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Synthesis of site specific 1, n^2-ethenodeoxyguanosine in a small oligonucleotide

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SYNTHESIS OF SITE SPECIFIC 1, N^2-ETHENODEOXYGUANOSINE IN A SMALL OLIGONUCLEOTIDE

by

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KAC

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LIST OF ABBREVIATIONS

MDA	Malondialdehyde
PGH_2	Prostaglandin A_2
DNA	Deoxyribonucleic acid
PyP	3-(β-D-pentofuranosyl)-pyrimido [1, 2-a] purine-10-(3H)-one
PyP-dR	3-(β-D-pentofuranosyl)-pyrimido [1, 2-a] purine-10-(3H)-one 2'-deoxyribose
7-Me-PyP	7-Methyl-3-(β-D-pentofuranosyl)-pyrimido [1, 2-a] purine-10-(3H)-one
α-Me-MDA	α-Methyl-malondialdehyde
RNA	Ribonucleic acid
7-Me-PyP-dR	2'-Deoxy-7-methyl-3-(β-D-pentofuranosyl)-pyrimido [1, 2-a] purine-10-(3H)-one
HPLC	High Performance Liquid Chromatography
BRL	Bethesda Research Laboratories
α-Me-TEP	α-Methyl-1, 1, 3, 3-tetraethoxypropane
Na-α-Me-MDA	Sodium α-methyl-malondialdehydate
dG	2'-Deoxyguanosine
DMSO	Dimethyl Sulfoxide
CH_3CN	Acetonitrile
C_{18}	Octadecyl bound to silica
UV	Ultraviolet
NMR	Nuclear Magnetic Resonance
THF	Tetrahydrofuran
TEAB	Triethylammonium bicarbonate
MeOH	Methanol
3'-MPdG	3'-Monophosphoryl-2'deoxyguanosine
7-Me-PyP-pdRp	3', 5'-Bisphosphoryl-2'-Deoxy-7-methyl-3-(β-D-pento-furanosyl)-pyrimido [1, 2-a] purine-10-(3H)-one
pdGp	3', 5'-Bisphosphoryl-2'-deoxyguanosine
TEAA	Triethylammonium acetate
pdεGp	3', 5'-Bisphosphoryl-1, N^2-etheno- 2'-deoxyguanosine
PEG	Polyethylene glycol

I. INTRODUCTION

A. Malondialdehyde (MDA)

MDA (1, 3 - propanedial) is a three carbon dialdehyde shown to be produced in significant quantities both *in vitro* and *in vivo*.[1] It was first detected as an end-product of lipid peroxidation, oxidation of unsaturated fatty acids[2] (Figure 1). MDA is also produced enzymatically as a side product of prostaglandin biosynthesis. Prostaglandins are synthesized *in vivo* by the oxidative cyclization of polyunsaturated fatty acid.[3,4] The cyclic peroxide (PGH_2), derived from arachidonic acid, is the key intermediate which degrades to MDA by two different prostaglandin synthases, thromboxane synthase[5] and prostacyclin synthase[6]. Thromboxane synthase generates one molecule of MDA for every prostaglandin, thromboxane A_2, formed (Figure 2).

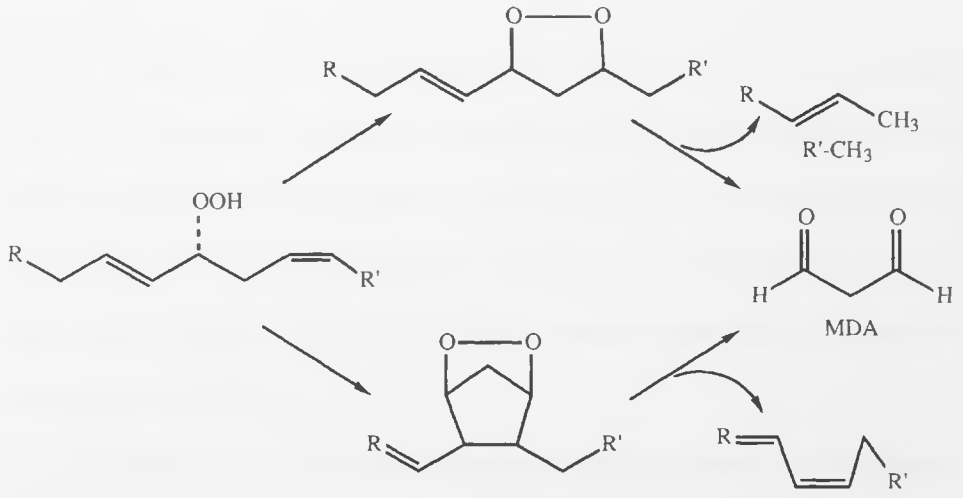


Figure 1. Non-enzymatic formation of MDA

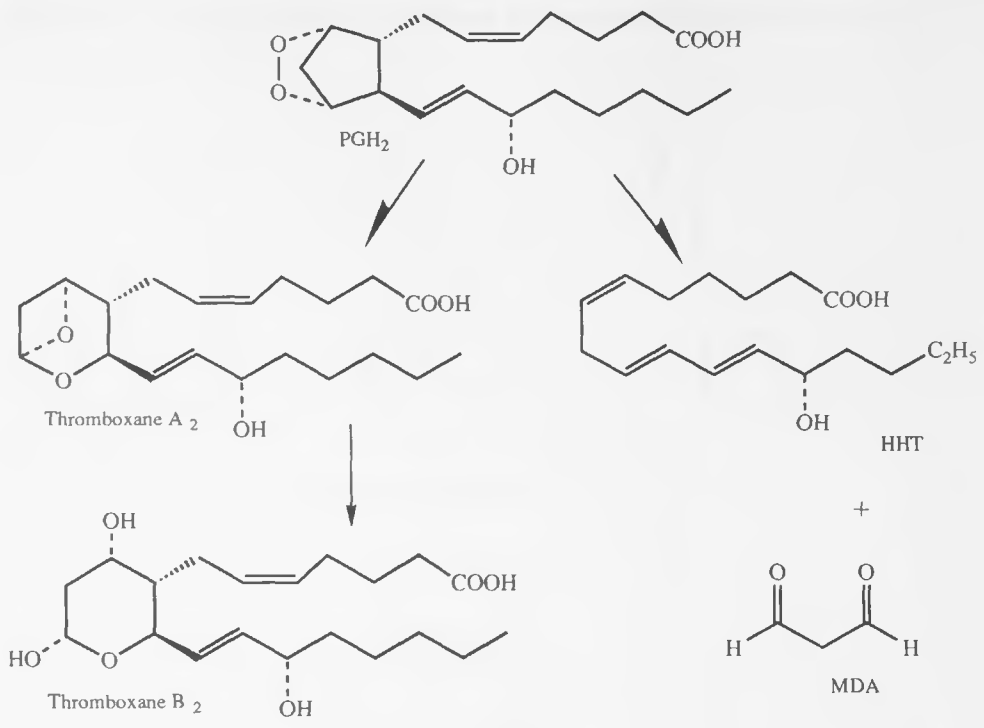


Figure 2. Enzymatic formation of MDA via thromboxane synthase

Chemical modifications of nucleosides followed by replication of the mutational event are believed to initiate carcinogenesis. MDA has been reported as a carcinogen[7], a mutagen[8] and as being toxic[7,9]. Its mutagenicity has been well characterized both in bacterial[10] and mammalian cells.[11] Because of its genotoxicity, mutagenicity, and reaction with nucleic acid bases[12], MDA has sparked a great deal of interest in its potential carcinogenicity.

Among structurally-related compounds, MDA is unique in that it produces frameshift mutations whereas the others such as methyl glyoxal and acrolein, induce base-pair substitutions.[13] MDA forms several different adducts with deoxyribonucleo-

sides but it is not certain which adduct causes the frameshift mutations (Figure 3).

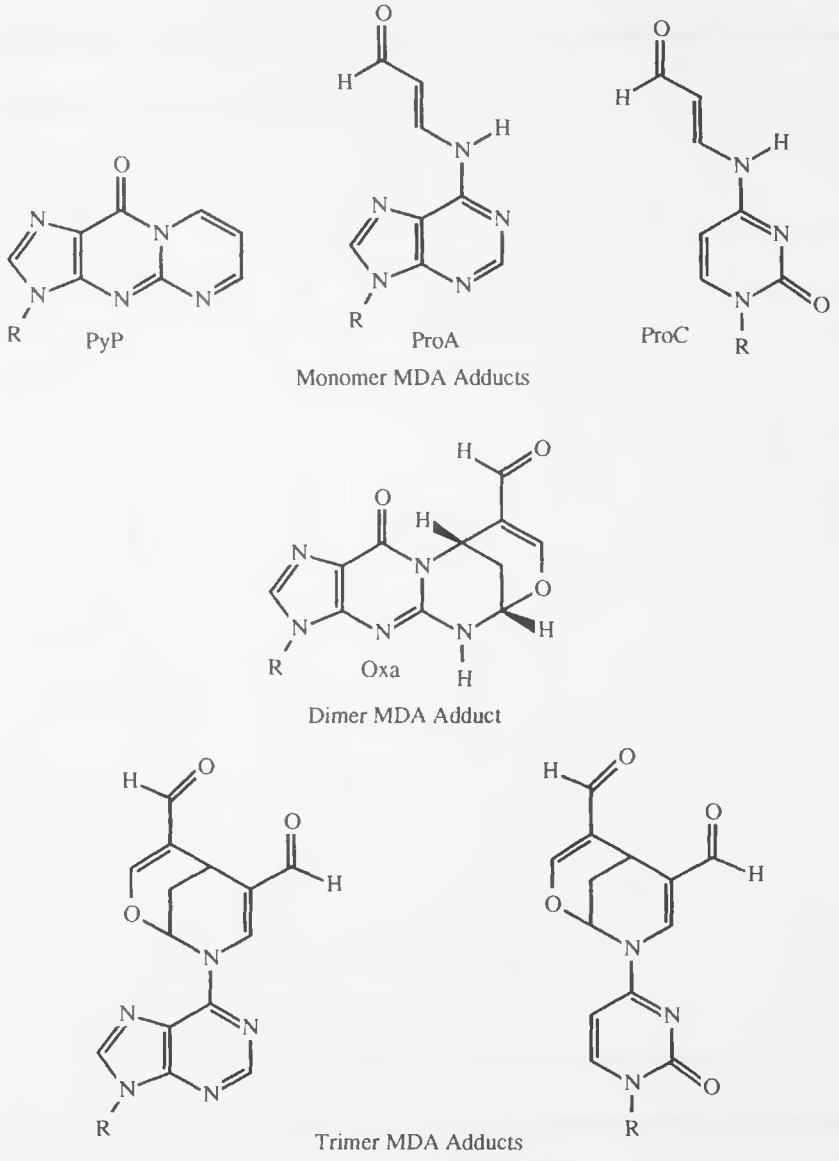


Figure 3. Structures of MDA adducts to guanosine, adenosine and cytosine

The adducts can be divided into two main categories; monomer and polymer. Monomer adducts are formed from a single molecule of MDA, while polymer adducts

are formed from polymers of MDA. MDA polymerizes due to its unique ability to exist as an enolate ion at physiological pH (pKa = 4.46 at 25°C[12]). The enolate ion serves both as an electrophile (elec) and a nucleophile (nuc) which readily condense to form dimers, trimers, and other polymers (refer to Figure 4).

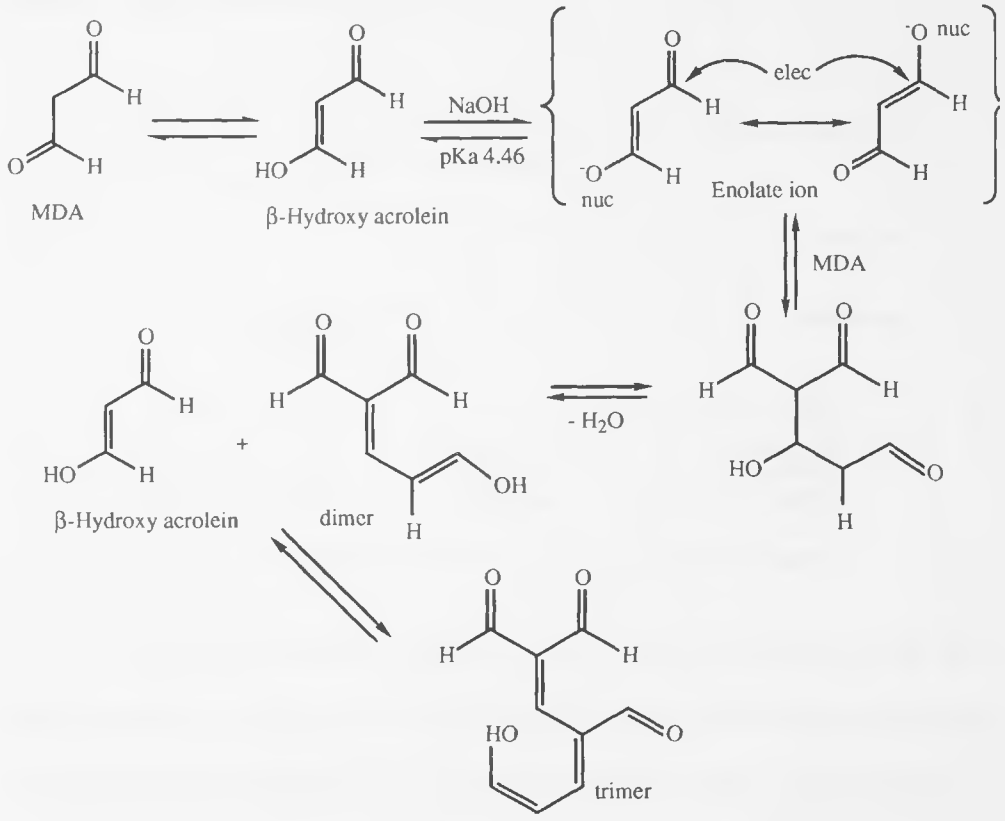


Figure 4. MDA dimer and trimer formation

Ideally, a single adduct on a replicating genome could simulate the *in vivo* process by which MDA mutagenesis would occur. With recent advances in oligonucleotide chemistry and gene splicing, techniques have been developed which construct biologically active genomes with individual DNA adducts at defined sites. This technique is

known as site-specific mutagenesis.

The most efficient method for constructing a singly-modified genome utilizes a gapped heteroduplex, an approach developed by Romano and coworkers[14]. The gap in the genome provides the sequence into which a singly adducted complementary oligonucleotide is ligated (refer to Figure 5).

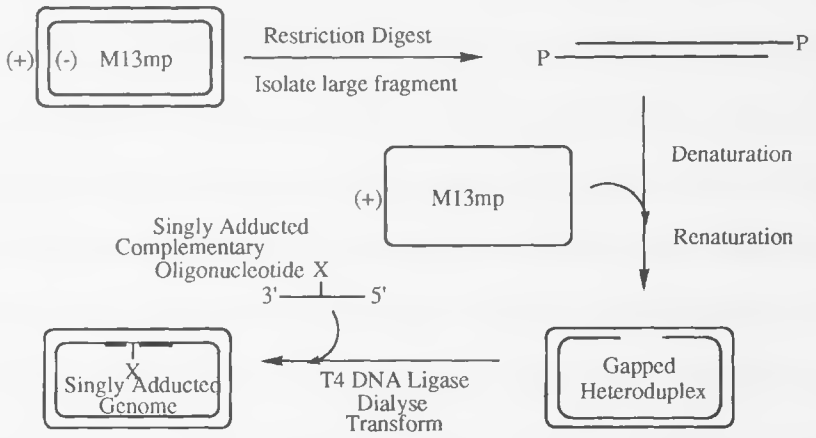


Figure 5. M13 gapped heteroduplex approach to site-specific mutagenesis

This study involves the synthesis of a small oligonucleotide with a single MDA derived adduct at a specific site. Once constructed the nucleotide could then be inserted into the gapped heteroduplex[15] for site-specific mutagenesis studies. From this technique, many questions dealing with the mechanism of mutagenesis could be answered: Of the numerous MDA adducts which adduct causes frameshift mutations? Is a specific site on the genome important for mutations?

The adduct of interest was rationalized as the 3-β-D-*erythro*-pentofuranosyl-pyrimido[1, 2-*a*]purin-10(3H)-one (PyP in Figure 3) adduct because of the following observations: 1. β-substituted acroleins with good leaving groups cause frameshift muta-

tions and are thus believed to have a similar mechanism of adduct formation as MDA[16]; 2. Acridine dyes, planar aromatic molecules, which are very similar in structure to PyP, also cause frameshift mutations. 3. Of the four bases in DNA, 2'-Deoxyguanosine is the most reactive towards MDA; and 4. The adducts found *in vitro* formed from dimers and trimers of MDA do not seem plausible for an *in vivo* study since MDA is very reactive.

Constructing the PyP oligonucleotide proposes many synthetic challenges. MDA adducts to deoxyguanosine are very unstable. They are sensitive to both acidic and basic conditions. MDA in solution pH > 5 is in its ionic form (pKa = 4.46 at 25°C)[17]. Once in the enolate form as β-hydroxy acrolein, MDA can form dimers and trimers (Figure 4) to result in additional adduct formation. (Refer to Figure 3 for adducts of dimer[18] and trimer [19] MDA). Yields for PyP-dR are reportably poor, 1.86%.[12] As a result of the numerous problems mentioned with MDA, 7-Me-PyP appears a good candidate to study. α–Me-MDA (used to form the 7-Me-PyP), unlike MDA, does not form polymers (dimers and trimers). The structure of 7-Me-PyP is very similar to PyP and therefore appears a viable substitution for PyP (Figure 6).

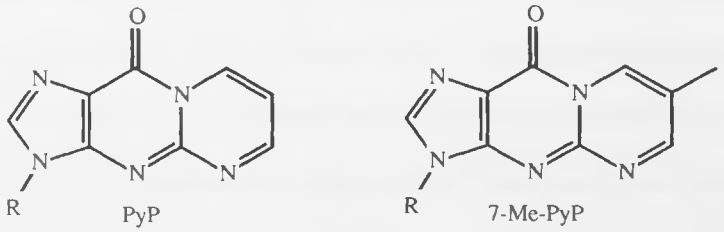


Figure 6. MDA adduct PyP structural comparison with 7-Me-PyP

B. T_4-RNA Ligase

The construction of the oligonucleotide with the site specific adduct wasœ accomplished enzymatically by a method developed by Brennan and Gumport[20] utilizing T_4-RNA ligase. The technique is especially useful for the synthesis of oligonucleotides with base labile adducts, such as PyP or 7-Me-PyP, since the reaction is carried out in a physiological pH. The uniqueness of the enzyme, T_4-RNA ligase, is its low selectivity which enables it to ligate DNA and adducted DNA as well as RNA.[21]

T_4-RNA ligase catalyzes the ligation of a donor, 5'-phosphate to an acceptor, 3'-OH. The acceptor must be at least 3 residues in length. The donor can be as small as one nucleoside, preferably 5', 3'-bisphosphorylated. The 3'-phosphate on the donor inhibits the ligation from continuing and prevents multiple ligations of the donor. The 3'-OH on the acceptor molecule is required for ligation.[22] Shown below is a simple equation for the ligation of the deoxyribonucleotide substrates employed.

$$\underset{\text{(acceptor)}}{\text{dCpdGpdC}} + \underset{\text{(donor)}}{\text{pdG*p}} \xrightarrow{T_4\text{-RNA Ligase}} \text{dCpdGpdCpdG*p}$$

The mechanism of T_4-RNA ligation[21] appears analogous to DNA ligation.[23] Two key intermediates are involved in the ligation process. Both are covalent complexes and have been isolated; the adenylylated enzyme[24] and the transfer of the adenyl group of the enzyme to the 5'-phosphate donor to form an ado-5'diphosphate-5'-oligonucleotide.[25,26] The mechanism is illustrated in Figure 7 with the substrates of interest.

The substrates employed are the adduct as mentioned, 7-Me-PyP-dR with phosphates in the 5' and 3' positions, and the trimer, dCpdGpdC. dCpdGpdC was

selected due to the occurrence of frameshift mutations in *Salmonella typhimuriunm hisD3052*, "GC"-rich regions.[27] With the use of these substrates an adducted oligonucleotide, dCpdGpdCpdεGp, can be utilized for further studies in site-specific mutagenesis.

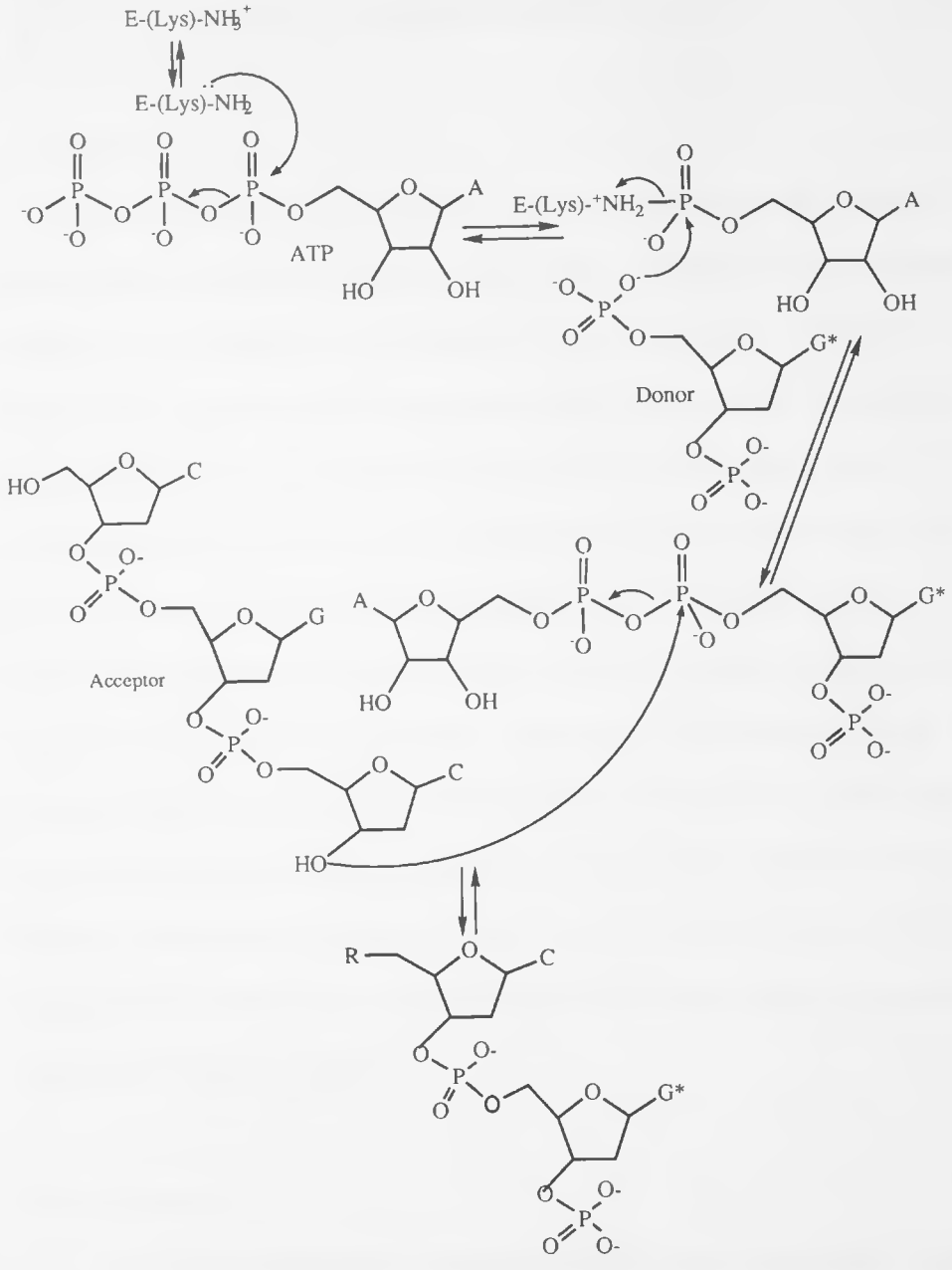


Figure 7. Mechanism of T_4-RNA ligation, E = T4-RNA Ligase, G* = Ethenoguanine

II. EXPERIMENTAL

A. Materials

Benzoyl chloride and crotonaldehyde were purchased from Aldrich. All HPLC grade solvents were obtained from American Scientific. 2-Methyl-1, 1, 3, 3- tetraethoxy propane was a gift from K. Stone, Wayne State University, Detroit. 3', 5'-Bisphosphoryl-2'-deoxyguanosine (Na^+) was purchased from Pharmacia LKB. Adenylate kinase (myokinase grade V, pig muscle dialyzed in 50 mM HEPES-NaOH, 5,000 units/ml), bovine serum albumin, creatine phosphokinase (type 1 rabbit muscle, 1750 units/ml), dithiothreitol, HEPES-NaOH, hexamine cobalt (III) chloride, phosphocreatine, pyrophosphoryl chloride (CAUTION: extremely corrosive), spermine, and triethylamine were all purchased from Sigma. Adenosine-5'-triphosphate (Na^+) and 2'-deoxyguanosine were obtained from United States Biochemical Corp. T_4-RNA ligase was purchased from three different companies with three different specific activities: Pharmacia (9000 units/ml), Bethesda Research Laboratories (6000 units/ml) and New England Biolabs (4000 units/ml). All other reagents were reagent grade and purchased from standard chemical companies.

B. Instrumentation

HPLC was performed on a Varian Vista 5000 LC with a Varian 2050 variable wavelength absorbance monitor. Fluorescence was monitored by the Kratos Fluorescence Detector. The columns used were a Beckman Ultrasphere ODS (5μ, C_{18}, 4.6 mm x 25 cm) for analytical purposes and an Altex Ultrasphere ODS (5μ, C_{18}, 10

mm x 25 cm) which was used for preparatory or large scale purifications. All analytical columns were run at a flow rate of 1 ml/min unless otherwise specified.

^{1}H-NMR (Proton Nuclear Magnetic Resonance) were recorded on a General Electric QE 300 Fourier transform spectrometer. dCdGdC was synthesized on a Beckman System 1 plus DNA Synthesizer at the Molecular Biology Center at Wayne State University.

C. Synthesis

1. α-Methyl Malondialdehyde (α-Me-MDA)[28]

In a 10 ml round bottom flask, 1.176 g (5.0 mmol) α-Me-TEP, 1 ml 1 M HCl, and 2.5 ml distilled H_2O were combined. The solution was stirred for 1 hr at 70°C. After cooling, 5 M NaOH was added dropwise to pH 9. The reaction mixture was evaporated *in vacuo* and the residue dissolved in 10 ml anhydrous ethanol. The solution was filtered to remove NaCl and concentrated *in vacuo* . The crude product was dissolved in a minimal amount of ethanol and benzene added dropwise to precipitate the sodium salt of α-Me-MDA. The mixture was stored at 0°C overnight and the white precipitate was filtered and washed with cold ethanol to yield 0.968 g Na-α-Me-MDA (70%).

^{1}H-NMR (300MHz, D_2O): δ (ppm) 1.54 (s, 3H, $-CH_3$), 8.45 (s, 2H, -CHO).

2. 2'-Deoxy-7-methyl-3-(β-D-pentofuranosyl)-pyrimido [1, 2-a] purine-10-(3H)-one (7-Me-PyP-dR).

The procedure was adapted from Stone[29] in which Na-α–Me-MDA 0.120 g

(1.40 mmol) was dissolved in 1 ml distilled H_2O and acidified to pH 3.5. α-Me-MDA was extracted three times with 4 ml of Et_2O. The ether extracts were combined and evaporated *in vacuo*, without heating. 2'-Deoxyguanosine (dG), 50.5 mg (0.189 mmol), was dissolved in 4 ml DMSO in a 10 ml round bottom flask and a few mg $MgSO_4$ added. The reaction was stirred for 24 hr at 50°C then placed on a high vacuum line (0.3 torr) to remove the DMSO.

The crude product was chromatographed on a Baker C_{18}, reverse phase MPLC column with 15% CH_3CN/H_2O at a flow rate of 4 ml/min . Fractions were collected by monitoring for yellow color and fluorescence. The reaction mixture was separated by HPLC on an analytical reverse phase C_{18} column (4 x 250 mm). Three UV-active (254 nm) peaks were resolved with 20 min of H_2O followed by a gradient of 0-15% CH_3CN/H_2O in 10 min. The first peak eluting at 23.5 min corresponded to starting material. The second peak eluted at 28.7 min and possessed fluorescence and NMR properties indicative of 7-Me-PyP. The third peak eluted at 32.5 min and possessed spectral properties characteristic of 7-Me-PyP-dR. The isolated yield was 25%. ^{1}H-NMR (300MHz, D_2O): δ (ppm) 2.4 (s, 3H, $-CH_3$), 2.6 (m, 1H, $H^{2"}$,), 2.8 (m, 1H, $H^{2'}$), 3.75 (m, 2H, $H^{5',5"}$), 4.2 (dt, 1H, $H^{4'}$), 4.6 (dt, 1H, $H^{3'}$), 6.4 (t, 1H, $H^{1'}$), 8.4 (s, 1H, H^2), 8.9 (s, 1H, H^6), 9.1 (s, 1H, H^8)

3. Bisphosphorylation Attempts on 2'-Deoxyguanosine

In a 5 ml pear-shaped round bottom flask, 7.3 mg dG (27.3 μmol), dried over P_2O_5 in a vacuum desicator overnight, 3ml dry THF, dry molecular sieves (3Å) and a

stir bar were cooled to -40°C (ethylene glycol/CO_2 bath). Pyrophosphoryl chloride, 100 µl (0.73 mmol, ρ = 1.85 g/ml) was then added (under N_2). After 5 hr, the reaction was cooled to -78°C (acetone/CO_2) and quenched with frozen 0.5 M triethylammonium bi-carbonate (TEAB), pH 7.5 then allowed to warm to room temperature.

The sample was evaporated *in vacuo*, redissolved in MeOH and evaporated *in vacuo* again (to remove excess TEAB). The crude product was diluted in 0.1 M TEAB, pH 7.5, filtered and run on an HPLC. The gradient used was 0-30% 0.1M TEAB pH 7.5/CH_3CN in 20 min on a Beckman reverse phase C_{18} analytical column (4 x 250 mm). Three major peaks were detected by UV (254 nm) and determined based on purchased standards (run on the same gradient) to be: 14.2 min, 3'-dGMP; 15.0 min, 5'-dGMP and at 15.6 min., dG.

4. Synthesis of β-Benzoyloxy-α-methylacrolein

In a dry 10 ml round bottom flask, 0.253 g (2.3 mmol) Na-α-Me-MDA, dried over P_2O_5 in a vacuum desicator, was suspended in 6 ml anhydrous THF. Benzoyl chloride, 0.326 g (2.3 mmol, 0.27 ml) was slowly added with vigorous stirring at ambient temperature. The mixture stirred for 1hr then the solid was filtered off (NaCl). The filtrate was evaporated *in vacuo* . The solid was redissolved in ether, washed quickly with cold sat'd $NaHCO_3$ and once with cold H_2O, dried with $MgSO_4$, and immediately filtered. White needles formed in the filtrate. The solid, β-benzoyloxy-α-methylacrolein, was filtered and weighed at 1.2 mg. The filtrate was evaporated *in vacuo* and recrystallized in ether/hexane. The second precipitate, was filtered for a total

yield of 0.2316 g (53%). ^{1}H-NMR (300MHz, $CDCl_3$): δ (ppm) 2.0 (s, 3H, -CH_3), 7.6 (m, 2H, *m*-Ph-H), 7.7 (dd, 1H, *p*-Ph-H), 8.2 (dd, 2H, *o*-Ph-H), 8.3 (s, 1H, C=CH), 9.6 (s, 1H, -CHO). The mass spectrum was also indicative of the product containing a molecular ion at 190, Ph-C≡O^+ at 105 and the phenyl cation at 77.

5. Attempted Synthesis of 7-Me-PyP-dR from β-Benzoyloxy-α-methylacrolein

In a 5 ml pear shaped round bottom flask, dG 31.0 mg (0.116 mmol, dried over P_2O_5 in a vacuum desicator) 0.5 ml dry DMSO, a few mg $MgSO_4$ and 27.2 mg β-benzoyl-oxy-α-methylacrolein (0.143 mmol) were combined and stirred for 4 days under N_2. The mixture was then vortexed and filtered to remove the benzoic acid and $MgSO_4$. The solution was placed on the rotary evaporator attached to a high vacuum pump to remove the DMSO.

A short MPLC column containing Baker reverse phase C_{18} packing (2.5 x 30 cm) eluted with 15% CH_3CN/H_2O at a flow rate of 4 ml/min the crude product. A peak eluted after 45 min which contained the characteristic yellow fluorescence of 7-Me-PyP-dR. The peak was collected and subsequently run on a reverse phase C_{18} Beckman analytical HPLC column (4 x 250 mm), 0-30% CH_3CN/0.1M TEAA pH 6.5 in 30 min. A band at 20 min coeluted with a 7-Me-PyP-dR standard. Unfortunately, ^{1}H-NMR revealed mostly TEA and benzoic acid. The product was quantitatively too small to distinguish.

6. Attempted Synthesis of 7-Me-PyP-pdRp by β-Benzoyloxy-α-methylacrolein

3', 5'-Bisphosphoryl-2'-deoxyguanosine (pdGp, 8.1 mg, 16 μmol) was placed in a 5 ml pear shaped round bottom flask with 0.5 ml dry DMSO, under N_2. β-Benzoyloxy-α-methylacrolein, 24.8 mg, was then added along with a few mg of $MgSO_4$. The reaction stirred at room temperature. After two days, the fluorescence characteristic of 7-Me-PyP formation was not evident so 3 ml of 0.1 M TEAA, pH 6.5 was added (in case benzoic acid formation was depurinating the starting material). After six days, the filtered reaction mixture was run on reverse phase C_{18} analytical HPLC column (4 x 250 mm), 0-30% CH_3CN/0.1 M TEAA pH 6.5 in 30 min. Fluorescence, indicative of the 7-Me-PyP adducts, (λ_{ex} = 342 nm, λ_{em} = 370 nm), was not detected.

7. Attempted Synthesis of 7-Me-PyP-pdRp from α-Me-MDA

pdGp (20.5 mg, 40 μmol) was placed in a 10 ml round bottom flask with 450 μl DMSO. (The bisphosphate did not dissolve completely). Na^+-α-Me-MDA, 150 mg, was hydrolyzed to the free acid form. The salt was dissolved in 1 ml H_2O and the pH lowered to 4.0. The acid, α-Me-β-hydroxyacrolein, was then extracted with 3 x 3 ml ether and the ether extracts were evaporated *in vacuo* without warming. DMSO (150 μl) was added to the α-Me-β-hydroxyacrolein. The α-Me-β-hydroxyacrolein/DMSO (50 μl) was added to the pdGp along with a few mg of $MgSO_4$. The reaction mixture

was then heated to 37°C.

After three days, the crude product was run on the HPLC reverse phase C_{18} preparatory column. The gradient used was 0-10% CH_3CN/ 0.1M TEAB (pH 7.5) in 30 min. Various peaks were collected and analyzed by ^{1}H-NMR. Peaks at 16.6 and 17.6 min appeared to contain Oxa and PyP yet the ^{1}H-NMR were not conclusive due to the low mass.

8. Synthesis of 3', 5'-Bisphosphoryl-2'-deoxy-1, N^2- ethenoguanosine (pdεGp)

2, 3-Epoxybutanal was prepared by the method of Wellman and co-workers.[30] It was obtained in a 40% yield and its ^{1}H-NMR corresponded to literature values.[31] ^{1}H-NMR (300MHz, $CDCl_3$): δ (ppm) 1.5 (d, 3H, $-CH_3$), 3.1 (m, 1H, H^3), 3.3 (m, 1H, H^2), 9.0 (d, 1H, -CHO).

3', 5'-Bisphosphoryl-2'-deoxyguanosine (10.3 mg, .02 mmoles) was placed in a 5 ml pear shaped round bottom flask. 2, 3-Epoxybutanal (10 µl) was added. Distilled water (2.6 ml) was then added and the pH was raised to 10. The reaction stirred vigorously for 3.5 hr at room temperature. Afterwards, the solution was neutralized with 0.1 M HCl, evaporated *in vacuo*, redissolved in 0.1 M TEAB pH 7.5, filtered and run on an HPLC. The crude mixture was separated on a Rainin DYNAMAX reverse phase C_{18} column (21.4 x 250 mm) with a gradient of 0 to 9% CH_3CN/0.1M TEAB pH 7.5 in 35 min.

The major peak eluting at 31.2 min appeared to be product, pdεGp, by ^{1}H-NMR. The product was reinjected onto the DYNAMAX HPLC reverse phase C_{18} col-

umn, collected then tested for purity on a C_{18} analytical column. The overall yield of the reaction was 35% (0.01695 mmoles). The ^{1}H-NMR was indicative of the ethenoguanosine adducts previously cited in literature.[32] ^{1}H-NMR (300MHz, D_2O) δ (ppm): 2.7 (m, 1H, $H^{2"}$), 3.0 (m, 1H, $H^{2'}$), 4.2 (d, 2H, $H^{5',5"}$), 4.5 (m, 1H, $H^{4'}$), 5.1 (m, 1H, $H^{3'}$), 6.5 (m, 1H, $H^{1'}$), 7.4 (d, 1H, H^7), 7.6 (d, 1H, H^6), 8.4 (s, 1H, H^2).

9. Synthesis of dCpdGpdCpdεGp

The T_4-RNA ligase reaction procedure was followed from Gumport[33-35] in which the nucleotides (pdεGp, 4 mM, ε_{285} ~12x103[32]), oligo (dCpdGpdC, 1mM), spermine (8 mM), phosphocreatine (40 mM), ATP (0.4 mM), and HCC (10 mM) were dried under vacuum in a 1.5 ml screw-top Eppendorf plastic tube. Upon completion of drying, the following were added to a total volume of 10 µl: 50 mM HEPES-NaOH (pH 7.9), 10 µg/ml BSA, 20 mM DTT, 5 mM $MnCl_2$, and H_2O (nano-pure), myokinase (~1690 units), phospho-creatine kinase (1750 units) and T_4-RNA Ligase(15-50 units). The reaction tubes were incubated at 17°C for 1-10 days (Heated H_2O bath in the cold room, tubes were submerged to avoid a change in concentration by evaporation or condensation).

Samples were prepared for the HPLC by first stopping the ligation with EDTA (9 µl EDTA for every 1 µl aliquote of reaction mixture) then precipitating the enzyme with CH_3CN/H_2O with a total percentage of 50% acetonitrile (e.g. For a 1 µl aliquote of ligation mixture, 9 µl EDTA, 15 µl H_2O and 25 µl CH_3CN were added). The Eppendorf tubes were spun for 5 min to pellet the enzyme. The supernatant was then removed and placed into a new tube and dried. The residue was redissolved in 50 µl H_2O and

injected onto the HPLC.

The mixture was separated on a reverse phase C_{18} analytical HPLC column. The solvent system used was a shallow gradient from 0-12% CH_3CN/50 mM TEAB pH 7.3 in 30 min. then a sharp increase from 12-30% CH_3CN/50 mM TEAB pH 7.3 in 5 min. The column was washed with 100% CH_3CN for 10 min after every injection. For a typical chromatigraph refer to Figure 8.

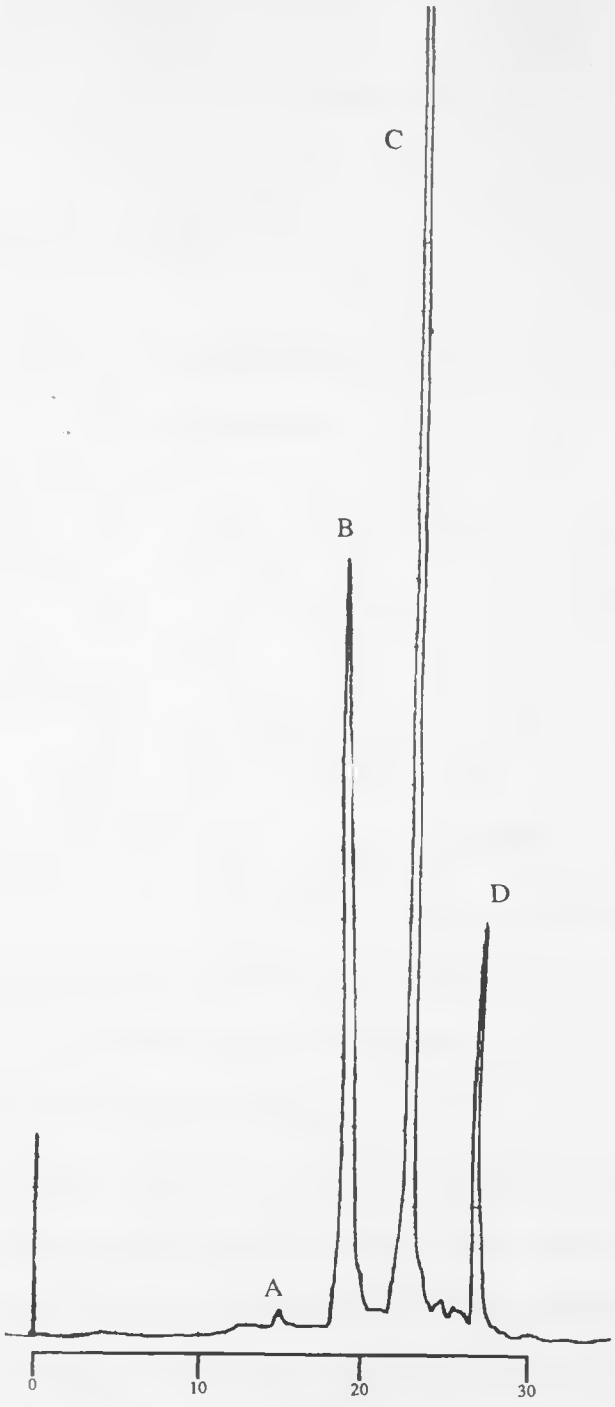


Figure 8. UV profile of reverse-phase C_{18} column separation. Samples were run with a gradient from 0-12% CH_3CN/50 mM TEAB pH 7.3 in 30 min. A = ATP, B = pdeGp, C = dCpdGpdC, D = dCpdGpdCpdGp

III. RESULTS

A. Synthesis of 7-Me-PyP-dR

α-Me-MDA was hydrolyzed and combined with 2'-deoxyguanosine to produce 7-Me-PyP-dR (refer to Figure 9).

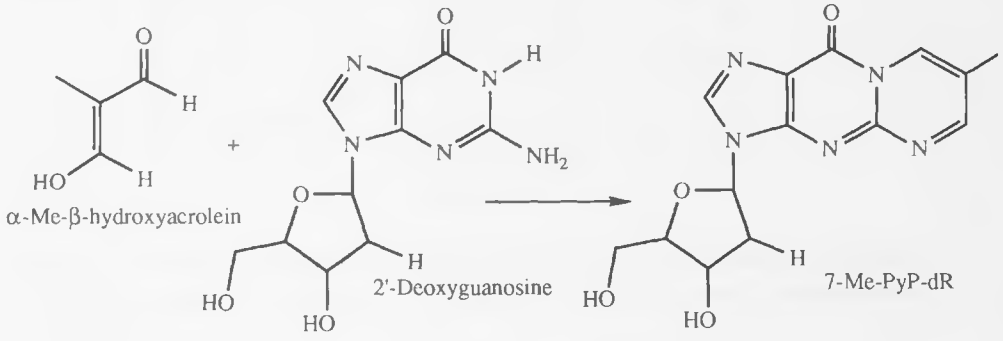


Figure 9. Synthesis of 7-Me-PyP-dR

Various conditions were utilized in order to maximize the yield of 7-Me-PyP-dR with as little depurination as possible. Two molecules of water are eliminated in the formation of PyP, therefore materials were included to enhance the removal of water (e.g. molecular sieves (3Å) and $MgSO_4$).

Apparently, water was not the only thing trapped in the molecular sieves. Reactions which contained molecular sieves (3Å) yielded less of the 7-Me-PyP-dR, the desired product, than reactions containing $MgSO_4$ (refer to Figure 10). Any reactions containing the molecular sieves also produced more depurinated product than 7-Me-PyP-dR (refer to Figure 11). $MgSO_4$ seemed to enhance the formation of product before it depurinated (refer to Figure 12).

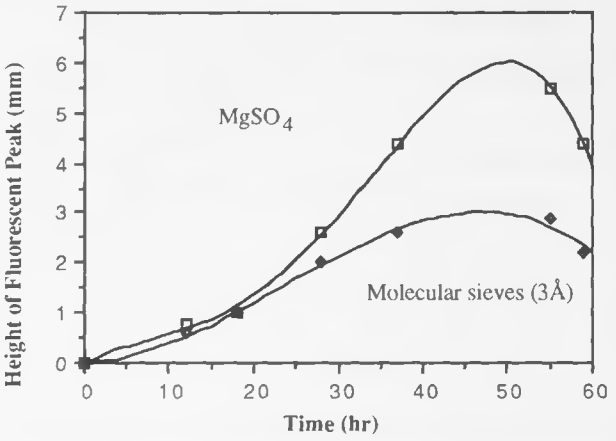


Figure 10. 7-Me-PyP-dR formation influenced by $MgSO_4$ and Molecular sieves (3Å). Formation is denoted by height of fluorescent (λ_{ex}= 342, λ_{em}= 370) Peaks eluting from C_{18} analytical HPLC column.

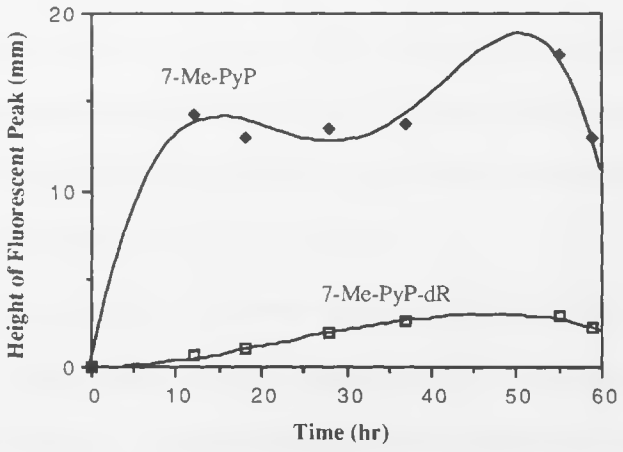


Figure 11. Molecular sieves enhanced production of depurinated over deoxyribose, 7-Me-PyP.

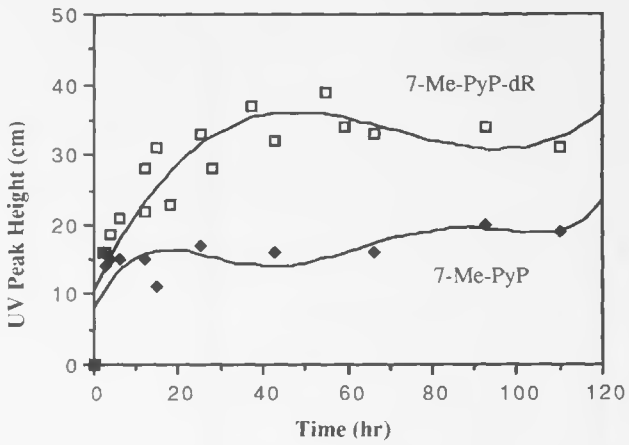


Figure 12. $MgSO_4$ enhanced production of 2-'deoxyribose (dR) over depurinated, 7-Me-PyP

A second approach to increasing the product yield was to vary the temperature. Heat seemed to increase the amount of product, but with the increase in temperature time became a crucial factor. At 50°C a maximum yield was obtained in one day but after six hours depurination became faster than product formation (refer to Figure 13).

Concentrating the reactants in DMSO improved the yield of 7-Me-PyP-dR. The estimated solubility of dG in DMSO was 0.115 g/ml and the estimated solubility of α-Me-β-hydroxyacrolein was 0.272 g/ml. The optimal concentrations were found to be 1 mM dG and 10 mM α-Me-β-hydroxyacrolein.

Maximum yield for 7-Me-PyP-dR was obtained under the following conditions; $MgSO_4$ addition, maintain temperature at 50°C for 6 hr, and highly concentrated reactants. The yield of 25% was much greater than a previously published yield of 1.86% for the formation of PyP-dR.[12]

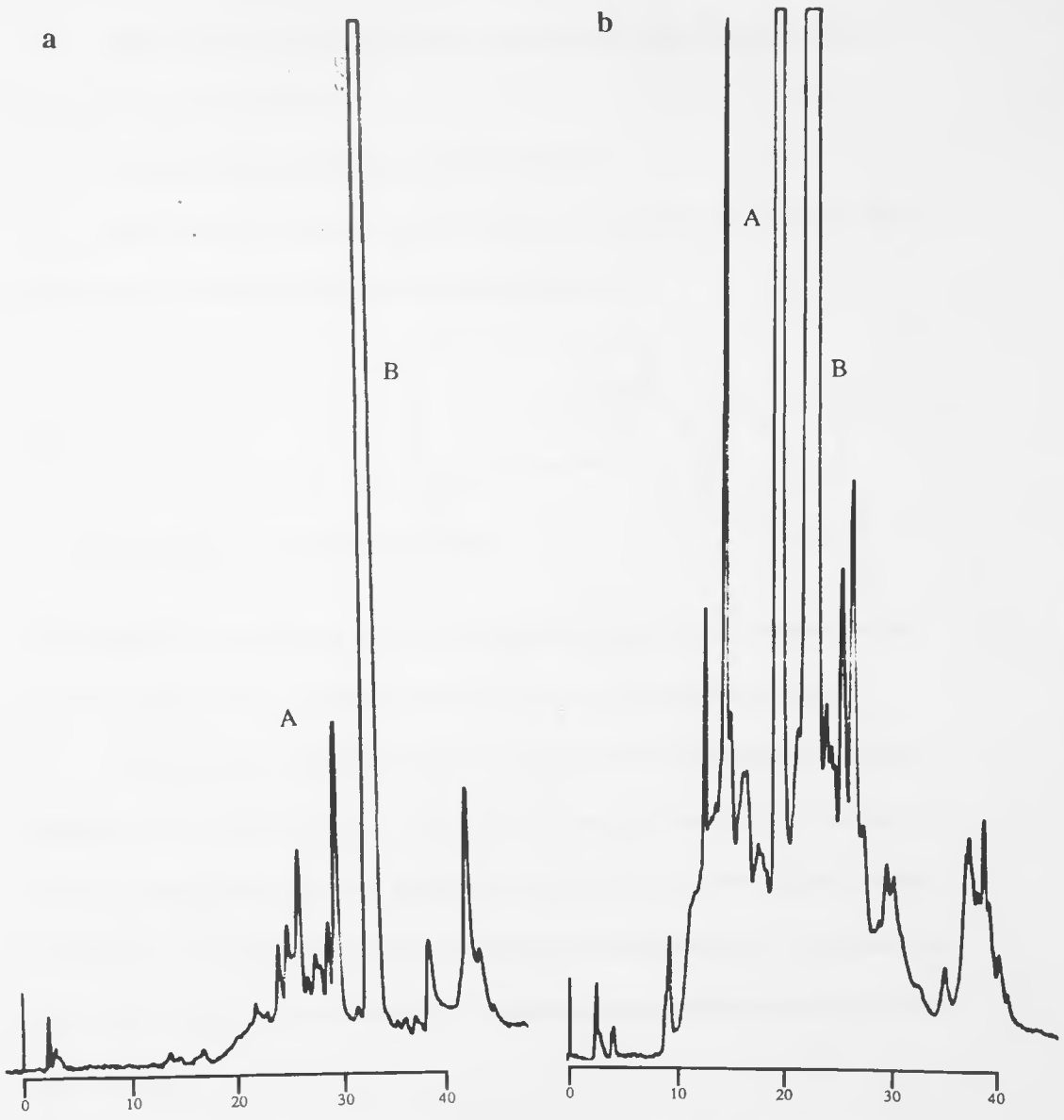


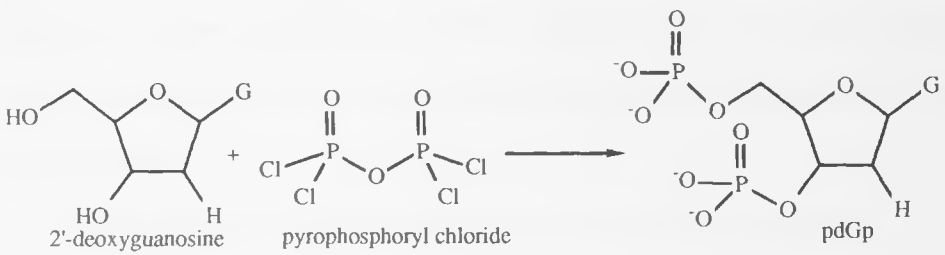
Figure 13. Time dependent depurination of 7-Me-PyP-dR at 50°C. Fluorescent profiles (λ_{em}= 370, λ_{ex}= 342 nm) on C_{18} Analytical column (flow = 1 ml/min) at a) 2 hr (0-15% CH_3CN/H_2O from 20-30 min) and b) 72 hr (0-15% CH_3CN/H_2O in 20 min). A = 7-Me-PyP, B = 7-Me-PyP-dR.

B. Attempts at the Synthesis of 7-Me-PyP-pdRp

Three different approaches for the preparation of 7-Me-PyP-pdRp were pursued. These are as follows:

1. Bisphosphorylation Attempts on 7-Me-PyP-dR.

Barrio, *et. al.*,[36] predicted the bisphosphorylation of dG and 7-Me-PyP-dR by pyrophosphoryl chloride as shown in the following reaction:



Once phosphorylated, pdGp or 7-Me-PyP-pdRp could be used as the substrate for the T_4-RNA ligase reaction. Numerous reactions were run to test this hypothesis.

Initial reactions employed Barrio's procedure for the bisphosphorylation of guanosine and 7-Me-PyP-R[36] with 7-Me-PyP-dR as starting material (see Experimental Section). Most experiments, upon addition of pyrophosphoryl chloride, turned instantly to a brown tar. This was due to incomplete drying of starting material. Of the reactions that worked, usually only two products were detected, starting material and depurinated product (14a).

Fearing that the adduct maybe enhancing the depurination, deoxyguanosine was used as a starting material instead of the 7-Me-PyP-dR. Results were still negative; deoxyguanosine did not form bisphosphate but depurinated. In order to discover the

reason for depurination, the pH of the reaction mixture before quenching the reaction mixture was tested (the mixture contained only the starting materials, dG and pyrophosphoryl chloride). The pH was found to be less than 2. Therefore, it seemed obvious that it was the low pH that caused the depurination and that this pathway was not a viable extension to form the substrate for the ligase reaction, 7-Me-PyP-pdRp from the 2'-deoxyribose, 7-Me-PyP-R.

Subsequent attempts were made to modify the procedure by using anhydrous solvents and greater concentrations of buffer. Even the best results did not lead to the desired bisphosphate product, but only produced the monophosphorylated product (refer to Figure 14b).

2. Attempted Synthesis of 7-Me-PyP-dR from α-Me-MDA and pdGp.

The procedure followed was similar to the above synthesis of 7-Me-PyP-dR in which α-Me-MDA was hydrolyzed to its acid form, α-Me-β-hydroxyacrolein, and added to pdGp in DMSO. Likewise a few milligrams of $MgSO_4$ were also added. Unlike the deoxyribose reaction the temperature was lower with a longer reaction time (37°C and 3 days) due to the lower stability of the phosphates. Unfortunately, the yields were too low to get conclusive identification of 7-Me-PyP-pdRp.

3. Attempted Synthesis of 7-Me-PyP-pdRp from β-Benzoyloxy-α-methylacrolein and pdGp.

Based on the fact that good leaving groups in the β-position of acroleins enhance the mutagenicity and therefore possible reactivity with nucleotides, β-Benzoyloxy-α-methylacrolein was used as a starting material to increase the yield of 7-Me-PyP-pdRp. The appearance of the adduct was not evident based on fluorescence

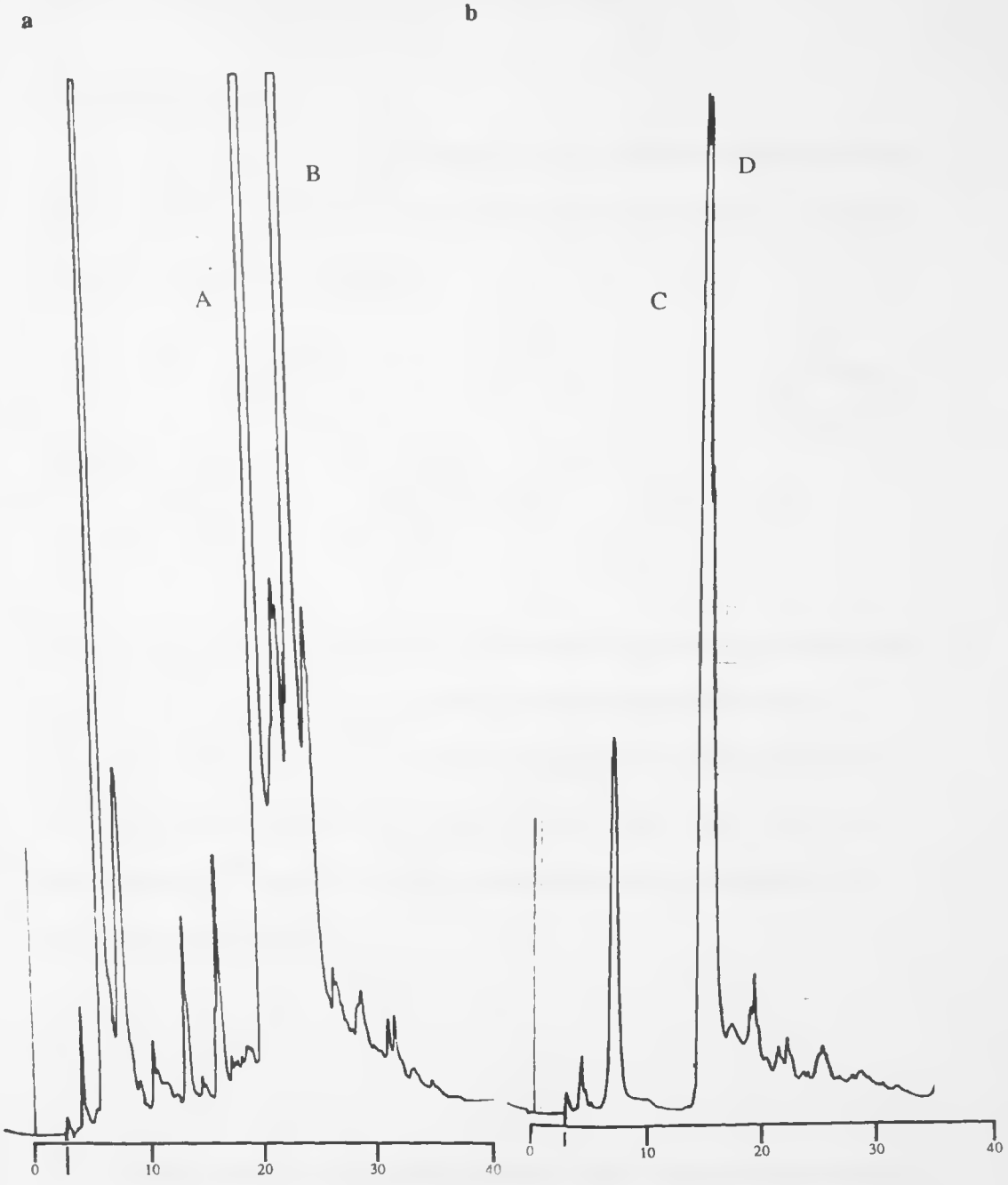
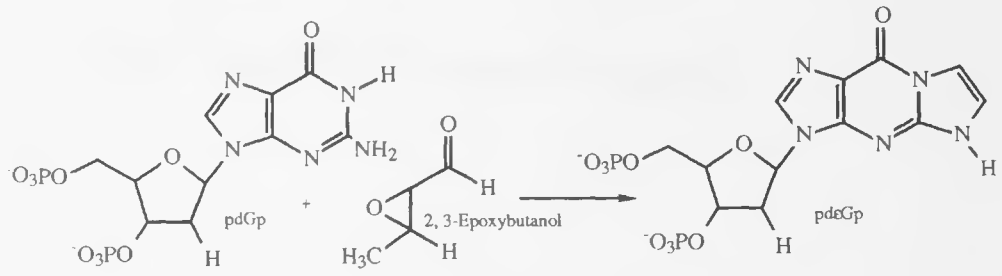


Figure 14. Bisphosphorylation UV profiles from C18 Analytical Column (flow = 1 ml/min) with a) 7-Me-PyP-dR (0-20% CH_3CN/0.1 M TEAA pH 6.5 in 30 min. Note only starting material, A, and depurinated, B, were formed) and b) 2'-deoxyguanosine (dG) in which only monophosphorylated dG was formed (0-30% CH_3CN/0.1 M TEAA pH 6.5 in 20 min. A = 7-Me-PyP, B = 7-Me-PyP-dR C = monophosphorylated dG, D = dG.

and in the ^{1}H-NMR, only benzoic acid was distinguishable.

C. Synthesis of pdεGp

A new substrate for the ligase reaction, pdεGp, an adduct similar in structure to 7-Me-PyP-pdRp, was synthesized. The adduct was prepared by adding 2, 3 - epoxybutanol to pdGp in the following reaction:



Purification of the deoxynucleotide was somewhat more difficult than previously reported[32] because the phosphorylated adduct did not precipitate from solution as the unphosphorylated product does. After HPLC separation the ^{1}H-NMR revealed the major product to be the desired adduct, pdεGp, in a 35% yield. In the ^{1}H-NMR, the sugar protons, $H^{5', 5''}$, and $H^{3'}$ are shifted downfield indicative of the attached 3', 5'-phosphates (refer to Figure 15).

D. T_4-RNA Ligase

Several authors have successfully utilized T_4-RNA ligase for the formation of a small adducted oligonucleotide at a specific site.[36,20] The mechanism involves the

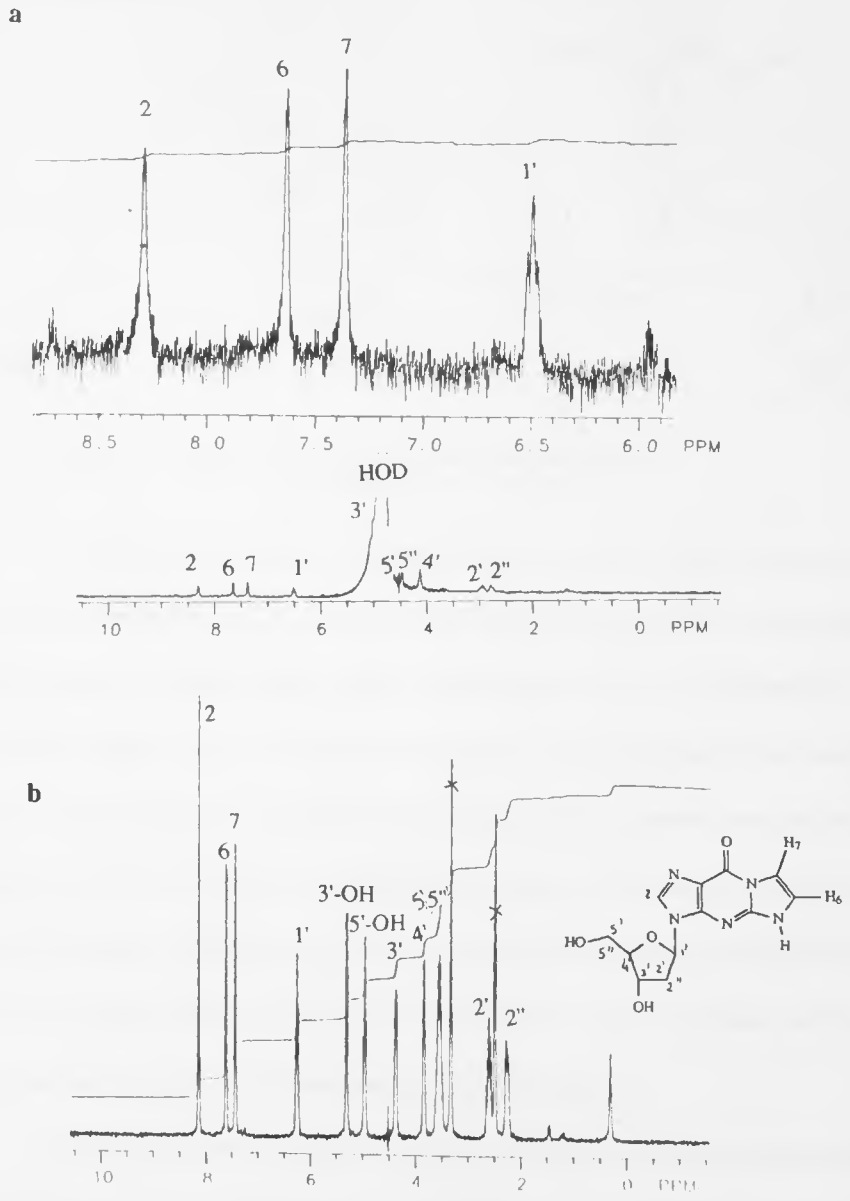


Figure 15. ^{1}H-NMR comparison between a) pdεGp in D_2O and b) dεG in DMSO-d6

ligation of the donor (pdεGp or G*) to the 3'-OH of the acceptor molecule (dCdGdC) as shown in Figure 16.

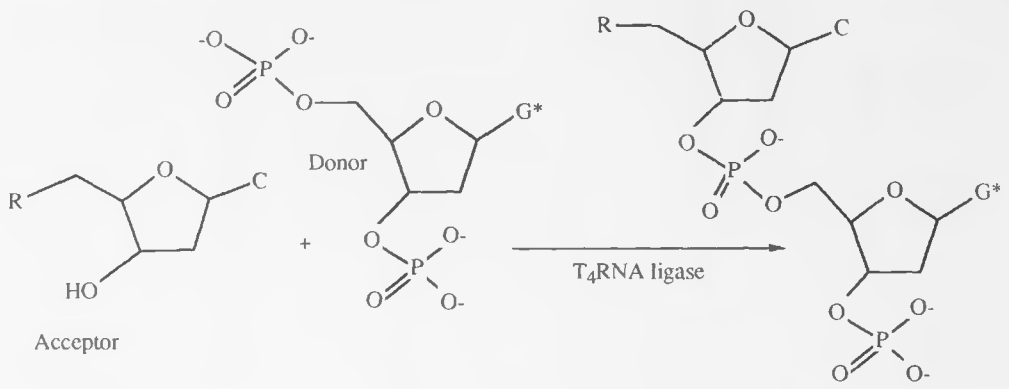


Figure 16. Enzymatic synthesis of an oligodeoxyribonucleotide by T_4-RNA Ligase, R = HO-dCpdG

To determine the extent of successful ligation, every ligation experiment contained a standard reaction (a separate tube) which contained pdGp as the acceptor instead of adducted bisphosphate, pdεGp. Initial experiments were designed to determine if pdεGp was a substrate for the enzyme. These experiments indicated that pdεGp is a poor substrate. The yield for dCpdGpdCpdεGp appeared close to 1% (refer to Figure 17). To test if pdεGp was inhibiting the ligase, pdGp was run in the same tube along with pdεGp. Both ligation products, dCpdGpdCpdGp and dCpdGpdCpdεGp did appear to be formed (refer to Figure 18). Thus pdεGp was not a complete inhibitor of the ligase but a competitive substrate with pdGp for ligation.

Even though pdεGp was not completely inhibiting the enzyme, the yields for the ligation were low and did not run to completion. The pdεGp was repurified on HPLC to remove any impurities that may be inhibiting the ligation. Triethylamine impurities were found in the 0.1M TEAB buffer and removed by distilling the TEA over

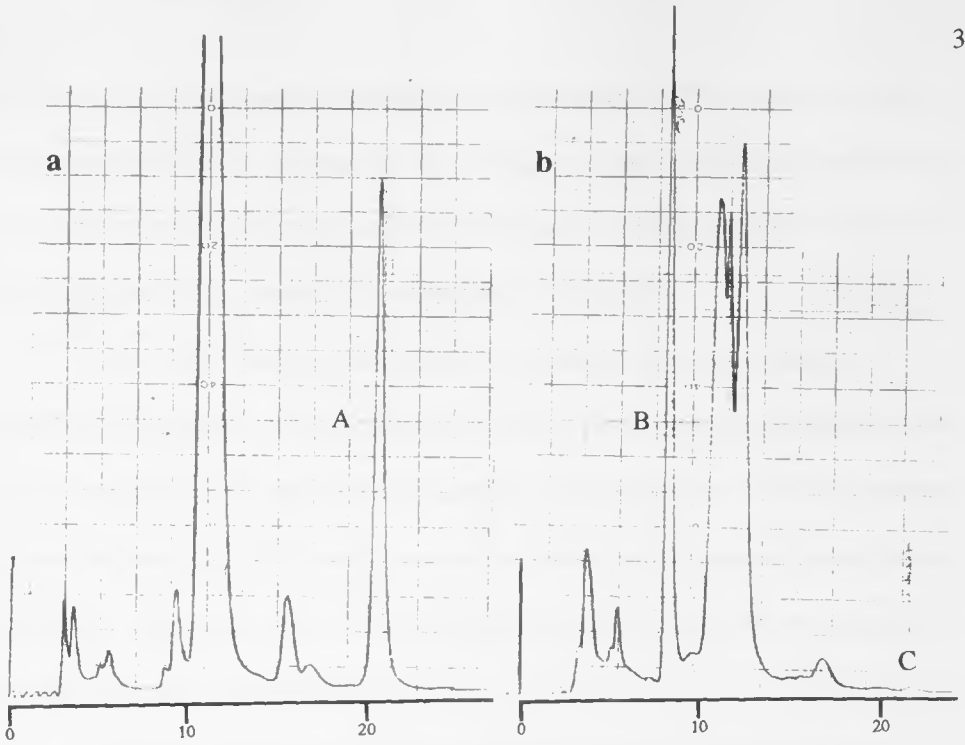


Figure 17. T_4-RNA ligation of a) pdGp and b) pdεGp with dCdGdC
A = dCdGdCdGp, B = dCdGdC, C = dCdGdCdεGp?

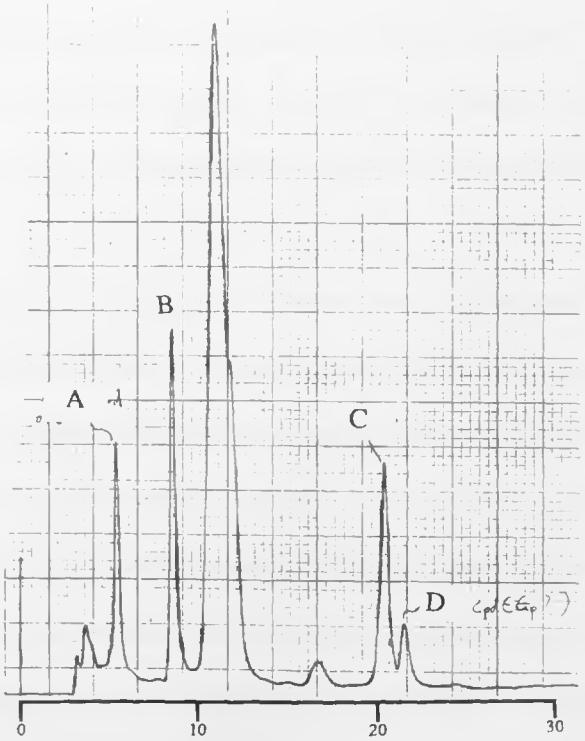


Figure 18. T_4-RNA Ligation with both pdGp and pdεGp. A = 5'-Adenylated donor?,
B = dCdGdC, C = dCdGdCdGp, D = dCdGdCdεGp?

~0.5%$ClSO_3$ onto KOH pellets, redistilling and collecting at 89°C. Buffers were prepared just prior to use since storage at 4°C overnight resulted in detectable impurities by UV and fluorescence spectroscopy. After purification of pdεGp, its yield of ligation to form dCpdGpdCpdεGp seemed to increase slightly (Figure 19).

Several other attempts were made to increase the yield of the reaction. Tessier's modifications[37] of an added polymer (25% polyethylene glycol, PEG) and low ATP concentrations were applied to the technique. Twenty-five percent PEG increased the yield slightly but the ATP modification of removing the regeneration system (phosphocreatine, creatine phosphokinase, and adenylate kinase omitted from reaction mixture) didn't appear to change the yield.

Attempts to scale up the standard reaction (2x and 10x) did not increase product yield. With an increase of 10 fold, no product was observed. In the 2 fold experiment, after 2 days, the product reached about 10% then dropped. The addition of more enzyme appeared to inhibit ligation. The yield for any reaction (either substrate, pdGp or pdεGp) was below 50% and variable depending on the specific activity of the enzyme.

T_4-RNA ligase purchased from different companies produced very different yields. Yields directly correlated with the specific activity of the enzyme purchased. Ligations involving the Pharmacia enzyme (27 units, 9000 units/ ml) gave the best yields as denoted in reactions with the "standard", pdGp and $d(pA)_4$. Bethesda Research labs (BRL, 18 units, 6000 units/ml) had slightly lower yields while Biolabs (12 units, 4000 units/ml) enzyme had a very low yield in comparison (refer to Figure 20). Although Biolabs ligase gave very poor yields the enzyme was not as sensitive to amine

impurities as the other ligases, BRL and Pharmcia.

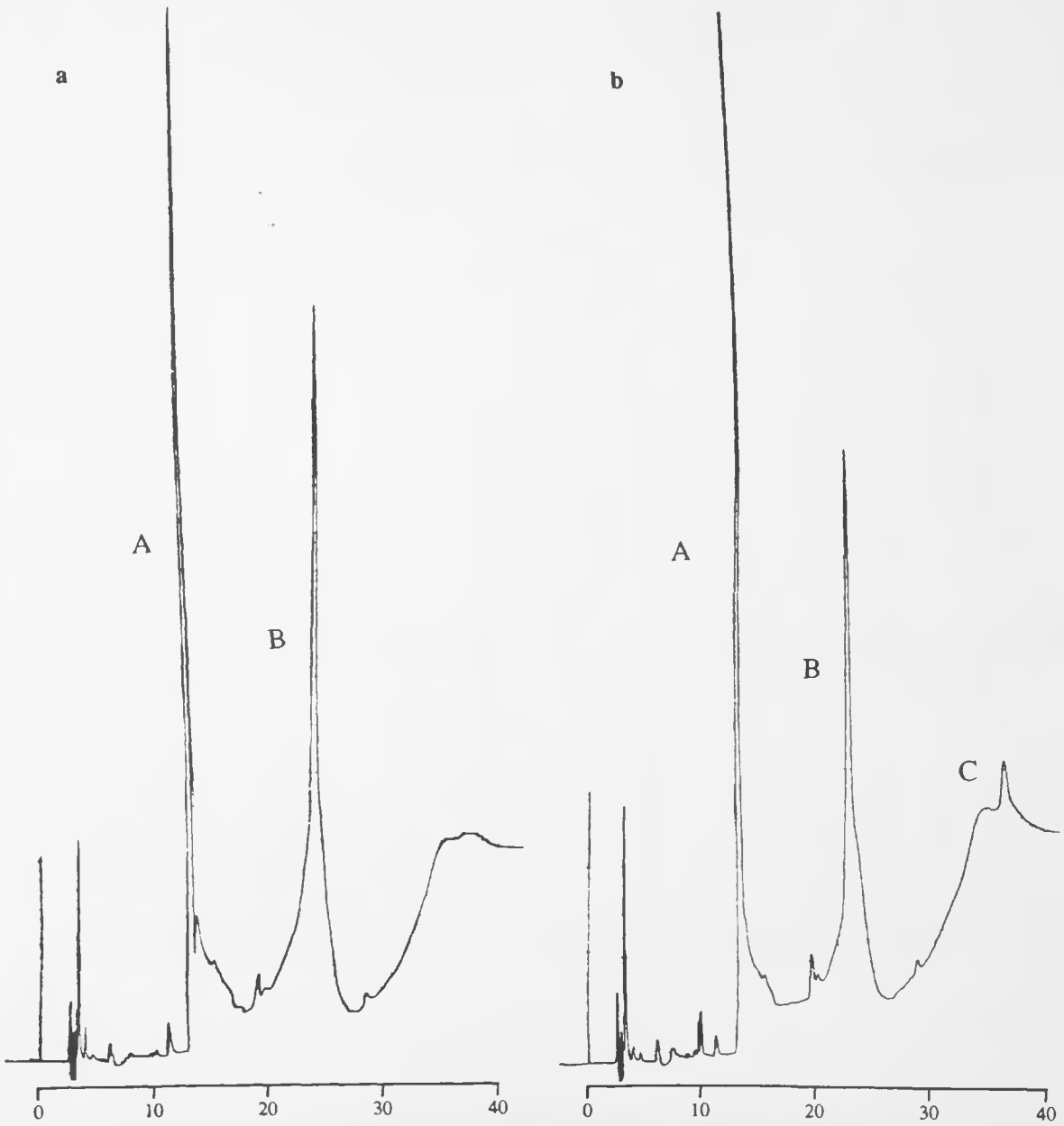


Figure 19. UV profiles (λ = 250) of the formation of dCpdGpdCpdεGp by T_4-RNA Ligase on Spherisorb S5 SAX column, 10-40% 1 M KH_2PO_4, 5% MeOH/5% MeOH in 20 min. a) Reaction at time = 0. b) Reaction after 7 days. Note formation of C at 38 min. A = pdεGp, B = dCpdGpdC, C = dCpdGpdCpdεGp.

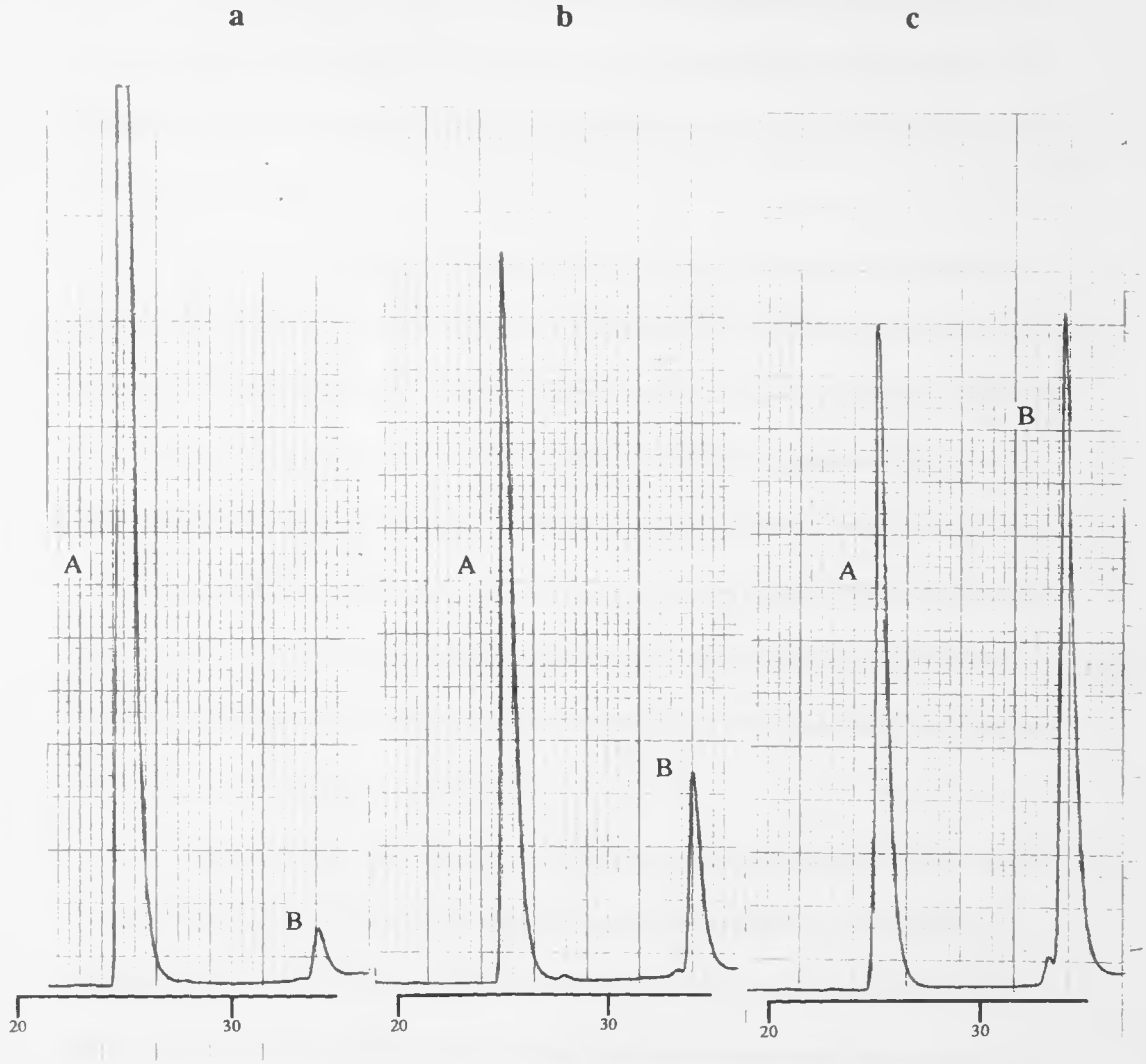


Figure 20. T_4-RNA Ligase comparison between a) Biolabs b) BRL and c) Pharmacia, A = d(pA)4, B = dApdApdApdApdGp (ligation product). Spherisorb S5 SAX column 10-40% 1 M KH_2PO_4, 5% MeOH/ 5% MeOH in 40 min.

IV. DISCUSSION

Two different strategies were applied to the synthesis of 7-Me-PyP-pdRp, the T_4-RNA ligase donor substrate: Phosphorylating the adduct and modifying the 3', 5'-bisphosphorylated deoxyguanosine. Phosphorylating the adduct would be preferred due to economic reasons.

Phosphorylation of 7-Me-PyP-dR was proposed as a plausible reaction, because 7-Me-PyP-ribose can be successfully phosphorylated.[36] Initially, experiments were conducted to synthesize 7-Me-PyP-dR because the adduct's preparation gives few side products and good yields, and 3', 5' -bisphospho-2'-deoxyguanosine is very expensive ($250/50mg, Pharmacia). The yield for the synthesis of 7-Me-PyP-dR was increased greatly over previous literature for PyP-dR from 1.86%[12] to 25%. This was accomplished by the addition of $MgSO_4$, the use of a high concentration of reactants, and by optimizing the temperature and duration such that maximum yield was obtained with as little depurination as possible.

$MgSO_4$ is known to complex or trap water. Molecular sieves (3Å), also known for their ability to trap water, were tested and compared to $MgSO_4$ for enhanced production of 7-Me-PyP-dR. Molecular sieves did not work nearly as well as $MgSO_4$ (refer to Figure 10). In fact molecular sieves yield more depurinated product than 7-Me-PyP-dR (refer to Figure 11). $MgSO_4$, on the other hand, produces more 7-Me-PyP-dR than the depurinated form, 7-Me-PyP (refer to Figure 12). Either entrapment of water by $MgSO_4$ acts as a driving force for the condensation, the elimination of water helps to prevent depurination, or it is a combination of the two that enables the $MgSO_4$ to

increase the yield of the 7-Me-PyP-dR. Molecular seives do not accomplish this same phenomena probably because they trap MDA.

In order for 7-Me-PyP-dR to be utilized as substrate for the enzymatic reaction it had to be phosphorylated. Although Barrio, *et. al.*[36] has proposed bisphosphorylation of dG and 7-Me-PyP-dR our attempts with their method were futile. The pH for the reaction was too low thus the product depurinated (refer to Figure 14a). Any attempts to modify the procedure, such as utilizing solvents or buffers, did not result in the bisphosphorylated but only the monophosphorylated product (refer to Figure 14b).

The approach was then turned towards adducting pdGp. The reaction of MDA with pdGp yielded virtually no product. Basu[16] has shown that increasing the leaving group ability of a series of acrolein substituted on the β-carbon increased their mutagenicity. Therefore β-benzoyloxyacrolein, a compound with a very good leaving group on the β-carbon of acrolein, was used in the attempted synthesis of 7-Me-PyP-pdRp. The benzoic acid that formed from the β-leaving group proved to be more detrimental to the stability of the purine than beneficial to the yield. Neither starting material nor product, only benzoic acid, was detected in the ^{1}H-NMR.

Due to the lack of success with PyP, etheno-2'-deoxyguanosine, dεG, an adduct with a very similar structure to the PyP base was employed (Figure 22). pdεGp was readily obtained by the reaction of pdGp with 2, 3-epoxybutanal with an overall yield of 35%. The ^{1}H-NMR revealed a downfield shift of the all the sugar protons except $H^{4'}$ indicating that both phosphates were present (refer to Figure 15a,b). The ligase acceptor, dCpdGpdC, was readily synthesized on a DNA sequencer and purified by C-18 analytical HPLC. These substrates were then used for ligation.

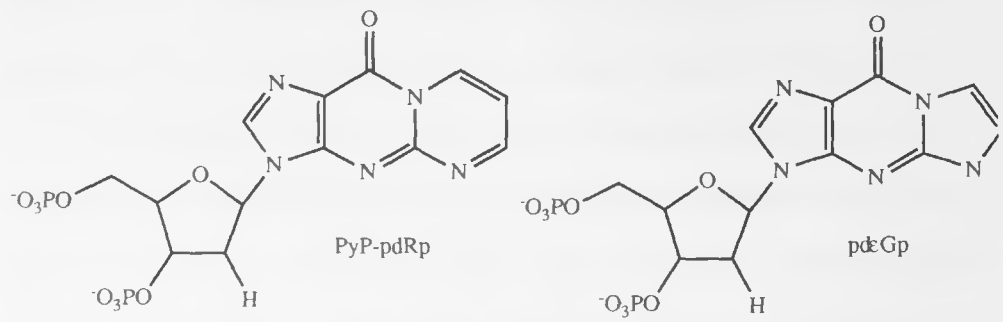


Figure 22. MDA adduct, pd(PyP)p, structure comparison with pdεGp

Many problems arose in the ligation reactions. Not only was the enzyme, T_4-RNA ligase, difficult to purchase with a high specific activity but a method of detection had to be developed. Originally the reaction mixture was separated with an ion-exchange column (Micropak and Spherisorb S5 SAX) but soon after use the retention times changed drastically. Without consistent retention times it was difficult to distinguish product peaks from impurities; results became incomparable. It is uncertain why column performance was inconsistent; perhaps the EDTA added to chelate the Mg^{+2} affected the column. Techniques established by other researchers[36] proved infeasible because the anion exchange columns were unobtainable (Rainin or Phase Sep S5 SAX column on back-order for 9 months). Using a readily available Beckman C-18 Analytical column and ion-pairing, HPLC separation between substrates and products was obtained (refer to Figure 8).

The ligase reaction did show some promise. There appears to be product, a peak at 38 min that increases over time (refer to Figure 19). The pdεGp is obviously not as good a substrate as the standard, dG. Reactions with the substrates under the same conditions produce a greater percent ligation of dG than pdεGp (refer to Figure 17). As

Figure 18 shows, pdεGp does not completely inhibit the ligase and once the amine impurities were removed the ligation increased (compare Figure 17 with Figure 19).

The substrate ligation not only seemed to be dependent on the purity of the substrate but also changed for the various enzymes (different suppliers of the T_4-RNA ligase). The enzyme with the highest specific activity, Pharmacia's, performed ligation to the greatest extent (refer to Figure 20). The Pharmacia ligase was also more sensitive to impurities then the other enzyme suppliers, Biolabs and BRL, tested. With any impurities present (detected by ^{1}H-NMR), ligation for the Pharmacia enzyme dropped from about 50% ligation to less then 10% while the Biolabs enzyme only ligated about 3% but was insensitive to the presence of impurities (results not shown).

Unfortunately, not enough material was produced to enable complete characterization. Several variations of the method were attempted to improve the mass and yield of the product, dCpdGpdCpdεGp, (e.g. scaling up the reaction mixture and adding PEG) yet none were successful. The substrate, pdεGp, is a poor substrate in comparison to dG with only about 10% ligation compared to almost 50% found for dG.

Often noted in HPLC profiles for the ligation of pdεGp but not the standard, pdGp, is thought to be AMP-pdεGp (refer to Figure 7 and Figure 18). Perhaps the reason pdεGp is a poor substrate is due to this structure. Uhlenbeck and Gumport[35] noted that once the donor is adenylylated, ATP and the enzyme are no longer required for ligation to occur. Perhaps the yields can be improved if the adenylated donor, AMP-pdεGp, could be isolated and reacted with the acceptor, dCpdGpdC. Gumport[21] also noted that base helps to shift the equilibrium from adenylylated donor, AMP-pdεGp, to product.

pH studies of the reaction could denote optimum pH for maximum yields. If yields could be improved such that the identification of the product was evident, the ligation of dCpdGpdC to pdεGp by T_4-RNA ligase would be a useful method.

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VI. ABSTRACT

SYNTHESIS OF SITE SPECIFIC 1, N^2-ETHENODEOXYGUANOSINE IN A SMALL OLIGONUCLEOTIDE

by

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Malondiadehyde (MDA) is a known carcinogen and mutagen produced by lipid peroxidation and prostaglandin synthesis *in vivo*. MDA is unique from structurally related compounds, methyl glyoxal and acrolein, because it produces frameshift mutations whereas the latter compounds induce base-pair substitutions. MDA forms several adducts with deoxyguanosine but it is not certain which one causes the frameshift mutations. Ideally, a single adduct on a replicating genome could simulate the *in vivo* process by which MDA mutagenesis would occur. This technique is known as site-specific mutagenesis. The approach of choice for site-specific mutagenesis experiment is the gapped heteroduplex method which requires the formation of a small oligonucleotide with an adduct at a defined site. The construction of the oligonucleotide with the site specific adduct was attempted by ligating an adducted bisphosphate to the oligonucleotide, dCpdGpdC, with T_4-RNA ligase. The synthesis of 3', 5'-bisphosphoryl-7-

methyl-3-(β-D-pentofuranosyl)-pyrimido [1, 2-a] purine-10-(3H)-one-2'-deoxyribose (7-Me-PyP-pdRp) was attempted. Although modification of the synthesis of 7-methyl-3-(β-D-pentofuranosyl)-pyrimido [1, 2-a] purine-10-(3H)-one-2'-deoxyribose, the precusor to 7-Me-PyP-pdRp, with $MgSO_4$ and minimal concentrations of reactants improved the yield to 25%, the bisphosphate could not be formed. An adduct similar in structure to the MDA adduct was employed. The adduct, formed from 2, 3-epoxybutanal under basic conditions in a 35% yield, was 3', 5'-bisphosphoryl-1, N^2-etheno-2'-deoxyguanosine.

Substrates, pdεGp and dCpdGpdC, for the T_4-RNA ligase were successfully prepared. Ligation appeared successful, based on UV profiles, except possitive identification of the oligonucleotide product, dCpdGpdCpdεGp, was not possible due to low yields. Any modifications to increase the yield such as scale up attempts and polyethylene glycol addition were ineffective.

VII. AUTOBIOGRAPHICAL STATEMENT

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