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SEPARATION OF DIASTEREOMERS OF METHYLPHOSPHONATE DINUCLEOTIDES

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Abstract: A chiral derivatizing agent, l-menthyl chloroformate, has been used as a 3'-OH blocking group to facilitate the resolution of diastereomers of methylphosphonate dinucleotides by silica gel column chromatography.

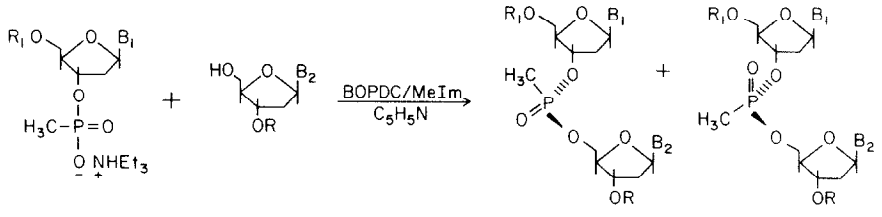
We have been interested in the synthesis of oligonucleotide analogs for the purpose of probing DNA-protein interactions[1]. One interesting class of analogs are the methylphosphonates, wherein the phosphodiester moiety is replaced by the non-ionic methylphosphonyl group[2]. Miller and coworkers[3,4] have used these analogs as models to study the conformation and biological function of nucleic acids. More recently, methylphosphonate analogs have been used as probes for DNA-protein contact points[5] and in the study of B-Z DNA transitions[6].

The introduction of a methyl group creates chirality at phosphorus, since the methyl group may occupy either a pseudoaxial, or a pseudoequatorial position, corresponding to the S or the R configuration. Synthesis of methylphosphonates is hampered by their low reactivity towards coupling reagents. In addition, there are no coupling reagents that would bring about stereospecific methylphosphonate bond formation, and hence the diastereomers must be separated after each coupling reaction. Agarwal and Riftina[2] and Miller et al.[3] have developed methods for the synthesis of methylphosphonate oligonucleotides, using acetate as a 3'-OH protecting group, but the method gave low yields, and not all diastereomers were easily separable. Recently, we have reported the use of N,N-bis(2-oxo-3-oxazolidinyl) phosphorodiamidic chloride (BOPDC)[7] as a coupling reagent in the synthesis of oligonucleotides by the triester method[8]. The excellent yields obtained with this reagent suggested to us that it might also be useful in the synthesis of methylphosphonate-containing oligonucleotides. Indeed, with this coupling agent we were able to synthesize a few methylphosphonate dinucleotides with yields of 65 to 70%.

A method was still needed to facilitate the separation of the diastereomers; we therefore explored the use of a chiral derivatizing agent in conjunction with BOPDC. Our choice of l-menthyl chloroformate was based on the following considerations:

a) the l-menthyl group has been used with excellent results for the resolution of optical isomers[9,10]; b) the alkyl group is fairly hydrophobic, and its presence should interfere differentially with the binding of the oligonucleotide to silica gel; c) l-menthyl chloroformate is commercially availat'e, and d) the chloroformate reacts readily with sugar hydroxy groups, yielding stable carbonate derivatives. The use of this reagent for the separation of eight diastereomeric pairs of dinucleotides is described below.

The protection of the 3'-OH was performed using Schotten-Baumann conditions: The 5'-OH and amino-protected nucleoside (0.5mmol) and dimethyl-aminopyridine (1mmol) were dissolved in dry methylene chloride (10ml). l-menthyl chloroformate (1.0mmol) was added to the solution, and the reaction mixture was stirred at R.T. overnight. After neutralization with 5% aqueous bicarbonate, the product was extracted into chloroform, and the organic layer was washed, first with 1% monobasic sodium phosphate, and then with water. After evaporation to dryness, the product was detritylated with 2% benzenesulfonic acid in methanol:chloroform (3:7 v/v). The final product was purified by chromatography using a silica gel column and a chloroform-methanol gradient as the eluent. All of the 3'-OH derivatized nucleosides were obtained in 75 to 85% overall yield. The products were judged pure by silica gel TLC (10% methanol/methylene chloride) and by RP-18 TLC (acetone/water 7:3 v/v). A peak of 1720cm^{-1} in the IR spectrum was assigned to the -O-CO-O- group.



1 2 3a 3b

R = l-menthoxycarbonate
R_1 = dimethoxytrityl
B_1 = T, C^{Bz} ; B_2 = T, C^{Bz}, A^{Bz}, G^{ib}

3a S
3b R

The coupling reactions were done according to the reported procedure[8]. The triethylammonium salt 1 was coupled with the 5'-OH of the amino- and 3'-OH-protected nucleoside 2, in the presence of BOPDC. The reaction mixture was stirred at 37°C for three hours, after which the solvent was removed under high vacuum. The residue was dissolved in methylene chloride and extracted with 5% aqueous sodium bicarbonate. The organic layer was washed with water and evaporated to dryness. The pairs of diastereomers (3a and 3b) were separated by column chromatography using

silica gel and a gradient of acetone in methylene chloride. The eluent fractions were analyzed by TLC on silica gel, using acetone/methylene chloride (3:7 v/v for T-T, T-C and T-G, and 1:1 v/v for the other dimeric pairs). The relative yields of the fast and slow components vary between 1.5 and 1, depending on the dinucleotide.

The method described above was applied to the synthesis and separation of eight diastereomeric pairs, listed in Table 1. In six cases complete separation of the diastereomers was achieved. Only the T-C and T-G pairs failed to give baseline resolution, although the separation was sufficient to give pure diastereomers. Space-filling models indicate that for any given conformation of the dinucleotide, the menthyl group can come into direct contact with the methyl group in one isomer, but not in the other. This may be a factor in the enhanced separation obtained using a 3' menthyl group.

Table 1

Dinucleotide	R_f value		Dinucleotide	R_f value	
DMT-TpT-R	0.64	0.52	DMT-CpT-R	0.60	0.41
DMT-TpA-R	0.44	0.34	DMT-CpA-R	0.40	0.30
DMT-TpC-R	0.69	0.63	DMT-CpC-R	0.68	0.56
DMT-TpG-R	0.31	0.27	DMT-CpG-R	0.43	0.25

Abbreviations: DMT = 4,4'-dimethoxytrityl; T = 2'-deoxythymidine; C = 4-N-benzoyl-2'-deoxycytidine; G = 2-N-isobutyryl-2'-deoxyguanosine; A = 6-N-benzoyl-2'-deoxyadenosine; R = menthoxycarbonate; p = methylphosphonate.

Removal of the menthyl group, without cleaving the base protecting group, is difficult but can be accomplished as described below. Under these conditions, the methylphosphonyl linkage was fairly stable with less than 5% hydrolyzed after prolonged treatment (2hrs). Good yields of pure 3' deblocked diastereomeric pairs were isolated, and NMR spectra showed that both the base-protecting groups and the methylphosphonate linkage remained intact.

A typical deblocking reaction is as follows: To a precooled solution of fully blocked dimer (0.12mmol), in 1ml of methanol/pyridine (1:1 v/v), we added 0.4ml of 2N aqueous NaOH, and the reaction mixture was stirred at 4°C. Progress of the reaction was monitored by periodic withdrawal of aliquots and analysis by silica gel TLC, developed in methanol/methylene chloride (1:9 v/v) solvent. Deblocking is usually complete in 20 to 40 min. Methylene chloride was then added to the mixture,

and the organic layer washed with water. The solvent was evaporated, and the residue chromatographed on a silica gel column, using a gradient of methanol in methylene chloride. Appropriate fractions were pooled, and the dinucleotide precipitated by adding the solution into petroleum ether. Yields of the isolated dimers were generally about 60%. The dimers migrated as a single spot, both on silica gel and on RP-18 TLC. Stereochemical purity of the products was judged by NMR spectroscopy. In the spectra of all dimers, the PCH_3 resonances appear as a doublet between 1.45 and 1.57ppm[11], characteristic of two-bond hetero coupling between P and H, with a coupling constant of J_{PCH3} = 17.0 Hz[12]. There is a small (up to 0.07ppm) difference in the chemical shifts between the methyl resonances of the two diastereomers.

In summary, we have shown that methylphosphonate dinucleotide stereoisomers can be separated by using a 3' menthyl protecting group. Currently we are developing methods to determine the absolute configuration of the methylphosphonate linkage. The synthetic strategy could be extended to tetramers or hexamers with either all S or all R stereochemistry at the phosphonate linkage. We have already synthesized by this method a few hexadecamers with methylphosphonates at defined positions[5]. Availability of such oligonucleotide analogs provides an opportunity to study nucleic acid structure and protein-nucleic acid interactions.

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