Active Site Directed Inhibition of Estrone Sulfatase by Nonsteroidal Coumarin Sulfamates

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Estrogens are the major mitogens involved in promoting the growth of tumors in endocrine-dependent tissues, such as the breast and endometrium. Although plasma estrogen concentrations are similar in women with or without breast cancer, breast tumor estrone (E1, **1**, Figure 1) and estradiol levels are significantly higher than in normal breast tissue or blood. As *in situ* synthesis of estrogen is thought to make an important contribution to the high levels of estrogens in tumors, specific inhibitors of estrogen biosynthesis should be of potential value for the treatment of endocrine-dependent tumors.

In the past two decades, much effort has been devoted to the development of inhibitors of the aromatase pathway which converts the androgen precursor androstenedione to estrone. However, there is now convincing evidence that the estrone sulfatase (E1-STS) pathway, i.e. the hydrolysis of estrone sulfate (E1S, **2**, Figure 1) to E1, as opposed to the aromatase pathway is the major source of estrogen in breast and endometrial tumors.[1,2] This theory is supported by the only modest reduction of plasma estrogen concentration in postmenopausal women with breast cancer treated by aromatase inhibitors, such as aminoglutethimide and 4-hydroxyandrostenedione[3,4] and the relatively high plasma E1S concentration in these aromatase inhibitor-treated patients. The long half-life of E1S in blood (10−12 h) compared with the unconjugated estrogens (20 min),[5] and high levels of steroid sulfatase activity in liver and normal and malignant breast tissues, also lend support to this theory.[6]

Estrone 3-*O*-sulfamate (EMATE, **3**, Figure 1) is the most potent E1-STS inhibitor developed to date (>99% inhibition of E1-STS activity in intact MCF-7 cells at 0.1 μM) and inhibits the enzyme in a time- and concentration-dependent manner, indicating that it acts as an active site directed inactivator.[7,8] Although EMATE was originally designed for the inhibition of E1-STS, it also inhibits dehydroepiandrosterone sulfatase (DHA-STS), the enzyme which has a pivotal role in regulating the biosynthesis of the estrogenic steroid androstenediol.[8,9] There is now substantial evidence to suggest that androstenediol may be of even greater importance as a promotor of breast tumor growth.[10] EMATE is also active *in vivo* as almost complete

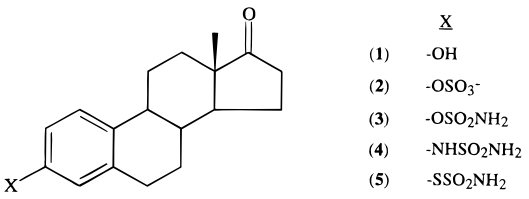
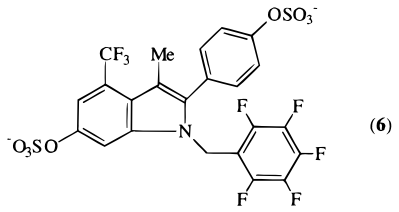


Figure 1. Structures of estrone (**1**), estrone sulfate (**2**), EMATE (**3**), and steroid sulfamates **4** and **5**.



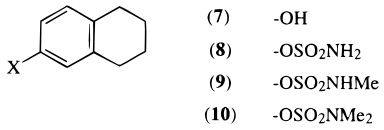


Figure 2. Structures of a phenylindole sulfate steroid sulfatase inhibitor (**6**), THN (**7**), and THN sulfamates **8**−**10**.

inhibition of rat liver E1-STS (99%) and DHA-STS (99%) activities resulted when it was administered either orally or subcutaneously.[11] The bridging O atom of the sulfamate moiety in EMATE is crucial for activity. Thus, when the 3-O atom is replaced by other heteroatoms (Figure 1) as in estrone 3-*N*-sulfamate (**4**) and estrone 3-*S*-sulfamate (**5**), these analogues are only weak non-time-dependent inactivators.[12]

Although optimal potency for inhibition of E1-STS may have been attained in EMATE, a steroid nucleus nevertheless possesses several disadvantages: first, as a consequence of the mechanism of action of this agent, there is a strong likelihood of estrone being released during sulfatase inhibition,[8,12] and second, a recent report demonstrated that, unexpectedly, EMATE and its estradiol congener possess potent estrogenic activity.[13] Moreover, other recently discovered potential therapeutic applications of this remarkable compound mean that it will be highly advantageous to develop potent and orally active irreversible *nonsteroidal* counterparts, which are nonestrogenic and do not possess the capability of being metabolized to compounds with hormonal activity. Thus, EMATE has been shown to have a memory-enhancing effect in rats.[14] Studies in mice have suggested an association between DHA-STS activity and the regulation of part of the immune response which may also occur in humans.[15,16] If a nonsteroidal E1-STS inhibitor could also inhibit DHA-STS, it not only would be more suitable for therapeutic use in breast cancer but also would be more appropriate for other nonmalignant conditions, such as the prevention of autoimmune diseases, when drugs may need to be administered from an early age.

The first attempt to develop such inhibitors was made with the synthesis of sulfate derivatives of 2-phenylindoles, of which one example (**6**, Figure 2) had an IC_{50} of 80 μM, albeit as a *competitive* inhibitor. Such compounds are expected to be substrates for steroid sulfatase, and one of these sulfates was indeed hydrolyzed to the free phenol upon incubation with the enzyme.[17]

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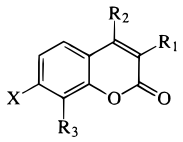
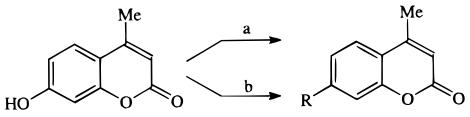


	X	R_1	R_2	R_3
(**11**)	-OH	H	H	H
(**12**)	$-OSO_3^-$	H	CH_3	H
(**13**)	$-OSO_2NH_2$	H	H	H
(**14**)	$-OSO_2NH_2$	H	CH_3	H
(**15**)	$-OSO_2NH_2$	CH_3	CH_3	CH_3
(**16**)	$-OSO_2NH_2$	H	CF_3	H

Figure 3. Structures of 7-hydroxycoumarin (**11**), 7-(sulfooxy)-4-methylcoumarin (**12**), and coumarin sulfamates **13**−**16**.



	R
(**12**)	$-OSO_3^-$
(**14**)	$-OSO_2NH_2$

Figure 4. Route a: Sulfation of 7-hydroxy-4-methylcoumarin; pyridine/SO_3-pyridine complex, NaOH in MeOH. Route b: Sulfamoylation of 7-hydroxy-4-methylcoumarin; NaH/DMF, H_2NSO_2Cl in toluene.

Our first series of nonsteroidal inhibitors was based upon tetrahydronaphth-2-ol (**7**, THN) derivatives (**8**−**10**, Figure 2) which were A/B ring mimics of EMATE. In intact MCF-7 breast cancer cells, it was found that **8** showed E1-STS inhibition activity, although much weaker than EMATE, and the methylated derivatives **9** and **10** were much weaker still.[7] We have now established that **8** only acts as a weak time- and concentration-dependent inhibitor (data not shown). Hence, preincubation of placental microsomes with **8** at 10 μM for 60 min resulted in less than 10% inhibition of E1-STS activity.

In pursuit of alternative nonsteroidal mimics of EMATE, we chose the monohydroxylated coumarin structure, whose ring system is ubiquitous in Nature and in many pharmaceuticals. Our core coumarin, 7-hydroxycoumarin (**11**, Figure 3), should mimic the A and B rings of EMATE and differs from THN at the B ring by possessing an α,β-unsaturated lactone in place of a saturated cyclic hydrocarbon. We anticipated that the extended conjugation of such coumarin structures over THN should enhance the overall activity by virtue of lowering the relative pK_a of the leaving phenol released during enzyme inactivation. Thus, we first synthesized 7-(sulfooxy)-4-methylcoumarin (**12**) to examine if coumarin sulfates are substrates for E1-STS. 7-Hydroxy-4-methylcoumarin in dry pyridine was treated with sulfur trioxide−pyridine complex (2 equiv)[18] under N_2 (Figure 4a). After removal of pyridine, basification, precipitation, and crystallization of the residue from methanol/ether (1:1), pure **12** was obtained. To examine whether **12** could act as a substrate for E1-STS, 100 μg of the compound was incubated for 1 h with placental microsomes in the absence or presence of EMATE (10 μM). The unconjugated coumarin formed at the end of the incubation was extracted with diethyl ether. After evaporation of solvent, the residue was examined by TLC using ethyl acetate/methanol (80:20) as eluent, in which the coumarin sulfate **12** and 7-hydroxy-4-methylcoumarin had R_f values of 0.79 and 0.95, respectively. Only unconjugated 7-hydroxy-4-methylcoumarin was detected after incubation of **12** with placental microsomes. The inclusion of EMATE in the reaction mixture completely abolished the hydrolysis of **12** by E1-STS, indicating that the coumarin sulfate is indeed a substrate for the sulfatase.

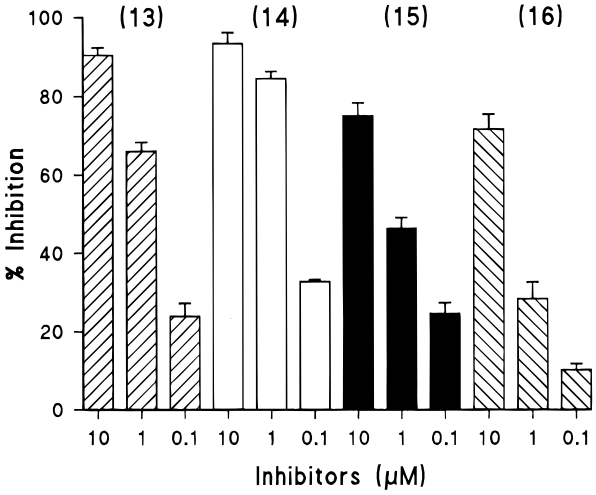


Figure 5. Dose-dependent inhibition of estrone sulfatase in intact MCF-7 breast cancer cells by coumarin 7-*O*-sulfamate (**13**), 4-methylcoumarin 7-*O*-sulfamate (**14**), 3,4,8-trimethylcoumarin 7-*O*-sulfamate (**15**), and 4-(trifluoromethyl)coumarin 7-*O*-sulfamate (**16**). Assays were performed essentially as previously described.[7,8] Monolayers of intact MCF-7 cells in 25 cm^2 flasks were incubated for 20 h at 37 °C with [^{3}H]estrone sulfate (2 nM) and coumarin sulfamates at 0.1−10 μM. Estrone sulfatase activity was determined by measuring the total amount of ^{3}H-labeled estrone and estradiol formed. Sulfatase activity in untreated cells was 100−200 fmol/20 h/10^6 cells. Each point represents the mean ± sd of triplicate measurements.

Having demonstrated that 7-(sulfooxy)-4-methylcoumarin (**12**) was a substrate for E1-STS, it was reasoned that sulfamate derivatives of coumarin were likely to act as sulfatase inhibitors. Coumarin 7-*O*-sulfamate (**13**), 4-methylcoumarin 7-*O*-sulfamate (**14**), 3,4,8-trimethylcoumarin 7-*O*-sulfamate (**15**), and 4-(trifluoromethyl)coumarin 7-*O*-sulfamate (**16**) were then synthesized and evaluated for E1-STS inhibition. Compounds **13**−**16** were prepared by first treating a solution of the appropriate coumarin in anhydrous DMF with sodium hydride (1 equiv) at 0 °C under N_2. Sulfamoyl chloride (ca. 1.5 equiv), stored as a standard solution in dry purified toluene,[12] was then added, and after the mixture was warmed to room temperature overnight and the reaction quenched, the crude product, after workup for each coumarin sulfamate, was purified by flash chromatography and recrystallization (yields, 24−60%). All new compounds were fully characterized by spectroscopic and combustion analysis. The synthesis of 4-methylcoumarin 7-*O*-sulfamate (**14**) is shown in Figure 4b.

These coumarin sulfamates were then tested for their ability to inhibit E1-STS activity using intact MCF-7 breast cancer cells or placental microsomes (100 000 g fraction) essentially as previously described.[7,8,19] The free parent coumarins of all coumarin sulfamates prepared were devoid of E1-STS inhibitory activity when tested up to 10 μM. In contrast, all four coumarin sulfamates **13**−**16** inhibited estrone sulfatase inhibitory activity in a dose-dependent manner (Figure 5), and the inhibition at 10 μM ranged from 71.5% for **16** to 93.3% for **14**. The IC_{50} for inhibition of E1-STS by **14**, the most effective inhibitor, measured using intact MCF-7 cells was 380 nM. As with EMATE, **14** also inhibited E1-STS activity in a time- and concentration-dependent manner in a biphasic fashion (Figure 6), indicating a similar mechanism of action (potential chemical modi

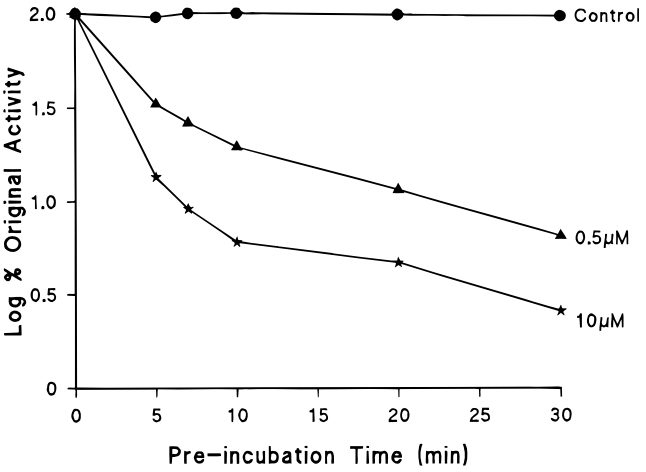


Figure 6. Time- and concentration-dependent inactivation of estrone sulfatase by 4-methylcoumarin 7-*O*-sulfamate (**14**). Placental microsomes (200 μg) were preincubated with **14** (control, ●; 0.5 μM, ▲; and 10 μM, ★) for 0−30 min at 37 °C followed by incubation with dextran−charcoal for 10 min at 4 °C. Dextran−charcoal was sedimented by centrifugation and portions of the supernatants were then incubated with [^{3}H]-estrone sulfate (20 μM) for 1 h at 37 °C to assess remaining sulfatase activity. Duplicate experiments were run at each concentration, but assays for residual activity were taken at different times in each experiment.

fication of two active site residues). At 10 μM, **14** reduced the original E1-STS activity by 95% after preincubating the enzyme with the inhibitor for 20 min. Also, **14** inhibited placental microsomal DHA-STS activity by 93.6% at the same concentration (data not shown).

In order to examine if **14** possessed estrogenic activity and also to test its ability to inhibit E1-STS *in vivo*, it was administered to rats (1 mg/kg subcutaneously in propylene glycol for 5 days) 14 days after ovariectomy had been performed. Administration of **14** did not result in any significant increase in the uterine weight in these rats, showing that **14** was devoid of any estrogenic agonist properties, in contrast to EMATE. Thus, the results, as (uterine weight × 100)/total body weight, expressed as mean ± sd for control, EMATE and **14** were 0.04 ± 0.01, 0.13 ± 0.02, and 0.04 ± 0.02, respectively. The E1-STS activity in the uteri obtained from these animals, determined as described,[11] was inhibited by 89.4% compared with the activity in untreated animals. Preliminary data obtained in a similar fashion to those described previously[11] also demonstrate potent oral activity in rats for **14**, similar to that observed for EMATE.

In summary, nonsteroidal inhibitors have been developed that, while potent *in vivo* as sulfatase inhibitors, are completely devoid of estrogenic activity. Of the coumarin sulfamates tested, 4-methylcoumarin 7-*O*-sulfamate (**14**), together with coumarin 7-*O*-sulfamate (**13**), appear most active *in vitro*, with **14** being shown to act as a time- and concentration-dependent inhibitor like EMATE. These coumarin sulfamates therefore represent key lead compounds for the optimization of nonsteroidal sulfatase inhibition. The further development of such inhibitors should enable the therapeutic use of sulfatase inhibitors to be broadened, not only for endocrine-dependent cancers, but also for other conditions, such as autoimmune diseases.

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Supporting Information Available: Experimental details for **14** (2 pages). Ordering information is given on any current masthead page.

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