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Inhibition of steroid sulfatase with 4-substituted estrone and estradiol derivatives

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ABSTRACT

Steroid sulfatase (STS) catalyzes the desulfation of biologically inactive sulfated steroids to yield biologically active desulfated steroids and is currently being examined as a target for therapeutic intervention for the treatment of breast cancer. We previously demonstrated that 4-formyl estrone is a time- and concentration-dependent inhibitor of STS. We have prepared a series of 4-formylated estrogens and examined them as irreversible STS inhibitors. Introducing a formyl, bromo or nitro group at the 2-position of 4-formylestrone resulted in loss of concentration and time-dependent inhibition and a considerable decrease in binding affinity. An estradiol derivative bearing a formyl group at the 4-position and a benzyl group at the 17β-position yielded a potent concentration and time-dependent STS inhibitor with a K_I of 85 nM and a k_{inact} of 0.021 min^{-1} (k_{inact}/K_I of 2.3×10^5 M^{-1} min^{-1}). Studies with estrone or estradiol substituted at the 4-position with groups other than a formyl group revealed that good reversible inhibitors can be obtained by introducing small electron withdrawing groups at this position. An estradiol derivative with fluorine at the 4-position and a benzyl group at the 17β-position yielded a potent, reversible inhibitor of STS with an IC_{50} of 40 nM. The introduction of relatively small electron withdrawing groups at the 4-position of estrogens and their derivatives may prove to be a general approach to enhancing the potency of estrogen-derived STS inhibitors.

1. Introduction

Breast cancer kills more women in the United States than any cancer except lung cancer.[1] The majority of breast tumors are estrogen receptor positive and are dependent upon estrogens for survival. This has led researchers to examine hormone therapy as a means of arresting breast tumor growth. Current hormone therapy consists mainly of either ER modulators which block the action of estrogens on breast cancer cells or of selective inhibitors of enzymes involved in the biosynthesis of estrogens such as aromatase and 17β-hydroxysteroid dehydrogenase.[2] This latter approach lowers the amount of estrogens in the body. Several aromatase inhibitors have shown some clinical success and are now on the market.[3] Of the enzymes that have been targeted for hormone therapy, steroid sulfatase (STS) is the only enzyme that is not directly involved in estrogen biosynthesis. STS catalyzes the desulfation of biologically inactive sulfated steroids such as estrone sulfate (E1S), into biologically active desulfated steroids such as estrone (**1**, E1) (Fig. 1).[4] The water soluble steroidal sulfates function as circulating reservoirs of inactive steroid precursors which are then converted into their active form by STS in cells.

Numerous inhibitors of STS have been made.[5] The vast majority are aryl sulfamates.[5] These compounds are irreversible, suicide inhibitors though their precise mode of action has yet to be determined. EMATE (**2**), the first of this class to be discovered, is a very potent STS inhibitor with an IC_{50} of 56 nM with purified STS[5c]; however, it is estrogenic and therefore not a suitable candidate for drug development (Fig. 2). Since the discovery of EMATE, many potent, non-estrogenic, sulfamate-based STS inhibitors have been prepared.[5] 667-Coumate (**3**) is an example of such a compound (IC_{50} = 350 nM in intact MCF-7 cells[5c]) and has been examined in Phase I clinical trials for treating breast cancer.[6] Reversible STS inhibitors have also been developed.[5] Some of these compounds, such as 17-α-substituted E2 derivatives of type **4**, are potent inhibitors.[5]

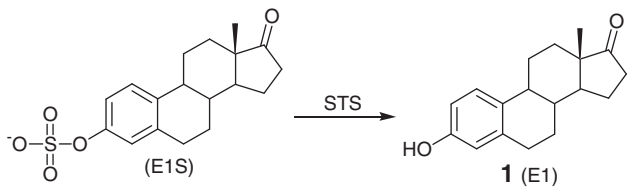

Figure 1. Desulfation of estrone sulfate (E1S) to estrone (**1**, E1) catalyzed by STS.

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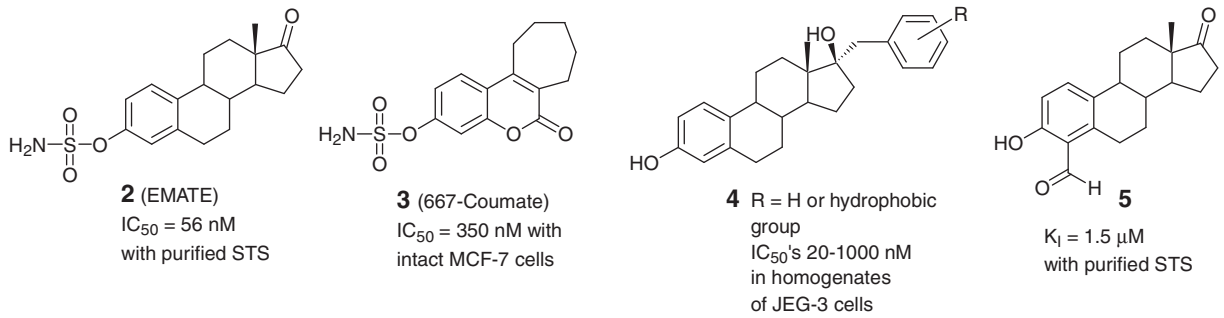


Figure 2. Irreversible and reversible inhibitors of STS.

We recently showed that 4-formylestrone (**5**, Fig. 2), is a time- and concentration-dependent inhibitor of STS.[7] Extensive dialysis of the **5**-inactivated STS revealed the inhibition to be almost irreversible. STS could be protected against inactivation by estrone phosphate, a competitive STS inhibitor indicating that inactivation with **5** was a result of a reaction with active site residues.[7] As part of our efforts to learn more about the mechanism of inhibition and to develop more potent STS inhibitors, we report here the synthesis and evaluation of other A-ring formylated estrogen derivatives as irreversible STS inhibitors as well as our studies on other 4-substituted estrone (E1) and E2 derivatives as reversible STS inhibitors.

2. Results and discussion

2.1. Inhibition with formyl-substituted estrogens

We first examined formyl-substituted E1 derivatives **6** and **7** (Fig. 3) as STS inhibitors. These and all subsequent inhibition studies were performed using purified STS in Tris buffer at pH 7.0 containing 0.01% Triton X-100 and using 4-methylumbelliferyl sulfate (4-MUS) as the substrate.[8] In contrast to compound **5**, compounds **6** and **7** did not exhibit time- and concentration-dependent inhibition of STS at 10 μM, further emphasizing the importance of having the formyl group at the 4-position. The poor solubility of these compounds in our assay buffer prevented us from performing studies at higher concentrations.

Next we examined 2,4-diformylestrone (**9**) as an STS inhibitor (Fig. 4). The synthesis of this compound was not readily accomplished. Attempts to prepare it by direct bisformylation of E1 or by formylation of **5** using $MgCl_2$/paraformaldehyde, Et_3N[9] or hexamethylenetetramine in TFA[10] failed. We were able to dihydroxymethylate E1 using paraformaldehyde/NaOH; however, the triol product **8** was unstable and could not be purified by chromatography since it decomposed during silica gel purification whether in the presence or absence of triethylamine. Therefore crude **8** was subjected to MnO_2 which gave compound **9** in a 15% yield over two steps (Fig. 4). Although the yield was low, sufficient quantities could readily be obtained for inhibition studies. Compound **9** was considerably more soluble in aqueous solution than **6** and **7**. However, **9** did not exhibit time-dependent inhibition of STS and was a poor reversible STS inhibitor with an IC_{50} value of 224 μM. To determine if other 2-substituted derivatives of compound **5** behaved in a similar manner we prepared the 2-nitro and 2-bromo derivatives **10** and **11** (Fig. 4). These two derivatives of **5** were chosen because we found that 2-nitroestrone (**12**, IC_{50} = 17 μM) and 2-bromoestrone (**13**, IC_{50} = 40 μM) are better STS inhibitors than E1 itself (IC_{50} = 51 μM) and the nitro and bromo groups could be easily introduced by nitration or bromination of **5** (Fig. 4). Neither **10** nor **11** exhibited time- and concentration-dependent STS inhibition and both were poor reversible inhibitors of STS with **10** exhibiting less than 50% inhibition at 50 μM[11] and **11** having an IC_{50} of 145 μM. It appears that substitution of **5** at the 2-position, at least with relatively small electron withdrawing groups, has a detrimental effect on the binding affinity and abolishes time-dependency on inhibition.

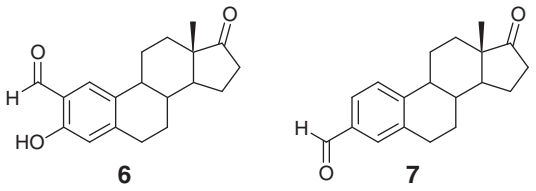


Figure 3. 2-Formylestrone (**6**) and 1,3,5(10)-triene-17-one-3-carbaldehyde (**7**).

Since 17-substituted E2 derivatives of type **4**, are potent inhibitors of STS we examined compounds **15** and **16** as STS inhibitors (Fig. 5). These compounds were prepared as a mixture by direct formylation of **14**[12] but could be separated by careful silica gel flash chromatography (Fig. 5).

Compound **15** proved to be a very good time- and concentration-dependent STS inhibitor (Fig. 6) and more or less pseudo-first order behavior was exhibited at all concentrations of **15** over the 50 min time course of the experiment (Fig. 6). In contrast compound **5** exhibited pseudo-first order behavior only over the first 5 min of the reaction unless the concentration of **5** was greater than 1 μM.[7] From the data in Figure 6, a Kitz-Wilson plot was generated (inset Fig. 6) and a K_I of 85 nM and k_{inact} of 0.021 min^{-1} were obtained for **15** from which a k_{inact}/K_I of 2.3×10^5 M^{-1} min^{-1} was derived. A Kitz-Wilson analysis of the data obtained from the first 5 min of STS inactivation with compound **5** gave a K_I of 1.5 μM and a k_{inact} of 0.13 min^{-1} and a $k_{inact}/K_I = 1 \times 10^5$ M^{-1} min^{-1}.[7] The greater affinity of **15** for STS compared to **5** may be due the benzyl group projecting into a tunnel between the two hydrophobic alpha helices that insert STS into the membrane of the endoplasmic reticulum.[13] Such an interaction has been proposed to account for the substantially increased affinity of compounds of type **4** over estradiol.[14] Like compound **5**, inactivation of STS by compound **15** could be prevented by estrone-3-phosphate, a competitive inhibitor of STS,[15] indicating that inactivation was occurring at the active site (Fig. 7). The 2-formyl derivative **16** was a very poor time- and concentration-dependent STS inhibitor with only 15% activity lost over 60 min at 10 μM.[16] As was observed with the formylated E1 (**5** and **6**), inhibition with the formylated 17-benzyl E2 derivatives was more pronounced when the formyl group was at the 4-position. To determine if the inhibition of STS by **15** was reversible a solution of STS was treated with 1 μM **15** for 60 min (95% STS inactivation) followed by extensive dialysis, and then examined for STS activity. Only 14% of the activity was recovered. In contrast, about 2–3% activity was recovered with compound **5**.[9]

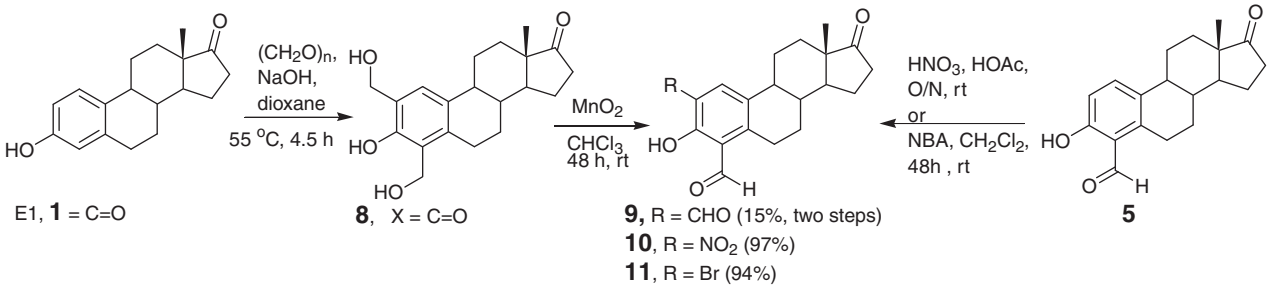


Figure 4. Synthesis of 4-formyl estrone derivatives **9**–**11**.

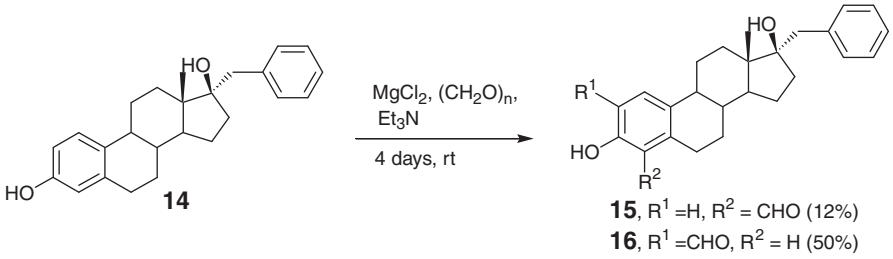


Figure 5. Synthesis of **15** and **16**.

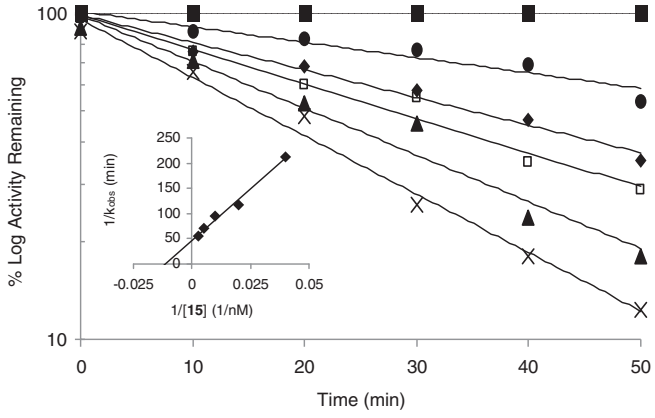


Figure 6. Time- and concentration-dependent inhibition of STS with compound **15**. [**15**] = ■ 0, ● 25, •50, □ 100, ▲ 200, ×400 nM. Kitz-Wilson plot is shown as the inset. See the Section 4 for details.

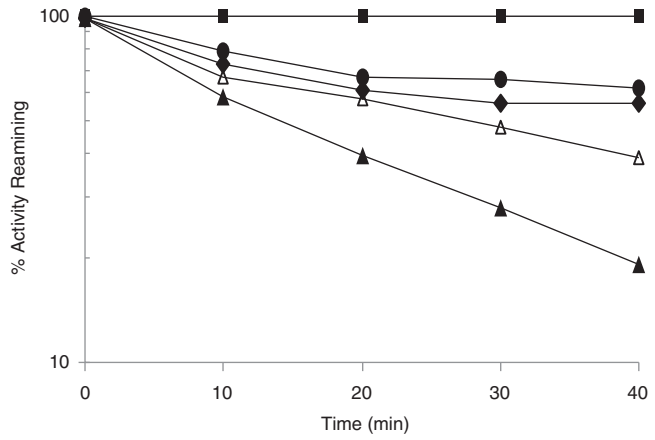


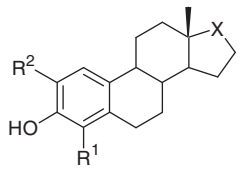
Figure 7. Time- and concentration-dependent inhibition of STS with 400 nM **15** in the presence varying amounts of estrone-3-phosphate at 0 (▲), 2.5 (△), 5(♦), 25 (●) μM. A control was conducted in the absence of **15** and estrone-3-phosphate (■). See the Section 4 for details.

Aldehyde-based inhibitors of enzymes are well-known and many inhibit by forming Schiff bases with basic residues such as lysine and arginine.[17] These adducts can sometimes be trapped as stable amines by reacting the imine adducts with reducing agents such as $NaBH_4$ or $NaCNBH_4$.[18] The active site of STS has several lysine and arginine residues such as Lys-368, Lys-134 and Arg-79 that may be capable of Schiff base formation.[13] In addition, Arg-98 which lies at the entrance to the active site may also be capable of this type of adduct formation.[13] However, we have not been able to identify a modified residue upon treatment of **5**- or **15**-inactivated STS with $NaBH_4$ or $NaCNBH_4$ followed by deglycosylation, proteolytic digestion and MS analysis of the resulting peptides. It is possible that $NaBH_4$ or $NaCNBH_3$ is unable to access the modified residue. This is not an unreasonable assumption since if Schiff base formation is indeed occurring it appears to be almost irreversible suggesting that access by water for the reverse reaction may be limited. Certain aldehyde-based inhibitors are also known to form adducts with the serine residue of serine proteases.[19] Similarly, inhibition of STS with **5** and **15** may occur via formation of a stable covalent adduct with the active site formyl glycine hydrate. Such an adduct would be expected to revert to reactants upon denaturation and/or proteolytic digestion.

2.2. STS inhibition with other 4-substituted estrogens

A comparison of the K_I's of compounds **5** (1.5 μM) and **15** (85 nM) with the IC_{50}'s or K_i's of E1 (IC_{50} = 51 μM, Table 1) and compound **14** (K_i = 250 nM)[20] suggested to us that the presence of the formyl group at the 4-position of the A-ring significantly enhances binding affinity.[21] This prompted us to examine other 4-substituted E1 and E2 derivatives as reversible STS inhibitors (Table 1). The 4-hydroxymethyl (**17**), 4-aminomethyl (**18**), 4-amino (**19**) and 4-vinyl (**20**) E1 derivatives were very poor inhibitors (Table 1). Interestingly, the 4-CO_2H derivative, compound **21**, was also a poor STS inhibitor. If an amino-bearing residue is involved in forming a Schiff base with the formyl group of **5** or **15**, then it would not be unreasonable to assume that compound **21** would be a good inhibitor as it could potentially form electrostatic interactions with the amino group. The fact that **21** is a very poor

Table 1
Inhibition of STS with 4-substituted estrogen derivatives



Compound	R^1	R^2	X	IC_{50} (μM)[a]
5	CHO	H	C=O	1.5 μM (K_I)
15	CHO	H	C(OH)Bn (β)	85 nM (K_I)
E1	H	H	C=O	51
17	CH_2OH	H	C=O	ND[b]
18	CH_2NH_2	H	CHOH (β)	ND[c]
19	NH_2	H	CHOH (β)	ND[d]
20	$CH{=}CH_2$	H	C=O	ND[e]
21	CO_2H	H	C=O	215
22	Br	H	C=O	4.8
23	F	H	C=O	3.3
24	CN	H	C=O	6.7
25	NO_2	H	C=O	2.4
26	NO_2	H	CHOH (β)	2.8
12	H	NO_2	C=O	17
27	NO_2	NO_2	C=O	26

[a] Errors are ±5%. ND – not determined.
[b] 46% inhibition at 50 μM.
[c] 4% inhibition at 50 μM.
[d] 43% inhibition at 50 μM.
[e] 45% inhibition at 50 μM.

inhibitor argues somewhat against the Schiff base inhibition mechanism for compounds **5** and **15** although such an argument must be taken with caution as the electrostatic interaction would clearly depend upon the ionization states of the respective acid and amine.

Substitution of the 4-position of E1 with relatively small electron withdrawing groups such as Br, CN, F or NO_2 groups resulted in a significant increase in inhibitory potency (compounds **22–25**). The 4-nitro E1 derivative **25** was the best of this series with an IC_{50} of 2.4 μM. The 4-nitro E2 derivative **26** was slightly less potent. The lower IC_{50}'s of compounds **22**–**26** may be due to an increase in the concentration of the phenolate anion which may favor binding to STS. However, the IC_{50}'s do not exhibit a linear relationship with the pK_a's of *ortho*-substituted phenols.[22] Moreover, the 2-nitro derivative **12**, which would most likely have a pK_a similar to that of the 4-nitro derivative **25,** was seven-fold less potent than **25** and the 2,4-dinitro derivative **27** was about 10-fold less potent than **25**. Taken together, these results suggest that the effect exerted on the IC_{50}'s by these groups may also be attributed to factors such as H-bonding ability and/or steric interactions or other interactions. We investigated the mode of inhibition for compound **25** and found it to be a non-competitive inhibitor with K_i of 1.4 μM.[23]

To determine whether an electron withdrawing group at the 4-position of the A-ring would also increase the potency of 17-substituted E2 derivatives of type **4** we examined the 4-fluoro E2 derivative **28** as an STS inhibitor. (Fig. 8) This compound was readily prepared by reacting 4-fluoroestrone with benzylmagnesium bromide. The IC_{50} of this compound was found to be 40 nM which is seven-fold lower than that of compound **14**. We investigated the mode of inhibition for compound **28** and found it to be exhibit linear mixed-type inhibition with K_i of 30 nM and an αK_i of 90 nM.[23] It has been shown that E1 and compound **14** also exhibit linear mixed-type inhibiton.[20] So it appears that E1, compounds **14**, **25**, **28** and most probably **22**–**24**, are capable of binding at sites both within and outside the active site. Based on these results, it is possible that compounds **5** and **15** may also interact with a site or sites outside the active site. However, time-dependent inhibition of STS

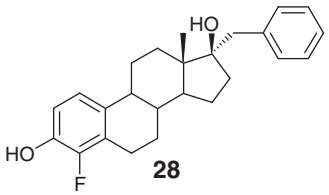


Figure 8. Structure of compound **28**.

by **5** and **15** can be protected by estrone-3-phosphate, a purely competitive STS inhibitor. This suggests that if **5** and **15** also bind at a site(s) outside the active site then the binding at this external site(s) is reversible and may or may not affect enzyme activity or, if time-dependent and essentially irreversible, has no effect on enzyme activity.

3. Conclusions

In summary, we have shown that introducing small electron withdrawing substituents at the 2-position of 4-formyl estrone results in loss of concentration and time-dependent inhibition and a considerable decrease in inhibitor binding affinity. Nevertheless, potent time- and concentration-dependent inhibition of STS can be obtained by introducing a formyl group into the 4-position of 17β-benzyl estradiol (compound **15**). The mechanism by which this and 4-formyl estrone (**5**) inhibit STS is still unknown though attempts to identify a modified residue upon treatment of **15**-inactivated STS are continuing. Studies with estrogen derivatives substituted at the 4-position with groups other than a formyl revealed good reversible, non-competitive inhibitors can be obtained by introducing a small electron withdrawing group at this position. Introducing a fluorine into the 4-position of 17β-benzyl estradiol yielded a potent reversible non-competitive inhibitor of STS. The introduction of a relatively small electron withdrawing group at the 4-position of estrogens and their derivatives may prove to be a general approach to enhancing the potency of estrogen-derived STS inhibitors.

4. Experimental

4.1. General

All starting materials and reagents were obtained from Aldrich Chemical Company. THF was distilled from sodium-benzophenone. CH_2Cl_2 was distilled from calcium hydride under nitrogen. Silica gel chromatography was performed using silica gel (60 Å, 230–400 mesh) obtained from Silicycle (Laval, Quebec, Canada). ^1H, ^{13}C, and ^{19}F NMR spectra were recorded on a Bruker Avance 300 spectrometer. For NMR spectra obtained using $CDCl_3$ as the solvent, chemical shifts (δ) for ^1H NMR spectra are reported relative to internal Me_4Si (δ 0.0 ppm), chemical shifts for ^{13}C spectra are relative to the residual solvent peak (δ 77.0 ppm, central peak), and chemical shifts for ^{19}F NMR are relative to a trichlorofluoromethane (δ 0.0 ppm) external standard. Low-resolution (LRMS) and high-resolution (HRMS) electron impact (EI) mass spectra were obtained on a JEOL HX110 double focusing mass spectrometer. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Compounds **12**,[24] **13**,[25] **14**,[12] and **17**,[9] **18**,[9] **19**,[26] **20**,[9] **21**,[9] **22**,[27] **23**,[28] **24**,[29] **25**,[24] **26**[30] and **27**[31] were prepared according to literature procedures. Steroid sulfatase from human placenta was purified as previously described.[32] Fluorimetry was performed using a Spectramax GeminiXS plate reader (Molecular Devices, CA) at 25 °C.

4.2. Syntheses

4.2.1. Estra-1,3,5(10)-triene-17-one-3-carbaldehyde (7)

To a solution of 3-hydroxymethylestrone[33] (400 mg, 1.41 mmol) in CH_2Cl_2 (30 mL) at rt was added pyridinium chlorochromate (600 mg, 2.80 mmol, 2 equiv). The reaction was stirred for 100 min then filtered through Celite. The filtrate was washed with H_2O and brine, then dried (Na_2SO_4), filtered and concentrated to give a brown solid. Purification of the residue by flash chromatography (ethyl acetate–hexane, 1:5, then ethyl acetate–hexane–CH_2Cl_2, 1:5:3, then ethyl acetate–hexane, 1:1) gave pure **7** as a white solid (377 mg, 95%). Mp: 183–185 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.93 (s, 1H, CHO), 7.63 (d, J = 8.1 Hz, 1H, H-2), 7.59 (s, 1H, H-4), 7.44 (d, J = 8.0 Hz, 1H, H-1), 3.00–2.95 (m, 2H), 2.55–2.31 (m, 3H), 2.20–1.95 (m, 4H), 1.69–1.44 (m, 6H), 0.91 (s, 3H, CH_3, H-18); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 220.3, 192.1, 147.0, 137.4, 134.2, 130.1, 127.1, 126.0, 50.4, 47.7, 44.8, 37.6, 35.7, 31.4, 29.1, 26.1, 25.5, 21.5, 13.7; LRMS (EI) m/z (%) 282 (M^+, 100), 238 (43), 225 (24); HRMS (EI) calcd for $C_{19}H_{22}O_2$ 282.1620; found 282.1624.

4.2.2. 2,4-Diformylestra-1,3,5(10)-triene-17-one (9)

A suspension of E1 (1.08 g, 4.00 mmol), paraformaldehyde (300 mg, 10.0 mmol, 2.5 equiv) and NaOH (fine powder, 100 mg, 2.50 mmol, 0.625 equiv) in dioxane (3 mL) was heated at 55 °C for 4.5 h before cooling to rt. After diluting with H_2O, the reaction mixture was acidified with 0.5 M HCl and then stirred for 2 min. The precipitate was collected by suction filtration and washed thoroughly with H_2O then dried under high vacuum to give 1.01 g of crude triol **9** as white solid. To a solution of **9** (1.0 g) in $CHCl_3$ (50 mL) was added activated MnO_2 (4.80 g, 55.2 mmol, 13.8 equiv) and the resulting mixture was stirred for 2 days at rt. After passing through a Celite pad, the filter cake was rinsed with $CHCl_3$ and the filtrate was washed with H_2O and brine then dried (Na_2SO_4), filtered and concentrated. The residue was purified by flash chromatography (ethyl acetate–hexane, 1:3 to 1:2.5 to 1:2) to give **9** as a yellow solid (88 mg, 15% over 2 steps starting from E1). Mp: 212–213 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 12.27 (s, 1H, OH), 10.44 (s, 1H, CHO), 10.21 (s, 1H, CHO), 7.88 (s, 1H, H-1), 3.40 (dd, J = 18.3 Hz, J = 5.4 Hz, 1H, H-6), 3.22–3.10 (m, 1H), 2.61–2.37 (m, 2H), 2.25–1.89 (m, 5H), 1.63–1.38 (m, 6H), 0.87 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 220.0, 194.3, 190.3, 163.6, 148.4, 134.1, 132.2, 121.5, 119.1, 50.1, 47.7, 43.6, 37.0, 35.7, 31.3, 26.7, 26.0, 25.7, 21.4, 13.7; LRMS (EI) m/z (%) 326 (M^+, 100), 298 (99), HRMS (EI) calcd for $C_{20}H_{22}O_4$ 326.1518; found 326.1516.

4.2.3. 4-Formyl-2-nitroestra-1,3,5(10)-triene-17-one (10)

To a solution of **5**[9] (90 mg, 0.30 mmol) in acetic acid (30 mL) at 45 °C was added conc. HNO_3 (0.20 mL, 2.8 mmol, 9.3 equiv). The reaction mixture was stirred overnight at rt. After removal of acetic acid in vacuo, the residue was purified by flash chromatography (ethyl acetate–hexane, 1:1) to give of **10** as a yellow solid (100 mg, 97%). Mp: 184–186 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 11.99 (br s, 1H, OH), 10.52 (s, 1H, CHO), 8.15 (s, 1H, H-1), 3.40 (dd, J = 18.6 Hz, J = 5.4 Hz, 1H), 3.16 (ddd, J = 18.3 Hz, J = 11.1 Hz, J = 6.9 Hz, 1H), 2.53–1.90 (m, 5H), 1.65–1.30 (m, 6H), 0.87 (s, 3H, CH_3, H-18); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 220.0, 192.7, 156.1, 149.8, 133.4, 132.9, 128.2, 121.3, 50.1, 47.6, 43.6, 36.7, 35.7, 31.2, 27.4, 26.0, 25.6, 21.4, 13.7; LRMS (EI) m/z (%) 343 (M^+, 75), 325 (M-CO, 100), 308 (7), 367 (10), 115 (11), 97 (12); HRMS (EI) calcd for $C_{19}H_{21}NO_2$ 343.1420; found 343.1419.

4.2.4. 4-Formyl-2-bromoestra-1,3,5(10)-triene-17-one (11)

To a solution of **5**[9] (300 mg, 1.01 mmol) in dry CH_2Cl_2 (30 ml) was added *N*-bromoacetamide (NBA, 167 mg, 1.21 mmol, 1.2 equiv) under Ar at rt. The resulting mixture was stirred at rt for 2 days. The mixture was concentrated and purified by flash chromatography (CH_2Cl_2) to give the product as a yellow amorphous solid (355 mg, 94%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 12.56(s, 1H, OH), 10.32 (s, 1H, CHO), 7.71 (s, 1H, H-1), 3.34 (dd, J = 5.4, 11.5 Hz, 1H), 3.04–3.18 (m, 1H), 2.51 (dd, J = 8.8, 18.1 Hz, 1H), 1.95–2.28 (m, 6H), 1.40–1.70 (m, 6H), 0.91 (s, 3H, CH_3, H-18). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 220.1, 195.2, 157.6, 139.3, 138.1, 132.8, 118.1, 108.9, 50.0, 47.7, 43.5, 37.2, 35.8, 31.4, 26.1, 25.8, 25.12, 21.4, 13.8; LRMS (EI) m/z (%) 378 (M+2, 99), 376 (M+, 100); HRMS (EI) calcd for $C_{19}H_{21}BrO_3$ 376.0674; found 376.0670.

4.2.5. 2-Formyl-17α-benzyl-17β-hydroxyestra-1,3,5(10)-triene (15) and 2-formyl-17α-benzyl-17β-hydroxyestra-1,3,5(10)-triene (16)

Dry $(CH_2O)_n$ (1.16 g, 38.7 mmol, 7.0 equiv), and dry $MgCl_2$ (3.15 g, 33.1 mmol, 6.0 equiv) were added to a dry round-bottom flask under Ar. To this was added dry THF (100 mL) followed by dry triethylamine (4.6 mL, 33 mmol, 6.0 equiv). The resulting mixture was stirred at rt for 10 min. Compound **14**[12] (2.0 g, 5.5 mmol) was added and the reaction mixture was stirred at rt for 4 days. The mixture was diluted with EtOAc, acidified with 1N HCl, and the resulting mixture was stirred 10 min and then extracted with EtOAc. The combined extracts were washed with H_2O and brine and then dried (Na_2SO_4), filtered, and concentrated. ^{1}H NMR of the crude product revealed that the ratio of 2-formyl and 4-formyl was 4.5:1. The crude product was purified by flash chromatography (CH_2Cl_2) to give **15** (257 mg, 12% yield) and **16** (1.07 g, 50% yield) as white amorphous solids. Characterization data for **15**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 11.98 (s, 1H, ArOH), 10.37 (s, 1H, CHO), 7.49 (d, J = 8.9 Hz, 1H, H-1), 7.34–7.22 (m, 5H), 6.79 (d, J = 8.8 Hz, 1H, H-2), 3.32 (dd, J = 5.7 Hz, J = 17.2 Hz, 1H, H-6), 3.18–3.09 (m, 1H, H-6), 2.91 (d, J = 13.2 Hz, 1H, PhC*H*H), 2.65 (d, J = 13.2 Hz, 1H, PhCH*H*), 2.36–2.30 (m, 1H), 2.29–2.17 (m, 1H), 2.07–1.93 (m, 2H), 1.80–1.24 (m, 10H), 0.96 (s, 3H CH_3). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 195.6, 161.4, 139.6, 138.2, 135.5, 131.7, 131.0, 130.5, 128.1, 126.4, 117.5, 115.7, 82.9, 49.2, 46.7, 43.8, 42.4, 38.8, 33.7, 31.4, 27.0, 26.6, 25.6, 23.2, 14.5. LRMS (EI) m/z (%) 390 (M^+, 23), 298 (100), 281 (45); HRMS: Calcd for $C_{26}H_{30}O_3$; 390.2195, found: 390.2198. Characterization data for **16**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 10.77 (s, 1H, ArOH), 9.81 (s, 1H, CHO), 7.43 (s, 1H, H-1), 7.35–7.23 (m, 5H), 6.70 (s, 1H, H-2), 2.95–2.87 (m, 3H, 1H from PhC*H*H), 2.66 (d, J = 13.2 Hz, 1H, PhCH*H*), 2.42–2.36 (m, 1H), 2.25–2.19 (m, 1H), 2.03–1.92 (m, 2H), 1.83–1.24 (m, 10H), 0.97 (s, 3H CH_3). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 196.1, 159.3, 148.1, 138.2, 132.8, 131.0, 130.5, 128.2, 126.4, 119.0, 117.0, 82.9, 49.5, 46.8, 43.3, 42.4, 39.3, 33.7, 31.2, 30.2, 27.1, 26.3, 23.3, 14.5; LRMS (EI) m/z (%) 390 (M^+, 18), 298 (100), 281 (42); HRMS (EI): Calcd for $C_{26}H_{30}O_3$; 390.2195, found: 390.2198.

4.2.6. 4-Fluoro-17α-benzyl-17β-hydroxyestra-1,3,5(10)-triene (28)

This was prepared using the procedure developed by Poirier and coworkers for the synthesis of 17β-benzyl derivatives of E2.[12] To a solution of **23**[28] (0.022 g, 0.076 mmol) in dry THF (2 mL) at 0 °C (ice bath) was added a 2 M solution of benzylmagnesium chloride (0.381 mL, 0.76 mmol) in ether and stirred for 24 h during which time the reaction was allowed to warm to room temperature. The mixture was quenched with sat. NH_4Cl and extracted with EtOAc. The organic layer was washed with brine, dried (Na_2SO_4) and concentrated. The residue was dissolved in MeOH (2 mL), cooled to and $NaBH_4$ (5.7 mg, 0.152 mmol) was added at 0 °C, and stirred for 2 h at rt. The reaction was quenched with water and the methanol was removed by rotary evaporation. The mixture was extracted with EtOAc, washed with brine, dried (Na_2SO_4) and concentrated. The residue was purified by flash chromatography (ethyl acetate–hexane, 1:4) to give **28** as a white amorphous solid (0.0158 g, 55% yield). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.31–7.24 (m,

5H), 6.96 (d, J = 8.3 Hz, 1H, H-1), 6.78 (t, J = 8.3 Hz, 1H, H-2), 3.00–2.88 (m, 1H, H-6), 2.78–2.61 (m, 1H, H-6′), 2.91 (d, J = 13.2 Hz, 1H, PhC*H*H), 2.65 (d, J = 13.2 Hz, 1H, PhCH*H*), 2.40–2.30 (m, 1H), 2.25–2.15 (m, 1H), 2.23–1.91 (m, 2H), 1.80–1.22 (m, 10H), 0.95 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 149.0 (d, J = 240 Hz), 140.9 (d, J = 15.8 Hz), 138.2, 133.6 (d, J = 3.0 Hz), 131.0, 128.1, 126.3, 124.5 (d, J = 14.7 Hz), 120.8 (d, J = 4.0 Hz), 113.7 (d, J = 2.0 Hz), 83.0, 49.4, 46.7, 43.7, 42.4, 39.1, 33.7, 31.3, 29.7, 26.5, 26.3, 22.3 (d, J = 4.5 Hz), 14.4; ^{19}F NMR ($CDCl_3$, 75 MHz) δ − 145.5; LRMS (EI) m/z (%) 380 (M^+, 15%), 288 (100%), 271 (55%), 231 (20%), 177 (20%); HRMS (EI) calcd for $C_{25}H_{29}FO_2$; 380.2152, found 380.2152.

4.3. Inhibition studies

4.3.1. IC_{50} determinations

Inhibitor solutions (20 μL) of various concentrations of inhibitor in DMSO/0.1 M Tris–HCl pH 7.0 (1:1) were added to the wells of a 96-well microtiter plate containing 140 μL of 0.1 M Tris–HCl, pH 7.0, and 20 μL a 2 mM solution of 4-methylumbelliferyl sulfate (4-MUS) in 0.1 M Tris–HCl, pH 7.0. The reaction was initiated by the addition of 20 μL of an 80 nM solution of STS in 20 mM Tris–HCl, pH 7.4, 0.1% Triton X-100. The final concentration of 4-MUS was 200 μM.[32] STS activity was measured by monitoring the production of fluorescent 4-methylumbelliferone (λ_{ex} = 360 nm, λ_{emit} = 460 nm) for 10 min. All reactions were performed in triplicate. The activity of STS in the presence of inhibitor was compared to the activity of STS in the absence of inhibitor, and a percent activity was calculated. This percent activity was plotted on a semi-log graph against the log concentration of the inhibitor, and fitted to the equation: vi = vo/[1+([*I*]/IC_{50})*S*] + *B* where vi = initial rate of reaction at inhibitor concentration [*I*]; vo = velocity in the absence of inhibitor; *B* = background activity; *S* = slope factor using Grafit from Erithacus Software (Surrey, U.K.). Errors are on the order of ±5%.

4.3.2. K_i determinations

Various solutions of inhibitors **25** and **28** were prepared in DMSO/0.1 M Tris–HCl pH 7.0 (1:1). These solutions (20 μL) were added to the wells of a black 96-well microtiter containing 160 μL of varying concentrations of 4-MUS in 0.1 M Tris–HCl pH 7.0 (1:1). The reaction is initiated by the addition of 20 μL of 80 nM STS in buffer containing 20 mM Tris–HCl, 0.1% Triton X-100, pH 7.4. STS activity was monitored as described in Section 4.3.1. A positive control was done in a similar manner, with the exception of adding STS and replacing the volume with 20 μL of 20 mM Tris–HCl, 0.1% Triton X-100, pH 7.0. All reactions were performed in triplicate. The initial rates of the reaction, obtained as relative fluorescent units over time (RFU/s) for each 4-MUS concentration, were plotted as a Lineweaver-Burk graph using Excel 2007. The slopes and intercepts of the Lineweaver-Burk plots were replotted based on the equations for non-competitive inhibition to obtain the desired K_i values.

4.3.3. Determination of time and concentration dependent inhibition of STS with inhibitor 15

Stock solutions of compound **15** were prepared in DMSO/0.1 M Tris–HCl, pH 7.0 (1:1). These solutions (20 μL) were added to an Eppendorf tube containing 160 μL of 0.1 M Tris–HCl, pH 7.0 and 20 μL of 3.1 μM STS in 20 mM Tris–HCl, 0.1% Triton X-100 pH 7.4. Aliquots (4 μL) were withdrawn every 10 min and added to a well containing 196 μL of 4 mM 4-MUS in 0.1 M Tris–HCl, pH 7.0. This was repeated in triplicate. STS activity was determined as described in Section 4.3.1. A similar procedure was carried out for a control which contained 20 μL of DMSO/0.1 M Tris–HCl pH 7.0 (1:1) in place of the inhibitor solutions. The percent activity remaining as a function of time was plotted as a semilog graph. The slopes of these plots are the observed pseudo-first order rate constants (k_{obs}) for inhibition. Eq. 1 was used to calculate the inhibition constant, K_I.

$$K_{obs} = \frac{k_{inact}}{1 + \frac{K_I}{[I]}} \quad (1)$$

A plot of the reciprocals of the observed inhibition rate constants (k_{obs}) against the reciprocals of the inhibitor concentrations gave a straight line, from which the apparent dissociation constant for inhibition, K_I, was determined from the intersection on the x-axis at $-1/K_I$ and the rate constant for inactivation, k_{inact}, was determined from the intersection on the y-axis (i.e., $1/k_{inact}$).

4.3.4. Time and concentration-dependent inhibition of STS by inhibitor 15 in the presence of estrone-3-phosphate (protection experiments)

Protection experiments with estrone-3-phosphate were carried out using the same procedures as described in Section 4.3.3 with the exception that varying amounts of estrone-3-phosphate (2.5, 5, and 25 μM). The concentration of **15** was 400 nM.

4.4. Dialysis experiment

A 200 μL solution containing 1 μM of **15** and 277 nM STS in 0.1 M Tris–HCl, 5% DMSO, 0.1% Triton X-100, pH 7.0 was incubated for 60 min at room temperature. Aliquots (4 μL) were withdrawn at t = 0 and t = 60 min and added to the wells of a 96-well microtiter plate containing 196 μL of 4 mM 4-MUS. STS activity is monitored as described previously. This was performed in triplicate. The remaining 176 μL was transferred to a dialysis bag and dialyzed against 1 L of Tris–HCl buffer (0.1 M, pH 7.0), 0.1% Triton X-100. Aliquots (4 μL) were withdrawn at 540, 1020, and 1500 min and STS activity was determined as described in Section 4.3.3. After withdrawing aliquots at 540 and 1020 min the dialysis buffer was changed. A control was performed in an identical manner except no inhibitor was present.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.08.046.

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