Estrone Sulfatase: Probing Structural Requirements for Substrate and Inhibitor Recognition†

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ABSTRACT: The enzyme-catalyzed desulfation of steroids is a transformation that plays an important role in steroid biosynthesis. Conversion of steroid sulfates to unconjugated steroids may provide a source of steroids for processes such as steroid transport and the growth and proliferation of breast cancer. Steroid sulfatase catalyzes the hydrolysis of 3β-hydroxysteroid sulfates. To identify structural features important in enzyme−inhibitor interaction, a variety of steroidal and non-steroidal phosphate esters were synthesized and tested as inhibitors of steroid sulfatase activity. We report that the basic structure for enzyme−inhibitor binding does not include the steroid nucleus. Furthermore, the hydrophobicity of the non-steroidal phosphates was determined to be an important factor for optimal inhibition. The monoanionic form of the phosphorylated compounds was found to be the inhibitory species. The best non-steroidal inhibitor of steroid sulfatase activity was *n*-lauroyl tryamine phosphate with a K_i of 3.6 μM and 520 nM at pH 7.5 and 7.0. The poorest non-steroidal based inhibitor of sulfatase activity was tetrahydronaphthyl phosphate with a K_i of 870 and 360 μM at pH 7.5 and 7.0.

Sulfation and desulfation are ubiquitous biological events that play an important role in metabolic and regulatory processes. For example, it has been reported that sulfation is the most common of all tyrosine modifications, with as much as 1% of the tyrosyl residues of total protein in an organism being sulfated (Baeuerle & Huttner, 1985; Huttner, 1982, 1987). The sulfation and desulfation of other biological molecules, such as cerebrosides, glycosaminoglycans, neurotransmitters, and steroids is also quite common. Despite this, sulfatases, particularly those that play a role in the metabolism and processing of steroids, remain among the most poorly understood of the hydrolytic enzymes. The sulfated forms of many steroids, particularly estrone and dehydroepiandrosterone (DHEA), greatly predominate over the unconjugated steroids in the circulatory system. Indeed, with the exception of cholesterol, DHEA sulfate is the most prevalent of the plasma steroids. In addition, these sulfated species have particularly long half-lives in plasma (Hobkirk, 1985). Interestingly, it was recently reported that sterol sulfates are good inhibitors of protein tyrosine kinase $pp60^{v\text{-}src}$, thus raising the possibility that steroid sulfates may potentially play a role in the regulation of protein kinase activity (Fu & Schmitz, 1994). Steroid sulfatase (E.C. 3.1.6.2), catalyzes the hydrolysis of 3-β-hydroxysteroid sulfate esters such as dehydroepiandrosterone sulfate (DHEA sulfate) and of aromatic steroid sulfates such as estrone sulfate.

Over one-third of all breast cancers require stimulation by estrogens or other steroids for optimal growth. Estradiol is thought to be the estrogen responsible for the growth stimulation of most hormone-dependent breast tumors (Carlström, 1984). There are two potential sources for the biosynthesis of intracellular estradiol: (1) peripheral sources, such as ovaries and adrenal glands via the intermediacy of estrone sulfate, and (2) intratumoral biosynthesis from steroid precursors, such as androstenedione (Carlström, 1984). Estrone sulfate is the most abundant estrogen in plasma, with serum levels of this conjugated steroid reported to be two to ten times higher than the levels of estrone (Loriaux et al., 1971; Pasqualini et al., 1989; Santen et al., 1986). Estrone sulfate also appears to be a major source of estrogen for growing breast tumors (Naitoh et al., 1989; Santen et al., 1986). Evidently, estrone sulfate concentrations in blood sera are sufficient to supply growing tumors with estradiol (Santen et al., 1986; Utsumi, 1989; Naitoh et al., 1989). The level of estrone sulfatase activity found in breast cancer tissue is substantially higher than levels in healthy breast tissue. Interestingly, this is even true for breast cancer cells that are steroid receptor-negative and therefore should not require estrone sulfatase for growth (Naitoh et al., 1989; Pasqualini et al., 1990; Santen et al., 1986; Utsumi, 1989). The role of estrone sulfatase (if any) in promoting the growth of receptor-negative breast tumors is currently obscure.

Efforts to block the biosynthesis of estrogens have centered mainly on the successful development of aromatase inhibitors. In contrast, there are correspondingly few reports of estrone sulfatase inhibitors. The antiestrogens ICI 164384, tamoxifen, and its metabolites have been reported to be noncompetitive inhibitors of steroid sulfatase activity (Santner & Santen, 1993). A variety of steroidal phosphonates and phosphonate derivatives, as well as estrone phosphate, have been shown to be competitive inhibitors of steroid sulfatase activity (Howarth et al., 1993; Purohit et al., 1994; Duncan et al., 1993; Li et al., 1995; Anderson et al., 1995). Steroid sulfamates have recently been demonstrated to be time dependent inactivators, as well as competitive inhibitors of steroid sulfatase activity (Howarth et al., 1994; Purohit et al., 1995). In addition, sulfamate derivatives of tetrahydronaphth-2-ol have been shown to be poor inhibitors of

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steroid sulfatase activity. Based on this observation, the authors conclude that the entire steroid skeleton is required for optimal inhibition of sulfatase activity (Howarth et al., 1994). The development of potent steroid sulfatase inhibitors may be of therapeutic benefit, as estrone production is not completely abolished by aromatase inhibitors, which reduce plasma concentrations of estrone and estrone sulfatase by only 50% (Santen et al., 1978; Dowsett et al., 1987). Furthermore, at physiological concentrations of estrone sulfate and androstenedione, hormone-dependent breast tumor production of estrone from estrone sulfate is ten times faster than the rate of estrone production via the aromatase pathway (Santen et al., 1986).

In this paper we report the activity of several new types of inhibitors of steroid sulfatase activity. In addition, as elucidated from our inhibitor studies, we report that the basic structural motif that results in tight binding to the enzyme does not require a steroid nucleus. This finding raises the possibility that enzyme has physiologically relevant substrates other than simple steroid sulfates, which in turn suggests other possible roles for the activity of this enzyme in receptor-negative breast tumors.

MATERIALS AND METHODS

Materials

Triton X-100, peroxide content <1 ppm, was purchased from Boehringer Mannheim. [^{3}H]-6,7-Estrone sulfate and [^{14}C]-4-estrone were obtained from Du Pont-New England Nuclear. Fresh human placenta from live births were obtained from the Labor and Delivery Ward at Bloomington Hospital, Bloomington, IN. Diethylaminoethyl cellulose was purchased from Whatman LabSales, Inc. *p*-Acetylphenyl sulfate was synthesized according to the literature procedure described by Milsom and co-workers (1972). A BCA Protein Assay Reagent kit was purchased from Pierce. Estrone sulfate was synthesized and purified using the procedure of Birnböck (Birnböck & von Angerer, 1990). 1,3-Bis[tris(hydroxymethyl)methylamino]propane was purchased from Aldrich Chemical Company, Inc. All other chemicals were of ACS reagent grade. Assay temperature was controlled using a Neslab Instruments, Inc. circulating waterbath (±0.1 °C). Absorbance readings were measured using either a Cary 3 UV−vis spectrophotometer from Varian Analytical Instruments or a UV160U UV−vis spectrophotometer from Shimadzu Scientific Instruments, Inc. and 1 cm path length quartz cuvettes, type 18B from Uvonic Instruments, Inc.

Methods

Preparation of Steroid Sulfatase. Steroid sulfatase was extracted from fresh human placenta using the extraction procedure of Vaccaro and co-workers (1987). Extracts 3 through 6 were further purified by ion-exchange chromatography using diethylamino cellulose, the buffer system, and gradient described by Vaccaro and co-workers (1987).

General Procedure for the Determination of the pK_a of Phosphates by ^{31}P NMR. A solution of 1,3-bis[tris(hydroxymethyl)methylamino]propane (600 mg) and Triton X-100 (300 μL), in distilled water (9.5 mL) was titrated to the appropriate pH by the addition of glacial acetic acid. The phosphate ester (0.0134 mmol) was then dissolved in the solution (2.0 mL), and the pH and spectrum were recorded. H_3PO_4 was employed as an external reference. A solution prepared by substituting D_2O for H_2O was used to lock and shim the spectrometer. The ^{31}P resonance chemical shift was observed and then plotted as a function of pH. The pK_a value for each compound was determined by analysis of the plot using the single pK_a determination curve fitter found in the computer program Grafit.

Steroid Sulfatase Assay and Inhibition Studies. Enzyme assays were carried out in 1.5 mL polypropylene microcentrifuge tubes. 70 μL of 20 mM Tris-HCl buffer, pH 7.4, containing 0.1% (w/v) Triton X-100, 0.02% (w/v) NaN_3, and 0.3 unit (nmol of *p*-acetylphenyl sulfate hydrolyzed/min) of partially purified steroid sulfatase free of phosphatase activity was pre-incubated for 10 min at 37 °C with 490 μL of 0.22 M bis-Tris propane−acetate buffer (pH indicated), containing inhibitor at a variety of concentrations. 140 μL of buffer (pre-equilibrated to 37 °C), containing *p*-acetylphenyl sulfate at a variety of concentrations, was added to the pre-incubation mixture. 200 μL aliquots were removed at $T = 0$, 10, and 20 min and quenched into 500 μL of 1.4 M NaOH. Absorbance readings for *p*-acetylphenoxide were measured at 325 nm (ϵ = 21 000 M^{-1} cm^{-1}). The K_i and type inhibition for each inhibitor, at the indicated pH, were determined from Lineweaver−Burk plots and secondary replots of the slopes of Lineweaver−Burk plots versus the corresponding inhibitor concentration (Lineweaver & Burk, 1934; Engel, 1981).

Kinetic Analysis of Alternative Substrates. Enzyme assays were carried out in 1.5 mL polypropylene microcentrifuge tubes. 0.3 unit (nmol *p*-acetylphenyl sulfate hydrolyzed/min) of steroid sulfatase (specific activity 10.3 units/g of protein) was pre-incubated for 10 min at 37 °C with 490 μL of 0.22 M bis-Tris propane−acetate buffer, pH 7.5. 140 μL of buffer, pre-equilibrated to 37 °C, containing various concentrations of substrate, was added to the mixture. 200 μL aliquots were removed at $T = 0$, 5, and 10 minutes and quenched into 500 μL of 1.4 M NaOH. Absorbance readings for the resulting phenoxide were measured at 325 nm (ϵ = 21 000 M^{-1} cm^{-1}). The K_m and V_{max} were determined by Lineweaver−Burk plot analysis (Lineweaver & Burk, 1934).

Synthesis of Inhibitors and Substrates

General Methods. Unless run under aqueous conditions, all reactions were conducted in dried glassware under an atmosphere of dry argon or nitrogen. Diethyl ether and tetrahydrofuran were distilled from sodium benzophenone ketyl. Methylene chloride was distilled from calcium hydride. Pyridine was distilled from barium oxide. Phenol was dissolved in methylene chloride, dried over magnesium sulfate, filtered, and concentrated *in vacuo*. Zinc was amalgamated by combining mossy zinc with mercuric chloride (0.5 mol %) in water, perturbing occasionally for 0.5 h, filtering, and washing once with water (Read & Wood, 1955). *p*-Octyl phenol was both synthesized and obtained commercially from Aldrich. ^{1}H spectra were recorded on either a Varian XL-300 or a Varian XL-400 MHz spectrometer at 300 or 400 MHz, respectively. ^{13}C spectra were recorded on either a Varian XL-300 or a Varian XL-400 MHz spectrometer at 75 or 100.5 MHz, respectively. ^{31}P NMR spectra were recorded on either a Nicolet NT-360 MHz spectrometer at 146 MHz or a 300 MHz Gemini-3000

spectrometer at 121.4 MHz (85% H_3PO_4 was used as the external reference). ^{19}F spectra were recorded on a Nicolet NT-360 spectrometer at 339 MHz using $CHCl_2F$ at −80.88 ppm as the external reference. IR spectra were run on a Perkin-Elmer 298 spectrophotometer. Silica Gel 60 (230−400 mesh, EM science) was employed for flash column chromatography purifications. Sodium ion exchange columns were prepared by washing the column with 8−10 resin volumes of 1 N sodium hydroxide followed by washing with distilled water until the eluent was neutral.

General Procedure for Synthesis of p-Acyl Phenols. Aluminum chloride (7.0 g, 53 mmol) was added quickly to a solution of phenol (2.50 g, 26.6 mmol) in CH_2Cl_2 (10.0 mL) at 0 °C. The slurry was stirred at 0 °C for 0.5 h before the acid chloride (29.3 mmol) was added dropwise. The solution was stirred for 0.5 h at 0 °C and then at ambient temperature overnight. The slurry was cautiously diluted with iced 1 N HCl (30 mL) and extracted with Et_2O (3 × 30 mL). The organic layers were combined and extracted with 2 N NaOH (3 × 30 mL). The aqueous layers were combined, acidified with 1 N HCl until the pH was 2, and then extracted with Et_2O (3 × 75 mL). The organic layers were combined and washed with saturated $NaHCO_3$ (3 × 20 mL), dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by either column chromatography or recrystallization from hexane to afford a white solid (Malthete *et al*., 1981).

p-Butanoyl Phenol. Yield = 80%. Spectral data identical to those previously reported (Booth *et al*., 1980).

p-Hexanoyl Phenol. Yield = 74%. Spectral data identical to those previously reported (Sucrow *et al*., 1985).

p-Octanoyl Phenol. Yield = 100%. ^1H NMR ($CDCl_3$, 400 MHz): δ 7.91 (2H, d, J = 8.49 Hz), 6.92 (2H, d, J = 8.81 Hz), 2.91 (2H, t, J = 7.55 Hz), 1.71 (2H, m), 1.30 (8H, m), 0.86 (3H, t, J = 6.91 Hz). ^{13}C NMR ($CDCl_3$, 100.5 MHz): δ 200, 160, 130, 129, 115, 38, 31, 29, 28, 25, 22, 14. CI/CH_4 = 200.1452 (Malthete *et al*., 1981).

p-Decanoyl Phenol. Yield = 39%. ^1H NMR ($CDCl_3$, 400 MHz): δ 7.92 (2H, d, J = 8.81 Hz), 6.88 (2H, d, J = 8.81 Hz), 2.91 (2H, t, J = 7.55), 1.71 (2H, m), 1.30 (12H, m), 0.86 (3H, t, J = 6.02 Hz). ^{13}C NMR ($CDCl_3$, 100.5 MHz): δ 200.18, 160.46, 130.49, 115.09, 38.02, 31.52, 29.00, 24.48, 22.32, 18.74, 13.77 (Ralston *et al*., 1942).

General Procedure for Synthesis of p-Alkyl Phenols. *p*-Acyl phenol (3.62 mmol) dissolved in ethanol (1.5 mL) was added to a flask charged with amalgamated zinc (2.4 g, 36 mmol), water (3.0 mL), and concentrated HCl (3.0 mL). The reaction was boiled under reflux overnight. After cooling to ambient temperature, the solution was cautiously diluted with saturated $NaHCO_3$ (10 mL) and extracted with Et_2O (3 × 5 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated *in vacuo* to afford a crude yellow solid. Purification by column chromatography (3:1 hexane/ethyl acetate) gave the title compounds as white solids (Read & Wood, 1955).

p-Octyl Phenol. *p*-Octyl phenol was purchased commercially from Aldrich or synthesized. Yield = 88% (91% based on recovered starting material). ^1H NMR ($CDCl_3$, 300 MHz): δ 7.02 (2H, d, J = 8.20 Hz), 6.72 (2H, d, J = 8.59 Hz), 2.53 (2H, t, J = 8.20 Hz), 1.59 (2H, m), 1.28 (8H, m), 0.90 (3H, t, J = 7.42 Hz). ^{13}C NMR ($CDCl_3$, 100.5 MHz): δ 129.43, 115.01, 35.05, 31.90, 29.49, 22.68, 14.12.

General Procedure for the Synthesis of Sulfates

Method A. Sulfur trioxide−pyridine complex (318 mg, 2.00 mmol), was added to a solution of the phenol derivative (1.00 mmol) in pyridine (4.5 mL). After 2 h the pyridine was removed under high vacuum. The residual brown solid was transferred onto iced aqueous KOH (2 g in 10 mL of distilled water), diluted with H_2O (40 mL), washed with Et_2O (5 × 50 mL), washed with $CHCl_3$ (3 × 15 mL), acidified with 1 N H_2SO_4 (until pH was 4), and washed with Et_2O (7 × 50 mL). $Ba(OH)_2$ was added to adjust the pH to 12 and the aqueous solution was stored at 4 °C overnight. The resulting precipitate ($BaSO_4$) was removed by filtration, the filtrate was passed down a Dowex AG cation exchange column (proton form), and the column was rinsed with distilled water. NaOH (1 N) was added to the column eluent until the pH was 12. Water was removed by rotary vacuum evaporation until a precipitate formed. The mixture was stored at 4 °C overnight, and the white precipitate was then collected by filtration.

Method B. Sulfur trioxide−pyridine complex (318 mg, 2.00 mmol), was added to a solution of the phenol derivative (1.00 mmol) in pyridine (4.5 mL). After 2 h the pyridine was removed *in vacuo*. The brown solid was purified by column chromatography (15% methanol in CH_2Cl_2) and converted to the sodium salt with a Dowex AC cation 50w-x8 ion exchange column (sodium form) to afford a white solid.

p-Acetylphenyl Sulfate, ***11***. Prepared by method A. Yield = 70%. ^1H NMR (CD_3OD, 300 MHz): δ 8.00 (2H, d, J = 8.59 Hz), 7.44 (2H, d, J = 8.21 Hz), 2.58 (3H, s). ^{13}C NMR (D_2O, 75 MHz): δ 200, 130.70, 121.14, 26.28.

p-Butanoylphenyl Sulfate, ***12***. Prepared by method A. Yield = 87%. ^1H NMR (CD_3OD, 300 MHz): δ 8.00 (2H, d, J = 8.20 Hz), 7.45 (2H, d, J = 8.20 Hz), 2.98 (2H, t, J = 7.81 Hz), 1.77 (2H, m), 1.00 (3H, t, J = 7.42 Hz). ^{13}C NMR (D_2O, 75 MHz): δ 200, 130.47, 121.22, 40.56, 17.91, 13.03.

p-Hexanoylphenyl Sulfate, ***13***. Prepared by method B. Yield = 73%. ^1H NMR (CD_3OD, 300 MHz): δ 7.90 (2H, d, J = 9.38 Hz), 7.30 (2H, d, J = 8.20 Hz), 2.89 (2H, t, J = 7.42 Hz), 1.59 (2H, m), 1.24 (4H, m), 0.83 (3H, t). ^{13}C NMR (CD_3OD, 75 Mz): δ 203, 186, 160, 136, 132, 121, 56, 41, 34, 27, 26, 18.

p-Octanoylphenyl Sulfate, ***14***. Prepared by method B. ^1H NMR (D_2O, 400 MHz): δ 7.83 (2H, d, J = 8.49 Hz), 7.44 (2H, d, J = 8.80 Hz), 2.83 (2H, t, J = 7.55 Hz), 1.16 (10H, m), 0.78 (3H, t, J = 6.60 Hz). Compound **14** proved to be too insoluble to obtain a ^{13}C NMR spectrum.

General Procedure for the Synthesis of Phosphate Esters

Method A. The phenol derivative (3.06 mmol) was combined with 4′,4′-dimethylaminopyridine (DMAP, 46 mg, 0.38 mmol) in pyridine (1.0 mL). Diethyl chlorophosphate (1.1 mL, 7.65 mmol) was then added dropwise. The solution was stirred overnight, diluted with Et_2O (50 mL), washed with 25% $NaHSO_4$ (3 × 7 mL), followed by saturated $NaHCO_3$ (3 × 5 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated to a yellow oil. Purification by column chromatography (3:1 hexane/ethyl acetate) gave the phosphorylated compound.

Method B. The phosphate esters were prepared using the method reported by Stowell and Widlanski (1995). I_2 (0.964

g, 3.80 mmol) was added to a solution of CH_2Cl_2 (13 mL) and either $P(OMe)_3$ or $P(OBn)_3$ (0.500 mL, 4.24 mmol) at 0 °C. After the solution turned colorless, it was added to a solution of the phenol derivative (3.47 mmol), CH_2Cl_2 (35 mL), and pyridine (1.13 mL, 14.0 mmol) at −35 °C. After 5 min, the reaction was diluted with CH_2Cl_2 (35 mL), washed with saturated $NaHSO_4$ solution (3 × 30 mL), and washed with phosphate buffer pH 7 (1 × 30 mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash chromatography using 1:1 hexane/ethyl acetate as eluant.

Diethyl p-Octanoylphenyl Phosphate. Prepared by method A. Yield = 38%. ^{1}H NMR (CD_3OD, 300 MHz): δ 8.03 (2H, d, J = 8.20 Hz), 7.31 (2H, d, J = 8.20 Hz), 4.24 (4H, m), 2.99 (2H, t, J = 7.42 Hz), 1.70 (2H, m), 1.35 (12H, m), 0.89 (3H, t, J = 7.03 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 199.17, 154.30, 133.94, 130.15, 119.95, 64.90, 38.59, 31.77, 29.30, 24.42, 22.69, 16.15, 14.15. ^{31}P NMR ($CDCl_3$, 146 MHz): δ −6.219.

Dibenzyl p-Octylphenyl Phosphate. Prepared by method B. Yield = 98%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.32 (10H, m), 7.03 (4H, m), 5.11 (2H, d), 2.58 (2H, t), 2.58 (2H, m), 1.30 (10H, m), 0.90 (3H, t). ^{31}P NMR ($CDCl_3$, 146 MHz): δ −9.508.

Diethyl p-Decanoylphenyl Phosphate. Prepared by method A. Yield = 39%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.97 (2H, d, J = 8.20 Hz), 7.30 (2H, d, J = 7.81 Hz), 4.23 (4H, m), 2.92 (2H, t, J = 7.42 Hz), 1.65 (2H, m), 1.38 (18H, m), 0.89 (3H, t, J = 7.03 Hz). ^{13}C NMR ($CDCl_3$, 100.5 MHz): δ 199.17, 154.30, 133.94, 130.51, 119.95, 64.90, 38.59, 31.77, 29.21, 24.42, 22.69, 16.18, 14.15. ^{31}P NMR ($CDCl_3$, 146 MHz): δ −6.225. IR: 3010, 2940, 2880, 1690, 1610, 1390, 1230, 1040, 950 cm^{-1}.

Diethyl p-Decylphenyl Phosphate. Prepared by method A. Yield = 33%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.08 (4H, s), 4.17 (4H, m), 2.52 (2H, t, J = 7.55 Hz), 1.53 (2H, m), 1.28 (20H, m), 0.84 (3H, t, J = 6.92 Hz). ^{13}C NMR ($CDCl_3$, 100.5 MHz): δ 148.48, 139.42, 129.30, 119.50, 64.30, 35.06, 31.75, 31.35, 29.30, 22.53, 15.90, 13.95. ^{31}P NMR ($CDCl_3$, 146 MHz): δ −5.420. IR: 2940, 2880, 1510, 1460, 1390, 1360, 1280, 1220, 1030, 960 cm^{-1}.

Diethyl Estrone Phosphate. Prepared by method A. Yield = 78%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.21 (d, 1H, J = 8.4 Hz), 6.94 (m, 2H), 4.20 (m, 4H), 2.88 (q, 2H, J = 3.6 Hz), 2.48 (dd, 1H, J = 8.4, 18.8 Hz), 2.37 (m, 1H), 2.24 (m, 1H), 2.19−1.90 (m, 4H), 1.66−1.31 (m, 10H), 0.89 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 220.75, 148.61, 138.21, 136.40, 126.51, 119.89, 117.12, 99.40, 64.43 (d, J = 6.2 Hz), 50.34, 47.89, 43.98, 37.98, 35.80, 31.47, 29.37, 26.28, 25.52, 21.52, 16.06 (d, J = 7.1 Hz), 13.78. ^{31}P NMR ($CDCl_3$, 146 MHz): δ −5.46.

Dimethyl Dehydroepiandrosterone Phosphate. Prepared by method B. Yield = 75%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.41 (d, 1H, J = 5.2 Hz), 4.19 (m, 1H), 3.76 (s, 3H), 3.73 (s, 3H), 2.55−2.37 (m, 3H), 2.18−1.78 (m, 6H), 1.38−1.19 (m, 2H), 1.18−0.97 (m, 5H), 0.864 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 220.91, 139.47, 122.25, 78.09 (d, J = 6.2 Hz), 54.09, 51.59, 50.01, 47.44, 39.75 (d, J = 3.5 Hz), 36.73, 36.46, 35.77, 31.36, 31.31, 30.69, 29.42, 29.38, 21.80, 20.27, 19.24, 13.48. ^{31}P NMR ($CDCl_3$, 146 MHz): δ 1.03. HRMS for $C_{21}H_{33}O_5P$ [M + H], calcd 397.4650; found 397.2148.

Diethyl Tetrahydronaphthyl Phosphate. Prepared by method A. Yield = 74%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.08−6.83 (m, 3H), 4.33−4.08 (m, 4H), 2.87−2.62 (m, 4H), 1.91−1.67 (m, 4H), 1.33 (t, 6H). ^{13}C NMR ($CDCl_3$, 75 H Mz): δ 147.57 (d, J = 7.7 Hz), 137.64, 132.75, 129.19, 119.13 (d, J = 4.9 Hz), 116.24 (d, J = 4.8 Hz). ^{31}P NMR ($CDCl_3$, 146 MHz): δ −5.32.

Diethyl n-Lauroyl Tyramine Phosphate. Prepared by method A. Yield = 58%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.07 (s, 4H), 4.19 (m, 4H), 3.38 (m, 2H), 2.71 (t, 2H, J = 7.24 Hz), 2.05 (t, 2H, J = 7.74 Hz), 1.52 (m, 2H), 1.29 (t, 2H, J = 7.23 Hz), 1.19 (m, 18H), 0.80 (t, 3H, J = 6.60 Hz). ^{13}C NMR ($CDCl_3$, 100.5 MHz): δ 173.18, 149.11, 135.74, 129.76, 119.85, 64.37, 40.39, 36.50, 34.82, 31.72, 29.21, 25.62, 22.49, 22.49, 15.93, 13.92.

General Procedure for Deblocking Phosphate Esters. Trimethylsilyl bromide (TMSBr, 730 μL, 5.5 mmol) was added dropwise to a solution of the phosphate triester (0.55 mmol) in CH_2Cl_2 (1.0 mL). The solution was stirred overnight. Volatiles were removed, and the off-white residue was stirred in methanol for 0.5 h. Volatiles were again removed, and the off-white solid was dissolved in water or methanol and passed down a Dowex ion exchange column (sodium form) to afford a white solid as the final product.

*Estrone Phosphate, **1***. Yield = 96%. ^{1}H NMR (CD_3OD, 400 MHz): δ 7.17 (d, 1H, J = 9.2 Hz), 6.98 (m, 2H), 2.87 (t, 2H, 4.4 Hz), 2.54−2.36 (m, 2H), 2.30−1.82 (m, 5H), 1.72−1.35 (m, 6H), 0.92 (s, 3H). ^{13}C NMR (CD_3OD, 75 MHz): δ 152.97, 138.29, 134.84, 126.72, 121.32 (d, J = 4.4 Hz), 118.79 (d, J = 4.9 Hz), 51.66, 45.42, 39.82, 36.74, 32.81, 30.42, 27.72, 27.08, 22.50, 14.27. ^{31}P NMR (CD_3OD, 146 MHz): δ −0.98.

*Dehydroepiandrosterone Phosphate, Triethylammonium salt, **2***. The ester phosphate was deblocked as previously described. The residue was dissolved in THF (1 mL), a 1:1 solution of triethylamine:methanol (1 mL) was added, and the product was concentrated under reduced pressure. CH_3CN (2 × 3 mL) was added, and the product was concentrated *in vacuo* to produce a white solid (0.223 g, 96%). ^{1}H NMR (CD_3OD, 400 MHz): δ 5.40 (d, 1H, J = 5.2 Hz), 3.95 (m, 1H), 2.55−2.41 (m, 2H), 2.31 (t, 1H, J = 12 Hz), 2.19−1.47 (m, 12H), 1.39−1.20 (m, 2H), 1.17−0.98 (m, 5H), 0.89 (s, 3H). ^{13}C NMR (CD_3OD, 100.5 MHz): δ 223.53, 142.21, 122.30, 76.29 (d, J = 5.2), 53.01, 51.75, 47.32, 41.60, 38.38, 37.78, 36.68, 32.75, 32.68, 31.87, 31.09, 31.05, 22.80, 21.45, 19.87, 13.94, 9.09. ^{31}P NMR (CD_3OD, 146 MHz): δ 2.31.

*p-Octylphenyl Phosphate, **5***. Yield = 92%. ^{1}H NMR (CD_3OD, 400 MHz): δ 7.14 (2H, d, J = 8.00 Hz), 7.09 (2H, d, J = 9.60 Hz), 2.57 (2H, t, J = 3.2 Hz), 1.58 (2H, m), 1.28 (10H, m), 0.89 (3H, t, J = 8.00 Hz). ^{31}P NMR (CD_3OD, 146 MHz): δ −3.508. Compound **5** was too insoluble to obtain a ^{13}C NMR spectrum.

*p-Decylphenyl Phosphate, **6***. Yield = 100%. ^{1}H NMR (CD_3OD, 400 MHz): δ 7.10 (4H, s), 2.56 (2H, t, J = 8.17 Hz), 1.58 (2H, m), 1.28 (14H, m), 0.86 (3H, t, J = 6.92 Hz). ^{31}P NMR (CD_3OD, 360 MHz): δ −3.302. Compound **6** was too insoluble to obtain a ^{13}C NMR spectrum.

*p-Octanoylphenyl Phosphate, **7***. Yield = 57% (recovering starting material yield = 72%). ^{1}H NMR (CD_3OD, 400 MHz): δ 7.98 (2H, d, J = 8.80 Hz), 7.55 (2H, d, J = 8.80 Hz), 2.98 (2H, t, J = 7.24 Hz), 1.68 (2H, m), 1.31 (8H, m), 0.90 (3H, t, J = 6.92 Hz). ^{13}C NMR (CD_3OD, 100.5 MHz): δ 201.67, 157.56, 134.04, 131.10, 121.20, 39.33,

32.94, 30.37, 25.69, 23.70, 14.43. ^{31}P NMR (CD_3OD, 146 MHz): δ −4.184.

*p-Decanoylphenyl Phosphate, **8***. Yield = 93%. ^{1}H NMR (CD_3OD, 400 MHz): δ 7.99 (2H, d, J = 8.81 Hz), 7.33 (2H, d, J = 8.80 Hz), 2.97 (2H, t, J = 7.54 Hz), 1.70 (2H, m), 1.30 (12H, m), 0.89 (3H, 6.61 Hz). Compound **8** was too insoluble to obtain ^{13}C or ^{31}P NMR spectra.

*n-Lauroyltyramine Phosphate, **9***. Yield = 100%. ^{1}H NMR (CD_3OD, 400 Mz): δ 7.12 (2H, d, J = 8.80 Hz), 7.05 (2H, d, J = 8.71 Hz), 3.42 (2H, t, J = 7.23 Hz), 2.69 (2H, t, J = 7.24 Hz), 2.09 (2H, t, J = 7.23 Hz), 1.70 (2H, t, J = 7.23 Hz), 1.25 (16H, m), 0.82 (3H, t, J = 6.61 Hz). ^{13}C NMR ($CDCl_3$, 100.5 MHz): δ 176.32, 151.69, 136.58, 130.80, 121.33, 95.16, 41.96, 37.12, 35.82, 33.07, 30.44, 27.06, 24.21, 23.74, 14.46. ^{31}P NMR (CD_3OD, 146 MHz): δ −3.51.

*Tetrahydronaphthyl Phosphate, **10***. The diethyl ester was deblocked as described above. The residue was then dissolved in THF (0.5 mL), a 1:1 solution of triethylamine/methanol (0.5 mL) was added, and the product was concentrated under vacuum. CH_3CN (2 × 0.5 mL) was added, and the product was concentrated *in vacuo* to afford a white solid (0.055 g, 93%). ^{1}H NMR (CD_3OD, 400 MHz): δ 6.91 (bs, 3H), 3.21 (q, 6H, J = 7.5 Hz), 2.69 (m, 4H), 1.75 (m, 4H), 1.27 (m, 9H). ^{13}C NMR (CD_3OD, 75 MHz): δ 152.03, 138.86, 132.82, 130.54, 121.49 (d, $J_{C,P}$ = 5.1 Hz), 118.91 (d, $J_{C,P}$ = 4.5 Hz), 47.33, 30.48, 29.70, 24.53, 24.27, 9.04. ^{31}P NMR (CD_3OD, 146 MHz): δ −2.45.

General Procedure for the Synthesis of Phosphorofluoridates. Triethylamine (0.30 mL, 2.1 mmol) and pyridine (0.30 mL, 5.7 mmol) were added to the appropriate phosphate ammonium salt (0.260 mmol). The mixture was stirred until the phosphate had dissolved, and then (70:30) HF−pyridine (0.060 mL, 1.60 mmol) was added. The reaction was stirred for 10 min, and then a solution of DCC (113 mg, 0.548 mmol) in pyridine (5.7 mmol) was added. After the solution had been stirred at 50 °C for 20 h, the reaction was quenched with brine. The solution was then filtered and rinsed with H_2O. The filtrate was washed with petroleum ether (3 × 1 mL) and extracted with CH_2Cl_2 (3 × 1 mL). The CH_2Cl_2 extracts were combined, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The compound was dissolved in distilled water and passed down a Dowex AG 50W-X8 Na^+ form cation exchange column. The desired product was concentrated *in vacuo* to give a white solid.

*Estrone Phosphorofluoridate, Sodium Salt, **3***. Yield = 57%. ^{1}H NMR (D_2O, 300 MHz): δ 7.04 (d, 1H, J = 8.4 Hz), 6.72 (m, 2H), 2.63 (m, 2H), 2.42−2.26 (m, 1H), 2.18 (m, 1H), 2.08−1.56 (m, 5H), 1.55−1.03 (m, 6H), 0.69 (s, 3H). ^{13}C NMR (CD_3OD, 100 MHz): δ 223.23, 151.48, 139.07, 136.74, 121.17 (d, J = 4.4 Hz), 118.52 (d, J = 4.4 Hz), 51.53, 45.29, 39.58, 36.69, 32.75, 30.41, 27.49, 26.97, 22.46, 14.29. ^{31}P NMR (CD_3OD, 146 MHz): δ −9.91 (d, $J_{F,P}$ = 936 Hz). ^{19}F NMR (CD_3OD, 339 MHz): δ −77.52 (d, $J_{F,P}$ = 939 Hz).

*Dehydroepiandrostrone Phosphorofluoridate, Sodium Salt, **4***. Yield = 69%. ^{1}H NMR (CD_3OD, 400 MHz): δ 5.40 (d, 1H, J = 5.2 Hz), 4.02 (m, 1H), 2.50−2.30 (m, 3H), 2.16−1.91 (m, 4H), 1.90−1.75 (m, 2H), 1.72−1.41 (m, 6H), 1.36−1.20 (m, 2H), 1.14−0.94 (m, 5H), 0.89 (s, 3H). ^{13}C NMR (CD_3OD, 100 MHz): δ 221.23, 140.55, 121.25, 76.13, 51.66, 50.08, 45.66, 40.05, 36.95, 36.53, 35.83, 31.42, 31.36, 30.73, 29.57, 21.83, 20.28, 19.32, 13.50. ^{31}P NMR (CD_3OD, 146 MHz): δ −5.37 (d, $J_{P,F}$ = 932.1 Hz). ^{19}F NMR (CD_3OD, 139 MHz): δ −75.35 (d, $J_{P,F}$ = 931.1 Hz).

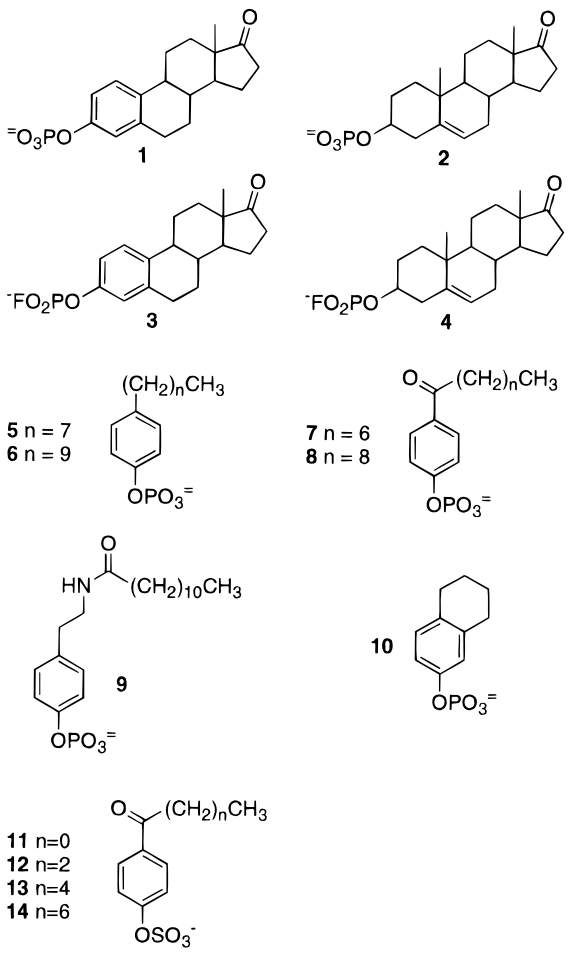

FIGURE 1: Structures of steroid sulfatase inhibitors and substrates.

RESULTS

Synthesis of Inhibitors and Substrates. The new inhibitors and substrates described in this paper were synthesized by phosphorylation or sulfation of *p*-acylphenols and *p*-alkylphenols. These acylphenols are easily prepared by Friedel−Crafts acylation of phenol (with the requisite acid chloride). Conversion to the sulfate or phosphate ester is then readily accomplished by sulfation with sulfur trioxide−pyridine or phosphorylation with diethyl chlorophosphate. The desired alkyl phenols, all known compounds, were prepared by Clemmenson reduction of the corresponding acyl phenol. These compounds were also readily phosphorylated with either diethyl chlorophosphate or the recently reported alkylphosphite/I_2 method (Stowell & Widlanski, 1995).

K_i Determination for the Inhibition of Steroid Sulfatase Activity. The inhibition of steroid sulfatase activity by each compound (see Figure 1 for structures) was examined as follows: the K_i for each compound at the indicated pH was routinely determined by secondary replot of the slopes from Lineweaver−Burk plots versus corresponding inhibitor concentrations (Engel, 1981). The slopes of Lineweaver−Burk plots (Figure 2A and 2B) generated from initial rates of *p*-acetylphenyl sulfate hydrolysis in the presence and absence of varying concentrations of inhibitor were plotted as a function of the corresponding concentration of inhibitor (see

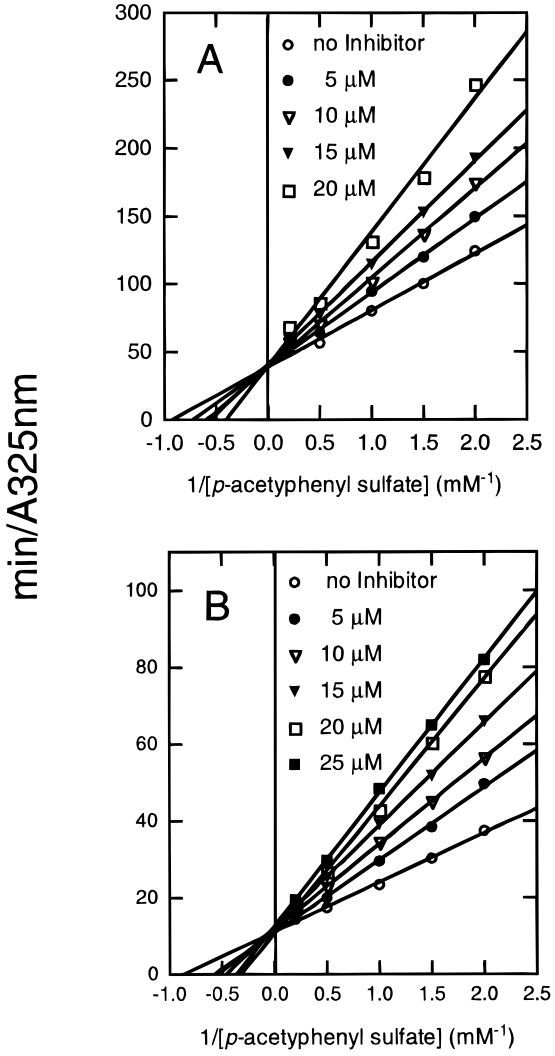

FIGURE 2: Lineweaver−Burke plots of the effects of dehydroepiandrosterone phosphofluoridate (A) and *p*-octylphenyl phosphate (B) on steroid sulfatase activity at pH 7.5. See Materials and Methods for reaction procedures and conditions.

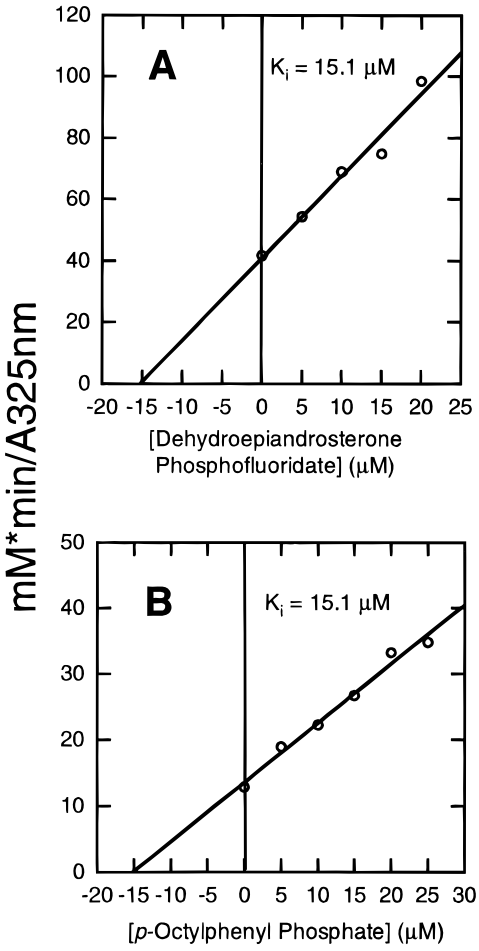

FIGURE 3: Secondary replots of slopes from Lineweaver−Burk plots versus the corresponding inhibitor concentrations to determine the K_i of dehydroepiandrosterone phosphofluoridate (A) and *p*-octylphenyl phosphate (B) for steroid sulfatase at pH 7.5. K_i was determined as described in Materials and Methods.

Figure 3A and 3B). In each case inhibition was found to be strictly competitive.

pH Dependent Inhibition of Steroid Sulfatase Activity by Steroidal Phosphates and Derivatives. Steroidal phosphates were found to be competitive inhibitors of steroid sulfatase activity at pH 7.5. The pH dependence of this inhibition was examined in order to determine whether steroid sulfatase was preferentially inhibited by the monoanionic or dianionic phosphate species. The K_i for both estrone phosphate and dehydroepiandrosterone phosphate decreases substantially when the pH is reduced. Inhibition of steroid sulfatase activity by estrone phosphate was weakest at pH 8.5 with a K_i value of 52.3 μM and strongest at pH 6.0 with a K_i value of 170 nM. Inhibition of steroid sulfatase activity by dehydroepiandrosterone phosphate also demonstrated a similar trend with K_i values of 110 nM and 4.1 μM at pH 5.5 and 8.0, respectively. The inhibition of steroid sulfatase activity by estrone phosphofluoridate and dehydroepiandrosterone phosphofluoridate was investigated at pH 6.0 and 7.5 (see Table 1). The K_i for estrone phosphofluoridate was determined to be 14.7 and 13.7 μM and the K_i for dehydroepiandrosterone phosphofluoridate was found to be 11.3 and 17.6 μM at pH 6.0 and 7.5, respectively. In contrast to the steroidal phosphates, the K_i for the steroidal phosphofluoridates did not vary significantly as a function of changing pH.

Inhibition of Steroid Sulfatase Activity with Non-Steroidal Phosphates. To delineate structural features important in enzyme−substrate (inhibitor) interaction, several non-steroidal phosphate esters were synthesized (structures shown in Figure 1) and examined for their ability to inhibit steroid sulfatase activity. The phosphate esters contained various features of the steroid nucleus. The K_i for each inhibitor was determined at pH 7.0 and 7.5 (results shown in Table 2) in each case the inhibition was observed to be pH dependent. In this series, tetrahydronaphthyl phosphate was observed to be the weakest inhibitor of steroid sulfatase activity, with a K_i of 360 and 870 μM at pH 7.0 and 7.5. *n*-Lauroyl tyramine phosphate was the strongest inhibitor at pH 7.0 with a K_i of 420 nM. Inhibition of steroid sulfatase activity by *n*-lauroyl tyramine phosphate ($K_i = 3.6$ μM) was observed to be comparable to the inhibition displayed by *p*-decylphenyl phosphate at pH 7.5 ($K_i = 3.2$ μM).

Determination of K_m and V_{max} for Chromogenic Aryl sulfates of Increasing Hydrophobicity. A commonly used unnatural substrate for assaying steroid sulfatase activity is *p*-acetylphenyl sulfate (Dodgson & Spencer, 1953; Dodgson et al., 1953; Milsom et al., 1972). The observation that increasing the hydrophobicity of non-steroidal aryl phosphates corresponded to a decrease in K_i led to the hypothesis

Table 1: K_i's for Compounds **1**−**4** (Each K_i Value Represents the Mean of at Least Two Independent Determinations)[a]

	pH						
compound	5.5	6.0	6.5	7.0	7.5	8.0	8.5
1		0.17 ± 0.01	0.34 ± 0.09	0.89 ± 0.15	5.0 ± 0.7	15.0 ± 1.1	52.3 ± 1.1
2	0.11 ± 0.04	0.14[a]	0.13 ± 0.01	0.89 ± 0.15	2.0 ± 0.02	4.1[a]	
3		14.7[a]			13.7 ± 1.6		
4		12.2 ± 0.9			17.6 ± 2.5		

[a] Data are the result of one determination. Conditions and procedures are described in Materials and Methods.

Table 2: K_i's for Compounds **5**−**10** (Each K_i Value Represents the Mean of at Least Two Independent Determinations)

	pH	
compound	7.0	7.5
5	5.1[a]	17.0 ± 1.9
7	20.0 ± 2.4	64.0 ± 1.0
10	360[a]	870 ± 0.13
8	7.0[a]	15.0[a]
9	0.520 ± 0.09	3.6[a]

[a] Data are the result of one determination. Conditions and procedures are described in Materials and Methods.

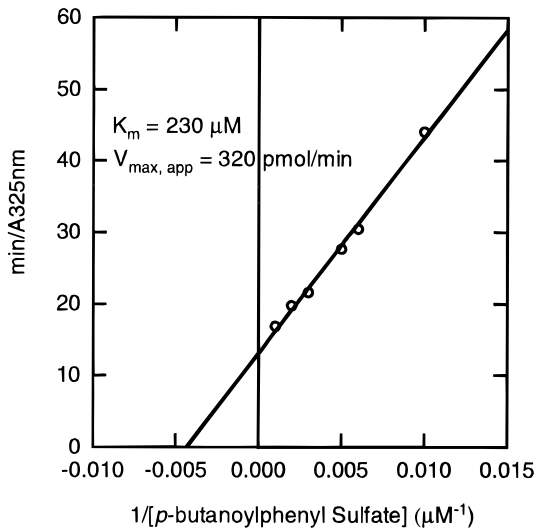

FIGURE 4: Lineweaver−Burk plot to determine K_m and $V_{max,app}$ for *p*-butanoylphenyl sulfate as a substrate of steroid sulfatase activity. Conditions and reaction procedure were as described in Materials and Methods.

that a similar trend might be observed for substrates of steroid sulfatase. We surmised that increasing the hydrophobicity of the *p*-acetylphenyl sulfate might result in decreasing the K_m. To ascertain the effect of increasing the hydrophobicity of steroid sulfatase substrates, the compounds shown in Figure 1 were synthesized and tested as substrates for steroid sulfatase. The Michaelis constants K_m and V_{max} were determined from Lineweaver−Burk plots (see Figure 4). The K_m for *p*-acetylphenyl sulfate of 1.0 mM is the highest in this series of compounds (**11**−**14**) compared to the lowest K_m value of 73.7 μM for *p*-octanoylphenyl sulfate. While K_m varies significantly as a function of increasing hydrophobicity V_{max} remains fairly constant. A summary of K_m and V_{max} data is shown in Table 3. The ϵ for the unconjugated form of each substrate was experimentally determined and found to be 21 000 M^{-1} cm^{-1}.

Determination of pK_a for Estrone Phosphate, Dehydroepiandrosterone Phosphate, n-lauryltyramine Phosphate, p-Decylphenyl Phosphate, and p-Decanoylphenyl Phosphate by ^{31}P NMR. The pK_a values for the steroidal phosphates and three non-steroidal phosphates were determined by ^{31}P NMR (Table 4). Chemical shift data for each compound were obtained and then plotted as a function of corresponding pH. The data were analyzed using the single pK_a determination curve fitter in the computer program Grafit.

Table 3: K_m's of Compounds **11**−**14**[a]

compound	K_m (mM)	$V_{max,app}$ (pmol/min)
11	1.0 ± 0.1	280 ± 6
12	0.220 ± 0.016	310 ± 13
13	0.082 ± 0.001	240 ± 7
14	0.074 ± 0.004	250 ± 6

[a] Conditions and procedures are described in Materials and Methods.

Table 4: pK_a Values for Compounds **1**, **2**, **5**, **7**, and **9**[a]

compound	pK_a	compound	pK_a
1	5.9	**7**	6.0
2	7.0	**9**	6.3
5	6.8		

[a] Conditions and procedures are described in Materials and Methods.

DISCUSSION

We previously reported that estrone phosphate and dehydroepiandrosterone phosphate are tight binding inhibitors of steroid sulfatase activity (Anderson et al., 1995). It was determined that the monoanionic form of the steroidal phosphate preferentially bound to steroid sulfatase. Though steroid sulfatase was strongly inhibited by the steroid phosphates, in no case was hydrolysis of the inhibitors observed. Thus it seems that the sulfatase can readily distinguish between the phosphoryl group and the sulfuryl group only with respect to catalysis and not with respect to binding.

One possible explanation for the tight binding of the steroidal phosphates is that the phosphate ester mimics an intermediate or transition state of the normal reaction. If the mechanism utilized by the sulfatase is an associative type of process that proceeds via the direct attack of a bound water molecule on the sulfate ester, the peripheral hydroxyl group of the phosphate monoanion may be well suited to mimic this attacking water molecule and engage in hydrogen bonding to an active site base. Dibbelt and Kuss have suggested that there is an active site histidine residue that has a pK_a of 5.8 (Dibbelt & Kuss, 1991). This histidine residue is a likely candidate for the active site base. Matching of the pK_a's of this active site base and estrone phosphate (5.9) might enhance the strength of this putative hydrogen bond (Cleland & Kreevoy, 1994; Schwartz et al., 1995). It has been suggested that the enzyme catalyzes sulfate ester hydrolysis via the intermediacy of a sulfated enzyme (Purohit et al., 1995). Presumably, even in this

mechanism an active site base would be necessary to activate a water molecule for enzyme desulfation. Of course, it is always possible that the tight binding of the steroidal phosphates is an entirely fortuitous result. Such ambiguities cannot be ruled out simply on the basis of the results that we have obtained.

In light of the crystallographic studies of sulfate- and phosphate-binding proteins (Pflugrath & Quiocho, 1985; Luecke & Quiocho, 1990) and theoretical studies (Kanyo & Christianson, 1991), we were surprised to find that an enzyme that catalyzes the hydrolysis of sulfate esters was so powerfully inhibited by phosphate esters. While the inhibition of sulfatase activity by steroidal phosphates is unexpected and has only been recently demonstrated for steroid sulfatase, some recent findings show that sulfate esters may sometimes function as reasonable mimics of phosphate esters, particularly as inhibitors of kinase and phosphatase activities (Li et al., 1995; Anderson, Myers, Widlanski, unpublished results). For example, tyrosine dephosphorylation of the insulin receptor has been shown to be inhibited by a sulfopeptide and protein tyrosine kinase $pp60^{v\text{-}src}$ can be inhibited by steroid sulfates (Liotta et al., 1994; Fu & Schmitz, 1994). In addition, we have recently shown that purple acid phosphatase activity is inhibited by *p*-decanoylphenyl sulfate (Anderson, Myers, Widlanski, unpublished results).

To delineate structural features involved in binding, we synthesized a variety of non-steroidal phosphate esters and assayed their ability to inhibit steroid sulfatase activity. The only essential molecular determinants important for steroid sulfatase−inhibitor binding were determined to be the A ring from the steroid nucleus and an appropriately positioned charged group. We found that the potency of steroid sulfatase inhibition could be greatly enhanced by increasing the hydrophobicity of the basic aryl phosphate motif. The aryl phosphate derivatives all displayed the same pH-dependence for sulfatase inhibition that we previously reported for steroid phosphates. For example, *p*-decylphenyl phosphate (**6**) is a competitive inhibitor with K_i values of 3.2 μM and 960 nM at pH 7.0 and 7.5. The K_i values are similar to those found for estrone phosphate (5.0 μM and 890 nM) at the same pH.

The data presented in Table 2 support the idea that the primary motif necessary for enzyme−inhibitor binding is the phosphorylated A ring. The potency of this binding interaction is clearly enhanced by increasing the hydrophobicity of the inhibitor. For example, the K_i values for *p*-octylphenyl phosphate and *p*-decylphenyl phosphate (compounds **5** and **6**) were determined to be 17.0 and 3.2 μM. This shows that increasing inhibitor hydrophobicity yields a more potent steroid sulfatase inhibitor. The simple addition of an ethylene group to *p*-octylphenyl phosphate results in a 5-fold increase in inhibition. We also tested tetrahydronaphthyl phosphate (**10**) as an inhibitor, because it contained both the A and B rings of the steroid nucleus. In the series of non-steroidal phosphates, this molecule was found to be the poorest inhibitor of steroid sulfatase activity (K_i = 360 and 870 μM at pH 7.0 and 7.5). This result was not unexpected, as Potter and co-workers have previously reported that sulfamate derivatives of tetrahydronaphth-2-ol are poor inhibitors of steroid sulfatase activity (Howarth et al., 1994). However, it is also clear that the C and D rings of the steroid are not required for recognition and probably only contribute to binding by virtue of enhancing the ability of these compounds to partition into a nonpolar (i.e., bilayer or micelle) environment.

Because the hydrophobicity of the phosphorylated inhibitors appeared to be an important determinant in binding, we surmised that this could also be a relevant factor for substrate−enzyme interaction. To test this idea, we synthesized increasingly hydrophobic derivatives of *p*-acetyl phenyl sulfate (**11**−**14**) and tested them as substrates for steroid sulfatase. The phenol product for each of these substrates is a chromogenic molecule with a λ_{max} of 323 nm and an $\epsilon_{(325nm)}$ of 21 000 cm^{-1} M^{-1}. Due to the chromogenic nature of the products, these molecules were ideal for quickly testing our hypothesis that increasingly hydrophobic steroid sulfatase substrates would have correspondingly lower K_m values.

Increasing the hydrophobicity of these steroid sulfatase substrates does indeed give rise to a reduction in K_m (Table 3). On going from *p*-acetyl phenyl sulfate to *p*-octanoylphenyl sulfate the K_m changes by a factor of 17.5. As, K_m decreases, the hydrophobicity of the substrate increases and V_{max} remains relatively unchanged. The likeliest explanation for this observation is that increasing the hydrophobicity of the substrate (or inhibitor) enhances partitioning into a nonpolar environment (i.e., micelle or bilayer) and presents the sulfatase with a locally higher substrate/inhibitor concentration. While substrate hydrophobicity plays a role in determining substrate K_m, we were surprised that the K_m value for these substrates does not approach the value for the natural substrate estrone sulfate (3.5 μM).

The behavior of these alternative substrates was not completely analogous to that observed for the steroidal and non-steroidal phosphorylated inhibitors. While the binding affinity of the inhibitory phosphates (i.e., **5** and **6**) correlates to their hydrophobicity and ultimately surpasses the binding potency of steroid sulfates, the K_m for the artificial substrates (**11**−**14**) never achieves that observed for the steroid sulfates. An obvious difference between these two sets of molecules (**5**, **6**, and **11**−**14**) is the presence of the benzylic ketone. For substrates, a benzylic ketone is required in this position if the product is to be chromogenic. However, for steric or electronic reasons the presence of this keto moiety may affect binding.

To further probe the possibility that the keto moiety affects binding, we synthesized and assayed two hydrophobic aryl phosphates (**7**−**8**) that contain a benzylic keto group analogous to that found in substrates (**11**−**14**), but are otherwise identical to inhibitors **5** and **6**. These inhibitory phosphates do have significantly higher K_i values than compounds lacking the keto functionality (i.e., **5** and **6**). The K_i's for *p*-octanoylphenyl phosphate and *p*-decanoylphenyl phosphate were determined to be 64 and 15 μM, at pH 7.5. By comparison, the K_i's for *p*-octylphenyl phosphate and *p*-decylphenyl phosphate are 17 and 3.2 μM. Thus, the presence of a benzylic ketone decreases the binding affinity of the inhibitors by a factor of approximately 4 in comparison to inhibitors lacking the ketone. However, the interpretation of this experiment is not completely straightforward.

The addition of an acyl group *para* to the phosphate ester may have an affect on the pK_a of the phosphate ester. Lowering of this value would likely reduce the effectiveness of the phosphorylated inhibitors because the monoanion is the inhibitory species. A comparison of the pK_a of *p*-octanoylphenyl phosphate (**7**) (6.0) and *p*-octylphenyl phos-

phate (**5**) (6.8) reveals that the addition of a benzylic ketone does indeed perturb the pK_a value of these inhibitors. For compounds containing the benzylic ketone, the lower pK_a may therefore correlate with a lower concentration of the monoanionic form of the inhibitor, resulting in poorer inhibition and higher K_i values. Thus, it is clear that a keto moiety in the benzylic position of both substrates (sulfate esters) and inhibitors (phosphate esters) decreases binding potential. However, the effect appears to be more significant for the sulfate esters.

To further test the potential for the sulfatase to interact with the side chain of the inhibitors, we synthesized and assayed a compound that contained a hydrophobic side chain appended to a phosphorylated tyramine group. In this molecule, the polar functionality (the amide) is further removed from the benzene ring so that it is not in conjugation. This compound was the best inhibitor of the sulfatase yet tested, with a K_i of 520 nM at pH 6.0 and a K_i of 3.6 μM at pH 7.5. Thus, an amide further removed from the benzene ring is not detrimental to inhibition. This result suggests that there is unlikely to be extensive interaction between the enzyme and the hydrophobic side chain of the inhibitor at positions several ångströms from the A ring. Therefore, it is probable that the role of the side chain is largely to increase partitioning of the inhibitor into the membrane.

The observation that hydrophobic aryl sulfates other than steroids function as good substrates for steroid sulfatase raises a question about the possibility of other sulfated molecules functioning as *in vivo* substrates of the sulfatase. For example, it is possible that steroid sulfatase may also catalyze the hydrolysis of the sulfotyrosyl residues of membrane-bound or membrane-associated proteins. Conversely, the inhibition of the sulfatase by hydrophobic aryl phosphates suggests a potential mechanism for *in vivo* regulation of this activity. *In vivo* sulfatase activity may be inhibited by a phosphorylated metabolite, protein, or even by phosphorylation of a residue on the sulfatase itself.

We have shown that steroid sulfatase activity is inhibited by monoanionic steroid phosphates. A series of simple non-steroidal phosphate and sulfate esters did indeed prove to be inhibitors/substrates of the sulfatase, thus demonstrating that the steroid nucleus as a whole is unnecessary for enzyme−inhibitor binding. Ultimately, these observations should enable us to define more clearly the nature of the substrate recognition process utilized by this enzyme. In addition, this study provides clear design parameters for new types of sulfatase inhibitors that are likely to be simple, highly potent, nonestrogenic, and useful in delineating the role of the sulfatase *in vivo*.

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