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Structure–activity relationship determination within a group of substituted phenyl sulfamate based compounds against the enzyme oestrone sulfatase

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Abstract

The enzyme oestrone sulfatase (ES) is responsible for the conversion of the stored (sulfated) form of oestrogens to the active form, namely oestrone. In our continuing quest to synthesize potent inhibitors of oestrone sulfatase and to determine the structural requirements for such inhibition, we have synthesized and evaluated several derivatives of phenyl sulfamate. We report the results of the synthesis and biochemical evaluation of a series of 3- and 4-aminosulfonated derivatives of phenol in an effort to investigate the role of the acid dissociation constant (pK_a), and therefore the stability of the phenoxide ion, on the inhibitory activity of compounds against this enzyme. The results showed that there was a strong correlation between the observed pK_a and inhibitory activity within the aminosulfonated compounds considered. This suggested that in the inhibition of oestrone sulfatase by these compounds, pK_a was an important physicochemical property, and as such, the stability of the O^- ion was a crucial factor in the inhibition, and therefore the drug design process.

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Introduction

In the treatment of hormone-dependent breast cancer, extensive research has been undertaken to produce compounds that are both potent and selective inhibitors of the cytochrome P450 enzyme aromatase. Although the third generation of aromatase inhibitors (Feutrie & Bonneterre 1999; Brodie & Njar 2000) are able to lower the plasma levels of the female sex hormones (which have been implicated in the initiation/progression of hormone-dependent breast cancers) to as low as 98%, hormone-dependent breast cancer tissue continues to be stimulated by oestrogens. This is believed to be due to the activity of the enzyme oestrone sulfatase, which converts the stored (sulfated) form of the oestrogens to the active (non-sulfated) forms (Figure 1), thereby increasing the level of oestrogens in plasma, as well as breast tissue, and therefore sustaining tumour growth. A number of steroidal inhibitors (Howarth et al 1994; Reed et al 1996; Woo et al 1998) have been investigated as potent inhibitors of this enzyme, including oestrone-3-*O*-sulfamate (EMATE) (IC50 = 65 pM) (Figure 2)—a time- and concentration-dependent irreversible inhibitor. However, this compound has been shown to possess potent oestrogenic properties, and as a result, the investigation into non-steroidal inhibitors has intensified and has resulted in compounds such as 4-methylcoumarin-7-*O*-sulfamate (COUMATE) (Woo et al 1996) which is a time- and concentration-dependent irreversible inhibitor (Figure 2). COUMATE has been derivatized further, resulting in a series of tricyclic compounds (Woo et al 2000), such as 667-, 668- and 669-COUMATE (Figure 2 shows 666- and 667-COUMATE). The potent inhibitors, in general, contain an aminosulfonate moiety that is involved in the irreversible inhibition of oestrone sulfatase. From the consideration of the results obtained with the known sulfamate-containing steroidal and non-steroidal inhibitors, a 'definitive model' was proposed (Woo et al 1998) where it was suggested that the most fundamental and basic requirements for inhibition was the phenolic ring attached via the phenolic oxygen atom to the sulfamate group—it was presumed that the oxygen atom

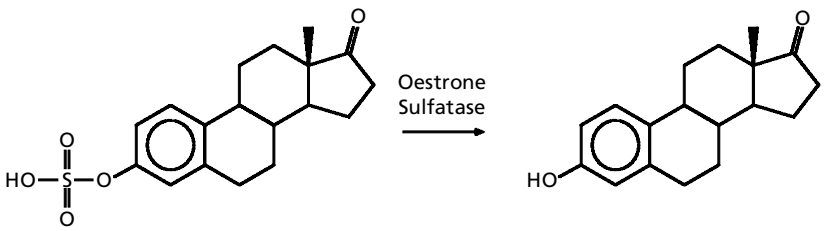

Figure 1 Action of the enzyme oestrone sulfatase on oestrone sulfate.

was required for strong hydrogen bonding to the active site with the result that the O—S bond of the aminosulfonate group was weakened.

In an effort to overcome the lack of detailed information about the active site of oestrone sulfatase, to probe the nature of the proposed pharmacophore, and to rationalize the inhibitory activity of the aminosulfonate-based compounds, we initiated a series of structure–activity relationship (SAR) determination studies (Ahmed et al 1998, 1999; Ahmed & James 1999). From the results of our initial molecular modelling and SAR studies and a review of potential mechanisms for oestrone sulfatase, we proposed that the stability of the O^- anion (i.e. pK_a) may be an important factor in the determination of the overall inhibitory activity within the sulfamate-containing inhibitors. We report the initial results of our study to verify our previous model and conclusions, and as such, the synthesis of a number of *o*-, *m*- and *p*-substituted phenol derivatives containing sulfamate groups has been described. Biochemical evaluation and pK_a determination of these compounds have been carried out also, in an effort to determine the effect of pK_a on the irreversible inhibitory activity. The choice of the phenolic structure was based upon our observations from other studies undertaken within our laboratories, where we have shown that logP may also be a factor. The choice of the substituted phenols

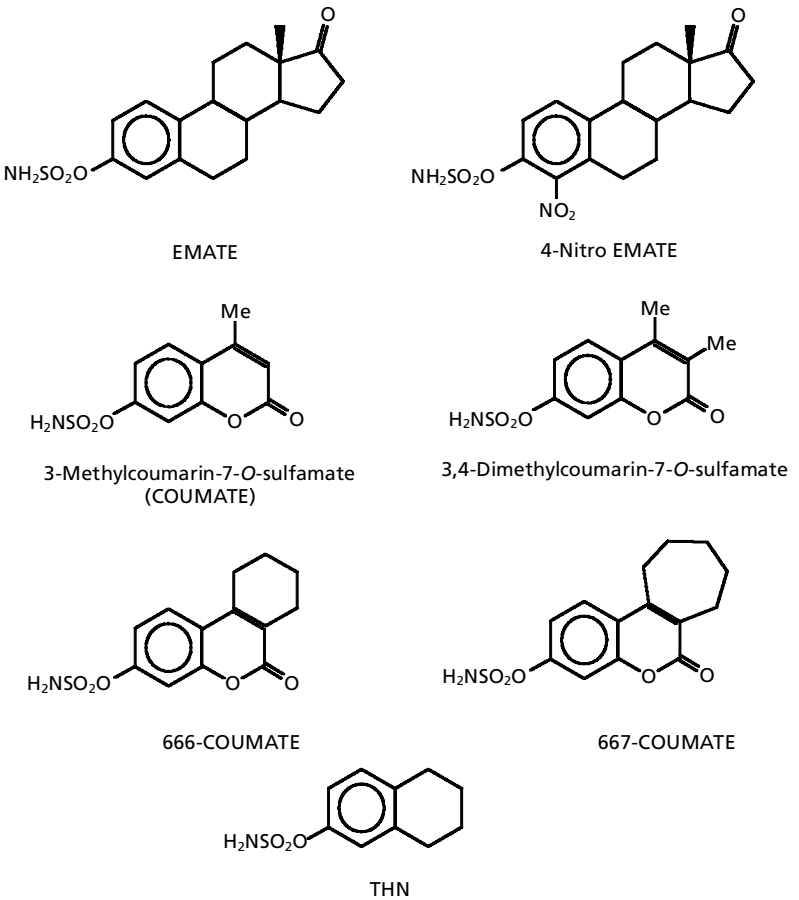

Figure 2 Known steroidal and non-steroidal inhibitors of oestrone sulfatase.

therefore minimized the logP problem and as such allowed us to consider the effect of pK_a alone on the inhibition of oestrone sulfatase.

Materials and Methods

Materials

Chemicals were purchased from Sigma-Aldrich Company Ltd (Poole, UK), and checked for purity by ^{1}H and ^{13}C NMR (Brucker 300 MHz and 75.5 MHz, respectively). Infrared spectrometry was carried out on a Perkin-Elmer Fourier transform-Paragon 1000 IR. Mass spectroscopy data were obtained from the EPSRC mass spectrometry service centre at the University of Wales College Swansea, UK, using a VG ZAB-E instrument. Low resolution mass spectra were obtained on a Walters 2700 sample manager connected to a Micromass LCT. Melting points are uncorrected and were undertaken on a BUCHI 512 or a Gallenkamp Instrument.

Aminosulfonyl chloride

Methanoic acid (1 L, 26.5 mmol) was added dropwise to chlorosulfonyl isocyanate (2.3 mL, 26.5 mmol), under an atmosphere of nitrogen gas, at 0 °C. After evolution of gas had ceased, anhydrous toluene (20 mL) was added and the solution stirred for 1 h. Insoluble by-products were removed by filtration, and the resulting solution of aminosulfonyl chloride, in toluene, was used immediately in the synthesis of the aminosulfonated phenols.

*Phenyl sulfamate (**1**)*
Sodium hydride (NaH) (80% dispersion in mineral oil, 0.12 g, 4 mmol) was added to a stirred solution of phenol (0.3 g, 3.2 mmol) in dimethyl formamide (DMF) (20 mL) under an atmosphere of nitrogen gas at 0 °C. After evolution of hydrogen had ceased, aminosulfonyl chloride in toluene (10 mL, ~10 mmol) was added in one portion and the reaction allowed to stir for 10 h. The reaction was then quenched with saturated sodium bicarbonate ($NaHCO_3$) solution (50 mL), extracted into dichloromethane (DCM) (2 × 50 mL), washed with water (3 × 30 mL) and dried over anhydrous magnesium sulfate ($MgSO_4$). The mixture was filtered and the solvent removed under vacuum, to give a yellow oil which was purified using flash chromatography to give **1** (0.14 g, 25.4%) as a white solid (mp 77.6–81.2 °C; $R_f = 0.32$; diethyl ether/petroleum ether 40–60 °C (60:40)). $\nu_{(max.)}$ (Film) cm^{-1}: 3421.1 and 3307.8 (NH), 1367.5 and 1177.2 (S═O). δ_H (*CDCl₃*) 7.43–7.25 (5H, m, ArH), 5.24 (2H, s, NH_2). δ_C (*CDCl₃*) 150.024, 129.923, 127.306, 122.142. MS (M^+) calculated mass 173.014665, obtained mass 173.015633.

*3-Methylphenyl sulfamate (**2**)*
Compound **2** was synthesized following the same procedures as for **1** except that NaH (60% dispersion in mineral oil, 0.25 g, 6.25 mmol) was added to a stirred solution of 3-cresol (0.5 g, 4.62 mmol) in DMF (10 mL). Aminosulfonyl chloride in toluene (10 mL, ~10 mmol) was added after 30 min. Removal of the solvent under vacuum yielded an orange oil, which was purified using flash chromatography to give **2** (0.32 g, 37%) as a white solid (mp 85.8–87.4 °C; $R_f = 0.42$; diethyl ether/petroleum ether 40–60 °C (50:50)). $\nu_{(max.)}$ (Film) cm^{-1}: 3390.5, 3286.4 (NH_2), 1375.2, 1188.2 (S═O). δ_H (*CDCl₃*) 7.35–7.04 (4H, m, ArH), 5.09 (2H, s, NH_2) 2.37 (3H, s, CH_3). δ_c (*CDCl₃*): 150.037, 140.346, 129.556, 128.102, 122.545, and 118.901 (C—Ar), 21.257 (CH_3). MS m/z 188 (M^+1).

*3-Fluorophenyl sulfamate (**3**)*
Compound **3** was synthesized following the same procedures as for **1** except that NaH (60% dispersion in mineral oil, 0.3 g, 7.5 mmol) was added to a stirred solution of 3-fluorophenol (0.5 mL, 5.6 mmol) in DMF (10 mL). Aminosulfonyl chloride in toluene (10 mL, ~10 mmol) was added after 30 min. Removal of the solvent under vacuum yielded an oil, which was purified using flash chromatography to give a colourless oil which solidified on standing to give **3** (0.32 g, 29.9%) as a white solid (mp 49–53 °C; $R_f = 0.34$; diethyl ether/petroleum ether 40–60 °C (50:50)). $\nu_{(max.)}$ (Film) cm^{-1}: 3367.9, 3290.3 (NH_2), 1391.1, 1191.9 (S═O). δ_H (*CDCl₃*) 7.40–7.01 (4H, m, ArH), 5.42 (2H, s, NH_2). δ_c (*CDCl₃*): 164.058–161.138 (d, J = 240 Hz, F-CAr), 150.502–150.358 (d), 130.829–130.706 (d), 117.951–117.906 (d), 114.723–114.445 (d), 110.436–110.109 (d) (C—Ar); MS m/z EI 191 (M^+).

*3-Chlorophenyl sulfamate (**4**)*
Compound **4** was synthesized following the same procedures as for **1** except that NaH (60% dispersion in mineral oil, 0.5 g, 12.5 mmol) was added to a stirred solution of 3-chlorophenol (1 g, 7.78 mmol) in DMF (10 mL). Aminosulfonyl chloride in toluene (20 mL, ~20 mmol) was added after 30 min. Removal of the solvent under vacuum yielded an oil, which was purified using flash chromatography to give **4** (0.56 g, 34.6%) as a white solid (mp 67.3–72.0 °C; $R_f = 0.45$; diethyl ether/petroleum ether 40–60 °C (50:50)). $\nu_{(max.)}$ (Film) cm^{-1}: 3375.5, 3270.3 (NH_2), 1370.9, 1171.2 (S═O). δ_H (*d-Acetone*) 7.51–7.28 (4H, m, ArH), 7.29 (2H, s, NH_2). δ_c (*d-Acetone*): 152.349, 134.806, 131.633, 127.495, 123.295, 121.784 (C—Ar).

*3-Bromophenyl sulfamate (**5**)*
Compound **5** was synthesized following the same procedures as for **1** except that NaH (60% dispersion in mineral oil, 0.4 g, 10 mmol) was added to a stirred solution of 3-bromophenol (1 g, 5.8 mmol) in DMF (10 mL). Aminosulfonyl chloride in toluene (20 mL, ~20 mmol) was added after 30 min. Removal of the solvent under vacuum yielded an oil, which was purified using flash chromatography to give **5** (0.53 g, 36.4%) as a white solid (mp 88–90.1 °C; $R_f = 0.43$; diethyl ether/petroleum ether 40–60 °C (50:50)). $\nu_{(max.)}$ (Film) cm^{-1}: 3342.1, 3282.2 (NH_2), 1378.0, 1160.1 (S═O). δ_H (*d-Acetone*) 7.54–7.32 (4H, m, ArH), 7.29 (2H, s, NH_2). δ_c (*d-Acetone*): 151.842, 131.943, 130.447, 126.162, 122.474, and 122.219 (C—Ar).

*3-Cyanophenyl sulfamate (**6**)*
Compound **6** was synthesized following the same procedures as for **1** except that NaH (60% dispersion in mineral oil, 0.5 g, 12.5 mmol) was added to a stirred solution of 3-cyanophenol (1 g, 8.4 mmol) in DMF (10 mL). Aminosulfonyl chloride in toluene (20 mL, ~20 mmol) was added after 30 min. Removal of the solvent under vacuum yielded an oil, which was purified using flash chromatography to give **6** (0.5 g, 30.1%) as a white solid (mp 104–107 °C; $R_f = 0.4$; diethyl ether/petroleum ether 40–60 °C (60:40)). $\nu_{(max.)}$ (Film) cm^{-1}: 3371.1, 3283.5 (NH_2), 2238.3 (CN), 1388.0, 1189.1 (S═O). δ_H (*d-Acetone*) 7.76–7.65 (4H, m, ArH), 7.30 (2H, s, NH_2). δ_c (*d-Acetone*): 151.258 (CN), 131.909, 131.285, 128.289, 126.622, 118.169, and 114.014 (C—Ar). MS m/z 199 (M^+1).

*3-Nitrophenyl sulfamate (**7**)*
Anhydrous potassium carbonate (dried at 100 °C, 0.8 g, 5.8 mmol) was rapidly added to a solution of 3-nitrophenol (0.8 g, 5.8 mmol) in sodium-dried toluene (20 mL), and allowed to stir at 40 °C for 30 min. Aminosulfonyl chloride in toluene (10 mL, ~10 mmol) was then added in one portion and the reaction allowed to stir at room temperature for three days. The reaction was then quenched in $NaHCO_3$ (50 mL), extracted into DCM (2 × 50 mL), washed (3 × 30 mL water) and dried ($MgSO_4$). Removal of the solvent under vacuum yielded an oil, which was purified using flash chromatography to give **7** (0.32 g, 29.9%) as an off white solid (mp 116–119 °C; $R_f = 0.32$; diethyl ether/petroleum ether 40–60 °C (50:50)). $\nu_{(max.)}$ (Film) cm^{-1}: 3411.0, 3309.6 (NH_2), 1530.8, 1203.3 (NO_2), 1376.4, 1184.6 (S═O). 300 MHz δ_H (*d-Acetone*) 8.27–7.76 (4H, m, ArH), 7.41 (2H, s, NH_2). MS m/z 219 (M^+1) also 437 ($2M^+1$ dimer).

*4-Methylphenyl sulfamate (**8**)*
Compound **8** was synthesized following the same procedures as for **1** except that NaH (60% dispersion in mineral oil, 0.25 g, 6.25 mmol) was added to a stirred solution of 4-cresol (0.5 g, 4.62 mmol) in DMF (10 mL). Aminosulfonyl chloride in toluene (10 mL, ~10 mmol) was added after 30 min. Removal of the solvent under vacuum yielded an orange oil, which was purified using flash chromatography to give **8** (0.37 g, 42.8%) as a white solid (mp 79–81 °C; $R_f = 0.42$; diethyl ether/petroleum ether 40–60 °C (50:50)). $\nu_{(max.)}$ (Film) cm^{-1}: 3384.0, 3279.9 (NH_2), 1356.4, 1173.6 (S═O). δ_H (*CDCl₃*) 7.19 (4H, s, ArH), 5.12 (2H, s, NH_2) 2.35 (3H, s, CH_3). δ_c (*CDCl₃*): 147.892, 137.302, 130.368, and 121.799 (C—Ar), 20.872 (CH_3). MS m/z EI 187 (M^+) 107 (base peak) CI 205 (MNH_4^+).

*4-Fluorophenyl sulfamate (**9**)*
Compound **9** was synthesized following the same procedures as for **1** except that NaH (60% dispersion in mineral oil, 0.3 g, 7.5 mmol) was added to a stirred solution of 4-fluorophenol (0.5 g, 4.5 mmol) in DMF (10 mL). Aminosulfonyl chloride in toluene (10 mL, ~10 mmol) was added after 30 min. Removal of the solvent under vacuum yielded an oil, which was purified using flash chromatography to give **9** (0.32 g, 29.9%) as a white solid (mp 87–89 °C; $R_f = 0.34$; diethyl ether/petroleum ether 40–60 °C (50: 50)). $\nu_{(max.)}$ (Film) cm^{-1}: 3365.9, 3288.9 (NH_2), 1388.7, 1156.5 (S═O). δ_H (*d-Acetone*) 7.39–7.18 (4H, m, ArH), 7.11 (2H, s, NH_2). δ_c (*CDCl₃*): 163.117–159.889 (d, J = 240 Hz, F-CAr), 147.319–147.285 (d), 125.007–124.890 (d), 117.018–116.707 (d) (C—Ar). MS ($M^+ + NH_4$) calculated mass 209.0396, obtained mass 209.0398.

*4-Chlorophenyl sulfamate (**10**)*
Compound **10** was synthesized following the same procedures as for **1** except that NaH (60% dispersion in mineral oil, 0.5 g, 12.5 mmol) was added to a stirred solution of 4-chlorophenol (1 g, 7.78 mmol) in DMF (10 mL). Aminosulfonyl chloride in toluene (20 mL, ~20 mmol) was added after 30 min. Removal of the solvent under vacuum yielded an oil, which was purified using flash chromatography to give **10** (0.65 g, 40.2%) as a white solid (mp 98.4–101.2 °C; $R_f = 0.45$; diethyl ether/petroleum ether 40–60 °C (50:50)). $\nu_{(max.)}$ (Film) cm^{-1}: 3388.0, 3277.0 (NH_2), 1354.5, 1168.4 (S═O). δ_H(*d-Acetone*) 7.50–7.33 (4H, dd, J = 8 Hz, 50 Hz, ArH), 7.22 (2H, s, NH_2). δ_c (*d-Acetone*):149.872, 132.461, 130.330, and 124.819 (C—Ar); MS m/z EI 225, 227 ($M^+ + NH_4$).

*4-Bromophenyl sulfamate (**11**)*
Compound **11** was synthesized following the same procedures as for **1** except that NaH (60% dispersion in mineral oil, 0.2 g, 5 mmol) was added to a stirred solution of 4-bromophenol (0.5 g, 2.89 mmol) in DMF (10 mL). Aminosulfonyl chloride in toluene (10 mL, ~10 mmol) was added after 30 min. Removal of the solvent under vacuum yielded a clear yellow oil, which was purified using flash chromatography to give **11** (0.29 g, 39.8%) as a white solid (mp 113–116 °C; $R_f = 0.37$; diethyl ether/petroleum ether 40–60 °C (60:40)). $\nu_{(max.)}$ (Film) cm^{-1}: 3377.0, 3274.6 (NH_2), 1349.6, 1172.7 (S═O). δ_H (*CDCl₃*) 7.57–7.20 (4H, dd, J = 9 Hz, 95 Hz, ArH), 5.02 (2H, s, NH_2). δ_c (*CDCl₃*): 150.561, 133.355, 125.142, and 120.065 (C—Ar). MS (M^+-1) calculated mass 250.9252, obtained mass 250.9254.

*4-Cyanophenyl sulfamate (**12**)*
Compound **12** was synthesized following the same procedures as for **1** except that NaH (60% dispersion in mineral oil, 0.5 g, 12.5 mmol) was added to a stirred solution of 4-cyanophenol (1 g, 8.4 mmol) in DMF (10 mL). Aminosulfonyl chloride in toluene (20 mL, ~20 mmol) was added after 30 min. Removal of the solvent under vacuum yielded an oil, which was purified using flash chromatography to give **12** (0.56 g, 33.7%) as a white solid (mp 157.9–160.5 °C; $R_f = 0.33$; diethyl ether/petroleum ether 40–60 °C (60:40)). $\nu_{(max.)}$ (Film) cm^{-1}: 3359.1, 3228.1 (NH_2), 2232.4 (CN), 1378.1, 1157.3 (S═O). δ_H(*d-Acetone*) 7.92–7.50 (4H, dd, J = 8 Hz, 109 Hz, ArH), 7.40 (2H, s, NH_2). δ_c (*d-Acetone*):154.446 (CN), 134.806, 123.965, 118.424, and 111.026 (C—Ar). MS m/z 199 (M^+1).

*4-Nitrophenyl sulfamate (**13**)*
Compound **13** was synthesized following the same procedure as for **7** except potassium carbonate (0.5 g, 3.61 mmol) was added to 4-nitrophenol (0.5 g, 3.6 mmol) in toluene (20 mL). Aminosulfonyl chloride in toluene (10 mL, ~10 mmol) was added after 30 min. Removal of the solvent under vacuum yielded an off-white solid, which was purified using flash chromatography to give **13** (0.26 g, 33%) as a pale yellow solid (mp 110.4–111.2 °C; $R_f = 0.34$; diethyl ether/petroleum ether 40–60 °C (60:40)). $\nu_{(max.)}$ (Film) cm^{-1}: 3384.6 and 3306.9 (NH), 1351.3 and 1180.0 (S=O). δ_H ($CDCl_3$) 8.33–7.48 (4H, dd, J = 9 Hz, 246 Hz, ArH), 5.81 (2H, s, NH_2). δ_C ($CDCl_3$) 134.911, 132.845, 125.671, 122.675 (C—Ar). MS M/z 218(M^+), 139 (Base peak).

pK_a determination

Although the pK_a values for some of the phenol compounds were available in the literature, the values for the majority of phenolic compounds were lacking. As such, we concluded that the determination of values for all of the parent compounds would be a better approach. The evaluation of the pK_a of the starting phenols involved a spectroscopic technique (Harwood & Moody 1989) which considered the change in UV absorption by the phenolic group under acidic, buffer, and basic conditions. That is, a solution of borax buffer (pH 9) was prepared using sodium tetraborate decahydrate (9.54 g in 1 L), to which hydrochloric acid (92 mL, 0.1 M) was added.

Approximately 3–4 mg of the phenolic compound was added to 100 mL borax buffer. The solution was mixed thoroughly and the UV spectra determined (between 350 and 250 nm). The absorbance value was adjusted to approximately 1 involving the addition of buffer or the phenolic compound. The solution was filtered (to remove any undissolved phenol) and 20 mL of the stock solution was made up to 25 mL with either HCl (2 M), borax buffer, or NaOH (2 M). The UV spectrum of each solution was determined and using the absorption values at a single chosen wavelength, the mole fraction (x) was calculated. The pK_a was then calculated using the equation:

$$pK_a = pH + ((1 - x)/x)$$

Biochemical evaluation of inhibitory activity

Initial screening and the IC50 determinations
The biochemical evaluation of the inhibitory activity of the series of compounds was undertaken in triplicate using the previously reported method of Selcer et al (1996). The total assay volume was 1 mL. [^{3}H]Oestrone sulfate (25 μL, 50 mM/tube; 750 000 dpm) and the inhibitors (various concentrations/tube, 25 μL) dissolved in ethanol were added to a 10-mL assay tube, and the ethanol removed with a stream of nitrogen. Tris-HCl buffer (0.05 M, pH 7.2, 0.2 mL) was added to each tube. Placental microsomes (10.42 mg mL^{-1}) were then diluted with Tris-HCl buffer (115 μg mL^{-1}). The microsomes and assay tubes were pre-incubated in a shaking water bath for 5 min at 37 °C before the addition of the microsomes (0.8 mL) to the tubes. After 10-min incubation (at 37 °C), toluene (4 mL) was added to quench the assay, and the tubes placed on ice. The quenched samples were vortexed for 45 s and centrifuged (3000 rev min^{-1}, 10 min). Toluene (1 mL) was removed and added to 5 mL scintillation fluid (Triton X). The samples were counted for 3 min. Control samples with no inhibitor were incubated simultaneously. Blank samples were obtained by incubating with boiled microsomes. In the determination of the IC50 values, linear regression analysis by the least-square method was used to produce the line of best fit; as such, we considered correlation coefficients greater than 0.9 to suggest a relationship between the inhibitory activity and log of the concentration of inhibitor. The resulting best fit equation was used to determine the IC50 value.

The compounds were hypothesized to be irreversible inhibitors.

Determination of mode of action
The irreversible inhibition was determined using the procedure described by Purohit et al (1995) using EMATE (10 μM), COUMATE (100 μM), **6** (700 μM) and **7** (700 μM). Placental microsomes (18 mg mL^{-1}, 55 μL) were incubated with each of the inhibitors (25 μL in ethanol, removed with a stream of nitrogen) in Tris-HCl buffer (50 mm, pH 7.2, 945 μL) at 37 °C for 10 min. A control tube with no inhibitor was incubated simultaneously (100% tubes). A 100-μL sample, in triplicate, was taken from each sample and tested for oestrone sulfatase activity using the procedure above, except that 900 μL Tris-HCl buffer was added to the assay tubes. A second 100-μL sample, in triplicate, was subjected to dialysis at 4 °C for 16 h, with regular changes of Tris-HCl buffer. The microsomes were then removed from the dialysis tubing and tested for oestrone sulfatase activity as described above.

Results and Discussion

Chemistry

In the synthesis of the 3- and 4-substituted phenol derivatives, a modified literature procedure (Woo et al 1998) (Figure 3) was followed and was found to proceed well and in good yield without any major problems. However, in the synthesis of the 2-substituted inhibitors, we discovered that, in general, the aminosulfonation reaction could not be undertaken easily. The lack of product was due, presumably, to intramolecular hydrogen bonding or steric factors between the substituent and the phenolic OH group. It was therefore decided not to consider the 2-derivatives of phenol. It should be noted that although the 2- and 4-nitro derivatives of EMATE (however, not their synthesis) have been reported, our attempts to synthesize the ortho-nitro or the 2,6-di-nitro derivatives were not successful. An alternative route involves the direct nitration of the phenyl sulfamate. This method, however, resulted in a mixture of compounds, none of which were found to be the nitrated sulfamate.

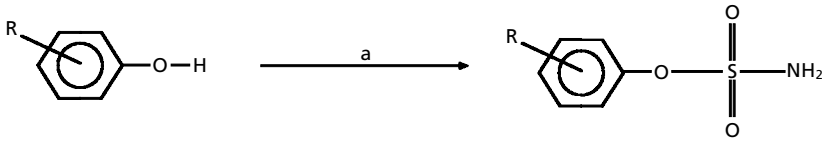

Figure 3 Synthesis of aminosulfonate derivative of the phenol based compounds (a = NaH (or anhydrous K_2CO_3)/toluene/H_2NSO_2Cl).

pK_a studies

Table 1 shows the experimental values for the pK_a of the phenols together with the reported literature values (where available). A plot of the experimental vs reported pK_a values was undertaken in an effort to check the accuracy of our technique and it was observed that the experimental values correlated well with the literature values ($r^2 = 0.99$).

Biochemical evaluation and structure–activity relationship

Table 2 shows the initial screening data together with the IC50 values obtained for the synthesized compounds. The results of the irreversible inhibition evaluation showed that the enzyme did not recover its catalytic activity on undertaking dialysis of the protein to remove any loosely bound molecules i.e. **6** and **7** were indeed irreversible inhibitors.

The analysis of the inhibitory data showed that, whilst the majority of the compounds were weak inhibitors (such as the 4-methyl- or 4-fluoro-derivatives which possessed IC50 values $> 10\,000\,\mu$M), a number possessed good inhibitory activity. For example, the 3-cyanophenyl (**6**) and 3-nitrophenyl (**7**) sulfamate compounds possessed IC50 values of 190.5 and 120 μM, respectively. Consideration of the relative potencies of these compounds in comparison with the previously reported potent inhibitors showed that they were weaker inhibitors than COUMATE and EMATE. In comparison, **6** and **7** were weaker than COUMATE by a factor of ~16 and 10, respectively.

Table 1 The experimentally determined and reported pK_a value of parent substituted phenols.

Group	Compound	Substitution	pK_a (experimental)	pK_a (literature)
H	**1**	–	9.86 ± 0.21	9.89
CH_3	**2**	3	10.0 ± 0.12	10.01
F	**3**	3	9.16 ± 0.12	NA
Cl	**4**	3	9.00 ± 0.08	8.85
Br	**5**	3	8.94 ± 0.1	NA
CN	**6**	3	8.54 ± 0.11	NA
NO_2	**7**	3	8.66 ± 0.23	8.28
CH_3	**8**	4	10.2 ± 0.07	10.17
F	**9**	4	9.84 ± 0.16	NA
Cl	**10**	4	9.36 ± 0.18	9.18
Br	**11**	4	9.29 ± 0.17	NA
CN	**12**	4	8.02 ± 0.09	NA
NO_2	**13**	4	7.3 ± 0.16	7.15

Values are mean ± s.d.; NA indicates literature data not available.

Table 2 The synthesized compounds, the initial screening inhibition data and IC50 values.

Compound	Percentage inhibition	IC50 (μM)	Relative potency
1	29.7[a] ± 2.2	>10 000	–
2	39.5[a] ± 1.2	>10 000	–
3	79.6[a] ± 9.1	2089 ± 50	174.1
4	62.0[b] ± 0.9	537 ± 21.2	44.8
5	79.2[b] ± 5.1	257 ± 6.3	21.4
6	84.3[b] ± 7.4	190.5 ± 4.3	15.9
7	90.4[b] ± 9.2	120 ± 3.9	10
8	27.4[a] ± 4.4	>10 000	–
9	37.0[a] ± 4.3	>10 000	–
10	37.0[b] ± 3.6	1584.8 ± 66.1	132.1
11	50.8[b] ± 1.7	912 ± 12.4	76
12	74.4[b] ± 6.3	300 ± 3.3	25
13	82.5[b] ± 6.7	330 ± 10.3	27.5
COUMATE	99.5[a] ± 1.9	12 ± 0.16	1
667-COUMATE	99.9[b] ± 3.3	0.21 ± 0.03	0.02
EMATE	99.8[b] ± 1.2	0.5 ± 0.01	0.04

The values shown are the means ± s.d.; [a]at inhibitor concentration of 10 000 μM; [b]at inhibitor concentration of 1000 μM; relative potency is with respect to COUMATE.

A detailed consideration of Table 2 showed that a relationship did exist between the IC50 and pK_a (of the parent phenol). The relationship between pK_a and IC50 were mathematically modelled using a quadratic relationship and the validity of this relationship was confirmed using an analysis of variance ($P < 0.05$ denoted significance). If the two series were considered separately, however, a much stronger correlation was observed between pK_a and IC50 values, with an optimum pK_a value of between 7.5–8.6. The optimum value was important since it provided us with a factor which could be used in the design of novel (and potentially more potent) inhibitors of oestrone sulfatase.

The observation that the inhibitory activity increased with a decrease in the pK_a can be rationalized through the stability of the phenoxide ion in the overall process of the hydrolysis of the sulfamate group. It was observed that electron-donating groups (such as the methyl- or amino-derivative) destabilized the phenoxide ion, whereas the electron-withdrawing groups stabilized it, and as a result, increased potency. It is interesting to note that whilst the fluoro-derivative might have been expected to possess

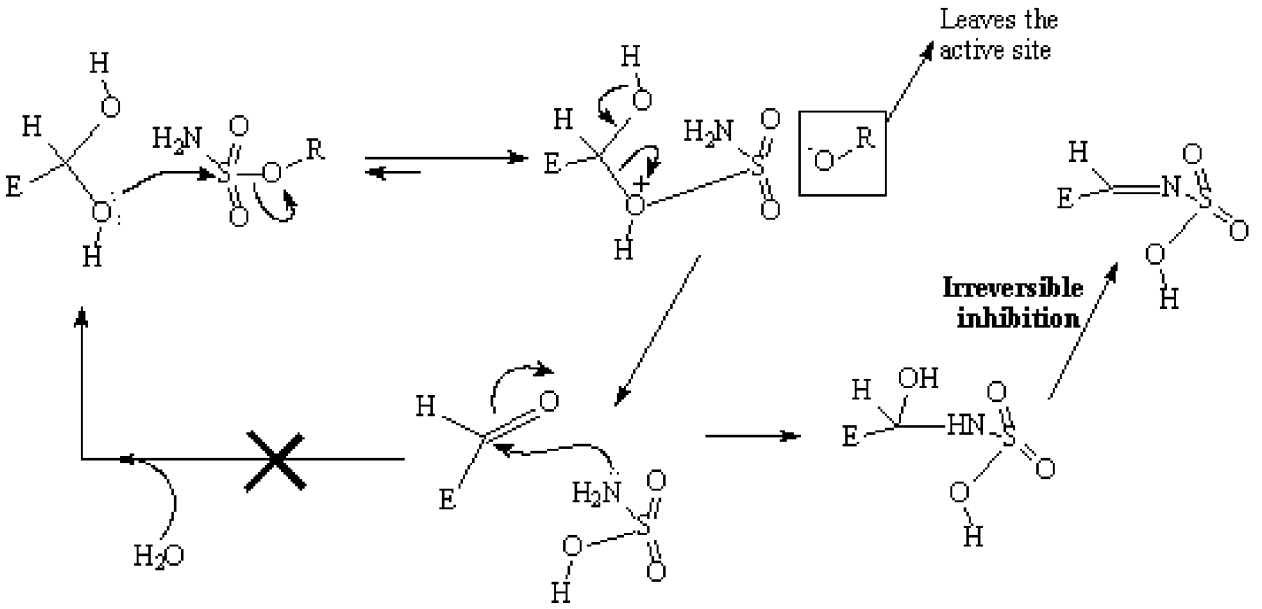

Figure 4 Proposed mechanism of irreversible inhibition of oestrone sulfatase by sulfamate based compounds involving the initial cleavage of the S—OR bond allowing the release of sulfamic acid.

good inhibitory activity (due to the high electronegativity of fluorine) these derivatives were, however, relatively poor inhibitors. On the other hand, the 4-bromo derivatives might have been expected to be poor inhibitors due to the low electronegativity of bromine (in comparison with Cl or F), however they were the most potent of the halogenated compounds. The observation that the potency of the halogen-substituted phenol followed the order Br > Cl > F can be rationalized through the consideration of the volume of space which is available for atoms such as bromine to stabilize the overall negative charge on the phenoxide oxygen. Clearly, the inhibitory activity of the cyano- and the nitro-phenol derivatives were due to the strong electron-withdrawing ability of these two groups.

The observation that inhibitory activity was related to the stability of the phenoxide ion has important implications with regards to the proposed mechanisms of oestrone sulfatase. For example, Woo et al (1996) hypothesized that hydrogen bonding between the phenoxy oxygen atom and the active site was a major factor in the weakening of the S—OAr bond. From the results of our investigation, this involvement of the hydrogen bond would appear to be inappropriate. It is the stability of the phenoxide ion which was observed to be the more important factor and which therefore suggested the cleavage of the S—OAr bond, as opposed to H-bonding. Woo et al (2000) proposed an alternative mechanism whereby the NH_2 moiety of the sulfamate group attacked the carbonyl group within the oestrone sulfatase active site. Furthermore, this mechanism did not require the cleavage of the S—OAr bond until after the irreversible production of the imine, and as such, this mechanism contradicts the results of this study. Recently, we reported (Ahmed et al 2002) an alternative mechanism (Figure 4) for the irreversible inhibition of oestrone sulfatase by sulfamate-based compounds, where we proposed that cleavage of the S—OAr bond was the crucial step to the inhibition of this enzyme and occurred before the formation of the imine product.

Conclusion

The results of the biochemical evaluation showed that a relationship existed between the irreversible inhibition of oestrone sulfatase and the pK_a of the parent phenol. That is, when the hydrolysis (sulfatase) reaction occurred, the resulting phenoxide ion (and in particular its stability) was a major factor in determining the inhibitory activity of the aminosulfonate containing inhibitors.

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