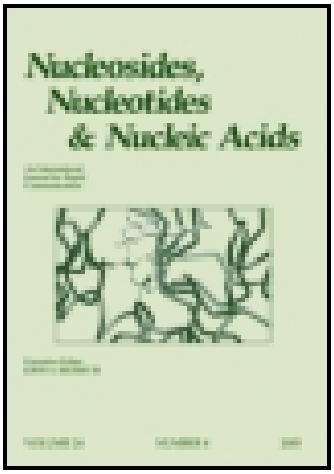

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Synthesis and Separation of Dinucleoside Phenylphosphonate Diastereomers

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SYNTHESIS AND SEPARATION OF DINUCLEOSIDE PHENYLPHOSPHONATE DIASTEREOMERS+

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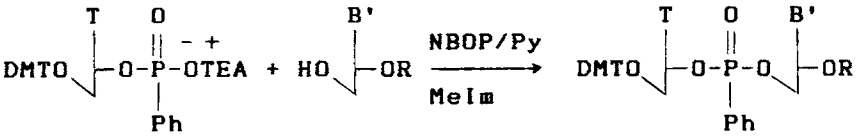
ABSTRACT: Dinucleoside phenylphosphonates have been synthesized in excellent yields using 6-Nitrobenzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (NBOP) as a coupling reagent. A general method for the resolution of two diastereomers of phenylphosphonate dimers has been developed.

In recent years, oligonucleoside methylphosphonates have been used *inter alia* as antisense probes in regulating gene expression[1,2]. These analogues have nonionic internucleotide linkage resulting from the replacement of one of the phosphate oxygens by a methyl group, thus rendering the phosphorus chiral with R or S configuration. The methylphosphonate bond closely resembles the conformation of phosphodiester bond but is resistant to nuclease hydrolysis[3]. Consequently, these analogues have attracted considerable research interest[4,5,6]. Curiously very little information is available on the corresponding phenylphosphonate analogues. Agarwal and Riftina[7] have reported the synthesis of dithymidyl phenylphosphonate in 60% yield and they have not resolved the two diastereomers. In this paper we report the synthesis and, for the first time, resolution of dinucleoside phenylphosphonate diastereomers.

+ CDRI Communication Number 5167.

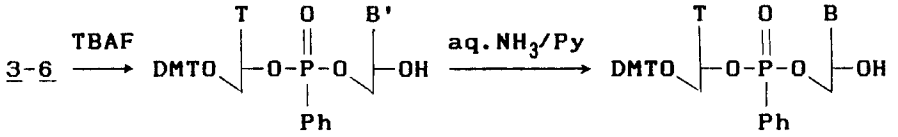
Synthesis of oligodeoxynuclotides containing phosphonate linkage is complicated by two factors viz. 1) low reactivity of the phosphonate diester towards coupling reagents and 2) difficulty in the separation of phosphonate diastereomers after each coupling[8]. In the case of nucleoside methylphosphonates this difficulty has been circumvented by using stereospecific coupling methods[9,10], but only with limited success due to their poor yields. Recently we have developed and used NBOP as a coupling reagent in triester-based oligonucleotide synthesis with excellent results[11]. As an obvious extension of that work we used NBOP to synthesize phenylphosphonate dimers. We have obtained excellent results with NBOP in the case of phenylphosphonate dinucleotides as well. As expected coupling proceeded rapidly and without any side reactions. We have synthesized a few dinucleotides in almost quantitative yields. But still a method was needed for the separation of diastereomers. Earlier we have reported improved resolution of methylphosphonate dinucleoside diastereomers using a chiral handle viz. l-menthyloxycarbonyl group at the 3'-OH[12]. However, using this approach resolution of dinucleoside phenylphosphonate diastereomers was not successful. Subsequently we found that diastereomer resolution could be achieved after removing the 3'-OH protecting group. In order to demonstrate the generality of this strategy we have synthesized and resolved all the thymidyl phenylphosphonate dinucleoside diastereomers (7-10). Since removal of the l-menthyloxycarbonyl group is somewhat difficult, and it was no longer required for the resolution of diastereomers, we have used silyl protecting group at the 3'-hydroxyl in subsequent coupling reactions (Scheme 1).

Suitably protected phosphonate salt 1, hydroxyl component 2 and N-methylimidazole were stirred with NBOP at room temp for 15 min. After the usual workup, the product was purified by flash silica gel chromatography using a linear gradient of methanol (0-5%) in chloroform. Appropriate fractions were pooled and concentrated and the product was precipitated from petroleum ether. The isolated coupling



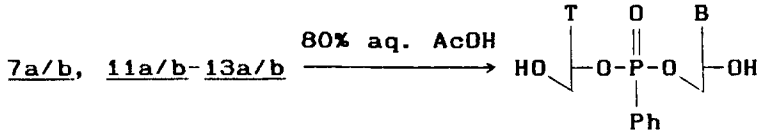
1 2

3, B' = T
4, B' = C^{Bz}
5, B' = A^{Bz}
6, B' = G^{iBu}
Py= Pyridine
MeIm= *N*-Methylimidazole
R= *tert*-butyldimethylsilyl

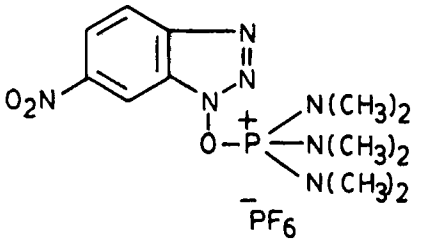


7a/b, B' = T
8a/b, B' = C^{Bz}
9a/b, B' = A^{Bz}
10a/b, B' = G^{iBu}
TBAF= Tetrabutylammonium-fluoride

11a/b, B= C
12a/b, B= A
13a/b, B= G



14a/b, B= T
15a/b, B= C
16a/b, B= A
17a/b, B= G



6-Nitrobenzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (NBOP).

SCHEME-1

TABLE 1

Comp. No.	%Yield	R_f value(A)	^{31}P NMR	Mol.wt.	FABMS(M-H)$^-$
7a	46	0.37	21.3403	908	907
7b	44	0.34	20.6141	908	907
8a	46	0.44	25.1043	997	996
8b	45	0.38	24.4637	997	996
9a	42	0.42	25.2079	1021	1020
9b	40	0.40	24.6427	1021	1020
10a	45	0.30	24.5137	1003	1002
10b	43	0.22	23.8430	1003	1002

yields of the compounds 3, 4, 5, and 6 are 95%, 92%, 90% and 88% respectively. Selective and quantitative removal of the silyl group was achieved using TBAF. Here it may be mentioned that during the fluoride mediated removal of the silyl group phenylphosphonate linkage was found to be stable as monitored by TLC. The reaction mixture showed two spots on TLC corresponding to the two diastereomers. There was no spot corresponding to either of the hydrolytic products. Earlier the tBDMS group has been used by Callahan et al[13] to protect the 3'-OH of methylphosphonate dinucleotides. During the removal of this group by TBAF, they did not observe cleavage of the methylphosphonate linkage and our results corroborate their findings. The desilylated dimers (7-10) were passed through silica gel column to separate diastereomers. We have obtained base line resolution of the diastereomers reported here. Only in the case of compound 9 we obtained a few fractions containing both isomers. In all the dinucleotides reported here the ratio of isomers a (faster moving on silica gel TLC) & b (slower moving on silica gel TLC) was nearly equal. Stereochemical purity of the isomers has been ascertained by ^{31}P NMR, wherein only one signal due to the phosphonate residue was observed for each diastereomer and they are separated by approximately 0.7 ppm (Table 1).

It is well documented that 1:1 (v/v) pyridine-aq. ammonia at 4° C for four days selectively removes the base protecting groups without hydrolyzing the methyl phosphonate bond[14] . Therefore we have used the same conditions for selective and

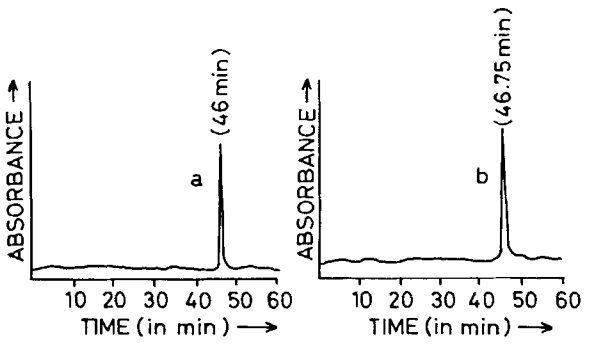


Fig.1

complete removal of the base protecting groups from compounds 8-10. Complete removal of the base protecting groups including the isobutyryl group from dG has been confirmed by TLC and FABMS (see experimental, compounds 11 a/b to 13 a/b). Under these deblocking conditions we again did not observe any cleavage of the phenylphosphonate bond. Finally the 4,4'-dimethoxytrityl group was removed by treatment with 80% acetic acid. After workup the individual isomers were lyophilized and obtained as white solids (see experimental, compounds 14 a/b to 17 a/b). The purity of the completely deblocked dimers has been checked by RP-18 HPLC. HPLC profile of Tp(Ph)T (isomers a and b) is shown in (Fig-1). All the intermediate compounds along with the final dinucleotides gave the correct molecular ion peak and the expected fragmentation pattern in negative ion FABMS[15].

The present findings may be summarized as follows: 1) Nucleoside phenylphosphonates can be synthesized in excellent yields using NBOP reagent. 2) We have resolved for the first time phenylphosphonate dinucleoside diastereomers. 3) Availability of pure isomers would be useful in structural and biochemical studies of nucleic acids.

EXPERIMENTAL

General :

All solvents were freshly distilled and dried before use. Nucleosides were purchased from Cruachem, Scotland. TLC was

carried out on Polygram SIL G/UV_{254} precoated silica gel plates, Macherey-Nagel, Germany, using 8% methanol in chloroform (solvent A) and 15% methanol in chloroform (solvent B). Flash column chromatography was done using silica gel purchased from Acme Synthetic Chemicals, India. HPLC was carried out on Shimadzu LC-6A with a Dupont RP-18 column using a linear gradient of acetonitrile in 0.1M TEAA (pH 7.1). ^{31}P NMR spectra were recorded on a Bruker WM-400 MHz spectrometer using 1% H_3PO_4 in D_2O as an external standard. FAB mass spectra were obtained from a Jeol SX-102/DA 6000 mass spectrometer.

Synthesis of 5'-O-dimethoxytritylthymidine-3'-O-phenylphosphonate triethylammonium salt (1) :

Triazole (12.6 mmol, 820 mg) was dried by repeated coevaporation with dioxane. It was then taken in dioxane (30 mL) and triethylamine (12.6 mmol, 1.75 mL) was added to it. The reaction was stirred for 10 min. and to this was added phenylphosphonic dichloride (6.0 mmol, 850 µL) when triethylammonium chloride precipitated out as white powder. The reaction mixture was stirred for a total of 30 min. and then filtered into predried 5'-*O*-dimethoxytritylthymidine (5 mmol, 2.72 g) dissolved in pyridine (30 mL). The reaction mixture was stirred at room temp for an hour and then quenched with excess of an ice-cold mixture of water and TEA. The reaction mixture was concentrated under reduced pressure. The residue was dissolved in chloroform and the organic layer washed with water, dried over anhydrous sodium sulphate and evaporated to a foam. The product (1) was dissolved in a small amount of chloroform and added to pet. ether with stirring to obtain as a white powder (Yield 90%), R_f 0.06(A).

General procedure for coupling reaction :

A mixture of 5'-*O*-dimethoxytritylthymidine-3'-*O*-phenylphosphonate triethylammonium salt (1.1 mmol, 862 mg), a suitably protected hydroxyl component (1.0 mmol) and *N*-methylimidazole (1.65 mmol, 131 µL) were dried by repeated

coevaporation with pyridine. The residue was dissolved in pyridine (15 mL). To this was added NBOP (1.65 mmol, 803 mg) and the reaction mixture stirred at room temp for 15 min. After completion of the reaction, the solvent was removed under reduced pressure. The residue was dissolved in chloroform and washed with 5% aq. sodium bicarbonate, water and saturated brine. The organic layer was dried over sodium sulphate and concentrated. The product was purified by flash silica gel chromatography using a linear gradient of methanol (0-5%) in chloroform. Appropriate fractions were pooled and concentrated. The pure product was dissolved in a small amount of chloroform and added to pet. ether with stirring to obtain as a white powder.

Compound	Isolated % Yield	R_f (A)
DMT-Tp(Ph)T-tBDMS (3)	95	0.63
DMT-Tp(Ph)C'-tBDMS (4)	92	0.74
DMT-Tp(Ph)A'-tBDMS (5)	90	0.74
DMT-Tp(Ph)G'-tBDMS (6)	88	0.60

General procedure for removal of tBDMS group. :

Fully protected dinucleotides (3-6, 0.5 mmol) were stirred with 1M solution of TBAF in THF (0.75 mmol, 0.75 mL) at room temp for 30 min. The reaction mixture was then evaporated to dryness under reduced pressure. The residue was dissolved in chloroform and washed successively with water and saturated brine, dried over anhydrous sodium sulphate and evaporated. The product which was obtained as a mixture of diastereomers was resolved by flash silica gel column chromatography using a linear gradient of methanol (0-5%) in chloroform. Appropriate fractions were pooled and concentrated. Each isomer was dissolved in a small amount of chloroform and added to pet. ether with stirring to obtain as a white powder. Yields are given in Table 1.

General procedure for removal of base protecting groups :

Compounds 8-10 (0.2 mmol each of isomers a & b) were treated with a 1:1 (v/v) mixture of pyridine-25% aq. ammonia

(15 mL) at 4°C for 4 days. The reaction mixture was evaporated under reduced pressure. The product was purified by flash silica gel chromatography using a linear gradient of methanol (0-5%) in chloroform. Appropriate fractions were pooled and concentrated. Each isomer was dissolved in a small amount of chloroform and added to pet. ether with stirring to obtain as a white powder in quantitative yield.

Compound	R_f (A)	FABMS $[M-H]^-$
DMT-Tp(Ph)C (11a)	0.19	892
DMT-Tp(Ph)C (11b)	0.14	892
DMT-Tp(Ph)A (12a)	0.17	916
DMT-Tp(Ph)A (12b)	0.14	916
DMT-Tp(Ph)G (13a)	0.08	932
DMT-Tp(Ph)G (13b)	0.05	932

General procedure for removal of the 4,4'-dimethoxytrityl group.

Compounds 7, 11, 12 and 13 (isomers a and b respectively, 0.1 mmol) were treated with 80% acetic acid (2 mL) and the reaction mixture stirred below 10°C for 30 min. Detritylation was over within this time as monitored by TLC (solvent A). Acetic acid was completely removed by coevaporation with methanol on Savant Speed Vac concentrator. The residue was dissolved in water and repeatedly washed with ether to remove 4,4'-dimethoxytritanol. The aqueous layer was lyophilized to give completely deblocked dimers as white powder.

Compound	Isolated % Yield	R_f (B)	FABMS$[M-H]^-$
Tp(Ph)T (14a)	96	0.53	605
Tp(Ph)T (14b)	94	0.52	605
Tp(Ph)C (15a)	97	0.14	590
Tp(Ph)C (15b)	95	0.13	590
Tp(Ph)A (16a)	95	0.34	614
Tp(Ph)A (16b)	95	0.33	614
Tp(Ph)G (17a)	92	0.11	630
Tp(Ph)G (17b)	93	0.11	630

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