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2-Difluoromethyloestrone 3-*O*-sulphamate, a highly potent steroid sulphatase inhibitor

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Abstract

Steroid sulphatase is a target enzyme of growing therapeutic importance. The synthesis and in vitro biological evaluation of three novel 2-substituted analogues of oestrone 3-*O*-sulphamate (EMATE), an established steroid sulphatase inhibitor, are described. One inhibitor, 2-difluoromethyloestrone 3-*O*-sulphamate (**6**), was found to have an IC_{50} of 100 pM and be some 90-fold more potent than EMATE in inhibiting steroid sulphatase activity in a placental microsomal preparation, rendering this agent the most potent steroidal STS inhibitor in vitro reported to date. Lowering of the pK_a value of the leaving parent steroid phenol by the 2-difluoromethyl group during irreversible enzyme sulphamoylation most likely facilitates the potent inactivation of steroid sulphatase by (**6**). However, our preliminary molecular docking studies using the X-ray crystal structure of steroid sulphatase suggest that F·····H interactions between the 2-difluoromethyl group of (**6**) and hydrogen bond donor residues lining the catalytic site of STS might also contribute to the high potency observed for (**6**).

Keywords: Steroid sulphatase; Breast cancer; 2-Difluoromethyl oestrone sulphamate

The inhibition of the steroid sulphatase (STS) pathway, which involves the hydrolysis of the biologically inactive oestrone sulphate (E1S) to oestrone, is now recognised to be a new form of endocrine therapy for hormone-dependent breast cancer (HDBC). There is strong evidence to suggest that an STS inhibitor acting alone or in conjunction with an aromatase inhibitor might render a more effective oestrogen ablation in postmenopausal women suffering from HDBC. The recent entry of the non-steroidal STS inhibitor 667COUMATE [1] into a Phase I trial is a promising advance. The results of a follow-on Phase II trial will allow the role of STS inhibition in the management of HDBC to be assessed.

The first potent STS inhibitor discovered was the steroidal oestrone 3-*O*-sulphamate (EMATE) [2]. The high potency observed for EMATE has been attributed to its structural mimicry of the natural substrate E1S and its unique manner of enzyme inactivation which is time- and concentration-dependent [3]. However, EMATE was subsequently shown to be highly oestrogenic in the rat and this undesirable property of EMATE renders this agent as not suitable for use in the treatment of HDBC.

As part of a programme to reduce the oestrogenicity of EMATE and to further improve its potency as an STS inhibitor, one of the initial strategies was to introduce substituents such as 2-propenyl, *n*-propyl, nitro, methoxy [4]; halogens, cyano, methylsulphanyl, and ethyl [5] to the A-ring of EMATE at the 2- and/or 4-positions. The general structure–activity relationship derived from these studies for the 2-substituted analogues of EMATE indicated that compounds with electron-withdrawing substituents on the A-ring showed either comparable (e.g., 2-NO_2) or higher potency (e.g., 2-halogens, 4-nitro) than EMATE in vitro. In comparison, those analogues with bulkier aliphatic substituents were found to be weaker STS inhibitors. Overall, the most successful A-ring modified analogue of EMATE was 2-methoxyoestrone 3-*O*-sulphamate. Although this agent is considered to be equipotent to EMATE, it has

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the important advantage over EMATE of being non-oestrogenic [4].

To further expand the series of 2-substituted analogues of EMATE and investigate the effects of these substituents on the STS inhibitory activity of EMATE, we report in this work the synthesis of the novel analogues 2-methyloestrone 3-*O*-sulphamate (**10**, Scheme 1), 2-difluoromethyloestrone 3-*O*-sulphamate (**6**), and 2-difluoromethyloestradiol 3-*O*-sulphamate (**7**). The 2-methyl derivative (**10**) has not yet been reported and the STS inhibitory activity of this compound will further bolster the structure–activity relationship for those members of the series bearing lower alkyl substituents at the 2-position of the A-ring. The introduction of fluorine atoms in the 2-methyl group of (**10**) to give derivative (**6**) was designed to introduce an electron-withdrawing effect on the A-ring of the inhibitor, rendering the parent 2-difluoromethyloestrone (**5**) a better leaving group. Our previous work on non-steroidal STS inhibitors [1,6] has already demonstrated that lowering the pK_a values of phenols (Ar–OH) will enhance the potency of their respective sulphamates ($ArOSO_2NH_2$) against STS. It has been reasoned that the sulphamoylation of important catalytic amino acid residue(s) lining the active site, which requires the prior cleavage of the H_2NSO_2–O bond of the irreversible inhibitor, is facilitated in phenol sulphamate esters whose phenolic groups have better leaving ability. In common with other candidates of the series, sulphamates (**6**), (**7**), and (**10**) are tested in a placental microsomal preparation which is an established in vitro assay for determining the inhibitory activities of potential STS inhibitors.

Materials and methods

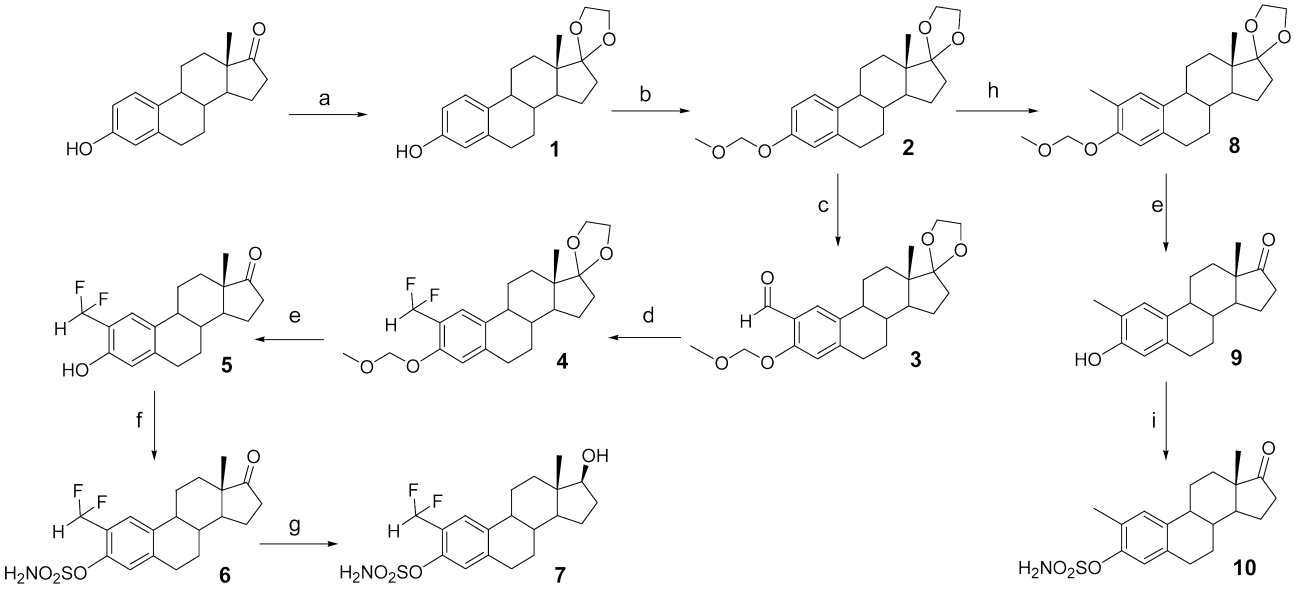
Materials

All chemicals were either purchased from Aldrich Chemical/Fluka (Gillingham, Dorset, UK) or Lancaster Synthesis (Morecambe, Lancashire, UK. All organic solvents, of A.R. grade, were supplied by Fisher Scientific (Loughborough, UK) and stored over 4 Å molecular sieves. A solution of sulphamoyl chloride in toluene (ca. 0.7 M) was prepared as described by Woo et al. [7]. An appropriate volume of this solution was freshly concentrated in vacuo immediately before use. Sulphamoylation of 2-methyloestrone (**9**) was carried out according to the procedure described by Okada et al. [8].

Thin Layer Chromatography (TLC) was performed on pre-coated plates (Merck TLC aluminium sheets silica gel 60 F_{254}, Art. No. 5554). Flash column chromatography was performed on silica gel (Sorbsil C60). IR spectra were determined by a Perkin–Elmer 782 infrared spectrophotometer and peak positions are expressed in cm^{-1}. ^1H and ^{19}F NMR spectra of compounds were recorded with Jeol JMN-GX270 and JMN-GX400 NMR spectrometers, and chemical shifts are reported in parts per million (ppm, δ) relative to residual undeuterated solvent unless stated otherwise. FAB-mass spectra were recorded at the Mass Spectrometry Service Centre, University of Bath, using *m*-nitrobenzyl alcohol (NBA) as the matrix. Elemental analyses were performed by the Microanalysis Service, University of Bath, and are within ± 0.4% of theory unless otherwise stated. Melting points were determined using a Reichert–Jung Thermo Galen Kofler block and are uncorrected.

LC/MS system

Waters 2790 Alliance, ZQ MicroMass spectrometer and PDA detector; Ionisation technique: APCI; Column: Waters "Symmetry" C18 (packing: 3.5 μm), 4.6 × 100 mm; Elution: Gradient (Flow Rate): 50:50 MeOH/H_2O (0.5 mL/min) to 95:5 MeOH/H_2O (1 mL/min) over 10 min. *HPLC* System: Waters 717 with Autosampler and PDA detector; Column: Waters Radialpak C18 8 × 100 mm; Mobile phase: MeOH/H_2O (70:30) isocratic; Flow rate: 1 mL/min.



Scheme 1. Synthesis of sulphamates (**6**), (**7**), and (**10**). (a) ethylene glycol/toluene, *p*-TSA, Dean and Stark conditions, 16 h; (b) NaH/DMF, 0 °C, MOMCl, overnight at r.t.; (c) *s*-BuLi/THF, DMF, −78 to 0 °C; (d) Deoxo-fluor (3 eq.)/CH_2Cl_2, reflux 16 h; (e) AcCl/MeOH, 15 min at r.t.; (f) CH_2Cl_2, DBMP, H_2NSO_2Cl; (g) THF-MeOH, $NaBH_4$, 0 °C; (h) s-BuLi/THF, MeI, −78 to 0 °C; and (i) H_2NSO_2Cl/DMA.

Chemistry

The synthesis of sulphamates (**6**), (**7**), and (**10**) was carried out according to Scheme 1 and their experimental data together with intermediates (**4**), (**5**), (**8**), and (**9**) are reported as follows. For intermediates (**1**–**3**), their synthetic details will be reported elsewhere.

*2-Difluoromethyl-3-O-methoxymethylene-17,17-ethylenedioxy-1,3,5[10] oestratriene (**4**).* To a stirred solution of 2-formyl-3-*O*-methoxymethylene-17,17-ethylenedioxy-1,3,5[10]estratriene (**3**, 1.0 g, 2.58 mmol) in anhydrous dichloromethane (8 mL) was added dropwise [bis(2-methoxyethyl)amino]sulphur trifluoride (Deoxo-fluor, 1.53 mL, 7.73 mmol). After stirring and refluxing under N_2 for 16 h, the reaction mixture was cooled and poured into a saturated aqueous solution of $NaHCO_3$ (100 mL). The aqueous layer was extracted with dichloromethane (4×100 mL) and the combined organic extracts were dried (Na_2SO_4), filtered, and concentrated in vacuo. The crude oil that was obtained was fractionated by flash chromatography (ethyl acetate/hexane, 1:9) to give (**4**) as a yellow oil (505 mg, 1.24 mmol, 48%); R_f 0.66 (ethyl acetate/hexane, 2:3); δ_H (400 MHz, $CDCl_3$) 0.89 (s, 3H, C_{18}–CH_3), 1.26–2.38 (m, 13H), 2.86 (m, 2H, C_6–H_2), 3.48 (s, 3H, –$CH_2OC\underline{H}_3$), 3.94 (m, 4H, C_{17}–OCH_2CH_2O), 5.19 (s, 2H, –$C\underline{H}_2OCH_3$), 6.87 (s, 1H, ArH), 6.93 (t, 1H, *J* 55.8 Hz, –CHF_2) and 7.48 (s, 1H, ArH); δ_F (376 MHz, $CDCl_3$) −113.7 (dd, *J* 55 and 296 Hz), −114.6 (dd, *J* 55 and 296 Hz). This product was used for the next reaction without further purification.

*2-Difluoromethyloestrone (**5**).* Acetyl chloride (3 mL) was added dropwise to methanol (7.8 mL) at 0 °C and (**4**) (505 mg, 1.24 mmol) was added to the resulting mixture at room temperature. After sonicating briefly and then standing for 15 min, the reaction mixture was quenched with water (40 mL). The resulting mixture was extracted with ethyl acetate (2×50 mL) and the combined organic extracts washed with water (2×100 mL), brine (2×100 mL), dried ($MgSO_4$), filtered and concentrated in vacuo. The yellow solid that was obtained was recrystallised from ethyl acetate and hexane to give (**5**) as pale yellow crystals (192 mg, 600 μmol, 48%); m.p. 208–210 °C; R_f 0.47 (ethyl acetate/hexane, 2:3); ν_{max} (KBr) 3500–3100, 3000–2800, 1715, 1610, 1500, 1420; δ_H (400 MHz, $CDCl_3$) 0.94 (s, 3H, C18–CH_3), 1.26–2.55 (m, 13H), 2.87 (m, 2H, C6–H_2), 5.11 (t, 1H, $J \sim 2.3$ Hz, OH), 6.62 (s, 1H, ArH), 6.82 (t, 1H, *J* 55.4 Hz, CHF_2) and 7.31 (s, 1H, ArH); δ_F (376 MHz, $CDCl_3$) −111.4 (dd, *J* 54 and 296 Hz), −112.2 (dd, *J* 55 and 296 Hz); LRMS: (FAB+) *m/z* (rel. intensity) 320.1 [100, M^+], (FAB−) *m/z* (rel. intensity) 473.2 [40, $(M + NBA)^+$], 319.1 [100, $(M - H)^-$]; HRMS (FAB+) *m/z* 320.15796 (M^+), calcd. for $C_{19}H_{22}F_2O_2$ 320.15879. Found: C 71.0, H 6.97; calcd. for $C_{19}H_{22}F_2O_2$ C 71.23, H 6.92%. HPLC [λ_{max}: 286 nm]: $t_R = 5.7$ min.

*2-Difluoromethyloestrone 3-O-sulphamate (**6**).* Sulphamoyl chloride in toluene (ca. 0.7 M, 2.36 mL) was added to a stirred mixture of 2-difluoromethyloestrone (**5**, 106 mg, 0.33 mmol) and 2,6-di-*tert*-butyl-4-methylpyridine (DBMP, 204 mg, 0.99 mmol) in anhydrous dichloromethane (10 mL) under an atmosphere of N_2 at room temperature. The progress of the reaction was monitored by thin layer chromatography. After 1.25 h, water (40 mL) was added to quench the reaction and the resulting mixture was extracted with dichloromethane (40 mL). The organic portion was then washed with brine (3×50 mL), dried ($MgSO_4$), filtered, and evaporated. The pink oil that was obtained was fractioned by flash chromatography (hexane/ethyl acetate, 3:2) to give (**6**) as off-white crystals (110 mg, 0.26 mmol, 83%); m.p. 179–181 °C; R_f 0.50 (hexane/ethyl acetate, 3:2); ν_{max} (KBr) 3450–3010, 3000–2800, 1700, 1390, 1190; δ_H (400 MHz, $CDCl_3$) 0.92 (s, 3H, C18–CH_3), 1.24–2.56 (m, 13H), 2.96 (m, 2H, C6–H_2), 4.94 (s, 2H, NH_2), 6.90 (t, 1H, *J* 54.3 Hz, CHF_2), 7.25 (s, 1H, ArH) and 7.57 (s, 1H, ArH); δ_F (376 MHz, $CDCl_3$) −112.7 (dd, *J* 54 and 301 Hz), −113.7 (dd, *J* 54 and 301 Hz); LRMS: (FAB+) *m/z* (rel. intensity) 400.0 [70, $(M + H)^+$], (FAB−) *m/z* (rel. intensity) 398.0 [100, $(M - H)^-$]; HRMS (FAB+) *m/z* 400.13818 $(M + H)^+$, calcd. for $C_{19}H_{24}F_2NO_4S$ 400.13941. Found: C 57.4, H 6.05, N 3.37; calcd. for $C_{19}H_{23}F_2NO_4S$ C 57.13, H 5.80, N 3.51%. HPLC [λ_{max}: 221 nm]: $t_R = 3.6$ min.

*2-Difluoromethyloestradiol 3-O-sulphamate (**7**).* To a stirred mixture of methanol (3 mL), tetrahydrofuran (3 mL) and 2-difluoromethyloestrone 3-*O*-sulphamate (**6**, 75 mg, 190 μmol) at ice/water temperature was added $NaBH_4$ (28.4 mg, 0.75 mmol). The reaction was monitored by TLC and quenched after 25 min with aqueous ammonium chloride (10 mL). The resulting mixture was extracted with ethyl acetate (3×50 mL) and the combined organic extracts were washed with brine (3×50 mL), dried ($MgSO_4$) and evaporated. The crude syrup obtained was crystallised from ethyl acetate and hexane to give (**7**) as off-white crystals (50 mg, 124 μmol, 65%); m.p. 172–179 °C; R_f 0.20, (ethyl acetate/hexane, 2:3); ν_{max} (KBr) 3500, 3280, 3000–2800, 1360, 1190; δ_H (400 MHz, CD_3OD) 0.78 (s, 3H, C18–CH_3), 1.15–2.39 (m, 13H), 2.91 (m, 2H, C6–H_2), 3.67 (dd, 1H, *J* 8.5 Hz, C17–H_α), 7.00 (t, 1H, *J* 55.4 Hz, CHF_2), 7.19 (s, 1H, ArH), and 7.56 (s, 1H, ArH); δ_F (376 MHz, DMSO-d_6) −111.3 (dd, *J* 52 and 296 Hz), −112.1 (dd, *J* 52 and 296 Hz); LRMS: (FAB+) *m/z* (rel. intensity) 401.0 [30, M^+], (FAB−) *m/z* (rel. intensity) 400.1 [100, $(M - H)^-$]; HRMS (FAB+) *m/z* 401.14721 $(M)^+$, calcd. for $C_{19}H_{25}F_2NO_4S$ 401.14724. HPLC [λ_{max}: 286 nm]: $t_R = 3.5$ min (purity: >96%). LC/MS: $t_R = 3.26$ min (purity: >95%), *m/z* (AP−) 400.2 (45%), 321.2 (100%).

*17,17-Ethylenedioxy-2-methyl-3-O-methoxymethylene-1,3,5[10]-estratriene (**8**).* A solution of 17,17-ethylenedioxy-3-*O*-methoxymethylene-1,3,5[10]-estratriene (**2**, 2.19 g, 6.12 mmol) in THF (40 mL) was cooled in a dry ice/acetone bath for 0.25 h and then treated with *s*-butyl lithium (1.3 M in cyclohexane, 14.1 mL, 18.3 mmol, 3 eq.) in a dropwise manner over 4 h. After stirring for a further 4 h at this temperature, iodomethane (1.15 mL, 18.3 mmol) was added and the reaction was allowed to warm to room temperature overnight before the reaction was quenched with saturated ammonium chloride solution (10 mL). Diethyl ether (100 mL) was added to the resulting mixture and the organic fraction upon separation was washed with water (250 mL), brine (2×350 mL), dried ($MgSO_4$), and evaporated. The crude product that obtained was fractionated by flash chromatography (hexane/ethyl acetate, 3:1) to give (**8**) as a colourless oil (1.59 g, 4.28 mmol, 70%); R_f 0.36 (hexane/ethyl acetate, 3:1); δ_H (400 MHz, $CDCl_3$) 0.88 (s, 3H, C18–CH_3), 2.21 (s, 3H, Ar–CH_3), 1.30–2.12 (m, 11H), 2.16–2.30 (m, 1H), 2.35–2.46 (m, 1H), 2.88 (m, 2H, C6–CH_2), 3.48 (s, 3H, OCH_3), 3.87–3.96 (m, 4H, OCH_2CH_2O), 5.16 (s, 2H, OCH_2O), 6.77 (s, 1H, ArH), and 7.08 (s, 1H, ArH). This fraction was used without further purification.

*2-Methyloestrone (**9**).* Acetyl chloride (8 mL) was added dropwise to methanol (19 mL) at 0 °C and the resulting mixture was warmed to and kept at room temperature for 5 min. To this acidic solution was added the crude product (**8**) (1.5 g, 4.03 mmol) and after sonicating briefly and then standing for 15 min, the reaction mixture was quenched with water (40 mL). The resulting mixture was extracted with ethyl acetate (2×100 mL) and the combined organic extracts were washed with water (2×200 mL), brine (2×200 mL), dried ($MgSO_4$), filtered, and evaporated. The white solid that was obtained was fractionated by flash chromatography (ethyl acetate/hexane, 3:7–2:3 gradient) to give (**9**) as white crystals (974 mg, 3.43 mmol, 85%); m.p. 232–236 °C (Lit. [9] 232–233 °C); δ_H (270 MHz, $CDCl_3$) 0.91 (s, 3H, C18–CH_3), 1.26–2.90 (m, 18H), 4.59 (s, 1H, OH), 6.53 (s, 1H, ArH) and 7.04 (s, 1H, ArH); LC/MS: $t_R = 6.73$ min (purity: >97%), *m/z* (AP−) 283.37 (100%); HRMS (FAB+) *m/z* 284.17757 (M^+), calcd. for $C_{19}H_{24}O_2$ 284.17763.

*2-Methyloestrone 3-O-sulphamate (**10**).* To a solution of 2-methyloestrone (**9**, 150 mg, 5.28 μmol) in *N,N*-dimethylacetamide (1.5 mL) at room temperature and under an atmosphere of N_2 was added sulphamoyl chloride in toluene (ca. 0.7 M, 1.85 mL). After stirring overnight at room temperature the reaction mixture was diluted with ethyl acetate (30 mL) and the organic layer was washed with brine (4×20 mL), dried ($MgSO_4$), filtered, and evaporated. The white residue that obtained was recrystallised from acetone/hexane to give (**10**) as creamy crystals (130 mg, 3.58 μmol, 68%); m.p. 218–222 °C; δ_H (270 MHz, DMSO-d_6, referenced to TMS) 0.43–1.33 (m, ~11H), 1.39 (s, 3H, C18–CH_3), 1.43–1.63 (m, ~2H), 1.67 (s, 3H, CH_3), 1.98 (s, 2H,

C6–H_2), 6.16 (s, 1H, ArH), 6.37 (s, 1H, ArH), and 7.10 (s, 2H, OSO_2NH_2); LC/MS: $t_R = 6.01$ min (purity: >95%), *m/z* (AP−) 363.3(18%), 362.3(100%), 283.3(54%); HRMS (FAB+) *m/z* 363.15095 (M^+), calcd. for $C_{19}H_{25}NO_4S$ 363.15043.

Biology

The ability of the EMATE derivatives to inhibit sulphatase activity was examined using a placental microsomal (100,000 g) fraction. To determine the IC_{50}s for the inhibition of oestrone sulphatase, activity was measured in the presence of inhibitor using [6,7-^{3}H]E1S (51 Ci/mmol, NEN-Dupont, Boston, MA) adjusted to 20 μM with unlabelled E1S (Sigma). After incubation of the substrate ± inhibitor with the microsomes for 1 h, the product formed from E1S was isolated with toluene. [4-^{14}C]Oestrone (1×10^4 dpm; Amersham, Aylesbury, UK) was used to monitor procedural losses with product formation being quantified using scintillation spectrometry. The sensitivity of this assay, which was determined by incubating ^{3}H E1S in the absence of microsomes, was 1.5 ± 0.7% of control (no inhibitor) activity.

Modecular modelling

The X-ray crystal structure of STS was obtained from the protein data bank (PDB) using the code 1P49.pdb. The structure was loaded into Molecular Operating Environment Software (MOE, 2003.02, Chemical Computing Group Inc., Canada) and the MMFF94 forcefield chosen and hydrogen atoms were added. Heavy atoms were fixed and the hydrogen positions minimised. Compound (**6**), prepared as its mono-anion by removing a N-proton from the sulphamate moiety, was docked to the resultant PDB structure using Gold v2.1 (Cambridge Crystallographic Database) running on an SGI octane2. The Ca^{2+} ion was chosen as the atom to target during docking runs.

Results and discussion

The synthesis of 2-difluoromethyloestrone 3-*O*-sulphamate (**6**, Scheme 1) was achieved in three steps starting from the protected formyl oestrone (**3**). The method described by Lal et al. [10] for the fluorination of simple aromatic compounds was applied to the fluorination of the formyl group of (**3**). Lal et al. reported that the fluorination of benzaldehyde using 1.7 equivalents of Deoxo-fluor ([bis(2-methoxyethyl)amino]sulphur trifluoride) at room temperature in dichloromethane was accomplished after 16 h. These conditions, however, proved to be too mild for the fluorination of the protected formyl oestrone (**3**) as shown by the recovery of mainly starting material after work-up. When the reaction mixture was refluxed for 4 h, a new spot was detected by monitoring with thin layer chromatography. Upon work-up and fractionation of the crude by flash column chromatography, the desired product 2-difluoromethyl-3-*O*-methoxymethylene-17,17-ethylenedioxy-1,3,5[10]oestratriene (**4**) was obtained, albeit in a poor 12% yield. The fluorination reaction was repeated but at reflux for 16 h in the presence of 3 equivalents of Deoxo-fluor. These more vigorous conditions gave (**4**) in a more useful 48% yield. The 2-difluoromethyloestrone derivative (**4**) was then deprotected under acidic conditions generated by mixing acetyl chloride with methanol. Upon recrystallisation of the crude residue 2-difluoromethyloestrone (**5**) was obtained in 48% yield. The sulphamoylation of (**5**) was carried out in the presence of DBMP and sulphamoyl chloride in toluene to give 2-difluoromethyloestrone 3-*O*-sulphamate oestrone (**6**) in 83% yield. The oestradiol derivative (**7**) was prepared using the established method of reduction of the 17-carbonyl group of (**6**) with sodium borohydride. It is interesting to note that the ^{19}F NMR spectra of fluorinated compounds (**4**–**7**) all showed two double doublets between −111 and −115 ppm with coupling constants corresponding to geminal H–F and F–F couplings. This observation indicates that the two geminal fluorine atoms are magnetically non-equivalent, suggesting that the rotation of the 2-difluoromethyl group is somehow restricted. It is not clear whether intra-molecular hydrogen bonding between the 2-fluoromethyl group and the hydroxyl/sulphamate group of the compounds is involved, but this seems likely.

Patton [9] described the synthesis of 2-methyloestrone via the hydrogenolysis of 2-diethylaminoethyloestrone. In our approach, the 2-position of the protected oestrone (**2**, Scheme 1) was lithiated by treatment with *s*-BuLi at −78 °C, and the resulting anion was quenched with iodomethane to give the protected 2-methyloestrone (**8**, Scheme 1) in 70% yield as a single regioisomer. This regioselective *ortho*-lithiation of 3-*O*-methoxymethylene protected oestrogen derivatives was pioneered by Pert et al. [11]. The subsequent deprotection of (**8**) and the sulphamoylation of 2-methyloestrone (**9**) to give 2-methyloestrone 3-*O*-sulphamate (**10**) were carried out in the usual manner.

The IC_{50} values of sulphamates (**6**), (**7**), and (**10**) for the inhibition of the STS activity in a placental microsome preparation are shown in Table 1. For comparison, the IC_{50} values for EMATE, oestradiol 3-*O*-sulphamate (E2MATE) and a related analogue with an electron-withdrawing group at the 2-position, 2-chloro-oestrone 3-*O*-sulphamate (2-Cl-EMATE), are included as references.

It is clear that 2-methyloestrone 3-*O*-sulphamate (**10**) is a weak inhibitor of STS, some 1000-fold less potent than EMATE. In comparison with the inhibitory activities reported for other 2-alkylEMATE congeners, the potency of (**10**) is lower than that of 2-ethyloestrone 3-*O*-sulphamate ($IC_{50} = 820$ nM, placental microsomes

Table 1
IC_{50} values for EMATE, E2MATE, 2-Cl-EMATE, and sulphamates (**6**), (**7**), and (**10**) determined using placental microsomes

Inhibitor	IC_{50} ± SD (nM)
EMATE	9.1 ± 0.5
E2MATE	16.0 ± 1.4
2-Cl-EMATE[a]	3.4 ± 0.02
6	0.1 ± 0.01
7	0.7 ± 0.05
10	10,700 ± 800

[a] The synthesis of this compound will be reported elsewhere.

[5]) but higher than that of 2-*n*-propyloestrone 3-*O*-sulphamate ($IC_{50} = 29\,\mu M$, placental microsomes [4]). This finding appears to suggest that the hydrophobic interactions between lower alkyl groups and amino acid residues surrounding the 2-position of A-ring of the inhibitor in the active site are optimised with an ethyl group.

A methyl group is often viewed to be isosteric and isolipophilic with the chloro atom [12]. While 2-Cl-EMATE (Table 1) was about 2.5-fold more potent than EMATE, (**10**) was found to be some 3000-fold weaker as an STS inhibitor than 2-Cl-EMATE. This finding further supports the suggestion that the high potency observed for 2-Cl-EMATE (and for other 2-halogenated EMATEs as well) is a direct result of the electron-withdrawing effect of the chlorine atom on the A-ring of the inhibitor. The hyperconjugative electron-donating effect of the methyl group in (**10**) on the A-ring is reflected by the pK_a value of 10.31 for 2-methylphenol as calculated using Advanced Chemistry Development (ACD) Software Solaris v.4.67. The predicated pK_a values using the ACD software for 2-chlorophenol and phenol are 8.50 and 9.86, respectively.

In order to broaden the investigation into the effect of electron-withdrawing substituents on the inhibitory activities of 2-substituted analogues of EMATE, two fluorine atoms were introduced to (**10**) to give the 2-difluoromethyl derivative of EMATE (**6**, Scheme 1). The rationale for the difluorination of the methyl group of (**10**) is that the pK_a value for 2-fluoromethylphenol as calculated by ACD software is 8.65 which is reasonably close to that of 2-chlorophenol (i.e., 8.50, vide supra). This suggests that both the pK_a value and the leaving ability of 2-difluoromethyloestrone (**5**) should be similar to those of 2-chloro-oestrone, rendering an interesting comparison between the inhibitory activity of (**6**) and that of 2-Cl-EMATE.

As shown in Table 1, the IC_{50} value for 2-difluoromethylEMATE (**6**) against the STS activity in placental microsomes was found to be 100 pM which is about 90-fold lower than that of EMATE. The oestradiol congener (**7**) was 7-fold weaker than (**6**) as an STS inhibitor although E2MATE was about 2-fold weaker than EMATE (Table 1). The remarkable level of inhibitory activity exhibited by (**6**) suggests that this agent is the most potent steroidal STS inhibitor in vitro reported to date, exceeding significantly the level of potency achieved in vitro by 2-Cl-EMATE (Table 1) and several other highly potent D-ring modified derivatives of EMATE reported recently [13,14].

The increase in potency observed for (**6**) compared to that of (**10**) can be attributed to the better leaving ability of (**5**) than (**9**) because of the presence of two fluorine atoms. However, the exceptionally high potency of (**6**) compared to 2-Cl-EMATE would not be obviously accounted for by this parameter alone.

While the use of fluorine substitution to extend the biological half-life of bioactive compounds and eliminate the formation of toxic metabolites is a widely accepted paradigm in the drug optimisation process, the involvement of the fluorine group in hydrogen bonding remains a controversial subject. However, it has been recognised that fluorine in certain circumstances appears to act as a hydrogen bond acceptor [15–20] and enhances the biological activity of ligands [19–21].

The X-ray crystal structure of human STS has been published recently [22]. We previously published a homology model of the enzyme [23] and docked in oestrone 3-sulphate and some reversibly binding mimics. Using the new crystal structure data (PDB code: 1P49.pdb) we docked in oestrone 3-sulphate and EMATE (data not shown) and found that high scoring docking was possible and that broad correlation with the earlier homology model was achieved. Amongst the amino acid residues lining the catalytic region of the active site five of them have been identified to be Lys^{134}, Lys^{368}, His^{136}, His^{290}, and His^{346}. Although it is not clear if these hydrogen bond donor groups indeed reside in the vicinity of the 2-position of the A-ring of steroid, they are nonetheless putative candidates for interacting with the 2-difluoromethyl group of (**6**). In order to explore the premise that F·····H interactions, inter alia, could contribute to the high potency observed for (**6**), the crystal structure of human STS was constructed with MOE and inhibitor (**6**) docked into the enzyme active site as its mono-anionic form ($\sim OSO_2NH^-$) using Gold v2.1 running on an SGI Octane2. The docking results (Fig. 1) indicate that the sulphamate group of (**6**), as anticipated, interacts with the catalytic site of STS and its Ca^{2+} atom in a similar manner to the sulphate group of E1S. It should be emphasised that this dock produces a putative complex *prior to* the presumed irreversible inactivation step.

Theoretical studies have indicated that C–F·····H–C interactions are of no significant value because they are weak with energies similar to those of van der Waals complexes [15]. However, although short contacts between fluorine and the acidic hydrogens of HO or HN are rare amongst the organofluorine compounds deposited in the Cambridge Structural Database System, it has been suggested that C–F·····H–O and C–F·····H–N interactions of 2.5 Å or lower might provide some beneficial stabilising effects between a ligand and its target [15]. As indicated by the docking results depicted in the Fig. 1, one of the fluorine atoms of (**6**) is about 2.27 and 2.78 Å away from Lys^{368} and His^{290}, respectively, whereas the other one is 3.80 Å away from His^{485}. However, in a dynamic situation these contacts between fluorine atoms of (**6**) and the hydrogen bond donor residues might indeed be closer than what have been depicted in the rigid docking model. For this reason, the docking results presented here suggest that, inter alia, F·····H interactions might have a significant contribution to the exceptional high potency

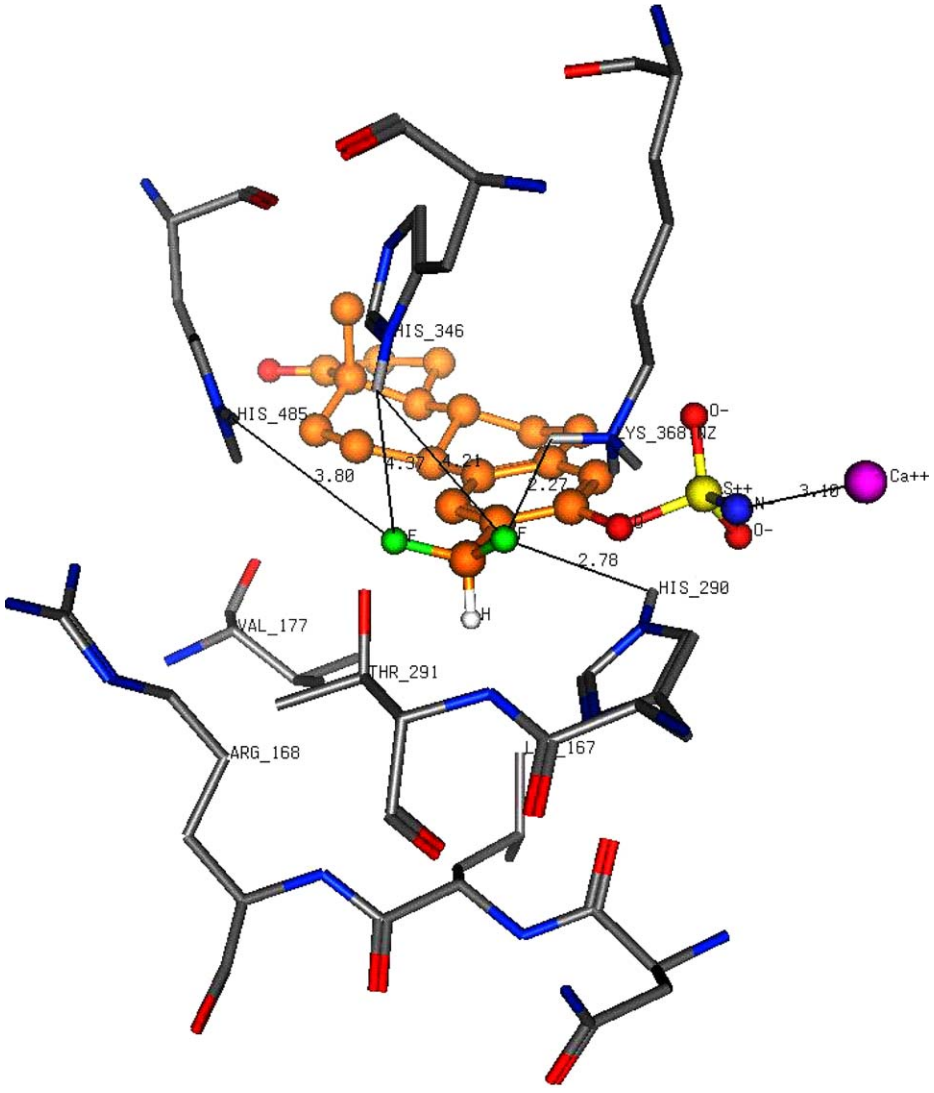


Fig. 1. The docking of the mono-anionic form ($\sim OSO_2NH^-$) of inhibitor (**6**) into the active site of the crystal structure of human STS (PDB code: 1P49.pdb). The sulphamate group interacts, as anticipated, with the catalytic site of STS and its Ca^{2+} atom (purple ball) in a similar manner to the sulphate group of E1S. Distances between selective atoms are shown suggesting that putative F·····H interactions might occur between one of the fluorine atoms of (**6**) with Lys^{368} (2.27 Å) and His^{290} (2.78 Å).

observed for (**6**). It can be envisaged that the subsequent synthesis and docking of other congeners of (**6**) such as 2-monofluoromethyl- and 2-trifluoromethyloestrone 3-*O*-sulphamates should help in the development of a clearer structure–activity relationship for this type of STS inhibitors.

In conclusion, the synthesis and in vitro biological evaluation of three novel 2-substituted analogues of EMATE have been described. One inhibitor, 2-difluoromethyloestrone 3-*O*-sulphamate (**6**), was found to be some 90-fold more potent than EMATE, rendering this agent the most potent steroidal STS inhibitor in vitro reported to date. While the lowering of the pK_a value of 2-difluoromethyloestrone, by its 2-difluoromethyl group, relative to the unsubstituted congeners most probably facilitates the inactivation of STS by (**6**), our preliminary docking studies also suggest that putative F·····H interactions between the 2-difluoromethyl group of (**6**) and hydrogen bond donor residues lining the catalytic site of STS might contribute to the high potency observed for (**6**). These results contribute to the development of structure–activity relationships for this class of potential novel therapeutic agents.

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