evaporated to dryness and the semisolid residue was eluted from a flash column (250 g, cyclohexane–EtOAc, gradient, 3:1 → 1:1) to afford crude **37**: yield, 929 mg. A portion (102 mg) of this material was further purified on a silica gel thick plate (2000 μm, $CHCl_3$–EtOAc, 95:5) to afford **37** as an amber glass: yield, 29 mg.

Further development of the above flash column with cyclohexane–EtOAc (1:1) afforded crude **36** (1.56 g), which after an additional flash column [125 g, $CHCl_3$ → $CHCl_3$–MeOH (98:2)] gave **36** as an amber semisolid: yield, 1.28 g. This material was essentially homogeneous by TLC, but the ^1H NMR spectrum indicated the presence of trace amounts of unidentified phenyl containing impurities.

Ethyl [6-Amino-4-(2-formylhydrazino)-5-nitropyridin-7-yl]carbamate (39). A suspension of **38** (2.5 g, 9.8 mmol)[7] in butyl formate (250 mL) was heated at reflux with stirring. A clear solution was formed after 1 h, and after 2 h the resulting mixture was cooled to room temperature. The product was collected by filtration and dried in vacuo over P_2O_5: yield, 2.67 g.

Ethyl [6-Amino-4-(2-methylhydrazino)-5-nitropyridin-2-yl]carbamate (40). To a partial solution of **39** (4.3 g, 15 mmol) in anhydrous THF (850 mL) at room temperature was added 2 M $BH_3{\cdot}SMe_2$ in THF (35 mL) with stirring. After 2.5 h the dark mixture was filtered (Celite), and the filtrate was evaporated to dryness in vacuo. This residue was dissolved in MeOH (100 mL) and reevaporated to remove some of the borane reagent. This residue was dissolved in preheated 2-propanol (50 mL), cooled to room temperature, and then refrigerated. The solid was collected by filtration, washed with hexane (150 mL), and dried in vacuo over P_2O_5: yield, 2.0 g.

The combined filtrate and wash from above gave another crop of impure **40**: yield, 1.0 g (24%). Further recrystallization from 2-propanol resulted in a low recovery of **40**. The solid recovered from alcoholic solutions appeared to contain the corresponding 4-(methylazo)pyridine [mass spectrum, 269 $(M + 1)^+$].

Ethyl (5-Amino-1,2-dihydro-3-phenylpyrido[3,4-*e*]-*as*-triazin-7-yl)carbamates (47–49) and Ethyl (5-Amino-3-phenylpyrido[3,4-*e*]-*as*-triazin-7-yl)carbamate (50). Method A. The hydrogenation of ethyl (6-amino-4-hydrazino-5-nitropyridin-2-yl)carbamate (**38**, 570 mg, 2.23 mmol)[7] was performed as previously reported to give a product containing **43** and the hydrochloride of **42** (670 mg, 82% purity by HPLC).[13] This sample and methyl benzimidate hydrochloride (430 mg, 2.51 mmol) were dissolved in H_2O (10 mL) containing Et_3N (0.5 mL) and stirred at room temperature for 19 h. The red precipitate was collected by filtration, washed with H_2O (10 mL), and extracted with hot EtOH (50 mL): yield, 132 mg. The ^1H NMR spectrum in DMSO-d_6 showed a 5:1 ratio of **47–50**. The EtOH filtrate was cooled to afford a second crop (234 mg), which contained a 1:7 ratio of **47–50**. In another experiment an aqueous solution of the hydrochloride of **42** (from hydrogenation of 1.0 g of **38**) was reacted with methyl benzimidate hydrochloride (700 mg) in the absence of Et_3N. The red product was washed well with H_2O, which gave an essentially pure sample of the hydrochloride of **50**: yield, 237 mg.

Method B. A solution of **40** (135 mg, 0.500 mmol) in MeOH (20 mL) containing Raney nickel (0.5 g, weighed wet, washed 1 × H_2O and 2 × MeOH) was hydrogenated at room temperature and atmospheric pressure for 1 h. The catalyst was removed by filtration (Celite) and washed with MeOH (5 mL), and to the combined filtrate and wash containing **44** was added methyl benzimidate hydrochloride (118 mg, 0.690 mmol). After stirring for 16 h the dark reaction mixture was evaporated to dryness and the residue was washed with Et_2O: yield, 142 mg. This sample showed multiple spots by TLC, but the mass spectrum [327 $(M + 1)^+$] indicated the presence of **48**. Column chromatography (silica gel, 10 g; 95:5 and 9:1 $CHCl_3$–MeOH) gave multiple red bands, which were combined and recolumned (silica gel, 3 g; $CHCl_3$) to give one major red band: yield, 41 mg. This product was identified as **50** by TLC and its mass spectrum.

Method C. The hydrogenation of ethyl (6-amino-4-(1-methylhydrazino)-5-nitropyridin-2-yl)carbamate (**41**, 1.0 g, 3.7 mmol)[13] in 1:1 H_2O–EtOH (200 mL) gave a mixture of **45** [mass spectrum, 241 $(M + 1)^+$] and the hydrazine cleavage product [mass spectrum, 226 $(M + 1)^+$]. Reaction of a solution of this mixture in H_2O with methyl benzimidate hydrochloride gave only a low yield of recovered cleavage product. In another approach the preparation of **46** from **41** was performed as previously reported.[13] A sample of crude **46** (80 mg) was dissolved in hot EtOH (10 mL) and maintained at the temperature for 1 h. After the addition of H_2O (5 mL), the solution was cooled for 16 h to deposit a mixture containing **50** rather than **49** as a major component [TLC; mass spectrum, 311 $(M + 1)^+$].

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Inhibition of Enzymes of Estrogen and Androgen Biosynthesis by Esters of 4-Pyridylacetic Acid

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A variety of esters of 4-pyridylacetic acid have been prepared by base mediated exchange from the methyl ester. Several of the esters of alcohols that contained a cyclohexyl ring were potent inhibitors of human placental aromatase and of the rat testicular 17α-hydroxylase/C_{17-20}lyase complex. The most potent agents found against both enzyme complexes were the borneyl, isopinocampheyl, and 1-adamantyl esters. These were over 100 times more potent than aminoglutethimide against aromatase and of greater potency than ketoconazole against hydroxylase/lyase. Potency against either enzyme complex was reduced if the ester function was borne on the cyclohexyl ring in an axial rather than an equatorial position. Some differential selectivity could be introduced since whereas methyl substitution adjacent to the carbonyl group reduced the inhibition of aromatase, it increased that against hydroxylase/lyase.

The aromatase enzyme complex performs the last steps in the biosynthesis of estrogens. Since a proportion of breast tumors are dependent upon estradiol for their growth, depletion of circulating estradiol levels by inhibition of this enzyme provides an approach to the treatment of hormone-dependent breast cancer.[1] For this purpose,

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the aromatase inhibitor aminoglutethimide (1) is in clinical use.[2] We have developed an analogue, 3-ethyl-3-(4-pyridyl)piperidine-2,6-dione (**2**),[3-5] having comparable potency to 1 but having the advantages of (i) not additionally inhibiting the cholesterol side chain cleavage (CSCC) enzyme so causing the requirement for corticosteroid replacement therapy, (ii) being devoid of sedative activity, and (iii) having a more favorable metabolic profile than 1. This analogue is presently undergoing clinical trial.

Recently workers at Farmitalia Carlo-Erba[6] reported that the cyclohexyl ester (**3**) of *R*-2-(4-aminophenyl)propanoic acid is a potent inhibitor of aromatase. By analogy with the relation of **2** to **1**, our search for selective aromatase inhibitors of high potency led us to investigate the substitution of 4-pyridyl for 4-aminophenyl in **3**.

(1) (2) (3) (4)

During the course of our work, Farmitalia Carlo-Erba, having presumably followed similar reasoning, reported that cyclohexyl 4-pyridylpropanoate and related compounds[7] are aromatase inhibitors. We report our results which concentrate on the esters of 4-pyridylacetic acid rather than of the propanoic acid, and show that the cyclohexyl 4-pyridylacetate (4) is a very potent inhibitor of human placental aromatase but that even greater potency is found with esters of bulkier alcohols, yet all the compounds lack selectivity in additionally giving strong inhibition of the 17α-hydroxylase/C_{17-20}lyase complex that is essential for the biosynthesis of androgens.

Results and Discussion

Synthesis. In the first step of our reported synthesis of the individual enantiomers of **2**,[8] *n*-butyllithium-mediated ester exchange[9] was used to convert methyl 4-pyridylacetate into the ester with a chiral secondary alcohol auxiliary derived from camphor. This same method was employed to prepare the variety of esters listed in Table I and worked well for the preparation of esters from primary and secondary alcohols. Tertiary alcohols reacted slowly, and somewhat lower yields were then obtained. The phenyl ester could not, however, be prepared in this way since lithium phenoxide will not displace the more basic methoxide. Condensation of 4-pyridylacetic acid and phenol by trifluoroacetic anhydride was used instead.

Mono- or dimethylation of the cyclohexyl ester between the carbonyl group and pyridyl moiety was carried out in view of the reported benefit[10] in the (aminophenyl)acetic esters upon adding a methyl group and was readily carried

Table I. Preparation of 4-Pyridylacetic Acid Esters

R—O—C(=O)—CH_2—(4-pyridyl)

	R	yield,[a] %	form	mp	anal.
4	cyclohexyl	96	HCl	154–6	C,H,N,Cl
5	*trans*-4-methylcyclohexyl	93	HCl	166–8	C,H,N,Cl
6	*cis*-4-methylcyclohexyl	65	HCl	166–8	C,H,N,Cl
7	4-ethylcyclohexyl[c]	93	HCl	172–4	C,H,N,Cl
8	*trans*-4-*tert*-butylcyclohexyl[d]	40	free base	69–70	C,H,N
9	1-methylcyclohexyl	40	HCl	179–81	C,H,N,Cl
10	cyclopentyl	85	HCl	146–8	C,H,N,Cl
11	3-methylcyclohexyl[b,c]	92	HCl	172–4	C,H,N,Cl
12	(−)-menthyl	85	free base	61–3	C,H,N
13	(+)-menthyl	86	free base	62–3	C,H,N
14	*exo*-norborneyl[b]	96	HCl	151–2	C,H,N,Cl
15	*endo*-norborneyl[b]	91	HCl	182–4	C,H,N,Cl
16	(−)-borneyl	92	free base	28–30	C,H,N
17	(+)-borneyl	80	free base	28–30	C,H,N
18	(1*S*,2*S*,3*S*,5*R*)-(+)-isopinocampheyl	92	HCl	206–7	C,H,N,Cl
19	(1*R*,2*R*,3*R*,5*S*)-(−)-isopinocampheyl	96	HCl	205–7	C,H,N,Cl
20	1-adamantyl	11	free base[e]	69–70	C,H,N
21	phenyl	73	$HOCOCF_3$	96–98	C,H,N
22	isopropyl	88	HCl	155–6	C,H,N,Cl
23	3-methylbut-1-yl	80	HCl	141–3	C,H,N,Cl
24	4-methylpent-2-yl[b]	83	HCl	168–70	C,H,N,Cl

[a] With the sole exception of the phenyl ester (see text) products were formed by transesterification from the methyl ester and yields are those obtained for the free base after chromatography. [b] Alcohol used and the ester were a mixture of enantiomers. [c] Alcohol used and the ester were a mixture of cis and trans epimers. [d] The alcohol used was a mixture of cis and trans isomers but the trans isomer crystallized from the product mixture. The yield is that for the pure isomer obtained. [e] To remove unreacted alcohol it was necessary to form the hydrochloride and then break it down with 1 M K_2CO_3.

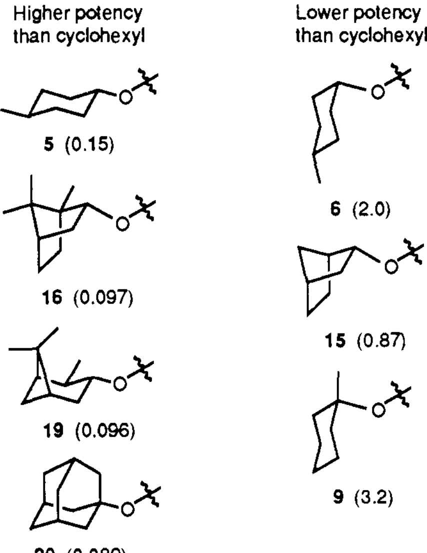

Figure 1. Comparison of aromatase inhibitory activity of esters formed from 4-pyridylacetic acid and some cyclohexyl ring containing alcohols (IC_{50} against aromatase).

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Table II. Enzyme Inhibition Data

	R	IC_{50} (95% confidence limits), μM human placental aromatase[a]	rat testicular C_{17-20}lyase	rat testicular 17α-hydroxylase	ratio of lyase/aromatase potency
	Variation of the Alkoxy Moiety (R–O–C(=O)–CH₂–4-pyridyl)				
4	cyclohexyl	0.30 (0.28–0.32)	20 (18–24)	15 (14–17)	67
5	*trans*-4-methylcyclohexyl	0.15 (0.12–0.18)	4.4 (3.9–4.9)	4.2 (3.7–4.8)	29
6	*cis*-4-methylcyclohexyl	2.0 (1.2–2.7)	36 (33–40)	25 (24–26)	18
7	4-ethylcyclohexyl	0.12 (0.10–0.13)	2.7 (2.6–2.9)	2.0 (1.9–2.1)	23
8	*trans*-4-*tert*-butylcyclohexyl	0.31 (0.27–0.35)	3.0 (2.7–3.4)	2.1 (1.9–2.4)	10
9	1-methylcyclohexyl	3.2 (2.4–3.9)	11 (9–15)	10 (8–13)	3.5
10	cyclopentyl	2.3 (1.5–3.0)	131 (114–153)	77 (67–90)	57
11	3-methylcyclopentyl	2.0 (1.3–2.7)	32 (29–35)	28 (25–31)	16
12	(−)-menthyl	0.31 (0.25–0.38)	5.1 (4.6–5.9)	4.5 (4.2–4.8)	16
13	(+)-menthyl	0.24 (0.21–0.27)	15 (13–17)	12 (11–12)	63
14	*exo*-norborneyl	0.34 (0.25–0.42)	41 (37–45)	31 (29–34)	120
15	*endo*-norborneyl	0.87 (0.75–0.99)	46 (39–55)	45 (41–50)	53
16	(−)-borneyl	0.097 (0.091–0.103)	2.2 (2.0–2.5)	1.5 (1.4–1.6)	23
17	(+)-borneyl	0.29 (0.25–0.33)	0.54 (0.48–0.61)	0.59 (0.54–0.64)	1.9
18	(1*S*,2*S*,3*S*,5*R*)-(+)-isopinocampheyl	0.12 (0.09–0.15)	0.28 (0.24–0.34)	0.26 (0.24–0.27)	2.3
19	(1*R*,2*R*,3*R*,5*S*)-(−)-isopinocampheyl	0.096 (0.090–0.102)	1.8 (1.6–2.1)	1.7 (1.5–2.0)	19
20	1-adamantyl	0.089 (0.085–0.093)	0.61 (0.53–0.73)	0.56 (0.52–0.59)	6.9
21	phenyl	13.9 (12.7–15.1)	>100	>100	-
22	isopropyl	>20	>100	>100	-
23	3-methylbut-1-yl	11.8 (10.0–13.6)	>100	>100	-
24	4-methylpent-2-yl	9.7 (9.3–10.1)	17 (15–19)	13 (13–14)	1.8
	Methylated Compounds (cyclohexyl–O–C(=O)–C(R¹)(R²)–4-pyridyl)				
25	R^1=H R^2=Me	2.1 (1.9–2.3)	2.2 (2.0–2.3)	2.0 (1.9–2.1)	1.0
26	R^1=R^2=Me	13.0 (12.0–13.1)	4.5 (4.0–5.1)	3.6 (3.3–3.9)	0.34

[a] Under the same experimental conditions aminoglutethimide gave IC_{50} = 14 μM (95% confidence limits 13–15 μM).

out by treatment with potassium hydride and iodomethane.

Inhibition of Human Placental Aromatase. The abilities of the esters of 4-pyridylacetic acid to inhibit the aromatisation of tritium labeled androstene-3,17-dione by human placental microsomes are given in Table II. Several of the compounds had values of IC_{50} better than 0.5 μM and all of these had a cyclohexyl ring present. The cyclopentyl ester (**10**) was about 7-fold weaker an inhibitor than the cyclohexyl ester (4). The phenyl ester **21** and esters of acyclic aliphatic alcohols **22–24** possessed little inhibitory activity. A comparison of the structures of some of the cyclohexane ring-containing esters in given in Figure 1. The best inhibitors **5**, **7**, **16**, and **18–20** (IC_{50} ≤0.15 μM) each possess a methyl or methylene group in a region of space that by examination of molecular models is about 11 Å away from the pyridine nitrogen atom. Three of the esters containing a cyclohexyl ring (**6**, **9**, and **15**) had significantly lower potency than the parent cyclohexyl compound. The *endo*-norborneyl group in **15** projects only as far as a cyclopentyl group as a result of the boat conformation of the cyclohexyl ring imposed by the ring fusion. This explains the relatively low activity of **15** but the dimethylene bridge (shown as projecting downward in Figure 1) nevertheless causes some benefit as **15** is more potent than the cyclopentyl analogue and this bridge is also present in the potent (−)-borneyl ester **16**. The *cis*-4-methylcyclohexyl analogue **6** exists in a preferred conformation in which the oxygen substituent, rather than the methyl substituent, is borne on the cyclohexyl ring in the axial position due to the greater covalent radius of the methyl group. This feature is apparent from the ^{1}H NMR spectrum where in **6** the multiplet signal for C*H*O at δ 5.00 (width at half height 10 Hz) compares with that in the conformationally locked *cis*-*tert*-butyl analogue (δ 5.02, width 7 Hz) and is unlike that in the trans isomer **5** (δ 4.68, width 21 Hz) where the greater width of the multiplet in **5** reflects the large axial–axial coupling. A result of this conformation of **6** is that the cyclohexyl ring does not project in the direction required for optimal inhibitory activity (see Figure 1). The poor activity of the 1-methylcyclohexyl analogue **9** can similarly be ascribed to the methyl group causing the oxygen substituent to adopt the axial position. In this compound steric bulk of the methyl group cannot provide an explanation for the low potency since the 1-adamantyl ester **20** has a methylene in the same position and is very potent.

Differences between the potencies of esters from enantiomeric alcohols reflect the chirality of the enzyme pocket in which the compounds bind. There was a 3-fold difference in activity between the (−)- and (+)-borneyl esters (**16** and **17**) but the menthyl (**12** and **13**) and isopinocampheyl (**18** and **19**) ester pairs gave a much smaller activity differential. These latter pairs differ in the position of an isopropyl or methyl group at the ring positions adjacent to the site of oxygen attachment and these sites are presumably not important for activity. In the borneyl esters, one or other of the geminal dimethyl groups is presumably important for optimal activity (compare **15**) and these will be in a different region of space for the two enantiomers.

The introduction of a methyl substituent adjacent to the carbonyl group to give the cyclohexyl pyridylpropionate **25** reduced aromatase inhibitory activity by a factor of 7 whereas for the corresponding (4-aminophenyl)acetic acid ester similar methyl incorporation has been reported to

increase potency and by over 50-fold.[10] This unexpected contrast might reflect the greater distance between the introduced methyl groups and the basic nitrogen in the aminophenyl series. Introduction of a second methyl group to give **26** decreased the aromatase inhibition further.

The kinetics of inhibition by the cyclohexyl and adamantyl esters 4 and **20** were, as determined by Lineweaver–Burke reciprocal plots, consistent with competitive displacement of the steroidal substrate from the aromatase enzyme. Values of inhibition constant K_i were 0.045 and 0.015 μM for 4 and **20**, respectively. In comparison, aminoglutethimide has K_i = 1.8 μM. Further evidence of a mechanism of action involving displacement of the steroid is that after preincubation of the microsomal preparation with androstenedione, addition of the ester caused an increase in absorption at 425 nm and a decrease at 385 nm in the electronic spectrum. This "Type II" spectrum is consistent with conversion of the heme iron from a high-spin to low-spin state[13] and thus to displacement of the androstenedione. This same property was found for the aromatase inhibitors 4-cyclohexylaniline,[14] *d*-aminoglutethimide,[14] and the pyridylglutarimide **2**.[3]

Inhibition of the Cholesterol Side Chain Cleavage (CSCC) Enzyme. Inhibition of the CSCC enzyme by aminoglutethimide is considered a disadvantage for breast cancer treatment since corticosteroids are depleted and need to be replaced by additional therapy. The cyclohexyl ester 4 was studied for the inhibition of the CSCC enzyme and found to have an IC_{50} value of 180 μM. Under the same conditions, aminoglutethimide has an IC_{50} value of 60 μM. Therefore, although 4 did weakly inhibit the CSCC enzyme, by virtue of the much greater aromatase inhibition compared with aminoglutethimide, there is a good selectivity for aromatase.

Inhibition of Rat Testicular 17α-Hydroxylase and C_{17-20}Lyase. Our interest in the inhibition of enzymes of androgen biosynthesis with a view to finding new drugs for the treatment of prostate cancer led us to test the compounds against the 17α-hydroxylase/C_{17-20}lyase complex from rat testes. The assay measures the effect of the compounds on the rate of conversion of radiolabeled progesterone to 17α-hydroxyprogesterone and thereon to androstenedione. It was found that these esters markedly inhibited these enzymatic steps and values of inhibitory potency (IC_{50}) are listed in Table II. For all of the compounds tested, the inhibition of the lyase was comparable to that of the hydroxylase. This may be explained by the esters denying access of the natural substrate to a binding site common for the two catalytic activities. Indeed, evidence has been reported for both the hydroxylase and lyase steps being associated with a single enzyme[15] and the presence of a single steroid binding site has been inferred in the reported finding that there is not free exchange of the 17α-hydroxy intermediate.[16] The IC_{50} value of 4 was found to increase with an increasing concentration of the natural enzyme substrate progesterone and this is consistent with competitive inhibition.

Upon variation of the alcoholic moiety of the esters, it was found that the most potent agents (having IC_{50} <10 μM, i.e. **5**, **7**, **8**, **12**, **16–20**) all contained a cyclohexyl ring and the placement of a 4-alkyl substituent on the ring (**4** → **5**) was beneficial. The cyclopentyl ester **10** had only one quarter the potency of **4** and the phenyl ester **21** and the esters of acyclic alcohols **22–23** were noninhibitory. In all these respects, the structure–activity relationships paralleled those against aromatase but there are important differences however which show that the hydroxylase/lyase complex is different from the aromatase enzyme in the structural requirements for inhibition. Thus, the ester with one acyclic alcohol **24** in which the chain could extend as far as a cyclohexyl ring was as effective an inhibitor as **4** and methyl group introduction into the 1-position of the cyclohexyl ring as in **9** improved inhibition slightly, whereas aromatase inhibition was markedly decreased. Furthermore, where enantiomeric pairs were prepared (menthyl, borneyl, and isopinocampheyl) the more inhibitory enantiomer against hydroxylase/lyase was the less inhibitory against aromatase. For the most potent ester found, **18**, the IC_{50} value against the lyase step is estimated at 0.28 μM. This compound is significantly more potent than ketoconazole, an imidazole-based drug which has been clinically used in prostate cancer treatment[17] and in which the principal locus of action has been ascribed to C_{17-20}lyase inhibition[18] against which it has IC_{50} = 10 μM. The most marked difference in the structure–activity relationships between the different enzyme complexes is found upon methylation adjacent to the ketone function. Addition of one methyl group in **25** increased inhibition of the hydroxylase/lyase by 8-fold unlike against aromatase, where activity was decreased. A second methyl group (in **26**) slightly reduced activity but this compound gave the best selectivity of those described for the hydroxylase/lyase over aromatase.

Conclusions

We have described a series of easily prepared esters of 4-pyridylacetic acid that are potent competitive inhibitors of key enzymes of steroid hormone biosynthesis. There are several common structural elements that enhance inhibition against the two enzyme complexes, aromatase and 17α-hydroxylase/lyase and these are presumably ones that give the esters a greater structural similarity to a steroid. In this respect the pyridyl esters described may provide a versatile framework for the inhibition of enzymes of steroid hormone biosynthesis, but raises the matter of how there can be selectivity against a given enzyme. This problem has been addressed in the following paper by Laughton and Neidle in which molecular modeling techniques have been used to compare the pyridyl esters with the natural enzyme substrates.

Potent inhibition of aromatase is promising for the potential development of the use of pyridylacetic acid esters in the treatment of breast cancer. However, since all the esters also inhibit 17α-hydroxylase, cortisol would be depleted and cortisol replacement therapy could be needed at the concentrations of drug needed to achieve complete suppression of estrogen synthesis. Indeed, no compounds giving much better selectivity for aromatase over hydroxylase/lyase than the cyclohexyl ester could be found.

The inhibition of androgen biosynthesis by virtue of the hydroxylase/lyase inhibition indicates that such compounds could be useful for the treatment of prostate cancer since a high proportion of such tumors are dependent upon androgens for growth.[19] Additional inhibition of aroma-

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tase may be undesirable since androgens are retained but the promising selectivity in the dimethyl derivative **26** may provide a basis for the design of selective inhibitors of the hydroxylase/lyase complex. Ideally however, only the lyase and not the hydroxylase should be inhibited to avoid the depletion of cortisol and this may not be possible with competitive inhibitors such as the esters described.

Lastly, it should be mentioned that the compounds described, being esters, can be expected to be susceptible to metabolic degradation by esterases and this would limit their utility in vivo. Indeed, we have found that the compounds are hydrolyzed by hog liver esterase, though the hindered dimethylated ester **26** was degraded some 5-fold more slowly than either the parent cyclohexyl ester **4** or the monomethylated derivative **25**. In this respect it is noteworthy that improved resistance to hydrolysis coincides with improved inhibition of the hydroxylase/lyase. In the case of aromatase inhibition, the workers at Farmitalia Carlo-Erba have shown that the amide corresponding to **25**, where –O– has been replaced by –NH–, is effective at reducing circulating estrogen levels in PMSG-pretreated rats.[7] Also, in the aminophenyl series, this substitution does not affect aromatase inhibitory potency in vitro.[6] Thus, if need be, similar substitution could be envisioned for the potent compounds containing terpene moieties described here. In any case, the compounds described should provide a basis for the design of improved inhibitors of steroid hormone biosynthesis.

Experimental Section

Chemical Methods. Standard Procedures. ^{1}H NMR spectra (250 MHz) were recorded on a Bruker AC250 spectrometer. Chromatography refers to column chromatography on silica gel (Merck Art. 15111) with the solvent indicated applied at a positive pressure of 0.5 atm. Melting points were determined with a Kofler hot-stage instrument and are uncorrected. THF refers to anhydrous tetrahydrofuran.

Preparation of Esters of 4-Pyridylacetic Acid, 4–20 and 22–24. A stirred solution of the alcohol (11 mmol) in THF (10 mL) under N_2 was cooled with an ice–salt bath. *n*-BuLi (6.9 mL of a 1.6 M solution in hexane, 11 mmol) was added followed, after 5 min, by a solution of methyl 4-pyridylacetate (prepared in 76% yield by treatment of 4-pyridylacetic acid with excess MeOH containing HCl, but see also ref 20) (1.51 g, 10 mmol) in THF (3 mL), and the mixture then allowed to attain room temperature. After 4 h (primary or secondary alcohols) or 16 h (tertiary alcohols) the mixture was partitioned between Et_2O (2 × 40 mL) and H_2O (40 mol), and the ether layers were concentrated. Chromatography of the residue gave on elution with 50:50:1 light petroleum, bp 60–80 °C/Et_2O/NEt_3 the required ester, yield given in Table I. The esters gave δ_H ($CDCl_3$) inter alia signals in the ranges 3.57–3.67 (s, 2, CH_2), 4.61–5.06 (m, 1 or 2, OC*H* if present), 7.18–7.31 (d, *J* = 6.0–6.2 Hz, 2, pyridine ring), 8.49–8.58 (d, 2, pyridine ring). Those esters that were solid at room temperature (**10**, **11**, **14**, **15**) were recrystallized from cold light petroleum (bp 60–80 °C). Those esters that were oils were converted into the hydrochloride by passing HCl through an Et_2O solution. The solid hydrochlorides were recrystallized from dioxane or Et_2O–dioxane and finally sublimed at 100 °C (0.1 mmHg). Melting points are recorded in Table I.

Phenyl 4-Pyridylacetate (21). A mixture of 4-pyridylacetic acid hydrochloride (9.32 g, 53.7 mmol), phenol (6.06 g, 64.4 mol), and $(CF_3CO)_2O$ (9.86 mL, 69.8 mmol) in CH_2Cl_2 (100 mL) was stirred at ambient temperature. After 24 h, the mixture was poured into H_2O (300 mL) and Et_2O (300 mL) which was basified with K_2CO_3. On concentration of the Et_2O layer to 50 mL the trifluoroacetate salt of the phenyl ester 18 crystallized (8.39 g, 73% yield): ^{1}H NMR ($CDCl_3$) δ_H 4.11 (s, 2, CH_2), 7.06–7.11 (m, 2, *o*-Ph), 7.18–7.31 (m, 1, *p*-Ph), 7.37–7.44 (m, 2, *m*-Ph), 7.80 (d, *J* = 6.5 Hz, 2, pyridine), 8.81 (d, *J* = 6.5 Hz, 2, pyridine).

Cyclohexyl (*R*,*S*)-2-(4-Pyridyl)propanoate (25). A solution of 4 (732 mg, 3.34 mmol) in THF (3 mL) was added to a stirred suspension of KH (35% wt dispersion in oil, 383 mg, 3.34 mmol) in THF (10 mL) under N_2 at 0 °C. After 10 min, MeI (380 mg, 2.68 mmol) was added, and after 1 h at 20 °C, work up and chromatography as described for the preparation of the ester 4 gave **22** (492 mg, 63%) as an oil (^{1}H NMR ($CDCl_3$) δ_H 1.15–1.80 (m, 10, cyclohexyl), 1.49 (d, *J* = 7.1 Hz, 3, $CHCH_3$), 3.67 (q, *J* = 7.1 Hz, 1, $CHCH_3$), 4.76 (m, 1, OC*H*), 7.23 (d, *J* = 6.1 Hz, 2, pyridine), 8.54 (d, *J* = 6.1 Hz, 2, pyridine)) and was converted to the hydrochloride, mp 129–130 °C. Anal ($C_{14}H_{20}ClNO_2$) C, H, N, Cl.

Cyclohexyl 2-Methyl-2-(4-pyridyl)propanoate (26). A solution of 4 (639 mg, 2.92 mmol) in THF (2 mL) was added to a stirred suspension of KH (35% wt dispersion in oil, 736 mg, 6.42 mmol) in THF (6 mL) under N_2 at 0 °C. After 10 min, MeI (828 mg, 5.84 mmol) was added, and after 1 h at 20 °C, work up and chromatography as described for the preparation of the ester 4 gave **23** (587 mg, 82%) as an oil (^{1}H NMR ($CDCl_3$) δ_H 1.19–1.9 (m, 10, cyclohexyl), 1.56 (s, 6, CH_3), 4.78 (m, 1, OCH), 7.24 (d, *J* = 6.2 Hz, 2, pyridine), 8.54 (d, *J* 6.2 Hz, 2, pyridine)) and was converted into the hydrochloride, mp 161–163 °C. Anal ($C_{15}H_{22}ClNO_2$) C, H, N, Cl.

Enzyme Preparation and Assay Procedures for the Aromatase and CSCC Enzymes. The reagents and conditions for the assays of the CSCC and aromatase enzymes were those previously described.[21] The aromatase enzyme was obtained from the microsomal fraction of human placenta. Activity was monitored by measuring the ^3H_2O formed during the conversion of [1β,2β-^{3}H]-labeled androstenedione to estrogens. Activated charcoal followed by centrifugation was used to separate the ^3H_2O from the steroids. The k_m value for androstenedione was 0.038 μM, so the final substrate concentration in the assay was 0.38 μM. The mitochondrial fraction of bovine adrenal cortex provided the source of the CSCC enzyme. Activity was assayed by measuring the [^{14}C]isocaproic acid released from the substrate [26-^{14}C]-cholesterol. Chromatography on alumina was used to adsorb the steroid while the isocaproic acid filtered through the column. A final substrate concentration of 14 μM (k_m = 4.5 μM) was employed.

All assays were run in duplicate at 37 °C and samples were removed at 5-min intervals up to a total incubation time of 15 min to ensure linearity of product formation. The compounds to be tested were dissolved in dimethyl sulfoxide and added to the assay tubes to give 2% of the total volume. Dimethyl sulfoxide alone was added to control tubes. The IC_{50} value is the concentration of inhibitor required to reduce the enzyme activity to 50% of the control value at the final substrate concentration stated above. For the IC_{50} determinations, the rates of reaction were determined at five concentrations of each inhibitor and the data were fitted by linear regression to the Dixon equation.[22] The results are given as IC_{50} (the concentration of the compound required to inhibit the reaction by 50%) with 95% confidence limits.

17α-Hydroxylase/C_{17-20}Lyase Assay. This assay was essentially carried out by the reported procedure.[23] 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, 1 μM [^{3}H]progesterone in 50 μM sodium phosphate buffer pH 7.4. The reaction was carried out at 37 °C, started by the addition of the enzyme, and terminated by the addition of 2 volumes of MeCN–MeOH (1:2) containing unlabeled steroids ca. 100 μM. The reaction products were separated by HPLC as described.[23] For some assays the fractions of interest were collected and counted in a Packard Liquid Scintillation counter, while for the rest the effluent was mixed on-line 1:1 with Ecoscint A (National Diagnostics) and

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monitored continuously by a Berthold LB506C radiochemical detector fitted with a 1-mL flow cell. Hydroxylase activity was measured as the production of 17α-hydroxyprogesterone, androstenedione, and testosterone while lyase activity was measured as the production of androstenedione and testosterone alone.

All the compounds were tested at least four concentrations and the enzyme activities measured were fitted by linear regression to the Dixon equation.[22] The results are given as IC_{50} (the concentration of the compound required to inhibit the reaction by 50%) with 95% confidence limits.

The IC_{50} for 4 was estimated at several concentrations of progesterone in order to ascertain the nature of the enzyme inhibition.[22]

Comparative Hydrolysis Rates of 4, 25, and 26 by Hog Liver Esterase. Samples of hog liver esterase (2 μg, Boehringer) were preincubated for 2 min in 10 mM sodium borate buffer, pH 8.0 (0.9 mL) at 30 °C. Reactions were started in parallel by the addition of solutions of the esters (1 μmol) in the above buffer (0.1 mL). Aliquots (0.1 mL) were taken at intervals of 0, 5, 10, and 15 min and added to ice-cold CH_3CN (0.1 mL). Samples (0.05 mL) were analyzed by HPLC by using a 15-cm Apex octadecyl (5-μm) stationary phase and 10 mM sodium phosphate buffer (pH 6.8)/CH_3CN 1:1 as mobile phase at a flow rate of 1.5 mL min^{-1}. The pyridylacetic acids formed were detected by their UV absorption at 254 and 229 nm. No hydrolysis took place when the esters were incubated in the absence of enzyme. In the 15-min period, the ester 4 was hydrolyzed to an extent of 12% and graphs of amounts of the 4-pyridylacetic acid formed against time were essentially linear. Rates of hydrolysis of the esters expressed in nmol/min ± SEM (determined from regression analysis) were for 4, 7.3 ± 0.2; **25**, 8.2 ± 1.0; **26**, 1.3 ± 0.3.

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Inhibitors of the P450 Enzymes Aromatase and Lyase. Crystallographic and Molecular Modeling Studies Suggest Structural Features of Pyridylacetic Acid Derivatives Responsible for Differences in Enzyme Inhibitory Activity

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Derivatives of 4-pyridylacetic acid are known to be inhibitors of the cytochrome P450 enzymes aromatase and lyase (17α-hydroxylase/C_{17-20}lyase), and are therefore of interest in the treatment of hormone dependent breast and prostate cancers. We report the determination of the crystal structure of one such derivative, the 4-*tert*-butyl cyclohexyl ester, and molecular modeling studies on two related inhibitors, the cyclohexyl ester and its α-methyl derivative. These latter two compounds show a marked difference in their relative activities against aromatase and lyase. Two models are proposed for the interaction of these molecules with the target enzymes on the basis of their ability to adopt conformations that partially mimic steroid substrates. From these models an explanation can be advanced for the fact that, compared with the unmethylated analogue, the (racemic) α-methylated compound is seven times poorer as an inhibitor of aromatase but seven times better as an inhibitor of lyase. The model proposed for binding to aromatase places the α-carbon of the ester group in the position occupied by C(2) of steroid substrates. In contrast, that proposed for binding to lyase places this atom in the position occupied by C(17) of steroid substrates. The introduction of steric bulk at C(2) is known to be unfavorable for aromatase inhibition, while its introduction at C(17) may lead to a better mimicry of the steroid D-ring and so improve lyase inhibition.

Introduction

A proportion of breast tumors require the presence of estradiol for their growth. The reduction of estrogen levels therefore represents a desirable goal in the treatment of such cancers. One approach to this has been the use of inhibitors of the enzyme aromatase, which is responsible for the last step in the biosynthesis of the estrogens from the androgens.[1,2] Aromatase is a cytochrome P-450 enzyme, as are several other of the steroid-processing enzymes; one of the major problems that has been encountered in the design and use of aromatase inhibitors has been a lack of selectivity in their inhibitory action. For instance the aromatase inhibitor aminoglutethimide also inhibits the P-450 enzyme desmolase (cholesterol side chain cleavage enzyme); this enzyme is necessary for the synthesis of all steroid hormones and therefore corticosteroid replacement therapy must accompany its use.[3] However, inhibitors of other P-450 enzymes, if selective, are also of interest. For example inhibitors of the enzyme 17α-hydroxylase/C_{17-20}lyase, which is involved in the biosynthesis of the androgens from pregnanes, may be of use in the treatment of androgen-dependent prostate cancer.[1,4]

In the absence of an X-ray crystal structure for aromatase, studies of the natural substrates of the enzyme and of its steroidal and non-steroidal inhibitors have lead to the development of a model for the active site.[5]

Two features of this model, those utilized by the natural substrates, are a hydrophobic region that interacts with the steroid skeleton and a hydrogen-bond donor that interacts with an acceptor grouping in the C(3) position of the steroid. Two further features of the model are the heme and an additional hydrophobic pocket extending from the region occupied by C(4) of steroid substrates.

There is no direct interaction between the heme and natural substrates, as this site is required to bind the di-

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