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Derivation of a possible transition-state for the reaction catalysed by the enzyme Estrone Sulfatase (ES).

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Abstract We have determined a possible transition-state for the reaction catalysed by the enzyme Estrone Sulfatase (ES) - as a representation of the active site. Using the derived structure, we have superimposed several steroidal and non-steroidal inhibitors in an attempt to rationalise the inhibitory activity of a number of potent inhibitors.

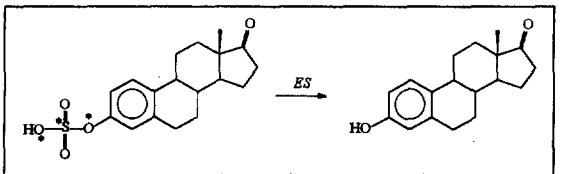

Figure 1. Action of ES on estrone sulfate.

The use of aromatase (AR) inhibitors in the treatment of hormone dependent breast cancer has not shown the expected decrease in the plasma levels of the female sex hormones (which have been implicated in the initiation/progression of breast cancers). This has been shown to be due to the activity of the enzyme estrone sulfatase (ES), the enzyme responsible for the conversion of the stored (sulfated) form of the estrogens (E1S) to the active form[1] (E1) (Figure 1). As such, in postmenopausal women this conversion allows the supply of E1 to the tumour via a non-AR related route.

Although a number of mechanisms have been proposed for the de-sulfatation of estrone sulfate, that of Anderson et al[2] is the most favoured (Figure 2) as it also allows the potent irreversible inhibition of inhibitors such as EMATE (IC_{50}=65pM)[3] (Figure 3) to be explained. EMATE has been found to be a time and concentration dependent irreversible inhibitor. However, it has also been shown to possess potent estrogenic properties, and as such, a number of other compounds are at present under investigation. These include non-steroidal, as well as steroidal, compounds. The investigation of non-steroidal inhibitors has intensified recently as this type of compound has potential benefits over the steroidal type in that they may totally lack estrogenic properties.

In an effort to overcome the lack of information regarding the ES active site, we have initiated a series of structure-activity relationship determination studies involving the consideration of inhibition data available for known steroidal and non-steroidal inhibitors of this enzyme and the modelling of the inhibitors. We therefore

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present in this report the initial results of the molecular modelling studies involving the determination of the transition-state of the reaction catalysed by ES.

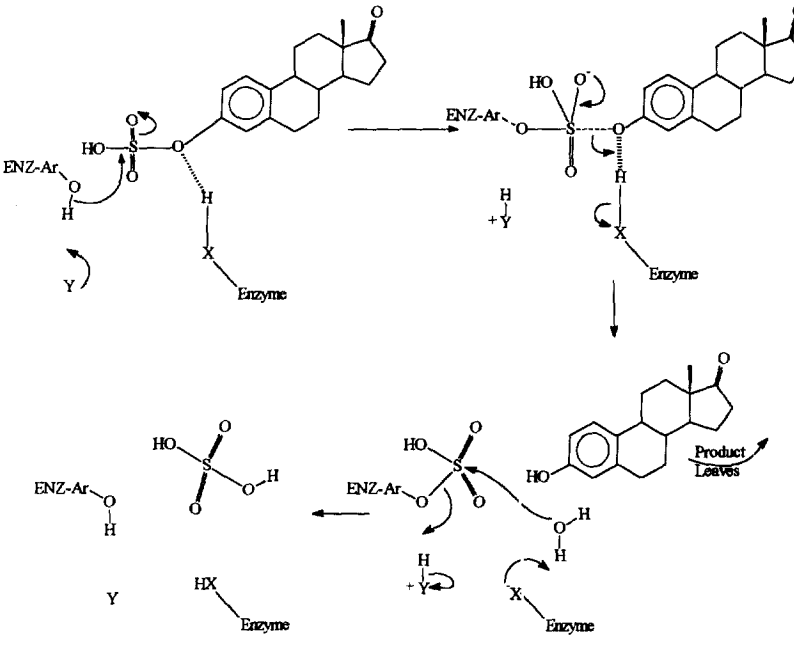

Figure 2. Proposed mechanism for the de-sulfatation of estrone sulfate[2].

In the construction of the theoretical representation of the ES active site, the structures of the substrate, and two histidine residues [one molecule (labelled as X in Figure 2) is presumed to undergo hydrogen bonding with the phenolic oxygen of E1S whilst the remaining histidine molecule (labelled as Y in Figure 2) is presumed to undergo protonation of the imidazole nitrogen allowing the formation of the ENZ-Ar-O$^-$, which then attacks the S of the sulfonate group] and the tyrosine amino acid residue which have been proposed by Anderson et al[2] to exist at the active site, were all constructed within the CACHE[4] molecular modelling software on an IBM PC compatible microcomputer. The completed structures were then refined performing a pre-optimisation calculation in mechanics using augmented MM2[5], followed by a geometry optimisation in Mopac[6] using AM1 parameters[7]. In order to determine the transition state, the hydrogen of the histidine zwitterion was attached to the sulfonate group of estrone sulfate, via a weak bond. Similarly, another hydrogen atom was attached to the phenolate oxygen atom of the substrate. The initial structures were minimised using the MM2 and Mopac/AM1 routines (reactant & product files)[6,7]. The saddle point for the reaction was then calculated and the resulting transition-state structure refined by performing a minimise gradient calculation using Mopac and AM1 parameters. The molecule's vibrational transitions were calculated in order to 'verify' the transition-state (within Mopac using AM1 parameters). A single negative vibration (-1211.58cm^{-1}) was observed upon viewing the molecule file, resulting in the final structure for the ES active site (Figure 4).

The structures of estrone sulfate, EMATE and the steroidal and non-steroidal inhibitors considered within the present study (Figure 3) were all constructed within the Alchemy III[8] molecular modelling software on an IBM PC compatible microcomputer. The completed structures were then subjected to an initial minimisation using the conjugate-gradient algorithm until the gradient fell below 10^{-6} resulting, in general, in 500 or more iterations per structure. Conformational analysis was performed (using the systematic search method with energy windows of 20-40 kcal mol^{-1} and bond rotation between 20-50°) on flexible parts of the inhibitors using Powersearch[8] in order to determine the low energy conformers. On the assumption that the shape of estrone sulfate would reflect the nature of the binding site of ES, the lowest energy conformers (an energy window of ΔE=5kcal/mol was used in determining the conformers to be used) of the inhibitors were superimposed by specification of three or more points on the sulfonate group of the substrate (Figure 1) on the transition state in the fitting process - we concluded[12] from the large number and variety of non-steroidal inhibitors which possess potent activity, that the carbon backbone of the steroidal structure may not be important to the initial inhibitory activity of these compounds, as such the only requirement appeared to be the sulfamate group - which has recently been supported by Woo et al[10].

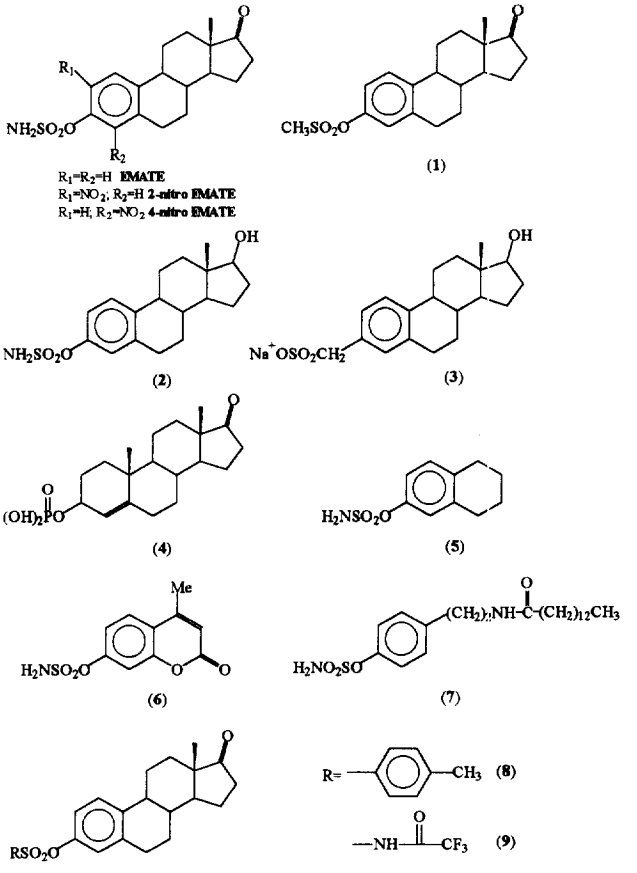

Figure 3. Some of the inhibitors of ES considered within the present study.

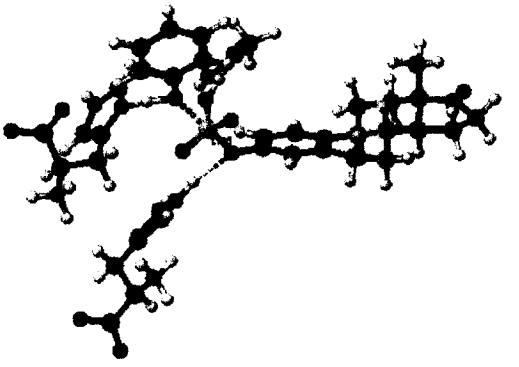

Figure 4. Calculated transition-state for the de-sulfatation reaction undertaken by ES.

Consideration of the overall structure shows that the amino acid residues postulated to be involved in the overall reaction mechanism are positioned close to the sulfonate and the C(3) position of the steroidal backbone, such that the C(2) is hindered - the nearest *amino acid* atom to steroid C(2) being 2.5Å (Figure 4). However, consideration of the C(4) position shows that it is slightly less hindered than the C(2) - the nearest amino acid atom to steroid C(4) being 6.1Å. This observation is therefore consistent with recent experimental data[9] which shows that the 2-nitro derivatives of EMATE are poorer inhibitors than the 4-nitro substituted derivatives. We therefore propose that these groups are involved in steric interaction with the amino acid residues leading to destabilisation of the enzyme-inhibitor complex. The superimpositioning of the low energy conformers of the nitro derivatives of EMATE shows that interaction is likely between the nitro group on the C(2) of the steroid and the active site located hydrogen bonding groups [Figure 5 shows the two derivatives superimposed onto the transition-state with the 2-nitro EMATE resulting in C(2)-NO_2 to the nearest amino acid atom distance of 2.7Å and the 4-nitro EMATE resulting in C(2)-NO_2 to the nearest amino acid atom distance of 4.3Å]. This result is further supported by compounds such as the 2-alkyl derivatives of EMATE which are found to be some 36,000 times less active than the 4-nitro EMATE, the superimpositioning of these types of compound therefore suggests that close interaction between the different groups is possible.

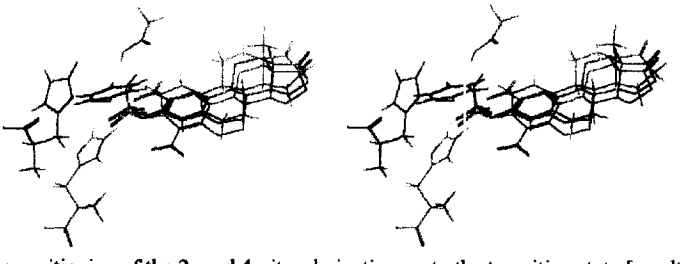

Figure 5. Superimpositioning of the 2- and 4-nitro derivatives onto the transition state [resulting in substrate C(17)=O to inhibitor C(17)=O distances of 3.4Å and 4.9Å respectively].

When the steroidal inhibitors were superimposed onto the sulfonate group of the transition state, it was discovered that the positions of the steroidal backbone of the different steroidal inhibitors did not correspond well. For example, when EMATE (IC_{50}=65pm, Figure 6) was superimposed using the sulfonate group, the

C(17) carbonyl group of the two respective structures were found to result in inhibitor C(17)=O to estrone sulfate C(17)=O distance of 3.5Å, with root mean square fit value of 0.27.

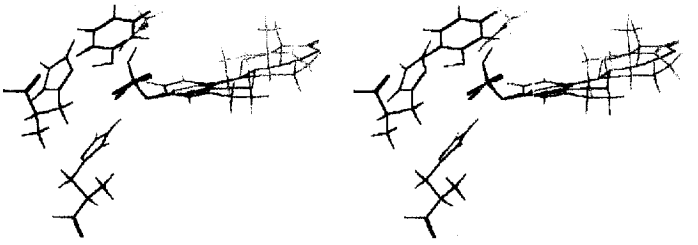

Figure 6. Low energy conformer of EMATE superimposed onto the transition-state.

The consideration of alternative estrone based inhibitors show similar results. The low energy conformers of the weaker inhibitors based upon estradiol [i.e. compounds containing a β hydroxy group instead of the C(17)=O] (2 and 3), show that the inhibitors superimpose in a similar manner to EMATE, resulting in a substrate C(17)=O to C(17)-βOH group distance of 3.9Å. From the consideration of the potent steroidal inhibitors, in particular, the positioning of the C(17)=O group of these compounds with respect to the transition-state, we conclude that the most relevant part of the steroid-derived structure is the possession of a C(3) sulfonate/sulfamate group. We therefore postulated that the C(17) position may not be involved in any interaction with the active site – using this hypothesis we have recently synthesised a number of highly potent compounds (comparable to EMATE in their potency and which have been submitted for patenting) that do not possess groups that may be postulated to mimic the C(17) area of the steroid.

The validity of the above hypothesis is further supported by the results obtained from the superimpositioning study using the recently reported non-steroidal inhibitors. Compound **5** has an IC_{50} of 1μM and does not possess any polar groups which can undergo interaction with the C(17) region of the active site. Alternative compounds exist [for example, COUMATE (**6**) (IC_{50}= 380nM)[10] and **7** (IC_{50}= 55.8nM)[11]] which *do* possess groups capable of undergoing hydrogen bonding with the region of the active site corresponding to the steroid C(17) position, but are found to be great distances away from the appropriate hydrogen bonding group at the active site. With compound **7**, it was discovered that the lowest energy conformer possessed an inhibitor amide carbonyl group to active site C(17) hydrogen bonding group distance of 4.8Å (Figure 7). Although this distance is not considered close enough for hydrogen bonding, this compound possesses potent inhibitory activity. As such, the results of the investigation of the non-steroidal compounds would appear to suggest the existence of another factor in the potent activity of this type of inhibitor, possibly logP.

The compounds considered thus far contain the less hindered sulfamate moiety (OSO_2NH_2). However, there are sulfamate derivatives such as those in inhibitors **8** and **9** which possess bulky groups (Figure 3). These compounds have not been considered previously as it was not clear how these inhibitors could be accommodated within the active site, indeed, these compounds were initially presumed to be weak inhibitors.

However, on evaluation, they were found to possess potent activity. With our representation of the active site, we believe that the activity of these compounds can now be rationalised. That is, we discovered that on undertaking conformational analysis of the rotatable bonds within the sulfamate group, conformers were found (which were within a ΔE~5kcal/mol range) which allowed the bulky group to be accomodated within the active site without undergoing close interaction between the components of the active site and the inhibitors (Figures 8) - the inhibitors' and substrate backbone did not superimpose well. To our understanding, this is the first report where the activity of these groups has been rationalised.

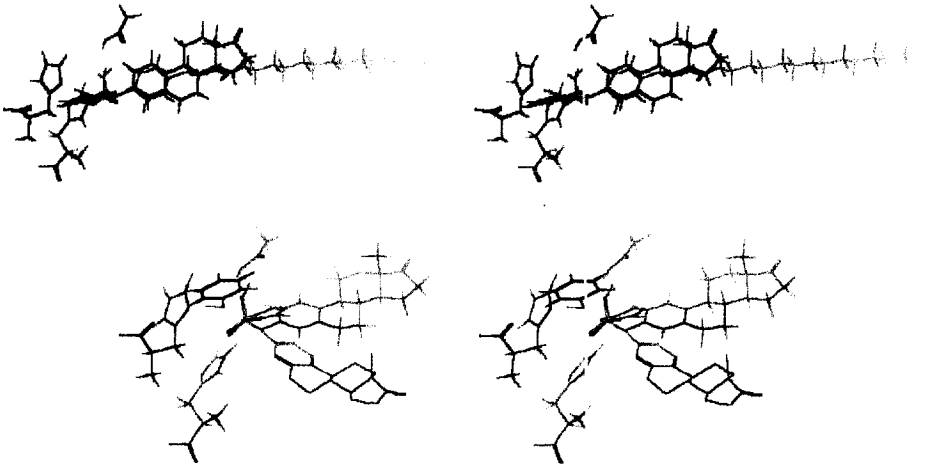

Figure 8. Low energy conformer of 8 superimposed onto the transition-state.

In conclusion, we believe that the derivation of the transition-state of the de-sulfatation reaction catalysed by ES has resulted in a good working model of its active site. The study also suggests that the mimicking of the sulfonate group is of greater importance than the overlap of the steroid backbone.

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