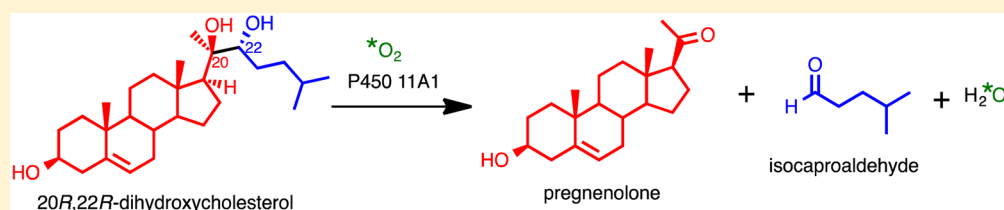


# Isotope-Labeling Studies Support the Electrophilic Compound I Iron Active Species, $\text{FeO}^{3+}$ , for the Carbon–Carbon Bond Cleavage Reaction of the Cholesterol Side-Chain Cleavage Enzyme, Cytochrome P450 11A1

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**S** Supporting Information



**ABSTRACT:** The enzyme cytochrome P450 11A1 cleaves the C20–C22 carbon–carbon bond of cholesterol to form pregnenolone, the first 21-carbon precursor of all steroid hormones. Various reaction mechanisms are possible for the carbon–carbon bond cleavage step of P450 11A1, and most current proposals involve the oxoferryl active species, Compound I ( $\text{FeO}^{3+}$ ). Compound I can either (i) abstract an O–H hydrogen atom or (ii) be attacked by a nucleophilic hydroxy group of its substrate, 20R,22R-dihydroxycholesterol. The mechanism of this carbon–carbon bond cleavage step was tested using <sup>18</sup>O-labeled molecular oxygen and purified P450 11A1. P450 11A1 was incubated with 20R,22R-dihydroxycholesterol in the presence of molecular oxygen (<sup>18</sup>O<sub>2</sub>), and coupled assays were used to trap the labile <sup>18</sup>O atoms in the enzymatic products (i.e., isocaproaldehyde and pregnenolone). The resulting products were derivatized and the <sup>18</sup>O content was analyzed by high-resolution mass spectrometry. P450 11A1 showed no incorporation of an <sup>18</sup>O atom into either of its carbon–carbon bond cleavage products, pregnenolone and isocaproaldehyde. The positive control experiments established retention of the carbonyl oxygens in the enzymatic products during the trapping and derivatization processes. These results reveal a mechanism involving an electrophilic Compound I species that reacts with nucleophilic hydroxy groups in the 20R,22R-dihydroxycholesterol intermediate of the P450 11A1 reaction to produce the key steroid pregnenolone.

## INTRODUCTION

Steroid hormone (e.g., progesterone, mineralocorticoids, glucocorticoids, androgens, estrogens) biosynthesis begins with the conversion of cholesterol (Scheme 1, 1) to pregnenolone (Scheme 1, 4), which is catalyzed by cytochrome P450 (P450) 11A1. This reaction proceeds in three steps (Scheme 1). The first two steps involve hydroxylation reactions at the C22- and C20-positions of cholesterol, and the third step is a carbon–carbon bond cleavage process. Alternatively, the 20R,22R-dihydroxycholesterol intermediate (Scheme 1, 3) has been proposed to arise from cholesterol through a C20,C22-epoxide intermediate, which in turn is hydrolyzed by water to form the 1,2-diol moiety.<sup>1</sup> Another early proposal was that a C20-hydroperoxide is formed and then is converted to 20R,22R-dihydroxycholesterol.<sup>2</sup> However, the C20- and C22-hydroxy groups of 20R,22R-dihydroxycholesterol (Scheme 1, 3) both come from molecular oxygen, which was shown from an incubation of cholesterol with adrenal microsomes in the presence of a mixture of <sup>16</sup>O<sub>2</sub> and <sup>18</sup>O<sub>2</sub>.<sup>3</sup> Therefore, the sequential hydroxylation mechanism catalyzed by Compound I ( $\text{FeO}^{3+}$ ) to form 20R,22R-dihydroxycholesterol (Scheme 1, 1 to 3) is favored through the <sup>18</sup>O-

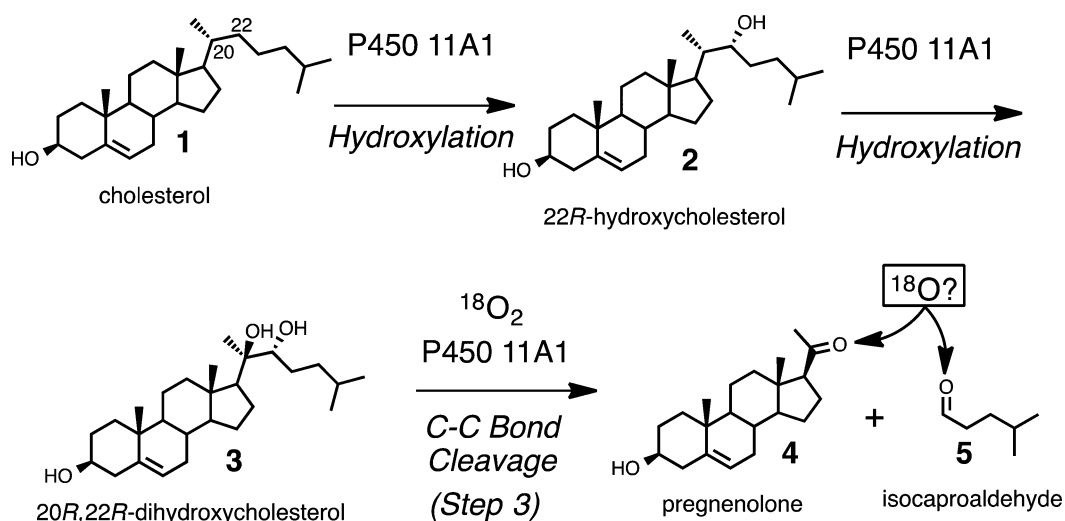
labeling studies. Moreover, through electron paramagnetic resonance and electron nuclear double resonance (EPR and ENDOR) studies, Compound I has directly been shown to be the active iron species in both the hydroxylation and C–C bond cleavage steps.<sup>4,5</sup> (The role in the C–C cleavage is supported by a 50% yield of the product pregnenolone from 20R,22R-dihydroxycholesterol.<sup>5</sup>) Despite knowledge that Compound I is the active iron intermediate in the C20–C22 bond cleavage step (Scheme 1, 3 to 4 and 5), it is still not clear *how* Compound I cleaves the side chain of cholesterol.<sup>6</sup> Several possible Compound I mechanisms for this C20–C22 lyase step are viable, and we have addressed the issue with <sup>18</sup>O-labeling studies.

Four mechanistic proposals can potentially explain the carbon–carbon bond cleavage step of P450 11A1 using Compound I as the active iron species (Scheme 2).<sup>6–10</sup> The four mechanisms can be divided into two types of reactivity: (i) one involves the *nucleophilic attack of the hydroxy group* of the substrate (Scheme 2A, 3) on the Compound I species of P450

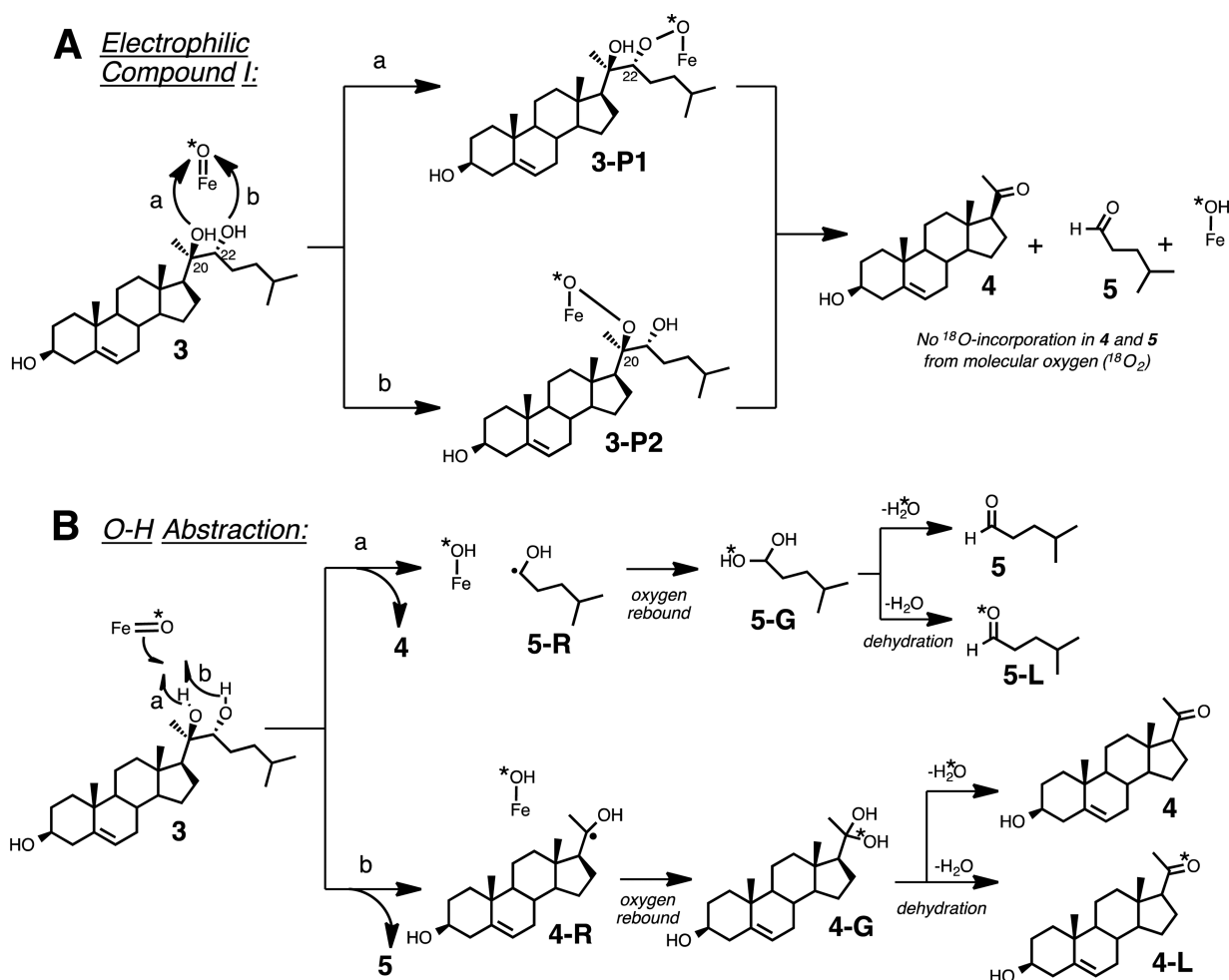
Received: May 6, 2016

Published: August 29, 2016

Scheme 1. Three Steps Catalyzed by P450 11A1, Converting Cholesterol to Pregnenolone, the First Steroid Precursor Found in Steroid Hormone Biosynthesis



Scheme 2. Four Compound I Mechanisms Grouped into Two Categories Depicting the C–C Bond Cleavage Step of P450 11A1: (A) Involves an Electrophilic Compound I Species, while (B) Shows O–H Hydrogen Atom Abstraction by Compound I; (A) Shows No Incorporation of an Oxygen into the Products (5 and 4) from Molecular Oxygen ( $^*\text{O}_2$ ), while (B) Indicates Oxygen Incorporation from Molecular Oxygen into Products 5-L and 4-L [ $^*\text{O} = ^{18}\text{O}$ ]



11A1 (Scheme 2A),<sup>11</sup> and (ii) the second type involves the O–H hydrogen atom abstraction of the substrate (Scheme 2B, 3) by Compound I (Scheme 2B).<sup>9</sup> In the latter case, where an O–H

hydrogen atom abstraction occurs, an oxygen atom (e.g.,  $^{18}\text{O}$ ) from molecular oxygen (e.g.,  $^{18}\text{O}_2$ ) would be incorporated into one of the products (Scheme 2B, 4-L or 5-L) after the oxygen

rebound step. On the other hand, in the mechanisms involving nucleophilic attack of the substrate on Compound I (i.e., “electrophilic Compound I”), no oxygen atom from molecular oxygen is incorporated into either of the products, pregnenolone or isocaproaldehyde (Scheme 2A, 4 and 5). Assuming that the dehydration step (Scheme 2B) is not stereospecific and that the resulting product isotopologues will be in an equivalent mixture (1:1 of 5:5-L or 4:4-L), we used  $^{18}\text{O}$ -labeling experiments to determine whether P450 11A1 undergoes an electrophilic Compound I mechanism (Scheme 2A) or an O–H abstraction mechanism (Scheme 2B) in the C–C bond cleavage step to form pregnenolone and isocaproaldehyde from 20R,22R-dihydroxycholesterol (Scheme 2, 3).

## EXPERIMENTAL SECTION

**Chemical Synthesis: General.** The synthesis of 20R,22R-dihydroxycholesterol was accomplished following a modified route from a previously reported procedure.<sup>12</sup> As done previously, compounds were visualized using either ceric ammonium molybdate stain (CAM, Hanessian's stain: ammonium molybdate (4.8% w/v), ceric ammonium molybdate (0.2% w/v), and  $\text{H}_2\text{SO}_4$  (6% v/v) in  $\text{H}_2\text{O}$ )<sup>13</sup> or a UV lamp (254 nm) on TLC plates with fluorescent indicator ( $\text{F}_{254}$ ).  $\text{D}_2\text{O}$  (99.9% enriched) was purchased from Sigma-Aldrich. Bruker NMR spectrometers (600 or 400 MHz) were used to acquire NMR spectra of synthesized compounds in the Vanderbilt facility. The proton and carbon chemical shifts of  $\text{CDCl}_3$  were referenced to  $\delta$  7.26 and 77.16 ppm, respectively, and solvent impurities in the spectra of the purified compounds were identified from previously reported chemical shifts.<sup>14</sup> The  $\text{D}_2\text{O}$  chemical shift in the proton NMR spectrum was referenced to  $\delta$  4.79 ppm. Bruker TopSpin 3.2 software was used to process the NMR spectra. Other experimental details regarding the synthesis of 20R,22R-dihydroxy-[21,21,21- $^3\text{H}_3$ ]-cholesterol and 4-methylpentan-1-ol picolinoyl ester (e.g., NMR spectra and  $R_f$  values of synthetic intermediates) are reported in the Supporting Information. *tert*-Butyldimethylsilyl (TBDMS) chloride was purchased from Oakwood Chemical (Columbia, SC). Silica gel (SiliaFlash F60, for purification of compounds) was purchased from Silicycle (Quebec, Canada). The ethylpyridine acetate standard (compound 8d-0) was synthesized according to our previous study.<sup>15</sup>

**Synthesis of Pregnenolone-3-OTBDMS Ether (6).** Pregnenolone (4, 5.0 g, 16 mmol), *tert*-butyldimethylsilyl chloride (6.6 g, 44 mmol, 2.8 mol equiv), and imidazole (1.0 g, 15 mmol, 0.9 mol equiv) were dissolved in  $\text{CH}_3\text{CN}$  (100 mL). The reaction was stirred for 17 h at room temperature. The reaction mixture was diluted with  $\text{H}_2\text{O}$  (200 mL) and extracted with ethyl acetate (300 mL). The organic extracts were combined and concentrated. The crude material was purified via flash column chromatography (100% hexanes to 20% ethyl acetate in hexanes, v/v) to afford pregnenolone-3-OTBDMS ether (6) (5.7 g, 13 mmol, 84%) as a white solid.  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta$  5.32 (broad s, 1H), 3.51–3.45 (m, 1H), 2.53 (apparent t,  $J = 9.0$  Hz, 1H), 2.31–2.23 (m, 1H), 2.21–2.14 (m, 2H), 2.12 (s, 3H), 2.07–2.03 (m, 1H), 2.02–1.96 (m, 1H), 1.84–1.79 (m, 1H), 1.75–1.59 (m, 4H), 1.59–1.52 (m, 3H), 1.52–1.40 (m, 3H), 1.28–1.19 (m, 1H), 1.18–1.11 (m, 1H), 1.06 (td,  $J_1 = 13.1$  Hz,  $J_2 = 3.0$  Hz, 1H), 1.00 (s, 3H), 0.99–0.94 (m, 1H), 0.89 (broad s, 9H), 0.62 (s, 3H), 0.06 (s, 6H).

**Synthesis of Dithiane 7.** *n*-Butyllithium (3.68 mL of a 2.5 M solution in hexanes, 9.2 mmol, 2.0 mol equiv) was added dropwise to dithiane (1.11 g, 9.2 mmol, 2.0 mol equiv) in THF (15 mL) at  $-30$  °C under an atmosphere of argon. The mixture was stirred for 10 min at the same temperature, and the reaction was cooled to  $-78$  °C. The reaction mixture was stirred for 2 h, and the temperature was gradually warmed to  $-20$  °C. Pregnenolone-3-OTBDMS ether 6 (2.0 g, 4.6 mmol, 1.0 mol equiv) in THF (15 mL) was added at  $-78$  °C, and the reaction was stirred for 1 h. The reaction was quenched with the addition of  $\text{H}_2\text{O}$  (100 mL) and stirred for an additional 10 min at room temperature. The reaction mixture was diluted with ethyl acetate (200 mL), and the organic layer was extracted and concentrated under reduced pressure. The crude material was purified by flash column chromatography (100%

mL increments of 100% hexanes to 10% ethyl acetate in hexanes (v/v) to 20% ethyl acetate/hexanes (v/v)) to afford dithiane 7 (550 mg, 0.64 mmol, 14%) as a white solid.  $R_f$  0.50 (hexanes:ethyl acetate, 4:1, v/v).  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta$  5.24 (m, 1H), 4.07 (broad s), 3.45–3.37 (m, 1H), 2.91–2.85 (m, 1H), 2.85–2.73 (m, 2H), 2.21–2.15 (m, 1H), 2.12–2.06 (m, 1H), 2.06–1.99 (m, 1H), 1.94–1.87 (m, 1H), 1.86–1.70 (m, 3H), 1.69–1.61 (m, 1H), 1.61–1.53 (m, 1H), 1.52–1.39 (m, 4H), 1.36 (s, 3H), 1.27–1.15 (m, 1H), 1.15–1.06 (m, 1H), 1.02–0.96 (m, 1H), 0.93 (s, 3H), 0.82 (broad s, 5H), 0.80 (broad s, 7H),  $-0.02$  (s, 6H);  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ )  $\delta$  141.3, 120.9, 76.6, 72.4, 61.2, 56.7, 55.0, 50.0, 42.9, 42.7, 40.1, 37.3, 36.5, 32.0, 31.7, 31.4, 31.3, 30.8, 26.0, 25.9, 24.1, 23.7, 21.6, 20.9, 19.4, 18.1, 13.3,  $-4.6$ .

**Synthesis of Aldehyde 8.** A modified procedure previously reported<sup>16</sup> was used.  $\text{HgCl}_2$  (345 mg, 1.27 mmol, 2.0 mol equiv) and  $\text{HgO}$  (139 mg, 0.64 mmol, 1.0 mol equiv) were added to a solution of dithiane 7 (550 mg, 0.64 mmol, 1.0 mol equiv) in  $\text{CH}_3\text{CN}$  (10 mL) and  $\text{H}_2\text{O}$  (0.1 mL). The reaction mixture was heated under reflux for 2.5 h. The reaction mixture was filtered through a short pad of silica gel with ethyl acetate (1 L). The filtrate was concentrated, and the crude material was purified via flash column chromatography to afford the aldehyde 8 (90 mg, 0.20 mmol, 31%) as a white solid.  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta$  9.56 (s, 1H), 5.31–5.30 (m, 1H), 3.50–3.45 (m, 1H), 2.29–2.23 (m, 1H), 2.18–2.11 (m, 2H), 1.99–1.93 (m, 1H), 1.82–1.75 (m, 2H), 1.74–1.68 (m, 2H), 1.68–1.59 (m, 2H), 1.57–1.41 (m, 7H), 1.35 (s, 3H), 1.33–1.22 (m, 9H), 1.22–1.08 (m, 2H), 1.07–1.01 (m, 2H), 1.00 (s, 3H), 0.98–0.91 (m, 2H), 0.88 (s, 9H), 0.81 (d,  $J = 6.9$  Hz, 3H), 0.79 (s, 3H), 0.05 (s, 6H).

**Synthesis of Grignard Adduct 9.** Isopentylmagnesium bromide (21.7 mL, 2.5 M solution in diethyl ether, 43 mmol, 20 mol equiv) was added to a solution of aldehyde 8 (1.0 g, 2.2 mmol, 1.0 mol equiv) in THF (100 mL) at  $-78$  °C. The reaction mixture was stirred for 1.5 h. The reaction was diluted with  $\text{H}_2\text{O}$  (100 mL) at  $-78$  °C, and the reaction mixture was left to warm to room temperature. The reaction mixture was extracted with  $\text{CH}_2\text{Cl}_2$  (2  $\times$  250 mL). The combined organic extracts were concentrated and purified by flash column chromatography (300 mL increments of 100% hexanes to 10% ethyl acetate in hexanes (v/v) to 30% ethyl acetate in hexanes (v/v)) to yield the diol 9 (220 mg, 0.41 mmol, 19%) as a white solid.  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta$  5.32–5.31 (m, 1H), 3.51–3.46 (m, 1H), 3.41–3.37 (m, 1H), 2.29–2.24 (m, 1H), 2.21 (d,  $J = 3.2$  Hz, 1H), 2.17 (ddd,  $J_1 = 13.2$  Hz,  $J_2 = 5.1$  Hz,  $J_3 = 2.3$  Hz, 1H), 2.12 (dt,  $J_1 = 12.5$  Hz,  $J_2 = 3.6$  Hz, 1H), 2.00–1.95 (m, 1H), 1.85–1.79 (m, 2H), 1.75–1.69 (m, 1H), 1.68–1.61 (m, 1H), 1.58–1.40 (m, 11H), 1.26–1.22 (m, 2H), 1.22 (s, 3H), 1.21–1.18 (m, 1H), 1.17–1.11 (m, 1H), 1.06–1.03 (m, 1H), 1.02–0.96 (m, 1H), 1.00 (s, 3H), 0.94–0.92 (m, 1H), 0.90 (s, 3H), 0.88 (broad s, 12H), 0.06 (s, 6H).

**Synthesis of 20R,22R-Dihydroxycholesterol (3).** To a stirring solution of diol 9 (220 mg, 0.41 mmol) in  $\text{CH}_3\text{OH}:\text{CH}_2\text{Cl}_2$  (40 mL, 1:1, v/v) was added conc HCl (12 M, 0.02 mL). The reaction was stirred for 15 min, and the mixture was concentrated under reduced pressure. The resulting crude material was purified by flash column chromatography (100% hexanes to 50% hexanes/ethyl acetate, v/v) to yield 20R,22R-dihydroxycholesterol (3, 50 mg, 0.12 mmol, 29%) as a white solid.  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta$  5.34–5.33 (m, 1H), 3.55–3.46 (m, 1H), 3.39–3.35 (m, 1H), 2.33–2.26 (m, 2H), 2.25–2.19 (m, 1H), 2.12 (dt,  $J_1 = 12.7$  Hz,  $J_2 = 3.5$  Hz, 1H), 1.99–1.94 (m, 1H), 1.94–1.92 (m, 1H), 1.86–1.79 (m, 3H), 1.67–1.59 (m, 2H), 1.58–1.38 (m, 10H), 1.20 (s, 3H, C-21), 1.19–1.10 (m, 3H), 1.10–1.03 (m, 1H), 1.00 (s, 3H, C-19), 0.90 (d,  $J = 7$  Hz, 6H), 0.89 (s, 3H) ( $^1\text{H}$  NMR characterization matched the previously reported synthesis<sup>12</sup>);  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ )  $\delta$  140.9, 121.7, 77.5, 76.5, 71.8, 56.8, 54.9, 50.2, 43.3, 42.4, 40.3, 37.4, 36.6, 36.5, 31.9, 31.7, 31.4, 29.3, 28.2, 24.1, 23.1, 22.5, 22.1, 20.5, 19.5, 13.7.

**Synthesis of [21,21,21- $^2\text{H}_3$ ]-Pregnenolone-3-OTBDMS Ether (6a).** NaH (0.89 g, 35 mmol, 3.1 mol equiv) was added as a solid to a solution of pregnenolone-3-*O*-*tert*-butyldimethylsilyl ether 6 (4.9 g, 11 mmol, 1.0 mol equiv) in THF (200 mL) at 0 °C. The reaction flask was evacuated and backfilled with argon.  $\text{D}_2\text{O}$  (5 mL) was added, and the reaction was allowed to stir for 8 days at room temperature. The reaction mixture was concentrated and purified by flash column chromatography (100%



hexanes to 10% ethyl acetate in hexanes (v/v) to 30% ethyl acetate in hexanes (v/v) to afford [21,21,21-<sup>2</sup>H<sub>3</sub>]-pregnenonolone-3-*O*-*tert*-butyldimethylsilyl ether (**6a**, 4.0 g, 9.2 mmol, 84%) as a white solid. The proton NMR spectrum showed a small peak corresponding to the 21-methyl protons ( $\delta$  2.12), which integrated to 0.31, while the 18-methyl protons ( $\delta$  0.63) integrated to 3.00 and the 17-proton ( $\delta$  2.53) integrated to 0.96. <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>)  $\delta$  5.32–5.31 (m, 1H), 3.52–3.44 (m, 1H), 2.53 (apparent t,  $J$  = 8.8 Hz, 1H), 2.31–2.21 (m, 1H), 2.21–2.13 (m, 2H), 2.12 (s, 0.3H), 2.11–2.01 (m, 1H), 2.07–1.96 (m, 2H), 1.82 (dt,  $J_1$  = 13.2 Hz,  $J_2$  = 3.4 Hz, 1H), 1.76–1.40 (m, 8H), 1.29–1.02 (m, 3H), 1.00 (s, 3H), 0.99–0.93 (m, 1H), 0.89 (s, 9H), 0.63 (s, 3H), 0.06 (s, 6H).

**Synthesis of Isocaproaldehyde (5) via Swern Oxidation.** Dimethyl sulfoxide (20.2 mL, 284 mmol, 2.9 mol equiv) was added to oxalyl chloride (12.6 mL, 147 mmol, 1.5 mol equiv) in CH<sub>2</sub>Cl<sub>2</sub> (500 mL) at –78 °C. After 10 min, 4-methylpentan-1-ol (12.3 mL, 10 g, 97.9 mmol) was added. Triethylamine (41 mL, 294 mmol, 3.0 mol equiv) was added with the use of an addition funnel. After 1 h, the reaction was diluted with H<sub>2</sub>O (100 mL). The reaction mixture was washed with HCl (1 M, 3 × 300 mL), and the organic layer was dried with anhydrous MgSO<sub>4</sub> and concentrated to yield isocaproaldehyde (**5**) as a clear oil (9 g, 90 mmol, 92%). The purity of the crude material was determined by <sup>1</sup>H NMR and used for subsequent experiments (i.e., *gem*-diol/aldehyde equilibrium in D<sub>2</sub>O and yeast alcohol dehydrogenase reduction rates). <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>)  $\delta$  9.77 (t,  $J$  = 2.0 Hz, 1H), 2.42 (td,  $J_1$  = 7.4 Hz,  $J_2$  = 1.9 Hz, 2H), 1.62–1.54 (m, 1H), 1.52 (apparent q,  $J$  = 7.4 Hz, 2H), 0.90 (d,  $J$  = 6.6 Hz, 6H); <sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>)  $\delta$  203.1 (C1), 42.1 (C2), 30.9 (C3, –CH<sub>2</sub>–), 27.8 (C4, –CH–), 22.5 (C5 and C6, –CH<sub>3</sub>).

**Determination of the *gem*-Diol/Aldehyde Equilibrium of Isocaproaldehyde.** An <sup>1</sup>H NMR spectrum of a solution of isocaproaldehyde in potassium phosphate buffer (D<sub>2</sub>O) was acquired. The following buffer solution was prepared: 100 mM potassium phosphate in H<sub>2</sub>O (pH = 7.8, 1 mL) was lyophilized, and D<sub>2</sub>O (1 mL) was added (pD 7.4).<sup>17–19</sup> A solution (0.07 mL) of isocaproaldehyde (2.7 mg/1 mL of D<sub>2</sub>O) was added to buffer A (0.63 mL), and the <sup>1</sup>H NMR spectrum was acquired on a 600 MHz NMR spectrometer.

**Enzyme Incubations.** <sup>18</sup>O<sub>2</sub> gas (99% <sup>18</sup>O), yeast alcohol dehydrogenase (369 units/mg, one unit converted 1  $\mu$ mol of ethanol to acetaldehyde min<sup>–1</sup> at pH 8.8 and 25 °C, CAS Registry No. 9031-72-5), glucose 6-phosphate dehydrogenase (Type XV, 210 units/mg, one unit oxidized 1  $\mu$ mol of glucose 6-phosphate to 6-phospho-D-gluconate min<sup>–1</sup> in the presence of NADP<sup>+</sup> at pH 7.4 at 25 °C, CAS Registry No. 9001-40-5), and NADH were purchased from Sigma-Aldrich. A 310 mM aqueous solution of (2-hydroxypropyl)- $\beta$ -cyclodextrin ( $\beta$ -CD) was made for solubilization of substrates in enzymatic incubations.<sup>20</sup> An OLIS/Aminco DW2 spectrophotometer (On-Line Instrument Systems, Bogart, GA) was used to measure P450 concentrations.<sup>21</sup>

**Human Cytochrome P450 11A1 Expression.** A codon-optimized P450 11A1 plasmid was purchased from Genewiz (South Plainfield, NJ) (nucleotide sequence provided in [Supporting Information](#)). The P450 11A1 plasmid (pCWori<sup>+</sup> vector<sup>22</sup>) was co-expressed with another plasmid containing the *Escherichia coli* molecular chaperone GroEL/ES in *E. coli* JM109 cells in Luria–Bertani (LB) media (100 mL) containing ampicillin (100  $\mu$ g/mL) and kanamycin (50  $\mu$ g/mL). This pre-culture was incubated overnight at 37 °C and 220 rpm. The scaled-up expression of P450 11A1 (i.e., bulk culture) was grown in 4.5 L (6 × 750 mL/2.8 L flask) of Terrific Broth (TB) media supplemented with the same concentrations of ampicillin and kanamycin as the pre-culture, along with bactopeptone (2 g/L), trace elements (250  $\mu$ L/L of culture),<sup>23</sup> NaCl (1 mM), thiamine (1 mM), glycerol (4 mL/L), and the pre-culture (dilution of 1:100, v/v). The bulk cultures were incubated at 37 °C and 225 rpm until the OD<sub>600</sub> reached 0.7–0.75 (~6 h). P450 expression was induced by the addition of isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) (1.0 mM),  $\delta$ -amino-levalulinic acid (1.0 mM), and arabinose (1.0 mM). The induced cultures were incubated for 42 h at 28 °C and 190 rpm in a Multifors incubator. The cells were harvested by centrifugation (3500g for 20 min).

**Cytochrome P450 11A1 Purification.** All steps were carried out at 4 °C. The cell pellets from bulk cultures (6 L) were resuspended in 300 mL of TES buffer (100 mM Tris-acetate (pH 7.5) containing 0.5 M

sucrose and 0.5 mM EDTA) for each pellet derived from 1 L of bulk culture. Lysozyme (2 mg/L of bulk culture) was added, and the suspension was incubated on ice for 60 min and then centrifuged at 3500g for 20 min. The spheroplasts were resuspended in sonication buffer (50 mL of 100 mM potassium phosphate (pH 7.5) containing 16% glycerol (v/v), 9 mM magnesium acetate, 100  $\mu$ M dithiothreitol (DTT)), 1.0 mM phenylmethylsulfonyl fluoride (PMSF), and two protease inhibitor tablets (Roche) for each 1 L of bulk culture. After sonication at 0 °C, the contents were centrifuged at 12000g for 15 min. The recovered supernatant was centrifuged at 140000g for 60 min. The pellet was collected, homogenized, and stirred in solubilization buffer (600 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol (v/v), 0.1 mM EDTA, 10 mM  $\beta$ -mercaptoethanol, 0.5 M KCl, and 1% (w/v) sodium 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)) overnight at 4 °C. The resulting solution was centrifuged at 140000g for 60 min, and the supernatant was loaded onto a Ni-NTA (nitriloacetic acid) column (1.5 × 8 cm), which had been previously equilibrated with 100 mM potassium phosphate buffer (pH 7.5) containing glycerol (20%, v/v), 10 mM  $\beta$ -mercaptoethanol, 0.5 M KCl (0.5 M), and sodium CHAPS (1% w/v). The column was washed with the same equilibration buffer containing 20 mM imidazole and 2.8 mM pentaethylene glycol monoocetyl ether (C8E5) detergent. The concentration of the imidazole was raised to 200 mM in the same buffer to elute P450 11A1. The eluted P450 11A1 fractions (based on A<sub>418</sub>) were combined and dialyzed against 2 L of dialysis buffer (100 mM potassium phosphate (pH 7.5) containing 20% glycerol (v/v)) three times for 24 h. The concentration of P450 11A1 was measured using ferrous vs ferrous-CO difference spectra,<sup>21</sup> and a typical yield of the protein was 135 nmol. The dialyzed protein was divided into 150  $\mu$ L aliquots and stored at –20 °C until use for incubations.

**Adrenodoxin (Adx) Expression.** *E. coli* DH5 $\alpha$  cells were transformed with the plasmid pBA1159 for bovine Adx expression.<sup>24–27</sup>

A pre-culture was prepared by inoculating a single colony into 10 mL of LB media containing 100  $\mu$ g/mL of ampicillin and grown at 37 °C with gyrorotary shaking at 220 rpm. The pre-culture (5 mL) was then added to bulk culture medium (500 mL of TB media containing ampicillin (100  $\mu$ g/mL), thiamine (340  $\mu$ g/mL), and trace elements<sup>23</sup> (0.025% v/v). The cultures were incubated at 37 °C with shaking at 220 rpm for 4 h. IPTG (1 mM) was added to induce Adx expression. The induced cultures were incubated for 30 °C for 24 h. The cells were pelleted by centrifugation (3000g) for 5 min, and the pelleted cells were sonicated in 10 mM potassium phosphate buffer (pH 7.4). The sonicated material was centrifuged at 100000g for 30 min to remove cell debris, and the supernatant was used for the purification step.

**Adx Purification.** The supernatant (*vide supra*) was loaded onto a DEAE-Sepharose column (2.5 × 20 cm) that had been equilibrated with 50 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and washed with 1 L of the equilibration buffer. The bound Adx was eluted with the same buffer containing NaCl (170 mM then 300 mM). The fractions that absorbed at 414 nm from the 170 mM pool were highly purified, based on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (15%, w/v, gel). The fractions from the 170 mM pool, (pooled based on A<sub>414</sub> readings) were dialyzed twice against 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and applied to a DEAE-Sepharose column (2.5 × 10 cm). The column was washed with the dialysis buffer and eluted with the same buffer containing NaCl (500 mM). Fractions containing adrenodoxin were combined based on A<sub>414</sub> measurements. The impure fractions from the DEAE steps were purified further with gel filtration chromatography on a Sephadex G-75 (2.5 × 100 cm) using 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA ( $\leq$ 20 mL per run). The total yield (from 6 L) was 25.7  $\mu$ mol of Adx, which was stored in 1 mL aliquots at –70 °C.

**Adrenodoxin Reductase (ADR) Expression.** *E. coli* DH5 $\alpha$  cells were transformed with the plasmid containing bovine ADR<sup>28</sup> in a pCWori<sup>+</sup> expression vector<sup>22,26,27</sup> with a (His)<sub>6</sub> tag added to the C-terminus of the cDNA to facilitate protein purification. LB media (10 mL containing 100  $\mu$ g/mL ampicillin) was inoculated with a single colony and incubated overnight at 37 °C with gyrorotary shaking at 220

rpm. The overnight culture (5 mL) was used to inoculate 500 mL of TB media containing ampicillin (100  $\mu\text{g}/\text{mL}$ ), thiamine (340  $\mu\text{g}/\text{mL}$ ), and trace elements (0.025%, v/v).<sup>23</sup> The cultures were incubated at 37 °C and 220 rpm for 4 h. ADR expression was induced with the addition of 1.0 mM IPTG. Following induction, the induced cultures were incubated at 30 °C and 220 rpm for another 24 h.

**ADR Purification.** The cell cultures were harvested by centrifugation (3000g) for 10 min. The cell pellet was sonicated in potassium phosphate buffer (100 mM, pH 7.4) containing EDTA (0.1 mM), glycerol (20% v/v), and protease inhibitor tablets (Roche). The sonicated solution was centrifuged (100000g) for 30 min to remove the cell debris. The supernatant was loaded on a Ni-NTA column, which was then washed with the sonication buffer containing 20 mM imidazole. ADR was eluted with the sonication buffer containing 200 mM imidazole. The eluted ADR was dialyzed against 40 volumes of 100 mM potassium phosphate buffer (pH 7.4) containing glycerol (20% v/v) and 0.1 mM EDTA three times to remove the imidazole and then stored at -80 °C.

**Cytochrome P450 17A1.** Human P450 17A1 was expressed and purified as described elsewhere.<sup>15</sup>

**Cytochrome  $b_5$  and NADPH-P450 Reductase.** Recombinant human cytochrome  $b_5$ <sup>29</sup> and NADPH P450 rat reductase<sup>30</sup> were expressed in *E. coli* and purified as described previously.

**P450 11A1 Coupled Assay with Yeast Alcohol Dehydrogenase: Isocaproaldehyde Analysis.** In preliminary assays, we measured a  $k_{\text{cat}}$  of 4.5  $\text{s}^{-1}$  and a  $K_m$  (isocaproaldehyde) of 32  $\mu\text{M}$  for the reduction of isocaproaldehyde by yeast alcohol dehydrogenase (utilizing NADH oxidation at 340 nm). P450 11A1 (450 pmol), Adx (168 pmol), ADR (708 pmol), yeast alcohol dehydrogenase (5 mg) and  $L\text{-}\alpha\text{-}1,2\text{-dilauroyl-sn-glycero-3-phosphocholine}$  (50  $\mu\text{g}$ ) were added to a Thunberg tube containing 800  $\mu\text{L}$  of 100 mM potassium phosphate buffer (pH 7.4) followed by the addition of either cholesterol (52 nmol) or 20R,22R-dihydroxycholesterol (142 nmol), which was dissolved in a solution of  $\beta\text{-CD}$  (310 mM). The cap of the Thunberg tube contained the NADPH-regenerating system<sup>31</sup> (100  $\mu\text{L}$  of the following mixture: 8.3  $\mu\text{mol}$  of glucose 6-phosphate, 4  $\mu\text{g}$  of glucose 6-phosphate dehydrogenase, 333 nmol of NADP<sup>+</sup> plus NADH (100  $\mu\text{L}$  of a 100 mM solution). The Thunberg tube was purged with argon on a gas train by alternate evacuation with reduced pressure and backfilling with argon (repeated 10 times). Before injection of <sup>18</sup>O<sub>2</sub> gas, the tube was evacuated with reduced pressure for 3 min. After the <sup>18</sup>O<sub>2</sub> gas was injected, the contents of the tube and cap were mixed, and the incubation was shaken at 50 rpm and 37 °C. After 15 min, the tube was placed on ice, and CH<sub>2</sub>Cl<sub>2</sub> (5 mL) was added. The organic layer was extracted and taken to dryness under a stream of N<sub>2</sub>.

**Derivatization with Picolinoyl Chloride.** A solution of picolinoyl chloride in THF (200  $\mu\text{L}$  of a 5 mg/mL solution) was added to the dried incubation extracts from the P450 11A1-yeast alcohol dehydrogenase-<sup>18</sup>O<sub>2</sub> reaction (*vide supra*). The solution was mixed using a vortex device for 5 s and dried under a stream of N<sub>2</sub>. The samples were dissolved in CH<sub>3</sub>CN (75  $\mu\text{L}$ ) for LC-MS analysis.

**P450 11A1 Coupled Assay with P450 17A1: Pregnenolone Analysis.** P450 11A1 (900 pmol), Adx (840 pmol), ADR (900 pmol), P450 17A1 (5.6 nmol), cytochrome  $b_5$  (11 nmol), NADPH P450 reductase (6.2 nmol), and  $L\text{-}\alpha\text{-}1,2\text{-dilauroyl-sn-glycero-3-phosphocholine}$  (50  $\mu\text{g}$ ) were added to a Thunberg tube containing 2 mL of 50 mM potassium phosphate buffer (pH 7.4). Cholesterol (1, 160 nmol in 1.5  $\mu\text{mol}$   $\beta\text{-CD}$ ) or 20R,22R-dihydroxy-[21,21,21-<sup>2</sup>H<sub>3</sub>]-cholesterol (3a, 190 nmol in 1.5  $\mu\text{mol}$  of  $\beta\text{-CD}$ ) was added to the Thunberg tube. The cap contained the NADPH-regenerating system.<sup>31</sup> The Thunberg tube was purged with argon on a gas train by evacuation with reduced pressure and backfilling with argon (repeated 10 times). Before injection of <sup>18</sup>O<sub>2</sub> gas, the tube was evacuated with reduced pressure for 3 min. After the <sup>18</sup>O<sub>2</sub> gas was injected, the contents of the tube and cap were mixed, and the incubation was shaken at 50 rpm at 37 °C. After 60 min, the tube was placed on ice, and CH<sub>2</sub>Cl<sub>2</sub> (5 mL) was added. HCl (3 M, 1 mL, chilled to 0 °C) was added, and the solution was mixed with a vortex device and centrifuged at 1500g for 1 min. The organic layer was extracted and dried with anhydrous MgSO<sub>4</sub> (~50 mg). The MgSO<sub>4</sub> was filtered using a Pasteur pipet with a cotton plug. Diazo reagent (*vide infra*) was added to

the CH<sub>2</sub>Cl<sub>2</sub> extract of the reaction mixture, and the resulting solution was dried under a stream of nitrogen. The samples were dissolved in CH<sub>3</sub>CN (75  $\mu\text{L}$ ) and analyzed by LC-MS.

**Derivatization with Diazo Reagent.** The diazo reagent was generated by the addition of NaOH (2 mL of a 30% w/v solution in H<sub>2</sub>O) to the nitrosoarene precursor in diethyl ether (~5 mg in 2 mL) in a borosilicate screw cap vial.<sup>15,32</sup> The two layers were mixed with a vortex device, and the diethyl ether layer was extracted and dried with anhydrous MgSO<sub>4</sub> (~50 mg). The organic layer was filtered through a Pasteur pipet with a cotton plug and was used immediately to treat the incubation extracts prepared above. **Caution: Nitrosoarene and diazo compounds are carcinogenic and explosive! Extra care was used when handling the compounds. Double-glove protection and proper personal protective equipment (safety glasses and a laboratory coat) are required. No ground glass joints were used when handling these compounds. A solution of acetic acid (50 mL) chilled on ice was set aside to rinse all of the glassware to react any remaining diazo compound generated. All of the experiments were performed in a fume hood.**

**Derivatization of P450 11A1/P450 17A1 Incubation Extracts with Picolinoyl Chloride.** The CH<sub>3</sub>CN solution remaining after the LC-MS analyses was added to a solution of picolinoyl chloride in THF (200  $\mu\text{L}$  of a 5 mg/mL solution). The solution was mixed using a vortex device for 5 s and dried under a stream of N<sub>2</sub>. The samples were dissolved in CH<sub>3</sub>CN (75  $\mu\text{L}$ ) for LC-MS analysis. These samples were used to analyze the deuterium content of 17-hydroxypregnenolone picolinoyl esters (Supporting Information).

**LC-MS Analysis: General.** An Acquity UPLC system was used for the LC-MS analysis of compounds from the enzymatic incubations. Thermo XCalibur Qual Browser 2.2 software (ThermoFisher Scientific) was used to process LC-MS data. An LTQ Orbitrap XL mass spectrometer was used to detect derivatized products in the ESI positive mode with a 100 000 resolution setting. LTQ Tune Plus software was used to calibrate and tune the instrument. The mass window scanned was  $m/z$  50–800.

**Calibration of the Mass Spectrometer (LTQ Orbitrap XL).** The mass spectrometer was calibrated before each sequence using Pierce LTQESI positive ion calibration solution (ThermoFisher) containing a mixture of caffeine, MRFA (the peptide methionine–arginine–phenylalanine–alanine), and Ultramark 1621 in CH<sub>3</sub>CN, CH<sub>3</sub>CO<sub>2</sub>H, and CH<sub>3</sub>OH by direct infusion (10  $\mu\text{L}/\text{min}$ ). The mass spectrometer was first tuned for MRFA ( $m/z$  524), and then the tube lens voltage was adjusted to 145 V to fragment caffeine ( $m/z$  195 to 138). The tune conditions were as follows: sheath gas flow rate, 19; aux gas flow rate, 3; sweep gas flow rate, 0; spray voltage, 4.00 kV; capillary temperature, 300 °C; capillary voltage, 4.00 V; tube lens, 145.00 V. The exact masses used to calibrate the instrument were ( $m/z$ ) 138.066190, 195.087652, 524.264964, 1121.997024, 1221.990636, 1321.984249, 1421.977862, 1521.971475, 1621.965088, and 1721.958701.

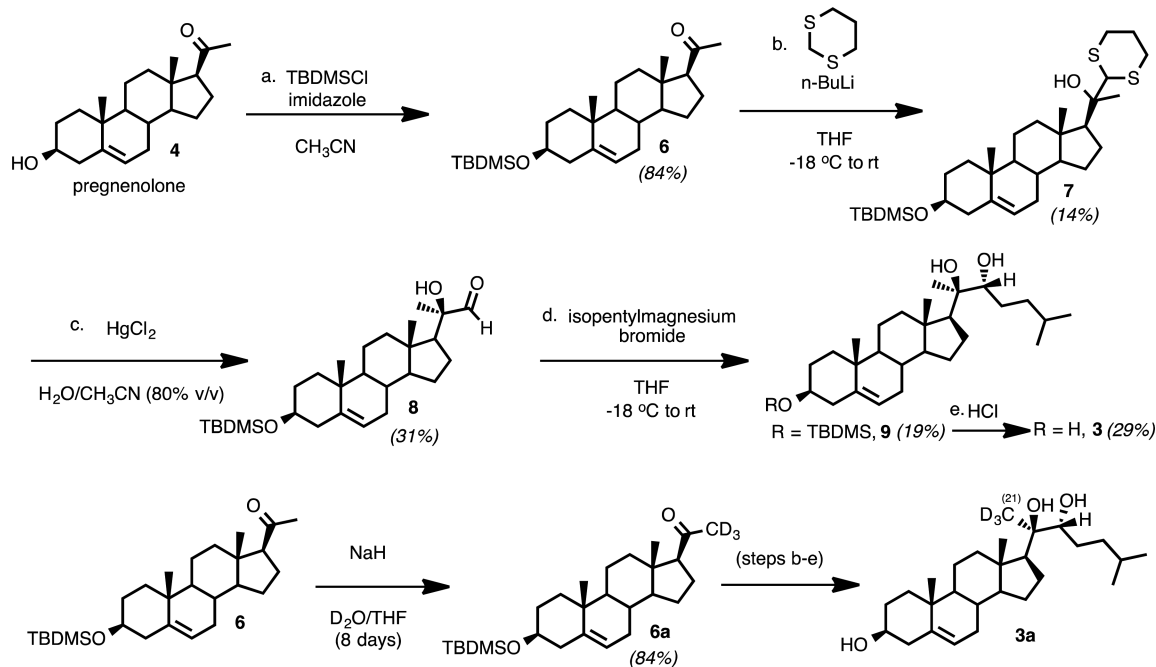
**Mass Spectrometer Tune Conditions.** The following tune settings were used to detect the picolinoyl ester of the derivatized isocaproaldehyde product (compound 15) in the APCI positive mode with the following settings (acquired after tuning with the synthetic standard, Supporting Information): vaporizer temperature, 450 °C; sheath gas flow rate, 50; aux gas flow rate, 5; sweep gas flow rate, 5; discharge current, 5.00  $\mu\text{A}$ ; capillary temperature, 275 °C; capillary voltage, 12.00 V; tube lens, 50.00 V.

The tune settings used to detect pregnenolone in the APCI positive mode were as follow: vaporizer temperature, 450 °C; sheath gas flow rate, 50; aux gas flow rate, 5; sweep gas flow rate, 5; discharge current, 5.00  $\mu\text{A}$ ; capillary temperature, 275 °C; capillary voltage, 43.00 V; tube lens, 55.00 V.

The pyridine acetate of the derivatized acetic acid product (compound 18) was detected in ESI positive mode with the following tune settings (acquired after tuning with the synthetic standard): sheath gas flow rate, 15; aux gas flow rate, 5; sweep gas flow rate, 0; spray voltage, 4.00 kV; capillary temperature, 300 °C; capillary voltage, 16.00 V; tube lens, 30.00 V.

The tune settings for detecting picolinoylated pregnenolone and monopicolinoylated-20,22-dihydroxycholesterol (Supporting Information) using ESI positive mode were as follows: sheath gas flow rate, 8;

**Scheme 3. Synthesis of 20R,22R-Dihydroxycholesterol (3) and 20R,22R-Dihydroxy-[21,21,21-<sup>2</sup>H<sub>3</sub>]-cholesterol (3a) Substrates for <sup>18</sup>O<sub>2</sub> Incubation with P450 11A1**



aux gas flow rate, 0; sweep gas flow rate, 0; spray voltage, 5.00 kV; capillary temperature, 275 °C; capillary voltage, 24.00 V; tube lens, 70.00 V.

**Liquid Chromatography (LC) Methods: General.** A Phenomenex Kinetex octylsilane reversed-phase column (2.6 μm, C<sub>8</sub>, 100 Å, 100 mm × 2.1 mm) was used to separate all of the analytes. For every LC method, the column temperature was held at 40 °C. In order to maintain consistent retention times in each run in the sequence, the column was equilibrated with the initial gradient conditions for at least 10 min until the delta value (i.e., maximum and minimum difference in pressure) was <30 psi. Two to three sequential injections of the standards were made until the retention times remained stable. LC-MS spectra were acquired in full scan mode: *m/z* 100–1000 (for yeast alcohol dehydrogenase coupled assays), *m/z* 50–700 or 50–800 (for P450 17A1 coupled assays). ICIS peak algorithm detection was used. Peaks in LC-MS data were acquired in profile mode.

**LC Method To Detect Picolinoyl Ester of Isocaproaldehyde Derivative 15: Method A.** The LC method used to detect the derivatized isocaproaldehyde product (compound 15) and pregnenolone were run with solvents A (10 mM ammonium acetate, H<sub>2</sub>O:CH<sub>3</sub>CN, 95:5, v/v) and B (10 mM ammonium acetate, CH<sub>3</sub>CN) with the following gradient: 95% A from 0 to 1.0 min, 95% A to 100% B from 1.0 to 4.0 min, 100% B from 4.0 to 5.0 min, 100% B to 95% A from 5.0 to 5.5 min, 95% A (all v/v) from 5.5 to 8.0 min with a flow rate of 0.3 mL/min.

**LC Method To Detect Pyridine Acetate Derivative 18: Method B.** The LC method to detect the derivatized acetic acid product (compound 18) and picolinoylated pregnenolone was run with solvents A (10 mM ammonium formate, H<sub>2</sub>O) and B (10 mM ammonium formate, CH<sub>3</sub>CN:H<sub>2</sub>O, 95:5, v/v) with the following gradient: 100% A from 0 to 1.0 min, 100% A to 100% B from 1.0 to 4.0 min, 100% B from 4.0 to 5.2 min, 100% B to 100% A from 5.2 to 5.3 min, 100% A (all v/v) from 5.3 to 8.0 min with a flow rate of 0.3 mL/min.

## RESULTS

**Overall Strategy.** In order to determine if an oxygen atom from molecular oxygen is incorporated into either of the C–C bond cleavage products of P450 11A1 (Scheme 2, 4 and 5), incubations were performed using 20R,22R-dihydroxycholesterol in the presence of purified P450 11A1 and <sup>18</sup>O<sub>2</sub>.

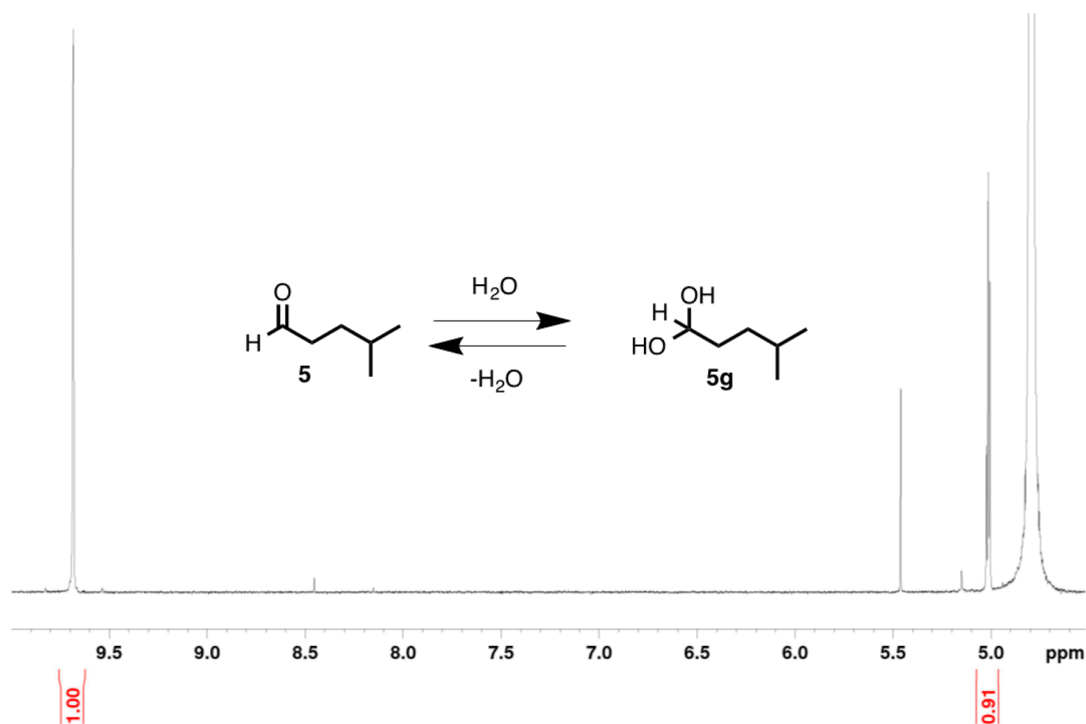
Because the carbonyl oxygens in pregnenolone and isocaproaldehyde are readily exchangeable with water, it was necessary to convert the enzymatic products (Scheme 2, 4 and 5) to different chemical entities so that the oxygens would be incapable of exchanging with water. The derivatized C–C bond cleavage products (4 and 5) would be detected by LC-MS for analysis of <sup>18</sup>O content.

**Synthesis of Substrates: 20R,22R-Dihydroxycholesterol (3) and Its Deuterated Isotopologue, 20R,22R-Dihydroxy-[21,21,21-<sup>2</sup>H<sub>3</sub>]-cholesterol (3a).** The synthesis of 20R,22R-dihydroxycholesterol (3) commenced with pregnenolone as the starting material (Scheme 1). The 3-hydroxy group was protected as a TBDMS ether (Scheme 3, 7). One-carbon homologation of pregnenolone-TBDMS ether (7) with a 1,3-dithiane anion nucleophile followed. The resulting dithiane (8) was converted to the aldehyde (9) with mercury-promoted hydrolysis. Grignard addition onto the aldehyde with isopentylmagnesium bromide yielded the 1,2-diol (10). The C3-oxygen was deprotected by removal of the TBDMS group in CH<sub>3</sub>OH with HCl to furnish 20R,22R-dihydroxycholesterol (3). The <sup>1</sup>H NMR spectrum matched a previously reported spectrum.<sup>33</sup>

Additionally, 20R,22R-dihydroxy-[21,21,21-<sup>2</sup>H<sub>3</sub>]-cholesterol (3a) was obtained by synthesizing [21,21,21-<sup>2</sup>H<sub>3</sub>]-pregnenolone-3-*tert*-butyldimethylsilyl ether (6a) and performing the same steps to access 20R,22R-dihydroxy-[21,21,21-<sup>2</sup>H<sub>3</sub>]-cholesterol from the deuterated silyl ether (Scheme 3). Pregnenolone-3-OTBDMS (6) was deuterated on the C21 position by reacting with NaH and D<sub>2</sub>O in THF at room temperature for 8 days (Scheme 3, 6 to 6a). These reaction conditions resulted in the regioselective deuteration of the C21 position over the C17 position through the formation of the kinetic enolate (see Supporting Information for <sup>1</sup>H NMR spectrum).

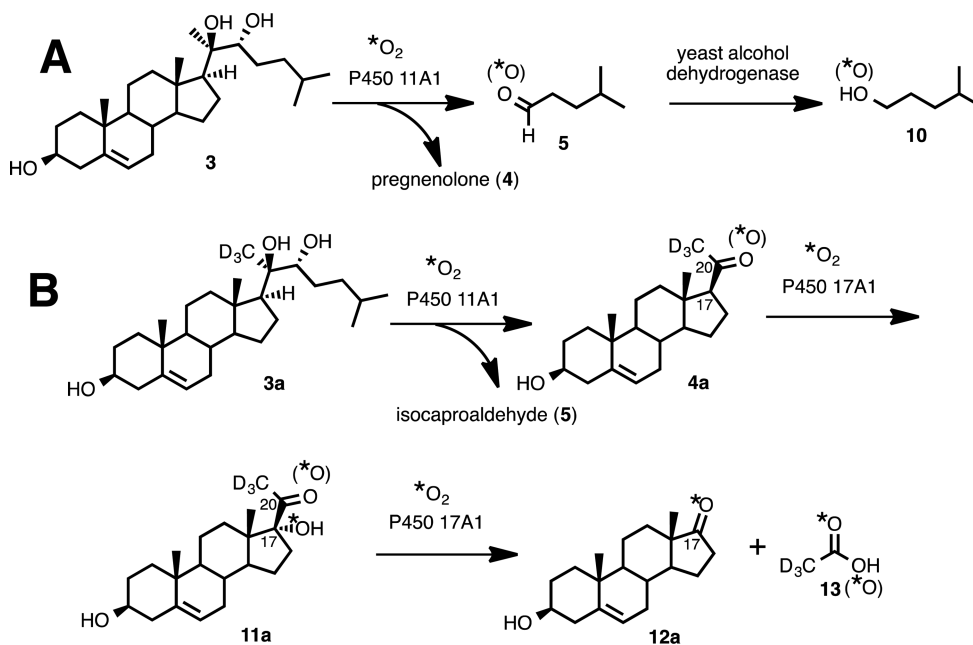
**gem-Diol/Aldehyde Equilibrium of Isocaproaldehyde (5) in D<sub>2</sub>O.** The equilibrium between the aldehyde and *gem*-diol forms of isocaproaldehyde in D<sub>2</sub>O was measured by proton





**Figure 1.**  $^1\text{H}$  NMR spectrum of isocaproaldehyde (**5**) in potassium phosphate buffer in  $\text{D}_2\text{O}$  (pD 7.4). The aldehyde ( $\delta$  9.68) and the *gem*-dihydroxymethine ( $\delta$  5.01, triplet,  $J = 5.4$  Hz) protons are shown (600 MHz).

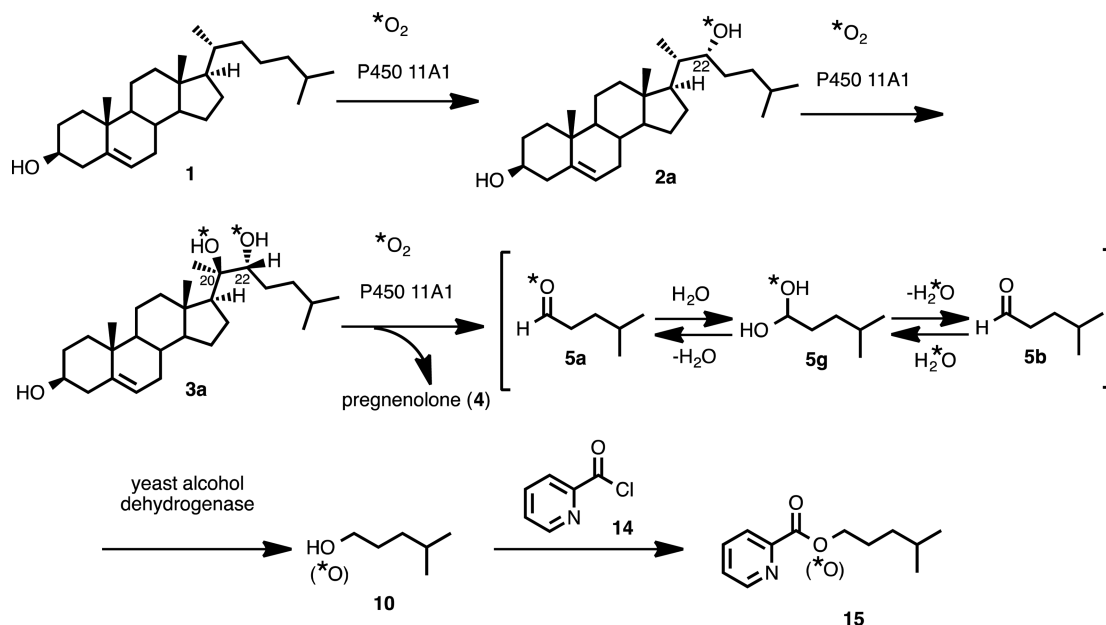
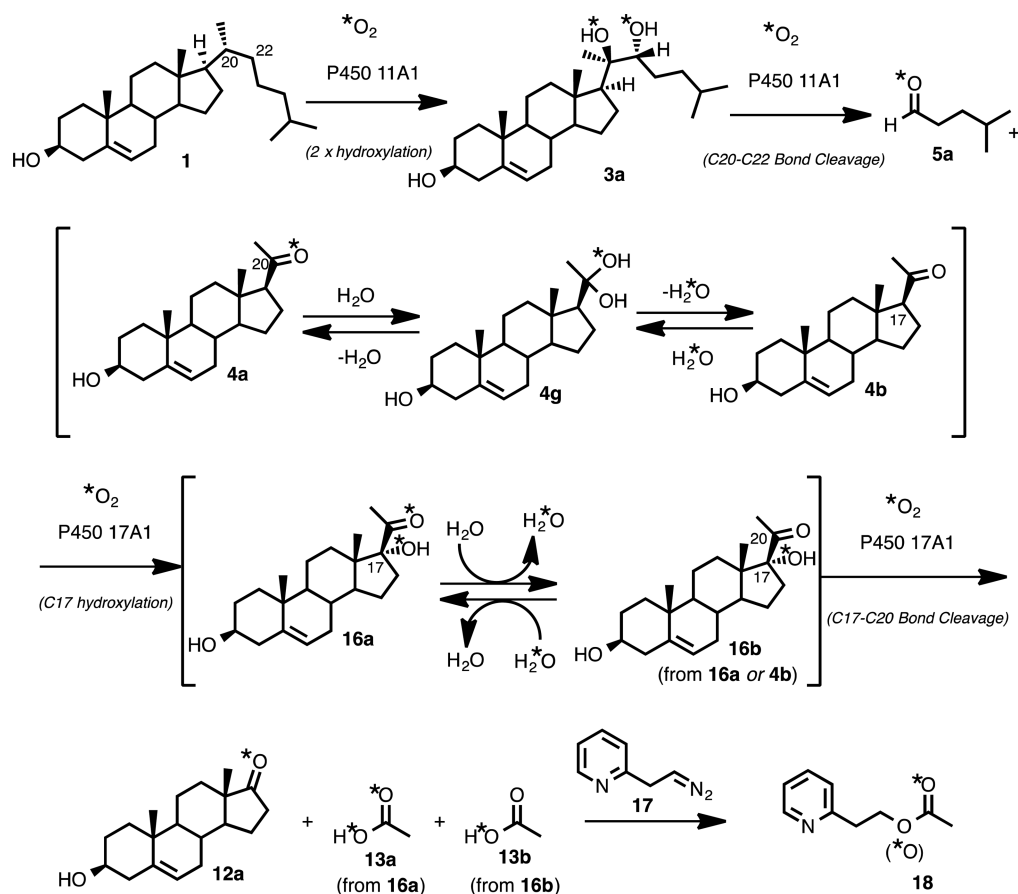
**Scheme 4. Coupled-Enzyme Assays for P450 11A1: Oxygen Trapping Experiments Used To Avoid Exchange of the Carbonyl Oxygens with Water in (A) Isocaproaldehyde (**5**) and (B) Pregnenolone (**4**), the Carbon–Carbon Bond Cleavage Products of P450 11A1**



NMR. Similar to the case of 19-oxoandrostenedione,<sup>13</sup> the *gem*-diol and aldehyde were in a 0.9:1.0 ratio in  $\text{D}_2\text{O}$  (Figure 1). The fact that isocaproaldehyde is hydrated in water was important in the context of our  $^{18}\text{O}_2$  experiments. The purpose of this study is to distinguish between the two types of mechanisms presented in Scheme 2 (i.e., electrophilic Compound I and O–H abstraction). In other words, in order to determine whether or not P450 11A1 incorporates an  $^{18}\text{O}$  atom into isocaproaldehyde from molecular

oxygen ( $^{18}\text{O}_2$ ) (i.e., if Scheme 2Ba supported), the aldehyde oxygen must be trapped at a state where it cannot exchange with  $\text{H}_2\text{O}$ .

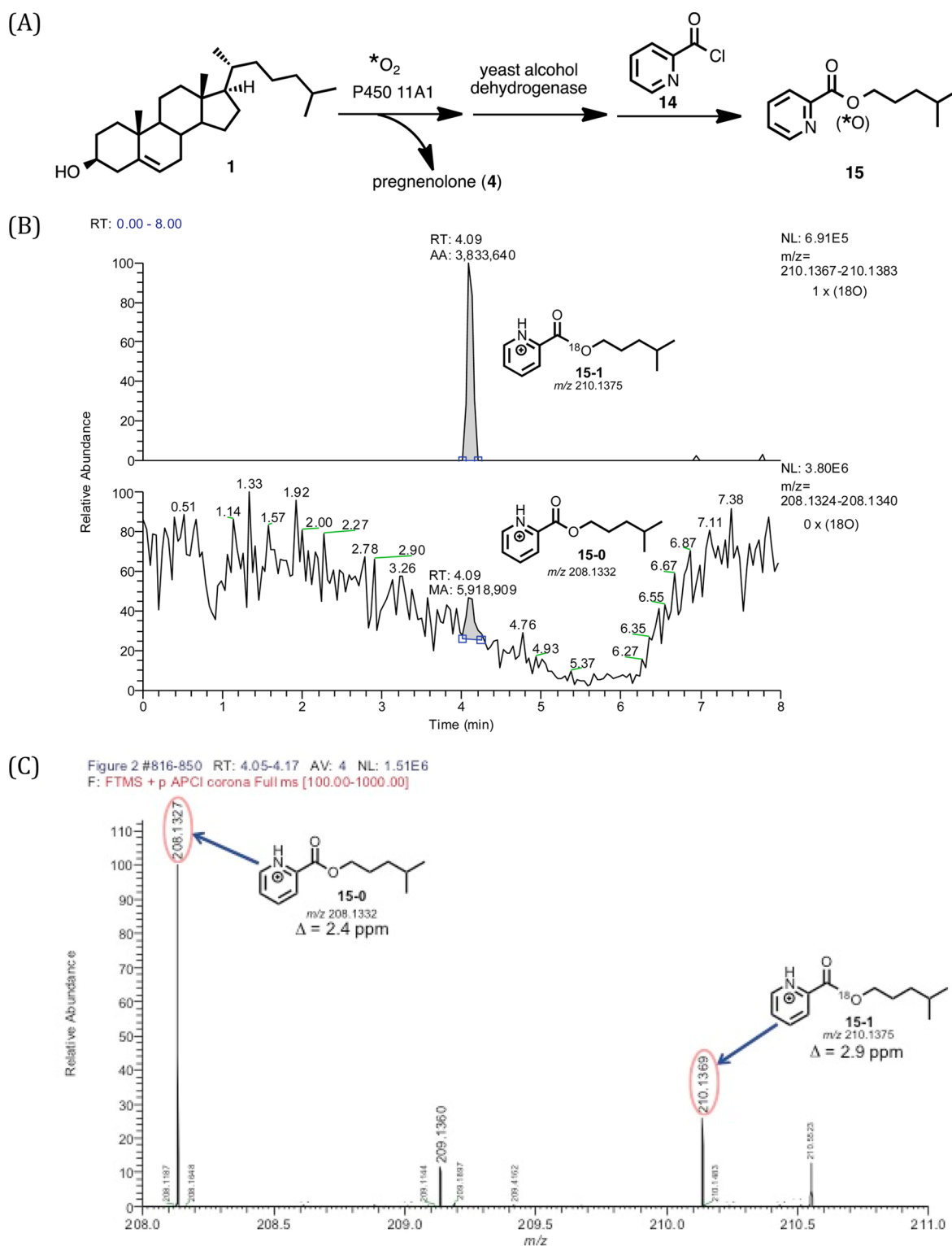
**Design of Coupled Assays To Avoid Exchange of Carbonyl Oxygen Atoms of Isocaproaldehyde and Pregnenolone with  $\text{H}_2\text{O}$ .** The carbonyl oxygens in steroids can be exchanged readily with  $\text{H}_2\text{O}$ .<sup>13</sup> As shown in the proton NMR spectrum of isocaproaldehyde (one of the lyase products

Scheme 5. Positive Control Assay for Monitoring the  $^{18}\text{O}$  Atom Content in the Derivatized Isocaproaldehyde Product 15Scheme 6. Positive Control Assay for Monitoring the  $^{18}\text{O}$  Atom Content in the Pregnenolone Product (4a)

of P450 11A1) in  $\text{D}_2\text{O}$ , the aldehyde exists in equilibrium in the *gem*-diol form (Figure 1). Moreover, exchange kinetics of ketones and aldehydes with water have also been determined experimentally to occur on a time scale from seconds to minutes (i.e., less than 1 h)<sup>34,35</sup> while the incubations in this study were run for 15 to 60 min in this study. Therefore, coupled enzyme

assays were necessary to trap the carbonyl oxygens of the reaction products of P450 11A1, pregnenolone and isocaproaldehyde (Scheme 4, 4 and 5), so that no exchange would occur with the water medium. Two types of coupled assays (yeast alcohol dehydrogenase and P450 17A1) were performed depending on the product (i.e., isocaproaldehyde or pregnenolone) analyzed.

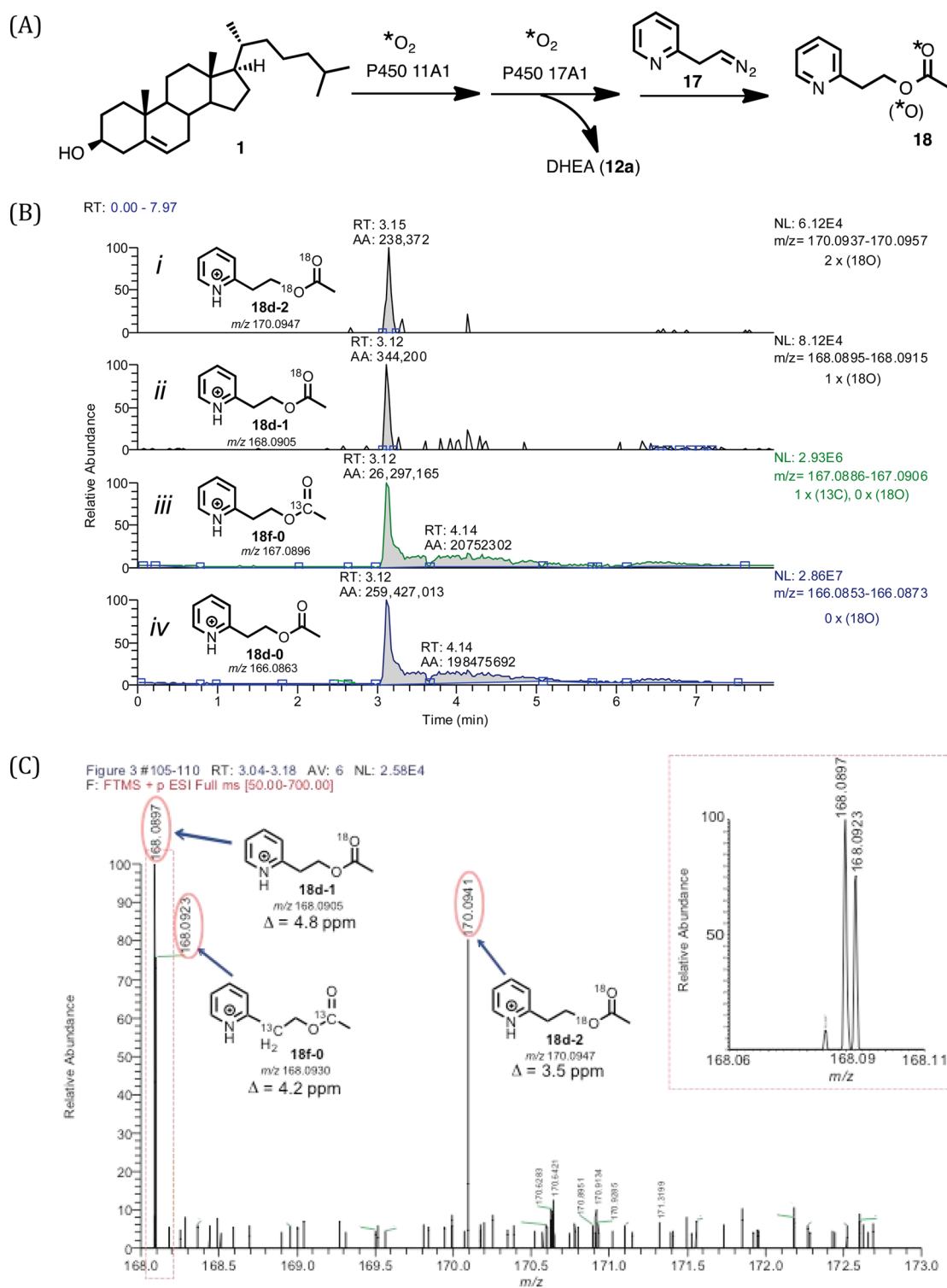




**Figure 2.**  $^{18}\text{O}_2$  incubation of P450 11A1 coupled with yeast alcohol dehydrogenase to reduce isocaproaldehyde to the alcohol followed by derivatization with picolinoyl chloride to the ester (positive control with cholesterol). Schemes showing the incubation/derivatization conditions for (A) the cholesterol substrate. Part (B) is the selected ion chromatograms of the derivatized picolinoyl ester containing one  $^{18}\text{O}$  atom ( $m/z$  210.1375, **15-1**) and no  $^{18}\text{O}$  atom ( $m/z$  208.1332, **15-0**). Chromatogram information: mass tolerance window, 4 ppm; full scan MS,  $m/z$  100–1000 range; APCI corona positive mode. (C) is the mass spectrum of the detected peak at  $t_R$  4.05–4.17 min. The peak of interest eluted at  $t_R$  4.09 min.

**Yeast Alcohol Dehydrogenase Trapping of the Oxygen in Isocaproaldehyde.** In order to trap the oxygen in the isocaproaldehyde product (Scheme 2, 5), we used yeast alcohol dehydrogenase to convert the aldehyde to the resulting alcohol

(Scheme 4A, 5 to 10),<sup>36</sup> which was then derivatized as the picolinoyl ester (Scheme 5, 10 to 15) and analyzed by mass spectrometry. The picolinoyl ester products would either have an  $^{18}\text{O}$  atom or  $^{16}\text{O}$  atom, depending on whether or not P450 11A1

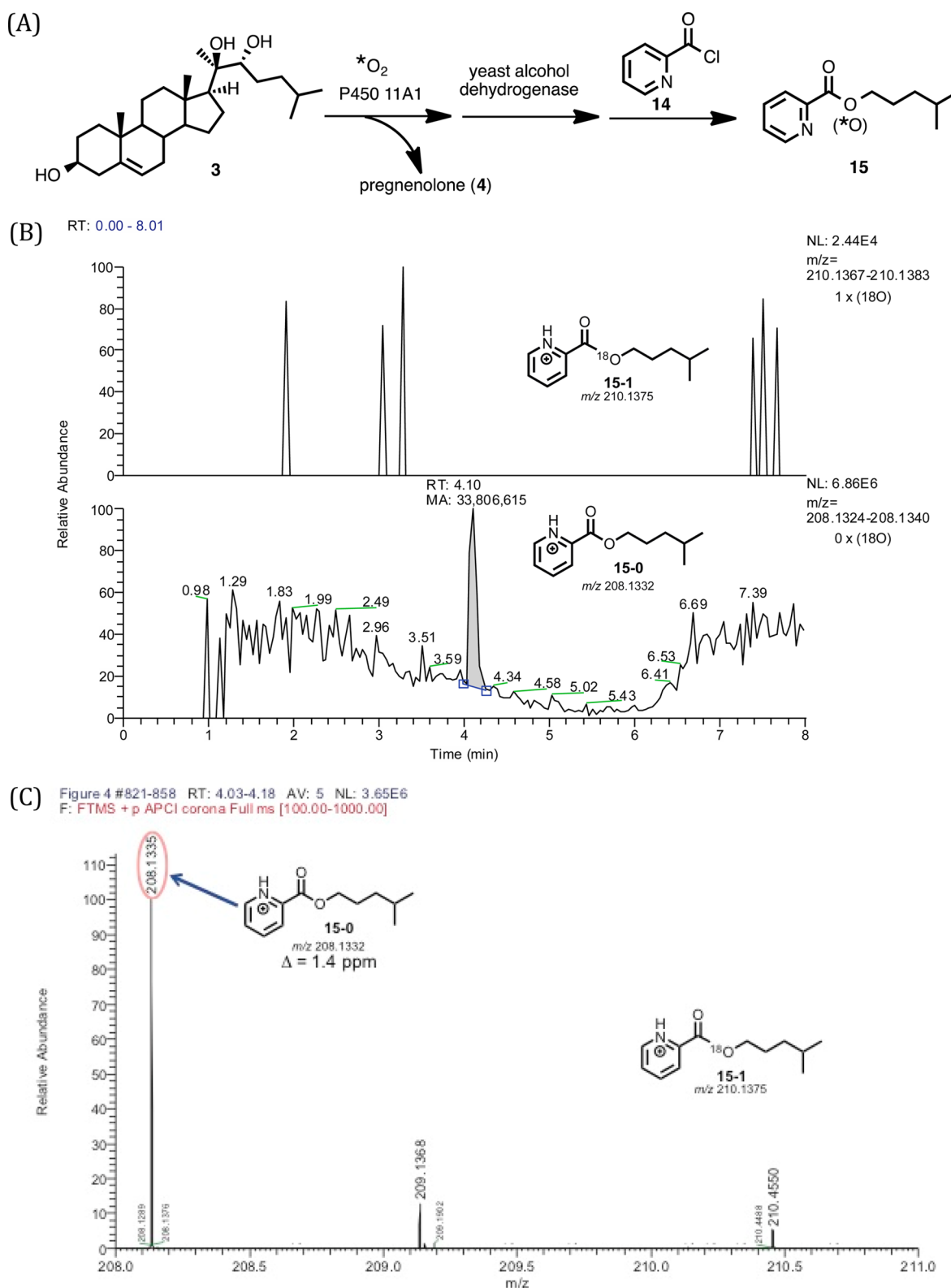


**Figure 3.** Positive control experiment of P450 11A1/P450 17A1 coupled assay. (A) Scheme showing positive control incubation of cholesterol with P450 11A1 in the presence of  $^{18}\text{O}_2$  followed by derivatization with the pyridine-containing diazo reagent. (B) Selected ion chromatograms of the pyridine acetate isotopologues (*i-iv*). (C) Mass spectra of the pyridine acetate isotopologues of the positive control incubation showing the presence of two  $^{18}\text{O}$  atoms (**18d-2**) and confirming retention of  $^{18}\text{O}$  atoms in the coupled assay. The expanded box shows the peaks detected (**18d-1** and **18f-0**) in the  $m/z$  168.06–168.11 range. Information for the chromatogram: full scan MS,  $m/z$  50–700 range; mass tolerance window, 6 ppm; ESI positive-ion mode. The peak of interest eluted at  $t_R$  3.15 min.

incorporated an oxygen atom into isocaproaldehyde. Derivatization of the alcohol to the picolinoyl ester enhanced the ionization of the compounds through protonation of the pyridine nitrogen in the ESI positive mode. The presence of an  $^{18}\text{O}$  atom in the derivatized product would support the O–H abstraction

mechanism (Scheme 2B-b), while the absence of an  $^{18}\text{O}$  atom would support the electrophilic Compound I mechanism (Scheme 2A).

**Use of P450 17A1 To Trap the C20-Oxygen in Pregnenolone.** Conversely, in order to analyze the carbonyl



**Figure 4.** P450 11A1/yeast alcohol dehydrogenase incubation of 20R,22R-dihydroxycholesterol (**3**) in the presence of  $^{18}\text{O}_2$  followed by derivatization. (A) Scheme showing incubation followed by derivatization with picolinoyl chloride (**14**). (B) Selected ion chromatograms monitoring for picolinoyl esters containing one  $^{18}\text{O}$  atom (**15-1**) and no  $^{18}\text{O}$  atom (**15-0**). (C) Mass spectrum showing the absence of  $^{18}\text{O}$ -incorporated picolinoyl ester isotopologue (**15-1**). The peak of interest is at  $t_R$  4.10 min.

oxygen in the pregnenolone product of P450 11A1 (Scheme 2, **4**), a coupled assay with P450 17A1 was performed to convert pregnenolone into acetic acid and dehydroepiandrosterone (DHEA) (Scheme 4B). The carbon–carbon bond cleavage

product of P450 11A1 is pregnenolone, which is a substrate for P450 17A1. The transformation catalyzed by P450 17A1 occurs in two steps: (i) hydroxylation at the C17-position of pregnenolone and (ii) cleavage of the 17,20-carbon–carbon

bond of 17 $\alpha$ -hydroxypregnenolone to form DHEA and acetic acid (Scheme 4B, 4a to 12a and 13). We have shown that P450 17A1 incorporates a single oxygen ( $^{18}\text{O}$ ) atom from molecular oxygen ( $^{18}\text{O}_2$ ) into the acetic acid product in the lyase step (ii) when 17 $\alpha$ -hydroxy-[21,21,21- $^3\text{H}_3$ ]-pregnenolone is used as the substrate.<sup>15</sup> Therefore, in order to determine  $^{18}\text{O}$  incorporation into pregnenolone by P450 11A1, the derivatized acetic acid product was analyzed for incorporation of one or two  $^{18}\text{O}$  atoms. If the derivatized acetate product contained two  $^{18}\text{O}$  atoms, then the mechanism shown in Scheme 2B-a would be supported. One of the  $^{18}\text{O}$  atoms would have come from P450 11A1 and the second  $^{18}\text{O}$  atom would have come from P450 17A1. However, if there were only one  $^{18}\text{O}$  atom incorporated into the acetate product, then the electrophilic Compound I mechanism (Scheme 2A) is supported. In this case, the  $^{18}\text{O}$  atom would only come from P450 17A1.

**Positive Control Experiments Using Cholesterol Indicated Retention of the Oxygen Atom in the Carbonyl-Containing Products.** Two separate positive control experiments were performed to verify minimal exchange of the carbonyl oxygens in either the isocaproaldehyde (Scheme 5, 5a to 5b) or pregnenolone (Scheme 6, 4a to 4b or 14a to 14b) product with the water medium. If the last step does not incorporate  $^{18}\text{O}$  label into either of the P450 11A1 products (i.e., if Scheme 2A is supported by the results), then the possibility of  $\text{H}_2\text{O}$  exchanging with the carbonyl groups of pregnenolone and isocaproaldehyde (Scheme 2B, 4-L and 5-L) to yield products lacking an  $^{18}\text{O}$  label must be ruled out. Therefore, these positive control experiments are especially crucial in the case that the P450 11A1 incubation with 20R,22R-dihydroxycholesterol and  $^{18}\text{O}_2$  does not incorporate an  $^{18}\text{O}$  label into either pregnenolone or isocaproaldehyde (i.e., the experimental results support the scenario in Scheme 2A).

Both positive control experiments involve the use of cholesterol as the substrate for P450 11A1 instead of 20R,22R-dihydroxycholesterol (3) (Schemes 5 and 6). These experiments are critical in establishing oxygen retention in the products during the enzyme incubation and derivatization conditions. In previous mechanistic studies with P450 19A1, we showed that the aldehyde oxygen of 19-[ $^{18}\text{O}$ ]-labeled 19-oxoandrostenedione readily exchanges with water in pH 7.4 phosphate buffer (estimated  $t_{1/2} < 1$  s).<sup>13</sup> The use of cholesterol guarantees incorporation of  $^{18}\text{O}$  atoms into isocaproaldehyde (Scheme 5) and pregnenolone (Scheme 6).

**Positive Control: Yeast Alcohol Dehydrogenase.** The positive control experiment for  $^{18}\text{O}$  incorporation into isocaproaldehyde involved the use of cholesterol (Scheme 5, 1) as the starting material with P450 11A1 coupled with yeast alcohol dehydrogenase in the presence of  $^{18}\text{O}_2$ . The resulting alcohol product was derivatized with picolinoyl chloride and analyzed by LC-MS (Scheme 5, 10 to 15). The detection of an  $^{18}\text{O}$ -labeled picolinoyl ester product would show that the oxygen atom from isocaproaldehyde does not exchange completely with the water medium (Scheme 5, 5a to 5b) during the conversion from the aldehyde to alcohol by yeast alcohol dehydrogenase (Scheme 5, 5a or 5b to 10).

LC-MS analysis of 4-methylpentanol picolinoyl ester (Scheme 5, 15) derived from the yeast alcohol dehydrogenase-coupled incubation of P450 11A1 with cholesterol and  $^{18}\text{O}_2$  confirmed the presence of an  $^{18}\text{O}$  atom in the product (Figure 2B). This observation was important in establishing that the yeast alcohol dehydrogenase reaction was efficient enough to trap the  $^{18}\text{O}$  atom in the initial isocaproaldehyde product (Scheme 5, 5a)

before exchange with the water medium could occur (Scheme 5, 5a to 5b).

**Positive Control: P450 17A1.** In order to verify the retention of the oxygen in the C20-position of pregnenolone, a similar control experiment was performed using cholesterol (Scheme 6, 1) as the starting material. Additionally, analysis of the C20-oxygen in the pregnenolone product formed from P450 11A1 was achieved by P450 17A1-mediated conversion of pregnenolone into acetic acid *in situ* (Scheme 6). The presence of two  $^{18}\text{O}$  atoms in the acetic acid product (Scheme 6, 13a) would confirm that at least some, if not all, of the oxygen atoms are retained during the P450 11A1/P450 17A1 coupled assay (Scheme 6).

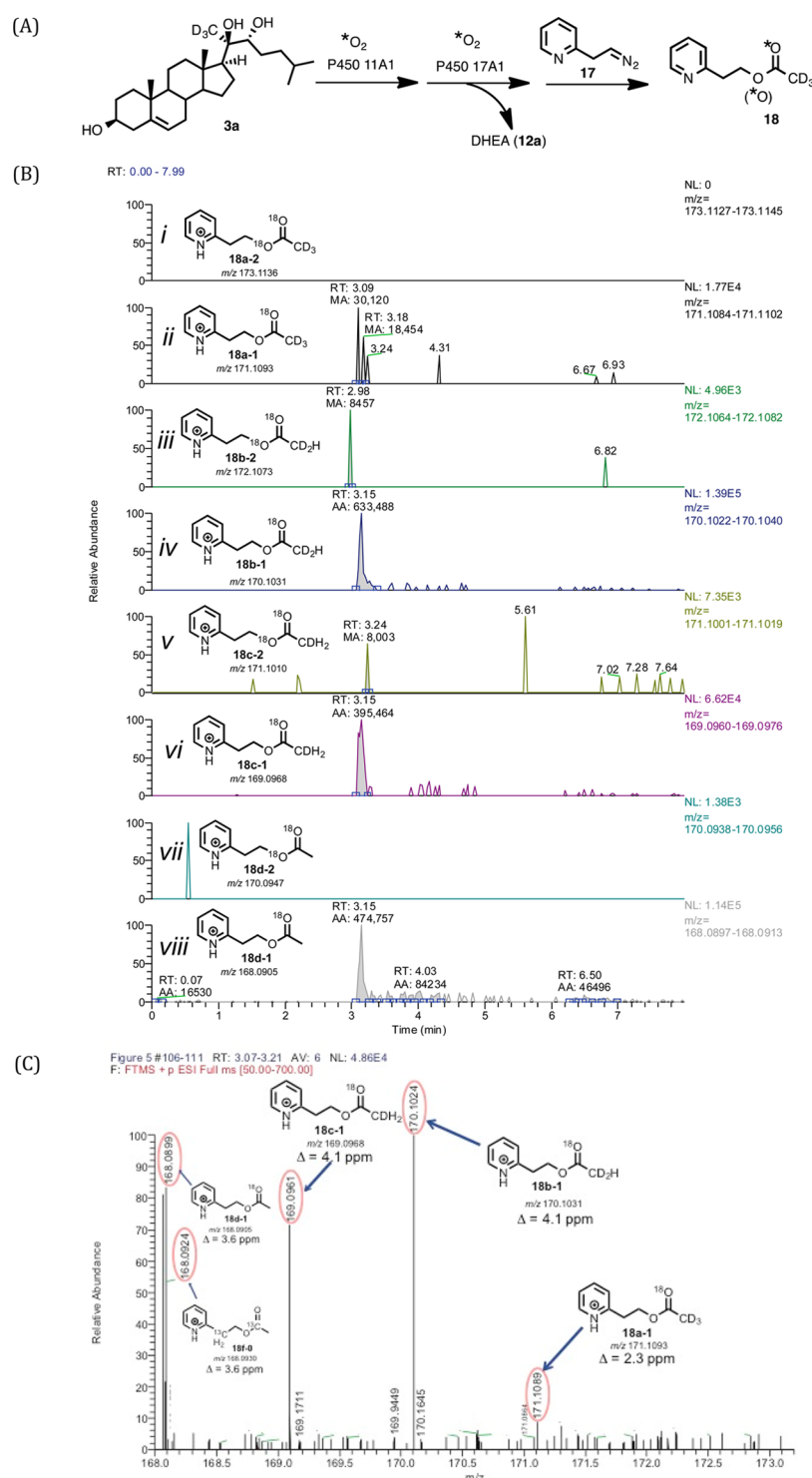
LC-MS analysis of the pyridine acetate isotopologues from the positive control experiment showed the detection of an acetate containing two  $^{18}\text{O}$  atoms (Figure 3B, 18d-2). There was also a pyridine acetate isotopologue containing only one  $^{18}\text{O}$  atom in the positive control experiment. The ratio of the acetate isotopologues containing two  $^{18}\text{O}$  atoms and one  $^{18}\text{O}$  atom was 0.7 to 1.0 (Figure 3B, the ratio of 18d-2:18d-1). The detection of the acetate with a single  $^{18}\text{O}$  atom indicates the possibility of some exchange of the C20-oxygen in the 21-carbon steroid products (Scheme 4, 4a to 4b or 16a to 16b) during the enzyme-coupled assay. However, the LC-MS analysis of the synthetic pyridine acetate standard with no  $^{18}\text{O}$  label (Supporting Information), which was prepared by treating acetic anhydride with the corresponding alcohol,<sup>15</sup> did show the presence of some acetate containing one  $^{18}\text{O}$  atom (0.003%  $^{18}\text{O}$ ). Therefore, the source of the mono- $^{18}\text{O}$ -containing acetate detected in the positive control experiment (Figure 3B,C, 18d-1) may be from the acetate derived from background acetic acid. Nevertheless, the positive control experiment confirmed that we were still able to detect the acetate isotopologue containing two  $^{18}\text{O}$  atoms (Figure 3B), indicating that the P450 17A1 reactions (i.e., 17 $\alpha$ -hydroxylation and C17,C20-lyase) are fast enough to trap the original C20-oxygen in pregnenolone generated from P450 11A1 (Scheme 6, 4a). In other words, the positive control experiment verified that the transformation from 4a to 4b (Scheme 6) does not completely occur before P450 17A1 converts pregnenolone to acetic acid (Scheme 6, 4a to 13a).

With the results from both positive control experiments using cholesterol as the substrate, we have established that our enzyme-coupled assay systems are efficient enough to trap the carbonyl oxygens in the C20–C22 bond cleavage products of P450 11A1 (i.e., isocaproaldehyde and pregnenolone). We now present the results from the  $^{18}\text{O}$ -labeling experiments using the direct lyase substrate, 20R,22R-dihydroxycholesterol.

**P450 11A1 Incubation Coupled with Yeast Alcohol Dehydrogenase: Analysis of  $^{18}\text{O}$  Incorporation from Molecular Oxygen ( $^{18}\text{O}_2$ ).** The yeast alcohol dehydrogenase-P450 11A1 coupled incubation with 20R,22R-dihydroxycholesterol in the presence of  $^{18}\text{O}_2$  showed no incorporation of an  $^{18}\text{O}$  atom derived from molecular oxygen ( $^{18}\text{O}_2$ ) (Figure 4B). The lack of  $^{18}\text{O}$  incorporation in this experiment, combined with the knowledge that the yeast alcohol dehydrogenase enzyme traps the initial carbonyl oxygen in isocaproaldehyde as the alcohol (Scheme 5, 5a to 10) before it can exchange with water, rules out the C20 O–H abstraction mechanism that is followed by oxygen rebound onto the cleaved side chain (Scheme 2Ba).

**P450 11A1 Incubation Coupled with P450 17A1: Analysis of  $^{18}\text{O}$ -Incorporation from Molecular Oxygen ( $^{18}\text{O}_2$ ).** Because of the background acetic acid problem revealed in the positive control experiment regarding the detection of  $^{18}\text{O}$ -



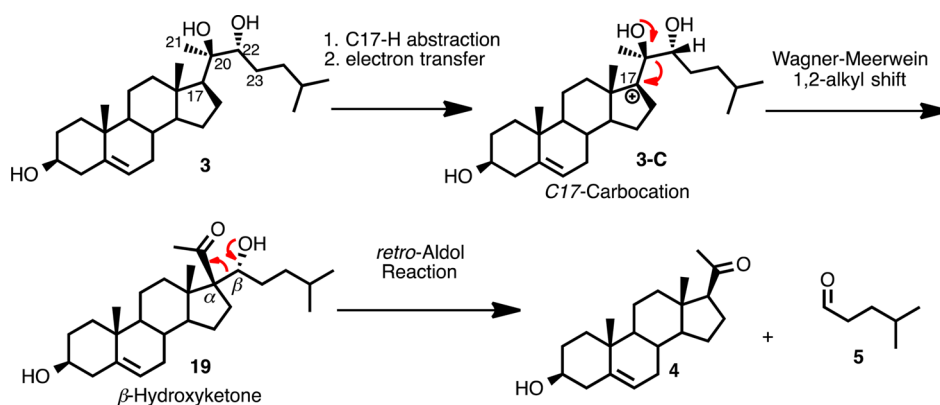


**Figure 5.** P450 11A1/P450 17A1 incubation of 20R,22R-dihydroxy-[21,21,21- $^2\text{H}_3$ ]-cholesterol (**3a**) in the presence of  $^{18}\text{O}_2$  followed by derivatization. (A) Scheme showing the incubation of P450 11A1 with 20R,22R-dihydroxy-[21,21,21- $^2\text{H}_3$ ]-cholesterol coupled with P450 17A1 in the presence of  $^{18}\text{O}_2$  followed by derivatization with the pyridine-containing diazo reagent. (B) Chromatogram showing different pyridine isotopologues generated from the incubation (*i*–*viii*). (C) Mass spectral analysis of pyridine acetate isotopologues from P450 11A1 incubation of 20R,22R-dihydroxy-[21,21,21- $^2\text{H}_3$ ]-cholesterol with P450 17A1 in  $^{18}\text{O}_2$ . No isotopologues with two  $^{18}\text{O}$  atoms were detected (*i*, *iii*, *v*, *vii*). Full-scan MS,  $m/z$  50–700 range; mass tolerance, 6 ppm; ESI positive mode. The peak of interest is at  $t_R$  3.15 min.

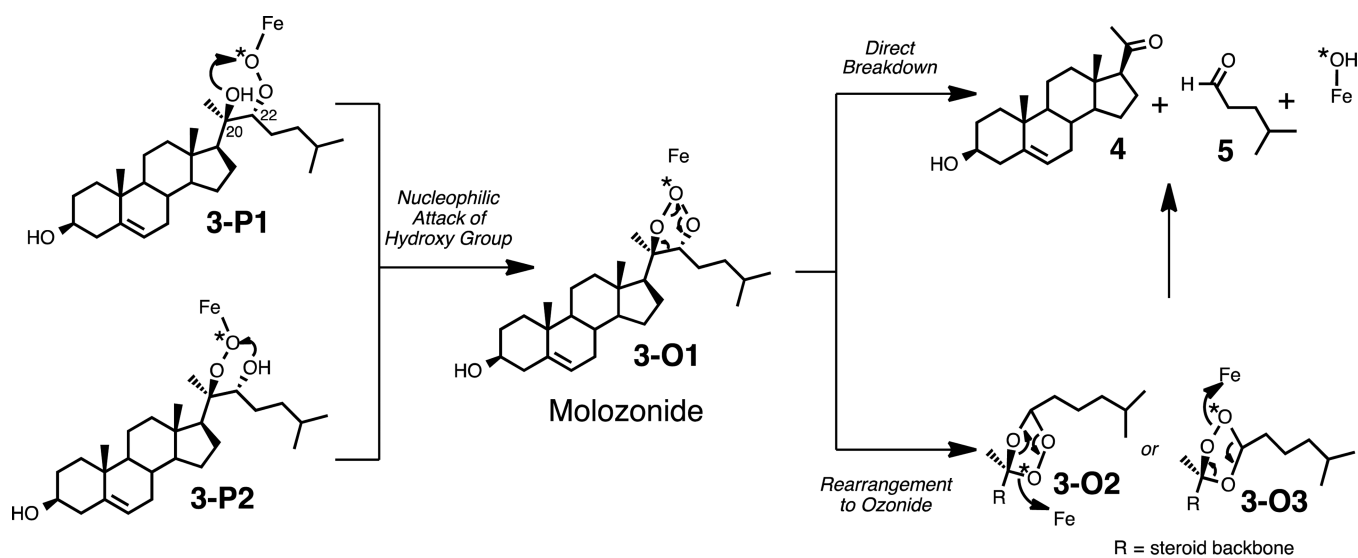
containing acetate (Figure 3, **18d-1**), we used 20R,22R-dihydroxy-[21,21,21- $^2\text{H}_3$ ]-cholesterol in the P450 11A1 incubation (Figure 5A, **3a**) when probing the C20,C22-lyase reaction. The synthesis did not yield a completely (i.e., 100%) 21,21,21- $d_3$ -incorporated 20R,22R-dihydroxycholesterol, and analysis of the

substrate by LC-MS revealed a mixture of isotopologues containing three, two, and one deuteriums (Supporting Information, **3a**). This isotopic mixture was inconsequential in our analysis because we employed HRMS (i.e., resolution of the LTQ Orbitrap XL mass spectrometer set at 100 000). With such

Scheme 7. Alternative Mechanistic Proposal Involving C–H Abstraction as the Initial Step for the C20–C22 Carbon–Carbon Bond Cleavage of P450 11A1



Scheme 8. Alternative Mechanism for C–C Bond Cleavage Involving a Molozonide Intermediate (3-O1)



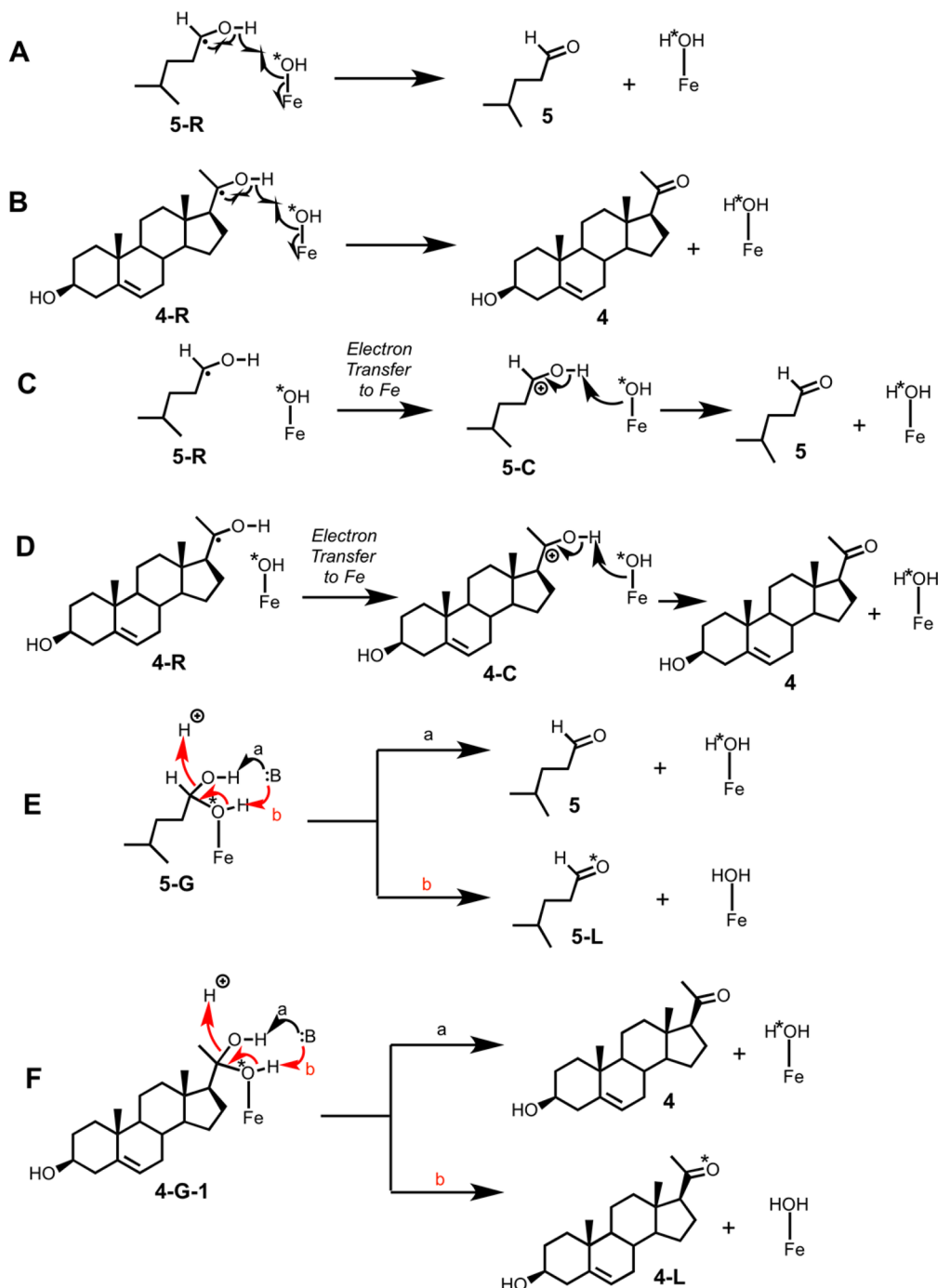
high resolution, we were able to clearly distinguish between the mass differences of a pyridine acetate isotopologue with two deuteriums (**18b-0**,  $m/z$  168.0988) vs one  $^{18}\text{O}$  atom (**18d-1**,  $m/z$  168.0905) vs two  $^{13}\text{C}$  atoms (**18f-0**,  $m/z$  168.0924), with a difference in mass of 49 ppm (**18b-0** vs **18d-1**) and 11 ppm (**18d-1** vs **18f-0**; i.e., minimum resolving powers ( $m/\Delta m$ )<sup>37,38</sup> of 20 000 and 88 500 are required for the first two isotopologues (**18b-0** and **18d-1**) and the last two isotopologues (**18d-1** and **18f-0**), respectively. As shown in Figure 3C (and in the inset), the two isotopologues, **18d-1** and **18f-0**, were cleanly resolved when the resolving power was set to 100 000. The LC-MS analysis of the P450 11A1-P450 17A1 coupled incubation with 21,21,21- $d_3$ -incorporated 20R,22R-dihydroxycholesterol (Figure 5A, **3a**) in the presence of  $^{18}\text{O}_2$  revealed that none of the pyridine acetate isotopologues contained two  $^{18}\text{O}$  atoms (Figure 5B,C, **18**). This observation, combined with the fact that the positive control experiment (Figure 3) confirmed the retention of the C20-carbonyl oxygen in pregnenolone through the trapping process (Scheme 6, **4a** to **16a**), eliminates the C20–C22 bond cleavage mechanism involving the C22 O–H abstraction and oxygen rebound into pregnenolone (Scheme 2Bb).

The results from both sets of coupled assays (i.e.,  $^{18}\text{O}_2$  incubation of P450 11A1 with either yeast alcohol dehydrogenase or P450 17A1, Figures 4B and 5B) showed that an  $^{18}\text{O}$  atom

from molecular oxygen ( $^{18}\text{O}_2$ ) was not incorporated into the C20–C22 bond cleavage products (i.e., pregnenolone or isocaproaldehyde). Thus, P450 11A1 cleaves the carbon–carbon bond of 20R,22R-dihydroxycholesterol through an electrophilic Compound I mechanism (Scheme 2A).

## DISCUSSION

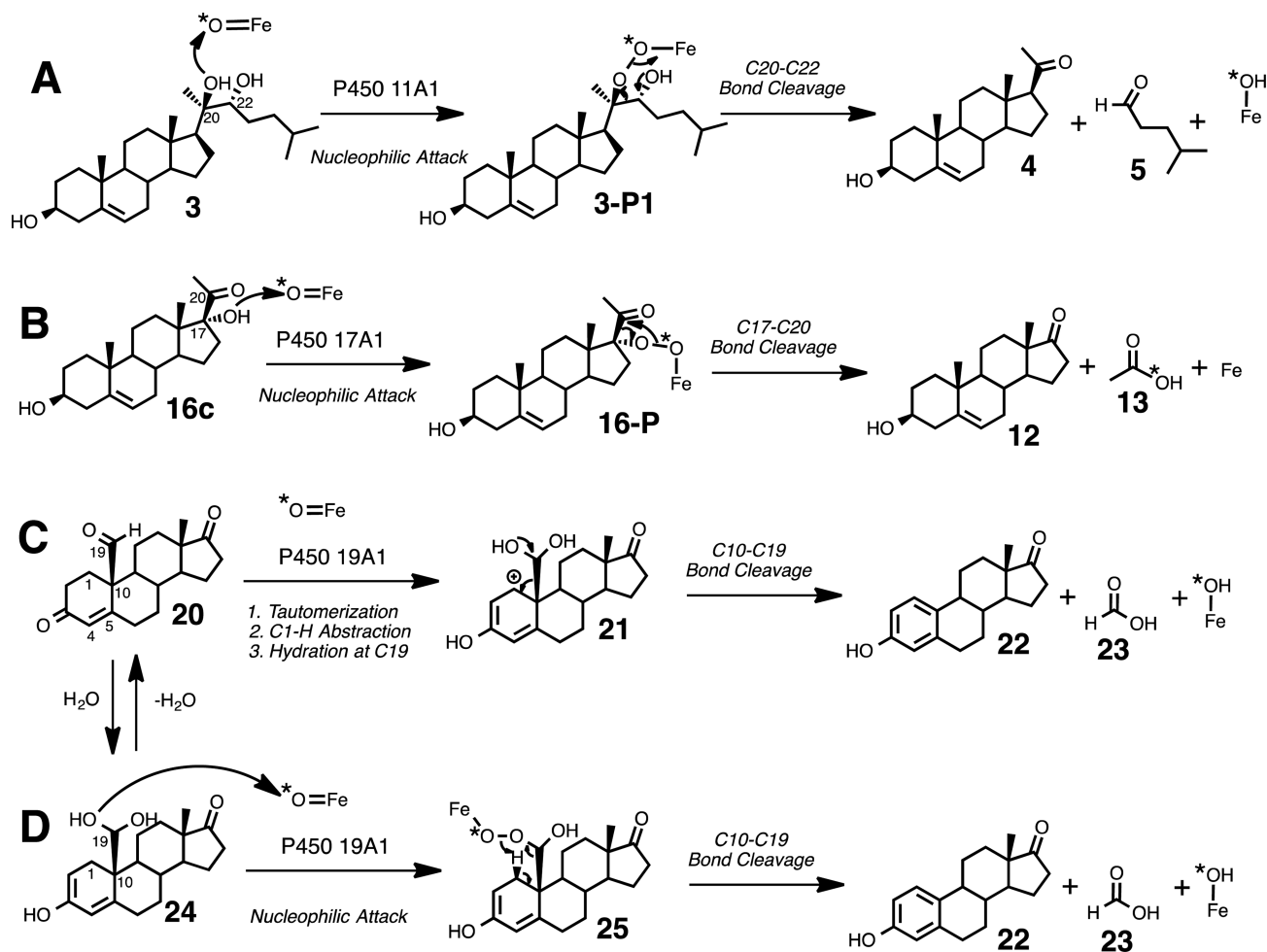
The incubations with P450 11A1 and its substrate 20R,22R-dihydroxycholesterol in the presence of  $^{18}\text{O}_2$  revealed that an oxygen atom from molecular oxygen was not incorporated into either pregnenolone (Figure 5) or isocaproaldehyde (Figure 4), supporting an electrophilic Compound I mechanism (Scheme 2A). The positive control experiments (Figures 2 and 3) confirmed retention of the oxygen atom in the carbonyl functional groups of the products during the coupled assays (i.e., yeast alcohol dehydrogenase to reduce isocaproaldehyde and P450 17A1 to analyze pregnenolone). These observations rule out a carbon–carbon bond cleavage mechanism that involves O–H abstraction followed by oxygen rebound (Scheme 2B) and support an electrophilic Compound I mechanism (Scheme 2A). Nonetheless, it is difficult to determine whether it is the 22R-hydroxy group (Scheme 2Aa) or 20R-hydroxy group (Scheme 2Ab) of 20R,22R-dihydroxycholesterol that attacks Compound I.

Scheme 9. Alternate Possibilities Considered To Explain the  $^{18}\text{O}$  Incorporation Results

#### A Potential Additional C–H Abstraction Mechanism.

P450 11A1 has been shown to oxygenate at different sites on other sterol substrates besides the C20- and C22-positions. For example, ergosterol is hydroxylated on the C23-position by P450 11A1 after epoxidation of the C20–C22 double bond.<sup>39</sup> Additionally, vitamin D<sub>2</sub> is a substrate for P450 11A1, where it catalyzes the hydroxylation of the C20- and C17- carbons.<sup>40</sup> The possibility of P450 11A1 reacting at adjacent positions (i.e., C17 and C23) on its substrate suggested another possible mechanism for C20–C22 bond cleavage, as shown in Scheme 7. In this scenario, the C17 $\alpha$ -H hydrogen atom is abstracted and an electron transfer occurs that forms a tertiary carbocation, which can undergo a 1,2-alkyl shift to form a  $\beta$ -hydroxy ketone (Scheme

7, 19). A subsequent retro-aldol reaction would then result in the formation of the final products, pregnenolone and isocaproaldehyde (Scheme 7, 19 to 4 and 5). Despite this mechanism being consistent with the  $^{18}\text{O}$ -labeling results, the possibility is ruled out by the detection of a tetradeuterated pregnenolone product (Supporting Information, 4x to 4z) when 20R,22R-dihydroxy-[17,21,21,21- $^2\text{H}_4$ -cholesterol was incubated with P450 11A1. Another piece of evidence discounting this proposed mechanism (Scheme 7) is that only the hydroxycholesterol intermediates (Scheme 1, 2 and 3) have been isolated from a reaction with P450 11A1 and cholesterol.<sup>33</sup> However, another possible mechanism initiated by abstraction of the C23-hydrogen atom cannot be eliminated.

Scheme 10. Summary of the Mechanisms of Three P450 Systems Supported by the  $^{18}\text{O}$ -Labeling Studies Discussed in the Text: P450 11A1 (A), P450 17A1 (B), and P450 19A1 (C,D)

**An Electrophilic Compound I Mechanism Involving a Molozonide Intermediate.** Interestingly, the substrate for the carbon–carbon bond cleavage reaction contains two adjacent alcohol groups (i.e., a 1,2-diol) at the site of bond breakage (C20- and C22-positions), which suggests another possible mechanism. Furthermore, any “monohydroxylated” substrate such as 22R-hydroxycholesterol (Scheme 1, 2) results in a second hydroxylation by P450 11A1 to form 20R,22R-dihydroxycholesterol (Scheme 1, 3) and not a carbon–carbon bond cleavage reaction. This observation suggests that the second hydroxy group near the site of C–C bond cleavage may play a role in the lyase reaction. In other words, the C–C bond cleavage reaction does not occur with 22R-hydroxycholesterol (Scheme 1, 2) to yield pregnene-3,20-diol and isocaproaldehyde. As shown in Scheme 8, which shows the peroxide intermediate (Scheme 8, 3-P1) after initial reaction between electrophilic Compound I and 20R,22R-dihydroxycholesterol (Scheme 2A), a nucleophilic attack by the second hydroxy group may result in a molozonide intermediate (Scheme 8, 3-O1), which can directly break down to the C–C bond cleavage products (Scheme 8, 4 and 5). This mechanism involving the direct breakdown of the molozonide intermediate (Scheme 8, 3-O1) would be consistent with the observation that the cleaved products (Scheme 8, 3-O1 to 4 and 5) do not contain  $^{18}\text{O}$ -label from molecular oxygen ( $^{18}\text{O}_2$ ). In the field of chemical synthesis, ozonolysis is a common way to cleave C–C bonds of alkene functional groups through a molozonide to

ozonide transformation.<sup>41</sup> However, the decomposition of the ozonide intermediates (Scheme 8, 3-O2 and 3-O3) may result in the incorporation of an  $^{18}\text{O}$  label in the products (Scheme 8, 4 and 5). Since we did not observe any incorporation of an  $^{18}\text{O}$  atom into the isocaproaldehyde product (Scheme 8, 5), we can rule out the ozonide (Scheme 8, 3-O3 to 5) reaction pathway, but the direct breakdown of the molozonide is still viable (Scheme 8, 3-O1 to 5).

**Other Alternative Compound I Proposals.** Some alternate mechanistic possibilities can be considered (Scheme 9). In parts A–D of Scheme 9, mechanisms are presented in which there is no oxygen rebound but instead a proton-coupled electron transfer (formal removal of a hydrogen atom) occurs to yield the carbonyl products. This mechanism, formally analogous to a carbon desaturation, would be consistent with the  $^{18}\text{O}$  labeling results. Such mechanisms have been considered before, for at least 30 years, but no evidence has been obtained to support this possibility. Doing the most relevant experiments has not been possible with P450 11A1, in that the only types of labeling experiments possible with the substrate cholesterol are described here and the ability to catalyze other reactions is limited.<sup>27</sup> P450 11A1 does catalyze a number of other reactions, particularly with vitamin D,<sup>42,43</sup> but none of these involve the formation of carbonyls. Reactions with other P450s can be considered, e.g., the P450 19A1 reaction that converts its aldehyde substrate to the carboxylic acid (i.e., the reactions in



Scheme 9A–D do not occur).<sup>13</sup> The 19-oxoandrostenedione substrate yielded a mixture of 19-oic androgen product isotopologues that had one <sup>18</sup>O atom and no <sup>18</sup>O atom. If the carbonyl forming reaction of P450 19A1 occurred in the fashion shown in Scheme 9A–D, then we would not have seen any <sup>18</sup>O-incorporated in the 19-oic androgen products.<sup>13</sup>

Some earlier work with other P450s showed varying results for incorporation of label from <sup>18</sup>O<sub>2</sub> into carbonyl products.<sup>44–47</sup> However, all of these studies cited were done under incubation conditions extending from minutes to hours, conditions under which carbonyl oxygens are known to rapidly exchange with water,<sup>13,34,35,48,49</sup> and none of these results can be considered valid. Surprisingly, the rapid oxygen exchange was already well-established in the P450 field, and an enzymatic trapping procedure had been utilized in <sup>18</sup>O incorporation studies in which aldehydes were generated.<sup>50</sup> Although we cannot completely discount the alcohol “desaturation” possibility (Scheme 9A–D), we believe that there is currently no real precedent for this in P450s.

Another possibility that can be considered is shown in Scheme 9E,F, in which recombination of the Compound II form of P450 with the carbinol radical occurs to give an iron linked *gem*-diol (Scheme 9, 4G-1). Base-catalyzed removal of a proton from the hydroxyl not linked to iron (Scheme 9 E,F, path a) would lead to unlabeled carbonyl products. In our Introduction, we started with the assumption that the dehydration step (Scheme 2B) is not stereospecific. We are inclined against this mechanism (Scheme 9E,F), in that formation of P450 iron-bound *gem*-diols does not have precedent.

## CONCLUSIONS

In summary, isotope labeling experiments combined with coupled enzyme assays support an electrophilic Compound I mechanism (Scheme 2A, i.e., nucleophilic attack of a hydroxyl on the electrophilic FeO<sup>3+</sup>), proposed elsewhere<sup>6–10</sup> and also suggested in structural<sup>11</sup> and EPR/ENDOR<sup>5</sup> studies, and rule out an initial O–H hydrogen atom abstraction mechanism (Scheme 2B) in the carbon–carbon bond cleavage step of P450 11A1 to form pregnenolone from cholesterol. The electrophilic Compound I mechanism is consistent with a mechanism we have proposed in the P450 17A1-catalyzed C17–C20 bond cleavage reaction of 17 $\alpha$ -hydroxypregnenolone to form dehydroepiandrosterone and acetic acid (Scheme 10B, 16c to 12 and 13).<sup>15</sup> A similar mechanism probably occurs in the P450 1A2-catalyzed carbon–carbon bond cleavage process of nabumetone,<sup>51</sup> where an  $\alpha$ -hydroxy ketone intermediate comparable to 17 $\alpha$ -hydroxypregnenolone (16c) was isolated from the incubation. On the contrary, previous <sup>18</sup>O-labeling studies suggested that P450 19A1 does not undergo the electrophilic Compound I mechanism as it catalyzes the cleavage of the C10–C19 bond in androgens to form estrogens and formic acid. Instead, the enzyme abstracts the C1 $\beta$ -H hydrogen atom of its 19-oxo androgen substrate as the initial step.<sup>13</sup> Furthermore, the direct C–C bond cleavage substrate of P450 19A1 (e.g., Scheme 10C, 20, 19-oxoandrostenedione) differs from those of P450 11A1 (e.g., Scheme 10A, 3, 20R,22R-dihydroxycholesterol) and P450 17A1 (e.g., Scheme 10B, 16c, 17 $\alpha$ -hydroxypregnenolone) in that there is no free hydroxy group present in the case of P450 19A1. However, to remain consistent with the electrophilic Compound I mechanisms proposed for P450s 11A1 and 17A1, we show an alternative mechanism for P450 19A1, which generates a ferric peroxy hemiacetal intermediate (Scheme 10D, 25). This tetrahedral intermediate is similar to the one proposed in the

mechanism involving the nucleophilic attack of the ferric peroxide species onto the aldehyde (20)<sup>15</sup> except that in Scheme 10D, the <sup>18</sup>O label from molecular oxygen (<sup>18</sup>O<sub>2</sub>) is not directly attached to the C19-carbon (Scheme 10D, 25). This mechanism for P450 19A1 (Scheme 10D) involves the formation of an alcohol substrate through hydration of the aldehyde (Scheme 10, 20 to 24), and the resulting *gem*-diol (Scheme 10D, 24) conducts a nucleophilic attack on Compound I. The <sup>18</sup>O-labeling studies in this system<sup>9</sup> are consistent with the mechanism shown in Scheme 10D (i.e., lack of incorporation of <sup>18</sup>O label in the formic acid product (23) from <sup>18</sup>O<sub>2</sub>). The formation of this tetrahedral intermediate (Scheme 10D, 25) has been explored without enzyme present, where the hydroperoxy hemiacetal intermediate was generated *in situ* by treating 19-oxoandrostenedione with hydrogen peroxide.<sup>52</sup> However, an estrogen product was not formed in the study, and instead the  $\Delta^{4,5}$ -double bond was epoxidized to yield 19-oxo-androst-4 $\beta$ ,5 $\beta$ -epoxydione. The authors concluded that the presence of the enzyme drives the reaction toward formation of the estrogen (i.e., Scheme 10, 22) rather than the epoxide.

The peroxy/molozone mechanism we propose has some precedent in the work of Hochberg et al.,<sup>53,54</sup> as pointed out by Ortiz de Montellano and De Voss.<sup>9</sup> In the Hochberg work,<sup>53</sup> a crude adrenal mitochondrial preparation efficiently (23% yield) converted the substrate analogue (20R)-20-(*p*-tolyl)-5-pregne-3 $\beta$ ,20-diol to pregnenolone (and its product progesterone), in the presence of the cofactor NADPH. In other work by Hochberg,<sup>54</sup> a similar reaction occurred with the 20-cyclopropylcarbinyl analogue to yield pregnenolone and cyclopropanecarboxylic acid. Experiments with non-hydroxylated analogues did not yield these steroid products. The results can be explained by the heterolytic addition of FeO<sup>3+</sup> to the 20-hydroxyl group to form a Fe–O–O–C bond (as in Scheme 2, 3-P2), which decomposes to yield pregnenolone.<sup>9</sup>

With the experimental evidence disclosed in this report and more data from other systems (i.e., P450s 17A1 and 19A1<sup>6,13,15,55</sup>), we can begin to unambiguously classify how P450 enzymes cleave carbon–carbon bonds. Several questions still remain about the nature of the active oxygenated species involved in catalysis by mammalian P450 51A1, the lanosterol 14 $\alpha$ -demethylase, and its microbial orthologues.<sup>6,55</sup>

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b04437.

Codon optimization and construction of expression vector for human P450 11A1, enzyme-catalyzed conversion of isocaproaldehyde to 4-methylpentan-1-ol, detection of naturally abundant mono-<sup>18</sup>O-isotope incorporated ethylpyridine acetate from synthetic standard, LC-MS analysis of picolinoylated 20,22-dihydroxy-[21,21,21-<sup>2</sup>H<sub>3</sub>]-cholesterol, LC-MS analysis of picolinoylated pregnenolone using 20,22-dihydroxy-[17,21,21,21-<sup>2</sup>H<sub>4</sub>]-cholesterol as the starting material, and experimental data for synthesized chemicals (PDF)

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## Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

Supported in part by National Institutes of Health grants R37 CA090426 and R01 GM118122. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. I-J.J. was supported by the Vanderbilt International Scholars Program.

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