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Diethoxy N, N-diisopropyl Phosphoramidite as an Improved Capping Reagent in the Synthesis of Oligonucleotides Using Phosphoramidite Chemistry

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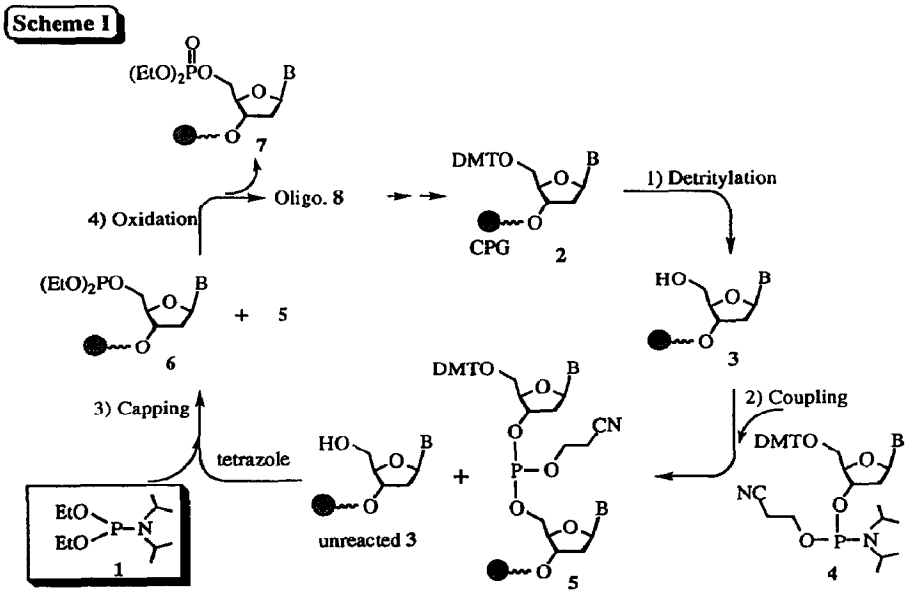
Abstract: *The use of diethoxy N, N-diisopropyl phosphoramidite (DDP) as a capping reagent for the synthesis of oligonucleotides, in conjunction with phosphoramidite chemistry, results in overall improvement in quality and yield.*

In large-scale synthesis of oligonucleotides (10 μmol - 1 mmol scale), using phosphoramidite chemistry, considerable efforts have been expended in enhancing the efficiency of the synthesis such as the use of improved coupling, oxidizing and deblocking reagents.[1,2] However, practical difficulties, still remain both in terms of yield and quality of the final oligonucleotide. Of nuisance value has been the presence of "N - 1 and N - 2" sequences (1-10%) which result in batch to batch variations in the quality of the product oligonucleotide.[3] These failure sequences originate due to incomplete coupling of the 5'-OH group of the controlled pore glass (CPG)-bound nucleotide with the incoming 3'-phosphoramidite. A "capping" step after coupling or oxidation of the newly formed internucleotidic phosphite linkage is known to be beneficial, in terms of preventing side reactions and truncation of failure sequences to aid in product purification. In this regard, we tried several variants of the presently used reagent combinations of acetic anhydride/N-methyl imidazole or acetic anhydride/DMAP, but there was no significant reduction in the failure sequences of a synthesized oligonucleotide (as evaluated by capillary electrophoresis and anion-exchange chromatography). This led us to rationalize that the capping groups may be lost from a capped oligonucleotide or CPG-bound monomer upon exposure to the various reagents and moisture accumulated during the synthesis cycle.[4a] The "uncapped sites" could in turn participate in side reactions leading to failure sequences. We therefore investigated the use of a new capping reagent with better reactivity and which would form more stable capped products. Described herein is the use of diethoxy-N,N-diisopropyl phosphoramidite (DDP) (**1**) as an alternative capping reagent [4b, 6-9, 10] for the synthesis of oligonucleotides using phosphoramidite chemistry.

The general strategy for the incorporation of the capping cycle, using DDP,[11] during the oligonucleotide synthesis, is revealed in Scheme I. The capping step is introduced after the formation of the internucleotide phosphite linkage in **5** following the standard coupling reaction (*via* **2-4**). The capping reaction in the present case involves phosphitylation of the 5'-OH group of the failed sequence **3** to give the product **6**. During the oxidation cycle of the synthesis, **6** will get oxidized to the stable capped phosphotriester **7** along with the desired **8**. A typical cycle for the synthesis of an oligonucleotide (10 μmol scale), using DDP is shown in Table 1. After completion of the synthesis, the work-up, purification and isolation of the purified oligonucleotide was done using standard protocols.

In order to validate the use of the reagent in routine oligonucleotide synthesis, we used DDP in the synthesis of 15-mer and 25-mer oligonucleotides [Phosphoric diester oligonucleotides 5'-CATCTCTCTCCTCCT-3' (15-mer) and 5'-GTATCGCAACGAAGTCACTGGTCTT-3' (25-mer)], on a 10 μmol scale on a Milligen/Biosearch 8700 DNA synthesizer.



For evaluation of oligonucleotides obtained, using acetylation capping and phosphorylation capping protocols, other synthesis conditions (such as detritylation, coupling, oxidation and ammonium hydroxide treatment) were kept unchanged. For acetylation capping, the capping program used was as recommended by the manufacturer. In case of the phosphorylation capping, the program for capping cycle used was as in Table 1. We performed several comparative experiments involving: (1) acetylation capping method, (2) phosphorylation capping method and (3) phosphorylation capping with an additional phosphorylation (pre-cap) of the CPG-bound nucleoside monomer prior to the synthesis.

Table 1. Synthesis Cycle for 10 μmol Scale

Step	Reagent/solvent	Function	Time in sec per cycle (repeat)
1	CH_3CN	Wash	20
2	2.5% Dichloroacetic acid/CH_2Cl_2	Detritylation	25 (4 X)
3	CH_3CN	Wash	20 (2 X)
4	Nucleoside/tetrazole/CH_3CN	Coupling	25• (24 X)
5	CH_3CN	Wash	20
6	DDP (0.1M in CH_3CN) /0.45M tetrazole	Capping	12* (15 X)
7	0.1M I_2/THF:Pyr:H_2O (90:5:5)	Oxidation	46
8	CH_3CN	Wash	20 (4 X)

• delivery for 5 sec, reaction time, 20 sec; * delivery for 2 sec, reaction time 10 sec.

Following the synthesis (5'-DMT-on), the oligonucleotide was isolated by treatment of CPG with 28-30% NH_4OH (55°C, 10 h). It was then detritylated using 80% acetic acid. A sample of the crude oligonucleotide was analyzed by ion-exchange chromatography.[12] Figure 1 shows the typical ion-exchange chromatographic profile of a 15-mer oligonucleotide using the acetylation capping reagent and the DDP protocols. As is evident from Fig. 1 and Table 2, there is significant overall improvement in the yield and quality of the oligonucleotides synthesized using DDP as the capping reagent.

To ascertain if heterocyclic base modifications occur in the oligonucleotide during capping, while using DDP, we prepared a 25-mer oligonucleotide (containing random mixture A,T,G,C bases) and subjected the crude oligonucleotide to enzymatic digestion with snake venom *phosphodiesterase* and *alkaline phosphatase*.[13] The products were analyzed by HPLC. No base modifications were detected in the oligonucleotide while employing the phosphitylation capping protocol in synthesis (data not shown).

Table 2. HPLC Analysis of the crude 15- and 25-mer oligonucleotides[12,13]

Method	25-mer		15-mer	
	Overall Yield (%)[$]	Failure seq. (%)	Overall yield (%)	Failure seq.(%)
Acetylation Capping	81	19	84	16
Phosphorylation Capping	87	13	92	8
Pre-cap and phosph-orylation capping	91	9	nd[•]	nd

[$]Estimated as the ratio of area of product peak to combined areas of all peaks including product peaks.[13] Average coupling efficiency in all cases was > 99%. [•]nd, Not determined.

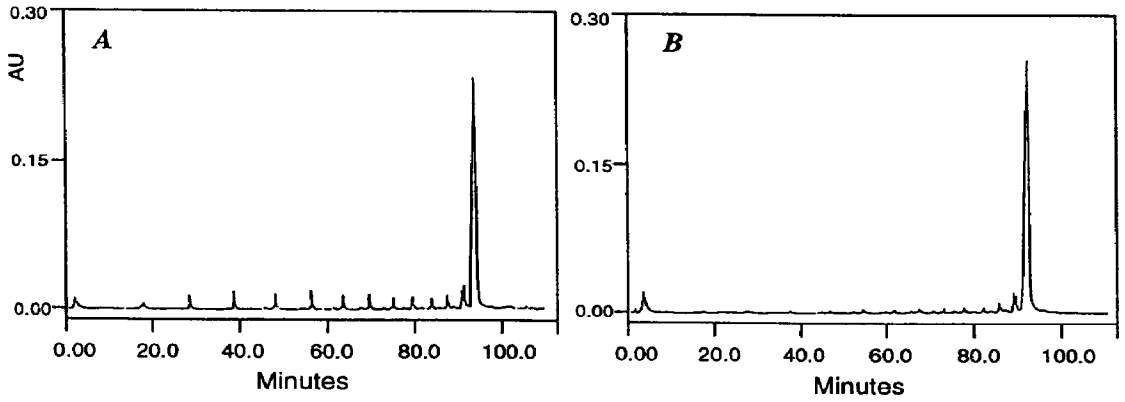


Figure 1. HPLC profiles of a crude oligonucleotide (15-mer) synthesized on 10 μmol scale: Panel A, using the acetylation capping protocol in current use; Panel B, using the new phosphorylation capping protocol.

In summary, by using the phosphorylation capping treatment using DDP, we have demonstrated that there is: (a) improvement in overall yield of the final desired oligonucleotide, (b) reduction in the failure sequences and (c) an additional beneficial effect in the incorporation of a pre-capping step in the synthesis cycle in the synthesis of oligonucleotides.

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11. The commerical phosphoramidite **1** was distilled under reduced pressure (b.p. 38-40 °C/1.5 mm Hg). Like other phosphoramidites, it has to be stored under anhydrous conditions at -20 °C. Capping solutions were freshly prepared before use.
12. HPLC analyses were performed on a Waters 600E high performance liquid chromatograph, equipped with a Waters 996 Photodiode Array Detector, on a GEN-PAK FAX (4.6 x 100 mm) ion-exchange column (Waters). The column was eluted with a linear gradient of buffer **A**, 25 mM Tris-HCl, 10% CH_3CN, pH, 8.5 and buffer **B**, 25 mM Tris-HCl, 1M LiCl, 10% CH_3CN, pH, 8.5; gradient: buffer **A** from 100% to 50%, buffer **B** from 0% to 50%, within 0 to 110 min; flow rate, 0.5 mL/min; column temperature, 65 °C.
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