Estrone 3-Sulfate Mimics, Inhibitors of Estrone Sulfatase Activity: Homology Model Construction and Docking Studies†

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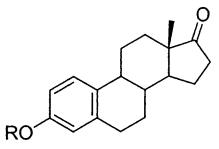
Received August 20, 2002; Revised Manuscript Received October 2, 2002

ABSTRACT: Steroid sulfatase (STS) is a new target for the endocrine therapy of breast cancer. To ascertain some of the requirements for inhibition of estrone sulfatase activity, a number of novel analogues of estrone 3-*O*-sulfate possessing sulfate surrogates were synthesized and evaluated as inhibitors of estrone sulfatase (STS) in comparison to a lead inhibitor, estrone-3-*O*-methylthiophosphonate (E1-3-MTP). Using a selective enzyme digestion, one of the diastereoisomers of this compound, (R_p)-E1-3-MTP, could be prepared and evaluated. From structure−activity studies, we show that chirality at the phosphorus atom, hydrophobicity, basicity, size, and charge all influence the ability of a compound to inhibit estrone sulfatase activity. Of these, hydrophobicity seems to be the most important since simple, active nonsteroidal inhibitors, based on 5,6,7,8-tetrahydronaphth-2-ol (THN), can be prepared, provided that they are lipophilic enough to partition into a nonpolar environment. Also, a negatively charged group is favorable for optimal binding, although it appears that the presence of a potentially cleavable group can compensate for lack of charge in certain cases. A homology model of STS has been constructed from the STS sequence, and molecular docking studies of inhibitors have been performed to broaden the understanding of enzyme/inhibitor interactions. This model clearly shows the positions of the key amino acid residues His136, His290, Lys134, and Lys368 in the putative catalytic region of the formylglycine at position 75, with residues Asp35, Asp36, Asp342, and Gln343 as ligands in the coordination sphere of the magnesium ion. Docking studies using the substrate and estrone-3-sulfate mimics that are active inhibitors indicate they are positioned in the area of proposed catalysis, confirming the predictive power of the model.

Breast cancer is the most prevalent type of cancer in Western countries and approximately one-third of breast tumors are hormone-dependent (*1*). There is considerable evidence derived from epidemiological, clinical, and experimental studies to suggest that estrogens have a central role in supporting the growth of hormone-dependent tumors (*2*). In postmenopausal women, in whom breast cancer most commonly occurs, breast tumor concentrations of estrogens are much higher than estrogen concentrations in the plasma (*3*−*5*). While retention of estrogens in breast tumors by high-affinity binding proteins will contribute to the level of estrogens in breast tumors, estrogen concentrations are similar in both receptor-positive and receptor-negative tumors (*6*). It is therefore likely that local formation of estrogens from estrogen precursors makes an important contribution to the estrogen content of breast tumors.

It is now generally appreciated that estrone sulfate (E1S)[1] (**2**) (Figure 1) is the major source of these estrogens in postmenopausal women and that the action of estrone sulfatase, the enzyme responsible for the hydrolysis of E1S



1 R = H
2 R = SO_3H
3 R = PS(OH)Me
4 R = $SO_2C_6H_4CH_3$
5 R = SO_2NH_2

FIGURE 1: Structures of estrone (**1**), estrone sulfate (**2**), estrone-3-*O*-methylthiophosphonate (E1-3-MTP) (**3**), estrone-3-*p*-toluenesulfonate (**4**), and estrone-3-*O*-sulfamate (EMATE) (**5**).

(**2**) to estrone (**1**), plays a pivotal role in regulation of its production. These conclusions are supported by the findings that (a) 10 times more estrone originates from E1S in breast cancer tissue than from intratumoral aromatization of androstenedione by aromatase (*7*), (b) aromatase activity in breast tumors is a millionfold lower than estrone sulfatase activity (*8*), (c) plasma concentrations of E1S are much higher than unconjugated estrone (*9*), and (d) the half-life of E1S is considerably longer than that of unconjugated estrone (*10*, *11*). Therefore, since E1S is an important source of estrogens in postmenopausal women, it is possible that development of potent inhibitors of estrone sulfatase activity

† This work was supported by Imperial College Exploitation Ltd. and Sterix Ltd.

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[1] Abbreviations: adiol, 5-androstene-3β,17β-diol; ASA, arylsulfatase A; ASB, arylsulfatase B; DHEA-S, dehydroepiandrosterone sulfate; EMATE, estrone-3-*O*-sulfamate; E1-3-MTP, estrone-3-*O*-methylthiophosphonate; E1S, estrone sulfate; MMFF, Merck molecular force field; MOE, molecular operating environment; SVPDE, snake venom phosphodiasterase; STS, steroid sulfatase; TMS, tetramethylsilane; THN, 5,6,7,8-tetrahydronaphth-2-ol.

10.1021/bi020543g CCC: $22.00 © 2002 American Chemical Society
Published on Web 11/21/2002

could be of considerable therapeutic value in the treatment of hormone-dependent breast cancer.

Although the significance of the estrone sulfatase pathway in the biosynthesis of estrogens in postmenopausal women has been recognized for some time, there is now a growing realization that another steroid, 5-androstene-3β,17β-diol (adiol), is maybe of even greater importance as a promoter of breast tumor growth (*12*). This androgenic hormone can bind to the estrogen receptor with high affinity and stimulate the growth of estrogen-positive breast cancer cells even in the presence of an aromatase inhibitor (*12*−*14*), thereby showing that it does not need to undergo conversion to an estrogen to cause tumor growth. In postmenopausal women, isotopic infusion studies have revealed that more than 90% of adiol originates from dehydroepiandrosterone sulfate (DHEA-S) (*15*). This can be converted either into DHEA by DHEA sulfatase, with subsequent metabolism to adiol, or into adiol sulfate, with subsequent hydrolysis to adiol. Thus, in postmenopausal women, it is becoming ever more apparent that, in order to increase their response to endocrine therapy, it will be necessary to inhibit formation of this steroid as well. It is still unclear whether estrone sulfatase and DHEA sulfatase are the same or different enzymes, but from transient transfection of a placental steroid sulfatase cDNA into COS-1 cells, it was found that the expressed protein was able to hydrolyze both E1S and DHEA-S (*16*). This, therefore, implies that inhibition of steroid sulfatase activity in vivo should reduce not only the formation of estrone from E1S but also the production of adiol from DHEA-S.

Several classes of estrone sulfatase inhibitors have been reported in the past few years and include danazol (*17*), sulfate derivatives of 2-phenylindoles (*18*), steroid sulfates (*19*), steroid sulfonyl halides (*20*), steroid phosphates (*19*, *21*, *22*), and most recently a series of nonsteroidal phosphates of which *n*-lauroyltyramine phosphate (*22*) was shown to be the most active. We have also developed and reported on a wide range of reversible and irreversible estrone sulfatase inhibitors including a series of phosphonate derivatives of estrone and tetrahydronaphth-2-ol (THN) (*23*, *24*), with estrone-3-methylthiophosphonate (E1-3-MTP) (**3**) being the most effective reversible inhibitor in this series and our first lead compound. For the sulfonate derivatives of estrone and THN studied (*25*), estrone-3-*p*-toluenesulfonate (**4**) was the best inhibitor, and finally, for the irreversibly inhibiting sulfamate derivatives of estrone and THN (*26*, *27*), estrone-3-*O*-sulfamate (EMATE) (**5**) is the most potent inhibitor reported to date. In a placental microsomal preparation, EMATE was found to inhibit estrone sulfatase activity by >99% at 10 μM, and it had an IC_{50} value of 80 nM (*28*). Subsquent enzyme kinetic studies confirmed that EMATE inhibits estrone sulfatase in a time- and concentration-dependent manner, which indicates that it acts as an active site-directed inactivator (*26*, *28*). EMATE and its estradiol congener have been shown to possess estrogenic activity (*29*), and this has rendered EMATE unsuitable for use in the treatment of hormone-dependent breast cancer. While estrone release may be a direct consequence of its proposed mechanism of action (*30*, *31*) during sulfatase inhibition, the enhanced estrogenicity of EMATE seems most likely to result from its properties as a prodrug of the steroid (*29*). Thus, in our pursuit of compounds to be used in the treatment of hormone-dependent breast cancer, we required other potential inhibitors of estrone sulfatase activity. For one new class of irreversibly acting nonsteroidal sulfamates, of which 667COUMATE is an example, we have discussed extensively the various possible mechanisms of action for irreversible interaction with the active site of the enzyme (*31*). There is, nevertheless, still considerable interest in the development of reversible inhibitors of steroid sulfatase.

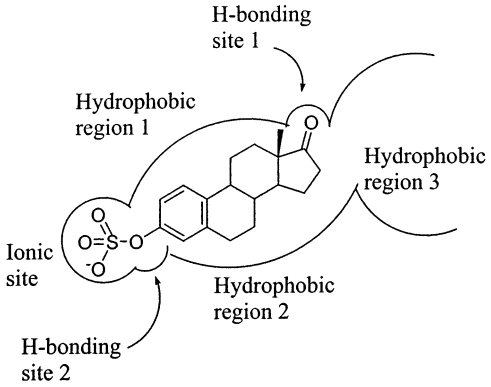


FIGURE 2: Proposed structure for the active site of estrone sulfatase based on its recognition of E1S.

It has been postulated that high-affinity binding to estrone sulfatase is facilitated by the presence of the following groups, which are then able to form important interactions with amino acid residues at the active site of the enzyme: (1) an oxygen anion or an uncharged but highly electronegative substituent at the central ester atom, which is available for ionic interactions; (2) an oxygen atom or sterically and/or electronically similar link between the ring and the sulfonate or the analogous ester moiety, which is able to be involved in hydrogen-bonding interactions; (3) a large carbon skeleton, such as the steroidal structure, which can provide hydrophobic interactions; and (4) a C-17 carbonyl group for hydrogen bonding (*19*, *30*). A simplistic representation is depicted (Figure 2). However, it can be seen from the homology model that the ionic site is in the region of the magnesium ion coordinated by Asp35, Asp36, Asp342, and Gln343 in the area of the formylglycine residue at position 75; other important residues in the H-bonding site 2 region are His136, His290, Lys134, and Lys360. Anderson and co-workers have implied from their studies that the only real essential molecular determinants important for steroid sulfatase inhibitor binding are the A ring of the steroidal nucleus and an appropriately positioned negatively charged group (*22*). However, contrary to both of these hypotheses, EMATE (**5**) is a neutral compound which has neither a negatively charged group nor a highly electronegative substituent at the central ester atom but is, as yet, the best inhibitor of estone sulfatase activity currently discovered. Clearly, therefore, there is a need to investigate further the criteria necessary to ensure tight binding to the enzyme active site. Thus, starting with our first lead inhibitor of E1-3-MTP, we have conducted a structure−activity relationship (SAR) study designed to focus upon the recognition of E1S by estrone sulfatase. Herein we present the synthesis of a number of novel estrone 3-sulfate surrogates, the resulting SAR data obtained, and the development of a homology model for steroid sulfatase to which candidate inhibitors can be docked and scored.

MATERIALS AND METHODS

Materials

All organic solvents were of AR grade and were supplied by Fisons plc (Loughborough, U.K.) and were dried according to the procedures described by Perrin and Armarego (*32*). Estrone and 5,6,7,8-tetrahydronaphth-2-ol were purchased from Sigma and Aldrich Chemical Co., respectively, and were dried prior to use under vacuum. The snake venom phosphodiesterase used was phosphodiesterase I (EC 3.1.41) type VIII-S from *Crotalus durissus terrificus* venom in a 50% glycerol solution containing 5 mM Tris-HCl (pH 7.5) as supplied by Sigma Chemical Co. (activity: 5−15 units/mg of protein at pH 8.9 at 25 °C).

Thin-layer chromatography (TLC) was performed on precoated plates (Merck TLC aluminum sheets, silica $60F_{254}$, article no. 5554). Products were visualized either by UV light or by spraying the plate with phosphomolybdic acid in methanol followed by heating. Flash chromatography refers to the method of Still et al. (*33*) and was carried out using Sorbsil C60 silica gel.

^{1}H, ^{13}C, and ^{31}P NMR spectra were run on JEOL FX90Q and GX270 NMR spectrometers. ^{1}H and ^{13}C chemical shifts were measured in parts per million relative to tetramethylsilane (TMS), and ^{31}P chemical shifts were measured in parts per million relative to external 85% H_3PO_4. *J* values are given in hertz. Melting points (uncorrected) were determined using a Reichert-Jung Thermo Galen Kofler block. Microanalysis was performed by the University of Bath microanalysis service. Mass spectra were recorded at the University of Bath Mass Spectrometry Service and at the SERC Mass Spectrometry Service Centre. Optical rotations were measured using an Optical Activity Ltd. AA-10 polarimeter, and $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. Synthetic compounds were tested for activity in MCF-7 cells as described previously (*24*, *26*, *28*).

Methods

Inhibition of Sulfatase Activity. The ability of compounds to inhibit estrone sulfatase activity in intact MCF-7 breast cancer cells at various concentrations was assessed as described previously (*24*, *26*, *28*). Briefly, intact monolayers of MCF-7 breast cancer cells were incubated for 20 h at 37 °C with [^{3}H]E1S (5 pmol, 7×10^5 dpm, 60 Ci/mmol, NEN-Du Pont, Boston, MA) in serum-free minimal essential medium (2.5 mL) with or without inhibitors (0.1−10 μM). After incubation, medium (1 mL) was removed and product estrone separated from E1S by solvent partition using toluene (5 mL). [4-^{14}C]Estrone (7×10^3 dpm, 52 mCi/mmol. Amersham International, U.K.) was used to correct for procedural losses. An aliquot of the organic phase was added to scintillation fluid and the ^{3}H and ^{14}C content measured by scintillation spectrometry. The mass of E1S hydrolyzed was calculated from the ^{3}H counts detected (corrected for the volume of medium and organic solvent used and for recovery of 4-^{14}C counts) and the specific activity of the substrate.

Two methods were employed to examine whether the most potent inhibitor detected in this series, **3**, was acting in a reversible or irreversible manner to inhibit estrone sulfatase activity. MCF-7 cells were incubated with **3** or **5**, a known irreversible inhibitor, for a 2 h period. After incubation cells were washed with phosphate-buffered saline (PBS, 5 mL × 5) and assayed for remaining activity. For the second method placental microsomes, which are a rich source of estrone sulfatase activity, were incubated with **3** or **5** for 30 min at 37 °C followed by dialysis for 16 h at 4 °C. After dialysis remaining estrone sulfatase was assayed.

Molecular Modeling. Molecular modeling was performed on a Silicon Graphics Octane 2 with a dual R12000 processor. The molecular operating environment (MOE) from the Chemical Computing Group Inc. was used for model building. Docking studies and scoring were performed using FlexX in Sybyl version 6.8 chemical information software from Tripos Inc.

Synthetic Chemistry

*Preparation of Thiophosphonate Derivatives. (A) Preparation of O-(2-Cyanoethyl)thiophosphonate Diesters. (1) Estrone-3-O-(2-cyanoethyl)methylthiophosphonate (**7**).* Methylthiophosphonic dichloride (2.90 mL, 27.74 mmol, 3 equiv) was added dropwise to a stirred solution of estrone (2.50 g, 9.25 mmol, 1 equiv) in anhydrous pyridine (40 mL) at 0 °C under nitrogen. The reaction mixture was allowed to warm to room temperature, and stirring was continued for a further 24 h. The reaction mixture was then cooled to 0 °C, and anhydrous 2-cyanoethanol (3.79 mL, 55.47 mmol, 6 equiv) was added dropwise. The reaction was again allowed to warm to room temperature, whereupon stirring was continued for another 24 h. Subsequently, the reaction mixture was poured into water (100 mL), and the resulting aqueous solution was extracted with ethyl acetate (4 × 75 mL). The combined organic extracts were dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo. Final traces of pyridine were removed by repeated coevaporation with toluene (3 × 30 mL). Purification of the crude material was effected by flash chromatography (98:2 chloroform:methanol) to afford **7** as a pale yellow oil, which solidified on standing (2.14 g, 55%): mp 88−90 °C; R_f 0.59 (98:2 chloroform:methanol); ^{1}H NMR ($CDCl_3$) δ 0.91 (s, 3H, C18-C*H*$_3$), 1.40−1.75 (m, 7H), 1.94−2.60 (series of m, 6H), 2.04 (d, 3H, J = 15.39, P-C*H*$_3$), 2.67−2.78 (m, 2H), 2.92 (m, 2H), 4.30 (m, 2H, OC*H*$_2$CH$_2$CN), 6.92 (br d, 2H, J = 13.73, C2-*H* and C4-*H*), 7.26 (d, 1H, J = 9.16, C1-*H*); ^{13}C NMR ($CDCl_3$) δ 13.78 (q, C18-*C*H$_3$), 19.69 (t), 21.50 (t), 22.64 (q, P-*C*H$_3$), 25.66 (t), 26.24 (t), 29.32 (t), 31.46 (t), 35.78 (t), 37.88 (d), 44.01 (d), 47.87 (s, *C*13), 50.34 (d), 60.91 (t, O*C*H$_2$CH$_2$CN), 61.00 (s), 118.61 (d), 121.44 (d), 126.53 (d), 137.14 (s), 138.34 (s), 147.94 (s), 220.69 (s, *C*O) (CN signal was too weak to assign); ^{31}P NMR ($CDCl_3$) (^{1}H-decoupled) δ 95.16 and 95.19 (ratio of diastereoisomers 1.4:1); MS (EI, 70 eV) *m*/*z* (%) 417 (M^+) (4), 312 (59), 270 (10), 259 (17), 242 (17), 206 (48), 165 (31), 148 (72), 132 (25), 112 (90), 95 (62), 83 (38), 71 (55), 57 (100), 43 (62), 29 (19).

*(2) Estrone-3-O-(2-cyanoethyl)phenylthiophosphonate (**8**).* This was prepared in an identical manner to that of **7**. Phenylthiophosphonic dichloride (784 μL, 5.05 mmol, 3 equiv), estrone (0.46 g, 1.68 mmol, 1 equiv), pyridine (10 mL), and 2-cyanoethanol (1.38 mL, 20.21 mmol, 12 equiv) were used. The crude material was purified by flash chromatography (98:2 chloroform:methanol) to afford **8** as a pale yellow viscous oil which semicrystallized on standing (0.59 g, 73%): R_f 0.65 (98:2 chloroform:methanol); ^{1}H NMR

($CDCl_3$) δ 0.89 (s, 3H, C18-CH_3), 1.40−1.64 (m, 7H), 1.91−2.54 (series of m, 6H), 2.69−2.76 (m, 2H), 2.85−2.88 (br m, 2H), 4.20−4.40 (m, 2H, OCH_2CH$_2$CN), 6.84 (s, 2H, C2-*H* and C4-*H*), 7.20 (d, 1H, J = 8.97, C1-*H*), 7.47−7.63 (m, 3H, P-C$_6H_5$), 7.88−8.07 (m, 2H, P-C$_6H_5$); ^{13}C NMR ($CDCl_3$) δ 13.69 (q, C18-*C*H$_3$), 19.62 (t), 21.41 (t), 25.53 (t), 26.14 (t), 29.19 (t), 31.36 (t), 35.71 (t), 37.75 (d), 43.88 (d), 47.74 (s, *C*13), 50.21 (d), 61.04 (t, OCH$_2$CH$_2$CN), 116.57 (s, *C*N), 118.49 (d), 121.40 (d), 126.30 (d), 128.31 (s), 128.54 (d), 131.07 (d), 131.23 (d), 132.92 (d), 136.84 (s), 138.11 (s), 147.87 (s), 220.62 (s, *C*O); ^{31}P NMR ($CDCl_3$) (^{1}H-decoupled) δ 85.764; MS (CI, isobutane) *m*/*z* (%) 480 (1) ([M + H]$^+$), 437 (3), 281 (100), 256 (10), 228 (13), 212 (6), 194 (6), 174 (4), 141 (7), 94 (17), 69 (7).

(3) 5,6,7,8-Tetrahydronaphth-2-(2-cyanoethyl)methylthiophosphonate (***17***). This was synthesized according to the procedure used to form **7**. Methylthiophosphonic dichloride (1.10 mL, 10.10 mmol, 3 equiv), 5,6,7,8-tetrahydronaphth-2-ol (0.50 g, 3.38 mmol, 1 equiv), pyridine (10 mL), and 2-cyanoethanol (1.38 mL, 20.20 mmol, 6 equiv) were used. Purification of the crude product was effected using flash chromatography (chloroform) to give **17** as a yellow oil (0.74 g, 74%): R_f 0.43 (chloroform); ^{1}H NMR ($CDCl_3$) δ 1.63−1.79 (m, 4H, C6-H_2 and C7-H_2), 2.02 (d, 3H, J = 15.38, P-CH_3), 2.60−2.83 (m, 6H, C5-H_2 and C8-H_2 and OCH$_2$CH_2-CN), 4.15−4.44 (series of m, 2H, *O*CH_2CH$_2$CN), 6.86 (d, 2H, J = 7.50, C1-*H* and C3-*H*), 7.02 (d, 1H, J = 7.88, C4-*H*); ^{13}C NMR ($CDCl_3$) δ 20.00 (t), 20.91 (q, P-*C*H$_3$), 22.73 (t), 22.95 (t), 29.10 (t), 29.55 (t), 61.14 (t, OCH$_2$CH$_2$CN), 116.59 (s, *C*N), 118.64 (d), 121.59 (d), 130.00 (d), 134.32 (s), 138.64 (s), 147.27 (s); ^{31}P NMR ($CDCl_3$) (^{1}H-decoupled) δ 94.923; MS (EI, 70 eV) *m*/*z* (%) 295 (93), 164 (26), 149 (36), 131 (100), 115 (14), 95 (30), 83 (26), 54 (30), 41 (16).

(B) Preparation of Ammonium Salts of Thiophosphonates. (1) Ammonium Salt of Estrone-3-methylthiophosphonate (***3***). A solution of aqueous ammonia (25 mL, specific gravity 88) was added to **7** (0.55 g, 1.32 mmol), and the resulting suspension was heated at 65 °C for 5 h. Subsequently, the solvent was removed in vacuo, and the white residue was azeotroped with toluene (3 × 30 mL) in order to remove the final traces of water. Minor colored impurities were removed from the orange/cream waxy solid by treatment of a solution of this compound in methanol with activated charcoal. Upon solvent evaporation in vacuo, **3** was obtained as a cream foamy solid (0.46 g, 91%); ^{1}H NMR (CD_3OD) δ 0.91 (s, 3H, C18-CH_3), 1.40−3.40 (series of m, 18H), 1.75 (d, 3H, J = 14.65, P-CH_3), 6.99 (br d, 2H, J = 7.88, C2-*H* and C4-*H*), 7.20 (br d, 1H, J = 8.24, C1-*H*); ^{13}C NMR (CD_3-OD) δ 14.27 (q, C18-*C*H$_3$), 16.81 (t), 22.45 (t), 24.70 (q, P-*C*H$_3$), 26.95 (t), 27.57 (t), 30.42 (t), 32.76 (t), 36.68 (d), 39.57 (s, *C*13), 45.34 (d), 51.57 (d), 120.11 (d), 122.77 (d), 126.85 (d), 136.13 (s), 138.53 (s), 151.8 (s), 223.51 (s, *C*O); ^{31}P NMR (CD_3OD) (^{1}H-decoupled) δ 74.43; MS (FAB$^-$, NBA) 363 [(M − H)$^-$]; MS (FAB$^+$, NBA) 365 [(M + H)$^+$].

(2) Ammonium Salt of Estrone-3-phenylthiophosphonate (***9***). This was prepared in an identical manner to that of **3**. Compound **8** (0.32 g, 0.68 mmol) and aqueous ammonia (10 mL, specific gravity 0.88) were used. Minor colored impurities were removed from the product obtained by treatment of a solution of this compound in methanol with activated charcoal. Upon solvent evaporation in vacuo, **9** was afforded as a pale yellow foamy solid (0.18 g, 61%): ^{1}H NMR (CD_3-OD) δ 0.89 (s, 3H, C18-CH_3), 1.42−1.75 (m, 7H), 1.84−2.77 (series of m, 6H), 2.84−2.87 (br m, 3H), 3.20 (t, 2H, J = 6.96), 6.81 (br d, 2H, J = 8.79, C2-*H* and C4-*H*), 7.11 (br d, 1H, J = 8.79, C1-*H*), 7.39 (br s, 3H, P-C$_6H_5$), 7.92−7.95 (br m, 2H, P-C$_6H_5$); ^{13}C NMR (CD_3OD) δ 14.34 (q, C18-*C*H$_3$), 16.74 (t), 22.51 (t), 26.99 (t), 27.64 (t), 30.46 (t), 32.83 (t), 36.78 (d), 39.64 (s, *C*13), 45.41 (d), 51.64 (d), 117.77 (d), 120.25 (d), 122.90 (d), 126.76 (d), 128.54 (s), 128.74 (d), 131.27 (d), 131.69 (d), 131.85 (d), 136.03 (s), 138.43 (s), 151.77 (s), 223.80 (s, *C*O); ^{31}P NMR (CD_3OD) (^{1}H-decoupled) δ 66.54; MS (FAB$^-$, NBA) 425 [(M − H)$^-$]; MS (FAB$^+$, NBA) 427 [(M + H)$^+$].

(3) Ammonium Salt of 5,6,7,8-Tetrahydronaphth-2-methylthiophosphonate (***18***). This was synthesized according to the procedure used to form **3**. Compound **17** (0.33 g, 1.12 mmol) and aqueous ammonia (16 mL, specific gravity 0.88) were used. Again minor colored impurities were removed from the product obtained by treatment of a solution of this compound in chloroform with activated charcoal. Upon solvent evaporation, **18** was obtained as a pale yellow foamy solid (0.28 g, 98%): ^{1}H NMR ($CDCl_3$) δ 1.75 (quintet, 4H, J = 3.30, C6-H_2 and C7-H_2), 1.97 (d, 3H, J = 15.75, P-CH_3), 2.70 (br d, 4H, J = 4.03, C5-H_2 and C8-H_2), 6.89 (d, 2H, J = 8.97, C1-*H* and C3-*H*), 6.98 (d, 1H, J = 7.88, C4-*H*); ^{13}C NMR ($CDCl_3$) δ 20.98 (q, P-*C*H$_3$), 22.74 (t), 22.98 (t), 28.67 (t), 29.29 (t), 118.58 (d), 121.53 (d), 129.85 (d), 134.01 (s), 138.43 (s), 147.62 (s); ^{31}P NMR ($CDCl_3$) (^{1}H-decoupled) δ 76.52; MS (FAB$^-$, NBA) 241 [(M − H)$^-$]; MS (FAB$^+$, NBA) 243 [(M + H)$^+$].

Preparation of Phosphonate Derivatives. (A) Estrone-3-methylphosphonate (***10***). This was prepared according to the procedure described by Cox et al. (*34*). Methylphosphonic dichloride (0.74 g, 5.55 mmol, 3 equiv) was added to a stirred solution of estrone (0.51 g, 1.88 mmol, 1 equiv) in anhydrous pyridine (7 mL) at 0 °C under nitrogen. The reaction mixture was allowed to warm to room temperature, and stirring was continued for a further 24 h. Subsequently, the reaction mixture was poured onto ice (20 mL), and the resulting aqueous solution was extracted with ethyl acetate (4 × 15 mL). The combined organic extracts were dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo to afford a pale brown gum. Final traces of pyridine were removed by repeated coevaporation with toluene (3 × 20 mL). K_2HPO_4 buffer (0.25 M, pH 8.69, 7 mL) was added to the residue, and the mixture was warmed gently until a white, cloudy solution was obtained. The aqueous solution was washed with ethyl acetate (3 × 6 mL) before being rendered acidic (pH 2) by dropwise addition of a 2 M (aqueous) solution of hydrochloric acid whereupon the desired compound **10** precipitated out of solution. The white solid was collected by suction filtration and dried over phosphorus pentoxide. Recrystallization from methanol:water (1:1) afforded **10** as a white crystalline solid (0.32 g, 48%): mp 104−106 °C; ^{1}H NMR (DMSO-d_6) δ 0.83 (s, 3H, C18-CH_3), 1.37−1.70 (m, 6H), 1.45(d, 3H, J = 17.22, P-CH_3), 1.75−2.50 (series of m, 7H), 2.84 (br m, 2H), 6.91 (br d, 2H, J = 13.02, C2-*H* and C4-*H*), 7.26 (br d, 1H, J = 8.25, C1-*H*); ^{13}C NMR (DMSO-d_6) δ 12.32 (q, C18-*C*H$_3$), 13.59 (q, P-*C*H$_3$), 21.24 (t), 25.49 (t), 25.98 (t), 29.06 (t), 31.43 (t), 35.48 (t), 37.65 (d), 43.62 (d), 47.42 (s, *C*13), 49.66 (d), 117.93 (d), 120.49 (d), 126.56 (d), 135.71 (s), 137.91 (s), 148.66 (s), 219.75 (s, *C*O); ^{31}P NMR (DMSO-d_6) (^{1}H-

decoupled) δ 24.47; MS (FAB$^-$, NBA) 347 [(M − H)$^-$]; MS (FAB$^+$, NBA) 349 [(M + H)$^+$]. Anal. ($C_{19}H_{25}PO_4$) C, H, N.

*(B) Estrone-3-ethylphosphonate (**11**)*. This was prepared in an identical manner to that of **10**. Ethylphosphonic dichloride (409.81 μL, 3.84 mmol, 3 equiv), estrone (0.35 g, 1.28 mmol, 1 equiv), and pyridine (8 mL) were used. The product obtained was purified by precipitation from chloroform by addition of pentane whereupon **11** was afforded as a white crystalline solid (0.32 g, 70%): mp 208−210 °C; ^{1}H NMR ($CDCl_3$) δ 0.89 (s, 3H, C18-C*H*$_3$), 1.19 (dt, 3H, J = 20.89, J = 7.51, P-CH$_2$C*H*$_3$), 1.35−1.66 (m, 6H), 1.74−2.56 (series of m, 9H), 2.86 (m, 2H), 6.93 (m, 2H, C2-*H* and C4-*H*), 7.19 (d, 1H, J = 8.43, C1-*H*), 9.73 (br s, exchangeable, 1H, O*H*); ^{13}C NMR ($CDCl_3$) δ 6.21 (q, P-CH$_2$C*H*$_3$), 13.80 (q, C18-*C*H$_3$), 17.76, 19.88 (dt, J = 144.30, P-*C*H$_2$CH$_3$), 21.55 (t), 25.75 (t), 26.34 (t), 29.35 (t), 31.54 (t), 35.81 (t), 38.05 (d), 44.05 (d), 47.91 (s, *C*13), 50.42 (d), 117.93 (d), 120.67 (d), 126.48 (d), 136.36 (s), 138.16 (s), 148.04 (s), 220.66 (s, *C*O); ^{31}P NMR ($CDCl_3$) (^{1}H-decoupled) δ 33.71; MS (FAB$^-$, NBA) 361 [(M − H)$^-$]; MS (FAB$^+$, NBA) 363 [(M + H)$^+$]. Anal. ($C_{20}H_{27}O_4P\cdot 0.75H_2O$) C, H, N.

*(C) Estrone-3-phenylphosphonate (**12**)*. This was prepared according to the same procedure used to form **10**. Phenylphosphonic dichloride (786.67 μL, 5.55 mmol, 3 equiv), estrone (0.50 g, 1.85 mmol, 1 equiv), and pyridine (12 mL) were used. The product obtained was purified by precipitation from dichloromethane by addition of pentane to yield **12** as a white crystalline solid (0.58 g, 77%): mp 200−202 °C; ^{1}H NMR ($CDCl_3$) δ 0.88 (s, 3H, C18-C*H*$_3$), 1.25−1.70 (m, 6H), 1.91−2.54 (series of m, 7H), 2.78 (br m, 2H), 6.85 (d, 2H, J = 7.69, C2-*H* and C4-*H*), 7.08 (d, 1H, J = 8.06, C1-*H*) 7.52 (m, 3H), 7.83 (m, 2H), 9.62 (br s, exchangeable, 1H, O*H*); ^{13}C NMR ($CDCl_3$) δ 13.78 (q, C18-*C*H$_3$), 21.52 (t), 25.67 (t), 26.27 (t), 29.22 (t), 31.51 (t), 35.78 (t), 37.93 (d), 43.98 (d), 47.87 (s, *C*13), 50.40 (d), 117.85 (d), 120.69 (d), 126.32 (d), 128.06 (s), 128.20 (d), 128.44 (d), 131.46 (d), 131.61 (d), 132.55 (d), 136.24 (s), 138.03 (s), 148.16 (s), 220.66 (d, *C*O); ^{31}P NMR ($CDCl_3$) (^{1}H-decoupled) δ 17.16; MS (FAB$^-$, NBA) 409 [(M − H)$^-$]; MS (FAB$^+$, NBA) 411 [(M + H)$^+$]; Anal. ($C_{24}H_{27}O_4P\cdot H_2O$) C, H, N.

*(D) 5,6,7,8-Tetrahydronaphth-2-methylphosphonate (**19**)*. This was prepared using the same method as had been used to make **10**. Methylphosphonic dichloride (0.67 g, 5.06 mmol, 3 equiv), 5,6,7,8-tetrahydronaphth-2-ol (0.25 g, 1.69 mmol, 1 equiv), and pyridine (2 mL) were used. Compound **19** was obtained as a pale yellow oil (0.06 g, 15%): ^{1}H NMR ($CDCl_3$) δ 1.58 (d, 3H, J = 17.95, P-C*H*$_3$), 1.75−1.80 (quintet, 4H, J = 3.30, C6-*H*$_2$ and C7-*H*$_2$), 2.73 (br d, 4H, J = 3.66, C5-*H*$_2$ and C8-*H*$_2$), 6.90 (d, 2H, J = 6.60, C1-*H* and C3-*H*), 7.00 (d, 1H, J = 8.79, C4-*H*), 12.45 (br s, exchangeable, 1H, O*H*); ^{13}C NMR ($CDCl_3$) δ 10.22, 12.39 (dq, J = 147.60, P-*C*H$_3$), 22.80 (t), 23.03 (t), 28.70 (t), 29.32 (t), 117.80 (d), 120.76 (d), 130.06 (d), 133.76 (s), 138.60 (s), 147.58 (s); ^{31}P NMR ($CDCl_3$) (^{1}H-decoupled) δ 30.25; MS (FAB$^-$, NBA) 225 [(M − H)$^-$]; MS (FAB$^+$, NBA) 227 [(M + H)$^+$].

*(E) 5,6,7,8-Tetrahydronaphth-2-phenylphosphonate (**20**)*. This was prepared in an identical manner to that of **10**. Phenylphosphonic dichloride (718 μL, 5.06 mmol, 3 equiv), 5,6,7,8-tetrahydronaphth-2-ol (0.25 g, 1.69 mmol, 1 equiv), and pyridine (12 mL) were used. Compound **20** was afforded as a pale yellow, viscous oil (0.22 g, 46%): ^{1}H NMR ($CDCl_3$) δ 1.67 (t, 4H, J = 3.20, C6-*H*$_2$ and C7-*H*$_2$), 2.60 (d, 4H, J = 6.78, C5-*H*$_2$ and C8-*H*$_2$), 6.77 (m, 3H, C1-*H*, C3-*H* and C4-*H*), 7.32−7.51 (m, 3H), 7.76−7.84 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 22.74 (t), 23.00 (t), 28.64 (t), 29.19 (t), 117.74 (d), 120.79 (d), 127.94 (s), 128.12 (d), 128.35 (d), 129.84 (d), 131.43 (d), 131.59 (d), 132.40 (d), 133.44 (s), 138.34 (s), 147.73 (s); ^{31}P NMR ($CDCl_3$) (^{1}H-decoupled) δ 16.51; MS (FAB$^-$, NBA) 287 [(M − H)$^-$]; MS (FAB$^+$, NBA) 289 [(M + H)$^+$].

*(F) Triethylammonium Salt of Estrone-3-hydrogen Phosphonate (**13**)*. Imidazole (1.79 g, 26.34 mmol) was dissolved in dry acetonitrile (17 mL) and cooled to 0 °C. Phosphorus trichloride (0.69 mL, 7.95 mmol) was added to the solution, and the mixture was stirred for 15 min. Subsequently, anhydrous triethylamine (3.88 mL, 27.84 mmol) was added to the slurry, and again the reaction was left to stir for 15 min. Finally, a suspension of estrone (0.50 g, 1.85 mmol) in dry acetonitrile (17 mL) was added slowly to the reaction mixture, and the reaction was allowed to warm to room temperature, whereupon stirring was continued for a further 20 h. The reaction was then cooled once again to 0 °C, and distilled water (12.5 mL) was added dropwise. The solution was left to warm to room temperature, and stirring was continued for another hour. The reaction mixture was concentrated in vacuo, and the residue was coevaporated first with triethylamine (50 mL) and then with toluene (3 × 30 mL) to give a beige waxy solid. This material was dissolved in chloroform (20 mL) and washed with water (20 mL). The aqueous layer was reextracted with chloroform (3 × 20 mL), and the combined organic layers were dried over anhydrous Na_2SO_4. Removal of the solvent in vacuo yielded a white glassy solid. Purification was effected using flash chromatography (90:10 chloroform:methanol with 1% triethylamine) to afford **13** as a white glassy solid (0.55 g, 70%): R_f 0.30 (90:10 chloroform:methanol with 1% triethylamine); ^{1}H NMR (CD_3OD) δ 0.88 (s, 3H, C18-C*H*$_3$), 1.29 [t, 9H, J = 7.33, HN$^+$(CH$_2$C*H*$_3$)$_3$], 1.41−1.68 (m, 6H), 1.91−2.52 (series of m, 7H), 2.84 (m, 2H), 3.14 [q, 6H, J = 7.33, HN$^+$(*C*H$_2$-CH$_3$)$_3$], 6.87 (d, 2H, J = 9.34, C2-*H* and C4-*H*), 7.19 (d, 1H, J = 8.43, C1-*H*), 7.75 [br s, 1H, *H*N$^+$(CH$_2$CH$_3$)$_3$], 8.04 (d, 1H, J = 60.26, *H*-P); ^{13}C NMR (CD_3OD) δ 9.47 [q, HN$^+$-(CH$_2$*C*H$_3$)$_3$], 14.59 (q, C18-*C*H$_3$), 22.74 (t), 27.24 (t), 27.76 (t), 30.68 (t), 33.02 (t), 36.98 (t), 39.83 (d), 45.51 (d), 47.78 [t, HN$^+$(*C*H$_2$CH$_3$)$_3$], 51.73 (d), 79.85 (s, *C*13), 119.43 (d), 122.43 (d), 127.70 (d), 136.60 (s), 139.37 (s), 152.00 (s), 223.45 (s, *C*O); ^{31}P NMR (CD_3OD) (^{1}H-coupled) δ 2.882 and −2.882 (d, J = 630.40, *P*-H); MS (FAB$^-$, NBA) 333 [(M − H)$^-$]; MS (FAB$^+$, NBA) 335 [(M + H)$^+$].

*Preparation of S-Methylthiophosphonate Diesters. (A) Estrone-3-S-(methyl)methylthiophosphonate (**14**)*. Compound **3** (0.14 g, 0.38 mmol) was dried by repeated coevaporation with anhydrous methanol (3 × 5 mL). Subsequently, the compound was dissolved in a further quantity of dry methanol (2 mL), and a large excess of methyl iodide (4.63 mL, 74.44 mmol) was added dropwise. The reaction mixture was then left to stir at room temperature for 29 h. The excess methyl iodide and solvent were removed in vacuo to give a brown viscous oil. Purification of this crude product was effected using flash chromatography (95:5,chloroform: methanol) and yielded **14** as a yellow viscous oil (0.14 g; 100%): R_f 0.39 (95:5 chloroform:methanol); ^{1}H NMR

($CDCl_3$) δ 0.91 (s, 3H, C18-CH_3), 1.40−1.75 (m, 6H), 1.96 (d, 3H, J = 15.38, P-CH_3), 1.93−2.56 (series of m, 7H), 2.30 (d, 3H, J = 13.55, S-CH_3), 2.91 (m, 2H), 7.00 (br s, 2H, C2-*H* and C4-*H*), 7.24 (d, 1H, J = 8.79, C1-*H*); ^{13}C NMR ($CDCl_3$) δ 12.32 (q, S-*C*H_3), 13.75 (q, C18-*C*H_3), 18.29 and 19.91 (dq, J = 110.2, P-*C*H_3), 21.54 (t), 25.69 (t), 26.27 (t), 29.39 (t), 31.49 (t), 35.81 (t), 37.95 (d), 44.05 (d), 47.91 (s, *C*13), 50.37 (d), 117.97 (d), 120.79 (d), 126.56 (d), 136.94 (s), 138.34 (s), 147.97 (s), 220.60 (s, *C*O); ^{31}P NMR (CD_3OD) (^{1}H-decoupled) δ 53.32; MS (EI, 70 eV) *m*/*z* (%) 378 [M^+] (100), 321 (10), 269 (20), 167 (10), 49 (39), 129 (13), 109 (24), 94 (71), 83 (74), 71 (41), 57 (83), 43 (81), 29 (30); HRMS calculated 378.1419, found 378.1419 [M^+].

*(B) Estrone-3-S-(methyl)phenylthiophosphonate (**15**)*. This was prepared in an identical manner to that of **14**. Compound **9** (0.21 g, 0.47 mmol), dry methanol (2 mL), and methyl iodide (2.62 mL, 42.00 mmol) were used. The crude material was purified by flash chromatography (98:2 chloroform:methanol) to afford **15** as a pale yellow, glassy solid (0.10 g, 49%): R_f 0.40 (98:2 chloroform:methanol); ^{1}H NMR ($CDCl_3$) δ 0.91 (s, 3H, C18-CH_3), 1.40−1.72 (m, 6H), 1.94−2.56 (series of m, 7H), 2.20 (d, 3H, J = 14.10, S-CH_3), 2.90 (m, 2H), 7.06 (s, 2H, C2-*H* and C4-*H*), 7.27 (d, 1H, J = 10.81, C1-*H*), 7.49−7.64 (m, 3H), 7.96−8.04 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 12.10 (q, S-*C*H_3), 13.78 (q, C18-*C*H_3), 21.54 (t), 25.72 (t), 26.27 (t), 29.39 (t), 31.49 (t), 35.81 (t), 37.95 (d), 44.05 (d), 47.91 (s, *C*13), 50.37 (d), 118.05 (d), 120.88 (d), 126.59 (d), 128.54 (d), 128.77 (d), 130.36 (s), 131.33 (d), 131.49 (d), 132.85 (d), 136.81 (s), 138.37 (s), 148.26 (s), 220.75 (s, *C*O); ^{31}P NMR ($CDCl_3$) (^{1}H-decoupled) δ 43.76; MS (EI, 70 eV) *m*/*z* (%) 440 [M^+] (100), 269 (40), 171 (71), 141 (18), 115 (20), 97 (25), 77 (32), 55 (13); HRMS calculated 440.1575, found 440.1575 [M^+].

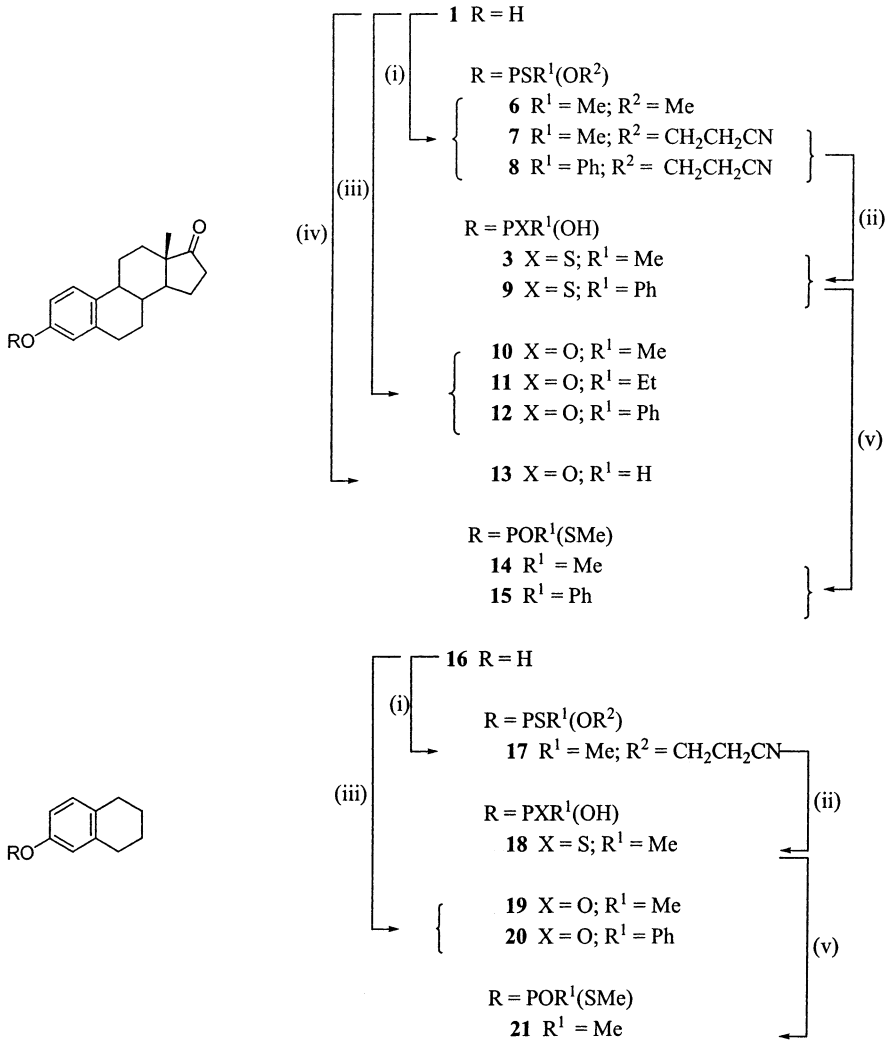
*(C) 5,6,7,8-Tetrahydronaphth-2-S-(methyl)methylthiophosphonate (**21**)*. This was synthesized according to the procedure used to form **14**. Compound **18** (0.15 g, 0.60 mmol), dry methanol (1 mL), and methyl iodide (3.38 mL, 54.23 mmol) were used. Purification of the crude product was effected by flash chromatography (chloroform) to give **21** as a yellow/brown viscous oil (0.12 g, 78%): R_f 0.39 (chloroform); ^{1}H NMR ($CDCl_3$) δ 1.77 (quintet, 4H, J = 3.34, C6-H_2 and C7-H_2), 1.94 (d, 3H, J = 15.57, P-CH_3), 2.29 (d, 3H, J = 13.37, S-CH_3), 2.73 (br d, 4H, J = 6.41, C5-H_2 and C8-H_2), 6.96−7.03 (m, 3H, C1-*H*, C3-*H*, and C4-*H*); ^{13}C NMR ($CDCl_3$) δ 12.19 (q, S-*C*H_3), 18.16 and 19.78 (dq, J = 110.2, P-*C*H_3), 22.74 (t), 22.96 (t), 28.67 (t), 29.35 (t), 117.80 (d), 120.82 (d), 130.03 (d), 134.02 (s), 138.66 (s), 147.84 (s); ^{31}P NMR ($CDCl_3$) (^{1}H-decoupled) δ 52.41; MS (EI, 70 eV) *m*/*z* (%) 256 [M^+] (100), 241 (27), 209 (18), 178 (16), 147 (76), 131 (52), 109 (37), 91 (40), 77 (13), 57 (16), 41 (22), 28 (15); HRMS calculated 256.0687, found 256.0687 [M^+].

*Preparation of O-Alkylthiophosphonate Diesters. Estrone-3-O-(methyl)methylthiophosphonate (**6**)*. Methylthiophosphonic dichloride (580.40 μL, 5.55 mmol, 3 equiv) was added dropwise to a stirred solution of estrone (0.50 g, 1.85 mmol, 1 equiv) in anhydrous pyridine (1 mL, 12.36 mmol, 6.68 equiv) and dry dichloromethane (18.50 mL) at 0 °C. The solution was allowed to warm to room temperature, and stirring was continued for a further 26 h. Subsequently, the reaction was cooled to 0 °C, and a solution of dry methanol (0.50 mL, 10.90 mmol, 5.89 equiv) in anhydrous pyridine (7 mL) was added dropwise. The reaction was allowed to warm to room temperature once more, and stirring was continued overnight. The reaction mixture was then poured into water (20 mL), and the resulting aqueous solution was extracted with ethyl acetate (4 × 15 mL). The combined organic extracts were dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo. Final traces of pyridine were removed by repeated coevaporation with toluene (3 × 20 mL) to yield a beige waxy solid which fumed in air. Purification was effected by flash chromatography (96:4 dichloromethane:methanol) to afford **6** as a cream waxy solid (0.42 g, 61%): R_f 0.55 (96:4 dichloromethane:methanol); ^{1}H NMR ($CDCl_3$) δ 0.90 (s, 3H, C18-CH_3), 1.46−1.62 (m, 6H), 1.96 (d, 3H, J = 15.38, P-CH_3), 1.90−2.54 (series of m, 7H), 2.90 (m, 2H), 3.78 (d, 3H, J = 14.11, OCH_3), 6.85 (d, 2H, J = 7.33, C2-*H* and C4-*H*), 7.23 (d, 1H, J = 8.43, C1-*H*); ^{13}C NMR ($CDCl_3$) δ 13.75 (q, C18-*C*H_3), 20.39 (q), 21.49 (t), 22.09 (q), 25.62 (t), 26.22 (t), 29.32 (t), 31.46 (t), 35.73 (t), 37.90 (d), 43.97 (d), 47.84 (s, *C*13), 50.34 (d), 118.49 (d), 121.31 (d), 126.33 (d), 136.65 (s), 138.04 (s), 148.35 (s), 220.58 (s, *C*O); ^{31}P NMR ($CDCl_3$) (^{1}H-decoupled) δ 95.03; MS (EI, 70 eV) *m*/*z* (%) 378 [M^+] (17), 256 (29), 234 (100), 192 (13), 171 (27), 160 (23), 149 (19), 140 (13), 128 (19), 109 (100), 93 (23), 85 (14), 71 (26), 57 (46), 43 (31), 28 (17).

*Preparation of the Phosphoroamidate Derivative. 5,6,7,8-Tetrahydronaphth-2-phenylphosphoroamidate (**22**)*. Phenylphosphonic dichloride (316 μL, 2.23 mmol, 1.1 equiv) was added dropwise to a stirred solution of 5,6,7,8-tetrahydronaphth-2-ol (0.30 g, 2.02 mmol, 1 equiv) in anhydrous pyridine (164 μL, 2.02 mmol, 1 equiv) and dry dioxane (10 mL) at 0 °C. Subsequently, the reaction mixture was allowed to warm to room temperature, and stirring was continued for a further 24 h. The reaction mixture was then cooled to 0 °C, and dry gaseous ammonia was bubbled through the solution for approximately 10−15 min. The reaction mixture was poured into water (20 mL), and the aqueous phase was extracted with ethyl acetate (6 × 15 mL). The combined organic extracts were dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo. Final traces of pyridine and dioxane were removed by repeated coevaporation with toluene (3 × 20 mL). Purification of the crude material was effected by flash chromatography (92:8 chloroform:methanol with 1% triethylamine) to afford **22** as a cream-colored solid which was further purified by precipitation from chloroform by addition of pentane (0.29 g, 50%): R_f 0.36 (98:2 chloroform:methanol with 1% triethylamine); ^{1}H NMR ($CDCl_3$) δ 1.73 (quintet, 4H, J = 3.21, C6-H_2 and C7-H_2), 2.68 (br s, 4H, C5-H_2 and C8-H_2), 3.36 (br s, exchangeable, 2H, NH_2), 6.83−6.95 (m, 3H, C1-*H*, C3-*H* and C4-*H*), 7.40−7.56 (m, 3H), 7.85−7.94 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 22.80 (t), 23.06 (t), 28.67 (t), 29.35 (t), 117.77 (d), 120.79 (d), 128.25 (d), 128.48 (d), 129.97 (d), 131.23 (d), 131.39 (d), 132.11 (d), 133.44 (s), 138.56 (s), 148.03 (s) [one aromatic C(s) too weak to observe]; ^{31}P NMR ($CDCl_3$) (^{1}H-decoupled) δ 20.54; MS (EI, 70 eV) *m*/*z* (%) 287 [M^+] (16), 148 (68), 120 (100), 91 (21), 77 (14), 28 (12). Anal. ($C_{16}H_{18}NO_2P$) C, H, N.

Stereospecific Enzymatic Digestion of the S_p-Diastereoisomer of (R_p,S_p)-Estrone-3-methylthiophosphonate (6) Using Snake Venom Phosphodiesterase. A solution of (R_p,S_p)-**3**

Scheme 1: Synthesis of Steroidal and Nonsteroidal Inhibitors of Estrone Sulfatase[a]



[a] Reagents and conditions: (i) (a) R^1PSCl_2/pyridine, 24 h, (b) R^2OH, 24 h; (ii) NH_4OH, 65 °C, 5 h; (iii) (a) R^1POCl_2/pyridine, 24 h, (b) H_2O; (iv) (a) imidazole/PCl_3/Et_3N/CH_3CN, 20 h, (b) H_2O; (v) MeI/MeOH, 29 h.

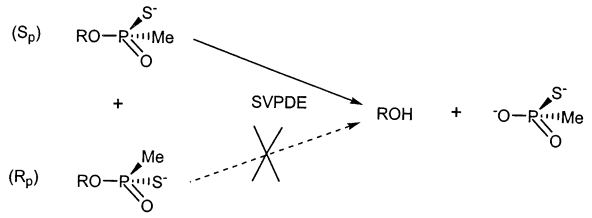
(6.60 mM) in Tris-HCl buffer (200 mM, pH 8.75) containing magnesium chloride (2 mM) was prepared. This solution was then incubated at 37 °C with snake venom phosphodiesterase (0.31 unit). The enzymatic digestion of (S_p)-**3** was monitored periodically by extraction of an aliquot (50 μL) and observing by HPLC using a Cyclobond I β-cyclodextrin column and a mobile phase gradient of 70−80% of buffer B (buffer A, 0.02 M ammonium sulfate; buffer B, 0.02 M ammonium sulfate in 75%:25% methanol:water). After 4−5 days, the enzyme was found to be denaturated. Therefore, the solution of (R_p,S_p)-**3** was treated every 4−5 days with additional quantities of snake venom phosphodiesterase (0.31 unit) until the remaining concentration of (S_p)-**3** was less than 5%, as evaluated by HPLC. This took a total of 33 days. Subsequently, the enzymatic digestion was stopped by addition of chloroform (1 mL). The resulting solution was mixed thoroughly, and the denaturated enzyme was precipitated by centrifuging. The supernatant was collected and evaporated to dryness in vacuo. The residue was triturated with chloroform, and the combined organic extracts were concentrated in vacuo. Final purification of (R_p)-**3** was achieved on an ion-exchange column of Q-Sepharose using a gradient of triethylammonium bicarbonate buffer (pH 7.40) ranging from 25 to 500 mM. Pure (R_p)-**3** eluted at ca. 300 mM buffer and was recovered in >97% optical purity, as determined by HPLC, in quantitative yield as its triethylammonium salt: $[\alpha]_D = +50.5°$ (*c* 0.99, MeOH).

RESULTS

Synthesis of Thiophosphonates. We have previously reported elsewhere the preparation of thiophosphonates **3**, **9**, and **18** (Scheme 1) in communication form (*23*), but we now present here the full details of their synthesis. Thiophosphonates **3**, **9**, and **18** were prepared from either estrone (**1**) or THN (**16**) using a two-step pathway. This involved first formation of the corresponding *O*-(2-cyanoethyl)thiophosphonate diesters [i.e., **7**, **8**, and **17** (Scheme 1) respectively] from the alcohol by treatment with the appropriate alkylthiophosphonic dichloride, followed by quenching the intermediate monophosphonic chloride generated with 2-cyanoethanol. Subsequent β-elimination of the cyanoethyl moiety was readily accomplished using aqueous ammonia to afford the ammonium salts of the desired thiophosphonates, as a mixture of diastereoisomers, in yields ranging from 61% to 98% after purification.

*Enzymatic Digestion of the S_p-Diastereoisomer of (R_p,S_p)-Estrone-3-methylthiophosphonate (***3***) (Scheme 2).* E1-3-MTP (**3**) (Scheme 1) synthesized by the above pathway exists as

Scheme 2: Stereospecific Enzymatic Digestion of (S_p)-E1-MTP Using Snake Venom Phosphodiesterase (SVPDE)[a]



[a] R = estrone.

a mixture of diastereoisomers since the phosphorus atom is chiral. To assess the ability of each diastereoisomer of E1-3-MTP (**3**) to inhibit estrone sulfatase activity, the mixture of two diastereoisomers prepared from our pathway had to be separated. The individual resonances due to the two diastereoisomers were not even observed by ^{31}P NMR spectroscopy (δ_P 74.45 ppm). The only technique by which they could both be seen was by HPLC (Figure 3a). In the hope of being able to assess the ability of at least one of the two diastereoisomers to inhibit sulfatase activity and in order to assign the absolute configuration at phosphorus of the diastereoisomers giving rise to the two peaks observed by HPLC, we decided to digest enzymatically one of the diastereoisomers from the mixture (Scheme 2). Enzymatic methods have been applied to the assignment of the absolute configurations of nucleoside phosphorothioates (*35*). Snake venom phosphodiesterase (SVPDE) is known to hydrolyze specifically the S_p-diastereoisomer of (4-nitrophenyl)phenyl-phosphonothioate (*36*) and the R_p-diastereoisomer of 5′-*O*-adenosyl 5′-*O*-uridyl phosphorothioate[2] (*37*). Therefore, by analogy, we expected the S_p-diastereoisomer of E1-3-MTP (**3**) to be hydrolyzed by SVPDE. Thus, the mixture of diastereoisomers of E1-3-MTP (**3**) was incubated with SVPDE, and the digestion was monitored periodically by HPLC. After 33 days, the peak with the longer retention time had completely disappeared (Figure 3b), and this enabled the assignment of the peak with the shorter retention time to the R_p-diastereoisomer and the peak with the longer retention time to the S_p-diastereoisomer. Subsequently, the enzyme was denatured, and the isolated supernatant was evaporated in vacuo. The organic products were separated from the buffer salts used in the enzyme assay by trituration of the residue with chloroform. Final purification of the R_p-diastereoisomer was achieved by ion-exchange chromatography using a gradient of triethylammonium bicarbonate buffer (Figure 4). Pure (R_p)-**3** was recovered in quantitative yield as its triethylammonium salt and in >97% optical purity (by HPLC).

Synthesis of Phosphonates. Phosphonates **10**, **11**, **12**, **19**, and **20** were prepared from either estrone (**1**) or THN (**17**) using adaptations of the method of Cox et al. (*34*) (Scheme 1), in which a solution of the alcohol was treated with the appropriate alkylthiophosphonic dichloride. Following an aqueous workup and purification, the desired phosphonates were obtained in yields ranging from 1% to 77% with the lowest yield reported for the synthesis of THN-2-methyl-phosphonate (**19**).

[2] The spatial configuration around phosphorus is idential in these two substrates. The RS sequence rules, however, dictate different assignments of absolute configuration.

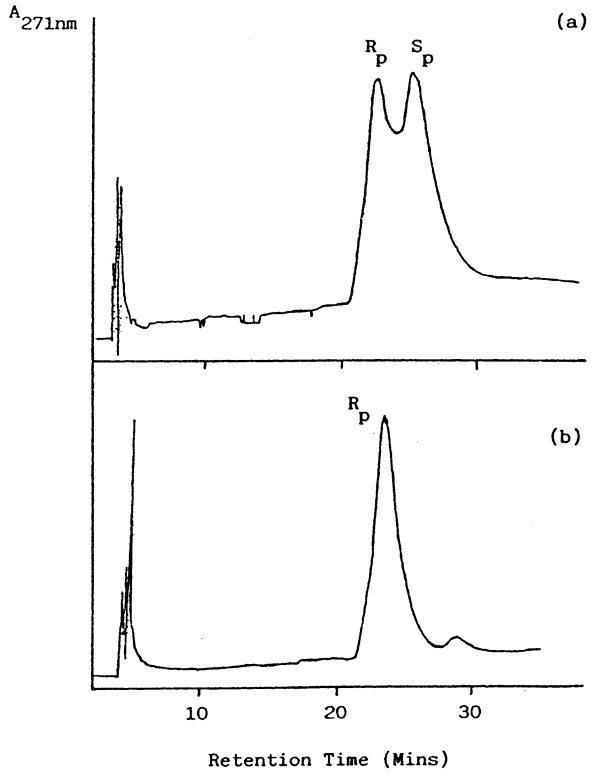


FIGURE 3: HPLC analysis of (a) (R_p,S_p)-E1-3-MTP and (b) (R_p)-E1-3-MTP (after digestion of the S_p isomer by snake venom phosphodiesterase) using a Cyclobond I β-cyclodextrin column. Buffer A was 0.02 M aqueous ammonium sulfate, and buffer B was 0.02M ammonium sulfate in 75%:25% methanol:water. A linear gradient of 70−80% buffer B was used over 30 min at a flow rate of 1 mL/min. The eluents were monitored at 280 nM.

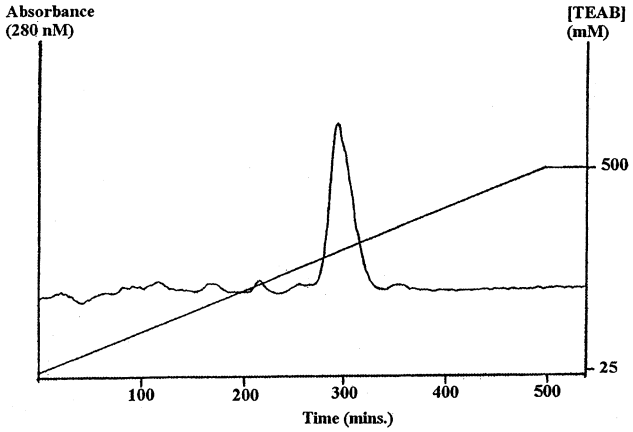
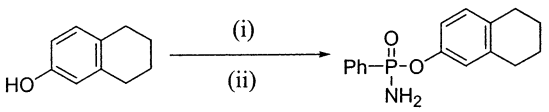


FIGURE 4: Ion-exchange chromatography for the purification of (R_p)-E1-3-MTP using a Q-Sepharose column and a triethylammonium bicarbonate buffer (pH 7.4) over a gradient of 25−500 mM.

The hydrogen phosphate of estrone derivative **13** was prepared according to the method of Garegg et al. (*38*) (Scheme 1). First estrone (**1**) was treated with phosphorus triimidazole, made in situ from phosphorus trichloride and imidazole. Subsequent hydrolysis of the intermediate phosphoramidite formed resulted in spontaneous isomerization of a P−OH bond to give the triethylammonium salt of the desired hydrogen phosphonate in 70% yield after purification.

Synthesis of O-(Methyl)- and S-(Methyl)thiophosphonate Diesters. The *O*-(methyl)methylthiophosphonate diester **6** was prepared in an identical manner to that of the *O*-(2-cyanoethyl)thiophosphonate diesters **7** and **8**, whereby

Scheme 3: Synthesis of THN Phenylphosphoroamidate[a]



16 **22**

[a] Reagents and conditions: (i) $PhPOCl_2$/pyridine/dioxane, 24 h; (ii) NH_3(g).

Table 1: Percentage Inhibition of Estrone Sulfatase Activity in Intact MCF-7 Breast Cancer Cells for E1-3-MTP (**3**) and Compounds **6**−**15**, **17**−**21**, and **22** at Various Concentrations[a]

	% inhibition		
compound	10 μM	1 μM	0.1 μM
(R_p,S_p)-**3**	95	74	52
(R_p)-**3**	33	15	−
10	41	−	−
(R_p,S_p)-**9**	42	0	−
11	53	−	−
12	42	−	−
13	80	−	0
6	−31	−	−
7	−52	−	0
8	75	−	0
14	28	−	0
15	0	0	−
17	−35	−	−
(R_p,S_p)-**18**	31	−	−
19	22	−	−
20	8	−	−
21	92	−	16
22	83	31	−

[a] Mean results at duplicate concentrations are shown. The coefficient of variation for the assay was <10%. (−) = not determined. Negative values indicate apparent enzyme stimulation.

estrone was first treated with methanethiophosphonic dichloride, but the intermediate estrone methanethiomonophosphonic choride thus produced was quenched with methanol rather than 2-cyanoethanol. *O*-(Methyl)methylthiophosphonate diester **6** was afforded in a 61% yield after purification. The *S*-(methyl)thiophosphonate diesters **14**, **15**, and **21** (Scheme 1) were synthesized from the corresponding thiophosphonates **3**, **9**, and **18** (Scheme 1), respectively, by treatment with an excess of methyl iodide in methanol. Following purification, the desired *S*-(methyl)thiophosphonate diesters were obtained in yields ranging from 49% to 100%.

Synthesis of Phosphoroamidate. The phenylphosphoramidate derivative of THN (**22**) (Scheme 3) was prepared from THN (**16**) by treatment with phenylphosphonic dichloride followed by quenching the intermediate phenylmonophosphonic chloride with gaseous ammonia. After purification **22** was obtained in a 50% yield

Inhibition of Sulfatase Activity. The ability of compounds **3**, **6**−**15**, **17**−**21** (Scheme 1), and **22** (Scheme 3) to inhibit estrone sulfatase activity in intact MCF-7 breast cancer cells at various concentrations was assessed as described previously (*24*, *26*, *28*). The results are presented in Table 1. Of this series the R_p,S_p mixed diastereoisomers of **3** proved to be the most potent inhibitor of estrone sulfatase activity with an IC_{50} in the 100 nM region. Of the other compounds tested at 10 μM, **8**, **13**, **21**, and **22** were among the most potent, inhibiting activity by 75%−92%. These compounds are, therefore, more potent than danazol, one of the first known estrone sulfatase inhibitors, which at 10 μM only inhibited activity by 40% in MCF-7 cells.

As **3** was the most potent inhibitor in this series, it was selected for further testing to ascertain its mode of inhibition of estrone sulfatase activity. When **3** and **5** were incubated with MCF-7 cells for a 2 h period followed by extensive washing with PBS, estrone sulfatase activity was almost fully restored in cells previously incubated with **3** with activity at 88% of pretreatment levels. In contrast, in cells exposed to **5**, a known irreversible inhibitor, activity remained almost completely inactivated (>99%) after removal of the compound and extensive washing of the cells. Similar results were obtained when placental microsomes were incubated with **3** and **5**. Whereas activity was almost fully restored (98%) after incubation of **3** with placental microsomes post dialysis, it remained almost completely inactivated after incubation of **5**. These findings confirm that compounds such as **3**, and other members of this series with relatively low inhibitory potencies, are only acting as reversible inhibitors of estrone sulfatase activity. This is in contrast to the mechanism of action of **5**, which is a very potent inhibitor, that acts as an irreversible inhibitor. Subsequent kinetic studies confirmed that whereas **5** was acting as an inhibitor in a time- and concentration-dependent manner, **3** was only a competitive inhibitor.

DISCUSSION

There are currently two schools of thought in the literature over the extent of key structural features required to ensure tight binding of a compound to the active site of estrone sulfatase. One view favors an entire "estrone"-type skeleton (*19*, *30*) while the other favors just the A ring (*22*). Nevertheless, both indicate that the other essential molecular determinant for tight binding is a negatively charged group (*19*, *22*). However, this latter point is confounded by the best inhibitor synthesized to date, EMATE (**5**) (Figure 1), which is a neutral compound. With the unexpected finding that EMATE possesses estrogenic activity, thereby rendering it unsuitable for use in the treatment of hormone-dependent breast cancer, we felt that it was necessary to evaluate further the structural requirements important in enzyme−inhibitor interaction, so that future inhibitors of estrone sulfatase activity could be developed.

Returning to our initial lead inhibitor, E1-3-MTP (**3**) (Figure 1), we realized that a number of important SAR questions had yet to be addressed. First, E1-3-MTP (**3**) synthesized by our pathway (Scheme 1) (*23*, *24*) exists as a mixture of diastereoisomers. A key question, therefore, is whether only one or both are active. Also, E1-3-MTP (**3**) contains a methylthiophosphonate functionality, and so other factors that warrant investigation are the role of (a) the sulfur atom and (b) the methyl group in the observed inhibition. Finally, E1-3-MTP (**3**) is a negatively charged compound and so contains the crucial structural feature for an estrone sulfatase inhibitor reported by both Li et al. (*19*) and Anderson et al. (*22*). However, in the light of our discovery of EMATE (**5**) (Figure 1), we felt that a subsequent exploration on charge and its requirement for inhibition was justified. In this paper, we attempt to answer these questions and present here our findings thus far. In line with our previous work, we have also prepared and examined a number of the corresponding THN analogues.

To assess the ability of each diastereoisomer of E1-3-MTP (**3**) to inhibit estrone sulfatase activity, the mixture of two diastereoisomers prepared from our pathway had to be separated. Therefore, to examine at least one of the two diastereoisomers as an inhibitor sulfatase activity, we chose to digest enzymatically the S_p-diastereoisomer from the mixture using SVPDE. The ability of the remaining (R_p)-E1-3-MTP to inhibit estrone sulfatase activity was then examined using intact MCF-7 cells. From Table 1, it can be seen that (R_p)-**3** had an inhibitory activity of only 33% at 10 μM and 15% at 1 μM whereas (R_p,S_p)-**3** showed inhibitions of 95% and 74% at the same concentrations, indicating that (S_p)-**3** is clearly the most potent inhibitor of the diastereomeric pair.

To focus on the necessity of the sulfur atom in E1-3-MTP (**3**) for inhibition, the corresponding methylphosphonate **10** (Scheme 1) was prepared. However, on examining the ability of **10** to inhibit estrone sulfatase activity in intact MCF-7 cells, only a 41% inhibition was observed at 10 μM compared to the 95% inhibition exhibited by El-3-MTP (**3**) at the same concentration (Table 1). On going from E1-3-MTP (**3**) to **10**, not only has the charge distribution changed, since in **3** the negative charge resides mainly on the sulfur atom and in **10** it is delocalized over both oxygen atoms, but more importantly the hardness of the base has increased. This will have consequences for binding affinity if the active site contains a soft acid since this will favor forming interactions with the softer base **3** rather than the harder base **10**. It has recently been identified that histidine and lysine are two of the amino acids at the active site of the sulfatase family (*39*), and so it is conceivable that the active site soft acid influencing the binding of **3** and **10** is lysine (pK_a 10). Thus, it appears that the hardness of the base has some significance over the extent of inhibition obtained.

To ascertain the role of the methyl group in the thiophosphonate moiety of E1-3-MTP (**3**) in the inhibition of estrone sulfatase, higher and lower homologues within the thiophosphonate and phosphonate families were prepared, i.e., estrone-3-phenylthiophosphonate (**9**), estrone-3-ethylphosphonate (**11**), estrone-3-phenylphosphonate (**12**), and estrone-3-hydrogenphosphonate (**13**) (Scheme 1). Their abilities to inhibit estrone sulfatase activity were subsequently assessed in intact MCF-7 cells. From Table 1, it can be seen that all showed modest inhibition of estrone sulfatase activity at 10 μM, although they were weaker inhibitors than (R_p,S_p)-**3**. It should be noted that the extent of inhibition observed for (R_p,S_p)-**9** was identical to that obtained for its phosphonate analogue **12** at 10 μM (42%). The hydrogen phosphonate derivative **13** displayed the highest level of inhibition (80% at 10 μM) within this range of compounds.

For the thiophosphonates the extent of inhibition decreased on going from the methyl derivative to the phenyl derivative i.e., (R_p,S_p)-**3** > (R_p,S_p)-**9**. The reason for this finding is that presumably the larger phenyl group cannot be accommodated as easily at the active site, and this hypothesis is confirmed by the docking studies in the homology model we describe. Furthermore, since (R_p,S_p)-**9** and **12** displayed exactly the same activities, unlike (R_p,S_p)-**3** and **10**, it appears that the increased steric hindrance introduced by the phenyl group has a greater influence over binding affinity for these compounds than the softness of the base. The ethylphosphonate **11** was found to be a slightly better inhibitor of estrone sulfatase activity than the methylphosphonate **10**, which could be attributed to the fact that an ethyl group is more hydrophobic than a methyl group and so would be available for additional interactions of this type at the active site. Alternatively, the greater inhibitory activity exhibited by **11** could be a result of this increased hydrophobicity enhancing its ability to partition into a nonpolar environment (i.e., micelle or bilayer). Finally, the high degree of inhibition exhibited by the hydrogen phosphonate derivative **13** is possibly due to the facts that the hydrogen is first smaller than the methyl group of **10**, which enables **13** to fit better into the active site as our model would predict, and second it is involved in hydrogen-bonding interactions at the active site. As mentioned above, one of the amino acids demonstrated to be present in the active site of estrone sulfatase is histidine (*28*, *42*), and so it is conceivable that it is this histidine residue which is participating in a hydrogen-bonding interaction with the hydrogen of **13**. Therefore, it appears that the size of the inhibitor plays an important part in binding affinity.

The requirement of charge for inhibition of estrone sulfatase was assessed by examining the *O*-(2-cyanoethyl)-thiophosphonate diester intermediates **7** and **8** (Scheme 1), formed as intermediates in the synthetic pathways to E1-3-MTP (**3**) and estrone-3-phenylthiophosphonate (**9**), respectively, the *O*-(methyl)methylthiophosphonate diester **6** (Scheme 1), the *S*-(methyl)thiophosphonate diesters **14** and **15** (Scheme 1) as estrone sulfate surrogates, to inhibit estrone sulfatase activity in intact MCF-7 cells. In addition, the phosphoramidate **22** was prepared (Scheme 3). As can be seen from Table 1, all of these compounds proved to be weaker inhibitors of estrone sulfatase activity than (R_p,S_p)-**3**. Surprisingly, the *O*-(methyl)- and *O*-(2-cyanoethyl)- methylthiophosphonate diesters of **3** (i.e., **6** and **7**) even stimulated estrone sulfatase activity at this concentration. The reason the *S*-(methyl)methylthiophosphonate diesters **14** and **15** displayed only 28% and 0% inhibition at 10 μM is possibly a consequence of two factors: first, these compounds are uncharged and so are unable to participate in any ionic interactions with amino acids at the active site, and second, by introducing a methyl group at the sulfur, the size of the compound has increased. As mentioned above for (R_p,S_p)-**9**, it appears that a phenyl group cannot be as easily accommodated at the active site of estrone sulfatase, and by going from (R_p,S_p)-**9** to **15** the steric hindrance and polarity have increased yet further, hence resulting in the total loss of inhibitory activity. Therefore, from **14** and **15**, it seems that binding affinity is dictated by size; these observations are supported by the theories of Li et al. (*19*) and Anderson et al. (*22*).

The reason diesters **6** and **7** apparently activate estrone sulfatase activity remains currently a mystery. Since little is known about the structure of this enzyme, it is difficult to rationalize these observations. However, it appears that these compounds are able to modify the active site to favor substrate binding, but whether this is achieved by binding at the substrate site (competitive activator) or at another "allosteric" site (noncompetitive activator) is unclear, given our lack of structural knowledge to date (vide infra for another possible interpretation). Finally, the best inhibitor of estrone sulfatase activity from all the neutral compounds studied here was the *O*-(2-cyanoethyl)phenylthiophosphonate

diester **8**. Surprisingly, it exhibited an inhibitory activity of 75% at 10 μM, which was far higher than the inhibition observed for the parent phenylthiophosphonate (R_p,S_p)-**9** at the same concentration, although at 0.1 μM it showed no activity at all (Table 1). The reason for this observation could be threefold: First, since it is a neutral compound with a hydrophobic β-cyanoethyl moiety, this will help it to partition easier into a nonpolar environment (i.e., micelle or bilayer) compared to (R_p,S_p)-**9**. Second, the β-cyanoethyl group, on binding, could be involved in hydrophobic interactions with residues at the active site. Last, at the active site, a basic amino acid such as histidine could conceivably induce β-elimination of the β-cyanoethyl group, implying that **8** might act like a prodrug of (R_p,S_p)-**9**. The latter might mean that **8** may stay longer at the active site, hence contributing to its enhanced inhibitory properties with respect to (R_p,S_p)-**9**. Thus, from **8**, it seems that the presence of a hydrophobic, cleavable group can make up for some of the steric problems introduced with the phenyl group.

Continuing our search for simpler, nonsteroidal inhibitors of estrone sulfatase activity as a result of the discovery that EMATE (**5**) possessed estrogenic properties, we have also examined a number of the corresponding THN analogues. THN (**16**) (Scheme 1) mimics the A and B rings of the steroidal nucleus. The particular derivatives investigated were the *O*-(2-cyanoethyl)methylthiophosphonate diester **17** (Scheme 1), the methylthiophosphonate (R_p,S_p)-**18** (Scheme 1), the methyl- and phenylphosphonates **19** and **20**, respectively (Scheme 1), the *S*-(methyl)methylthiophosphonate **21** (Scheme 1), and the phenylphosphoroamidate **22** (Scheme 3). From Table 1, it can be seen that, at 10 μM, (R_p,S_p)-**18**, **19**, and **20** were all weaker inhibitors of estrone sulfatase activity than their respective estrone derivatives i.e., (R_p,S_p)-**3**, **10**, and **12**. These results were in accord with our observation that THN-*O*-2-sulfamate is a much weaker inhibitor of estrone sulfatase activity than EMATE (i.e., at 10 μM, it was found to inhibit estrone sulfatase activity by 97% and at 1 μM by 47%) (*26*, *27*). Anderson et al. have also reported that THN-phosphate was the poorest inhibitor of steroid sulfatase activity in their studies (*22*). The reason (R_p,S_p)-**18**, **19**, and **20** are all weaker inhibitors of estrone sulfatase activity than their corresponding estrone derivatives is possibly because they are negatively charged and not hydrophobic enough to partition into a nonpolar (i.e., bilayer or micelle) environment. The importance of hydrophobicity for optimal inhibition has recently been highlighted (*22*).

Contrary to the above, the *S*-(methyl)methylthiophosphonate diester **21** of THN exhibited greater inhibition of estrone sulfatase activity than its corresponding estrone analogue (i.e., at 10 μM, **21** inhibited estrone sulfatase activity by 92%, compared to 28% for **14**) (Table 1). Unlike (R_p,S_p)-**18**, **19**, and **20**, this compound is neutral, rendering it better able to partition into a nonpolar environment. Additionally, since **21** is smaller than **14**, it can fit into the active site better, and although it is unable to make ionic interactions with amino acid residues at the active site, the extra methyl group on the sulfur atom is available for hydrophobic interactions. Thus, it appears that simple, nonsteroidal potent inhibitors of estrone sulfatase activity can be prepared, providing they are sufficiently hydrophobic to partition into a nonpolar environment. This conclusion is further supported by the finding that another neutral analogue of THN, phenylphosphoramidate **22**, is also a potent inhibitor of estrone sulfatase activity (i.e., at 10 μM, **22** inhibited estrone sulfatase activity by 83%) (Table 1). On binding to the enzyme, the amino group of **22** is available to participate in hydrogen-bonding interactions with suitable amino acids such as histidine at the active site.

Finally, like its respective estrone derivative **7**, the *O*-(2-cyanoethyl)methylthiophosphonate diester **17** was apparently found to stimulate estrone sulfatase activity at 10 μM (Table 1). This result cannot as yet be unequivocally explained, given the current lack of structural information on this enzyme. However, it is of interest to note that **17**, as **6** and **7**, is a diester. This means that all the compounds that apparently stimulate estrone sulfatase activity are neutral in character. It is therefore quite possible that the stimulatory effects observed for these compounds are due to an increase in membrane fluidity, as a result of disruption, thus increasing the availability of substrate. This is explained on the basis that steroid sulfatase is a membrane-bound enzyme and is known to be operating in cells in an unsaturated mode with respect to a physiological substrate concentration. Therefore, when the membrane fluidity is potentially disrupted in the presence of a hydrophobic compound, such as the ones described here, ingress of exogenous polar E1S is facilitated more readily, and the enzyme is consequently able to operate more efficiently in respect of substrate turnover, thus giving rise to an apparent stimulation by the neutral compound. Such an effect on plasma membrane lipid mobility has been noted previously for medroxyprogesterone acetate on CAMA-1 mammary cancer cells (*40*).

Modeling Studies. In contrast to other enzymes involved in estrogen biosynthesis, such as the aromatase complex, little is known about the structure of estrone sulfatase (STS), although the sequence of human STS has been cloned and expressed (*41*). Although an X-ray crystal structure of STS has not yet been published, the crystal structures of human arylsulfatase A (ASA) and human arylsulfatase B (ASB) have been reported (*39*). In addition, the active site formylglycine generated by posttranslational modification of the cysteine at position 69 (C69) has been mutated to serine in ASA and ASB (*39*), and to date two crystal structures of ASA with active site mutations, C69S and C69B, have been reported together with the mutant P426L (*42*) and their mechanism of action reviewed (*43*). As the sulfatases are members of a well-conserved gene family with high sequence homology (*44*), the construction of a homology model from the STS sequence was performed with the view to obtaining a model for molecular docking studies of inhibitors to broaden the understanding of enzyme/inhibitor interactions.

FUGUE has been developed for sequence−structure homology recognition using environment-specific substitution tables and structure-dependent gap penalties (*45*). Given the sequence of any protein, FUGUE will calculate and score with confidence levels the homology of the sequence to crystal structures in the Protein Data Bank (PDB). To ensure the protein with highest homology to STS was identified from which the homology model would be built, the STS sequence was submitted to FUGUE. This process gave ASA and a mutant C69S, PDB codes 1AUK (*39*) and 1E3C (*42*), with the highest score and 99% confidence levels. In addition, the molecular operating environment (MOE) (*46*) was used, taking the STS sequence file to perform a similar search,

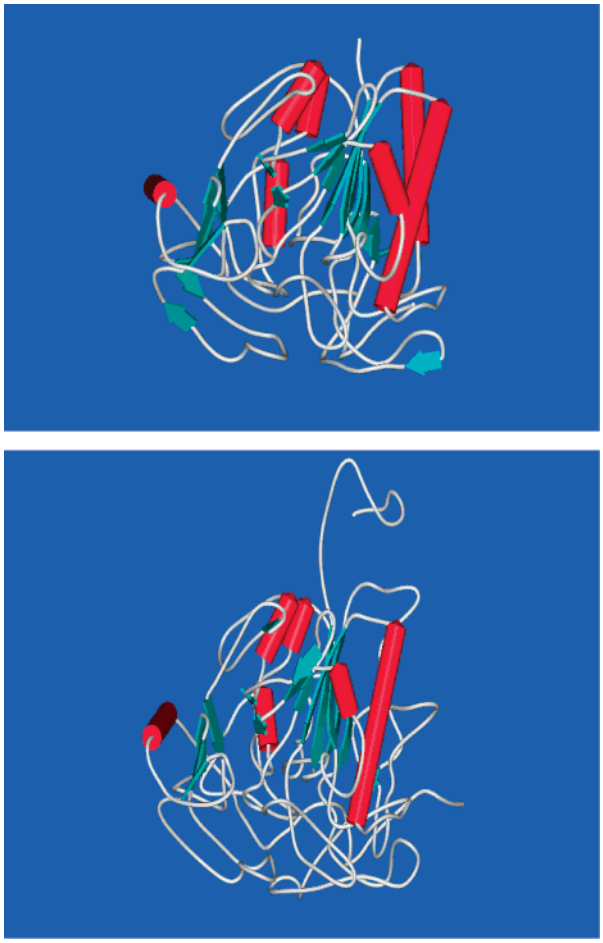


FIGURE 5: Homology model of STS (bottom) and crystal structure of ASA from 1AUK.pdb (top).

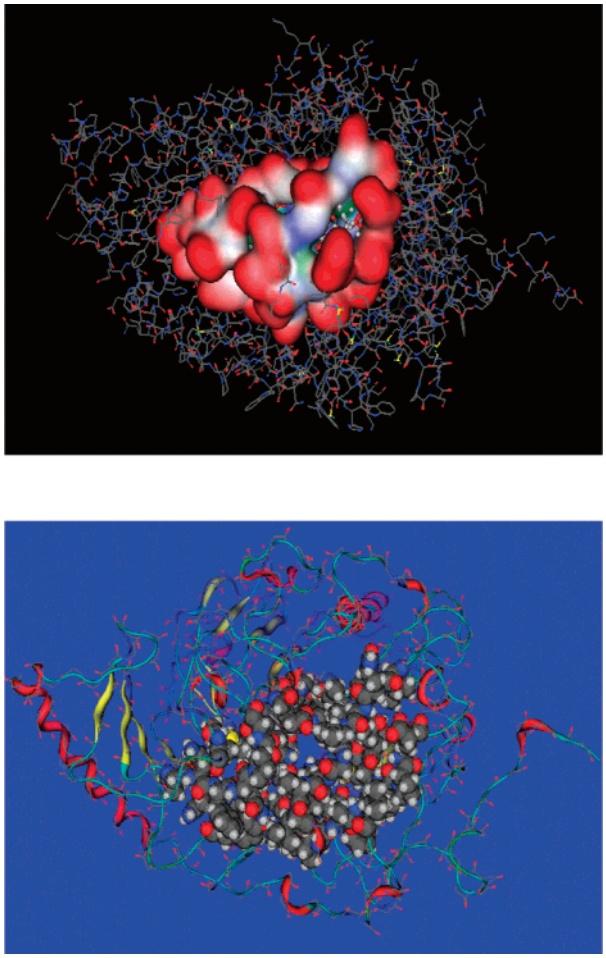


FIGURE 6: Active site on the STS model identified using MOE: small red and white spheres (top) and hole for docking to (bottom).

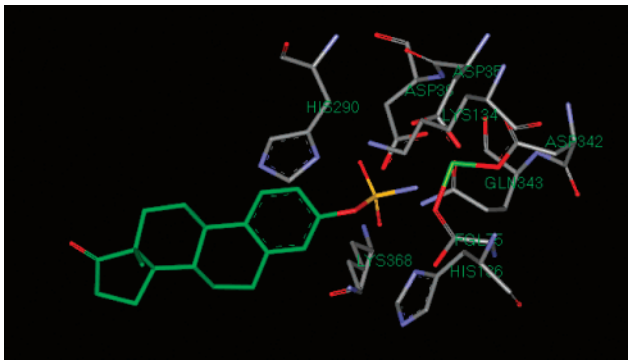


FIGURE 7: EMATE (**5**) in green and selected active site residues (magnesium ion in light green).

and again the output identified ASA, 1AUK (*39*), with the highest homology (~40%). The X-ray crystal structure of ASA using 1AUK.pdb as a template to build the homology model against was chosen. The model was built using MOE (*46*) and minimized with the Merck molecular force field (MMFF94) (*47*), the C75 residue was then changed to L-C_α-formylglycine, and the structure was minimized to give the homology model of STS which is depicted in Figure 5, showing similar secondary features to ASA. It must be borne in mind that ASA is a soluble protein and not membrane bound, whereas STS is a membrane-bound protein and changes to the protein structure in the membrane-bound region would not be taken into account in the homology model.

The active site in this STS model was identified using MOE (*46*) and is indicated in Figure 6.

The active site pocket was used to position EMATE (**5**) and to reminimize the structure with MMFF94 prior to use of the model for docking studies, the active site formylglycine was hydrated to give the diol, and this model was used for docking studies. Both diastereoisomers of E1-3-MTP were docked into the model using MOE with the sulfur atom bearing a negative charge, estrone sulfate (**2**) was docked as the anion, estrone phosphate as both the monoanionic and dianionic species (*22*), and EMATE (**5**) as the neutral form. As MOE has no scoring function, FlexX (*48*) was then used to dock and score (R_p)-E1-3-MTP (**3**), (S_p)-E1-3-MTP, the substrate estrone sulfate (**2**), the potent sulfamate-based STS inhibitor EMATE (**5**), and estrone phosphate; these results are depicted in Table 2. The scores are based on similar orientation in the active site of the homology model, and negative scores are predictive of good binding affinity. In general, the larger the negative score the higher the predicted affinity and the greater the inhibitory potency of the ligand. From the docking study (R_p)-E1-3-MTP (**3**) gave the least negative score, and this observation is encouraging as (R_p)-E1-3-MTP (**3**) is approximately 2 orders of magnitude less potent than (R_p,S_p)-E1-3-MTP, which shows 52% inhibition at 0.1 μM.

The results of the docking studies on the model with the hydrated form of the formylglycine group using EMATE

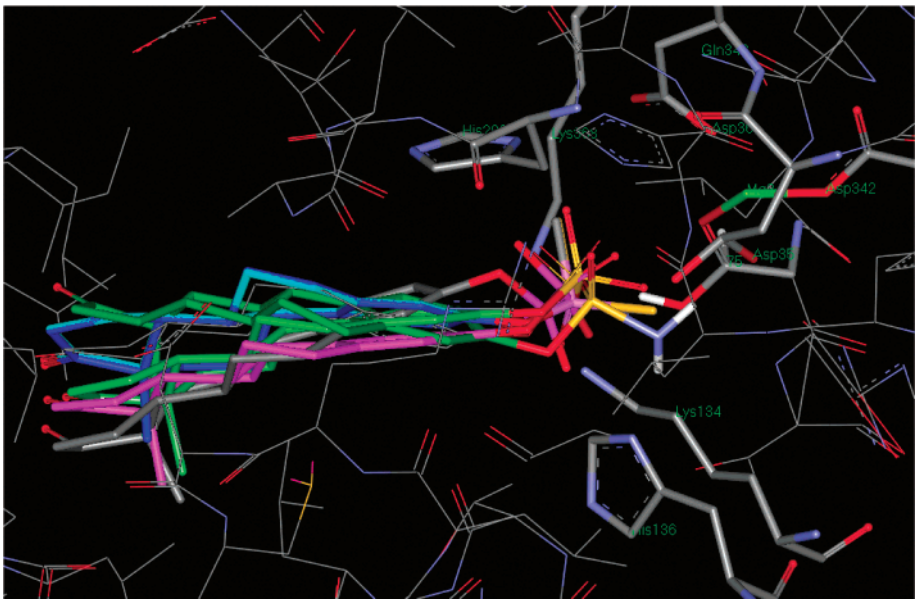
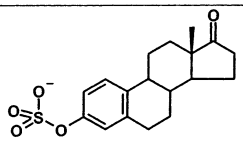
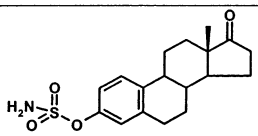
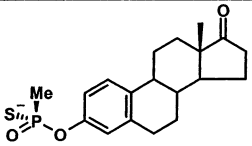
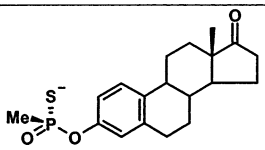
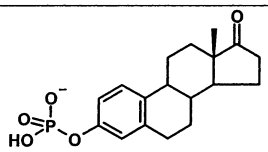
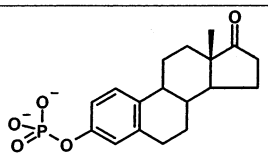


FIGURE 8: (S_p)-E1-3-MTP (light blue), (R_p)-E1-3-MTP (dark blue), estrone sulfate (**2**) (lime), estrone phosphate monoanion (gray), estrone phosphate dianion (pink), and EMATE (**5**) (green).

Table 2: Docking Scores of Substrate and Selected Inhibitors

Compound	Structure	FlexX Score[a,b]
Estrone Sulfate (E1S) (**2**)		-22.2
EMATE (**5**)		-24.8
S*p*-E1-3-MTP		-9.1
R*p*-E1-3-MTP (**3**)		-8.7
Estrone phosphate monoanion		-18.5
Estrone phosphate dianion		-21.6

[a] Large negative scores correspond to high predicted affinities. [b] Score on formylglycine hydrated as the diol.

(**5**) as the ligand are shown in Figure 7, with selected active site residues highlighted. Key residues identified around the active site region which may be involved in catalysis are His136, His290, Lys134, and Lys368. Three aspartic acid residues, Asp35, Asp36, and Asp342, and Glu343 are coordinated to the central magnesium ion together with the hydrated L-C_α-formylglycine residue at position 75 (FG75). It has been suggested by Dibbelt et al. (*49*) that an active site histidine with a pK_a of 5.8 acts as an active site base. As shown in Figure 7, His136 is in the proximity of residue 75 and indeed may act as the active site base for the deprotonation of the hydrated formylglycine.

The docked orientations of (S_p)-E1-3-MTP, E1S (**2**), EMATE (**5**), and a related compound from another study, estrone phosphate (*22*), are depicted in Figure 8. All four compounds are able to adopt a similar binding mode with the sulfate, sulfamate, methylthiophosphonate, or phosphate groups pointing toward the central magnesium ion and the L-C_α-formylglycine residue or its hydrated form at position 75 in a sterically congested area. The oxygen atom attached to the A ring of the steroid and the attached sulfur or phosphorus atoms are in the proximity of the proposed catalytic residues of His290, Lys134, and Lys368. The methyl group on (S_p)-E1-3-MTP appears in a sterically restricted region, and this would explain why the phenyl analogue (**9**) is much less active than **3**. Interestingly, although of similar size, the methyl phosphate (**10**) is also much less active than **3**, and as discussed earlier **10** is a harder base than **3**, and part of the reason for the observed lower activity may be the less favorable interaction with one of the soft acid residues, Lys134 or Lys368 (see Figure 8).

Through this work we have shown that chirality, hydrophobicity, basicity, size, and charge all influence the ability of a compound to inhibit estrone sulfatase activity. Of these, hydrophobicity is probably the most important since simple, nonsteroidal potent inhibitors can be prepared, provided that they are sufficiently hydrophobic to partition into a nonpolar environment. Our investigations with neutral estrone derivatives here have largely shown that a negatively charge group

is favorable for optimal binding, supporting the results of Li et al. (*19*) and Anderson et al. (*22*). This appears to contradict our earlier findings with EMATE except that one neutral compound studied lends itself to the theory that the presence of a potentially cleavable group can compensate for the lack of a charged moiety. This agrees with the proposed mechanism of action of EMATE, which involves cleavage of the sulfamate group (*30*, *31*). It is not possible at this stage to explain in detail why a few of the compounds mentioned in this paper apparently stimulate enzyme activity although a qualitative rationalization is offered. While EMATE is a potent, irreversible inactivator of estrone sulfatase, and indeed all subsequent such sulfamates that have been synthesized by ourselves and others demonstrate this property, it is quite feasible to imagine that, perhaps in other positive indications for sulfatase inhibition, reversible inhibitors, such as those described here, could be more appropriate and are worthy of further investigation. These observations have thus enabled us to establish some of the factors governing the recognition of E1S by estrone sulfatase and, importantly, to develop a homology model of the sulfatase with predictive power. All of this may be exploited in the design of future reversible and irreversible inhibitors of estrone sulfatase activity for potential therapeutic benefit.

ACKNOWLEDGMENT

The authors thank Mr. G. J. Cooper for technical assistance.

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BI020543G