTTTGTAAAAATGGATTCTGTAATTAGAGAAACTAGTAGATTGAGAA
ATGATTTTATAGGTTTACCACATAAAAATATTTCATCTAAAACTATAACTTTATCAG
GTGGTGCAATGATTAGACCAGGTGAAAGAGCTTATGTTAATGCTTATTCTAATCAT
AGAGATGGTACAATTCAAAAAGTAACAGATAATCTTAAATCTTTTGAACCTTATAG
ATTTGTTAATCAAGATAGAAATAGTACTAAAATTGGTGAAGATTTTATATTTTTCGG
TATGGGTAAACATGCTTGTCCAGGTAGATGGTTTGCTATTCAAGAAATTAAGACAA

TAATTGCAATGATGATTAGATCTTATCAATTGTCTGCTTTAGGTCCAGTTACTTTTC

CAACTGATGATTATTCTAGAATTCCAATGGGTAGATTCAAAATTGTTCCAAGAAAA

TAA

Table S8. Codon optimized CPR gene sequence.

Sequence (5′-3′)
ATGGATTTGCCAACAGCTACTGATATTAATGAAAAACCAAAATTATCTAAAGAAGA
ACAAGATCCTAGAAATTTTGTAAAATTAATGAATGATCAAAATAGAAATGAATTGA
TAATATTTTATGGTAGTCAAACTGGTACTGGTGAAGATTATGCACAAAGATTAGGT
AAAGAATGTAAAAAACGTTTTAATATTCAACCAATGGTAGCTGATCTAGAAAATTA
TGATTTGGGTTACTTAGATACTTTGCCTAAAGAAACAATTGCAGTTTTTGTTATTAG
CACCTATGGAGAAGGAGATCCAACTGATTCTGCAGTTAATTTTTGGGAATTGTTAA
ATAAGGATGTTCCAACATTTTCTAAAGGTTGTGCTGTTGAAAGACCTTTGAAAGAT
TTGAGATATTTCGTTTTTGGTTTAGGTAATAGAACATATGAATATTTTAATGGTGCA
GCAATTGGTGTTGATAAACAATTAACTCAATTAGGAGCTACAAGATTAGGTGAAGT
TGGTATGGGAGATGATGATAATTCTCTTGAAGATGATTTTATTCAATGGCAAGATC
AAGTTTGGCCATTGTTAGCTGATGCTTTAGCTACTTCAACTGATACTGTTGATGAAC
AAGCTCAAGCTCAACATGCTTATAAAGTTATGATGGGTCAAGAAAAGGAAGATGA
ATCTTTCTATTATATGGGTGAATTAGGAGATACACAATTAACAACATGGTCTGCTA
AGAGACCTTATCCAGCTCCTGTTAAAATTCATGACTTAACTCCAGCTTCTAGAGATC
AAAGACATTGTTTACATTTGGATGTTGATTTGTCTAATTCTAATATAAGTTATACTA
CTGGAGATCATTTGGGGATATGGCCAACTAATAATGAAGATGAAGTTTTCTTAGTC
TCTTCTTTATTTGGTTGGAATGATGCTTATTTGGATCAAGTTATCAATGTAGTTCCA
ACTGATTCAACTAACAAACCACCATTTCCTCAACCAACTACATTAAGATCAGCTTT
AAGACACTATCTTGATATAGCACAATTGCCATCTAGATCTACTTTAGACTTGCTTTT
ACCTTCTTGCTCAAATGATAGTTTAAAATCATTTTTACAAAATTTGGTTAATGATAA
GGATGAACATAAAAGAGTAGTTTTAGATCAAGTAAGAAACTTAGGTCAATTATTAT
CTTTCGCATTAGAAACTATTGGTTCTACTACTACTGATGGTGCTTTGAAAGATATTC
CAGTAGAAGTTGTTTTAGAATGTTATAGCAGATTGCAACCTAGATATTATTCTATTT
CATCAAGTAGTAGTGAAAGTGCTACTACTGTATCTGCAACAGCTGTTACACTTAAG
TATAACCCAACTCCTGATAGAACTGTTTATGGTGTTAACACTAATTACCTATGGGCA
ATTCATCAATCTATGTCATCAACACCATCATCTGATGTTCCAAAATATGTTGTTGAT

GGACCAAGACAACAATACTTAATTACAAAGGAAGCAAATTCTGATTCTATAAAAAT

TAAGATTCCAGTTCATATTAGAAAATCCACTTTTAGATTACCTCCATCAAGTTCTAC

ACCTGTTATTATGGTTGGTCCTGGTACTGGTGTAGCTCCATTTCGTGGTTTTGTTAG

AGAAAGAGTGTATCAAAAACAAGTTTTAGGTGAAGATGTTGGTGCTACTGTTTTAT

TTTTCGGTTGTAGAAGATCTACTGAAGATTATCTTTATGCTGATGAATGGCCTAGAT

TGTTTAAATCCTTAGGTAATGGTCCATCTAGAATTATTACTGCTTTCTCAAGAGAAT

CTGAAGAGAAAAAAGTTTATGTTCAACAAAGATTGGCTGAACATGGTCAAGAAAT

GTGGGATTTGCTAGCTAATCAAGGTGCTTATTTTTATGTTTGTGGAGATGCTAAATA

TATGGCTAAAGATGTTCAACAAACTGTAATTGATATGGCTAAATCATTTGGTGGTTT

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Figures

Enzyme kinetic parameters analysis of P450s (FigS1-S6)

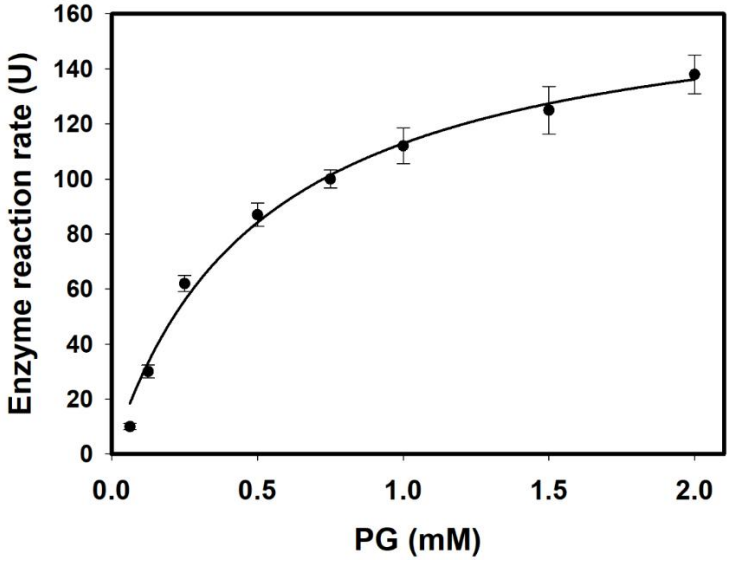

Figure S1. Kinetic analysis of VD16-BM3.

The value of V_{max} and K_m are shown in Table S8.

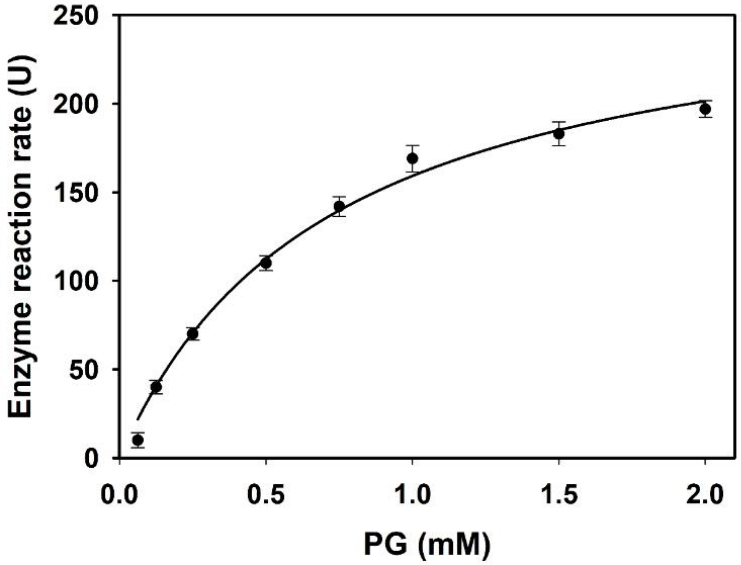

Figure S2. Kinetic analysis of VD17-BM3.

The value of V_{max} and K_m are shown in Table S8.

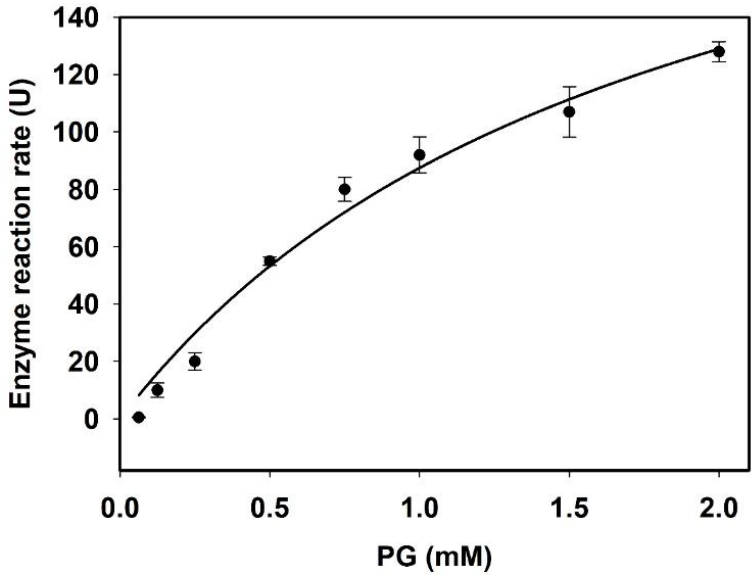

Figure S3. Kinetic analysis of VD21-BM3.

The value of V_{max} and K_m are shown in Table S8.

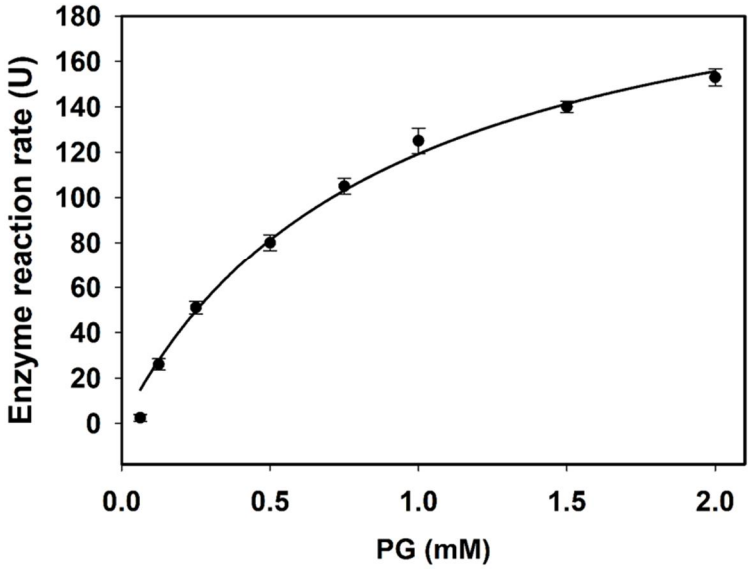

Figure S4. Kinetic analysis of VD1721-BM3.

The value of V_{max} and K_m are shown in Table S8.

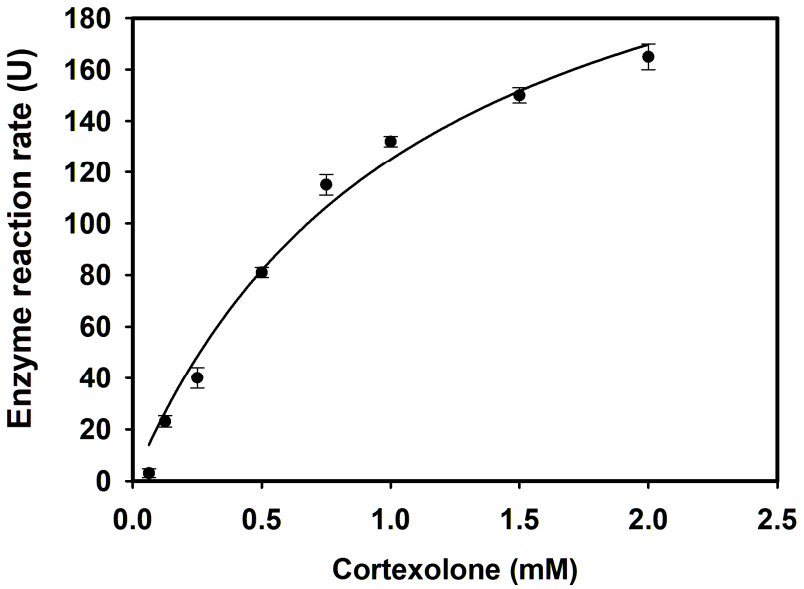

Figure S5. Kinetic analysis of FA11a-BM3.

The value of V_{max} and K_m are shown in Table S8.

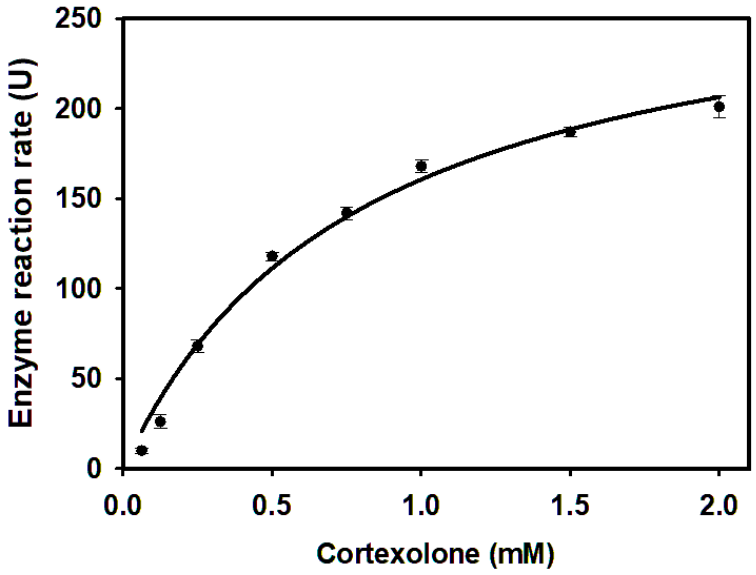

Figure S6. Kinetic analysis of *Ao*CYP5311B2-*Ao*CPR.

The value of V_{max} and K_m are shown in Table S8.

Mechanism analysis for CYP5311B2 and FA11a-BM3 (S7)

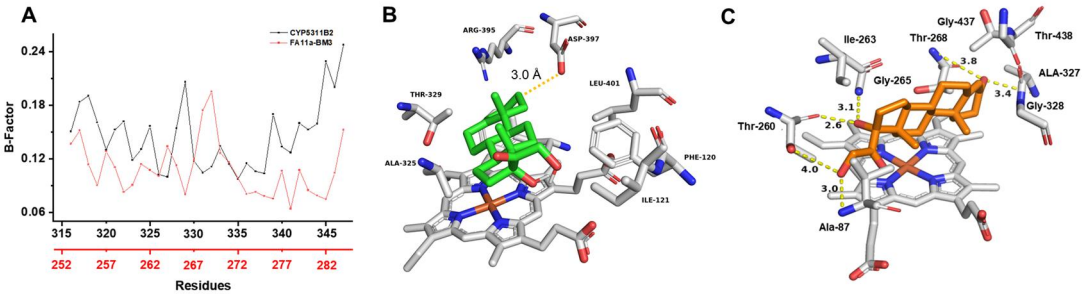

Figure S7 (A) B-Factor analysis for the heme α-helix of FA11a-BM3 and CYP5311B2 Residues from 315 to 345 were the α-helix near heme center of CYP5311B2. Residues from 252 to 282 were the α-helix near heme center of FA11a-BM3. (B) Docking results of CYP5311B2 and **c**. (C) Docking results of FA11a-BM3 and **c**.

Protein characterization (FigS8-S14)

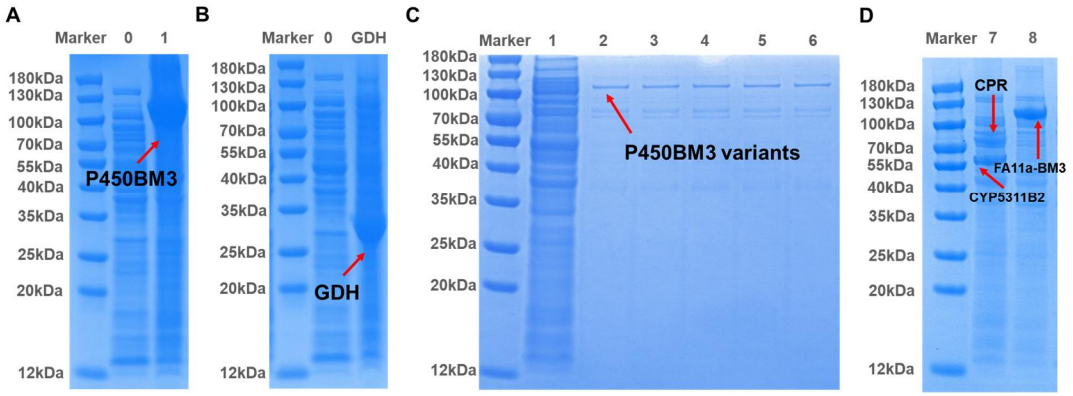

Figure S8. SDS PAGE of P450BM3s (117 kDa), GDH (31 kDa), *Ao*CYP5311B2 (60 kDa), and *Ao*CPR (76 kDa).

0: Extraction of *E. coli* BL21(DE3)-pET28a-1. GDH: Extraction of *E. coli* BL21(DE3)-pETDuet-*gdh*. 1: Extraction of *E. coli* BL21(DE3)-pET28a-P450BM3. C-1: protein samples were diluted to 10 mg/mL. 2: Purified VD16-BM3. 3: purified VD17-BM3. 4: purified VD21-BM3. 5: purified VD1721-BM3. 6: purified FA11a-BM3. 7: Extraction of *E. coli* BL21(DE3)- pETDuet-CYP5311B2-CPR. 8: Extraction of *E. coli* BL21(DE3)-pETDuet-FA11a-BM3.

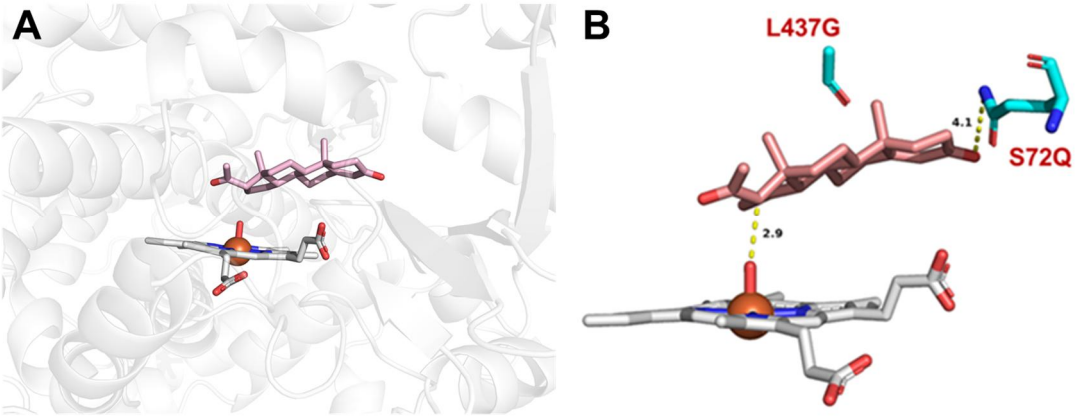

Figure S9. PG posed in VD17-BM3 variants.

(A) Overall structure of PG docked into the VDBM3-S72QL437G (**VD17-BM3**) for 17α-(OH)-PG production. (B) Q72 and G437 of **VD17-BM3** locate around PG. H-bond

was formed by PG and Q72 in 4.1Å. The distance between C-17 of PG and Heme's Fe=O is 2.9Å.

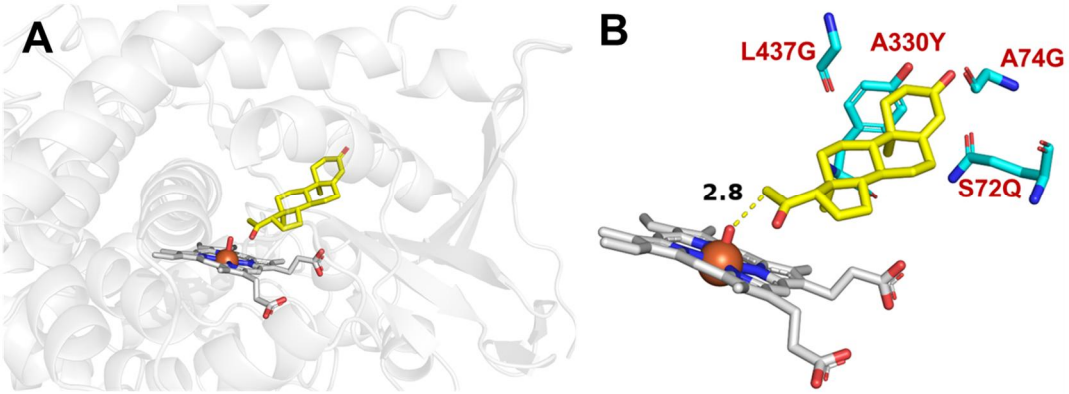

Figure S10. PG posed in VD21-BM3 variants.

(A) Overall structure of PG docked into the VDBM3-S72Q/A74G/L437G/A330Y (**VD21-BM3**) for 21α-(OH)-PG production. (B) S72Q, A74G, L437G, and A330Y of **VD21-BM3** locate around PG. The distance between C-21 of PG and Heme's Fe=O is 2.8Å.

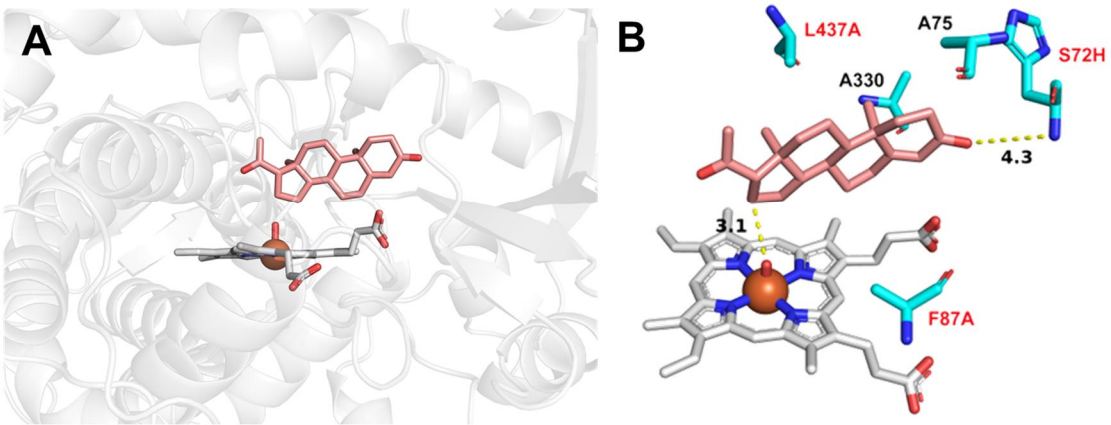

Figure S11. PG posed in VD16-BM3 variants.

(A) Overall structure of PG docked into the VDBM3-S72H/L437A (**VD16-BM3**) for 16β-(OH)-PG production. (B) S72H and L437A of **VD16-BM3** locate around PG. The distance between C-16 of PG and Heme's Fe=O is 3.1Å.

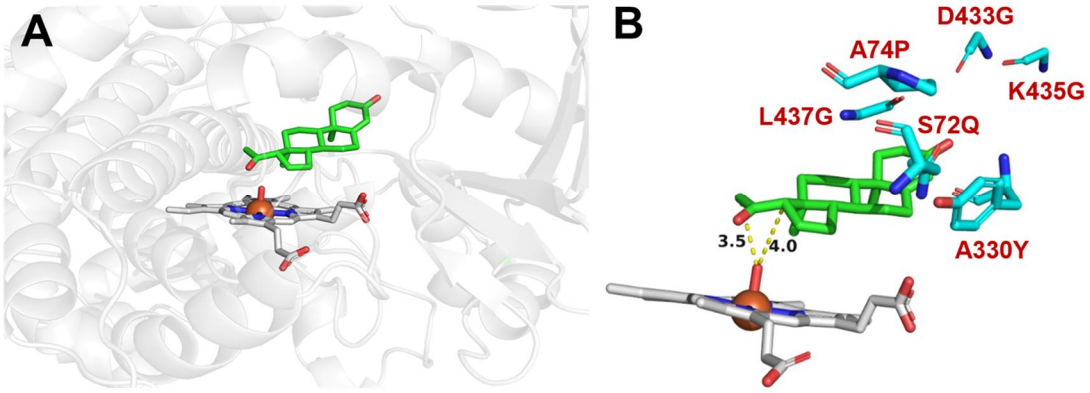

Figure S12. PG posed in VD1721-BM3 variants.

(A) Overall structure of PG docked into the VDBM3-S72Q/A74G/L437G/A330Y/K435G/L433G (**VD1721-BM3**) for 17,21α-(2OH)-PG production. (B) S72Q, A74G, L437G, A330, K435G, and L433G of **VD21-BM3** locate around PG. The distances between C-17/C-21 of PG and Heme's Fe=O are 4.0/3.5Å.

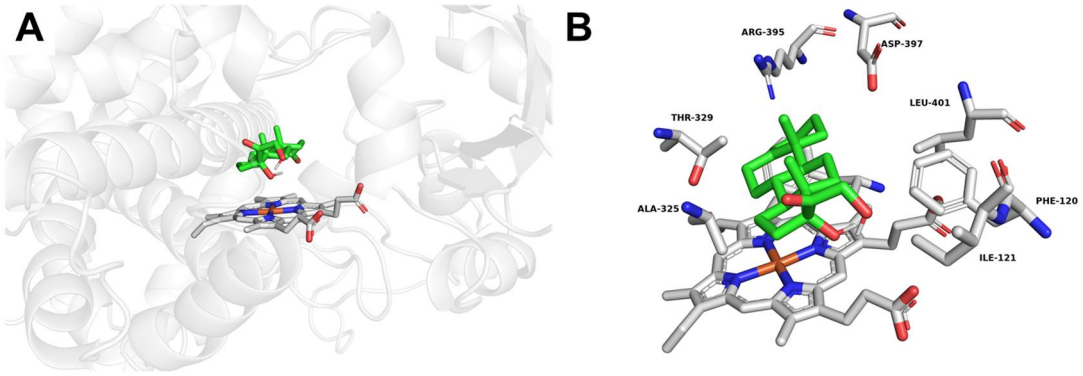

Figure S13. 17,21α-(2OH)-PG posed in CYP5311B2 variants.

(A) Overall structure of 17,21α-(2OH)-PG docked into the **CYP5311B2** for hydrocortisone production. (B) Residues of CYP5311B2 around 17,21α-(2OH)-PG in 4 Å.

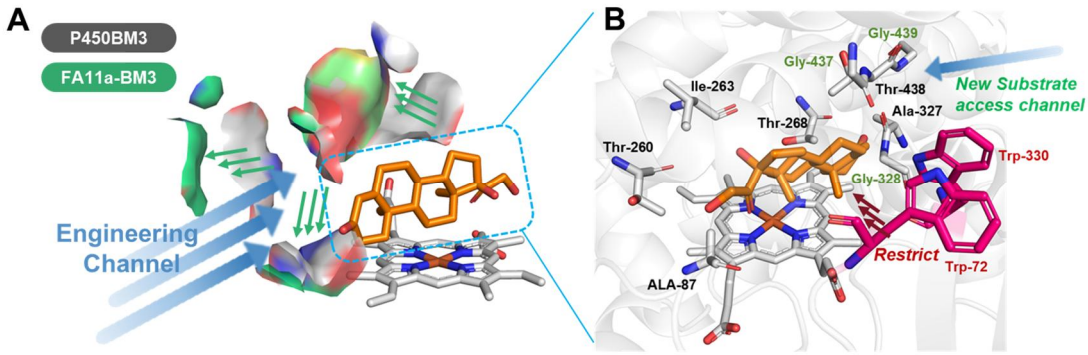

Figure S14. 17,21α-(2OH)-PG posed in FA11a-BM3 variants.

(A) Display of substrate channel surfaces for P450BM3 (grey rendering) and FA11a-BM3 (green rendering). (B) Details for c docked into FA11a-BM3 heme pocket.

HPLC analysis for PG hydroxylation (FigS15-S21)

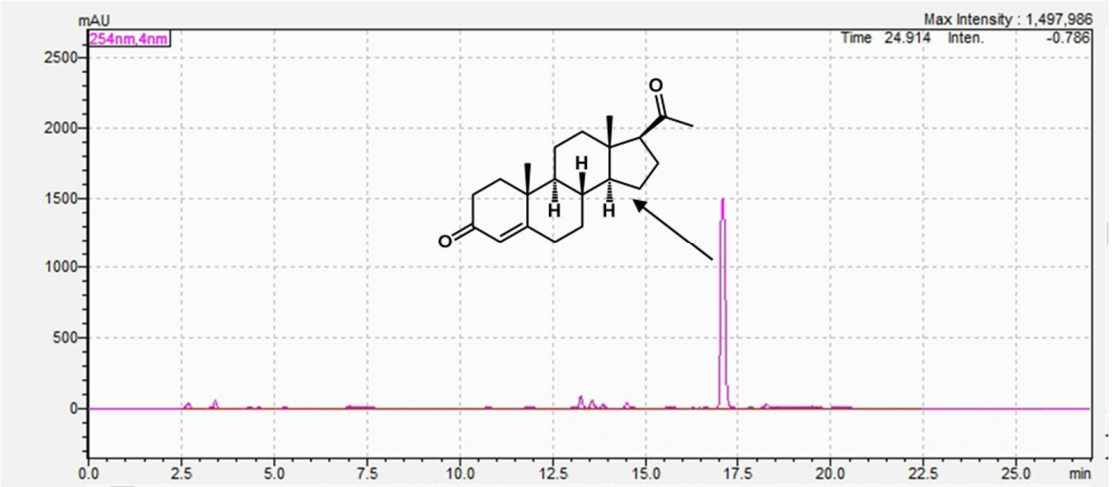

Figure S15. HPLC analysis for PG. The retention time of PG was 17.4min.

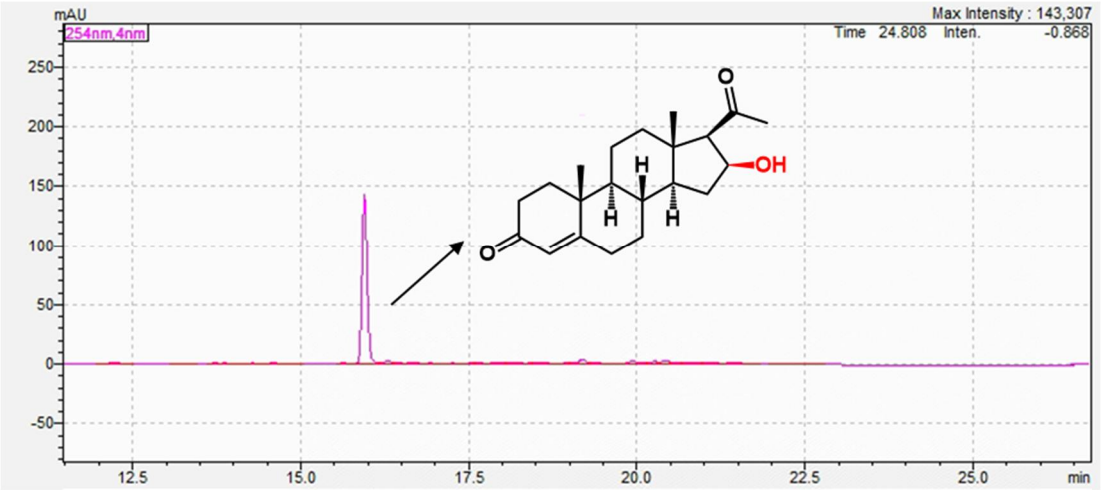

Figure S16. HPLC analysis for 16β-(OH)-PG. The retention time of 16β-(OH)-PG was 15.2min.

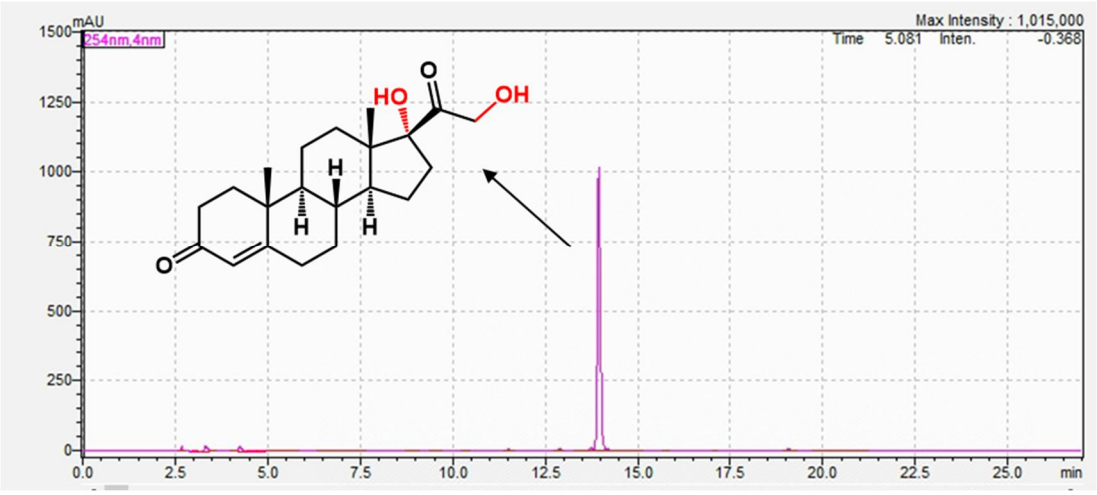

Figure S17. HPLC analysis for 17α,21-(OH)-PG. The retention time of 17α,21-(OH)-PG was 14.1min. The retention time of 16β-(OH)-PG was 15.2min.

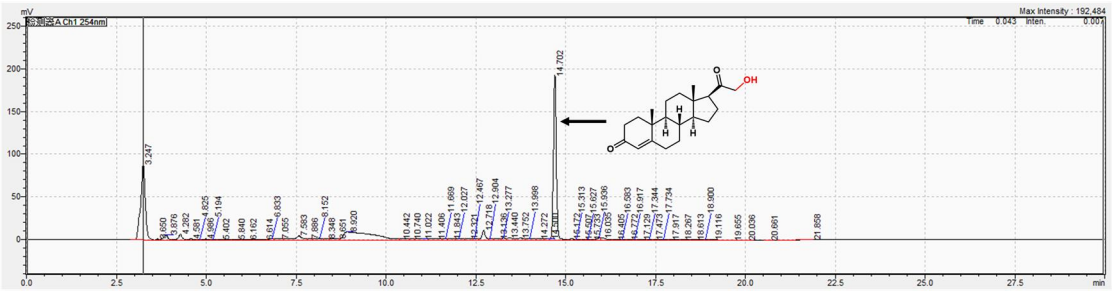

Figure S18. HPLC analysis for 21-OH-PG bioproduction. The retention time of 21-(OH)-PG was 14.7min.

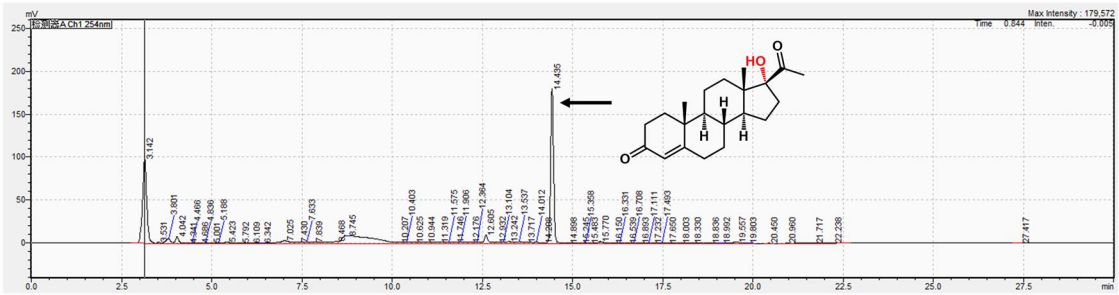

Figure S19. HPLC analysis for 17α-(OH)-PG bioproduction. The retention time of hydrocortisone was 14.5min.

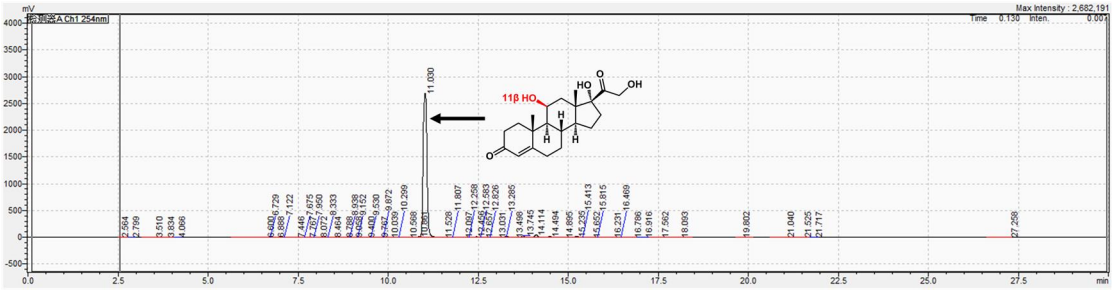

Figure S20. HPLC analysis for hydrocortisone bioproduction. The retention time of hydrocortisone was 11.03min.

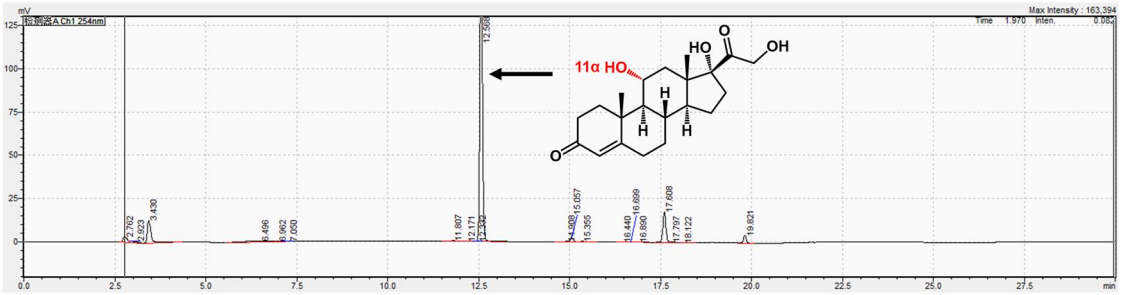

Figure S21. HPLC analysis for 11α-hydrocortisone bioproduction. The retention time of 11α-hydrocortisone was 12.5min.

GC-MS analysis for substrates and products (FigS22-S27)

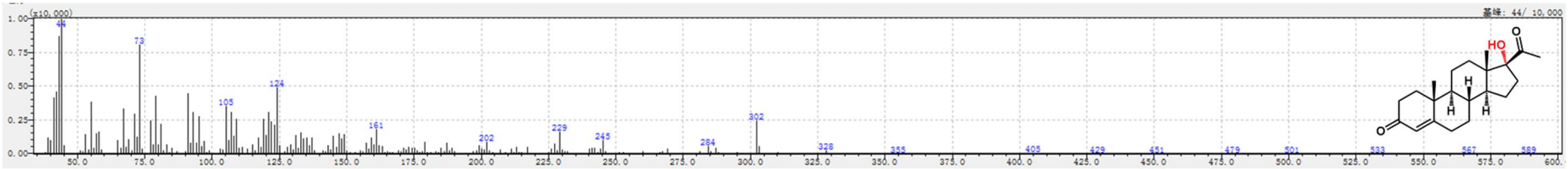

Figure S22. GCMS analysis for 17α-(OH)-PG.

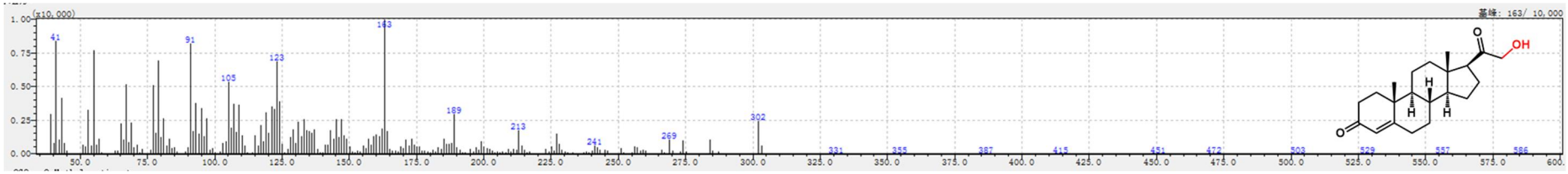

Figure S23. GCMS analysis for 21-(OH)-PG.

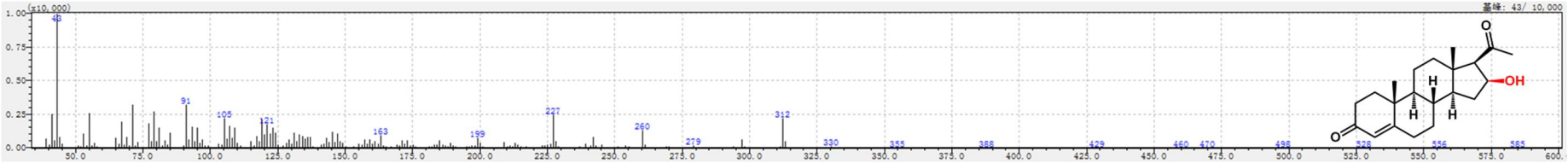

Figure S24. GCMS analysis for 16β-(OH)-PG.

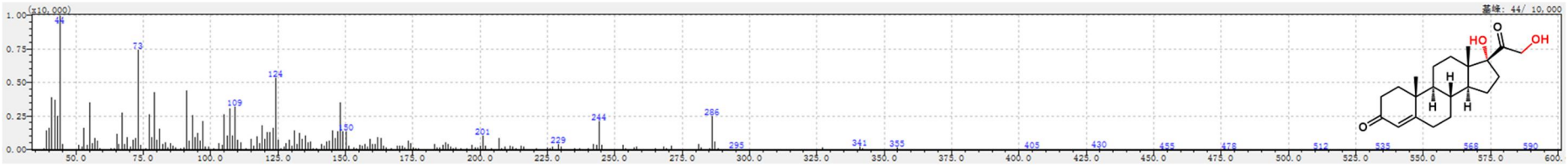

Figure S25. GCMS analysis for 17α,21-(2OH)-PG.

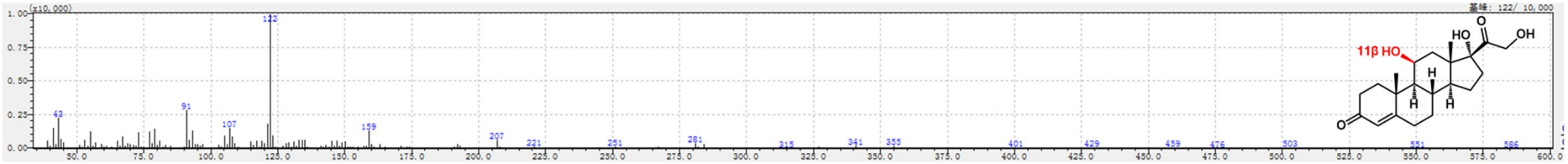

Figure S26. GC-MS analysis for hydrocortisone.

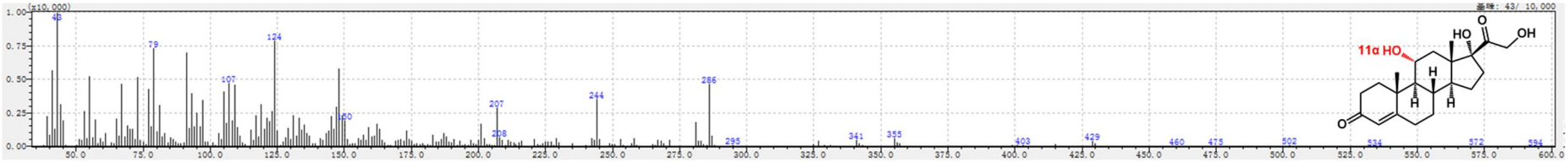

Figure S27. GCMS analysis for 11α-hydrocortisone.

NMR analysis (FigS28-S39)

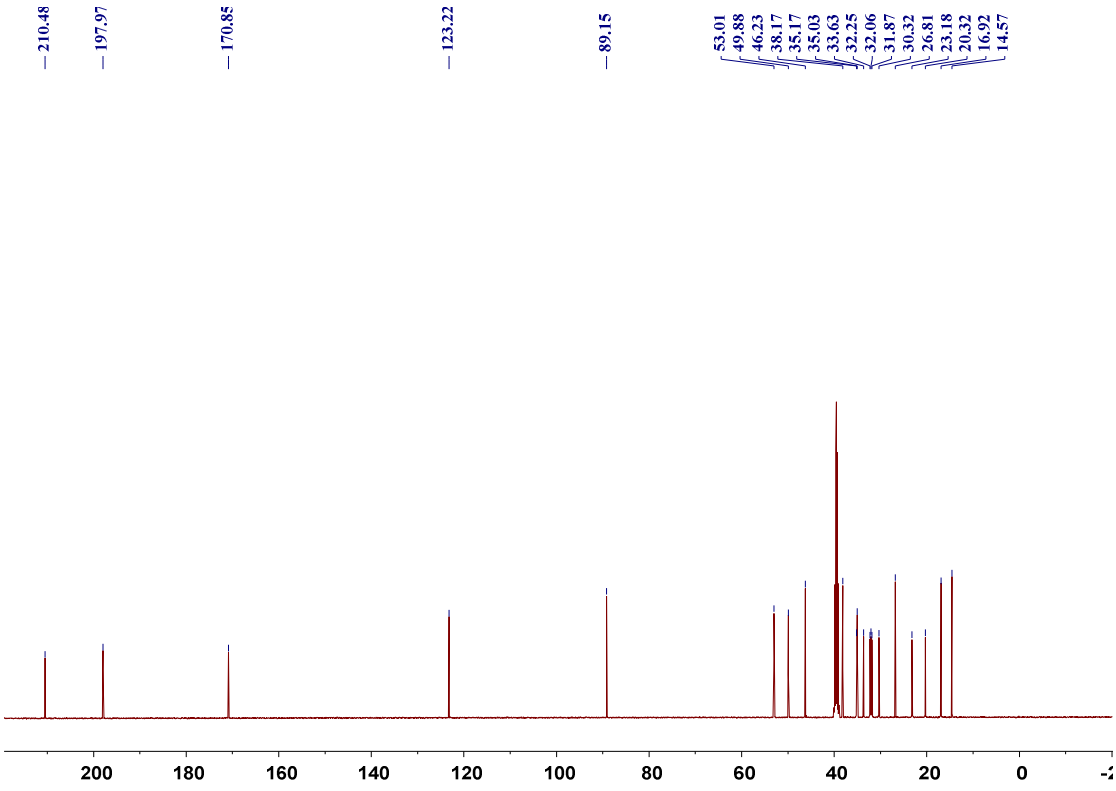

Figure S28. ^{13}C NMR (151 MHz, $CDCl_3$) for 17α-hydroxy-PG.

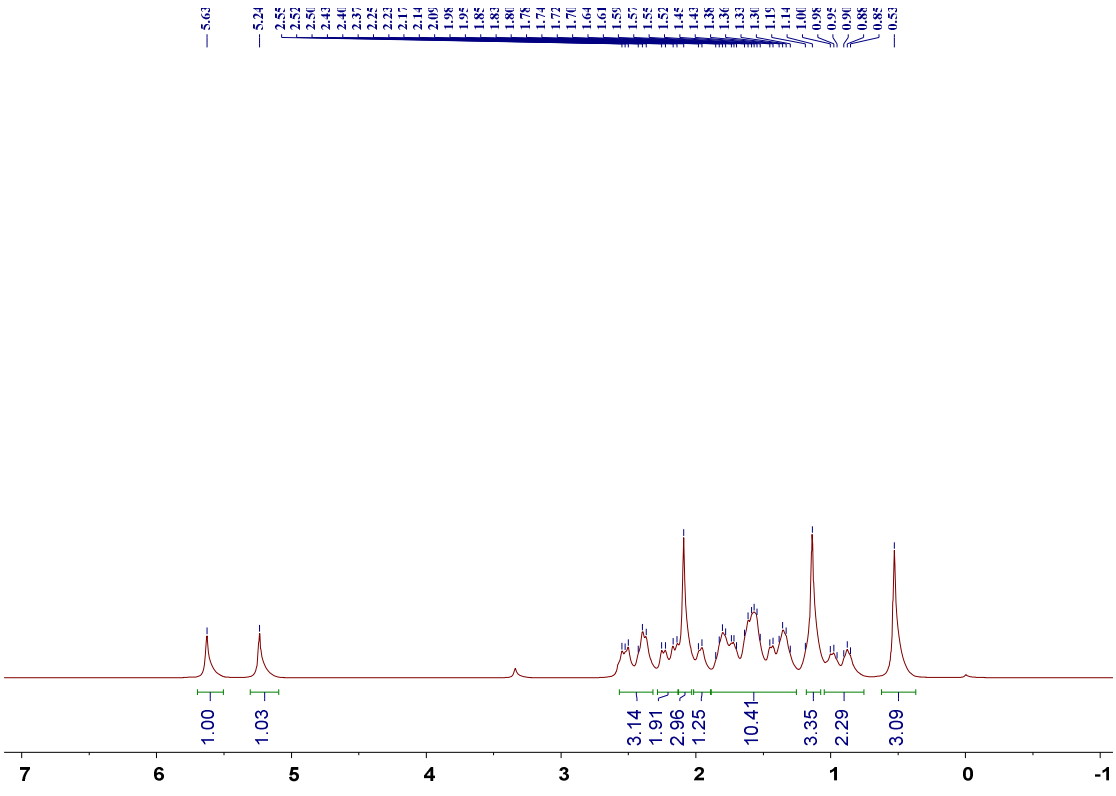

Figure S29. ^{1}H NMR (400 MHz, $CDCl_3$) for 17α-hydroxy-PG.

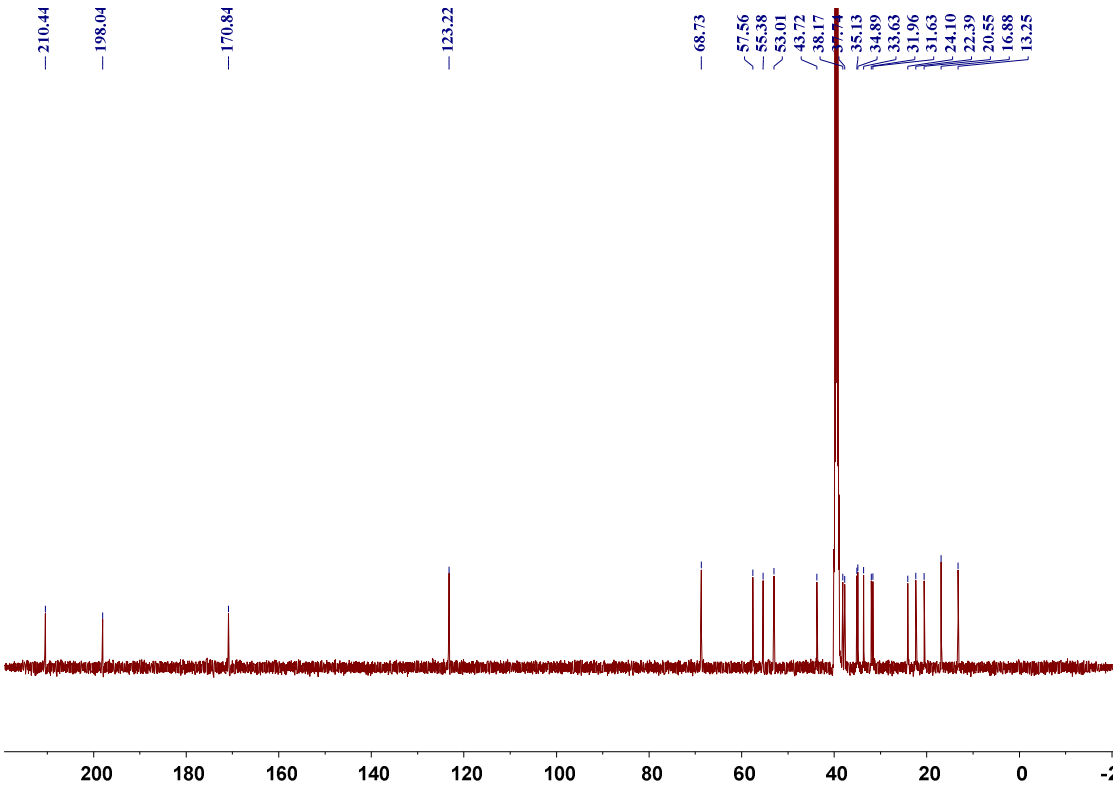

Figure S30. ^{13}C NMR (151 MHz, $CDCl_3$) for 21-hydroxy-PG.

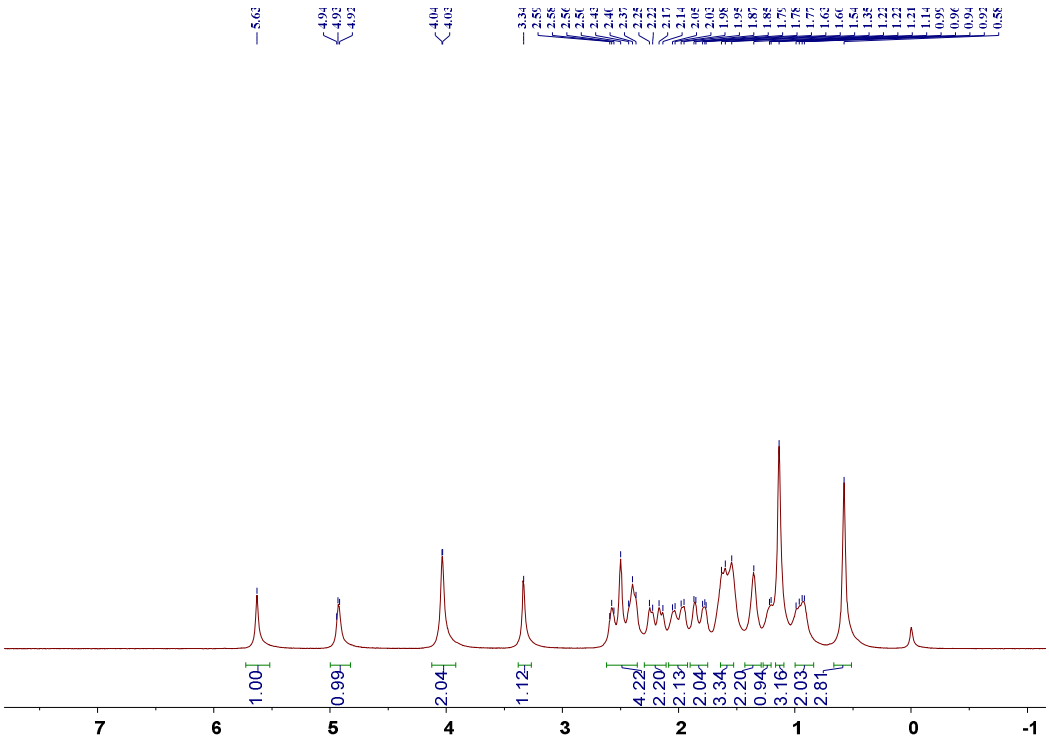

Figure S31. ^{1}H NMR (400 MHz, $CDCl_3$) for 21-hydroxy-PG.

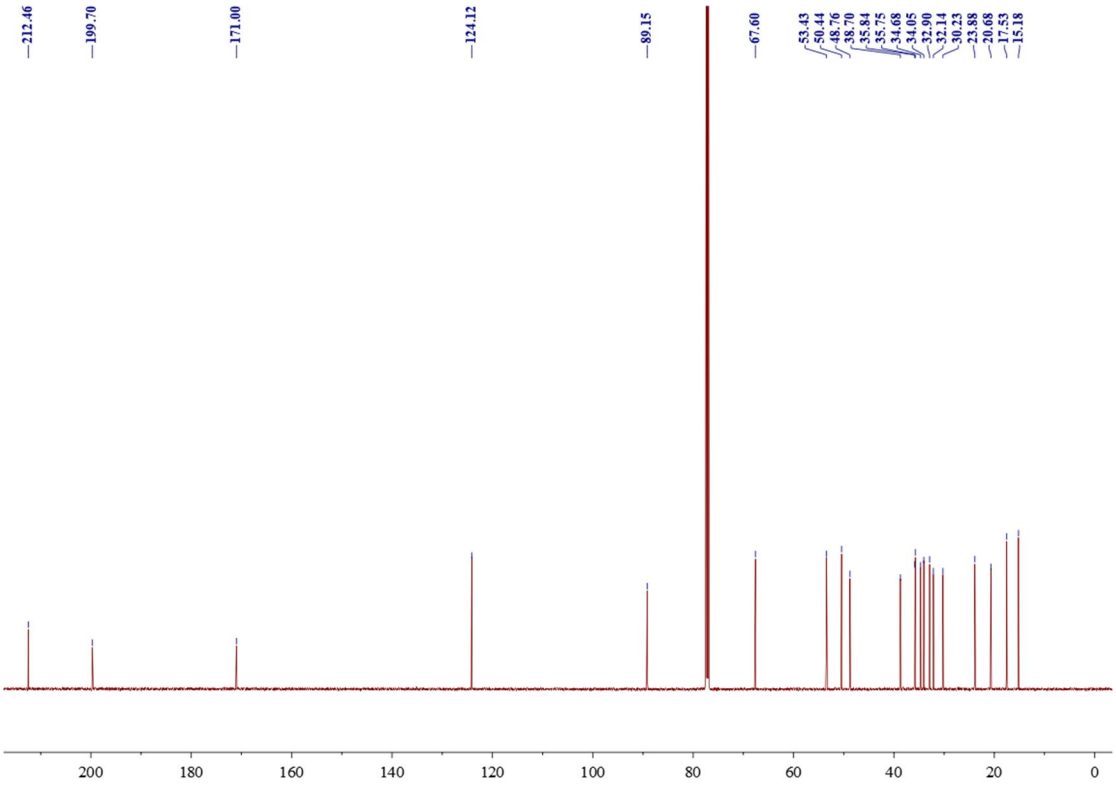

Figure S32. ^{13}C NMR (151 MHz, $CDCl_3$) for cortexolone.

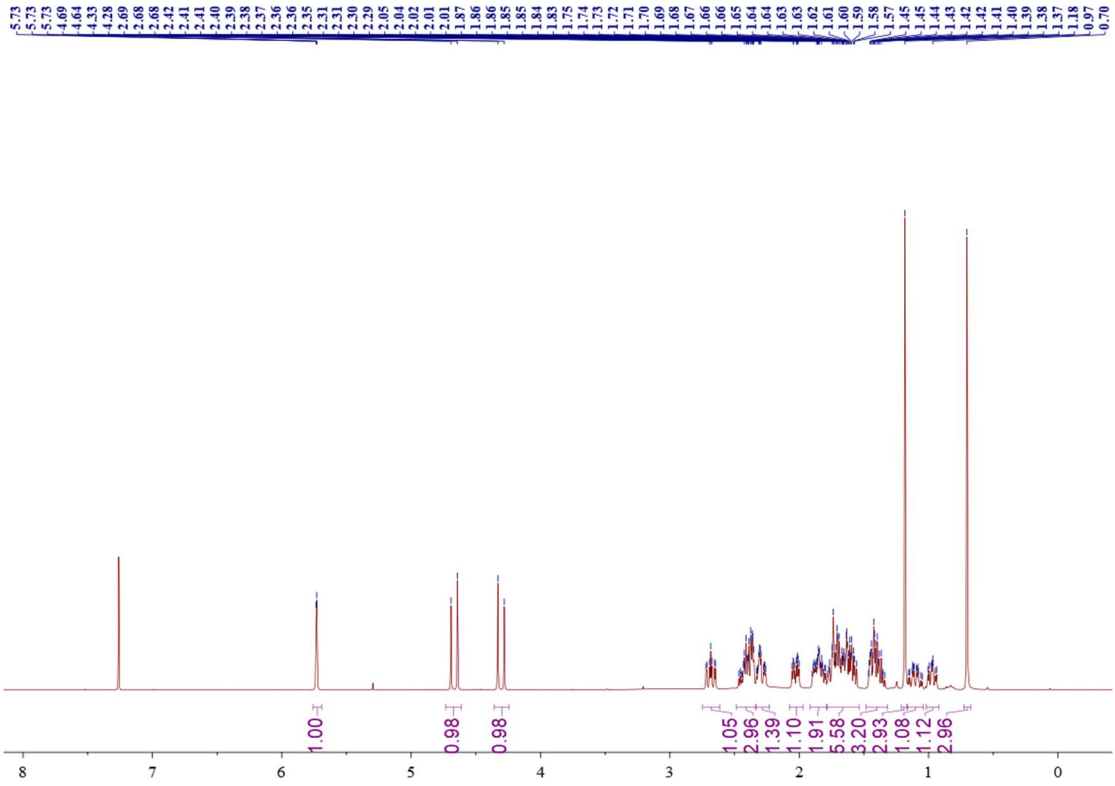

Figure S33. ^{1}H NMR (400 MHz, $CDCl_3$) for cortexolone.

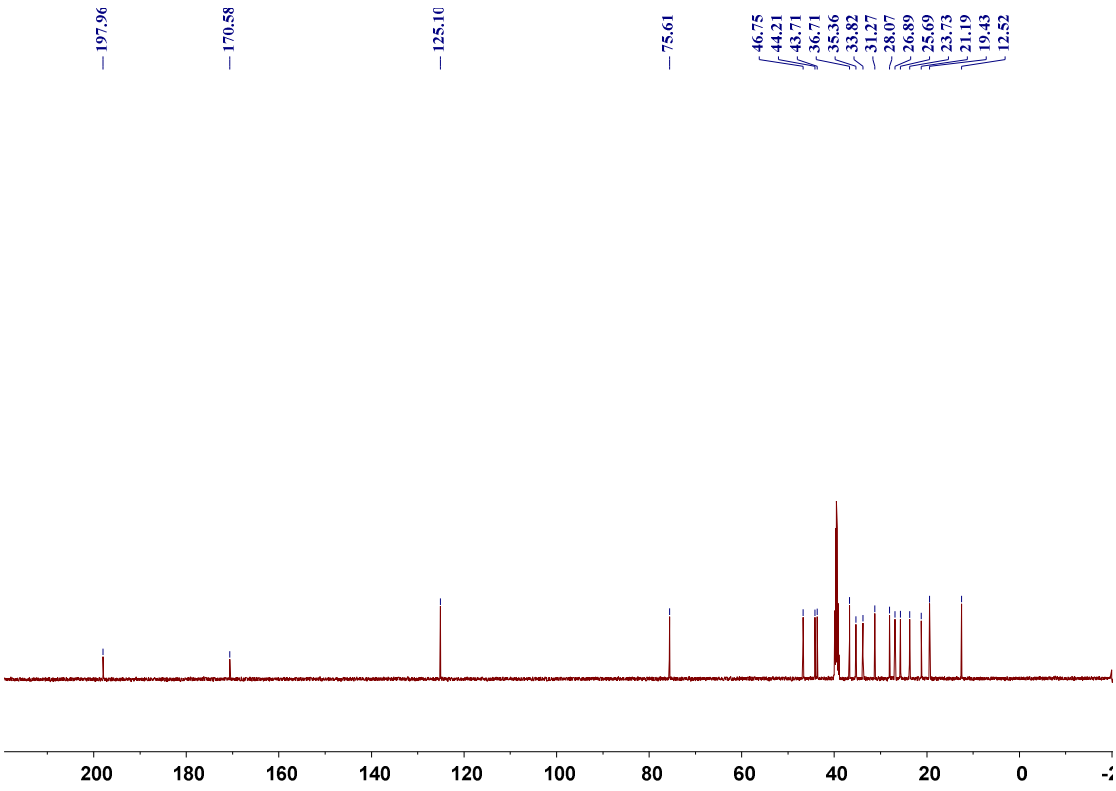

Figure S34. ^{13}C NMR (151 MHz, $CDCl_3$) for 16β-hydroxy-PG.

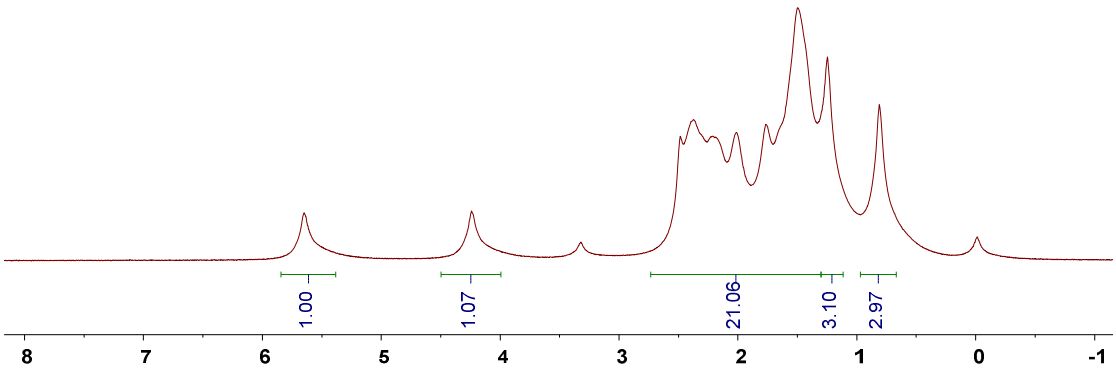

Figure S35. ^{1}H NMR (400 MHz, $CDCl_3$) for 16β-hydroxy-PG.

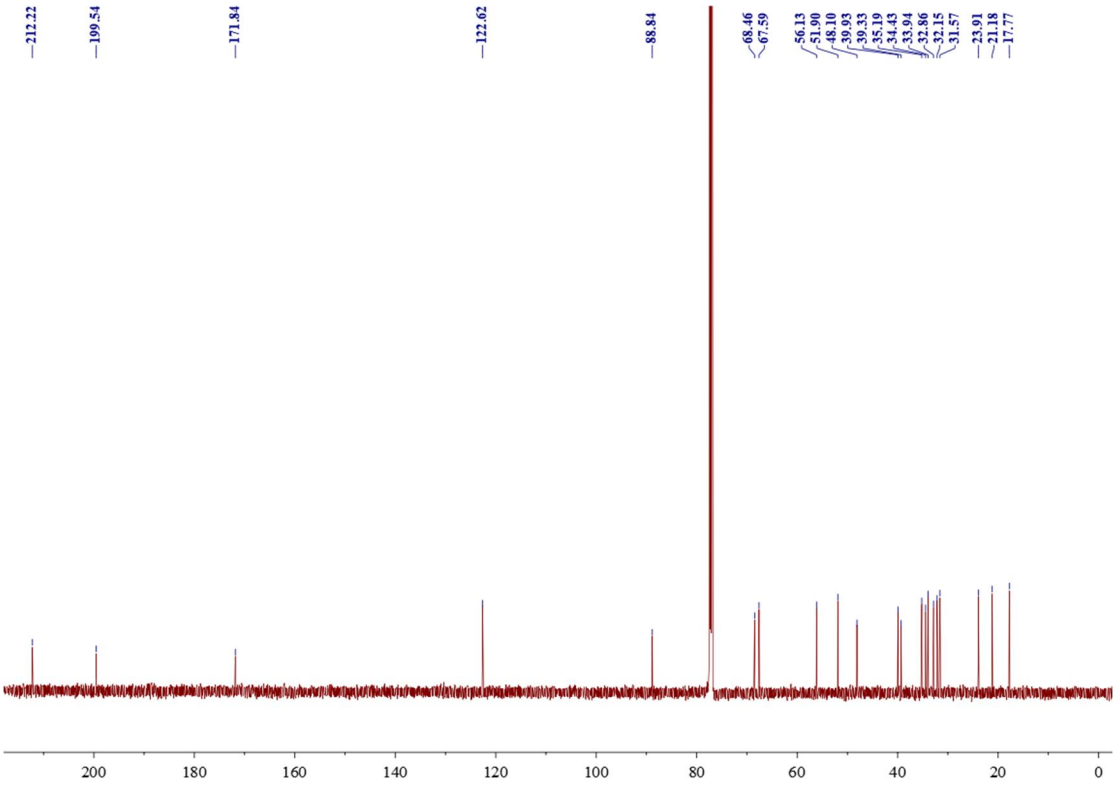

Figure S36. ^{13}C NMR (151 MHz, $CDCl_3$) for hydrocortisone.

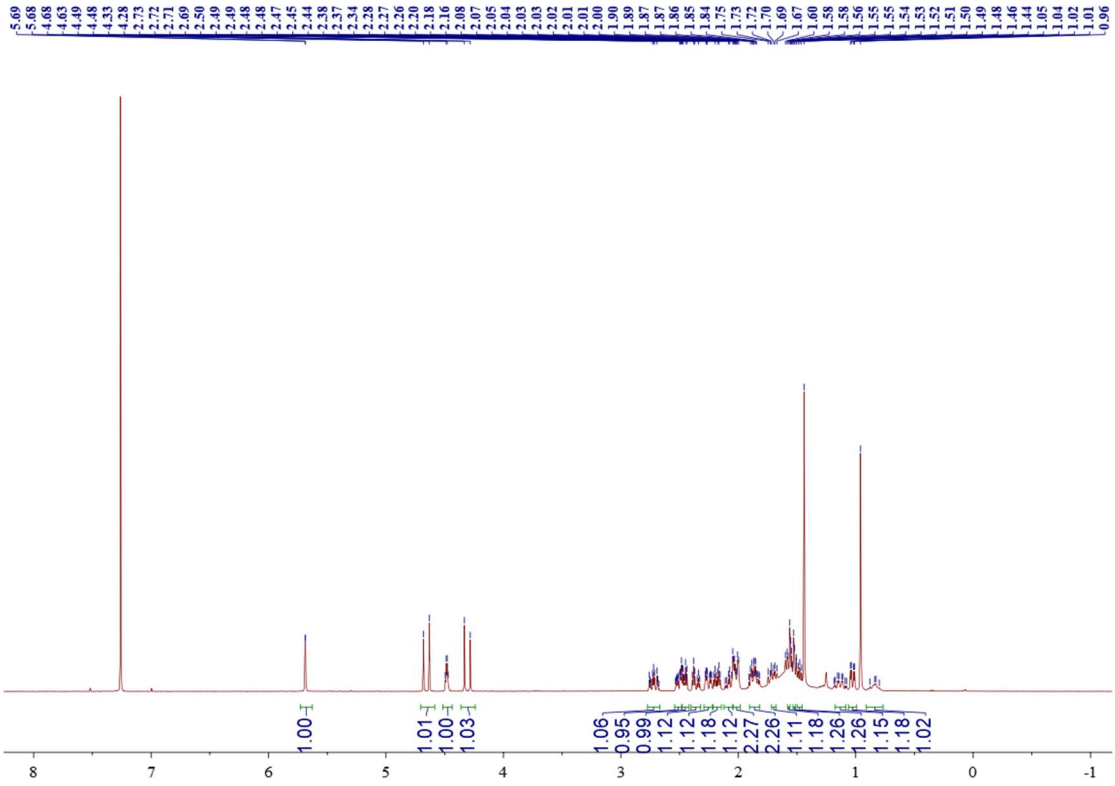

Figure S37. ^{1}H NMR (400 MHz, $CDCl_3$) for hydrocortisone.

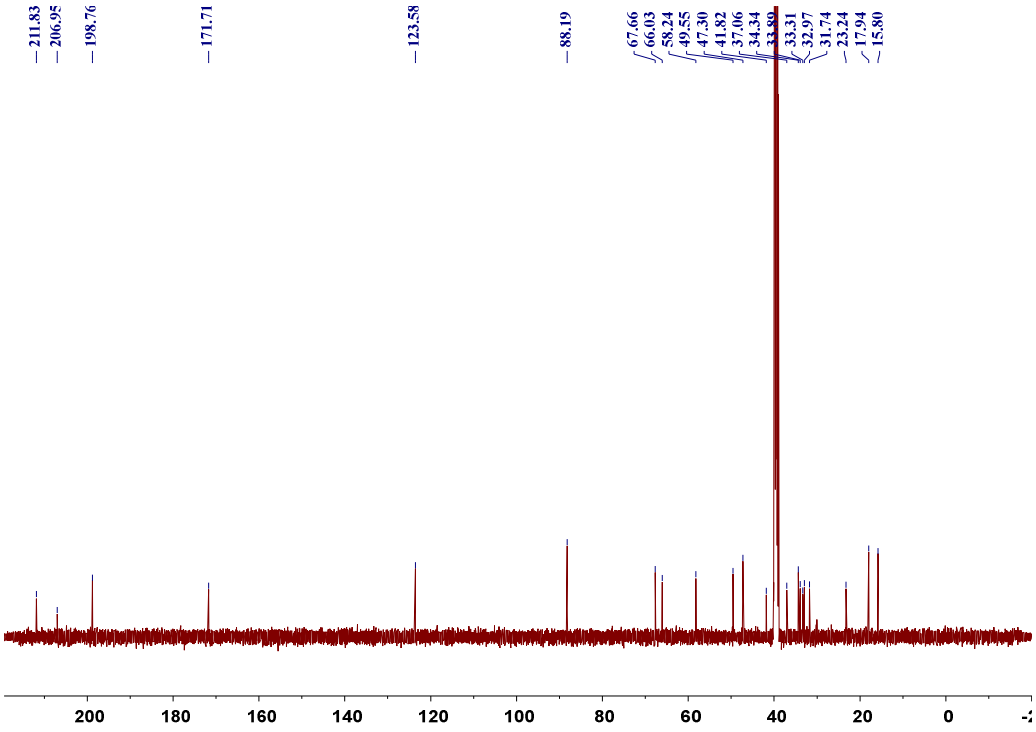

Figure S38. ^{13}C NMR (151 MHz, $CDCl_3$) for 11α-hydrocortisone.

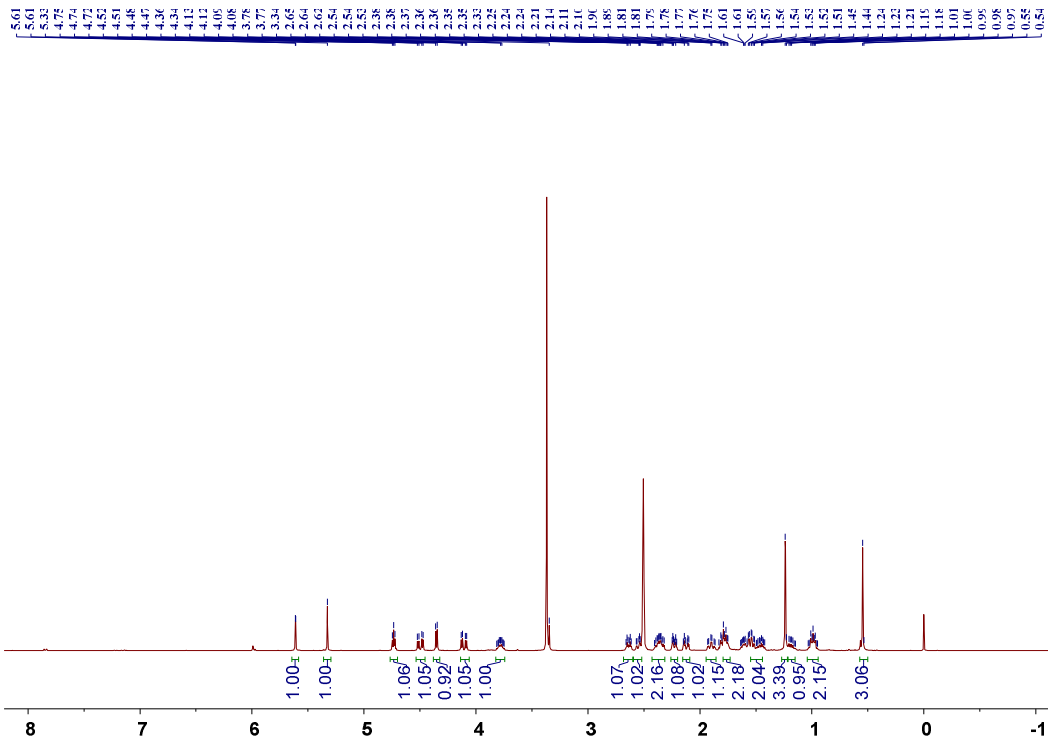

Figure S39. ^{1}H NMR (400 MHz, $CDCl_3$) for 11α-hydrocortisone.

MD simulations for P450s and substrate complexes in 100 ns (FigS40-43)

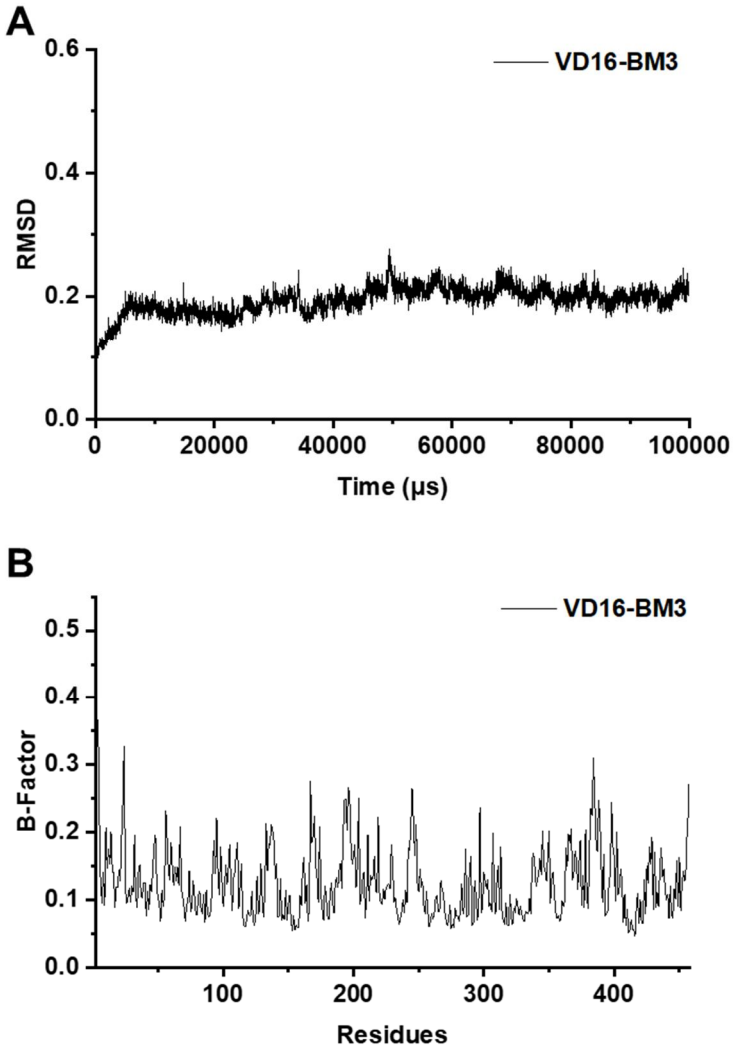

Figure S40 RMSD and B-Factor analysis for VD16-BM3.

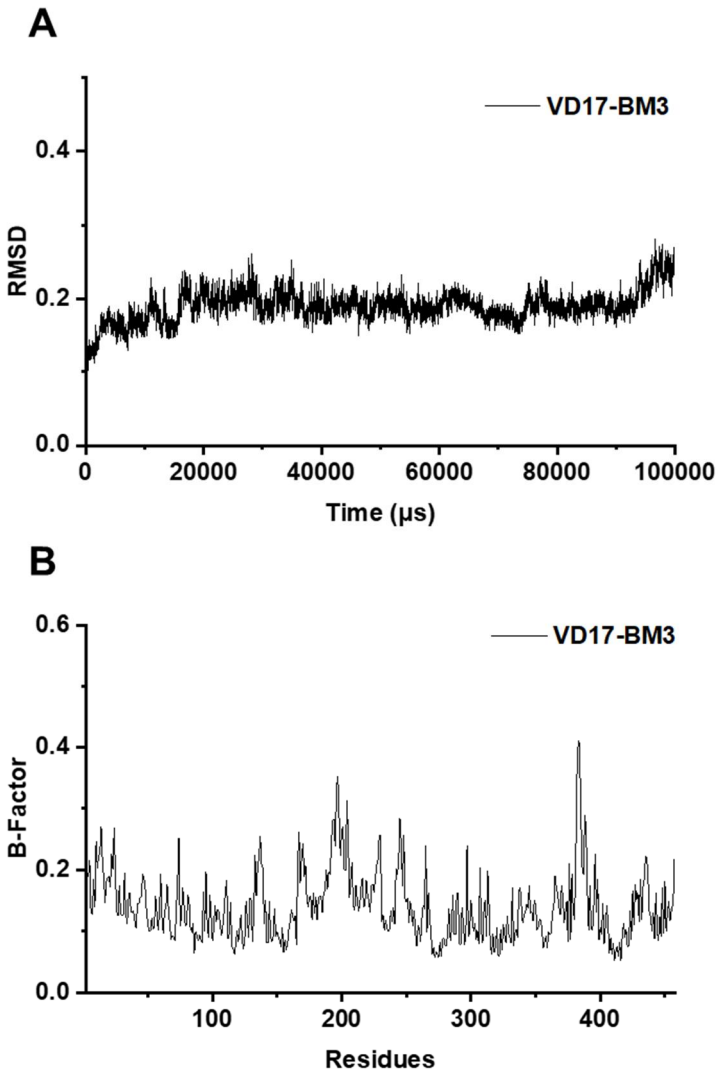

Figure S41 RMSD and B-Factor analysis for VD17-BM3.

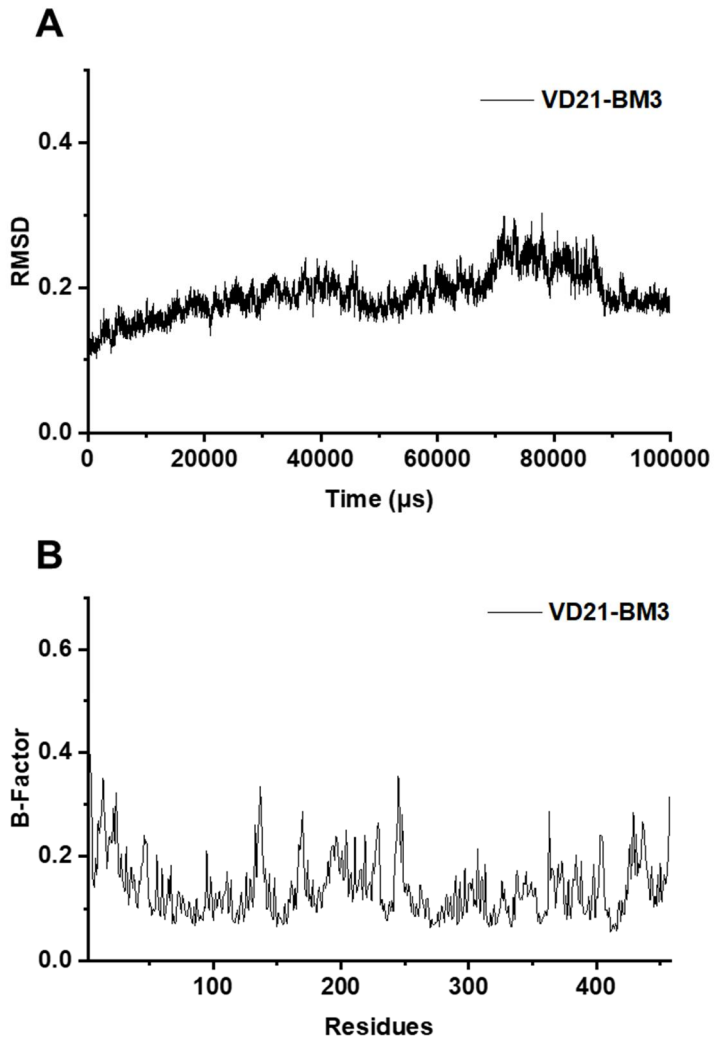

Figure S42 RMSD and B-Factor analysis for VD21-BM3.

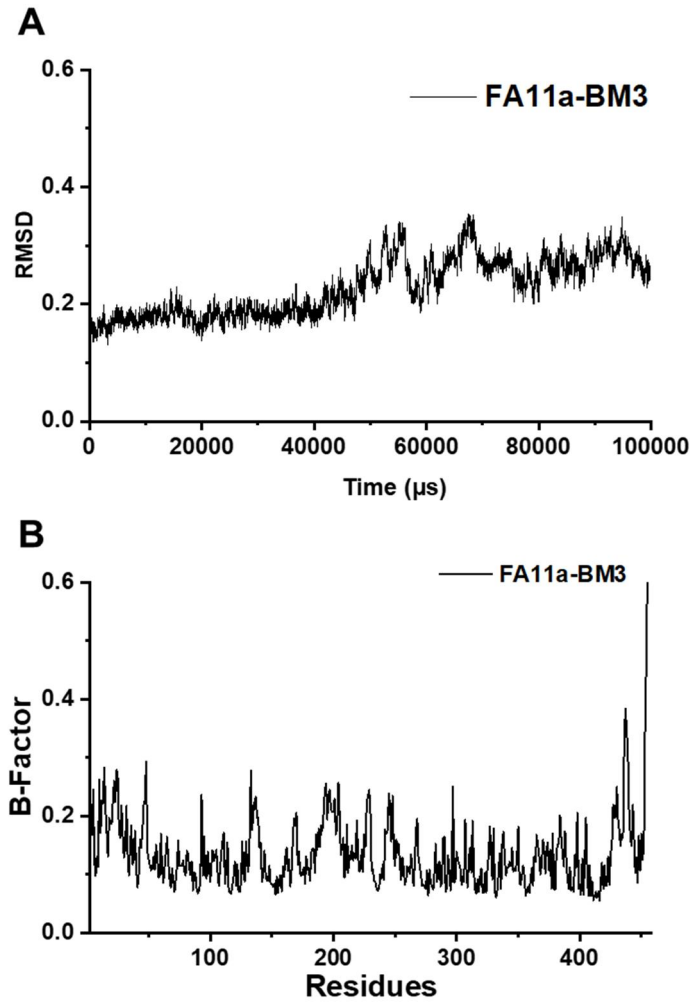

Figure S43 RMSD and B-Factor analysis for FA11a-BM3.

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