1. NONENZYMATIC CLEAVAGE OF SINGLE-STRANDED DNA TO NUCLEOTIDE RESOLUTION

2. NOVEL BASE SPECIFIC DNA CLEAVAGE REACTIONS

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To Sheila

And

My Parents

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ABSTRACT

PART 1

A new strategy for the complementary-addressed modification of nucleic acids was investigated involving the enzymatic incorporation of a modified 2'-deoxynucleotide 5'-triphosphate molecule into oligonucleotide strands. These modified 2'-deoxynucleotide 5'-triphosphate compounds (and hence the strands into which they were incorporated) carried a latent reactive group in the form of a methylthioether function. The methylthioether function was activated by treatment with cyanogen bromide to enable alkylation of a complementary nucleic acid strand. The alkylation was shown to involve methyl group transfer and upon piperidine treatment resulted in the cleavage of the DNA at essentially a single residue on the target strand. The system was found to be capable of cleaving oligonucleotides as well as long pieces (5386 bases) of single-stranded DNA to nucleotide resolution.

PART 2

Two novel base specific DNA cleavage reactions were discovered and investigated. The first is an A specific reaction caused by K_2PdCl_4 at low pH. The second is a photochemical reaction with "GG" specificity caused by some nitroaromatic and Co(III) compounds. Reaction of DNA with K_2PdCl_4 at low pH followed by a piperidine workup produces specific cleavage at adenine residues. Product analysis revealed the K_2PdCl_4 reaction involves selective depurina-

tion at adenine, affording a gapping reaction analogous to the other chemical DNA sequencing reactions. Adenine residues methylated at the exocyclic amine (N6) react with lower efficiency than unmethylated adenine in an identical sequence. This simple protocol specific for A may be a useful addition to current chemical sequencing reactions. Photolysis of DNA in the presence of 4-nitroveratrole, 3-nitroanisole or Co(III) compounds such as $Co(III)(NH_3)_6$ followed by a piperidine workup produces cleavage of the DNA with "GG" specificity, that is the 5'-G of 5'-GG-3' sequences is preferentially attacked. Product analysis revealed that the aromatic guanine base is decomposed into numerous fragments, and the nitroaromatic compound is apparently not consumed in the reaction. An electron transfer mechanism is proposed to account for the photochemical reaction at 5'-GG-3' sequences.

TABLE OF CONTENTS

PART 1: NONENZYMATIC CLEAVAGE OF SINGLE-STRANDED DNA TO NUCLEOTIDE RESOLUTION

INTRODUCTION 2

BACKGROUND
- DNA Structure And Properties 5
- Complementary Addressed Modification Of Nucleic Acids. 8
- Functional Groups Used 11

DESIGN OF SYSTEM
- New Approaches To The Synthesis Of Hunter Strands 22
- Reasons For Choosing An Alkylation Type Of Cleavage Reaction 26
- Reasons For Choosing A Methylthioether Group As The Latent Alkylating Function
  - SAM 28
  - The CNBr Peptide Cleavage Reaction 32
  - Design Of The Modified 2'-Deoxyuridine 5'-Triphosphate Compounds Carrying A Methylthioether Function 34

RESULTS AND DISCUSSION
- Synthesis
  - Modified 2'-Deoxyuridine 5'-Triphosphate Compounds Carrying A Methylthioether Function 35
- Klenow Enzyme Incorporation Reactions 40
- The CNBr Cleavage Reaction
  - Optimum Conditions And Specificity 52
  - Base Specificity Of The Cleavage Reaction 61
  - Linker Length Dependence 62
  - Nature Of The 5' Termini Produced In The Cleavage Reaction 67
  - Nature Of The Guanine Species Produced In The Cleavage Reaction 71
  - Proposed Reaction Mechanism 85
  - Computer Modeling 88
- Cleavage Of ΦX174 Single-Stranded DNA 100

Cleavage Of ΦX174 Single-Stranded DNA Using An Allylamine Oligonucleotide Post-Synthetically Modified With Methylthioether
Synthesis Of **9** 106
Cleavage Of ΦX174 Single-Stranded DNA Using Oligonucleotide 12 109
Attempted Cleavage Of A Double-Stranded Sequence Through Formation Of A Triple-Strand 113
Attempted Complementary-Addressed Modification Of Nucleic Acids Using 1,10-Phenanthroline Derivatives 115
Copper-Thiol Cleavage Reactions 117
Cobalt(III) Photochemistry 117
Attempted Directed Alkylation Using A Cofactor 117

CONCLUSION 121

EXPERIMENTAL PROCEDURES 124

REFERENCES 159

PART 2: NOVEL BASE SPECIFIC DNA CLEAVAGE REACTIONS

INTRODUCTION 167

THE K_2PdCl_4 REACTION AT ADENINE
Background
Chemical DNA Sequencing 169
Metal-Ion DNA Interactions 171
Results And Discussion
The Protocol For The K_2PdCl_4 Cleavage Reaction At Adenine Residues 176
Product Analysis 181
Mechanistic Studies 186
Proposed Mechanism 188

A NOVEL PHOTOCHEMICAL DNA CLEAVAGE REACTION WITH "GG" SPECIFICITY
Background
Nitroanisole Photochemistry 194
Photochemistry Of Co(III) Complexes And DNA 197
Results And Discussion
The Nitroaromatic "GG" Reaction 198
Product Analysis 204
Proposed Mechanism 209
The Co(III) Photochemical "GG" Reaction 212
Attempted Complementary-Addressed Cleavage Of Single-Stranded DNA Using A Nitroaromatic Group As A Cleaving Function 215

CONCLUSION 219

EXPERIMENTAL PROCEDURES 221

REFERENCES 238

LIST OF FIGURES

Figure 1 The Watson-Crick Base Pairs.....6
Figure 2 The B Form DNA Helix.....7
Figure 3 Alkylated Products Obtained Upon Reaction Of [^{14}C]methyl Methanesulfonate With Double Stranded DNA.....27
Figure 4 The Proposed Mechanism Of Cleavage Of Peptides At Methionine Residues By CNBr.....32
Figure 5 Synthetic Scheme For Preparation Of Compound **2**.....36
Figure 6 Synthetic Schemes For Preparation Of Compounds **3-5**....37
Figure 7 Ion-Exchange HPLC Traces Of Pure Compound **2**.....39
Figure 8 Sequences Of Synthetic Oligonucleotides.....41
Figure 9 Klenow Enzyme Fill-In Reactions Using High And Low Concentrations Of Klenow Enzyme.....43
Figure 10 Klenow Enzyme Fill-In Reactions Using **2** And Low Klenow Concentrations.....46
Figure 11 Scheme For The Method Used To Produce An Oligonucleotide 3 With A Single Residue Of **2** On The 3' End.....49
Figure 12 Klenow Enzyme Fill-In Reactions Revealing Direct Evidence For Incorporation Of **2** Into Oligonucleotide 3.....50
Figure 13 Scheme For The Complemetary-Addressed Cleavage Of Oligonucleotide 1 Using An Enzymatically Incorporated Residue Of **2** and CNBr.....53
Figure 14 CNBr Cleavage Reactions Of The Oligonucleotide1,2 Duplex Using An Enzymatically Incorporated Residue Of **2**.....55
Figure 15 More CNBr Cleavage Reactions Of The Oligonucleotide 1,2 Duplex Using An Enzymatically Incorporated Residue Of **2**.....57
Figure 16 CNBr Cleavage Reactions Of The Oligonucleotide5,2 Duplex Using An Enzymatically Incorporated Residue Of **2**.....63
Figure 17 CNBr Cleavage Reactions Of The Oligonucleotide1,2 Duplex Using An Enzymatically Incorporated Residue Of **2,4** or **5**.....65
Figure 18 Scheme For The Method Used To Produce The Oligonucleotide 6,7 Duplex Labelled With ^{32}P At The 3' End Of Oligonucleotide 6 Carrying A Single Residue Of 2 Filled-Into Oligonucleotide 7.....68
Figure 19 CNBr Cleavage Reactions Of The Oligonucleotide6,7 Duplex Using An Enzymatically Incorporated Residue Of **2**.....69

Figure 20 Scheme For The Method Used To Isolate And Identify Any Methylated Guanine Produced In The Complementary-Addressed CNBr-Methylthioether Reaction.....72
Figure 21 HPLC Chromatogram Of The Ethanol Precipitation Supernatant Obtained In The Large Scale CNBr Cleavage Reaction Using **2**.....74
Figure 22 HPLC Chromatogram Of The Ethanol Precipitation Supernatant Obtained In A Control Of The Large Scale CNBr Cleavage Reaction Using T (No **2**).....76
Figure 23 HPLC Chromatogram Of Commercially Prepared Guanine, Adenine And N7-Methylguanine.....78
Figure 24 13-24 Minute Portion Of The Chromatogram In Figure 21.....80
Figure 25 13-24 Minute Portion Of The Chromatogram In Figure 22.....81
Figure 26 13-24 Minute Portion Of The Chromatogram In Figure 21.....82
Figure 27 13-24 Minute Portion Of The Chromatogram In Figure 22.....83
Figure 28 13-24 Minute Portion Of The Chromatogram Produced By Commercially Prepared Adenine And N7-Methylguanine.....84
Figure 29 Large Scale CNBr Cleavage Reaction Of The Oligonucleotide 8,9 Duplex.....86
Figure 30 Proposed Mechanism For The CNBr-Methylthioether Cleavage Reaction Using A Piperidine Workup.....89
Figure 31 Computer Generated Illustration Of The Filled-In Oligonucleotide 1,2 Duplex Containing A Sinlge Residue Of **2**.....91
Figure 32 Closeup View Of The Same Computer Illustration Shown In Figure 31.....92
Figure 33 Closeup View Of The Same Computer Illustration Shown In Figure 31 And Figure 32.....93
Figure 34 Same Illustration As Figure 33, But Viewed Fronm A Perspective Rotated 90°.....94
Figure 35 Closeup View Of The Same Computer Illustration Shown In Figure 31.....95
Figure 36 Some Relevant Distances And Angles Derived From The Computer Generated Model Depicted In Figures 31-35.....97
Figure 37 Scheme For The Complementary-Addressed Methylation And Cleavage Of Single-Stranded ΦX174 Using An Enzymatically Incorporated Residue Of **2** And CNBr.....102

Figure 38 Cleavage Of Single-Stranded ΦX174 Using An Enzymatically Incorporated Residue Of **2** And CNBr.....103
Figure 39 Synthetic Scheme For The Preparation Of Compounds **6 - 9** 107
Figure 40 Side Reaction Encountered In The Synthesis Of Compound **7** And The Procedure Used To Avoid It.....108
Figure 41 Scheme For The Complementary-Addressed Methylation And Cleavage Of Single-Stranded ΦX174 Using Oligonucleotide 12 Post-Synthetically Modified With Methylthioether.....110
Figure 42 Cleavage Of Single-Stranded ΦX174 Using Oligonucleotide 12 Post-Synthetically Modified With Methylthioether.....111
Figure 43 Scheme For The Attempted Complementary-Addressed Methylation And Cleavage Of Double-Stranded DNA Through Formation Of A Triple-Stranded Structure.....114
Figure 44 Synthetic Schemes For The Preparation Of Compounds **10 - 12** 116
Figure 45 Scheme For The Proposed Directed Alkylation Reaction Using A Cofactor 119
Figure 46 400 MHz ^{1}HNMR Spectrum Of Compound **2** In D_2O 129
Figure 47 400 MHz ^{1}HNMR Spectrum Of Compound **7** In d_6-DMSO 136
Figure 48 The Reactions Used For Chemical Sequencing According To The Method Of Maxam And Gilbert.....170
Figure 49 Comparison Of The pK_a's And Stability Constants For Metal Binding To The N1 And N7 Positions Of The Purines.....174
Figure 50 Synthetic Scheme For The Preparation Of Compound **13** 177
Figure 51 Gel A Comparison Of The G, G+A and K_2PdCl_4 A Reaction On A 517 Base Pair Restriction Fragment Of DNA. Gel B The G and K_2PdCl_4 A Reaction On A 517 Base Pair Restriction Fragment Of DNA Illustrating A Straightforward New Sequencing Strategy.....178

Figure 52 Gel A Comparison Of The Electrophoretic Mobilities Of The Fragments Produced By The G, G+A and K_2PdCl_4 A Reaction On A High Resolution Gel. Gel B Comparison Of The DMS Reactions Conducted At pH 2.0 In The Presence And Absence Of K_2PdCl_4 182
Figure 53 HPLC Chromatogram Produced By The K_2PdCl_4 A Reaction On 50 μg Calf Thymus DNA Showing That Free Adenine Is The Only Released Product Of The Reaction 184
Figure 54 Comparison Of The K_2PdCl_4 Reaction Cleavage Efficiency Of N6-Methyladenine With Unmethylated Adenine In Analogous Sequences 189
Figure 55 Densitometry Of The Middle Region Of Gel In Figure 54 191
Figure 56 The Proposed Mechanistic Scheme For The K_2PdCl_4 Reaction At A vs. G 192
Figure 57 Cleavage Of Double-Stranded DNA With "GG" Specificity Produced By Photolysis In The Presence Of 4-Nitroveratrole 199
Figure 58 Comparison Of The Cleavages Of Double-Stranded DNA Produced By Photolysis In The Presence Of 4-Nitroveratrole, 4-Nitroanisole, 3-Nitroanisole and 2-Nitroanisole 201
Figure 59 Synthetic Schemes For The Preparation Of Compounds **14-20** 203
Figure 60 HPLC Chromatograms Of Reactions Run On 50 μg Calf Thymus DNA Showing That Guanine Is Selectively Destroyed Upon Photolysis In The Presence Of 6.5 mM 3-Nitroanisole 205
Figure 61 HPLC Chromatograms Of Reactions Run On 2',3',5'-Tri-O-Acetylguanosine Showing That The Aromatic Guanine Base Is Decomposed Into Numerous Products Upon Photolysis In The Presence Of 1 Equivalent Of 3-Nitroanisole 207
Figure 62 Proposed Mechanism Of The "Double-G" Reaction 210
Figure 63 Cleavage Of Double Stranded DNA With "GG" Specificity Produced By Photolysis In The Presence Of $Co(III)(NH_3)_6$ 213
Figure 64 Synthetic Schemes For The Preparation Of Compounds **21-23** 216
Figure 65 Molecules Pepared By Paul Brown Which Carry A 3-Nitroanisole Or 4-Nitroveratrole Group 218

LIST OF ABBREVIATIONS

A adenine
α-^{32}P-dGTP α-^{32}P-2'-deoxyguanosine 5'-triphosphate
AMB ammonium bicarbonate buffer
AMP adenosine 5'-monophosphate
ATP adenosine 5'-triphosphate
bp base pairs
C cytosine
CAP calf alkaline phosphatase
Ci curie
***cis*-DPP** *cis*-diamminedichloroplatinum(II)
cm centimeter
CNBr cyanogen bromide
CPK Corey-Pauling-Koltun
CT DNA sonicated, deproteinized calf thymus DNA
d doublet
D_2O deuterium oxide
dATP 2'-deoxyadenosine 5'-triphosphate
DCC 1,3-dicyclohexylcarbodiimide
dCTP 2'-deoxycytidine 5'-triphosphate
ddGTP 2',3'-dideoxyguanosine 5'-triphosphate
(dien)Pd(II) ((diethylenetriamine)Pd(II)Cl)Cl
dGTP 2'-deoxyguanosine 5'-triphosphate
DMF N,N-dimethylformamide
DMS dimethyl sulfate
DMSO dimethyl sulfoxide
DNA deoxyribonucleic acid
DTT dithiothreitol
dUTP 2'-deoxyuridine 5'-triphosphate
EDTA ethylenediaminetetraacetic acid
EI electron ionization
ESR electron spin resonance spectroscopy
eV electron volts
FAB fast atom bombardment
G guanine
g gram
γ-^{32}P-ATP γ-^{32}P-adenosine 5'-triphosphate
GMP guanosine 5'-monophosphate
^{1}H NMR proton nuclear magnetic resonance spectroscopy
h hour
HPLC high performance liquid chromatography
Hz hertz
IR infrared absorption spectroscopy
M molar
m multiplet
μg microgram
mg milligram
MHz megahertz
min minute
μl microliter
ml milliliter
mM millimolar
mm millimeter
mmol millimole
MP melting point
mR/h milliroentgen per hour

mRNA messenger RNA
MWCO molecular weight cut-off
NaOAc sodium acetate
NHS N-hydroxysuccinimide
nm nanometer
nmol nanomole
RNA ribonucleic acid
rpm revolutions per minute
rRNA ribosomal RNA
s singlet
SAM (-)S-adenosyl-L-methionine
T thymine
t triplet
TAG 2',3',5',-tri-O-acetyl-guanosine
TBE Tris-borate, EDTA electrophoresis buffer
TEA triethylamine
TEAB triethylammonium bicarbonate buffer
TLC thin layer chromatography
TMP thymidine 5'-monophosphate
Tris tris(hydroxymethyl)aminomethane
tRNA transfer RNA
TTP thymidine 5'-triphosphate
UV ultravolet
UV-vis ultraviolet-visible absorption spectroscopy

PART 1

NONENZYMATIC CLEAVAGE OF SINGLE-STRANDED DNA TO NUCLEOTIDE RESOLUTION

INTRODUCTION

A system capable of selective, programmed modification, especially cleavage, of single-stranded DNA could have many important applications in molecular biology. For example, such a system might greatly facilitate the *in vitro* manipulations of large pieces of DNA which are unavoidably encountered in the analysis of eukaryotic genomes. Although eukaryotic DNA is double-stranded, techniques are commonly employed which allow the transfer of these eukaryotic sequences into single-stranded vectors such as M13, a phage which can be grown in bacterial hosts.[1] These single-stranded vector systems are convenient because they enable certain site-directed mutagenesis techniques[2] as well as sequencing with the Sanger method.[3]

The single-stranded DNA (and double-stranded DNA for that matter) manipulation systems currently used are somewhat limited because they depend on restriction enzymes for DNA cleavage. The recognition sequences of these enzymes are quite specific and are not always present in a particular DNA sequence of interest. Conversely, an investigator might need to cleave a very long piece of DNA in a single location and there might be no restriction enzyme with the appropriate specificity. It would therefore be useful to produce a system in which the specificity of cleavage was programmed by the investigator.

Ideally, a sequence specific single-stranded DNA cleavage system should satisfy at least four criteria. 1) The single-stranded DNA cleavage system must be capable of producing cleavage to nucleotide

resolution since genetic manipulation techniques require precise cutting and ligation of DNA.[4] 2) The cleavage reaction must produce termini on the DNA fragments which are useful for ligation or other enzymatic manipulations. Useful termini would be 3' and 5' hydroxyl and/or phosphate,[4] because no sugar or base fragments are tolerated by the phosphatase and ligase enzymes. 3) The system must be relatively easy to use and quick. Time-consuming synthesis or prolonged procedures should be avoided, and in general the system should utilize techniques similar to those already in current practice. 4) The cleavage efficiency must be high enough to allow for the isolation of relatively large amounts of cleaved product. The first part of this thesis deals with the design, synthesis and reactions of a system intended to meet the above criteria.

An extensive survey of pertinent literature is presented in the following section which covers what has been done in the general area of complementary-addressed modification of nucleic acids. To date, no complementary-addressed system has been demonstrated to meet all four of the above criteria for general use as *in vitro* single-stranded DNA cleaving agents.

We chose to pursue a new strategy of producing nucleic acid strands capable of complementary-addressed modification involving the enzymatic incorporation of modified 2'-deoxynucleotide 5'-triphosphate molecules into oligonucleotide strands.[5] These modified 2'-deoxynucleotide 5'-triphosphate compounds (and hence the strands into which they were incorporated) carried a latent reactive

group in the form of a methylthioether function. The methylthioether function was activated (by treatment with cyanogen bromide) to enable alkylation of a complementary (or "target") nucleic acid strand. The alkylation reaction was shown to involve methyl group transfer and upon piperidine treatment resulted in the cleavage of the DNA at the site of alkylation. Importantly, cleavage was observed at essentially a single residue on the target strand. DNA fragments with electrophoretic mobilities consistent with 3' and 5' phosphate termini were exclusively generated by the cleavage reaction. The system was shown to be capable of cleaving short oligonucleotides as well as long pieces (>5000 bases) of single-stranded DNA to nucleotide resolution.

It is hoped that some of the lessons learned from these studies might someday be applied to methylation and cleavage of double-stranded DNA as well.

BACKGROUND

DNA Structure And Properties A fundamental property of strands of nucleic acids is their ability to recognize and form stable complexes with complementary strands. Such recognition and binding is the result of the specific hydrogen bonds described by Watson and Crick.[6] Adenine forms two hydrogen bonds with thymine (or uracil) and guanine forms three hydrogen bonds with cytosine (see Figure 1). Furthermore, strands of nucleic acids, whether DNA or RNA, can have defined secondary structures built around this specific base pairing.

Double-stranded DNA usually adopts the highly ordered right-handed B form (see Figure 2).[7] This type of DNA structure has 10.5 residues per turn of the helix with a 3.4 Å distance between adjacent stacked bases. The bases in a base pair of B form DNA are not rigorously planar but exhibit some propeller twist, usually around 11°-17° in magnitude.[6] There is a narrow minor groove and wider major groove running along the B form helix with each groove having different and characteristic functional groups placed there by the base and sugar moieties of the residues. The two DNA strands are antiparallel in B form DNA since one strand has the deoxyribose sugars oriented in the 3'-5' direction while in the complementary strand they are oriented in the 5'-3' direction.

RNA molecules can have more complex, yet highly ordered structures such as those observed with tRNA molecules.[8] As with DNA, the RNA structures are primarily built around specific base

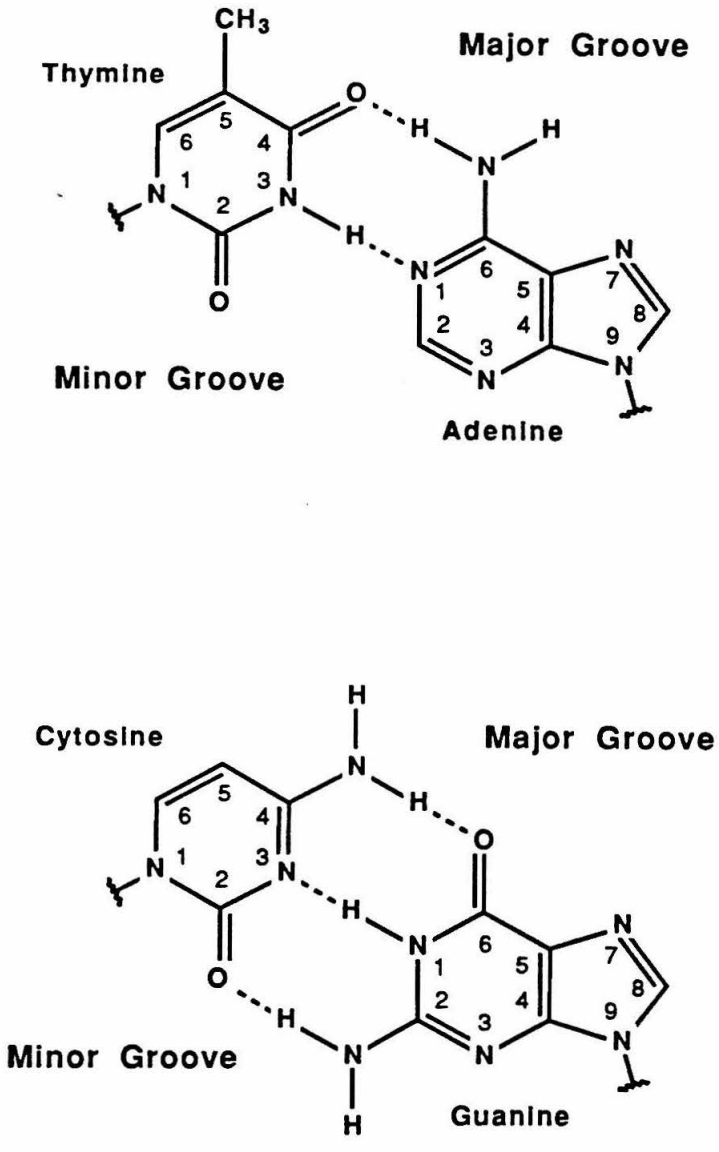


Figure 1 The Watson-Crick base pairs showing all of the hydrogen bonds responsible for the sequence recognition properties of DNA. The location of functional groups in the minor or major grooves of right-handed B form DNA is also indicated.

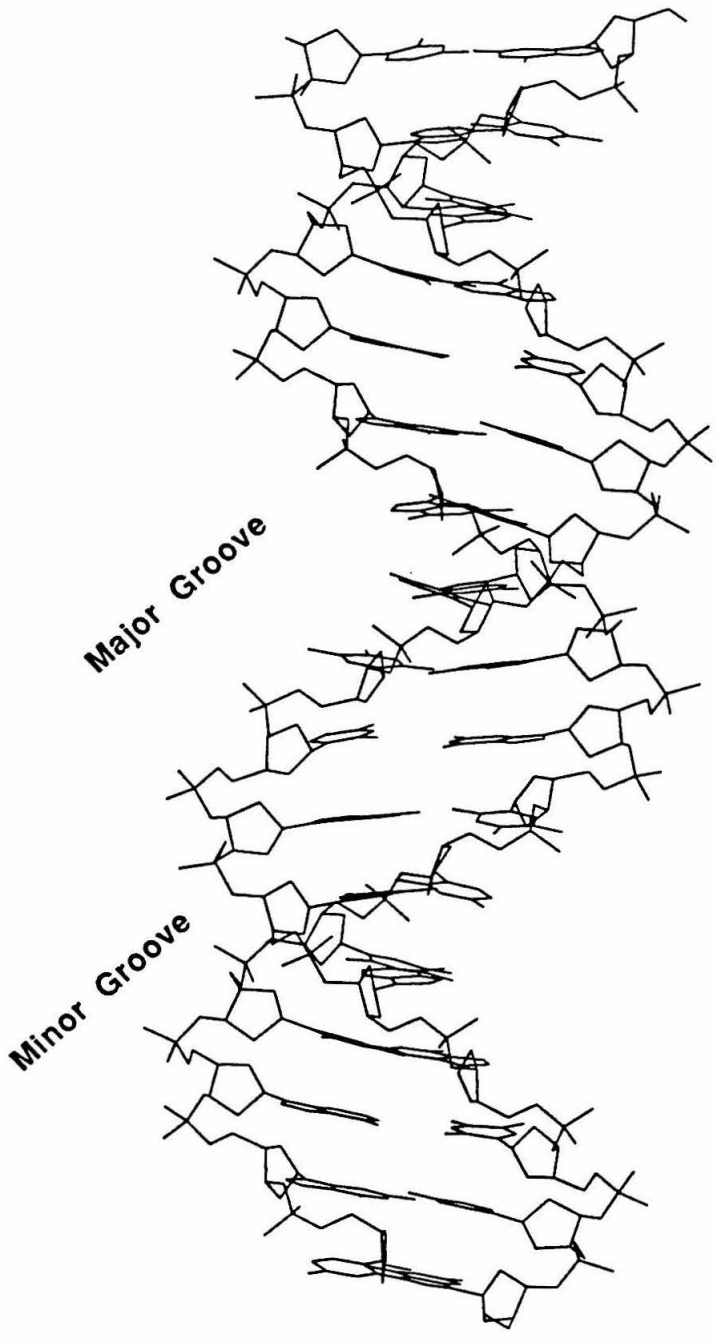


Figure 2 The B form DNA helix illustrating the location of the minor and major grooves.

pairing, however much of the RNA base pairing is of the intrastrand variety.

The kinetics of the process by which a nucleic acid double helix is formed from two isolated strands have been studied. A generally accepted model explains the hybridization process as being analogous to the closing of a zipper.[9] There is a relatively slow step of forming a nucleation site, followed by rapid elongation to give the completed double helix. Kinetic analysis has indicated that the nucleation site consists of a relatively unstable complex of around three base pairs and subsequent base pairing greatly stabilizes the duplex formation in a rapid and cooperative process.[9] The process is cooperative because the presence of stacked bases in the growing double helix makes the formation of new stacked bases more energetically favorable, thus the double helix, once started, is rapidly elongated.

Complementary-Addressed Modification Of Nucleic Acids The unique recognition, folding and hybridization properties of nucleic acids just described are largely responsible for their prominent role in the chemistry of living systems. On the other hand, these same properties can be exploited in the designs of synthetic reagents capable of reacting with nucleic acids in important ways. The placing of reactive groups on nucleic acids for the purpose of modifying complementary strands was first described in 1967.[10] Since then, many different derivatized nucleic acid strands have been prepared, each designed to deliver a reactive group to a specific location of a complementary nucleic acid structure. Such comple-

mentary-addressed sequence specific modification has been used to attempt the elucidation of tRNA and rRNA structure, mutate specific genes *in vitro*, affect cellular processes *in vivo* and modify and/or cleave single-stranded oligonucleotides or DNA.[11]

Oligonucleotides are ideal molecules for the selective delivery of reactive groups because of the fast and cooperative nature of the hybridization process, the profound accuracy in recognition and the expected defined secondary structure of two complementary strands. Because of the kinetics and fidelity of the hybridization reaction, oligonucleotides with complementary DNA sequences can be simply mixed, heated slightly if needed, and duplexes will be formed in virtually quantitative yield and with the complementary sequences exactly lined up. Furthermore, the highly ordered secondary structure presumably produced upon hybridization (B form DNA helix for example) can be exploited to promote reactions simply by placing any reactive groups in predictable close proximity to each other.

Two Different Approaches Until now, two different approaches have been taken to produce the derivatized strands of nucleic acids used for complementary-addressed modification reactions. The most widely used approach involves the reactions of heterobifunctional reagents.[10] One function of the reagent covalently links it to an existing nucleic acid strand (creating a so-called "hunter strand"), while the other function is used as the group which modifies the complementary (or "target") strand. Reagents have been attached to the 3' and 5' ends of DNA and RNA, to random or specific

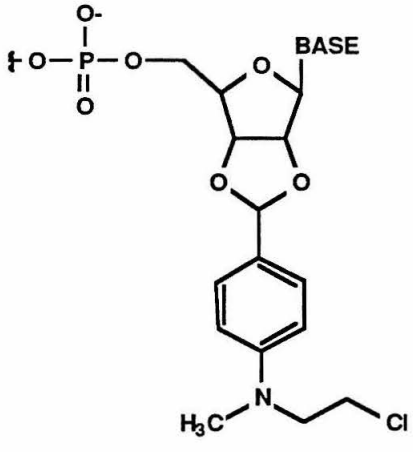
sites along a strand and to unusual bases such as 4-thiouridine at specific sites in an RNA molecule.[11] Clearly, the functional groups on the heterobifunctional reagent must be chosen and positioned carefully or premature reaction or inactivation can take place. The ideal reagent contains a highly reactive first group which quantitatively attaches the reagent to the hunter strand and a latent second reactive group which is activated only after the hunter strand has hybridized to the target strand. These heterobifunctional reagents are limited in terms of their general application; different strategies are required to attach the reagent to the 3' or 5' end of an oligonucleotide, and mixtures of modified oligonucleotides have usually resulted when the reagents were attached to bases in the middle of the hunter strand.

A second approach to the synthesis of hunter strands involves the covalent attachment of a latent reactive functional group onto a base followed by chemical incorporation of the modified nucleoside into a synthetic nucleic acid strand.[12] This approach has the considerable advantage of being able to control the exact placement of the modified base(s) anywhere along the nucleic acid strand. Furthermore, if the modified base can be used in automated oligonucleotide synthesis, the production of hunter strands can be greatly facilitated. However, the conditions used during chemical oligonucleotide synthesis, deprotection and purification are harsh enough to place limitations on the nature of the latent reactive functions.[13-16] In order to be used with this chemical synthesis approach, the reactive function must be chemically stable (in at least protected form) to oxidiz-

ing, acidic and basic conditions as well as electrophilic and nucleophilic reagents.

Functional Groups Used The functional groups that have been used most often for complementary-addressed modification of nucleic acids are alkylating agents. Of these, the nitrogen mustard derivatives (chloroethyl amines) have been the most thoroughly investigated. Reagents have also been prepared which utilize photochemical, inorganic and redox functions.[11]

The first heterobifunctional reagent reported and the one studied in the most detail is an aromatic nitrogen mustard linked to the 3' end of a ribonucleic acid strand via an acetal linkage to the 2' and 3' position of the 3' terminal residue.[10] This type of linkage insured

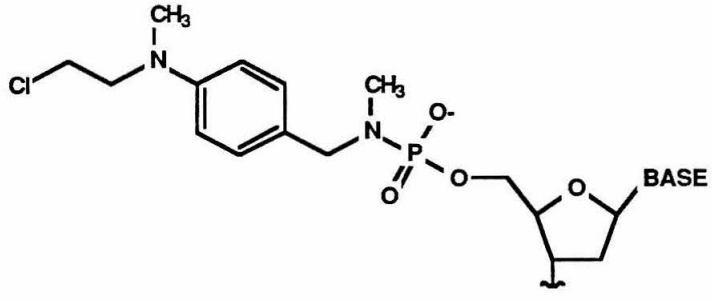


that only a single functional group was attached per hunter strand and only at the 3' end. These modified nucleic acid strands were synthesized from the oligonucleotide and benzaldehyde derivative of the reagent in a quantitative condensation reaction.[11] The hunter strands thus produced were quickly purified by gel filtration chromatography and used before the chloroethyl amine moiety hy-

drolyzed or reacted with other hunter strands. Fortunately, once hybridized to the target strand, the interstrand alkylation reaction was apparently highly favored and occurred with remarkable rates relative to less desirable types of reaction.[17,18] Reaction on the target strand with guanine, cytosine and adenine residues was reported.[19]

Nucleic acid strands carrying this reagent have been reported to help elucidate tRNA[20-22] and rRNA[19,23] structure, alkylate mRNA *in vivo*,[24] as well as cleave single stranded oligo-[17,18] and polynucleotides.[25] Gel electrophoresis of the products obtained upon reaction with single-stranded DNA revealed that >80% of the target strands were modified when a large excess of the hunter strand was used. Several adjacent bases were modified on the target strand, up to four residues on either side of the location of the center of modification.[25]

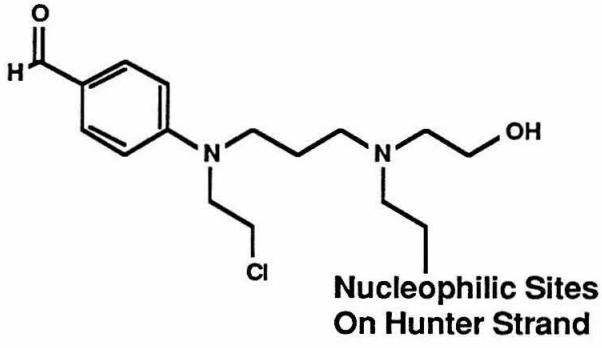
A similar nitrogen mustard derivative was attached to the 5' end of a nucleic acid strand.[26] An oligonucleotide modified with this



group reacted with and caused the cleavage of a 365 base single-stranded DNA fragment.[27] The cleavage was centered around the location of the reactive group in the sequence of the 365 base target that was complementary to the sequence of the hunter strand. The

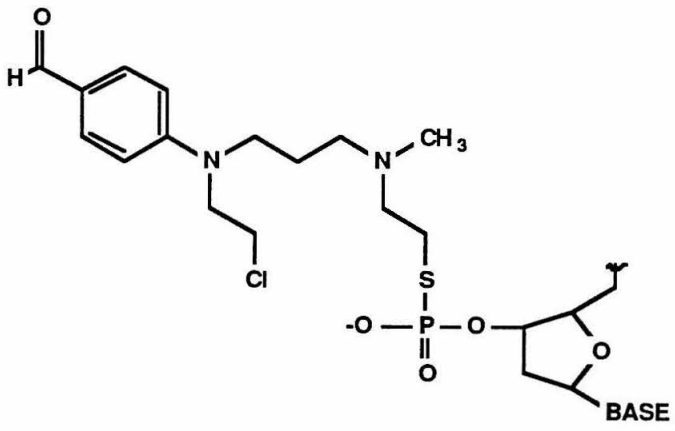
cleavage occurred to an equal extent at three adjacent guanine residues.

More sophisticated versions of the aromatic nitrogen mustard derivatives were reported.[28] These functional groups were designed



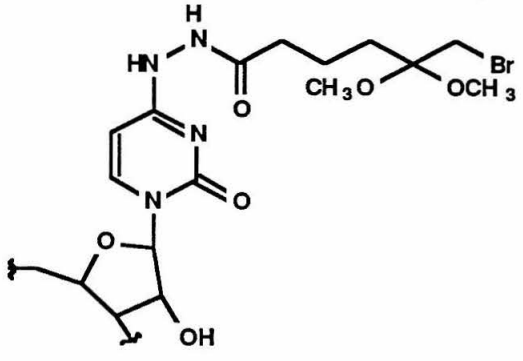
to allow activation of the alkylating moiety after the hunter strand was hybridized to the target. The formyl group at the para position on the ring served to deactivate the 2-chloroethylamine function. Once the hunter strand bearing this group was hybridized to the target strand, treatment of the system with sodium borohydride apparently reduced the formyl group which had the effect of activating the chloroethyl amine function enabling alkylation of the target strand. A heterobifunctional reagent bearing this group was attached at random to nucleophilic sites on mRNA early transcripts (at a loading of 3-5% reagent per transcript nucleotide) of the phage T7.[28] These modified transcripts were hybridized to T7 DNA and incubated following sodium borohydride treatment. The targeted DNA was used to transfect *E. coli* and 3 out of the 24 plaques produced contained mutations in the targeted gene.

The same functional group was attached to the 3' end of an oligonucleotide via a thiophosphate linkage.[29] This oligonucleotide



was shown to be capable of efficiently alkylating a complementary target strand once the system was treated with sodium borohydride.

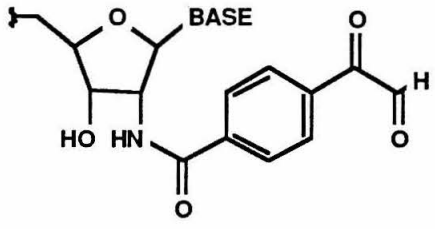
Another alkylating group capable of activation was reported prior to the nitrogen mustard reagents just discussed. The latent re-



active group consisted of an α-bromo ketal[30] which was attached to the C4 position of random cytosine residues in the hunter strands via an acylhydrazide linkage.[31] After hybridization to the target strand, treatment of the system with acid (pH 2.5) apparently hydrolyzed the ketal unmasking the reactive a-bromo ketone alkylating function. Using a filter binding assay, RNA transcript hunter strands carrying

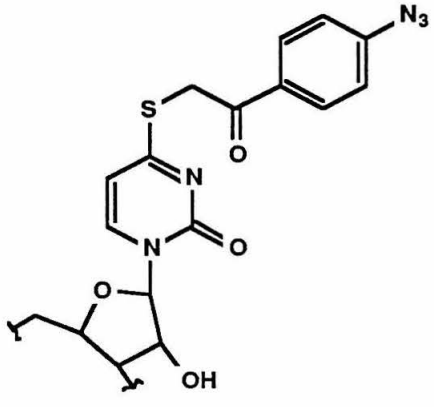
25% of the cytosines derivatized were found to form stable crosslinks to T7 DNA with a 20% efficiency.[31]

An interesting alkylating function was attached to the 3' end of an oligonucleotide via a 2' amido linkage. The alkylating function



consisted of a phenylglyoxal moiety and the system was used to study the 16 S RNA of the 30 S ribosome subunit.[32] An mRNA model oligonucleotide carrying the alkylating function was observed to covalently attach to the 16 S RNA in a highly specific reaction.

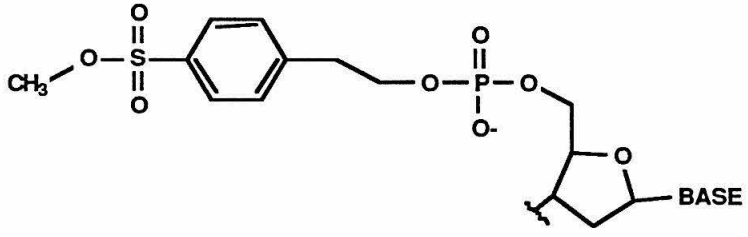
Highly specific hunter strands have been produced by taking



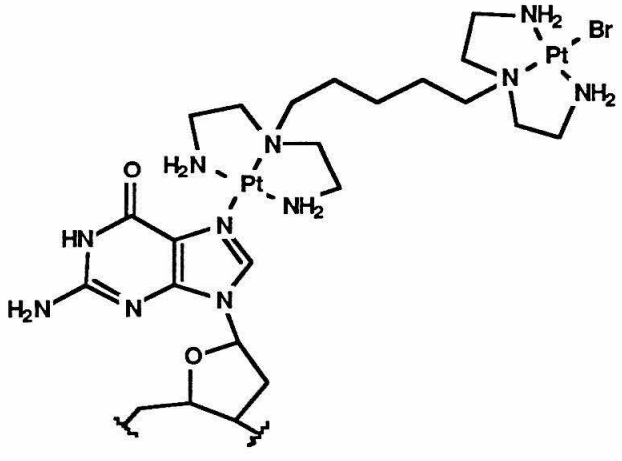
advantage of the presence of unique bases at certain positions of RNA molecules. For example, a phenylazide function was attached specifically to a single 4-thiouridine residue at position 8 of $tRNA_1^{Val}$ molecules.[33] These modified tRNA molecules were ir-

radiated (350 nm light) in the presence of ribosomes and the 16 S RNA of the 30 S ribosome subunit was specifically crosslinked with an efficiency of 20%.

A methylating reagent was reported, based on a methyl phenylsulfonate.[34] The reagent was attached to the 5' end of a short

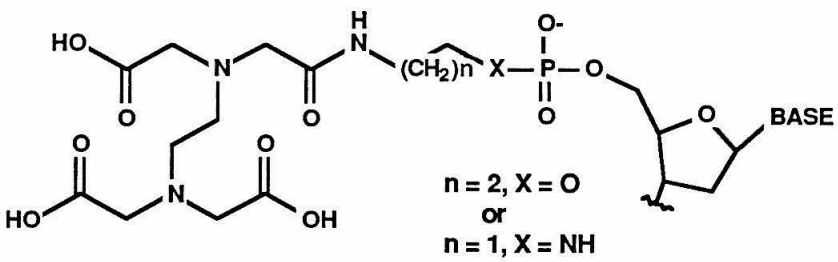


oligonucleotide (solubility considerations during the synthesis made it difficult to use long oligonucleotides) and the product was purified by gel chromatography just prior to use. The modified oligonucleotide was incubated with rRNA at low temperature and significant methyl transfer onto the rRNA was observed. The methylated RNA was not analyzed to determine where the modification was occurring.



A reagent has been described based on the binding of platinum(II) complexes to the N7 position of guanine residues.[35] The reagent was bound to the hunter strand presumably at the only G residue. This modified strand was then incubated with a complementary oligonucleotide and a crosslinked product was apparently obtained.

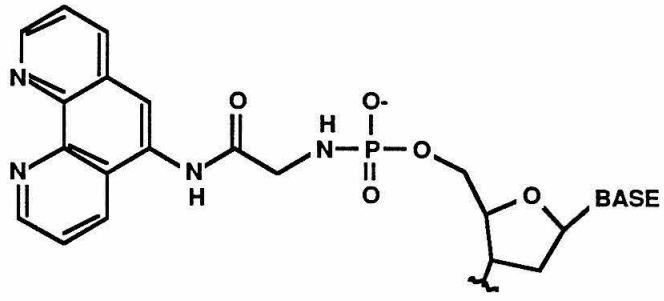
EDTA-Fe(II) in the presence of O_2 and a reducing agent has been shown to be an effective method of causing the cleavage of DNA, probably through the formation of hydroxyl radicals which attack nearby sugar groups on the DNA backbone.[36] Two similar systems have been reported in which oligonucleotides were post-syn-



thetically modified with an EDTA reagent and then used for complementary-addressed modification of target strands. In both cases, EDTA anhydride was reacted with oligonucleotides carrying a primary amine on the 5' end. In the first report, an octathymadilate strand carrying the EDTA group was observed to specifically degrade poly(dA), but in curiously low yield.[37] In the second report, the EDTA-carrying hunter strand (with a heterogeneous base sequence) was hybridized to a complementary oligonucleotide, and the target was degraded specifically at the location of the EDTA with about 15%

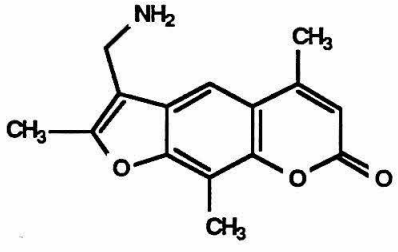
efficiency.[38] A total of 8-10 residues were attacked revealing the nonselective nature of the cleavage reaction.

A similar system based on the phenanthroline ligand attached to the 5' end of an oligonucleotide has been reported.[39] The cleav-



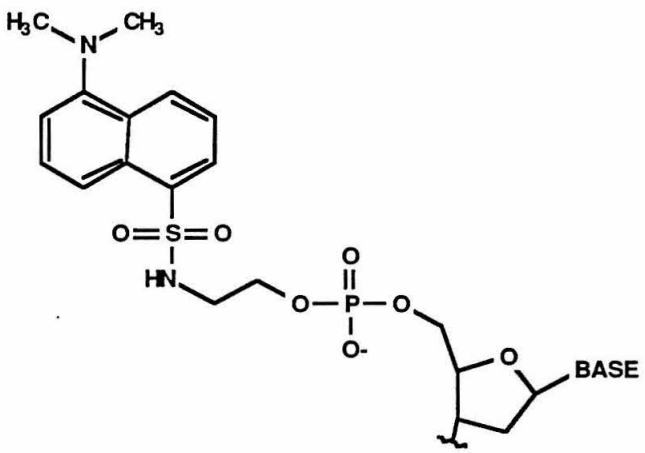
age reaction utilized cupric ion and thiol, and just like with EDTA-Fe(II), several bases on the target strand were attacked.

Photochemical reagents offer the advantage of being inert until



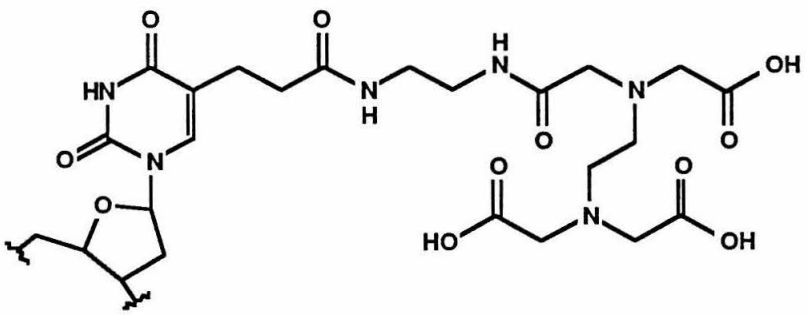
photolyzed, so the modification reaction can be timed to occur only after the hunter strand has hybridized to the target. For example, rRNA was photolyzed in the presence of 4'-aminomethyl-4,5',8-trimethylpsoralen to produce monoadducts on the rRNA strand at a frequency of about four psoralen molecules per strand.[40] This modified strand was then incubated with a large supercoiled plasmid carrying the rRNA gene and efficient hybridization was observed. Upon

further irradiation, the psoralen molecules created covalent interstrand crosslinks selectively in the region of the genome complementary to the rRNA with around 50% efficiency. A dansyl group



covalently linked to the 5' end of an oligonucleotide probe has also been reported to modify a complimentary strand upon irradiation.[41]

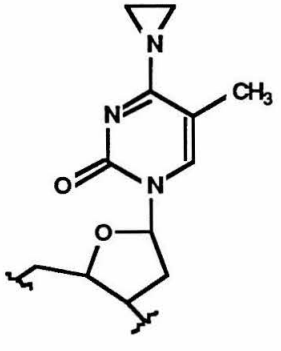
In this laboratory, the Fe(II)-EDTA group has been used in a



system which illustrates the feasibility of the second approach of producing hunter strands through chemical synthesis with a modified base. A protected EDTA group was covalently attached to the 5 position of uridine and used to produce modified oligonucleotides via

the phosphoramidite method.[12] A 167 base pair double-stranded DNA fragment was heat-denatured then quickly cooled in the presence of oligonucleotide carrying EDTA. Upon incubation with Fe(II) and dithiothreitol (DTT), cleavage was observed on the fragment only in the region complementary to the oligonucleotide, centered around the location of the EDTA moiety. Several bases on either side of the EDTA group were attacked.

This work has more recently been expanded to include automated synthesis of oligonucleotides carrying the EDTA group. These modified probes have now been used to specifically cleave large pieces (7000 base pair) of single-stranded phage DNA[42] as well as investigate triple-stranded formation among oligonucleotides and restriction fragments.[43] The observed cleavage of double-stranded DNA through triple-strand formation has brought complementary-addressed modification into a whole new arena, wherein single-stranded nucleic acid strands are not the only possible targets.



A variation of the chemical synthesis with a modified base approach was reported to produce an oligonucleotide carrying an aziri-

dine group attached to the 4 position of a 5-methylcytosine residue.[44] The oligonucleotide was chemically synthesized with a modified 5-methylcytosine base substituted in the 4 position with triazole. After synthesis of the oligonucleotide by the solid phase method, the triazole was exchanged with ethylenimine. The oligonucleotide now carrying the aziridine was removed from the solid support, deprotected, purified and hybridized to a complementary target strand. When the target strand contained a normal cytosine residue opposite the base carrying the ethylenimine, a covalent interstrand crosslink was obtained after a 4 day incubation.

The preceding examination of the literature has illustrated several important points about complementary-addressed modification of nucleic acid strands. First, oligonucleotides carrying reactive groups were shown to be capable of specific and efficient modification of complementary strands. Second, it was advantageous to use latent groups which were activated after hybridization, thus avoiding premature deactivation or reaction with other hunter strands. A very robust latent reactive group (EDTA) also allowed for production of modified bases which were conveniently placed anywhere along oligonucleotide strands by chemical synthesis, not just at the ends or randomly distributed among the various bases. Finally, although important applications have been demonstrated and more will undoubtedly be found, to date no reactive oligonucleotide system has been proven capable of reaction with and cleavage of a complementary strand to nucleotide resolution while satisfying all four of the criteria listed in the introduction.

DESIGN OF SYSTEM

New Approaches To The Synthesis Of Hunter Strands

The work described in the preceding literature review illustrated how heterobifunctional reagents were limited when it came to providing a universal approach to the production of nucleic acid strands carrying reactive groups at precise positions along the strand. Different strategies were required for attaching the reagents to the 3' or 5' ends. Furthermore, placing the reactive groups at the ends of the hunter strands did not produce modification of the target strands to nucleotide resolution. These terminal linkages placed the reactive group close to conformationally labile, single-stranded regions of DNA target strands possibly explaining the observed modification and cleavage of several adjacent bases.

The second approach of using modified bases in chemical synthesis has the considerable advantages which derive from precise control over placement of the latent reactive group(s) along an oligonucleotide strand. However, some of the latent reactive groups we wished to investigate might not be robust enough to quantitatively withstand the chemical oligonucleotide synthesis procedures.

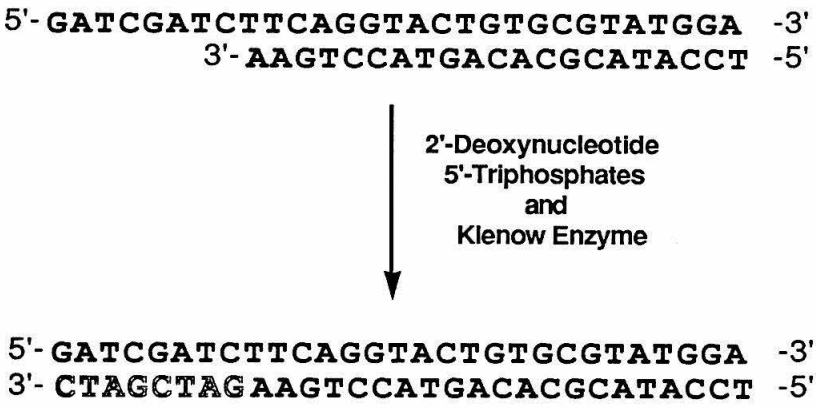
An approach described here, utilizing an enzyme reaction to place a modified nucleotide triphosphate molecule into specific positions of a hunter strand,[5] retains several of the advantages inherent in the chemical synthesis approach while hopefully allowing the use of some relatively sensitive latent cleavage functions. The enzymatic approach could be used to specifically place the modified base at different but discrete positions along an oligonucleotide strand, so

just like with the chemical synthesis approach, the modified base could be precisely placed in the middle of the hunter strand. The reactive group would therefore be in a presumably double-helical region of DNA when the hunter strand was hybridized to the target strand. This expected secondary structure could be exploited to specifically align the reactive group with a single residue on the target strand, thus increasing the specificity of the modification reaction. The enzyme reaction would be carried out under mild conditions, namely room temperature and neutral pH, allowing use of relatively sensitive latent reactive functional groups. The enzymatic techniques that would be used to produce the hunter strands (*vida infra*) are similar to those currently employed for Sanger-sequencing[3] or 3' end-labelling of restriction fragments,[4] thus criterion 3) listed in the introduction should be satisfied by such procedures.

For the above reasons, using modified 2'-deoxynucleotide 5'-triphosphate molecules enzymatically incorporated into oligonucleotide strands was chosen as the primary approach to producing hunter strands in order to explore different complementary-addressed cleavage strategies as described in the following sections of this thesis.

The enzyme most capable of producing hunter strands is the Klenow fragment of DNA polymerase 1. The Klenow fragment of DNA polymerase 1 or so-called Klenow enzyme is produced by limited proteolysis (using subtilisin) of DNA polymerase 1.[45] The Klenow enzyme catalyzes a "fill-in" reaction in which 2'-deoxynucleotide 5'-triphosphate molecules are incorporated in the 5'-3' direction into a

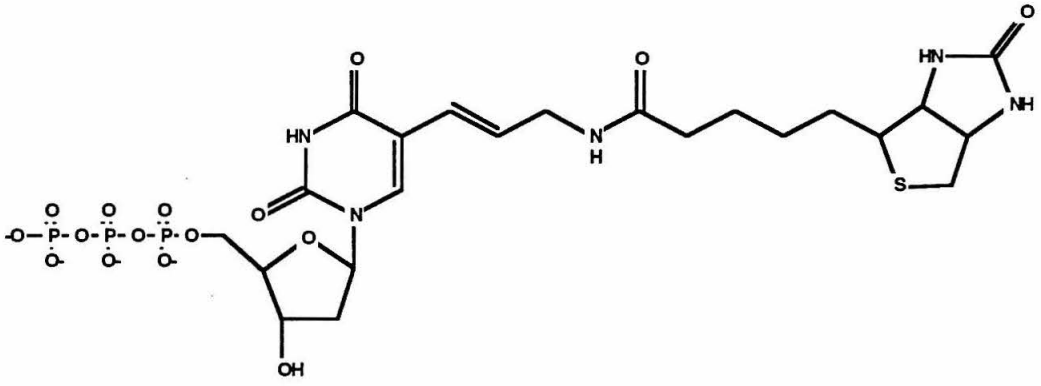
DNA strand.[45] The enzyme places the new base opposite its complement (A to T, G to C, etc.) with virtually perfect accuracy. Bases



are added along the growing strand until the 5' end of the complementary (or so-called "template") strand has been reached, thus DNA duplexes with 5' overhang ends are required for the reaction. The enzymatic reaction also requires a "primer" oligonucleotide containing a 3'-OH group onto which 2'-deoxynucleotide 5'-triphosphate molecules are placed as the strand is filled-in. The Klenow enzyme contains a 3'-5' editing function which apparently assists in providing the observed high fidelity of base incorporation.

The feasibility of the using the Klenow enzyme for the synthesis of nucleic acid strands capable of complementary-addressed modification was indicated in the literature by the successful enzymatic synthesis of oligonucleotides carrying biotin groups.[46] The biotinylated oligonucleotide probes have been used in non-radioactive alternatives to ^{32}P end-labelling procedures during electrophoretic analysis of nucleic acids. According to the procedures, modified 2'-

deoxyuridine 5'-triphosphate molecules (carrying the biotin moiety



covalently attached at the 5 position of the uridine ring) were enzymatically incorporated into the DNA probe by the Klenow fragment of DNA polymerase 1. The modified 2'-deoxyuridine 5'-triphosphate molecule carrying the rather bulky biotin group was placed opposite only A residues in a template strand by the Klenow enzyme, so it is reasonable to assume the enzyme will be able to place 2'-deoxyuridine 5'-triphosphate molecules carrying other groups of similar size at the 5 position of the uridine opposite A residues in a template strand.

Using the Klenow enzyme approach of incorporating modified 2'-deoxyuridine 5'-triphosphate molecules into hunter strands has at least one apparent limitation; it is impossible to place a modified base on or near the 5' end of a strand. To overcome this limitation, a fourth type of approach (conceptually analogous to the preparation of aziridine oligonucleotides[44] discussed earlier) was investigated whereby a modified base was synthesized carrying a primary amine function in protected form. The modified base was placed in an oligonucleotide strand via automated chemical synthesis. Once

deprotected, the amine function was used as a specific target for a heterobifunctional reagent. This approach offers absolute flexibility and control over the placement of the reactive group on the hunter strand as well as the convenience of automated synthesis without the limitations on reactive groups usually encountered with chemical synthesis procedures.

Reasons Behind Choosing An Alkylation Type Cleavage Reaction Of all the possible types of DNA modification reactions, we have concentrated on alkylation reactions because they have several useful properties.

The key criterion mentioned in the introduction is 1), the requirement of cleavage to nucleotide resolution. Such specificity could be achieved by placing a nonspecific reactive group in a precise geometry next to a single site on a target strand or by placing a reactive group with specificity close to several sites, only one of which can lead to reaction. The latter approach is simpler to achieve thanks to the inherent specificity of alkylation reactions on DNA.

Alkylation reactions are highly specific when it comes to where on the different bases alkylation occurs.[47] For example, Figure 3 lists the percent obtained of each of the different products resulting from reaction of [^{14}C]methyl methanesulfonate with double-stranded DNA.[48] The most striking feature of the data is the high reactivity of the N7 position of guanine relative to other sites in double-stranded DNA. The N7 position of guanine resides in the major groove of B form DNA while the next most reactive site, N3 of adenine, is in the minor groove. The second most reactive site in the

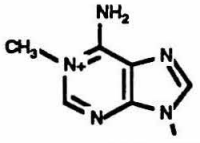
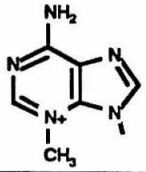
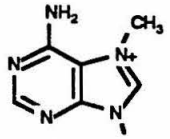
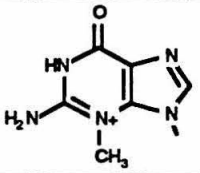
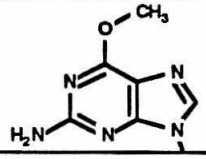
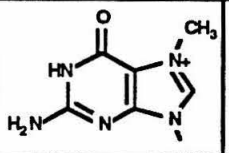
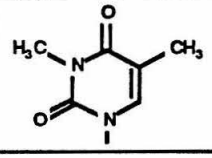
Site Of Methylation	Percent Of Total Methylated ± S. D.
	1.89 ± .12
	11.3 ± .38
	1.83 ± .09
	.62 ± .03
	.31 ± .03
	81.4 ± 2.6
	.08 ± 0

Figure 3 Alkylated products obtained upon reaction of [^{14}C]methyl methanesulfonate with double-stranded DNA. Data is from reference 48.

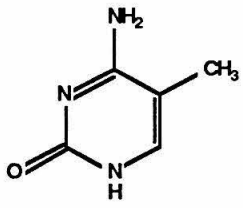
major groove is N7 of adenine, but that site is almost two orders of magnitude less reactive than N7 of guanine. Therefore, placing a methylating group in the major groove of double-stranded DNA should have an inherent base specificity of almost two orders of magnitude!

Treatment of DNA containing N7-methylguanine residues with piperidine causes DNA strand scission at the site of the methylated base.[49] The DNA strands produced contain exclusively 3' and 5' phosphate ends at the site of cleavage. Therefore, criterion 2) in the introduction could be satisfied by cleavage of DNA resulting from complementary-addressed methylation at N7 of guanine followed by a piperidine treatment.

The complementary-addressed alkylation of nucleic acids[11] was proven to be quite facile and occurred with relatively high yields. We can therefore expect that criterion 4) mentioned in the introduction can also be satisfied by using alkylating reagents.

Reasons For Choosing A Methylthioether Group As The Latent Alkylating Function; SAM The choice of a methylthioether group as a latent alkylating function was inspired by the pathways used for *in vivo* methylation of DNA.

Specifically methylated DNA has been found in both prokaryotes and eukaryotes. Methylated bases are found in prokaryotes such as *E. coli* to the extent of 1% 5-methylcytosine (relative to all cytosine) and 2.1% N6-methyladenine (relative to all adenine).[50] In



5-Methylcytosine

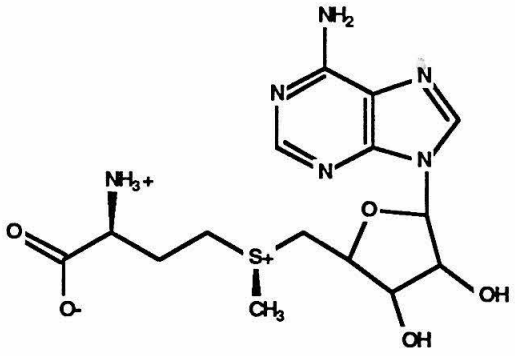
N6-Methyladenine

eukaryotes, 5-methylcytosine is the only methylated base detected and it can comprise up to 9% or more of all cytosine.[51]

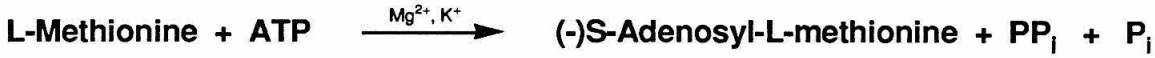
In prokaryotic systems, the methylated bases are an essential component of the restriction endonuclease-methylase system used to prevent invasion by foreign DNA.[52] Bacterial chromosomal DNA has certain sequences methylated. The bacterial cell also contains a restriction endonuclease which recognizes and cleaves that same DNA sequence, but the bacteria's own DNA is not attacked because of the methylation. Invading DNA (from a phage for example) will most likely not be methylated in the proper sequence so the phage DNA is cleaved by the restriction endonuclease and the DNA invasion is thwarted.

In eukaryotes, specific methylation is a field of intense investigation, and is currently thought to be a mechanism by which gene expression is regulated, especially during embryonic development.[53] For example, methylation of one of the two X chromosomes during early development has been indicated as a mechanism by which X inactivation occurs in female mammalian cells.[54]

All DNA methylation enzyme systems studied so far use the same cofactor as the source of the methyl group transferred, (-)S-

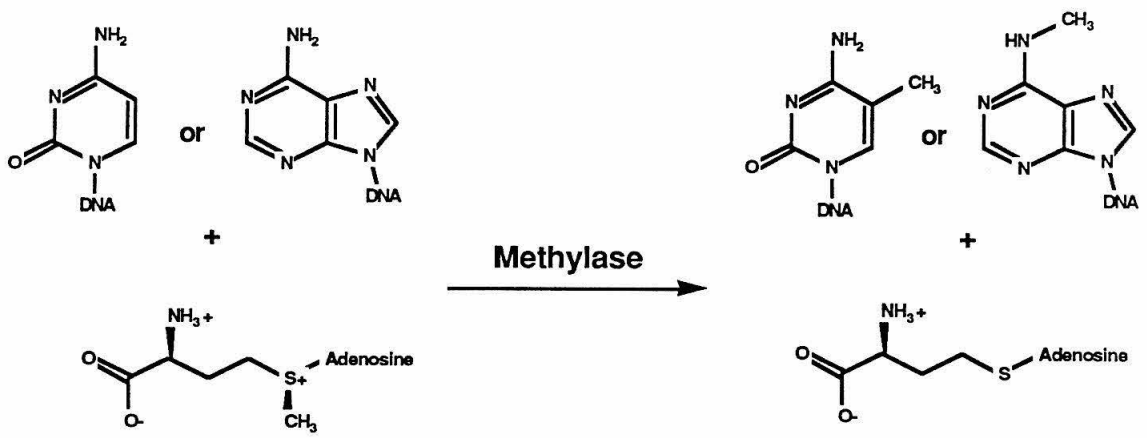


adenosyl-L-methionine (SAM).[55] SAM is synthesized through an enzymatic reaction of the stoichiometry shown below.[56] The enzyme



responsible is called S-adenosyl-L-methionine synthetase (EC 2.5.1.6).[57] The chiral sulfonium of SAM is produced exclusively as the (-) or S isomer, but it racemizes in aqueous solution ($k_{race.}$ = 8 x 10^{-6} s^{-1} at pH 7.5 and 37°C)[58] and the (+) or R isomer is inactive in enzymatic methyl transfer reactions. SAM also hydrolyzes at neutral pH to yield homoserine and 5'-deoxy-5'-(methylthio)adenosine[59] ($k_{hyd.}$ = 6 x 10^{-6} s^{-1} at pH 7.5 and 37°C).[58] These spontaneous deactivation processes (racemization and hydrolysis) require that the SAM produced be used quickly in methylation reactions. This is an important consideration because of the extremely high energy cost to the cell of synthesizing SAM.[60]

The enzymatic DNA methylation reaction follows the general stoichiometry outlined below. In the case of cytosine methylation, a



covalent enzyme-cytosine intermediate has been proposed.[61] The reaction is expected to follow an S_N2 pathway with inversion of the methyl group in analogy to other enzymatic methylations using SAM.[62]

Interestingly, free SAM has been found to methylate DNA in small but detectable amounts under physiologically relevant conditions (1×10^{-5} M SAM, 4 h, 37°C) in the absence of any methylase.[63] Not surprisingly, N7-methylguanine was found to be the primary product of the nonenzymatic reaction.

The CNBr Peptide Cleavage Reaction The methylthioether group of methionine can be thought of as a latent alkylating group in another well known reaction, namely the cyanogen bromide (CNBr) cleavage of peptides or proteins.[64,65] Treatment of a methionine containing peptide or protein with CNBr under acidic conditions causes specific peptide cleavage at the site of the methionine. The products of the reaction are methylthiocyanate, aminoacylpeptide and peptidyl homoserine lactone. A mechanism of cleavage has been

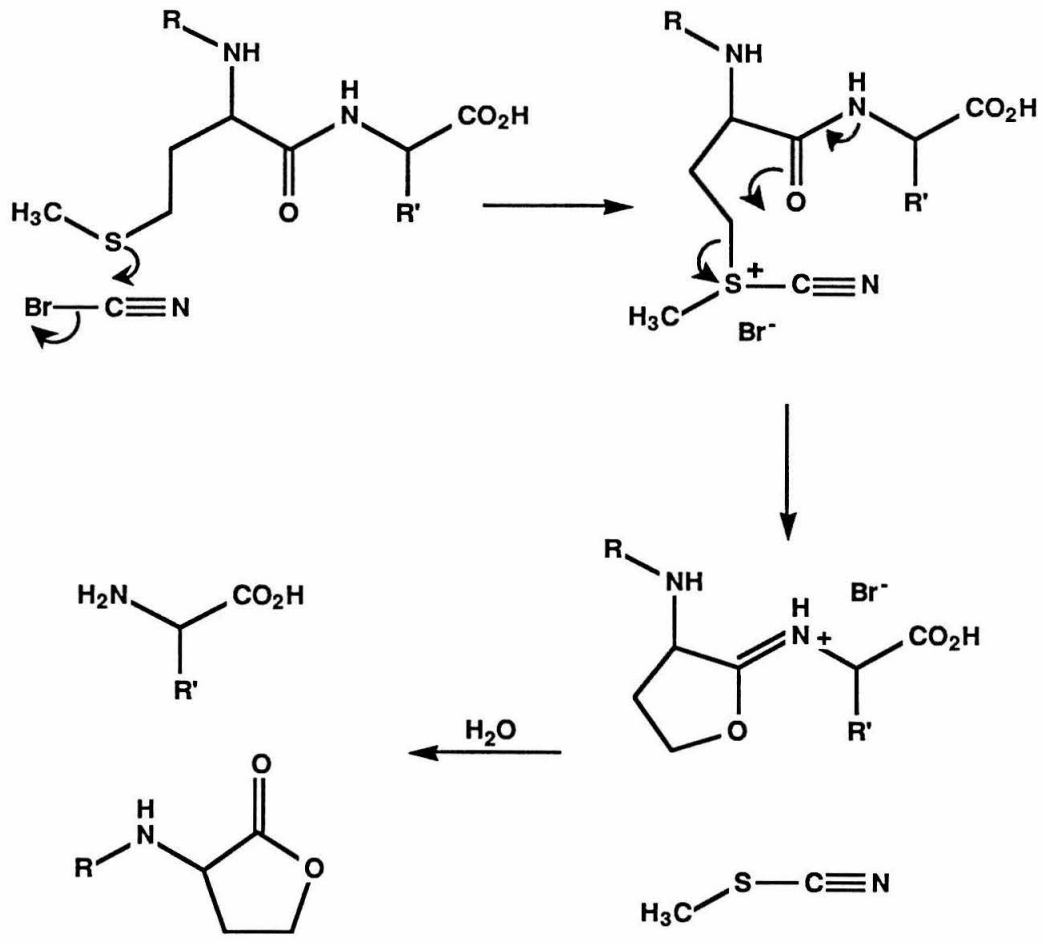


Figure 4 The mechanism proposed in reference 65 for the cleavage of peptides at methionine residues by CNBr.

proposed (see Figure 4). The proposed cyanosulfonium bromide intermediate has not been isolated or characterized. The CNBr cleavage reaction at methionine has been an important tool in the specific fragmentation of proteins to facilitate sequence determination.

The CNBr peptide cleavage reaction was in fact based upon the much older (first reported in 1877) reaction of dialkylthioethers with CNBr.[66,67] The CNBr treatment cleaves the dialkylthioethers pro-

ducing an alkylthiocyanate and an alkyl bromide.

It is reasonable to assume that under the proper conditions, the cyanosulfonium bromide intermediate produced upon reaction with CNBr and a methylthioether could lead to a methyl transfer reaction in analogy to SAM. Of course undesirable reaction pathways such as the intramolecular lactonization observed with the methionine reaction must be accounted for and hopefully circumvented.

The preceding discussions of DNA alkylation, SAM and the CNBr peptide cleavage reactions have indicated the reasons behind our choosing a methylthioether as a latent alkylating (methyl transfer) group to be used for complementary-addressed modification of DNA. The original scheme I devised for activating the methylthioether function on a hunter strand involved enzymatic activation to the methyl-adenosyl sulfonium with S-adenosyl-L-methionine synthetase in the presence of ATP. The enzyme's substrate specificity has been thoroughly investigated and it appears as though an ester

linkage from the carboxyl group of methionine to an alcohol function on the hunter strand would be acceptable as substrate to the enzyme.[68] Unfortunately, this enzymatic activation was never attempted because of the difficulty encountered in obtaining a pure sample of S-adenosyl-L-methionine synthetase. CNBr treatment was therefore used as the activation method as described in the following section of this thesis.

Design Of The Modified 2'-Deoxyuridine 5'-Triphosphate Compounds Carrying A Methylthioether Function 5-allylamino-2'-deoxyuridine 5'-triphosphate was chosen as the precursor to all of the modified bases which were enzymatically incorporated into hunter strands and investigated for cleavage activity because the synthesis has been described,[46] and the Klenow enzyme is known to accept as substrate molecules of this form.[46] The side chain of compound **2** (*vida infra*) was designed using a CPK model of right-handed B form DNA assuming a methyl transfer reaction to the N7 position of guanine(s) on the target strand. A side chain with two methylene units between the carbonyl group and the sulfur atom was chosen because this length appeared to allow facile methyl transfer reaction on the DNA while not providing the proposed five-membered ring transition state required for the unwanted peptide bond cleavage reaction.

RESULTS AND DISCUSSION

Synthesis

Modified 2'-Deoxyuridine 5'-Triphosphate Compounds Carrying A Methylthioether Function The modified 2'-deoxyuridine 5'-triphosphate (dUTP) compounds (**2**, **4** and **5**) were synthesized using strategies analogous to methods in the literature[46] (see Figures 5 and 6). dUTP was treated with mercuric acetate at pH 6 and 60°C. The product 5-mercurinucleotide 5'-triphosphate was collected as a flocculent white solid that was precipitated from aqueous solution by the addition of cold ethanol. The isolated yield was observed to increase when the ethanolic mixture was allowed to precipitate at 4°C overnight. The key intermediate **1** was produced by reacting the 5-mercurinucleotide 5'-triphosphate with allylamine in the presence of K_2PdCl_4 at pH 5.4. The product **1** was isolated as the ninhydrin-positive fraction eluted between .4 M and .5 M triethylammonium bicarbonate (pH 7.6) on a DEAE Sephadex column. The methylthioether derivatives **2**, **4** and **5** were produced by reacting the N-hydroxysuccinimide esters of the appropriate methylthioether acid compounds with **1** at pH 8.85. The reaction was monitored by subjecting small aliquots of the reaction to a quantitative ninhydrin test.[69] The reaction was stopped when the ninhydrin test revealed the absence of any primary amine groups which would have indicated unreacted **1**.

The crude product **2**, **4** or **5** was initially isolated as the short wave UV absorbing fraction that eluted between .7 M and .9 M triethylammonium bicarbonate (pH 7.6) on a DEAE Sephadex column.

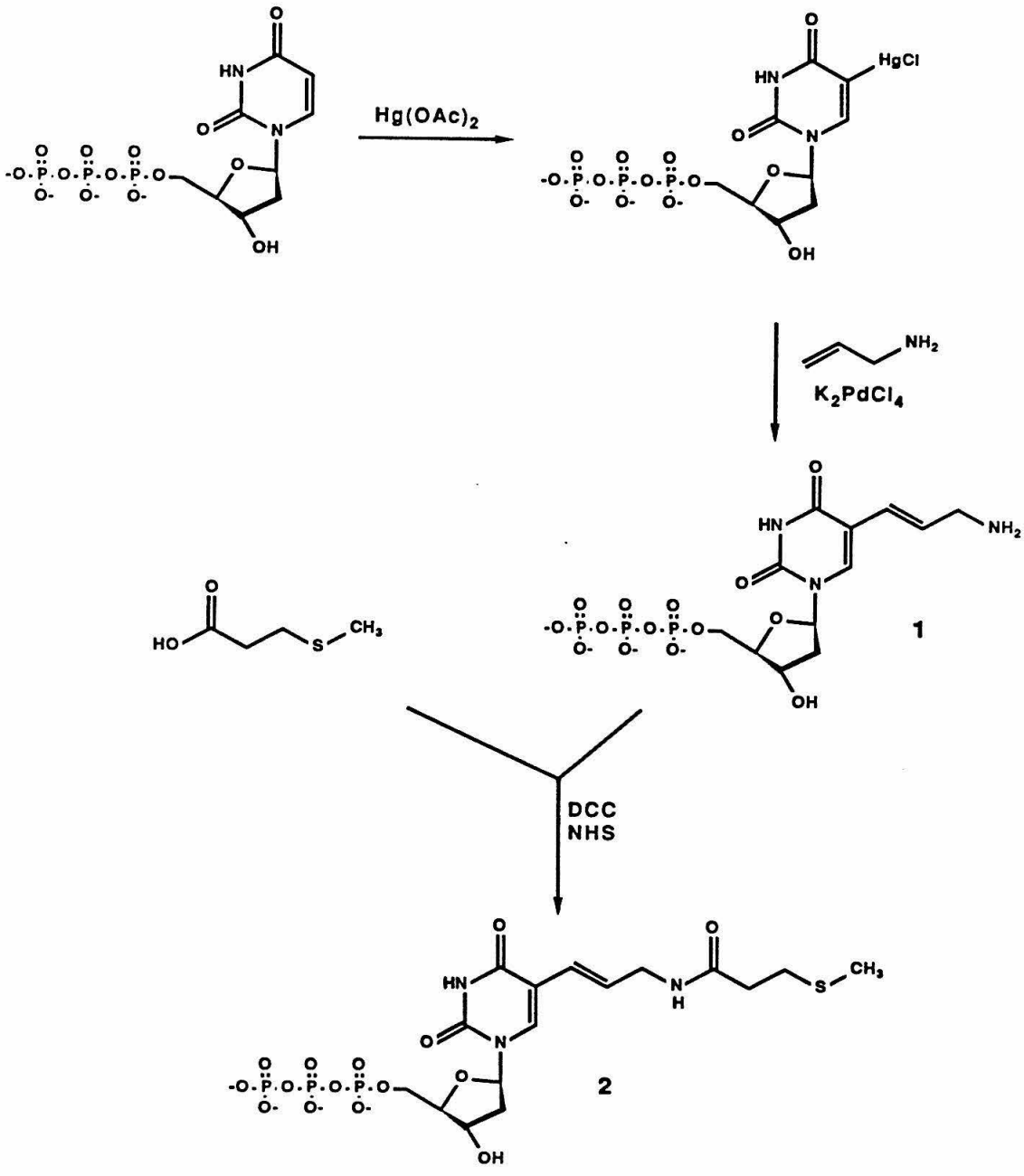


Figure 5 Synthetic scheme for preparation of compounds **1** and **2**.

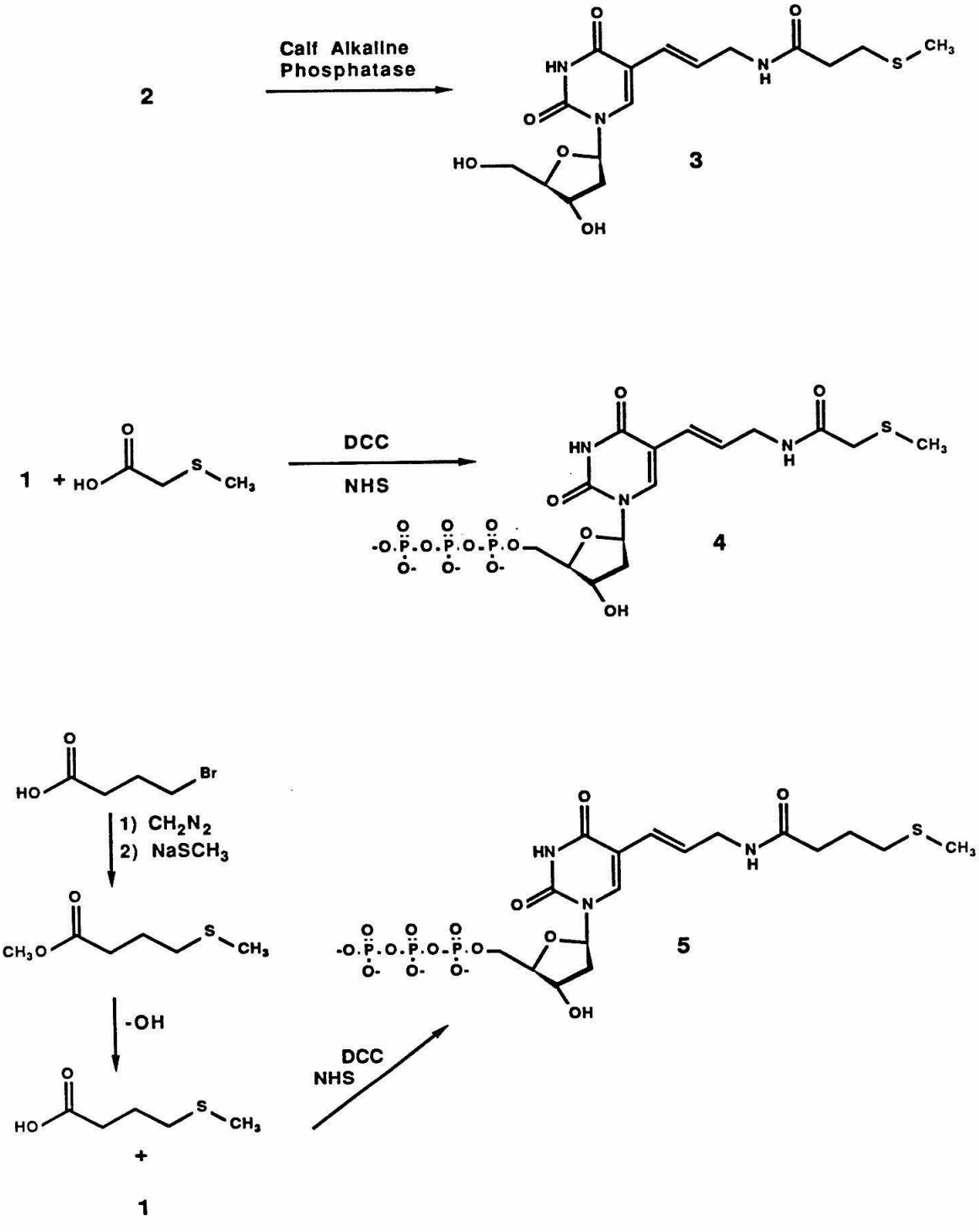


Figure 6 Synthetic schemes for preparation of compounds **3**,**4** and **5**.

The pure **2**, **4** or **5** was obtained by chromatographing the crude product on an analytical anion exchange HPLC column eluted with a linear gradient of 0-.3 M ammonium bicarbonate (pH 7.6) buffer. To prevent the volatile ammonium bicarbonate from bubbling out of solution and thereby crippling the HPLC pumps and/or detector, the ammonium bicarbonate buffer was placed in an ice bucket and kept at 0°C during the chromatography. The purified **2**, **4** or **5** was lyophilized for several days to remove water and buffer. The lyophilized material was always stored under vacuum to prevent air oxidation of the methylthioether function.

UV-Vis, IR and ^{1}H NMR spectroscopy were used to confirm the structures of the modified dUTP compounds. As additional proof of structure, **2** was dephosphorylated by the enzyme calf alkaline phosphatase. High resolution mass spectral analysis proved the resulting nucleoside (**3**) had a molecular formula that was identical to that predicted for **3**.

The key to the synthesis of **2**, **4** or **5** was the HPLC purification procedures designed to remove even traces of contaminating dUTP derivatives. It was feared that compounds with less steric bulk at the 5 position such as 2'-deoxyuridine 5'-triphosphate (dUTP) or **1** might be preferred substrates of the Klenow enzyme, so that these compounds would have to be eliminated before **2**, **4** or **5** would be accepted as substrate by the enzyme.

The purity of **2**, **4** and **5** was confirmed by reinjection into the HPLC (see Figure 7). These HPLC conditions would have cleanly sep-

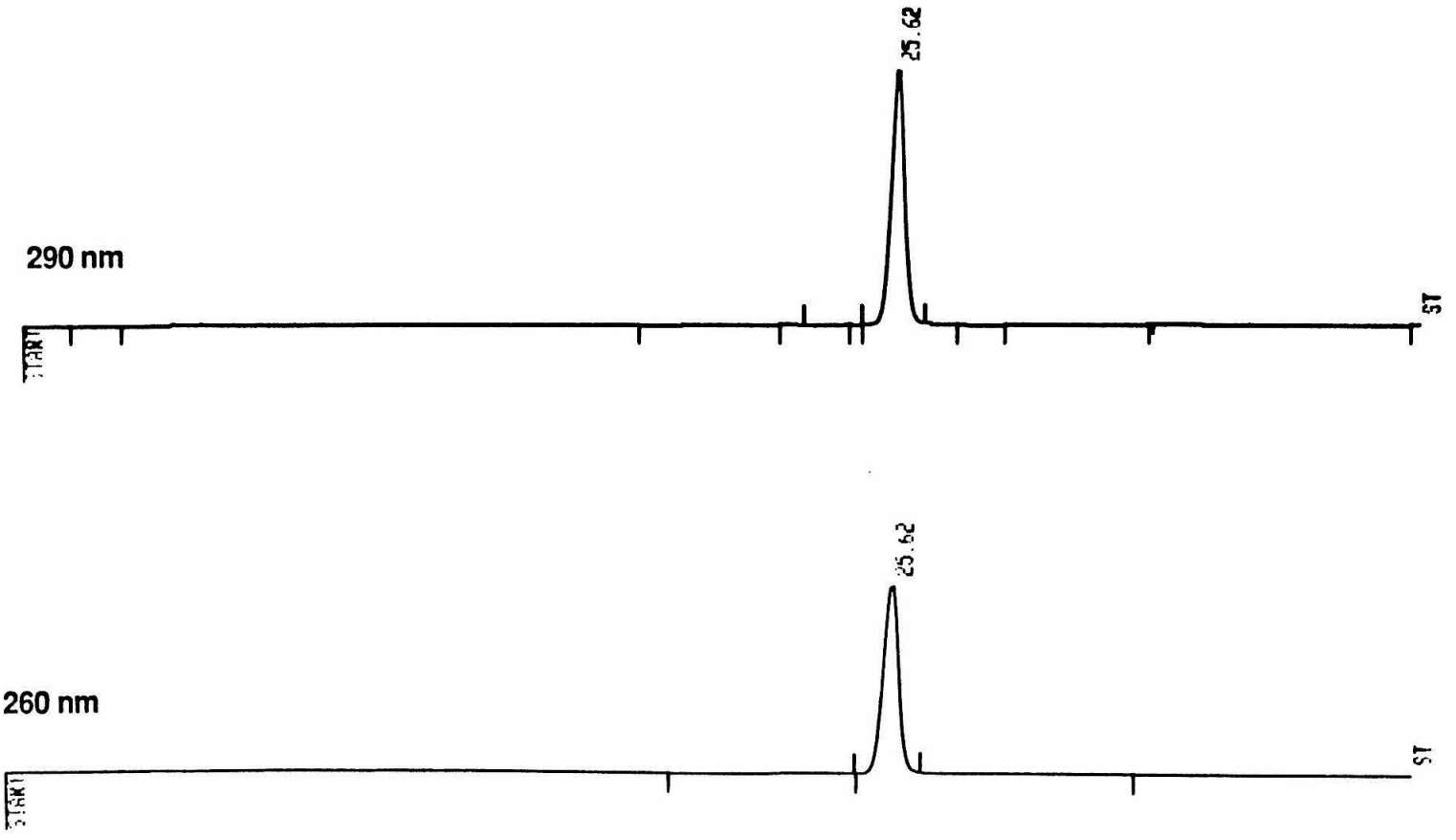


Figure 7 Ion-exchange HPLC traces of pure compound **2** simultaneously monitored at 260 nm and 290 nm. Approximately 100 µg of **2** was loaded onto a 4.6 mm x 25 cm Synchropak Q300 anion exchange column eluted with a 30 minute linear gradient of 0-.3M ammonium bicarbonate (pH 7.6) at a flow rate of .75 ml/min.

arated dUTP and **1** from **2**, **4** or **5**. Furthermore, the fact that the HPLC peak maxima were always at exactly the same retention time when the analysis was simultaneously monitored at two different wavelengths (260 nm and 290 nm) indicated that the material under the peak was homogeneous.

The ^{1}H NMR spectra were also diagnostic as to the purity of **2**, **4** and **5** (see Figure 46 in the Experimental section). Of particular interest in the spectrum is the sharp singlet at ~8 ppm arising from the C6 proton. The chemical shift of this proton was found to be very sensitive to the nature of the constituent at C5. As such, a sharp singlet ~8 ppm with no nearby signals was taken as a first indication of relatively pure material.

Klenow Enzyme Incorporation Reactions

It has been previously reported in the literature that dUTP compounds modified in the 5 position with a biotin moiety were accepted as substrate by the Klenow fragment of the DNA polymerase 1 enzyme.[46] These modified dUTP compounds were placed exclusively opposite A residues during the 5'-3' DNA fill-in reaction catalyzed by the enzyme.

The ability of compound **2** to act as substrate for the Klenow enzyme was thoroughly investigated. Oligonucleotides 1 and 2 (see Figure 8) were used to study the reaction. Oligonucleotides 1 and 2 were always annealed to each other by placing them in a 60°C water bath which was immediately removed from heat and allowed to cool slowly to room temperature over one hour. Note that the sequences

5'-CTGTCTGGGGAGTCTCAGCAGTAGTCGTCATCAG-3'
Oligonucleotide 1

5'-CTGATGACGACTACTGCTGA-3'
Oligonucleotide 2

5'-TTTTTCTCTCTCTC-3'
Oligonucleotide 3

5'-AGAGAGAGAGAAAAACCCCCC-3'
Oligonucleotide 4

5'-CTGTCGGGAGAGTCTCAGCAGTAGTCGTCATCAG-3'
Oligonucleotide 5

5'-TTTGTAGTTGTAGAT-3'
Oligonucleotide 6

5'-CATCTACAAC-3'
Oligonucleotide 7

5'-CTGTGTTCGTAGTCTCAGCAGTAGTCGTCA-3'
Oligonucleotide 8

5'-TGACGACTACTGCTGAGA-3'
Oligonucleotide 9

5'-GCAAAGTAAGAGCTTCTCGAGCTGCGCAAGGATAGGTCG-3'
Oligonucleotide 10

5'-ATTAAGCCACTTCTCCTC-3'
Oligonucleotide 11

5'-AAGCCACTTCNCCTCATCCAA-3'
Oligonucleotide 12
N = 5-(3-amino-*trans*-1-propenyl)deoxyuridine

Figure 8 Sequences of synthetic oligonucleotides.

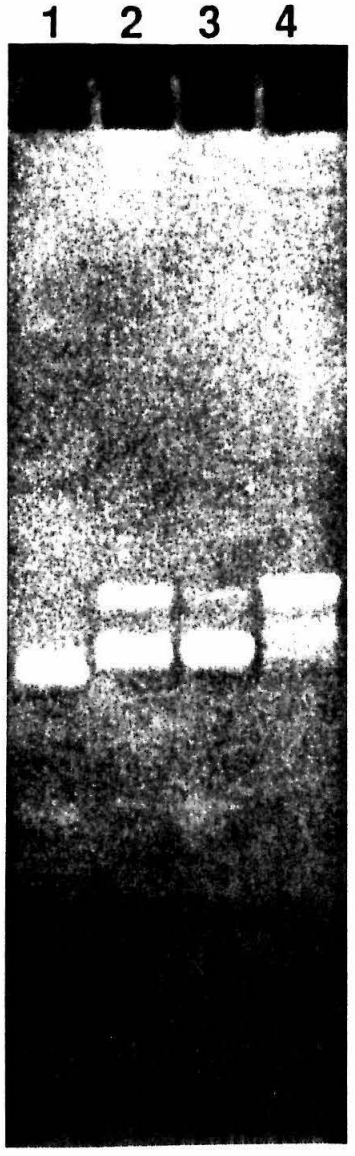
of oligonucleotides 1 and 2 were specifically designed to preclude the two strands from annealing in any way different from the desired duplex.

Non-denaturing 15% polyacrylamide gels were found to separate filled-in from incompletely filled-in duplexes to nucleotide resolution. The DNA was visualized in the gels either by staining with ethidium bromide or simply by looking for UV-absorbing bands (the DNA appeared dark) when irradiated with short wave UV light above a fluorescent TLC plate.

Conditions were sought wherein the Klenow enzyme would place no base opposite the single A in the overhang region of the oligonucleotide 1 and 2 duplex unless a modified dUTP compound or thymidine 5'-triphosphate (TTP) was added to the reaction. It was surprising to me that high concentrations of Klenow enzyme were found to place a base opposite this A even when only dATP (2'-deoxyadenosine 5'-triphosphate), dCTP (2'-deoxycytidine 5'-triphosphate) and dGTP (2'-deoxyguanosine 5'-triphosphate) were added to the reaction (see lane 2 in Figure 9). This misincorporation was found to be independent of all reactant concentrations (dCTP, dATP, dGTP and buffers) except that of the enzyme. Therefore, assuming the enzyme was 100% accurate in placing appropriate bases opposite the A, the enzyme preparation apparently had the unexpected ability of producing TTP-like molecules from the reaction mixture. Klenow enzyme is known to have a 3'-5' exonuclease activity[45] so it is reasonable to assume that at high enzyme concentrations some

Figure 9

Klenow enzyme fill-in reactions using high and low concentrations of Klenow enzyme. 2 mm x 160 mm x 160 mm 15% nondenaturing polyacrylamide gel stained with ethidium bromide. In each reaction the oligonucleotide 1,2 duplex was formed by heating .1 nmol of oligonucleotide 1 and .12 nmol oligonucleotide 2 to 60°C then slowly cooling in a water bath. The Klenow enzyme reactions were carried out at room temperature for 1.5 h. **Lane 1** Oligonucleotide 1,2 duplex. **Lane 2** Oligonucleotide 1,2 duplex filled-in with .8 mM each of dCTP, dATP and dGTP with 12.5 units of Klenow enzyme. **Lane 3** Oligonucleotide 1,2 duplex filled-in with .8 mM each of dCTP, dATP and dGTP with 5 units of Klenow enzyme. **Lane 4** Oligonucleotide 1,2 duplex filled-in with .8 mM each of dCTP, dATP, dGTP and TTP with 5 units of Klenow enzyme.



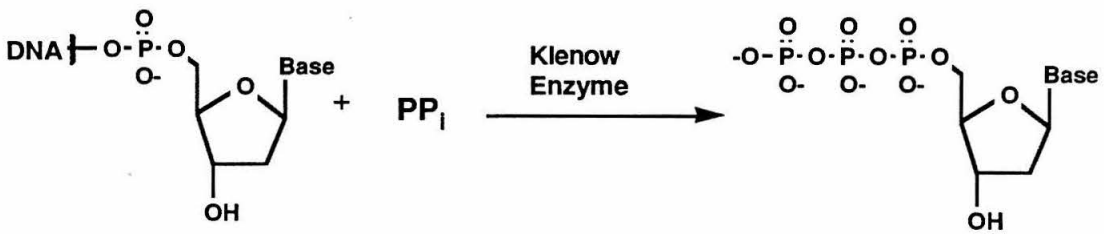
5'-CTGTCTGGGGAGTCTCAGCAGTAGTCGTCATCAG-3'
3'-GACAGACCCCTCAGAGTCGTCATCAGCAGTAGTC-5'

5'-CTGTCTGGGGAGTCTCAGCAGTAGTCGTCATCAG-3'
3'-CAGAGTCGTCATCAGCAGTAGTC-5'

5'-CTGTCTGGGGAGTCTCAGCAGTAGTCGTCATCAG-3'
3'-AGTCGTCATCAGCAGTAGTC-5'

thymidine residues were being removed along with the other 2'-deoxynucleotide residues from the 3' ends of oligonucleotides 1 and 2. How could the liberated T residues be used by the Klenow fragment in the fill-in reactions?[4]

When there is not the appropriate dNTP in the reaction mixture to allow the usual fill-in reaction, an "idling turnover" activity has been observed with the Klenow enzyme which produces 2'-deoxynucleotide 5'-triphosphate molecules from pyrophosphate (liberated in the usual Klenow incorporation reaction) and a 3'-terminal

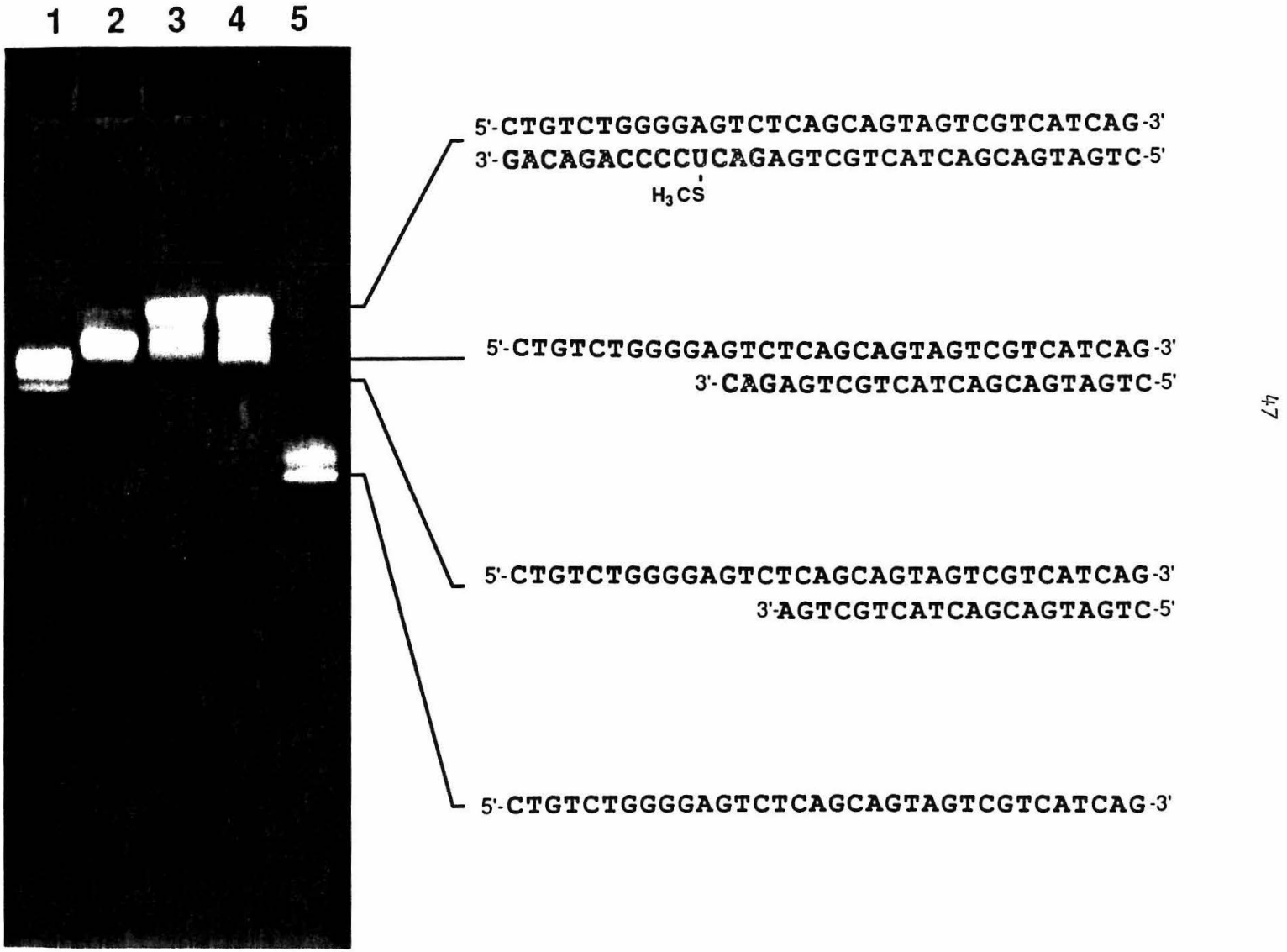


residue on the template DNA.[70] Therefore, the misincorporation I observed opposite the A at high Klenow enzyme concentrations was apparently caused by this type of "idling turnover" synthesis of TTP from the T residues already on the probes. The misincorporation reaction was avoided in my fill-in reactions by using low Klenow enzyme concentrations and shorter reaction times.

Figure 10 shows how using the low concentrations of Klenow, no base was incorporated opposite the single A in the overhang region (lane 2) of the oligonucleotide 1,2 duplex unless a modified 2'-deoxyuridine 5'-triphosphate (lane 3) or TTP (lane 4) was present. Lane 1 is a control that was run with Klenow enzyme but no 2'-deoxynucleotide 5'-triphosphate molecules added and lane 5 is another

Figure 10

Klenow enzyme fill-in reactions using **2** and low Klenow concentrations. 2 mm x 160 mm x 160 mm 15% nondenaturing polyacrylamide gel stained with ethidium bromide. In each reaction the oligonucleotide 1,2 duplex was formed by heating .1 nmol oligonucleotide 1 and .1 nmol oligonucleotide 2 to 60°C then slowly cooling in a water bath. The Klenow reactions were carried out using 3 units of enzyme each at 37°C for 20 minutes. **Lane 1** Oligonucleotide 1,2 duplex. **Lane 2** Oligonucleotide 1,2 duplex filled-in with .8 mM each of dCTP, dATP and dGTP. **Lane 3** Oligonucleotide 1,2 duplex filled-in with .8 mM each of dCTP, dATP, dGTP and **2**. **Lane 4** Oligonucleotide 1,2 duplex filled-in with .8 mM each of dCTP, dATP, dGTP and TTP. **Lane 5** Oligonucleotide 1.


1 2 3 4 5
5'-CTGTCTGGGGAGTCTCAGCAGTAGTCGTCATCAG-3'
3'-GACAGACCCCUCAGAGTCGTCATCAGCAGTAGTC-5'
H3CS
5'-CTGTCTGGGGAGTCTCAGCAGTAGTCGTCATCAG-3'
3'-CAGAGTCGTCATCAGCAGTAGTC-5'
5'-CTGTCTGGGGAGTCTCAGCAGTAGTCGTCATCAG-3'
3'-AGTCGTCATCAGCAGTAGTC-5'
5'-CTGTCTGGGGAGTCTCAGCAGTAGTCGTCATCAG-3'

control that contains only oligonucleotide 1. Gels of this type were taken as indirect evidence that the modified 2'-deoxynucleotide 5'-triphosphate was specifically incorporated opposite the A in the overhang region of the oligonucleotide 1,2 duplex.

Direct evidence was obtained for Klenow enzyme incorporation of **2** into oligonucleotide strands by using oligonucleotides 3 and 4. The experiment is described in Figure 11. The oligonucleotide 3,4 duplex contained only a single base 5' overhang, namely the 5' terminal A residue of oligonucleotide 4. The Klenow enzyme was therefore able to place a single residue of **2** or thymidine (T) onto the 3' end of oligonucleotide 3 when **2** or TTP were present respectively. Since oligonucleotide 4 was six bases longer than oligonucleotide 3, the two oligonucleotides were easily separated on a denaturing 20% polyacrylamide gel (see Figure 12). Furthermore, since the **2** or T residue was placed at the end of the oligonucleotide 3, this residue had a relatively large effect on the electrophoretic mobility of the oligonucleotide. Lane 1 of Figure 12 was the result of reaction of the oligonucleotide 3,4 duplex with Klenow enzyme in the presence of TTP. Lane 2 is the same as lane 1 except **2** was used in place of TTP. Lane 3 contains unreacted oligonucleotide 3. The change in electrophoretic mobility of the band corresponding to the filled-in oligonucleotide 3 in lanes 1 and 2 was taken as direct evidence for the enzymatic incorporation of **2** by the Klenow enzyme.

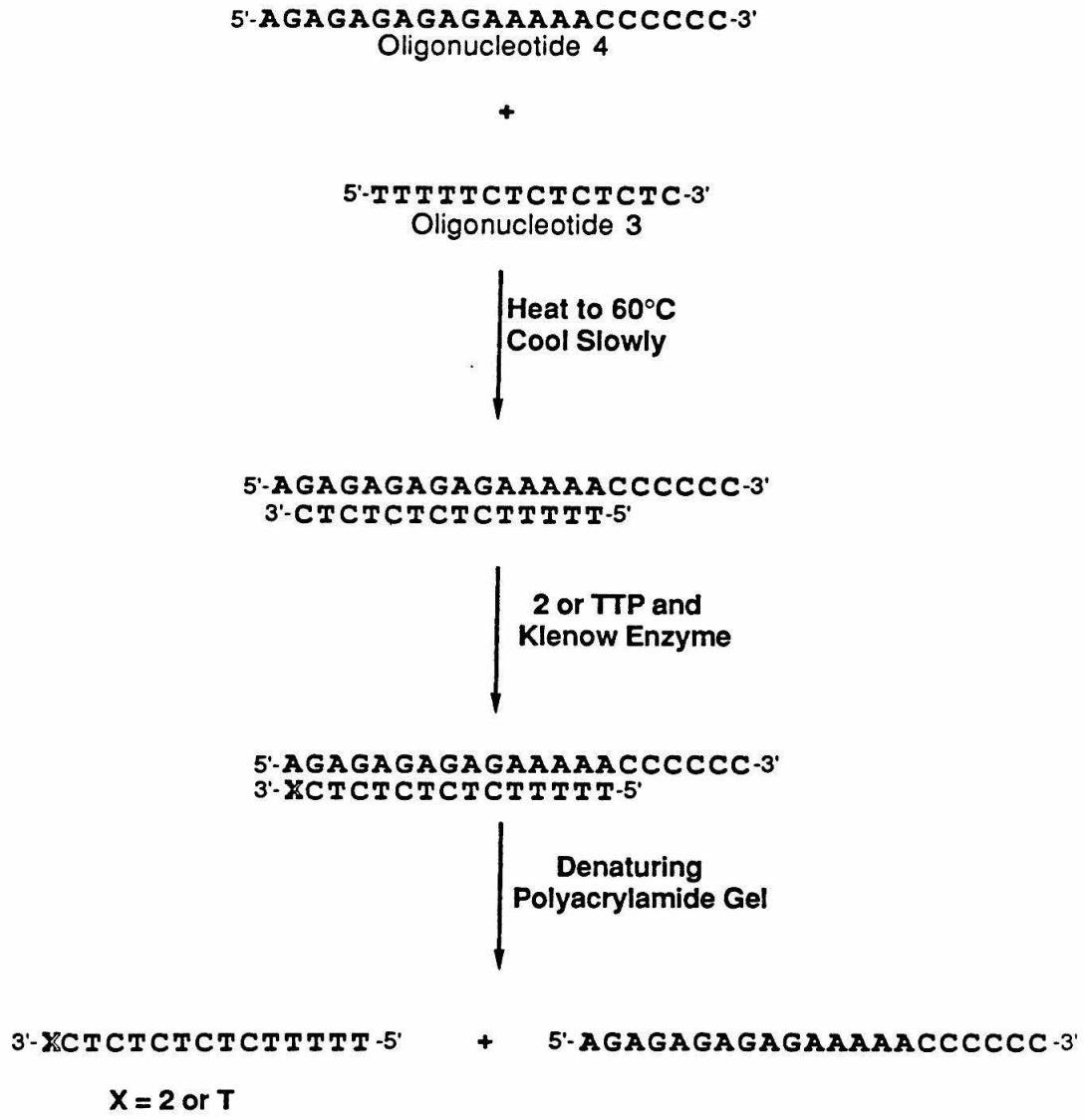
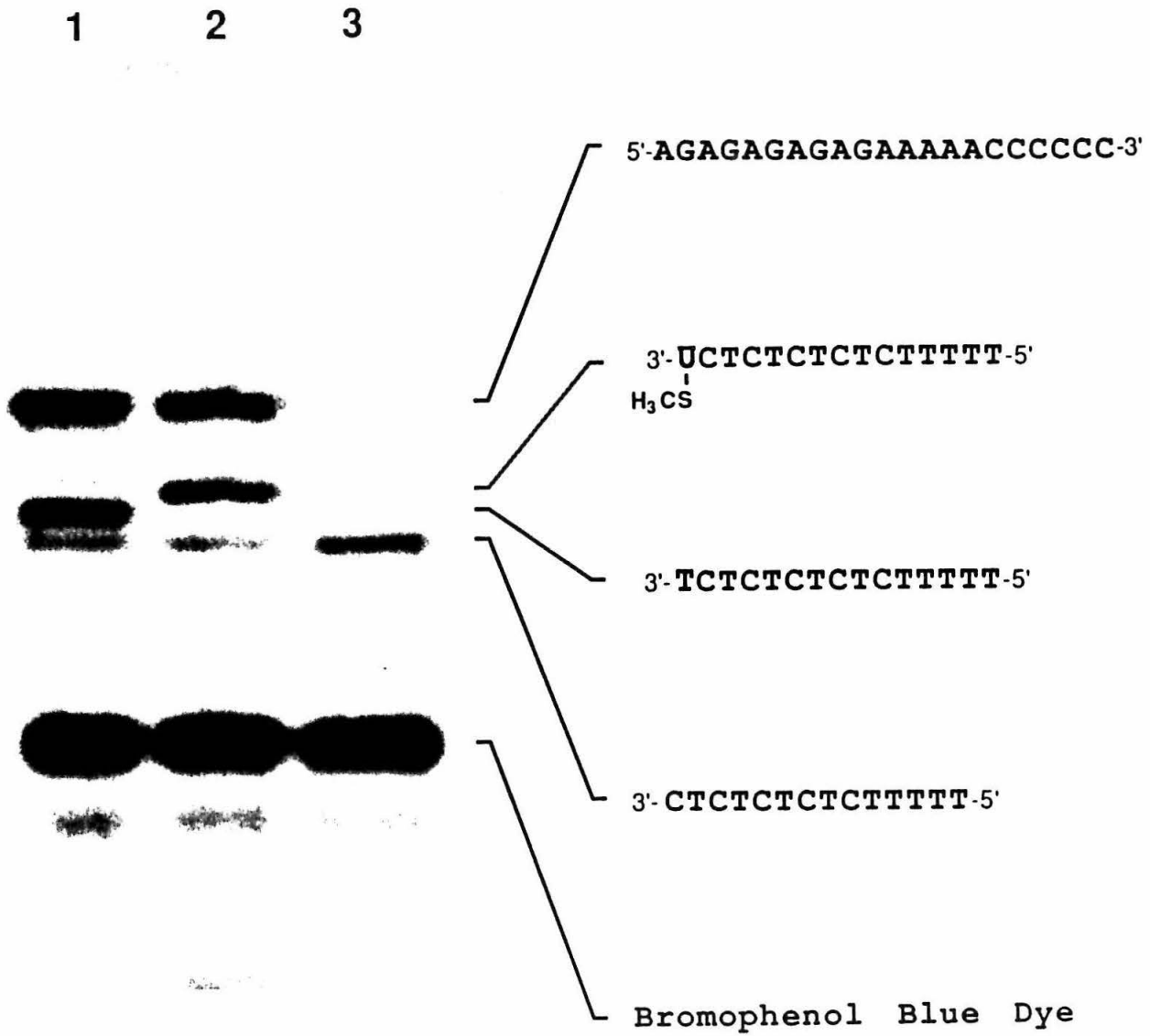


Figure 11 Scheme for the method used to produce an oligonucleotide 3 carrying a single residue of **2** on the 3' end.

Figure 12

Klenow enzyme fill-in reactions revealing direct evidence for incorporation of **2** into oligonucleotide 3. 2 mm x 160 mm x 160 mm 15% denaturing polyacrylamide gel visualized by short wave UV light. In each reaction the oligonucleotide 3,4 duplex was formed by heating 1 nmol oligonucleotide 3 and 1 nmol oligonucleotide 4 to 60°C then slowly cooling in a water bath. The Klenow enzyme reactions were carried out using 18 units of enzyme at 25°C for 20 minutes. The reactions were ethanol precipitated then dissolved in formamide loading buffer, heated to 90°C for 3 min, chilled in ice for 5 min then loaded onto the gel. **Lane 1** Oligonucleotide 3,4 duplex filled-in in the presence of 1 mM dTTP. **Lane 2** Oligonucleotide 3,4 duplex filled-in in the presence of 1 mM **2**. **Lane 3** Oligonucleotide 3.


1
2
3
5'-AGAGAGAGAGAAAAACCCCCC-3'
3'-UCTCTCTCTCTTTTT-5'
H3CS
3'-TCTCTCTCTCTTTTT-5'
3'-CTCTCTCTCTTTTT-5'
Bromophenol Blue Dye

The CNBr Cleavage Reaction

Optimum Conditions And Specificity The specificity and optimum conditions of the complementary-addressed cleavage reaction using **2** and CNBr were initially investigated using the oligonucleotide 1,2 duplex (see Figure 13). The sequences of oligonucleotides 1 and 2 were designed to prevent improper annealing reactions as well as allow analysis of the specificity of the cleavage reaction. Five potentially reactive guanine residues were placed on oligonucleotide 1 adjacent to where the single residue of **2** was incorporated on oligonucleotide 2. Four of these guanine residues were to the 5' side of **2** because assuming a right-handed B form helix, [7] the latent reactive methylthioether group attached to **2** should be located in the major groove adjacent to the reactive N7 positions of the guanine residues only to the 5' side. The helix structure should prevent reaction to the 3' side or intrastrand cleavage (see computer modeling section).

Oligonucleotide 1 was labelled with ^{32}P at the 5' end by the standard enzymatic reaction catalyzed by T4 polynucleotide kinase.[4] The labelled oligonucleotide 1 was annealed to oligonucleotide 2 as usual. A Klenow enzyme fill-in reaction (using a low enzyme concentration) was carried out in the presence of dATP, dCTP, dGTP and **2** or TTP. The filled-in, radioactively labelled duplexes were isolated as the uppermost band on a non-denaturing 15% polyacrylamide gel such as that in Figure 10. The bands were excised from the gel, the oligonucleotide duplexes were eluted out of the gel slices with .2 M

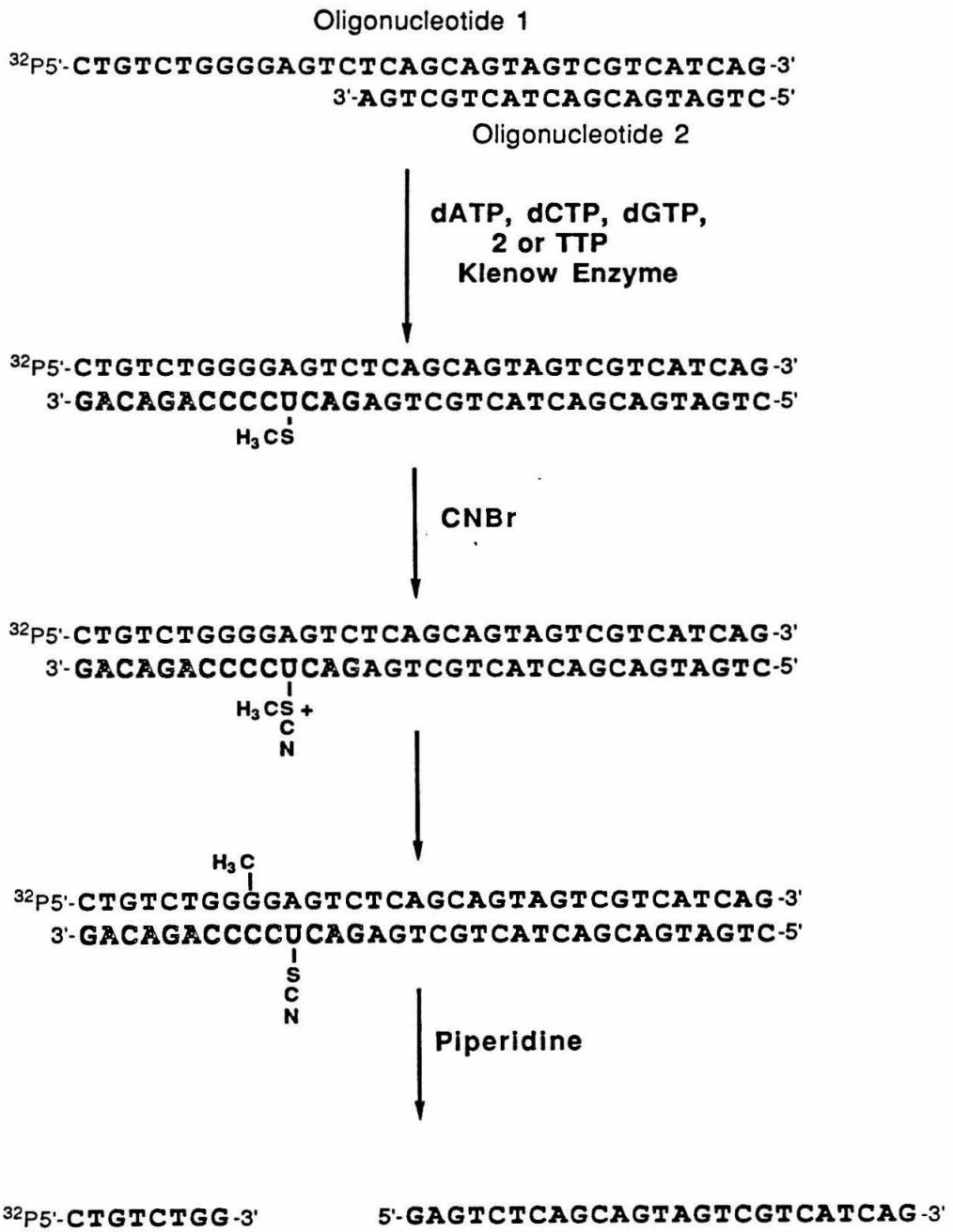


Figure 13 Scheme for the complementary-addressed cleavage of oligonucleotide 1 using an enzymatically incorporated residue of **2** and CNBr.

NaCl solution at room temperature and the resulting solutions were dialyzed against sodium phosphate buffer (.25 mM, pH 7.5).

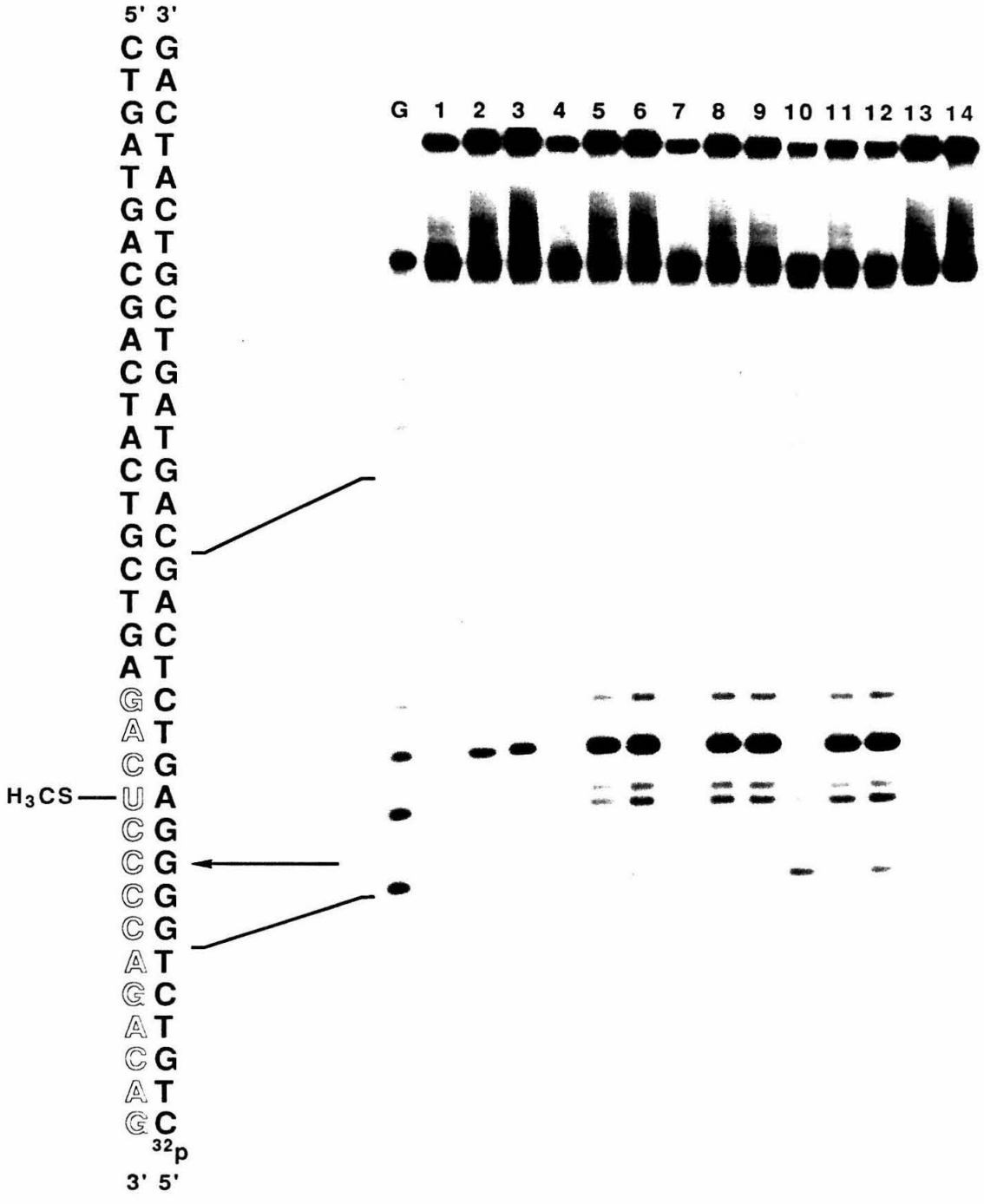
Isolating the duplexes by the above method insured a 1:1 stoichiometry of oligonucleotide 1 to filled-in oligonucleotide 2. Furthermore, since the duplexes were never denatured after the fill-in reaction, every strand of oligonucleotide 1 used in the subsequent CNBr cleavage reactions was hybridized to a filled-in oligonucleotide 2 strand carrying a single residue of **2**. These duplexes could therefore be used to assign the absolute cleavage efficiency of **2** in the complementary-addressed cleavage reaction using CNBr. To date, every other complementary-addressed system has been investigated using an excess of hunter strand, so an absolute cleavage efficiency has never before been unambiguously reported.

The isolated oligonucleotide duplexes, containing a single residue of **2** or T in the filled-in region, were exposed to CNBr under various conditions and then treated with piperidine at 90°C. The samples were loaded onto denaturing 20% polyacrylamide sequencing-type gels. The gels were autoradiographed and the results analyzed (see Figures 14 and 15). The following general conclusions were drawn from the data produced on several gels.

CNBr concentrations of up to 40 mM could be used with no detectable nonspecific reaction with the DNA bases. As was anticipated, the "soft" CN electrophile apparently prefers "soft" nucleophiles such as the methylthioether of **2** over the "harder" nucleophiles of DNA such as the aromatic amines. Of course this profound specificity of

Figure 14

CNBr cleavage reactions of the oligonucleotide 1,2 duplex using an enzymatically incorporated residue of **2**. Autoradiogram of a 20% denaturing polyacrylamide gel. Radioactively-labelled, filled-in oligonucleotide 1,2 duplexes containing a single residue of **2** or T were prepared and isolated according to procedures detailed in the experimental section. The cleavage reactions were run by placing 2 mR/h of the duplex along with 1 μg of CT DNA in 10 μl of a solution containing 5 mM NaCl, 30 mM buffer (sodium phosphate pH 5.5 or 7.5 or potassium phthalate pH 4.5) and 30 mM CNBr. The reactions were incubated at the specified temperature for the specified amount of time then 2 μl of 50% aqueous piperidine was added and the solutions were heated at 90°C for 20 min. The samples were then lyophilized to dryness, the residue redissolved in formamide loading buffer and .2 mR/h was loaded onto each lane of the gel. The reactions run in lanes 1,4,7 and 10 used oligonucleotide 1,2 duplexes filled-in with a regular T residue, the reactions run in all other lanes used duplexes containing a residue of **2**. **G** Maxam-Gilbert G reaction. **Lane 1** pH 7.5, 37°C, 48 h. **Lane 2** pH 7.5, 37°C, 24 h. **Lane 3** pH 7.5, 37°C, 48 h. **Lane 4** pH 5.5, 25°C, 48 h. **Lane 5** pH 5.5, 25°C, 24 h. **Lane 6** pH 5.5, 25°C, 48 h. **Lane 7** pH 5.5, 37°C, 48 h. **Lane 8** pH 5.5, 37°C, 24 h. **Lane 9** pH 5.5, 37°C, 48 h. **Lane 10** pH 4.5, 37°C, 48 h. **Lane 11** pH 4.5, 37°C, 24 h. **Lane 12** pH 4.5, 37°C, 48 h. **Lane 13** pH 7.6, no CNBr, 37°C, 48 h. **Lane 14** pH 7.5, no CNBr, 37°C, 48 h, no piperidine treatment. The histogram shows the location of the ^{32}P label as well as the positions filled-in by the Klenow enzyme (outlined bases). The position of the residue of **2** (H_3CS-U) in relation to the observed guanine cleavage site (arrow) is also indicated.


5' 3'
C G
T A
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A T
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T A
G C
C G
T A
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C G
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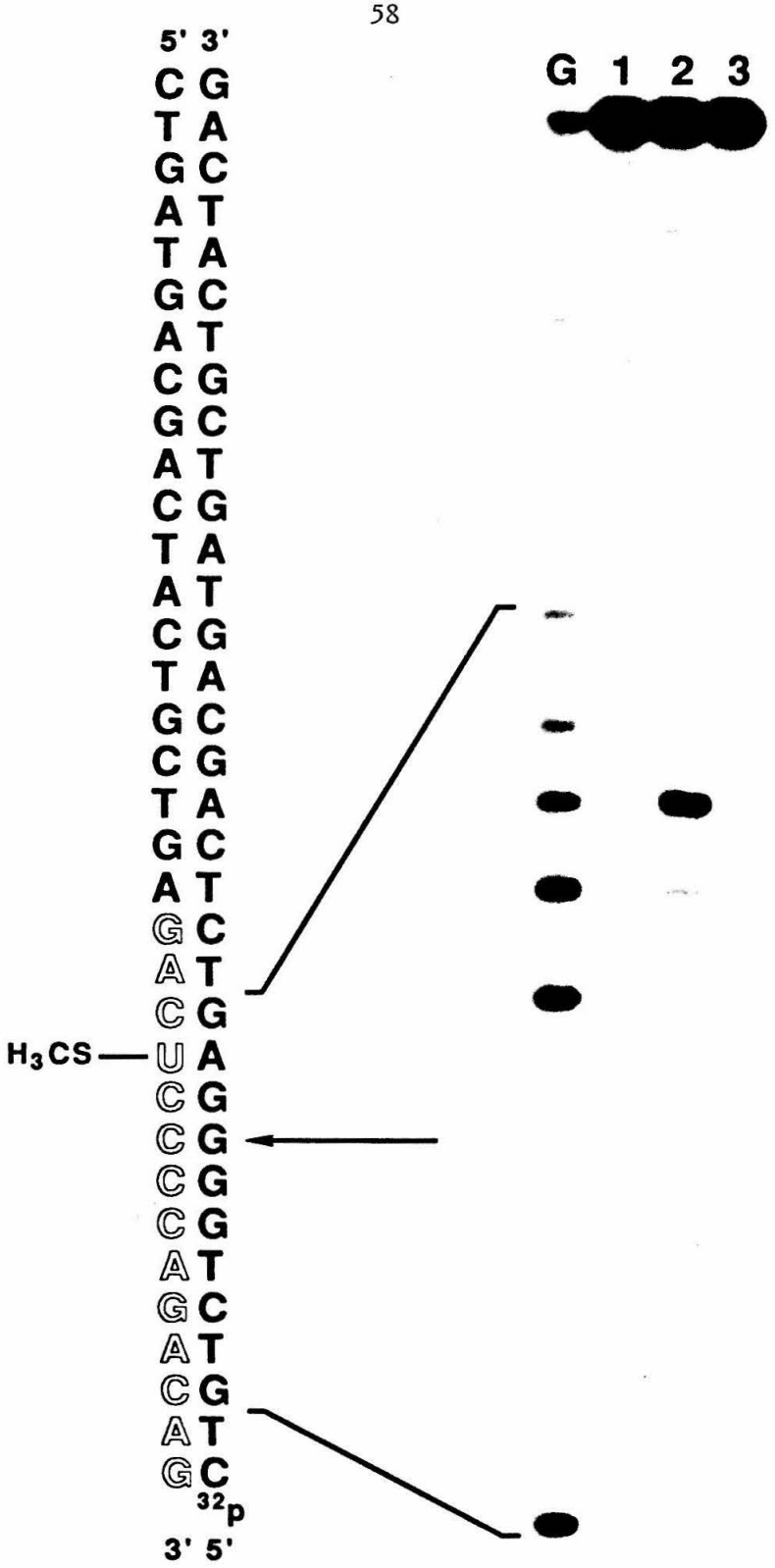
G C
A T
C T
A G
C C
C G
C G
C G
A T
G C
A T
C G
A T
G C
32p
3' 5'
H3CS
G 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 15

CNBr cleavage reactions of the oligonucleotide 1,2 duplex using an enzymatically incorporated residue of **2**. Autoradiogram of a 20% denaturing polyacrylamide gel. Radioactively-labelled, filled-in oligonucleotide 1,2 duplexes containing a single residue of **2** or T were prepared and isolated according to procedures detailed in the experimental section. The cleavage reactions were run by placing 2 mR/h of the duplex along with 1 μg of CT DNA in 10 μl of a solution containing 5 mM NaCl, 25 mM NaOAc pH 5.5 with or without 20 mM CNBr. The reactions were incubated at room temperature (25°C) for 5.5 h then 2 μl of neat piperidine was added and the solutions were heated at 90°C for 20 min. The samples were then lyophilized to dryness, the residue redissolved in formamide loading buffer and .2 mR/h was loaded onto each lane of the gel. **G** Maxam-Gilbert G reaction. **Lane 1** Oligonucleotide 1,2 duplexes filled-in with a regular T residue, reacted with 20 mM CNBr. **Lane 2** Oligonucleotide 1,2 duplexes filled-in with a residue of **2**, reacted with 20 mM CNBr. **Lane 3** Oligonucleotide 1,2 duplexes filled-in with a residue of **2**, no CNBr added during reaction. The histogram shows the location of the ^{32}P label as well as the positions filled-in by the Klenow enzyme (outlined bases). The position of the residue of **2** (H_3CS-U) in relation to the observed guanine cleavage site (arrow) is also indicated.


5' 3'
C G
T A
G C
A T
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C G
T A
G C
C G
T A
G C
A T
G C
A T
C G
H3CS — U A
C G
C G
C G
A T
G C
A T
C G
A T
G C
32p
3' 5'
G 1 2 3

the CNBr reaction was a fundamental requirement of the CNBr activated methylthioether alkylation strategy.

A single G on the complementary strand two base pairs to the 5' side of the single residue of **2** was the primary site of cleavage revealing unprecedented specificity. The adjacent G residues were cleaved with an efficiency which was 0.08 as high. This pronounced specificity is the most significant result of the study and could pave the way for similar systems to be developed for use in molecular biological manipulations. Some possible reasons for the observed high degree of reaction specificity will be discussed in the computer modeling section.

No specific cleavage was observed when the oligonucleotide duplex contained a T residue in place of the single residue of **2** or when the CNBr treatment was omitted (see lanes 1 and 3 in Figure 15). These controls demonstrate that the methylthioether is activated by CNBr to form a species (presumably a cyanosulfonium intermediate) capable of specifically reacting with the base(s) of a complement strand.

The efficiency of the cleavage produced by **2** increased with decreasing pH. This finding was not surprising because of the nature of the expected cyanosulfonium intermediate formed during the reaction. Such an intermediate should be highly susceptible to attack by -OH so that lowering the pH should decrease the rate of this proposed hydrolysis side reaction by lowering the concentration of -OH.

The oligonucleotide duplexes could withstand a pH as low as 5.5 without noticeable depurination. In acid solution it is well known that DNA is depurinated because protonating the purine aromatic nitrogen atoms weakens the glycosidic bond, facilitating hydrolysis and thus depurination.[49] The depurinated sites in DNA are cleaved by a piperidine treatment[49] (such as that used in the CNBr cleavage reaction), so the acid depurination caused an unwanted nonspecific cleavage reaction when the reactions were run at a pH below 5.5.

With a CNBr concentration of 25 mM, the reaction was complete in 8-12 hours. During the reaction, it was important to keep the reaction vessels tightly sealed, otherwise the volatile CNBr quickly evaporated, greatly slowing the rate of reaction.

A 10% aqueous piperidine treatment of 20 minutes at 90°C was sufficient to produce maximal specific cleavage with minimal background cleavage. Since heating is known to cause the spontaneous depurination of DNA, the length of time for the piperidine-heat treatment was shortened as much as possible. Also to prevent depurination, the oligonucleotides and oligonucleotide duplexes were always eluted from gel slices at room temperature even though literature procedures called for elution from gels with prolonged heat treatments.

The maximal cleavage efficiency (determined by scintillation counting and/or densitometry) was ~12%. The reasons for this low cleavage efficiency remain unclear. The flexibility of the linker between the methylthioether and the uridine base might allow other

unreactive conformations, facilitating the proposed hydrolysis side reaction. The expected cyanosulfonium intermediate would have a full positive charge so it might be attracted to the negatively charged DNA phosphate backbone, away from the desired N7-guanine site again facilitating the possible hydrolysis reaction. Another possibility is that the side chain of **2** can not adopt the optimum geometry required for facile reaction with the N7-guanine position (these issues are addressed in the computer modeling section).

The CNBr-methylthioether cleavage reaction produced oligonucleotide fragments that migrated on a high resolution gel consistent with having 3'-phosphate termini indicating an alkylation type of reaction mechanism. The observed specific cleavage always resulted in a band which exactly comigrated with a band in the reference G lane. The G lane was produced by the standard dimethyl sulfate reaction known to produce fragments with 3'- and 5'-phosphate termini.[49] Oligonucleotide fragments with 3'-hydroxyl termini and 3'-sugar or base fragment termini would have run noticeably different on these denaturing 20% gels.[71]

Base Specificity Of The Cleavage Reaction The base specificity of the CNBr-methylthioether cleavage reaction was investigated by using an oligonucleotide duplex produced by annealing 5'-^{32}P-end-labelled oligonucleotide 5 with oligonucleotide 2. Two **2** or T residues were enzymatically incorporated into the overhang region and the completely filled-in duplexes were gel purified as usual. The gel used to analyze the CNBr reaction showed that A was not at-

tacked in a manner analogous to G (see Figure 16) during the complementary-addressed reaction using **2** and CNBr.

This preference for reaction at the N7 position of guanine relative to the N7 position of adenine is consistent with the known preferences of S_N2 type alkylating agents at these sites (see Figure 3) and therefore implies an S_N2 alkylation mechanism. Interestingly, the guanine residues adjacent to the target A residue were cleaved with a higher efficiency than observed with analogous guanine residues on oligonucleotide 1. This suggests that the proposed reactive cyanosulfonium species was sufficiently long-lived to enable increased reaction at less favorable sites.

Linker Length Dependence Compounds **4** and **5** were synthesized to determine the optimum linker length between the uridine and methylthioether functions. Both **4** and **5** were enzymatically incorporated into an oligonucleotide 1,2 duplex. The CNBr cleavage reaction revealed that **2** appears to possess the optimum linker length (see Figure 17). Interestingly, **5** with one methylene unit longer than **2** demonstrated the same specificity but approximately 60% less cleavage efficiency than **2**.

It should be pointed out that both **4** and **5** could have different reactivities than **2**, so that the observed cleavage efficiency might not simply reflect linker length. Compound **4** could be less reactive due to the methylthioether group being α to the carbonyl, and compound **5** could be deactivated through an intramolecular lactonization pathway analogous to the CNBr peptide cleavage mechanism. In

Figure 16

CNBr cleavage reactions of the oligonucleotide 5,2 duplex using an enzymatically incorporated residue of **2**. Autoradiogram of a 20% denaturing polyacrylamide gel. Radioactively-labelled, filled-in oligonucleotide 5,2 duplexes containing two residues of **2** or T were prepared and isolated according to procedures detailed in the experimental section. The cleavage reactions were run by placing 2 mR/h of the duplex along with 1 μg of CT DNA in 10 μl of a solution containing 5 mM NaCl, 25 mM sodium phosphate pH 5.5 with or without 20 mM CNBr. The reactions were incubated at room temperature (25°C) for 5.5 h then 2 μl of neat piperidine was added and the solutions were heated at 90°C for 20 min. The samples were then lyophilized to dryness, the residue redissolved in formamide loading buffer and .2 mR/h was loaded onto each lane of the gel. **G** Maxam-Gilbert G reaction. **Lane 1** Oligonucleotide 5,2 duplexes filled-in with two regular T residues, reacted with 20 mM CNBr. **Lane 2** Oligonucleotide 5,2 duplexes filled-in with two residues of **2**, reacted with 20 mM CNBr. **Lane 3** Oligonucleotide 5,2 duplexes filled-in with two residues of **2**, no CNBr added during reaction. The histogram shows the location of the ^{32}P label as well as the positions filled-in by the Klenow enzyme (outlined bases). The positions of the residues of **2** (H_3CS-U) in relation to the observed guanine cleavage sites (arrows) are also indicated. Adenine did not react in a manor analogous to guanine in the reaction.

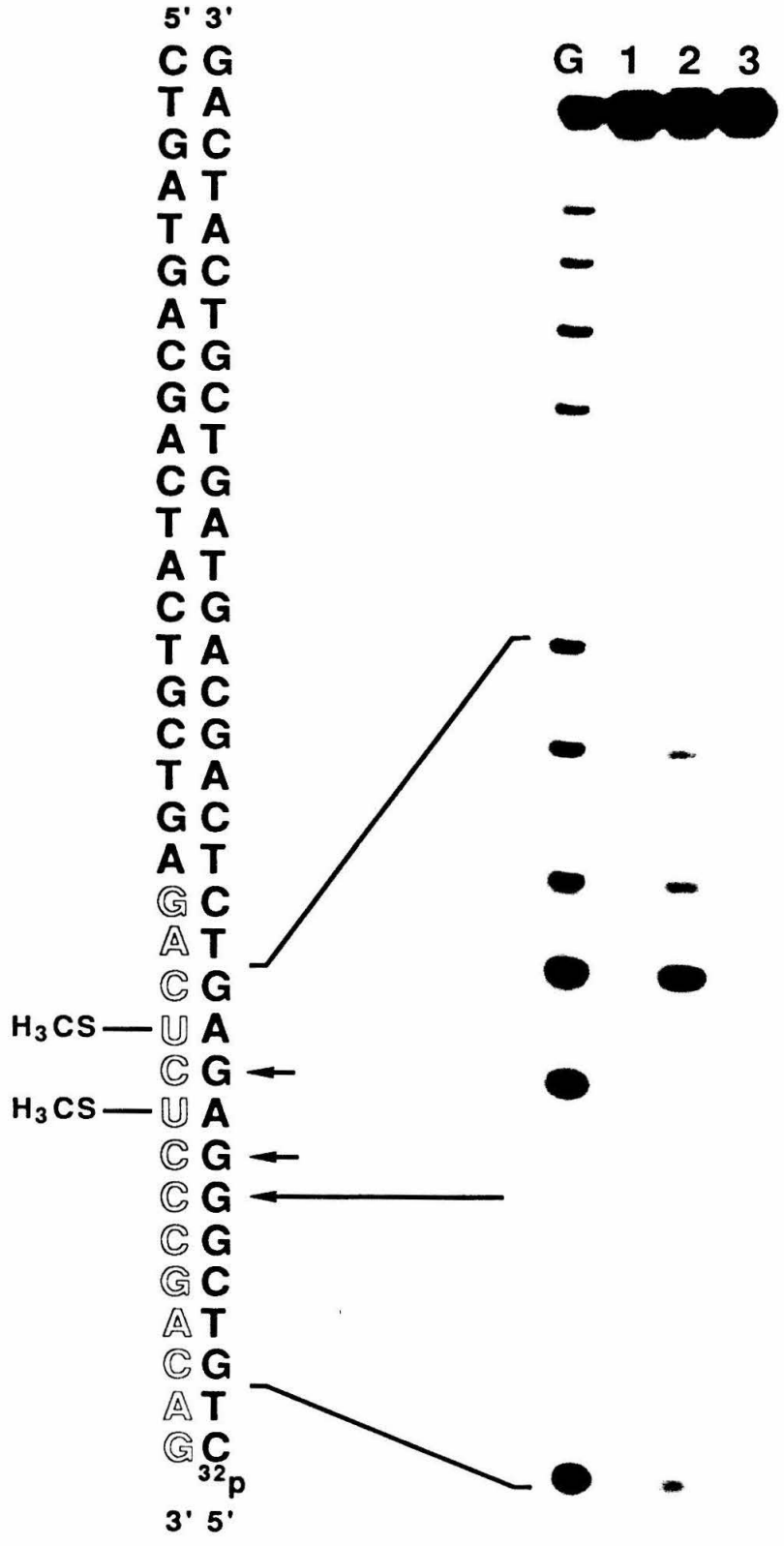
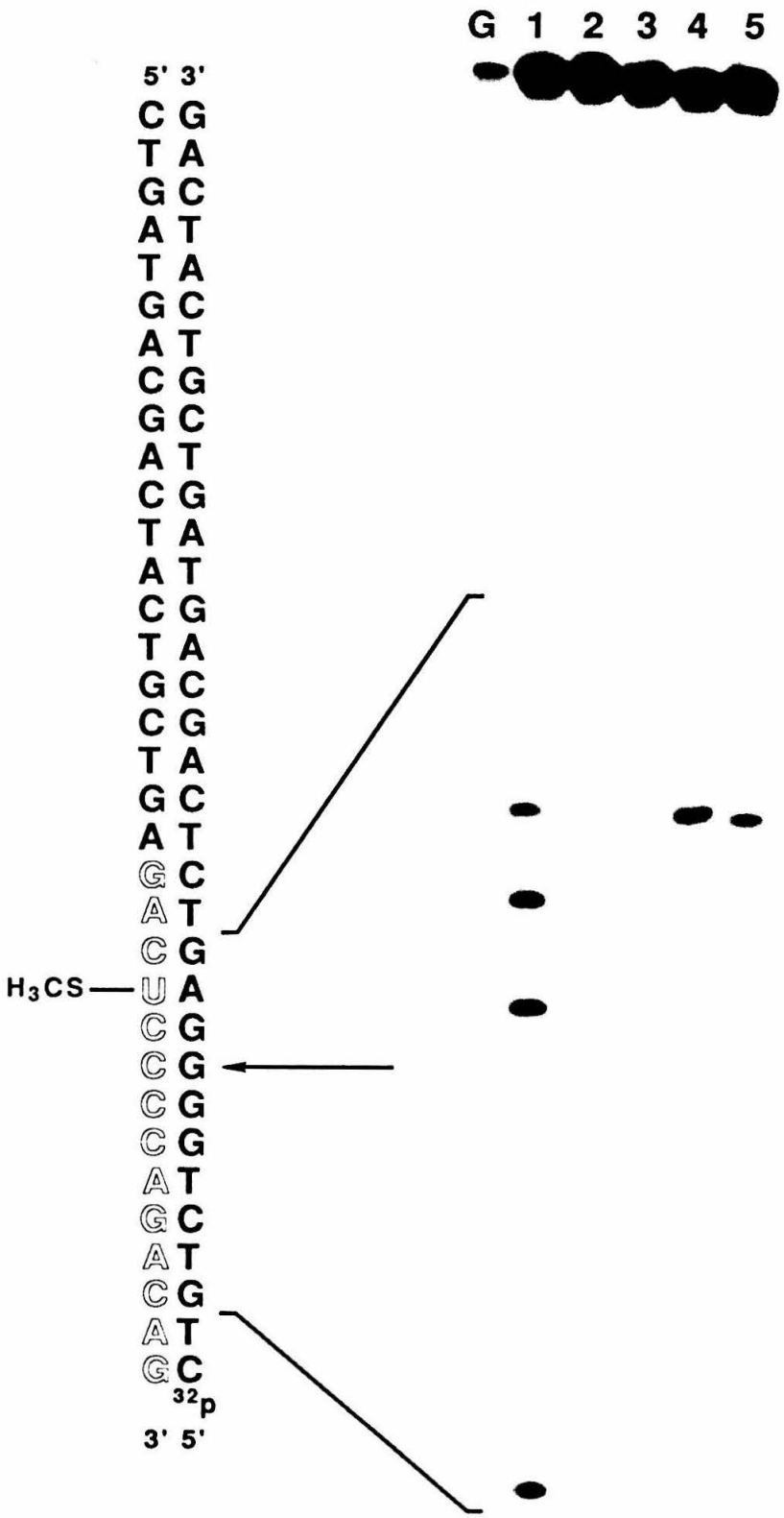

5' 3'
C G
T A
G C
A T
T A
G C
A T
C G
G C
A T
C G
T A
A T
C G
T A
G C
C G
T A
G C
A T
G C
A T
C G
H3CS — U A
C G
H3CS — U A
C G
C G
C G
G C
A T
C G
A T
G C
32p
3' 5'
G 1 2 3

Figure 17

CNBr cleavage reactions of the oligonucleotide 1,2 duplex using an enzymatically incorporated residue of **2**,**4** or **5**. Autoradiogram of a 20% denaturing polyacrylamide gel. Radioactively-labelled, filled-in oligonucleotide 1,2 duplexes containing a single residue of **2**,**4**,**5** or T were prepared and isolated according to procedures detailed in the experimental section. The cleavage reactions were run by placing 2 mR/h of the duplex along with 1 μg of CT DNA in 10 μl of a solution containing 5 mM NaCl, 25 mM NaOAc pH 5.5 with or without 10 mM CNBr. The reactions were incubated at room temperature (25°C) for 5.5 h then 2 μl of neat piperidine was added and the solutions were heated at 90°C for 20 min. The samples were then lyophilized to dryness, the residue redissolved in formamide loading buffer and .2 mR/h was loaded onto each lane of the gel. **G** Maxam-Gilbert G reaction. **Lane 1** Oligonucleotide 1,2 duplexes filled-in with a regular T residue, reacted with 10 mM CNBr. **Lane 2** Oligonucleotide 1,2 duplexes filled-in with a residue of **4**, reacted with 10 mM CNBr. **Lane 3** Oligonucleotide 1,2 duplexes filled-in with a residue of **2**, reacted with 10 mM CNBr. **Lane 4** Oligonucleotide 1,2 duplexes filled-in with a residue of **5**, reacted with 10 mM CNBr. **Lane 5** Oligonucleotide 1,2 duplexes filled-in with a residue of **2**, no CNBr added during the reaction. The histogram shows the location of the ^{32}P label as well as the positions filled-in by the Klenow enzyme (outlined bases). The position of the residue of **2**,**4** or **5** (H_3CS-U) in relation to the observed guanine cleavage site (arrow) is also indicated.


G 1 2 3 4 5
5' 3'
C G
T A
G C
A T
T A
G C
A T
C G
G C
A T
C G
T A
A T
C G
T A
G C
C G
T A
G C
A T
G C
A T
C G
H3CS U A
C G
C G
C G
C G
A T
G C
A T
C G
A T
G C
32p
3' 5'

any case, **2** appeared to be the molecule most efficient at causing specific cleavage.

Nature Of The 5' Termini Produced In The Cleavage Reaction The nature of the 5' termini produced in the complementary-addressed cleavage reaction using **2** and CNBr was investigated with oligonucleotides 6 and 7. These oligonucleotides were annealed together as usual then filled-in with Klenow enzyme in the presence of dATP, dCTP, α-^{32}P-dGTP and **2** (see Figure 18). Since there was only one C residue in a 5' overhang region, the filled-in duplex contained a single ^{32}P located at the 3' terminus of oligonucleotide 6, while a single residue of **2** was incorporated into oligonucleotide 7. The duplex was purified on a non-denaturing 15% polyacrylamide gel and isolated as usual. The CNBr cleavage reaction showed the predicted single cleavage at the G residue on oligonucleotide 6 two bases to the 5' side of the residue of **2**. On the high resolution denaturing 20% polyacrylamide gel, the mobility of the resulting fragment was identical to the same fragment produced by the dimethyl sulfate G reaction known to produce 5'-phosphate termini[49] (see Figure 19) again consistent with an alkylation mechanism of the CNBr-methylthioether reaction.

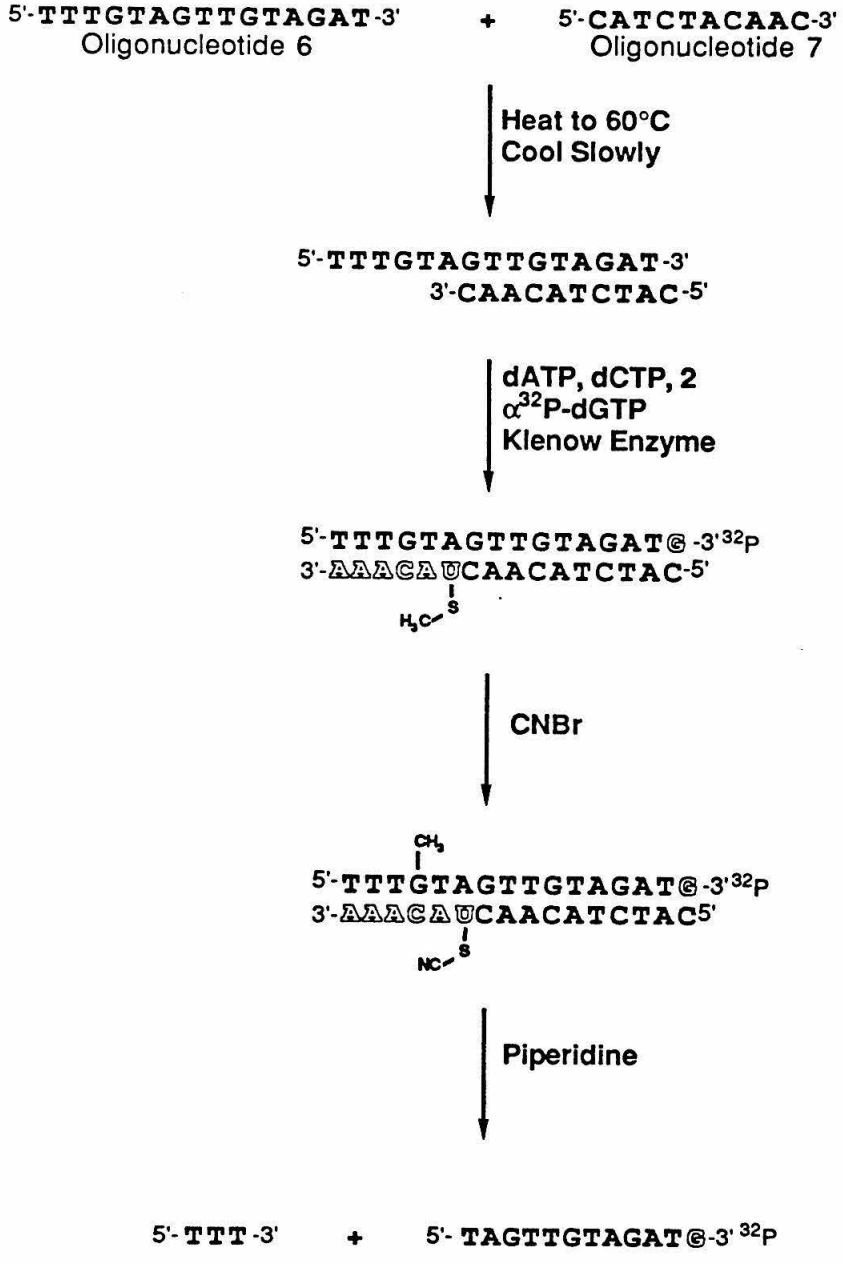
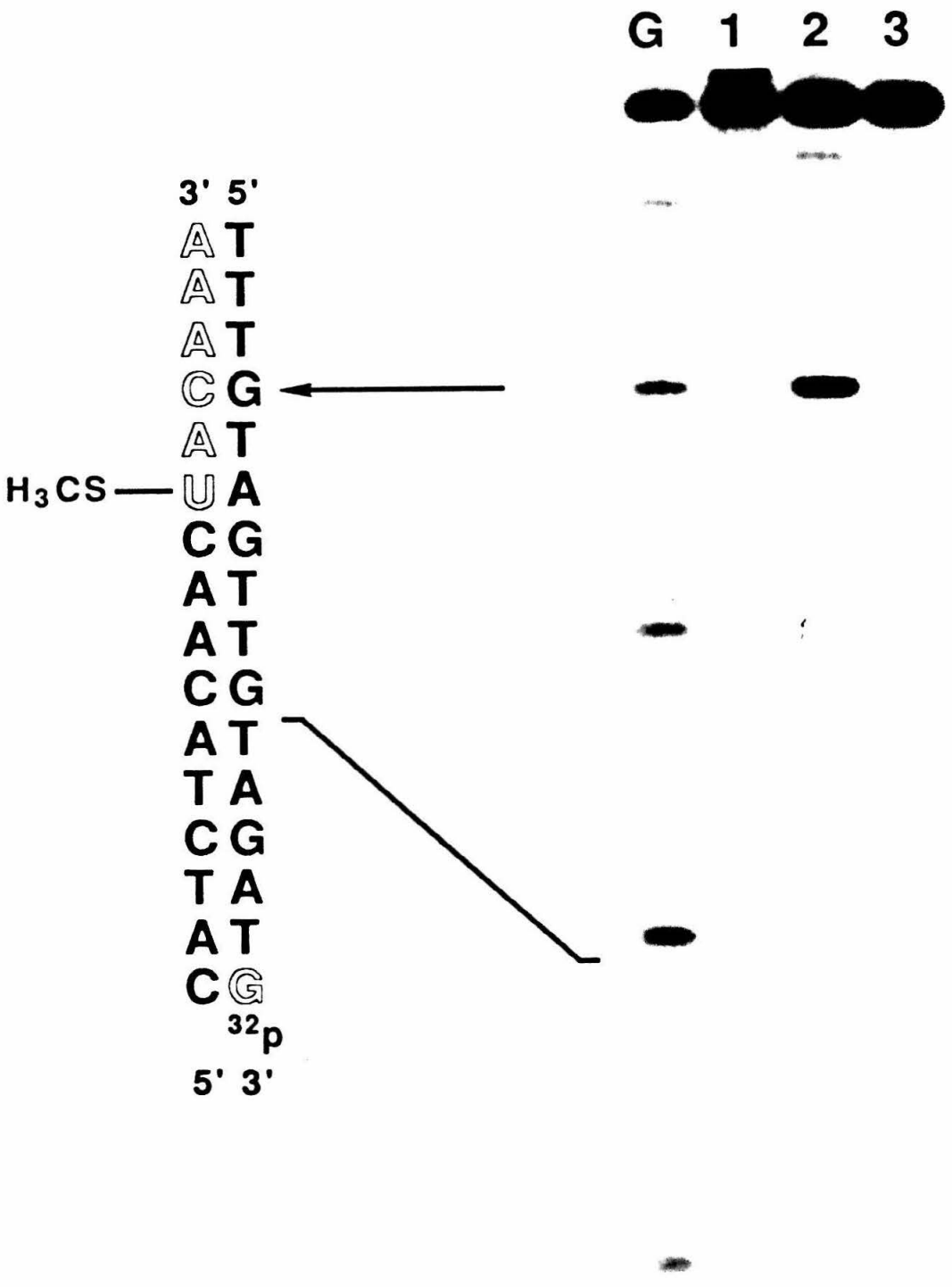


Figure 18 Scheme for the method used to produce the oligonucleotide 6,7 duplex labelled with ^{32}P at the 3' end of oligonucleotide 6 carrying a single residue of **2** filled-into oligonucleotide 7.

Figure 19

CNBr cleavage reactions of the oligonucleotide 6,7 duplex using an enzymatically incorporated residue of **2**. Autoradiogram of a 20% denaturing polyacrylamide gel. Oligonucleotide 6,7 duplexes, labelled with ^{32}P at the 3' end of oligonucleotide 6, containing a single residue of **2** or T were prepared and isolated according to procedures detailed in the experimental section (see Figure 18 for scheme). The cleavage reactions were run by placing 2 mR/h of the duplex along with 1 μg of CT DNA in 10 μl of a solution containing 5 mM NaCl, 25 mM NaOAc pH 5.5 with or without 20 mM CNBr. The reactions were incubated at room temperature (25°C) for 12 h then 2 μl of neat piperidine was added and the solutions were heated at 90°C for 20 min. The samples were then lyophilized to dryness, the residue redissolved in formamide loading buffer and .2 mR/h was loaded onto each lane of the gel. **G** Maxam-Gilbert G reaction. **Lane 1** Oligonucleotide 6,7 duplexes filled-in with a regular T residue, reacted with 20 mM CNBr. **Lane 2** Oligonucleotide 6,7 duplexes filled-in with a residue of **2**, reacted with 20 mM CNBr. **Lane 3** Oligonucleotide 6,7 duplexes filled-in with a residue of **2**, no CNBr added during reaction. The histogram shows the location of the ^{32}P label as well as the positions filled-in by the Klenow enzyme (outlined bases). The position of the residue of **2** (H_3CS-U) in relation to the observed guanine cleavage site (arrow) is also indicated.


G 1 2 3
3' 5'
A T
A T
A T
C G
A T
H3CS — U A
C G
A T
A T
C G
A T
T A
C G
T A
A T
C G
32p
5' 3'

Nature Of The Guanine Species Produced In The Cleavage Reaction The nature of the guanine species produced in the complementary-addressed cleavage reaction using CNBr and **2** was investigated by carrying out a large scale enzyme fill-in reaction using **2** and oligonucleotides 8 and 9. A scheme was designed and optimized to recover and identify any methylated G residues which might result from the large scale CNBr reaction (see Figure 20).

10 nmol each of oligonucleotide 8 and 9 were annealed together as usual. 10 mR/h of 5'-^{32}P-end-labelled oligonucleotide 8 was used to allow for a gel analysis of the large scale cleavage reaction. 1 μmol each of dATP, dGTP, dCTP and **2** or TTP were added to a solution containing the oligonucleotide 8,9 duplex followed by 250 units of Klenow enzyme. After the Klenow reaction was completed, the filled-in duplexes were purified on a non-denaturing 15% polyacrylamide gel and the uppermost band was excised from the gel. The oligonucleotide duplexes were eluted from the gel with .2 M NaCl and dialyzed to remove the salt. In all, 4.04 nmol of filled-in duplex was recovered (based on an extinction coefficient for completely double-stranded DNA of ε_{260} = 6700/phosphate[72]) containing a single residue of **2** incorporated into oligonucleotide 8. The volume of the sample was reduced *in vacuo* to 50 μl then sodium acetate pH 5.5 and CNBr were added to a final concentration of 25 mM each and a final volume of 100 μl. The reaction was allowed to proceed for 36 hours at room temperature then the solvent and CNBr were removed *in vacuo* and the residue was redissolved in 100 μl water.

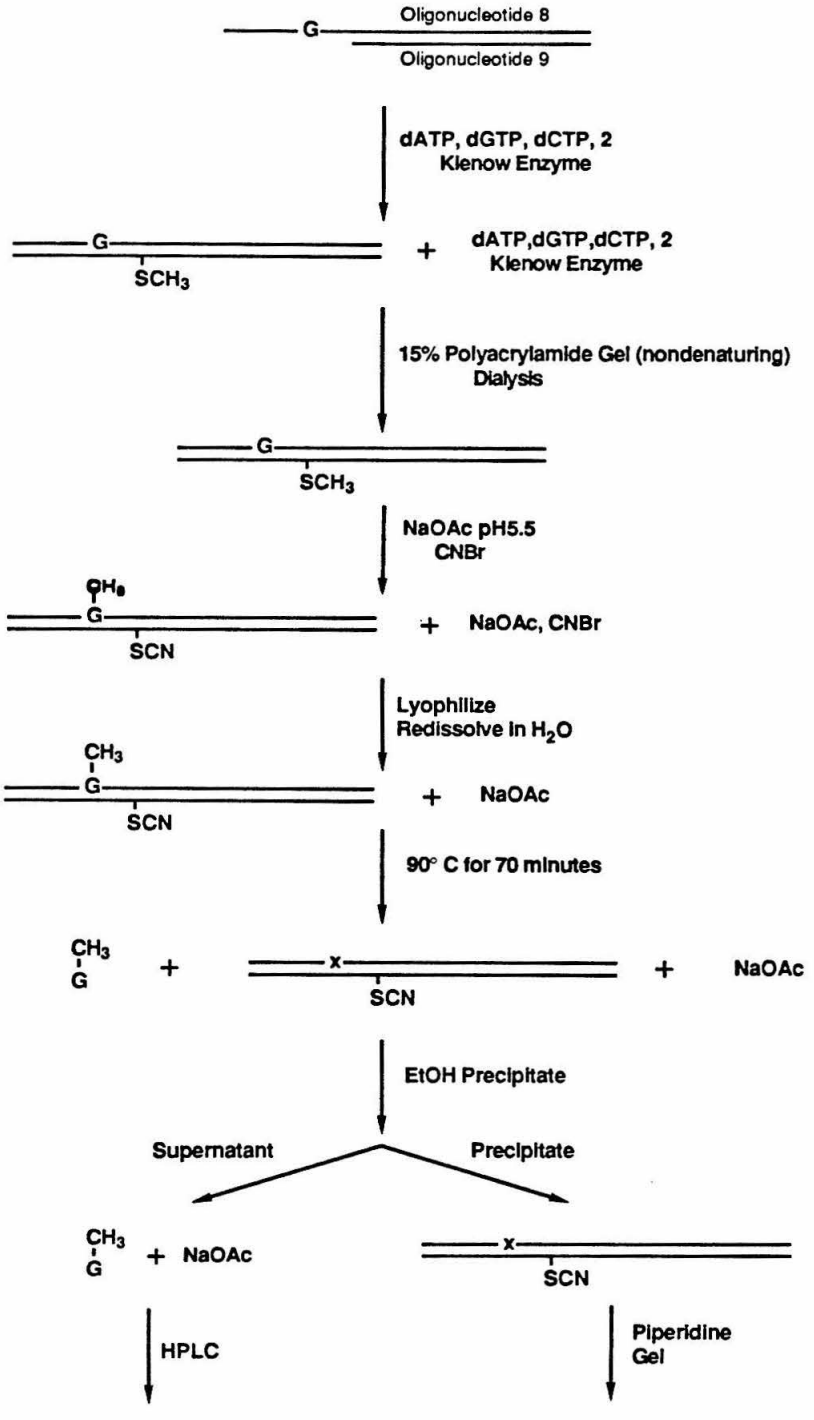


Figure 20 Scheme for the method used to isolate and identify any methylated guanine produced in the complementary-addressed CNBr-methylthioether reaction.

The key to this product isolation scheme was removing any methylated G from the oligonucleotide duplexes, and this was accomplished by heating the sample at 90°C for 70 minutes.[48] Notice that the sodium acetate pH 5.5 was still present. This heat treatment at pH 5.5 was shown to quantitatively liberate N7-methylguanine from oligonucleotide strands methylated with dimethyl sulfate (data not shown).

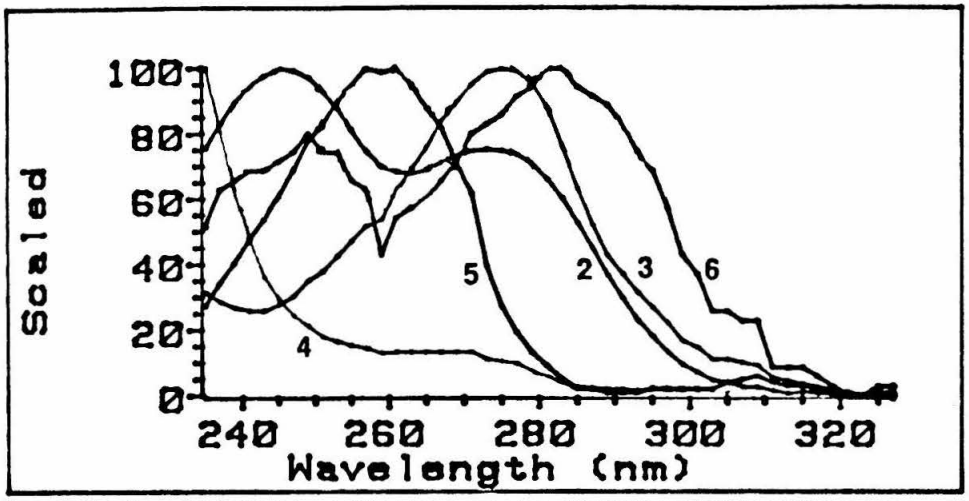
The sample was then cooled to room temperature and 300 µl of ethanol was added. In model reactions, 85% of N7-methylguanine remained in solution while the oligonucleotide duplexes were precipitated when a similar ethanol precipitation was performed on solutions containing known amounts of commercially prepared N7-methylguanine and 5 nmol of oligonucleotide 8,9 duplex. This 85% yield could not be increased by adjusting conditions and was very reproducible. The ethanol precipitation step was necessary to separate the oligonucleotide from any reaction product so it was conducted despite the non-quantitative yield.

The supernatant from the ethanol precipitation was dried *in vacuo* then the residue was redissolved in 28 µl of 10 mM ammonium acetate pH 5.5. 25 µl of this was injected onto a reverse phase HPLC column and diode array detection was used to monitor the results. As a control, the above reaction scheme was repeated using TTP instead of **2**.

Figures 21 and 22 are the HPLC chromatograms of the ethanol precipitation supernatants obtained in the large scale CNBr cleavage

Figure 21

HPLC chromatogram (monitored at 270 ±30 nm) of the ethanol precipitation supernatant obtained in the large scale CNBr cleavage reaction using oligonucleotide 8,9 duplexes containing a single residue of **2** in the filled-in region. The filled-in oligonucleotide 8,9 duplexes were prepared and the CNBr reaction was conducted as described in the experimental section (see Figure 20). The sample was eluted on a Vydac 201HS5415 4.6 mm x 15 cm reverse phase column with a 30 min linear gradient of 0-2% acetonitrile in 10 mM ammonium acetate pH 5.5 at a flow rate of .5 ml/min. The UV-Vis spectra of the different peaks are recorded in the upper left panel.


Scaled
100
80
60
40
20
0
240
260
280
300
320
Wavelength (nm)
1
2
3
4
5
6

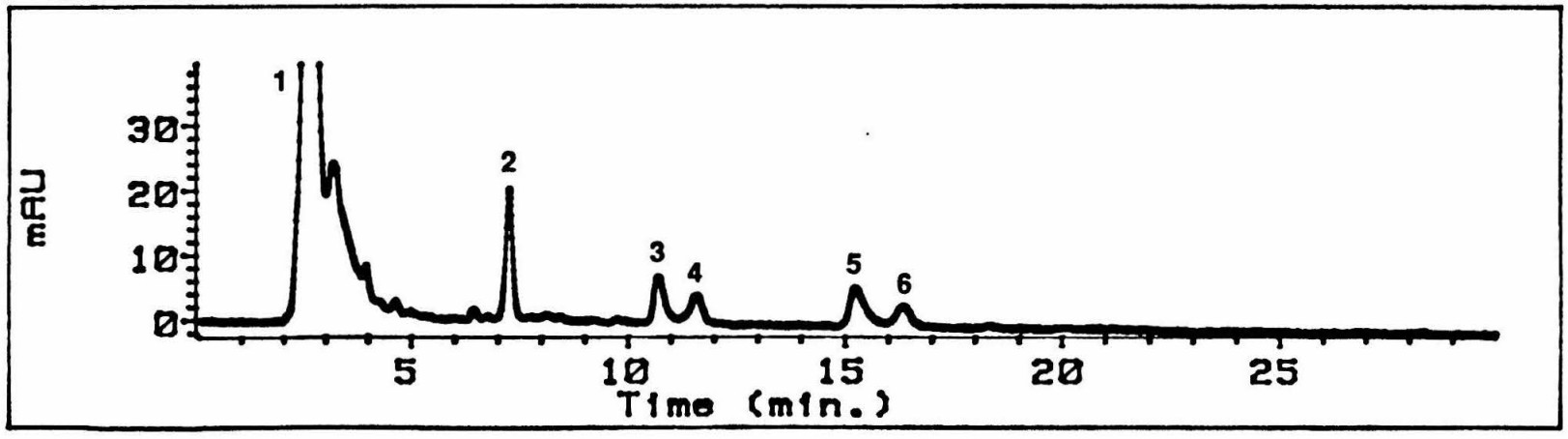
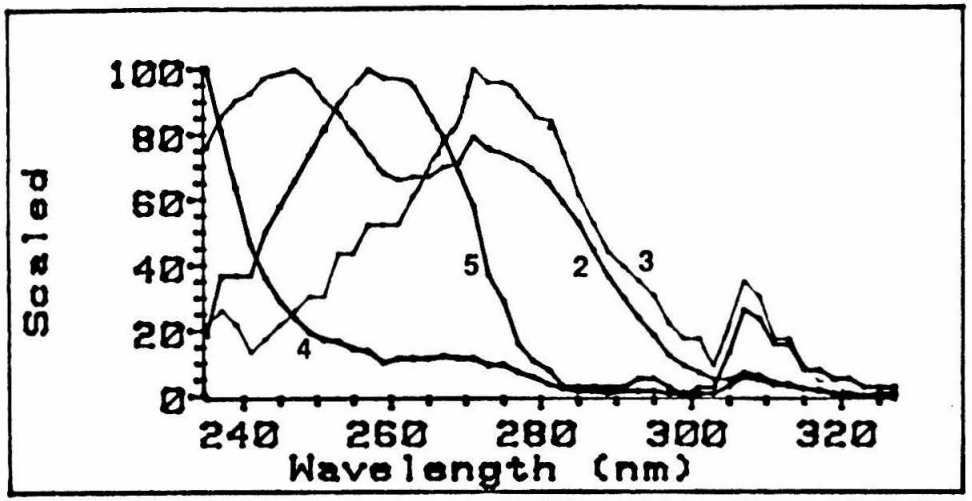
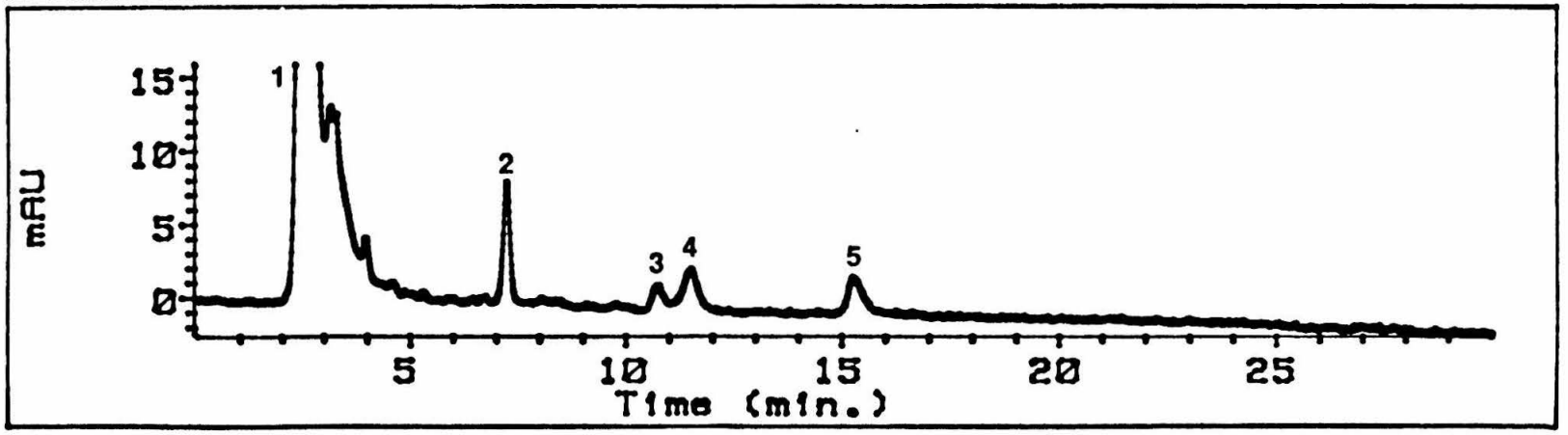

mAU
30
20
10
0
5
10
15
20
25
Time (min.)
1
2
3
4
5
6

Figure 22

HPLC chromatogram (monitored at 270 ±30 nm) of the ethanol precipitation supernatant obtained in a control of the large scale CNBr cleavage reaction using oligonucleotide 8,9 duplexes containing a single residue of T in the filled-in region (no **2**). The filled-in oligonucleotide 8,9 duplexes were prepared and the CNBr reaction was conducted as described in the experimental section (see Figure 20). The sample was eluted on a Vydac 201HS5415 4.6 mm x 15 cm reverse phase column with a 30 min linear gradient of 0-2% acetonitrile in 10 mM ammonium acetate pH 5.5 at a flow rate of .5 ml/min. The UV-Vis spectra of the different peaks are recorded in the upper left panel.


Scaled
100
80
60
40
20
0
240
260
280
300
320
Wavelength (nm)
1
2
3
4
5


mAU
15
10
5
0
5
10
15
20
25
Time (min.)
1
2
3
4
5

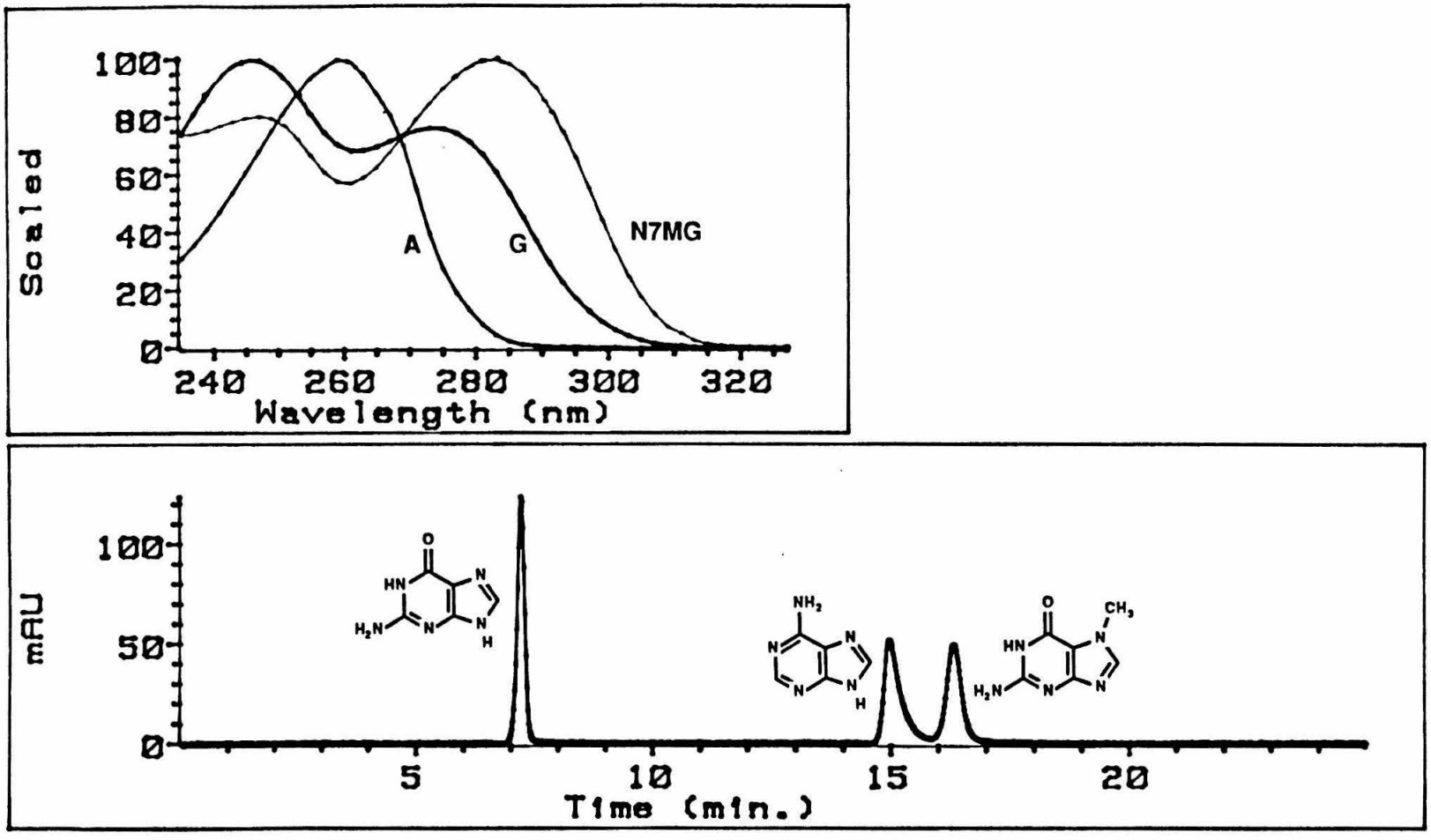


Figure 23 HPLC chromatogram (monitored at 270 ±30 nm) of commercially prepared guanine, adenine and N7-methylguanine. The sample was eluted exactly as described for Figures 21 and 22. The UV-Vis spectra of the different compounds are recorded in the upper left panel.

reactions using oligonucleotide 8,9 duplexes containing **2** and TTP respectively. The ultraviolet-visible absorption (UV-Vis) spectra of the different peaks are recorded in the upper left panel of the figures. The two chromatograms are virtually identical except for the presence of peak 6 in Figure 21 which is not present in Figure 22. Therefore, this peak represents the only observed reaction product of the complementary-addressed cleavage reaction using CNBr and **2**. Figure 23 is the HPLC chromatogram produced by injecting a mixture of commercially prepared guanine, adenine and N7-methylguanine. By comparing the retention times and UV-Vis spectra of the commercial standards with those of the peaks in Figures 21 and 22 the following unambiguous assignments were made. Peaks 2 and 5 in Figures 21 and 22 are guanine and adenine respectively. These purines were probably released during the heat treatment. Peak 1 is probably residual buffer and peaks 3 and 4 have not been identified. Peak 6 in Figure 21 is N7-methylguanine! Therefore, the only unique, isolated product of the complementary-addressed cleavage reaction using CNBr and **2** is N7-methylguanine.

Figures 24 and 25 are the 13-23 minute portions of Figures 21 and 22 respectively. Figure 26 confirms the identity of peak 6 in Figure 21 as N7-methylguanine by superimposing the UV-Vis spectrum of a commercial sample of N7-methylguanine with that from peak 6. Figure 27 confirms the identity of peak 5 in Figure 22 (and Figure 21 for that matter) as adenine by superimposing the UV-Vis spectrum of a commercial sample of adenine with that from peak 5.

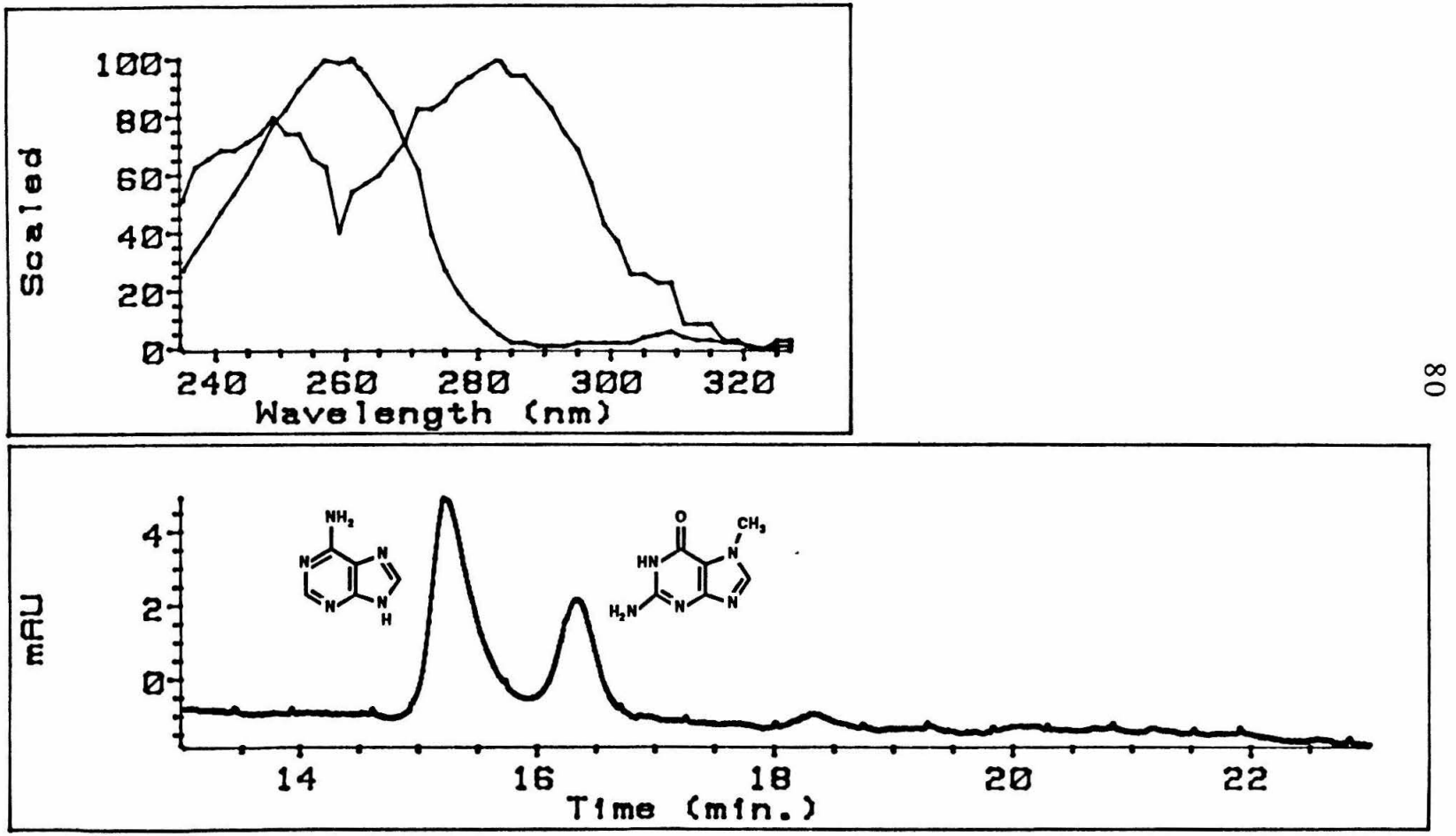


Figure 24 13-23 minute portion of the chromatogram in Figure 21.

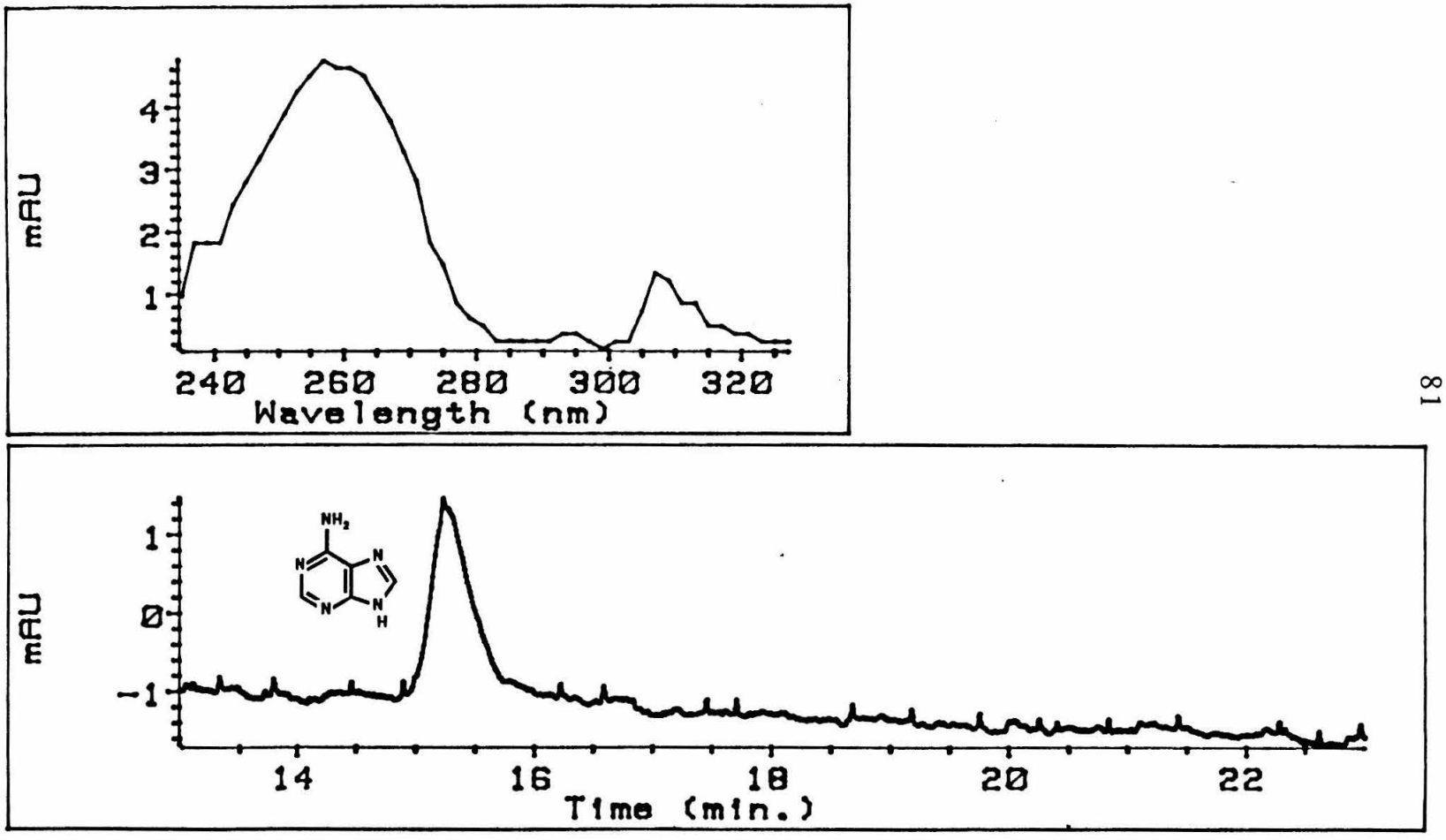


Figure 25 13-23 minute portion of the chromatogram in Figure 22.

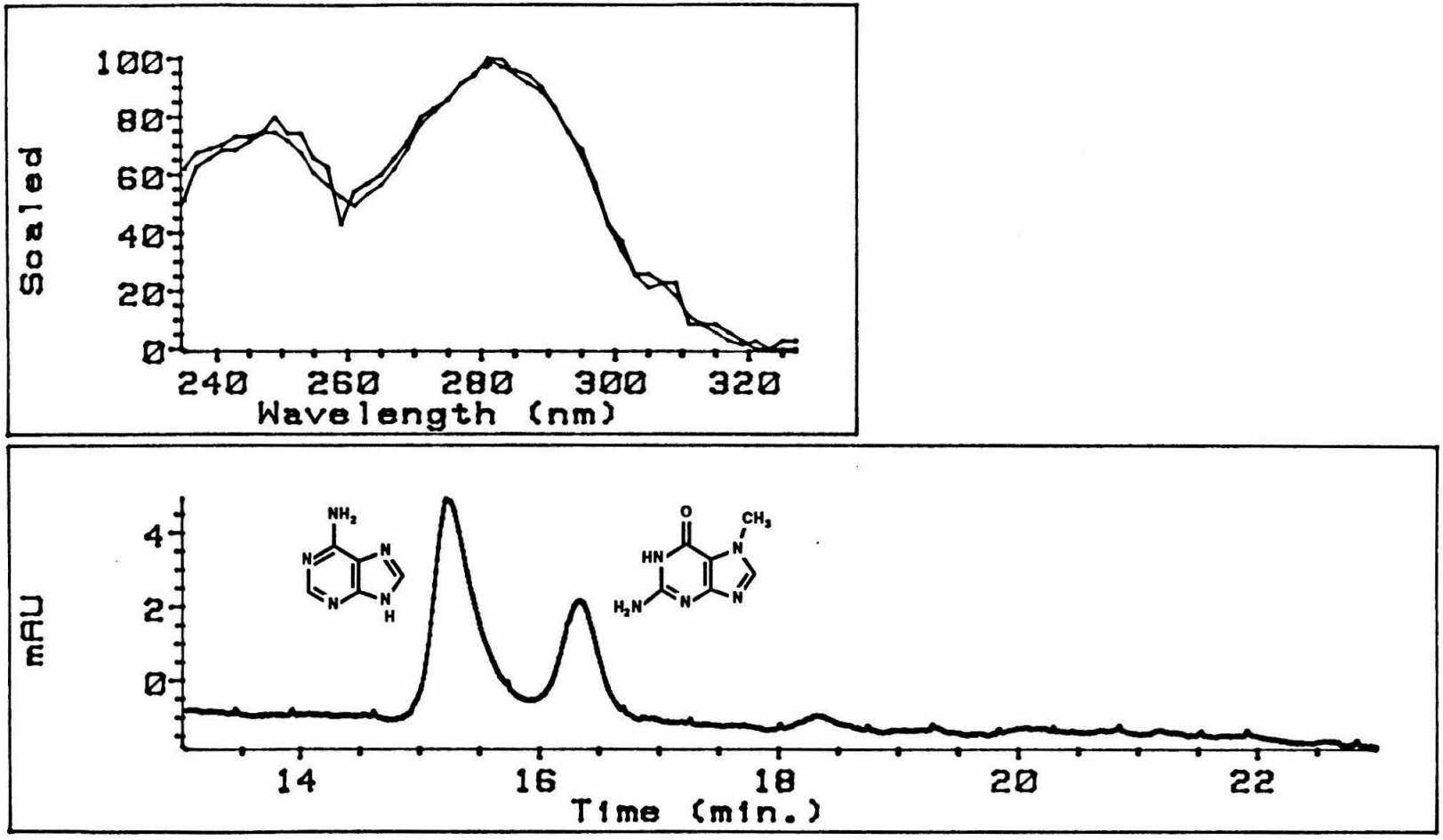


Figure 26 13-23 minute portion of the chromatogram in Figure 21. The UV-Vis spectrum of commercially prepared N7-methylguanine is superimposed upon the spectrum from the 16.3 minute peak in the chromatogram.

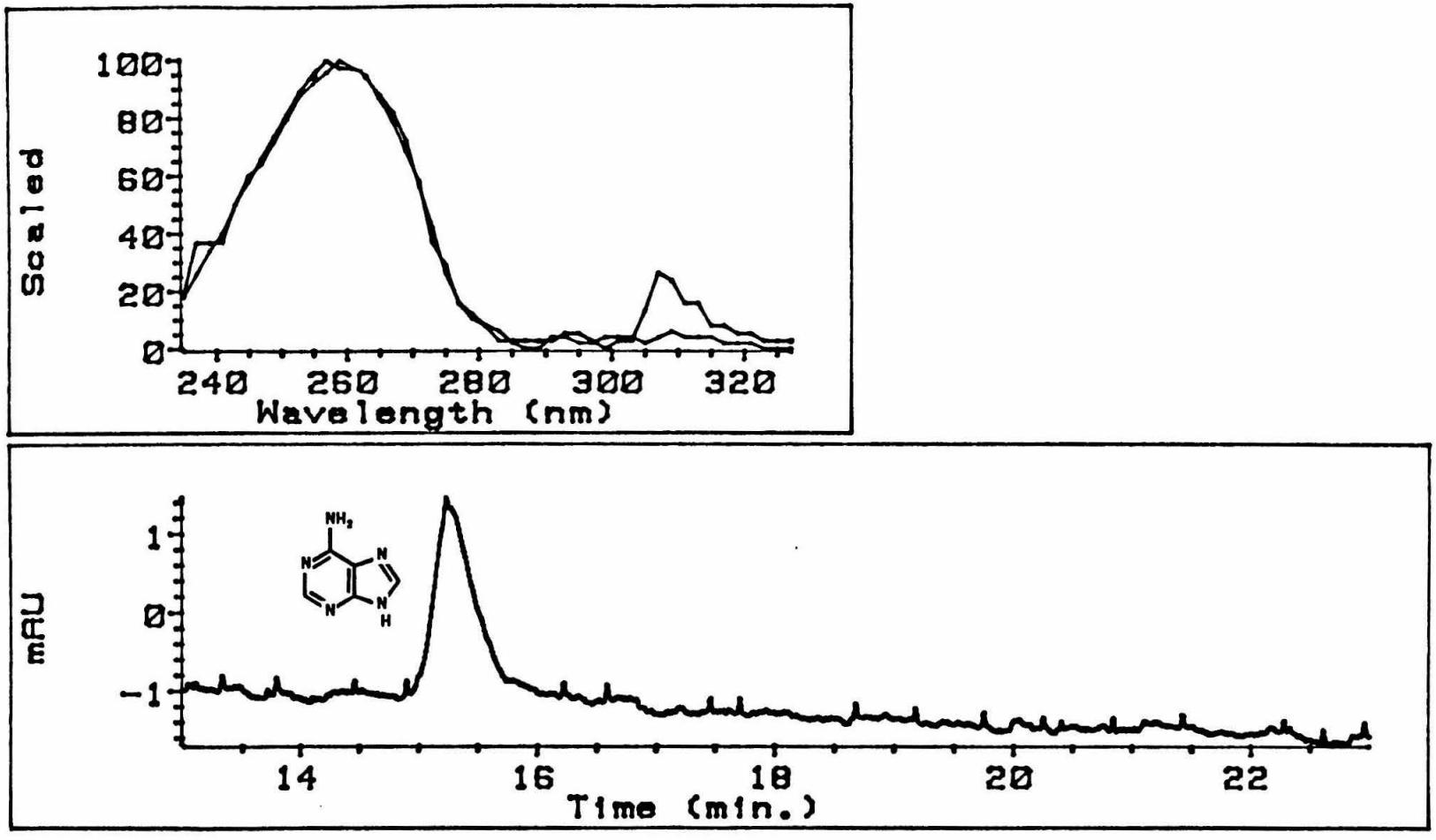


Figure 27 13-23 minute portion of the chromatogram in Figure 22. The UV-Vis spectrum of commercially prepared adenine is superimposed upon the spectrum from the peak in the chromatogram.

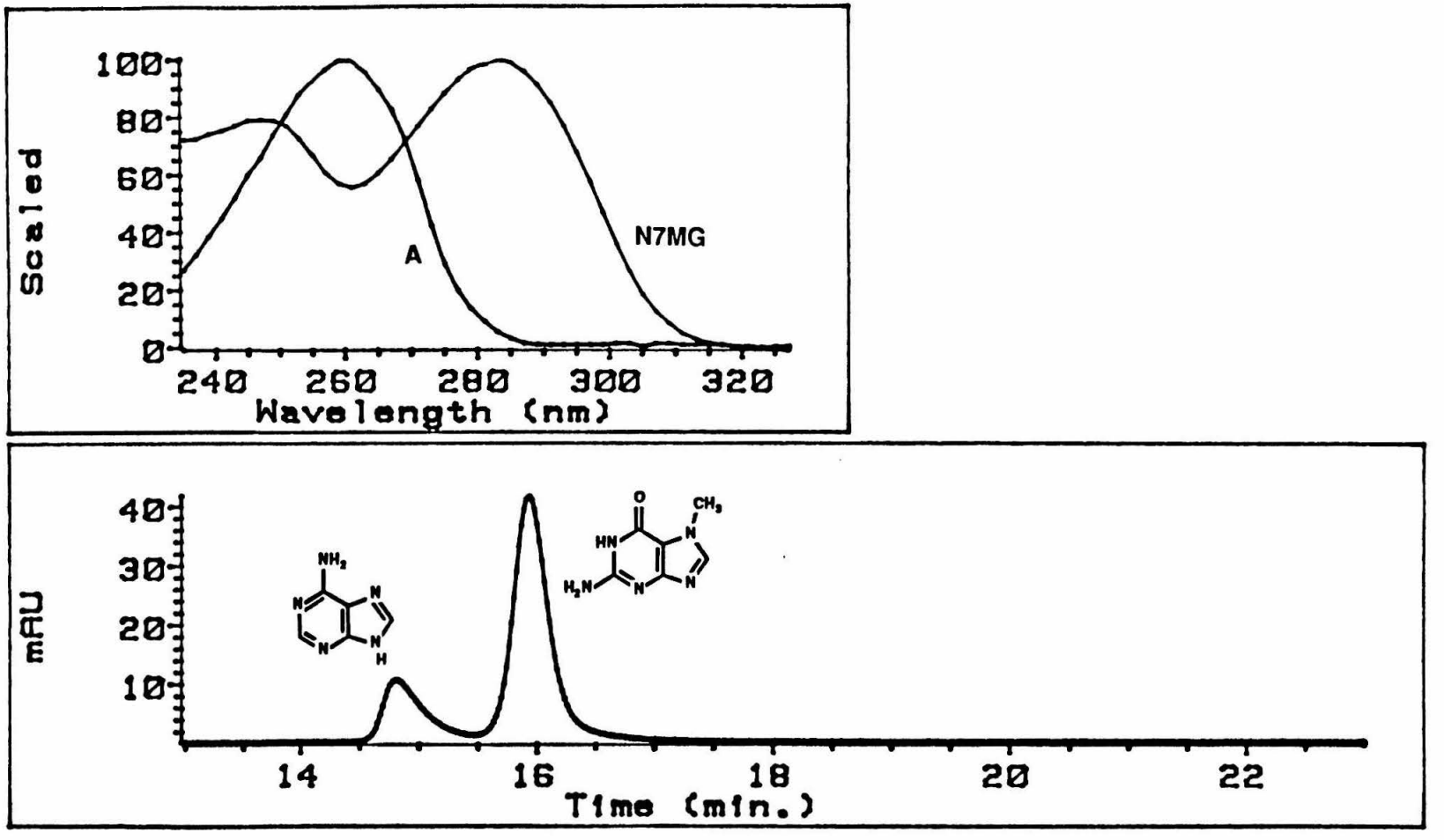


Figure 28 13-23 minute portion of a chromatogram produced by commercially prepared adenine and N7-methylguanine. The sample was eluted exactly as described for Figures 21 and 22. The UV-Vis spectra of the two compounds are recorded in the upper left panel.

Figure 28 can be used for comparison with Figures 24-27 and is the 13 to 23 minute portion of a chromatogram produced by injection of commercially prepared adenine and N7-methylguanine.

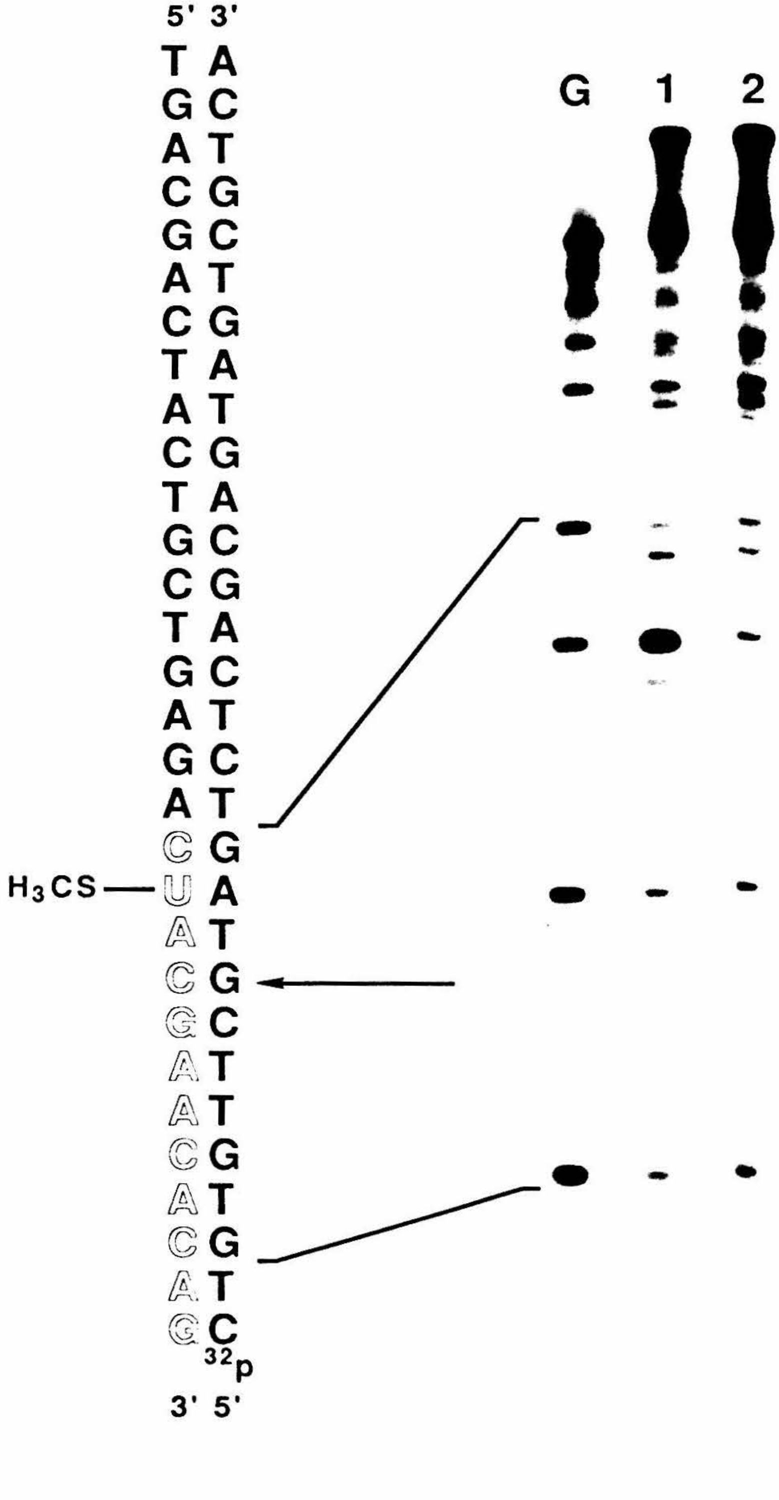
Known amounts of commercially prepared N7-methylguanine were injected in calibration runs and the integrations of those peaks were used to quantitate the amount of N7-methylguanine in peak 6 of Figure 21. The integration of peak 6 in Figure 21 indicated that it represents .295 nmol N7-methylguanine. Correcting for the expected 15% loss during the ethanol precipitation and the 3 µl of solution not injected into the HPLC, .39 nmol of N7-methylguanine was produced during the CNBr reaction.

The precipitated oligonucleotides from the large scale reactions were loaded onto a denaturing 20% polyacrylamide gel (see Figure 29). Densitometry performed on the autoradiograph of the gel revealed that the single G residue on the complementary strand two bases to the 5' side of the residue of **2** was cleaved with 12% efficiency.

Proposed Reaction Mechanism Methyl transfer is clearly the predominant mechanism of complementary-addressed reaction by CNBr and **2** since the .39 nmol of N7-methylguanine calculated to have been produced in the reaction accounts for 82% of the expected reaction product (based on the 12% cleavage efficiency observed on the gel, Figure 29). This 82% rate could actually be higher owing to the way the concentration of the purified 8,9 duplex was calculated. An ε_{260} = 6700/phosphate was used to compute the purified

Figure 29

Large scale CNBr cleavage reactions of the oligonucleotide 8,9 duplex. Autoradiogram of a 20% denaturing polyacrylamide gel. The precipitated oligonucleotide 8,9 duplexes from the large scale CNBr reaction were dissolved in 10% aqueous piperidine and heated to 90°C for 30 minutes. The samples were lyophilized to dryness, the residue redissolved in formamide loading buffer and .2 mR/h was loaded onto each lane of the gel. **G** Maxam-Gilbert G reaction. **Lane 1** Oligonucleotide 8,9 duplex filled-in with a residue of **2**. **Lane 2** Oligonucleotide 8,9 duplex filled-in with a regular T residue. The histogram shows the location of the ^{32}P label as well as the positions filled-in by the Klenow enzyme (outlined bases). The position of the residue of **2** (H_3CS-U) in relation to the observed guanine cleavage site (arrow) is also indicated. Densitometry revealed that the absolute cleavage efficiency of the reaction was 12%.


5' 3'
T A
G C
A T
C G
G C
A T
C G
T A
A T
C G
T A
G C
C G
T A
G C
A T
G C
A T
C G
H3CS— U A
A T
C G
G C
A T
A T
C G
A T
C G
A T
G C
32p
3' 5'
G
1
2

oligonucleotide duplex concentration, but this number was derived from long pieces of double-stranded DNA exposed to normal salt concentrations.[72] The isolated oligonucleotide 8,9 duplex absorption was measured under low salt conditions which might have caused partial denaturing of the strands, thus increasing the observed absorbance at 260 nm.[3] If this was the case, less than 4.04 nmol of filled-in duplex was actually recovered from the fill-in reaction and the percentage of N7-methylguanine produced relative to expected would be higher than reported.

The apparent production of 3'- and 5'-phosphate termini after piperidine treatment is consistent with the methyl transfer mechanism. A detailed mechanism can be proposed for the complementary-addressed methylation and cleavage reaction caused by **2**, CNBr and a piperidine workup (see Figure 30). The proposed scheme accounts for all of the observed reaction products and was based on the previously proposed scheme for piperidine promoted cleavage at methylated guanine residues of DNA.[49]

Computer Modeling An oligonucleotide duplex was built using the DNA builder in Biograf version 1.32. The sequence corresponded to the 17 base pairs in the filled-in oligonucleotide 1,2 duplex centered around the location of the single residue of **2**. The duplex was constructed with a regular T residue in place of the residue of **2** and the structure was subjected to 5000 steps of energy minimization followed by a dynamics run (10,000 steps, initial temperature 1,000°C and final temperature 300°C).

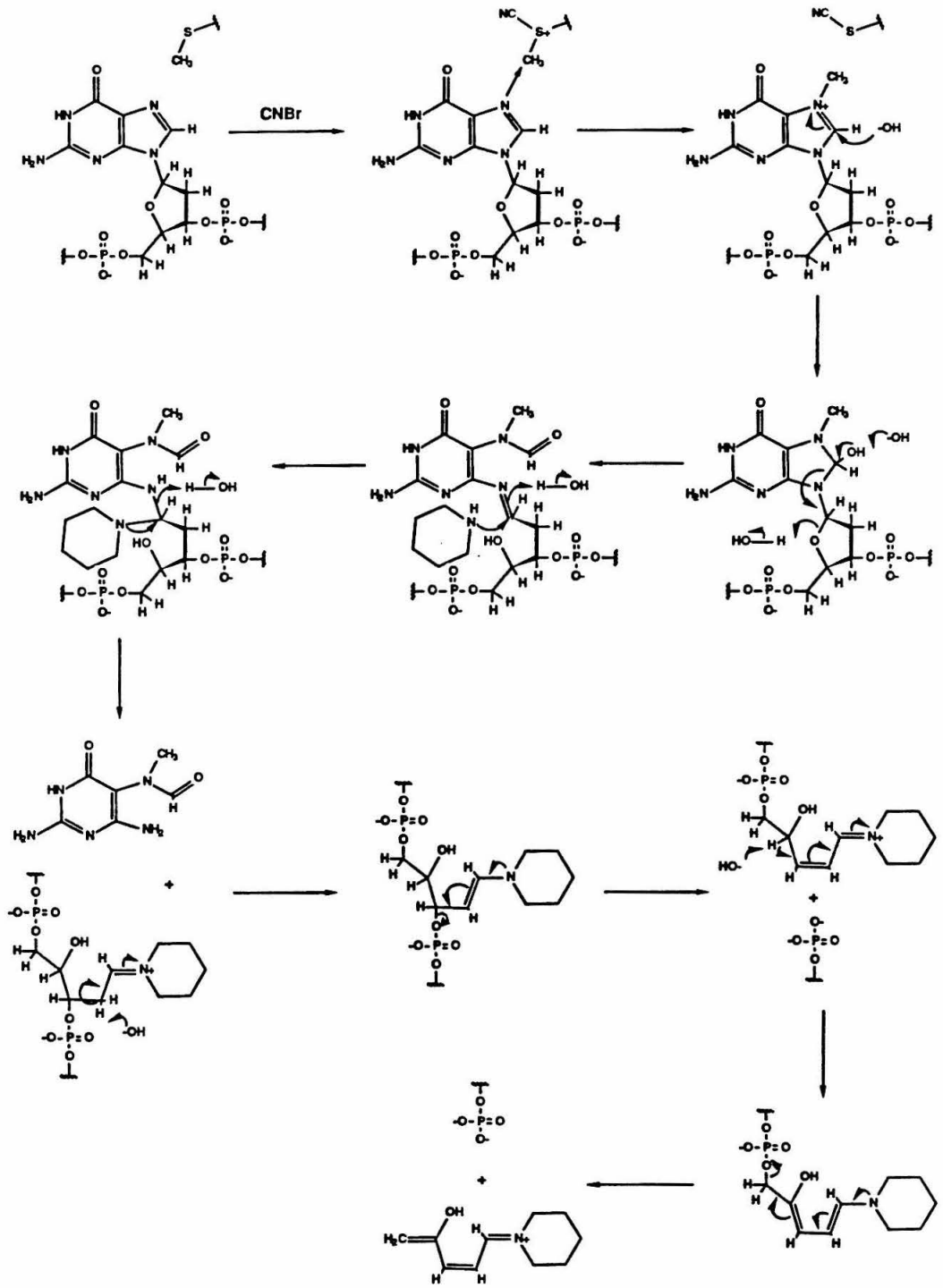


Figure 30 Proposed mechanism for the CNBr-methylthioether cleavage reaction using a piperidine workup.

The resulting duplex was modified by building the side chain from **2** onto the appropriate T residue. In order to approximate a linear trajectory of attack, the sulfur atom of the side chain of **2** was covalently linked to the N7 position of the reactive guanine residue. The covalent linkage was accomplished via a S-C-C-N series of bonds wherein the two carbon atoms were sp^1 hybridized. Using the C-C triple bond insured a linear geometry of the linkage as well as a relatively close S-N distance (4.1 Å). The geometry of the side chain was optimized by fixing the positions of all the atoms on the oligonucleotide duplex and only allowing the side chain atoms to move during energy minimization using the Dreiding default parameters. The optimized geometry contained reasonable bond angles and distances. The sp^1 carbon bonded to the N7 position of guanine was removed and the sp^1 carbon bonded to sulfur was exchanged for a methyl group. The S-C bond length for the methyl group was not readjusted.

Figure 31 shows the oligonucleotide duplex with the attached side chain from the residue of **2**. Figure 32 is a close-up shot of the same duplex showing the details around the side chain. Figure 33 is the same view as Figure 32 except only the residue of **2** and the reactive guanine are depicted. Figure 34 is the same as Figure 33, but viewed from a 90° rotated perspective. Figure 35 is the same as Figure 32 but only adjacent guanine residues are included. These figures serve to illustrate a possible linear geometry of the N7-guanine, methyl group and sulfur atom as well as the normal bond angles and distances of the entire side chain which are possible in such a confor-

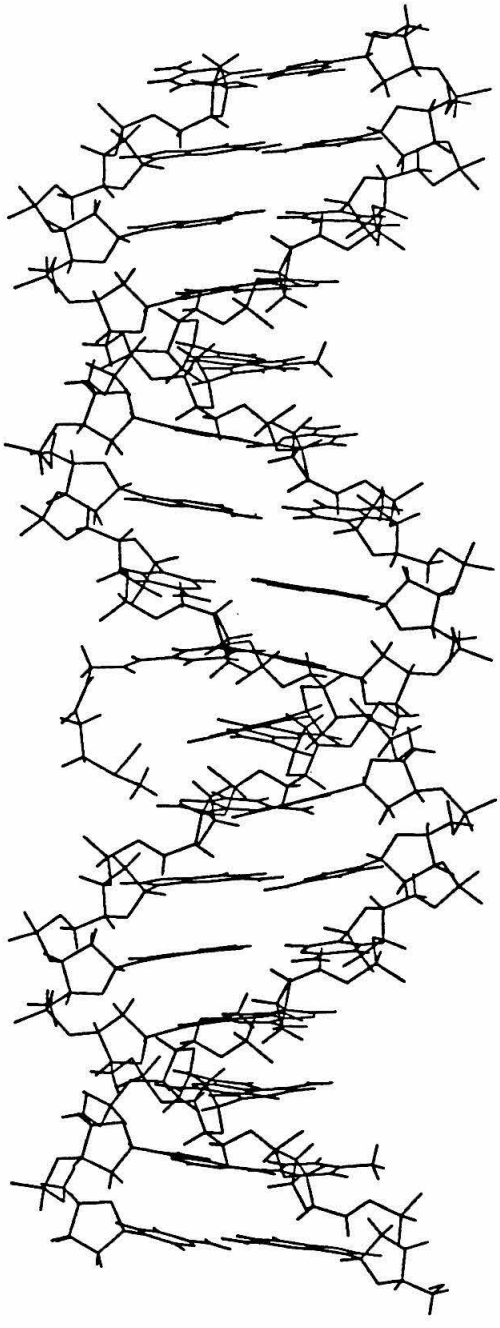


Figure 31 Computer generated illustration of the filled-in oligonucleotide 1,2 duplex containing a single residue of **2**. The side chain of **2** is visible in the major groove of the duplex in the center of the figure. The picture was generated as described in the text.

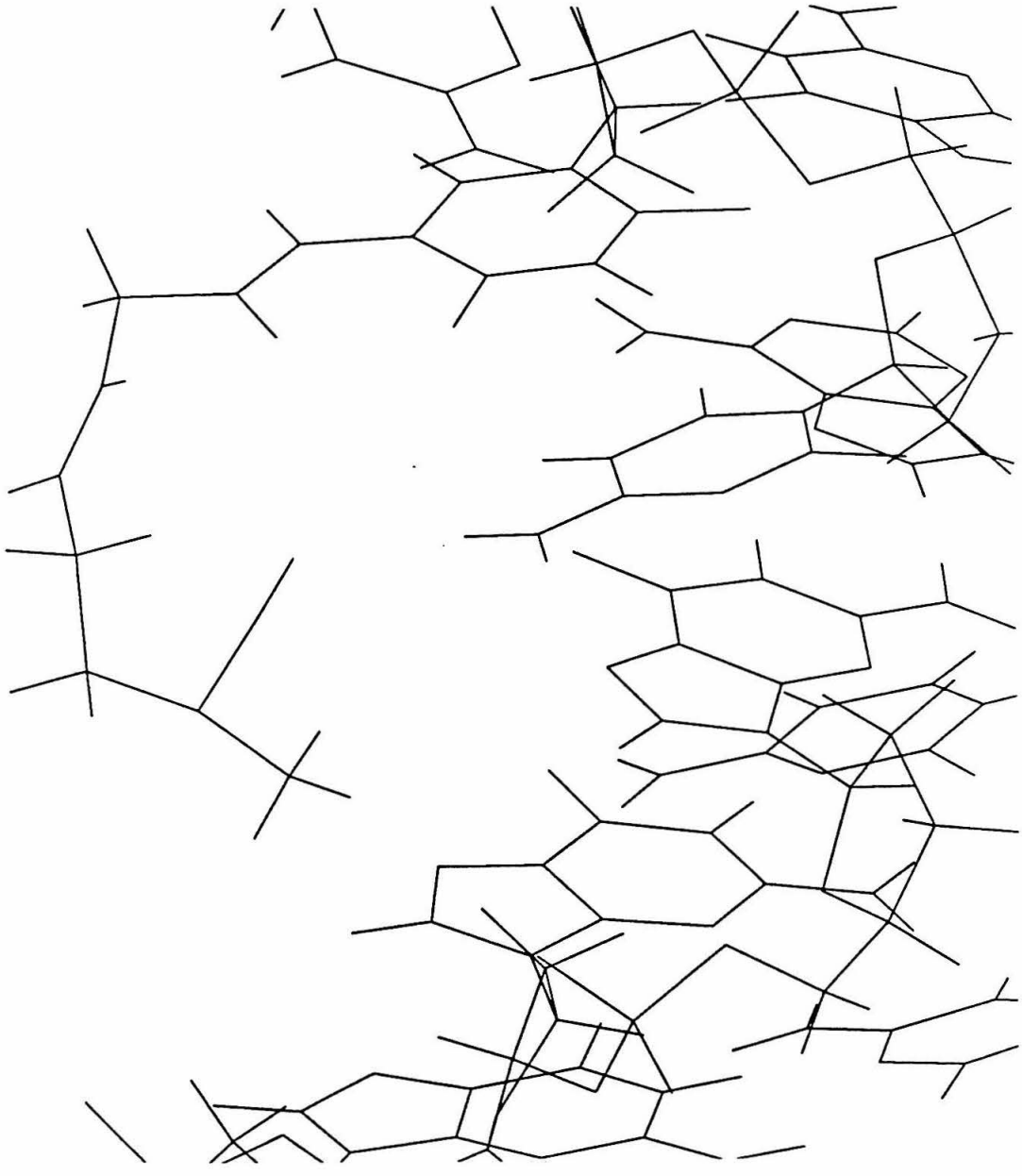


Figure 32 Closeup view of the same computer generated illustration shown in Figure 31. A possible orientation of the side chain of **2** just prior to a methylation reaction is depicted.

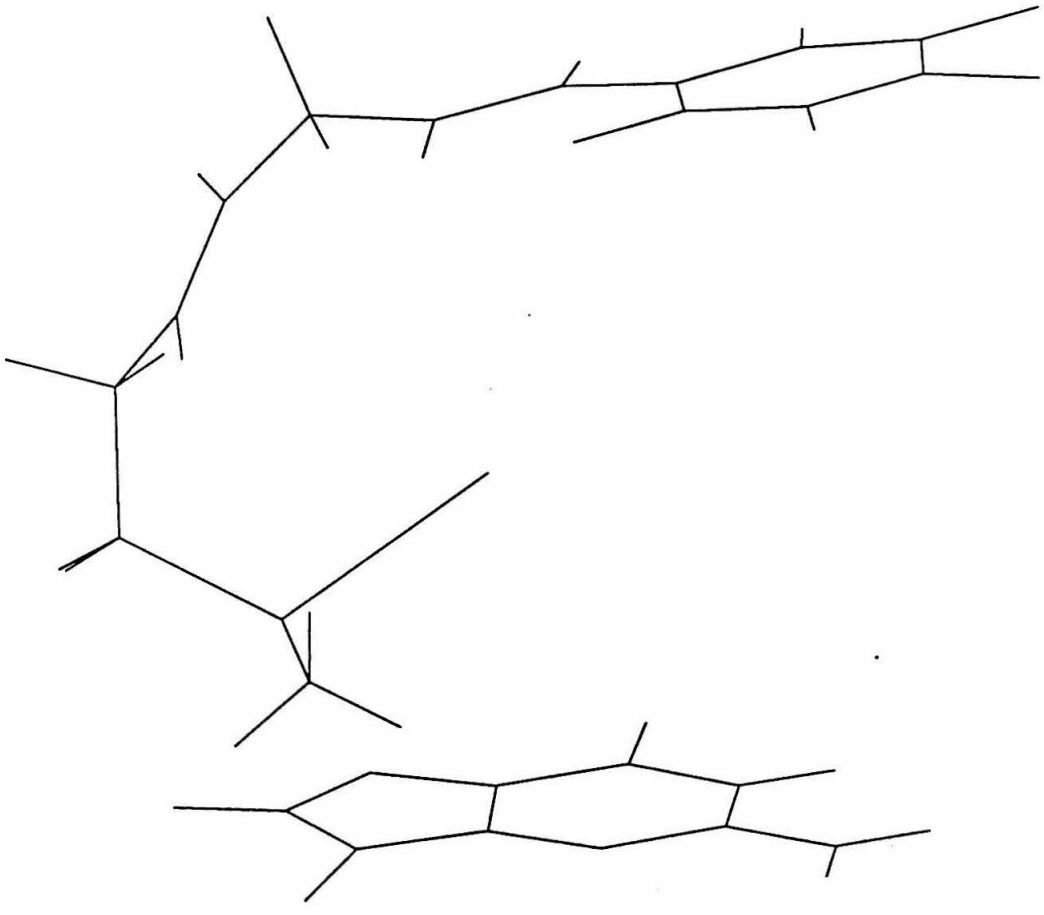


Figure 33 Closeup view of the same computer generated illustration shown in Figures 31 and 32. Only the residue of **2** and the guanine residue observed to react are drawn . The linear N7 of guanine-methyl group-sulfur atom geometry is evident.

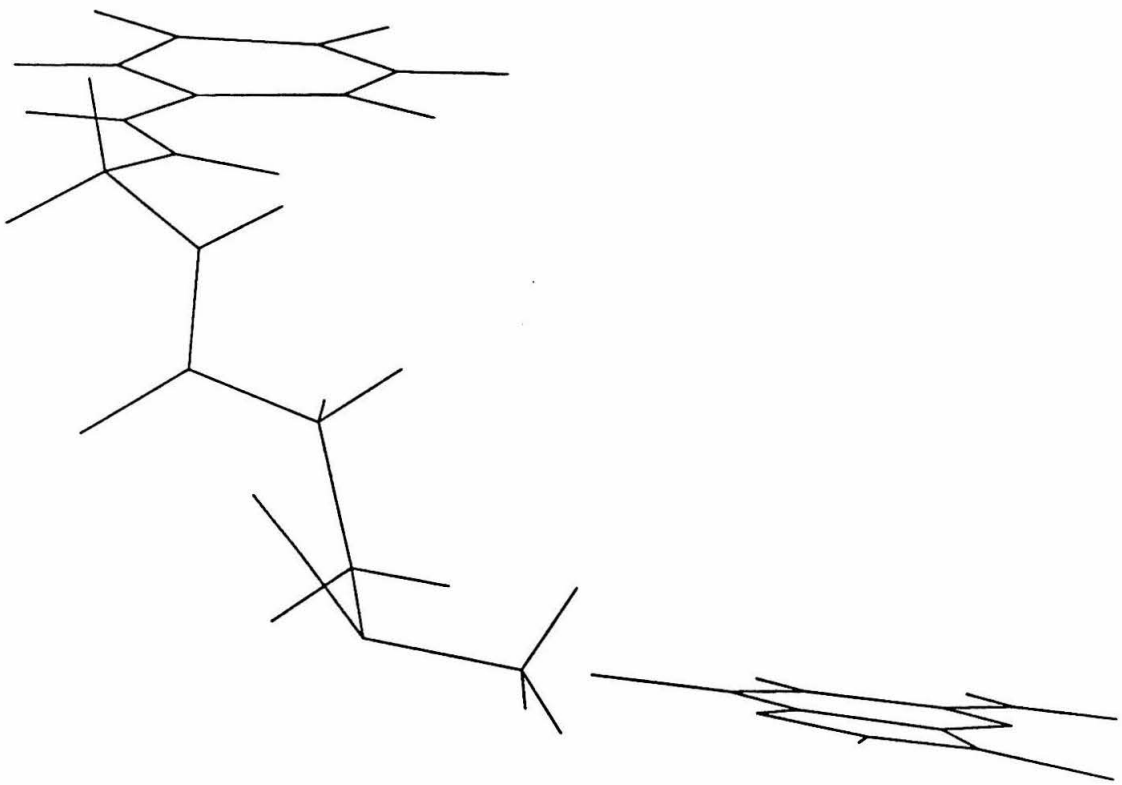


Figure 34 Same illustration as Figure 33, but viewed from a perspective rotated 90°.

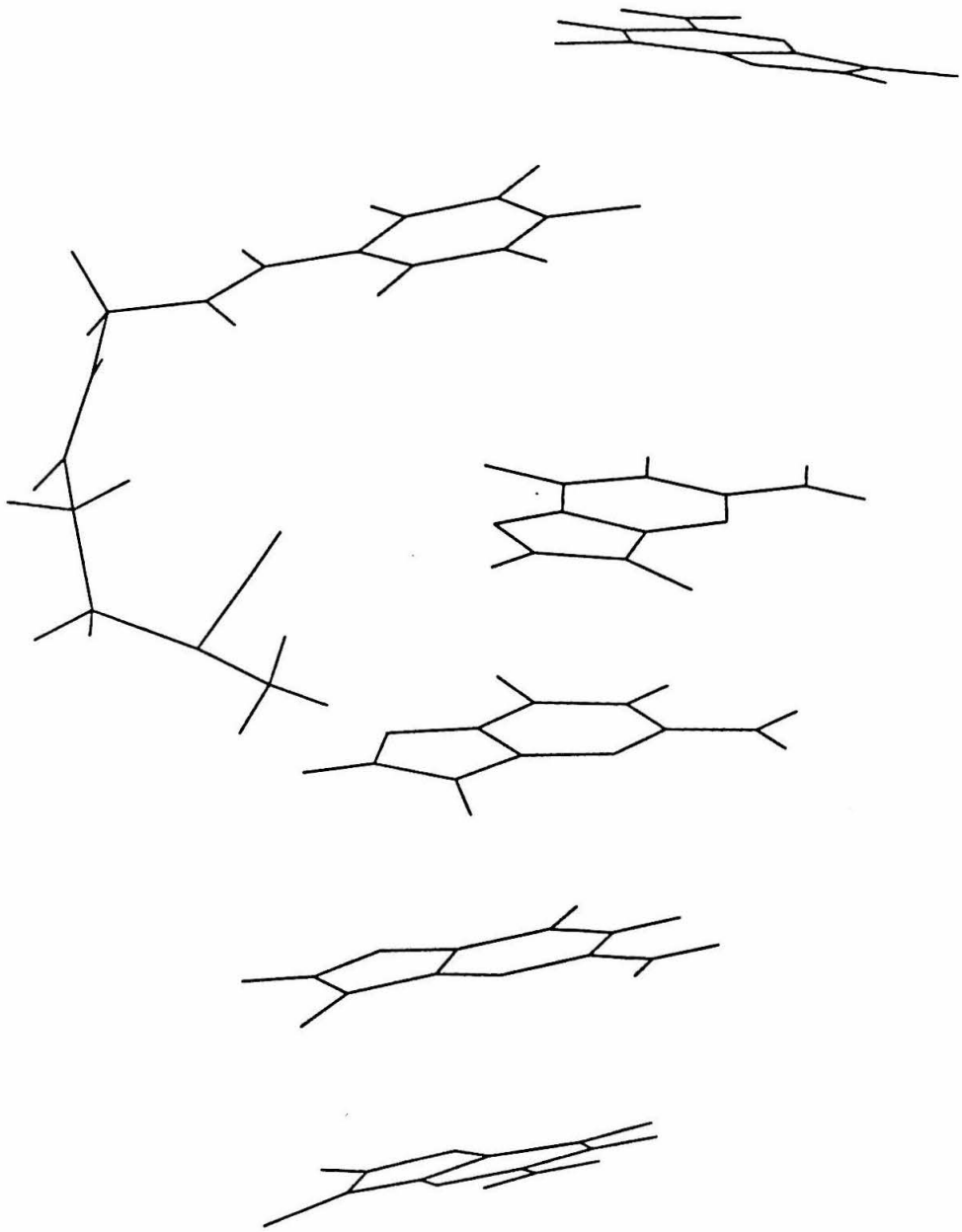
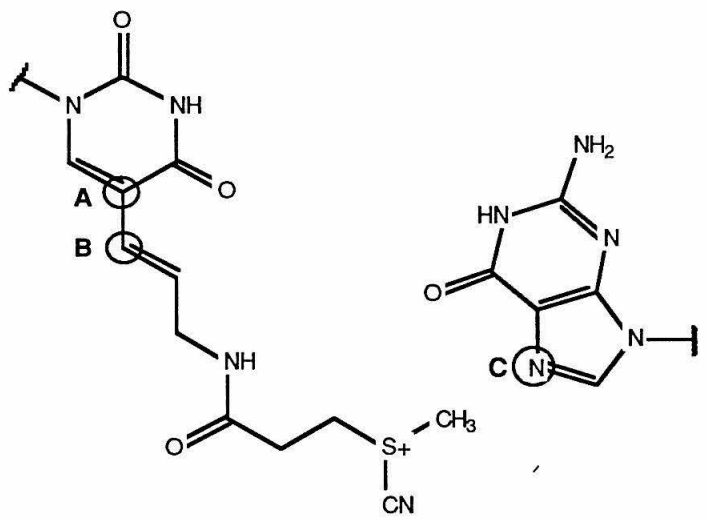


Figure 35 Closeup view of the same computer generated illustration shown in Figure 31. Only the residue of **2** and adjacent guanine residues are drawn.

mation. Furthermore, since figures 31-35 were intended to depict a possible geometry exhibited just prior to a methyl transfer event, several observations about the structure should be relevant to the observed reaction.

Analysis of the geometry of the bases in the computer generated model of the oligonucleotide duplex revealed how the methyl transfer reaction might be facilitated by the propeller twist of the bases. As is most clearly seen in Figure 34, the 5 position of the residue of **2** is predicted to point downward toward the N7 of guanine with which it reacts, and similarly the reactive N7 position of guanine is predicted to point upward toward the residue of **2**. This geometry reduces the severity of the angle the side chain of **2** must accommodate to allow the methyl transfer reaction.

The differences in distances and geometries (relative to **2**) predicted for the adjacent guanine residues helps to explain the single base reaction specificity observed with the oligonucleotide 1,2 duplex (see Figure 15). Figure 36 contains a simplified depiction of the residue of **2** and a guanine residue which is meant to represent any of the guanine residues adjacent to **2** in the computer generated duplex. The table in Figure 36 lists relevant distances and geometries derived from Figure 35. Of course these angles and distances should be thought of as merely probable averages because the duplex is certainly flexible and can attain altered conformations in solution. Nevertheless, it is informative to compare the distances and angles for guanine residues 8,9 and 10; all of which were observed to react



G Residue Position	B - C Distance (Å)	A - B - C Angle	Relative Reactivity
12	10.1	35°	-
10	8.5	66°	0.08
9	8.7	82°	1.0
8	10.7	89°	0.08
7	13.8	89°	-

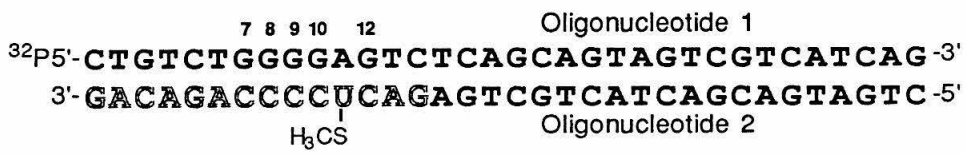


Figure 36 Some relevant distances and angles derived from the computer generated model depicted in Figures 31-35.

to some extent. The distances from the atom labelled as "B" on **2** to the atoms labelled "C" on the guanines (the N7 position) are 10.7 Å, 8.7 Å and 8.5 Å respectively for residues 8,9 and 10. The angles made by the atoms labelled "A" "B" and "C" are 89°, 82° and 66° respectively for 8,9 and 10. These angles and distances represent the angles and distances the side chain would have to span during the methyl transfer reaction. The distances for guanine residues 9 and 10 are similar but the angle is substantially smaller for 10. Conversely, the angles for residues 8 and 9 are similar, but the distance to residue 8 is longer. Therefore, the single base specificity for reaction at residue 9 observed in the methyl transfer reaction derives from angular as well as distance considerations.

The severe angle (35°) and large distance (10.1 Å) that would be required for a methyl transfer reaction to residue 12 in the model probably explain why the reaction occurs exclusively to the 5' side of **2**. The observed reaction to the 5' side could therefore be thought of as direct evidence for the right-handed nature of the 1,2 duplex in solution.

A similar analysis revealed that intrastrand cleavage is also unlikely because of the angles involved. This agrees with the observed lack of any intrastrand cleavage (data not shown).

The side chain was constrained in the computer modeling studies to provide a linear approach of the H_3C-S- to the N7 of guanine residue 9. This constraint was based on the known preference of a tetrahedral carbon for backside attack[73] and the rigorous angular

requirements of the methyl transfer reaction using sulfonium compounds. A series of compounds has been reported capable of intramolecular methyl transfer from sulfonium species to nucleophiles held in defined geometries.[74] In these studies, intramolecular methyl transfer was observed only when the nucleophile-H_3C-S- geometry allowed a linear transition state and even a 20° deviation from the desired 180° precluded reaction.

The computer model building studies failed to produce a reasonable structure that would allow backside attack from the reactive N7 of guanine onto the methylene carbon atom α to the sulfonium group on the side chain of **2**. The analogous α methylene carbon atom is known to react during the CNBr-methionine peptide cleavage reaction.[65] Thus the model building studies were consistent with the observed methyl transfer mode of reaction.

Studying the side chain of **2** in Figures 32-35 could help to explain the observed 12% cleavage efficiency seen in the methyl transfer reaction. The side chain appears fairly rigid due to the double bond and rigid peptide linkage, but the three methylene groups still allow a wide variety of different conformations, only a small percentage of which could satisfy the geometrical requirements of the methyl transfer reaction. Furthermore, the positive charge on the presumed sulfonium intermediate should tend to pull the sulfonium group towards the negatively charged DNA phosphate backbone and therefore away from the reactive guanine residue. All of these "undesirable" conformations should increase the rate of

deactivation via hydrolysis of the sulfonium group and thereby help to lower the cleavage efficiency to the observed 12%.

Covalently constraining the methylthioether side chain into a conformation conducive to the methyl transfer reaction should dramatically increase the efficiency of the reaction. A possible approach would involve linking the allylamine and methylthioether functions to ortho positions of an aromatic ring. Positively charged groups could be attached at other locations on the aromatic ring to help position the ring close to the DNA phosphate backbone and thereby place the methylthioether function close to the N7 position of a targeted guanine residue.

Another approach to increasing the efficiency of the complementary-addressed methylation reaction might be to decrease the reactivity of the sulfonium species, thereby slowing the hydrolysis deactivation reaction. An enzymatically prepared S-adenosylmethyl sulfonium or an aromatic cyanomethyl sulfonium species should have the desired decrease in reactivity.

Cleavage Of ΦX174 Single-Stranded DNA

The application of the complementary-addressed CNBr reaction using **2** to methylate and cleave large pieces of single-stranded DNA was investigated using linearized, single-stranded ΦX174 DNA. A method was developed for enzymatic incorporation of a single residue of **2** into an oligonucleotide capable of causing the cleavage of ΦX174 DNA to nucleotide resolution.

ΦX174 virion DNA is a single-stranded circular DNA of 5386 bases.[75] The DNA was linearized at the unique Xho 1 restriction site using oligonucleotide 10. Oligonucleotide 10 was annealed to the ΦX174 DNA using the usual gentle heat treatment then the DNA was digested with Xho 1 restriction endonuclease. The linearized ΦX174 DNA was end-labelled with ^{32}P at the 5' end using the standard T4 polynucleotide kinase enzyme reaction.[4] This DNA was purified by a denaturing 5% polyacrylamide gel.

A single residue of **2** was enzymatically incorporated into oligonucleotide 11 which was complementary to a sequence in the ΦX174 DNA seventy bases from the 5' end (see Figure 37). Oligonucleotide 11 was annealed to the ΦX174 DNA then a Klenow enzyme fill-in reaction was conducted in the presence of dATP, dCTP, 2',3'-dideoxyguanosine 5'-triphosphate (ddGTP) and **2** or TTP. Using the ddGTP as a chain terminator[3] insured that the Klenow reaction stopped precisely after adding eight residues so that only a single residue of **2** was incorporated into the filled-in oligonucleotide 11.

Sodium acetate (pH 5.5) and CNBr were added to final concentrations of 25 mM and 16 mM respectively. The reactions were incubated at room temperature for 18 hours then 10% by volume of neat piperidine was added and the reactions were heated at 90°C for 20 minutes. The site-specific cleavage was analyzed on a denaturing 8% polyacrylamide gel (see Figure 38).

Cleavage was observed on the ΦX174 DNA at the expected single G residue two bases to the 5' side of where the single residue of **2**

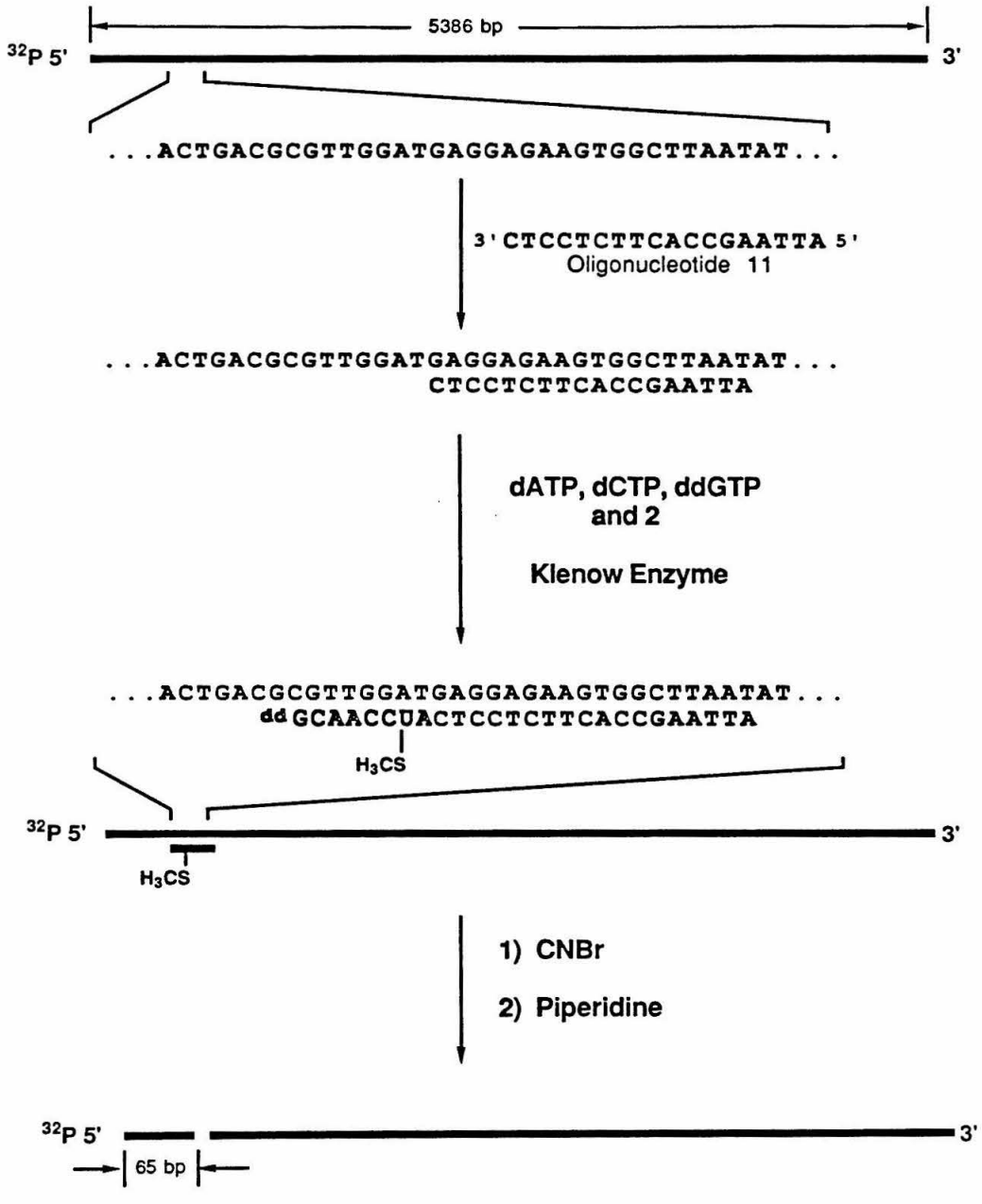
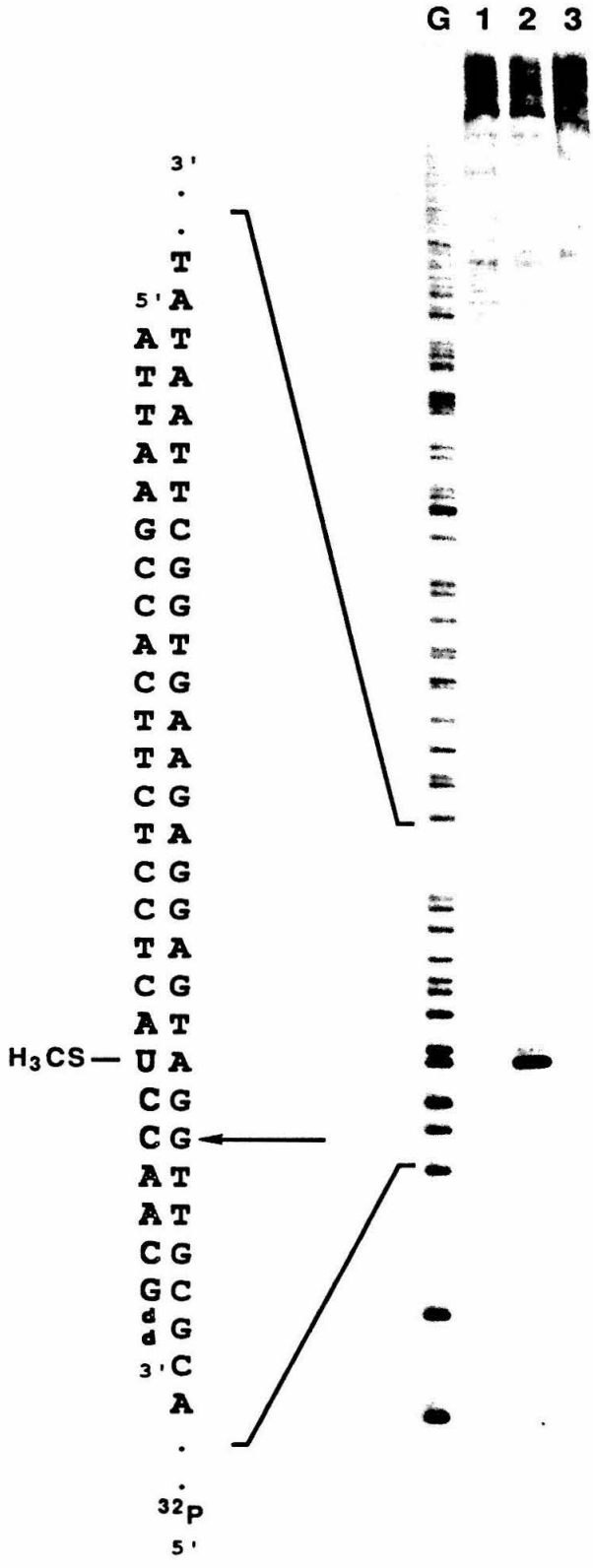


Figure 37 Scheme for the complementary-addressed methylation and cleavage of single-stranded ΦX174 DNA using an enzymatically incorporated residue of **2** and CNBr.

Figure 38

Cleavage of single-stranded ΦX174 DNA using an enzymatically incorporated residue of **2** and CNBr. Autoradiogram of a dried, denaturing 8% polyacrylamide gel. ΦX174 was linearized and labelled with ^{32}P at the unique Xho 1 restriction site as described in the experimental section. Oligonucleotide 11 was annealed to 5 mR/h of the ΦX174 DNA in a 10 μl solution then a Klenow enzyme fill-in reaction was conducted in the presence of 4 units Klenow fragment, dATP, dCTP, ddGTP (used as a chain terminator) and **2** or TTP. After the Klenow enzyme reactions, NaCl and NaOAc pH 5.5 solutions were added to final concentrations of 5 mM and 25 mM respectively. 1 μg CT DNA was added and the reactions were incubated at room temperature for 18 h with or without 15 mM CNBr. 3 μl of neat piperidine was then added and the samples were heated at 90°C for 20 min. The samples were lyophilized to dryness, the residue redissolved in formamide loading buffer and .5 mR/h was loaded onto each lane of the gel. **G** Maxam-Gilbert G reaction. **Lane 1** TTP was used in the Klenow enzyme fill-in reaction, the resulting solution was reacted with 15 mM CNBr. **Lane 2** **2** was used in the Klenow enzyme fill-in reaction, the resulting solution was reacted with 15 mM CNBr. **Lane 3** **2** was used in the Klenow enzyme fill-in reaction, the resulting solution was reacted <u>without</u> CNBr. The histogram shows the positions filled-in by the Klenow enzyme (outlined bases). The position of the residue of **2** (H_3CS-U) in relation to the observed guanine cleavage site (arrow) is also indicated. Densitometry revealed that the absolute cleavage efficiency of the reaction was 9%.


G 1 2 3
3'
5'
H3CS—
32P
5'

was placed. The observed cleavage efficiency was 9%. Cleavage was also observed at an adjacent G residue, but with 0.08 the cleavage efficiency observed at the main site of cleavage.

This methylation and cleavage of ΦX174 DNA at essentially a single nucleotide using an enzymatically incorporated residue of **2** and a chain terminating 2',3'-dideoxynucleotide 5'-triphosphate represents a relatively simple and general approach to the programmed cleavage of large pieces of single-stranded DNA. The protocol is similar to and no more difficult than the Klenow enzyme procedures in routine use for Sanger sequencing[3] and 3'-end-labelling during Maxam-Gilbert sequencing.[49] The CNBr and piperidine procedures are less involved than the Maxam-Gilbert base specific cleavage protocols.[49] The CNBr reaction using **2** requires a target sequence of the form 3'-AN_1GN_2-5' where N_1 and N_2 could be any base (N_1,N_2 = A should be avoided if possible) and the G residue is the predicted site of cleavage. As demonstrated in Figure 38, this reaction specificity coupled to the sequence specificity inherent in oligonucleotide delivery give this system potential for use in the programmed cleavage of even large pieces of single-stranded DNA to nucleotide resolution.

Ideally, one could design reagents which have different tether "reaches". Altering the distance and geometry between the uridine base and the methylthioether function could produce reagents capable of specific cleavage of sequences of the form 3'-AG-5' or 3'-AN_1N_2G-5'. Such reagents would allow consideration of more poten-

tial sites of cleavage along a DNA strand, thus adding flexibility to the system.

Cleavage Of ΦX174 Single-Stranded DNA Using An Allylamine Oligonucleotide Post-Synthetically Modified With Methylthioether.

Synthesis Of 9 The synthesis of **9** was accomplished using methods analogous to those for the synthesis of a 2'-deoxyuridine derivative carrying a covalently attached EDTA moiety[12] (see Figure 39). The mercurinucleoside **6** was prepared by treating 2'-deoxyuridine with mercuric acetate.[76] The production of the N-trifluoroacetylallylamino derivative **7** turned out to be more difficult than expected (see Figure 40).

Reacting **6**, N-trifluoroacetylallylamine and K_2PdCl_4 in methanol/water for 6 hours produced an inseparable mixture of 70% **7**, 30% impurity which was identified by ^1H NMR as the methanol addition product known to occur in this type of reaction.[76] Synthesis of pure **7** was finally accomplished by conducting the reaction in isopropanol/water and stirring for 3 days instead of 6 hours. The final purification consisted of vapor diffusion recrystallization with hexane from a concentrated ethyl acetate solution.

The 5' hydroxyl group of **7** was protected by treatment with 4,4'-dimethoxytriphenylmethyl chloride in pyridine. The product **8** was purified by flash chromatography then used to prepare **9** by treatment with N,N-diisopropylmethylphosphonamidic chloride.

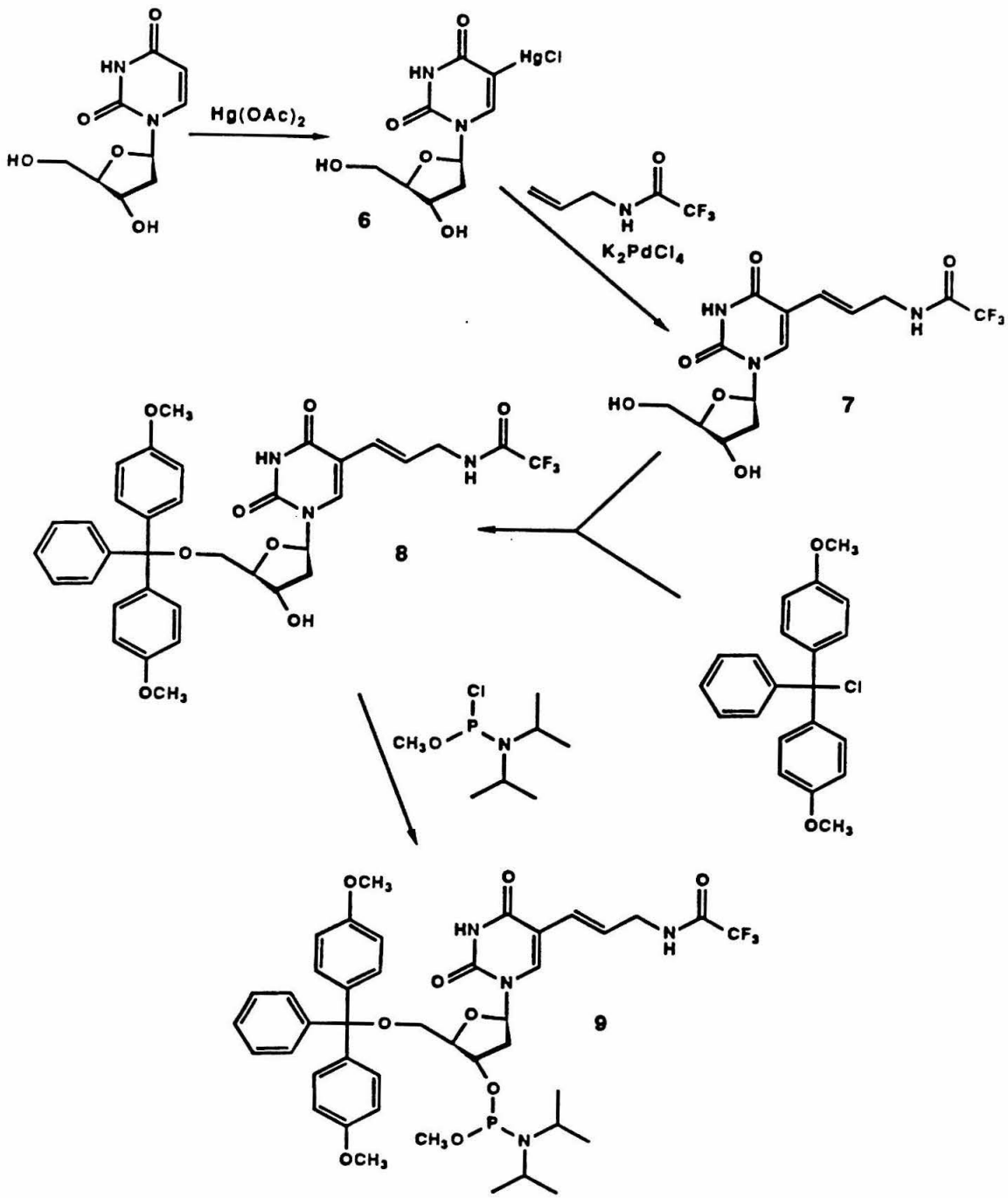


Figure 39 Synthetic scheme for preparation of compounds **6-9**.

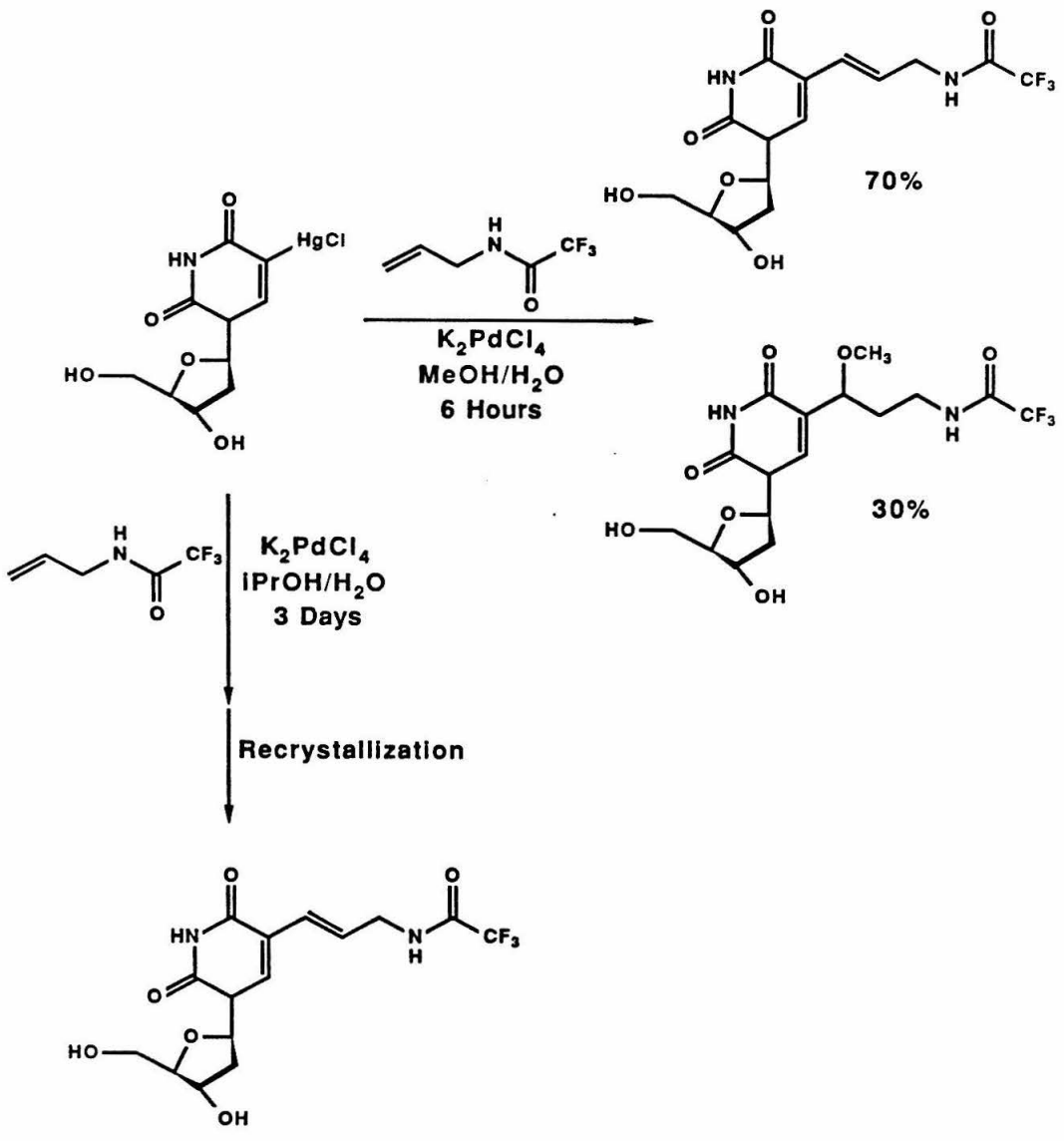


Figure 40 Side reaction encountered in the synthesis of compound 7 and the procedure used to avoid it.

9 was used in automated oligonucleotide synthesis to produce oligonucleotide 12. Based on the reactions of model compounds (data not shown), it was assumed that the trifluoroacetyl group was removed during the prolonged ammonia treatment (24 hours at 55°C) used during the standard oligonucleotide deprotection procedures.

Cleavage Of ΦX174 Single-Stranded DNA Using Oligonucleotide 12 Purified oligonucleotide 12 was treated with the N-hydroxysuccinimide ester of 3-methylthiopropionic acid for 11 hours at room temperature. The resulting oligonucleotide was annealed to linearized single-stranded ΦX174 DNA 5' end-labelled with ^{32}P as before at the Xho 1 site (see Fig 41). Sodium acetate (pH 5.5) and CNBr were added to final concentrations of 30 mM and 16 mM respectively. The reactions were incubated for 18 hours at room temperature and treated with piperidine as usual. A denaturing 8% polyacrylamide gel revealed specific cleavage at the expected G residue 2 bases to the 5' side of the position of the single residue of **9** (see lane 4 Figure 42). The observed cleavage efficiency was well below that observed using an enzymatically incorporated residue of **2** (see lane 2 Figure 42).

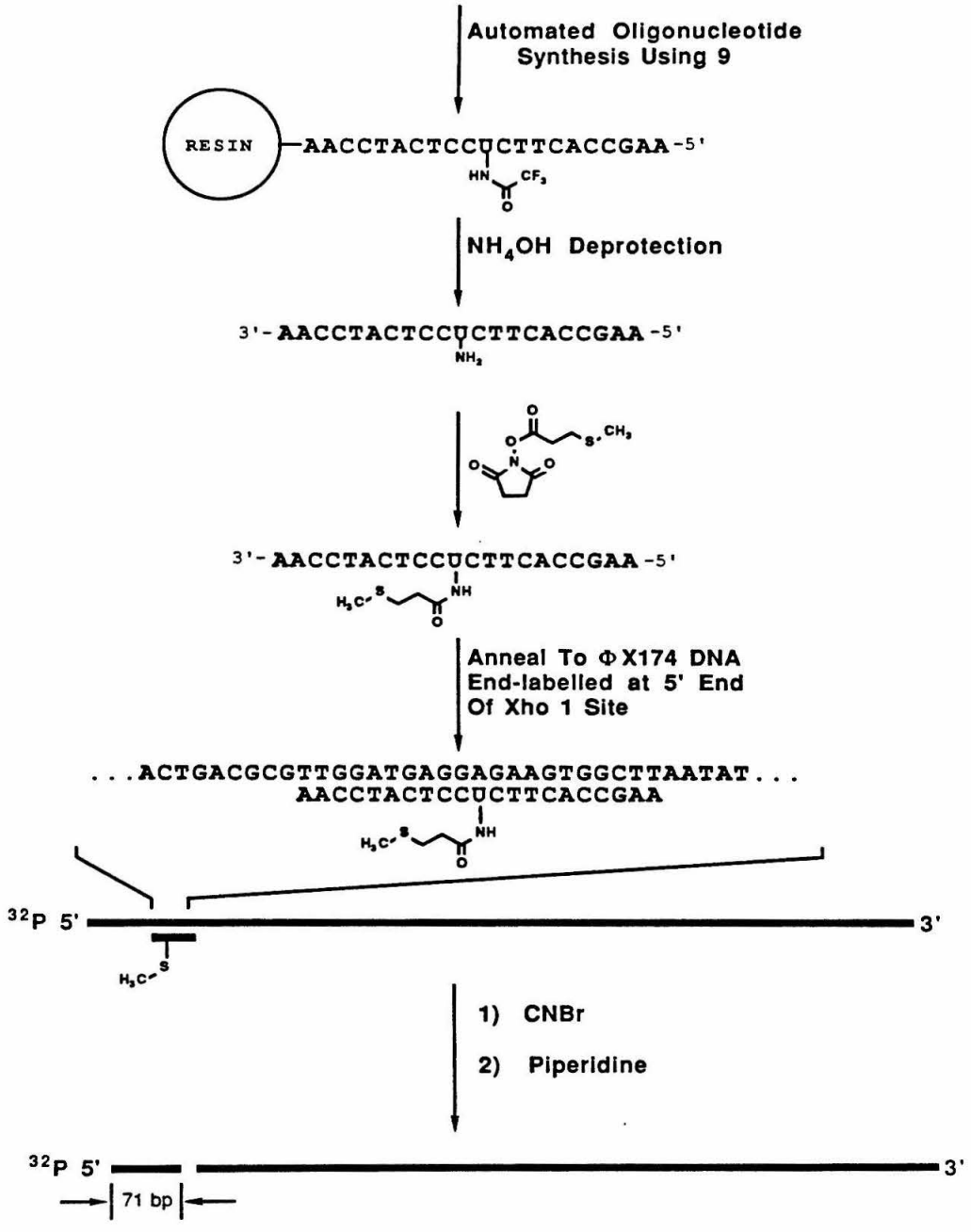
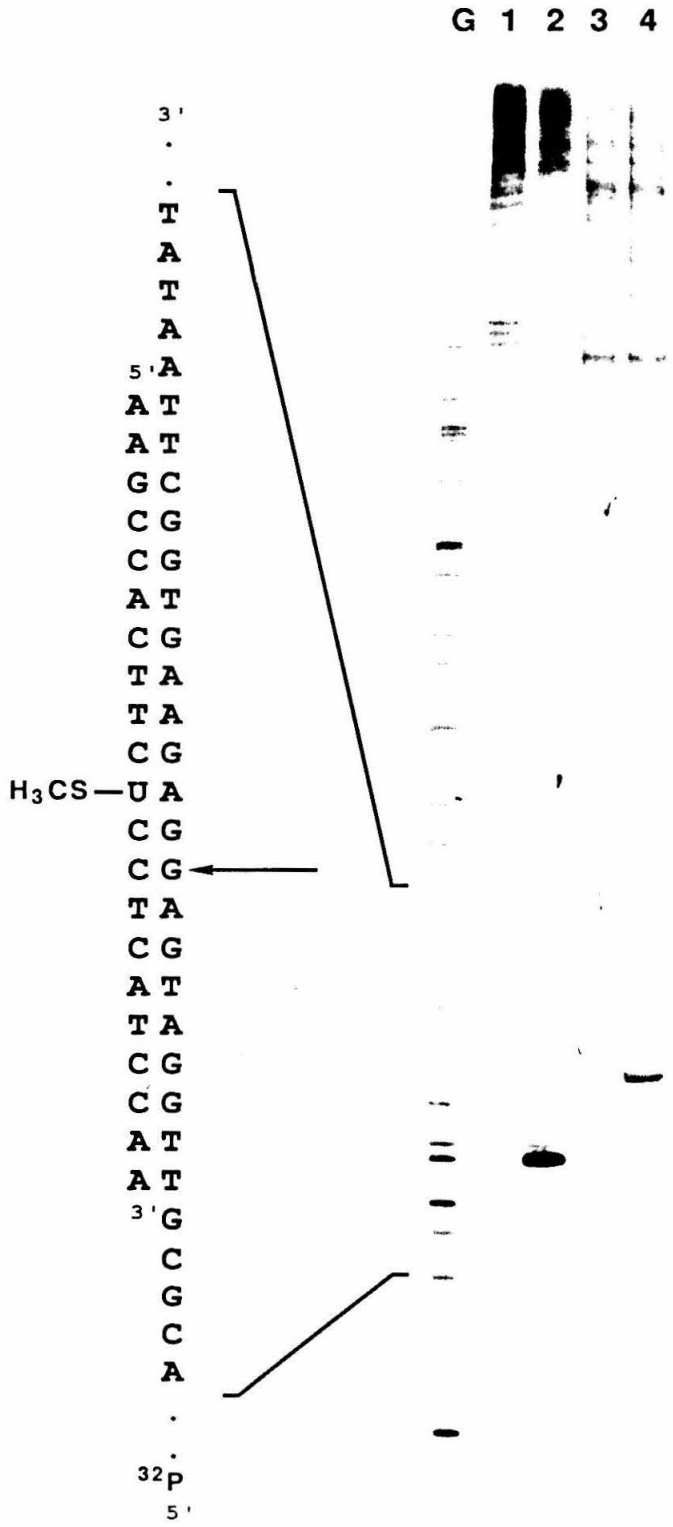


Figure 41 Scheme for the complementary-addressed methylation and cleavage of single-stranded ΦX174 DNA using oligonucleotide 12 post-synthetically modified with methylthioether.

Figure 42

Cleavage of single-stranded ΦX174 DNA using oligonucleotide 12 post-synthetically modified with methylthioether. Autoradiogram of a dried, denaturing 8% polyacrylamide gel. ΦX174 was linearized and labelled with ^{32}P at the unique Xho 1 restriction site as described in the experimental section. Oligonucleotide 12 was treated with the N-hydroxysuccinimide ester of 3-methylthiopropionic acid for 11 h at room temperature. The resulting oligonucleotide (**Lane 4**) or oligonucleotide 11 (**Lane 3**) was annealed to the ΦX174 DNA and NaCl and NaOAc pH 5.5 solutions were added to final concentrations of 5 mM and 25 mM respectively. 1 μg CT DNA was added and the reactions were incubated at room temperature for 18 h with 15 mM CNBr. 2 μl of neat piperidine was then added and the samples were heated at 90°C for 20 min. The samples were lyophilized to dryness, the residue redissolved in formamide loading buffer and .5 mR/h was loaded onto each lane of the gel. **G** Maxam-Gilbert G reaction. **Lane 1** Same as Lane 1 of Figure 38. **Lane 2** Same as Lane 2 of Figure 38. **Lane 3** Oligonucleotide 11 was annealed to the ΦX174 DNA prior to the CNBr reaction. **Lane 4** Oligonucleotide 12 that had reacted with the N-hydroxysuccinimide ester of 3-methylthiopropionic acid was annealed to the ΦX174 DNA prior to the CNBr reaction The histogram shows the position of the residue hopefully modified with methylthioether (H_3CS-U) in relation to the observed guanine cleavage site (arrow).


G 1 2 3 4
3'
T
A
T
A
5' A
A T
A T
G C
C G
C G
A T
C G
T A
T A
C G
H3CS—U A
C G
C G
T A
C G
A T
T A
C G
C G
A T
A T
3' G
C
G
C
A
32P
5'

Attempted Cleavage Of A Double-Stranded Sequence Through Formation Of A Triple-Strand

Oligonucleotide 3 carrying a single residue of **2** on the 3' end was prepared as described in Figure 11 and isolated from a denaturing 20% gel like that in Figure 12.

A 628 base pair restriction fragment was prepared and 5' end-labelled which contained a region known to allow triple-strand formation with oligonucleotides of the same sequence as oligonucleotide 3.[43] It was expected from CPK model building studies that triple-strand formation with the oligonucleotide 3 carrying the residue of **2** on the 3' end would place the methylthioether function in position to alkylate a G residue adjacent to the triple-strand site (see Figure 43). Unfortunately, using various conditions known to promote triple-strand formation[43,77], no specific cleavage was observed at the expected position on the 628 base pair fragment.

Attempted Complementary-Addressed Modification of Nucleic Acids Using 1,10-Phenanthroline Derivatives

In an attempt to increase the yield of a complementary-addressed alkylation reaction, compounds **10** and **12** were synthesized. These 1,10-phenanthroline derivatives could potentially be used for cleavage of DNA through the action of cupric ion and thiol, cobalt(III) and UV light or directed alkylation using a cofactor. It was this last possibility of directed alkylation using a cofactor that especially motivated the synthesis of compounds **10** and **12**.

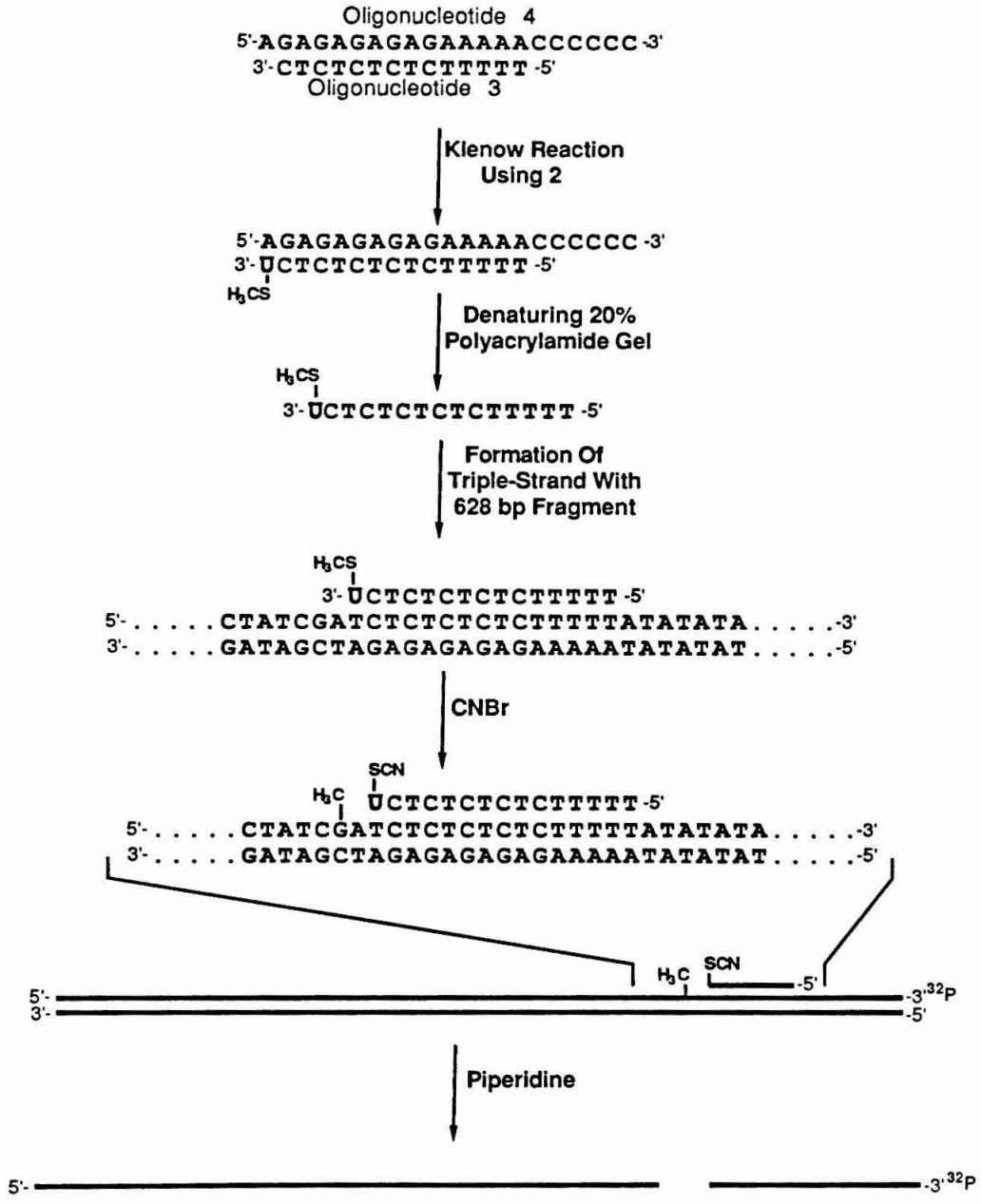


Figure 43 Scheme for the attempted complementary-addressed methylation and cleavage of double-stranded DNA through formation of a triple-stranded structure.

Compound **10** was synthesized by coupling 1,10-phenanthroline-2-carboxylic acid to compound **1** (see Figure 44). As usual, the reaction was monitored using the quantitative ninhydrin reaction. After intitial purification on a DEAE Sephadex column, pure **10** was isolated using ion exchange HPLC.

Compound **12** was prepared from reaction of 5-isothiocyanato-1,10-phenanthroline (compound **11**) with compound **1**. The product was purified by a combination of DEAE Sephadex chromatography and reverse phase HPLC.

Both compounds **10** and **12** were accepted as substrate by the Klenow enzyme judging from gels similar to that shown in Figure 10. As before, the enzyme fill-in reactions were carried out with the oligonucleotide 1,2 duplex, thus the 1,10-phenanthroline group was incorporated into the duplexes near several guanine residues in the major groove of a presumed B form helix.

Copper-Thiol Cleavage Reactions The oligonucleotide 1,2 duplex containing a single enzymatically incorporated residue of **12** was incubated with cupric sulfate (2-10 mM) and 3-thiopropionic acid (1-5 mM). A small but reproducible amount of cleavage on the complementary strand was observed, with the cleavage spread over several residues. This cleavage was consistent with that previously reported for an oligonucleotide carrying a 1,10-phenanthroline group on the 5' end.[39]

Cobalt(III) Photochemistry The oligonucleotide 1,2 duplex containing **12** was incubated with various concentrations of *cis*-β-

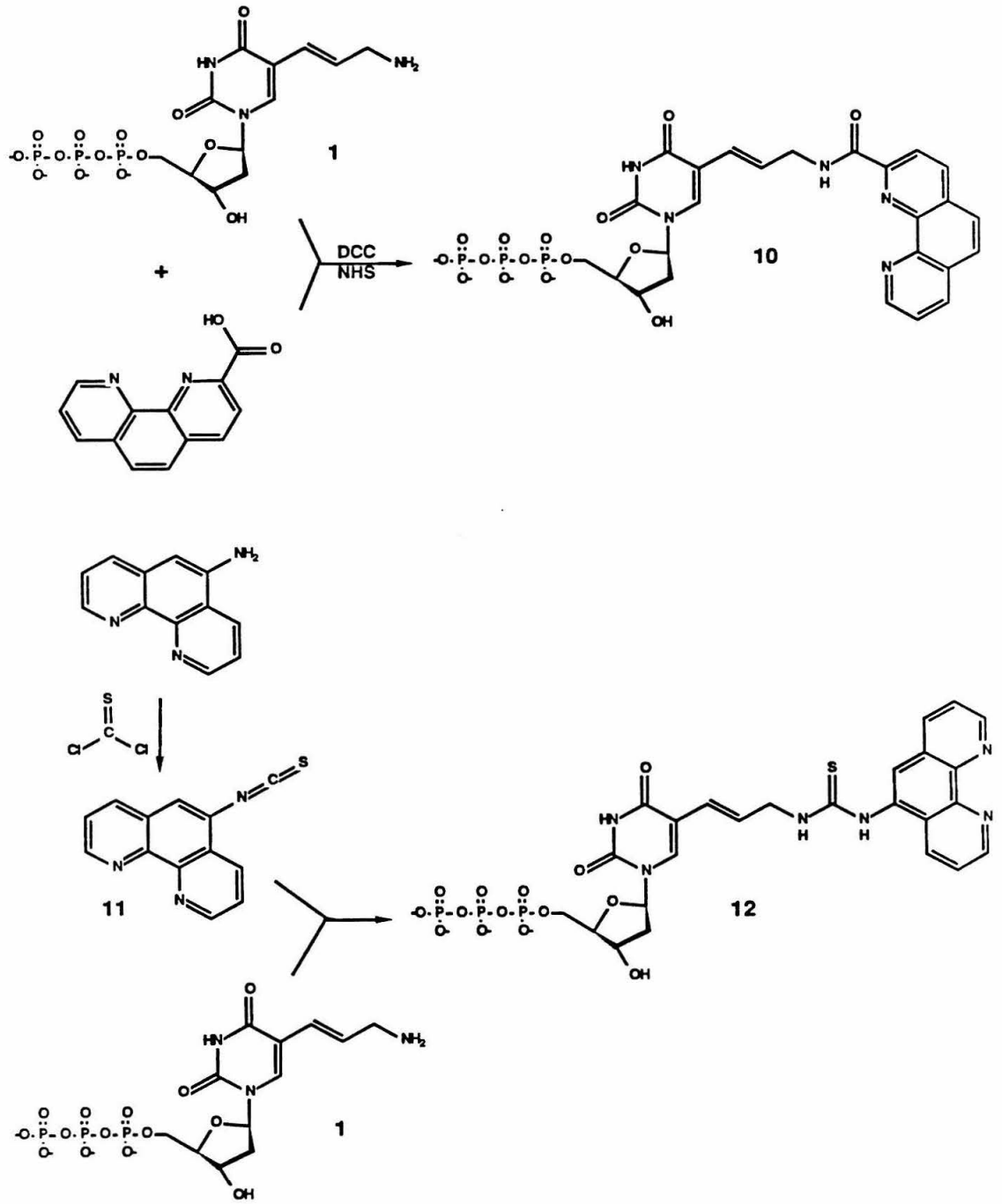


Figure 44 Synthetic schemes for the preparation of compounds **10** and **12**.

Co(III)(trien)(H_2O)$(OH)^{2+}$ in hopes of forming the β-Co(trien) chelate with the residue of **12**. The *cis*-β-Co(III)(trien)(H_2O)(OH) was prepared by stirring β-Co(III)(trien)(carbonate) with 1 N HCL then neutralizing to pH 7 following the cessation of CO_2 evolution.[78] It was anticipated that a compound **12**-β-Co(III)(trien) complex would be capable of site specific cleavage of DNA upon photolysis similar to other Co(III) complexes of 1,10-phenanthroline derivatives reported to cleave DNA in the presence of UV light.[79] Various combinations of incubation and photolysis protocols failed to produce complementary-addressed DNA cleavage reproducibly. However, it was discovered that *cis*-β-Co(III)(trien)(H_2O)(OH) and Co(III)$(NH_3)_6$ by themselves efficiently cleave DNA with "GG" specificity upon photolysis (*vida infra*).

Attempted Directed Alkylation Using A Cofactor The main motivation for synthesizing compounds **10** and **12** was to attempt directed alkylation reactions using cofactors. The proposed scheme involved binding of a four-coordinate metal such as cupric ion to the 1,10-phenanthroline group of **10** or **12** on a hunter strand. Subsequent coordination of an alkylating agent cofactor to the vacant site(s) on the metal atom would hopefully precisely position as well as activate the alkylating agent (see Figure 45). Although not catalytic in a formal sense, the 1,10-phenanthroline-metal complexes should be kinetically labile enough to allow several different alkylating agent molecules to coordinate and thereby potentially react with the target sequence during the course of the reaction.

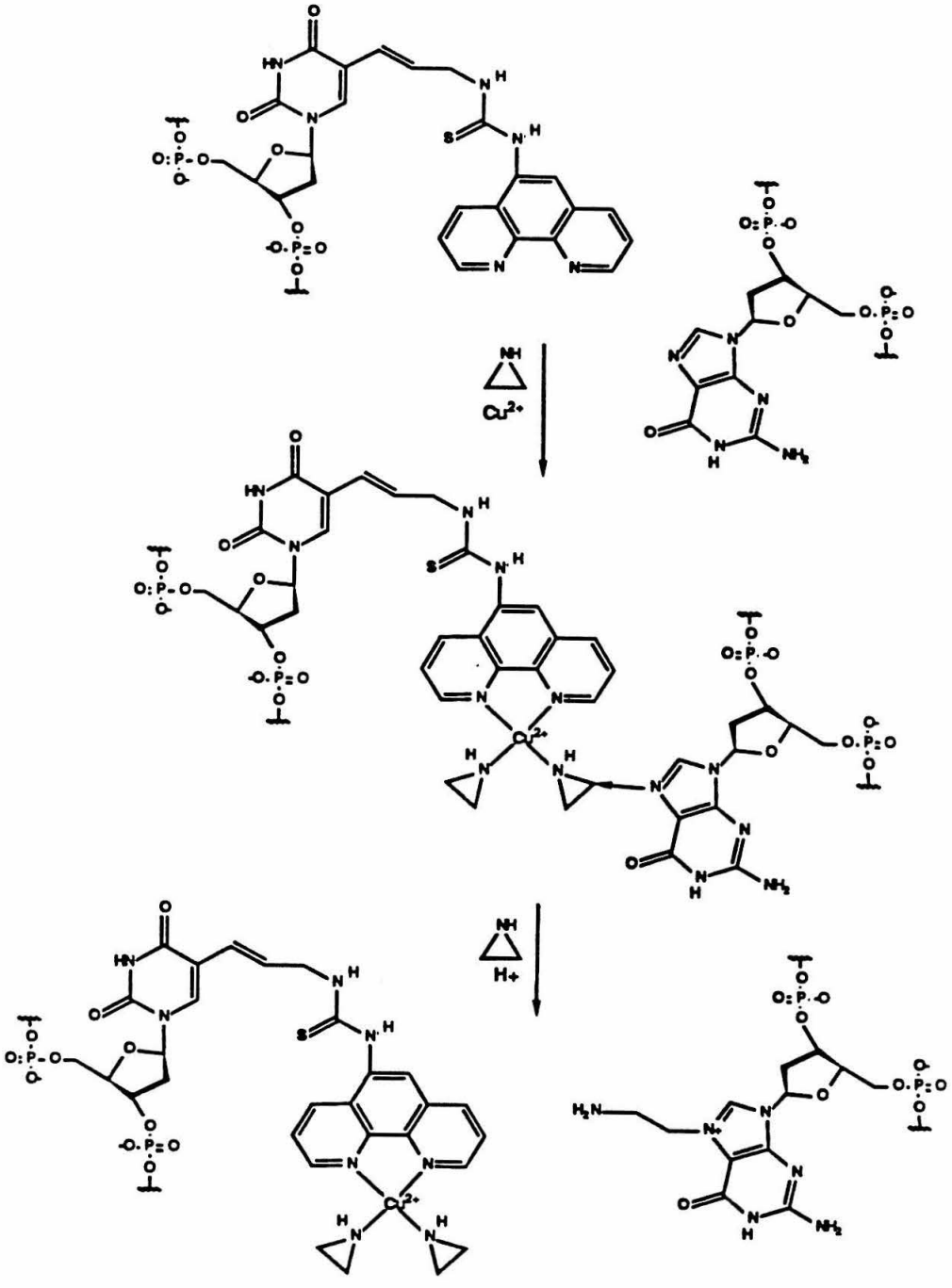


Figure 45 Scheme for the proposed directed alkylation reaction using a cofactor.

This alkylating agent "turnover" should allow for a high alkylation efficiency in spite of a low inherent efficiency of the individual alkylating molecules.

Oligonucleotide duplexes containing an enzymatically incorporated residue of **10** or **12** were used to investigate the directed alkylation reaction using cofactors. Several different metals were tried as well as a number of potentially coordinating alkylating agent cofactors. Aziridine, N-(2-hydroxyethyl)aziridine and ethylene sulfide were all examined for alkylating ability in the reaction. It was hoped that these cofactor alkylating agents would bind to the metal atom (which was coordinated to the 1,10-phenanthroline group of **10** or **12**) and thereby be held in position to be attacked by nucleophilic sites on the target strand. Furthermore, binding to the positively charged metal atom was expected to enhance the electrophilic properties of the alkylating agents so that the metal-alkylating agent coordination could be viewed as an activation event. No combination of metal atom, coordinating alkylating agent cofactor and pH was found to be capable of site-specific alkylation and cleavage of any of the adjacent guanine residues of the oligonucleotide 1,2 duplex with either **10** or **12**.

A different strategy was tried involving methionine and CNBr. Amino acids are known to coordinate metal ions such as cupric ion with high affinity ($K_d = 10^{-12}$ M[80]) so it was hoped that methionine would bind to a metal atom coordinated to a residue of **10** or **12**. Addition of CNBr would then activate the methylthioether group to-

ward alkylation of the target strand. This strategy also failed to produce any site specific alkylation activity with the oligonucleotide 1,2 duplex.

The absence of any observed alkylation in the proposed directed alkylation reaction using **10** or **12** could be explained in a number of ways. The metal atom may not have been complexing the 1,10-phenanthroline group and/or alkylating agent cofactors. 1,10-phenanthroline-metal-DNA phosphate backbone complexation is a likely competing reaction. It is also possible that the side chains of **10** and/or **12** might not have been flexible enough to allow the geometry required for an alkylation reaction.

Despite the disappointing results, this directed alkylation using cofactors could potentially be turned into a powerful method of highly efficient and specific alkylation once proper adjustments in system design have been made.

CONCLUSION

The studies discussed in the preceding section of this thesis have served to illustrate the capabilities of some new strategies for complementary-addressed modification of single-stranded DNA.

First and most important, complementary-addressed methylation and cleavage to nucleotide resolution was accomplished using as the reactive group a methylthioether function activated with CNBr. The specificity inherent in the CNBr activated methylthioether methylation reaction coupled to the specificity of oligonucleotide delivery produced a system capable of cleaving the 5,386 base ΦX174 genome at essentially a single residue!

Cleavage was always observed at a guanine residue 2 bases to the 5' side of the modified residue carrying the methylthioether group. Adjacent guanine residues reacted with an efficiency only 0.08 as high as that of the primary guanine reaction site. A similar reaction at N7 of adenine did not occur. Computer modeling studies explained the observed single-base specificity of the reaction in terms of the distance and angle the reactive side chain is required to span in order to allow reaction. This unprecedented specificity serves to illustrate the possible importance of placing a reactive group in the center of a hunter strand, so that when it is hybridized to a target strand the resulting secondary structure can be exploited to promote a highly specific reaction.

HPLC analysis of the product of the reaction proved that methyl transfer was the predominant pathway of reaction and that N7 of guanine was the site of methylation on the target strand. High

resolution gel electrophoresis showed that a piperidine treatment of the specifically methylated target strand produced oligonucleotide fragments that migrated consistent with having 3' and 5' phosphate termini in analogy to the dimethyl sulfate reaction used for Maxam-Gilbert chemical DNA sequencing.

The enzymatic approach to the incorporation of modified bases carrying latent reactive groups such as methylthioether proved to be a convenient and reliable method of producing hunter strands. Modified 2'-deoxynucleotide 5'-triphosphate molecules carrying methylthioether groups or even functions as large as 1,10-phenanthroline were accepted as substrate by the Klenow enzyme. The modified bases were selectively incorporated into the middle or 3' end of hunter strands opposite A residues on a template (or target) strand. The protocol developed for the enzymatic fill-in reaction of modified bases is similar to (and no more difficult than) the protocols already in routine use for Sanger-sequencing and 3'-end-labelling. This enzymatic approach was proven flexible enough to allow for strategies which produced hunter strands capable of specifically methylating and cleaving single-stranded oligonucleotides as well as very long (5386 bases) pieces of single-stranded DNA.

The absolute efficiency of the complementary-addressed cleavage reaction using a CNBr activated methylthioether function was ~12%. Hopefully, future side chain designs will be able to increase the absolute efficiency of the reaction.

In conclusion, the Klenow enzyme-CNBr-methylthioether system described in this thesis for complementary-addressed methylation and cleavage of single-stranded DNA satisfied the first three of the four criteria mentioned in the introduction for general use as a single-stranded DNA cleavage strategy. These studies should serve as a foundation upon which future work will build a more efficient system capable of programmed specific cleavage of single-stranded DNA.

EXPERIMENTAL PROCEDURES

^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-GX400 FT 400 MHz spectrometer and are reported in parts per million (ppm) from tetramethylsilane. ^{19}F nuclear magnetic resonance spectra were recorded on a JEOL FX 90Q FT spectrometer and are reported in ppm from CCl_3F. Ultraviolet-visible (UV-Vis) spectra were recorded on a Cary 219 Spectrophotometer or a Beckman model 25 Spectrophotometer. Infrared spectra (IR) were recorded on a Shimadzu model IR-435 infrared spectrometer. Mass spectral determinations (high resolution positive ion fast atom bombardment (FAB)) were performed at the Midwest Center for Mass Spectrometry at Lincoln, Nebraska; a National Science Foundation Regional Instrumentation Facility (Grant No. CHE 8211164).

Preparative high pressure liquid chromatography (HPLC) was performed with two Altex 110A pumps, an Altex model 420 controller, a Beckman model 165 variable wavelength two channel detector and two Hewlett-Packard 3390A integrators. Analytical HPLC was performed with a Hewlett-Packard 1090 liquid chromatograph with diode array detection and a Hewlett-Packard 79994A analytical work station with a Colorpro 8 pen plotter. Preparative ion-exchange HPLC was performed on a Synchrom Inc. Synchropak Q300 4.6 mm x 25 cm anion exchange column using an Upchurch Uptight precolumn packed with Q300 material. Preparative reverse phase HPLC was performed on an Altex Ultrasphere ODS (5 micron) C_{18} 4.6 mm x 25

cm column using an Uptight precolumn packed with Beckman ODS pellicular material. Analytical reverse phase HPLC was performed on a Vydac 201HS5415 4.6 mm x 15 cm C_{18} column using no precolumn. Fast protein liquid chromatography (FPLC) of synthetic oligonucleotides was performed with two Pharmacia P-500 pumps, an LCC-500 controller, UV-2 dual path monitor and Frac-100 fraction collector.

Densitometry was performed by an LKB Ultroscan XL laser densitometer and scintillation counting was performed by a Beckman LS 3801 scintillation counter. Lyophilization was performed on a Labconco lyophilizer fitted with a liquid nitrogen trap or a Savant Speedvac Concentrator. 8% polyacrylamide gels were dried on a Bio-Rad model 483 slab dryer and all autoradiography was performed with Kodak X-Omat AR film. Photographs of preparative polyacrylamide gels were taken with Polaroid type 667 film.

Computer modeling studies were carried out using an Evans and Sutherland PS340E/μVax II system using Biograf version 1.32 software. Geometry optimization was performed by running energy minimization using the Biograf Dreiding default parameters.

Polyacrylamide gels were run in 1X TBE electrophoresis buffer (89 mM Tris-borate pH 8.3, 2mM EDTA). The lyophilized samples were redissolved in 1X Formamide loading buffer (80% v:v formamide in water, 50 mM Tris-borate pH 8.3, 1 mM EDTA, .1% w:v bromophenol blue) before loading onto all denaturing polyacrylamide gels and 1/8 volume of 10X glycerol loading buffer (30% v:v

glycerol in water, .1% w:v bromophenol blue) was added to each sample to be loaded onto a non-denaturing polyacrylamide gel.

All chemicals were the best available grade and used without further purification unless stated otherwise. All water was pretreated with an organic removal cartridge (Corning) and doubly distilled. For the reactions involving oligonucleotides or natural DNA; all buffers, reaction vessels and pipette tips (Rainin) were autoclaved just prior to use.

5-(3-amino-*trans*-1-propenyl)-2'-deoxyuridine 5'-triphosphate (1) Prepared essentially according to literature methods.[46] 2'-Deoxyuridine 5'-triphosphate (300 mg, .54 mmol; Sigma) and $Hg(OAc)_2$ (.9 g, 2.7 mmol; Aldrich) were placed in 60 ml .1N NaOAc pH 6.0. and the mixture was stirred under argon at 60°C for four hours. After cooling on ice, LiCl (228 mg, .54 mmol) was added and the solution was extracted 6X 75 ml ethyl acetate to remove excess $HgCl_2$. The 5-mercurinucleotide 5'-triphosphate was precipitated by the addition of 120 ml cold ethanol and the mixture was placed at 4°C overnight. The next morning, the flocculent white precipitate was collected by centrifugation into a pellet. The pellet was washed 2X 50 ml cold ethanol then dried *in vacuo*. The 5-mercurinucleotide 5'-triphosphate was used without further purification. The 5-mercurinucleotide 5'-triphosphate was dissolved in 40 ml .1N NaOAc pH 5.4 and 4.5 ml of freshly prepared 2 M allylammonium acetate pH 5.4 The allylammonium acetate solution was made just prior to use by slowly adding .75 ml redistilled allyl

amine (13.3 M; Aldrich Gold Label) to 4.25 ml ice cold 4.0 M acetic acid. K_2PdCl_4 (140 mg, .43 mmol: Aldrich) was added to the nucleoside-allylammonium acetate solution under argon producing a black solution and black precipitate. After 18 hours at room temperature, the mixture was filtered (45 micron, Millipore) and the filtrate was diluted to 185 ml with water. The solution was loaded onto an 18 x 200 mm chromatography column packed with DEAE-Sephadex A-25-120 (Pharmacia) pre-equilibrated with .05 M triethylammonium bicarbonate buffer (TEAB) pH 7.6. The column was eluted under slight air pressure with 60 ml each of .1 M, .2 M, .3 M, .4 M, .5 M, .6 M, .7 M, .8 M, .9 M, and 1.0 M TEAB pH 7.6. The product **1** was identified as the only ninhydrin positive compound eluting between .4 M and .5 M triethylammonium bicarbonate. The product (188 mg, 42%) was used without further purification. NMR (D_2O) δ 8.05 (1H, s, H_6), 6.42 (1H, d, J=15.9 Hz, -HC=), 6.22 (2H, m, =CH-, H_1'), 4.48 (1H, m, H_3'), 4.15 (2H, m, H_5') 4.02 (1H, m, H_4'), 3.52 (2H, d, J=6 Hz, CH_2), 2.22 (2H, m, H_2').

5-[3-[[3-(Methylthio)propionyl]amino]-*trans*-1-propenyl]-2'-deoxyuridine 5'-triphosphate (2) 3-Methylthio-propionic acid (26.4 mg, .22 mmol; Tokyo Kasei) was placed in 5.0 ml DMF (freshly distilled over CaH at reduced pressure) under argon along with 1,3-dicyclohexylcarbodiimide (DCC) (49 mg, .24 mmol; Aldrich) and N-hydroxysuccinimide (NHS) (38 mg, .32 mmol; Aldrich, recrystallized from ethyl acetate). The solution was stirred at room temperature for 12 h then filtered. To the filtrate was added a solu-

tion of **1** (50 mg, .06 mmol) in 5 ml .1 M sodium borate buffer pH 8.85. Aliquots (40 µl) were periodically removed and subjected to a quantitative ninhydrin test.[69] The reaction was stopped when the ninhydrin test detected no more **1** (3-6 h) as evidenced by an absorbance with λ_{max}= 570 nm. The reaction was diluted with 100 ml water and placed upon an 18 x 200 mm DEAE-Sephadex A-25-120 column pre-equilibrated with .05 M TEAB pH 7.6. The column was eluted under slight air pressure with 40 ml each of .1 M, .2 M, .3 M, .4 M, .5 M, .6 M, .7 M, .8 M, .9 M, 1.0 M and 1.2 M TEAB. The crude product **2** was eluted between .7 M and .8 M TEAB as the last UV absorbing compound and the pooled fractions were lyophilized to dryness. The crude **2** was redissolved in water and purified by preparative ion exchange HPLC on a Synchropak Q300 anion exchange column monitored at 260 and 290 nm. Compound **2** was the major component eluted with a 30 minute linear gradient of 0-0.3 M ammonium bicarbonate (AMB) pH 7.6 and a flow rate of .75 ml/min (see Figure 7). To avoid bubbles forming in the HPLC pump piston chambers, the AMB buffer was chilled in ice during the chromatography. The appropriate fractions were pooled and lyophilized to dryness to yield the ammonium salt of pure **2** (23 mg, 55%). NMR (D_2O) δ 7.8 (1H, s, H_6), 6.28-6.35 (1H, m, =CH-), 6.18-6.23 (2H, m, -HC=, H_1'), 4.53-4.55 (1H, m, H_3'), 4.08-4.12 (3H, m, H_5', H_4'), 3.8 (2H, d, J=5 Hz, CH_2), 2.66 (2H, t, J=7 Hz, -CH_2-S), 2.49 (2H, t, J=7 Hz, CH_2), 2.25-2.28 (2H, m, H_2') 1.99 (3H, s, S-CH_3). UV-Vis (H_2O) λ_{max} = 239 nm (ε= 10,600), λ_{min}= 267 nm (ε= 4100), λ_{max}= 289 nm (ε= 7000).

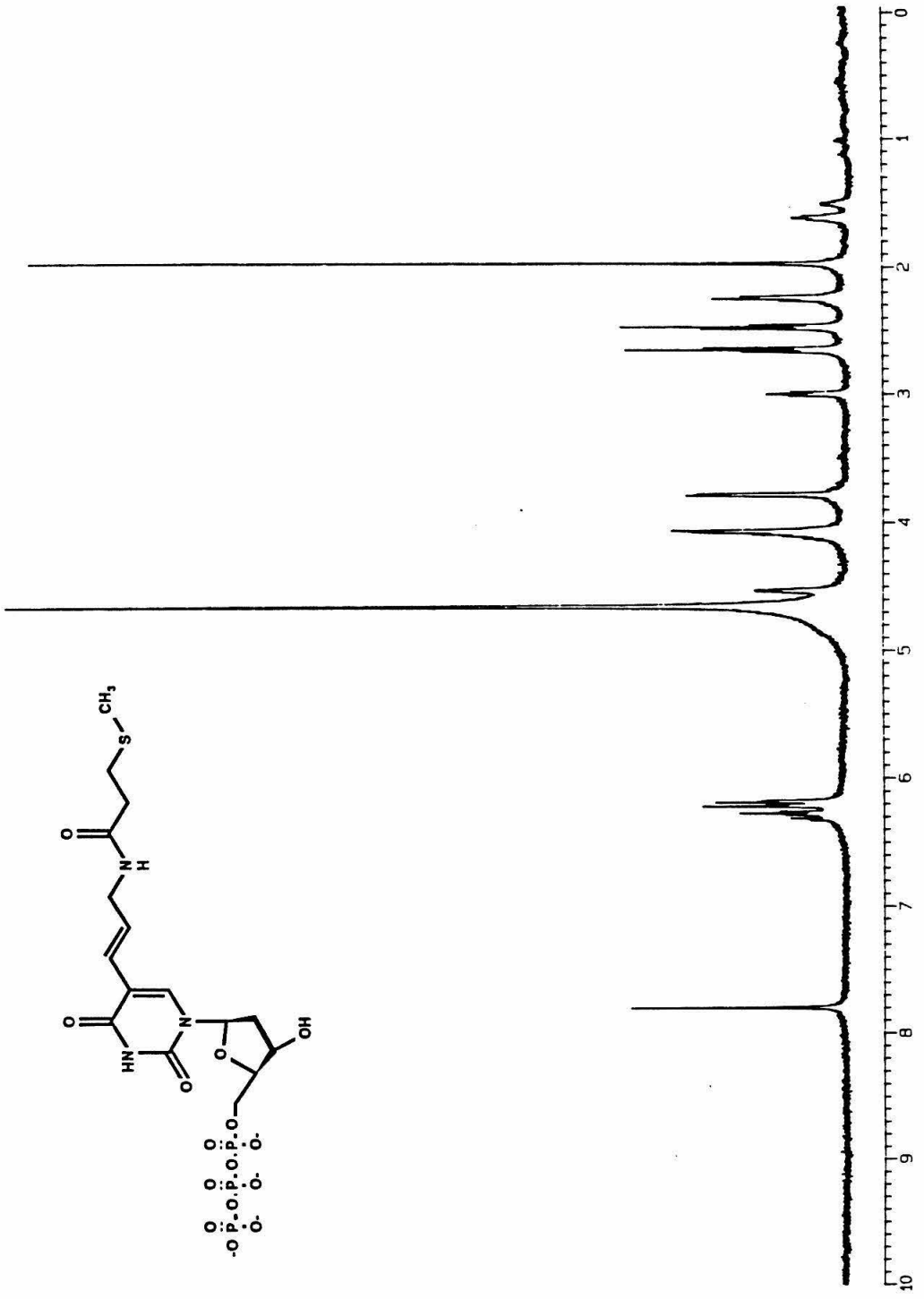


Figure 46 400 MHz ^{1}H NMR spectrum of compound **2** in D_2O.

IR (KBr) 1650, 1260, 1090, 1040, 800.

5-[3-[[3-(Methylthio)propionyl]amino]-*trans*-1-propenyl]-2'-deoxyuridine (3) **2** (4 mg, 6 μmol) was precipitated from 50 μl .4 M NaOAc pH 8.0 by the addition of 150 μl ethanol. The precipitate was collected by centrifugation and redissolved in 135 μl water. 15 μl 10X CAP buffer (.5 M Tris-HCl pH 9.0, 10 mM $MgCl_2$, 1 mM $ZnCl_2$) and 15 μl calf alkaline phosphatase (CAP) (10 units/μl, Boehringer Mannheim Biochemicals) were added. The reaction was incubated at 37°C for 3 h. The product was isolated by preparative reverse phase HPLC as the major component absorbing at 290 nm when eluted with a 30 minute linear gradient of 0-15% acetonitrile in water. The appropriate fractions were pooled and lyophilized to yield pure **3**. NMR (D_2O) δ 7.74 (1H, s, H_6), 6.12-6.17 (3H, m, -HC=, =CH-, H_1'), 4.32 (1H, m, H_3'), 3.88 (1H, m, H_4'), 3.77 (2H, d, J=3 Hz, CH_2), 3.63-3.69 (2H, m, H_5'), 2.66 (2H, t, J= 6.8 Hz, -CH_2-S), 2.46 (2H, t, J= 6.8 Hz, CH_2), 2.25 (2H, m, H_2'), 1.97 (3H, s, S-CH_3). MS (high resolution positive ion FAB) calculated for $C_{16}\ H_{23}\ N_3\ O_6\ S$ m/z= 385.1309; found M+H= 386.138586.

5-[3-[[2-(Methylthio)acetyl]amino]-*trans*-1-propenyl]-2'-deoxyuridine 5'-triphosphate (4) 2-Methylthioacetic acid (12.7 mg, .12 mmol; Fluka) was placed in 5.0 ml DMF (freshly distilled over CaH at reduced pressure) under argon along with DCC (25 mg, .13 mmol) and NHS (19 mg, .16 mmol, recrystallized from ethyl acetate). The solution was stirred at room temperature for 12 h then filtered. To the filtrate was added a solution of **1** (50 mg, .06

mmol) in 5 ml .1 M sodium borate buffer pH 8.85. Aliquots (40 µl) were periodically removed and subjected to the quantitative ninhydrin test. The reaction was stopped after 3.5 h since there was no more detectable **1**. The reaction was diluted with 100 ml water and placed upon an 18 x 160 mm DEAE-Sephadex A-25-120 column pre-equilibrated with .05 M TEAB pH 7.6. The column was eluted under slight air pressure with 40 ml each of .1 M, .2 M, .3 M, .4 M, .5 M, .6 M, .7 M, .8 M, .9 M, 1.0 M and 1.2 M TEAB. The crude product **4** was eluted between .7 M and .8 M TEAB as the last UV absorbing compound and the pooled fractions were lyophilized to dryness. The crude **4** was redissolved in water and purified by preparative ion exchange HPLC on a Synchropak Q300 column monitored at 260 nm and 290 nm. Compound **4** was the major component eluted with a 30 minute linear gradient of 0-0.3 M AMB pH 7.6 and a flow rate of .75 ml/min. To avoid bubbles forming in the HPLC pump piston chambers, the AMB buffer was chilled in ice during the chromatography. The appropriate fractions were pooled and lyophilized to dryness to yield the ammonium salt of pure **4**. NMR (D_2O) δ 7.82 (1H, s, H_6), 6.29-6.38 (1H, m, =CH), 6.18-6.27 (2H, m, -HC=, H_1'), 4.53-4.56 (1H, m, H_3'), 4.04-4.18 (3H, m, H_5', H_4'), 3.82 (2H, d, CH_2), 3.16 (2H, s, $-CH_2-S$), 2.23-2.26 (2H, m, H_2') 1.99 (3H, s, $S-CH_3$).

5-[3-[[4-(Methylthio)butanyl]amino]-*trans*-1-propenyl]-2'-deoxyuridine 5'-triphosphate (5) 4-Bromobutyric acid (500 mg, 3 mmol; Aldrich) was dissolved in 20 ml dry

ether and cooled to 0°C. Diazomethane (produced from Diazald and the Aldrich diazomethane kit) was added dropwise until the yellow color of the diazomethane persisted. The solvent was removed to yield pure methyl-4-bromobutyrate. NMR ($CDCl_3$) δ 3.68 (3H, s, CH_3), 3.47 (2H, t, J= 6.7 Hz, CH_2), 2.52 (2H, t, J= 7.1 Hz, CH_2), 2.18 (2H, m, -CH_2-). Methyl-4-bromobutyrate (250 mg, 1.4 mmol) and sodium thiomethoxide (98.1 mg, 1.4 mmol; Fluka) were placed in 30 ml dry acetonitrile under argon. The mixture was stirred for 2 h then filtered and the solvent was removed *in vacuo*. The product was purified by flash chromatography on a 3 cm x 18 cm silica gel column eluted with 35% hexane in chloroform to yield pure methyl-4-(methylthio)butyrate (195 mg, 94%). Thin layer chromatography (TLC) (40% hexane in chloroform) R_f= .25 visualized as a yellow spot by $KMnO_4$. NMR ($CDCl_3$) δ 3.68 (3H, s, CH_3), 2.54 (2H, t, J= 7 Hz, CH_2), 2.45 (2H, t, J= 7.3 Hz, CH_2), 2.09 (3H, s, S-CH_3), 1.93 (2H, m, -CH_2-). Methyl-4-(methylthio)butyrate (70 mg, .47 mmol) was placed in 5 ml water, 1 ml methanol and .70 ml 10 N HCl. The reaction was heated at 60°C under argon for 36 h. The methanol was removed *in vacuo*, the solution was extracted 4X 10 ml chloroform, the organic layer was dried over sodium sulfate and the solvent was removed *in vacuo* to yield pure 4-(methylthio)butyric acid. NMR ($CDCl_3$) δ 2.56 (2H, t, J= 6.8 Hz, CH_2), 2.51 (2H, t, J= 7.2 Hz, CH_2), 2.09 (3H, s, S-CH_3), 1.94 (2H, m, -CH_2-). 4-(Methylthio)butyric acid (40 mg, .3 mmol) was placed in 5.0 ml DMF (freshly distilled over CaH at reduced pressure) under argon along with DCC (62 mg, .3 mmol) and NHS (40 mg,

.35 mmol, recrystallized from ethyl acetate). The solution was stirred at room temperature for 12 h then filtered. To the filtrate was added a solution of **1** (50 mg, .06 mmol) in 5 ml .1 M sodium borate buffer pH 8.85. Aliquots (40 µl) were periodically removed and subjected to the quantitative ninhydrin test. The reaction was stopped after 3.5 h since there was no more detectable **1**. The reaction was diluted with 100 ml water and placed upon an 18 x 160 mm DEAE-Sephadex A-25-120 column pre-equilibrated with .05 M TEAB pH 7.6. The column was eluted under slight air pressure with 40 ml each of .1 M, .2 M, .3 M, .4 M, .5 M, .6 M, .7 M, .8 M, .9 M, 1.0 M and 1.2 M TEAB. The crude product **5** was eluted between .8 M and .9 M TEAB as the last UV absorbing compound and the pooled fractions were lyophilized to dryness. The crude **5** was redissolved in water and purified by preparative ion exchange HPLC on a Synchropak Q300 column monitored at 260 nm and 290 nm. Compound **5** was the major component eluted with a 30 minute linear gradient of 0-0.3 M AMB pH 7.6 and a flow rate of .75 ml/min. To avoid bubbles forming in the HPLC pump piston chambers, the AMB buffer was chilled in ice during the chromatography. The appropriate fractions were pooled and lyophilized to dryness to yield the ammonium salt of pure **5**. NMR (D_2O) δ 7.81 (1H, s, H_6), 6.29-6.34 (1H, m, =CH-), 6.16-6.22 (2H, m, -HC=, H_1'), 4.55-4.57 (1H, m, H_3'), 4.08-4.14 (3H, m, H_5', H_4'), 3.79 (2H, d, CH_2), 2.42 (2H, t, CH_2), 2.23-2.26 (4H, m, H_2', CH_2) 1.96 (3H, s, S-CH_3), 1.77 (2H, m, -CH_2-).

5-mercuri-2'-deoxyuridine (6) The 5-mercurinucleoside was prepared according to literature methods.[76] 2'-Deoxyuridine (2.28 g, 10 mmol; Sigma) was placed in 13 ml water along with $Hg(OAc)_2$ (3.38 g, 10.6 mmol). The solution was stirred at 50°C for 16 h then NaCl (1.5 g, 25 mmol) in 10 ml water was added and the water was removed *in vacuo*. The flocculent white powder was rinsed 5X methanol, 1X ether then dried *in vacuo* to yield the 5-mercurinucleoside **6** (4.083 g, 92%) which was used without further purification.

5-[3-[(trifluoroacetyl)amino]-*trans*-1-propenyl]-2'-deoxyuridine (7) Allylamine (3.0 ml, 40 mmol) and trifluoroacetic anhydride (7.5 ml, 53 mmol; Aldrich) were placed in 40 ml dry ether at 0°C under argon. After stirring for 1 h, 10 ml water and 50 ml ether were added. The organic layer was washed with saturated sodium bicarbonate solution until a neutral pH was obtained. The aqueous layers were combined and the solvent was removed *in vacuo*. The pure N-trifluoroacetyl allylamine (5.76 g, 95%) was obtained by distillation at reduced pressure. N-trifluoroacetyl allylamine (1 g, 6.75 mmol) and **6** (500 mg, 1.08 mmol) were placed in 10 ml water and 10 ml isopropanol. K_2PdCl_4 (355 mg, 1.1 mmol) was added and the mixture was stirred for three days at room temperature. The solution was dried *in vacuo* then chromatographed on a 3.5 cm x 20 cm silica column eluted with 15% methanol in methylene chloride. The pooled fractions containing the crude product were dried *in vacuo* then the white brittle foam was redissolved in 12 ml ethyl acetate

and crystallized by vapor diffusion with hexane for five days at -20°C. White crystals of pure **7** (103 mg, 25%) were obtained. Melting point 167-169°C. TLC (15% methanol in methylene chloride) R_f= .45 visualized with short wave UV. ^1H NMR (d_6-DMSO) δ 11.45 (1H, s, N_3), 9.67 (1H, t, NH), 8.06 (1H, s, C_6), 6.43- 6.50 (1H, m, =CH-), 6.14-6.22 (2H, m, H_1', -HC=), 5.24 (1H, d, J= 4.3 Hz, OH_3'), 5.11 (1H, t, J= 5.5 Hz, OH $_5$'), 4.23-4.26 (1H, m, H_3'), 3.88 (2H, t, J= 5.5 Hz, H_5'), 3.79 (1H, m, H_4'), 3.54-3.65 (2H, m, CH_2), 2.08-2.18 (2H, m, H_2'). MS (high resolution positive ion FAB) calculated for $C_{14}H_{16}N_3O_6F_3$ m/z= 379.0992, found M+H 380.106952.

5-[3-[(trifluoroacetyl)amino]-*trans*-1-propenyl]-2'-deoxyuridine 5'-[4,4'-(dimethoxy)triphenylmethyl] (8) **7** (227 mg, .64 mmol) was placed in 1.0 ml pyridine (freshly distilled over CaH). 4,4'-dimethoxytriphenylmethyl chloride (238 mg, .70 mmol; Aldrich) was added and after 5 h methanol (.5 ml) was added. After 30 min the mixture was concentrated to a gum *in vacuo* and redissolved in methylene chloride. Pure **8** (245 mg, 56%) was obtained as a white brittle foam after chromatography on a silica column eluted with 4% methanol in methylene chloride. TLC (8% methanol in methylene chloride) R_f= .4 visualized by short wave UV. ^1H NMR ($CDCl_3$) δ 10.05 (1H, s, N_3), 7.83 (1H, s, C_6), 6.87-7.41 (13H, m, phenyl) 6.42 (1H, t, H_1'), 6.30 (1H, m, =CH-), 5.38 (1H, d, J= 15 Hz, -HC=), 4.57-4.63 (1H, m, H_3'), 4.09 (1H, m, H_4'), 3.75 (6H, s, OCH_3), 3.30- 3.52 (4H, t, m, H_5', CH_2), 2.30-2.55 (2H, m, H_2'). ^{19}F NMR ($CDCl_3$) δ 75.72 (s, CF_3). MS (high resolution positive ion FAB)

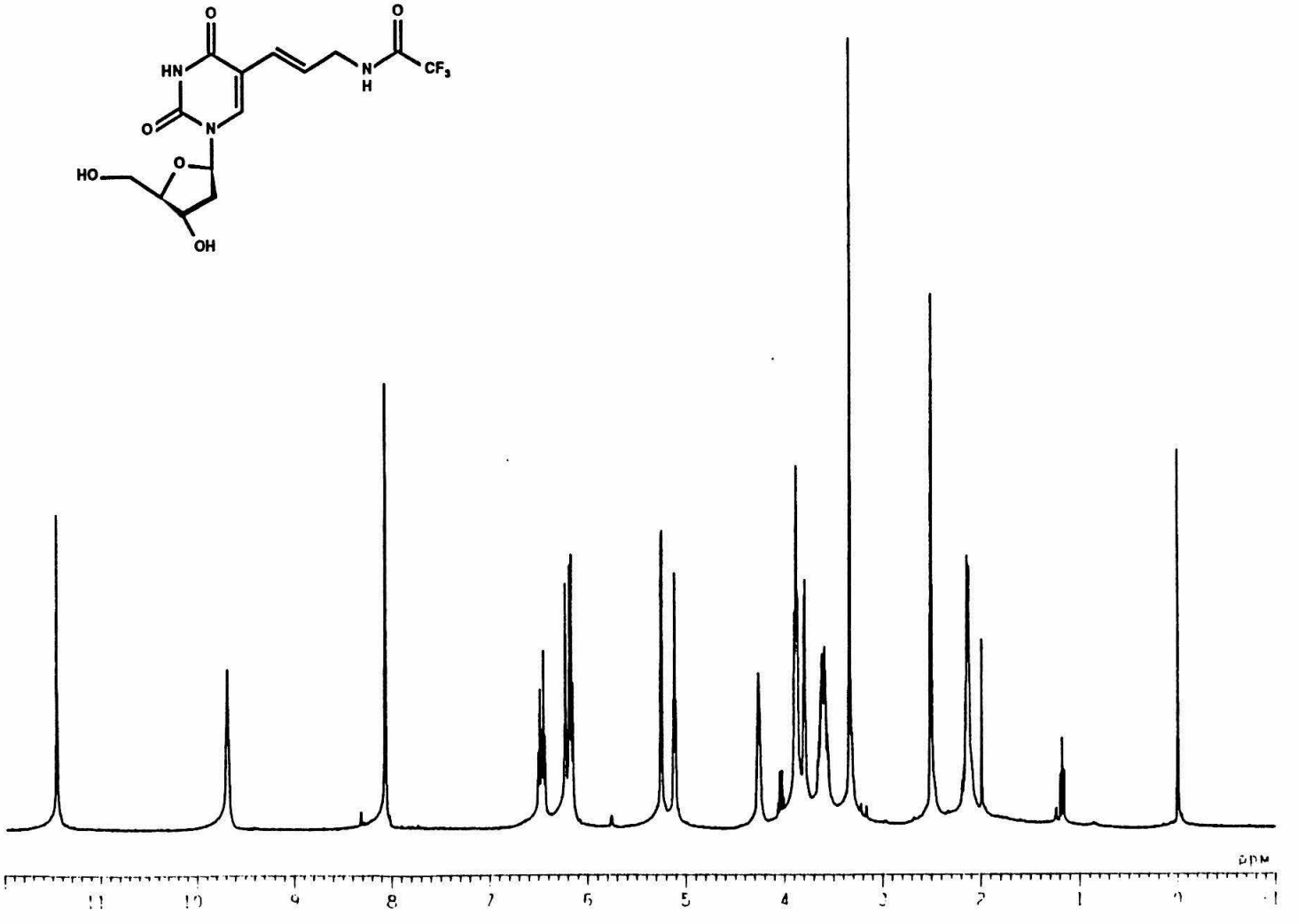


Figure 47 400 MHz ^{1}H NMR spectrum of compound **7** in d_6-DMSO.

calculated for $C_{35}H_{34}N_3O_8F_3$ m/z= 681.2299, found M+Li= 688.245919.

5-[3-[(trifluoroacetyl)amino]-*trans*-1-propenyl]-2'-deoxyuridine 5'-[4,4'-(dimethoxy)triphenylmethyl] 3'-N,N-diisopropylmethoxyphosphoramidite (9) **8** (242 mg, .35 mmol) was placed in 7.75 ml methylene chloride (freshly distilled over CaH) along with diisopropylethylamine (187 µl, 1.07 mmol). N,N-Diisopropylmethylphosphonamidic chloride (140 µl, .7 mmol; Aldrich) was added and the reaction was stirred for 2.5 h under argon. The reaction was quenched with 3.5 ml dry methanol then 20 min later 7 ml ethyl acetate was added and the solution was washed 2X 10 ml saturated sodium bicarbonate and 1X 10 ml saturated NaCl. The organic layer was dried over sodium sulfate and placed on high vacuum overnight. The resulting white brittle foam was placed in 3.5 ml dry acetonitrile and used for automated oligonucleotide synthesis using the standard reaction program. TLC (5% methanol, 1% triethylamine, 94% methylene chloride) R_f= .2 visualized by short wave UV.

5-[3-[[2-carboxy-1,10-phenanthroline]amino]-*trans*-1-propenyl]-2'-deoxyuridine 5'-triphosphate (10) 2-carboxy-1,10-phenanthroline (67.2 mg, .3 mmol; prepared by Rick Ikeda), DCC (61.8 mg, .3 mmol) and NHS (40 mg, .35 mmol) were stirred in 23 ml DMF under argon overnight at room temperature. The next morning, **1** (50 mg, .06 mmol) dissolved in 5 ml .1 M sodium borate buffer pH 8.85 was added to the mixture and the reaction was monitored with the quantitative ninhydrin test as usual. After three hours the

reaction was complete so the mixture was diluted with 100 ml water and placed upon an 18 x 200 mm DEAE-Sephadex A-25-120 column pre-equilibrated with .05 M TEAB pH 7.6. The column was eluted under slight air pressure with 40 ml each of .1 M, .2 M, .3 M, .4 M, .5 M, .6 M, .7 M, .8 M, .9 M, 1.0 M and 1.2 M TEAB. The crude product **10** was eluted between 1.0 M and 1.2 M TEAB as the last UV absorbing compound and the pooled fractions were lyophilized to dryness. The crude **10** was redissolved in water and purified by preparative ion exchange HPLC on a Synchropak Q300 anion exchange column monitored at 260 and 290 nm. Compound **10** was the major component eluted with a 30 minute linear gradient of 0-0.3 M ammonium bicarbonate (AMB) pH 7.6 and a flow rate of .75 ml/min. To avoid bubbles forming in the HPLC pump piston chambers, the AMB buffer was chilled in ice during the chromatography. The appropriate fractions were pooled and lyophilized to dryness to yield the ammonium salt of pure **10**. NMR (D_2O) δ 8.98 (1H, s, Ar), 8.43 (1H, d, J=8.2 Hz, Ar), 8.38 (1H, d, J=8.5 Hz, Ar), 8.17 (1H, d, J=8.2 Hz, Ar), 7.8 (4H, m, H_6, Ar), 6.58 (1H, m, =CH-), 6.38 (1H, d, J=16 Hz, -HC=), 6.18 (1H, t, J=6.9 Hz, H_1'), 4.53-4.58 (1H, m, H_3'), 4.15-4.19 (5H, m, H_4', H_5', $-CH_2-$), 2.25-2.28 (1H, m, H_2'). UV-vis (H2O) λ_{max} = 232, λ_{min} = 257, λ_{max} = 279. IR (KBr) 3400, 1700, 1380, 1260, 1100, 800.

5-[3-[[5-thiouryl-1,10-phenanthroline]amino-*trans*-1-propenyl]-2'-deoxyuridine 5'-triphosphate (12) 5-amino-phenanthroline (100 mg, .52 mmol, Polysciences, Inc.) and calcium

carbonate (68 mg, .68 mmol) were placed in 10 ml acetonitrile and heated to 60°C. Thiophosgene (50 µl, .63 mmol, Aldrich) was added and the solution was stirred under argon for 4 h. The solvent was removed *in vacuo* then the residue was redissolved in 4 ml DMF and filtered. To the filtrate was added 150 ml ether and the precipitated **11** (69 mg, 56%) was isolated by centrifugation. NMR (d_6-DMSO) δ 9.37 (1H, s), 9.29 (1H, s), 8.94 (1H, d, J=7.7 Hz), 8.87 (1H, d, J=8.0 Hz), 8.53 (1H, s) 8.19 (2H, m). IR (KBr) 3400, 2800, 2080, 1725, 1590, 1530, 1495, 1455, 1385, 1230, 900, 720. MS (HREI) calculated for $C_{13}H_7N_3S$ m/z = 237.03621, found m/z = 237.0359. **11** (45 mg, .19 mmol) and **1** (50 mg, .06 mmol) were dissolved in 7 ml DMF and 5 ml .1 M sodium borate buffer pH 8.85 and the solution was heated to 60°C. The reaction was monitored with the quantitative ninhydrin test as usual. After 30 min the reaction was complete so the mixture was diluted with 100 ml water and placed upon an 18 x 200 mm DEAE-Sephadex A-25-120 column pre-equilibrated with .05 M TEAB pH 7.6. The column was eluted under slight air pressure with 40 ml each of .1 M, .2 M, .3 M, .4 M, .5 M, .6 M, .7 M, .8 M, .9 M, 1.0 M and 1.2 M TEAB. The crude product **12** was eluted between 1.1 M and 1.2 M TEAB as the last UV absorbing compound and the pooled fractions were lyophilized to dryness. The crude **12** was redissolved in water and purified by reverse phase HPLC using an Altex Ultrasphere ODS column eluted with a 20 min linear gradient of 0-20% acetonitrile in 8 mM ammonium bicarbonate pH 7.8. The appropriate fractions were pooled and lyophilized to yield the

ammonium salt of pure **12**. NMR (D_2O) δ 9.05 (2H, m, Ar), 8.7 (2H, m, Ar), 8.05 (1H, s, Ar), 7.85-8.00 (3H, m, H_6, Ar), 6.4 (1H, m, =CH-), 6.2-6.3 (2H, m, -HC=, H_1'), 4.53-4.58 (1H, m, H_3'), 4.2 (2H, m, $-CH_2-$), 4.1 (2H, m, H_4', H_5'), 2.25-2.28 (1H, m, H_2') IR (KBr) 3200, 1690, 1540, 1460, 1420, 1235, 1070, 900, 800, 530.

Automated Oligonucleotide Synthesis Oligonucleotides (1 μmol scale) were synthesized by solid phase methods on a Beckman System 1 Plus DNA Synthesizer using the standard reaction program, phosphoramidite bases and all other reagents supplied by the manufacturer. The synthesis was monitored by following the amount of dimethoxytrityl group released after each deprotection cycle. Average yields per coupling exceeded 98%.

The oligonucleotides were removed from the resin deprotected using the manufacturer's protocol. Following the synthesis, the reaction column was dried *in vacuo* then the resin was removed from the column and placed in a 4 ml vial containing 1.5 ml 1:2:2 thiophenol:triethylamine:dioxane or the Beckman demethylation reagent. After 24 h at room temperature, the liquid was carefully removed and the resin was washed 3X 1.5 ml methanol and 3X 1.5 ml ether. The resin was dried *in vacuo* then 1.5 ml concentrated ammonia was added. After 2 h at room temperature, the ammonia solution was removed (not discarded) and another 1.5 ml concentrated ammonia was added. After 2 h more at room temperature, this second ammonia solution was removed from the resin and placed with the first. The combined solutions were dried under a

stream of argon and the residue was redissolved in 1.5 ml concentrated ammonia. The 4 ml vial was sealed and the solution was heated at 55°C for 24 h. The liquid was then dried by an argon stream and the residue was redissolved in 1.5 ml water and extracted 3X 1 ml ether. The aqueous layer was concentrated on the Speedvac in preparation for purification.

Purification And Isolation Of Synthetic Oligonucleotides

.25 μmole of oligonucleotide per injection was purified by FPLC on a Mono-Q 5 mm x 10 cm column eluted at .5 ml per minute with a 90 minute linear gradient of 10-90% solvent B in solvent A (A= 50 mM Tris-HCl pH 7.0, 20% Acetonitrile; B= 1.0 M KCl, 50 mM Tris-HCl pH 7.0, 20% Acetonitrile).

Alternatively, .25 mmole of deprotected oligonucleotide was purified by loading (in formamide loading buffer) onto two lanes of a 2 mm x 20 cm x 38 cm 15% polyacrylamide gel (1:20 crosslinked, 42% urea). The gel was run at 750 volts for 6-12 h. The oligonucleotide bands were visualized with a hand held UV light (short wave) and the desired band excised from the gel. The gel slice was crushed then placed in the minimum amount of .2 M NaCl and eluted overnight at room temperature. The mixture was filtered and the supernatant containing the oligonucleotide was collected.

In either case (FPLC or gel isolation), the purified oligonucleotide was dialyzed at 4°C in Spectropore 7, 7.6 mm diameter 2000 molecular weight cutoff (MWCO) dialysis tubing (Spectrum Medical Industries) against 3X 4000 ml of .25 mM NaPhosphate pH 7.6, .02

mM EDTA. The concentrations of the purified oligonucleotides were determined by measuring their UV absorbance at 260 nm in a 1 cm path length cell and using the equation:

$$[\text{Oligonucleotide}] = \frac{\text{Absorbance at 260 nm}}{(10{,}000)(\text{number of bases in oligonucleotide})}$$

Oligonucleotides were checked for purity by determining their UV absorbance at 280 nm and were only used if the $A_{260}/A_{280} > 1.8$.[4]

5'-End-labelling Of Oligonucleotides Purified oligonucleotide (.30 nmol) was placed in 34 µl water and 5 µl 10X kinase buffer (.7 M Tris-HCl pH 7.6, .1 M $MgCl_2$). 5 µl of 50 mM dithiothreitol (DTT) was added followed by 3 µl of γ-^{32}P-adenosine 5'-triphosphate (γ-^{32}P-ATP) (> 7,000 Ci./mmol, New England Nuclear) and 2 µl of polynucleotide kinase (10 units/µl, New England Biolabs). The reaction was incubated for 45 min at 37°C, then another 2 µl of the polynucleotide kinase was added. After another 45 min at 37°C, the reaction was ethanol precipitated by adding 5 µl (10% volume) of 4 M sodium acetate then 150 µl (3X volume) ethanol. The reaction was chilled in dry ice for 5 min then spun in a mini-centrifuge (Eppendorf model 5412, Brinkman) at 12,000 revolutions per min for 8 min at 4°C. The supernatant was removed and the resulting radioactive pellet was washed with 50 µl 70% aqueous ethanol then dried briefly (2 min) *in vacuo*.

Annealing of Oligonucleotides The newly 5'-^{32}P-labelled oligonucleotide pellet was redissolved in 50 µl water and 18 µl 10X

Klenow buffer (60 mM Tris-HCl pH 7.4, 500 mM NaCl, 60 mM $MgCl_2$). The complementary oligonucleotide (.30 nmol) was added followed by enough water to make a final volume of 120 µl. The solution was placed in a water bath (80 ml of water in a beaker) which was pre-heated to 60°C. The water bath was removed from heat and the oligonucleotides were incubated in the water bath long enough for the temperature to fall to room temperature (1 h).

Analytical Scale Klenow Fragment Fill-in Reaction On Annealed Oligonucleotides; Enzymatic Incorporation Of 2, 4 And 5 1/3 of the newly annealed oligonucleotide solution was used for each fill-in reaction. To the 40 µl of oligonucleotide duplex solution was added 6 µl of 50 mM DTT followed by 15 µl of a solution containing 3 mM each of 2'-deoxyguanosine 5'-triphosphate (dGTP), 2'-deoxyadenosine 5'-triphosphate (dATP) and 2'-deoxycytidine 5'-triphosphate (dCTP). Depending on the product desired, 5 µl of a 10 mM solution of **2** (or **4** or **5**) or thymidine 5'-triphosphate (TTP) was added followed by 3 µl of the Klenow fragment of DNA polymerase 1 (1 unit/µl, Boehringer Mannheim Biochemicals). The reaction was incubated at 37°C for 20 min and quenched by the addition of 8 µl of glycerol loading buffer.

Purification And Isolation Of Filled-in Oligonucleotide Duplexes. The quenched Klenow fragment reaction was loaded onto two 1 cm wide lanes of a 2 mm x 160 mm x 160 mm 15% non-denaturing polyacrylamide gel (1-20 crosslink, no urea). The gel was

electrophoresed at 240 volts until the bromophenol blue dye had reached 2 cm from the bottom of the gel (4-5 h).

The oligonucleotide duplexes were visualized by staining with ethidium bromide. To stain the oligonucleotide duplexes in the gel, the glass plates were removed and the gel was gently shaken in 200 ml of a 5 μg/ml ethidium bromide solution for 20 min. The gel was rinsed with water and placed on a short wave UV transilluminator (Fotodyne Model 3-3000). The uppermost band corresponded to the completely filled-in duplexes so this band was excised from the gel, crushed and placed in a 1.5 ml bullet vial.

Alternatively, the oligonucleotide duplexes could be visualized in the gel simply be removing the glass plates and placing the gel upon a fluorescent TLC plate. The duplexes appeared as dark bands when illuminated with a hand-held short wave UV light. The uppermost band was excised from the gel, crushed and placed in a 1.5 ml bullet vial.

To the crushed gel slices containing the filled-in oligonucleotide duplexes was added the minimum amount of .2 M NaCl solution required to cover the material (300-750 μl). This was incubated at room temperature under argon for 8 h. The mixture was filtered and the filtrate was placed in 2000 MWCO dialysis tubing and dialyzed against 2X 4000 ml .25 mM NaPhosphate pH 7.5, .02 mM EDTA at 4°C under a blanket of argon. The DNA solutions derived from gels that were stained with ethidium bromide were extracted 2x 200 μl n-

butanol (to remove the ethidium bromide) before being placed in the dialysis tubing.

Cyanogen Bromide (CNBr) Cleavage Reactions Using Oligonucleotide Duplexes Containing 2, 4 or 5 2 mR/h of the radioactively-labelled, filled-in duplex was placed in a .5 ml bullet vial along with 1 µl 50 mM NaCl, 2.5 µl 100 mM sodium acetate (NaOAc) pH 5.5 and 1 µl of a 1 mg/ml solution of sonicated calf thymus DNA (CT DNA)(Sigma). The total volume was adjusted to 8 µl with water then 2 µl of 100 mM CNBr was added. The reaction was incubated in the sealed vial (usually 6-24 h) at room temperature then 2 µl of 50% aqueous piperidine (Sigma) was added. The reaction was heated at 90°C for 20 min then frozen in dry ice and lyophilized to dryness. The residue was dissolved in 20 µl formamide loading buffer. The formamide solution was heated at 90°C for 3 min then chilled in an ice bath for 5 min. 2 µl of the chilled formamide solution (approximately .2 mR/h) was loaded per lane of a .4 mm x 32 cm x 38 cm 20% denaturing polyacrylamide gel (1:20 crosslinked, 42% urea). The gel was electrophoresed at 1400 volts until the bromophenol blue reached the desired location (the bromophenol blue runs with an oligonucleotide of 8 base pairs in length). After electrophoresis, one glass plate was removed, the gel was covered with plastic film (Saran Wrap) and autoradiographed.

The cleavage efficiency of the reaction was determined both by densitometry of the autoradiograms and by scintillation counting. The scintillation counting was performed by running a gel with 2

mR/h total radioactivity in each lane. The area of the gel containing the cleaved band was excised as was the area of the gel representing uncleaved oligonucleotide or background reaction. The gel pieces were crushed then placed in 2 ml 15% hydrogen peroxide solution and heated at 55°C for 12 h. This treatment was used to break up the polyacrylamide and allow the radioactivity to get into solution. 18 ml of Safety Solve (Research Products International, Incorporated) was added and the samples were counted. The scintillation counting results agreed with the densitometry results suggesting the accuracy of each method.

Large Scale Klenow Fragment Fill-in Reaction Using Oligonucleotides 8 and 9. 150 µl of 68 µM oligo 8 and 75 µl of 138 µM oligo 9 were placed in a 1.5 ml bullet vial along with 10 mR/h of 5'-^{32}P-end-labelled oligonucleotide 8. 75 µl of 10X Klenow buffer was added and the solution was placed in an 80 ml water bath at 60°C and allowed to cool to room temperature over 1 h. 300 µl of a solution with 3 mM each of dATP, dGTP, dCTP was added followed by 100 µl of 10 mM **2** or 100 µl of 10 mM TTP. 50 µl of 5 units/µl Klenow fragment was added and the reaction was incubated at 37°C for 25 min. 85 µl of glycerol loading buffer was added to quench the reaction and the entire sample was loaded onto seven continuous (the lane teeth were removed) 1 cm wide lanes of a 2 mm x 160 mm x 160 mm 15% non-denaturing polyacrylamide gel (1-20 crosslink, no urea). The gel was electrophoresed at 240 volts for 3 h. The oligonucleotide was visualized with a hand held short wave UV light

and the uppermost band corresponding to the completely filled-in duplex was excised from the gel and crushed.

500 µl of .2 M NaCl was added to the crushed gel slices and the mixture was incubated at room temperature under argon for 6 h. The supernatant was removed and 600 µl .2 M NaCl was added to the gel fragments. After 8 h the supernatant was again removed and the combined supernatants were dialyzed against 4000 ml water for 10 h under a blanket of argon at 4°C. 4.04 nmol of duplex filled-in with **2** and 2.67 nmol of duplex filled-in with TTP was recovered (based on ε_{260} = 6700/phosphate for double-stranded DNA[72]).

Large Scale CNBr Cleavage Reaction And Isolation Of N7-Methylguanine. The filled-in oligonucleotide duplex solution was lyophilized to 50 µl then 25 µl of 100 mM NaOAc pH 5.5 and 25 µl of 100 mM CNBr were added. The solution was incubated for 36 h at room temperature. The solvent was evaporated with a stream of argon then the sample was placed under high vacuum for 3 h. The pellet was then redissolved in 100 µl water and heated at 90°C for 70 min to liberate any N7-methylguanine from the oligonucleotide duplex.[48] 300 µl ethanol was added and after 15 min at room temperature, the mixture was spun at 12,000 rpm for 10 min. The supernatant was removed and evaporated to dryness. The residue was redissolved in 28 µl 10 mM ammonium acetate pH 5.5 and injected onto the HPLC.

Analytical HPLC Of Reaction Products 25 µl of the solution containing the residue from the ethanol supernatant was loaded onto

a Vydac 201HS5415 4.6 mm x 15 cm C_{18} column using no precolumn. The sample was eluted with a .5 ml/min flow rate and a 30 min linear gradient of 0-2% acetonitrile in 10 mM ammonium acetate pH 5.5. The amount of N7-methylguanine observed in the reaction was quantified by comparing the integrated peak area with the peak areas of standard samples of commercially prepared N7-methylguanine (Sigma) of known amount.

Gel Analysis Of Reaction Products The oligonucleotide pellet obtained in the ethanol precipitation step of the large scale CNBr cleavage reaction and product isolation was redissolved in 60 µl water. 30 µl (2 mR/h) of this solution was placed in a .5 ml bullet vial and 3 µl neat piperidine was added. The reaction was heated at 90°C for 20 min then frozen, lyophilized to dryness and the residue was redissolved in 20 µl formamide loading buffer. The loading buffer solution was heated at 90°C for 3 min then cooled in ice for 5 min and 2 µl was loaded onto a .4 mm x 32 cm x 38 cm 20% denaturing polyacrylamide gel (1:20 crosslinked, 42% urea). The gel was autoradiographed at -70°C (with no intensifier screen) and the amount of site specific cleavage (12%) was determined by densitometry.

Linearization And 5'-End-labelling Of ΦX174 At The Xho 1 Site 10 µg of single-stranded ΦX174 virion DNA (New England Biolabs) was ethanol precipitated then redissolved in 70 µl water and 10 µl 10X Xho 1 buffer (1.5 M NaCl, 100 mM Tris-HCl pH 8.0, 100 mM $MgCl_2$). 10 µl of oligonucleotide 10 (10 µM) was added

and the solution was placed in an 80 ml water bath preheated to 60°C. The solution was allowed to cool to room temperature in the water bath (1 h) then 10 µl of 50 mM DTT and 7 µl of Xho 1 restriction endonuclease (15 units/µl, New England Biolabs) were added. After 4 h at 37°C, the DNA was ethanol precipitated then redissolved in 80 µl water and 10 µl 10X CAP buffer. 3.5 µl of CAP (19 units/µl) was added and the reaction was incubated at 37°C for 45 min. The solution was extracted 2X 100 µl phenol (freshly equilibrated with Tris base to a final pH of 7.0), 1X 100 µl chloroform (1:24 chloroform:isoamyl alcohol) and 3X 900 µl ether. The DNA was then ethanol precipitated and dissolved in 70 µl water and 10 µl 10X kinase buffer (700 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$). The pellet was allowed to dissolve at 4°C for 10 h. 3 µl of γ-^{32}P-ATP, 10 µl of 50 mM DTT and 7 µl polynucleotide kinase (10 units/µl) were added and the solution was incubated at 37°C for 45 min. 10 µl neat piperidine was added and the solution was heated at 90°C for 30 min (this piperidine treatment was used to remove any depurinated DNA strands from the DNA so the cleavage gels would have less unwanted background cleavage). The DNA was ethanol precipitated then redissolved in 30 µl formamide loading buffer, heated at 90°C for 2 min, cooled in ice for 5 min and loaded onto a 2 mm x 160 mm x 160 mm 5% denaturing polyacrylamide gel (1:20 crosslinked, 50% urea) and electrophoresed at 240 volts for 5 h. The DNA in the gel was stained with ethidium bromide by removing both glass plates and gently shaking the gel in 200 ml of a 5 µg/ml ethidium bromide solution for

20 min. The gel was rinsed with water and placed on a short wave UV transilluminator. The uppermost band corresponded to the linearized, 5'-end-labelled DNA so this band was excised from the gel and placed in an Elutrap (Schleicher and Schuell). The DNA was eluted from the gel slice for 5 h at 100 volts. The isolated DNA solution was extracted 2x 200 µl n-butanol then placed in 2000 MWCO dialysis tubing and dialyzed against 2x 4000 ml .25 mM NaPhosphate pH 7.5, .02 mM EDTA at 4°C.

Klenow Fragment Fill-in And CNBr Cleavage Reactions Using ΦX174 DNA And 2 2 mR/h of the 5'-end-labelled ΦX174 DNA linearized at the Xho 1 site was placed in a .5 ml bullet vial along with 1 µl of 10X Klenow buffer and 1 µl of oligonucleotide 11 (10 µM). The solution was placed in an 80 ml water bath that was preheated to 60°C and allowed to cool to room temperature in the water bath (1 h). 1 µl of 50 mM DTT was added along with 3 µl of a solution containing 3 mM each of dATP, dCTP and 2',3'-dideoxyguanosine 5'-triphosphate (ddGTP) (Boehringer Mannheim Biochemicals). 1 µl of 10 mM **2** (or TTP for the control reaction) was added followed by 2 µl of 2 units/µl Klenow fragment and the reaction was incubated at room temperature for 1 h.

7.5 µl of 100 mM NaOAc pH 5.5, 20 mM NaCl was then added along with 1 µl of 1 mg/ml CT DNA and 9 µl of 50 mM CNBr. The reaction was incubated in the sealed vial at room temperature for 18 h then 3 µl of neat piperidine was added and the solution was heated at 90°C for 20 min. The sample was frozen, lyophilized to dryness

and the residue was redissolved in 20 µl formamide loading buffer. The loading buffer solution was heated at 90°C for 3 min then cooled in ice for 5 min and 2 µl was loaded onto a .4 mm x 32 cm x 38 cm 8% denaturing polyacrylamide gel (1:20 crosslinked, 50% urea). The gel was transferred to a piece of filter paper (3MM Chr, Whatman), dried by heating to 80°C *in vacuo* (model 438 slab dryer, Bio-Rad) for 45 min and autoradiographed. The site specific cleavage efficiency was determined by densitometry.

CNBr Cleavage Of ΦX174 DNA Using An Allylamine-Oligonucleotide Post-synthetically Modified With Methylthioether 3-Methylthiopropionic acid (26.4 mg, .22 mmol) was placed in 5.0 ml dry acetonitrile under argon along with DCC (49 mg, .24 mmol) and NHS (38 mg, .32 mmol, recrystallized from ethyl acetate). The solution was stirred at room temperature for 12 h then filtered. The solvent was removed from the filtrate *in vacuo*. The residue was redissolved in 5.0 ml DMF and 10 µl of this solution was added to 50 µl of .1M sodium borate buffer pH 8.85, containing 1 nmol of oligonucleotide 12 (the oligonucleotide contained a single allylamine group on the 11th residue from the 5' end). This reaction was incubated at room temperature for 11 h under argon. The oligonucleotide was then ethanol precipitated and the pellet was redissolved in 400 µl water.

1 µl of the 400 µl solution containing the modified oligonucleotide 12 was added to 2 mR/h of 5'-^{32}P-end-labelled ΦX174 DNA linearized at the Xho 1 site. The volume of the reaction was adjusted

to 5 µl with water and 1 µl 10X Klenow buffer (to maintain pH during annealing) was added. The solution was placed in an 80 ml water bath preheated to 60°C. The annealing reaction was allowed to cool to room temperature in the water bath (1 h) and 1 µl of 1 mg/ml CT DNA, 1 µl of .1M NaCl, 7.5 µl 100 mM NaOAc pH 5.5 and 6 µl 50 mM CNBr were added. The reaction was incubated in the sealed vial at room temperature for 18 h then 3 µl of neat piperidine was added and the solution was heated at 90°C for 20 min. The sample was frozen, lyophilized to dryness and the residue was redissolved in 20 µl formamide loading buffer. The loading buffer solution was heated at 90°C for 3 min then cooled in ice for 5 min and 2 µl was loaded onto a .4 mm x 32 cm x 38 cm 8% denaturing polyacrylamide gel (1:20 crosslinked, 50% urea). The gel was transferred to a piece of filter paper (3MM Chr, Whatman), dried by heating to 80°C *in vacuo* for 45 min and autoradiographed. The site specific cleavage efficiency was determined by densitometry.

Preparation Of Oligonucleotide 3 Carrying A Terminal Methylthioether Residue For Attempted Triple-Stranded Cleavage Reaction 20 µl of 48 µM oligonucleotide 3 and 20 µl of 48 µM oligonucleotide 4 were added to 30 µl water and 10 µl 10X Klenow buffer in a .5 ml bullet vial. The solution was placed in an 80 ml water bath preheated to 60°C. The annealing reaction was allowed to cool to room temperature in the water bath (1 h) then 10 µl 10 mM **2** was added followed by 9 µl of 2 units/µl Klenow fragment. The reaction was incubated at room temperature for 20 min then

ethanol precipitated. The oligonucleotide pellet was redissolved in 20 µl formamide loading buffer, heated at 90°C for 2 minutes, cooled in ice for 5 min then loaded onto a 2 mm x 20 cm x 20 cm 15% denaturing polyacrylamide gel (1:20 crosslinked, 50% urea) electrophoresed at 320 volts for 3 h. The DNA on the gel was visualized with a hand-held short wave UV light and the band corresponding to the filled-in oligonucleotide 3 (middle band on gel) was excised from the gel. The gel slice was crushed, 500 µl of .2 M NaCl was added and the mixture was incubated at room temperature for 8 h under argon. The mixture was filtered, the filtrate placed in 1000 MWCO dialysis tubing (Spectropore 7, Spectrum Medical Industries) and dialyzed against 2X 4000 ml .25 mM NaPhosphate pH 7.5, .02 mM EDTA.

Attempted Cleavage Of Double-Stranded DNA Through Formation Of A Triple-Stranded Structure 11 µg of plasmid pDMAG10 (prepared by Dave Mendel) in 90 µl water was placed in a 1.5 ml bullet vial along with 10 µl 10X EcoR1 buffer (1 M Tris-HCl pH 7.5, .5 M NaCl, .1M $MgCl_2$) and 2 µl 20 units/µl EcoR1 restriction endonuclease (New England Biolabs) was added. The reaction was incubated at 37°C for 3 h then ethanol precipitated. The DNA pellet was redissolved in 40 µl water and 5 µl 10X Klenow buffer then 2 µl 10 mM TTP, 2 µl α-^{32}P-2'-deoxyadenosine 5'-triphosphate (>3000 Ci/mmol, Amersham) and 2 µl of 5 units/µl Klenow fragment were added. The reaction was incubated at 37°C for 20 min then ethanol precipitated. The pellet was redissolved in 40 µl water and 5 µl 10X

Bgl 1 buffer (.66 M NaCl, .1 M Tris-HCl pH 7.4). 3 µl of 8 units/µl of Bgl 1 restriction endonuclease (New England Biolabs) was added and the solution was incubated at 37°C for 5 h. 8 µl of glycerol loading buffer was added to quench the reaction and the sample was loaded onto a 1 cm wide lane of a 2 mm x 160 mm x 160 mm 5% non-denaturing polyacrylamide gel (1-20 crosslink, no urea). The gel was electrophoresed at 240 volts for 3 h. The band corresponding to the 628 base pair fragment was excised from the gel, the DNA was isolated with the Elutrap then placed in 2000 MWCO dialysis tubing and dialyzed against 2X 4000 ml .25 mM NaPhosphate pH 7.5, .02 mM EDTA.

2 mR/h of the 628 base pair DNA fragment and 1 µl of 10 µM oligonucleotide 3 containing a single residue of **2** (on the 3' end) were placed in each of several .5 ml bullet vials. 1.5 µl of 200 mM NaOAc pH 5.5 or 6.5 was added along with 1 µl of 1 M NaCl and various combinations of spermidine, $Co(III)(NH_6)Cl_3$, ethylene glycol, ethanol, tetrahydrofuran, $MgCl_2$, and $CuCl_2$ (all are reagents known to promote triple helix formation[43,77]). 2.5 µl of 100 mM CNBr was added and the sealed reactions were incubated at 0°C for 24 h. 2 µl of 50% aqueous piperidine was then added and the solutions were heated at 90°C for 20 min, frozen in dry ice, lyophilized to dryness and the residue redissolved in 20 µl formamide loading buffer. The loading buffer solution was heated at 90°C for 3 min then cooled in ice for 5 min and 2 µl was loaded onto a .4 mm x 32 cm x 38 cm 8% denaturing polyacrylamide gel (1:20 crosslinked, 50% urea). The gel

was transferred to a piece of filter paper (3MM Chr, Whatman), dried by heating to 80°C *in vacuo* for 45 min and autoradiographed.

Attempted Complementary-Addressed Cleavage Reactions Using 10 and 12. Oligonucleotide 1,2 duplexes were prepared containing a single enzymatically incorporated residue of **10** or **12** exactly as described for **2**.

The Copper-Thiol Reaction The copper-thiol cleavage reactions were run in a 10 μl volume by incubating .2 mR/h of oligonucleotide 1,2 duplex containing a residue of **12** with 100 μM CT DNA, 2-10 μM cupric sulfate and 1-5 mM 3-thiopropionic acid for 1 h at room temperature. The samples were frozen in dry ice, lyophilized to dryness and the residue redissolved in 2 μl formamide loading buffer. The loading buffer solution was heated at 90°C for 3 min then cooled in ice for 5 min and the 2 μl was loaded onto a .4 mm x 32 cm x 38 cm 20% denaturing polyacrylamide gel (1:20 crosslinked, 42% urea). The gel was electrophoresed and autoradiographed as usual. 1-5 mM DTT and 1-5 mM sodium ascorbate were also tried as reducing agents, but 3-thiopropionic acid was the only reagent which allowed any cleavage.

Attempted Photochemical Cleavage Using Co(III) Derivatives Photochemical cleavage using Co(III) complexes of the 1,10-phenanthroline group of **12** in oligonucleotide 1,2 duplexes was attempted. Attempts were made to synthesize the Co(III)-**12** complexes by incubating various concentrations of *cis*-β-Co(III)(trien)(H_2O)(OH) (.01-10 mM) with the oligonucleotide duplex

(.5 mR/h) in various buffers (1-10 mM sodium cacodylate pH 7.0, 1-10 mM borate buffer pH 8-10 and sodium acetate pH 5.5) for various lengths of time (16-30 h) at room temperature. These samples were dialyzed against 4 l of .5 mM sodium phosphate pH 7.5 at 4°C for 36 h. The samples were then photolyzed at room temperature in a quartz cuvette for various amounts of time with a focused beam of light from a high pressure Hg-Xe photolysis lamp (Oriel model 6140) filtered through a pyrex filter ($\lambda \geq 300$ nm). After photolysis, 2 µl neat piperidine was added to each 20 µl reaction and the samples were heated to 90°C for 20 min, frozen in dry ice, lyophilized to dryness and the residue redissolved in 2 µl formamide loading buffer. The loading buffer solution was heated at 90°C for 3 min then cooled in ice for 5 min and the 2 µl was loaded onto a .4 mm x 32 cm x 38 cm 20% denaturing polyacrylamide gel (1:20 crosslinked, 50% urea). The gel was electrophoresed and autoradiographed as usual.

The *cis*-β-Co(III)(trien)(H_2O)(OH) was prepared just prior to use by placing β-Co(III)(trien)(carbonate) (72 mg, .2 mmol; Sigma) in 5.0 ml 1.0 N HCl. The solution was stirred for 1 h then the pH was raised to 7.0 by the addition of concentrated NaOH.[78]

Attempted "Catalytic" Alkylation The "catalytic" alkylation reactions were attempted using oligonucleotide 1,2 duplexes containing a single enzymatically incorporated residue of **10** or **12**. .5mR/h samples of duplex were placed in .5 ml reaction vials containing 1-10 mM sodium cacodylate or borate buffer pH 7.0-10.0

and 100 μM CT DNA. 2 μM-2 mM cupric sulfate, cupric acetate, zinc sulfate or nickel sulfate was added and the reactions were incubated for 0-1 h. To these solutions were added aziridine (1 μM-2 mM), N-(2-hydroxyethyl)aziridine (10 μM-1 mM, Aldrich) or ethylene sulfide (50 μM-2 mM, Aldrich). The total volume of the reactions was 10 μl. The reactions were incubated for 8-24 h at room temperature. 2 μl of 50% aqueous piperidine was added to each reaction and the samples were heated to 90°C for 20 min, frozen in dry ice, lyophilized to dryness and the residue redissolved in 2 μl formamide loading buffer. The loading buffer solution was heated at 90°C for 3 min then cooled in ice for 5 min and the 2 μl was loaded onto a .4 mm x 32 cm x 38 cm 20% denaturing polyacrylamide gel (1:20 crosslinked, 50% urea). The gel was electrophoresed and autoradiographed as usual.

The aziridine was prepared just prior to use by placing β-chloroethylamine hydrochloride (1.0 g, 8.7 mmol; Aldrich) in 10 ml water. NaOH (.66 gm, 16.5 mmol) in 20 ml water was slowly added. The solution was heated in an oil bath and the volatile fraction containing aziridine was distilled off of the reaction and collected in an ice cooled flask.

The attempted cleavage reactions using methionine and CNBr were run exactly as above except at pH 5.5 in 10 mM sodium acetate. 2 μM-2 mM methionine was added in place of the aziridine, N-(2-hydroxyethyl)aziridine or ethylene sulfide. 25 mM CNBr was

added after 0-1 h and the reactions were incubated at room temperature for 16-24 h. The rest was done the same as above.

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PART 2

NOVEL BASE SPECIFIC DNA CLEAVAGE REACTIONS

INTRODUCTION

During the course of the studies on complementary-addressed modification of single-stranded DNA, two new base-specific cleavage reactions were discovered. The first reaction involves unprecedented cleavage of DNA at A residues by K_2PdCl_4 at pH 2.0 followed by a piperidine treatment. Product analysis revealed that the reaction is the result of adenine specific depurination, thus the K_2PdCl_4 reaction is a "gapping" reaction compatible with the other sequencing reactions currently used with Maxam-Gilbert chemical sequencing methods.[1] N6-methyladenine was shown to react with K_2PdCl_4 35% less efficiently than normal adenine in similar sequences. A mechanism is proposed for the reaction wherein Pd(II) binds to the N7 position of adenine then protonation at N1 results in glycosidic bond hydrolysis and thus depurination. This simple K_2PdCl_4 A specific reaction should be a useful addition to current chemical DNA sequencing procedures and is a strong candidate for use in automated sequenators.

The second reaction that was discovered is a photochemical reaction which produces cleavage of double-stranded DNA at the 5'-G of 5'-GG-3' or 5'-GA-3' sequences. Nitroveratrole, m-nitroanisole and $Co(III)(NH_3)_6Cl_3$ were all shown to be capable of the reaction. Preliminary product analysis of the reaction between m-nitroanisole and 2',3',5'-tri-O-acetylguanosine revealed that the mechanism does not involve simple alkylation and numerous 2',3',5'-tri-O-acetylguanosine decomposition products were observed. The m-nitroanisole was apparently not irreversibly altered by the reaction. A mechanism is proposed based on an electron transfer pathway leading to oxidative

decomposition of the guanine base. Various 2'-deoxynucleotide molecules with covalently attached m-nitroanisole or nitroveratrole groups were tried in photochemical complementary-addressed cleavage reactions, but none produced any site specific cleavage on DNA target strands.

THE K_2PdCl_4 REACTION AT ADENINE

BACKGROUND

Chemical DNA Sequencing Chemical DNA sequencing according to the method of Maxam and Gilbert utilizes base specific chemical modification reactions followed by a piperidine workup which causes phosphodiester bond cleavage at the site of the modified base.[1,2] To date reactions have been reported which are capable of causing the cleavage of DNA at G,[1,3] G+A,[2] A>G,[1] A>C,[1] C,[1] C+T,[1] and T[3] residues (see Figure 48).

For example, treatment of DNA with dimethyl sulfate (DMS) results in methylation at N7 of guanine and N3 of adenine (see Figure 3) in about a 7:1 ratio respectively.[4] A piperidine treatment then opens the N7-methylguanine ring (via attack at C8) leading to hydrolysis of the glycosidic bond. Subsequent β-elimination reactions produce 3' and 5' ends on the cleaved strand[2] (see Figure 30). Since the entire base and sugar are removed from the DNA strand, this reaction (like all the Maxam-Gilbert reactions) is termed a "gapping" reaction. Because of the N6-amino group, N3-methyladenine is somewhat less susceptible to base hydrolysis so a relatively short piperidine treatment of methylated DNA results in a G specific sequencing lane.

On the other hand, N3-methyladenine is substantially more susceptible to acid catalyzed glycosidic bond hydrolysis than N7-methylguanine, so a DMS reaction followed by a quick acid then base treatment results in an A>G sequencing lane.[1] This difference in N3-

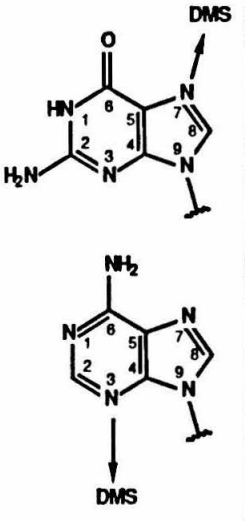
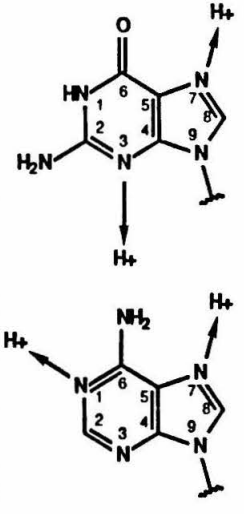
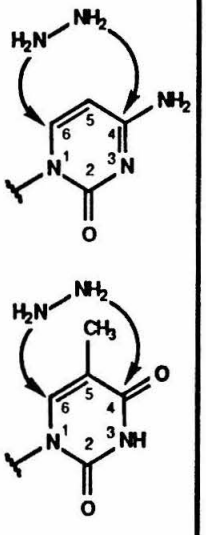
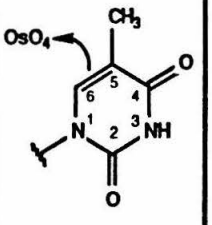
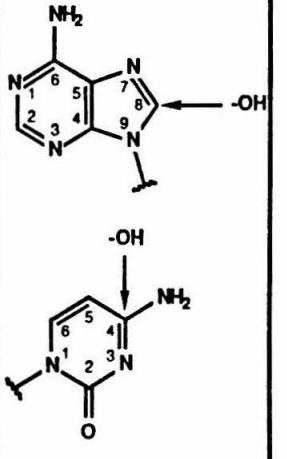
REACTION	G, A>G	A+G	C,C+T	T	A>C
REAGENT	DMS	H+	N_2H_4	OsO_4	-OH
POSITION(S) OF ATTACK					
PIPERIDINE TREATMENT	YES	YES	YES	YES	YES

Figure 48 The reactions used for chemical sequencing according to the method of Maxam and Gilbert.[2]

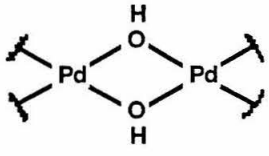
methyladenine versus N7-methylguanine reactivity is the result of the different protonation states at the N1 positions of both compounds. The N1 position of guanine is protonated at neutral pH (pK_a = 9.2)[5] and when protonated at N1, the guanine ring is overall neutrally charged. Therefore, since there are no other sites which protonate in mild acid,[5] the N7-methylguanine ring carries only the positive charge from the methyl group at N7. In contrast, N1 of adenine is protonated at mildly acidic pH (pK_a = 3.6),[5] and when protonated, a net positive charge is added to the aromatic adenine ring. Therefore, in mild acid the N1 position of N3-methyladenine is protonated thereby placing a second positive charge in the adenine ring (one from the protonation at N1 and one from the methyl group at N3) helping to make the ring a good leaving group thus facilitating the hydrolysis of the glycosidic bond. A quick base treatment breaks the DNA backbone at the depurinated sites (along with some unavoidable C8 hydrolysis of N7-methylguanine residues) so an A>G lane is produced. This same difference in reactivity at low pH between adenine and guanine will be discussed later in relation to the proposed mechanism of the K_2PdCl_4 reaction.

The other base specific cleavage reactions use a similar strategy of exploiting a unique reactivity to selectively disrupt the aromatic ring of certain base(s), then a piperidine treatment leads to strand scission and a gapping reaction.

Metal Ion-DNA Interactions Thanks in part to the success of Pt(II) compounds as chemotheraputic agents,[6,7] the interactions

of DNA with metal ions have been thoroughly investigated. *Cis*-diamminedichloroplatinum(II) (*cis*-DPP) has shown activity against several human malignancies and is particularly effective for treating testicular cancer.[7] Considerable evidence suggests that DNA is the primary target of *cis*-DPP *in vivo*.[7] Product isolation[8] and spectroscopic studies[9] have shown that *cis*-DPP binds to DNA strands mainly through intrastrand crosslinks made by bridging adjacent N7 positions of a G-G sequence. Bonds to N7 of adenine have also been detected.[8,10] Other metals such as ruthenium(II)[11] have been shown to bind at N7 of guanine in DNA and numerous Pd(II) complexes[12-21] have been shown to interact with DNA or DNA bases.

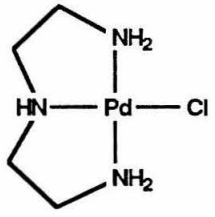
Pd(II), like Pt(II), has a d_8 valence electronic configuration and prefers square planar, four-coordinate complexes in aqueous solution.[22] Pd(II) is about 10^5 times more kinetically labile than Pt(II),[23] possibly explaining why Pt(II) complexes are better chemotheraputic agents. In aqueous solution, metal-chloride bonds in Pd(II)-chloride complexes such as K_2PdCl_4 are hydrolyzed by



water leading to multimeric bridging hydroxide species.[23] HCl is released in the reaction explaining why K_2PdCl_4 is an acid. Protonation of the bridging OH ligands below pH 5.0 breaks up the multimeric structures producing monomeric aquo complexes.[23] The

H_2O in these complexes can be readily exchanged for preferred ligands such as amines or thiols.[22]

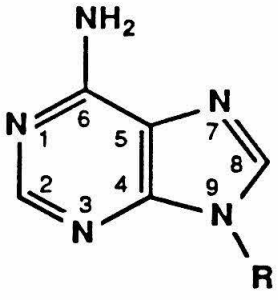
The interaction of ((diethylenetriamine)Pd(II)Cl)Cl ((dien)Pd(II))



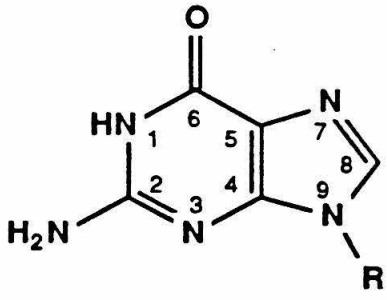
(dien)Pd(II)

with purine nucleosides and nucleotides has been studied by NMR.[5,24-26] Pd(II) bonds to the N1 or N7 positions have been identified and the respective stability constants determined (see Figure 49).[5] For example, in the case of adenosine 5'-monophosphate (AMP) the stability constants determined for (dien)Pd(II) binding to N1 and N7 are about the same. For guanosine 5'-monophosphate (GMP) the stability constants for binding to N1 and N7 are also about the same, but they are ~10^3 times higher for GMP relative to AMP. In contrast, Cu(II) slightly prefers binding at N1 positions and protons markedly prefer binding at N1.[5] The important point is that different species have drastically different preferences and metals like Pd(II) bind to the imidazole N7 positions just as strongly as they bind to the much more basic N1 positions.

The binding of Pd(II) to the different purine binding sites is pH dependent because the Pd(II) must compete with protons for binding.[5] Therefore at all but basic pH, N7 is essentially the only site of



Adenine



Guanine

Base	Position	pK_a (a)	Stability Constant log	
			Cu(II) (a)	dienPd(II) (b)
Adenine	N1	3.6	1.7	5.00
	N7	-1.6	1.3	5.04
Guanine	N1	9.2	4.2	7.86
	N7	2.2	2.8	8.09

(a) Measured for the nucleoside
(b) Measured for the nucleotide 5'-monophosphate

Figure 49 Comparison of the pK_a's and stability constants for metal binding to the N1 and N7 positions of the purines. The data is from reference 5.

Pd(II) binding to guanine because the N1 site is protonated. For adenine, protonation at N1 only occurs at acidic pH so that Pd(II) binding to N7 only predominates below pH ~2.[5]

Crystallographic studies have served to further illustrate the importance of Pd(II) and Pt(II) binding to N7 positions of purines as well as indicate the possible presence of ligand to purine hydrogen bonds. A crystal structure of Pd(II) with 6-mercapto-9-benzylpurine revealed Pd(II) bonds to the N7 position and sulfur atom.[27] A $Pt(II)(Cl)_3$ complex with adenine contained a single metal to adenine bond at the N7 position.[28] Interestingly, this crystal structure showed a hydrogen bond between a chloride ligand on the Pt(II) and a hydrogen atom on the N6 of adenine. Evidence for a similar type of hydrogen bond was found for Pd(II) and Pt(II) amine complexes of nucleotide 5'-monophosphate compounds in solution.[26,29] A proton on the coordinated amine apparently forms a hydrogen bond with an oxygen atom on the phosphate. A ligand to base or phosphate hydrogen bond could help stabilize specific metal-purine complexes on DNA during the K_2PdCl_4 reaction.

RESULTS AND DISCUSSION

The K_2PdCl_4 reaction which cleaves DNA at adenine residues was discovered serendipitously during studies conducted to investigate the diazo group as a cleaving function for complementary-addressed modification of DNA. Compound **13** was synthesized using the strategy outlined in Figure 50. The structure of **13** was confirmed by NMR, UV-vis and IR. **13** was incorporated into oligonucleotide 1,2 duplexes using the standard Klenow enzyme reaction and the resulting filled-in duplexes were purified on a 15% nondenaturing polyacrylamide gel. Photolysis (λ = 320 ± 10 nm) of the oligonucleotide 1,2 duplexes containing a residue of **13** failed to produce any cleavage on the complementary strand. Therefore, transition metals known to decompose diazo compounds were also tried in an effort to "activate" the diazo group.[30] $CuCl_2$, $Cu(OAc)_2$ and K_2PdCl_4 were all examined for activity, but no site specific cleavage was observed. Limited cleavage at adenine residues in the lanes with K_2PdCl_4 was observed. Since specific cleavage of DNA only at adenine was unprecedented, the K_2PdCl_4 reaction was investigated in detail and a reproducible protocol for an A specific sequencing lane was developed.

The Protocol For The K_2PdCl_4 Cleavage Reaction At Adenine Residues Figure 51 shows the results of reacting a ^{32}P end-labelled restriction fragment of DNA with K_2PdCl_4 at pH 2.0 followed by heating in aqueous piperidine. Lanes 1 and 4 of gel A are DMS G reactions carried out on the DNA fragment that was 5' and

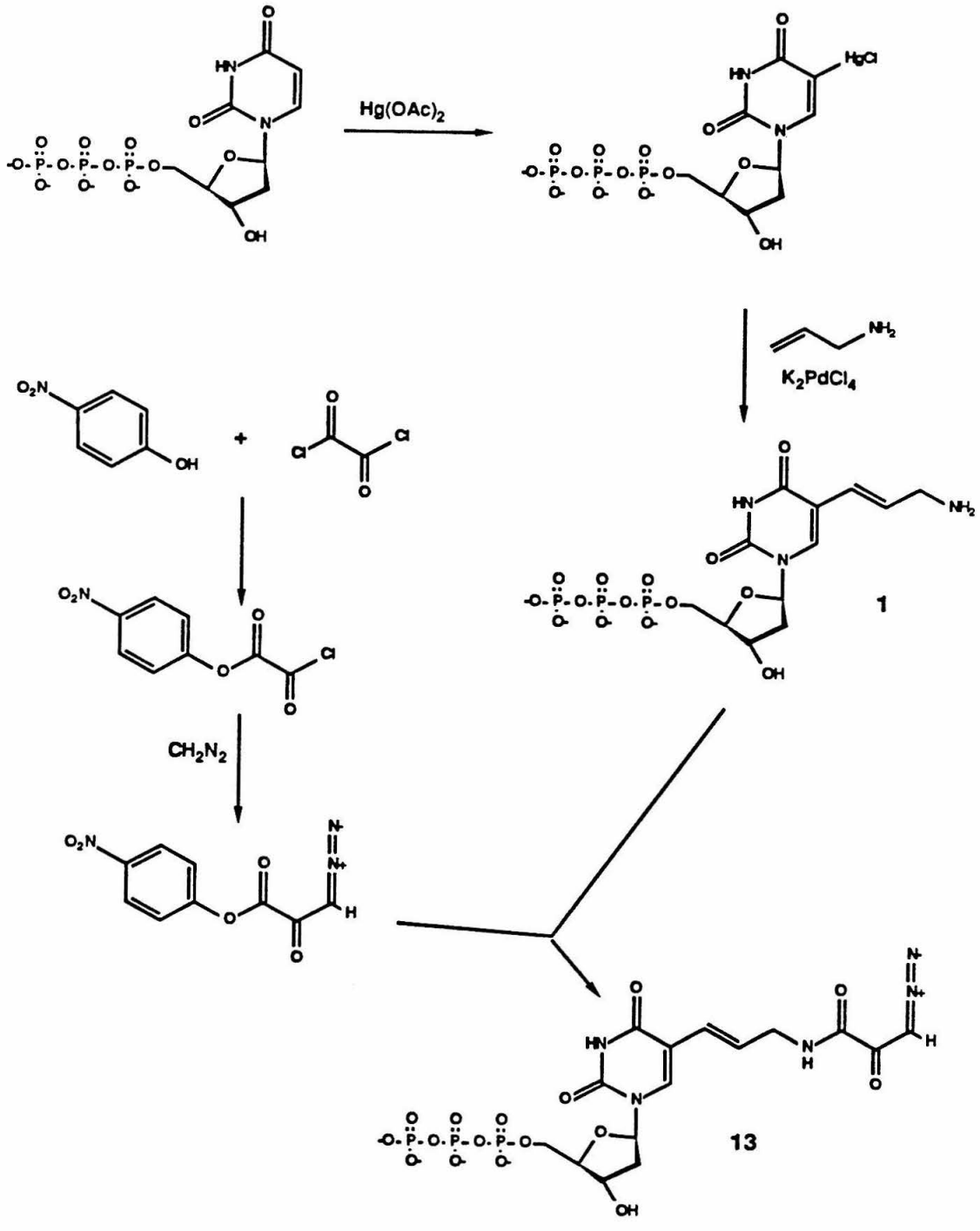
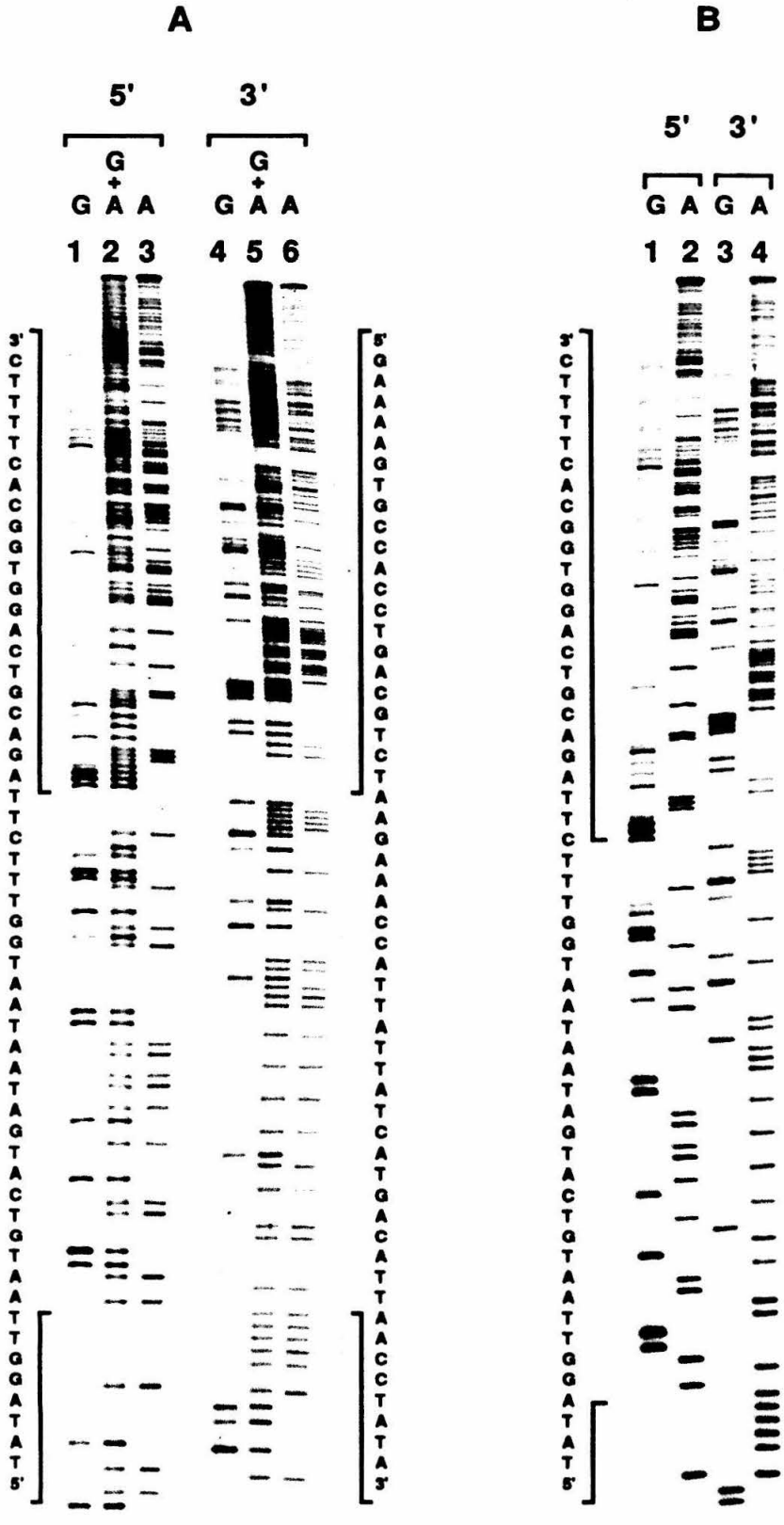


Figure 50 Synthetic scheme for the preparation of compound **13**.

Figure 51

Gel A Comparison of the G, G+A and K_2PdCl_4 A reaction on a 517 base pair restriction fragment of DNA. Autoradiogram of a dried 8% denaturing polyacrylamide gel. Lanes 1-3 contain reactions on the 517 base pair fragment labelled with ^{32}P at the 5' end. Lanes 4-6 contain reactions of the 517 base pair fragment labelled with ^{32}P on the 3' end. **Lanes 1 and 4** Maxam-Gilbert G reaction. **Lanes 2 and 5** Maxam-Gilbert G+A reaction. **Lanes 3 and 6** K_2PdCl_4 A reaction.

Gel B The G and K_2PdCl_4 A reaction on a 517 base pair restriction fragment of DNA illustrating a straightforward new sequencing strategy. The entire DNA sequence of the fragment can be read in analogy to a Sanger type sequencing gel. Autoradiogram of a dried 8% denaturing polyacrylamide gel. Lanes 1 and 2 contain reactions on the 517 base pair fragment labelled with ^{32}P at the 5' end. Lanes 3 and 4 contain reactions of the 517 base pair fragment labelled with ^{32}P on the 3' end. **Lanes 1 and 3** Maxam-Gilbert G reaction. **Lanes 2 and 4** K_2PdCl_4 A reaction.


A
5'
3'
G
+
A
G A A
1 2 3
G A A
4 5 6
3'CTTTTCACGGTGGACTGCAGATTCTTTGGTAATAATAGTACTGTAATTGGATAT5'
5'GAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATA3'
B
5' 3'
G A G A
1 2 3 4
3'CTTTTCACGGTGGACTGCAGATTCTTTGGTAATAATAGTACTGTAATTGGATAT5'

3'-labelled respectively at the same restriction site. Lanes 2 and 5 are the standard formic acid catalyzed Maxam-Gilbert G+A lanes[2] and lanes 3 and 6 are K_2PdCl_4 A lanes. Comparison with the G+A and G lanes clearly demonstrates that the K_2PdCl_4 reaction produces uniform cleavage exclusively at all A residues.

Gel B of Figure 51 illustrates how the K_2PdCl_4 A reaction can be used in conjunction with the DMS G reaction to allow a new straightforward sequencing strategy. The DNA fragment is end-labelled with ^{32}P at the same restriction site on both the 5' and 3' ends. A K_2PdCl_4 A reaction and DMS G reaction are conducted on the fragments and the four reactions are loaded adjacent to each other on a sequencing gel. In gels of this type, each base position is represented by a cleavage band in only one lane so the complete DNA sequence can be quickly and unambiguously read, similar to a sequencing gel run according to the Sanger method.[31]

The K_2PdCl_4 reaction protocol consists of incubating the DNA fragment in 2 mM K_2PdCl_4, 20 mM HCl/NaCl pH 2.0 for 30 to 45 minutes at room temperature. The reaction is stopped with thiol to coordinate the Pd(II) which otherwise interferes with electrophoresis of the DNA fragments. Standard G stop solution containing 2-mercaptoethanol can be used.[2] The DNA is then ethanol precipitated (the bright yellow Pd(II)-thiol complexes stay mostly in the supernatant) and the resulting pellet is redissolved in 10% aqueous piperidine. The piperidine solution is heated at 90°C for 30 minutes then frozen and lyophilized. The residue is redissolved in formamide

loading buffer, then the sample is loaded onto a polyacrylamide sequencing gel and electrophoresed as usual. Except for the K_2PdCl_4 pH 2.0 treatment of the DNA instead of reaction with DMS, the A reaction protocol is identical to the G reaction protocol so the reactions are conveniently run side by side.

Product Analysis The electrophoretic mobility (on a high resolution 20% polyacrylamide gel) of the fragments produced by the K_2PdCl_4 reaction are identical to the A cleavage fragments produced by the standard G+A reaction indicating that the K_2PdCl_4 reaction produces 3' and 5' phosphate termini (see Figure 52, gel A). Fragments with hydroxyl or sugar fragment termini run noticeably different on these gels.[32] The K_2PdCl_4 A reaction can therefore be used in conjunction with all of the previously discovered base-specific reactions used for complete DNA sequence determination.

An HPLC analysis of the K_2PdCl_4 reaction on calf thymus DNA is shown in Figure 53. Trace 1 is a control G+A depurination reaction run by heating calf thymus DNA in .1N HCl at 70°C for 45 minutes. Trace 2 is another control showing the products of the K_2PdCl_4 reaction after 0 minutes (the 2-mercaptoethanol was added to the reaction before the K_2PdCl_4). Trace 3 is the result of reacting calf thymus DNA with 2.5 mM K_2PdCl_4 for 3 hours at room temperature. Comparison of trace 3 with the other two traces shows that free adenine is the only observed reaction product of the K_2PdCl_4 reaction. The other peaks in the chromatograms can be identified as well and the large peaks in traces 2 and 3 at 23 and 39 minutes are 2-mer

Figure 52

Gel A Comparison of the electrophoretic mobilities of the fragments produced by the G, G+A and K_2PdCl_4 A reaction on a high resolution gel. Autoradiogram of a 20% denaturing polyacrylamide gel. Lanes 1-3 contain reactions on the 517 base pair fragment labelled with ^{32}P at the 3' end. Lanes 4-6 contain reactions of the 517 base pair fragment labelled with ^{32}P on the 5' end. **Lanes 1 and 4** Maxam-Gilbert G reaction. **Lanes 2 and 5** K_2PdCl_4 A reaction. **Lanes 3 and 6** Maxam-Gilbert G+A reaction.

Gel B Comparison of DMS reactions conducted at pH 2.0 in the presence and absence of K_2PdCl_4. Adenine specific cleavage is only observed in the lane with K_2PdCl_4 (lane 1) indicating an "adenine enhancement" type of mechanism for the K_2PdCl_4 reaction. Autoradiogram of a dried, 8% denaturing polyacrylamide gel. The reactions were carried out at pH 2.0 on the 517 base pair DNA fragment labelled with ^{32}P on the 5' end using the usual K_2PdCl_4 A reaction conditions and piperidine workup, except 1 µl DMS was added to each reaction and the reactions were stopped (with the usual thiol stop solution) after only 5 min at room temperature. **Lane 1** The DMS reaction at pH 2.0 in the presence of 2 mM K_2PdCl_4. **Lane 2** The DMS reaction at pH 2.0 with no K_2PdCl_4 present.

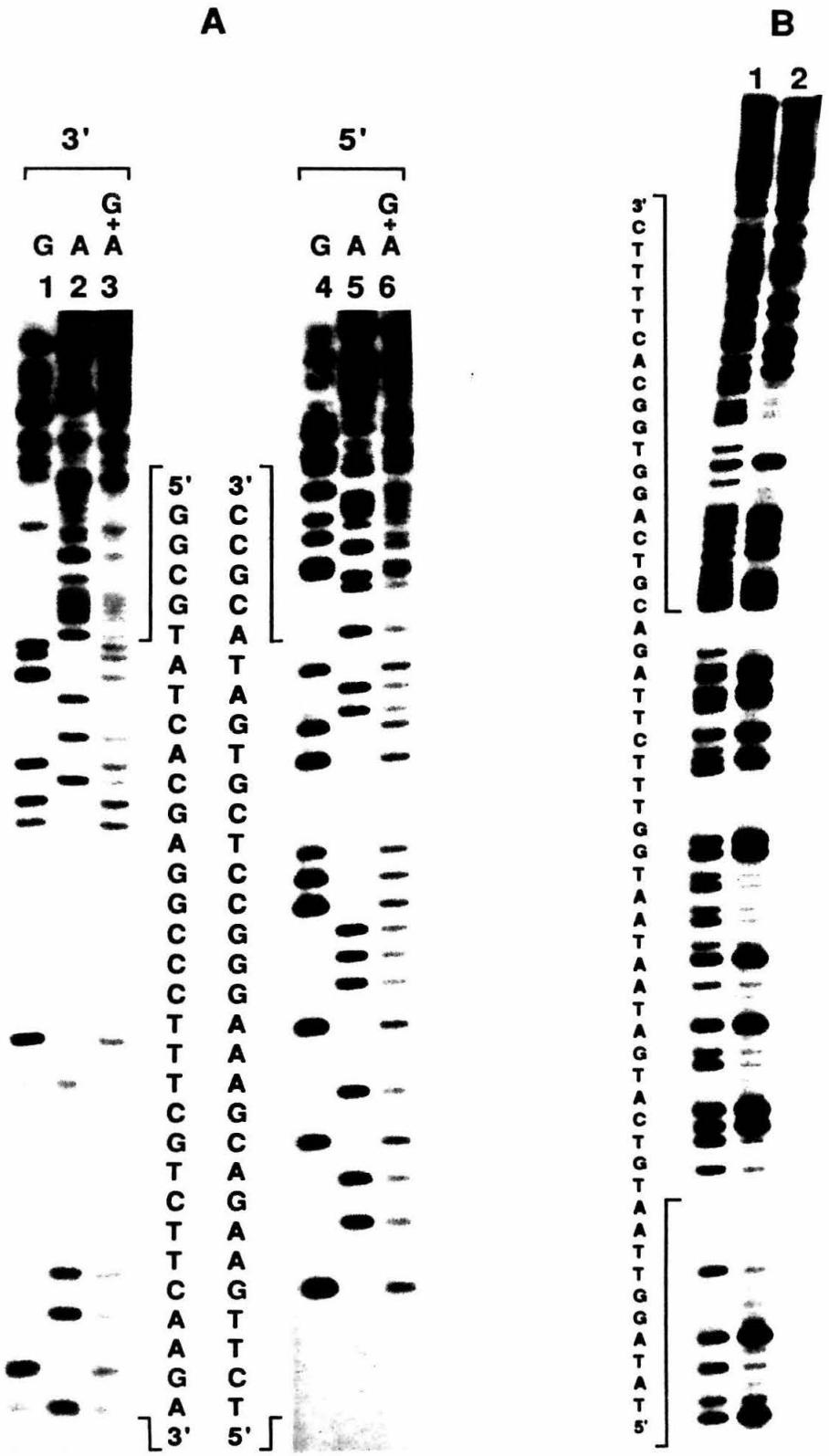
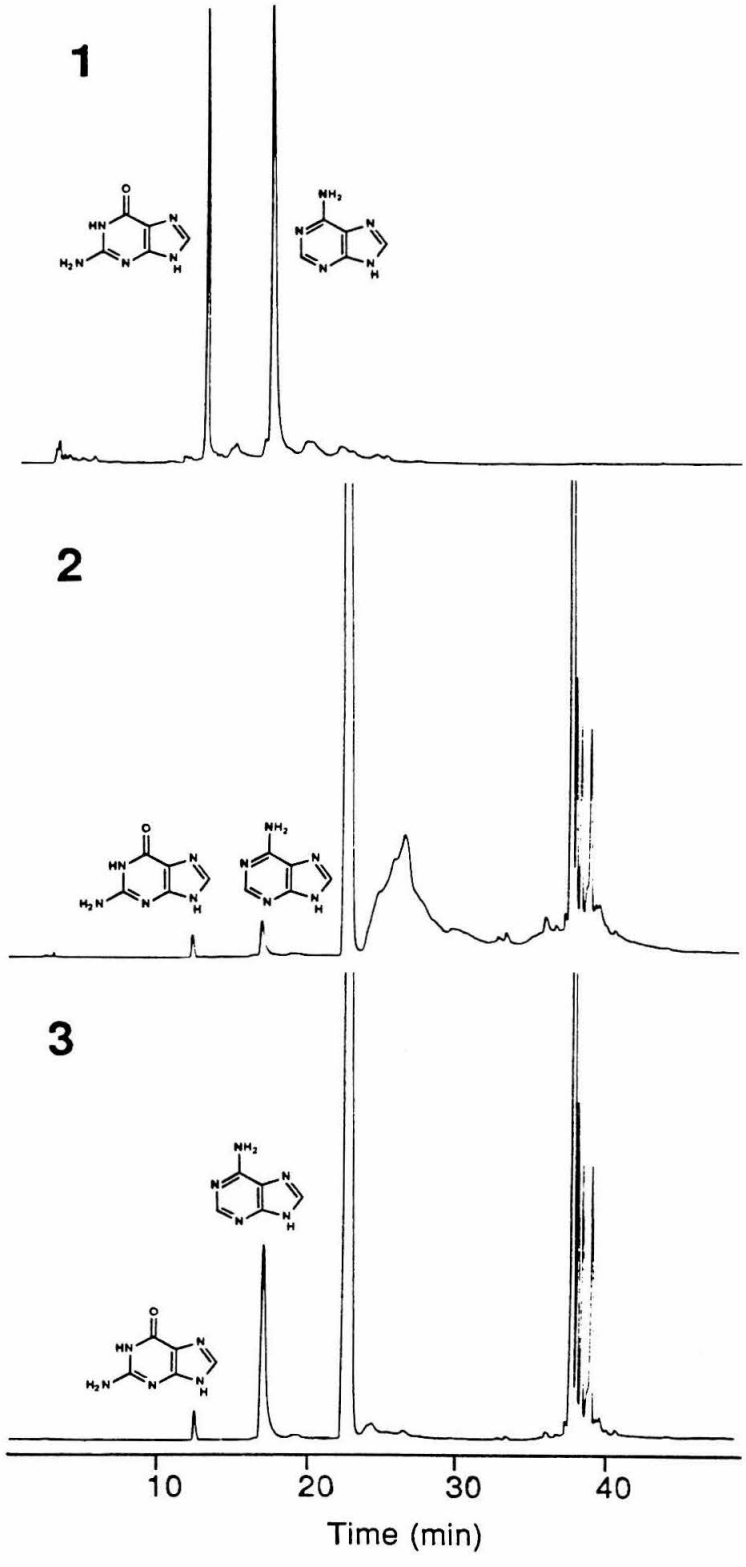

A
3'
5'
G
A
G+A
1 2 3
4 5 6
5' GGCGTATTCACGAGGCCCTTTCGTCTTCAAGA 3'
3' CCGCATAGTGCTCCGGGAAGCAGAAGTTCT 5'
B
1 2
3' CTTTTCACGGTGGACTGCAGATTCTTTGGTAATAATAGTACTGTAATTGGATAT 5'

Figure 53

HPLC chromatogram (monitored at 254 nm) produced by the K_2PdCl_4 A reaction on 50 μg calf thymus DNA showing that free adenine is the only released product of the reaction. In each case the reactions were frozen in dry ice, lyophilized to dryness and the residue was redissolved in 25 μl of 8 mM ammonium acetate pH 5.5. The samples were chromatographed on an Altex Ultrasphere ODS HPLC column eluted with a 30 minute linear gradient of 0-15% acetonitrile in 8 mM ammonium acetate pH 5.5 followed by 15-75% acetonitrile in 15 minutes with a flow rate of .75 ml/min. **Chromatograph 1** An acid catalyzed G+A reaction produced by heating the DNA in .1 N HCl at 70°C for 45 minutes. **Chromatograph 2** A control reaction in which the 2-mercaptoethanol was added to the DNA before the K_2PdCl_4 and the sample was immediately frozen. **Chromatograph 3** The DNA was reacted with 2.5 mM K_2PdCl_4 at room temperature for 3 hours then 2-mercaptoethanol was added to stop the reaction.


1
2
3
10
20
30
40
Time (min)

captoethanol and Pd(II)-thiol complexes respectively. The broad peak ~25 minutes in trace 2 is unreacted calf thymus DNA.

Mechanistic Studies The observed K_2PdCl_4 reaction which causes adenine specific depurination of DNA could be produced by suppressing G reaction in the otherwise G+A acid catalyzed depurination reaction, by causing an enhanced A specific depurination reaction or a combination of both effects. Since Pd(II) has been shown to bind primarily at the N7 of GMP at neutral to acidic pH (with a higher stability constant than for AMP[26]), it is possible that the Pd(II) is binding to the N7 position of G and thereby preventing the standard acid catalyzed depurination reaction at G. If the Pd(II) is less efficient at preventing depurination at A, an A specific reaction would be produced. Alternatively, the K_2PdCl_4 could be causing enhanced depurination at A through a reaction which does not occur to the same extent (or at all) at G.

The "G suppression" mechanism is discounted and the "adenine enhancement" mechanism is indicated as the dominant mechanism of the K_2PdCl_4 reaction by the cleavage observed in the reaction of DNA with DMS at pH 2.0 in the presence and absence of K_2PdCl_4 (see Figure 52, gel B). The G reaction by DMS (known to involve a methylation reaction at the N7 position)[4] was at best partially inhibited by the K_2PdCl_4 and A cleavage only occurred in the K_2PdCl_4 reaction. Other HPLC and gel results have verified that the K_2PdCl_4 causes strongly enhanced reaction at adenine residues in acidic pH relative to the same pH conditions without K_2PdCl_4.[33]

Therefore, the primary effect of K_2PdCl_4 during the reaction is to enhance specific depurination at adenine residues.

Since the K_2PdCl_4 is causing enhanced depurination at adenine residues, the Pd(II) is apparently binding to the adenine in a manner which assists the hydrolysis of the glycosidic bond at low pH. The absence of significant neighboring base dependence on the observed A cleavage (see Figure 51) indicates that the depurination reaction caused by Pd(II) probably does not involve bridging bonds between adenine and adjacent bases. In a formal sense, Pd(II) could be binding to any of the "exposed" nitrogen lone pairs on adenine, that is at N3, N6 or N7. If the Watson-Crick base pairing is disrupted (a reasonable assumption at pH 2.0) then binding to N1 is also possible. Although it has been proposed in the literature,[34] direct coordination to N6 is not likely, even in conjunction with binding to N1 or N7.[35] Therefore, either N1, N3 or N7 is most likely to be the site of Pd(II) binding during the K_2PdCl_4 reaction.

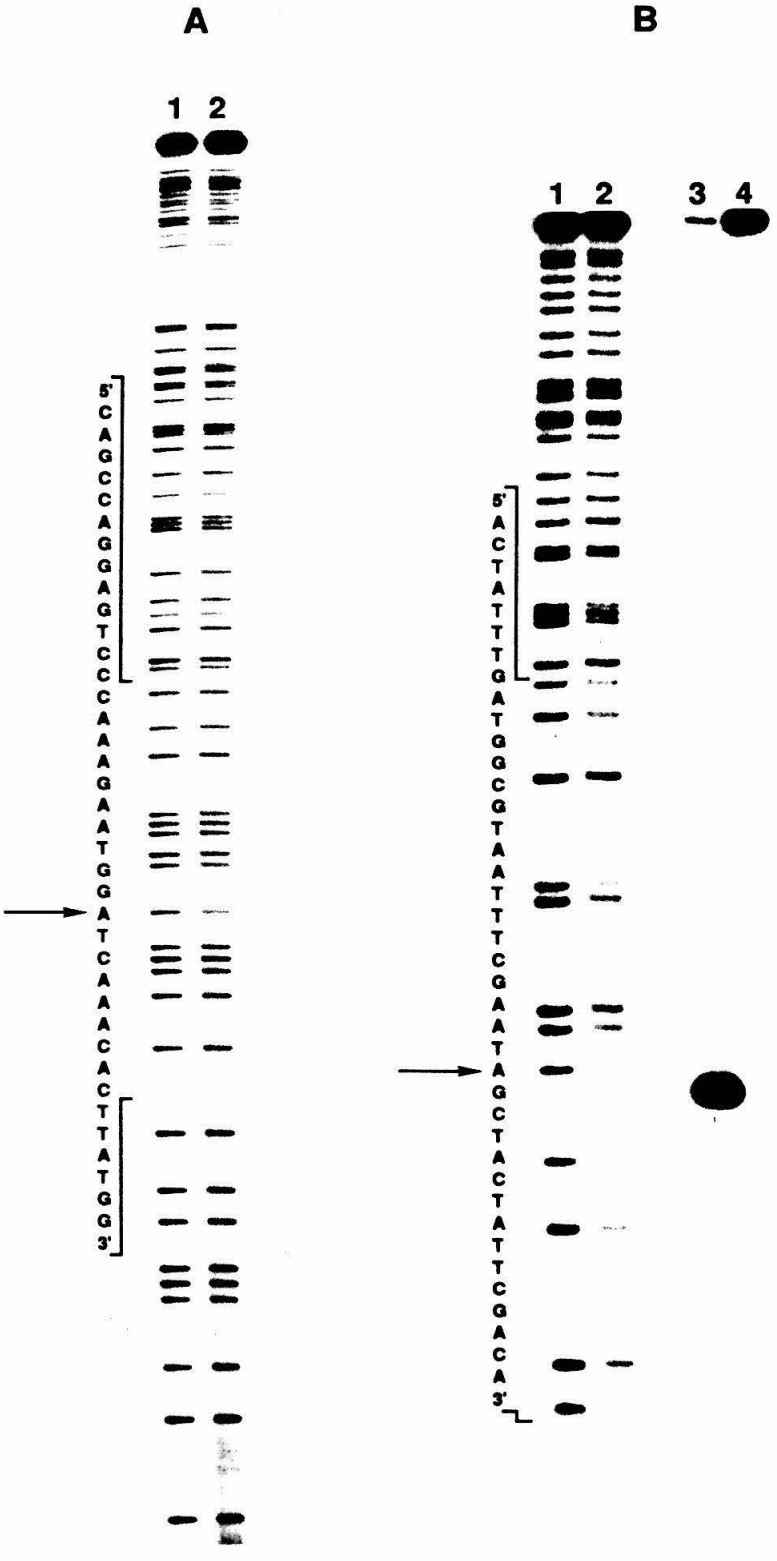
The naturally occurring N6-methyladenine was used to help determine where on adenine the Pd(II) is binding during the K_2PdCl_4 reaction. Sets of analogous DNA restriction fragments, one containing N6-methyladenine at a single position and the other containing only unmethylated adenine, were produced in two ways. In one case, one restriction fragment was prepared from lambda DNA amplified in a strain of *E. coli* which methylates the A in the sequences 5'-GATC-3' and the analogous fragment was prepared from lambda DNA amplified in a strain of *E. coli* (dam-, dcm-) which

does not methylate the adenines (Figure 54, gel A). Alternatively, *in vitro* methylation using Taq 1 methylase and SAM was used to methylate a specific position of a restriction fragment (Figure 54, gel B). The K_2PdCl_4 reaction was run on the restriction fragments and in both cases, cleavage efficiency was decreased by 35% at the N6-methyladenine relative to unmethylated adenine in the analogous sequence (see Figure 55). Assuming that a methyl group on the exo-cyclic N6 amine does not noticeably effect the nucleophilic properties of the adenine ring nitrogen atoms, the observed 35% decrease in cleavage efficiency at N6-methyladenine probably results from the methyl group on N6 creating a steric barrier to binding by Pd(II) at N1 or N7 (binding to N3 would not be effected by a steric barrier at N6). Therefore, the adenine depurination reaction apparently in-volves Pd(II) binding to the expected N1 or N7 positions. N1 (pK_a = 3.6) should be largely protonated at pH 2, so N7 emerges as the likely site of Pd(II) binding[5] during the K_2PdCl_4 reaction.

Proposed Mechanism A reasonable mechanism can be proposed for the K_2PdCl_4 A reaction at low pH which is consistent with all of the experimental results. Binding of Pd(II) to N7 of adenine residues would be expected to introduce significant positive charge into the aromatic adenine ring. Simultaneous protonation at N1 would place even more positive charge in the adenine ring thereby weakening the glycosidic bond enough to allow hydrolysis and release of the adenine-Pd(II) complex (see Figure 56). A similar depurination reaction is not likely at guanine residues, since even at

Figure 54

Comparison of the K_2PdCl_4 reaction cleavage efficiency of N6-methyladenine with unmethylated adenine in analogous sequences of DNA. Autoradiograms of dried 8% denaturing polyacrylamide gels. **Gel A** The 254 base pair restriction fragments of lambda DNA (labelled with ^{32}P on the 3' end at the Sal 1 site) with or without N6-methyladenine residues were prepared according to methods described in the Experimental Procedures section. **Lane 1** The K_2PdCl_4 reaction carried out on a 254 base pair fragment of lambda DNA containing only unmethylated adenine. **Lane 2** The K_2PdCl_4 reaction carried out on an analogous 254 base pair fragment of lambda DNA containing N6-methyladenine at the location indicated by the arrow.
Gel B A 167 base pair fragment of DNA was prepared with or without a single N6-methyladenine residue. **Lane 1** The K_2PdCl_4 reaction carried out on the 167 base pair fragment containing only unmethylated adenine. **Lane 2** The K_2PdCl_4 reaction carried out on the 167 base pair fragment methylated *in vitro* using the Taq 1 methylase. **Lane 3** Control showing the Taq 1 restriction endonuclease digestion of the 167 base pair fragment which contains only unmethylated adenine. **Lane 4** Control showing the Taq 1 endonuclease digestion of the 167 base pair fragment methylated *in vitro* using the Taq 1 methylase. The location of the N6-methyladenine residue is indicated by the arrow.


A
1 2
5'CAGCCAGGAGTCCCAAAGAATGGATCAAACACTTATGG3'
B
1 2 3 4
5'ACTATTTGATGGCGTAATTTCGAATAGCTACTATTCGACA3'

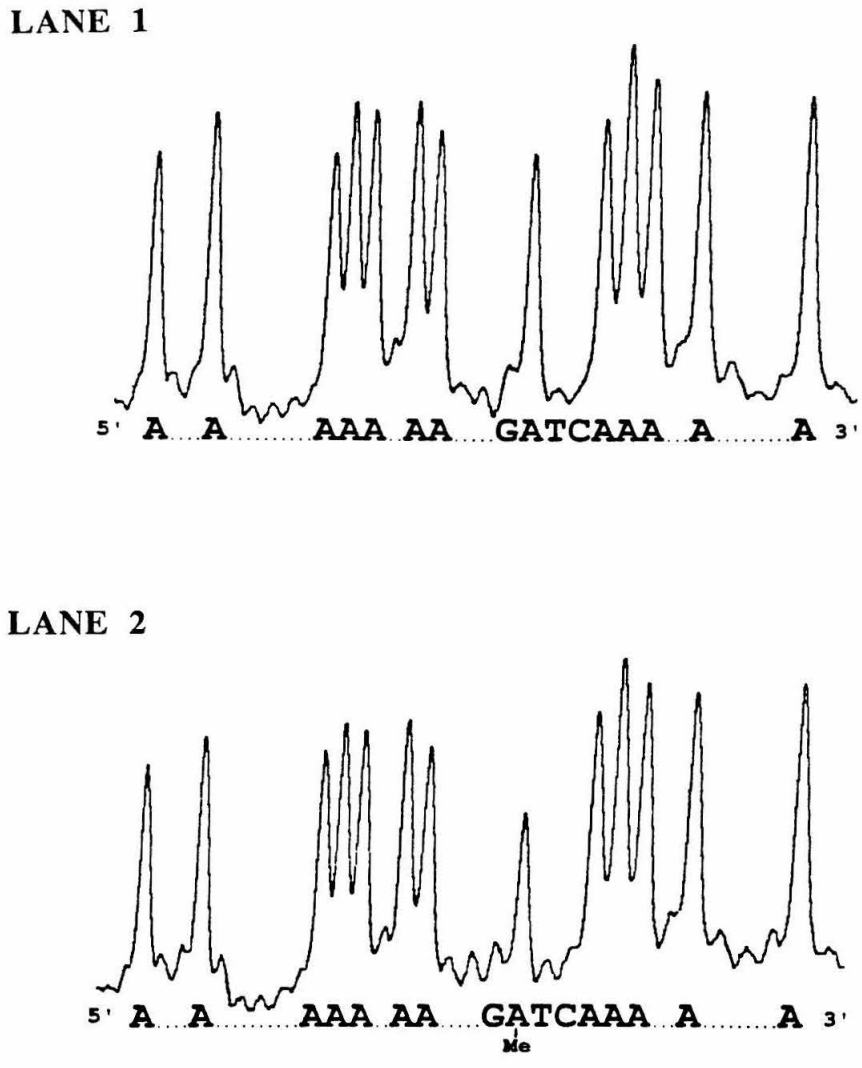


Figure 55 Densitometry of the middle region of Gel A in Figure 54 showing that the base position with N6-methyladenine (arrow, lane 2) reacts with a 35% lower efficiency than unmethylated adenine (lane 1) located in the same sequence.

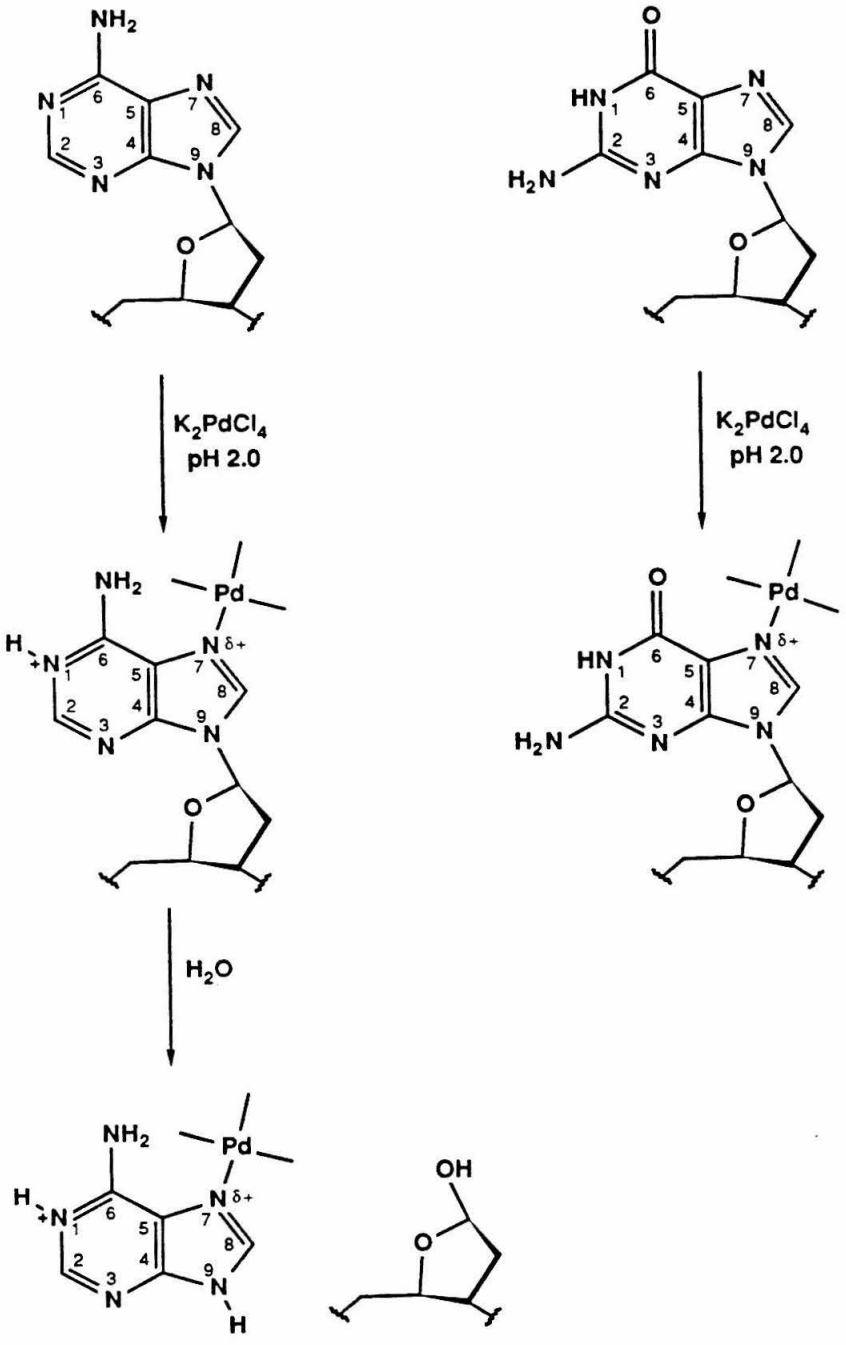


Figure 56 The proposed mechanistic scheme for the K_2PdCl_4 reaction at A vs. G.

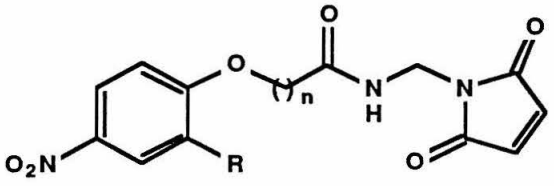
neutral pH the N1 position is protonated without creating net positive charge on the guanine ring. Without this extra positive charge, the guanine residue glycosidic bond is not weakened enough (even with Pd(II) bound at N7) to allow hydrolysis. The difference in observed reaction at adenine residues relative to guanine residues therefore derives mostly from the difference in the protonation states of the N1 positions.

This proposed mechanism is analogous to the likely mechanism of the Maxam-Gilbert A>G reaction which involves an acid treatment following DMS alkylation at guanine and adenine residues.[1] The Pd(II) binding can be considered as almost a reversible type of "alkylation" of the DNA bases, wherein the Pd(II) can be removed from the bases (through coordination to thiol) before the piperidine treatment.

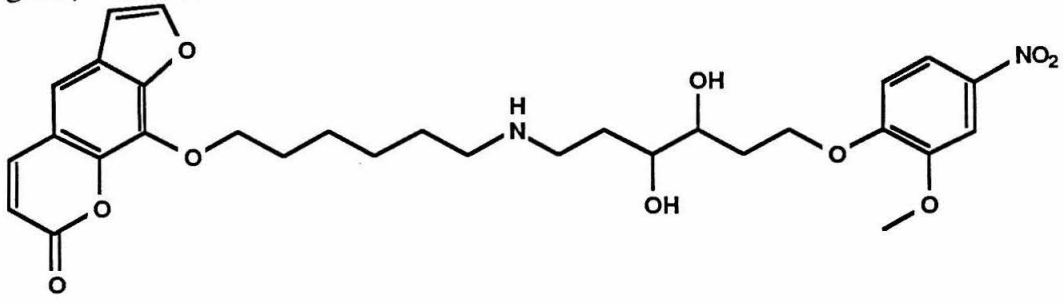
A NOVEL PHOTOCHEMICAL DNA CLEAVAGE REACTION WITH "GG" SPECIFICITY

BACKGROUND

Nitroanisole Photochemistry The use of nitroanisole and 4-nitroveratrole derivatives as photochemically activated DNA cleaving agents was suggested by the literature precedent for use of these compounds as protein photoaffinity labels and crosslinking agents. Cantor *et. al.* reported a series of bifunctional reagents consisting of a maleimide unit (a thiol specific reagent) tethered to a photoactivated 4-nitroanisole or 4-nitroveratrole group.[37] Fetal hemoglobin was

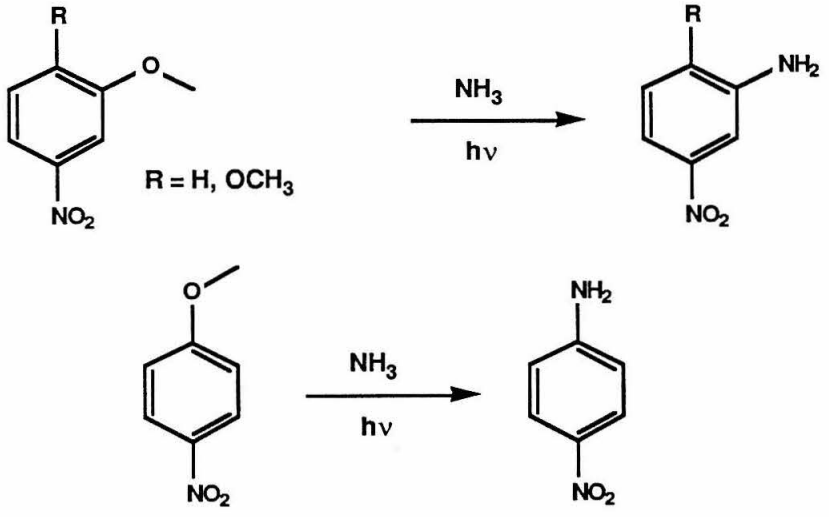


incubated then photolyzed in the presence of the reagent containing R=OCH_3 and n=3. A protein subunit-subunit crosslink was observed with an overall yield of 80%. In our laboratory, a bifunctional reagent using a 4-nitroveratrole group was investigated which was designed to photochemically crosslink DNA to protein.[38] The reagent consisted of a psoralen function (a DNA specific photochemical reagent) linked via a cleavable diol tether to the 4-nitroveratrole



moiety. Photolyzing intact bacteriophage T7 in the presence of the reagent apparently caused significant protein-DNA crosslinking, but attempted cleavage of the crosslink with periodate failed to release enough protein for analysis.

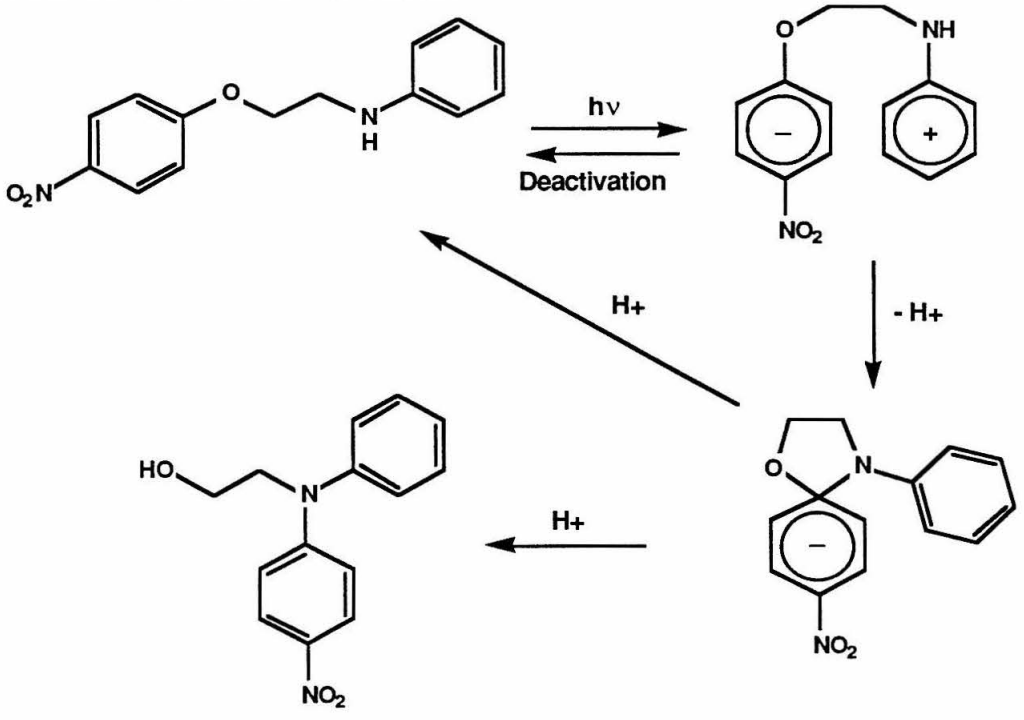
Both of the above reagents were based on the previously reported nucleophilic aromatic photosubstitution reactions of 4-nitroveratrole and nitroanisole compounds.[39-50] Upon photolysis in the presence of nucleophiles (-OH, -CN, NH_3, H_2NCH_3 etc.) 4-nitroveratrole, 3-nitroanisole and 4-nitroanisole exchange a methoxy group



with nucleophile. In the case of 4-nitroveratrole, the photochemical substitution selectively meta to the nitro group is in contrast to the observed thermal hydrolysis of the methoxy group para to the nitro function.[39] This photochemical selectivity has been of great theoretical interest.[48,49,51-55]

The mechanism of the reactions between 3-nitroanisole (or 3,5-dinitroanisole) and nucleophiles such as -OH or triethylamine (TEA)

have been studied in detail using flash photolysis techniques.[56-58] The reactions were found to be more complex than a simple nucleophilic attack onto a photoexcited state of the nitroaromatic compound. In the first stage of the reaction, the photoexcited triplet state of the nitroaromatic was found to form a charge transfer complex with a nucleophile such as -OH. This charge transfer complex led to a σ-complex (a Meissenheimer intermediate) which either decayed back to starting materials or to the substitution product. Interestingly, when TEA was the nucleophile, the charge transfer complex resulted in efficient electron transfer producing the anion of the nitroaromatic and the TEA cation.



As described in the schematic above, an electron transfer to a photoexcited nitroaromatic has also been proposed to explain the

observed intermediates in the intramolecular photo-Smiles rearrangement.[59]

Photochemistry Of Co(III) Complexes And DNA Upon photolysis in the charge transfer to metal absorption band (usually UV to near UV), the Co(III) atom in Co(III) complexes characteristically undergoes one electron reduction with the corresponding formation of a cation radical from one electron oxidation of a coordinated ligand.[60] The Co(II) species thus produced is kinetically labile and quickly exchanges ligands. The cation radical fragment of the ligand can go on to react with other components of the system.[60,61]

Several Co(III) complexes have demonstrated the ability to photochemically damage DNA. Such complexes include Co(III)-bleomycin,[62,63] tris(4,7-diphenyl-1,10-phenanthroline)Co(III),[64] tris(1,10-phenanthroline)Co(III),[65] tris(ethylenediamine)Co(III)[65] and $Co(III)(NH_3)_6$.[65] Upon photolysis these compounds produced single strand breaks in DNA and the cleavage did not require a chemical workup (heating in piperidine etc.). As of now, there is not a complete understanding of the mechanism(s) by which this damage occurs.

RESULTS AND DISCUSSION

The Nitroaromatic "GG" Reaction In hopes of discovering a photochemically activated DNA alkylating agent, DNA was photolyzed with near UV light in the presence of 4-nitroveratrole (2-10 mM) followed by a piperidine workup. Highly efficient and specific cleavage was observed at certain G residues (see Figure 57). Cleavage occurred primarily at the 5'-G of 5'-GG-3' sequences and to a somewhat lesser extent the 5'-G of 5'-GA-3' sequences. Other G residues were cleaved with minimal efficiency and no C,T or A residues were cleaved.

The three nitroanisole isomers were also investigated for cleavage activity, and only 3-nitroanisole was able to cause the photochemical "GG" reaction (see Figure 58). The specificity and efficiency of the cleavage produced by 3-nitroanisole are apparently identical to those of 4-nitroveratrole. The control reactions in lanes 9 and 10 of Figure 58 demonstrate that both light and nitroaromatic are required for the reaction. Other gels revealed that the "GG" reaction with 4-nitroveratrole or 3-nitroanisole is completely quenched by as little as .5 mM Tris-HCl. Furthermore, the cleavage efficiency of 4-nitroveratrole was found to be directly proportional to its absorbance in the near UV (300 nm - 420 nm).

In order to better understand the reaction, several nitroanisole derivatives were prepared (see Figure 59). Surprisingly, of all the derivatives, only compound **19** was able to produce the "GG"

Figure 57

Cleavage of double-stranded DNA with "GG" specificity produced by photolysis in the presence of 4-nitroveratrole. Autoradiogram of an 8% denaturing polyacrylamide gel. Lanes 1-3 contain reactions on the 517 base pair DNA fragment labelled with ^{32}P at the 3' end. Lanes 4-6 contain reactions on the 517 base pair DNA fragment labelled with ^{32}P on the 5' end. **G** Maxam-Gilbert G reaction. **Lanes 1 and 3** Photolysis (λ=355±10 nm) for 15 min in the presence of 10 mM 4-nitroveratrole. **Lanes 2 and 4** Photolysis (λ=355±10 nm) for 30 min in the presence of 10 mM 4-nitroveratrole. The 20 µl total volume photolysis reactions contained 10% by volume acetonitrile (because of the poor solubility of 4-nitroveratrole in pure water), 100 µg sonicated calf thymus DNA, 2 mM sodium cacodylate pH 7.5, 25 mM NaCl and .05 mM EDTA. After photolysis, all the reactions were ethanol precipitated then redissolved in 10% aqueous piperidine, heated at 90°C for 30 minutes, frozen, lyophilized and loaded onto the gel.
The lengths of the arrows on the histograms indicate the relative amount of cleavage observed at the different base locations as determined by densitometry. The preference for cleavage at the 5'-G of 5'-GG-3' or 5'-GA-3' sequences is apparent.

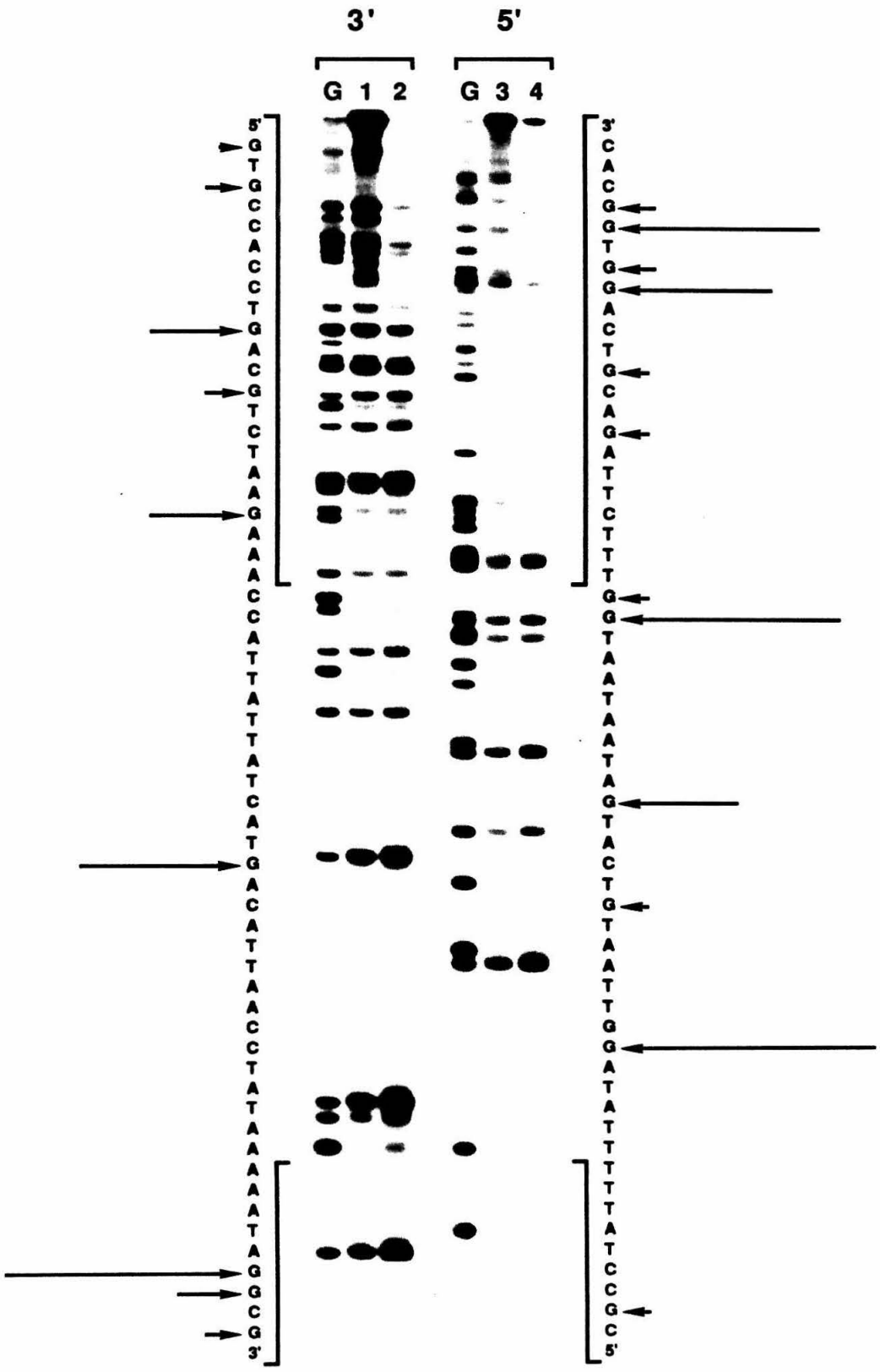
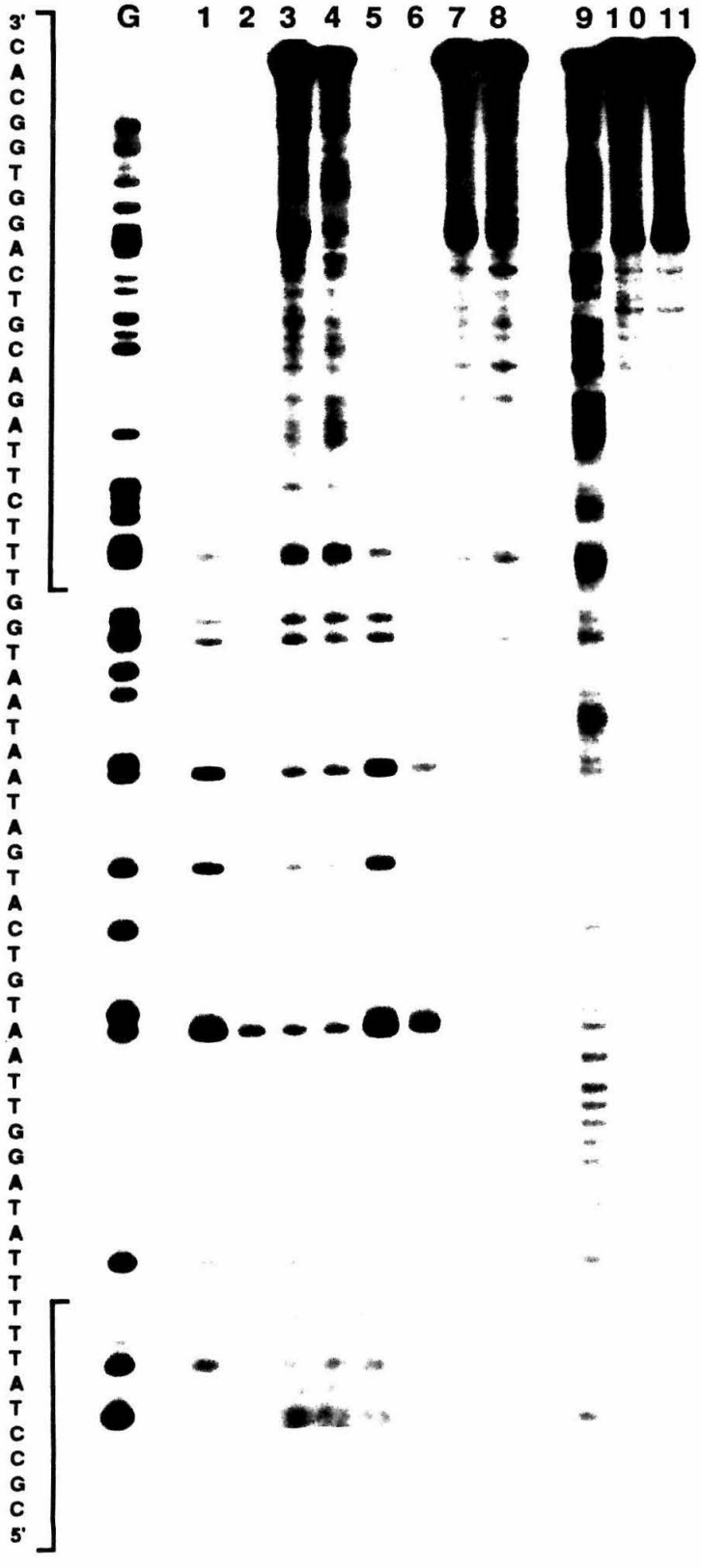

3'
5'
G 1 2
G 3 4
5'GTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGC3'
3'CACGGGTGGACTGCAGATTCTTTGGGTAATAATAGTACTGTAATTGGATATTTTTATCCGC5'

Figure 58

Comparison of the cleavages of double-stranded DNA produced by photolysis in the presence of 4-nitroveratrole, 4-nitroanisole, 3-nitroanisole and 2-nitroanisole. Autoradiogram of an 8% denaturing polyacrylamide gel. All of the lanes contain reactions on the 517 base pair DNA fragment labelled with ^{32}P on the 5' end. **G** Maxam-Gilbert G reaction. **Lanes 1 and 2** Photolysis (λ=330±10 nm) for 15 and 30 min respectively in the presence of 2 mM 4-nitroveratrole. **Lanes 3 and 4** Photolysis (λ=330±10 nm) for 15 and 30 min respectively in the presence of 2 mM 4-nitroanisole. **Lanes 5 and 6** Photolysis (λ=330±10 nm) for 15 and 30 min respectively in the presence of 2 mM 3-nitroanisole. **Lanes 7 and 8** Photolysis (λ=330±10 nm) for 15 and 30 min respectively in the presence of 2 mM 2-nitroanisole. **Lane 9** Control with photolysis (λ=330±10 nm) of DNA for 30 min with no added nitroaromatic. **Lane 10** Control with incubation of DNA with 2 mM 4-nitroveratrole for 30 min in the dark. **Lane 11** Control with DNA that was neither photolyzed nor incubated in the presence of a nitroaromatic compound. The 20 µl total volume photolysis reactions contained 10% by volume acetonitrile (because of the poor solubility of the nitroaromatics in pure water), 100 µg sonicated calf thymus DNA, 2 mM sodium cacodylate pH 7.5, 25 mM NaCl and .05 mM EDTA. After photolysis, all the reactions were ethanol precipitated then redissolved in 10% aqueous piperidine, heated at 90°C for 30 minutes, frozen, lyophilized and loaded onto the gel.


G 1 2 3 4 5 6 7 8 9 10 11
3' C A C G G T G G A C T G C A G A T T C T T T G G T A A T A A T A G T A C T G T A A T T G G A T A T T T T T A T C C G C 5'

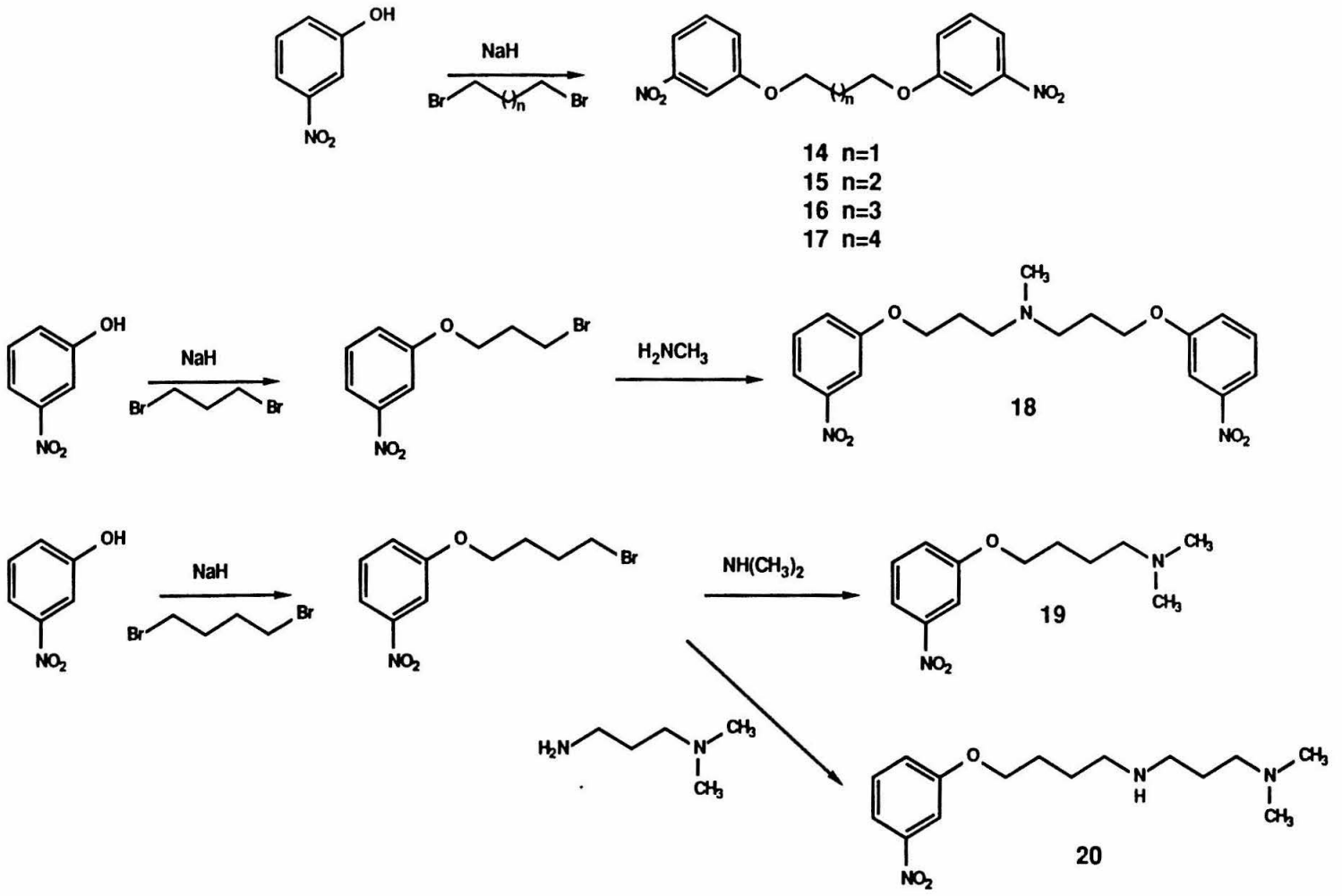
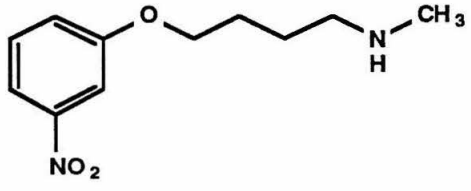


Figure 59 Synthetic schemes for the preparation of compounds **14-20**.

reaction, and none of the others exhibited photochemical DNA cleavage ability of any specificity.

Product Analysis HPLC analysis was used to help identify the product(s) of the "GG" reaction. Calf thymus DNA was photolyzed in the presence of 3-nitroanisole or **19**. The DNA was then digested with nucleases (DNAse 1 and nuclease P1) and phosphatase (alkaline phosphatase) to yield the nucleosides quantitatively which were analyzed by reverse phase HPLC. Alternatively, the DNA was heated in .1 M HCl at 90°C for 45 minutes to liberate guanine and adenine which were again examined by HPLC (see Figure 60). Using either workup, only the guanine (or guanosine) peak was observed to decrease in size upon photolysis of the DNA with 3-nitroanisole or **19**. No new peaks which absorbed at 254 nm were observed in the chromatograms except when **19** was used.

This same new peak absorbing at 254 nm was observed when **19** was photolyzed by itself (no DNA) and turned out to be demethylated **19.** This structure was confirmed by isolating the material



under the new peak and comparing its NMR spectrum and HPLC retention time with those of a synthetic sample.

To investigate further the reaction between the nitroaromatic compounds and the guanine bases, 2',3',5'-tri-O-acetylguanosine

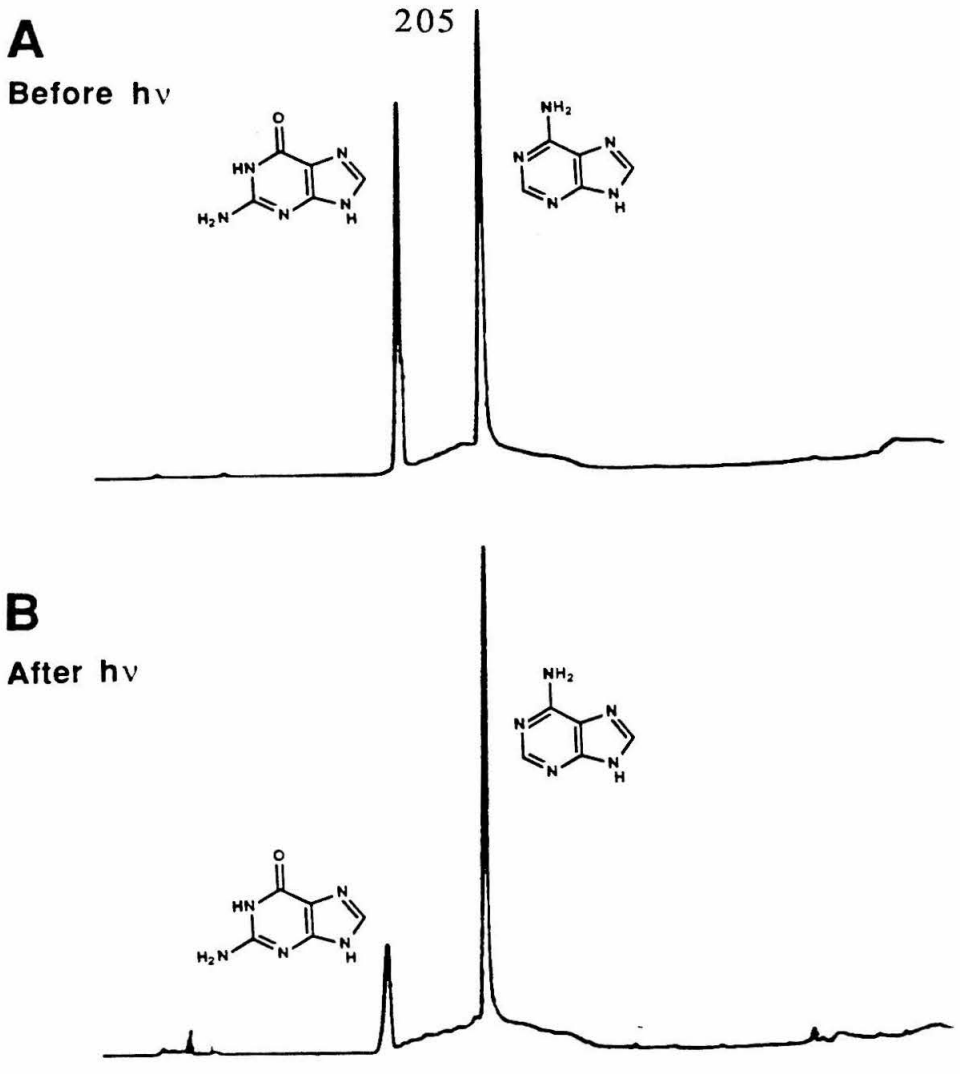


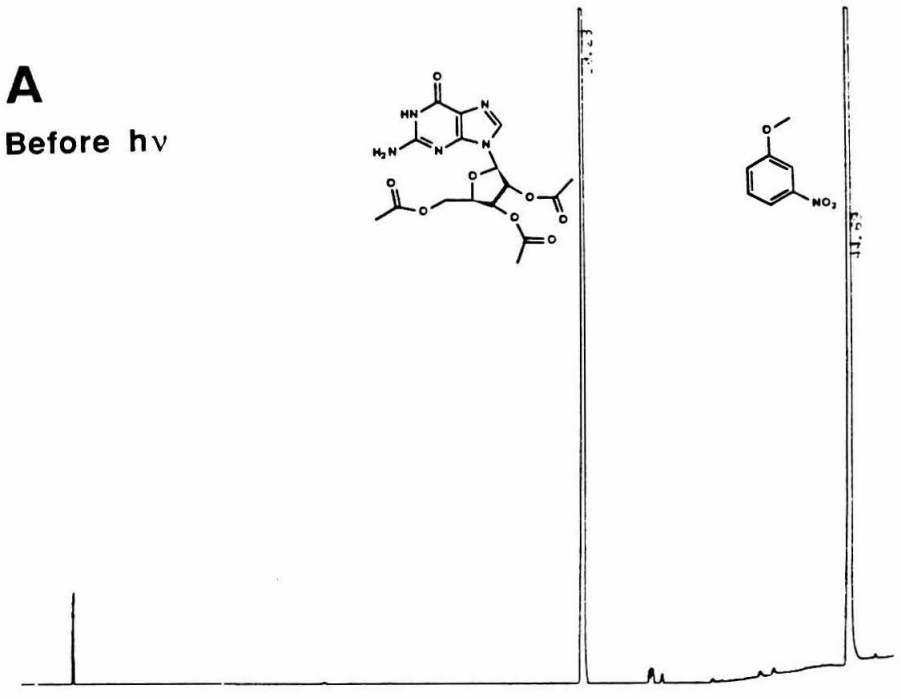
Figure 60 HPLC chromatograms (monitored at 254 nm) of reactions run on 50 μg calf thymus DNA showing that guanine is selectively destroyed upon photolysis (λ=330±10 nm for 8 h) in the presence of 6.5 mM 3-nitroanisole. The reactions were worked up by heating in .1 N HCl at 90°C for 30 min (this treatment quantitatively liberates purine residues) followed by neutralization to pH 5.5 with Tris base. The samples were chromatographed on a reverse phase C_{18} HPLC column eluted with a 20 minute linear gradient of 10-45% methanol in 5 mM sodium phosphate pH 5.5 using a flow rate of .75 ml/min. **Chromatograph A** A control reaction which was not photolyzed. **Chromatograph B** The same as chromatograph A except the sample was photolyzed. Note, the 3-nitroanisole elutes much later and is not shown on these chromatographs.

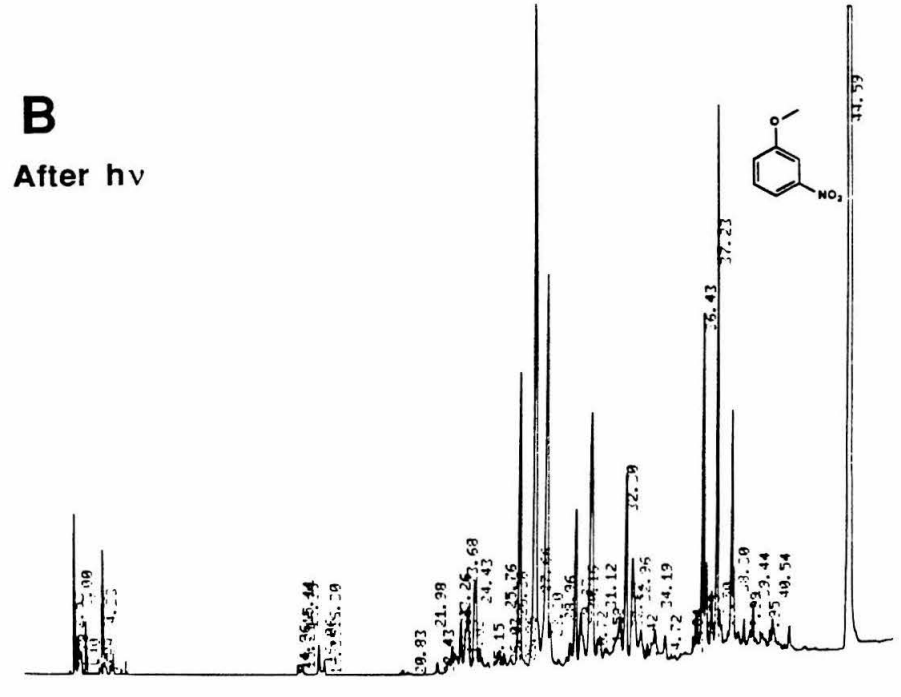
(TAG) was photolyzed in the presence of one equivalent of 3-nitroanisole and the products of the reaction were analyzed by HPLC (see Figure 61). The TAG peak was greatly diminished upon photolysis, but the amount of 3-nitroanisole was not detectably reduced. The photoexcited 3-nitroanisole was apparently catalytically decomposing the TAG.[66] Numerous new peaks appeared which must represent the TAG decomposition products. The great majority of these products absorbed 220 nm UV light but not 254 nm UV light indicating that the aromatic guanine ring was decomposed, leaving only peptide fragments. This decomposition of the guanine ring leading to peptides is reminiscent of the products reported for the electrochemical oxidation of guanine.[67,68] Controls were run wherein TAG or 3-nitroanisole were photolyzed ($\lambda \geq 300$ nm) alone. In these controls, the TAG remained unchanged and the 3-nitroanisole photolysis produced a small amount of 3-nitrophenol, a product not observed during the reaction with TAG.

The reaction between TAG and 3-nitroanisole was also examined by placing a 1:1 ratio of the compounds in an NMR tube and photolyzing the sample. Upon photolysis, the NMR peaks corresponding to the TAG protons were greatly decreased in size relative to the 3-nitroanisole protons. No new large signals were observed, but rather numerous uninterpretable small ones appeared. No methanol was detected to be a product of the reaction so a nucleophilic displacement of the methoxy group on 3-nitroanisole was apparently not even a preliminary step in the reaction.

Figure 61

HPLC chromatograms (monitored at 220 nm) of reactions run on 2',3',5,-tri-O-acetylguanosine (TAG) showing that the aromatic guanine base is decomposed into numerous products (probably peptide fragments) upon photolysis (λ=330±10 nm for 8 h) in the presence of 1 equivalent of 3-nitroanisole. The 3-nitroanisole is apparently not consumed in the reaction. The samples were chromatographed on a reverse phase C_{18} HPLC column eluted with a 20 minute linear gradient of 10-45% methanol in 5 mM sodium phosphate pH 5.5 followed by a 15 minute linear gradient or 45-75% methanol using a flow rate of .75 ml/min. **Chromatograph A** A control reaction which was not photolyzed. **Chromatograph B** The same as chromatograph A except the sample was photolyzed.


A
Before hν
NO2


B
After hν
NO2
37.23
34.59

Proposed Mechanism The above experiments have shown that the expected nucleophilic aromatic photosubstitution reaction[37,49] of 3-nitroanisole (and 4-nitroveratrole) is not the mechanism of the "GG" reaction, but rather in analogy to previously reported electrochemical results,[67] an oxidative decomposition of the guanine base is apparently occurring. The oxidative decomposition is most likely the result of photoinduced electron transfer from the aromatic guanine ring to the nitroaromatic. Electron transfer to nitroaromatics has been observed in analogous reactions.[56-58] An electron transfer pathway would most easily explain the demethylation side reaction observed with compound **19** in analogy to the enzymatic[69,70], chemical[71] and electrochemical[72] N-dealkylation reactions thought to involve electron transfer. An electron transfer pathway could also explain the apparent catalytic role of the nitroaromatic, which presumably is acting as a photoactivated electron "shuttle", not a simple electrophile.

The "GG" specificity of the 4-nitroveratrole and 3-nitroanisole photoreaction observed on double-stranded DNA can be explained with the proposed electron transfer mechanism once a key assumption is made (see Figure 62). The key assumption is that electron transfer occurs along the π system (*i.e.* between the π electrons of adjacent stacked bases) of the chiral DNA structure[73,74] faster in the 5'-3' direction than the 3'-5' direction.

The "GG" reaction is probably initiated by electron transfer from the 3'-guanine base (the ionization potential of guanine is

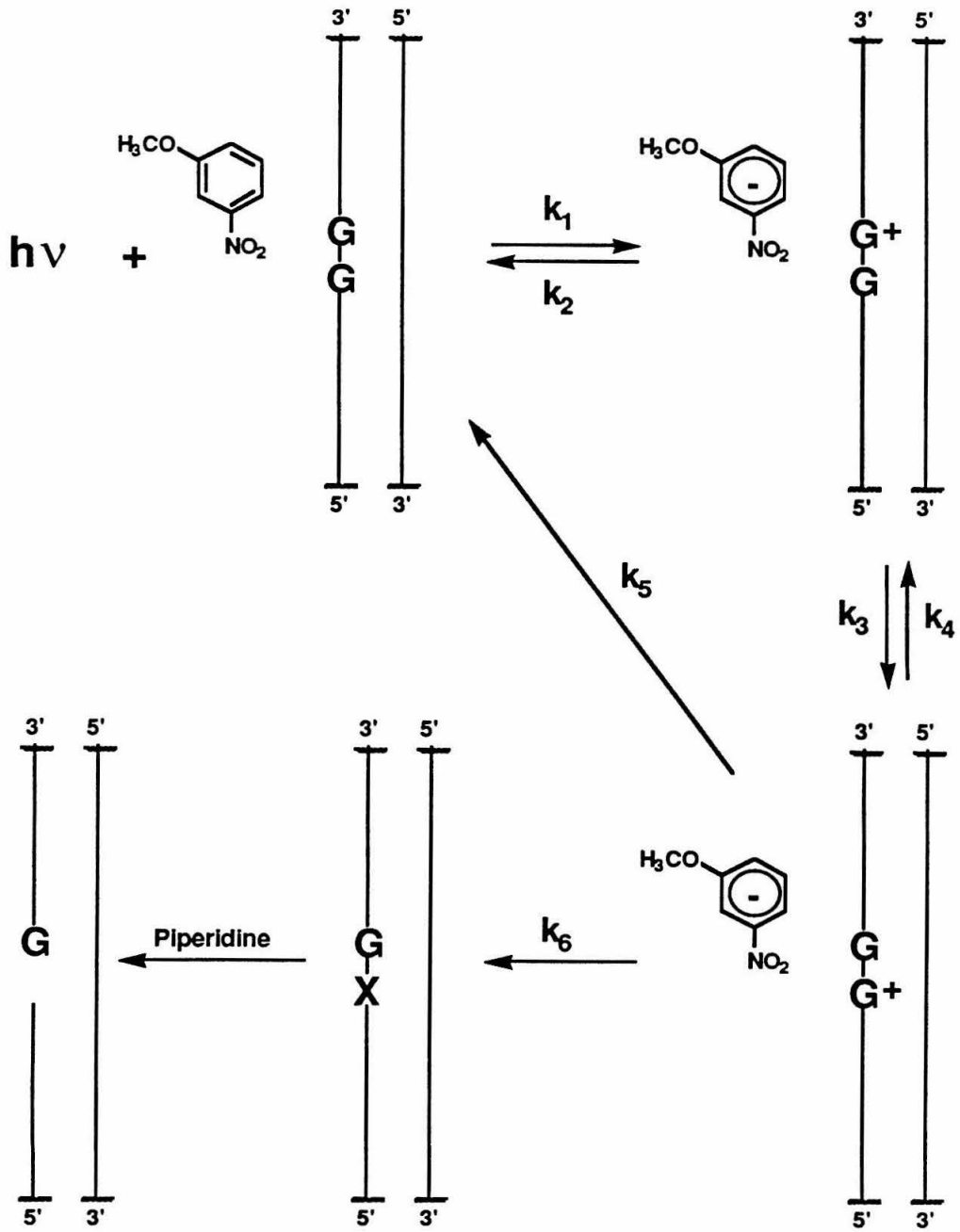


Figure 62 Proposed mechanism of the "GG" reaction. It is assumed that $k_3 > k_4$. In order to allow "GG" specificity, $k_2 \geq k_1$, $k_3 \geq k_2$ and $k_6 \geq k_5$.

8.0 ± .2 eV[75]) in the 5'-GG-3' sequence to the photoexcited nitroaromatic compound producing a 3'-guanine cation radical and the radical anion of the photoexcited nitroaromatic. An electron from the 5'-guanine is then transferred to the 3'-guanine cation radical (5'-3' electron transfer is assumed to be very rapid) resulting in a 5'-guanine cation radical and the original 3'-guanine base. The 5'-guanine cation radical apparently decomposes in a way which leads to a piperidine labile site, and the nitroaromatic anion must somehow "shuttle" its "extra" electron to an unknown acceptor. The reaction does not work in reverse (initial attack of the 5'-guanine base) causing the decomposition of the 3'-guanine base because the 3'-5' electron transfer is assumed to be not fast enough.

The reaction can also work when adenine (the ionization potential of adenine is 8.3 ± .1 eV[75]) is the 3'-base, but with less frequency probably because of the higher ionization potential of adenine relative to guanine. The pyrimidine bases apparently act as "insulators" (the ionization potentials of cytosine and thymine are 9.0 ± .1 eV and 8.95 ± .1 eV respectively[75]), since a guanine base with a cytosine or thymine base to the 3' side does not react to a significant extent.

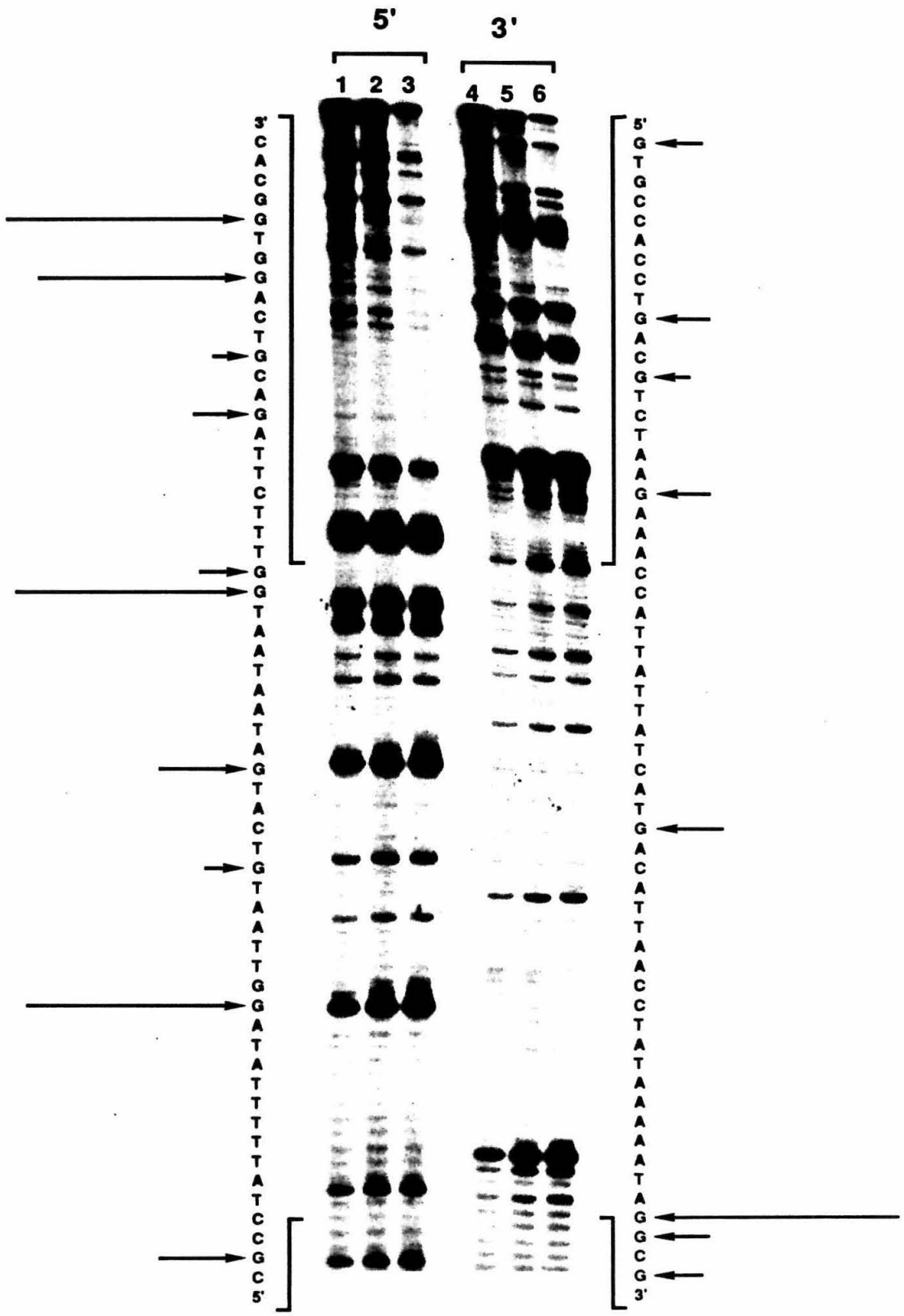
The "GG" specificity (as opposed to nonspecific reaction at all purines) implies that the "charge separation" produced by the 5'-3' electron transfer reaction along the DNA facilitates the guanine base decomposition reaction. That is, the 5'-guanine cation radical, resulting from 5'-3' electron transfer, apparently leads to base de-

composition much more often than the cation radical of a base (the 3'-guanine or adenine for example) which serves as the original electron donor to the photoexcited nitroaromatic. This is probably because the electron back transfer rate from the nitroaromatic anion to the original electron donor cation is relatively fast compared to the base decomposition rate. On the other hand, the 5'-guanine cation radical produced by the 5'-3' electron transfer is relatively far away from the nitroaromatic anion, so electron back transfer to the 5'-guanine cation radical is apparently slowed to the point that the guanine base decomposition reaction can readily occur. For this scheme to be viable, the 5'-3' electron transfer from a 5'-guanine base must be fast enough to compete with the electron back transfer to the original electron donor cation.[76]

The $Co(III)(NH_3)_6$ Photochemical "GG" Reaction Much detailed mechanistic work (flash photolysis, ESR, guanine decomposition product identification etc.) will be required before the proposed mechanism of specific DNA cleavage illustrated in Figure 62 can be confirmed. However, strong additional evidence for at least the electron transfer component of the mechanism was obtained by photolyzing $Co(III)(NH_3)_6$ with double-stranded DNA followed by a piperidine workup. $Co(III)(NH_3)_6$ has been indicated in the literature to be a photoactivated DNA cleaver,[65] but the specificity of cleavage was not reported. I found that photolyzing ($\lambda \geq 300$ nm) $Co(III)(NH_3)_6$ (1 mM) in the presence of double-stranded DNA followed by a piperidine workup produced a cleavage reaction at

Figure 63

Cleavage of double-stranded DNA with "GG" specificity produced by photolysis in the presence of $Co(III)(NH_3)_6$. Autoradiogram of an 8% denaturing polyacrylamide gel. **Lanes 1,2 and 3** Photolysis ($\lambda \geq 300$ nm) of the 517 base pair DNA fragment (labelled on the 5' end) for 10, 20 and 40 min respectively in the presence of 200 μM $Co(III)(NH_3)_6$. **Lanes 4,5 and 6** Photolysis ($\lambda \geq 300$ nm) of the 517 base pair DNA fragment (labelled on the 3' end) for 10, 20 and 40 min respectively in the presence of 200 μM $Co(III)(NH_3)_6$. The 20 μl total volume photolysis reactions contained 100 μg sonicated calf thymus DNA, 10 mM sodium cacodylate pH 7.0 and 20 mM NaCl. After photolysis, all the reactions were ethanol precipitated then redissolved in 10% aqueous piperidine, heated at 90°C for 30 minutes, frozen, lyophilized and loaded onto the gel.
The lengths of the arrows on the histograms indicate the relative amount of cleavage observed at the different base locations as determined by densitometry. The preference for cleavage at the 5'-G of 5'-GG-3' or 5'-GA-3' sequences is apparent.


5'
3'
1 2 3
4 5 6
3' CACGGTGGACTGCAGATTCTTTGGTAATAATAGTACTGTAATTGGATATTTTTATCCGC 5'
5' GTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCG 3'

guanine residues with specificity markedly similar to the 4-nitroveratrole or 3-nitroanisole cleavage (see Figure 63). The only similarities in the structures and/or chemistries of Co(III)$(NH_3)_6$ and the nitroaromatics are the characteristic electron transfer pathways of their photochemistries,[56-60] thus lending support for the electron transfer mechanism of the "GG" reaction. *Cis*-β-Co(III)(trien)(H2O)(OH) was observed to produce the "GG" reaction as well. Other inorganic as well as organic molecules should be capable of photoexcited electron transfer involving similar reduction potentials, so this "GG" reaction may turn out to be a relatively general phenomenon.

Attempted Complementary-Addressed Cleavage Of Single-Stranded DNA Using A Nitroaromatic Group As A Cleaving Function The 3-nitroanisole and 4-nitroveratrole groups appeared to be ideal candidates for use as photoactivated cleavage functions of complementary-addressed reagents. The group is chemically inert until photoactivated and appears to be catalytic, so extremely high cleavage efficiencies should be possible. Furthermore, the observed "GG" cleavage reaction is so specific that cleavage to nucleotide resolution should be possible.

For these reasons, compounds **21-23** were synthesized according to the schemes outlined in Figure 64.[77] All of these compounds were accepted as substrate by the Klenow enzyme and incorporated into oligonucleotide 1,2 duplexes (see Figure 8 for sequences). Unfortunately, upon prolonged photolysis ($\lambda \geq 300$ nm) and a piperi

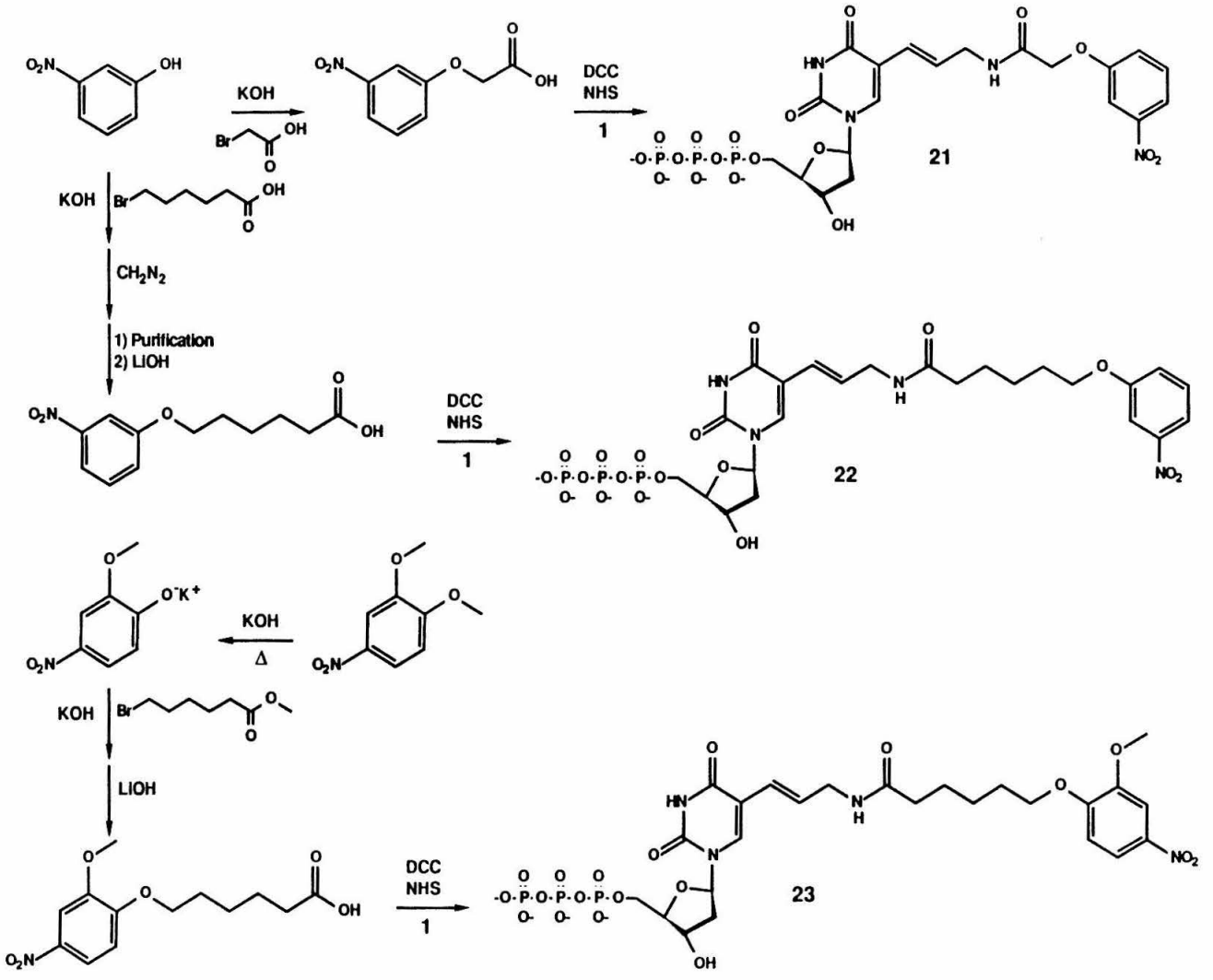


Figure 64 Synthetic schemes for the preparation of compounds **21-23**.

dine workup, no specific cleavage of the target DNA strand was observed with any of the compounds.

Paul Brown in our laboratory also synthesized several compounds with the 4-nitroveratrole or 3-nitroanisole group in hopes of producing site specific cleavage of DNA upon photolysis (see Figure 65). The modified nucleoside compound was chemically incorporated into an oligonucleotide strand that was complementary to oligonucleotide 1, but unfortunately upon photolysis no site specific cleavage was observed. The distamycin derivatives were examined for cleavage activity on a restriction fragment of double-stranded DNA known to have several distamycin binding sites (the 517 base pair fragment of pBR322), but again upon photolysis no site specific cleavage was observed.

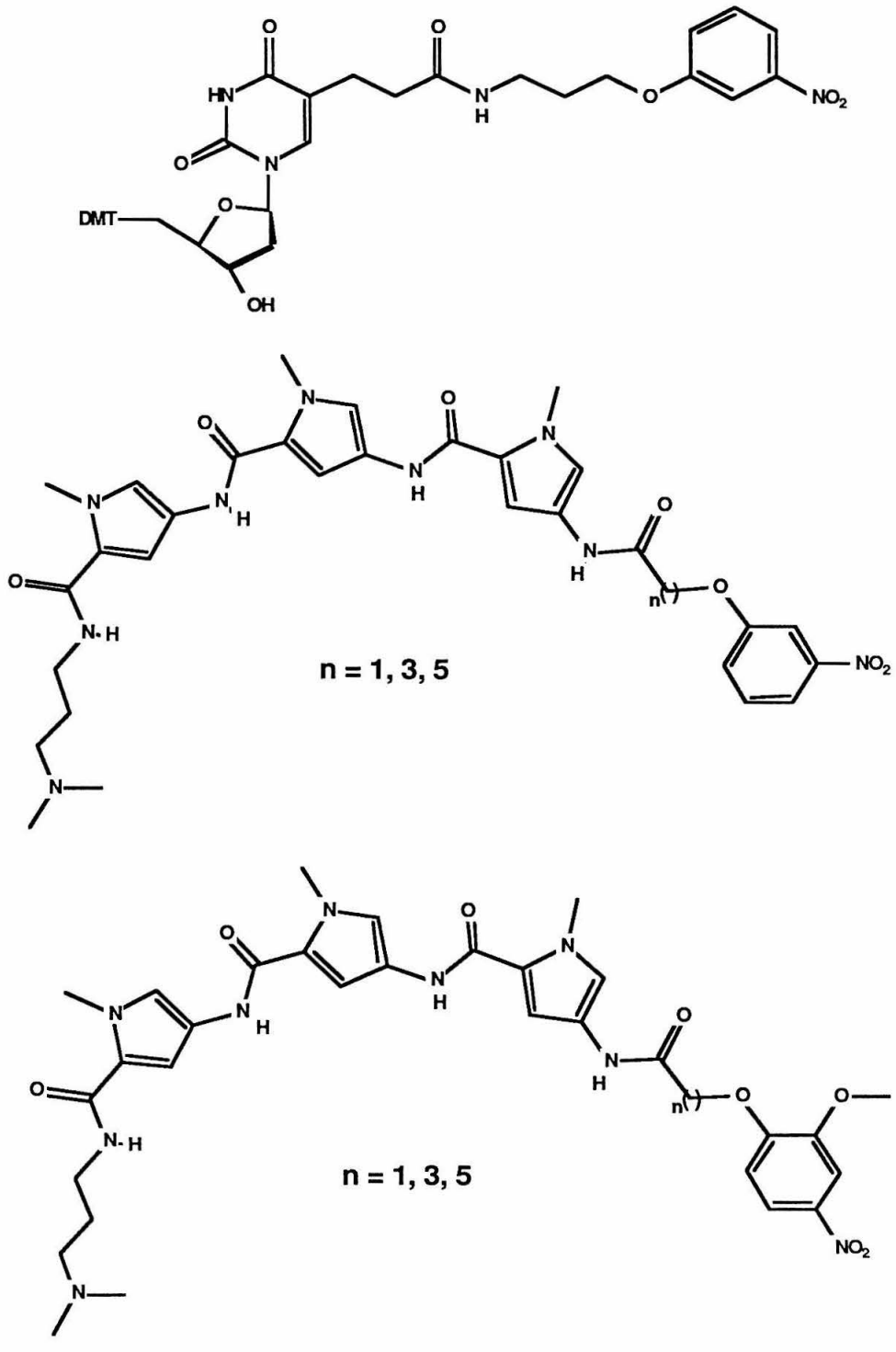


Figure 65 Molecules prepared by Paul Brown which carry a 3-nitroanisole or 4-nitroveratrole group.

CONCLUSION

While working in the general area of complementary-addressed modification and cleavage of single-stranded DNA, two new base specific DNA cleavage reactions were discovered. The first was discovered serendipitously (the K_2PdCl_4 reaction at adenine), and the second (the "GG" reaction) had somewhat more rational origins. Both reactions apparently involve novel chemistry on the DNA.

The reaction of K_2PdCl_4 with DNA at low pH turned out to be a convenient and reliable method for the production of an adenine specific chemical sequencing lane. The reaction probably involves binding of Pd(II) to N7 and protonation at N1 of adenine which results in specific depurination of adenine. The DNA is then cleaved at the depurinated sites by piperidine and heat. This A specific reaction may be timely with regard to developing chemistry for automated sequencing requiring four base specific chemical methods.

The "GG" reaction caused by a photoexcited nitroaromatic or Co(III) compound displays a novel specificity. A preliminary product analysis of the reaction between guanine and a nitroaromatic revealed that the reaction apparently does not involve the expected aromatic photosubstitution reaction, but rather the nitroaromatic is acting in a catalytic manner. Guanine was selectively decomposed during the reaction leaving numerous fragments which are probably peptides. A mechanism involving electron transfer is proposed to explain the "GG" specificity and the apparent catalytic nature of the nitroaromatic molecule. Such an electron transfer mechanism is not

unreasonable considering what is known about the characteristic electron transfer pathways of the photochemistry of both the nitroaromatics and the Co(III) compounds.

The reasons for the failure of compounds **21-23** (and the molecules prepared by Paul Brown) to produce complementary-addressed cleavage of DNA remain unclear. The long tether of compounds **22** and **23** in particular should have made a wide variety of geometries possible between the 4-nitroveratrole or 3-nitroanisole group and the target bases. Despite the disappointing results, the mechanism of the "GG" reaction should be more thoroughly investigated, and hopefully what is learned could be applied to the design of new complementary-addressed reagents which can fully utilize the "cleavage potential" of the photochemical "GG" reaction.

EXPERIMENTAL PROCEDURES

5-[3-[[3-diazo-2-oxopropionyl]amino]-*trans*-1-propenyl]-2'-deoxyuridine 5'-triphosphate (13) 4-nitrophenol (1 g, 6.5 mmol, Aldrich Gold Label) was dissolved in 25 ml freshly distilled oxalyl chloride (Aldrich) under Ar and heated at reflux overnight. The excess oxalyl chloride was removed *in vacuo* then the residue was placed in 8 ml dry ether and filtered under Ar. The solvent was removed from the filtrate. The filtrate residue was redissolved in 12 ml ether and ethereal diazomethane (produced with the Aldrich diazomethane kit) was added dropwise. After the yellow color of diazomethane persisted, the solution was filtered (under Ar!) and the precipitate was collected and dried *in vacuo* to yield *p*-nitrophenyl 3-diazo-2-oxo-propionate (300 mg, 19%) (The preceding procedure followed that of Lawton *et. al.*[78] as modified by Paul Brown). *p*-Nitrophenyl 3-diazo-2-oxo-propionate (45 mg, .2 mmol) was placed in 5.0 ml DMF (freshly distilled over CaH at reduced pressure) under argon along with **1** (50 mg, .06 mmol) in 5 ml .1 M sodium borate buffer pH 8.85. Aliquots (40 µl) were periodically removed and subjected to the quantitative ninhydrin test. The reaction was stopped after 18 h since there was no more detectable **1**. The reaction was diluted with 100 ml water and placed upon an 18 x 160 mm DEAE-Sephadex A-25-120 column pre-equilibrated with .05 M TEAB pH 7.6. The column was eluted under slight air pressure with 40 ml each of .1 M, .2 M, .3 M, .4 M, .5 M, .6 M, .7 M, .8 M, .9 M, 1.0 M and 1.2 M TEAB. The crude product **13** was

eluted between .5 M and .6 M TEAB as the last UV absorbing compound and the pooled fractions were lyophilized to dryness. The crude **13** was redissolved in water and purified by preparative ion exchange HPLC on a Synchropak Q300 column monitored at 260 nm and 290 nm. Compound **13** was the major component eluted (at ~19 min) with a 30 minute linear gradient of 0-0.3 M AMB pH 7.6 and a flow rate of .75 ml/min. To avoid bubbles forming in the HPLC pump piston chambers, the AMB buffer was chilled in ice during the chromatography. The appropriate fractions were pooled and lyophilized to dryness to yield the ammonium salt of pure **13**. ^{1}H NMR (D_2O) δ 7.81 (1H, s, H6), 6.31-6.36 (1H, m, =CH-), 6.19-6.24 (2H, m, -HC=, H1'), 4.54-4.58 (1H, m, H3'), 4.09-4.14 (3H, m, H5', H4'), 3.89 (2H, d, J= 5.6 Hz, CH_2), 2.25-2.30 (2H, m, H2'). UV-Vis (H_2O) λ_{max}= 246 nm, λ_{min}= 272 nm, λ_{max}= 298. IR (KBr) 3200, 2100 (characteristic diazo stretch), 1690, 1620, 1530, 1420, 1380, 1340, 1080, 900, 800.

1,X-bis-(3-nitrophenoxy)-alkane Compounds (14-17) 3-nitrophenol (500 mg, 3.6 mmol, Aldrich) was placed in 3 ml DMF under Ar and cooled to 0°C in an ice bath. NaH (86 mg, 3.6 mmol) was added and the mixture was stirred for 2.5 h at 0°C. 1,3-dibromopropane was added and the solution was stirred at room temperature for 2 days. The reaction was quenched with 50 ml water and the yellow precipitate was collected and chromatographed on silica gel eluted with methylene chloride to yield pure **14** (252 mg, 22%). Compounds **15-17** were prepared in the same way with the appropriate dibromoalkane substituted for 1,3-dibromopropane. Isolated

yields ranged from 20-70%. **1,3-bis-(3-nitrophenoxy)propane (14)** TLC (methylene chloride) R_f = .5 visualized with short wave UV. ^1H NMR ($CDCl_3$) δ 7.8 (4H, m, Ar), 7.3 (4H, m, Ar), 4.3 (4H, t, J= 7.5 Hz, CH_2-), 2.3 (2H, m, J= 7.5 Hz, -CH_2-). UV-Vis (H_2O) λ_{max}= 276 nm, 335 nm. IR (KBr) 3080, 1620, 1580, 1510, 1400, 1470, 1240, 1090, 1010, 810, 730. MS (EI) calculated for $C_{15}H_{14}N_2O_6$ m/z= 318; found 318. **1,4-bis-(3-nitrophenoxy)butane (15)** TLC (methylene chloride) R_f = .75 visualized with short wave UV. ^1H NMR ($CDCl_3$) δ 7.8 (4H, m, Ar), 7.3 (4H, m, Ar), 4.15 (4H, m, CH_2-), 2.1 (4H, m, -CH_2-). UV-Vis (H_2O) λ_{max}= 276 nm, 335 nm. IR (KBr) 3080, 2940, 1615, 1580, 1520, 1470, 1340, 1280, 1240, 1090, 1030, 1010, 860, 810, 730. MS (EI) calculated for $C_{16}H_{16}N_2O_6$ m/z= 332; found 332. **1,5-bis-(3-nitrophenoxy)pentane (16)** TLC (methylene chloride) R_f = .8 visualized with short wave UV. ^1H NMR ($CDCl_3$) δ 7.8 (4H, m, Ar), 7.3 (4H, m, Ar), 4.0 (4H, t, J= 5.5 Hz, CH_2-), 1.8 (6H, m, -CH_2-). UV-Vis (H_2O) λ_{max}= 276 nm, 335 nm. IR (KBr) 3020, 1620, 1580, 1510, 1400, 1470, 1240, 1090, 1010, 810, 730. MS (EI) calculated for $C_{17}H_{18}N_2O_6$ m/z= 346; found 346. **1,6-bis-(3-nitrophenoxy)hexane (17)** TLC (chloroform) R_f = .7 visualized with short wave UV. ^1H NMR ($CDCl_3$) δ 7.8 (4H, m, Ar), 7.3 (4H, m, Ar), 4.06 (4H, t, J= 6.3 Hz, CH_2-), 1.9 (4H, m, -CH_2-), 1.6 (4H, m, -CH_2). UV-Vis (H_2O) λ_{max}= 276 nm, 335 nm. IR (KBr) 3010, 2900, 1620, 1580, 1510, 1470, 1390, 1240, 1090, 1010, 810, 730. MS (EI) calculated for $C_{18}H_{20}N_2O_6$ m/z= 360; found 360.

N-Methylamino-bis-[3-(3-nitrophenoxy)propane] (18) 3-nitrophenol (500 mg, 3.6 mmol) was placed in 3 ml DMF under Ar and cooled to 0°C in an ice bath. NaH (86 mg, 3.6 mmol) was added and the mixture was stirred for 2.5 h at 0°C. 1,3-dibromopropane (5 ml, 49 mmol) was added and after 3 days, the solvent was removed *in vacuo* and the 1-bromo-3-(3-nitrophenyloxy)propane was purified by chromatography on silica gel eluted with 5% methanol in methylene chloride to yield a golden oil (200 mg, 24%). 1-bromo-3-(3-nitrophenyloxy)propane (200 mg, .77 mmol) was dissolved in 2 ml DMF along with triethylamine (268 µl, 1.92 mmol). Methylamine (40% aqueous solution, 30 µl, .39 mmol) was added and the solution was stirred under Ar for 3 days. The product was purified by chromatography on silica gel eluted with 10% methanol in methylene chloride to yield **18**. TLC (10% methanol/methylene chloride) R_f = .5 visualized with short wave UV. ^1H NMR ($CDCl_3$) δ 7.7 (4H, m, Ar), 7.3 (4H, m, Ar), 4.06 (4H, t, CH_2-), 2.58 (4H, t, CH_2-), 2.3 (3H, s, CH_3) 1.98 (4H, m, -CH_2-). UV-Vis (H_2O) λ_{max}= 376 nm, 335 nm. IR (KBr) 2990, 1620, 1520, 1350, 1240, 730. MS (EI) calculated for $C_{19}H_{23}N_3O_6$ m/z= 389; found 389.

1-(N,N-Dimethylamino)-4-(3-nitrophenoxy)butane (19) 3-nitrophenol (500 mg, 3.6 mmol) was placed in 3 ml DMF under Ar and cooled to 0°C in an ice bath. NaH (86 mg, 3.6 mmol) was added and the mixture was stirred for 2.5 h at 0°C. 1,4-dibromopropane (5 ml, 42 mmol) was added and after 3 days, the solvent was removed *in vacuo* and the 1-bromo-4-(3-nitrophenoxy)butane was purified

by chromatography on silica gel eluted with 1:1 Cyclohexane: methylene chloride to yield a golden oil (661 mg, 67%). 1-bromo-4-(3-nitrophenoxy)butane (300 mg, 1.1 mmol) was placed in 3 ml acetonitrile and dimethylamine (40% aqueous solution, 678 µl, 6 mmol) was added. The solution was stirred under Ar for 24 h at room temperature then the product was purified by chromatography on a 2.0 cm x 12 cm silica gel column eluted with 30% methanol in methylene chloride. Pure **19** was obtained after recrystallization from methanol (257 mg, 97%). MP 186-7°C TLC (30% methanol/methylene chloride) R_f = .6 visualized with short wave UV. ^1H NMR ($CDCl_3$) δ 7.8 (4H, m, Ar), 7.4 (4H, m, Ar), 4.1 (2H, t, J= 5.8 Hz, CH_2-), 3.16 (2H, t, J= 8.1 Hz, CH_2-), 2.9 (6H, s, CH_3) 2.15 (2H, m, -CH_2-) 1.97 (2H, m, -CH_2-). IR (KBr) 2920, 2660, 1530, 1475, 1350, 1280, 1240. MS (Pos Ion FAB) calculated for $C_{12}H_{18}N_2O_3$ m/z= 238; found M+H= 239. Synthetic 1-(N-methylamino)-4-(3-nitrophenoxy)butane was prepared exactly as above except methylamine was used in place of dimethylamine and the product was purified by a silica gel column eluted with 6% (3% ammonia in water) in methanol. TLC (6% (3% ammonia in water) in methanol) R_f = .5 visualized with short wave UV. ^1H NMR ($CDCl_3$) δ 7.8 (4H, m, Ar), 7.4 (4H, m, Ar), 4.06 (2H, t, J= 6.3 Hz, CH_2-), 2.67 (2H, t, J= 7.2 Hz, CH_2-), 2.5 (3H, s, CH_3), 1.89 (2H, m, CH_2), 1.68 (2H, m, -CH_2-).

1-[3-(N,N-dimethylamino)propylamino]-4-(3-nitrophenoxy)butane (20) 1-bromo-4-(3-nitrophenoxy)butane (300 mg, 1.1 mmol) was placed in 3 ml acetonitrile and 1-amino-3-N,N-

dimethylaminopropane (1.4 ml, 950 mmol) was added. The solution was stirred under Ar for 24 h at room temperature then the product was purified by chromatography on a 2.0 cm x 12 cm silica gel column eluted with 6% (3% ammonia in water) in methanol (75 mg, 23%). TLC (6% (3% ammonia in water) in methanol) R_f = .7 visualized with short wave UV. ^1H NMR ($CDCl_3$) δ 7.8 (4H, m, Ar), 7.4 (4H, m, Ar), 4.05 (2H, t, J= 6.4 Hz, CH_2-), 2.67 (4H, m, CH_2-), 2.31 (2H, t, J= 7.2 Hz, CH_2-)2.22 (6H, s, CH_3) 1.88 (2H, m, -CH_2-) 1.65 (4H, m, -CH_2-). IR (KBr) 2920, 1530, 1350, 1240. MS (Pos Ion FAB) calculated for $C_{13}H_{25}N_3O_3$ m/z= 295; found M+H= 296.

5-[3-[[2-(3-nitrophenoxy)acetyl]amino]-*trans*-1-propenyl]-2'-deoxyuridine 5'-triphosphate (21) This compound was first prepared by Marius Sutter. 3-Nitrophenoxyacetic acid (prepared by Marius Sutter, 175 mg, .81 mmol) was placed in 10 ml acetonitrile along with NHS (101 mg, .88 mmol) and DCC (181 mg, .88 mmol) and the solution was stirred overnight. The mixture was then filtered and the solvent was removed from the filtrate *in vacuo*. The residue was redissolved in 2 ml DMF. To this DMF solution was added a 20 ml solution containing **1** (70 mg, .11 mmol) in .1 N sodium borate buffer pH 8.5. After 6 h at room temperature, the reaction was diluted with 100 ml water and placed upon an 18 x 160 mm DEAE-Sephadex A-25-120 column pre-equilibrated with .05 M TEAB pH 7.6. The column was eluted under slight air pressure with 40 ml each of .1 M, .2 M, .3 M, .4 M, .5 M, .6 M, .7 M, .8 M, .9 M, 1.0 M and 1.2 M TEAB. The crude product **21** was eluted between .7 M and .8

M TEAB as the last UV absorbing compound and the pooled fractions were lyophilized to dryness. The product was purified by prep HPLC (eluted at ~17 min on a 22 mm x 25 cm preparative C_{18} reverse phase HPLC column with a 25 min linear gradient of 0-25% acetonitrile in 5 mM ammonium acetate pH 5.5 with a flow rate of 10 ml/min) to yield the ammonium salt of pure **21** (22 mg, 26%). ^1H NMR (D_2O) δ 7.81 (3H, m, H6, Ar), 7.4 (2H, m, Ar), 6.31-6.36 (2H, m, =CH-, -HC=), 6.2 (1H, d, H1'), 4.54-4.58 (1H, m, H3'), 4.1 (3H, m, H5', H4'), 3.9 (2H, d, CH_2), 2.25-2.30 (2H, m, H2'). IR (KBr) 3200, 1625, 1545, 1455, 1380, 1290, 1030.

5-[3-[[6-(3-nitrophenoxy)hexyl]amino]-*trans*-1-propenyl]-2'-deoxyuridine 5'-triphosphate (22). 3-Nitrophenol (2.78 gm, 20 mmol) was placed in 20 water along with 6-bromohexanoic acid (3.8 gm, 20 mmol). To this was added dropwise 2.0 M KOH (20 ml, 40 mmol). The solution was heated at reflux for 6 h then brought to pH 2.0 with HCl and extracted 2x 50 ml ether. The ether layer was washed 1x 50 ml with .5 M sodium bicarbonate solution then acidified to pH 2.0 with HCl. Attempts at recrystallization and chromatography failed to produce pure material so 1.0 gm of the crude acid was placed in 20 ml ether at 0°C. Ethereal diazomethane (generated with the Aldrich diazomethane kit) was added until the yellow color persisted. Pure methyl 6-(3-nitrophenoxy)hexanoate was isolated by chromatography on a 4 x 20 cm silica gel column eluted with 2% acetonitrile in methylene chloride. TLC (2% acetonitrile in methylene chloride) R_f= .75, visualized with short wave UV.

^{1}H NMR ($CDCl_3$) δ 7.8 (4H, m, Ar), 7.4 (4H, m, Ar), 4.06 (2H, t, J= 6.5 Hz, CH_2-), 3.65 (3H, s, CH_3), 2.37 (2H, t, J= 7.4 Hz, CH_2-), 1.84 (2H, m, -CH_2-), 1.7 (2H, m, -CH_2-), 1.55 (2H, m, -CH_2-). Methyl 6-(3-nitrophenoxy)hexanoate (462 mg, 1.74 mmol) was dissolved in 60 ml 1:1 acetonitrile: water and 6 m of 1.0 N LiOH was added. The solution was heated to 60°C for 20 min then acidified to pH 2.0 with HCl, extracted 5x 50 ml ether, dried over sodium sulfate and the solvent was removed *in vacuo* to yield pure 6-(3-nitrophenoxy)hexanoic acid (432 mg, 98%). ^{1}H NMR ($CDCl_3$) δ 7.8 (4H, m, Ar), 7.4 (4H, m, Ar), 4.04 (2H, t, J= 6.4 Hz, CH_2-), 2.42 (2H, t, J= 7.5 Hz, CH_2-), 1.86 (2H, m, -CH_2-) 1.76 (2H, m, -CH_2-), 1.56 (2H, m, -CH_2-). 6-(3-nitrophenoxy)hexanoic acid (125 mg, .49 mmol) was placed in 15 ml acetonitrile along with NHS (63 mg, .55 mmol) and DCC (113 mg, .55 mmol) and the solution was stirred overnight. The mixture was then filtered and the solvent was removed from the filtrate *in vacuo*. The residue was redissolved in 2 ml DMF. To this DMF solution was added a 20 ml solution containing **1** (70 mg, .11 mmol) in .1 N sodium borate buffer pH 8.5. After 6 h at room temperature, the reaction was diluted with 100 ml water and placed upon an 18 x 160 mm DEAE-Sephadex A-25-120 column pre-equilibrated with .05 M TEAB pH 7.6. The column was eluted under slight air pressure with 40 ml each of .1 M, .2 M, .3 M, .4 M, .5 M, .6 M, .7 M, .8 M, .9 M, 1.0 M and 1.2 M TEAB. The crude product **22** was eluted between .9 M and 1.0 M TEAB as the last UV absorbing compound and the pooled fractions were lyophilized to dryness. The product was purified by prep HPLC

(eluted at ~20 min on a 22 mm x 25 cm preparative C_{18} reverse phase HPLC column with a 30 min linear gradient of 0-30% acetonitrile in 5 mM ammonium acetate pH 5.5 with a flow rate of 10 ml/min) to yield the ammonium salt of pure **22** (32 mg, 36%). ^1H NMR (d_6-DMSO) δ 8.55 (1H, s, NH), 8.07 (1H, s, H6), 7.70-7.85 (2H, m, Ar), 7.45-7.60 (2H, m, Ar), 6.50-6.56 (1H, m, -HC=), 6.41 (1H, d, -HC=), 6.1 (1H, m, H1'), 4.42-4.47 (1H, m, H3'), 4.2 (1H, m), 4.1 (2H, t, CH_2), 3.97 (1H, m), 3.8-3.85 (2H, m, H5'), 3.65-3.75 (1H, m, H4'), 2.1-2.17 (2H, m, H2'), 1.73-1.78 (2H, m, CH_2), 1.5-1.57 (2H, m, CH_2), 1.37-1.42 (2H, m, CH_2). IR (KBr) 3200, 1675, 1545, 1455, 1330, 1070.

5-[3-[[6-(2-methoxy-4-nitrophenoxy)hexyl]amino]-*trans*-1-propenyl]-2'-deoxyuridine 5'-triphosphate (23)

Nitroveratrole (5 g, 27 mmol) was dissolved in 100 ml 2.0 N KOH and the solution was refluxed for 16 h. Upon cooling, bright orange-red crystals formed and these were isolated by filtration, washed with ether and dried *in vacuo* to yield potassium 2-methoxy-4-nitrophenoxide (4.1 g, 73%) (The preceding procedure was developed by Paul Brown). Meanwhile, 6-bromohexanoic acid (1.0 g, 5.1 mmol) was dissolved in 60 ml ether at 0°C and ethereal diazomethane was added (generated with the Aldrich diazomethane kit) until the yellow diazomethane color persisted. The ether solution was washed 4x 50 ml 10% sodium bicarbonate solution, dried over sodium sulfate and the solvent was removed *in vacuo* to yield pure methyl 6-bromohexanoate (1.06 g, 98%). ^1H NMR ($CDCl_3$) δ 3.58 (3H, s, CH_3), 3.33 (2H, t, CH_2), 2.24 (2H, t, CH_2), 1.8 (2H, m, CH_2), 1.57 (2H, m, CH_2),

1.36 (2H, t, CH_2). Potassium 2-methoxy-4-nitrophenoxide (240 mg, 1.12 mmol) was dissolved in 20 ml freshly distilled DMF along with methyl 6-bromohexanoate (200 mg, .96 mmol) and the solution was stirred under Ar for 72 h. The mixture was filtered and the solvent was removed *in vacuo*. The residue was chromatographed on a 22 mm x 200 mm silica gel column eluted with 4% methanol in methylene chloride to yield pure methyl 6-(2-methoxy-4-nitrophenoxy)hexanoate (165 mg, 58%). MP 63-64°C TLC (5% methanol in chloroform) R_f= .9 visualized with short wave UV. ^1H NMR ($CDCl_3$) δ 7.9 (1H, m, Ar), 7.73 (1H, d, J= 2.7 Hz, Ar), 6.9 (1H, d, J= 9 Hz, Ar), 4.11 (2H, t, J= 6.6 Hz, CH_2), 3.95 (3H, s, OCH_3), 3.68 (3H, s, CH_3), 2.37 (2H, t, J= 7.5 Hz, CH_2), 1.91 (2H, m, CH_2), 1.74 (2H, m, CH_2), 1.54 (2H, m, CH_2). Methyl 6-(2-methoxy-4-nitrophenoxy)hexanoate (140 mg, .47 mmol) was dissolved in 16 ml 1:1 water: acetonitrile and 1.7 ml 1.0 N LiOH was added. The solution was heated to 60°C for 20 min then the acetonitrile was removed *in vacuo* and the pH was adjusted to 2 with HCl. The pH 2 solution was extracted 5x 20 ml chloroform, the organic layer was dried over sodium sulfate and the solvent was removed *in vacuo* to yield pure 6-(2-methoxy-4-nitrophenoxy)hexanoic acid (131 mg, 98%). ^1H NMR ($CDCl_3$) δ 7.84 (1H, m, Ar), 7.68 (1H, d, J= 3 Hz, Ar), 6.81 (1H, d, J= 9.2 Hz, Ar), 4.04 (2H, t, J= 6.6 Hz, CH_2), 3.87 (3H, s, OCH_3), 2.36 (2H, t, J= 7.5 Hz, CH_2), 1.86 (2H, m, CH_2), 1.68 (2H, m, CH_2), 1.51 (2H, m, CH_2). 6-(2-methoxy-4-nitrophenoxy)hexanoic acid (35 mg, .11 mmol) was placed in 3 ml DMF along with NHS (18 mg, .15 mmol) and DCC (27 mg, .13 mmol) and

the solution was stirred under Ar overnight. The mixture was then filtered and the solvent was removed from the filtrate *in vacuo*. The residue was redissolved in 2 ml DMF. To this DMF solution was added a 20 ml solution containing **1** (50 mg, .08 mmol) in .1 N sodium borate buffer pH 8.5. After 10 h at room temperature, the reaction was diluted with 100 ml water and placed upon an 18 x 160 mm DEAE-Sephadex A-25-120 column pre-equilibrated with .05 M TEAB pH 7.6. The column was eluted under slight air pressure with 40 ml each of .1 M, .2 M, .3 M, .4 M, .5 M, .6 M, .7 M, .8 M, .9 M, 1.0 M and 1.2 M TEAB. The crude product **23** was eluted between .5 M and .6 M TEAB as the last UV absorbing compound and the pooled fractions were lyophilized to dryness. The crude **23** was redissolved in water and purified by preparative ion exchange HPLC on a Synchropak Q300 column monitored at 260 nm and 350 nm. Compound **23** was the major component eluted (at ~23 min) with a 30 minute linear gradient of 0-0.3 M AMB pH 7.6 and a flow rate of .75 ml/min. To avoid bubbles forming in the HPLC pump piston chambers, the AMB buffer was chilled in ice during the chromatography. The appropriate fractions were pooled and lyophilized to dryness to yield the ammonium salt of pure **23** (25 mg, 37%). UV-Vis (H_2O) λ_{max}= 242, 297, 350(sh). ^1H NMR (d_6-DMSO) δ 8.3 (1H, s, NH), 7.9 (1H, s, H6), 7.89 (1H, m, Ar), 7.81 (1H, d, Ar), 7.11 (1H, d, Ar), 6.42-6.46 (1H, m, -HC=), 6.27 (1H, d, -HC=), 6.09 (1H, m, H1'), 4.34-4.35 (1H, m, H3'), 4.2 (1H, m), 4.03 (2H, t, CH_2), 3.97 (1H, m), 3.9 (3H, m, OCH_3), 3.8-3.85 (2H, m, H5'), 3.65-3.75 (1H, m, H4'), 2.05-2.09 (2H, m, H2'), 1.66-1.7

(2H, m, CH_2), 1.44-1.5 (2H, m, CH_2), 1.3-1.35 (2H, m, CH_2). IR (KBr) 3200, 1675, 1505, 1455, 1330, 1220, 1070.

Preparation Of DNA Restriction Fragments The 517 and 167 base pair DNA fragments from pBR322 were prepared by literature methods with Eco R1 and Rsa 1 restriction endonucleases.[79] 15 μg of pBR322 was digested with Eco R1 restriction endonuclease (New England Biolabs) using the procedure recommended by the manufacturer. One half of the DNA was labelled with ^{32}P at the 3' ends using α-^{32}P-dATP in the standard Klenow enzyme (Boehringer Mannheim Biochemicals) reaction.[2] The other half was labelled with ^{32}P at the 5' ends using the standard polynucleotide kinase (New England Biolabs) reaction.[2] The resulting fragments were digested with Rsa 1 using the procedure suggested by the manufacturer. The 517 base pair fragments were isolated as the second fastest moving band on a non-denaturing 5% polyacrylamide gel and the 167 base pair fragments were isolated as the fastest moving band. The appropriate bands were cut out of the gel, the gel slices were crushed and the DNA was eluted with elution buffer (.25% sodium dodecyl sulfate, 1 mM EDTA, 10 mM $MgCl_2$ and 500 mM ammonium acetate) overnight at room temperature. The DNA was dialyzed against 2x 4000 ml 1 mM NaPhosphate pH 7.5 at 4°C.

The 167 base pair fragments containing a single N6-methyladenine were prepared by placing 5'-^{32}P-end-labelled 167 base pair fragment (5.5 mR/h) in 14 μl water followed by 2 μl 10X Taq 1 methylase buffer (1.0 M NaCl, 100 mM Tris-HCl pH 8.4, 60 mM

$MgCl_2$), 2 µl 60 mM 2-mercaptoethanol, 2 µl 5 mM SAM and 10 units Taq 1 methylase (New England Biolabs). The reaction was heated at 65°C for 1 h then 2x ethanol precipitated.

The 254 base pair fragment of bacteriophage lambda DNA was prepared from N6-methyladenine free lambda DNA (λC1857Sam7 amplified in dam-, dcm- *E. coli*, Pharmacia) and lambda DNA (λC1857Sam7, Pharmacia) containing N6-methyladenine at all 5'-GATC-3' sequences. In either case, 50 µg of the DNA was digested with Sal 1 restriction endonuclease (New England Biolabs) using the procedure suggested by the manufacturer. The DNA was labelled with ^{32}P at the 3' end by incorporating α-^{32}P-dATP and α-^{32}P-TTP using the standard Klenow enzyme reaction. The DNA was digested with Xho 1 restriction endonuclease (New England) again using the manufacturer's suggested procedure. The 254 base pair fragments were isolated as the fastest moving radioactive band on a 5% nondenaturing polyacrylamide gel. The appropriate bands were cut out of the gel and eluted from the gel slices with an Elutrap (Schleicher and Schuell). The DNA was dialyzed against 2x 4000 ml 1 mM NaPhosphate pH 7.5 at 4°C.

Cleavage Of DNA At Adenine With K_2PdCl_4 The ^{32}P labelled fragment (5 mR/h) was placed in 160 µl of H_2O along with 1 µg of sonicated calf thymus DNA. 40 µl of a solution containing 10 mM K_2PdCl_4 (Alfa or Aldrich) and 100 mM HCl/NaCl, pH 2.0 was added (this solution was prepared by adjusting the pH of 200 mM HCl with 1 N NaOH to pH 2.0 followed by dilution with water to a fi-

nal concentration of 100 mM HCl/NaCl and addition of the appropriate amount of K_2PdCl_4). The reaction was incubated at room temperature for 30-45 minutes and stopped by adding 50 µl of a thiol stop solution containing 1.5 M NaOAc, 1.0 M 2-mercaptoethanol (Aldrich) and 20 µg/ml of calf thymus DNA. 750 µl of ethanol was added and the solution was chilled in dry ice for 10 minutes then spun at 12,000 rpm for 6-10 minutes. The supernatant was removed, the DNA pellet washed with 70% ethanol and dried briefly *in vacuo*. The pellet was redissolved in 50 µl 10% aqueous piperidine, heated at 90°C for 30 minutes, frozen in dry ice and lyophilized to dryness. The lyophilized DNA was dissolved in formamide loading buffer and 0.5 mR/h was loaded onto each lane of a .4 mm x 32 cm x 38 cm 8% polyacrylamide gel (1:20 crosslinked, 50% urea). The gel was electrophoresed for the desired amount of time then transferred to a piece of filter paper and dried (using a Bio-Rad model 483 slab dryer) then autoradiographed. Densitometry was performed on the autoradiographs using a laser densitometer (LKB Ultroscan XL).

The DMS reactions run at pH 2.0 with and without K_2PdCl_4 were conducted as above but with a couple of changes. 1 µl of DMS was added to the reactions with or without the K_2PdCl_4, and the thiol stop solution was added after only 5 min incubation at room temperature.

HPLC Analysis Of K_2PdCl_4 Reaction Products In each reaction, 50 µg of calf thymus DNA was dissolved in 50 µl water. A G+A control reaction was run by heating the DNA in .1 N HCl for 45 min-

utes at 75 °C then the pH was neutralized with .1 M NaOH and the sample was frozen in dry ice and lyophilized. The K_2PdCl_4 reaction was run by adding 5 μl of 25 mM K_2PdCl_4 to the DNA and the solution was incubated at room temperature for 3 h. The reaction was stopped by adding 2 μl of 2-mercaptoethanol and the sample was frozen and lyophilized. In a control reaction the 2-mercaptoethanol was added to the DNA before the K_2PdCl_4 and the solution was immediately frozen and lyophilized. In each case, the lyophilized residue was redissolved in 50 μl 8 mM ammonium acetate pH 5.5 and chromatographed on an Altex Ultrasphere ODS column eluted with a 30 minute linear gradient of 0-15% acetonitrile in 8 mM ammonium acetate pH 5.5 followed by a 15 min linear gradient of 15-85% acetonitrile.

Photolysis Reactions The photolyses used in the reactions of DNA with nitroaromatics (4-nitroveratrole, 3-nitroanisole etc.) or Co(III) complexes ($Co(III)(NH_3)_6$ etc.) were conducted in one of two ways. In some cases, the sample was placed in the beam from a monochrometer (Oriel model 240) (the sample was 1.5 cm from the monochrometer so that the diameter of the beam was 2 cm at the sample) which was attached to the high pressure Hg-Xe photolysis lamp (Oriel model 6140). When more intense illumination was desired, the monochrometer was removed and the beam was filtered through 10 cm of water to remove IR then filtered with a pyrex filter (.5 cm pyrex glass) so that only light with $\lambda \geq 300$ nm was allowed to

reach the sample. This light was focused with a lens so again the beam diameter was 2.0 cm at the sample.

The sample (20 µl total volume) was usually photolyzed by either method in a 1 mm x 1 cm x 4.5 cm quartz cuvette (Fisher Scientific). During photolysis the sample was kept at room temperature with a vigorous stream of compressed air. When low temperature was desired, the quartz cuvette was not used, but rather the sample (20 µl total volume) was placed in a quartz NMR tube that had been cut off 5 cm from the bottom. A small septum was used to seal the sample in the tube and the tube was immersed in a quartz photolysis finger dewar filled with water at the desired temperature (usually 0°C).

HPLC Analysis Of The Photochemical Reaction Between Calf Thymus DNA And 3-Nitroanisole Or 19. 1 mg of **19** was dissolved in a 1.0 ml solution containing CT DNA (sonicated, deproteinized, 4.78 mg/ml). This solution was placed in a pyrex testube and photolyzed using the monochrometer at 330 ± 10 nm for 8 h. After photolysis, the reactions were worked up in one of two ways.

To 50 µl of the above photolyzed solution 10X DNAse buffer (100 mM Tris-HCl pH 7.9, 100 mM $MgCl_2$, 100 mM KCl, 50 mM $CaCl_2$) and 50 µl water were added followed by 25 units of DNAse (Sigma). After 4 h at 25°C, the pH of the solution was adjusted to 5.5 with HCl then 10 µl of 50 mM zinc sulfate was added. 5 µg of Nuclease P1 (Boehringer Mannheim Biochemicals) was then added and the reaction was incubated at 37°C for 12 h. 200 mM Tris base was added to

a final pH of 8.0 followed by 45 units of alkaline phosphatase (Boehringer Mannheim Biochemicals). After 1 h at 37°C, the solution was injected onto a 4.6 mm x 25 cm C_{18} reverse phase HPLC column (Altex Ultrasphere ODS) eluted with a 20 min linear gradient of 10-45% methanol in 5 mM sodium phosphate pH 5.5 followed by a 15 min linear gradient of 45-75% methanol with a flow rate of .75 ml/min and monitored at 254 nm and 220 nm or 290 nm.

Alternatively, 50 µl of the photolyzed sample was hydrolyzed in .1 N HCl for 30 min at 90°C then the pH of the solution was adjusted to 5.5 with Tris base and the solution was injected onto the HPLC and eluted as described above.

HPLC Analysis Of The Photochemical Reaction Between 2',3',5'-tri-O-acetylguanosine And 3-Nitroanisole 2',3',5'-tri-O-acetylguanosine (4.0 mg, .011 mmol, recrystallized from methanol, Sigma) and 3-nitroanisole (1.7 mg, .011 mmol, recrystallized from methanol, Aldrich) were dissolved in 1.5 ml of 1:1 acetonitrile: water and photlyzed for 6 h with the pyrex filter setup oo up to 16 h with the monochrometer set up. In either case, 50 µl aliquots were analyzed by reverse phase HPLC using the same elution conditions described above.

NMR experiments were run with 2',3',5'-tri-O-acetylguanosine (4.0 mg, .011 mmol, recrystallized from methanol) and 3-nitroanisole (1.7 mg, .011 mmol, recrystallized from methanol) in 1.0 ml d_3-CH_3CN. The sample was sealed in an NMR tube and photolyzed as usual. NMR spectra were taken before and after the photolysis.

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