Pergamon

Tetrahedron Letters, Vol. 37, No. 3, pp. 331-334, 1996
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0040-4039/96 $15.00 + 0.00

0040-4039(95)02165-5

A Novel Phosphitylating Reagent for *In Situ* Generation of Deoxyribonucleoside Phosphoramidites

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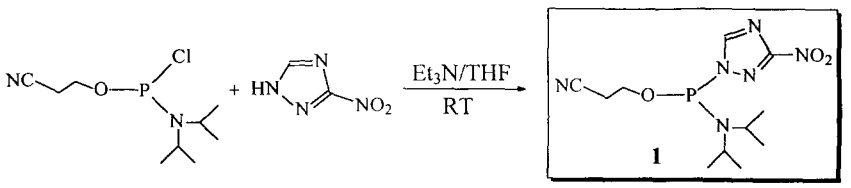
Abstract: A new phosphitylating reagent, *2-cyanoethoxy(N,N-diisopropylamino)3-nitro-1,2,4-triazolylphosphine* (1), has been prepared and effectively used in *in situ* generation of 5'-DMT-nucleoside phosphoramidites and automated syntheses of oligonucleotides.

Oligonucleotides have become indispensable tools in modern molecular biology, being used in a wide variety of techniques ranging from diagnostic probing methods, PCR to antisense inhibition of gene expression.[1] This widespread use of oligonucleotides has led to an increasing demand for rapid, inexpensive and efficient methods for synthesizing oligonucleotides. The automated solid-phase synthesis of oligonucleotides using phosphoramidites is the most popular method for most applications.[2] However, the preparation and purification of monomeric nucleoside phosphoramidites are still a major limiting factor for cost efficient synthesis of oligonucleotides. There are also the problems associated with the purification of monomeric nucleoside phosphoramidites along with those related to the stability and radioactivity of phosphoramidites.[3] For example, Bodepudi *et al.*[3a] have reported that the preparation of phosphoramidites from 2'-deoxy-7,8-dihydro-8-oxoguanosine, following the standard procedure, resulted in extensive decomposition of the phosphoramidites during the purification due to their instability and sensitivity to water. One potential approach to overcome these problems is to generate the phosphoramidite *in situ*. In such attempts, Beaucage *et al.*[4] reported the selective activation of bis-(pyrrolidino)methoxyphosphine by 4,5-dichloroimidazole. Subsequently, Barone *et al.*[5] described the selective activation of bis-(*N,N*-dialkylamino)alkoxyphosphines by 1H-tetrazole or its *N,N*-diisopropylammonium salt. Although such phosphorodiamidites have been used for commercial scale production of nucleoside phosphoramidites, these *in situ* methodologies have not been widely applied in the synthesis of oligonucleotides so far, because an additional purification is preferred.

We would like to report herein a novel phosphitylating reagent, *2-cyanoethoxy(N,N-diisopropylamino)3-nitro-1,2,4-triazolylphosphine* (1), for *in situ* generation of nucleoside phosphoramidites and automated syntheses of oligonucleotides.

The compound **1** was prepared in an excellent yield from chloro(2-cyanoethoxy)-*N,N*-diisopropylaminophosphine and 3-nitro-1,2,4-triazole in the presence of triethylamine (Scheme 1).[6]



Scheme 1.

The *in situ* generation of deoxyribonucleoside phosphoramidites was first studied using ^{31}P NMR spectroscopy. The reaction was carried out at room temperature using **1** (0.15 mmol, 47.1 mg) and 5'-DMT-thymidine (0.15 mmol, 81.7 mg) in 0.7 mL of $CDCl_3$. The NMR results indicated that **1** reacted quickly with 5'-DMT-thymidine, however, the reaction did not give the desired product (the 5'-DMT-thymidine phosphoramidite) selectively under this condition (Figure 1a). This was because a molecule of 3-nitro-1,2,4-triazole (a weak acid) was also released from the reaction, which activated the diisopropylamino group on the resulting nucleoside phosphoramidites to react with another molecule of 5'-DMT-nucleoside to form the 3'-3' dimer[7]. To solve this problem, a second or tertiary amine was added in the reaction to trap the 3-nitro-1,2,4-triazole molecule released. The *in situ* generation of nucleoside phosphoramidites was then carried out using **1** and 5'-DMT-nucleosides (dA^{Bz}, dC^{Bz}, dG^{iBu} and T) in the presence of diisopropylethylamine or diisopropylamine. Typically, a solution of 5'-DMT-thymidine (81.7 mg, 0.15 mmol) and *N,N*-diisopropylethylamine (0.026 mL, 0.15 mmol) in $CDCl_3$ (0.7 mL) was added to 0.15 mmol of **1** (47.1 mg). After stirring for 10 min at room temperature, the solution was transferred into a NMR tube and examined by ^{31}P NMR spectroscopy. As Figure 1b showed, the corresponding 5'-DMT-thymidine phosphoramidite was formed exclusively within 10 min in a ratio of 97 to 3 as compared to 3'-3'-dimer.

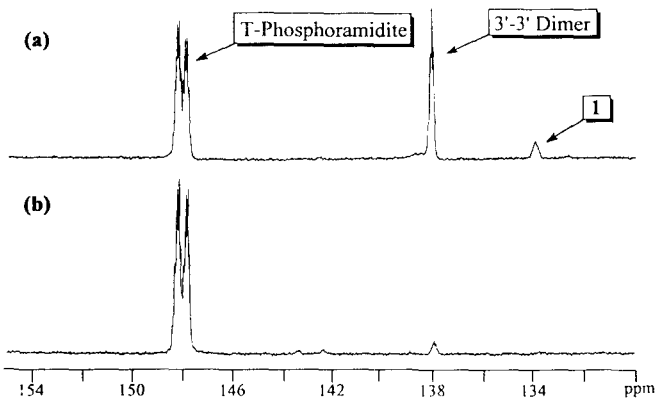
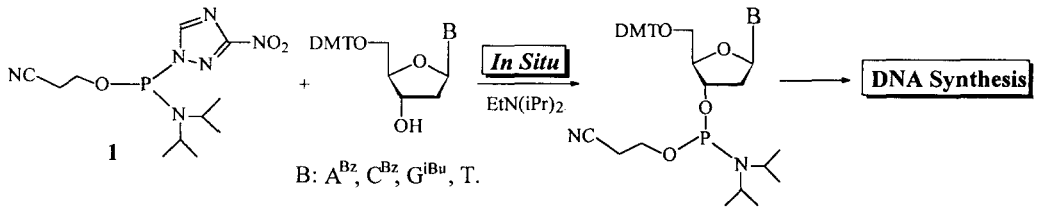


Figure 1. ^{31}P NMR spectra of the phosphoramidite generated *in situ*: (a) without amine; (b) with $EtN(iPr)_2$.

To evaluate their effectiveness in automated solid-phase DNA synthesis, we prepared *in situ* 0.1 M solutions of nucleoside phosphoramidites (dA^{Bz}, dC^{Bz}, dG^{iBu} and T) using **1** and 5'-DMT-nucleosides (dA^{Bz}, dC^{Bz}, dG^{iBu} and T) in the presence of diisopropylethylamine (Scheme 2).[8]



Scheme 2.

Two oligonucleotides were synthesized:[9] (a) The 25mer of the oligonucleotide phosphorothioate, 5'-dCTCTCGCACCCATCTCTCTCCTTCT-3' (**GEM® 91**),[10] was synthesized using the four phosphoramidites (dA^{Bz}, dC^{Bz}, dG^{iBu} and T) prepared *in situ*; (b) The 25mer of the oligonucleotide phosphodiester (**GEM® 91** sequence) was synthesized using the dC^{Bz} phosphoramidite prepared *in situ* (used in 13 of 24 coupling steps) along with three other commercial 2-cyanoethoxy(*N*,*N*-diisopropylamino) nucleoside phosphoramidites (dA^{Bz}, dG^{iBu} and T) obtained from PerSeptive Biosystems. The results showed that these oligonucleotides can be synthesized using the phosphoramidite prepared *in situ* in an average stepwise yield of better than 98%. The result was very comparable with those where the four commercial nucleoside phosphoramidites were used. The CE analysis results of these oligonucleotides are given in Figure 2.

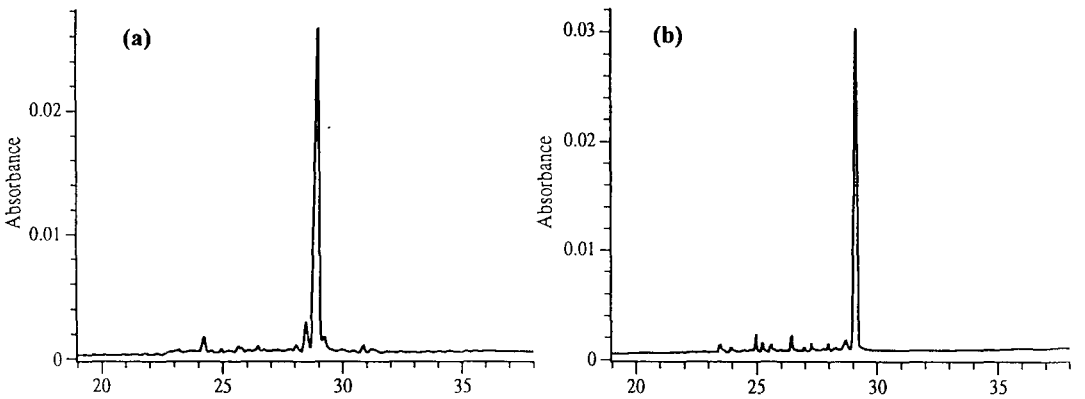


Figure 2. Gel-capillary electrophoresis analysis of the 25mer oligonucleotides: (a) phosphorothioate (**GEM® 91**); (b) phosphodiester (**GEM® 91** sequence).

In summary, our study shows that the phosphitylating reagent **1** reacts quickly with deoxyribonucleosides under weak basic condition without an additional activation step, and generates

chemoselectively the corresponding deoxyribonucleoside phosphoramidites *in situ*. It is also relatively stable and easy to handle. The *in situ* methodology using **1** can be easily adapted in the current phosphoramidite approach for the synthesis of oligonucleotides. This method may open a new avenue in large-scale syntheses of oligonucleotide analogues and in the syntheses of oligonucleotides containing modified nucleosides[11] as well as radioisotopically labeled nucleosides.

Acknowledgment. We thank Mr. J. X. Tang and Mr. P. Dawidczyk for their expert technical assistance.

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6. To a stirred solution of 3-nitro-1,2,4-triazole (9.64 g, 84.5 mmol) and triethylamine (14.1 mL, 10.26 g, 101.4 mmol) in THF (200 mL) was added dropwise chloro-2-cyanoethoxy-*N,N*-diisopropylaminophosphine (20.0 g, 84.5 mmol) at room temperature. The mixture was stirred overnight at room temperature. The reaction mixture was filtered to remove the resulting salt, and the solvent was removed under the reduced pressure to give the crude product as a pale brown oil (24.9 g, 95%). After standing at room temperature, the oil becomes a pale yellow wax-like solid. ^{1}H NMR ($CDCl_3$) δ 8.39 (s, 1H), 4.09 (m, 2H), 3.51 (m, 2H), 2.81 (m, 2H), 1.19 (d, J = 6.0 Hz, 6H), 1.07 (d, J = 9.0 Hz, 6H); ^{31}P NMR ($CDCl_3$) δ 133.9.
7. A 3'-3' dimer was also synthesized by the reaction of *bis*(*N,N*-diisopropylamino)-2-cyanoethoxyphosphine (1.0 eq.), 5'-DMT-T (2.05 eq.) and Tetrazole (2.05 eq.). ^{31}P NMR ($CDCl_3$) δ 138.2.
8. (a) General procedure: To 2.0 mmol of 5'-DMT-deoxyribonucleoside in THF (10.0 mL) was added a solution of **1** (0.66 g, 2.1 mmol) and *N,N*-diisopropylethylamine (0.38 mL, 0.28 g, 2.2 mmol) in CH_3CN (10.0 mL) at room temperature. The mixture was stirred for 10 min, and the phosphoramidite solution (0.1 M) was ready to use. There was no precipitate found in the phosphoramidite solution prepared *in situ* using **1**. (b) In a similar manner, we carried out the synthesis using **1**, 5'-DMT-thymidine and diisopropylethylamine in acetonitrile. After the solvent was removed, the reaction mixture was purified by silica-gel chromatography to give 5'-DMT-thymidine phosphoramidite in 94% yield.
9. The oligonucleotides were synthesized on a 0.2 or 1 μmol scale following the standard protocol in using an automated synthesizer (Millipore 8909 Expedite™, Bedford, MA). Eight-hour treatment with ammonium hydroxide at 65 °C was carried out to cleave the oligomer from the support and to deprotect nucleoside bases. The mixture was then filtered to remove the CPG. After the ammonium hydroxide solution was removed by Speed Vac, the remaining crude products were submitted for CE and IEX-HPLC analysis.
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(*Received in USA* 11 *October* 1995; *revised* 2 *November* 1995; *accepted* 10 *November* 1995)