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Evaluation of 7-Hydroxy-Flavones as Inhibitors of Oestrone and Oestradiol Biosynthesis

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A series of 4-aryl substituted 7-hydroxy-flavones were prepared using the three-step Baker–Venkataraman synthesis in good overall yields. The flavones were all evaluated *in vitro* for inhibitory activity against aromatase ($P450_{AROM}$, CYP19), using human placental microsomes, and for inhibitory activity against 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD-1) using human placental cytosol. The phenyl, 4-fluoro-phenyl and 4-bromo-phenyl derivatives displayed moderate inhibitory activity against $P450_{AROM}$ (IC_{50} 17.2, 13.5 and 10.1 μM, respectively), none of the flavones, including the standard genistein, displayed any inhibitory activity against 17β-HSD type 1 at 100 μM concentration.

Keywords: Flavones; Baker–Venkataraman synthesis; P450 aromatase; 17β-hyroxysteroid dehydrogenase; Inhibitors

INTRODUCTION

Oestrogens stimulate growth in hormone-dependent breast cancer, therefore modern strategies seek to remove the influence of hormones on tumour growth using two approaches. The first approach employs anti-oestrogens, which block the action of the hormone on its receptor,[1] the second approach, generally used in conjunction with anti-oestrogen therapy, aims to deplete circulating and tissue levels of the respective mitogenic hormone by inhibition of a specific target enzyme involved in its steroidogenic pathway:[2] for breast cancer–aromatase ($P450_{AROM}$)[3] or 17β-hydroxysteroid dehydrogenase (17β-HSD) type 1 isoenzyme[4] or the metabolic enzyme oestrogen sulphatase[5] (Fig. 1).

Despite the marked fall of oestrogen plasma levels in postmenopausal women, the breast tissue concentration of oestrogen is similar to that of premenopausal women, and much greater (5- to 45-fold) than in plasma. Aromatase activity has been reported in non-malignant and malignant breast tissue[6] and 17β-HSD type 1 is also present.[7] Oestrone sulphate, as a potential source of oestrone, does not appear to be taken up in breast tissue.[8]

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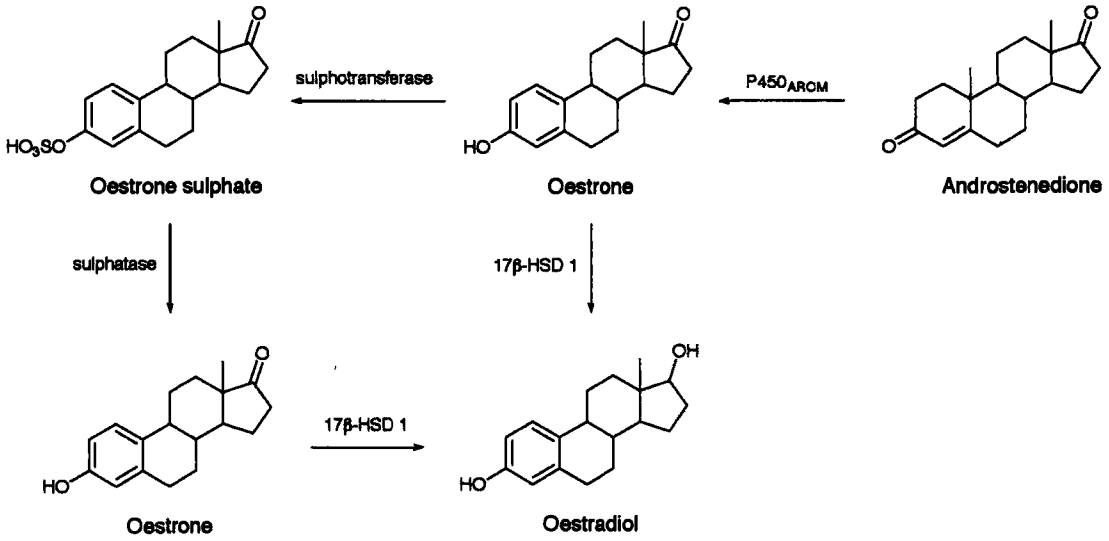


FIGURE 1 Enzymes involved in the production of the oestrogens–oestrone and oestradiol.

Flavones are of interest owing to their known pharmacological and pharmaceutical functions.[9] Many naturally occurring flavones, isoflavones and flavanones also display phytoestrogenic activity.[10] The phytoestrogenic activity of compounds such as genistein, daidzein and biochanin A (Fig. 2), led us to investigate the ability of simple 4-substituted flavones to inhibit key enzymes, $P450_{AROM}$ and 17β-HSD type 1, involved in oestrogen biosynthesis.

MATERIALS

^{1}H and ^{13}C NMR spectra were recorded with a Brucker Avance DPX300 spectrometer operating at 300 and 75 MHz respectively, in DMSO-d_6 with Me_4Si as internal standard. Chemical shifts are expressed in parts per million downfield from TMS. Microanalyses were determined by Medac Ltd., Surrey. Flash column chromatography was performed with silica gel 60 (230–400 mesh)

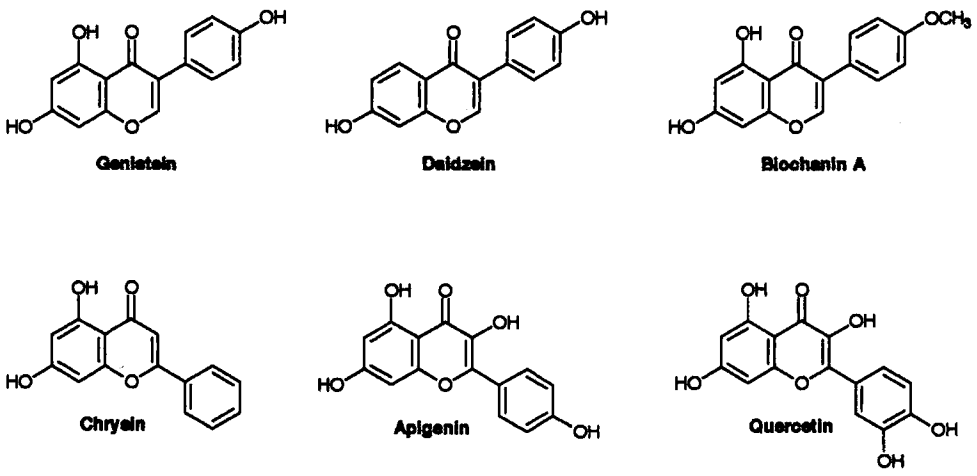


FIGURE 2 Naturally occurring flavones and isoflavones.

(Merck) and TLC was carried out on precoated silica plates (kiesel gel 60 F_{254}, BDH). Melting points were measured with a Gallenkamp Melting Point Apparatus and are reported uncorrected. All the reactions were carried out under nitrogen using anhydrous solvents.

[1,2,6,7-^{3}H]androstenedione (86.4 Ci/mmol–37 MBq/mL) and [2,4,6,7-^{3}H]oestrone (92 Ci/mmol–37 MBq/mL) were purchased from NEN-Dupont UK (Stevenage, Herts). Scintillation fluid was optiphase Hisafe from Fisons Chemicals (Loughborough, UK). The scintillation counter used was a LKB Wallac, 1217, Rack-beta. HPLC used a Milton Roy consta-Metric 3000 solvent delivery system linked to a LabLogic β-Ram detector, using a Bondapak C18 (3.9 × 300 mm) Waters column.

METHODS

Chemistry

Compounds **5a**, **5c**, **5e-h** and **6** were prepared according to reported methods.[11–13] Compounds **5b**, **5d**, **7** and **8** were prepared according to the following procedures. Numbering used for NMR characterisation is as shown in Fig. 3.

General Method for the Preparation of Benzoyl Esters 3

To a cold (0°C) solution of the benzoyl chloride **2** (10 mmol) in dry pyridine (10 mL) was added 2,4-dihydroxyacetophenone **1** (10 mmol). The reaction was stirred at room temperature until completion of the reaction (30 min to 12 h) then poured into ice water/1M aqueous HCl with vigorous stirring. The resulting precipitate was collected by filtration, washed with water and purified by recrystallisation.

2-ACETYL-1-[(4-FLUOROPHENYL)CARBONYLOXY]-5-HYDROXY-BENZENE **3b**

Ivory crystalline solid (95%), mp 126–128°C (MeOH). ^{1}H NMR δ 12.54 (s, 1H, OH), 8.25 (m, 2H, H-2′, H-6′), 7.85 (d, *J*=8.7 Hz, 1H, H-3), 7.24 (dd, *J*=1.9, 8.7 Hz, 2H, H-4, H-6), 6.90 (ddd, *J*=2.2, 8.7 Hz, 2H, H-3′, H-5′), 2.69 (s, 3H, CH_3); ^{13}C NMR δ 204.1 (C=O, C-8), 168.5, 165.1 (C, C-4′), 164.4 (C, C-5), 163.7 (C=O, C-7), 157.2 (C, C-1), 133.4, 133.3 (CH, C-2′, C-6′), 132.5 (CH, C-3), 125.6 (C, C-1′), 118.2 (C, C-2), 116.5, 116.2 (CH, C-3′, C-5′), 113.4 (CH, C-4), 111.7 (CH, C-6), 27.1 (CH_3).

2-ACETYL-5-HYDROXY-1-[(4-IODOPHENYL)CARBONYLOXY]BENZENE **3d**

Yellow crystalline solid (84%), mp 106–108°C (EtOH). ^{1}H NMR δ 12.24 (s, 1H, OH), 8.15 (m, 2H, H-2′, H-6′), 7.67 (d, *J*=8.7 Hz, 1H, H-3), 7.18 (m, 2H, H-3′, H-5′), 6.88 (m, 2H, H-4, H-6), 2.68 (s, 3H, CH_3); ^{13}C NMR δ 203.5 (C=O, C-7), 166.5 (C=O, C-8), 166.9 (C, C-5), 166.4 (C, C-1), 165.8 (C, C-4′), 159.8 (C, C-1′), 135.3 (CH, C-3), 133.8 (CH, C-3′), 133.6 (C, C-2), 116.4 (CH, C-5′), 114.6 (CH, C-2′), 113.2 (CH, C-6′), 109.6 (CH, C-4), 104.5 (C, C-6), 27.2 (CH_3).

General Method for the Preparation of Diketones 4

To a solution of the benzoyl ester (4 mmol) in dry pyridine (4 mL) was added pulverised KOH (6 mmol). The reaction mixture was heated at

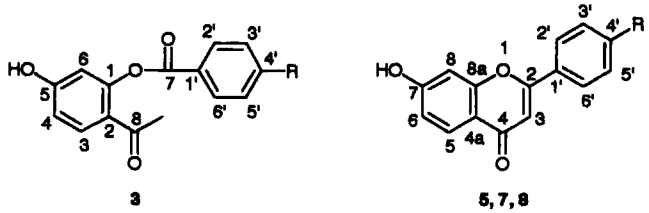


FIGURE 3 Numbering system for NMR characterisation.

50°C for 1 h then on cooling, aqueous acetic acid (10%, 4 mL) was added. The resulting precipitate was collected by filtration, dried and used in the next step without any further purification. *1-(2,4-Dihydroxyphenyl)-3-(4-fluorophenyl)-1,3-propanedione* **4b** (75%); *1-(2,4-dihydroxyphenyl)-3-(4-iodophenyl)-1,3-propanedione* **4d** (50%).

General Method for the Preparation of Flavones 5

A mixture of the crude diketone **4d** (1 mmol), conc. H_2SO_4 (0.45 mL) and glacial acetic acid (2 mL) was heated under reflux (110°C) for 2 h and then cooled to room temperature. The reaction mixture was poured into crushed ice and the resulting precipitate collected by filtration and recrystallised from MeOH.

2-(4-Fluorophenyl)-7-hydroxy-4*H*-4-chromenone **5b**

Yellow crystalline solid (48%), mp 240–242°C. ^{1}H NMR δ 10.86 (s, 1H, 7-OH), 8.14 (m, 2H, H-2′, H-6′), 7.89 (d *J*=8.7 Hz, 1H, H-5), 7.41 (m, 2H, H-3′, H-5′), 7.01 (d, *J*=2.2 Hz, 1H, H-8), 6.94 (dd, *J*=2.25, 8.7 Hz, 1H, H-6), 6.91 (s, 1H, H-3); ^{13}C NMR δ 176.7 (C=O, C-4), 166.0 (C, C-4′), 163.1, 162.7 (C, C-2, C-7), 157.8 (C, C-8a), 155.2 (C, C-4′), 129.2, 129.1 (CH, C-2′, C-6′), 128.2 (C, C-1′), 126.9 (CH, C-5), 116.7 (C, C-4a), 116.4 (CH, C-3′, C-5′), 115.4 (CH, C-6), 106.9 (CH, C-3), 102.9 (CH, C-8). Found: C, 69.89; H, 3.48%. $C_{15}H_9FO_3$ requires: C, 70.05; H, 3.52%.

7-Hydroxy-2-(4-iodophenyl)-4*H*-4-chromenone **5d**

Cream crystalline solid (86%), mp 288–290°C. ^{1}H NMR δ 10.87 (s, 1H, 7-OH), 7.92 (m, 2H, H-2′, H-6′), 7.85 (m, 3H, H-3, H-3′, H-5′), 6.99 (d, *J*=2.1 Hz, 1H, H-8), 6.94 (dd, *J*=2.3, 8.4 Hz, 2H, H-5, H-6); ^{13}C NMR δ 176.7 (C=O, C-4), 163.2, 161.5 (C, C-2, C-7), 157.8 (C, C-8a), 138.3 (CH, C-3′, C-5′), 131.2 (C, C-1′), 128.3 (CH, C-2′, C-6′), 126.9 (CH, C-5), 116.5 (C, C-4a), 115.5 (CH, C-6), 107.2 (CH, C-3), 102.9 (CH, C-8), 99.5 (C, C-4′). Found: C, 49.40; H, 2.56%. $C_{15}H_9IO_3$ requires: C, 49.48; H, 2.49%.

Ethyl-3-[4-(7-Hydroxy-4-oxo-4H-chromen-2-yl)phenyl]acrylate 7

A solution of *tetrakis*triphenylphosphine Pd(0) (95 mg, 0.082 mmol), Et_3N (0.4 mL, 2.87 mmol) in anhydrous 1,4-dioxane (15 mL) was heated at 100°C to produce a dark red solution and then cooled slightly. To this solution was added ethyl acrylate (0.45 mL, 4.12 mmol) followed by **5d** (0.3 g, 0.82 mmol) and Et_3N (0.15 mL) in anhydrous 1,4-dioxane (10 mL). The reaction mixture was refluxed for 24 h then cooled and the inorganic residues removed by filtration through a celite pad. The filtrate was concentrated under reduced pressure and the resulting residue purified by recrystallisation from ethanol to give the product as a yellow crystalline solid (0.113 g, 42%), mp 260–262°C. ^{1}H NMR δ 10.86 (s, 1H, 7-OH), 8.08 (d, *J*=8.3 Hz, 2H, H-2′, H-6′), 7.89 (d, *J*=8.6 Hz, 3H, H-5, H-6, H-8), 7.76 (m, 1H, C*H*=CH), 6.97 (m, 3H, H-3, H-3′, H-5′), 6.76 (d, *J*=16.0 Hz, 1H, CH=C*H*), 4.21 (q, *J*=7.0 Hz, 2H, CH_2), 1.27 (m, 3H, CH_3); ^{13}C NMR δ 176.7 (C=O, C-4), 166.4 (C=O, COEt), 163.2, 161.4 (C, C-2, C-7), 157.8 (C, C-8a), 143.4 (CH, C H=CH), 137.2 (C, C-4′), 133.0 (C, C-1′), 129.21 (CH, C-2′, C-6′), 126.9 (CH, C-2′, C-6′, C-5), 120.3 (CH, CH=C H), 116.6 (C, C-6), 115.5 (CH, C-4a), 107.5 (CH, C-3), 102.9 (CH, C-8), 60.5 (CH_2), 14.5 (CH_3). Found: C, 71.12; H, 4.75%. $C_{20}H_{16}O_5$ requires: C, 71.42; H, 4.97%.

3-[4-(7-Hydroxy-4-oxo-4H-chromen-2-yl) phenyl]acrylic Acid 8

A solution of **7** (64 mg, 0.20 mmol) in aqueous 2N sodium hydroxide (3 mL) was stirred at room temperature for 20 h and then at 80°C for 1 h. The reaction mixture was cooled in an ice-bath and acidified with aqueous HCl. The white precipitate formed was collected by filtration and

washed with water and acetone to give the product as a yellow powder (24 mg, 39%), mp 296–300°C. ^{1}H NMR δ 10.81 (s, 1H, 7-OH), 8.10 (d, J=8.2 Hz, 2H, H-2′, H-6′), 7.84 (d, J=8.4 Hz, 3H, H-5, H-6, H-8), 7.80 (m, 1H, C*H*=CH), 6.93 (m, 3H, H-3, H-3′, H-5′), 6.77 (d, J=15.4 Hz, 1H, CH=C*H*), 5.61 (s, 1H, OH); ^{13}C NMR δ 175.6 (C=O, C-4), 166.8 (C=O, CO_2H), 164.4, 161.5 (C, C-2, C-7), 158.9 (C, C-8a), 144.5 (CH, *C*H=CH), 137.9 (C, C-4′), 133.5 (C, C-1′), 129.7 (CH, C-2′, C-6′, C-5), 127.2 (CH, C-3′, C-5′), 121.0 (CH, CH=C*H*), 116.7 (C, C-4a), 115.5 (CH, C-6), 107.9 (CH, C-3), 102.8 (CH, C-8). Found: C, 70.24; H, 3.93%. $C_{18}H_{12}O_5$ requires: C, 70.13; H, 3.91%.

Preparation of Human Placental Cytosol and Microsomes

Freshly delivered full-term placenta was removed of connective tissue and then cut into small pieces and stored in ice. The placenta was washed with Tris pH 7.4 buffer, containing 25 mM KCl, 5 mM MgCl and 0.25 mM sucrose, and initially blended to a fine consistency using a commercial food blender. The blended placenta was then homogenised using a Potter-Elvejhem homogeniser. The homogenate was centrifuged at 12,000 rpm for 15 min at 4°C using a Sorvall OTD ultracentrifuge, then the supernatant ultracentrifuged at 38,000 rpm for 60 min at 4°C. The supernatant (cytosolic fraction) was stored in vials at −80°C, the remaining pellets (microsomal fraction) were dispersed in Tris buffer using a homogeniser then stored in vials at −80°C. Protein concentration of both the microsomal and cytosolic fractions was determined using the Pierce BCA protein assay.

Enzyme Assays

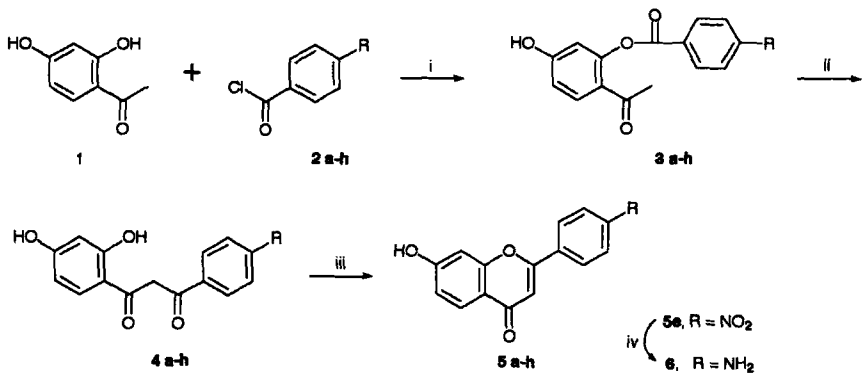
Aromatase

The classical ^3H_2O assay[14] was used to measure the effect of the flavones on aromatase activity using human placental microsomes. A solution of [1,2,6,7-^{3}H]androstenedione and androstenedione (0.5 μM final concentration) was incubated in test tubes at 37°C for 15 min with the human placental microsomal preparation (8.24 mg/mL, 30 μL), phosphate buffer (400 μL, 50 mM, pH 7.4) and NADPH (50 μL, 16 mM) in the presence of flavone (10 μL, 1 or 5 mmol–20 or 100 μM final concentrations, respectively) in EtOH or DMSO. Control experiments were run with EtOH or DMSO (10 μL) in place of the inhibitor. The reaction was quenched by the addition of aqueous $HgCl_2$ (30 μL, 1 mM) followed by an aqueous suspension of charcoal (1 mL, 1% by weight). The test tubes were centrifuged (15 min, 3000 rpm), then the supernatant liquid placed in a scintillation vial to which 1 mL of scintillation fluid was added. The ^3H_2O contained in each vial was then determined using a LKB Wallac, 1217, Rack-beta scintillation counter.

For IC_{50} values the general method described for determination of percentage inhibition was followed except that a range of concentrations of inhibitor were used. Calculation of IC_{50} was determined by plotting % inhibition *versus* Log [I] using Cricket Graph III 1.5f software.

β-HSD Type 1

A solution of [2,4,6,7-^{3}H]oestrone and oestrone (0.5 μM final concentration) was incubated in test tubes at 37°C for 15 min with the human placental cytosolic preparation (1.45 mg/mL, 10 μL), phosphate buffer (420 μL, 50 mM, pH 7.4) and NADPH (50 μL, 16 mM) in the presence of flavone (10 μL, 5 mmol, 100 μM final concentration) in EtOH or DMSO. Control experiments were run with EtOH or DMSO (10 μL) in place of the inhibitor. The reaction was quenched by the addition of EtOAc containing 0.05% w/v butylated hydroxyanisole (2 mL) with subsequent vortexing. The EtOAc layer was transferred into clean test tubes then the EtOAc removed by evaporation. The percentage inhibition was determined using the HPLC



SCHEME 1 Reagents and conditions: (i) pyridine, 30 min to 12 h; (ii) KOH, pyridine, 50°C, 20 min to 1 h; (iii) H_2SO_4, AcOH, 110°C, 2 h; (iv) 10% Pd/C, H_2, EtOH, 1 h. a, R=H; b, R=F; c, R=Br; d, R=I; e, R=NO_2; f, R=CH_3; g, R=n-C_4H_9; h, R=OCH_3.

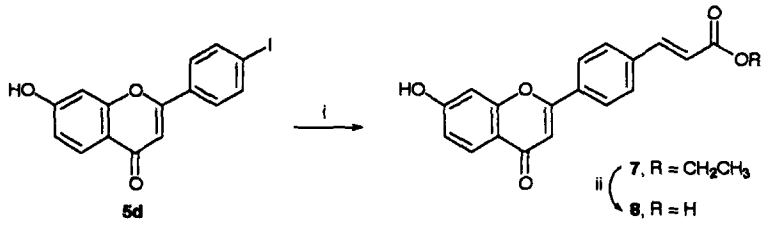
method using a β-RAM detector. HPLC system: column, Bondapak C18 3.9 × 390 mm Waters column; eluent, MeOH–H_2O–^{i}PrOH–CH_2Cl_2 18:17:3:2 v/v/v/v; flow, 1.3 mL/min (220–230 psi); wavelength, 280 nM. Elution of both oestrone (retention time ~20 min) and oestradiol (retention time ~16 min) was observed and the percentage present in each test tube calculated, by comparison with the control, inhibition of metabolism could be determined.

RESULTS AND DISCUSSION

Chemistry

Preparation of the 7-hydroxy-2-aryl-chromen-4-ones, **5a**, **5c**, **5d–h** and **6** have previously been described,[11–13] was achieved using the 3-step Baker–Venkataraman synthesis.[15,16] The first step involved reaction of 2,4-dihydroxyacetophenone **1** with the 4-substituted benzoyl chlorides **2** in pyridine to give the benzoyl esters **3**. The benzoyl chlorides **2a–h** were prepared by reaction of the corresponding acids with either thionyl chloride or oxalyl chloride and the moisture sensitive acid chlorides stored under nitrogen until required. Treatment of the benzoyl esters **3a–h** with KOH induced an intramolecular Claisen condensation forming the 1,3-diketones **4a–h**, which on heating in acetic acid containing sulphuric acid, cyclised to form the required flavones **5a–h** (Scheme 1). The 7-hydroxy-2-(4-aminophenyl)-chromen-4-one derivative was prepared by catalytic hydrogenation of the 4-nitrophenyl derivative.[3]

Sonogashira coupling[17] of the 4-iodophenyl flavone **5d** with ethyl acrylate in the presence of $Pd(Ph_3)_3$, CuI and triethylamine gave the acrylic acid ethyl ester substituted flavone **7**, which was subsequently saponified by treatment with 2N NaOH to give the acrylic acid derivative **8** (Scheme 2).



SCHEME 2 Reagents and conditions: (i) $(Ph_3P)_4Pd$, CuI, Et_3N, 1,4-dioxane, ethyl acrylate, 100°C, 24 h; (ii) 2N aq. NaOH, r.t., 20 h then 80°C, 1 h.

TABLE I Inhibition of human placental $P450_{AROM}$

Compound	R	% Inhibition		IC_{50} (μM)*
		100 μM	20 μM	
5a	H	70.8	56.6	17.2
5b	F	67.7	59.2	13.5
5c	Br	74.2	69.4	10.1
5d	I	50.3	–	~100
5e	NO_2	67.3	29.7	> 20
5f	CH_3	46.6	–	> 100
5g	n-C_4H_9	15.0	–	> 100
5h	OCH_3	49.6	–	~100
6	NH_2	76.6	31.8	> 20
7	CH=CH-CO_2Et	33.8	–	> 100
8	CH=CH-CO_2H	52.9	–	~100

*Concentration androstenedione, 0.5 μM. IC_{50} values are the average of two experiments.

Inhibitory Activity

The flavones were all evaluated *in vitro* for inhibitory activity against aromatase ($P450_{AROM}$, CYP19), using human placental microsomes, and for inhibitory activity against 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD-1) using human placental cytosol. As can be seen from Table I, the phenyl (**5a**), 4-fluoro-phenyl (**5b**) and 4-bromo-phenyl (**5c**) derivatives displayed moderate inhibitory activity against $P450_{AROM}$ (IC_{50} 17.2, 13.5 and 10.1 μM, respectively).

The 4-nitro (**5e**) and 4-amino (**6**) derivatives displayed comparable activity (approx. 30% inhibition at 20 μM) indicating no preference in the 4-position for either electron withdrawing or electron-donating functional groups, and no preference for groups capable of either ionic or hydrogen bonding. However, the activity of the 4-fluoro, and in particular bulky 4-bromo-derivative compared with the 4-methyl substituent (**5f**), might suggest the requirement for a H-acceptor in this position. The larger substitutents, butyl (**5g**) and the ethyl acrylate (**7**), were not well tolerated by the enzyme with very low inhibitory activity observed.

None of the flavones, including the standard genistein, displayed any inhibitory activity against 17β-HSD type 1 at 100 μM concentration. Genistein has shown activity against human testes microsomal 17β-HSD type 3 (IC_{50} 30.3 μM),[18] with this result demonstrating selectivity between the two isoenzymes.

Although the activity observed for **5a–c** was moderate, it was greater than that observed for aminoglutethimide (IC_{50} 18.5 μM). As such, these 4-substituted flavones are useful lead compounds for oestrogen-ablation therapy, suitable for further structure modification and evaluation.

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