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AN INVESTIGATION OF SEVERAL DEOXYNUCLEOSIDE PHOSPHORAMIDITES USEFUL FOR SYNTHESIZING DEOXYOLIGONUCLEOTIDES

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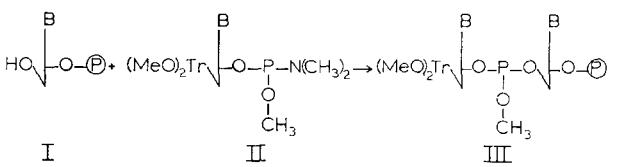
Various deoxynucleoside N,N-dialkylaminomethoxyphosphines were examined for stability and reactivity in deoxyoligonucleotide synthesis. Of those examined, the N-morpholino and N,N-diisopropylamino derivatives most completely satisfied all criteria.

Deoxynucleoside N,N-dimethylaminophosphoramidites (1) are extremely useful intermediates for polymer supported deoxyoligonucleotide synthesis (2). The key step is condensation of a deoxynucleoside covalently joined to silica gel (compound I) with the appropriate deoxynucleoside phosphoramidite (compound II). This reaction is catalyzed by 1H-tetrazole in acetonitrile. After oxidation and detritylation, repetitions of this cycle lead to deoxyoligonucleotides.



B = 1-Thyminyl; 1-(N-4-Benzoylcytosinyl); 9-(N-6-Benzoyladeninyl); or 9-(N-2-Isobutyrylguaninyl)

P = Derivatized Silica

$(MeO)_2Tr$ = Di-*p*-anisylphenylmethyl

Figure 1. Outline of Deoxyoligonucleotide Synthesis.

Although the N,N-dimethylaminophosphoramidites have been used successfully for synthesizing several hundred deoxyoligonucleotides (20 to 30 mononucleotides each), certain limitations prompted us to survey the chemical reactivity of related phosphoramidites. For example, when II was isolated as a solid by precipitation (1), the purity (based on ^{31}P-NMR) ranged from 60% to 95%. The major contaminant was the hydrolyzed product (the deoxynucleoside phosphonate). Additionally, when compound II was dissolved in acetonitrile without activation, variable stability ranging from hours to weeks was obtained. These observations prompted us to examine the deoxynucleoside diisopropylamino, morpholino, pyrrolidino, and 2,2,6,6-tetramethylpiperidino phosphoramidites as potential intermediates for deoxyoligonucleotide synthesis.

The synthesis of deoxynucleoside phosphoramidites begins with the preparation of compounds IV, V, VI and VII (Figure 2). Three routes have been used (A, B and C). Compounds IV and V were prepared via route A (1) and isolated in 81% and 78% yield, respectively. An alternative procedure utilizing silylamines was used to prepare IV and VI. The procedure, initially developed by S. Beaucage in this laboratory (3), involves dropwise addition of N-trimethylsilylmorpholine (4) or N-trimethylsilylpyrrolidine (Petrarch) to Cl_2POCH_3 (1:1.1 mol ratio) at 0°C

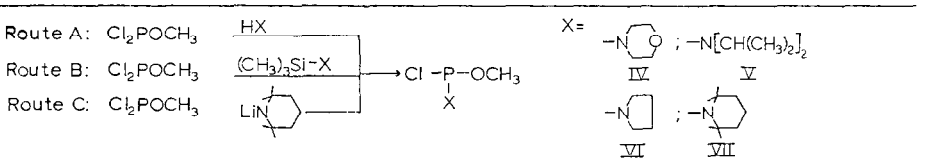


Figure 2. Chlorophosphite Synthetic Routes.

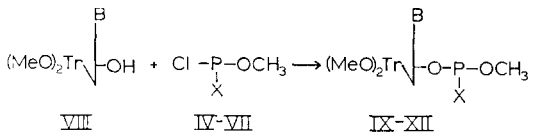
Table 1. Physical Chemical Data for Chloro-N,N-dialkylaminomethoxyphosphines

Compound	Boiling Point °C/mm Hg	δ-^{31}P (ppm)[a] $CDCl_3$	Elemental Analysis Calc.	Found
IV	52-54°C/0.1 mm Hg	-172.1	$C_5H_{11}NO_2PCl$	C, 32.54; H, 5.93; N, 7.89; P, 16.59; Cl, 19.40
V	35-36°C/0.02 mm Hg	-183.9	$C_7H_{17}NOPCl$	C, 42.59; H, 8.73; N, 7.13; P, 15.49; Cl, 17.74
VI	28-30°C/0.01 mm Hg	-182.7		
VII	61-64°C/0.02 mm Hg	-194.4		

[a] δ is relative to a sealed tube internal standard of 5% H_3PO_4 (aq.).

followed by distillation *in vacuo*. The isolated yields were 86% and 90%, respectively. Synthesis of compound VII (55% yield) was completed using route C (5).

Compounds IV, V, VI and VII were greater than 98% pure (by ^{31}P-NMR) (Table 1). Satisfactory elemental analysis of compound V was obtained only after distillation over cesium fluoride. CsF appeared to eliminate trace contamination of the appropriate amine hydrochloride while minimizing decomposition during the distillation. Of the chloridites so far examined, compounds IV and V are preferable. Both are less volatile and therefore less reactive with atmospheric moisture. They can be easily transferred in a syringe exposed to normal atmospheric conditions. Compound VI and chloro-N,N-dimethylaminomethoxyphosphine are the most volatile reagents. They fume extensively in moist air and care must be taken during transfers so that contact with the atmosphere is minimal. Compound VII exists as an extremely reactive solid at ambient temperatures. Syringe transfers as a liquid must therefore be completed at elevated temperatures.



IX: X = N-morpholino
X: X = N,N-diisopropyl
XI: X = N-pyrrolidino
XII: X = 2,2,6,6-tetramethyl-N-piperidino

Figure 3. Synthesis of Deoxynucleoside Phosphoramidites. B = 1-Thyminyl.

Deoxynucleoside N,N-dialkylaminophosphoramidites were prepared (Figure 3) using the following general method (1). $(MeO)_2$TrdT (VIII, 1.65 g, 3 mmol) was dissolved in 9 ml CH_2Cl_2 (dist. over Na_2CO_3) containing diisopropylethylamine (1.9 ml, 10 mmol). Compound IV (0.67 ml, 4.5 mmol) in a syringe was added rapidly to the magnetically stirred solution (20°C). After 10 min the solution was transferred with 150 ml ethylacetate (prewashed with satd. $NaHCO_3$) into a 500 ml extraction funnel. The solution was extracted once with 200 ml of satd. $NaHCO_3$ and the organic phase was conc. *in vacuo*. Compound IX was dissolved in hexane:chloroform (3:2) containing 5% morpholine (Aldrich Gold Label) and loaded on to a 50 g silica gel column (3 cm X 25 cm) prepared with the same solvent. The eluting solvent was hexane:chloroform (3:2) containing 5% morpholine. Fractions of 20 ml were collected every minute. Pooled fractions containing the product were concentrated and freed of morpholine by four coevaporations with n-butylether: benzene (1:10). The final product was dissolved in 25 ml benzene (dist. over CaH_2), precipitated (at r.t.) into pentane (500 ml), collected by filtration and dried *in vacuo* (0.05 mm Hg,

24 h). The yield of white solid was 90% (1.86 g, 2.7 mmol). This procedure, including the column chromatography step has also been used successfully to prepare the 3'-methoxymorpholinophosphine derivatives of $(MeO)_2$TrdbzA, $(MeO)_2$TrdbzC, and $(MeO)_2$TrdibG. Satisfactory C, H, N and P analyses were obtained for all four derivatives. The other deoxynucleoside phosphoramidites (compounds II, X-XII) are relatively unstable on silica gel and therefore are isolated as unpurified precipitates following aqueous extraction (1).

Analysis by ^{31}P-NMR of compounds II, IX-XII (Figure 4) indicates that IX (column purified) and X (unpurified precip.) can be isolated in essentially pure form (Panels A and B, 98% relative to phosphorus containing compounds). Isolated unpurified precipitates of compounds II, XI and XII, however, are quite heterogeneous (Panels C, D and E) and display NMR signals suggesting considerable hydrolysis (5-20%) of the phosphoramidites during the work-up. Additionally, compound XII was contaminated (20%) with the bis-(5'-di-*p*-anisylphenylmethylthymidine-3') methoxyphosphine (δ = -139). This impurity was unavoidable even when triethylamine was used as an acid scavenger and the nucleoside was added as a dilute solution. The stability of compounds IX and X in acetonitrile (no tetrazole) was also examined. After 42 days in a sealed tube of CD_3CN, compound IX (Panel A) was re-examined by ^{31}P-NMR. The results (Panel F) suggest that compound

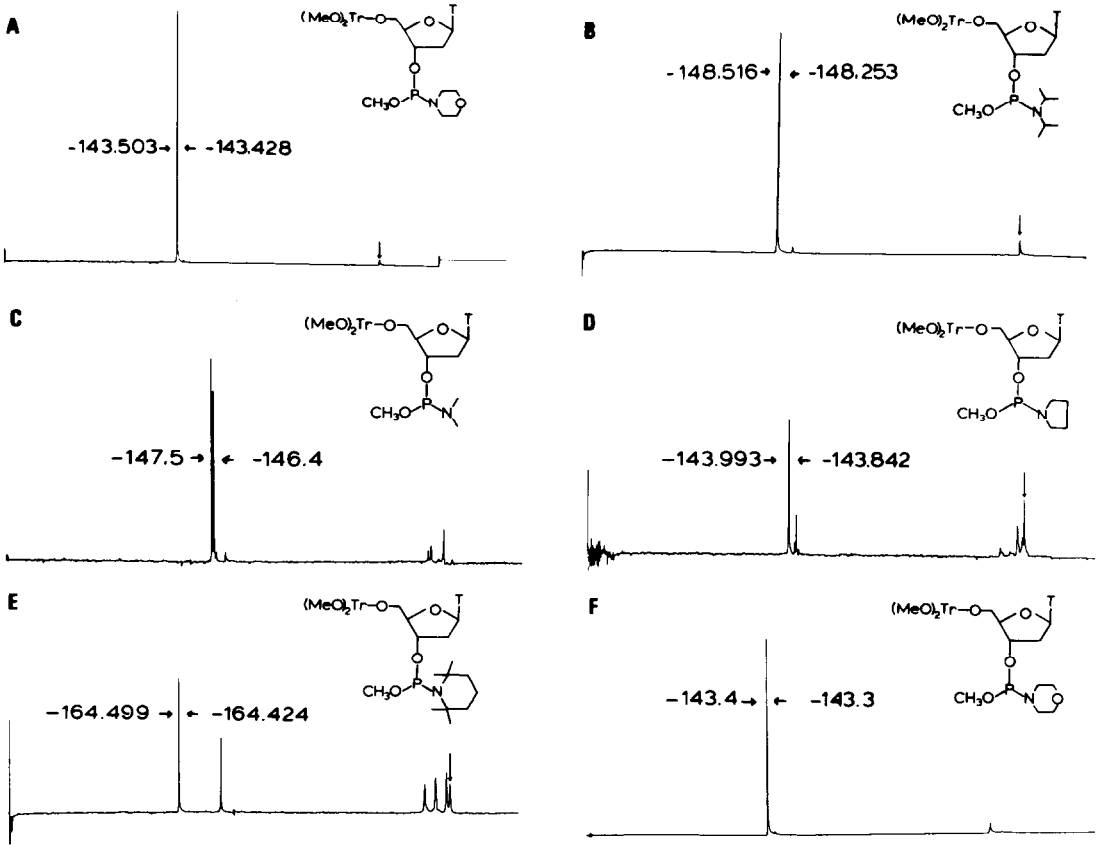


Figure 4. ^{31}P-NMR Spectra of Compounds II, IX-XII. The arrow indicates the signal from H_3PO_4 as an internal standard (capillary tube).

IX is extremely stable in this solvent since the product is still greater than 96% pure. Similar results with compound X were also obtained (85% pure after 40 days).

The activation and water content of various deoxynucleotide phosphoramidites were studied by NMR and by measuring reactivities in kinetic experiments. Compounds II, IX, X and XI (all 0.13 M) were separately activated with tetrazole (0.4 M) in CD_3CN:CH_3CN (1:1). Since the ^{31}P-NMR chemical shift of tetrazoylphosphoramidite (6) is at -126 ppm (3), the equilibrium concentration of the active deoxynucleoside tetrazoylphosphoramidite can be determined. Approximately 40% of II and 60% of XI were converted to the deoxynucleoside tetrazoylphosphoramidite. However compounds IX and X varied considerably in the fraction of deoxynucleoside tetrazoylphosphoramidites (5% and 95%, respectively). Only 5-10% hydrolysis products (-8.6 ppm) were observed in each experiment, indicating that the solvents and compounds were free of water. Aliquots (0.4 ml) of each equilibrium solution were allowed to react for 1 min with samples of compound I (125 mg each) containing 42 μmole deoxythymidne per gram derivatized silica suspended in 1.5 ml CD_3CN:CH_3CN (1:1). Each reaction mixture was quenched with THF:H_2O:lutidine (2:2:1) and oxidized to phosphate derivatives with I_2. After removal of silica gel and protecting groups (1), the relative abundance of d(TpT) and dT was determined by analytical hplc. Compounds II, X and XI reacted quantitatively with compound I to form d(TpT). The reaction with IX was 70% complete after one minute. Further expermiments with IX have demonstrated that this reaction, however, is complete after 5 min suggesting that longer reaction times or higher concentrations of IX are required for complete reaction.

These experiments indicate that compounds IX and X are the reagents of choice for synthesizing deoxyoligonucleotides. The respective chlorophosphines can be isolated in high yield and do not fume excessively in air. Based on ^{31}P-NMR data, IX can be isolated in essentially homogeneous form by silica gel column chromatography and X appears to be at least 98% pure simply by precipitation of the crude reaction mixture after an aqueous work-up. Both compounds are also stable for weeks in an acetonitrile solution, which is an important factor for extensive deoxyoligonucleotide synthesis. The N-morpholino and N,N-diisopropylaminophosphoramidite derivatives of all four common deoxynucleosides are currently being examined with excellent preliminary results in our polymer supported deoxyoligonucleotide synthesis procedure.

Acknowledgments

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