Pyridyl-Substituted Tetrahydrocyclopropa[a]naphthalenes: Highly Active and Selective Inhibitors of P450 arom

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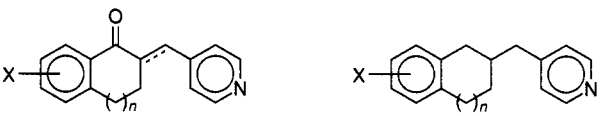
The synthesis and biological evaluation of substituted *exo*-1-(4-pyridyl)-1a,2,3,7b-tetrahydro-1*H*-cyclopropa[*a*]naphthalenes as inhibitors of estrogen biosynthesis is described [H (**1**); 4-OCH_3 (**2**); 5-OCH_3 (**3**); 6-OCH_3 (**4**); 1-CH_3, 6-OCH_3 (**5**); 4-OCH_3, 7-Br (**6**); 6-OCH_3, 5-Br (**7**); 4-OH (**8**); 5-OH (**9**); 6-OH (**10**)]. The synthetic key step—the formation of the cyclopropyl ring— was accomplished using the conditions of a modified Wolff–Kishner reduction (N_2H_5OH/KOH; ΔT) and yielded exclusively the exo-configurated diastereomers. The racemic compounds **1**–**10** showed an inhibition of human placental aromatase (P450 arom) exhibiting relative potencies (rp) from 3.7 to 303 (compounds **8** and **4**, respectively; rp of aminoglutethimide (AG) ≡ 1, fadrozole = 359). The enantiomers of **4** and **7** were separated by LPLC on tribenzoyl cellulose and by crystallization of the diastereomeric tartrates (**4**). (1a*S*,2*S*,7b*S*)-(+)-**4** (absolute configuration determined by X-ray crystallographic analysis) is the active P450 arom inhibiting enantiomer of **4** and shows a rp value of 617. Compound **4** is a reversible inhibitor showing a competitive type of inhibition and a type II difference spectrum. In vitro **4** influenced other steroidogenic P450 enzymes either not at all (bovine adrenal P450 scc) or only marginally (rat testicular P450 17, bovine adrenal P450 18). In ACTH-stimulated rat adrenal tissue, **4** was less active, inhibiting corticosterone and aldosterone formation compared to AG and fadrozole, respectively. In vivo **4** was not superior to AG as far as the inhibition of the uterotrophic activity of androstenedione (juvenile SD rats) and the reduction of the plasma estradiol concentration (pregnant mares' serum gonadotropin-primed SD rats) are concerned. Compound **4** shows marked antitumor activity in the dimethylbenzanthracene-induced mammary carcinoma of the SD rat: in the postmenopausal model it is at least as active as AG; in the premenopausal experiment it is clearly superior to AG. No induction of hepatic P450 enzymes was observed in the latter experiment. The rp value of **4** toward rat ovarian P450 arom, i.e., 23 (rp of AG ≡ 1), is markedly decreased compared to the human enzyme (rp value of 303). From this fact it must be concluded that **4** should be more active in the human than in the rat.

For almost two decades estrogen-dependent diseases such as breast cancer have been treated with estrogen antagonists like tamoxifen.[1] More recently the use of inhibitors of estrogen biosynthesis has been regarded as a promising alternative to antihormone treatment.[2] The P450 enzyme aromatase (P450 arom) is a very good target for drug (inhibitor) development because it catalyzes the last step in estrogen formation.[3,4] The only commercially available nonsteroidal inhibitor of P450 arom, aminoglutethimide (3-(4-aminophenyl)-3-ethylpiperidine-2,6-dione, AG) has been used for more than 10 years.[2] AG is a second-line drug because inhibition of P450 arom is moderate and selectivity is low,[2] i.e., it also inhibits other steroidogenic P450 enzymes leading to a depletion of glucocorticoid production.[2,5] For several years different groups have been concerned with the development of more active and selective inhibitors of P450 arom. Very recently, 4-hydroxyandrostenedione (formestane)[6,7] has been admitted to the market. Compared to AG the development of this steroidal compound is a remarkable step forward.[2] However, one has to keep in mind that formestane shows some disadvantages as well, such as the im route of application as well as additional effects which are typical of androgens, like the reduction of sex hormone binding globulin.[8] Several nonsteroidal compounds have been shown to strongly inhibit P450 arom,[9–26] some of which (fadrozole, vorozole, letrozole, and anastrozole) are presently in clinical evaluation.[2,27] But they are not void of adverse effects either, e.g., fadrozole is known to inhibit aldosterone plasma concentration at a dose of 0.6 mg/day.[28] In the classes of 4-(pyridylmethylene)- and 4-(pyridylmethyl)-substituted benzocycloalken-1-ones (Chart 1, type A) and 4-(pyridylmethyl)-substituted benzocycloalkenes (type B), we recently found very interesting compounds.[17,26,29] Depending on the type and position of substitutent on the benzene nucleus they are highly active and selective inhibitors of P450 arom.

Chart 1



The present paper describes further structural optimizations in this class, namely the development of pyridyl-substituted tetrahydrocyclopropanaphthalenes (Chart 2). The syntheses and structure–activity studies regarding in vitro activity (inhibition of P450 arom) of

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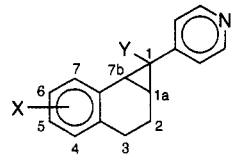
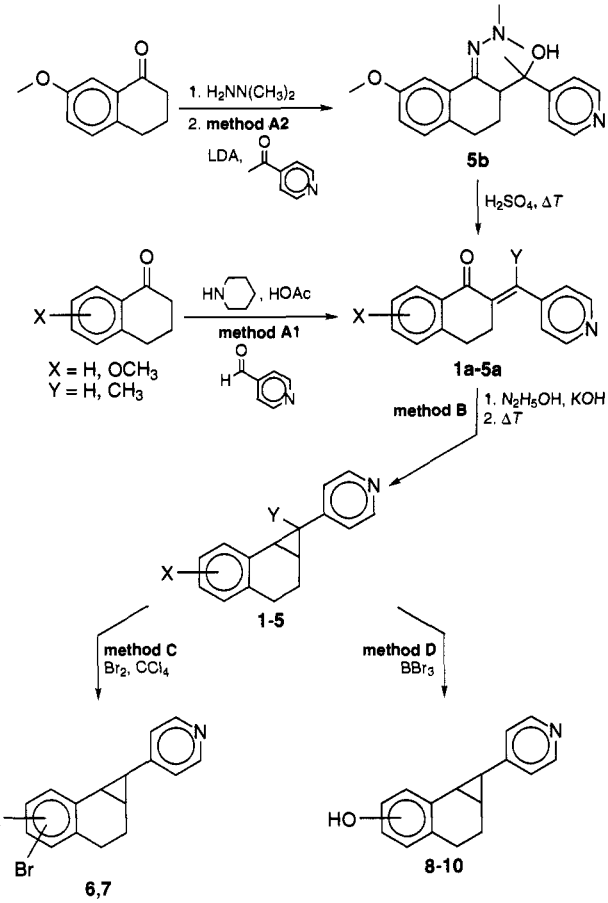
Chart 2



compd	X	Y	compd	X	Y
1	H	H	6	4-OCH_3, 7-Br	H
2	4-OCH_3	H	7	6-OCH_3, 5-Br	H
3	5-OCH_3	H	8	4-OH	H
4	6-OCH_3	H	9	5-OH	H
5	6-OCH_3	CH_3	10	6-OH	H

Scheme 1



compounds **1**–**10** will be described in this paper. Select compounds are further evaluated for selectivity (inhibition of P450 scc, P450 17, P450 18, corticoid formation, hepatic P450 enzymes) as well as in vivo efficacy (antiuterotrophic, estradiol plasma concentration reducing, and antitumor activity).

Chemistry

The starting materials for the syntheses of compounds **1**–**10** were 1-tetralone and the OCH_3-substituted 1-tetralones. The *E*-configurated pyridylmethylene compounds **1a**–**4a**[17] were obtained by aldol condensation of the corresponding tetralones with pyridine-4-carboxaldehyde using piperidine/acetic acid as recently described[17] (method A1, Scheme 1). As this reaction failed with 4-acetylpyridine as did a procedure using H_2SO_4 (54%) as catalyst, compound **5a** was synthesized starting from the *N*,*N*-dimethylhydrazone of 4-methoxy-1-tetralone, compound **5c**. Reaction of **5c** with LDA and 4-acetylpyridine (method A2, Scheme 1) led to the aldol compound **5b**, a mixture of two diastereomers. From the latter the 1-(4-pyridyl)ethylidene-substituted tetralone **5a** was synthesized by reaction with H_2SO_4 (54%) at 80 °C. Only one isomer was obtained in this reaction. ^{1}H-NMR studies (NOE experiments) revealed that the *E*-configuration has to be assigned to **5a**, as was also shown for **1a**–**4a**.[17]

The formation of the cyclopropane compounds **1**–**5** (Table 1) was accomplished by reaction of the tetralones **1a**–**5a** with N_2H_5OH/KOH in di(ethylene glycol) and subsequent heating at 200 °C (conditions of a modified Wolff–Kishner reduction; method B, Scheme 1), a reaction which has already been performed with 2-benzylidene-1-tetralone.[30] Intermediates are the corresponding hydrazones and probably the subsequently formed pyrazolines (3-substituted 4,5-dihydronaphtho-[1,2-*c*]pyrazolines). Interestingly, the identical reaction using correspondingly substituted indanone derivatives results in a mixture of 2-(pyridylmethylene)-substituted indans and 2-(pyridylmethyl)-substituted indenes (Hartmann and Bayer, unpublished results).

Only one of the two possible diastereomers, the *exo*-configurated compound (pyridyl substituent *exo* corresponding to the tetrahydronaphthalene moiety), was obtained. The configuration of compounds **1**–**5** was determined by ^{1}H- and ^{13}C-NMR spectroscopy (compounds **1**–**5** and compound **1**, respectively) as well as X-ray analysis (compound **1**).[31]

Bromination of the 4-OCH_3 and 6-OCH_3 compounds **2** and **4** was accomplished by reaction with Br_2/CCl_4 and yielded the 7-bromo as well as the 5-bromo compounds **6** and **7**, respectively (method C, Scheme 1, Table 1). The structures of the latter compounds, i.e., the positions of the bromo substituents, were assigned by ^{1}H-NMR spectroscopy. Ether cleavage of the OCH_3-substituted compounds **2**–**4** was performed with BBr_3 (method D, Scheme 1) yielding the hydroxy compounds **8**–**10** (Table 1).

The separation of the enantiomers of **4** was successfully performed by LPLC on the optically active sorbens tribenzoyl cellulose (method E, Table 1, Figure 1). Several injections gave milligram quantities of the enantiomerically pure compounds. The antipodes of compound **7** (Table 1) were prepared identically.

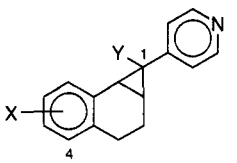
For the preparation of larger amounts of the enantiomers of **4**, a crystallization procedure was developed using (2*R*,3*R*)-(−)-dibenzoyltartaric acid (method F, Table 1). The levorotatory enantiomer (−)-**4** was obtained by repeated crystallization of the diastereomeric salts of **4** and the chiral acid (1:1) from ethanol. Compound (+)-**4** was prepared by repeated crystallization from acetonitrile of the salt obtained from the mother liquor of the aforementioned procedure.

The absolute stereochemistry of the cyclopropane moiety was determined by X-ray analysis of the (2*R*,3*R*)-(−)-dibenzoyltartrate of compound (−)-**4** (Figure 2).

Biological Properties

Inhibition of P450 arom in Vitro. The inhibitory activities of the compounds toward P450 arom were determined in vitro using human placental microsomes and [1β,2β-^{3}H]testosterone according to the method of

Table 1. 4-Pyridyl-Substituted Tetrahydrocyclopropanaphthalenes 1–10



compd	X	Y	mp, °C	formula[a]	method of prepn	recryst solvent	yield, %
1	H	H	79–80	$C_{16}H_{15}N$	B	*n*-hexane	60
2	4-OCH_3	H	96.5–97.5	$C_{17}H_{17}NO$	B	*n*-hexane	64
3	5-OCH_3	H	76–77	$C_{17}H_{17}NO$	B	*n*-hexane	52
4	6-OCH_3	H	96–97	$C_{17}H_{17}NO$	B	*n*-hexane	63
(+)-**4**	6-OCH_3	H	116.5–117.5	$C_{17}H_{17}NO$	E/F	*n*-hexane	25
(−)-**4**	6-OCH_3	H	117–117.5	$C_{17}H_{17}NO$	E/F	*n*-hexane	33
5	6-OCH_3	CH_3	92	$C_{18}H_{19}NO$	B	*n*-hexane	68
6	4-OCH_3, 7 Br	H	127	$C_{17}H_{16}NO$ Br	C	*n*-hexane	68
7	6-OCH_3, 5 Br	H	122–123	$C_{17}H_{16}NO$ Br	C	*n*-hexane	42
(+)-**7**	6-OCH_3, 5 Br	H	109.5–111	$C_{17}H_{16}NO$ Br	E		50
(−)-**7**	6-OCH_3, 5 Br	H	110–112.5	$C_{17}H_{16}NO$ Br	E		50
8	4-OH	H	>220	$C_{16}H_{15}NO$	D	EtOH	56
9	5-OH	H	207–208	$C_{16}H_{15}NO$	D	EtOH/H_2O	54
10	6-OH	H	>220	$C_{16}H_{15}NO$	D	EtOH/H_2O	63

[a] C, H, and N analyses were within ±0.4% of the theoretical values.

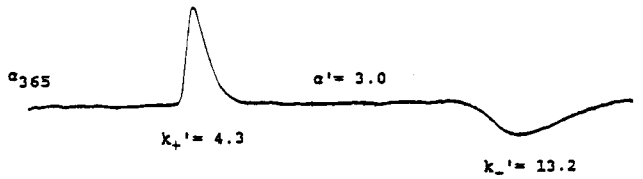


Figure 1. Separation of (+)- and (−)-**4** by LPLC. Polarimetric detection α_{365}; tribenzoylcellulose; MeOH. k': capacity factor of the dextrorotatory resp. levorotatory compound; ($k' = (t_i - t_o)/t_o$ with t_i = retention time and t_o = dead time). α': relative retention; $\alpha' = k'_-/k'_+$.

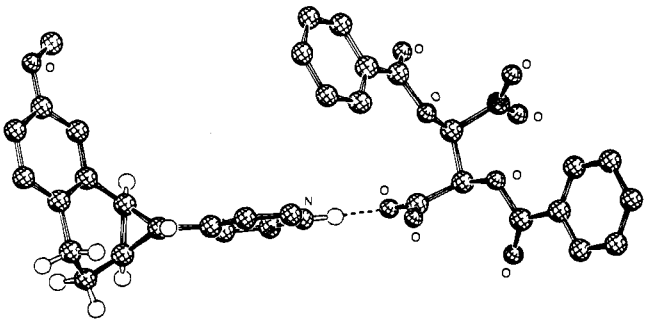


Figure 2. X-ray crystal structure of the (2*R*,3*R*)-(−)-dibenzoyltartrate of compound (1a*R*,1*R*,7b*R*)-*exo*-6-methoxy-1-(4-pyridyl)-1a,2,3,7b-tetrahydro-1*H*-cyclopropa[*a*]naphthalene.

Thompson and Siiteri.[32] The IC_{50} values and the potencies of the compounds, relative to AG, are given in Table 2.

Showing a relative potency (rp) of 100, the unsubstituted compound **1** is highly active. It exceeds its noncyclic analogue by a factor of 3 (rp value of 2-(4-pyridylmethyl)tetralin = 32[26]). As observed with the 2-(pyridylmethyl)tetralins, OCH_3 and OH substituents also strongly influence the activity of the cyclopropyl compounds, depending on their location at the benzene nucleus. In the 4-position, they strongly decrease activity (rp values of compounds **2** and **8**: 5.5 and 3.7, respectively). Located in the 5-position, the OCH_3 group increases activity, whereas the OH substituent decreases activity (rp values of compounds **3** and **9**: 268 and 56, respectively). In the case of the 6-position, both substituents strongly enhance activity (rp values of

Table 2. Inhibition of Human Placental Aromatase by Pyridyl-Substituted Tetrahydrocyclopropanaphthalenes 1–10

compd	X	Y	IC_{50},[a] μM	rp[b]
1	H	H	0.185	100
2	4-OCH_3	H	3.35	5.5
3	5-OCH_3	H	0.069	268
4	6-OCH_3	H	0.061	303
(+)-**4**	6-OCH_3	H	0.030	617
(−)-**4**	6-OCH_3	H	10	1.9
5	6-OCH_3	CH_3	0.37	50
6[c]	4-OCH_3, 7-Br	H	4.3	4.3
7	6-OCH_3, 5-Br	H	1.4	13
(+)-**7**	6-OCH_3, 5-Br	H	0.92	20
(−)-**7**	6-OCH_3, 5-Br	H	2.8	6.6
8	4-OH	H	5.0	3.7
9	5-OH	H	0.33	56
10	6-OH	H	0.066	280

[a] IC_{50} is the concentration of inhibitor required to give 50% inhibition. Concentration of testosterone: 2.5 μM. The given values are mean values of at least three experiments. The deviations were within ±5%. [b] Relative potency, calculated from the IC_{50} values and related to AG (IC_{50} of AG: 18.5 μM). [c] Tested as hydrobromide.

compounds **4** and **10**: 303 and 280, respectively). Compound **4** is the most active substance of this series. The activity of the racemic mixture is due to the dextrorotatory, (1a*S*,1*S*,7b*S*)-configurated enantiomer (+)-**4**, which exhibits a relative potency of 617, resulting in a compound which is similarly active as two recently developed inhibitors of P450 arom: fadrozole[18] and vorozole.[14,33] These compounds are presently in clinical evaluation[34,35] and have shown in our hands relative potencies of 359 and 395 (racemate), respectively. Compared to the (+)-enantiomer, the levorotatory enantiomer (−)-**4** is almost inactive; compared to the reference compound AG, however, (−)-**4** is still almost twice as active.

The exchange of the endo configurated hydrogen of the cyclopropyl moiety by a CH_3 group reduces aromatase inhibitory activity markedly (compound **5**, rp value of 50 compared to **4**, 303).

The same result is observed after introduction of a Br substituent in the 5-position of compound **4**, i.e., ortho to the 6-OCH_3 group (compound **7**, rp value of 13 compared to **4**, 303).

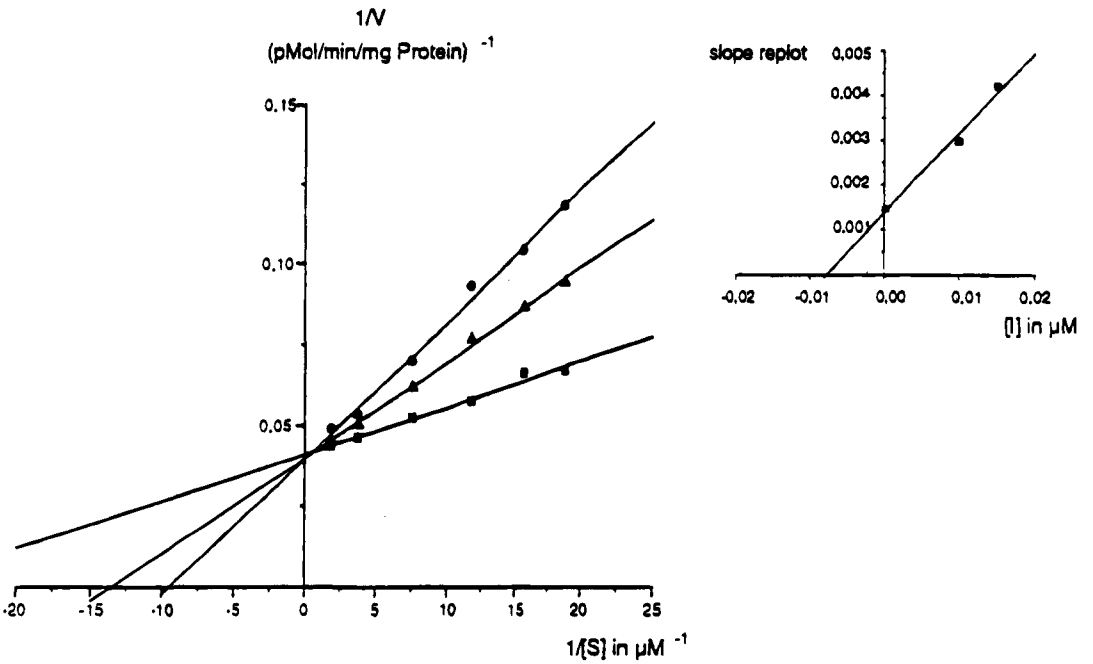


Figure 3. Lineweaver–Burk plot and slope replot of compound **4**. Substrate: testosterone; ■, control; ▲, 0.01 μM; ●, 0.015 μM (K_m value: 36 nM, K_i = 8 nM).

Interestingly the differences in activity between the enantiomers of **7** are less distinct as they are in compound **4** (rp value of (+)-**7** and (−)-**7**: 20 and 6.6, respectively).

The introduction of a bromine into the 7-position of the 4-OCH_3 compound **2** influences aromatase inhibition only marginally (rp values of **6** and **2**: 4.3 and 5.5, respectively).

As irreversible inhibition of aromatase is shown by some steroidal compounds, two select compounds (**4** and **10**) were examined for irreversible inhibition using the method of Brodie et al.[36] with modifications.[10] Enzyme activity was determined after incubation of human placental microsomes with NADPH and inhibitor (10 μM) for 30 min followed by treatment with dextran-coated charcoal. None of the compounds caused a reduction of enzyme activity (data not shown).

For getting a further insight into the mode of aromatase inhibition for this type of compounds, Lineweaver–Burk and difference spectroscopy experiments were performed with compound **4**. A competitive inhibition vs testosterone was observed (Figure 3). The linear slope replot indicates that there is only one specific binding site for the inhibitor at the enzyme.[37] The difference spectrum exhibiting a minimum at 395 nm and a maximum at 425 nm (type II difference spectrum; Figure 4) is typical of the interaction of the pyridyl N with the central iron ion of the cytochrome P450 component.[38]

Inhibition of P450 scc in Vitro. The inhibition of compounds **1**–**4**, **9**, and **10** toward P450 scc was determined in vitro using bovine adrenal mitochondria and [26-^{14}C]cholesterol according to a described procedure.[39] The compounds were tested in a relatively high concentration of 25 μM. In contrast to the reference compound AG, which inhibited the enzyme by 53%, the tested tetrahydrocyclopropanaphthalenes showed no inhibition of the enzyme (data not shown).

Inhibition of P450 17 in Vitro. Rat testicular microsomes were used as source of the enzyme, and nonlabeled progesterone served as substrate. The separation of the steroids was accomplished using the procedure recently described by us.[40] The inhibition values of select compounds are listed in Table 3. A very high concentration of 125 μM is necessary to achieve a marked effect. In spite of the structural differences of the test compounds, the inhibition values do not strongly vary from the range of 36–68%. While (+)-**4** shows an inhibition of 54%, its antipode (−)-**4** is almost as active

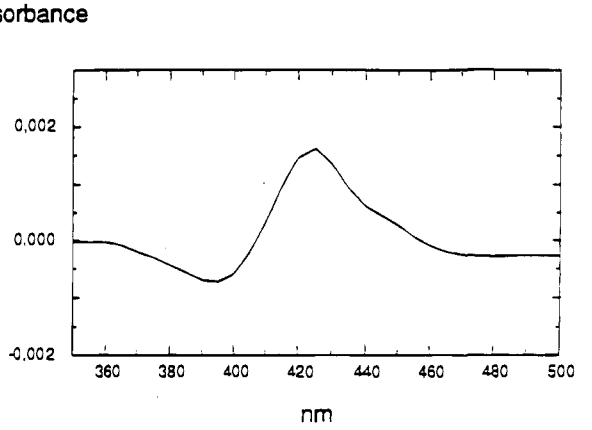


Figure 4. Difference spectrum obtained by the addition of compound **4** to solubilized high-spin aromatase (testosterone, 5 μM).

Table 3. Inhibition of Rat Testicular P_{450} 17 by Select Pyridyl-Substituted Tetrahydrocyclopropanaphthalenes

compd	X	Y	% inhibition[a]
1	H	H	36
2	4-OCH_3	H	51
4	6-OCH_3	H	47
(+)-**4**	6-OCH_3	H	54
(−)-**4**	6-OCH_3	H	45
5	6-OCH_3	CH_3	68
9	5-OH	H	41

[a] Concentration of inhibitor, 125 μM. Concentration of progesterone, 25 μM. Under identical experimental conditions ketoconazole caused an inhibition of 65%. All values are the mean of at least two determinations (deviations within ±5%).

Table 4. Inhibition of Corticosterone and Aldosterone Formation by Select Pyridyl-Substituted Tetrahydrocyclopropanaphthalenes[a]

compd	X	Y	IC_{50}, μM[c] corticosterone[b]	IC_{50}, μM[c] aldosterone[b]
1	H	H	nd	3
4	6-OCH_3	H	75	15
(+)-**4**	6-OCH_3	H	95	6
(−)-**4**	6-OCH_3	H	30	10
10	6-OH	H	76	7
AG			50	80

[a] In vitro, rat adrenal slices; stimulation by ACTH 1−24 (0.1 μg/mL) as well as a potassium containing buffer; incubation: 2 h, 37 °C. [b] Determination by RIA. [c] IC_{50} is the concentration required to give 50% inhibition.

inhibiting the enzyme by 45%. The absence of enantioselectivity is typical for poorly active compounds.[41]

Inhibition of Corticoid Formation in Vitro. The effects of select tetrahydrocyclopropanaphthalenes on corticosterone and aldosterone biosynthesis are shown in Table 4. The method developed by Häusler et al.[42] was applied using ACTH-stimulated rat adrenal fragments in potassium-containing buffer.

As can be seen from the IC_{50} values, the levorotatory compound (−)-**4**, the nonactive enantiomer regarding aromatase inhibition, is more active than its antipode and shows a stronger inhibition of corticosterone production compared to AG (IC_{50} values of 30 and 50 μM, respectively). Compounds **4**, (+)-**4**, and **10**, however, inhibited corticosterone formation to a less extend than did AG (IC_{50} values 75, 95, and 76 μM). They are about equiactive to fadrozole and vorozole, which in our hands inhibited corticosterone formation with IC_{50} values of 80 and 95 (racemate) μM, respectively.[21]

The tetrahydrocyclopropanaphthalenes are stronger inhibitors of aldosterone formation than AG (IC_{50} values between 3 and 15 μM as compared to 80 μM). They are approximately equiactive to vorozole (IC_{50} value of 12 μM as racemate[21]) but more favorable compared to fadrozole (IC_{50} value of 0.6 μM[21]). The marked inhibition of the aldosterone formation in adrenal slices by aromatase inhibitors is due to inhibition of 18-hydroxylase (P450 18).

For further examination, bovine adrenal mitochondria as a source of P450 18 and corticosterone as substrate were used. After termination of the incubation, a HPLC procedure and UV detection were applied for separation and quantification of the steroids. The inhibition values obtained with select tetrahydrocyclopropanaphthalenes and fadrozole correspond to the data of the organ culture experiment (inhibitor concentration 1 μM; percent inhibition of P450 18 by **1**, 55; **2**, 38; **4**, 45; **9**, 36; and fadrozole, 80).

Inhibition of Hepatic P450 Enzymes in Vitro. As inhibition of hepatic P450 enzymes can influence metabolism of endogenous and exogenous compounds, the effect of **4** was determined using rat hepatic microsomes as source of the enzymes[43] and *p*-nitroanisol and aminopyrine as substrates. Compound **4** showed a stronger inhibition of O- as well as N-demethylase compared to AG (inhibitor concentration 150 μM; percent inhibition of O-demethylase (*p*-nitroanisol) by **4**, 92, and AG, 12, as well as N-demethylase (aminopyrine) by **4**, 96, and AG, 40).

Inhibition of P450 arom in Vivo. In order to elucidate the in vivo aromatase inhibitory activity, select compounds were further tested. In juvenile female rats, androstenedione treatment stimulates uterine weight strongly. This effect is caused by ovarian aromatization of the androgen and can be dose-dependently antagonized by aromatase inhibitors.[44] Table 5 shows the antiuterotrophic effects of the tetrahydrocyclopropanaphthalenes **1**, **4**, **5**, and **7**. Compounds **5** and **7** turned out to be inactive in this in vivo model. Compounds **1** and **4**, however, showed a marked activity. They inhibited uterine growth by 68 and 61%, respectively, and thus are equipotent to AG, which showed 73% inhibition of the androstenedione-induced aromatase-mediated uterotrophic effect.[26]

Table 5. Effect of Select Pyridyl-Substituted Tetrahydrocyclopropanaphthalenes on the Androgen-Stimulated Uterine Growth[a]

treatment group	effect,[b] means ± SD	% inhibition
control	88 ± 32	
androstenedione[c]	202 ± 42	
1[d]	124 ± 23[e,f]	68
4[d]	132 ± 11[e,g]	61
control	76 ± 14	
androstenedione[c]	138 ± 12	
5[d]	139 ± 18	−1
control	80 ± 13	
androstenedione[c]	187 ± 8	
7[d]	176 ± 20	10

[a] Immature female SD rats. [b] Uterus wet weight (mg)/body weight (g) × 100. [c] 30 mg/kg. [d] 0.043 mmol/kg + androstenedione 30 mg/kg. [e] Significantly different from androgen stimulated control (Student's *t*-test). [f] $p < 0.01$. [g] $p < 0.05$.

The reduction of plasma estradiol (E_2) level of compounds **4** and **10** was determined using pregnant mares' serum gonadotropin (PMSG)-stimulated female rats according to the procedure of Brodie et al.[45] The E_2 concentration lowering effect of the compounds was determined 1 and 6 h after subcutaneous application of a single dose of inhibitor (8.61 μmol/kg) using conventional radioimmunoassay technique. After 1 h the effect of compounds **4** and **10** was similar to that of AG (**4**, 43% inhibition vs untreated control; **10**, 36%; AG, 37%; all significant ($p < 0.05$), $n = 5-7$, Student's *t* test). After 6 h, however, the new compounds showed no inhibitory activity in contrast to AG (**4**, 16%; **10**, 22%; AG, 53%).

Antitumor Activity. The antitumor activity of compounds **1** and **4** was determined using the dimethylbenzanthracene (DMBA)-induced mammary carcinoma of the SD rat.[46] This experimental breast tumor was used in two modifications: in a postmenopausal and in the standard premenopausal arrangement. The postmenopausal model makes use of ovariectomized, testosterone propionate treated, tumor bearing rats.[47] It mimics the endocrine situation of a postmenopausal or ovariectomized woman. As can be seen from the percent change of tumor area in Table 6a, the ovariectomy-induced regression can be fully overcome by the administration of testosterone propionate. This stimulation of tumor growth is due to aromatization in peripheral tissues[47] and consequently can be dose-dependently inhibited by aromatase inhibitors.[47]

As can be seen from Table 6a, compounds **1** and **4** show a strong antitumor effect. They are at least equieffective to AG, which was tested in equimolar dosage under identical conditions[26] (percent change of tumor area: ov, −96; ov + tp, 141; ov + tp + AG, 4).

Table 6. Effect of Select Pyridyl-Substituted Tetrahydrocyclopropanaphthalenes on the DMBA-Induced, Hormone-Dependent Mammary Carcinoma of the SD Rat: (a) Postmenopausal and (b) Premenopausal Model

treatment group[a]	no. of tumors		% of tumors with				% change of tumor area[h]
	B[b]	NT[c]	CR[d]	PR[e]	NC[f]	P[g]	
(a) Postmenopausal Model							
ov	28	0	82	18	0	0	−96
ov + tp	36	12	19	25	19	36	+92
ov + tp + **1**	32	1	47	28	16	9	−36[i]
ov + tp + **4**	33	5	52	15	15	18	−29[i]
(b) Premenopausal Model							
control	17	11	0	4	32	57	+169
4	25	10	0	20	43	37	+55[k]

[a] Each treatment group consisted of 8−10 animals; ov = ovariectomy, tp = testosterone propionate; the animals received a single dose (tp = 23.9 mg/kg; **1**, 9.5 mg/kg; **4**, 10.8 mg/kg) daily from Monday to Thursday, and a double dose on Friday, sc, as solution in olive oil. [b] At the beginning of the test. [c] Occurring during the test. [d] CR = complete remission, tumor not palpable. [e] PR = partial remission, reduction of initial tumor size ≥50%. [f] NC = no change, tumor size 51−150% of initial tumor size. [g] P = progression, tumor size >150% of initial tumor size. [h] Average on the 28th day of therapy. [i] Significantly different from tp-treated control group, $p < 0.025$, U test according to Wilcoxon, Mann, and Whitney. [k] Significantly different from control group, $p < 0.05$, Student's t-test.

In the conventional premenopausal model, the rats have intact ovaries which continuously stimulate tumor growth by estrogen formation. Inhibition of estrogen production leads via the hypothalamic pituitary feedback mechanism to a gonadotropin stimulation of the ovaries. Therefore, it is more difficult to treat intact, tumor-bearings rats. Nevertheless, compound **4** shows a strong antitumor effect (Table 6b). It is much more active than AG, which was tested under identical conditions at a five fold higher dosage[10] (percent change of tumor area: control, 298; AG, 50 mg/kg, 199).

As AG is known to induce hepatic P450 enzymes in the treatment of human breast cancer leading to an acceleration of its own metabolism[48]—one of several disadvantages of AG as a drug—we were interested in the question whether compound **4** shows some stimulatory effect in the premenopausal rat tumor test. Therefore, at the end of treatment, the V_{max} values of *p*-nitroanisol-O-demethylase and aminopyrine-N-demethylase were determined in liver microsomes. No significant difference was observed (control group, 1.04 ± 0.03, O-demethylase and 3.97 ± 0.40, N-demethylase; compound **4**, 1.01 ± 0.06 and 3.20 ± 0.12, Student's t-test, $p > 0.005$). This means that in this test compound **4** shows no induction of hepatic P450 enzymes.

With the exception of the premenopausal tumor experiment, the tested compounds were not stronger in activity in the rat models compared to AG, in spite of the fact that they were much more active inhibitors of human P450 arom in vitro. It is well-known that there are differences in the amino acid sequences of P450 arom from different species,[49] and this might lead to differing inhibitory activities of certain inhibitors. Using a microsomal P450 arom preparation from rat ovaries and applying the same experimental procedure as we did with the preparation from human placental origin, compound **4** showed an IC_{50} value of 0.265 μM and a rp value of 23 (IC_{50} of AG = 6.2 μM). This means that compound **4** is by a factor of 13 less active an inhibitor of rat P450 arom compared to the human enzyme (IC_{50} = 0.061 μM, rp = 303, see Table 2).

Discussion

The present study shows that aromatase inhibitory potencies of the 2-(4-pyridylmethyl)-substituted benzocycloalkenes and -alkenones[17,26] can be further enhanced by cyclopropyl formation. The most active compound of this study is (+)-**4**, showing an inhibitory potency in vitro of about 3 times higher than was observed for (+)-5-hydroxy-2-(4-pyridylmethyl)-1-tetralone (rp values 617 vs 213[17]). The difference spectroscopy and enzyme kinetic experiments described in this paper demonstrate that the inhibition of P450 arom is caused (a) by complex formation of the pyridyl N with the central iron ion of the heme and (b) by interaction with the substrate binding site at the apoprotein moiety of the enzyme. Thus compound **4** exhibits an identical mode of inhibition compared to the well-known P450 arom inhibitors of the azole type, i.e., fadrozole (f), vorozole (v), letrozole (l), and anastrozole (a), which are presently under clinical evaluation. It is striking, however, that there are marked structural differences between the title pyridyl-substituted compounds and the azole-type inhibitors, which are imidazolyl- (f) or triazolyl- (v, l, a) substituted diarylmethanes (f, a) or triarylmethanes (v, l). While the azole compounds bear chlorine or CN substituents at the benzene ring, the benzocycloalkenes are unique with respect to the OH or OCH_3 substituents which strongly influence aromatase inhibitory activity depending on their location at the benzene nucleus. The latter compounds therefore are valuable scientific tools for the elucidation of the topography of the active site of P450 arom. First molecular modeling experiments support the hypothesis that the OH or OCH_3 groups of the inhibitors interact as hydrogen bond acceptors with the 17β OH or 17 keto binding site of the steroidal substrates in the enzyme (Hartmann and Bartz, unpublished results).

For clinical use, the selectivity of enzyme inhibition is, beside inhibitory potency, another important criterion. Compound **4** does not inhibit P450 scc and only marginally influences the other steroidogenic P450 key enzymes, i.e., P450 17 and P450 18. With respect to inhibition of aldosterone formation it is superior to fadrozole. Bearing in mind the high in vitro aromatase inhibitory efficacy, however, the in vivo activity of **4** in the rat experiments was somewhat disappointing. With the exception of the premenopausal tumor model, which was inhibited by **4** stronger than by AG, compound **4** was not or only slightly superior to the commercially available compound. An unsatisfactory uptake into intact cells as a reason for the lack of in vivo activity is very unlikely because using human preadipocyte cell culture experiments compound **4** was shown to be 298 times more active than AG (Löffler, unpublished results). A possible explanation for the low in vivo activity in the rat is the finding that **4** inhibits rat ovarian P450 arom by a factor of 13 (!) less than it inhibits the human enzyme. Thus one can speculate that **4** might be much more active in the human than in the rat. First studies on the metabolism of compound **4** in the rat demonstrate an extensive hydroxylation at the aliphatic moiety of the enzyme (Schröder, unpublished results).

Experimental Section

Melting points were determined on a Büchi 510 apparatus or on a Kofler melting point apparatus Thermopan (Reichert) and are uncorrected. Elemental analyses were performed by

the Mikroanalytischen Laboratorien, University of Regensburg and University of the Saarland, and were within ±0.4% of the calculated values. ^{1}H-NMR spectra were measured on a Varian EM 360L (60 MHz) or Bruker AW 80 (80 MHz), WM 250 (250 MHz), AC 300 (300 MHz), and AM 400 (400 MHz) spectrometers. ^{13}C spectra were measured on a Bruker WH 90 (22.64 MHz) spectrometer. LPLC-equipment, Model Duramat pump, Chemie und Filter; detector, Perkin-Elmer PE 241 polarimeter. Column chromatography was performed on Merck Kieselgel 60 and on Macherey und Nagel Kieselgel MN 6; IR spectra were performed on a Beckman Acculab 7 spectrometer and a Perkin-Elmer PE 398 spectrometer.

Method A1. General Procedure for the Synthesis of the (*E*)-2-(4-Pyridylmethylene)-1-tetralones 1a–4a. A mixture of piperidine (2.00 g, 23.5 mmol), acetic acid (2.00 g, 33.3 mmol), 4-pyridinecarboxaldehyde (16.1 g, 150 mmol), and the corresponding 1-tetralones (100 mmol) was heated at 130 °C for 1.5 h. After removal of the lower boiling materials under reduced pressure at temperatures not exceeding 130 °C, the residue was treated with dichloromethane and extracted with 2 N HCl. The aqueous layer was neutralized with saturated $NaHCO_3$ solution, and the crude product was collected, washed with water, and dried in vacuo. Recrystallization from a suitable solvent was carried out in the absence of light and yielded compounds **1a–4a**.

7-Methoxy-1-tetralone Dimethylhydrazone (5c). A mixture of 7-methoxy-1-tetralone (5 g, 28 mmol), *asym-N,N*-dimethylhydrazine (6 mL, 80 mmol), *p*-toluenesulfonic acid (50 mg), and benzene (50 mL) was heated under a Dean–Stark trap until no more water was deposited. The solvent was evaporated, and the crude product was purified by bulb tube distillation (p < 1 Torr) to give 5.5 g (90%) of **5c**: mp 35–36 °C; ^{1}H-NMR (80 MHz, $CDCl_3$) δ 1.70–2.00 (m, 2H, CH_2), 2.30–2.50 (m, 10H, CH_2 and $N(CH_3)_2$), 3.81 (s, 3H, OCH_3), 6.77–7.68 (m, 3H, arom H); IR (KBr) 3050, 2940, 2820, 2770.

Method A2. Synthesis of 2-[1-Hydroxy-1-(4-pyridyl)-ethyl]-1-tetralone Dimethylhydrazone 5b. Diisopropylamine (1.6 g, 15.8 mmol) was dissolved in dry THF (10 mL). After the mixture was cooled to −78 °C, butyllithium (10 mL, 15.8 mmol) was added and the solution was stirred for 15 min. The dimethylhydrazone **5c** (3 g, 15.7 mmol) dissolved in dry THF (30 mL) was added dropwise to the mixture at room temperature, and stirring was continued for 1 h. After the mixture was cooled to −78 °C, a solution of 4-acetylpyridine (1.5 g, 13.7 mmol) in dry THF (10 mL) was added dropwise to the mixture and stirring was continued overnight. The solution was hydrolyzed with acetic acid (1 g, 13.7 mmol) in dichloromethane and partitioned between dichloromethane and water. The aqueous layer was separated and extracted with dichloromethane. The combined organic extracts were dried, and the solvent was evaporated to yield **5b** (3.1 g, 65%) as a 2:1 mixture of two diastereomers. The product was converted to **5a** without further purification: ^{1}H-NMR (80 MHz, DMSO) δ 1.40 and 1.45 (2s, 3H, CH_3, diastereomers), 1.80–2.90 (m, 5H, CH_2CH_2CH), 2.30 and 2.41 (2s, 6H, $N(CH_3)_2$, diastereomers), 3.70 (s, 3H, OCH_3), 5.50 (s, 1H, br, OH), 6.90–7.43 (m, 5H, arom H and pyridyl H), 8.40–8.60 (m, 2H, pyridyl H).

(*E*)-7-Methoxy-2-(4-pyridylethylidene)-1-tetralone (5a). **5b** (2.8 g, 8.3 mmol) was dissolved in sulfuric acid (22 mL, 54%), and the solution was heated at 80 °C for 1 h. The mixture was poured onto ice water and extracted with ether. After neutralization with 10% aqueous NaOH, the reaction solution was extracted with dichloromethane. The organic extracts were dried (Na_2SO_4) and treated with activated charcoal. Evaporation of the solvent gave the crude product which was purified by recrystallization from ethyl acetate (700 mg, 31%): mp 131–132 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.17 (s, 3H, CH_3), 2.97 (t, 2H, CH_2, J = 6 Hz), 3.04 (t, 2H, CH_2, J = 6 Hz), 3.77 (s, 3H, OCH_3), 7.02–7.06 (m, 3H, arom H-6 and pyridyl-H), 7.18 (d, 1H, arom H-5, J_o = 8.4 Hz), 7.40 (d, 1H, arom H-8, J_m = 2.8 Hz), 8.56 (dd, 2H, AA'XX', pyridyl H, J_o = 6 Hz, J_m = 1.5 Hz); IR (KBr) 3050, 2950, 2840, 1660. Anal. ($C_{18}H_{17}NO_2$) C, H, N.

Method B. General Procedure for the Synthesis of the *exo*-1-(4-Pyridyl)-1a,2,3,7b-tetrahydro-1*H*-cyclopropa[*a*]naphthalenes 1–5. A mixture of the enones **1a–5a** (15 mmol), powdered KOH (13.1 g, 234 mmol), hydrazine hydrate (15 mL, 247 mmol, 80%), and di(ethylene glycol) (200 mL) was heated at temperatures not exceeding 140 °C for 1.5 h. The water was distilled off, and the mixture was heated at 195–200 °C until no more nitrogen was formed. After cooling, the reaction solution was poured into water and extracted with dichloromethane. The organic extracts were washed with water and dried (Na_2SO_4). The solvent was evaporated, and the crude product was purified by column chromatography (SiO_2; ethyl acetate) and recrystallized from *n*-hexane.

Method C. General Procedure for the Synthesis of the Bromo Compounds 6 and 7. The corresponding tetrahydrocyclopropanaphthalenes **2** and **4** (410 mg, 1 mmol), shielded from light, were dissolved in CCl_4 and cooled to −15 °C. A solution of Br_2 in CCl_4 (80 mg, 1 mmol) was added dropwise to the reaction mixture, and stirring was continued for 1 h. The precipitate was suction filtered using a cold filtering flask, and the hydrobromide was obtained by fractional crystallization from acetone in the cold. The hydrobromide was dissolved in water, and saturated $NaHCO_3$ solution was added. Extraction with dichloromethane followed by evaporation of the solvent yielded compounds **5** and **7**. Purification was performed by recrystallization from *n*-hexane.

Method D. General Procedure for the Ether Cleavage Yielding the Hydroxy Compounds 8–10. A solution of the methoxy compounds **2–4** (5.0 mmol) in dry dichloromethane (150 mL), shielded from light, was cooled to −78 °C, and BBr_3 (4.38 g, 17.5 mmol) was added under N_2. After 30 min the cooling bath was removed, and stirring was continued for 4 h. Hydrolysis was carried out by dropwise addition of methanol (5 mL) and was completed by stirring for 30 min. The mixture was concentrated to about one-half of the volume. After cooling, the solid was filtered and washed with dichloromethane. In case of no or only small amounts of solid precipitating after hydrolysis, the reaction mixture was evaporated to dryness. The residue or the filtered solid was taken up in water and filtered. The product was precipitated by addition of saturated $NaHCO_3$ solution. The solid was collected, washed with water, and dried in vacuo. Further purification was performed by recrystallization from a suitable solvent.

Method E. Separation of the Enantiomers of the 1-(4-Pyridyl)-1a,2,3,7b-tetrahydro-1*H*-cyclopropa[*a*]naphthalenes 4 and 7 by LPLC. The separation was performed using the optically active sorbens tribenzoyl cellulose with MeOH as eluent.

(+)- and (−)-*exo*-6-Methoxy-1-(4-pyridyl)-1a,2,3,7b-tetrahydro-1*H*-cyclopropa[*a*]naphthalene ((+)-4 and (−)-4). **4** (12 mg) was dissolved in methanol (1 mL). Repeatedly, 200 μL of this solution was injected. $(+)_{365}$-**4**: k'_+ = 4.3, p = 100%; $[\alpha]_D$ = 208 ± 17 (c = 4.48 mg/mL; MeOH). $(-)_{365}$-**4**: k'_- = 13.2, p = 100%; $[\alpha]_D$ = −203 ± 16 (c = 5.31 mg/mL; MeOH). The separated enantiomers (yield 50% each) were recrystallized from *n*-hexane.

(+)- and (−)-*exo*-5-Bromo-6-methoxy-1-(4-pyridyl)-1a,2,3,7b-tetrahydro-1*H*-cyclopropa[*a*]naphthalene ((+)-7 and (−)-7). **7** (12 mg) was dissolved in methanol (1 mL). Repeatedly, 200 μL of this solution was injected. (+)-**7**: k'_+ = 5.1, p = 100%; $[\alpha]^{22}_{436}$ = +515 ± 39; $[\alpha]^{22}_{365}$ = +1184 ± 86 (c = 6.0 mg/mL; EtOH). (−)-**7**: k'_- = 50, p = 100%; $[\alpha]^{22}_{436}$ = −507 ±32; $[\alpha]^{22}_{365}$ = −1184 ± 82 (c = 6.0 mg/mL; EtOH).

Method F. Separation of (+)- and (−)-*exo*-6-Methoxy-1-(4-pyridyl)-1a,2,3,7b-tetrahydro-1*H*-cyclopropa[*a*]naphthalene via Tartrate Formation: (−)-*exo*-6-Methoxy-1-(4-pyridyl)-1a,2,3,7b-tetrahydro-1*H*-cyclopropa[*a*]naphthalene ((−)-4). **4** (2.51 g, 10 mmol) was dissolved in ethanol and an equimolar solution of (2*R*,3*R*)-(−)-*O*,*O*'-dibenzoyltartaric acid in ethanol was added. The tartrate salt of (−)-**4** crystallized within 1–2 h. The precipitate was recrystallized three times from ethanol. Liberation of the base was performed by adding saturated $NaHCO_3$ solution and extracting the aqueous phase with dichloromethane. Evaporation of the solvent and recrystallization from *n*-hexane yielded (−)-**4** (33%).

(+)-*exo*-6-Methoxy-1-(4-pyridyl)-1a,2,3,7b-tetrahydro-1*H*-cyclopropa[*a*]naphthalene ((+)-4). The mother liquor resulting from the crystallization of the tartrate salt of (−)-**4** was evaporated to obtain the tartrate salt of (+)-**4**. The salt was recrystallized three times from acetonitrile and the base was liberated as described before. Recrystallization from *n*-hexane yielded (+)-**4** (25%).

Biological Methods. Enzyme Preparations. The enzymes were prepared according to described methods: human placental P450 arom,[10] bovine adrenal P450 scc,[10] rat testicular P450 17,[40] and bovine adrenal P450 18.[50] For the preparation of rat ovarian P450 arom, the same procedure was applied as for the human enzyme.

Hepatic P450 enzymes were prepared by centrifugation of a homogenate of rat livers in sodium phosphate buffer (0.01 M, pH 7.4) containing 1.15% KCl at 105000*g* (60 min, 4 °C). The microsomes were resuspended in a minimum volume of sodium phosphate buffer (0.05 M, pH 7.4) containing 0.001 M EDTA and 20% glycerol and were stored at −70 °C.

Enzyme Assays. The enzyme assays were performed as described: human placental and rat ovarian P450 arom,[10] bovine adrenal P450 scc,[10] and rat testicular P450 17.[40] The irreversible inhibition of P450 arom was determined as described.[17]

For determination of P450 18 inhibition, the test compounds (1 μM) were incubated with corticosterone (200 μM), bovine adrenal mitochondria (0.5 mg protein/0.5 mL), $NADP^+$ (1 mM), glucose-6-phosphate (7 mM), and glucose-6-phosphate-dehydrogenase (1 IU/0.5 mL) in Tris-HCl buffer (0.05 M, pH 7.4) containing $MgCl_2$ (1.2 mM), KCl (0.6 mM), NaCl (140 mM), and $CaCl_2$ (2.5 mM) at 30 °C for 10 min. The incubation mixture was acidified by adding 250 μL of HCl (1 N) and extracted with ethyl acetate (1 mL). After washing with NaOH (1 N) and buffer, the solvent was evaporated. The steroids were dissolved in methanol and separated by HPLC using a Nucleosil 120-5 C 18 column and methanol/H_2O (1:1) as eluent (flow rate: 1 mL/min).

Inhibition of rat hepatic P450 enzymes was determined by incubation of the compounds (150 μM) with *p*-nitroanisole (1.2 mM) or aminopyrine (0.8 mM), rat hepatic microsomes (1 mg protein/0.86 mL), $MgCl_2$ (4.2 mM), $NADP^+$ (0.3 mM), glucose-6-phosphate (1.6 mM), glucose-6-phosphate-dehydrogenase (1 IU/0.86 mL) and in case of aminopyrine as substrate semicarbazide (7.5 mM) in sodium phosphate buffer (0.05 M, pH = 7.4) for 10 min at 37 °C. The amount of *p*-nitroanisol formed was determined photometrically at 405 nm after alkalization of the samples. The amount of formaldehyde formed by N-demethylation of aminopyrine was determined according to Nash.[51]

The V_{max} values were calculated according to Lineweaver and Burk[52] after incubation of liver microsomes from rats prepared after termination of the premenopausal tumor test as described above. *p*-Nitroanisol and aminopyrine were used in concentrations ranging from 0 to 1.2 and 10 mM, respectively.

The Lineweaver Burk plot[52] and the difference spectrum were obtained according to procedures published recently.[21] The assay for the determination of the corticoid formation was performed using the method recently described.[21]

In Vivo Methods. Inhibition of the Androgen-Stimulated Uterine Growth. The method of Bhatnagar et al.[44] was used with minor modifications.[26]

Inhibition of the Plasma E_2 Concentration. The test was performed as described using female PMSG-primed SD rats.[10]

DMBA-Induced, Hormone-Dependent Mammary Carcinoma. The tumor induction was performed as described.[10] Animals bearing at least one tumor greater than 140 mm^2 were classified in groups of 10. In the premenopausal model treatment was started, i.e., the rats were subcutaneously administered oil solutions of the test compound. In the postmenopausal model they were first ovariectomized and then treated with testosterone propionate and test compound. Measurement of tumor size[10] and determination of body weight were made once weekly. The therapy was terminated at the 28th day.

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Supplementary Material Available: Crystal data for the (2*R*,3*R*)-(−)-dibenzoyl tartrate of (1a*R*,1*R*,7b*R*)-(−)-**4** including tables listing atomic coordinates and equivalent isotropic displacement coefficients, bond lengths, bond angles, and anisotropic displacement coefficients, ^1H NMR data of 1−10, as well as ^{13}C NMR data of 1 (Tables 8 and 9, Figure 5 (9 pages)). Ordering information is given on any current masthead page.

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