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The Mechanism of the Irreversible Inhibition of Estrone Sulfatase (ES) Through the Consideration of a Range of Methane- and Amino-Sulfonate-Based Compounds

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Abstract—We report the results of our study into a series of simple phenyl and alkyl sulfamates and alkyl methanesulfonates as potential inhibitors of the enzyme estrone sulfatase (ES). The results of the study show that the substituted phenyl sulfamates are good irreversible inhibitors; the alkyl sulfamate compounds were found to lack inhibitory activity; whilst the large alkyl chain containing methanesulfonate-based compounds were found to possess weak reversible inhibitory activity. Using the results of the inhibition study, we postulate the probable mechanism for ES and suggest that an attack by the gem-diol is a major requirement *prior* to the hydrolysis of the sulfamate group, *following* which, attack on the active site C=O occurs and which therefore leads to the production of an imine type functionality, resulting in irreversible inhibition.

The enzyme estrone sulfatase (ES) converts the stored (sulfated) form of estrogens to the active (non-sulfated) form, thereby allowing the stimulation of estrogen-dependent tumours via a non-aromatase (AR) pathway (which is therefore not blocked by AR inhibitors). A number of steroidal and non-steroidal inhibitors[1,2] has been investigated as potent inhibitors of this enzyme, including estrone-3-*O*-sulfamate (EMATE) (a time- and concentration-dependent irreversible steroidal inhibitor with estrogenic properties) and coumarin-7-*O*-sulfamate (COUMATE) (also a time- and concentration-dependent irreversible non-steroidal inhibitor but lacking estrogenic properties)—both compounds, along with other potent inhibitors, contain a sulfamate moiety which is believed to be involved in the irreversible inhibition of ES (Fig. 1).

From the results of our molecular modelling studies[3,4] and a review of potential mechanisms for ES, we designed a number of substituted phenyl- and alkyl-sulfamates. We argued that if the recently proposed mechanism[5] was correct, a wide range of phenyl- and alkyl-sulfamated compounds would be expected to possess inhibitory activity involving the attack of the carbonyl group at the active site of ES by the NH_2 moiety of the sulfamate-based compounds. From our study on pK_a,[6] however, we concluded that the hydrolysis of the S–OR bond was an important step in the inhibition process (Fig. 2). This contradicted the recently proposed mechanism by Woo et al.[5] (2000) (where the authors suggested that the cleavage of the carbon backbone occurred *after* the imine production).

The consequence of the conclusion from our earlier study is, therefore, that if the hydrolysis of the sulfamic

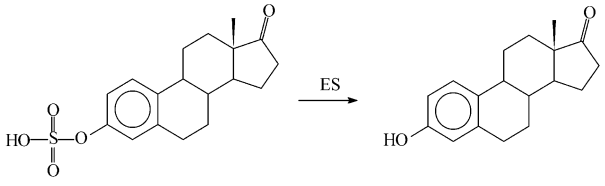

Figure 1. Reaction catalysed by ES.

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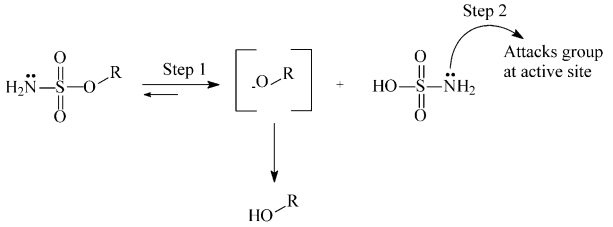


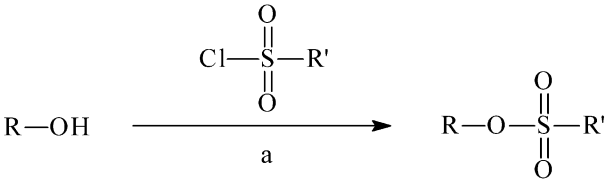
Figure 2. Hydrolysis of phenyl aminosulfonate containing compounds.

moiety (step 1, Fig. 2) occurs *prior* to the irreversible inhibiting step (step 2, Fig. 2), then the phenyl sulfamate based compounds should possess good inhibitory activity whilst the alkyl sulfamates would be expected to lack inhibitory activity (since the phenoxide ion would be expected to be more stable than the alkoxide anion thereby favouring the hydrolysis reaction). However, if the proposed mechanism of Woo et al.[5] was correct, then *all* sulfamate-based compounds would be expected to possess inhibitory activity since they all contain the NH_2 moiety using which they would be able to undertake attack of the C=O group in the formylglycine (FGly 69) residue. We therefore argued that the synthesis of a range of phenyl- and alkyl-sulfamated compounds would allow us to resolve the problem and to determine whether the hydrolysis of the S–OR bond plays any part in the inhibitory activity of compounds as irreversible inhibitors of ES.

Here, we report the synthesis and biochemical evaluation of a range of alkyl- and phenyl-sulfamates and a small range of methanesulfonate-based compounds of straight chain alkyl alcohols. From the consideration of the structure–activity relationship (SAR) determination of these compounds, we suggest an alternative and novel mechanism for inhibition of ES, a mechanism that can be used in the rationalisation of a wide range of irreversible *and* reversible inhibitors.

In the synthesis of the 4-aminosulfonated derivatives of 4-hydroxy phenol and alkyl alcohols, modified literature procedure[3,5] (Scheme 1) was followed and was found to proceed well and in good yield without any major problems. The synthesis of 2,2,2-trichloroethyl sulfamate[7] and 4-bromophenyl sulfamate[8] are given as examples.

The results of the biochemical evaluation[9–11] (Table 1) shows that, in general, all of the compounds considered within the present study are less potent than COUMATE and EMATE—it should be noted that the aim of the present study *was not* the synthesis of highly



Scheme 1. Synthesis of the phenyl and alkyl-sulfamates (R = alkyl alcohol or phenol; R′ = Me, NH_2, CH_3–Ph; a = NaH or K_2CO_3/toluene).

potent inhibitors but to evaluate the inhibitory activity of the alkyl- and phenyl-sulfamates in an attempt to gain further insight into the mechanism of inhibition by sulfamate based compounds. Detailed consideration of the inhibitory activity suggests that a marked difference is observed between the phenyl- and alkyl-sulfamate based compounds. That is, the former is found to possess greater inhibitory activity compared to the alkyl sulfamates, the latter being, in general, non-inhibitors (Table 1). A trend is also observed within the alkyl sulfamates, however, where α-substituted compounds such as **1** are found to be more potent than non-α-substituted compounds. Consideration of the pK_a of the parent alcohols of the α-substituted alkyl alcohols and phenols shows that these compounds possess a lower pK_a value than the non-substituted alkyl alcohols—the latter would therefore be expected to produce a less stable RO^- ion when compared to the α-substituted alcohols or indeed the phenols. The investigation into the mode of action[10,11] of all of the inhibitors which were initially observed to possess some inhibition, shows that the sulfamate-based inhibitors (both phenolic and alkyl alcohol-based compounds) are irreversible, that is, *the enzyme did not recover activity after incubation with the synthesised sulfamate (alkyl or phenyl)-based compounds*, whereas the methanesulfonate-based compounds, although very weak inhibitors, are found to be reversible in nature. This observation demonstrates that the sulfamate-based compounds that show a degree of inhibition do so through an irreversible interaction with the active site.

Table 1. Showing the initial screening inhibition data and IC_{50} values for a range of the synthesised compounds

Compd	R	R′	Percentage inhibition	IC_{50} (μmoles/litre)
1	Cl_3C–CH_2	NH_2	60.0[b]	750
2	Cl_2HC–CH_2	NH_2	30.0[b]	ND
3	ClH_2C–CH_2	NH_2	15.0[b]	ND
4	CH_3-$(CH_2)_5$–CH_2–	NH_2	0[a]	ND
5	CH_3-$(CH_2)_6$–CH_2–	NH_2	0[a]	ND
6	CH_3-$(CH_2)_7$–CH_2–	NH_2	0[a]	ND
7	CH_3-$(CH_2)_7$–CH_2–	CH_3	28.0[b]	ND
8	CH_3-$(CH_2)_{10}$–CH_2–	CH_3	17.0[b]	ND
9	Phenyl	NH_2	29.7[b]	> 10,000
10	4-Methylphenyl	NH_2	27.4[b]	> 10,000
11	3-Methylphenyl	NH_2	39.5[b]	2089 ± 50
12	4-Fluorophenyl	NH_2	37.0[b]	537 ± 21.2
13	3-Fluorophenyl	NH_2	79.6[b]	2089 ± 50.0
14	4-Chlorophenyl	NH_2	37.6[b]	1585 ± 66.1
15	3-Chlorophenyl	NH_2	62.0[b]	537 ± 21.2
16	4-Bromophenyl	NH_2	50.8[b]	912 ± 12.4
17	3-Bromophenyl	NH_2	75.1[b]	257 ± 6.3
18	4-Iodophenyl	NH_2	66.0[b]	560 ± 16.2
19	3-Iodophenyl	NH_2	89.4[b]	120 ± 1.2
20	4-Cyanophenyl	NH_2	74.4[b]	300 ± 3.3
21	3-Cyanophenyl	NH_2	84.3[b]	191 ± 4.3
22	4-Nitrophenyl	NH_2	82.5[b]	330 ± 10.3
23	3-Nitrophenyl	NH_2	90.4[b]	120 ± 3.9
COUMATE	4-Methyl coumarin	NH_2	99.5[b]	12 ± 0.16
EMATE	Estrone	NH_2	99.8[b]	0.5 ± 0.001

[a]At inhibitor concentration of 10 mM.
[b]At inhibitor concentration of 1 mM.
ND, not determined.

As previously mentioned, the mechanism of Woo et al.[5] (2000) suggests that all sulfamated compounds (both alkyl and phenyl) would be expected to possess inhibitory activity resulting from the attack of the C=O group within the active site by the NH_2 moiety of the sulfamate group. However, consideration of the results shows that the non-α-substituted alkyl sulfamate-based compounds possessed no inhibitory activity whatsoever, even up to concentrations of inhibitor exceeding 10 mmol/L. We can therefore only conclude that the proposed attack of the aldehydic group by the NH_2 group, as the first step of the mechanism, *does not occur*. It may be argued that the larger alkyl chains may be involved in some steric interaction which may result in weak (or a lowering of) inhibitory activity, however, we have recently shown good inhibitory activity in similar long alkyl chain containing phenolic compounds,[12] as such, we propose that the steric factor is not of great significance.

The consideration of the inhibitory activity and SAR data for a wide range of alkyl- and phenyl-sulfamate-based compounds, and more importantly the non-inhibitors, suggests that the initial step in the inhibition of ES by sulfamate based compounds is indeed the cleavage of the S–OR bond and not, as suggested by Woo et al.,[5] the attack of the C=O group by the NH_2 moiety of the sulfamate group (Fig. 2). We therefore propose that the order of events involves the cleavage of the S–OR bond, resulting in the formation of the sulfamate moiety as well as the RO^- ion (which is therefore related to the pK_a of the parent ROH, as such, phenol and α-substituted alkyl alcohol-based sulfamated compounds possess inhibitory activity). The production of the sulfamic acid moiety is *then* followed by the attack of the aldehydic group by the NH_2 of sulfamic acid resulting in the formation of an imine type structure (with the loss of a molecule of water) resulting in the irreversible inhibition of ES.

In conclusion, through the consideration of the inhibition data of a wide range of compounds, we have rationalised the potent (as well as the lack of) inhibitory activity within a range of sulfamate and methanesulfonate-based inhibitors of ES, thereby allowing a new mechanism for the irreversible inhibition of ES by sulfamate containing compounds to be proposed.

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7. **Synthesis of *2,2,2-trichloroethyl sulfamate* (1)**. Potassium carbonate (0.58 g, 4.20 mmol, 2 equiv) was added to a stirred solution of trichloroethanol (0.40 g, 4.2 mmol) in toluene (20 mL) under nitrogen, and warmed to 40 °C. Aminosulfonyl chloride in toluene (20 mL, ~20 mmol) was then added, and the reaction allowed to stir for 4 days at room temperature. The reaction was 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 produced **8** as an orange solid. Mp 46.5–49.2 °C (Yield 10%). $\nu_{(max)}$ (Film) cm^{-1}: 3397.5 and 3294.4 (NH), 1371.8 and 1189.1 (S=O). 300 MHz δ_H ($CDCl_3$) 5.45 (2H, s, NH_2) 4.46 (2H, s, CH_2–). δ_C ($CDCl_3$) 93.1567 (–$\underline{C}Cl_3$), 78.6485 (–$\underline{C}H_2CCl_3$).
8. **Synthesis of *4-bromophenyl sulfamate* (16)**. Compound **16** was synthesised following the same procedures as for compound **1** except that NaH (60% dispersion in mineral oil, 0.2 g, 5.00 mmol) was added to a stirred solution of 4-bromophenol (0.5 g, 2.89 mmol) in DMF (10 mL). Aminosulphonyl 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 (**16**) (0.29 g, 39.8%) as a white solid [mp 113–116 °C; R_f 0.37 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 ($CDCl_3$): 7.56 (2H, d, J=9 Hz, ArH), 7.19 (2H, d, J=9 Hz, ArH), 5.02 (2H, s, NH_2). δ_c ($CDCl_3$): 150.6, 133.4, 125.1, 120.1 (C–Ar). MS m/z found: MH^+ 250.9254, $(BrC_6H_6NO_3S)H^+$ requires 250.9252.
9. ***ES assay***. The total assay volume was 1 mL. ^{3}H-Estrone sulfate (25 μL, 50 μM/tube; 750,000 dpm) and the inhibitors (various concentrations) 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.
10. ***Irreversible ES assay***. The irreversible inhibition was determined using the procedure described by Purohit et al. (1998)[14] using EMATE (10 μM), COUMATE (100 μM) and sulfamated phenyl ketones (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.

11. ***Time and concentration dependency assay***. An experiment was conducted to determine whether the active compounds were inhibiting estrone sulfatase in a time and concentration dependent manner. Dilutions of each compound (in ethanol) were placed in 5 mL assay tubes and the ethanol removed with a stream of nitrogen. Tris–HCl buffer (50 mM, pH 7.2, 0.2 mL) was added and the tubes warmed in a shaking water bath (37 °C, 5 min). At the appointed time the microsomes were added (800 μL, 192 μg), and the assay tubes incubated for between 0 and 60 min. After incubation, dextran coated charcoal (1% w/v in Tris–HCl buffer, 500 μL) was added and the tubes allowed to shake at 37°C for a further 10 min. After this time the tubes were immediately centrifuged (1000 rpm, 3 min), and a portion of the supernatant (750 μL) added to pre-incubated 10 mL assay tubes containing estrone sulfate (25 μL (ethanol removed) to give final concentration 50 μM/tube, 750,000 dpm/tube) and Tris–HCl buffer (50 mM, pH 7.2, 250 μL). After 30 min of incubation at 37 °C, the assay tubes were quenched by the addition of toluene (4 mL) and placed on ice. Each tube was vortexed for 45 s, and centrifuged (3000 rpm) for 10 min. Aliquots (1 mL) of each toluene layer were added to Cocktail T (5 mL) and counted by liquid scintillation.

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