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Stereospecific removal of methyl phosphotriesters from DNA by an *Escherichia coli ada*$^+$ extract

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ABSTRACT

The *ada*$^+$ gene product, a DNA methyltransferase present in extracts from an *Escherichia coli* strain constitutive for the adaptive response, removes only half of the methyl phosphotriesters from alkylated DNA. Since DNA phosphotriesters occur in two isomeric configurations (denoted Rp and Sp), we examined whether this reflects a stereospecific mode of repair by the methyltransferase. Analysis by reverse-phase HPLC, phosphorus NMR and circular dichroism established that only triesters in the Sp configuration are acted upon by the *E. coli* extract.

INTRODUCTION

Internucleotide phosphate groups are a common site of attack by many of the more potent carcinogenic alkylating agents, especially those such as MNNG and ENU which display a high reactivity toward oxygen atoms in DNA[1]. The phosphotriesters so produced are refractory to exo- and endonucleases[2,3], and severely reduce the rate and extent of template-directed DNA and RNA synthesis *in vitro*[4,5]. These lesions persist undiminished in cellular DNA for at least 48 hours in both human fibroblasts[6] and Chinese hamster V79 cells[7], and have also been implicated in the induction of sister-chromatid exchange in CHO cells[8].

Until recently there was no convincing evidence to suggest that phosphotriesters could be repaired in either prokaryotic or eukaryotic cells. McCarthy *et al.*[9] have now shown that *Escherichia coli*, upon exposure to sublethal doses of MNNG, can develop an inducible repair response, mediated by a methyltransferase protein, towards alkylated phosphate groups. This protein (MW 39kd) acts in a stoichiometric fashion by simply transferring a methyl group to one of its own cysteine residues, in a manner analogous to the action of *E. coli* O^6-methylguanine-DNA methyltransferase.

A surprising property of the protein, however, is that it acts on only half of the methyl phosphotriesters, using methylated synthetic double-

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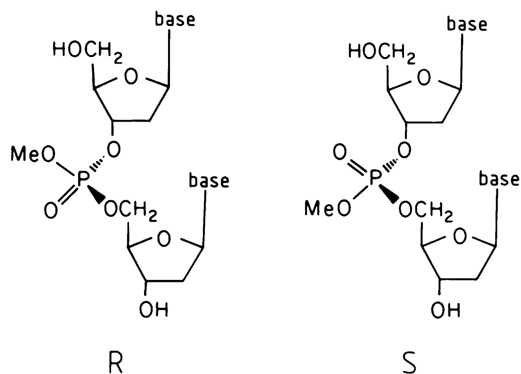


Figure 1. R- and S-phosphate (Rp and Sp) configurations of a methylated dinucleoside monophosphate.

stranded polymers or native DNA as substrate. We considered the possibility that this might reflect a stereospecific mechanism of action. The oxygen atoms in a phosphate group are tetrahedrally displaced about the phosphorus atom. Esterification of the phosphate group with three different moieties thus produces an isomeric pair which can be designated Rp and Sp (Fig. 1) after the manner of Cahn, Ingold and Prelog[10]; in the case of DNA phosphotriesters a diastereomeric pair is produced. In this communication we demonstrate, using poly(dT)(methylated):poly(dA) as the substrate, that only the Sp diastereoisomer of the methyl phosphotriester of TpT [i.e., Tp(Me)T] is subject to attack by the methyltransferase.

MATERIALS AND METHODS

Bacterial Cell Extracts

Two derivatives of *E. coli* B strain F26 were used: BS21 which is constitutive for the adaptive response[21], and BS23 which is an Ada^- mutant (kindly supplied by Dr. B. Sedgwick). Bacteria (3 g wet weight) were suspended in 12 ml of 70 mM HEPES-KOH (pH 7.8), 1 mM dithiothreitol and 5% glycerol and sonicated at 0°C with a Branson Sonifier Model 185. After centrifugation at 30,000 g for 15 min to remove cellular debris, extracts containing 7.5 mg protein per ml were stored in small aliquots at -70°C and once used were not refrozen.

Reagents

The following materials were purchased from the sources indicated: thymidine, 5'-(9-phenylxanthenyl)-thymidine and 1-(mesitylenesulphonyl)-3-nitro-

1,2,4-triazole from Cruachem Products, Oregon; 2-chlorophenyl dichlorophosphate and caesium fluoride from Aldrich; poly(dT) and poly(dA) from P-L Biochemicals; [^{3}H]-labeled *N*-methyl-*N*-nitrosourea from New England Nuclear; deoxyribonuclease I from Worthington; bacterial alkaline phosphatase and calf spleen phosphodiesterase from Sigma; and *Crotalus durissus* venom phosphodiesterase from Boehringer Mannheim.

Acetonitrile, pyridine and methanol were dried by heating, under reflux, with calcium hydride for 16 h, then distilled at atmospheric pressure, and stored over molecular sieves (Linde Type 4A).

Synthesis of Tp(Me)T

The 2-chlorophenyl phosphotriester of 5'-(9-phenylxanthenyl)-TpT was synthesized by the procedure of Chattopadhyaya and Reese[11]. To replace the chlorophenyl moiety with a methyl group, a modification of the method described by Ogilvie and Beaucage[12] was used as follows. Caesium fluoride (152 mg, 1 mmol) was added to a solution of the 5'-protected 2-chlorophenyl TpT (0.1 mmol) dissolved in a mixture of anhydrous methanol (1 ml) and dichloromethane (1 ml), and the reaction mixture was stirred at room temperature under dry nitrogen for 20 min. Solvent was then evaporated under reduced pressure, after which the residue was redissolved in dichloromethane (2 ml). Undissolved caesium fluoride was removed by filtration through a Kieselguhr pad. After evaporation of the dichloromethane, the residue was redissolved in chloroform (1 ml), and the product was purified by short column chromatography[13] on TLC grade silica gel (Merck 60H), eluting with chloroform/ethanol (19:1). The 9-phenylxanthenyl group was removed by hydrolysis in 80% acetic acid at 37°C for 30 min.

The diastereoisomers of Tp(Me)T were separated by HPLC on a Whatman Partisil 10 ODS-2 column (4.6 x 250 mm), eluting with 24% MeOH/H_2O at a flow rate of 0.8 ml/min.

Preparation of Poly(dT)(methylated):poly(dA)

5 A_{260} units of poly(dT) were dissolved in 0.2 ml of 0.3 M *N*-(2-hydroxyethyl)-piperazine-*N*'-3-propanesulphonic acid buffer (EPPS; pH 8.6), and to this was added 1 mCi of [^{3}H]-MNU contained in 0.05 ml of absolute alcohol. The mixture was incubated at 37°C in a tightly sealed Eppendorf tube for 2 h. The alkylated polymer was precipitated by addition of 0.05 ml 1 M sodium acetate (pH 5.0) and 0.6 ml of cold ethanol. After centrifugation, the pellet was dissolved in 1 ml of 70 mM HEPES-KOH (pH 7.8), 0.1 M NaCl, 1 mM EDTA and dialysed against a litre of this buffer at 4°C for 24 h, and then twice more against a litre of the same buffer without NaCl. Finally, the methylated

poly(dT) was annealed to 5 A_{260} units of poly(dA) to give a double-stranded substrate with a specific activity of approximately 1.25 x 10^6 cpm/mg.

Methyltransferase Assay

The procedure of McCarthy *et al.*[9] was followed except that proteinase K (1 mg/ml) was used in place of protease VI.

Analysis of Phosphotriester Removal

To about 5 x 10^3cpm of the methylated substrate in 0.19 ml 70 mM HEPES-KOH (pH 7.8), 1 mM EDTA, 1 mM dithiothreitol was added either 0.01 ml of bacterial lysate (0.075 mg protein) or 0.01 ml of 70 mM HEPES-KOH (pH 7.8), 1 mM dithiothreitol, 5% glycerol. After incubation of the reaction mixtures at 37°C for 10 min, the DNA was precipitated by addition of 0.021 ml of 2 M sodium acetate and 0.66 ml of cold ethanol. The pellet was dissolved in 0.15 ml of 70 mM HEPES-KOH (pH 7.8), 1 mM dithiothreitol, 25 mM $MgCl_2$ and the DNA was digested with 100 units of DNase I (dissolved in 0.05 ml H_2O) for 1 h at 37°C. Alkaline phosphatase (0.05 mg), venom phosphodiesterase (0.025 mg) and spleen phosphodiesterase (0.05 mg) were next added, and incubation continued for a further 3 h. Protein was removed by centrifugation following its precipitation by addition of 3 volumes of cold ethanol. The volume of the supernatant was reduced to approximately 0.03 ml before loading the labeled products, together with the UV marker for Tp(Me)T, onto the HPLC column. Chromatographic conditions were the same as those given above.

Phosphorus NMR Spectra

The purified isomers (ca. 1 mg) were each dissolved in 3 ml of DMSO-d_6 and 4 mg of 8-hydroxyquinoline was added. Spectra were recorded on a Bruker AM-500 spectrometer operating at 202 MHz for phosphorus. Sweep widths of 5000 Hz were used employing 8K of data points. The FID was exponentially multiplied using a line broadening of 2 Hz, zero filled to 32K, and transformed. The reference was external phosphoric acid (85%).

Optical Spectroscopy

Ultraviolet spectra were recorded on a Cary 17 spectrophotometer and a JASCO J40 CS spectropolarimeter was used for CD measurements. Temperature was monitored by a Comark electronic thermometer with the thermocouple probe inserted directly into the solution.

The CD spectra are reported in terms of a dissymmetry factor (g-factor)[14] defined by:

$$g_\lambda = \frac{\Delta A_\lambda}{A_{max}}$$

where ΔA_λ and A_{max} are the differential (between left- and right-handed cir-

cularly polarized light) and isotropic optical densities, respectively, of the same solution in the same path-length cell; whilst the former is at the quoted temperature, the latter is at 21°C. Since the g-factor is a dimensionless quantity, results can be obtained without having to determine the extinction coefficient of the sample. As it is the general shape of the CD versus temperature curve that is of interest, quoting spectra in terms of this g-factor is sufficient.

RESULTS

The purpose of this investigation was to ascertain whether the observation of McCarthy and coworkers[9] that only 50% of methyl phosphotriesters are repaired by the *E. coli* methyltransferase has a stereochemical basis and, if so, which of the two diastereoisomers is a substrate for the protein. This required (i) synthesis of Tp(Me)T and chromatographic separation of its two diastereomers, (ii) determination of their metabolic fate following incubation of poly(dT)(methylated):poly(dA) with an extract from *E. coli* strain BS21, and (iii) assignment of the configuration of each isomer.

Synthesis of Tp(Me)T and Isolation of the Isomers

The synthesis of the methyl ester of TpT was based on the transesterification protocol developed by Ogilvie and Beaucage[12] involving a fluoride-catalysed exchange of a phenyl group for a methyl group. In this instance, the 2-chlorophenyl group was used because it is a superior leaving group compared to the unsubstituted analogue, thereby reducing reaction times from 40 h to 20-30 min.

Reverse-phase HPLC provided almost baseline separation of the two diastereoisomers (Fig. 2a). Since the retention times of the isomers were very sensitive to small changes in the alcohol concentration in the eluant, optically detectable amounts of Tp(Me)T were always included in the assays as a UV marker.

Activity of *E. coli* Extract on Poly(dT)(methylated):poly(dA)

Figure 2a shows the elution profile of poly(dT)(methylated):poly(dA) digested by DNase I, venom and spleen phosphodiesterases and alkaline phosphatase. The total cpm for each peak and its ratio with respect to 3-methylthymidine (which is not demethylated by the bacterial extract) are given in Table 1. The assignment of O^2-methylthymidine, 3-methylthymidine and O^4-methylthymidine was based on similar chromatograms published by Ahmmed and Laval[15] and Dolan *et al.*[16]. Our ratio of 2:1 for triester:3-methylthymidine content in the methylated synthetic DNA differed substantially from the 12:1

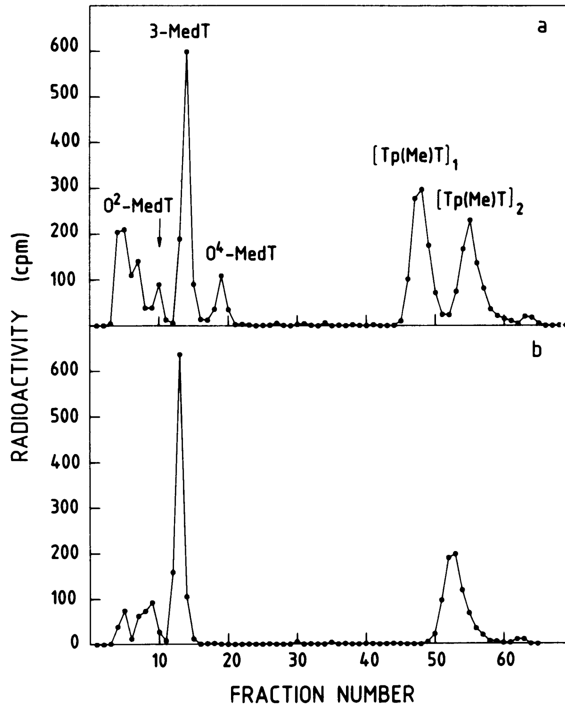


Figure 2. Reverse-phase HPLC analysis of indicated radiolabeled alkylation products released from poly(dT)(methylated):poly(dA) during incubation without (panel a) or with (panel b) a crude extract from *E. coli ada*$^+$ strain BS21, as revealed by subsequent digestion of the methylated substrate with DNase I, venom and spleen phosphodiesterases and alkaline phosphatase. Note that the incubation (10 min) with the bacterial extract (75 µg protein) resulted in the complete loss of O^4-methylthymidine (O^4-MedT) and [Tp(Me)T]$_1$ without any significant reduction in the levels of the remaining MNU-induced products, namely, O^2-methylthymidine (O^2-MedT), 3-methylthymidine (3-MedT) and [Tp(Me)T]$_2$.

Table 1. Removal of alkylated products from poly(dT)(methylated): poly(dA) by a crude extract of *E.coli* strain BS21

Alkylation product	Incubation without bacterial extract		Incubation with bacterial extract		Percent removed
	cpm	product/3-MedT	cpm	product/3-MedT	
3-MedT	891	1.000	915	1.000	–
O^2-MedT	141	0.158	155	0.169	0
O^4-MedT	196	0.220	4	0.004	98
[Tp(Me)T]$_1$	958	1.075	3	0.003	100
[Tp(Me)T]$_2$	798	0.895	765	0.836	7

ratio of McCarthy *et al.*[9] and presumably reflects the different procedures used to methylate DNA.

Analysis of the substrate, after incubation with a 5-10 fold excess of bacterial extract (as determined by the methyltransferase assay) prepared from the constitutive mutant and subsequent enzyme digestion (Fig. 2b), revealed the complete removal of the less retained triester diastereoisomer, $[Tp(Me)T]_1$, and O^4-methylthymidine, but only a small reduction of $[Tp(Me)T]_2$. Even when incubation with the extract was continued for 5 h, the original level of $[Tp(Me)T]_2$ was essentially maintained. Thus repair of methyl phosphotriesters by this extract was stereospecific. Incubation with the BS23 (Ada^- mutant) extract, on the other hand, did not affect either O^4-methylthymidine or phosphotriester content (data not shown).

Stereochemical Assignment of the Diastereoisomers

The configurations of $[Tp(Me)T]_1$ and $[Tp(Me)T]_2$ were established by two independent techniques, namely, phosphorus NMR and circular dichroism. We observed that purified $[Tp(Me)T]_1$ and $[Tp(Me)T]_2$, dissolved in DMSO, have chemical shifts of 0.45 ppm and 0.36 ppm, respectively. Potter *et al.*[17], in a study not involving separation of the isomers, had determined that under similar solvent conditions the phosphorus atom of the Sp isomer of Tp(Me)T resonates at lower field than that of the Rp isomer. $[Tp(Me)T]_1$ was therefore identified as the Sp diastereomer and $[Tp(Me)T]_2$ as the Rp isomer. The difference of 0.09 ppm was the same as that found by Potter and coworkers, although their chemical shift values for the two isomers (i.e., 0.69 and 0.60) were slightly different from ours, probably because of differences in concentration of the isomers and 8-hydroxyquinoline.

Confirmation of the NMR-derived designations was obtained from a circular dichroism analysis of the isolated isomers. The shape of a CD versus temperature curve is related to the cooperativity involved in the base stacking process[18], and it is therefore possible to qualitatively ascertain the relative ability of a particular dinucleoside monophosphate, within a well defined set, to adopt a fully stacked conformation. If the TpT conformation giving rise to base stacking in solution is a B-DNA-like structure, the phosphate oxygen atom, which on methylation yields the Rp isomer, is in closer proximity to the bases and sugar rings than the other phosphate oxygen, as is evident in a CPK space-filling model (Fig. 3a). NMR data obtained at room temperature[19] indicate that TpT exists predominantly in an extended conformation; however, the g_{max} versus temperature curve for the parent dinucleoside monophosphate (see Figs. 4 and 5) strongly suggests that a proportion of the

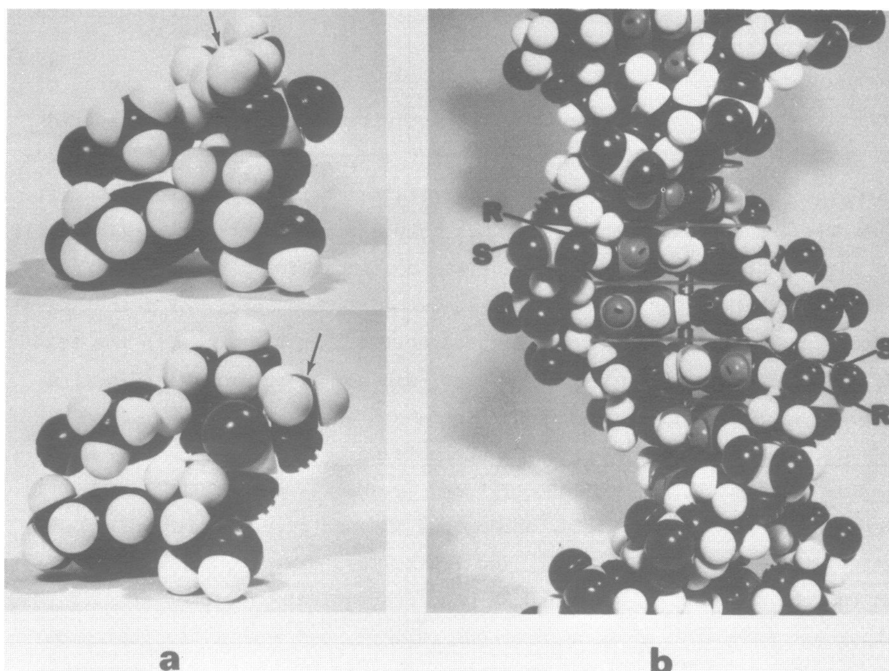
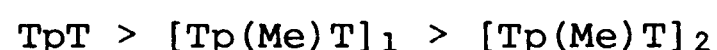


Figure 3. CPK space-filling models. (Panel a) The two diastereoisomers of Tp(Me)T in a pseudo-B-DNA configuration illustrating the greater steric crowding of the methyl group in the Rp-isomer (upper) than in the Sp-isomer (lower). Phosphotriester methyl groups are indicated by the arrows. (Panel b) View into the major groove of the B-DNA double helix, based on the 12 base-pair structure solved by Dickerson and coworkers[23]. The Rp-prochiral oxygens of the phosphate backbone are shown to lie in the major groove whereas the Sp oxygens protrude almost perpendicular from the helix.

molecules adopt a stacked structure especially at low temperatures. Hence the diastereoisomer that exhibits more disruption to base stacking with respect to the parent compound is in all likelihood the Rp isomer. A comparison of the g_{max} versus temperature curves (Fig. 5) indicates that the relative ability of the compounds to adopt the stacked state geometry is:



$$TpT > [Tp(Me)T]_1 > [Tp(Me)T]_2$$

On this basis $[Tp(Me)T]_2$, having the shallowest gradient, is assigned the Rp configuration and $[Tp(Me)T]_1$ the Sp one.

DISCUSSION

We have established that the removal of DNA methyl phosphotriesters by an extract from an *E. coli* strain constitutive for the adaptive response is

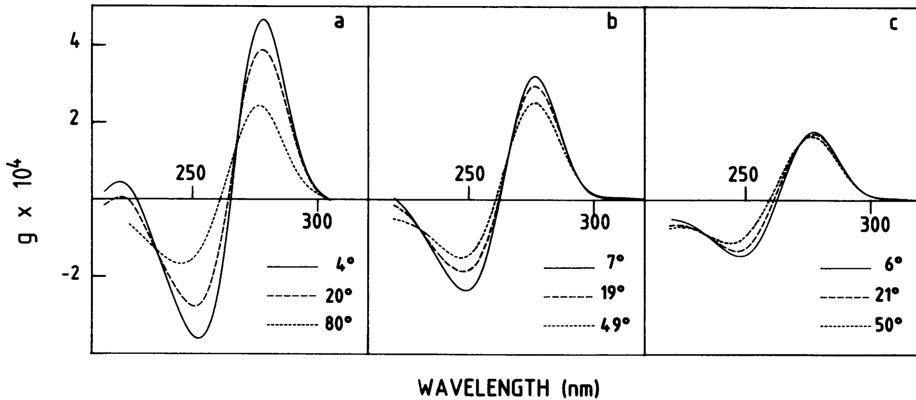


Figure 4. Variable temperature CD spectra recorded for the parent dinucleoside monophosphate TpT (panel a) and its two diastereoisomeric methyl phosphotriesters, $[Tp(Me)T]_1$ (panel b) and $[Tp(Me)T]_2$ (panel c).

mediated in a stereospecific manner, and that it is only the triesters in the Sp configuration that are subject to the action of the methyltransferase. During the course of our studies others have reported that this protein is the same as the one responsible for repairing O^6-methylguanine and O^4-methylthymine residues (i.e., the *ada*$^+$ gene product), but different cysteine resi-

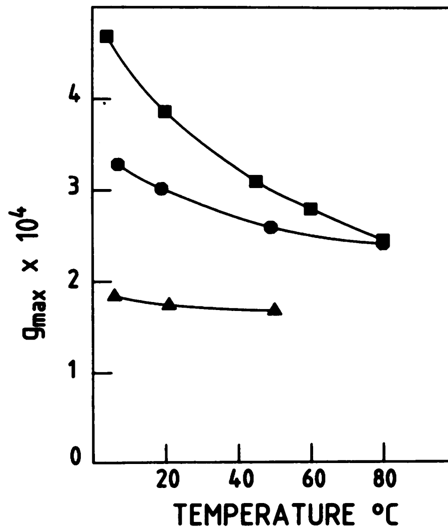


Figure 5. Positive maximum CD (g_{max}) versus temperature curves for TpT (■), $[Tp(Me)T]_1$ (●) and $[Tp(Me)T]_2$ (▲).

dues in the protein act as acceptors for the methyl groups from phosphotriesters and alkylated base residues.[20,22]

It is not clear why only the Sp triesters are repaired by the bacterial extract. Possibly a separate system for handling the Rp triesters exists *in vivo*, but is lost in the preparation of the extract. An alternative explanation is that the Rp esters are not as detrimental to the bacteria as the Sp isomers and consequently do not need to be removed. While phosphotriesters are not promutagenic lesions *per se*, they are known to inhibit a variety of enzymes involved in DNA processing (as outlined in the Introduction), and thus may reduce base-pairing fidelity. For those enzymes requiring the negative charge on the phosphate, such as exo- and endonucleases, either phosphotriester configuration must be considered deleterious. However, for enzymes that use the DNA as a template rather than as a substrate (e.g., polymerases and rec A), it is possible that an Sp isomer would be a greater impediment because its methyl group protrudes more prominently from the backbone (see Fig. 3b). An experiment by Miller and coworkers[4], in which DNA templates containing specific ethyl phosphotriester stereoisomers were prepared, revealed that one isomer inhibits *E. coli* DNA polymerase I more significantly than the other. Although the authors did not assign the configuration, the isomer which resulted in slower polymerisation was shown to be the less retained on an ODS-2 reverse-phase column. We (M.W. and A.F.D.) have found that for a series of ethyl phosphotriester diastereomers the Sp configuration was in all cases the less retained of the isomers (in preparation).

McCarthy and Lindahl[20] have proposed that the transfer of methyl groups from phosphotriesters to the Ada protein has a regulatory role in the adaptive response. If this is the sole purpose of the transfer then it would not be necessary to remove methyl groups from both isomers.

Clearly, further experimentation, both *in vivo* and *in vitro*, is required to determine the biological significance and metabolic fate of DNA phosphotriesters.

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