Synthesis and biochemical studies of estrone sulfatase inhibitors

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The synthesis and biochemical evaluation of estrone sulfatase inhibitors are described. Inhibitors were designed through modifications of the substrate estrone sulfate. An in vitro assay using the microsomal fraction isolated from human term placenta was used to evaluate sulfatase inhibitory activity. All the inhibitors (except sulfonyl chloride analog) exhibited low inhibitory activities in the screening assay. Sulfonyl chloride analog is a strong inhibitor, which caused 91.5% inhibition of the enzymatic activity at 300 μM. (*Steroids* **58**:106–111, 1993)

Keywords: steroids; estrone sulfatase; mammary carcinoma; sulfatase inhibitors; hormone-dependent

Introduction

It is estimated that approximately 30–40% of all breast cancers are estrogen dependent.[1] In postmenopausal advanced breast cancer patients, the estrogen levels of breast tumors are an order of magnitude higher than in plasma.[2,3] However, Bradlow detected no active uptake of estrogens by breast tumors, and he also pointed out that the circulating level of estrogens in postmenopausal women would not be sufficient to stimulate tumor growth.[4] Two pathways are proposed to occur in breast cancer cells to explain the high concentration of estrogens in breast tumors: (1) conversion of androstenedione to estrone by aromatase (aromatase pathway), and (2) conversion of estrone sulfate to estrone by estrone sulfatase (sulfatase pathway).[5,6] Recently, more attention has been directed toward the sulfatase pathway. Estrone sulfate is the most abundant circulating estrogen in women[7–11] and estrone sulfatase has been consistently found in human breast cancer cells.[5,6,12–21] It was reported that a high percentage of [^{3}H]estrone sulfate was converted to estradiol in different hormone-dependent mammary cancer cell lines (MCF-7, R-27, T-47D), but little or no conversion was found in the hormone-independent mammary cancer cell lines (MDA-MB-231, MDA-MB-436).[15,21] In addition, the conversion of [^{3}H]estrone sulfate to [^{3}H]estrone and [^{3}H]estradiol was also demonstrated by incubating the homogenates of mammary carcinoma tissue in vitro in 23 breast cancer patients.[14] Santen et al. evaluated estrogen production from breast tumor via the estrone sulfate to estrone (sulfatase) pathway and compared it with the androstenedione to estrone (aromatase) pathway. When comparing the sulfatase with aromatase activity in human tumors at physiological levels of substrates, the amount of estrone produced through sulfatase was 10 times higher than through the aromatase pathway (2.8 pmol estrone/g protein versus 0.27 pmol/g protein) in human breast tumors. Santen suggested that this sulfatase pathway is significant and perhaps the primary means of local estrogen production in breast tumor tissues.[5,6]

Preliminary results indicated the importance of estrone sulfate as a potential source of estrogen to support the growth of estrogen-dependent breast cancer. Potent inhibitors of estrone sulfatase may be potential therapeutic agents for the treatment of estrogen-dependent breast cancer. The structures of the proposed estrone sulfatase inhibitors are illustrated in Figure 1.

Experimental

General methods

Steroids were purchased from Sigma Chemical Company (St. Louis, MO) or Steraloids (Wilton, NH) and checked for purity by thin-layer chromatography or melting point (MP). Chemicals

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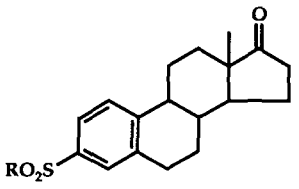


R = CH_3, O^-, NH_2, Cl, and F

Figure 1 Proposed estrone sulfatase inhibitors.

and silica gel were purchased from Aldrich Chemical Company (Milwaukee, WI). Biochemicals were obtained from Sigma Chemical Company. [6,7-^{3}H]Estrone sulfate (40–60 Ci/mmol) and [4-^{14}C]estrone (50–60 mCi/mmol) and [1β-^{3}H]4-androstene-3,17-dione (15–30 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Melting points were performed on a Thomas Hoover capillary melting point apparatus and were uncorrected. Infrared (IR) spectral data were obtained from a Perkin Elmer (Norwalk, CT) 1430 ratio recording spectrophotometer. Proton NMR spectra were obtained with either a Varian (San Fernando, CA) EM 360 NMR (60 MHz) or a Bruker (Billerica, MA) WH-300 (300 MHz) spectrophotometer. Elemental analyses were performed by Atlantic Microlab Inc. (Norcross, GA). Centrifugation was performed on a Damon CRU-5000 centrifuge and ultracentrifugation on a Beckman Type B ultracentrifuge. Radioactive samples were analyzed with a Packard Tri-Carb 4530 liquid scintillation counter using Insta-Gel (Packard Instrument Co., Downers Grove, IL) as the counting solution.

3-[(N,N-dimethylthiocarbamoyl)oxy]estra-1,3,5(10)-trien-17-one (2). To a solution of estrone **(1)** (15.0 g, 0.055 mol) in dry DMF (225 ml) under nitrogen was added a suspension of NaH (1.6 g, 0.066 mol) in DMF (2 ml). After the evolution of hydrogen had ceased, the solution was cooled in an ice bath and dimethylthiocarbamoyl chloride (10.6 g, 0.0833 mol) was added. The resulting solution was heated at 80 C for 1 hour, cooled to room temperature, and poured into cold water (300 ml). The crude product formed was then filtered, dried under vacuum, and purified by chromatography on a silica gel column eluted with methylene chloride to obtain **2** (16.7 g, 87%). An analytical sample was obtained by recrystallization from ethyl acetate: mp 210–212 C; IR (KBr) 1,735 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.93 (s, 3H, 18-CH_3), 3.35 (s, 3H, N-CH_3), 3.45 (s, 3H, N-CH_3), 6.7–7.4 (m, 3H, aromatic).

3-[(N,N-dimethylcarbamoyl)thio]estra-1,3,5(10)-trien-17-one (3). A suspension of **2** (16.7 g, 0.04 mol) in mineral oil (180 mL) was heated under nitrogen at 280 C for 5 hours. The reaction mixture was then cooled to room temperature, diluted with petroleum ether (400 ml), and filtered to obtain crude **3** (16 g, 95.8%), which was used directly for the next step. An analytical sample was obtained by column chromatography using silica gel and eluted with methylene chloride/hexane (10 : 1)/followed by recrystallization from ethyl acetate: mp 173–175 C; IR (KBr) 1,670, 1,730 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.9 (s, 3H, 18-CH_3), 3.07 (s, 6H, 2N-CH_3), 7.1–7.4 (m, 3H, aromatic).

3-Mercaptoestra-1,3,5(10)-trien-17-one (4). To a solution of **3** (5 g, 0.014 mol) in 95% ethanol (650 ml) was added NaOH (16 g), dissolved in water (70 ml), and the mixture was refluxed under nitrogen for 3 hours. The solution was then cooled to room temperature and the excess NaOH was neutralized using 8% HCl (150 ml). This was then concentrated under reduced pressure, poured into water (100 ml), filtered, and dried under vacuum. The crude product was then chromatographed on a silica column eluted with methylene chloride ethyl/acetate/hexane (10 : 1 : 1 v/v/v) to obtain **4** (3 g, 68%). An analytical sample was obtained by recrystallization from ethyl acetate: mp 195–197 C; IR (KBr) 1,735 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.92 (s, 3H, 18-CH_3), 3.36 (s, 1H, 3-SH), 7.04–7.26 (m, 3H, aromatic).

3-(Benzylthio)estra-1,3,5(10)-trien-17-one (6). To a solution of **4** (5 g, 0.0175 mol) in dry DMF (150 ml) was added a suspension of NaH (0.504 g, 0.021 mol) in dry DMF (5 ml) and was allowed to stir under nitrogen. After all the hydrogen had evolved, benzyl bromide (2.6 ml, 0.0218 mol) was added and the resulting mixture was allowed to stir at room temperature for 3 hours. The reaction mixture was then poured into cold water and the resulting precipitate was filtered and dried in vacuo. This crude product was then purified by column chromatography using silica gel and eluted with methylene chloride/ethyl acetate/hexane (10 : 1 : 1 v/v/v) to obtain **6** (5.2 g, 80%). An analytical sample was obtained by recrystallization from ethyl acetate: mp 127–129; IR (KBr) 1,730 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.9 (s, 3H, 18-CH_3), 4.1 (s, 2H, 3-SCH_2), 6.9–7.4 (m, 8H, aromatic). Analysis calculated for $C_{25}H_{28}OH$: C, 79.79; H, 7.49. Found: C, 79.56; H, 7.56.

Estra-1,3,5(10)-trien-17-one-3-sulfonyl chloride (7). Chlorine gas was bubbled for 5 minutes through a cool suspension of **6** (2 g, 0.0053 mol) in glacial acetic acid (100 ml) and water (13.2 ml). Cold water (300 ml) was added and the resultant mixture was extracted with ethyl acetate (200 ml). The water layer was further extracted with ethyl acetate (50 ml). The combined ethyl acetate portions were then washed well with 5% $NaHCO_3$, dried ($MgSO_4$), and evaporated under reduced pressure to obtain **7** (1.045 g, 55%) in a pure form: mp 153–155 C; IR (KBr) 1,740 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.93 (s, 3H, 18-CH_3), 7.26–7.81 (m, 3H, aromatic). Analysis calculated for $C_{18}H_{21}ClO_3S$: C, 61.27; H, 6.00. Found: C, 61.29; H, 6.02.

Estra-1,3,5(10)-trien-17-one-3-sulfonyl fluoride (10). To a solution of **7** (0.4 g, 1.13 mmol) in dioxane (8 ml) was added KF (0.15 g, 2.06 mmol) dissolved in water (2 ml) and the resultant mixture was heated at 50–55 C for 3 hours. This was then evaporated under reduced pressure and the residue obtained was chromatographed on a silica gel column and eluted with methylene chloride/hexane (10 : 1 v/v). An analytical sample was obtained by recrystallization from ethyl acetate to give **10** (0.29 g, 76%): mp 160–162 C; IR (KBr) 1,735 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.93 (s, 3H, 18-CH_3), 7.27–7.81 (m, 3H, aromatic). Analysis calculated for $C_{18}H_{21}FO_3S$: C, 64.26; H, 6.29. Found: C, 64.28; H, 6.28.

Estra-1,3,5(10)-trien-17-one-3-sulfonamide (9). Cold concentrated NH_4OH (7 ml) was added to a solution of **7** (0.2 g, 0.6 mmol) in cold acetone (7 ml). The resulting mixture was stirred at room temperature for 15 minutes, after which 30 ml of methylene chloride was added. This was then washed well with water followed by brine, dried with anhydrous $MgSO_4$, and evaporated under reduced pressure to get **9** (0.11 g, 58%): mp 228–230 C; IR (KBr), 3,240, 3,360, 1,715 cm^{-1}; ^{1}H NMR (DMSO-d6) δ 0.84 (s, 3H, 18-CH_3), 7.21 (s, 2H, -NH_2), 7.45–7.58 (m, 3H, aromatic). Analysis calculated for $C_{18}H_{23}NO_3S \times 0.25\ H_2O$: C, 63.97; H, 7.01, N 4.20. Found: C, 63.77, H. 6.96, N 4.07.

Estra-1,3,5(10)-trien-17-one-3-sulfonic acid, potassium salt (8). To a solution of **7** (0.6 g, 1.8 mmol) in dioxane was added aqueous K_2CO_3 (3.6 g in 40 ml) and the resulting solution was refluxed for 3 hours. Dioxane was then removed in vacuo and the residue

diluted with cold water and filtered to obtain **8** (0.32 g, 50.6%). An analytical sample was obtained by recrystallization from aqueous methanol: mp 292 C (decomposed); IR (KBr) 1,740 cm^{-1}; ^1H NMR (DMSO-d6) δ 0.83 (s, 3H, 18-CH_3), 7.20–7.34 (m, 3H, aromatic). Analysis calculated for $C_{18}H_{21}SO_4K$ × water: C, 55.36; H, 5.94. Found: C, 55.59; H, 5.96.

3-(Methylsulfinyl)estra-1,3,5(10)-trien-17-one (5). To a solution of **4** (0.858 g, 3 mmol) in dry DMF (40 ml) was added a suspension of NaH (0.086 g, 3.6 mmol) in dry DMF (3 ml). After the evolution of hydrogen had ceased, the solution was cooled in an ice bath and CH_3I (0.224 ml, 3.6 mmol) was added. The ice bath was then removed and the solution allowed to stir at room temperature for 3 hours. The solution was poured into 1% NaOH (60 ml) and the resulting precipitate was filtered. The crude product was washed well with water, dried in vacuo, and chromatographed on a silica gel column (methylene chloride) to obtain **5** (0.80 g, 90%): mp 121–123 C; IR (KBr) 1,735 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.92 (s, 3H, 18-CH_3), 2.47 (s, 3H, S-CH_3), 7.02–7.26 (m, 3H, aromatic). Analysis calculated for $C_{19}H_{24}OS$: C, 75.95; H, 8.05. Found: C, 76.00; H, 8.03.

3-(Methylsulfonyl)estra-1,3,5(10)-trien-17-one (11). A solution of mCPBA (0.86 g, 5 mmol) in $CHCl_3$ (10 ml) was added to a solution of **5** (0.5 g, 1.66 mmol) in $CHCl_3$ (5 ml) and was allowed to stir for 2 days at room temperature. The resulting precipitate was then filtered, and the filtrate was washed sequentially with water, $NaHCO_3$, and brine. This was then dried ($MgSO_4$), filtered, and evaporated under reduced pressure to obtain **11** (0.81, 85%): mp 175–177 C; IR (KBr) 1,735 cm^{-1}; ^1H NMR ($CDCL_3$) δ 0.93 (s, 3H, 18-CH_3), 3.03 (s, 3H, -$SO_2\underline{CH_3}$), 7.47–7.71 (m, 3H, aromatic). Analysis calculated for $C_{19}H_{24}O_3S$: C, 68.64; H, 7.28. Found: C, 68.76; H, 7.25.

Biochemical evaluation of inhibitors

Preparation of placental microsome. Human placentas were obtained immediately upon delivery from Mercy Hospital of Pittsburgh and stored on ice during transportation to the laboratory. The preparation of microsomes was performed according to the method of Ryan and Ohno.[22] All procedures were performed at 0–4 C. The placenta was cut free of connective tissue and large blood vessels with scissors. The tissue was then homogenized in a Waring blender (Waring Commercial, New Hartford, CT) with two parts of tissues to one part of homogenization buffer consisting of 0.05 M sodium phosphate, 0.25 M sucrose, and 0.04 M nicotinamide, pH 7. The homogenate was centrifuged at 10,000 × *g* for 30 minutes. The debris was discarded and the supernatant was centrifuged at 105,000 × *g* for 1 hour. The procedure was repeated once again and the resulting pellets were stored at −70 C. The pellets were used within 6 weeks after preparation.

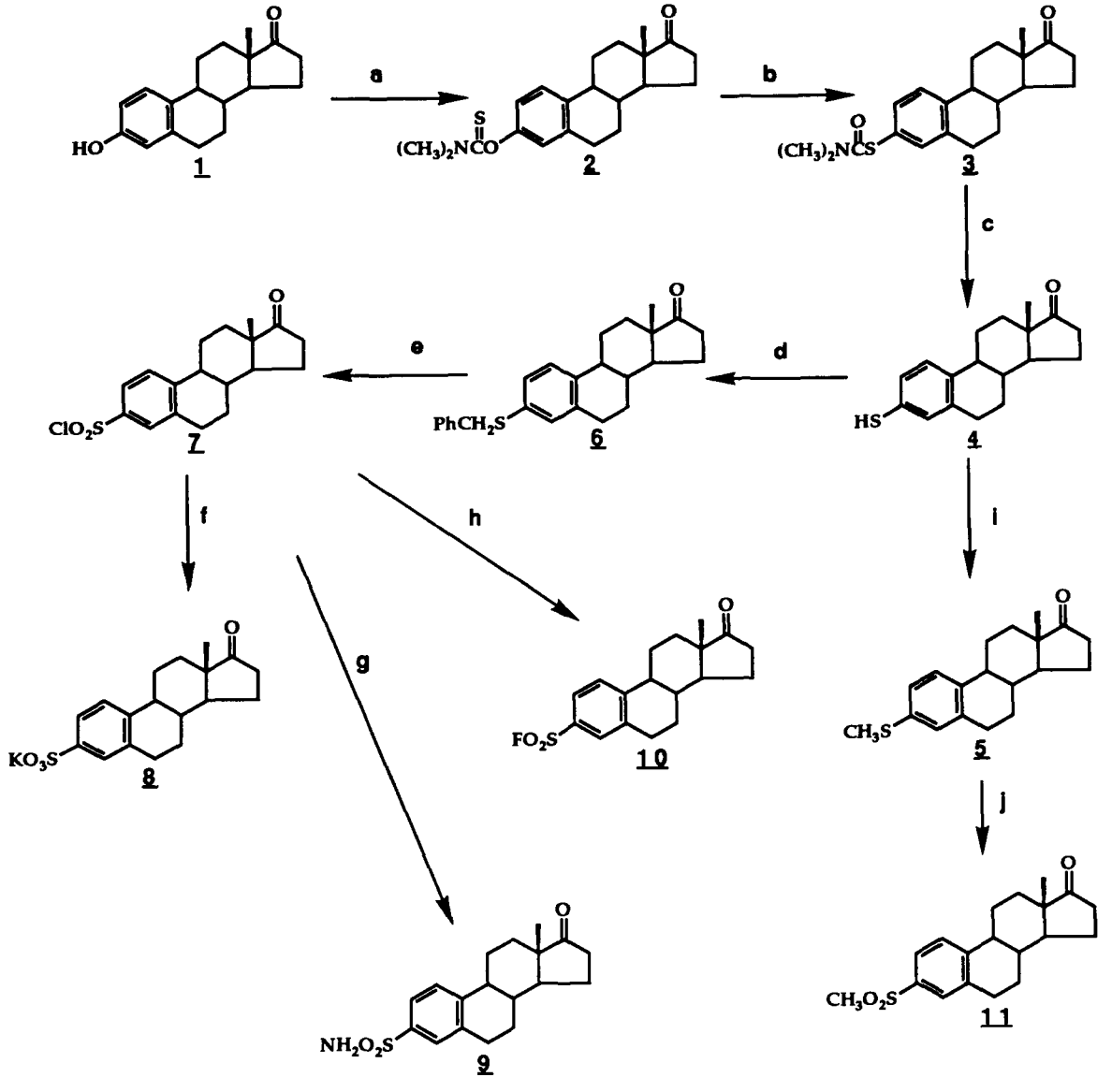
Screening assay procedure. [6,7-^3H]estrone sulfate (60 μM/tube; 1 μCi/tube) in ethanol and an inhibitor (60 or 300 μM/tube) in ethanol were added to a 5-ml test tube. The ethanol was removed with a stream of nitrogen. Tris-HCl buffer (0.05 M, pH 7.2, 0.8 ml) was added to each tube. Placental microsomes were then diluted with 0.05 M Tris-HCl buffer of pH 7.2 (1 mg of microsomal protein/ml of buffer). The microsomes and assay tubes containing steroids were preincubated for 5 minutes at 37 C in a water bath shaker. The assay began by the addition of the microsomes (0.2 ml) to the tubes. After 30 minutes of incubation at 37 C, 2 ml of ethyl acetate was added to quench the assay. [^{14}C]estrone (10,000 dpm/tube) was added concurrently with ethyl acetate as internal standard for the determination of extraction efficiency. Control samples with no inhibitor were incubated simultaneously. Blank samples were obtained by incubating boiled microsomes. Unconjugated estrone was extracted with 3 × 1 ml of ethyl acetate, and the ethyl acetate layer was combined, dried ($MgSO_4$), and removed. Estrone was purified by thin-layer chromatography using methylene chloride/ether (9 : 1 v/v), followed by a second chromatography using toluene-ethanol (13% ethanol, v/v). The zone corresponding to estrone was scraped off and extracted with ethanol. Aliquots were taken for determination of radioactivity. All the samples were run three times in triplicate with variation of less than 5%. Product formation for samples containing an inhibitor was compared with that of the control samples run simultaneously and are reported as percent inhibition of control samples.

For the K_m determination, the substrate (0.01–0.2 mM) was incubated with enzyme (0.2 mg microsomal protein per sample for 5 minutes), and the remainder of the procedure was completed as described for the screening assay. Under these conditions, the conversion of substrate to product did not exceed 12.5%, and the reaction velocity was constant.

Aromatase assay. Aromatase activity in human placental microsomes was assayed by a radiometric method developed by Siiteri and Thompson[23] in which the tritium from [1β-^3H]4-androstene-3,17-dione was released as ^3H_2O and used as an index of estrogen formation. Briefly, [1β-^3H]4-androstene-3,17-dione (300,000 dpm, 250 nM) and a concentration of inhibitor (60 μM) was preincubated with propylene glycol (100 μl), nicotinamide-adenine-dinucleotide phosphate (1.8 mM), and glucose 6-phosphate dehydrogenase (5 U) at 37 C for 5 minutes. The enzyme assay began with the addition of the microsomal suspension (3.0 ml, 0.05 mg) to the mixture of steroids and cofactors. The solution was incubated at 37 C for 15 minutes in a shaking water bath and was stopped by addition of $CHCl_3$ (5 ml), followed by vortexing of the samples for 20 seconds. The samples were then centrifuged for 10 minutes (1,000 × *g*). Aliquots of water (200 μl) were mixed with scintillation cocktail (5 ml), and the radioactivity was counted. Assays were run three times in triplicate with variation of less than 6% and control samples containing no inhibitor were run simultaneously. Blank samples were obtained by incubating boiled microsomes. Product formation for samples containing an inhibitor was compared with that of the control samples run simultaneously and are reported as percentage of activity of control samples.

Results and discussion

The synthesis of the proposed estrone sulfatase inhibitors was performed as shown in Scheme 1, which is similar to the synthetic scheme designed by Metcalf and co-workers for the synthesis of 5α-reductase inhibitors.[24] Treatment of estrone **1** with sodium hydride and dimethylthiocarbamoyl chloride in dimethylformamide (DMF) afforded O-aryl thiocarbamate **2**.[25] The thiocarbamate **2** was converted to S-aryl thiocarbamate **3** through Newman-Kwart rearrangement by heating **2** in heavy mineral oil (285–295 C) for 4 hours.[25] Thiophenol **4** was obtained by hydrolysis of **3** with sodium hydroxide in aqueous ethanol.[25] Sulfonyl chloride **7**, the most important intermediate in the synthetic scheme, was obtained through benzylation of the thiophenol to **6** followed by oxidation with chlorine in acetic acid-water.[26] The resulting sulfonyl chloride was hydrolyzed with aqueous potassium carbonate in dioxane to obtain the potassium salt of the sulfonic acid **8**. Treatment of **7** with ammonium hydroxide yielded

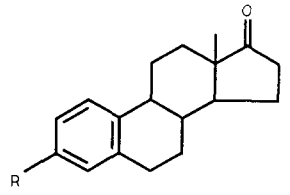


Scheme 1 (a) NaH, Me_2NCSCl; (b) 285 C; (c) NaOH, $EtOH/H_2O$; (d) NaH, $PhCH_2Br$; (e) Cl_2, $HOAc/H_2O$; (f) K_2CO_3, $dioxane/H_2O$; (g) NH_4OH; (h) KF, $dioxane/H_2O$; (i) NaH, CH_3I; (j) mCPBA.

sulfonamide **9**. Sulfonyl fluoride analog **10** was obtained by stirring **7** with potassium fluoride in dioxane. The methylsulfone analog **11** was obtained through methylation of thiophenol followed by oxidation with m-chloroperbenzoic acid (mCPBA). Inhibitor **8** could be obtained directly by reacting the S-aryl thiocarbamate **3** with hydrogen peroxide in formic acid.[27] Sulfonyl chloride **7** could then be obtained by reacting **8** with phosphorus pentachloride. However, this route was not adopted because of low yield.

The approach for the design of the proposed inhibitors is by removing the phenolic oxygen on the substrate estrone sulfate to eliminate the point of cleavage by the enzyme to form the anionic sulfonate analog (**8**), the neutral sulfonamide (**9**), and methylsulfone (**11**) analogs. The sulfonyl chloride (**7**) and sulfonyl fluoride (**10**) analogs were designed as active site–directed irreversible inhibitors. An in vitro screening assay similar to the one used by Brueggemeier and co-workers for the evaluation of aromatase inhibitors was used for the preliminary evaluation of the newly synthesized compounds.[28] Microsomal fraction of human placental tissue was used as the sulfatase enzyme system. The common assay for estrone sulfatase activity used [6,7-^{3}H]estrone sulfate as the substrate and analyzed the amount of [6,7-^{3}H]estrone formed. The screening assays were run in triplicate, and the amount of estrone formed was averaged. The values were then compared with control samples (without inhibitor) run simultaneously, and were reported as the percentage of inhibition of control samples. The substrate concentration was 60 μM, which is approximately five times the K_m (12

Table 1 Inhibition of estrone sulfatase in human placental microsomes.



Inhibitor	Substituent (R)	% Inhibition of estrone sulfatase [I] = 60 μM	[I] = 300 μM
7	$-SO_2Cl$	65.1	91.5
8	$-SO_3^-K^+$	22.0	40.2
9	$-SO_2NH_2$	18.9	45.3
10	$-SO_2F$	25.8	44.1
11	$-SO_2CH_3$	6.5	35.6

μM) for the enzyme preparation. Inhibitors were assayed at concentrations of 60 and 300 μM. Currently, it could not be determined from these screening assays whether the inhibitors inhibited the enzyme by binding to the active site of the enzyme unless competitive inhibition studies are performed. However, in the screening assays, the percent inhibitions were reduced when the substrate concentration was increased (results not shown). The results of the screening assays of the inhibitors are shown in Table 1. All the inhibitors (except sulfonyl chloride **7**) exhibit low inhibitory activities (% inhibition ranges from 35.6% to 45.3% at concentration of 300 μM) irrespective of their charges or hydrogen bonding capacities. Sulfonyl chloride analog **7** is a strong inhibitor, which caused 91.5% inhibition of the enzyme at 300 μM concentration. Inactivation of the enzyme through alkylation may explain its strong inhibitory activity. In addition, in order to investigate if the inhibition of sulfatase activities by the inhibitors was due to the disruption of the microsomal membrane, the enzymatic activity of aromatase (another enzyme present in the placental microsomes) was measured in the presence of inhibitors (60 μM) and compared with control (without inhibitor). The results of the aromatase assay (shown as percentage of aromatase activity of control) is shown in Table 2. The percentage of aromatase activity in the presence of inhibitors is 88.9–95.1%, which does not correlate with their abilities to inhibit estrone sulfatase.

Table 2 Effect of proposed inhibitors on aromatase activity.

Inhibitor	% Aromatase activity of control
7	90.5
8	88.9
9	93.3
10	90.3
11	95.1

The results indicated that the inhibitors did not disrupt the microsomal membrane. Inactivation studies on inhibitors **7** and **10** will be performed. In addition, competitive inhibition and cell culture studies on all inhibitors are underway.

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