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Synthesis of a non-hydrolyzable estrone sulfate analogue bearing the difluoromethanesulfonamide group and its evaluation as a steroid sulfatase inhibitor

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Steroid sulfatase (STS) catalyzes the hydrolyis of steroidal sulfates such as estrone sulfate (ES1) and is considered to be an attractive target in the treatment of steroid dependent cancers. A non-hydrolyzable estrone sulfate (ES1) analogue bearing an α,α-difluorosulfonamide moiety at the 3-position on the A-ring, compound **7**, was synthesized. Key to the success of this synthesis was the first use of the allyl group as a sulfonamide protecting group. The pK_a of this ES1 mimic in 0.1 M bis-tris propane, 10% DMSO was determined to be 8.05 using ^{19}F NMR. Compound **7** is a reversible inhibitor with a K_i similar to that of its sulfonate analogue at pH 7.0. It is more potent than its nonfluorinated sulfonamide analogue and, its inhibitory potency increases with increasing pH, a trend opposite to that of other STS inhibitors. Possible reasons for this are presented.

Introduction

The CF_2 moiety was proposed as a stable isoelectronic and isopolar replacement for bridging oxygen in phosphate esters over two decades ago.[1] Since then, numerous reports demonstrating the utility of this tactic for developing inhibitors of enzymes that bind or hydrolyze phosphate esters have appeared. Surprisingly, this strategy has rarely been exploited outside of phosphorus chemistry. For example, the sulfonamide group has been used extensively as a pharmacophore in medicinal chemistry.[2] However, α-fluorinated sulfonamides have been given only cursory attention. The presence of fluorines alpha to the sulfonamide leads to a linear acidity increase of approximately 1.5 pK_a units per fluorine as well as a significant increase in lipophilicity.[3] Relatively simple fluoromethane sulfonamides (F_3CSO_2NRR or HF_2CSO_2NRR) have been shown to possess anti-inflammatory and herbicidal properties.[4–7] Nevertheless, besides recent studies with carbonic anhydrase,[8,9] an enzyme known to exhibit a high affinity for a wide range of sulfonamides, such compounds have never been examined as enzyme inhibitors. One possible reason for this was a lack of a facile method for their preparation. Nevertheless, we recently demonstrated that such compounds can be prepared by electrophilic fluorination of appropriately protected sulfonamides and this represents the first rational syntheses of this class of compounds.[10]

Our interest in α-fluorosulfonamides stems mainly from our studies on the development of reversible inhibitors of steroid sulfatase (STS). Steroid sulfatase catalyzes the hydrolyis of steroidal sulfates such as estrone sulfate (ES1, **1**) and is a potential target in the treatment of steroid dependent cancers.[11] The majority of STS inhibitors reported to date are primary aryl sulfamates ($Ar–OSO_2NH_2$) which act as irreversible suicide inhibitors.[11] EMATE (**2**) is an example of this class of inhibitors.[11] The S–O bond must be hydrolyzed by the enzyme for irreversible inhibition to occur. Indeed, the S–O bond of EMATE is readily cleaved by STS, even though EMATE is an apparently neutral compound at physiological pH and the enzyme's natural substrate is an anionic sulfate ester. To account for STS's ability to readily bind and hydrolyze EMATE, as well as other sulfamates, it has been suggested that it is the conjugate base of EMATE, which is isoelectronic and isosteric to ES1, that binds to STS, even though the conjugate base is the minor species at physiological pH with the N-proton of EMATE having a pK_a of 9.5 in 70% aqueous methanol.[12–14] Nevertheless, such a hypothesis is consistent with the active site architecture of STS.[15] The active site contains a calcium ion as well as other cationic residues such as Lys134, Lys368, His290, His346 and His136 some of which are probably involved in interacting with the anion of the sulfate substrate.

Although the sulfamate inhibitors have garnered much attention, most primary sulfamates have limited chemical stability in solution and breakdown to form sulfamic acid and the phenolic portion of the inhibitor.[11] Consequently, it has been suggested that reversible, non-sulfamate inhibitors may be more amenable for drug development.[11] Unlike sulfamates, primary sulfonamides have not been extensively examined as STS inhibitors. Sulfonamide **4** is a reversible STS inhibitor and exhibits a K_i of 140 μM when using ^{35}S-dehydroepiandosterone sulfate as substrate at pH 7.0.[16] This K_i is approximately 3.5 times greater than that of sulfonate **3** (40 μM) and both have K_i values that are much greater than that of ES1 (0.9 μM) under the same conditions. On the basis of these studies and on studies with other sulfonate analogs, 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.[16]

We recently demonstrated that the CF_2 group can be used as a stable replacement for the bridging oxygen in estrone sulfate (ES1) in that compound **5** is a competitive STS inhibitor (K_i = 57 μM, pH 7.4, 0.1% Triton X-100) and was approximately 10-fold more potent than its non-fluorinated analogue **6**.[17,18] Since both **5** and **6** have pK_a values that are far below the pH at which the studies were performed (pH 7.4), we reasoned that their difference in potency was due to the fluorines interacting with residues in the active site perhaps by fluorine H-bonding with His290 which is believed to act as a general acid during the cleavage of the S–O bond.[15,18]

On the basis of our studies with compound **5**, and the possibility that it is the conjugate base of EMATE that binds to STS, we decided to prepare the difluoromethylene analogue of EMATE, **7**, and examine it as a reversible STS inhibitor. Its pK_a should be approximately three pK_a units less than that of a primary non-fluorinated sulfonamide (pK_a ~10–11)[3] and so a significant portion of its conjugate base should be present at physiological pH. Here we report the synthesis of

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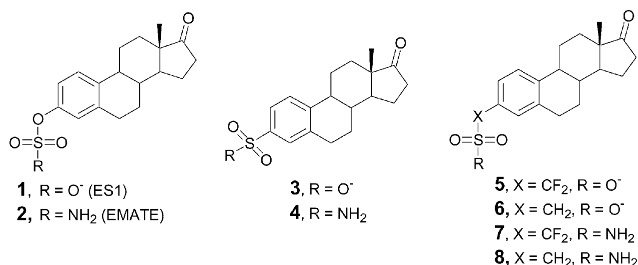
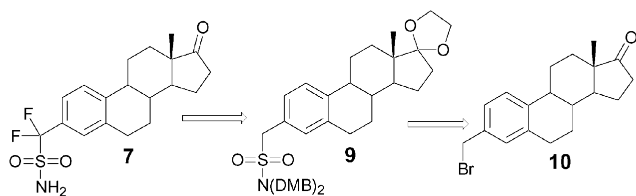
fluorosulfonamide **7** and introduce the diallylamino group as a new protecting group for sulfonamides. We also demonstrate that the pK_a of α-fluorosulfonamides can be determined using ^{19}F NMR. Finally, we report that compound **7** has a K_i similar to that of compound **5** at pH 7.0, is more potent than its nonfluorinated analog **8** and, its inhibitory potency increases with increasing pH, a trend opposite to that of other STS inhibitors.



Results and discussion

Synthesis

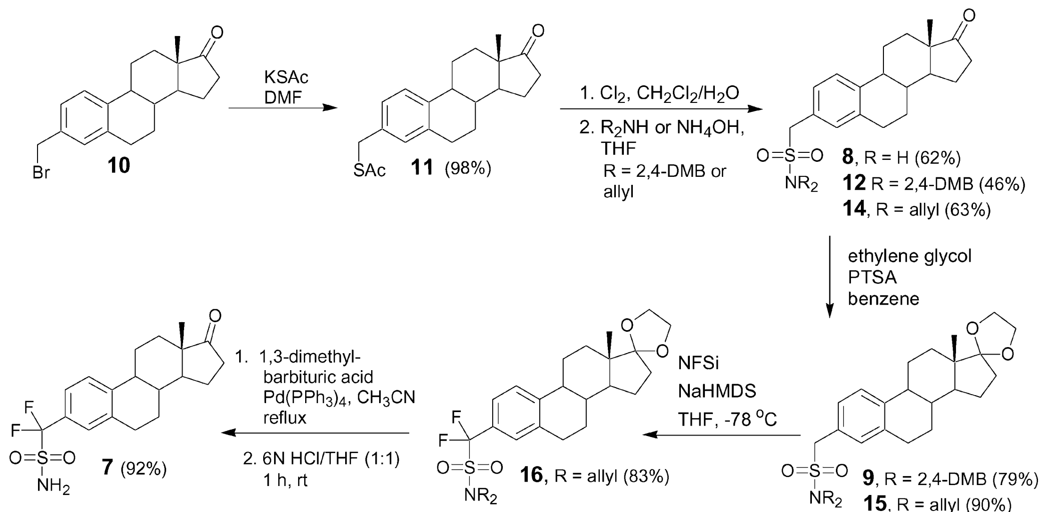
In our initial report on electrophilic fluorination of sulfonamides, we introduced the 2,4-dimethoxybenzyl (DMB) group as a new sulfonamide protecting group.[10] Benzylic sulfonamides protected with this group underwent electrophilic fluorination at the α-position using Na or KHMDS as base and *N*-fluorobenzenesulfonimide (NFSi) as fluorinating agent usually in high yields and the DMB group was easily removed using TFA–CH_2Cl_2. Therefore, we expected that **7** could be constructed by electrophilic fluorination of sulfonamide **9** followed by deprotection (Scheme 1). We anticipated that sulfonamide **9** could be constructed starting from known benzyl bromide **10**[17] (Scheme 1).



Scheme 1

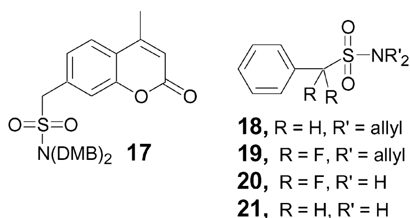
To prepare sulfonamide **9**, bromide **10** was reacted with potassium thioacetate to give thioester **11** in 98% yield (Scheme 2). This species was subjected to oxidative chlorination to give the sulfonylchloride which was then reacted with ammonium hydroxide to give the nonfluorinated sulfonamide **8** in 62% yield. The sulfonyl chloride could also be reacted with bis(2,4-dimethoxybenzyl)amine which gave sulfonamide **12** in 46% yield (2 steps). Protection of the ketone moiety at the 17-position in **12** as a cyclic ketal was accomplished using ethylene glycol–catalytic PTSA which gave compound **9** in 79% yield.

We initially attempted the electrophilic fluorination of **9** using conditions we previously developed for benzylic sulfonamides (2.2 equiv. NaHMDS, 2.5 eq. NFSi, −78 °C–rt).[10] However, no fluorinated product was obtained and only unreacted starting material was recovered or unidentified byproducts. Other bases (Li or KHMDS, BuLi, MeLi, NaH), changes in temperature and reaction times gave similar outcomes. These results were entirely unexpected considering that the coumarin **17** was shown to undergo electrophilic fluorination in 53% yield.[10,19] We reasoned that the α-position might be very sterically hindered due to both the DMB protecting group and the B-ring of the steroid. Therefore, other less sterically demanding sulfonamide protecting groups were considered. Only a small number of protecting groups have been developed for sulfonamides. We previously demonstrated that two of them, the benzyl and 4-methoxybenzyl groups, were very difficult to remove from α,α-difluorosulfonamides and so these groups were not considered as possible replacements for the DMB group in **9**.[10] The dimethylpyrrole group has been used as a protecting group for sulfonamides.[20] However, this group is removed under harsh conditions (refluxing concentrated TFA in H_2O) and we expected that the presence of fluorines would make this group even more difficult to remove as was the case with the 4-methoxybenzyl group.[10] Therefore, we turned to the allyl group which has been used extensively for the protection of amines and esters. Although diallyl sulfonamides are known compounds, the allyl group has never been used as a sulfonamide protecting group. Nevertheless, we anticipated that it's relatively small size would enable us to fluorinate the corresponding steroidal sulfonamide and it could be removed under conditions that have been developed for its removal from amines and esters. Model allyl sulfonamide **18** was readily fluorinated to give sulfonamide **19** in 86% yield using NaHMDS–NFSi. However, attempts to deprotect sulfonamide **19** using $TolSO_2Na$, dimedone or 2-thiobenzoic acid as allyl scavengers in the presence of Rh or Pd catalysts were unsuccessful or proceeded very slowly giving low yields of monodeprotected products after prolonged reaction times. Guibe and coworkers have reported that allyl groups can be removed from amines using 1,3-dimethylbarbituric acid as the allyl scavenger and $Pd(PPh_3)_4$ in CH_2Cl_2 at 30 °C.[21] These conditions resulted in a very slow monodeprotection of **19**. However, by performing the reaction in refluxing CH_3CN, the deprotected product **20** was obtained



Scheme 2

in 93% yield. These results prompted us to apply allyl protection to the synthesis of **7**. Oxidative chlorination of **11** followed by reaction of the sulfonyl chloride with diallyl amine gave sulfonamide **14** in 63% yield. The ketone was protected as before in 90% yield and the resulting compound **15** subjected to NaHMDS–NFSi. This gave the fluorinated sulfonamide **16** in 83% yield which supported our hypothesis that steric factors played a key role in preventing the electrophilic fluorination of sulfonamide **9**. The allyl protecting group in sulfonamide **16** was readily removed using 1,3-dimethylbarbituric acid in the presence of cat. $Pd(PPh_3)_4$ in refluxing CH_3CN. Treating the resulting deprotected product with HCl in THF gave compound **7** in an outstanding 92% yield (two steps).



Inhibition studies

The inhibition of STS with compound **7** was determined at pH 7.0, the optimal pH of the enzyme, in the presence of 0.01% Triton X-100, 10% DMSO at 25 °C using methylumbelliferyl sulfate (MUS) as substrate. The inhibition was reversible and not time dependent which is consistent with previous observations that the irreversible inhibition found with sulfamate STS inhibitors, such as EMATE, requires cleavage of the S–O bond.[11] Compound **7** exhibited mixed inhibition, which is common with many steroidal STS inhibitors and substrates, with a K_i of 82 μM.[16] Under these conditions, sulfonate **5** also exhibits mixed inhibition with a K_i of 73 μM. These values are close to the K_m (95 μM) recently reported for estrone sulfate in 0.1 M Tris-HCl, 0.1% Triton X-100, pH 7.5.[22] The nonfluorinated sulfonamide **8** exhibited mixed inhibition with a K_i of 350 μM at pH 7.0.

To learn more about the effect of the ionization state of **7** on STS inhibition we first determined its pK_a. ^{19}F NMR was used since this technique allowed us to ascertain its pK_a under conditions (0.1 M bis-tris propane, 10% DMSO, 25 °C) similar to those of the inhibition studies (0.1 M Tris buffer, 10% DMSO, 25 °C) and only very small quantities were required. ^{19}F NMR has been used to determine the pK_a values of a wide variety of fluorinated carboxylic acids and it has been shown that accurate pK_a values can be determined even when the total chemical shift difference between neutral and completely deprotonated species is less than 0.2 ppm.[23] Using ^{19}F NMR, excellent titration curves were obtained for **7** (for example, see Fig. 1). From this data, a pK_a of 8.05 ± 02 was determined. We also determined the pK_a of compound **20** to be 8.00 ± 02 under the same conditions using ^{19}F NMR. This is 0.3 pK_a units higher than that determined for **20** in purely aqueous solution at 37 °C using potentiometric titration[8] and this difference is probably due to the presence of the DMSO, buffer and the different temperature (25 *vs.* 37 °C). Thus, at pH 7.0 under our assay conditions, 10% of sulfonamide **7** exists as the conjugate base. The pK_a of sulfonamide **21** in aqueous solution has recently been determined by potentiometric titration to be 10.5.[8] Thus, under our conditions, the pK_a of sulfonamide **8** is probably about 10.8, 0.3 pK_a units higher than **21**. Therefore, at pH 7.0, less than 0.02% of **8** exists as the conjugate base.

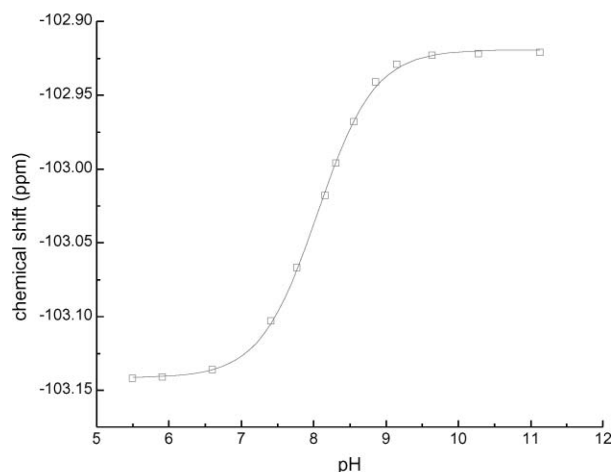


Fig. 1 Titration curve for compound **7** using ^{19}F NMR.

To see if the potency of the inhibitors changed as pH increased, we determined their inhibition of STS between pH 7.0 and 8.8. The K_m for MUS increases over this pH range (Table 1). Other STS substrates also exhibit an increase in K_m with increasing pH.[24] Between pH 8.0–8.8, sulfonate **5** exhibited mainly competitive inhibition, and its K_i increased by approximately 2-fold with increasing pH (Table 1). Since sulfonate **5** is completely ionized over the pH range of this study, then its increase in K_i with pH is most likely due to a change in the ionization state of a residue important for binding. The K_i for the nonfluorinated sulfonamide **8** at pH 8.8 also increased 1.4-fold compared to that at pH 7.0. At all pHs, the fluorinated sulfonamide **7** exhibited mixed inhibition. The K_i for the fluorinated sulfonamide **7** did not change significantly between pH 7.0–8.0. However, above pH 8.0, the K_i decreased and at pH 8.8 it was almost 3-fold lower (28 μM) than at pH 7.0 (Table 1) and 18-fold lower than sulfonamide **8** at pH 8.8.

At pH 7.0, the sulfonamide **7** has an approximately 4.3-fold greater affinity for STS than compound **8**, a modest difference considering the concentration of the conjugate base of inhibitor **7** at physiological pH is at least 500 times greater than that of inhibitor **8**. This would suggest that, at pH 7.0, STS does not have a strong preference for binding to the conjugate base of **7** and has a modest affinity for the neutral form of **7**. The 4.3-fold difference could be due to a variety of factors such as the fluorines interacting with residues in the active site. The K_i for **7** does indeed decrease as the pH increases from 7.0 to 8.8 which is what one would expect if the anion exhibited a greater affinity for the enzyme. However, the decrease is not as large (3-fold) as one would expect based solely on the difference in the concentrations of the neutral and anionic forms of **7** (63-fold higher at pH 8.8 compared to pH 7.0) and if STS did not bind or had an extremely poor affinity for the neutral form. However, our studies with the nonfluorinated sulfonamide **8**

Table 1 Effect of pH on the K_m of MUS and the inhibition of STS with compounds **5**, **7** and **8**

Compound	pH	K_i or K_m (μM)
MUS	7.0	145 ± 7[a]
MUS	8.0	338 ± 18[a]
MUS	8.5	830 ± 57[a]
MUS	8.8	990 ± 70[a]
5	7.0	73 ± 7[b,c]
5	8.0	111 ± 22[b,d]
5	8.5	145 ± 55[b,d]
5	8.8	147 ± 50[b,d]
7	7.0	82 ± 8[b,c]
7	8.0	74 ± 12[b,c]
7	8.5	40 ± 6[b,c]
7	8.8	28 ± 1[b,c]
8	7.0	350 ± 22[b,c]
8	8.0	387 ± 28[b]
8	8.8	503 ± 45[b]

[a] K_m. [b] K_i. [c] Mixed inhibition. Only the K_i for binding to free enzyme is shown. K_i values for binding the ES complex are higher yet follow the same trend as the K_i values reported in the above table. [d] Competitive inhibition.

and with sulfonamide **4**[16] indicate that STS will bind neutral sulfonamides but not with an affinity equal to that of sulfates such as ES1. Thus, STS may have just a modest preference for binding the conjugate base of sulfonamides over their neutral form. The lack of a significant change in K_i for **7** between pH 7.0 and 8.0 may be due to competing processes in which an increase in the concentration of the anionic form (and hence a decrease in K_i) is offset by an unfavorable change in the ionization state of a residue important for binding as is suggested by the increase in K_i with pH found with sulfonate **5** and sulfonamide **8**. The 18-fold difference in K_i values between compounds **7** and **8** at pH 8.8 could be due to differences in the concentrations of their respective conjugate bases as well as other factors such as the fluorines in **7** interacting with residues in the active site. It is interesting to note that sulfonamide **7** and sulfonate **5** exhibit very similar K_i values at pH 7.0. However, at pH 8.8, sulfonamide **7** exhibits an almost 5-fold higher affinity for STS than sulfonate **5**. This would suggest that the anionic sulfonamide moiety of **7** has a greater affinity than the anionic sulfonate moiety in **5** for STS.

Although it appears that the ionization state of **7** is not a major factor in the binding of **7** to STS, this may not necessarily be the case with EMATE. The K_I of EMATE has been determined to be 670 nM using a radiometric assay and crude microsomal preparations of STS at an unspecified pH and so appears to have a considerably greater affinity for STS than compound **7**.[25] Although the pK_a of EMATE in purely aqueous solution is probably lower than that reported in 70% aqueous methanol (9.5), its pK_a is certainly greater than that of inhibitor **7**. If it is the conjugate base of EMATE that interacts with STS, then this would suggest that the anionic sulfamoyl group binds with an affinity that is dramatically greater than the conjugate base of **7**. It is possible that the difluoromethylene linkage in **7** actually hinders the anionic nitrogen from interacting optimally with active site residues while this may not a problem with EMATE. However, comparing K_I values of irreversible inhibitors to K_i values of reversible inhibitors should be approached with caution due to the possibility that the inactivation step by the irreversible inhibitor is completely or partly rate limiting.[26] Nevertheless, our results intimate that the possibility that EMATE may bind to STS as the neutral species cannot be entirely discounted.

The observation that **7** binds better at basic pH may be significant for crystallographic studies. Although the crystal structure of STS has been reported, the structure of an STS-inhibitor complex has not.[15] Such a structure would be very useful for rational inhibitor design. However, one of the potential difficulties in obtaining the structure of an STS-inhibitor complex is that the enzyme is crystallized at pH 8.5 where its affinity for a specific inhibitor may not be optimal as was the case with sulfonate **5**.[27] However, since **7** binds to STS with a greater affinity at basic pHs, then this compound or a derivative of it may enable one to obtain the structure of an STS inhibitor complex.[28]

In summary, we have described the synthesis of sulfonamide **7**, a novel non-hydrolyzable estrone sulfate or EMATE analogue bearing an α,α-difluorosulfonamide moiety at the 3-position on the A-ring. Key to the success of this synthesis was the use of the allyl group as a sulfonamide protecting group. This is the first report of the use of the allyl group as a protecting group for sulfonamides. Very few PGs for sulfonamides have been reported and this group may be very useful not only in the preparation of α-fluorinated sulfonamides but sulfonamides in general. We have also demonstrated that the pK_a of α-fluorosulfonamides can be determined by ^{19}F NMR. We have shown that compound **7** is a reversible STS inhibitor with a greater affinity for STS than its nonfluorinated analogue **8**. Their difference in affinity is pH dependent and may be due to a combination of the fluorines interacting with specific residues in the active site and differences in ionization state of the sulfonamide moiety and enzymatic residues. The fact that **7** binds better at basic pH is also significant for crystallographic studies and such studies are in progress.

Experimental

General methods

All starting materials and reagents were obtained from the Aldrich Chemical Company. THF was distilled from sodium-benzophenone. Silica gel chromatography was performed using silica gel 60 Å (230–400 mesh) obtained from Silicycle (Laval, Quebec, Canada). CH_2Cl_2 was distilled from calcium hydride under nitrogen. DMF was distilled under reduced pressure from calcium hydride onto freshly activated 4 Å sieves under argon. CH_3CN was distilled from calcium hydride under nitrogen. For syntheses, ^{1}H, ^{13}C and ^{19}F NMR spectra were recorded on a Bruker Avance 300 spectrometer. Chemical shifts (δ) for ^{1}H NMR are reported in ppm relative to the internal standard tetramethylsilane (TMS). ^{13}C NMR spectra run are reported in ppm relative to the $CDCl_3$ ($\delta = 77.0$) central peak. ^{19}F NMR are reported in ppm relative to $CFCl_3$ (external). Low-resolution (LRMS) and high-resolution (HRMS) electron impact (EI) mass spectra were obtained on a Micromass 70-S-250 sector mass spectrometer. Infrared spectra were obtained on a Perkin Elmer Spectrum RX Fourier transform spectrophotometer. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Steroid sulfatase was purified as previously described.[29]

Thioacetic acid [3-methylestra-1,3,5-(10)-triene-17-one] ester (11). Potassium thioacetate (3.80 g, 33.3 mmol) was added to a solution of **10** (8.30 g, 23.9 mmol) in DMF (250 ml) and stirred for 16 h. After removal of DMF, the residue was diluted with H_2O (80 ml) and EtOAc (120 ml). The layers were separated and the aqueous layer extracted with EtOAc (2 × 40 ml). The combined organic extracts were washed with H_2O (2 × 60 ml) and brine (60 ml), dried (Na_2SO_4) and evaporated. Purification of the residue by flash chromatography on silica gel using 25% ethyl acetate–hexane as eluent gave pure thioacetate **11** as a white solid (8.00 g, 98%). Mp 78–79 °C; ν_{max}(film)/cm^{-1} 2929, 1739, 1691 and 1133; δ_H (300 MHz, $CDCl_3$) 0.89 (3H, s, CH_3), 2.32 (3H, s, $CH_3C(=O)$), 4.04 (2H, s, CH_2S), 7.00 (1H, s, ArH), 7.05 (1H, d, *J* 8.1, ArH), 7.20 (1H, d, *J* 8.0, ArH); δ_C (75 MHz, $CDCl_3$) 13.9, 21.7, 25.8, 26.5, 29.4, 30.5, 31.6, 33.1, 35.9, 38.1, 44.4, 48.0, 50.5, 125.8, 126.3, 129.4, 135.1, 136.9, 139.0, 195.3, 220.9; *m*/*z* 342 (20, M$^+$), 299 (9), 267 (100); *m*/*z* 342.1657 (M$^+$ $C_{21}H_{26}O_2S$ requires 342.1654).

***N*,*N*-Bis-(2,4-dimethoxybenzyl)-[estra-1,3,5-(10)-triene-17-one]-3-methanesulfonamide (12).** H_2O (18 ml) was added to a solution of **11** (1.20 g, 3.51 mmol) in CH_2Cl_2 (40 ml). The mixture was cooled in an ice bath and Cl_2 was bubbled through the solution slowly. When TLC showed the reaction was complete, the solution was purged with N_2 for 10 min, then cold CH_2Cl_2 (50 ml) was added. After separation of the CH_2Cl_2 layer, the aqueous layer was extracted with cold CH_2Cl_2 (30 ml). The combined organic layers were dried (Na_2SO_4) and evaporated to give 1.30 g estra-1,3,5-(10)-triene-17-one-3-methanesulfonyl chloride as a white foam (ν_{max}(film)/cm^{-1} 2932, 1738, 1372 and 1170; δ_H ($CDCl_3$) 0.89 (3H, s, CH_3), 4.80 (2H, s, CH_2S), 7.18 (1H, s, ArH), 7.22 (1H, d or dd, *J* 9.2, ArH), 7.34 (1H, d, *J* 8.1, ArH)). A solution of bis(2,4-dimethoxybenzyl)amine (1.50 g, 4.73 mmol) in THF (10 ml) was added slowly to a solution of the crude sulfonyl chloride (920 mg, 2.51 mmol) in THF (100 ml) at 0 °C. The mixture was stirred 0.5 h at rt, after which it was evaporated and purified by flash chromatography on silica using 50% ethyl acetate–hexane as eluent to give pure sulfonamide **12** (751 mg, 46%) as a white foam. ν_{max}(film)/cm^{-1} 2935, 1738, 1614, 1589, 1506, 1329, 1292, 1209, 1158, 1125 and

1040; δ_H (300 MHz, $CDCl_3$) 0.79 (3H, s, CH_3), 3.70 (12H, s, OCH_3), 3.96 (2H, s, CH_2S), 4.16 (4H, s, CH_2N), 6.35 (2H, 2s overlapping, ArH), 6.76 (1H, s, ArH), 7.12 (1H, d, *J* 8.0, ArH), 7.20 (1H, d, *J* 8.7, ArH); δ_C (75 MHz, $CDCl_3$) 13.8, 21.6, 25.7, 26.4, 29.3, 31.6, 35.8, 44.3, 38.0, 45.3, 47.9, 50.4, 55.2 (2C), 55.4 (2C), 58.6, 98.3 (2C), 104.3 (2C), 117.2 (2C), 125.5, 127.0, 128.1, 131.2 (2C), 131.4, 136.6, 139.9, 158.4 (2C), 160.6 (2C), 220.5; *m/z* 647 (16, M^+), 582 (1), 497 (4), 432 (10), 404 (4), 316 (39), 267 (28), 178 (39), 151 (100), *m/z* 647.2917 (M^+ $C_{37}H_{45}O_7NS$ requires 647.2917).

Estra-1,3,5-(10)-triene-17-one-3-methanesulfonamide (8). Concentrated NH_4OH (10 mL) was added over 10 min to a solution of estra-1,3,5-(10)-triene-17-one-3-methanesulfonyl chloride (prepared from **11**, 600 mg, 1.64 mmol) in THF (100 ml) at 0 °C and the reaction was stirred overnight. H_2O (80 ml) was added and the mixture was extracted with CH_2Cl_2 (120 ml). The organic layer was washed with brine (30 ml), dried (Na_2SO_4) and evaporated. Purification of the residue by flash chromatography on silica using 60% ethyl acetate–hexane as eluent gave pure sulfonamide **8** (353 mg, 62%) as a white solid. Mp 189–191 °C; ν_{max}(film)/cm^{-1} 3370, 3265, 2903, 1732, 1335, 1163 and 1130; δ_H (300 MHz, $CDCl_3$) 0.87 (3H, s, CH_3), 4.22 (2H, s, CH_2S), 6.02 (2H, s, NH_2), 7.11 (1H, s, ArH), 7.16 (1H, d, *J* 6.4, ArH), 7.26 (1H, d, *J* 7.4, ArH); δ_C (75 MHz, $CDCl_3$) 13.3, 21.3, 25.6, 26.4, 29.1, 31.8, 35.2, 38.2, 44.4, 47.6, 50.3, 60.2, 125.4, 128.2, 131.4, 136.5, 139.8, 218.6; *m/z* 347 (11, M^+), 342 (<1), 284 (1), 267 (100), 105 (10); *m/z* 347.1556 (M^+ $C_{19}H_{25}O_3NS$ requires 347.1555).

***N*,*N*-Diallyl-[estra-1,3,5-(10)-triene-17-one]-3-methanesulfonamide (14).** A solution of estra-1,3,5-(10)-triene-17-one-3-methanesulfonyl chloride (prepared from **11**, 1.50 g, 4.39 mmol) in THF (50 ml) was added over 20 min to a solution of diallylamine (2.0 mL) in THF (120 ml) at 0 °C. The mixture was stirred 1.5 h at rt, after which it was quenched with H_2O (100 ml) and extracted with EtOAc (2 × 100 ml). The combined extracts were washed with H_2O (2 × 100 ml), brine (100 ml), dried (Na_2SO_4) and evaporated. Purification of the residue by silica gel chromatography using 33% ethyl acetate–hexane as eluent gave pure sulfonamide **14** (1.18 g, 63% from **7**) as a colorless oil. ν_{max}(film)/cm^{-1} 2928, 1737, 1642, 1340, 1149 and 929; δ_H (300 MHz, $CDCl_3$) 0.80 (3H, s, CH_3), 3.59 (4H, d, *J* 6.1 CH_2N), 4.05 (2H, s, CH_2S), 5.07 (2H, s, CH_2=), 5.12 (2H, s, CH_2=), 5.64–5.47 (2H, m, C*H*=CH_2), 7.00–7.10 (2H, d and s overlapping, ArH), 7.18 (1H, d, *J* 7.9 Hz, ArH); δ_C (75 MHz, $CDCl_3$) 13.9, 21.6, 25.7, 26.4, 29.3, 31.6, 35.9, 38.0, 44.3, 47.9, 49.6 (2C), 50.4, 58.6, 119.1 (2C), 125.7, 126.6, 128.1, 131.3, 133.2 (2C), 136.9, 140.3, 220.5; *m/z* 427 (1, M^+), 336 (6), 322 (3), 267 (100); *m/z* 427.2177 (M^+ $C_{25}H_{33}O_3NS$ requires 427.2181).

***N*,*N*-Bis(2,4-dimethoxybenzyl) 17,17-ethylenedioxyestra-1,3,5-(10)-triene-3-methanesulfonamide (9).** Ethylene glycol (3 mL) and PTSA (30 mg, 0.17 mmol) were added to a solution of **12** (560 mg, 0.87 mmol) in benzene (60 ml). The mixture was heated under reflux for 4 h using a Dean–Stark trap. The reaction was allowed to cool and then extracted with Et_2O (60 ml). The extract was washed with H_2O (2 × 30 ml), brine (30 ml), dried (Na_2SO_4) and evaporated. Purification of the oily residue by flash chromatography on silica using 45% ethyl acetate–hexane as eluent gave pure **9** (472 mg, 79%) as a white foam. ν_{max}(film)/cm^{-1} 2930, 1614, 1505, 1456, 1329, 1209, 1120 and 1046; δ_H (300 MHz, $CDCl_3$) 0.86 (3H, s, CH_3), 3.75 (s, 6H, OCH_3), 3.78 (s, 6H, OCH_3), 3.84–3.96 (4H, m, OCH_2CH_2O), 4.01 (2H, s, CH_2S), 4.20 (4H, s, CH_2N), 6.41–6.47 (4H, d and s overlapping, ArH), 6.77 (1H, s, ArH), 6.93 (1H, d, *J* 7.8, ArH), 7.19 (1H, d, *J* 8.4, ArH), 7.22 (2H, d, *J* 8.4, ArH); δ_C (75 MHz, $CDCl_3$) 14.4, 22.5, 26.0, 27.0, 29.4, 30.8, 34.3, 38.8, 44.1, 45.4, 46.2, 49.5, 55.2, 55.5, 58.8, 65.7, 65.3, 93.4, 104.2, 117.4, 119.4, 125.6, 126.6, 127.9, 131.3, 136.9, 140.6, 158.4, 160.6; *m/z* 691 (1, M^+), 476 (7), 316 (56), 151 (100), 99 (29); *m/z* 691.3176 (M^+ $C_{39}H_{49}O_8NS$ requires 691.3179).

***N*,*N*-Diallyl 17,17-ethylenedioxyestra-1,3,5-(10)-triene-3-methanesulfonamide (15).** Ethylene glycol (5 mL) and PTSA (280 mg, 1.47 mmol) were added to a solution of **14** (1.18 g, 2.50 mmol) in benzene (60 ml). The mixture was heated under reflux for 4 h using a Dean–Stark trap. After cooling, the benzene layer was washed with H_2O (2 × 30 ml), brine (30 ml), dried (Na_2SO_4) and evaporated. The colorless oil residue was purified by flash chromatography on silica using 28% ethyl acetate–hexane as eluent to give pure **15** (1.17 g, 90%) as a colorless oil. ν_{max}(film)/cm^{-1} 2938, 2873, 1642, 1338, 1158, 1146, 1104, 1044 and 928; δ_H (300 MHz, $CDCl_3$) 0.82 (3H, s, CH_3), 3.62 (4H, d, *J* 6.1, CH_2N), 3.77–3.92 (4H, m, OCH_2CH_2O), 4.08 (2H, s, CH_2S), 5.12 (2H, d, *J* 15.0, CH_2=), 5.13 (2H, d, *J* 11.7, CH_2=), 5.50–5.67 (2H, m, C*H*=CH_2), 7.03 (1H, s, ArH), 7.07 (1H, d, *J* 8.0, ArH), 7.22 (1H, d, *J* 8.0, ArH); δ_C (75 MHz, $CDCl_3$) 14.4, 22.4, 26.0, 26.9, 29.5, 30.7, 34.3, 38.8, 44.0, 46.1, 49.5 (2C), 49.6, 58.7, 64.6, 65.3, 119.1 (2C), 119.3, 125.7, 126.2, 127.9, 131.3, 133.2 (2C), 137.2, 141.0; *m/z* 471 (13, M^+), 406 (6), 380 (2), 311 (100), 99 (88); *m/z* 471.2448 (M^+ $C_{27}H_{37}O_4NS$ requires 471.2443).

***N*,*N*-Diallyl difluoro [17,17-ethylenedioxyestra-1,3,5-(10)-triene]-3-methanesulfonamide (16).** NaHMDS (1.0 M in THF, 4.8 mL, 4.80 mmol) was added over 30 min to a solution of **15** (940 mg, 1.85 mmol) and NFSi (1.60 g, 5.08 mmol) in THF (30 mL) at −78 °C. The mixture was stirred for 2 h at −78 °C, after which it was quenched with sat. NH_4Cl (8 ml), diluted with H_2O (40 ml) and extracted with EtOAc (2 × 120 ml). The combined extracts were washed with H_2O (2 × 80 ml), brine (80 mL), and dried (Na_2SO_4) and evaporated. Purification of the residue by flash chromatography on silica gel using 30% ethyl acetate–hexane as eluent gave pure difluorosulfonamide **16** (844 mg, 83%) as a white solid. Mp 109–110 °C; ν_{max}(film)/cm^{-1} 2939, 2874, 1367, 1175, 1102 and 1044; δ_H (300 MHz, $CDCl_3$) 0.84 (3H, s, CH_3), 3.77–3.89 (4H, m, OCH_2CH_2O), 3.92 (4H, d, *J* 6.2, CH_2N), 5.21 (2H, d, *J* 17.6, CH_2=), 5.23 (2H, d, *J* 9.8, CH_2=), 5.84–5.69 (2H, m, C*H*=CH_2), 7.32–7.42 (3H, m, ArH); δ_C (75 MHz, $CDCl_3$) 14.4, 22.4, 25.9, 26.8, 29.5, 30.7, 34.2, 38.5, 44.3, 46.1 (2C), 49.5, 49.9, 64.6, 65.3, 119.2, 119.9 (2C), 122.3 (t, *J* = 281 Hz), 124.3 (t, *J* 5.7), 125.7, 126.0 (t, *J* 23), 127.6 (t, *J* 6.3), 132.7 (2C), 137.3, 144.7; δ_F (282 MHz, $CDCl_3$) −101; *m/z* 507 (4, M^+), 433 (6), 381 (5), 347 (100), 99 (38); *m/z* 507.2249 (M^+ $C_{27}H_{35}O_4NF_2S$ requires 507.2255).

Difluoro [estra-1,3,5-(10)-triene-17-one]-3-methanesulfonamide (7). $Pd(PPh_3)_4$ (240 mg, 0.207 mmol) was added to a solution of **16** (540 mg, 1.07 mmol) and 1,3-dimethylbarbituric acid (2.00 g, 14.1 mmol) in dry CH_3CN (15 ml). The mixture was heated under reflux in an atmosphere of argon for 18 h, after which it was cooled and diluted with water (50 ml). The mixture was extracted with ether (3 × 50 ml) and the combined extracts washed with H_2O (2 × 50 ml), dried (Na_2SO_4) and evaporated to leave a yellow oil. 6N HCl (15 ml) was added to the solution of the residue in THF (50 ml). The mixture was stirred for 1 h at rt, after which it was diluted with H_2O (50 ml) and extracted with ethyl acetate (3 × 50 ml). The combined extracts were washed with H_2O (50 ml), brine (50 ml), dried (Na_2SO_4) and evaporated. Purification of the residue by flash chromatography on silica gel using 40% ethyl acetate–hexane as eluent gave difluorosulfonamide **7** as a pale yellow solid which was recrystallized from CH_2Cl_2–hexane to give **7** (372 mg, 92%) as a white crystalline solid. Mp 165–167 °C; ν_{max}(film)/cm^{-1} 3262, 2934, 1732, 1368, 1185 and 1089; δ_H (300 MHz, $CDCl_3$) 0.88 (3H, s, CH_3), 5.27 (2H, s, NH_2), 7.34–7.46 (3H, m, ArH); δ_C (75 MHz, $CDCl_3$) 13.9, 21.7, 25.6, 26.2, 29.3, 31.5, 35.9, 37.8, 44.5, 48.1, 50.5, 120.7 (t, *J* 281), 124.6 (t, *J* 6.3), 125.1 (t, *J* 25), 125.8, 127.8 (t, *J* 5.7), 137.3, 144.4, 221.5; δ_F (282 MHz,

$CDCl_3$) −102; *m/z* 383 (1, M^+), 303 (100); *m/z* 383.1373 (M^+ $C_{19}H_{23}O_3NF_2S$ requires 383.1367).

***N*,*N*-Diallyl-1,1-difluoro-1-phenylmethanesulfonamide (19).** NaHMDS (1.0 M in THF, 20 mL, 20 mmol) was added over 1 h to a solution of *N*,*N*-diallyl phenylmethanesulfonamide (**18**, 2.01 g, 8 mmol) and NFSi (6.00 g, 19.2 mmol) in THF (30 ml). The mixture was stirred for 2.5 h at −78 °C, after which it was quenched with sat. NH_4Cl (10 ml) and extracted with EtOAc (2 × 200 ml). The combined extracts were washed with H_2O (2 × 100 ml), brine (30 mL), and dried (Na_2SO_4) and evaporated. Purification of the residue by flash chromatography on silica gel using 20% ethyl acetate–hexane as eluent gave pure **19** (1.97 g, 86%) as a colorless liquid. ν_{max}(film)/cm^{-1} 3084, 2929, 1644, 1453, 1368, 1274, 1175, 1126, 1089 and 930; δ_H (300 MHz, $CDCl_3$) 3.93 (4H, d, *J* 6.5 CH_2), 5.23 (2H, d, *J* 15.7, CH_2=), 5.27 (2H, d, *J* 8.8, CH_2=), 5.70–5.86 (2H, m, C*H*=CH_2), 7.43–7.57 (3H, m, ArH), 7.66 (2H, d, *J* 7.4, ArH); δ_C (75 MHz, $CDCl_3$) 50.0 (2C), 119.9 (2C), 122.0 (t, *J* 280), 127.2 (t, *J* 6.3), 128.6 (2C), 129.1 (t, *J* 22), 132.0, 132.5 (2C); δ_F (282 MHz, $CDCl_3$) −101; *m/z* 287 (1, M^+), 208 (7), 196 (7), 194 (8), 127 (100); *m/z* 287.0800 (M^+ $C_{13}H_{15}O_2NF_2S$ requires 287.0792).

1,1-Difluoro-1-phenylmethanesulfonamide (20). $Pd(PPh_3)_4$ (184 mg, 0.156 mmol) and 1,3-dimethylbarbituric acid (1.42 g, 10.0 mmol) were added to a solution of **19** (287 mg, 1 mmol) in dry CH_3CN (20 ml). The mixture was heated under vigorous reflux under an atmosphere of argon for 16 h, after which it was cooled and diluted with water (50 ml). The mixture was extracted with ether (3 × 50 ml) and the combined extracts were washed with H_2O (2 × 50 ml), dried (Na_2SO_4) and evaporated. Purification of the residue by flash chromatography on silica gel using 33% ethyl acetate–hexane as eluent gave pure difluorosulfonamide **20** (194 mg, 93%) as a white solid. ^1H, ^{13}C and ^{19}F NMR spectra were identical to those reported previously.[10]

Titration of 7 and 20 using ^{19}F NMR. 60 µL of a 3.5 mM solution of **7** or **20** in DMSO were added to a 5 mm NMR tube containing 540 µL of 100 mM bis-tris propane (pH 5.5–11.13). A coaxial insert (Wilmad, NJ, USA, WGS-5BL) containing D_2O was inserted for the purpose of obtaining a lock. ^{19}F NMR spectra were acquired at 25 ± 1 °C on a Bruker Avance 500 at 470.25 MHz. Proton decoupling was not applied. 400 scans were recorded for each spectrum with an interpulse delay of 3 s. Each experiment was performed in duplicate. Under these conditions, the ^{19}F NMR spectrum of **20** appears as a singlet while **7** appears as a doublet. pK_a values were obtained by performing a non linear curve fit to the equation $y = A_2 + (A_1 - A_2)/1 + \exp(x - x°/dx)$, where y is the chemical shift, x is the pH, A_1 and A_2 represent the upper and lower limits of the chemical shifts and x_0 represents the pK_a, to the data using the program ORIGIN (Origin Lab Corp. Northampton, MA, USA). For **7**, identical pK_a values were obtained from plots of the chemical shifts of either peak or their average *versus* pH.

K_i determinations

An appropriate volume of a MUS stock solution in 0.1 M TrisHCl at the desired pH was added to the wells of a 96-well microtiter plate. 0.1 M TrisHCl buffer at the appropriate pH was added to bring the total volume up to 80 µL such that the concentrations of MUS were 104–625 µM (for studies at pH 7.0–8.0) or 125–3125 µM (for studies at pH 8.5 and 8.8). 10 µL of an inhibitor stock solution in DMSO were added. The assay was initiated by the addition of 10 µL of STS (11 mU mL^{-1}) in 20 mM TrisHCl, pH 7.4, 0.1% Triton X-100. The final assay volume was 100 µL. For studies conducted at pH 7.0 and 8.0, the final concentration of MUS was 83.3–500 µM while at pH 8.5 and 8.8, the concentration of MUS was 100–2500 µM. For compounds **5** and **7**, the final concentration of inhibitor was 50, 100 or 200 µM. For compound **8**, the final concentration of inhibitor was 100, 200 or 400 µM. The final concentration of buffer was 92 mM TrisHCl, 0.01% Triton X-100, 10% DMSO and the final enzyme concentration was 1.1 mU mL^{-1}. The production of 4-methylumbelliferone was monitored over 10–15 min using a Spectramax GeminiXS plate reader (excitation 360 nm, emission 460 nm) at 25 °C. Each reaction was performed in triplicate. 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 values were calculated from replots of the slopes or intercepts of the Lineweaver–Burk graphs according to the equations for mixed and competitive inhibition.[30]

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