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THE PROOLIGONUCLEOTIDE APPROACH : II. SYNTHESIS AND STABILITY STUDIES OF CHIMERIC OLIGONUCLEOTIDE MODELS

Isabelle Barber, Guilhem Tosquellas, François Morvan, Bernard Rayner and Jean-Louis Imbach*

Laboratoire de Chimie Bio-Organique, URA 488 du CNRS,

Université de Montpellier II, Place Eugène-Bataillon, 34095 Montpellier Cedex 5 France

Abstract: Alkylation of a central gap of three phosphorothioate linkages into a dodecathymidine methylphosphonate with different iodoalkyl acylates yielded the corresponding neutral oligonucleotides. Upon incubation of these prooligonucleotides in cell extracts, the bioreversible alkyl acylate masking groups were selectively removed by carboxyesterases present in the milieu.

In a previous paper[1] based on model dithymidine phosphorothiolate triesters bearing bioreversible protecting groups [pivaloyloxymethyl (POM), S-acetylthiomethyl (MeSATM) and S-acetylthioethyl (MeSATE)], we demonstrated that these compounds could lead selectively to dithymidine phosphorothioates upon carboxyesterase mediated hydrolysis in total CEM cell extracts (a T lymphoblastoid cell line). This observation seemingly opens the way for the possible design of oligo prodrugs, providing that such an approach can be extended to oligonucleotides (oligos) bearing several phosphorothiolate internucleotide linkages.

As compared to the parent ionic phosphorothioate oligos, neutral phosphorothiolate prooligos are expected *i)* to be more enzymatically stable against degradative enzymes due to the fact that no phosphotriesterase activity has been reported in eukariotic cells *ii)* to show less unspecific binding to extra and intracellular proteins[2-6] due to the suppression of the oligo's negative charges *iii)* to enhance the transport of the oligos across the cell membrane, as it has been reported, that a diminution in the number of negative charges on an oligo may increase its uptake[7-10] *iv)* to present a completely different bioavailability pattern in relation with their route of administration.

The solid phase synthesis of neutral phosphorothiolate oligos is a challenging problem as one has to preclude any basic treatment in order to avoid hydrolysis to the corresponding phosphodiester oligo[11]. Therefore, starting from preformed phosphorothioate oligos, a post-synthetic alkylation was performed.

To evaluate the validity of this prooligo approach and also to carefully study the protection/deprotection processes, we decided initially to concentrate our efforts on a dodecathymidine chimeric prooligo containing a gap of three central consecutive phosphorothioate internucleotidic linkages between two methylphosphonate flanks. As a bioreversible protecting group, we first selected MeSATM and POM, as we have previously shown[1] that their iodo derivatives can readily alkylate phosphorothioate linkages.

Starting from methylphosphonamidite and phosphoramidite synthons, the chimeric dodecamer **1** was synthesized on a solid support (Fig. 1) and was purified by HPLC on a C18 reverse phase column.

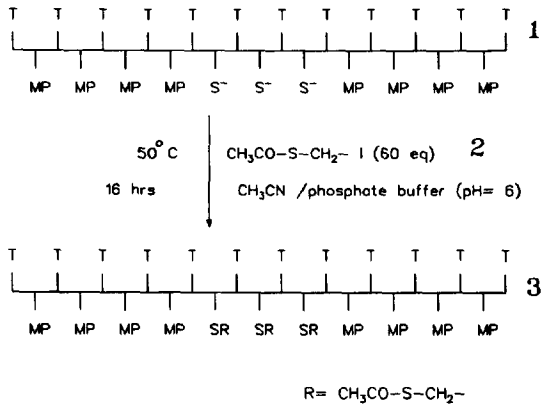

Figure 1 : Alkylation of the chimeric dodecamer **1**, MP : methylphosphonate linkage.

Alkylation of **1** with MeSATM iodide **2** was carried out at pH 6 and was monitored by HPLC. As illustrated in Fig. 2, after two hours (2B), one observes the formation of the mono and dialkylated as well as the accumulation of the trialkylated product **3**. After 16 hrs (2C) the fully alkylated chimeric oligo **3** was formed in nearly quantitative yield and was obtained after purification by HPLC.

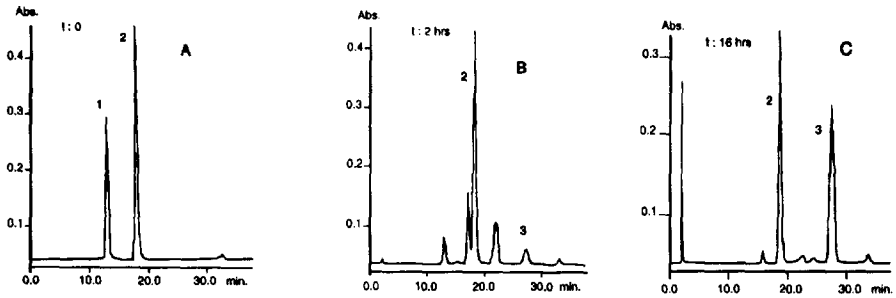

Figure 2: . HPLC profiles of alkylation of **1** with MeSATM iodide **2** to yield **3**

The stability of **3** was evaluated in culture medium (RPMI 1640 + 10% heat unactivated fetal calf serum) and also in total CEM cell extracts (HPLC monitored, oligo concentration 10μM). In the culture medium, a single alkyl group was removed with a $t_{1/2}$ of 40 min with presumably formation of phosphorothioate and phosphodiester internucleoside bonds. It is noteworthy that on a dimeric model basis, the $t_{1/2}$ of the corresponding dithymidine MeSATM phosphorothiolate triester was found to be 60 min with obtention of a mixture of phosphorothioate and phosphodiester linkages[1]. In contrast, in total CEM cell extract, the half-life of **3** is very short (< 5 min) and the corresponding chimeric parent oligo **1** is obtained selectively ($t_{1/2}$ of appearance, 18 min).

From this first experiment it could be concluded that **1** may be too unstable for cell culture experiments. However, from the observed data using CEM cell extracts, it appears to comply with the requirements of a prooligonucleotide approach.

As a result we decided to evaluate the potentiality of the POM protecting group, as we have previously demonstrated[1], on a dimeric model, that the stability of the corresponding phosphorothiolate triester in culture medium is greater than that of the MeSATM equivalent ($t_{1/2}$: 360 min and 60 min respectively).

Also, as we had planned to further study the cellular uptake of such neutral chimeric prooligos, we decided to synthesize directly the corresponding 5'-biotin derivatized prooligo **8**.

The chimeric oligo **4** bearing an hexamethyleneamino link on the terminal 5' position was synthesized with an automated DNA synthesizer using the same chemistry as for **1**. In the next step, **4** was condensed with the biotin N-hydroxysuccinimide ester **5** (HPLC monitoring) to give the corresponding conjugated chimeric oligo **6**, after purification by HPLC. Further selective alkylation of the central phosphorothioate linkages was performed with the pivaloyloxymethyl iodide derivative **7** (POM-I) in acetonitrile/water/lutidine at room temperature with HPLC monitoring. The expected biotin conjugated prooligo **8** was isolated after purification by HPLC[12].

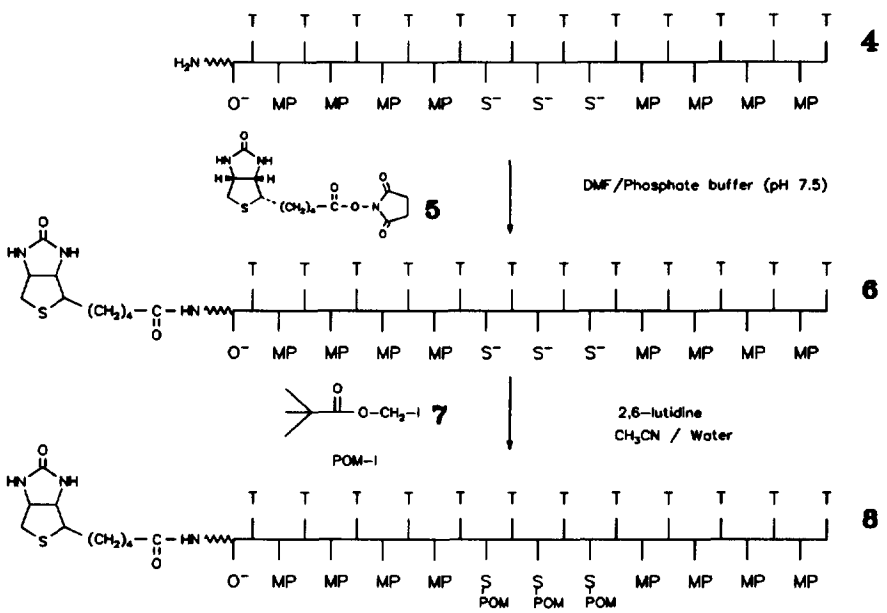

Figure 3 : Synthesis of a fully S-alkylated chimeric dodecathymidine **8**, MP : methylphosphonate linkage.

The half-life of **8** was shown to be 4.5 hrs in culture medium and 1.8 hrs in human serum. It is noteworthy that, in both media, the parent oligo **6** was not recovered and from the HPLC data, one could assume that concomitant ionic phosphodiester and phosphorothioate formation had occurred. In total CEM cell extracts, it was possible to follow, by HPLC, the successive removal of the POM groups. It was observed that the first protecting group was removed very rapidly ($t_{1/2} < 2$ min), whilst the deprotection of the remaining POM groups proceeded much more slowly, *i.e.* the half-life for complete apparition of the fully deprotected chimeric oligo **6** being 2.4 hrs (figure 4).

The observed differences in the kinetics of POM deprotection can be explained easily, with reference to the two main structural features which influence the activity of mamalian esterases, namely the nature of the substrate functionality and the lipophilicity of the molecules. In this respect, polar or charged compounds are not the prefered substrate for these enzymes[13-14]. Thus, one can hypothesize that progressive carboxyesterase mediated deprotection of the gap decreases the affinity of the chimeric oligo as an enzyme substrate due to the successive increase in negative charge.

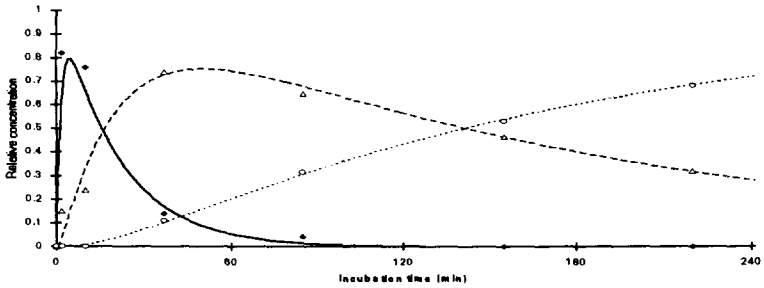

Figure 4 : Kinetics of deprotection of **8** in cell extract. ♦ : mono-deprotected product; ∆ : di-deprotected product; o: tri-deprotected product, oligo **6**.

Therefore, on the basis of this preliminary data, one can conclude that the choice of the bioreversible carboxyesterase mediated protecting group is of prime importance in the design of further prooligo models in terms of the kinetics of deprotection. This very promising prooligonucleotide approach may open up a wide field of investigation as such a concept can be applied to any kind of phosphodiester or/and phosphorothioate oligonucleoside.

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12) To a solution of **6** (0.8 mM) in acetonitrile water (270 µl, 1:1, v/v), pivaloyloxymethyl iodide (690 mM in acetonitrile, 15 µl) and 2,6-lutidine (460 mM in acetonitrile, 15 µl) were added. The resulting mixture was stirred for 3 hrs at RT and then oligo **8** was purified by HPLC on C18 reverse phase column (50 mM TEAAc as buffer). Finally triethylammonium salts were removed on Sep-Pack (C18).
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