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In situ activation of bis-dialkylaminophosphines – a new method for synthesizing deoxyoligonucleotides on polymer supports

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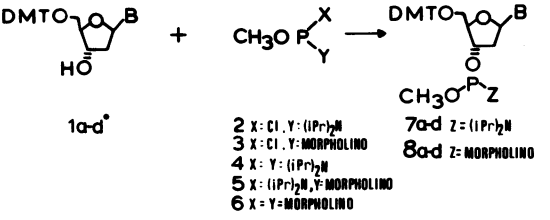
ABSTRACT

Deoxynucleoside phosphoramidites can be prepared in good yield from deoxynucleosides, bis-dialkylaminophosphines, and the corresponding dialkylamine hydrotetrazolide or tetrazole as catalysts. These phosphoramidites generated *in situ* lead to the direct synthesis of deoxyoligonucleotides on polymer supports.

INTRODUCTION

The current phosphite triester methodology for deoxyoligonucleotide synthesis requires the condensation of deoxynucleoside phosphoramidites **7a-d** or **8a-d**, activated by tetrazole, with the 5'-hydroxyl group of a deoxynucleoside or deoxyoligonucleotide attached covalently to a polymer support (1-4). Although these phosphoramidites can be prepared by existing methods (5,6) from the appropriately protected deoxynucleosides **1a-d**, the chlorophosphines **2** and **3** used in forming **7a-d** and **8a-d**, respectively, are difficult to prepare and easily react with trace amounts of water. Moreover, the high reactivity of **2** and **3** and the concomitant production of insoluble amine hydrochloride salts preclude their use for any strategy involving the *in situ* generation of deoxynucleoside phosphoramidites for deoxyoligonucleotide synthesis on solid supports (7). Because of our interest in the latter approach, we were prompted to investigate the relative stability and reactivity of the aminophosphines **4**, **5**, and **6** towards phosphodiester formation. We wish to report that the phosphoramidites **7a-d** and **8a-d** can be prepared in good yields by the reaction of suitably protected deoxynucleosides **1a-d** and bis-dialkylaminophosphines **4** or **6** using amine salts **9** and **10**, respectively, as catalysts. This method was applied to a

synthesis of a deoxyoligonucleotide directly on a solid support via an *in situ* approach.



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* a, B = T ; b, B = A^{bz} ; c, B = G^{ib} ; d, B = C^{bz}
DMT = dimethoxytrityl

MATERIALS AND METHODS

Reagents and Solvents

Polynucleotide kinase and *Eco*RI were obtained from BRL. Deoxynucleosides were purchased from Vega Biochemicals and protected according to the procedure of Jones (8). Methyldichlorophosphite was obtained from Aldrich and distilled before use. Diisopropylamine, triethylamine, and morpholine were purified by distillation from calcium hydride.

Anhydrous solvents were obtained as follows: dichloromethane by distillation first from phosphorous pentoxide and then calcium hydride, and acetonitrile by successive distillations from calcium hydride, phosphorous pentoxide, and then calcium hydride.

General Procedures

^{1}H NMR shifts were recorded on a Varian EM-390 spectrometer and the chemical shifts (ppm) are reported relative to an internal standard of tetramethylsilane. ^{31}P NMR spectra were recorded on a Bruker WM-250 spectrometer (101.2 MHz) and the chemical shifts (ppm) are reported relative to an external capillary standard of 85% H_3PO_4. The mass spectra were determined on a Varian MAT CH5 mass spectrometer.

Preparation of bis-(diisopropylamino)methoxyphosphine (4)

To 14 g (0.11 mol) of methyldichlorophosphite in 500 ml of diethylether at -10°C under a nitrogen atmosphere was added 96 g

(0.95 mol, 9 eq) of diisopropylamine over 1 h. The reaction was allowed to warm to room temperature and stirred an additional 16 h. Removal of the amine hydrochloride salt by filtration and evaporation of the solvent gave a pale yellow liquid. The crude material was fractionally distilled twice from calcium hydride (to remove residual amine salts) to afford 21.5 g (77%) of **4** as a colorless liquid: b.p. 40°-42°C, 50 μ Hg; ^{31}P NMR (CH_2Cl_2) δ = 130.1; ^{1}H NMR ($CDCl_3$) δ 1.15 (d, 6, $CH(C\underline{H}_3)_2$), 1.13 (d, 6, $CH(C\underline{H}_3)_2$), 3.35 (d, 3, J_{P-H} = 12 Hz, $POCH_3$) and 3.3-3.7 (m, 2, $C\underline{H}(CH_3)_2$); mass spectrum m/e (rel intensity) 262 (21, M^+) 161 (100), 120 (22), 87 (22), and 77 (21).

Preparation of (diisopropylaminomorpholino)methoxyphosphine (**5**)

To a mixture of 3.0 g (15.7 mmol) of **2** and 1.53 g (15.7 mmol, 1 eq) of triethylamine in 100 ml of diethylether at -10°C under a nitrogen atmosphere was added 1.32 g (15.7 mmol, 1 eq) of morpholine over 15 min. While still cold, the reaction mixture was poured into saturated aqueous sodium bicarbonate and the organic layer was then washed with brine and dried over sodium sulfate. Evaporation of the solvent followed by fractional distillation of the crude liquid from calcium hydride afforded 1.91 g (77%) of **5**: b.p. 64-66°C, 50 μ Hg; ^{31}P NMR (CH_2Cl_2) δ = 126.6; ^{1}H NMR ($CDCl_3$) δ 1.13 (d, 6, $CH(C\underline{H}_3)_2$), 1.19 (d, 6, $CH(CH_3)_2$), 2.6-3.0 (m, 4, $N(CH_2)_2$), 3.43 (d, 3, J_{P-H} = 12 Hz, $POCH_3$) and 3.3-3.8 (m, 4, $O(CH_2)_2$); mass spectrum m/e (rel intensity) 248 (46, M^+), 161 (49), 87 (29), 77 (25), and 44 (21).

Preparation of bis-(morpholino)methoxyphosphine (**6**)

To 744 g (50 mmol) of methyldichlorophosphite in 200 ml of diethylether at -10°C under a nitrogen atmosphere was added 26.0 g (300 mmol, 6 eq) of morpholine over 1 h. The reaction mixture was allowed to warm to room temperature and stirred an additional 16 h. Removal of the amine salt by filtration and evaporation of the solvent gave a colorless liquid. The crude material was fractionally distilled from calcium hydride to afford 9.5 g (81%) of **6**: b.p. 82-84°C, 15 μ Hg; ^{31}P NMR (CH_2Cl_2) δ = 131.3; ^{1}H NMR ($CDCl_3$) δ 2.7-3.3 (m, 4, $N(CH_2)_2$), 3.43 (d, 3, J_{P-H} = 12 Hz, $POCH_3$) and 3.5-3.8 (m, 4, $O(CH_2)_2$); mass spectrum m/e (rel intensity) 234 (20, M^+), 148 (100), 116 (24), 92 (15), and 58 (22).

The Following is a Typical Experimental Procedure for the Preparation of Phosphoramidites

To a mixture of 544 mg (1.0 mmol) of **1a** and 86 mg (0.5 mmol, 0.5 eq) of **9** in 5 ml of dry dichloromethane under a nitrogen atmosphere was added 282 mg (1.1 mmol, 1.1 eq) of phosphine **4**. After 1 h the reaction mixture was poured into saturated aqueous sodium bicarbonate and the organic layer was washed with brine and dried over sodium sulfate. Evaporation of the solvent gave a white foam which was then taken up in 3 ml of dichloromethane and precipitated in 300 ml of cold hexanes (-78°C). The resultant suspension was filtered cold and dried under vacuum to afford 700 mg (87%) of a 1:1 diastereomeric mixture of **7a** as an amorphous solid. The phosphoramidites **8a-d** were prepared by a similar procedure and were purified by flash chromatography on silica gel affording **8a-d** in 63% to 70% yields.

^{31}P NMR Spectral Data of Phosphoramidites.

7a: δ = 148.5, 148.1; **7b:** δ = 148.5, 148.3; **7c:** δ = 148.3, 148.1; **7d:** δ = 148.6, 148.1.

8a: δ = 143.4, 143.2; **8b:** δ = 143.4; **8c:** δ = 143.7, 143.2; **8d:** δ = 143.7, 143.4.

RESULTS AND DISCUSSION

Very reactive bis-aminophosphines, such as methylphosphoroditetrazolide (9) or the corresponding bis-triazolylphosphine (10), have been shown to be applicable to phosphoramidite synthesis. Recently, Beaucage (11) has shown that deoxynucleoside phosphoramidites can be prepared *in situ* utilizing bis-(pyrrolidino) methoxyphosphine and 4,5-dichloroimidazole as a mild acid catalyst. Independently, we have observed similar results when **4** and **6** are activated with the corresponding amine hydrotetrazolides **9** and **10**, but with much more selectivity of activation. Moreover, the reaction has been shown to be catalytic in either tetrazole or the salt.

The ease of phosphine preparation is exemplified by the direct procedure used for the production of **4**. Excess diisopropylamine was added to a solution of methyldichlorophosphite. Removal of the amine salt and fractional distillation of the crude liquid afforded a 77% yield of **4**. The bis-aminophosphine **4**

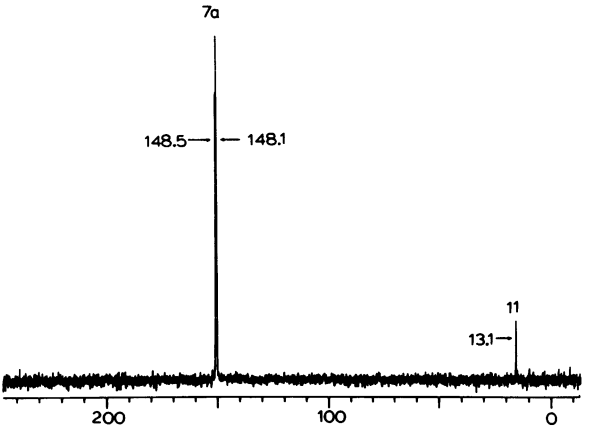


Fig. 1. The ^{31}P NMR spectrum of **7a** in dichloromethane.

is very stable when stored at -10°C. Even with repeated sampling of the phosphine, the ^{31}P NMR spectrum of **4** was unchanged after one month. When oxygen was bubbled through a solution of **4** in dichloromethane for 24 h, the ^{31}P NMR indicated approximately 8% degradation and 11% hydrolysis of **4**.

The bis-morpholinophosphine **6** was prepared in a similar manner (80% yield) and had stability comparable to **4**. The mixed phosphine **5** could not be obtained cleanly in the same one pot procedure as above; however, treatment of **2** with one equivalent of morpholine and triethylamine gave a 77% yield of **5**, contaminated with about 2% of the thermodynamically more stable phosphine **6**.

The reaction of deoxynucleosides **1a-d** with **4** and **9** gave **7a-d** in 82% to 92% isolated yield after precipitation from cold hexanes. A typical procedure for the preparation of **7a** required the addition of 1.1 equivalents of **4** to a mixture of **1a** and 0.5 equivalents of **9** in dichloromethane. The TLC showed the reaction to be complete in about 20 min. After an aqueous work-up and precipitation of the material in cold hexanes, **7a** was obtained in 87% yield as an amorphous solid. The ^{31}P NMR spectrum of **7a** (Fig. 1) showed two signals corresponding to a 1:1 diastereomeric mixture of phosphoramidites. The spectral data of **7a-d** was found to be identical with that of authentic samples of phosphoramidites prepared according to the procedure of McBride and

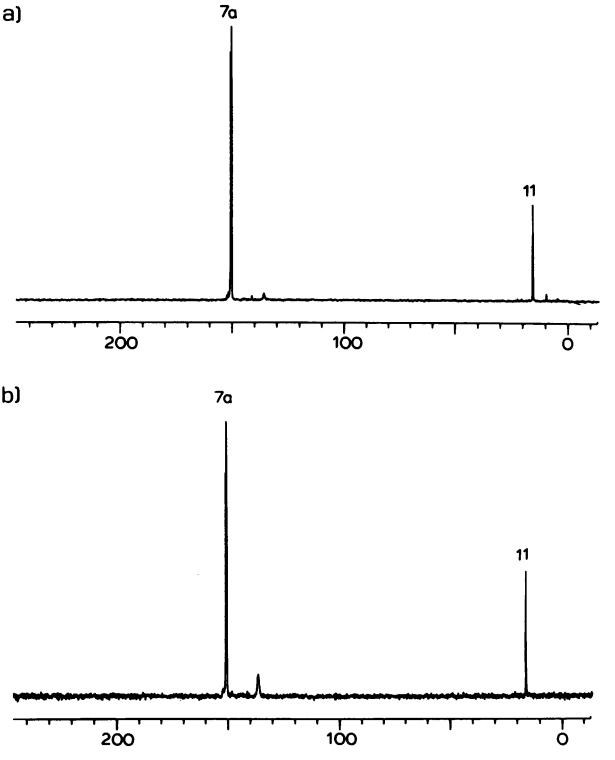
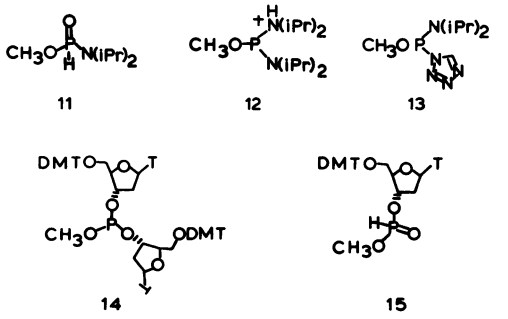


Fig. 2. The ^{31}P NMR spectrum of the reaction mixture (**1a**, 1.1 eq of **4**, and 0.5 eq of **9**) in (a) dichloromethane and (b) acetonitrile.

Caruthers (6). The ^{31}P NMR spectrum of the above reaction mixture clearly demonstrated the selectivity of the phosphitylation (Fig. 2 a,b). In addition to the major peaks characteristic of **7a**, there was a minor peak at 13.1 ppm assigned to phosphoamidous acid **11** resulting from hydrolysis of **4** by traces of water present in the reaction mixture. We did not attempt to remove traces of **11**, since it is inert and not deleterious to the chemistry. Even when tetrazole was used to activate **4**, the reaction of **4** with water was much faster than the analogous reaction of **7a** with water (6,12). Therefore, compound **4** can behave as a dessicant for the *in situ* approach to DNA synthesis. A small, broad signal centered at 134.1 ppm cannot be rationalized at this time; however, we speculate that this signal is due to the presence of reactive intermediates such as **12** or **13**. This broad signal was



observed in mixtures of **4** and **9** in acetonitrile as well as dichloromethane. Of considerable importance was the lack of any signals corresponding to either the 3', 3'-triphosphite **14** or the phosphonus acid **15** (resulting from hydrolysis of **7a**), indicating the phosphoramidite was not activated. We observed less than 1% of **14** or **15** in the ^{31}P NMR spectrum after accumulation of 2000 scans (Fig. 2a). To demonstrate further the selectivity of using amine hydrotetrazolides as catalysts, a dichloromethane solution of **7a** in the presence of 0.5 equivalents of **9** and excess absolute ethanol showed less than 5% of the triphosphite by TLC. If an excess of **9** (2 eq) was used to initiate the reaction of **1a** with **4**, then less than 3% each of **14** and **15** was formed.

Under catalytic conditions (0.1 eq of **9**) in either acetonitrile or dichloromethane, the major product observed (Fig. 3) was the diphosphite **16** (13). As the amount of **9** used to initiate the

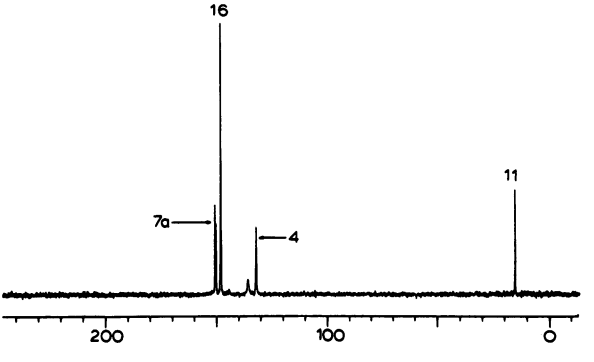
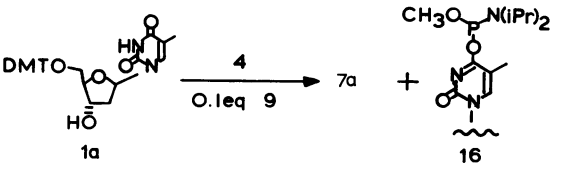


Fig. 3. The ^{31}P NMR spectrum of the reaction mixture (**1a**, 1.1 eq of **4**, and 0.1 eq of **9**) after 40 min in dichloromethane.



reaction was increased, the formation of **16** decreased until, at 0.5 equivalents of added **9**, only **7a** was formed by ^{31}P NMR analysis. More interestingly, a mixture of **1a** and **4** with no added catalyst resulted in a slow reaction (two days) to form **7a** presumably via **16** as an intermediate. Apparently, the imide function of **1a** acts catalytically with **5** to form **16** as the initial product which then reacts slowly with **1a** to form **7a**.

To understand further the relative reactivities of the amino groups and their corresponding salts, we studied the reaction of the mixed phosphine **5** with **1a** in the presence of **9** and **10** (prepared by treatment of a solution of tetrazole in acetonitrile with an excess of the appropriate amine followed by filtration and drying under vacuum) in acetonitrile by ^{31}P NMR. We anticipated the possibility of protonation of the diisopropyl group over the morpholino group in **5** to give primarily the morpholino phosphoramidite **8a**. This was the case when 1.0 equivalents of tetrazole was used, affording a mixture of **7a**/**8a** in a ratio of 1:20. However, in the presence of 0.5 equivalents of either **9** or

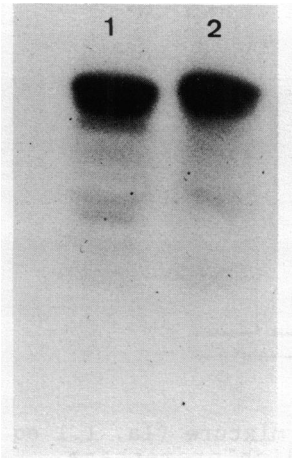


Fig. 4. The gel electrophoresis pattern (20% polyacrylamide/7 M urea) of the crude, radiolabeled synthetic oligomers prepared by the standard phosphite method (lane 1), and by the *in situ* procedure (lane 2).

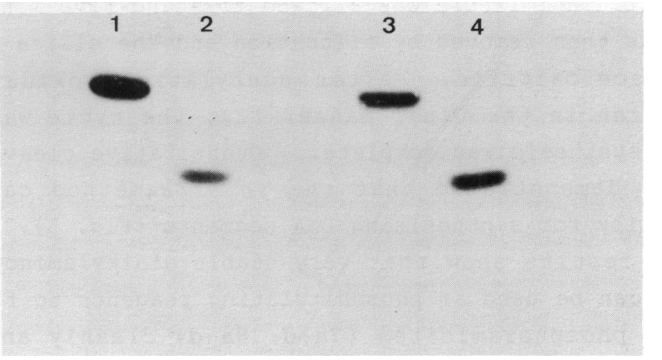


Fig. 5. The gel electrophoresis pattern of *Eco*RI digestion of DNA prepared by the standard method (lanes 1 and 2) and by the *in situ* method (lanes 3 and 4).

10, a ratio of 1:10 (**7a**/**8a**) was observed. In the latter case, the product distribution seemed to be determined primarily by salt catalyzed transamidation of the phosphine and/or of the phosphoramidite (14).

Phosphoramidites **1a-d** prepared using this approach were tested as synthons by constructing d(GGGAATTCCC), a self-complementary segment containing the *Eco*RI recognition sequence. The deoxyoligonucleotide was synthesized and deprotected using standard procedures (2), and isolated by gel electrophoresis in 57% yield. The average coupling yield (measured spectrophotometrically from the dimethoxytrityl cation, λ_{max} 498 nm, ε = 7.2 X 10^4) was greater than 95%. After end-labeling with [γ-^{32}P]ATP and T4-kinase (Fig. 4), the self-complementary segment was found to be degraded completely with *Eco*RI (Fig. 5) indicating that the synthesis was satisfactory (15).

Compound **4** was also tested as part of an *in situ* synthesis strategy. Phosphoramidites **7a-d** were each prepared as 0.1 M solutions in dry acetonitrile containing **4** (1.0 eq) and **9** (0.5 eq). The segment d(GGGAATTCCC) was then prepared in 50% isolated yield (average coupling yield was 94%) using the following procedure: To a suspension of the appropriately derivatized silica support (16) in 250 µl of a 0.4 M solution of tetrazole in acetonitrile was added 450 µl (45 µmol, 20 eq) of a 0.1 M solution of the appropriate deoxynucleoside phosphoramidite gener-

ated *in situ*. The mixture was allowed to stand five minutes. The solution was then removed by filtration and the silica was washed with dry acetonitrile. After acetylation, oxidation, and detritylation in the usual manner (2), the cycle was repeated until the synthesis was complete. Quantitative cleavage at the *Eco*RI site demonstrated that the *in situ* method can be used satisfactorily for synthesizing DNA segments (Fig. 5).

These results show that very stable dialkylaminophosphines (**4** and **6**) can be used as phosphitylating reagents to form deoxynucleoside phosphoramidites (**7a-d**, **8a-d**) cleanly and in good yields. The reactions are catalytic in either tetrazole or the corresponding amine hydrotetrazolides (**9** or **10**) and selectively lead to the formation of only the 3'-deoxynucleoside phosphoramidites without concurrent hydrolysis to the phosphonus acid or synthesis of the 3'-3' dinucleoside phosphite. This selectivity renders these reagents attractive candidates for the *in situ* generation of phosphoramidites useful for DNA synthesis on solid supports, and may have applicability to an automated polynucleotide synthesizer.

ACKNOWLEDGEMENTS

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