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STEREOSPECIFIC BIO-REVERSIBILITY OF DINUCLEOSIDE S-ALKYL PHOSPHOROTHIOLATES TO DINUCLEOSIDE PHOSPHOROTHIOATES

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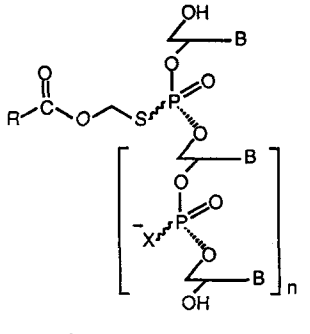
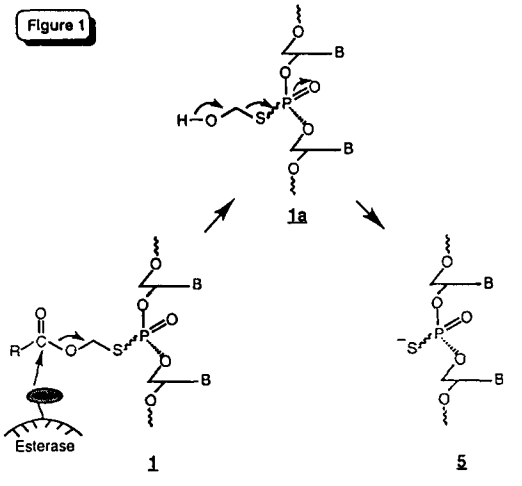
Abstract: In model studies towards prodrugs of oligonucleoside phosphorothiolates eg., **1**, the dinucleoside S-alkyl phosphorothiolates **3a-c** were prepared by chemoselective alkylation of d[TpsT] with the iodoalkyl acylates **8a-c**. Upon incubation with serum or *porcine liver esterase*, stereospecific conversion of R_p and S_p **3c** to **2** was observed along with the desulfurized product **4**.

The therapeutic potential of oligonucleoside phosphorothioates (PS oligos) as antisense-mediated inhibitors of gene expression is well recognized.[1,2] It is presently believed that the cellular uptake of PS oligos occurs by receptor-mediated endocytosis.[3] In our earlier studies we have shown that the cellular uptake of PS oligos was increased by incorporating non-ionic internucleotidic linkages in PS oligos.[4] We therefore envisioned that bio-reversible analogs,[5,6] of PS oligos, *e.g.*, **1**, that carry one or more of the lipophilic acyloxyalkyl ester groups, might serve as useful *prodrug* forms of the parent phosphorothioate **5**.



1 X= O, S

Figure 1



1a **1** **5**

As a wide variety of compounds, eg., **8a-c**, with different R groups can be prepared using the general procedure of Scheme 1,[6h,i] and since chemoselective alkylations of oligonucleotides had been previously reported, [7a,b] the

synthetic targets of **1** appeared feasible. Instead of applying this concept in the synthesis of the more complex target *viz.*, **1**, we initially chose to do model studies on simpler dinucleoside analogs, so that the experience gained from this endeavor could be applied towards the target prodrug **1**. Additionally, in the present study, our objective was to select an R group for **1** which: (a) would confer stability (against nucleases and esterases) to **1** in serum; (b) would provide sufficient lipophilicity to **1** so that it traverses the cellular membranes and other physiologic barriers by passive diffusion (c) would allow intracellular esterases to hydrolyze the carboxylic ester group in **1**, to regenerate the parent phosphorothioate **5** (Fig. 1) and (d) would confer favorable pharmaceutical properties for formulation of **1** into a suitable dosage form. We report herein the synthesis and stability towards hydrolysis of dinucleoside S-alkyl phosphorothiolates **3a-c** in human serum and their hydrolysis mediated by esterases.

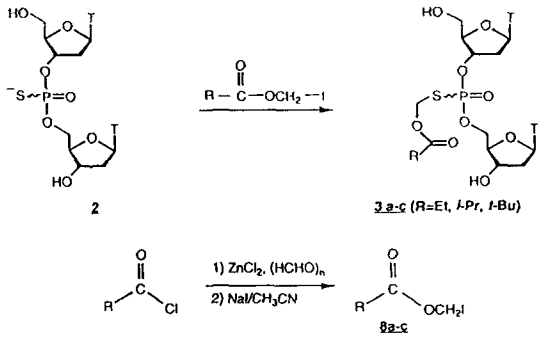
To carry out our model studies, we synthesized d(TpsT) **2** on a 10 x 10 μmole scale, using phosphoramidite chemistry,[8] on an automated DNA synthesizer. Oxidation of the internucleotidic phosphite linkage was carried out using 3*H*-1,2-benzodithiole-3-one-1,1-dioxide, to generate the phosphorothioate linkage.[9a,b] After usual work-up, **2** was obtained as an R_p:S_p diastereomeric mixture (R_p:S_p ratio of **2** was 60:40 based on ^{31}P-NMR and HPLC analysis).[10a,b] The chemo-selective alkylation[7a,b] of the diastereomeric mixture of **2** with iodomethyl pivalate gave S-pivaloyl phosphorothiolate **3c** (R_p:S_p, 60:40).[11a]

To evaluate their stability and bio-reversibility, the R_p:S_p mixture of triesters **3c** (R_p:S_p, 60:40) were incubated with human serum. Hydrolytic stability was monitored by quantifying the product and reactant peaks by HPLC. We observed a slow and *stereospecific* hydrolytic conversion of S_p triester **3c** to the diester S_p **2** ($t_{1/2}$ = 335 min) and R_p triester **3c** (to R_p **2**) ($t_{1/2}$ = 1980 min) (*stereospecificity* implied, in this case, is that S_p **3c** is converted to S_p **2** and R_p **3c** to R_p **2**) . In addition, the formation of the phosphoric diester **4** (*ca.* 5.0 %) as a by-product was seen. Nuclease-mediated hydrolytic products (*i.e.*, mononucleosides) were not seen during the assay period. In accordance with the behavior of other acyloxyalkyl carboxylic esters,[5,6] the hydrolysis, of **3c** to **2**, was anticipated to occur by enzyme-mediated nucleophilic attack on the *ester carbonyl* carbon followed by cleavage of the acyloxy alkyl group (Figure 1). As an additional support of this mechanism we prepared the benzoyl analog of **3** (R = Ph) and were pleased to observe the formation of **2** and benzoic acid, the by-product of serum-mediated hydrolysis.

Although it is well documented[10b] that R_p and S_p nucleoside phosphorothioates display differential susceptibilities, towards hydrolysis of the chiral phosphodiester bond, against *snake venom phosphodiesterase I* and *nuclease P1*, the *selective* hydrolysis and desulfurization of stereoisomeric S-alkyl phosphorothiolates, *eg.*, **3c** by *factors* (*eg.*, esterases) present in serum, is not reported. These initial results were intriguing especially because the chiral phosphorous center appeared to be far away from the carbonyl ester function to provide stereodifferentiation in hydrolysis of **3c** by the ubiquitous esterases present in serum.

We therefore synthesized[11a] R_p and S_p triesters **3a-c** (Scheme 1) and investigated their serum-mediated hydrolytic profile. During the synthesis, no evidence of epimerization at the chiral phosphorous center was noted. The esters **3a-c** were soluble in aqueous buffers (pH 7.0) and stable indefinitely, even when stored in aqueous buffers (pH 7.0), at 0-5°C. Their stability studies were carried out at ambient temperature.[11b]

Scheme 1



When exposed to human serum, the S_p esters of **3a-c** were hydrolyzed faster compared to the R_p esters of **3a-c** ($kS_p > kR_p$) (Table 1, for details). Interestingly, at their respective $t_{1/2}$ of hydrolysis, whereas only 95% of the S_p and R_p pivaloyl analogs of **3c** gave the expected S_p **2** and R_p **2** respectively (rest being the desulfurized product **4**), 99% of the S_p and R_p isomers of **3a**, **3b** gave S_p **2** and R_p **2** respectively (rest being **4**).

Table 1. Hydrolysis of **3a-c** by human serum.

Compound	R_t (min)	$t_{1/2}$ (min)	k (X 10^{-2} min^{-1})$	% 2@
R_p **3a**	38.7	40	1.73	99 (R_p)
S_p **3a**	39.3	11	6.30	99 (S_p)
R_p **3b**	41.6	82	0.85	99 (R_p)
S_p **3b**	42.2	28	2.47	99 (S_p)
R_p **3c**	44.3	1980	0.04	95 (R_p)
S_p **3c**	44.9	335	0.21	95 (S_p)

@estimated at $t_{1/2}$ of hydrolysis, remaining being **4**. The hydrolysis mixture contained *ca.* 0.6 A_{260} units of substrates **3a-3c**, 20 µL human serum (GIBCO, BRL), in 60 µL of tris buffer (pH 7.0, 25 mM) at 37°C. At each time point, 10µL aliquots of incubation mixture was diluted with 140 µL buffer A and analyzed by reverse-phase HPLC, (Waters 600E instrument) using 8NV C_{18} 4µ Radial Pak cartridge column, gradient (100% A to 60% B over 60 minutes) of buffer A (0.1M $CH_3CO_2NH_4$) and buffer B (80:20, CH_3CN:0.1 M $CH_3CO_2NH_4$), flow rate 1.5 mL/min. Retention times (R_t) of R_p **2**, were 24.2; S_p **2**, 25.4; and **4**, 21.0 minutes respectively. $ First-order rate constant. Appropriate controls were done.

We also studied the hydrolysis of **3b-c** by *porcine liver esterase* (a mixture of seven isozymes) as a mimic of the intracellular esterases. It is pertinent to mention that many *esterases* especially *serine proteases* are inhibited by organophosphorous compounds.[12a] It was indeed difficult to predict *a priori* whether 3a-c would be substrates for the esterases. We did not study **3a** because the serum studies had shown this analog to be quite labile. In the event, it was gratifying to note that incubation of **3b-c** with porcine liver esterase also resulted in their stereospecific bio-reversibility to **2**. As expected, the half lives were dependent on the amount of esterase used in the study. Interestingly, inverse stereochemical preference was noted in the hydrolysis *i.e.*, R_p was hydrolyzed faster than S_p (Table 2).

Table 2. Hydrolysis of **3b-c** by *porcine liver carboxy esterase.*

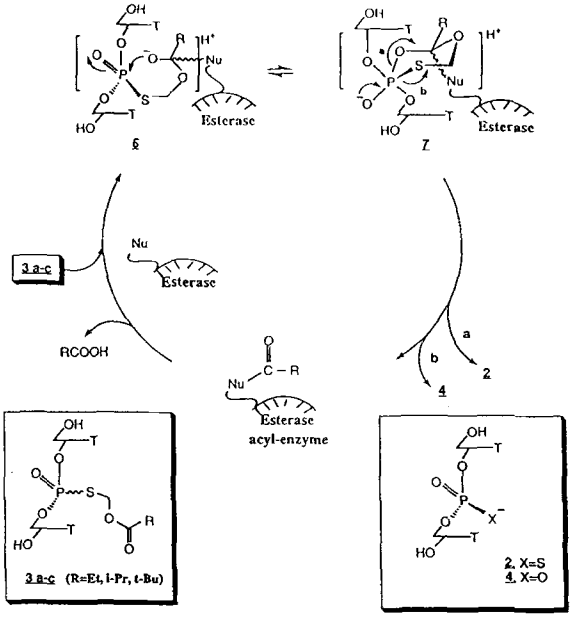
Compound	R_t (min)	$t_{1/2}$ (min)	% 2@
R_p **3b**	41.6	42	99 (R_p)
S_p **3b**	42.2	64	99 (S_p)
R_p **3c**	44.3	220	97 (R_p)
S_p **3c**	44.9	715	98 (S_p)

@estimated at $t_{1/2}$ of hydrolysis, remaining being **4**. The hydrolysis mixture contained *ca.* 0.6 A_{260} units of substrates **3b-3c**, 5μL (0.015 unit) of *porcine liver carboxyl esterase* in 60 μL of tris buffer (pH 7.0, 25 mM) at 37°C. At each time point, 10μL aliquots of incubation mixture was diluted with 140 μL buffer A and analyzed by reverse-phase HPLC, as described under Table 1. Appropriate controls were done.

Finally it is important to rationalize why storage of **3a-c** in buffer at ambient temperature gave predominantly the desulfurized product **4** whereas the enzyme-mediated hydrolysis gave predominantly the desired product **2** - an indicator of true bio-reversibility of the analogs **3a-c**. A simple explanation would be that **4** is derived by direct nucleophilic attack (by water) on phosphorous followed by displacement of the S-alkyl group where as **2** would be derived by attack (enzyme-mediated) on the carbonyl group (Scheme 1). Another way to rationalize this observation would be to postulate a common hydrolytic pathway for the origin of **2** and **4** involving an initial nucleophilic attack (*e.g.*, by serine hydroxyl group) by the enzyme *or* water on the ester carbonyl to generate the acyl-enzyme intermediate **6** (Fig. 2). Intramolecular attack of the oxyanion of **6**, on the juxtapositioned phosphorothiolate moiety, would give the cyclic intermediate **7** (S_p faster than R_p) which could undergo fragmentation either by *path a* to give phosphorothioate diester **2** or by *path b* to give the desufurized product **4**. In case of the less hindered S_p and R_p esters **3a-b**, *path a* is favored over *path b* 99:1, with the preference reduced to 95:5 for the more hindered **3c**. That this pathway may be operative is indicated by the reported rate

to 95:5 for the more hindered **3c**. That this pathway may be operative is indicated by the reported rate enhancement in the hydrolysis of phosphate and phosphonate esters which have an appropriately positioned carbonyl group.[12a-e] Additional experiments in progress in our laboratory may help in elucidating the intricacies of this transformation. Irrespective of the actual course of the reaction, both the steric bulk at the ester carbonyl group as well as the stereochemistry at the phosphorous center of **3a-c** dictate the hydrolytic rate and profile of products.

Figure 2



With the knowledge gained from these model studies, we have recently completed the synthesis of acyloxyester analogs of d[TpsT]5, a 10-mer phosphorothioate analog and a 25-mer antisense oligonucleotide. The stability, cellular uptake, bio-reversibility, toxicity and antiviral evaluations of these oligonucleotide analogs are in progress and will be reported in due course. The herein described strategy could lead to the design of novel *prodrugs* of oligonucleoside phosphorothioates.[13]

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10. (a) The assignment of R_p and S_p configuration for the diastereomers of **2** was done[10b] using *phosphodiesterase I* (*type II*, SIGMA) which stereospecifically hydrolyses R_p **2**, and *nuclease P1* which hydrolyses S_p **2**. ^{31}P-NMR (D_2O) (trimethyl phosphate): δ *ppm* R_p **2**, 52.6; S_p **2**, 52.2.; (b) Connolly, B. A.; Potter, B. V. L.; Eckstein, F.; Pingoud, A.; Grotjahn, L. *Biochemistry*, **1984**, *23*, 3443-53.

11. (a) The esters **3a-c** were synthesized by reacting R_p or S_p **2** (50 A_{260} units) (0.5 mL Tris buffer 250 mM, pH 7.0) with the corresponding iodoalkyl acylates[6h,i] (2 mmoles) in acetonitrile (3 mL), at 37°C for 3-4 hr. The reaction mixture was quenched with sodium bisulfite (0.5%, 100 µL), evaporated to dryness *in vacuo* and subjected to preparative reverse-phase HPLC using conditions described under Table 1. The solvent was removed *in vacuo* and the esters, **3a-c** thus obtained (isolated yields 60-70% based on **2**), were used as such for further studies. Typical ^{31}P-NMR (D_2O) (trimethyl phosphate): δ *ppm* R_p **3c**, 24.8; S_p **3c** 25.8. (b) Estimated $t_{1/2}$ in Tris buffer (25mM, pH7.0, 22°C) R_p or S_p **3a** > 5days; R_p or S_p **3b** > 10 days; R_p or S_p **3c** > 30 days (as monitored by HPLC). *Interestingly, the predominant hydrolytic product in all cases was the desulfurized diester* ***4***.

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13. RPI dedicates this paper to Palghat R. Raghu, on the occasion of his 66th birthday.

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