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Effects of fluorine substitution on substrate conversion by cytochromes P450 17A1 and 21A2†

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Cytochromes P450 17A1 (CYP7A1) and 21A2 (CYP21A2) catalyze key reactions in the production of steroid hormones, including mineralocorticoids, glucocorticoids, and androgens. With the ultimate goal of designing probes that are selectively metabolized to each of these steroid types, fluorinated derivatives of the endogenous substrates, pregnenolone and progesterone, were prepared to study the effects on CYP17A1 and CYP21A2 activity. In the functional assays, the hydroxylase reactions catalysed by each of these enzymes were blocked when fluorine was introduced at the site of metabolism (positions 17 and 21 of the steroid core, respectively). CYP17A1, furthermore, performed the 17,20-lyase reaction on substrates with a fluorine installed at the 21-position. Importantly, none of the substitutions examined herein prevented compound entry into the active sites of either CYP17A1 or CYP21A2 as demonstrated by spectral binding assays. Taken together, the results suggest that fluorine might be used to redirect the metabolic pathways of pregnenolone and progesterone to specific types of steroids.

Introduction

Endogenous steroid hormones are produced by a series of metabolic pathways that stem from a common ancestor, cholesterol. At the core of these enzymatic reactions are the heme-containing cytochromes P450 17A1 (CYP17A1) and 21A2 (CYP21A2), which differentiate steroids into one of three distinct classes (*i.e.*, mineralocorticoids, glucocorticoids, or androgens) early in their biosynthesis (Fig. 1).[1,2] CYP17A1 performs two unique reactions: C17 hydroxylation of pregnenolone and progesterone, followed by C17–C20 bond cleavage to give dehydroepiandrosterone and androstenedione, respectively. The initial 17α-hydroxylase reaction catalysed by CYP17A1 produces precursors to glucocorticoids, which regulate glucose metabolism, and androgens, the male sex hormones. The latter 17,20-lyase activity is additionally required to complete androgen biosynthesis. Thus, CYP17A1 is a target for hormone-dependent prostate cancer.[3,4] CYP21A2 also catalyses a key hydroxylation reaction, but at the 21-position of progesterone and its 17α-hydroxylase product. This reaction is essential in the production of mineralocorticoids (which influence electrolyte and fluid balance) in addition to glucocorticoids.

Given that CYP17A1 and CYP21A2 operate at key points in steroidogenesis, selectively blocking the sites of metabolism on pregnenolone and progesterone is expected to dramatically influence the metabolic pathways of these feedstock steroids. For example, a substrate for CYP17A1 but not CYP21A2 would only be channelled toward androgens. Conversely, mineralocorticoids would be produced from precursors that are not metabolized by CYP17A1. Thus, specific classes of steroids could be enriched, which would be useful for studying the biosynthesis and activities of these hormones.

Incorporation of fluorine into probe molecules can modify biological activity in mechanistically informative ways.[5,6] In this case, introducing a fluorine atom at C17 or C21 is expected to direct substrates toward CYP21A2 or CYP17A1 metabolism, respectively. To this end, Yoshimoto *et al.* previously described several different halogenated compounds that were used to study the activities of CYP17A1 and CYP21A2 in yeast microsomes.[7] Along with structural biological approaches,[8,9] this work has helped profile how changes near C17 of substrates affects enzymatic function. In the current study, we sought to expand this approach by focusing on how enzyme kinetics and binding respond to C17 and C21 fluorination.

To accomplish this objective, we prepared a series of pregnenolone and progesterone substrates along with their predicted metabolites (*i.e.*, product standards for identification

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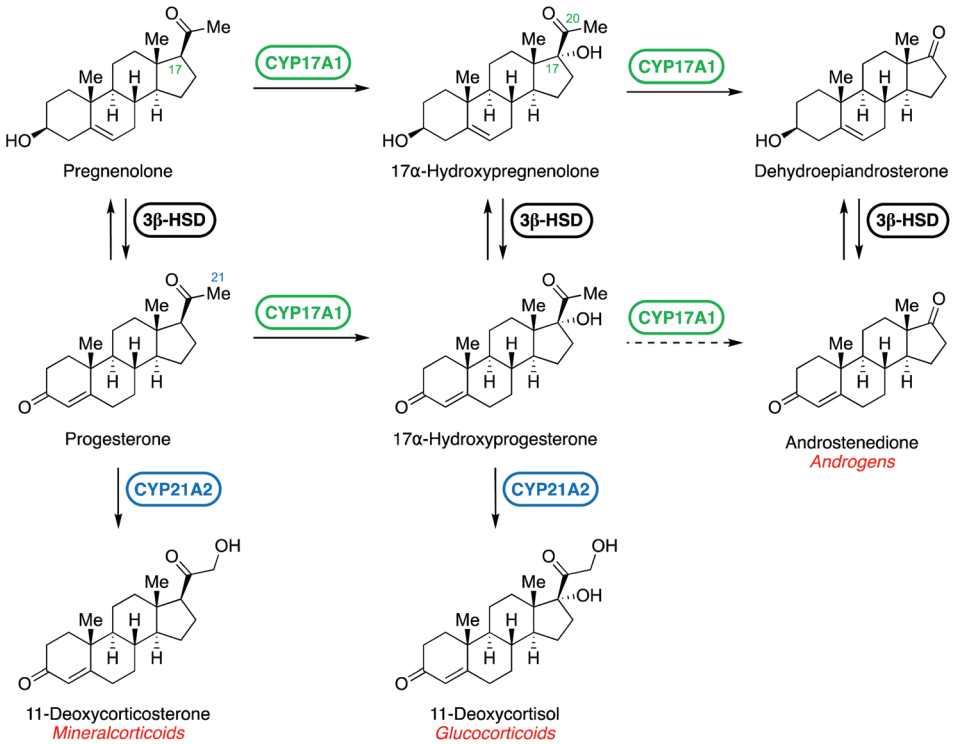


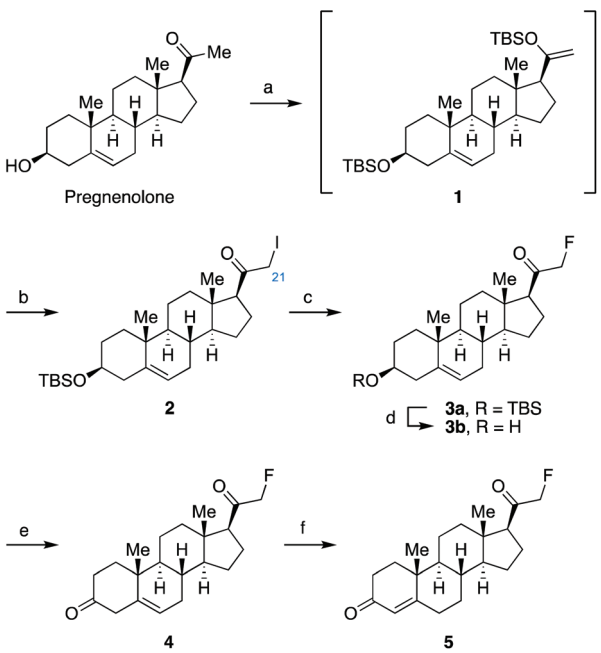
Fig. 1 CYP17A1 and CYP21A2 channel precursors to either mineralocorticoids, glucocorticoids, or androgens (red text) in steroid metabolism. 3β-Hydroxysteroid dehydrogenase (3β-HSD) interconverts Δ^5-3β-hydroxy- and Δ^4-3-oxosteroids. Lower conversion of 17α-hydroxyprogesterone by CYP17A1 is indicated by the dashed arrow.

and quantification). CYP17A1 and CYP21A2 are known to display low promiscuity compared to other cytochrome P450 enzymes, and so the scope of our study was limited to mono-fluorinated substrates to minimize confounding effects, such as steric hindrance. Notably, the van der Waals radii of fluorine (1.47 Å) is closer to oxygen than hydrogen (1.52 and 1.20 Å, respectively). We then evaluated the synthetic substrates for conversion by CYP17A1 and CYP21A2 and quantified the effects fluorine substitutions had on their binding affinity to each enzyme.

Results and discussion

Synthesis of fluorinated substrates and product standards

For the first set of compounds, we introduced a single fluorine atom at the 21-position of the steroid core (Scheme 1). Starting from pregnenolone itself, silyl enol ether **1** was prepared by adapting known reaction conditions.[10] This intermediate was then treated with *N*-iodosuccinimide (NIS) to install an iodine at C21 (**2**) in 87% yield over two steps. Nucleophilic substitution using tetrabutylammonium difluorotriphenylsilicate (TBAT) then delivered the fluorinated **3a**, which was subsequently deprotected with HF·pyridine (**3b**). With this material in hand, we prepared the corresponding progesterone analogue by pyridinium chlorochromate (PCC) oxidation followed by acid-catalysed isomerization (**4** then **5**).



Scheme 1 Introduction of a fluorine at position 21 of pregnenolone and progesterone. Reagents and conditions: (a) TBSOTf, NEt_3, CH_2Cl_2, −78 °C, 1 h; (b) NIS, CH_2Cl_2, rt, 2 h, 87% over 2 steps; (c) TBAT, MeCN, reflux, 19 h, 74%; (d) HF·pyridine, CH_2Cl_2, 0 °C, 1 h, 86%; (e) PCC, CH_2Cl_2, rt, 5 h, 56%; (f) oxalic acid dihydrate, MeOH, 80 °C, 2 h, 85%.

Since both of the 21-fluorosteroids (**3b** and **5**) were predicted to act as substrates of CYP17A1, we also synthesized standards of their 17α-hydroxylase products. Initially, a variation of the previous route was planned, starting from commercially available 17α-hydroxypregnenolone. While troubleshooting preliminary reactions however, we discovered that this material was sold in *ca.* 80% purity (determined by ^{1}H NMR), which could not be improved by chromatography or recrystallization. As a result, alternative strategies were devised and executed to access these compounds (Scheme 2).

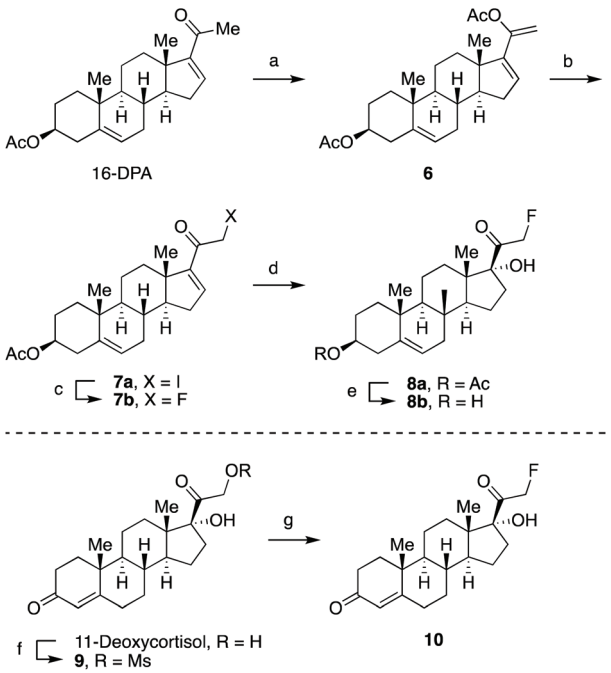
Following work published by Djerassi *et al.*, 16-dehydropregnenolone acetate (16-DPA) was transformed into enol acetate **6**, which was reacted with NIS to afford the α-iodo ketone **7a**.[11] As before, nucleophilic substitution with TBAT provided the C21 fluorinated version of 16-DPA (**7b**). We then converted this intermediate to its α-hydroxy ketone (**8a**) in 66% yield using a variation of the Mukaiyama hydration developed by Magnus *et al.*[12] Acetate deprotection ultimately afforded the target compound **8b**, which was isolated directly from the reaction mixture by vacuum filtration. We also used the last two steps of this route to access authentic samples of 17α-hydroxypregnenolone starting from 16-DPA (see ESI, Scheme S1†).

The 17α-hydroxylase product of **5**, by comparison, was much more straightforward to prepare. Adopting a strategy by Pomper *et al.*,[13] we trapped the more reactive 21-hydroxy group of 11-deoxycortisol as its mesylate (**9**). This leaving group was later displaced with TBAT to deliver **10** in 40% overall yield.

For the second series, we replaced the hydrogen atom at position 17 of pregnenolone and progesterone with a fluorine. While routes to both compounds are known, our efforts to reproduce earlier work were either unsuccessful or avoided altogether because hazardous reagents, like ClO_3F, were used.[7,14–16] Consequently, we devised a new approach that took inspiration from work published by Stavber *et al.*[17] (Scheme 3). Specifically, pregnenolone was treated with *N*-fluorobenzenesulfonimide (NFSI) to directly produce the α-fluoro ketone **11** in 24% yield (4.0 g scale). This material was then converted into the related progesterone analogue (**12**) by an Oppenauer oxidation in just two steps, a notable improvement over the previous route (five steps).[7]

Similar to before, we did not expect that the 17α-fluoro group of **11** and **12** would block catalysis by CYP21A2. Accordingly, methods to prepare the 21-hydroxylase products of these fluorinated steroids were also pursued (Scheme 4). Initially, we developed a pilot route for the synthesis of 21-hydrxoypregnenolone (see ESI, Scheme S2†) using a previous intermediate (**2** from Scheme 1). However, this strategy relied on functionalizing C21 *via* a silyl enol ether, whose 17α-fluorinated counterpart could not be prepared from **11**. So instead, we installed the hydroxyl group by way of a radical halogenation.[18] Thus, TBS-protected **13** was treated with molecular iodine in the presence of an initiator (AIBN) to afford crude **14**. KOAc was then used to displace the intermediate iodine, giving **15** in 61% yield over two steps.

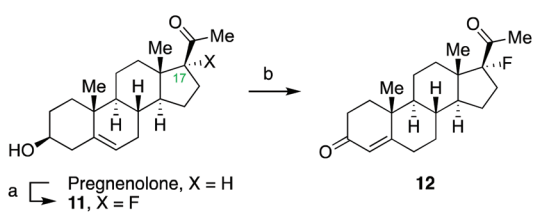
Removal of the TBS group on **15** gave **16a**, but attempts to deprotect the acetate using standard conditions (K_2CO_3 in MeOH) led to a complex mixture of products. Alternatively, treatment of **16a** with $Sc(OTf)_3$, which was previously reported to selectively cleave acetates adjacent to a carbonyl group,[19] afforded the predicted 21-hydroxylase product (**16b**) in 36% yield. For the corresponding 17α-fluoroprogesterone analogue, oxidation followed by isomerization yielded **17a**, which was deprotected with $Sc(OTf)_3$ to give **17b**.



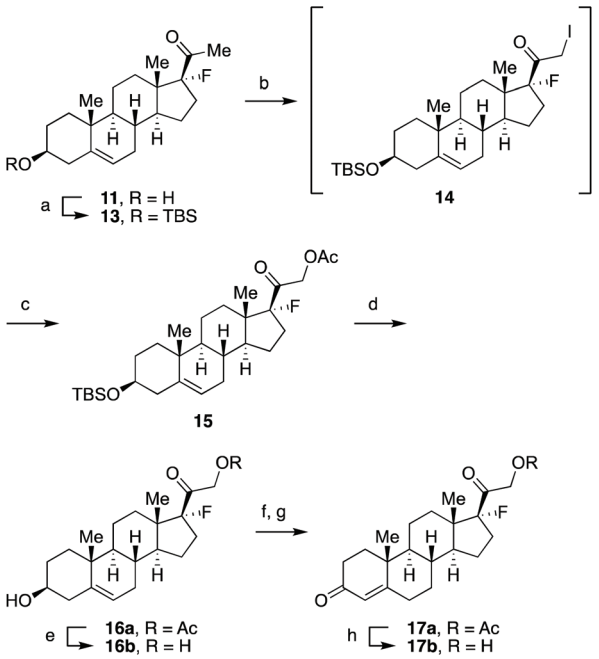
Scheme 2 Routes to the 17α-hydroxylase products of 21-fluoropregneneolone and -progesterone. Reagents and conditions: (a) isopropenyl acetate, *p*TsOH·H_2O, reflux, 4 h, 77%; (b) NIS, CH_2Cl_2, rt, 2 h, 68%; (c) TBAT, MeCN, reflux, 24 h, 75%; (d) $Mn(dpm)_3$, O_2, $PhSiH_3$, i-PrOH, 0 °C, 4 h, then $P(OEt)_3$, 0 °C, 2 h, 66%; (e) K_2CO_3, MeOH, rt, 18 h, 89%; (f) MsCl, NEt_3, THF, 0 °C to rt, 1.5 h, 91%; (g) TBAT, MeCN, reflux, 24 h, 44%.

Functional evaluation of CYP17A1 and CYP21A2 against fluorinated substrates

We then set out to quantify how different sites of fluorination affected the kinetics of CYP17A1 and CYP21A2 action on our collection of substrates. Briefly, compounds were incubated



Scheme 3 Introduction of a fluorine at position 17 of pregnenolone and progesterone. Reagents and conditions: (a) NFSI, MeOH, reflux, 5 h, 24%; (b) $Al(Oi\text{-}Pr)_3$, cyclohexanone, toluene, reflux, 3 h, 48%.



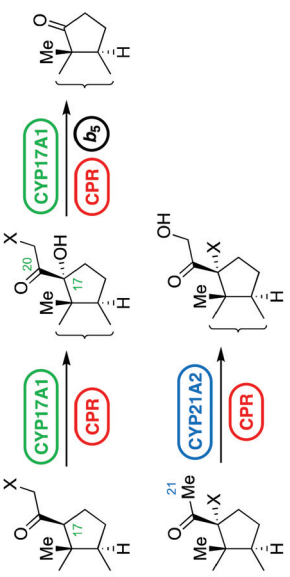
Scheme 4 Routes to the 21-hydroxylase products of 17α-fluoropregnenolone and -progesterone. Reagents and conditions: (a) TBSCl, DMAP, imidazole, CH_2Cl_2, rt, 16 h, 95%; (b) I_2, AIBN, CaO, THF, MeOH, rt, 3 h; (c) KOAc, acetone, reflux, 18 h, 61% over 2 steps; (d) HF·pyridine, CH_2Cl_2, 0 °C, 1 h, 93%; (e) $Sc(OTf)_3$, MeOH, H_2O, MW, 80 °C, 4 h, 36%; (f) PCC, CH_2Cl_2, rt, 5 h, 63%; (g) oxalic acid dihydrate, MeOH, 80 °C, 2 h, 90%; (h) $Sc(OTf)_3$, MeOH, H_2O, MW, 80 °C, 4 h, 57%.

with either enzyme along with NADPH-cytochrome P450 reductase (CPR), which is essential for the catalytic activities of CYP17A1 and CYP21A2. Substrate conversion was followed by LCMS, and products were confirmed using commercially available standards or those synthesized as above (see ESI† for details).

Initially, we investigated the 17α-hydroxylase reaction catalysed by CYP17A1 on the native substrates, pregnenolone and progesterone (Table 1, entries 1 and 3). While hydroxylation was observed for these control substrates, no products were detected by LCMS for either of the 17α-fluorosteroids (**11** and **12**), which agrees with previous work by Yoshimoto *et al.*[7] Nonetheless, this result is somewhat surprising given that CYP17A1 can install a hydroxy group at alternative positions on the steroid core generating minor products, such as 16α-hydroxyprogesterone.[20–22] By comparison, CYP17A1 did perform the 17α-hydroxylase reaction on substrates bearing a fluorine atom at the 21-position. Interestingly, this substitution lowered the K_m value of **3b** by 2.4-fold compared to pregnenolone (entries 1 and 2). Nearly identical kinetic parameters, however, were observed between progesterone and its 21-fluorinated analogue, **5** (entries 3 and 4).

To study the 17,20-lyase reaction, cytochrome b_5 (b_5) was added to the previous enzyme system (*i.e.*, CYP17A1 and CPR) to augment C17–C20 bond cleavage. In agreement with previous studies,[23] 17α-hydroxypregnenolone was a better sub-

Table 1 Effects of fluorine substitution on the kinetic parameters of CYP17A1 and CYP21A2



Products were not detected by LCMS for the following substrates:

CYP17A1
- 17α-Fluoroprogesterone (**12**)

CYP21A2
- Pregnenolone
- 17α-Hydroxypregnenolone

CYP17A1 and CYP21A2
- 21-Fluoropregnenolone (**3b**)
- 21-Fluoroprogesterone (**5**)

Activity	Entry	Substrate	X	Product[a]	k_{cat} [b] (min^{-1})	K_m [b] (μM)
17α-Hydroxylase	1	Pregnenolone	H	17α-Hydroxypregnenolone	1.2 [1.2, 1.3]	1.0 [0.8, 1.3]
	2	21-Fluoropregnenolone (**3b**)	F	21-Fluoro-17α-hydroxypregnenolone (**8b**)	0.88 [0.84, 0.93]	0.42 [0.30, 0.58]
	3	Progesterone	H	17α-Hydroxyprogesterone	2.3 [2.0, 2.6]	5.0 [3.0, 8.1]
	4	21-Fluoroprogesterone (**5**)	F	21-Fluoro-17α-hydroxyprogesterone (**10**)	2.3 [2.2, 2.4]	6.3 [5.4, 7.4]
17,20-Lyase	5	17α-Hydroxypregnenolone	H	Dehydroepiandrosterone	0.23 [0.20, 0.25]	0.20 [0.12, 0.32]
	6	21-Fluoro-17α-hydroxypregnenolone (**8b**)	F		0.17 [0.16, 0.18]	0.60 [0.48, 0.74]
	7	17α-Hydroxyprogesterone	H	Androstenedione	0.059 [0.052, 0.067]	4.3 [2.8, 6.6]
	8	21-Fluoro-17α-hydroxyprogesterone (**10**)	F		0.031 [0.027, 0.036]	3.4 [2.1, 5.4]
21-Hydroxylase	9	Progesterone	H	11-Deoxycorticosterone	0.22 [0.20, 0.23]	0.12 [0.087, 0.16]
	10	17α-Hydroxyprogesterone	OH	11-Deoxycortisol	3.7 [3.5, 4.0]	1.00 [0.79, 1.3]
	11	17α-Fluoroprogesterone (**12**)	F	17α-Fluoro-21-hydroxyprogesterone (**17b**)	0.064 [0.059, 0.070]	0.071 [0.047, 0.11]

[a] Detection limits for the observed and unobserved products are presented in Table S2 (see ESI†). [b] The 95% confidence intervals are reported in brackets ($n = 2$).

strate in the 17,20-lyase reaction than its progesterone counterpart (Table 1, entries 5 and 7). This trend was also observed for the 21-fluorosteroids (entries 6 and 8); although, the K_m for **8b** was 3-fold greater than 17α-hydroxypregnenolone (entries 5 *vs.* 6), while the k_{cat} for **10** was 1.9-fold smaller than 17α-hydroxyprogesterone (entries 7 *vs.* 8). In addition, no products were formed in the 17,20-lyase reaction when a fluorine was substituted for the 17α-hydroxy group (*i.e.*, **11** and **12**).

Switching to the 21-hydroxylase activity of CYP21A2, none of the predicted products were detected for pregnenolone or any of its derivatives by LCMS. This result was expected seeing as CYP21A2 demonstrates high specificity for its native substrates, progesterone and its 17α-hydroxylase product.[24] Thus, 11-deoxycorticosterone and 11-deoxycortisol were both observed in these assays (Table 1, entries 9 and 10). Similar to CYP17A1, product formation was undetectable when a fluorine was introduced at the normal site of hydroxylation, in this case C21 on progesterone (**5**). Consequently, the only fluorinated analogue of progesterone that did act as a substrate for the 21-hydroxylase reaction placed a fluorine at the 17-position of the steroid core (entry 11). However, this substitution dramatically lowered catalysis by 3.4-fold compared to its parent substrate (entries 9 *vs.* 11), once again highlighting the specificity of CYP21A2.

Spectral binding of compounds to CYP17A1 and CYP21A2

Based on the functional assays alone, it was unclear whether fluorine blocked catalysis directly or disrupted binding in the enzyme active site such that catalysis could not occur. To help distinguish between these two possibilities, we performed a series of spectral binding assays using CYP17A1 or CYP21A2 to characterize the interaction between ligand and enzyme. Notably, all of the monofluorinated pregnenes prepared in this study displayed a shift in the UV–Vis difference spectra from *ca.* 420 to *ca.* 395 nm (see ESI, Fig. S2†). This so-called type I binding is consistent with each ligand displacing the water molecule coordinated to the heme iron. Such binding was also observed for most of the endogenous steroids. The only interaction that showed no significant change in the UV–Vis difference spectrum was between 17α-hydroxypregnenolone and CYP21A2. However, the absence of a spectral shift cannot be used to rule out ligand binding, as some compounds can occupy the cytochrome P450 active site in such a way that water remains bound to the iron.[25] Regardless, these experiments demonstrated that a single fluorine atom at either C17 or C21 does not prevent ligand entry into the CYP17A1 or CYP21A2 active sites.

Dissociation constants between enzyme and compound were also measured from these assays using a single binding site model (see ESI† for details). For CYP17A1, we observed that pregnenolone (Table 2, entry 1) and progesterone (entry 6) generally had lower binding affinities (larger K_d values) than their corresponding fluorinated analogues (entries 2, 3, and 8). The exception to this trend was the 21-fluorosteroid **5**, whose dissociation constant was similar to progesterone (entries 6 *vs.* 7). Interestingly, a single fluorine atom at the 21-position of 17α-hydroxypregnenolone resulted in greater binding affinity for CYP17A1 compared to the parent ligand (entries 4 *vs.* 5), while the opposite was found for 17α-hydroxyprogesterone and its counterpart (entries 9 *vs.* 10). Even with a 1.8-fold larger K_d value, 21-fluoro-17α-hydroxyprogesterone was still turned over in the 17,20-lyase reaction (*vide supra*).

In contrast to CYP17A1, the introduction of a fluorine into the structure of pregnenolone at either position had little effect on its dissociation constant for CYP21A2 (Table 2, entries 1–3). Progesterone, on the other hand, exhibited a higher sensitivity to substitution, with particularly decreased affinity upon fluorination at the 21 position (entries 6 *vs.* 7 and 8). Unexpectedly, the K_d values for pregnenolone and its analogues (entries 1–3) were *ca.* 3-fold lower than 17α-hydroxyprogesterone (entry 9), a native substrate.

While the structural basis for the trends observed in Table 2 is beyond the scope of this work, we have used techniques in computational molecular modelling to dock the fluorinated analogues of the endogenous substrates into the activity sites of CYP17A1 and CYP21A2 (data not included). These studies, however, provided ambiguous results and did not suggest any key differences in the binding interactions.

Table 2 Effects of fluorine substitution on binding affinity to CYP17A1 and CYP21A2

		K_d [a] (μM)	
Entry	Ligand	CYP17A1	CYP21A2
1	Pregnenolone	<0.1[b]	1.6 [0.55, 4.6]
2	21-Fluoropregnenolone (**3b**)	0.053 [0.045, 0.062]	1.8 [1.5, 2.1]
3	17α-Fluoropregnenolone (**11**)	<0.022[c]	1.6 [1.2, 2.1]
4	17α-Hydroxypregnenolone	0.21[b]	—
5	21-Fluoro-17α-hydroxypregnenolone (**8b**)	0.14 [0.13, 0.16]	2.7 [2.1, 3.5]
6	Progesterone	0.23[b]	0.23 [0.14, 0.38]
7	21-Fluoroprogesterone (**5**)	0.18 [0.15, 0.22]	1.6 [1.4, 2.0]
8	17α-Fluoroprogesterone (**12**)	0.032 [0.026, 0.039]	0.51 [0.44, 0.59]
9	17α-Hydroxyprogesterone	0.33[b]	4.6 [2.9, 7.2]
10	21-Fluoro-17α-hydroxyprogesterone (**10**)	0.59 [0.47, 0.74]	14 [10, 19]

[a] The 95% confidence intervals are reported in brackets (n = 1). [b] Literature values.[8,26] [c] This ligand is a very tight binder, which precludes definition of a dissociation constant with similar accuracy to the other experiments.

Conclusions

To summarize this work, we report syntheses of a collection of fluorinated substrates and product standards that were evaluated for catalysis by CYP17A1 and CYP21A2. In the functional assays, hydroxylation by CYP17A1 and CYP21A2 was not observed by LCMS when fluorine was introduced at C17 or C21, respectively. Moreover, the 17,20-lyase reaction catalysed by CYP17A1 still took place on substrates that incorporated fluorine at position 21. While different sites of fluorination could have prevented ligand entry into the CYP17A1 and CYP21A1 active sites, spectral binding assays revealed that such substitutions were instead blocking catalysis directly. In certain cases, a single fluorine atom unexpectedly resulted in greater binding affinity between ligand and enzyme (*e.g.*, **11** and **12** for CYP17A1). Looking to the future, the results presented herein provide valuable insight as to how fluorine might be used to redirect the metabolic pathways of pregnenolone and progesterone.

Author contributions

C. D. V. synthesized and characterized all compounds. A. G. B. and R. Y. recombinantly expressed and purified the enzymes used in this study. Preliminary functional assays were performed by R. Y., and A. G. B. determined the final kinetic parameters. In addition, A. G. B. undertook the spectral binding assays and obtained dissociation constants for each compound. E. E. S. and J. A. conceived the study and assisted the other authors in data analysis. The initial draft of this article was written by C. D. V., and all authors participated in its revision.

Conflicts of interest

The authors declare no competing financial interest.

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