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FIRST SYNTHESIS OF ALTERNATING SATE-PHOSPHOTRIESTER / PHOSPHODIESTER PROOLIGONUCLEOTIDES ON SOLID SUPPORT

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Abstract :Prooligonucleotides with alternating S-Acyl-ThioEthyl (SATE) phosphotriester and phosphodiester linkages or a gap were synthesized by elimination of cyanoethyl group with non-nucleophilic base DBU. Their half-lives in the presence of Pig Liver Esterases (PLE) and in total cell extract were determined.

The main drawback for therapeutic oligonucleotides (ODN) is connected with their polyanionic structure which implies poor uptake, enzymatic degradation and unfavorable pharmacokinetic parameters [1,2]. To overcome such drawbacks, we proposed to apply the prodrug concept to ODN using an enzymolabile protection to temporary mask the phosphodiester negative charges [3-5].

For this purpose, we developed an automated synthesis of prooligothymidines (with oxo or thiono phosphotriesters) using thymidine phosphoramidite building blocks bearing a S-pivaloyl-thioethyl (tBuSATE) enzymolabile protecting group [6] and a photo-cleavable spacer [7] that avoiding any nucleophilic treatment during the release of the prooligonucleotide from solid support.

As fully tBuSATE masked prooligos were highly lipophilic and hence poorly soluble, the aim of this work was to synthesize partially protected prooligos having negatives charges in predetermined positions to increase their solubility. We report in this communication the automated synthesis on solid support of prooligos bearing alternating phosphotriester tBuSATE and phosphodiester or phosphorothioate linkages, and their stability in presence of pig liver esterases (PLE) and in total cells extract (TCE).

The synthesis of such alternating tBuSATE prooligos could be designed using the standard phosphoramidite approach provided that cyanoethyl (CNE) elimination conditions do affect neither

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the tBuSATE phosphotriester linkage nor the solid support anchoring. As CNE groups are removed by a β-elimination mechanism, we decided to explore the ability of DBU - a strong non nucleophilic base in anhydrous media- to selectively eliminate the CNE groups while keeping intact the tBuSATE groups.

First, we synthesized on the photolabile solid support [7] an hexathymidylate **4** (Fig.1) to determine under which conditions CNE groups can be completely and selectively eliminated by DBU.

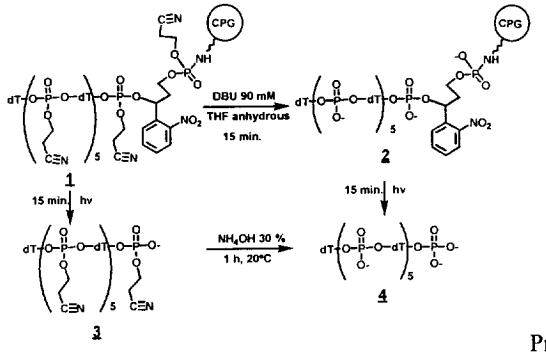
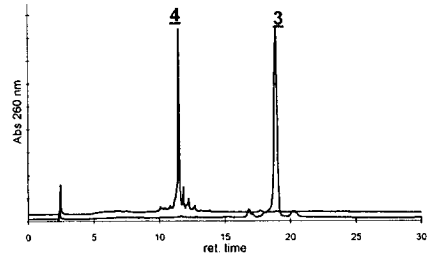


Figure 1: Hexamer deprotection



Profile 1: HPLC reverse phase profiles of the crude hexamers

The 3'-phosphate hexathymidine **4** was obtained following two different reaction schemes (Figure 1). In one hand, a 15 min DBU treatment (90 mM in dry THF at RT) of **1** led to hexathymidylate phosphodiester **2** still anchored on the solid support and then a 15 min photolysis [7] yielded **4** (Profile 1). In another hand, **1** was first cleaved from solid support by photolysis to give the CNE protected oligo **3** and then treatment with concentrated ammonia (1h at RT) yielded **4** (Profile 1). HPLC co-injection of the both samples of **4** showed that the same product was obtained and that no CNE group remained after the DBU treatment.

Thus at least six CNE protecting groups can be eliminated from an oligo under the aforementioned conditions without affecting the anchorage on the solid support.

The DBU-deprotection conditions being set, we synthesized then two alternating prododecathymidines **7** and **8** (Figure 2). Prooligo **7** was constituted with alternating phosphorothioate/tBuSATE-phosphotriester, and prooligo **8** was the phosphodiester/tBuSATE-thionophosphotriester analog. They were synthesized on a 2 μmole scale using β–cyanoethyl and tBuSATE thymidine phosphoramidites [6] and the same photo-cleavable solid support previously

reported [7]. Phosphodiester and phosphotriester backbones were obtained by oxidation with *tert*-butyl hydroperoxide [8], and phosphorothioate and thionophosphotriester backbones by sulfurization with Beaucage reagent [9].

Two samples of fully protected oligos **5** and **6** were treated with DBU for 21 and 120 min, then photolysis was performed during 15 min to release the alternating prooligos. These structures allow us to easily distinguish by ^{31}P-NMR the fate of each kind of phosphorus containing linkage *i.e.* phosphotriester (≈ -2 ppm), thionophosphotriester (≈ 67 ppm), phosphodiester (≈ -0.6 ppm), phosphorothioate diester (≈ 56 ppm) and 3'-terminal phosphorothioate monoester (≈ 46 ppm)

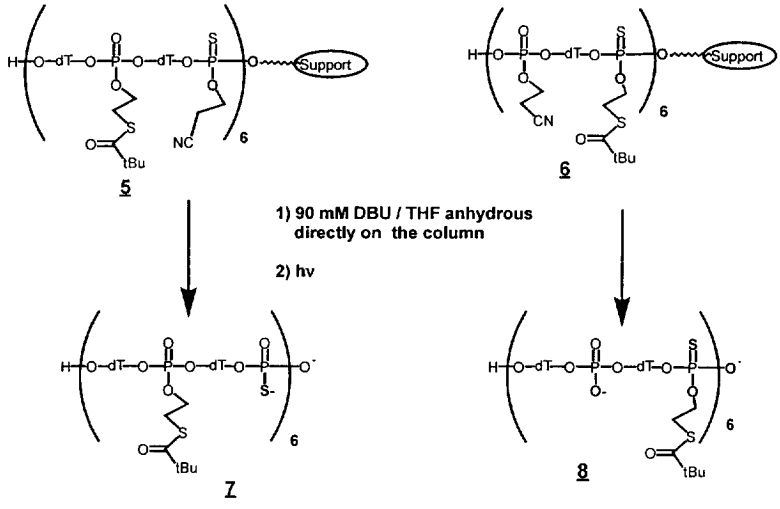


Figure 2: Synthesis of alternating tBuSATE prooligos

The six CNE groups on the thionophosphotriesters of **5** were completely removed within 120 min of DBU treatment. Indeed, after 21 min a weak signal at 67.6 ppm corresponding to the cyanothionophosphotriester was still present but has disappeared after 120 min. This extended DBU treatment did not induce the removal of tBuSATE since the integration of the signal at -1.6 ppm (phosphotriester) did not decrease between 21 and 120 min.

Under the same experimental conditions, the complete elimination of CNE groups on phosphotriesters of **6** (within 21 min) proceeded faster than on previous thionophosphotriesters (within 120 min) and was comparable to the time of deprotection of **1**. The observed differences in the rate of CNE elimination can be explained with the higher electronegativity of oxygen with respect to sulfur.

Noteworthy that MeSATE analogs can not be synthesized since the corresponding phosphotriesters were degraded by DBU. It follows that alternating tBuSATE prooligos bearing up to seven negative charges can be obtained.

For comparison purpose alternating prooligos (fully oxo **9** and fully thiono **10**) and gap prooligos with either neutral methylphosphonate wings **11** or charged wings but with nuclease resistant 2'-O-methyl phosphodiester **12** were synthesized (Fig. 3). These prooligos were characterized by mass spectrometry (ESI) [10].

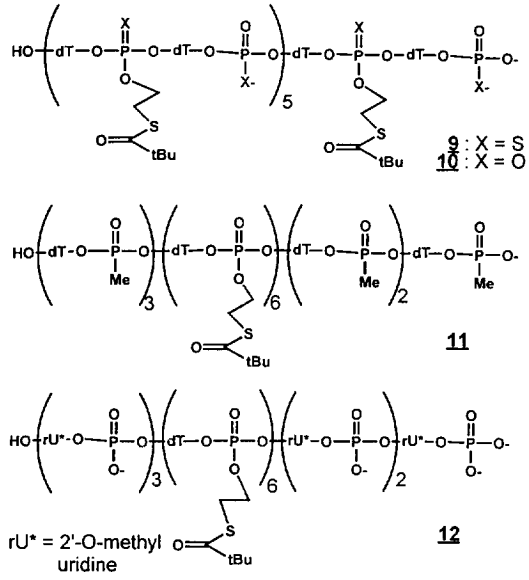


Figure 3 : Schematic representation of prooligos **9** to **12**.

The substrate capacity for carboxyesterases of the prooligos **7**-**12**, exhibiting various structures (alternating or gap with charged or neutral wings) was then evaluated. Their half-live in presence of pig liver esterases (PLE) and in total CEM cell extract (TCE) were determined, using HPLC [11]. The half-life values for the first hydrolysis, reported in Table, brought out the difference of substrate capacity between alternating (**7** to **10**) and gap (**11** and **12**) prooligos.

Our result suggests that the esterase activities of PLE are very sensitive to the presence of negative charges adjacent to the SATE groups since all alternating prooligos (**7** to **10**) were not substrate while gap prooligos (**11** and **12**) were hydrolyzed. This discrepancy could be explained by the fact that only in gap structures carboxyesterases can hydrolyze a SATE group surrounded by neutral phosphotriesters. Hence, the difference of structure appears to be determinant for a prooligo

to be a PLE substrate. This fact is not surprising since it was reported that most carboxyesterases have higher affinity for lipophilic esters than for polar or charged substrates [12]. The overall lipophilicity of each prooligo may be reflected by their retention time (R_T) on reverse phase HPLC under the same conditions (Table) [13]. Considering the gap prooligos, **11** exhibits the highest lipophilicity and a 35 min half-life for the first hydrolysis of a SATE group and **12** was hydrolyzed seven-fold faster ($t_{1/2}$ 5min). Our result suggests that when a prooligo possesses a too high lipophilicity its substrate capacity for carboxyesterases decreases. We already reported similar results with prooligos bearing pivaloyloxymethyl (POM) enzymolabile groups with either neutral or charged wings [14,15].

Prooligos	Half - life		HPLC C_{18} Retention Time (min)
	PLE	TCE	
7	Stable	3 h	51.9
8	Stable	2.5 h	51.7
9	Stable	17 h	58.4
10	Stable	1 h	48.8
11	35 min	1.2 h	60.5
12	5 min	0.25 h	54.7

Table : Half-life for the first hydrolysis of prooligos **7** to **12** with PLE (8 units/ml) and in TCE, and HPLC retention time on reverse phase C18 column (Nucleosil 5 µm, 150 x 4.6, elution : 50 mM TEAAc pH 7 for 7 min, then 0% to 80% acetonitrile in 50 mM TEAAc for 77 min).

In TCE, all the prooligos were substrate of the enzymes, but for a same range of lipophilicity gap prooligos (**11** or **12**) were hydrolyzed faster than the alternating prooligos (**7** to **10**). Indeed, **12** (R_T 54.7 min) which exhibited a R_T value intermediate between those of **7** (R_T 51.9 min) and **9**, (R_T 58.4 min) was hydrolyzed faster ($t_{1/2}$ 0.25h) than **7** ($t_{1/2}$ 3h) and **9** ($t_{1/2}$ 17h). Likewise **11** (R_T 60.5 min, $t_{1/2}$ 1.2h) was hydrolyzed faster than **9** (R_T 58.4 min, $t_{1/2}$ 17h). Furthermore, for each type of structure (alternating or gap), the higher the lipophilicity of prooligo, the lower the rate of hydrolysis for the first SATE group.

These data showed that carboxyesterases activities of PLE and TCE are different and are highly disturbed by negative charges adjacent to the SATE group. Our results suggest that both the local hydrophobicity surrounding the SATE group and the global lipophilicity of the prooligos are important factors for the design of prooligos to be unmasked by carboxyesterases.

It is now possible to synthesize prooligos partially substituted with tBuSATE protecting groups in any given positions. After labeling, such compounds, of various lipophilicities, are under evaluation for their ability to be taken up into intact cells. Preliminary data along this line are encouraging.

Acknowledgment
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