Supplementary information for:

Selective steroidogenic cytochrome P450 haem iron ligation using steroid-derived isonitriles.

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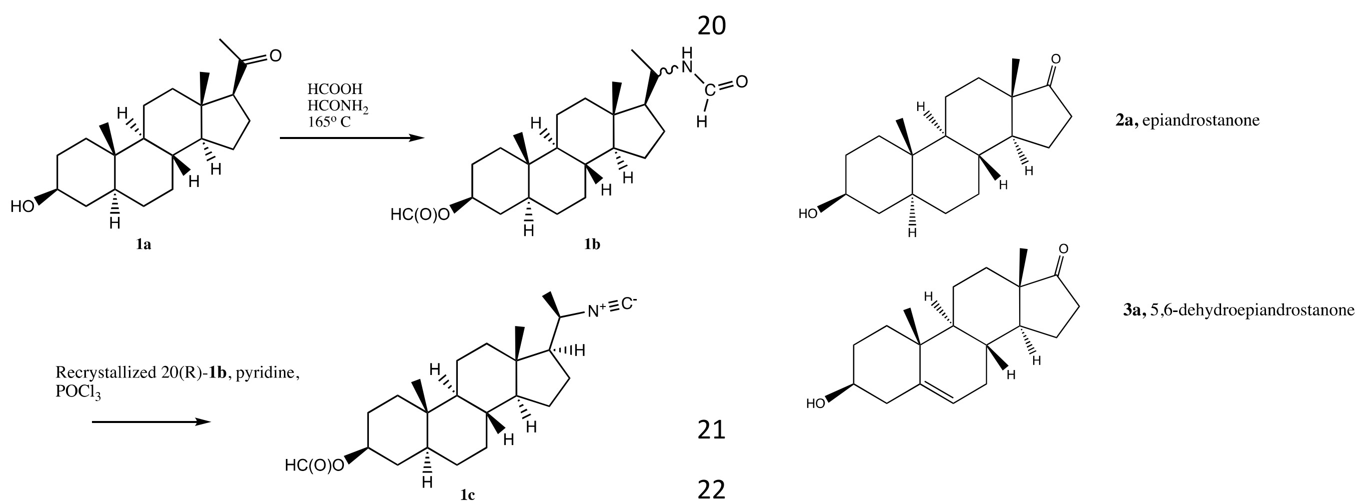
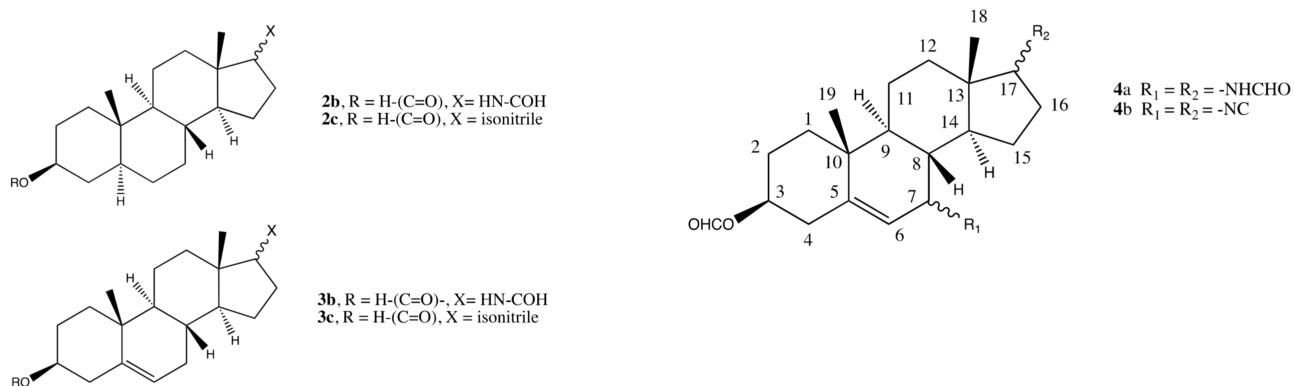
Supplementary Methods

S1) Synthesis and characterization of compounds 1-4





3β-formyl-(R)-(20-amidoformyl)pregnane **1b.** 2g of 3β-hydroxy-5α-pregnan-20-one **1a** (3.2 mmole) (CAS 516-55-2, Oakwood Chemical, Estill SC) was added to 4 mL of 95% formic acid and 4.8 mL of formamide in a Pyrex test tube equipped with a magnetic stir bar. The test tube was stoppered with glass wool and heated to 165 °C on an aluminum heating block with stirring and held at temperature for 3 hours. After cooling, the two-phase mixture was mixed with sufficient benzene to dissolve the solid upper layer. The organic layer was filtered to remove unreacted **1a**, which is relatively insoluble in benzene, then washed 2x with saturated $NaHCO_3$ solution, dried over anhydrous Na_2SO_4, filtered, evaporated and recrystallized from benzene. Reversed phase HPLC of the first crop of recrystallized material (C_{18} column, acetonitrile/water gradient 20/80 -> 90/10, detection at 210 nm) showed evidence for several products, and ^1H,^{13}C NMR of the isolated fractions confirmed that R and S epimers of the formamide are present in ~2:1 proportion. Furthermore, two conformational isomers were observed at slow exchange om the ^1H chemical shift time scale for both epimers, presumably due to slow interconversion of the *cis* and *trans* NH-CHO forms (only the more upfield

^{1}H shifts for the two forms are reported below). Finally, the 3β-hydroxy group was esterified by formic acid in ~90% of the first recrystallization, based on NMR signal intensities.

The second crop of crystals from benzene, 0.78 g fine needles were obtained, m.p. 175-180 °C, was determined by NMR to be essentially pure 20-(R)-**1b**, and was used for the isonitrile synthesis.

***)^{1}H NMR** (d_6-benzene): H1 (0.74, 1.50); H2 (0.72,1.50); H3 (4.83); H4 (1.31,1.55); H5 (0.86); H6, 1.06; H7, 1.31, 1.54; H8, (R_{20}, 1.13; S_{20} 1.80); H9 (0.42); H11 (S_{20}, 1.10, 1.35; R_{20} α, 1.44; R_{20} β, 0.87); H12, 1.76, 1.47; H14, 0.77; H15 (S_{20} α 1.50, S_{20} β, 2.34; R_{20} α, 1.31; R_{20} β, 1.11); H16 (S_{20} α 1.03, S_{20} β, 1.59; R_{20} α, 1.72; R_{20} β, 0.82); H17 (S_{20}, 0.98 R_{20}, 0.87); H18 (S_{20}, 0.56; R_{20}, 0.63); H19 (0.59); H20 (S_{20}, 4.02; R_{20}, 4.06); H21 (S_{20}, 0.97; R_{20}, 0.85), formamide NH (S_{20}, 5.28, exch. 4.04; R_{20}, 5.86, exch. 4.19); formamide H(CO) (S_{20}, 7.59; R_{20}, 7.74); formate H(CO) (7.74)

^{13}C NMR (d_6-benzene): C1, 33.3; C2, 38.0; C3, 74.5; C4, 35.5; C5, 45.0; C6, 29.8; C7, 35.5; C8, 36.6; C9, 55.6; C10, 37.4; C11, 25.2; C12, 29.0; C13 (R_{20}, 42.9; S_{20},45.9); C14, 57.6; C15, (S_{20}, 21.6; R_{20}, 22.5); C16 (R_{20}, 40.9; S_{20}, 40.0); C17 (R_{20}, 57.7; S_{20}, 56.4); C18 (R_{20}, 13.8; S_{20}, 12.6); C19, 13.3; C20 (R, 46.4; S, 50.4); C21 (R_{20}, 22.7; S_{20}, 23.3); formamide carbonyl (R_{20}, 160.4; S_{20}, 163.7); formate carbonyl (161.3).

^{15}N NMR (in d_6-benzene): formamide R_{20}, 136.1; S_{20}, 102.6.

HRMS (1b): calculated for $C_{23}H_{38}NO_3$ (M+1), 376.2852, observed, 376.2835

Peaks eluted from C18 reverse phase HPLC (acetonitrile/water gradient), 72% ACN and 73% ACN.

3β-formyl-(R)-(20-isonitrilo)pregnane **<u>1c</u>.** (Identified as compound **1** in the Communication) After drying over P_2O_5 in a vacuum dessicator, 0.265 mg (0.8 mmol) of recrystallized 20-(R)-**1b** was dissolved in 0.8 mL (3.2 mmole) of dry pyridine under N_2 with stirring and cooled in an ice bath. 80 μL (0.8 mmol) of $POCl_3$ (Sigma) was added slowly dropwise. After all of the $POCl_3$ was added, the ice bath was removed, and the reaction allowed to proceed for ~2 h. The reaction mixture slowly darkened, and when no further color change was observed, the reaction was quenched with the addition of ice chips and 1 mL of saturated $NaHCO_3$ solution. The reaction mixture was extracted with diethyl ether (5 mL x3), the aqueous layer discarded and the organic layer filtered through anhydrous Na_2SO_4. Solvent was removed by a gentle stream of N_2 without heating, and excess pyridine removed using a SpeedVac. The resulting solid was examined by IR spectroscopy to confirm the presence of the isonitrile group, which exhibits a sharp absorption band at 2138 cm^{-1}.

^{1}H NMR <u>1c</u> (d_6-benzene): H1, 0.70, 1.43; H2, 1.41,1.73; H3, 4.79; H4, 1.27,1.51; H5, 0.85; H6, 1.01,1.05; H7, 0.69, 1.45; H8, 1.01; H9, 0.32, 0.39; H11, 1.06, 1.30; H12, 0.76, 1.52; H14 (R_{20}, 0.68); H15 (R_{20} α, 1.45; R_{20} β, 1.84); H16 (R_{20} α, 1.42; R_{20} β, 0.72); H17 (R_{20}, 1.12); H18 (R_{20}, 0.34); H19, 0.56; H20 (R_{20}, 3.11); H21 (R_{20}, 0.75).

^{13}C NMR <u>1c</u> (d_6-benzene): C1, 37.1; C2, 28.1; C3, 73.2; C4, 34.7; C5, 44.9; C6, 28.9; C7, 32.5; C8, 35.4; C9, 54.5; C10, 36.9; C11, 21.4; C12, 32.7; C13 (R_{20}, 43.0; S_{20}, 42.3); C14 (R_{20}, 56.3); C15 (R_{20}, 26.9); C16 (R_{20}, 39.1); C17 (R_{20}, 55.8); C18 (R_{20}, 12.5); C19, 12.4; C20 (52.4); C21 (R_{20}, 22.4); isonitrile C (R_{20}, 158.1 (broad)).

HRMS (<u>1c</u>): calculated for $C_{22}H_{35}NO_2$ (M+1), 358.2747, observed, 358.2741

<u>1c</u> was eluted from C18 reverse phase HPLC (acetonitrile/water gradient), 95% ACN.

76 *3β-formyl-(R,S)-(17-amidoformyl)androstane* **2b.** 1g of 3β-hydroxy-5α-androstan-17-one **1** (2.9 mmole)
77 (CAS 481-29-8, Sigma) was added to 2 mL of 95% formic acid and 2.4 mL of formamide in a Pyrex test tube
78 equipped with a magnetic stir bar. The test tube was stoppered with glass wool and heated to 175 ^oC on an
79 aluminum heating block with stirring and held at temperature for 4 hours. After cooling, the solid mass was
80 extracted with CH_2Cl_2. After removal of solvent from the extract, the product was recrystallized from a
81 minimal amount of ethanol and CH_2Cl_2 by slow evaporation. Based on relative NMR signal intensities, the
82 recrystallized product is approximately 95:5 S_{17}:R_{17} epimers.

83 **^{1}H NMR 2b** (d_6-benzene): H1, 1.38, 1.59; H2, 1.52,1.80; H3, 4.77; H4 1.64,1.69; H5, 1.12; H6, 1.39,1.77; H7,
84 0.86, 1.63; H8, 1.32; H9, 0.62; H11, 1.20, 1.51; H12, 0.95, 1.55; H14, (R_{17}, 1.03; S_{17} 0.93) H15 ($R_{17,}$ 1.15, 1.67;
85 S_{17}, 1.17,1.61); H16 ($R_{17,}$ 2.21,1.29; $S_{17,}$ 1.96,1.22); H17 (R_{17}, 3.31; S_{17}, 3.10); H18 (R_{17}, 0.71; S_{17}, 0.64); H19
86 0.78; formamide 17-HN (R_{17}, 7.09; $S_{17,}$ 7.15); formamide 17-HCO (R_{17}, 7.86; $S_{17,}$ 7.93); 3-HCO, 7.96.

87 **^{13}C NMR 2b** (d_6-benzene): C1, 33.9; C2, 27.3; C3, 73.5; C4, 36.7; C5, 44.5; C6, 31.2; C7, 31.5; C8, 35.5; C9, 54.1;
88 C10, 35.5; C11, 20.5; C12, 36.5; C13 (R_{17}, 44.8; $S_{17,}$ 42.9); C14 ($R_{17,}$ 50.0; $S_{17,}$ 52.4); C15 (R_{17}, 24.6; $S_{17,}$ 23.4); C16
89 (R_{17}, 29.8; $S_{17,}$ 28.3); C17 (R_{17}, 61.6; $S_{17,}$ 63.0); C18 (R_{17}, 18.1; $S_{17,}$ 11.6); C19, 12.1; formamide carbonyl (R_{17},
90 165.2; S_{17}, 165.1); 3-CHO, 160.9.

91 **^{15}N NMR 2b** (d_6-benzene): formamide S_{17}, 115.6; R_{17}, 121.7.

92 **HRMS (2b):** calculated for $C_{21}H_{34}NO_3$ (M+1), 348.2539, observed, 348.2526

93 **2b** eluted from C18 reverse phase HPLC (acetonitrile/water gradient), 70% ACN (R_{17}) and 72% ACN (S_{17}).

94 *3β-formyl-(R,S)-(17-amidoformyl)androst-5,6-ene* **3b.** 1g of 3β-hydroxy-5α-androst-5,6-ene-17-one **3a** (2.9
95 mmole, CAS 481-29-8, Sigma) was added to 2 mL of 95% formic acid and 2.4 mL of formamide in a Pyrex test
96 tube equipped with a magnetic stir bar. The test tube was stoppered with glass wool and heated to 175 ^oC
97 on an aluminum heating block with stirring and held at temperature for 6 hours. After cooling, the solid mass
98 was extracted with CH_2Cl_2. The product was recrystallized from a minimal amount of hexanes and CH_2Cl_2 by
99 slow evaporation, m.p. 255 ^oC, By NMR, product formamide was determined to be >9:1 S_{17} :R_{17}.

100 **^{1}H NMR 3b** (d-chloroform): H1, 1.16, 1.89; H2 1.65, 1.90; H3, 4.73; H4, 2.35 ; H6, 5.40; H7, 1.58, 2.01; H8,
101 1.32; H9, 1.16; H11, 1.35, 1.63; H12, 1.08,1.75; H14, (R_{17}, 1.05; S_{17} 1.13); H15 (R_{17}, 1.44, 1.61; S_{17}, 1.40,
102 1.58); H16 (R_{17} α 1.51, R_{17} β, 2.09; S_{17} α, 1.36; S_{17} β, 2.14); H17 (S_{17}, 3.26; R_{17}, 3.41); H18, 0.73; H19, 1.04;
103 formamide 17-HN (R_{17}, 6.10; $S_{17,}$ 5.56); formamide 17-HCO (R_{17}, 8.03; $S_{17,}$ 8.20); 3-HCO, 8.04.

104 **^{13}C NMR 3b** (d-chloroform): C1, 36.9; C2, 27.5; C3, 73.8; C4, 42.2; C5, 139.4; C6, 122.6; C7, 31.5; C8, 35.4;
105 C9, 53.9; C10, 37.32; C11, 20.25; C12, 36.2; C13 (R_{17}, 42.8; $S_{17,}$ 42.9); C14 ($R_{17,}$ 52.8; $S_{17,}$ 52.8); C15 (R_{17}, 20.4;
106 $S_{17,}$ 20.5); C16 (R_{17}, 28.8; $S_{17,}$ 28.6); C17 (R_{17}, 62.6; S_{17}, 57.5); C18, 11.7; C19, 19.12; formamide carbonyl (R_{17},
107 164.2; S_{17}, 164.3); 3-CHO, 160.4.

108 **^{15}N NMR 3b** (d-chloroform): formamide S_{17}, 114.2; R_{17}, 120.2.

109 **HRMS (3b):** calculated for $C_{21}H_{32}NO_3$ (M+1), 346.24382, observed, 346.2365

110 Peaks eluted from C18 reverse phase HPLC (acetonitrile/water gradient), 68% ACN (S_{17}) and 70% ACN (R_{17}).

111 Isonitrile derivatives **2c** and **3c** (identified as compounds **2** and **3** in the Communication) were
112 prepared from compounds **2b** and **3b** as **1d** above. Presence of the isonitrile group was confirmed

113 in each case by a strong narrow infrared absorbance band at 2138 cm^{-1} in the products after removal
114 of solvent and excess pyridine under vacuum at room temperature.

115

116 **^{1}H NMR (<u>2c</u>)** (d6-DMSO): H1, 1.12, 1.79; H2 1.56, 1.86; H3, 4.77; H4, 1.64, 1.44; H5, 1.27; H6, 1.28, 1.34; H7,
117 0.96, 1.71; H8, 1.44; H9, 1.09; H11, 1.36, 1.65; H12, 1.77, 2.25; H14, 0.76; H15, 1.36, 1.69; H16, 1.23, 1.80;
118 H17 (S_{17}, 3.82; R_{17}, 3.61); H18, (S_{17}, 0.82; R_{17}, 0.90); H19, 1.10; 3-HCO, 8.31.

119 **^{13}C NMR (<u>2c</u>)** (d6-DMSO): C1, 36.5; C2, 27.7; C3, 73.4; C4, 34.3; C5, 44.5; C6, 28.0; C7, 31.7; C8, 36.3; C9,
120 51.4; C10, 36.0; C11, 23.8; C12, 29.4; C13 (R_{17}, 50.0; $S_{17,}$ 43.6); C14, 53.8; C15 20.7; C16 (R_{17}, 45.1; $S_{17,}$ 35.9);
121 C17 (R_{17}, 62.6; S_{17}, 62.1); C18, 13.0; C19, 12.5; isonitrile C (S_{17}, 156.5; R_{17}, 156.1); 3-CHO, 162.3.

122 **^{15}N NMR (<u>2c</u>)** (d6-DMSO): isonitrile S_{17}, 180.0; R_{17}, 193.4.

123 **<u>2c</u> HRMS (**identified as compound **2** in the communication**)**: calculated for $C_{21}H_{32}NO_2$ (M+1), 330.2433,
124 observed 330.2417

125 Peak eluted from C18 reverse phase HPLC (acetonitrile/water gradient), 88% ACN.

126 **^{1}H NMR (<u>3c</u>)** (d6-DMSO): H1, 1.21, 1.97; H2 1.70, 1.72; H3, 4.65; H4, 2.42, 2.42; H6, 5.47; H7, 1.72, 2.08; H8,
127 1.53; H9, 1.10; H11, 1.55, 1.72; H12, 1.28, 1.84; H14, 1.37; H15, (S_{17}, 2.07, 1.72; R_{17}, 2.36, 1.84); H16, (S_{17},
128 1.28, 1.84; R_{17}, 1.22, 1.78); H17 (R_{17}, 3.60; S_{17}, 3.82); H18, 0.86; H19, 0.90; 3-HCO, 8.27.

129 **^{13}C NMR (<u>3c</u>)** (d6-DMSO): C1, 36.9; C2, 23.7; C3, 73.6; C4, 37.7; C5, 140.4; C6, 122.8; C7, 31.9; C8, 32.1; C9,
130 50.1; C10, 33.7; C11, 20.5; C12, 35.7; C13 (S_{17}, 45.5; $R_{17,}$ 43.5); C14, 50.2; C15 (S_{17}, 31.8; $R_{17,}$ 31.4); C16 (S_{17},
131 35.8; $R_{17,}$ 36.8); C17 (S_{17}, 62.7; R_{17}, 62.3); C18, (S_{17}, 16.7; $R_{17,}$ 12.6); C19, 19.5; isonitrile C (S_{17}, 156.2; R_{17},
132 156.9); 3-CHO, 162.3.

133 **^{15}N NMR (<u>3c</u>)** (d6-DMSO): isonitrile S_{17}, 183.9; R_{17}, 179.4.

134 **<u>3c</u> HRMS** calculated for $C_{20}H_{30}NO$ (M+1, free 3-OH)**,** 300.2327, found 300.2308

135 Peak eluted from C18 reverse phase HPLC (acetonitrile/water gradient), 80% ACN.

136 *3β-formyl-(R,S)-(7,17-bis-amidoformyl)androst-5,6-ene* **<u>4a</u>** was prepared as with compounds **1-3**, starting
137 from androstene-3β-hydroxy-5,6-dehydro-7,17-dione (CAS 566-19-8, Steraloids, Inc., Newport, RI).

138 **^{1}H NMR <u>4a</u>** (d_6-acetone): H1, 1.26, 1.94; H2 1.68, 1.88; H3, 4.87; H4, 1.95, 1.92; H6, 5.80; H7, 4.49; H8, 1.87;
139 H9, 1.35; H11, 1.51, 1.79; H12, 1.34, 1.83; H14, 1.47; H15, 1.61, 2.11; H16 α 1.31, 1.29; β, 1.81; H17
140 (epimer 1, 3.45; epimer 2, 4.01); H18, 0.87; H19, 1.1; formamide 7-NH, 7.37; 17-NH, 7.17; formamide 7-
141 HCO 8.18; 17-HCO, 8.24; 3-HCO, 8.19.

142 **^{13}C NMR <u>4a</u>** (d_6-acetone): C1, 33.1; C2, 24.1; C3, 73.3; C4, 33.2; C5, 142.6; C6, 127.2; C7, 49.3; C8, 37.7; C9,
143 48.6; C10, 34.6; C11, 20.6; C12, 36.8; C13 43.7; C14, 52.8; C15 28.2; C16 (epimer 1, 37.1; epimer 2,, 36.1);
144 C17 (epimer 1, 56.9; epimer 2, 62.9); C18, 11.5; C19, 17.8; formamide carbonyl (7-, 160.3; 17-, 160.8); 3-
145 CHO, 162.3.

146 **^{15}N NMR <u>4a</u>** (d_6-acetone): 7-N, 135.3; 17-N, 125.6

147 *3β-formyl-(R,S)-(7,17-bis-isonitrilo)androst-5,6-ene* **4b** The crude 7,17-diformamide **4a** was dried over P_2O_5
148 in vacuum and used to prepare the isonitrile as above. After 3x extraction of the crude neutralized reaction
149 mixture with diethyl ether, the dehydration of **4a** to **4b** was found to be essentially complete by NMR. Based
150 on integration of the C7 1H peak, the major isonitrile at that position is produced in ~4:1 over the minor
151 epimer. NOEs between 7H, 9H and 14H that the major epimer isonitrile at the 7 position is in the *R*-
152 configuration. At the 17 position, the isonitrile is ~10:1 *S*-configuration, based on NOEs between the 17,
153 15 and 16 (but not 18-CH_3) protons. Some evidence of dehydration by loss of the 3-hydroxyl group is present
154 in the isonitrile spectrum, in the form of new vinyl protons (presumably at 3- and 4- positions). The presence
155 of the isonitrile was confirmed by IR spectroscopy, with a strong absorption band at 2138 cm^{-1}.

156 **^{1}H NMR 4b** (d_6-DMSO): H1, 1.14, 1.87; H2 1.79, 1.97; H3, 4.77; H4, 1.87, 1.87; H6, 5.37 (R), 5.28 (S); H7, 4.30
157 (R), 4.28 (S); H8, 1.93; H9, 1.16; H11, 1.47, 1.75; H12, 1.27, 1.84; H14, 1.42; H15, 1.83, 2.30; H16, 1.28, 1.84
158 (S), 1.27, 1.82 (R); β, 1.81; H17, 3.65 (S), 3.74 (R); H18, 0.95 (S), 0.91 (R); H19, 1.06; 3-HCO, 8.27.

159 **^{13}C NMR 4b** (d_6-DMSO): C1, 33.0; C2, 25.3; C3, 73.4; C4, 33.0; C5, 144.4; C6, 119.7 (R), 117.8 (S); C7, 55.3
160 (R), 55.7 (S); C8, 39.3; C9, 46.7; C10, 34.6; C11, 20.5; C12, 35.3; C13, 44.2; C14, 52.8; C15 29.4; C16, 35.4 (S),
161 35.3 (R); C17, 61.9 (S), 61.9 (R)); C18, 12.7 (S), 12.6 (R); C19, 18.5; 3-CHO, 162.8, 7-N**C**, 156.6, 17-N**C**, 156.6.

162 **^{15}N NMR 4b** (d_6-DMSO): 7-**N**C, 185.0; 17-**N**C, 177.4

163 HRMS for $C_{22}H_{28}N_2O_2$ (M+1), 353.2230, found 353.2224.

164 Peak eluted from C18 reverse phase HPLC (acetonitrile (ACN)/water gradient), 70% ACN.

165 ***) Note on NMR assignments and spectra:** All NMR assignments are numbered according to the accepted
166 IUPAC system for gonane and steranes, shown in the structure above for compound **4**. Relevant NMR
167 spectra used for resonance assignment of all isonitrile compounds 1-4 are deposited as Supplementary
168 Data 1.

169 **NMR spectroscopy**. All two-dimensional NMR experiments used for resonance and stereochemical
170 assignments were performed on a Bruker NEO spectrometer at the Landsman Research Facility (Brandeis
171 Univ.) operating at 800.13 MHz (^{1}H), 201.19 MHz (^{13}C) and 81.08 MHz (^{15}N). All ^{1}H and ^{13}C chemical shifts
172 are reported in ppm relative to tetramethylsilane; ^{15}N shifts are reported in ppm relative to anhydrous
173 ammonia. For assigning ^{1}H and ^{13}C correlations, ^{1}H,^{13}C-HSQC and HMBC experiments were performed.
174 Formamide ^{15}N resonances were assigned using natural abundance ^{1}H,^{15}N-HSQC, and ^{1}H,^{15}N-HMBC used for
175 assigning isonitrile ^{15}N resonances. Stereochemistry at reaction centers was established by analysis of ^{1}H,^{1}H
176 NOESY experiments, based on the known stereochemistry of steroid starting materials, except in the case of
177 **1c**, in which the crystal structure of the CYP17A1-**1c** complex provided the stereochemical relationship at C_{20}.

178 **Infrared spectroscopy.** Infrared spectra were recorded on a diamond anvil-equipped Nicolet FTIR
179 spectrometer.

180 **High-resolution mass spectrometry.** High-resolution mass spectra were obtained at the Brandeis University
181 interdepartmental mass spectrometry facility on a Bruker timsTOF Pro mass spectrometer operating in
182 positive ion mode in tandem with liquid chromatographic separation of analytes.

183

184

185 **S2) Ligand binding and inhibition assays**

186 **Reduction and reoxidation assays.** Reduction of the isonitrile-bound P450 complex was observed by taking
187 a baseline absorbance reading from 400 to 500 nm using a UV-visible scanning spectrophotometer, of a 1cm
188 quartz cuvette containing 1 µM P450, with the selected isonitrile compound at a saturating concentration
189 (*i.e.,* ≥ concentration needed to reach ΔA_{max}), in the same buffer used for binding assays (100 mM potassium
190 phosphate buffer (pH 7.4), 20% glycerol, and 100 mM sodium chloride). A small quantity of sodium dithionite
191 (spatula tip-full) was then added to the cuvette, and the spectra were recorded periodically over time to
192 observe the formation of peaks indicating the reduction (~426 and 456 nm) and subsequent reoxidation
193 (~435 nm) of the complex. The total time course for Figure 6B was 103 minutes and 37 seconds.

194 **CYP17A1 Inhibition assay**. (Adapted from DeVore, NM, Scott, EE. (2012). *Nature, 482*(7383), 116-119.)
195 Metabolic activity of CYP17A1 was evaluated by measuring 17α-hydroxylation of progesterone as detected
196 by HPLC with UV detection at 240 nm. A 1:4 ratio of CYP17A1 to recombinant NADPH-cytochrome P450
197 reductase was mixed and incubated on ice for 20 minutes. This mixture was added to buffer (50 mM Tris, pH
198 7.4, 5 mM $MgCl_2$) containing 11.5 mM progesterone and either abiraterone (0-1 µM) or **1c** (0-50 µM).
199 Reaction vials were warmed to 37 °C for three minutes, then catalysis was initiated by adding NADPH to a
200 final concentration of 1 mM. After 10 minutes, metabolism was quenched by adding 300 µL of 20%
201 trichloroacetic acid and placed on ice. The reaction vials were centrifuged to pellet the precipitated protein,
202 then the supernatant was injected onto a C18 column (Phenomenex, Luna, 50 x 4.6 mm) for HPLC. The 30-
203 minute HPLC method ran at 0.8 mL/min and started with a mobile phase of 60% acetonitrile, 40% water with
204 0.2% acetic acid for five minutes, increased to 80% acetonitrile over 10 minutes, held at 80% acetonitrile for
205 five minutes, 100% acetonitrile for five minutes, then returned to 60% acetonitrile to prepare for the next
206 sample. Metabolite elution was normalized to β-estradiol as an internal standard. A standard curve of known
207 product concentrations was used to convert normalized area under curve to amount of product produced. A
208 four-parameter variable-slope equation ($Y=Y_{min} + (Y_{max}-Y_{min})/(1+(IC_{50}/X)$^(Hill Slope))) was used to fit the data
209 and calculate IC_{50} values for inhibitors using GraphPad Prism 9.

210 **CYP3A4 nifedipine metabolism assay**. (Adapted from Bart, AG, Scott, EE. (2017). *J. Biol. Chem.,* 292(51),
211 20818–20833.) Metabolic activity of CYP3A4 was evaluated by measuring metabolism of nifedipine to
212 dehydronifedipine, as detected by HPLC with UV detection at 254 nm. Reactions were carried out in amber
213 microcentrifuge tubes (NFP is light sensitive) in a final volume of 150 µl. A 1:2 ratio of CYP3A4 to recombinant
214 NADPH-cytochrome P450 reductase was mixed and incubated at room temperature for 20 minutes. This
215 mixture was added to buffer (40 mM HEPES, 30 mM $MgCl_2$, pH 7.4) containing 0.1 mM NFP and increasing
216 concentrations of **1c** (0-100 µM). Reaction vials were warmed to 37 °C for three minutes, then catalysis was
217 initiated by adding NADPH to a final concentration of 1 mM. After 20 minutes, metabolism was quenched
218 by adding 50 µL acetonitrile and placed on ice. The reaction vials were centrifuged at 5000 xg for 5 minutes
219 to pellet the precipitated protein, then the supernatant was injected onto a C18 column (Phenomenex, Luna,
220 50 x 4.6 mm) for HPLC. Separation on HPLC was obtained using a mobile phase of 45%/55% water/methanol
221 for 40 minutes. A standard curve of known product concentrations was used to convert the area under the
222 curve to the amount of product produced.
223

224 **S3) Crystallization of 1-CYP17A1 complex and X-ray diffraction**

225 **Protein Crystallization and Structure Determination.** CYP17A1 was saturated with 3β-formyl-5α
226 pregnanolone-20(*R*)-isonitrile (verified by 430 nm Soret peak) and prepared for crystallization via hanging

227 drop vapor diffusion. A protein solution of 30 mg/mL CYP17A1 in 50 mM Tris-HCl (pH 7.4), 20% glycerol, 500
228 mM NaCl, and 0.2% Emulgen 913 was equilibrated against 0.1 M Tris-HCl (pH 8.5), 0.25 M $LiSO_4$, 30% PEG
229 3350, and 7% sucrose at 22 degrees C. Crystals appeared after 48 hours. Crystals were cryoprotected in
230 mother liquor supplemented with 24% glycerol and flash cooled in liquid nitrogen. Diffraction data was
231 collected at 100 K at the Standford Synchrotron Radiation Laboratory beamline 12-2. Data were processed
232 to 2.2 Angstroms using XDS[1] and Scala[2]. The structure was solved by molecular replacement using PHASER[3]
233 with a search model based on CYP17A1 complexed with 3-keto-5a-abiraterone (PDB 6WW0). Iterative model
234 building and structure refinement were accomplished using COOT[4] and Phenix.refine[5]. Validation of this
235 structure was performed in Phenix[5]. X-ray data statistics are provided in Table S1. All figures were made
236 using PyMOL[6]. Full coordinates are available as Supplemental Data 2.

237

238 **Table S1. X-ray data collection, refinement, and validation statistics.** Highest-resolution shell shown in
239 parentheses. Software used for structure refinement and visualization are referenced below.

	CYP17A1 bound to 3β-formyl-5α-pregnenolone-(R) C20-isonitrile
Data collection	
Space group	$P2_12_12_1$
Cell dimensions	
A, *b*, *c* (Å)	86.13, 152.27, 171.59
(°)	90.00, 90.00, 90.00
Resolution (Å)*	2.20
Redundancy*	13.4 (12.5)
R_{pim}*	0.075 (1.453)
Mn(I/sd)*	6.8 (0.9)
CC ½*	0.997 (0.382)
Completeness* (%)	99.6 (99.1)
Total Reflections*	1,536,253 (206,362)
Unique Reflections*	114,766 (16,489)
Refinement	
Resolution (Å)	38.96 - 2.20
No. reflections	113,087
R_{work} / R_{free}	0.216 / 0.259
Number of non-hydrogen atoms / B factor	
Protein	14871 / 55.8
Ligand	156 / 62.3
Heme	172 / 45.3
Solvent	268 / 49.6
R.M.S deviations	
Bond lengths (Å)	0.005
Bond angles (º)	0.73
Coordinate error (Maximum-likelihood) (Å)	0.30
Ramachandran plot: preferred/allowed/outliers (%)	96.7 / 3.2 / 0.11

240
241

242 **S4) Supplementary References**

243

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