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OESTRONE 3-*O*-(*N*-ACETYL)SULPHAMATE, A POTENTIAL MOLECULAR PROBE OF THE ACTIVE SITE OF OESTRONE SULPHATASE.†

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Abstract: *N,N*-Dialkylated derivatives of the steroid sulphatase inhibitor, oestrone 3-*O*-sulphamate (EMATE) are weak reversible inhibitors of the enzyme. *N*-Acetylated-EMATE (**8**), but not the benzoyl derivative, inhibits the enzyme irreversibly, albeit less potently than EMATE and will allow hitherto difficult radiolabelling on the sulphamate group to facilitate investigation of the enzyme inactivation mechanism. © 1997 Elsevier Science Ltd.

The contribution of oestrogens to the growth of breast tumours has long been recognized.[1] In postmenopausal women, in whom breast cancer most frequently occurs at a time when the production of oestrogens by the ovaries has ceased, oestrogens continue to be produced extraglandularly in adipose tissue, and also in normal and malignant breast tissues. It was originally thought that oestrogens within breast tumour originated mainly via the aromatase pathway, where the androgen precursor androstenedione can be converted

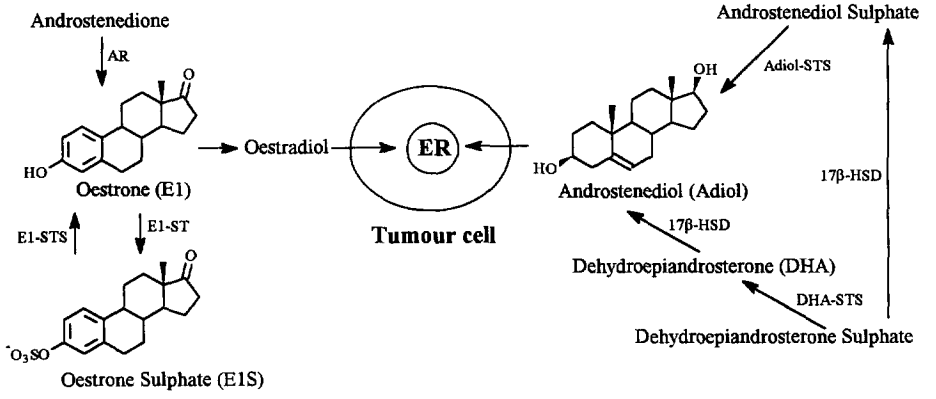

Fig. 1 The origin of oestrogenic steroids in postmenopausal women. AR, aromatase; ST, sulphotransferase STS, sulphatase; 17β–HSD, 17β-hydroxysteroid dehydrogenase; ER, oestrogen receptor.

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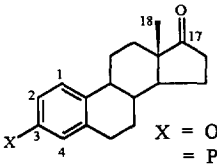
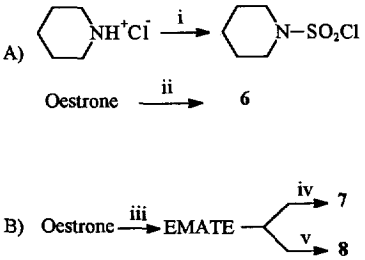
into oestrone (E1) by aromatase (Fig. 1). However, there is now growing evidence to suggest that oestrone sulphate (E1S) in plasma and tissues acts as a reservoir for the formation of E1 by the action of oestrone sulphatase (E1-STS) and that the E1-STS pathway (Fig. 1) is the major source of breast tumour oestrogen,[2,3] accounting for the high concentrations of oestrogens in such tissues. The importance of the E1-STS pathway is reflected by the disappointing results with aromatase inhibitors in recent clinical studies.[4-8]

Oestrone 3-*O*-sulphamate (EMATE, Fig. 2) is the most potent steroidal E1-STS inhibitor synthesized to date. *In vitro*, EMATE inhibits E1-STS activity by > 99% at 0.1 μM in intact MCF-7 breast cancer cells and in a time- and concentration-dependent manner in placental microsomes preparation, indicating that it acts as an irreversible inhibitor.[9,10] Subsequent studies have also shown that EMATE inhibits dehydroepiandrosterone sulphatase (DHA-STS),[10,11] the enzyme which regulates the biosynthesis of the oestrogenic steroid androstenediol (Adiol, Fig. 1). There is now strong evidence to suggest that androstenediol may be of considerable importance as a promotor of breast tumour growth.[12] EMATE is active *in vivo*, inhibiting rat liver E1-STS and DHA-STS activities almost completely when given either orally or subcutaneously.[13] Thus, it is anticipatated that steroid sulphatase inhibitors like EMATE, when used alone or complemented by aromatase inhibitors in treating hormone-dependent breast tumours, will render oestrogen ablation and hence tumour regression more effectively by reducing not only the formation of E1 from E1S but also the synthesis of Adiol from dehydroepiandrosterone sulphate.



X =	OSO_2NH_2	EMATE
=	PS(OH)Me	1
=	$NHSO_2NH_2$	2
=	SSO_2NH_2	3
=	OSO_2NHMe	4
=	OSO_2NMe_2	5
=	OSO_2N⬡	6
=	$OSO_2N(CH_2Ph)_2$	7
=	$OSO_2NHCOMe$	8
=	$OSO_2NHCOPh$	9
=	OSO_2NHEt	10
=	OSO_2NHCH_2Ph	11
=	$OSO_2NHCOEt$	12

Fig. 2 Structures of compounds **1** - **12**.



Scheme 1

A) Synthesis of *N*-piperidinosulphamoyl chloride and **6**.
i, SO_2Cl_2, 45°C, 24 h;
ii, NaH/DMF, *N*-piperidinosulphamoyl chloride.

B) Synthesis of EMATE, **7** and **8**.
iii, NaH/DMF, H_2NSO_2Cl;
iv, NaH/DMF, benzyl bromide;
v, NaH/CH_2Cl_2, acetyl chloride.

Whilst the biological activities of EMATE have been studied extensively, relatively little is known about its structure-activity relationships. The sulphamate group of EMATE is indispensable for its inhibitory activity and the analogue oestrone 3-methylthiophosphonate (**1**, Fig. 2) was only a reversible E1-STS inhibitor.[14] It has

been shown that the high potency of EMATE and its irreversible time-dependent nature of inhibition absolutely require a bridging oxygen atom in the sulphamate group ($H_2NSO_2\underline{O}$-). Hence, oestrone 3-*N*-sulphamate (**2**, Fig. 2) and oestrone 3-*S*-sulphamate (**3**) are only weak non-time-dependent inactivators.[15] Moreover, a significant reduction in the inhibitory activity of EMATE results when the *N*-atom of the sulphamate group is increasingly methylated. Hence, in an MCF-7 cell preparation, oestrone 3-*O*-(*N*-methyl)-sulphamate (**4**, Fig 2) and oestrone 3-*O*-(*N,N*-dimethyl)sulphamate (**5**) inhibited E1-STS by 80 and 50%, respectively at 0.1 μM whereas EMATE showed > 99% inhibition at the same concentration. Both **4** and **5** were also not found to be irreversible inhibitors.[9]

Studies on the mechanism of irreversible enzyme inactivation by EMATE are presently hindered because of the difficulty of introducing a radiolabelled motif on the sulphamate group. In order to probe further the structure-activity relationships for the sulphamate group of EMATE, focus upon its tolerance to further modification, and introduce a group amenable to radiolabelling, we alkylated and acylated the *N*-atom to give oestrone 3-*O*-*N*-(piperidino)sulphamate (**6**, Fig. 2), oestrone 3-*O*-(*N,N*-dibenzyl)sulphamate (**7**) and oestrone 3-*O*-(*N*-acetyl)sulphamate (**8**). We report here the evaluation of these EMATE analogues for E1-STS inhibition.

Compound	Test Concentration (μM)	% Inhibition (Mean ± S.D., n = 3)
EMATE	0.1	50 ± 3
	0.2	82 ± 7
	1.0	95 ± 3
6	20	13 ± 4
	50	17 ± 3
	100	20 ± 5
7	20	43 ± 6
	50	63 ± 4
	100	75 ± 4
8	0.2	30 ± 2
	1.0	63 ± 3
	10	92 ± 7
	25	>99 ± 5

Table 1 Direct inhibition of oestrone sulphatase in placental microsomes by **6** - **8**, and EMATE. Assays were performed essentially as previously described.[9,14] The substrate, [^{3}H]-E1S (4 x 10^5dpm, 5 pmol) adjusted to a final concentration of 20 μM with unlabelled E1S, ± inhibitor was incubated with placental microsomes (100 μg protein) at 37^{0}C for 30 min. The product formed was isolated from the mixture by extraction with toluene (4 ml). [4 -^{14}C]-E1, (7 x 10^3 dpm) was used to monitor procedural losses.

In comparison with EMATE, both compounds **6** and **7** were weak E1-STS inhibitors although **7** was better by nearly four-fold (Table 1). None of these compounds, however, was found to be an irreversible inhibitor (data not shown) showing that *N,N*-disubstitution of the sulphamate group abolishes completely the time-dependent inhibitory action of EMATE. The *N*-acetyl derivative of EMATE, **8** was the best inhibitor, inactivating E1-STS by > 99% at 25 μM, but EMATE inhibited the enzyme almost completely at 1.0 μM

(Table 1). However, importantly, **8** exhibited time- and concentration-dependent inhibition of E1-STS in a similar biphasic manner to EMATE (Fig. 3B), although the inactivation profile was not as efficient as that for EMATE (Fig. 3A).

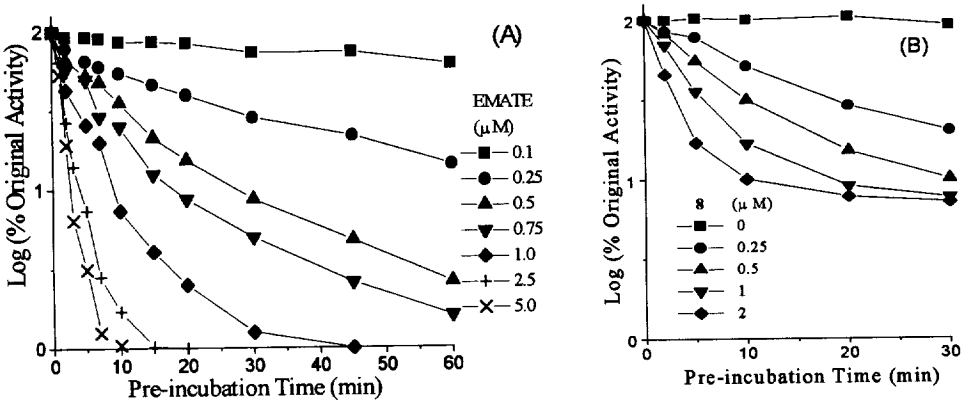


Fig. 3 Time- and concentration-dependent inactivation of oestrone sulphatase by A) EMATE and B) oestrone 3-*O*-(*N*-acetyl)sulphamate, **8**. Placental microsomes (200 μg) were preincubated with inhibitor at various concentrations for 0 - 60 min (EMATE) and 0 - 30 min (**8**) at 37°C followed by incubation 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]-E1S (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.

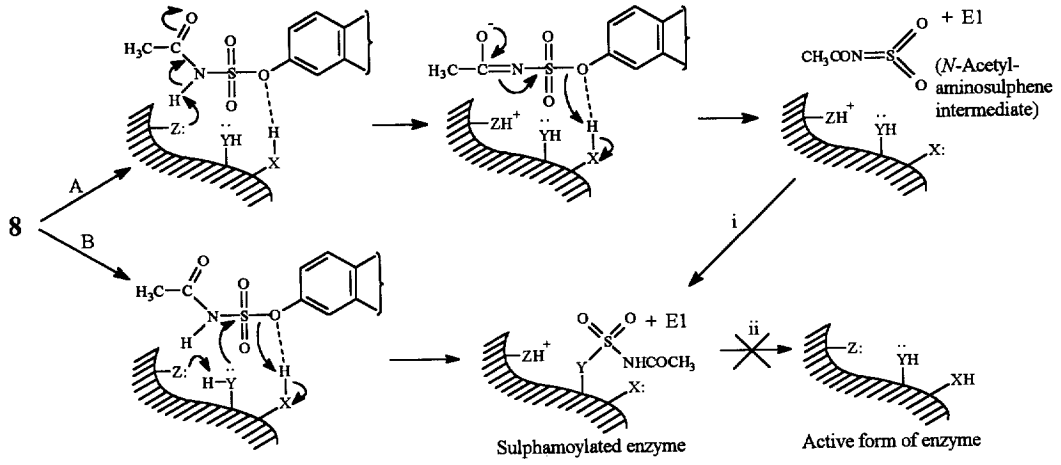


Fig. 4 Proposed mechanism of action of **8** in the inhibition of E1-STS: A) via an *N*-acetylaminosulphene intermediate, and B) via a nucleophilic attack by an amino acid residue in the active site. i) attacks by a nucleophilic amino acid residue in the active site - selective or random sulphamoylation. ii) no hydrolysis of the sulphamoylated E1-STS by water to regenerate the active form of the enzyme. X, Y and Z : amino acid residues. -------- , hydrogen bonding.

When EMATE binds to E1-STS, we propose that inactivation of the enzyme commences when either an essential amino acid residue, which may be sulphated during catalysis, or an important neighbouring residue, becomes irreversibly sulphamoylated. Our recent work, based on the analysis of the pH dependence of E1-STS activity and of enzyme inactivation by EMATE, suggested that this essential amino acid residue may be a histidine or a tyrosine.[11] It is reasonable to expect that **8** sulphamoylates E1-STS in much the same manner as EMATE (Fig. 4). Two mechanisms for this sulphamoylation are feasible: it may either be mediated by a) the formation of a reactive electrophilic *N*-acetylaminosulphene intermediate, which rapidly sulphamoylates the active site, from the anion of EMATE via an E1cb process, possibly initiated by an enzyme catalysed proton abstraction from the *N*-acetylamine on the sulphamate group (Fig. 4A); or b) a direct nucleophilic attack by an amino acid residue at the sulphur atom of the sulphamate group (Fig. 4B). Both mechanisms will lead to a sulphamoylated enzyme intermediate which, we propose, cannot be hydrolysed by water to regenerate the active form of the enzyme, in the same manner or as rapidly as, the sulphoenzyme intermediate resulting from the hydrolysis of oestrone sulphate, thus rendering the enzyme irreversibly inhibited.

On closer examination, one might expect **8** to be a better inhibitor than EMATE since the electron-withdrawing effect of the *N*-acetyl group should lower the p*K*a of the *N*-proton and hence enhances its susceptibility towards abstraction. However, since **8** was found to be a weaker time-dependent E1-STS inhibitor than EMATE, this reduction in potency may therefore be the result of the increased steric bulk of the *N*-acetyl group. The implication that an *N*-acyl derivative of EMATE can be more potent than an *N*-alkyl derivative of similar size but possess time-dependent inactivation capacity means that we can focus upon two important points in the future synthesis of other *N*-substituted analogues of EMATE, namely: Does increased steric bulk at the *N*-atom decrease activity? Does *N*-acylation facilitate a more effective irreversible enzyme sulphamoylation by lowering the p*K*a of the *N*-proton? In an attempt to deal with these questions, we synthesised oestrone 3-*O*-(*N*-benzoyl)sulphamate (**9**, Fig. 2) and preliminary results showed that **9** was *not* an irreversible inhibitor of E1-STS although it is still a reasonable reversible inhibitor. Other *N*-substituted analogues of EMATE such as **10** - **12** will undoubtedly address the above questions more effectively.

The implication that sulphamate **8** is a time- and concentration-dependent E1-STS inhibitor will allow the hitherto very difficult radiolabelling with ^{14}C or ^{3}H on the sulphamate moiety to facilitate investigation of the enzyme inactivation mechanism and demonstrate enzyme sulphamoylation unequivocally. Indeed, we have recently prepared ^{14}C-(**8**) labelled at the carbonyl carbon of the *N*-acetyl group. Hence, it should be feasible to identify the important catalytic residues of E1-STS, the amino acid composition of which can then be further elucidated by amino acid modifying agents and site-directed mutagenesis once a partially purified enzyme or an expressed enzyme preparation is available.

It is not unexpected that compounds **6** and **7** are only reversible inhibitors of E1-STS. The absence of an abstractable proton on a fully substituted sulphamate group precludes the mechanism depicted in Fig. 4A. Also,

the alternative mechanism depicted in Fig. 4B might not be operable as the substituents on the *N*-atom of **6** and **7**, through their steric bulk could have either misaligned or shielded the *S*-atom of the sulphamate group from the required position in the active site for attack by a nucleophilic amino acid residue. Thus, the weak non-time-dependent inhibition of E1-STS shown by **6** and **7** most likely results purely from a competitive interaction of these agents with the enzyme.

In summary, the synthesis and preliminary biological activity of a potential new probe of steroid sulphatase mechanism is described, which should allow a deeper understanding of the powerful new class of sulphamate-based inhibitors.

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