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β-CYANOETHYL N,N-DIALKYLAMINO/N-MORPHOLINOMONOCHLORO PHOSPHOAMIDITES, NEW PHOSPHITYLATING AGENTS FACILITATING EASE OF DEPROTECTION AND WORK-UP OF SYNTHESIZED OLIGONUCLEOTIDES

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Summary: β-Cyanoethyl monochlorophosphoamidites of the secondary amines N,N-dimethylamine, N,N-diisopropylamine and morpholine have been prepared which showed quantitative phosphitylation of the protected deoxynucleosides. The phosphitylated deoxynucleosides have successfully been used in the formation of internucleotidic bonds. β-Cyanoethyl nucleoside-3'-N,N-diisopropylphosphoamidites have satisfactorily been used for the synthesis of the oligodeoxynucleotide d(CGGTACCG).

Since the introduction of the phosphite triester method in oligonucleotide synthesis by Letsinger,[1] various alkyl/aryl phosphorodichloridites[2] have been explored for the preparation of reactive nucleosidephosphoromonochloridite intermediates. Recently, because of the very reactive, unstable character of the phosphorodichloridites and reactive nucleoside intermediates, methyl N,N-dialkylamino/N-morpholino phosphoamidites have been introduced as fairly stable monofunctional phosphitylating reagents by Caruthers[3] and Adams[4].

The efficiency and success of oligonucleotide synthesis not only depends on the efficient synthetic methods but also on the ease of work-up procedure, e.g. removal of protecting groups from the phosphate moiety and exocyclic amino groups, quantitative cleavage of oligonucleotides from the carrier and subsequent purification without causing significant undesirable side reactions or loss of material. The methyl groups, which are in use for phosphate protection in the phosphite triester approach are usually removed by treatment with (I) a mixture of thiophenol : triethylamine : dioxane and (II) t-butylamine-methanol mixture,[5] while the exocyclic amino groups at the bases are removed with concentrated aqueous ammonia. These deprotections usually take more time than the actual synthesis of oligodeoxynucleotides in fully protected form in solid phase synthesis. The long time taken for deprotection and the contamination by thiophenol of the deprotected material dictates the need for a better phosphate protecting group in phosphite triester approach. This protecting group should be such, that it can be removed in a short time under mild basic conditions during cleavage of the oligomeric chain from the polymer and deacylation of the amino groups. Furthermore, it would be desirable if deprotection is not accompanied by the formation of

relatively large amounts of salts and other side or reaction products. A group which fullfills these criteria, is the ß-cyanoethyl group. It had been used in phosphate triester approach by Letsinger[6] and Cramer[7], and has now been chosen by us for protection of the phosphorous atom using the phosphite triester method. This group can indeed be removed very easily from the internucleotidic phosphate moiety with concentrated aqueous ammonia during the final deprotection of oligomer from polymer and de-N-acylation. All operations could be performed in a single step and a shorter time.

In this communication we describe (I) the preparation of monofunctional ß-cyanoethyl N,N-dialkylamino/N-morpholino phosphoromonochloridites, (II) their use in the formation of internucleotidic bond and (III) synthesis of an oligodeoxynucleotide following phosphite triester approach in solid support synthesis.

A general method of preparation of ß-cyanoethyl monochlorophosphoamidites and 5'-O,N-protected nucleoside-3'-ß-cyanoethyl phosphoamidites is represented in scheme 1. A solution of N-trimethylsilylated secondary amine (0.1 mol) or free secondary amine (0.2 mol) in ether (30 ml) was added to a solution of ß-cyanoethyl phosphorodichloridite (0.1 mol) in ether (60 ml) over 1 to 1 1/2 hours at -20° C under inert atmosphere. After 20 hours of stirring at room temperature, the amine hydrochloride was removed and the solution was concentrated under exclusion of moisture in vacuo. The concentrated residue was finally distilled. The N,N-dimethyl and N,N-diisopropyl amine derivatives distilled at 90-92° C/0.60 mm and 103-104° C/0.08 mm, respectively. The physical characteristics of the monochlorophosphoamidites are given in [8].

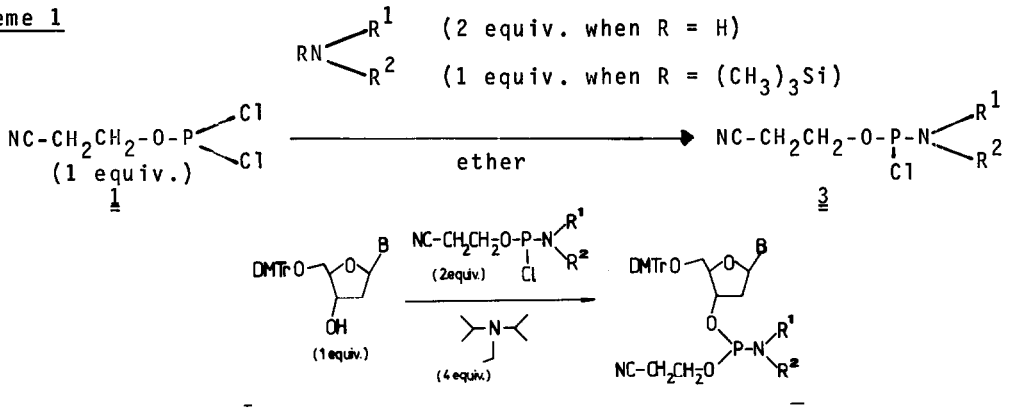
The nucleoside phosphoamidites were prepared according to the literature[3] with some modifications: THF was used as solvent, the amine hydrochloride was removed prior to aqueous work-up. Finally these nucleoside derivatives were precipitated as white powder from hexane at -70° C. According to ^{31}P-NMR spectra these products were approximately more than 90% pure without any further purification.

ß-Cyanoethyl monochloro-N-morpholinophosphoamidite could not be distilled due to the thermal decomposition. The crude derivative was found to be more than 94% pure and the nucleoside morpholinophosphoamidites prepared from this were also about 90% pure. In this case the only impurity detected from ^{31}P-NMR spectra was a 3'-3'-dimer.

When 5'-unprotected nucleosides anchored to controlled pore glass beads[9] were treated with these three different classes of ß-cyanoethyl nucleoside phosphoamidites (20-25 equ.) in the presence of sublimed tetrazole (75-80 equ.) in acetonitrile, the condensations were complete in less than 30 minutes and in each case coupling yield was more than 94%.

In order to find the general application of these derivatives in oligodeoxynucleotide synthesis, we synthesized the octamer d(CGGTACCG) in overall

Scheme 1



a) $R^1=R^2=CH_3$ b) $R^1=R^2=$ H_3C H_3C :CH- c) $R^1+R^2=$ morpholino

B = Thymine, 2-(methyl)benzoylcytosine,[12] isobutyrylguanine, benzoyladenine

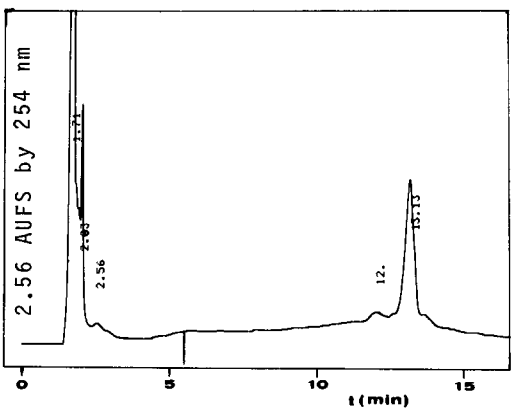


Figure 1a

Figure 1b

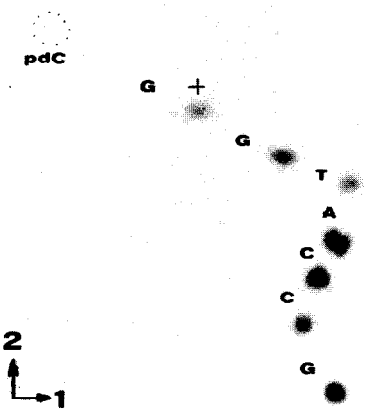


Figure 1c

Figure 1:
a) Purification of the resulting octamer after deprotection from the solid support by HPLC as DMT-d(CGGTACCG) with step linear gradient from 10-25% in 5 minutes and 25-29% CH_3CN in 30 minutes in 0.1 M TEAA, pH 7.0, at room temperature.
b) Electrophoresis of the octamer (lane 1) on a 20% polyacrylamide gel containing 7 M urea after HPLC (figure 1a). Lane 2: homo-oligo-dT chain length standard. *: $d(pT)_3$.
c) Two-dimensional fingerprint of a partial snake venom phosphodiesterase digest of the octamer d(CGGTACCG); 1: cellulose acetate electrophoresis, 2: homochromatography. +: xylene cyanol (blue marker).

yield of 55% using 5'-O,N-protected nucleoside-3'-ß-cyanoethyl N,N-diisopropyl phosphoamidites. Removal of the oligomer from the glass beads and of N- and P-protecting groups was performed in one step by treating the polymer with concentrated aqueous ammonia at 50° C for 16 hours. After evaporation, the residue was dissolved in HPLC-starting buffer, filtered through millipore filter and directly purified by reversed phase HPLC. The oligonucleotide was characterized by length sizing on 20% polyacrylamide gel using homo-oligo-d(T) chain length standard[10] and sequence analysis by "mobility shift" method (figure 1)[11].

In conclusion, we would like to mention that the ß-cyanoethyl N,N-dialkylaminophosphoromonochloridites are fairly easy to prepare and handle, the active nucleoside derivatives prepared from these reagents are stable for more than six months. Removal of ß-cyanoethyl groups from the internucleotidic phosphate moiety is possible in a short time during concomitant de-N-acylation and cleavage of oligomer from the polymer support thus simplifying and facilitating work-up and purification. These advantages should make these new derivatives very useful in the synthesis of oligodeoxynucleotides.

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8) Physical characteristics of monochloro ß-cyanoethyl phosphoamidites: 3a: ^{31}P-NMR: δ = 175.97 with respect to 80% H_3PO_4 in acetone-d_6; ^{1}H-NMR: ($CDCl_3$) in ppm: 4.01, 4.17 (2t, P-OCH_2, 2H), 2.71 (t, -CH_2CN, 2H), 2.7 (d, $N(CH_3)_2$, 6H); m^+/e: 180, 182 (m+2), 145 (-Cl), 136 (-C_2H_6N), 110 (-C_3H_4NO). 3b: ^{31}P-NMR: δ = 179.82; ^{1}H-NMR ($CDCl_3$): 4.02, 4.2 (2t, P-OCH_2, 2H), 3.8 (m, N(CH), 2H), 2.77 (t, -CH_3CN, 2H), 1.29 (d, N-$C(CH_3)_2$, 12H); m^+/e: 236, 238 (m+2), 201 (-Cl), 116 (-C_3H_4NO), 136 (-$C_6H_{14}N$). 3c: ^{31}P-NMR: δ = 168.22; ^{1}H-NMR ($CDCl_3$): 3.96, 4.1 (2t, P-O-CH_2, 2H), 3.67 (d, $O(CH_2)_2$, 4H), 3.17 (m, P-$N(CH_2)_2$, 4H), 2.74 (t, CH_2CN, 2H).
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