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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF DIASTEREOMERIC PHOSPHOROTHIOATE ANALOGUES OF OLIGODEOXYRIBONUCLEOTIDES AND OTHER BACKBONE-MODIFIED CONGENERS OF DNA

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SUMMARY

Diastereomeric phosphorothioate analogues of oligodeoxyribonucleotides, which were synthesized by an automated, solid-phase, phosphoramidite-coupling method, were conveniently separated by reversed-phase high-performance liquid chromatography on a μBondapak C_{18} column with a gradient of acetonitrile in triethylammonium acetate buffer. These synthetic and chromatographic methods were also used to obtain diastereomerically pure bis-phosphorothioate, alkanephosphonate, and O-alkyl phosphotriester analogues of DNA for exploratory studies of stereochemistry and phosphorolytic enzymes.

INTRODUCTION

The availability of improved chemical methods for the automated synthesis of oligodeoxyribonucleotides, which are now widely used in physicochemical and molecular biological studies[1], led us to investigate new synthetic procedures for conveniently obtaining backbone-modified analogues of DNA. Such analogues are useful for elucidating the interactions of DNA with proteins/enzymes[2–4], and may have some clinical value as targeting/therapeutic agents[5]. Among the methods for purifying oligodeoxyribonucleotides[1], anion-exchange and reversed-phase high-performance liquid chromatography (HPLC) have become increasingly popular, and we anticipated that the reversed-phase HPLC approach would allow for both the purification of backbone-modified analogues of DNA and the separation of the diastereomeric forms of these products, which result from the "local chirality" at each asymmetrically substituted internucleotide phosphorus center. Prior to the investigations de-

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scribed in this report, the synthesis and separation of diastereomeric backbone-modified DNA fragments had been limited to mostly dinucleotides having either a phosphorothioate[2], methanephosphonate[6–8], phosphotriester[9], or phosphoramidate[10] linkage. The focus here on phosphorothioate analogues of DNA was stimulated, in part, by pioneering studies of Eckstein[2], Bryant and Benkovic[11] and Brody and Frey[12], who found that nucleolytic enzymes catalyze the hydrolysis of phosphorothioate linkages in a stereoselective manner, which indicated that phosphorothioate analogues of oligodeoxyribonucleotides were useful stereochemical probes for the mechanisms of enzyme-catalyzed hydrolysis of DNA.

EXPERIMENTAL*

Automated synthesis of backbone-modified analogues of oligodeoxyribonucleotides

5'-Dimethoxytrityl-3'-methoxy-N,N-diisopropylaminophosphine deoxynucleosides[13] having either N-benzoyl (for A and C) or N-isobutyryl (for G) blocking groups were used as received (Applied Biosystems, Foster City, CA, U.S.A.). The corresponding O-isopropylphosphomorpholidite reagents were synthesized by B. Uznański according to ref. 14. "Long-chain alkylamine"-type CPG[15], having 20–40 μmol/g of 3'-linked 5'-DMT deoxynucleoside (either Applied Biosystems or American BioNuclear, Emeryville, CA, U.S.A.) was used as the solid support for automated synthesis with a Model 380A DNA synthesizer (Applied Biosystems), which was equipped with three column ports for independently programmable parallel syntheses on scales of either 1 μmol or 10 μmol of support-bound nucleoside. The essential features of the chemical steps for trichloroacetic acid-catalyzed detritylation, 1H-tetrazole-catalyzed coupling of the phosphoramidite, capping of residual 5'-HO groups, oxidation of the intermediate phosphite, O-demethylation of the backbone, cleavage from the support, and deblocking of the bases have been previously described[16,17]. The 10 μmol-scale syntheses included a post-detritylation neutralization step with 2,6-lutidine[18]. Apparent coupling yields were generally >97%, based on a colorimetric assay for the DMT cation in 0.1 *M* *p*-toluenesulfonic acid in acetonitrile at 530 nm. The last coupling cycle either excluded or included detritylation to provide either 5'-DMT or 5'-HO product, respectively. Phosphorothioate linkages were introduced by reaction of the support-bound phosphite intermediate with elemental sulfur, as previously described[19]. To minimize the hydrolytic decomposition of alkanephosphonate linkages, which were introduced by reaction of the support-bound phosphite intermediate with either 5 *M* methyl iodide–acetonitrile[18] (60–100°C, 10 h) or saturated DMT chloride–acetonitrile[18] (60°C, 8 h), ethylenediamine–absolute ethanol[20] (1:1) was used for removal of the product from the solid support (25°C, 2 h) and subsequent deblocking of the bases (25°C, 7 h).

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* Abbreviations used: DMT = dimethoxytrityl; A = deoxyadenosine; G = deoxyguanosine; C = deoxycytidine; T = thymidine; CPG = controlled-pore glass; TEAA = triethylammonium acetate; SVPDE = snake venom phosphodiesterase; PS = phosphorothioate; PS (subscript) = phosphorothioate linkage; PMe and Me (subscript) = methylphosphonate linkage; DMT (subscript) = dimethoxytrityl-phosphonate linkage; POiPr (subscript) = O-isopropyl phosphotriester linkage; PF (subscript) = phosphorofluoridate; POEt (subscript) = O-ethyl phosphotriester; PO = phosphodiester linkage.

Chromatography

The HPLC system consisted of two Waters Model 6000A solvent pumps, a Model 720 gradient programmer/system controller, a Model 480 variable-wavelength UV detector (set at 254 nm), a Model 710 automatic injector, and a Waters μBondapak C_{18} column (30 cm × 7.8 mm, 10-μm packing) (Waters Assoc., Milford, MA, U.S.A.). Data were recorded and integrated with a Waters Model 730 signal recorder–integrator. Acetonitrile was HPLC grade (Burdick & Jackson, Muskegon, MI, U.S.A.). TEAA buffer (0.1 *M*, pH 7.0) was prepared from triethylamine ("Gold Label" grade, Aldrich, Milwaukee, WI, U.S.A.) and glacial acetic acid (>99.9%, "aldehyde-free", J. T. Baker, Phillipsburg, NJ, U.S.A.) with deionized water, and was vacuum-filtered before use.

Characterization of backbone-modified analogues of oligodeoxyribonucleotides

The isolated peak fractions from the reversed-phase HPLC column were partially concentrated under a stream of nitrogen to remove acetonitrile, and were then taken to dryness in a vacuum centrifuge (Savant Instruments, Hicksville, NY, U.S.A.). Chromatographed products having a 5'-DMT group were detritylated with 3% aqueous acetic acid (pH 2.5–2.7) at 25°C for 5–10 min. This was followed by extraction with ethyl acetate and then removal of water in the vacuum centrifuge. Selected 5'-HO oligodeoxyribonucleotide analogues were analyzed by ^{1}H and/or ^{31}P Fourier-transform NMR spectroscopy, which provided chemical-shift and integrated-signal intensity data that were consistent with the expected structures. Selected 5'-HO oligodeoxyribonucleotide analogues were also analyzed by positive fast-atom bombardment mass spectrometry (FAB-MS), which gave *m/z* values for the expected molecular ions. Base composition was determined by a modified version[18] of the formic acid-catalyzed degradation method[21], which involved heating at 120°C for 12 h and then HPLC analysis: μBondapak C_{18} column and 2% acetonitrile in TEAA at 4 ml/min, detection of the released cytosine (4.76 min), guanine (8.21 min), thymine (10.18 min), and adenine (15.26 min) bases at 280 nm, and normalization[21] of peak areas by comparison to a standard sample of GGAATTCC[17].

Enzyme-catalyzed hydrolyses

Incubations of either the oligodeoxyribonucleotide analogues or their digests with either SVPDE (E.C. 3.1.4.1, from *Crotalus adamanteus*, Type II, Sigma, St. Louis, MO, U.S.A.) and nuclease P1 (from *Penicillium citrinum*, Sigma), or EcoRI endonuclease (Bethesda Research Labs., Gaithersburg, MD, U.S.A.) or alkaline phosphatase (Sigma) were performed according to reported procedures[18,22].

RESULTS

Phosphorothioate analogues of oligodeoxyribonucleotides

Recent stereochemical studies[23] of the formation of chiral internucleotide linkages by phosphoramidite-coupling in the solid-phase synthesis of oligodeoxyribonucleotide analogues have demonstrated that O-methyl N,N-diisopropylphosphoramidites (and presumably other types of phosphoramidite reagents) undergo 1H-tetrazole-catalyzed coupling to support-bound 5'-HO chain-termini to give intermediary phosphites with epimerization at phosphorus (Fig. 1). The subsequent con-

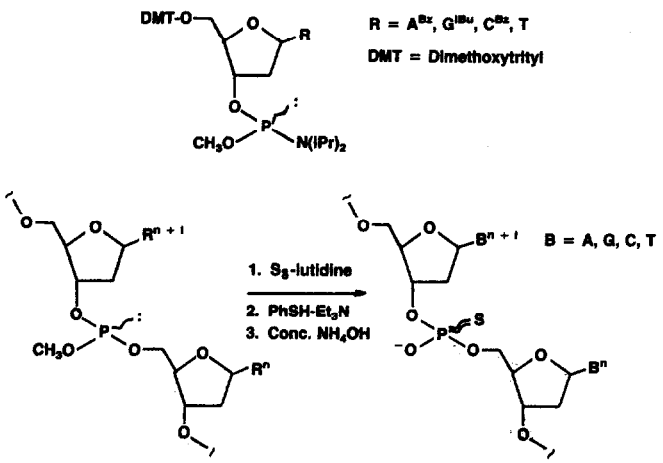

Fig. 1. Generalized structures for phosphoramidite reagents (top), a support-bound dinucleoside O-methyl phosphite intermediate (lower left), and a phosphorothioate internucleotide linkage (lower right), each having *Rp* and *Sp* absolute configurations at phosphorus. Bz = N-benzoyl; iBu = N-isobutyryl; PhSH = thiophenol; Et_3N = Triethylamine.

version of the phosphite moieties into P-chiral-modified internucleotide linkages thus yields oligodeoxyribonucleotide analogues as a set of 2^n diastereomeric products, where n is the number of P-chiral-modified internucleotide linkages. Our initial synthesis of various dinucleoside phosphorothioates ($N_{PS}N'$) via sulfurization of the support-bound epimeric phosphites with S_8 in 2,6-lutidine afforded diastereomeric pairs of 5'-DMT derivatives which, in each case, were eluted from the μBondapak C_{18} column as an apparently single peak by a 1%/min linear gradient of acetonitrile in TEAA (0.1 *M*, pH 7.0) that began at acetonitrile–TEAA (20:80)*. Collection of these 5'-DMT materials, followed by acetic acid-catalyzed removal of the DMT group gave 5'-HO dimers, which were now readily separable into a pair of fast- and slow-eluted diastereomers (Table I) upon gradient elution from the reversed-phase column, starting at acetonitrile–TEAA (5:95). 5'-Dimethoxytritylated trimer through hexamer analogues of oligodeoxyribonucleotides, having two phosphorothioate moieties that were either adjacent or had either one or two intervening phosphodiester linkage(s), were eluted either as an apparently single peak or as a pair of peaks, and in rare instances gave rise to three components (Table I). Collection of the separated components, followed by detritylation and gradient elution of the resultant 5'-HO materials led to the isolation of the set of four diastereomers that were expected in each case (Table I). The presence of phosphorothioate linkages in these analogues was established by ^{31}P NMR spectrometry and FAB-MS, in selected cases, and in all cases by means of separate treatments with SVPDE[11,24,25] and nuclease P1[26], fol-

* Under these HPLC conditions, the benzamide byproduct that results from ammoniolysis of N-benzoylated A and C moieties is eluted at approximately 5.5–6.5 min, depending on the particular μBondapak C_{18} column. The benzamide peak is gradually eluted faster as the column "ages"; however, when the retention time for benzamide eventually decreased to less than approximately 4.5–5.0 min, the column was found to be inadequate for high-performance separations, as judged by reanalysis of previously studied samples and by comparing it with a new column.

TABLE I

SEPARATION OF DIASTEREOMERS OF MONO- AND BIS-PHOSPHOROTHIOATE (PS) ANALOGUES OF DI- TO OCTAMERIC OLIGODEOXYRIBONUCLEOTIDES BY REVERSED-PHASE HPLC

HPLC of 5'-DMT and 5'-HO analogues on a μBondapak C_{18} column (30 cm × 7.8 mm) with a linear gradient of acetonitrile in 0.1 *M* TEAA (pH 7.0) at a flow-rate of 4 ml/min, starting at acetonitrile–TEAA (20:80) for 5'-DMT (gradient 1%/min), and acetonitrile–TEAA (5:95) for 5'-HO (gradient 0.5%/min), unless specified otherwise. With entries such as $T_{PS}T_{PS}T$, it should be understood that the 5'-DMT component at 21.9 min gave, after detritylation, a pair of 5'-HO diastereomers at 21.5 and 22.5 min, while the 5'-DMT component at 22.9 min similarly afforded a pair of diastereomers at 20.0 and 22.2 min.

Formula, 5' → 3'	*Elution time (min)*		*Absolute configuration at phosphorus*
	5'-DMT	*5'-HO*	
$T_{PS}T$	12.5*	13.4**	*R*
		14.2	*S*
$T_{PS}A$	12.3*	12.3**	*R*
		13.1	*S*
$T_{PS}G$	–	10.6**	*R*
		11.3	*S*
$A_{PS}T$	22.0	13.3**	*R*
		14.1	*S*
$A_{PS}A$	16.0***	16.5	*R*
		18.3	*S*
$G_{PS}G$	10.1*	12.5	*R*
		14.0	*S*
$G_{PS}C$	10.4*	12.7	*R*
		13.9	*S*
$G_{PS}A$	10.6*	10.8**	*R*
		11.6	*S*
$C_{PS}C$	16.8***	9.9	*R*
		11.6	*S*
$C_{PS}G$	10.9*	11.0	*R*
		12.0	*S*
$T_{PS}TT$	20.5	19.4	*S*
	21.5	19.1	*R*
$TT_{PS}T$	18.7	17.8	*R*
		19.4	*S*
$AT_{PS}T$	12.5	14.8	*R*
		16.2	*S*
$T_{PS}T_{PS}T$	21.9	21.5	*S,R*
		22.5	*S,S*
	22.9	20.0	*R,R*
		22.2	*R,S*
$A_{PS}A_{PS}A$	18.5	29.5	*R,R*
		31.3	*R,S*
		31.7	*S,R*
		33.3	*S,S*

(*Continued on p. 268*)

TABLE I (*continued*)

Formula, 5' → 3'	*Elution time (min)*		*Absolute configuration at phosphorus*
	5'-DMT	*5'-HO*	
$G_{PS}G_{PS}G$	23.5	22.2	*S,R*
		23.9	*S,S*
	24.6	20.4	*R,R*
		22.5	*R,S*
$G_{PS}G_{PS}A$	17.5	13.2**	*S,R*
	18.2	13.6	*S,S*
	19.1	12.6	*R,R*
		13.1	*R,S*
$C_{PS}C_{PS}C$	18.0	10.7§	*R,R*
		12.0	*S,R*
		13.1	*R,S*
		14.2	*S,S*
$TTT_{PS}T$	18.4	27.2	*R*
		29.3	*S*
$TAT_{PS}A$	14.2***	19.0	*R*
		19.8	*S*
$T_{PS}ATA$	15.6***	20.4	*S*
	16.3	19.9	*R*
$T_{PS}TT_{PS}T$	16.0***	24.1	*S,R*
		24.9	*S,S*
	17.0	23.7	*R,R*
		24.6	*R,S*
$T_{PS}AT_{PS}A$	15.3***	22.0	*S,R*
		22.8	*S,S*
	16.0	21.5	*R,R*
		22.3	*R,S*
$G_{PS}GC_{PS}C$	18.0*	17.0	*S,R*
		18.1	*S,S*
	18.8	16.0	*R,R*
		17.5	*R,S*
$TTT_{PS}T_{PS}T$	17.2	32.1	*R,R*
		33.3	*S,R*
		33.6	*R,S*
		34.5	*S,S*
$T_{PS}ATA_{PS}A$	15.3	20.9§§	*S,R*
		22.0	*S,S*
	16.0	20.2	*R,R*
		21.3	*R,S*
$TTT_{PS}TTT$	16.5	14.1**	*R*
		14.4	*S*
$GC_{PS}GCGC$	10.9	20.7	*R*
		21.5	*S*

TABLE I (*continued*)

Formula, 5' → 3'	*Elution time (min)*		*Absolute configuration at phosphorus*
	5'-DMT	*5'-HO*	
$TTT_{PS}T_{PS}TT$	16.1	32.5	*R,R*
		33.2	*S,R*
		33.7	*R,S*
		34.6	*S,S*
$C_{PS}CTTAAGG$	13.2	12.8	*S*
	14.2	12.3	*R*
$CAAGTTA_SG$	16.5	11.4	–
		12.2	

* Gradient 0.33%/min.
** Gradient 1%/min.
*** Gradient 1.33%/min.
§ Gradient 0.25%/min.
§§ Gradient 0.17%/min.

TABLE II

SEPARATION OF DIASTEREOMERS OF MONO- AND BIS-PHOSPHOROTHIOATE (PS) ANALOGUES OF THE OCTAMER GGAATTCC BY REVERSED-PHASE HPLC

See Table I for details of the standard HPLC conditions for 5'-DMT and corresponding 5'-HO analogues.

Formula, 5' → 3'	*Elution time (min)*		*Absolute configuration at phosphorus*
	5'-DMT	*5'-HO*	
$G_{PS}GAATTCC$	18.4*	18.2	*R*
	19.8	18.9	*S*
$GG_{PS}AATTCC$	19.2*	14.7**	*R*
	21.8	14.7	*S*
$GGA_{PS}ATTCC$	11.4	17.7	*R + S*
$GGAA_{PS}TTCC$	11.2	18.2	*R + S*
$GGAAT_{PS}TCC$	10.9	17.4	*R*
		17.9	*S*
$GGAATT_{PS}CC$	11.2	16.9	*R*
		17.7	*S*
$GGAATTC_{PS}C$	11.9	17.1	*R*
		17.6	*S*
$G_{PS}G_{PS}AATTCC$	13.2	15.6	*S,R*
	13.6	15.8	*S,S*
	14.0	15.3	*R,R*
		15.6	*R,S*

* Gradient 0.5%/min, flow-rate 5 ml/min.
** Gradient 1%/min.

lowed, if necessary, by addition of alkaline phosphatase, and then identification by means of HPLC retention times of the undigested dinucleoside phosphorothioate, $N_{PS}N'$. The enzymatic reactions also allowed the assignment of the absolute configuration at each phosphorothioate center. Undigested dimers with the *Rp* absolute configuration were found in the nuclease P1 digest of a given substrate, while the corresponding digest obtained with SVPDE was devoid of detectable $N_{PS}N'$. Conversely, SVPDE led to (*Sp*)-$N_{PS}N'$, while nuclease P1 gave a product mixture devoid of detectable $N_{PS}N'$. This method of stereochemical characterization was successfully applied to longer oligomers (see below); however, in the aforementioned cases with two adjacent phosphorothioate centers, it was found[22] that SVPDE did not hydrolyze the *Rp*,*Sp* and *Sp*,*Sp* diastereomers, and in cases with a $G_{PS}G$ unit the stereodifferentiation by SVPDE was problematic[22].

The influence of the location of a phosphorothioate internucleotide linkage upon the separability by HPLC of the diastereomers was initially examined by using the seven possible phosphorothioate analogues of the octamer GGAATTCC, first as the 5'-DMT derivative and then as the 5'-HO material, after collection and detritylation of the former peak(s) (Table II).

While it was not possible to fractionate the *Rp* and *Sp* diastereomers of either $GGA_{PS}ATTCC$ or $GGAA_{PS}TTCC$ by the presently reported tandem HPLC conditions, the ability to separate the *Rp* and *Sp* diastereomers of $G_{PS}GAATTCC$ and $GG_{PS}AATTCC$ (Fig. 2) allowed a preliminary investigation of these self-complementary oligonucleotide analogues as possible substrates for EcoRI endonuclease, which cleaves between G and A in duplex DNA having the sequence GAATTC. As reported in a preliminary communication[19], it was found that the *Rp*, but not the *Sp* diastereomer of the former octamer, was cleaved, and that neither of the two diastereomers of the latter octamer was a good substrate for EcoRI, relative to the normal oxo-substrate. In these studies with EcoRI, HPLC proved to be a convenient and sensitive method for analyzing the incubation mixtures, following heat-denaturation of EcoRI and treatment with alkaline phosphatase to afford AATTCC, which was readily available as an authentic HPLC standard via its chemical synthesis.

The enhanced separability of the diastereomers of 5'-DMT-$G_{PS}GAATTCC$,

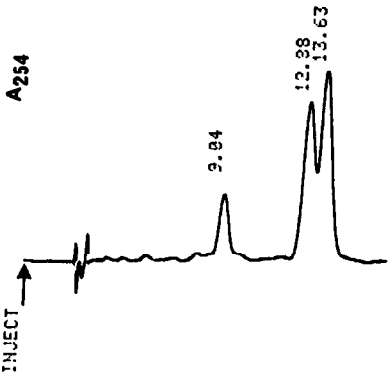

Fig. 2. HPLC tracing recorded for a sample that contained the fast- (12.88 min) and slow-eluted (13.63 min) diastereomers of $GG_{PS}AATTCC$ as well as some GGAATTCC (9.04 min). See Table I for chromatographic details; gradient 0.0625%/min at a flow-rate of 5 ml/min starting at acetonitrile–TEAA (10:90).

TABLE III

SEPARATION OF DIASTEREOMERS OF DECA- TO HEXADECAMERIC 5'-TERMINAL MONOPHOSPHOROTHIOATE (PS) ANALOGUES OF OLIGODEOXYRIBONUCLEOTIDES BY REVERSED-PHASE HPLC

See Table I for details of the standard HPLC conditions for 5'-DMT and corresponding 5'-HO analogues.

Formula, 5' → 3'	*Elution time (min)*		*Absolute configuration at phosphorus*
	5'-DMT	*5'-HO*	
$G_{PS}GGAATTCCC$	23.5*	17.7**	*R*
	24.6	18.1	*S*
$T_{PS}GCATACGAC$	15.4***	20.6	*S*
	16.3	20.7	*R*
$T_{PS}ATATCGATATA$	11.2§	23.2	*S*
	12.2	22.4	*R*
$T_{PS}ATATATCGATATATA$	10.1§	23.3	*S*
	11.1	22.9	*R*

* Gradient 0.33%/min.
** Flow-rate 5 ml/min.
*** Gradient 0.5%/min for 10 min, then isocratic.
§ Gradient 2%/min for 5 min, then isocratic.

relative to the other members of this homologous series of octameric monophosphorothioate analogues, led to the examination of examples of longer 5'-DMT-$N_{PS}R$ oligomers (Table III). It was found[22] that even a hexadecamer of this type could be separated into its *Rp* and *Sp* diastereomers by gradient elution from a C_{18} column. In contrast to these results, other relatively long oligomers (Table IV), having a

TABLE IV

MONO- AND POLY-PHOSPHOROTHIOATE (PS) ANALOGUES OF 5'-DMT OLIGODEOXYRIBONUCLEOTIDES PURIFIED BUT NOT STEREOCHEMICALLY RESOLVED BY REVERSED-PHASE HPLC

See Table I for details of the standard HPLC conditions for 5'-DMT analogues.

Formula, 5' → 3'	*Elution time (min)*
$TATATAT_{PS}ATATA$	8.5*
$TATATG_{PS}CATATA$	13.0
$GAATTCCCAAGTTA_SG$	13.8**
$GGGTCCCCCACTATCTC_{PS}C$	9.5
$GGGTCCCCCACTATCT_{PS}C_{PS}C$	9.5
$GGGTCCCCCACTA_{PS}T_{PS}C_{PS}T_{PS}C_{PS}C$	9.7
$ATATAGCGC_{PS}GCGCTATAT$	10.4
$ATATAG_{PS}C_{PS}G_{PS}C_{PS}G_{PS}C_{PS}G_{PS}CTATAT$	10.4
$TGTATCTGGT_{PS}GCATATCGAC$	11.4
$ATTATGGGTCGCCAA_{PS}ACCTGG$	11.1
$A_{PS}G_{PS}A_{PS}A_{PS}C_{PS}C_{PS}T_{PS}C_{PS}T_{PS}A_{PS}G_{PS}G_{PS}T_{PS}C_{PS}A_{PS}A_{PS}G_{PS}G$	14–26**
$TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT_{PS}T$ (1 10 20 30 40 50)	9.8

* Gradient 2.5%/min.
** Gradient 0.8%/min for 5 min, then isocratic.

TABLE V

SEPARATION OF DIASTEREOMERS OF METHYL (Me) AND 4,4'-DIMETHOXYTRITYL (DMT) PHOSPHONATE ANALOGUES OF OLIGODEOXYRIBONUCLEOTIDES

HPLC on a μBondapak C_{18} column (30 cm × 7.8 mm) with a linear gradient of 1%/min acetonitrile in 0.1 *M* TEAA (pH 7.0) at a flow-rate of 4 ml/min, starting at acetonitrile–TEAA (5:95), unless specified otherwise.

Formula, 5' → 3'	*Elution time (min)*		*Absolute configuration at phosphorus*	*Formula, 5' → 3'*	*Elution time (min)*		*Absolute configuration at phosphorus*
	5'-DMT	*5'-HO*			*5'-DMT*	*5'-HO*	
$T_{Me}T$	–	14.1	*R* (?)	$T_{DMT}C$	–	16.1**	–
		14.8	*S* (?)			16.6	–
$A_{Me}T$	–	15.0	*R*	$C_{DMT}C$	–	14.4**	–
		16.2	*S*			15.0	–
$TTTTT_{Me}TTTTT$	23.3*	4.5	–	$A_{DMT}A$	–	17.1**	–
		4.8	–			18.0	–
$TTTTTTTTT_{Me}T$	24.2*	15.4	*R*	$GG_{DMT}AATTCC$	–	15.2***	–
		15.6	*S*			15.8	–
$T_{DMT}T$	–	18.8**	–	$GGAA_{DMT}TTCC$	–	15.1***	–
		20.0				16.5	

* Gradient 0.5%/min, flow-rate, 3 ml/min, starting at acetonitrile–TEAA (20:80).
** Gradient 1%/min for 10 min, then isocratic, starting at acetonitrile–TEAA (30:70).
*** Gradient 2%/min for 10 min, followed by gradient 1%/min, starting at acetonitrile–TEAA (5:95).

phosphorothioate linkage that was not adjacent to the 5′-DMT group, failed to show separation of diastereomeric species. Conversion of these 5′-DMT compounds into their corresponding 5′-HO oligomers did not lead to separation of diastereomers.

The presence of one or more phosphorothioate linkages in dimers through octamers led to slower elution of 5′-DMT (and 5′-HO) phosphorothioate analogues relative to their oxo-counterparts (data not reported in Tables I and II), which proved to be advantageous since reversed-phase HPLC could be used as a one-step method for removal of truncated sequences and other byproducts, removal of low levels of undesired oxo-contaminant, and fractionation of the diastereomeric phosphorothioate-containing products. With longer sequences, the separation of a 5′-DMT monophosphorothioate from its 5′-DMT oxo-contaminant was not studied by HPLC, due to interference caused by the presence of other as yet unidentified 5′-DMT-bearing byproducts. On the other hand, the cumulative effect of the presence of multiple phosphorothioate linkages (Tables IV) upon retention time was clearly evident from the chromatographic behavior of a 19-mer and its perthio-counterpart, which, in principle, consisted of 2^{18} diastereomers. This mixture was eluted (14–26 min) as a broad distribution of overlapping peaks that appeared 4–16 min after the oxo-compound (10 min).

With regard to the possible correlation of reversed-phase HPLC elution times and absolute configuration at phosphorus in monophosphorothioate analogues of oligodeoxyribonucleotides, if was found (Tables I–III) that 5′-HO oligomers having the *Rp* configuration were generally eluted faster than their counterparts having the *Sp* configuration. Interestingly, this order of elution was usually reversed by the presence of the 5′-DMT group.

Alkanephosphonate analogues of oligodeoxyribonucleotides

Reaction of support-bound 5′-DMT dithymidyl (3′ → 5′) O-methyl phosphite with methyl iodide in acetonitrile at 100°C for 3 h gave, via an Arbusov-type reaction, a 70% yield of dithymidyl (3′ → 5′) methanephosphonate ($T_{Me}T$) as a roughly equimolar mixture of fast- and slow-eluted diastereomers (Table V), which were collected and individually characterized by ^{31}P NMR spectroscopy and FAB-MS[18]. The diastereomers of $A_{Me}T$ were similarly prepared and were fractionated by HPLC, while reactions with DMT chloride in acetonitrile at 60°C for 8 h led to HPLC isolation and structural characterization the fast- and slow-eluted diastereomers of dithymidyl (3′ → 5′) 4,4′-dimethoxytriphenylmethanephosphonate ($T_{DMT}T$), as well as the P-DMT diastereomers of other dimers and octamers, all of which are listed in Table V.

In contrast to the diastereomers of $GGAA_{PS}TTCC$, which were unseparable by reversed-phase HPLC, it was possible to fractionate $GGAA_{DMT}TTCC$ into its fast- and slow-eluted diastereomers. More striking examples of the separability of alkanephosphonate-containing oligonucleotide analogues were provided by two decamers of T having a methanephosphonate internucleotide linkage. In the first of these cases, 5′-DMT-$T_4T_{Me}TT_4$ was found to be eluted as an apparently single peak at 23.3 min. This was collected, detritylated, and again analyzed by HPLC as 5′-HO material. This 5′-HO material was separated into three components that were eluted at 3.63, 4.50, and 4.76 min, which were tentatively identified as the oxo-contaminant (T_{10}) and the fast- and slow-eluted diastereomers of $T_4T_{Me}TT_4$. The second case

involved the synthesis of 5'-DMT-$T_8T_{Me}T$, which led to HPLC elution of the oxo-contaminant (T_{10}, 23.4 min) and an apparently single peak for the phosphonate product (24.2 min). Detritylation of this phosphonate product led to HPLC elution of overlapping peaks (15.4 and 15.6 min, "fast":"slow" = 34:66) which were collected and identified by means of ^{31}P NMR spectroscopy as a mixture of the expected diastereomeric products, $T_8T_{Me}T$ [δ_{PMe} 35.19 (major isomer), δ_{PMe} 35.13 (minor isomer), δ_{PO} −1.65, ratio PMe:PO = 1:8]. The structural assignment for the mixture of products was confirmed by tandem digestions with nuclease P1 and alkaline phosphatase, followed by heat-denaturation of the enzymes and then HPLC analysis, which showed the presence of fast- and slow-eluted $T_{Me}T$ that presumably (see below) have the *Rp* and *Sp* configurations, respectively. The 40:60 ratio measured for the (*Rp*)-$T_{Me}T$:(*Sp*)-$T_{Me}T$ digestion-fragments indicated that the minor fast-eluted and major slow-eluted $T_8T_{Me}T$ products correspond to the *Rp* and *Sp* diastereomers, respectively.

The aforementioned assignment of *Rp* and *Sp* absolute configurations to the fast- and slow-eluted diastereomers of $T_{Me}T$, respectively, was based on the following correlation with $A_{Me}T$, which served as a stereochemical "anchor" compound. Samples of the fast- and slow-eluted diastereomers of $A_{Me}T$, which were obtained in our laboratory by the solid-phase method of synthesis, were shown by means of HPLC to have essentially the same retention times as those which we measured for authentic samples of (*Rp*)- and (*Sp*)-$A_{Me}T$, respectively. The latter diastereomer had been unambiguously stereochemically defined through X-ray crystallographic measurements by Chacko *et al.*[27]. If one assumes that the type of correlation between the relative retention times of the diastereomers of 5'HO-$N_{PS}N'$ and absolute configurations at phosphorus likewise obtains for 5'HO-$N_{Me}N'$, then the fast and slow elution behavior of (*Rp*)- and (*Sp*)-$A_{Me}T$, respectively, imply that the fast- and slow-eluted diastereomers of $T_{Me}T$ and other $N_{Me}N'$ compounds also have *Rp* and *Sp* configurations, respectively. It should be emphasized that extension of this correlation involving methanephosphonates to one which also includes fundamentally different types of alkanephosphonates (*e.g.*, $N_{DMT}N'$) is not warranted at this time.

O-Isopropyl phosphotriester analogues of oligodeoxyribonucleotides

An interesting, novel, and potentially general method for the solid-phase synthesis of O-alkyl phosphotriester analogues of oligodeoxyribonucleotides involves the conversion of a phosphorothioate internucleotide linkage into a phosphotriester moiety by reaction with, *e.g.*, 2,4-dinitrofluorobenzene to give a phosphorofluoridate intermediate that can be trapped with an alcohol. The feasibility of this synthetic route was initially tested by the separate reactions of (*Rp*)- and (*Sp*)-$T_{PS}T$ with 2,4-dinitrofluorobenzene in pyridine. This led to an epimeric mixture of the expected phosphorofluoridates ($^1J_{PF} \approx$ 900 Hz) which was converted by *in situ* ethanolysis to the corresponding O-ethyl phosphotriester, $T_{POEt}T$. The product was isolated by elution (18.9 min) from a C_{18} column with 1%/min acetonitrile *vs.* TEAA (initial acetonitrile–TEAA ratio is 5:95) and was identified by FAB-MS. While studies of the extension of this method to longer-chain, support-bound, base-protected O-alkyl phosphotriester analogues are currently in progress, an alternative procedure for direct synthesis was developed, based on a new class of coupling reagents, *viz.*, O-isopropylphosphomorpholidites[14]. The products made by this alternative procedure

TABLE VI

SEPARATION OF DIASTEREOMERS OF DI- TO OCTAMERIC O-ISOPROPYL PHOSPHOTRIESTER (POiPr) AND CORRESPONDING THIONOPHOSPHOTRIESTER ANALOGUES OF OLIGODEOXYRIBONUCLEOTIDES BY REVERSED-PHASE HPLC

HPLC on a μBondapak C_{18} column (30 cm × 7.8 mm) with a linear gradient of 1%/min acetonitrile in 0.1 *M* TEAA (pH 7.0) for 10 min at a flow-rate of 4 ml/min, starting at acetonitrile–TEAA (20:80), unless specified otherwise. Absolute configuration at phosphorus in parentheses.

Formula, 5′ → 3′	*Elution time (min)*		*Formula, 5′ → 3′*	*Elution time (min)*	
	5′-DMT	*5′-HO*		*5′-DMT*	*5′-HO*
$G_{POiPr}G$	–	16.1 16.6	$A_{POiPr}TA_{POiPr}T$	23.1§	13.7 14.1
$T_{POiPr}T$	–	36.0 (*R*)* 38.5 (*S*)		23.5	13.6 14.0
$T_{P(S)OiPr}T$	–	21.3 (*R*)** 24.3 (*S*)	$G_{POiPr}GAATTCC$	16.7 22.2	16.0§§ 16.6
$G_{POiPr}C$ (no separation)	–	16.3	$GG_{POiPr}AATTCC$	12.7 13.4	14.3 (*S*)§§ 13.9 (*R*)
$A_{POiPr}A$	–	11.4*** 11.8	$GG_{P(S)OiPr}AATTCC$	14.0 14.4	15.8 (*S*)§§ 15.5 (*R*)
$A_{POiPr}T$ (no separation)	–	13.0***	$GGA_{POiPr}ATTCC$	8.4§§§	13.1§§ 13.7
$A_{POiPr}G$	–	17.9 18.4	$GGAA_{POiPr}TTCC$	9.3§§§ 10.4	15.3§§ 14.4
$A_{POiPr}C$	–	18.6 19.0			

* Acetonitrile–water (13:87).
** Acetonitrile–water (22:78).
*** Gradient 3%/min for 5 min, followed by gradient 0.25%/min, initial acetonitrile–TEAA composition is 5:95.
§ Gradient 2%/min, initial acetonitrile–TEAA composition is 5:95.
§§ Initial acetonitrile–TEAA composition is 5:95.
§§§ Gradient 0.25%/min, starting at acetonitrile–TEAA (25:75).

are listed in Table VI and include electronically neutral dimers, "mixed-linkage" octamers, and a representative bis-phosphotriester, as well as thiono triesters that were obtained by phosphite sulfurization (see above). In view of the non-stereoselective 1H-tetrazole-mediated coupling mechanism found for O-methyl N,N-diispropyl phosphoramidites[23], it was not surprising that the analogous coupling of O-isopropylphosphomorpholidites afforded diastereomeric mixtures of dimer products, which were separated as 5′-HO species by reversed-phase HPLC in five of the seven cases studied. The four diastereomers of the bis-phosphotriester $A_{POiPr}TA_{POiPr}T$ were also separated; however, in this case it was necessary to fractionate first two pairs of the 5′-DMT derivatives and then further to resolve each of these pairs as 5′-HO species. Notable among the octamers was the chromatographic behavior of $GGAA_{POiPr}TTCC$, the diastereomers of which were separated as either 5′-DMT or 5′-HO species (Fig. 3).

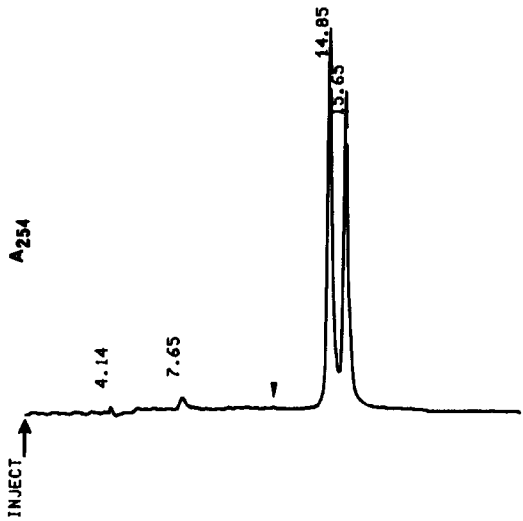

Fig. 3. HPLC tracing recorded for a sample that contained the fast- (14.85 min) and slow-eluted (15.65 min) diastereomers of $GGAA_{POiPr}TTCC$; the arrowhead indicates the elution time (12.1 min) for GGAATTCC. See Table I for chromatographic details; gradient, 1%/min at a flow-rate of 4 ml/min starting at acetonitrile–TEAA (5:95).

A method for the assignment of absolute configuration at stereogenic centers in phosphotriester analogues of oligodeoxyribonucleotides had not been reported previously and was therefore addressed by devising the configurational correlation outlined in Fig. 4 for an O-isopropyl phosphotriester internucleotide linkage[14]. This correlation is based upon highly stereoselective conversions of a thionophosphotriester into both the phosphotriester of interest and a phosphorothioate product, having a configuration which is either known or can be readily assigned by enzymatic procedures[19,22]. The correlation was first studied by conducting two parallel syntheses for separate oxidation and sulfurization to afford, respectively, fast- and slow-eluted diastereomers of $T_{POiPr}T$, and fast- and slow-eluted diastereomers of its thiono coun-

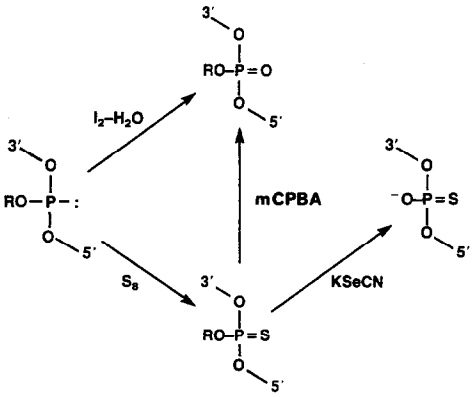

Fig. 4. Generalized scheme for the chemical correlation of the absolute configuration of a phosphotriester linkage with the known absolute configuration of a phosphorothioate linkage, via stereoretentive conversions involving a common thionophosphotriester precursor.

terpart. Stereoretentive conversion of the latter pair of diastereomers into the former pair of diastereomers was achieved with *m*-chloroperbenzoic acid (mCPBA), while potassium selenocyanate (KSeCN) was found to convert stereoselectively the slow- and fast-eluted thiono compounds into (*Rp*)- and (*Sp*)-$T_{PS}T$, respectively. Other independent experiments were used to establish that the O-dealkylation reaction with KSeCN proceeds with retention of configuration at phosphorus[14]. By extension, it was concluded that (*Rp*)-$T_{PS}T$ was derived from the slower-eluted *Sp* diastereomer of the thionophosphotriester, which also gave rise to the faster-eluted *Rp* diastereomer of $T_{POiPr}T$ via the mCPBA-mediated reaction. These findings for $T_{POiPr}T$ and $T_{P(S)OiPr}T$ thus revealed that the relative reversed-phase HPLC elution times for diastereomeric phosphotriesters was not correlated with similar mobility data for their stereochemically related thiono phosphotriesters, which differ only in the replacement of P=O by P=S moieties.

DISCUSSION

Traditional methods for the purification of fully deprotected synthetic oligodeoxyribonucleotides by either polyacrylamide gel electrophoresis or by low-pressure ion-exchange chromatography have limitations with regard to either large-scale purifications or separations of products that differ by virtue of either base composition, base sequence, or other subtle structural features, such as the intra-chain location and absolute configuration of modified P-chiral internucleotide linkages. Highly efficient reversed-phase columns have therefore proven to be well-suited to a wide variety of standard as well as specialized applications, which deal with either fully deprotected 5′-HO or lipophilic 5′-DMT oligodeoxyribonucleotides and which generally use "volatile" eluents, such as acetonitrile–TEAA, that are convenient and permit ion-pair chromatography[28,29]. Although there are now many reported examples that demonstrate the selectivity of reversed-phase HPLC toward oligodeoxyribonucleotides having differences among the bases, there is much less information concerning constitutional isomers[30] and stereochemical aspects of backbone-modified analogues of oligodeoxyribonucleotides, such as those described in this report. The results obtained for the relatively large number of phosphorothioate-containing analogues revealed some noteworthy features and patterns. Selectivity of the C_{18} bonded phase toward constitutional isomers having reverse sequences or different locations of the phosphorothioate (PS) linkage was evidenced by the chromatographic behavior of compounds such as $T_{PS}A$ *vs.* $A_{PS}T$, and $TAT_{PS}A$ *vs.* $T_{PS}ATA$. Depending on the length of the oligomer and the ratio of PS–PO internucleotide linkages, the incorporation of PS moieties into the backbone of oligodeoxyribonucleotides sometimes led to significantly longer (but never shorter) elution times, relative to the corresponding non-sulfurated "oxo" compound. The reason(s) for this fundamental difference in chromatographic behavior is (are) not known at the present time, but may be tentatively associated with the substantially lower pK_a of monothioic *vs.* oxy acids of phosphorus, which strongly influences the ion-pair adsorption process[28,29]. For 5′-HO monophosphorothioates the order of elution of diastereomers was *Rp* followed by *Sp*; however, the presence of a 5′-DMT group caused a reversal of this order. Except for dimers, the presence of a 5′-DMT group also led to enhanced separation of *Rp* and *Sp* diastereomers in those cases having a proximate

phosphorothioate linkage, *viz.*, 5'-DMT-N_{PS}R. For octamers and especially longer sequences, it was generally not possible to fractionate either 5'-DMT or 5'-HO diastereomers which had the stereogenic phosphorothioate group located either near the middle or toward the 3'-terminus of the molecule. While the physiochemical reasons which underlie the aforementioned elution orders of the diastereomers, and the remarkable separability of diastereomers of 5'-DMT-N_{PS}R are not known at the present time, these empirical observations are nevertheless useful. For example, it is now reasonable to approach the construction of relatively long, diastereomerically pure 5'-HO-RN_{PS}N'R' compounds by a convergent synthesis that involves enzymatically joining oligonucleotide fragment R and the 5'-HO diastereomers derived from the presumably HPLC-separable oligonucleotide fragment 5'-DMT-N_{PS}N'R'.

In comparison to the phosphorothioate-containing analogues, very little stereochemical information is presently available for methanephosphonate analogues; however, the data suggests that the order of elution of the diastereomers is the same for both classes of compounds: *R*p followed by *S*p. It remains to be seen whether this correlation is verified by further examples, and —perhaps less likely— whether it extends in a simple fashion to the diastereomers of N_{DMT}N' wherein the dimethoxytrityl-bearing phosphonate linkage is structurally quite different from either PMe or PS. In any event, the presently reported P-Me and P-DMT alkane phosphonate analogues revealed that *R*p and *S*p diastereomers of relatively long, *i.e.*, octa- to decameric molecules can be separated by reversed-phase HPLC, even in those cases were the stereogenic (modified) linkage is either centrally located or located near the 5'-end. This chromatographic behavior, which does not apply to phosphorothioate-containing analogues, was also evidenced by the separation of the diastereomers of the phosphotriester $GGA_{POiPr}ATTCC$ and $GGAA_{POiPr}TTCC$. This suggests that uncharged, lipophilic, stereogenic moieties, such as P-Me, P-DMT, and P-OiPr are better able to interact stereodifferentially with the bonded C_{18} phase, relative to charged, hydrophilic, stereogenic moieties, such as the phosphorothioate group. If additional examples support this rationalization, then it may be possible to design new types of coupling reagents, leading to backbone-attached groups that have strong stereodifferentiating interactions with a reversed-phase HPLC column, as a route to previously unseparable diastereomers of oligodeoxyribonucleotide analogues.

That subtle structural factors can influence the reversed-phase HPLC separation of diastereomers of backbone-modified oligodeoxyribonucleotide analogues was evidenced by the relative mobilities of the *R*p and *S*p diastereomers of O-isopropyl phosphotriesters and their thiono triester counterparts that differ only by replacement of P=O by P=S. The electronic and steric differences between these two types of stereogenic triester moieties would, *a priori*, seem to be minor with regard to stereodifferentiation of diastereomers by the bonded C_{18} phase; yet, these differences led to opposite patterns of relative mobility for the dimer case. This underscores the caution which is necessary when considering empirical configurational correlations involving diastereomers and physical measurements, such as HPLC elution times. Strictly speaking, these correlations need not necessarily hold, even among members of a homologous family of compounds.

In summary, the presently reported chromatographic studies of structurally diverse backbone-modified oligodeoxyribonucleotide analogues have provided indi-

cations of the scope of reversed-phase HPLC for the separation of stereoisomers of these compounds. At the same time, it has been possible to define some of the practical limitations of the separations. While the majority of compounds that are reported here were separated into diastereomerically pure or highly-enriched forms, the unsuccessful cases found among the poly-anionic phosphorothioate analogues represent one of the more challenging problems for future studies. Our preliminary results have indicated that strong-anion-exchange HPLC may be useful in this connection; however, ion-pair chromatography may prove to be more successful. Our continuing work in this area will be reported elsewhere.

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REFERENCES

1 K. Itakura, J. J. Rossi and R. B. Wallace, *Ann. Rev. Biochem.*, 53 (1984) 323.
2 F. Eckstein, *Angew. Chem., Int. Ed. Engl.*, 22 (1983) 423.
3 P. S. Miller, S. Chandrasegaran, D. L. Dow, S. M. Pulford and L. S. Kan, *Biochemistry*, 21 (1982) 5468.
4 S. A. Noble, E. F. Fisher and M. H. Caruthers, *Nucl. Acids. Res.*, 12 (1984) 3387.
5 P. F. Torrence, J. Imai, K. Lesiak, J.-C. Jamoulle, H. Sawai, J. Warinnier, J. Balzarini and E. De Clercq, in E. De Clercq and R. T. Walker (Editors), *Targets for the Design of Antiviral Agents*, Plenum Press, New York, 1984, p. 259.
6 P. S. Miller, J. Yano, E. Yano, C. Carroll, K. Jayaraman and P. O. P. Ts'o, *Biochemistry*, 18 (1979) 5134.
7 J. Engels and A. Jäger, *Biochemistry*, 21 (1982) 912.
8 N. D. Sinha, V. Grossbruchhaus and H. Köster, *Tetrahedron Lett.*, 24 (1983) 887.
9 P. S. Miller, K. N. Fang, N. S. Kondo and P. O. P. Ts'o, *J. Amer. Chem. Soc.*, 93 (1971) 6657.
10 B. Uznański, W. Niewiarowski and W. J. Stec, *Tetrahedron Lett.*, 23 (1982) 4289.
11 F. R. Bryant and S. J. Benkovic, *Biochemistry*, 18 (1979) 2825.
12 R. S. Brody and P. A. Frey, *Biochemistry*, 20 (1981) 1245.
13 L. J. McBride and M. H. Caruthers, *Tetrahedron Lett.*, 24 (1983) 245.
14 W. J. Stec, G. Zon, K. A. Gallo, R. A. Byrd, B. Uznański and P. Guya, *Tetrahedron Lett.*, in press.
15 S. P. Adams, K. S. Kavka, E. J. Wykers, S. B. Holder and G. R. Galluppi, *J. Amer. Chem. Soc.*, 105 (1983) 661.
16 M. H. Caruthers, S. L. Beaucage, C. Becker, W. Efcavitch, E. F. Fisher, G. Galluppi, R. Goldman, P. deHaseth, F. Martin, M. Matteucci and Y. Stabinsky, in J. K. Setlow and A. Hollaender (Editors), *Genetic Engineering Principles and Methods*, Vol. 4, Plenum Press, New York, 1982, p. 1.
17 M. S. Broido, G. Zon and T. L. James, *Biochem. Biophys. Res. Commun.*, 119 (1984) 663.
18 W. J. Stec, G. Zon, W. Egan, R. A. Byrd and L. R. Phillips, *J. Org. Chem.*, in press.
19 W. J. Stec, G. Zon, W. Egan and B. Stec, *J. Amer. Chem. Soc.*, 106 (1984) 6077.
20 P. S. Miller, C. H. Agris, A. Murakami, P. M. Reddy, S. A. Spritz and P. O. P. Ts'o, *Nucl. Acids. Res.*, 11 (1983) 6225.
21 H. J. Fritz, D. Eick and W. Werr, in H. G. Gassen and A. Lang (Editors), *Chemical and Enzymatic Synthesis of Gene Fragments*, Verlag Chemie, Deerfield Beach, FL, 1982, p. 199.
22 W. J. Stec and G. Zon, *Tetrahedron Lett.*, 25 (1984) 5275.
23 W. J. Stec and G. Zon, *Tetrahedron Lett.*, 25 (1984) 5279.

24 P. M. J. Burgers, F. Eckstein and D. H. Hunneman, *J. Biol. Chem.*, 254 (1979) 7476.
25 P. M. J. Burgers, B. K. Sathyanarayana, W. Saenger and F. Eckstein, *Eur. J. Biochem.*, 100 (1979) 585.
26 V. B. L. Potter, B. A. Connolly and F. Eckstein, *Biochemistry*, 22 (1983) 1369.
27 K. K. Chacko, K. Linder, W. Saenger and P. S. Miller, *Nucl. Acids Res.*, 11 (1983) 2801.
28 A. Sokolowki, N. Balgobin, S. Josephson, J. B. Chattopadhyaya and G. Schill, *Chem. Scr.*, 18 (1981) 189.
29 W. Jost, K. Unger and G. Schill, *Anal. Biochem.*, 119 (1982) 214.
30 H. Schott and H. Eckstein, *J. Chromatogr.*, 296 (1984) 363.