In some cases, 0.10-mL aliquots were quenched in 1 N HCl (1.8 mL) prior to injection. Unreacted carbamate and 4-methoxyphenol concentrations were determined by HPLC analysis with a C-18 reverse-phase column using either a gradient mobile phase of 95% dilute H_3PO_4 (1.0 mL of 85% H_3PO_4 in 1.0 L of H_2O)–5% CH_3CN to 5% dilute H_3PO_4–95% CH_3CN over 30 min or isocratic elution with a mobile phase of 87.5% dilute H_3PO_4–12.5% CH_3CN, flow = 1.0 or 3.0 mL/min. The carbamates are stable at the pH of the mobile phase, and therefore injection into the HPLC effectively stops the reaction. The detector was set at 220 nm. The half-life is the time required for 50% conversion of carbamate to 4-methoxyphenol and was calculated by using first-order kinetics. Results in Table I are the average of at least two separate determinations.

Stability of *N*-Methyl-*N*-[2-(methylamino)ethyl]carbamic Acid 4-Methoxyphenyl Ester Hydrochloride (1a) in Murine Plasma. A solution of the carbamate hydrochloride **1a** (0.27 mg) in 0.10 mL of dilute H_3PO_4 (1 mL of 85% H_3PO_4 in 1 L of H_2O) was added to a magnetically stirred mixture of fresh murine plasma (1.6 mL) and pH 7.4 phosphate buffer (0.40 mL) preheated to 37 °C to give a carbamate concentration of 5×10^{-4} M. This solution was maintained at 37 °C and pH 7.4 with a Radiometer Copenhagen pH stat. At various intervals, aliquots (25 μL) were removed, quenched in 7% $HClO_4$ (0.20 mL) to stop the reaction, and shaken. After centrifugation (14000 *g*, 8 min) clear supernatant was pipetted from the insoluble pellet and analyzed by the same HPLC method used in the buffer reactions.

Acknowledgment. We are indebted to J. Moreau for elemental analyses, Y. Lee for the pK_a determinations, S. Pitzenberger, J. Murphy, and S. Varga for NMR spectra, and D. Frankenfield for supplying the murine plasma. We also thank W. Randall for helpful advice on determining half-lives of the carbamates and V. Finley for preparation of the manuscript.

Registry No. **1a**, 122734-28-5; **1a** (base), 122734-36-5; **1b**, 122734-30-9; **1b** (base), 122734-37-6; **1c**, 122754-64-7; **1d**, 122734-29-6; **1e**, 123183-68-6; **1f**, 123183-69-7; **1g**, 122734-33-2; **1h**, 123183-70-0; **2**, 150-76-5; **4a**, 112257-19-9; **4c**, 122734-34-3; **4d**, 122734-32-1; **4d**·fumarate, 123183-73-3; **4e**, 121492-06-6; **4e**·fumarate, 123183-74-4; **4f**, 57260-73-8; **4f**·fumarate, 123183-75-5; **4g**, 75178-96-0; **4g**·fumarate, 123183-76-6; **4h**, 123183-72-2; **5a**, 122734-31-0; **5c**, 122734-35-4; **5d**, 123183-77-7; **5e**, 123183-78-8; **5f**, 123183-79-9; **5g**, 123183-80-2; **5h**, 123183-81-3; **6**, 57561-39-4; **7**, 123183-82-4; **8**, 123183-71-1; di-*tert*-butyl dicarbonate, 24424-99-5; ethylenediamine, 107-15-3; *N*-methylethylenediamine, 109-81-9; 4-methoxyphenyl chloroformate, 7693-41-6; *N,N'*-dimethylimidazolidinone, 80-73-9; *N,N'*-dimethylethylenediamine, 110-70-3; *N*-methylethanolamine, 109-83-1; *N,N'*-diethylethylenediamine, 111-74-0; 1,3-propanediamine, 78-90-0; *N,N'*-dimethyl-1,3-propanediamine, 111-33-1.

Synthesis and Biochemical Studies of 7-Substituted 4,6-Androstadiene-3,17-diones as Aromatase Inhibitors[1]

Pui-Kai Li and Robert W. Brueggemeier*

College of Pharmacy and OSU Comprehensive Cancer Center, 500 West 12th Avenue, The Ohio State University, Columbus, Ohio 43210. *Received March 6, 1989*

Inhibitors of aromatase, the cytochrome P-450 enzyme complex responsible for the biosynthesis of estrogens, may be useful as therapeutic agents for the treatment of estrogen-dependent disease states such as breast and endometrial cancer. Several 7α-thio-substituted androstenediones have proven to be potent inhibitors of aromatase in vitro and in vivo. Recent research efforts have focused on designing aromatase inhibitors with both substitution at C-7 and extended linear conjugation in rings A and B of the steroid nucleus. The targeted compounds, 7-substituted 4,6-androstadiene-3,17-diones **4–10**, were prepared by the addition of either Grignard or lithium reagents to 3,3:17,17-bis(ethylenedioxy)-5-androsten-7-one (**3**). Inhibitory activities of the compounds were evaluated in vitro by enzyme kinetic studies employing the microsomal fraction isolated from human term placenta. 7-Benzyl- and 7-phenethyl-4,6-androstadiene-3,17-dione analogues are effective inhibitors with apparent K_i's of 60.9–174 nM, while the 7-phenyl analogue exhibited an apparent K_i of 1.424 μM. Thus, several 7-substituted 4,6-androstadiene-3,17-diones were prepared and exhibited good competitive inhibition of aromatase in vitro in human placental microsomes.

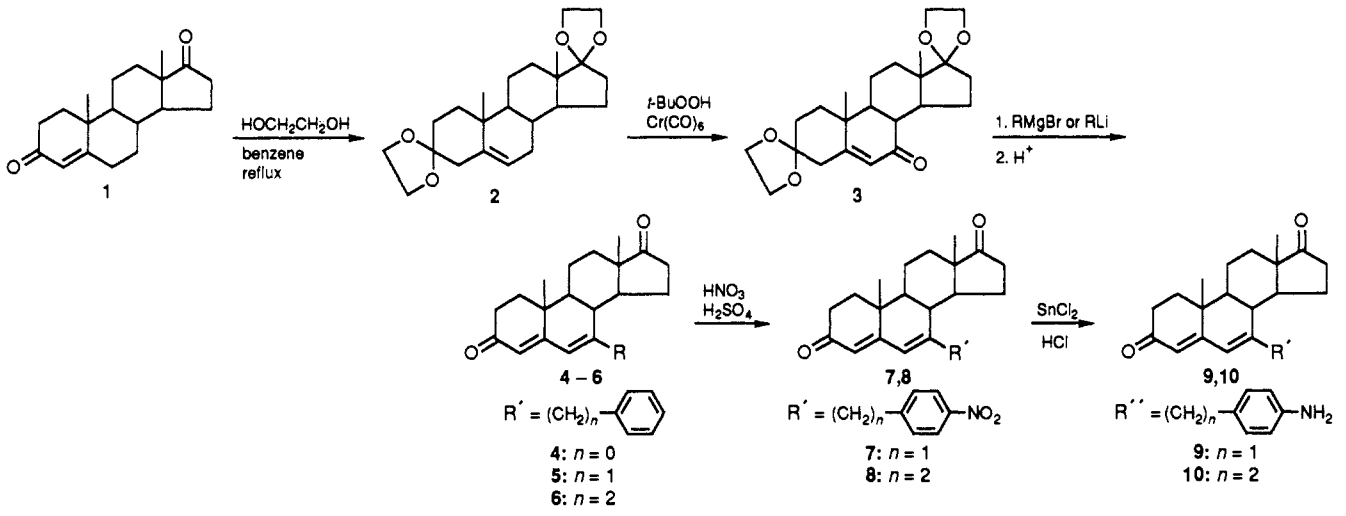
Aromatase is the cytochrome P-450 enzyme complex responsible for the conversion of androgens to estrogens. Estrogens are involved in reproductive processes and are also implicated in estrogen-dependent disease states such as breast and endometrial cancers. Thus, inhibitors of aromatase may be useful in controlling these physiological processes and disease states. The aromatase inhibitors 4-hydroxyandrostenedione and aminoglutethimide have demonstrated therapeutic effectiveness in the treatment of hormone-dependent breast tumors in both animals[2-4] and humans.[5-7]

Previous work from our laboratory has illustrated that several 7α-thio-substituted derivatives of androstenedione were effective inhibitors of aromatase.[8-12] Among the compounds synthesized, 7α-[(4'-aminophenyl)thio]-4-androstene-3,17-dione (7α-APTA) was found to be one of the most potent inhibitors with an apparent K_i of 18 nM. These aromatase inhibitors have also demonstrated activity in inhibiting aromatase activity in MCF-7 cells[13] and in reducing tumor volumes in the DMBA-induced rat

(1) A portion of this work was presented: 70th Annual Meeting of the Endocrine Society, New Orleans, LA, June 8–11, 1988; Abstract 616.
(2) Brodie, A. M. H.; Schwarzel, W. C.; Shaikh, A. A.; Brodie, H. J. *Endocrinology* **1977**, *100*, 1684.
(3) Brodie, A. M. H.; Marsh, D. A.; Brodie, H. J. *J. Steroid Biochem.* **1979**, *10*, 423.
(4) Brodie, A. M. H.; Brodie, H. J.; Garrett, W. M.; Hendrickson, J. R.; Marsh, D. A.; Tsai-Morris, C.-H. *Biochem. Pharmacol.* **1982**, *31*, 2017.
(5) Santen, R. J.; Santner, S.; Davis, B.; Veldhuis, J.; Samojlik, E.; Ruby, E. *Clin. Endocrinol. Metab.* **1978**, *47*, 1257.
(6) Coombes, R. C.; Goss, P.; Dowsett, M.; Gazet, J.-C.; Brodie, A. M. H. *Lancet* **1984**, *2*, 1237.
(7) Sjoerdsma, A. *Clin. Pharmacol. Ther.* **1981**, *30*, 3.
(8) Brueggemeier, R. W.; Floyd, E. E.; Counsell, R. E. *J. Med. Chem.* **1978**, *21*, 1008.
(9) Brueggemeier, R. W.; Snider, C. E.; Counsell, R. E. *Cancer Res. (Suppl.)* **1982**, *42*, 3334s.
(10) Darby, M. V.; Lovett, J. A.; Brueggemeier, R. W.; Groziak, M. P.; Counsell, R. E. *J. Med. Chem.* **1985**, *28*, 803.
(11) Snider, C. E.; Brueggemeier, R. W. *J. Steroid Biochem.* **1985**, *22*, 325.
(12) Snider, C. E.; Brueggemeier, R. W. *J. Biol. Chem.* **1987**, *262*, 8685.
(13) Brueggemeier, R. W.; Katlic, N. E. *Cancer Res.* **1987**, *47*, 4548.

0022-2623/90/1833-0101$02.50/0

Scheme I



mammary tumor model.[14,15]

Androstenedione derivatives with extended linear conjugation in ring A and/or B are effective inhibitors.[16] For example, 4,6-androstadiene-3,17-dione has the same affinity for the enzyme aromatase as the substrate androstenedione. The introduction of substituents at C_7 may lead to enhanced affinity of these analogues for the aromatase complex. Furthermore, replacement of the carbon–sulfur bond with a carbon–carbon bond would yield analogues with similar lipophilic character and eliminate potential metabolic oxidation of the thioether linkage. Thus, compounds with both features (extended linear conjugation in ring A and/or B and a carbon–carbon bond at C_7) may result in potent inhibitors. The studies described in this paper focus on the preparation and biochemical evaluation of 7-substituted 4,6-androstadiene-3,17-diones.

Chemistry

The synthesis of the 7-substituted 4,6-androstadiene-3,17-dione derivatives was carried out as shown in Scheme I. The scheme began with the preparation of 3,3:17,17-bis(ethylenedioxy)-5-androstene (**2**) from androstenedione and ethylene glycol in refluxing benzene and *p*-toluenesulfonic acid as catalyst. The most important synthetic intermediate, 3,3:17,17-bis(ethylenedioxy)-5-androsten-7-one (**3**), was synthesized by allylic oxidation of **2**. Several oxidizing agents were attempted. The first attempt involved reaction with sodium chromate tetrahydrate in an acetic anhydride/acetic acid mixture at 40 °C for 24 h. Compound **3** was obtained in a very low yield (15%) due to the acidic nature of the reaction medium. Reaction with chromium trioxide and dimethylpyrazole as oxidizing agent was also attempted, but the workup procedure was tedious and the yield was low (21%). Satisfactory results for the synthesis of **3** were obtained by using *tert*-butyl hydroperoxide (*t*-BuOOH) in the presence of chromium hexacarbonyl catalyst, $Cr(CO)_6$, in refluxing acetonitrile,[17] which gave a yield of 55%.

The next step in the synthesis of 7-substituted 4,6-androstadiene-3,17-diones **4–6** was incorporation of C_7 substituents into the steroid. Originally, introduction of the aryl groups on C_7 was attempted by reacting 3,3:17,17-bis(ethylenedioxy)-5-androsten-7-one (**3**) with Wittig reagents. Subsequent hydrolysis of the ketal protecting groups would then give the corresponding 7-substituted 4,6-androstadiene-3,17-dione. However, addition did not occur when 3,3:17,17-bis(ethylenedioxy)-5-androsten-7-one (**3**) reacted with benzylphosphonium bromide and sodium hydride (NaH) in benzene at 70 °C for 24 h. The failure of the reaction is more likely to be due to the CH_2Ph group, in view of the known ability of $Ph_3P^+CH_2^-$ to react with the 5-en-7-one system.[18]

An alternate route of incorporating an aryl group at C-7 position employed the use of a strong nucleophile. 3,3:17,17-Bis(ethylenedioxy)-5-androsten-7-one (**3**) was reacted with either aryl Grignard or aryllithium reagents to give the 7-aryl-7-hydroxy-3,3:17,17-bis(ethylenedioxy)-5-androstene, which was hydrolyzed without isolation. Methanol–sulfuric acid was used for the hydrolysis of the two ethylene ketal groups, with concomitant dehydration of the 7-hydroxy groups to yield 7-substituted 4,6-androstadiene-3,17-diones.

The configurations of the aryl and the hydroxy groups in 7-aryl-7-hydroxy-3,3:17,17-bis(ethylenedioxy)-5-androstenes were not elucidated; however, attack from the less hindered α face would favor the assignment of 7α-aryl-7β-hydroxy-3,3:17,17-bis(ethylenedioxy)-5-androstene. Upon hydrolysis and concomitant dehydration, only the desired 7-substituted 4,6-androstadiene-3,17-dione was obtained. The 7α-aryl-7β-hydroxy-5-ene system would be expected to yield an exocyclic double bond upon dehydration when the aryl groups are benzyl or phenethyl, since the hydroxyl group can achieve a suitable coplanar trans relationship with a hydrogen from the aryl groups. The exocyclic double bond formed rearranges to form the 4,6-dien-3-one system, which is the thermodynamically more stable product.

Compounds **7** and **8** were synthesized in a one-step process. Nitration of **5** and **6** with nitric acid and sulfuric acid in a modification of a procedure of Anselm and Zuckmayer[19] led to the formation of **7** and **8**, respectively. Only the para-substituted products were isolated, and purification was carried out by recrystallization using ethyl acetate/hexane. Amino analogues **9** and **10** were synthesized from their nitro analogues **7** and **8**, respectively,

(14) Brueggemeier, R. W.; Li, P.-K.; Snider, C. E.; Darby, M. V.; Katlic, N. E. *Steroids* **1987**, *50*, 163.
(15) Brueggemeier, R. W.; Li, P.-K. *Cancer Res.* **1988**, *48*, 6808.
(16) Schwarzel, W. C.; Kruggel, W. G.; Brodie, H. J. *Endocrinology* **1973**, *93*, 866.
(17) Pearson, A. J.; Chen, Y.-S.; Han, G. R.; Hsu, S.-Y.; Tapan, R. *J. Chem. Soc., Perkin Trans. 1.* **1985**, 267.
(18) Sondheimer, F.; Mechoulam, R. *J. Am. Chem. Soc.* **1958**, *80*, 3087.
(19) Anselm, F.; Zuckmayer, F. *Ber. Dtsch. Chem. Ges.* **1899**, *32*, 3283.

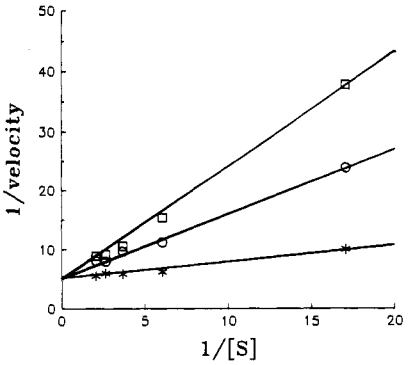


Figure 1. Double reciprocal plot of aromatase inhibition by inhibitor 8. Varying concentrations of androstenedione were incubated with aromatase at single inhibitor concentrations of 0 μM (*), 0.3 μM (○), or 0.6 μM (□). Each point represents the average of two determinations with a variation of less than 7%.

by reduction of the nitro group with stannous chloride and hydrochloric acid.[20]

Biochemical Results

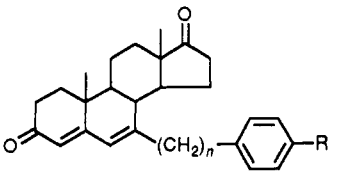
7-Substituted 4,6-androstadiene-3,17-diones **4–10** were evaluated in vitro by enzyme kinetic studies using human placental microsomes. Aromatase activity in human placental microsomes was assayed by the radiometric method developed by Siiteri and Thompson[21] in which the tritium in [1β-^{3}H]-4-androstene-3,17-dione was transferred into water during aromatization. The amount of ^3H_2O released was used as an index of estrogen formation. All the inhibitors were evaluated at concentrations ranging from 0 to 600 nM in initial velocity studies performed under limiting enzyme concentrations. In these microsomal assays, substrate concentrations were varied while the inhibitor concentration remained constant. Each substrate concentration was run in duplicate and the results of the studies were plotted in a typical Lineweaver–Burk or double-reciprocal plot as 1/velocity vs 1/[substrate]. The apparent K_i of the inhibitor is an index of the affinity of the enzyme for the inhibitor and is determined by a weighted regression-analysis computer program.[22] The results for compound 8 are shown in the Lineweaver–Burk plot (Figure 1) and the apparent K_i's of compounds **4–10** are shown in Table I. In these studies, the apparent K_m for androstenedione was found to be 0.051 ± 0.009 μM. Also each inhibitor demonstrated competitive inhibition, as determined from the Lineweaver–Burk plots and V_{max} intercepts.

Discussion

The inhibitors exhibited a wide range of inhibitory activity, with apparent K_i's ranging from 60.9 nM to 1.424 μM. 7-Benzyl- and 7-phenethyl-4,6-androstadiene-3,17-diones **5–10** are effective inhibitors of aromatase and exhibited apparent K_i's ranging from 60.9 to 174 nM. 7-Phenyl-4,6-androstadiene-3,17-dione (4), on the other hand, is a poor inhibitor, with an apparent K_i of 1.424 μM.

Previous studies illustrated that aromatase has considerable tolerance for androstenedione and testosterone derivatives with bulky 7α-substituents.[8-12] The reason for the low inhibitory activity of inhibitor 4 may be that the 7-phenyl group of the inhibitor can only adopt a pseudo-β-position, whereas the 7-benzyl and 7-phenethyl groups

Table I. Aromatase Inhibition by Various 7-Substituted 4,6-Androstadienediones[a]



compound	n	R	K_i, nM	inhibition
4 7-phenyl-ADD	0	H	1424	competitive
5 7-benzyl-ADD	1	H	61	competitive
6 7-phenethyl-ADD	2	H	174	competitive
7 7-(nitrobenzyl)-ADD	1	NO_2	94	competitive
9 7-(aminobenzyl)-ADD	1	NH_2	88	competitive
8 7-(nitrophenethyl)-ADD	2	NO_2	95	competitive
10 7-(aminophenethyl)-ADD	2	NH_2	88	competitive

[a] K_m for androstenedione = 51 nM (SE = 9 nM).

of 4,6-androstadiene-3,17-diones **5–10** can orient themselves in a way that the phenyl rings can protrude into the 7α pocket. In 7-benzyl-4,6-androstadiene-3,17-dione, addition of polar substituents (NO_2 or NH_2) on the phenyl ring result in inhibitors **7** and **9** with lower inhibitory activity. However, the opposite result was observed for 7-phenethyl-4,6-androstadiene-3,17-diones in which addition of polar substituents (NO_2 or NH_2) on the phenyl ring resulted in inhibitors **8** and **10** with increased inhibitory activity. Additional factors other than geometry may be involved in enhancing the affinity of the inhibitors to the enzyme.

Thus, several 7-substituted 4,6-androstadiene-3,17-dione analogues were prepared and exhibited good competitive inhibition of aromatase in vitro in human placental microsomes. The most effective inhibitors were those with extended linear conjugation, the 4,6-dien-3-one functionality, and flexible 7-substitution, such as the benzyl or phenethyl moiety. However, these inhibitors are not as effective as the previously reported 7α-thio-substituted androstenediones.[14] Nevertheless, the 7-substituted 4,6-androstadiene-3,17-diones may offer an advantage in vivo over the 7α-thio-substituted analogues by providing greater metabolic stability. Further evaluation of these new analogues in cell-culture systems and in vivo will provide additional information on the efficacy of these new androstadienediones as potential aromatase inhibitors for the treatment of estrogen-dependent cancers.

Experimental Section

General Procedures. Steroids were purchased from Searle Laboratories (Skokie, IL) or Steraloids (Wilton, NH) and checked for purity by thin-layer chromatography or melting point. Chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI). Dioxane was dried with calcium hydride and distilled from sodium. Silica gel was purchased from E. Merck (Darmstadt, Germany) and aluminum oxide (basic) was from Fischer Scientific (Fair Lawn, NJ). TLC plates were purchased from Analtech Inc. (Newark, NE). Biochemicals were obtained from Sigma Chemical Co. [1β-^{3}H]-4-Androstene-3,17-dione was purchased from New England Nuclear (Boston, MA). Melting points were obtained on a Thomas-Hoover capillary melting point apparatus and were uncorrected. IR spectral data were recorded on a Beckman IR 4230 spectrophotometer. UV data were obtained from a Beckman DU-8 spectrophotometer. NMR spectra were obtained with either a Bruker HX-90E NMR spectrometer (90 MHz), a Bruker WP-80DS NMR spectrometer (80 MHz), or an IBM AF/250 spectrometer in the pulse mode. Mass spectra were obtained at The Ohio State University Chemical Instrumentation Center using a Kratos MS-30 mass spectrometer. Elemental analyses were performed by Galbraith Lab. Inc., Knoxville, TN. Centrifugation was performed on a Sorvall RC2-B centrifuge and a Beckman L5-50B ultracentrifuge was used for ultracentrifugation. Ra-

(20) Shen, T. Y.; Walford, G. L.; Witzel, B. E.; Ruyle, W. V. Ger. Offen. 2,031,223, 1971; *Chem. Abstr.* **1971**, *74*, 125270x.
(21) Siiteri, P. K.; Thompson, E. A. *J. Steroid Biochem.* **1975**, *6*, 317.
(22) Cleland, W. W. *Method Enzymol.* **1979**, *63*, 103.

dioactive samples were analyzed with a Beckman LS 6800 scintillation counter using Formula 963 (New England Nuclear) as the counting solution.

3,3:17,17-Bis(ethylenedioxy)-5-androstene (2). A mixture of 4-androstene-3,17-dione (20 g, 70 mmol), ethylene glycol (100 mL), *p*-toluenesulfonic acid (0.5 g), and benzene (400 mL) was heated under reflux for 48 h in a 1-L round-bottom flask equipped with a Dean–Stark trap. Saturated sodium bicarbonate solution (50 mL) was added to the cooled mixture and the benzene layer was separated, washed with water (2 × 100 mL), dried (Na_2SO_4), and concentrated to dryness under reduced pressure. Recrystallization from ethanol gave pure **2** (18.1 g, 70.3%): mp 169–170 °C (lit.[26] mp 171–173.5 °C); IR (KBr) 2940, 1440, 1380, 1310, 1100 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (s, 3 H, C_{18}), 1.05 (s, 3 H, C_{19}), 3.82–3.96 (m, 8 H, $-OCH_2CH_2O-$), 5.27–5.41 (m, 1 H, C_6).

3,3:17,17-Bis(ethylenedioxy)-5-androsten-7-one (3). To a solution of 3,3:17,17-bis(ethylenedioxy)-5-androstene (**2**, 1 g, 2.7 mmol) in acetonitrile (CH_3CN, 30 mL) were added *t*-BuOOH (1 mL, 9.23 mmol) and $Cr(CO)_6$ (0.135 g, 0.16 mmol). The mixture was refluxed for 24 h and then cooled to room temperature. Water (20 mL) was added, and the product was extracted with ethyl acetate. The ethyl acetate layer was dried (Na_2SO_4) and concentrated to dryness under reduced pressure to give a yellowish solid (0.89 g), which was purified by column chromatography using silica gel. Elution with ethyl acetate/hexane and recrystallization with ethanol/water yielded a slight yellowish solid: mp 206–207 °C; IR (KBr) 3030, 2950, 2890, 1670, 1295, 1100 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (s, 3 H, C_{18}), 1.22 (s, 3 H, C_{19}), 3.75–4.05 (m, 8 H, $-OCH_2CH_2O-$), 5.65 (d, 1 H, C_6); MS *m/e* (relative intensity) 388 (M^+, 0.040), 373 (0.094), 100 (0.363), 99 (1.00), 86 (0.116). Anal. ($C_{23}H_{32}O_5 \cdot 0.25H_2O$) C, H.

7-Phenyl-4,6-androstadiene-3,17-dione (4). To a solution of 3,3:17,17-bis(ethylenedioxy)-5-androsten-7-one (**3**, 2.0 g, 7.1 mmol) in THF (50 mL) was added phenylmagnesium chloride (0.5 M, 30 mL, 15 mmol) dropwise in 30 min under argon. The solution was allowed to stir at room temperature for 2 h and then poured into saturated aqueous NH_4Cl solution and extracted with ether (2 × 100 mL). The ether layer was washed with saturated NaCl solution and dried (Na_2SO_4), and the solvent was removed to furnish brown, gummy crystals (2.2 g). The crystals were dissolved in dioxane (30 mL) containing 5% H_2SO_4 (5 mL) and the resulting solution was stirred at room temperature for 24 h. The solution was then neutralized with saturated $NaHCO_3$ and extracted with $CHCl_3$. The $CHCl_3$ layer was separated and dried (Na_2SO_4), and the solvent was evaporated to give a yellowish solid (1 g). Recrystallization from hexane yielded yellowish crystals of **4** (0.8 g, 42.5%): mp 187–188 °C; UV (methanol) λ_{max} = 249 nm, 310 nm; IR (KBr) 3030, 2960, 2930, 2850, 1735, 1640, 1605, 1365, 1230, 770 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.02 (s, 3 H, C_{18}), 1.21 (s, 3 H, C_{19}), 5.77 (s, 1 H, C_4), 6.18 (d, 1 H, C_6), 7.26–7.40 (m, 5 H, aromatic); MS *m/e* (relative intensity) 360 (M^+, 0.732), 212 (1.00), 197 (0.254), 179 (0.171), 178 (0.165), 149 (0.210), 107 (0.156), 91 (0.157). Anal. ($C_{25}H_{28}O_2$) C, H.

7-Benzyl-4,6-androstadiene-3,17-dione (5). Compound **3** (10 g, 26 mmol) was treated with benzylmagnesium bromide in hexane (2.5 M, 50 mL, 125 mmol) in a manner similar to that of **4**. Compound **5** was isolated as a yellowish solid (4.3 g, 44.6%): mp 80–83 °C; UV (methanol) λ_{max} = 250 nm, 296 nm; IR (KBr) 3020, 2940, 2860, 1740, 1655, 1620, 1375, 750 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.92 (s, 3 H, C_{18}), 1.13 (s, 3 H, C_{19}), 5.43 (s, 1 H, C_4), 5.89 (s, 1 H, C_6), 7.15–7.40 (m, 5 H, aromatic); MS *m/e* (relative intensity) 374 (M^+, 0.351), 283 (0.679), 265 (0.100), 173 (0.112), 131 (0.120), 106 (0.200), 91 (1.00). Anal. ($C_{26}H_{30}O_2$) C, H.

7-Phenethyl-4,6-androstadiene-3,7-dione (6). Compound **3** (7 g, 18 mmol) was treated with phenethyllithium synthesized by the procedure of Screttas[23] in THF (0.5 M, 50 mL, 25 mmol) in a manner similar to that of **4**. Compound **6** was isolated as yellowish crystals (1.8 g, 25.7%): mp 121–122 °C, UV (methanol) λ_{max} = 251 nm; IR (KBr) 2980, 2875, 1740, 1645, 1615, 1370, 800 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.99 (s, 3 H, C_{18}), 1.01 (s, 3 H, C_{19}), 5.65 (s, 1 H, C_4), 6.08 (s, 1 H, C_6), 7.16–7.39 (m, 5 H, aromatic); MS *m/e* (relative intensity) 388 (M^+, 0.053), 149 (0.161), 129 (0.173), 111 (0.102), 97 (0.131), 91 (0.532). Anal. ($C_{27}H_{32}O_2$) C, H.

7-(4′-Nitrobenzyl)-4,6-androstadiene-3,17-dione (7). A 60% aqueous sulfuric acid solution (60 mL) was mixed with concentrated nitric acid (12 mL) at 0 °C. 7-Benzyl-4,6-androstadiene-3,17-dione (**5**, 1.29 g, 3.5 mmol) dissolved in acetic acid (5 mL) was added slowly to the solution over 20 min at 0 °C and stirring was continued for 24 h. The mixture was neutralized with saturated $NaHCO_3$ and extrated with ethyl acetate (EtOAc) (2 × 150 mL). The EtOAc layer was separated, washed with water (200 mL), and dried (Na_2SO_4), and the solvent was evaporated to give an oil. Column chromatography with ethyl acetate/petroleum ether afforded product **7** (0.9 g, 62.3%): mp 115–117.5 °C, UV (methanol) λ_{max} = 250 nm, 292 nm. IR (KBr) 2970, 2860, 1740, 1660, 1620, 1600, 1370, 795 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.90 (s, 3 H, C_{18}), 1.08 (s, 3 H, C_{19}), 3.74 (d, 2 H, benzyl), 5.59 (s, 1 H, C_4), 5.86 (s, 1 H, C_6), 7.32–7.36 (d, 2 H, $C_{2'}$, J = 8.5 Hz), 8.19–8.23 (d, 2 H, $C_{3'}$, J = 8.5 Hz); MS *m/e* (relative intensity) 419 (M^+, 0.459), 389 (0.242), 283 (1.00), 271 (0.149), 106 (0.400). Anal. ($C_{26}H_{29}NO_4$) C, H.

7-(4′-Nitrophenethyl)-4,6-androstadiene-3,17-dione (8). A 60% aqueous sulfuric acid solution (30 mL) was mixed with concentrated nitric acid (6 mL) at 0 °C. 7-Phenethyl-4,6-androstadiene-3,17-dione (**6**, 0.6 g, 1.54 mmol) in acetic acid (5 mL) was added slowly over 20 min at 0 °C and stirring was continued for 24 h. The mixture was treated in a manner similar to that of **7** to afford product **8** (0.4 g, 59.7%): mp 130–131.5 °C, UV (methanol) λ_{max} = 250 nm, 295 nm. IR (KBr) 2940, 2870, 1735, 1660, 1620, 1350, 805 cm^{-1}. ^1NMR ($CDCl_3$) δ 1.01 (s, 3 H, C_{18}), 1.02 (s, 3 H, C_{19}), 5.66 (s, 1 H, C_4), 6.08 (s, 1 H, C_6), 7.35–7.38 (d, 2 H, $C_{2'}$, J = 8.5 Hz), 8.16–8.20 (d, 2 H, $C_{3'}$, J = 8.5 Hz); MS *m/e* (relative intensity) 433 (M^+, 0.500), 416 (0.433), 398 (0.142), 297 (0.192), 285 (0.205), 269 (0.229), 106 (1.00), 91 (0.159). Anal. $C_{27}H_{31}NO_4$) C, H.

7-(4′-Aminobenzyl)-4,6-androstadiene-3,17-dione (9). 7-(4′-Nitrobenzyl)-4,6-androstadiene-3,17-dione **7** and stannous chloride dihydrate (1.4 g, 6.2 mmol) were dissolved in a solution of ethanol (10 mL) and concentrated hydrochloric acid (1.5 mL). The reaction mixture was stirred under reflux for 30 min and then allowed to cooled to room temperature. The mixture was poured into ice water (50 mL), and the solution was extracted with dichloromethane (2 × 30 mL). The dichloromethane solution was washed with saturated $NaHCO_3$ solution and water, dried (Na_2SO_4), and concentrated to an oil. The oil was chromatographed on a silica column eluted with ethyl acetate/petroleum ether (3:1) to afford a yellowish solid, which was recrystallized by ethyl acetate/hexane to yield yellowish crystals (0.09 g, 48.4%): mp 189–191 °C, UV (methanol) λ_{max} = 251 nm, 296 nm; IR (KBr) 3470, 3380, 3030, 2980, 2950, 1740, 1660, 1620, 1530, 1370 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.92 (s, 3 H, C_{18}), 1.08 (s, 3 H, C_{19}), 3.53 (s, 2 H, benzyl), 3.6–3.8 (br s, 2 H, NH_2), 5.59 (s, 1 H, C_4), 5.92 (s, 1 H, C_6), 6.64–6.68 (d, 2 H, $C_{3'}$, J = 8.3 Hz), 6.90–6.93 (d, 2 H, $C_{2'}$, J = 8.3 Hz); MS *m/e* (relative intensity) 389 (M^+, 0.527), 283 (0.080), 119 (0.088), 106 (1.00), 43 (0.500). Anal. ($C_{26}H_{31}NO_2$) C, H.

7-(4′-Aminophenethyl)-4,6-androstadiene-3,17-dione (10). 7-(4′-Nitrophenethyl)-4,6-androstadiene-3,17-dione (**8**, 0.2 g, 0.46 mmol) and stannous chloride (1.4 g, 6.2 mmol) were dissolved in a solution of ethanol (10 mL) and concentrated hydrochloric acid (1.5 mL). The reaction mixture was treated in a manner similar to that of **9** to yield a yellowish crystal (0.12 g, 64.5%): mp 160–161.5 °C, UV (methanol) λ_{max} = 251 nm, 297 nm; IR (KBr) 3450, 3360, 2980, 2950, 1740, 1645, 1620, 1530, 1380. ^1H NMR ($CDCl_3$) δ 0.97 (s, 3 H, C_{18}), 1.00 (s, 3 H, C_{19}), 3.6 (br s, 2 H, NH_2), 5.62 (s, 1 H, C_4), 6.04 (s, 1 H, C_6), 6.64–6.67 (d, 2 H, $C_{3'}$, J = 8.3 Hz), 6.95–6.98 (d, 2 H, $C_{2'}$, J = 8.3 Hz); MS *m/e* (relative intensity) 403 (M^+, 0.082), 279 (0.017), 252 (0.019), 106 (1.00), 91 (0.107). Anal. ($C_{27}H_{33}NO_2$) C, H.

Biochemical Methods. Preparation of Placental Microsomes. Human placenta were obtained immediately upon delivery from the Ohio State University Hospital and stored on ice during transportation to the laboratory. The preparation of microsomes was performed according to the method of Ryan.[24] All procedures were carried out at 0–4 °C. The placenta was cut free of connective tissue and large blood vessels with scissors. The tissue was then

(23) Screttas, C. G.; Screttas, M. M. *J. Org. Chem.* **1978**, *43*, 1046.

(24) Ryan, K. J. *J. Biol. Chem.* **1959**, *234*, 268.

homogenized in a cold Waring blender with two parts of tissues to one part of homogenization buffer consisting of 0.05 M sodium phosphate, 0.25 M sucrose, and 0.04 M nicotinamide, pH 7. The homogenate was centrifuged at 10000g for 30 min. The debris was discarded and the supernatant was centrifuged at 105000g for 1 h. The microsomal pellet obtained was resuspended in 0.1 M sodium phosphate buffer, pH 7, and centrifuged at 105000g for 1 h. The procedure was repeated once again and the resulting pellet was stored at -70 °C until needed. Protein concentrations were determined by the method of Lowry.[25]

Competitive Inhibition Studies. Aromatase activity in human placental microsomes was assayed by a radiometric method developed by Siiteri and Thompson[21] in which the tritium from [1β-^{3}H]-4-androstene-3,17-dione was released as ^3H_2O and used as an index of estrogen formation. The procedures for evaluation of inhibition are similar to those previously reported by Brueggemeier et al.[14] [1β-^{3}H]4-Androstene-3,17-dione (300000 dpm), various concentration of 4-androstene-3,17-dione (60–500 nM), and a concentration of inhibitor (0–600 nM) were preincubated with propylene glycol, (100 μL), NADP (1.8 mM), glucose 6-phosphate (2.85 mM) and glucose-6-phosphate dehydrogenase (5 units) at 37 °C for 5 min. Placental microsomes (0.07–0.1 mg) were diluted to 3.0 mL with 0.1 M sodium phosphate buffer, pH 7, and warmed to 37 °C for 5 min. The enzyme assay began with the addition of the microsomal suspension (3.0 mL) to the mixture of steroids and cofactors. The solution was incubated at 37 °C for 15 min in a shaking water bath and was stopped by addition of $CHCl_3$ (5 mL), followed by vortexing of the samples for 20 s. The samples were then centrifuged for 10 min (1000g). Aliquots of water (200 μL) were mixed with scintillation cocktail (5 mL) and the radioactivity was counted. Assays were run in duplicate and control samples containing no inhibitor were run simultaneously. Blank samples were obtained by incubating boiled microsomes. Results were analyzed by a weighted regression-analysis computer program.[22]

Acknowledgment. This work was supported in part by NIH Grant R01 DK-40255, NIH Grant P30 CA-16058, and American Cancer Society Grant BC-482.

Registry No. 1, 63-05-8; 2, 4966-70-5; 3, 122623-16-9; 4, 122623-17-0; 5, 122623-18-1; 6, 122623-19-2; 7, 122623-20-5; 8, 122623-21-6; 9, 122623-22-7; 10, 122623-23-8; ethylene glycol, 107-21-1; benzylmagnesium bromide, 1589-82-8; phenethyllithium, 42808-98-0.

(25) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
(26) Herzog, H. L.; Jevnik, M. A.; Tully, M. E.; Hershberg, E. B. *J. Am. Chem. Soc.* **1953**, *75*, 4425.

cis-Diamineplatinum(II) Complexes Containing Phosphono Carboxylate Ligands as Antitumor Agents

L. Steven Hollis,* Arthur V. Miller, Alan R. Amundsen, John E. Schurig,† and Eric W. Stern

Engelhard Corporation, Research and Development, Menlo Park CN-40, Edison, New Jersey 08818, and Bristol-Myers Corporation, Pharmaceutical Research and Development, Wallingford, Connecticut 06492. *Received March 31, 1989*

A series of platinum complexes of the form *cis*-M[PtA_2(PC)] (I) has been prepared and tested for antitumor activity in mice. Compounds in this series contain either two monodentate amine ligands (A), such as NH_3 or isopropylamine, or one bidentate diamine (A_2), such as ethylenediamine, 1,2-diaminopropane, or 1,2-diaminocyclohexane. The PC ligand is a bidentate, O-bound, phosphono carboxylate chelate of the form $-O_2C(CR_1R_2)_nPO_3-$, where n = 0 or 1 and R_1 and R_2 are chosen from H, methyl, ethyl, propyl, butyl, phenyl, or pentanoic acid substituents. The resulting complexes (I) were prepared as the free acids (M = H) or as sodium salts (M = Na). Members of this series have demonstrated good activity in a number of tumor screens. A total of 18 platinum–phosphono carboxylate (Pt-PC) complexes were tested against Sarcoma 180 ascites (S180a) in CFW mice, with 13 analogues showing activity above the 50% ILS level. Antitumor activity was also observed vs L1210 leukemia in CDF_1 mice, where six of the 12 compounds tested gave ILS values in the 60–160% range, and vs M5076 reticulum cell sarcoma (sc tumor, iv drug), where four of the four compounds tested gave ILS and T–C values comparable to that of cisplatin. Each of the Pt-PC complexes was characterized by NMR (^{195}Pt, ^{13}C, and ^{31}P), HPLC, and elemental analysis. These compounds, which are anionic at neutral pH, display excellent solubility and stability in aqueous media, such as phosphate-buffered saline and fetal calf serum. On the basis of a comparative study of BUN and serum creatinine levels in treated mice, representative complexes from this series are also less kidney toxic than cisplatin. The results of these studies demonstrate that the platinum–phosphono carboxylate complexes are a promising new class of antitumor agents.

Cisplatin is an effective anticancer agent that is presently used in the treatment of testicular, ovarian, and bladder carcinomas.[1,2] In addition, cisplatin is widely used in combination with other antitumor agents, such as VP16, doxorubicin, and bleomycin, in treating small-cell lung carcinoma and head and neck cancers.[3] The limited activity that cisplatin displays against such major forms of the disease as breast and colon cancer has stimulated the search for new platinum-based antitumor agents.[1a] Furthermore, the adverse effects that are observed in patients receiving cisplatin, such as nephrotoxicity, myelosupression, neurotoxicity, and emesis, have inspired efforts to develop new agents that will display improved toxicological properties.[4] Research in this field has produced a number of promising new compounds that show good activity and reduced toxicity in a variety of animal tumor screens and in clinical trials.[5,6] While several of these second-generation platinum compounds, such as carboplatin, *cis*-[Pt-

† Bristol-Myers Corp.

(1) (a) Carter, S. K. In *Platinum Coordination Complexes in Cancer Chemotherapy*; Hacker, M. P.; Doyle, E. B.; Krakoff, I. H., Eds.; Martinus-Nijhoff Publishing: Boston, 1984; p 359. (b) Loehrer, P. J.; Einhorn, L. H. *Ann. Intern. Med.* **1984**, *100*, 704.
(2) (a) Nicolini, M., Ed. *Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy*; Martinus-Nijhoff Publishing: Boston, 1988. (b) Prestayko, A. W., Crooke, S. T., Carter, S. K., Eds. *Cisplatin Current Status and New Developments*; Academic Press, Inc.: New York, 1980. (c) McBrien, D. C. H., Slater, T. F., Eds. *Biochemical Mechanisms of Platinum Antitumor Agents*; IRL Press: Oxford, England, 1986.
(3) (a) Paccagnella, A.; Favaretto, G.; Fiorentino, M. V. In ref 2a, p 384. (b) Choksi, A.; Hong, W. K. In ref 2a, p 375. (c) Fiorentino, M. V.; Ghiotto, C. In ref 2a, p 415. (d) Hacker, M. P.; Roberts, J. D. In ref 2a, p 163.

0022-2623/90/1833-0105$02.50/0