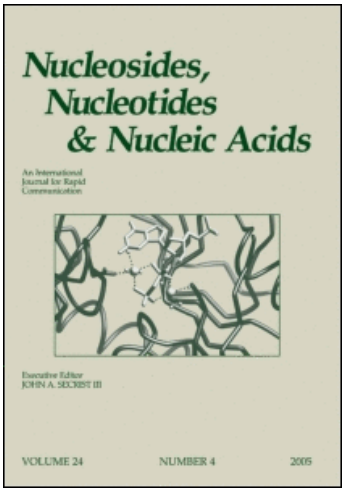

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Use of the 2-(4-Pyridyl)Ethyl Protecting Group in the Synthesis of DNA Fragments Via Phosphoramidite Intermediates

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USE OF THE 2-(4-PYRIDYL)ETHYL PROTECTING GROUP IN THE SYNTHESIS OF DNA FRAGMENTS VIA PHOSPHORAMIDITE INTERMEDIATES

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ABSTRACT 2-(4-Pyridyl)ethyl is a new protecting group for the internucleotidic bonds in the synthesis of deoxyribooligonucleotides by the phosphoramidite approach. This group is stable to alkali and acid conditions, and can be removed easily by two step procedures under mild conditions. The synthesis of deoxyribooligonucleotides by using phosphoramidite units containing 2-(4-pyridyl)ethyl group is also described.

Recently, the phosphoramidite approach is utilized as an important procedure for the synthesis of oligonucleotides on a solid support.[1)] Bis(diisopropylamino)alkoxyphosphines or diisopropylamino-alkoxychlorophosphines are appropriate phosphitylating agents for their synthesis.[1,2)] On the other hand, several protecting groups with better removal properties have recently been proposed for internucleotidic bonds.[3)] However, most of the phosphate protecting groups are unstable under alkaline conditions.

In a previous paper,[4)] we reported that the 2-(2-pyridyl)-ethyl (2-PYE) group was a suitable protecting group for the internucleotidic bonds and removed via two step procedures with concomitant loss of the amino and hydroxyl protecting groups. It is now found that 2-(4-pyridyl)ethyl (4-PYE) group is much more effective for the protecting group of internucleotidic bonds than the 2-PYE group.

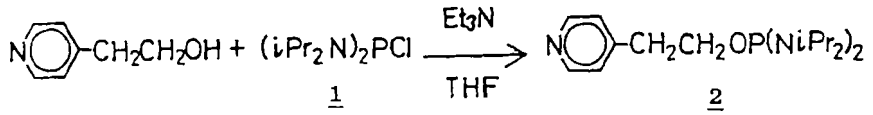
The phosphoramidite unit (5) containing 4-PYE group can be prepared according to the procedure described previously[5)].

TABLE 1. Yields and ^{31}P-NMR Spectra Analysis of 5

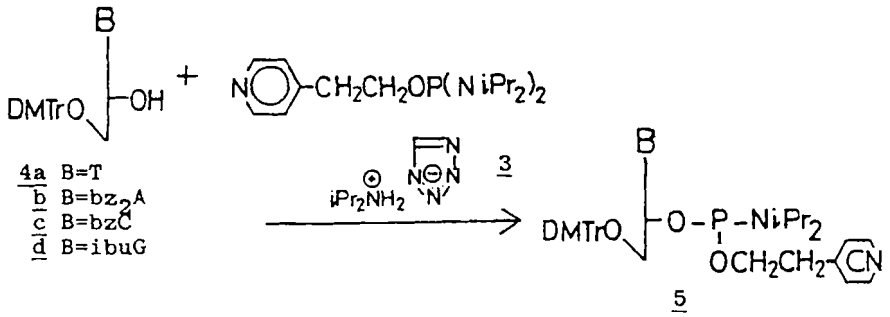
Compound	yield (%)	^{31}P-NMR Chemical Shift (ppm)
5a	87	147.6, 147.2
5b	86	147.9, 147.6
5c	84	148.0, 147.6
5d	80	145.8, 144.1

The chemical shifts are given in ppm relative to the external standard 85% H_3PO_4.

Reaction of bis(diisopropylamino)chlorophosphine (1) and 2-(4-pyridyl)ethanol in the presence of triethylamine gave the phosphitylating agent 2, which can be further activated in the presence of 3[5]. It was further reacted with the nucleosides (4) to give the phosphoramidite units (5) in good yields (TABLE 1).

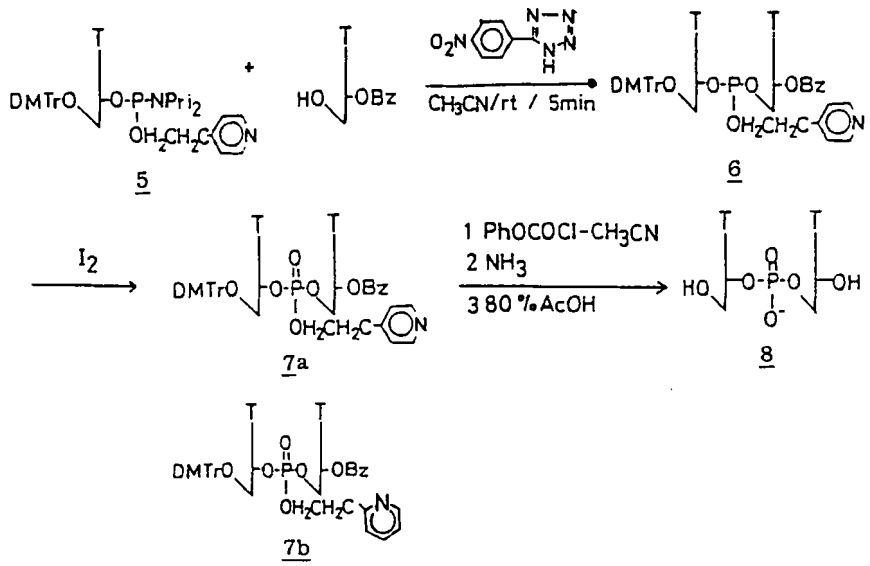


1 2



5

The ^{31}P-NMR of 5 showed two characteristic signals corresponding to a diastereomeric mixture. Further, the reaction of 5a (1.1 molar equiv.) and 3'-O-benzoylthymidine (1.0 molar equiv.) in the presence of 5-p-nitrophenyltetrazole[6] in CH_3CN led to the formation of the 3'-5'-O-dinucleoside phosphite triester (6). After 5 min and the usual workup, the residue was oxidized with 0.1 M I_2 in THF-pyridine-H_2O (40:20:1) to give the dimer 7 in 85% yield.



The protection of internucleotidic bonds with 2-(4-pyridyl)-ethyl group would be expected to be removable under two step procedures via β-elimination. The dimer 7 was treated first with phenyl chloroformate in CH_3CN at room temperature for 6 h and then with conc. ammonia at room temperature for 6 h. The results obtained are given in TABLE 2. Compared with 2-(2-pyridyl)ethyl group, it is noted that the removal of the 4-PYE group was achieved easily by use of between 3 and 5 molar equiv. of phenylchloroformate to 7. For this reason, whereas the 2-PYE group was only partly phenyloxycarbonylated and subsequently cleaved with alkaline treatment, [after removal of the DMTr group, yield of d-TpT (7) was 50%], the 4-PYE group was transformed completely and the unblocked dimer 7 was obtained almost quantitatively.

The phosphoramidite intermediates 5 were used in the synthesis of a dodecamer DNA sequence, d-AGCAAAAGCAGG, complementary to the 3'-terminal of v-RNA segments as a specific primer for the preparation of Influenza Virus Strands.[7] The synthesis was performed on a controlled pore glass (9)[8] (50 mg, 30 μmol/g, n=0) in a column type reactor fitted with sintered glass. Dedimethoxytritylation was carried out by treatment with 3% Cl_3CCOOH in CH_2Cl_2 for 2 min.

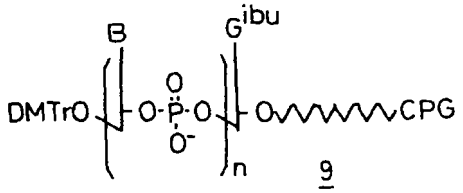
After thorough washing and drying of the column in vacuo, the phosphoramidite units (5) (20 equiv.) and 5-p-nitrophenyltetrazole[6] (60 equiv.) dissolved in CH_3CN (600 μl) was added with a syringe under nitrogen atmosphere. The reaction was completed in 5 min followed by the oxidation with 0.1 M I_2 in THF-

TABLE 2. Comparision of the 2-(4-Pyridyl)ethyl Group with 2-(2-Pyridyl)ethyl Group.

Dimer	PhOCOCl (molar equiv.)	Yields(%) of 8
7a	3	50
7a	5	62
7a	10	80
7a	20	88
7b	3	91
7b	5	89
7b	10	92
7b	20	90

The dimers were treated first with phenyl chloroformate in CH_3CN at room temperature for 6 h and then with conc. ammonia at room temperature for 6 h.

pyridine-H_2O (40:20:1, v/v). The elongation cycle is summarized in TABLE 3. The rate of the synthesis of deoxyribo-



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oligonucleotides using 5-p-nitrophenyltetrazole was much faster than 1H-tetrazole.[6,9] The average yield per elongation step was 95% for deoxyribooligonucleotide as calculated from trityl cation.

After the synthesis of dodecamer, the CPG was treated first with phenyl chloroformate in CH_3CN at room temperature for 6 h and then with conc. ammonia at 60 °C for 12 h to remove the 4-PYE and heterocyclic amino protecting groups, and to release the oligonucleotides from the CPG. The 5'-protected oligonucleotide was separated by reversed phase C_{18} silica gel column (FIG. 1). The fractions of main peak were collected, evaporated and treated with 80% AcOH for 15 min to remove the DMTr group. The unblocked oligomer was purified by TSKgel DEAE-2SW (FIG. 2).[10] From 1.5 μmol of the resin, 28 OD units of d-AGCAAAAGCAGG was

TABLE 3. Steps Involved in One Elongation Cycle

Step	Solvent and Reagent	Amount (ml)	Time (min)	Operation Times
1	3% Cl_3CCOOH in CH_2Cl_2	2	1	2
2	CH_2Cl_2	5		2
3	CH_3CN	5		2
4	vacuum		5	
5	Phosphoramidite (20 equiv.) and p-NP-tetrazole (40 equiv.) in CH_3CN	0.5	5	1
6	CH_3CN	3		2
7	0.1 M I_2 in THF-pyridine-H_2O (40:20:1)	2	1	2
8	CH_3CN	5		2
9	CH_2Cl_2	5		1

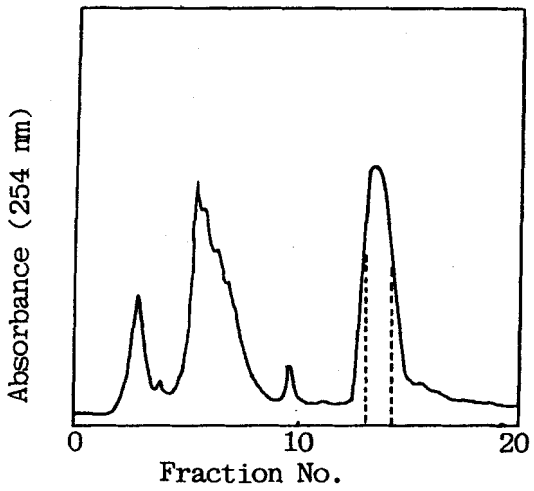

Fig. 1. Reversed Phase Chromatography on a Column (0.5 X 12 cm) of C-18 Silica Gel (35-105 u, Waters) Using a Linear Gradient of CH_3CN (0-30%) in 0.05M Triethylammonium Acetate (Total 200 ml).

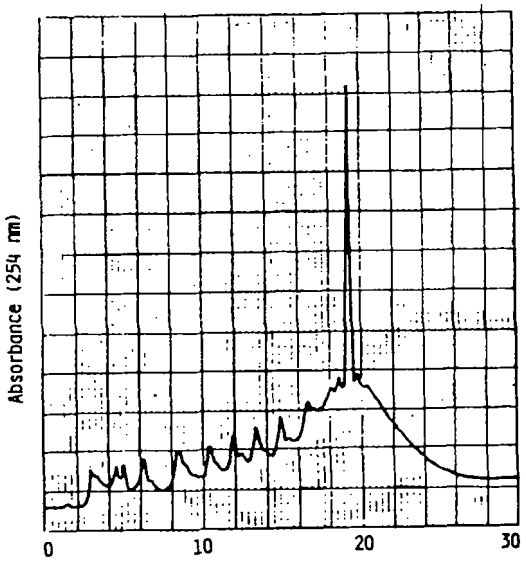

Fig. 2. Purification of the 12 mer. Anion Exchange HPLC Using a TSKgel DEAE 2SW Column with a Linear Gradient of Ammonium Formate (From 0.1M to 1.5M During 40 min) in 20% CH_3CN.

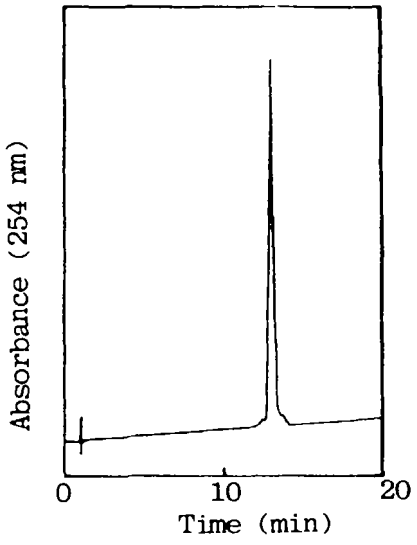

Fig. 3. Analysis of the 12 mer. Reversed Phase HPLC Using a TSKgel Oligo-DNA RP Column with a Linear Gradient of CH_3CN (From 7 to 15% During 25 min) in 0.1M Triethyl-ammonium Acetate (pH 7.0).

Fig. 4. Electrophoresis on 20% Polyacrylamide Gel of d-AGCAAAAGCA-GG (Track 1), Synthesized by the Phosphiramidite Method and d-AGCAA-AAGCAGG (Track 2), Synthesized by the Phosphotriester Method.

obtained. The main peak was found to be homogeneous by reversed phase C_{18} HPLC (FIG. 3) and by electrophoresis (FIG. 4). The proportions of nucleosides and nucleoside 5'-phosphates were analyzed by the reversed phase C_{18} HPLC after hydrolysis of the unblocked oligomer with snake venom phosphodiesterase and found to agree with the calculated value.

In conclusion, 2-(4-pyridyl)ethyl group should prove to be superior to 2-(2-pyridyl)ethyl as a protecting group for the internucleotidic bonds in the phosphoramidite approach to deoxyribooligonucleotides.

EXPERIMENT

Thin-layer chromatography (t.l.c) was performed on plates of Kieselgel $60F_{254}$ (Merck). Column chromatography was performed on silica gel (BW-300; Fuji Davison Co. Ltd.). Aminopropyl CPGs (pore size 500A , particle size 90-125 μm) was purchased from Merck Co. Ltd. Reversed-phase column chromatography was performed on alkylated silica gel (C_{18}, 55-105 μ, Waters Associates Inc.). HPLC was performed on a Shimazu LC-6A chromatography system. Anion exchange chromatography was performed on TSKgel DEAE-2SW. Reversed phase HPLC was carried out on TSKgel oligo-DNA RP. Deoxyribonucleosides were purchased from Yositomi-seiyaku Co. Ltd.

Preparation of the Phosphoramidite Units

To a solution of bis(diisopropylamino)chlorophosphine (400 mg, 1.5 mmol) in dry Et_2O (1 ml), Et_3N (200 μl, 1.5 mmol)) and 2-(4-pyridyl)ethanol (168 μl, 1.5 mmol) was added. After 16 h, petroleum ether was added to the mixture, and the reaction

mixture was left to stand overnight in a refrigerator. After the removal of triethylammonium hydrochloride, all the volatile components of the filtrate were removed under high vacuum. The ^{31}P-NMR spectroscopy of the crude phosphitylating agent 2 showed two peaks: a major peak at 121.8 ppm assigned to 2 and a minor peak at 140 ppm assigned to 1. The phosphitylating agent 2 thus obtained was treated with 5'-O-dimethoxytritylthymidine (4a) (545 mg, 1.0 mmol) in the presence of 3 (171 mg, 1.0 mmol) in dry CH_2Cl_2 (5 ml). After 30 min, the solution was washed with aqueous saturated solution of NaCl (3 X 5 ml). The organic layer was dried over Na_2SO_4 and evaporated in vacuo. The residue was applied to a column of silica gel and eluted with a mixture of CH_2Cl_2-ethyl acetate-Et_3N (45:45:5,v/v). The appropriate fractions containing 5a was concentrated under a high vacuum and 5a was isolated as a white powder (689 mg, 87%) by precipitation from cold hexane (-78 °C). In a similar manner, other phosphoramidite units (5) were obtained as shown in TABLE 1.

Synthesis of Dimer, d-DMTrTp(4-PYE)TOBz (7a)

The phosphoramidite unit 5a (1.068 g, 1.4 mmol) was treated with 3'-O-benzoylthymidine (345 mg, 1.0 mmol) in the presence of 5-p-nitrophenyltetrazole (280 mg, 4.0 mmol) in dry CH_3CN for 5 min. To the reaction mixture, 0.1 M I_2 in THF-pyridine-H_2O (40:20:1, v/v) (16 ml) was added and the mixture was kept for 5 min. After the usual work-up, the resulting residue was applied to a column of silica gel and eluted with a stepwise gradient of MeOH (0-5%) in CH_2Cl_2 to give the fully protected dimer (7) (892 mg, 85%). Rf 0.52 (CH_2Cl_2-MeOH, 9:1, v/v); UV max(MeOH) 278 (sh), 268 nm.

Removal of 2-(2- or 4-Pyridyl)ethyl Groups from 7a,b

The fully protected dimer (7a,b) (1.0 molar equiv) were treated with phenyl chloroformate (3-20 molar equiv) in CH_3CN at room temperature for 6 h. The solution was evaporated in vacuo and the residue was dissolved in conc. ammonia and the mixture was kept at room temperature for 6 h. The solution was concentrated and 80% AcOH. After 15 min, the solution was evaporated in vacuo and the residue was dissolved in water and washed with ether. The deblocked dimer d-TpT (8) was isolated after paper chromatographic separation using Whattman 3MM paper with iso-PrOH-conc.ammonia-H_2O (7:1:2, v/v). The yields of 8 were summarized in TABLE 2. The dimer was degraded by nuclease P1 to give d-T and d-pT in the ratios of 1.00:1.09.

Synthesis of Deoxyribooligonucleotides on Polymer Support

The nucleoside resin (1.5 μmol) was placed on sintered glass filter capped with a serum cap. The condensation reaction and washings have been done according to TABLE 3. The reactor was flushed with N_2 gas and the solvent was added with syringe. At the condensation step, a CH_3CN solution of the phosphoramidite units (30 μmol) and 5-p-nitrophenylterazole (60 μmol) was added through the serum cap by syringe. The excess of reagents was removed by flushing with N_2 gas. After the synthesis, the resin was washed with CH_3CN, ether, and dried.

Deblocking and Purification

The resin was treated with phenyl chloroformate (0.23 ml, 1.9 mmol) in CH_3CN (2 ml) at room temperature for 24 h. The solution was evaporated in vacuo and the residue was dissolved in conc. ammonia (5 ml). The mixture was kept at 60 °C for 12 h. The resin was filtered off and washing with H_2O. The combined filtrate was evaporated and the residue was applied on a reversed phase C_{18} silica gel column (FIG. 1). The appropriate fractions containing oligomers bearing DMTr group was collected and evaporated in vacuo and then treated with 80% AcOH (10 ml) at room temperature for 15 min. The solvent was concentrated and redissolved in a small volume of water. The aqueous layer was washed with ether and evaporated. The residue was applied on a TSKgel DEAE 2-SW HPLC column (FIG. 2). Elution was performed with a linear gradient of ammonium formate (0.1-1.5 M) in 20% CH_3CN. The pure oligomer, d-AGCAAAAGCAGG (10) with 28 OD units was obtained. The purity of the product was tested by analytical HPLC (FIG. 3) and 20% polyacrylamide gel electrophoresis (FIG. 4). Base analysis by digestion with snake venom phosphodiesterase gave d-A, d-pA, d-pC, and d-pG in a ratio of 1.00:4.89:2.10:3.85.

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