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Synthesis and Evaluation of 4-Substituted-4-androstene-3,17-dione Derivatives as Aromatase Inhibitors

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The synthesis and biological evaluation of 4-amino-, 4-alkoxy-, 4-aryloxy-, 4-alkyl- and 4-aryl-4-androstenedione derivatives as inhibitors of estrogen synthetase (aromatase) are described. Inhibitory activity of synthesized compounds was assessed using a human placental microsomal preparation as the enzyme source and [1β-^{3}H]androstenedione as substrate. Synthesized compounds exhibiting aromatase inhibitory activity were evaluated further under initial velocity conditions to determine apparent K_i values. Several compounds were effective competitive inhibitors and have apparent K_i values ranging from 38 to 1290 nM, with the apparent K_m for androstenedione being 47 nM. Alkylation or arylation of 4-N, S, or O-substituted steroids results in compounds that are effective competitive inhibitors that are devoid of time-dependent inactivation and that the free pair of electrons on N, S, or O is not an essential requirement for 4-substituted androstenedione derivatives to be effective aromatase inhibitors. The results obtained from this investigation are consistent with our previous studies which show that aromatase has a hydrophobic pocket in the active site around the C-4α region of androstenedione.

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INTRODUCTION

Aromatase is a cytochrome *P*450 dependent enzyme that catalyzes the aromatization of androgens to estrogens [1], and hence plays a key role in endocrine physiology and estrogen-dependent disease. In post-menopausal women, the major source of estrogen is thought to be the peripheral conversion of androgens to estrogens [2]. Although much of this conversion occurs in fat and muscle cells [3], certain human breast cancers have the capacity to convert androgens to estrogens [4–7]. In view of the fact that estrogens play a significant role in the growth and maintenance of mammary tumors, inhibition of estrogen synthesis by aromatase inhibitors may be one significant approach in the treatment of breast cancer.

Numerous aromatase inhibitors, analogs of 4-androstenedione, have been described [8, 9], including 4-hydroxy [10], 19-ethynyl [11], and 1-methylene [12] derivatives, which have been evaluated clinically. It is interesting to note that 4-hydroxyandrostenedione (4-OHA) [13], 4-aminoandrostenedione [14] and 4-mercaptoendrostenedione [15, 16] have a free pair of electrons and were all found to be excellent competitive inhibitors as well as time-dependent inactivators. However, the 4-(alkylthio)- and 4-(arylthio)androstenedione analogs have been found to be effective competitive inhibitors [17–19], but were not time-dependent inactivators [20], underlining the importance of the free -SH group. Whether the free pair of electrons on the oxygen, sulfur or nitrogen analogs of the 4-substituted substrate is essential for aromatase inhibitory activity is yet to be determined. To further expand on our earlier studies in probing the size of the hydrophobic pocket around the C-4 of androstenedione, and to address the importance of a free -NH_2, SH- and -OH at C-4, the synthesis of a series of 4-amino, 4-(O-alkyl), 4-(O-aryl), 4-(alkyl), and 4-(aryl)-4-androstene-3,17-dione derivatives has been carried out and their resultant *in vitro* aromatase inhibitor activity determined.

EXPERIMENTAL

Melting points were determined on a Fisher–Jones melting point apparatus and are uncorrected. Infrared spectra were obtained on a Nicolet 5DXC FT-IR spectrometer. Nuclear magnetic resonance were

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obtained with a GE 300 MHz spectrometer using TMS as internal standard. TLC was performed on a pre-coated silica gel plate (Silica Gel GF; Analtech, Inc., Newark, NJ). Silica gel (200–400 mesh, Aldrich Chemical Company, Milwaukee, WI) was used for all column chromatography. Ultracentrifugation was performed on a Beckman L2-65B ultracentrifuge. Radioactivity was determined on a Beckman LS-100 liquid scintillation counter. Androstenedione was purchased from Steraloids (Wilton, NH). All other chemicals were reagent grade and were obtained from Aldrich Chemical Company or Sigma Chemical Company (St Louis, MO).

4-Hydroxy-4-androstene-3,17-dione (3)

The synthesis of **3** was carried out using androstenedione (**1**) as described by Mann and Pietrzak [21].

4-Methoxy-4-androstene-3,17-dione (4a)

To a solution of 300 mg of **3** in THF (20 ml) was added 112 mg (1 mmol) of potassium *t*-butoxide and the solution was stirred at 0° for 5 min. To the cooled solution was added 1.42 g (10 mmol) of methyl iodide and the reaction was stirred at room temperature for an additional 6 h. After addition of 15 ml of saturated NH_4Cl, the mixture was extracted with CH_2Cl_2 (200 × 2), and the combined organic layers were washed with water, dried (Na_2SO_4) and concentrated to afford 230 mg of crude oil that was purified on a silica gel column (ethylacetate : hexane; 1 : 2.5). The fractions corresponding to product were pooled and recrystallized from acetone–hexane to give pure crystals of **4a** (167 mg, 56%); m.p. 136–138°C ^{1}H-NMR ($CDCl_3$) δ 0.92 (s, 3H, 18-CH_3), 1.22 (s, 3H, 19-CH_3), 3.60 (s, OCH_3), IR: 1735, 1677, 1608 cm^{-1}.

4-Ethoxy-4-androstene-3,17-dione (4b)

Yield 39%; m.p. 157–159°C (from acetone–hexane); IR (KBr) 1739, 1680, 1605 cm^{-1}; ^{1}H-NMR δ 0.92 (3H, s, 18-Me), 1.22 (s, 3H, 19-Me), 1.28 (t, 3H, 4-$CH_2\underline{CH}_3$), 3.76–3.85 (m, 2H, 4-$O\underline{CH}_2CH_3$). Anal. ($C_{21}H_{30}O_3$) C, H.

4-Propoxy-4-androstene-3,17-dione (4c)

Yield 62%; oil; IR (KBr) 1739, 1706, 1611 cm^{-1}; ^{1}H-NMR δ 0.92 (s, 3H, 18-Me), 0.97 (t, 3H, 4-$OCH_2CH_2\underline{CH}_3$), 1.22 (s, 3H, 19-Me), 3.63–3.73 (m, 2H, 4-$O\underline{CH}_2CH_2CH_3$).

4-Butoxy-4-androstene-3,17-dione (4d)

Yield 47.5%; oil; IR (KBr) 1739, 1683, 1614 cm^{-1}; ^{1}H-NMR δ 0.92 (s, 3H, 18-Me), 0.94 (t, 3H, 4-$O(CH_2)_3\underline{CH}_3$), 1.21 (s, 3H, 19-Me), 3.66–3.77 (m, 2H, 4-$O\underline{CH}_2(CH_2)_2CH_3$).

4-Benzyloxy-4-androstene-3,17-dione (4e)

Yield 52%; m.p. 147–149°C. IR (KBr) 1738, 1678, 1608 cm^{-1}; ^{1}H-NMR δ 0.87 (s, 3H, 18-Me), 1.12 (3, 3H, 19-Me), 4.84 (q, 2H, 4-$O\underline{CH}_2Ph$), 7.34 (m, 5H, Ph). Anal. ($C_{26}H_{32}O_3$) C, H.

4-Phenoxy-4-androstene-3,17-dione (4f)

Commercial copper (I) iodide (238 mg, 1.25 mmol) was stirred with di-*n*-butyl sulfide (350 mg, 0.42 ml, 2.4 mmol) for 30 min at 80°C, and the resulting amber liquid was filtered and stored at room temperature. To a cooled solution (−78°C) of iodo[bis(di-*n*-butylsulfide)] copper (1.2 mmol) in THF (20 ml) was added methyllithium (0.71 ml, 1.4 M, 1 mmol) resulting in the immediate formation of a bright yellow methyl copper precipitate. The solid was compacted down by centrifugation at room temperature and the supernatant removed by cannula. Fresh ether (15 ml) was added to the methyl copper and the solution kept at −78°C. A solution of **3** (302 mg, 1 mmol) in THF (20 ml) and pyridine (20 ml) was added to the methyl copper solution (1 mmol) and the mixture was stirred for 2 h at room temperature after which iodobenzene (300 mg, 1.5 mmol) was added and the reaction was refluxed for 5 h. The reaction mixture was poured over 50 g of ice containing 20 ml of conc. HCl, extracted with CH_2Cl_2, and the organic extracts were combined, washed with water, dried (Na_2SO_4) and concentrated to give 172 mg of a foamy product of **4f**. Recrystallization from acetone–hexane gave 138 mg of pure **4f** (45% yield); m.p. 148–149°C. IR (KBr) 2945, 1739, 1683, 1165 cm^{-1}. ^{1}H-NMR δ 0.93 (s, 3H, 18-Me), 1.33 (s, 3H, 19-Me), 6.79–7.29 (m, 5H, Ph). Anal. ($C_{25}H_{30}O_3$) C, H.

4-(4′-Methyl-phenoxy)-4-androstene-3,17-dione (4g)

This compound was prepared as described for **4f**. Yield: 34.4%; m.p. 148–149°C. IR (KBr) 1739, 1683, 1614 cm^{-1}. ^{1}H-NMR δ 0.92 (s, 3H, 18-Me), 1.32 (s, 3H, 19-Me), 2.26 (s, 3H, Ph-CH_3), 6.69–7.05 (dd, 4H, Ph). Anal. ($C_{26}H_{32}O_3$) C, H.

3β-Acetoxy, 17β-acetoxy-5α-androstane-4α,5α-epoxide (6)

m-Chloroperbenzoic acid (4.3 g, 20 mmol) was added to a solution of 3β,17β-acetoxy-4-androstene (7.18 g, 19.2 mmol) in CH_2Cl_2 (300 ml), and the mixture was stirred at room temperature for 8 h in the dark. The reaction mixture was washed with $Na_2S_2O_3$ solution, 5% $NaHCO_3$ solution, water, dried (Na_2SO_4) and evaporated to give **6** in 83% yield. IR (KBr) 2970, 2950, 1737, 1450, 1376 cm^{-1} ^{1}H-NMR δ 0.81 (s, 3H, 18-Me), 1.13 (s, 3H, 19-Me), 2.04, 2.08 (s, s, 6H, $\underline{CH}_3$-CO-), 2.88 (s, 1H, 4-H), 4.59 (t, 1H, 17-H), 4.96 (t, 1H, 3-H).

Grignard reactions of the 4α,5α-epoxide

To a solution of 300 mg (0.78 mmol) of androstan-4α,5α-epoxy-3β,17β-diacetate (**6**) in 30 ml of THF was added 20 molar equivalent of Grignard reagent. (RMgBr: R = methyl, ethyl, *n*-propyl, *n*-butyl, benzyl, phenyl, or toluyl) in THF (15 ml) and the reaction

mixture was stirred at 0°C for 10 min followed by refluxing under nitrogen for 8 h. The reaction mixture was cooled, quenched with a saturated solution of NH_4Cl and the aqueous layer extracted with CH_2Cl_2. The combined organic layers were washed with water, dried (Na_2SO_4) and evaporated to dryness leaving a residue which was purified by column chromatography and/or recrystallization from acetone/hexane to yield 4β-substituted 5α-androstane-3β,5α,17β-triols. (7).

*4β-Methyl-5α-androstane-3β,5α,17β-triol (**7a**)*

Yield 56%, m.p. 210–211°C; ^{1}H-NMR δ 0.71 (s, 3H, 18-Me), 0.96 (s, 3H, 19-Me), 0.98 (d, 3H, 4-Me), 3.53–3.58, 4.33–4.35 (m, m, 2H, 3-H, 17-H).

*4β-Ethyl-5α-androstane-3β,5α,17β-triol (**7b**)*

Yield 85%; m.p. 219–220°C; ^{1}H-NMR δ 0.88 (s, 3H, 18-Me), 0.94 (s, 3H, 19-Me), 0.98 (t, 3H, 4-CH_2CH_3), (q, 24, 4-CH_2CH_3), 3.52–3.61 (m, 1H, 17-H), 4.38–4.50 (m, 1H, 3-H).

*4β-Propyl-5α-androstane-3β,5α,17β-triol (**7c**)*

Yield 51.6%; m.p. 138–139°C; ^{1}H-NMR δ 0.72 (s, 3H, 18-Me), 0.89 (t, 3H, 4-$CH_2CH_2CH_3$), 0.95 (s, 3H, 19-Me), 3.62 (t, 1H, 17-H), 4.36–4.42 (m, 1H, 3-H).

*4β-Butyl-5α-androstane-3β,5α,17β-triol (**7d**)*

Yield 81.4%; m.p. 147–148°C; ^{1}H-NMR δ 0.72 (s, 3H, 18-Me), 0.88 (t, 3H, 4-$(CH_2)_3CH_3$), 0.95 (s, 3H, 19-Me), 3.63 (t, 1H, 17-H), 4.35–4.42 (m, 1H, 3-H).

*4β-Benzyl-5α-androstane-3β,5α,17β-triol (**7e**)*

Yield 69%; oil; ^{1}H-NMR δ 0.71 (s, 3H, 18-Me), 1.10 (s, 3H, 19-Me), 3.59 (t, 1H, 17-H), 4.36–4.42 (m, 1H, 3-H), 7.19–7.25 (m, 5H, Ph).

*4β-Phenyl-5α-androstane-3β,5α,17β-triol (**7f**)*

Yield 88.5%; oil; ^{1}H-NMR δ 0.62 (s, 3H, 18-Me), 0.81 (s, 3H, 19-Me), 3.55–3.70 (m, 1H, 17-H), 4.57–4.69 (m, 1H, 3-H), 7.19–7.47 (m, 5H, Ph).

*4β-Tolyl-5α-androstane-3β,5α,17β-triol (**7g**)*

Yield 92%; oil; ^{1}H-NMR δ 0.63 (s, 3H, 18-Me), 0.84 (s, 3H, 19-Me), 3.17 (d, 1H, 4-H), 3.57–3.68 (m, 1H, 17-H), 4.57–4.69 (m, 1H, 3-H), 7.06–7.36 (m, 4H, Ph).

Oxidation of 4β-substituted 3β,5α,17β-triols (7)

To a chilled solution of 250 mg of 4-substituted 5α-androstane-3β-5α,17β-triol (7) in 30 ml of acetone was added 0.1 ml of Jones reagent and the solution stirred at 0°C. The reaction was monitored by TLC. After 5–7 min the reaction was quenched with 10 ml of MeOH followed by addition of 5 ml of saturated $NaHCO_3$ and 15 ml of H_2O. Extraction with CH_2Cl_2, followed by washing the organic layer with brine, water, drying over $NaSO_4$ and evaporation of the solvent gave crude product. Recrystallization from acetone–hexane gave the 4β-substituted 5α-androstane-5α-ol-3,17-diones (8).

*4β-Methyl-5α-androstane-5α-ol-3,17-dione (**8a**)*

Yield 61%; m.p. 193–195°C, ^{1}H-NMR δ 0.88 (s, 3H, 18-Me), 1.17 (s, 3H, 19-Me), 1.23 (d, 3H, 4-CH_3).

*4β-Ethyl-5α-androstane-5α-ol-3,17-dione (**8b**)*

Yield 86%; m.p. 213–214°C. ^{1}H-NMR δ 0.85 (s, 3H, 18-Me), 0.88 (t, 3H, 4-CH_2CH_3), 1.18 (s, 3H, 19-Me).

*4β-Propyl-5α-androstane-5α-ol-3,17-dione (**8c**)*

Yield 79%; m.p. 192–193°C; ^{1}H-NMR δ 0.86 (s, 3H, 18-Me), 0.88 (t, 3H, 4-$CH_2CH_2CH_3$), 1.18 (s, 3H, 19-Me).

*4β-Butyl-5α-androstane 5α-ol-3,17-dione (**8d**)*

Yield 90%; m.p. 144–145°C; ^{1}H-NMR δ 0.86 (s, 3H, 18-Me), 0.86 (t, 3H, 4-$CH_2(CH_2)_2CH_3$), 1.18 (s, 3H, 19-Me).

*4β-Benzyl-5α-androstane-5α-ol-3,17-dione (**8e**)*

Yield 49%; m.p. 238–240°C; ^{1}H-NMR δ 0.89 (s, 3H, 18-Me), 1.29 (d, 2H, 4-CH_2Ph), 1.30 (s, 3H, 19-Me), 7.07–7.27 (m, 5H, Ph).

*4β-Phenyl-5α-androstane-5α-ol-3,17-dione (**8f**)*

Yield 71%; m.p. 199–203°C, ^{1}H-NMR δ 0.8 (s, 3H, 18-Me), 0.94 (s, 3H, 19-Me), 7.16–7.29 (m, 5H, Ph).

*4β-Toluyl-5α-androstane-5α-ol-3,17-dione (**8g**)*

Yield 72%; m.p. 229–231°C; ^{1}H-NMR δ 0.81 (s, 3H, 18-Me); 0.94 (s, 3H, 19-Me), 2.30 (s, 3H, Ph-CH_3), 3.59 (s, 1H, 4-H); 7.07 (s, 4H, Ph).

Dehydration of 5α-Hydroxy steroids (8)

To a solution of the 4β-substituted 5α-hydroxy-androstane-3,17-dione (8) in 30 ml of EtOH was added 50 mg of TsOH and the solution refluxed with stirring for 3 h. Following evaporation of the solvent from the reaction mixture, the residue was dissolved in CH_2Cl_2 and the organic layer was washed with 5% $NaHCO_3$, brine, water, dried (Na_2SO_4), and evaporated to dryness to yield a crude solid. Purification on column chromatography (silica gel, EtOAc–Hexane; 1:3) followed by recrystallization from acetone–hexane gave the corresponding 4-substituted 4-androstene-3,17-diones (9).

*4-Methyl-4-androstene-3,17-dione (**9a**)*

Yield 48%; m.p. 150–151°C; ^{1}H-NMR δ 0.92 (s, 3H, 18-Me), 1.20 (s, 3H, 19-Me), 1.78 (s, 3H, 4-Me). IR (KBr) 1739, 1664, 1615 cm^{-1}. Anal. ($C_{20}H_{28}O_2$) C, H.

*4-Ethyl-4-androstene-3,17-dione (**9b**)*

Yield, 61%; m.p. 173–174°C; ^{1}H-NMR δ 0.87 (t, 4-$CH_2\underline{CH}_3$), 0.91 (s, 3H, 18-Me), 1.19 (s, 3H, 19-Me) IR (KBr) 1739, 1667, 1618 cm^{-1}. Anal. ($C_{21}H_{30}O_2$) C, H.

*4-Propyl-4-androstene-3,17-dione (**9c**)*

Yield, 57%, m.p. 134–135°C; ^{1}H-NMR δ 0.87 (t, 3H, 4-$CH_2CH_2\underline{CH}_3$), 0.92 (s, 3H, 18-Me), 1.19 (s, 3H, 19-Me). IR (KBr) 1743, 1661 cm^{-1}. Anal. ($C_{22}H_{32}O_2$) C, H.

*4-Butyl-4-androstene-3,17-dione (**9d**)*

Yield, 95%; oil; ^{1}H-NMR δ 0.90 (t, 3H, 4-$CH_2CH_2CH_2\underline{CH}_3$), 0.91 (s, 3H, 18-Me), 1.19 (s, 3H, 19-Me), IR (KBr) 1739, 1669, 1615 cm^{-1}.

*4-Benzyl-4-androstene-3,17-dione (**9e**)*

Yield, 85%; oil; ^{1}H-NMR δ 0.91 (s, 3H, 18-Me), 1.25 (s, 3H, 19-Me), 3.73 (s, 2H, 4-$\underline{CH}_2$Ph), 7.07–7.28 (m, 5H, Ph). IR (KBr) 1736, 1664 cm, 1614 cm^{-1}.

*4-Phenyl-4-androstene-3,17-dione (**9f**)*

Yield, 63%; m.p. 233–234 C; ^{1}H-NMR δ 0.93 (s, 3H, 18-Me), 1.32 (s, 3H, 19-Me), 7.02–7.35 (m, 5H, Ph). IR (KBr) 1737, 1668, 1614 cm^{-1}. Anal. ($C_{25}H_{30}O_2$) C, H.

*4-Toluyl-4-androstene-3,17-dione (**9g**)*

Yield, 34%; m.p. 284–286 C; ^{1}H-NMR δ 0.93 (s, 3H, 18-Me), 1.31 (s, 3H, 19-Me), 2.35 (s, 3H, Ph-$\underline{CH}_3$), 6.90–7.16 (dd, 4H, Ph). IR (KBr) 1742, 1670, 1614 cm^{-1}. Anal. ($C_{26}H_{32}O_2$) C, H.

*4-Methanesulfonyloxy-4-androstene-3,17-dione (**10**)*

To a solution of 500 mg of compound 3 in 50 ml of dry CH_2Cl_2 was added 2 ml of Et_3N and 0.1 ml of methanesulfonyl chloride at 0 C, and the reaction mixture was stirred at 0°C for 0.5 h. After washing the organic layer with water and drying over Na_2SO_4, evaporation of the solvent gave 490 mg of crude solid which was not purified further and was used immediately for the next reaction.

*Amination of mesylate (**11**)*

To a chilled solution (dry ice–acetone) of 200 mg of **11** in 10 ml of THF was bubbled ammonia, methylamine or dimethylamine. The reaction mixture was heated in a steel bomb for 4 h at 110°C after which the organic layer was washed with water, dried over Na_2SO_4 and evaporated to dryness to give crude product. Purification over a column of silica gel followed by recrystallization from EtOAc–hexane gave pure 4-amino products.

*4-Amino-4-androstene-3,17-dione (**11a**)*

Yield 27%; m.p. 195–197°C; ^{1}H-NMR δ 0.91 (s, 3H, 18 Me), 1.18 (s, 3H, 19 Me), 3.50 (broad, 2H, NH_2); IR (KBr) 3515, 3392, 2940, 2888, 2856, 1737, 1665, 1615 cm^{-1}. Anal. ($C_{19}H_{27}NO_2$) C, H,N.

*4-Methylamino-4-androstene-3,17-dione (**11b**)*

Yield 37%; m.p. 198–200°C; ^{1}H-NMR δ 0.92 (s, 3H, 18 Me), 1.20 (s, 3H, 19 Me), 2.55 (s, 3H, NH $\underline{CH}_3$), 3.87 (broad, 1H, 4-N$\underline{H}$ CH_3); IR (KBr) 3371, 2972, 2966, 2925, 2888, 2854, 1726, 1666, 1609, 1483, 1210 cm^{-1}. Anal. ($C_{20}H_{29}NO_2$) C, H,N.

*4-Dimethylamino-4-androstene-3,17-dione (**11c**)*

Yield 51%; m.p. 194–196°C; ^{1}H-NMR δ 0.92 (s, 3H, 18-Me), 1.19 (s, 3H, 19-Me), 2.59 (s, 6H, N($\underline{CH}_3)_2$); IR (KBr) 2967, 2945, 2920, 2898, 2876, 2830, 1740, 1669, 1615, 1452, 1053 cm^{-1}. Anal. ($C_{21}H_{31}NO_2$) C, H,N.

Biochemical methods—enzyme preparation

Microsomes were obtained from human placentas after normal deliveries and prepared as described previously [18]. Following isolation of the microsomal pellets (washed twice), they were lyophilized and stored at −20°C. These preparations can be kept for 6 months without loss of activity.

Screening assay procedure

The method of Thompson and Siiteri [1], as modified by Reed and Ohno [22], was used in our studies. This assay quantitates the production of [^{3}H]H_2O released from [1β-^{3}H]androstenedione after aromatization. All enzymatic studies were performed in 0.1 M phosphate buffer, pH 7.4, at a final incubation volume of 3.0 ml. The incubation mixture contained 2.5 mM glucose 6-phosphate; 0.5 mM NADP; 7 unit glucose-6-phosphate dehydrogenase; various concentrations of inhibitors ranging from 10^{-5} to 10^{-8} M; 0.25 μM (0.25 μCi) [1β-^{3}H]androstenedione; 1.0 mM EDTA; 10 mM phosphate buffer; and 0.15 mg of lyophilized human placental microsomes. Incubations were terminated by addition of 5 ml of $CHCl_3$, followed by vortexing for 40 s. After centrifugation at 15,000 ***g*** for 5 min, a 0.5 ml aliquot was removed and added to scintillation mixture for determination of ^3H_2O production.

Time-dependent inactivation studies were carried out as described by Covey and Hood [23].

K_i assay procedure

This procedure is essentially similar to that employed in the screening assay, except that the substrate concentration was varied from 0.005–0.25 μM and using only 0.025–0.03 mg of microsomal protein that results in a constant initial velocity, even at the lowest substrate concentration. Control samples were incubated at 0 min. Each inhibitor was examined at two concentrations (0.1 and 0.3 μM).

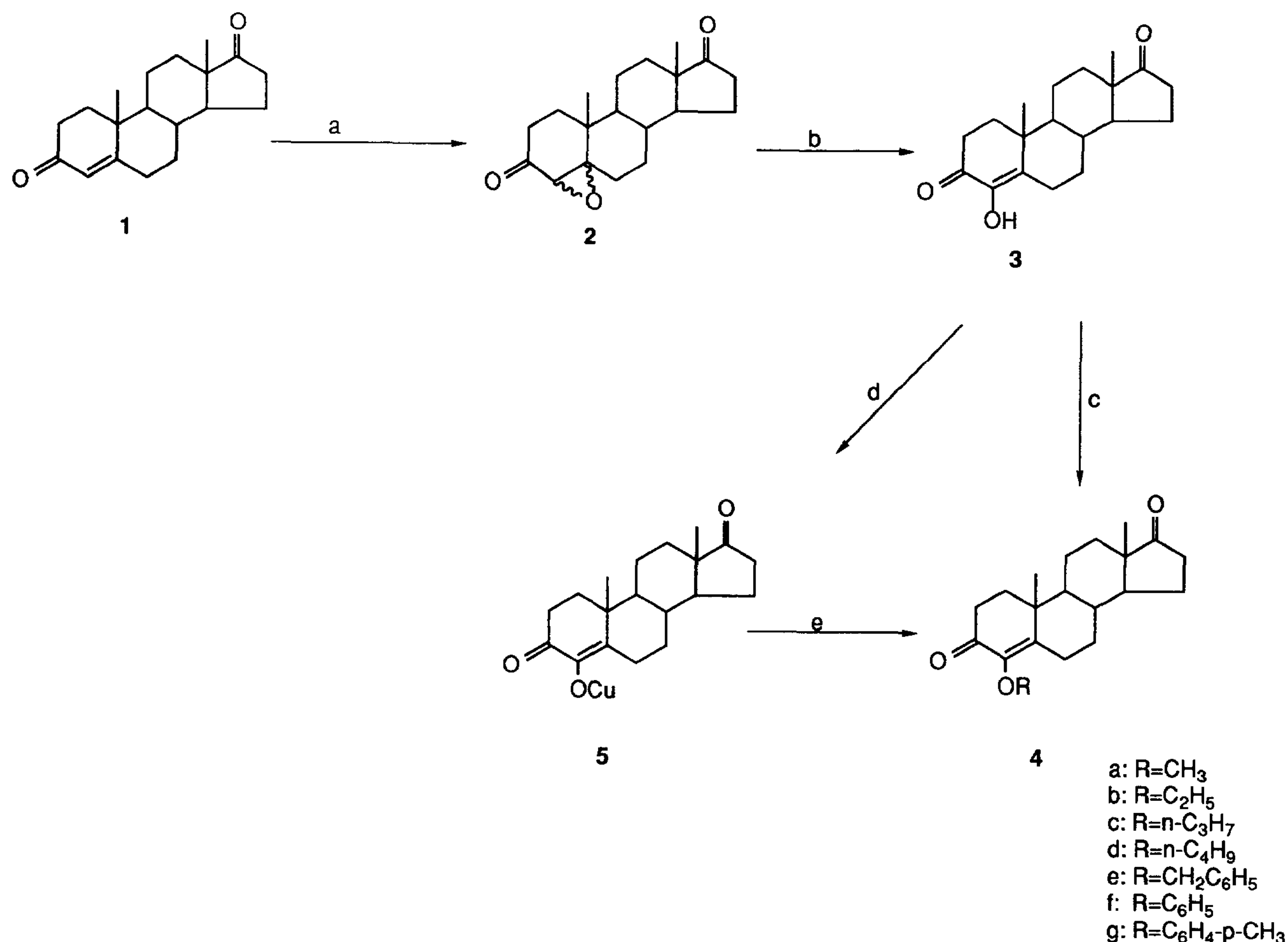
RESULTS

The synthesis of the 4-(alkoxy)-4-androstene-3,17-dione derivatives was carried out as shown in Scheme 1. Epoxidation of **1** with 30% H_2O_2 under basic conditions gave a mixture of the α- and β-epoxides (**2**) in a ratio of 6:1 β:α. Treatment of **2** with 2% conc. H_2SO_4 in glacial acetic acid gave 4-hydroxyandrostene-3,17-dione (4-OHA) in yields ranging between 50–60% as described previously [21, 24]. The synthesis of **4a–4e** was carried out by treatment of **3** with t-BuOK in dry THF and alkyl bromide to afford the 4-alkoxy series (**4a–4e**) in good yields. The 4-aryloxy analogs **4f** and **4g** were synthesized by preparing the 4-copper alkoxide (**5**) followed by treatment with the aryliodide to yield the corresponding ethers as described by Whitesides *et al.* [25].

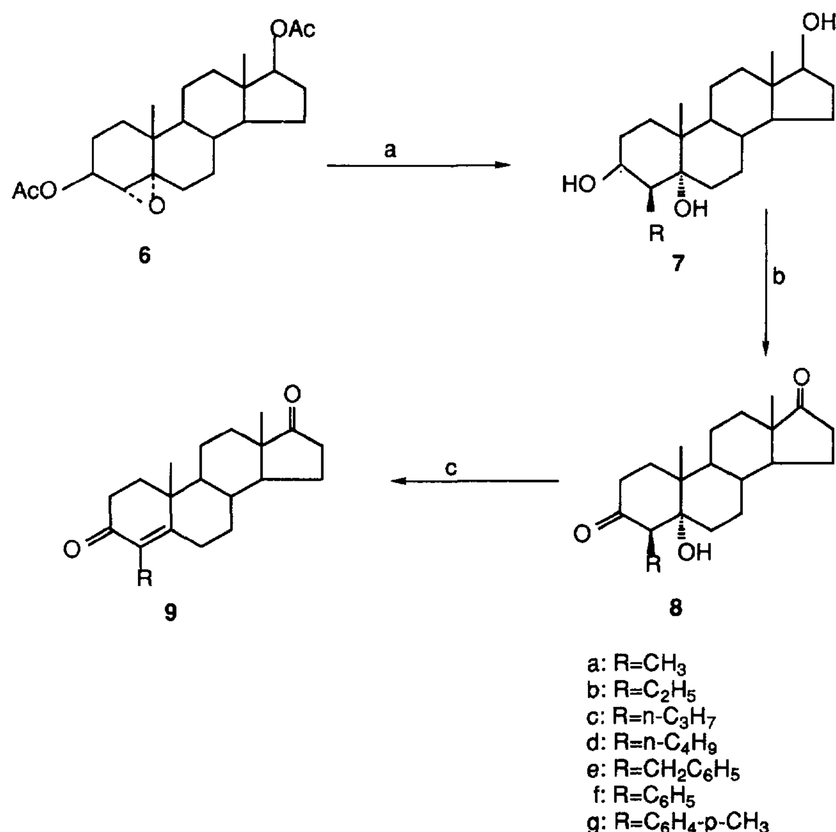
The synthesis of the 4-alkyl and 4-aryl androstenediones was carried out using the intermediate 4α,5α-epoxy-5α-androstene-3β,17β-diol acetate (**6**) (Scheme 2). Reaction of the epoxide **6** with alkyl and aryl Grignard reagents in THF with heating under reflux gave the corresponding 4β-substituted triols **7** which were oxidized by Jones reagent to give the 4β-substituted 5α-hydroxy 3,17-diones **8** in excellent yields. Jones oxidations should be carried out at 0°C in acetone for not more than 5–7 min, otherwise a very complex mixture of at least 4–5 products will be obtained. Treatment of the 5α-ols **8** with TSOH in refluxing ethanol gave the corresponding 4-substituted 4-ene-3-one steroids **9** in very good yields.

The synthesis of the 4-amino substituted androstenediones (**11a–c**) was carried out as shown in Scheme 3. Attempts at obtaining compound **11a** by reaction of the mesylate **10** with sodium azide as described previously [26] gave a complex mixture of products. Therefore, we used a different procedure in which the mesylate **10** was treated with ammonia, methylamine, or dimethylamine and the reaction mixture was heated at 110°C in a steel bomb to give after purification over silica gel, pure **11a**, **11b**, and **11c** in 27, 37, and 51% yield, respectively.

The enzyme used in this study was obtained from twice-washed human placental microsomes, as described previously by Ryan [27], and lyophilized to minimize loss of enzyme activity. Aromatase activity in the placental microsomes was determined by the radiometric method developed by Thompson and Siiteri [1]



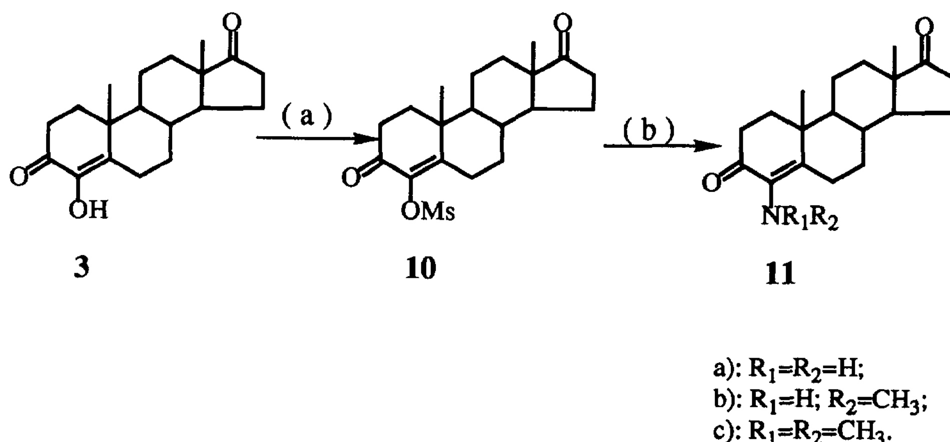
Scheme 1. Reagents: (a) H_2O_2/NaOH, CH_2Cl_2, H_2O; (b) H_2SO_4, AcOH; (c) t-BuOK, THF, RBr; (d) CH_3Cu; (e) RI.



Scheme 2. Reagents: (a) RMgBr, THF; (b) Jones reagent; (c) TsOH, EtOH.

as modified by Reed and Ohno [22]. This assay quantitates the production of [^3H_2O] released from [1β-^{3}H]4-androstenedione after aromatization. The substrate concentration was 0.25 μM, which is approx. 5 times the K_m value for the enzyme preparation. Aromatization was measured at several inhibitor concentrations ranging from 10^{-5} to 10^{-8} M.

Table 1 summarizes the results of the initial screening assays for the 4-substituted androstenedione derivatives synthesized in this study. For comparative purposes, we have included several compounds previously reported as effective aromatase inhibitors: 4-OHA, 4-thiophenyl-4-androstenedione (4-TPAD) and aminoglutethimide (AG). Compounds exhibiting effective inhibition in the initial screening assay were evaluated further in order to characterize the nature of their interaction with the catalytic site. Aromatization was measured at several concentrations of the inhibitor in the presence of increasing concentrations of androstenedione. All of the inhibitors tested exhibited clear-cut competitive-type inhibition. The double reciprocal plots of the results obtained for **9e** and **4f** are



Scheme 3. Reagents: (a) MsCl, NEt_3, THF; (b) HNR_1R_2, THF.

Table 1. Results of screening of inhibition of aromatase by 4-substituted 4-androstenediones

Compound	R	% Inhibition*	K_i†	Inhibition‡
4a	OCH_3	31	159	Competitive
4b	OCH_2CH_3	22	182	Competitive
4c	$OCH_2CH_2CH_3$	29	580	Competitive
4d	$O(CH_2)_3CH_3$	24	218	Competitive
4e	$OCH_2C_6H_5$	48	204	Competitive
4f	OC_6H_5	51	412	Competitive
4g	OC_6H_4-*p*-CH_3	2	ND	
9a	CH_3	65	119	Competitive
9b	CH_2CH_3	43	128	Competitive
9c	$CH_2CH_2CH_3$	34	130	Competitive
9d	$CH_2(CH_2)_2CH_3$	26	220	Competitive
9e	$CH_2C_6H_5$	76	38	Competitive
9f	C_6H_5	19	826	Competitive
9g	C_6H4-*p*-CH_3	22	950	Competitive
11a	NH_2	17	ND	
11b	$NHCH_3$	14	468	Competitive
11c	$N(CH_3)_2$	5	1290	Competitive
4-MA	SH	51	97	Competitive
4-OHA	OH	85	47	Competitive
4-TPAD	SC_6H_5	89	36	Competitive
AG		58	ND	

*All incubations were carried out with 0.75 μM inhibitor and 0.25 μM [1β-^{3}H]androstenedione.
Values are reported for average of three experiments.
†Apparent K_m for androstenedione, 47 nM.
‡Inhibition type was determined by Lineweaver–Burke plot.
4-MA, 4-mercaptoandrostenedione; 4-OHA, 4-hydroxyandrostenedione; 4-TPAD, 4-thiophenyl-4-androstenedione; AG, aminoglutethimide; ND, not determined.

shown in Figs 1 and 2.. The apparent inhibition constants (K_i) of 38 and 412 nM were observed for **9e** and **4f**, respectively.

The 4-benzyl (**9e**), 4-phenyl (**9f**), 4-methoxy (**4a**), 4-phenoxy (**4f**), 4-(*N*-methyl amino) (**11b**), and 4(*N*,*N*-dimethyl amino) (**11c**) 4-androstenedione inhibitors were then tested for their ability to act as time-dependent inactivators of microsomal aromatase. However, none of these compounds were found to be time-dependent inactivators of aromatase (data not shown).

DISCUSSION

Previous studies in our laboratory [17, 18] dealing with 4-(thioalkyl)- and 4-(thioaryl)-derivatives of androstenedione showed that aromatase has a tightly fitted hydrophobic binding pocket in the active site around the C-4 region of 4-androstenedione that is capable of accommodating bulky substituents not to exceed 5.5 Å in length and 4.3 Å in width. The results obtained from this investigation showed that all new compounds displayed moderate to good binding affinity for aromatase and support our earlier observation on the size of the hydrophobic pocket around C-4 of androstenedione. It is worth noting that the 4-alkyl/aryl substituents were in general more potent than their corresponding 4-alkoxy/aryloxy analogs

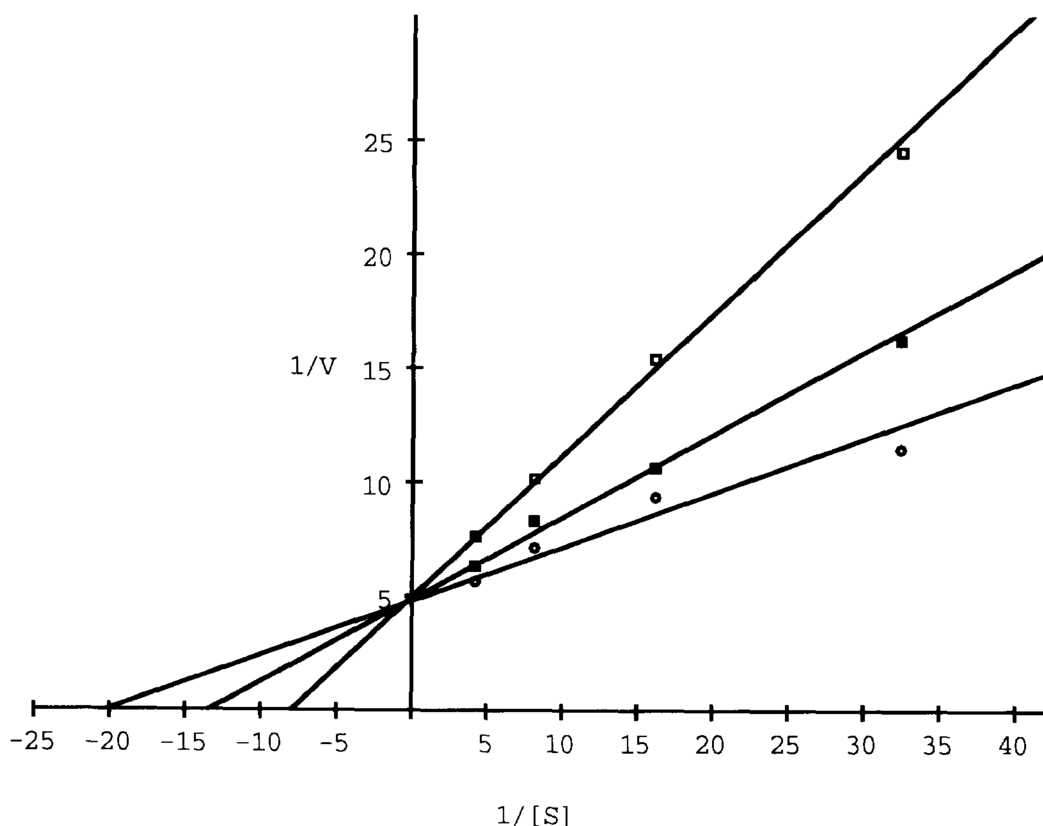


Fig. 1. Lineweaver–Burke analysis of the inhibition of aromatase by 4-phenoxy-4-androstenedione (4f). Inhibitor concentrations were expressed as 0 nM (○), 250 nM (■), and 500 nM (□).

(Table 1). Furthermore, these compounds were somewhat less potent than their corresponding 4-thiosubstituted analogs [17, 18]; also compare compounds **4f** and **9e** with 4-TPAD (Table 1).

The 4-O-alkyl/aryl substituted analogs (**4a–4g**) were found to be less effective than the 4-alkyl/aryl derivatives (**9a–9g**) as inhibitors of estrogen biosynthesis. Increasing the alkyl side-chain in the 4-alkyl series results in a decrease in enzyme inhibition, while increasing the chain length in the 4-ether series (**4a–4d**) does not seem to make any significant difference in inhibitory activity. All aryl substituted analogs (**4e, 4f, 9e**, 4-TPAD) analyzed in this study and in previous studies [17, 18] showed considerably higher binding affinity than their corresponding S [17, 18], O (**4a–4d**), or methylene (**9b–9d**) alkyl substituted analogs which suggest that restricted motion using rigid molecules leads to compounds that bind more effectively in the hydrophobic pocket. It is interesting to note that the 4-phenyl substituted analogs **9f** and **9g** have very low inhibitory activity compared to **4f, 9e** and 4-TPAD. Molecular modeling studies and energy minimization analyses of **4f, 9e, 9f** and 4-TPAD showed that two local minima are observed for **4f, 9e** and 4-TPAD with the 4-phenylether (**4f**), 4-benzyl (**9e**), and 4-phenylthioether (4-TPAD) substituents oriented below the steroid A-ring. On the other hand, the 4-phenyl (**9f**) substituent adopts a different orientation in which the phenyl ring is in a pseudo β-position. Thus, it is quite possible that the reason for the low inhibitory activity of **9f** may be that the 4-phenyl group of the inhibitor can only adopt a pseudo β-orientation, whereas the 4-benzyl, 4-phenylether and 4-phenylthioether groups of 4-androstenedione can orient themselves in such a way that the phenyl rings protrude into the 4α-pocket. The results obtained from this study clearly show that substituents that adopt a 4α-orientation are effective inhibitors of aromatase and strongly support the existence of a well defined hydrophobic pocket around the 4α-position of the steroid molecule.

The compounds 4-hydroxyandrostenedione, 4-mercaptoandrostenedione and 4-aminoandrostenedione have been shown to be effective competitive inhibitors of aromatase. In addition, all three compounds have been found to cause time-dependent inactivation of aromatase [13, 16]. These results suggest that a free OH, SH or NH_2 group may be of importance in imparting time-dependent inactivation of this enzyme. To explore the importance of a free $-NH_2$ -OH or -SH group we synthesized O-alkyl/aryl, S-alky/aryl and N-alkyl substituted androstenedione derivatives and determined their time-dependent inactivation of aromatase. The results of these studies showed that formation of ether or thioether analogs

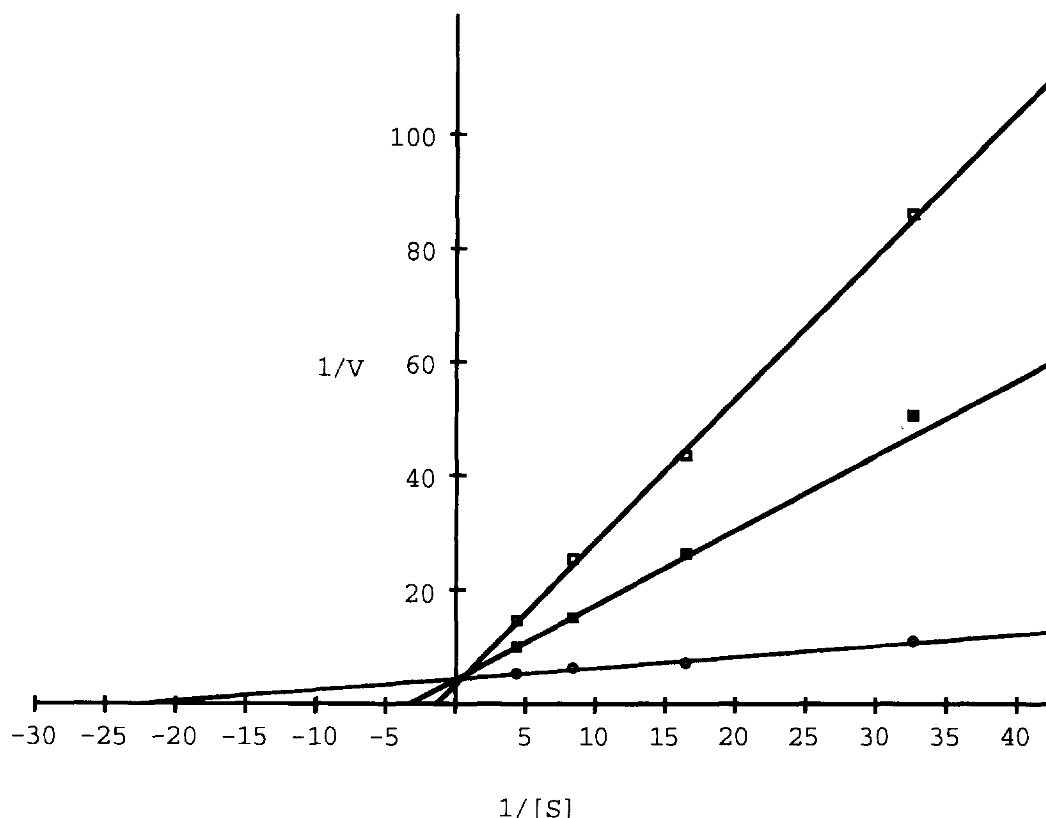


Fig. 2. Lineweaver–Burke analysis of the inhibition of aromatase by 4-benzyl-4-androstenedione (9e). Each point represents an average of two samples. The variation between duplicate samples was less than 5% in all cases. Inhibitor concentrations were expressed as 0 nM (○), 250 nM (■), and 500 nM (□).

results in compounds that are effective competitive inhibitors but did not bring about aromatase inactivation. Data obtained from 4-(*N*-methylamino) and 4(*N*,*N*-dimethylamino)-4-androstenedione showed also that *N*-alkylation leads to compounds that are competitive inhibitors but are devoid of causing time-dependent inactivation of aromatase. Thus, these results clearly show that N, S, or O-alkylation of 4-substituted androstenedione results in compounds incapable of inactivating aromatase, underlining the importance of the free -OH, -SH, or $-NH_2$ group. Although some investigations have been carried out to delineate the mechanism of 4-OHA, 4MA, and 4-aminoandrostenedione as time-dependent inactivators of aromatase, these studies have not yet provided a satisfactory mechanism to account for their action.

The high inhibitory activity displayed by the 4-substituted alkyl and aryl androstenediones (**4e**, **4f**, **9a**, **9b**) show that a free pair of electrons in the 4-N, S, or O-substituted analogs is not an essential requirement for aromatase inhibition. It is worth noting that the 4-benzyl analog (**9e**) is somewhat less active than the 4-thiophenyl analog (4-TPA) and more active than the 4-phenylether (**4f**) analog.

In conclusion, the results obtained from this investigation support our earlier studies which show that the enzyme can tolerate bulky substituents at the 4-position. Furthermore, these results show that the enzyme pocket is located in the 4α-region of 4-androstenedione. Alkylation or arylation of 4-N, S, or O-substituted steroids results in compounds that are effective competitive inhibitors that are devoid of time-dependent inactivation and that the free pair of electrons on N, S, or O is not an essential requirement for 4-substituted androstenedione derivatives to be effective aromatase inhibitors but may be necessary for inactivation of aromatase.

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