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Synthesis and evaluation of novel 4-amino-4,6-androstadiene-3,17-dione: An analog of formestane

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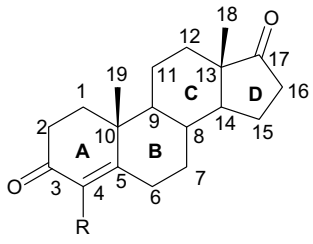
ABSTRACT

Synthesis of 4-amino-4,6-androstadiene-3,17-dione **7**, an analog of formestane used in breast cancer therapy as an aromatase inhibitor, from 4-acetoxy-4-androstene-3,17-dione **2** is described. This is the first report of a 4-amino diene (4,6) system in this series of molecules. The new (**7**) and reported molecules were screened by the National Cancer Institute (NCI, Bethesda, USA) for in vitro antitumor activity against 60 human cancer cell lines. Molecule **7** showed best activity against breast cancer cell line (MCF-7).

Mammary tumors represent the most widespread type of malignant neoplasms and are the second major cause of mortality in women.[1] About half of these malignancies require a source of estrogens for their growth and development. Estrogens are biosynthesized from androgens by the microsomal cytochrome P-450 enzyme system termed aromatase. Aromatase represents an enzyme complex made up of two major components: a flavoprotein (NADPH-cytochrome P-450 reductase) and a specific form of cytochrome P-450 commonly known as aromatase cytochrome P-450. This protein is involved in the binding of C-19 atom of the steroid substrate and catalyzes the multistep reaction resulting in the aromatization of the ring A of the steroid.[2] Current endocrine therapy relies upon the use of either antiestrogens, which act on tumor cells directly or an alternative strategy consisting of the application of aromatase inhibitors (AI) which suppress production of estrogens in the organ. A potent aromatase inhibitor would be a possible alternative to endocrine ablation in the treatment of advanced estrogen dependent mammary carcinoma. Since aromatase catalyses the final step in the biosynthesis of estrogens, the potent aromatase inhibitor formestane **1** (4-hydroxy-4-androstene-3,17-dione, 4-OHA) has been proven very effective in the treatment of advanced estrogen dependent breast cancer (Fig. 1).[3]



1. R = OH, Formestane (4-OHA)
2. R = OAc, Formestane acetate (4-OAcA)

Figure 1.

The mechanism of the inhibitory effect of 4-OHA on aromatase has not been completely elucidated. An inactivation mechanism, which involves the formation of a covalent bond between the enzyme and position 4 of the steroid substrate has been proposed by Covey et al.[4] Protonation of the hydroxy group at position 4 of the substrate and its elimination as a water molecule occurs in the enzyme bound intermediate. Inactivation of the enzyme follows aromatization of the ring A of the steroid. Although 4-OHA is a potent aromatase inhibitor, it suffers from a low bioavailability and short term action and manifests in vivo activity only on subcutaneous administration mainly due to its high rate of glucuronidation.[5] These limitations of 4-OHA has lead to the quest for the synthesis and analysis of its various analogues with improved

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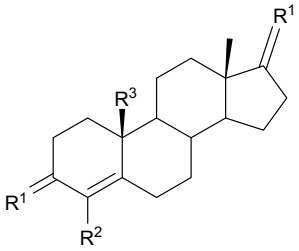
aromatase inhibitory activity with fewer limitations. The chemical synthesis of new AI's is a big challenge employing original approaches based on a combination of several methods. Various structural modifications of 4-OHA[6] have consisted of the introduction of substituents into ring A or at adjacent positions with simultaneous preservation, in the majority of cases, of polar functional groups at C(3), and C(17). The sites for substitution have been C (1), C (4), C (6), C (7) and C (19).

Highly active 4-amino androstenediones present special interest. They appeared to be efficient aromatase inhibitors. Studies of structure activity relationship revealed the enhanced rate and selectivity of aromatase inhibition upon the introduction of 1,2- and 6,7-double bonds into the molecules of 4-amino androstenediones. Further conjugation of the 3-keto-4-ene system caused more rapid inactivation of aromatase in rat ovarian microsomes than the parent compound 4-OHA. Conjugated systems such as 4-amino 1,4,6-triene-3,17-dione have been synthesized by a patented and lengthy chemical procedure and evaluated in detail,[7] but no attempts have been made in the past to synthesize 4-amino-4,6-3,17-dione molecule (**7**). Considering the significance of extended conjugation in 4-OHA in breast cancer therapy, herein we report a new methodology for the synthesis of **7** which will facilitate the design of novel aromatase inhibitors for the treatment of breast cancer.

Attempts to synthesize reported 4-hydroxy-4,6-androstadiene-3,17-dione **5** from 4-acetoxy-4-androstenene-3,17-dione **2** using triethyl orthoformate and *p*-toulenesulfonic acid[8] as well as direct oxidation using the patented procedure[9] with chloranil and acetic acid in toulene were unsuccessful. These procedures work well when the 4-hydroxy group is protected with an electron donating group but not with parent 4-OHA (**1**) or its acetyl derivative (**2**).

We used an alternative approach (Scheme 1) by synthesizing a stable 4-acetoxy-6-bromo-4-androstene-3,17-dione **3** by reacting 4-OHA with *N*-bromosuccinamide in refluxing carbon tetrachloride for 20 min. in 94% yield. The ^{1}H NMR spectrum showed a multiplet at 5.48 ppm for one proton (C6), while this carbon appears at 42.12 ppm in the ^{13}C NMR. The HRMS showed peak at M^++Na at 445.09 which confirms the structure of **3**. Compound **3** yielded 4-acetoxy-4,6-androstadiene-3,17-dione **4** on treatment with sodium iodide in acetone. The double bond formation in **4** is evident



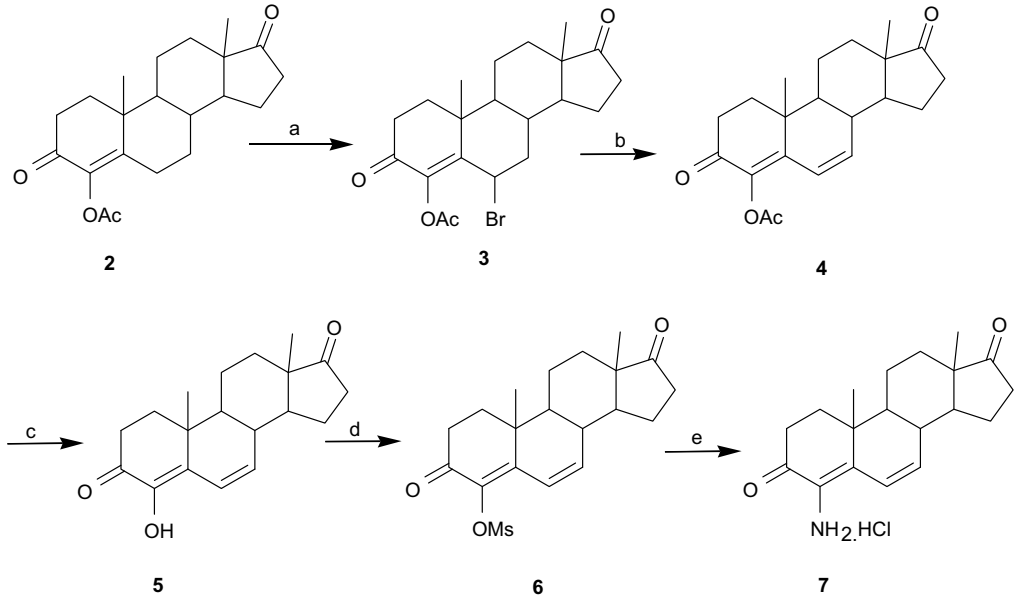
8. R^1 = S, R^2 = OAc, R^3 = CH3
9. R^1 = O, R^2 = H, R^3 = CH_2F
10. R^1 = O, R^2 = OC_6H_5 , R^3 = CH_3
11. R^1 = O, R^2 = OCH_2CH_3 , R^3 = CH_3

Figure 2.

from the presence of 6,7 olefinic protons (5.15 and 5.65 ppm) in the ^{1}H NMR. The yield of **4** after column chromatography was 74%. The final step of deprotection of the acetyl group was done by reacting **4** with 2.5 M KOH in methanol: dichloromethane (20:1) at room temperature for 2 h to give **5** as a colorless solid after silica gel column chromatography. The ^{1}H NMR spectrum showed the olefinic protons at C6 and C7 in **5** at 6.92 and 6.03 ppm, the ^{13}C NMR showed the C6 and C7 carbons at 115.13 and 135.69 ppm, respectively. Conversion of **5** to the methanesulfonyl derivative **6** followed by treatment with NH_4OH (28% NH_3) yielded the title compound **7** as an oil, which was precipitated out as salt by passing HCl gas to the DCM solution of the oil.

We also synthesized some compounds **8-11** (Fig. 2) for comparing the in vitro activity with our amino compound **7**. All the synthesized analogs have been evaluated by the National Cancer Institute (NCI, Bethesda, USA), on all the 60 human cancer cell lines organized into subpanel derived from nine different human cancer types[10]: leukemia, melanoma, lung, colon, renal, ovarian, breast, prostate and CNS, at one dose (10^{-5} M concentration) primary anti-cancer assay (Table 1).

A compound is considered active when it reduces the mean growth percentage of any cell lines to 32% or less (negative numbers indicate cell kill). Compound **7** have shown significant activity



Scheme 1. Reagents and conditions: (a) NBS/CCl_4, reflux, 20 min., 94% (b) NaI/acetone, refluxed, 4 h, 74% (c) 2.5 M KOH, MeOH: CH_2Cl_2 (20:1), rt, 2 h, 26% (d) MsCl, Py, 0 °C, 1 h, 76% (e) NH_4OH, 1,4-dioxane, 96 h.

Table 1
In vitro one dose primary anticancer assay for compounds

Panel/Cell line	7	8	9	10	11
NSC lung cancer					
A549/ATCC	103.34	103.40	99.26	100.25	97.79
EKVX	97.99	65.58	94.85	104.06	111.14
HOP-62	111.88	102.14	123.96	106.47	103.52
HOP-92	61.94	83.39	113.59	87.06	92.93
NCI-H226	96.30	113.39	111.29	96.55	98.58
NCI-H23	74.07	100.35	106.48	97.57	101.11
NCI-H322M	88.38	92.32	101.55	106.72	95.97
NCI-H460	83.77	99.49	108.88	77.42	83.36
NCI-H522	76.59	89.15	87.35	87.31	91.00
Colon cancer					
COLO 205	121.26	110.35	134.97	115.44	132.65
HCC-2998	96.01	126.51	139.86	128.78	125.75
HCT-116	6.15	86.59	110.19	99.87	103.20
HCT-15	72.99	105.90	106.17	94.74	110.45
HT29	51.17	111.91	116.40	100.27	98.16
KM12	39.52	109.64	117.01	115.72	123.89
SW-620	15.36	107.43	125.89	120.45	120.57
Breast cancer					
BT-549	82.63	94.20	97.23	110.10	93.87
HS578T	112.26	117.57	127.96	103.50	118.13
MCF7	−15.97	97.31	130.43	103.22	108.56
MDA-MB- 231/ATCC	100.87	107.68	117.71	109.86	106.73
MDAMB-435	112.52	116.00	100.91	113.61	104.79
NCI/ADR-RES	68.71	104.07	107.98	90.34	97.45
T-47D	73.66	102.16	39.44	116.70	117.94
Ovarian cancer					
IGROV1	49.46	36.69	62.42	55.73	85.75
OVCAR-3	64.57	105.69	119.07	121.39	128.63
OVCAR-4	61.98	102.43	98.85	100.72	96.98
OVCAR-5	107.69	108.39	118.37	97.47	101.66
OVCAR-8	44.03	89.90	94.26	93.48	98.96
SK-OV-3	120.27	118.54	120.50	117.40	111.30
Leukemia					
CCRF-CEM	6.62	92.56	88.21	90.45	91.63
HL-60(TB)	52.53	97.15	112.8	105.95	112.2
K-562	−26.94	68.18	93.48	86.08	94.09
MOLT-4	57.20	114.9	106.1	105.63	98.59
RPMI-8226	−18.11	57.95	—	87.07	—
SR		88.29	118.7	66.91	104.9
Renal cancer					
786-0	89.28	97.68	105.6	100.78	103.4
A498	99.19	110.9	111.1	103.00	105.6
ACHN	88.16	110.1	119.9	105.72	105.1
CAKI-1	84.08	112.9	111.0	112.48	115.2
RXF 393	95.21	104.6	67.71	107.93	117.9
SN12C	88.53	180.9	113.5	90.07	110.6
TK-10	85.59	97.59	114.8	122.99	100.6
UO-31	64.59	79.41	87.76	76.98	85.82
Melanoma					
LOX IMVI	60.57	95.59	96.65	101.44	100.3
M14	79.51	101.6	102.8	102.62	103.8
MALME-3M	70.81	75.28	94.17	120.52	87.47
SK-MEL-2	74.41	88.46	97.19	82.37	94.48
SK-MEL-28	103.4	119.4	116.5	119.81	135.2
SK-MEL-5	69.71	100.9	108.7	94.23	99.39
UACC-257	86.69	114.4	97.04	108.45	117.1
UACC-62	81.02	105.6	114.6	87.46	90.78
Prostate cancer					
DU-145	62.36	115.4	116.2	107.32	123.2
PC-3	46.51	89.28	—	89.25	96.50
CNS cancer					
SF-268	89.03	111.7	109.8	113.44	128.3
SF-295	105.1	124.3	106.3	124.31	114.0
SF-539	82.75	105.8	105.8	106.75	111.3
SNB-19	102.8	110.1	109.3	114.92	112.3
SNB-75	94.19	98.00	90.07	91.11	79.71
U251	54.19	102.0	111.4	91.35	

by reducing the growth of cells in wide range of cell lines (nine panels). The most sensitive cell lines are colon cancer HCT-116 (6.15), SW-620 (15.36), KM12 (39.52), Breast cancer MCF-7 (−15.97), Ovarian cancer OVCAR-8 (44.03), Leukemia CCRF-CEM (6.62), K-562 (−26.94), SR (−18.11) and also Prostate cancer PC-3 (46.51) as compared to the other compounds.

In conclusion we have synthesized a new 4-amino-4,6-androstadiene-3,17-dione (**7**)[11] compound, that exhibits potential in vitro cytotoxicity in number of cancer cell lines. The detailed anticancer activity and mechanism of action of 7 will be published in due course.

Acknowledgments

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10. (a) Grever, M. R.; Schepartz, S. A.; Chabner, B. A. *The National Cancer Institute: Cancer Drug Discovery and Development Programme. Seminars in Oncology* **1992**, *196*, 622; b The human tumor cell lines of the cancer screening panel are grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. Cells are inoculated into 96-well microtiter plates in 100 μL at plating densities ranging from 5000 to 40,000 cells/well. After cell inoculation, the microtiter plates are incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of drugs. The drugs are solubilized in dimethyl sulfoxide at 400-fold the desired final concentration and stored frozen prior use. At the time of drug addition, an aliquot of frozen concentrate is thawed and diluted to twice the desired final concentration with complete medium containing 50 μg/mL gentamicin. Additional four, 10-folds or half log serial dilutions are made to provide a total five drug concentrations plus control. Aliquots of 100 μL of these different drugs dilutions (single dose of 10^{-5} M) are added to the appropriate microtiter wells already containing 100 μL of medium, resulting in the required final drug concentration. Following drug addition, the plates are incubated for an additional 48 h at 37 °C C, 5% CO_2, 95% air and 100% relative humidity. Cells are fixed in situ by the gentle addition of 50 μL of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The supernatant is discarded, and plates are washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 μL) at 0.4% (w/v) in 1% acetic acid is added to each well, and plates are incubated for 10 min at room temperature. After staining unbound dye is removed by washing (five times with 1% acetic acid) and plates air dried. Bound stain is subsequently solubilized with 10 mM trizma base, and the absorbance is read on an automated plate reader at a wavelength of 515 nm.
11. *4-Acetoxy-6-bromo-4-androstene-3,17-dione (3):* To a solution of **2** (120 mg, 0.35 mmol) in carbon tetrachloride (20 ml) was added NBS (240 mg, 1.35 mmol) and benzoyl peroxide (13 mg, 0.05 mmol). The reaction mixture was stirred and refluxed for 20 min. After being cooled to room temperature, the mixture was filtered and the filtrate was washed with a solution of aqueous sodium bicarbonate and water. The organic phase was dried with sodium sulfate and concentrated under reduced pressure. The residue was purified by column chromatography on SiO_2 using hexane-ethyl acetate (2:1) as eluent. Evaporation of the appropriate fraction yielded **3** as colorless solid (140 mg, 94%).%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.48(1 H, t, 6-**H**), 2.30(3 H, s, COC***H*₃**), 1.61(3 H, s, 19-C***H*₃**), 0.98(3 H, s, 18-C***H*₃**). ^{13}C NMR ($CDCl_3$, 125 MHz) δ

219.78(C-17), 190.60(C-3), 167.97(*C*OCH$_3$), 150.04(C-4), 141.01(C-5), 52.64(C-9), 50.27(C-14), 42.12(C-6), 29.85(C-8), 22.45(CO*C*H$_3$), 20.40(C-19), 13.79(C-18). ES-MS (in positive ionization) *m*/*z* calcd for $C_{21}H_{27}BrO_4Na$ 445.11 (M + Na), measured 445.1.

4-Acetoxy-4,6-androstadiene-3,17-dione (4): To a solution of **3** (630 mg, 1.49 mmol) in acetone (150 ml) was added sodium iodide (7.0 g, 46.70 mmol). The reaction mixture was stirred and refluxed for 4 h. The solvent was removed under reduced pressure. The residue was partitioned between dichloromethane (150 mL) and an aqueous sodium thiosulfate solution. Dichloromethane was washed with a solution of aqueous sodium thiosulfate (2 × 50 mL), water, dried with sodium sulfate and concentrated under reduced pressure. The residue was purified by column chromatography on SiO_2 using hexane-ethyl acetate (2:1) as eluent. Evaporation of the appropriate fraction yielded **4** as colorless solid (377 mg, 74%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.15 and 5.65 (2 H, m, 6-**H** and 7-**H**), 2.38(3 H, s, COC***H*****$_3$**), 1.26(3 H, s, 19-C**H$_3$**), 0.92(3 H, s, 18-C**H$_3$**). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 219.74(C-17), 191.43(C-3), 168.31(*C*OCH$_3$), 148.02(C-4), 140.67(C-5), 124.74(C-7), 123.88(C-6), 53.13(C-9), 50.96(C-14), 29.42(C-8), 22.42(CO*C*H$_3$), 20.25(C-19), 13.93(C-18). ES-MS (in positive ionization) *m*/*z* calcd for $C_{21}H_{26}O_4Na$ 365.18 (M + Na), measured 365.2.

4-Hydroxy-4,6-androstadiene-3,17-dione (5): To a solution of **4** (35 mg, 0.1 mmol) in methanol-dichloromethane (20:1) (20 ml) was added a solution of 2.5 M KOH in water (1.5 mL). The reaction mixture was stirred for 2 h at ambient temperature. The solvent was removed under reduced pressure. The residue was purified by column chromatography on SiO_2 using hexane-ethyl acetate (3:1) as eluent. Evaporation of the appropriate fraction yielded **5** as colorless solid of (8 mg, 26%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.92(1 H, dd, $J_{7,6}$ = 8.5 Hz, $J_{7,8}$ = 3.5 Hz, 7-**H**), 6.15(1 H, br, s, O**H**), 6.03(1 H, dd, $J_{6,7}$ = 8.5 Hz, $J_{6,8}$ = 3.5 Hz, 6-**H**), 1.15(3 H, s, 19-C**H$_3$**), 0.90(3 H, s, 18-C**H$_3$**). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 220.21(C-17), 184.01(C-3), 146.26(C-4), 140.64(C-5), 135.69(C-7), 115.13(C-6), 51.43(C-9), 49.26(C-14), 30.74(C-8), 21.66(C-19), 13.55(C-18). ES-MS (in positive ionization) *m*/*z* calcd for $C_{19}H_{24}O_3Na$ 323.17 (M+Na), measured 323.2.

4-Methanesulfonyl-4,6-androstadiene-3,17-dione (6): To a cool solution of **4** (125 mg, 0.42 mmol) in dichloromethane-triethylamine (30:1) (15 ml) was added methanesulfonyl chloride (0.035 mL, 0.45 mmol). After the reaction mixture was stirred at 0 °C for 1 h, ice-water (20 mL) was added. The organic solvent was separated, washed with water, dried with sodium sulfate and concentrated under reduced pressure. The residue was purified by column chromatography on SiO_2 using hexane-ethyl acetate (2:1) as eluents. Evaporation of the appropriate fraction yielded a yellowy solid (121 mg, 76%). EI-MS (in positive ionization) *m*/*z* calcd for $C_{20}H_{26}O_5S$ 378.15(M^+), measured 378.1498.

4-Amino-4,6-androstadiene-3,17-dione (7): NH_4OH [NH_3 (28%), 16 mL] was added to a stirred solution of **5** (96 mg, 0.25 mmol) in dry 1, 4-dioxane (8 mL), and the reaction was allowed to stir at room temperature for 96 h. The solvents were removed in vacuo, the oil was redissolved in dry dichloromethane and to it HCL gas was passed, the salt of **7** precipitated out. EI-MS (in positive ionization) *m*/*z* calcd $C_{19}H_{25}NO_2$ 299.1(M^+), measured 300.16 (M+1).