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The Development of A-ring Modified Analogues of Oestrone-3-*O*-sulphamate as Potent Steroid Sulphatase Inhibitors with Reduced Oestrogenicity

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Steroid sulphatases regulate the formation of oestrogenic steroids which can support the growth of endocrine-dependent breast tumours. The development of potent steroid sulphatase inhibitors could therefore have considerable therapeutic potential. Several such inhibitors have now been developed of which the most potent to date is oestrone-3-*O*-sulphamate (EMATE). Unexpectedly, this inhibitor proved to be a potent oestrogen. In an attempt to reduce the oestrogenicity, whilst retaining the potent sulphatase inhibitory properties associated with this type of molecule, a number of A-ring modified derivatives were designed and synthesized. A-ring modified compounds included the 2-methoxy, 2/4-nitro, 2/4-*n*-propyl and 2/4-allyl EMATE analogues. The ability of these derivatives to inhibit oestrone sulphatase activity was examined using placental microsomes. The allyl-substituted EMATE derivatives were more potent inhibitors than the propyl analogues but were all considerably less potent than EMATE. In contrast, the 2-methoxy and 2/4-nitro analogues were potent sulphatase inhibitors with 4-nitro EMATE being 5 times more active than EMATE. The 4-nitro, 2-methoxy, 4-*n*-propyl and 4-allyl derivatives were also tested *in vivo* for their oestrogenicity and ability to inhibit sulphatase activity. While both 4-nitro and 2-methoxy EMATE were potent inhibitors *in vivo*, 2-methoxy EMATE had no stimulatory effect on uterine growth in ovariectomized rats. The identification of a potent steroid sulphatase inhibitor lacking any oestrogenicity, such as 2-methoxy EMATE, should be of considerable value in evaluating the potential of steroid sulphatase inhibition for breast cancer therapy. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

The steroid sulphatase pathway makes an important contribution to the formation of oestrogens in breast tumours [1–3]. At least ten times as much oestrone is formed by the sulphatase pathway, from oestrone sulphate (E1S), as via the aromatase route in which oestrone is synthesized from androstenedione [4] although the precise amounts of oestrone formed *in vivo* from each pathway and in each organ remains to be established. In addition, there is an increasing awareness that aromatase inhibitors do not block the synthesis of another steroid, androst-5-ene-3β,17β-diol (Adiol) which is a potent oestrogen [5–8]. Most of the Adiol synthesized in postmenopausal women is derived from dehydroepiandrosterone (DHA) which in turn is formed from DHA-sulphate (DHA-S) [9]. There is evidence that the hydrolysis of oestrone sulphate (E1S) and DHA-S is mediated by the same sulphatase [10].

Inhibition of the steroid sulphatase pathway could therefore be of considerable therapeutic value in women with endocrine-dependent tumours of the breast or endometrium. A number of steroid sulphatase inhibitors have been developed including

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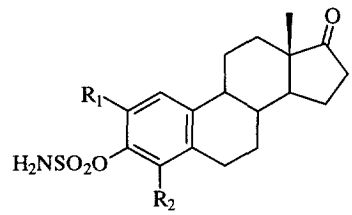
oestrone-3-*O*-methylthiophosphonate [11] and oestrone-3-sulphonyl chloride [12]. The most potent inhibitors identified so far which are active *in vivo* are a series of oestrone sulphamate derivatives of which the most active is oestrone-3-*O*-sulphamate (EMATE, 1) [13–15]. Unexpectedly, EMATE was recently found to possess potent oestrogenic properties, being five times more active than ethinyloestradiol when administered orally to rats [16]. However, the reason why EMATE is such a potent oestrogen remains to be elucidated.

Due to the sensitivity of endocrine-dependent tumours of the breast and endometrium to oestrogens, EMATE would be unsuitable for use in such conditions. In an attempt to reduce the oestrogenicity of EMATE whilst retaining the potent sulphatase inhibitory properties associated with this type of molecule, a number of modifications have been made to the A-ring of the steroid nucleus (Fig. 1). It has previously been shown that the substitution of the aromatic ring at C-2 and/or C-4 of the steroid nucleus by nitro, *n*-propyl or allyl groups greatly reduces their oestrogenicity compared with the parent compound [17, 18]. In addition, 2-methoxy EMATE was also synthesized and tested since 2-methoxyoestradiol, a natural metabolite of oestradiol, inhibits angiogenesis and suppresses tumour growth [19].

The resulting A-ring modified compounds have been tested for their ability to inhibit steroid sulphatase activity using placental microsomes. Potential lead compounds were also tested *in vivo*, not only to examine their ability to inhibit sulphatase activity, but also to test their oestrogenicity using an ovariectomized rat uterus weight gain assay.



R_1	R_2		
H	H	**1**	(EMATE)
CH_3O	H	**2**	(2-methoxy-EMATE)
NO_2	H	**3**	(2-nitro-EMATE)
H	NO_2	**4**	(4-nitro-EMATE)
$CH_3CH_2CH_2$	H	**5**	(2-n-propyl-EMATE)
H	$CH_3CH_2CH_2$	**6**	(4-n-propyl-EMATE)
$CH_3CH_2CH_2$	$CH_3CH_2CH_2$	**7**	(2,4 di-n-propyl-EMATE)
$H_2C{=}CHCH_2$	H	**8**	(2-allyl-EMATE)
H	$H_2C{=}CHCH_2$	**9**	(4-allyl-EMATE)
$H_2C{=}CHCH_2$	$H_2C{=}CHCH_2$	**10**	(2,4-diallyl-EMATE)

Fig. 1. Structures of oestrone-3-*O*-sulphamate (EMATE) and A-ring modified analogues.

MATERIALS AND METHODS

Chemical synthesis

2-Methoxy oestrone and oestrone were purchased from Sigma (Poole, Dorset, U.K.). 2-Nitro- and 4-nitro oestrones were prepared by nitrating oestrone according to the method of Tomson and Horwitz [20]. The synthesis of 2- and 4-alkyl oestrone was carried out by the method of Patton [21]. The sulphamoylation reaction was performed by reacting the parent steroids with sulphamoyl chloride after treatment with 1 eq. of sodium hydride. EMATE was synthesized as previously described [13].

All sulphamates prepared have been thoroughly characterized spectroscopically and have good combustion analyses. Full synthetic details will be reported elsewhere.

Placental microsome assay

The ability of the EMATE derivatives to inhibit sulphatase activity was examined using a placental microsomal (100,000*g*) fraction. To determine the IC_{50}s for the inhibition of oestrone sulphatase, activity was measured in the presence of inhibitor using [6,7-^{3}H]E1S (51 Ci/mmol, NEN-Dupont, Boston, MA) adjusted to 20 μM with unlabelled E1S (Sigma). After incubation of the substrate $\pm$ inhibitor with the microsomes for 1 h, the product formed from E1S was isolated with toluene. [4-^{14}C]Oestrone (1×10^4 dpm; Amersham, Aylesbury, U.K.) was used to monitor procedural losses with product formation being quantified using scintillation spectrometry. The sensitivity of this assay, which was determined by incubating ^{3}H E1S in the absence of microsomes, was $1.5 \pm 0.7\%$ of control (no inhibitor) activity.

Nature of steroid sulphatase inhibition

To investigate the nature of sulphatase inhibition by two of the EMATE analogues, intact MCF-7 cells were pre-treated with the inhibitors (at 1 and 10 μM concentrations) for 2 h at 37°C. After removal of the medium and washing the cells 5 times with phosphate buffered saline (PBS), the remaining sulphatase activity was assayed using [6,7-^{3}H]E1S (2–3 nM) as previously described [14, 22].

In vivo studies

Ovariectomized female rats (200–250 g) were obtained from Harlan-Olac (Bicester, Oxon, U.K.). 14 days after ovariectomy, groups of rats, with three rats per compound tested, were treated p.o. with vehicle (propylene glycol) or 2.0 mg/kg of inhibitor per day for 5 days. At the end of the dosing schedule ani-

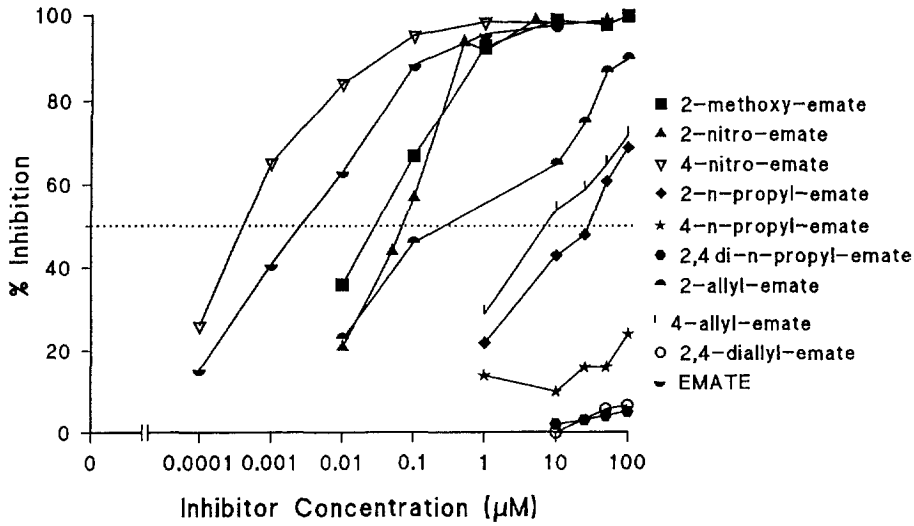


Fig. 2. Dose–response curves for inhibition of oestrone sulphatase activity in placental microsomes by oestrone-3-*O*-sulphamate (EMATE) and A-ring modified derivatives. Each point represents the mean of triplicate measurements for which the coefficients of variation were <10%.

mals were killed using an approved procedure 24 h after administration of the last dose.

Oestrone sulphatase activity in tissues

Liver tissue was obtained from rats and frozen on solid carbon dioxide and stored at −20°C until assayed [15].

Uterotrophic studies

Uteri were excised of fat and weighed and the body weights of animals were also recorded. Results were expressed as (uterine weight × 100)/total body weight.

Statistics

The significance of differences in uterine weights and oestrone sulphatase activity in tissues for control and treated animals were assessed using Student's *t*-test.

RESULTS

A-ring modifications

The ability of the A-ring modified EMATE derivatives (compounds **2** to **10**) to inhibit oestrone sulphatase activity was examined in placental microsomes (Fig. 2). Of the different analogues tested, the 4-nitro (**4**) derivative was the most efficient inhibitor and was more active than EMATE. At a concentration of 0.01 μM, 4-nitro EMATE inhibited microsomal sulphatase activity by 84.3% compared with 63.3% for EMATE. The 2-methoxy (**2**) and 2-nitro (**3**) analogues were also efficient inhibitors and at 0.1 μM inhibited activity by 67 and 57%, respectively. In this assay the 2- (**8**) and 4-allyl (**9**) substituted compounds, whilst being only relatively weak inhibitors (55 and 29% inhibition at 1.0 μM) were superior to the 2-*n*- (**5**) and 4-*n*-propyl (**6**) derivatives (22 and 14% inhibition at 1.0 μM). The 2,4-di-*n*-propyl (**7**) and 2,4-diallyl (**10**) analogues were devoid of any inhibitory activity.

The IC_{50}s for the inhibition of oestrone sulphatase were calculated from the results obtained using placental microsomes (Table 1). The IC_{50}s for the 2-methoxy and 2- and 4-nitro analogues were 30, 70 and 0.8 nM, respectively, compared with an IC_{50} of 4 nM for EMATE. Values for 2- and 4-allyl EMATE were approximately eleven times lower than the corresponding values for the 2-*n*- and 4-*n*-propyl EMATES.

Nature of inhibition

It was previously shown that EMATE acts as an irreversible, active site-directed inhibitor. The ability of three of the A-ring modified derivatives to act in such a manner was therefore also examined (Fig. 3). For this, intact cells were pre-treated with inhibitor for 2 h at 37°C and subsequently washed 5 times with

Table 1. IC_{50}s for A-ring modified derivatives of oestrone-3-O-sulphamate (EMATE) determined using placental microsomes

Inhibitor	IC_{50} (μM)
1 EMATE	0.004
2 2-methoxy	0.03
3 2-nitro	0.07
4 4-nitro	0.0008
5 2-*n*-propyl	29
6 4-*n*-propyl	>100
7 2,4-di-*n*-propyl	>100
8 2-allyl	2.5
9 4-allyl	9.0
10 2,4-diallyl	>100

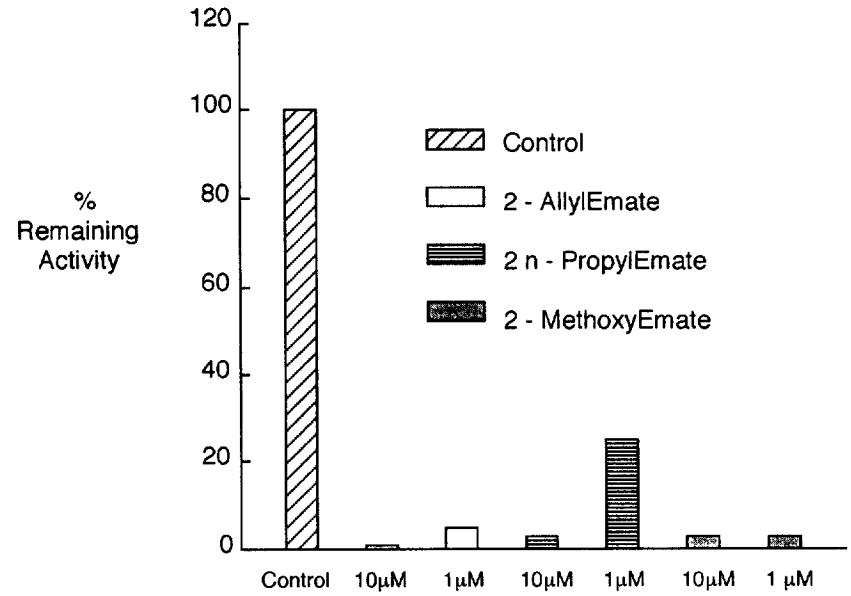


Fig. 3. Irreversible inhibition of oestrone sulphatase activity in MCF-7 cells by 2-allyl-oestrone-3-*O*-sulphamate (2-allyl-EMATE), 2-*n*-propyl-EMATE and 2-methoxy-EMATE. Intact MCF-7 cells were pretreated with inhibitors, at 1.0 and 10.0 μM concentrations, for 2 h at 37°C and subsequently washed 5 times with phosphate buffered saline. Cells were then reassayed for remaining oestrone sulphatase activity.

PBS and then assayed for remaining sulphatase activity. The 2-*n*-propyl (**5**), 2-allyl (**8**) and 2-methoxy (**2**) EMATE derivatives which were tested all acted as irreversible inhibitors with no recovery of activity at 10 μM. At 1 μM there was a 25% recovery of sulphatase activity in cells pre-treated with the 2-*n*-propyl derivative but not for the 2-allyl or 2-methoxy analogues.

In vivo studies

Having identified a number of A-ring modified EMATE derivatives which retained a range of sulphatase inhibitory properties, the 2-methoxy (**2**), 4-nitro (**4**), 4-*n*-propyl (**5**) and 4-allyl (**9**) were selected for *in vivo* testing. In animals receiving vehicle only, liver sulphatase activity was 352 ± 11 nmol/h/mg protein. EMATE and the 2-methoxy and 4-nitro derivatives (tested at 2 mg/kg/day for 5 days) all inhibited rat liver sulphatase activity by 95% or more (Fig. 4). In contrast, while active *in vivo*, the 4-*n*-propyl and 4-allyl derivatives were less potent, inhibiting sulphatase activity by 70 and 40%, respectively. E1S which was administered to animals as a positive control for the oestrogenicity bioassay had no inhibitory effect on *in vivo* sulphatase activity. Both E1S and EMATE stimulated uterine growth in the ovariectomized animals by 169 and 414%, respectively, compared with that of animals receiving vehicle only (Fig. 5). 4-Nitro EMATE also increased uterine growth (by 158%), to a similar extent to that of E1S, but 50% lower than the stimulation resulting from the administration of EMATE. In contrast, the 2-methoxy, 4-*n*-propyl and 4-allyl derivatives were devoid of oestrogenicity.

DISCUSSION

Steroid sulphatases regulate a number of important physiological and pathological functions in the body [23, 24]. There is currently considerable interest in developing a sulphatase inhibitor that will be safe to use in human studies [25, 26]. The A-ring modified EMATE derivatives were synthesized and tested in an attempt to identify an inhibitor which retained the potent sulphatase inhibitory properties of EMATE, which is orally active in rats and humans [15, 27] while being devoid of the oestrogenicity associated with this compound [16]. Of the analogues tested *in vitro*, 4-nitro EMATE was the most potent, being 5 times more active than EMATE. However, *in vivo*, at the concentration tested, sulphatase activity was almost completely inhibited by both compounds. Further studies, employing a range of lower doses, will be required in order to fully evaluate the comparative potencies of these inhibitors *in vivo*. While 50% less oestrogenic than EMATE, 4-nitro EMATE did still stimulate uterine growth in the ovariectomized rat. 2-Nitro EMATE, although a relatively potent inhibitor when tested *in vitro*, proved to be unstable and was therefore not selected for further testing.

The IC_{50} for 2-methoxy EMATE was 8 times higher than for EMATE. However, *in vivo* this compound proved to have a similar inhibitory potency to that of EMATE but was completely devoid of any

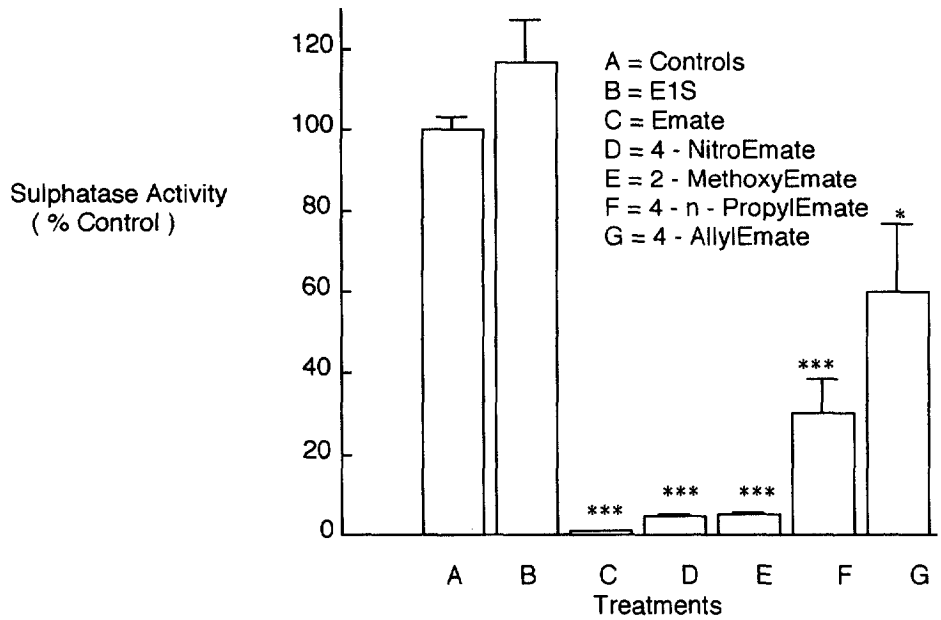


Fig. 4. Effect of oestrone-3-*O*-sulphamate (EMATE) and selected A-ring modified analogues of EMATE on *in vivo* liver sulphatase activity in the rat. Steroids were administered orally in propylene glycol, 2 mg/kg/day for 5 days. Oestrone sulphatase activity was measured in samples of liver obtained 24 h after administration of the last dose (mean ± SD, $n = 3$) (*$p < 0.05$, ***$p < 0.001$ compared with controls).

oestrogenicity as monitored by the uterine growth assay. This analogue would therefore appear to be a potential new lead compound for developing as a potent steroid sulphatase inhibitor. As 2-methoxyoestradiol has previously been shown to inhibit tumour angiogenesis and the growth of Meth-A sarcomas and B16 melanomas *in vivo* [19], this compound is worthy of further investigation.

The other A-ring modified EMATE derivatives (i.e. 2/4-*n*-propyl, 2,4-di-*n*-propyl and 2/4-allyl)

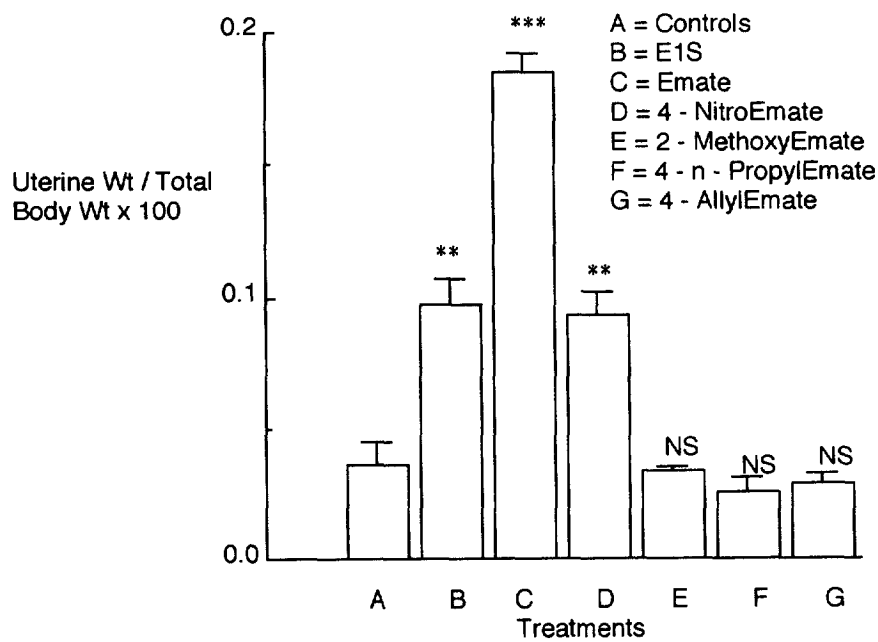


Fig. 5. Effect of oestrone sulphate (E1S, 50 μg/day), oestrone-3-*O*-sulphamate (EMATE, 1 mg/kg/day) and selected A-ring modified analogues of EMATE on uterine growth in the ovariectomized rat. Steroids were administered orally in propylene glycol, 2 mg/kg/day for 5 days. Uterine weights were measured 24 h after administration of the last dose (mean ± SD, $n = 3$) ($p < 0.01$, ***$p < 0.001$; NS, not significant compared with controls).**

were considerably less potent inhibitors than EMATE. *In vitro*, 4-allyl EMATE was more potent than the 4-*n*-propyl analogue while *in vivo* their relative potencies were reversed. It is not apparent why the relative potencies of these compounds differ *in vitro* and *in vivo* but may reflect differences in the metabolism which they undergo.

To investigate whether changes to the A-ring altered the nature of inhibition, three of the derivatives, the 2-allyl, 2-*n*-propyl and 2-methoxy EMATES, were examined for their ability to irreversibly inhibit sulphatase activity. As previously found for EMATE [13], the 2-allyl, 2-*n*-propyl and 2-methoxy EMATES acted as irreversible sulphatase inhibitors. In keeping with the somewhat higher potency of 2-allyl EMATE than the 2-*n*-propyl analogue as an inhibitor detected *in vitro*, this analogue also appeared to be more effective as an irreversible inhibitor when tested at 1 μM.

In a study, which employed the Allen–Doisy vaginal smear bioassay, Patton and Dmochowski [17] some years ago reported a number of modifications to the oestrogen steroid nucleus which reduced its oestrogenicity. Replacement of the hydrogen at C-2 by a propyl or allyl group greatly reduced the oestrogenicity of these compounds. Substitution with the alkyl groups at the C-4 position resulted in compounds which were even less oestrogenic than their C-2 isomers. Similar studies revealed that while 4-nitro-oestradiol was 200 times less active than oestradiol, the 2-nitro derivative was considerably less potent than the C-4-nitro isomer.

Patton and Dmochowski concluded from their study that the decrease in oestrogenicity which resulted from substitution with alkyl groups at the C-2 and/or C-4 positions was probably due to the bulkiness of the groups. No doubt this also applies to the ability of the EMATE derivatives to act as sulphatase inhibitors. They also postulated that steric interaction between the alkyl group at C-4 and a 6-hydrogen atom may distort the conformation of the molecule. Such a mechanism could impede the ability of the C-4 alkyl EMATE derivatives to gain entry into the active site of the sulphatase enzyme. In addition, it was noted that the rotation of the alkyl groups at C-4 would be restricted whilst their rotation at C-2 would be relatively unrestricted. They postulated that this could account for differences in the oestrogenicity of the C-2 and C-4 alkyl oestrogens. Again, such a mechanism may also account for the differences in sulphatase-inhibitory potency between the C-2 and C-4 alkyl substituted EMATE derivatives. The marked difference in the oestrogenicity of 4-nitro and 2-nitro oestradiol was thought to result from the deleterious effect of intramolecular hydrogen bonding of the 2-nitro group with the C-3 hydroxyl group. Due to the steric interaction with a 6-hydrogen atom, such bonding does not occur with the 4-nitro group, making the 4-nitro compound a considerably more potent oestrogen than its corresponding C-2 isomer. It is possible that intramolecular hydrogen bonding between the 2-nitro group and sulphamoyl moiety at the C-3 position of EMATE also occurs. This may therefore account for the lower potency of 2-nitro EMATE as a steroid sulphatase inhibitor compared with that of 4-nitro EMATE.

In summary, a number of A-ring modified derivatives of EMATE have been synthesized and tested *in vitro* and *in vivo* in an attempt to identify a potent steroid sulphatase inhibitor devoid of any oestrogenic properties. 4-Nitro EMATE and 2-methoxy EMATE were both potent inhibitors *in vitro* and *in vivo*. However, while the oestrogenicity of 4-nitro EMATE was reduced compared with that of EMATE, 2-methoxy EMATE had no oestrogenic effects on uterine growth in the ovariectomized rat. In view of the anti-proliferative and anti-angiogenic properties associated with 2-methoxyoestradiol [19], the sulphamoylated derivative of this steroid may have considerable therapeutic potential.

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