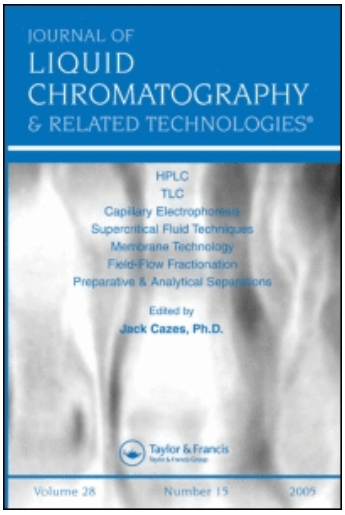

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CHROMATOGRAPHIC PURIFICATION OF NON-IONIC METHYLPHOSPHONATE OLIGODEOXYRIBONUCLEOSIDES

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ABSTRACT

A procedure is described for the purification of synthetic methylphosphonate oligodeoxyribonucleosides. Syntheses were done on an automated instrument with the trityl group on in the last cycle. The oligomers were manually cleaved from the solid support and purified by reverse-phase HPLC in order to remove shorter fragments produced during incomplete couplings. After detritylation, the oligomers were repurified by reverse-phase HPLC. Typically, an overall yield of 25% or more was obtained.

INTRODUCTION

Methylphosphonate oligodeoxyribonucleosides (MP-DNA) are chemically modified strands of DNA in

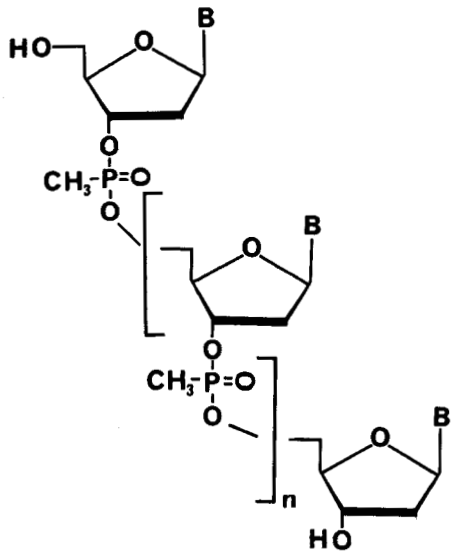


Figure 1. Structural representation of MP-DNA.

which the internucleoside linkages are non-ionic (figure 1). This type of DNA analog was introduced by Miller, Ts'o and colleagues (1-5). It has the properties of nuclease resistance and permeability through cell membranes, while still retaining sequence-specific hybridization to complementary strands of DNA. As such, MP-DNA has potential therapeutic value for translation arrest of the mRNA of viruses and other pathogens.

The monomers (methylphosphonamidites) for the automated synthesis of MP-DNA are now commercially available (6). The coupling procedure is essentially the same as for the synthesis of the

usual oligodeoxyribonucleotides. A mixed oligomer containing both phosphodiester and methylphosphonate linkages can be synthesized, but this report concerns oligomers having only non-ionic linkages. Completely non-ionic MP-DNA requires new procedures for handling and purification. The commonly used procedures for the purification of chemically synthetic DNA are gel electrophoresis, reverse-phase HPLC and anion-exchange HPLC. Anion-exchange chromatography and the usual gel electrophoresis procedures are obviously not applicable. Although the addition of sodium dodecyl sulfate to the polyacrylamide gel does allow migration, the use of reverse-phase HPLC was found to be the most useful technique for purifying MP-DNA. This procedure is herein presented.

MATERIALS AND METHODS

Reagents:

Methylphosphonamidite monomers ("A, T, C and G"), as well as coupling reagents were purchased from Applied Biosystems, Inc. (Foster City, CA). Anhydrous dimethyl formamide (DMF) and ethylene diamine were obtained from Aldrich Chemicals, Inc. (Milwaukee, WI). Anhydrous (200 proof) ethanol was

from Pharmaco (Bayonne, NJ). The DMF was dried over calcium hydride prior to use as a diluent for the monomers. HPLC grade acetonitrile, water, triethylamine and acetic acid were used. "T" and "A" methylphosphonamidites (0.25 g) were each dissolved in 3.5 ml of anhydrous acetonitrile (Applied Biosystems, Inc.), whereas "C" and "G" were each dissolved in 1 ml of anhydrous DMF followed by 2 ml of anhydrous acetonitrile.

Equipment:

Synthesis was done on an Applied Biosystems Model 380A-updated instrument. The chromatography system was a Varian Model 5000 instrument with a Model 9060 photodiode array detector.

Synthesis:

Results obtained with two different MP-DNA's are described in this report. One is a 24-mer consisting only of "T"s, and the other is the 24-mer "5'-CTCCATTTCTTGCTCTCCTCTGTC-3'." The dimethoxytrityl (DMT) blocking group was left on after the last cycle of synthesis. Rather than automatically releasing the completed MP-DNA from the solid support with ammonia, the support was removed and incubated at 55^{o} C for 1 hour in 400μl

of anhydrous ethanol/ethylene diamine (1:1). This process cleaves the oligomer from the support and deblocks the protecting groups from the bases, while causing minimal hydrolysis of the alkali-sensitive methylphosphonate bonds. The supernatant was collected and the support was extracted twice with 400μl aliquots of anhydrous ethanol. The fractions were pooled and evaporated to a sticky solid under vacuum. The crude MP-DNA was dissolved in 1 ml of DMF/mobile phase A (1:1) (see below) for chromatographic purification. Aliquots were diluted into the same solution for measurement of the absorbance at 260 nm. The detritylation procedure is described in the Results section.

Chromatography:

A Bakerbond (Baker, Inc., Phillipsburg, NJ) reverse-phase (C18) column (3μm, 120 A, 4.6 X 50 mm) was eluted at a flow rate of 1 ml/min at room temperature. Mobile phase A was 5% acetonitrile/95% 0.1 M triethylammonium acetate, pH 7.0 and mobile phase B was 95% acetonitrile/5% 0.1 M triethylammonium acetate, pH 7.0. Chromatography was carried out with the following gradient: 10 min isocratically with mobile phase A followed by a linear gradient of 2%/min increase of mobile phase B for 50 min. The detector output at 258 nm was recorded.

RESULTS AND DISCUSSION

Synthesis:

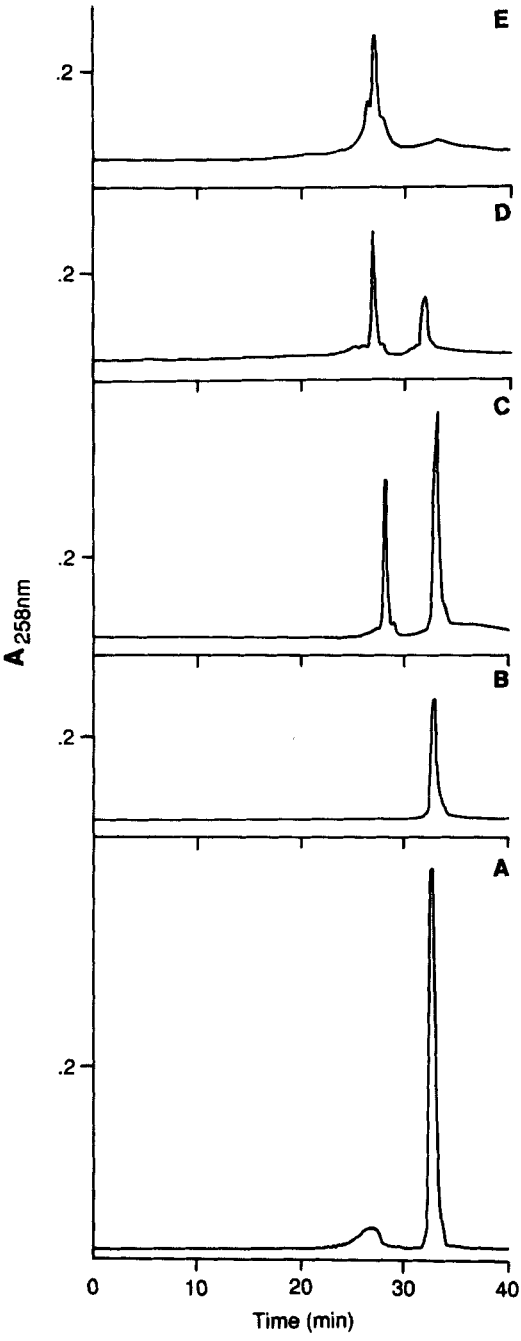
According to the trityl cation assay, the coupling at each cycle (generally in the range of 95% to 99%) appeared to procede less efficiently than for the usual oligodeoxyribonucleotide synthesis. It should be cautioned that without drying the DMF over calcium hydride, the monomers are unstable and synthesis becomes even less efficient. The DMT group was left on at the 5'-terminus after the final cycle to aid in the reverse-phase purification. The rationale is that all the shorter oligomers resulting from incomplete coupling at each cycle are automatically capped by acetylation of the 5'-terminus in the instrument. The exceptional hydrophobicity of the trityl group causes the completed oligomer to elute much later than all the non-tritylated oligomers. Even though the MP-DNA is considerably less polar than the usual DNA, the trityl group was found to still produce a significant shift in the elution position (see below).

Detritylation and Chromatographic Purification Steps:

Chromatographic analysis of the crude products (figs. 2A and 3A) revealed a major peak

supposedly representing the tritylated oligomer and an earlier-eluting minor peak representing shorter (mostly n-1) oligomers. In the case of the mixed-base 24-mer, the failed sequences represent about one-third of the total (figure 3A), whereas they represent a smaller fraction for the 24-mer of only "T." The tritylated forms were collected and subjected to detritylation.

In the automated instrument, detritylation is accomplished by a short pulse (2 min) of 3% trichloroacteic acid in methylene chloride, whereas manual detritylation is often done in 80% acetic acid for 1 hour. Figures 2 and 3 illustrate how reverse-phase HPLC may be used to monitor the progress of detritylation. The use of 80% acetic acid was not sufficient to effect detritylation (figure 2B). This failure was most likely due to a combination of insolubility of the non-polar MP-DNA, as well as insufficient acid strength, since the combination of DMF and 80% acetic acid (1:1) did produce partial detritylation (figures 2C and 2D). However, complete detritylation was achieved by treatment with 3% trichloroacetic acid in methylene chloride for 2 min at room temp. The favored detritylation procedure was found to be suspending the dried crude oligomer in 200 μl of 3% trichloroacetic acid in methylene chloride and vigorously mixing for 2 min. This is followed by the addition of 2 ml of diethyl ether and 1 ml of water. The upper organic phase is discarded and the lower phase is taken to dryness, dissolved in



DMF/mobile phase A and rechromatographed. Further research on the detritylation process, including monitoring for depurination, is in progress.

This purification/detritylation process is shown again for the mixed-base 24-mer MP-DNA in figure 3. Rechromatography of the major peak shown in Panel A demonstrates the removal of incompletely synthesized (mostly n-1) oligomers (Panel B). When this material is treated under the optimal conditions, essentially complete detritylation is achieved (Panel C). This material then constitutes the purified MP-DNA.

It should be noted that a jagged peak is obtained for each purified MP-DNA (figures 2E and 3C). One possible explanation is chemical damage

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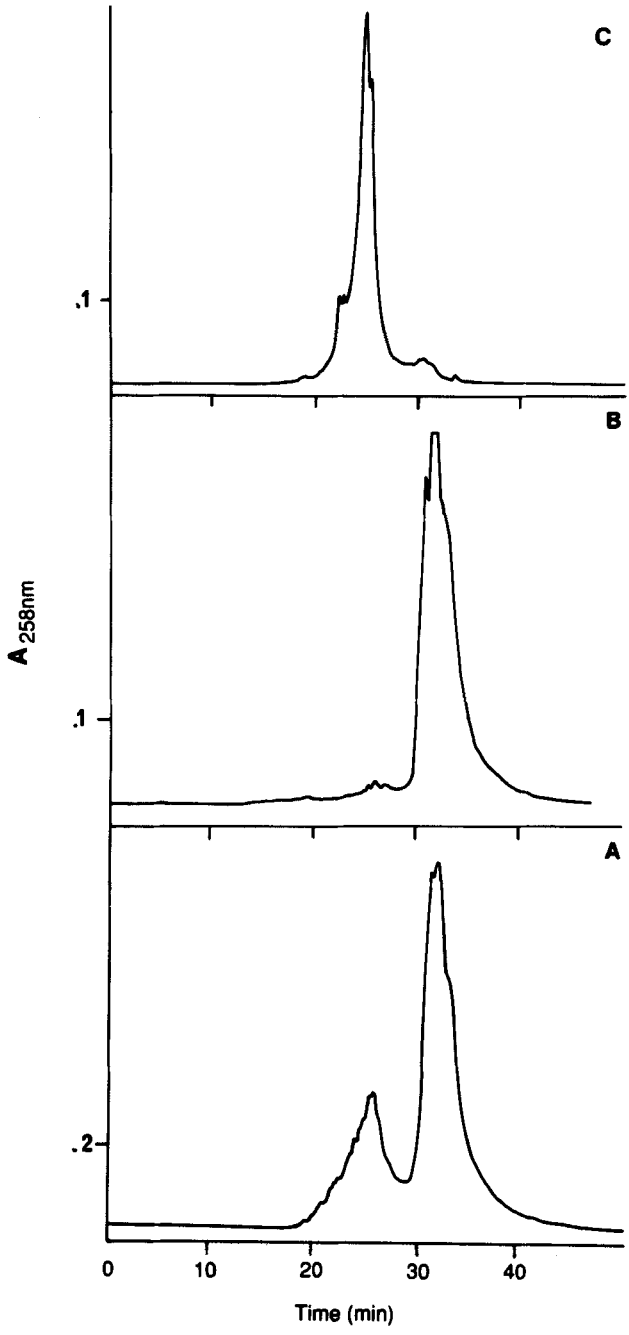
Figure 2. Chromatographic purification of a 24-mer MP-DNA consisting of only "T." Reverse-phase chromatography of the crude product, after cleavage from the solid support and base deprotection, is shown in Panel A. Fractions corresponding to the major peak, representing the tritylated oligomer, were pooled, dried and treated with 80% acetic acid for 1 hour at room temp. Chromatography (Panel B) indicated the failure of detritylation. The peak from Panel B was dried and further treated with DMF/80% acetic acid (1:1) for 1 hour and rechromatographed (Panel C). Less than half of the material had been detritylated. The two peaks were pooled, dried and treated with DMF/80% acetic acid for an additional hour. Rechromatography (Panel D) indicated that a majority of the oligomer had been detritylated. The two peaks were pooled, dried and treated with 3% trichloroacetic acid in methylene chloride, as described in the text. Rechromatography (Panel E) indicated nearly complete detritylation.



to the MP-DNA, such as depurination, from the strong acid during the detritylation step. This, of course, is not possible for the MP-DNA having only the pyrimidine "T." Another explanation is the partial resolution of the stereoisomers, which result from the chiral center at each internucleoside linkage (figure 1); there are 2^{23} stereoisomers for a 24-mer.

Yield:

The chromatographic runs of the crude products (figures 2A and 3A) illustrate how well the couplings proceeded. In the case of the 24-mer with only "T"'s (figure 2A), the relative amount of incomplete oligomers was low, in agreement with the trityl cation assay that indicated a coupling efficiency of 99% per cycle. In the case of the mixed-base 24-mer (figure 3A), incomplete

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Figure 3. Chromatographic purification of a mixed-base 24-mer MP-DNA. Reverse-phase chromatography of the crude product, after cleavage from the solid support and base deprotection, is shown in Panel A. The major peak represents tritylated oligomer, while the minor peak corresponds to non-tritylated, shorter (n-1) oligomers. Rechromatography of the major peak is shown in Panel B. This rechromatographed material was dried and detritylated with 3% trichloroactic acid in methylene chloride. Rechromatography (Panel C) demonstrated that detritylation had gone essentially to completion.

oligomers represented about one-third of the crude product, in agreement with the trityl cation assay that indicated coupling yields of some cycles as low as 95%.

The chromatographic recovery of either a tritylated or detritylated MP-DNA was determined using injections of 1 and 10 A_{260} units. Recoveries were typically above 50%. The overall yield of the desired MP-DNA, as determined by A_{260} units (and after correction for the content of incomplete sequences) was typically about 25%. Thus, losses occurred during both the chromatographic and intermediate handling steps. From our experience with other strands of MP-DNA, not presented in this report, it should be possible to routinely achieve higher overall yields. These findings may be compared with the results obtained by Miller, et al. (3) using a similar procedure, upon which our own approach is based. That report, however, is not directly comparable, since they worked with shorter (less than 10-mers) sequences of MP-DNA.

Conclusion:

Since the therapeutic utility of these backbone-modified oligodeoxyribonucleotides has yet to be evaluated, it is possible that completely non-ionic forms of lengths around 20 bases may be required. Accordingly, the most

facile procedures for purification and handling of these substances must be developed. This report addresses these fundamental issues.

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