Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 3001–3005

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Determination and Use of a Transition State for the Enzyme Estrone Sulfatase (ES) from a Proposed Reaction Mechanism

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Received 8 March 2001; revised 20 August 2001; accepted 5 September 2001

Abstract—Using the postulated mechanism for the enzyme estrone sulfatase (ES), we have determined a possible transition state for the reaction catalysed by ES as a representation of the active site. Using the derived structure, we have undertaken the molecular modelling of several steroidal and non-steroidal inhibitors in an attempt to rationalise the inhibitory activity of a number of potent inhibitors.

In our search for potent inhibitors of the enzyme estrone sulfatase (ES), we have initiated a number of structure–activity relationship studies which have allowed us an initial insight into the structural requirements for the inhibition of ES,[1] the enzyme responsible for the conversion of the stored (sulfated) form of estrogen (E1S) to the active form (E1) (Fig. 1).

Although a number of mechanisms have been proposed for the de-sulfatation reaction catalysed by ES,[2,3] these have now shown to be incorrect since the initial attacking group is now believed to be a gem-diol[4] (Fig. 2) rather than the previously presumed tyrosine[2] [the tyrosine based mechanism was favoured since it allowed the rationalisation of the mode of action of potent irreversible inhibition by compounds such as estrone-3-sulfamate (EMATE) ($IC_{50} = 65$ pM)[3] (Fig. 3)]. 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, including a wide variety of non-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 lack estrogenic properties.

In an effort to overcome the lack of information regarding the ES active site, we have initiated a series of molecular modelling studies involving the determination of a representation of the ES active site. Here, we present the initial results of our modelling study involving the determination of the transition-state of the reaction catalysed by ES.

In the construction of the probable transition state as a representation of the ES active site, the structures of the substrate, and formylglycine (FGly69) and histadine (N229) residues which have been proposed by von Bulow et al.[4] to exist at the active site, were all constructed within the CACHE[5] 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,[6] followed by a geometry optimisation in Mopac[7] using AM1 parameters.[8] In order to determine the transition state, the oxygen atom of the formylglycine residue was attached to the sulfonate group of estrone sulfate via a weak bond. Similarly, another hydrogen atom [from histadine residue 229 (Fig. 2)] was attached to the phenolate oxygen atom of the substrate—it is postulated that this group may be involved in the donation of a hydrogen atom to the resulting

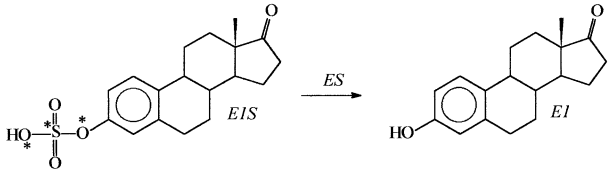

Figure 1. Action of ES on estrone sulfate.

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PII: S0960-894X(01)00607-2

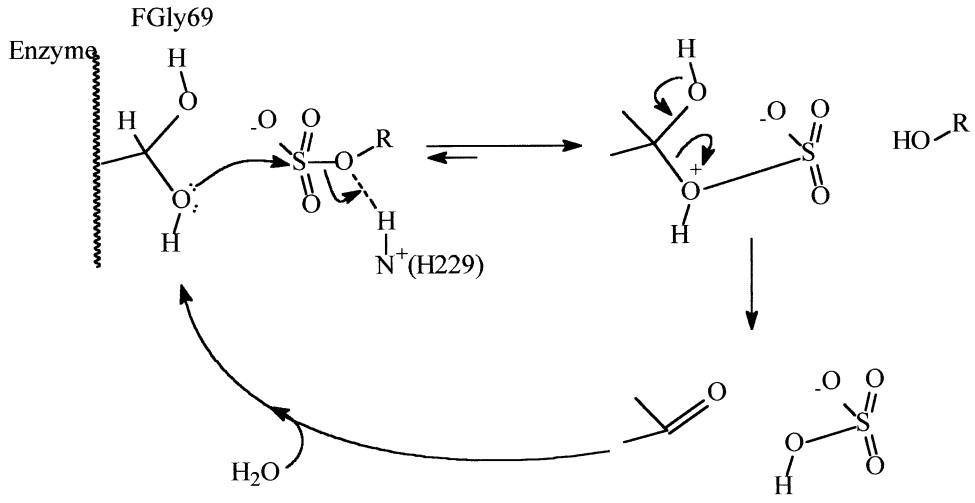

Figure 2. Proposed mechanism for the de-sulfatation of estrone sulfate.

phenoxide ion. The initial structures were minimised using the MM2 and Mopac/AM1 routines (reactant and product files).[7,8] 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 ($-502.21\,cm^{-1}$) was observed upon viewing the molecule file, resulting in the final structure of the transition state (Fig. 4).

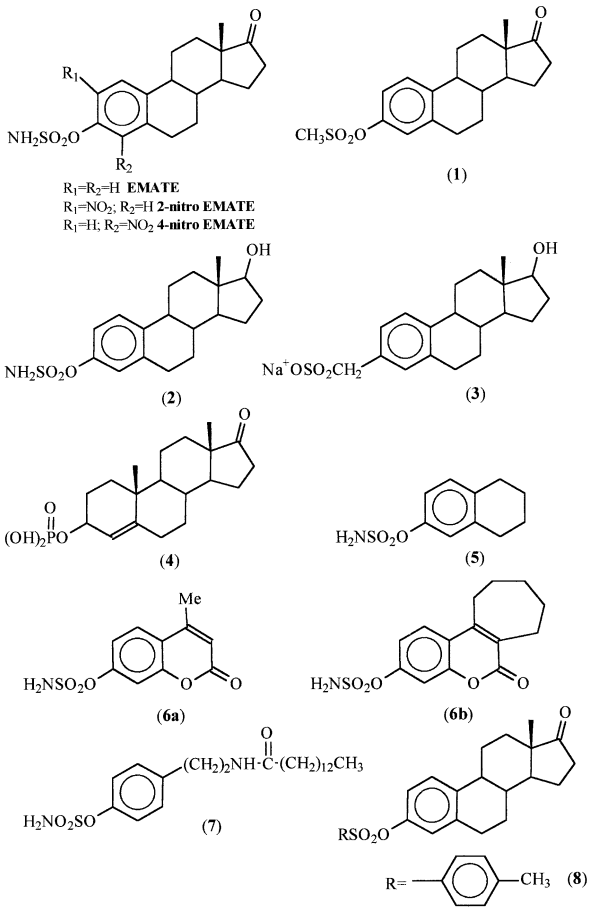

Figure 3. Inhibitors of ES considered within the present study.

The structures of E1S, EMATE and the steroidal and non-steroidal inhibitors considered within the present study (Fig. 3) were all constructed within the Alchemy III[9] 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 and 50°) on flexible parts of the inhibitors using Powersearch[9] in order to determine the low energy conformers. On the assumption that the shape of E1S would reflect the nature of the binding site of ES, the lowest energy conformers of the inhibitors were superimposed by specification of three or more points on the sulfonate group of the substrate (Fig. 1) on the transition state in the fitting process (an energy window of $\Delta E = 5$ kcal/mol was used in determining the conformers to be used). The superimpositioning points were chosen since the sulfamate group is common to most of the potent inhibitors and, as such, we postulated that this group is important in mimicking the natural substrate's binding to the active site.

From the results of the consideration of structure–activity relationship studies,[1] we concluded (from the large number and variety of non-steroidal inhibitors which possess potent activity) that the carbon backbone of the compounds may not be crucial to their inhibitory activity. As such, we postulated that the only requirement appeared to be the sulfamate group—which has recently been supported by Woo et al.[10]

Consideration of the derived transition state shows that the amino acid residues postulated to be involved in the reaction mechanism are positioned close to the sulfonate and that the C(3) position of the steroidal backbone such that the C(2) is hindered [the nearest amino

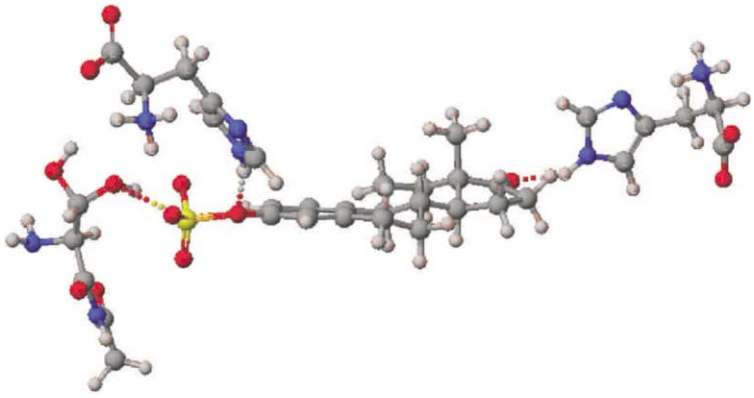

Figure 4. Calculated transition-state for the de-sulfatation reaction undertaken by ES.

acid atom to steroid C(2) being 2.1 Å (Fig. 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 3.5 Å. This observation is therefore consistent with experimental data[11] 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 [Fig. 5 shows the two derivatives superimposed onto the transition-state with the 2-nitro EMATE resulting in a C(2)–NO_2 to the nearest amino acid atom distance of 2.1 Å and the 4-nitro EMATE resulting in a C(2)–NO_2 to the nearest amino acid atom distance of 3.5 Å; that is, steric interaction is possible between the nitro group and the enzyme active site residues in the case of 2-nitro EMATE which is absent in the 4-nitro EMATE]. 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 the 2-alkyl derivative shows that high levels of steric interaction is possible between the alkyl group and the enzyme active site residues.

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} = 65 pM, Fig. 6) was superimposed using the sulfonate group, the C(17) carbonyl group of the two respective structures were found to result in an inhibitor C(17)=O to E1S C(17)=O distance of 1.6 Å, with root mean square fit value of 0.17.

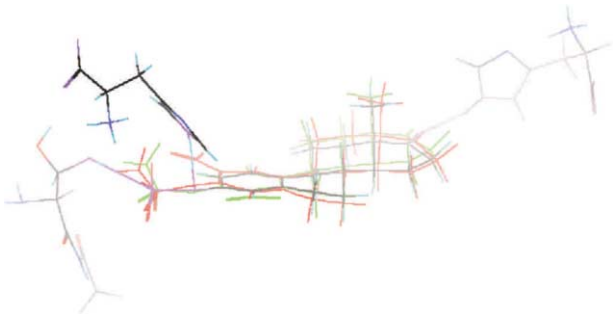

Figure 5. Superimpositioning of the 2-nitro EMATE (red) and 4-nitro EMATE (green) derivatives onto the transition state.

The consideration of alternative estrogen based inhibitors shows 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 3.7 Å. From 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 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 reported non-steroidal inhibitors. Compounds such as **5** have IC_{50} values of 1 μM but, however, do not possess any polar groups which can mimic the steroidal C(17) region of the steroid backbone. Alternative compounds exist [e.g., COUMATE (**6a**) (IC_{50} = 380 nM);[10] 667-COUMATE (**6b**) (IC_{50} = 8 nM)[12] and **7** (IC_{50} = 55.8 nM)[13]] 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. Indeed, **6b** possesses a heptyl cyclic moiety which does not mimic the C(17)=O in any way, however, it is the most potent compound known to date and is now entering phase II clinical trials. Furthermore, superimpositioning of **6b** onto the transition state

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

Figure 7. Low-energy conformer of **6b** superimposed onto the transition state.

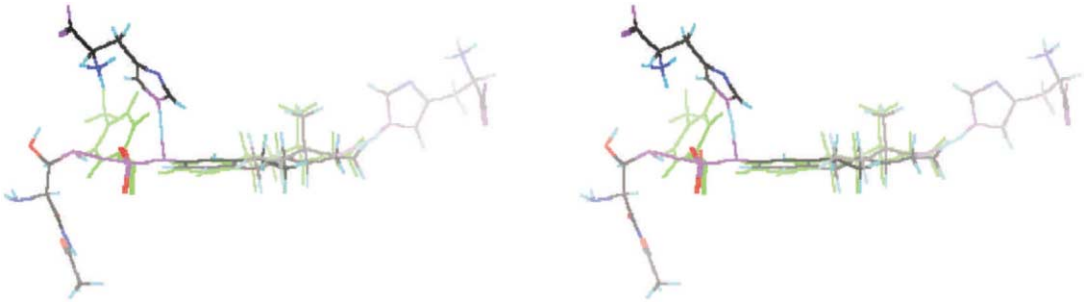

Figure 8. Low-energy conformer of **8** superimposed onto the transition state.

shows that the lowest energy conformer possessed an inhibitor carbonyl group to active site C(17) hydrogen bonding group distance of 6.4 Å (Fig. 7), a distance which is considered to be too large for strong interaction.

The compounds considered thus far contain the less hindered sulfamate moiety (OSO_2NH_2). However, there are sulfamate derivatives, such as inhibitor **8**, which possess a bulky group attached to the sulfonate group (Fig. 3). These types of compound have not been considered previously as it was unclear as to how they could be accommodated within the active site; indeed, these compounds were initially presumed to be weak inhibitors. On evaluation they were found to possess good inhibitory activity, the mode of action of **8** has not, however, been rationalised. We believe that with our representation of the active site, the activity of these bulky group-containing 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 $\Delta E \sim 5$ kcal/mol range) which allowed the bulky group to be accommodated within the active site without undergoing close interaction between the components of the active site and the inhibitors (Fig. 8).

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 and is a good aid to drug design and discovery. The study also suggests that the mimicking of the sulfonate group is of greater importance than the overlap of the steroid backbone.

References and Notes

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