Hydroxyperfluoroazobenzenes: Novel Inhibitors of Enzymes of Androgen Biosynthesis

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In a search for inhibitors of 17α-hydroxylase–$C_{17,20}$-lyase and testosterone–5α-reductase, target enzymes in the development of drugs to treat hormone-dependent prostatic cancer, we have identified certain compounds chemically derived by the hydrolysis of decafluoroazobenzene (**4**) as novel inhibitors for these two enzymes. Hydrolysis of **4** gave the known 4-hydroxynonafluoroazobenzene (**1**) and the novel 2-hydroxynonafluoroazobenzene (**2**). By AlI_3 demethylation of 4,4′-dimethoxyoctafluoroazobenzene (**5**) or by hydrolysis of **4** under phase-transfer conditions 4,4′-dihydroxyoctafluoroazobenzene (**3**) was obtained. Compounds **1** and **2** were inhibitors of the hydroxylase (IC_{50} values, respectively, 30 and 63 μM) and of the lyase (IC_{50} values 33 and 16 μM) steps on the pathway of androgen biosynthesis. The 2-hydroxy compound **2** underwent spontaneous conversion into octafluorodibenz[*b,f*][1,4,5]oxadiazepine (**6**) which had IC_{50} values, respectively, of 50 and 15 μM for the hydroxylase and lyase steps and which contributed to the observed activity of **2**. Effective inhibitors of the 5α-reductase were **1** (K_i 10 μM) and **3** (K_i 4 μM): the activities of **1** and **3** were markedly pH dependent, with respective IC_{50} values of 14 and 5 μM at pH 7.4 and of 2 and 0.8 μM at pH 6.6.

Inhibitors of various enzymatic steps in the pathways of androgen biosynthesis have been used to treat hormone-dependent prostatic carcinoma, both experimentally and in the clinic. Important target enzymes are 17α-hydroxylase–$C_{17,20}$-lyase which converts pregnenolone and progesterone via their 17-hydroxy derivatives into dehydroepiandrosterone and androstenedione, respectively, and testosterone–5α-reductase which converts testosterone into 5α-dihydrotestosterone (DHT). Thus, ketoconazole, an imidazole-based antifungal agent which has recently been used in the clinic to treat prostatic carcinoma,[1] is believed to act by inhibiting 17α-hydroxylase–$C_{17,20}$-lyase.[1,2] Although the most potent inhibitors of testosterone–5α-reductase so far found are the 4-azasteroid derivatives developed by Rasmusson and co-workers,[3] no compound having the 5α-reductase as its principal locus of action has yet been established in the clinic.

As part of a program designed to develop new agents for the treatment of hormone-dependent prostatic carcinoma we have sought new inhibitors of these enzymes. Here, we report on a novel class of inhibitors of the 17α-hydroxylase–$C_{17,20}$-lyase and 5α-reductase, namely the hydroxyperfluoroazobenzenes. The compounds described are the known[4] 4-hydroxynonafluoroazobenzene (**1**), the novel 2-hydroxynonafluoroazobenzene (**2**) and 4,4′-dihydroxyoctafluoroazobenzene (**3**), and a dibenzoxadiazepine derived by ring closure of **2**. Our interest in these compounds was prompted by our finding[5] that the antiprostatic agent bifluranol[6] (*erythro*-3,3′-difluoro-4,4′-dihydroxy-α-ethyl-α'-methylbibenzyl) and some analogues including the 3,3′,5,5′-tetrafluoro derivative were inhibitors of 17α-hydroxylase–$C_{17,20}$-lyase. The present compounds, particularly **3**, have some analogous structural features. It was also hoped that the multiple fluorine substitution, coupled with the choice of an azo group as a linkage between the phenolic rings would enhance the acidity of the hydroxyl functions and aid solubility at physiological pH.

Results and Discussion

Synthesis. The compounds described in this paper were prepared from decafluoroazobenzene (**4**) for which we recently reported a convenient new synthesis.[7]

By use of the published procedure[4] to prepare 4-hydroxynonafluoroazobenzene (**1**), namely hydrolysis of **4** by potassium hydroxide in refluxing *tert*-butyl alcohol we isolated an additional hydroxynonafluoroazobenzene. Its ^{19}F NMR spectrum contained, in addition to signals for the pentafluorophenyl residue, two pairs of similarly coupled 1 F multiplets consistent with four contiguous fluorine atoms leading to its assignment as the 2-hydroxy isomer **2**. In addition, the relatively low frequency of two of these multiplets shows the fluorine atoms meta to the azo linkage are retained, so excluding the possibility of it being the 3-isomer.

Prolonged reaction under the foregoing conditions gave no evidence for further hydrolysis of **1** to 4,4′-dihydroxyoctafluoroazobenzene (**3**) nor did other hydrolytic conditions (e.g., phase-transfer catalysis using M sodium hydroxide) hydrolyze **4** beyond the mono-hydroxy stage. The formation of the phenolate anion strongly deactivates the system to further nucleophilic attack. We consequently chose to investigate as an alternative approach to **3** the demethylation of the known[8] 4,4′-dimethoxyoctafluoroazobenzene (**5**). The combined electron-withdrawing effect of the fluorine and *p*-azo substituents would tend to deactivate the methoxy substituents to dealkylation by Lewis acids. Indeed, the reaction of aluminium chloride[9] with **5** was sluggish, and the yield of product **3** was poor. The more powerful[10] Lewis acid aluminium iodide however cleanly demethylated **5** to give **3** in high yield. Preparation of **3** by this route required in our hands a careful chromatographic separation of the precursor **5** from other methoxylated byproducts formed[8] from the methanolysis

(1) Rajfer, J.; Sikka, S. C.; Rivera, F.; Handelsman, D. J. *J. Clin. Endocrinol. Metab.* **1986**, *63*, 1193.
(2) Ayub, M.; Levell, M. J. *J. Steroid Biochem.* **1987**, *28*, 521.
(3) Rasmusson, G. H.; Reynolds, G. F.; Steinberg, N. G.; Walton, E.; Patel, G. F.; Liang, T.; Cascieri, M. A.; Cheung, A. H.; Brooke, J. R.; Berman, C. *J. Med. Chem.* **1986**, *29*, 2298.
(4) Birchall, J. M.; Haszeldine, R. N.; Kemp, J. E. G. *J. Chem. Soc. (C)* **1970**, 449.
(5) Barrie, S. E.; Rowlands, M. G.; Foster, A. B.; Jarman, M. *J. Steroid Biochem.* **1989**, *33*, 1191.
(6) Pope, D. J.; Gilbert, A. P.; Easter, D. J.; Chan, R. P.; Turner, J. C.; Gottfried, S.; Parke, D. V. *J. Pharm. Pharmacol.* **1981**, *33*, 297.
(7) Deadman, J. J.; Jarman, M.; McCague, R.; McKenna, R.; Neidle, S. *J. Chem. Soc., Perkin Trans. 2* **1989**, 971.
(8) Burdon, J.; Morton, C. J.; Thomas, D. F. *J. Chem. Soc.* **1965**, 2621.
(9) Burdon, J.; Damodaran, V. A.; Tatlow, J. C. *J. Chem. Soc.* **1964**, 763.
(10) Satchell, D. P. N.; Satchell, R. S. *Chem. Rev.* **1969**, *69*, 251.

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Table I. Inhibition of 17α-Hydroxylase and $C_{17,20}$-Lyase: Percent Inhibition at 100 μM and Concentration Giving 50% Inhibition of Enzyme Activity (IC_{50}) ± SE

compd	position of OH group(s)	% inhibition at 100 μM concentration[a]		IC_{50} values, μM[b]	
		17α-hydroxylase	$C_{17,20}$-lyase	17α-hydroxylase	$C_{17,20}$-lyase
1	4	76 ± 1	71 ± 2	30 ± 3	33 ± 2
2	2	62 ± 2	83 ± 1	63 ± 5	16 ± 1
3	4,4′	15 ± 5	14 ± 5	-	-
4	-	4 ± 2	0	-	-
6	-	67 ± 2	88 ± 1	50 ± 5	15 ± 2
7	-	8 ± 4	13 ± 4	-	-
ketoconazole	-	62[c] ± 3	48[c] ± 3	6 ± 0.4	11 ± 1
azobenzene	-	0	0	-	
4-hydroxyazobenzene	4	6 ± 1	0	-	-

[a] Determined in triplicate. [b] IC_{50} ± SE calculated from Dixon plot by using linear regression. [c] These values are for inhibition at 10 μM concentration.

of **4**. We therefore reinvestigated the possibility of a direct hydrolysis of **4**, mindful of the difficulty of performing a second nucleophilic displacement on the anion generated from **1**. The successful approach used 50% aqueous sodium hydroxide, in which hydration of the hydroxide ion is minimal,[11] increasing its reactivity towards electrophiles. Reaction with **4** under conditions of phase-transfer catalysis using toluene as solvent gave the bis tetra-*n*-butylammonium salt of **3** from which **3** itself could be generated by treatment with sulphuric acid in a more convenient and higher yielding synthesis than the preparation via **5**.

Enzyme-Inhibitory Activity. 17α-Hydroxylase–$C_{17,20}$-Lyase. The removal of the two-carbon substituent on C_{17} in the degradation of pregnenolone or progesterone to dehydroepiandrosterone and androstenedione, respectively, occurs in two steps. For progesterone, the intermediate is 17α-hydroxyprogesterone, and the present assay quantifies this intermediate as well as the substrate and endproduct, allowing the inhibitory potency of the test compounds toward the 17α-hydroxylase and $C_{17,20}$-lyase steps to be discriminated. The inhibitory potencies toward the two enzymatic steps in the degradation of progesterone to androstenedione are shown in Table I. It would, in principle, be desirable to inhibit the lyase step selectively. 17α-Hydroxyprogesterone, being the immediate precursor of cortisol, is the final branching point in the pathways leading to corticosteroids. Hence inhibition of the hydroxylase step depletes cortisol, and replacement therapy could therefore be needed.

Since the hydroxylase and lyase steps are thought to be mediated by a single enzyme,[12] selective inhibition of one or other step was not expected. Surprisingly, then, 2-hydroxynonafluoroazobenzene (**2**) proved a markedly selective inhibitor for the lyase step. The ratio between the IC_{50} values for lyase–hydroxylase inhibition was ca. 1:4, whereas 4-hydroxynonafluoroazobenzene (**1**) was a virtually equipotent inhibitor of the two steps.

This surprising selectivity of **2** prompted a closer study which revealed that the compound was unstable at the pH of the assay, as indicated by HPLC analyses during incubation in the buffer used for assay. These showed that **2** was consumed with a half-life of 24 min and a single new product formed which had half the retention time on the reverse-phase HPLC column. Unlike **2** which absorbed 1.8 times more strongly at 280 nm than 254 nm, the new product absorbed 0.6 times more weakly at the longer wavelength. By reaction of **2** with buffered (pH 7.4) aqueous ethanol, which needed to be conducted at high dilution owing to the limited solubility of **2**, it was possible to prepare sufficient product for characterization. Its mass spectrum and elemental analysis showed that this product had formed from **2** by the loss of hydrogen fluoride and thus it may be assigned as the novel dibenzoxadiazepine (**6**) formed by S_NAr displacement of fluoride by ionized hydroxyl on the opposing phenyl ring in the cis isomer of **2**. The structural assignment as **6** is supported by the presence of only four signals in the ^{19}F NMR spectrum. Under more basic conditions, **6** could not be obtained since it reacted further by displacement by ethoxide of the fluorine substituents para to the azo linkage to give the diethoxy derivative (**7**). The characteristics of the dibenzoxadiazepine **6** on HPLC, in terms of retention time and ratio of absorbance at 254 and 280 nm, matched those of the product formed during the incubation. Also, from the extinction coefficients determined at either of the two wavelengths for synthetic **2** and **6**, together with relative peak areas monitored during the incubation, it could be calculated that, after 1 h, the amount of **6** formed accounted for 70% of the original amount of **2** with 16% of **2** remaining. The inhibitory activity of **6** toward the hydroxylase and lyase steps was similar to that of **2** with a 5-fold discrimination in favor of lyase inhibition. In view of the rate of formation of **6** from **2** at pH 7.4 and 37 °C, **6** must at least contribute as an inhibitory component under the conditions of the assay of **2**. Compound **7** proved noninhibitory.

Compound **1** appeared to be a noncompetitive inhibitor with respect to progesterone. The IC_{50} values, determined at a progesterone concentration of 0.3 μM, 28 ± 2 μM for hydroxylase and 29 ± 2 μM for lyase, are insignificantly different from those (Table I) obtained at a substrate concentration of 1 μM. For competitive inhibition, the IC_{50} value would be lower at the lower substrate concentration.[13] Preliminary results (not shown) indicate that compound **2** behaves likewise. There appears to be a requirement for the fluorine substituents since azobenzene and 4-hydroxyazobenzene were noninhibitory, but, interestingly in view of the activity of **6**, the parent compound **4** was almost devoid of inhibitory activity. The introduction of two hydroxyl functions, as in **3**, produced a very weak inhibitor.

Testosterone–5α-Reductase. The structure–activity relationships for inhibition of the 5α-reductase (Table II) differed markedly from those for inhibition of the hydroxylase–lyase inasmuch as the 2-hydroxy derivative **2** was inactive, whereas the 4,4′-dihydroxy derivative **3** was a good inhibitor. There was a marked increase in the activity of **3** and of the 4-hydroxy derivative **1** on lowering

(11) Landini, D.; Maia, A.; Podda, G. *J. Org. Chem.* **1982**, *47*, 2264.
(12) Nakajin, S.; Hall, P. F. *J. Biol. Chem.* **1981**, *256*, 3871.
(13) Dixon, M.; Webb, E. C. *Enzymes*, 2nd ed.; Academic Press: New York, 1964.

Table II. Inhibition of Testosterone-5α-Reductase: Concentration Giving 50% Inhibition of Enzyme Activity (IC_{50}) ± SE and Apparent K_i values

compd	position of OH group(s)	IC_{50}, μM (pH 7.4)	IC_{50}, μM (pH 6.6)	K_i, μM (pH 7.4)
1	4	14 ± 1.1	2 ± 0.1	10
2	2	nd[a]	b	na[c]
3	4,4′	5 ± 0.3	0.79 ± 0.06	4
4	-	b	nd	na
progesterone	-	2.6 ± 0.20[d]	1.6 ± 0.11	nd
azobenzene	-	b	nd	na
4-hydroxyazobenzene	4	b	nd	na

[a] nd = not determined. [b] No inhibition at the highest concentration tested (20 μM). [c] na = not applicable. [d] Published value,[17] 62% inhibition at 3 μM (pH 7.4).

the pH from 7.4 to 6.6. That progesterone, tested for comparison, showed only a small increase in potency suggests a change in the ratio between ionized and unionized species for **1** and **3**, and that the *un-ionized* form is the active component.

The type of inhibition was investigated for **1** and **3** at pH 7.4 with use of Lineweaver–Burk plots and was found to be noncompetitive. There is a requirement for both the fluorine and a hydroxyl group since **4**, azobenzene, and 4-hydroxyazobenzene were not inhibitors.

Other Enzyme Inhibition Tests and Conclusion. Compounds **1** and **2** were also tested for inhibitory activity towards other enzymes in the steroidogenic pathways, namely the cholesterol side-chain cleavage (CSSC) and aromatase enzyme complexes. Aromatase is an important target enzyme in the development of drugs to treat hormone-dependent breast cancer, mediating the conversion of androstenedione and testosterone into estrone and estradiol, respectively. Its inhibition could cause an accumulation of androstenedione, partly negating the intended androgen depletion. Inhibitors of the CSSC enzyme complex can deplete both corticosteroids and androgen precursors and necessitate corticosteroid replacement therapy if used therapeutically. Aromatase, the CSSC enzyme, and the hydroxylase–lyase enzyme are all cytochrome P_{450} type enzymes and inhibitors of one such enzyme can be inhibitors of others, aminoglutethimide being a well-known example.[14] None of the compounds **1**–**3** inhibited aromatase. Compound **2** was a weak inhibitor of CSSC (IC_{50} 92 μM), whereas **1** was not inhibitory.

In conclusion the presently described compounds represent a novel type of inhibitor for two enzymes which are important targets for the development of new drugs to treat prostatic cancer. Reasonable discrimination in inhibitory activity between the two target enzymes and between the hydroxylase–lyase and two other important P_{450} type enzymes involved in steroidogenesis is exemplified, as well as selectivity between the individual steps in the hydroxylase-lyase pathway.

Experimental Section

^1H NMR spectra (250 MHz) (internal Me_4Si = δ 0) and ^{19}F NMR spectra (235 MHz) (internal C_6F_6 = δ −163) were determined with use of a Bruker AC 250 spectrometer, and UV/visible spectra on a Pye-Unicam SP8-150 spectrophotometer. Mass spectra (electron impact, 70 eV) were obtained by direct insertion with a VG 7070H spectrometer and VG 2235 data system. Analytical HPLC was conducted with a 15 cm × 4.6 mm stainless steel column packed with 5 μm APEX ODS. Detection was at 254 and 280 nm and quantitation was carried out with a Trilab (Trivector, Sandy, Beds.). Melting points were determined with a Reichert micro hot stage apparatus and are uncorrected. For the biochemical studies, thin-layer chromatography (TLC) was carried out on glass plates (20 × 20 cm) coated with Merck Kieselgel 60 and were scanned for radioactivity with a Berthold LB 283 Linear Analyzer. High-performance liquid chromatography (HPLC) was carried out on a Waters instrument fitted with a U6K injector and M440 detector. Elemental analyses were determined by CHN Analysis Ltd., S. Wigston, Leicester, England.

4-Hydroxynonafluoroazobenzene (1) and 2-Hydroxynonafluoroazobenzene (2). A solution of decafluoroazobenzene[7] (**4**; 10.8 g, 0.03 mol) and KOH (3 g, 0.053 mol) in *t*-BuOH (150 mL) was heated under reflux for 2 h. To the cooled mixture was added 1 M HCl (5 mL), the mixture was concentrated and made a solution in CCl_4 which was treated with silica gel (Merck, Art. No. 15111) and concentrated, and the residue was applied to a column (1 kg, 50 cm^2 cross section) of this silica gel. Preparative HPLC (eluant light petroleum, bp 60–80 °C) gave first unreacted **4** (2.3 g, 21%) then **2** (0.46 g, 4.3%) which gave orange crystals, mp 159–161 °C, from CCl_4; mass spectrum, m/z 360 ($M^{\bullet+}$, 100); UV spectrum λ_{max} (EtOH) 325 (ε 15 000 mol^{-1} dm^3 cm^{-1} and 430 sh nm (3360); ^{19}F NMR ($CDCl_3$) δ −169.8 (dt, 1, $J_{3,5}$ = 5.4, $J_{4,5}$ = $J_{5,6}$ = 21.4 Hz, F-5), −164.4 (ddd, 1, $J_{3,5}$ = 5.4, $J_{3,6}$ = 8.7, $J_{3,4}$ = 20.1 Hz, F-3), −161.5 (dt, 2, *m*-F in C_6F_5), −149.1 (t, 1, J = 21.0 Hz, *p*-F in C_6F_5), −148.8 (m, 2, *o*-F in C_6F_5), −146.7 (ddd, 1, $J_{4,6}$ = 6.0, $J_{3,6}$ = 8.7, $J_{5,6}$ = 21.4 Hz, F-6), −145.0 (dt, J = 6.0, 21 Hz, F-4). Anal. ($C_{12}HF_9N_2O$) C, H, N; F: calcd, 47,48; found, 46.87. Further elution with Et_2O then Et_2O–MeOH (1:1) gave **1** (7.21 g, 67%) as red needles from CCl_4, mp 166–167 °C (lit.[4] mp 164 °C).

4,4′-Dihydroxyoctafluoroazobenzene (3). (a) From Decafluoroazobenzene (4). To a stirred solution of **4** (10 g, 27.6 mmol) in toluene (150 mL) cooled by a water bath at 18 °C was added a solution of NaOH (75 g) in H_2O (150 mL) followed by *n*-Bu_4NHSO_4 (18.7 g, 55.2 mmol). After 5 min the solution became viscous and, after a further 15 min, was diluted with toluene (150 mL), followed by H_2O (150 mL), whereupon a granular orange solid separated. This was recovered by filtration, washed with toluene (100 mL), followed by 2-propanol (100 mL), thoroughly drained, and recrystallized from 2-propanol to give the bis tetra-*n*-butylammonium salt of **3** (14.5 g): ^1H NMR [$(CD_3)_2SO$] δ 0.93 (t, 24, $J_{4,3}$ = 7.15 Hz, butyl H-4), 1.30 (quintet, 16, $J_{4,3}$ = $J_{3,2}$, butyl H-3), 1.56 (1, br quintet, butyl H-2), 3.6 (br t, 16, $J_{1,2}$ = 8 Hz, butyl H-1); ^{19}F NMR δ −172.1 (F-3,5), −158.2 (F-2,6) AA′XX′ system, coupling constants determined for free acid only; see below. An analytical sample was obtained by recrystallization from aqueous EtOH. Anal. ($C_{44}H_{72}N_4F_8O_2$) C, H, N, F.

Crystallization of this product (200 mg) from HOAc–H_2O (1:1) gave orange rods (105 mg), mp 158–160 °C, which analyzed as the monohydrate of the mono tetra-*n*-butylammonium salt of **3**. Anal. ($C_{28}H_{39}F_8N_3O_3$) C, H, N, F.

To obtain **3** as the free phenol, the crude bis tetra-*n*-butylammonium salt (12 g) was partitioned between 1 M H_2SO_4 (120 mL) and Et_2O (240 mL), the organic phase was washed with 1 M H_2SO_4 (120 mL) and then with H_2O (2 × 120 mL), and the residue from the concentrated organic phase was recrystallized from EtOH–H_2O (1:2) to give **3** (4.77 g, 58% based on **4**): mp 222–223 °C; mass spectrum m/z 358 (69, $M^{\bullet+}$), 193 ([M − HOC_6F_4]$^+$, 51), 165 ($HOC_6F_4^+$, 100); ^{19}F NMR [$(CD_3)_2SO$] δ −163.0 (F-3,5), −152.4 (F-2,6); AA′XX′ system, $J_{2,3}$ = 21.0 Hz, $J_{2,5}$ = 6.3 Hz, $J_{2,6}$ = 7.0 or 4.8 Hz, $J_{3,5}$ = 4.8 or 7.0 Hz. Anal. ($C_{12}H_2F_8N_2O_2$) C, H, N, F.

(b) From 4,4′-Dimethoxyoctafluoroazobenzene (5). To a refluxing solution of **5**[6] (1.1 g, 3.04 mmol) in CS_2 (70 mL) was added a solution of AlI_3 (3.72 g, 9.12 mmol) in CS_2 (10 mL), and after 15 min the mixture was poured onto ice and treated with Na_2SO_3 (1 g) and concentrated HCl (10 mL). After extraction with Et_2O (5 × 100 mL) crude **3** (0.37 g) was obtained as orange needles from MeCN. Analytical HPLC (solvent H_2O–MeCN–AcOH, 50:50:1) revealed **3** (t_R 92 s, 89.5%) and another component (t_R 155 s, 8.8%) of the same MW (mass spectrum) and which was therefore probably the cis isomer of **3**. Recrystallization from aqueous EtOH gave pure **3**, identical with material prepared by method a above.

Octafluorodibenz[*b*,*f*][1,4,5]oxadiazepine (6). To a stirred aqueous solution of pH 7.64 obtained by mixing 0.1 M $Na_2HPO_4·H_2O$ (81 mL) and 0.1 M $NaH_2PO_4·2H_2O$ (19 mL) was added dropwise a solution of **2** (170 mg, 0.472 mmol) in EtOH (60 mL).

(14) Lønning, P. E.; Kvinnsland, S. *Drugs* **1988**, *35*, 685.

After 18 h the initially red-orange solution had turned pale yellow. Water (100 mL) was added, and the solution was extracted with Et_2O (3 × 50 mL) to yield upon concentration of the extracts the title compound (120 mg, 75%) which gave yellow needles (25 mg) from Et_2O, forming plates at 110 °C and subliming at 176–178 °C: mass spectrum m/z 340 (80, $M^{\bullet+}$); UV spectrum λ_{max} (EtOH) 240 sh (ϵ 6150 mol^{-1} dm^3 cm^{-1}), 328 (6960) and 422 nm (640). ^{19}F NMR ($CDCl_3$) δ −158.4 [m, 2, F-3 (para to O)], −156.6 [m, 2, F-1 (ortho to O)], −147.7 [dt, 2, F-4 (ortho to N)], −144.4 [m, 2, F-2 (para to N)]. Anal. ($C_{12}F_8N_2O$) C, N.

2,9-Diethoxyhexafluorodibenz[*b*,*f*][1,4,5]oxadiazepine (7). To a stirred solution obtained by treating 0.1 M $Na_2HPO_4 \cdot H_2O$ (150 mL) with Na_3PO_4 to pH 11.5 was added dropwise a solution of **2** (80 mg, 0.22 mmol) in EtOH (50 mL). After 25 h the solution was diluted with H_2O (250 mL) and concentrated HCl (25 mL) then extracted with Et_2O (3 × 250 mL) to give the title compound as an orange powder (23 mg, 26%) from CCl_4–light petroleum (bp 60–80 °C): mp 91–94 °C; mass spectrum, m/z 392 (100, $M^{\bullet+}$); ^{19}F NMR ($CDCl_3$) δ −155.9 (d, 2, J = 21.2 Hz, F-3), −154.2 (d, 2, J = 8.3 Hz, F-1), −147.3 (dd, 2, J = 8.3, 21.2 Hz, F-4). Anal. ($C_{16}H_{10}F_6N_2O_3$) C, H, N.

Enzyme Preparation and Assay Procedures. 17α-Hydroxylase–$C_{17,20}$-Lyase. The 17α-hydroxylase–$C_{17,20}$-lyase activities were measured as previously described.[5] The enzyme source was a microsomal preparation from rat testes. The assay mixture contained 250 μM NADPH, 10 mM glucose-6-phosphate, 3 U/mL glucose-6-phosphate dehydrogenase, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 1 μM [^{3}H]progesterone in 50 mM sodium phosphate buffer (pH 7.4). The reaction was carried out at 37 °C, started by the addition of the enzyme, and terminated after 10 min by the addition of 2 vol MeCN–MeOH (1:2) containing unlabeled steroids ca. 100 μM. The steroids were separated by HPLC, and the fractions of interest were collected and counted. $C_{17,20}$-Lyase activity corresponds to the production of testosterone and androstenedione, while the 17α-hydroxylase activity corresponds to the production of testosterone, androstenedione, and 17α-hydroxyprogesterone. The control activities were 1.6 and 8.7 nmol/h per mg protein, respectively. The test compounds were added in ethanol, bringing the concentration of ethanol in the assay to 5%. Control tubes received ethanol only. To determine the IC_{50} value for a compound (the concentration required to reduce the enzyme activity to 50% control) the enzymatic rates were measured in triplicate at not less than three concentrations of compound. The ranges of concentration employed were as follows: for compound **1**, 10–200 μM; for **2**, 10–100 μM; for **6**, 1–100 μM, and for ketoconazole 1–10 μM. The data were fitted by linear regression to the Dixon equation

$$1/V_i = 1/V_o + IC/(V_o IC_{50})$$

where V_o is the uninhibited rate, V_i is the rate in the presence of inhibitor concentration IC. The correlation coefficients for the line fitting were greater than 0.95.

HPLC Analysis during Incubation of 2. A solution of **2** (20 μL; 5 mM) in EtOH was added to 50 mM sodium phosphate buffer (pH 7.4; 0.98 mL), and the solution was maintained at 37 °C in the dark. Aliquots of 100 μL were removed into 100 μL of ice-cold EtOH and analyzed with 60:40 ethanol–0.1% acetic acid as running solvent on a 10-cm Apex (C18, 5 μm) column at a flow rate of 1 mL min^{-1}. Peaks of retention time 10.5 min (corresponding to **6**) and 20.6 min (corresponding to **2**) were detected. Respective relative areas measured at 254 nm were as follows: 0 min (initial time at which the solution was sampled) = 104/161; 30 min = 220/82; 60 min = 285/36; 120 min = 291/not detected. Measured at 280 nm these were as follows: 0 min = 67/287; 30 min = 133/165; 60 min = 169/59; 120 min = 167/8.7. A graph of ln(area for **2**) against time was linear giving a first order half life of decay of 24 min. Extinction coefficients ϵ (mol^{-1} dm^3 cm^{-1}) determined from synthetic materials in the same solvent mixture as used for HPLC were 2340 at 254 nm and 4310 at 280 nm, for **2**, and 4330 at 254 nm and 2630 at 280 nm, for **6**, and were used to determine the relative amounts **6** vs **2** present. When measured at 254 nm, relative amounts of material **2**–**6** were at 0 min 74:26 (total = 100), at 60 min 16:70 (total = 86), and at 120 min 0:70 (total = 70). Measuring at 280 nm, for the same times gave ratios 73:27 (total = 100), 15:70 (total = 85), and 2:68 (total = 70).

Testosterone-5α-Reductase. Ventral prostates from adult Wistar rats were homogenized in 3 vol of 50 mM potassium phosphate buffer (pH 7.4), containing 0.33 M sucrose and 1 mM dithiothreitol. Subcellular fractions were prepared by differential centrifugation according to ref 15. The crude nuclear fraction, containing 70% of the total 5α-reductase activity with a specific activity, expressed as DHT formed, of 5.3 pmol/min per mg was utilized as a source of the enzyme.

Enzyme activity was monitored radiometrically by measuring the conversion of 4-[^{14}C]testosterone into 4-[^{14}C]DHT. Assay tubes (total volume of 0.5 mL), at 37 °C, contained 5 μM testosterone with 0.5 μCi of 4-[^{14}C]testosterone (57 mCi/mmol), 1 mM NADPH, and 1 mM dithiothreitol in 50 mM potassium phosphate buffer (pH 7.4), and incubations were started by the addition of 50 μL of the nuclear fraction (ca. 1.5 mg). Assays were stopped after 30 min by adding ethyl acetate (3 mL) containing 1 mM $HgCl_2$ (0.3 mL) and cold carrier steroids (ca. 20 μg). After centrifugation at 1000g for 15 min, the ethyl acetate layer was removed and concentrated under nitrogen, and the concentrate was chromatographed on silica gel TLC plates in CH_2Cl_2–EtOAc (9:1). Radiochromatogram scanning revealed DHT as the only radioactive product and permitted quantification of the enzyme reaction.

The enzyme displayed a broad optimum pH range from 7.0 to 7.5. Lineweaver–Burk plots were linear with an average apparent K_m value of 1.4 μM (± 0.5, SE, four determinations). All subsequent assays were performed at pH 7.4 with a final substrate concentration of 5 μM and under conditions of linearity with respect to time and protein concentration. For inhibition experiments, compounds were added in DMSO (2% of total volume) to give concentrations of 1, 2, 5, 10, and 20 μM. For controls, solvent only was added and all assays were performed in duplicate. Kinetic analysis of the inhibitors was carried out by Lineweaver–Burk plots with the method of least-squares analysis being used to obtain a linear fit to the data. A secondary plot of reciprocal maximum velocity against inhibitor concentration generated the K_i values.

Other Enzyme Assays. Assays for inhibition of cholesterol side-chain cleavage and aromatase were carried out as described previously.[16]

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(15) Liang, T.; Cascieri, M. A.; Cheung, A. H.; Reynolds, G. F.; Rasmusson, G. H. *Endocrinology* **1985**, *117*, 571.
(16) Foster, A. B.; Jarman, M.; Leung, C.-S.; Rowlands, M. G.; Taylor, G. N. *J. Med. Chem.* **1983**, *26*, 50.
(17) Patwardhan, V. V.; Lanthier, A. *J. Steroid Biochem.* **1975**, *6*, 137.