2-Substituted Estradiol Bis-sulfamates, Multitargeted Antitumor Agents: Synthesis, In Vitro SAR, Protein Crystallography, and In Vivo Activity†

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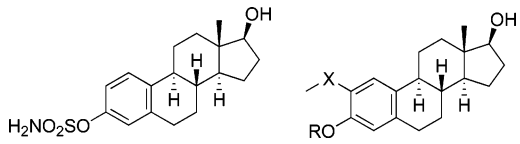
The anticancer activities and SARs of estradiol-17-*O*-sulfamates and estradiol 3,17-*O*,*O*-bis-sulfamates (E2bisMATEs) as steroid sulfatase (STS) inhibitors and antiproliferative agents are discussed. Estradiol 3,17-*O*,*O*-bis-sulfamates **20** and **21**, in contrast to the 17-*O*-monosulfamate **11**, proved to be excellent STS inhibitors. 2-Substituted E2bisMATEs **21** and **23** additionally exhibited potent antiproliferative activity with mean graph midpoint values of 18−87 nM in the NCI 60-cell-line panel. **21** Exhibited antiangiogenic in vitro and in vivo activity in an early-stage Lewis lung model, and **23** dosed p.o. caused marked growth inhibition in a nude mouse xenograft tumor model. Modeling studies suggest that the E2bisMATEs and 2-MeOE2 share a common mode of binding to tubulin, though COMPARE analysis of activity profiles was negative. **21** was cocrystallized with carbonic anhydrase II, and X-ray crystallography revealed unexpected coordination of the 17-*O*-sulfamate of **21** to the active site zinc and a probable additional lower affinity binding site. 2-Substituted E2bisMATEs are attractive candidates for further development as multitargeted anticancer agents.

Introduction

In previous studies we have established the ability of 3-*O*-sulfamoylated estrogens such as estrone 3-*O*-sulfamate (EMATE)[a] to act as potent irreversible inhibitors of steroid sulfatase (STS),[1,2] a clinical target for the treatment of hormone-dependent breast cancer.[3,4] This activity is believed to arise from either nucleophilic attack of the sulfamate group onto the conserved formylglycine residue in the STS active site or, most likely, transfer of the sulfamate group either to a nucleophilic residue in the active site of STS or by generation of a highly electrophilic sulfonylamine species, to irreversibly arrest the catalytic function of the enzyme.[5] Further investigations on the biological activities of these compounds revealed that estrogen 3-*O*-sulfamates are also highly active reversible inhibitors of carbonic anhydrase II, an enzyme which is highly expressed in red blood cells, through a coordination of the monoanionic form of the sulfamate moiety to the zinc atom in the enzyme active site.[6] This interaction is believed to underlie the high oral bioavailability observed for estradiol 3-*O*-sulfamate (**1**, E2MATE: Figure 1) (which is atypical of estrogen derivatives), wherein reversible uptake by red blood cells and interaction with carbonic anhydrase II results in a bypassing of first-pass liver metabolism.[7]



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2 X = O, R = H
3 X = O, R = SO_2NH_2
4 X = CH_2, R = SO_2NH_2
5 X = S, R = SO_2NH_2

Figure 1. Chemical structures of estradiol 3-*O*-sulfamate (E2MATE, **1**), 2-methoxyestradiol (**2**), and 2-substituted E2MATE compounds **3**−**5**.

The discovery of the antiproliferative and antiangiogenic effects of 2-methoxyestradiol (**2**, 2-MeOE2; Figure 1) and a number of closely related compounds has stimulated considerable interest in the potential of such molecules as clinical agents for the treatment of cancer.[8] The antiproliferative effects of 2-MeOE2 are independent of the estrogen receptor (ER) status of the treated cancer cell line and, though a precise mechanistic picture remains to be established, appear to stem from its ability to disrupt the dynamics of tubulin polymerization.[9] At high concentrations 2-MeOE2 has been shown to down-regulate HIF-1α and HIF-2α protein expression in tumor cells.[10] This causes subsequent down-regulation of the mRNA expression for the HIF-1α-regulated genes Glut-1, $VEGF_{165}$ and endothelin-1. The effects on HIF, however, occur downstream of the interaction with microtubules, and it has recently been suggested that such effects occur on treatment with all microtubule-disrupting agents.[11] Various analogues of 2-MeOE2 modified in the A-ring (C-2),[12−15] B-ring (C-6),[13] and D-ring (C-14, C-15, C-16, C-17)[14,16,17] display significantly enhanced in vitro antiproliferative activity, though translation of their activity into the in vivo context is complicated by conjugative inactivation (e.g. sulfation of the 3- and/or 17-hydroxyl groups) and metabolic oxidation of their 17β-hydroxyl group to the inactive C-17 ketones mediated by 17β-hydroxysteroid dehydrogenase

† Protein crystal data: Protein Data Bank accession code 2GD8.

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[a] Abbreviations: EMATE, estrone 3-*O*-sulfamate; E2MATE, estradiol 3-*O*-sulfamate; E2bisMATE, estradiol 3,17-*O*,*O*-bis-sulfamate; STS, steroid sulfatase; CA, carbonic anhydrase; hCAII, human carbonic anhydrase II; ER, estrogen receptor; E2S, estradiol 3-*O*-sulfate; HDBC, hormone-dependent breast cancer; HIF, hypoxia-inducible factor; DMA, *N*,*N*-dimethylacetamide.

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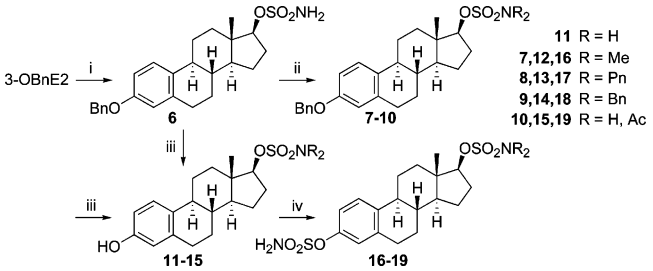
type II.[18] The importance of conjugation and metabolism was illustrated in a recent clinical trial where a dose escalation of up to 6 g/day of 2-MeOE2 was used in an attempt to achieve satisfactory plasma concentrations.[19]

Investigations on the effects of 2-substitution on sulfatase inhibitory activity in the estrogen 3-*O*-sulfamate series and the effects of 3-*O*-sulfamoylation on the antiproliferative activity of 2-MeOE2 led us to discover a series of 2-substituted estrogen 3-*O*-sulfamates as a class of antitumor agents notable for their multiple mechanisms of action.[20,21] Thus, in addition to irreversibly inhibiting STS-mediated conversion of E1S to E1 at concentrations in the nanomolar range, compounds such as 2-methoxyestradiol 3-*O*-sulfamate (**3**, 2-MeOE2MATE; Figure 1) displayed potent antiproliferative effects against a range of estrogen-independent human cancer cell lines in vitro. As with 2-MeOE2 the antiproliferative activities of the 2-substituted estrogen 3-*O*-sulfamates appear to arise from their ability to disrupt the paclitaxel-induced polymerization of tubulin, resulting in a G2/M phase cell cycle arrest and apoptosis.[22] As would be expected of microtubule-disrupting drugs, 2-substituted estradiol 3-*O*-sulfamates, among other effects, induce tumor suppressor protein p53 and phosphorylation of BCL-2 and BCL-XL.[22] Significantly, the antiproliferative effects of 2-MeOE2MATE were expressed at much lower concentrations than that required for 2-MeOE2, and furthermore, the cell cycle arrest induced by the sulfamate compound proved irreversible, whereas the effects of 2-MeOE2 treatment were reversible.

Structure−activity studies of 2-substituted estrogen 3-*O*-sulfamates showed that optimal antiproliferative effects were obtained with methoxy, ethyl, or methylsulfanyl substitution at the 2-position (i.e. 2-CH_3X, where X = O, CH_2, or S, e.g., compounds **3**, **4**, and **5**; Figure 1).[21] In contrast, in the absence of a 3-*O*-sulfamate group 2-ethoxy and 2-(1-propenyl) substitution is reportedly optimal.[12] The sulfamoylated estradiol derivatives proved far more potent than the nonsulfamoylated derivatives against the proliferation of a wide range of cancer cell lines, with, for example, 2-MeOE2MATE proving to be 8-fold more active against the proliferation of MCF-7 cells than 2-MeOE2. Comparison of the two molecules across the NCI screening panel further highlighted the enhancement in antiproliferative activity afforded by incorporation of the 3-*O*-sulfamate group, with 2-MeOE2MATE exhibiting a mean GI_{50} (mean graph midpoint, MGM) across the NCI panel of 110 nM (cf. 2-MeOE2, 1.3 μM).[21] Substitution of the sulfamate nitrogen was not well tolerated and caused a dramatic reduction in antiproliferative activity. In striking contrast to the nonsulfamoylated 2-substituted estrogen series the sulfamoylated estrone derivatives also proved to be active against the proliferation of cancer cells, with 2-ethylestrone 3-*O*-sulfamate (2-EtEMATE) proving to be over 160-fold more active than 2-ethylestrone. This result clearly indicated that, although 2-substituted estrogen 3-*O*-sulfamates exhibit mechanistic similarities with 2-substituted estradiols, they are not prodrugs of the parent estrogens and are instead a novel class of anticancer agents with a contrasting structure−activity relationship. Additionally, the antiangiogenic effects of 2-substituted EMATEs have also been shown to be superior to those of 2MeOE2 in in vitro model systems, with significantly reduced concentrations of the sulfamate derivatives (with respect to the corresponding estradiol derivatives) being required to inhibit, for example, HUVEC growth and in vitro angiogenesis.[23] Evaluation of the antitumor effects of 2-substituted estradiol 3-*O*-sulfamates in in vivo models reinforced the findings of in vitro studies. 2-Methoxyestrone 3-*O*-sulfamate (2-MeOEMATE) inhibited the growth of NMU-induced mammary tumors in rats,[24] while a separate study showed that 2-MeOE2MATE and 2-EtE2MATE dosed orally at 20 mg/kg inhibited the growth of MDA-MB-435 xenografts (ER^- human breast cancer cells) in female mice by 33% and 40%, respectively, and that this inhibition persisted after the cessation of dosing.[21]

Scheme 1. Synthesis of 3,17-*O*,*O*-Bis-sulfamate Derivatives of Estradiol[a]



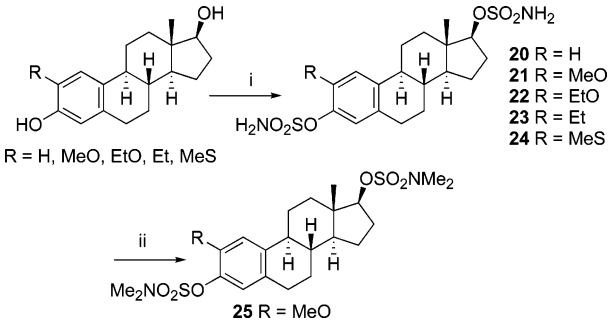
[a] Reagents and conditions: (i) *t*-BuOK, H_2NSO_2Cl, 0 °C to rt; (ii) NaH, DMF, then RX; (iii) H_2, Pd/C, EtOH/THF; (iv) DBMP, H_2NSO_2Cl, CH_2Cl_2, 0 °C to rt.

Preliminary studies had established that D-ring modification of 2-substituted estradiol 3-*O*-sulfamates could significantly modulate both the antiproliferative and sulfatase inhibitory activities of these compounds.[25] In particular, it was found that hydrogen-bonding interactions around C-17 were key to high antiproliferative activity. As part of a wider structure−activity program, we thus set out to establish the effect of introduction of a second sulfamate group at C-17 on both STS inhibition and antiproliferative activity, since an aliphatic sulfamate ester moiety is unlikely to be rapidly removed in vivo. Such a substitution was anticipated to both satisfy H-bonding requirements proximal to the D-ring and also block undesirable conjugation at C-17. We report here synthetic routes to 2-substituted estradiol bis-sulfamates and those with various simple substitutions at the 17-sulfamate group. In vitro and in vivo results demonstrate the superiority of the simple 2-substituted bis-sulfamates with respect to both the corresponding monosulfamates and their parent estradiol derivatives. In addition, a novel interaction of 2-methoxyestradiol 3,17-*O*,*O*-bis-sulfamate with carbonic anhydrase II is demonstrated through X-ray crystallography on a human carbonic anhydrase II (hCAII)−ligand complex.

Chemistry

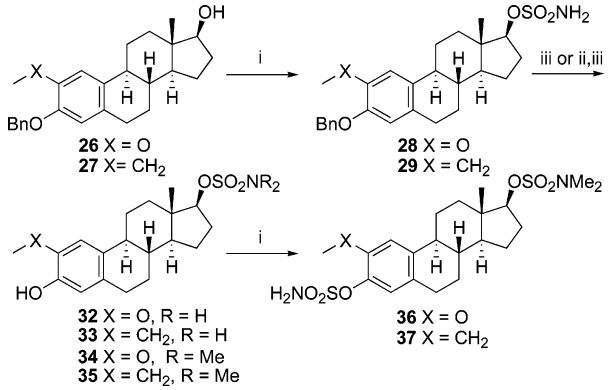
The synthesis of 3,17-*O*,*O*-bis-sulfamates was first investigated in the estradiol series as we were interested to develop the chemistry with readily available starting materials and also to assess the STS inhibitory activities of these compounds. Elaboration of these compounds was achieved in a straightforward and generally high-yielding manner (Scheme 1). Thus, to generate differentially substituted sulfamate groups at the 3- and 17- positions, 3-*O*-benzylestradiol was sulfamoylated to afford the 17-*O*-sulfamated estradiol **6** using potassium *tert*-butoxide and sulfamoyl chloride in DMF solution. Alkylation of **6** by reaction with the appropriate alkyl or benzyl halide proceeded smoothly to give the *N*,*N*-dialkylsulfamate compounds **7**−**9** in excellent yield (89−99%). Acylation was also achieved under similar conditions, with acetyl chloride used to obtain the monoacetylated sulfamate derivative **10**, albeit in a modest 46% yield. Compounds **6**−**10** were then converted into the corresponding phenols **11**−**15** under standard hydrogenolytic conditions, which in turn were sulfamoylated with the hindered pyridine base 2,6-di-*tert*-butyl-4-methylpyridine (DBMP) and

Scheme 2. Synthesis of 2-Substituted Estradiol 3,17-*O*,*O*-Bis-sulfamates[a]



[a] Reagents and conditions: (i) DMA, H_2NSO_2Cl, 0 °C to rt; (ii) NaH, DMF, MeI, 38%.

Scheme 3. Synthesis of 2-Substituted Estradiol 17-*O*-Sulfamates, 2-Substituted Estradiol 17-*O*-(*N*,*N*-Dimethyl)sulfamates, and 2-Substituted 3-*O*-Sulfamoylestradiol 17-*O*-(*N*,*N*-Dimethyl)sulfamates[a]



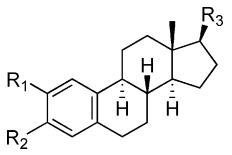
[a] Reagents and conditions: (i) DMA, H_2NSO_2Cl, 0 °C to rt; (ii) NaH, DMF, MeI (for **34** and **35** only); (iii) H_2, Pd/C, THF, EtOH.

sulfamoyl chloride in dichloromethane solution[26] to give the bis-sulfamates **16**−**19**.

Bis-sulfamoylation of estradiol proved to be straightforward, with reaction of estradiol and excess sulfamoyl chloride in dimethylacetamide[27] delivering high yields of estradiol 3,17-*O*,*O*-bis-sulfamate (**20**, E2bisMATE) directly in excellent yield (Scheme 2). A series of 2-substituted estradiol 3,17-*O*,*O*-bis-sulfamates was then synthesized in an analogous manner from the appropriate 2-substituted estradiols. Thus, 2-methoxyestradiol 3,17-*O*,*O*-bis-sulfamate (**21**, 2-MeOE2bisMATE), 2-ethoxyestradiol 3,17-*O*,*O*-bis-sulfamate (**22**, 2-EtOE2bisMATE), 2-ethylestradiol 3,17-*O*,*O*-bis-sulfamate (**23**, 2-EtE2bisMATE), and 2-methylsulfanylestradiol 3,17-*O*,*O*-bis-sulfamate (**24**, 2-MeSE2bisMATE) were prepared in a rapid manner and in good yield as shown in Scheme 2. 2-MeOE2bisMATE (**21**) was alkylated under basic conditions to give the bis(*N*,*N*-dimethyl)sulfamate derivative **25** in a straightforward manner.

A small series of 17-*O*-sulfamate, 17-*O*-(*N*,*N*-dimethyl)-sulfamate, and 3-*O*-sulfamoyl-17-*O*-(*N*,*N*-dimethyl)sulfamate derivatives of 2-MeOE2 and 2-ethylestradiol (2-EtE2) was next targeted. To sulfamoylate the 17-hydroxyl group in a selective manner, the more acidic 3-hydroxyl group was benzylated by reacting the appropriate 2-substituted estradiol with benzyl bromide at room temperature in DMF solution. The resultant 2-substituted 3-*O*-benzylestradiol derivatives **26** and **27** were then reacted with sulfamoyl chloride in DMA to give the 17-*O*-sulfamates **28** and **29** (Scheme 3); samples of these compounds were then methylated to give the *N*,*N*-dimethylsulfamate derivatives **30** and **31**. The 2-methoxy and 2-ethyl 17-*O*-sulfamates **28**−**31** were then deprotected to give the free phenols **32**−**35** by catalytic hydrogenation. Sulfamoylation of the 3-hydroxy 17-*O*-(*N*,*N*-dimethyl)sulfamate derivatives **34** and **35** afforded the unsymmetrically substituted bis-sulfamate derivatives **36** and **37**. A set of 2-methoxy- and 2-ethyl-estradiol analogues bearing 17-*O*-sulfamoyl or 17-*O*-(*N*,*N*-dimethyl)-sulfamoyl groups in combination with a 3-hydroxyl or 3-*O*-sulfamoyl group were thus available for evaluation.

Table 1. STS Inhibitory Activity of 3-*O*-, 17-*O*-, and 3,17-*O*,*O*-Sulfamoylated Estradiol Derivatives in Placental Microsomes



compd no.	R_1	R_2	R_3	IC_{50}(STS)[a]	inhibition (%) at 10 μM
1	H	OSO_2NH_2	OH	16 nM	
11	H	OH	OSO_2NH_2	5 μM	65
12	H	OH	OSO_2NMe_2		8.7 ± 3.2
13	H	OH	OSO_2NPent_2		0
14	H	OH	OSO_2NBn_2		1.4 ± 3.1
15	H	OH	OSO_2NHAc		31 ± 3
20	H	OSO_2NH_2	OSO_2NH_2	18 nM	
16	H	OSO_2NH_2	OSO_2NMe_2		96 ± 0.3
17	H	OSO_2NH_2	OSO_2NPent_2	190 nM	93 ± 1
18	H	OSO_2NH_2	OSO_2NBn_2		38.2 ± 2.9
19	H	OSO_2NH_2	OSO_2NHAc		90.9 ± 0.3
21	MeO	OSO_2NH_2	OSO_2NH_2	39 nM[31]	
23	Et	OSO_2NH_2	OSO_2NH_2	1 μM	
24	MeS	OSO_2NH_2	OSO_2NH_2	320 nM	

[a] IC_{50} figures are the mean values obtained from experiments performed in triplicate, SEM < ±7%.

Results and Discussion

To ascertain the effect of C-17 sulfamoylation on the STS inhibitory activity of the estradiol 3-*O*-sulfamate derivatives, selected compounds were assayed using the radiometric assay developed by Purohit et al.[28] wherein the STS-mediated conversion of tritiated E1S to E1 by placental microsomes is determined. Unsurprisingly, the 17-*O*-sulfamate estradiol derivatives **11**−**15** lacking a 3-*O*-sulfamate group and with the sulfamate linked to a saturated ring displayed only minimal inhibitory activity towards the enzyme, with the nonsubstituted sulfamate **11** giving an IC_{50} of 5 μM, while the substituted derivatives caused, at best, <10% inhibition at a concentration of 10 μM (Table 1). Previous studies have shown that a nonsubstituted aryl 3-*O*-sulfamate group that mimics the sulfate group of the enzyme's natural substrate is a requisite for potent irreversible inhibition.[1,5,28] The 3,17-*O*,*O*-bis-sulfamate derivatives **16**−**20** proved more active than the 17-*O*-monosulfamates, with E2bisMATE (**20**) proving to be a potent inhibitor of STS (IC_{50} = 18 nM). As can be seen from the activity of the 3,17-*O*,*O*-(*N*,*N*-dimethyl)bis-sulfamate (**16**), 3,17-*O*,*O*-(*N*,*N*-dipentyl)bis-sulfamate (**18**), and 3,17-*O*,*O*-(*N*-acetyl)bis-sulfamate (**19**) derivatives substitution on the 17-*O*-sulfamate group is relatively well tolerated, although the larger benzyl-substituted compound **17** proved to be significantly less active. It was thus apparent that in this series large substituents, though not substitution per se, on the C-17 sulfamate are poorly tolerated in the active site of this enzyme and, furthermore, that the 3-*O*-sulfamate group is required for potent irreversible inhibition of STS.[5] In earlier studies we have shown that introduction of a substituent at the 2-position can modulate the STS inhibitory

Table 2. Antiproliferative Activities of Estradiol Derivatives Against MCF-7 Human Breast Cancer Cells

compd no.	2-substituent	3-substituent	17-substituent	GI_{50}(MCF-7) (μM)
20	H	OSO_2NH_2	OSO_2NH_2	>10
2[a]	MeO	OH	OH	2.35
3[a]	MeO	OSO_2NH_2	OH	0.36
32	MeO	OH	OSO_2NH_2	>10
21	MeO	OSO_2NH_2	OSO_2NH_2	0.25
34	MeO	OH	OSO_2NMe_2	>10
36	MeO	OSO_2NH_2	OSO_2NMe_2	>10
25	MeO	OSO_2NMe_2	OSO_2NMe_2	>100
22	EtO	OSO_2NH_2	OSO_2NH_2	10
4a[a]	Et	OH	OH	10.5
4[a]	Et	OSO_2NH_2	OH	0.07
33	Et	OH	OSO_2NH_2	>10
23	Et	OSO_2NH_2	OSO_2NH_2	0.07
35	Et	OH	OSO_2NMe_2	>10
37	Et	OSO_2NH_2	OSO_2NMe_2	4.41
5a[a]	MeS	OH	OH	3.96
5[a]	MeS	OSO_2NH_2	OH	0.43
24	MeS	OSO_2NH_2	OSO_2NH_2	0.19

[a] Data for these compounds are taken from ref 20. GI_{50} figures are the mean values obtained from experiments performed in triplicate, SEM = ±7%.

activity of estrone 3-*O*-sulfamate derivatives, with high activity being conferred by electron-withdrawing groups.[29,30] The high STS inhibitory activity displayed by 2-MeOE2bisMATE (**21**) was thus not surprising, nor was the decrease in activity observed across the 2-substituted series **21** > **24** > **23** on the basis of the electronic properties of these substituents. It would thus appear that, in addition to their antiproliferative and antiangiogenic effects discussed below, estradiol 3,17-*O*,*O*-bis-sulfamates could have therapeutic potential for the treatment of hormone-dependent breast cancer (HDBC) by inhibition of STS and the resultant lowering of circulatory and intratumoral estrogen levels.[4]

As a first-stage predictor of anticancer activity the compounds were evaluated for their ability to inhibit the proliferation of MCF-7 cells, an ER^+ human breast cancer cell line. Previous experience had shown that results from this assay were predictive of the antiproliferative effects against ER^+ and ER^- cancer cells already obtained with 2-MeOE2MATE. The results of this assay are presented in Table 2; data for 2-substituted estradiols and selected 3-*O*-sulfamate derivatives are included for comparison.[21] As can be seen estradiol 3,17-*O*,*O*-bis-sulfamate (**20**) showed only modest activity as an antiproliferative agent, with a concentration of 10 μM causing a 32% inhibition of MCF-7 proliferation. The 3-*O*-sulfamate derivatives **3**, **4**, and **5** are, respectively, 8-, 150- and 9-fold more active than the corresponding estradiol derivatives **2**, **4a**, and **5a**.[21] In contrast, introduction of a single sulfamate group at the 17-position proved deleterious to activity, with 2-methoxyestradiol 17-*O*-sulfamate (**32**, 2-MeOE2-17MATE) proving to be over 4 times less active than 2-MeOE2. 2-Ethylestradiol 17-*O*-sulfamate (**33**) also proved to be inactive. The *N*-alkylated 17-monosulfamates 2-methoxyestradiol 17-*N*,*N*-dimethylsulfamate (**34**) and 2-ethylestradiol 17-*N*,*N*-dimethylsulfamate (**35**) also proved to be devoid of significant antiproliferative activity. It could thus be concluded that although 17-*O*-sulfamoylation of 2-methoxyestradiol may serve to block inactivating conjugation and metabolism at this position, its deleterious effects on antiproliferative activity are profound. It is also apparent, albeit not surprisingly, that estradiol 17-*O*-sulfamates do not act as prodrugs of the parent estradiols under the conditions of the assay.

The bis-sulfamoylated derivatives **21**, **23**, and **24**, in contrast, displayed equal or greater activity in this assay than the corresponding 3-*O*-sulfamates. 2-MeOE2bisMATE (**21**) (GI_{50} = 0.25 μM) was found to be nearly 10-fold more active in this assay than 2-MeOE2 (**2**) and also slightly more active than 2-MeOE2MATE (**3**) (GI_{50} = 0.36 μM). Similarly, 2-MeSE2bisMATE (**24**) (GI_{50} = 0.19 μM) proved to be 20-fold more active than 2-MeSE2 and 2-fold more active than the corresponding monosulfamate **5**. As in the 3-*O*-sulfamate series, the most potent compound in vitro proved to be the 2-ethyl derivative, with 2-EtE2bisMATE (**23**) causing 50% inhibition of cell proliferation at 70 nM; in the in vitro context the mono- and bis-sulfamate derivatives of 2-EtE2 proved to be equipotent. In contrast, 2-EtOE2bisMATE (**22**) (GI_{50} = 10 μM) proved to be less active than the corresponding monosulfamate (GI_{50} = 0.61 μM[21]), a result which illustrates the steric limitations on the size of the 2-substituent which we had previously observed in the monosulfamate series.[21]

The effect of C-17 sulfamate substitution on the activity of the bis-sulfamates was evident from the activities of 2-methoxy- and 2-ethylestradiol-3-*O*-sulfamoyl-17-*O*-(*N*,*N*-dimethyl)sulfamates (**36** and **37**) whose respective activities were >40- and 63-fold less than those of the corresponding unsubstituted bisMATEs **21** and **23**. Unsurprisingly, the tetramethylsulfamate **25** also proved devoid of antiproliferative activity, as we had previously observed that alkylation of the 3-*O*-sulfamate group was detrimental to in vitro antiproliferative activity in the monosulfamate series,[21] although we would anticipate activity of such a compound in vivo. It thus appears that incorporation of a nonsubstituted sulfamate at C-17 is well tolerated at the site of action of the 2-substituted estradiol 3-*O*-sulfamates, though alkylation of this group is not tolerated. Whether the detrimental effect of alkylation is attributable to the removal of H-bond donor capabilities of the group or simply due to the increased steric size is not clear, although precedent from 17-oximo derivatives of 2-ethylestrone 3-*O*-sulfamates suggests that steric considerations are likely the most important factor.[25]

A selection of these compounds was made for screening in the NCI human cancer cell line panel, thus allowing an evaluation of activity across various cancer phenotypes.[32] Data obtained in eight individual cell lines from this assessment are presented in Table 3. The cell line data presented are on the grounds of the availability of comparative data for 2-MeOE2 which were obtained from the literature.[12] The MGM data represent the mean concentration required to cause 50% growth inhibition in all the cell lines successfully evaluated and thus indicate the relative activity of compounds across the panel. MGM (μM) values are restricted by definition by the range of concentrations at which the assays are performed, in this case 10^{-4} to 10^{-8} M; thus, a numerical value of 0.01 is maximal and would be obtained for a compound which causes 50% growth inhibition at concentrations of 10 nM or less in all cell lines.

As can be seen, the relative activities of these compounds across the panel correlate well with those obtained in the preliminary MCF-7 assay discussed above as illustrated in the 2-methoxy-substituted series of compounds, wherein mean concentrations of 1.3 μM, 110 nM, and 87 nM of the estradiol **2**, 3-*O*-sulfamate **3**, and bis-sulfamate **21** compounds, respectively, were required to effect 50% growth inhibition across the panel. The activity of the 2-alkoxybisMATEs was seen to decrease with an increase in the steric size of the alkoxy group, with the 2-ethoxy compound **22** proving to be 34-fold less potent than the 2-methoxy compound **21**. As in the MCF-7 assay the 2-ethyl compounds proved to be the most potent, with an MGM value of 18 nM obtained for 2-EtE2bisMATE (**23**). Signifi-

Table 3. GI_{50} (μM) and MGM (μM) Values Obtained from the NCI Screening Panel[a]

	lung HOP-62	colon HCT-116	CNS SF-539	melanoma UACC-62	ovarian OVCAR-3	renal SN12-C	prostate DU-145	breast MDA-MB-435	MGM
2	0.7	0.47	0.32	0.36	0.21	0.95	1.8	0.08	1.3
3	0.21	0.066	0.044	0.039	0.026	0.32	0.19	0.02	0.11
32	15	3.82	4.81	4.2	2.5	11.8	18.3	0.646	6.4
21	0.051	0.045	0.036	<0.01	<0.01	0.126	0.083	<0.01	0.087
22	1.3	2.3	6.5	1.8	15.6	7.2	2.7	34.5	2.95
4	<0.01	<0.01	<0.01	<0.01	<0.01	0.04	<0.01	<0.01	0.016
23	<0.01	N/A	<0.01	<0.01	<0.01	<0.028	<0.01	<0.01	0.018
20	23.5	18.7	12.4	17.4	18.1	22.8	38.7	16.6	14.5
18	2.41	10.5	2.44	2.39	10.8	7.53	3.42	4.57	

[a] Data for **2**,[12] **3**, and **4**[21] were taken from the literature.

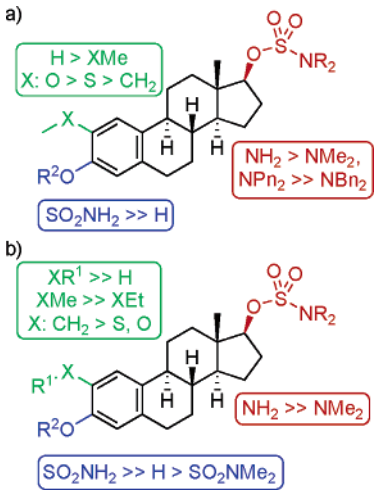


Figure 2. In vitro SAR of E2bisMATE derivatives as (a) STS inhibitors and (b) antiproliferative agents.

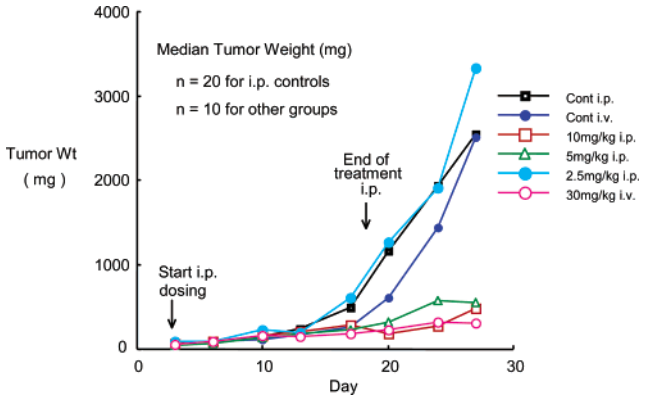


Figure 3. Activity of 2-MeOE2bisMATE (**21**) in the NCI Lewis lung model.

cantly, the compounds identified in the preliminary MCF-7 assay proved to have high activity against the whole range of cancer cell types present in the panel. In contrast to the 2-substituted bis-sulfamate derivatives the C-2 unsubstituted estradiol bis-sulfamates **18** and **20** displayed only modest activity. A COMPARE analysis[33] of data obtained for **23** against the publicly available NCI screening data afforded only 12 positive correlations, with the highest Pearson correlation coefficient being 0.67 (>0.6 is considered a positive correlation), which was obtained for the known microtubule disruptor Auristatin A though, significantly, no correlation was seen with the 2-substituted estradiol derivatives. Similar results were obtained for the COMPARE analysis of **21**.

A summary of the in vitro SAR of the E2bisMATE derivatives as STS inhibitors and antiproliferative agents is presented in Figure 2. STS activity is conferred by the 3-*O*-sulfamate group in conjunction with either no substituent at C-2, thus allowing unhindered interaction/reaction of the sulfamate group at the enzyme active site, or electronegative C-2 substituents, which can either participate in electrostatic interactions with the proximal residues of the active site or render the sulfamate group more reactive to nucleophilic attack. Substitution of the C-17 sulfamate group with alkyl groups is relatively well tolerated. In contrast, substitution at C-2 is seen to be essential for high antiproliferative activity in the bisMATE series, with the optimal substituent being the CH_3X group (X = O, S, CH_2). Here, lipophilic interactions appear to dominate as the observed in vitro activity is inversely related to the H-bond acceptor capability of the 2-substituent and, furthermore, a radical reduction in antiproliferative activity is seen with increasing steric size. Removal of the 3-*O*-sulfamate group results in a drastic reduction in antiproliferative activity as does methylation of the 17-*O*-sulfamate nitrogen.

Having obtained highly promising activity in the NCI panel, 2-MeOE2bisMATE was selected for further evaluation by the NCI. The NCI antiangiogenesis resource uses in vitro HUVEC growth inhibition, cord formation, and chemotaxis assays to select compounds with antiangiogenic activity.[34] 2-MeOE2bisMATE (**21**) gave IC_{50} values of 0.70, 0.13, and 0.40 μM in these respective assays, thus indicating the potential antiangiogenic activity of this molecule is in agreement with results reported elsewhere.[23]

On the basis of its in vitro activity, 2-MeOE2bisMATE was also selected for evaluation in the NCI hollow fiber assay, which allows an assessment of the ability of subcutaneous and intraperitoneally dosed candidate compounds to inhibit proliferation of a selection of human cancer cells in vivo. This assay entails intraperitoneal (ip) and subcutaneous (sc) implanting of hollow poly(vinylidene fluoride) containing a culture of one of twelve human tumor cell line fibers in mice.[35] Each tumor line is thus examined in the ip and sc environments. The mice are then treated ip at two dose levels (25 and 37.5 mg/kg, 25% and 37.5%, respectively, of the MTD) on a daily basis for 4 days, after a further day the fibers are collected for analysis, and the analyzed compound is awarded a score of 2 if a 50% reduction in viable cell mass compared to that of the vehicle control samples is observed. In this assay 2-MeOE2bisMATE obtained an ip score of 22 and an sc score of 6 (i.e., 50% growth inhibition was observed in eleven of the ip implanted fibers and three of the sc implanted fibers; the maximum possible score is 96), thus surpassing the threshold score of 20 assigned as a positive indication of in vivo efficacy.

2-MeOE2bisMATE (**21**) was then selected for evaluation in the early-stage Lewis lung carcinoma model, a mouse model of non-small cell lung cancer.[36] A plot of the data obtained is presented in Figure 3 and clearly shows the ability of 2-MeOE2bisMATE to inhibit tumor growth. In this study dosing was commenced 3 days after implantation and maintained for 15 days. The ip cohort (2.5, 5, and 10 mg/kg) was dosed daily

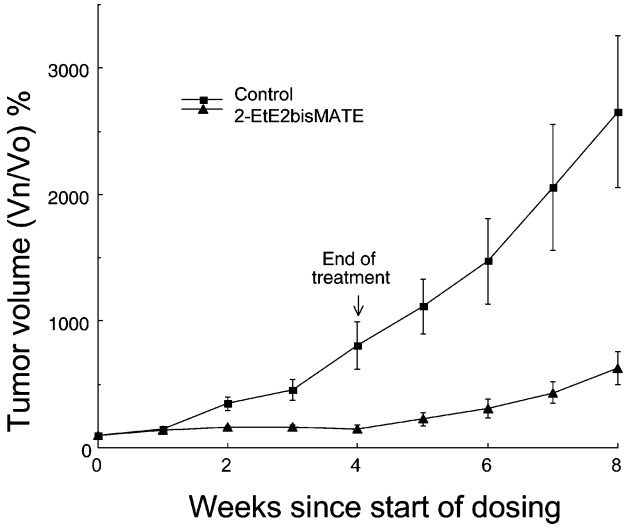


Figure 4. In vivo effect of 2-EtE2bisMATE (**23**) on the growth of MDA-MB-435 tumor xenografts in female nude mice. Vehicle (THF/propylene glycol) or compound was administered orally (20 mg/kg) once daily for 28 days. Tumor volumes were monitored at weekly intervals for the duration of drug administration and continued for a further 4 weeks postdosing (means ± SEM, $n = 12$) ($p < 0.01$ vs controls at the end of the study, Student's t test).

(vehicle 10% DMSO in saline), and the iv (30 mg/kg) cohort was dosed every 3 days (vehicle 12.5% EtOH, 12.5% cremophore, 75% saline). Inhibition of tumor growth was seen for all doses apart from the 2.5 mg/kg ip dose. Significantly, no weight loss was observed on treatment with **21**.

An assessment of the ability of 2-EtE2bisMATE (**23**) to inhibit tumor growth in vivo was performed using xenografts derived from MDA-MB-435 (ER^-) human breast cancer cells transplanted into female mice. As can be seen in Figure 4, oral administration of 2-EtE2bisMATE (20 mg/kg, qd, 28d) caused an 84% inhibition of tumor growth over the dosing period. No significant weight loss or other signs of toxicity were observed in the treated animals. Interestingly, significant tumor growth inhibition was still observed after cessation of drug treatment at 4 weeks. The in vivo activity of **23** in this model was essentially equivalent to that observed for 2-MeOE2bisMATE (**21**), which inhibited tumor growth by 86%.[37] The bis-sulfamates were thus significantly more active than the analogous 3-*O*-monosulfamates **3** and **4**, which, in the same model, at the same dose, caused 33% (2-MeOE2MATE, **3**) and 40% (2-EtE2MATE, **4**) inhibition of tumor growth over the treatment period.[21] 2-MeOE2 caused no inhibition of tumor growth in this model at this dose.[37] The enhanced activity of 2-EtE2bis-MATE (**23**) with respect to the corresponding monosulfamate **4** may well reflect the susceptibility of the monosulfamate compound to accelerated clearance and deactivation by conjugation of the C-17 hydroxyl group. These preliminary in vivo studies with nonoptimized dosing underline the potential of the 2-substituted estradiol bis-sulfamates for further development as therapeutic agents for the treatment of cancer.

An important mechanistic element of the antiproliferative effects of both the 2-substituted estradiol 3-*O*-sulfamates and their estradiol counterparts is their ability to disrupt normal microtubule dynamics.[22] Furthermore, the 2-substituted estradiol 3-*O*-sulfamates are believed, like 2-methoxyestradiol,[9] to bind to tubulin in a competitive manner with colchicine. It has previously been established that treatment of MDA-MB-231 cells with **21** caused a complete loss of microtubule structure, thus supporting the postulate that this compound, like the monosulfamate derivatives, functions as a microtubule disruptor.[38] We were thus

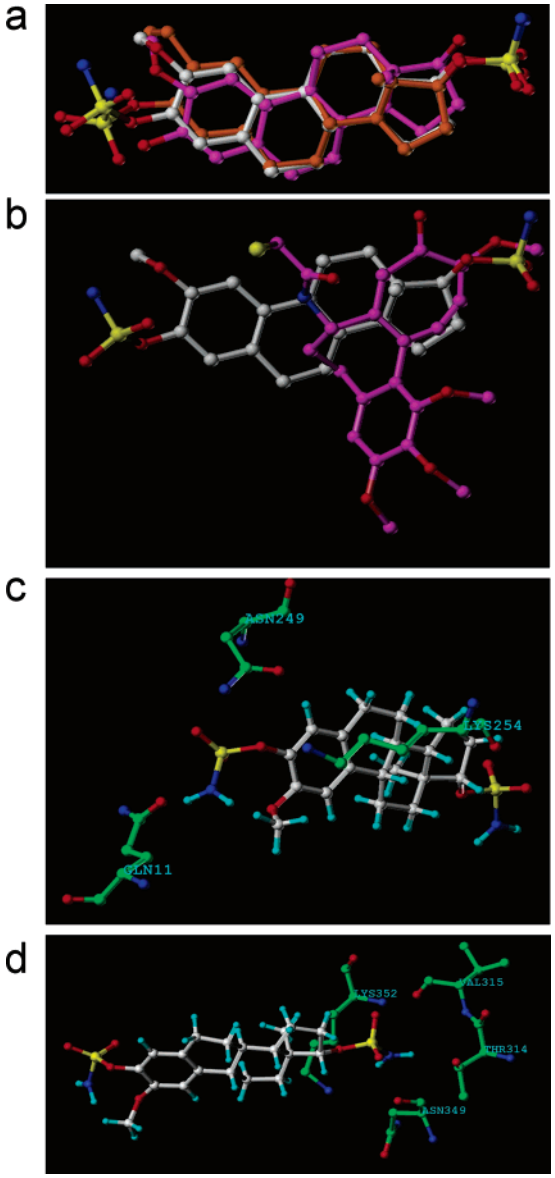


Figure 5. (a) Overlays of the highest scoring docks of 2-MeOE2 (**2**) (violet), 2-MeOE2bisMATE (**21**) (silver), and 2-EtE2bisMATE (**23**) (amber) into the colchicine binding site of tubulin. (b) Overlay of the highest scoring dock of **21** (silver) and the crystallographic ligand DAMA (violet). (c) Residues with the potential to H-bond to the 3-*O*-sulfamate group of **21**. (d) Residues with the potential to H-bond to the 17-*O*-sulfamate group of **21**.

drawn to study the computational docking of 2-MeOE2bisMATE (**21**) and 2-EtE2bisMATE (**23**), as well as 2-MeOE2 (**2**), to tubulin around the colchicine binding site, an approach facilitated by the publication of the crystal structure (Protein Data Bank (PDB) accession code 1SA0) of a colchicine derivative bound to a microtubule stabilized on a stathmin-like domain.[39]

Comparison of the best scoring docks (Figure 5a) of 2-methoxyestradiol (violet) (**2**) and the 2-methoxyestradiol (silver) (**21**) and 2-ethylestradiol (amber) (**23**) bis-sulfamate derivatives shows that all three share the same binding mode. Unsurprisingly, the sulfamate groups as well as the steroid skeleton of **21** and **23** occupy the same regions. The steroidal D-ring of **21** overlies the seven-membered aromatic ring of the colchicine-derived ligand *N*-deacetyl-*N*-(2-mercaptoacetyl)colchicine (DAMA), with the sulfamate **21** and hydroxyl group of DAMA apparently occupying the same region of space (Figure 5b). The possibility for H-bonding around the steroidal A-ring is shown for **21** in

Figure 5c; here the proximal Asn249 and Lys254 residues are available for H-bonding to the sulfamate NH_2 (and for the OH of 2-MeOE2 (**2**) (not shown)), while Gln11 could potentially form a H-bond donor/acceptor pair interaction with the sulfamate group. The residues proximal to the C-17 sulfamate of **21** are shown in Figure 5d; once again potential H-bond acceptor functions are available for interaction with the sulfamate NH_2 group (Asn349 and Val315), while Lys352 and Thr314 appear well placed to donate H-bonds to the oxygen atoms of the sulfamate group (Lys 352 appears the most likely to interact with the 17-hydroxyl group of 2-MeOE2 (**2**) (not shown)).

While the results of this docking study are not unequivocal and are speculative in the absence of experimental support for interaction of **21** and **23** with the colchicine binding site, the potential of the sulfamate group to offer further H-bonding interactions with respect to 2-MeOE2 around both the A- and D-rings is clear and is reflected by the higher docking scores obtained in this computational study. Perhaps more importantly, insights obtained from this docking could direct point mutation studies with the aim of generating resistant cell lines, especially when the in vitro results on analogous molecules indicate that the H-bond acceptor properties of the C-17 substituent are key to antiproliferative activity.[25]

The ability of estrogen 3-*O*-sulfamates to reversibly inhibit carbonic anhydrase (CA) is now well established and arises from the coordination of the monoanionic form of the sulfamate moiety to the zinc ion of the active site.[6] This interaction therefore offers the possibility of sequestration of the estrogen sulfamate into red blood cells and the consequent bypassing of first-pass liver metabolism.[7] Having ascertained that 2-MeO-E2bisMATE (**21**) reversibly inhibits hCAII (IC_{50} = 379 nM)[40] and also that this compound displays high oral bioavailability (85%) in rats, we were drawn to investigate its interaction with carbonic anhydrase II[37] in more detail.

We recently reported the X-ray crystal structures of two classes of sulfamate ester drugs bound to carbonic anhydrase II, STX64, a compound from clinical trial,[4,41] and two examples of a dual sulfatase aromatase inhibitor,[42] and were thus drawn to examine the interaction of 2-MeOE2bisMATE (**21**) and hCAII (Figure 6) using the same method. Crystals of the hCAII−**21** adduct obtained by cocrystallization were isomorphous with those of the native protein, allowing for the determination of the crystallographic structure by difference Fourier techniques. The model was refined using the CNS program to crystallographic *R*-factor and *R*-free values of 0.195 and 0.212, respectively. The statistics for data collection and refinement are presented in the Supporting Information. The overall quality of the model was high, with 100% of the non-glycine residues located in the allowed regions of the Ramachandran plot. Inspection of the electron density maps in the enzyme active site, at various stages of the crystallographic refinement, allowed location of two inhibitor molecules in the active cavity of the enzyme (Figure 6a,b), with one interacting with the zinc ion of the active site and a second lying close to the protein surface. Unexpectedly, the first ligand was not coordinated to Zn through its 3-*O*-arylsulfamate, as had been previously observed,[6,41,42] but through the 17-*O*-sulfamate.

Several polar and hydrophobic interactions stabilized the first inhibitor molecule within the hCAII active site (Figure 6c). Indeed, the ionized N atom of the 17-*O*-sulfamate moiety of **21** was coordinated to the catalytic Zn^{2+} ion with a tetrahedral geometry (N---Zn^{2+} = 1.93 Å), displacing the hydroxide ion usually present in the active site of the uninhibited enzyme.[43] In addition, this nitrogen atom also makes a hydrogen bond with the hydroxyl group of Thr199 (N---ThrOG = 2.75 Å), which in turn interacts with the Glu106OE1 atom (2.58 Å). On the other hand, one of the oxygen atoms of the coordinated sulfamate moiety is hydrogen bonded to the backbone amide of Thr199 (ThrN---O = 3.01 Å), whereas the other one is at a distance of 3.15 Å from the Zn^{2+} ion. The organic scaffold of the inhibitor is oriented toward the hydrophobic part of the active site cleft, establishing a large number of strong Van der Waals interactions (<4.5 Å) with residues Gln92, Val121, Phe131, Val135, Leu198, Thr200, and Pro202. Finally, the 3-*O*-sulfamate group interacts poorly with the enzyme and is very poorly defined in the electron density maps (Figure 6b).

The observation that this inhibitor molecule was bound to the zinc ion in the active site with the 17-*O*-sulfamate group, rather than the 3-*O*-sulfamate group (Figure 6c), as elsewhere as the monoanion, is rather surprising. In fact, the ionization of the alkyl sulfamate of C-17 is disfavored relative to ionization of the aryl sulfamate of the 3-position. This may arise because the 2-substituent can potentially render less favorable the interaction of the 3-*O*-sulfamate group with the zinc ion, causing a steric hindrance with proximal residues in the active site; indeed, we noticed in the earlier structures of the *o*-bromo derivative of a dual sulfatase aromatase inhibitor that the affinity of the brominated ligand was 5-fold lower than that of the non-brominated compound.[42]

Comparison of the CAII inhibitory activity of 2-MeO-E2bisMATE (**21**) (IC_{50} = 379 nM) with that of EMATE (IC_{50} = 42 nM,[40] which binds through its aryl sulfamate[6]), 2-MeO-EMATE (IC_{50} = 376 nM[40]), and 2-MeOE2-17MATE (**32**) (IC_{50} = 526 nM[40]) shows that the inhibitory activity of 2-MeO-E2bisMATE (**21**) is greater than that of 2-MeOE2-17MATE (**32**), where coordination can occur solely through the cyclic alkyl sulfamate at C-17 as in that observed in the corresponding structure of a loosely related sugar sulfamate.[44] Furthermore, since 2-MeOEMATE is significantly less active than EMATE as a CAII inhibitor, methoxy substitution at C-2 hinders the interaction of aryl sulfamates with CAII. Finally, since 2-MeO-EMATE acts as an inhibitor of CAII through its 3-*O*-sulfamate group, it seems likely that 2-MeOE2bisMATE binds to CAII through both its 3-*O*- and 17-*O*-sulfamate groups and that these interactions are likely to underlie the high oral bioavailability of **21** that is atypical of estradiol derivatives and contrasts strongly with that of 2-MeOE2.

The second binding site located for the ligand close to the usual binding pocket does not include sulfamate coordination and was not seen in the earlier study on the related ligand EMATE.[6] Presumably, while unique, this represents a much lower affinity interaction with the enzyme, as confirmed by the rather low occupation factor (0.61), and is most likely a function of the high ligand concentration used in the crystallization.

Thus, in the present study we have synthesized a series of novel estradiol bis-sulfamate derivatives substituted at C-2 and on the nitrogen of both the 3-*O*- and 17-*O*-sulfamate groups. Introduction of a second sulfamate group on the 17-hydroxyl group of the potent irreversible STS inhibitor E2MATE afforded E2bisMATE, a molecule with slightly reduced STS inhibitory activity relative to that of the parent compound. Substitution of the C-17 sulfamate group nitrogen of E2bisMATE with large alkyl or benzyl groups proved deleterious to the STS inhibitory activity. The STS inhibition effected by the novel C-2-substituted E2bisMATEs varied with the electronic properties of the substituent, though the IC_{50} values remained in the nanomolar range. It could thus be concluded that the STS inhibitory properties of bis-sulfamoylated estradiol derivatives

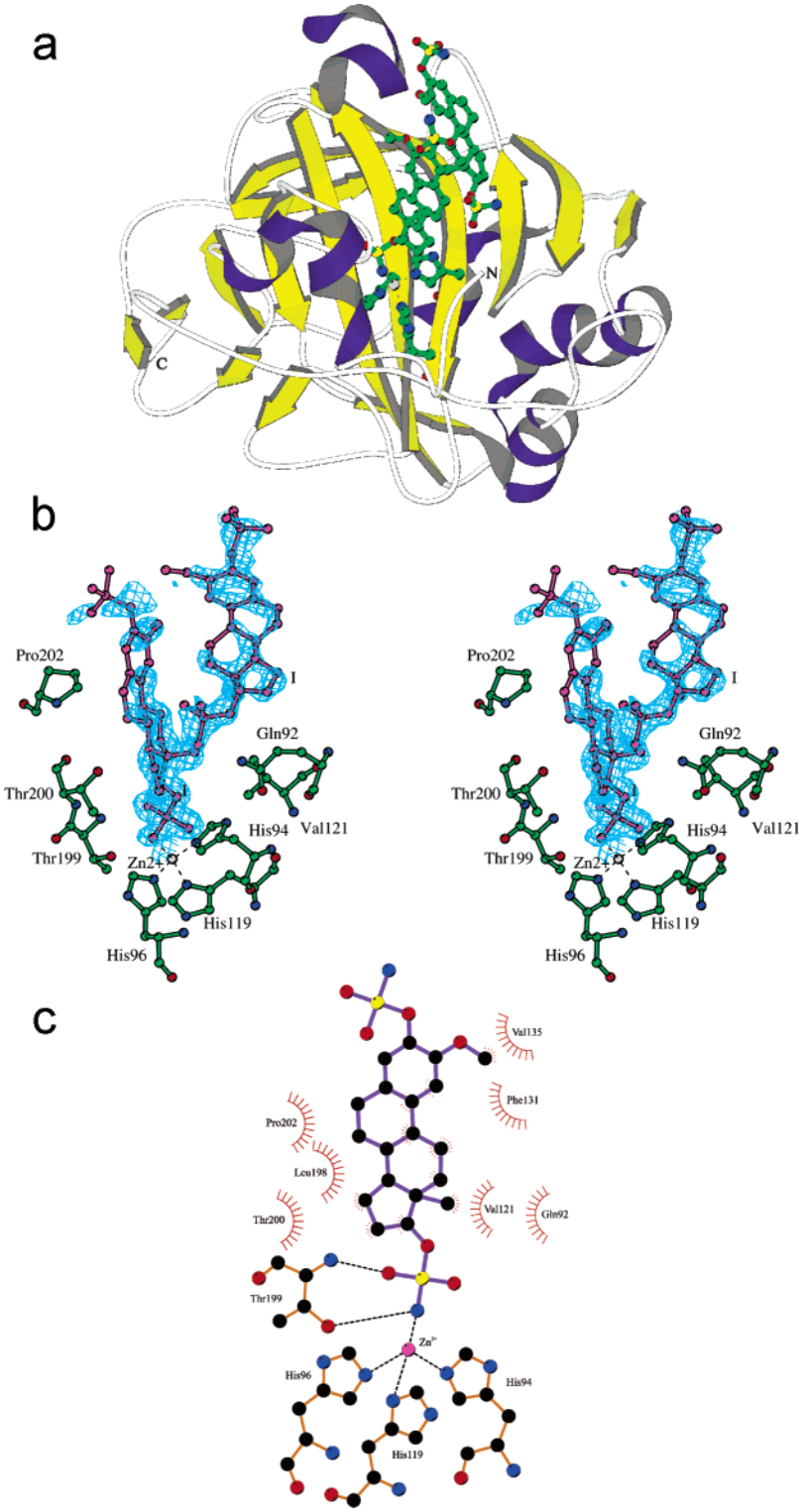


Figure 6. (a) Ribbon diagram of the hCAII−**21** complex. The two inhibitor molecules, metal-coordinating residues His94, His96, and His119, and the zinc ion are represented in ball and stick format. (b) Stereoview of the active site region in the hCAII−**21** complex. The simulated annealing omit $|2F_o - F_c|$ electron density map, relative to the two inhibitor molecules, is shown. Residues coordinating the metal ion and participating in recognition of the inhibitor molecules are also reported. (c) Detailed schematic representation of **21** within the hCAII active site.

highlight their potential for application against hormone-dependent breast cancer.

In addition to these findings a number of 2-substituted estrogen 3,17-*O*,*O*-bis-sulfamates exhibited high antiproliferative activity in vitro against a wide range of hormone-independent tumor cell lines. Notably, these compounds were typically over 10-fold more potent than the parent estradiols as evidenced by the respective MGM values of 87 and 18 nM obtained from the NCI panel for 2-MeOE2bisMATE (**21**) and 2-EtE2bisMATE (**23**) (cf. 2-MeOE2, 1.3 μM). In addition to antiproliferative activity 2-MeOE2bisMATE also exhibited significant inhibitory activity in in vitro assays of angiogenesis. The antiproliferative activity of these molecules, believed to principally derive from their ability to disrupt normal microtubule dynamics, was also evidenced in the in vivo context, with significant growth inhibition observed with ip dosing of 2-MeOE2bisMATE in

the NCI hollow fiber assay and the early-stage Lewis lung model. Furthermore, oral administration of 2-MeOE2bisMATE and 2-EtE2bisMATE caused tumor regression and prolonged growth inhibition upon cessation of dosing in an MDA-MB-435 xenograft model. Computational docking studies suggest that the 2-substituted estradiol bis-sulfamates share a mode of binding to tubulin which is common with 2-MeOE2 but can exploit further H-bonding interactions around the steroidal A- and D-rings. To further investigate the origins of the high oral bioavailability displayed by this class of molecule,[37] a crystal structure of 2-MeOE2bisMATE with CAII was obtained, and it was confirmed for the properly bound ligand of the two located in the structure that one of the sulfamate groups interacts with the zinc atom in the active site of CAII, although the orientation was unexpected and against established precedent for aryl sulfamate derivatives.

In conclusion, the studies detailed herein highlight compounds such as 2-MeOE2bisMATE (**21**) and 2-EtE2bisMATE (**23**) as potent inhibitors of in vivo tumor growth that may target the tumor cells directly by microtubule disruption. Translation of the antiangiogenic activity observed in in vitro screens to the in vivo context could extend the therapeutic potential of these molecules, as could their ability to inhibit steroid sulfatase. The compounds are straightforward to synthesize, display high oral bioavailability, and are resistant to deactivating metabolism and conjugation. Studies are presently under way to progress these agents toward clinic trial.

Experimental Section

Chemistry. All chemicals were purchased from Aldrich Chemical Co. (Gillingham, U.K.), Fluka (Gillingham, U.K.), or Lancaster Synthesis (Morecambe, U.K.). Organic solvents of AR grade were supplied by Fisher Scientific (Loughborough, U.K.) and used as supplied. Anhydrous *N*,*N*-dimethylformamide (DMF) and *N*,*N*-dimethylacetamide (DMA) were purchased from Aldrich and stored under a positive pressure of N_2 after use. THF was distilled from sodium with benzophenone indicator. Sulfamoyl chloride was prepared by an adaptation of the method of Appel and Berger[45] and was stored in the refrigerator under a positive pressure of N_2 as a solution in toluene as described by Woo et al.[46] An appropriate volume of this solution was freshly concentrated in vacuo immediately before use. E1 was purchased from Sequoia Research Products (Oxford, U.K.). Reactions were carried out at room temperature (rt) unless otherwise stated. Flash column chromatography was performed on silica gel (MatrexC60).

^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian Mercury VX400 NMR spectrometer at 400 and 100 MHz, respectively, and chemical shifts are reported in parts per million (ppm, δ) relative to the peak for tetramethylsilane (TMS) as an internal standard. Mass spectra were recorded at the Mass Spectrometry Service Center, University of Bath, U.K., or at the EPSRC National Mass Spectrometry service, Swansea, U.K. FAB-MS were carried out using *m*-nitrobenzyl alcohol (NBA) as the matrix. Elemental analyses were performed by the Microanalysis Service, University of Bath. Melting points were determined using a Reichert-Jung Thermo Galen Kofler block and are uncorrected.

Molecular Modeling. The ligands for docking were built using the Sybyl7.1[47] molecular modeling environment from Tripos Inc., assigned with Gasteiger−Huckel charges, and then minimized using the MMFF94s force field as implemented within Sybyl7.1. The crystal structure 1SA0[39] was cleaned also using Sybyl7.1 by first removing the crystallographic ligand and cofactors, then adding the requisite hydrogen atoms to the protein, and finally carrying out a backbone-constrained minimization of all hydrogen atoms and side chain atoms to allow for internal hydrogen bond formation and removal of any internal clashes. The AMBER FF99 force field with Gasteiger−Huckel charges was used for the constrained minimization as implemented within Sybyl7.1. The ligands were then docked into this cleaned form of the 1SA0 crystal structure using the GOLD version 2.2 docking package from the Cambridge Crystallographic Data Centre (CCDC).[48−52] The site for docking was defined as an 11 Å radius centered on atom number 5208 (the C^{d2} atom of Leu 255) of the crystal structure which fully encapsulates the colchicine binding site. Each ligand was docked into the protein a total of 25 times and the docking fit scored with the GoldScore scoring function; early termination based on the rmsd of the docked poses was disabled. Validation was carried out by docking the crystallographic ligand DAMA into the protein, with the highest scoring dock proving to be consistent with that of the crystallographic ligand (rmsd between the crystallographic and docked colchicine 0.75 Å).

Protein Crystallography. The hCAII−**21** complex was obtained by adding a 5 M excess of the inhibitor to a 10 mg/mL protein solution in 100 mM Tris−HCl, pH 8.5. Crystals of the complex were obtained at 22 °C using the hanging drop vapor diffusion method. Drops of 2 μL, prepared by mixing 1 μL of complex solution with an equal volume of precipitant solution (2.6 M $(NH_4)_2SO_4$, 0.3 M NaCl in 100 mM Tris−HCl (pH 8.4), and 5 mM 4-(hydroxymercury)benzoate), were suspended over wells containing 1.0 mL of precipitant. In about 2 days crystals of about 0.2 mm × 0.2 mm × 0.3 mm were grown. A complete dataset was collected at 1.46 Å resolution from a single crystal, at 100 K, at the synchrotron source Elettra in Trieste, Italy, using a Mar CCD detector. Prior to cryogenic freezing, the crystals were transferred to the precipitant solution with the addition of 15% (v/v) glycerol. Diffracted intensities were processed using the HKL crystallographic data reduction package (Denzo/Scalepack).[53] A total of 142133 reflections were measured and reduced to 40728 unique reflections. Crystal parameters and data processing statistics are summarized in tabular form in the Supporting Information.

The structure of the native hCAII (PDB code 1CA2)[43] was used as a starting model for rigid body refinement in CNS.[54] Water molecules were removed from the starting model prior to structure factor and phase calculations. Fourier maps calculated with $3F_o - 2F_c$ and $F_o - F_c$ coefficients showed prominent electron density features in the active site region. After an initial refinement, limited to the enzyme structure, a model for two inhibitor molecules was easily built up and introduced into the atomic coordinate set for further refinement. Many cycles of manual rebuilding using the program O[55] and positional and temperature refinement using the program CNS[54] were necessary to reduce the crystallographic *R*-factor and *R*-free values (in the 20.00−1.46 Å resolution range) to 0.195 and 0.212, respectively. The statistics for refinement are summarized in the Supporting Information.

Biology. The effects of compounds on cell growth were determined using a microtiter plate assay (Promega, Madison, WI). STS inhibition was assayed by the method of Purohit et al.[28] The ability of 2-EtE2bisMATE (**23**) to inhibit tumor growth in vivo was examined using xenografts derived from MDA-MB-435 (ER^-) human breast cancer cells transplanted into female mice with 12 mice per group. These studies were carried out by AntiCancer Inc. (San Diego, CA). Treatments were initiated when tumor volumes reached 100−200 mm.^{3} Drugs were dissolved in a minimum volume of tetrahydrofuran (THF), diluted with propylene glycol, and administered at 20 mg/kg orally, daily for 28 days. The length (l) and width (w) of the tumors were measured at weekly intervals, from which tumor volumes were calculated using the formula (lw^2/2). Monitoring of tumor volumes continued for a further 28 day period after the end of drug administration. Lewis lung studies were carried out by the developmental therapeutics program of the NCI.[56]

3-*O*-Benzylestradiol 17-*O*-sulfamate (6). An ice cold solution of 3-*O*-benzylestradiol[57] (1.20 g, 3.31 mmol) in DMF (35 mL) was treated with potassium *tert*-butoxide solution (4 mL, 4 mmol, 1 M in THF) and then, after 0.25 h, sulfamoyl chloride (24 mL, 16.3 mmol, 0.7 M in toluene) in a dropwise manner. The reaction was allowed to warm to rt over 2 h and then recooled to 0 °C before addition of saturated ammonium chloride (15 mL) and water (60

mL). The solution was extracted into ethyl acetate (3 × 100 mL), and the combined organic layers were washed with brine (3 × 100 mL), dried, and evaporated to give a yellow solid (1.48 g). Recrystallization (ethyl acetate/hexane) gave **6** as white crystals (1.29 g, 88% yield): mp 108−109 °C; ^{1}H NMR δ 7.26−7.45 (5H, m), 7.19 (1H, d, J = 8.6 Hz), 6.78 (1H, dd, J = 8.6 and 2.3 Hz), 6.71 (1H, d, J = 2.3 Hz), 5.03 (2H, s), 4.55−4.80 (2H, br), 4.51 (1H, dd, J = 8.2 and 8.2 Hz), 2.80−2.90 (2H, m), 1.15−2.40 (13H, m), 0.87 (3H, s); MS [FAB$^+$] m/z 441.2 (M$^+$, 41), 91.1 (100); HRMS [FAB$^+$] m/z found 441.1977, calcd 441.1974. Anal. ($C_{25}H_{31}NO_4S$) C, H, N.

3-*O*-Benzylestradiol 17-*O*-(*N*,*N*-dimethyl)sulfamate (7). A solution of **6** (300 mg, 0.68 mmol) in DMF (10 mL) was treated with sodium hydride (60 mg, 1.36 mmol) and then methyl iodide (169 μL, 2.72 mmol). The reaction was stirred for 16 h and then diluted with ethyl acetate (50 mL) and water (30 mL). The organic layer was then separated, washed with brine (3 × 50 mL), dried, and evaporated to give sulfamate **7** as a pale yellow solid (310 mg, 99%): mp 117−121 °C; ^{1}H NMR δ 7.25−7.45 (5H, m), 7.19 (1H, d, J = 8.4 Hz), 6.78 (1H, dd, J = 8.4 and 2.7 Hz), 6.72 (1H, d, J = 2.7 Hz), 5.03 (2H, s), 4.46 (1H, dd, J = 8.2 and 7.9 Hz), 2.87 (6H, s), 2.75−2.90 (2H, m), 1.15−2.40 (13H, m), 0.86 (3H, s); MS [FAB$^+$] m/z 469.2 (M$^+$, 50), 345.2 (46), 91.1 (100); HRMS [FAB$^+$] m/z found 469.2284, calcd 469.2287.

3-*O*-Benzylestradiol 17-*O*-(*N*,*N*-dipentyl)sulfamate (8). Sulfamate **6** (300 mg, 0.68 mmol) in DMF (10 mL) was reacted with pentyl bromide (0.45 mL, 3.62 mmol) and sodium hydride (73 mg, 1.81 mmol) as described for the synthesis of **7**. Column chromatography (hexane to 9:1 hexane/ethyl acetate) gave **8** as a white solid (490 mg, 92%): mp 55−56 °C; ^{1}H NMR δ 7.26−7.48 (5H, m), 7.19 (1H, d, J = 8.2 Hz), 6.78 (1H, dd, J = 8.2 and 2.7 Hz), 6.71 (1H, d, J = 2.7 Hz), 5.03 (2H, s), 4.42 (1H, dd, J = 8.5 and 8.4 Hz), 3.10−3.25 (4H, m) 2.75−2.90 (2H, m), 1.10−2.40 (25H, m), 0.91 (6H, t, J = 7.8 Hz), 0.83 (3H, s); MS [FAB$^+$] m/z 581.2 (M$^+$, 28); HRMS [FAB$^+$] m/z found 581.3522, calcd 581.3539.

3-*O*-Benzylestradiol 17-*O*-(*N*,*N*-dibenzyl)sulfamate (9). Sulfamate **6** (300 mg, 0.68 mmol) in DMF (10 mL) was reacted with benzyl bromide (0.33 mL, 2.72 mmol) and sodium hydride (60 mg, 1.36 mmol) as described for the synthesis of **7**. Column chromatography (5:1 hexane/ethyl acetate) gave **9** as a white solid (380 mg, 89%): mp 92−93 °C; ^{1}H NMR δ 7.28−7.45 (15H, m), 7.19 (1H, d, J = 8.6 Hz), 6.78 (1H, dd, J = 8.6 and 2.3 Hz), 6.71 (1H, d, J = 2.3 Hz), 5.03 (2H, s), 4.48 (1H, dd, J = 9.0 and 8.8 Hz), 4.40 (2H, d, J = 15.6 Hz), 4.26 (2H, d, J = 15.6 Hz), 2.78−2.93 (2H, m), 1.16−2.34 (13H, m), 0.78 (3H, s); MS [FAB$^+$] m/z 621.3 (M$^+$, 34), 245.2 (56), 91.1 (100); HRMS [FAB$^+$] m/z found 621.2906, calcd 621.2913.

3-*O*-Benzylestradiol 17-*O*-(*N*-acetyl)sulfamate (10). A solution of **6** (300 mg, 0.68 mmol) in dichloromethane (20 mL) was treated with sodium hydride (27 mg, 0.68 mmol) and acetyl chloride (53 μL, 0.75 mmol). The reaction mixture was stirred for 16 h before evaporation of solvent. Column chromatography (2:1 to 1:1 hexane/ethyl acetate) afforded recovered starting material (100 mg, 33%) and the desired sulfamate **10** (150 mg, 46%) as a white solid: mp 82−85 °C; ^{1}H NMR δ 8.70 (1H, br), 7.28−7.46 (5H, m), 7.18 (1H, d, J = 8.6 Hz), 6.78 (1H, dd, J = 8.6 and 2.9 Hz), 6.72 (1H, d, J = 2.9 Hz), 5.03 (2H, s), 4.68 (1H, dd, J = 8.4 and 8.4 Hz), 2.77−2.93 (2H, m), 2.23 (3H, s), 1.20−2.36 (13H, m), 0.89 (3H, s); MS [FAB$^-$] m/z 482.2 ((M$^+$ − H)$^-$, 100); HRMS [FAB$^+$] m/z found 483.2079, calcd 483.2079.

Estradiol 17-*O*-sulfamate (11). A solution of **6** (205 mg, 0.46 mmol) in THF (1 mL) and ethanol (5 mL) was treated with 10% Pd/C (30 mg) and then stirred under a H_2 atmosphere for 16 h. The catalyst was removed by filtration, and the solvent was evaporated to afford **11** as a white solid (160 mg, 98%). A sample of this material was crystallized from ethyl acetate/hexane: mp 168−169 °C; ^{1}H NMR (DMSO-d_6) δ 9.00 (1H, s), 7.39 (2H, s), 7.04 (1H, d, J = 8.4 Hz), 6.50 (1H, dd, J = 8.4 and 2.5 Hz), 6.44 (1H, d, J = 2.5 Hz), 4.33 (1H, dd, J = 8.4 and 8.2 Hz), 2.65−2.76 (2H, m), 1.15−2.32 (13H, m), 0.76 (3H, s); ^{13}C NMR δ 155.0, 137.1, 130.0, 126.1, 114.9, 112.7, 87.4, 48.6, 43.3, 42.6, 39.3, 35.9, 29.0, 27.5, 26.7, 25.7, 22.6, 11.6; MS [ES$^-$] m/z 350.5 ((M$^+$ − H)$^-$, 100). Anal. ($C_{18}H_{25}NO_4S\cdot0.5H_2O$) C, H, N.

17β-*O*-(*N*,*N*-Dimethylsulfamoyl)estradiol (12). A solution of **7** (310 mg, 0.66 mmol) in methanol (20 mL) and THF (5 mL) was treated with 10% Pd/C (150 mg) and then stirred under a H_2 atmosphere for 16 h. The catalyst was removed by filtration, and the solvent was evaporated to afford **12** as a white solid (205 mg, 82%): mp 170−173 °C; ^{1}H NMR δ 7.11 (1H, d, J = 8.6 Hz), 6.63 (1H, dd, J = 8.6 and 2.7 Hz), 6.56 (1H, d, J = 2.7 Hz), 4.63 (1H, dd, J = 9.4 and 7.8 Hz), 3.48 (2H, br), 2.87 (6H, m), 2.74−2.90 (2H, m), 1.18−2.36 (14H, m), 0.86 (3H, s); MS [FAB$^+$] m/z 379.2 (M$^+$, 16); HRMS [FAB$^+$] m/z found 379.1818, calcd 379.1817.

17β-*O*-(*N*,*N*-Dipentylsulfamoyl)estradiol (13). A solution of **8** (460 mg, 0.79 mmol) in methanol (20 mL) and THF (7 mL) was treated with 10% Pd/C (150 mg) and then stirred under a H_2 atmosphere for 2.25 h at rt. The catalyst was removed by filtration, and the solvent was evaporated to afford **13** as a white solid (310 mg, 80%): mp 93−96 °C; ^{1}H NMR δ 7.14 (1H, d, J = 8.2 Hz), 6.62 (1H, dd, J = 8.2 and 2.7 Hz), 6.56 (1H, d, J = 2.7 Hz), 4.58 (1H, br), 4.42 (1H, dd, J = 8.5 and 8.3 Hz), 3.12−3.24 (4H, m), 2.76−2.86 (2H, m), 1.18−2.34 (15H, m), 0.91 (6H, t, J = 7.0 Hz), 0.83 (3H, s); MS [FAB$^+$] m/z 491.3 (M$^+$, 16), 255.2 (100); HRMS [FAB$^+$] m/z found 491.3074, calcd 491.3069.

17β-*O*-(*N*,*N*-Dibenzylsulfamoyl)estradiol (14). A solution of **9** (200 mg, 0.38 mmol) in methanol (20 mL) was treated with 10% Pd/C (100 mg) and then stirred under a H_2 atmosphere for 7 h at rt. The catalyst was removed by filtration, and the solvent was evaporated to afford **14** as a white solid (230 mg, 84%): mp 60−63 °C; ^{1}H NMR δ 7.28−7.39 (10H, m), 7.14 (1H, d, J = 8.2 Hz), 6.63 (1H, dd, J = 8.2 and 2.7 Hz), 6.56 (1H, d, J = 2.7 Hz), 4.49 (1H, dd, J = 8.5 and 8.3 Hz), 4.32 (4H, s), 2.76−2.84 (2H, m), 1.27−2.34 (14H, m), 0.78 (3H, s); MS [FAB$^-$] m/z 530.2 ((M$^+$ − H)$^-$, 56%), 276.1 (100); HRMS [FAB$^+$] m/z found 531.2423, calcd 531.2443.

Estradiol-17β-*O*-(*N*-acetyl)sulfamate (15). A solution of **10** (110 mg, 0.23 mmol) in methanol (10 mL) was treated with 10% Pd/C (50 mg) and then stirred under a H_2 atmosphere for 5 h at rt. The catalyst was removed by filtration, and the solvent was evaporated to afford **15** as a white solid (90 mg, 87%): mp 130−134 °C dec; ^{1}H NMR δ 7.11 (1H, d, J = 8.6 Hz), 6.63 (1H, dd, J = 8.6 and 2.7 Hz), 6.56 (1H, d, J = 2.7 Hz), 4.63 (1H, dd, J = 9.4 and 7.8 Hz), 3.48 (2H, br), 2.70−2.90 (2H, m), 2.13 (3H, s), 1.18−2.34 (13H, m), 0.88 (3H, s); MS [FAB$^+$] m/z 393.2 (M$^+$, 86); HRMS [FAB$^+$] m/z found 393.1613, calcd 393.1610.

3-*O*-Sulfamoylestradiol 17β-*O*-(*N*,*N*-dimethyl)sulfamate (16). A solution of **12** (180 mg, 0.47 mmol) in dichloromethane (5 mL) was treated with DBMP (292 mg, 1.42 mmol) and then sulfamoyl chloride (3.49 mL, 2.37 mmol). The reaction mixture was stirred for 19 h and then diluted with ethyl acetate (50 mL) and water (40 mL). The separated organic layer was then washed with brine (3 × 40 mL), dried, and evaporated. Column chromatography (3:1 hexane/ethyl acetate) gave **16** as a white solid (195 mg, 90%): mp 140−142 °C; ^{1}H NMR δ 7.31 (1H, d, J = 8.6 Hz), 7.08 (1H, dd, J = 8.6 and 2.7 Hz), 7.04 (1H, d, J = 2.7 Hz), 4.89 (2H, br), 4.69 (1H, dd, J = 8.6 and 8.6 Hz), 2.88 (6H, s), 2.80−2.92 (2H, m), 1.20−2.38 (13H, m), 0.86 (3H, s); MS [FAB$^+$] m/z 458.2 (M$^+$, 18), 334.2 (100); HRMS [FAB$^+$] m/z found 458.1537, calcd 458.1545. Anal. ($C_{20}H_{30}N_2O_6S_2$) C, H, N.

3-*O*-Sulfamoylestradiol 17β-*O*-(*N*,*N*-dipentyl)sulfamate (17). Phenol **13** (260 mg, 0.54 mmol) in dichloromethane (15 mL) was reacted with DBMP (330 mg, 1.61 mmol) and sulfamoyl chloride (3.95 mL, 2.69 mmol) as described for the synthesis of **16**. Column chromatography (5:1 hexane/ethyl acetate) gave **17** as a white solid (270 mg, 89%): mp 105−107 °C; ^{1}H NMR δ 7.31 (1H, d, J = 8.6 Hz), 7.08 (1H, dd, J = 8.6 and 2.7 Hz), 7.04 (1H, d, J = 2.7 Hz), 4.89 (2H, br), 4.43 (1H, dd, J = 8.3 and 7.8 Hz), 3.12−3.26 (4H, m), 2.82−2.93 (2H, m), 1.18−2.38 (25H, m), 0.91 (6H, t, J = 7.0 Hz), 0.83 (3H, s); MS [FAB$^-$] m/z 569.2 ((M$^+$ − H)$^-$, 100); HRMS [FAB$^+$] m/z found 569.2739, calcd 569.2719. Anal. ($C_{28}H_{45}N_2O_6S_2$) C, H, N.

3-*O*-Sulfamoylestradiol 17*β*-*O*-(*N*,*N*-dibenzyl)sulfamate (18). Phenol **14** (200 mg, 0.38 mmol) in dichloromethane (10 mL) was reacted with DBMP (230 mg, 1.13 mmol) and sulfamoyl chloride (2.77 mL, 1.88 mmol) as described for the synthesis of **16**. Column chromatography (eluant 4:1 to 3:2 hexane/ethyl acetate) gave **18** as a white foam (194 mg, 84.5%): mp 58−62 °C; ^{1}H NMR δ 7.28−7.38 (11H, m), 7.08 (1H, dd, J = 8.4 and 2.3 Hz), 7.04 (1H, d, J = 2.3 Hz), 4.88 (2H, br), 4.49 (1H, dd, J = 8.4 and 8.3 Hz), 4.32 (4H, s), 2.85−2.92 (2H, m), 1.28−2.32 (13H, m), 0.78 (3H, s); MS [FAB$^+$] m/z 611.1 (M^+ + H, 7), 334.1 (100); HRMS [FAB$^-$] m/z found 609.2091, calcd 609.2093 ((M^+ − H)$^-$). Anal. ($C_{32}H_{38}N_2O_6S_2$) C, H, N.

3-*O*-Sulfamoylestradiol 17*β*-*O*-(*N*-acetyl)sulfamate (19). A solution of **15** (67 mg, 0.17 mmol) in DMF (5 mL) was treated with DBMP (105 mg, 0.51 mmol) and then sulfamoyl chloride (1.25 mL, 0.85 mmol) and was stirred for 7 h. The reaction mixture was then partitioned between ethyl acetate (50 mL) and water (40 mL) andthe organic layer separated, then washed with brine (3 × 40 mL), dried, and evaporated. Column chromatography (2:1 hexane/ethyl acetate) gave **19** as a pale orange solid (27 mg, 34%): mp 76−80 °C dec; ^{1}H NMR δ 8.12 (1H, br), 7.28 (1H, d, J = 8.6 Hz), 7.08 (1H, dd, J = 8.6 and 2.7 Hz), 7.04 (1H, d, J = 2.7 Hz), 4.95 (2H, br), 4.69 (1H, dd, J = 8.7 and 8.4 Hz), 2.80−2.86 (2H, m), 2.24 (3H, s), 1.20−2.38 (13H, m), 0.89 (3H, s); MS [FAB$^+$] m/z 472.2 (M^+, 20); HRMS [FAB$^-$] m/z found 471.1257 calcd 471.1260 ((M^+ − H)$^-$).

Estradiol 3,17-*O*,*O*-bis-sulfamate (20). Estradiol (270 mg, 1 mmol) was added to an ice cold solution of sulfamoyl chloride (3 mmol) in DMA (1.5 mL). The resulting mixture was stirred for 3 h at room temperature, then diluted with ethyl acetate (20 mL), then washed with water (3 × 20 mL) and brine (20 mL), dried, and evaporated to give a white foam. Column chromatography ($CHCl_3$/acetone 9:1) afforded the desired bis-sulfamate **20** as a colorless oil which was crystallized from ethyl acetate/hexane to give white crystals (300 mg, 70%): mp 200−201 °C; ^{1}H NMR (DMSO-d_6) δ 7.95 (2H, s), 7.40 (2H, s), 7.35 (1H, d, J = 8.7 Hz), 7.01 (1H, dd, J = 8.7 and 2.4 Hz), 6.96 (1H, d, J = 2.4 Hz), 4.34 (1H, dd, J = 8.9 and 7.9 Hz), 2.78−2.88 (2H, m), 1.20−2.40 (13H, m), 0.77 (3H, s); ^{13}C NMR δ 148.0, 138.1, 126.7, 121.9, 119.3, 87.4, 48.7, 43.5, 42.6, 37.8, 35.9, 30.7, 29.0, 27.5, 26.4, 25.5, 22.6, 11.6; MS [ES$^-$] m/z 429.5 ((M^+ − H)$^-$, 100). Anal. ($C_{18}H_{26}N_2O_6S$) C, H, N.

2-Methoxyestradiol 3,17-*O*,*O*-bis-sulfamate (21). 2-MeOE2 (**2**) (1.00 g, 3.31 mmol) was added to an ice cold solution of sulfamoyl chloride (13.2 mmol) in DMA (6.5 mL). The resulting mixture was stirred for 3 h at room temperature and then cooled to 0 °C, at which stage a dense white suspension formed. The reaction mixture was then transferred into a separating funnel, diluted with ethyl acetate (100 mL), then washed with water (2 × 100 mL) and brine (2 × 100 mL), dried, and evaporated to give a white foam. Column chromatography (8:1, then 4:1, and then 1:1 $CHCl_3$/acetone) afforded the desired bis-sulfamate **21**. Crystallization (ethyl acetate/hexane) gave white crystals (1.22 g, 79%): mp 180−182 °C; ^{1}H NMR (DMSO-d_6) δ 7.83 (2H, s), 7.42 (2H, s), 6.99 (1H, s), 6.99 (1H, s), 4.34 (1H, dd, J = 8.4 and 8.2 Hz), 3.77 (3H, s), 2.70−2.80 (2H, m), 1.15−2.45 (13H, m), 0.78 (3H, s); MS [ES$^-$] m/z 459 ((M^+ − H)$^-$, 100), 379 (25). Anal. ($C_{19}H_{28}N_2O_7S_2$) C, H, N.

2-Ethoxyestradiol 3,17-*O*,*O*-bis-sulfamate (22). 2-Ethoxyestradiol (180 mg, 0.57 mmol) was reacted with sulfamoyl chloride (2.5 mmol) in DMA (1.5 mL) as described for the synthesis of **21**. Column chromatography (chloroform/acetone gradient) gave the desired sulfamate **22** (230 mg, 85%) as a white powder: mp 180−181 °C; ^{1}H NMR δ 7.02 (1H, s), 6.90 (1H, s), 5.11 (2H, s), 4.84 (2H, s), 4.51 (1H, dd, J = 8.4 and 7.5 Hz), 4.07−4.15 (2H, m), 2.78−2.83 (2H, m), 1.20−2.34 (16H, m including 1.42 (3H, t, J = 3.5 Hz)), 0.87 (3H, s); MS [APCI$^-$] m/z 473.19 ((M^+ − H)$^-$, 100). Anal. ($C_{20}H_{30}N_2O_7S_2$) C, H, N.

2-Ethylestradiol 3,17-*O*,*O*-bis-sulfamate (23). 2-Ethylestradiol (240 mg, 0.80 mmol) was reacted with sulfamoyl chloride (2.4 mmol) in DMA (1.5 mL) as described for the synthesis of **21**. After workup the crude product was purified by column chromatography (9:1 chloroform/acetone) and gave the desired bis-sulfamate **23** as a white crystalline solid (334 mg, 91%): mp 190−192 °C; ^{1}H NMR (DMSO-d_6) δ 7.92 (2H, s), 7.38 (2H, s), 7.19 (1H, s), 6.98 (1H, s), 4.32 (1H, dd, J = 9.0 and 7.8 Hz), 2.75−2.81 (2H, m), 2.62 (2H, q, J = 7.4 Hz), 1.18−2.40 (H, m), 1.12 (3H, t, J = 7.4 Hz), 0.77 (3H, s); ^{13}C NMR δ 145.9, 137.7, 134.8, 133.3, 126.4, 121.4, 87.3, 48.6, 43.5, 42.6, 37.8, 35.9, 28.6, 27.5, 26.5, 25.5, 23.6, 22.5, 14.8, 11.5; MS [ES$^-$] m/z 457.4 ((M^+ − H)$^-$, 100). Anal. ($C_{20}H_{30}N_2O_6S_2$) C, H, N.

2-Methylsulfanylestradiol 3,17-*O*,*O*-bis-sulfamate (24). 2-Methylsulfanylestradiol (279 mg, 0.87 mmol) was reacted with sulfamoyl chloride (2.2 mmol) in DMA (1.5 mL) as described for the synthesis of **21**. Column chromatography (9:1 chloroform/acetone) gave the desired bis-sulfamate **24** (250 mg, 60%) as a foam. Crystallization (ethyl acetate/hexane) gave white crystals: mp 176−178 °C; ^{1}H NMR (DMSO-d_6) δ 8.01 (2H, s), 7.39 (2H, s), 7.17 (1H, s), 7.06 (1H, s), 4.32 (1H, dd, J = 8.2 and 8.2 Hz), 2.75−2.82 (2H, m), 1.20−2.45 (16H, m including 2.40 (3H, s)), 0.77 (3H,s); ^{13}C NMR δ 145.0, 138.2, 134.1, 128.2, 124.0, 121.1, 87.3, 48.5, 43.5, 42.6, 37.7, 35.9, 28.5, 27.5, 26.4, 25.5, 22.6, 14.8, 11.6; MS [ES$^-$] m/z 475.4 ((M^+ − H)$^-$, 100); HRMS [ES$^-$] m/z found 475.1041, calcd 475.1037 ((M − H)$^-$). Anal. ($C_{19}H_{28}N_2O_6S_3 \cdot H_2O$) C, H, N.

2-Methoxy-3,17-*O*,*O*-bis(*N*,*N*-dimethylsulfamoyl)estradiol (25). A solution of **21** (150 mg, 0.33 mmol) in DMF (10 mL) was treated with sodium hydride and then, after 10 min, methyl iodide (178 μL, 2.87 mmol). The reaction was stirred for 16 h, then diluted in ethyl acetate (25 mL), and quenched with water (25 mL). The organic layer was separated, then washed with water (2 × 25 mL) and brine (25 mL), dried, and evaporated. The crude product was crystallized from acetone/hexane to give **25** as white crystals (64 mg, 38%): mp 140−143 °C; ^{1}H NMR ($CDCl_3$, TMS = 0) δ 7.04 (1H, s), 6.86 (1H, s), 4.45 (1H, dd, J = 7.9 and 7.8 Hz), 3.86 (3H, s), 2.97 (6H, s), 2.87 (6H, s), 2.76−2.84 (2H, m), 1.23−2.35 (13H, m), 0.87 (3H, s); ^{13}C NMR δ 149.0, 139.0, 137.0, 129.2, 123.5, 110.1, 89.2, 56.2, 49.4, 44.3, 43.4, 38.7, 38.6, 38.1, 36.6, 28.6, 27.9, 27.0, 26.2, 23.1, 11.8; HRMS [FAB$^+$] m/z found 516.1964, calcd 516.1964. Anal. ($C_{23}H_{36}N_2O_7S_2$) C, H, N.

2-Methoxy-3-*O*-benzylestradiol (26). A solution of 2-MeOE2 (5.48 g, 18.2 mmol) in ethanol (100 mL) was refluxed with potassium carbonate (22.7 g, 7.5 mmol) and benzyl bromide (6.51 mL, 55 mmol) for 8 h. The reaction was then diluted with ethyl acetate (150 mL), washed with water (3 × 100 mL) and brine (50 mL), dried, and evaporated. Column chromatography (9:1 to 3:1 hexane/ethyl acetate) afforded **26** as a white foam (6.2 g, 86%) which was then precipitated from chloroform/hexane to give a white powder: mp 75−78 °C (lit.[58] mp 89−90 °C); ^{1}H NMR (DMSO-d_6) δ 8.32 (1H, s), 7.28−7.47 (5H, m), 6.84 (1H, s), 6.71 (1H, s), 5.01 (2H, s), 4.51 (1H, m), 3.73 (3H, s), 2.65−2.75 (2H, m), 1.10−2.38 (13H, m), 0.68 (3H, s, 18-CH_3); MS [APCI$^+$] m/z 392.5 (M^+, 100). Anal. ($C_{26}H_{32}O_3$) C, H, N.

2-Ethyl-3-*O*-benzylestradiol (27). A solution of 2-EtE2 (1.2 g, 4 mmol) in ethanol (30 mL) was refluxed with potassium carbonate (4.69 g, 34 mmol) and benzyl bromide (1.42 mL, 12 mmol) for 8 h as described for the synthesis of **26**. The crude oil was purified by column chromatography (3:1 hexane/ethyl acetate) to give **27** as a colorless glassy solid (1.2 g, 76%): mp 73−75 °C; ^{1}H NMR δ 7.29−7.44 (5H, m), 7.09 (1H, s), 6.61 (1H, s), 5.02 (2H, s), 3.68−3.76 (1H, m), 2.75−2.90 (2H, m), 2.66 (2H, q, J = 7.4 Hz), 1.14−2.39 (17H, m including 1.21 (3H, t, J = 7.4 Hz)), 0.77 (3H, s); ^{13}C NMR δ 154.3, 137.6, 134.8, 132.1, 130.1, 128.3, 127.5, 126.9, 126.1, 111.8, 81.9, 69.8, 50.1, 44.1, 433, 39.0, 36.8, 30.7, 29.8, 27.4, 26.5, 23.6, 23.2, 14.8, 11.2; MS [ES$^-$] m/z 389.6 ((M^+ − H)$^-$, 100). Anal. ($C_{27}H_{34}O_2$) C, H, N.

2-Methoxy-3-*O*-benzylestradiol 17-*O*-sulfamate (28). 26 (900 mg, 2.3 mmol) was added to a solution of sulfamoyl chloride (4.6 mmol) in DMA (3 mL) as described for the synthesis of **21**. Column chromatography (3:2 hexane/ethyl acetate) gave the desired sulfamate **28** (860 mg, 79%) as white crystals: mp 169−171 °C; ^{1}H NMR δ 7.26−7.46 (5H, m), 6.82 (1H, s), 6.62 (1H, s), 5.10 (2H,

s), 4.51 (1H, dd, J = 8.2 and 8.2 Hz), 3.86 (3H, s), 2.67−2.84 (2H, m), 1.19−2.40 (13H, m), 0.87 (3H, s); MS [FAB$^-$] m/z 470.3 ((M$^+$ − H)$^-$, 100); HRMS [FAB$^+$] m/z found 471.2084, calcd 471.2079. Anal. ($C_{26}H_{33}NO_4S$) C, H, N.

2-Ethyl-3-*O*-benzylestradiol 17-*O*-sulfamate (29). 27 (900 mg, 2.3 mmol) was added to a solution of sulfamoyl chloride (4.6 mmol) in DMA (6 mL) as described for the synthesis of **19**. Column chromatography (2:1 hexane/ethyl acetate) gave the desired sulfamate **29** (950 mg, 88%) as a white powder: mp 144−147 °C; ^{1}H NMR δ 7.28−7.45 (5H, m), 7.09 (1H, s), 6.63 (1H, s), 5.04 (2H, s), 4.67 (2H, s), 4.51 (1H, dd, J = 8.8 and 7.7 Hz), 2.80−2.88 (2H, m), 2.66 (2H, q, J = 7.4 Hz), 1.15−2.40 (16H, m including 1.21 (3H, t, J = 7.4 Hz)), 0.87 (3H, s); MS [ES$^-$] m/z 468.3 ((M$^+$ − H)$^-$, 100). Anal. ($C_{27}H_{35}NO_4S\cdot H_2O$) C, H, N.

2-Methoxy-3-*O*-benzylestradiol 17-*O*-(*N*,*N*-dimethyl)sulfamate (30). A solution of **28** (300 mg, 0.63 mmol) in DMF (10 mL) at rt was treated with sodium hydride (55 mg, 1.38 mmol) and then methyl iodide (133 μL, 2.14 mmol). After 16 h the reaction was diluted with ethyl acetate (50 mL) and water (30 mL). The organic layer was separated, washed with water (3 × 50 mL), then dried, and evaporated. The crude oil was then crystallized (ethyl acetate/hexane) to give the desired product **30** as a white solid (300 mg, 94%): mp 153−156 °C; ^{1}H NMR δ 7.25−7.46 (5H, m), 6.83 (1H, s), 6.62 (1H, s), 5.10 (2H, s), 4.46 (1H, dd, J = 8.2 and 8.2 Hz), 3.86 (3H, s), 2.87 (6H, s), 2.66−2.85 (2H, m), 1.19−2.38 (13H, m), 0.86 (3H, s); ^{13}C NMR δ 147.5, 146.4, 137.3, 132.4, 128.6, 128.4, 127.7, 127.2, 114.5, 109.6, 89.4, 71.0, 56.3, 49.2, 44.0, 43.4, 38.6, 38.6, 36.5, 29.0, 27.8, 27.1, 26.2, 23.0, 11.6; MS [FAB$^+$] m/z 499.3 (M$^+$, 100); HRMS [FAB$^+$] m/z found 499.2393, requires 499.2392. Anal. ($C_{28}H_{37}NO_5S$) C, H, N.

2-Ethyl-3-*O*-benzylestradiol 17-*O*-(*N*,*N*-dimethyl)sulfamate (31). A solution of sulfamate **29** (520 mg, 1.11 mmol) in DMF (10 mL) was reacted with sodium hydride (98 mg, 2.43 mmol) and methyl iodide (234 μL, 2.4 mmol) as described for the synthesis of **30**. Column chromatography (4:1 hexane/ethyl acetate) yielded a colorless oil which solidified on contact with ethyl acetate to give **31** (440 mg, 80%) as a white solid: mp 152−154 °C; ^{1}H NMR δ 7.26−7.44 (5H, m), 7.09 (1H, s), 6.62 (1H, s), 5.03 (2H, s), 4.45 (1H, dd, J = 9.2 and 7.9 Hz), 2.86 (6H, s), 2.78−2.86 (2H, m), 2.65 (2H, q, J = 7.4 Hz), 1.20−2.38 (13H, m), 1.20 (3H, t, J = 7.4 Hz), 0.85 (3H, s); ^{13}C NMR δ 154.5, 137.7, 134.8, 131.8, 130.3, 128.5, 127.6, 127.0, 126.2, 111.8, 89.5, 69.8, 49.2, 43.8, 43.5, 38.6, 38.6, 36.6, 29.6, 27.8, 27.1, 26.1, 23.5, 23.0, 14.7, 11.7; MS [APCI$^+$]-m/z 498.4 (M$^+$ + H, 7), 373.5 (100). Anal. ($C_{29}H_{39}NO_4S$) C, H, N.

2-Methoxyestradiol 17-*O*-sulfamate (32). A solution of **28** (103 mg, 0.22 mmol) in THF (1 mL) and ethanol (6 mL) was treated with Pd/C (18 mg, 10%) and hydrogen (1 atm) for 16 h as described for the synthesis of **11**. The resultant solid was crystallized (ethyl acetate/hexane) to give **30** as fine needles (65 mg, 77%): mp 180−182 °C; ^{1}H NMR δ 6.76 (1H, s), 6.63 (1H, s), 5.43 (1H, s) 4.70 (2H, s), 4.50 (1H, dd, J = 9.4 and 8.2 Hz), 3.85 (3H, s), 2.72−2.82 (2H, m), 1.16−2.34 (14H, m), 0.86 (3H, s); ^{13}C NMR δ 144.6, 143.5, 131.2, 129.3, 114.6, 108.0, 90.8, 56.0, 49.1, 44.0, 43.3, 38.4, 36.4, 28.8, 27.7, 27.1, 26.3, 23.0, 11.7; MS [ES$^-$] m/z 380.5 ((M$^+$ − H)$^-$, 100). Anal. ($C_{19}H_{27}NO_5S$) C, H, N.

2-Ethylestradiol 17-*O*-sulfamate (33). A solution of **29** (156 mg, 0.33 mmol) in THF (1 mL) and methanol (5 mL) was reacted with 10% Pd/C (30 mg) and hydrogen (1 atm) for 16 h as described for the synthesis of **11**. The resultant white powder was crystallized (ethyl acetate/hexane) to give **33** as white crystals (98 mg, 78%): mp 181−182 °C; ^{1}H NMR ($CDCl_3$/CD_3OD) δ 6.97 (1H s), 6.44 (1H, s), 4.41 (2H, dd, J = 8.8 and 8.0 Hz), 2.68−2.78 (2H, m), 2.54 (2H, q, J = 7.4 Hz), 1.16−2.32 (H, m), 1.15 (3H, t, J = 7.4 Hz), 0.80 (3H, s); ^{13}C NMR δ 151.8, 134.9, 131.2, 127.7, 126.1, 114.9, 90.2, 49.0, 43.7, 43.2, 38.5, 36.3, 29.1, 27.6, 27.1, 26.0, 23.0, 23.0, 14.4, 11.6; MS [APCI$^-$] m/z 378.4 ((M$^+$ − H)$^-$, 100). Anal. ($C_{20}H_{29}NO_4S$) C, H, N.

2-Methoxyestradiol 17-*O*-(*N*,*N*-dimethyl)sulfamate (34). A solution of **30** (364 mg, 0.73 mmol) in THF (6 mL) and methanol (20 mL) was reacted with 10% Pd/C (100 mg) and hydrogen (1 atm) for 16 h as described for the synthesis of **11**. Column chromatography (4:1 hexane/ethyl acetate) afforded **34** as a white solid (175 mg , 59%): mp 151−155 °C; ^{1}H NMR δ 6.76 (1H, s), 6.63 (1H, s), 5.43 (1H, s), 4.46 (1H, dd, J = 9.2 and 8.1 Hz), 3.85 (3H, s), 2.86 (6H, s), 2.74−2.82 (2H, m), 1.17−2.32 (13H, m), 0.85 (3H, s); ^{13}C NMR δ 144.6, 143.5, 131.4, 129.3, 114.6, 108.0, 89.5, 56.0, 49.2, 44.0, 43.4, 38.6, 38.6, 36.5, 28.9, 27.8, 27.1, 26.3, 23.0, 11.7; MS [FAB$^+$] m/z 409.3 (M$^+$, 100); HRMS [FAB$^+$] m/z found 409.1938, calcd 409.1923. Anal. ($C_{21}H_{31}NO_5S$) C, H, N.

2-Ethylestradiol 17-*O*-(*N*,*N*-dimethyl)sulfamate (35). A solution of **31** (340 mg, 0.68 mmol) in THF (10 mL) and methanol (20 mL) was reacted with 10% Pd/C (30 mg) and hydrogen (1 atm) for 16 h as described for the synthesis of **11**. **35** was obtained as a white solid (270 mg, 98%): mp 163−165 °C; ^{1}H NMR δ 7.04 (1H, s), 6.50 (1H, s), 4.65 (1H, s), 4.45 (1H, dd, J = 8.0 and 7.8 Hz), 2.87 (6H, s), 2.76−2.84 (2H, m), 2.60 (2H, q, J = 7.4 Hz), 1.25−2.38 (13H, m), 1.23 (3H, t, J = 7.4 Hz) and 0.86 (3H, s); MS [ES$^-$] m/z 406.2 ((M − H)$^-$, 20); HRMS [FAB$^+$] m/z found 407.2130, calcd 407.2130. Anal. ($C_{22}H_{33}NO_4S$) C, H, N.

2-Methoxy-3-*O*-sulfamoylestradiol 17β-*O*-(*N*,*N*-dimethyl)-sulfamate (36). A solution of **34** (133 mg, 0.325 mmol) in dichloromethane (10 mL) was added at rt to DBMP (200 mg, 0.975 mmol) and then sulfamoyl chloride (2.39 mL, 1.625 mmol) as described for the synthesis of **16**. Chromatography (eluent hexane/ethyl acetate, 3:2) afforded 40 mg (30%) of recovered starting material and the desired sulfamate **36** (105 mg, 66%) as a white solid: mp 181−186 °C; ^{1}H NMR δ 7.04 (1H, s), 6.92 (1H, s), 4.95 (2H, br), 4.46 (1H, dd, J = 9.4 and 7.8 Hz), 3.86 (3H, s), 2.88 (6H, s), 2.76−2.84 (2H, m), 1.20−2.34 (13H, m), 0.87 (3H, s); MS [FAB$^+$] m/z 488.2 (M$^+$, 100); HRMS [FAB$^+$] m/z found 488.1661, calcd 488.1651.

2-Ethyl-3-*O*-sulfamoylestradiol 17-*O*-(*N*,*N*-dimethyl)sulfamate (37). Sulfamoyl chloride (0.56 mmol) was dissolved in DMA (1 mL) at 0 °C before addition of **35** (50 mg, 0.12 mmol) as described for the synthesis of **19**. Column chromatography (5% acetone in chloroform) gave **37** as a clear colorless oil which was precipitated from acetone/hexane to give a white powder (31 mg, 52%): mp 178−180 °C; ^{1}H NMR δ 7.16 (1H, s), 7.06 (1H, s), 4.98 (2H, s), 4.46 (1H, dd, J = 8.6 and 8.2 Hz), 2.87 (6H, s), 2.80−2.86 (2H, m), 2.69 (2H, q, J = 7.4 Hz), 1.25−2.36 (13H, m), 1.21 (3H, t, J = 7.4 Hz) and 0.85 (3H, s); ^{13}C NMR δ 146.0, 138.9, 135.6, 133.6, 126.9, 121.3, 89.3, 49.4, 44.0, 43.4, 38.6, 38.2, 36.5, 29.2, 27.9, 26.0, 23.2, 23.1, 14.8, 11.8; HRMS [FAB$^+$] m/z found 486.1858, calcd 486.1858. Anal. ($C_{22}H_{34}N_2O_6S_2\cdot 0.5H_2O$) C, H, N.

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Supporting Information Available: Elemental analysis results and crystallographic data for the hCAII−**21** complex. This material is available free of charge via the Internet at http://pubs.acs.org.

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