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THE PROOLIGONUCLEOTIDE APPROACH. III: SYNTHESIS AND BIOREVERSIBILITY OF A CHIMERIC PHOSPHORODITHIOATE PROOLIGONUCLEOTIDE

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Abstract: Alkylation of a central gap of three phosphorodithioate linkages into a dodecathymidine methylphosphonate with methylacylthioethyl iodide (Me-SATE-I) yielded the corresponding neutral oligonucleotide. Upon incubation of the resulting non ionic prooligonucleotide in cell extracts, the bioreversible Me-SATE masking groups were selectively removed by carboxyesterases present in the milieu.

The artificial control of gene expression by oligonucleotides (oligos) is limited by different hurdles, the two main of them being nuclease resistance and cellular uptake. The problems concerning nuclease resistance may be solved by the use of modified oligos (i.e. phosphorothioate oligos) [1-3]. Nevertheless none modified oligo possesses yet, all the qualities required for antisense therapy. Furthermore, due to their polyanionic character and their high molecular weight oligos are slowly taken up by cells and the physiological mechanisms of oligos internalization are not well understood [4]. The neutral methylphosphonate oligos showed better uptake than wild type oligos but they are unable to elicit RNase H activity and present reduced binding capacities with the targeted RNA strand [5,6]. Thus, their use as antisense agent is restrained. However, they could be used in a chimeric approach which combines methylphosphonate and phosphodiester or phosphorothioate internucleosidic linkages [7]. Increased uptake could be achieved by coupling oligos with poly-L-lysine [8], cholesteryl moiety [9] or by encapsulation into liposomes [10]. Another possibility is to temporarily neutralize the negative charges of oligos with bioreversible protecting groups in order to form oligo prodrugs (prooligos). The resulting neutral prooligos could be expected *i)* to be enzymatically stable against degradative enzymes *ii)* to show less unspecific binding to extra and intracellular proteins [4,6,11-14] *iii)* to present a completely different bioavailability pattern in relation with their route of administration.

We already demonstrated [15,16] that the prooligonucleotide approach could be envisaged on the basis of the observed stability data in biological media of a 12-mer chimeric prooligonucleotide bearing a gap of three masked phosphorothioate linkages and two neutral methylphosphonate flanks. On this model, two different protecting groups removed by carboxyesterases were evaluated; the pivaloyloxymethyl (POM) and the S-methyl acetylthiomethyl (Me-SATM) [16]. However, both POM and Me-SATM protecting groups may present some limitations in term of the observed stabilities in biological milieu [15,16]. Then, we decided to explore the behavior of S-AcylThioEthyl (SATE) protecting groups. Since iodo-SATEs (**2a-c**) are less reactive than iodo-POM or iodo-Me-SATM, we chose to use a phosphorodithioate model because we previoulsy showed that their alkylation is more efficient than that of phosphorothioates. Furthermore, preliminary data on Me-SATE dithymidine phosphorothioate and dithioate [15], have shown that the kinetics of deprotection in various biological medium seems to be suitable for the prooligonucleotide approach.

In this communication, is reported the stability of a dithymidine phosphorodithioate model, bearing various SATE protecting groups, differing on the nature of the alkyl chain (R_1, fig. 1), methyl (Me-SATE), *tert*-butyl (tBu-SATE) and phenyl (Ph-SATE), in culture medium and in total cell extract as a mimic of intracellular medium. Then we extended our study on the most appropriate protecting group for the chimeric phosphorodithioate methylphosphonate 12-mer prooligonucleotide **4** (Fig. 3).

Starting from 5'- and 3'-dimethoxytrityl thymidine, the dithymidine phosphorodithioate **1** was synthesized following an already reported procedure [17,18] using the H-phosphonate method in solution. The iodoalkyl acylates **2a-c** were obtained as described in the literature[19] from the corresponding acid chloride, sodium iodide and episulfide. The triesters **3a-c** were synthesized by alkylation of **1** with **2a-c** (20 mol. eq.) in 200 mM phosphate buffer pH 6:acetonitrile (1:1, v/v) at 55°C. The rate of alkylation of **1** was slightly dependent on the nature of the alkylating agent, a 70 % of alkylation was achieved after 80, 96 and 118 hours for **2a**, **2b** and **2c** respectively. The triesters **3a-c** were purified, as diastereoisomeric mixtures, by silica gel chromatography and characterized by ^{31}P-NMR and mass spectrometry [20].

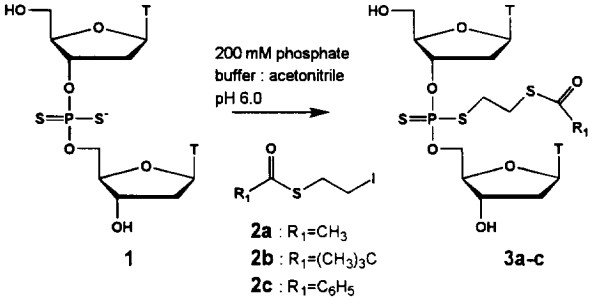

Figure 1 : synthesis of **3a-c** by alkylation of dithymidine phosphorodithioate

The stability and the fate of **3a-c** upon incubation condition were determined in three different biological media; in culture medium (RPMI 1640 containing 10% of heat inactivated fetal calf serum) and in total CEM-SS cell extract, as mimics for cell culture assays, and in gastric juice for eventual *in vivo* experimentations.

The decomposition of **3a-c** ($c = 5\ 10^{-5}$ M) was monitored by HPLC using a reported cleaning method [21] and the half-life of each compound was determined (Table 1).

As control, we firstly checked that each dimer was substrate of porcine liver esterase (PLE). Their half-lives were less than 5 min. in presence of 8U of PLE /ml.

	Half-life		
Medium	**3a**	**3b**	**3c**
Culture Medium (RPMI 1640 + 10% FCS)	6.0 h / 9.8 h	42.7 h*	46.5 h*
Total Cell Extract	< 5 min.	0.9 h / 1.5 h	0.6 h / 1.3 h
Gastric Juice	7 days	stable^{+}	stable^{+}
Porcine Liver Esterase	< 2 min.	< 4 min.	5 min.

* same value for the both diastereoisomers,: $^{+}$No degradation observed up to 30 days

Table 1 : half-lives of **3a-c,** Rp and Sp diastereoisomers, in different biological media

The observed decomposition data deserve the following comments. In culture medium and in cell extract, selective formation of a phosphorodithioate diester exclude any nucleophilic attack on the phosphorous atom which corroborate literature data on the susceptibility towards hydrolysis of thionophosphorothiolate triesters [22]. It follows that the observed degradation in both media proceeds through a Cα-S bond breakage mechanism, presumably induced by carboxyesterases (Fig. 2). Compounds **3a-c** are diastereoisomeric mixtures as corroborated by the presence of two peaks of roughly equivalent intensity in HPLC profiles. In several cases, a different rate of hydrolysis was observed. The rate of decomposition between two diastereoisomers may vary by a factor of two. Such an enantiomeric dependance has been previously reported for the hydrolysis of Rp and Sp methylphosphonothioate mediated by acetylcholine esterase [23,24].

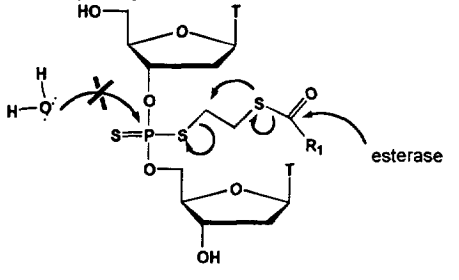

Figure 2 : Proposed mechanism for hydrolysis of **3a-c**.

In gastric juice, **3a-c** are highly stable, the slight degradation observed for **3a** may arise either from the acid hydrolysis of the thioester function or from some residual carboxyesterase activity. In addition, this observed stability in low pH conditions indicates that the phosphotriester functionality should be stable in the acidic endosome or lysosome compartments.

In a second step, a model dodecathymidine **5** containing a central gap of three phosphorodithioate triesters and two methylphosphonate flanks was synthesized. The Me-SATE bioreversible protecting group was selected since the corresponding dimer **3a** is stable enough in culture medium and the Me-SATE is rapidly removed in total cell extract. Furthermore the corresponding trialkylated prooligo can be expected to be less insoluble than the ones with tBu-SATE or Ph-SATE protecting groups. The synthesis of the model dodecathymidine was performed by post-synthetic alkylation of oligo **4** (Fig. 3). [25]

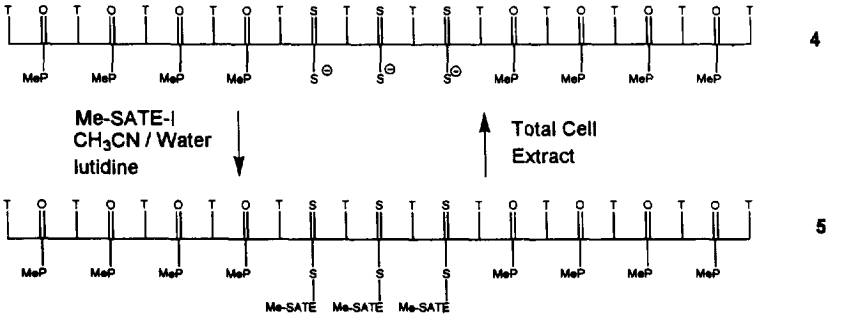

Figure 3 : Alkylation of **4** with Me-SATE iodide to yield **5** and bioreversible demasking in total cell extract.

The stability of **5** was evaluated in culture medium and in total cell extract and its half-lives are reported in table 2, as well as our previous results obtained with the POM protecting group on a three phosphorothioate gap with methylphosphonate flanks **6** [16]. HPLC profiles of **5** at different time of incubation in total cell extract are shown on figure 4.

	Half-life	
Medium	**5**	**6**
Culture Medium (RPMI 1640 + 10% FCS)	32 h	4.5 h
Total Cell Extract	15 min.	< 5 min.

Table 2 : half-lives of **5** and **6** in culture medium and in total cell extract.

In both media, oligo **5** selectively leads to oligo **4** with a good stability in culture medium ($t_{1/2}$ 32 h). In cell extract, a short $t_{1/2}$ was obtained for the hydrolysis of the first Me-SATE protecting group ($t_{1/2}$ 15

min) and the $t_{1/2}$ for the appearence of oligo **4** being 80 min. As shown on table 2, the Me-SATE protecting group seems more suitable than the previously reported POM one [16] for a prooligonucleotide approach, as it presents more stability under *in vitro* assay conditions.

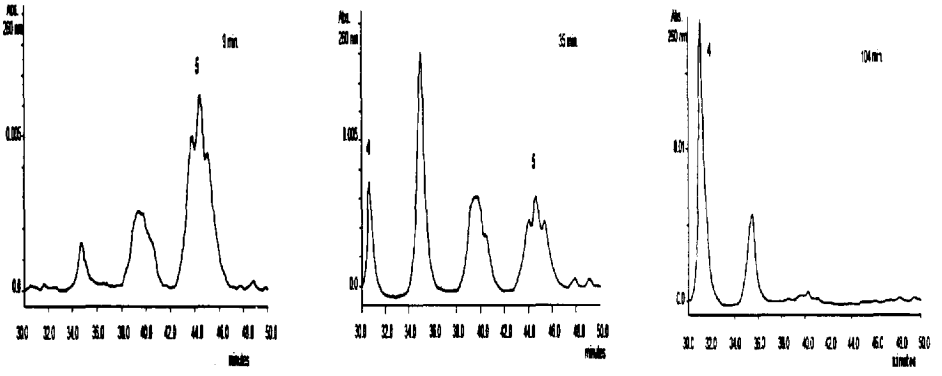

Figure 4 : HPLC profiles of **5** after 9 min., 35 min. and 104 min. incubation in total cell extract.

In addition, both models **5** and **6** selectively lead to the formation of respectively the phosphorodithioate and the phosphorothioate gap which could potentially induced cleavage of the targeted mRNA by Rnase H.

It follows from the observed stability data that the prooligonucleotide approach may be envisaged on the basis of the herewith reported data for the ME-SATE bioreversible protecting group. Works along this line are in progress and will be reported elsewhere.

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20. MS (FAB +) $[M+H]^+$:**3a** : 681, **3b** :723, **3c** : 743; ^{31}P-NMR (ppm) relative to external 66% H_3PO_4, **3a** : 96.8 and 97.2, **3b** : 97.1 and 97.3, **3c** : 96.6 and 97.2.
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25. **4** : ^{31}P-NMR 36.3 and 114.1 ppm for respectively methylphosphonate and phosphorodithioate linkages. **5** : ^{31}P-NMR broad peaks centered at 35.8 and 97.6 ppm for respectively methylphosphonate and thionophosphorothiolate linkages.

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