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Short Communication

Normal-phase high-performance liquid chromatographic resolution of 5′-O-protected deoxynucleoside methylphosphonamidites

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Abstract

High-performance liquid chromatography has been used for the analytical and preparative resolution of some nucleoside methylphosphonamidites. The pure diastereomers were required for a study on the mechanism of the coupling reactions of these compounds.

1. Introduction

The use of linkage-modified oligonucleotide analogues as agents for the antisense control of gene expression has received considerable attention recently [1], and many phosphorus-modified and dephospho internucleotide linkages have been examined. One of the more intensively studied classes of analogues is the oligonucleoside methylphosphonates. These appear to show considerable promise as antisense agents. Unfortunately, substitution of methyl for a non-bridging oxygen creates a new asymmetric centre at phosphorus. Considerable experimental [2] and theoretical [3] evidence indicates that Rp-configured oligomers are much more effective binders than their Sp-configured counterparts. It would therefore be useful to have access to a synthetic route which permits the stereospecific synthesis of these important molecules. Leznikowski *et al.* [2] have developed and we have modified [4] such a route, which is based on the displacement of an alkoxide from a resolved nucleoside methylphosphonate. It requires, however, the use of a nucleoside alkoxide, and as such, is not amenable to automation. In addition, coupling yields are too low for the efficient preparation of oligomers more than perhaps eight units in length. We are interested in the use of the more reactive nucleoside phosphonamidites in a stereospecific synthesis. In particular, we are exploring the use of resolved phosphonamidites and non-nucleophilic catalysts in coupling reactions. This report describes a procedure for the resolution of these phosphonamidites.

2. Experimental

HPLC was performed on a Waters system using a 600E system controller, 484 tunable absorbance detector and 470 scanning fluorescence detector. All solvents were premixed, and

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Table 1
Analytical resolution of methylphosphonamidites **1a**

B	Flow (ml/min)	t_{R1} (min)	t_{R2} (min)	R_s
Thymidine	2.0	10.5	511.8	51.20
N^4-Benzoylcytosine	8.0	1.40	1.70	1.28
N^6-Benzoyladenine	2.0	5.77	6.61	1.03
N^2-Isobutyrylguanine	8.0	1.65	1.98	1.06

B = nucleoside heterocyclic base, protected at the exocyclic amino group where appropriate. t_{R1} and t_{R2} refer to the retention times of the first- and second-eluting peaks, respectively. 5′-OH protecting group is dimethoxytrityl. See Experimental for further details regarding column and operating conditions.

hexane–dichloromethane mixtures were sparged with helium for 10 min at 100 ml/min, to avoid excessive solvent evaporation. A Waters μPorasil (300 × 3.9 mm, 10 μm particle size, 125 Å pore size) column was used for analytical runs, and preparative chromatography was done on a larger μPorasil (300 × 19 mm 15–20 μm particle size, 125 Å pore size). Detection was achieved by monitoring absorbance at 254 nm for analytical runs, and by monitoring fluorescence at 511 nm (excitation at 254 nm) for preparative runs. The mobile phase velocity for analytical runs is given in Table 1, and was 45 ml/min for preparative runs. For preparative separations, 250 μl of a solution of **1b** (140 mg/ml, in the mobile phase) was injected. Diastereomeric purity was checked by NMR and analytical HPLC. 5′-O-Dimethoxytritylmethylphosphonamidites **1a** were purchased from ABN (Hayward, CA, USA). 5′-O-Monomethoxytritylmethylphosphonamidites were prepared in this laboratory, and used for reasons of economy. ^{31}P NMR chemical shifts are reported relative to 85% phosphoric acid.

3. Discussion

We initially tried a procedure reported by Lebedev *et al.* [5], in which was described such a resolution. In that report, an Alltech 2.25 RSil 10-μm silica gel HPLC column was treated with a solution of triethylamine (1%) in chloroform–ethanol (99:1). Subsequent washing of the column with an ethanol–chloroform (2.5:97.5) solution, followed by the loading of the phosphonamidites and elution with ethanol–chloroform (1:99) reportedly gave resolution, with quantitative recovery, on a preparative scale.

In our initial attempts to reproduce this separation, we achieved excellent resolution on an analytical scale, using a Waters μPorasil analytical column. Unfortunately, this initial success was short-lived, as we noted rapid degradation of column performance after only a few injections. Plate count dropped from *ca.* 6000 to *ca.* 3000, with a corresponding decrease in resolution. The manufacturers of the column indicated that treatment of the stationary phases with triethylamine could lead to such a degradation in column performance. Although partial recovery of column performance was possible by passing a stream of helium through the warm (60°C) column, we were not satisfied. We desired a procedure which would reliably resolve the compounds without risk of degradation of the expensive columns.

We therefore decided to try other methods for this resolution. Preliminary experiments to perform the separation on a reversed-phase column (Waters C-18 μNovapak radial-pak cartridge) failed, although it has been reported that a similar system can be used to resolve a nucleoside 5′-O-dimethoxytritylmethoxyphosphoramidite [6]. Returning to the solvent system reported by Lebedev [5], we observed that, in the absence of triethylamine pretreatment, unresolved diastereomers were recovered in quantitative yield, with very short retention times (<1 min). Stengele and Pfleiderer [7] have reported the resolution of cyanoethylphosphoramidite diastereomers without the use of base pretreatment.

Lowering solvent polarity (1% to 0% ethanol in dichloromethane, at a constant mobile phase velocity of 1.00 ml/min) led to an increase in retention times, but no resolution. Varying mobile phase velocity for a given composition did not improve the situation. This increased retention times, but significant band broadening also occurred.

As a result, solvent composition was altered to improve both resolution of the diastereomers and to prevent column degradation. Using a mobile phase composed of hexane–dichloromethane (60:40), we achieved a small degree of resolution, but with unacceptably long retention times (>30 min). This led to poor recovery of the amidites, apparently due to degradation on the column. Addition of absolute ethanol, and further refinement of the system, led to a solvent composition of hexane–dichloromethane–ethanol (59.7:39.8:0.5). This mobile phase gave excellent resolution with reasonably short retention times (Table 1). Purine phosphonamidites gave noticeably poorer resolution than did their pyrimidine counterparts. This seems largely due to peak tailing and was also noted by Lebedev, in the case of guanosine phosphonamidites. Guanosine methylphosphonamidites are only poorly soluble in this mobile phase.

Preparative HPLC used the stationary and mobile phases described above. For preparative purposes, we used 5′-O-monomethoxytrityl-protected phosphonamidites. Injecting about 35 mg per run, we were able to achieve partial resolution of the diastereomers. Fluorescence detection was used to monitor the eluate. Although baseline resolution was not achieved, we were able to isolate diastereomerically pure material by collecting three fractions: baseline to apex, apex to apex, and apex to baseline. The first and last fractions contained pure material, while the middle peak was composed of unresolved isomers. Evaporation of the solvent gave the pure methyl phosphonamidites as white powders. Yields and other data are given in Table 2. Representative analytical chromatograms and ^{31}P NMR spectra are shown in Figs. 1 and 2. A representative preparative chromatogram is given in Fig. 3. We were unable to isolate significant amounts of diastereomerically pure 2′-deoxyguanosine methylphosphonamidites on a preparative scale. Variation of flow-rate and ethanol concentration failed to give preparative resolution, apparently because of extensive peak tailing.

In conclusion, we have developed a system for the resolution of protected nucleoside methylphosphonamidites which allows for reasonably straightforward isolation of the diastereomers. The present method avoids damage to the stationary phases of the columns, and the desired compounds are recovered as powders, avoiding the need for precipitation as a last step.

4. Acknowledgements

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Table 2
Preparative resolution of methylphosphonamidites **1b**

B	t_R (min)	^{31}P NMR (C^2H_3CN)	Recovery (%)	Total recovery (%)
Thymidine	9.571	20.92	32.0	84
	11.42	120.52	28.6	
N^4-Benzoylcytosine	9.13	123.27	33.7	86
	10.64	121.02	31.8	
N^6-Benzoyladenine	5.89	121.38	28.8	94
	7.64	119.96	28.8	

t_R = retention time, recovery refers to percentage of injected material recovered stereochemically pure, total recovery refers to percentage of injected material recovered, including unresolved diastereomers. Flow-rate was 45 ml/min. 5′-OH protecting group is monomethoxytrityl. See Experimental for further details on column and operating conditions.

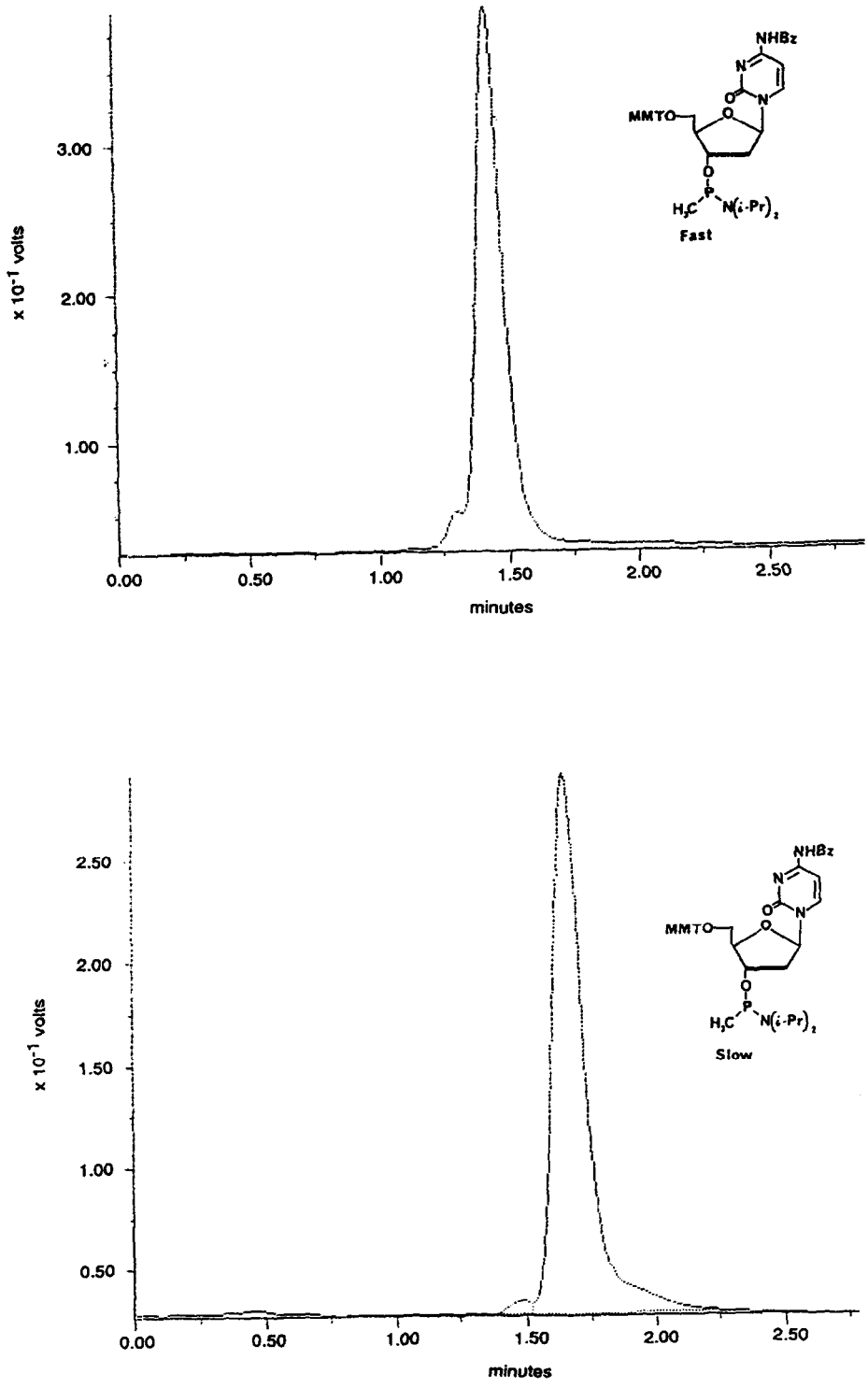

Fig. 1. Analytical chromatograms of resolved phosphonamidites.

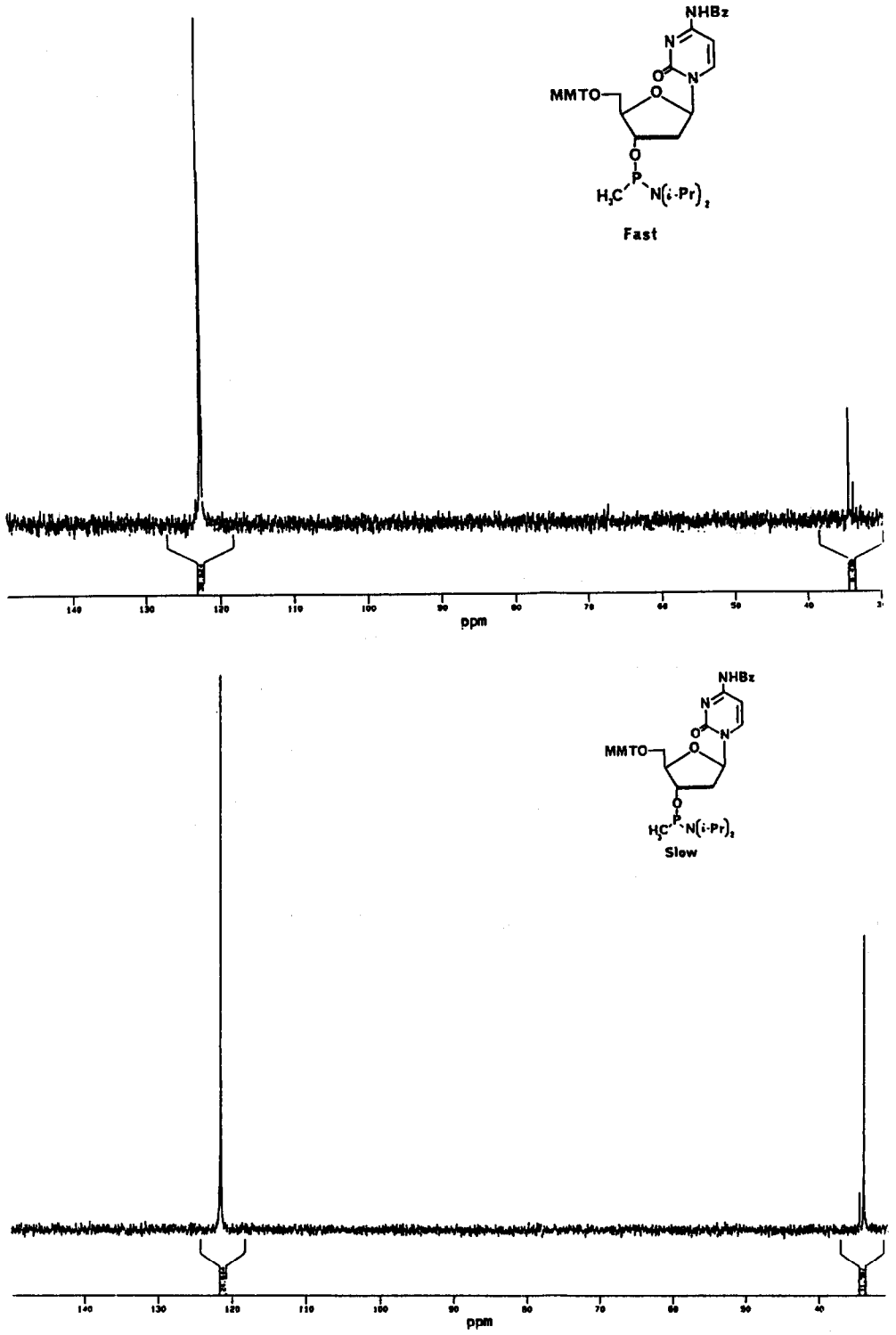

Fig. 2. ^{31}P NMR spectra of resolved phosphonamidites.

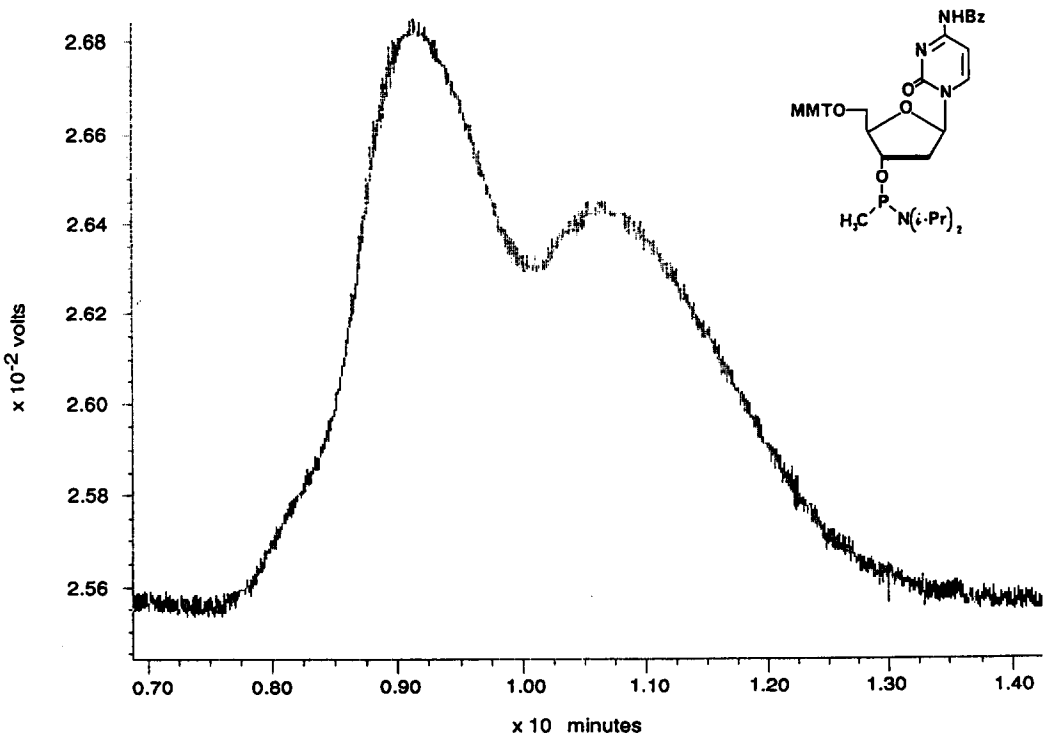

Fig. 3. Preparative chromatogram of N-benzoylcytidine phosphonamidite.

5. References

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