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Note

Reversed-phase high-performance liquid chromatography of protected oligodeoxynucleotides

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Reversed-phase high-performance liquid chromatography (HPLC) has proved to be a valuable tool[1] in the preparation and analysis of both synthetic and natural oligonucleotides. The majority of reports in this area have concerned data on deprotected oligomers and not much information has been revealed on the protected materials[2].

We report here our studies on such molecules and the effect of various protecting groups.

EXPERIMENTAL

A Waters ALC202 HPLC instrument comprising two Model 6000A pumps linked to a Model 660 solvent programmer was used exclusively. Injection of 2–10 μl of a freshly prepared methanol–chloroform solution of the oligomer onto the Waters μBondapak C_{18} column was via a septumless Valco 7000 loop injector. Detection at 260 nm was via a Tracor 970A variable wavelength spectrometer onto a Texas Instrument flat bed x–y recorder. A linear gradient of 25–100% (30 min, 2 ml/min) of acetonitrile in aqueous 0.1 M ammonium acetate (pH 6.8) was used. The aqueous solutions were made up with Baker HPLC grade water and degassed twice by filtration through a Millipore 0.45-μm filter.

RESULTS AND DISCUSSION

During our synthetic studies on the synthesis of 18-mers of D- and L-deoxyuridine[3] via the phosphotriester procedure, it was found that the smaller protected oligomers had reasonable mobility and distinguishable R_F values on conventional silica gel thin-layer chromatography plates and columns. This technique became less satisfactory for units much above a hexanucleotide in length, and it was found[3] that reversed-phase plates and columns were a satisfactory alternative.

The reversed-phase aspect was examined closer and a detailed study was conducted on all the intermediates involved in our synthetic sequences.

We utilized a Waters μBondapak C_{18} HPLC column pioneered by Fritz *et al.*[4] for partially protected oligonucleotides (containing phosphodiester linkages).

The oligomers employed in the phophotriester synthesis[3] of the 18-mers could be divided into the four categories represented in Table I.

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TABLE I

RETENTION TIMES OF PROTECTED OLIGODEOXYNUCLEOTIDES

DMT = 5′-Dimethoxytrityl; p = terminal 3′-phosphate; Ar = *p*-chlorophenyl; ce = cyanoethyl; Ac = terminal 3′-acetyl; $[dU]_n$ = oligonucleotide chain with 3′ → 5′ phosphotriester linkages; [dU] = 2′-deoxyuridine.

n	*Retention time (min)*			
	DMTO[dU]$_n$p(OAr)-(Oce)	*HO[dU]$_n$p(OAr)-(Oce)*	*DMTO[dU]$_n$(OAc)*	*HO[dU]$_n$(OAc)*
1	18.1	6.7		1.8
2	18.4			
3	18.7	11.9	17.0	9.8
6	20.7		19.3	13.8
9			20.7	
12			21.7	18.2
18			23.3	20.1

The fully protected oligomers, possessing a 5′-dimethoxytrityl (DMT) group and a terminal 3′-phosphotriester, have moderate retention times which do not increase tremendously on going from the mononucleotide (n = 1, 18.1 min) to the hexanucleotide (n = 6, 20.7 min). The lipophilic DMT group and the terminal phosphotriester evidently have a much greater influence upon the retention on the column than does the overall length of the oligomer chain. The peaks showed multiplicity due to phosphorus diastereomers, which was very evident with the mononucleotide (Fig. 1) and more complex with the hexanucleotide (Fig. 2). (The retention time given in Table I is the average of the multiplicity of the peaks.) In fact, with the monomer DMTO[dU]p(OAr)(Oce), the two stereoisomers were almost fully resolved. This sep-

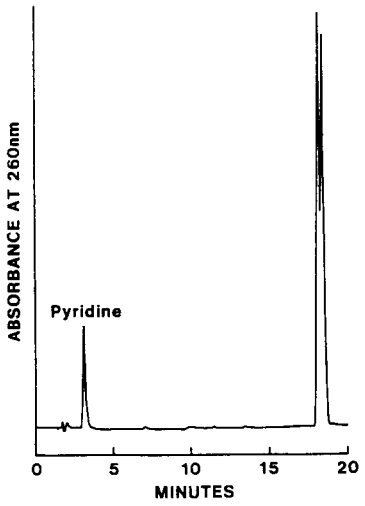

Fig. 1. μBondapak C_{18} reversed-phase HPLC profile of DMTO[dU]p(OAr)(Oce) using a linear gradient of 25–100% acetonitrile in 0.1 *M* ammonium acetate (30 min at 2 ml/min).

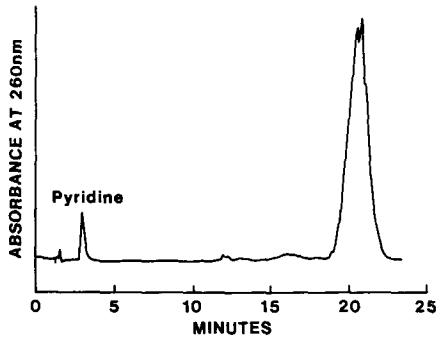

Fig. 2. μBondapak C_{18} reversed-phase HPLC profile of DMTO[dU]$_6$p(OAr)(Oce). Conditions as for Fig. 1.

aration could nearly be achieved on silica gel and some fractionation occurred during column chromatography. Separation and assignment of configuration of the stereoisomers of a monophosphotriester of thymidine has recently been achieved[5].

Removal of the DMT group, as shown in Table I, dramatically reduced the retention times as anticipated (Fig. 3). The multiplicity was still evident, but not quite as pronounced.

The majority of the oligomers prepared in our synthesis possessed a 5′-DMT and a 3′-OAc. The retention times of these materials may be compared to those having a terminal 3′-phosphotriester, the only structural difference being the group on the 3′-end. The corresponding oligomers having the 3′-OAc are eluted slightly ahead of those with the 3′-phosphotriester. The extra lipophilicity of the phosphate *p*-chlorophenyl and cyanoethyl groups is sufficient to effect this difference. Multiplicity of peaks in the smaller oligomers was again evident. The trimer has already been presented[3] and the hexamer (Fig. 4) and the octadecamer (Fig. 5) are shown here.

When the 5′-DMT group was removed, there was a dramatic reduction in

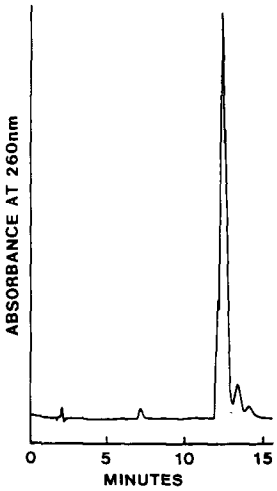

Fig. 3. μBondapak C_{18} reversed-phase HPLC profile of HO[dU]$_3$p(OAr)(Oce). Conditions as for Fig. 1.

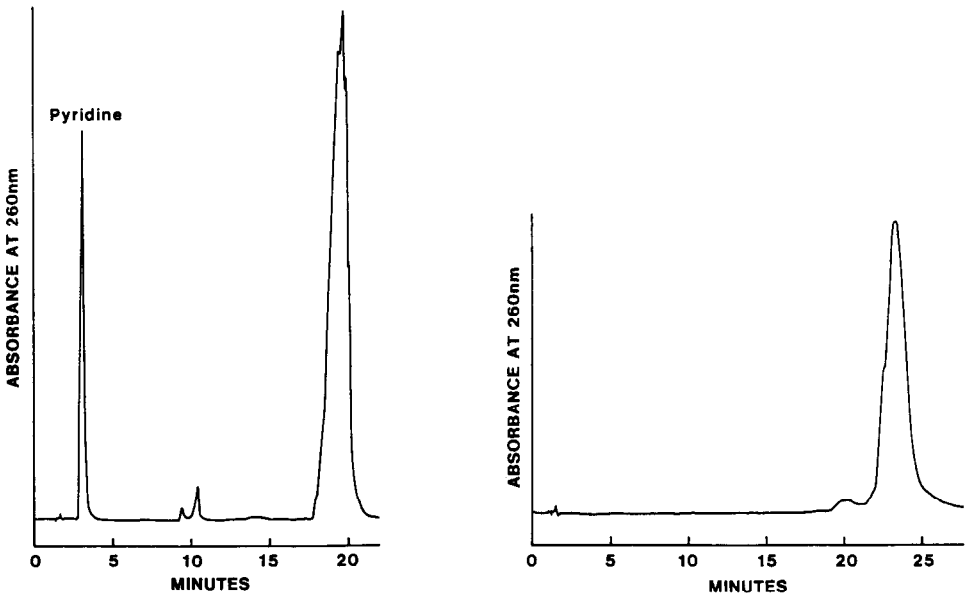

Fig. 4. μBondapak C_{18} reversed-phase HPLC profile of DMTO[dU]$_6$OAc. Conditions as for Fig. 1.

Fig. 5. μBondapak C_{18} reversed-phase HPLC profile of DMTO[dU]$_{18}$OAc. Conditions as for Fig. 1.

retention times, especially for the smaller units (Table I). As the oligomer size increased up to the 18-mer, this difference became less striking. The utility of the HPLC separation of DMT-containing oligomers *versus* those with a 5′-OH was put to good use in the final coupling reaction of our synthesis:

$$\underset{(1)}{\text{DMTO[dU]}_6\text{p(OAr)(Oce)}} + \underset{(2)}{\text{HO[dU]}_{12}\text{OAc}} \rightarrow \underset{(3)}{\text{DMTO[dU]}_{18}\text{OAc}}$$

During the coupling, the hexanucleotide (1) is converted to a phosphodiester (by removal of the cyanoethyl group) and as a consequence does not have any mobility on silica gel plates. However, both the reactant dodecamer (2) and the product octadecamer (3) had the same mobility. However, in the HPLC separation their retention times were 5 min apart. Hence, the reaction could be easily monitored and the yield of (3) optimized. Final preparative separation of (3) from unreacted (2) was achieved on a gravity RP-2 column[3].

We have extended our own work[3] with reversed-phase HPLC to the deprotection, purification, and enzymatic degradation of oligonucleotides.

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