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4- and 6-(*p*-Sulphamoylphenyl)androstenediones: Studies of aromatase inhibitor-based oestrone sulphatase inhibition

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ABSTRACT

4-(*p*-Sulphamoylphenyl)androstenedione (**3**) and 6α-*p*-sulphamoylphenyl analogues **12**–**14** were synthesised and tested as aromatase inhibitors as well as oestrone sulphatase inhibitors in human placental microsomes. All of the *p*-sulphamoylphenyl compounds synthesised were powerful inhibitors of aromatase with apparent K_i values ranging between 30 and 97 nM. In addition, the aromatase inhibitory activities of 6α-*p*-hydroxyphenyl compounds **9**–**11**, which may be produced from their respective sulphamoylphenyl compounds by action of oestrone sulphatase, were also high in a range of 23 and 75 nM of the K_i values. On the other hand, all of the sulphamoylphenyl compounds were poor inhibitors of oestrone sulphatase with more than about 200 μM of IC_{25} values. Although the present findings of the oestrone sulphatase inhibition are disappointing, such attempts may be valuable to develop a new class of drugs having a dual function, aromatase inhibitor and oestrone sulphatase inhibitor, for the treatment of oestrogen-dependent breast cancer.

In post-menopausal women with oestrogen-dependent breast cancer, the oestrogen concentration in tumour tissues is much higher than that circulating in plasma [1–5]. Peripheral and intratumoural synthesis of oestrogens has been initially considered almost exclusively through the aromatase pathway. The enzyme, aromatase, which converts androstenedione into oestrone, has therefore been among the prime targets for depleting oestrogen levels [5–7] (Fig. 1). The sole inhibition of this enzyme could not afford an effective reduction of oestrogenic stimulation to tumours, the reason being that other pathways are involved in oestrogen bio-synthesis [8,9]. The sulphatase pathway, where the inactive precursor oestrone sulphate is converted into oestrone by the enzyme, oestrone sulphatase, is now considered to be the main source for the local production of oestrogens in tumours, providing 10-fold more oestrone than the aromatase pathway [10–13]. Oestrone sulphate has been suggested to act as a reservoir for the synthesis of active steroids in post-menopausal women [14].

Inhibitors of aromatase are of interest in the treatment of established oestrogen-dependent breast cancer [7,15,16]. Various analogues of androstenediones have been synthesised as aromatase inhibitors by various laboratories. We have previously reported that 6α- and 6β-alkyl- [17,18] or 6-aryl- [19] substituted androstenediones and their 1,4-diene-, 4,6-diene- and 1,4,6-triene-derivatives [20–22] are potent competitive inhibitors of aromatase. On the other hand, 4-alkyl- and 4-aryl-substituted androstenedione derivatives have been reported to be moderate-to-powerful inhibitors of aromatase [23].

The most notable and potent of steroid sulphatase inhibitors is oestrone 3-sulphamate [24–26]. Oestrone 3-sulphamate has been reported to act as an active site-directed irreversible inhibitor of oestrone sulphatase [25]. Cleavage of a sulphamoyl moiety of oestrone 3-sulphamate is the prerequisite for the irreversible inactivation [27–29], and oestrone is released during the sulphatase inactivation. Oestrone formation is unsuitable for use in the treatment of oestrogen-dependent breast cancer.

We have then postulated that the aromatase inhibitor-based sulphamates are useful for the treatment of the breast cancer. Recently, 3-sulphamates of 2-, 4- or 6-substituted oestrogens, which act as aromatase inhibitors [30], have been synthesised and evaluated as aromatase inhibitor-based oestrogen sulphatase inhibitors; some of them are effective inhibitors of both aromatase and oestrone sulphatase [31]. In this article, considering that 4-phenyl- [23] and 6-phenyl- [19] androstenedione analogues are good aromatase inhibitors, we synthesised 4- and 6α-(*p*-sulphamoylpheny) derivatives of androstenedione analogues and examined them as aromatase inhibitors as well as oestrone sulphatase inhibitors.

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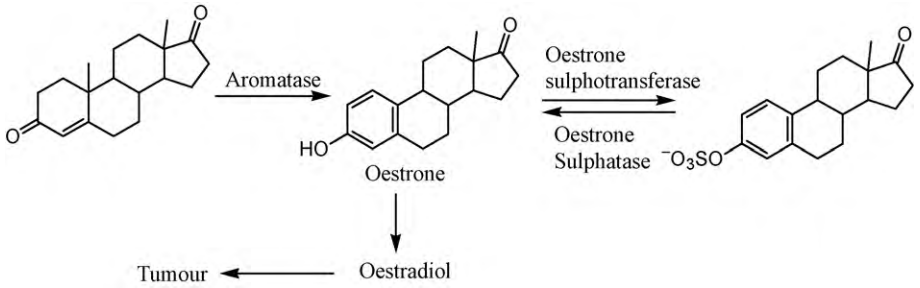

Fig. 1. Oestrogen formation by aromatase and oestrone sulphatase pathways in peripheral and breast tissue.

1. Experimental

1.1. Material and general methods

Dextran-coated charcoal was obtained from Sigma–Aldrich Corporation (St. Louis, MI, USA) and nicotinamide adenine dinucleotide phosphate (NADPH) was from Kohjin Co., Ltd. (Tokyo, Japan). [6,7-^{3}H]Oestrone was purchased from Perkin-Elmer Japan, Co. Ltd. (Yokohama, Japan) and [1β-^{3}H]androstenedione (25.4 Ci mmol^{-1}) (^{3}H distribution: β/α = 74.2/25.8) was from New England Nuclear Corporation (Boston, MA, USA). The compound 4-(*p*-methoxyphenyl)androstenedione (**1**) [23] and 3,3;17,17-bis(ethylenedioxy)androstan-5α,6α-epoxide (**4**) [17] were synthesised according to the previous methods. All other chemicals were purchased from Sigma–Aldrich Corporation (St. Louis, MI, USA).

Melting points were measured on a Yanagimoto melting-point apparatus (Kyoto, Japan) and are uncorrected. Infrared (IR) spectra were recorded on a Perkin-Elmer Fourier transform-infrared spectroscopy (FT-IR) 1725X spectrophotometer in a KBr pellet, and ultraviolet (UV) spectra were determined in 95% EtOH on a Hitachi 150-20 spectrophotometer (Tokyo, Japan). ^{1}H nuclear magnetic resonance (NMR) spectra were obtained in $CDCl_3$ solution with a JEOL LA 400 (400 MHz) and JEOL LA 600 (600 MHz) spectrometers (Tokyo, Japan) using tetramethylsilane as an internal standard and mass spectra (MS) (electron impact, (EI) mode) with a JEOL JMS-DX 303 spectrometer. Thin-layer chromatography (TLC) was performed on E. Merck pre-coated silica gel plates (silica gel 60F-254, Darmstadt, Germany). Column chromatography was conducted with silica gel 60, 70–230 mesh (E. Merck).

1.2. Synthesis of 4-(*p*-sulphamoylphenyl)androstenedione (**3**)

The compound 4-(*p*-methoxyphenyl)androst-4-ene-3,17-dione (**1**) (75 mg, 89 μmol) in 47% HBr in acetic acid (8 ml) was heated at 120 °C for 2 h, and the reaction mixture was poured into ice-water (100 ml). The product was extracted with ethyl acetate (EtOAc) (200 ml) and the organic layer was washed with 5% sodium bicarbonate ($NaHCO_3$) solution and water and dried (Na_2SO_4). Evaporation of the solvent gave the crude solid, which was recrystallised from acetone to yield 4-(*p*-hydroxyphenyl)androst-4-ene-3,17-dione (**2**) (32 mg, 42 μmol) in 48% yield, mp 287–289 °C. Compound **2**: IR (KBr): 3307 cm^{-1} (OH), 1720 and 1666 cm^{-1} (C=O); UV λ_{max}: 229 nm (ε = 13,000); ^{1}H NMR δ: 0.93 (3H, s, 18-Me), 1.31 (3H, s, 19-Me), 6.76 (2H, d, *J* = 8.7 Hz, aromatic protons), 6.85 (2H, d, *J* = 8.7 Hz, aromatic protons); MS *m*/*z*: 378 (M$^+$, 64), 200 (100), 188 (20), 172 (12). Anal. calculated for $C_{25}H_{30}O_3$: C, 79.33; H, 7.99. Found C, 79.31; H, 8.01.

Sodium hydride (60% oil suspension, 73 mg, 1.8 mmol) was separately added to stirred solutions of compound **2** (71 mg, 0.18 mmol) in anhydrous dimethylforamide (DMF) (6 ml) at 0 °C. Subsequently, sulphamoyl chloride (0.25 ml, 2.35 mmol) was added carefully, and the resulting mixture was allowed to stand at room temperature with stirring for 2 h. The reaction mixture was then poured in chilled 5% $NaHCO_3$ solution (200 ml), extracted with EtOAc (200 ml), washed with saturated NaCl solution, and dried with Na_2SO_4. Evaporation of the solvent gave an oily substance that was purified by column chromatography (hexane–EtOAc) and preparative thin-layer chromoatography (TLC) (hexane–EtOAc) followed by recrystallisation to give a solid. The solid was recrystallised from acetone to produce 4-(*p*-sulphamoy1phenyl)androst-4-ene-3,17-dione (**3**) in 26% yield (23 mg, 51 μmol), mp 240–244 °C. Compound **3**: IR (KBr): 1721 and 1663 cm^{-1} (C=O), 1387 and 1163 cm^{-1} (SO_2NH_2); UV λ_{max}: 230 nm (ε = 12,000); ^{1}H NMR δ: 0.97 (3H, s, 18-Me), 1.38 (3H, s, 19-Me), 7.03–7.06 (2H, d, *J* = 8.4 Hz, aromatic protons), 7.31–7.34 (2H, d, *J* = 8.7 Hz, aromatic protons); MS *m*/*z*: 457 (M$^+$, 4), 376 (64), 279 (5), 200 (100). Anal. calculated for $C_{25}H_{31}NO_5S$: C, 65.62; H, 6.83; N, 3.06. Found C, 65.33; H, 6.75; N, 3.00.

1.3. Synthesis of 6-(*p*-hydroxyphenyl)androstenedione derivatives ***9*–*11***

To a solution of the bis(ethylenedioxy)-5α,6α-epoxide (**4**) (500 mg, 1.28 mmol) in tetrahydrofuran (THF) (20 ml) was added 20 molar equivalent of *p*-methoxyphenylmagnesium bromide in THF (10 ml), and the mixture was heated under reflux for 4 h in a N_2 stream. After the solution was cooled, saturated NH_4Cl solution (100 ml) was added to this and the product was extracted with EtOAc (200 ml × 2). The combined organic layers were washed with water to neutrality, dried (Na_2SO_4), and evaporated to dryness leaving the residue, which was purified by column chromatography (hexane–EtOAc) and recrystallised from EtOAc to give 6β-(*p*-methoxyphenyl)-3,3;17,17-bis(ethylenedioxy)androstan-5α-ol (**5**) in 90% yield (1.64 g, 3.24 mmol), mp 211–212 °C. Compound **5**: IR (KBr): 3538 cm^{-1} (OH); ^{1}H NMR δ: 0.68 (3H, s, 19-Me), 0.96 (3H, s, 18-Me), 2.91 (1H, d, *J* = 5.9 Hz, 6α-H), 3.78 (3H, s, OCH_3), 3.84–4.06 (8H, m, 3-$O_2(CH_2)_2$ and 17-$O_2(CH_2)_2$), 6.79 (2H, d, *J* = 8.9 Hz, aromatic protons), 7.26–7.34 (2H, m, aromatic protons); MS *m*/*z*: 498 (M$^+$, 12), 480 (10), 121 (19), 99 (100). Anal. calculated for $C_{30}H_{42}O_6$: C, 72.26; H, 8.49. Found C, 72.37; H, 8.77.

3 M $HClO_4$ (1.13 ml) was added to a solution of compound **5** (1.62 g, 3.21 mmol) in THF (24 ml), and the reaction mixture was stirred at room temperature for 5 h. After this time, the mixture was diluted with EtOAc (200 ml), washed with 5% $NaHCO_3$ solution and water, dried (Na_2SO_4) and evaporated to yield the residue. The residue was purified by column chromatography followed by recrystallisation from acetone to produced 6β-(*p*-methoxyphenyl)-5α-hydroxyandrostane-3,17-dione (**6**) (1.32 g, 3.22 mmol, 98% yield), mp 201–203 °C. Compound **6**: IR (KBr): 3452 cm^{-1} (OH), 1738 and 1708 cm^{-1} (C=O); ^{1}H NMR δ: 0.98 (3H, s, 19-Me), 1.02 (3H, s, 18-Me), 2.86 (1H, d, *J* = 6.1 Hz, 6α-H), 3.81 (3H, s, OCH_3), 6.80–6.86 (2H, m, aromatic protons), 7.26–7.33 (2H, m, aromatic protons); MS *m*/*z*: 410 (M$^+$, 89), 392 (55), 339 (100),

241 (63). Anal. calculated for $C_{26}H_{34}O_4$: C, 76.06; H, 8.35. Found C, 76.07; H, 8.63.

Thionyl chloride (1.9 ml, 26 mmol) was added to a chilled solution of compound **6** (1.30 g, 3.20 mmol) in dry pyridine (19 ml), and the mixture was stirred for 3 min at 0 °C, poured into ice-water (100 ml), extracted with EtOAc (100 ml × 2). The combined organic layers were washed with water, dried (Na_2SO_4) and evaporated to afford the crude product, which was purified by column chromatography (hexane–EtOAc) and recrystallised from acetone–hexane, giving 6β-(*p*-methoxyphenyl)androst-4-ene-3,17-dione (**7**) (1.01 g, 2.58 mmol, 80% yield), mp 182–183 °C. Compound **7**: IR (KBr): 1737 and 1678 cm^{-1} (C=O); UV λ_{max}: 231 nm (ε = 18,400) and 240 nm (ε = 13,600); ^1H NMR δ: 0.70 (3H, s, 19-Me), 0.91 (3H, s, 18-Me), 3.76 (1H, d, J = 4.9 Hz, 6α-H), 3.81 (3H, s, OCH_3), 6.04 (1H, s, 4-H), 6.85–6.89 (2H, m, aromatic protons), 7.22–7.26 (2H, m, aromatic protons); MS m/z: 392 (M^+, 25), 377 (13). Anal. calculated for $C_{26}H_{32}O_3$: C, 79.56; H, 8.22. Found C, 79.42; H, 8.50.

Compound **7** (560 mg, 1.43 mmol) was dissolved in 95% EtOH (18 ml) and 1 M HCl (1.8 ml) was added to the solution. The mixture was heated under reflux for 12 h. After removing most of the solvent, the mixture was diluted with EtOAc (200 ml), washed with 5% $NaHCO_3$ solution and water, dried (Na_2SO_4) and evaporated. The residue was subjected to column chromatography followed by recrystallisation to afford 6α-(*p*-methoxyphenyl)androst-4-ene-3,17-dione (**8**) (432 mg, 1.10 mmol, 77% yield), mp 198–201 °C. Compound **8**: IR (KBr): 1737 and 1672 cm^{-1} (C=O); UV λ_{max}: 226 nm (ε = 18,800) and 240 nm (ε = 13,600); ^1H NMR δ: 0.95 (3H, s, 18-Me), 1.34 (3H, s, 19-Me), 3.52–3.58 (1H, m, 6β-H), 3.81 (3H, s, OCH_3), 5.20 (1H, d, J = 1.5 Hz, 4-H), 6.86 (2H, d, J = 2.0 Hz, aromatic protons), 6.89 (2H, d, J = 2.8 Hz, aromatic protons); MS m/z: 392 (M^+, 100), 377 (46), 336 (26), 121 (33). Anal. calculated for $C_{26}H_{32}O_3$: C, 79.56; H, 8.22. Found C, 79.66; H, 8.35.

Compound **8** (1.06 g, 2.70 mmol) in 48% HBr in AcOH (50 ml) was heated at 40 °C for 24 h, and the reaction mixture was similarly treated to the method described for the synthesis of compound **2**. The crude product was subjected to column chromatography followed by recrystallisation from acetone to give 6α-(*p*-hydroxyphenyl)androst-4-ene-3,17-dione (**9**) (650 mg, 1.73 mmol, 64% yield), mp 250–253 °C. Compound **9**: IR (KBr): 3423 cm^{-1} (OH), 1736 and 1656 cm^{-1} (C=O); UV λ_{max}: 226 nm (ε = 15,400) and 240 nm (ε = 12,500); ^1H NMR δ: 0.95 (3H, s, 18-Me), 1.34 (3H, s, 19-Me), 3.50–3.56 (1H, m, 6β-H), 5.24 (1H, d, J = 1.3 Hz, 4-H), 6.78 (2H, d, J = 8.6 Hz, aromatic protons), 6.95 (2H, d, J = 8.4 Hz, aromatic protons); MS m/z: 378 (M^+, 100), 363 (27), 322 (19), 107 (30). Anal. calculated for $C_{25}H_{30}O_3$: C, 79.33; H, 7.99. Found C, 79.61; H, 7.77.

A mixture of compound **9** (300 mg, 0.79 mmol), chloranil (284 mg, 1.16 mmol), *p*-toluenesulphanic acid (*p*-TsOH) monohydrate (6.9 mg, 0.36 mmol), and xylene (23 ml) was heated under reflux for 5 h and cooled to room temperature. The reaction mixture was subjected directly to a column of silica gel (20 g). Elution with hexane–EtOAc gave the crude product, which was subjected to recrystallisation to give 6-(*p*-hydroxyphenyl)androsta-4,6-diene-3,17-dione (**10**) (227 mg, 0.60 mmol, 76% yield), mp 217–218 °C. Compound **10**: IR (KBr): 3526 cm^{-1} (OH), 1737 and 1640 cm^{-1} (C=O); UV λ_{max}: 232 nm (ε = 9,600) and 280 nm (ε = 14,800); ^1H NMR δ: 1.00 (3H, s, 18-Me), 1.24 (3H, s, 19-Me), 5.69 (1H, s, 4-H), 6.07 (1H, d, J = 2.1 Hz, 7-H), 6.74–6.79 (2H, m, aromatic protons), 6.95–6.99 (2H, m, aromatic protons); MS m/z: 376 (M^+, 100), 361 (22), 343 (10), 228 (23). Anal. calculated for $C_{25}H_{28}O_3$: C, 79.75; H, 7.50. Found C, 79.76; H, 7.54.

A solution of compound **10** (200 mg, 0.53 mmol) and 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) (180 mg, 0.79 mol) in benzene (31 ml) was refluxed for 10 h, and then the reaction mixture was loaded onto a column of Al_2O_3, eluting with EtOAc gave the crude product, which was purified by a column chromatography (hexane–EtOAc) and recrystallised from acetone to afford 6-(*p*-hydroxyphenyl)androsta-1,4,6-triene-3,17-dione (**11**) (130 mg, 0.35 mmol, 61% yield), mp 254–255 °C. Compound **11**: IR (KBr): 3610 cm^{-1} (OH), 1742 and 1647 cm^{-1} (C=O), 1598 cm^{-1} (C=C); UV λ_{max}: 242 nm (ε = 19,000) and 290 nm (ε = 8,700); ^1H NMR δ: 1.02 (3H, s, 18-Me), 1.35 (3H, s, 19-Me), 5.98 (1H, s, 4-H), 6.06 (1H, s, 7-H), 6.31–6.35 (1H, m, 2-H), 6.76–6.80 (2H, m, aromatic protons), 7.02–7.06 (2H, m, aromatic protons), 7.15 (1H, d, J = 10.2 Hz, 1-H); MS m/z: 374 (M^+, 100), 359 (26), 211 (51), 107 (22). Anal. calculated for $C_{25}H_{26}O_3$: C, 80.18; H, 7.00. Found C, 79.99; H, 7.12.

1.4. Synthesis of 6-(p-sulphamoylphenyl)androstenedione derivatives **12**–**14**

Compounds **9**–**11** (0.30 mmol) were sulphamoylated by the similar procedure to the synthesis of the 4-sulphamoyloxy derivative **3** with sulphamoyl chloride (300 mg, 2.6 mmol) and NaH (3.0 mmol). The crude products were purified by column chromatography and recrystallised from MeOH to give the corresponding 6-(sulphamoylphenyl)androstene derivatives **12**–**14** in 23–30% yield.

Compound **12**: mp 199–203 °C. IR (KBr): 1736 and 1656 cm^{-1} (C=O), 1388 and 1157 cm^{-1} (SO_2NH_2); UV λ_{max}: 216 nm (ε = 14,000) and 240 nm (ε = 12,200); ^1H NMR δ: 0.96 (3H, s, 18-Me), 1.36 (3H, s, 19-Me), 3.61–3.66 (1H, m, 6β-H), 5.11 (1H, d, J = 1.8 Hz, 4-H), 7.15–7.19 (2H, m, aromatic protons), 7.26–7.32 (2H, m, aromatic protons); MS m/z: 378 (M^+, 100), 363 (29), 322 (14), 107 (25). Anal. calculated for $C_{25}H_{31}NO_5S$: C, 65.62; H, 6.83; N, 3.06. Found C, 65.24; H, 6.84; N, 3.06.

Compound **13**: mp 210–215 °C. IR (KBr): 1731 and 1641 cm^{-1} (C=O), 1387 and 1156 cm^{-1} (SO_2NH_2); UV λ_{max}: 290 nm (ε = 16,300); ^1H NMR δ: 1.01 (3H, s, 18-Me), 1.26 (3H, s, 19-Me), 5.53 (1H, s, 4-H), 6.12 (1H, d, J = 2.1 Hz, 7-H), 7.14–7.19 (2H, m, aromatic protons), 7.28–7.33 (2H, m, aromatic protons); MS m/z: 376 (M^+, 100), 361 (38), 319 (17), 228 (33). Anal. calculated for $C_{25}H_{29}NO_5S$: C, 65.91; H, 6.42; N, 3.07. Found C, 65.69; H, 6.23; N, 2.96.

Compound **14**: mp 198–204 °C. IR (KBr): 1729 and 1648 cm^{-1} (C=O), 1599 cm^{-1} (C=C), 1386 and 1158 cm^{-1} (SO_2NH_2); UV λ_{max}: 240 nm (ε = 16,000) and 308 nm (ε = 8,000); ^1H NMR δ: 1.04 (3H, s, 18-Me), 1.36 (3H, s, 19-Me), 5.90 (1H, d, J = 1.8 Hz, 4-H), 6.05 (1H, d, J = 2.1 Hz, 7-H), 6.31 (1H, dd, J = 1.8 and 10.2 Hz, 2-H), 7.17 (1H, d, J = 10.2 Hz, 1-H), 7.21–7.25 (2H, m, aromatic protons), 7.30–7.36 (2H, m, aromatic protons); MS m/z: 374 (M^+, 100), 289 (25), 276 (48), 250 (40). Anal. calculated for $C_{25}H_{27}NO_5S$: C, 66.20; H, 6.00; N, 3.09. Found C, 66.02; H, 5.70; N, 3.08.

1.5. Enzyme preparation

Human placental microsomes (sedimenting at 105,000 × g for 60 min) were obtained as described by Ryan [32]. They were washed once with 0.05 mM dithiothreitol solution, lyophilised, and stored at −20 °C until use.

1.6. Aromatase assay

Aromatase activity was measured essentially according to the original procedure of Siiteri and Thompson [33]. The screening assay for determination of IC_{50} value and the kinetic assay were carried out essentially according to the assay method described in our previous work [34]. Briefly, 20 μg of protein from the lyophilised microsomes, and 20-min-incubation period were used for the screening assay, and 20 μg of protein from the microsomes and a 5-min period were used for the kinetic assay. The assays were carried out at 37 °C in 67 mM phosphate buffer, pH 7.5, in the presence of NADPH (180 μM) under air.

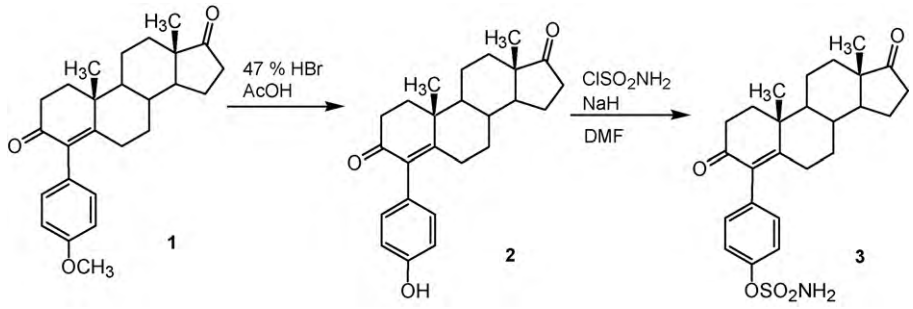

Fig. 2. Synthesis of 4-(*p*-sulphamoylphenyl)androstenedione (**3**).

Apparent K_i values were calculated using non-linear regression analysis with GraFit software [35].

1.7. Oestrone sulphate assay

Oestrone sulphatase activity was determined essentially by the standard literature method [25]. All enzymatic studies were carried out in 67 mM phosphate buffer, pH 7.5, at a final incubation volume of 0.5 ml. The incubation mixture contained 20 μM of (^{3}H)oestrone sulphate (2×10^5 dpm), 20 μg of protein of the lyophilised microsomes, various concentrations of inhibitors and 25 μM of MeOH. Incubations were carried out at 37 °C for 5 min and terminated by adding of 3 ml of toluene, followed by vortexing for 45 s. After centrifugation at 2500 revolutions per minute (rpm) for 5 min at 4 °C, an aliquot (1.0 ml) in duplicate was removed from the organic layer and added to scintillation mixture for determination of the (^{3}H)oestrone production. Control samples with no inhibitor were incubated simultaneously. Blank samples were obtained by incubating with boiled microsomes (95 °C for 10 min). The basal sulphatase activity (without the inhibitor) for the IC_{50} assay was

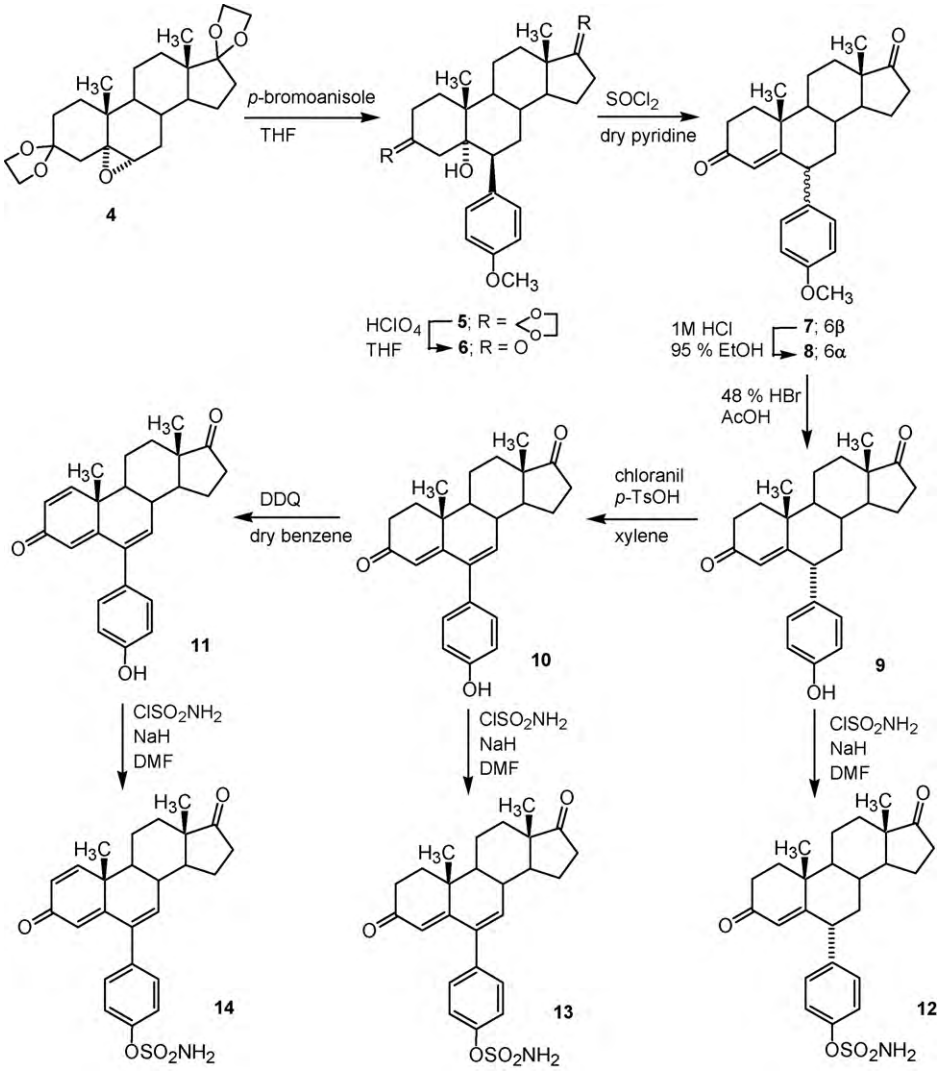

Fig. 3. Synthesis of 6-(*p*-sulphamoylphenyl)androstenediones (**12**–**14**).

found to be 3.6 nmol min^{-1} mg^{-1} protein (approximately 3.6% conversion of the used substrate).

2. Results and discussion

We initially synthesised 4- and 6-(*p*-sulphamoylphenyl) androstenediones (**3** and **12**–**14**) through the following pathways (Fig. 2). The compound 4-(*p*-methoxyphenyl)androstenedione (**1**) [23] was demethylated with 47% HBr in acetic acid to give 4-*p*-hydroxyphenyl compound **2**. Treatment of phenol **2** with sulphamoyl chloride in the presence of NaH in anhydrous DMF afforded the 4-*p*-sulphamoyl compound **3** in 13% yield from compound **1**.

6α-(*p*-Hydroxyphenyl)androstenedione (**9**) was synthesised from 3,3;17,17-bis(ethylenedioxy)androstan-5α,6α-epoxide (**4**) [17] in five steps in 34% yield (Fig. 3). Reaction of compound **4** with *p*-methoxyphenylmagnesium bromide in THF giving 6β-(*p*-methoxyphenyl)-5α-ol **5**, which was de-protected with $HClO_4$ in THF to yield 5α-hydroxy-3,17-diketone **6**. Dehydration of compound **6** with $SOCl_2$ in dry pyridine gave 6β-*p*-methoxyphenyl-4-ene-3-one **7** of which isomerisation with acid produced 6α-*p*-methoxyphenyl isomer **8** and finally demethylation of compound **8** with 48% HBr in acetic acid afforded 6α-*p*-hydroxyphenyl compound **9**, which has thermodynamically more stable quasi-equatorial conformation than the quasi-axial 6β-*p*-hydroxypnenyl isomer. Compound **9** was then converted into the 6α-*p*-sulphamoylphenyl compound **12** (23% yield), similarly to the synthesis of the sulphamoyl compound **3**. On the other hand, compound **9** was treated, under reflux, with chloranil in the presence of *p*-TsOH in xylene to yield 4,6-diene derivative **10** (76% yield) of which dehydrogenation with DDQ under reflux in dry benzene gave 1,4,6-triene compound **11** (61% yield). Both compounds **10** and **11** were finally converted into the 6-sulphamoylphenyl derivatives **13** and **14** in about 30% yields.

Spectral and analytical data of the new compounds were consistent with their respectively assigned structures.

We initially studied inhibition of aromatase in human placental microsomes by the sulphamoylphenyl derivatives of androstenedione and its 4,6-diene and 1,4,6-triene compounds, the sulphamates **3** and **12**–**14**. In addition to the above compounds, the aromatase inhibitory activities of *p*-hydroxyphenyl derivatives **2** and **9**–**11**, which may be produced by hydrolysis of the corresponding sulphamoylphenyl derivatives by oestrone sulphatase [27–29], were also examined. Aromatase activity in placental microsomes was determined using a radiometric assay in which tritiated water released from [1β-^{3}H]androstenedione in the incubation medium during aromatisation was measured [33]. To characterise the nature of inhibitor binding to the active site of aromatase, aromatisation was measured at several inhibitor concentrations and substrate concentrations. The results of these studies were plotted on a typical Lineweaver–Burk plot. The results are shown in Table 1. In the studies, the apparent K_m and V_{max} values for the substrate androstenedione were 33 nM and 110 pmol min^{-1} mg^{-1} protein, respectively. All of the sulphamates and the phenols, except for compound **2**, showed clear-cut competitive inhibition (Fig. 4). The apparent K_i values were obtained by Dixon plots. All of the steroids **3** and **9**–**14** examined in this study, were potent competitive inhibitors of human placental aromatase with the apparent K_is ranging from 23 to 97 nM. The K_i values were almost comparable with the K_m value of the substrate, ~33 nM. It has previously been reported that the affinities to aromatase of the non-polar 4- and 6-phenyl derivatives of androstenedione and its 4,6-diene and 1,4,6-triene analogues are very high and the K_i values are almost similar to the K_m value of the substrate [17,22,23]. This study indicated that a hydrophilic substitution such as a hydroxyl or sulphamolyl group of a para position of the phenyl ring at the C-4 and C-6 positions of androstenediones did not change the affinity to aromatase. Aromatase would tolerate such hydrophilic groups as phenol and sulphamate, at these positions.

Table 1
In vitro aromatase inhibition by *p*-hydroxyphenyl- and *p*-sulphamoylphenyl-substituted steroids.

Compound	IC_{50}, nM[a]	Apparent K_i, nM[b]
p-Hydroxyphenyl-substituted steroid		
4-Ene, **2**	>2000	–
6α-4-Ene, **9**	300	27 ± 1.1
6-4,6-Diene, **10**	240	23 ± 1.3
6-1,4,6-Triene, **11**	890	75 ± 2.4
p-Sulphamoylphenyl-substituted steroid		
4-Ene, **3**	990	97 ± 5.0
6α-4-Ene, **12**	400	39 ± 1.8
6-4,6-Diene, **13**	300	30 ± 2.1
6-1,4,6-Triene, **14**	680	56 ± 3.7

[a] Substrate: 300 nM of [1β-^{3}H]androstenedione.
[b] Apparent K_i values were obtained by Dixon plot in which K_m for androstenedione was 33 nM.

The inhibitory activities of oestrone sulphatase in placental microsomes by the 4- and 6-*p*-sulphamoylphenyl derivatives **3** and **12**–**14** were next examined, and the results are shown in Table 2. In

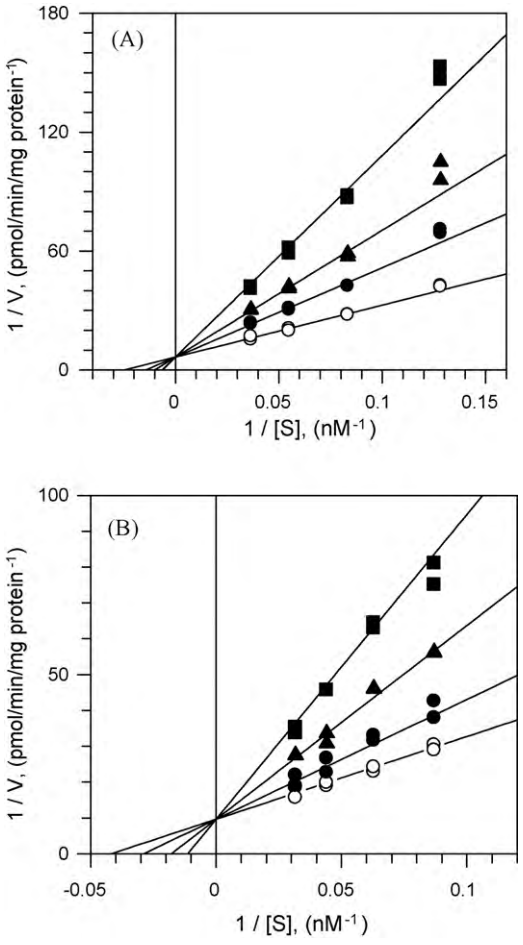

Fig. 4. Lineweaver–Burk plots of inhibition of human placental aromatase by *p*-hydroxyphenyl- and *p*-sulphamoylphenyl-substituted steroids with AD as the substrate. (A) Concentrations of *p*-hydroxyphenyl-substituted steroid **10**: (○) control (0 nM); (●) 20 nM; (▲) 40 nM; (■) 80 nM. (B) Concentrations of *p*-sulphamoylphenyl-substituted steroid **13**: (○) control (0 nM); (●) 10 nM; (▲) 30 nM; (■) 60 nM. The inhibition experiments with all the other steroids examined gave essentially similar to (A) and (B) (data not shown).

Table 2
In vitro sulphatase inhibition by *p*-sulphamoylphenyl-substituted steroids.

Compound	IC_{25}, μM[a]
4-Ene, **3**	190 ± 12
6α-4-Ene, **12**	390 ± 23
6-4,6-Diene, **13**	210 ± 9
6-1,4,6-Triene, **14**	200 ± 13
For comparison	
Oestrone 3-sulphamate	IC_{50} = 0.32 ± 0.0062

[a] Substrate: 20 μM of [^{3}H]oestrone sulphate.

addition to the above compounds, oestrone 3-sulphamate, a typical oestrone sulphatase inhibitor previously reported [24–26], was tested as a positive control. The oestrone sulphatase activity in the placental microsomes was determined by the radiometric method in which the amount of [^{3}H]oestrone released from the substrate [^{3}H]oestrone sulphate during the incubation was used as an index of the sulphatase activity [25]. In the studies, the apparent K_m for oestrone sulphate was found to be approximately 18 nM. Disappointingly, the *p*-sulphamoylphenyl derivatives **3** and **12**–**14** were very weak inhibitors of oestrone sulphatase. Then, 25% inhibition values of the control, IC_{25} values, of the sulphamates **3** and **12**–**14** were obtained to be ranging from 190 to 400 μM. The oestrone sulphatase activity was not effectively but slightly blocked by these sulphamates examined. The results reveal that the introduction of a *p*-sulphamoylphenyl moiety to C-4 or C-6 position of androstenedione analogues fail to obtain the effective inhibitory activity of oestrone sulphatase; the position is unsuitable for the inhibitor of oestrone sulphatase.

3. Conclusion

Compounds **3** and **12**–**14** introduced by a *p*-sulphamolyphenyl group at C-4 or C-6 position of androstenedione analogues were the powerful aromatase inhibitors but not effective oestrone sulphatase inhibitors. It seems that the position of the sulphamoylphenyl group other than the C-4 or C-6 position will be useful for causing a dual function, the aromatase inhibitor and the oestrone sulphatase inhibitor. Synthesis of other sulphamoylphenyl derivatives of androstenedione analogues, which are powerful for oestrone sulphatase, is now conducted in our laboratory.

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