adequate data of this type are not available for analogy studies. We therefore feel that experiments should be performed to create such data.

Acknowledgment. We are grateful for support from the Swedish Natural Science Research Council and the Institute of Applied Mathematics, Stockholm.

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Synthesis and Biochemical Evaluation of Inhibitors of Estrogen Biosynthesis[1]

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The synthesis and biochemical evaluation of various C_{19}-steroidal derivatives as inhibitors of estrogen biosynthesis are described. Steroids with substitutions on the A or B ring were synthesized by Michael addition of various thiol reagents to appropriate dienone intermediates. An in vitro assay employing the microsomal fraction isolated from human term placenta was used to evaluate aromatase inhibitory properties. Agents exhibiting high inhibitory activity were further evaluated in initial velocity studies (low product formation) to determine apparent K_i values. Several 7α-substituted androst-4-ene-3,17-diones were effective competitive inhibitors and have apparent K_i values equal to or less than the apparent K_m of 0.063 μM for the substrate androstenedione.

Inhibitors of estrogen biosynthesis have potential use as pharmacological tools and therapeutic agents. Such compounds can aid in the evaluation of the structural requirements of the enzymatic site, the purification of the aromatase enzyme, and the determination of estrogen function in biochemical processes. Therapeutically, aromatase inhibitors have potential use in the control of reproduction since a decrease in estrogen levels would result in insufficient uterine development. A more immediate use of inhibitors of estrogen biosynthesis would be in the treatment of disease states. A potent aromatase inhibitor would be a possible alternative to endocrine ablation in the treatment of advanced estrogen-dependent mammary carcinoma.

Four reports have appeared in the literature concerning inhibitors of estrogen biosynthesis. Schwarzel et al.,[3] Schubert et al.,[4] and Siiteri and Thompson[5] studied various available steroids for their ability to block the aromatization of androstenedione. The fourth report by Bellino et al.[6] examined bromoandrogens for their ability to inactivate the enzymatic site.

The objective of this research was to develop new agents as inhibitors of estrogen biosynthesis. At the outset, the research problem was considered to involve four steps: (1) to synthesize "lead" compounds as potential inhibitors; (2) to develop a screening assay for inhibitors and evaluate the feasibility of the study; (3) to synthesize additional inhibitors based on the screening results; and (4) to perform follow-up kinetic analysis on the more effective inhibitors.

Previous studies[3,7,8] indicated that C_{19} steroids resembling the substrate androstenedione most effectively interact with the active site of aromatase. On this basis, it was reasoned that effective inhibitors should retain the C_{19}-steroid nucleus as well as ketonic functions at the 3 and 17 positions. The introduction of various substituents

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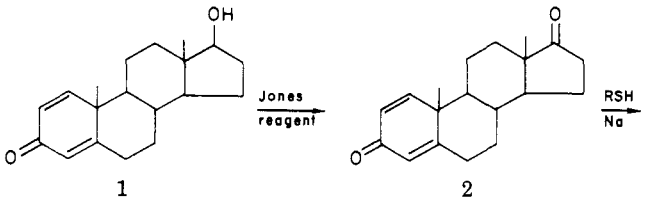
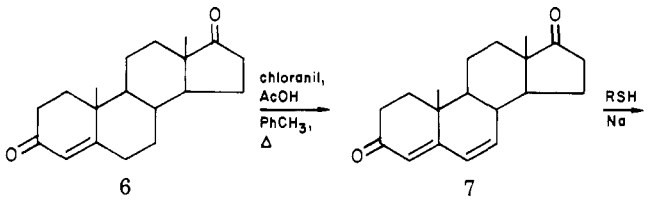
Scheme I



Scheme II



on the A or B ring of androstenedione could result in favorable and possibly enhanced interactions of the steroid derivatives with the enzyme. Since the effect of such substitution on binding to the enzyme was not known, model or lead compounds were synthesized and evaluated to determine the feasibility of this approach.

Chemistry. Synthetic efforts concentrated on the introduction of substituents at the C-1 or C-7 position of androstenedione. Steroids containing a 1α or 7α substituent have been reported in the literature and arise from a 1,4 or 1,6 addition of various nucleophiles to a 1,4-dien-3-one or a 4,6-dien-3-one steroid, respectively. Dodson and Tweit[9] and Schaub and Weiss[10] added certain alkylthiol and acylthiol reagents to 4,6-dien-3-one steroids. Later, several 7α-alkylthioandrostanes were synthesized by addition of the thiols using sodium methylate or a strongly basic anion-exchange resin.[11] During the course of this research, the synthesis of 7α- and 7β-alkyl steroids using various lithium dialkylcuprate reagents was reported.[12] 1α-Substitution has been achieved by the addition of cyanide ion[13] or nitroalkanes[14] to the 1,4-dien-3-one system.

The synthesis of androstenedione derivatives substituted on the A or B ring by the reaction of thiols with dienones was particularly attractive because of the ready availability of dienone and thiol starting materials. The sequences employed in the preparation of 1α- and 7α-substitutions are shown in Schemes I and II. 17β-Hydroxyandrosta-1,4-dien-3-one (1) was converted to the dione **2** using Jones reagent,[15] and androsta-4,6-diene-3,17-dione (**7**) was synthesized from androstenedione (**6**) using chloranil and acetic acid.[16] The procedure of Kaneko et al.[11] for thiol additions was modified for the synthesis, resulting in high yields of product. If the thiol reagent was a liquid, the dienedione was dissolved in excess thiol under nitrogen and metallic sodium was added to begin the reaction. If the thiol was a solid, dioxane was used as the solvent, again with the sodium added last. Thiol reagents not commercially available were prepared following reported procedures.[17,18]

Table I. Screening Results of Synthesized Compounds

	R	mp, °C	yield, %	% inhibn[a] 12.5 μM	% inhibn[a] 6.25 μM
	1α-substituted derivatives				
3	$-SC_6H_5$	190-191	85	24.2	21.2
4	$-SCH_2C_6H_5$	170-174	65	15.5	20.8
5	$-SCH_2C_6H_4$-p-$N(CH_2CH_3)_2$	219-221	39	36.3	32.2
	7α-substituted derivatives				
8	$-S(CH_2)_3CH_3$	152-153	58	74.1	62.4
9	$-SC_6H_5$	233-235	84	49.7	45.3
10	$-SCH_2C_6H_5$	206-208	98	84.1	66.7
11	$-SCH_2C_6H_4$-p-$N(CH_2CH_3)_2$	179-181	43	42.3	45.1
12	$-SCH_2CO_2CH_2CH_3$	135-137	37	72.4	
13	$-S(CH_2)_2C_6H_5$	130-131	47	82.8	75.7
14	$-S(CH_2)_3C_6H_5$	178-179	69	66.4	68.4
15	$-SC_6H_4$-p-OCH_3	261-263	33	64.9	35.3
16	$-SCH_2C_6H_4$-p-OCH_3	220-222	41	63.0	64.0
17	$-SC_6H_4$-p-NH_2	254-256	91	95.0	55.6

[a] Maximum variation of ±10%.

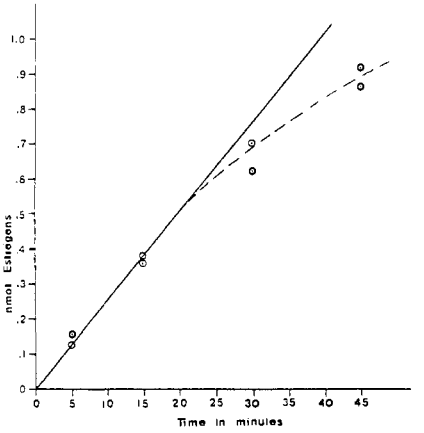


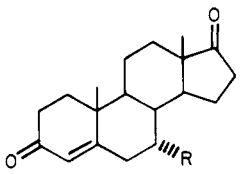
Figure 1. Linearity of screening assay.

The initial series of compounds synthesized (**3–11**) is listed in Table I. As a consequence of the preliminary screening results, synthesis continued on various 7α-substituted derivatives (**12–17**). Synthetic efforts focused on the synthesis of derivatives containing an aromatic moiety at the 7α position since numerous chemical substituents can be introduced on an aromatic ring which are less feasible with aliphatic substituents (e.g., photoaffinity groups, alkylating moieties, and radionuclides).

Biochemical Results. An in vitro screening assay was developed for the preliminary evaluation of the newly synthesized compounds. The enzyme system was isolated from the microsomal fraction of human placental tissue.[19] The common assay for enzymatic activity employed [^{14}C]androstenedione as the substrate and analyzed the amount of [^{14}C]estrone and [^{14}C]estradiol formed.[3,19] Recently, a ^3H_2O assay has been introduced which analyzes the amount of ^3H_2O released from [1β,2β-^{3}H]androstenedione during aromatization.[20,21] The [^{14}C]androstenedione assay was employed in our studies because of familiarity with that type of assay.

Based on the results of a preliminary kinetic study, a screening assay was developed to determine the possible inhibitory activity of the compounds synthesized. The androstenedione concentration was 1.25 μM, with the

Table II. K_i Values of Selected Inhibitors[a]



compd	R	app K_i, μM	inhibn
8	$-SCH_2CH_2CH_2CH_3$	0.057	competitive
10	$-SCH_2C_6H_5$	0.069	competitive
13	$-SCH_2CH_2C_6H_5$	0.062	competitive
16	$-SCH_2C_6H_4$-p-OCH_3	0.031	competitive
17	$-SC_6H_4$-p-NH_2	0.018	competitive

[a] Apparent K_m for androstenedione, 0.063 μM ± 0.003.

inhibitors screened at concentrations of 6.25 and 12.5 μM (5 and 10 times [s]). Figure 1 shows that under these conditions the formation of estrogens was linear with time for 20 min.

Table I contains the results of the screening assays for the initial series of compounds and the additional 7α-substituted androstenediones synthesized. The screening assays were run in duplicate, and the amount of estrogens formed was averaged. The values were then compared to control samples (no inhibitor present) run simultaneously and are reported as the percent inhibition of control samples. The term "inhibition" represents the ability of various compounds to block the conversion of [^{14}C]-androstenedione to [^{14}C]estrogens. It could not be determined from this preliminary study whether the compounds were true inhibitors or acting as alternate substrates.

The results of the screening assays are qualitative and serve to indicate which compounds are effective inhibitors. The conditions of these assays, where [s] = 1.25 μM and [I] = 12.5 μM, are well above the K_m and K_i values determined in later studies and thus are not truly representative of physiological conditions. The fact that these screening assays were performed at high concentrations can explain the large variation (maximum ± 10%) and the varying differences of inhibition at the two inhibitor concentrations. Nevertheless, this screening assay was an important "first approximation" of inhibitory activity and an important guide in choosing compounds for further examination.

Compounds exhibiting effective inhibition in the screening assays were evaluated more thoroughly to determine apparent K_i values. The data were plotted in both Michaelis–Menten and Lineweaver–Burk fashions. The apparent K_m for androstenedione and the apparent K_i for the inhibitors were determined from the Lineweaver–Burk plots and a weighted linear regression program.[22] In addition, the type of inhibition exhibited by the compounds was determined from the Lineweaver–Burk plots. The apparent K_i values of compounds evaluated in this manner are shown in Table II. In all studies, the apparent K_m for androstenedione was found to be 0.063 ± 0.003 μM. Also, each compound evaluated demonstrated competitive inhibition, as determined from the Lineweaver–Burk plots. Figures 2 and 3 are the reciprocal plots for compounds 13 and 17; all other compounds examined gave similar plots.

Compounds 8 and 10 of the initial series were selected as representative compounds for evaluation of their susceptibility to aromatization, i.e., for their ability to serve as alternate substrates. These agents were each incubated with aromatase under conditions similar to those employed

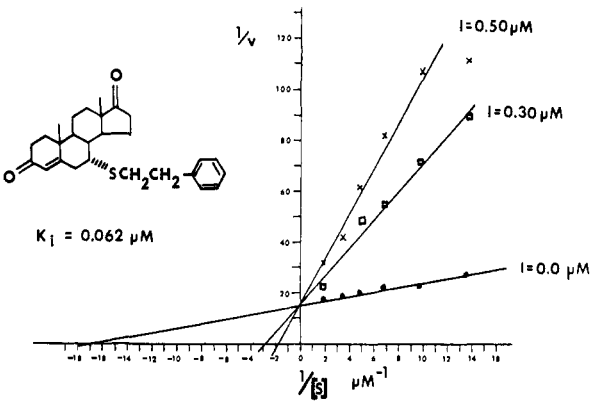


Figure 2. Lineweaver–Burk plot for compound 13. Velocity (v) is expressed as nanomoles of estrogens formed per milligram of microsomal protein per minute. Each point represents an average of two samples. The variation between duplicate samples was less than 5% in all cases. The maximum conversion of androstenedione to estrogens during the assays was 7.2% and was observed in the control samples where [s] = 0.515 μM.

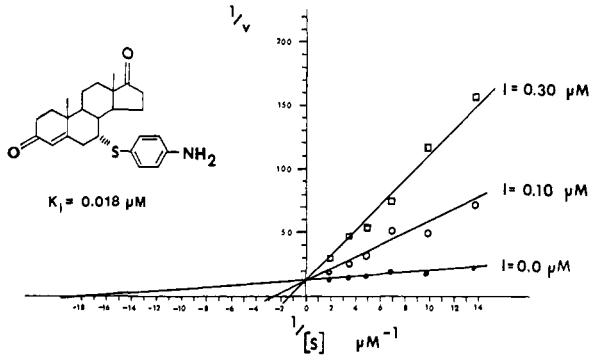


Figure 3. Lineweaver–Burk plot for compound 17. Velocity (v) is expressed as nanomoles of estrogens formed per milligram of microsomal protein per minute. Each point represents an average of two samples. The variation between duplicate samples was less than 5% in all cases. The maximum conversion of androstenedione to estrogens during the assays was 7.8% and was observed in the control samples where [s] = 0.515 μM.

in the screening assay. Following the workup, extracted steroids were separated by TLC. The presence of phenolic steroids on TLC plates was determined by UV absorption, iodine staining, and Folin–Ciocalteau's phenol reagent.[23] Phenolic products were detected in a control sample employing androstenedione as the substrate but not in the samples using compounds 8 or 10 as substrates. Derivatization of extracted steroids with dansyl chloride,[24] followed by TLC, confirmed these results. In vitro metabolism of these synthesized compounds has not been further examined at this time.

Finally, the 7α-butylthio- and 7α-benzylthioandrostenediones were evaluated in vivo for general hormonal activity. These inhibitors did not exhibit estrogenic, progestational, androgenic, or anabolic activity in standard animal screening assays.[25]

Conclusions

The 1α-substituted androstenediones exhibited poor inhibition of estrogen biosynthesis. Since the enzymatic oxidations of androstenedione to estrone occur at the C-19 and C-2 positions,[26] functionality near these regions might be expected to interfere with appropriate interaction of the substrate at the active site.

Several 7α-substituted compounds, on the other hand, were effective inhibitors of estrogen biosynthesis. The apparent K_i values of inhibitors analyzed ranged from

0.018 to 0.069 μM, with all compounds exhibiting competitive inhibition. Both alkyl and aryl substituents at the 7α position led to effective inhibitors. The most effective inhibitor tested was 7α-(4 -amino)phenylthioandrost-4-ene-3,17-dione (17) with an apparent K_i of 0.018 μM. To date, this is the most potent in vitro inhibitor of aromatase. Further in vitro[27] and in vivo studies with these inhibitors are currently in progress.

Experimental Section

A. Synthetic Methods. Infrared spectra of KBr pellets or neat samples were recorded on either a Perkin-Elmer 337 or a Perkin-Elmer 281 spectrophotometer. Proton magnetic resonance spectra were obtained with either a Varian A-60A or a Varian EM-360A spectrophotometer, in parts per million downfield from tetramethylsilane in $CDCl_3$. Mass spectra were recorded on a Du Pont 21-490 mass spectrometer. Melting points were determined on either a Thomas-Hoover capillary melting point apparatus or a Mel-Temp apparatus and are uncorrected unless otherwise indicated. Elemental analyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich., or Midwest Microlab, Ltd., Indianapolis, Ind. Analytical thin-layer chromatography was done with polyethylene-backed silica gel plates and polyethylene-backed alumina plates, obtained from Eastman Kodak, Rochester, N.Y., and both contained fluorescent indicator. Adsorbents for column chromatography were silica gel (60–200 mesh, Davison Chemical) and aluminum oxide (activity II–III, E. Merck). Dioxane and THF were dried by distillation from CaH_2; benzene and toluene were dried over sodium metal. Starting steroids were obtained from Steraloids, Wilton, N.H., or Searle Laboratories, Skokie, Ill. Other reagents were purchased from Aldrich Chemical Co., Milwaukee, Wis. Purity of all starting materials was determined by melting point, thin-layer chromatography, and infrared spectroscopy.

All compounds were synthesized by one of two methods, depending upon whether the thiol reagent was a solid or a liquid. All final compounds gave the expected IR and NMR spectra, and elemental analysis for C and H was obtained for each compound.

1α-Phenylthioandrost-4-ene-3,17-dione (3). Sodium metal (80 mg, 3.5 mmol) was added to a solution of **2** (560 mg, 2.0 mmol) in thiophenol (15 mL) with stirring under N_2. The reaction was warmed to 60 °C for 2 days, then poured into saturated aqueous NH_4Cl, and extracted with Et_2O. The Et_2O layer was dried (Na_2SO_4) and solvents were removed under vacuum to give a clear oil. Crystallizations from hexane gave the analytical sample, **9** (670 mg, 85%): mp 190–191 °C; IR (KBr) 3030, 2920, 2900, 2830, 1745, 1680, 1610, 1575 cm^{-1}; ^1H NMR δ 0.92 (s, 3 H, C_{18}), 1.41 (s, 3 H, C_{19}), 3.68 (t, 1 H, 1β), 5.97 (s, 1 H, vinyl), 7.3–7.7 (m, 5 H, phenyl). Anal. ($C_{25}H_{30}O_2S$) C, H.

7α-(4′-Amino)phenylthioandrost-4-ene-3,17-dione (17). Sodium metal (120 mg, 5.2 mmol), **7** (3.5 g, 12 mmol), and 4-aminothiophenol (5.0 g, 39.3 mmol) in anhydrous dioxane (25 mL) were combined and stirred for 5 days at 60 °C. The suspension was then poured into water to give a white solid. The solid was filtered and dissolved in CH_2Cl_2. The CH_2Cl_2 solution was treated with activated Norit and filtered through Celite 545, and solvent was removed. The residue was recrystallized from hexane–acetone to give crystals of 17 (4.45 g, 91%): mp 254–256 °C; IR (KBr) 3420, 3340, 3010, 2940, 2870, 1740, 1670, 1620, 1590 cm^{-1}; ^1H NMR δ 0.92 (s, 3 H, C_{18}), 1.21 (s, 3 H, C_{19}), 3.39 (m, 1 H, 7β), 3.82 (s, 2 H, amine), 5.76 (s, 1 H, vinyl), 6.5–7.4 (m, 4 H, aromatic). Anal. ($C_{25}H_{31}NO_2S$) C, H.

B. Biochemical Methods. Materials. Glucose 6-phosphate, glucose-6-phosphate dehydrogenase (type XV), $NADP^+$, and dansyl chloride were purchased from Sigma Chemical Co., St. Louis, Mo. All reagents and solvents were of analytical grade. Centrifugations were performed on a Sorval RC2-B centrifuge and a Beckman L3-40 ultracentrifuge.

Radioisotopes and LS Counting. [4-^{14}C]Androst-4-ene-3,17-dione, [6,7-^{3}H]estrone, and [6,7-^{3}H]estradiol were obtained from New England Nuclear, Boston, Mass. Radioactive chromatographic sheets were scanned on either a Berthold TLC Scanner LB-2723 or a Packard Radiochomatogram Scanner 7201. Samples were counted in 10 mL of a 1/1 mixture of PCS (Amersham/Searle, Arlington Hts., Ill.) and spectrograde *p*-xylene (Aldrich, Milwaukee, Wis.). Samples were counted with a Beckman LS-150 scintillation counter using double channel techniques with automatic quench correction. Channel counting efficiencies and ^{14}C dpm spillover into ^{3}H cpm (10.5%) were determined by standardization with [^{3}H]toluene and [^{14}C]toluene quenched standards.

Enzyme Preparation.[19] Placental tissue was obtained upon delivery, transported to the lab on ice, and processed at 4 °C. The tissue was teased free from fetal membrane and large blood vessels, minced with scissors, and homogenized for 60 s in a Waring blender with two parts of tissue to one part of homogenization buffer. The buffer consisted of 0.25 M sucrose, 0.05 M sodium phosphate, pH 7.0, and 0.04 M nicotinamide. The homogenate was centrifuged at 10000g for 30 min. The supernatant was then centrifuged at 105000g for 1 h to obtain the microsomal pellet. The pellet was resuspended in 0.1 M sodium phosphate buffer, pH 7.0, and recentrifuged at 105000g for 1 h. This washing procedure was repeated. The twice-washed microsomes were resuspended in 0.1 M sodium phosphate buffer, pH 7.0, and frozen at −20 °C until needed. Enzyme preparation was stable for 3–4 months at −20 °C. The amounts of microsomal protein present per milliliter of frozen suspension were determined by the method of Lowry[28] as modified by Miller.[29]

Screening Assay Procedure. Androstenedione (1.25 μM/flask; 79000 ^{14}C dpm/flask) in ethanol and an inhibitor (12.5 or 6.25 μM/flask) in ethanol were added to a 25-mL Erlenmeyer flask containing 100 μL of propylene glycol. The ethanol was then removed with a stream of nitrogen. $NADP^+$ (1.71 mM/flask) and glucose 6-phosphate (2.85 mM/flask) were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 (0.5 mL/flask). This solution was added to the flasks along with 1.0 unit (10 μL) of glucose-6-phosphate dehydrogenase. Prepared microsomes were thawed and diluted with 0.1 M sodium phosphate buffer (0.25 mg of microsomal protein/3.0 mL of buffer). The microsomes and assay flasks containing steroid and NADPH-generating system were preincubated for 5 min at 37 °C in a water bath shaker. The assay began by addition of the microsomes to the flask (3.0 mL/flask). After 15.0 min of incubation at 37 °C, ethyl acetate (4.0 mL) was added to quench the assay. Control samples with no inhibitor were incubated simultaneously, and blank samples were incubated for 0 min. [^{3}H]Estrone and [^{3}H]estradiol (80000 dpm each/flask) were added as internal standards for determination of extraction efficiency. Steroids were extracted with 3 vol of ethyl acetate, and the ethyl acetate layer was dried (Na_2SO_4) and removed. The residue was spotted on alumina plates and the plates developed in $CHCl_3$ and scanned for radioactivity. The band corresponding to estrogens was cut, placed in glass LCS vials, and counted to determine radioactivity.

K_i Assay Procedure. Various concentrations of androstenedione (0.075–0.515 μM/flask; 63000 ^{14}C dpm/nmol) and a single concentration of inhibitor in propylene glycol (100 μL) were incubated at 37 °C with $NADP^+$ (1.71 mM), glucose 6-phosphate (2.85 mM), glucose-6-phosphate dehydrogenase (1.0 unit), and microsomes (0.25 mg of microsomal protein) in 3.5 mL of buffer. The incubations were stopped with ethyl acetate at 8.0 min to ensure initial velocity conditions with <10% product formation. [^{3}H]Estrone and [^{3}H]estradiol (80000 dpm each/flask) were added and the extracted steroids were treated as before to determine radioactivity. Control samples with no inhibitor were incubated simultaneously, and blank samples were incubated for 0 min. Each inhibitor was examined at two or more concentrations (0.1, 0.3, 0.5 μM).

Acknowledgment. This investigation was supported by fellowships (R.W.B.) from the Rackham School for Graduate Studies and the American Foundation for Pharmaceutical Education. Supplies were provided in part by an award from The University of Michigan Cancer Research Institute. The authors also wish to thank Searle Laboratories for their generous gift of steroid starting materials and for performing the in vivo endocrine assays.

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(1) Taken in part from the dissertation presented by R. W. Brueggemeier, Aug 1977, to the Graduate School of The University of Michigan in partial fulfillment of the re-

quirements for the Doctor of Philosophy degree.
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11,12-Secoprostaglandins. 5. 8-Acetyl- or 8-(1-Hydroxyethyl)-12-hydroxy-13-aryloxytridecanoic Acids and Sulfonamide Isosteres as Inhibitors of Platelet Aggregation

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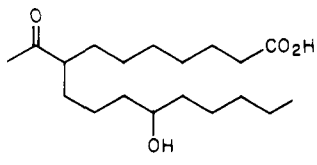
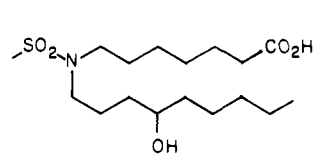
The synthesis of a series of 8-acetyl- (or 1-hydroxyethyl-) 12-hydroxy-13-aryloxytridecanoic acids and their sulfonamide isosteres is described. These compounds are formally derived from members of earlier reported series of modified secoprostaglandins by replacing the ω-butyl chain termini by substituted aryloxy groups. A number of these compounds are potent inhibitors of collagen-induced blood platelet aggregation in guinea pigs on oral administration.

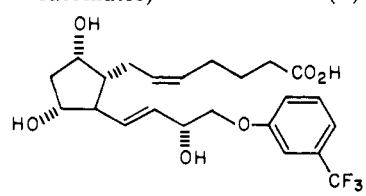
The first paper[2] in this series described the chemistry and biological properties of a series of acylhydroxyalkanoic acids that represent simplified analogues of the secoprostaglandins formally derived from the natural substances by scission of the cyclopentane ring between carbon atoms 11 and 12. Certain members of this series that most closely resemble the prostaglandins in structure (e.g., 8-acetyl-12-hydroxyheptadecanoic acid, 1) have shown a



1 (mixture of racemates)



(±)-2



(±)-3

number of the characteristic in vitro and in vivo biological actions of the prostaglandins of the E series. Subsequently, we showed that acids in several series[3,4] isosteric with these acylhydroxyalkanoic acids display similar biological properties. The most thoroughly studied of these series is that of the sulfonamide isosteres[4] of which 7-[*N*-(4-hydroxynonyl)methanesulfonamido]heptanoic acid (**2**) is an active representative.

An important development in prostaglandin analogue research was reported in 1974 by Crossley and Walpole and co-workers of ICI Pharmaceuticals Division.[5] They found that some analogues of $PGF_{2\alpha}$ in which the terminal butyl group is replaced by an aryloxy group are markedly more selective in action than the natural substance. In particular, the potency of active compounds (e.g., **3**) in terminating pregnancy is markedly increased over that of $PGF_{2\alpha}$ while the potency in stimulating contraction of smooth muscle is diminished.

It was of obvious interest to determine the effect of a similar structural modification on the activity of our secoprostaglandins. The synthesis and biological examination of analogues of **1** and **2** that contain aryloxy groups are the subjects of this paper. An aryloxy-substituted member of the 8-methylsulfonyl-12-hydroxyalkanoic acid series [8-methylsulfonyl-12-hydroxy-13-(4-fluorophenoxy)tridecanoic acid] was described previously.[3b]

Chemistry. The method of preparation of the 8-acetyl-12-hydroxy-13-aryloxytridecanoic acids (**8a–f**) and their 8-(1-hydroxyethyl) reduction products (**9a–d**) is outlined in Scheme I. The key intermediate **6** was obtained by epoxidation with *m*-chloroperbenzoic acid of ethyl 8-acetyl-12-decenoate (**5**) prepared through a con-

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