Synthesis and Biological Activity of the Superestrogen (*E*)-17-Oximino-3-*O*-sulfamoyl-1,3,5(10)-estratriene: X-ray Crystal Structure of (*E*)-17-Oximino-3-hydroxy-1,3,5(10)-estratriene

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Received December 21, 1998

Steroid sulfatases regulate the formation of estrogenic steroids which can support the growth of endocrine-dependent breast tumors. Therefore, the development of potent steroid sulfatase inhibitors could have considerable therapeutic potential. Several such inhibitors have now been developed including estrone 3-*O*-sulfamate (EMATE, **1**), which shows potent active site-directed inhibition. However, EMATE was subsequently shown to be also a potent estrogen. In an attempt to reduce the estrogenicity while retaining the potent sulfatase inhibitory properties associated with this type of molecule, (*E*)-17-oximino-3-*O*-sulfamoyl-1,3,5(10)-estratriene (**5**) (estrone oxime 3-*O*-sulfamate, OMATE) was synthesized. The X-ray crystal structure of (*E*)-17-oximino-3-hydroxy-1,3,5(10)-estratriene (**4**) (estrone oxime) demonstrated the presence of only one geometrical isomer [*anti*-isomer, (*E*)]. OMATE potently inhibited estrone sulfatase (E1-STS) activity and was similar to EMATE (>99% inhibition at 0.1 μM in MCF-7 breast cancer cells). It was also evaluated in vivo for its estrogenicity and ability to inhibit sulfatase activity. While it was equipotent with EMATE in vivo as a sulfatase inhibitor, it surprisingly had a stimulatory effect on uterine growth in ovariectomized rats about 1.5-fold greater than that of EMATE. Thus, OMATE possesses potential as a superestrogen and modification at C-17 is identified as a useful route for enhancement of estrogenicity in sulfamate-based estrogens.

Introduction

Estrogens have a key role in the growth and development of hormone-dependent breast tumors.[1,2] The origin of estrogenic steroids in postmenopausal women, in whom breast cancer most frequently occurs, is shown in Chart 1.

There is convincing evidence that the estrone sulfatase pathway (Chart 1), i.e., hydrolysis of estrone sulfate to estrone, as opposed to the aromatase pathway is the major route for in situ production of estrogens in breast tumors, accounting for the higher estrogen levels in breast tumors of postmenopausal women than those in plasma from the same individual.[3−5] Although estrone and estrone sulfate have generally been considered to be the major sources of estrogenic steroids, there is now substantial evidence to suggest that the estrogenic steroid androstenediol (Chart 1) may be of importance in the support of growth and development of hormone-dependent breast tumors.[6] Thus, inhibitors of estrone sulfatase (E1-STS) as well as the enzymes regulating the biosynthesis of androstenediol such as dehydroepiandrosterone sulfatase (DHA-STS) are potential drugs to treat estrogen-dependent cancers, such as that of breast, when used alone or in conjunction with aromatase inhibitors.

A number of steroid sulfatase inhibitors have been developed including estrone 3-*O*-sulfamate (EMATE, **1**), estrone 3-*O*-methylthiophosphonate[7] (**2**), and estrone 3-sulfonyl chloride (**3**) (Chart 2).[8] EMATE was the first steroid sulfatase inhibitor exhibiting potent active site-directed irreversible inhibition against both E1-STS and DHA-STS, both in vitro and in vivo orally or subcutaneously.[9−12] Unexpectedly, however, EMATE was found to be a potent estrogen, being ca. 5 times more estrogenic than ethinylestradiol when administered orally to rats.[13] The exact reasons why EMATE is such a potent estrogen remain to be elucidated. However, current evidence suggests that it is acting as a prodrug for estrone.

Due to the sensitivity of endocrine-dependent tumors of the breast and endometrium to estrogens, EMATE would be clearly unsuitable for use as a sulfatase inhibitor in such conditions. In attempts to reduce the estrogenicity of EMATE while retaining the potent sulfatase inhibitory properties associated with this type of molecule, we synthesized a number of A-ring-modified analogues of EMATE including the 2-methoxy, 2/4-nitro, 2/4-*n*-propyl, and 2/4-allyl EMATE analogues.[14] It has also previously been shown that modification of the D-ring of the steroid nucleus reduces estrogenicity compared with the parent compound. For example, it was found that removal of the oxygen function at C-17 of the D-ring of estrone reduced its estrogenicity by about 9 times in a mouse uterotrophic assay by the subcutaneous route.[15]

Modification to the D-ring of the estrone steroid

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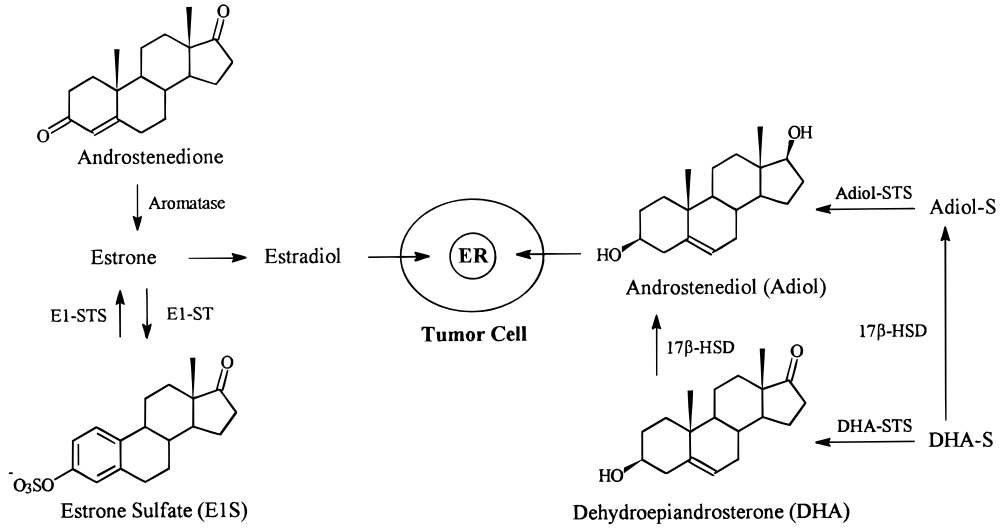
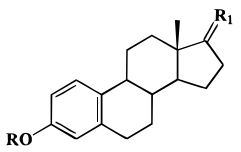
10.1021/jm980717l CCC: $18.00
Published on Web 07/24/1999

Chart 1. Origin of Estrogenic Steroids in Postmenopausal Women[a]



[a] E1, estrone; S, sulfate; ST, sulfotransferase; STS, sulfatase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; Adiol, androstenediol; DHA, dehydroepiandrosterone; ER, estrogen receptor.

Chart 2. Structures of Steroid Sulfatase Inhibitors (**1**−**3**, **5**) and Estrone Oxime (**4**)



	R	R_1
(**1**)	$-SO_2NH_2$	O
(**2**)	$-PS(OH)CH_3$	O
(**3**)	$-SO_2Cl$	O
(**4**)	-H	NOH
(**5**)	$-SO_2NH_2$	NOH

nucleus therefore offers an additional potential route for the development of potent sulfatase inhibitors lacking estrogenicity. 17-Oximino-3-hydroxy-1,3,5(10)-estratriene (**4**) (estrone oxime; (*E*)-stereochemistry assumed) had a weak estrogenic activity (about 40-fold less estrogenic than estrone)[16,17] but was found to possess no antiestrogenic activity.[17] Its relative binding affinity, determined using a calf cytosol preparation, was about 50% lower than the value for estrone.[18] While estrone oxime was considerably less potent than estrone in an estrogenic bioassay, it was only 2.5 times less active as a postcoital antifertility agent[18] suggesting some separation of its uterotrophic and antifertility effects. We therefore synthesized (*E*)-17-oximino-3-*O*-sulfamoyl-1,3,5(10)-estratriene (**5**) (estrone oxime 3-*O*-sulfamate, OMATE). We anticipated that, by introducing a sulfamoyl group as one of the structural requirements for E1-STS inhibitory activity, compound **5** might also possess a lower estrogenicity but retain inhibitory potency against steroid sulfatase.

We report here the synthesis of **5** and an X-ray crystal structure determination for its precursor **4**, to unambiguously assign the oxime stereochemistry. OMATE (**5**) was tested for its ability to inhibit E1-STS activity using placental microsomes, and its estrogenicity was evaluated in vivo using an ovariectomized rat uterine weight gain assay.

Materials and Methods

Chemical Synthesis. All chemicals were either purchased from Aldrich Chemical Co. (Gillingham, Dorset, U.K.) or Lancaster Synthesis (Morecambe, Lancashire, U.K.). All organic solvents, of AR grade, were supplied by Fisons plc (Loughborough, U.K.) and stored over 4 Å molecular sieves. Anhydrous dimethylformamide (DMF), used for all the sulfamoylation reactions, was purchased from Aldrich and was stored under a positive pressure of N_2 after use.

Sulfamoyl chloride was prepared by an adaptation of the method of Appel and Berger[19] and was stored as a solution in toluene as described by Woo et al.[12]

E1-S and E1 were purchased from Sigma Chemical Co. (Poole, U.K.). [6,7-^{3}H]E1-S (specific activity, 50 Ci/mmol) and [4-^{14}C]E1 (specific activity, 52 mCi/mmol) were purchased from New England Nuclear (Boston, MA). [6,7-^{3}H]E1 (specific activity, 97 Ci/mmol) was obtained from the Amersham International Radiochemical Center (Amersham, U.K.).

Thin layer chromatography (TLC) was performed on precoated plates (Merck TLC aluminum sheets silica gel 60 F_{254}, Art. No. 5554). Product(s) and starting material (SM) were detected by either viewing under UV light or treating with a methanolic solution of phosphomolybdic acid followed by heating. Flash column chromatography was performed on silica gel (Sorbsil C60). IR spectra were determined using a Perkin-Elmer 782 infrared spectrophotometer, and peak positions are expressed in cm^{-1}. ^{1}H NMR and DEPT-edited ^{13}C NMR spectra were recorded with JMN-GX400 NMR spectrometers, and chemical shifts are reported in parts per million (ppm, δ) relative to tetramethylsilane (TMS) as an internal standard. Mass spectra were recorded at the Mass Spectrometry Service Center, University of Bath. FAB-MS were carried out using *m*-nitrobenzyl alcohol (NBA) as the matrix, and elemental analyses were performed by the Microanalysis Service, University of Bath. Melting points were determined using a Reichert-Jung Thermo Galen Kofler block and are uncorrected.

(*E*)-17-Oximino-3-hydroxy-1,3,5(10)-estratriene or (*E*)-Estrone Oxime (4). To a solution of estrone (10 g, 36.98 mmol) in ethanol (300 mL) were added hydroxylamine hydrochloride (7.71 g, 111 mmol), sodium hydroxide (3.0 g, 75 mmol), and water (10 mL). The mixture was heated under reflux for 2 h and upon cooling poured into 1 N HCl (100 mL). The precipitate that formed was filtered, washed with cold water, and air-dried to give a white solid (10.13 g, 96%). For analysis, a sample was recrystallized from aqueous methanol (90%) to give **4** as colorless crystals: mp 249−251 °C (lit. mp 248−250 °C);[20] IR (KBr) 3500−2500 (OH), 1690 (−C=N−) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.85 (3H, s, C-18), 1.26−2.80 (14H, m), 3.17 (1.5H, d, J = 5.2 Hz, reduced to a singlet after D_2O exchanged, CH_3OH), 4.13 (0.5H, q, J = 5.2 Hz, exchanged with D_2O), 6.44 (1H, d, $J_{C-2-H,C-4-H}$ = 2.1 Hz, C-4-H), 6.50 (1H, dd, $J_{C-1-H,C-2-H}$ = 8.2 Hz and $J_{C-4-H,C-2-H}$ = 2.4 Hz, C-2-H), 7.04 (1H, d, $J_{C-2-H,C-1-H}$ = 8.5 Hz, C-1-H), 9.03 (1H, br s, exchanged with D_2O, C-3-OH) and 10.1 (1H, br s, exchanged with D_2O, C=N−OH); δ_C (DMSO-d_6, 400 MHz) 17.35 (q, C-18), 22.57 (t),

24.94 (t), 25.99 (t), 26.90 (t), 29.13 (t), 34.33 (t), 37.91 (d), 43.62 (d), 43.61 (s, C-13), 48.66 (q, CH_3 from methanol), 52.52 (d), 112.79 (d, C-2), 114.99 (d, C-4), 126.00 (d, C-1), 130.19 (s), 137.10 (s), 155.02 (s, C-3) and 167.99 (s, C=N); MS *m/z* (FAB+) 439.3 [15, $(M + H + NBA)^+$], 286.3 [100, $(M + H)^+$], 268.3 [20, $(M - H_2O)^+$]; MS *m/z* (FAB−) 437.3 [65, $(M - H + NBA)^-$], 284.2 [100, $(M - H)^-$]; Acc MS (FAB+) 286.18046, $C_{18}H_{24}NO_2$ requires 286.18072. Found: C, 73.5; H, 8.27; N, 4.48. 2($C_{18}H_{23}NO_2$)MeOH requires: C, 73.72; H, 8.36; N, 4.65.

(*E*)-17-Oximino-3-*O*-sulfamoyl-1,3,5(10)-estratriene or (*E*)-Estrone Oxime 3-*O*-Sulfamate (5). A stirred solution of estrone oxime (1.0 g, 3.504 mmol) in anhydrous DMF (20 mL) was treated with sodium hydride (60% dispersion, 280 mg, 7.0 mmol) at 0 °C under an atmosphere of N_2. After evolution of hydrogen had ceased, sulfamoyl chloride in toluene (excess, ca. 5 equiv) was added. The reaction mixture was stirred overnight at room temperature and poured into brine (150 mL), and the organic fractions were extracted into ethyl acetate (200 mL); after further exhaustive washing with brine, the organic layer was dried ($MgSO_4$), filtered, and evaporated in vacuo. Purification of the crude product (1.33 g) obtained by flash chromatography with chloroform/acetone gradient gave a white residue (312 mg) which was further purified by recrystallization from acetone/hexane (1:2) to give **5** as white crystals (289.5 mg, 25%): mp 174−176 °C; IR (KBr) 3400−3280 (NH_2), 1710(−C=N−), 1390 ($-SO_2-$) cm^{-1}; δ_H (DMSO-d_6, 400 MHz) 0.87 (3H, s, C-18), 1.29−2.85 (15H, m), 6.98 (1H, d, $J_{C-2-H,C-4-H} = 2.1$ Hz, C-4-H), 7.02 (1H, dd, $J_{C-1-H,C-2-H} = 8.5$ Hz and $J_{C-4-H,C-2-H} = 2.4$ Hz, C-2-H), 7.35 (1H, d, $J_{C-2-H,C-1-H} = 8.5$ Hz, C-1-H), 7.90 (2H, s, exchanged with D_2O, $-OSO_2NH_2$) and 10.12 (1H, br s, exchanged with D_2O, −C=N−OH); δ_C (400 MHz, DMSO-d_6)17.17 (q, C-18), 22.40 (t), 24.78 (t), 25.60 (t), 26.35 (t), 28.84 (t), 34.12 (t), 37.25 (d), 43.20 (s, C-13), 43.63 (d), 52.32 (d), 119.18 (d, C-2), 121.76 (d, C-4), 126.41 (d, C-1), 138.10 (s), 137.86 (s), 147.86 (s, C-3) 167.71 (s, C=N); MS *m/z* (FAB+) 518.2 [90, $(M + H + NBA)^+$], 365.2 [100, $(M + H)^+$], 285.2 [10, $(M + H - SO_2NH_2)^+$]; MS *m/z* (FAB−) 517.2 [40, $(M - H + NBA)^-$], 363.2 [100, $(M - H)^-$]; Acc MS (FAB+) 365.15451, $C_{18}H_{25}N_2O_4S$ requires 365.15350. Found: C, 59.2; H, 6.84; N, 7.03. $C_{18}H_{24}N_2O_4S$ requires: C, 59.32; H, 6.64; N, 7.69.

X-ray Crystallography. Crystal data: $C_{37}H_{50}N_2O_5$, M = 301.40, monoclinic; a = 7.067(10), b = 13.533(2), c = 17.077(5) Å; β = 96.10(3)°, U = 1624.0(6) A^3; space group $P2_1$, Z = 2, D_c = 1.233 g cm^{-3}; μ(Mo $K\alpha$) = 0.081 mm^{-1}, $F(000)$ = 652. Crystallographic measurements were made at 293(2) K on a CAD4 automatic four-circle diffractometer in the range $2.39° < \theta < 23.93°$. Data (2876 reflections) were corrected for Lorentz and polarization but not for absorption. The structure was solved by direct methods and refined using the SHELX (Sheldrick 1990, 1993)[21,22] suite of programs. Tables of geometric data, indicating H-bonding interactions, are available as Supporting Information.

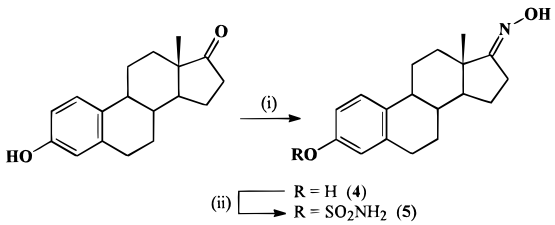
Biological Studies. The ability of estrone oxime to inhibit E1-STS activity in vitro and in vivo was determined as previously described.[7,10,11] Briefly, **5** was initially tested in intact monolayers of MCF-7 cells using [6,7-^{3}H]E1-S (4×10^5 dpm, 2 nM; NEW-DuPont, Boston, MA) and subsequently using placental microsomes using [6,7-^{3}H]E1-S (4×10^5 dpm) adjusted to 20 μM with unlabeled estrone. After incubation of cells or microsomes with substrate ± inhibitor, the product formed was isolated from the mixture with toluene.[7] For in vivo studies **5** was administered orally to ovariectomized rats (2 mg/kg/day for 5 days). E1-STS activity was measured in liver and uterine tissue samples obtained at the end of the treatment period.[11] Uterine weights were recorded and compared with those of animals receiving vehicle (propylene glycol) or EMATE.

Statistics. The significance of differences in uterine weights were assessed using Student's *t*-test.

Results and Discussion

Estrone oxime (**4**) was prepared in excellent yield by an adaptation of the method described by Bernard and Hayes[20] and was converted to the corresponding sulfamate **5** using standard methods[12] (Scheme 1). Of the two possible geometrical isomers for 17-oximes, Nagata et al.[23] and other groups[16,24] assumed formation of the *anti*-isomer, since they believed this form to be more stable than the *syn*-form and that the transition state leading to the *anti*-oxime may have an energy lower than that in the case of the *syn*-isomer.[24] TLC analysis of **4** showed a single spot, in different mobile phases, indicating the formation of one isomer. The ^{1}H NMR spectrum was also consistent with one isomer. In an attempt to confirm which isomer was obtained from this reaction, we attempted some NOE experiments. The results, however, were not conclusive, possibly due to the fact that the hydroxyl proton of the oxime is exchangeable. We therefore had to resort to X-ray crystallography as the only option to resolve the isomer question. A suitable crystal of estrone oxime (approximate dimensions 0.25 × 0.25 × 0.15 mm) was obtained using a slow recrystallization from aqueous methanol and was used for data collection.

Scheme 1. Synthetic Route to OMATE[a]



[a] Reagents and conditions: (i) NaOH/alc., $NH_2OH{\cdot}HCl$, 2 h, reflux; (ii) NaH/DMF, $ClSO_2NH_2$.

An ORTEX plot of the asymmetric unit of estrone oxime is shown in Figure 1a, along with the labeling scheme used. All four rings and the key features of the steroid are clearly visible. The asymmetric unit is seen to consist of two chirally similar molecules hydrogen bonded to each other via their oxime functionalities together with one molecule of methanol. The hydrogen attached to O2A is also involved in hydrogen bonding to the solvent oxygen. The geometric parameters for both molecules are similar within 2 estimated standard deviations. The absolute configuration could not be determined reliably from this structure, despite refining the Flack parameter both for the data presented and also for the inverted structure, but this was not necessary in this case. Figure 1a shows only one geometrical isomer [*anti*-isomer (*E*)]. All C−C bond lengths were in the range 1.351−1.556 Å, and all the C−O bond lengths were between 1.377 and 1.40 Å, with bond angles ranging from 99.6° to 128.21°. All C−N bond lengths were in the range 1.277−1.28 Å, and all N−O bond lengths were between 1.425 and 1.433 Å. An examination of the supramolecular array of **4** revealed that the lattice is dominated by polymeric strands along *b* due to extended hydrogen bonding. In addition to the interactions within the asymmetric unit, H2 also interacts with O1 of the molecule generated via the *x, 1+y, z* symmetry transformation. Also, the proximity of O5 as presented to O1A by the *x, −1+y,* z operation seems indicative of a further interaction between the methanolic proton (not located) and this latter oxygen. The hydrogen-bonding interactions in (*E*)-estrone oxime (**4**), viewed along the *a* axis, are shown in Figure 1b.

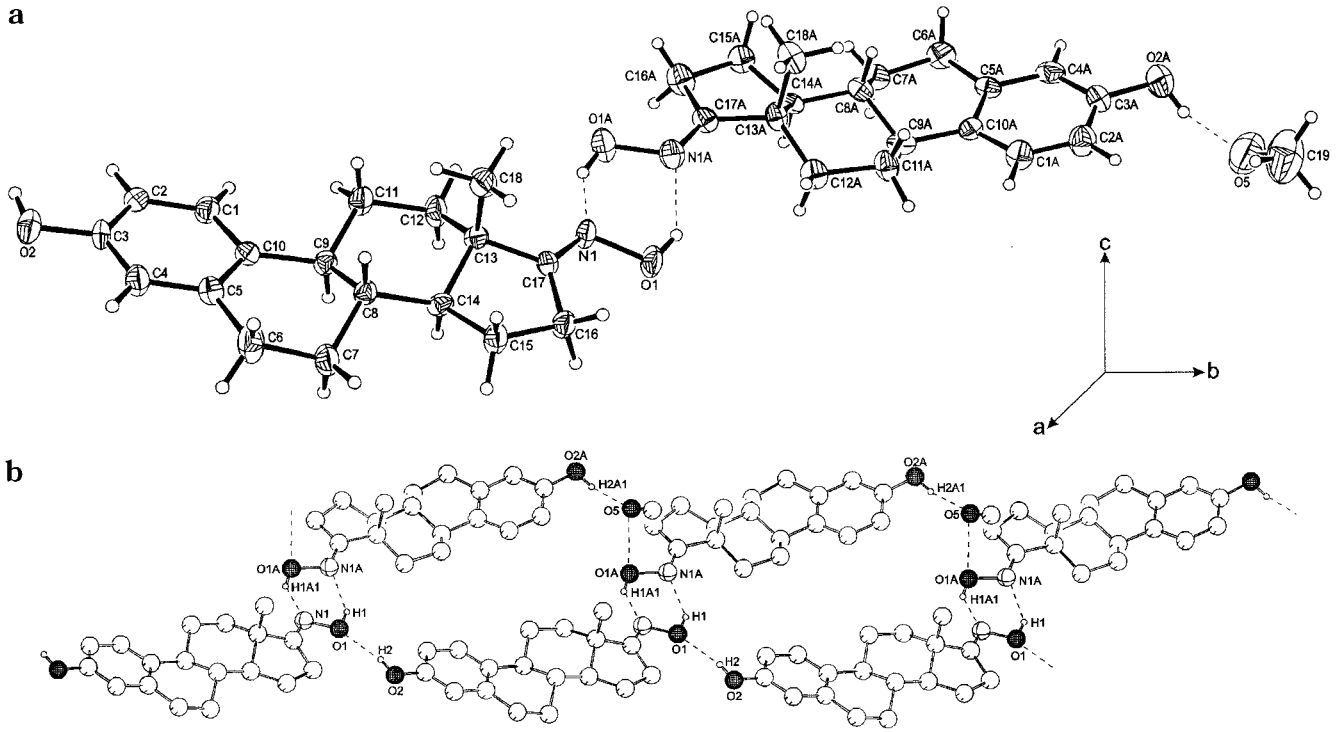


Figure 1. (a) ORTEX plot of asymmetric unit in the X-ray crystal structure of (*E*)-estrone oxime (**4**). Thermal ellipsoids are shown at the 30% probability level. (b) Hydrogen-bonding interactions in (*E*)-estrone oxime (**4**), viewed along the *a* axis (methanol molecules omitted for clarity).

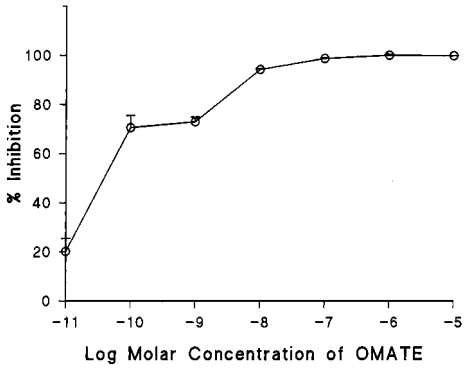


Figure 2. Inhibition of estrone sulfatase activity in intact MCF-7 breast cancer cells by OMATE.

Estrone 3-*O*-sulfamate (**1**) is one of the most potent steroidal E1-STS inhibitors yet synthesized. In vitro, EMATE inhibits E1-STS activity by >99% at 0.1 μM in intact MCF-7 breast cancer cells and in a time- and concentration-dependent manner in a placental microsomes preparation, indicating that it acts as an irreversible inhibitor.[9,10] Subsequent studies have also shown that EMATE inhibits DHA-STS,[10,25] the enzyme that regulates the biosynthesis of the estrogenic steroid androstenediol (Chart 1).

(*E*)-Estrone oxime 3-*O*-sulfamate (OMATE, **5**) was tested for its ability to inhibit estrone sulfatase activity in MCF-7 cells and placental microsomes. It was found to be a highly potent inhibitor in MCF-7 cells (Figure 2, 99% inhibition at 0.1 μM) and thus similar to EMATE in potency. The IC_{50} for inhibition of E1-STS by **5** in placental microsomes was found to be 80 nM, which compares with a value of 93 nM for EMATE.[10] In vivo by the oral route it also inhibited rat liver estrone sulfatase activity to a similar extent to that of EMATE (99%).[11] OMATE was also tested for its estrogenicity using an ovariectomized rat uterine weight gain assay. In ovariectomized rats receiving vehicle only, the average uterine weight (expressed as uterine weight/total body weight × 100) was found to be 0.036 ± 0.001 (mean ± SD). For animals receiving EMATE, there was a significant increase in uterine weight (0.11 ± 0.02, $p < 0.01$) and also for those administered OMATE (0.15 ± 0.01, $p < 0.001$). The difference in uterine weight between animals receiving EMATE or OMATE was also significant ($p < 0.05$). Surprisingly, therefore, this compound stimulated uterine growth with a potency about 50% greater than that of EMATE.

The reason this compound is more estrogenic than EMATE remains to be elucidated. As previously noted, studies carried out by Peters and colleagues[16] reported that estrone oxime was about 40 times *less* estrogenic than estrone on oral administration. However, orally administered estrogens are generally only poorly absorbed from the gastro-intestinal tract, and it was only with the development of synthetic estrogen, ethinylestradiol, that a potent orally active estrogen became available. It has recently been shown that the addition of a sulfamoyl group to estrone renders the product (EMATE) ca. 5 times more estrogenic than ethinylestradiol on oral administration.[13] Thus, the addition of a sulfamoyl group greatly increases the estrogenicity of estrone, and a further enhancement of activity by oral administration has now also been achieved by the introduction of an oxime function at the C-17 position of EMATE.

OMATE is also a potent sulfatase inhibitor, and since the sulfamoyl group is responsible for this activity and the superestrogenicity of OMATE, these activities cannot be separated. This is not likely to be a significant problem since the dose level for use as an estrogen will be considerably lower than that required for use as a sulfatase inhibitor.

It is likely, but remains to be confirmed, that the enhanced oral activity of EMATE, as compared with that of estrone, results from its passage through the liver without undergoing metabolism.[26] Once transit through the liver has been achieved and estrone is released from the prodrug, it will be subjected to the normal pathways of estrogen metabolism and inactivation, i.e., hydroxylation and conjugation. In vivo, there is evidence that after the oral administration of EMATE, estradiol rather than estrone is the major circulating estrogen released.[26] Estradiol can be oxidized to the less potent estrogen, estrone, by estradiol 17β-hydroxysteroid dehydrogenase, type II (E2 DH II), which is widely distributed in body tissues.[27] It is possible that the introduction of the oxime function hinders metabolic inactivation by E2 DH II, thus making OMATE more estrogenic in vivo than EMATE. 17β-Estradiol 3-*O*-sulfamate is now in clinical trial as a potential candidate for hormone replacement therapy and oral contraceptive applications.[26] If the oxime function of OMATE does indeed render it more resistant to metabolic inactivation than EMATE, or its estradiol equivalent, and thus more estrogenic, it, or the next generation of derivatives, may have some advantage over these compounds for the above applications.

In summary, therefore, we have taken a compound reported to be 40-fold less estrogenic than estrone and have converted it to a compound that is not only more estrogenic than estrone but also more potent than EMATE, itself a superestrogen. This illustrates further the dramatic enhancements of estrogencity that can be achieved by 3-*O*-sulfamoylation and demonstrates that still further gain can be made by modification at C-17.

Acknowledgment. This work was supported by Sterix Ltd. We thank Dr. L. W. L. Woo for discussion and a critical reading of the manuscript.

Supporting Information Available: Tables of geometric data. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM980717L