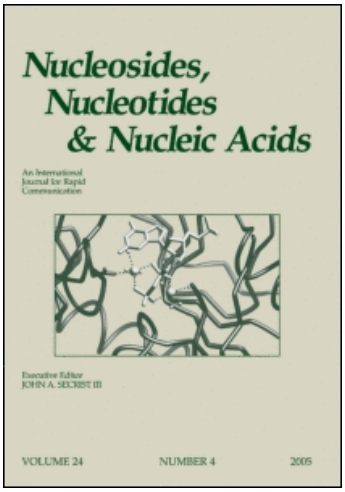

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DNA-Triesters - The Synthesis and Absolute Configuration Assignments at P-Stereogenic Centres

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DNA-TRIESTERS - THE SYNTHESIS AND ABSOLUTE CONFIGURATION ASSIGNMENTS AT P-STEREOGENIC CENTRES

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ABSTRACT

Decadeoxyribonucleotide GGGAATTCCC and nine diastereomeric pairs of its mono-O-ethyl ester analogues were synthesized via phosphoramidite approach using the combination of 5'-DMT-base protected (except T) nucleoside 3'-(2-cyanoethyl N,N-diisopropyl phosphoramidites) and 3'-(O-ethyl N,N-diisopropyl phosphoramidites). Under conditions of release from solid support and removal of base-protecting groups (25% NH_4OH, 25°C, 48 h) 2-cyanoethyl groups were removed while O-ethyl phosphate triester functions were practically intact. Isolation of products and separation of diastereomers were performed by means of RP-HPLC. Absolute configuration at P-stereogenic centres was established via degradation of decamers into corresponding dinucleoside O-ethyl phosphates and stereochemical correlation with dinucleoside phosphorothioates of known configuration at phosphorus. Decadeoxyribonucleotide mono-O-ethyl esters were used for mapping the contact points between DNA and Eco RI endonuclease - the restriction enzyme which recognizes canonical sequence .. GAATTC.. and cleaves unmodified DNA strands giving ..G and pAATTC.. .

INTRODUCTION

Recognition and interaction between double-stranded DNA and any protein responsible for biodegradation of DNA should be considered in principle of diastereomerism due to emphasized differences between "non-bridging" heterotopic oxygen atoms at the internucleotide phosphate bond, one of which is directed "inwards" DNA double helix, while the second one is directed "outwards" DNA. However, physical expression or consequences of diastereomeric interactions can be observed only after isotopic [1] or elemental [2] substitution of one of these atoms. Alkylation of internucleotide phosphate at any of two "non-bridging" heterotopic oxygen atoms, due to stereogenicity of phosphorus, generates the pair of diastereomers of DNA-triesters; number m of diastereomers depends on the number n of alkylated internucleotide phosphates ($m=2^n$).

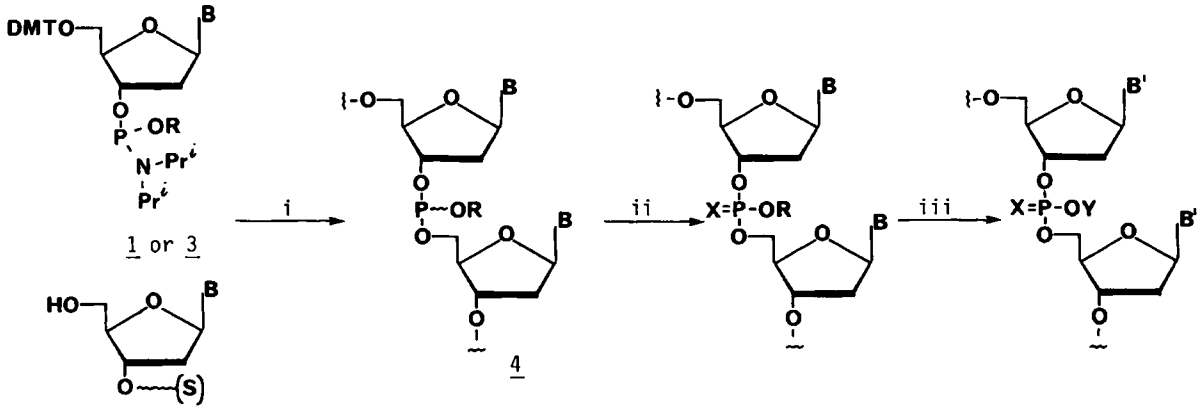
Alkylation of DNA was recognized as biologically important lesion as early as in the late fifties [3] but the detailed studies on the structure,stability and interactions of DNA-triesters with other biopolymers have been undertaken only recently [4,5]. The discovery of DNA-methyltrans-

ferase isolated from E.coli strain constitutive for the adaptive response, which diastereoselectively removes methyl group from DNA-O-methyl esters [6], generated an increase of interest in stereochemistry of DNA-triesters originally emphasized in pioneering works of Miller et.al [7] and Jensen and Reed [8].

Since the chirospecific synthesis and stereochemistry of biophosphates is the continuing goal of this Laboratory [9] we have focused our attention on the design of methods of synthesis and absolute configuration assignment at stereogenic P-centres of DNA-triesters [10,11]. In this paper we present the results of our studies on the synthesis of decamer GGGAATTCCC and nine pairs of diastereomers resulting from single modification of each internucleotide phosphate in this decamer by ethyl ester group, separation of diastereomers, assignment of absolute configuration at P-chiral centres and the influence of triester modification on the substrate ability of esterified decanucleotide for Eco RI endonuclease.

THE SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDES BEARING INTERNUCLEOTIDE O-ETHYL PHOSPHATE OR O-ETHYL PHOSPHOROTHIOATE FUNCTION

As it was mentioned in our earlier works [10,11,12], our approach to the synthesis of oligonucleotides bearing selectively esterified internucleotide phosphate is based on the modification of phosphoramidite strategy developed by Caruthers et al. [13]. Besides four appropriately protected nucleoside 3'-(O-2-cyanoethyl N,N-diisopropyl phosphoramidite) (1) [14] routinely used as building blocks for elongation of oligonucleotide chain bound *via* 3'-oxygen of "primer" nucleoside (2) to solid support, we have synthesized four protected nucleoside 3'-(O-ethyl N,N-diisopropyl phosphoramidites) (3) [11]. Condensation of 2 with 1 or 3 in the presence of tetrazole gives corresponding intermediary O-(2-cyanoethyl)- or O-ethyl phosphites (4) (Scheme 1). Phosphites 4 can be oxidized with either iodine solution in H_2O/2,6-lutidine/THF or saturated solution of sulfur in 2,6-lutidine to give phosphates (5) or phosphorothioates (6), respectively. When sequential growth of oligonucleotide chain is completed, cleavage of oligonucleotide from the support, removal of 2-cyanoethyl groups from internucleotide phosphates and phosphorothioates, and deprotection of bases are achieved by treatment of solid support-bound oligonucleotide with 25% NH_4OH for 48 h at 25°C. Under these conditions internucleotide phosphates and phosphorothioates protected with ethyl group remain untouched up to 85%. Two-step HPLC purification (RP-C_{18} μBondapak or ODS-Hypersil column with acetonitrile - triethylammonium bicarbonate buffer, pH 7.4, as an eluting system): first for 5'-DMT protected di(oligo)nucleotide and second for 5'-deprotected compound gives the purified desired di(oligo)deoxyribonucleotides (7), di(oligo)-deoxyribonucleoside phosphorothioates (8), di(oligo)deoxyribonucleoside



1 R=$CNCH_2CH_2$-
3 R=CH_3CH_2-

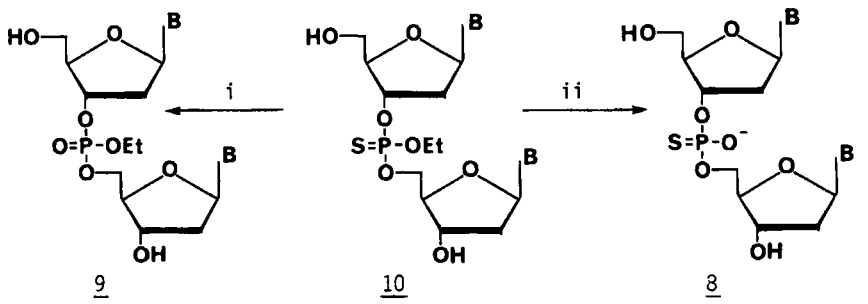
R=$CNCH_2CH_2$-

R=CH_3CH_2-

5	R=$CNCH_2CH_2$-,	X=O	7	X=O,	Y=H
6	R=$CNCH_2CH_2$-,	X=S	8	X=S,	Y=H
5	R=CH_3CH_2-,	X=O	9	X=O,	Y=CH_3CH_2-
6	R=CH_3CH_2-,	X=S	10	X=S,	Y=CH_3CH_2-

B = Thy, 4-benzoyl-Cyt, 6-benzoyl-Ade, 2-isobutyryl-Gua
B'= Thy, Cyt, Ade, Gua
i = tetrazole
ii = H_2O/I_2/2,6-lutidine/THF, X=O
S_8/2,6-lutidine, X=S
iii = 25% NH_4OH

SCHEME 1.



9 10 8

i = 3% H_2O_2 in CH_3CN-H_2O (1:1), 48 h, 25°C
ii = PhSH/Et_3N/dioxane, 6 h, 50°C

SCHEME 2.

O-ethyl phosphates (9) or di(oligo)deoxyribonucleoside O-ethyl phosphorothioates (10), respectively.

ASSIGNMENT OF ABSOLUTE CONFIGURATION AT STEREOGENIC PHOSPHORUS ATOM IN DINUCLEOSIDE O-ETHYL PHOSPHATES 9 AND DINUCLEOSIDE O-ETHYL PHOSPHOROTHIOATES 10

The overall procedure for assignment of absolute configuration at P-chiral centres in 9 and 10 is depicted in Scheme 2.
This procedure is based on two independent reactions which convert dinucleoside O-ethyl phosphorothioates 10 into dinucleoside phosphorothioates 8 and dinucleoside O-ethyl phosphates 9. Conversion 10→9 was achieved by treatment of 10 with 3% H_2O_2 at 25°C for 48 h [15]; this process is known to occur with retention of configuration [16]. Independently, each diastereomer of 10 was treated with tiophenol/triethylamine/dioxane solution (1:2:2) at 50°C for 6 h and resulting dinucleoside phosphorothioates 8 were examined as the substrates for nuclease P1, which is known as diastereoselective nucleolytic enzyme towards (Sp)-dinucleoside phosphorothioates [17]. Assignment of absolute configuration in the products of stereoretentive conversion 10→8 on this way allowed us to establish stereochemistry of our target molecules 9. The results of this correlation are listed in Table 1.

ASSIGNMENT OF ABSOLUTE CONFIGURATION AT P-ATOMS IN OLIGONUCLEOTIDE MONO-O-ETHYL ESTERS

As summarized in Table 2, decanucleotide GGGAATTCCC (7) and its mono-ethylated derivatives (9_1-9_9) were synthesized and separated into diastereomeric species. All of them bear canonical sequence ..GAATTC.. recognizable by Eco RI endonuclease. Absolute configuration at phosphorus

TABLE 1. Separation of diastereomers of dinucleoside O-ethyl phosphates $N_{P(O)(OEt)}N'$ (9) and dinucleoside O-ethyl phosphorothioates $N_{P(S)(OEt)}N'$ (10) by RP-HPLC

Comp. No.	Formula $N_{P(X)(OEt)}N'$	X	Retention time (min) 5'-DMT	Retention time (min) 5'-HO	Relative HPLC mobility and absolute configuration at phosphorus atom	
9a	N=dA, N'=dA	O	-	8.60[a]	*fast*	(Rp)
			-	9.60	*slow*	(Sp)
10a		S	7.00[b]	6.75[c]	*fast*[m]	(Sp)
			9.30	6.75	*slow*[m]	(Rp)
9b	N=dA, N'=dT	O	-	18.75[d]	*fast*	(Sp)
			-	20.00	*slow*	(Rp)
10b		S	-	30.00[e]	*fast*	(Sp)
			-	33.00	*slow*	(Rp)
9c	N=dC, N'=dC	O	-	12.00[f]	*fast*	(Rp)
			-	12.50	*slow*	(Sp)
10c		S	5.00[g]	4.00[h]	*fast*[m]	(Sp)
			5.50	4.00	*slow*[m]	(Rp)
9d	N=dG, N'=dA	O	-	4.85[a]	*fast*	(Rp)
			-	5.40	*slow*	(Sp)
10d		S	4.60[i]	4.30[c]	*fast*[m]	(Sp)
			6.00	4.30	*slow*[m]	(Rp)
9e	N=dG, N'=dG	O	-	4.50[a]	*fast*	(Rp)
			-	6.00	*slow*	(Sp)
10e		S	4.50[g]	3.30[h]	*fast*[m]	(Sp)
			5.50	3.30	*slow*[m]	(Rp)
9f	N=dT, N'=dC	O	-	14.20[f]	*fast*	(Rp)
			-	14.50	*slow*	(Sp)
10f		S	5.50[g]	3.50[h]	*fast*[m]	(Sp)
			6.50	3.50	*slow*[m]	(Rp)
9g	N=dT, N'=dT	O	-	23.25[j]	*fast*	(Sp)
			-	24.25	*slow*	(Rp)
10g		S	-	8.20[k]	*fast*	(Sp)
			-	8.75	*slow*	(Rp)

The synthesis of dinucleoside O-ethyl phosphates and dinucleoside O-ethyl phosphorothioates was performed on 1 µmole scale. Overall yield of the synthesis was from 4.5 to 7.0 A_{260} units (21-36%) for each diastereomer. Because some of diastereomeric of dinucleotide triesters were not separable as free 5'-HO species it was necessary to separate them before the detritylation step.
HPLC separation was achieved on two ways: **A** *- µBondapak C_{18} column (30 cm x 7.8 mm) at a flow-rate of 3.5 ml/min;* **B** *- ODS-Hypersil column (30cm x 4.6mm) at a flow-rate of 1.5 ml/min. Elution conditions: a/* **B**, *12% CH_3CNaq.; b/* **B**, *42% CH_3CNaq.; c/* **B**, *20% CH_3CNaq.; d/* **A**, *15% CH_3CNaq.; e/* **A**, *20% CH_3CNaq.; f/* **A**, *linear gradient 5-30% CH_3CN-0.1 M TEAB pH 7.4, 1.25%/min.; g/* **A**, *60% CH_3CNaq.; h/* **B**, *40% CH_3CNaq.; i/* **B**, *45% CH_3CNaq.; j/* **A**, *12% CH_3CNaq.; k/* **A**, *25% CH_3CNaq.; m/ relative HPLC mobilities of 5'-DMT protected compounds.*

TABLE 2. O-ethyl phosphotriester analogues of the decamer GGGAATTCCC: separation of their diastereomers by RP-HPLC and their digestion with Eco RI endonuclease

Comp. No	Formula 5'→3'	Retention time (min) 5'-DMT[a]	Retention time (min) 5'-HO[b]	Relative HPLC mobility[c] and absolute configuration at phosphorus	T_M[d] (°C)	Product of digestion with Eco RI[e]	Degree of digestion with Eco RI (%)
7	GGGAATTCCC	15.50	10.00		47.0	GGG	100
9_1	G#GGAATTCCC	19.00	11.00	*fast* (Rp)	43.0	-	-
		21.00	11.00	*slow* (Sp)	43.0	-	-
9_2	GG#GAATTCCC	19.50	10.50	*fast* (Rp)	46.5	-	-
		21.00	10.50	*slow* (Sp)	47.5	-	-
9_3	GGG#AATTCCC	20.00	10.75	*fast* (Rp)	45.0	-	-
		21.00	10.50	*slow* (Sp)	46.5	-	-
9_4	GGGA#ATTCCC	18.50	11.00	*fast* (Rp)	39.5	-	-
		20.00	10.50	*slow* (Sp)	46.5	-	-
9_5	GGGAA#TTCCC	19.00	11.50	*fast* (Rp)	40.0	-	-
		22.00	11.00	*slow* (Sp)	47.5	-	-
9_6	GGGAAT#TCCC	16.00	11.50	*fast* (Rp)	38.5	GGG	10
		18.00	11.30	*slow* (Sp)	47.5	GGG	100
9_7	GGGAATT#CCC	16.00	11.00	*fast* (Rp)	42.0	GGG	20
		18.00	11.25	*slow* (Sp)	48.0	GGG	10
9_8	GGGAATTC#CC	16.00	11.00	*fast* (Rp)	43.5	GGG	20
		18.00	11.00	*slow* (Sp)	47.0	GGG	15
9_9	GGGAATTCC#C	19.00	10.70	*fast* (Rp)	-	GGG	100
		20.00	10.70	*slow* (Sp)	47.0	GGG	100

= P(O)(OEt)

O-ethyl phosphotriester analogues of the decamer GGGAATTCCC were synthesized on 0.5 μmole scale using multicolumn solid phase synthesis approach [12]. Overall yield for each diastereomer was about 4-6 A_{260} units.

HPLC separation was achieved on a μBondapak C_{18} column (30 cm x 7.8 mm) with the gradient of acetonitrile-0.1 M TEAB pH 7.4 at a flow-rate of 3.5 ml/min.

a/ Gradient 5-30% CH_3CN-0.1 M TEAB (exp. 0.25) for 20 min. ; then isocratic.

b/ Gradient 5-30% CH_3CN-0.1 M TEAB 1.25%/min.

c/ Descriptors "fast" and "slow" refer to the diastereomers of decanucleotides separated as 5'-DMT derivatives.

d/ Melting temperature T_M was measured at λ_{max}=258 nm in buffer containing 10 mM Tris-Cl (pH 7.6) , 80 mM NaCl, 20 mM $MgCl_2$; decamer concentration A_{260}=0.5-0.7 unit/ml

e/ About 0.5 A_{260} unit of the oligonucleotide dissolved in 100 μl of the buffer containing 10 mM Tris-Cl (pH 7.6), 80 mM NaCl, 20 mM $MgCl_2$ was incubated with Eco RI endonuclease (100 units) at 16°C for 24 hr.(Without added enzyme O-ethyl phosphotriester oligonucleotides were completely resistant for the dealkylation).

in each diastereomer of decanucleotide O-ethyl ester was assigned by means of digestion of 9_1-9_9 with snake venom phosphodiesterase (SVPDE) followed by alkaline phosphatase [18]. Isolated dinucleoside O-ethyl phosphates, which were not degraded by this phosphodiesterase [19], were compared by coinjection with genuine samples of 9 (Table 1) by means of HPLC

e.g. $GGGA_{P(O)(OEt)}ATTCCC \xrightarrow{SVPDE} A_{P(O)(OEt)}A + 3G + 2T + 3C$

"*fast* (Sp)" "*slow* (Sp)"

SUBSTRATE ABILITY OF DECANUCLEOTIDES $\underline{9}_1$-$\underline{9}_9$ FOR ECO RI ENDONUCLEASE

Each diastereomer of $\underline{9}_1$-$\underline{9}_9$ was independently exposed on the action of Eco RI endonuclease under conditions, when decamer $\underline{7}$ was completely digested by the enzyme into GGG and pAATTCCC. The aliquots from each digestion, after protein denaturation, were analyzed on RP-HPLC. Since the attempted digestions were performed at 16°C (20-30° below the melting temperature for dimers $\underline{9}_1$-$\underline{9}_9$) it may be concluded, that the modified oligomers $\underline{9}_1$-$\underline{9}_9$ exist in duplex form what is the prerequisite for recognition by Eco RI restrictase. An inspection of Table 2, summarizing the results of these experiments, reveals that the presence of O-ethyl triester internucleotide function in the decanucleotide $\underline{9}_1$-$\underline{9}_8$ effectively protects "scissile" bond between G and A from the endonucleolytic action of Eco RI enzyme. This finding is consistent with the results of our earlier works with oligonucleotides bearing O-isopropyl functions [10,11] and, in principle, with results of Modrich et al. on alkylation interference with Eco RI endonuclease recognition [20]. Although the direct comparison of our results with those reported by Modrich is not possible (because specific recognition and binding can not be compared with results of "execution" of two former acts of interaction between DNA analogues and protein), our results confirm Modrich's data, that independently on the steric orientation of O-ethyl group ["inwards" major groove in (Rp)-esters and "outwards" DNA in (Sp)-esters] the enzyme does not cleave internucleotide bond between G and A in all compounds $\underline{9}_1$-$\underline{9}_5$. Internucleotide phosphate between A and A at neighbouring position to "scissile" bond has been shown by Modrich et al. as not being involved in specific binding to protein, while our experiments demonstrate that alkylation of this internucleotide phosphate "protects" oligomer from nucleolytic action of the enzyme (see Table 2; comp. no. $\underline{9}_4$). This may indicate that either this neighbouring phosphate in natural decamer $\underline{7}$ is involved in interaction with magnesium ions (which were absent in Modrich's experiments) or the presence of bulky ethyl group in both strands of $\underline{9}_4$ duplex prevents the formation of tight interface between DNA and protein. The diastereomer (Rp)-$\underline{9}_6$, like both diastereomers of $\underline{9}_7$ and $\underline{9}_8$, appeared to be rather poor substrates for Eco RI and it is exciting that (Sp)-$\underline{9}_6$ and both diastereomers of $\underline{9}_9$ were digested by this enzyme with the rate comparable with that observed for unmodified decamer $\underline{7}$. These results may indicate that nonamer GGGAATTCC fills the enzyme binding site and that there are "wholes" in this interface which allow to "escape" O-ethyl group from the protein network [like in the case of (Sp)-$\underline{9}_6$] what results in the cleavage of internucleotide bond between G and A. This interpretation implies, that negative charge on phosphate between T and T is not essential for specific binding, what is also in agreement with Modrich's conclusions [20]. Studies on the influence of esterified internucleotide bond present at any position of one

strand of DNA on the cleavage of complementary "unaltered" strand of heteroduplex composed of unmodified and esterified oligonucleotide are in progress.

CONCLUSIONS

1/ Protected nucleoside 3'-(O-2-cyanoethyl N,N-diisopropyl phosphoramidites) 1 and 3'-(O-ethyl N,N-diisopropyl phosphoramidites) 3 make feasible the synthesis of oligodeoxyribonucleotide mono-O-ethyl esters like 9 and oligonucleotide poly-O-ethyl esters (not discussed in this paper).
2/ Degradation of oligonucleotide mono-O-ethyl esters with SVPDE followed by alkaline phosphatase gives dinucleoside O-ethyl phosphates. Their absolute configuration can be assigned by the methodology depicted in Scheme 2.
3/ Assignment of absolute configuration at "esterified" internucleotide phosphate allows to discuss in stereochemical terms the orientation of O-alkyl function with respect to double-stranded DNA, presumably existing in B-conformation [21].
4/ Esterification of internucleotide phosphates (with excluded possibility of interference with base alkylation) protects canonical sequence ..GAATTC.. from endonucleolytic cleavage by means of Eco RI restrictase.
5/ "Protection" of internucleotide phosphates by alkylation of internucleotide bonds may results from hampering of the recognition by enzyme due to quenching of negative charge(s). Moreover, it is suggested that stereo-hindrance introduced by the presence of alkyl group prevents the formation of tight interface between DNA and the protein.
6/ Internucleotide phosphate between A and A in canonical structure ..GAATTC.. may be involved in interaction with magnesium ions necessary for execution of cleavage of oligonucleotide between G and A, catalyzed by the presence of Eco RI endonuclease.

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