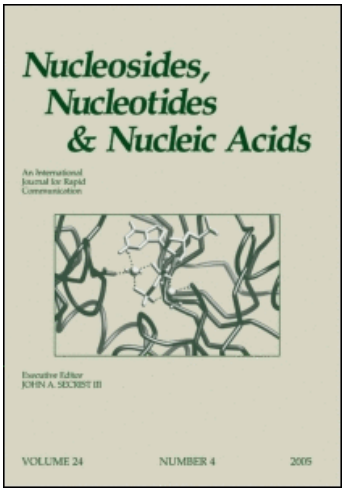

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6-N-1 Trobenzotriazol-1-Yloxytris(Dimethylamino)Phosphonium Hexafluorophosphate (NBOP): A New Coupling Reagent in Oligonucleotide Synthesis

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6-NITROBENZOTRIAZOL-1-YLOXYTRIS(DIMETHYLAMINO)PHOSPHONIUM HEXAFLUOROPHOSPHATE(NBOP): A NEW COUPLING REAGENT IN OLIGONUCLEOTIDE SYNTHESIS.+

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Abstract: 6-Nitrobenzotriazol-1-yloxytris(dimethylamino) phosphonium hexafluorophosphate (NBOP, 2) has been demonstrated as an efficient coupling reagent in oligonucleotide synthesis. The reagent is stable and gives high coupling yields without any side products.

Synthetic oligonucleotides have manifold applications in understanding biological processes[1]. More recently they have been used as antisense probes in regulating gene function[2]. This has necessitated parallel development in oligonucleotide synthesis methodology to obtain them in requisite purity and amounts. Chemical synthesis of oligonucleotides is generally accomplished by either phosphoramidite[3] or phosphotriester[4] method. A key step in phosphotriester synthesis is the use of a coupling reagent during nucleotide bond formation. The commonly used coupling reagents have some drawbacks:
1) arylsulfonyl chlorides and their azoles are unstable on prolonged storage, 2) reaction is slow and invariably leads to sulfonation of the hydroxyl group of the nucleoside component[5], 3) nucleoside bases, guanine and thymine, are modified[6]. Although coupling rate has been enhanced using nucleophilic catalysts[7], side reactions have not been

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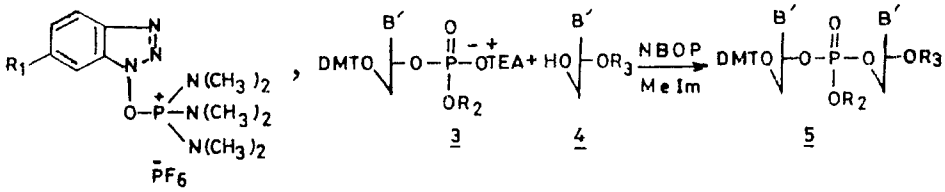

1 BOP, R_1= H
2 NBOP, R_1= NO_2

DMT = Dimethoxytrityl ; B' = Protected base
R_2 = 2-Chlorophenyl ; MeIm = *N*-Methylimidazole
R_3 = Benzoyl; 2-$ClC_6H_4OP(O)OCH_2CH_2CN$

SCHEME 1

completely eliminated. Therefore, we thought it worthwhile to explore new coupling reagents and our results are reported in this paper.

Earlier, we have reported the use of *N,N'*-bis(2-oxo-3-oxazolidinyl)phosphorodiamidic chloride[8] in oligonucleotide synthesis with improved coupling efficiency, but the reagent is unstable on prolonged storage. In order to obtain a nonhygroscopic reagent we focussed our attention on other phosphorous based compounds. We were attracted by one such reagent widely used in peptide synthesis viz. benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP, 1)[9]. With this reagent we failed to achieve coupling in presence of a nucleophilic catalyst even after 24 hrs. This could be due to lack of phosphodiester activation. In order to increase the reactivity, benzotriazoloxy residue of 1 was replaced with 6-nitrobenzotriazoloxy to obtain 2. Synthesis of 2 was carried out by a procedure similar to that for BOP reagent[10] except that KPF_6 was dissolved in dry acetonitrile before addition. The crude product 2 was recrystallized from acetone-ether to obtain pure NBOP (2) as a nonhygroscopic and stable solid in 45% yield. The structural assignment for 2 is principally supported by FABMS, NMR and IR studies. In the IR

spectrum of 2 characteristic peaks at 767, 841 and 1017 cm^{-1} were assigned to PF_6 and P-N group frequencies respectively. A comparison of the NMR spectra of 2 and 1 shows close similarities excepting for the aromatic protons which appear as a multiplet at 8.45 ppm and a singlet at 8.8 ppm counting for two and one aromatic proton respectively. However, in case of 1 there is a broad multiplet in the region 7.4-8.1 ppm for four aromatic protons. A doublet at 3.0 ppm (J= 11 Hz) was assigned to $N(CH_3)_2$ groups. FAB mass spectrum of 2 exhibited strong peak at mass number 342 $[M-PF_6]^+$. Fragmentation of this ion leading to 6-Nitrohydroxybenzotriazole was also observed at m/z 180. In the case of 2, as well as 1, conventional mass spectrometry failed to give satisfactory results.

As expected, NBOP proved to be superior to other known coupling reagents. Reactions with reagent 2 proceeded fast and they were complete in less than 15 min. Suitably protected phosphodiester (3, 1.1 mmol) and hydroxyl component (4, 1.0 mmol) in pyridine were reacted in the presence of 1.5 times excess of *N*-methylimidazole and NBOP at room temp for 15 min (scheme 1). All coupling reactions were routinely monitored by RP-18 TLC plates in 7:3 v/v acetone-water (solvent B). In this system unreacted hydroxyl component was not visible suggesting that coupling reactions using NBOP go to completion. More importantly, coupling proceeded without any side reactions either with the hydroxy group or the heterocyclic base moiety. This was deduced from the following experiment: suitably protected monomers having free 5'-OH were treated with NBOP under the conditions used for coupling. After the workup, the products were isolated and analyzed by spectral data. The spectral data (NMR and FAB mass) of the isolated product were superimposable with the hydroxyl component thereby confirming that NBOP mediated coupling reactions are free from side products. Suitability of reagent 2 in oligonucleotide synthesis has been demonstrated by making a few natural dimers and oligomers in excellent yields. Since all coupling reactions go to completion capping step has been eliminated.

TABLE 1

Oligodeoxynucleotide	% Yield
DMT-TpT-OBz (5)	94
DMT-CpG-OBz (6)	92
DMT-ApG-OBz (7)	93
DMT-TpCp* (8)	94
DMT-CpGp* (9)	91
DMT-TpApG-OBz (10)	92
DMT-CpGpG-OBz (11)	91
DMT-TpTpCp* (12)	93
DMT-ApTpCp* (13)	92
DMT-CpGpCpG-OBz (14)	92
DMT-CpGpCpGp* (15)	90
DMT-ApTpCpCpG-OBz (16)	91
DMT-CpGpCpGpCpG-OBz (17)	85
DMT-ApTpCpTpTpCp* (18)	86
DMT-ApTpCpTpTpCpTpT-OBz (19)	85

Abbreviations: DMT=4,4'-Dimethoxytrityl; Bz=Benzoyl; T=Thymidine; C=4-N-benzoyl-2'-deoxycytidine; G=2-N-isobutyryl-2'-deoxyguanosine; A=6-N-benzoyl-2'-deoxyadenosine; p=2-Chlorophenylphosphate; p*=2-$ClC_6H_4OP(O)OCH_2CH_2CN$; These abbreviations are based on a simplified scheme suggested by Reese[11]. Coupling yields refer to 1+2, 2+2, 3+2, 3+3, 4+2 and 6+2 fragment condensations in case of tri, tetra, penta, hexa and octamers respectively.

The data reported in table 1 corresponds to isolated yields of the products after purification by flash silica gel column chromatography. It can be seen that yields in the coupling reactions are essentially independent of the chain length.

All the protecting groups were removed from the final oligomers by the standard procedure[12]. The products were purified from FPLC and in each case the major peak with highest retention time was isolated. That this major peak was the required product was checked by synthesizing CGCGCG on a Pharmacia Gene Assembler Plus by the standard phosphoramidite method (data not shown) and coinjecting with the same hexamer synthesized by us using NBOP. The two were found to comigrate on FPLC. The FPLC profiles of the crude (A) and purified (B) octamer having the sequence ATCTTCTT are shown in fig 1. The clean FPLC profiles of the crude deblocked oligomers including the octamer (fig 1) demonstrate the effectiveness of

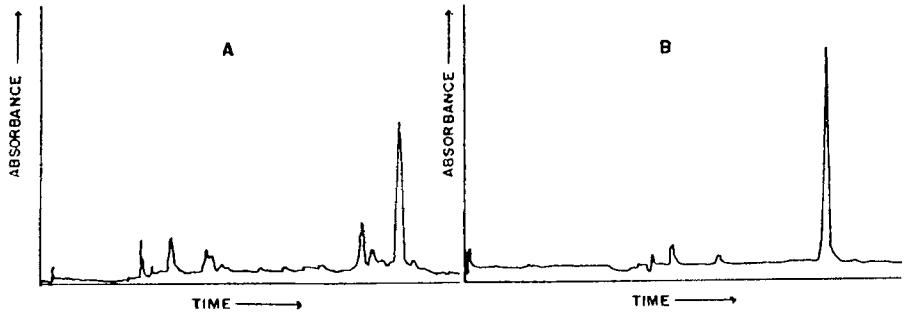

FIGURE-I

FPLC was performed on Pharmacia FPLC system using mono Q HR5/5 column at pH 12, flow rate 1 mL/min. using water ----> 1.0 M aq.NaCl linear gradient (35 min.)

NBOP as a coupling reagent. The effectiveness of NBOP is also evident from the fact that only 1.5 times excess of NBOP, as against 3 times and more in case of known coupling reagents, is sufficient to take the coupling reaction to completion. An additional desirable property of NBOP is that, unlike BOPDC or arylsulfonyl chlorides and their azole derivatives, it does not liberate any acidic component. Since this work was completed, we have successfully used NBOP in synthesizing four more hexamers containing methylphosphonate linkage at defined positions for DNA-protein interaction studies and the results will be published elsewhere.

The present findings may be summarized as follows:

1) reagent 2 is a stable and nonhygroscopic compound,
2) coupling yields are excellent without any side products,
3) it would be an ideal coupling reagent for solid phase oligonucleotide synthesis using phosphotriester chemistry.

EXPERIMENTAL

General :

All the solvents were freshly distilled and dried before use. Melting points were determined on a Toshniwal melting

point apparatus and are uncorrected. UV, IR, NMR and FAB mass spectra were recorded on Perkin Elmer Lambda 15 UV/VIS spectrophotometer, Perkin-Elmer 881 Infrared Spectrophotometer, Bruker WM 400 MHz spectrometer and Jeol SX-102/DA 6000 double focussing mass spectrometer respectively. TLC was performed on polygram SIL G/UV_{254} in 8% MeOH in $CHCl_3$ (Solvent A) and RP-18 TLC plates in 7:3 v/v acetone-water (Solvent B) from Macherey-Nagel, Germany. Synthesis of a hexamer CGCGCG for the purpose of comparison was carried out on Pharmacia Gene Assembler Plus using the standard protocol. FPLC was performed on Pharmacia FPLC system using Mono Q HR5/5 anion exchange column at pH 12 (aq.NaOH) and flow rate 1 mL/min.

Synthesis of NBOP :

To a stirred ice cold solution of HMPA (28.75 mmol, 5.0 mL) in anhydrous dichloromethane (10 mL) was added $POCl_3$ (28.75 mmol, 2.67 mL) and the mixture stirred for 5 min. To this was added a solution of 1-Hydroxy-6-nitrobenzotriazole (28.75 mmol, 5.17 g) and anhydrous triethylamine (57.5 mmol, 8.8 mL) in anhydrous dichloromethane (40 mL) and stirred for another 15 min. The reaction mixture was filtered into a solution of KPF_6 (28.75 mmol, 5.29 g) in acetonitrile (50 mL) and stirred for 5 min at room temperature. The solvent was removed under reduced pressure and the residue was poured into an ice cold mixture of water (400 mL) and triethylamine (43.12 mmol, 5.99 mL) and stirred for 2 min. The supernatant was decanted and the precipitate washed with cold water. The crude product was dried over P_2O_5 and recrystallised from acetone-ether.

Yield : 45%; mp: 170-171°C; IR(KBr): 841, 767 cm^{-1} (PF_6) and 1017 cm^{-1} (P-N); ^{1}NMR: 3.0 ppm [d, 18H, $N(CH_3)_2$, J= 11 Hz], 8.45 ppm [m, 2H, aromatic] and 8.80 ppm [s, 1H, aromatic]; FABMS:(m/z) 342 $[M-PF_6]^+$ & 180 [6-NO_2-HOBT]

General procedure for coupling :

Triethylammonium salt of suitably protected phosphodiester, 3, (1.1 mmol), hydroxyl component, 4,

(1 mmol) and *N*-methylimidazole (1.65 mmol) were repeatedly dried by coevaporation with anhydrous pyridine . The residue was dissolved in andhyrous pyridine (15 mL). To this was added NBOP (1.65 mmol) and the reaction mixture stirred for 15 min. After the reaction was complete, as monitored by TLC, the reaction mixture was evaporated under reduced pressure. The residue was dissolved in chloroform (100 mL), washed with 5% aq. sodium bicarbonate, water and saturated brine and dried over anhydrous sodium sulphate. The organic layer was evaporated and the crude product purified by flash silica gel column chromatography using a linear gradient of methanol (0-5%) in chloroform. Appropriate fractions were pooled and concentrated. The product was dissolved in small amount of chloroform and added dropwise to pet.ether with stirring to obtain a white powder.

Deblocking and Purification :

First, the fully protected oligomers (0.045 mmol) were treated with tetramethylguanidinium pyridine-2-aldoxime (3.15 mmol) in a mixture of dioxane-water (1:1 v/v) for 16 h at room temperture to remove phosphate protecting groups. The reaction mixture was dried on Speed Vac Concentrator (SVC). Next, this was treated with 25% aq.ammonia at 60°C for 16 h to remove base protecting groups. The reaction mixture was dried on SVC. The residue was dissolved in water and extracted with ether to remove the reagents and the benzamide and isobutyrylamide present in the reaction mixture. A portion (50 $A_{260}U$) of the crude product so obtained was passed through sephadex G-15 column. Finally, the oligomer obtained (36 $A_{260}U$) from sephadex column was treated with 80% aqueous acetic acid below 10°C for 15 min to remove dimethoxytrityl group. The acid was removed on SVC by repeated coevaporation with methanol. The residue was dissolved in water and washed with ether to remove the dimethoxytritanol. Finally completely deblocked oligomer was purified by HPLC using mono Q column.

Monitoring of side reactions :

3'-*O*-(*tert*-Butyldimethylsilyl)-N^2-isobutyryl-2'-deoxyguanosine (0.1 mmol) and *N*-methylimidazole were dried by

repeated coevaporation with anhydrous pyridine. The residue was dissolved in anhydrous pyridine (1 mL). NBOP (0.165 mmol) was added to it and the reaction mixture stirred overnight at room temp. The solvent was removed under reduced pressure. The residue was taken in chloroform (25 mL) and the organic layer washed with 5% aq. sodium bicarbonate, water and saturated brine. The chloroform layer was dried over anhydrous sodium sulphate and concentrated. The product obtained was purified by flash silica gel chromatography using a gradient of methanol (0-5%) in chloroform. TLC, ^{1}H NMR and the FABMS data of the isolated product was superimposable with that of the starting material.

R_f : 0.53 (normal silica gel TLC, solvent A)

^{1}H NMR (ppm) : 0.1 (s, 6H, $Si(CH_3)_2$, tBDMS)
: 0.09 (s, 9H, $C(CH_3)_3$, tBDMS)
: 1.2 (t, 6H, $-HC(C\underline{H}_3)_2$, isobutyryl)
: 2.0-5.0 (aliphatic protons, sugar)
: 6.1 (t, 1H, H-1', guanosine)
: 7.8 (s, 1H, H-8, guanosine)

FABMS : 452 $[M+H]^+$, 336 $[2'dG^{iBu}]^+$

The above experiment was also carried out with 3'-*O*-(*tert*-butyldimethylsilyl)thymidine.

R_f : 0.55 (normal silica gel TLC, solvent A)

^{1}H NMR (ppm) : 0.1 (s, 6H, $Si(CH_3)_2$, tBDMS)
: 0.9 (s, 9H, $C(CH_3)_3$, tBDMS)
: 1.9 (s, 3H, CH_3, thymidine)
: 2.0-5.0 (aliphatic protons, sugar)
: 6.1 (t, 1H, H-1', thymidine)
: 7.4 (s, 1-H, H-6, thymidine)

FABMS : 357 $[M+H]^+$

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