in vacuo (P_2O_5, 60 °C) to obtain a white solid (37 mg, 62%): mp 175–188 °C (softening ca. 135 °C); IR (KBr) ν 3480, 2960, 1740 sh, 1700 sh, 1660, 1615, 1585, 1510, 1485, 1470, 1420 w, 1400, 1350 w, 1315, 1270, 1220 cm^{-1}; NMR ($CDCl_3$ + DMSO-d_6, 3:1) δ 1.5–2.5 (m, CH_3CO, β- and γ-CH_2, C_5-CH_2, C_6-CH), 3.15 (m, δ-CH_2, C_7-CH_2), 3.73 (s, MeO), 3.85 (m, C_9-CH_2), 4.60 (m, α-CH, NH), 5.05 (s, $C_6H_5CH_2O$), 5.50 (m, NH), 6.38 (m, NH), 7.35 (m, $C_6H_5CH_2O$, C_3- and C_5-H), 7.56 (s, NH), 8.00 (d, J = 8 Hz, C_2- and C_6-H), 8.25 (m, NH), 8.53 (s, N^{10}-CHO). Anal. ($C_{32}H_{37}N_7O_8 \cdot 0.5H_2O$) C, H, N.

N^{α}-(5,6,7,8-Tetrahydro-5-deazapteroyl)-L-ornithine (5-dH$_4$PteOrn, 20). A solution of **36** (88 mg, 0.134 mmol) in glacial AcOH (3 mL) was treated with 30% HBr in AcOH (3 mL). A precipitate formed on contact, but dissolved almost immediately. The solution was left to stand at room temperature for 45 min and then evaporated to dryness under reduced pressure. The residue was triturated with Et_2O (10 mL), the Et_2O was decanted, and the residue was taken up in H_2O (2 mL) by basification to pH 10 with 2 N NaOH. An additional 5 mL of 2 N NaOH was added, and the solution was sparged with N_2 and heated in an oil bath kept at 80 °C for 30 min. The solution was cooled, and a small amount of brown solid was removed by filtration. The filtrate was adjusted to pH 6 with AcOH and subjected to preparative HPLC on C_{18} silica gel (analytical retention time 10 min; 1% AcOH, 5% EtOH, 1.0 mL/min). Pooled pure fractions were evaporated and the residue was freeze-dried to obtain a white solid (59 mg, 81%): mp >300 °C; IR (KBr) ν 3440, 2980, 2950, 1705, 1650 sh, 1615, 1580 sh, 1560, 1525, 1490 sh, 1415, 1350, 1315, 1285, 1235, 1205 cm^{-1}; UV λ_{max} (0.1 M HCl) 220 nm infl (ϵ 14,300), 279 (24,900), 302 infl (9,100); λ_{max} (pH 7.4 phosphate buffer) 219 nm (ϵ 35,100), 281, 31,700), 300 infl (21,200); λ_{max} (0.1 M NaOH) 278 nm (ϵ 26,500), 294 infl (21,700); NMR (CF_3CO_2H) δ 1.62 (m, β- and γ-CH_2, CH_3COOH), 2.10 (m, C_5-CH_2, C_6-CH), 2.6–3.3 (m, C_7-CH_2, C_9-CH_2, δ-CH_2), 4.40 (m, α-CH), 7.0–7.6 (m, C_2-, C_3-, C_5-, C_6-H). Anal. ($C_{20}H_{27}O_4 \cdot CH_3CO_2H \cdot 3H_2O$) C, H, N.

Acknowledgment. This work was supported by grants CA39867 (R.G.M., A.R.) and CA41461 (J.H.F.) from the National Cancer Institute, DHHS. The authors are indebted to Dr. G. Peter Beardsley and Ms. Barbara Moroson, Yale University School of Medicine, for carrying out some of the cytotoxicity assays, and to Mr. Scott Smith for his excellent technical assistance in carrying out GARFT and FPGS assays.

Registry No. **13**, 132343-86-3; **14**, 139347-01-6; **14**·NH_3, 139347-18-5; **15**, 139347-02-7; **15**·$^5/_4NH_3$, 139375-95-4; **16**, 139347-03-8; **16**·NH_3, 139347-19-6; (6*R*)-L-**17**, 139347-04-9; (6*R*)-L-**17**·$^4/_5NH_3$, 139347-20-9; (6*S*)-L-**17**, 139347-14-1; (6*S*)-L-**17**·$^4/_5NH_3$, 139347-21-0; (*R**,*R**)-(±)-**18**, 139347-05-0; (*R**,*R**)-(±)-**18**·$^7/_4NH_3$, 139347-22-1; (*R**,*S**)-(±)-**18**, 139347-15-2; (*R**,*S**)-(±)-**18**·$^7/_4NH_3$, 139347-23-2; (*R**,*R**)-(±)-**19**, 139347-06-1; (*R**,*R**)-(±)-**19**·$^1/_2NH_3$, 139347-24-3; (*R**,*S**)-(±)-**19**, 139347-16-3; (*R**,*S**)-(±)-**19**·$^1/_2NH_3$, 139347-25-4; (6*R*)-L-**20**, 139347-07-2; (6*R*)-L-**20**·HOAc, 139347-26-5; (6*S*)-L-**20**, 139347-17-4; (6*S*)-L-**20**·HOAc, 139347-27-6; **21**, 87373-56-6; **22**, 139347-08-3; **24**, 139347-09-4; **25**, 139347-31-2; **25**·$^1/_4NH_3$, 139347-38-9; **26**, 139347-32-3; **27**·$^3/_2$ oxalate, 139405-66-6; **27**·HCl, 139405-64-4; **28**·HCl, 139347-33-4; **29**, 139347-34-5; **30**, 139347-35-6; **31**, 139347-36-7; **32**, 139347-37-8; **33**, 139375-96-5; **34**, 139375-94-3; (*R**,*R**)-(±)-**35**, 139347-10-7; (*R**,*S**)-(±)-**35**, 139347-11-8; (6*R*)-L-**36**, 139347-12-9; (6*S*)-L-**36**, 139347-13-0; FPGS, 63363-84-8; GARFT, 9032-02-4; H-Orn(Z)-OMe, 62631-17-8; L-$H_2NCH(C H_2CH_2SO_3H)CO_2H$, 14857-77-3; L-$H_2NCH(CH_2CH_2SO_3H)$-COOMe, 139347-30-1; L-$H_2NCH(CH_2CH_2SO_3^-)CO_2^-$·2 ($PhCH_2$)-$Me_3N^+$, 139347-29-8; DL-$H_2NCH(CO_2H)CH_2CH_2PO_3H_2$, 20263-07-4; 4-$O_2NC_6H_4COCl$, 122-04-3; 4-$H_2NC_6H_4CO_2H$, 150-13-0; ($PhCH_2$)$Me_3N^+ \cdot MeO^-$, 122-08-7.

Synthesis and Evaluation of a New Series of Mechanism-Based Aromatase Inhibitors

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A series of new 4-(alkylthio)-substituted androstenedione analogues was designed as potential suicide inhibitors of aromatase on the basis of mechanistic considerations on the mode of action of the enzyme. Their synthesis and biological evaluation are described. Among the most interesting are the 4-[(difluoromethyl)thio]-, 4-[(fluoromethyl)thio]-, and 4-[(chloromethyl)thio]androstenediones **12**, **13**, and **14** with respective IC_{50}'s of 2.7, 0.8, and 0.94 μM. Compound **12** was a reversible inhibitor of aromatase while compounds **13** and **14** displayed time-dependent kinetics of inhibition with respective K_I's and half-times of inactivation of 30 nM and 3.75 min for **13** and 30 nM and 3 min for **14**. The inhibition of aromatase by **14** was NADPH-dependent, and was protected by the presence of substrate (0.5–1 μM), while β-mercaptoethanol (0.5 mM) failed to protect the enzyme from inactivation. Dialysis failed to reactivate aromatase previously inactivated by **14**. The mechanistic implications of these findings are discussed.

Introduction

Breast cancer is among the most common malignancies in women today. In the US alone about 130 000 cases are reported each year.[1] In the case of ER + tumors, ablative therapy is more and more frequently replaced by endocrine palliative treatment. Today, medical endocrine treatment of advanced postmenopausal breast cancer largely falls within three groups:[2] (a) antiestrogens, acting directly on the tumor cell via the receptor, (b) aromatase inhibitors, suppressing estrogen production, (c) progestins, of which the mechanism of action is still uncertain.[3]

The major pathway of estrogen production in postmenopausal women is the peripheral conversion of androgens to estrogens. The enzyme that catalyzes this conversion is a cytochrome P-450 dependent monooxygenase, aromatase. Although the mechanism of this reaction is still the subject of controversy, most authors agree today that

(1) Young, J. L., Jr. *Breast Cancer*; Alan R. Liss., Inc.: New York, 1989; I-5, chapter 1, pp 1–5.

(2) Lønning, P. E.; Dowsett, M.; Powles, T. J. Postmenopausal Estrogen Synthesis and Metabolism: Alterations Caused by Aromatase Inhibitors Used for the Treatment of Breast Cancer. *J. Steroid Biochem.* **1990**, *35*, 355–356.

(3) Lundgren, S.; Lønning, P. E.; Utaaker, E.; Aakvaag, A.; Kwinnsland, S. Influence of Progestins on Serum Hormone Levels in Postmenopausal Women with Advanced Breast Cancer. I. General Findings. *J. Steroid Biochem.* **1990**, *36*, 99–104.

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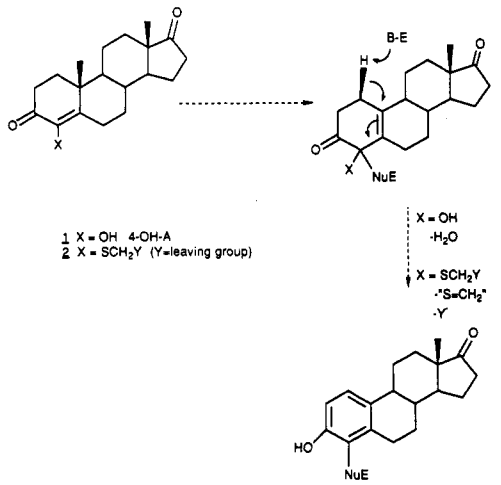
Scheme I



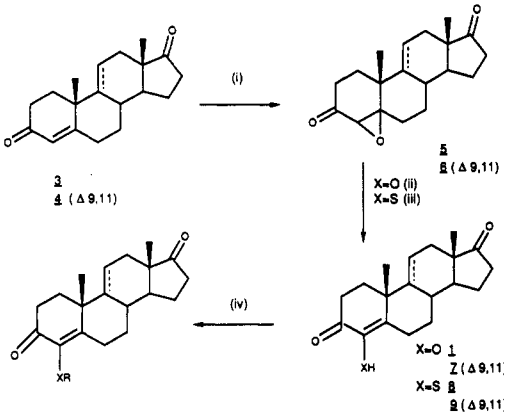
it probably takes place via three successive oxidations at carbon 19 of the steroid nucleus.[4] Numerous aromatase inhibitors have been synthesized and studied in vitro on human placental aromatase. Those include competitive as well as irreversible inhibitors.[4] Among the latter, the mechanism-based inhibitors, also referred to as suicide inhibitors, are of special interest due to their high selectivity.

One of the earliest suicide inhibitor of aromatase to be described was 4-hydroxyandrostene-3,17-dione (4-OH-A, 1).[5,6] The mechanism by which this compound inactivates the enzyme remains unknown. In 1982, Covey proposed a mechanism for the reaction of aromatization of androgens to estrogens that also rationalized the time-dependent inactivation observed with 4-OH-A (1).[7] This mechanism involved formation of a covalent bond between the enzyme and the 4-position of the steroid nucleus. In the case of 4-OH-A, the OH group (following rapid protonation) would leave as water instead of the normally departing enzyme nucleophile (Scheme I, X = OH).

At the time this mechanism was published, we reasoned that a compound like 2, if substrate of the enzyme, could give rise to a "double jeopardy suicide inhibition"[8] since elimination of the group in position 4, if it happened, would not only tie the enzyme onto the steroid nucleus but also generate within the enzyme cavity a molecule of thioformaldehyde (Scheme I, X = SCH_2Y). The latter could irreversibly alkylate the prosthetic heme group[9] or bind covalently to the protein due to its electrophilic character.[10]

Although this concept of freeing a little electrophilic entity within the enzyme has already been applied,[11] to

Scheme II[a]



[a] (i) H_2O_2/NaOH; (ii) H_2SO_4/AcOH; (iii) AcSH/dioxane, then MeOH/HCl; (iv) RX, tBuOK, THF.

Scheme III

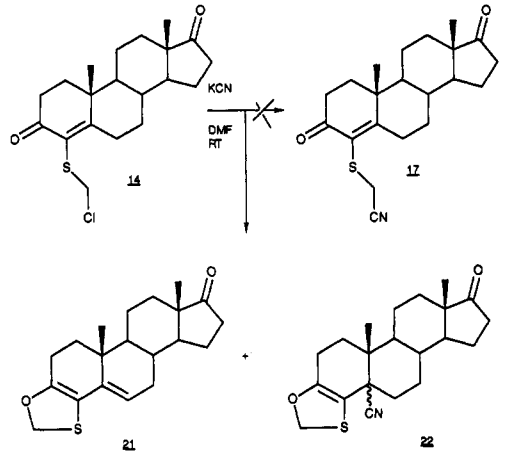


Table I. Synthesis of 4-Alkoxy and 4-(Alkylthio)androstenediones

X	R	Δ9,11	compd	% yield	ref
O	CH_3		10	48	14
O	CH_2OCH_3		11	53	-
S	CHF_2		12	52	17
S	CH_2F		13	21	17
S	CH_2Cl		14	36	17
S	CH_3		15	58	13a
S	CH_2NO_2		16	12	17
S	CH_2CN		17	57	17
S	CH_2SCH_3		18	47	17
S	CH_2OCH_3		19	31	17
S	CH_2Cl	yes	20	45	17

(4) (a) Wright J. N.; Akthar, M. 'Slow binding' Sixth-ligand Inhibitors of Cytochrome P-450 Aromatase. *Steroids* **1990**, *55*, 142–151. Wright, N. J.; Slatcher, G.; Akthar, M. *Biochem. J.* **1991**, *273*, 533–539. (b) Cole, P. A.; Robinson, C. H. Mechanism and Inhibition of Cytochrome P-450 Aromatase. *J. Med. Chem.* **1990**, *33*, 2933–2942. (c) Berg, D.; Plempel, M. Studies on Estrogen Biosynthesis using Radioactive and Stable Isotopes. In *Sterol Biosynthesis Inhibitors, Pharmaceutical and Agrochemical Aspects*; Series in Biomedicine; Ellis Horwood Ltd: Chichester, England, 1988; Chapter 22.

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(6) Covey, D. F.; Hood, W. F. Aromatase Enzyme Catalysis is Involved in the Patent Inhibition of Estrogen Biosynthesis Caused by 4-Acetoxy- and 4-Hydroxy-4-androstene-3,17-dione. *Mol. Pharmacol.* **1982**, *21*, 173–80.

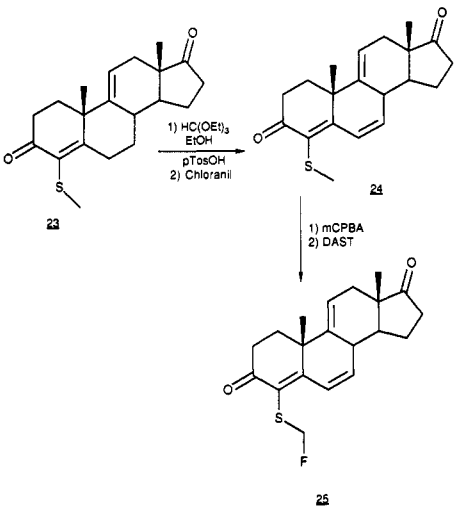
(7) Covey, D. F.; Hood, W. F. A New Hypothesis Based on Suicide Substrate Inhibitor Studies for the Mechanism of Action of Aromatase. *Cancer Res.* (suppl) **1982**, *42*, 3227s–3333s.

(8) This terminology was first used by D. F. Covey (ref 7) to design "an inactivator that would provide the enzyme with two successive opportunities to catalyze its own demise".

(9) Ortiz de Montellano, P. R. *Cytochrome P-450. Structure, Mechanism and Biochemistry*; Plenum Press: New York, London, 1986; Chapter 8.

(10) For a few pertinent references on thioformaldehyde, see: Lown, J. W.; Koganty, R. R. Formation of Novel 1,2-Oxathietanes from 2-Chloroethyl Sulfoxide Precursors and Their Reactions in Solution, Including Formal [σ2s + σ2a] Cycloreversions and Rearrangements. *J. Am. Chem. Soc.* **1986**, *108*, 3811–3818. Wazneh, L.; Guillemin, J. C.; Guenot, P.; Valley, Y.; Denis, J. M. Formation of Reactive Thioaldehydes by Vacuum Gas-Phase Dehydrocyanation of Thiocyanohydrins: Characterization by MS/MS Spectrometry. *Tetrahedron Lett.* **1988**, *29*, 5899–5900. Usov, V. A.; Timokhina, L. V.; Voronkov, M. G. The Synthesis and Properties of Thioaldehydes. *Russ. Chem. Rev.* **1990**, *59*, 378–395.

Scheme IV



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our knowledge it has not been illustrated with thioformaldehyde. To be of pharmacological interest, these compounds would have to demonstrate their time-dependent mechanism of inhibition.

Although 4-OH-A is a potent aromatase inhibitor, it suffers from a low bioavailability and duration of action mainly due to its high rate of glucuronidation.[12] We thought that 4-(alkylthio) substitution might also play favorably on those factors.

Several 4-(alkylthio)- and 4-(arylthio)androstenedione analogues have already been described.[13,14] Abul-Hajj showed that with alkyl chains under four carbons and phenyl, the compounds retained a good inhibitory activity.[13a] All the compounds exhibited competitive type inhibition. No heteroatom-substituted (alkylthio)-androstenedione have been reported so far.

Chemistry

The synthesis of the desired analogues is outlined in Scheme II. Treatment of androst-4-ene-3,17-dione (**3**) or androsta-4,9(11)-diene-3,17-dione (**4**) with hydrogen peroxide under basic conditions afforded the corresponding epoxides **5**[15] and **6**[16] as 5:1 and 3:2 mixtures of β:α isomers, respectively. Treatment of epoxides **5** and **6** under described conditions afforded 4-hydroxy or 4-mercaptoandrostenedione **1**,[5] **7**,[16] **8**,[14] and **9**.[17] Subsequent alkylation with the appropriately functionalized alkyl halides gave the 4-alkoxy- and 4-(alkylthio)androstenediones **10–20** (Table I).

Scheme V

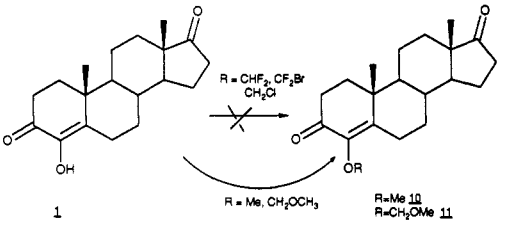

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Table II. Inhibition of Aromatase by 4-Alkoxy- and 4-Thio-Substituted Analogues of Androstenedione[a]

R	Δ9,11	compd	IC_{50}, μM	K_I, nM	k_{inact}, 10^{-3} s^{-1}	$t_{1/2}$, min
OH		**1**	0.37	27	2.3	5
OH	yes	**7**	0.35	ND	ND	ND
SH		**8**	3.5	ND	ND	ND
SH	yes	**9**	3.8	72	3.9	3
SCH_3		**15**	4.4	NTD	NTD	NTD
SCH_2Cl		**14**	0.94	30	3.8	3
SCH_2Cl	yes	**20**	0.86	27	4.8	2.4
SCH_2F		**13**	0.8	30	3.1	3.8
$SCHF_2$		**12**	2.7	NTD	NTD	NTD
SCH_2SMe		**18**	1.2	NTD	NTD	NTD
SCH_2OMe		**19**	10	NTD	NTD	NTD
OCH_2OMe		**11**	4.4	NTD	NTD	NTD
SCH_2CN		**17**	3.7	169	1.2	10

[a] Abbreviations: ND = not determined; NTD = not time-dependent.

Some attempts were made to find a route to fluoro compound **13** avoiding the use of not readily available bromofluoromethane[18] or fluoroiodomethane.[19] Thus, treatment of 4-[(chloromethyl)thio]androst-4-ene-3,17-dione (**14**) with fluoride ion or other nucleophiles (CN, I) failed to give rise to nucleophilic displacement of the chlorine atom but afforded oxygen-cyclized products. So, when **14** was allowed to react with potassium cyanide for 12 h at room temperature, compounds **21** and **22** were obtained with respectively 12 and 28% yield (Scheme III). On the other hand, it was possible to adapt the reaction described by McCarthy and co-workers[20] to the 4-(methylthio)androstenedione analogues. Thus, when **24**, obtained in the usual fashion from **23**, was treated with mCPBA followed by DAST [(diethylamido)sulfur trifluoride] in refluxing chloroform, the (fluoromethyl)thio analogue **25** was obtained in 43% overall yield (Scheme IV).

Interestingly, under conditions where 4-mercaptoandrostenedione was successfully haloalkylated, we were unable to obtain the oxygenated analogues (Scheme V). Although a few O-(difluoromethyl) enol ethers have been

(11) See, for instance: Donadio, S.; Perks, H. M.; Tsuchiya, K.; White, E. H. Alkylation of Amide Linkages and Cleavage of the C Chain in the Enzyme-Activated-Substrate Inhibition of α-Chymotrypsin with *N*-Nitrosamides. *Biochemistry* **1985**, *24*, 2447–2458 and refs therein.

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(16) Eder, U.; Neef, G.; Kerb, U.; Schillinger, E.; Toepert, M.; Beier, S.; Nishino, Y. German Patent, DE3213328A1, April 6th, 1982.

(17) Gourvest, J. F.; Lesuisse, D.; Philibert, D.; Vevert, J. P. French Patent 88, 16993, Dec 12, 1988.

(18) Haszeldine, R. N. The reactions of Metallic Salts of Acids with Halogens. Part III. Some Reactions of Salts of Fluorohalogenoacetates and of Perfluoro-acids. *J. Chem. Soc.* **1952**, 4259–4268.

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(20) McCarthy, J. R.; Peet, N. P.; LeTourneau, M. E.; Inbasekaran, M. (Diethylamino) Sulfur Triofluoride in Organic Synthesis. 2. The Transformation of Sulfoxides to α-Fluoro Thioethers. *J. Am. Chem. Soc.* **1985**, *107*, 735–737.

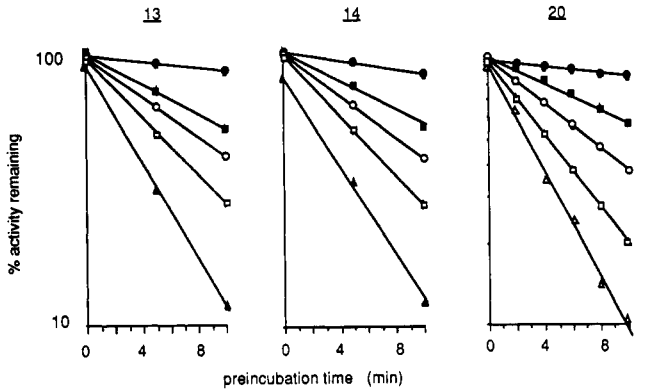

Figure 1. Time- and concentration-dependent loss of aromatase activity by 4-(alkylthio)androgens. The percent of activity remaining was calculated according to method 1 of Nelson[30] (see Experimental Section). [I] = 0 (●), 10^{-8} M (■), 3.33×10^{-8} M (○), 10^{-7} M (□), 3.33×10^{-7} M (△), 5×10^{-7} M (▲).

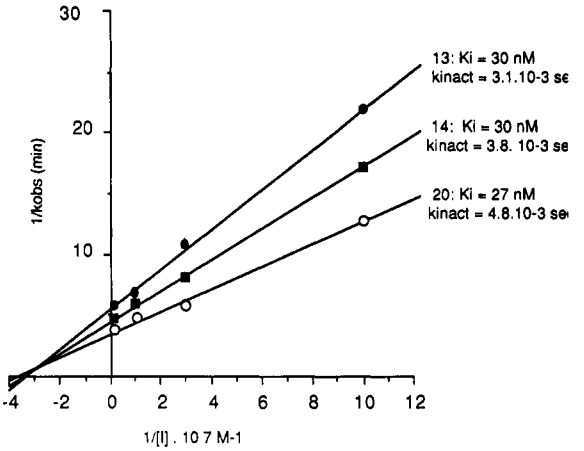

Figure 2. Double-reciprocal plots of the observed pseudo-first-order rate constants of inactivation (k_{obs}) versus inhibitor concentration for **13** (●), **14** (■), and **20** (○).

described in the chemistry of pyrimidine nucleosides[22] or β-diketo enolates,[23] we are only aware of two cases of such enol ethers on unactivated ketones.[24] On the other hand, under those conditions 4-OH-A was readily alkylated on oxygen with methyl iodide or bromomethyl methyl ether to afford compounds **10** and **11**, respectively (Scheme V). We believe the reason for the failure of the former reactions lies in the instability of the corresponding *O*-(haloalkyl) enol ether.[25] The methoxymethyl analogue **11** was already very sensitive to hydrolysis.

Biochemistry

Human placental microsomes were prepared as described.[26] The method of Thomson and Siiteri[27] was used for evaluating the newly synthesized compounds as aromatase inhibitors. A V_{max} of 75 pmol/mg per min and an apparent K_m of 50 nM for androstenedione were found for the microsomes of the placenta used for determinations of the K_I and k_{inact} values of the inhibitors.[28] The substrate concentration was 500 nM (10 times the K_m value for the enzyme preparation). Inhibitors were assayed at concentrations ranging from 10^{-5} to 10^{-8} M. From the obtained

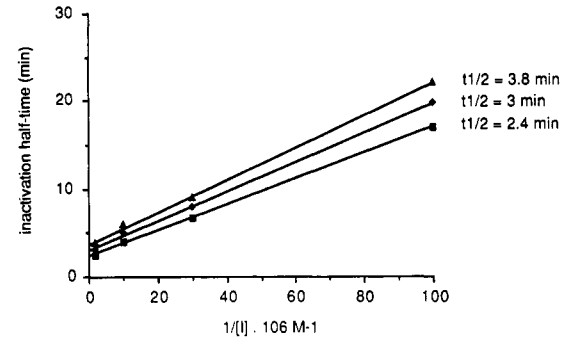

Figure 3. Plot of the half-time of inactivation versus 1/[I] for **13** (▲), **14** (◆), and **20** (■).

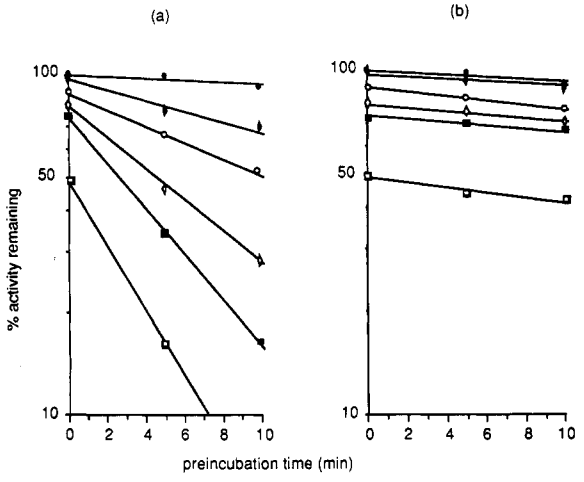

Figure 4. Interaction of **14** with aromatase in the presence (a) or in the absence (b) of NADPH. The percent of remaining activity was calculated according to method 2 of Nelson[30] (see Experimental Section). [I] = 0 (●), 10^{-8} M (◆), 10^{-7} M (○), 2.5×10^{-7} M (◇), 5×10^{-7} M (■), 7.5×10^{-7} M (□).

values, inhibitory concentrations to reduce the activity of the enzyme to 50% of the control value were calculated (IC_{50}). Table II presents the ensemble of those results. For comparative purposes we have included some compounds previously reported as aromatase inhibitors: 4-OH-A (**1**),[5] 4-(methylthio)androstene-3,17-dione (**15**),[13a] and 4-mercaptoandrostene-3,17-dione (**8**).[5,13,14]

The new compounds were also tested for their ability to act as suicide substrates of aromatase in human placental microsomes. For that purpose, each compound was first evaluated at various times and concentrations. For

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Table III. Protection Studies of the Inactivation of Aromatase by **14** in the Presence of Androstenedione or β-Mercaptoethanol

additive in the preincubation	% activity remaining		
	$t = 0$	$t = 5$ min	$t = 10$ min
	35 ± 3	11 ± 1.4	5.3 ● 0.3
AD (0.5 μM)	62.8 ± 5.3	49 ± 3.7	36.5 ± 2.8
AD (1 μM)	70 ± 6.4	59 ± 2.6	46 ± 3.2
β-MSH (0.5 mM)	32 ● 3.3	9.8 ● 0.7	5 ± 0.4
β-MSH (10 mM)	36.8 ± 1.4	17.3 ± 1.1	10.2 ± 0.9

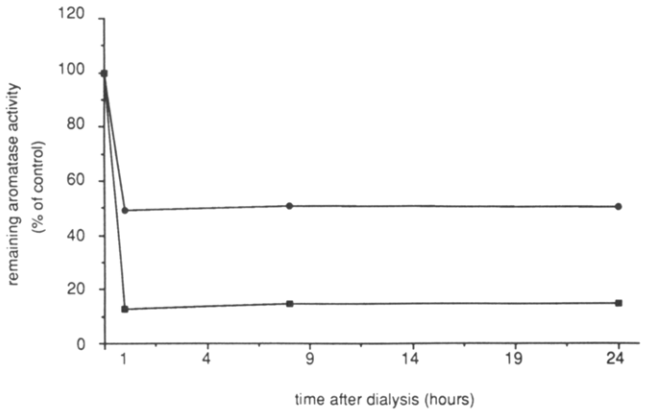

Figure 5. Irreversibility of the inactivation of aromatase by **14**. [14] = 5×10^{-7} M (●), 5×10^{-6} M (■).

the compounds that gave a time-dependent inactivation, kinetics were studied, with K_I k_{inact}, and $t_{1/2}$[28] calculated by the Kitz–Wilson method.[29] These results also figure in Table II.

Of all new thio-substituted androstenedione analogues tested, only compounds **13**, **14**, and **20** and to a lesser extent **17** were time-dependent inhibitors of aromatase. Compounds **13**, **14**, and **20** exhibited very comparable kinetics of inhibition (Figure 1). Inactivation kinetics were of pseudo-first-order, at least during the first 10 min. With the use of increasing concentrations of inhibitors, increasing values of k_{obs} were obtained. Double-reciprocal plots of k_{obs} versus inhibitor concentration yielded the K_I's and k_{inact}'s[29] (Figure 2). Finally, Figure 3 presents a plot of the half-time of inactivation, i.e. the time required to decrease the enzymatic activity by 50%, versus the reciprocal of the inhibitor concentration. The y intercept of the resulting line is the half-time of inactivation at infinite inhibitor concentration ($t_{1/2}$).

Preincubation of **14** in the absence of the required cofactor NADPH failed to give rise to time-dependent inhibition of aromatase (Figure 4). This pattern is to be expected from a competitive inhibitor. The inhibition was active site-directed since incubation of **14** in the presence of increasing concentrations of the substrate androstenedione protected the enzyme from inactivation (Table III). Addition of 0.5 mM β-mercaptoethanol in the preincubation medium had no effect on the rate of inactivation. Nevertheless, at high concentrations in β-mercaptoethanol (10 mM) some protection was observed (twice the enzymatic activity without β-mercaptoethanol) (Table III).

Finally, the covalent nature of inhibition was demonstrated by dialysis of the inactivated enzyme. The percent of activity remaining versus control[31] after 8 or 24 h of dialysis was identical to the one measured before dialysis (Figure 5).

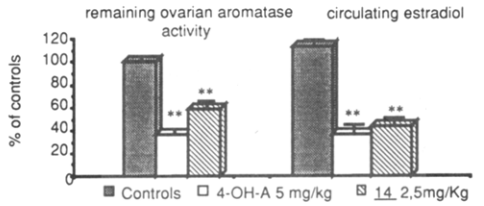

Figure 6. In vivo studies of **14** on PMSG-primed female rats, intravenous route.

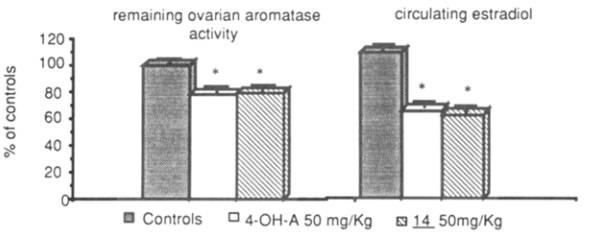

Figure 7. In vivo studies of **14** on PMSG-primed female rats, subcutaneous route.

The lead compound **14** was evaluated against 4-OH-A for in vivo activity. PMSG-primed female rats were treated either intravenously (Figure 6) or subcutaneously (Figure 7) with similar doses of 4-OH-A or **14**. After 2 h the animals were sacrificed and circulating estradiol levels were measured as the total blood fraction. The ovaries were collected, ovarian microsomes were prepared, and remaining aromatase activity was determined.[6] As shown in Figures 6 and 7, **14** displayed no improvement over 4-OH-A. Furthermore, like 4-OH-A, it also proved to be inactive at oral doses of 50 mg/kg.

Discussion

All new compounds inhibited aromatase, some of them with affinities comparable to that of 4-OH-A reflected in first approximation by IC_{50} values. The presence of a 9,11 double bond did not induce any appreciable change in the affinities (Table II, compare compounds **1** and **7**, **14** and **20**). Only compounds **13**, **14**, **17**, and **20** were time-dependent inhibitors of aromatase.

To truly characterize an inhibitor as mechanism-based, certain criteria must be met.[28] In all of the above experiments, chloro and fluoro compounds **13**, **14**, and **20** responded to these requirements. Pseudo-first-order time-dependent loss of enzymatic activity was observed with a rate of inactivation proportional to inhibitor concentration (saturation kinetics were observed) (Figures 1–3). The rate of inactivation was slower in the presence of increasing concentrations of substrate, and unaffected by the presence of exogenous nucleophiles (Table III). The less drastic loss of aromatase activity observed at high concentration of β-mercaptoethanol could be due to the known propensity of these small molecules to diffuse within the enzyme active site,[28] although one cannot exclude the possibility of some unspecific labeling. The inactivation required the presence of NADPH (Figure 4), implying that enzymatic activation of the inhibitor was required. Finally, enzyme activity did not return upon dialysis (Figure 5).

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(31) Dialysis of the fresh enzyme resulted in substantial losses of activity after 8 and 24 h. Those were integrated into the calculation of the percent of activity remaining in the inactivated enzyme.

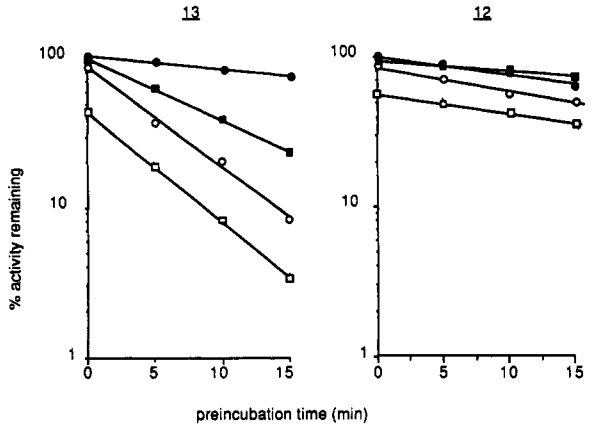
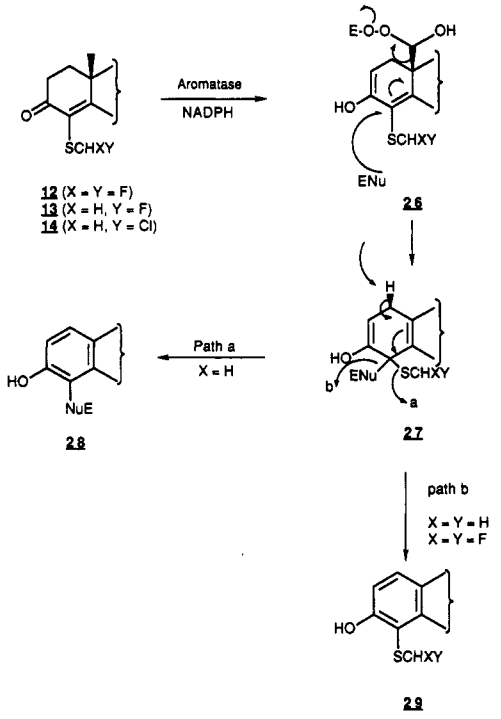


Figure 8. Time and concentration dependency of aromatase inhibition by **12** and **13**. The percent of activity remaining was calculated according to method 2 of Nelson[30] (see Experimental Section). [I] = 0 (●), 10^{-8} M (■), 10^{-7} M (○), 10^{-6} M (□).

Scheme VI

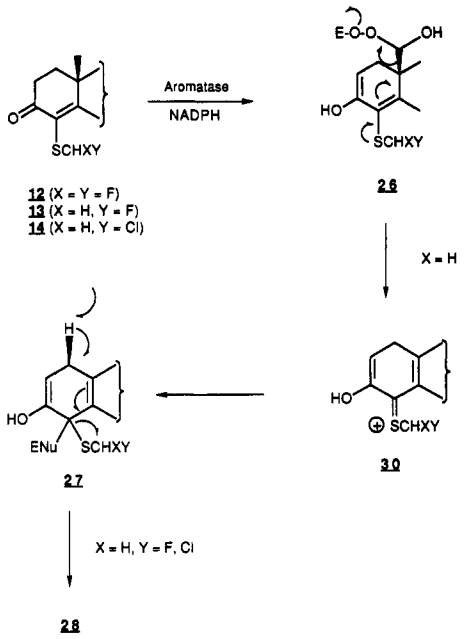


Still, the mechanism by which inactivation occurs remains unclear. The difference between mono- and difluoro analogues **13** and **12** is interesting. The IC_{50} value is multiplied by a factor 3 with the additional fluorine atom, showing that the enzyme might be sensitive to electronic effects in that position (Table II). More puzzling is the fact that **13** is an irreversible inhibitor of human placental aromatase while **12** is a competitive inhibitor. Figure 8 shows the time-dependent kinetics of both inhibitors.

The inactivation of aromatase by **13** and **14** could proceed according to Covey's mechanism as depicted in Scheme VI (path a). However, in order to explain the difference of behavior between SCH_3 and $SCHF_2$ (competitive) and SCH_2F (time-dependent) two conditions would have to be fulfilled: (i) the step of aromatization (**27** → **28**) would require the elimination of an halogen atom as the driving force (when X = Y = H elimination of the enzyme would become a more favorable alternative) (Scheme VI, path b); (ii) owing to the higher energy of the carbon–fluorine bond on CHF_2 than CH_2F,[33] the elimination of the enzyme on intermediate **27** (when X = Y = F) would again become a more favorable alternative for **12** (Scheme VI, path b). The ease of chemical hydrolysis of the carbon–fluoride bond via elimination of fluoride, suggesting the order $CF_3 < CHF_2 < CH_2F$ discussed by Santi,[34] fits well into this explanation. However, we are not aware of such drastically different kinetics enzymatic inhibition between mono- and difluoro compounds, when inactivation proceeds through elimination of a fluorine atom; i.e. α-(monofluoromethyl)dehydroputrescine and -ornithine are only slightly faster time-dependent inactivators than the difluoromethyl analogues.[35] Furthermore, several enzyme inactivators proceed through elimination of fluoride on a trifluoromethyl group.[36] More closely related to our case is the suicide inactivation of homoserine dehydratase by (trifluoromethyl)thionine[37] which proceeds through elimination of a CF_3S^- ion leading to carbonothioic difluoride (CSF_2) as a highly electrophilic entity.

Two alternative explanations cannot be discounted but seem unlikely to us: (i) compound **12** is not a substrate for the enzyme; precedents where molecules differing from an enzyme substrate by just one fluorine atom are not a substrate for the enzyme anymore have been described;[38]

Scheme VII



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(ii) the inactivation could take place via direct nucleophilic displacement of the halogen atom by the enzyme. Such displacements would be less favorable in the case of difluoromethyl analogue **12**. Studies of Hine[39] have established that the ease of nucleophilic displacement of halogen atoms goes in the sense $CH_3X > CH_2X_2 \gg CHX_3$. Nevertheless, this displacement would have to happen on an oxidized species since no time-dependent inactivation was seen in the absence of NADPH. Besides, one would have then expected a difference between the half-times of inactivation of monochloro and monofluoro compounds **14** and **13**, as often observed in the inactivation of protease enzymes by halo ketones.[40] The similarity of inactivation kinetics by **14** and **13** could either mean that the nucleophile involved in the direct displacement of the halogen atom is a strong one (i.e. cysteine[40]) or that the departure of this halogen is not rate-determining.[41]

Finally, another mechanism which could account for the data is depicted in Scheme VII.[42] Assistance of the lone pair of sulfur to the departure of formic acid in **26** would produce in a mechanism-based fashion the highly electrophilic species **30** which could then be trapped within the enzyme before aromatization to **28**. In the case of **12**, the intermediate **30** would not form with the more electron-withdrawing difluoro compound.

Conclusion

A new series of androstenedione analogues synthesized; they displayed good affinity for aromatase and some of them behaved like suicide inactivators of the enzyme. In view of the above results and the relatively easy access to radioisotopically labeled analogues of **13** and **14**, we feel that these compounds could be valuable tools in the study of the mechanism of aromatization of androgens to estrogens.

Experimental Section

Melting points were obtained on a Koffler hot stage and are uncorrected. ^1H nuclear magnetic resonance spectra (NMR) were recorded with Bruker AC300, AM250, and WH90 using $CDCl_3$ solution. Chemical shifts are reported in parts per million (ppm) with tetramethylsilane (TMS) as an internal standard. Coupling constant (J) are given in hertz; the abbreviations s, d, t, q, and m refer to singlet, doublet, triplet, quartet, and multiplet, respectively. Mass spectra were recorded on a MATT 311 (ionization potential, 3 kV; electron accelerating potential, 70 eV; ion source temperature, 200 °C). Infrared spectra were recorded on a Nicolet 20SX or 5SX using chloroform solutions. UV spectra were recorded with a Cary 2200. Thin-layer chromatography (TLC) was performed with DC Plastiekfolien Kieselgel 60 F254 (E. Merck). UV, iodine vapor, or sulfuric acid were used to visualize the developed plates. Column chromatography was performed with silica gel (Merck, 230–400 mesh grade). Androstenedione was obtained from Schering. Androsta-4,9(11)-diene-3,17-dione was synthesized by dehydration of 9α-hydroxyandrostenedione[43] (Gist-Brocades). Chemicals were obtained from Aldrich and used as received.

Protein concentrations were determined according to the method of Lowry.[44] The aromatase activity was measured by the release of tritium into the aqueous medium from [1β,2β-^3H]androstenedione (New England Nuclear Research Products, 41.8 Ci/mmol). Radioactivity was measured on a LKB1214 (liquid scintillation counter). NADPH tetrasodium salt, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma. An estradiol kit (ER155) was purchased from Baxter-Travenol and PMSG was obtained from Distrivet (Roussel-UCLAF Group).

4,5-Epoxyandrostane-3,17-dione (5). In a 1-L Erlenmeyer flask equipped with a magnetic stirbar, a septum, and an argon inlet was placed 20 g (69.9 mmol) of androstenedione **3** in a 1:10 mixture of CH_2Cl_2/MeOH. H_2O_2 (30% aqueous solution, 34 mL, 332.8 mmol) was added dropwise over a 30-min period to the solution. After stirring of the reaction mixture for 30 min at room temperature, the flask was cooled to 0 °C with an ice bath and 12.6 mL of 4 M aqueous NaOH was added dropwise over 30 min. The flask was left at 4 °C during 12 h, and then the mixture was neutralized (pH paper) by dropwise addition of a 1 N HCl solution. The reaction mixture was concentrated on the rotary evaporator to a volume of about 50 mL. The precipitate was filtered, washed with water, and dried under vacuum to afford a first crop of epoxide (14.75 g). About 500 mL of water was added to the filtrate and the aqueous phase extracted with CH_2Cl_2. The organic extracts were combined, dried ($MgSO_4$), and concentrated to afford a second crop of the epoxide as a white powder (4.29 g, total yield 90%). TLC (AcOEt/hexane 1:1): $R_f = 0.5$. NMR (integration shows a 5:1 mixture of β:α epoxides): δ 0.90 (s, 18-Me of β-isomer), 0.91 (s, 18-Me of α-isomer), 1.09 (s, 19-Me of α-isomer), 1.18 (s, 19-Me of β-isomer), 3.01 (s, H4 of β-isomer), 3.07 (s, H4 of α-isomer). IR: 1735, 1708 cm^{-1}. MS: m/e 302, 284, 274, 259, 230.

4,5-Epoxyandrost-9(11)-ene-3,17-dione (6). The procedure described above for the synthesis of epoxide **5** was used. Starting from 4.69 g (16.5 mmol), 4.95 g of epoxide **6** was obtained as a white powder (99% yield). TLC (AcOEt/hexane 1:1): $R_f = 0.5$. NMR (integration shows a 3:2 mixture of β:α epoxides): δ 0.87 (s, 18-Me), 1.24 (s, 19-Me of α-isomer), 1.33 (s, 19-Me of β-isomer), 2.97 (s, H4 of β-isomer), 3.08 (s, H4 of α-isomer), 5.5 (m, H11) IR: 1734, 1707, 912, 892, 881, and 867 cm^{-1}.

4-Hydroxyandrost-4-ene-3,17-dione (1). Epoxide **5** (6.81 g, 22.5 mm) was added in small portions to a well stirred mixture of concentrated H_2SO_4 (1.36 mL) and AcOH (68 mL). The mixture, which turned progressively orange, was then stirred for 4 h at room temperature under an inert atmosphere (argon). It was then poured into a beaker containing about 550 g of ice and allowed to stand at 4 °C during 12 h. The precipitate was filtered, washed with water and dried under vacuum to afford a first crop of 4-hydroxyandrost-4,9(11)-diene-3,17-dione (**7**) as a white powder (0.7 g). The filtrate was extracted with CH_2Cl_2; the organic extracts were combined, dried ($MgSO_4$), and concentrated on the rotary evaporator and chromatographed (AcOEt/hexane 1:9, 3:7, and 1:1). The first fraction was 4-hydroxyandrost-4-ene-3,17-dione (2.84 g, total yield 52%). Two recrystallizations from hot AcOEt afforded pure **1** as colorless needles. The second fraction of chromatography consisted of a mixture of 2α-acetoxyandrost-4-ene-3,17-dione[45] and 4α-acetoxy-5α-hydroxyandrostane-3,17-dione (1.77 g). Crystallization in ether afforded the latter (0.223 g, yield 3%, mp: 158 °C); the filtrate consisted mainly of the former (yield 23%). The third chromatographic fraction was 2α-hydroxyandrost-4-ene-3,17-dione[40] (0.58 g). **1**: TLC (AcOEt/hexane): $R_f = 0.51$. Mp: 212 °C. NMR: δ 0.99 (s, 18-Me), 1.2 (s, 19-Me), 6.1 (s, OH). IR: 3449, 1732, 1668, 1638 cm^{-1}. MS: m/e 302, 287, 266, 260, 147. UV (EtOH): 277 nM (ε = 12 700); (EtOH, 1 N NaOH) 326 nM (ε = 8700). Anal. ($C_{19}H_{26}O_3$): C, H. 2α-Acetoxyandrost-4-ene-3,17-dione: TLC (AcOEt/hexane 1:1): $R_f = 0.4$. NMR: δ 0.88 (s, 18-Me), 1.37 (s, 19-Me), 2.19 (s, OAc), 5.37

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(dd, J = 13.5 and 5.2, H2), 5.73 (s, H4), 5.85 (m, H11). 4α-Acetoxy-5α-hydroxyandrostane-3,17-dione: TLC (AcOEt/hexane 1:1): R_f = 0.4. Mp: 156 °C. NMR: δ 0.88 (s, 18-Me), 1.14 (s, 19-Me), 2.12 (s, OAc), 4.9 (m, H2). IR: 3585, 1755, 1734 cm^{-1}. MS: m/e 362, 344, 320, 302, 234, 219, 216. 2α-Hydroxyandrost-4-ene-3,17-dione: TLC (AcOEt/hexane 1:1): R_f = 0.29. NMR: δ 0.92 (s, 18-Me), 1.33 (s, 19-Me), 4.27 (dd, J = 13.5 and 6, H2), 3.44 (OH), 5.83 (s, H4). IR: 3645, 3500, 1679, 1615, 863 cm^{-1}. MS: m/e 302, 258. UV (EtOH): 240 nM (EtOH, 1 N NaOH), 235, 325 nM.

4-Hydroxyandrosta-4,9(11)-diene-3,17-dione (7). Using the procedure described for the synthesis of 1, 4.6 g of crude 7 were obtained from 4.95 g (16.4 mmol) of epoxide 6. Flash chromatography (AcOEt/hexane 1:9 then 3:7 then 1:1) gave 4-hydroxyandrostenedione as the first fraction (660 mg, yield 13.6%) (R_f = 0.49, AcOEt/hexane 1:1). One recrystallization from hot ethyl acetate afforded pure 7 as colorless needles (390 mg). The second fraction of chromatography was 2α-acetoxyandrost-4,9-(11)-diene-3,17-dione (500 mg, 9%). The third fraction of chromatography was mainly unpure 2α-hydroxyandrost-4,9-(11)-diene-3,17-dione. 7: TLC (AcOEt/hexane 1:1): R_f = 0.49. Mp: 211 °C. NMR: δ 0.88 (s, 18-Me), 1.36 (s, 19-Me), 6.09 (s, OH). IR: 3449, 1734, 1668, 1643 cm^{-1}. MS: m/e 300, 282, 257, 239, 227. UV (EtOH): 278 nM (ε = 13 000). UV (EtOH, 1 N NaOH): 328 nM (ε = 8950). Anal. ($C_{19}H_{24}O_3$): C, H. 2α-Acetoxyandrost-4-ene-3,17-dione: TLC (AcOEt/hexane 1:1): R_f = 0.37. NMR: δ 0.88 (s, 18-Me), 1.37 (s, 19-Me), 2.19 (s, OAc), 5.37 (dd, J = 13.5 and 5.2, H2), 5.73 (s, H4), 5.85 (m, H 11).

4-Mercaptoandrost-4-ene-3,17-dione (8). Thioacetic acid (6 mL) in dioxane (20 mL) was added to an ice-cooled solution of epoxide 5 (19.04 g, 63 mmol) in dioxane (200 mL). After 4 days of stirring at room temperature under argon, 6 mL of thioacetic acid was added to the dark mixture and it was stirred again for 24 h at room temperature. The mixture was concentrated on a rotary evaporator (equipped with a vacuum pump), then ethyl ether was added (about 600 mL), and the precipitate was filtered to yield 15.8 g of a yellow powder which was identified as 4-(acetylthio)-5-hydroxyandrost-4-ene-3,17-dione. The filtrate was concentrated under reduced pressure and flash chromatographed (AcOEt/hexane 1:1) to give 3.19 g more of the product (total yield 80%). NMR: δ 0.90 (s, 18-Me), 1.07 (s, 19-Me), 2.41 (s, SAc). IR: 3600, 1733, 1687 cm^{-1}. Mp: 206 °C. 4-(Acetylthio)-5-hydroxyandrost-4-ene-3,17-dione (5 g, 13.2 mmol) was dissolved in a 1:1 mixture of MeOH/THF (250 mL), 2.5 mL of concentrated HCl was added, and the mixture was stirred during 12 h at room temperature. Concentrated HCl (2 mL) was again added and the mixture stirred for 24 h more at room temperature. The mixture was then poured on water and crushed ice (about 500 mL) and the precipitate filtered and dried under vacuum to afford 3.52 g (84%) of 8 as a pale yellow powder. TLC (AcOEt/hexane 1:1): R_f 0.48. NMR: δ 0.92 (s, 18-Me), 1.21 (s, 19-Me), 4.76 (s, SH). IR: 2548, 1667, 1574 cm^{-1}. Mp: >260 °C. MS: m/e 318, 191, 155, 141, 128. UV (EtOH): 297 nM (ε = 5250); (EtOH, 0.1 N NaOH) 349 nM. Anal. ($C_{19}H_{26}O_2S$): C, H, S.

4-Mercaptoandrosta-4,9(11)-diene-3,17-dione (9). This compound was prepared as described for 4-mercaptoandrost-4-ene-3,17-dione. Yield: 28%. TLC (AcOEt/cyclohexane 1:1): R_f = 0.51. NMR: δ 0.88 (s, 18-Me), 1.36 (s, 19-Me), 2.85 (dt, $H6_{eq}$), 5.58 (m, H11), 4.77 (SH). Anal. ($C_{19}H_{24}O_2S$): C, H. Mp: 158 °C.

4-Methoxyandrost-4-ene-3,17-dione (10). A 25-mL flame-dried flask was equipped with an argon inlet and a magnetic stirbar, and 122 mg of 4-hydroxyandrost-4-ene-3,17-dione (1, 0.4 mmol) was introduced followed by 5 mL of THF. The solution was cooled down to 0 °C with an ice bath and 45.5 mg (0.4 mmol) of potassium *tert*-butoxide was added all at once. After 5 min of stirring at 0 °C, 0.4 mL (large excess) of methyl iodide was added dropwise and the mixture stirred for 10 min at room temperature. Water (10 mL) was added to the reacting mixture, the aqueous phase was extracted with CH_2Cl_2 (30 mL), and the organic extracts were combined, dried ($MgSO_4$), and concentrated to afford 101.3 mg of crude yellow oil. Flash chromatography (AcOEt/hexane 1:1) gave 10 as a colorless oil that crystallized in diethyl ether (60.7 mg, 48%). TLC (AcOEt/hexane 1:1): R_f = 0.49. NMR: δ 0.92 (s, 18-Me), 1.22 (s, 19-Me), 3.58 (s, OMe). IR: 1735, 1677, 1608 cm^{-1}. MS: m/e 316.

4-(Methoxymethyl)androst-4-ene-3,17-dione (11). A 100-mL flame-dried flask was equipped with an argon inlet and a magnetic stirbar and 1.35 g of 4-hydroxyandrost-4-ene-3,17-dione (1, 4.4 mmol) was introduced following by 40 mL of dry THF. The solution was cooled down to 0 °C with an ice bath and 605 mg (1.1 equiv) of potassium *tert*-butoxide was added all at once. After 30 min of stirring at 0 °C, 0.482 mL (1.1 equiv) of bromomethyl methyl ether was added dropwise and the mixture stirred for 30 min at room temperature. Saturated aqueous $NaHCO_3$ (40 mL) was added to the reacting mixture, the aqueous phase was extracted with CH_2Cl_2 (60 mL), and the organic extracts were combined, dried ($MgSO_4$), and concentrated. The crude yellow oil was rapidly flash chromatographed (AcOEt/hexane 3:7 then 1:1) to afford 11 as a pale yellow oil (800 mg, 53%). Two recrystallizations from diethyl ether gave pure 11 as white crystals. TLC (AcOEt/hexane (1:1): R_f = 0.66 NMR: δ 0.92 (s, 18-Me), 1.23 (s, 19-Me), 3.80 (s, OMe), 4.91 (AB, J = 6, OCH_2O). IR: 1735, 1675, 1609 cm^{-1}. Anal. ($C_{21}H_{30}O_4$): C, H.

4-[(Difluoromethyl)thio]androst-4-ene-3,17-dione (12). A 50-mL flame-dried flask was equipped with an argon inlet and a magnetic stirbar, and 1 g of 4-mercaptoandrost-4-ene-3,17-dione (8, 3.14 mmol) was introduced followed by 20 mL of dry THF. The solution was cooled down to 0 °C with an ice bath and 422.6 mg (1.2 equiv) of potassium *tert*-butoxide was added all at once. The mixture turned pale orange. After 5 min of stirring at 0 °C, chlorodifluoromethane was bubbled into the solution through the septum by means of a large needle and the mixture was then stirred for 12 h at room temperature. The mixture was concentrated under reduced pressure and the crude yellow oil was flash chromatographed (AcOEt/hexane 3:7 then 1:1) to afford 12 as a pale yellow oil (610 mg, 53%). One recrystallization from diethyl ether gave pure 12 as cubic orange crystals. TLC (AcOEt/hexane 1:1): R_f = 0.46. NMR: δ 0.93 (s, 18-Me), 1.29 (s, 19-Me), 6.91 (t, J = 59, CHF_2). IR 1735, 1680, 1559, 1050–1070 cm^{-1}. MS: m/e 368, 317, 285, 267. Mp: 129 °C. Anal. ($C_{20}H_{26}O_2SF_2$) C, H, F, S.

4-[(Fluoromethyl)thio]androst-4-ene-3,17-dione (13). A described procedure was applied for the synthesis of fluorobromomethane.[46] A 100-mL flame-dried flask was equipped with an argon inlet and a magnetic stirbar, and 2 g of 4-mercaptoandrost-4-ene-3,17-dione (8, 6.28 mmol) was introduced followed by 80 mL of dry THF. The solution was cooled down to 0 °C with an ice bath and 422.6 mg (1.2 equiv) of potassium *tert*-butoxide was added all at once. The mixture turned pale orange. After 5 min of stirring at 0 °C, bromofluoromethane was bubbled into the solution through the septum by means of a large needle and a stream of nitrogen during 2 h at room temperature. Saturated aqueous NH_4Cl (80 mL) was added to the reacting mixture, the aqueous phase was extracted with CH_2Cl_2 (120 mL), and the organic extracts were combined, dried ($MgSO_4$), and concentrated. The crude yellow oil was flash chromatographed (AcOEt/hexane 3:7) to afford 13 as a pale yellow oil (457 mg, 21%). One recrystallization from diethyl ether gave pure 13 as colorless crystals. TLC (AcOEt/hexane 1:1): R_f = 0.56. NMR: δ 0.93 (s, 18-Me), 1.28 (s, 19-Me), 5.49 (d, J = 53, CH_2F). IR: 1735, 1675, 1560, 1050–1070 cm^{-1}. MS: m/e 368, 317, 285, 267. Mp: 129 °C. Anal. ($C_{20}H_{27}O_2SF$): C, H, F, S.

The other 4-(alkylthio)androstenediones (14–20) were obtained similarly.

4-[(Chloromethyl)thio]androst-4-ene-3,17-dione (14). Starting from 4-mercaptoandrost-4-ene-3,17-dione (8, 1 g, 3.14 mmol), potassium *tert*-butoxide (422.6 mg, 1.2 equiv), and bromochloromethane (0.43 mL, 1.5 equiv) in 50 mL of dry THF, the product was obtained as a white powder after flash chromatography and crystallization from diethyl ether. Yield: 36%. TLC (AcOEt/hexane 1:1): R_f = 0.48. NMR: δ 0.93 (s, 18-Me), 1.3 (s, 19-Me), 3.65–3.8 (s, $H6_{eq}$), 4.81 (s, SCH_2Cl). IR 1734, 1675, 1555 cm^{-1}. MS: m/e 366, 330, 315, 300. Mp: 174 °C. Anal. ($C_{20}H_{27}O_2SCl$): C, H, S, Cl.

4-(Methylthio)androst-4-ene-3,17-dione (15). Starting from 4-mercaptoandrost-4-ene-3,17-dione (8, 1 g, 3.14 mmol), potassium

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tert-butoxide (387 mg, 1.1 equiv), and methyl iodide (0.4 mL) in 40 mL of dry THF, the product was obtained as off-white needles after flash chromatography and crystallization from diethyl ether. Yield: 58%. TLC (AcOEt/hexane 1:1): R_f = 0.55. NMR: δ 0.93 (s, 18-Me), 1.25 (s, 19-Me), 2.20 (s, SMe). IR: 1735, 1673, 1556 cm^{-1}. Mp: 134 °C. Anal. ($C_{20}H_{28}O_2S$): C, H, S.

4-[(Nitromethyl)thio]androst-4-ene-3,17-dione (16). Starting from 4-mercaptoandrost-4-ene-3,17-dione (8, 1 g, 3.14 mmol), potassium *tert*-butoxide (387 mg, 1.1 equiv), and bromonitromethane (0.263 mL, 1.2 equiv) in 40 mL of dry THF, the product was obtained as a dark oil after flash chromatography. It could not be crystallized. Yield: 12%. TLC (AcOEt/hexane 1:1): R_f = 0.5. NMR: δ 0.93 (s, 18-Me), 1.28 (s, 19-Me), 3.61 (ddd, $H6_{eq}$), 5.34 (AB, J = 12.5, CH_2NO_2). IR: 1735, 1677, 1553 cm^{-1}.

4-[(Cyanomethyl)thio]androst-4-ene-3,17-dione (17). Starting from 4-mercaptoandrost-4-ene-3,17-dione (8, 1 g, 3.14 mmol), potassium *tert*-butoxide (387 mg, 1.1 equiv), and bromoacetonitrile (0.263 mL, 1.1 equiv) in 40 mL of dry THF, the product was obtained as yellow crystals after flash chromatography and crystallization from diethyl ether. Yield: 57%. TLC (AcOEt/hexane 1:1): R_f = 0.52. NMR δ 0.93 (s, 18-Me), 1.31 (s, 19-Me), 3.51 and 3.60 (2 d, J = 17, SCH_2CN), 3.75 (dt, $H6_{eq}$). IR: 2232, 1735, 1675, 1556 cm^{-1}. Anal. ($C_{21}H_{27}O_2SN$): C, H, S, N.

4-[[(Methylthio)methyl]thio]androst-4-ene-3,17-dione (18). Starting from 4-mercaptoandrost-4-ene-3,17-dione (8, 1 g, 3.14 mmol), potassium *tert*-butoxide (387 mg, 1.1 equiv), and chloromethyl methyl thioether (0.313 mL, 1.2 equiv) in 40 mL of dry THF, the product was obtained as a yellow powder after flash chromatography and crystallization from diethyl ether. Yield: 47%. TLC (AcOEt/hexane 1:1): R_f = 0.53. NMR: δ 0.93 (s, 18-Me), 1.28 (s, 19-Me), 2.14 (s, SMe), 3.82 (AB, SCH_2S). IR: 1735, 1673, 1557 cm^{-1}. Anal. ($C_{21}H_{30}O_2S_2$): C, H, S.

4-[(Methoxymethyl)thio]androst-4-ene-3,17-dione (19). Starting from 4-mercaptoandrost-4-ene-3,17-dione (8, 1 g, 3.14 mmol), potassium *tert*-butoxide (387 mg, 1.1 equiv), and bromomethyl methyl ether (0.308 mL, 1.2 equiv) in 40 mL of dry THF, the product was obtained as an orange powder after flash chromatography and crystallization from diethyl ether. Yield: 31%. TLC (AcOEt/hexane 1:1): R_f = 0.5. NMR: δ 0.93 (s, 18-Me), 1.26 (s, 19-Me), 3.74 (s, OMe), 3.76 (ddd, $H6_{eq}$), 4.69 and 4.73 (2 d, SCH_2O). IR: 1735, 1674, 1558 cm^{-1}. Anal. ($C_{21}H_{30}O_3S$): C, H, S.

4-[(Chloromethyl)thio]androsta-4(9,11)-diene-3,17-dione (20). Starting from 4-mercaptoandrosta-4,9(11)-diene-3,17-dione (9, 200 mg, 0.6 mmol), potassium *tert*-butoxide (85 mg, 1.2 equiv, 0.8 mmol), and bromochloromethane (60 μL, 1.5 equiv, 0.9 mmol) in 10 mL dry THF, the product was obtained as a yellow solid after flash chromatography and crystallization from diethyl ether/pentane. Yield: 45%. TLC (AcOEt/cyclohexane 1:1): R_f = 0.73. NMR: δ 0.9 (s, 18-Me), 1.44 (s, 19-Me), 3.78 (dt, $H6_{eq}$), 4.81 (s, SCH_2Cl), 5.61 (m, H11). IR: 1559, 1675, 1736 cm^{-1}. Mp: 116 °C. Anal. ($C_{20}H_{25}O_2SCl$): C, H, S, Cl.

Synthesis of 17-Oxoandrost-3-eno[3,4-*b*][1,3]oxathiole-5-carbonitrile (22) and Androsta-3,5-dieno[3,4-*b*][1,3]oxathiol-17-one (21). 4-[(Chloromethyl)thio]androstene-3,17-dione (14) (210 mg, 0.57 mmol) and 55 mg (1.5 equiv) of potassium cyanide were stirred for 12 h at room temperature in 1 mL of DMF. The crude mixture was concentrated and flash chromatographed (AcOEt/hexane 3:7) to afford a first fraction of **21** (22 mg, 12%, R_f = 0.63, AcOEt/hexane 1:1) as a white solid. The second fraction was compound **22** (58 mg, 28%). **21**: IR: 1734, 1657, 1634 cm^{-1}. NMR: δ 0.92 (s, 18-Me), 1.03 (s, 19-Me), 5.16 (s, H5), 5.51 (s, CH_2S). **22**: IR: 1735, 1671, 1617 cm^{-1}. NMR (2:1 mixture of isomers): δ 0.88 (s, 18-Me), 0.99 and 1.29 (2 s, 19-Me), 5.57 and 5.59 (2 s, CH_2S).

4-(Methylthio)androsta-4,6(7),9(11)-triene-3,17-dione (24). 4-(Methylthio)androsta-4,9(11)-diene-3,17-dione (**23**) (300 mg, 0.9 mmol), 1.11 mL (7 mmol, 7.5 equiv) of triethyl orthoformate, 1.5 mg of *p*-toluenesulfonic acid, and 7.5 mL of ethanol were stirred for 2 h at room temperature, and then 0.4 mL of triethylamine was added. The mixture was poured into 50 mL of a saturated solution of $NaHCO_3$ and the aqueous phase extracted with CH_2Cl_2 (3 × 20 mL). The organic extracts were dried and concentrated to afford 200 mg of crude product which was used without further purification. The crude enol ether (200 mg, 0.6 mmol), chloranile (250 mg, 1 mmol, 1.7 equiv), and 9.3 mL of a 95:5 mixture of acetone/H_2O were stirred at room temperature for 12 h. The mixture was poured into 10 mL of a 10% aqueous solution of sodium thiosulfate, then 10 mL of a saturated solution of sodium bicarbonate was added, and the mixture was stirred for 1 h at room temperature. The aqueous phase was extracted with CH_2Cl_2, and the organic extracts were dried, concentrated, and flash chromatographed (AcOEt/cyclohexane 2:8) to afford 100 mg (33.5%) of **24** as a white foam. TLC (AcOEt/cyclohexane 1:1): R_f = 0.61. IR: 1738, 667, 1616, 1526 cm^{-1}. NMR: δ 0.96 (s, 18-Me), 1.33 (s, 19-Me), 3.08 (dq, J = 12, 2, H8), 6.63 (dd, J = 10, 2.5, H6), 7.53 (dd, J = 10, 2.5, H7), 5.56 (m, H11), 2.23 (s, SMe). Anal. ($C_{20}H_{22}O_2S$): C, H, S.

4-[(Fluoromethyl)thio]androsta-4,6(7),9(11)-triene-3,17-dione (25). To 300 mg (9 mmol) of compound **24** dissolved in 10 mL of CH_2Cl_2 was added 176 mg (1 mmol, 1.1 equiv) of mCPBA at 0 °C. After stirring for 40 min at that temperature, 5 mL of a saturated solution of $NaHCO_3$ was added and the aqueous phase was extracted with CH_2Cl_2. The organic extracts were dried and concentrated to afford 310 mg (98%) of sulfoxide as a white foam (R_f = 0.06, AcOEt/cyclohexane 1:1) which was used without further purification.

To 170 mg (0.5 mmol) of sulfoxide dissolved in 5 mL of chloroform and stirred at −78 °C was added 0.14 mL (1 mmol, 2 equiv) of DAST. The mixture was stirred for 12 h at room temperature and then refluxed for 12 h. A 5-mL portion of a saturated solution of $NaHCO_3$ was added and the aqueous phase extracted with CH_2Cl_2. The organic extracts were dried, concentrated, and flash chromatographed with AcOEt/cyclohexane 3:7 to afford 75 mg (43.8%) of the fluoro compound as a yellow solid. R_f = 0.6 (AcOEt/cyclohexane 1:1). NMR: δ 0.97 (s, 18-Me), 1.37 (s, 19-Me), 3.1 (dq, J = 11 and 2.5, H8), 5.35–5.75 (m, CH_2F), 5.60 (m, H11), 6.4 (dd, J = 10, 2.5, H6), 7.25 (dd, J = 10, 2.5, H7).

Preparation of Human Placental Microsomes. Placentae from term nonsmoking women were collected right after delivery, processed within 2 h, and stored at −20 °C. The microsomes were prepared as described by Ryan.[26] The final pellet obtained was resuspended in 50 mM phosphate buffer (pH 7.4), aliquoted, and stored in liquid nitrogen.

K_m and V_{max} Determination. To 970 μL of 0.05 M phosphate buffer containing glucose 6-phosphate (2.5 mM) and glucose 6-phosphate deshydrogenase (264 units/mL) were added successively 10 μL of microsomal suspension (protein concentration, 5 mg/mL) and 10 μL of [1β,2β-^{3}H]androstenedione to give final concentrations ranging from 50 to 1000 nM. The reaction was started by adding 10 μL of an aqueous solution of NADPH (10 mM). The tritium release assay was conducted for 10 min at 37 °C, after which the incubations were stopped by the addition of 300 μL of a 1 mM $HgCl_2$ solution followed by 1 mL of an aqueous suspension of 5% DCC (dextran-coated charcoal). The tubes were then vigorously vortexed. After decanting (4 °C) during 30 min, the tubes were centrifuged (10 min, 3500 rpm) and an aliquot (100 μL) of the supernatant was removed and mixed with 10 mL of LSC cocktail prior to LS counting. The K_m and V_{max} values were determined by subtracting the dpm values from incubations made without enzyme from their respective incubations made in the presence of enzyme, followed by the least square analysis of the double reciprocals of background-corrected results versus androstenedione concentrations. Each point was determined in triplicate.

IC_{50} Determinations. Various concentrations of inhibitor (as 10 μL/mL from stock solutions made in DMSO) ranging from 10^{-5} to 10^{-8} M were added to incubations containing 50 μg/mL of microsomal protein in phosphate buffer containing glucose 6-phosphate (2.5 mM) and glucose-6-phosphate dehydrogenase (264 units/mL). DMSO alone was added for the controls. The substrate [1β,2β-^{3}H]androstenedione (isotopic dilution 8) was added as 10 μL/mL of an ethanolic solution to reach a final concentration of $10K_m$. The reaction was started by addition of NADPH (100 μM final concentration). Tritium-release assays were conducted at 37 °C for 10 min. The percent of aromatase activity was determined by dividing the residual aromatase activity (DPM) in the presence of inhibitor after 10-min incubation by the activity of the enzyme without inhibitor at the same time and multiplying by 100. The 100% was then obtained for [I] = 0.

Time-Dependent Concentration-Dependent Inhibition Experiments. Incubations contained 50 μg/mL of microsomal

protein and 100 μM NADPH in phosphate buffer containing glucose 6-phosphate (2.5 mM) and glucose-6-phosphate dehydrogenase (264 units/mL); inhibitors were added as 10 μL/mL from stock solutions made in DMSO. Different concentrations of inhibitor were used. DMSO alone was added for the controls.

Microsomes, inhibitors, and the cofactors were preincubated together during different times (up to 10 min). Then 10 μL of an ethanolic solution of [1β,2β-^{3}H]androstenedione was added to a $10K_m$ substrate concentration. Tritium-release incubations were then conducted at 37 °C for 10 min as described above. Background radioactivity was determined in incubations lacking enzyme.

The two methods described by Nelson[30] for plotting the ln of the percent of activity remaining versus time were used. In method 1, which was used to calculate K_I and k_{inact} values, the percent of the activity remaining was determined by dividing the activity in the presence of inhibitor after preincubation by the activity in the presence of inhibitor at the beginning of the preincubation ($t = 0$) and multiplying by 100. The values of k_{obs} were obtained by plotting the ln of the percent activity remaining versus time of preincubation for each concentration of inhibitor. Least square analysis of the double-reciprocal plots of k_{obs} versus inhibitor concentrations allowed the K_I and k_{inact} values to be determined. In method 2, the percent of activity remaining was determined by dividing the activity in the presence of inhibitor after preincubation by the activity present at $t = 0$ with no inhibitor added and multiplying by 100.

Experiments without NADPH. Time-dependent experiments were done as described above, except the cofactor NADPH was omitted in the preincubation but was given at the same time as the substrate.

β-Mercaptoethanol (β-MSH) and Substrate Protection Experiments. They were conducted as described above for the time-dependent experiments including β-MSH (0.5 and 10 mM) or substrate (0.5 and 1 μM of nontritiated androstenedione) in the preincubation buffer. The results are expressed as described above and compared to controls (without β-MSH or without substrate in the preincubation).

Irreversibility of Inhibition Caused by 14. In 5 mL of total volume, at 37 °C, 10 mg of microsomal protein was incubated in phosphate buffer with 0.5 and 0.05 μM 14 in the presence of 5 mM NADPH; G6P (50 mM) and G6PDase (264 IU/mL) were added to ensure against depletion of reduced cofactor. After 2 h, each incubation was stopped in ice and an aliquot was checked for aromatase activity. The rest was dialyzed (4 °C) against 500 mL of incubating phosphate buffer. This was replaced every 2 h by fresh buffer and an aliquot was withdrawn for aromatase assay after 8 and 24 h of dialysis. The results are expressed as percent of control (obtained with no inhibitor present).

In Vivo Experiments. Female Wistar rats from IFFA-CREDO (Lyon, France) were fed an ad libitum diet and housed under conditions of controlled temperature and humidity. They were maintained on a 12 h light/12 h dark cycle. Female rats 60 days old were injected with PMSG (100 IU/rat every 2 days for 10 days). On day 11, compound 13 or 4-OH-A (1) was given subcutaneously (50 mg/kg), intravenously (5 and 2.5 mg/kg), or orally (50 mg/kg). 2 h later, the animals were sacrificed by decapitation. Total trunk blood was collected and the estradiol measured by RIA. The ovaries were collected and ovarian microsomes were prepared as described.[6] Aromatase activity was assessed as with human placental microsomes. (*P < 0.05, **P < 0.01[47]).

Acknowledgment. We wish to thank our colleagues and their collaborators in the Physical Chemistry Department and the Analytical Laboratory for their help in recording and interpreting the spectra and performing the elemental analysis, respectively. We thank Dr. J. Leclaire for stimulating discussions. We are grateful to the Maternité des Lilas for providing the placentae.

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Novel Anthraquinone Inhibitors of Human Leukocyte Elastase and Cathepsin G[1]

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A large series of variously substituted anthraquinones has been synthesized and assayed for inhibitory capacity against human leukocyte elastase (HLE) and cathepsin G (CatG), two serine proteinases implicated in diseases characterized by the abnormal degradation of connective tissue, such as pulmonary emphysema and rheumatoid arthritis. It was found that 2-alkyl-1,8-dihydroxyanthraquinone analogues are competitive inhibitors of HLE with IC_{50} values ranging from 4 to 10 μM, and also inhibit CatG with IC_{50} values ranging from 25 to 55 μM. Consequently, analogues containing the 2-alkyl-1-hydroxy-8-methoxyanthraquinone substitution pattern inhibit HLE to the same magnitude as for the compounds above, but show very little inhibition of CatG. Anthraquinones containing long, hydrophobic *n*-butyl carbonate moieties in the 1- and 8-positions in conjunction with a third hydrophobic substituent in the 2- or 3-position are highly selective for HLE, with K_i values in the range of 10^{-7} M. All of the inhibitors described are completely reversible, with no evidence of acyl-enzyme formation detected.

Introduction

Human leukocyte elastase (HLE) and cathepsin G (CatG) are two serine proteinases derived from the azurophil granules of human neutrophils and have been implicated in various pathological states associated with abnormal degradation of connective tissue, such as rheumatoid arthritis, adult respiratory distress syndrome, and pulmonary emphysema.[2] Both of these enzymes have been shown to attack lung elastin, which is the basis of their implication in abnormal connective tissue degradation. The most widely accepted explanation of the observed pathogenesis is the proteinase–antiproteinase imbalance[3] postulate, which reasons that a deficiency in the naturally occurring inhibitor, α-1-proteinase inhibitor (α-1-antitrypsin), allows extracellular proteinases to act unchecked, thus resulting in uncontrolled connective tissue destruction. This postulate is supported by the fact that persons with genetic deficiencies in the α 1-PI gene are strongly associated with development of pulmonary emphysema. Additionally, it has been shown[4] that the oxi-

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(1) This material was presented in April 1991 at the 201st National Meeting of the American Chemical Society in Atlanta, Georgia (Division of Medicinal Chemistry Abstract #36).

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0022-2623/92/1835-1597$03.00/0