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Lipocap: a Lipophilic Phosphoramidite-based Capping Reagent

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Abstract: *The use of O-β-cyanoethyl-O-octyl-N,N-diisopropyl phosphoramidite (**1**) (Lipocap) as a capping reagent in the automated solid phase synthesis of oligonucleotides is described. Its lipophilicity allows the easy separation of capped failure sequences from trityl-off full length material by reverse phase HPLC. Consequently, oligonucleotides are isolated with higher yield and shorter time.*

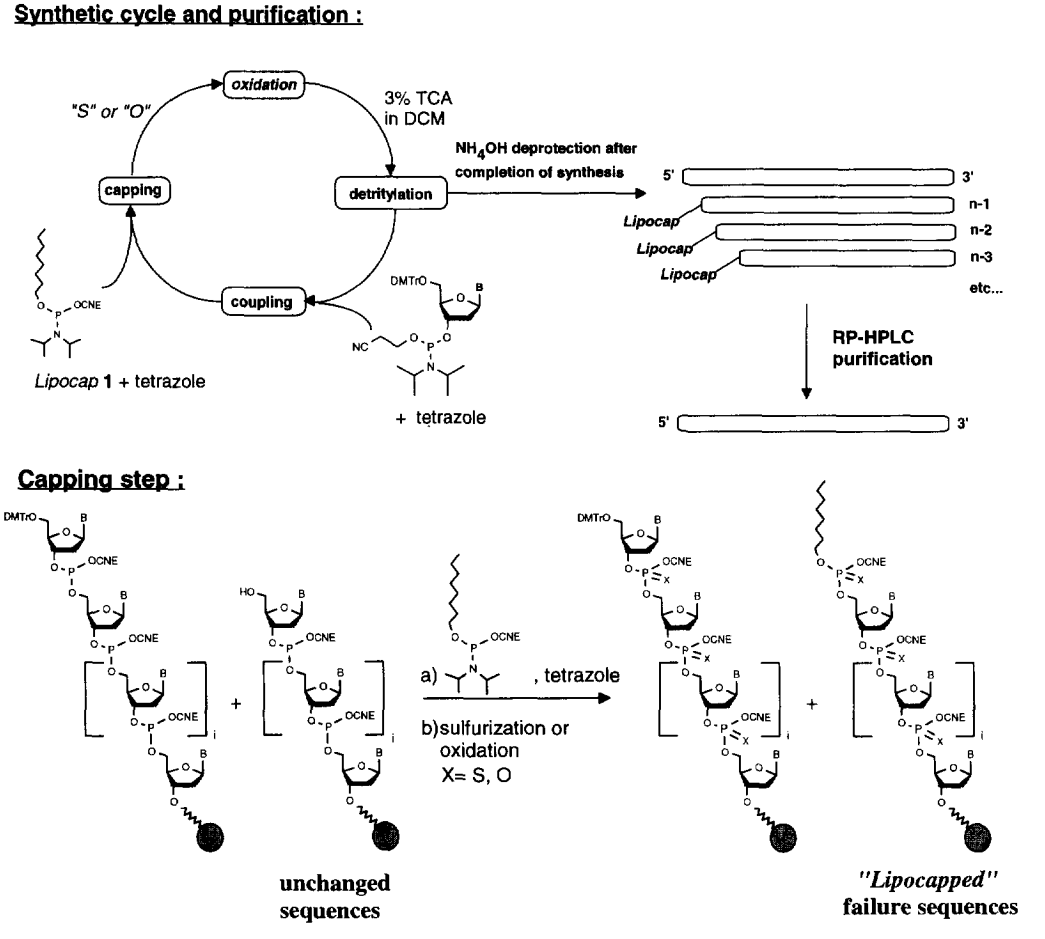
INTRODUCTION

In 1978, Zamecnik and Stephenson[1] demonstrated that synthetic oligonucleotides inhibited protein synthesis by specific interaction with the messenger RNA (m-RNA) coding for the protein. This approach, later called *antisense strategy*, triggered a considerable interest in oligonucleotides as a new class of potential therapeutic compounds[2]. Biological activity of oligonucleotides was demonstrated in animals[3] and in clinical studies[4]. As a consequence, the synthesis and purification of oligonucleotides has been a matter of intensive investigation and review[5].

The most commonly used approach for the synthesis of oligonucleotides is the solid phase method, using phosphoramidite chemistry[6]. Typically, the oligonucleotide is synthesized using the following standard cycle[4]: 1) deprotection of the 5'-terminal 4,4'-dimethoxytrityl group (DMT) of the support bound, growing oligonucleotide chain; 2) coupling of the next 3'-phosphoramidite building block the free 5'-OH; 3) capping of unchanged species and 4) oxidation of the phosphorus atom. This cycle is repeated as often as required for completion of the oligonucleotide assembly. Oligonucleotide cleavage from the support and removal of the base labile protecting groups is performed by ammonia treatment. In the classical procedure, the terminal 5'-protecting group is not removed (*trityl-on*) and the purification consists of a reverse phase HPLC separation of the trityl bearing full length oligonucleotide from all non lipophilic components. Although efficient and used on the multigram scale[7], this method displays two main disadvantages: a) it requires a post-purification chemical treatment (removal of DMT group with aq. acetic acid) and b) the relative instability of the DMT group leads to the loss of some full-length material due to unwanted detritylation during the purification procedure. Therefore, we explored the possibility of using a *lipophilic phosphitylating reagent*[8,9] which we call *Lipocap* as replacement of the standard acylation capping reagent during oligonucleotide synthesis. This should allow the efficient removal of *trityl-off* full length oligonucleotide from lipophilic truncated material by a simple RP-HPLC treatment. In this report, we describe the use of the capping reagent **1** (Scheme 2) for a *trityl-off* purification scheme.

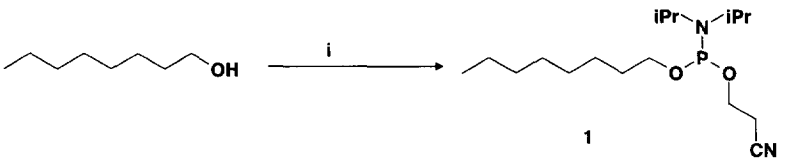
RESULTS AND DISCUSSION

The principle of this approach is illustrated in Scheme 1 and consists of the use of a lipophilic, ammonia stable capping reagent **1**. Unreacted material is capped with **1** using conventional phosphoramidite chemistry. The difference of this method from the conventional acetyl-capping is that it renders the failure sequences lipophilic, thus allowing their easy separation from full length *trityl-off* oligonucleotide. Since the purified oligonucleotide is recovered *trityl-off*, no further chemical treatment is required.



Scheme 1. *Lipocap /"trityl-off"* strategy.

1. Synthesis of Lipocap 1.- The phosphoramidite **1** was straightforwardly prepared by reacting O-β-cyanoethyl-*N,N,N',N'*-tetraisopropyl phosphorodiamidite with *n*-octanol in the presence of diisopropyl ammonium tetrazolide under argon atmosphere at room temperature for 20 hours. The product was obtained after flash chromatography as a colorless oil in 90% yield (Scheme 2).



Scheme 2. *Reagents and conditions*: i: O-β-cyanoethyl-*N,N,N',N'*-tetraisopropyl phosphordiamidite (1.1 equiv.)/diisopropyltetrazolide (1.2 equiv.), dry CH_2Cl_2, R.T., 20h under argon atmosphere.

2. *Oligonucleotide Synthesis.*- To be a useful capping reagent, it is necessary that the *Lipocap* **1** reacts in high yield with the free 5'-OH of the unchanged oligonucleotide chain. The phosphorothioate 20-mer **4** was synthesized using the phosphoramidite **1** as the capping reagent, a) with a total failure coupling[10] simulated at position #10, b) at position #20 and c) without failure coupling yielding respectively the oligonucleotides **2**, **3** and **4**. The data obtained are summarized in Table 1. The capillary gel electrophoresis (CGE) analysis of the crude phosphorothioate **4** displayed a quality equal to the standard procedure (see Figure 1). Furthermore, the use of the Lipocap **1** resulted in the complete blocking of chain elongation after pos. #10 and #20 in exp. a) and b), respectively. The compounds **2** to **4** were identified by MALDI-TOF MS[11] and RP-HPLC analysis of crude oligonucleotides **2** and **3** confirmed quantitatively the total blocking of chain elongation[12]. This demonstrates that the use of the phosphoramidite **1** as capping reagent on DNA synthesizers ensures a total blocking of failed sequences due to incomplete couplings, yielding crude materials consisting of full length material and lipophilic capped sequences.

Table 1. Identification (MALDI-TOF MS) of the products obtained using **1** as capping reagent.

Experiment	Product observed (5'→3')	MW observed (expected)
a)	**2** Octyl-sAs CsAsTs GsCsAs TsT	3030.7 (3033.0)
b)	**3** Octyl-sCsCs CsGsCs CsTsGs TsGsAs CsAsTs GsCsAs TsT	6241.1 (6236.2)
c)	**4** TsCsCs CsGsCs CsTsGs TsGsAs CsAsTs GsCsAs TsT	6347.2 (6348.2)

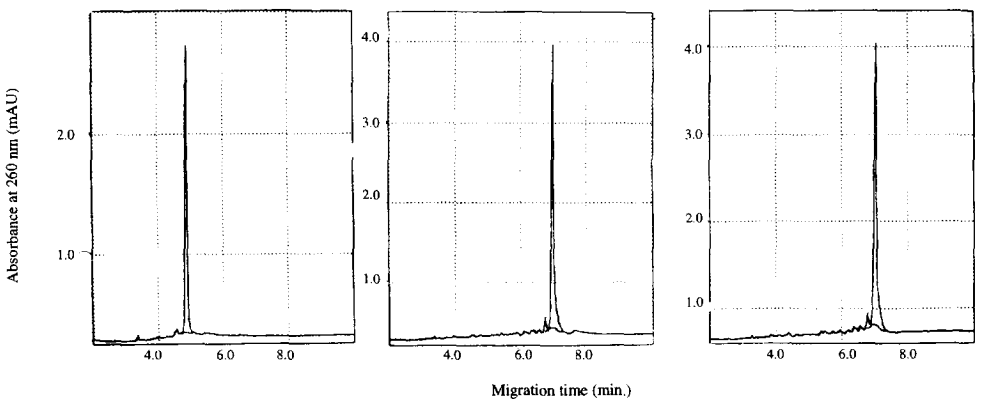


Figure 1: CGE scans of crude oligonucleotides **2**, **3** and **4** (from left to right) obtained by automated oligonucleotide synthesis using the lipophilic capping reagent **1**.

3. Oligonucleotide Purification.- In order to check the usefulness of the lipophilic capping reagent **1** as an aid in the oligonucleotide purification, the oligonucleotide **3** (19-mer, n-octyl capped) was co-injected with the 20-mer **4** (see Figure 2). As can be seen, an excellent resolution is obtained between the 20-mer (full-length material) and the 19-mer bearing the lipophilic capping group.

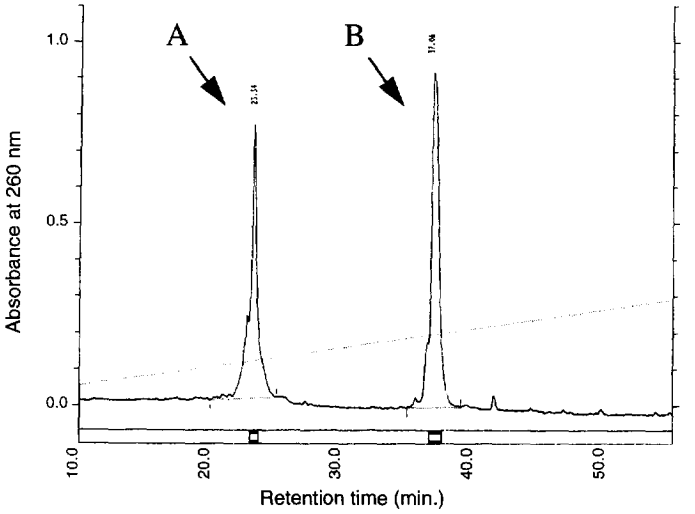


Figure 2: Co-injection of purified 20-mer **4** (peak A) and lipocapped 19-mer **3** (peak B) in RP-HPLC.

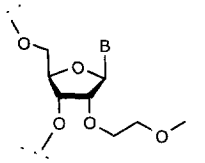
To establish the benefit resulting from the use of capping reagent **1** in routine oligonucleotide synthesis, we prepared the two 20-mer oligonucleotides **5** and **6** (Formula 1) using both the *lipocap/trityl-off* procedure and the classical *trityl-on* approach in "head to head" comparisons. The two oligonucleotides chosen for this study are a phosphorothioate antisense oligodeoxynucleotide **5** targeting c-raf mRNA and a mixed phosphodiester/phosphorothioate oligonucleotide of the same sequence containing deoxynucleotides and 2'-methoxyethoxy modified building blocks[13] **6**. Both oligonucleotides are of current interest as inhibitors of the proliferation of certain tumor cells[3].

(**5**): 5' - TsCsCs CsGsCs CsTsGs TsGsAs CsAsTs GsCsAs TsT

(**6**): 5' - T C C C G Cs CsTsGs TsGsAs CsAsT G C A T T

s: phosphorothioate linkage

underlined:



Formula 1. 20-mers **5** and **6** synthesized using the *lipocap/trityl-off* procedure and the classical *trityl-on* approach in "head to head" comparisons

Table 2. Procedure for the synthesis of **5** and **6** using *Lipocap* (**1**) as capping reagent.

Step	Reagent/Solvent	Function	Time in sec. per cycle
1	MeCN	Wash	20 (2x)
2	3% CCl_3COOH/CH_2Cl_2	Detritylation	40 (3x)
3	MeCN	Wash	20 (3x)
4	Phosphoramidite/tetrazole/MeCN	Coupling	65 (2x) + 125[a]
5	MeCN	Wash	20 (2x)
6	Lipocap/tetrazole/MeCN[b]	Capping	65 (2x)[c]
7	MeCN	Wash	20 (2x)
8	Stec's reagent[d] or 0.5M tBuOOH in DCM	Sulfurization /Oxidation	70 (3x)[c]
9	MeCN	Wash	20 (2x)

a) delivery = 5 sec. and reaction time = 60 or 300 sec. for 2'-methoxyethoxy building blocks or for unmodified phosphoramidites, respectively; b) Cap A solution: *Lipocap* (0.15 M in MeCN); Cap B solution: tetrazole (0.45 M in MeCN); c) delivery = 5 sec. and reaction time = 60 sec; d) according to ref. 14.

All syntheses were performed on a *Perkin Elmer* ABI 394B DNA synthesizer on the 10 µmole scale using either the standard procedure (acetic anhydride capping) or the modified procedure shown in Table 2. After ammonia deprotection the oligonucleotides were purified by RP-HPLC. *Trityl-on* fractions were deprotected by treatment with aqueous acetic acid (80% in water, v/v) according to the standard procedure. Table 3 summarizes the amounts obtained before and after purification for oligonucleotides (**5**) and (**6**) prepared either using conventional acetylation capping in combination with *trityl-on* RP-HPLC purification (entries 1 and 2), or using *Lipocap* (**1**) as capping reagent in combination with *trityl-off* RP-HPLC purification (entries 3 and 4). The purity of all materials obtained was controlled by CGE analysis (Figure 3). As can be seen, the *Lipocap* approach affords both oligonucleotides with equal purity but in a higher overall yield (52 and 50%) as compared to the classical procedure (47 and 41%)[15]. Taken together with the considerable time-saving in the purification step (no requirement of trityl removal), these data demonstrate the usefulness of (**1**) as a versatile capping reagent for oligonucleotide synthesis.

Table 3. Data of the head-to-head comparison at 10 µmol scale.

entry	method	compound	crude material	purified material	
			[OD_{260}]	[OD_{260}]	(%)
1	Acetic anhydride capping	**5**	1366	642	47
2	and *trityl-on* purification	**6**	1357	556	41
3	*Lipocap* capping	**5**	1334	696	52
4	and *trityl-off* purification	**6**	1250	625	50

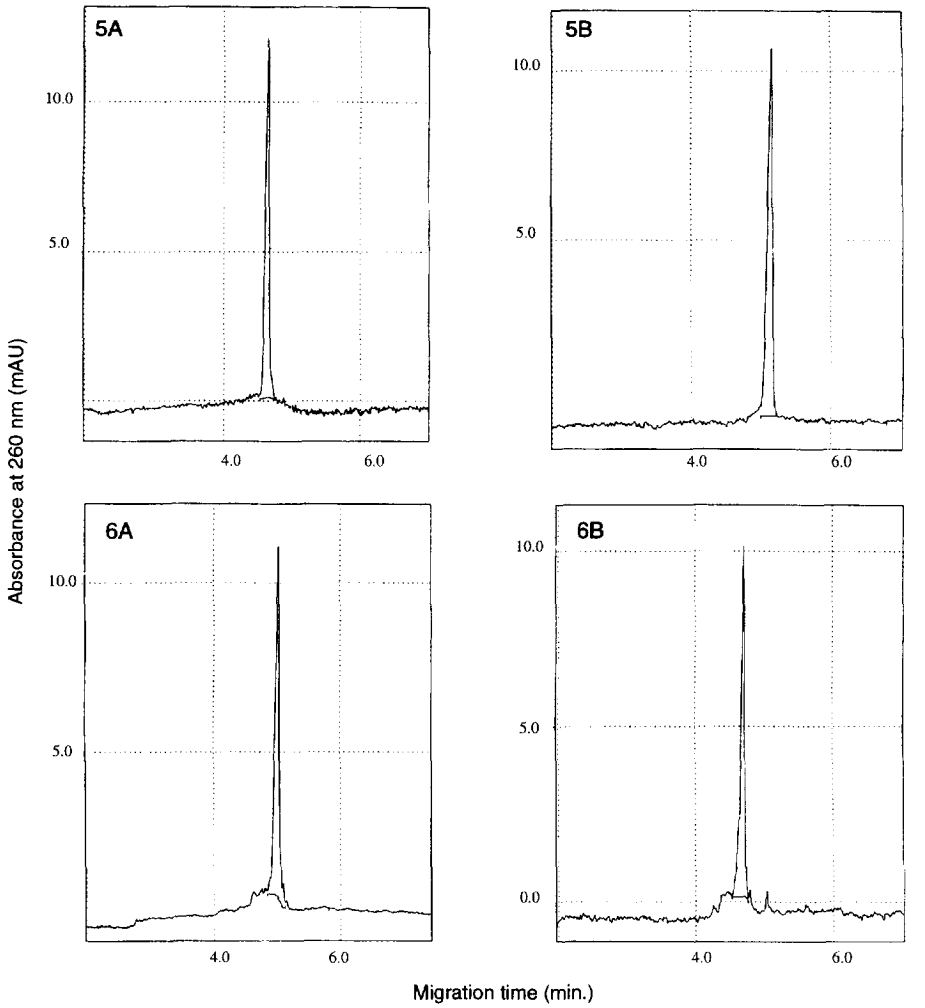


Figure 3. CGE analysis of oligonucleotides **5** and **6**, prepared with acetic anhydride capping and *trityl-on* purification (**5A** and **6A**) or *Lipocap* capping and *trityl-off* purification (**5B** and **6B**).

CONCLUSION

In summary, we have shown that the use of *Lipocap* **1**, as replacement of the standard capping reagents can easily be implemented in routine oligonucleotide synthesis. In comparison to the classical *trityl-on* strategy, its use allows the recovery of user-ready oligonucleotides after RP-HPLC purification in equivalent purity and higher yields. Consequently, by reducing the preparation time and increasing the overall yields, this capping reagent may find application in oligonucleotide synthesis in research as well as on the large scale.

EXPERIMENTAL PART

General. Anhydrous solvents were purchased from *Fluka*. Reagents for oligonucleotide synthesis were purchased from *Cruachem*. O-β-*N,N,N',N'*-tetraisopropyl phosphordiamidite was prepared according to ref. 16. Flash chromatography: silica gel 60 (70-230 mesh, *Merck*). NMR spectra were recorded on a *Bruker* Avance dpx 400 (400 MHz); chemical shifts δ in ppm *versus* $SiMe_4$ (=0 ppm, ^1H; internal standard) or H_3PO_4 (^{31}P; external standard) and coupling constants *J* in Hz.

Oligodeoxyribonucleotide synthesis.- All oligonucleotides were synthesized on an *Perkin Elmer* ABI394B DNA synthesizer using standard chemical protocols based on the O-β-cyanoethyl-phosphoramidite chemistry except for the 2'-methoxyethoxy modified building blocks, for which amidines were used as protecting groups[13]. Analytical HPLC runs were carried out with *Shandon* RP-ODS Hypersil column (5 mm, 125 mm x 4.6 mm) on a *Beckman* Gold liquid chromatography system. Semi-preparative HPLC runs were carried out with *Merck* LiChrospher WP 300 RP-18 (LichroCART 10 μm, 250 mm x 10 mm) on a *Merck-Hitachi* liquid chromatography system formed by a L-3000 Photo Diode Array Detector, a L-6200A Intelligent-Pump system, and a D-2500 Chromato-Integrator. Mass spectra were run on an LDI 1700 instrument (*Linear Scientific Inc.*). Capillary gel electrophoresis was performed on a *Beckman* P/ACE 5010.

O-β-*Cyanoethyl-O-octyl*-N,N-*diisopropyl phosphoramidite (1).*- O-β-Cyanoethyl-*N,N,N',N'*-tetraisopropyl phosphorodiamidite (76.8 mL, d=0.84, 214.4 mmol) was added to a suspension of diisopropyl ammonium tetrazolide (40 g, 233.6 mmol) in 600 mL of dry CH_2Cl_2 under argon atmosphere. Freshly distilled *n*-octanol (30.4 mL, d=0.824, 194.4 mmol) was dissolved in 100mL of dry CH_2Cl_2 and added dropwise over a period of 20 min. The suspension was stirred 20 h at room temperature. The suspension was washed with $NaHCO_3$ sat.. The organic layer was collected and washed with $NaHCO_3$ sat., dried over sodium sulfate, filtered and evaporated. The oily residue (63 g) was purified by flash chromatography using diethylether/hexane (1/10) containing 0.5% *N*-methylmorpholine. Yield: 57.12 g (90%), colorless oil. ^1H NMR (400 MHz, $CDCl_3$): δ 3.85 (m, 2H), 3.6 (m, 4H), 2.64 (t, *J*=7 Hz), 1.6 (m, 5H), 1.3 (br s, 8 H), 1.2 (m, 12H), 0.88 (m, 2H). ^{31}P NMR ($CDCl_3$): δ 146.269.

Synthesis of "lipocapped" oligonucleotides.- The oligonucleotides (**2**) to (**6**) were synthesized using standard chemicals and protocols in which the capping solutions were replaced by a 0.15 M solution of the *Lipocap* (**1**) in MeCN and a 0.45 M solution of tetrazole in MeCN.

Purification of "lipocapped" oligonucleotides.- HPLC runs were performed on *Shandon* RP-ODS Hypersil columns (analytical: 5 mm, 4.6 x 125 mm) at 1 mL/min or on *Merck* LiChrospher WP 300 RP-18 cartridges (LiChroCART 10 mm, 250 mm x 10 mm) at 6 mL/min, by using the following mobile phases: Buffer A (50 mM TEAA) and Buffer B (MeCN/50 mM TEAA, 7:3, v/v), both pH = 7.0. The *trityl-on* oligonucleotides **5** and **6** were eluted using the following program: 15% B to 55% B over 40 min; 55% B to 100% B over 1 min and 100% B for 10 min. The oligonucleotides (**2**) to (**6**) synthesized using the *Lipocap* strategy were eluted using the following program: 5% B to 35 % B over 60 min; 35% B to 100% B over 1 min and 100% B for 10 min.

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REFERENCES

1. Zamecnik, P.C.; Stephenson, M.L. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, pp. 280-284.
2. De Mesmaeker, A.; Haener, R.; Martin, P.; Moser, H.E. *Acc. Chem. Res.* **1995**, *28*, pp. 366-374 and references cited therein.
3. Altmann, K-H.; Dean, N.M.; Fabbro, D.; Freier, S.M.; Geiger, T.; Haener, R.; Huesken, D.; Martin, P.; Monia, B.P.; Mueller, M.; Natt, F.; Nicklin, P.; Phillips, J.; Pieles, U.; Sasmor, H.; Moser, H.E. *Chimia* **1996**, *50*, pp. 168-176.
4. Hodgson, J. *Nature Biotechnology* **1996**, *14*(7), pp. 815-816.
5. Eckstein, F. *Oligonucleotides and Analogues: A practical approach*; D. Rickwood; B.D. Hames Eds; IRL Press; Oxford, **1991**.
6. Beaucage, S.L.; Iyer, R.P. *Tetrahedron* **1992**, *48*, pp. 2223-2311.
7. Ravikumar, V.T.; Andrade, M.; Wyrzykiewicz, T.K.; Scozzari, A.; Cole, D.L. *Nucleosides & Nucleotides* **1995**, *14*(6), pp. 1219-1226.
8. Some phosphitylating agents bearing lipophilic chains were already described in the synthesis of 5'-phosphate-oligonucleotides : a) Garcia-Echevarria, C.; Haener, R. *Tetrahedron* **1996**, *52*, pp. 3933-3938; MacKellar, C.; Graham, D.; Will, D.W.; Burgess, S.; Brown, T. *Nucleic Acids Res.* **1992**, *20*(13), pp. 3411-3417.b) For a review see: Beaucage, S.L.; Iyer, R.P. *Tetrahedron* **1993**, *49*, pp.1925-1963 and references cited therein.
9. Phosphoramidite chemistry was already used for alternative capping reagents: Yu, D.; Tang, J.-Y.; Iyer, R.P.; Agrawal, S. *Tetrahedron Lett.* **1994**, *35*, pp. 8565-8568.
10. Total failure coupling was simulated by supplying the column with dry acetonitrile instead of expected phosphoramidite solution in the coupling step.
11. Pieles, U.; Zuercher, W.; Schaer, M.; Moser, H.E. *Nucleic Acids Res.* **1993**, *21*, 3191.
12. To estimate quantitatively the capping completion, two aliquots were taken directly after the failure coupling and at the end of the synthesis before the last detritylation. A partial capping would have resulted in the presence of *trityl-on* species in the crude material obtained from the second aliquot. Analysis by RP-HPLC revealed the absence of such species. Both crude materials looked identical and were characterized as the 9-mer (**2**).
13. 2'-Methoxyethoxy building blocks are amidine protected (see ref. 3). These protected groups require 10% ammonium acetate in the standard ammonia solution used for DNA deprotection. We have observed that the trityl group is occasionally partially removed under those conditions, inducing a decrease in the yields when *trityl-on* purification scheme is used.
14. Stec, W.J.; Uznanski, B.; Wilk, A.; Hirschbein, B.L.; Fearon, K.L.; Bergot B.J. *Tetrahedron Lett.* **1993**, *34*, pp. 5317-5320.
15. Our experience in the use of the *Lipocap* strategy at research scale (1 µmole scale) in routine oligonucleotide synthesis is that the purification yield can often reach 60%. This represents a relative increase of up to 50% as compared to the average yield observed with *trityl-on* strategy.
16. Bannwarth, W.; Trzeciak, A. *Helv. Chim. Acta* **1987**, *70*, pp. 175-186.

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