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Synthesis of halogenated pregnanes, mechanistic probes of steroid hydroxylases CYP17A1 and CYP21A2

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ABSTRACT

The human steroidogenic cytochromes P450 CYP17A1 (P450c17, 17α-hydroxylase/17,20-lyase) and CYP21A2 (P450c21, 21-hydroxylase) are required for the biosynthesis of androgens, glucocorticoids, and mineralocorticoids. Both enzymes hydroxylate progesterone at adjacent, distal carbon atoms and show limited tolerance for substrate modification. Halogenated substrate analogs have been employed for many years to probe cytochrome P450 catalysis and to block sites of reactivity, particularly for potential drugs. Consequently, we developed efficient synthetic approaches to introducing one or more halogen atom to the 17- and 21-positions of progesterone and pregnenolone. In particular, novel 21,21,21-tribromoprogesterone and 21,21,21-trichloroprogesterone were synthesized using the nucleophilic addition of either bromoform or chloroform anion onto an aldehyde precursor as the key step to introduce the trihalomethyl moieties. When incubated with microsomes from yeast expressing human CYP21A2 or CYP17A1 with P450-oxidoreductase, CYP21A2 metabolized 17-fluoroprogesterone to a single product, whereas incubations with CYP17A1 gave no products. Halogenated steroids provide a robust system for exploring the substrate tolerance and catalytic plasticity of human steroid hydroxylases.

1. Introduction

The human steroidogenic cytochromes P450 CYP17A1 (P450c17, 17α-hydroxylase/17,20-lyase) and CYP21A2 (P450c21, 21-hydroxylase) oxygenate the 17-position and 21-positions of progesterone, respectively. The hydroxylase activity of CYP17A1 leads to the production of the glucocorticoid cortisol, while the activity of CYP21A2 leads to the production of both mineralocorticoids and glucocorticoids. Deficiency of CYP21A2 (21-hydroxylase deficiency, 21OHD, also called congenital adrenal hyperplasia, CAH) is the most common autosomal recessive disease in human beings, with an incidence of 1:16,000 live births in the classical form [1] and at least 1:1000 in the nonclassical form [2]. Deficiency of CYP17A1 causes hypertension [3], and the CYP17A1 locus has been identified in genome-wide linkage studies as a candidate gene for primary hypertension [4]. In addition to its 17-hydroxylase activity, CYP17A1 also cleaves the 17,20-carbon carbon bond of 17-hydroxysteroids, and because this lyase activity produces 19-carbon androgens, this enzyme is a target to treat prostate cancer [5]. Abiraterone acetate, a potent and specific inhibitor of CYP17A1, received FDA approval for the treatment of castration-resistant prostate cancer unresponsive to docitaxel in early 2011, improving median survival from 10.9 to 14.8 months [6]. Consequently, these enzymes are critically important in human physiology and disease, and a better understanding of their chemistries might lead to better treatments of rare and common diseases.

Interestingly, when progesterone is the substrate, CYP17A1, both 17α- and 16α-hydroxylates in a 3:1 ratio [7]; however when pregnenolone is used as a substrate, 17-hydroxypregnenolone is the only hydroxylation product. CYP21A2 exclusively 21-hydroxylates progesterone and 17-hydroxyprogesterone, but we recently showed that the rationally designed CYP21A2 mutation V359G is primarily a progesterone 16α-hydroxylase [8]. Consequently, these biosynthetic enzymes, known for their high fidelity, also demonstrate intrinsic and latent catalytic plasticity.

Because neither the crystal nor the NMR structures are known for both enzymes, insight to structure-function relationships derives largely from computer modeling [9], site-directed mutagenesis [10], human genetics [11], and substrate analog studies. Halogenated substrate analogs have been employed as probes of cytochrome P450 function and as a strategy to prevent drug metabolism for many years. Limited data exist on how steroidogenic P450 enzymes handle halogenated substrates, and few efforts have comprehensively pursued their synthesis and characterization. Consequently, we developed methodologies to incorporate fluorine, chlorine, and bromine atoms on either the 17- or 21-positions of progesterone and pregnenolone, and we tested these compounds as substrates for CYP17A1 and CYP21A2.

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2. Experimental procedures

2.1. Materials and methods

NMR spectra were obtained using Varian instruments at frequencies for ^{1}H and ^{13}C as specified in the experimental detail. Chemical shifts were referenced to the chloroform peak in the ^{1}H NMR assigned at 7.26 ppm and in the ^{13}C NMR assigned at 77.16 ppm [12]. Reaction progress was determined either by TLC monitoring, or an aliquot was taken and analyzed by NMR. NMR was the only technique used to validate the structures of the new compounds. Pregnenolone was purchased from Waterstone Technologies, and all other reagents and solvents were purchased from Sigma Aldrich or Fisher. NMR spectra are provided in the supporting information.

2.2. Chemical syntheses

2.2.1. pregn-5-ene-21-N-pyridyl-3β-ol-20-one iodide (21-N-pyridinyl-pregnenolone iodide, compound **19**)

Iodine (6.0 g, 23.6 mmol, 1.3 mol eq) was added to a heated stirring solution of pregnenolone (6.0 g, 19.0 mmol) in pyridine, and the reaction was stirred at reflux for 2 h under a N_2 atmosphere. Additional iodine (3.0 g, 11.8 mmol) was added, and the resulting mixture was refluxed overnight. The reaction mixture was cooled to room temperature, diluted with ethyl acetate (100 mL), filtered through cotton, and dried under reduced pressure to afford the crude pyridinium salt **19** as a yellow powder, which was used without purification. ^{1}H NMR (500 MHz, CD_3OD) δ 8.78 (d, J = 5.5 Hz, 1H), 8.64 (t, J = 8.0 Hz, 1H), 8.14 (apparent t, J = 7.0 Hz, 2H), 5.76 (d, J = 17.5 Hz, 1H), 5.61 (d, J = 17.5 Hz, 1H), 5.34 (d, J = 5.5 Hz, 1H), 3.39 (ddd, J_1 = 15.5, J_2 = 10.5, J_3 = 4.5 Hz, 1H), 2.83 (apparent t, J = 9 Hz, 1H), 2.25–2.16 (m, 3H), 2.04–1.98 (m, 2H), 1.90–1.85 (m, 2H), 1.79–1.77 (m, 2H), 1.72–1.68 (m, 1H), 1.63–1.43 (m, 4H), 1.31–1.24 (m, 3H), 1.15–1.00 (m, 2H), 1.02 (s, 3H), 0.88–0.83 (m, 1H), 0.74 (s, 3H).

2.2.2. 3β-hydroxy-androst-5-ene-17β-carboxylic acid (etienic acid, compound **20**)

To the crude pyridinium salt **19** dissolved in THF (40 mL), NaOH pellets (5.2 g) were added, and the reaction was stirred at reflux. After 10 h, the reaction was cooled to RT, diluted with methanol (4 mL) and CH_2Cl_2 (30 mL), and washed with saturated aqueous $NaHCO_3$ solution (50 mL) to submerge the carboxylic acid in the aqueous layer. The aqueous layer was separated and concentrated via reduced pressure. The resultant solid was filtered through a cotton-plugged funnel with CH_2Cl_2 (100 mL), and the sodium salt **20a** was collected as a white powder (3.8 g, 11.2 mmol, 59% over two steps). The carboxylic acid **20** [13] is obtained by dispersing 20a in diethyl ether, washing with 10% HCl, and concentrating the organic extracts. ^{1}H NMR of carboxylic acid 20 (300 MHz, $CDCl_3$) δ 5.35 (d, J = 5.1 Hz, 1H), 3.53 (ddd, J_1 = 16.5, J_2 = 10.8, J_3 = 5.1 Hz, 1H), 2.40 (apparent t, J = 8.7 Hz, 1H), 2.34–2.23 (m, 2H), 2.11–1.94 (m, 3H), 1.90–1.82 (m, 3H), 1.47–1.80 (m, 7H), 1.34–1.23 (m, 2H), 1.23–1.07 (m, 1H), 1.02 (s, 3H), 1.04–0.94 (m, 1H), 0.75 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 177.8, 142.4, 122.4, 72.5, 57.7, 56.5, 51.8, 44.9, 43.1, 39.5, 38.7, 37.9, 33.5, 33.1, 32.4, 25.7, 24.8, 22.3, 20.1, 13.8.

2.2.3. 21,21,21-trifluoro-3β-hydroxy-pregn-5-en-20-one (21,21,21-trifluoropregnenolone, compound **13**) and 21,21,21-trifluoro-pregn-4-ene-3,20-dione (21,21,21-trifluoroprogesterone, compound **4**)

The carboxylic acid **20** (0.2 g, 0.6 mmol, 1.0 mol eq) in dry toluene (20 mL) was cooled to 0 °C. Pyridine (1.0 mL, 8.6 mmol, 14 mol eq) was added via syringe under a N_2 atmosphere followed by trifluoroacetic anhydride (1.2 mL, 8.6 mmol, 13.7 mol eq). The reaction was stirred at reflux for 16 h, and a second portion of trifluoroacetic anhydride (1.0 mL) was added via syringe. Once the trifluoroketone was verified by NMR and TLC, water (0.2 mL) was added, and the reaction continued to stir at reflux for 2 h. The reaction was cooled to room temperature, washed with H_2O (20 mL), and the aqueous layer was back-extracted with ethyl acetate (2 × 20 mL). The combined organic extracts were concentrated, and the crude material was purified via flash column chromatography (hexanes to 50% ethyl acetate in hexanes) to yield **13** as a light yellow solid (0.1 g, 0.27 mmol, 43%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.32 (d, J = 5.2 Hz, 1H), 3.50 (ddd, J_1 = 15.6, J_2 = 11.2, J_3 = 4.4 Hz, 1H), 2.93 (apparent t, J = 9.2 Hz, 1H), 2.30–2.10 (m, 3H), 2.02–1.93 (m, 3H), 1.89–1.71 (m, 4H), 1.63–1.41 (m, 5H), 1.38–1.18 (m, 2H), 1.10–1.02 (m, 1H), 1.00–0.93 (m, 1H), 0.98 (s, 3H), 0.72 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 193.9 (q, J_{CF} = 33.9 Hz), 140.9, 121.2, 115.5 (q, J_{CF} = 174.7), 71.6, 57.3, 56.7, 49.8, 47.1, 42.2, 38.04, 38.03, 37.3, 36.5, 32.1, 31.8, 31.6, 24.7, 21.1, 19.4, 13.5.

Compound **4** (21,21,21-trifluoroprogesterone) is obtained by Oppenauer oxidation of compound **13** as previously described [14] or the by the procedure in Sections 2.2.20 and 2.2.21.

2.2.4. 21,21,21-trichloro-3β-trichloroacetoxy-pregn-5-en-20-one (21,21,21-trichloropregnenolone-3-trichloroacetate, compound **23**)

To carboxylic acid **20** (1.09 g, 3.5 mmol) in CH_2Cl_2 (100 mL) was added pyridine (1.5 mL, 18.6 mmol, 5.4 mol eq) and trichloroacetic anhydride (1.0 mL, 8.9 mmol, 2.6 mol eq) under a N_2 atmosphere at 0 °C. The reaction was warmed to RT and stirred for 18 h. The resulting reaction mixture was concentrated via reduced pressure and purified via flash column chromatography (hexanes to 50% ethyl acetate in hexanes) to yield trichloroketone **23** (0.65 g, 1.2 mmol, 33%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.42 (m, 1H), 4.80–4.71 (m, 1H), 3.22 (apparent t, J = 8 Hz, 1H), 2.51–2.39 (m, 2H), 2.22–2.11 (m, 1H), 2.07–1.89 (m, 6H), 1.82–1.71 (m, 2H), 1.39–1.31 (m, 1H), 1.27–1.14 (m, 2H), 1.06 (s, 3H), 0.93 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.9, 161.5, 138.9, 123.3, 79.7, 56.9, 54.2, 49.8, 47.3, 38.7, 37.4, 36.9, 36.7, 32.1, 32.0, 31.4, 27.2, 25.4, 21.1, 19.5, 13.6.

2.2.5. methyl 3β-hydroxy-androst-5-ene-17β-carboxylate (etienic acid methyl ester, compound **24**)

To pyridinium iodide **19** (6.5 g, 12.5 mmol) dissolved in methanol (10 mL) was added sodium methoxide solution (5.4 M, 10 mL). The reaction was stirred for 48 h and loaded directly on a silica gel column (hexanes to 50% ethyl acetate in hexanes), and methyl ester 24 (3.3 g, 9.9 mmol, 79%) was purified as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36–5.34 (m, 1H), 3.67 (s, 3H), 3.56–3.49 (m, 1H), 2.37–2.24 (m, 3H), 2.21–2.09 (m, 2H), 2.00–1.94 (m, 2H), 1.89–1.65 (m, 4H), 1.53–1.38 (m, 4H), 1.35–1.23 (m, 3H), 1.15–1.05 (m, 2H), 1.00 (s, 3H), 0.67 (s, 3H).

2.2.6. methyl 3β-(t-butyl)dimethyl-siloxy-androst-5-ene-17β-carboxylate (etienic acid methyl ester-3-TBDMS-ether, compound **25**)

Solid t-butyldimethylsilyl chloride (0.61 g, 4.0 mmol, 1.3 mol eq) followed by imidazole (0.28 g, 4.2 mmol, 1.3 mol eq) was added to a stirring solution of methyl ester 24 (1.05 g, 3.2 mmol) in dimethylformamide (50 mL). The reaction was stirred for 1 h, and the resulting mixture was diluted with ethyl acetate (50 mL) and washed with water (at least 2 × 30 mL). The combined organic extracts were concentrated via reduced pressure and purified via flash column chromatography (hexanes to 50% ethyl acetate in hexanes), which also removed residual dimethylformamide and yielded TBDMS-ether **25** (0.70 g, 1.6 mmol, 50%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.31 (broad s, 1H), 3.66 (s, 3H),

3.50–3.44 (m, 1H), 2.34 (apparent t, J = 12 Hz, 1H), 2.30–2.19 (m, 2H), 2.19–2.09 (m, 2H), 2.05–1.94 (m, 2H), 1.85–1.65 (m, 5H), 1.60–1.37 (m, 4H), 1.32–1.22 (m, 2H), 1.14–1.04 (m, 2H), 0.99 (s, 3H), 1.00–0.89 (m, 2H), 0.88 (s, 12H), 0.66 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.7, 141.7, 121.0, 72.7, 61.5, 56.3, 55.3, 51.4, 50.3, 44.1, 42.9, 38.4, 37.5, 36.8, 32.2, 32.0, 26.1, 24.7, 23.8, 21.0, 19.6, 18.4, 13.5, 4.5.

2.2.7. 3β-(t-butyl)dimethyl-siloxy-androst-5-ene-17β-methanol, compound **26**

To methyl ester **25** (1.56 g, 3.5 mmol) in CH_2Cl_2 (100 mL) was added di-isobutyl aluminum hydride (3.0 mL, 16.8 mmol, 4.8 mol eq, neat) at −78 °C under a N_2 atmosphere. The reaction was stirred for 1 h, then ethyl acetate (10 mL) and a saturated aqueous solution of Rochelle's salt (10 mL) were added. The reaction mixture warmed to RT and stirred until the two layers separated (~1–2 h). The aqueous layer was extracted with ethyl acetate (3 × 40 mL), and the combined organic extracts were dried with $MgSO_4$ and concentrated under reduced pressure. The crude material was purified via flash column chromatography (hexanes to 50% ethyl acetate in hexanes) to yield alcohol **26** (1.28 g, 3.1 mmol, 84%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.31 (broad s, 1H), 3.71 (dd, J_1 = 12.0, J_2 = 8.0 Hz, 1H), 3.53 (dd, J_1 = 12.0, J_2 = 8.0 Hz, 1H), 3.53–3.44 (m, 1H), 2.30–2.25 (m, 1H), 2.25–2.15 (m, 1H), 2.05–1.95 (m, 1H), 1.85–1.75 (m, 3H), 1.75–1.40 (m, 10H), 1.00 (s, 3H), 0.89 (s, 9H), 0.66 (s, 3H), 0.06 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 141.7, 121.2, 72.7, 64.8, 56.4, 53.1, 50.6, 43.0, 41.8, 38.8, 37.5, 36.8, 32.2, 32.1, 31.8, 26.1, 25.7, 24.8, 20.9, 19.6, 18.4, 12.6, −4.4.

2.2.8. 3β-(t-butyl)dimethyl-siloxy-androst-5-ene-17β-carboxaldehyde, compound **27**

Alcohol **26** (1.03 g, 2.5 mmol) in CH_2Cl_2 (100 mL) was treated at RT with Dess-Martin periodinane (1.53 g, 3.6 mmol, 1.4 mol eq), and the reaction was stirred for 2 h. The reaction mixture was washed with saturated aqueous $NaHCO_3$ and extracted with ethyl acetate. The combined organic extracts were dried with $MgSO_4$ and concentrated under reduced pressure. The crude material was purified via flash column chromatography (hexanes to 50% ethyl acetate in hexanes) to yield aldehyde **27** (0.66 g, 1.6 mmol, 65%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.77 (d, J = 4 Hz, 1H), 5.31 (broad s, 1H), 3.51–3.43 (m, 1H), 2.38–1.97 (m, 9H), 1.85–1.71 (m, 6H), 1.35–1.21 (m, 3H), 1.00 (s, 3H), 0.88 (s, 9H), 0.76 (s, 3H), 0.05 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 205.2, 141.7, 120.9, 72.6, 63.0, 56.6, 50.3, 44.9, 42.9, 38.5, 37.5, 36.8, 32.2, 32.0, 31.6, 26.1, 26.0, 25.1, 21.2, 20.7, 19.6, 18.4, 13.9.

2.2.9. 21,21,21-tribromo-pregn-5-ene-3β-(t-butyl)dimethylsiloxy-20-ol, compound **28**

To a flask containing aldehyde **27** (0.78 g, 1.9 mmol) was added bromoform (1.4 mL, 16.0 mmol, 8.5 mol eq) followed by DBU (0.32 mL, 2.1 mmol, 1.1 mol eq). The reaction was stirred at RT for 24 h and partially purified via flash column chromatography (hexanes to 30% ethyl acetate in hexanes) to yield a mixture of the 20α- and 20β-carbinols **28** with unreacted **27** (0.56 g total weight), which was used directly for the next step. (See supporting information for NMR spectra.)

2.2.10. 21,21,21-tribromo-pregn-5-ene-3β-(t-butyl) dimethylsiloxy-20-one, compound **29**

To the mixture of 20α- and 20β-alcohols **28** with unreacted aldehyde **27** (0.56 g, 1.0 mmol) in CH_2Cl_2 (50 mL) was added Dess-Martin periodinane (0.60 g, 1.4 mmol, 1.3 mol eq). The reaction was stirred for 2 h and purified via flash column chromatography (hexanes to 50% ethyl acetate in hexanes) directly to yield ketone **29** (0.19 g, 0.28 mmol, 27%, 15% over two steps) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.31 (broad s, 1H), 3.53–3.44 (m, 1H), 3.37 (apparent t, J = 8 Hz, 1H), 2.32–2.11 (m, 2H), 2.10–1.94 (m, 2H), 1.86–1.75 (m, 2H), 1.65–1.35 (m, 5H), 1.25 (s, 3H), 1.02 (s, 3H), 0.96 (s, 3H), 0.88 (s, 9H), 0.05 (s, 6H); ^{13}C NMR (100 MHz, CD Cl_3) δ 192.1, 141.8, 120.9, 72.6, 56.9, 52.9, 50.0, 47.4, 42.9, 39.3, 37.5, 36.8, 33.0, 32.2, 32.1, 29.9, 26.1, 25.6, 21.2, 19.6, 18.4, 13.7, 9.9, −4.4.

2.2.11. 21,21,21-tribromo-pregn-5-en-3β-ol-20-one (21,21,21-tribromopregnenolone, compound **15**)

To a solution of **29** (0.19 g, 0.28 mmol) in CH_2Cl_2 (10 mL) and methanol (5 mL) was added camphorsulfonic acid (0.2 mg, 0.85 μmol, 0.003 mol eq). The reaction was stirred for 12 h and directly purified via flash column chromatography (hexanes to 50% ethyl acetate in hexanes) to afford tribromide **15** (90 mg, 0.16 mmol, 58%) as a white solid. Deprotection using p-toluenesulfonic acid was also successful, but longer reaction times led to formation of the dibromide (21,21-dibromopregnenolone) by mono-dehalogenation. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36 (m, 1H), 3.58–3.48 (m, 1H), 3.37 (apparent t, J = 8 Hz, 1H), 2.35–2.20 (m, 3H), 2.13–1.93 (m, 3H), 1.90–1.75 (m, 3H), 1.65–1.35 (m, 9H), 1.15–0.95 (m, 1H), 1.03 (s, 3H), 0.96 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.1, 141.0, 121.4, 71.8, 56.9, 52.9, 50.1, 47.4, 42.3, 39.3, 37.4, 36.7, 33.0, 32.2, 32.0, 31.7, 25.6, 21.2, 19.6, 13.7.

2.2.12. 21,21,21-tribromo-pregn-4-ene-3,20-dione (21,21,21-tribromoprogesterone, compound **6**)

To a solution of 21,21,21-tribromopregnenolone **15** (70 mg, 0.13 mmol) in CH_2Cl_2 (15 mL) was added Dess-Martin periodinane (55 mg, 0.13 mmol, 1.0 mol eq). After stirring for 3 h at RT, the reaction mixture was directly purified via flash column chromatography (hexanes to 50% ethyl acetate in hexanes) to yield 21,21,21-tribromoprogesterone **6** (32 mg, 0.06 mmol, 46%). The Δ^5-olefin isomerized to the Δ^4-isomer on the silica column. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.73 (s, 1H), 3.38 (apparent t, J = 8 Hz, 1H), 2.55–2.20 (m, 7 H), 2.10–1.95 (m, 4H), 1.92–1.80 (m, 3H), 1.55–1.35 (m, 2H), 1.30–1.15 (m, 2H), 1.17 (s, 3H), 0.99 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.6, 191.9, 170.9, 124.1, 56.0, 53.6, 52.7, 49.8, 47.3, 39.1, 38.7, 35.82, 35.78, 34.1, 32.87, 32.85, 32.1, 25.4, 21.1, 17.5, 13.8.

2.2.13. 21,21,21-trichloro-pregn-5-ene-3β-(t-butyl) dimethylsilyloxy-20-ol, compound **31**

To a flask containing aldehyde **27** (0.60 g, 1.4 mmol) was added chloroform (20 mL) followed by DBU (0.50 mL, 3.7 mmol, 2.6 mol eq). The reaction was stirred at RT for 72 h and directly purified via flash column chromatography (hexanes to 50% ethyl acetate in hexanes) to afford epimeric trichlorocarbinols **31** [15] (0.13 g, 17%) as a white solid. ^{1}H NMR (less polar 20-epimer, 400 MHz, $CDCl_3$) δ 5.31 (broad s, 1H), 4.27 (d, J = 4 Hz, 1H), 3.55–3.41 (m, 1H), 2.80 (d, J = 4 Hz, 1H), 2.39 (dd, J_1 = 8, J_2 = 4 Hz, 1H), 2.30–2.23 (m, 1H), 2.20–1.96 (m, 4H), 1.90–1.80 (m, 2H), 1.76–1.70 (m, 3H), 1.35–1.19 (m, 5H), 1.01 (s, 3H), 0.89 (s, 9H), 0.86 (s, 3H), 0.06 (6H); ^{1}H NMR (more polar 20-epimer, 400 MHz, $CDCl_3$) δ 5.32 (broad s, 1H), 4.02 (dd, J_1 = 9, J_2 = 6 Hz, 1H), 3.55–3.43 (m, 1H), 2.69 (d, J = 6 Hz, 1H), 2.32–2.14 (m, 5H), 2.05–1.91 (m, 4H), 1.85–1.65 (m, 8H), 1.35–1.15 (m, 3H), 0.89 (s, 9H), 0.86 (s, 3H), 0.06 (s, 6H).

2.2.14. 21,21,21-trichloro-pregn-5-ene-3β-(t-butyl) dimethylsilyloxy-20-one, compound **32**

Compound **32** was obtained from **31** in the similar fashion to section 2.2.10 (110 mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.32 (broad s, 1H), 3.52–3.44 (m, 1H), 3.22 (apparent t, J = 12 Hz, 1H), 2.32–2.10 (m, 4H), 2.10–1.95 (m, 2H), 1.90–1.65 (m, 6H), 1.65–1.40 (m, 6H), 1.01 (s, 3H), 0.92 (s, 3H), 0.88 (s, 9H), 0.05 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.0, 141.8, 120.8, 72.6, 57.0, 54.2, 50.0, 47.3,

42.9, 38.8, 37.5, 36.8, 32.18, 32.15, 32.06, 31.4, 26.1, 25.5, 21.2, 19.6, 18.4, 13.6, −4.4.

*2.2.15. 21,21,21-trichloro-pregn-5-en-3β-ol-20-one (21,21,21-trichloropregnenolone, compound **14**)*

To a solution of **32** (104 mg, 0.19 mmol) in THF (25 mL) was added p-toluenesulfonic acid monohydrate (66 mg, 0.35 mmol, 1.8 mol eq). The reaction was stirred for 2 h, and the product was purified via flash column chromatography (hexanes to 70% ethyl acetate in hexanes) to yield **14** (71 mg, 0.17 mmol, 87%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36 (broad s, 1H), 3.60–3.45 (m, 1H), 3.22 (apparent t, J = 12 Hz, 1H), 2.35–2.10 (m, 4H), 1.40–1.20 (m, 5H), 1.00 (s, 3H), 0.93 (s, 3H).

*2.2.16. 21,21,21-trichloro-pregn-4-ene-3,20-dione (21,21,21-trichloroprogesterone, compound **5**)*

The procedure to oxidize **14** was followed as in section 2.2.12 with isomerization on the silica gel column to yield **5** [16] (7 mg, 58%, over two steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.73 (broad s, 1H), 3.22 (apparent t, J = 12 Hz, 1H), 2.49–2.27 (m, 5H), 2.21–2.11 (m, 1H), 2.08–1.96 (m, 1H), 1.95–1.75 (m, 4H), 1.70–1.56 (m, 4H), 1.55–1.25 (m, 2H), 1.19 (s, 3H), 0.96 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.6, 191.8, 170.8, 124.1, 56.1, 53.6, 53.0, 47.1, 38.6, 35.84, 35.77, 34.0, 32.9, 32.1, 31.3, 25.3, 21.1, 17.5, 13.7.

*2.2.17. 5,6,17-tribromo-20-oxo-pregn-5-ene-3β-yl acetate, compound **36***

Pregnenolone (4.0 g, 12.6 mmol) and p-toluenesulfonic acid (2.4 g, 14.0 mmol, 1.1 mol eq) were refluxed in acetic anhydride (45 mL, 25 mol eq) until 20 mL of solvent was condensed out of the reaction via a Dean-Stark trap and a reflux condenser. The reaction was cooled to room temperature and then maintained at 0 °C for 1 h. The cold reaction solution was diluted with diethyl ether (25 mL) and washed with $NaHCO_3$ (sat. aqueous solution, 3 × 25 mL). The organic layer was concentrated via reduced pressure to yield enol acetate **33** as a brown solid, which was used without purification.

The crude enol acetate **33** (5.0 g, 12.5 mmol) in acetic acid (20 mL) with sodium acetate (2.0 g, 24.3 mmol, 1.9 mol eq) was treated with a mixture of bromine (1.3 mL, 25.4 mmol, 2.0 mol eq) in 5 mL H_2O, and the reaction was stirred for 1 h at room temperature. The reaction was quenched with saturated aqueous $Na_2S_2O_3$ (30 mL), extracted with ethyl acetate (3 × 15 mL), and concentrated under reduced pressure. The crude material was purified via flash column chromatography (10% ethyl acetate in hexanes) to yield the tribromide **36** as a white solid (5.2 g, 8.8 mmol, 70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.41–5.49 (m, 1H), 3.04 (ddd, J_1 = 16.0, J_2 = 12.0, J_3 = 3.6 Hz, 1H), 2.76 (ddd, J_1 = 15.6, J_2 = 12.4, J_3 = 4.0 Hz, 1H), 2.03 (dd, J_1 = 14.0, J_2 = 10.4 Hz, 1H), 2.35 (s, 3H), 2.23–2.31 (m, 2H), 2.04–2.15 (m, 2H), 2.02 (s, 3H), 1.78–1.99 (m, 5H), 1.69–1.75 (m, 2H), 1.56–1.65 (m, 3H), 1.44 (s, 3H), 1.21–1.32 (m, 2H), 0.78 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 201.3, 170.4, 87.4, 85.8, 71.9, 55.6, 49.7, 47.3, 46.6, 41.94, 41.91, 37.1, 36.6, 36.1, 35.4, 31.4, 27.5, 26.2, 22.8, 21.5, 21.4, 20.3, 14.4.

*2.2.18. 17-bromopregnenolone-3-acetate (17-bromo-20-oxo-pregn-5-ene-3β-yl acetate, compound **37**)*

Tribromide **36** (2.5 g, 4.2 mmol) and sodium iodide (1.5 g, 10 mmol, 2.4 mol eq) were dissolved in acetone (30 mL), and the reaction was stirred for 1 h. The reaction was quenched with $Na_2S_2O_3$ (20 mL sat. aqueous), mixed with brine (10 mL), and extracted with diethyl ether (3 × 25 mL). The organic extracts were combined and concentrated via reduced pressure, and the crude material was purified via silica column chromatography (10% ethyl acetate in hexanes) to yield **37** (1.4 g, 3.3 mmol, 77%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 (d, J = 5.2 Hz, 1H), 4.55–4.63 (m, 1H), 3.06 (ddd, J_1 = 16.0, J_2 = 12.0, J_3 = 4.0 Hz, 1H), 2.37 (s, 3H), 2.23–2.34 (m, 3H), 2.02, (s, 3H), 1.93–2.00 (m, 2H), 1.82–1.90 (m, 5H), 1.36–1.69 (m, 5H), 1.22–1.36 (m, 1H), 1.12–1.20 (m, 1H), 1.06 (dd, J_1 = 12.4, J_2 = 4.8 Hz, 1H), 1.01 (s, 3H), 0.76 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 201.6, 170.6, 139,7, 122.3, 86.2, 73.9, 51.1, 49.3, 47.0, 38.1, 37.0, 36.6, 36.1, 35.5, 32.4, 31.2, 27.8, 27.6, 23.1, 21.5, 21.2, 19.4, 14.0.

*2.2.19. 17-bromo-3β-hydroxy-pregn-5-en-20-one (17-bromopregnenolone, compound **12**)*

To a solution of acetate **37** (0.55 g, 1.26 mmol) dissolved in 5 mL CH_2Cl_2 and 45 mL methanol was added 0.2 mL of 12 M HCl, and the reaction was stirred overnight. The reaction mixture was concentrated via reduced pressure, and the crude product was purified via flash column chromatography (20% ethyl acetate in hexanes) to yield **12** (0.45 g, 1.14 mmol, 91%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.36 (d, J = 8.5 Hz, 1H), 3.48–3.59 (m, 1H), 3.08 (ddd, J_1 = 19.0, J_2 = 7.5, J_3 = 5.5 Hz, 1H), 2.38 (s, 3H), 2.23–2.34 (m, 3H), 2.03–2.07 (m, 1H), 1.97–2.02 (m, 2H), 1.82–1.94 (m, 4H), 1.39–1.71 (m, 3H), 1.04–1.36 (m, 5H), 1.01 (s, 3H), 0.78 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 201.6, 140.9, 121.1, 86.3, 71.5, 51.1, 49.3, 46.9, 42.2, 37.3, 36.4, 36.0, 35.5, 32.3, 31.6, 31.5, 27.5, 23.0, 21.1, 19.5, 13.9.

2.2.20. 17α-bromo-pregn-5-ene-3,20-dione (17-bromo-Δ^5-progesterone)

Alcohol **12** (0.51 g, 1.28 mmol) and Dess-Martin periodinane (0.52 g, 1.21 mmol, 0.9 mol eq) were dissolved in CH_2Cl_2 (5 mL) and stirred for 30 min at room temperature, then concentrated via reduced pressure and purified via flash column chromatography (10% ethyl acetate in hexanes) to yield the 3-ketosteroid intermediate (0.36 g, 0.92 mmol, 72%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.30 (apparent t, J = 2.8 Hz, 1H), 3.23 (dd, J_1 = 16.4, J_2 = 3.6 Hz, 1H), 3.02, (ddd, J_1 = 16.4, J_2 = 12.0, J_3 = 4.0 Hz, 1H), 2.78 (dd, J_1 = 16.4, J_2 = 2.0, Hz, 1H), 2.44 (ddd, J_1 = 13.6, J_2 = 13.6, J_3 = 6.0 Hz, 1H), 2.34 (s, 3H), 2.20–2.29 (m, 2H), 1.98–2.05 (m, 2H), 1.79–1.96 (m, 4H), 1.60–1.70 (m, 2H), 1.41–1.56 (m, 3H), 1.18–1.29 (m, 1H), 1.14 (s, 3H), 1.06–1.11 (m, 1H), 0.76 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 210.0, 201.4, 138.6, 122.4, 86.0, 51.0, 48.4, 48.3, 47.0, 37.6, 36.9, 36.8, 36.1, 35.4, 32.4, 31.5, 27.5, 23.0, 21.4, 19.3, 14.0.

*2.2.21. 17α-bromo-pregn-4-ene-3,20-dione (17-bromo-progesterone, compound **3**)*

17-Bromo-pregn-5-ene-3,20-dione (0.36 g, 0.92 mmol) was dissolved in 1:1 CH_2Cl_2:methanol (30 mL), and 0.1 mL of 12 M HCl was added. After stirring at RT for 1 h, the reaction mixture was concentrated via reduced pressure, and the crude material was purified via flash column chromatography (hexanes to 10% ethyl acetate in hexanes) to yield **3** [17] as a white solid (0.34 g, 0.87 mmol, 94%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.75 (s, 1H), 3.07 (ddd, J_1 = 17, J_2 = 12, J_3 = 4 Hz, 1H), 2.38 (s, 3H), 2.29–2.48 (m, 6H), 2.40 (s, 3H), 1.86–2.08 (m, 3H), 1.67–1.79 (m, 2H), 1.54–1.62 (m, 1H), 1.21 (s, 3H), 1.12–1.18 (m, 2H), 1.06 (ddd, J_1 = 10.0, J_2 = 10.0, J_3 = 3.2 Hz, 1H), 0.83 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 201.3, 199.4, 170.6, 124.1, 85.9, 52.9, 50.4, 47.0, 38.6, 36.05, 36.02, 35.7, 35.4, 34.0, 32.8, 31.7, 27.5, 22.9, 21.1, 17.5, 14.1.

*2.2.22. 17-fluoro-20-oxo-pregn-5-en-3β-yl acetate (17-fluoropregnenolone-3-acetate, compound **35**)*

Enol acetate **33** (0.10 g, 0.25 mmol), Selectfluor (0.25 g, 0.71 mmol, 2.8 mol eq) and sodium acetate (0.05 g) were weighed in a screw-cap vial and dissolved in acetonitrile (3 mL). The reaction was stirred at 60 °C for 2 h, then concentrated under reduced pressure and purified via flash column chromatography (10–50% ethyl acetate in hexanes) to afford fluoride **35** (56 mg, 0.15 mmol, 59%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 (m, 1H),

4.63–4.55 (m, 1H), 2.64–2.49 (m, 1H), 2.37–2.28 (m, 1H), 2.21 (d, J_{HF} = 8 Hz, 3H), 2.01 (s, 3H), 1.98–1.93 (m, 1H), 1.20–1.10 (m, 1H), 1.00 (s, 3H), 0.64 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.7 (d, J_{CF} = 132 Hz), 170.6, 139.7, 122.4 (d, J_{CF} = 60 Hz), 112.2, 110.3, 73.9, 51.6, 49.5, 38.2, 37.1, 36.7, 32.0, 31.3, 30.7, 27.7, 23.9, 21.5, 20.6, 19.4, 14.1 (d, J_{CF} = 20 Hz).

*2.2.23. 17-fluoro-3β-hydroxy-pregn-5-en-20-one (17-fluoropregnenolone, compound **10**)*

Methanolysis of acetate **35** as described in section 2.2.19, yielded compound **10** (20 mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37–5.34 (m, 1H), 3.58–3.50 (m, 1H), 2.66–2.49 (m, 1H), 2.36–2.23 (m, 1H), 2.23 (d, J_{HF} = 5.2 Hz, 3H), 2.09–1.95 (m, 2H), 1.90–1.72 (m, 3H), 1.72–1.60 (m, 2H), 1.35–1.25 (m, 1H), 1.15–1.05 (m, 1H), 1.01 (s, 3H), 0.67 (s, 3H).

*2.2.24. 17-fluoro-pregn-4-ene-3,20-dione (17-fluoroprogesterone, compound **1**)*

The procedures for oxidation and isomerization of **10** to **1** [18] were followed as described in sections 2.2.20 and 2.2.21 (10 mg, 45%, 2 steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.74 (broad s, 1H), 2.59 (dddd, J_{HF} = 40, J_1 = 12, J_2 = 4, J_3 = 2 Hz, 1H), 2.48–2.26 (m, 1H), 2.23 (d, J_{HF} = 5 Hz, 3H), 2.05–2.01 (m, 1H), 1.92–1.62 (m, 9H), 1.19 (s, 3H), 1.08–0.97 (m, 1H), 0.70 (s, 3H); additional resonances from impurities appeared in the 2.25–2.50 ppm region of the spectrum.

*2.2.25. 17-chloro-20-oxo-pregn-5-en-3β-yl acetate (17-chloropregnenolone-3-acetate, compound **34**)*

To enol acetate **33** (90 mg, 0.22 mmol) in CH_2Cl_2 (5 mL) in a screw cap vial was added N-chlorosuccinimide (46 mg, 0.34 mmol, 1.5 mol eq). The reaction was stirred at 55 °C for 2 h, concentrated under reduced pressure, and purified via flash column chromatography (10–50% ethyl acetate in hexanes) to afford chloride **34** (60 mg, 0.15 mmol, 69%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.39–5.38 (m, 1H), 4.67–4.56 (m, 1H), 3.09–2.98 (m, 1H), 2.32 (s, 3H), 2.10–1.95 (m, 3H), 2.04 (s, 3H), 1.95–1.84 (m, 4H), 1.75–1.61 (m, 3H), 1.30–1.05 (m, 4H), 1.02 (s, 3H), 0.74 (s, 3H).

*2.2.26. 17-chloro-3β-hydroxy-pregn-5-en-20-one (17-chloropregnenolone, compound **11**) and 17α-chloro-pregn-4-ene-3,20-dione (17-chloroprogesterone, compound **2**)*

Using procedures described in sections 2.2.19–2.2.21, methanolysis of acetate **34** gave **11** (15 mg, 80%), and oxidation/isomerization of 11 gave 2 [19] (10 mg, 67%, 2 steps). ^{1}H NMR of compound **11** (400 MHz, $CDCl_3$) δ 5.37–5.35 (m, 1H), 3.57–3.50 (m, 1H), 3.01 (ddd, J_1 = 15.4, J_2 = 11.5, J_3 = 2.9 Hz, 1H), 2.32 (s, 3H), 2.32–2.15 (m, 2H), 2.06–1.93 (m, 3H), 1.92–1.80 (m, 4H), 1.79–1.74 (m, 1H), 1.72–1.64 (m, 2H), 1.60–1.37 (m, 3H), 1.15–1.02 (m, 2H), 1.01 (s, 3H), 0.74 (s, 3H).

^{1}H NMR of compound **2** (400 MHz, $CDCl_3$) δ 5.75 (s, 1H), 3.07–2.96 (m, 1H), 2.45–2.26 (m, 3H), 2.32 (s, 3H), 2.09–1.66 (m, 11H), 1.19 (s, 3H), 1.06–0.97 (m, 2H), 0.91–0.79 (m, 3H), 0.77 (s, 3H).

*2.2.27. 20-oxo-pregn-5-en-3β-yl formate (pregnenolone-3-formate, compound **38**)*

Pregnenolone (6.0 g, 19.0 mmol) and formic acid (60 mL) were stirred for 30 min at 70 °C. The reaction was cooled to RT, diluted with diethyl ether (200 mL), and washed sequentially with water (50 mL) and $NaHCO_3$ (2 × 50 mL, saturated aqueous solution). The organic phase was concentrated and crystallized with acetone and diethyl ether to yield formate **38** (5.0 g, 14.0 mmol, 74%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (s, 1H), 5.38 (m, 1H), 4.77–4.67 (m, 1H), 2.52 (apparent t, J = 9 Hz, 1H), 2.36 (m, 2H), 2.21–2.10 (m, 3H), 2.10 (s, 3H), 2.05–1.95 (m, 2H), 1.90–1.86 (m, 2H), 1.71–1.57 (m, 5H), 1.51–1.39 (m, 4H), 1.01 (s, 3H), 0.61 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.57, 160.7, 139.4, 122.8, 73.9, 63.8, 56.9, 50.0, 44.1, 38.9, 38.1, 37.0, 36.7, 31.9, 31.8, 31.6, 27.8, 24.6, 22.9, 21.1, 19.4, 13.3.

*2.2.28. pregna-5,20-diene-3β,20-diyl 20-acetate 3-formate, compound **39***

To formate **38** (1.0 g, 2.8 mmol) in isopropenyl acetate (50 mL) with p-toluenesulfonic acid monohydrate (0.1 g) was refluxed for 20 h as water was removed with a Dean Stark trap. Longer times (>24 h) produced isomerization to the 17,20-enol acetate. The reaction was diluted with diethyl ether (50 mL) and washed with $NaHCO_3$ (3 × 40 mL, saturated aqueous solution). The combined organic extracts were concentrated and purified via flash column chromatography (hexanes to 50% ethyl acetate in hexanes) to afford enol acetate **39** (0.4 g, 1.0 mmol, 36%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (s, 1H), 5.38 (d, J = 4 Hz, 1H), 4.69 (s, 2H), 4.76–4.65 (m, 1H), 2.40–2.33 (m, 2H), 2.10 (s, 3H), 1.93–1.84 (m, 4H), 1.30–1.02 (m, 5H), 1.01 (s, 3H), 0.66 (s, 3H).

*2.2.29. 21-bromo-20-oxo-pregn-5-en-3β-yl formate (21-bromopregnenolone-3-formate, compound **40**)*

A solution of enol acetate **39** (135 mg, 0.35 mmol) and N-bromosuccinimide (0.062 g, 0.350 mmol, 1 mol eq) in methylene chloride (10 mL) was refluxed for 2 h. The reaction mixture was cooled, concentrated, and purified via flash column chromatography (hexanes to 50% ethyl acetate in hexanes) to yield 21-bromide **40** (40 mg, 0.10 mmol, 27%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (s, 1H), 5.40 (m, 1H), 4.77–4.68 (m, 1H), 3.93 (d, J = 12 Hz, 1H), 3.90 (d, J = 12 Hz, 1H), 2.83 (apparent t, J = 8 Hz, 1H), 1.35–1.11 (m, 8H), 1.02 (s, 3H), 0.66 (s, 3H).

*2.2.30. 21-bromo-pregn-5-en-3β-ol-20-one (21-bromopregnenolone, compound **18**)*

Formate **40** (40 mg, 0.1 mmol) was dissolved 5 mL each methanol and CH_2Cl_2, and 2 drops of 12 M HCl was added. The reaction was stirred for 1 h and directly purified via flash column chromatography (10–50% ethyl acetate in hexanes) to afford **18** [20] (30 mg, 0.08 mmol, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.35 (m, J = 8 Hz, 1H), 3.94 (d, J = 12 Hz, 1H), 3.90 (d, J = 12 Hz, 1H), 3.57–3.47 (m, 1H), 2.83 (apparent t, J = 8 Hz, 1H), 2.31–2.25 (m, 3H), 2.05–1.95 (m, 2H), 1.80–1.70 (m, 4H), 1.35–1.06 (m, 5H), 1.01 (s, 3H), 0.66 (s, 3H).

*2.2.31. 21-bromo-pregn-4-ene-3,20-dione (21-bromoprogesterone, compound **9**)*

To a solution of **18** (15.6 mg, 0.04 mmol) in CH_2Cl_2 (20 mL) was added Dess-Martin periodinane (17 mg, 0.04 mmol, 1 mol eq). The reaction was stirred for 2 h, and the mixture was directly purified via flash column chromatography (hexanes to 50% ethyl acetate in hexanes) to yield **9** [21] (2.0 mg, 13%). The Δ^5-olefin appeared to isomerize to the Δ^4-isomer on the silica column. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.74 (broad s, 1H), 3.93 (d, J = 12 Hz, 1H), 3.89 (d, J = 12 Hz, 1H), 2.84 (apparent t, J = 8 Hz, 1H), 2.49–2.17 (m, 6H), 1.19 (s, 3H), 1.12–0.85 (m, 5H), 0.70 (s, 3H).

*2.2.32. 20-oxo-pregna-5,16-dien-3β-yl formate, compound **42***

Formic acid (50 mL) and 16,17-dehydropregnenolone **41** (5.0 g, 19.2 mmol) were stirred at reflux for 30 min, cooled to RT, washed with H_2O, and extracted with diethyl ether (2 × 100 mL). The resulting mixture was crystallized with acetone and diethyl ether to yield formate **42** (5.46 g, 16.0 mmol, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (s, 1H), 5.37 (m, 1H), 4.77–4.65 (m, 1H), 2.52 (apparent t, J = 9 Hz, 1H), 2.40–2.32 (m, 2H), 2.23–2.10 (m, 3H), 2.10 (s, 3H), 2.05–1.82 (m, 4H), 1.75–1.40 (m, 5H), 1.25–1.10 (m, 3H), 1.01 (s, 3H), 0.61 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.6, 160.7, 139.4,

122.8, 63.8, 56.9, 50.0, 44.0, 38.9, 38.1, 37.0, 36.7, 31.9, 31.8, 27.8, 24.6, 22.9, 21.1, 19.4, 13.3.

2.2.33. pregna-5,16,20-triene-3β,20-diyl 20-acetate 3-formate, compound **43**

Ketone **42** (1.22 g, 3.56 mmol) and p-toluenesulfonic acid monohydrate (0.15 g, 0.8 mmol, 0.2 mol eq) were refluxed in isopropenyl acetate (50 mL) for 20 h as water was removed with a Dean-Stark apparatus. The reaction was cooled to RT and diluted with diethyl ether (100 mL). The solution was washed with $NaHCO_3$ (saturated aqueous solution, 2 × 50 mL), and the organic layer was washed with brine (2 × 25 mL), dried with $MgSO_4$, and concentrated via reduced pressure. The solid formed during concentration was washed with ice-cold methanol to yield the enol acetate **43** [22] (0.90 g, 2.34 mmol, 66%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (s, 1H), 5.82 (m, 1H), 5.40 (m, 1H), 5.06 (s, 1H), 4.78 (s, 1H), 4.78–4.65 (m, 1H), 2.39–2.36 (m, 2H), 2.18 (s, 3H), 2.15–2.10 (m, 1H), 2.04–1.99 (m, 1H), 1.95–1.86 (m, 3H), 1.70–1.47 (m, 8H), 1.19–1.13 (m, 1H), 1.07 (s, 3H), 0.98 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.2, 160.7, 149.7, 148.2, 139.7, 129.8, 122.7, 104.8, 102.3, 73.9, 57.0, 50.1, 46.1, 38.1, 36.9, 35.3, 31.6, 31.0, 30.2, 27.8, 21.0, 20.9, 19.3, 15.9.

2.2.34. 21-iodo-20-oxo-pregna-5,16-dien-3β-yl formate, compound **44**

To enol acetate **43** (0.33 g, 0.86 mmol) in CH_2Cl_2 (50 mL) was added N-iodosuccinimide (0.29 g, 1.29 mmol, 1.5 mol eq). The reaction was stirred at RT for 1 h and directly purified via flash column chromatography (hexanes to 50% ethyl acetate in hexanes) to afford iodide **44** (0.30 g, 0.64 mmol, 74%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.02 (s, 1H), 6.79 (s, 1H), 5.39 (broad s, 1H), 4.70–4.65 (m, 1H), 4.07 (d, J = 12 Hz, 1H), 4.02 (d, J = 12 Hz, 1H), 2.39–2.30 (m, 5H), 2.05–1.98 (m, 1H), 1.92–1.80 (m, 2H), 1.76–1.53 (m, 5H), 1.50–1.43 (m, 1H), 1.37 (ddd, J_1 = 12, J_2 = 12, J_3 = 5 Hz, 1H), 1.14 (apparent t, J = 12 Hz, 1H), 1.06 (s, 3H), 0.96 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 191.2, 160.7, 151.7, 145.7, 140.0, 122.4, 73.9, 56.1, 50.4, 46.5, 38.2, 36.9, 36.8, 34.4, 32.7, 31.6, 30.2, 27.8, 20.7, 19.3, 15.6, 3.8.

2.2.35. 21-fluoro-20-oxo-pregna-5,16-dien-3β-yl formate, compound **45**

A solution of iodide **44** (0.70 g, 1.45 mmol) in acetonitrile (50 mL) with AgF (0.54 g, 4.26 mmol, 3.4 mol eq) was stirred at RT for 36 h to complete the reaction. After addition of water, the mixture was extracted with ethyl acetate (2 × 25 mL), and the combined organic extracts were concentrated via reduced pressure. The crude material was purified via flash column chromatography (hexanes to 50% ethyl acetate in hexanes) to yield fluoride 45 [23] (0.39 g, 1.07 mmol, 76%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.04 (s, 1H), 6.78 (s, 1H), 5.4 (broad s, 1H), 5.16 (dd, J_{HF} = 24, J_1 = 8 Hz, 1H), 5.04 (dd, J_{HF} = 24, J_1 = 8 Hz, 1H), 4.80–4.65 (m, 1H), 2.48–2.29 (m, 5H), 2.20–1.95 (m, 4H), 1.95–1.80 (m, 4H), 1.07 (s, 3H), 0.96 (s, 3H).

2.2.36. 21-fluoro-3β-hydroxy-pregna-5,16-dien-20-one (16,17-dehydro-21-fluoropregnenolone, compound **46**)

Formate **45** (0.39 g, 1.07 mmol) was methanolyzed with 12 M HCl as in section 2.2.19 to give **46** (0.27 g, 0.81 mmol, 76% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.77 (s, 1H), 5.35 (m, 1H), 5.13 (dd, J_{HF} = 24, J_1 = 5 Hz, 1H), 5.04 (dd, J_{HF} = 24, J_1 = 5 Hz, 1H), 2.42–2.20 (m, 5H), 2.15–1.95 (m, 2H), 1.88–1.80 (m, 2H), 1.56–1.46 (m, 1H), 1.45–1.32 (m, 2H), 1.12–1.01 (m, 3H), 1.04 (s, 3H), 0.95 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 192.1 (d, J_{CF} = 16 Hz), 151.6, 145.4 (d, J_{CF} = 38 Hz), 141.5, 121.0, 83.4 (d, J_{CF} = 181 Hz), 71.8, 56.0, 50.6, 46.9, 42.4, 37.2, 36.8, 34.5, 33.0, 32.4, 31.7, 30.2, 20.8, 19.4, 16.0.

2.2.37. 21-fluoro-pregna-4,16-diene-3,20-dione (16,17-dehydro-21-fluoroprogesterone, compound **48**)

Using the procedure in section 2.2.12, **46** (0.27 g, 0.81 mmol) was oxidized to **47**, followed by isomerization as in section 2.2.21 to yield **48** (0.18 g, 0.54 mmol, 67%, 2 steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.78 (s, 1H), 5.74 (broad s, 1H), 5.15 (dd, J_{HF} = 24, J_1 = 12 Hz, 1H), 5.05 (dd, J_{HF} = 24, J_1 = 12 Hz, 1H), 2.50–2.25 (m, 5H), 2.16–2.10 (m, 1H), 2.06–2.00 (m, 1H), 1.92–1.85 (m, 1H), 1.82–1.50 (m, 6H), 1.46–1.33 (m, 3H), 1.22 (s, 3H), 0.98 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 210.2, 192.1 (d, J_{CF} = 16 Hz), 151.5, 145.3 (d, J_{CF} = 5 Hz), 139.3, 122.3, 104.9, 83.4 (d, J_{CF} = 183 Hz), 55.8, 49.7, 48.5, 47.0, 37.7, 37.2, 34.4, 33.0, 31.6, 30.3, 21.0, 19.2, 16.0.

2.3. Enzymology studies

2.3.1. CYP17A1 and CYP21A2 microsome incubation with halosteroid

Enzyme assays used human CYP21A2 and CYP17A1 with P450-oxidoreductase in microsomes from yeast expressing the recombinant proteins as described [24]. Test steroid (Fig. 1, 20–50 μM, delivered in <5 μL ethanol) and microsomes (5–10 μL, ~10 pmol P450) were incubated in 1 mL 50 mM potassium phosphate (pH 7.4) with 1 mM NADPH for 2–5 h at 37 °C. For control incubations with ketoconazole to inhibit P450 activity, microsomes and 100 μM ketoconazole were preincubated at room temperature for 15 min before the addition of steroid followed by NADPH. The incubations were terminated with the addition of CH_2Cl_2 (1 mL),

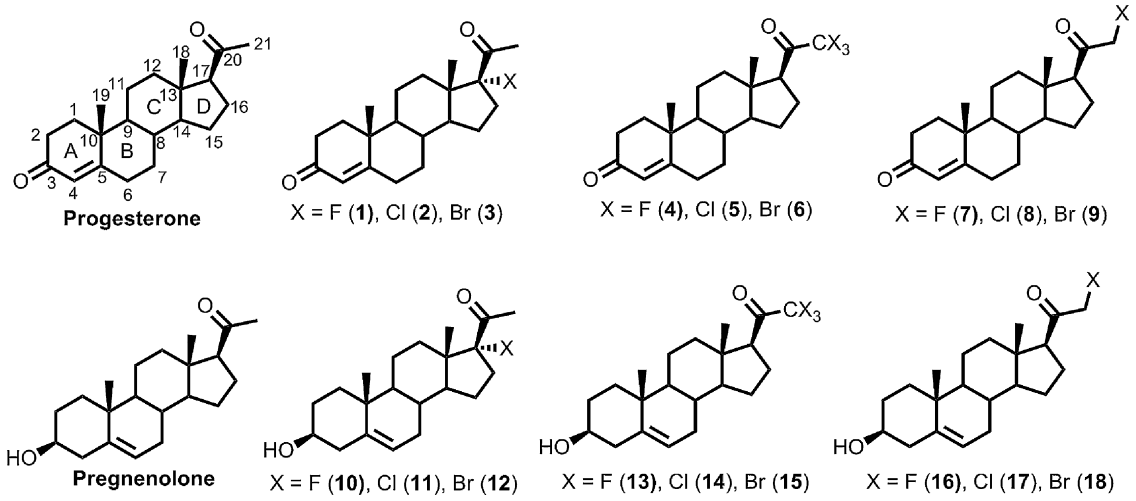
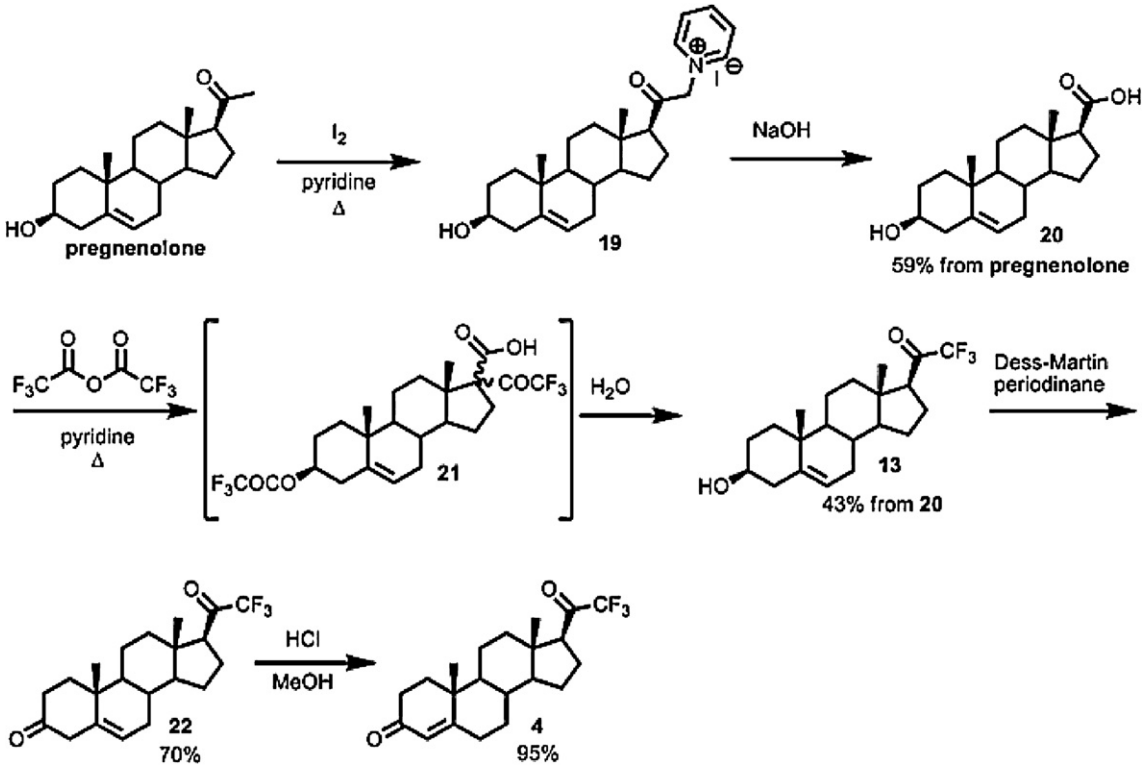


Fig. 1. Halogenated steroid substrates (**1**–**18**) synthesized for incubation with steroidogenic cytochromes P450, CYP17A1 and CYP21A2.



Scheme 1. Synthesis of 21,21,21-F_3-progesterone (**4**) via Zard methodology.

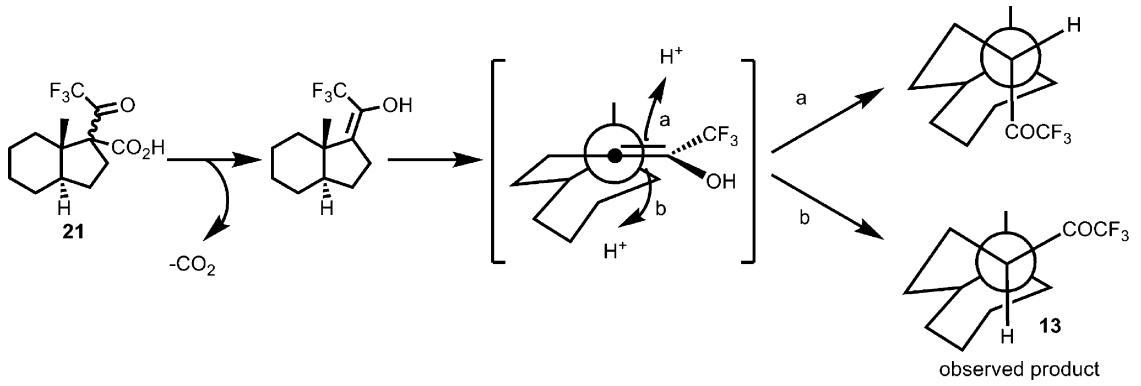
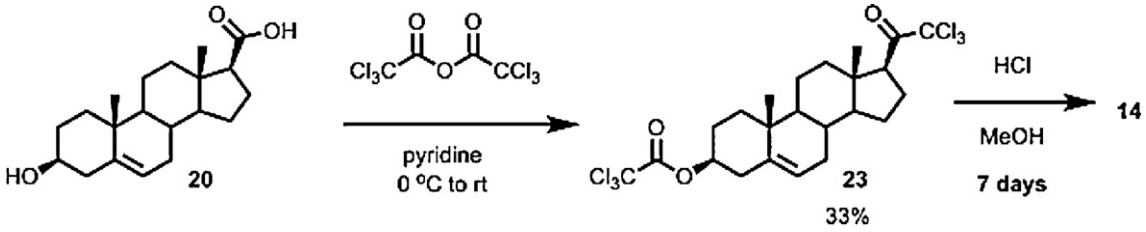


Fig. 2. Explanation of C-17 stereochemistry of 21,21,21-F_3-pregnenolone–AB ring system omitted for clarity.

extraction, and centrifugation. The organic extract was dried under nitrogen flow, dissolved in 50% aqueous methanol (70–90 μL), and analyzed by high performance liquid chromatography (HPLC).

2.3.2. Cholesterol oxidase treatment

For analysis of incubations with Δ^5,3β-hydroxy-steroids, substrates and products were converted to their Δ^4,3-ketosteroid congeners to generate a UV chromophore. The dried extracts were dissolved in 80 μL of ethanol and diluted in 0.2 mL of 50 mM potassium phosphate (pH 7.4) solution. To this solution, 1 unit of cholesterol oxidase in 40 μL water was added, and the incubation was shaken at 200 rpm and 30 °C for 5–10 h. The reaction was extracted with 1 mL of CH_2Cl_2, and the dried extracts were dissolved in 50% methanol for HPLC analysis.



Scheme 2. 21,21,21-Cl_3-pregnenolone-3-trichloroacetate (**23**) synthesis using trichloroacetic anhydride.

2.3.3. HPLC analyses

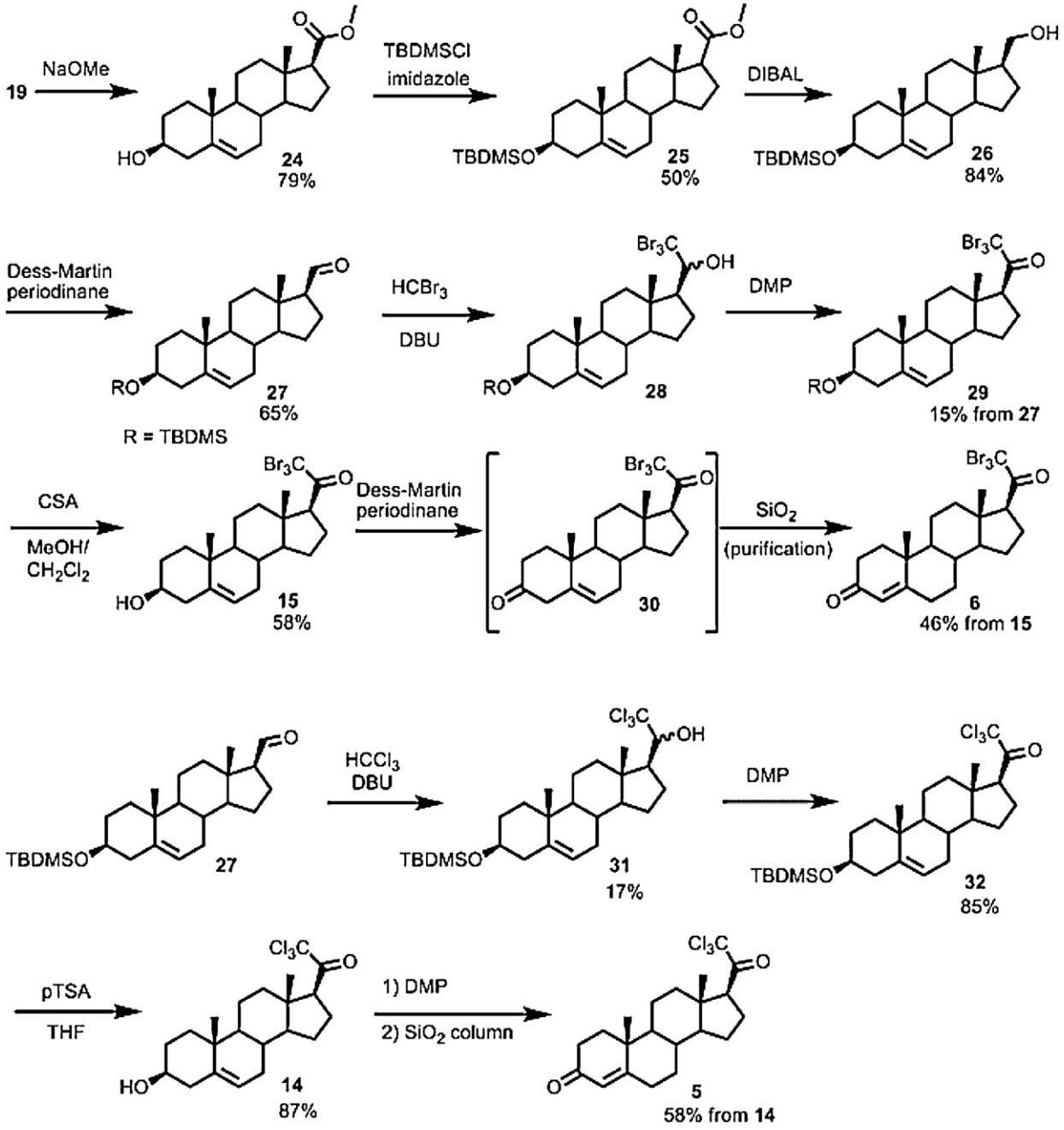
Steroids were resolved using a Waters Breeze 1525 HPLC equipped with autosampler and dual-wavelength UV-visible detector set at 254 nm. The stationary phase was a Symmetry 3.1 mm × 150 mm, 3.5 μm C_{18} (Waters) or either Kinetex 2.1 mm × 100 mm, 2.6 μm C_{18} or 3.0 mm × 50 mm, 2.6 μm, C_8 (Phenomenex) reverse-phase column maintained at 40 °C (30 °C for Kinetex 3.0 mm × 50 mm), and methanol–water gradients at 0.4 mL/min (0.6 mL/min for Kinetex 3.0 mm × 50 mm) comprised the mobile phase. For the Kinetex 3.0 mm × 50 mm C_8 column in each run, the gradient went from 27% methanol from 0 to 0.5 min, 39% methanol at 0.51 min, 44% methanol to 16 min, 60% methanol to 20 min, 71% methanol to 22 min, 75% methanol to 30 min, and back to 27% methanol to re-equilibrate for 3 min. Authentic standards and starting substrates were chromatographed before and/or after incubation products with each experiment.

3. Results

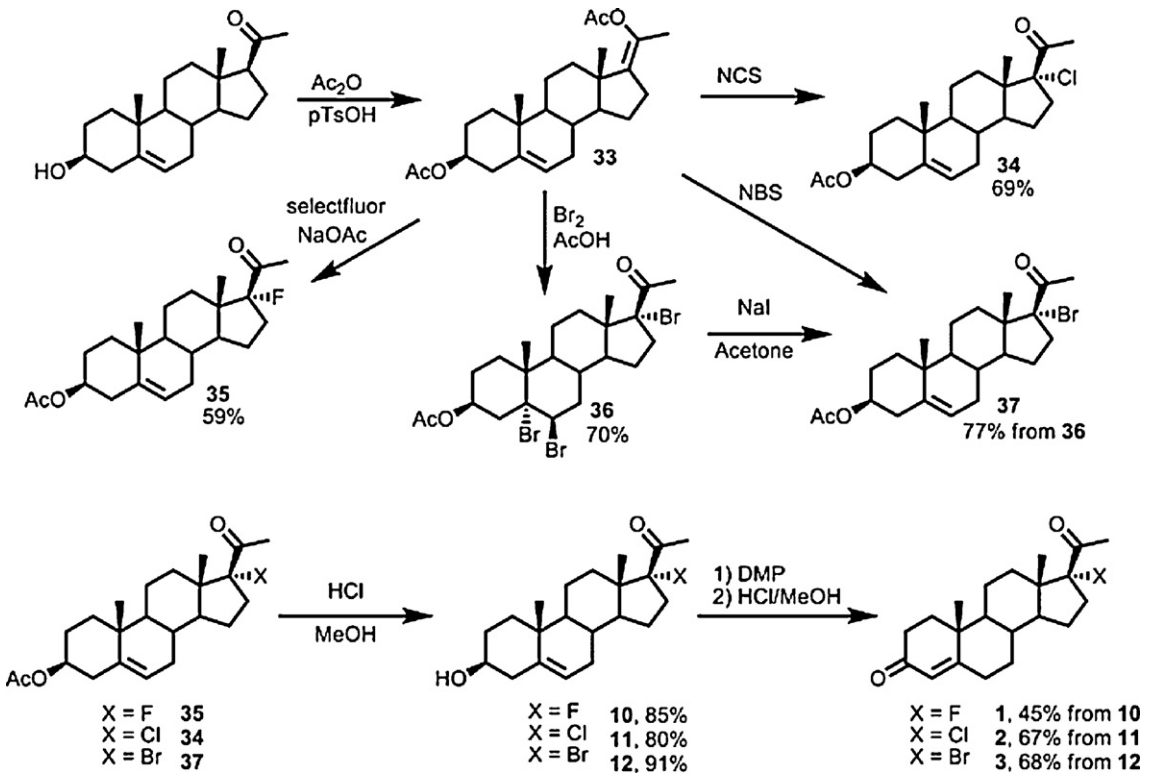
3.1. Synthesis of compounds

*3.1.1. 21,21,21-Trihalo progesterone compounds (**4**, **5**, **6**, **13**, **14**, **15**):*

The first approach to 21,21,21-trihalo compounds (Scheme 1) used the 20-carbon carboxylic acid **20**, derived from the pyridinium salt **19**, to attempt Zard's protocol of transforming carboxylic acids to trifluoromethyl-ketones [25]. Trifluoroacetic anhydride and pyridine converted the carboxylic acid **20** to the trifluoromethyl ketone **13** upon addition of water and heating. The β-ketoacid intermediate **21** is observable by NMR before the addition of water, and the decarboxylation proceeds with the desired stereochemistry at C-17, probably due to torsional strain in the protonation step (Fig. 2).

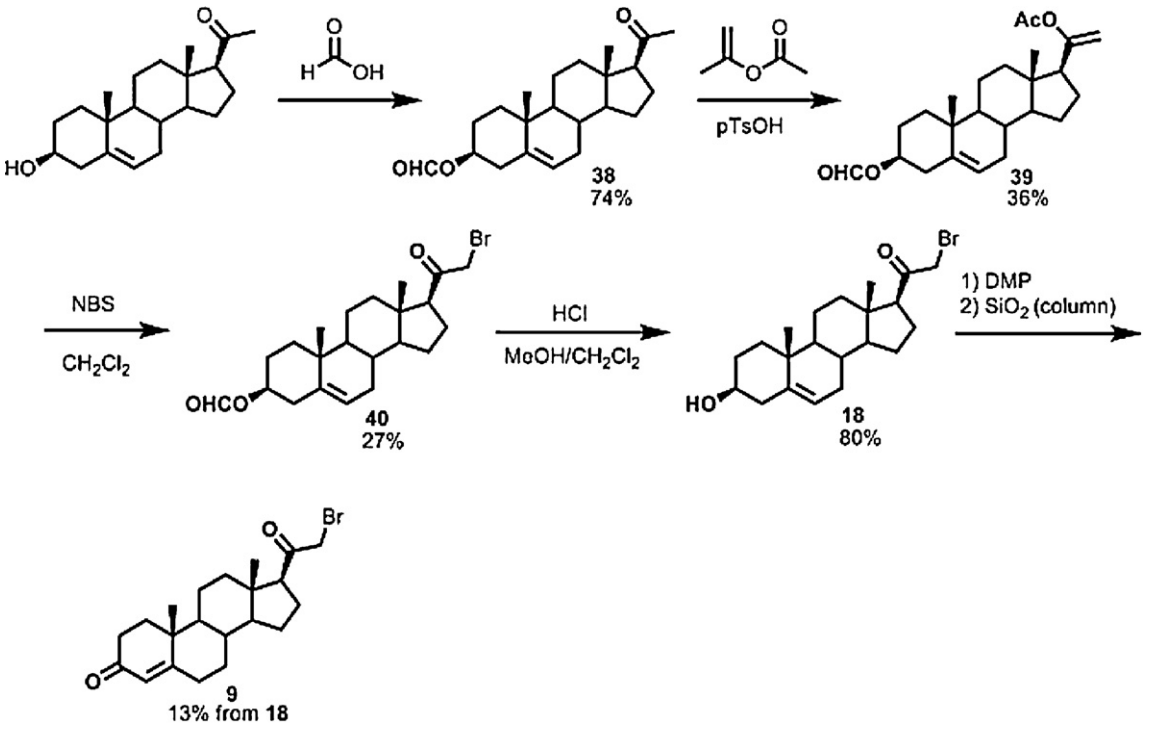


Scheme 3. 21,21,21-trihalopregnenolone (**14**,**15**) and 21,21,21-trihaloprogesterone (**5**,**6**) syntheses using haloform.



Scheme 4. 17-halopregnenolone (**10**-**12**) and 17-haloprogesterone (**1**-**3**) syntheses.

Interestingly, the trichloromethyl ketone **23** was also obtained in a similar manner by using either trichloroacetic anhydride or trichloroacetic acid chloride, except we found that the reaction must be done at 0 °C due to the reactive chloride leaving groups, and the product contained an incidental 3-trichloroacetate group (Scheme 2). Moreover, in the trichloro-case, the β-ketoacid decarboxylation occurred spontaneously, without the addition of water. The base-labile trichloroacetyl group mandated the deprotection of the 3-trichloroacetate group under acidic conditions in methanol, which took a week to complete and prompted investigation of alternative approaches. In a similar manner to the trichloromethyl ketone case, we attempted to use tribromoacetyl



Scheme 5. 21-monobromoprogesterone (**9**) synthesis.

chloride to form the tribromomethyl ketone, but too many side reactions were observed.

Due to difficulties with the trichloromethyl and tribromomethyl cases, we pursued a second route, which involved the addition of a trihalomethyl anion onto the 20-carbon aldehyde **27** (Scheme 3). One equivalent of DBU and excess bromoform was used to optimize yield and to prevent solidification of the reaction mixture [15]. Aldehyde **27** also served as a versatile intermediate to make alkyl substituents at the 21-position in addition to making the 21,21,21-trihalo compounds, including the 21,21,21-trichloroketone **14** by using the chloroform anion as the nucleophile. If 3-ketosteroids are sought, the TBDMS ether can be removed before the Dess-Martin periodinane-mediated oxidation step, and the resulting 3,20-diketones are isomerized to the Δ^4,3-ketosteroids under mildly acidic conditions, which avoids the harsher Oppenauer oxidation protocol when the compounds contain labile or reactive groups.

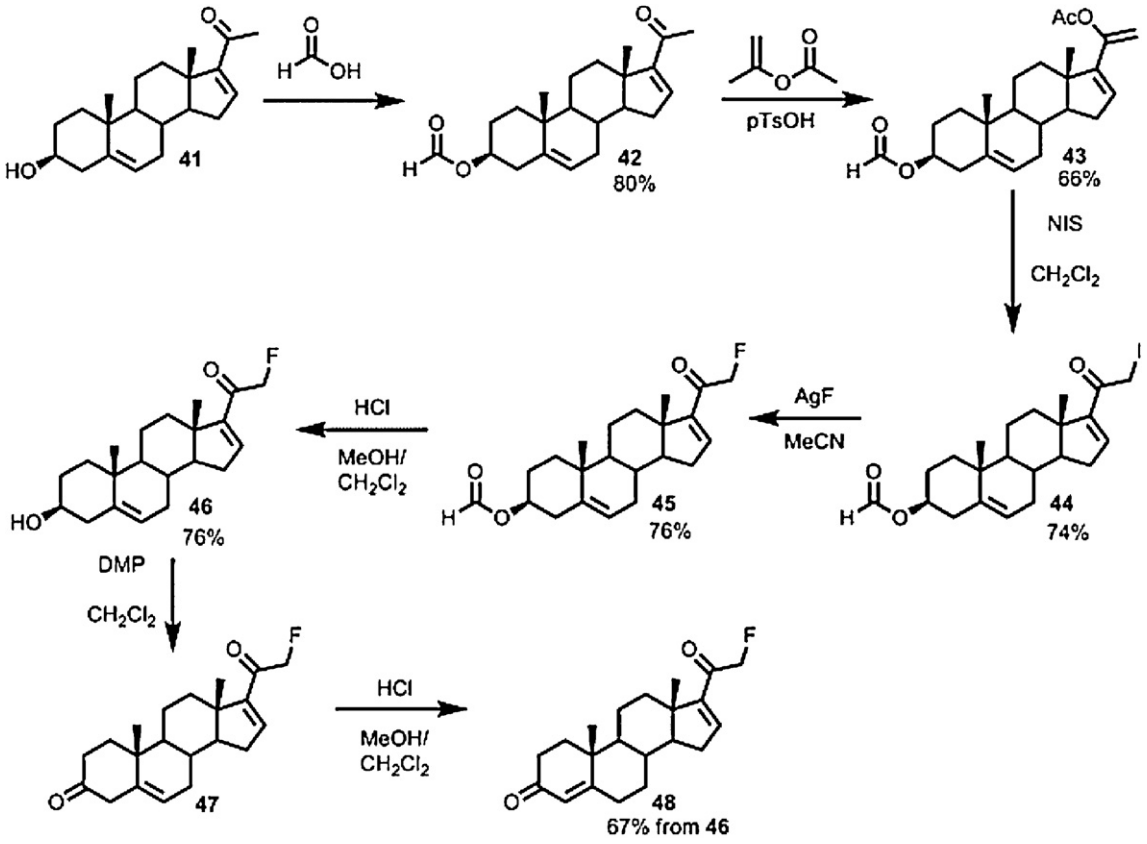
3.1.2. 17-Monohalo compounds (1,2,3,10,11,12)

The 17-monohalo compounds were afforded from a common intermediate – the 17,20-enol acetate, obtained in literature precedence [17] by refluxing pregnenolone in acetic anhydride and catalytic p-toluenesulfonic acid. The 17,20-enol acetate was a highly versatile intermediate, and we were able to use Selectfluor to fluorinate [26], NCS to chlorinate, and NBS to brominate the 17-position and access compounds **1**–**3** and **10**–**12**. As an alternative to NBS, we could repeat the reported protocol of bromine in acetic acid [17], but the alkene on the 5-position is dibrominated, adding an additional step in reforming the Δ^5-alkene with sodium iodide (Scheme 4). Presence of the 17-halogen did not complicate the facile oxidation and isomerization of pregnenolone halides to the corresponding progesterone series with Dess-Martin periodinane, followed by mild acid or spontaneous isomerization during purification on silica gel chromatography.

3.1.3. 21-Monohalo progesterone compounds (7,8,9)

Analogous to the 17-monohalo steroid series, one can envision that the 21-monohalo compounds can be synthesized by halogenating the less substituted 20,21-enol acetate intermediate (Scheme 5, compound **39**). Refluxing pregnenolone-3-formate with isopropenyl acetate and catalytic p-toluenesulfonic acid formed the 20,21-enol acetate as the kinetic product, which isomerized to the thermodynamic product (17,20-enol acetate) if refluxing was prolonged (3 days). We selectively halogenated the 20,21-enol acetate using N-iodo-, N-bromo-, and N-chlorosuccinimide to form the 21-iodo-, 21-bromo, and 21-chloropregnenolone formates, with chlorination requiring heat, providing routes to compounds **7**,**8**, and **9**.

We also sought to synthesize 21-fluoro-16,17-dehydroprogesterone to use as a potential substrate for CYP17A1. This compound was obtained from the same procedure used to access 21-monohalosteroids, except starting with 16,17-dehydropregnenolone (Scheme 6). Unlike in the 16,17-saturated case, refluxing 16,17-dehydropregnenolone-3-formate in isopropenyl acetate and catalytic p-toluenesulfonic acid cleanly yielded the 20,21-enol acetate, which was iodinated using N-iodosuccinimide. AgF was used to displace the iodide, followed by deformylation, oxidation, and isomerization, affording 21-fluorosteroid **48**.



Scheme 6. 16,17-dehydro-21-fluoroprogesterone (**48**) synthesis.

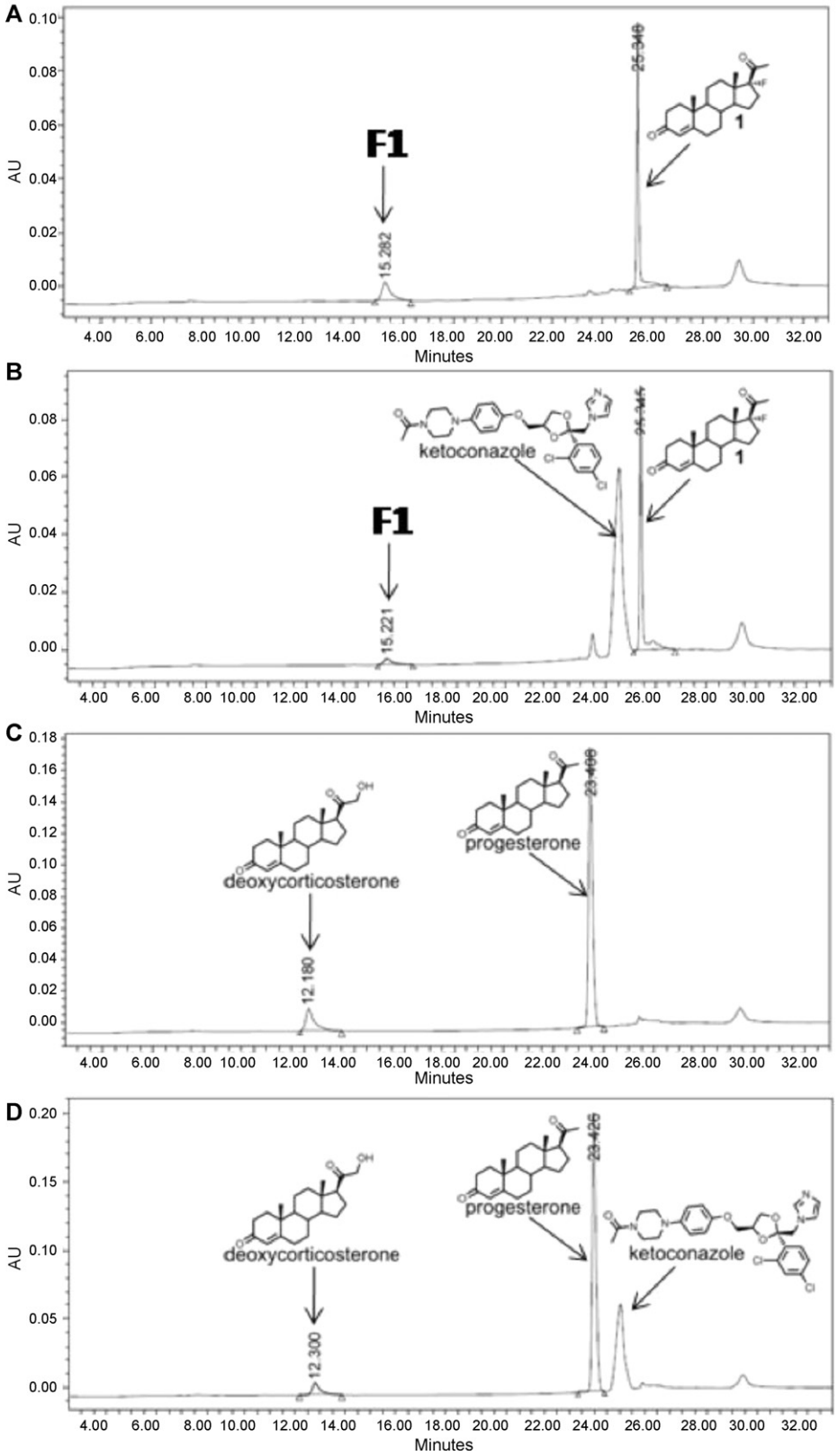


Fig. 3. *In vitro* studies of 17-fluoroprogesterone as a substrate for CYP21A2. HPLC traces of incubations: (A) 17-fluoroprogesterone (**1**) incubation with CYP21A2, (B) 17-fluoroprogesterone (**1**) incubation with CYP21A2 and ketoconazole, (C) progesterone incubation with CYP21A2, (D) progesterone incubation with CYP21A2 and ketoconazole. The formation and migration of compound **F1** is consistent with 17-fluoro-21-hydroxyprogesterone. Conversion of 17-fluoroprogesterone to **F1** was inhibited 65% by pre-incubation with ketoconazole, and conversion of progesterone to 11-deoxycorticosterone was inhibited 40% by ketoconazole using the same conditions. The peak at 30 min is due to the change in solvent gradient.

3.2. Halogenated steroids as substrates for human CYP17A1 and CYP21A2

Fluorinated small molecules have importance in the field of medicine to probe the biological function of compounds in vivo [27]. Consequently, we screened how the incorporation of fluorine atom(s) on either the 17-position or the 21-position of progesterone or pregnenolone altered metabolism by CYP17A1 or CYP21A2.

3.2.1. 17-fluorosteroids as substrates

When yeast microsomes containing CYP17A1 and P450-oxidoreductase were incubated with 17-fluoroprogesterone, under conditions where progesterone is largely metabolized to 17α- and 16α-hydroxylated products, no metabolism of 17-fluoroprogesterone **1** could be detected despite prolonged incubations (not shown). Similarly, CYP17A1-containing microsomes did not metabolize 17-fluoropregnenolone **10** to any demonstrable products, as assessed after conversion via cholesterol oxidase to their cognate Δ^4-steroids. In both experiments, which were repeated 3 or more times, only 17-fluoroprogesterone was recovered, without any convincing evidence of product formation (not shown). These results demonstrate that blocking metabolism at the 17-position of both pregnenolone and progesterone by fluorine replacement does not result in metabolic switching to an alternative C-H bond, such as the 16α-position, as might be predicted from the known progesterone 16α-hydroxylase activity of human CYP17A1 [7].

In contrast, when yeast microsomes containing CYP21A2 and P450-oxidoreductase were incubated with 17-fluoroprogesterone, under conditions where progesterone is metabolized to 11-deoxycorticosterone via 21-hydroxylation, comparable amounts of 17-fluoroprogesterone were reproducibly converted to an earlier-eluting peak, consistent with 21-hydroxylation (Fig. 3). This metabolism of progesterone and 17-fluoroprogesterone was comparably inhibited by ketoconazole, consistent with P450-mediated oxygenation (Fig. 3). This result suggests that 17-fluorination does not significantly impede substrate binding or alter catalysis by CYP21A2.

3.2.2. 21,21,21-trifluorosteroids as substrates

When 21,21,21-trifluoroprogesterone **4** was incubated with yeast microsomes containing CYP17A1 or CYP21A2, two earlier-eluting peaks were variably observed, but their formation was only partially inhibited by ketoconazole. The same pattern of product formation was observed from incubations with CYP17A1-containing microsomes and 21,21,21-trifluoropregnenolone **13**, followed by cholesterol oxidase treatment (not shown). Consequently, we are unable to conclude if the 21,21,21-trifluorosteroids are substrates for CYP17A1 and CYP21A2.

4. Discussion

In summary, by exploring novel and previously reported methodologies, we have synthesized halogenated progesterone analogs to probe the active sites of CYP17A1 and CYP21A2. Zard's methodology proved useful in accessing 21,21,21-trifluoropregnenolone **13** directly from the carboxylic acid intermediate, and we have found that trichloroketones are accessible from carboxylic acid precursors by using trichloroacetyl chloride as the electrophile. Moreover, nucleophilic bromoform addition was required to ultimately access 21,21,21-tribromopregnenolone **15**. Synthesis of a kinetic 20,21-enol acetate was required to regioselectively halogenate the 21-position of pregnenolone over the $\Delta^{5,6}$-alkene and 17-position.

Njar et al. have reported the synthesis of 21,21,21-trifluoropregnenolone through the addition of trifluoromethyl silane onto an aldehyde intermediate and have shown that 21,21,21-trifluoropregnanes are inhibitors of CYP17A1 [14]. We found some evidence that 21,21,21-trifluorinated substrates might also be metabolized by CYP17A1 and CYP21A2, but the inherent difficulties in these experiments precluded unambiguous conclusions. One explanation for these results is that the electron-withdrawing trifluoromethyl group shifts the hydration equilibrium from the C20 ketone of compound **4** to its geminal diol, which is too bulky to bind to either active site. In contrast, we reproducibly demonstrated that CYP21A2 but not CYP17A1 metabolized 17-fluoroprogesterone to a single ketoconazole-inhibited product, possibly 17-fluoro-21-hydroxyprogesterone. These data demonstrate that steroid halogenation might block normal sites of P450 oxygenation but not preclude substrate binding and turnover.

The synthetic methods described here enable the detailed study of 17- and 21-halogenated progesterone and pregnenolone substrate analogs as substrates, inhibitors, and mechanistic probes of steroidogenic cytochrome P450 enzymes. By varying the location and number of halogen atoms, as well as the specific halide employed, variations in steric, electronic, and reactivity properties are introduced in the steroid near the sites of reactivity. Although the classical cytochrome P450 reaction is the hydroxylation of C-H bonds in alkanes, these substituted pregnanes are also reagents to test the capacity of CYP17A1 and CYP21A2 to catalyze halide reductions, as described for other cytochromes P450 [28].

In addition, halogenated steroids have been successfully employed for a variety of purposes in science and medicine. As suggested above, halogenated steroids might be employed as reversible [29] or irreversible [30] enzyme inhibitors, by blocking catalysis and/or increasing affinity for the steroid. Halogenated steroids are also in use as agonists and antagonists of steroid hormone receptors, and halogenation also blocks positions of metabolism by hepatic enzymes such as CYP3A4. This modification increases the half-life of the potential drug, as seen in the potent glucocorticoid dexamethasone (9α-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione), with a half-life of over 36 h. Furthermore, fluorinated [^{18}F-] steroids are used as imaging agents for positron-emitted tomography (PET) studies [31,32], by binding to receptors for these steroids. Consequently, improved methods to selective halogenation of steroids might facilitate advances in several disciplines.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2011.09.007.

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