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Multiple Pathways for the Irreversible Inhibition of Steroid Sulfatase with Quinone Methide-Generating Suicide Inhibitors

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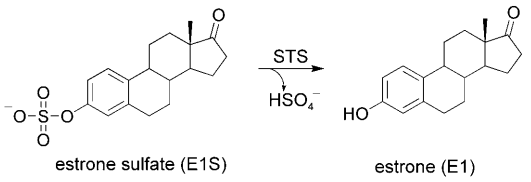
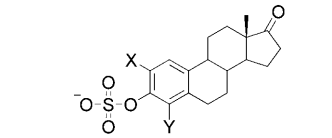
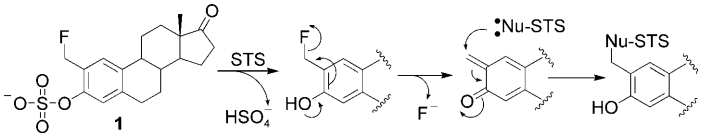
Breast cancer is one of the dominant forms of cancer in North American women. Endocrine therapies, such as estrogen receptor antagonists and aromatase inhibitors are being used to treat estrogen-dependent forms of this disease. More recently, inhibitors of steroid sulfatase (STS), which catalyzes the desulfation of biologically inactive sulfated steroids to biologically active steroids (Scheme 1), are also being examined as potential drugs for treating estrogen-dependent breast cancer, and a number of potent inhibitors have been developed.[1]



Scheme 1. Hydrolysis of estrone sulfate by STS.

Because of their potential as anticancer agents we initiated studies to develop STS inhibitors. Several years ago we suggested that estrone sulfate (E1S) derivatives **1**–**4** could potentially act as suicide inhibitors of STS.[2] Hydrolysis of the S–O bonds in **1**–**4** by STS would produce quinone methides in the active site that could react with residues required for catalysis, thus inactivating STS (as illustrated for compound **1** in Scheme 2). Herein we show that several of these compounds are suicide inhibitors of STS. We also demonstrate that the inhibition of STS by one of these compounds involves an unexpected process in which the main inactivation pathway does not involve reaction of a quinone methide with an active-site nucleophile, and this has led to the discovery of a novel and potent STS inhibitor.





Scheme 2. Proposed mechanism for inhibition of STS with compound **1**.

The synthesis of compounds **1** and **2** is described elsewhere.[2] Compounds **3** and **4** were prepared by using a similar approach (Scheme 3).[3] A trichloroethyl-protected sulfate group was introduced onto 4-formyl estrone (4-FE1)[4] by using trichloroethylsulfuryl chloride[2] to give compound **5**. Selective reduction of the aldehyde in **5** with $Zr(OiPr)_4$–BINOL complex gave hydroxymethyl compound **7**. Treatment of compounds **5** and **7** with DAST gave mono- and difluoromethyl compounds **6** and **8**, respectively, in good yield. Finally, deprotection of the sulfate group in **6** and **8** by catalytic transfer hydrogenolysis by using ammonium formate and Pd/C gave compounds **3** and **4**.

Compounds **1**–**4** were examined for time and concentration-dependent STS inhibition by incubating them with STS at pH 7.0 in 100 mM Tris buffer, withdrawing aliquots at various time intervals, diluting the aliquot into a solution of excess 4-methylumbelliferyl sulfate (4-MUS), a fluorogenic substrate, in the same buffer, and then following the STS activity by fluorimetry.[5] Both monofluoromethyl derivatives (**1** and **3**)[3] exhibited time and concentration-dependent inhibition. The inhibition occurred relatively rapidly within the first few minutes but then slowed and eventually reached a plateau as illustrated in Figure 1 A for compound **1**. However, at high concentrations of inhibitor (100 μM) inactivation continued until almost all activity was lost as illustrated for compound **1** in Figure 1 A. This behavior suggests multiple labeling events are required for irreversible inhibition, or the inhibitors are rapidly consumed and the ratio of the number of times that the inhibitors are turned over is much greater than each enzyme inactivation event (a high partition ratio).[6] In the initial five minutes, pseudo-first-order reaction rates were observed from which a K_i of 68 μM and a k_{inact} of 0.34 min^{-1} were derived for **1** by using the methods of Kitz–Wilson.[3,7] Subjecting the data obtained for compound **3** to a similar analysis yielded a K_i of 3.4 μM and a k_{inact} of 0.056 min^{-1}.[3] Addition of 5 mM β-mercaptoethanol (β-ME), a good nucleophile, had little or no effect on the rate of inactivation of STS with inhibitors **1** (Figure 1 A) and **3**;[3] this suggests that a reactive species was not accumulating in solution, entering the active site and inactivating STS.[8] STS could be

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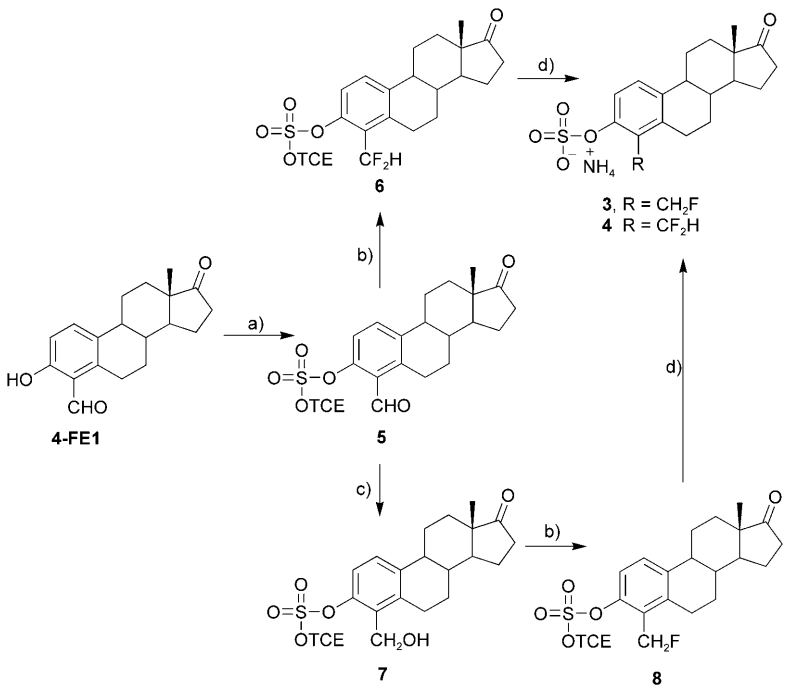


Scheme 3. Synthesis of compounds **3** and **4**. A) trichloroethylsulfuryl chloride. Et_3N, DMAP, THF, 14 h (99%); B) DAST, CH_2Cl_2, 0 °C, 1 h, RT, 3–12 h (83% for **6**, 92% for **8**); C) $Zr(OiPr)_4$, BINOL, toluene, 3.5 h (70%); D) 15–20 wt.% 10% Pd/C,HCO_2NH_4, MeOH, 24 h (86% for **3**, 85% for **4**).

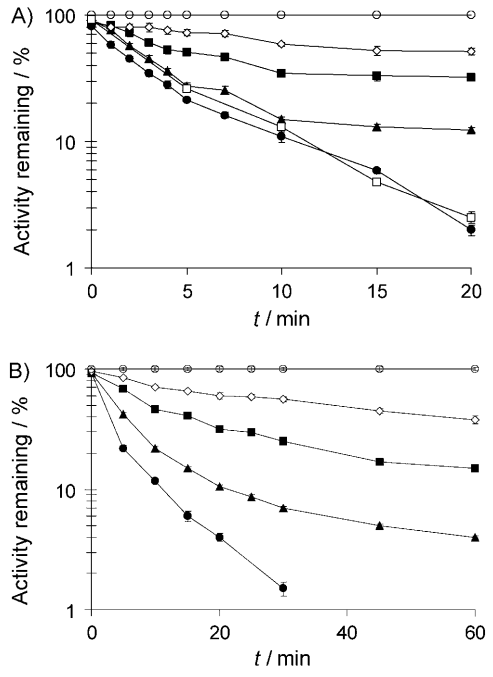


Figure 1. A) Inactivation of STS with inhibitor **1**. ○: 0 μM; ◇: 12.5 μM; ■: 25 μM; ▲: 50 μM; ●: 100 μM; □: 100 μM **1** + 5 mM β-ME. B) Inactivation of STS with 100 μM inhibitor **1** in the presence of E1P. ●: 0 μM E1P; ▲: 50 μM E1P; ■: 250 μM E1P; ◇: 500 μM E1P; ○: 0 μM **1**, 0 μM E1P.

protected against inactivation by inhibitors **1** (Figure 1B) and **3**[3] with estrone phosphate (E1P), a good competitive STS inhibitor;[9] this indicates that irreversible inhibition required active-site binding and enzymatic activation. Finally, after inactivation with inhibitors **1** or **3**, no STS activity could be recovered after extensive dialysis; this emphasizes the irreversibility of the inhibition.

Difluoromethyl derivatives **2** and **4** were screened for STS inhibition by using 1–10 μM of each compound. At these concentrations the 2-difluoromethyl derivative **2** did not exhibit time- and concentration-dependent inhibition, yet was readily consumed by STS as determined by HPLC.[3] In contrast, the 4-difluoromethyl derivative **4** displayed time and concentration-dependent STS inhibition. However, an initial lag phase was observed. This was followed by relatively rapid loss of activity, which did not follow pseudo first-order kinetics (Figure 2A). Moreover, incubation of **4** (10 μM) with STS in the presence of β-ME (5 mM) resulted in an appreciable decrease in the inactivation rate, no lag phase was observed, and the inactivation kinetics were pseudo-first order (Figure 2A). A small amount of activity (approximately 3%) could be recovered after extensive dialysis (10^{12}-fold dilution over 24 h), and E1P protected STS against inactivation (Figure 2B). Taken together,

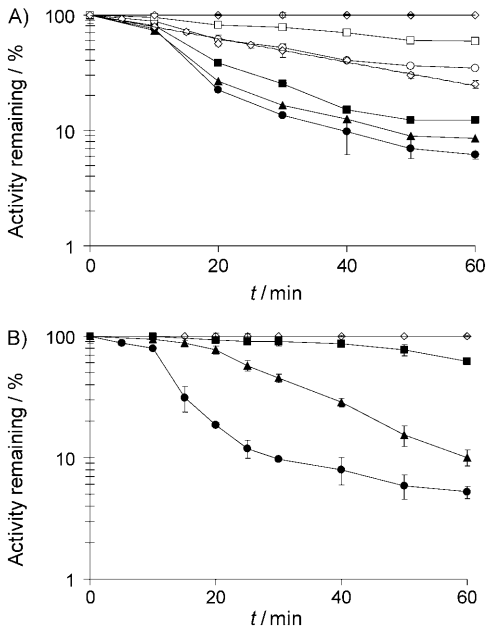
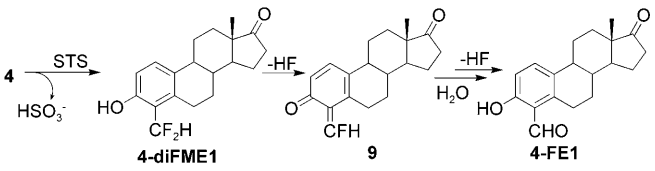


Figure 2. A) Inactivation of STS with inhibitor **4**. ◇: 0 μM; □: 1 μM; ○: 2.5 μM; ■: 5 μM **4**; ▲: 7.5 μM; ●: 10 μM; ◇: 10 μM **4** + 5 mM β-ME. B) Inactivation of STS with 10 μM inhibitor **4** in the presence of E1P. ●: 0 μM E1P; ▲: 1 μM E1P; ■: 5 μM E1P; ◇:0 μM **4**, 0 μM E1P.

these results suggest that upon cleavage of the S–O bond, a reactive species accumulates in solution and then enters the active site and inactivates the enzyme.

When STS hydrolyzes the sulfate group from inhibitor **4**, 4-difluoromethylestrone (4-diFME1, Scheme 4), the initial enzymatic hydrolysis product, can breakdown to form quinone methide **9** in the active site. The quinone methide could then react with an active-site residue(s) and inactiate STS. Alternatively, 4-diFME1 could diffuse out of the active site and breakdown to form compound **9** in solution, which could then react with water to give 4-formyl estrone (4-FE1, Scheme 4). HPLC analysis of the reaction of STS with inhibitor **4** revealed that 4-FE1 was indeed produced (Figure 3 C–E). In addition, we were also able to detect a peak corresponding to 4-diFME1. This species, whose presence was confirmed by spiking the STS-catalyzed reaction with an authentic sample of independently synthesized 4-diFME1[3] (Figure 3 F), is formed as an unstable intermediate and rapidly decomposes to give 4-FE1 (Figure 3 F and G). Because aldehydes have been known to act as highly potent and sometimes almost irreversible enzyme inhibitors,[10] we reasoned that inactivation of STS by compound **4** might be mainly due to inhibition by 4-FE1. Indeed, incubation of STS with just 4-FE1 resulted in time and concentration-dependent inhibition of STS (Figure 4 A). At low concentrations (<1 μM) of 4-FE1 the inactivation plateaus after about 40 min, whereas at higher concentrations (≥ 1 μM) pseudo-first-order behavior was observed throughout, and almost complete inactivation could be achieved within 60 min with just 5 μM. This behavior might be due to multiple labeling events that are both productive and unproductive towards inactivation. Extensive dialysis (10^{12}-fold dilution over 24 h) of the 4-FE1-inactivated enzyme resulted in the recovery of only 5% activity; this indicates that inhibition with 4-FE1 is almost irreversible, although it can best be classified as a slow-binding inhibitor with an extremely slow off-rate. A Kitz–Wilson analysis by using the initial reaction rates (first 30 min) yielded a K_i of 1.5 μM and a k_{inact} of 0.65 min^{-1} (k_{inact}/K_i of 4.3×10^6 $M^{-1}min^{-1}$).[3,11] E1P protected STS against inhibition by 4-FE1; this indicates that inactivation required a reaction with an active-site residue(s) (Figure 4 B). β-ME (5 mM) had no effect on the inhibition with 4-FE1, and this reveals that the effect of β-ME with compound **4** was due to the reaction of β-ME with quinone methide **9**.[3] Thus, the lag phase that is seen with inhibitor **4** is likely due to the time that is required for sufficient 4-FE1 to accumulate in solution and then enter the active site and inhibit STS.[12] The fact that some inhibition still occurs in the presence of 5 mM β-ME indicates that some inhibition by quinone methide **9** occurs, though it appears that inhibition by 4-FE1 is the dominant inhibitory



Scheme 4. Formation of 4-FE1 from inhibitor **4**.

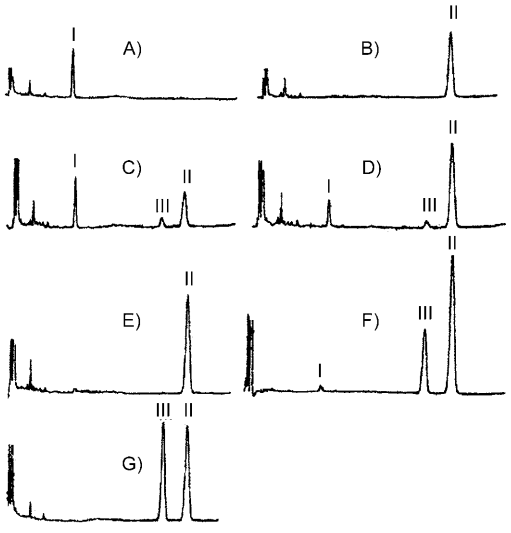


Figure 3. RP-HPLC analysis of the reaction of inhibitor **4**, with STS. A) 10 μM Inhibitor **4** in 0.10 M Tris, pH 7.0, 0.01% Triton X-100 (assay buffer). Peak I corresponds to inhibitor **4** ($t_R = 15$ min); B) 10 μM inhibitor 4-FE1 in assay buffer containing 2% DMSO. Peak II corresponds to 4-FE1 ($t_R = 38$ min). C)–E) 10 μM inhibitor **4** with STS in assay buffer after C) 5 min, D) 10 min, and E) 30 min of reaction. Peak III corresponds to 4-diFME1 ($t_R = 33$ min). F) Reaction of 10 μM inhibitor **4** with STS in assay buffer after 5 min spiked with 25 μM 4-diFME1. G) 25 μM 4-diFME1 in assay buffer. Although the 4-diFME1 was injected into the HPLC within 15 s of dissolving it in the assay buffer, some decomposition to 4-FE1 had occurred in this time interval as evidenced by the presence of peak II.

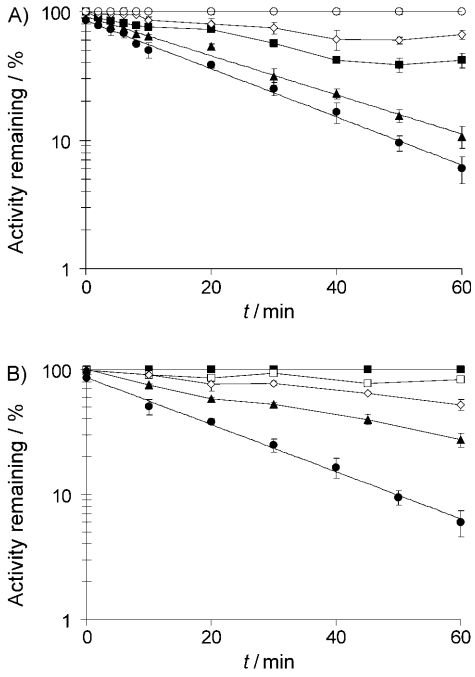


Figure 4. A) Inactivation of STS with inhibitor **4-FE1**. ○: 0 μM; ◇: 0.25 μM; ■: 0.5 μM; ▲: 1 μM; ●: 5 μM. B) Inactivation of STS with 5 μM **4-FE1** in the presence of E1P. ●: 0 μM E1P; ▲:2.5 μM E1P; ◇: 5 μM E1P; □: 25 μM E1P; ■: 0 μM 4-FE1, 0 μM E1P.

pathway. Inhibition of STS with A-ring-formylated estrones exhibits some specificity for the formyl group at the 4-position because incubation of STS with 10 μM 2-formylestrone (2-FE1) did not result in time- and concentration-dependent inhibition; this is consistent with compound **1**, which is a STS substrate that produces 2-FE1 as a product[3] and does not show any time or concentration-dependent inhibition of STS at 10 μM.[13] Although the site of STS modification by 4-FE1 has yet to be established, it is highly likely that it is forming a Schiff base with an active-site residue because it has been shown that certain aldehydes can function as almost irreversible enzyme inhibitors through the formation of stable Schiff base adducts with residues bearing side-chain amines.[10] Lys368 in the active site of STS is a strong candidate for Schiff base formation because it is in close proximity to the 4-position of estrone, which has been modeled into the active site of STS.[14] It is possible that the formyl group of 2-FE1 cannot attain the correct geometry or is too far removed from Lys368 to form a stable Schiff base. The active site has other nucleophilic residues, such as Lys134 and Arg79, that might also be capable of being labeled by 4-FE1 as well as by the quinone methides that are generated from inhibitors **1**, **3**, and **4**.

The finding that the monofluoro derivatives (**1** and **3**) function as classic suicide inhibitors, whereas compound **2** and, to a lesser extent compound **4**, do not, can be explained by the greater stability of the difluoromethyl estrones compared to the monofluoromethylestrones.[15] The more stable difluoromethyl estrones have more time to diffuse out of the active site before decomposition to the corresponding quinone methides. Difluoromethyl-based suicide inhibitors that operate by generation of quinone methides have been reported for other hydrolytic enzymes,[15,16] and some are currently being examined as activity-based probes for proteomic studies.[16] To our knowledge, this is the first example in which this class of inactivator functions by generating an aldehyde that acts as an almost irreversible inhibitor. These studies also underscore the need for a careful kinetic analysis of the inactivation process such that the inhibitory mechanism can be properly addressed.

In summary, we have shown that irreversible STS inhibitors can be obtained by using compounds that produce reactive quinone methides upon activation by STS.[17,18] We also demonstrate that the inhibition of STS by compound **4** involves an unexpected process in which the main inactivation pathway does not involve reaction of a quinone methide with an active-site nucleophile; this has led to the discovery of 4-FE1 as a potent, slow-binding inhibitor of STS. Efforts to improve the potency of 4-FE1, such as by introducing benzylic moieties to the 17-position,[19] are in progress. Further kinetic and mechanistic studies and investigations to determine which residues are being modified are also in progress and will be reported in due course.

Experimental Section

General procedure for the determination of time and concentration-dependent inhibition of STS by compounds 1–4 and 4-FE1: A solution of STS (2 μM, 10 μL) in Tris (20 mM, pH 7.4) containing Triton X-100 (0.1%) was added to solutions of various concentrations of the compounds (90 μL) in Tris (0.10 M, pH 7.0, for compounds **1**–**4**) or Tris (0.10 M, pH 7.0) containing DMSO (2%, for 4-FE1). Controls that did not contain inhibitor were performed for all experiments. These mixtures were allowed to incubate at 22 °C and aliquots (4 μL) were removed at various time intervals and added to the wells of a 96-well microtiter plate containing 4-methylumbelliferyl sulfate (4-MUS, 196 μL, 4 mM, ca. $20\times K_M$[5]) in Tris (0.10 M, pH 7.0) containing Triton X-100 (0.01%). The production of the fluorescent product, 4-methylumbelliferone (4-MU), was followed for 8 min ($\lambda_{ex}=360$ nm, $\lambda_{em}=460$ nm) by using a fluorimeter platereader at 22 °C. All determinations were carried out in triplicate, and errors are reported as the standard deviation. The percent activity of STS in the presence of inhibitor after each time interval was calculated as a percentage of activity in the absence of inhibitor. The percent activity remaining as a function of time was plotted as a semilog graph.

Time- and concentration-dependent inhibition of STS by inhibitors 1, 3, 4 and 4-FE1 in the presence of estrone phosphate (E1P): Studies with E1P were performed in the same manner as described above for the time- and concentration-dependent inhibition studies except various amounts of E1P were present in the incubation mixtures. The concentrations of the inhibitors were 100 μM for inhibitors **1** and **3**, 10 μM for inhibitor **4**, and 5 μM for 4-FE1.

Time- and concentration-dependent inhibition of STS by inhibitors 1, 3, 4 and 4-FE1 in the presence of β-mercaptoethanol (β-ME): Time- and concentration-dependent inhibition studies of STS by compounds **1**, **3** and **4** and 4-FE1 in the presence of β-ME were performed in the same manner as that described above for the time and concentration-dependent inhibition studies except 5 mM β-ME was present in the incubation mixtures. The concentrations of the inhibitors were 100 μM for compounds **1** and **3**, 10 μM for inhibitor **4**, and 5 μM for 4-FE1.

Dialysis experiments: For inhibitors **1**, **3** and **4**, STS (200 nM) was incubated with inhibitor (100 μM of **1** and **3**, 10 μM inhibitor **4**) in Tris (0.10 M, pH 7.0) containing Triton X-100 (0.01%) in a total volume was 200 μL. For 4-FE1, STS was incubated with inhibitor (4-FE1 5 μM) in Tris, (0.10 M, pH 7.0) containing Triton X-100 (0.01%) and DMSO (2%) in a total volume of 200 μL. The mixture was allowed to incubate for 1 h. A control was also performed in an identical manner except that it did not contain inhibitor. Aliquots (4 μL) were withdrawn, and the STS activity was determined in the usual manner. Almost no activity (less than 5%) remained for all of the mixtures. The remaining incubation mixtures were dialyzed in microdialysis units (1 L of 0.1 M Tris, pH 7, 0.1% Triton at 4 °C). The dialysis proceeded for 24 h with the dialysis buffer changed after 3, 6, and 9 h. After 24 h, aliquots (4 μL) were withdrawn from the incubation mixtures and diluted into MUS (4 mM) in Tris (0.1 M, 196 μL, pH 7) and the STS activity was followed in the usual manner.

HPLC analysis of the reaction of compound 4 with STS: Compound **4** (10 μM) was incubated with STS (200 nM) in Tris, (0.10 M, pH 7.0) containing Triton X-100 (0.01%). Aliquots were withdrawn after 5, 10 and 30 min incubation and injected into an HPLC equipped with a C-18 reversed-phase analytical column and a UV detector set at 270 nm. An isocratic gradient consisting of 55% of TFA (0.1%)/H_2O and 45% CH_3CN over 45 min at 1 mL min^{-1} was employed. The retention times for compounds **4**, 4-difluoromethylestrone (4-diFME1) and 4-FE1 are 15, 33, and 38 min respectively.

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Keywords: breast cancer therapy · cancer · inhibitors · steroid sulfatase · steroids

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[8] This does not mean that no quinone methides are being produced outside the active site. Because 2- and 4-hydroxymethylestrone, the products resulting from the reaction of the quinone methides derived from inhibitors **1** and **3** with water, were readily detectable by HPLC (see the Supporting Information) then some partitioning of the quinone methides or their precursors out of the active site is occurring.

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[11] This analysis treats the inactivation process as irreversible, which is reasonable because only a small amount of activity was recovered after 24 h of extensive dialysis.

[12] An alternative explanation for the lag phase and results with β-ME is that quinone methide **9** is accumulating then entering the active site and inhibiting STS. However, it is very unlikely that such a highly reactive species would accumulate in solution to any appreciable extent.

[13] We were unable to perform accurate studies with these inhibitors at concentrations greater than 10 μM due to the tendency of 2- and 4-FE1 to precipitate at higher concentrations. It is possible that 2-FE1 is an inhibitor of STS at concentrations greater than 10 μM.

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