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Inhibition of Estrone Sulfatase (ES) by Derivatives of 4-[(Aminosulfonyl)oxy] Benzoic Acid

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Abstract—In our search for potent inhibitors of the enzyme estrone sulfatase (ES), we have undertaken the synthesis and biochemical evaluation of a range of straight chain alkyl esters of 4-[(aminosulfonyl)oxy] benzoic acid. The results of the study show that the synthesised compounds possess greater inhibitory activity when compared to COUMATE, although they were all found to possess lower inhibitory activity with respect to EMATE. Furthermore, the data suggest a strong correlation between logP and IC_{50} and therefore adds further support to our previous report where we suggested a link between inhibitory activity and hydrophobicity.

In the treatment of hormone dependent breast cancer, extensive research has been undertaken to produce compounds which are both potent and selective inhibitors of the cytochrome P-450 enzyme aromatase (AR).[1,2] However, the enzyme estrone sulfatase (ES) provides another source of estrogens and is responsible for the conversion of the stored (sulfated) form of the estrogens to the active (non-sulfated) form (Fig. 1).

A number of steroidal inhibitors[3–5] have been investigated as potent inhibitors of this enzyme, including estrone-3-*O*-sulfamate (EMATE)—a time and concentration dependent irreversible inhibitor. However, this compound has been shown to possess potent estrogenic properties, and as a result, the investigation into non-steroidal inhibitors has intensified. This search has resulted in the coumarin derived compound, namely, 4-methylcoumarin-7-*O*-sulfamate (COUMATE) and other derivatives, such as, 667- and 669-COUMATE (Fig. 2). In general, the aminosulfonate moiety is believed to be involved in the irreversible inhibition of ES.

We have recently shown that the requirement of the phenyl group within both the steroidal and, in particular, the non-steroidal inhibitors, is necessary for the stabilisation of the phenoxide ion[6] and that the incorporation of electron-withdrawing groups within the phenyl ring can further increase the inhibitory activity. However, the requirement of logP and the nature of the role of this physicochemical property in the inhibition of ES has yet to be fully rationalised. From the consideration of the potential mechanism for the de-sulfatation reaction catalysed by ES (Fig. 3), we proposed that the role of logP was to aid the exit of the carbon backbone from the active site—which is presumably polar in nature so as to mimic (and therefore stabilise) the sulfate group.

Here, we report the initial results of a study in an attempt to consider the contribution of logP in the overall inhibition of ES. We have therefore undertaken: the synthesis of a number of straight chain esters of 4-sulfamated benzoic acid; biochemical evaluation (including the mode of action, that is, reversible or irreversible), and; the determination of the logP and pK_a values of the esters of 4-hydroxybenzoic acid in an effort

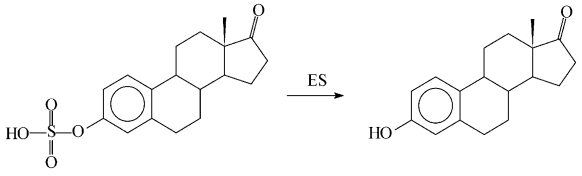

Figure 1. Action of the enzyme ES on estrone sulfate.

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to determine the effect of these two physicochemical properties on the inhibitory activity of the ester based compounds.

In the synthesis of the 4-aminosulfonated derivatives of benzoic acid, modified literature procedure[5,7] (Scheme 1) was followed and was found to proceed well and in good yield without any major problems. The syntheses of methyl 4-hydroxybenzoate[8] and methyl 4-[(aminosulfonyl)oxy]benzoate[9] are described as examples. The synthesised compounds were then evaluated for ES inhibition using standard literature method so as to determine the initial screening inhibition and IC_{50} values,[10] whilst the mode of action was determined using a method involving dialysis of bound/unbound inhibitor.[11]

In the calculation of the logP (Table 1) of the aminosulfonated compounds, we discovered that very little is known about the contribution of the sulfamate group towards the overall logP of the molecule. In an effort to simplify our logP calculations (using Projectleader™) and therefore remove any potential problems with the calculated values, we used the parent non-sulfamated compounds as a guide to the determination of the potential optimum logP.

Although the pK_a values exist for a small number of the phenols, we concluded that a more consistent approach would be the determination of the pK_a values of the parent phenols considered within our present study (Table 2). The determination of the pK_a of the starting phenols involved a spectroscopic technique[12] that considered the change in UV absorption by the phenolic group under: acidic (pH 2); pH9, and; basic (pH 11) conditions. The results of the pK_a determination are shown in Table 2 and are found to be within narrow range (between 8.0 and 8.5).

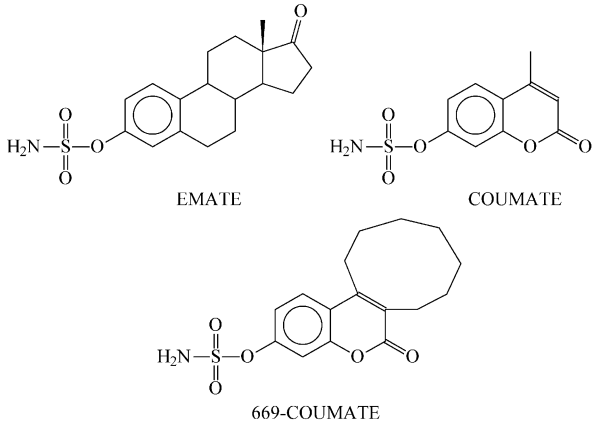


Figure 2. Structures of EMATE and two coumarin-based inhibitors.

The results of the determination of the logP, IC_{50} and pK_a values are shown in Tables 1 and 2, respectively. The compounds were also evaluated for their mode of action—it was discovered that all the synthesised compounds were irreversible inhibitors of ES. A more detailed consideration of logP and IC_{50} data from the current study shows that within a given series of compounds (where pK_a is within a narrow range), the logP of the inhibitors is an important factor in determining the inhibitory activity (Fig. 4). Within the series of compounds considered, an 'optimum' logP value is observed at about 3.8 (for the parent phenolic compound). It is interesting to note that the calculated logP of the carbon backbone of a number of the known potent inhibitors of ES is also close to the optimum observed within the current study. For example, the carbon backbones of two of the most potent inhibitors known to date [EMATE and 669-COUMATE (Fig. 2)] are calculated to possess logP values of 3.8 and 3.4, respectively.

In conclusion, the results of the present study show that logP is an important factor in determining the inhibitory activity of the sulfamate containing inhibitors of ES. Results of our previous studies have also shown that pK_a (which is related to the overall stability of the $^{-}$OR ion) is also a factor to be considered. Within the present study, we have maintained the pK_a at optimum value through the use of esters of 4-hydroxybenzoic acid as the starting compound. As a result, we have synthesised some highly potent compounds which have served as good lead compounds. As such, this is the first report to show that these two factors can be combined in the design of highly potent inhibitors of ES. Furthermore, we have synthesised, through the consideration of the physicochemical factors, some of the most potent non-steroidal compounds known to date (the most potent, **14**, being only 6.8 times weaker than EMATE, and 3.5 times more potent than COUMATE), only the tricyclic

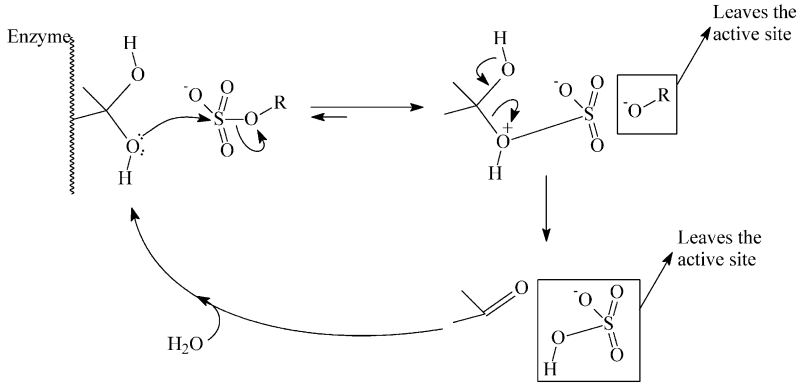
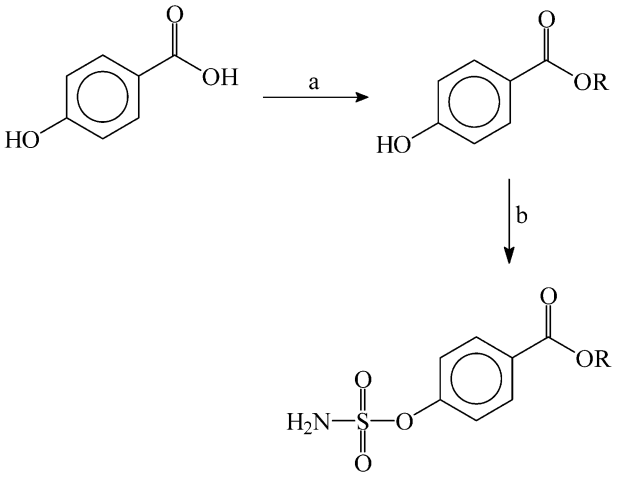


Figure 3. Mechanism of action of ES on estrone sulfate.



Scheme 1. Synthesis of the 4-sulfamate derivative of the substituted benzoic acid [a = ROH/Δ/toluene; b = NaH/H_2NSO_2Cl/toluene] (R = Me to Dec).

Table 1. Showing the synthesised compound, the calculated logP (of the non-sulfamated 4-hydroxybenzoate derivatives) and the mean IC_{50} values ($n = 3$)

Compd	R	Calculated logP	Percentage inhibition %		IC_{50}/μM
			[I] = 50 μM	[I] = 10 μM	
2	CH_3	1.49	74.7	25.4	31.6±1.23
4	C_2H_5	1.84	63.4	25.6	31.6±1.95
6	C_3H_7	2.30	76.9	42.6	13.2±0.4
8	C_4H_9	2.70	ND	48.3	10.5±0.28
10	C_5H_{11}	3.10	86.2	64.4	5.9±0.44
12	C_6H_{13}	3.49	ND	72.6	3.8±0.16
14	C_7H_{15}	3.89	83.7	69.5	3.4±0.25
16	C_8H_{17}	4.29	80.4	63.0	5.0±0.26
18	C_9H_{19}	4.68	76.2	ND	4.8±0.17
20	$C_{10}H_{21}$	5.08	ND	36.5	22.4±0.48
EMATE	—	3.80	ND	68.6%	0.5±0.001
COUMATE	—	1.60	83.1	47.6%	12±0.16

ND = not determined.

Table 2. Calculated pK_a of parent phenols of compounds

Compd	R	λ_{max} Ab (nm)	pK_a
2	CH_3	296	8.28±0.07
4	C_2H_5	296	8.22±0.09
6	C_3H_7	296	8.03±0.11
8	C_4H_9	295	8.07±0.09
10	C_5H_{11}	296	8.47±0.13
12	C_6H_{13}	296	8.52±0.15
14	C_7H_{15}	295	8.27±0.1

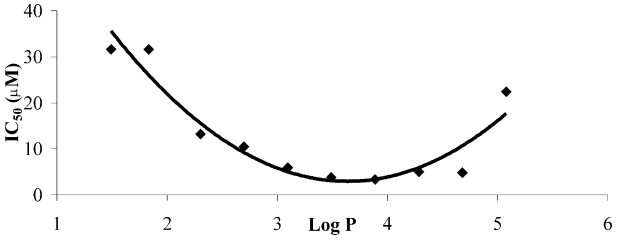


Figure 4. Plot of IC_{50} versus logP of the synthesised compounds.

derivatives of COUMATE (namely, 667- to 6612-COUMATE) are stronger inhibitors of ES.

Acknowledgements

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8. Methyl-4-hydroxybenzoate (**1**): Concd H_2SO_4 (3 mL) was added to a suspension of 4-hydroxy benzoic acid (3 g, 21.74 mmol) in methanol (20 mL) and the solution refluxed for 1 h. After cooling to room temperature, NaOH (~15 mL, pH 13) was added to neutralise the solution. The resulting mixture was allowed to stand for 15 min, before being poured into a cool beaker, and made up to 500 mL with water. The white precipitate was filtered, and dried (80 °C), to give (**1**) (3.3 g, 99.9%) as a white crystalline solid [mp 112–115 °C; R_f 0.47 diethyl ether/petroleum ether 40–60 °C (50/50)]. $\nu_{(max.)}$ (film) cm^{-1}: 3263.0 (OH) 1688.2 (C=O). δ_H ($CDCl_3$): 7.95 (2H, d, $J = 8$ Hz, ArH), 6.89 (2H, d, $J = 8$ Hz, ArH), 6.06 (1H, s, OH), 3.90 (3H, s, CH_3). δ_c ($CDCl_3$): 167.2 (C=0), 160.0, 131.8, 122.3, 115.1 (CAr), 52.0 (CH_3). GCMS t_R 9.176 m/z 152 (M^+).
9. Methyl 4-[(aminosulfonyl)oxy]benzoate (**2**): Sodium hydride (NaH) (60% dispersion in mineral oil, 0.16 g, 4 mmol) was added to a stirred solution of (**1**) (0.5 g, 3.29 mmol) in dimethyl formamide (DMF) (20 mL) under an atmosphere of nitrogen gas at 0 °C. After evolution of hydrogen had ceased (after 30 min), 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 (**2**) (0.24 g, 31.6%) as a pure white solid [mp 118–121 °C; R_f 0.24 diethyl ether/petroleum ether 40–60 °C (50/50)]. $\nu_{(max.)}$ (film) cm^{-1}: 3376.1, 3274.0 (NH_2), 1704.3 (C=O), 1376.7, 1156.9 (S=O). δ_H ($CDCl_3$): 8.08 (2H, d, $J = 9$ Hz, ArH), 7.41(2H, d, $J = 9$ Hz, ArH), 5.10 (2H, s, NH_2), 3.93 (3H, s, H_3C–). δ_c ($CDCl_3$): 165.2 (C=O), 154.0, 149.8, 131.6, and 121.9 (CAr), 52.4 (CH_3). MS m/z found: M^+ 231.0198, $(C_8H_9NO_5S)^+$ requires 231.0201.
10. ES assay: The total assay volume was 1 mL. ^{3}H-estrone sulfate (25 μL, 20 μM/tube; 300,000 dpm) and the inhibitors (50 μM/tube) 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 were then diluted with Tris–HCl buffer (115 μg/mL). The microsomes and assay tubes were pre-incubated for 5 min at 37 °C in a shaking water bath prior to the addition of the microsomes (0.8 mL) to the tubes. After 20 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 rpm, 10 min). 1 mL of toluene was removed and added to 5 mL scintillation cocktail (TRITONX). The aliquots were counted for 3 min. All samples were run in triplicate. Control samples with no inhibitor were incubated simultaneously. Blank samples were obtained by incubating with boiled microsomes. It should be noted that EMATE and COUMATE were synthesised within our laboratories using published procedures and used within our assays as the standard compounds.

11. Irreversible ES assay: The irreversible inhibition was determined using EMATE (10 μM), COUMATE (100 μM), **12** (700 μM) and **14** (700 μM). Placental microsomes (18 mg/mL, 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). An aliquot (100 μL) in triplicate, was taken from each sample and tested for ES activity using the procedure above, except that 900 μL of Tris–HCl buffer was added to the assay tubes. A second aliquot (100 μL) 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 ES activity as described above.

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