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The difluoromethylene group as a replacement for the labile oxygen in steroid sulfates: a new approach to steroid sulfatase inhibitors

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Abstract—Several estrone sulfate and estradiol sulfate analogues, in which the sulfate group was replaced with an α,α-difluoromethylenesulfonate group or an α,α-difluoromethylenetetrazole group, were examined as inhibitors of steroid sulfatase (STS). These compounds were 4.5–10.5 times more potent than their non-fluorinated analogues. Moreover, the presence of the fluorines changed the mode of inhibition from mixed to competitive. The inhibitor bearing the α,α-difluoromethylenetetrazole group exhibited an affinity for STS approaching that of the natural STS substrate, estrone sulfate. Possible reasons for the enhanced affinity of the fluorinated compounds compared to their non-fluorinated counterparts are discussed.

Approximately one-third of breast tumors in postmenopausal women require stimulation by estrogens for optimal growth.[1] Consequently, certain enzymes responsible for the biosynthetic production of such estrogens are being evaluated as targets for the treatment of hormonally-dependent breast cancer. Until fairly recently, two such enzymes, aromatase and 17β-hydroxysteroid dehydrogenase, have been the main focus of inhibitor/drug development. However, more recently it has been proposed that another enzyme, steroid sulfatase [STS, also known as estrone sulfatase or arylsulfatase C (ASC)], may also be a potential target for therapeutic intervention.[2] STS catalyzes the hydrolytic desulfation of steroidal sulfates, such as estrone sulfate (E1-S) or dehydroepiandrosterone sulfate (DHEAS), to the corresponding steroids and inorganic sulfate. Studies have suggested that these sulfated steroids, by the reaction of STS, function as reservoirs for the formation of estrogens in breast tumors.[2] Over the last several years, a number of reports have appeared describing inhibitors of STS.[3] In one report, Li and co-workers examined a number of sulfonate analogues as STS inhibitors.[4a] Among these compounds were sulfonates **1** and **2**, in which the labile sulfate ester oxygen of E1-S and estradiol sulfate (E2-S) was replaced with a non-hydrolyzable methylene group. However, these compounds were found to be poor inhibitors exhibiting mixed inhibition with K_i values of 140 and 130 μM, respectively, and have an affinity for STS that is considerably poorer than that of substrates ES-1 and ES-2. On the basis of these studies and on studies with other sulfonate analogues,[4b] it was concluded that an oxygen atom or an electronically similar link between the aryl moiety and the sulfur atom is essential for high affinity binding.[4a]

The difluoromethylenephosphonic acid group (DFMP), R-$CF_2PO_3^{-2}$, has been used extensively in the design of inhibitors of enzymes that hydrolyze or bind phosphate esters (R-*O*-PO_3^{-2}).[5] In some instances, compounds bearing the -$CF_2PO_3^{-2}$ were dramatically better inhibitors of phosphatases than the analogous non-fluorinated analogues (R-$CH_2PO_3^{-2}$).[5f] The increased potency of the DFMP's compared to their non-fluorinated analogues has been attributed to the lower pK_a values of the fluorinated phosphonic acids and/or H-bonding interactions between the fluorines and residues in the active sites.[5a–f] In the case of STS, the low affinity of **1** and **2**, compared to ES-1 and ES-2, is probably not a result of differences in pK_a values of the sulfate and

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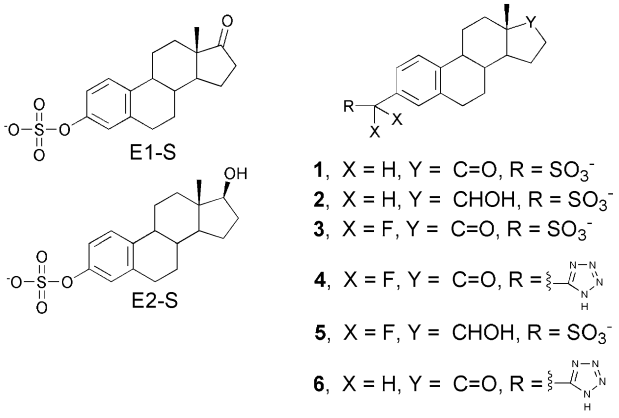
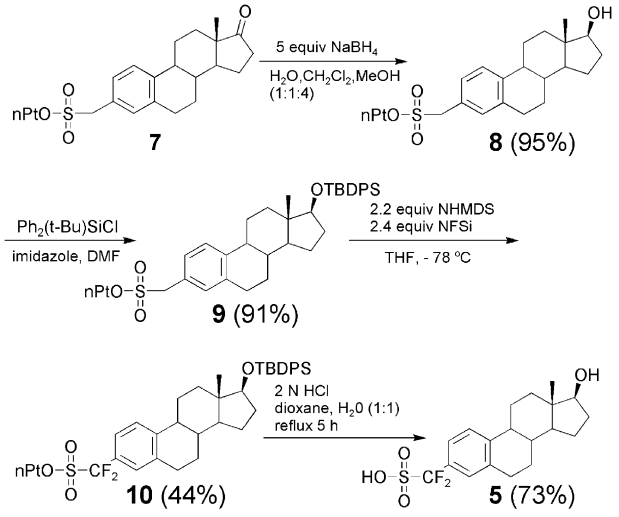
sulfonate moieties since both should be completely ionized at the pH under which the studies were performed. The poor affinity of the sulfonates is probably a result of loss of an interaction between the labile ester oxygen of the sulfates and STS. Therefore, we decided to examine the difluoromethylene moiety as a replacement for the labile oxygen in anticipation that the fluorines would partake in specific interactions with residues in the active site, in a manner similar to that found with difluoromethylenephosphonic acid inhibitors of certain protein tyrosine phosphatases.[5f] To determine this, we prepared two E1-S analogues, the difluoromethylenesulfonic acid **3** and the difluoromethylenetetrazole **4**.[6] Tetrazole **4** was prepared because it has been reported that the tetrazole group could serve the role of the sulfate moiety at the CCK receptor in vitro in certain analogues of CCK-8 sulfotyrosine-bearing peptides.[7] Therefore, it was hypothesized that the tetrazole group might also be a good sulfate surrogate in steroidal sulfates. Here we report the results of inhibition studies with compounds **3** and **4**, as well as with compound **5**, which is the 17-hydroxy analogue of **3**, and with the non-fluorinated compounds **1** and **6**, with highly purified STS.



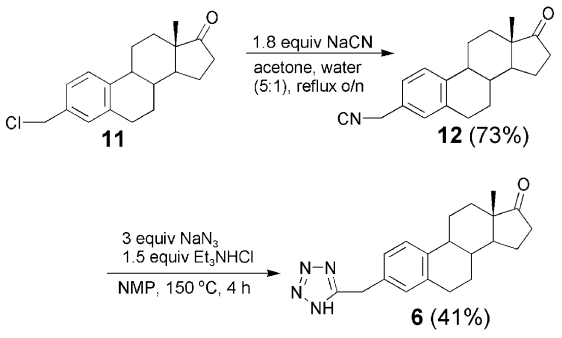
Compounds **3** and **4** were prepared as previously reported.[6] Compound **5**, which bears a hydroxy group at the 17-position in the D-ring, was prepared as outlined in Scheme 1. The starting material, compound **7**, was described previously.[6] The key step was the electrophilic fluorination of sulfonate **9** using *N*-fluorobenzenesulfonimide.[8] Compound **6** was prepared as outlined in Scheme 2. The starting material, **11**, was prepared as previously described.[6] Conversion of nitrile **12** to tetrazole **6** turned out to be somewhat challenging. Standard conditions for converting nitriles to tetrazoles, such as $NaN_3/NH_4Cl/\Delta$[9] or $Me_3Si{-}N_3/(Bu)_2SnO/\Delta$,[10] resulted in little or no conversion. However, it was found that by using the procedure of Bernstein and Vacek,[11] which involved heating a mixture of **12**, NaN_3 and Et_3NHCl in *N*-methylpyrrolidinone at 150 °C, tetrazole **6** could be obtained in reasonable yield.

Human placental STS was purified to apparent homogeneity (one band on an SDS gel using silver staining) using the procedure of Hernandez-Guzman et al.[12] Inhibition studies were performed as follows. To the wells of a 96-well plate was added an appropriate amount of a stock solution of **1** or **3**–**6** in DMSO to assay buffer which consisted of 0.33 M Tris–HCl, 0.1% Triton, pH 7.4. To this was added an appropriate amount of a stock solution of 4-methylumbelliferyl sulfate (4-MUS) in 0.33 M Tris–HCl, pH 7.4. These mixtures were incubated at 37 °C for 2 min. The reaction was initiated by adding 10 μL of purified STS in 20 mM Tris–HCl, 0.1% Triton, pH 7.4. The final concentration of STS was 0.005 mg/mL. The total volume was 100 μL, the final concentration of DMSO was 10% and the final concentration of Triton was 0.08%. The production of 4-methylumbelliferone was monitored over 10–15 min using a Spectramax GeminiXS plate reader (excitation 359 nm, emission, 451 nm) at 37 °C. Each reaction was performed in triplicate. The concentration range over which the inhibitors were tested ranged from approximately 0.5- to 3-fold K_i. Controls were performed in an identical manner but did not contain STS. Initial rates (v) were determined by taking the slopes of plots of the change in relative fluorescence units with time. These data were plotted as Lineweaver–Burk graphs and K_i and αK_i values were calculated from replots of the slopes or intercepts of the Lineweaver–Burk graphs according to the equations for mixed and competitive inhibition.[13]

Li et al. have reported that compound **1** exhibits mixed inhibition with purified STS and has a K_i value of 140 μM.[4a,14] Similar results were reported for com-



Scheme 1.



Scheme 2.

pound **2**. We also prepared compound **1** using the methodology of Li et al. and examined it as an STS inhibitor. Under our assay conditions, compound **1** also exhibited mixed inhibition with a K_i of $600 \pm 74\,\mu M$ and an αK_i of $1378 \pm 75\,\mu M$. The difference between our result and that of Li et al. is most probably due to the differences in assay conditions. STS activity in Li et al.'s studies was determined radiometrically using E1-^{35}S as substrate at pH 7.0 in 100 mM Tris–acetate in the absence of Triton and DMSO while our studies were conducted using 4-MUS as substrate in 333 mM Tris–HCl buffer at pH 7.4 with 0.08% Triton and 10% DMSO. Interestingly, fluorosulfonate **3** did not exhibit mixed inhibition but instead displayed only competitive inhibition with a K_i of $57 \pm 6\,\mu M$ (Fig. 1). Compound **5** was also a competitive inhibitor with a slightly lower K_i value ($37 \pm 5\,\mu M$). Tetrazole inhibitor **6** displayed mixed inhibition with a K_i of $72.7 \pm 1.4\,\mu M$ and an αK_i of $213 \pm 38\,\mu M$. However, its fluoro analogue, compound **4**, exhibited only competitive inhibition with a K_i of $16 \pm 3\,\mu M$ (Fig. 2).

Several aspects of the above results are notable. First, tetrazoles **4** and **6** are 3.5–8.3 times more potent than their sulfonate analogues, **1** and **3**, respectively. At pH 7.5 in Tris buffer and in the presence of Triton, E1-S has been reported to have a K_m of 21 μM.[15] Under the same conditions, E1-S has also been reported to competitively

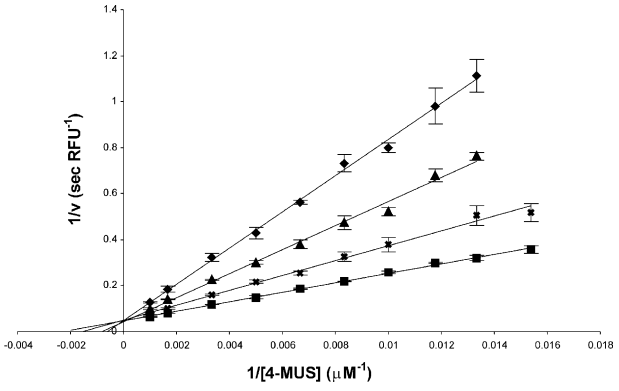


Figure 1. Inhibition of STS by sulfonate **3**. Assays were conducted as described in the text in the presence of 0 μM (■), 30 μM (x), 75 μM (▲), and 150 μM (◆) compound **3**.

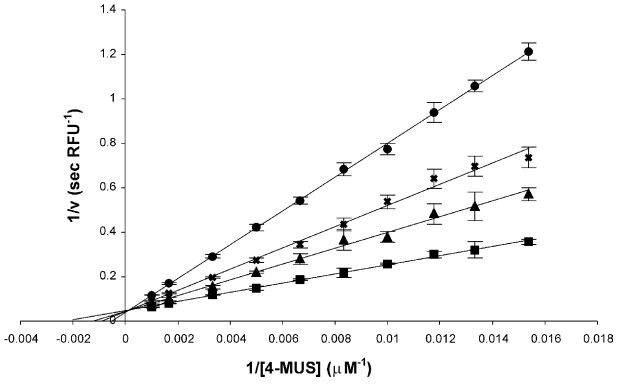
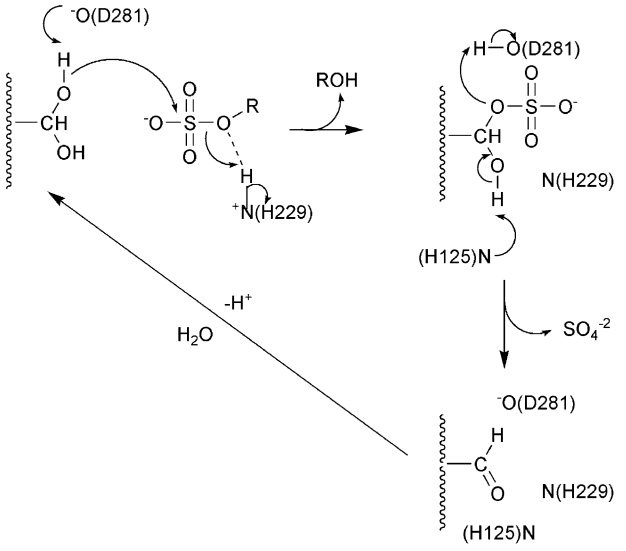


Figure 2. Inhibition of STS by tetrazole **4**. Assays were conducted as described in the text in the presence of 0 μM (■), 10 μM (▲), 25 μM (x), and 50 μM (●) compound **4**.

inhibit STS desulfation of 4-MUS with a K_i of 60 μM.[15] Thus, it appears that compound **4** exhibits an affinity for STS approaching that of the natural substrate. To our knowledge, the only other report describing the use of the tetrazole group as a sulfate mimetic was by Tilley et al. who used the tetrazole group to mimic the sulfate group in sulfotyrosine-bearing CCK-8 peptide analogues.[7] None of the tetrazole-bearing peptides exhibited an affinity for the CCK receptor equal to that of the analogous sulfated peptides.[7] The superior affinity of the tetrazole derivatives over the analogous sulfonates in the present study is to our knowledge unprecedented and is surprising when one considers that the two groups are, from a structural point of view, quite different. Modeling studies will be necessary to determine how the tetrazole group is interacting with STS.[16]

The second notable result is that replacing the bridging methylene unit in **1** and **6** with a difluoromethylene group results in a change in the mode of inhibition from mixed inhibition to solely competitive inhibition. Li has reported that estrone sulfate (ES-1) exhibits mixed-type inhibition when using DHEA-^{35}S as a substrate.[4a] Li also noted that subtle changes in the sulfate group in ES-1 can yield STS inhibitors that display inhibition patterns that are different from ES-1.[4a] For example, estrone phosphate and estrone-3-methylsulfonate are purely competitive inhibitors.[4a] The CF_2 group may nullify the non-competitive binding mode as well as increase the competitive binding mode such that the non-competitive binding is relatively insignificant (or both).

The third, and perhaps the most significant result, is that replacing the bridging methylene unit (in **1** and **6**) with a difluoromethylene group results in a 4.5–10.5-fold increase in inhibitor potency. Since sulfonates **1** and **3**, and tetrazoles **4** and **6**, are ionized at physiological pH,[17] it stands to reason that enhanced affinity of the fluorinated compounds **3** and **4** is probably not due to the increased acidity of these compounds. It is more likely that their enhanced affinity is due to interactions of the fluorines with residues in the active site. One possibility is that the fluorines are hydrogen bonding with residues in the active site. Although fluorine is capable of acting as an H-bond acceptor, the subject of fluorine hydrogen bonds involving C–F is a subject of much controversy.[18a–c] Nevertheless, it appears that such H-bonds can form in certain instances[19] although the optimal strength of such bonds is still unknown. What residue(s) in STS might be involved in such an H-bond with compounds **3** and **4**? The mechanism of STS has not yet been studied in detail. Most of the work that has been done on the mechanism of aryl sulfatases (AS's) has been performed on arylsulfatase A (ASA).[20] All AS's known to date have a formylglycine residue in the active site resulting from a post-translational modification of a serine or cysteine residue. The crystal structure of human ASA shows that the formylglycine exists as a hydrate which is stabilized by coordination to a Mg^{+2} ion and H-bonds to Asp-281 and His-125. On the basis of the STS crystal structure and other studies, a mechanism has been proposed for ASA (Scheme



Scheme 3.

3).[20,21] One of the hydroxyls of the formylglycine hydrate attacks the sulfur atom of the substrate. It has been hypothesized that the ester oxygen of the substrate is involved in H-bonding with Lys-302 and His-229. His-229 is a key residue, since it is believed to assist in the cleavage of the S–O bond of the substrate by acting as a general acid. The sulfate group is then eliminated from the hydrate, resulting in formation of formyl glycine which is then rehydrated.

In the crystal structure of STS, the formylglycine is hydrated and the hydrate is sulfated.[16] This is similar to that found in the crystal structure of arylsulfatase B (ASB).[22] Nine out of 10 catalytically important residues are identical in STS, ASA and ASB. The exception is Gln-343 in STS as opposed to an Asn in ASA and ASB and these residues function as ligands to the cation. Superimposition of these nine α-carbon atom positions by least squares minimization resulted in root mean squared deviation of 0.39 Å between STS and either ASA or ASB.[16] Thus, the mechanism for STS is most likely very similar to that proposed for ASA. His-229 in ASA, the residue that is believed to be involved in H-bonding to the sulfate ester oxygen, corresponds to His-290 in STS. Ghosh and co-workers have modeled E1-S in the STS active site with its sulfate moiety superimposed with the crystallographically observed sulfate of the sulfated formylglycine hydrate. The oxygen of the labile S–O bond is within H-bonding distance of one of the imidazole nitrogens of His-290. It has been proposed that His-290 acts as a general acid in a manner analogous to that proposed for His-229 in ASA.[16] Thus, it is possible that the fluorines in **3** and **4** enhance binding by H-bonding with His-290. Such H-bonding would not be without precedent. The enhanced affinity of difluoromethylenephosphonic acid inhibitors of protein tyrosine phosphatase 1B, compared to their non-fluorinated analogues, has been attributed at least in part to H-bonding with an N–H backbone proton.[5f]

Although α,α-difluoromethylenephosphonic acids have been used as inhibitors and probes of enzymes and proteins that bind or hydrolyze phosphate esters for many years, the analogous approach to sulfatase inhibitor design has never been pursued. To our knowledge, this is the first demonstration of the use of fluoromethylenesulfonic acids as enzyme inhibitors. In this case, the fluorinated compounds were considerably better inhibitors than their non-fluorinated analogues and changed the mode of inhibition from mixed to competitive, demonstrating the utility of this approach to sulfatase inhibitor design. The use of a tetrazole to mimic the sulfate group is also worthy of note since the tetrazole-bearing compounds were superior inhibitors to the sulfonate analogues. We expect that the approaches to sulfatase inhibitor design outlined here will also be useful for preparing inhibitors and probes of other proteins that bind or hydrolyze sulfate esters.

Acknowledgements

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