Synthesis, biological evaluation, and stability studies of raloxifene mono- and bis-sulfamates as dual-targeting agents

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Abstract

All three possible sulfamate derivatives of the selective estrogen receptor modulator Raloxifene (bis-sulfamate **7** and two mono-sulfamates **8-9**) were synthesized and evaluated as inhibitors of the clinical drug target steroid sulfatase (STS), both in cell-free and in cell-based assays, and also as estrogen receptor (ER) modulators. Bis-sulfamate **7** was the most potent STS inhibitor with an IC_{50} of 12.2 nM in a whole JEG3 cell-based assay, with the two mono-sulfamates significantly weaker. The estrogen receptor-modulating activities of **7-9** showed generally lower affinities compared to Raloxifene HCl, diethylstilbestrol and other known ligands, with mono-sulfamate **8** being the best ligand (Ki of 1.5 nM) for ERα binding, although **7** had a Ki of 13 nM and both showed desirable antagonist activity. The antiproliferative activities of the sulfamate derivatives against the T-47D breast cancer cell line showed **7** as most potent (GI_{50} = 7.12 µM), comparable to that of Raloxifene. Compound **7** also showed good antiproliferative potency in the NCI-60 cell line panel with a GI_{50} of 1.34 µM against MDA-MB-231 breast cancer cells. Stability testing of **7-9** showed that bis-sulfamate **7** hydrolyzed by desulfamoylation at a surprisingly rapid rate, initially leading selectively to **8** and finally to Raloxifene **3** without formation of **9**. The mechanisms of these hydrolysis reactions could be extensively rationalized. Conversion of Raloxifene (**3**) into its bis-sulfamate (**7**) thus produced a promising drug lead with nanomolar dual activity as an STS inhibitor and ERα antagonist, as a potential candidate for treatment of estrogen-dependent breast cancer.

Keywords

Estrogen receptor; Raloxifene; Steroid sulfatase; Enzyme inhibition; Sulfamate.

1. Introduction

Diseases such as cancer are usually a result of multiple factors that, due to inherent complexity and the ability to develop resistance, are difficult to treat. They require therapeutic intervention in various cellular processes to ensure remission and prevent relapse [1, 2]. Various adverse molecular mechanisms are involved in cancer drug resistance, with single targeted therapies being more prone to acquire them [2]. On the other hand, the use of multidrug cocktails can suffer from adverse effects because of drug-drug interactions, and compliance can pose a challenge that limits

the options available. Formulations containing two or more agents in a regimen were made to improve patient compliance and comfortability. However, multidrug formulations suffer from limited dosing flexibility and pose pharmacokinetic challenges. Thus, the design of single small molecules capable of interfering with two or more pathological pathways might present a solution for the treatment of complex diseases. Additionally, the cost and risk of developing multi-targeting agents do not differ from developing single target agents [2] and, moreover, present dose flexibility that multicomponent agents lack [1, 2]. However, design of such agents with ideal potencies without sacrificing drug-likeness physicochemical properties remains a significant challenge for medicinal chemists [1-6].

Steroid sulfatase (STS) catalyzes the hydrolysis of inactive steroidal sulfate esters, giving rise to active forms that bind to estrogen and androgen receptors (ER and AR). Therefore, STS has emerged as an attractive drug target for the treatment of hormone-dependent diseases such as breast and endometrial cancers [7-11] and endometriosis [10]. The pharmacophore of an STS inhibitor is usually based upon an aryl sulfamate moiety that is fused to a hydrophobic polycyclic system [11-14]. Irosustat (STX64, **1**, Figure 1), a first-in-class STS inhibitor, has entered various clinical studies, most recently the IRIS phase II combination clinical trial in breast cancer, where the drug was dosed with an aromatase inhibitor [15]. In addition, a phase II randomized study versus megestrol acetate was carried out for treatment of advanced endometrial cancer [16]. Furthermore, a phase I trial in prostate cancer [17] was conducted. The original STS inhibitor E2MATE (**2**, Figure 1) was studied in a phase II clinical trial in combination with norethindrone acetate for the treatment of endometriosis [10]. The therapeutic value of STS inhibition in hormone-dependent diseases has now been validated and this supports the design of synergistic dual action agents to improve its effectiveness, especially in the light of the positive results of the combination IRIS trial, where efficacy was demonstrated. Efforts have so far been directed towards designing such multi-targeting agents involving STS inhibition, for example a drug that blocks both STS and another additional enzyme that takes part in the synthesis of estrogen (i.e. aromatase [7], or 17β-hydroxysteroid dehydrogenase [18]) [10], and which has proven to be a successful strategy, even in *in vivo* models [19-22].

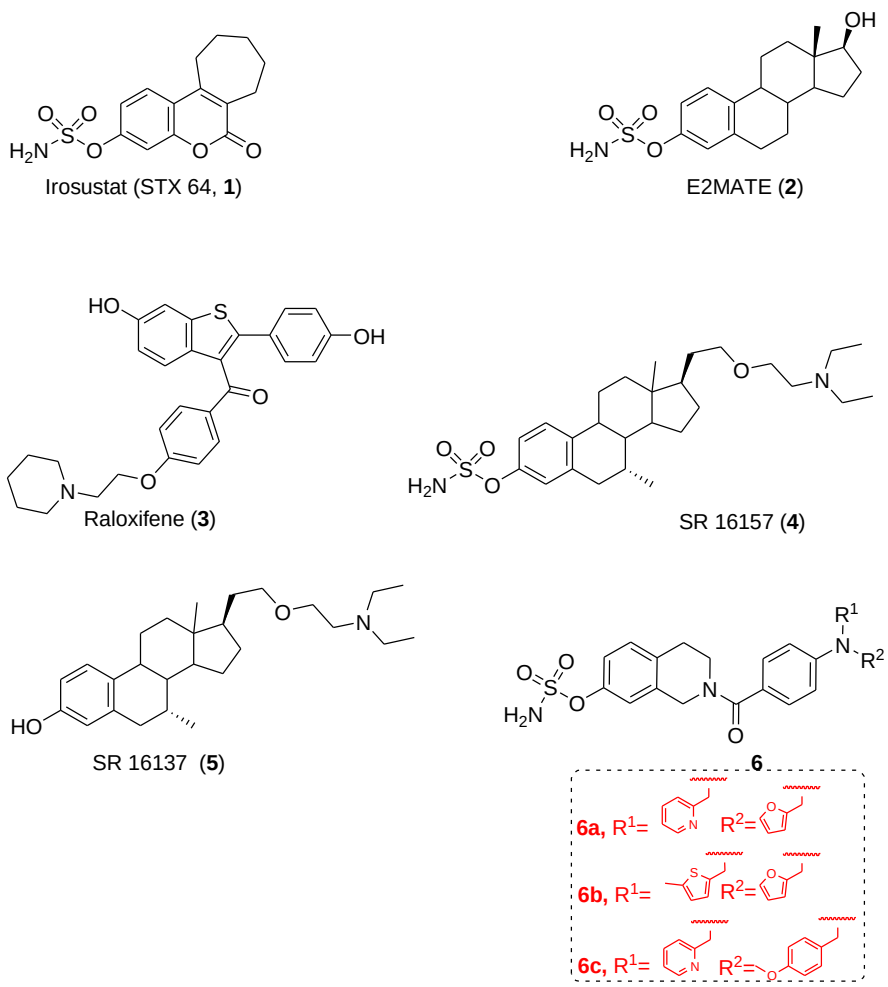


Figure 1. Structures of Irosustat (**1**), E2MATE (**2**), Raloxifene (**3**), SR 16157 (**4**), SR 16137 (**5**) and six derivatives (**6a**, **6b**, and **6c**).

In the present work, we explored Raloxifene-based sulfamate analogues as new potential multi-targeting drug leads. Raloxifene **3** (Figure 1) is a selective estrogen receptor modulator (SERM) and is approved for post-menopausal osteoporosis, as well as for invasive breast cancer in post-menopausal women [23, 24]. Raloxifene has two hydroxyl groups and sulfamoylation of either one or both hydroxyl groups would yield aryl sulfamate derivatives, that should irreversibly inhibit STS. Metabolism of these, if at all, could in principle lead to Raloxifene **3** being released synergistically as an antiestrogenic agent. However, sulfamates are reported not to be effective as prodrugs [25] and any search should in principle address intact single agents highly active at both STS and ER. Nevertheless, a report by Rasmussen et.al. discussed the discovery of SR 16157 (**4**, Figure 1), a sulfamate-containing STS inhibitor, which releases the potent desulfamoylated SR 16137 (**5**, Figure 1) with SERM activity upon STS inhibition [26]. They concluded that SR 16157

was 10 times more potent than SR 16137 in terms of growth inhibition *in vitro*, which they attributed to the inhibition of STS by SR 16157, and the subsequent release of the antiestrogenic SERM, SR 16137 [26]. Preclinical studies of SR 16137 exhibited acceptable toxicological and pharmacokinetic properties [27]. A different design strategy was reported by Ouellet et.al., where sulfamate-possessing tetrahydroisoquinoline derivatives **6a-c** (Figure 1) were reported with dual STS inhibitory and intrinsic SERM activities [28, 29]. In this study the three possible Raloxifene sulfamate derivatives **7-9** (Figure 2) were synthesized and evaluated against STS in both cell-free and whole-cell assays, and their estrogen receptor ER binding and antagonist activities studied and antiproliferative activities against T-47D breast cancer cells. Additionally, bis-sulfamate **7** was evaluated widely against cancer cells in the NCI-60 panel. Finally, given known controversies regarding pro-drug activities of sulfamate esters, the chemical stability of these sulfamate derivatives was comprehensively studied.

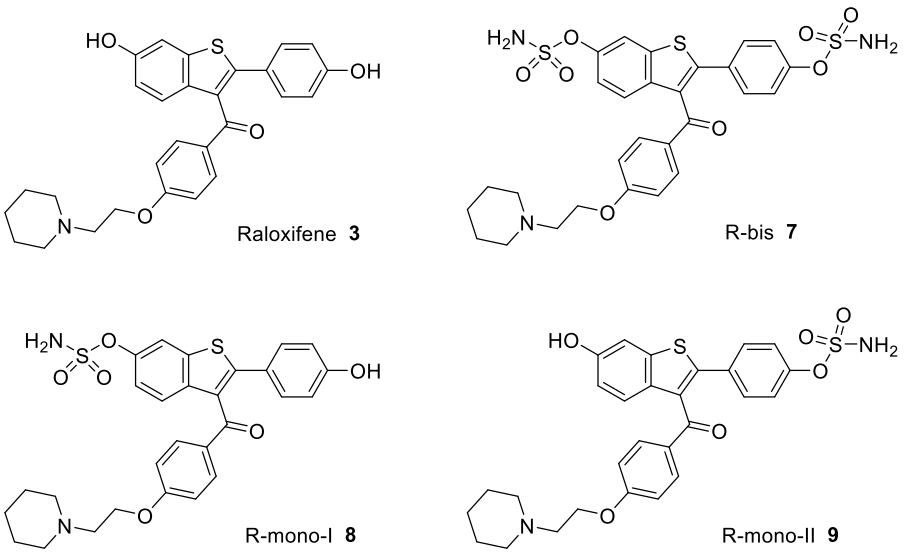


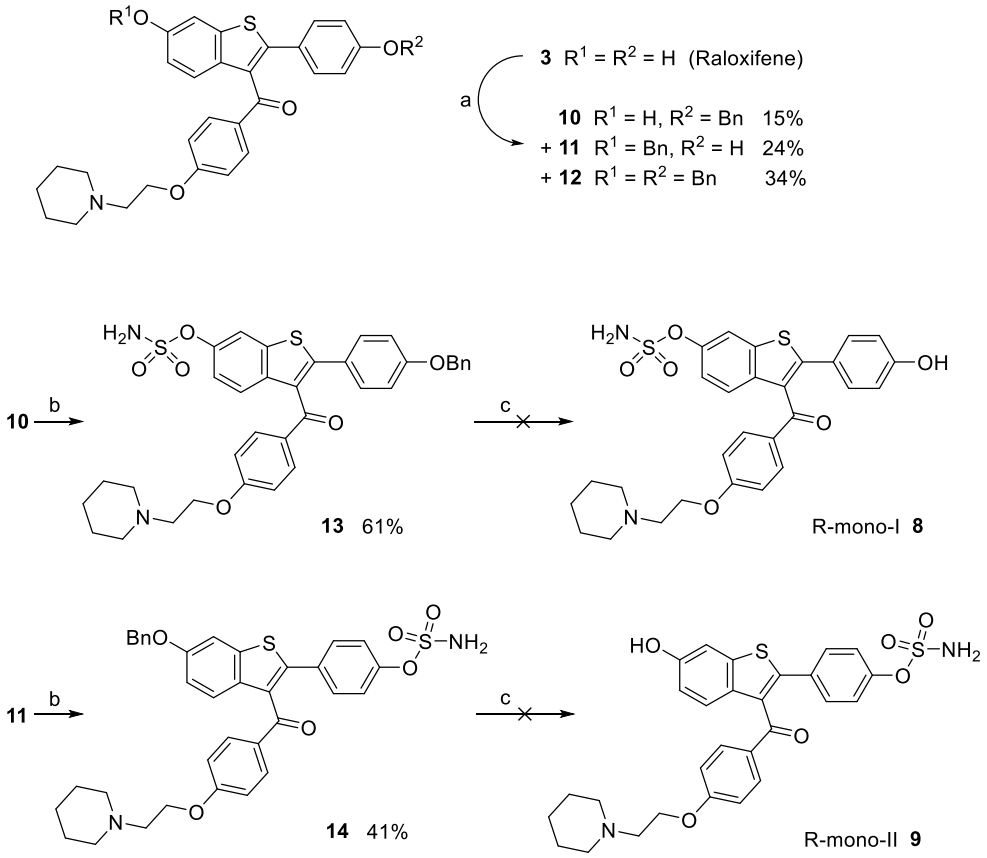
Figure 2. Structures of Raloxifene (**3**) and the target sulfamates **7-9**.

2. Results and discussion

2.1. Chemistry

To explore synthetic routes to the derivatives R-mono-I **8** and II **9** we first sought to prepare mono-protected Raloxifene derivatives that would then be subjected to sulfamoylation followed by deprotection to give rise to the desired mono-sulfamoylated compounds (Scheme 1). Thus,

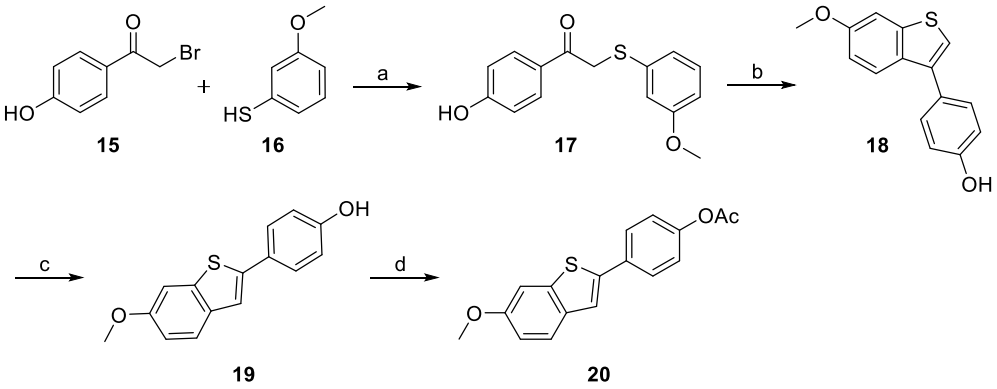
initially Raloxifene **3** was treated with sodium hydride and benzyl bromide (1 equiv.) at room temperature in *N,N'*-dimethylformamide (DMF) to deliver the mono-benzyl derivatives **10** and **11** in low yields of 15% and 24%, respectively along with the di-benzyl derivative **12** (34%) and a recovery of 11% of the starting material **3** (Scheme 3). Compounds **10** and **11** were then treated with sulfamoyl chloride in presence of 2,6-di-*tert*-butyl-4-methylpyridine (DBMP) in toluene and dichloromethane at room temperature to produce the mono-benzylated sulfamates **13** and **14** in moderate yields of 61% and 41%, respectively. We then planned to synthesize the mono-sulfamoylated compounds **8-9**. Unfortunately, subjecting compounds **13** and **14** to palladium-catalyzed hydrogenation in methanol gave a complex reaction mixture in both cases and no desired product was isolated.



Scheme 1. Attempted synthesis of Raloxifene mono-sulfamates **8-9**. *Reagents and conditions*: a) NaH, BnBr, DMF, rt; b) NaH, H_2NSO_2Cl, DMF, rt; c) Hydrogen, Pd/C (5%), MeOH, rt.

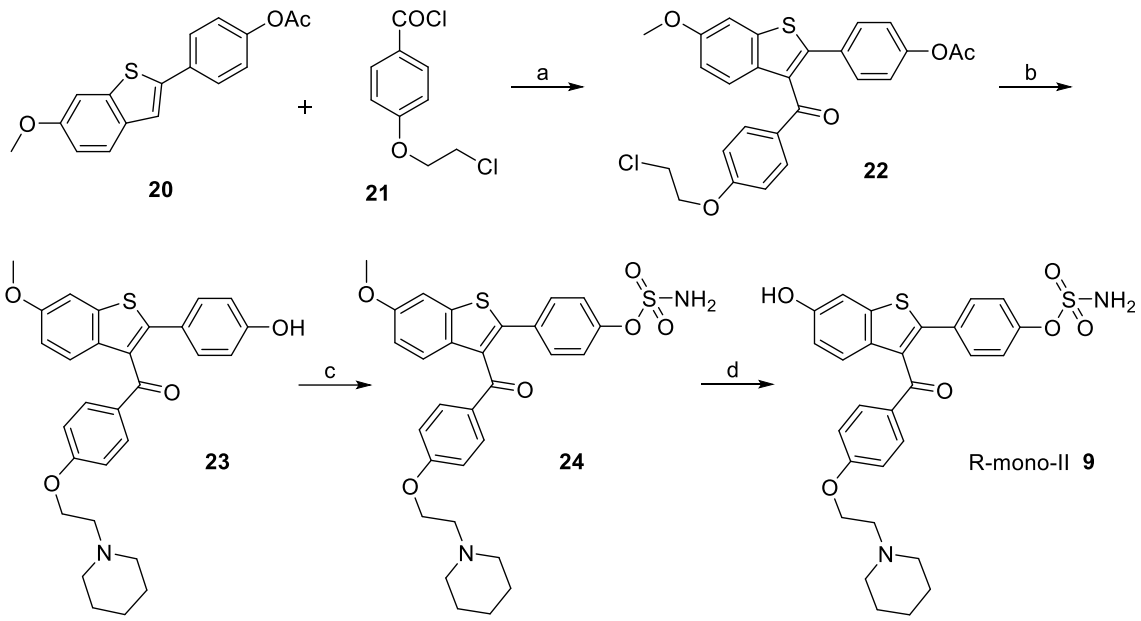
Therefore, we decided to synthesize the desired compounds from simple starting materials in a way that would allow access to the heterocyclic Raloxifene core structure with two different protecting groups on the phenols [30]. Thus, treatment of thiol **16** with aqueous potassium

hydroxide solution followed by the addition of **15** in ethyl acetate gave the thiol ether **17**. Subsequent treatment of the latter with aluminum chloride in dichloromethane afforded compound **18** (Scheme 2). Isomerization of **18** using methanesulfonic acid in toluene at 90 °C afforded the desired regioisomer **19** [31]. The free hydroxyl group was then converted into an acetoxy group using acetic anhydride in the presence of triethylamine to give the orthogonally-protected benzothiophene core structure **20** [32].



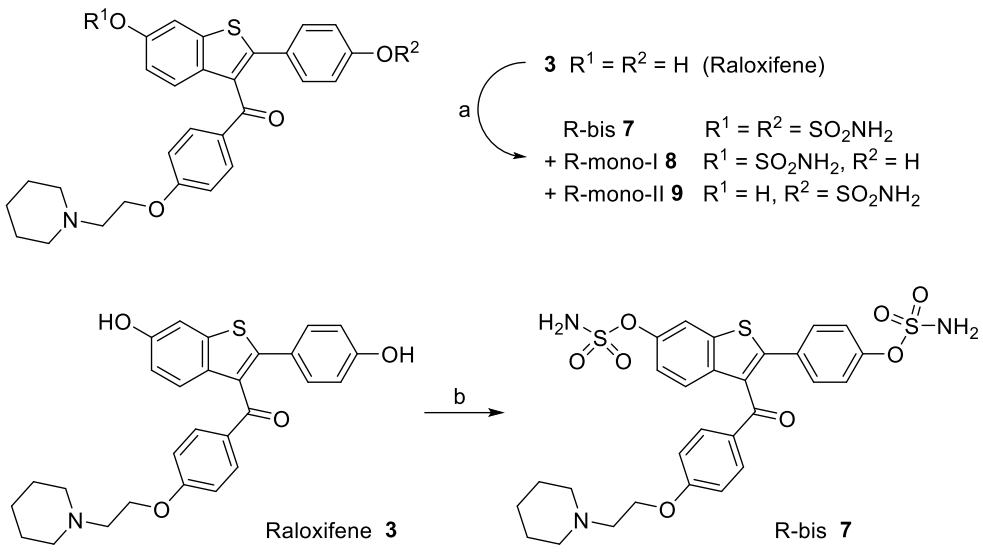
Scheme 2. Synthesis of the orthogonally-protected benzothiophene core structure **20**. *Reagents and conditions*: a) KOH, H_2O, EtOAc, rt, 72%; b) $AlCl_3$, CH_2Cl_2, 0 °C to rt, 54%; c) CH_3SO_3H, toluene, 90 °C, 74%; d) Ac_2O, Et_3N, EtOAc, reflux, 58%.

A Friedel-Craft's acylation of **20** was then performed using **21** [33] and aluminum chloride to yield compound **22**. Treatment of **22** with piperidine followed by simultaneous deprotection of the acetyl protecting group delivered the mono methyl Raloxifene **23**. Sulfamoylation of the free hydroxyl group of **24** followed by subsequent demethylation of the methoxy group of **24** delivered the desired mono-sulfamoylated R-mono-II **9** (Scheme 3).



Scheme 3. Synthesis of Raloxifene mono-sulfamate R-mono-II **9**. *Reagents and conditions*: a) $AlCl_3$, CH_2Cl_2, 0 °C; b) Piperidine, 1,4-dioxane, rt to 85 °C; c) H_2NSO_2Cl, NaH, DMA, rt; d) Thiourea, $AlCl_3$, CH_2Cl_2, reflux.

This long synthetic route to R-mono-II **9** led us to devise another more concise and simpler synthetic plan to synthesize the desired regioisomeric derivative R-mono-I **8**. Thus, treatment of Raloxifene **3** with sodium hydride and sulfamoyl chloride in DMF gave a mixture of the bis-sulfamoylated compounds R-bis **7,** R-mono-I **8** and R-mono-II **9** (Scheme 4), which were separated using preparative HPLC. R-bis **7** was also synthesized by reacting Raloxifene with a much larger excess of sulfamoyl chloride and sodium hydride (Scheme 4). With the three sulfamates now in hand it was possible to evaluate their biological activities.



Scheme 4. Synthesis of Raloxifene sulfamates R-mono-I **8** and R-bis **7**. *Reagents and conditions*: a) H_2NSO_2Cl (3 equiv.), NaH (1.5 equiv.), DMF, rt; b) H_2NSO_2Cl (10 equiv.), NaH (2 equiv.), DMF, rt.

2.2. Biological studies

2.2.1. Inhibitory effects of the target compounds against steroid sulfatase (STS) enzyme:

The sulfamoylated Raloxifene derivatives were tested against both JEG3 lysate and whole JEG3 cells to determine their inhibitory activity against STS, using Raloxifene **3** and irosustat as positive controls (Table 1). Raloxifene **3** and its HCl salt were inactive or had an insignificant activity in both assays. This is expected as they do not have the sulfamate warhead required for STS inhibition. Pleasingly, the bis-sulfamate R-bis **7** was found to be the most potent with IC_{50} values of 96.6 nM against the STS enzyme in JEG3 cell lysate and 12.2 nM in a whole cell assay. Moreover, it exhibited stronger inhibitory activity than both mono-sulfamoylated derivatives, with *ca*. 21.5-fold stronger potency in JEG3 lysate, and 27.2-fold higher potency in whole cell assays. Moreover, R-mono-I **8** was 1.6-fold more active in STS assay, and 1.8-fold in the cell-based assay in comparison to R-mono-II **9**, which indicates that the sulfamate position on the benzothiophene ring is more advantageous for the STS inhibition than when positioned on the terminal phenyl ring.

Table 1. Inhibition of Raloxifene hydrochloride, Raloxifene **3**, R-bis **7**, R-mono-I **8**, and R-mono-II **9** against STS in JEG3 lysate and whole cell assays.

Compound	STS inhibition (nM)	
	Lysate: IC_{50} (95% CI)[a]	Whole Cell: IC_{50} (95% CI)[a]
Raloxifene HCl	Inactive	20-25% inhibition at 50-100 µM

Raloxifene **3**	Inactive	Inactive
R-bis **7**	96.6 (76.4 to 122.2)	12.2 (9.8 to 14.8)
R-mono-I **8**	2081 (1897 to 2283)	332 (324 to 339)
R-mono-II **9**	3398 (3097 to 3728)	607 (593 to 626)
Irosustat	6.4	2.1

[a] 95% confidence interval.

2.2.2. Estrogen Receptor alpha (ERα) coactivator binding assay:

Table 2 illustrates results of the ERα coactivator binding assay of Raloxifene HCl and its sulfamoylated derivatives R-bis **7**, R-mono-I **8**, and R-mono-II **9** in comparison to diethylstilbestrol, which has a high affinity towards estrogen receptors ER [34] (see Supplementary Information). Accordingly, R-mono-I **8** was found to be the most potent of these sulfamate derivatives of Raloxifene. However, it was less potent by *ca.* 21-fold than Raloxifene HCl and 7.4-fold than diethylstilbestrol against ERα. Furthermore, the mono-sulfamoylated R-mono-I **8** was 8.6-fold more potent than the bis-sulfamoylated Raloxifene derivative R-bis **7**. Interestingly, R-mono-II **9** did not bind to ERα, suggesting that the placement of the sulfamate group on the phenyl ring is not tolerated for receptor binding, which might explain the difference in potency between R-mono-I **8** and R-bis **7**. During the initial evaluation of R-mono-II **9** at concentrations of 0.1 µM and 1 µM, the percentage inhibition values were only 15.6% and 61.1%, respectively. Therefore, ERα binding of R-mono-II **9** was not determined.

Table 2. Ki and IC_{50} values of Raloxifene HCl and its sulfamates (R-bis **7** and R-mono-I **8**) in comparison to diethylstilbestrol.[a]

Compound	ERα Binding	
	Ki (nM)	IC_{50} (nM)
Raloxifene HCl	0.07 ± 0.01	0.24 ± 0.02
R-bis **7**	13.0 ± 0.8	45.0 ± 1.0
R-mono-I **8**	1.5 ± 0.02	5.2 ± 0.2
Diethylstilbestrol	0.2 ± 0.02	0.7 ± 0.03

[a] Results are expressed as the means of duplicate assays ± SEM.

Table 3 illustrates the activities of Raloxifene HCl and the sulfamoyated derivatives **7-9** in the ERα coactivator assay expressed as percentages and compared to known agonists and antagonists of ERα. Raloxifene HCl exhibited 100% antagonism at 20 nM, while R-bis **7** and R-mono-I **8** only exhibited comparable antagonism at a 50-fold higher concentration. Interestingly, R-mono-I **8** exhibited 18% agonism in contrast to other derivatives, where agonism was expressed as a negative value.

Table 3. ERα coactivator assay of Raloxifene HCl, sulfamoylated derivatives **7-9**, E1, E3, diethylstilbestrol, fulvestrant, and 4-OH tamoxifen expressed as percentages.

Compound	ERα coactivator assay	
	Antagonism	Agonism
Raloxifene HCl	100% at 20 nM	-14%
R-bis **7**	95% at 1 µM	-17%
R-mono-I **8**	94% at 1 µM	18%
R-mono-II **9**	27% at 10 µM	-13%
E1	-	EC_{50} = 1.4 nM
E3	-	EC_{50} = 3.0 nM
Diethylstilbestrol	-	EC_{50} = 2.5 nM
Fulvestrant	IC_{50} = 13.0 nM	-
4-OH Tamoxifen	IC_{50} = 3.8 nM	-

2.2.3. Antiproliferative activity against T-47D cells

All three target compounds **7-9**, in addition to Raloxifene **3**, were also evaluated for their antiproliferative activity against the T-47D breast cancer cell line (Table 4). The growth medium was charcoal-stripped fetal bovine serum (FBS) to eliminate estrogen and was provided with estradiol sulfate (E2S) [14]. Overall, both mono-sulfamates R-mono-I **8** and R-mono-II **9** showed relatively modest or weak mean inhibition at 10 µM with 40% and 12%, respectively. R-bis **7** on the other hand exhibits the strongest antiproliferative activity with a GI_{50} of 7.12 µM, comparable to that of Raloxifene **3**. This is in correlation with its stronger potency against STS.

Table 4. Antiproliferative activity of R-bis **7**, R-mono-I **8**, R-mono-II **9**, and Raloxifene **3** against T-47D cells grown in charcoal-stripped FBS, stimulated by E2S.[a]

Compound	Mean % inhibition at 10 µM concentration	GI_{50} (µM)
R-bis **7**	75% ± 3%	7.12 ± 0.46
R-mono-I **8**	40% ± 2%	N.D.[b]
R-mono-II **9**	12% ± 1%	N.D.[b]
Raloxifene **3**	78% ± 2%	6.89 ± 0.35

[a] Results are expressed as the means of triplicate assays ± SEM. [b] N.D. not determined.

2.2.4. Antiproliferative activity of R-bis **7** in the NCI-60 cell assay:

Bis-sulfamate R-bis **7** was selected for further anti-cancer evaluation in the NCI-60 cell line assay. Data from nine cell lines are presented (Table 5) along with the mean activity across the whole panel (mean graph midpoint, MGM value). In most cases, R-bis **7** displays GI_{50} values around 10-20 µM. However, much better GI_{50} values of between 1-2 µM are observed against few cell lines. GI_{50} values of 1.47 µM against UACC-62 (melanoma), 1.61 µM against SN12C (renal)

and 1.34 µM against MDA-MB-231 (breast) are the best potencies observed for R-bis **7** in these assays (Table 5).

Table 5. Antiproliferative activity of R-bis **7** in µM against various cancer cell lines from the NCI-60 cell line panel.[a]

Leukemia CCRF-CEM	**Lung HOP-62**	**Colon HCT-116**	**CNS SF-539**	**Melanoma UACC-62**
9.02	14.8	14.6	13.1	1.47
Ovarian OVCAR-4	**Renal SN12C**	**Prostate DU-145**	**Breast MDA-MB-231**	**MGM**
10.6	1.61	16.0	1.34	10.0

[a] Results are GI_{50} values in µM and the mean of three determinations. The MGM represents the mean concentration that caused 50% growth inhibition in all 60 cell lines.

Overall, R-bis **7** shows potent STS inhibitory and its anti-proliferative activity against T-47D cells is comparable to that of its parent compound Raloxifene **3**. However, its ERα binding with Ki and IC_{50} values of 13.0 ± 0.8 nM and 45.0 ± 1.0 nM, respectively, is much weaker (about 200-fold). Crystal structures of the ER protein with various ligands including Raloxifene **3** have been published [34]. The weaker activity of R-bis **7** may indicate that space in between the two distant binding domains that usually bind to the two hydroxyl groups in **3** might not easily extend further to accommodate the additional two sulfamoyl groups. R-mono-I **8** with only one sulfamoyl group and Ki and IC_{50} values of 1.5 ± 0.02 nM and 5.2 ± 0.2 nM, respectively, displays activities in between the ones **7** and **3**. However, the binding mode of R-bis **7** at the ER is currently unknown and could possibly be very different to the one known for Raloxifene **3**. Mono-sulfamates with dual potent STS inhibitory and intrinsic SERM properties have been described previously [34] and the best of these *N*-benzoyl-tetrahydroisoquinoline sulfamates [34] show similar dual activities as R-bis **7**. However, molecular modelling suggests that the sulfamoyled heterocyclic unit points towards residues that are also known to bind to the piperidine ring of Raloxifene **3**.

2.2.5. Stability studies of Raloxifene sulfamate derivatives **7-9** in wet DMSO-d_6

Compound **7** surprisingly did not seem particularly stable in solution media, with suspected desulfamoylation taking place. Thus, we decided to carry out a stability study in wet DMSO-d_6 using NMR spectroscopy. Hydrolysis of the sulfamoyl group of **8** led to Raloxifene **3** as the only product with 70% of **8** left after 6 days and only 18% after 45 days at the end of the study (Figure 3, Table S1). Hydrolysis of the bis-sulfamate **7** occurs at a very fast rate, and after 6 days, only 27%

of the starting material remained and 35% of the mono-sulfamate **7** and 18% of Raloxifene **3** were formed (Table 7). Interestingly, hydrolysis of **7** did not produce mono-sulfamate **9**. However, there was a fourth product visible in this complex reaction mixture and it seems that an equilibrium was involved that possibly slowed down the hydrolysis of **7** to **8**. The aromatic and heteroaromatic rings bearing the two sulfamoyloxy groups and the ketone are electron-deficient. Therefore, it seems reasonable to assume that a water molecule could add to the ketone function to form a ketone hydrate which in turn eliminates its electron-withdrawing effect from the benzothiophene 3-position and form a less electron-deficient heteroaromatic ring system than **7** (Figure 3). The amount of the postulated R-bis ketone hydrate **25** increased to about 20% as its highest concentration after 6 days with the concentration of **8** peaking slightly later after 10 days at 37%. After that, concentrations of both components slowly decreased. Starting from day 30 onwards, there was nothing left of compound **7** and only about 6% of the postulated R-bis ketone hydrate **25** was detected (Figure 4, Table S2). At the end of the experiment after 45 days, the tube contained a mixture of 88% Raloxifene **3** and 11% mono-sulfamate **8**. Surprisingly, the overall reaction of the two hydrolysis steps of the bis-sulfamate **7** via **8** leading to Raloxifene **3** occurred at a slightly faster rate than the one-step hydrolysis of **7** to Raloxifene **3**. However, both hydrolysis reactions of either **7** or **8** lead to Raloxifene **3** as the final product. Raloxifene **3** itself is known to be stable under strong acidic and neutral conditions but it degrades very quickly in strong alkaline solutions [35].

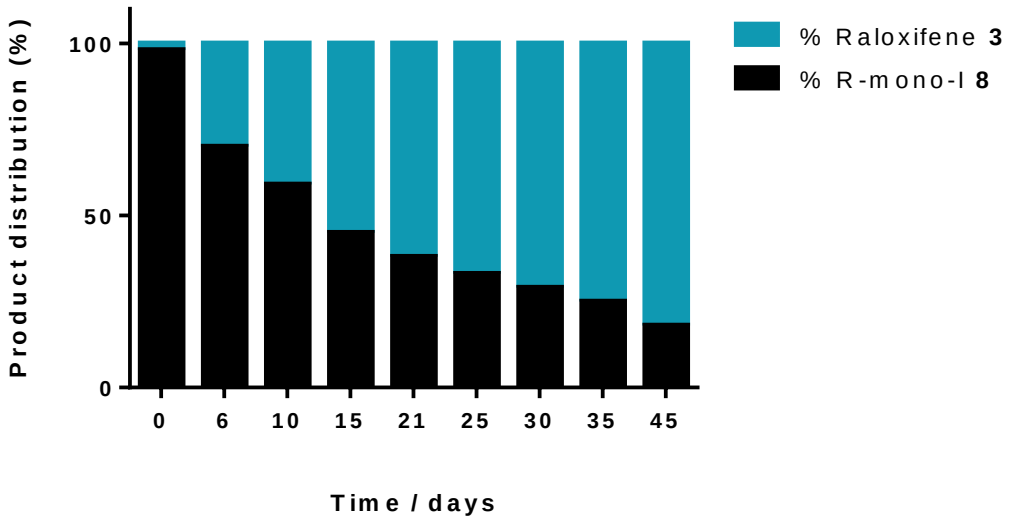


Figure 3. Time-dependent product distribution for the hydrolysis of R-mono-I **8** in wet DMSO-d_6 at room temperature followed by ^{1}H NMR.

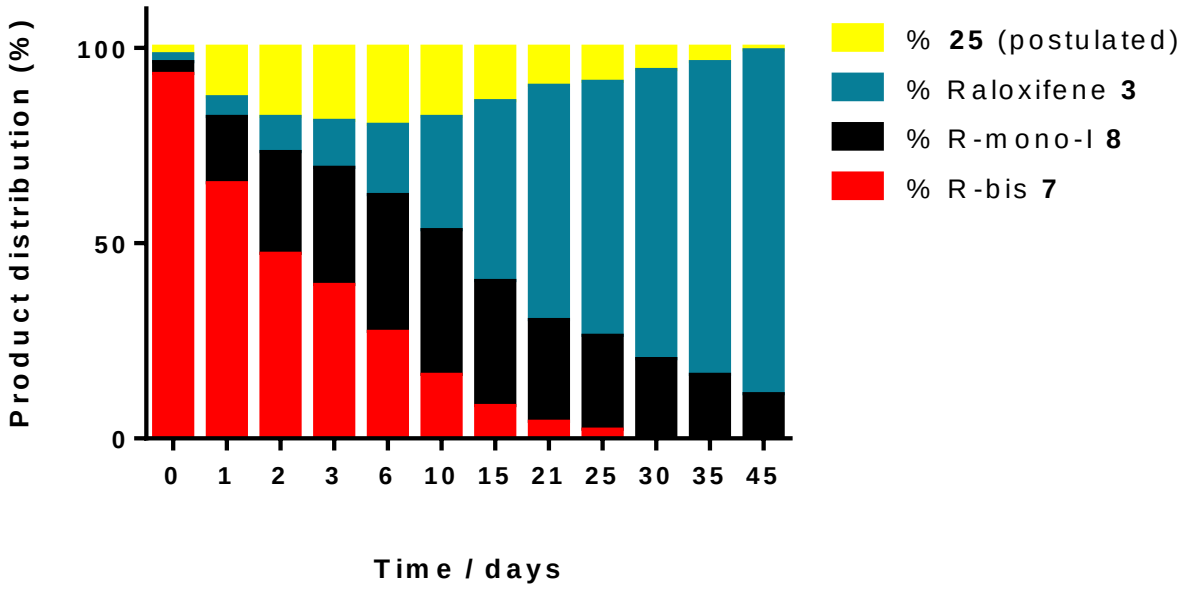


Figure 4. Time-dependent product distribution for the hydrolysis of R-bis **7** in wet DMSO-d_6 at room temperature followed by ^{1}H NMR.

The reaction of mono-sulfamate **9** in wet DMSO-d_6 also gave only a single product over the duration of the experiment (Figure 5, Table S3). However, Raloxifene **3** was not observed in this reaction but Raloxifene *S*-oxide (**27**) was formed instead. Remarkably, there was already 11% of product **27** present right at the start of the reaction. The ^{1}H NMR spectrum was recorded less than two hours after the sample preparation. Overall, the reaction seems to occur at a similar rate to that of **8**, leading to only one product. At the end of the experiment after 45 days, 88% of Raloxifene *S*-oxide (**27**) was formed along with 12% of the remaining mono-sulfamate **9**. For this hydrolysis experiment, the contents of the NMR tube were worked up and purification by column chromatography was performed. The main product was isolated and characterized. We also tested whether Raloxifene *N*-oxide **28** was formed during this reaction but the NMR data did not agree with the published data [36].

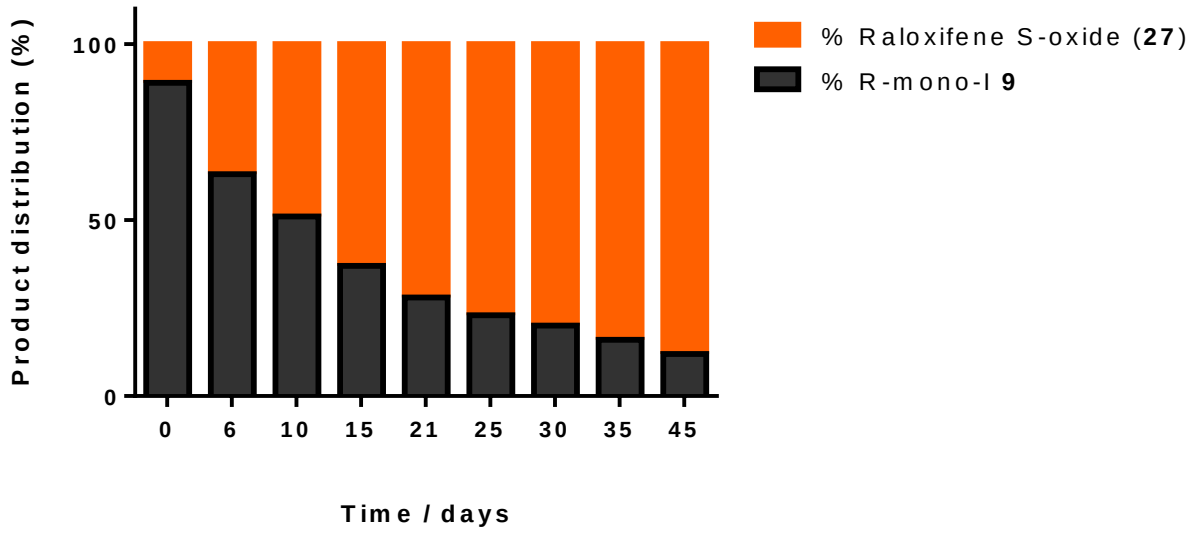


Figure 5. Time-dependent product distribution for the oxidation and hydrolysis of R-mono-I **9** in wet DMSO-d_6 at room temperature followed by ^{1}H NMR.

2.2.6. Explanation of the observed reaction pattern:

To understand the hydrolysis patterns of the different Raloxifene sulfamate derivatives, we examined the resonance interactions of both sulfamoyl groups with the aromatic and heteroaromatic ring systems. The benzothiophene sulfamate is in resonance with the *para*-phenyl sulfamate. However, this *para*-phenyl sulfamate is in addition in resonance with the ketone at the 3-positon of the benzothiophene ring. In compound R-bis **7**, the sulfamoyl groups are electron-withdrawing groups making the aryl rings very electron-deficient. Since the ketone function is at the 3-position of the benzothiophene ring, it selectively destabilizes the *para*-phenyl sulfamoyl group leading to its selective hydrolysis to form R-mono-I **7** without any formation of R-mono-II **9** being observed (Figure 6). The resulting electron-donating hydroxyl group present in **8** could potentially stabilize the remaining sulfamoyl group, but more likely resonates with the ketone at the 3-position. Without resonance stabilization, hydrolysis to Raloxifene **3** should occur.

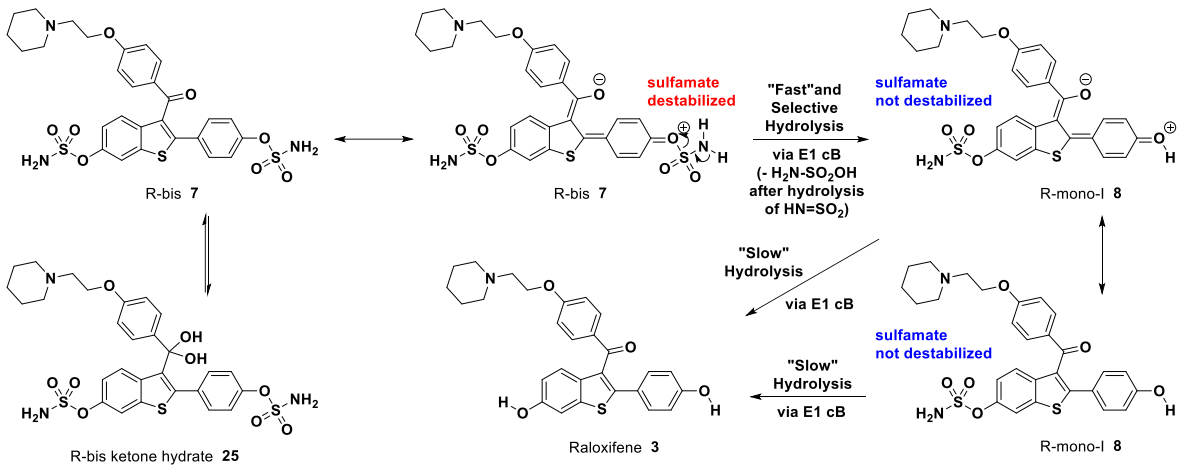


Figure 6. Raloxifene sulfamates **7** and **8** and their hydrolysis to Raloxifene **3**.

In R-mono-II **9**, the ketone function at the 3-position of the benzothiophene ring destabilizes the *para*-phenyl sulfamate in the same way as was described above for compound R-bis **7**. However, R-mono-II **9** seems completely stable towards hydrolysis as the electron-donating hydroxyl group overrides the electron-withdrawing effect of the ketone function and hence stabilizes the sulfamoyl group (Figure 7). Thus, hydrolysis does not occur and therefore the formation of Raloxifene **3** was not observed. However, the presence of the hydroxyl group facilitates slow oxidation of the sulphur atom resident at the benzothiophene ring, through the presence of molecular oxygen in the solution. Initially R-mono-II *S*-oxide **26** must be formed. In contrast to R-mono-II **9**, however, R-mono-II *S*-oxide **26** is resistant to hydrolysis and was not detected in the ^{1}H NMR spectrum. The various resonance structures of R-mono-II *S*-oxide **26** can explain its instability. The hydroxyl group in **26**, rather than stabilizing the sulfamoyl group, resonates with the *S*-oxide function leading to various resonance structures. In two of the possible resonance structures the sulfamate is not stabilized. Hydrolysis from these two resonance structures would be possible. However, it does not seem very likely that these two resonance structures would suffer the very fast hydrolysis that is required to keep the concentration of R-mono-II *S*-oxide **26** below visibility in the ^{1}H NMR spectra. However, a third possible resonance structure leaves a much-destabilized *para*-phenyl sulfamate and renders it extremely hydrolysis labile to deliver Raloxifene S-oxide (**27**) as the only product (Figure 7). As wet DMSO-d_6 was used as a reagent/solvent combination for these experiments, the hydrolysis of the sulfamoyl group(s) most likely occurs via an E1 cB mechanism [37-40].

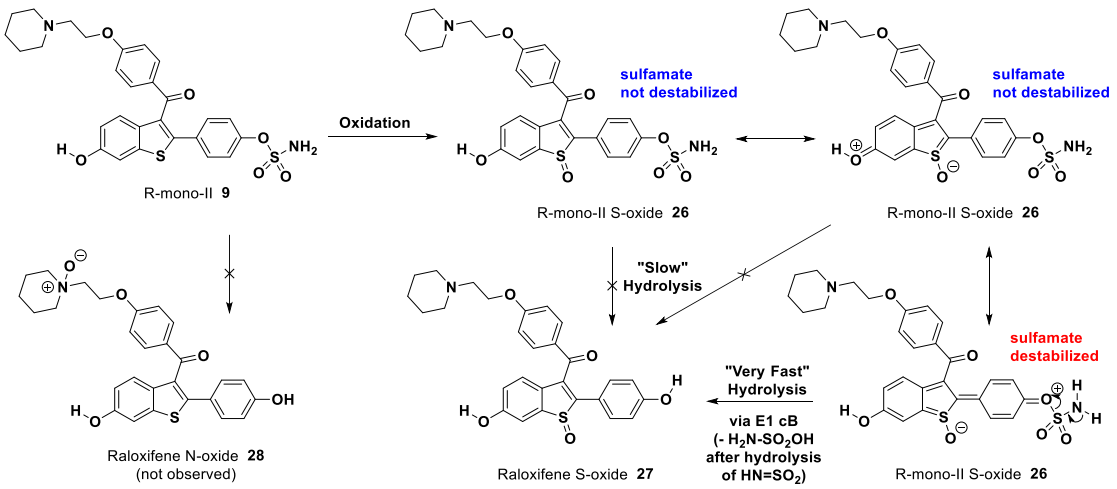


Figure 7. Raloxifene sulfamate R-mono-II (**9**) and its oxidation and subsequent hydrolysis to Raloxifene *S*-oxide (**27**).

While such instability of the most potent STS inhibitor **7** might *prima facie* be of potential concern in any drug development programme, we believe its rapid hydrolysis rate may not necessarily be important practically. Such sulfamate derivatives, including the clinical drugs irosustat and E2MATE are known to be sequestered almost completely in red blood cells after oral administration, by reversible binding to carbonic anhydrase II, with the sulfamoyl group then being protected from first pass metabolism [7-10, 41]. The heightened relative potency of **7** against STS may also be a function of the higher instability of this ligand as there is generally a correlation with the required ease of transfer of a sulfamate group to the hydrated formyl glycine residue with concomitant irreversible STS inhibition [10].

3. Conclusion

In summary, dual STS inhibition and ERα receptor antagonism is a potential approach for treatment of estrogen-dependent breast cancer, and it is believed to be more effective compared to a single SERM [26-29]. We now report the synthesis and biological evaluation of Raloxifene mono-sulfamates (R-mono-I **8** and R-mono-II **9**) and Raloxifene bis-sulfamate R-bis **7**, as new potential multi-targeting agents with potent STS inhibitory activity, as well as crucial stability aspects. These ligands were designed to act as dual inhibitors of STS enzyme and as potential ERα receptor antagonists with low nanomolar potency. It is interesting that these compounds exhibited lower IC_{50}s against STS activity in JEG3 cells compared to cell lysate. This suggests these compounds may be binding to other proteins within this lysate potentially compromising their

activity. Furthermore, as these inhibitors had higher activity in intact cells, this may suggest that enzyme preparations may not be predictive of therapeutic action. The most promising compound, bis-sulfamate **7**, exhibited an excellent inhibitory effect against STS with an IC_{50} value of 96.6 nM in JEG3 lysate and 12.2 nM in the cell-based assay, as well as high affinity to the ERα receptor as shown in the ERα coactivator binding assay, with a Ki value of 13.0 ± 0.8 nM and an IC_{50} of 45.0 ± 1.0 nM. This compound, while not as potent as single agents such as Raloxifene HCl (Ki = 0.07 ± 0.01 nM, IC_{50} = 0.24 ± 0.02 nM) and diethylstilbestrol (Ki = 0.2 ± 0.02 nM, IC_{50} = 0.7 ± 0.03 nM) that possess high affinity towards the estrogen receptor, still exhibited desirable and potent activities. Compound **7** also exhibited 95% ERα antagonism at 1 µM concentration in a coactivator assay, albeit being almost 50-fold weaker in concentration compared to Raloxifene HCl (100% at 20 nM), with fulvestrant (IC_{50} = 13.0 nM) and 4-OH tamoxifen (IC_{50} = 3.8 nM) also showing a negative agonism. Antiproliferative activity against T-47D breast cancer cell lines was also exhibited by **7** (GI_{50} = 7.12 ± 0.46 µM) that was comparable to Raloxifene (GI_{50} = 6.89 ± 0.35 µM). Evaluation of **7** in the NCI-60 cancer cell line assay displayed an MGM value of 10.0 µM. However, a few cell lines like UACC-62 and MDA-MB-231 responded better with GI_{50} values of 1.47 µM and 1.34 µM, respectively. Further studies to investigate the molecular mechanism(s) of action against the triple negative MDA-MB-231 are recommended to be conducted in the future. Stability testing of the three Raloxifene sulfamates **7**-**9** showed that bis-sulfamate **7** hydrolyzed at a very fast rate, initially leading selectively to mono-sulfamate **8** and finally Raloxifene **3**, without any formation of **9**. The mechanisms of all hydrolysis reactions were discussed, and the observed formation of products rationalized. However, the rapid hydrolysis rate for bis-sulfamate **7** should not be a serious concern for potential future *in vivo* studies, as such sulfamates are generally sequestered in red blood cells through the sulfamoyl group with the drug protected from metabolism. In conclusion, **7** is a promising novel lead with dual activity as an STS inhibitor and ERα antagonist and is a potential development candidate for treatment of estrogen-dependent breast cancer. The next step would be comprehensive *in vivo* studies. Moreover, lessons learned from this study could also be of wider value for any future elaboration of the general idea of a dual SERM-STS inhibitor based around the sulfamate approach.

4. Experimental

4.1. **General**

All solvents and reagents were purchased from commercial sources and used without further purification. Sulfamoyl chloride was either purchased as a solid reagent or prepared by an adaptation of the method of Appel and Berger [42] and was stored in the refrigerator under positive pressure of N_2 as a solution in toluene as described in the literature [43]. For column chromatography, silica gel with pore size of 0.040–0.063 mm (230–400 mesh) and reagent grade solvents were used for purification of the final compounds. Some intermediate compounds were only confirmed by LC-MS and used without further purification in the next step. ^{1}H NMR and ^{13}C NMR spectra were recorded with either a Bruker Avance 500 NMR spectrometer (Bruker, Billerica, MA, USA) at 500 or 125 MHz, respectively, or a Varian Mercury VX 400 NMR spectrometer (Varian, Palo Alto, CA, USA) at 400 or 100 MHz, respectively. Chemical shifts are reported in parts per million (ppm) relative to the corresponding residual solvent peak as internal standard (^{1}H NMR: DMSO-*d*$_6$: 2.50 ppm; CD_3OD: 3.31 ppm; $CDCl_3$: 7.26 ppm; ^{13}C NMR: DMSO-*d*$_6$: 39.52 ppm; CD_3OD: 49.00 ppm; $CDCl_3$: 77.16 ppm). Coupling constants *J* are recorded to the nearest 0.1 Hz. Mass spectra were either recorded at the Mass Spectrometry Service Centre, University of Bath, UK or at Research Institute for Medical and Health Sciences, University of Sharjah, United Arab Emirates. FAB-MS was carried out using *m*-nitrobenzyl alcohol (NBA) as the matrix or was performed using LC-MS analyzer (Waters Corporation, MA, USA) at research institute for medical and health science, University of Sharjah. Melting points were determined using a Stuart SMP3 or SMP30 melting point apparatus.

4.2. **Chemistry**

4.2.1. Synthesis of [6-hydroxy-2-(4-*O*-benzylphenyl)benzo[*b*]thien-3-yl][4-[2-(1-piperidinyl)ethoxy] phenyl]methanone (**10**), [6-*O*-benzyl-2-(4-hydroxyphenyl)benzo[*b*]thien-3-yl][4-[2-(1-piperidinyl)ethoxy] phenyl]methanone (**11**), and [6-*O*-sulfamoyl-2-(4-*O*-sulfamoyl-phenyl)benzo[*b*]thien-3-yl][4-[2-(1-piperidinyl)ethoxy] phenyl]methanone (**12**)

A solution of Raloxifene (**3**, 1.25 g, 2.64 mmol) in DMF (50 mL) was treated at 0 °C under nitrogen atmosphere with NaH (60% dispersion in oil, 0.16 g, 6.6 mmol) and stirred for 30 min at rt. Benzyl bromide (0.29 mL, 2.64 mmol) was added dropwise at 0 °C to the red dark reaction

mixture. The solution was stirred at rt for 3 h. Then CH_2Cl_2 (50 mL) and brine (50 mL) were added, and the mixture was extracted with CH_2Cl_2 (3 × 50 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by flash column chromatography ($CHCl_3$/MeOH 99:1 to 85:15) gave **12**, **10**, and **11**. **12** as a yellow oil (580 mg, 34%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.79-7.72 (m, 2H, 2 × ArH), 7.52 (d, 1H, *J* = 9.0 Hz, H^4 of benzothiophene), 7.48-7.28 (m, 13H, 13 × ArH), 7.03 (dd, 1H, *J* = 2.3, 9.0 Hz, H^5 of benzothiophene), 6.85-6.79 (d, 2H, *J* = 8.6 Hz, 2 × ArH), 6.79-6.73 (m, 2H, 2 × ArH), 5.13 (s, 2H, 6-OC$\underline{H}_2$Ph), 4.98 (s, 2H, 4-OC$\underline{H}_2$Ph), 4.08 (d, 2H, *J* = 5.9 Hz, NCH$_2$C$\underline{H}_2$O), 2.74 (t, 2H, *J* = 5.9 Hz, NC$\underline{H}_2$CH$_2$O), 2.56-2.38 (m, 4H, N(C$\underline{H}_2$)$_2$), 1.64-1.54 (m, 4H, C$\underline{H}_2$CH$_2$C$\underline{H}_2$), 1.48-1.37 (m, 2H, CH$_2$C$\underline{H}_2$CH$_2$). M/S *m/z* (+ve FAB, rel. int.): 654.0 [100, $[M + H]^+$], 327.2 (7), 255.1 (8), 112.1 (30), 98.0 (58), 84 (12). HRMS (+ve FAB) *m/z* calcd for $C_{42}H_{40}NO_4S$ $[M + H]^+$ 654.2678, found 654.2675.

Compound **10** was isolated as a yellow oil (220 mg, 15%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.67 (d, 2H, *J* = 9.0 Hz, 2 × ArH), 7.42-7.25 (m, 8H, 8 × ArH), 7.17 (d, 1H, *J* = 2.0 Hz, H^7 of benzothiophene), 6.85-6.72 (m, 3H, 2 × ArH and H^5 of benzothiophene), 6.60 (d, 2H, *J* = 9.0 Hz, 2 × ArH), 4.98 (s, 2H, 4-OC$\underline{H}_2$Ph), 4.11 (t, 2H, *J* = 5.5 Hz, NCH$_2$C$\underline{H}_2$O), 2.82 (t, 2H, *J* = 5.5 Hz, NC$\underline{H}_2$CH$_2$O), 2.70-2.50 (m, 4H, C$\underline{H}_2$NC$\underline{H}_2$), 1.64-1.54 (m, 4H, C$\underline{H}_2$CH$_2$C$\underline{H}_2$), 1.54-1.38 (m, 2H, CH$_2$C$\underline{H}_2$CH$_2$). M/S *m/z* (+ve FAB, rel. int.): 564.1 [100, $[M + H]^+$], 112.1 (30), 98.0 (64), 84 (10). M/S *m/z* (-ve FAB, rel. int.): 562.2 [100, $(M-H)^-$], 495.1 (56), 340.0 (56), 235.0 (48), 188.0 (50). HRMS (+ve FAB) *m/z* calcd for $C_{35}H_{34}NO_4S$ $[M + H]^+$ 564.2209, found 564.2205.

Compound **11** was isolated as a yellow oil (350 mg, 24%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.68 (d, 2H, *J* = 8.6 Hz, 2 × ArH), 7.59 (d, 1H, *J* = 9.0 Hz, H^4 of benzothiophene), 7.48-7.28 (m, 6H, 6 × ArH), 7.03 (dd, 1H, *J* = 2.3, 9.0 Hz, H^5 of benzothiophene), 6.60 (d, 2H, *J* = 9.0 Hz, 2 × ArH), 6.54 (d, 2H, *J* = 8.2 Hz, 2 × ArH), 5.13 (s, 2H, 6-OC$\underline{H}_2$Ph), 4.03 (t, 2H, *J* = 5.5 Hz, NCH$_2$C$\underline{H}_2$O), 2.74 (t, 2H, *J* = 5.5 Hz, NC$\underline{H}_2$CH$_2$O), 2.65-2.44 (m, 4H, N(C$\underline{H}_2$)$_2$), 1.68-1.54 (m, 4H, C$\underline{H}_2$CH$_2$C$\underline{H}_2$), 1.52-1.38 (m, 2H, CH$_2$C$\underline{H}_2$CH$_2$). M/S *m/z* (+ve FAB, rel. int.): 564.1 [100, $[M + H]^+$], 112.1 (24), 98.0 (45), 84 (12). HRMS (+ve FAB) *m/z* calcd for $C_{35}H_{34}NO_4S$ $[M + H]^+$ 564.2209, found 564.2198.

4.2.2. Synthesis of [6-*O*-sulfamoyl-2-(4-*O*-benzyl-phenyl)benzo[*b*]thien-3-yl][4-[2-(1-piperidinyl)ethoxy] phenyl]methanone (**13**)

DMBP (197 mg, 0.96 mmol) was added at rt to a stirred solution of **10** (180 mg, 0.32 mmol) in CH_2Cl_2 (10 mL) under nitrogen atmosphere. Then a solution of sulfamoyl choride (0.68 M in toluene, 2.35 mL, 1.60 mmol) was added *via* syringe and the reaction mixture was stirred at rt overnight. Water (50 mL) and CH_2Cl_2 (60 mL) were added and upon separation, the organic layer was washed with brine (3 × 50 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by flash column chromatography ($CHCl_3$/MeOH 95:5 to 90:10) afforded **13** as a yellow oil (126 mg, 61%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.81 (d, 1H, *J* = 2.0 Hz, H^7 of benzothiophene), 7.70 (d, 2H, *J* = 9.0 Hz, 2 × ArH), 7.58 (d, 1H, *J* = 9.0 Hz, H^4 of benzothiophene), 7.42-7.28 (m, 7H, 7 × ArH), 7.23 (dd, 1H, *J* = 2.0, 8.6 Hz, H^5 of benzothiophene), 6.82 (d, 2H, *J* = 9.0 Hz, 2 × ArH), 6.74 (d, 2H, *J* = 9.0 Hz, 2 × ArH), 4.99 (s, 2H, 4-OC$\underline{H}_2$Ph), 4.17 (brs, 2H exch. D_2O, NH_2), 2.74 (t, 2H, *J* = 5.8 Hz, NC$\underline{H}_2CH_2O$), 2.60-2.40 (m, 4H, N(C$\underline{H}_2)_2$), 1.66-1.54 (m, 4H, C$\underline{H}_2CH_2C\underline{H}_2$), 1.50-1.38 (m, 2H, $CH_2C\underline{H}_2CH_2$). M/S *m/z* (+ve FAB, rel. int.): 643.1 [100, $[M + H]^+$], 112.1 (32), 98.0 (68). HRMS (+ve FAB) *m/z* calcd for $C_{35}H_{35}N_2O_6S_2$ $[M + H]^+$ 644.1970 found 644.1966.

4.2.3. Synthesis of [6-*O*-benzyl-2-(4-*O*-sulfamoyl-phenyl)benzo[*b*]thien-3-yl][4-[2-(1-piperidinyl)ethoxy]phenyl]methanone (**14**)

DMBP (393 mg, 1.92 mmol) was added to a stirred solution of **11** (360 mg, 0.64 mmol) in CH_2Cl_2 (20 mL) under nitrogen atmosphere at rt. Sulfamoyl choride (0.68 M in toluene, 4.69 mL, 3.20 mmol) was then added *via* syringe and the reaction mixture was stirred overnight at rt. Water (50 mL) was added and the mixture was extracted with CH_2Cl_2 (60 mL). The organic layer was then washed with brine (3 × 50 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by flash column chromatography ($CHCl_3$/MeOH 95:5 to 90:10) afforded **14** as a yellow oil (167 mg, 41% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.82 (d, 1H, *J* = 9.0 Hz, H^4 of benzothiophene), 7.60 (d, 2H, *J* = 8.6 Hz, 2 × ArH), 7.48-7.25 (m, 8H, 7 × ArH and H^7 of benzothiophene), 7.11 (dd, 1H, *J* = 2.3, 9.0 Hz, H^5 of benzothiophene), 7.09 (d, 2H, *J* = 9.0 Hz, 2 × ArH), 6.65 (d, 2H, *J* = 9.0 Hz, 2 × ArH), 5.15 (s, 2H, 6-OC$\underline{H}_2$Ph), 4.93 (brs, 2H exch. D_2O, NH_2), 4.04 (t, 2H, *J* = 5.5 Hz, $NCH_2C\underline{H}_2O$), 2.73 (t, 2H, *J* = 5.5 Hz, NC$\underline{H}_2CH_2O$), 2.60-2.40 (m,

4H, N(C<u>H</u>$_2$)$_2$), 1.65-1.54 (m, 4H, C<u>H</u>$_2$CH$_2$C<u>H</u>$_2$), 1.52-1.40 (m, 2H, CH$_2$C<u>H</u>$_2$CH$_2$). M/S *m/z* (+ve FAB, rel. int.): 643.1 [100, $[M + H]^+$], 112.1 (28), 98.0 (52), 84 (10). HRMS (+ve FAB) *m/z* calcd for $C_{35}H_{35}N_2O_6S_2$ $[M + H]^+$ 644.1970 found 644.1958.

4.2.4. Synthesis of 1-(4-hydroxyphenyl)-2-((3-methoxyphenyl)thio)ethan-1-one (**17**)

Compound **16** (36 g, 0.26 mol) was added dropwise to a stirred solution of KOH (19.88 g, 0.35 mol) in water (182 mL) at rt. After 10 min, compound **15** (66 g, 0.31 mol) dissolved in ethyl acetate (330 mL) was added dropwise over a period of 30 min at rt. The reaction mixture was stirred for 2 h at rt. The mixture was then separated. The organic layer was collected and washed with sodium bicarbonate solution (200 mL), dried (Na_2SO_4), filtered and concentrated *in vacuo* to afford 70 g of crude compound. Purification by recrystallization in methanol (50 mL) afforded **17** (51.84 g, 0.189 mol, 72%) as a white solid. Mp: 109-111 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.47 (brs, 1H, OH), 7.91 (d, 2H, *J* = 8.8 Hz), 7.20 (t, 1H, *J* = 8.4 Hz), 6.90-6.84 (m, 4H), 6.73 (d, 1H, *J* = 2.0 Hz), 4.56 (s, 2H, CO-CH$_2$-), 3.72 (s, 3H, OCH$_3$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 192.5 (CO), 162.5, 159.5, 137.2, 131.3, 129.8, 126.9, 120.0, 115.3, 113.2, 111.5, 55.1 (OCH$_3$); LC-MS: 273.25 $[M - H]^-$.

4.2.5. Synthesis of 4-(6-methoxybenzo[*b*]thiophen-3-yl)phenol (**18**)

Compound **17** (50 g, 0.182 mmol) was dissolved in CH_2Cl_2 (150 mL) and added dropwise to a stirred solution of $AlCl_3$ (29 g, 0.22 mol) in CH_2Cl_2 (375 mL) over a period of 30 min at 0-5 °C. The reaction mixture was then allowed to warm to rt and stirred for 12 h at rt. The mixture was cooled to <5 °C, quenched with 1N $HCl_{(aq)}$ (100 mL) and stirred for 15 min. The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (2 × 100 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated *in vacuo* to afford **18** (26.13 g, 0.1 mol, 54%) as a white solid. Mp: 129-132 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.61 (brs, 1H, OH), 7.72 (d, 1H, *J* = 9.2 Hz), 7.61 (d, 1H, *J* = 2.4 Hz), 7.43-7.39 (m, 3H), 7.04 (dd, 1H, *J* = 2.4, 9.2 Hz), 6.89 (d, 2H, *J* = 8.4 Hz), 3.84 (s, 3H, OCH$_3$); ^{13}C NMR (100 MHz, DMSO-d_6): δ 157.0, 141.7, 136.8, 131.4, 129.4, 126.3, 123.2, 120.0, 115.6, 114.4, 105.6, 55.5 (O<u>C</u>H$_3$); LC-MS: 255.20 $[M - H]^-$.

4.2.6. Synthesis of 4-(6-methoxybenzo[*b*]thiophen-2-yl)phenol (**19**)

Methanesulfonic acid (22.4 g, 0.23 mol) was added to a stirred solution of compound **18** (50 g, 0.195 mmol) in toluene (600 mL) at rt. The reaction mixture was heated to 95-100 °C and stirred for 4-5 h. Then *n*-heptane (225 mL) was added, and the reaction mixture was stirred for 1 h at 90 °C. *i*-PrOH (400 mL) was added and the mixture was stirred for 30 min at 80 °C. The reaction mixture was cooled to 0 °C and stirred for 1 h. The solid was filtered, washed with 30% *i*-PrOH in n-heptane (100 mL) and dried *in vacuo* to afford **19** (37 g, 0.15 mol, 74%) as an ash solid. Mp: 172-175 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.78 (brs, 1H, ArOH), 7.66 (d, 1H, J = 8.8 Hz, ArH), 7.55-7.51 (m, 4H, 4 × ArH), 6.97 (dd, 1H, J = 2.4, 8.8 Hz, ArH), 6.86-6.82 (m, 2H, 2 × ArH), 3.82 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 157.7, 156.9, 141.1, 139.7, 134.7, 127.2, 124.9, 123.9, 117.3, 116.0, 114.4, 105.2, 55.5 (O<u>C</u>H$_3$); LC-MS: 255.35 $[M - H]^-$.

4.2.7. Synthesis of 4-(6-methoxybenzo[*b*]thiophen-2-yl)phenyl acetate (**20**)

To a stirred solution of compound **19** (25 g, 0.1 mol) dissolved in EtOAc (250 mL), Et_3N (24.6 g, 0.244 mol) and Ac_2O (29.8 g, 0.29 mol) were slowly added at rt. The reaction mixture was then heated to 60-65 °C and stirred for 3 h. The mixture was cooled to rt, diluted with EtOAc (200 mL), and washed with $NaHCO_3$ (200 mL), water (200 mL), and brine (200 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated *in vacuo* to afford **20** (17.26 g, 0.058 mol, 58%). Mp: 181-183 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.68-7.64 (m, 3H, 3 × ArH), 7.41 (s, 1H, ArH), 7.30 (d, 1H, J = 2.4 Hz, ArH), 7.14 (d, 2H, J = 8.8 Hz, 2 × ArH), 6.98 (dd, 1H, J = 2.4, 8.8 Hz, ArH), 3.89 (s, 3H, OCH_3), 2.33 (s, 3H, CH_3CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.6 (CO), 157.6, 150.3, 141.1, 140.6, 134.8, 132.4, 127.3, 124.4, 122.2, 119.3, 114.7, 104.9, 55.7 (OCH_3), 21.3 (<u>C</u>H$_3$CO); LC-MS: 257.28 $[M - CH_3CO + 2\ H]^+$.

4.2.8. Synthesis of 4-(3-(4-(2-chloroethoxy)benzoyl)-6-methoxybenzo[*b*]thiophen-2-yl)phenyl acetate (**22**)

Compound **21** (10 g, 46 mmol) dissolved in CH_2Cl_2 (50 mL) was added dropwise to a stirred solution of compound **20** (8 g, 26.8 mmol) dissolved in CH_2Cl_2 (80 mL) over a period of 30 min at 0-5 °C, followed by portion wise addition of $AlCl_3$ (6.7 g, 50 mmol) over a period of 10 min at 0-5 °C and stirring for 2 h at 0-5 °C. The reaction mixture was quenched with HCl (10%,

25 mL) at <5°C and stirred for 15 min. The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (2 × 100 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated *in vacuo* to afford 25 g of crude material that was recrystallized in MeOH (50 mL) to afford **22** (1.28 g, 2.67 mmol, 10%). Mp: 149-152 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.76 (d, 2H, *J* = 8.0 Hz, 2 × ArH), 7.55 (d, 1H, *J* = 8.8 Hz, ArH), 7.43 (d, 2H, *J* = 9.2 Hz, 2 × ArH), 7.34 (s, 1H, ArH), 6.98-6.96 (m, 3H, 3 × ArH), 6.78 (d, 2H, *J* = 8.4 Hz, 2 × ArH), 4.22 (t, 2H, *J* = 6.0 Hz, $ClCH_2C\underline{H}_2O$), 3.89 (s, 3H, OCH_3), 3.79 (t, 2H, *J* = 6.0 Hz, $ClC\underline{H}_2CH_2O$), 2.26 (s, 2H, CH_2CO); ^{13}C NMR (100 MHz, $CDCl_3$): δ 193.0 (CO), 169.2 (CO), 162.5, 158.0, 150.8, 141.7, 140.4, 133.8, 133.0, 132.5, 131.7, 131.2, 124.4, 121.9, 115.2, 114.8, 114.4, 104.5, 68.1 ($ClCH_2\underline{C}H_2O$), 55.8 ($O\underline{C}H_3$), 41.7 ($Cl\underline{C}H_2CH_2O$), 21.3 ($\underline{C}H_3CO$); LC-MS: 481.30 $[M + H]^+$.

4.2.9. Synthesis of (2-(4-hydroxyphenyl)-6-methoxybenzo[*b*]thiophen-3-yl)(4-(2-(piperidin-1-yl)ethoxy)phenyl)methanone (**23**)

Piperidine (5.31 g, 62 mmol) was added to a stirred solution of compound **22** (3 g, 6.25 mmol) in 1,4-dioxane (15 mL) at rt and was stirred at 85 °C for 1 h. The reaction mixture was cooled to room temperature then quenched with ice cold water (50 mL) and extracted with MeOH/CH_2Cl_2 (1:9, 2 × 100 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated *in vacuo* to give 4.5 g of crude compound. Purification by column chromatography (CH_2Cl_2/MeOH 95:5) afforded compound **23** (2 g, 4.1 mmol, 66%). Mp: 239-242 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.79 (brs, 1H, ArOH), 7.93 (d, 1H, *J* = 8.8 Hz, ArH), 7.71 (d, 2H, *J* = 8.8 Hz, 2 × ArH), 7.62 (s, 1H, ArH), 7.51 (d, 2H, *J* = 8.8 Hz, 2 × ArH), 7.32 (d, 1H, *J* = 9.2 Hz, ArH), 7.07 (d, 2H, *J* = 8.8 Hz, 2 × ArH), 6.82 (dd, 2H, *J* = 2.0, 6.8 Hz, 2 × ArH), 4.25 (brs, 2H, $NCH_2C\underline{H}_2O$), 3.75 (s, 3H, OCH_3), 3.00-2.60 (m, 6H, $NC\underline{H}_2CH_2O$, $N(C\underline{H}_2)_2$), 1.57-1.42 (m, 6H, $CH_2CH_2CH_2$); LC-MS: 488.41 $[M + H]^+$.

4.2.10. Synthesis of 4-(6-methoxy-3-(4-(2-(piperidin-1-yl)ethoxy)benzoyl)benzo[*b*]thiophen-2-yl)phenyl sulfamate (**24**)

Compound **23** (1 g, 2.05 mmol) was dissolved in DMA (15 mL). NaH (164 mg, 4.1 mmol) was added portion wise at 0-5 °C followed by sulfamoyl chloride (1.18 g, 10 mmol). The reaction mixture was then stirred at rt for 5 h. The reaction mixture was quenched with ice cold water (100 mL), stirred for 10 min, and the resultant solid was filtered, washed with cold water (10 mL) and

dried *in vacuo* to afford compound **24** (0.52 g, 0.92 mmol, 45%) as a yellow solid. Mp: 206-209 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.10 (brs, 2H, NH_2), 8.02 (d, 1H, J = 8.8 Hz, ArH), 7.88 (s, 1H, ArH), 7.84-7.79 (m, 2H, 2 × ArH), 7.75-7.71 (m, 2H, 2 × ArH), 7.40-7.33 (m, 3H, 3 × ArH), 7.09 (d, 2H, J = 8.4 Hz, 2 × ArH), 4.37 (brs, 2H, $NCH_2C\underline{H}_2O$), 3.77 (s, 3H, OCH_3), 2.67-2.55 (m, 6H, $NC\underline{H}_2CH_2O$, $N(C\underline{H}_2)_2$), 1.67-1.46 (m, 6H, $CH_2CH_2CH_2$); LC-MS: 567.32 $[M + H]^+$.

4.2.11. Synthesis of 4-(6-hydroxy-3-(4-(2-(piperidin-1-yl)ethoxy)benzoyl)benzo[*b*]thiophen-2-yl)phenyl sulfamate (R-mono-II **9**)

$AlCl_3$ (1.7 g, 12.8 mmol) was added to a stirred solution of thiourea (725 mg, 9.5 mmol) in CH_2Cl_2 (20 mL) at rt. After 10 min, compound **24** (1.8 g, 3.18 mmol) dissolved in CH_2Cl_2 (100 mL) was added dropwise over a period of 30 min, then heated to 40-45 °C and stirred for 3-4 h. The reaction mixture was quenched with ice cold water (100 mL) and stirred for 10 min. Then, saturated $NaHCO_3$ (20 mL) was added, and the mixture was extracted with MeOH/CH_2Cl_2 (1:9, 2 × 100 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated *in vacuo* to give 2 g of crude compound. Purification by column chromatography (CH_2Cl_2/MeOH 97:3 to 95:5) afforded R-mono-II **9** (600 mg, 1.09 mmol, 34%). M.p. = 117-120 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.85 (d, 1H, J = 8.5 Hz, ArH), 7.78-7.40 (m, 6H, 6 × ArH)), 7.34 (d, 2H, J = 8.5 Hz, 2 × ArH), 7.10 (d, 1H, J = 8.5 Hz, ArH), 7.05 (d, 2H, J = 8.5 Hz, 2 × ArH), 4.20 (t, 2H, J = 5.5 Hz, $NCH_2C\underline{H}_2O$), 2.81 (brs, 2H, $NC\underline{H}_2CH_2O$), 2.56 (brs, 4H, $N(CH_2)_2$), 1.54-1.50 (m, 4H, $C\underline{H}_2CH_2C\underline{H}_2$), 1.43-1.34 (brs, 2H, $CH_2C\underline{H}_2CH_2$); ^{13}C NMR (125 MHz, DMSO-d_6): δ 193.5 (CO), 162.4, 153.7, 149.8, 139.7, 139.5, 134.0, 132.2, 131.7, 130.2, 127.1, 123.0, 120.0, 118.8, 115.9, 115.8, 114.4, 65.3 ($NCH_2\underline{C}H_2O$), 56.7 ($N\underline{C}H_2CH_2O$), 54.0 ($N(\underline{C}H_2)_2$), 24.9 ($\underline{C}H_2CH_2\underline{C}H_2$), 23.3 ($CH_2\underline{C}H_2CH_2$); LC-MS/MS: 553.25 $[M + H]^+$; HPLC purity: 97.86%.

4.2.12. Synthesis of 2-(4-hydroxyphenyl)-3-(4-(2-(piperidin-1-yl)ethoxy)benzoyl)benzo[*b*]thiophen-6-yl sulfamate (R-mono-I **8**)

NaH (65% in mineral oil, 58.5 mg, 1.59 mmol) was added portion wise to a solution of Raloxifene **3** (500 mg, 1.06 mmol) dissolved in DMF (5 mL) at rt and stirred for 15 min. The reaction mixture was cooled to 0 °C, sulfamoyl chloride (366 mg, 3.18 mmol) was added and stirred for 16 h at rt. The reaction mixture was quenched with ice cold water (20 mL) and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with cold water (2 × 10

mL), dried (Na_2SO_4), filtered and concentrated *in vacuo* to give 700 mg of crude compound. The crude mixture contained R-bis **7**, R-mono-I **8** and R-mono-II **9**. Purification by preparative HPLC (Kromasil C18 (250*4.6 mm, 5 µm column; mobile phase A: 0.01 M ammonium acetate in 1000 mL water, mobile phase B: acetonitrile; flow rate: 0.8 mL/min; column temperature: 40 °C; Gradient: (A/B): 0/10,10/75,15/80,25/80,26/10,30/10; Diluent: MeCN/water 3:1) afforded R-mono-I **8** after a retention time of 10.971 min (95 mg, 0.172 mmol, 16%). M.p. = 177-180 °C; ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ 8.01 (d, 1H, *J* = 2.2 Hz, H^7 of benzothiophene), 7.66 (d, 2H, *J* = 8.8 Hz, 2 × ArH), 7.51 (d, 1H, *J* = 8.8 Hz, H^4 of benzothiophene), 7.30 (dd, *J* = 2.2, 8.8 Hz, H^5 of benzothiophene), 7.24 (d, 2H, *J* = 8.7 Hz, 2 × ArH), 6.93 (d, 2H, *J* = 8.9 Hz, 2 × ArH), 6.72 (d, 2H, *J* = 8.7 Hz, 2 × ArH), 4.08 (t, 2H, *J* = 5.9 Hz, $NCH_2C\underline{H}_2O$), 2.62 (t, 2H, *J* = 5.9 Hz, $NC\underline{H}_2CH_2O$), 2.43-2.34 (m, 4H, $N(CH_2)_2$), 1.51-1.41 (m, 4H, $C\underline{H}_2CH_2C\underline{H}_2$), 1.39-1.31 (m, 2H, $CH_2C\underline{H}_2CH_2$); LC-MS/MS: 553.29 $[M + H]^+$; HPLC purity: 98.66%.

4.2.13. Synthesis of 3-(4-(2-(piperidin-1-yl)ethoxy)benzoyl)-2-(4-(sulfamoyloxy)phenyl)benzo[*b*]thiophen-6-yl sulfamate (R-bis **7**)

A) To a solution of Raloxifene **3** (100 mg, 0.21 mmol) dissolved in DMF (2 mL), NaH (65% dispersed in mineral oil, 25.3 mg, 0.63 mmol) was added portion wise at rt and stirred for 15 min. The reaction mixture was cooled to 0 °C, sulfamoyl chloride (120.7 mg, 1.05 mmol) was added and the mixture was stirred at rt for 16 h. The reaction mixture was quenched with ice cold water (20 mL) and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with cold water (2 × 10 mL), dried (Na_2SO_4), filtered and concentrated *in vacuo*. Purification by column chromatography (EtOAc followed by EtOAc/MeOH 20:1) afforded R-bis **7** (66 mg, 0.105 mmol, 50%).

B) Raloxifene **3** (264 mg, 0.56 mmol) was dissolved in DMF (8 mL) and treated with NaH (60% dispersion in mineral oil, 84.4 mg, 2.11 mmol) at 0 °C under nitrogen atmosphere and then stirred for 0.5 h at rt. A solution of sulfamoyl chloride (0.7 M in toluene, 3.61 mL, 2.53 mmol) was added dropwise at 0 °C to the dark-red reaction mixture. The mixture was stirred for 2 h at rt and then diluted with EtOAc (50 mL) and brine (50 mL). The mixture was extracted with EtOAc/MeOH 80:20 (3 × 50 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by flash column chromatography ($CHCl_3$/MeOH 95:5 to 85:15) gave R-bis

7 (200 mg, 56%). A second flash column chromatography using the same conditions was performed, followed by a recrystallization from acetone/hexane to obtain R-bis **7** as a white solid (70 mg, 19%) after drying at the high vacuum pump for 12 h at 60 °C.

M.p. = 174-176 °C; ^{1}H NMR (400 MHz, CD_3OD): δ 7.97 (d, *J* = 2.2 Hz, 1H, H^7 of benzothiophene), 7.77 (d, *J* = 8.9 Hz, 1H, H^4 of benzothiophene), 7.71 (d, *J* = 8.9 Hz, 2H, 2 × ArH), 7.45 (d, *J* = 8.8 Hz, 2H, 2 × ArH), 7.38 (dd, *J* = 2.2, 8.9 Hz, 1H, H^5 of benzothiophene), 7.18 (d, *J* = 8.8 Hz, 2H, 2 × ArH), 6.91 (d, *J* = 8.9 Hz, 2H, 2 × ArH), 4.40-4.31 (m, 2H, $NCH_2C\underline{H}_2O$), 3.55-3.50 (m, 2H, $NC\underline{H}_2CH_2O$), 3.25 (brs, 4H, $N(CH_2)_2$), 1.88 (brs, 4H, $C\underline{H}_2CH_2C\underline{H}_2$), 1.69 (brs, 2H, $CH_2C\underline{H}_2CH_2$); ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ 8.10 (brs, 4H, 2 × NH_2), 8.09 (d, *J* = 2.3 Hz, 1H, H^7 of benzothiophene), 7.74 (d, *J* = 8.9 Hz, 2H, 2 × ArH), 7.55-7.49 (m, 3H, 2 × ArH and H^4 of benzothiophene), 7.34 (dd, *J* = 2.3, 8.8 Hz, H^5 of benzothiophene), 7.28 (d, *J* = 8.8 Hz, 2 × ArH), 7.03 (d, *J* = 8.9 Hz, 2 × ArH), 4.46-4.36 (m, 2H, $NCH_2C\underline{H}_2O$), 3.45-3.37 (m, 2H, $NC\underline{H}_2CH_2O$), 2.99 (brs, 4H, $N(CH_2)_2$), 1.79-170 (m, 4H, $C\underline{H}_2CH_2C\underline{H}_2$), 1.57 (brs, 1H, one of $CH_2C\underline{H}_2CH_2$), 1.45 (brs, 1H, one of $CH_2C\underline{H}_2CH_2$); LC-MS/MS: 632.04 $[M + H]^+$; IR (KBr disc, cm^{-1}): 2918, 2851, 1724, 1595, 1160. HRMS (ESI+) calcd for $C_{28}H_{30}N_3O_8S_3$ $[M + H]^+$ 632.1190; found 632.1185. Microanalysis calcd (%) for $C_{28}H_{30}N_3O_8S_3 \cdot 2H_2O$: C, 50.36; H, 4.98; N, 6.29; found (%): C, 50.20; H, 4.96; N, 5.98.

4.2.14. Synthesis of (6-hydroxy-2-(4-hydroxyphenyl)-1-oxidobenzo[*b*]thiophen-3-yl)(4-(2-(piperidin-1-yl)ethoxy)phenyl)methanone (**27**, Raloxifene S-oxide)

R-mono-II **9** (4.4 mg, 8.0 µmol) was placed in a clean NMR tube and DMSO-*d*$_6$ (non-anhydrous, 0.6 mL) was added. ^{1}H NMR spectra were recorded at various time points for a duration of 45 days. To ensure that the reaction could progress without interruption, the NMR tube with its content was kept at rt (~20 °C) for the whole duration of 45 days. The contents of the tube were poured into EtOAc (50 mL). The tube was then washed several times with EtOAc (~2 mL each time) and the combined organic layers were washed with water (50 mL), dried (anhydrous Na_2SO_4), filtered and concentrated *in vacuo*. The residue (~4 mg) was dissolved in CH_2Cl_2/MeOH (50:50; ~0.4 mL), divided into three equal portions and each portion was separately subjected to column chromatography using a glass pipette (500 mg SiO_2: CH_2Cl_2 to CH_2Cl_2/MeOH 90:10 to 85:15 to 80:20) to give **27** (Raloxifene *S*-oxide: 2.7 mg, 68%) as a pale-yellow solid. ^{1}H NMR

(400 MHz, DMSO-d_6): δ 10.08 (brs, 1H, OH), 9.72 (brs, 1H, OH), 7.76 (d, J = 8.7 Hz, 1H, H^4 of benzothiophene), 7.72 (d, J = 9.0 Hz, 2H, 2 × ArH), 7.54 (s, 1H, H^7 of benzothiophene), 7.49 (d, J = 8.7 Hz, 2H, 2 × ArH), 7.04 (d, J = 9.0 Hz, 2H, 2 × ArH), 7.03 (d, J = 8.6 Hz, 1H, H^5 of benzothiophene), 6.80 (d, J = 8.7 Hz, 2H, 2 × ArH), 4.16 (t, J = 5.8 Hz, 2H, $NCH_2C\underline{H}_2O$), 2.70 (t, J = 5.8 Hz, $NC\underline{H}_2CH_2O$), 2.48-2.42 (m, 4H, $N(CH_2)_2$), 1.52-1.47 (m, 4H, $C\underline{H}_2CH_2C\underline{H}_2$), 1.40-1.34 (m, 2H, $CH_2C\underline{H}_2CH_2$). ^{13}C/deptQ NMR (100 MHz, DMSO-d_6): δ 193.6 (C_q), 162.6 (C_q), 157.6 (C_q), 152.9 (C_q), 141.5 (C_q), 138.6 (C_q), 134.2 (C_q), 131.7 (2 × CH), 130.0 (C_q), 127.1 (2 x CH), 126.3 (CH), 124.7 (C_q), 119.0 (C_q), 117.2 (CH), 115.9 (2 × CH), 115.3 (CH), 114.4 (2 x CH), 65.8 ($NCH_2\underline{C}H_2O$), 57.1 ($N\underline{C}H_2CH_2O$), 54.3 ($N(\underline{C}H_2)_2$), 25.4 ($\underline{C}H_2CH_2\underline{C}H_2$), 23.8 ($CH_2\underline{C}H_2CH_2$). HRMS (ESI+) calcd for $C_{28}H_{28}NO_5S$ $[M + H]^+$ 490.1683; found 490.1682.

Note: Unfortunately, Raloxifene *S*-oxide (**27**) was isolated in less pure form than was expected from the crude material. This may indicate that **27** is slightly unstable under the conditions that were used for purification.

4.3. Cell-free STS enzyme assay

It was carried out utilizing the procedure reported in our previously published article [14].

4.4. Whole-cell STS enzyme assay

It was carried out utilizing the procedure reported in our previously published article [14].

4.5. Antiproliferative screening against T-47D breast cancer cell line

It was carried out utilizing the procedure reported in our previously published article [14].

4.6. Estrogen Receptor alpha (ERα) coactivator binding assay:

The purpose of this experiment was to evaluate the affinity of compounds for the human estrogen receptor alpha (ERα) expressed in transfected Sf9 cells, determined in a radioligand binding assay. Full length receptor (10 ng) was incubated for 120 min at 22 °C with 0.5 nM [^{3}H]Estradiol in the absence or presence of the test compound in a buffer containing 10 mM Tris/HCl (pH 7.4), 10 % glycerol, 1 mM dithiothreitol (DTT), and 0.1% bovine serum albumin (BSA). Nonspecific binding was determined in the presence of 1 µM diethylstilbestrol.

Following incubation, the samples were filtered rapidly under vacuum through glass fiber filters (GFB, Packard) presoaked with 0.3% polyethyleneimine (PEI) and rinsed several times with ice-cold 50 mM tris/HCl using a 96-sample cell harvester (Unifilter, Packard). The filters were dried then counted for radioactivity in a scintillation cocktail (Microscint 0, Packard). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is diethylstilbestrol, which was tested in each experiment at several concentrations to obtain a competition curve from which its IC_{50} is calculated.

4.7. Stability studies

The hydrolysis of Raloxifene bis-sulfamate R-bis **7** and Raloxifene mono-sulfamate derivatives R-mono-I **8** and R-mono-II **9** was studied by ^{1}H NMR using a batch of wet DMSO-d_6. Compounds **7** (5.3 mg, 7.9 µmol), **8** (3.4 mg, 6.2 µmol) and **9** (4.4 mg, 8.0 µmol) were each placed in an NMR tube and DMSO-d_6 (non-anhydrous, 0.6 mL) was added to each tube. At the start of the hydrolysis reaction, the water/substrate ratio worked out as ~30:1 for R-bis **7**, ~40:1 for R-mono-I **8**, and ~25:1 for R-mono-II **9**. All three NMR tubes containing the sample solutions were kept at room temperature (21-25 °C) throughout the duration of the study. ^{1}H NMR spectra of all samples were recorded at various time points for a duration of 45 days. The product distributions were calculated for all recorded ^{1}H NMR spectra of all three samples (for selected ^{1}H NMR spectra see supporting information). For R-mono-II **9**, the product was isolated (see 4.2.13.)

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Supplementary data

Supplementary material including the NMR, LC-MS, and HPLC charts of the target compounds, dose-response curves of STS inhibition, ERα, and antiproliferative assays, and ^{1}H

NMR example spectra of each stability study along with general compound ratio calculation formula.

The authors declare no conflict of interest.

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