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Hydrophobicity, a Physicochemical Factor in the Inhibition of the Enzyme Estrone Sulfatase (ES)

Sabbir Ahmed,* Karen James, Caroline P. Owen, Chirag K. Patel and Mijal Patel

School of Chemical and Pharmaceutical Sciences, Kingston University, Penrhyn Road, Kingston upon Thames, Surrey, KT1 2EE, UK

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Abstract—We report the initial structure–activity relationship study (SAR) (in particular log P) of a series of compounds based upon 4-sulfamated phenyl ketones as potent inhibitors of the enzyme estrone sulfatase (ES). The results of the study show that these compounds are irreversible inhibitors of ES and that they are more potent than COUMATE, but weaker than EMATE. Analysis of the SAR data shows a strong correlation between IC_{50} and log P but also supports our previous study, which suggests a very strong relationship between pK_a and IC_{50}.

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 use of AR inhibitors does not result in the inhibition of all of the biosynthetic processes which lead to estrogen formation. That is, the enzyme estrone sulfatase (ES) converts the stored (sulfated) form of the estrogens to the active (non-sulfated) form (Fig. 1), thereby allowing the stimulation of tumours via a non-AR pathway (which is not blocked by AR inhibitors).

A number of steroidal inhibitors[3,4] has been investigated as potent inhibitors of this enzyme, including estrone-3-sulfamate (EMATE) (a time- and concentration-dependent irreversible steroidal inhibitor) and the 4-methylcoumarin-7-*O*-sulfamate derivative (COUMATE) (an irreversible non-steroidal inhibitor) (Fig. 2). However, since EMATE has been shown to possess potent estrogenic properties, the investigation into non-steroidal inhibitors has intensified.

In general, the potent inhibitors contain an aminosulfonate moiety which is believed to be involved in the irreversible inhibition of ES. From the consideration of the results obtained with the known sulfamate containing steroidal and non-steroidal inhibitors, a 'definitive model' was proposed where it was suggested that the most fundamental and basic requirements for inhibition were the phenolic ring, and a bridging oxygen atom joining the phenyl ring to the sulfamate group.[5] We have recently shown that the stabilisation of the phenoxide ion[6] and the incorporation of electron-withdrawing groups within the phenyl ring can result in a significant increase in the inhibitory activity of compounds, thus alkyl sulfamates, being poorer acids (in comparison to phenols), are weaker inhibitors of ES.[6]

In an effort to overcome the lack of detailed information regarding the active site of ES, probe the nature of

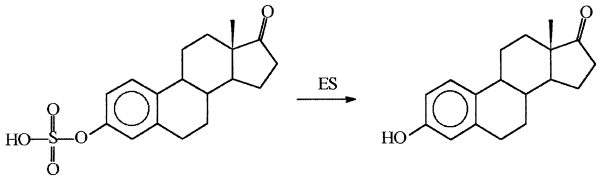
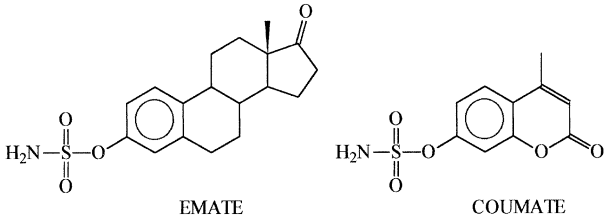


Figure 1. Action of the enzyme ES on estrone sulfate.



EMATE COUMATE

Figure 2. Structures of EMATE and COUMATE.

*Corresponding author. Tel.: +44-208-547-2000; fax: +44-208-547-7562; e-mail: ch_s534@kingston.ac.uk

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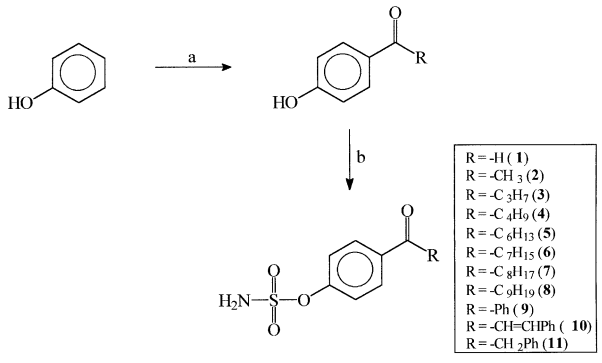
the proposed pharmacophore and rationalise the inhibitory activity of the aminosulfonate based compounds, we initiated a series of structure–activity relationship (SAR) determination studies. From the results of our initial molecular modelling study and a consideration of potential mechanisms for ES, we concluded that log P may also be an important factor in the inhibition process. That is, we concluded that the role of the carbon backbone is to favour the formation of the RO^- ion (hence the requirement for the aromatic moiety) *and* the expulsion of the RO^- due to the high log P of the carbon backbone within RO^-. We also hypothesised that as a result of the high log P requirement, the reaction catalysed by ES 'appears' to be an irreversible reaction.

In order to verify our hypothesis with respect to the importance of log P, we undertook a design process so as to incorporate the increasing log P, whilst restricting the pK_a of the parent phenol, and we predicted that sulfamated phenyl ketones would possess the appropriate characteristics. Here, we report the initial results of our study where we have undertaken: the synthesis of derivatives of 4-sulfamated phenyl ketones; the in vitro biochemical evaluation of the synthesised compounds; and the evaluation of the mode of action, that is reversible or irreversible inhibition.

Chemistry

In the synthesis of the 4-aminosulfonated derivatives of 4-hydroxyphenyl ketones, a modified literature procedure[7–9] (Scheme 1) was followed and was found to proceed well and in good yield without any major problems. The synthesis of 4-nonanoylphenyl sulfamate is given as an example[10]—it should be noted that since 4-hydroxynonanophenone is commercially available, it was not synthesised via the initial Friedel–Crafts acylation reaction.

The results of the biochemical evaluation[11] of the synthesised sulfamated phenyl ketones (as well as EMATE and COUMATE within our assays for comparison) are shown in Table 1 together with the relative potencies against the latter two compounds. Consideration of the results shows that these compounds are potent inhibitors of ES, with compound **7** being only 6.8 times weaker than EMATE. In comparison to COUMATE, we observe that a number of the synthesised compounds are equipotent or are stronger inhibitors than COUMATE; indeed compounds **5**, **6** and **7** are 2.4, 2.1 and 3.5 times more potent, respectively. These three compounds would therefore appear to be some of the most potent non-steroidal compounds known *to date*. The synthesised compounds were further evaluated to determine their mode of action.[12] It was discovered that the enzyme did not recover after the incubation with the synthesised compounds, that is the compounds are irreversible inhibitors of ES (Fig. 3).



Scheme 1. Synthesis of the 4-sulfamate derivative of the substituted benzoic acid [a = acid chloride/$AlCl_3$/DCM; b = NaH/H_2NSO_2Cl/toluene].

From the consideration of the initial SAR, the data from the biochemical evaluation of these inhibitors suggest that log P is indeed a factor in the inhibitory activity of compounds against ES—this is therefore the first report to show a high level of correlation between the inhibitory activity and the log P of the parent phenol (Fig. 4). Furthermore, the data suggest that there is an apparent optimum log P and thus there is alkyl chain length limit (between 6 and 8) beyond which the potency of the inhibitors decreases. The decrease in inhibitory activity with further increase in alkyl chain length may be as a result of: steric interaction between the alkyl chain and a part of the enzyme active site, thereby resulting in the destabilisation of the enzyme–inhibitor complex; or the log P of the overall sulfamated compound being too large, that is the high log P of the sulfamated compound disfavours the entry of the sulfamated compound into the ES active site, thereby lowering the inhibitory activity. That the hypothesis regarding the size of compound **8** may be a potential factor in the decreasing inhibitory activity can be observed when the compounds under consideration are superimposed onto estrone (Fig. 5). It is found that the overall length of compounds, such as **8**, exceeds that of estrone, resulting in a potential increase in interactions between parts of the active site and the alkyl chain. Whilst the steric factor may be important, we strongly believe that within the range of compounds synthesised, it is the greater hydrophobicity of **8**, in comparison to the lower alkyl chain containing inhibitors, which results in reduced inhibitory activity, that is the high hydrophobicity of this inhibitor disfavours its entry into the active site.

Compounds **9** and **10** appear to possess unusually weak inhibitory activity compared to, for example, compounds **4** and **5**, although all four inhibitors possess similar log P values. We believe that whilst the hydrophobicity factor is an important one, the pK_a factor is of even greater importance and highlights our previous hypotheses[6] regarding the stability of the phenoxide ion resulting from the hydrolysis of the sulfamate group. That is, within compounds **9** and **10**, the electron withdrawing C=O group attached directly to the sulfamated phenyl ring is connected to an electron-rich phenyl ring. In the case of **10**, this involves the conjugation of the overall π system. We suggest that the ability of the C=O group to attract electrons from a 'second' and alternative source of electrons lowers the stability of the phenoxide ion in comparison to compounds such as **8**.

Table 1. Inhibition data for compounds **1**–**11** and the relative potencies of some of the synthesised compounds in comparison to EMATE and COUMATE

Compound	R	IC_{50} (μM)	Potency wrt EMATE	Potency wrt COUMATE	Calculated log P of parent phenol
1	H	254±10.1	0.0019	0.0472	1.4
2	CH_3	302±6.7	0.0016	0.0397	1.1
3	C_3H_7	39.8±1.4	0.0126	0.3015	2.1
4	C_4H_9	20.9±0.38	0.0239	0.5742	2.5
5	C_6H_{13}	5.0±0.36	0.1000	2.4000	3.3
6	C_7H_{15}	5.6±0.19	0.0893	2.1429	3.7
7	C_8H_{17}	3.4±0.13	0.1471	3.5294	4.1
8	C_9H_{19}	13±0.05	0.0385	0.9231	4.4
9	Ph	63±1.83	0.0079	0.1905	2.9
10	CH=CHPh	263±5.5	0.0019	0.0456	3.4
11	CH_2Ph	33±1.1	0.0152	0.3636	2.9
EMATE	—	0.5±0.001	1	24	3.9
COUMATE	—	12±0.16	0.0417	1	1.7

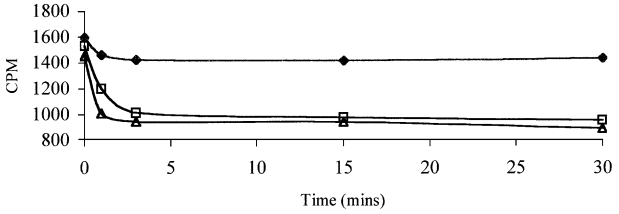


Figure 3. Plot of counts per min (CPM) versus time to show the time- and concentration-dependent inhibition of ES by compound **5**: 0 μmol/L (◆), 2 μmol/L (□) and 20 μmol/L (△) concentrations.

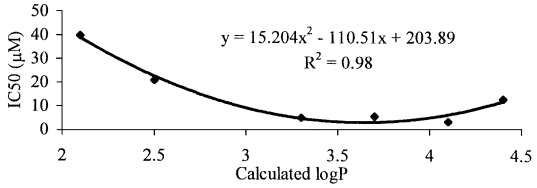


Figure 4. Plot of IC_{50} versus calculated log P of parent phenol.

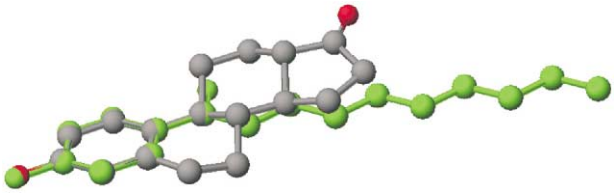


Figure 5. Superimpositioning of compound **8** onto estrone to show the length of the alkyl chain.

That our hypothesis may be valid is supported by the greatly increased inhibitory activity observed in compound **11** (compared to **9** and **10**), where the two π systems are now separated by a CH_2 spacer group which discontinues the conjugation, thereby allowing the C=O to withdraw electrons *solely* from the sulfamated phenyl ring system. However, the slightly weaker inhibitory activity of compound **11** compared to compound **4** suggests that a steric factor may also be involved.

In conclusion, we have synthesised a range of sulfamated phenyl ketones, which have proved to be some of the most potent non-steroidal compounds known to date; only the recently reported 667-COUMATE is known to be of greater potency. The compounds are therefore good lead compounds in the search for more potent non-steroidal inhibitors and have allowed us further insight into the structural features required for increased inhibitory activity.

References and Notes

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10. Chemistry: 4-Nonanoylphenyl sulfamate (**7**): Sodium hydride (NaH) (60% dispersion in mineral oil, 0.18 g, 4.5 mmol) was added to a stirred solution of 4-hydroxynonophenone (1 g, 4.27 mmol) in dimethyl formamide (DMF) (10 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 solidified on addition of water. The crude product was purified using flash chromatography to give **7** (0.32 g, 23.9%) as a white solid [mp 102–104 °C; R_f 0.57 ether/petroleum ether 40–60 °C (70/30)]. $\nu_{(max)}$ (Film) cm^{-1}: 3389.0, 3289.0 (NH_2), 1682.3 (C=O), 1377.9, 1181.8 (S=O). δ_H ($CDCl_3$): 7.99 (2H, d, J=9 Hz, ArH), 7.42 (2H, d, J=9 Hz, ArH), 5.17 (2H, s, NH_2), 2.94 (2H, t, J=7 Hz, $COCH_2CH_2$), 1.72 (2H, m, $COCH_2CH_2CH_2$), 1.35 (10H, m, $COCH_2CH_2[CH_2]_5CH_3$),

0.88 (3H, t, $J=7$ Hz, $-CH_3$). δ_C (acetone-d_6): 154.6 (C=O), 138.3, 136.1, 130.4, 122.8 (C–Ar) 38.8, 32.3, 29.7, 23.0 (CH_2), 14.1 (CH_3). MS m/z obtained MH^+ 314.1422, $(C_{15}H_{23}NO_4S)H^+$ requires 314.1426.

11. ES assay: In the biochemical evaluation, the standard literature method was used.[8] 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.

12. Irreversible ES assay: The irreversible inhibition was determined using the procedure described by Purohit et al. (1998)[9] 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.