Bioorganic & Medicinal Chemistry Letters, Vol.3, No.2, pp. 313-318, 1993
Printed in Great Britain

0960-894X/93 $6.00 + .00

PHOSPHONATES AND THIOPHOSPHONATES AS SULFATE SURROGATES: SYNTHESIS OF ESTRONE 3-METHYLTHIOPHOSPHONATE, A POTENT INHIBITOR OF ESTRONE SULFATASE

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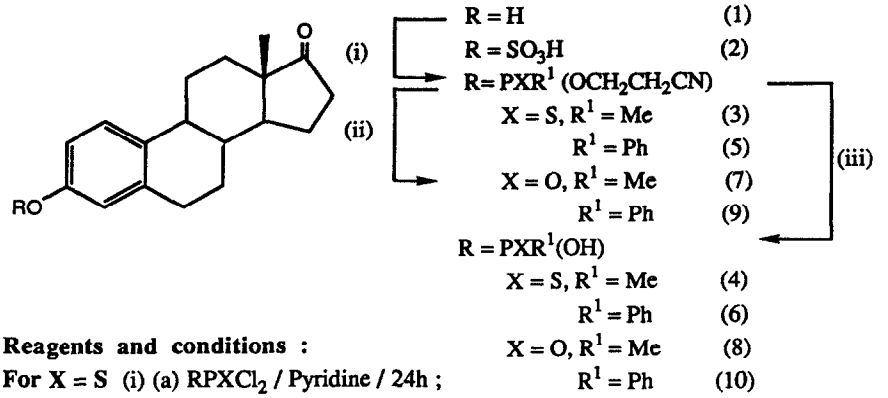
(*Received in USA* 4 *November* 1992)

Abstract: Estrone 3-phosphonates and thiophosphonates have been synthesised as analogues of estrone 3-sulfate and exhibit potent activity as competitive inhibitors of estrone sulfatase, a potential therapeutic target in treatment of breast cancer.

Breast cancer is the most prevalent type of cancer in Western countries and approximately one third of breast tumors are hormone-dependent[1]. There is considerable evidence derived from epidemiological, clinical and experimental studies to suggest that estrogens have a central role in supporting the growth of hormone-dependent tumors[2] and that *in situ* formation from estrogen precursors is likely to be a major contributor to the estrogen content of breast tumors. It has also been proposed that estrone sulfatase (steroid sulfatase, aryl sulfatase C, E.C.3.1.6.2), the enzyme complex responsible for the conversion of estrone sulfate **(2)** to estrone **(1)** [Scheme 1], may have a key role in regulating estrogen formation in breast tumors due to findings in relation to blood levels of estrone sulfate[3] and estrone sulfatase activity[4].

The development of specific inhibitors of estrogen synthesis could be an important advance in the treatment available for breast cancer. Until now considerable effort has gone into producing efficient inhibitors of aromatase activity, which converts androstenedione to estrone, and compounds such as aminoglutethimide and 4-hydroxyandrostenedione have been developed. While these compounds greatly reduce peripheral aromatase activity, plasma estrone and estrone sulfate concentrations are only reduced by some 50%[5,6]. It is therefore possible that inhibitors of estrone sulfatase activity, used alone or together with an aromatase inhibitor, may enhance the response to this type of endocrine therapy. To this



Reagents and conditions :
For X = S (i) (a) $RPXCl_2$ / Pyridine / 24h ;
(b) $HOCH_2CH_2CN$ / 24 h ;
(ii) NH_4OH / 65°C / 5h
For X = O (iii) (a) $RPXCl_2$ / Pyridine / 24h ;
(b) H_2O

Scheme 1: Synthesis of estrone sulfate analogues

end, we have attempted to develop an efficient inhibitor of estrone sulfatase which has resulted in a search for surrogates of the sulfate group. We report here on phosphonate and thiophosphonate analogues of estrone sulfate synthesised for this purpose and, in particular, on estrone-3-methylthiophosphonate (E1-3-MTP) (**4**) [Scheme 1]. We believe that this is the *first* example of the use of a methylthiophosphonate group as a replacement for sulfate. An approach to a sulfonate analogue of cholesterol 3-sulfate has recently been outlined[7].

E1-3-MTP (**4**) was initially prepared according to Cox *et al*[8]. However, a number of problems were encountered with this synthesis, especially in the purification of the final acidic product and a better route was therefore sought. We have thus devised a synthesis of the ammonium salt of E1-3-MTP (**4**) from estrone by a two step pathway [Scheme 1] involving first the formation of the 2-cyanoethylthiophosphonate diester (**3**) by reaction of estrone with methanethiophosphonic dichloride followed by quenching the intermediate estrone methanethiomonophosphonic chloride with 2-cyanoethanol. The resulting protected neutral P(V) intermediate is similar to those used in oligonucleotide synthesis[9], and it allowed for effective purification. ß-Elimination of the cyanoethyl moiety with ammonia afforded the desired material in 73% yield as a mixture of diastereomers.

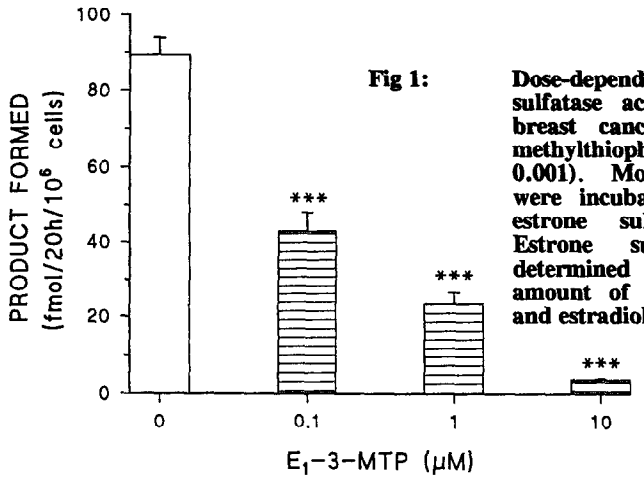
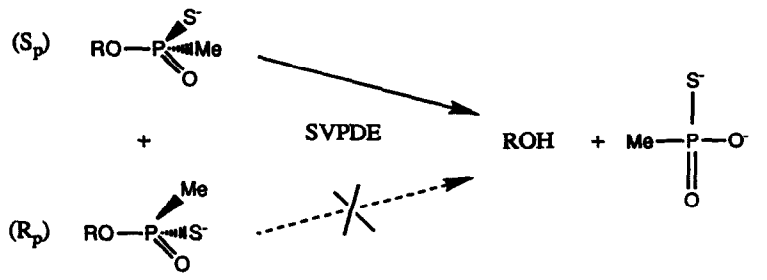


Fig 1: **Dose-dependent inhibition of estrone sulfatase activity in intact MCF-7 breast cancer cells by estrone 3 methylthiophosphonate (*** : $p < 0.001$). Monolayers of MCF-7 cells were incubated for 20h with [^{3}H] estrone sulfate and E1-3-MTP. Estrone sulfatase activity was determined by measuring the total amount of tritium labelled estrone and estradiol formed.**

The ability of E1-3-MTP (**4**) to inhibit estrone sulfatase activity was examined using MCF-7 breast cancer cells and placental and breast tumor cytosol preparations. At 10μM, E1-3-MTP (**4**) was found to inhibit estrone sulfatase activity in intact MCF-7 cells by 96% and at 1μM and 0.1μM by 74% and 52% respectively [Fig 1]. The K_i values for (**4**) in breast tumor and placental preparations were found to be 33μM and 15μM respectively. The IC_{50} values for (**4**), measured in both placental and breast tumor preparations, were 43μM and 36μM respectively.

The E1-3-MTP (**4**) synthesised by this pathway exists as a mixture of two diastereoisomers, since both the C_{18}-carbon atom and the phosphorus atom are chiral. A key question is obviously whether only one isomer is active, or both. However, separation of the two diastereoisomers in order to access the ability of each diastereoisomer to inhibit estrone sulfatase activity has proved to be very difficult due to the remoteness of the two chiral



Scheme 2: Enzymatic digestion of (R_p)-E1-3MTP (R = Estrone)

centres and individual resonances due to the two isomers were not observed by ^{31}P NMR spectroscopy (δ_p, 74.45ppm). The only technique by which both diastereoisomers could be observed was by HPLC using a ß-cyclodextrin chiral stationary phase with a mobile phase gradient of 70 - 80% of solution B [solution A: 0.02M ammonium sulphate; solution B: 0.02M ammonium sulphate in 75% methanol : 25% water] (Fig 2a). In order to assign the absolute configuration at phosphorus of diastereomers giving rise to the two peaks observed by HPLC and in the hope of being able to assess the ability of at least one of the two diastereoisomers to inhibit estrone sulfatase activity, we decided to digest enzymatically one of the diastereoisomers from the mixture (Scheme 2). Enzymatic methods have been applied to the assignment of the absolute configurations of nucleoside phosphorothioates[10]. Snake venom phosphodiesterase (SVPDE) is known to hydrolyse specifically the Sp diastereoisomer of 4-nitrophenyl phenyl phosphonothioate[11] and the Rp diastereosiomer of 5'-O-adenosyl 5'-O-uridyl phosphorothioate[12].* Therefore, by analogy, we expected the Sp diastereoisomer of E1-3-MTP (**4**) to be hydrolysed by SVPDE. Thus, the mixture of diastereoisomers was incubated with SVPDE for 33 days at 37°C and was monitored periodically by HPLC. After this time the peak with the longer retention time had completely disappeared [Fig 2b]. This enabled us to conclude that the peak with the shorter retention time could be assigned to the Rp diastereoisomer whilst that with the longer retention time was derived from the Sp diastereosiomer.

Subsequently, the enzyme digest was stopped by addition of chloroform, centrifuged and the supernatant was evaporated to dryness *in vacuo*. Trituration of the residue with chloroform allowed the organic products to be isolated from the buffer salts used in the enzyme assay. Final purification of the R_p diastereoisomer was achieved on an ion-exchange column of Q-Sepharose using a gradient of triethylammonium bicarbonate buffer (pH 7.4) ranging from 25mM to 500mM, and pure R_p-(**4**), eluting at ca. 300mM buffer, was recovered in > 97% optical purity (by HPLC) in quantitative yield as

* The spatial configuration around phosphorus is identical in these two substrates. The RS sequence rules, however, dictate different assignments of absolute configuration.

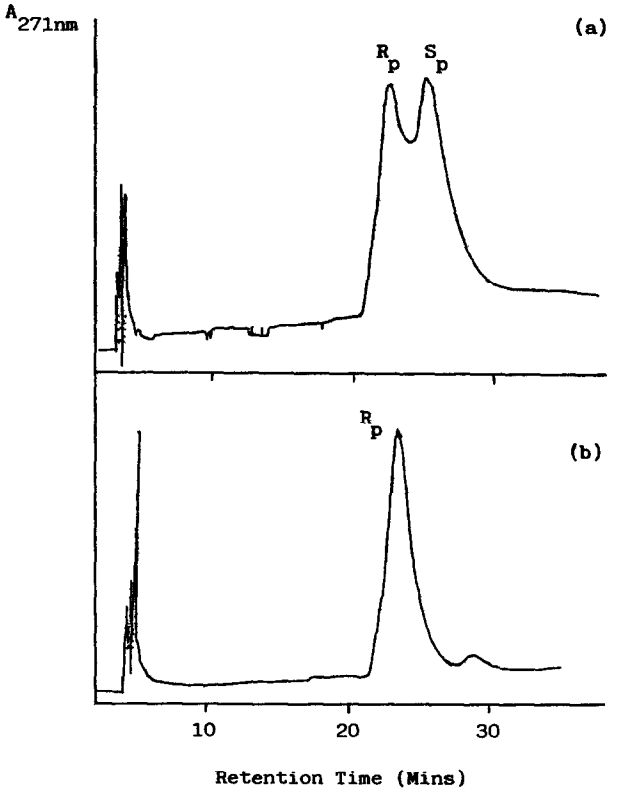


Fig 2: HPLC analysis of (a) (R_p,S_p)-E1-MTP and (b) (R_p)-E1-MTP after digestion of the S_p isomer by snake venom phosphodiesterase

the triethylammonium salt {$[\alpha]_D$ = +50.5° (c 0.99, MeOH)}. The ability of the R_p-(+)-E1-3-MTP to inhibit estrone sulfatase was assessed using MCF-7 cells as before. R_p-(4) had an inhibitory activity only 28% of that of (R_p,S_p)-(4), indicating that S_p-(4) is clearly the most potent inhibitor of the diastereomeric pair.

Using similar synthetic methodology (Scheme 1) we also synthesised the related phenylphosphonothioate (6), methylphosphonate (8), and phenylphosphonate (10) from the appropriate precursors (5), (7) and (9) respectively. These compounds had the relative activities indicated in the Table from which it is clear that E1-3-MTP (4) is the most potent inhibitor.

Compound	Inhibition %
R_p,S_p-(4)	96
R_p-(4)	27
R_p,S_p-(6)	42
(8)	54
(10)	70

Table: Inhibition of estrone sulfatase by estrone sulfate analogues at 10μM in intact MCF-7 cells. See legend to Fig 1 for details of assay procedure.

In summary, we have shown that E1-3-MTP **(4)**, as a mixture of diastereoisomers, efficiently inhibits estrone sulfatase activity in MCF-7 breast cancer cells as well as in placental and breast tumor cytosol preparations. Both diastereoisomers can be observed by HPLC and their absolute configurations have been determined using enzymatic methodology. R_p-(4) Was purified and was found to be a reasonable inhibitor of estrone sulfatase in MCF-7 breast cancer cells, but the S_p-isomer is clearly more potent.

ACKNOWLEDGEMENTS

This work was supported by IMPEL Exploitation Ltd. B.V.L.P. is a Lister Institute Fellow. We thank S. Alston for manuscript preparation.

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