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Acid Dissociation Constant, a Potential Physicochemical Factor in the Inhibition of the Enzyme Estrone Sulfatase (ES)

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Abstract—We report the initial results of the synthesis and biochemical evaluation of a series of aminosulfonate based compounds of phenol and the determination of the pK_a of the parent phenol in an attempt to investigate the role of this physicochemical factor in the irreversible inhibition of the enzyme estrone sulfatase (ES). The results of the study show that there is a strong correlation between the observed pK_a and inhibitory activity. We postulate that the stability of the phenoxide ion, as indicated by the acid dissociation constant, is an important factor in the irreversible inhibition of this enzyme. © 2001 Published by Elsevier Science Ltd.

Introduction

The enzyme estrone sulfatase (ES) converts the stored (sulfated) form of the estrogens to the active forms and has recently become a major therapeutic target against hormone dependent breast cancer. A number of steroidal inhibitors[1–3] have been investigated as potent inhibitors of this enzyme, including estrone-3-*O*-sulfamate (EMATE). However, this compound has been shown to possess potent estrogenic properties, and as a result, the investigation of non-steroidal inhibitors has intensified. The potent inhibitors, in general, contain an aminosulfonate moiety which is 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[4] 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 was required for strong hydrogen bonding to the active site with the result that the O–S bond of the amino sulfonate group was weakened.

In an effort to overcome the lack of detailed information regarding the active site and to probe the nature of the proposed pharmacophore, we initiated a series of structure–activity relationship (SAR) determination studies. Initially, we undertook the determination of: the transition state of the desulfatation reaction (Fig. 1) and the charge density of EMATE and its derivatives (2-nitro-, 4-nitro- and 2,4-dinitro-). Whilst the potential transition state allowed us to design potentially new compounds (involving the superimpositioning of potential inhibitors onto the derived transition state), the calculation of the electron density and partial charges (the electron density isosurface was generated by an extended Hückel wavefunction, using a geometry determined by mechanics optimization using augmented MM2 parameters) allowed us to consider the previously proposed mechanisms for ES and thus the role of the phenoxide ion (and therefore the involvement of pK_a) as a potential factor in the inhibition process. That is, the results of the charge density calculation and partial charge calculations showed that the sulfur atom of the sulfamate group is susceptible to nucleophilic attack. From this we concluded that the phenoxide ion stability may be an important factor in aminosulfonate containing inhibitors (Fig. 2).

In an effort to verify our conclusion with respect to the phenoxide ion, we designed and synthesized a number of *o*-, *m*- and *p*-substituted phenol derivatives containing the aminosulfonate group. Here, we report the initial results of the synthesis, biochemical evaluation,

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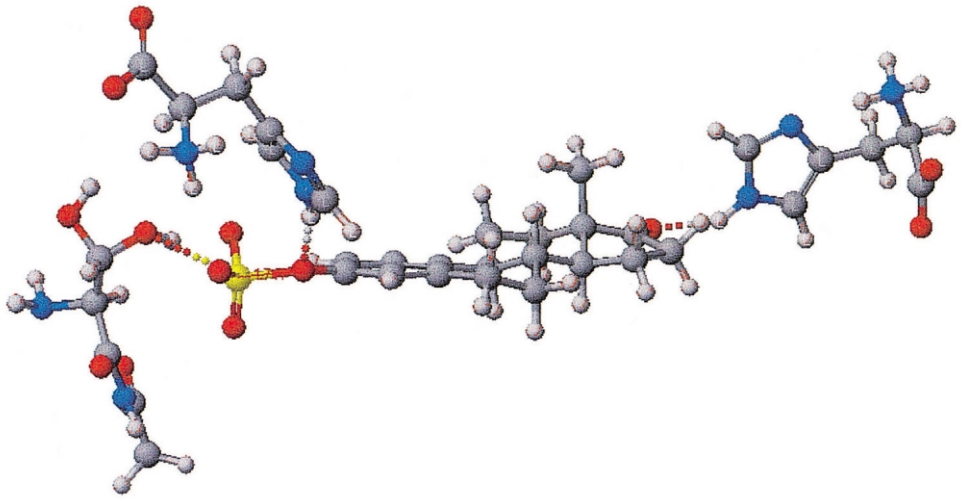


Figure 1. The transition-state for the desulfatation reaction catalysed by ES.

pK_a determination and the SAR determination study for these compounds.

In the synthesis of the 3- and 4-substituted phenol derivatives, a modified literature procedure[5–7] (Scheme 1) was followed and was found to proceed well and in good yield without any major problems—the synthetic procedures for the phenyl sulfamate (**1**) is outlined below as an example.[9] In the synthesis of the 2-substituted sulfamate compounds, a number of compounds proved difficult to synthesize (presumably due to steric hindrance or intramolecular hydrogen bonding) and as a result the 2-substituted compounds were not produced.

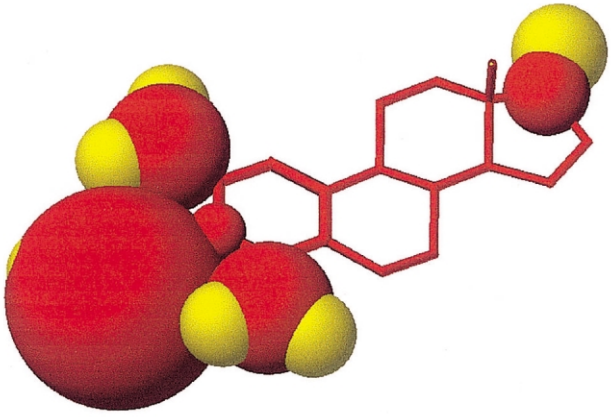
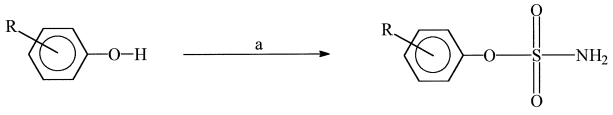


Figure 2. To show partial charges for 2,4-dinitroEMATE.

Although the pK_a values exist for a small number of the phenols, we concluded that a better, more consistent approach would be the determination of the pK_a values of all of the parent phenols considered within our present study. The determination of the pK_a of the starting phenols involved a spectroscopic technique[8] which considered the change in UV absorption by the phenolic group under acidic, pH 9, and basic conditions.



Scheme 1. Synthesis of aminosulfonate derivatives of the phenol based compounds [a = NaH (or anhydrous K_2CO_3)/toluene/H_2NSO_2Cl].

Table 1. Showing the synthesized compound and the determined pK_a, IC_{50} values and relative potency as compared to COUMATE

Compound number	Group	Substitution	pK_a	IC_{50}/μM	Relative potency
1	H	—	9.98	>10,000	—
2	CH_3	3	10	>10,000	—
3	F	3	9.16	2089	174
4	Cl	3	9	537	45
5	Br	3	8.95	257	21
6	CN	3	8.54	190.5	16
7	NO_2	3	8.28	120	10
8	CH_3	4	10.2	>10,000	—
9	F	4	9.8	>10,000	—
10	Cl	4	9.5	1584.8	132
11	Br	4	9.29	912	76
12	CN	4	8.02	300	25
13	NO_2	4	7.15	330	27.5
	COUMATE	—	—	12	1
	EMATE	—	—	0.1	0.008

The results of the pK_a determination and biochemical evaluation are shown in Table 1. In an effort to consider the inhibitory activity of the compounds under consideration, we undertook the evaluation of two of the known potent inhibitors against ES, namely, EMATE and coumarin-7-*O*-sulfamate (COUMATE). From the IC_{50} values, we can clearly see that a large range of inhibitory activity exists against ES, from compounds which are clearly non-inhibitors (e.g., the 3- and 4-methyl derivatives), to compounds (3-nitrophenyl sulfamate) which are only 10 times weaker than the most potent non-steroidal compound COUMATE (which possesses an IC_{50} of 12 μM). All the synthesized inhibitors were observed to possess irreversible inhibition (the method used in irreversible inhibition determination is outlined within the References and Notes).

From the consideration of the overall results for both 3- and 4-substituted phenols, it can be observed that a relationship exists between the inhibitory activity of the sulfamate compounds and the pK_a of the parent phenol (Fig. 3). Furthermore, the data (Table 1) may also suggest that compounds containing groups (i.e., electron withdrawing groups such as nitro and cyano groups) that are able to stabilize the phenoxide ion may result in more potent inhibitors than those containing groups which destabilize the phenoxide ion (i.e., electron donating groups such as methyl). This trend can be observed clearly when the two series of compounds are considered separately, that is, a higher correlation coefficient is obtained for the plot of IC_{50} versus pK_a for the 4-substituted compounds alone ($R^2 = 0.99$) (Fig. 4), compared to $R^2 = 0.73$ for both series of data (Fig. 3).

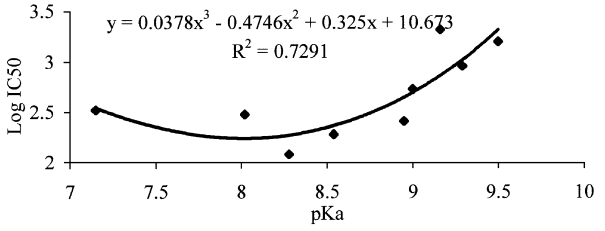


Figure 3. Plot of IC_{50} versus pK_a for all the 3- and 4-substituted phenols.

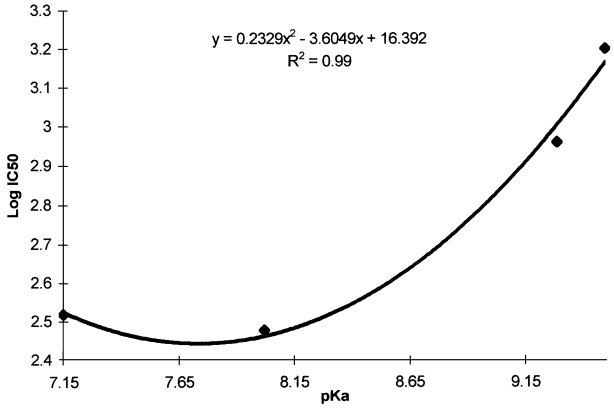


Figure 4. Plot of log IC_{50} versus pK_a for the 4-substituted phenols.

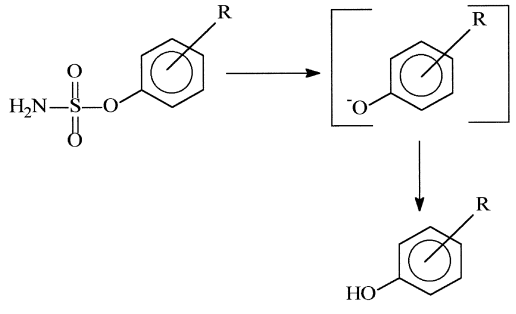


Figure 5. Hydrolysis of aminosulfonates.

In conclusion, the results of the biochemical evaluation show that a relationship may exist between the inhibitory activity and the pK_a of the parent phenol. That is, when the hydrolysis reaction occurs (Fig. 5), the resulting phenoxide ion (and in particular its stability) is possibly a factor in determining the inhibitory activity of the sulfamate containing inhibitors.

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9. Chemistry: Synthesis of phenyl sulfamate (**1**): NaH (80% dispersion in mineral oil, 0.12 g, 4.0 mmol) was added to a stirred solution of phenol (0.30 g, 3.19 mmol) in DMF (20 mL) under nitrogen 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 overnight. 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 a yellow oil, which was run through a column to give **1** (0.14 g, 25.4%) as a pure white solid mp 77.6–81.2 °C. $R_f = 0.32$ [diethyl ether/petroleum ether 40–60 °C (6:4)]. $\nu_{(max)}$ (Film) cm^{-1}: 3421.1 and 3307.8 (NH), 1367.5 and 1177.2 (S=O). 300 MHz δ_H ($CDCl_3$) 7.43–7.25 (5H, m, ArH), 5.24 (2H, s, NH_2). δ_C ($CDCl_3$) 150.024, 129.923, 127.306, 122.142. MS (M^+) calculated mass 173.014665, actual mass 173.015633.
ES assay: The biochemical evaluation of the series of compounds was undertaken in triplicate using the previously reported method of Selcer.[3] The total assay volume was 1 mL. ^3H estrone sulfate (25 μL, 20 μM/tube; 300,000dpm) and the inhibitors (of varying 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.

Irreversible ES assay: The irreversible inhibition was determined using the procedure described in ref 1 using EMATE (10 μM), COUMATE (100 μM), **12** (400 μM) and **13** (400 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 estrone sulfatase 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 duplicate, 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 estrone sulfatase activity as described above.