Supporting Information

Why Human Cytochrome P450c21 is a Progesterone 21-Hydroxylase

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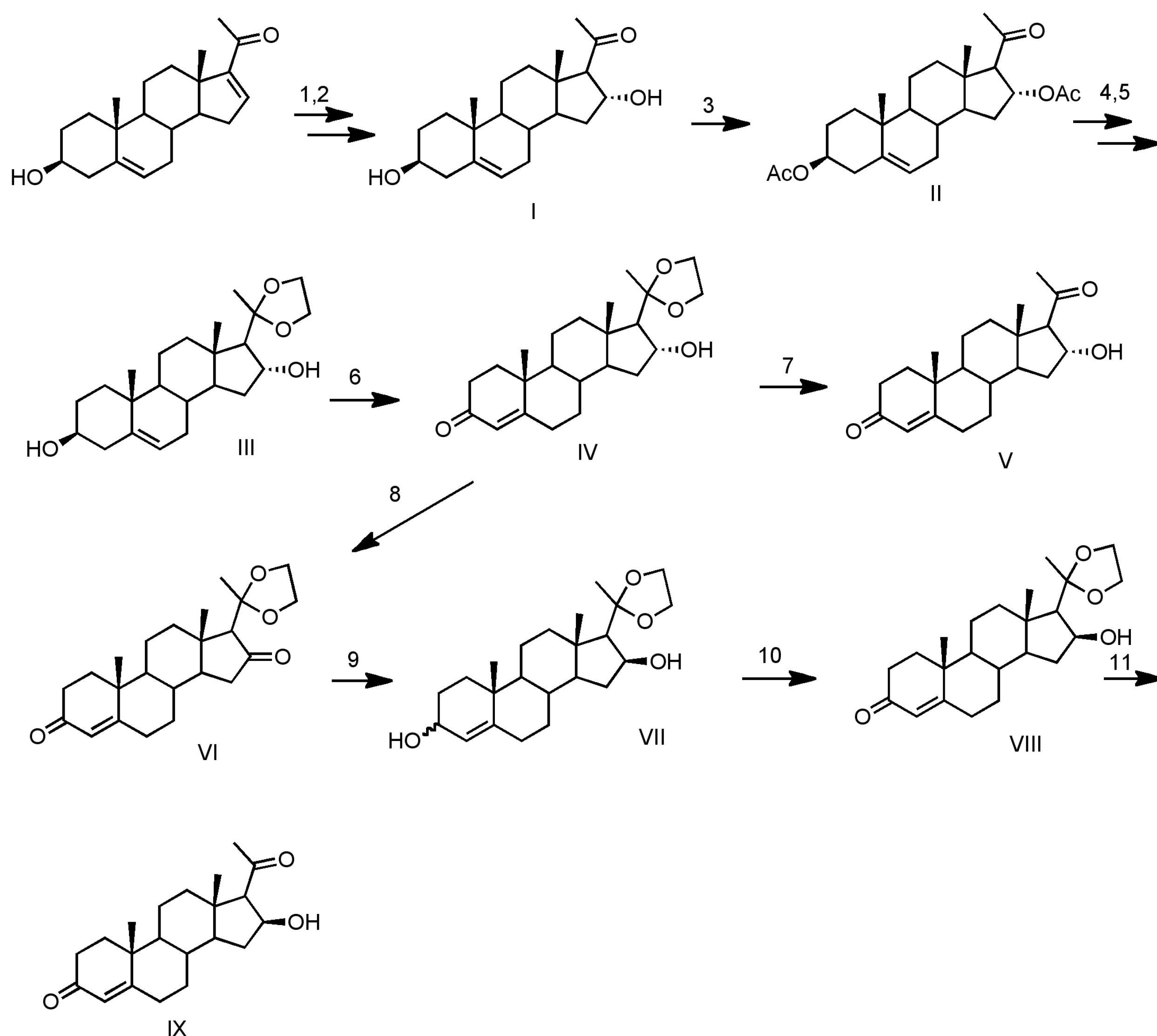
Chemical Syntheses:

Preparation of Pregn-4-ene-16α,17α-diol-3,20-dione. The diol was prepared by refluxing pregn-4-ene-16α,17α-diol-3,20-dione acetonide (6 mg, Steraloids) with 2 mg p-toluenesulfonic acid in methanol (4 mL) for 12 h, followed by concentration under reduced pressure. The product was extracted into ethyl acetate, which was washed with 10% $NaHCO_3$ solution and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The product was purified on a pipette column of silica gel using 60% ethyl acetate in hexanes to afford a white solid (4 mg, 74%), mp 210-212 C (lit. mp 215-217 C) (*1*).

Preparation of Pregn-4-ene-16β-ol-3,20-dione (16β-hydroxyprogesterone). Scheme I illustrates the synthetic strategy, yielding first 16α-hydroxyprogesterone, which is converted to the 16β-epimer via oxidation and stereoselective reduction with inversion of configuration:

Scheme I



Methods: 1) 35% H_2O_2/10% NaOH/MeOH; 2) NH_2NH_2-H_2O/HCl; 3) Ac_2O/pyridine/DMAP; 4) ethylene glycol/triethylorthoformate/TsOH/benzene 70C; 5) K_2CO_3/MeOH reflux; 6) N-methylpiperidone/Al(OiPr)$_3$; 7) 8% H_2SO_4/MeOH reflux; 8) Dess-Martin reagent/DCM; 9) $LiAlH_4$/Et_2O reflux; 10) same as 6; 11) same as 7.

Pregn-5-ene-3β,16α-diol-20-one (16α-hydroxypregnenolone, **I**)
Starting from 16-dehydropregnenolone (pregna-5,16-diene-3β-ol-20-one, 1 g, 3.20 mmol) and following the literature protocol (*2, 3*) via 16,17-epoxidation and reductive cleavage, the two-step conversion gave **I** (194 mg, 18% overall yield).

^{1}H NMR (400 MHz, $CDCl_3$): δ 0.63 (s, 3H), 0.94-1.16 (m, 4H), 1.37-1.89 (m, 18H), 1.91-2.07 (m, 2H), 2.13-2.34 (m, 4H), 2.51 (d, J=6.4Hz, 1H), 3.52-3.59 (m, 1H), 4.81-4.84 (m, 1H), 5.28-5.36 (m, 1H).

^{13}C NMR (500 MHz, $CDCl_3$): δ 14.54, 19.60, 20.08, 31.62, 31.75, 32.0, 35.44, 36.62, 37.42, 39.02, 42.46, 45.08, 50.06, 54.56, 71.37, 72.25, 73.75, 121.25, 141.06, 208.75.

Pregn-5-ene-3β,16α-diacetoxy-20-one (16α-hydroxypregnenolone diacetate, **II**)
To a solution of **I** (164 mg, 0.4 mmol) in pyridine (5 mL) was added acetic anhydride (0.2 mL) and dimethylaminopyridine (4 mg), and the reaction was stirred at RT for 24 h. After completion of the reaction. saturated NH_4Cl was added at 0C, and the products were extracted into diethyl ether, which was washed with 1N HCl, dried over $MgSO_4$, and concentrated under reduced pressure. Column chromatography on fine silica gave **II** (172 mg, 84%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 0.64 (s, 3H), 0.99 (s, 3H), 1.39-1.69 (m, 8H), 1.74-1.95 (m, 4H), 1.98 (s, 3H), 2.01 (s, 3H), 2.14 (s, 3H), 2.65 (d, J=6.4 Hz, 1H), 4.52-4.65 (m, 1H), 5.31-5.38 (m, 1H), 5.43-5.52 (m, 1H).

^{13}C NMR (500 MHz, $CDCl_3$): δ 14.51, 19.48, 20.75, 21.24, 21.52, 27.86, 31.38, 31.74, 31.82, 33.64, 36.62, 37.04, 38.26, 38.94, 44.75, 49.76, 54.62, 70.24, 73.75, 75.78, 122.12, 139.76, 170.74, 170.86, 206.26.

Pregn-5-ene-20-[1,3-dioxolan-2-yl]-3β,16α-diol (16α-hydroxypregnenolone 20-ethylene ketal, **III**)

To a solution of **II** (112 mg, 0.27 mmol) in benzene (6 mL) was added ethylene glycol (0.2 mL), triethylorthoformate (1 mL), and *p*-toluenesulfonic acid (4 mg). After refluxing for 2h, the reaction mixture was extracted with ethyl acetate, washed with saturated $NaHCO_3$ solution, concentrated, and subjected to saponification (*4*) using methanol and 1.5 M K_2CO_3 (1 mL). After completion of reaction, methanol was removed under reduced pressure, and the products were extracted with diethyl ether, which was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was subjected to column chromatography on fine silica to give **III** (70 mg, 77%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 0.77 (s, 3H), 0.81-0.91 (m, 2H), 0.94-1.38 (m, 12H), 1.38-1.66 (m, 9H), 1.73-1.87 (m, 3H), 1.89-2.03 (m, 2H), 2.14 (m, 2H), 2.52 (bs, 1H), 3.44-3.58 (m, 1H), 3.87-3.97 (m, 2H), 3.97-4.10 (m, 1H), 4.35-4.44 (m, 1H), 5.28-5.38 (m, 1H)

^{13}C NMR (400 MHz, $CDCl_3$): δ 14.38, 19.24, 20.72, 24.21, 29.96, 31.76, 32.04, 33.16, 36.82, 37.36, 41.14, 42.02, 50.21, 54.04, 62.91, 65.84, 68.28, 71.94, 72.78, 112.24, 121.78, 140.89.

Pregn-4-ene-20-[1,3-dioxolan-2-yl]-16α-ol-3-one (16α-hydroxyprogesterone 20-ethylene ketal, **IV**)

To a solution of **III** (70 mg, 0.19 mmol) in toluene (12 mL) was added 1 mL of N-methylpiperidone, and 2 mL of the solvent was distilled out using a Dean Stark apparatus. Next, aluminium isopropoxide (142 mg, 0.7 mmol) was added, and the reaction was refluxed for additional 6 h. After completion of reaction, the solution was transferred into separating funnel and extracted with ethyl acetate, which was dried over $MgSO_{4,}$ concentrated under reduced pressure, and chromatographed on fine silica to give **IV** (58 mg, 83%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 0.805(s, 3H), 0.91-1.14(m, 3H), 1.16(m, 3H), 1.20-1.88(m, 15H), 1.88-2.06(m, 2H), 2.18-2.48(m, 4H), 2.52(bs, 1H), 3.88-3.97(m, 2H), 3.97-4.10(m, 2H), 4.34-4.42(m, 1H), 5.71(bs, 1H).

Pregn-4-ene-16α-ol-3,20-dione (16α-hydroxyprogesterone, **V**)

To a solution of **IV** (23.2 mg, 0.061 mmol) in methanol (4 mL) was added 8% H_2SO_4 (0.16 mL), and the mixture was refluxed for 3 min (*5*). The methanol was evaporated under reduced pressure, and the residue was extracted with diethyl ether, which was dried over $MgSO_4$, concentrated under reduced pressure, and chromatographed on fine silica to give **V** (18 mg, 88%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 0.69 (s, 3H), 0.96-1.30 (m, 6H), 1.40-1.87 (m, 12H), 1.96-2.10 (m, 2H), 2.15-2.50 (m, 6H), 2.54 (d, J=6.4 Hz, 1H), 4.82-4.94 (m, 1H), 5.75 (bs, 1H).

^{13}C NMR (400 MHz, $CDCl_3$): δ 14.70, 17.55, 20.84, 31.90, 35.32, 53.68, 53.90, 72.19 (C-17), 73.74 (C-16), 124.28 (C-4), 170.92 (C-5), 199.71 (C-3), 208.65 (C-20).

Pregn-4-ene-20-[1,3-dioxolan-2-yl]-3,16-dione (16-ketoprogesterone-20-ethyleneketal, **VI**)

To a solution of **IV** (25.5 mg, 0.068 mmol) in dichoromethane (6 mL) was added was Dess Martin reagent (34.8 mg, 0.082 mmol), and the reaction was stirred for 1 h at RT. After completion of reaction, the product was purified by column chromatography directly on fine silica to give **VI** (21.4 mg, 84%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 0.97 (s, 3H), 1.02-1.30 (m, 8H), 1.32-1.82 (m, 14H), 1.90-2.08 (m, 3H), 2.20-2.48 (m, 7H), 3.89-4.02 (m, 4H), 5.73 (bs, 1H).

Pregn-4-ene-20-[1,3-dioxolan-2-yl]-3,16β-diol (**VII**)

To a solution of **VI** (20 mg, 0.054 mmol) in dry diethyl ether (6 mL) was added lithium aluminium hydride (4.3 mg, 0.113 mmol) in ether over 1 min (*5*). The mixture was refluxed for 1 h and quenched with saturated NH_4Cl at 0C. The products were extracted with diethyl ether, which was dried over $MgSO_4$, concentrated under reduced pressure, and chromatographed on fine silica to give **VII** (16.2 mg, 80%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 0.64-1.80 (m, 24H), 1.90-2.13 (m, 3H), 2.16-2.30 (m, 2H), 3.78 (d, J=4.4 Hz, 1H), 3.90-4.08 (m, 4H), 4.14 (bs, 1H), 4.35-4.54 (m, 1H), 5.28 (bs, 1H).

Pregn-4-ene-20-[1,3-dioxolan-2-yl]-16β-ol-3-one (16β-hydroxyprogesterone 20-ethylene ketal, **VIII**)

Oppenauer oxidation of compound **VII** (13.4 mg, 0.035 mmol) was performed as described for compound **IV**, yielding **VIII** (8.4 mg, 63%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.01 (s, 3H), 1.15-1.53 (m, 10H), 1.56 (s, 3H), 1.61-2.12 (m, 3H), 2.18-2.50 (m, 6H), 3.78 (d, J=3.7Hz, 1H), 3.90-4.08 (m, 4H), 4.38-4.50 (m, 1H), 5.68-5.76 (m, 1H).

Pregn-4-ene-16β-ol-3,20-dione (16β-hydroxyprogesterone, **IX**)

Deprotection of compound **VIII** (6.4 mg, 0.017 mmol) with 8%H_2SO_4 was performed as described for compound **V**, yielding **IX** (4.8 mg, 85%).

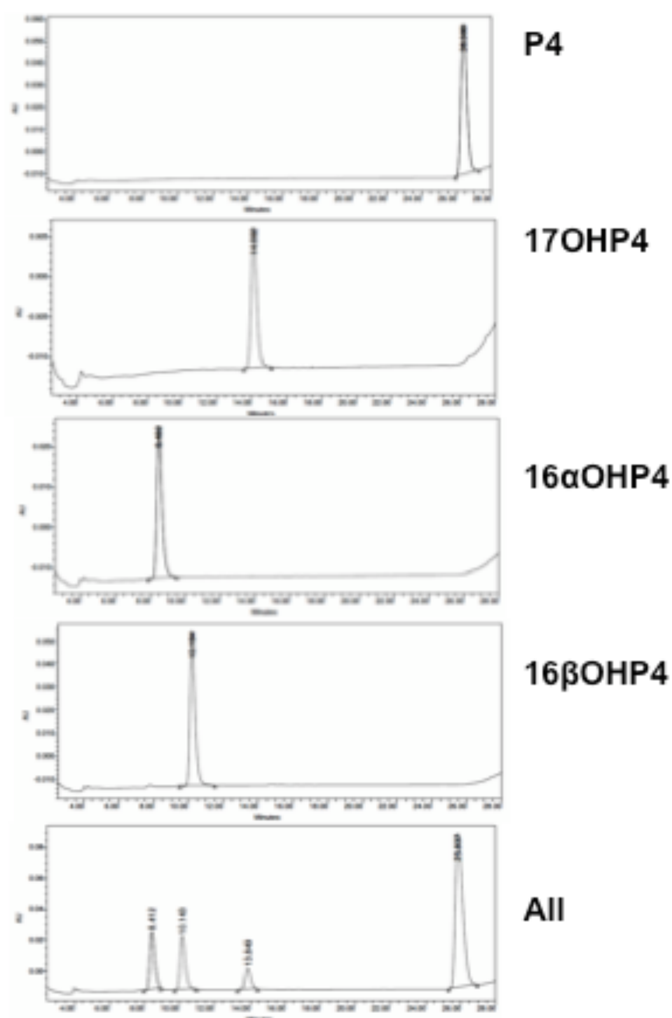
^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87-1.15 (m, 6H), 1.16-1.80 (m, 16H), 1.81-1.96 (m, 2H), 1.96-2.13 (m, 3H), 2.18-2.56 (m, 10H), 4.54-4.66 (m, 1H), 4.75 (bs, 1H), 5.75 (bs, 1H).

^{13}C NMR (500 MHz, $CDCl_3$): δ 15.48, 17.58, 20.58, 29.95, 31.64, 32.52, 32.56, 33.98, 35.42, 36.89, 38.72, 38.96, 43.92, 53.56, 53.68, 66.13 (C-16), 72.39 (C-17), 124.25 (C-4), 170.90 (C-5), 199.68 (C-3), 213.27 (C-20). The downfield shift of C-20 and upfield shift of C-16 in 16β-hydroxyprogesterone relative to the corresponding resonances in 16α-hydroxyprogesterone is probably due to intramolecular hydrogen bonding between the 16β-hydroxyl group and the 20-ketone, which is sterically precluded in 16α-hydroxyprogesterone.

Table S1. Activity inhibition by ketoconazole and reactive oxygen scavengers

Microsomes	Ketoconazole	Catalase	SOD
CYP21A2 Wild-type	98%	<1%	<1%
CYP21A2 V359A	100%	<1%	<1%
CYP21A2 V359G	100%	<1%	<1%

Microsomes containing CYP21A2 (wild-type or mutations, 100 μg protein) were incubated in 50 mM sodium phosphate pH 7.4 with 100,000 cpm [^{3}H]-P4 at 1 μM and 1 mM NADPH at 37C for 120 min. Inhibition was measured by triplicate incubations with ketoconazole (0.1 mM), catalase (0.1 mg/mL), or superoxide dismutase (SOD, 0.1 mg/mL). Inhibition is expressed as percent inhibition of activity in the absence of test substance. Similar results were obtained using [^{3}H]-17OHP4 as substrate. Microsomes from yeast transformed with empty expression vector yielded none of the identified metabolites of P4 or 17OHP4. These results demonstrate that product formation is catalyzed by the cytochrome P450 and not by other proteins present in the yeast microsomes or formed by reactive oxygen species released to the buffer.



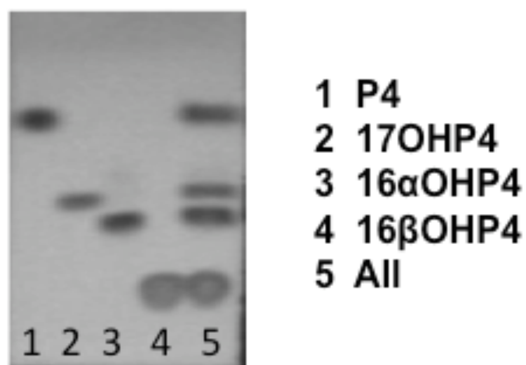
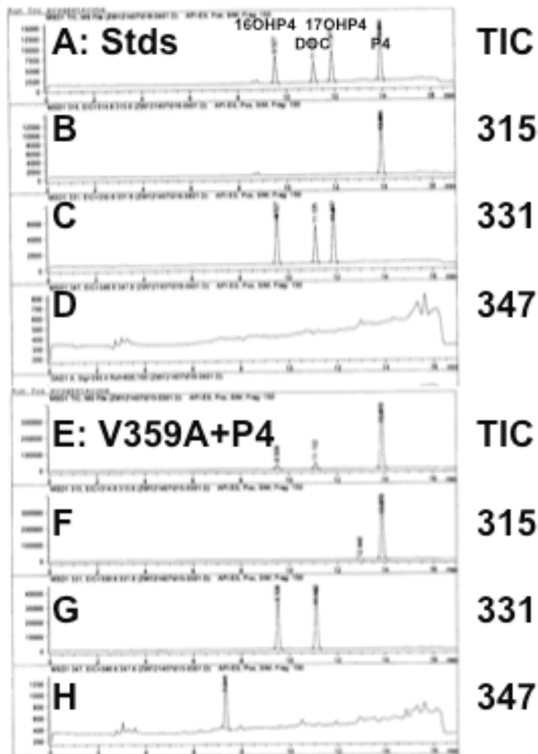


Figure S1. Reverse-phase HPLC chromatogram (top) and TLC (3:1 chloroform: ethyl acetate) of P4, 17OHP4, 16α-hydroxyprogesterone (16αOHP4), and 16β-hydroxyprogesterone (16βOHP4), and a mixture of the four steroids. The two 16-hydroxy epimers are easily distinguished by the longer retention time (HPLC) and higher R_f (TLC) of 16βOHP4, which are probably due to intramolecular hydrogen bonding between the 16β-hydroxyl group and the 20-ketone in 16βOHP4, which is sterically precluded in 16αOHP4.

Incubation with Progesterone



Incubation with 17OHProgesterone

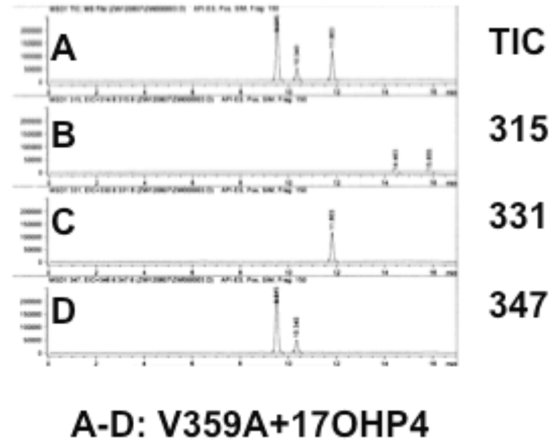


Figure S2. Liquid chromatography-mass spectrometry tracings of progesterone (P4) and 17-hydroxyprogesterone (17OHP4) products following incubation with microsomes from yeast expressing human CYP21A2 mutation V359A. Selected ion monitoring (SIM) was used to monitor $[M+H]^+$ ions of *m/z* = 315 (P4), 331 (mono-hydroxylated P4, including 17OHP4, DOC, and 16OHP4), and 347 (di-hydroxylated P4, including 11-deoxycortisol). Incubations were performed as described in the manuscript and injected into an Agilent 1100 LC/MSD SL single quadropole instrument (Agilent Technologies, Palo Alto, CA) with API-ES in positive ion mode. Samples were loaded onto a pre-column (Zorbax C_8, 4.6 x 12.5 mm, 5 μm, Agilent) at 1 ml/min for 1 min with 100% water containing 5 mM NH_4Ac, and then back flushed onto the analytical column at 1 ml/min (Zorbax C_{18}, 4.6 x 50 mm, 5 μm, Agilent). The mobile phase consisted of methanol (A) and water (B), both containing 5 mM (NH_4Ac). The following gradient was run for a total of 17 min: 0 – 2 min, 55% (A); 2 – 14 min, 55% to 90% (A); 14 – 17 min, 55% (A). MS parameters were as follows: gas temperature 350°C, nebulizer pressure 30 psig, drying gas (nitrogen) 12 L/min, VCap 4000V, fragmentor voltage 150V. At left, panels **A**-**D** show standards detected with total ion current (TIC, **A**) or SIM of *m/z* = 315 (**B**), 331 (**C**), or 347 (**D**). Panels **E**-**F** show products of P4 incubated with microsomes containing mutation V359A and detected with total ion current (TIC, **E**) or SIM of *m/z* = 315 (**F**), 331 (**G**), or 347 (**H**). These results confirm the identity of the early-eluting product as 16OHP4. At right, panels **A**-**D** show products of 17OHP4 incubated with microsomes containing mutation V359A and detected with total ion current (TIC, **A**) or SIM of *m/z* = 315 (**B**), 331 (**C**), or 347 (**D**). These results confirm that both products of 17OHP4 metabolism are di-hydroxylated progesterone derivatives, isobaric to the major product and earlier-eluting compound, 11-deoxycortisol.

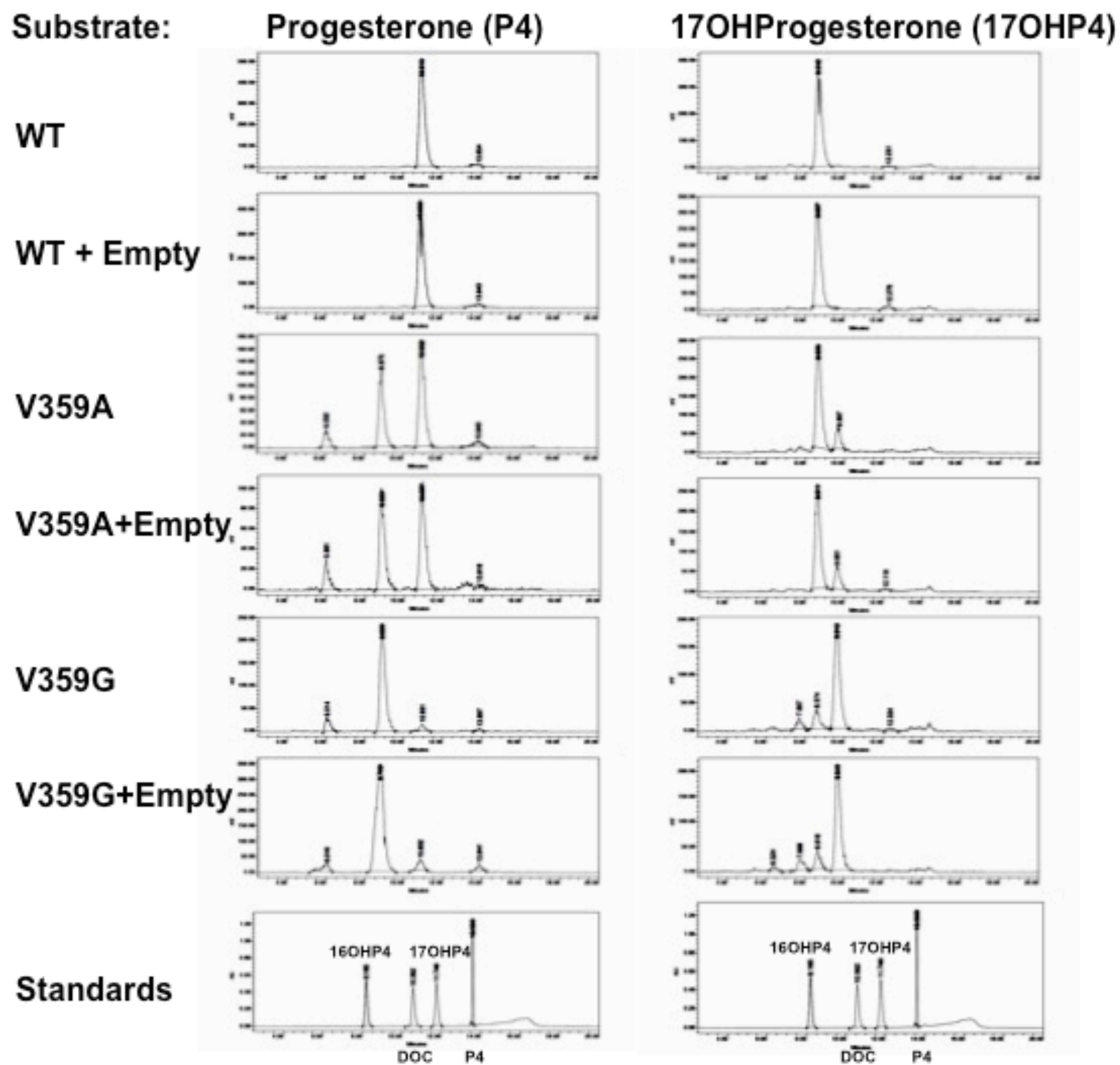


Figure S3. HPLC chromatograms of [^{3}H]-P4 or -17OHP4 following prolonged incubations with microsomes from yeast expressing Wild-type (WT) human CYP21A2 or mutations V359A and V359G. The prolonged incubations (120 min at 37C with 100 μg microsomes per conditions of Table S1 to allow complete substrate consumption) demonstrate that the product distribution does not further change over time, indicating that new metabolites are not subsequent metabolites of the 21-hydroxylated steroids DOC and 11-deoxycortisol. Paired incubations (+Empty) show that same products are obtained when products are further incubated another 120 min with 100 μg microsomes from mock-transformed yeast, demonstrating no further metabolism of steroids by endogenous yeast enzymes.

Homo sapiens|NP_001122062.1	A E V L R L R P V V P L A L P H
Bos taurus|NP_777064.1	A E V L R L R P V V P L A L P H
Canis lupus familiaris|NP_001003335.1	A E V L R L R P V V P L A L P H
Sus scrofa|NP_999598.1	A E V L R L R P V V P L A L P H
Macaca mulatta|NP_001181556.1	A E V L R L R P V V P L A L P H

Figure S4. Alignment of CYP21A2 sequence from 5 species shows absolute conservation of V359 (underlined) and surrounding residues.

References

1. Kym, P. R., Carlson, K. E., and Katzenellenbogen, J. A. (1993) Progestin 16α,17α-dioxolane ketals as molecular probes for the progesterone receptor: synthesis, binding affinity, and photochemical evaluation, *J Med Chem 36*, 1111-1119.
2. Batres, E. (1961) Steroids. CLVI. Ring D unsaturated 16-methylated corticosteroids, *J Org Chem*, 871–878.
3. Salvador, J. A. R., Leita, A. J. L., Melob, L., and Hanson, J. R. (2005) Hydrazine hydrate induced reductive cleavage of α,β-epoxy ketones: an efficient procedure for the preparation of β-hydroxy ketones, *Tetrahedron Lett 46*, 1067-1070.
4. Viger, A., Coustal, S., Perard, S., and Marquet, A. (1988) Synthesis of inhibitors of aldosterone biosynthesis, *Tetrahedron 44*, 1127-1134.
5. Bernstein, S., Heller, M., and Stolar, S. M. (1955) Steroidal cyclic ketals. XVI. 16-hydroxylated steroids. II. The preparation of 16-keto- and 16β-hydroxyprogesterone, *J Am Chem Soc 77*, 5327-5330.