Potent active site-directed inhibition of steroid sulphatase by tricyclic coumarin-based sulphamates

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Background: There is now abundant evidence that inhibition of steroid sulphatase alone or in conjunction with inhibition of aromatase may enhance the response of postmenopausal patients with hormone-dependent breast cancer to this type of endocrine therapy. Additionally, sulphatase inhibition has been proposed to be of potential therapeutic benefit in the immune system and for neuro-degenerative diseases. After the finding that our first highly potent active site-directed steroid sulphatase inhibitor, oestrone-3-*O*-sulphamate (EMATE), was highly oestrogenic, we proposed non-steroidal coumarin sulphamates such as 4-methylcoumarin-7-*O*-sulphamate (COUMATE) as alternative non-steroidal steroid sulphatase inhibitors. In this work, we describe how tricyclic coumarin-based sulphamates have been developed which are even more potent than COUMATE, are non-oestrogenic and orally active. We also discuss potential mechanisms of action.

Results: 4-Ethyl- (**4**), 4-(*n*-propyl)- (**6**), 3-ethyl-4-methyl- (**8**), 4-methyl-3-(*n*-propyl)coumarin-7-*O*-sulphamate (**11**); the tricyclic derivatives 665COUMATE (**13**), 666COUMATE (**15**), 667COUMATE (**17**), 668COUMATE (**20**) and the tricyclic oxepin sulphamate (**22**) were synthesised. In a placental microsome preparation, all of these analogues were found to be more active than COUMATE in the inhibition of oestrone sulphatase, with the most potent inhibitor being 667COUMATE which has an IC_{50} of 8 nM, some 3-fold lower than that for EMATE (25 nM). In addition, 667COUMATE was also found to inhibit DHEA-sulphatase some 25-fold more potently than EMATE in a placental microsome preparation. Like EMATE, 667COUMATE acts in a time- and concentration-dependent manner, suggesting that it is an active site-directed inhibitor. However, in contrast to EMATE, 667COUMATE has the important advantage of not being oestrogenic. In addition, we propose several diverse mechanisms of action for this active site-directed steroid sulphatase inhibitor in the light of recent publications on the crystal structures of human arylsulphatases A and B and the catalytic site topology for the hydrolysis of a sulphate ester.

Conclusions: A highly potent non-steroidal, non-oestrogenic and irreversible steroid sulphatase inhibitor has been developed. Several mechanisms of action for an active site-directed steroid sulphatase inhibitor are proposed. With 667COUMATE now in pre-clinical development for clinical trial, this should allow the biological and/or clinical significance of steroid sulphatase inhibitors in the treatment of postmenopausal women with hormone-dependent breast cancer and other therapeutic indications to be fully evaluated.

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Keywords: Breast cancer; Coumarin sulphamate; Mechanism of inhibition; Non-steroidal steroid sulphatase inhibitor

Received: **14 July 1999**
Revisions requested: **22 September 1999**
Revisions received: **6 July 2000**
Accepted: **2 August 2000**

Published: **18 August 2000**

Chemistry & Biology 2000, **7**:773–791

1074-5521/00/$ – see front matter
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PII: S1074-5521(00)00023-5

Introduction

In postmenopausal women with breast cancer, about one-third of patients have hormone-dependent tumours, the growth and development of which require the stimulation of oestrogens. Studies have shown that endocrine therapy plays an important role in the treatment of this type of breast cancer and that hormonal manipulation, through the inhibition of enzymes within the steroid biosynthetic cascade, may be one route to controlling the disease.

The aromatase enzyme, which converts androstenedione to oestrone (Figure 1), has been the prime target for reducing oestrogen levels. Several potent, well-tolerated and highly selective aromatase inhibitors are currently in clinical use. However, the clinical response of treated patients to the latest generation of aromatase inhibitors has been disappointing and this has cast doubt on the efficacy of treating hormone-dependent breast cancer (HDBC) by aromatase inhibitors alone without inhibiting concurrently the activ-

ities of other enzymes involved in the production of oestrogenic steroids [1,2]. There is now abundant evidence to suggest that the hydrolysis of oestrone sulphate (E1S, Figure 1) to oestrone (E1, Figure 1) by oestrone sulphatase (E1-STS) is the main source of oestrogens to tumours [3–9]. In addition, the production of androstenediol (Adiol), and to a lesser extent oestradiol (E2), via dehydroepiandrosterone (DHEA) (Figure 1) could also significantly contribute to the oestrogenic stimulation of hormone-dependent breast tumours [10–13]. Adiol, an androgen which binds to oestrogen receptor and has oestrogenic properties, originates from DHEA sulphate (DHEA-S) once it has been hydrolysed to DHEA (Figure 1) by DHEA sulphatase (DHEA-STS) [12–16]. Therefore, steroid sulphatase inhibitors, when used alone or in concert with an aromatase inhibitor, may enhance the response of hormone-dependent breast tumours to this type of endocrine therapy by reducing not only the formation of E1 from E1S but also the synthesis of other oestrogenic steroids such as Adiol via DHEA from DHEA-S.

In addition to its oncological role there is now evidence to suggest that steroid sulphatase may regulate part of the immune response [17,18]. In an animal model, it has been shown that inhibition of steroid sulphatase can modulate the immune response and have a beneficial effect in collagen-induced rheumatoid arthritis [19]. Recent studies have also suggested that inhibition of steroid sulphatase can potentiate the memory enhancing properties of DHEA-S [20–22].

Oestrone-3-*O*-sulphamate (EMATE, Figure 2) was our first highly potent steroid sulphatase inhibitor which exhibits active site-directed inhibition against E1-STS and DHEA-STS, both in vitro, and in vivo when administered orally or subcutaneously [23–28]. However, despite its potent biological activities, it is now unlikely that EMATE will be developed for clinical trials in the treatment of HDBC. A recent study has demonstrated that EMATE and its congener, oestradiol-3-*O*-sulphamate, are more oestrogenic than ethinyloestradiol when administered orally in the rat [29]. Although the mechanism for this oestrogenicity has not been fully elucidated, this undesirable property of EMATE strongly reinforced the preference for administering endocrine therapy via *non-steroidal* agents, which may not themselves, or through their metabolites, exert unwanted and/or unpredictable endocrinological effects.

We have now established that non-steroidal coumarin sulphamates such as coumarin-7-*O*-sulphamate (**1**, Figure 2) and 4-methylcoumarin-7-*O*-sulphamate (COUMATE, Figure 2) are steroid sulphatase inhibitors [28,30]. Although the orally active COUMATE is less potent than EMATE as an active site-directed inhibitor, it has the crucial advantage of being *non-oestrogenic* [31]. We have recently studied structure–activity relationships for COUMATE which have shown, inter alia, that the coumarin ring system of COUMATE is pivotal for its inhibitory activities [28]. In addition, one analogue of COUMATE, 3,4-dimethylcoumarin-7-*O*-sulphamate (**2**, Figure 2), was found to be some 12-fold more potent than COUMATE as an E1-STS inhibitor in intact MCF-7 breast cancer cells (IC_{50} = 30 nM) [28].

It is possible that the higher potency of **2** observed in comparison with **1** and COUMATE might be attributed to its stronger binding to the enzyme active site via a hydrophobic interaction provided by the methyl groups at the 3- and 4-positions. Since coumarin sulphamates such as **2** are presumably A/B ring mimics of EMATE, it is predicted that analogues with substituents of increased hydrophobicity at the 3- and/or 4-positions might be more potent steroid sulphatase inhibitors. Previous studies in this field have already highlighted an association between the hydrophobicity of a steroid sulphatase inhibitor and its inhibitory activity. Hence, the potency of non-steroidal, single-aryl ring steroid sulphatase inhibitors such as *p*-alkylphenyl phosphates [32] and (*p*-*O*-sulphamoyl)-*N*-alkanoyl tyramines [33,34] has been shown to increase as the length of the alkyl group increases. Recently, such association has also been demonstrated in steroid sulphatase inhibitors which are derivatives of E2. Compounds such as 17β-(*N*-alkylcarbamoyl)oestradiol-3-*O*-sulphamates [35], 17β-(*N*-alkanoyl)oestradiol-3-*O*-sulphamates [35] and 17α-benzyl derivatives of oestradiol-3-*O*-sulphamate [36] have been found to be potent. To explore any association between potency and hydrophobicity in two-ring compounds such as coumarin sulphamates, and also as part of our ongoing programme to develop an alternative *non-steroidal* and *non-oestrogenic* candidate to EMATE for clinical trials, we report here (i) the replacement of the methyl group of COUMATE at the C4-position, and that of **2** at the C3-position with an ethyl and *n*-propyl group; and in particular also (ii) the synthesis of several novel tricyclic derivatives of coumarin sulphamates. Hence, 4-ethylcoumarin-7-*O*-sulphamate (**4**, Scheme 1), 4-(*n*-propyl)coumarin-7-*O*-sulphamate (**6**), 3-ethyl-4-methylcoumarin-7-*O*-sulphamate (**8**), 4-methyl-3-(*n*-propyl)coumarin-7-*O*-sulphamate (**11**); the tricyclic derivatives 665COUMATE (**13**, Scheme 2; for the chemical name see Materials and methods), 666COUMATE (**15**), 667COUMATE (**17**), 668COUMATE (**20**) and the tricyclic oxepin sulphamate (**22**) were synthesised and examined for inhibitory activities against E1-STS in vitro.

Some efforts have been made recently to understand the mechanism of action of steroid sulphatase and how some inhibitors of this enzyme might work [25,28,32,37–39]. In the light of recent publications on the crystal structures of human arylsulphatase A and arylsulphatase B and the catalytic site topology for the hydrolysis of sulphate group [40–

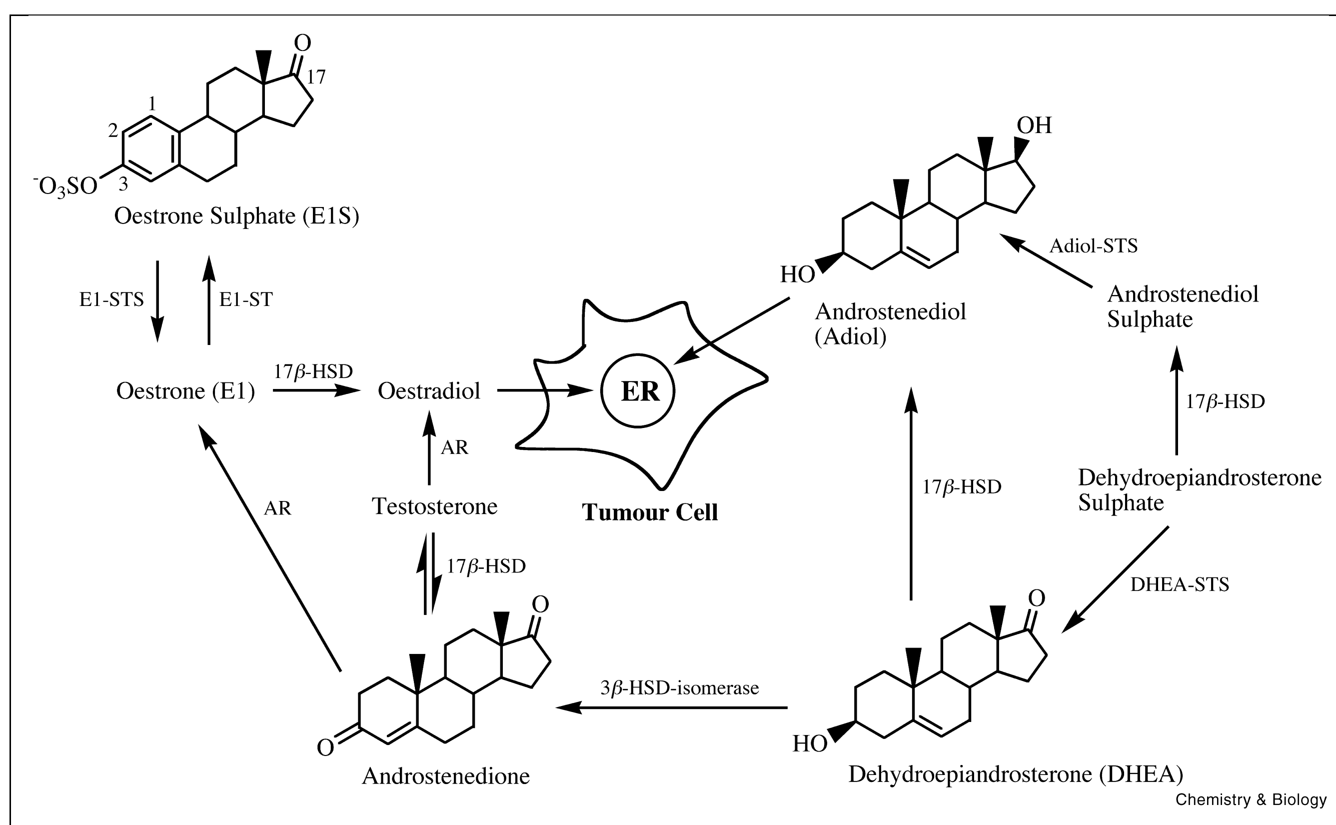


Figure 1. The origin of oestrogenic steroids in postmenopausal women: AR, aromatase; ST, sulphotransferase; STS, sulphatase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 3β-HSD-isomerase, 3β-hydroxysteroid dehydrogenase Δ^5,Δ^4-isomerase; ER, oestrogen receptor.

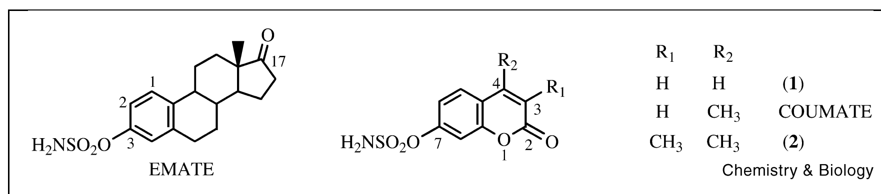
43], we also reappraise here our previously proposed mechanism of action for an active site-directed steroid sulphatase inhibitor [28,37,38].

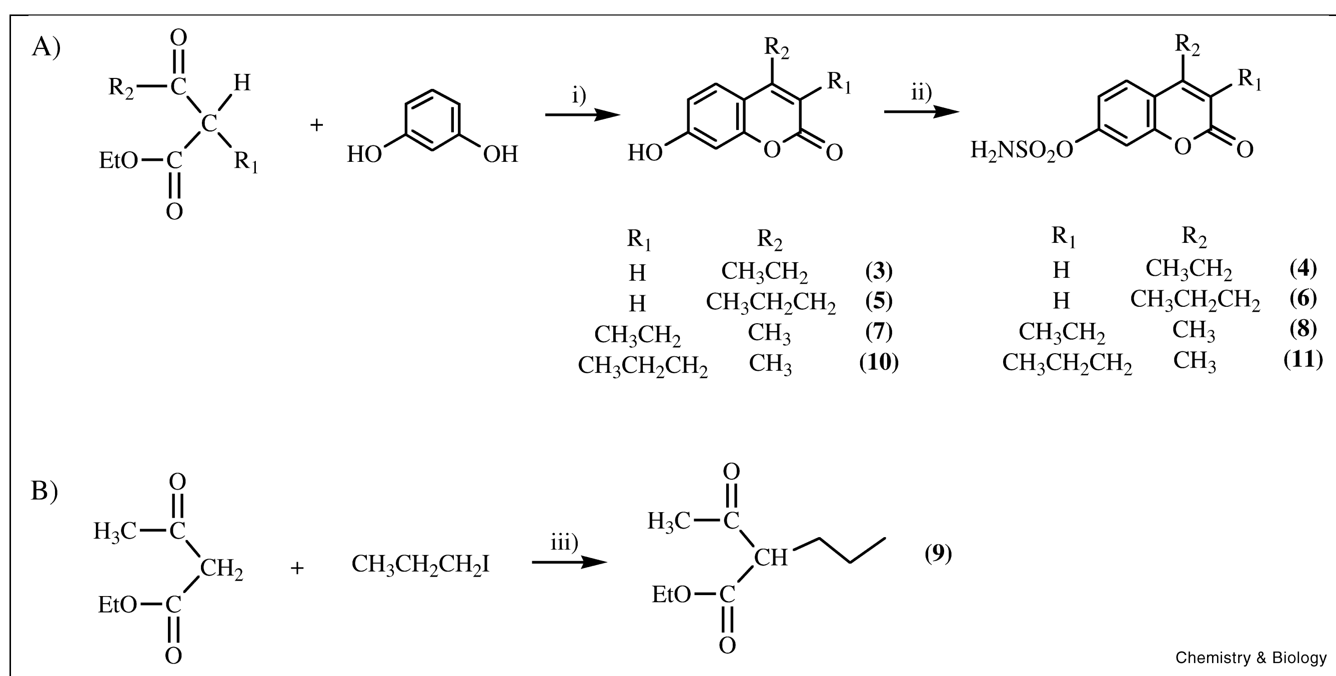
Results and discussion

Since the unexpected discovery that the potent steroid sulphatase inhibitor EMATE is also a highly active oestrogen, we have been exploring alternative phenolic-based non-steroidal ring structures whose sulphamates might exhibit steroid sulphatase inhibition but be devoid of oestrogenicity. Our interest in the coumarin ring system stemmed from the reported oestrogenicity of coumestrol (**23**, Figure 3), a plant derived tetracyclic coumaric compound [44,45]. Structurally, this phytooestrogen noticeably bears a resemblance to the oestrogens because of an apparently crude mimicry of the steroid skeleton. Therefore, the coumarin moiety of the molecule might be a mimic of the A/B ring of oestrogens. We then reasoned that sulphamates of simple bicyclic coumarins might exhibit steroid sulphatase inhibition but they themselves or their starting coumarins are unlikely to be oestrogenic. Indeed, this reasoning was corroborated with COUMATE (Figure 2), which was found to be an effective, orally active, non-oestrogenic active site-directed E1-STS inhibitor [28,30,31]; and also subsequently with its 3-methyl derivative, 3,4-dimethylcoumarin-7-*O*-sulphamate (**2**, Figure 2) [28].

The parent coumarins for this study were prepared by Pechmann reaction between resorcinol and the corresponding β-ketoester (Schemes 1A and 2A) in the presence of an

Figure 2. Structures of EMATE, coumarin-7-*O*-sulphamate (**1**), COUMATE and 3,4-dimethylcoumarin-7-*O*-sulphamate (**2**).



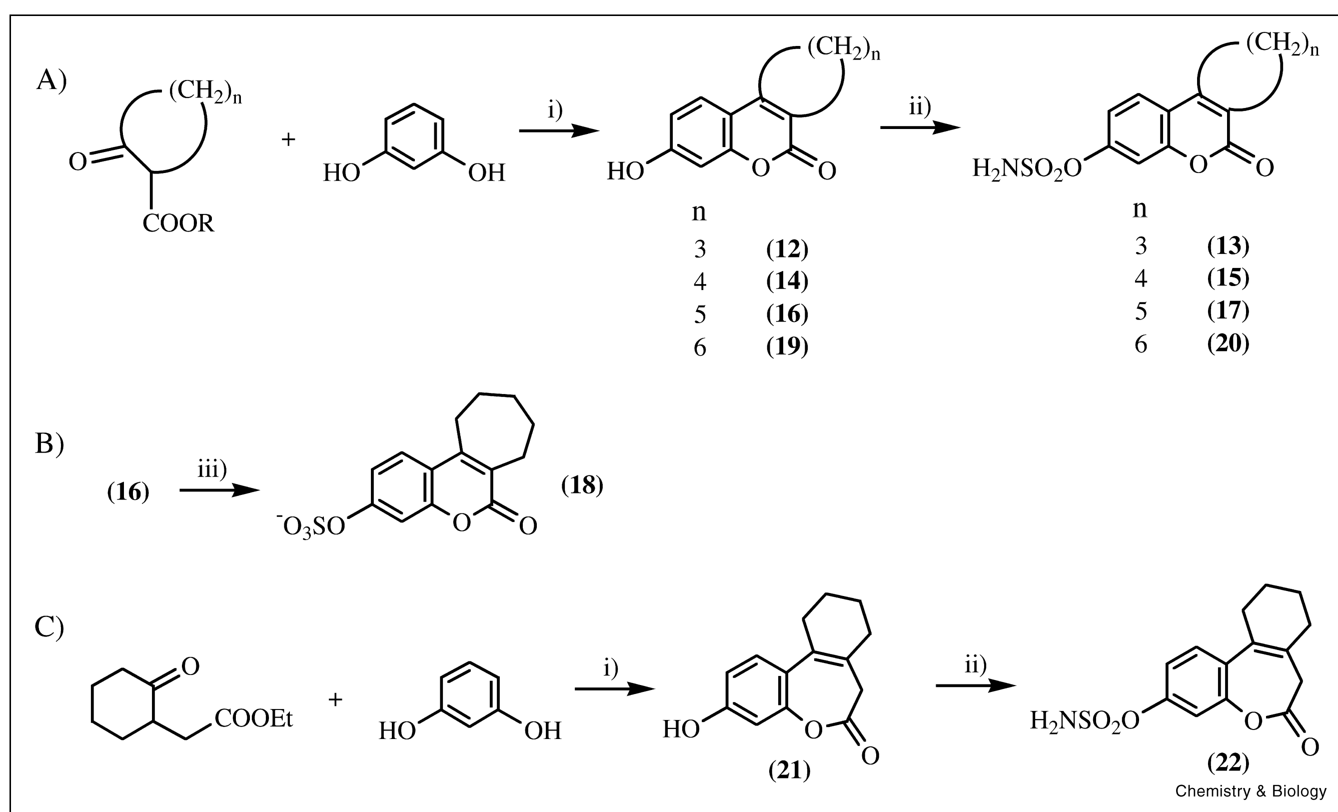


Scheme 1. (A) Structures and synthesis of coumarins (**3**, **5**, **7** and **10**) and their corresponding sulphamates (**4**, **6**, **8** and **11**). **(B)** Synthesis of ethyl (2-*n*-propyl)acetoacetate (**9**). (i) CF_3COOH/conc. H_2SO_4 (1:1), 0°C→r.t.; (ii) NaH/DMF, H_2NSO_2Cl; (iii) K_2CO_3/$(Bu)_4N^+Cl^-\cdot xH_2O/H_2O/CHCl_3$, r.t.

equimolar mixture of trifluoroacetic acid and concentrated sulphuric acid [46] as the condensing agent. For those coumarins which had been reported in the literature, our modified method has produced comparable yields. Since many of these parent coumarins were characterised almost 50 years ago, we now report their hitherto unavailable spectroscopic data.

It is interesting to note that, in contrast to lower members of the series whose yields are $>60\%$, the tricyclic coumarin **19** (Scheme 2A) was only isolated in around 25–33% yield (two attempts) with a recovery of almost half of the methyl 2-oxocyclooctane carboxylate used initially for the reaction. When the higher 6613COUMARIN (**24**, Figure 3) was prepared from the corresponding 13-membered cyclic β-ketoester in a similar manner, the crystalline product was isolated in 58% yield (data not shown). Although the mechanism for the relatively poor yield of **19** has not been fully elucidated, we suspect that the ring size of its starting 8-membered cyclic β-ketoester could be a contributory factor. It has been shown that when the carbonyl carbon of a 5-, 6-, 7-, 8- or 13-membered cyclanone becomes sp^3(C–OH) hybridised, the highest I-strain is observed in the resulting saturated cyclic molecule of cyclooctanone [47,48]. Since an equivalent $sp^2 \rightarrow sp^3$ conversion at the carbonyl carbon of the β-ketoester is also involved upon the penultimate Friedel–Crafts cyclisation step of the Pechmann reaction, it is anticipated that the formation of **19** is the most disfavoured, accounting for its poorest yield in our series of tricyclic coumarins.

The inhibitory activities of the 3-alkyl-4-methyl- and 4-alkylcoumarin sulphamates against E1-STS activity in placental microsomes are shown in Table 1. For the purpose of comparison, the inhibitory activities of **1**, COUMATE and **2** obtained from our previous work [28] are also included. It is clear that all these derivatives (**4**, **6**, **8** and **11**) were found to be more potent E1-STS inhibitors than COUMATE. The substitution at the 3-position of the coumarin ring with alkyl groups of longer chain length proved to be more productive than that at the 4-position as shown by the overall higher potencies of the inhibitors in the former series. Although the methyl group at C4 of the inhibitors in the 3-alkyl series must also be a contributory factor to the higher potency observed, it is apparent that the hydrophobic interactions between the amino acids in the active site, which naturally recognise the steroid scaffold, and the alkyl substituents are more effective when these substituents are placed at the C3 position of the coumarin ring. Since we have proposed that coumarin sulphamates are steroid sulphatase inhibitors by virtue of their structural mimicry of the A/B ring of EMATE, it is conceivable that the positioning of alkyl substituents with high rotational degrees of freedom at the 4-position of the coumarin ring may be counterproductive. The active site of steroid sulphatase, like many other enzymes with a steroid



Scheme 2. (A) Structures and synthesis of tricyclic coumarins (**12**, **14**, **16** and **19**) and their corresponding sulphamates (**13**, **15**, **17** and **20**). **(B)** Sulphation of coumarin **16**. **(C)** Synthesis of the tricyclic oxepin (**21**) and its sulphamate (**22**). (i) CF_3COOH/conc. H_2SO_4 (1:1), 0°C → r.t.; (ii) NaH/DMF, H_2NSO_2Cl; (iii) $Me_3N{\cdot}SO_3$, 1 M NaOH (aq).

as substrate, is expected to have limited accommodation for substituents at the C1/C11/C12 edge of the steroid scaffold. For this reason, the relatively weaker inhibitions shown by the inhibitors in the 4-alkyl series can be attributed to their less favourable binding to the enzyme active site.

Our prediction that coumarin sulphamate analogues with substituents of increased hydrophobicity at the 3- and/or 4-positions should be more potent steroid sulphatase inhibitors has been further confirmed by the even higher potency observed in most of the tricyclic coumarin sulphamates, e.g. both **17** and **20** gave almost complete inhibition at 1 μM in a placental microsome preparation (Table 2). The best inhibitor in this series is **17** (667COUMATE) whose IC_{50} value for the inhibition of E1-STS activity in placental microsomes is 8 nM (Figure 4A) which is some three-fold more potent than EMATE in the same assay (IC_{50} = 25 nM) (Figure 4A). Like EMATE, but much more effectively, **17** also inhibited the hydrolysis of DHEA-S by DHEA-STS in a placental microsome preparation (Figure 4B) with an IC_{50} of 4.5 nM (cf. 110 nM for EMATE [25]).

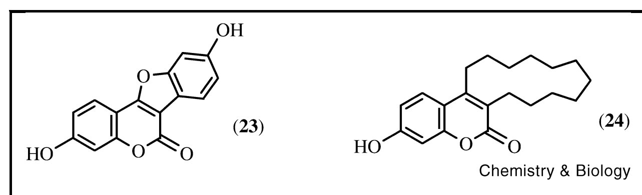


Figure 3. Structures of coumestrol (**23**) and 6613COUMARIN (**24**).

The time- and concentration-dependent inactivation of the E1-STS activity in placental microsomes by **17** is shown in Figure 5A. As with EMATE, the inhibition by **17** is biphasic, indicating that the inhibitor shares a similar mechanism of action to that proposed for EMATE, which we have postulated acts via irreversible sulphamoylation of one or more residues in the enzyme active site [28]. The double-reciprocal plot of the first-order rates of inactivation versus the concentrations of **17** is shown in Figure 5B. The apparent K_i for **17** was found to be 40 nM which is significantly lower than that for EMATE (670 nM) [25]. This suggests that the lower IC_{50} value observed for **17** in com-

Table 1

Inhibition of oestrone sulphatase activity in placental microsomes by 4-alkylcoumarin sulphamates (4 and 6) and 3-alkyl-4-methylcoumarin sulphamates (8 and 11) at various concentrations.

Compound	% Inhibition ± S.D. of E1-STS activity in placental microsomes at various concentrations		
	0.1 μM	1 μM	10 μM
1 (C3-H, C4-H)[a,b]	–	–	78 ± 1
COUMATE (C3-H, C4-Me)[b]	< 10	63 ± 1	93 ± 1
2 (C3-Me, C4-Me)[b]	35 ± 1	88 ± 2	97 ± 4
4 (C3-H, C4-Et)	35 ± 1	88 ± 1	> 99
6 (C3-H, C4-*n*-propyl)	42 ± 2	94 ± 1	96 ± 1
8 (C3-Et, C4-Me)	57 ± 1	96 ± 1	> 99
11 (C3-*n*-propyl, C4-Me)	83 ± 1	97 ± 1	> 99

[^{3}H]Oestrone sulphate (4×10^5 dpm), adjusted to 20 μM with unlabelled substrate, with or without the inhibitor at various concentrations, was incubated with placental microsomes (125 μg of protein/ml) for 30 min. The product formed was isolated by extraction into toluene with [4-^{14}C]oestrone (7×10^3 dpm) being used to monitor procedural losses. Each value represents the mean ± S.D. of triplicate measurements.

[a] > 90% at 50 μM.

[b] Results reproduced from [28].

parison with EMATE could be attributed to a higher affinity of **17** for the enzyme active site. As calculated by the method of Kitz and Wilson [49], the overall rate constant for the decrease in activity was found to be 3.45×10^{-3} s^{-1} for **17**. To date, the only successful approach to the design of steroid sulphatase inhibitors which are more potent than EMATE, the benchmark inhibitor of this field, has been by incorporating hydrophobic substituents at the C17 position of EMATE [35,36]. Although a series of (*p*-sulphamoyl)-*N*-alkanoyl tyramines have also shown good inhibitory activities, the best compound in this series (*N*-tetradecanoyl) has been reported to be less potent than EMATE [35]. We demonstrate here, therefore, for the first time that superior inhibition over EMATE can be achieved with an inhibitor that is essentially non-steroidal by nature.

Although the coumarin moiety of **17** is expected to mimic the A/B ring of EMATE, its third ring could not be described as a close mimic of the C/D ring of EMATE when its preferred conformation is examined. Our molecular modelling of **17** (Graphics 1) shows that its 7-membered ring is largely in the chair form, which is similar to that of cycloheptene [50], with the C=C moiety taking the place of one of the ring carbon atoms in the cyclohexane chair. Despite this lack of absolute conformational resemblance of **17** to EMATE, particularly in the C/D ring regions, the hydrophobic interactions between its cycloalkene system and neighbouring amino acid residues in the enzyme active site must be effective and favourable. As a consequence, the active site sulphamoylation, and hence inactivation, of E1-STS by **17** must be facilitated. For **20**, despite a higher hydrophobicity by virtue of an extra methylene group, its lower potency than **17** observed could be attributed to the highly strained multi-conformational cyclooctene system. It is possible that the binding conformation of the cyclooctene system of **20** might have less optimal interactions with the amino acid residues of the enzyme active site than the cycloheptene system of **17**.

Table 2

Inhibition of oestrone sulphatase activity in placental microsomes by tricyclic coumarin sulphamates (13, 15, 17 and 20) and tricyclic oxepin sulphamate (22) at various concentrations.

Compound	% Inhibition ± S.D. of E1-STS activity in placental microsomes at various concentrations		
	0.01 μM	0.1 μM	1 μM
13 (665COUMATE)[a]	< 10	37 ± 1	91 ± 2
15 (666COUMATE)[a]	< 10	63 ± 1	93 ± 1
17 (667COUMATE)	48 ± 2	91 ± 1	> 99
20 (668COUMATE)	17 ± 2	89 ± 1	> 99
22	< 10	31 ± 5	94 ± 2

For details of the assay, refer to the legend of Table 1.

[a] > 99% at 10 μM.

We have demonstrated previously that 7-(sulphooxy)-4-methylcoumarin is a substrate for E1-STS [28]. However, it is important to demonstrate the same with 667COUMARIN sulphate (**18**, Scheme 2B), which we synthesised by treating a solution of **16** (Scheme 2) in sodium hydroxide (~1 M) with sulphur trioxide-trimethylamine complex. When **18** was incubated with placental microsomes in the absence of EMATE (see Materials and methods), only the free coumarin **16** was detected. However, hydrolysis of **18** by E1-STS was completely abolished by the inclusion of EMATE in the reaction (data not shown). These results therefore indicate that the tricyclic coumarin sulphate **18** is a substrate for this enzyme.

The oestrogenic activity of **17** was also studied. Like COUMATE [31], when administered orally, **17** was found to be devoid of oestrogenicity as shown by its inability to stimulate uterine growth in ovariectomised rats [78]. With this lack of oestrogenicity, together with its higher active site-directed inhibitory activity against E1-STS than EMATE in vitro and also its ease of synthesis, **17** is now

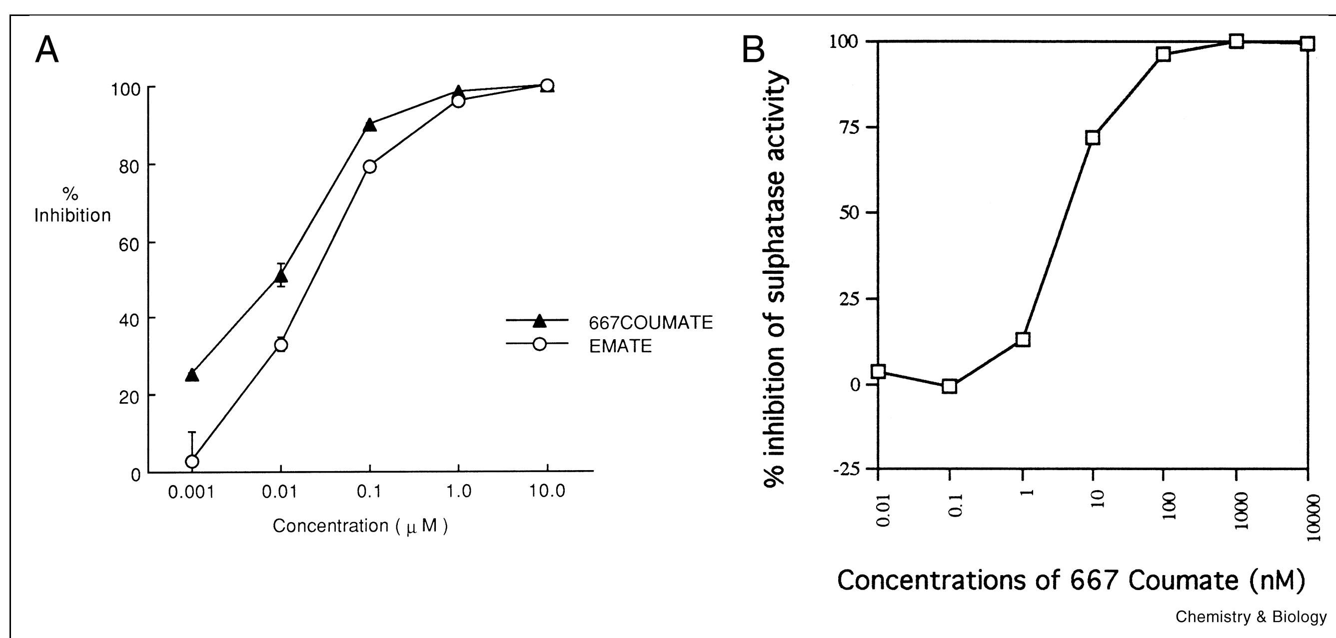


Figure 4. (A) Dose–response curves for inhibition of oestrone sulphatase activity in placental microsomes by 667COUMATE (**17**, ▲) and EMATE (○). Method as described for Table 1. **(B)** Dose–response curves for inhibition of DHEA-STS activity in placental microsomes by 667COUMATE (**17**). Method as described for Table 1 except [^{3}H]DHEA-S (4×10^5 dpm) adjusted to 20 μM with unlabelled DHEA-S was used.

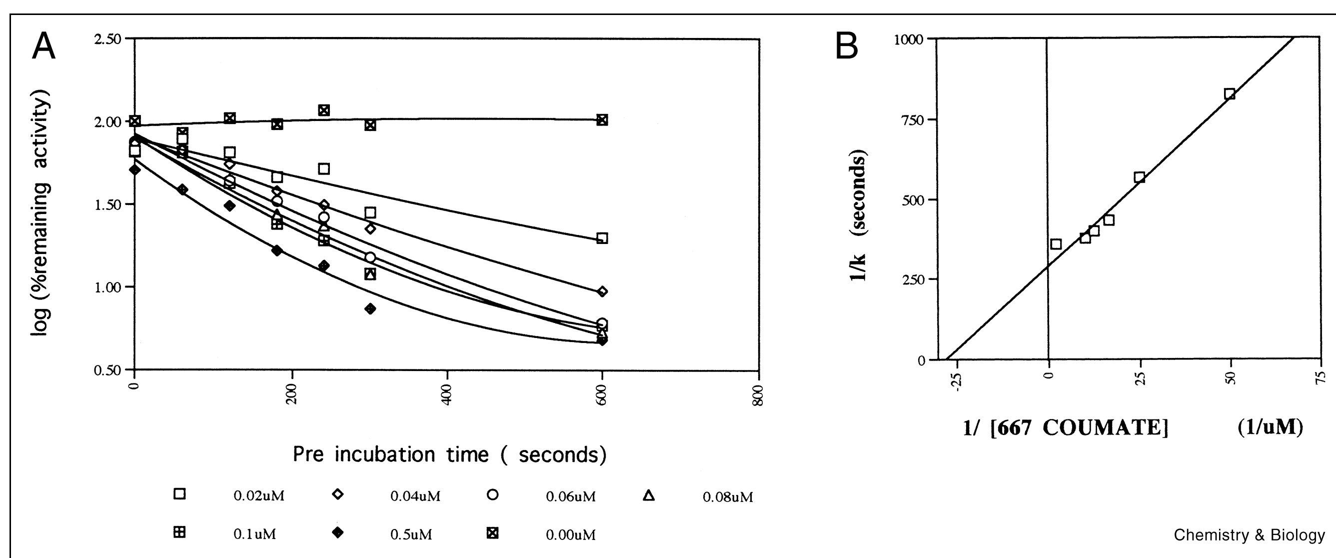
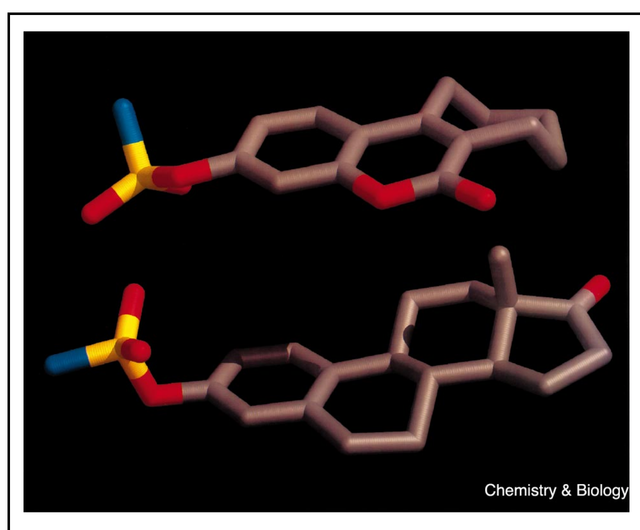


Figure 5. (A) Time- and concentration-dependent inactivation of oestrone sulphatase by 667COUMATE (**17**). Placental microsomes (200 μg of protein) were preincubated with the inhibitor at 0–0.5 μM for 0–10 min at 37°C followed by incubation with dextran–charcoal for 10 min at 4°C. Dextran–charcoal was sedimented by centrifugation, and portions of the supernatants were then incubated with [^{3}H]oestrone sulphate (20 μM) for 1 h at 37°C to assess remaining sulphatase activity. Duplicate experiments were run at each concentration, but assays for residual activity were taken at different times in each experiment. **(B)** Double reciprocal plot of inactivation rate constant versus 667COUMATE concentration.



Graphics 1. Molecular modelling of 667COUMATE (**17**, top) and EMATE on SYBYL® (Tripos Associate).

in formal pre-clinical development for phase I clinical trial for the treatment of postmenopausal women with HDBC.

In a limited structure–activity relationship study for our tricyclic coumarin sulphamates, we enlarged the middle ring of 666COUMATE (**15**, Scheme 2) from the α,β-unsaturated δ-lactonic to the unconjugated ε-lactonic moiety of **22** (Scheme 2). It is clear that the potency of **15** was significantly reduced by such structural modification as shown by the weaker inhibitory activity of **22** (Table 2). This result is consistent with the findings from our recent structure–activity relationships study on COUMATE which have shown a similar detrimental effect to the potency of the parent compound when the conjugation of the coumarin ring is either disrupted or removed entirely [28]. Our explanation for this effect is that the breaking of the S–O–Ar bond in the sulphamate group of coumarin sulphamates such as COUMATE during sulphamoylation of E1-STS is assisted by the extended conjugation present in the coumarin motif. Such conjugation improves the leaving group ability of coumarins by lowering the pK_a of the phenol (our recent finding that the 4-nitro analogue of EMATE is more potent than EMATE as a steroid sulphatase inhibitor [51] has already suggested that the inhibitory activity of an aryl sulphamate could be improved by enhancing the leaving group ability of the parent phenol, i.e. lowering of pK_a). Any disruption or removal of conjugation in the ring system of coumarins, including that in **14**, should therefore render analogues with poorer leaving group abilities (i.e. a higher pK_a value for the parent phenol) whose sulphamates are expected to be less active as E1-STS inhibitors. However, the susceptibility of the unconjugated β,γ-unsaturated ε-lactonic moiety of **22** to hydrolysis in phosphate buffered saline (pH = 7.4) used in the bioassay could explain the relatively weak inhibition shown by this inhibitor. It is possible that the tricyclic structure of **22**, supposedly required for good inhibitory activity, is already lost prior to the binding of the inhibitor to the enzyme active site.

Like other irreversible steroid sulphatase inhibitors reported to date, 667COUMATE possesses a phenol sulphamate ester which we have proposed to be the pharmacophore for inhibitors acting mechanistically like EMATE [28]. The mechanism(s) through which the sulphamate group irreversibly inactivates steroid sulphatase have already been broadly proposed and discussed without the knowledge of the enzyme active site (part of the difficulty in obtaining the crystal structure of steroid sulphatase lies in the crystallisation of this membrane-bound enzyme). However, two recent publications on the crystal structures of the soluble enzymes arylsulphatase A (ASA) [42] and arylsulphatase B (ASB) [40], and related articles [41,43,52,53] have provided valuable new insights into the conserved active site of sulphatases in general and on how a sulphatase enzyme might hydrolyse its substrates. We earlier proposed the involvement of histidine and tyrosine residues in the catalytic mechanism of steroid sulphatase and its inhibition [25]. With the greater understanding of the generic active site sulphatase topology we now feel it is crucial to reappraise these ideas and provide a focus for further mechanistic work.

Eleven mammalian sulphatases have been identified: eight in lysosomes, e.g. ASA and ASB, and three associated with microsomal membranes, e.g. steroid sulphatase. These sulphatases are members of a highly conserved gene family sharing extensive amino acid sequence homology [52,54], a unique post-translational modification that is essential for sulphate ester cleavage [52,55,56] as well as a high degree of structural similarity [40,42]. In all different sulphatases whose amino acid sequences have been resolved so far, including steroid sulphatase, the active site amino acid residues are conserved [40,52]. Presumably, enzyme specificity is derived from differential binding of the group attached to sulphate in the various different types of sulphatase. The post-translational modification in functional sulphatases involves the oxidation of a conserved cysteine residue to L-C_α-formylglycine (FGly) [52]. However, such modification does not take place in sufferers of a rare disorder known as multiple sulphatase deficiency whose sulphatases are all severely impaired [40,52].

From the crystal structure analysis of ASB, the post-translational modification of cysteine occurs at residue 91 which is replaced by a sulphate adduct of FGly [∼CH(OH)–OSO_3^-] [40]. The amino acid residues that encompass FGly91 and constitute the enzyme active site are Asp53, Asp54, Pro93, Ser94, Arg95, Lys145, His147, His242,

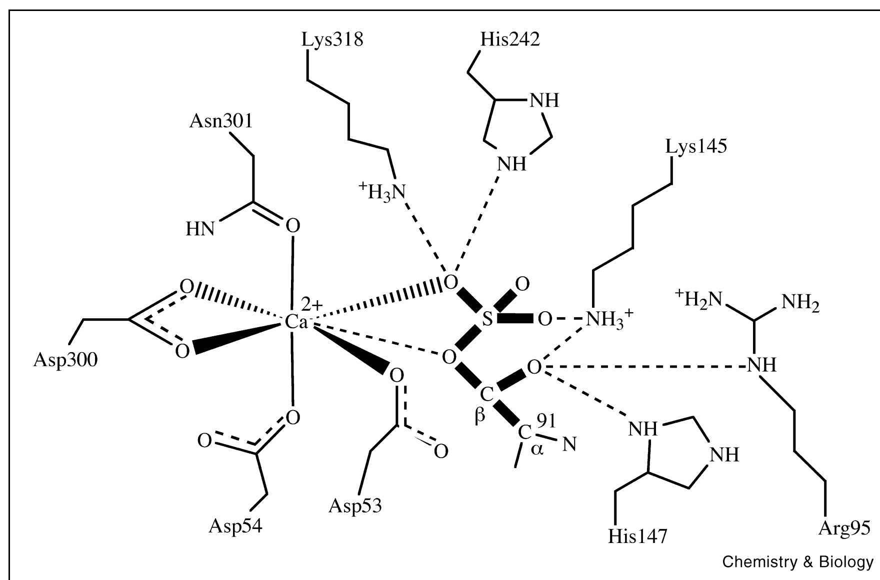


Figure 6. Sketch view of the catalytic site of arylsulphatase B showing hydrogen-bonded interactions (dashed lines) that stabilise the sulphate ester. The seven-coordinate metal ion is on the left. A salt bridge interaction between Lys145 and the O (carboxyl) atom of Asp53, which is not coordinated to the metal ion, and a number of charges and double bonds are omitted from the figure for clarity (adapted from [40] with permission).

Asp300 and Lys318 with Ca^{2+} being identified as the coordinating metal ion (Figure 6) [40]. For ASA, the predicted Cys69 is replaced by an aldehyde hydrate [$\sim CH(OH)_2$] which coordinates to a Mg^{2+} ion via one of its oxygen atoms [42]. The conserved residues lining the active site pocket of ASA are Asp29, Asp30, *Lys123, *Ser150, His125, *His229, Asp281, Asn282, *Lys302 (residues marked with an asterisk are in close proximity to the sulphate group of the substrate when it enters the active site) [42]. It is anticipated that similar features are found in the active site of steroid sulphatase in which a FGly residue, coordinated by a metal ion, is embedded in a cluster of conserved amino acid residues. An electrostatic potential survey on ASA and ASB has shown a common feature that the FGly residue is embedded within a positively charged substrate binding pocket [40,42]. The putative interpretation for this observation is that the nitrogen-containing amino acid residues lining the active site are protonated, which through hydrogen bonding to the oxygen atoms of the substrate might help stabilise the sulphate ester (Figure 6).

With the different resting forms of FGly observed in the crystals of ASA and ASB, two different catalytic mechanisms for desulphation of substrate are proposed [40,42]. For ASA [42], the reaction cycle is proposed to start with an attack on the sulphur atom of the substrate by one of the *gem*-diol oxygens, displacing the substrate alcohol as a consequence (transesterification) (Figure 7, ASA). The regeneration of free FGly from the sulphate adduct is supposed to involve the elimination of the sulphate group induced by the second, non-esterified hydroxyl group. The free FGly so formed is then rehydrated back to an aldehyde hydrate which is the resting form observed in ASA. For ASB [40], the reaction sequence could be initiated and proceed in a similar manner to that of ASA but differs in that the reaction is arrested at the sulphate adduct, which is apparently the resting form observed in the crystal. However, it is also entirely possible that the reaction sequence is initiated by one of the nucleophilic oxygen atoms in the sulphate of the substrate attacking the carbonyl group of FGly (Figure 7, ASB) to form a sulphate ester and this was proposed by Bond et al. [40]. The displacement of the substrate as an alcohol by water then follows rendering the sulphate adduct of FGly.

In order to discriminate between the two hypothetical mechanisms proposed above, the cysteine residue in the precursor protein of ASA and ASB was replaced, using site-directed mutagenesis, by a serine ($\sim CH_2$–OH) which is not oxidised to FGly in the cell lines used for the expression [41]. It was found that whilst the generated mutants are able to cleave ^{35}S-labelled substrate, only one catalytic half-cycle was completed with the formation of a sulphoserine ($\sim CH_2–O^{35}SO_3^-$). This sulphoserine-containing enzyme was found to be stable at pH 5 but the sulphate group was hydrolysed slowly at alkaline pH. These characteristics resemble those of alkaline phosphatase, whose phosphoserine intermediate is also stable at pH 5, but is hydrolysed at alkaline pH with no desulphation at acidic pH. This apparent trapping of the serine mutants at the sulphoserine intermediate is attributed to the absence of a second *gem*-hydroxyl group. In wild-type ASA, the non-esterified hydroxyl group is proposed to polarise the C_β–$\underline{O}$ bond of the sulphate adduct [$\sim C_\beta H(OH)–\underline{O}SO_3^-$] and hence assist its desulphation and the release of FGly. These findings have thus presented a strong case for the transesterification sequence described for ASA as the gen-

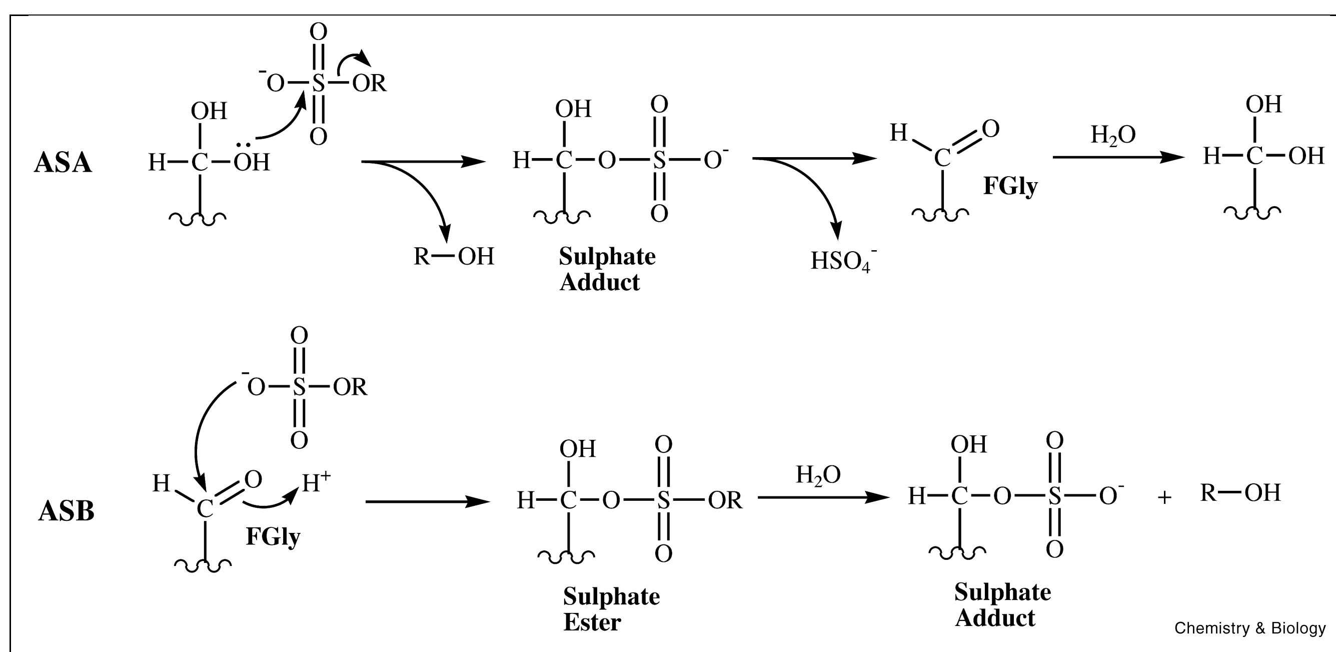


Figure 7. Proposed reaction scheme for sulphate ester cleavage by arylsulphatase A (ASA) and arylsulphatase B (ASB). FGly: formylglycine.

eral mechanism through which sulphatases, including steroid sulphatase, desulphate their substrates. If formation of the sulphated FGly were initiated by addition of the sulphate ester to the oxo-group as proposed for ASB in Figure 7, no catalytic reaction should, in principle, be observed in the serine mutant of ASB.

Although 667COUMATE has not been shown to be active against other sulphatase enzymes apart from steroid sulphatase, based on the above insights into the mechanisms for ASA- and ASB-catalysed sulphate hydrolysis (Figure 7) and given that the essential residues for sulphatase activity are conserved within the sulphatase family, several mechanisms for the inhibition of steroid sulphatase activity by 667COUMATE (or other related active site-directed sulphamates such as EMATE) are conceivable (Figs. 8–10). The mechanisms we propose here thus replace the more schematic ones [28,37,38] that were put forward previously, and we consider all potential possibilities.

Involvement of FGly (Figure 8)

Since it has been proposed by Bond et al. that the resting sulphated derivative of FGly observed in the crystals of ASB could be in equilibrium with its free form [40], a potential nucleophilic attack on the formyl group by the lone-pair electrons of the N-atom of the sulphamate group of 667COUMATE could be envisaged. The hemiaminal-type intermediate (**I**, Figure 8) so formed could then be hydrolysed to give 667COUMARIN and the sulphamoylated enzyme **II**, which upon dehydration gives an imino sulphamoylated enzyme **IV** (Figure 8, path A). Alternatively, **IV** could be formed via hydrolysis of the ester **III** after the dehydration of the hemiaminal intermediate **I** (Figure 8, path B). We propose that **II, III** and **IV** and possibly even **I** are 'dead-end' products and no regeneration of FGly as in ASB (Figure 7, ASB) is therefore anticipated. The formation of an azomethine adduct similar to **III** or **IV** is certainly not unprecedented for non-enzymatic reaction. In the course of sulphamoylating a solution of 2-nitrophenol in dimethylformamide (DMF) after deprotonation with sodium hydride, an azomethine adduct of 2-nitrophenol-*O*-sulphamate and DMF, was isolated [28]. The mechanism proposed for its formation was similar to the one described here in which the reaction is initiated by an addition of the sulphamate to the carbonyl group of DMF and progressed further by dehydration of the hemiaminal formed.

It cannot be ruled out that the nucleophilic attack on the carbonyl group of FGly is initiated not by a neutral sulphamate but by its mono-anionic form ($\sim OSO_2NH^-$, Figure 8). Previous studies on various sulphamates have shown that the N-proton is fairly acidic with a pK_a value in the range of 7–11, e.g. 10.53 for phenol sulphamate (50% aqueous ethanol) [57], ca. 9.5 for EMATE (70% aqueous MeOH) [58], 7.29 for 4-nitrophenol-*O*-sulphamate (aqueous buffers) [59] and 7.05 for *N*-phenyl-4-nitrophenol-*O*-sulphamate (50% aqueous acetonitrile) [60]. As an-

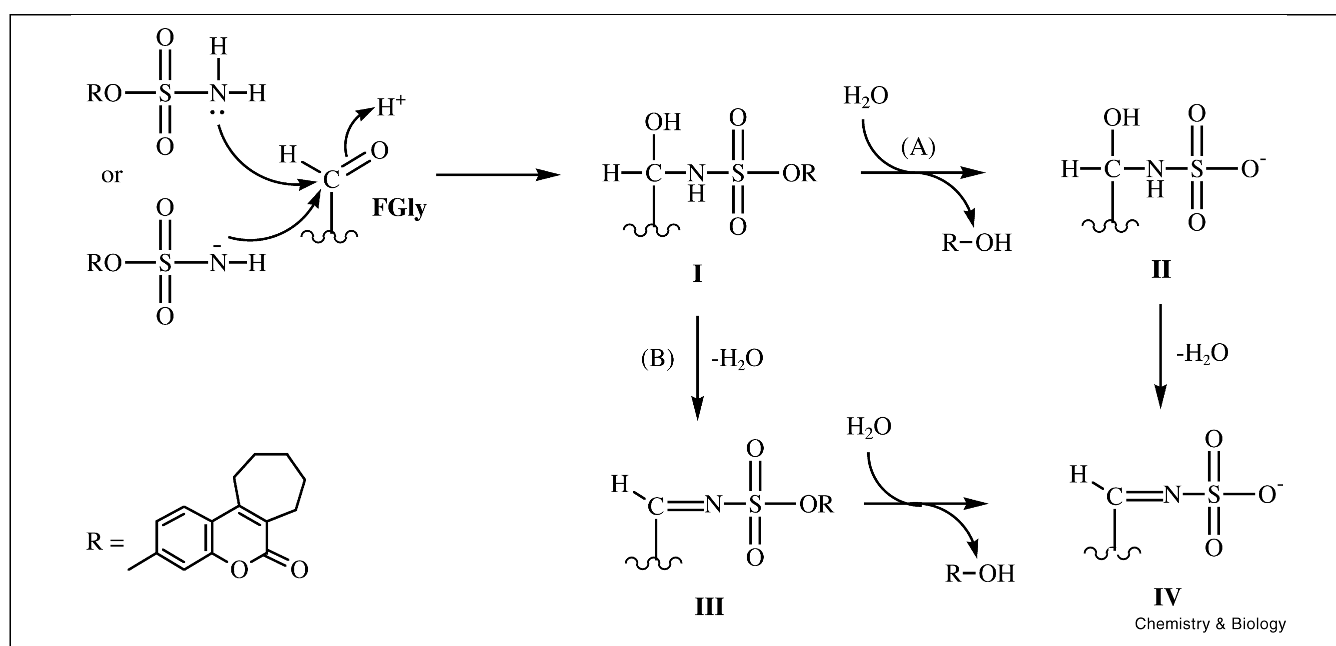


Figure 8. Proposed mechanism of steroid sulphatase inhibition by 667COUMATE involving the conserved formylglycine (FGly) residue in the enzyme active site. Structures **I**, **II**, **III** and **IV** are proposed to be 'dead-end' products.

ticipated, the pK_a of 667COUMATE in 50% aqueous methanol as determined by potentiometric titration was found to be 9.1 ± 0.1 (see Materials and methods). It is known that in a mixed solvent system such as 50% aqueous methanol the alcohol weakens the strength of an acid and the pK_a value actually measured could therefore be one or more units higher than that observed in water [61]. This implies that at physiological pH a significant proportion of the weak acid 667COUMATE could be in its conjugate base form. The fact that the sulphamate N-proton is fairly acidic also renders it susceptible to potential proton abstraction by basic amino acid residues in the enzyme active site. Given that several lysine and histidine residues are amongst the conserved amino acids found in the active sites of sulphatases, it is also highly conceivable that N-deprotonation of 667COUMATE takes place and the resulting anionic species then acts as a nucleophile attacking the FGly. The prospect of the involvement of an anionic species of 667COUMATE and other related sulphamate-based inhibitors in the inactivation of steroid sulphatase is perhaps more fitting to what has been shown in the crystal structures of ASA and ASB where the FGly residue is located in a positively charged substrate binding pocket coordinated by a bivalent metal ion. Since the sulphamoyl group is presumably acting as a sulphate surrogate, its anionic form ($\sim OSO_2NH^-$) should presumably interact more favourably with the enzyme active site and hence compete more effectively against E1S for binding than the neutral form.

Involvement of the aldehyde hydrate (*gem*-diol residue) (Figure 9)

Since an aldehyde hydrate was interpreted from the crystal of ASA [42], it is possible that one of oxygens of the *gem*-diol attacks the sulphur atom of the sulphamate group of 667COUMATE or other related irreversible inhibitors displacing the starting phenol and forming a sulphamoylated enzyme (**I**, Figure 9) as a result. This would be directly analogous to the mechanism proposed for the desulphation of substrate by ASA (see Figure 7, ASA). However, it is difficult to envisage convincingly that the sulphamoylated enzyme **I** is potentially a dead-end product and unable to proceed further since there is no apparent reason why the sulphamate cannot be expelled by the other free hydroxyl group of the *gem*-diol in a manner proposed for the regeneration of the aldehyde hydrate in ASA (see Figure 7, ASA).

Random specific or non-specific sulphamoylation (Figure 10)

It is also possible that irreversibly inhibiting sulphamate esters, including 667COUMATE, could inhibit steroid sulphatase in a more random manner by a specific or non-specific sulphamoylation of amino acid residues in the active site. Evidence for the involvement of two amino acid residues in the inactivation process is provided by the biphasic nature of inactivation exhibited by EMATE and that two ionisable groups with pK_a values of 7.2 and 9.8 are identified upon analysis of the pH dependence of en-

zyme activity and of enzyme inactivation by EMATE [25]. The finding that rose bengal inhibited oestrone sulphatase activity in a dose-dependent manner strongly suggested that a histidine was involved in the catalytic mechanism and also tyrosine was suspected since the pH dependence of the inactivation process correlated with a residue of pK_a 9.8 [25]. While the presence of two histidines in the enzyme active site has been confirmed, it is now known that the conserved active site residues throughout the entire family of sulphatases do not include tyrosine and so some other residue of similar pK_a must therefore be responsible. Since the pK_a of the hydroxyl groups of the *gem*-diol residue should be comparable to that of acetaldehyde hydrate at 13.6 [42], the most fitting residue in line with our current knowledge is therefore lysine (e.g. Lys145 and Lys318 of ASB) which has a pK_a value of ~10 in proteins [62]. We can thus reinterpret our previous findings on the pH dependence of enzyme activity and of enzyme inactivation by EMATE [25] in terms of histidine and lysine residues. A recent report which examined active site mutants of arylsulphatase A has confirmed the importance, inter alia, of the two lysine residues for sulphate group binding and a histidine residue for catalysis [43].

Two mechanisms for such random specific or non-specific sulphamoylation of active site amino acid residues are conceivable. Firstly, via a direct nucleophilic attack at the sulphur atom of 667COUMATE by an amino acid residue in the enzyme active site (Figure 10, path A). This mechanism is analogous to Figure 9 except that the sulphamate group of the inhibitor is attacked by a nucleophilic amino acid residue other than the hydrated FGly. Secondly, and more interestingly, via the formation of an electrophilic sulphonylamine species involving an E1*cB* process on the sulphamoyl group, possibly initiated by an enzyme-catalysed N-proton abstraction and stimulated by H-bonding to the bridging O-atom or coordination of the bridging O-atom to a bivalent metal ion (Figure 10, path B). With what is now known about the topology of the active sites of ASA and ASB, the attacking nucleophile in mechanism A and the N-proton abstracting amino acid residue in mechanism B could well be either one of the conserved lysine or histidine residues lining the active site of steroid sulphatase. It is impossible to imagine that the reactive sulphonylamine released as proposed in mechanism B could diffuse out of the enzyme active site and be attacked by a remote nucleophile. Given that lysine and histidine are neighbouring amino acid residues of clear importance in the catalytic mechanism, the products of inactivation would most likely be sulphamidated enzyme intermediates (Figure 11A,B) which, we propose, could be dead-end products. From literature precedent and our experience with sulphamides, e.g. oestrone 3-*N*-sulphamide [37] and some coumarin sulphamides (unpublished observation), they are

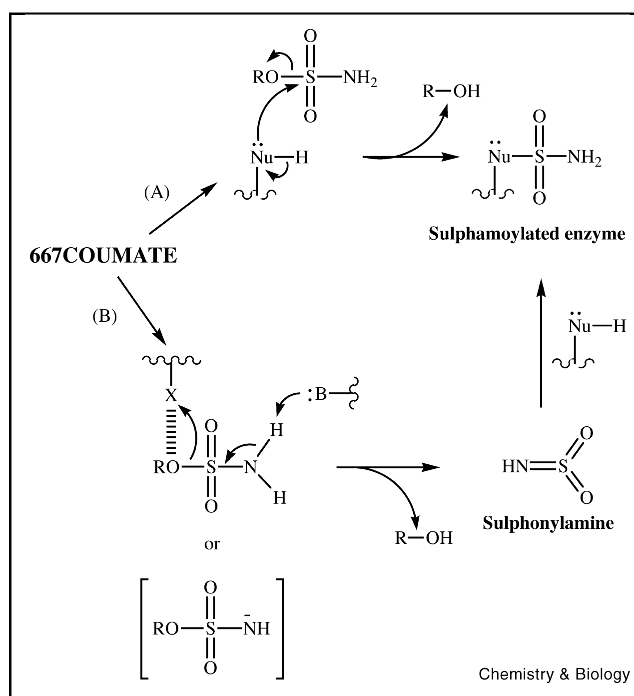


Figure 10. Proposed random specific or non-specific sulphamoylation by 667COUMATE of an essential amino acid residue in the steroid sulphatase active site. Path A: via an attack by a nucleophilic amino acid residue in the active site other than the *gem*-diol residue (:Nu-H). Path B: via the generation of a sulphonylamine species. No regeneration of the enzyme active form from the sulphamoylated intermediate is expected. :B, a proton-abstracting amino acid residue; X, a hydrogen bond-donating amino acid residue or a coordinating metal ion. Dashed line: hydrogen bonding.

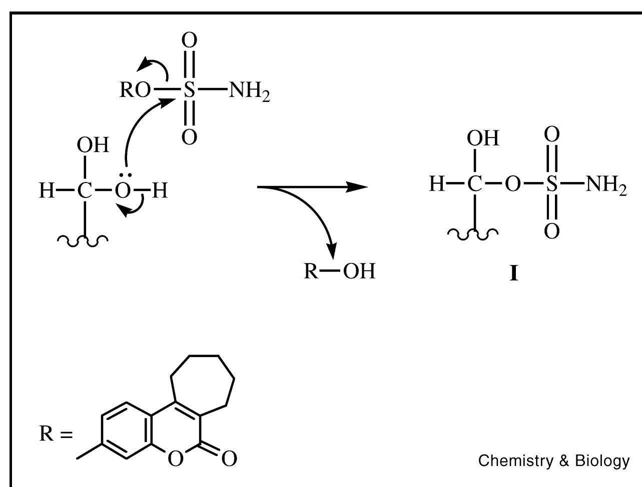


Figure 9. Proposed mechanism of steroid sulphatase inhibition by 667COUMATE via a nucleophilic attack on the sulphamoyl group by the hydrated form of formylglycine residue (aldehyde hydrate) in the enzyme active site. Structure **I** is proposed to be a 'dead-end' product.

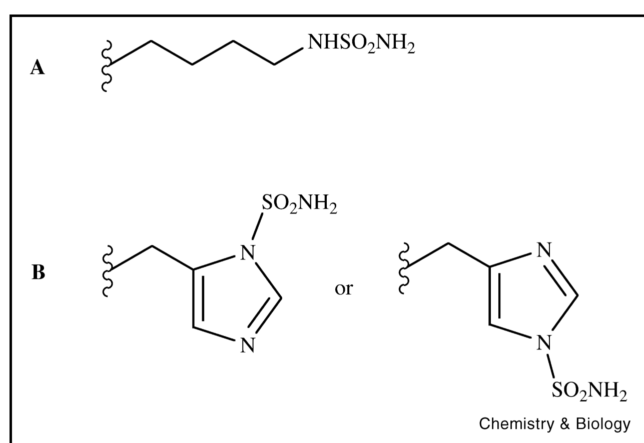


Figure 11. Proposed irreversible inactivation of steroid sulphatase via the formation of sulphamidated lysine **(A)** or histidine **(B)** residue in the enzyme active site.

inactive and stable entities and are therefore excellent candidates for a dead-end product of enzyme inactivation, more so than the proposed *gem*-diol sulphamate of FGly (**I**, Figure 9).

Since the isolation of a sulphamidated steroid sulphatase after treatment of the enzyme with an irreversible inhibitor like 667COUMATE has not yet been achieved, we felt it would be pertinent to the above proposed mechanisms if a transfer of sulphamoyl group from a coumarin sulphamate to an amine mimicking lysine or histidine residue could be demonstrated. Hence, experiments were performed in which a solution of coumarin sulphamate in anhydrous acetonitrile was stirred at room temperature with an excess of either imidazole (cf. histidine) or 2,2-diphenylethylamine (cf. lysine) in an atmosphere of nitrogen (see Materials and methods for details). With 2,2-diphenylethylamine used as the base, the isolated product was found to be the expected *N*-(2,2-diphenylethyl)sulphamide (**25**, $Ph_2CHCH_2NHSO_2NH_2$). Similar aminolysis reactions of various phenyl sulphamates by different types of amines in either aqueous or non-aqueous organic media have also demonstrated the formation of sulphamides as products [57,60,63–65]. Kinetic studies for these reactions have generally supported an E1*cB*-like elimination mechanism involving an sulphonylamine intermediate ($RN=SO_2$) [57,59,60,63–65]. When imidazole was used as the base, no corresponding sulphamide was detected but instead a salt (**26**) between imidazole and sulphamic acid was isolated. This result thus indicates that under our reaction conditions, upon an E1*cB*-like elimination of the sulphamoyl group, adventitious water in the solvent most likely quenches the sulphonylamine formed to give sulphamic acid which then forms a salt with imidazole. However, it is entirely possible that in a more restricted environment like that within the enzyme active site, where competing water molecules might not be in close proximity, a nearby histidine residue could well be the target of the electrophilic sulphonylamine species to give a sulphamidated enzyme intermediate as the dead-end product.

All the above mechanisms proposed for the inactivation of steroid sulphatase by an active site-directed sulphamate-based inhibitor including 667COUMATE and EMATE remain to be investigated and many questions have to be answered before any of these mechanisms can be confirmed. Nonetheless, we now have good models for understanding how inhibition of sulphatase by sulphamate esters may proceed, and these could be tested by experiment in the future once purified soluble steroid sulphatase is available.

In conclusion, a highly potent, orally active, non-steroidal, non-oestrogenic and irreversible steroid sulphatase inhibitor has been developed. Based on current knowledge of the conserved active site topology of sulphatases, several mechanisms of action for a sulphamate-based active site-directed steroid sulphatase inhibitor are proposed. With 667COUMATE now in pre-clinical development for Phase I clinical trial, this will allow the biological and/or clinical significance of steroid sulphatase inhibitors in the treatment of postmenopausal women with HDBC to be fully evaluated.

Significance

Breast cancer kills more women than any other forms of cancer. In postmenopausal women, in whom breast cancer most frequently occurs, about one-third of tumours are hormone-dependent, and their growth and development can be impeded when the tumour cells are deprived of oestrogenic stimulation. Endocrine therapy has proven to be effective in controlling hormone-dependent breast cancer (HDBC) and one important strategy is to reduce the production of oestrogens in extraglandular tissues and also within the tumour by inhibiting the conversion of androstenedione to oestrone (E1) with aromatase inhibitors. However, there is now convincing evidence that the disappointing overall response rate observed in postmenopausal women treated with the latest highly potent and selective aromatase inhibitors is due to the lack of a concurrent inhibition of oestrone sulphatase and dehydroepiandrosterone (DHEA) sulphatase. The former enzyme catalyses the hydrolysis of oestrone sulphate (E1S) to E1, whereas the latter plays an important role in regulating the production of oestrogenic steroids such as androstenediol. Hence, steroid sulphatase inhibitors, when used alone or in combination with an aromatase inhibitor, may enhance the response of hormone-dependent breast tumours to this type of endocrine therapy by reducing not only the formation of E1 from E1S but also the synthesis of oestrogenic steroids from DHEA sulphate via DHEA.

In addition to its oncological role there is now evidence that steroid sulphatase may regulate part of the immune response and that inhibition of the enzyme can modulate the immune response and have a beneficial effect in collagen-induced rheumatoid arthritis. Recent studies have also suggested that inhibition of steroid sulphatase might have a role in the treatment of Alzheimer's disease.

We have now developed a non-steroidal, non-oestrogenic, orally active and highly potent active site-directed steroid sulphatase inhibitor. This compound, 667COUMATE, which is a tricyclic coumarin-based sulphamate, is now in formal pre-clinical development for Phase I clinical trial wherein the role of steroid sulphatase inhibitors in the treatment of HDBC can be fully evaluated for the first time. With our current knowledge of the structure–activity relationship for steroid sulphatase inhibitors and also a better understanding of how these inhibitors work, it is anticipated that even more powerful steroid sulphatase inhibitors than 667COUMATE will be developed. The clinical use of these compounds will not only supplement current therapies in combating breast cancer but also allow the potential use of steroid sulphatase inhibitors in other therapeutic areas to be explored.

Materials and methods

Materials

All chemicals were either purchased from Aldrich Chemical Co. (Gillingham, Dorset, UK) or Lancaster Synthesis (Morecambe, Lancashire, UK). All organic solvents, of A.R. grade, were supplied by Fison plc (Loughborough, UK) and stored over 4 Å molecular sieves. Anhydrous dimethylformamide (DMF), used for all the sulphamoylation reactions, was purchased from Aldrich and was stored under a positive pressure of N_2 after use. Sulphamoyl chloride was prepared by an adaptation of the method of Appel and Berger [66] and was stored as a solution in toluene as described by Woo et. al. [28,37]. An appropriate volume of this solution was freshly concentrated in vacuo immediately before use.

E1S and E1 were purchased from Sigma Chemical Co. (Poole, UK). [6,7-^{3}H]E1S (specific activity, 50 Ci/mmol) and [4-^{14}C]E1 (specific activity, 52 mCi/mmol) were purchased from New England Nuclear (Boston, MA, USA).

Thin layer chromatography (TLC) was performed on pre-coated plates (Merck TLC aluminium sheets silica gel 60 F_{254}, Art. No. 5554). Product(s) and starting material were detected by viewing under UV light (all parent coumarins also give an intense purple fluorescence at 366 nm) and/or treating with a methanolic solution of phosphomolybdic acid followed by heating. Preparative TLC was performed on pre-coated plates (Merck TLC silica gel 60 F_{254}, 20×20 cm, layer thickness 2 mm, Art. No. 5717) and bands were visualised under UV light. Flash column chromatography was performed on silica gel (Sorbsil C60). IR spectra were determined by a Perkin-Elmer 782 infrared spectrophotometer, and peak positions are expressed in cm^{-1}. ^{1}H and *DEPT*-edited ^{13}C NMR spectra of compounds were recorded with a Jeol JMN-GX270 and JMN-GX400 NMR spectrometers, and chemical shifts are reported in parts per million (ppm, δ) relative to tetramethylsilane (TMS) as an internal standard. FAB mass spectra were recorded at the Mass Spectrometry Service Centre, University of Bath, using *m*-nitrobenzyl alcohol (NBA) as the matrix. Elemental analyses were performed by the Microanalysis Service, University of Bath. Melting points were determined using a Reichert-Jung Thermo Galen Kofler block and are uncorrected.

Biological studies were performed essentially as described previously [24,25,67]. For more details, see legends of individual figures or tables. To examine whether the sodium salt of 6-oxo-3-sulphooxy-8,9,10,11-tetrahydro-7*H*-cyclohepta-[*c*]][1]benzopyran (**18**, Scheme 2) could act as a substrate for E1-STS, 100 μg of the compound was incubated for 1 h with placental microsomes in the absence or presence of EMATE (10 μM). The product formed at the end of the incubation was extracted into diethyl ether. After evaporation of solvent, the residue obtained was examined by TLC using ether/methanol (2:1) as eluent. In this mobile phase, the tricyclic sulphate, **18** and the parent 3-hydroxy-6-oxo-8,9,10,11-tetrahydro-7*H*-cyclohepta-[*c*]][1]benzopyran (**16**, Scheme 2) have R_f values of 0.6 and 0.86 respectively.

pK_a determination of 667COUMATE

A 5 mM solution of 667COUMATE (**17**) in water/methanol (1:1) at room temperature was prepared and its pH read (WPA Linton Cambridge UK, CCMD625 pH meter). The titrant (50 mM KOH) was then added in equal portions. The pH was recorded after each addition when equilibrium has been reached (after stirring). The titration was completed within 10 min after the first addition of titrant. The pK_a was determined according to the procedure of Albert and Serjeant [61].

Synthesis

*4-Ethyl-7-hydroxycoumarin (**3**).* Resorcinol (1.21 g, 11.0 mmol) was dissolved in hot ethyl propionylacetate (1.52 g, 10.0 mmol). To this stirred mixture at ice-water temperature was added dropwise a mixture of trifluoroacetic acid (1.70 ml, 22.0 mmol) and concentrated sulphuric acid (2.2 ml, 22.0 mmol) at such a rate that the reaction temperature was kept below 10°C (about 30 min). The reaction mixture was then allowed to warm to room temperature and thereupon stirred for an additional 3 h before being quenched cautiously with ice-water. After stirring the suspension that formed for 1 h, the bright yellow precipitate was collected by suction filtration, washed exhaustively with water and then re-dissolved into acetone. The resulting solution was heated with activated charcoal, filtered and evaporated to give a light orange residue which was dried azeotropically with isopropyl alcohol. The crude product that obtained was purified by recrystallisation from acetone/hexane (2:3) to give **3** as creamy crystals (806 mg, 4.24 mmol, 42%); m.p. 175–178°C [literature value [68] 177°C (ethanol)]; δ_H (400 MHz, DMSO-d_6) 1.22 (3H, t, J=7.3 Hz, CH_3), 2.77 (2H, q, J=7.3 Hz, CH_2), 6.09 (1H, s, C3-H), 6.71 (1H, d, $J_{C6\text{-}H,\,C8\text{-}H}$=2.4 Hz, C8-H), 6.80 (1H, dd, $J_{C8\text{-}H,\,C6\text{-}H}$=2.4 Hz and $J_{C5\text{-}H,\,C6\text{-}H}$=8.4 Hz, C6-H), 7.64 (1H, d, $J_{C6\text{-}H,\,C5\text{-}H}$=8.8 Hz, C5-H) and 10.53 (1H, s, exchanged with D_2O, OH); MS (FAB+) *m/z* (rel. intensity) 190.8 [100, $(M+H)^+$]; MS (FAB−) *m/z* (rel. intensity) 342.8 [60, $(M-H+NBA)^-$], 188.8 [100, $(M-H)^-$]. Found: C, 69.4; H, 5.35; $C_{11}H_{10}O_3$ requires C, 69.43; H, 5.30%.

*4-Ethylcoumarin-7-O-sulphamate (**4**).* To a stirred solution of **3** (400 mg, 1.96 mmol) in anhydrous DMF (5 ml) at 0°C under an atmosphere of N_2 was added sodium hydride (60% dispersion, 78 mg, 1.96 mmol) followed by sulphamoyl chloride (3 eq.) after the evolution of hydrogen had ceased. The reaction mixture was stirred under N_2 at room temperature overnight and then poured into water (150 ml). The resulting mixture was extracted with ethyl acetate (150 ml) and the organic portion separated was washed with brine (5×100 ml), dried ($MgSO_4$), filtered and evaporated in vacuo at 40°C. The crude product that obtained was recrystallised from ethyl acetate/hexane (1:1) to give **4** as white crystals (278 mg, 1.03 mmol, 49%); m.p. 168–169°C; δ_H (400 MHz, DMSO-d_6) 1.25 (3H, t, J=7.3 Hz, CH_3), 2.85 (2H, q, J=7.3 Hz, CH_2), 6.37 (1H, s, C3-H), 7.29 (1H, dd, $J_{C8\text{-}H,\,C6\text{-}H}$=2.4 Hz and $J_{C5\text{-}H,\,C6\text{-}H}$=8.8 Hz, C6-H), 7.33 (1H, d, $J_{C6\text{-}H,\,C8\text{-}H}$=2.4 Hz, C8-H), 7.93 (1H, d, $J_{C6\text{-}H,\,C5\text{-}H}$=8.8 Hz, C5-H) and 8.24 (2H, s, exchanged with D_2O, OSO_2NH_2); MS (FAB+) *m/z* (rel. intensity) 270.1 [100, $(M+H)^+$], 191.1 [10, $(M+H-HNSO_2)^+$]; MS (FAB−) *m/z* (rel. intensity) 422.1 [15, $(M+NBA)^-$], 268.0 [100, $(M-H)^-$], 189.1 [35, $(M-H_2NSO_2)^-$]; HRMS (FAB+) *m/z* 270.04448 [$(M+H)^+$], calcd for

$C_{11}H_{12}NO_5S$ 270.04362. Found: C, 48.9; H, 4.06; N, 5.04; $C_{11}H_{11}NO_5S$ requires C, 49.06; H, 4.12; N, 5.20%.

*7-Hydroxy-4-(n-propyl)coumarin (**5**).* This was prepared from ethyl butyrylacetate (1.61 g, 10.0 mmol) in a similar manner to the preparation of **3**. The crude product that obtained was purified by recrystallisation from acetone/hexane (1:2) to give **5** as light yellow crystals (a total of 1.26 g, 6.17 mmol, 62%); m.p. 135–137°C [literature value [69] 130°C (ethanol)]; δ_H (400 MHz, DMSO-d_6) 0.97 (3H, t, J=7.5 Hz, CH_3), 1.63 (2H, sextet, J=7.5 Hz, –$CH_2\underline{CH_2}CH_3$), 2.71 (2H, t, J=7.6 Hz, –$\underline{CH_2}CH_2CH_3$), 6.08 (1H, s, C3-H), 6.71 (1H, d, J=2.4 Hz, C8-H), 6.80 (1H, dd, J=2.4 and 8.4 Hz, C6-H), 7.65 (1H, d, J=8.5 Hz, C5-H) and 10.53 (1H, s, exchanged with D_2O, OH); MS (FAB+) *m/z* (rel. intensity) 408.7 [15, $(2M)^+$], 204.8 [100, $(M+H)^+$]; MS (FAB−) *m/z* (rel. intensity) 406.8 [20, $(2M-H)^-$], 356.8 [20, $(M-H+NBA)^-$], 202.8 [100, $(M-H)^-$]. Found: C, 70.5; H, 6.00; $C_{12}H_{12}O_3$ requires C, 70.56; H, 5.93%.

*4-(n-Propyl)coumarin-7-O-sulphamate (**6**).* This was prepared from **5** (400 mg, 1.96 mmol) in a similar manner to the preparation of **4**. The crude product that obtained was purified by recrystallisation from ethyl acetate/hexane (1:1) to give **6** as white 'cotton-like' crystals (242 mg, 854 µmol, 44%); m.p. 174–178°C; TLC (chloroform/ethyl acetate, 2:1) R_f 0.32, cf. R_f 0.41 (**5**); δ_H (270 MHz, DMSO-d_6) 0.99 (3H, t, J=7.4 Hz, CH_3), 1.66 (2H, sextet, J=7.5 Hz, –$CH_2\underline{CH_2}CH_3$), 2.80 (2H, t, J=7.5 Hz, –$\underline{CH_2}CH_2CH_3$), 6.38 (1H, s, C3-H), 7.29 (1H, dd, J=2.4 and 8.6 Hz, C6-H), 7.33 (1H, d, J=2.4 Hz, C8-H), 7.95 (1H, d, J=8.6 Hz, C5-H) and 8.25 (2H, s, exchanged with D_2O, OSO_2NH_2); MS (FAB+) *m/z* (rel. intensity) 284.1 [100, $(M+H)^+$], 205.2 [8, $(M+H-HNSO_2)^+$]; MS (FAB−) *m/z* (rel. intensity) 436.1 [15, $(M-H+NBA)^-$], 282.1 [100, $(M-H)^-$], 203.1 [36, $(M-H_2NSO_2)^-$]; HRMS (FAB+) *m/z* 284.05950 [$(M+H)^+$], calcd for $C_{12}H_{14}NO_5S$ 284.05927. Found: C, 50.8; H, 4.64; N, 4.81; $C_{12}H_{13}NO_5S$ requires C, 50.87; H, 4.63; N, 4.95%.

*3-Ethyl-7-hydroxy-4-methylcoumarin (**7**).* This was prepared from ethyl 2-ethylacetoacetate (1.63 g, 10.0 mmol) in a similar manner to the preparation of **3**. The crude product that obtained was purified by recrystallisation from isopropyl alcohol/hexane (3:5) to give **7** as white crystals (a total of 1.24 g, 6.07 mmol, 61%); m.p. 201–203°C [literature value [70] 196–197°C]; δ_H (400 MHz, DMSO-d_6) 1.03 (3H, t, J=7.5 Hz, CH_3), 2.36 (3H, s, C4-CH_3), 2.54 (2H, q, J=7.3 Hz, CH_2), 6.68 (1H, d, J=2.4 Hz, C8-H), 6.79 (1H, dd, J=2.4 and 8.4 Hz, C6-H), 7.60 (1H, d, J=8.8 Hz, C5-H) and 10.38 (1H, br s, exchanged with D_2O, OH); MS (FAB+) *m/z* (rel. intensity) 409.2 [10, $(2M+H)^+$], 205.2 [100, $(M+H)^+$]; MS (FAB−) *m/z* (rel. intensity) 407.2 [16, $(2M-H)^-$], 357.2 [36, $(M+NBA)^-$], 203.2 [100, $(M-H)^-$]; HRMS (FAB+) *m/z* 205.08669 [$(M+H)^+$], calcd for $C_{12}H_{13}O_3$ 205.08647. Found: C, 70.2; H, 5.93; $C_{12}H_{12}O_3$ requires C, 70.56; H, 5.93%.

*3-Ethyl-4-methylcoumarin-7-O-sulphamate (**8**).* This was prepared from **7** (400 mg, 1.96 mmol) in a similar manner to the preparation of **4**. The crude product that obtained was purified by recrystallisation from ethyl acetate/hexane (1:1) to give **8** as white crystals (204 mg, 720 µmol, 37%); m.p. 179–181°C; δ_H (270 MHz, DMSO-d_6) 1.06 (3H, t, J=7.5 Hz, CH_3), 2.44 (3H, s, C4-CH_3), 2.61 (2H, q, J=7.5 Hz, CH_2), 7.28 (2H, m, C6-H and C8-H), 7.89 (1H, d, J=8.4 Hz, C5-H) and 8.21 (2H, br s, exchanged with D_2O, OSO_2NH_2); MS (FAB+) *m/z* (rel. intensity) 284.1 [100, $(M+H)^+$], 205.1 [14, $(M+H-HNSO_2)^+$]; MS (FAB−) *m/z* (rel. intensity) 436.1 [12, $(M-H+NBA)^-$], 282.1 [100, $(M-H)^-$], 203.1 [28, $(M-H_2NSO_2)^-$]; HRMS (FAB+) *m/z* 284.05984 [$(M+H)^+$], calcd for $C_{12}H_{14}NO_5S$ 284.05927. Found: C, 50.9; H, 4.67; N, 4.80; $C_{12}H_{13}NO_5S$ requires C, 50.87; H, 4.63; N, 4.95%.

*Ethyl 2-(n-propyl)acetoacetate (**9**).* Keto ester **9** was synthesised according to the method of Barbry et al. [71]. A mixture of potassium carbonate (14 g, 0.10 mol), ethyl acetoacetate (4.3 g, 33 mmol), 1-iodopropane (5.7 g, 33.7 mmol), tetrabutylammonium chloride hydrate (10 g), water (50 ml) and chloroform (50 ml) was stirred at room temperature for 40 h. The aqueous layer was then separated, acidified with 5 M hydrochloric acid and extracted with ether. The organic portions were combined, concentrated and the residual tetrabutylammonium chloride was precipitated by addition of ether. After filtration, the ethereal filtrate was dried ($MgSO_4$) and evaporated to give a yellow liquid which was fractionated by distillation. The fraction which boiled at 93°C at ∼ 15 mm Hg [literature value [72] 106–107°C, 22 mm Hg] was collected (2.13 g) and ^1H NMR has indicated that this colourless liquid contained about 5% of ethyl acetoacetate and 95% of **9**; δ_H (270 MHz, $CDCl_3$) 0.93 (3H, t, J=7.3 Hz, CH_3), 1.28 (5H, m, –$CH_2\underline{CH_2}CH_3$ and CH_3), 1.83 (2H, m, –$\underline{CH_2}CH_2CH_3$), 2.23 (3H, s, CH_3CO), 3.42 (1H, t, J=7.3 Hz, –CH) and 4.20 (2H, q, J=7.3 Hz, $COOC\underline{H_2}CH_3$); MS (EI) *m/z* (rel. intensity) 172.1 (25, M^+), 143.1 [50, $(M-Et)^+$], 130.1 [28, $(M+H-CH_3CO)^+$], 115.1 (23), 101.1 (40), 85.0 (20), 69.0 (30), 55.0 (30), 43.0 [100, $(CH_3CO)^+$]. This crude **9** was used for the next reaction without further purification.

*7-Hydroxy-4-methyl-3-(n-propyl)coumarin (**10**).* This was prepared from **9** (1.72 g) in a similar manner to the preparation of **3**. The yellow/brown crude product that obtained was fractionated by flash chromatography (chloroform/acetone, 20:1 to 1:1 gradient). The product isolated was further purified by recrystallisation from ethyl acetate/hexane (2:3) to give **10** as creamy crystals (813 mg, 3.73 mmol, ca. 37%); m.p. 160–173°C [literature value [73] 171–173°C (aq. ethanol)]; δ_H (400 MHz, DMSO-d_6) 0.92 (3H, t, J=7.3 Hz, CH_3), 1.45 (2H, sextet, J=7.5 Hz, –$CH_2\underline{CH_2}CH_3$), 2.36 (3H, s, C4-CH_3), 2.51 (2H, t, J=7.7 Hz, –$\underline{CH_2}CH_2CH_3$), 6.67 (1H, d, J=2.4 Hz, C8-H), 6.79 (1H, dd, J=2.4 and 8.4 Hz, C6-H), 7.60 (1H, d, J=8.8 Hz, C5-H) and 10.38 (1H, br s, exchanged with D_2O, OH); MS (FAB+) *m/z* (rel. intensity) 437.2 [10, $(2M+H)^+$], 219.2 [100, $(M+H)^+$]; MS (FAB−) *m/z* (rel. intensity) 435.2 [12, $(2M-H)^-$], 371.1 [15, $(M+NBA)^-$], 217.1 [100, $(M-H)^-$]; HRMS (FAB+) *m/z* 219.10300 [$(M+H)^+$], calcd for $C_{13}H_{15}O_3$ 219.10212. Found: C, 71.7; H, 6.49; $C_{13}H_{14}O_3$ requires C, 71.53; H, 6.47%.

*4-Methyl-3-(n-propyl)coumarin-7-O-sulphamate (**11**).* This was prepared from **10** (300 mg, 1.38 mmol) in a similar manner to the preparation of **4**. The grey syrupy crude product that obtained was fractionated by flash chromatography (chloroform/ethyl acetate, 20:1 to 1:1 gradient). The product that isolated was further purified by recrystallisation from ethyl acetate/hexane (1:3) to give **11** as white crystals (137 mg, 461 µmol, 34%); m.p. 139–142°C; δ_H (270 MHz, DMSO-d_6) 0.93 (3H, t, J=7.3 Hz, CH_3), 1.48 (2H, m, –$CH_2\underline{CH_2}CH_3$), 2.44 (3H, s, C4-CH_3), 2.58 (2H, t, J=7.5 Hz, –$\underline{CH_2}CH_2CH_3$), 7.27 (2H, m, C6-H and C8-H), 7.89 (1H, d, J=8.4 Hz, C5-H) and 8.21 (2H, s, exchanged with D_2O, OSO_2NH_2); MS (FAB+) *m/z* (rel. intensity) 298.1 [100, $(M+H)^+$]; MS (FAB−) *m/z* (rel. intensity) 593.2 [25, $(2M-H)^-$], 296.1 [100, $(M-H)^-$], 217.1 [45, $(M-HNSO_2)^-$]; HRMS (FAB+) *m/z* 298.07332 [$(M+H)^+$], calcd for $C_{13}H_{16}NO_5S$ 298.07492. Found: C, 52.4; H, 5.08; N, 4.67; $C_{13}H_{15}NO_5S$ requires C, 52.51; H, 5.09; N, 4.71%.

*7-Hydroxy-4-oxo-2,3-dihydro-1H-cyclopenta-[c]][1]benzopyran (**12**).* This was prepared from methyl 2-oxocyclopentane carboxylate (1.47 g, 10.0 mmol) in a similar manner to the preparation of **3**. The crude product that obtained was purified by recrystallisation from isopropyl alcohol/hexane (1:1) to give **12** as pale pink/white crystals (1.40 g, 6.92 mmol, 69%); m.p. 248–251°C [literature value [74] 243.5°C (aq. ethanol)]; δ_H (270 MHz, DMSO-d_6) 2.09 (2H, quintet, J∼7.6 Hz, C2-H_2), 2.71 (2H, t, J∼7.3 Hz, C3-H_2), 3.02 (2H, t, J∼7.7 Hz, C1-H_2), 6.74 (1H, d, $J_{C8-H, C6-H}$=2.2 Hz, C6-H), 6.79 (1H, dd, $J_{C6-H, C8-H}$=2.2 Hz and $J_{C9-H, C8-H}$∼8.4 Hz, C8-H), 7.43 (1H, d, $J_{C8-H, C9-H}$=8.4 Hz, C9-H) and 10.43 (1H, br s, exchanged with D_2O, OH); MS (FAB+) *m/z* (rel. intensity) 203.2 [100, $(M+H)^+$]; MS (FAB−) *m/z* (rel. intensity) 355.2 [36, $(M+NBA)^-$], 201.1 [100, $(M-H)^-$]. Found: C, 71.0; H, 4.90; $C_{12}H_{10}O_3$ requires C, 71.26; H, 4.99%.

*4-Oxo-2,3-dihydro-1H-cyclopenta-[c]][1]benzopyran-7-O-sulphamate (**13**).* This was prepared from **12** (500 mg, 2.47 mmol) in a similar manner to the preparation of **4**. The crude product that obtained was purified by recrystallisation from acetone/hexane (3:5) to give **13** as white crystals (403 mg, 1.43 mmol, 58%); m.p. 193–195°C; δ_H (400 MHz, acetone-d_6) 2.22 (2H, quintet, $J\sim$7.6 Hz, C2-H_2), 2.84 (2H, m, C3-H_2), 3.15 (2H, m, C1-H_2), 7.29 (1H, dd, $J_{C6\text{-}H,\,C8\text{-}H}\sim$2.2 Hz and $J_{C9\text{-}H,\,C8\text{-}H}$ = 8.7 Hz, C8-H), 7.33 (1H, d, $J_{C8\text{-}H,\,C6\text{-}H}$ = 2.1 Hz, C6-H), 7.35 (2H, br s, exchanged with D_2O, OSO_2NH_2) and 7.67 (1H, d, $J_{C8\text{-}H,\,C9\text{-}H}$ = 8.2 Hz, C9-H); MS (FAB+) *m/z* (rel. intensity) 282.0 [100, $(M+H)^+$], 202.1 [10, $(M-HNSO_2)^+$]; MS (FAB−) *m/z* (rel. intensity) 434.1 [22, $(M+NBA)^-$], 280.1 [100, $(M-H)^-$], 201.1 [26, $(M-H_2NSO_2)^-$]; HRMS (FAB+) *m/z* 282.04493 [$(M+H)^+$], calcd for $C_{12}H_{12}NO_5S$ 282.04362. Found: C, 51.3; H, 3.84; N, 4.90; $C_{12}H_{11}NO_5S$ requires C, 51.24; H, 3.94; 4.98%.

*3-Hydroxy-6-oxo-7,8,9,10-tetrahydro-dibenzo[b,d]pyran (**14**).* This was prepared from ethyl 2-cyclohexanone carboxylate (1.79 g, 10.0 mmol) in a similar manner to the preparation of **3**. The crude product that obtained was purified by recrystallisation from isopropyl alcohol/hexane (3:2) to give **14** as creamy crystals (1.45 g, 6.71 mmol, 67%); m.p. 200–202°C [literature value [75] 203–204°C (aq. ethanol)]; δ_H (270 MHz, DMSO-d_6) 1.74 (4H, m, C8-H_2 and C9-H_2), 2.38 (2H, m, C7-H_2), 2.74 (2H, m, C10-H_2*), 6.69 (1H, d, $J_{C2\text{-}H,\,C4\text{-}H}$ = 2.2 Hz, C4-H), 6.78 (1H, dd, $J_{C4\text{-}H,\,C2\text{-}H}$ = 2.4 Hz and $J_{C1\text{-}H,\,C2\text{-}H}$ = 8.6 Hz, C2-H), 7.53 (1H, d, $J_{C2\text{-}H,\,C1\text{-}H}$ = 8.4 Hz, C1-H*) and 10.34 (1H, s, exchanged with D_2O, OH); δ_C (100.4 MHz, DMSO-d_6) 20.77 (t), 21.16 (t), 23.39 (t), 24.51 (t), 101.83 (d), 111.87 (s), 112.56 (d), 118.34 (s), 124.93 (d), 147.58 (s), 152.94 (s), 159.76 (s) and 160.90 (s); MS (FAB+) *m/z* (rel. intensity) 217.2 [100, $(M+H)^+$]; MS (FAB−) *m/z* (rel. intensity) 369.2 [30, $(M+NBA)^-$], 215.1 [100, $(M-H)^-$]. Found: C, 72.0; H, 5.60; $C_{13}H_{12}O_3$ requires C,72.21; H, 5.59%. *An NOE interaction was observed between these protons.

*6-Oxo-7,8,9,10-tetrahydro-dibenzo[b,d]pyran-3-O-sulphamate (**15**).* This was prepared from **14** (300 mg, 1.39 mmol) in a similar manner to the preparation of **4**. The crude product that obtained was purified by recrystallisation from acetone/hexane (1:2) to give **15** as white crystals (204 mg, 691 μmol, 50%); m.p. 184.5–186.5°C; δ_H (270 MHz, acetone-d_6) 1.84 (4H, m, C8-H_2 and C9-H_2), 2.50 (2H, m, C7-H_2), 2.85 (2H, m, C10-H_2), 7.3 (4H, m, 2H exchanged with D_2O, C2-H, C4-H and OSO_2NH_2) and 7.78 (1H, d, $J_{C2\text{-}H,\,C1\text{-}H}\sim$8 Hz, C1-H); MS (FAB+) *m/z* (rel. intensity) 296.3 [100, $(M+H)^+$], 217.2 [13, $(M+H-HNSO_2)^+$]; MS (FAB−) *m/z* (rel. intensity) 448.1 [14, $(M+NBA)^-$], 294.1 [100, $(M-H)^-$], 215.1 [28, $(M-H_2NSO_2)^-$]; HRMS (FAB+) *m/z* 296.05921 [$(M+H)^+$], calcd for $C_{13}H_{14}NO_5S$ 296.05927. Found: C, 53.0; H, 4.50; N, 4.60; $C_{13}H_{13}NO_5S$ requires C, 52.87; H, 4.44; N, 4.75%.

*3-Hydroxy-6-oxo-8,9,10,11-tetrahydro-7H-cyclohepta-[c]][1]benzopyran (**16**).* This was prepared from methyl 2-oxocycloheptane carboxylate (1.72 g, 10.0 mmol) in a similar manner to the preparation of **3**. The crude product that obtained was purified by recrystallisation from ethyl acetate/hexane (7:4) to give **16** as creamy crystals (1.47 g, 6.38 mmol, 64%); m.p. 189–190°C [literature value [76] 188.5–189.5°C (ethanol)]; δ_H (400 MHz, DMSO-d_6) 1.49 (2H, m), 1.58 (2H, m), 1.83 (2H, m), 2.76 (2H, m, C7-H_2), 2.93 (2H, m, C11-H_2), 6.70 (1H, d, J = 2.4 Hz, C4-H), 6.78 (1H, dd, J = 2.4 and 8.7 Hz, C2-H), 7.71 (1H, d, J = 8.8 Hz, C1-H) and 10.41 (1H, br s, exchanged with D_2O, OH); MS (FAB+) *m/z* (rel. intensity) 461.2 [13, $(2M+H)^+$], 231.1 [100, $(M+H)^+$]; MS (FAB−) *m/z* (rel. intensity) 383.3 [25, $(M+NBA)^-$], 229.2 [100, $(M-H)^-$]. Found: C, 73.1; H, 6.16; $C_{14}H_{14}O_3$ requires C, 73.01; H, 6.13%.

*6-Oxo-8,9,10,11-tetrahydro-7H-cyclohepta-[c]][1]benzopyran-3-O-sulphamate (**17**).* This was prepared from **16** (400 mg, 1.74 mmol) in a similar manner to the preparation of **4**. The crude product that obtained was fractionated by flash chromatography (chloroform/ethyl acetate, 8:1 to 2:1, gradient) and the product that isolated was further purified by recrystallisation from ethyl acetate/hexane (3:5) to give **17** as white crystals (310 mg, 1.00 mmol, 58%); m.p. 169–171°C; TLC (chloroform/ethyl acetate, 4:1) R_f 0.33, cf. R_f 0.52 (**18**); IR (KBr) 3310, 3060, 2930, 2860, 1690, 1610, 1390, 1190; δ_H (270 MHz, acetone-d_6) 1.61 (2H, m), 1.70 (2H, m), 1.94 (2H, m), 2.90 (2H, m, C7-H_2), 3.07 (2H, m, C11-H_2), 7.29 (2H, m, C2-H and C4-H), 7.36 (2H, br s, exchanged with D_2O, OSO_2NH_2) and 7.95 (1H, d, $J\sim$9 Hz, C1-H); MS (FAB+) *m/z* (rel. intensity) 310.1 [100, $(M+H)^+$], 230.1 [10, $(M-HNSO_2)^+$]; MS (FAB−) *m/z* (rel. intensity) 462.3 [15, $(M+NBA)^-$], 308.2 [100, $(M-H)^-$], 229.2 [36, $(M-H_2NSO_2)^-$]; HRMS (FAB+) *m/z* 310.07529 [$(M+H)^+$], calcd for $C_{14}H_{16}NO_5S$ 310.07492. Found: C, 54.35; H, 4.96; N, 4.53; $C_{14}H_{15}NO_5S$ requires C, 54.36; H, 4.89; N, 4.53%.

*6-Oxo-3-sulphooxy-8,9,10,11-tetrahydro-7H-cyclohepta-[c]][1]-benzopyran (**18**).* To a stirred solution of **16** (100 mg, 434 μmol) in sodium hydroxide ($\sim$1 M, 1.5 ml) at room temperature was added sulphur trioxide–trimethylamine complex (126 mg, 868 μmol). After being stirred for 3 days, the reaction mixture was fractionated by flash chromatography (ether; then ether/methanol, 4:1 to 1:1, gradient) to give **18** as dull white residue (110 mg); TLC (ether/methanol, 2:1) R_f 0.6, cf. R_f 0.86 (**16** in $\sim$1 M NaOH); δ_H (400 MHz, DMSO-d_6) 1.50 (2H, m), 1.60 (2H, m), 1.84 (2H, m), 2.79 (2H, m, C7-H_2), 2.98 (2H, m, C11-H_2), 7.15 (1H, dd, J = 2.3 and 8.7 Hz, C2-H), 7.18 (1H, d, J = 2.1 Hz, C4-H) and 7.81 (1H, d, J = 8.8 Hz, C1-H); MS (FAB−) *m/z* (rel. intensity) 641.1 [18, $(2M-Na)^-$], 485.1 [16, $(M-H+NBA)^-$], 309.1 [100, $(M-Na)^-$], 229.1 [26, $(M-Na-SO_3)^-$]; HRMS (FAB−) *m/z* 309.04310 [$(M-Na)^+$], calcd for $C_{14}H_{13}O_6S$ 309.04329.

*3-Hydroxy-6-oxo-7,8,9,10,11,12-hexahydro-cycloocta-[c]][1]benzopyran (**19**).* This was prepared from methyl 2-oxocyclooctane carboxylate (1.02 g, 5.0 mmol) and resorcinol (610 mg, 5.5 mmol) in a similar manner to the preparation of **3**. After quenching the reaction mixture with ice-water, the crude product was extracted into ethyl acetate (150 ml). The organic portion which separated was then further washed with brine (5×100 ml), dried ($MgSO_4$), filtered and evaporated. The orange liquid/solid obtained was then fractionated by flash chromatography (ethyl acetate/hexane, 1:4 to 2:1 gradient) and **19** that isolated (405 mg, 1.66 mmol, 33%) was recrystallised from ethyl acetate/hexane (5:2) to give fine white crystals (256 mg); TLC (ethyl acetate/hexane, 1:1) R_f 0.42, cf. $R_f > 0.7$ (keto ester); m.p. 232–234°C; δ_H (400 MHz, DMSO-d_6) 1.38 (2H, m), 1.49 (2H, m), 1.60 (2H, m), 1.74 (2H, m), 2.71 (2H, t, $J\sim$5.6 Hz, C7-H_2), 2.99 (2H, t, $J\sim$6.3 Hz, C12-H_2), 6.72 (1H, d, J = 2.4 Hz, C4-H), 6.81 (1H, dd, J = 2.3 and 8.7 Hz, C2-H), 7.66 (1H, d, J = 8.8 Hz, C1-H) and 10.42 (1H, br s, exchanged with D_2O, OH); MS (FAB+) *m/z* (rel. intensity) 245.1 [100, $(M+H)^+$]; MS (FAB−) *m/z* (rel. intensity) 397.3 [30, $(M+NBA)^-$], 243.2 [100, $(M-H)^-$]. Found: C, 73.6; H, 6.67; $C_{15}H_{16}O_3$ requires C, 73.74; H, 6.61%. About 500 mg of the starting keto ester was retrieved.

*6-Oxo-7,8,9,10,11,12-hexahydro-cycloocta-[c]][1]benzopyran-3-O-sulphamate (**20**).* This was prepared from **19** (421 mg, 1.72 mmol) in a similar manner to the preparation of **4**. The crude product that obtained was fractionated by flash chromatography (chloroform/ethyl acetate, 8:1 to 4:1, gradient) and the product that isolated was further purified by recrystallisation from ethyl acetate/hexane (1:2) to give **20** as white crystals (305 mg, 943 μmol, 55%); m.p. 174–175.5°C; δ_H (270 MHz, acetone-d_6) 1.47 (2H, m), 1.55 (2H, m), 1.68 (2H, m), 1.86 (2H, m), 2.84 (2H, t, $J\sim$6.5 Hz, C7-H_2), 3.10 (2H, t, $J\sim$6.5 Hz, C12-H_2), 7.28 (2H, m, C2-H and C4-H), 7.37 (2H, br s, exchanged with D_2O, OSO_2NH_2) and 7.93 (1H, d, $J\sim$9 Hz, C1-H); MS (FAB+) *m/z* (rel. intensity) 324.1 [100, $(M+H)^+$], 244.1 [13, $(M-HNSO_2)^+$]; MS (FAB−) *m/z* (rel. intensity) 476.3 [64, $(M+NBA)^-$], 323.2 (58, M^-), 243.3 [100, $(M-H_2NSO_2)^-$]; HRMS (FAB+) *m/z* 324.08971 [$(M+H)^+$], calcd for

$C_{15}H_{18}NO_5S$ 324.09057. Found: C, 55.8; H, 5.39; N, 4.39; $C_{15}H_{17}NO_5S$ requires C, 55.71; H, 5.30; N, 4.33%.

*3-Hydroxy-6-oxo-6,7,8,9,10,11-hexahydrodibenz[b,d]oxepin (**21**).* This was prepared from ethyl (2-oxocyclohexyl)acetate (1.90 g, 10.0 mmol) in a similar manner to the preparation of **3**. The brown crude product that obtained was fractionated by flash chromatography to give **21** as a creamy residue (439 mg, 1.91 mmol, 19%); TLC (chloroform/ethyl acetate, 4:1) R_f 0.46, cf. 0.19 (resorcinol); MS (FAB+) *m/z* (rel. intensity) 461.3 [12, $(2M+H)^+$], 230.1 (100, M^+); MS (FAB−) *m/z* (rel. intensity) 383.2 [40, $(M-H+NBA)^-$], 229.2 [100, $(M-H)^-$]; HRMS (FAB+) *m/z* 230.09451 $(M)^+$, calcd for $C_{14}H_{14}O_3$ 230.09429. The ^1H NMR spectrum of **21** in $CDCl_3$ agrees with that reported by Hua et al. [77]. The phenolic **21** was sulphamoylated without further purification.

*6-Oxo-6,7,8,9,10,11-hexahydrodibenz[b,d]oxepin-3-O-sulphamate (**22**).* This was prepared from **21** (389 mg, 1.69 mmol) in a similar manner to the preparation of **4**. The brown syrup that obtained was fractionated by flash chromatography (chloroform/ethyl acetate, 8:1 to 2:1, gradient) and the product that isolated was further purified by recrystallisation from ethyl acetate/hexane (1:1) to give **22** as white crystals (186 mg, 601 μmol, 36%); m.p. 183–186°C; δ_H (400 MHz, acetone-d_6) 1.76 (4H, m, C9-H_2 and C10-H_2), 2.35 (2H, br s, CH_2), 2.53 (2H, v br s, CH_2), 2.85 (2H, s, C7-H_2), 7.17 (1H, d, $J=2.4$ Hz, C4-H), 7.24 (3H, reduced to 1H upon exchange with D_2O, dd, $J=2.6$ and 8.7 Hz, C2-H and OSO_2NH_2) and 7.65 (1H, d, $J=8.8$ Hz, C1-H). Found: C, 54.5; H, 4.97; N, 4.54; $C_{14}H_{15}NO_5S$ requires C, 54.36; H, 4.89; N, 4.53%.

*N-(2,2-Diphenylethyl)sulphamide (**25**).* To a stirred solution of 4-(*n*-propyl)coumarin-7-*O*-sulphamate (**6**) (100 mg, 353 μmol) in anhydrous acetonitrile under nitrogen at room temperature was added 2,2-diphenylethylamine (4 eq.) and the progress of the reaction was monitored by TLC (products were detected by treating with a methanolic solution of phosphomolybdic acid followed by heating). The disappearance of **6** was completed after 48 h with the formation of 7-hydroxycoumarin-4-(*n*-propyl)coumarin (**5**) and sulphamide **25**. Upon evaporation of the reaction mixture in vacuo, the light yellow syrup that resulted in ethyl acetate (50 ml) was washed with dilute hydrochloric acid (2×30 ml) and then water to neutral. The organic layer was left to evaporate in the fume cupboard and the creamy residue that obtained was fractionated on preparative TLC eluted with chloroform/acetone (4:1) to give sulphamide **25** (R_f 0.41, cf. R_f 0.56 for **5**) as creamy residue (52 mg, 188 μmol, 53%); IR (KBr) 3360, 3260, 1430, 1350, 1160; δ_H (270 MHz, DMSO-d_6) 3.50 (2H, t, $J\approx7$ Hz, CH_2NH), 4.22 (1H, t, $J\approx7$–8 Hz, CH), 6.47 (1H, t, $J\approx5$ Hz, exchanged with D_2O, NH), 6.57 (2H, s, exchanged with D_2O, SO_2NH_2) and 7.2–7.4 (10H, m, Ar); MS (FAB+) *m/z* (rel. intensity) 277.1 [100, $(M+H)^+$], 181.1 [97, $(M+H-H_2NSO_2NH_2)^+$], 97.1 [52, $(H_3NSO_2NH_2)^+$]; MS (FAB−) *m/z* (rel. intensity) 429.2 [65, $(M+NBA)^-$], 275.2 [100, $(M-H)^-$], 95.0 [38, $HNSO_2NH_2)^-$]; HRMS (FAB+) *m/z* 277.10133 [$(M+H)^+$], calcd for $C_{14}H_{17}N_2O_2S$ 277.10108.

*Sulphamic acid and imidazole salt (**26**).* The reaction was carried out in a similar manner to **25** except that imidazole (4 eq.) was used. White precipitate was detected after the reaction mixture was stirred for 17 h and the disappearance of coumarin sulphamate **6** was completed after 4 days. The white precipitate formed was filtered, washed several times with fresh acetonitrile and air-dried to give salt **26** as white residue (43 mg, 261 μmol, 70%); m.p. 152–154°C; IR (KBr) 3300, 3240, 3150, 3000, 2860, 1590; δ_H (400 MHz, DMSO-d_6) 7.40 (2H, s) and 8.45 (1H, s); MS (FAB+) *m/z* (rel. intensity) 375.2 [18, (imidazole+H+2NBA)$^+$], 222.1 [100, (imidazole+H+NBA)$^+$], 69.0 [16, (imidazole+H)$^+$]; MS (FAB−) *m/z* (rel. intensity) 402.0 [35, $(H_2NSO_2O+2NBA)^-$], 249.0 [100, $(H_2NSO_2O+NBA)^-$], 95.9 [65, $H_2NSO_2O^-$]. Found: C, 22.0; H, 4.29; N, 25.2; $C_3H_7N_3O_3S$ requires C, 21.82; H, 4.27; N, 25.44%. The melting point, the IR and MS spectra of **26** were comparable to those of the white solid (m.p. 148–154°C) precipitated upon addition of a solution of sulphamic acid in DMF dropwise to a solution of imidazole in acetonitrile.

Acknowledgements

This work was supported by The Cancer Research Campaign and Sterix Ltd. We thank Mr K. Smith for the molecular modelling study. We also thank Dr H.A.M. Hejaz for useful discussions.

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