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THE PROOLIGONUCLEOTIDE APPROACH. I : ESTERASE-MEDIATED REVERSIBILITY OF DITHYMIDINE S-ALKYL-PHOSPHOROTHIOLATES TO DITHYMIDINE PHOSPHOROTHIOATES

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Abstract: Alkylation of dithymidine phosphorothioate and phosphorodithioate with various iodoalkyl acylates afforded the corresponding uncharged S-alkyl phosphoromono- and di-thioates respectively. Upon incubation of these triesters in CEM cell extracts, the bioreversible alkyl acylate masking groups were selectively and rapidly removed by carboxyesterases present in the milieu, yielding the starting dinucleoside diesters.

The prodrug concept is well established and has been widely used in medicinal chemistry. In this respect, our group has recently developed a pronucleotide approach and has shown that it is possible to intracellularly deliver 5'-nucleotides, thus bypassing the first metabolic step of antiviral nucleosides[1-5]. This has been achieved through the use of bioreversible phosphate protecting groups yielding neutral phosphotriesters which, after uptake, are enzymatically more readily removed inside the cells than in the culture medium (Fig. 1).

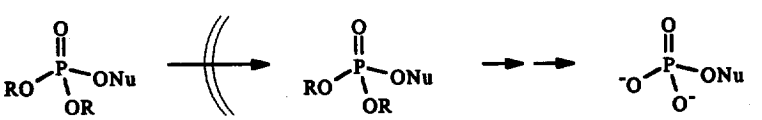


Fig 1: The pronucleotide approach

The problem we would like to address in this paper concerns the possibility of extending such an approach to oligonucleotides (oligos)[6], in order to bring about their selective intracellular release after deprotection. In other words, is it possible to design prooligonucleotides (prooligos) and, if so, what would be the biological consequences? One would expect greater stability in biological media (against nucleases) than the parent oligo as no phosphotriesterase seems to be reported in serum or in mammalian cells. One would also

expect increased uptake into cells due to their increased lipophilicity[7,8]. In addition, one can hypothesize a completely different bioavailability for a prooligo (as compared to its parent oligo) which could confer its pharmaceutical benefit and may open the way to various types of administration (i.e. vo). Obviously, this challenging approach raises numerous questions to be answered as the prooligo must be as stable as possible in the culture medium (pH~7.2, 37°C) but once in the cytosol decompose to the parent oligo.

From a chemical point of view, the key step of such an approach is based on the selective hydrolysis of a 3',5'-dinucleosidyl phosphotriester to its corresponding phosphodiester (Fig. 2, X= O).

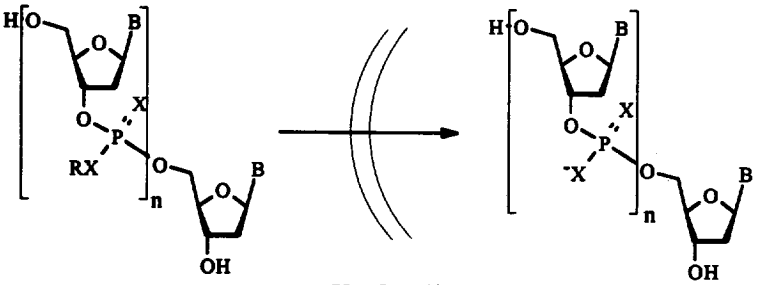


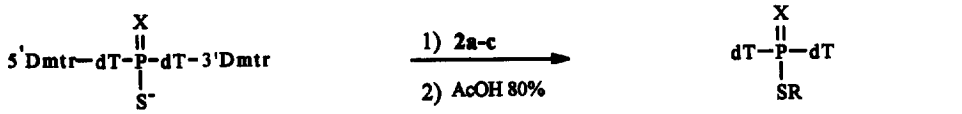
Fig.2: The prooligonucleotide approach

In addition, the synthesis of oligonucleoside phosphotriesters would necessitate the design of a new synthetic strategy avoiding any basic medium treatment due to the susceptibility of phosphotriesters to nucleophilic attack at the phosphorus centre. In a first step, to overcome this problem and to simplify matters, we decided to form the phosphotriester functionalities after the oligonucleotide synthesis. This choice implies that we will deal with more readily alkylated phosphorothioates[9] rather than with phosphate diester internucleoside linkages. In this respect, it has been previously reported that a single internal phosphorothioate diester linkage within an oligodeoxynucleotide can be selectively alkylated[10,11]. However, phosphorothiolate triesters are more prone to hydrolysis than are phosphate diesters[12] or triesters[13]. Therefore, before constructing any preliminary prooligo model, we will have to determine the nature of the phosphorothioate bioreversible protecting group which could be used not only in terms of its ease of introduction but also regarding its stability in serum and cell extracts.

This first paper reports the synthesis and the evaluation of various bioreversible protecting groups on the basis of the dinucleotide phosphorothioate model as shown on Fig. 3. Based on our previous work on the pronucleotide approach, we first selected three bioreversible phosphorothioate protecting groups namely, pivaloyloxymethyl (POM), S-acetylthiomethyl (MeSATM) and S-acetylthioethyl (MeSATE) that we have shown to be removable by carboxyesterases. The rational being that after carboxyesterase-mediated removal of the acyl group, the resulting phosphorothiolate triester will spontaneously yield the phosphorothioate diester through a selective C_{α}-S bond breakage process (Fig.4).

Therefore, starting from 5'- and 3'-dimethoxytrityl thymidines, we synthesized the dithymidine phosphorothioate **1a** using the H-phosphonate approach in solution[14]. This compound was obtained as a diastereoisomeric mixture (Rp and Sp) as expected. The iodoalkyl acylates **2a-c** were obtained as described in

the literature[15-17] and reacted with **1a** in acetonitrile, in the presence of 2,6-lutidine[18]. TLC monitoring of the reaction indicated that the rate of alkylation of **1a** was strongly dependent on the nature of the alkylating agent. Using a ten fold excess of iodoalkyl acylate **2a,2b** or **2c**, virtually complete alkylation was achieved after 30 min, 20 h and 20 days at room temperature respectively.



1a X= O
1b X= S

2a $tBuCOOCH_2$-I (POM-I)
2b CH_3COSCH_2-I (MeSATM-I)
2c $CH_3COS(CH_2)_2$-I (MeSATE-I)

3a R= POM X= O
3b R= MeSATM X= O
3c R= MeSATE X= O
3d R= MeSATE X= S

Fig.3: Synthesis of triesters **3a-3d**

Then, the acid-labile 5'- and 3'-O-Dmtr protecting groups were removed upon treatment with 80% aqueous acetic acid and the dithymidine phosphorothiolate triesters **3a-c** were isolated by column chromatography on silica gel and characterized by ^{31}P-, H-NMR and mass spectrometry. We have also found that the acid treatment did not induce any side reaction. In addition, the diastereoisomeric ratio of **3a-c** (HPLC and ^{31}P-NMR) was found to be the same as for the starting compound **1a**.

The next step was to evaluate the fate of **3a-c** in a culture medium and also in total cell extracts, both media being at neutral pH. The results are summarized in Table I.

Table I. Half-lives of **3a-c** in culture medium and in cell extracts along with the identification of their decomposition products.

Compound	RPMI 1640 + 10% FCS		CEM cell extracts	
	$t_{1/2}$ (min)	% **4a***	$t_{1/2}$ (min)	% **4a***
3a	360	20	40	100
3b	60	80	< 5	100
3c	420	50	< 5	100

*molar % of dithymidine phosphorothioate (**4a**) was estimated after complete hydrolysis of the starting compound, the other product being dithymidine phosphate (**4b**) which was partially degraded by nucleases present in the milieu. In a typical experiment, 0.5 mM substrate **3a**, **3b** or **3c** (0.1 mL in 5% aqueous dimethylsulfoxide) and either RPMI 1640 supplemented with 10% heat-deactivated foetal calf serum (FCS, Gibco) (0.9 mL) or CEM cell supernatant[3] (0.9 mL) were added and incubated at 37°C. At various times of incubation, 0.08 mL aliquots were analyzed by ISRP-HPLC[3].

The results lead up to the following conclusions:

i) In culture medium compounds **3a** and **3c** were more stable than **3b**. Both dithymidine phosphorothioate (**4a**) and phosphate (**4b**) diesters were detected in variable amounts depending on the nature of the bioreversible protecting group. Formation of **4b** arises from nucleophilic attack at the phosphorus centre[13] whereas formation of **4a** may be explained by a C_α-S bond breaking process through a carboxyesterase mediated mechanism, as shown on Fig. 5 (it should be noted that even if FCS was heat unactivated some carboxyesterate activity may remain [2,3]). This is corroborated by the fact that, upon incubation in RPMI and in the absence of FCS, **3a** was exclusively transformed into the corresponding phosphate diester **4b**. In contrast, incubation of **3b** ($t_{1/2}$ 220 min) under the same conditions afforded a mixture of **4a** and **4b**. In this later case, formation of **4a** may be tentatively explained through nucleophilic attack at C_α by a nucleophile present in RPMI 1640 followed by subsequent C_α-S bond cleavage.

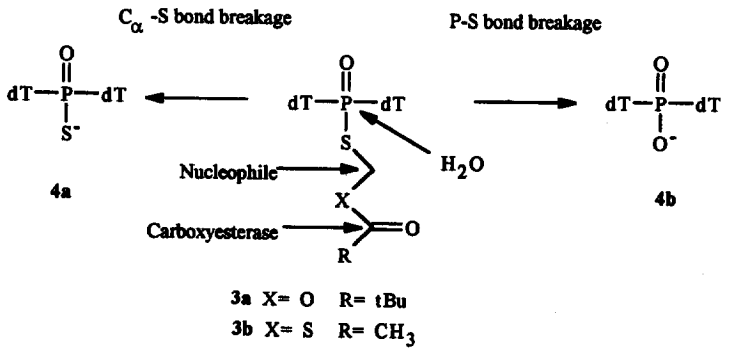


Fig 4: Possible decomposition pathways of dithymidine phosphorothiolates as exemplified with a POM or MeSATM bioreversible protecting group.

ii) In total CEM cell extracts, **3a-c** were selectively and rapidly hydrolized to the expected phosphorothioate diester **4a** through, presumably, a carboxyesterase mediated process as shown in Fig. 5. Such a mechanism has been fully demonstrated when the same bioreversible protecting groups were applied to the pronucleotide approach[1-5]. It is also noteworthy that **3a**, which exhibits a sterically hindered carbonyl group, was less rapidly transformed to **4a** than **3b** or **3c**.

Based on this data, the MeSATE bioreversible protecting group seems to be of more interest as it presents a higher extracellular/intracellular stability ratio than the other two. We therefore decided to extend this study to a more readily alkylable phosphorodithioate internucleotide linkage as the corresponding triester is expected to be less susceptible to nucleophilic attack at the phosphorus centre[13]. We have therefore synthesized the corresponding phosphorodithioate derivative **1b** (Fig. 3) following a reported procedure[19,20] and have studied its stability under the same experimental conditions as used previously.

Under similar conditions, complete alkylation of **1b** was achieved in 48 h instead of 20 days for **1a**. In addition, **3d** exhibited half-lives of 480 min and of less than 5 min in culture medium and total cell extracts respectively. Furthermore, only phosphorodithioate diester formation was observed in both media which excludes any side nucleophilic attack at the phosphorus centre, thus corroborating the literature data[13].

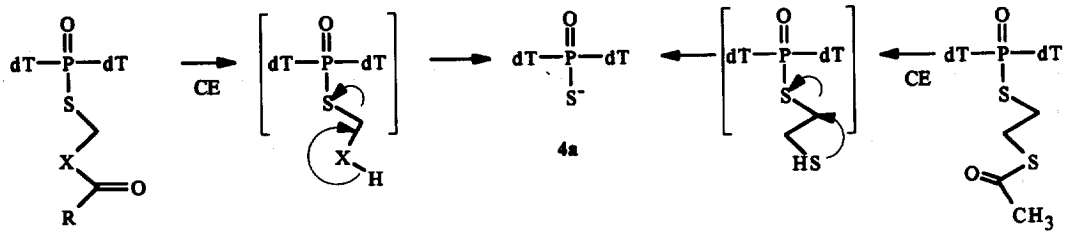


Fig. 5: Decomposition pathway of the bioreversible phosphate protecting groups in cell extracts (CE : carboxyesterase)

From this preliminary study, one can conclude on the basis of their stability ratio in culture medium versus cell extract that POM and MeSATE bioreversible phosphate protecting groups could be useful for evaluating the benefit of the pronucleotide approach. However, their introduction on rather long (i.e. 20 mer) all-phosphorothioate oligonucleotides using such a post-synthesis alkylation process may lead to difficulties in insuring complete alkylation of every internucleoside link. In addition, one can expect that the half-life of any oligonucleotide containing several phosphorothioate triester modifications to be shorter than that of the dimeric model. Hence, the use of chimeric oligonucleotide analogs combining phosphorothioate and methylphosphonate linkages[21] could represent a good compromise. Further studies are in progress in order to optimize the nature of the bioreversible protecting group in terms of reactivity and pharmacokinetic parameters.

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References and notes.

1. Puech, F.; Gosselin, G.; Lefebvre, I.; Pompon, A.; Aubertin, A.-M.; Kirn, A.; Imbach, J.-L. *Antiviral Res.* **1993**, *22*, 155.
2. Périgaud, C.; Gosselin, G.; Lefebvre, I.; Girardet, J.-L.; Benzaria, S.; Barber, I.; Imbach, J.-L. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 2521.
3. Pompon, A.; Lefebvre, I.; Imbach, J.-L.; Kahn, S.; Farquhar, D. *Antiv. Chem. & Chemother.* **1994**, *5*, 91.
4. Périgaud, C.; Aubertin, A.-M.; Benzaria, S.; Pélicano, H.; Girardet, J.-L.; Maury, G.; Gosselin, G.; Kirn, A.; Imbach, J.-L. *Biochem. Pharmacol.* **1994**, *48*, 11.
5. Benzaria, S.; Girardet, J.-L.; Périgaud, C.; Aubertin, A.-M.; Pélicano, H.; Maury, G.; Gosselin, G.; Kirn, A.; Imbach, J.-L. *Nucleic Acids Symposium Series* **1994**, 129.

6. We have presented part of this work at the " 3rd Cambridge Symposium : oligonucleotides and analogues " Sept. 5-8, 1993, Cambridge, England. During the preparation of this manuscript an independent publication reporting on some dinucleoside S-alkylphosphorothiolates bearing bioreversible POM protecting groups has just appeared (see ref. 9).
7. Spiller, D. G.; Tidd, D. M. *Anti - Cancer Drug Design* **1992**, *7*, 115.
8. Giles, R. V.; Spiller, D. G.; Tidd, D. M. *Anti - Cancer Drug Design* **1993**, *8*, 33.
9. Iyer, R. P.; Yu, D.; Agrawal, S. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2471.
10. Fidanza, J.; Ozaki, H.; McLaughlin, L. W. *J. Am. Chem. Soc.* **1992**, *114*, 5509.
11. Gish, G., Eckstein, F. *Science* **1988**, *240*, 1520.
12. Weistheimer, F. M. *Science* **1987**, *235*, 1173.
13. Cox, J. R.; Ramsay, O. B. *Chem. Rev.* **1964**, *64*, 317.
14. Stawinski, J.; Thelin, M. *Nucleosides, Nucleotides* **1990**, *9*, 129.
15. Miyake, A.; Yamaoka, M. *Eur. Pat. 0143601A2* **1985**,
16. Farrington, G. K.; Kumar, A.; Wedler, F. C. *Oppi Briefs* **1989**, *21*, 390.
17. Belsner, K.; Hoffman, H. M. R. *Synthesis* **1982**, 239.
18. Compounds **3a-c** were obtained (47-73%) according to the following typical two-step procedure. **1a** (Rp + Sp, 0.05 mmol) in acetonitrile (10mL) was reacted with **2a** (0.5 mmol) in the presence of 2,6-lutidine (0.25mmol) at room temperature for 30 min (20 h and 5 days were necessary for less reactive **2c** and **2b** respectively). The products were partitioned between 1M aqueous triethylammonium hydrogencarbonate and methylene chloride (CH_2Cl_2). Non volatile material from the organic layer was chromatographed on a silica gel column using 0-4% methanol in CH_2Cl_2 as eluent. A solution of the resulting pure bis dimethoxytritylated **3a** in acetic acid- methanol- water (8/1/1, 5mL) was stirred at room temperature for 5 h, evaporated *in vacuo* and the residue was chromatographed on a silica gel column (0-10% methanol in CH_2Cl_2). After lyophilization from dioxane, **3a** (Rp + Sp) was obtained (73%) as a colorless powder. ^{31}P-NMR (dimethylsulfoxide) (85% H_3PO_4 in D_2O) δ ppm 27.21 and 27.31. FAB-mass spectrum (3-nitrobenzylalcohol) 677 $(M+H)^+$
19. Stawinski, J.; Thelin, M.; Westman, E. *J. Org. Chem.* **1990**, *55*, 3503.
20. Stawinski, J.; Thelin, M.; Zain, R. *Tetrahedron Lett.* **1989**, *30*, 2157.
21. Zhou, L.; Morocho, A. M.; Chen, B.; Cohen, J. S. *Nucl. Acids Res.* **1994**, *22*, 453.

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