

**DESIGN AND SYNTHESIS OF MODIFIED DEOXYNUCLEOSIDES, SITE  
SPECIFICALLY DAMAGED OLIGONUCLEOTIDES, AND REPAIR INHIBITING  
CHEMOTHERAPEUTIC AGENTS**

by

**MARSHALL LEE MORNINGSTAR**

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Signature of Author \_\_\_\_\_  
Department of Chemistry  
July 7, 1994

Certified by \_\_\_\_\_  
John M. Essigmann  
Thesis Advisor

Certified by \_\_\_\_\_  
Gerald N. Wogan  
Thesis Advisor

Accepted by \_\_\_\_\_  
Dietmar Seyferth  
Chairman, Departmental Committee on Graduate Students

MASSACHUSETTS INSTITUTE  
OF TECHNOLOGY


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
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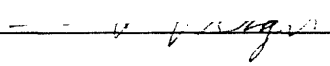
### Committee Page

This doctoral thesis has been examined by a Committee of the Department of Chemistry as follows:

Professor Steven R. Tannenbaum  \_\_\_\_\_ Chairman

Professor John M. Essigmann  \_\_\_\_\_ Thesis Supervisor

Professor Gerald M. Wogan  \_\_\_\_\_ Thesis Supervisor

Professor Scott C. Virgil  \_\_\_\_\_

# **DESIGN AND SYNTHESIS OF MODIFIED DEOXYNUCLEOSIDES, SITE SPECIFICALLY DAMAGED OLIGONUCLEOTIDES, AND REPAIR INHIBITING CHEMOTHERAPEUTIC AGENTS**

by

**Marshall Lee Morningstar**

Submitted to the Department of Chemistry on July 7, 1984 in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Organic Chemistry

## **Abstract**

This dissertation describes accomplishments in three areas of cancer research: the development of a new method to detect carcinogen DNA adducts, the synthesis of modified oligonucleotides for structural and biological studies, and the development of candidate cancer chemotherapeutic agents designed to damage DNA and to inhibit their own DNA repair selectively in tumor cells.

In an effort to facilitate progress in the evaluation of DNA adduct measurements as biomarkers of genotoxic exposure, a new method was developed to detect and to quantify DNA adducts. The method, which we have termed Adduct Detection by Acylation with Methionine (ADAM), involves the acylation of DNA adducts with a protected methionine derivative, t-butoxycarbonyl-L-methionine N-hydroxysuccinimidyl ester (TBM-NHS). This dissertation describes the optimization and the structure determination of the acylation products of 2'-deoxyguanosine (dGuo), used as a prototypical deoxynucleoside, and N-(deoxyguanosin-8-yl)-4-aminobiphenyl (dGuo-8-ABP), the major DNA adduct formed after exposure of cells to 4-aminobiphenyl, a known bladder carcinogen. Under optimized reaction conditions, a major product (greater than 88% isolated yield after chromatography) resulted from the TBM acylation of either dGuo or dGuo-8-ABP.

In an attempt to define the relationship between the structures and the biological consequences of chemical carcinogens and ionizing radiation, a series of site-specifically modified oligonucleotides was synthesized. My efforts have led to the synthesis of four site-specifically modified oligonucleotides that have undergone, or currently undergoing, biological experiments to determine the mutagenicity of the different DNA lesions. Two methods of synthesis were utilized to create the modified oligonucleotides: chemical modification of a preformed oligonucleotide or solid phase DNA synthesis utilizing a modified phosphoramidite. The site-specifically modified oligonucleotides that were synthesized contained the following lesions: dGuo-8-ABP, 7,8-dihydroguanine-8-one ( $G^{8-oxo}$ ),

7,8-dihydroadenin-8-one ( $A^{8-oxo}$ ), and 5-hydroxy-deoxyuridine (oh5dU). Preliminary studies are also described on the synthesis of a fifth modified oligonucleotide containing 5-hydroxy-deoxycytidine (oh5dC).

As part of ongoing studies to improve the selectivity of anticancer drugs that mediate their genotoxicity as a consequence of reacting with DNA, novel chemotherapeutic compounds were designed, synthesized and tested for biological activity. The designed chemotherapeutic agents consist of a chemically reactive DNA damaging domain linked to a second domain that attracts a tumor specific protein. The initial step in the functioning of these chemotherapeutic agents is the covalent modification of DNA by the warhead domain. Although damage occurs in both cancer and noncancerous cells, we propose that the selective binding of a tumor specific protein in cancerous cells can prevent the repair of the DNA lesion. The end result would be the higher relative toxicity of the drug in a cancer cell than in a noncancerous cell. Initial studies involved the synthesis and testing of a modified ss DNA capable of binding with high affinity to a targeted protein and capable of covalently modifying a ds DNA target. Although a designed compound could be synthesized, covalent modification of a ds DNA probe did not occur. Ensuing studies with a psoralen-linked biotin molecule did result in a DNA lesion that demonstrated *in vitro* that the binding of a targeted protein in the vicinity of a DNA lesion can inhibit the repair of DNA damage. Recent studies have involved the synthesis of a series of genotoxic compounds utilizing an estradiol recognition domain. These genotoxic compounds were designed to cause enhanced toxicity solely in tumor cells by exploiting the property of breast tumor cells to overexpress the estrogen receptor (ER) which can bind to and protect the designed DNA lesions.

Thesis supervisor: Professor John M. Essigmann

Title: Professor of Chemistry and Professor of Toxicology

Thesis supervisor: Gerald N. Wogan

Title: Professor of Chemistry and Professor of Toxicology



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## List of Abbreviations

A <sup>8-oxo</sup>	7,8-dihydro-8-oxoadenine
4-ABP	4-aminobiphenyl
ADAM	Adduct Detection by Acylation with Methioine
BOC	t-butoxycarbonyl
CBZ	benzyloxycarbonyl
cisplatin	<i>cis</i> - diamminedichloroplatinum (II)
CPG	control pore glass
dGuo	2'-deoxyguanosine
dGuo-8-ABP	N-(deoxyguanosin-8-yl)-4-aminobiphenyl
dUg	deoxyuracil glycol
DCC	dicylcohexylcarbodiimide
DIC	disopropylcarbodiimide
DMAP	dimethylaminopyridine
DMT	4,4'-dimethoxytrityl
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
ER-	estrogen receptor negative
ER+	estrogen receptor positive
FA	Fanconi's anemia
FAB-MS	fast atom bombardment-mass spectroscopy
G <sup>8-oxo</sup>	7,8-dihydro-8-oxoguanine
GC-MS	gas chromatography-mass spectroscopy
Hg-dC	5-mercuri-2'-deoxycytidine
HMG	high mobility group
HOBt	1-hydroxybenzotriazole
HPLC	high pressure liquid chromatography
hUBF	human upstream binding transcription factor
LAEDA	lithium acetylide, ethylene diamine
LCA	long chain amine



M%	molar equivalents
Me <sub>2</sub> SO	dimethylsulfoxide
NHS	N-hydroxysuccinimide
NOE	Nuclear Overhauser Effect
oh <sup>5</sup> dC	5-hydroxy-deoxycytidine
oh <sup>5</sup> dU	5-hydroxy-deoxyuridine
PCR	polymerase chain reaction
RIA	radioimmunoassay
SIMS	secondary ion mass spectroscopy
tBDMS	t-butyldimethylsilyl
TBM	t-butoxycarbonyl-L-methionine
TBM-NHS	t-butoxycarbonyl-L-methionine,N-hydroxysuccinimidyl ester
[ <sup>35</sup> S]-TBM-NHS	t-butoxycarbonyl-L-[ <sup>35</sup> S]methionine,N-hydroxysuccinimidyl ester
TCA	trichloroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TMP	2,4,6-trimethylphenyl
TMS	trimethylsilyl
TSP	tumor specific protein
XP	Xeroderma pigmentosum

## INTRODUCTION

Ionizing and UV radiation, chemotherapeutic drugs and environmental chemicals can damage various cellular constituents including proteins, lipids, RNA and DNA (Miller and Miller, 1981; Singer and Grunberger, 1983; Skipper and Tannenbaum, 1990; Perera, 1988). The resulting cellular damage can affect the growth, survival and proliferation of a cell (Hutchinson, 1989). DNA damage that eludes cellular repair mechanisms in particular has special significance because these lesions can destroy or heritably alter essential genetic information. The resulting genetic changes can convert a cell irreversibly to an altered neoplastic phenotype (Wogan, 1989). In an attempt to understand and to treat cancer, this thesis describes studies undertaken 1) to develop a new method to detect carcinogen-DNA damage, 2) to synthesize substrates with which to understand the biological consequence of particular DNA lesions, and 2) to develop a novel cancer treatment based on the inhibition of DNA damage selectively in tumor cells.

In an attempt to provide qualitative and quantitative information on human exposure to carcinogens, several DNA adduct detection methods have been developed (Beach and Gupta, 1992; Kaderlik et al., 1992). Results of a number of studies involving cells in culture and pilot trials involving animals and humans suggests that if the biologically critical DNA adduct can be determined and measured, there is a good correlation between the level of that DNA adduct and the frequency of induced mutations (Perera, 1988). As part of an ongoing effort in the Wogan laboratory to determine which chemical lesions form within DNA and to ascertain how prevalent the resulting lesions are, I report in the first chapter of this

dissertation the development and validation of a new method to detect DNA adducts. Our objective was to develop a method for detecting carcinogen-DNA adducts that could be broadly applicable to carcinogens from diverse chemical classes, have sensitivity adequate to detect adduct levels resulting from ambient human exposures, be capable of identifying individual adducts as well as mixtures resulting from complex exposures, have potential for quantifying adduct recovery, and utilize readily available reagents and equipment. The developed method, which we have termed Adduct Detection by Acylation with Methionine (ADAM), involves the acylation of DNA adducts with a protected methionine derivative, t-butoxycarbonyl-L-methionine N-hydroxysuccinimidyl ester (TBM-NHS). The first section of this dissertation describes the optimization and the structural determination of the acylation products of 2'-deoxyguanosine (dGuo), used as a prototypical deoxynucleoside, and N-(deoxyguanosin-8-yl)-4-aminobiphenyl (dGuo-8-ABP), the major DNA adduct formed after exposure of cells to 4-aminobiphenyl, a known bladder carcinogen.

A second aspect of my research has involved the preparation of biological substrates with which to investigate the mutagenic potential of oxidative and aromatic amine DNA damage. While DNA adduct measurements can provide evidence that a particular chemical has reacted with DNA, the biological importance of the lesion is, however, usually unknown. A crucial obstacle hindering an understanding of the importance of some DNA damaging agents arises from the general property of carcinogenic agents to form a variety of DNA adducts (Wood and Essigmann, 1991). By using viral or plasmid DNA singly modified with a specific adduct, the *in vivo* mutagenic potential for a particular DNA lesion can be determined. As part of ongoing efforts in the Essigmann laboratory to define the

relationship between DNA adducts and the biological consequences of chemical carcinogens and ionizing radiation, I report in the second chapter of this dissertation the synthesis of four site-specifically modified oligonucleotides for use in *in vivo* mutagenesis experiments and *in vitro* repair studies. My efforts have led to the synthesis of site-specifically modified oligonucleotides containing dGuo-8-ABP, 7,8-dihydroguanine-8-one ( $G^{8-oxo}$ ), 7,8-dihydroadenine-8-one ( $A^{8-oxo}$ ), and 5-hydroxy-deoxyuridine (oh5dU). Preliminary studies are also described on the synthesis of a fifth modified oligonucleotide containing 5-hydroxy-deoxycytidine (oh5dC).

A third aspect of my research has involved the development of a novel cancer treatment strategy. A crucial aspect of the proposed strategy is the specific binding of a protein to a DNA lesion. A number of studies have reported the existence of proteins in eucaryotic cells that bind to DNA damaged by chemical or physical agents, including platinum crosslinks (Donahue et al., 1990; Pil and Lippard, 1992; Brown et al., 1993; Treiber et al., 1994), ultraviolet light induced photoproducts (Feldberg and Grossman, 1976; Chu and Chang, 1988; Hirschfeld et al., 1990) and 1, $N^6$ -ethanoadenine (Rydberg et al., 1991). Additional studies suggest that protein binding to a DNA lesion can shield the lesion from repair, thereby increasing the biological efficacy of a genotoxic compound (Donahue et al., 1990; Brown et al., 1993; Treiber et al., 1994). Presumably, a therapeutic compound designed to react with DNA and concurrently be tightly bound by a tumor specific protein would provide a means of achieving a favorable therapeutic index. In principle, if the binding took place only in a tumor cell, the complex formed between the designed compound and a tumor specific protein would selectively slow the rate of repair of the

lesion in the tumor cell. The end result would be a higher relative toxicity of the drug in cancer cells compared to normal cells. I report in the third chapter of this dissertation the design and synthesis of model compounds to test our hypothesis. A key feature of our strategy, which we have termed Fatal Engineering, is the ability to control the specificity and affinity of the protein recognition domain in these candidate drugs. This ability should allow us to exploit the different molecular changes that are characteristic of various malignancies, including the expression or overexpression of oncogene-encoded or suppressor gene-encoded proteins.

## I. DEVELOPMENT OF ADDUCT DETECTION BY ACYLATION WITH METHIONINE (ADAM)<sup>1</sup>

### A. Introduction

Electrophilic genotoxic carcinogens and ionizing radiation can form a variety of covalent adducts with cellular macromolecules (Singer and Grunberger, 1983). Measurement of the levels of such adducts in DNA and proteins can provide important qualitative and quantitative information about biologically effective doses of genotoxic agents to which individuals have been exposed (Perera, 1988; Wogan, 1989; Wogan, 1992). Information acquired through such investigations would be useful in identifying individuals within populations at high risk for cancer by virtue of exposure. In addition, the measurement of molecular biomarkers provides a means to assess the efficacy of molecular interventions such as chemoprotective agents (Lippman et al., 1990).

In an effort to facilitate progress in utilizing DNA adduct measurements as biomarkers of genotoxic exposure, Dr. Gerald Wogan, Dr. Fayad Sheabar, and I sought to develop a new analytical procedure to detect carcinogen-DNA adducts. The methodology we developed is based on chemical derivatization of mononucleoside adducts by acylation with

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<sup>1</sup>. Portions of this section have been taken *verbatim* from Sheabar et al., 1994 and Sheabar, F.Z., Morningstar, M.L., and Wogan, G.N. (1994) Chemical derivatization of nucleosides by acylation with methionine: An experimental strategy for analysis of carcinogen-DNA adducts. *Chem. Res. Tox.* (accepted for publication).

an activated methionine reagent. The following sections describe my efforts as a member of the Wogan laboratory to develop and validate this new method for the measurement of carcinogen-DNA adducts. The two main objectives of my research were: 1) to synthesize standards of carcinogen-DNA adducts, including tritium-labeled, and 2) to synthesize and to elucidate the structure and regiochemical assignment of the acylation reaction products.

## **B. Background and Basis of Biomonitoring**

### **a. Human Biomonitoring**

Human exposure to xenobiotics occurs as a result of chemicals present in the environment (Perera et al., 1992), occupational settings (Melick et al., 1971), and food (Ames et al., 1990; Gold et al., 1992). One approach to estimate an individual's exposure to toxicants is accomplished by questionnaires, personal external monitors, and measurement of chemicals in the ambient environment. Unfortunately, extrapolating this information to calculate the chemical fraction each individual encounters has been difficult. The complexity arises from the heterogeneous nature of most environment exposures and because many chemicals require subsequent metabolic activation in order to cause molecular damage (Miller and Miller, 1981). In particular, the capability for metabolic activation varies between individuals (Harris et al., 1982; Harris et al., 1984), in different organs (Autrup et al., 1982; Selkirk et al., 1983), and probably in the same organ of one person over time. Consequently, assessing individual chemical exposures and risks by these methods has been very imprecise predicting only the approximate dose received by an individual.

A second approach for assessing the exposure of people to chemicals involves the measurement of biomarkers, internal markers of chemical exposure (Groopman and Kensler, 1993; reviewed in Biomarkers in Human Cancer-Part I (1992) and Part II (1993)).

Biomarkers provide unequivocal identification of chemical exposure and offer a means to measure the amount of activated chemical directly interacting with critical cellular targets. Three strategies have been employed to assess this internal dose of chemical agents. One method involves indirect measurement of biological responses such as mutations, sister chromatid exchange, chromosome aberrations, and abnormalities in cells and tissues of exposed individuals. The other two methods involve the direct measurement chemical exposure. One method involves the detection of carcinogens, their metabolites, and/or derivatives in body fluids or excreta (Wogan and Gorelick, 1985; Shuker and Farmer, 1992; Simic, 1992). The other method involves the detection of the products of a chemical's reaction with cellular macromolecules such as DNA, RNA, or protein (Tannenbaum, 1990; Perera et al., 1992; Kaderlik et al., 1992). Although all damaged macromolecules can potentially compromise cellular integrity, adducts within DNA have special significance because of their potential to initiate events leading to mutation and/or malignant transformation (Ozturk et al., 1991; Bressac et al., 1991). Consequently, efforts in this laboratory have focused on the measurement of carcinogen-DNA adducts in target tissues or accessible surrogate cells.



## **b. DNA Adducts**

Carcinogen-DNA addition products (DNA adducts) are formed from the covalent binding of reactive mutagens and carcinogens to sites on all four DNA bases as well as the phosphate and sugar backbone of DNA. The products of reaction can involve exocyclic amino and oxygen groups, purine or pyrimidine ring nitrogens, ribose carbons, or phosphate oxygens. The factors that influence the relative reactivity of an electrophilic compound toward specific sites in DNA are the electrophilicity of the chemical mutagen (O'Connor et al., 1975), the nucleophilicity of the DNA sites (Lawley et al., 1975), the steric accessibility of a reagent to a nucleophilic DNA site (Lawley et al., 1975), electrostatic interactions (Abnnon and Verly, 1972), and van der Waals interactions between the electrophile and the DNA (Swenson and Kadlubar, 1980). The relative contribution of these factors varies with different compounds. Consequently, a variety of DNA lesions can result from a single carcinogen or mutagen. However, the stability of the resulting DNA lesions varies as a consequence of the type of adduct or as a consequence of enzymatic repair (Singer and Grunberger, 1983; Swenson and Kadlubar, 1980). Some adducts involving covalent modification of the purine N7 and N9 positions and pyrimidine N1 position decompose spontaneously through chemical depurination or depyrimidination. Some adducts such as phosphotriesters and glycosidic adducts are transiently stable and ultimately decompose slowly. Other adducts are stably attached to DNA but are removed enzymatically by DNA repair. However, a proportion of chemically stable DNA adducts is not repaired (or not rapidly repaired) and, as such, remains fixed to DNA over long periods of time. In these cases, the modified nucleotides can cause mutations.

### **c. Methods for Detecting Carcinogen-DNA Adducts**

A variety of analytical techniques have been devised to measure DNA adducts (Wogan, 1989; Kaderlik et al., 1992). These methods include immunoassays (Poirier, 1993), <sup>32</sup>P-postlabeling (Beach et al., 1992), and physiochemical methods (Weston, 1993). While each of the analytical methods has some specific advantages for detecting particular DNA adducts, none of the currently available techniques is capable of accurately detecting every class of DNA adducts. Some of the advantages and limitations for each method are briefly described below.

#### **i. Radiolabeled Carcinogens**

One widely used method to study the interaction of chemical carcinogens with DNA uses radiolabeled carcinogens (Baird, 1979). By treating animals with radiolabeled carcinogens or reacting DNA *in vitro* with the reactive form of a carcinogen, DNA modified at 1 adduct/10<sup>8</sup> nucleotides can be detected when a carcinogen of high specific activity (> 25 Ci/mmol) is used. Accordingly, this method depends on the availability of highly radioactive carcinogens. Since human exposure rarely if ever involves radiolabeled carcinogens, this method is not applicable to the detection of ambient chemical adducts in humans.

#### **ii. Ultraviolet absorbance and Fluorescence Spectroscopy**

Detection of DNA adducts by ultraviolet and fluorescence spectroscopy depend on the absorption and, in the case of fluorescence, reemission of light. Because of the strong

absorbance of most DNA bases at 254 and 280 nm, ultraviolet absorbance is useful for the detection of a large number of carcinogen-DNA adducts. Unfortunately, this method, when compared with other available methods, is relatively insensitive (1 adduct/ $10^4$  nucleotides using 2600  $\mu\text{g}$  of DNA). In contrast, fluorescence spectroscopy can be very sensitive depending on the adduct under study and the instrumentation used (Weston and Bowman, 1991). The use of low temperature techniques and synchronous scanning, for instance, can detect 1 adduct/ $10^7$  nucleotides using 100  $\mu\text{g}$  of DNA and allow unambiguous adduct identification. However, this improvement depends on adduct fluorescence and the use of expensive instrumentation. One drawback for both ultraviolet absorbance and fluorescence spectroscopy is the limitation for providing structural information concerning unknown DNA adducts. Other disadvantages include the lack of quantitation due to broad peaks in many samples studied and cross-detection of other unrelated chromophores.

### **iii. Immunoassays**

Immunoassays take advantage of the specificity of antibody-antigen reactions to detect DNA adducts. Consequently, immunoassays are limited to the detection of DNA adducts for which antibodies are available (Phillips, 1990; Santella, 1988). Two commonly used techniques for DNA adduct detection are the radioimmunoassay (RIA) or the enzyme-linked immunosorbent assay (ELISA). Both assays depended the detection of an antigen-antibody complex. In order to detect the antigen-antibody complex by RIA, a radiolabeled tracer with specific activity of  $> 10 \text{ Ci/mmol}$  is required (Muller and Rajewsky, 1981). Consequently, RIA is limited to carcinogen-DNA adducts where a radioactive tracer is available. Although ELISA can be done when a radiolabeled tracer is not available, greater

intra- and inter-assay variability occurs because of the multiple steps involved in assaying for binding (Poirier, 1993). Some of the factors affecting the sensitivity of ELISA or RIA detection of carcinogen-DNA adducts include: 1) the antibody affinity; 2) the physical state of the biological sample (native, denatured, or digested DNA); 3) the amount of the sample that can be assayed without altering the standard curve; and 4) in the case of RIA, the specific activity of a radiolabeled tracer (Poirier, 1993). Sufficient sensitivity to assay human DNA adducts for both methods generally requires antisera with affinity constants in the range of  $10^8$  to  $10^9$  liters/mole. Before a particular antibody can be used in either immunoassay, however, extensive characterization is required to insure that unknown cross-reactivity with other chemicals does not occur. Unfortunately, the very high antibody specificity to a particular antigen can be a disadvantage. When studying a spectrum of related adducts, a battery of antibodies is needed.

#### **iv. Gas Chromatography / Mass spectrometry**

Gas chromatography / mass spectrometry (GC-MS) involves the separation of volatile compounds by gas chromatography and subsequent detection by mass spectrometry. Because nucleosides are not suitable for gas chromatography, a carcinogen-DNA adduct must be derivatized prior to analysis. One method commonly used involves the release of the adduct from DNA by acid treatment and chemical derivatization of the adduct with trimethylsilyl groups (Dizdaroglu, 1986). To increase the sensitivity of detection, selected-ion mode of data collection is often employed. This method offers high selectivity and sensitivity (1 adduct/ $10^8$  nucleotides) (Kaderlik et al., 1992). Unfortunately, the selected-ion mode limits the structural data obtained for a peak and consequently cannot be used for the

detection of unknown DNA adducts. Other drawbacks of this method include: 1) large amounts of DNA are required for analysis; 2) that the DNA adduct under study must be stable to enzymatic digestion or, commonly, acid treatment; 3) that the DNA be stable to chemical derivatization; 4) that the derivatized DNA adduct be sufficiently volatile for gas chromatography; and 5) the relatively high equipment cost.

#### **v. Electrochemical Conductivity**

Electrochemical conductivity involves the detection of voltage passed through a solution (Dahmen, 1986). Therefore, electrical conductance is limited to determining the concentration of chemicals capable of accepting an electrical charge. Since most carcinogen-DNA adducts can not accept an electrical charge, the types of DNA adducts that can be detected by this method are limited. In addition, one class of DNA adducts that can be detected by this method, oxidized DNA bases, is commonly generated during DNA extraction (Kasai et al., 1986). Therefore, the accurate quantitation of this adduct is problematic.

#### **vi. Atomic Absorbance Spectroscopy**

Atomic absorbance spectroscopy is based on the absorbance of radiation by free atoms released by high temperature combustion. The high temperatures of combustion limits this technique to the detection of metal ions. While this assay is very sensitive for detecting certain metals (1 adduct/ $10^7$  nucleotides for  $200\mu\text{g}$  of DNA) (Weston, 1993), the determination of different metals requires different preparations and cannot be done simultaneously. In addition, since the adduct is destroyed prior to detection by the high

temperature of combustion, it is not possible with this method to determine the specific adduct that was present. Consequently, this method is limited to detecting the total amount of metal bound to a sample.

#### **vii. $^{32}\text{P}$ -postlabeling**

$^{32}\text{P}$ -postlabeling involves enzymatic derivatization of nucleic acid monomer or dinucleotide with a radioactive reagent, chromatographic separation of the derivatized products, and then measurement of the radioactivity of the separated products. The protocol originally developed for  $^{32}\text{P}$ -postlabeling involved 1) enzymatic digestion of DNA to deoxynucleoside 3'-monophosphates; 2) enzymatic phosphorylation of the 5'-hydroxyl group with [ $\gamma$ - $^{32}\text{P}$ ]ATP; 3) chromatographic separation of the labeled products; and 4) quantitation of adducts by measurement of radioactivity (Randerath et al., 1981; Gupta et al., 1982). In order to increase the sensitivity of adduct measurement, the following changes to the original  $^{32}\text{P}$ -postlabeling protocol were made: 1) the use of high specific activity [ $\gamma$ - $^{32}\text{P}$ ]ATP, [ $\gamma$ - $^{35}\text{S}$ ]ATP, or different ATP concentrations; 2) improved separation of labeled unmodified nucleotides (the original separation technique used thin layer chromatography, TLC); and 3) purification of adducts from hydrolyzed DNA before postlabeling (Gorelick, 1993; Beach and Gupta, 1992; Randerath et al., 1985). With these improvements, the sensitivity of the  $^{32}\text{P}$ -postlabeling method is between 1 adduct per  $10^7$  to  $10^{10}$  adducts/nucleotide using 1 to 10  $\mu\text{g}$  of DNA.

Although  $^{32}\text{P}$ -postlabeling is widely used (Reddy et al., 1981; Reddy et al., 1984; Randerath et al., 1981; Randerath et al., 1989; Lo et al., 1982; Haseltine et al., 1983), a

variety of inherent methodological problems limits the usefulness of this technique. An important limitation of the postlabeling method is that accurate quantitative and qualitative measurements are achievable only if appropriate authentic adducts are available for use in recovery determinations and as chromatographic standards. Results for a number of different DNA adducts indicate that recoveries are variable and generally not quantitative. For example, the overall adduct recovery, reported as the percentage of a known amount of the adduct standard recovered as the  $^{32}\text{P}$ -labeled derivative, can vary from as high as 89-92% for O<sup>6</sup>-methylguanine-DNA (Wilson et al., 1988) to as low as 10-15% for fluoranthene-DNA (Gorelick and Wogan, 1989). An additional limitation to accurate quantitation arises due to poor chromatography resulting in incomplete separation of adducts. Even when adducts are separable, analysis of different chemically modified DNA often shows one or more radiolabeled compound after chromatography, originally seen as "spots" on TLC. Detected radioactivity does not always correspond to adducts; rather, radioactivity may be associated with metabolites (Masento et al., 1989), naturally occurring ribonucleotides (Beach and Gupta, 1992), and components extracted from polypropylene test tubes (Beach and Gupta, 1992). Only by applying "correction" factors has this method been useful for determining DNA adduct levels.

#### **d. Summary**

Analytical methods have been developed that are capable of detecting and quantifying levels of covalent adducts of DNA. Unfortunately, the different methods have specific advantages for the detection of only certain classes of DNA adducts. Human biomonitoring,

however, requires a method that is sensitive enough to detect the effects of subtle, low-level exposures to complex chemical mixtures of unknown composition. The following sections discuss the validation of a new method of sufficient sensitivity and specificity to detect ambient levels of DNA adducts.



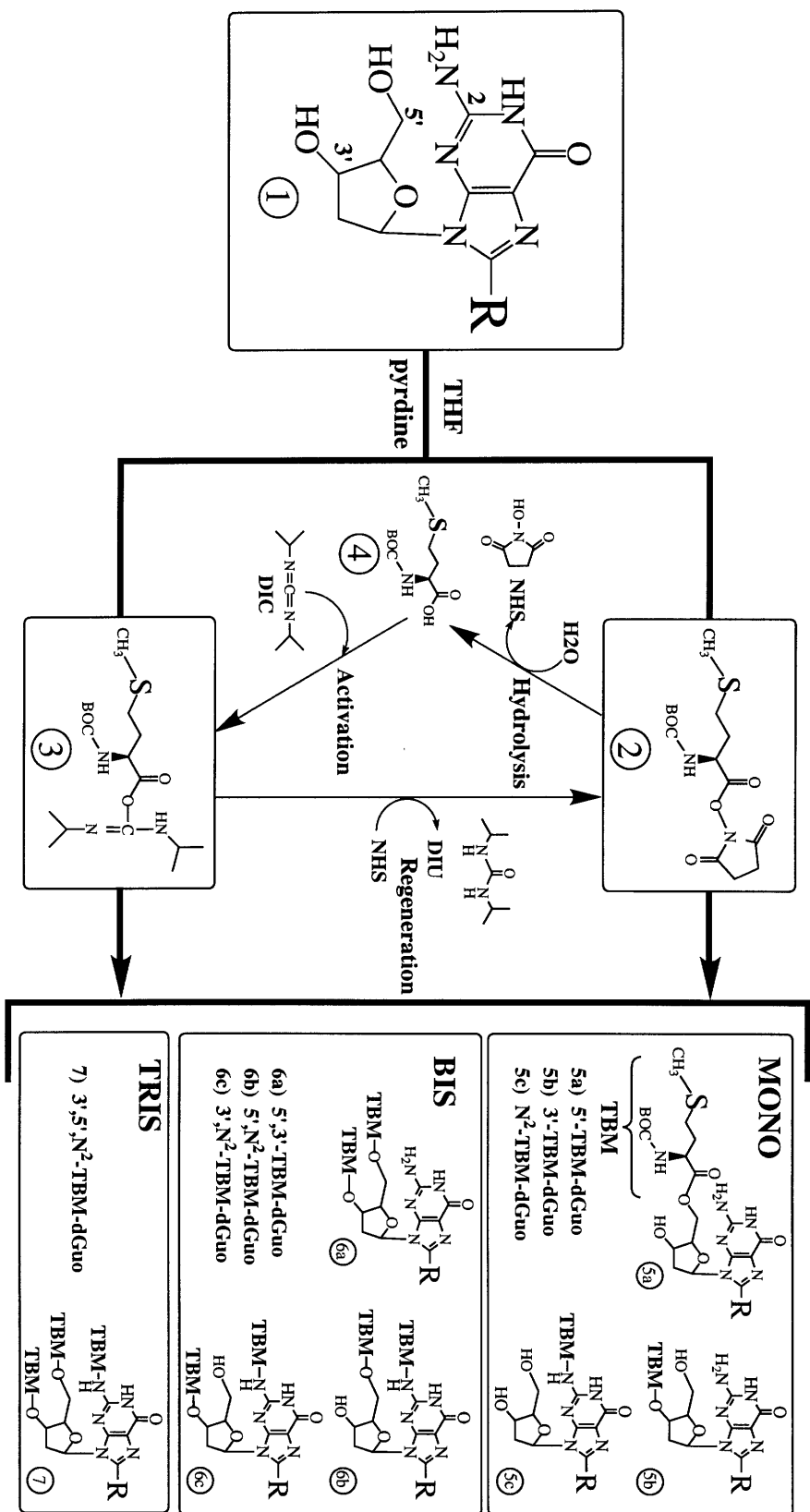
## **C. Design of Adduct Detection by Acylation with Methionine (ADAM)**

### **a. Introduction**

The features that we sought to incorporate into a new analytical procedure for detecting carcinogen-DNA adducts included the following: 1) broad applicability to detect carcinogens from diverse chemical classes; 2) sensitivity adequate to detect adduct levels resulting from ambient exposures; 3) the ability to identify individual adducts as well as mixtures resulting from complex exposures; 4) the potential for quantifying adduct recovery; 5) the ability to allow structural identification of specific carcinogen adducts; and 6) the utilization of readily available reagents and equipment. A strategy under development in the Wogan laboratory to fulfill these requirements involves the chemical acylation of carcinogen-modified deoxynucleosides (Figure 1).

The following sections in this chapter examine the chemical basis for the peracylation of nucleosides with BOC-methionine as a means to label carcinogen-DNA adducts. The first section begins by examining the crucial labeling reagent, [<sup>35</sup>S]t-butoxycarbonyl-L-methionine, N-hydroxysuccinimidyl ester ([<sup>35</sup>S]TBM-NHS). The second section examines the advantages and possible disadvantages of methionine acylation of nucleosides as the method of attaching a radioactive label. The particular points examined include: i) the peracylation of nucleosides; ii) the synthesis of amino acid esters of ribonucleosides; iii) the synthesis of amino acid esters of deoxynucleoside; and iv) the extent of methionine racemization upon activation. The third section discusses carbodiimide activation. We have experimentally established that the addition of diisopropylcarbodiimide to the acylation procedure for pmol or less of nucleoside is necessary for good yields.

**Figure 1.** Adduct Detection by Acylation with Methionine (ADAM). (1) R=H: dGuo, R=ABP: dGuo-8-ABP, (2) TBM-NHS, (3) O-acyl-isourea-BOC-methionine, (4) BOC-methionine, (5) mono-TBM acylation products, (6) bis-TBM acylation products, (7) tris-TBM acylation products.



**b. [<sup>35</sup>S]t-butoxycarbonyl-L-methionine, N-hydroxysuccinimidyl ester**

**i. Applications of [<sup>35</sup>S]TBM-NHS**

[<sup>35</sup>S]TBM-NHS (also known as [<sup>35</sup>S]Sulphur labeling reagent, <sup>35</sup>SLR) is sold by Amersham as a general purpose <sup>35</sup>S-labelling reagent. Amersham reports that the [<sup>35</sup>S]TBM-NHS will react rapidly with  $\epsilon$  amino group of lysine and the  $\alpha$  amino group of the N-terminal amino acid of a polypeptide. Slower reaction is supposed to take place with the thiol groups of cysteine, the indole groups of tryptophan, and the guanidyl group of arginine, although no data are available from Amersham to support these product claims.

Because the [<sup>35</sup>S]TBM-NHS reagent has been commercially available for only a few years, the number of literature examples where this reagent is used is limited (Boxberg, 1988; Stockes, 1986; Rabilloud and Therre, 1986). The first reported use of [<sup>35</sup>S]TBM-NHS was as a protein labeling reagent (Rabilloud and Therre, 1986). The authors found that labeling proteins with [<sup>35</sup>S]TBM-NHS was more sensitive than silver staining, a common method for detecting proteins. The authors determined that as much as 10<sup>6</sup> cpm could be incorporated into as little as 5  $\mu$ g of protein. Two years later, Boxberg confirmed that [<sup>35</sup>S]TBM-NHS was a sensitive means to detect proteins. Boxberg determined that the [<sup>35</sup>S]TBM-NHS reagent increased the sensitivity of detection compared with silver staining by 100-fold, allowing detection as little as 6 pg of protein.

In contrast to the random modification of proteins with [<sup>35</sup>S]TBM-NHS, Knaus et al. (1992) showed that [<sup>35</sup>S]TBM-NHS can be used to synthesize a small [<sup>35</sup>S] radiolabeled molecule. Knaus et al. reacted [<sup>35</sup>S]TBM-NHS with the alkyl amine, 1,4-dihydro-2,6-

dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridine carboxylic acid 2-aminoethyl)ethyl ester in ethyl acetate. The [ $^{35}\text{S}$ ] radiolabeled molecule, [ $^{35}\text{S}$ ]sadopine, was obtained in > 85 % yield. Based on this result, we predicted that the reaction of [ $^{35}\text{S}$ ]TBM-NHS with amino (and possibly hydroxyl groups) in a DNA adduct would be an effective method to radiolabel a carcinogen-deoxynucleoside.

## **ii. Reagent choice**

The [ $^{35}\text{S}$ ]t-butoxycarbonyl-L-methionine, N-hydroxysuccinimidyl ester ([ $^{35}\text{S}$ ]TBM-NHS) (2) was postulated to be a useful labeling reagent for the detection of modified nucleosides for six reasons. First, the acylation of nucleosides is a high yielding reaction (Schaller et al., 1963). High yields are required in order to insure that the method being developed can detect low levels of DNA modification. Second, a chemical acylation procedure would be applicable for all chemical classes of adducts (both bulky and non-bulky) since it depends only on the availability of free hydroxyl and/or amine groups on the nucleoside and/or carcinogen moiety. Third, the  $^{35}\text{S}$  TBM-NHS reagent is commercially available at high specific activity (> 1,000 Ci/mmol). For comparison, the highest commercial specific activity for [ $^{32}\text{P}$ ]-ATP is 6,000 Ci/ mmol. Fourth, the long half-life of  $^{35}\text{S}$  (87.1 days) and relatively low energy of radiation makes the  $^{35}\text{S}$  reagent easier to handle than a  $^{32}\text{P}$  compound. In contrast to  $^{32}\text{P}$  compounds that require shielding to prevent radiation exposure to laboratory personnel,  $^{35}\text{S}$  compounds required no shielding for safe handling. In addition, the low-energy  $\beta$  emissions of  $^{35}\text{S}$  result in sharper autoradiographic bands compared to those generated by  $^{32}\text{P}$ , allowing more resolution for the analysis of a two-dimensional TLC, for instance. Fifth, the lower-energy emissions of  $^{35}\text{S}$  cause less

radiolysis to occur. In practice, this means that  $^{35}\text{S}$  -reaction products can be stored at  $-20^\circ\text{C}$  for several weeks without significant degradation (Current Protocols in Molecular Biology). Sixth, the  $^{35}\text{S}$  TBM-NHS reagent is not prone to racemization and is stable to a variety of reaction conditions. The following section examines this last point in detail.

### **iii. Reagent Stability**

A basic criterion for the ADAM procedure is that the acylation reaction should lead to a single product. The fulfillment of this intention is endangered by the racemization of the labeling reagent. The reversible loss and gain of the  $\alpha$  proton of an amino acid results in the loss of chiral purity. Coupling of racemized amino acid with a chiral nucleoside results in the formation of two diastereoisomers. Because the resulting diastereoisomers may result in two chromatographically separable products, the quantitation and identification of the acylation products would become more problematic. Fortunately, the alkoxycarbonyl protected amino acids (eg. BOC-amino acids), except under very harsh conditions (Bailey 1990), do not racemize.

The BOC protecting group is stable to mild acid and base, oxidizing and reducing reagents, and a variety of nucleophiles (Greene and Wuts, 1991). If the protecting group employed to block the  $\alpha$  amino group were not stable to the acylation conditions, then the activated methionine labeling reagent could react with itself forming polymethionine polymers. Since the ADAM procedure does not need to manipulate the methionine labeling reagent subsequent to acylation, the only requirement of a nitrogen protecting group is that it be stable over time and to a variety of reaction conditions involving bases and

temperature. Only under strongly acidic conditions (eg. treatment with trifluoroacetic acid) is the BOC group removed (Bodanszky, 1993).

#### **iv. Summary**

[<sup>35</sup>S]TBM-NHS has been useful for the labeling of proteins and small molecules. Although all of the literature examples of using [<sup>35</sup>S]TBM-NHS involve the labeling of an amine, we postulated that this reagent could be used to acylate carcinogen-DNA adducts. The chemical and physical properties that appear to be ideal for a labeling reagent for carcinogen-DNA adducts include the easy of handling, the high specific activity, and the chemical stability of the reagent. The following section discusses the rationale for expecting the acylation of nucleosides with [<sup>35</sup>S]TBM-NHS to be a high yielding reaction.

### **c. Acylation of Nucleosides**

#### **i. Peracylation of Nucleosides**

Since the pioneering studies of Todd and coworkers (Hayes et al., 1954; Anderson et al., 1954) on the acylation of deoxynucleosides, over thirty acyl groups have been shown to react with the exocyclic amino groups of cytosine, adenine, and guanine (Beaucage and Iyer, 1992). In most cases, the acyl blocking groups were introduced by the peracylation of the parent nucleoside. The yields for these procedures is generally good (> 70%) (Jones et al., 1984). Therefore, the (per)acylation of deoxynucleosides would be an effective means of attaching a radioactive or fluorescent probe suitable for subsequent detection and quantitation.

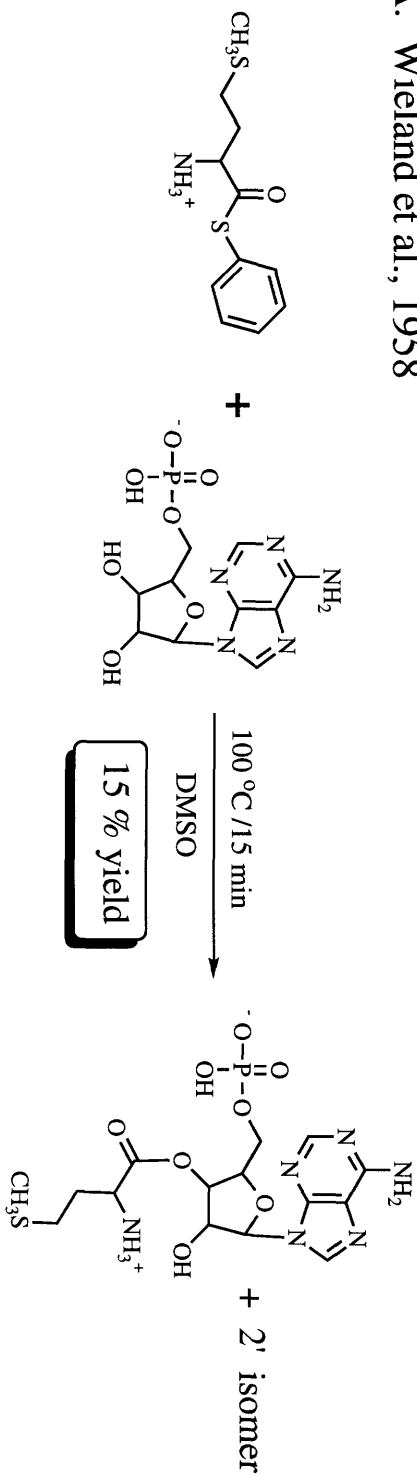
#### **ii. Acylamino esters of ribonucleosides**

Particularly relevant to the development of a methionine acylation procedure involving modified nucleosides are studies on the synthesis of acylamino acid esters of ribonucleosides. Synthetic acylamino acid esters of ribonucleosides have been synthesized and tested as substrates in order to understand the biochemistry of peptidyltransfer (Harris and Symons, 1973; Krayevsky et al., 1975). The first synthetic method for the preparation of 2'(3')-O-aminoacyl ribonucleoside 5'-phosphates was by Wieland and coworkers (Wieland et al., 1958) (Figure 2A). Treatment of a ribonucleoside 5'-phosphate (pA, pG, pC, and pU) with amino phenyl thioester hydrochlorides (or hydrobromides) afforded the 2'(3')-O-amino derivatives of the four common ribonucleoside 5'-phosphates. The disadvantages of this method include the high temperatures <100 °C required for reaction,

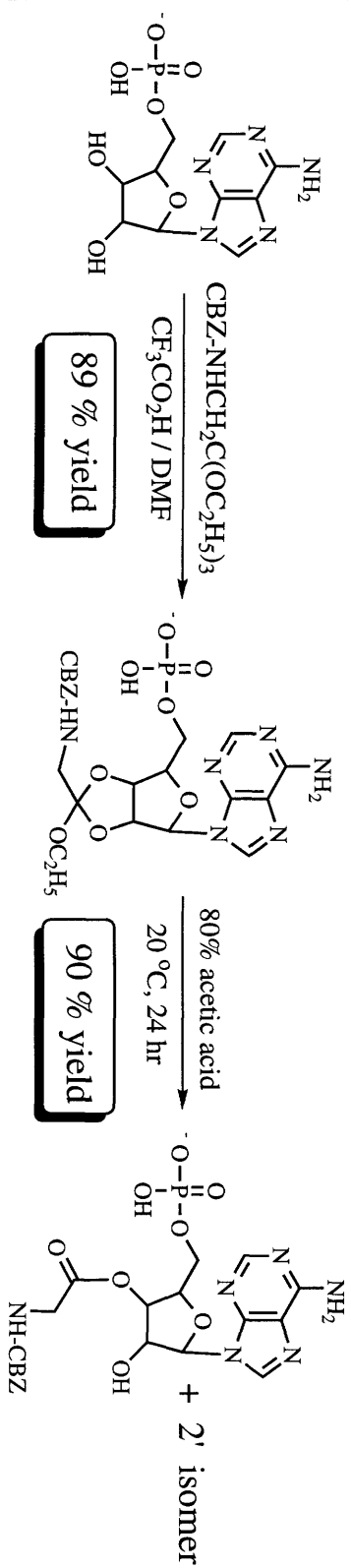


**Figure 2.** Literature methods for the synthesis of O-acylamino acid esters of ribonucleotides. A) acylation of pA with methionine phenyl thioester (Wieland et al., 1958).  
B) acylation of pA with N-benzyloxycarbonylglycine (CBZ-NH<sub>2</sub>C(OC<sub>2</sub>H<sub>5</sub>)<sub>3</sub>) via a two step acid catalyzed procedure (Zemlicka and Chladek, 1966)

A. Wieland et al., 1958



B. Zemlicka and Chladek, 1966



small yield of aminoacyl-nucleotides (2-17%), and the contamination of the aminoacyl-nucleotides with a number of by-products.

One technique that is suitable only for glycine involves the trifluoroacetic acid catalyzed coupling of N-benzyloxycarbonylglycine ethyl ortho ester with a variety of ribonucleosides and ribonucleotides (Zemlicka and Chladek, 1966; Zemlicka and Chladek, 1968; Chladek and Zemlicka, 1968; Chladek and Zemlicka, 1967) (Figure 2B). The product from acid catalyzed coupling was a cyclic 2',3' orthoester acylamino-nucleoside. A second step was then required to hydrolyze the cyclic 2',3' orthoester acylamino-nucleotide to the 2'(3') acylamino-nucleoside. The rather drastic conditions of condensation (DMF with CF<sub>3</sub>COOH) and hydrolysis (80% acetic acid, 20 °C, 24 h) would racemize any amino acid other than glycine.

An alternative method for the synthesis of 2'(3')-O-aminoacyl derivatives involves the reaction of N-benzyloxycarbonylamino anhydrides with 5' protected ribonucleotides (Rammler and Khorana, 1963) (Figure 3A). The symmetrical N-benzyloxycarbonyl-DL-phenylalanine anhydride used in their study was prepared by carbodiimide activation. Reaction of N-benzyloxycarbonyl-DL-phenylalanine anhydride with 5'-O-trityl (or 5'-O-di-p-methoxytrityl) ribonucleosides resulted in greater than 60% conversion to 2'(3')-O-aminoacyl derivatives. However, because the anhydride used in the acylation procedure was a racemate, it is uncertain whether any racemization occurred during the coupling step.

One method that is useful for all amino acids and does not lead to racemization involves the formation of BOC amino imidazole esters (Gottikh et al., 1970). Gottikh and coworkers determined that BOC-amino imidazole ester can be synthesized from BOC amino acids without racemization. Subsequent reaction of the BOC-amino imidazole esters with all four ribonucleoside 5'-phosphates occurs in modest yields without racemization (Figure 3B). The solvent medium providing the highest yield of BOC-amino acyl ribonucleoside esters was determined by Gottikh et al., as a 5:1 mixture of water:DMF. Because the BOC-amino acid imidazole ester was hydrolyzed to some extent by the water present, a ten-fold excess of BOC-amino acid-imidazole to nucleotide was required for optimal yields (as high as 88% for pU). For nucleotides soluble under completely anhydrous conditions, only a two-fold excess of BOC-amino acid imidazole esters was required for the same yield.

Gottikh and coworkers additionally showed that the acylation products and yield changed with the choice of solvent system (Gottikh et al., 1970). With pC, it was demonstrated that if the content of strongly solvating aprotic solvents ( $\text{Me}_2\text{SO}$ , DMF, formamide, or pyridine) was increased to more than 30%, the reaction with BOC-amino imidazole esters resulted mainly in acylation of the amino group of the cytidine residue. On the other hand, if the content of weakly-solvating aprotic solvents (acetonitrile, THF, acetone) was increased above 50%, the reaction of BOC-amino imidazole esters took place only at the ribose hydroxyls. This result illustrates the importance of solvent choice when attempting to per-acylate a deoxynucleoside.

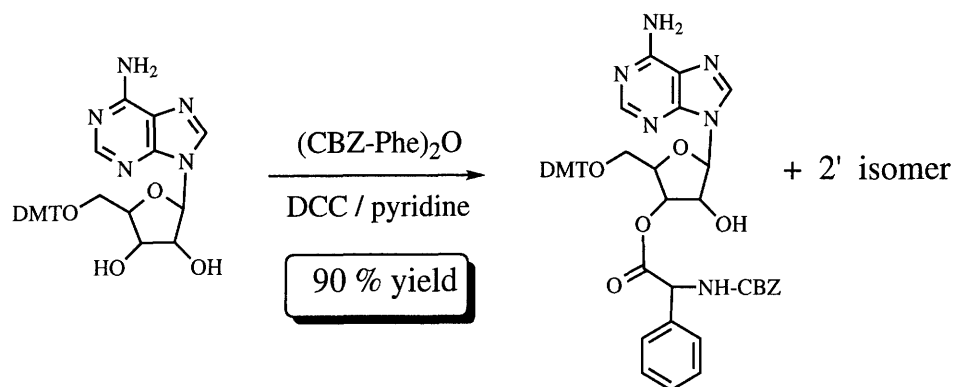
Seven years later, Azhayev et al. (1977) reported the same procedure but in larger quantities than the small scale preparations of Gottikh et al. (1970) (Figure 3C). Azhayev et al. additionally reported the acylation of ribonucleotides with BOC-methionine imidazole ester and N-formyl-methionine imidazole ester. The extent of acylation with BOC-methionine imidazole ester varied from as low as 22% yield for pC to 50% yield with pU. While Azhayev et al. found that no racemization occurred when preparing BOC-methionine imidazole ester, differing degrees of racemization occurred with N-formyl-L-methionine with three *in situ* carboxyl activating reagents. In the case of p-toluenesulfonyltriazole, no racemization was observed. Partial racemization was encountered with mesitylenesulfonyltriazole. In the case of dicyclohexylcarbodiimide (DCC), complete racemization occurred. These results illustrate the importance of the  $\alpha$  amino protecting group for preventing racemization of amino acids. In the procedure under development in small yield of aminoacyl-nucleotides (2-17%), and the contamination of the aminoacyl-nucleotides with a number of by-products.

### **iii. Acylamino esters of deoxynucleosides**

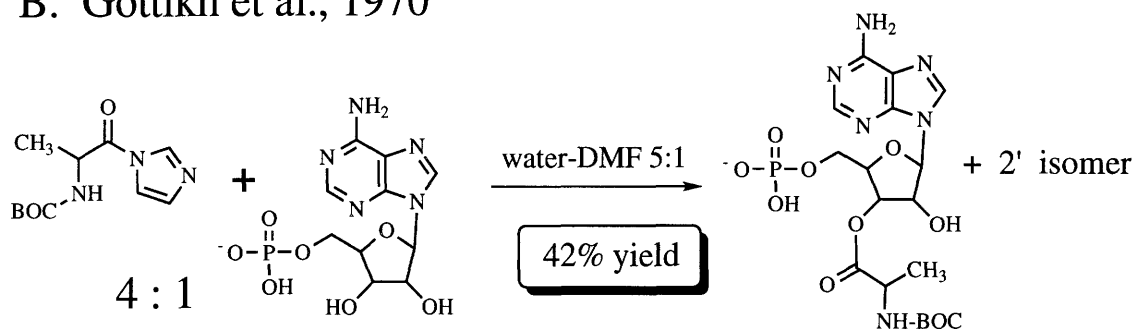
In direct support of our postulation that amino acid acylation would be an effective method to label modified deoxynucleosides, Sanda et al. (1987) and Hirohashi et al. (1987) showed that a modified deoxynucleoside, 2'-deoxy-5-fluorouridine, could react with *in situ* activated BOC-gly. Using DCC activation, 5' protected derivatives of 2'-deoxy-5-fluorouridine were acylated with BOC-gly.

**Figure 3.** Literature methods for the synthesis of O-acylamino acid esters of ribonucleotides. A) acylation of 5'-O-di-p-methoxytrityl adenosine with N-benzyloxycarbonyl-DL-phenylalanine anhydride (Rammler and Khorana, 1963). B) acylation of pA with BOC-alanine imidazole ester (Gottikh et al., 1970). C) acylation of pA with BOC-methionine imidazole ester (Azhayev et al., 1977).

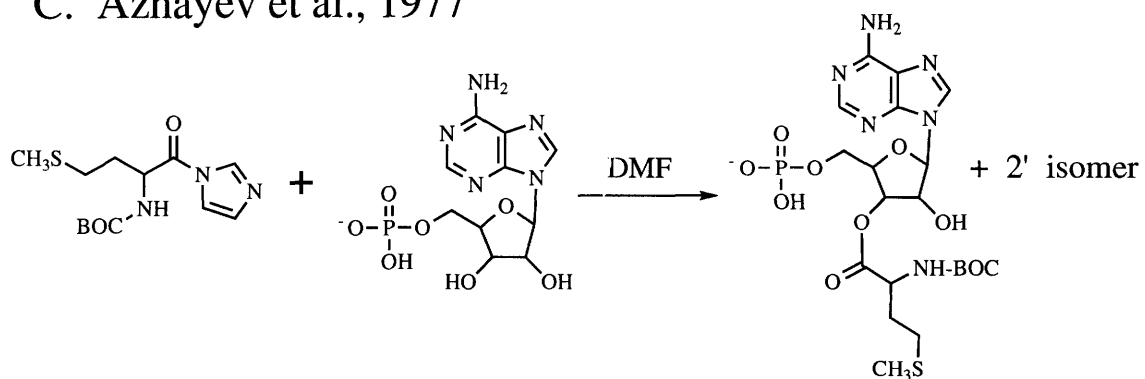
### A. Rammler and Khorana



### B. Gottikh et al., 1970



### C. Azhayev et al., 1977



#### **iv. Summary**

The acylation with an activated BOC-methionine derivative appears to be a facile method of attaching a radiolabel to modified nucleosides based on three lines of research. First, the work of Sanda et al. (1987) and Hirohashi et al. (1987) indicates that modified deoxynucleosides can be acylated with a BOC protected amino acid. Second, the work by Azahayev et al. (1977) indicates that imidazole activated BOC-methionine, in particular, can serve as the acylating reagent. Third, Gottikh et al. (1970) found no racemization occurring during activation or subsequent acylation of ribonucleosides with a BOC protected methionine derivative. Based on these findings, we have proposed that the acylation of modified deoxynucleosides with an appropriately activated BOC-methionine reagent, [<sup>35</sup>S]TBM-NHS, will result in facile labeling.

In terms of developing a general method to label a wide variety of modified deoxynucleosides, the study of Gottikh et al. indicates that solvent choice will be a crucial factor to optimize. In particular, Gottikh et al. found that the site of reaction with ribonucleosides changes with solvent conditions. Since we are attempting to peracylate modified deoxynucleosides, a solvent that is ideal for acylation of exocyclic amino groups and sugar hydroxyls simultaneously needs to be chosen.

#### **d. Carbodiimide Activation**

As was seen in the previous section, carbodiimide activation is a useful method to activate amino acids for coupling to ribo- and deoxyribonucleosides. Carbodiimide



activation can be used to prepare amino acid anhydrides (Rammner and Khorana, 1963) for subsequent acylation of ribonucleotides or to activate amino acids *in situ* for reaction with deoxynucleosides (Sanda et al., 1987; Hirohashi et al. 1987). The following section examines the advantages and limitations of carbodiimide activation.

#### **i. Formation of activated carbonyl**

The introduction of carbodiimides (Sheehan and Hess, 1955; Khorana, 1953), DCC and diisopropylcarbodiimide (DIC) as reagents for the formation of the peptide bond, was a major improvement in the synthesis of peptides. The novel feature of these coupling reagents was that they could be added to the mixture of the carboxyl and amine components. With these reagents, carboxyl activation and coupling with the amine of another amino acid proceed concurrently. The process of activation involves reaction of a carboxylic acid with a carbodiimide to form an O-acylisourea. This product results from the attack of one of the oxygens of the carboxylic acid onto the central carbon of the carbodiimide. This initial attack is enhanced by protonation of one of the nitrogens of the carbodiimide  $N=C=N$  bond. The carbonyl of the resulting O-acylisourea is then activated towards displacement by hydroxyls and amines (Bodanszky, 1993). The O-acyl-isourea intermediate, in addition, is somewhat basic thus increasing the reactivity of coupling via general base catalysis. Subsequent to displacement by a nucleophile, the isourea rearranges to a urea (dicyclohexylurea and diisopropylurea) which is a particularly stable molecule whose formation is also a driving force for the reaction. In the case of carboxyl activation by carbodiimides in the absence of a nucleophile, a second bond forming reaction occurs which leads to the formation of symmetrical anhydrides (Schlusser and Zahn, 1962). As shown by

the work of Rammler and Khorana (1963), symmetrical anhydrides are useful for acylation of ribonucleotides.

## **ii. Drawbacks of carbodiimide activation**

Carbodiimide couplings have four drawbacks. One drawback is that in certain solvents, such as methylene chloride or acetonitrile, the acylurea formation is suppressed (Sheehan et al., 1956). A second drawback of overactivation in the reactive intermediate is the loss of chirality in the case of amino acid activation (Konig and Geiger, 1970; Bodanszky, 1993). The extent of racemization increases with temperature and is more pronounced in polar solvents such as DMF than in methylene chloride (Weyland et al., 1963). A third drawback is that the nitrogen center of the N=C bond on the O-acylisoureas competes with other nucleophiles for the acyl residue (Figure 4A). The rearrangement of the intermediate O-acylisourea to produce N-acylureas, results in the loss of acylating amino acid. A fourth drawback is that amines can react with carbodiimides to yield stable guanidine derivatives under certain conditions (Figure 4B).

## **iii. Addition of auxiliary nucleophiles**

Racemization, N-acylureas and guanidine formation can be suppressed by the addition of auxiliary nucleophiles such as 1-hydroxybenzotriazole (HOBt) (Konig and Geiger, 1970) or N-hydroxysuccinimide (NHS) (Wunsch and Dress, 1966; Zimmerman and Anderson, 1967). The HOBt or NHS can react with the carbodiimide or anhydride intermediates to yield semi-stable HOBt or NHS esters. The presence of these nucleophiles in the reaction mixture reduces the concentration of the O-acylisourea and thereby the extent of

racemization. The resulting NHS ester are less reactive to nucleophilic displacement and, as such, can be isolated and stored (Anderson et al., 1963). In the case of some NHS esters, the material can be recrystallized from alcohol (Bodanszky and Ondetti, 1966). The slightly acid nature of HOBt and NHS also prevent proton abstraction from the  $\alpha$  carbon atom. In the case of a chiral acid, such as an amino acid, these additives can thus contribute to the conservation of chiral purity. Finally, the inclusion of HOBt or NHS in a DIC coupling reaction efficiently shortens the lifetime of the overactivated O-acyl-isourea intermediate and thus diminishes the extent of O- $\rightarrow$  N acyl migration leading to N-acylureas.

#### **iv. Summary**

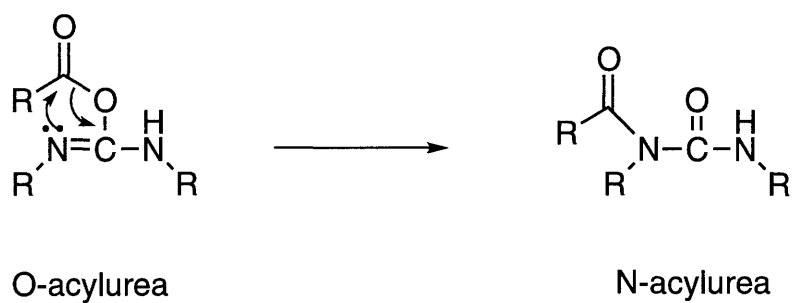
Although carbodiimide activation has some drawbacks such as the potential to racemize an amino acid substrate or to react unproductively, under optimal conditions carbodiimide activation is an effective method of ester/amide bond formation.

**Figure 4.** Side reactions involving DIC.

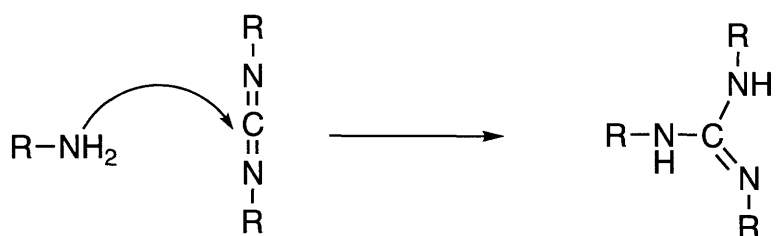
A. rearrangement of O-acylisourea to N-acylurea.

B. reaction of amine with carbodiimide

## A. N-acylurea Formation



## B. Guanidine Formation



## **D. Results and Discussion**

### **a. Introduction**

While a number of analytical methods can detect certain classes of DNA adducts, a general, inexpensive method capable of detecting all classes of DNA adducts is not available. Consequently, work was begun in the Wogan laboratory to develop a new method for detecting DNA adducts. The approach taken was to develop optimal conditions to peracylate nucleosides with [<sup>35</sup>S]TBM-NHS, an <sup>35</sup>S labeling reagent previously used in the detection of proteins. In support of our approach, a number of researchers have established that ribonucleotides can be acylated in good yields with protected amino acids. A study by Azhayev et al. (1977), in particular, showed that all four ribonucleotide 5'-phosphates can be acylated with BOC-methionine imidazole ester in fair to good yields. Consequently, the reaction of TBM-NHS with deoxynucleosides was envisioned be an effective method to label carcinogen-DNA adducts.

As depicted in Figure 1, a mixture of products can result from the acylation of deoxynucleosides. In order to simplify detection and quantitation, ideally only one product should result from the acylation procedure. Therefore, efforts in the Wogan laboratory have focused on developing conditions whereby all reactive sites on a carcinogen-DNA adduct are acylated. In addition to simplifying detection and quantitation, peracylation would allow the

attachment of more than one radiolabel to a target molecule. Consequently, the radioactive signal for a peracylated molecule would be greater.

The following sections examine the development of optimal conditions to peracylate dGuo and dGuo-8-ABP, a carcinogen-DNA adduct. Our studies began by examining the peracylation of dGuo. It was postulated that the optimal reaction conditions developed for the acylation of dGuo could then be applied to modified deoxynucleosides. In addition, the physical characterization of dGuo would later help in understanding the NMR and other physical characteristics of acylmethionyl-carcinogen-deoxynucleosides.

#### **b. Deoxyguanosine Acylation**

The following sections discuss the development of the optimal conditions for acylation of dGuo with TBM. The first section compares the peracylation of mmol quantities of dGuo with TBM by two methods: 1) reaction with TBM activated *in situ* with DIC; and 2) reaction with activated TBM-NHS. The second section compares the conditions for the peracylation of mmol quantities of dGuo with the conditions needed for the peracylation of pmol (or less) quantities of dGuo. Finally, the third section discusses the characterization of the acylation products.

##### **i. Acylation dGuo with BOC-methionine and DIC**

The first conditions chosen to peracylate dGuo involved *in situ* activation of TBM with DIC using the conditions of Sanda et al. (1987) and Hirohashi et al. (1987). Under these

conditions a mixture of products, as detected by TLC or HPLC, were obtained (Figure 5). Conditions examined to force the reaction to a single product included time of reaction (4 to 14 hr), temperature (room to 70 °C), ratio of reagents (5 to 10 fold excess BOC-Met and DIC/dGuo), solvent (Me<sub>2</sub>SO, pyridine, THF), and addition of base (triethylamine) or catalyst (DMAP). Optimal reaction conditions involved the reaction of dGuo (0.40 g, 1.40 mmol), BOC-Met (1.76 g, 7.07 mmol, 500 M%), DIC (1.00 ml, 6.43 mmol, 460 M%) and DMAP (0.354 g, 2.90 mmol, 207 M%) in THF (20 ml) and Me<sub>2</sub>SO (2 ml) at 70 °C. The reaction, as monitored by HPLC or TLC, showed only one product, peak 4, after 4 hr. Peak 4 could be purified by normal phase silica chromatography in 97 % yield.

Crucial to forcing the reaction to one product was the choice of solvent and temperature. When conducted at low temperature (room temperature to 50 °C), the acylation reaction yielded a mixture of peaks (starting material, peak1, peak 3 and peak 4). By increasing the reaction temperature, a single product (peak 4) resulted within 4 hr. However, unless a polar solvent such as Me<sub>2</sub>SO was added, the dGuo failed to dissolve and, consequently, no reaction occurred. Unfortunately, the reaction in Me<sub>2</sub>SO was not effective because of byproducts arising from the reaction of the DCC with the Me<sub>2</sub>SO solvent (March, 1985). A mixed solvent of pyridine and Me<sub>2</sub>SO was somewhat effective, but consistently higher yields of recovered product occurred with THF and Me<sub>2</sub>SO as the solvent.



## ii. Acylation of dGuo with TBM-NHS

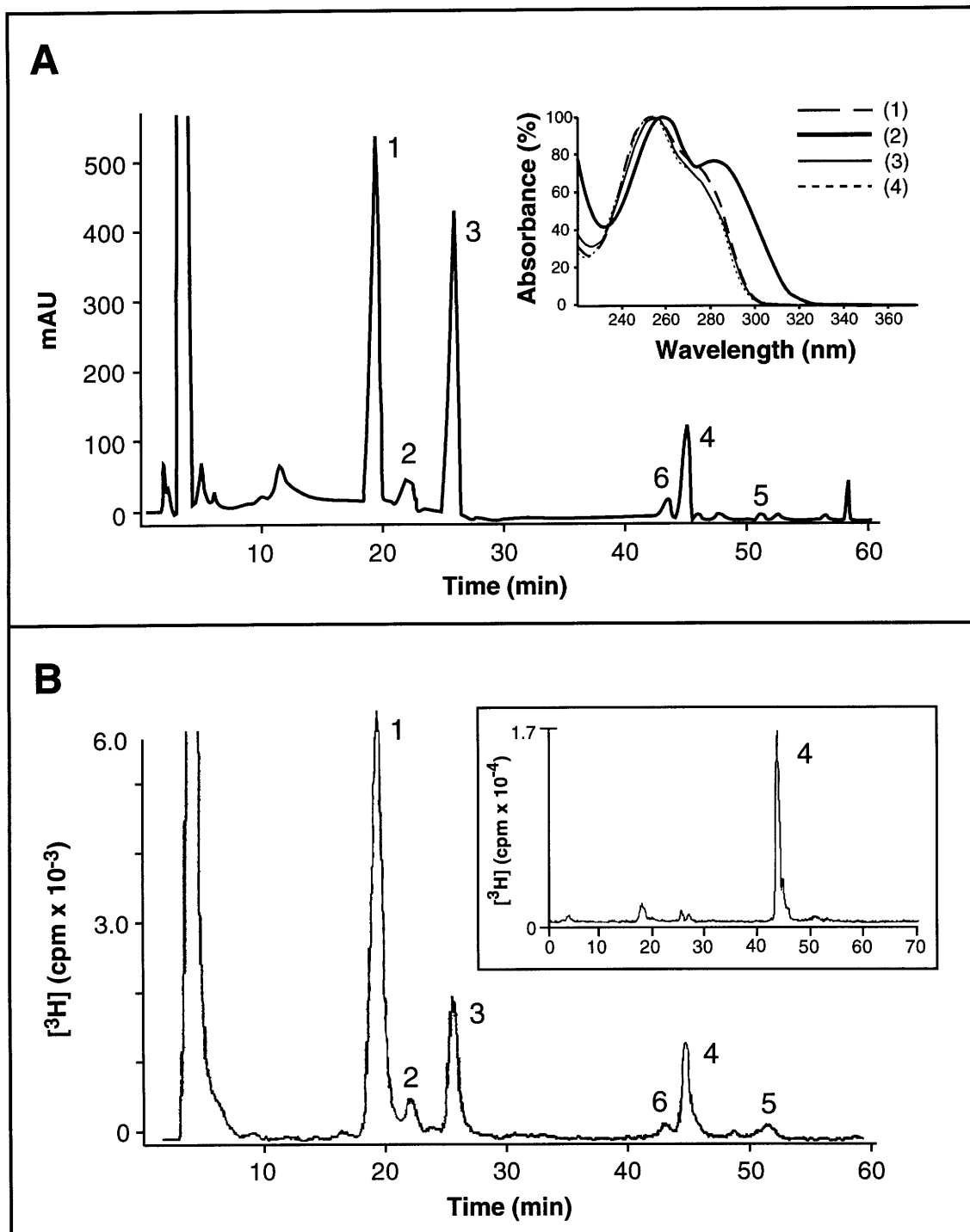
Having developed optimal conditions for the acylation of dGuo with TBM activated *in situ*, the same reaction conditions of solvent, time, and temperature were repeated with TBM-NHS as the acylating species. As seen with *in situ* activated TBM, one major product as detected by TLC or HPLC resulted after 4 hr. The same major product, peak 4, was isolated in 94 % yield after chromatography. However, in addition to this major peak, a minor less polar product, peak 5, was isolated in 2 % yield.

Based on the retention time peak 5 was proposed to result from additional acylation over that found with peak 4. In an attempt to synthesize larger quantities of the material eluting under peak 5, peak 4 was resubjected to acylation with TBM-NHS. Because of the increased solubility of peak 4 in THF as compared to dGuo, Me<sub>2</sub>SO was not added as a cosolvent. Peak 4 (1.35 g) was treated with TBM-NHS (0.95 g, 2.74 mmol) and DMAP (0.21 g) in 15 ml THF and heated at 70 °C. No reaction occurred even after 24 hr at 70 °C as monitored by HPLC. Although conversion of peak 4 to peak 5 did not occur, peak 4 proved to be completely stable at 70 °C for prolonged time (24 hr).

## iii. Acylation of [<sup>3</sup>H]dGuo with TBM-NHS

Under similar conditions of solvent, time, and temperature as developed for mmol scale acylation of dGuo, experiments conducted by F. Sheabar with pmol or less of dGuo or [<sup>3</sup>H]dGuo established that a similar elution profile resulted (Figure 5). Unfortunately, the extent of conversion of dGuo to acylated products was not identical. In order to achieve the same extent of conversion of dGuo to acylated dGuo products when conducted on pmol

**Figure 5.** HPLC profile of TBM acylated products of A) dGuo and B) [ $^3\text{H}$ ]dGuo.



scale conditions, three modifications of the reaction conditions were required which included: 1) the amount of acylating reagent; 2) the requirement for DIC; and 3) the choice of solvent.

A major difference between the acylation of pmol and mmol quantities of dGuo was the amount of acylating reagent used. While only a 5 fold excess of acylating reagent was used with mmol quantities of dGuo, over  $10^4$  molar equivalents were necessary to achieve the same extent of acylation in the case of pmol quantities of [ $^3\text{H}$ ]dGuo. Complete acylation of the [ $^3\text{H}$ ]dGuo did not occur with less than  $5 \times 10^4$  molar equivalents.

A second difference between mmol and pmol acylation reactions was the requirement for DIC, in the case of pmol reactions. In the case of mmol acylation of dGuo, DIC was required only for the activation of unactivated TBM. In contrast, the extent of conversion of pmol quantities of dGuo using TBM-NHS without DIC was only 19%. By including DIC when TBM-NHS was used as the acylating reagent in pmol scale reactions, the extent of acylation increased to 88%.

The third difference between acylation of mmol and pmol quantities of dGuo was the choice of solvent. In the case of mmol reactions,  $\text{Me}_2\text{SO}$  was required to insure that the dGuo dissolved. Because a 50 fold or greater dilution was used with pmol reactions, the poor solubility of dGuo in THF was not a problem. Consequently,  $\text{Me}_2\text{SO}$  was not required as a cosolvent for pmol reactions. However, the addition of a basic cosolvent did increase

the extent of conversion for pmol reactions. Two solvents that have proved to be very effective in increasing the yield are triethylamine and pyridine.

With these changes in the acylating conditions [TBM-NHS ( $2 \times 10^4$  molar equivalents), DIC ( $1 \mu\text{l}$ ), THF ( $50 \mu\text{l}$ ), and triethylamine ( $1 \mu\text{l}$ ) at  $75^\circ\text{C}$  for 2 hr], the acylation efficiency for dGuo exceeded 90% as detected by radioactivity measurement. As was determined for mmol scale acylation of dGuo, peak 4 was the major acylation product accounting for 88% of the total radioactivity eluting under the peaks derived from acylation. Subsequent experiments have demonstrated that the temperature used can be decreased to  $37^\circ\text{C}$  by increasing the molar excess of TBM-NHS slightly and using pyridine instead of triethylamine as a solvent. When acylation of pmol or smaller quantities of dGuo were incubated for 2 hr at  $37^\circ\text{C}$  with TBM-NHS ( $5 \times 10^4$  molar equivalents), DIC ( $1 \mu\text{l}$ ), pyridine ( $50 \mu\text{l}$ ), and THF ( $50 \mu\text{l}$ ), the conversion of starting nucleoside to acylated products exceeded 95%. Similar to the mmol scale acylation of dGuo with TBM-NHS, the major product was peak 4 in greater than 82% yield.

#### **iv. Structure Determination of Acylation Products of dGuo**

The structural identity of the acylation products (Figure 5, peaks 1 to 6) was determined by UV,  $^3\text{H}$  radioactivity, liquid SIMS, FAB-MS,  $^1\text{H}$  NMR, and  $^{13}\text{C}$  NMR. The first information used to assign to the structure of the TBM acylation products was UV spectra. The UV spectrum of the acylation products (peaks 1-6), irrespective whether conducted on mmol or pmol scale, revealed that the products all had UV absorption spectra characteristic of guanine. In fact, the UV absorbance spectrum of peaks 1, 3, and 4 were

almost identical (Figure 5, inset). The  $\lambda_{\text{max}}$  determined for all three products was 255 nm. In contrast, a shift to 260 nm in the UV  $\lambda_{\text{max}}$  was observed for peaks 2, 5, and 6. In addition, the shoulder seen at 270 nm for peaks 1, 3, and 4 became a distinctly separate peak with  $\lambda = 280$  nm. This bathochromic shift, a shift to longer wavelengths, was attributed to a change in electronic character of the purine ring. Since attachment of TBM to the 5' or 3' hydroxyls would not be expected to change the UV absorbance spectra, peaks 2, 5 and 6 were proposed to be substituted at the N<sup>2</sup> position of the purine ring.

HPLC experiments conducted by F. Sheabar involving acylation of [<sup>3</sup>H]dGuo help to establish that the products of acylation contained the deoxyribose portion of dGuo. As seen in Figure 5, the products of acylation coeluted irrespective of the detection method (<sup>3</sup>H or UV absorbance). This coelution meant that the products of acylation must have a purine chromophore (for UV detection), a sugar (since the tritium in [<sup>3</sup>H]dGuo was in the 1' and 2' positions in the deoxyribose group of dGuo), and the methionine labeling reagent. Assuming that no rearrangements took place, seven products would be possible: three mono-TBM-dGuo products (5'- ; 3'- ; and N<sup>2</sup>-), three bis-TBM-dGuo products (5',3'- ; 3',N<sup>2</sup>- ; and 5',N<sup>2</sup>-), and one tris-TBM-dGuo product (5',3',N<sup>2</sup>-) (Figure 1).

Definitive proof for the extent of acylation for peaks 1 to 5 was obtained by mass spectroscopy. In order to insure that the reaction products detected when conducted in pmol and mmol scale were the same, peaks from both methods were collected and submitted for mass spectroscopy using different instruments. Peaks 1 to 4 from a nmol scale reaction were submitted for liquid SIMS using a two sector MAT 731 secondary ion mass

spectrometer while peaks 1, 3, 4, and 5 from a mmol scale reaction were submitted for FAB-MS using a Finnigan mass spectrometer. Mass spectra of the acylation products from both methods and samples agreed with respect to the molecular weight of the parent ion. To insure the identity of the products of acylation, the exact molecular weights were determined for the parent ions of peaks 1, 3, 4, and 5.

The mass spectra of the polar acylation products, peaks 1 to 3, all showed parent proton and sodium molecular ions of 499 and 521 m/z, respectively. The parent proton molecular weight of 499 m/z corresponds to that of a mono-TBM-dGuo product (Figure 1, R=H: **5a,b,c**). In the case of peaks 1 and 3, the exact high resolution result (499.1972 and 499.1977, respectively) agreed with the expected exact molecular weight for  $[M+H]^+$  of 499.1975 for a mono-TBM-dGuo product. The fragmentation pattern for peaks 1 to 3, unfortunately, did not allow unambiguous assignment as to which peak corresponded to the 5', 3' or N<sup>2</sup> product. However, based on the bathochromic shift seen for peak 2, this product was tentatively assigned as the N<sup>2</sup>-mono-TBM-dGuo product (Figure 1, R=H: **5c**).

The mass spectrum determined for peak 4 showed a parent proton and sodium molecular ions of 730 and 752 m/z, respectively. This parent proton molecular weight corresponds to that of a bis-TBM-dGuo product (Figure 1, R=H: **6a,b,c**). The exact high resolution result (730.2909) additionally agreed with the expected exact molecular weight of  $[M+H]^+ = 730.2904$  for a bis-TBM-dGuo product. Since no bathochromic shift was seen in the UV spectra of this acylation product, peak 4 was tentatively assigned as the 5',3'-bis-TBM-dGuo product (Figure 1, R=H: **6a**).

Finally, the mass spectrum for peak 5 showed a parent proton and sodium molecular ions of 961 and 983, respectively. This molecular weight corresponds to that of a tris-TBM-dGuo product (Figure 1, R=H: 7). The exact high resolution result (961.3841) additionally agreed with the expected exact molecular weight of  $[M+H]^+ = 961.3833$  for a tris-TBM-dGuo product. In the case of this product, the molecular weight allowed us to unambiguously assign this product as the product of acylation at the N<sup>2</sup>, 5', 3' positions of dGuo (Figure 1, R=H: 7).

Confirmation of the regiochemical assignments peaks 1, 2, 3 and 4 depended on NMR. In assigning the regiochemistry of the mono-TBM-dG and bis-TBM-dGuo products, the chemical shifts of the H3', H5' protons and C3' and C5' carbons were used. The <sup>1</sup>H NMR of peak 1 showed a 0.6 ppm down field shift of the H5' signal relative to underivatized dGuo. In contrast, the <sup>1</sup>H NMR of peak 3 showed a 1 ppm down field shift of the H3' proton. This down field shift in the <sup>1</sup>H NMR were postulated to reflect the attachment of an electron withdrawing group on the different hydroxyl groups of dGuo. Therefore, peak 1 was tentatively assigned as the 5'-mono-TBM-dGuo product and peak 3 was tentatively assigned as the 3'-mono-TBM-dGuo product. The unambiguous assignment of peaks 1 and 3 was accomplished with <sup>13</sup>C NMR. The <sup>13</sup>C NMR of peak 1 showed a 3 ppm down field shift of C5' carbon relative to dGuo, confirming the regiochemical assignment of this peak as 5-TBM-dGuo. With peak 3, the C3', in contrast, shifted 5 ppm down field relative to dGuo, confirming the regiochemical assignment of this peak as 3'-TBM-dGuo.



Based on the FAB-MS and the absence of a UV bathochromic shift, peak 4 had been tentatively assigned as the 5',3'-bis-TBM-dGuo product (Figure 1, R=H: **6a**). The <sup>1</sup>H NMR confirmed this product as a bis-TBM-dGuo product. In addition, the distinct chemical shifts seen for BOC and thio methyl groups for the 5'-mono-TBM-dGuo (1.40 and 2.01 ppm, respectively) and the 3'-mono-TBM-dGuo (1.45 and 2.10 ppm, respectively) products were both seen in this sample (1.39, 1.44, 2.00, and 2.09 ppm). In addition, the H3' signal seen for this product was identical to the chemical shift for H3' seen in the 3'-mon-TBM-dGuo product; whereas, the H5' signal was identical to the chemical shift for the H5' proton in the 5'-mono-TBM-dGuo product. This result strongly indicated that acylation had taken place at both hydroxyls. Finally, <sup>13</sup>C NMR confirmed this product as the 5',3'-bis-TBM-dGuo product. Except for the tertiary carbon of the BOC group and the  $\alpha$  carbon of methionine, which were not separable at 300 MHz, all the <sup>13</sup>C resonances expected for 5',3'-bis-TBM-dGuo product were seen.

In the case of peak 5, the earlier FAB-MS result indicated that this peak was a tris-TBM-dGuo product. The <sup>13</sup>C and <sup>1</sup>H NMR confirmed this assignment. In the <sup>1</sup>H NMR of peak 5, the chemical shifts determined for the thiomethyl and BOC methyls were identical with the resonances seen with the 5',3'-bis-TBM-dG product. The <sup>13</sup>C chemical shifts for the ribose portion of this molecule were similar to those seen for the 5',3'-TBM-dGuo product, indicating attachment of TBM groups to both the 5' and 3' hydroxyls. However, both the <sup>13</sup>C and <sup>1</sup>H NMR showed changes in the purine resonances that were not seen for the 5', 3' and 5',3'-TBM-dG products. Specifically, a downfield shift of the H8 proton of 0.3 ppm occurred. In addition, the C6, C2, C4, C8 and C5 purine resonances that were

almost identical in 5'-TBM-dG, 3'-TBM-dG, and 5,3'-bis-TBM-dG products shifted both upfield and down field with this product. Based on the bathochromic shift and the shift in the purine resonances in the  $^{13}\text{C}$  and  $^1\text{H}$  NMR, peak 5 was unambiguously assigned as the 5',3',N<sup>2</sup>-tris-TBM-dGuo product (Figure 1, R=H: **7**).

In the case of peak 6, insufficient material was formed for unambiguous structural determination. Based on the UV,  $^3\text{H}$  radioactivity, and relative retention time, this peak was tentatively assigned as a bis-TBM-dGuo product (Figure 1, R=H: **6b, c**).

#### v. Summary

Whether done on a mmol or pmol scale, dGuo was acylated in greater than 94% yield with BOC-Met activated as an activated NHS ester or as an activated O-acylurea formed by *in situ* with DIC. The optimal reaction conditions for mmol acylation involved using greater than 500 M% of acylating reagent (TBM-NHS or BOC-Met + DIC), THF and Me<sub>2</sub>SO as a solvents and heating at 70 °C for 4 hr. The optimal conditions for pmol acylation involved using TBM-NHS (5 x 10<sup>4</sup> molar equivalents), DIC (1 μl), THF (50 μl), and pyridine (50 μl) at 37 °C for 2 hr. Under nonoptimal conditions, a mixture of products, peaks 1-6, resulted. Under optimized conditions, one major product, peak 4, resulted in both cases.

Structures of acylation products, peaks 1-5, were unambiguously assigned by UV, FAB-MS,  $^1\text{H}$  and  $^{13}\text{C}$  NMR. Peaks 1-3 were determined to be mono-TBM-dGuo products: 5', N<sup>2</sup>, and 3', respectively. Peak 4, the major product of acylation under optimized conditions, was determined to be the 5',3'-bis-TBM-dGuo product. Peak 5, a minor

product formed under optimized conditions, was determined to be the N<sup>2</sup>,5',3'-tris-TBM-dGuo product. The only peak not assigned was peak 6. Based on the retention time, this peak probably corresponded to one of the two bis-TBM-dGuo products not assigned.

### **c. Acylation of a DNA Adduct**

Using the optimal reaction conditions developed for dGuo, acylation was next tested with a prototypical carcinogen-DNA adduct. In order to insure that the newly developed method could peracylate all the reactive sites normally present on a deoxynucleoside, we choose to study a DNA adduct that did not involve covalent attachment to exocyclic groups. Having optimized acylation reaction conditions for dGuo, we chose to study a C8 guanine adduct, N-(deoxyguanosin-8-yl)-4-aminobiphenyl (dGuo-8-ABP). The dGuo-8-ABP adduct accounts for 80 % of the total ABP-DNA adducts resulting from exposure to 4-aminobiphenyl, a known bladder carcinogen (Melick et al., 1955; Melick et al., 1971). The biological importance of this DNA lesion is discussed in more detail in Chapter II of this dissertation.

#### **i. Synthesis of dGuo-8-ABP and [<sup>3</sup>H]dGuo-8-ABP**

The carcinogen-DNA adduct, dGuo-8-ABP, was synthesized according to the procedure of Famulok et al. (1989). In addition, in order to detect the dGuo-8-ABP and the TBM acylation products when optimizing the acylation reaction in amounts insufficient to be observed by UV absorption, the synthesis of a radiolabeled dGuo-8-ABP was required. Three methods were examined to synthesize a tritium labeled carcinogen-nucleoside: 1) *in*

*vitro* reaction of N-acetoxy-[<sup>3</sup>H]aminobiphenyl with dGuo; 2) *in vitro* reaction of N-acetoxy-aminobiphenyl with [<sup>3</sup>H]dGuo; and 3) *in vivo* adduct formation with 4-[<sup>3</sup>H]aminobiphenyl.

The first method used to synthesize tritium labeled dGuo-8-ABP involved reaction of N-acetoxy-[<sup>3</sup>H]aminobiphenyl with dGuo. The first step involved tritium labeling of 4-nitrobiphenyl (**8**) according to Breeman et al. (1978) and Thissen et al. (1980). The 4-[<sup>3</sup>H]nitrobiphenyl (**9**) was then reduced with an ammonium sulfide solution to N-hydroxy-[<sup>3</sup>H]aminobiphenyl (**10**) according to the procedure by Lee and King (1981). Activated N-acetoxy-[<sup>3</sup>H]aminobiphenyl (**11**) was prepared by reaction of the N-hydroxy-[<sup>3</sup>H]aminobiphenyl (**10**) with acetylcyanide at -45 °C. Unfortunately, as detected by HPLC, subsequent reaction of the N-acetoxy-[<sup>3</sup>H]aminobiphenyl (**11**) with dGuo failed to yield dG-8-[<sup>3</sup>H]ABP (**12**), reflecting the small extent of reaction (4 % yield) when only one equivalent of N-acetoxy-aminobiphenyl is added to dGuo (Famulk et al., 1989).

Failure to synthesize dGuo-8-[<sup>3</sup>H]ABP through the above route led us to synthesize [<sup>3</sup>H]dG-8-ABP (Figure 6b). The rationale for this approach was that synthesis of N-acetoxy-aminobiphenyl could be done on a large scale as compared to limited quantities of N-acetoxy-[<sup>3</sup>H]aminobiphenyl (**11**) that could be prepared. Consequently, the formation of [<sup>3</sup>H]dGuo-8-ABP could be forced, in theory, to 100 % conversion by the addition of a very large excess of N-acetoxy-aminobiphenyl to [<sup>3</sup>H]dGuo. The [<sup>3</sup>H]dGuo was prepared by F. Sheabar by dephosphorylation of [<sup>3</sup>H]dGTP (0.323 nmol, 31 Ci/mmol) using calf intestinal and bacterial alkaline phosphatases. The [<sup>3</sup>H]dGuo was then reacted with a large excess of

N-acetoxy-4-aminobiphenyl (1 mmol). After 3.5 hr, additional N-acetoxy-aminobiphenyl (1 mmol) was added. The [ $^3\text{H}$ ]dGuo-8-ABP was isolated in 29% yield after chromatography.

*In vivo* adduct formation involved treating nine month old female Fischer rats with 4- [ $^3\text{H}$ ]aminobiphenyl (5 mg, 200 mCi/mmol). 4- [ $^3\text{H}$ ]aminobiphenyl prepared by reduction of commercially available 4- [ $^3\text{H}$ ]nitrobiphenyl with hydrazine and Pd/C. Treatment of animals and isolation of carcinogen labeled DNA was done by F. Sheabar.

## **ii. Acylation of dGuo-8-ABP with TBM-NHS**

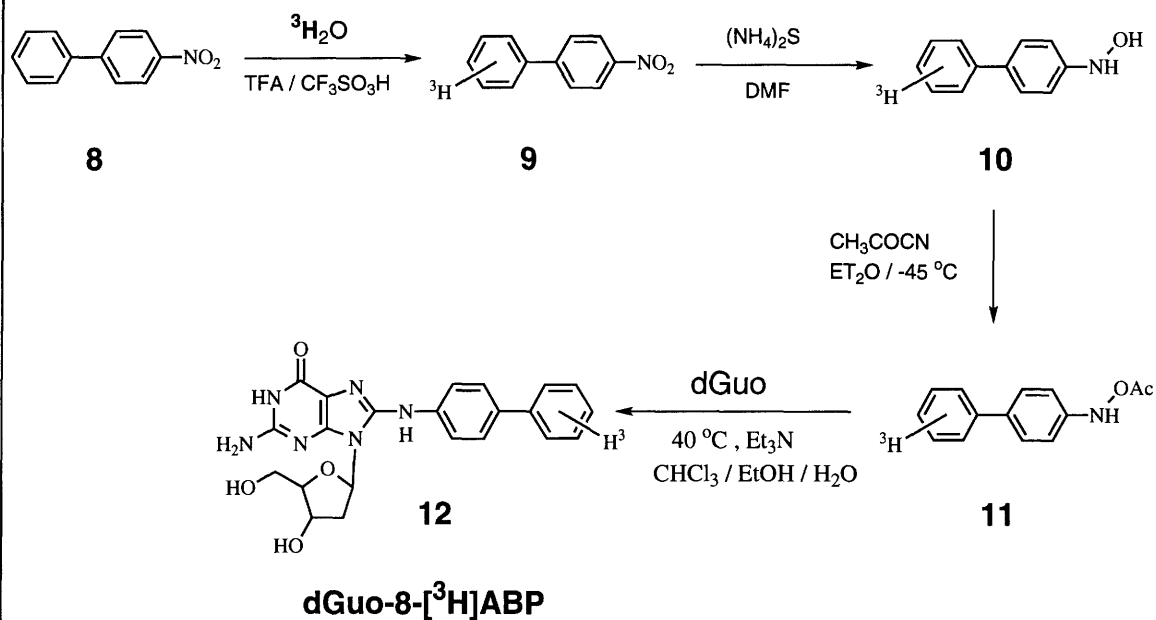
Using the optimal ratio of reactants, solvent, time, and temperature conditions developed for TBM-NHS acylation of mmol quantities of dGuo, acylation of dGuo-8-ABP yielded a mixture of products after 4 hr (Figure 7). After adding additional TBM-NHS (810 M%), a major peak, peak 3, resulted after 3 hr additional incubation. Peak 3 was isolated in 88 % yield after silica gel chromatography. When monitored by normal phase-HPLC, peak 2 was found to consist of two products. Synthetic standards of these two products of peak 2 were synthesized by using less TBM-NHS (400 M%) and lowering the temperature to 50 °C. In order to synthesize a large quantity of peak 4 for structural characterization, acylation of dGuo-8-ABP was done with a very large excess of TBM-NHS (730 M%) with only a minimum of solvent [ $\text{Me}_2\text{SO}$  (100  $\mu\text{l}$ ) and triethylamine (100  $\mu\text{l}$ )]. Triethylamine was specifically included in this reaction in order to increase the nucleophilicity of the  $\text{N}^2$  amino group by general base catalysis. Under these conditions, the reaction still produced a mixture of peaks 3 and 4.

**Figure 6.** Synthesis of tritium labeled dGuo-8-ABP.

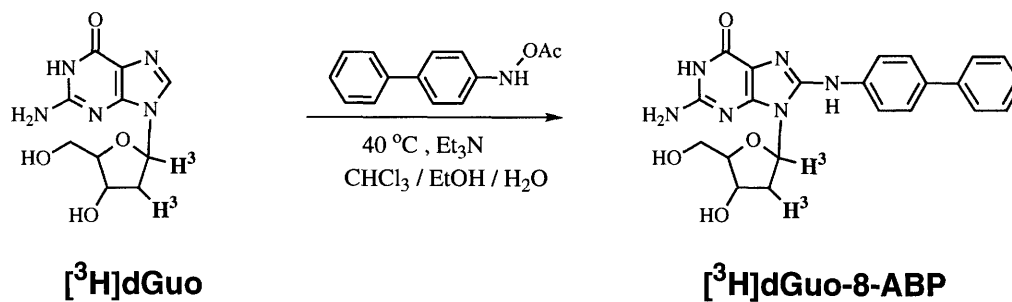
A) Synthesis of dGuo-8- $^3\text{H}$ ABP.

B) Synthesis of  $^3\text{H}$ dGuo-8-ABP.

### A. Synthesis of dGuo-8-[<sup>3</sup>H]ABP



### B. Synthesis of [<sup>3</sup>H]dGuo-8-ABP



### **iii. Acylation of [<sup>3</sup>H]dGuo-8-ABP with TBM-NHS**

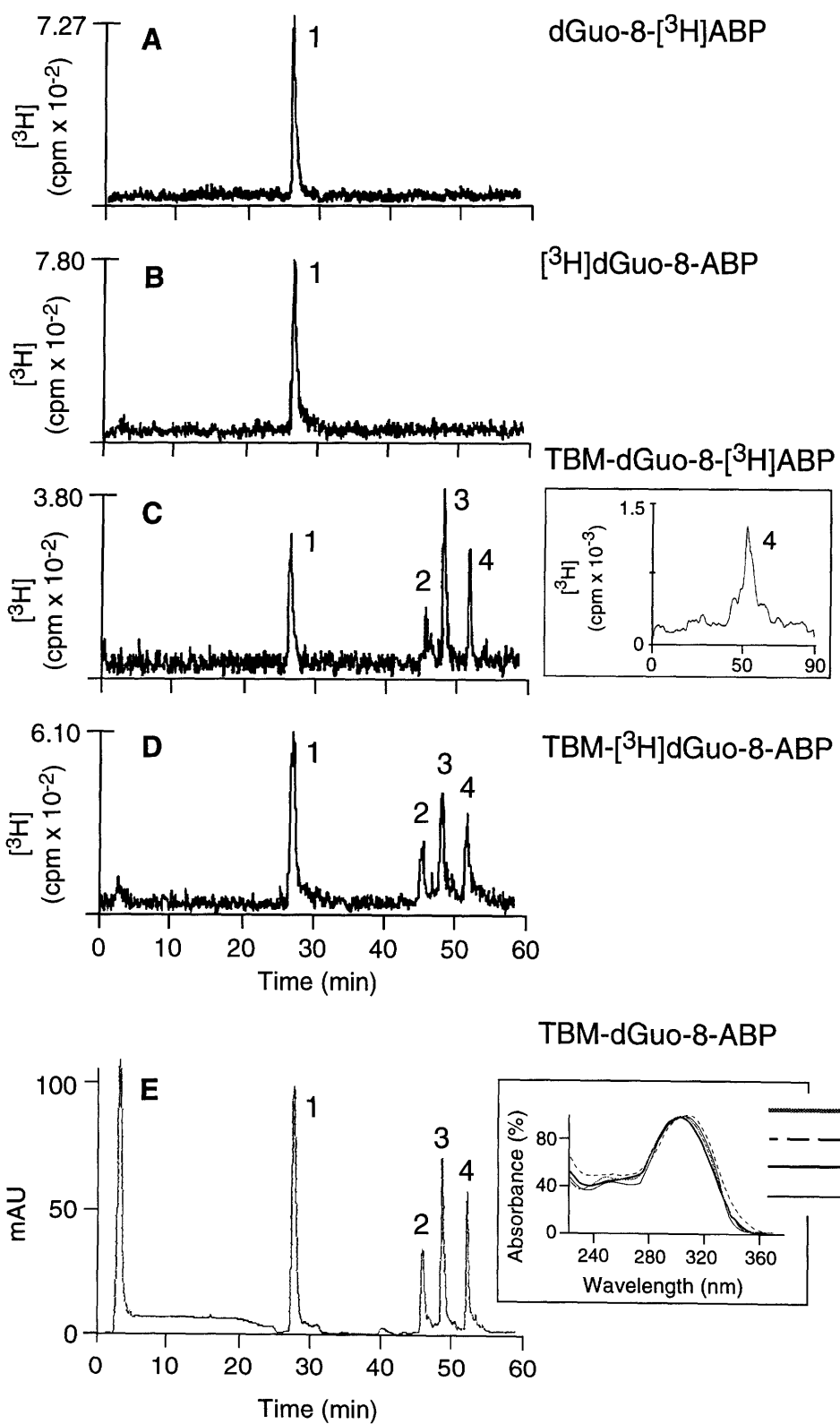
As was the case with the acylation of pmol quantities of dGuo, optimal acylation conditions developed for mmol quantities were not ideal for the acylation of pmol quantities of dGuo-8-ABP. Under non-optimal conditions, a mixture of products resulted (Figure 7). However, if the optimal conditions developed for the acylation of pmol quantities of [<sup>3</sup>H]dGuo were used, the extent of acylation was greater than 95 % (F. Sheabar, personal communication). The major acylation product was peak 4, representing greater than 80 % of acylated products.

### **iv. Acylation of [<sup>3</sup>H]dGuo-8-ABP with [<sup>35</sup>S]TBM-NHS**

Having developed optimal conditions to acylate a DNA adduct in pmol scale, the acylation procedure was repeated with the crucial <sup>35</sup>S labeling reagent, [<sup>35</sup>S]TBM-NHS. As depicted in Figure 7, the acylation profile obtained with [<sup>35</sup>S]TBM-NHS is identical to the acylation profile when conducted with TBM-NHS. It was necessary to demonstrate a linear relationship between the amount of adduct subjected to the acylation reaction and the <sup>35</sup>S incorporated into the acylated products (Sheabar et al., 1994). In a series of experiments conducted by F. Sheabar, [<sup>3</sup>H]dGuo-8-ABP (10 fmol to 30.6 pmol) was acylated with [<sup>35</sup>S]TBM-NHS and the incorporation of <sup>35</sup>S was determined. As required for our method, the amount of <sup>35</sup>S incorporated in the acylated products was linearly ( $r = 0.992$ ) related to the amount of adduct reacted. A linear relationship ( $r = 0.971$ ) also existed between the amount of dGuo-8-[<sup>3</sup>H]ABP isolated from rat liver DNA (formed as the result of treatment of female Fischer rats with 4-[<sup>3</sup>H]aminobiphenyl) introduced into the acylation reaction and



**Figure 7.** HPLC profile of TBM-acylated products of dGuo-8-ABP.



the amount of  $^{35}\text{S}$  in the acylated products over the range of 10 fmol to 7.8 pmol (Sheabar et al., 1994).

**v. Structure Determination of Acylation Products of dGuo-8-ABP**

Structural information concerning the acylated products of dGuo-8-ABP was obtained as described above for dGuo. Similar to the results obtained for the TBM-acylated products of dGuo, the UV spectra of the TBM acylation products of dGuo-8-ABP were similar, whether conducted on mmol or pmol scale. In the case of the underivatized dGuo-8-ABP, peak 1, or TBM-acylated dGuo-8-ABP, peaks 2 and 3, the the UV absorbance spectra were identical (Figure 7). However, in the case of peak 4, the  $\lambda_{\text{max}}$  shifted from 300 nm to 310 nm and the guanine absorbance at 254 nm shifted to 274 nm. Based on this bathochromic shift, peak 4 was tentatively assigned as a product resulting from acylation of the  $\text{N}^2$  amino group.

TBM acylation and chromatographic separation of different  $^3\text{H}$  labeled dGuo-8-ABP adducts confirmed that the nucleoside remained intact to the acylation procedure. As seen in Figure 7, dGuo-8- $^3\text{H}$ ABP (containing  $^3\text{H}$  in the ABP moiety) and  $^3\text{H}$ dGuo-8-ABP (containing  $^3\text{H}$  in the deoxyribose), as detected by  $^3\text{H}$  radioactivity, coeluted with the acylation products of dGuo-8-ABP, as detected by UV absorbance. Furthermore, when  $^{35}\text{S}$ TBM-NHS was used instead of TBM-NHS, the products of acylation again coeluted as determined by  $^{35}\text{S}$  radioactivity. This data indicated that the products of acylation retained all three structural components of the adduct: the sugar ( $^3\text{H}$  in the 1'- and 2'- positions in the sugar moiety of dGuo), the guanine base (UV spectrum), the carcinogen (UV spectrum

and  $^3\text{H}$  in the 4-ABP) (Figure 7C, D and E), and the methionine labeling reagent ( $^{35}\text{S}$  in the methionine). Under normal phase chromatography, it was subsequently determined that peak 2 was not homogeneous. The two fractions were attributed to regioisomers of the TBM group.

Preliminary characterization of the acylation products of dGuo-8-ABP was obtained by mass spectroscopy. Based on the FAB-MS and liquid SIMS data, peaks 2, 3 and 4 were unambiguously assigned as mono-, bis- and tris-acylated dGuo-8-ABP products, respectively. As was done with the dGuo acylation products, material made in mmol quantities was examined by FAB-MS whereas material made in nmol quantities was examined by liquid SIMS. The same  $[\text{M}+\text{H}]^+$  peak was obtained for coeluting material by both methods. In the case of the two fractions of peak 2, the same parent molecular ion of 666 m/z was determined. This molecular weight corresponded to that of a mono-TBM-dGuo-8-ABP products (Figure 1, R = 4-ABP: **5a,b,c**). Determination of the high resolution molecular weight of the proton molecular ion for the more polar and less polar fractions of peak 2 (666.2714 and 666.2707, respectively) confirmed that the two fractions were both mono-TBM-dGuo-8-ABP products (expected exact molecular weight 666.2710).

In contrast to peak 2, peaks 3 and 4 appear to be homogenous by normal and reversed phased HPLC using different gradients. The proton molecular ion detected for peak 3 by both means of mass spectroscopy was 897 m/z, corresponding to the m/z of bis-TBM-dGuo-8-ABP product (Figure 1, R = 4-ABP: **6a,b,c**). The exact high resolution result determined for the proton molecular ion (897.3635) agreed with the expected exact

molecular weight for  $[M+H]^+ = 897.3639$ . Finally, in the case of peak 4, the proton molecular ion of 1128 m/z for a tris-TBM-dGuo-8-ABP product was detected (Figure 1, R=ABP: 7). The exact high resolution result (1128.4577) agreed with the expected molecular weight for  $[M+H]^+ = 1128.4568$ .

The regiochemical assignment for peaks 2, 3, and 4 was again based on  $^1\text{H}$  and  $^{13}\text{C}$  NMR. As was done for the TBM-dGuo products, the H3' and H5' protons were used to assign regiochemistry. In the case of the more polar fraction of peak 2, the H5' protons shifted 0.5 ppm downfield without a corresponding change in the chemical shift for the H3' ribose protons. In the case of the other fraction, the H3' proton shifted 0.9 ppm downfield without a change in the chemical shift of the H5' protons. Consequently, the more polar fraction was tentatively assigned as the 5'-TBM-dGuo-8-ABP product (Figure 1, R=ABP: 5a) and the less polar component of peak 2 as the 3'-TBM-dGuo-8-ABP product (Figure 1, R=ABP: 5b). Comparison of the  $^{13}\text{C}$  NMR of these two compounds confirmed the assignment made by  $^1\text{H}$  NMR. All the carbon resonances of these two acylation products were identical except for changes in the C3' and C5' signals. In the case of the more polar fraction of peak 2, a downfield chemical shift of 3 ppm for C5' occurred. In contrast, in the case of the less polar fraction, a downfield chemical shift of 4.5 ppm for C3' occurred. Based on the chemical shifts of the C5' and C3' resonances as compared to the underivatized dGuo-8-ABP, the two components of peak 2 were definitely assigned as the 5'-TBM-dGuo-8-ABP product (most polar fraction) and 3'-TBM-dGuo-8-ABP product (the next fraction).

In the case of peak 3, liquid SIMS and FAB-MS indicated that this peak corresponded to a bis-TBM-dGuo-8-ABP product.  $^1\text{H}$  and  $^{13}\text{C}$  NMR confirmed the mass spectroscopy structural assignment and allowed the regiochemistry to be assigned. The  $^{13}\text{C}$  NMR of this acylation product showed a 2 ppm downfield shift for both C5' and C3'. Similarly, the  $^1\text{H}$  NMR showed downfield shift in both the H5' and H3' protons. Based on the absence of a UV bathochromic shift and the downfield shifts seen in both the  $^1\text{H}$  and  $^{13}\text{C}$  NMR, peak 3 was determined to be the 5',3'-bis-TBM-dGuo-8-ABP product (Figure 1, R=ABP: **6a**).

In the case of peak 4, the liquid SIMS, FAB-MS,  $^1\text{H}$  and  $^{13}\text{C}$  NMR all indicated that this peak was a tri-acylated product. As described earlier, the UV spectra of this material also showed a bathochromic shift. Based on our study of N<sup>2</sup>,5',3'-TBM-dGuo, the bathochromic shift reflected the attachment of a TBM group to the purine ring. As was the case with N<sup>2</sup>,5',3'-TBM-dGuo, the  $^{13}\text{C}$  NMR showed upfield and downfield shifts for the purine carbon resonances. However, peak 4 could not be unambiguously assigned as the N<sup>2</sup>,5',3'-tris-TBM-dGuo-8-ABP product (Figure 1, R=ABP: **7**) because an additional reactive site is present in this molecule, i.e., a secondary amine between the ABP and guanine moieties. Based on the steric size of TBM-NHS and the sterically crowded nature of this site of reaction, we have tentatively assigned this product as the N<sup>2</sup>,5',3'-tris-TBM-dGuo-8-ABP product (Figure 1, R=ABP: **7**).

#### vi. Summary

Under nonoptimal conditions, the acylation of dGuo-8-ABP with TBM-NHS resulted in a mixture of unacylated starting material (peak 1) and acylated products (peaks 2,3,and 4)

(Figure 7). Under optimized mmol conditions, the peracylation of dGuo-8-ABP occurred in greater than 88% yield. The major product was peak 3. In contrast, acylation of pmol quantities using TBM-NHS ( $5 \times 10^4$  molar equivalents), DIC (1  $\mu$ l), THF (50  $\mu$ l) and pyridine (50  $\mu$ l) at 37 °C for 2 hr, the acylation efficiency was greater than 95% with the major product, peak 4, accounting for greater than 80 % of the acylation products.

The acylation products, peaks 2, 3 and 4, were unambiguously assigned by UV, liquid SIMS, FAB-MS,  $^3\text{H}$ ,  $^{35}\text{S}$ ,  $^1\text{H}$ ,  $^{13}\text{C}$  NMR. Peak 2 was determined to consist of two products, 5'-mono-TBM-dGuo-8-ABP and 3'-mono-TBM-dGuo-8-ABP. Peak 3, the major peak under mmol conditions, was determined to be 5',3'-bis-TBM-dGuo-8-ABP. Peak 4, the major product under pmol conditions, was determined to be N<sup>2</sup>,5',3'-tris-TBM-dGuo-8-ABP.

## E. Conclusions

My efforts in the Wogan lab form the basis for a new method for the detection of carcinogen-DNA adducts. The method we have termed adduct detection by acylation with methionine, ADAM. The new method involves the chemical derivatization of deoxynucleosides with [<sup>35</sup>S]TBM-NHS, subsequent separation of acylation products by HPLC, and quantification of the products by radioactivity. The chemical acylation of dGuo and dGuo-8-ABP was studied in detail in order to establish optimal reaction conditions; conditions for chromatographic separation of the reaction products; and the structural and regiochemical identity of all the reaction products formed during acylation. Under optimized reaction conditions, a major product (greater than 88% isolated yield after chromatography) resulted from the TBM acylation of dGuo and dGuo-8-ABP.

The chemical identities of products of TBM acylation were determined through the use of liquid SIMS, FAB-MS, UV, <sup>3</sup>H and <sup>35</sup>S radioactivity, <sup>1</sup>H and <sup>13</sup>C NMR. The regiochemical identities of peaks 1-5 (Figure 5) resulting from the TBM acylation of dGuo were 5'-mono-TBM-dGuo; N<sup>2</sup>-mono-TBM-dGuo; 3'-mono-TBM-dGuo; 5',3'-bis-TBM-dGuo; and N<sup>2</sup>,5',3'-tris-TBM-dGuo, respectively. The regiochemical identities of peaks 2-4 (Figure 7) resulting from reaction of the TBM acylation of dGuo-8-ABP were 5'-mono-TBM-dGuo-8-ABP; 3'-mono-TBM-dGuo-8-ABP; 5',3'-bis-TBM-dGuo-8-ABP; and N<sup>2</sup>,5',3'-tris-TBM-dGuo-8-ABP, respectively. It is interesting to note that the relative elution order of the acylation products of dGuo and dGuo-8-ABP by RP-HPLC was the



same. In addition, this relative order of elution did not change when the chromatography was done under normal phase conditions.

## F. Future Work

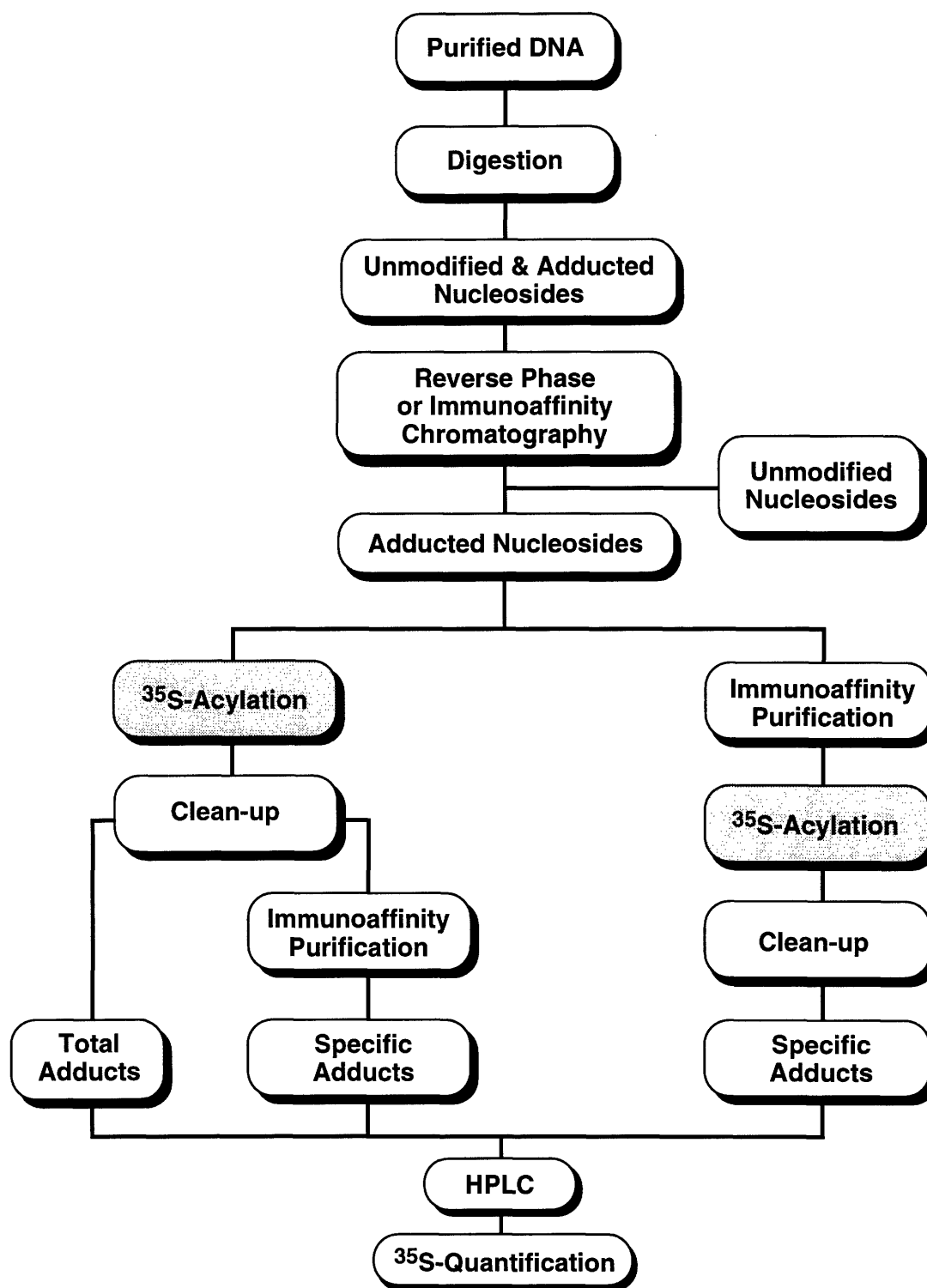
One important advantage we foresee for the ADAM procedure over all the methods currently available is the ability to compare total chemical exposure and specific chemical exposure. As depicted in Figure 8, by changing the method for adducted nucleoside purification, different acylated products can be analyzed. For example, in order to determine total adduct levels, adducted nucleosides would be acylated as a mixture of adducts. After HPLC separation of the acylation products, the total adduct level can be determined. In parallel, specific carcinogen adducts could be purified by employing immunoaffinity purification with monoclonal antibodies, when available, to separate a particular adduct of interest.

Possible changes to improve the ability of the ADAM procedure to detect chemical damage include the labeling of 5'-mononucleotides and/or the labeling of DNA base fragments. One of the biggest problems for the accurate quantification of DNA adducts by ADAM is the possible occurrence of multiple products. As was seen with dGuo and dGuo-8-ABP, the amount of reaction with exocyclic amino groups can vary dramatically depending on the chemical modification. Khorana and coworkers, however, found that acetylation of the N<sup>2</sup> amino group with acetic anhydride occurred quantitatively in the case of pyridinium deoxyguanosine-5'-phosphate (Ralph et al., 1963). The authors proposed that the acetylation of the exocyclic amino group in this nucleotide occurred faster than that in deoxyguanosine due to the intramolecular transfer of the acetyl group from the acetyl

phosphate mixed anhydride. Therefore, the acylation of 5'-monophosphates might be a way to increase the extent of acylation of exocyclic amino groups and lead to the formation of only one product from TBM acylation. An alternative change to improve the detection of DNA adducts would involve the labeling of DNA base fragments instead of carcinogen-deoxynucleosides adducts. This change might be necessitated due to the instability of some DNA adducts to the acylation procedure or by the desire to examine excision repair products from in urine, for example. In either case, as long as the carcinogen-base fragment has hydroxyl or amine groups, reaction of [<sup>35</sup>S]TBM-NHS should be an effective means of detection.

As an appendix to this last point, the ADAM procedure should be useful for the detection of carcinogen derivatives other than DNA adducts. Chemical acylation with [<sup>35</sup>S]TBM-NHS of carcinogens, their metabolites, and/or derivatives in body fluids or excreta would be possible as long as hydroxyl, amine, and possibly thiol groups are present on the molecule of interest. One class of damage, in particular, that would be suitable for detection would be protein-carcinogen adducts. Subsequent to enzymatic hydrolysis, reaction with TBM-NHS with a carcinogen-amino acid adduct via the  $\alpha$  amino group of the amino acid or hydroxyl or amines present on either the amino acid or carcinogen moiety should be an effective method to measure this type of chemical damage. By using a similar procedure as depicted in Figure 8, the total protein adducts and specific adduct exposure could be determined.

**Figure 8.** Flow diagram for carcinogen-nucleoside adduct detection by acylation with methionine.



## G. Experimental

### a. General Procedures and Materials

Unless otherwise indicated, all reagents and solvents were used as obtained without further purification. The following solvents were purified as follows: THF and  $\text{CH}_2\text{Cl}_2$  were distilled from  $\text{CaH}_2$  and pyridine and triethylamine was distilled from sodium. Chemicals were purchased as follows: dGuo from Sigma Chemical Co.; 4-aminobiphenyl and DIC from Aldrich Chemical Co.; 2'-deoxy-1',2'-[ $^3\text{H}$ ]guanosine 5'-triphosphate ([ $^3\text{H}$ ]dGTP) (31-37 Ci/mmol) from Amersham Co.; alkaline phosphatase from calf intestine from Boehringer Mannheim Co.; TBM-NHS from Accurate Chemicals and Scientific Corp. and Fluka Chemical Corp.; triethylamine from Fluka Chemical Corp. Authentic standards of N-(deoxyguanosin-8-yl)-4-aminobiphenyl (dGuo-8-ABP) and N-(deoxyguanosin-8-yl)-4-[ $^3\text{H}$ ]aminobiphenyl (dGuo-8-[ $^3\text{H}$ ]ABP) were gifts from Dr. F. Kadlubar, National Center for Toxicological Research, Jefferson, Arkansas. Silica gel TLC plates were visualized with UV illumination followed by charring with 5% anisaldehyde in (95:5:1) EtOH-AcOH- $\text{H}_2\text{SO}_4$ . Silica gel (230-400 mesh) was used for normal phase chromatographic separations. All reactions were performed under an argon atmosphere except when working in aqueous media.

Liquid secondary ion mass spectroscopy was obtained with a two sector MAT 731 secondary ion mass spectrometer combined with a Teknivent Vector/One workstation. Fast

atom bombardment (FAB-MS) was obtained with a Finnigan 8200 mass spectrometer. 2-nitrobenzyl alcohol (NBA) was used as the matrix for both methods. High-resolution mass spectral (HRMS) data are reported in units of  $m/z$  for  $M^+$  or the highest mass fragment derived from  $M^+$ .  $^1\text{H}$  and  $^{13}\text{C}$  NMR were recorded on Varian VXR-300, VXR-301, or Bruker AC-250 MHz spectrometers. Chemical shifts for  $^1\text{H}$  and  $^{13}\text{C}$  are expressed in parts per million downfield from  $\text{CDCl}_3$  (7.24 ppm or 77.00 ppm) or  $\text{CD}_3\text{OD}$  (3.30 and 35.00 ppm), respectively. Data are reported as follows: chemical shift, integration, multiplicity, and assignment.

Reversed phase HPLC analyses were performed on a Hewlett Packard model 1090 M liquid chromatograph equipped with a photodiode array detector, Radiomatic flow-through radioactivity detector model A250 and Microsorb  $5\mu$  C18 column (Rainin Instruments Co.) or with a Beckman 114M solvent delivery system with Beckman 421A controller with Hewlett Packard model 1040A photodiode array detector and either a Beckman  $\text{C}_{18}$  ultrasphere column or Si ultrasil column. Gradient I: a linear gradient of 30-70% acetonitrile in 0.1 M ammonium acetate over 20 min, 70-100% acetonitrile over 2 min and 100% acetonitrile over 7 min; gradient II: a linear gradient of 0-10 % acetonitrile in 0.1 M ammonium acetate over 30 min and 10-50 % over 30 min; gradient III: isocratic elution with 40 % methanol/ $\text{H}_2\text{O}$  over 13 min, a linear gradient of 40-60 % over 10 min, isocratic elution with 60 % over 12 min, a linear gradient of 60-100 % over 20 min and isocratic elution with 100% methanol over 25 min; gradient IV: isocratic elution with 100 %  $\text{CH}_2\text{Cl}_2$  for 5 min and then linear gradient of 100 % to 75 %  $\text{CH}_2\text{Cl}_2$ /methanol over 30 min. Flow rate was 1 mL per min for all gradients and the column temperature was 35 °C.

## b. Methods

### Synthesis of mono-TBM-dGuo with TBM-NHS

dGuo (1.74 g, 6.10 mmol), TBM-NHS (2.10 g 6.06 mmol, 100 M%), and DMAP (0.52 g) were suspended in THF (20 ml) and Me<sub>2</sub>SO (2 ml) and heated (70 °C). After 20 min, the reaction became clear. After 4 hr with heat, the reaction was concentrated to near dryness and the residue redissolved in CH<sub>2</sub>Cl<sub>2</sub> (100 ml) and washed with NaHCO<sub>3(sat)</sub> (100 ml) and NaCl<sub>(sat)</sub> (100 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The 5'-TBM-dGuo-8-ABP and 3'-TBM-dGuo-8-ABP were separated by silica gel chromatography (100 g) sequentially eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (96:4), CH<sub>2</sub>Cl<sub>2</sub>/MeOH (92:8), and CH<sub>2</sub>Cl<sub>2</sub>/MeOH (88:12). The major product isolated was 5'-TBM-dGuo, although most of the starting material had not reacted. Small amounts of 3'-TBM-dGuo and 5',3'-TBM-dGuo were isolated. The mono-TBM products were repurified with an isocratic gradient of 50% MeOH/Water on a Vydac C<sub>18</sub> preparative column using a Waters Model 590 pump at 16 ml/min with a Waters 484 Tunable Absorbance Detector set at 254 nm.

**5'-t-BOC-methionine-2'-deoxyguanosine ester (5'-TBM-dGuo)** (rf 0.50 CH<sub>2</sub>Cl<sub>2</sub>/MeOH 85:15). <sup>1</sup>H NMR (CD<sub>3</sub>OD): 7.87 (1H, s, H8), 6.26 (1H, t, H1'), 4.1-4.5 (5H, m, H3', H4', H5', Hα Met), 2.75 (1H, m, H2'), 2.39-2.53 (3H, m, H2', Hβ Met), 2.01 (3H, s, CH<sub>3</sub>S), 1.8-2.0 (2H, m, Hχ Met), 1.40 (9H, s, BOC). <sup>13</sup>C NMR (CD<sub>3</sub>OD): 174.32 (C1, Met), 159.95 (C6), 159.88 (BOC), 155.42 (C2), 153.06 (C4), 138.05 (C8), 118.27 (C5), 86.13 (C1'), 85.80 (C4'), 81.06 (OC(CH<sub>3</sub>)<sub>3</sub>), 72.97 (C3'), 66.44 (C5'), 54.37 (Cα Met),



40.74 (C2'), 32.33 (C $\chi$  Met), 31.31 (C $\beta$  Met), 29.05 (C(CH<sub>3</sub>)<sub>3</sub>), 15.53 (CH<sub>3</sub>S). FAB-MS expected exact molecular weight for [M+H]<sup>+</sup> 449.19750, high resolution result 499.1972.

$\lambda_{\max}$  = 255 nm (methanol)

**3'-t-BOC-methionine-2'-deoxyguanosine ester (3'-TBM-dGuo)** (rf 0.64 CH<sub>2</sub>Cl<sub>2</sub>/MeOH 85:15) <sup>1</sup>H NMR (CD<sub>3</sub>OD): 7.95 (1H, s, H8), 6.26 (1H, dd, H1'), 5.47 (1H, d, H3'), 4.30 (1H, dd, H $\alpha$  Met), 4.15 (1H, d, H4'), 3.81 (2H, d, H5'), 2.91 (1H, m, H2'), 2.49-2.63 (3H, m, H2', H $\beta$  Met), 2.10 (3H, s, CH<sub>3</sub>S), 1.97-2.08 (2H, m, H $\chi$  Met), 1.45 (9H, s, BOC). <sup>13</sup>C NMR (CD<sub>3</sub>OD): 173.45 (C1, Met), 159.95 (C6), 159.07 (BOC), 155.42 (C2), 152.15 (C4), 138.09 (C8), 118.21 (C5), 87.03 (C1'), 86.25 (C4'), 80.72 (OC(CH<sub>3</sub>)<sub>3</sub>), 77.62 (C3'), 66.44 (C5'), 54.10 (C $\alpha$  Met), 38.57 (C2'), 31.82 (C $\chi$  Met), 31.25 (C $\beta$  Met), 28.74 (C(CH<sub>3</sub>)<sub>3</sub>), 15.35 (CH<sub>3</sub>S). FAB-MS expected exact molecular weight for [M+H]<sup>+</sup> 449.19750, high resolution result 499.1977.  $\lambda_{\max}$  = 255 nm (methanol)

### Synthesis of bis-TBM-dGuo and tris-TBM-dGuo with TBM-NHS

dGuo (0.40 g, 1.40 mmol), TBM-NHS (2.60 g 7.51 mmol, 540 M%), and DMAP (0.343 g, 2.81 mmol, 200 M%) were suspended in THF (20 ml) and Me<sub>2</sub>SO (2 ml) and heated (70 °C). After 4 hr, the reaction was concentrated to near dryness and the residue redissolved in CH<sub>2</sub>Cl<sub>2</sub> (100 ml) and washed with NaHCO<sub>3(sat)</sub> (100 ml) and NaCl<sub>(sat)</sub> (100 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The bis-TBM-dGuo-8-ABP and tris-TBM-dGuo-8-ABP were separated by silica gel chromatography (100 g) sequentially eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (96:4), CH<sub>2</sub>Cl<sub>2</sub>/MeOH (92:8), and CH<sub>2</sub>Cl<sub>2</sub>/MeOH

(88:12). The major product isolated was 5',3'-bis-TBM-dG (0.96 g 1.32 mmol 94% yield) with a negligible amount of the N<sup>2</sup>,5',3'-tris-TBM-dG product (4 mg, 2% yield). The bis- and tris-TBM products were repurified with an isocratic gradient of 75 % and 85 % MeOH/Water, respectively, on a Vydac C<sub>18</sub> preparative column using a Waters Model 590 pump at 16 ml/min with a Waters 484 Tunable Absorbance Detector set at 254 nm.

**5', 3'-t-BOC-methionine-2'-deoxyguanosine diester (5',3'-bis-TBM-dGuo)** (rf 0.50 CH<sub>2</sub>Cl<sub>2</sub>/MeOH 90:10) <sup>1</sup>H NMR (CD<sub>3</sub>OD): 7.87 (1H, s, H8), 6.27 (1H, t, H1'), 5.46 (1H, m, H3'), 4.25-4.49 (5H, m, H $\alpha$  Met, H4', H5'), 3.29 (1H, m, H2'), 2.44-2.65 (5H, m, H2', H $\beta$  Met), 2.09 (3H, s, CH<sub>3</sub>S), 2.00 (3H, s, CH<sub>3</sub>S), 1.87-2.08 (4H, m, H $\chi$  Met), 1.44 (9H, s, BOC), 1.39 (9H, s, BOC). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 172.33 (C1, Met), 171.90 (C1, Met), 159.00 (C6), 155.51 (BOC), 153.78 (C2), 151.26 (C4), 136.35 (C8), 117.65 (C5), 84.55 (C1'), 82.07 (C4'), 80.31 (OC(CH<sub>3</sub>)<sub>3</sub>), 80.18 (OC(CH<sub>3</sub>)<sub>3</sub>), 75.86 (C3'), 64.64 (C5'), 52.83 (C $\alpha$  Met), 36.62 (C2'), 31.70 (C $\chi$  Met), 31.56 (C $\chi$  Met), 30.12 (C $\beta$  Met), 30.01 (C $\beta$  Met), 28.63 (C(CH<sub>3</sub>)<sub>3</sub>), 28.34 (C(CH<sub>3</sub>)<sub>3</sub>), 15.59 (CH<sub>3</sub>S), 15.40 (CH<sub>3</sub>S). FAB-MS expected exact molecular weight for [M+H]<sup>+</sup> 730.29041, high resolution result 730.2909.  $\lambda_{\text{max}}$  =255 nm (methanol)

**N<sup>2</sup>, 5', 3'-t-BOC-methionine-2'-deoxyguanosine triester (N<sup>2</sup>, 5', 3'-tris-TBM-dGuo)** (rf 0.75 CH<sub>2</sub>Cl<sub>2</sub>/MeOH 90: 10) <sup>1</sup>H NMR (CD<sub>3</sub>OD): 8.12 (1H, s, H8), 6.36 (1H, t, H1'), 5.46 (1H, m, H3'), 4.23-4.39 (6H, m, H $\alpha$  Met, H4', H5'), 3.30 (1H, m, H2'), 2.44-2.70 (7H, m, H2', H $\beta$  Met), 2.11 (3H, s, CH<sub>3</sub>S), 2.00 (3H, s, CH<sub>3</sub>S), 1.87-2.15 (6H, m, H $\chi$  Met), 1.45 (9H, s, BOC), 1.39 (9H, s, BOC). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 176.79 (C1, Met), 173.90 (C1,

Met), 173.63 (C1, Met), 158.18 (C6), 158.11 (BOC) (2 signals), 157.98 (BOC), 157.40 (BOC), 150.02 (C2), 149.15 (C4), 140.10 (C8), 122.20 (C5), 86.44 (C1'), 83.70 (C4'), 81.05 (OC(CH<sub>3</sub>)<sub>3</sub>), 80.82 (OC(CH<sub>3</sub>)<sub>3</sub>), 80.76 (OC(CH<sub>3</sub>)<sub>3</sub>), 76.79 (C3'), 65.66 (C5'), 56.08 (C $\alpha$  Met), 55.96 (C $\alpha$  Met), 54.05 (C $\alpha$  Met), 37.50 (C2'), 32.39 (C $\chi$  Met), 32.02 (C $\chi$  Met), 31.78 (C $\chi$  Met), 31.23 (C $\beta$  Met), 31.15 (C $\beta$  Met), 31.02 (C $\beta$  Met), 28.73 (C(CH<sub>3</sub>)<sub>3</sub>), 15.37 (CH<sub>3</sub>S), 15.20 (CH<sub>3</sub>S). FAB-MS expected exact molecular weight for [M+H]<sup>+</sup> 961.38333, high resolution result 961.3841.  $\lambda_{\text{max}}$  = 260 nm,  $\lambda$  = 280 nm (methanol)

### Synthesis of bis-TBM-dG with BOC-Met and DIC

dGuo (0.40 g, 1.40 mmol), BOC-Met (1.76 g, 7.07 mmol, 500 M%), DIC (1.00 ml, 6.43 mmol, 460 M%) and DMAP (0.354 g, 2.90 mmol, 207 M%) were suspended in THF (20 ml) and Me<sub>2</sub>SO (2 ml) and heated (70 °C). After 4 hr, the reaction was concentrated to near dryness and the residue redissolved in CH<sub>2</sub>Cl<sub>2</sub> (100 ml) and washed with NaHCO<sub>3(sat)</sub> (100 ml) and NaCl<sub>(sat)</sub> (100 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The bis-TBM-dGuo was purified by silica gel chromatography (100 g) sequentially eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (96:4), CH<sub>2</sub>Cl<sub>2</sub>/MeOH (92:8), and CH<sub>2</sub>Cl<sub>2</sub>/MeOH (88:12). The major product isolated was 5',3'-TBM-dG (0.96 g 1.36 mmol 97% yield).

### Synthesis of N-(deoxyguanosin-8-yl)aminobiphenyl (dGuo-8-ABP)

N-acetoxy-aminobiphenyl (19.27 mmol) dissolved in  $\text{CH}_2\text{Cl}_2$  (approximately 20 ml) was added dropwise to dGuo (0.60 g) dissolved in 1:1 EtOH:H<sub>2</sub>O (10 ml) at 40 °C. During the addition the  $\text{CH}_2\text{Cl}_2$  solution, the dGuo began to precipitate from solution and required additional EtOH (20 ml) for solubility. As monitored RP-HPLC, no difference in the ratio of dGuo to dGuo-8-ABP occurred from 20 min following addition of N-acetoxy-aminobiphenyl to 1 hr. After 3 hr, the reaction was diluted with  $\text{CH}_2\text{Cl}_2$  (100 ml) and H<sub>2</sub>O (50 ml). The aqueous solution was collected and the organic solution was washed with H<sub>2</sub>O (50 ml). The combined aqueous solution was washed with  $\text{CH}_2\text{Cl}_2$  (2 x 20 ml) and concentrated to dryness. The residue was redissolved in a minimum of MeOH and chilled (4 °C) overnight to precipitate dGuo. The filtrate was collected and chilled again to precipitate additional dGuo. The 8-ABP-dGuo was purified on a Vydac C<sub>18</sub> prep column using a Waters Model 590 pump at 16 ml/min with a Waters 484 Tunable Absorbance Detector set at 254 nm. An isocratic gradient of 15% B was used. The solvents were A: 1:1 MeOH/H<sub>2</sub>O B: MeOH. (12.2 mg, 1.3% yield)

**N-(deoxyguanosin-8-yl)aminobiphenyl** <sup>1</sup>H NMR (CD<sub>3</sub>OD): 7.70 (2H, d), 7.53 (4H, q), 7.37 (2H, t) 7.24 (t, 1H, H5"), 6.45 (dd, 1H, H1'), 4.57 (d, 1H, H3'), 4.02 (d, 1H, H4'), 3.90 (ddd, 2H, H5'), 2.70 (m, 1H, H2'), 2.17 (dd, 1H, H2'). <sup>13</sup>C NMR (CD<sub>3</sub>OD): 158.223 (C6), 154.14 (C2), 151.87 (C4), 146.32 (C1"), 142.04 (C5"), 141.14 (C8), 135.42 (C8"), 129.60 (C7"), 128.00 (C6"), 127.47 (C4" ), 127.27 (C3"), 119.46 (C2"),

113.62 (C5), 88.76 (C1'), 84.83 (C4'), 73.03 (C3'), 62.70 (C5'), 39.87 (C2').  $\lambda_{\text{max}} = 300$  nm (methanol)

### Synthesis of 4-[<sup>3</sup>H]aminobiphenyl

4-[<sup>3</sup>H]nitrobiphenyl (1.449  $\mu\text{mol}$ , 6.9 mCi/mmol) dissolved in 95:5 ethanol: isopropanol (1 ml) treated with hydrazine (100  $\mu\text{l}$ ) and palladium on activated carbon (1 mg). After 18 hr, additional hydrazine (40  $\mu\text{l}$ ) and Pd/C (1 mg) were added. After 20 min. the reaction was filtered to remove the palladium, dried *in vacuo*, redissolved in methanol/water (40:60), and chromatographed by RP-HPLC using gradient I.

### Synthesis of dGuo-8-[<sup>3</sup>H]ABP

4-nitrobiphenyl (1.10 g, 5.81 mmol) was dissolved in <sup>3</sup>H<sub>2</sub>O (1 ml, 5 Ci/mmol), trifluoroacetic acid (7.8 ml, 11.60 g, 55.22 mmol), and trifluoromethanesulphonic acid (600  $\mu\text{l}$ ). After 24 hr at reflux, the reaction was diluted with water (20 ml) and the solid filtered. The washed [<sup>3</sup>H]4-nitrobiphenyl was dissolved in DMF (50 ml) and treated with ammonium sulfide (20 wt% in water) (50 ml). After 4 hr, the reaction was diluted with CH<sub>2</sub>Cl<sub>2</sub> (100 ml) and washed with H<sub>2</sub>O (50 ml). The aqueous solution was back extracted with CH<sub>2</sub>Cl<sub>2</sub> (25 ml) and the combined organic solution dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated using a stream of N<sub>2</sub> gas. The activated [<sup>3</sup>H]N-hydroxy-aminobiphenyl was then suspended in Et<sub>2</sub>O (5 ml) and treated with acetyl cyanide at -45 °C. After 1 hr, the suspension was dissolved with CH<sub>2</sub>Cl<sub>2</sub> (25 ml) and washed with NaCl<sub>(sat)</sub> (50 ml). The organic solution was dried with

Na<sub>2</sub>SO<sub>4</sub>, and filtered. The [<sup>3</sup>H] N-acetoxy-aminobiphenyl dissolved in CH<sub>2</sub>Cl<sub>2</sub> was then added to dGuo (1.0 g) dissolved in water: ethanol: TEA (4: 4: 1) (10 ml). Unfortunately, as monitored by RP-HPLC, no dG-8-[<sup>3</sup>H]ABP was detected.

### **Synthesis of [<sup>3</sup>H]dGuo-8-ABP**

[<sup>3</sup>H]dGuo was prepared by dephosphorylation of [<sup>3</sup>H]dGTP (0.323 nmol, 31 Ci/mmol) for 1 hr at 37 °C in TE buffer (50 mM Tris-HCl, 50 mM EDTA) (pH 8.5) using calf intestine and bacterial alkaline phosphatase (1 and 1.12 unit, respectively). Dephosphorylation was monitored by RP-HPLC using gradient II and compared to the retention time of authentic standards of dGTP, dGMP, and dGuo. The enzymes were removed by filtration through a Millipore ultrafree-MC low-binding cellulose filter (nominal molecular weight limit, 30,000). N-acetoxy-4-aminobiphenyl (1 mmol) was added to the [<sup>3</sup>H]dGuo dissolved in a 4:4:1:1 mixture of water/ethanol/triethylamine/ chloroform (5.0 ml). The formation of [<sup>3</sup>H]dGuo-8-ABP was monitored by reverse phase HPLC using gradient II. After 3.5 hr, additional N-acetoxy-aminobiphenyl (1 mmol) was added. After 4.5 hr, the aqueous phase was separated and the organic phase washed with H<sub>2</sub>O (9 x 5 ml). The combined aqueous fractions were concentrated *in vacuo*, redissolved in methanol and stored overnight at -20 °C to precipitate any organic impurities. The adduct was purified using gradient III and rechromatographed to verify its purity. About chromatography, [<sup>3</sup>H]dGuo-8-ABP was recovered in 29% yield.

### Synthesis of 5',3'-bis-TBM-dGuo-8-ABP with TBM-NHS

dGuo-8-ABP (0.069 g, 0.16 mmol), TBM-NHS (0.48 g 1.39 mmol, 870 M%), and DMAP (0.045 g, 0.36 mmol, 230 M%) were suspended in THF (5 ml) and Me<sub>2</sub>SO (0.5 ml) and heated at 70 °C. After 4 hr, additional TBM-NHS (0.45 g, 1.30 mmol, 810 M%) was added to the refluxing solution. After 7 hr, the solution was concentrated and the residue redissolved in CH<sub>2</sub>Cl<sub>2</sub> (25 ml) and washed with NaHCO<sub>3(sat)</sub> (15 ml) and NaCl<sub>(sat)</sub> (15 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The reaction was purified by silica gel chromatography (50 g) sequentially eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (98:2) and CH<sub>2</sub>Cl<sub>2</sub>/MeOH (94:6) to yield 5',3'-bis-TBM-dGuo-8-ABP (0.13 g 0.14 mmol 88 % yield)

**5',3'-t-BOC-methionine-N-(deoxyguanosin-8-yl)aminobiphenyl ester (5',3'-bis-TBM-dGuo-8-ABP)** (rf 0.66 CH<sub>2</sub>Cl<sub>2</sub>/MeOH 90 :10) <sup>1</sup>H NMR (CD<sub>3</sub>OD): 7.56-7.40 (6H, m, Ar), 7.32 (2H, t), 7.22 (t, 1H, H5''), 6.30 (t, 1H, H1'), 5.50 (m, 1H, H3'), 4.70-4.20 (5H, m, H $\alpha$  Met, H4', H5'), 3.5 (1H, m, H2'), 2.60-2.36 (5H, m, H2', H $\beta$  Met), 2.07 (3H, s, CH<sub>3</sub>S), 1.95 (3H, s, CH<sub>3</sub>S), 2.10-1.81 (4H, m, H $\chi$  Met), 1.43 (9H, s, BOC), 1.41 (9H, s, BOC). <sup>13</sup>C NMR (CDCl<sub>3</sub> / CD<sub>3</sub>OD): 172.17 (C1, Met), 171.74 (C1, Met), 157.36 (C6), 155.62 (BOC), 155.40 (BOC), 152.08 (C2), 150.34 (C4), 144.90 (C1''), 140.33 (C5''), 140.07 (C8), 134.36 (C8''), 128.39 (C7''), 127.35 (C6''), 126.40 (C4''), 126.23 (C3''), 117.84 (C2''), 113.50 (C5), 83.95 (C1'), 81.62 (C4'), 80.15 (OC(CH<sub>3</sub>)<sub>3</sub>), 80.00 (OC(CH<sub>3</sub>)<sub>3</sub>), 75.32 (C3'), 64.30 (C5'), 53.30 (C $\alpha$  Met), 52.53 (C $\alpha$  Met), 34.52 (C2'), 31.39 (C $\chi$  Met), 30.96 (C $\chi$  Met), 29.92 (C $\beta$  Met), 29.81 (C $\beta$  Met), 28.07 (C(CH<sub>3</sub>)<sub>3</sub>) (same

Hz) 15.24 (CH<sub>3</sub>S), 15.05 (CH<sub>3</sub>S). FAB-MS expected exact molecular weight for [M+H]<sup>+</sup> 897.36391, high resolution result 897.3635.  $\lambda_{\text{max}}$  = 300 nm,  $\lambda$  = 254 nm (methanol)

### Synthesis of mono-TBM-dGuo-8-ABP with TBM-NHS

dGuo-8-ABP (0.17 g, 0.39 mmol), TBM-NHS (0.54 g 1.56 mmol, 400 M%), and DMAP (0.071 g, 0.58 mmol, 150 M% ) were suspended in THF (5 ml) and Me<sub>2</sub>SO (0.5 ml) and heated at 50 °C. After 1 1/2 hr, the reaction was concentrated to dryness. The mono-TBM-dGuo-8-ABP products were separated by silica gel chromatography (50 g) sequentially eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (96:4), CH<sub>2</sub>Cl<sub>2</sub>/MeOH (92:8), and CH<sub>2</sub>Cl<sub>2</sub>/MeOH (88:12).

**5'-t-BOC-methionine-N-(deoxyguanosin-8-yl)aminobiphenyl ester (5'-TBM-dGuo-8-ABP)** (rf 0.56 CH<sub>2</sub>Cl<sub>2</sub>/MeOH 90:10) <sup>1</sup>H NMR (CD<sub>3</sub>OD/CDCl<sub>3</sub>): 7.52-7.40 (6H, m, Ar), 7.37 (2H, t), 7.27 (t, 1H, H5"), 6.15 (t, 1H, H1'), 4.40-4.35 (3H, m, H4', H5'), 4.3-4.0 (2H, H3', H $\alpha$  Met), 3.1 (1H, m, H2'), 2.50-2.10 (3H, m, H2', H $\beta$  Met), 2.10-1.70 (2H, m, H $\chi$  Met), 1.92 (3H, s, CH<sub>3</sub>S), 1.35 (9H, s, BOC). <sup>13</sup>C NMR (CDCl<sub>3</sub> / CD<sub>3</sub>OD): 173.93 (C1, Met), 158.35 (C6), 157.68 (BOC), 153.64 (C2), 152.14 (C4), 146.62 (C1"), 141.72 (C5"), 141.32 (C8), 135.67 (C8"), 129.57 (C7"), 128.36 (C6"), 127.52 (C4" ), 127.26 (C3"), 119.37 (C2"), 114.23 (C5), 85.48 (C1'), 85.24 (C4'), 80.66 (OC(CH<sub>3</sub>)<sub>3</sub>), 72.60 (C3'), 66.04 (C5'), 53.82 (C $\alpha$  Met), 38.42 (C2'), 31.91 (C $\chi$  Met), 30.83 (C $\beta$  Met), 28.66 (C(CH<sub>3</sub>)<sub>3</sub>), 15.27 (CH<sub>3</sub>S), 15.05 (CH<sub>3</sub>S). FAB-MS expected exact molecular weightfor [M+H]<sup>+</sup> 666.27100, high resolution result 666.2714.  $\lambda_{\text{max}}$  = 300 nm,  $\lambda$  = 254 nm (methanol)



**3'-t-BOC-methionine-N-(deoxyguanosin-8-yl)aminobiphenyl ester (3'-TBM-dGuo-8-ABP)** (rf 0.43 CH<sub>2</sub>Cl<sub>2</sub>/MeOH 90 :10) <sup>1</sup>H NMR (CD<sub>3</sub>OD /CDCl<sub>3</sub>): 7.56-7.40 (6H, m), 7.32 (2H, t), 7.22 (t, 1H, H5''), 6.30 (t, 1H, H1'), 5.50 (m, 1H, H3'), 4.70-4.20 (4H, m, H $\alpha$  Met, H4', H5'), 3.5 (1H, m, H2'), 2.50-2.15 (3H, m, H2', H $\beta$  Met), 2.10-1.80 (2H, m, H $\chi$  Met), 2.07 (3H, s, CH<sub>3</sub>S), 1.95 (3H, s, CH<sub>3</sub>S), 1.43 (9H, s, BOC), 1.41 (9H, s, BOC). <sup>13</sup>C NMR (CDCl<sub>3</sub> / CD<sub>3</sub>OD): 173.58 (C1, Met), 158.41 (C6), 157.93 (BOC), 153.95 (C2), 151.77 (C4), 146.24 (C1''), 142.37 (C5''), 141.89 (C8), 135.52 (C8''), 129.64 (C7''), 128.09 (C6''), 127.54 (C4''), 127.35 (C3''), 119.70 (C2''), 113.86 (C5), 886.34 (C1'), 84.82 (C4'), 80.78 (OC(CH<sub>3</sub>)<sub>3</sub>), 77.63 (C3'), 62.69 (C5'), 53.84 (C $\alpha$  Met), 36.56 (C2'), 31.72 (C $\chi$  Met), 31.12 (C $\beta$  Met), 26.18 (C(CH<sub>3</sub>)<sub>3</sub>), 15.49 (CH<sub>3</sub>S), 15.49 (CH<sub>3</sub>S). FAB-MS expected exact molecular weight for [M+H]<sup>+</sup> 666.27100, high resolution result 666.2707.  $\lambda_{\text{max}}$  = 300 nm,  $\lambda$  = 254 nm (methanol)

### Synthesis of tris-TBM-dGuo-8-ABP with TBM-NHS

dGuo-8-ABP (0.072 g, 0.166 mmol) and TBM-NHS (0.42 g, 1.21 mmol, 730 M%) were dissolved in Me<sub>2</sub>SO (100  $\mu$ l) and triethylamine (100  $\mu$ l) and heated (80 °C). After 5 1/2 hr, additional TBM-NHS (0.186 g, 0.537 mmol, 325 M%), Me<sub>2</sub>SO (100  $\mu$ l) and triethylamine (100  $\mu$ l) were added. After 10 hr at 80 °C, the reaction was diluted with CH<sub>2</sub>Cl<sub>2</sub> (30 ml) and washed with NaHCO<sub>3(sat)</sub> (10 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated to dryness. The tris-TBM-dGuo-8-ABP was separated from bis-TBM-dGuo-8-ABP by silica gel chromatography (40 g) eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (96:4).

**N<sup>2</sup>,5',3'-tris-t-BOC-methionine-N-(deoxyguanosin-8-yl)aminobiphenyl ester (N<sup>2</sup>,5',3'-tris-TBM-dGuo-8-ABP)** (rf 0.53 CH<sub>2</sub>Cl<sub>2</sub>/MeOH 95:5) <sup>1</sup>H NMR (CD<sub>3</sub>OD /CDCl<sub>3</sub>): 7.60-7.45 (6H, m, Ar), 7.39 (2H, t), 7.25 (t, 1H, H5''), 6.33 (br, 1H, H1'), 5.39 (br, 1H, H3'), 4.45-4.20 (6H, m, H $\alpha$  Met, H4', H5'), 2.70-2.20 (8H, m, H2', H $\beta$  Met), 2.09 (3H, s, CH<sub>3</sub>S), 2.08 (3H, s, CH<sub>3</sub>S), 2.06 (3H, s, CH<sub>3</sub>S), 2.00-1.90 (6H, m, H $\chi$  Met), 1.52 (9H, s, BOC), 1.41 (18H, s, BOC). <sup>13</sup>C NMR (CDCl<sub>3</sub> / CD<sub>3</sub>OD): 172.77(C1, Met), 172.30 (C1, Met), 171.93 (C1, Met), 156.00 (C6), 154.66 (BOC), 152.78 (C2), 148.20 (C4), 140.27 (C1''), 138.90 (C5''), 139.5 (C8), 134.68 (C8''), 128.24 (C7''), 127.10 (C6''), 126.61 (C4''), 126.09 (C3''), 118.27 (C2''), 118.18(C5), 84.01 (C1'), 81.57 (C4'), 80.02 (OC(CH<sub>3</sub>)<sub>3</sub>), 79.85 (OC(CH<sub>3</sub>)<sub>3</sub>), 79.68 (OC(CH<sub>3</sub>)<sub>3</sub>), 75.07 (C3'), 62.0 (C5'), 52.32 (C $\alpha$  Met), 51.30 (C $\alpha$  Met), 51.05 (C $\alpha$  Met), 34.37 (C2'), 31.0 (C $\chi$  Met), 30.8 (C $\chi$  Met), 29.7 (C $\chi$  Met), 29.6 (C $\beta$  Met), 29.4 (C $\beta$  Met), 28.6 (C $\beta$  Met), 27.70 (C(CH<sub>3</sub>)<sub>3</sub>), 27.62 (C(CH<sub>3</sub>)<sub>3</sub>), 27.31 (C(CH<sub>3</sub>)<sub>3</sub>), 14.63 (CH<sub>3</sub>S), 14.49 (CH<sub>3</sub>S), 14.41 (CH<sub>3</sub>S). FAB-MS expected exact molecular weight for [M+H]<sup>+</sup> 1128.45683, high resolution result 1128.4577.  $\lambda_{\text{max}}$  = 310 nm,  $\lambda$  = 274 nm (methanol).

## **II. SYNTHESIS OF SITE-SPECIFICALLY MODIFIED OLIGONUCLEOTIDES**

### **A. Introduction**

Site-specifically modified oligonucleotides are important tools that allow precise physical and biological evaluation of DNA lesions (Basu and Essigmann, 1988). NMR spectroscopic and X-ray crystallographic studies of site-specifically modified oligonucleotides, for instance, provide unambiguous structural data on the conformational changes resulting from a chemical lesion. Moreover, site-specifically modified oligonucleotides can be used to characterize the types, amounts, and genetic requirements for mutations induced by chemical carcinogens and radiation (Fowler et al., 1982; Green et al., 1984; Loechler et al., 1984; Singer and Essigmann, 1991). In addition, site-specifically modified oligonucleotides can be used to rank the toxic potentials of DNA lesions formed by antitumor compounds (Bradley et al., 1993). In an attempt to define the relationship between the structures and the biological consequences of chemical carcinogens, antitumor drugs and ionizing radiation, the Essigmann laboratory has examined the mutagenic activity and repair of modified oligonucleotides. My efforts have focused on the synthesis of modified oligonucleotides.

### **B. Synthesis of Modified Oligonucleotides**

Three methods are commonly used to synthesize a site-specifically modified oligonucleotide: i) enzymatic synthesis, ii) chemical or physical modification of a preformed

oligonucleotide, and iii) total synthesis. The choice of method depends on the chemical nature and stability of a particular adduct or lesion to be studied.

**a. Enzymatic Synthesis of a Modified Oligonucleotide**

One method useful for the synthesis of some modified oligonucleotides involves enzymatic incorporation of a modified deoxynucleotide. The enzymes that have been utilized for this approach include DNA polymerase (Walker and Uhlenbeck, 1975), bacteriophage T4 RNA ligase (Brennan and Gumport, 1985; Wood et al., 1990) and terminal deoxynucleotidyl transferase (Hatahet et al., 1993). Although the method of incorporation involves an enzyme-catalyzed step, chemical synthesis may nevertheless be required to prepare the necessary substrate for study. One disadvantage of utilizing enzymes to synthesize modified oligonucleotides is that the substrate specificity of the various enzymes employed may limit the compounds that can be incorporated. For instance, only a limited number of chemically modified triphosphates are suitable substrates for DNA polymerase (Hatahet et al., 1993). In the case of RNA ligase and terminal deoxynucleotidyl transferase, the versatility of these methods has not been established. T4 RNA ligase and terminal deoxynucleotidyl transferase additionally have the problem that the modified nucleoside can only be added to the 3' end of an oligonucleotide. Having a modified deoxynucleotide on the end of an oligonucleotide chain can interfere with subsequent incorporation of the oligonucleotide into a viral genome for genetic studies.

## **b. Modification of a Preformed Oligonucleotide**

A second method for the synthesis of modified oligonucleotides involves the treatment of an oligonucleotide with a reactive DNA damaging agent (Basu and Essigmann, 1988). In most cases, this method involves treating an oligonucleotide with a chemical carcinogen or its activated metabolite. This method is useful for readily synthesized electrophilic reagents, such as epoxides, alkylators (alkyl halides, etc.), and oxidants (osmium tetroxide, peroxide, etc.), or radiation-induced lesions such as DNA photoproducts induced by UV light.

One of the limitations of this method is the preparation of the reactive form of the carcinogen. In many cases of DNA damage, the reactive form of a carcinogen is not available. Even when the reactive carcinogen is available, if a desired DNA sequence has more than one reactive site, multiple products can and do arise (Basu and Essigmann, 1988). Purification of the desired product from other reaction products, consequently, is problematic. To prevent the formation of multiple products, a DNA sequence with only one reactive site is often chosen. Therefore, this method may not be able to generate the DNA lesion in a preferred (i.e., biologically relevant) sequence.

## **c. Total Synthesis of Modified Oligonucleotides**

The total synthetic approach is the preferred method to prepare modified oligonucleotides because of the ease of automated DNA synthesis (Basu and Essigmann, 1988). This method enables the facile production of sufficient amounts ( $\mu\text{g}$  to mg quantities)

of modified oligonucleotide for full structural characterization (NMR or X-ray diffraction studies) and in-depth biological experiments. An additional advantage of the total synthetic approach is the ability to control adduct placement in an oligonucleotide. The ability to place a DNA adduct systematically in different sequence contexts is useful for determining the effect of the local chemical environment on the biological activity of a DNA lesion.

The major limitation of a total synthetic approach is the availability and stability of carcinogen-modified nucleosides. As was the case with an enzymatic procedure for synthesizing modified oligonucleotides, the nucleoside adduct of interest may not be available or may require multiple steps to synthesize. Even after preparing the desired carcinogen-nucleoside adduct, additional steps are required to prepare modified phosphoramidites (Beaucage and Iyer, 1992) or phosphotriesters (Reese, 1978). The preparation of a reactive monomer (or dimer) can involve multiple steps of protection deprotection and activation. In addition, when synthesizing a modified deoxynucleoside phosphoramidite or phosphotriester, reactive groups on the carcinogen moiety must be blocked prior to oligonucleotide synthesis. An additional source of problems may arise as a consequence of the conditions employed for DNA synthesis. Synthesis of a modified oligonucleotide may involve repeated treatments of mild acid (trichloroacetic acid), strong oxidants (iodine), and reactive electrophiles (phosphorylating reagent), depending on the position of the modified nucleoside in the DNA chain. In addition, after the desired modified oligonucleotide is obtained, deprotection of blocking groups and removal of the oligonucleotide from a resin support in the case of solid phase DNA synthesis commonly involves treatment of a modified oligonucleotide with concentrated ammonium hydroxide

and heat (60 °C). Consequently, in order for a modified oligonucleotide to be synthesized by a total synthetic approach, the adduct of interest must be stable to acidic, basic, oxidizing and electrophilic reagents.

### C. 4-Aminobiphenyl

#### a. Biological relevance of 4-aminobiphenyl.

4-aminobiphenyl (4-ABP) has been shown to be a potent bladder carcinogen in humans (Melick et al., 1955; Melick et al., 1971). In a study that examined chemical workers industrially exposed to 4-ABP, a striking 19 of 171 (11%) workers developed bladder tumors within 17 years of industrial exposure (Melick et al., 1955). A follow-up study of 315 exposed workers revealed that approximately 17% of this larger group had developed bladder cancers (Melick et al., 1971). Despite the cessation over 40 years ago of the industrial use of 4-ABP as an antioxidant in rubber manufacturing, exposure of people to 4-ABP continues today owing to cigarette smoking, coal-derived synthetic fuels, and to other as yet unknown sources (Talaska et al., 1991; Byrant et al., 1987; Kadlubar et al., 1988; Guerin and Buchanan, 1987).

Three major DNA adducts have been found subsequent to ABP exposure *in vivo* (Kadlubar et al., 1982; Beland and Kadlubar, 1985). The primary adduct formed is N-(deoxyguanosin-8-yl)-4-aminobiphenyl (dGuo-8-ABP) (Figure 9A). Less frequently occurring adducts are the N-(deoxyguanosin-N<sup>2</sup>-yl)-4-aminobiphenyl and N-(deoxyadenosin-8-yl)-4-aminobiphenyl (Figure 9A).

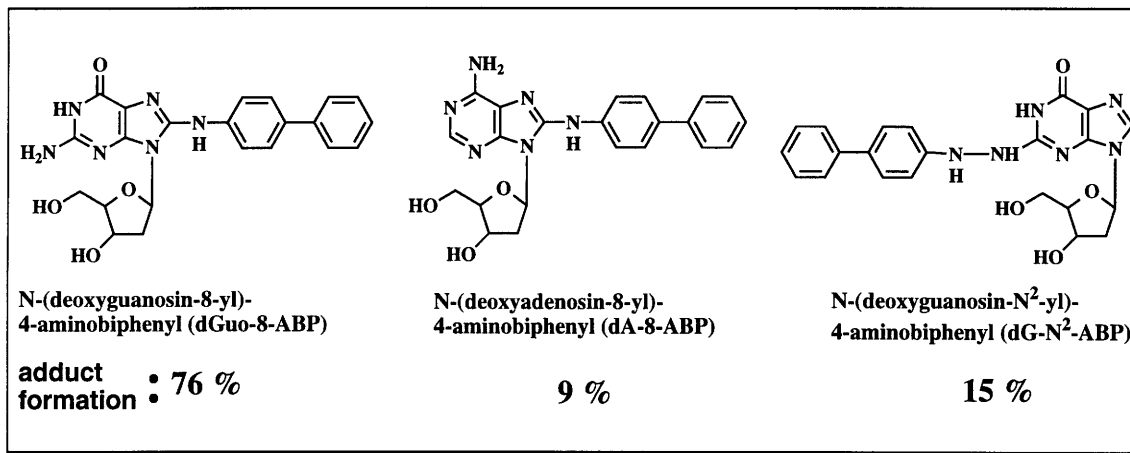


**Figure 9.** 4-ABP DNA adducts.

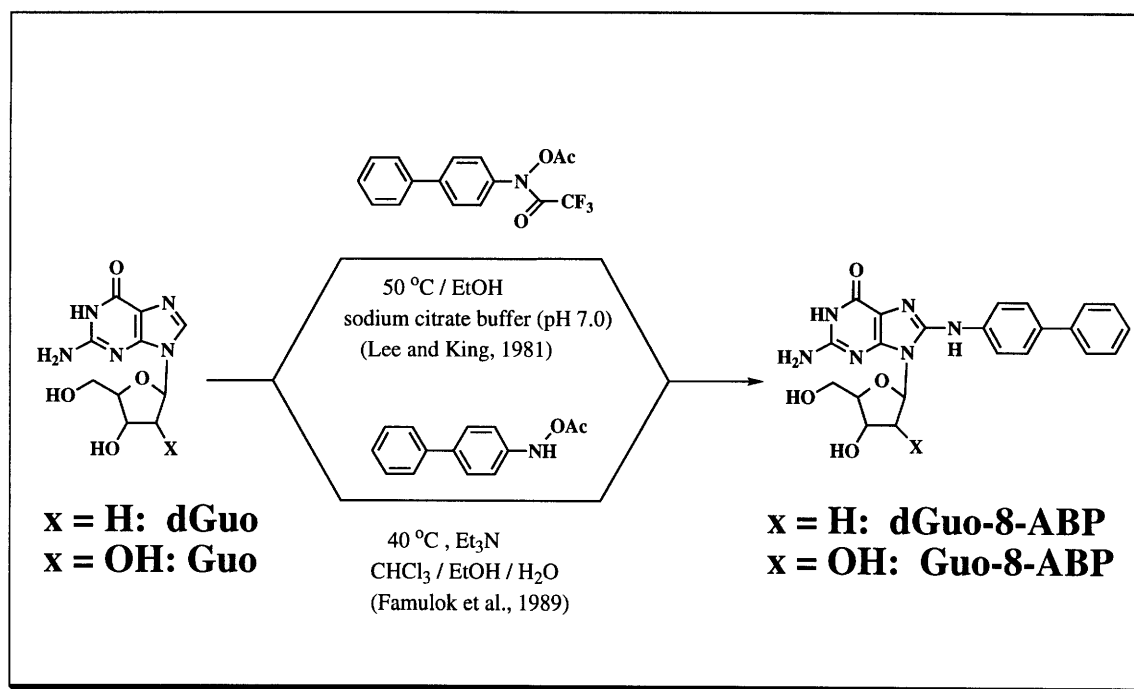
A) Structures and relative proportion of 4-ABP DNA adducts.

B) Two methods for synthesizing dGuo-8-ABP, the major 4-ABP DNA adduct.

## A. 4-ABP *in vivo* DNA adducts



## B. Synthesis of dGuo-8-ABP



An earlier study conducted in the Essigmann lab showed that a single ABP adduct, dGuo-8-ABP, when present in only one strand of a ds M13 genome, is weakly mutagenic (mutation frequency < 0.02 %) in *E. coli* (Lasko et al., 1988). The reasons postulated to explain the low observed mutagenic frequency of the dGuo-8-ABP lesion included: 1) the dGuo-8-ABP may not be mutagenic, 2) the dGuo-8-ABP lesion may not have been mutagenic in the sequence context evaluated, 3) the dGuo-8-ABP may have been rapidly repaired, and 4) the dGuo-8-ABP selectively inhibited replication of the adducted strand. In order to determine which of these possibilities were most feasible, S. Verghis began studies of the mutagenic effects of the dGuo-8-ABP lesion in ss DNA. A site-specifically modified genome was constructed using a modified tetramer, 5'-d(TG<sup>8-ABP</sup>CA), synthesized by F. Kadulbar and coworkers at the National Center for Toxicological Research. The method used to synthesize the modified tetramer was that of Lee and King (1981). Unfortunately, before biological testing of the site-specifically modified genome was complete, it became evident that the modified oligonucleotide had undergone some degradation since its synthesis. Since degradation products would confuse the biological results, a new modified tetramer was required for biological study. Since the modified oligonucleotide synthesized according to the procedure of Lee and King (1981) had undergone degradation, possibly as a consequence of unknown contamination, I undertook the synthesis of the same modified oligonucleotide, 5'-d(TG<sup>8-ABP</sup>CA), by the method of Famulok et al. (1989).

#### **b. Synthetic routes to dGuo-8-ABP**

Only two methods have been published on how to modify DNA with aminobiphenyl *in vitro*. The first published method involved the reaction of N-acetoxy-N-trifluoroacetyl-4-

aminobiphenyl with guanosine (Lee and King, 1981) (Figure 9B). Although the product of this reaction was the modified ribonucleotide, N-(guanosin-8-yl)-4-aminobiphenyl, this electrophilic reagent was subsequently shown to react with deoxynucleotides as part of a plasmid or short strands of DNA (Lasko et al., 1988). The procedure by Famulok et al. (1989), by contrast, involves the reaction of N-acetoxy-4-aminobiphenyl as the electrophilic species (Figure 9B). The N-acetoxy-4-aminobiphenyl reagent has been implicated as one of the reactive metabolites of 4-ABP *in vivo* leading to DNA modification (Flammang et al., 1988).

**c. Synthesis of an aminobiphenyl modified tetranucleotide**

As outlined in Figure 10, the N-acetoxy-aminobiphenyl can be prepared from commercially available 4-nitrobiphenyl in two steps. The first step involved reducing 4-nitrobiphenyl to N-hydroxy-aminobiphenyl with  $(\text{NH}_4)_2\text{S}$ . The N-hydroxy-aminobiphenyl can then be acylated with acetyl cyanide at low temperature ( $-40\text{ }^\circ\text{C}$ ) to yield N-acetoxy-aminobiphenyl. Although the N-acetoxy-aminobiphenyl, upon standing at room temperature as a solid rapidly degraded, the reactive electrophile can be stored temporarily as a dichloromethane solution at room temperature for a few hours or at  $-80\text{ }^\circ\text{C}$  for a few days.

The modified oligonucleotide, 5'-d(TG<sup>8-ABP</sup>CA) was synthesized in 30 to 40% yield by adding a large excess of N-acetoxy-aminobiphenyl (3.8 mmol) to unmodified tetramer (10  $\mu\text{mol}$ ). The ABP-modified oligonucleotide prepared by this method coeluted with the major peak present in the ABP-modified oligonucleotide used previously in this laboratory.

Subsequent to purification by reversed phase HPLC, the identity of the modified oligonucleotide was confirmed by FAB-MS and nucleoside digestion analysis. In the case of the negative ion mass spectrum of the modified oligonucleotide (calculated molecular weight = 1340 m/z), the major ion detected was 1339 corresponding to the [M-H]<sup>-</sup> ion. The nucleoside composition of the modified oligonucleotide was subsequently determined by enzymatic hydrolysis, reversed phase HPLC, and UV detection. Peak area analysis of the digestion products of the oligonucleotide, d[<sup>8</sup>ABP-TGCA], showed the proper ratio of dT, dC, dA and dGuo-8-ABP (Verghis, 1992; Verghis, Morningstar, and Essigmann, manuscript in preparation).

#### **d. Summary of Biological Results<sup>2</sup>**

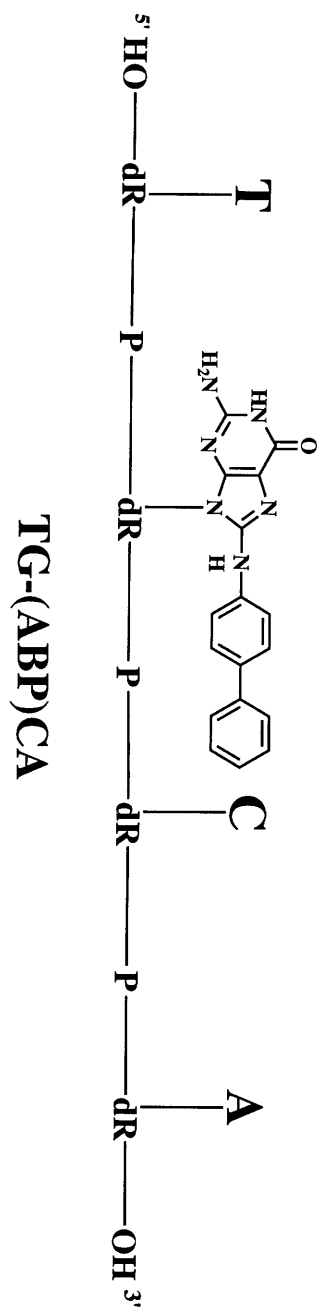
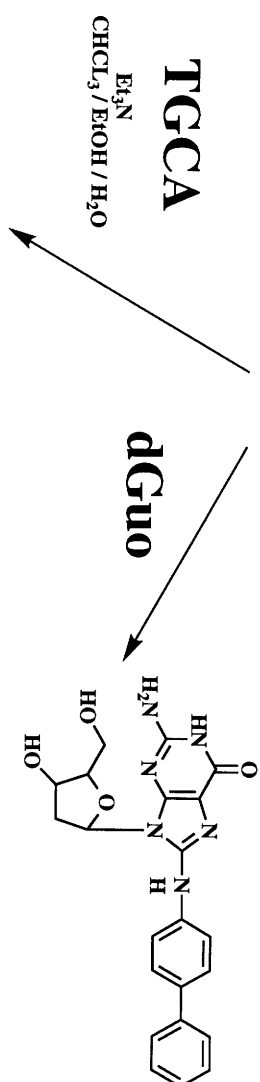
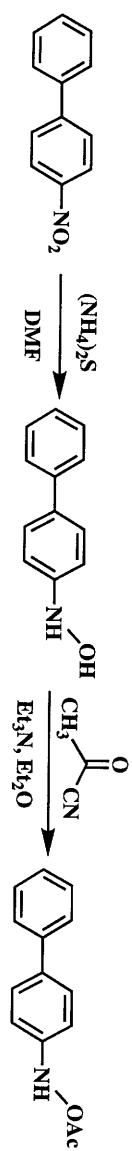
The modified oligonucleotide was subsequently incorporated into an M13 bacteriophage vector and the genetic effects of the dGuo-8-ABP lesion determined. The targeted mutation frequency of a single dGuo-8-ABP adduct in an M13 ss genome was determined to be approximately 0.01 % (Verghis, 1992). This value is similar to the low mutability determined for the same ABP lesion in ds DNA (Lasko et al., 1988). As mentioned earlier, the study of the 4-ABP lesion in ss DNA was undertaken to determine whether the low mutagenic frequency seen in ds DNA was because the dGuo-8-ABP lesion was 1) not mutagenic, 2) not mutagenic in the sequence context evaluated, 3) rapidly repaired, or 4) inhibitory to replication of the adducted strand selectively. The results from the most recent ss DNA experiment rule out the third and fourth possibilities as the reason for the low

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<sup>2</sup> The biological results were obtained by S. Verghis.

mutagenic frequency detected. Specifically, DNA repair enzymes function preferentially, or exclusively, on ds DNA. Facile repair of the adduct in ss DNA is therefore unlikely to account for the low mutagenicity of the dGuo-8-ABP lesion. Similarly, since the adduct was situated in ss DNA, no unmodified strand was available to undergo preferential replication as compared to the adducted strand. The mutagenicity results, therefore, must reflect actual replication past the dGuo-8-ABP lesion. It was concluded that the dGuo-8-ABP lesion was only a weakly mutagenic DNA lesion, at least in the sequence context evaluated.

Figure 10. Synthesis of a ABP-modified tetranucleotide.





#### **D. Purine oxidative lesions:**

#### **8-oxoguanine and 8-oxoadenine<sup>3</sup>**

##### **a. Introduction to Oxidative Damage**

A high rate of spontaneous oxidative DNA damage occurs as a consequence of aerobic metabolism, lipid peroxidation and immune responses (Ames and Gold, 1991). Some of the reactive oxidizing species produced include superoxide, nitric oxide, hydroxyl radicals, and hydrogen peroxide (Ames 1983; Cross et al., 1987; Floyd, 1990; Halliwell et al., 1990; Ames and Gold, 1991). In addition to the effects of endogenous reactive oxygen species, oxidative DNA damage can also occur if cells are exposed to carcinogens that generate oxygen radicals, including ionizing radiation and some chemical oxidants (von Sontag 1987; Halliwell et al., 1990). It has been estimated that the genome of a human cell receives on average about  $10^4$  oxidative hits/day (Ames and Gold, 1991). Some of the DNA lesions that result from oxidative damage include strand breaks, base modifications, protein-DNA cross-links, and AP sites (Hutchinson, 1985). As a protection against this oxidative damage, cells possess many defenses, including chemical antioxidants, DNA repair enzymes, catalase, and superoxide dismutase (Sies 1986; Frei et al., 1988; Breimer, 1990). Since some adducts escape repair, oxidative DNA damage can accumulate with time. These persistent DNA lesions are thought to contribute to mutations found in the DNA. In fact, oxidative DNA damage is believed by some to be a major contributor to both the aging process and various

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<sup>3</sup> Portions of this section have been taken *verbatim* from Wood et al. (1992).

age-related degenerative diseases, including cancer (Ames, 1983; Cross et al., 1987; Floyd, 1990; Halliwell et al., 1990; Ames and Gold, 1991).

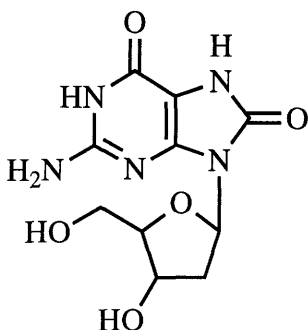
**b. Biological importance of 7,8-dihydro-8-oxoguanine ( $G^{8-oxo}$ )**

One of the predominate oxidative lesions detected in human tissues samples is 7,8-dihydro-8-oxoguanine ( $G^{8-oxo}$ ) (Figure 11). This lesion, originally detected in  $\gamma$  and X-irradiated DNA, has subsequently been shown to be mutagenic *in vitro* (Kuchino et al., 1987; Shibutani et al., 1991) and *in vivo* (Wood et al. 1990; Moriya et al., 1991; Cheng et al., 1992). A possible association between  $G^{8-oxo}$  and neoplastic transformation has been suggested (Floyd, 1990). The predominant mutation induced by  $G^{8-oxo}$ , a G to T transversion, was determined in this laboratory (Wood et al., 1990) and subsequently confirmed in others (Moriya et al., 1991; Cheng et al., 1992; Shibutani et al., 1991).

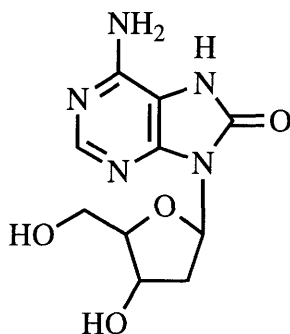
Although it has been the subject of less study, 7,8-dihydro-8-oxoadenine ( $A^{8-oxo}$ ) (Figure 11) has also been detected in DNA irradiated *in vitro* (Fuciarelli et al., 1989; Gajewski et al., 1990) as well as in urine and liver samples from *in vivo* sources (Stillwell et al., 1989; Malins and Haimanot, 1990). In irradiated DNA, this adduct is present at approximately one-third to one-half of the level of  $G^{8-oxo}$  (Fuciarelli et al., 1989; Stillwell et al., 1989). Structural studies performed on  $A^{8-oxo}$  (Cho and Evans 1991) have shown that this lesion, like  $G^{8-oxo}$ , exists predominantly as the 6,8-diketo tautomer at physiological pH. In addition, both modified bases are in the syn glycosidic conformation as contrast to the normal anti conformation in B DNA. Since these two structural features appear central to

**Figure 11.** Oxidized purines:  $G^{8-oxo}$  and  $A^{8-oxo}$ .

# Oxidized Purines



**7,8-dihydro-8-oxoguanine (G<sup>8-oxo</sup>)**



**7,8-dihydro-8-oxoadenine (A<sup>8-oxo</sup>)**

the known miscoding properties of G<sup>8-oxo</sup> (Culp et al., 1989; Cho et al., 1990; Kouchakdjian et al., 1991; Oda et al., 1991), it was speculated that the A<sup>8-oxo</sup> might also be a premutagenic lesion. We speculated that the presence of A<sup>8-oxo</sup> in DNA may explain the appearance of mutations at AT base pairs in the genomes of cells or viruses that have been exposed to ionizing radiation or chemical oxidation (Glickman et al., 1980; Grosovsky et al., 1988; Miles and Meuth 1989; McBride et al., 1991). In order to study the mutagenic potential of the A<sup>8-oxo</sup> lesion, an oligonucleotide containing the A<sup>8-oxo</sup> was required.

### c. Synthesis of A<sup>8-oxo</sup> Phosphoramidite

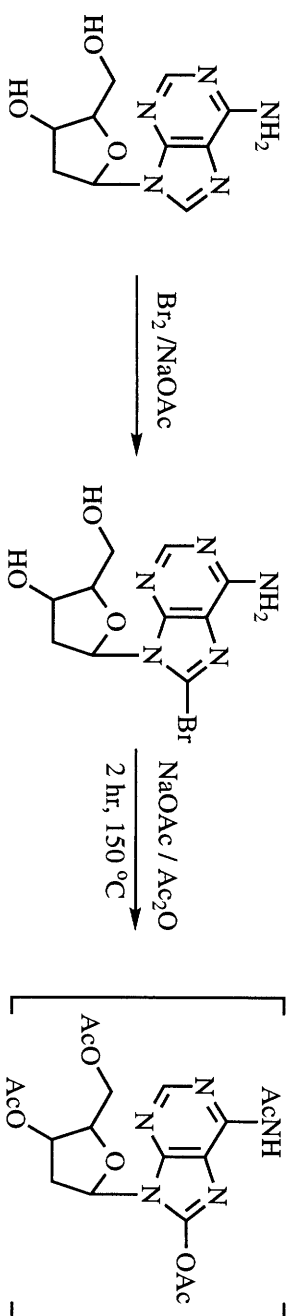
Although the structurally similar G<sup>8-oxo</sup> lesion was incorporated into a modified oligonucleotide by an enzymatic method (Wood et al., 1990), the sequence context chosen to study A<sup>8-oxo</sup>, 5'-d(GCTA<sup>8-oxo</sup>G), was not amenable to the same enzymatic procedure. Additionally, treatment of unmodified GCTAG with oxidizing conditions that would produce the A<sup>8-oxo</sup> lesion would additionally result in a mixture of oxidized purine and pyrimidine residues. Consequently, a total synthetic approach was taken to synthesize GCTA<sup>8-oxo</sup>G.

The 5'-d(GCTA<sup>8-oxo</sup>G) oligonucleotide was synthesized by the method of Guy et al. (1988) with a few modifications (Figure 12). The first step of the procedure involved oxidation of the C8 position of dA by bromination (Guy et al., 1988; Long et al., 1967; Ikehara and Uesugi, 1969). The next step of the procedure involved simultaneous displacement of the bromide with NaOAc and protection of the 5' and 3' hydroxyls of the nucleoside. Unfortunately, the method by Guy et al. (1988) involving phenoxyacetyl

anhydride protection was not high yielding when preformed in the Essigmann lab. The method was more reproducible when acetic anhydride was used instead of the phenoxyacetyl anhydride (Ikehara and Maruyama, 1975). After 2.5 hr at reflux, the reaction yielded a mixture of tetra- and penta-acetylated dA<sup>8-oxo</sup>. By varying the amount and molarity of sodium hydroxide added, the tetra- and penta-acetylated dA<sup>8-oxo</sup> could be converted quantitatively to either dA<sup>8-oxo</sup> or N<sup>2</sup>-acetyl-dA<sup>8-oxo</sup>. The following steps (protection of the 5' hydroxyl with dimethoxytrityl and formation of the 3' phosphoramidite) were done according to standard literature procedures (for a review on phosphoramidite synthesis, see Beaucage et al., 1992; Gait 1984).

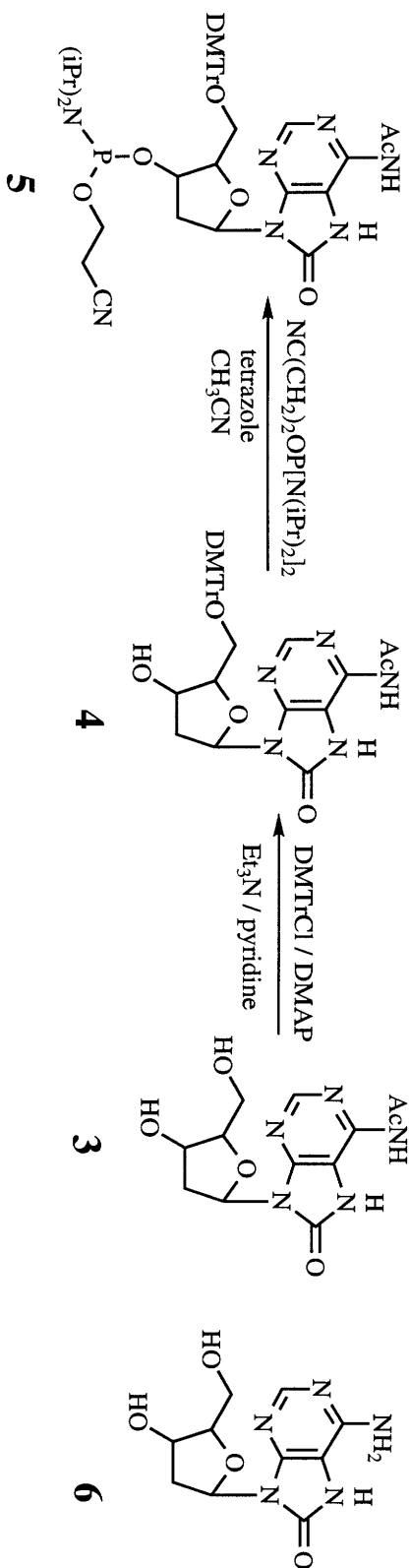
Purification of the fully protected dA<sup>8-oxo</sup> phosphoramidite proved to be problematic. Chromatographic conditions whereby decomposition of the activated phosphoramidite did not occur could not be developed. Bodepudi et al. (1991) similarly found that partial hydrolysis (5 to 20 %) during aqueous work-up and substantial decomposition occurred with dG<sup>8-oxo</sup> phosphoramidite. Consequently, the crude reaction dA<sup>8-oxo</sup> phosphoramidite product was used for solid phase DNA synthesis. This procedure was inefficient and resulted in poor coupling between the modified phosphoramidite and the 5' hydroxyl group of the oligonucleotide undergoing elongation. This process, however, did permit the synthesis of enough of the site-specifically modified oligomer for characterization and genetic evaluation.

**Figure 12.** Preparation of A<sup>8-oxo</sup> phosphoramidite.



## 2'-deoxyadenosine

**2**



**4**

**3**

**6**



**d. Characterization of d(GCTA<sup>8-oxo</sup>G)**

The identity of the modified oligonucleotide was confirmed by mass spectrometry and nucleoside digestion analysis. The observed values  $(M-H)^- = 1518.5$  and  $(M-H + Na)^- = 1541.2$ , as determined by matrix-assisted laser desorption and time of flight mass spectrometry, agreed with the calculated  $m/z = 1518.1$  for d(GCTA<sup>8-oxo</sup>G). The oligonucleotide composition was subsequently determined by enzymatically hydrolyzing the oligomer and examining the digestion products by RP-HPLC. Peak area analysis of the UV chromatogram indicated that the modified oligonucleotide, d(GCTA<sup>8-oxo</sup>G), contained the proper ratio of dT, dC, dG, and dA<sup>8-oxo</sup> (Wood et al., 1992).

**e. Discussion of the mutagenicity of A<sup>8-oxo</sup>**

Biological evaluation of d(GCTA<sup>8-oxo</sup>G) was performed simultaneously with d(GCTAG<sup>8-oxo</sup>), previously synthesized in this laboratory, to determine the relative mutagenicities of A<sup>8-oxo</sup> and G<sup>8-oxo</sup>. To determine the relative genetic effects of A<sup>8-oxo</sup> and G<sup>8-oxo</sup> *in vivo*, bacteriophage genomes containing either of the two lesions were constructed and used to transform a DNA repair proficient strain of *E.coli*. Mutant progeny were scored and the nature of the mutations determined by DNA sequencing. Analysis of phage DNA from mutant plaques obtained by plating immediately after transformation (infective centers assay) revealed that G<sup>8-oxo</sup> induced G to T transversions at an apparent frequency of 0.3%; this finding agreed with previous work of this lab (Wood et al., 1990). The frequency and spectrum of mutations derived from the A<sup>8-oxo</sup> modified DNA were almost indistinguishable

from those generated from an adenine-containing control genome. No evidence was obtained to indicate that the A<sup>8-oxo</sup> adduct induced any targeted base substitution mutations. It was estimated that A<sup>8-oxo</sup> was at least an order of magnitude less mutagenic than G<sup>8-oxo</sup> in *E. coli* cells with normal DNA repair capabilities.

A possible conclusion from this study was that A<sup>8-oxo</sup> does not contribute significantly to the spectrum of mutations that occur at AT base pairs in oxidatively-damaged DNA. A possible reason for the lack of mutagenicity of A<sup>8-oxo</sup> was that these studies were performed in repair proficient *E. coli* cells. It is conceivable that the A<sup>8-oxo</sup> adduct was removed from the M13 genome by an *E. coli* repair enzyme(s) prior to DNA replication, preventing the fixation of mutations. Another possibility is that A<sup>8-oxo</sup> may be one of many lesions that is mutagenic only in bacterial cells that are expressing the SOS response.

#### **f. Introduction to G<sup>8-oxo</sup> oligonucleotides**

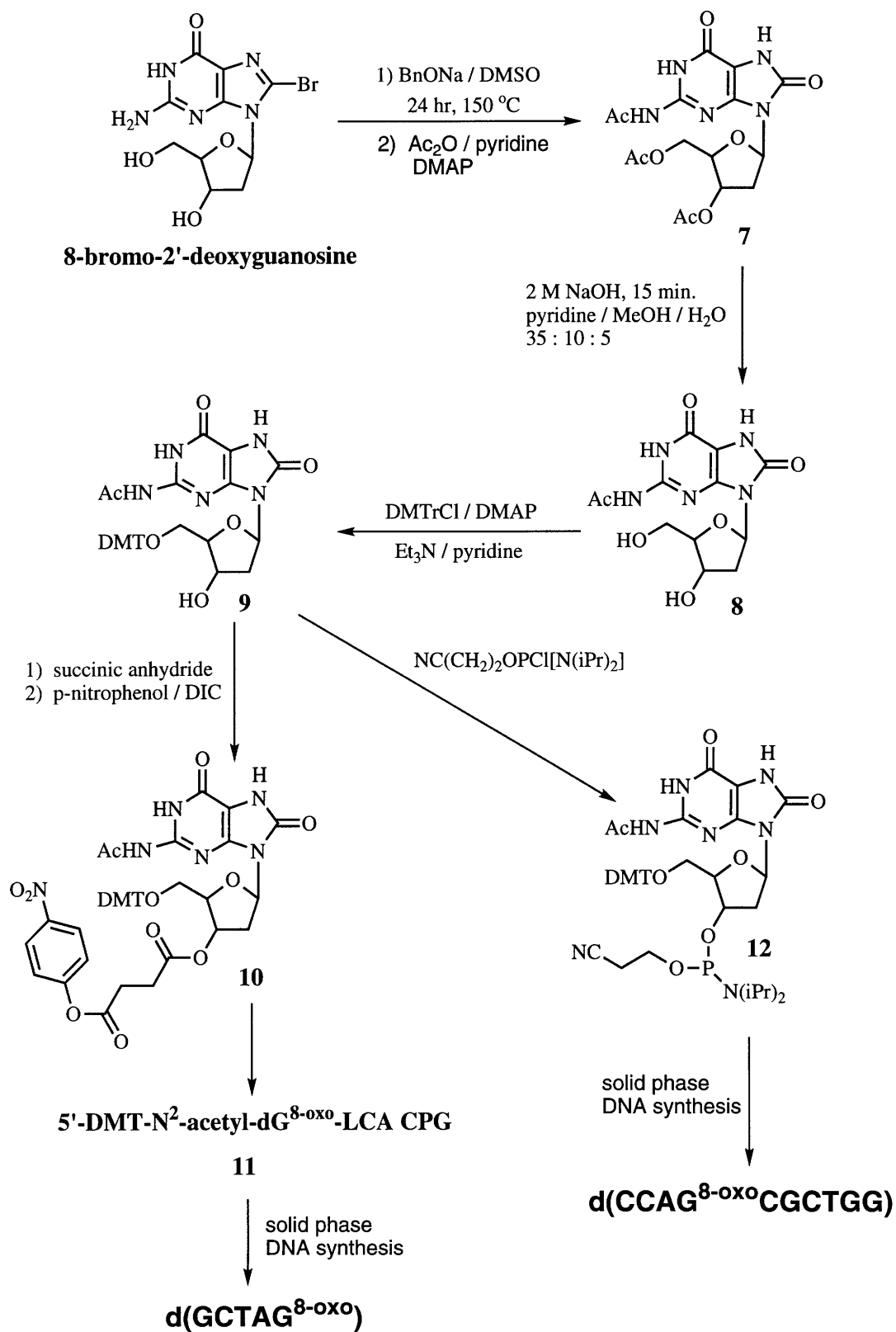
In order to understand whether the low mutagenic potential of A<sup>8-oxo</sup> in the previous mutagenicity experiment was due to facile DNA repair, work was started in the Essigmann lab by L. Miller to compare A<sup>8-oxo</sup> and G<sup>8-oxo</sup> in a repair deficient cell line. In order to compare the results obtained in the previous study with any new mutagenic results in a repair-deficient cell strain, the same M13 genomes would be required. This requirement meant that the desired G<sup>8-oxo</sup> containing oligonucleotide would again require the sequence d(GCTAG<sup>8-oxo</sup>). Since a large amount of oligonucleotide was need for these new studies, it was determined that the relatively inefficient enzymatic method previously used to generate a

G<sup>8-oxo</sup> containing oligonucleotide would not allow the production of a sufficient amount of oligonucleotide. Since the G<sup>8-oxo</sup> is the 3' terminal nucleotide of the desired oligonucleotide, a modified resin containing G<sup>8-oxo</sup> would be required if solid phase DNA synthesis was employed.

**g. Background on the synthesis of G<sup>8-oxo</sup> resin**

At the time this work was begun, three groups had reported the synthesis of dG<sup>8-oxo</sup> phosphoramidites. We postulated that the derivatives used to prepare the modified phosphoramidite would also be suitable for the preparation of a G<sup>8-oxo</sup> resin. Kuchino et al., (1987) had developed a method to prepare N<sup>2</sup>-acetyl-8-methoxy-5-O-monomethoxytrityl-2'-deoxyguanosine 3'-(2-chlorophenyl)phosphate. This phosphoramidite results in the synthesis of 8-methoxy modified oligonucleotides. The methyl group on the C8 hydroxyl can subsequently be removed in the last stage of the oligonucleotide synthesis by treatment with triethylamine in aqueous DMF. A second method involved the preparation of 3'-O-(diisopropylamino-2-cyanoethoxyphosphinyl)-5'-O-dimethoxytrityl-N<sup>2</sup>-isobutyryl-8-oxo-2'-deoxy-7H-guanosine (Bodepudi et al., 1991; Bodepudi et al., 1992). This method does not require a deprotection step to yield an G<sup>8-oxo</sup> oligonucleotide. A third method by Roelen et al. (1991) reported a similar procedure to Bodepudi et al. However, Roelen et al. found that the final step used by Bodepudi et al., the phosphitylation, did not occur without protection of the N7 position with a diphenylcarbamoyl protecting group. Nevertheless, both Bodepudi et al. and Roelen et al. found that the 4,4'-dimethoxytrityl-N<sup>2</sup>-protected-8-oxo-deoxynucleoside could be prepared in good yields. This protected G<sup>8-oxo</sup> molecule appeared to be ideal for the preparation of a G<sup>8-oxo</sup> resin.

**Figure 13.** Synthesis of G<sup>8-oxo</sup> resin and G<sup>8-oxo</sup> phosphoramidite.



#### **h. Synthesis of G<sup>8-oxo</sup> oligonucleotides**

The protected 4,4'-dimethoxytrityl-N<sup>2</sup>-acetyl-8-oxo-deoxynucleoside monomer was prepared by adapting the procedures of Bodepudi et al. (1991) and Roelen et al. (1991) (Figure 13). The method involved oxidation of the C8 position with bromine (Lin et al., 1985), displacement of the C8 bromide with sodium benzylate (Bodepudi et al., 1991), and subsequent protection of the N<sup>2</sup> amino and 5' hydroxyl groups with acetic anhydride (Roelen et al., 1991). In contrast to the earlier dA<sup>8-oxo</sup> synthesis in this laboratory, the bromination of dGuo occurred very rapidly (Long et al., 1967). Also in contrast to the dA<sup>8-oxo</sup> synthesis, the displacement of the C8 bromine from 8-Br-dGuo involved reaction with sodium benzylate (Lin et al., 1985) whereas the displacement of the C8 bromide of 8-Br-dA occurred with sodium acetate, a poorer nucleophile. In my hands, the procedure of Lin et al. led to a mixture of 8-benzyloxy-deoxyguanosine and 8-oxo-deoxyguanosine. The ratio of the products varied depending on the aqueous content of the DMSO used in the reaction. Procedurally, I found that separation of the two nucleosides difficult as unprotected monomers and routinely separated the two compounds after peracylation with acetic anhydride. The purified tetra-acetyl dG<sup>8-oxo</sup> was subsequently deprotected with NaOH (5M) in methanol to yield the N<sup>2</sup> protected nucleoside monomer, N<sup>2</sup>-acetyl-dG<sup>8-oxo</sup>. Subsequent protection of the 5' hydroxyl was done selectively with dimethoxytritylchloride in pyridine to yield the 5'-dimethoxytrityl-N<sup>2</sup>-acetyl-dG<sup>8-oxo</sup> (Roelen et al., 1991). A succinate linker arm for attaching the G<sup>8-oxo</sup> to a resin was appended to the 5'-dimethoxytrityl-N<sup>2</sup>-acetyl-dG<sup>8-oxo</sup> with succinic anhydride and dimethylaminopyridine in pyridine (Chow et al., 1981). The crude 3'-succinate-5'-dimethoxytrityl-N<sup>2</sup>-acetyl-dG<sup>8-oxo</sup>

was subsequently treated with DIC and p-nitrophenol to form the 3'-succinate-p-nitrophenyl ester 5'-dimethoxytrityl-N<sup>2</sup>-acetyl-dG<sup>8-oxo</sup>. Modified G<sup>8-oxo</sup> resin was prepared by mixing the 3'-succinate-p-nitrophenyl ester 5'-dimethoxytrityl-N<sup>2</sup>-acetyl-dG<sup>8-oxo</sup> with long chain amine control pore glass resin. The extent of loading of the nucleoside onto the solid support was established according to a described procedure (Gait et al., 1982). The determined loading was approximately 4 to 5  $\mu$ mol of G<sup>8-oxo</sup>/g resin. Modified G<sup>8-oxo</sup> resin columns were subsequently prepared and used for solid phase DNA synthesis. The desired d(GCTAG<sup>8-oxo</sup>) was prepared by normal solid phase DNA synthesis.

Preliminary characterization of the d(GCTAG<sup>8-oxo</sup>) oligonucleotide was performed by enzymatic digestion and HPLC analysis of the digestion products, as described previously for the d(GCTA<sup>8-oxo</sup> G) oligonucleotide. Peak area analysis of the UV chromatogram obtained from digested d(GCTAG<sup>8-oxo</sup>) indicated that the oligonucleotide compound contained dC, dG, dT, dA and dG<sup>8-oxo</sup>. Unfortunately, the amount of dG relative to dG<sup>8-oxo</sup> was slightly off. The reason for this discrepancy was not determined. Because of the unexpected departure of the postdoctoral fellow responsible for the biological studies, research on this project stopped before mutagenicity data were obtained in repair deficient cell lines.

#### **i. Synthesis of dG<sup>8-oxo</sup> phosphoramidite**

Although further study of the G<sup>8-oxo</sup> lesion has not been done in this laboratory, as part of a collaborative effort with L. Williams at Emory University, a dodecamer, d(CCA G<sup>8-oxo</sup>CGC TGG), was synthesized for X-ray analysis. In order to synthesize the necessary

phosphoramidite, the 5'-dimethoxytrityl-N<sup>2</sup>-acetyl-dG<sup>8-oxo</sup> used in the synthesis of a G<sup>8-oxo</sup> resin was phosphitylated with 2-cyano N,N diisopropyl chlorophosphoramidite (Figure 13). Unfortunately, the prepared phosphoramidite did not couple well, in agreement with Roelen et al. (1991). However, suitable amounts of the dodecamer, 5'-d(CCA G<sup>8-oxo</sup>CGC TGG), was synthesized and purified for X-ray analysis. No structural data from this oligonucleotide are available at this time.



**E. Pyrimidine oxidative lesions:**  
**5-hydroxyuridine and 5-hydroxycytidine**

**a. Importance of oxidized cytidines**

Although our previous study (Wood et al., 1992) confirmed that dG<sup>8-oxo</sup> causes G to T transversions, the lesion responsible for the major oxidative mutation at GC bases (GC->AT transitions) has not been established. Since it appears that oxidative damage of dG does not explain the predominance of GC to AT transitions, we began the study of dC oxidative lesions. Recent work by Wagner et al. (1992) established the steady state levels of three oxidative damage products of dC (Figure 14). Wagner et al. determined that 5-hydroxy-deoxycytidine (oh<sup>5</sup>dC) and deoxyuracil glycol (dUg) occurred at similar levels to those of dG<sup>8-oxo</sup> in rat liver kidney and brain DNA. A third oxidative lesion, 5-hydroxy-deoxyuracil (oh<sup>5</sup>dU) which is formed to an equal extent by various oxidative processes (e.g.,  $\gamma$ -radiation, UV, H<sub>2</sub>O<sub>2</sub> with Fe or Cu), however, occurred less often. The abundant existence of oh<sup>5</sup>dC, oh<sup>5</sup>dU and dUg in the genome implies that these lesions are not completely or rapidly repaired by the organism, and thus they may contribute to mutagenesis.

As a means to understand the potential mutagenic effects of oxidative cytosine lesions, work was begun in the Essigmann laboratory to develop site-specifically modified oligonucleotides containing oh<sup>5</sup>dU, oh<sup>5</sup>dC and dUg. The synthesis of a dUg containing

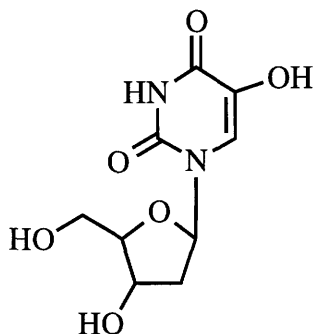
oligonucleotide was undertaken by D. Mathur. The approach used involved preferential oxidation of the uracil in the mono-uracilated oligonucleotide 5'-d(GUAGC) with osmium tetroxide. This method was used previously in this laboratory for the oxidation of a thymine to thymine glycol (Basu et al., 1989). In contrast, treatment of an oligonucleotide with oxidizing reagents capable of forming  $oh^5dU$  and  $oh^5dC$  would lead to the production of multiple products. Based on the stability of  $oh^5dU$  and  $oh^5dC$  to oxidizing, acidic and basic conditions (Beltz et al., 1955; Shen et al., 1966; Zorbach et al., 1968; Udea, 1960), a total synthetic approach appeared more suitable for the preparation modified oligonucleotides containing these lesions.

**b. Synthesis of a  $oh^5dU$  containing oligonucleotide**

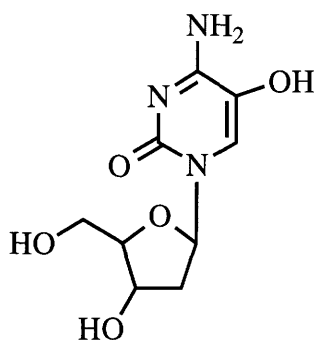
The synthesis of a modified oligonucleotide containing  $oh^5dU$  began with the preparation of the oxidized nucleoside. Three literature methods have been published for the preparation of  $oh^5dU$ . The first method developed to prepare  $oh^5dU$  involved the addition of aqueous bromine to dU, followed by treatment of the resulting dihydro intermediate with lead oxide (Beltz et al., 1955). A second method to synthesize  $oh^5dU$  involves formation of 5-bromo-dU and subsequent base catalyzed displacement of the bromide (Shen et al., 1966). A third method of synthesis involves the addition of aqueous bromine to dU resulting in formation of dUg and subsequent elimination with pyridine to form  $oh^5dU$  (Zorbach et al., 1968; Udea, 1960). This last method is the highest yielding (47 %) method for synthesizing  $oh^5dU$  and was the method used in this laboratory to prepare  $oh^5dU$ .

**Figure 14.** Oxidized cytosine products.

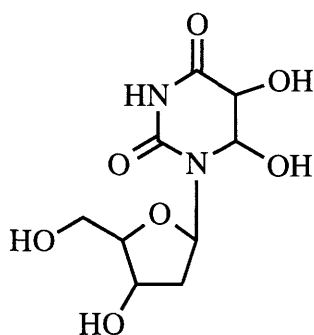
# Oxidized Pyrimidines



**5-hydroxy-deoxyuridine (<sup>oh</sup>5dU)**

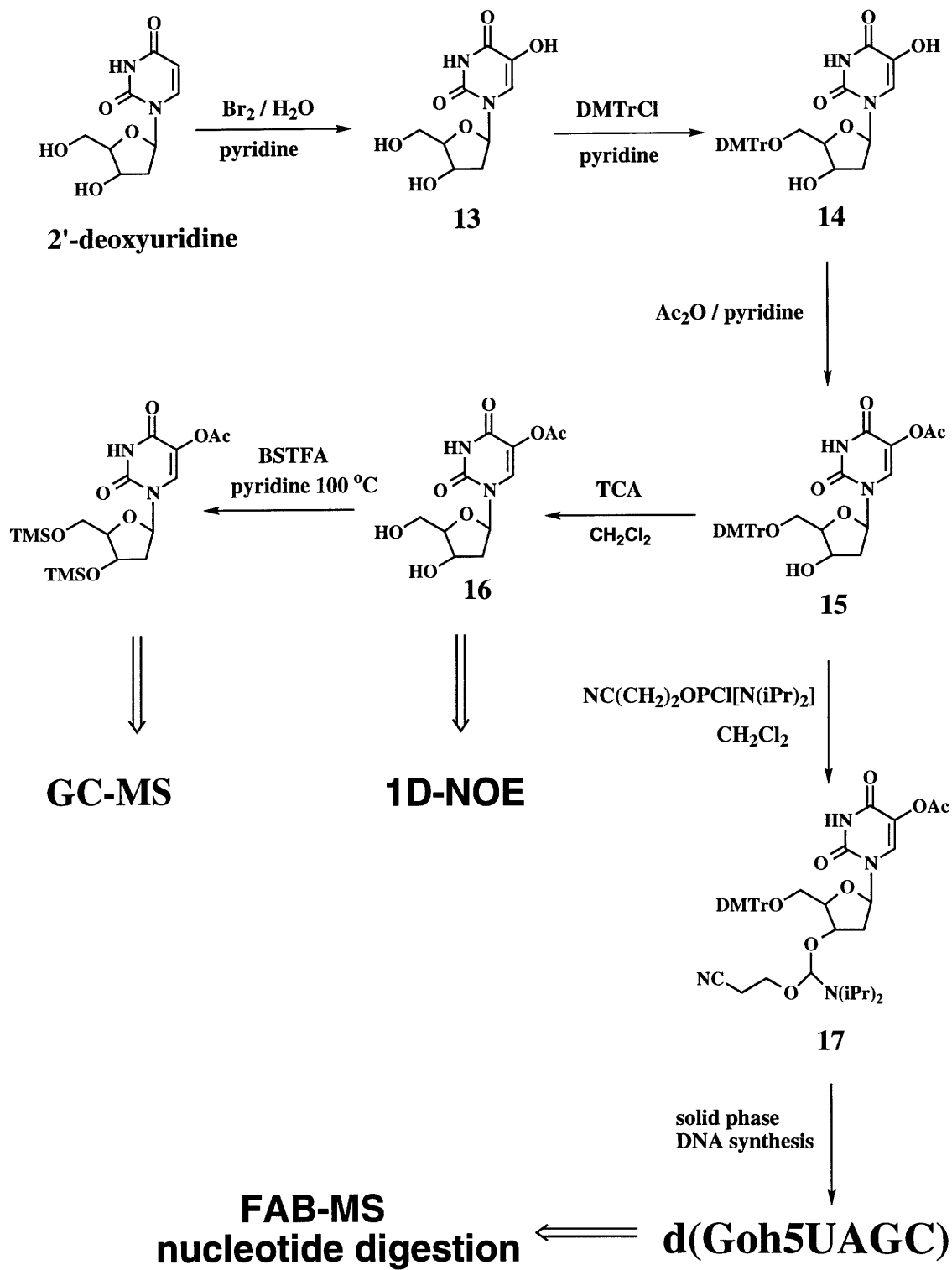


**5-hydroxy-deoxycytidine (<sup>oh</sup>5dC)**



**deoxyuracil glycol (dUg)**

**Figure 15.** Synthesis of oh<sup>5</sup>dU phosphoramidite.



The key problem in synthesizing a  $\text{oh}^5\text{dU}$  phosphoramidite was the problem of selectively blocking and activating the various hydroxyl groups. Based on the general reactivity of deoxynucleosides, the 5' hydroxyl was expected to be more reactive than the 3' hydroxyl (Gait, 1984). However, the reactivity of the 5-hydroxyl as compared to the 5' or 3' hydroxyl was not known. The reactivity of the three hydroxyl groups was determined by sequential treatment of the  $\text{oh}^5\text{dU}$  (**13**) with different protecting groups (Figure 15). Treatment of the  $\text{oh}^5\text{dU}$  (**13**) with DMTCl yielded a mono-DMT product. Based on the reactivity of DMTrCl with nucleosides, this product was proposed to be the 5'-DMT- $\text{oh}^5\text{dU}$  product (**14**). Treatment of 5'-DMT- $\text{oh}^5\text{dU}$  with one equivalent of acetic anhydride resulted in a mono acetylation product, either 5'-DMT-5-acetoxy-dU or 5'-DMT-3'-acetoxy- $\text{oh}^5\text{dU}$ . The regiochemistry of this product was subsequently determined by gas chromatography-mass spectroscopy (GC-MS) (Figure 16) and NMR spectroscopy (Figure 17).

The initial evidence for the regiochemical assignment of the mono-acetylated 5-DMT- $\text{oh}^5\text{dU}$  product came from GC-MS. If the acetate were on the riboside portion, the uracil base fragment determined by GC-MS would not contain an acetoxy group; however, if the acetate were on the uracil portion, the uracil base fragment would contain an acetoxy group. Because the 5'-DMT group would not be stable for gas chromatography, the DMT group was deprotected with trichloroacetic acid. The deprotected nucleoside was then treated with N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) to persilylate the molecule for GC-MS analysis. GC-MS of bis-TMS-acetoxy- $\text{oh}^5\text{dU}$  showed the presence of  $m/z=314$  and 299 ( $\text{M-CH}_3$ )<sup>+</sup> (Figure 16). The 314 molecular ion corresponds to a bis-TMS-acetoxy-uridine base

fragment; therefore, the product from mono-acetylation must have been 5'-DMT-5-acetoxy-dU. This result was then confirmed by a one dimensional Nuclear Overhauser Effect (1D-NOE) (Figure 17). If the product were 5'-DMT-3'-acetoxy-oh<sup>5</sup>dU, no NOE would be expected between a 3'-acetoxy group and the H6 proton. As seen in Figure 17, a positive NOE was found with H6. Unfortunately, because one of the H2' protons was also excited a positive NOE was additionally seen with H3'. This NOE result, consequently, can not prove that the product of acylation occurred only at the 5-OH, nevertheless, the 5'-DMT-3'-acetoxy-oh<sup>5</sup>dU product would not be consistent with the NOE data. Therefore, based on the GC-MS and this NOE result, the product from the mono-acetylation of 5'-DMT-oh<sup>5</sup>dU (14) must have been 5'-DMT-5-acetoxy-dU (15).

Since the 5'-DMT-5-acetoxy-dU product (15) was properly protected for solid phase DNA synthesis, subsequent formation of the 3'-phosphoramidite did not require additional manipulation of the protecting groups. Treatment of the 5'-DMT-5-acetoxy-dU product (15) with 2-cyano-N,N-diisopropylaminechlorophosphoramidite resulted in the necessary 3'-phosphoramidite (17). The desired oligonucleotide G<sup>oh5</sup>UAGC was prepared by solid phase DNA synthesis. The coupling efficiency of 5'-DMT-5-acetoxy-dU 3'-[(2-cyanoethyl) N,N-diisopropylphosphoramidite] was determined by dimethoxytrityl cation quantification to be greater than 65% (not optimized).

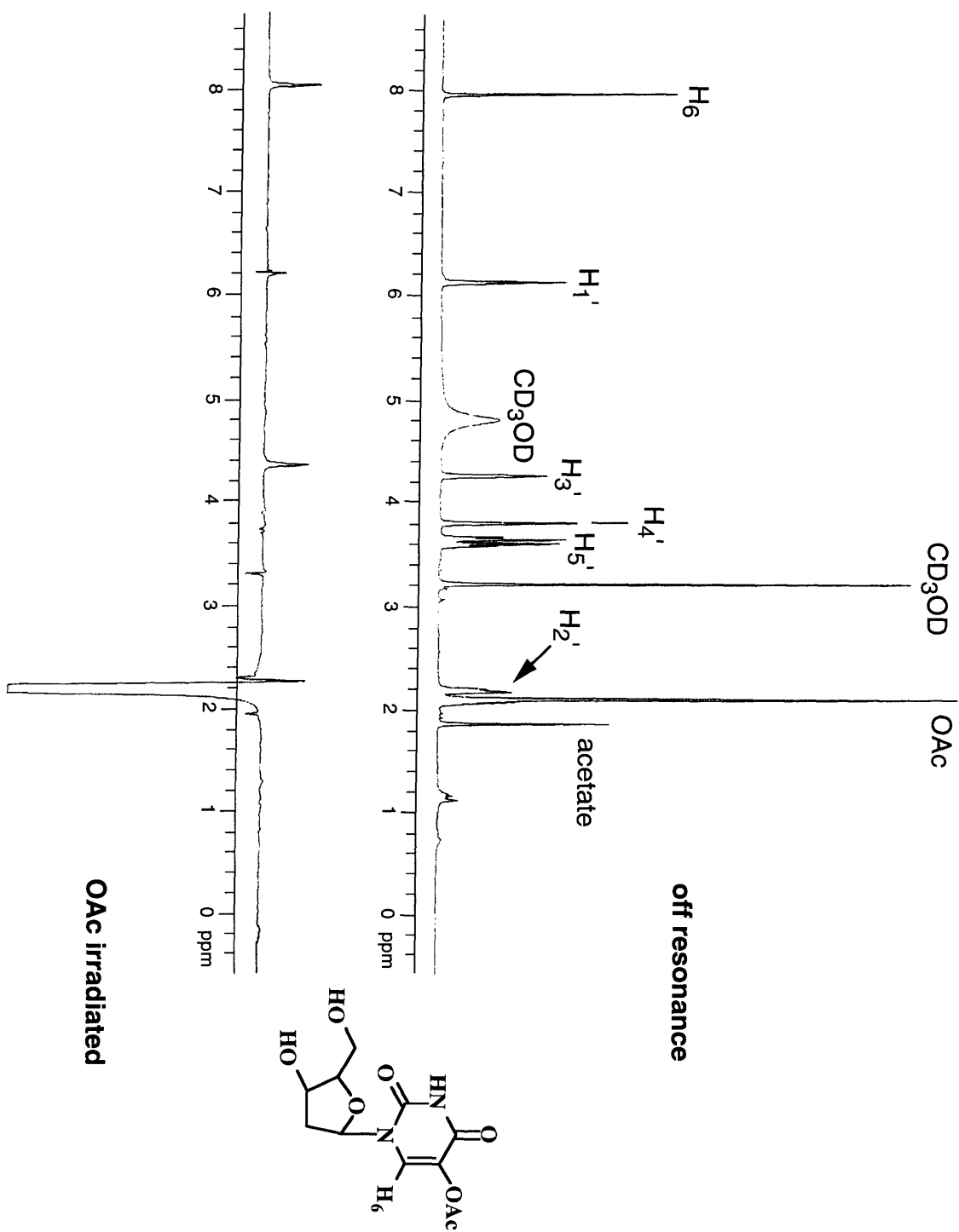
The identity of the purified oligonucleotide containing <sup>oh5</sup>dU was established by a combination of enzymatic DNA digestion followed by HPLC analysis and laser assisted matrix desorption mass spectrometry. Laser assisted matrix desorption of the modified



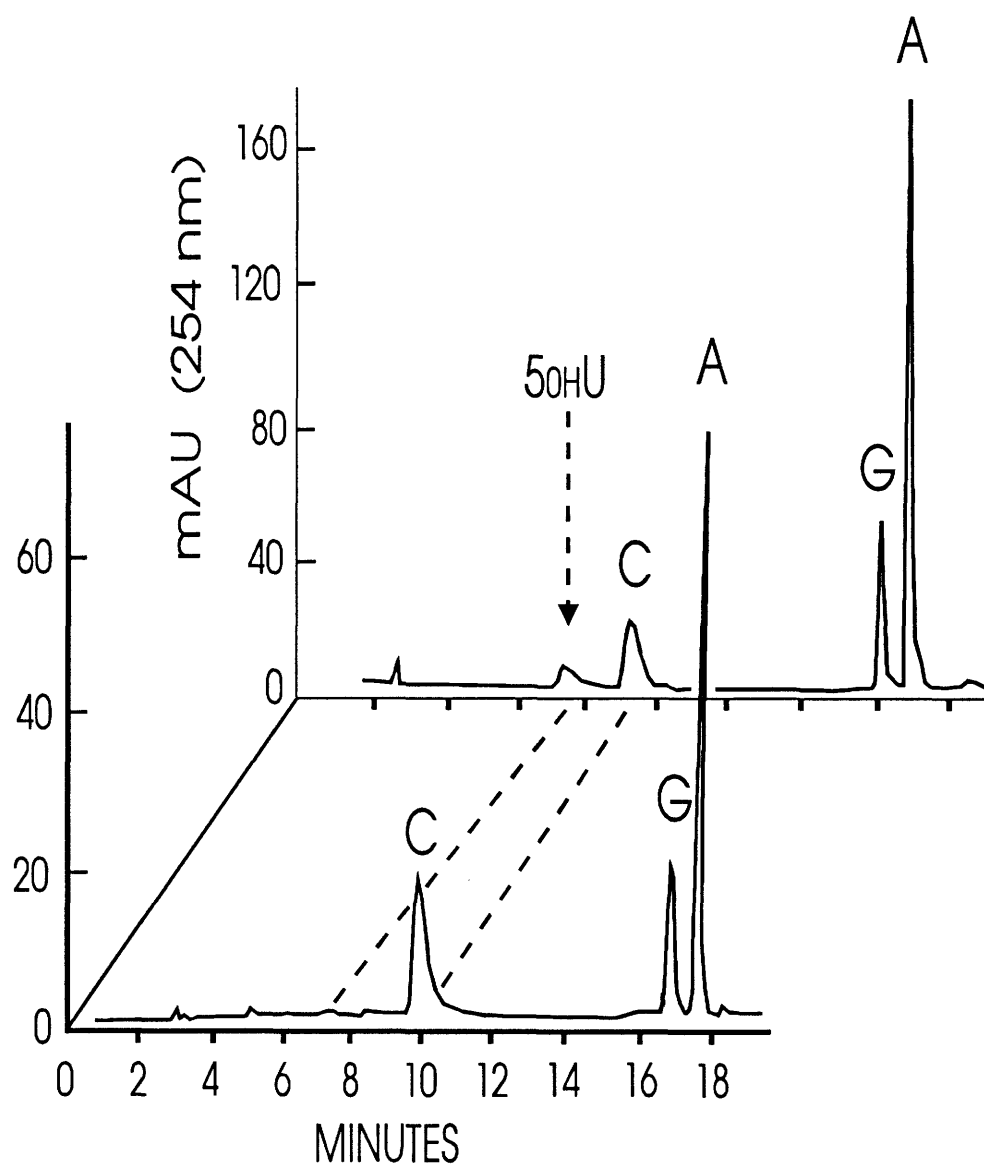
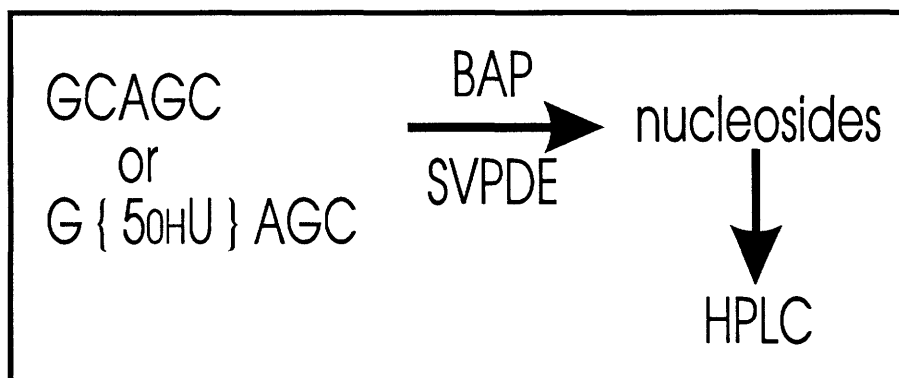
**Figure 16.** GC-MS of mono-acetylated 5-DMT-oh<sup>5</sup>dU.



**Figure 17.** 1D-NOE of 5-acetoxy-dU.



**Figure 18.** Enzymatic digestion of G<sup>oh5</sup>UAGC.



oligonucleotide showed a parent molecular ion of  $m/z=1504$ , in agreement with the calculated molecular weight for the modified oligonucleotide ( $m/z=1504.1$ ). Subsequent enzymatic digestion of the modified oligonucleotide and HPLC analysis confirmed the presence of the modified base,  $^{oh^5}dU$  (Figure 18). At this time, no biological results are available for this modified oligonucleotide.

**c. Preliminary studies to synthesize  $^{oh^5}dC$  containing oligonucleotides.**

Synthesis of  $^{oh^5}dC$  containing oligonucleotide is more difficult than synthesizing a  $^{oh^5}dU$  oligonucleotide for two reasons: 1)  $^{oh^5}dC$  has one additional reactive site, the  $N^4$  amino group, that must be blocked prior to oligonucleotide synthesis; and 2) it is unknown under what conditions  $^{oh^5}dC$  would deaminate to  $^{oh^5}dU$ . Three approaches have been utilized to synthesize  $^{oh^5}dC$  containing oligonucleotides. The first approach involved the oxidation of a protected cytosine. If the  $N^4$  amino group was protected prior to formation of the 5-hydroxy, the resulting functional groups in an  $N^4$ -protected- $^{oh^5}dC$  would be the same as the  $^{oh^5}dU$  nucleoside, the 5',3' and 5-hydroxy groups. Subsequent protection and activation steps developed for  $^{oh^5}dU$  could, in theory, be used to synthesize a  $N^4$  protected  $^{oh^5}dC$  phosphoramidite. Unfortunately, of the two methods examined to oxidize  $N^6$ -benzoyl-dA: bromine/water and pyridinium bromide perbromide, the oxidation products did not appear to have yielded a 5-hydroxy product. Based on this inability to oxidize  $N^6$ -benzoyl-dA, studies were undertaken to examine the potential of differentially protecting  $^{oh^5}dC$ .

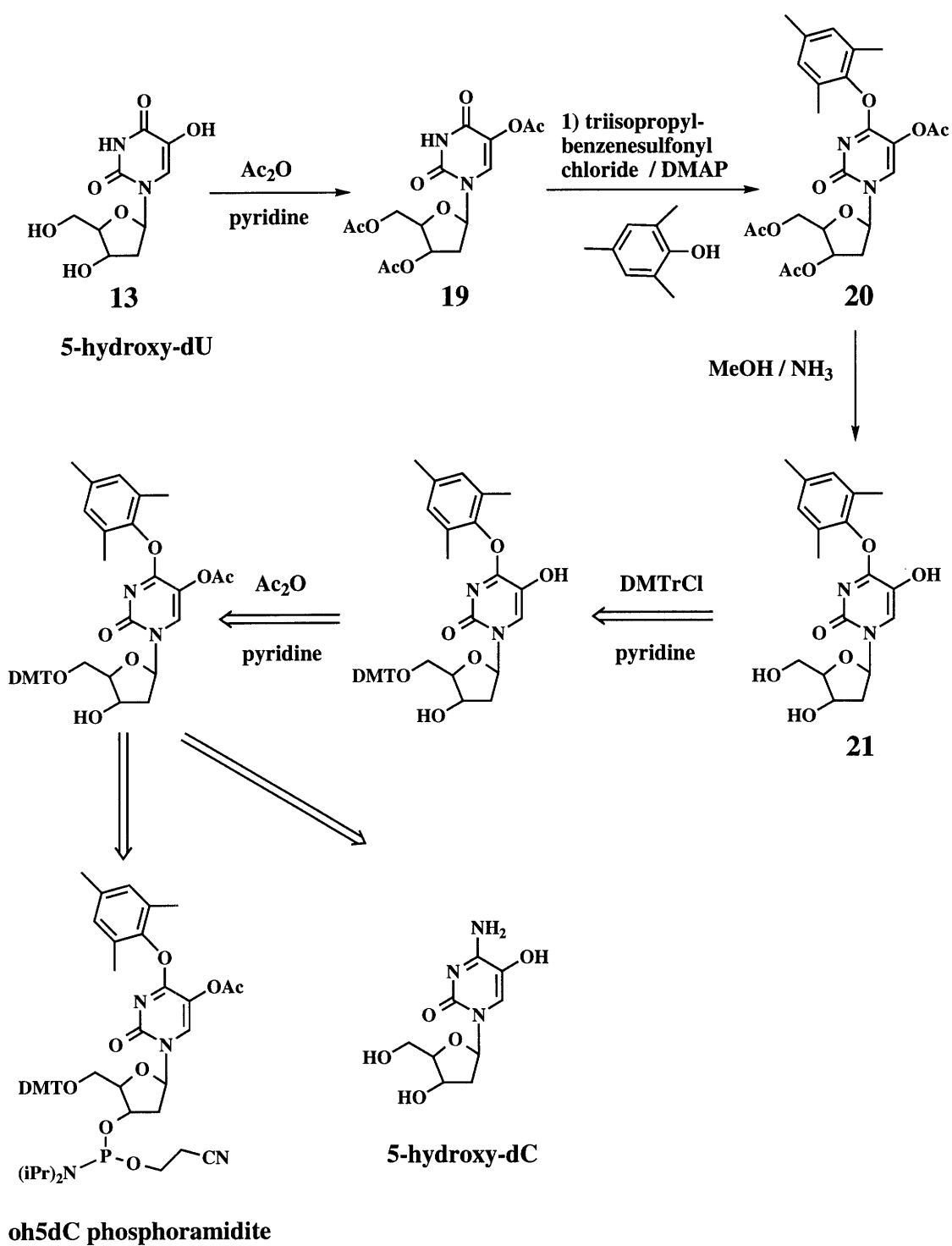
A procedure involving differential protection of oh<sup>5</sup>dC began with the synthesis of oh<sup>5</sup>dC. A similar oxidation procedure as with oh<sup>5</sup>dU was used (Eaton and Hutchinson, 1973). Unfortunately, preliminary studies involving peracylation of oh<sup>5</sup>dC with acetic anhydride or benzoyl chloride commonly led to multiple products. The production of multiple products seemed to indicate that some deamination was taking place while the N<sup>6</sup> amino group was being protected. These studies seemed to indicate that even if a protected N<sup>4</sup>-oh<sup>5</sup>dC could be synthesized, the oh<sup>5</sup>dC nucleotide might not be stable to oligonucleotide synthesis. Consequently a new approach was needed that would avoid the potential problem of deamination of the oh<sup>5</sup>dC nucleoside.

Only preliminary studies have been done on a third approach to synthesize a oh<sup>5</sup>dC modified oligonucleotide. As depicted in Figure 19, this approach involved the *in situ* generation of oh<sup>5</sup>dC from oh<sup>5</sup>dU. In 1986, Zhou and Chattopadhyaya reported the *in situ* site-specific modification of uracil to cytosine. The method involved displacement of 2,4,6-trimethylphenyl (TMP) or 2-nitrophenyl from the O4 position of uridine with ammonia. Sproat et al. (1989) and MacMillan and Verdine (1990) subsequently reported the displacement of these O4 activated uridines with a variety of alkylamines. We reasoned that synthesis of a TMP-oh<sup>5</sup>dU phosphoramidite would allow us to synthesize oh<sup>5</sup>dC containing oligonucleotides. Initial studies indicate that a TMP-5-hydroxy-dU derivative can be made in five steps. The current method begins by protection of oh<sup>5</sup>dU with acetic anhydride. The O4 position of oh<sup>5</sup>dU can subsequently be activated with triisopropylbenzenesulfonyl chloride and blocked with TMP. Using a methanolic ammonia solution, the acetate protecting groups can be removed without displacing the O4-TMP group. Work is continuing to validate this



approach. It is proposed that a reactive phosphoramidite could be synthesized in three more steps: 1) protection of the 5' hydroxyl with a dimethoxytrityl group, 2) protection of the 5 hydroxyl with an acetate protecting group, as done in the  $oh^5dU$  synthesis, and 3) formation of the 3' phosphoramidite.

**Figure 19.** Proposed synthetic pathway for <sup>oh5</sup>dC.



## F. Conclusions

The previous sections have described the synthesis of four modified oligonucleotides and an approach to synthesize a fifth modified oligonucleotide. The oligonucleotides were made in order to determine the mutagenic and structural consequences of an aromatic amine carcinogen and three different oxidative DNA lesions. The methods utilized to synthesize the modified oligonucleotide involved either the modification of a preformed oligonucleotide, in the case of 4-ABP, or solid phase DNA synthesis utilizing a modified phosphoramidite. In the case of the ABP oligonucleotide, reaction of N-acetoxy-aminobiphenyl with an oligonucleotide containing only one guanine residue was a facile method for the preparation of the modified oligonucleotide containing the major aminobiphenyl adduct. Subsequent biological study of the modified oligonucleotide by S. Verghis showed that the major ABP adduct was not mutagenic in ss DNA in the sequence context studied. Synthesis of two purine oxidation products, G<sup>8-oxo</sup> and A<sup>8-oxo</sup>, were made by total synthetic procedures. Subsequent biological studies conducted by M. Wood and A. Esteve established that the A<sup>8-oxo</sup> lesion was weakly mutagenic. Ongoing studies by L. Williams are attempting to establish the structural consequences of G<sup>8-oxo</sup> by X-ray crystallography. In contrast to the problems synthesizing oxidized purine lesions, a <sup>oh</sup>5dU phosphoramidite was readily synthesized and utilized in solid phase DNA synthesis. Ongoing studies are attempting to determine the mutagenic potential of this lesion. Further studies are required before a <sup>oh</sup>5dC oligonucleotide can be synthesized. One strategy that appears hopeful involves the *in situ* generation of <sup>oh</sup>5dC via a O<sup>4</sup> modified <sup>oh</sup>5dU oligonucleotide.

## G. Experimental

### a. General Procedures and Materials

Unless otherwise specified, all chemicals were bought from Aldrich or Sigma chemical companies. Long chain amine (LCA) control pore glass was bought from CPG, inc. (Fairfield, NJ). THF and CH<sub>2</sub>Cl<sub>2</sub> were distilled from CaH<sub>2</sub> and pyridine and triethylamine were distilled from sodium. Silica gel TLC plates were visualized with UV illumination followed by charring with 5% anisaldehyde in ethanol-acetic acid-sulfuric acid (95:5:1). Silica gel (230-400 mesh) was used for normal phase chromatographic separations. All reactions were performed under an argon atmosphere except when working in aqueous media.

Mass spectrometry data were obtained by matrix-assisted laser desorption and time of flight mass spectroscopy with 2-nitrobenzyl alcohol as the matrix. GC-MS was performed on a Model 5971A mass selective detector interfaced to a Model 5890 Series II gas chromatograph (both from Hewlett Packard). The injection port, ion source and interface were maintained at 240 °C. Separations were carried out on fused silica column (12.5 M x 0.20 mm) coated with cross-linked methyl silicone gum phase (film thickness 0.33 μm) (Hewlett Packard). Helium was the carrier gas at an inlet pressure of 37.5 psi. Scan mode of monitoring was performed at 70 eV using the electron-impact mode of ionization. The A<sup>8-oxo</sup> nucleoside base fragment was obtained by hydrolyzing the dA<sup>8-oxo</sup> nucleoside with 0.5 ml of formic acid (98%) in an evacuated and sealed tube at 150 °C for 30 min follow by

lyophilization and subsequent conversion to the trimethylsilyl (TMS) derivative in a Teflon-capped vial with 40  $\mu$ l of N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) acetonitrile/pyridine (2:1 v/v) at 85 °C for 30 min. After cooling the derivatized products were injected directly onto the GC without further treatment.

$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on Varian VXR-300, VXR-301, or Bruker AC-250 MHz spectrometers. Chemical shifts are expressed in parts per million downfield from  $\text{CDCl}_3$  (7.24 ppm or 77.00 ppm),  $\text{CD}_3\text{OD}$  (3.30 ppm and 35.00 ppm), and  $\text{Me}_2\text{SO}$  (2.49 ppm and 39.5) for  $^1\text{H}$  and  $^{13}\text{C}$ , respectively. Data are reported as follows: chemical shift, integration, multiplicity, and regiochemical assignments.

Reversed phase HPLC analyses were performed with a Beckman  $\text{C}_{18}$  ultrasphere or Phenomenex Ultramex 5- $\mu\text{m}$   $\text{C}_{18}$  columns. Chromatography was done with either a Beckman 114M solvent delivery system, Beckman 421A controller, and Hewlett Packard model 1040A photodiode array detector or with a Beckman Ultrasphere  $\text{C}_{18}$  column using a Nec PC-8300 controller, Beckman Analog Interface Module 406, Beckman 110B solvent Delivery Module, Nelson Analytical 760 series Interface, and Gateway 2000 computer. The compounds were monitored at 280 and 254 nm. The flow rate was 1 ml/min. Gradient I: a linear gradient from 0-50% acetonitrile in 0.1 M ammonium acetate pH 5.8.

Oligodeoxynucleotides were synthesized by solid phase DNA synthesis on an Applied Biosystems DNA synthesizer using phosphoramidite chemistry. The modified phosphoramidite could be added either manually (for less than 10  $\mu\text{mol}$  synthesizes) or

automatically for large scale preparation. Manual coupling of a modified phosphoramidite involved: 1) halting the oligonucleotide synthesis before the modified phosphoramidite was to be added; 2) adding the phosphoramidite dissolved in CH<sub>3</sub>CN (100  $\mu$ l) and ABI tetrazole/CH<sub>3</sub>CN solution (100  $\mu$ l) via two disposable 1 ml syringes; and 3) resuming automated DNA synthesis after the phosphoramidite coupling steps. Automatic coupling involved dissolving the phosphoramidite with CH<sub>3</sub>CN and attaching the solution to the X port on the DNA synthesizer. Subsequent to oligonucleotide synthesis in either case, the oligonucleotide was cleaved from the resin with NH<sub>4</sub>OH<sub>(con)</sub> (3 x 5 ml). The ammonium hydroxide solution containing the oligonucleotide was heated overnight at 60 °C. The deprotected oligonucleotide was purified by gel electrophoresis or RP-HPLC. The base composition of the modified oligonucleotide was determined by RP-HPLC after digestion with bacterial alkaline phosphatase and snake venome phosphodiesterase.

Coupling yields were determined by dimethoxytrityl cation quantification. The procedure involved 1) mixing the oligonucleotide dimethoxytrityl eluent (0.5 ml) with 0.1M toluene sulfonic acid dissolved in acetonitrile (4.5 ml); 2) diluting the dimethoxytrityl solution/acetonitrile solution (0.1 ml) with 0.1M toluene sulfonic acid dissolved in acetonitrile (0.9 ml); and 3) measuring the UV absorbance at  $\lambda=298$  nm .

## **b. Methods**

### **5'-d(TG<sup>8-ABP</sup>CA) (1)**

The tetranucleotide, TGCA (10  $\mu$ mol, ~0.18mg), prepared by solid phase DNA synthesis on

an Applied BioSystems DNA Synthesizer, was dissolved in 30 ml of  $\text{CHCl}_3$  / EtOH /  $\text{H}_2\text{O}$  (7:4:4) and triethylamine (1.0 ml). After warming to 40 °C, N-acetoxy-aminobiphenyl (0.86 g, 3.79 mmol) was added in 5 portions at approximately 15 min intervals. After 2.75 hr, the dark red solution was diluted with  $\text{CH}_2\text{Cl}_2$  (20 ml) and  $\text{H}_2\text{O}$  (5 ml). The aqueous layer was collected and washed  $\text{CH}_2\text{Cl}_2$  (2 x 25 ml). The combined aqueous solution was concentrated *in vacuo*. HPLC analysis using gradient I indicated that 30-40% of the d(TGCA) had reacted. The d(TG<sup>8-ABP</sup>CA) was purified by reversed phase HPLC using gradient I. After HPLC purification the d(TG<sup>8-ABP</sup>CA) was desalted using the same HPLC column and a linear  $\text{H}_2\text{O}$ ,  $\text{CH}_3\text{CN}$  gradient. Laser assisted matrix desorption mass spectrometry was used to analyze the d(TG<sup>8-ABP</sup>CA). The tetranucleotide was calculated to have a molecular weight of 1340. The negative ion spectrum showed the major  $[\text{M}-\text{H}]^-$  ion to be  $m/z = 1339$ .

### **8-Bromo-2'-deoxyadenosine (2).**

Deoxyadenosine (2.03 g 8.08 mmol) suspended in 20 ml of 1 M NaOAc buffer (pH 5.4) was treated with aqueous bromine  $\text{Br}_2$ /sodium acetate buffer (37 ml) prepared by dissolving  $\text{Br}_2$  (2 ml) in sodium acetate buffer (40 ml). After 2 hr, solid  $\text{NaHSO}_3$  was added until the intense brown color of the reaction solution faded. The pH of the solution was adjusted to pH 7.0. The solution was left to chill at -20 °C for 20 min. The brown solid was collected by filtration, washed with water, and dried on kugelrohr (60 °C, 0.3 mm Hg) overnight to yield 1.72 g, 5.21 mmol (65%) of 8-Bromo-2'-deoxyadenosine (2). (TLC rf 0.42 MeOH/ $\text{CH}_2\text{Cl}_2$  9:1) mp > 250 °C;  $^1\text{H}$  NMR ( $(\text{CD}_3)_2\text{SO}$ ): 2.19 (1H, m, H2"), 3.24 (1H, m,



H2'), 3.47 (1H, m, H5"), 4.62 (1H, m, H5'), 3.87 (1H, d, H4'), 4.47 (1H, d, H3'), 5.28 (1H, dd, 5'-OH), 5.33 (1H, d, 3'-OH), 6.28 (1H, t, H1'), 7.50 (2H, br, 6NH<sub>2</sub>), 8.10 (1H, s, H2). <sup>13</sup>C NMR ((CD<sub>3</sub>)<sub>2</sub>SO): 36.96 (C2'), 62.04 (C5'), 71.08 (C3'), 86.28 (C4'), 88.25 (C1'), 119.0 (C5), 127.0 (C4), 149.5(C6), 152.2 (C2), 154.9 (C8). UV (CH<sub>3</sub>OH) λ<sub>max</sub> = 265 nm (ε = 15,500) [no apparent shift in λ<sub>max</sub> with pH].

**2'-Deoxy-7,8-dihydro-N<sup>6</sup> -acetyl adenosin-8-one (3).**

The 8-bromo-2'-deoxyadenosine (**2**) (0.80 g, 2.42 mmol), dimethylaminopyridine (0.08 g, 0.655 mmol), and sodium acetate (1.63 g, 19.87 mmol), pre-dried under high vacuum on a kugelrohr at 0.5 mm Hg and 100 °C, were suspended in acetic anhydride (23 ml). After refluxing for 2 hr, the reaction was cooled to room temperature and quenched with Na<sub>2</sub>CO<sub>3(con)</sub> (50 ml). The aqueous solution was extracted with EtOAc (2 x 50 ml) and the combined organic solution was washed with Na<sub>2</sub>CO<sub>3(con)</sub> (2 x 75 ml), dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, concentrated, and evaporated twice from toluene. The residue was suspended in CH<sub>2</sub>Cl<sub>2</sub> and filtered.

The crude mix acetylated material was dissolved in 100 ml of pyridine/ MeOH/ water (35/10/5) and treated with 2 N NaOH (5 ml). After 15 min, the reaction (pH = 11.5) was quenched by the addition of AcOH<sub>(con)</sub> until pH 7.0. The solution was concentrated to dryness and evaporated twice from toluene. The solid was suspended in CH<sub>2</sub>Cl<sub>2</sub> and filtered. The collected solid was suspended in water, chilled to -20 °C for 15 min, and filtered again. The white solid was dried on a kugelrohr (60 °C and 0.5 mm Hg) to yield 1.01 g, 3.27 mmol (63%) of 2'-deoxy-7,8-dihydro-N<sup>6</sup> -acetyl adenosin-8-one (**3**). (TLC rf

0.46 MeOH/ CH<sub>2</sub>Cl<sub>2</sub> 95:5); mp 247-250; <sup>1</sup>H NMR ((CD<sub>3</sub>)<sub>2</sub>SO): 2.06 (1H, m, H2'), 2.12 (2H, s, N<sup>6</sup>Ac), 3.05 (1H, qt, H2'), 3.43 (1H, dd, H5''), 3.59 (1H, dd, H5'), 3.78 (1H, m, H4'), 4.41 (1H, br, H3'), 4.80 (1H, br, OH), 5.22 (1H, br, OH), 6.20 (1H, t, H1'), 8.39 (1H, s, H2), 10.27 (1H, br, NH), 10.83 (1H, br, NH). <sup>13</sup>C NMR ((CD<sub>3</sub>)<sub>2</sub>SO) 23.11 (Ac), 35.58 (C2'), 62.20 (C5'), 71.10 (C3'), 81.25 (C4'), 87.47 (C5'), 110.98 (C5), 138.03 (C4), 149.61 (C2), 150.16 (C8), 150.96 (C6), 169.4 (Ac). UV (CH<sub>3</sub>OH) λ<sub>max</sub> 290 nm (ε = 11,000) (pH 7.2); 290 nm (ε = 11,000) (pH 1.0); 307 nm (ε = 13,000), 271 nm (ε = 6,400) (pH 12).

**2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-7,8-dihydro-N<sup>6</sup>-acetyl adenosin-8-one (4).**

2'-Deoxy-7,8-dihydro-N<sup>6</sup>-acetyl adenosin-8-one (3) (0.30 g, 0.970 mmol), 4,4'-dimethoxytrityl chloride (0.68 g, 2.01 mmol, 200M%), and dimethylaminopyridine (0.002 g) were suspended in pyridine (10 ml). Upon addition of triethylamine (0.400 ml, 2.87 mmol, 300 M%), the brown solution turns a red color and within 30 min, the solution became a clear light brown. After 4.5 hr, additional dimethoxytritylchloride (0.35 g, 1.03 mmol, 100 M%) and Et<sub>3</sub>N (0.150 ml, 1.08 mmol, 110 M%) were added. After 7 hr, the reaction mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> (30 ml) and quenched with NaHCO<sub>3(sat)</sub> (40 ml). The organic layer was collected and the aqueous layer back extracted with CH<sub>2</sub>Cl<sub>2</sub> (30 ml). The combined organic solution was washed with 40 ml NaCl<sub>(sat)</sub> (40 ml), dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated to dryness. 2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-7,8-dihydro-N<sup>6</sup>-acetyl adenosin-8-one (4) was purified by silica gel chromatography (35 g) by eluting with MeOH/CH<sub>2</sub>Cl<sub>2</sub> (5:95) and > 1% Et<sub>3</sub>N to yield 0.484 g (0.790 mmol, 81%). (TLC R<sub>f</sub> 0.27

MeOH/ CH<sub>2</sub>Cl<sub>2</sub> 97:3); mp 133-136; <sup>1</sup>H NMR (CD<sub>3</sub>OD): 2.11 (1H, m, H2"), 2.12 (3H, s, Ac), 3.04 (1H, m, H2'), 3.14 (2H, m, 2 x H5'), 3.70 (6H, d, 6H, 2 x CH<sub>3</sub>O), 3.92 (1H, q, H4'), 4.48 (1H, q, H3'), 6.20 (1H, t, H1'), 6.78 (4H, m, DMT), 7.18 (7H, m, DMT), 7.34 (2H, d, DMT), 8.20 (1H, s, H2), 10.20 (1H, br, NH), 10.82 (1H, br, NH). <sup>13</sup>C NMR (CD<sub>3</sub>OD): 23.81 (Ac), 37.48 (C2'), 56.16 (DMT-OMe), 66.22 (C5'), 73.80 (C3'), 83.618 (C4'), 87.76 (DMT), 88.02 (C1'), 111.55 (C5'), 114.36 (Ph-OMe C3"), 128.11 (Ph C4"), 129.02 (Ph C2"), 129.84 (Ph C3"), 131.78 (Ph-OMe C2"), 137.93 (Ph-OMe C1"), 140.05 (C4), 147.10 (Ph C1"), 151.82 (C2) 152.58 (C8), 153.589 (C6), 160.45 (Ph-OMe C4"), 172.68 (Ac); UV (CH<sub>3</sub>OH) λ<sub>max</sub> 285 nm (ε =12,600) (pH 8.25); 284 nm (ε =13,000) (pH 1.0); 305 nm (ε =12,600), 270 nm (ε =900) (pH 12).

**2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-7,8-dihydro-N<sup>6</sup>-acetyl adenosin-8-one 3'[(2-cyanoethyl) N,N-diisopropylphosphoramidite] (5).**

2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (50 μl) was added to 2'-deoxy-5'-O-(4,4'-dimethoxytrityl)-7,8-dihydro-N<sup>6</sup>-acetyl adenosin-8-one (4) (73.5 mg, 120 μmol) dissolved in CH<sub>3</sub>CN (500 μl) with tetrazole (50 μl of a 3 M soln in CH<sub>3</sub>CN). After 2 hr, additional 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (50 μl) was added. The crude reaction mixture was used for DNA synthesis. (TLC rf =0.42 MeOH/ CH<sub>2</sub>Cl<sub>2</sub> 97:3)

**2'-Deoxy-7,8-dihydroadenosin-8-one (6).**

8-Bromo-deoxyadenosine (**2**) (0.87 g, 2.64 mmol), dimethylaminopyridine, and sodium acetate (2.11 g, 25.72 mmol), pre-dried on high vacuum on kugelrohr at 0.5 mm Hg and 100 °C, were suspended in Ac<sub>2</sub>O (40 ml). After 2 1/2 hr at reflux, the solution was cooled to room temperature and quenched with Na<sub>2</sub>CO<sub>3(sat)</sub> (100 ml) and extracted EtOAc (2 x 50 ml). The organic solution was washed Na<sub>2</sub>CO<sub>3(sat)</sub> (2 x 50 ml), dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, concentrated, and evaporated twice from toluene.

Sodium hydroxide (10 M, 5 ml) was added to the crude mix acetylated material dissolved in 50 ml of pyridine/ MeOH/ water (35/10/5). After 19 hr at room temperature, the reaction (pH 11.5) was quenched with AcOH<sub>(conc.)</sub> until pH 7.0. The solution was concentrated to dryness and evaporated twice from toluene. The 2'-deoxy-7,8-dihydroadenosin-8-one (**6**) was purified by silica chromatography (20 g) by eluting with 20% MeOH/CH<sub>2</sub>Cl<sub>2</sub>. (TLC rf 0.26 MeOH/ CH<sub>2</sub>Cl<sub>2</sub> 90:10); mp > 235 °C; <sup>1</sup>H NMR (CD<sub>3</sub>OD): 2.12 (1H, dd, H2"), 2.92 (1H, m, H2'), 3.68 (1H, dd, H5"), 3.83 (1H, dd, H5'), 4.02 (1H, s, H4'), 4.55 (1H, d, H3'), 6.37 (1H, dd, H1'), 7.94 (1H, s, H2). <sup>13</sup>C NMR (CD<sub>3</sub>OD): 39.14 (C2'), 64.54 (C5'), 74.04 (C3'), 84.72 (C4'), 89.49 (C1'), 111.61 (C5), 148.55 (C4), 149.63 (C2), 150.00 (C6), 157.78 (C8); UV (0.1 M NH<sub>4</sub>OAc) λ<sub>max</sub> 260 nm (ε = 11,000) (pH 6.85); UV (CH<sub>3</sub>OH) λ<sub>max</sub> 270 nm (ε = 10,000) (pH 8.6); 283 nm (ε = 14,000) (pH 1.0); 269 nm (ε = 8,500), 289 nm (ε = 9,400) (pH 12).

**2'-Deoxy-7,8-dihydro-N<sup>2</sup>,3',5'-triacetylguanosin-8-one (7).**

Sodium benzyolate was prepared by chilling benzyl alcohol (10 ml, 96.6 mmol) with an ice bath and then adding NaH (0.080 g, 3.33 mmol). After gas evolution ceased, DMSO (10 ml) and 8-bromo-deoxyguanosine (3.00 g, 8.67 mmol) were added. After 24 hr at 110 °C, the mixture was diluted with acetone whereby a brown solid precipitated from solution. The crude 2'-deoxy-7,8-dihydro-guanosin-8-one was washed with CH<sub>2</sub>Cl<sub>2</sub> and acetone and dried under a high vacuum.

The crude 2'-deoxy-7,8-dihydro-guanosin-8-one was dissolved in pyridine (50 ml) and treated with acetic anhydride (20 ml). After 2.5 hr at 80 °C, the reaction was concentrated to dryness, diluted with CH<sub>2</sub>Cl<sub>2</sub> (100 ml) and washed with Na<sub>2</sub>CO<sub>3(sat)</sub> (40 ml) and NaCl<sub>(sat)</sub> (40 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The 2'-deoxy-7,8-dihydro-N<sup>2</sup>,3',5'-triacetylguanosin-8-one (7) was purified by silica gel chromatography (50 g) by eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (96:4) (0.87 g, 2.12 mmol, 25 % yield) (TLC rf 0.29 CH<sub>2</sub>Cl<sub>2</sub>/MeOH 95/5) <sup>1</sup>H NMR (CDCl<sub>3</sub>): 9.51 (1H, s, NH), 6.38 (1H, dd, H1'), 5.42 (1H, m, H3'), 4.83 (1H, m, H5'), 4.44-4.36 (2H, m, H4', H5''), 2.97 (1H, m, H2''), 2.38 (1H, m, H2'), 2.32 (2H, s, NHAc), 2.13 (3H, s, OAc), 2.12 (3H, s, OAc).

**2'-Deoxy-7,8-dihydro-N<sup>2</sup>-acetylguanosin-8-one (8).**

Sodium hydroxide (5 M, 0.50 ml) was added to 2'-deoxy-7,8-dihydro-N<sup>2</sup>,3',5'-triacetylguanosin-8-one (7) (0.70 g, 1.71 mmol) dissolved in MeOH (10 ml). After 20 min

at room temperature, the reaction was quenched by adding acetic acid<sub>(con)</sub> (0.50 ml) and the solution was concentrated to dryness. The 2'-deoxy-7,8-dihydro-N<sup>2</sup>-acetylguanosin-8-one (**8**) was purified by silica gel chromatography (10 g) by eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (90:10). (0.33 g, 1.01 mmol, 60 % yield). (TLC rf 0.27 CH<sub>2</sub>Cl<sub>2</sub>/MeOH 90/10). <sup>1</sup>H NMR (CDCl<sub>3</sub>): 9.51 (1H, s, NH), 6.38 (1H, dd, H1'), 5.42 (1H, m, H3'), 4.83 (1H, m, H5'), 4.44-4.36 (2H, m, H4', H5''), 2.97 (1H, m, H2''), 2.38 (1H, m, H2'), 2.32 (2H, s, NHAc).

**2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-7,8-dihydro-N<sup>2</sup>-acetylguanosin-8-one (9).**

2'-Deoxy-7,8-dihydro-N<sup>2</sup>-acetylguanosin-8-one (**8**) (0.33 g, 1.01 mmol), dimethoxytritylchloride (0.704 g, 2.1 mmol), and DMAP (0.08 g, 0.65 mmol) were dissolved in pyridine (5 ml) and triethylamine (0.50 ml). After 12 hr at room temperature, the reaction mixture was concentrated to dryness. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (50 ml) and washed with NaHCO<sub>3(sat)</sub> (20 ml) and NaCl<sub>(sat)</sub> (20 ml). The organic solution was dried with NaSO<sub>4</sub>, filtered and concentrated. The 2'-deoxy-5'-O-(4,4'-dimethoxytrityl)-7,8-dihydro-N<sup>2</sup>-acetylguanosin-8-one (**9**) was purified by silica gel chromatography by eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (92:8) (0.70 mmol, 69 % yield) (TLC rf 0.52 CH<sub>2</sub>Cl<sub>2</sub>/MeOH 90:10) <sup>1</sup>H NMR (CDCl<sub>3</sub>/CD<sub>3</sub>OD): 7.45-7.14 (9H, m, Ar), 6.80-6.74 (4H, m, Ar), 6.64 (1H, dd, H1'), 4.65 (1H, m, H3'), 3.99 (1H, m, H4'), 3.85 (6H, s, DMTOCH<sub>3</sub>), 3.38-3.30 (2H, m, H5' and H5''), 3.14 (1H, m, H2''), 2.19 (1H, m, H2'), 2.03 (3H, s, NHAc). <sup>13</sup>C (CDCl<sub>3</sub>): 173.4, 158.1, 144.9, 135.9, 151.6, 146.9, 145.1, 129.9, 129.8, 127.9, 126.6, 113.0, 103.7, 85.8, 85.4, 81.1, 71.2, 65.0, 55.1, 36.2, 23.8.

**2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-7,8-dihydro-N<sup>2</sup>-acetylguanosin-8-one 3'-succinate p-nitrophenyl ester (10).**

2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-7,8-dihydro-N<sup>2</sup>-acetylguanosin-8-one (**9**) (0.45 g, 0.719 mmol), succinic anhydride (0.386g, 3.86 mmol, 540 M%), and dimethylaminopyridine (0.136 g, 1.11 mmol, 155 M%) were dissolved in pyridine (5 ml) and triethylamine (1.0 ml). After 13 hrs at room temperature, p-nitrophenol (0.50 g, 3.59 mmol, 500 M%) and diisopropylcarbodiimide (0.600 ml, 3.84 mmol, 530 M%). After 2.5 hr, p-nitrophenol (0.22 g, 1.59 mmol, 220 M%) and DIC (0.400 ml, 2.56 mmol, 355 M%) were added. After an additional 5.5 hr, the reaction mixture was concentrated to dryness. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (50 ml) and washed with NaHCO<sub>3(sat)</sub> (20 ml) and NaCl<sub>(sat)</sub> (20 ml). The organic solution was dried with NaSO<sub>4</sub>, filtered and concentrated. The residue was suspended in CH<sub>2</sub>Cl<sub>2</sub> and the diisopropylurea was filtered. The resulting solution was used without purification.

**2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-7,8-dihydro-N<sup>2</sup>-acetylguanosin-8-one 3'-succinate-LCA resin (11).**

2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-7,8-dihydro-N<sup>2</sup>-acetylguanosin-8-one 3'-succinate p-nitrophenyl ester (**10**) was added to long chain amine (LCA) control pore glass (2.00 g) suspended in pyridine (5 ml). After 4 hr, the reaction mixture was filtered, washed with DMF, MeOH, and CH<sub>2</sub>Cl<sub>2</sub>, and then suspended in pyridine (5 ml) and treated with acetic anhydride (1.0 ml) and DMAP (0.51g). After 2 hrs, the reaction was filtered and washed

with DMF, MeOH, and CH<sub>2</sub>Cl<sub>2</sub>. Dimethoxytrityl quantification was done by treating a portion of the resin with 70% HClO<sub>4</sub> (5.14 ml) dissolved in MeOH (4.6 ml) and measuring the absorbance at 498 nm. The loading was determined by using the relationship: bound dG<sup>8-oxo</sup> μmol/g = A<sup>498 nm</sup> x 14.3 [volume HClO<sub>4</sub> (ml)/ mg resin]. The loading was determined to be 4 to 5 μmol/g.

**2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-7,8-dihydro-N<sup>2</sup>-acetylguanosin-8-one 3'-[(2-cyanoethyl) N,N-diisopropylphosphoramidite] (12).**

2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (400 μl) and diisopropylethylamine (400 μl) were added to 2'-deoxy-5'-O-(4,4'-dimethoxytrityl)-7,8-dihydro-N<sup>2</sup>-acetylguanosin-8-one (**9**) (0.745 g, 1.17 mmol) dissolved in THF (5 ml) and CH<sub>2</sub>Cl<sub>2</sub> (5 ml). After 1 hr, 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (400 μl) was added. After 2 hr, the reaction was concentrated and the 2'-deoxy-5'-O-(4,4'-dimethoxytrityl)-7,8-dihydro-N<sup>2</sup>-acetylguanosin-8-one 3'-[(2-cyanoethyl) N,N-diisopropylphosphoramidite] (**12**) was purified by silica gel chromatography (20 g) eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (96:4). (TLC rf = 0.29 MeOH/ CH<sub>2</sub>Cl<sub>2</sub> 97:3).

**5-Hydroxy-2'-deoxyuridine (13).**

A bromine saturated aqueous solution (150 ml) was added to deoxyuridine (5.29 g, 23.2 mmol) dissolved in 50 ml H<sub>2</sub>O until a persistent yellow color developed. After 10 min, the excess bromine was quenched with Na<sub>2</sub>SO<sub>3</sub> until the reaction mixture became clear and



colorless. The aqueous solution was then poured into pyridine (200 ml) chilled to 0 °C. The ice bath was removed after 10 min, and the reaction mixture was allowed to stir at room temperature. After stirring overnight, the solvents were removed under vacuum in a rotary evaporator. The residue was redissolved in H<sub>2</sub>O (50 ml) and washed with CH<sub>2</sub>Cl<sub>2</sub> (2 x 50 ml). The aqueous solution was concentrated and the resulting viscous syrup was purified by silica gel chromatography by eluting with 20 to 80% MeOH/CH<sub>2</sub>Cl<sub>2</sub>. The slightly yellow solid obtained was crystallized from MeOH at 4 °C. The yield was determined to be 41% (2.26 g, 9.28 mmol). <sup>1</sup>H (CD<sub>3</sub>OD): 7.45 (1H, s, H6), 6.30 (1H, t, H1'), 4.37 (1H, d, H3'), 3.88 (1H, d, H4'), 3.74 (2H, m, H5' and H5''), 2.19 (2H, m, H2' and H2'').

**5'-O-(4,4'-Dimethoxytrityl)-5-hydroxy-2'-deoxyuridine (14).**

5-Hydroxy-2'-deoxyuridine (**13**) (1.08g 4.43 mmol) and dimethoxytritylchloride (2.25g 6.64 mmol) were dissolved in pyridine (20 ml) and stirred at room temperature. After 4 hr, the solvent was removed in vacuo, and the residue redissolved in CH<sub>2</sub>Cl<sub>2</sub> (50 ml) and washed with NaHCO<sub>3(sat)</sub> (20 ml) and NaCl<sub>(sat)</sub> (20 ml). The organic solution was dried with NaSO<sub>4</sub>, filtered and concentrated. The residue was purified by silica gel chromatography by eluting with 4% MeOH/CH<sub>2</sub>Cl<sub>2</sub> to yield 1.44g 2.59 mmol of 5'-O-(4,4'-dimethoxytrityl)-5-hydroxy-2'-deoxyuridine (**14**). (TLC rf 0.15 CH<sub>2</sub>Cl<sub>2</sub>/MeOH 90:10) <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.90 (1H, s, H6), 7.40-7.20 (9H, m, DMT), 6.83 (4H, d, DMT), 6.36 (1H, t, H1'), 4.60 (1H, br, H3'), 4.06 (1H, br, H4'), 3.80 (6H, s, DMT-OCH<sub>3</sub>), 3.34 (2H, s, H5' and H5''),

2.50-2.27 (2H, m, H2' and H2''). HRMS calculated for  $C_{30}H_{30}N_2O_8$  (M+) 546.2002, found 546.1999.

**5'-O-(4,4'-dimethoxytrityl)-5-acetoxy-2'-deoxyuridine (15).**

Acetic anhydride (200  $\mu$ l, 2.12 mmol, 120 M%) was added to 5'-O-(4,4'-dimethoxytrityl)-5-hydroxy-2'-deoxyuridine (**14**) (0.97 g, 1.74 mmol) dissolved in pyridine (10 ml). After 1.5 hr at room temperature, the reaction mixture was concentrated and the residue was redissolved in  $CH_2Cl_2$  (100 ml) and washed  $NaHCO_{3(sat)}$  (40 ml) and  $NaCl_{(sat)}$  (40 ml). The organic solution was dried with  $Na_2SO_4$ , filtered and concentrated. The 5'-O-(4,4'-dimethoxytrityl)-5-acetoxy-2'-deoxyuridine (**15**) was purified by silica gel chromatography (25 g) by eluting with 4 % MeOH/ $CH_2Cl_2$ . (TLC rf 0.35  $CH_2Cl_2$ /MeOH 95:5)  $^1H$  NMR ( $CDCl_3$ ): 7.90 (1H, s, H6), 7.40-7.20 (9H, m, DMT), 6.83 (4H, d, DMT), 6.36 (1H, t, H1'), 4.60 (1H, br, H3'), 4.06 (1H, br, H4'), 3.80 (6H, s, DMT- $OCH_3$ ), 3.34 (2H, s, H5' and H5''), 2.50-2.27 (2H, m, H2' and H2''), 1.89 (3H, s, Ac).  $^{13}C$  NMR ( $CDCl_3$ ): 168.26, 158.61, 158.12, 149.31, 149.29, 144.29, 135.47, 135.29, 131.23, 129.94, 129.83, 128.04, 127.96, 127.92, 127.59, 127.04, 123.82, 113.33, 87.06 (RO-OC-(Ph)<sub>3</sub>), 86.39 (C1'), 85.29 (C4'), 72.08 (C3'), 63.36 (C5'), 55.23 (DMT- $OCH_3$ ), 41.35 (C2'), 19.83 (Ac). HRMS calculated for  $C_{32}H_{32}N_2O_9$  (M+) 588.21078, found 588.2108.

**5-Acetoxy-2'-deoxyuridine (16).**

Acetic anhydride (60  $\mu$ l, 0.64 mmol, 100 M%) was added to 5'-O-(4,4'-dimethoxytrityl)-5-hydroxy-2'-deoxyuridine (**14**) (0.35 g, 0.63 mmol) dissolved in pyridine (5 ml). After 1.5 hr at room temperature, the reaction mixture was concentrated and the residue redissolved in  $\text{CH}_2\text{Cl}_2$  (5 ml). Three successive aliquots of a trichloroacetic acid solution (200  $\mu$ l, 200  $\mu$ l, and 1.0 ml, respectively) (ABI reagent) were added at 15 min intervals before complete deprotection of the dimethoxytrityl group occurred. The organic solution was concentrated to dryness *in vacuo*. The 5-acetoxy-2'-deoxyuridine (**16**) was purified by silica gel chromatography (10 g) eluting with 8 % MeOH/ $\text{CH}_2\text{Cl}_2$ . (0.15 g, 0.52 mmol, 83% yield) (TLC rf 0.34  $\text{CH}_2\text{Cl}_2$ /MeOH 90:10)  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 8.02 (1H, s, H6), 6.19 (1H, t, H1'), 4.34 (1H, d, H3'), 3.88 (1H, d, H4'), 3.72 (2H, m, H5' and H5''), 2.26 (1H, m, H2'), 2.18 (3H, s, Ac), 2.14 (1H, m, H2'').

**5'-O-(4,4'-Dimethoxytrityl)-5-acetoxy-2'-deoxyuridine 3'-[(2-cyanoethyl) N,N-diisopropylphosphoramidite] (17).**

2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (35  $\mu$ l) and triethylamine (35  $\mu$ l) are added to 5'-O-(4,4'-dimethoxytrityl)-5-acetoxy-2'-deoxyuridine (**15**) dissolved in  $\text{CH}_2\text{Cl}_2$ . After 1.5 hr, the reaction was purified by silica gel chromatography (8 g) eluting with 2 % MeOH/ $\text{CH}_2\text{Cl}_2$  and trace triethylamine. (85 mg, 0.110 mmol, 91% yield).

## 5'-d(G<sup>oh5</sup>UAGC)

The oligodeoxynucleotide sequence of d(G<sup>oh5</sup>UAGC) was synthesized on a Applied Biosystems DNA synthesizer by using the modified phosphoramidite (17). The modified trimer sequence, d(AGC), was accomplished by interrupting the normal oligonucleotide preparation prior to addition of the modified phosphoramite (17). The modified phosphoramidite was then added manually. The oligonucleotide synthesis was continued at the wash step in the standard synthesis program and the final phosphoramidite added. The coupling efficiency of the modified phosphoramidite was determined to be 65 % (not optimized) as determined by dimethoxytrityl quantification. The oligodeoxynucleotides were deprotected in concentrated aqueous ammonia overnight at 60 °C and subsequently purified by RP-HPLC.

The base composition of the oligonucleotide was determined by reversed phase HPLC after digestion of the compounds with bacterial alkaline phosphatase and snake venom phosphodiesterase. Peak area analysis of the chromatogram obtained from the adducted molecule, 5'-d(G<sup>oh5</sup>UAGC) indicated that this compound contained dC, dG, and dT in the same relative proportions as d(GCAGC), synthesized and purified at the same time as a control. Unlike the latter oligonucleotide, however, the adducted pentamer contained a compound with identical retention time and UV spectrum to a oh<sup>5</sup>dU standard.

### **5-Hydroxy-2'-deoxycytidine (18).**

A bromine saturated aqueous solution (150 ml) was added to deoxycytidine (5.29 g, 23.2 mmol) dissolved in 50 ml H<sub>2</sub>O until a persistent yellow color developed. After 10 min, the excess Br<sub>2</sub> was quenched with Na<sub>2</sub>SO<sub>3</sub> until the reaction mixture became clear and colorless. The aqueous solution was then poured into pyridine (200 ml) chilled to 0 °C. The ice bath removed after 10 min, and the reaction mixture was allowed to stir at room temperature. After stirring overnight, the solvents were removed under vacuum in a rotary evaporator. The residue was redissolved in H<sub>2</sub>O (50 ml) and washed with CH<sub>2</sub>Cl<sub>2</sub> (2 x 50 ml). The aqueous solution was concentrated and the resulting viscous syrup was purified by silica gel chromatography by eluting with 20 to 80% MeOH/CH<sub>2</sub>Cl<sub>2</sub>. The slightly yellow solid obtained was crystallized from MeOH at 4 °C. The yield was determined to be 41% (2.26 g, 9.28 mmol). <sup>1</sup>H (CD<sub>3</sub>OD): 8.62 (1H, s, H6), 6.20 (1H, t, H1'), 4.39 (1H, d, H3'), 3.98 (1H, d, H4'), 3.79 (2H, m, H5' and H5''), 2.19 (1H, m, H2'), 2.10 (1H, m H2'').

### **3',5',5-Triacetyl-2'-deoxyuridin-5-one (19).**

Acetic anhydride (4.00 ml, 42 mmol) and dimethylaminopyridine (35 mg, 0.28 mmol) were added to 5-hydroxy-2'-deoxyuridine (**13**) dissolved in pyridine (40 ml). After 5 hr at room temperature, the reaction mixture was concentrated, and the residue was redissolved in CH<sub>2</sub>Cl<sub>2</sub> (100 ml) and washed with H<sub>2</sub>O (50 ml), NaHCO<sub>3(sat)</sub> (50 ml), and NaCl<sub>(sat)</sub> (50 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The 3',5',5-triacetyl-2'-deoxyuridin-5-one (**19**) was purified by silica gel chromatography (40 g) by

eluting with 4 % MeOH/CH<sub>2</sub>Cl<sub>2</sub>. (rf 0.65 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 95:5) <sup>1</sup>H NMR (CDCl<sub>3</sub>): 9.13 (1H, br, NH), 7.52 (1H, s, H6), 6.35 (1H, t, H1'), 5.18 (1H, d, H3'), 4.4-4.2 (3H, m, H4', H5'), 2.54 (1H, m, H2'), 2.28 (3H, s, Ac), 2.15 (1H, m, H2'), 2.10 (3H, s, Ac), 2.08 (3H, s, Ac). HRMS calculated for C<sub>15</sub>H<sub>18</sub>N<sub>2</sub>O<sub>9</sub> (M+) 370.10123, high resolution result for m/z 370 (370.1010)

**3',5',5-Triacetyl-4-O-(2,4,6-trimethylphenyl)-2'-deoxyuridin-5-one (20).**

Triisopropylsulfonyl chloride (2.00g, 6.62 mmol, 180 M%) and triethylamine (2.00 ml, 14.38 mmol, 390 M%) were added to 3',5',5-triacetyl-2'-deoxyuridin-5-one (**19**) (1.37 g, 3.70 mmol) dissolved in CH<sub>2</sub>Cl<sub>2</sub> (12 ml). After 1.5 hr, additional triisopropylsulfonyl chloride (2.00g, 6.62 mmol, 180 M%) was added. After 6 hr, trimethylphenol (3.00 g, 22.06 mmol, 600 M%) and trimethylamine (approximately 2 ml). After 20 min, the reaction mixture was concentrated to dryness, and the residue was redissolved in CH<sub>2</sub>Cl<sub>2</sub> (100 ml) and washed with NaHCO<sub>3(sat)</sub> (50 ml), and NaCl<sub>(sat)</sub> (50 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The 3',5',5-triacetyl-4-O-(2,4,6-trimethylphenyl)-2'-deoxyuridin-5-one (**20**) was purified by silica gel chromatography (100 g) eluting with 2 % MeOH/CH<sub>2</sub>Cl<sub>2</sub>. (1.00 g, 2.05 mmol, 55 M%). (TLC rf 0.44 CH<sub>2</sub>Cl<sub>2</sub>/MeOH 98:2) <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.79 (1H, s, H6), 6.80 (2H, s, Ar), 6.18 (1H, dd, H1'), 5.18 (1H, m, H3'), 4.33-4.28 (3H, m, H4', H5', H5"), 2.74 (1H, m, H2'), 2.28 (3H, s, OAc), 2.21 (3H, s, OAc), 2.14 (1H, m, H2"), 2.06 (6H, s, ArCH<sub>3</sub>), 2.02 (3H, s, ArCH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 170.38, 170, 26, 168.64, 163.93, 153.70, 146.37, 135.61, 134.37, 129.62, 129.38, 121.94, 87.28, 83.02, 74.17, 63.73, 38.89, 20.87, 20.74, 20.67,

20.23, 12.28. HRMS calculated for  $C_{24}H_{28}N_2O_9$  ( $M^+$ ) 488.1795, high resolution result for  $m/z$  488 (488.1797)

**4-O-(2,4,6-trimethylphenyl)-2'-deoxyuridin-5-one (21).**

Ammonia saturated MeOH (5 ml) was added to 3',5',5-triacetyl-4-O-(2,4,6-trimethylphenyl)-2'-deoxyuridin-5-one (0.75 g, 1.53 mmol) dissolved in MeOH (5 ml). After stirring overnight at room temperature, the reaction was concentrated to dryness. The 4-O-(2,4,6-trimethylphenyl)-2'-deoxyuridin-5-one (**21**) was purified by silica gel chromatography (40 g) by eluting with 8 % MeOH/ $CH_2Cl_2$ . (0.55 g, 1.51 mmol, 100 % yield) (rf 0.36  $CH_2Cl_2$ /MeOH 90:10).  $^1H$  NMR ( $CD_3OD$ ): 8.04 (1H, s, H6), 6.80 (1H, s, Ph H), 6.22 (1H, t, H1'), 4.38 (1H, m, H3'), 3.98 (1H, m, H4'), 3.78 (2H, m, H5'), 2.40 (1H, m, H2'), 2.25 (3H, s, p-Ph- $CH_3$ ), 2.1 (1H, m, H2'), 2.08 (6H, s, o-Ph- $CH_3$ ). HRMS calculated for  $C_{18}H_{22}N_2O_6$  ( $M^+$ ) 362.14779, high resolution result for  $m/z$  362 (362.1477)

### III. FATAL ENGINEERING<sup>4</sup>

#### A. Introduction

The following sections of this dissertation describe the design, synthesis and preliminary biological results obtained during the development a new class of compounds that hopefully will prove useful for the treatment of cancer. The candidate chemotherapeutic agents have been designed to exploit biochemical differences between tumor and normal cells with the ultimate aim of selectively increasing the cytotoxicity for the tumor cell population. The new compounds consist of a chemically reactive DNA damaging domain linked to a protein recognition group. By choosing a protein recognition domain that attracts proteins uniquely or selectively expressed in tumor cells, we postulate that it will be possible to block the repair of a cytotoxic adduct selectively in those cells, sensitizing them to the genotoxic agent. A key feature of this strategy is the ability to control the specificity and affinity of the molecular recognition groups in these drugs. We have termed this strategy for the design of programmable drugs "Fatal Engineering."

My efforts as part of the Essigmann laboratory to develop and validate this new strategy for the treatment of cancer is described below. The first section establishes the background and basis for a drug design strategy based on the inhibition of DNA repair as an

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<sup>4</sup> Portions of this section have been taken *verbatim* from Chen, Z.-H., Morningstar, M.L., Croy, R.G., and Essigmann, J. M. (1993) Fatal Engineering: A new strategy for the rational design of antitumor drugs. *MIT Technology Disclosure*, August 16, 1993.



effective means to target tumor cells. The second section examines in detail the DNA damaging domains and protein recognition groups utilized in the developed compounds. The third section details the method of synthesis and any biological results thus far obtained on the designed compounds.

## **B. Background and Basis of Fatal Engineering**

Drugs that damage DNA are important therapeutic tools used in cancer treatment (Holland and Frei, 1982). Damage inflicted on the genomes of cancer cells can destroy genetic information essential for their growth and survival. The toxic effects of such drugs unfortunately also affects normal, non-cancerous cells. As a result severe dose-limiting toxicity can arise. The ability of cancer cells to become drug resistant also limits the efficacy of many chemotherapeutic agents used to treat human malignancies (Holland and Frei, 1982). The mechanisms by which a tumor cell becomes resistant to chemotherapeutic agents include diminished drug uptake, changes in drug metabolism and, in the case of agents that act by damaging DNA, enhanced DNA repair. One approach to increase the therapeutic effectiveness of DNA damaging compounds is to prevent abnormal cells selectively from repairing and recovering from genetic damage as a consequence of a DNA damaging agent. We are attempting to exploit the interaction of cellular proteins with DNA lesions as a means to achieve this objective. The rationale for this study comes from two independent biochemical observations: a) DNA repair rates affect the toxicity of a DNA damaging agent; and b) cellular proteins can influence DNA repair rates by shielding the

DNA damaging agent after it has been allowed to react with the genome of a cell. These two observations are examined in detail in the following two sections.

**a. The rate of repair affects the toxicity of a DNA damaging agent**

The study of repair defective cell lines and cells from humans with inherited DNA repair disorders demonstrates that diminished DNA repair rates enhance the toxicity of a DNA damaging agent. In patients with defects in the repair of certain types of DNA damage (two examples include Fanconi's anemia and Xeroderma pigmentosum), the lethality of DNA damaging drugs is substantially increased when compared to cells with normal abilities to repair DNA damage. Treatment of Fanconi's anemia cells, for instance, with drugs that crosslink DNA cause a ten-fold increase of genotoxic effects as compared to repair proficient cells (Ishida et al., 1982). The rate of repair of the damaged sites in DNA in Fanconi's anemia cells occurs at about one half the rate of normal cells. Thus, a reduction in the repair rate by a factor of two results in a ten-fold enhancement of lethality. Treatment of Fanconi's anemia and Xeroderma pigmentosum cells with cisplatin similarly causes a four fold increase in the sensitivity to the drug in both repair defective cell types (Dijit et al., 1988). Studies with repair deficient rodent cells also demonstrate that heightened sensitivity to DNA alkylating drugs correlates with the inability to repair DNA damage caused by bifunctional alkylating agents (Hoy et al., 1985). These results underscore the important role that DNA repair plays in cell survival and suggest that drugs that are capable of inhibiting repair processes can enhance lethality.

**b. Shielding of DNA adducts by proteins affects DNA repair**

The proposition that the shielding of DNA adducts increases the genotoxic effects of a DNA damaging agent comes from recent studies of the antitumor drug *cis*-diamminedichloroplatinum(II) (cisplatin). It is widely believed that cisplatin achieves antitumor activity by covalently binding to DNA, forming adducts that inhibit DNA polymerases (Bruhn et al., 1992). The principal adducts are the *cis*-[Pt(NH<sub>3</sub>)<sub>2</sub>{d(GpG)-N7(1),-N7(2)}] and *cis*-[Pt(NH<sub>3</sub>)<sub>2</sub>{d(ApG)-N7(1),-N7(2)}] intrastrand crosslinks (reviewed in Sherman and Lippard, 1987). The *cis*-[Pt(NH<sub>3</sub>)<sub>2</sub>{d(GpNpG)-N7(1),-N7(3)}] intrastrand crosslink, cisplatin interstrand crosslinks and monoadducts also form at a lower frequency. The genotoxicity of the cisplatin DNA adduct, *cis*-[Pt(NH<sub>3</sub>)<sub>2</sub>{d(GpG)-N7(1),-N7(2)}] was demonstrated in *E. coli* by Bradley et al. (1993). The survival of a constructed double stranded genome containing this adduct decreased to 22% as compared to an unplatinated control genome. A similar study of the same cisplatin lesion using a single stranded genome revealed that the same cisplatin lesion decreases survival to only 5% of a control (Yarema, 1994). These results indicate that as few as one DNA lesion can affect survival. It is not surprising, therefore, that the 1,000-10,000 cisplatin adducts per chromosome that form in cancer patients undergoing chemotherapy (Reed et al., 1993) are highly toxic to a tumor cell.

Recent findings indicate that binding by cellular proteins to the cisplatin-DNA adducts may increase the genotoxic effects of this drug for tumor cells. Specifically, a group of proteins has been identified that bind expressly to DNA containing the cisplatin *cis*-

[Pt(NH<sub>3</sub>)<sub>2</sub>{d(GpG)-N7(1),-N7(2)}] and the structurally similar *cis*-[Pt(NH<sub>3</sub>)<sub>2</sub>{d(ApG)-N7(1),-N7(2)}] intrastrand crosslinks (Donahue et al., 1990; Pil and Lippard 1992; Bruhn et al., 1992; Clugston et al., 1992; Hughes et al., 1992). A common DNA binding motif, the high mobility group (HMG) box has been revealed in most cisplatin binding proteins discovered so far. The HMG proteins, however, do not bind to DNA modified with the chemotherapeutically inactive *trans*-diamminedichloroplatinum(II) and monofunctionally coordinated platinum complexes. Recognition of the *cis*-[Pt(NH<sub>3</sub>)<sub>2</sub>{d(GpG)-N7(1),-N7(2)}] and the *cis*-[Pt(NH<sub>3</sub>)<sub>2</sub>{d(ApG)-N7(1),-N7(2)}] intrastrand crosslinks by HMG proteins results from a specific DNA conformation that arises as a consequence of cisplatin adducts in the DNA. These adducts cause unwinding of the DNA duplex by 13° and induce bending of 32-35° (Bellon et al., 1991). Based on the specific binding of protein to only the clinically active forms of cisplatin, Donahue et al. (1990) speculated that a protein-cisplatin adduct complex could be responsible for the clinical efficacy of cisplatin. Two mechanisms were proposed for the role of the protein complex in effecting the cytotoxicity of cisplatin: 1) the blocking of repair by the bound protein, and 2) the diversion of the bound protein from its normal cellular function. The first model would result in cisplatin lesions persisting in the genome of a cell and, as a consequence, DNA replication and transcription could be blocked. The second model would result in metabolic imbalances, especially if the bound protein were a crucial regulatory protein. Both models lead to eventual cell death. The models are not mutually exclusive.

Both models are supported by the work of Brown et al. (1993) and Treiber et al. (1994). Treiber et al. determined that the *cis*-[Pt(NH<sub>3</sub>)<sub>2</sub>{d(GpG)-N7(1),-N7(2)}] intrastrand

crosslink acts as a high affinity binding target for the human upstream binding transcription factor, hUBF. The binding of hUBF to the cisplatin adduct was proposed to disrupt the regulation of rRNA synthesis by titrating the hUBF from its normal binding site, the ribosomal DNA promoter. The binding of hUBF to the DNA lesion would have the additional effect of shielding the adduct from repair enzymes, in support of the first model.

In yeast, recent findings by Brown et al. (1993) provide the most direct evidence to date that the binding and possible shielding of cisplatin lesions from repair by HMG proteins can impact cell survival. These workers report that the elimination of one of four detected yeast proteins that bind to cisplatin-damaged DNA results in a two-fold decrease in the toxicity of cisplatin. This finding indicates that the binding of a protein presumably not responsible for the repair of cisplatin lesions significantly affects the toxicity of the drug.

### **C. The Fatal Engineering Concept**

The proposed mode of action of a Fatal Engineering compound is depicted in Figure 20. The initial step is covalent modification of DNA by the warhead portion of a Fatal Engineering compound. At this stage, both cancer and noncancerous cells would be damaged. In normal cells, the natural repair process would remove the lesion from DNA (Figure 20A), resulting in the diminished toxicity of the lesion. In contrast, in cancerous cells (Figure 20B), tumor specific proteins would associate with the molecular recognition domain of the DNA associated genotoxic compound. The complex formed between the Fatal Engineering compound and the tumor specific protein would, in principle, slow the

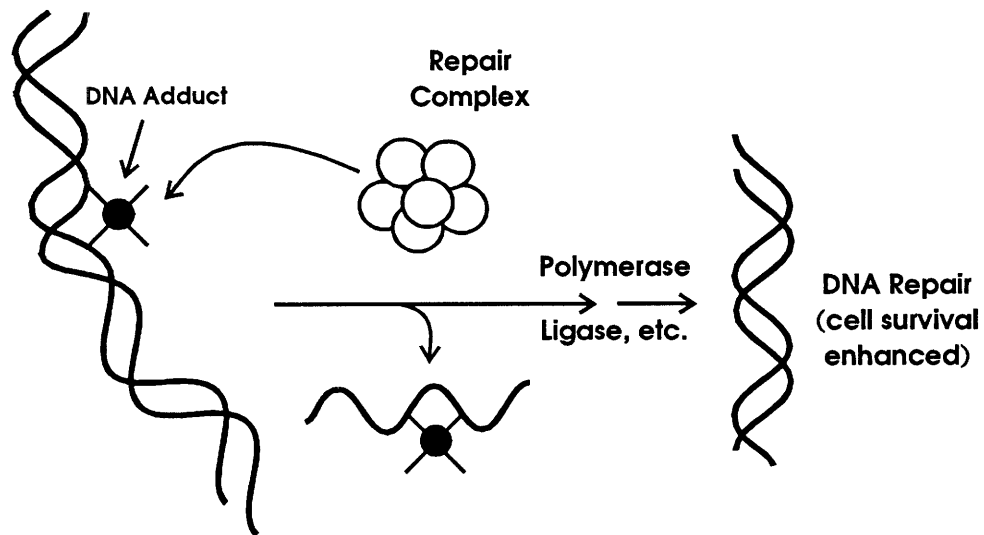
rate of repair of the lesion. The end result would be the higher relative toxicity of the drug in the cancer cell than in the noncancerous cell. Thus, a favorable therapeutic index would be achieved.

A key feature of Fatal Engineering is the ability to control the specificity and affinity of the protein recognition domain in these candidate drugs. This ability should allow us to exploit the different molecular changes that are characteristic of various malignancies, including the expression or overexpression of oncogene-encoded or suppressor gene-encoded proteins. Although our research has focused on developing a new cancer therapy, this approach could also be applied to any disease that results in the expression of foreign or unique proteins in the nucleus of a cell (e.g., viral infections, including that caused by human immunodeficiency virus). My contribution to this research has involved the design and synthesis of trifunctional Fatal Engineering compounds to validate this approach. The compounds that were made utilized either a psoralen or an aryl nitrogen mustard as cytotoxic warheads. The protein recognition domains that were used include: a ss DNA aptamer, biotin, and estradiol. The key features of each domain and the chemical properties of the molecules utilized are detailed in the following section.

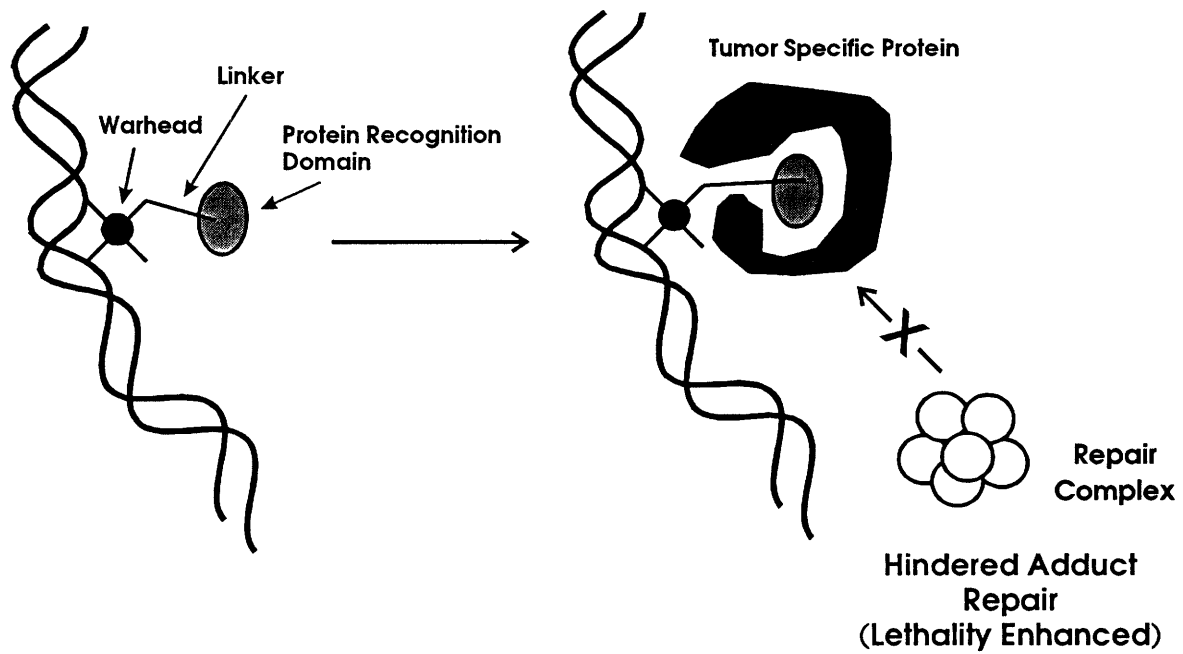
**Figure 20.** The conceptual basis for Fatal Engineering. Fatal Engineering attempts to exploit the differential repair of lethal DNA damage by the selective shielding of DNA damage. The specific shielding arises from the binding of proteins either selectively expressed or specifically present in abnormal cells to a complementary recognition domain of a Fatal Engineering compound. As a consequence of this shielding, the repair of the damaged segment of DNA is hindered resulting in increased genotoxicity.

A Fatal Engineering compound has three domains: 1) a DNA damaging domain or warhead; 2) a linker arm; and 3) a protein recognition domain. The DNA damaging domains so far explored as Fatal Engineering compounds are psoralen and an aryl nitrogen mustard. A ss DNA thrombin aptamer, biotin and estrogen have been tested as recognition domains for thrombin, streptavidin, and estradiol receptor proteins, respectively.

### A. Absence or Low Expression of Adduct Binding Protein



### B. Expression of Overexpression of Adduct Binding Protein





## **D. Design of Fatal Engineering Compounds**

### **a. DNA Damaging Domains**

The selection of the DNA warhead is limited to compounds that form repairable lesions. This requirement arises because the compounds under development exploit differential repair as a means to induce selective genotoxicity in tumor cells. Consequently, a chemical could be an effective warhead if: 1) the chemical can be activated by cellular metabolism or other means to a reactive species that covalently links the drug molecule to cellular DNA; and 2) the DNA adduct thus formed can be efficiently repaired by cellular enzymes. Fortunately, a large number of DNA damaging agents exist that meet these criteria (Holland and Frei, 1982; Brednel et al., 1984; Nielsen et al., 1991).

We postulated that by comparing the effects of two differing mechanisms for DNA adduct formation, we could determine which factors, such as reactivity, sequence selectivity and stability are important determinants of therapeutic efficacy. The two DNA damaging agents investigated as part of a Fatal Engineering compound were trimethylpsoralen and an aryl nitrogen mustard. The general chemical properties of these two warheads are discussed in detail in the following two sections.

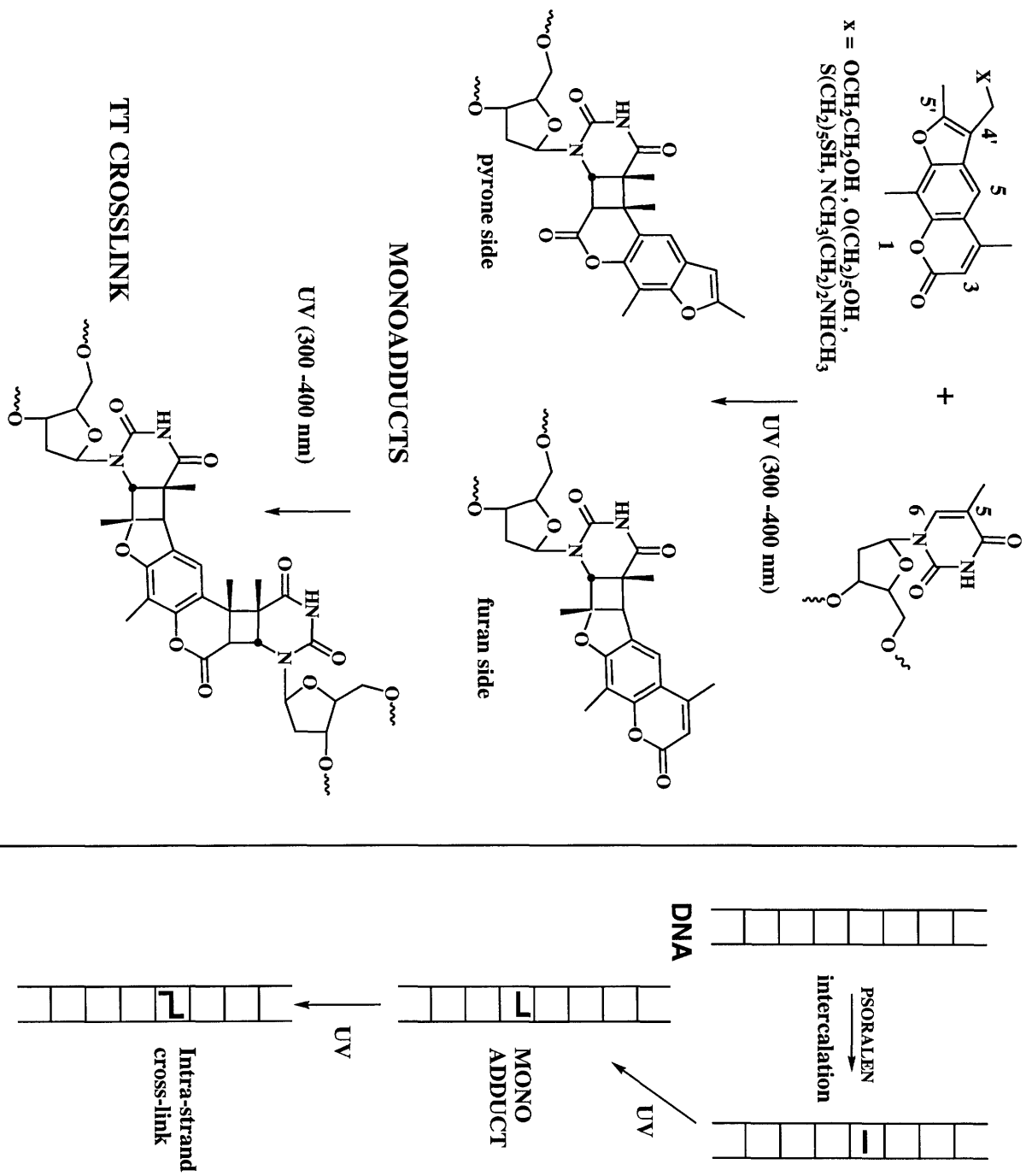
#### **i. Psoralen**

Psoralens are bifunctional photoreagents that form covalent bonds with the pyrimidine bases of nucleic acids in the presence of near UV light (Song et al., 1979; Hearst et al.,

1984). In Figure 21, the specific chemical reactions involved are illustrated along with a macroscopic view of the crosslinking process. The mechanism of the photoaddition of a psoralen to a DNA helix involves several steps. The initial step is the intercalation of the psoralen into the nucleic acid helix in the absence of near UV light. The necessity for intercalation before photochemical reactivity forbids the formation of intrastrand crosslinking. The intercalation step is reversible and normally does not lead to stable DNA lesions. When an intercalated psoralen absorbs a photon of wavelength between 300 and 400 nm, it is sensitized to react by cycloaddition at either the 3,4 double bond of the pyrone ring or the 4',5' double bond of the furan ring. If an adjacent pyrimidine is correctly positioned, a cyclobutane ring results from reaction with the 5,6 double bond of the pyrimidine (Straub et al., 1981; Kanne et al., 1982). Subsequent formation of a DNA crosslink to the opposite strand of DNA occurs only if two conditions are met (Hearst, 1989). First, the monoadduct formed must be a furan-side monoadduct. Second, a pyrimidine must be adjacent to the psoralen monoadduct on the opposite strand. The resulting mono- and crosslinked adducts are chemically stable (Cimino et al., 1985) and can result in inhibition of DNA replication and cellular proliferation (Cimino et al., 1985; Ben-Hur et al., 1984).

The molecular mechanism involved in the removal of the psoralen damage is fairly well understood (Brendel and Ruhland, 1984). Repair of psoralen monoadducts occurs via an excision repair pathway whereas repair of interstrand crosslinks occurs by a combination of excision and recombination (Cole et al., 1978; Cole and Sinden 1975). Although a crosslink is more effective in cell killing, the monofunctional adducts also result in genotoxicity (Cole

**Figure 21.** Mechanism of the photoreaction of psoralen with DNA. The first step is a thermal reaction involving the intercalation of the psoralen in the helix. In the second step, a cyclobutane ring can form with the pyrone or furan sides of psoralen after the absorption of a photon. The third step is the formation of the second cyclobutane ring upon absorption of a second photon. Covalent crosslinking between two strands of the DNA helix only occurs if the first cyclobutane ring formed is between the furan side of the psoralen.



et al., 1978). The repair of psoralen damage, however, does not occur in cells deficient in DNA repair (e.g., Fanconi's anemia and Xeroderma pigmentosum) (Brendel and Ruhland, 1984). In the absence of repair, these cells are rapidly killed. In fact, in a bacterial double-mutant *uvrArep*, a single crosslink on average results in cell killing (Bridges, 1983). Therefore, a psoralen DNA damaging domain would fulfill the primary requirement of a Fatal Engineering warhead - the formation of a repairable lethal DNA lesion.

## **ii. Nitrogen Mustards**

The other warhead utilized in our experiments was an aryl nitrogen mustard. All mustards contain at least one 2-chloroethyl group attached to a nucleophilic sulfur or nitrogen atom. Sulfur and nitrogen mustards can be synthesized with up to two or three 2-chloroethyl groups, respectively. By varying the substituents attached to the sulfur or nitrogen, chemicals with different selectivity and/or chemical stability have been synthesized (Wilman and Connor, 1983). Mustards that have two or more reactive arms can covalently crosslink DNA. One advantage of the nitrogen mustards as compared to the sulfur mustards is that a third substituent other than 2-chloroethyl can be added while retaining the ability to crosslink DNA. Mustards, however, are generally non-selective in their attack on biological material, reacting with structural proteins, enzymes, and DNA (Warkwick 1963; Wheeler 1967). The mustards are primarily activated to react by the process of entering a cell due to the difference between intracellular and extracellular chloride ion concentration.

The major lesions resulting from alkylation of nucleic acids by nitrogen mustards form through their reaction with the N7 position of guanine (Figure 22). Less extensive

reaction occurs at the N1 position of cytosine and the O<sup>6</sup> position of guanine (Lawley, 1966; Lawley and Thatcher, 1970). Crosslinking of two strands of DNA commonly results with polyfunctional mustards through the N7 position of guanine in one strand of DNA and the N7 position of guanine in the other strand (Lawley, 1966) (Figure 22). For almost 30 years, this product was thought to arise from the sequence 5'-d(GC). The distance between N7 guanine atoms in this dinucleotide pair appeared to be optimal for reaction with both arms of the nitrogen mustard. Recently it has been demonstrated that the crosslink in fact arises from the sequence 5'-d(GpNpC) (Grueneberg et al., 1991; Hopkins et al., 1991). This finding was unexpected because the tether that links the N7 atoms of the guaninyl crosslink is several angstroms too short to accommodate the spacing of the N7 atoms in the normal duplex DNA sequence 5'-d(GpNpC). This result suggests that distortion of the double helix must accompany formation of such a crosslink.

The reaction mechanism of adduct formation by aryl nitrogen mustards has also been subjected to several revisions. A variety of mechanisms ( $S_N1$ ,  $S_N2$ , 'internal  $S_N2$ ') have been proposed (Wilman et al., 1983) (Figure 22). The currently accepted mechanism of reaction of aryl nitrogen mustards involves two competitive pathways (Benn et al., 1970). One pathway involves direct  $S_N2$  displacement of chloride by a nucleophile. The second pathway involves a reactive aziridinium ion intermediate. The aziridinium ion is preferred except in the case of very powerful nucleophiles. Because the aziridinium pathway of activation is reversible, the relatively high chloride concentration present in blood decreases the *in vivo* rate of alkylation by aryl nitrogen mustards. Inside cells, however, the chloride concentration decreases whereby both methods of activation occur. As a result, aryl

nitrogen mustards are useful chemotherapeutic agents because of their increased reactivity in the intracellular environment.

The ability to crosslink DNA appears to be essential to the effectiveness of many nitrogen mustards (Colvin, 1982; Hemminki et al., 1984; Kohn, 1981). Although the interstrand crosslink might be considered the most dangerous lesion, the removal of the intrastrand and monofunctional lesions appear to be equally important for cell survival (Hoy et al., 1985). As a consequence, the ability of a nitrogen mustard to form a crosslink in DNA does not appear to be a critical requirement for the purpose of designing a Fatal Engineering compound.

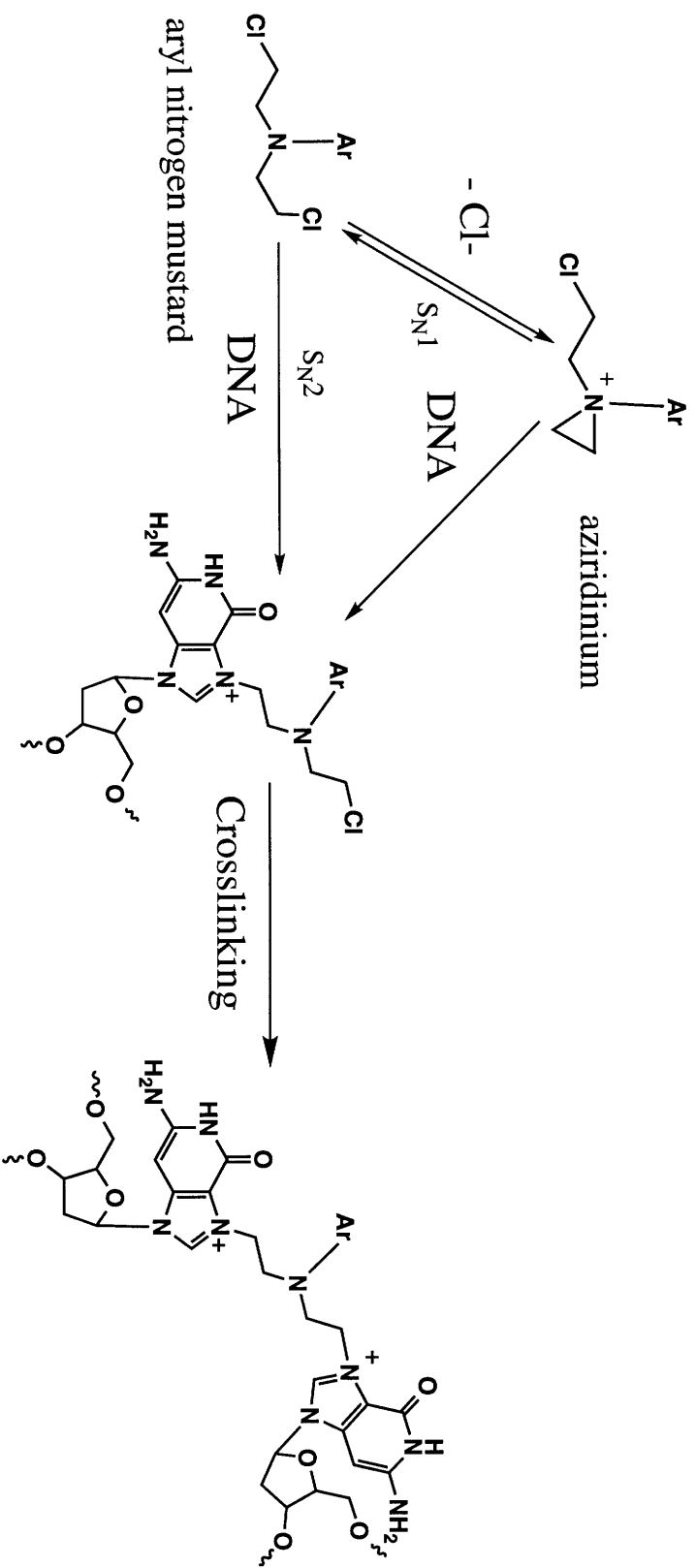
#### **b. Introduction to Protein Recognition Domains**

Three properties are likely to be necessary for a protein recognition domain to function effectively as part of a Fatal Engineering compound. The first property is that the protein recognition domain must selectively attract a tumor specific protein. The second property is that the protein-DNA adduct complex must be sufficiently stable to prevent displacement of the protein by repair enzymes. The third property is that the protein-DNA lesion must be sufficiently large to prevent access of DNA repair enzymes to the DNA lesion.

In order to determine the optimal parameters for these three factors, our initial studies examined the interaction of model proteins with known recognition domains. The synthesis

**Figure 22.** Mechanism of the crosslinking by aryl nitrogen mustards with DNA. Two competitive pathways of reaction result in reaction with DNA. One pathway involves direct  $S_N2$  displacement while the second pathway involves  $S_N1$  displacement and formation of a aziridinium species. Reaction with DNA can result in the alkylation of N7 of guanine. A common crosslink found with DNA occurs between guanines in the sequence 5'-d(GpNpC).





Mono Adducts

G,G cross-link

and study of compounds containing model protein recognition domains has allowed the *in vitro* testing and optimization of spacer arm length, warhead reactivity, solubility, stability, and - the crucial test - DNA repair. These initial findings have facilitated the design and development of a second generation of compounds to undergo biological evaluation. The following sections discuss some of the advantages of the three protein recognition domains that have been under study: a ss DNA aptamer, biotin, and estrogen.

#### **i. Aptamers**

The first recognition domain we examined as part of a Fatal Engineering compound was the anti-thrombin aptamer. The term "aptamer" is derived from the Latin word *aptus* meaning to fit. The term was introduced by Ellington and Szostak (1990) when they reported on the preparation of ss RNA molecules that bind with high affinity to organic dyes. Starting with a large random pool of oligonucleotides ( $10^{13}$  molecules), this technology selects for ss DNA or RNA that can bind specifically to, at least in theory, any target molecule. The selection cycle is a reiterative process involving isolation of DNA or RNA sequences that bind to the desired protein or target molecule and amplification of those sequences by polymerase chain reaction (PCR). Recently, these and other researchers have generated high affinity complexes between ss DNA or RNA and proteins such as thrombin (Bock et al., 1992) and U1-snRNP-A (Tsai et al., 1991), or small molecules such as tryptophan (Famulok et al., 1992).

One goal of ongoing research in our laboratory is the selection of an aptamer with high affinity for a mutated or overexpressed tumor encoded protein. A complex between such

an aptamer incorporated into a Fatal Engineering compound and its target protein could result in selective interference with repair of DNA lesions in tumor cells. As a test for this strategy, a Fatal Engineering compound was designed and synthesized using a known aptamer. The aptamer chosen was a ss DNA that bound to human thrombin which is admittedly a non-tumor specific protein. Our goal was to test for the ability of a thrombin aptamer linked to a DNA adduct to block repair *in vitro* in the presence and absence of thrombin.

## **ii. Biotin**

As a second method to test the concept of Fatal Engineering, two Fatal Engineering compounds were designed with biotin, a small molecule, as the protein recognition domain. Biotin is a prosthetic group that acts as a carrier of activated CO<sub>2</sub> for various enzymes that catalyze the fixation of CO<sub>2</sub> into cellular metabolites. Avidin is a tetrameric glycoprotein found in egg white that has exceptionally high affinity for biotin. Avidin or streptavidin, the unglycosylated form of avidin, binds one biotin molecule per subunit with a  $K_d = 10^{-15}$  M. This dissociation constant is five orders of magnitude smaller (i.e., tighter binding) than typical protein-nucleic and protein-protein interactions. As a consequence of this tight binding, the avidin-biotin system has provided the basis for a number of sensitive and specific detection methods (Green, 1975; Guesdon et al., 1979; Bayer and Wilchek, 1980). This ligand-protein recognition complex was of value to validate the Fatal Engineering approach. Although the resulting Fatal Engineering compound would have no activity in tumor cells since its molecular target, avidin, is not present in any human cells, the biotin-avidin system would allow us to examine *in vitro* whether avidin, a 60 KD protein, is

sufficient to block access to a DNA lesion by cellular repair enzymes. Furthermore, derivatives of biotin with differing affinities for avidin are known (Green, 1975) that would allow us to determine what affinity is required for a protein-DNA lesion to prevent DNA repair.

### **iii. Estrogen**

The high affinity of the estrogen receptor (ER) for estradiol and other estrogenic compounds served as our third ligand-protein recognition complex. In contrast with the biotin-avidin system, this ligand-protein combination would provide a clinically relevant model with which to test the Fatal Engineering strategy for drug design. Estradiol is the most potent of the naturally occurring estrogens. In mammals, endogenous estrogens are formed primarily by the ovaries, placenta, testes and adipose tissue acting to stimulate estrus, secondary sexual characteristics, and systemic effects such as growth and maturation of bones. Key to utilizing this system as part of a Fatal Engineering compound is that estrogens function *in vivo* by binding to a nuclear receptor protein. Proteins of this family of transcriptional regulators are activated by the binding of small lipophilic ligands such as steroid and thyroid hormones, retinoids and vitamin D3 (Green et al., 1988; Evans, 1988; Beato 1989; Gronemeyer, 1991). In the case of the ER, binding of estradiol which can freely diffuse across the cell membrane to the ER leads to ER dimerization and subsequent tight binding of the ER dimer to its specific DNA target, the estrogen responsive element (Parker, 1993; Gronemeyer, 1993; Baniahamad and Tsai, 1993). A DNA lesion that contained an estrogen recognition domain might be capable of interacting or "hijacking" the estrogen receptor resulting in the targeting of the protein to a DNA lesion. By exploiting

the differential expression of the ER in breast cancer patients described below, the designed compound could produce a DNA lesion with increased toxicity to breast cancer cells.

In breast cancer patients, the expression of estrogen receptor can vary significantly. Breast cancers having receptor levels greater than 10 fmol/mg protein are classified as estrogen receptor positive (ER+) and considered candidates for hormonal therapy (Pollow et al., 1990). The reported range of ER concentration in breast cancers varies between undetectable levels (ER-) to 4,000 fmol/mg protein (ER+). Of ER+ patients, 50-60% of the patients respond positively to endocrine therapy (DeSombre et al., 1979). Unfortunately, the patients with the highest ER levels (> 108 fmol/ mg), comprising 47% of the ER+ breast cancers do not respond to endocrine therapy. These ER+ patients have as poor a prognosis as ER- patients (Thorpe et al., 1993). Therefore, a drug that targeted the overexpression of ER as a means of enhanced genotoxicity would be an effective treatment for these unresponsive ER+ patients.

One very important advantage of this strategy beyond the ability target ER+ breast cancer cells is that systemic toxic side effects should be minimal. ER levels are very low in hematopoietic tissues such as the spleen and bone marrow. Consequently, a Fatal Engineering compound would be repaired in these cells. This finding means that the bone marrow toxicity that limits the dose of many anticancer drugs would not occur with an estrogen-Fatal Engineering compound. In terms of normal estrogen responsive tissues such as the uterus and ovaries, the mean concentration of ER in tumors in older patients (> 50 years of age) is two- to ten-fold greater. Therefore, a drug that targeted the overexpression

of ER as a means of enhanced genotoxicity would not adversely effect normal estrogen responsive tissues. Consequently, a compound that selectively utilized the overexpression of ER as a means to block the repair of DNA damage might have enhanced toxicity only in tumor cells.

Useful to the testing of the Fatal Engineering concept *in vivo* is the availability of cell lines that either overexpress or do not express the estrogen receptor. These cell lines can allow us to compare *in vivo* the toxic effects of our designed drugs in the presence and absence of ER. The effectiveness of the proposed drugs should not be affected by the possible abnormal function of the overexpressed ER. The Fatal Engineering strategy only requires that the overexpressed ER retain its ability to bind with estrogen tightly. By retaining the ability to bind with estrogen, the resulting protein-drug-DNA complex would function as the desired block to repair.

### **c. Introduction to Linker Arms**

The function of the linker arm is to connect the DNA warhead to the protein recognition domain. The ideal linker arm would function to balance the protection of the DNA lesion against the binding of each domain with their respective targets. Factors such as how far the protein recognition domain is presented away from the DNA helix are expected to effect the affinity of the protein for the protein recognition domain. If the linker arm is not of sufficient length, the DNA helix may adversely affect protein binding by steric hindrance or electrostatic repulsion and as a consequence the protein-drug-DNA

complex may not be of sufficient stability to block repair. In addition to an optimal distance for presenting the recognition domain, the spanning residues should be designed to increase the binding affinity of the tumor specific protein to the Fatal Engineering DNA lesion.

Other factors affecting the choice of linker include the ability to position correctly the protein recognition domain for subsequent binding by the tumor specific protein, stability to cellular enzymes, and the ability to help target our compounds to DNA. Preliminary studies using DNA models and molecular modeling have been used to guide us in the design of an optimal linker arm. Our present studies however have focused first on determining an ideal recognition domain and DNA warhead then developing an optimal linker. The following section details one approach we undertook to understand the constraints of the linker arm.

### **Sulfur-Mercury as a Method of Linking**

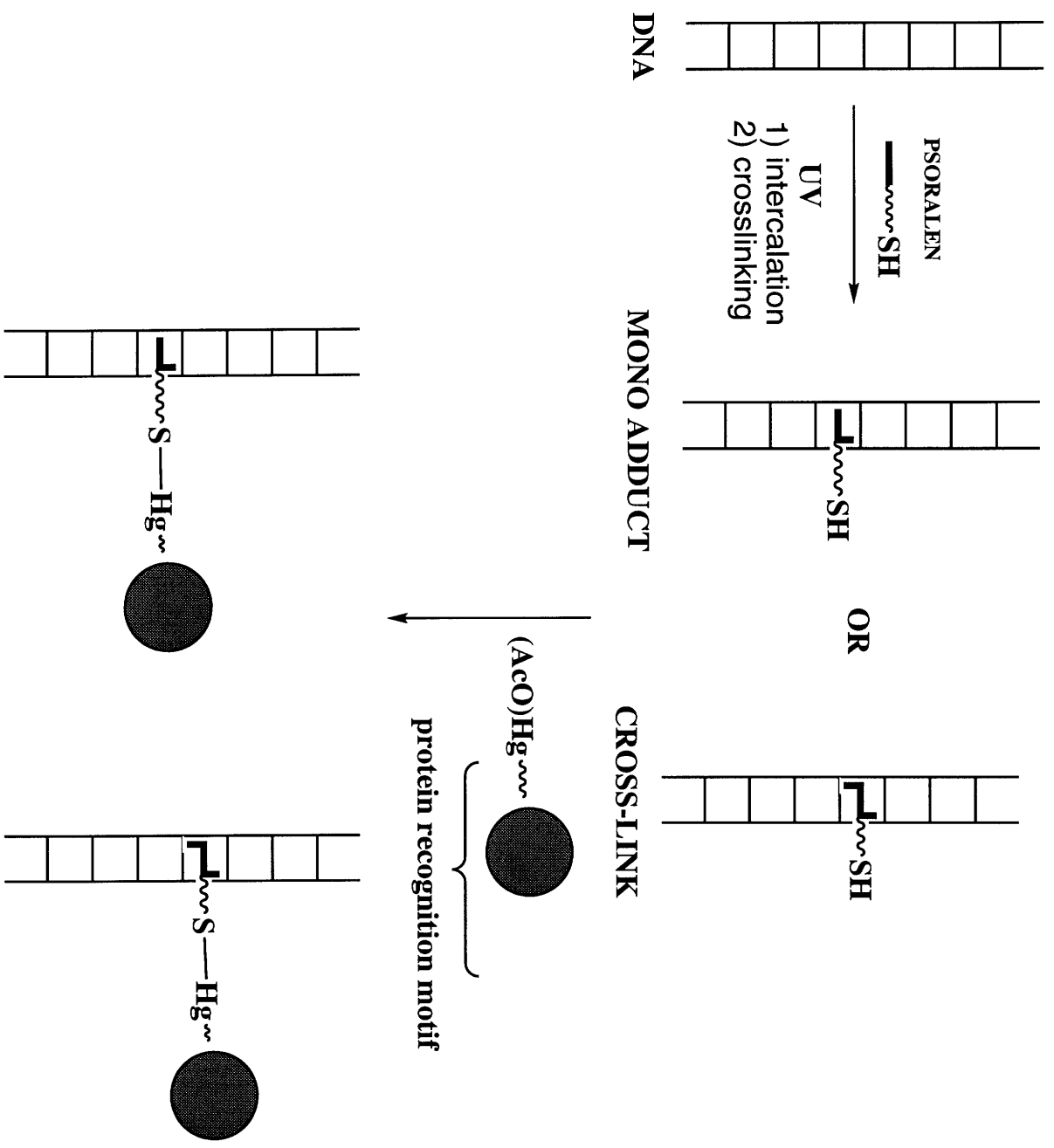
The approach we took to examine the linker requirements was to form a linker between a pre-existing DNA lesion and a protein recognition domain. If a covalent link could be formed between the warhead, already bound to DNA, and the recognition domain, the requirements such as length, positioning, and linker composition could be determined and later optimized. This modular synthetic approach would additionally allow the attachment and testing of different protein recognition domains attached to the same DNA adduct. As a result, the optimal recognition domain for a particular DNA lesion could more rapidly be joined and tested. The only requirement the DNA warhead would have in addition to the ability to damage DNA would be that it contained a functional arm that could later be used

to link to the protein recognition domain. The method studied as the chemical link involved a sulfur-mercury coupling chemistry.

This strategy was based on previous studies using the affinity of mercurated oligonucleotides for sulfur as a means of purification (Dale et al., 1975). Dale et al. showed that the introduction of as few as one mercury atom per 200 bases permits the selective and quantitative retention of the mercurated polymer probe on columns of sulfhydryl-agarose. In our work, a derivatized psoralen with a free thiol was chosen as the chemical warhead and a mercurated aptamer as the recognition domain. As depicted in Figure 23, the strategy involved 1) the photo-crosslinking of DNA with a thiol-psoralen, and 2) the attempted coupling of a mercurated aptamer recognition domain to the DNA lesion. The advantages of this strategy are threefold. First, a psoralen warhead would fulfill the requirement of a repairable lesion. Second, a variety of psoralens could be synthesized with different side chains that would allow us to determine the optimal length and chemical composition of the spacer arm needed to form a mercury sulfur couple. Third, the site of attachment of the mercury linkage to the aptamer could be changed, allowing us to optimize the site of coupling to the protein recognition domain. We postulated that the knowledge gained from this two step approach of forming a Fatal Engineering lesion could be subsequently used in the design and synthesis of a second generation of bifunctional Fatal Engineering compounds.



**Figure 23.** Sulfur-mercury as a method of coupling the recognition and DNA damaging domains together. The first step involved photo-crosslinking of a psoralen DNA damaging domain to ds DNA. The second step would involve coupling of a ss DNA aptamer to the psoralen lesion via a thiol side chain on the psoralen and a mercurated nucleotide specifically located in the DNA aptamer.



## **E. Design and Synthesis of Candidate Fatal Engineering Drugs**

### **a. Introduction**

The following sections describe in detail the design and synthesis of several DNA modifying compounds designed to block their own repair only in the presence of specific proteins. The compounds include a) one psoralen-ss DNA aptamer, b) three thiol-psoralens, c) two psoralen linked biotins, d) one psoralen linked estrogen, and e) three nitrogen mustard linked estrogens. Biological results, when available for a specific compound, are also presented.

### **b. Psoralen Linked Thrombin DNA Aptamer**

The recognition domain for the first Fatal Engineering compound utilized the unusual ability of some ss DNA or RNA molecules, aptamers, to bind selectively to proteins or small molecules. Our work attempted to exploit this ability as a means to cause a DNA adduct to interact selectively with a tumor specific protein. In the synthesized molecule, the DNA damaging warhead was psoralen, a repairable lesion. The resulting aptamer-psoralen was designed to form a ds DNA lesion that presented a recognition domain for subsequent protein binding (Figure 20). The Fatal Engineering effect would arise if repair were selectively blocked only in the presence of the protein complementary to the recognition domain. In order to evaluate this approach quickly, we started with a known aptamer-protein combination.

The earlier studies of Bock et al. (1992) had determined the necessary ss DNA sequence for binding to human thrombin. Sequence data from 32 thrombin aptamers (selected from an initial pool of approx  $10^{13}$  molecules) showed a highly conserved 14-17 base region. Several of the selected DNA sequences had binding affinities of  $K_d = 200$  nM for thrombin. In contrast, the original pool of DNA showed little affinity for thrombin. The sequence chosen for our oligonucleotide, 5'-CAG GTT GGT GTG GTT GGG-3', contains the consensus sequence determined by Bock et al. of GGT TGG (N)3 GGT TGG. In addition to the consensus sequence of Bock et al., a 5'-dinucleotide "linker," CpA, was added to our compound in an attempt to keep the attached DNA warhead from interfering with the aptamer structure.

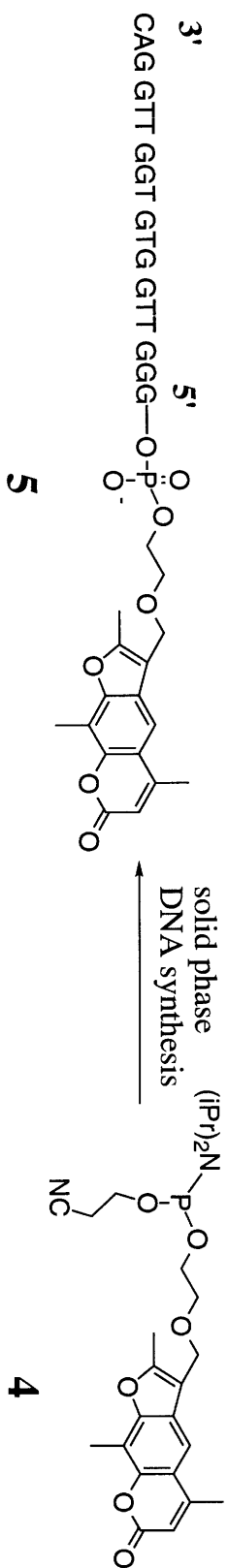
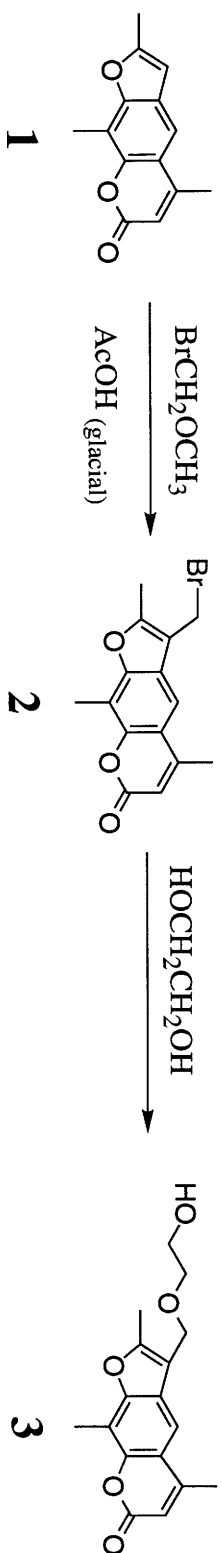
Part of the rationale behind the use of psoralen as the warhead for a DNA aptamer stems from the wealth of knowledge available on the synthesis of DNA with attached molecular reporters. A large number of oligonucleotides has been synthesized with attached intercalators, photoreactive and cleavage agents (Thuong et al., 1991; Uhlmann et al., 1990; Englisch et al., 1991). These DNA conjugates can result in site specific covalent modification of DNA if designed to act as a second strand in a DNA duplex or as a third strand in a triple helix. In the case of our compound, we sought to allow it to react randomly with ds DNA. Therefore, we intentionally designed our molecule not to have either of these means to react with DNA in a sequence-specific manner.

Synthesis of the first candidate Fatal Engineering drug was based on a literature method (Pieles et al., 1989). This procedure results in an oligonucleotide with a 5'-terminal

psoralen (**5**) (Figure 24). The starting material for the molecular warhead was 4,5',8-trimethylpsoralen (**1**). Side chains were appended onto the psoralen core via a literature procedure by Isaacs et al. (1977) involving chloro- or bromomethylation. The resulting 4'-chloro- or 4'-bromomethyl-4,5',8-trimethylpsoralens (**2**) are unstable and hydrolyze rapidly. The crude 4'-bromomethyl-4,5',8-trimethylpsoralen (**2**), if used immediately, reacts in high yields with a variety of sulfur, nitrogen, and oxygen nucleophiles (e.g., ethylene glycol, pentanedithiol, pentanediol, and dimethyl ethylene diamine). The linking arm of ethylene glycol was appended by heating the 4'-bromomethyl-4,5',8-trimethylpsoralen (**2**) in neat glycol. The resulting glycol-psoralen (**3**) was synthesized in greater than 80% yield for two steps. Next, the necessary phosphoramidite (**4**) was formed by reaction of the glycol-psoralen (**3**) with 2-cyanoethyl N,N-diisopropyl chlorophosphoramidite. The yields determined for each step did not vary from literature values.

The desired aptamer-psoralen (**5**) was synthesized by modified solid phase DNA synthesis. The synthesis procedure involved first preparing the 3' resin linked oligonucleotide, 5'-CAG GTT GGT GTG GTT GGG-3', by normal solid phase DNA synthesis (Gait 1984). The phosphoramidite (**4**) was then added by manual coupling. Manual coupling involved the removal of the column containing the resin bound oligonucleotide and manual injection of the phosphoramidite (**4**) in the presence of tetrazole / CH<sub>3</sub>CN. Following manual coupling, the column was placed again on the Applied Biosystems DNA synthesizer and subjected to an abbreviated coupling routine that did not include a trichloroacetic acid treatment. The oligonucleotide-psoralen was then cleaved from the resin and deprotected by heating the resin to 50-60 °C overnight in concentrated

**Figure 24.** Method of synthesis of thrombin aptamer. The phosphoramidite (4) necessary for solid phase DNA synthesis was synthesized in three steps from trimethylpsoralen occurring to the procedure of Pieleles et al. (1989).



ammonium hydroxide. The resin was filtered and the ammonia solution evaporated yielding crude aptamer-psoralen (5). The resulting oligonucleotide-psoralen (5) was purified by polyacrylamide gel electrophoresis by Z. Chen. The presence of the psoralen was determined by digestion of the DNA by snake venom phosphodiesterase I and alkaline phosphatase. The resulting aptamer digest was separated by HPLC and the presence of the psoralen verified by comparison to a known standard (Z. Chen, personal communication).

With the purified oligonucleotide-psoralen (5) in hand, the first question we sought to answer was whether the attachment of the psoralen to the 5' end of aptamer would interfere with the ability of the aptamer to interact with thrombin. Block et al. (1992) had shown previously that binding of a thrombin selected aptamer to thrombin slowed the catalytic activity of this enzyme. In the case of an unselected aptamer or a scrambled consensus sequence control, no inhibition of thrombin activity was seen by Block and coworkers. Fibrin formation, however, was six times slower in the presence of the selected aptamer. The assay done in our lab by K. Fu showed that similar inhibition to clotting occurred in the presence of the psoralen-aptamer (5). This result indicated that the 5' attachment of the psoralen warhead to the thrombin aptamer did not functionally alter the aptamer structure. Since the recognition domain was still intact, the next question we sought to answer was whether the psoralen-oligonucleotide would react with ds DNA. A ds DNA that contained only one site suitable for DNA crosslinking was chosen as a target for intercalation and photo-crosslinking with the aptamer-psoralen (5). Unfortunately, under a variety of reaction conditions, no covalent attachment occurred (Z. Chen, personal communication).



There are several possible explanations for this initial failure to form a Fatal Engineering lesion. It is possible that compound (5) failed to react with DNA because of unfavorable interactions between the psoralen-aptamer and ds DNA, possibly due to electrostatic repulsion between the aptamer and the DNA target. Previously, Takasugi et al. (1991) showed greatly decreased crosslinking ability if a single mismatch were introduced in to the triple helix binding site of a triple helix forming psoralen-oligonucleotide. In fact, under conditions of high salt where triple helix formation was abolished or when the triple helix recognition site had a five base pair change, no reaction occurred between the ds DNA target and their psoralen-oligonucleotide. This last result clearly indicates the need to have a driving force to bring and to hold the ds DNA and reactive oligonucleotide together. Even in the case where the aptamer and ds DNA might bind well, the psoralen might be positioned such that it is unable to intercalate and/or to crosslink. As discussed previously, the ability of psoralen to crosslink is dependent on it achieving a specific orientation in the ds DNA. Alternatively, the intercalation of the psoralen may have occurred into the aptamer structure making the psoralen unavailable. As a consequence, the psoralen would again be unavailable to interact with the target. Because it was our long term goal to use a DNA aptamer as a protein recognition domain, we subsequently tried to form the same type of lesion by a two-step approach using a mercury sulfur bond.

#### **c. Thiol-Psoralens and Mercurated DNA**

The inability of the psoralen-aptamer combination to intercalate and crosslink ds DNA productively led us to develop our next compound. We postulated that if the initial

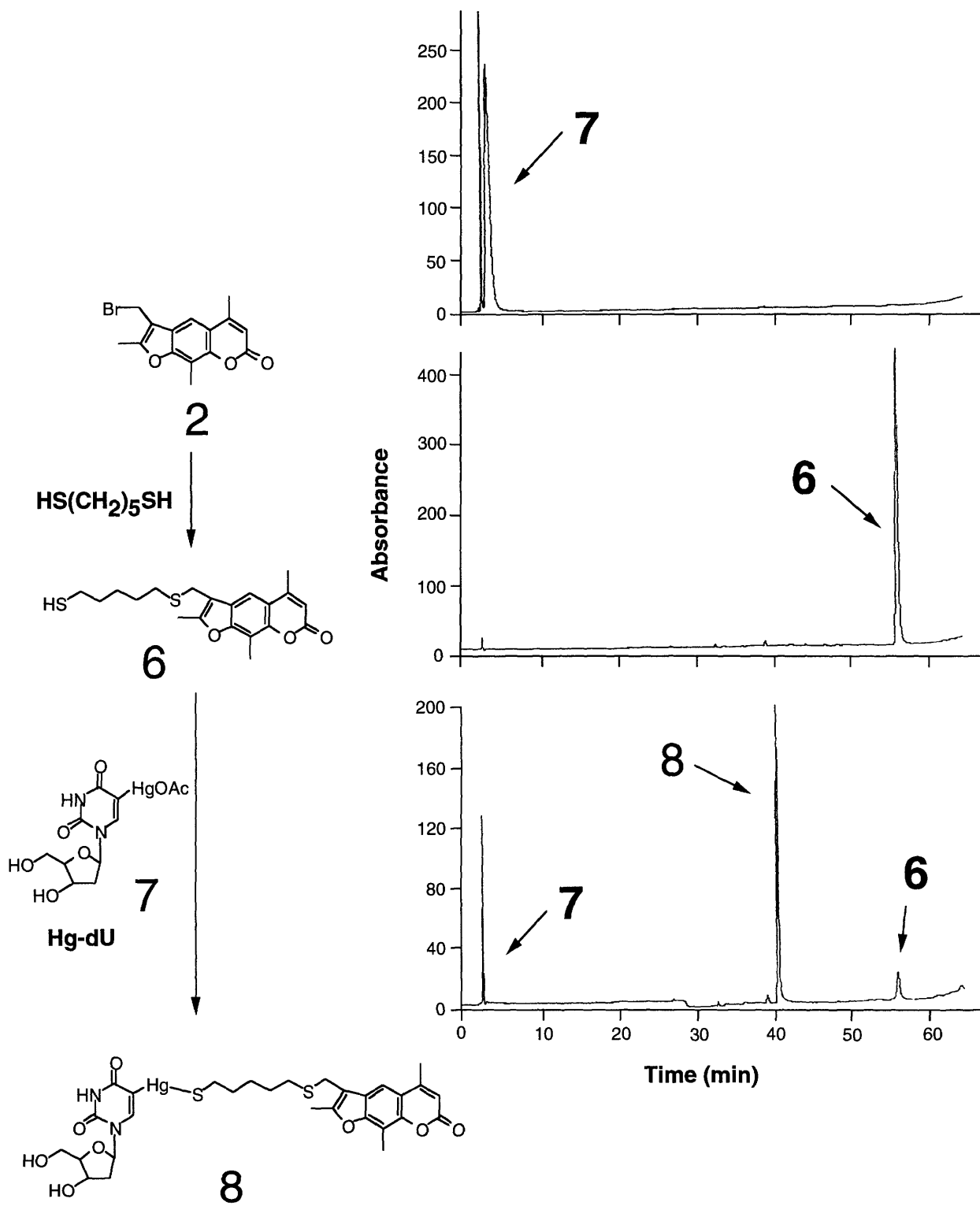
intercalation step where the warhead is allowed to form a DNA adduct were done in the absence of the aptamer, a DNA lesion would form. We additionally postulated that we could attach the desired recognition domain to the initial adduct to yield the desired Fatal Engineering lesion. The strategy chosen to link the two components together was by way of a mercury-sulfur bond (see discussion on linkers) (Figure 23). With this strategy, the first step would be to photo-crosslink the target ds DNA with a psoralen containing a side chain with a terminal sulfur. Following modification of DNA, a mercury-sulfur bond could link the mercurated aptamer recognition domain to the psoralen lesion. The advantages of this strategy would include: 1) the easier optimization of the initial psoralen warhead, 2) the ability to optimize the psoralen-aptamer link by moving a mercurated nucleotide to different places on the aptamer structure, and 3) characterization of linker requirements, such as length and chemical nature.

To test the approach described above, four psoralens were synthesized with side chains of varying length and composition but always ending in sulfur. The first thiol psoralen synthesized was the 4'-(thioxypenthoxyethyl)-trimethylpsoralen (**6**) (Figure 25). This psoralen was synthesized in one step by heating for 90 min 4'-bromomethyl-4,5',8-trimethylpsoralen (**2**) in neat 1,5 pentanedithiol. In order to ensure that the DNA crosslinked thiol-psoralen would react with a mercurated aptamer, reaction of the uncrosslinked thiol-psoralen (**6**) with a mercurated nucleoside, 5-mercuri-2'-deoxycytidine (Hg-dC) (**7**) was tested first. The extent of reaction between an equal molar solution of thiol psoralen (**6**) and Hg-dC (**7**) in 1:1 CH<sub>3</sub>CN: H<sub>2</sub>O at room temperature was monitored by reversed phase HPLC by using a diode array UV detector. A gradient starting with 10%

acetonitrile / 0.1 M ammonium acetate buffer and increasing to 100% acetonitrile over 50 min was used. With this gradient, the mercurated nucleoside eluted in the void volume after injection, whereas, the thiol psoralen (**6**) did not elute until the gradient had reached 100% acetonitrile. Examination of the reaction by HPLC after just a few minutes of reaction showed the presence of only one new peak of intermediate retention time (approximately 40 min). The UV profile of this new peak showed absorbances expected for both the psoralen and mercurated nucleoside. Isolation of the peak from the HPLC eluent and examination of the effluent by atomic absorption spectroscopy indicated that this new peak contained mercury. This preliminary data tentatively established that the free thiol psoralen (**6**) could react with a mercurated nucleoside as monomers in an acetonitrile/water solution to generate compound (**8**).

The next question was whether the thiol psoralen (**6**) could crosslink DNA. Using a DNA probe containing a single AT site, the thiol psoralen (**6**) in the presence of near UV light could crosslink ds DNA but with ten-fold less efficiency at equimolar concentration than the unmodified parental psoralen (Z. Chen, personal communication). The crosslinked material was then tested for reaction with a mercurated nucleotide, Hg-dCTP. Unfortunately, under all the conditions studied, no reaction was observed (Z.Chen, personal communication). We attributed this disappointing result to either or both of the following: 1) electrostatic repulsion of the nucleotide triphosphate and the ds DNA, or 2) a non-optimal linker arm.

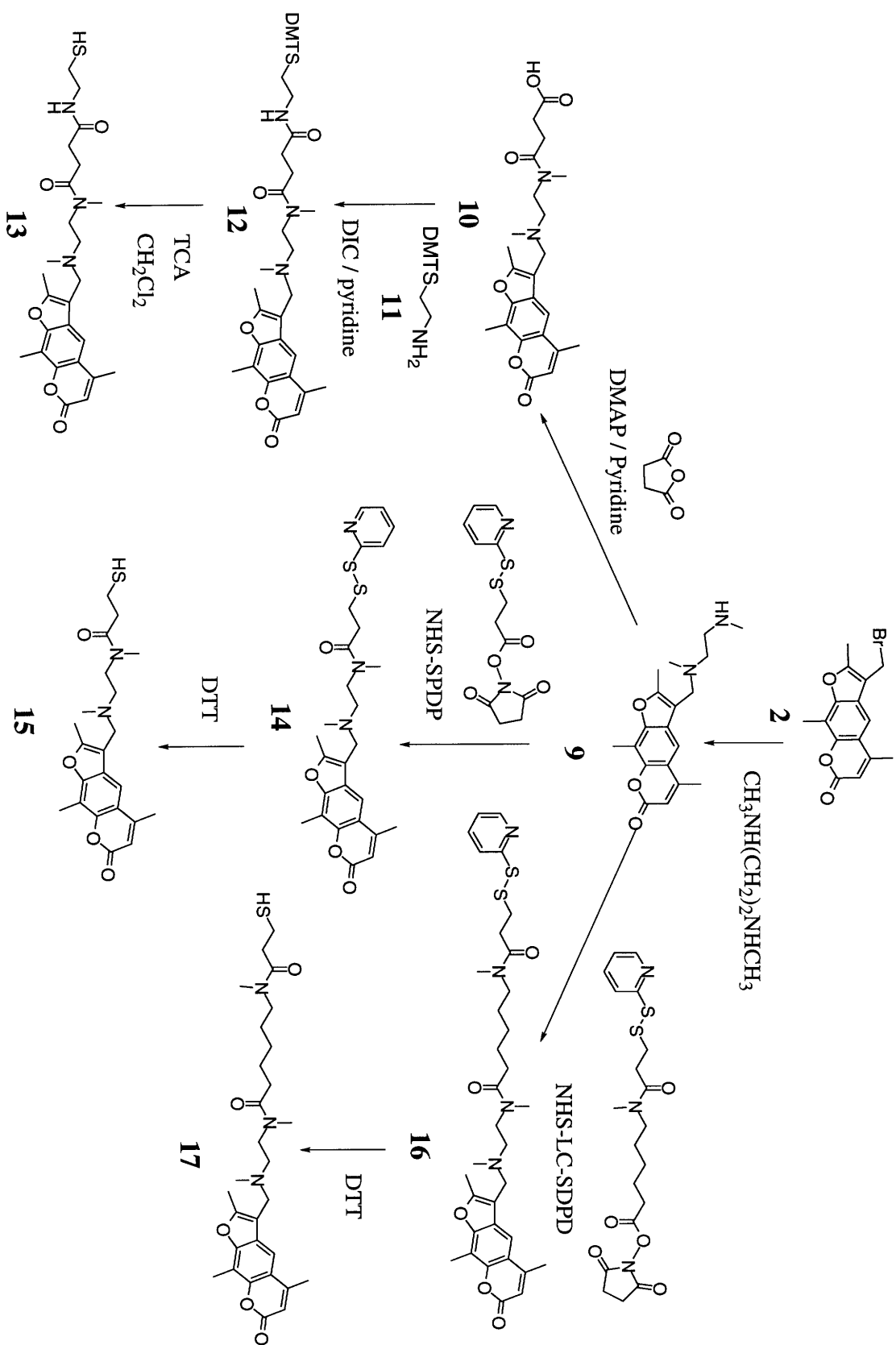
**Figure 25.** Thiol psoralen reaction with 5-mercuri-2'-deoxycytidine. The thiol (**6**) was synthesized in one step from the activated 4'-bromo-4,5',8-trimethylpsoralen (**2**) in neat 1,5-pentanedithiol. Reaction of the thiol-psoralen (**6**) with 5-mercuri-deoxycytidine (Hg-dC) resulted in the formation of a new peak as detected by HPLC.



In order to determine if the previous negative result were due to the length of the linker arm, three new psoralens with differing arm lengths and compositions were designed and synthesized (Figure 26): 4'-[[[(methylamino)ethyl]methylamino] methyl]trimethylpsoralen cysteaminy succinate ester (**13**), 4'-[[[(methylamino)ethyl]methylamino]methyl]trimethylpsoralen 6-[3-(2-pyridyldithio) propionamido]hexanoate ester (**17**), and 4'-[[[(methylamino)ethyl]methylamino]methyl] trimethylpsoralen) succinimidyl 3-(2-pyridyldithio)propionate (**15**). The starting material for all three compounds was the unstable 4'-bromomethyl-trimethylpsoralen (**2**). In order to increase the water solubility of the warhead, a new linker arm was added to the psoralen warhead: N,N-dimethyl ethylene. The [[[(Methylamino)ethyl]methylamino]methyl] trimethylpsoralen (**9**) had been synthesized previously and had showed greater solubility in water (Goldenberg et al., 1988).

Using the dialkylamine psoralen (**9**), the 4'-[[[(methylamino)ethyl]methylamino]methyl]trimethylpsoralen cysteaminy succinate ester (**13**) was synthesized in three steps. Treatment of the dialkylamine-trimethylpsoralen (**9**) with succinic anhydride, DMAP and pyridine resulted in the dialkylamine-trimethylpsoralen succinate (**10**). Unprotected cysteamine (2-mercaptoethylamine) could not be coupled directly to the terminal carboxylic acid of the psoralen (**10**) because a thiol ester would result instead of the desired amide with terminal thiol. To prohibit this reaction, the thiol of cysteamine was blocked with a dimethoxytrityl (DMT) group. Coupling of the DMT-cysteamine (**11**) with the psoralen (**10**) was done via a DIC activated ester. The final product, 4'-[[[(methylaminoethyl)methyl]trimethylpsoralen cysteaminy succinate ester(**13**), was obtained by treating the DMT-psoralen(**12**) with TCA/CH<sub>2</sub>.

**Figure 26.** Synthesis of thiol psoralens varying in chemical composition and length.





The synthesis of 4'-[[[(methylamino)ethyl]methylamino]methyl] trimethylpsoralen 6-[3-(2-pyridyldithio) propionamido] hexanoate ester (**17**), and 4'-[[[(methylamino)ethyl]methylamino]methyl]trimethylpsoralen) succinimidyl 3-(2-pyridyldithio)propionate (**15**) utilized a similar coupling strategy as done for 4'-[[[(methylamino)ethyl]methylamino]methyl]trimethylpsoralen cysteaminy succinate ester (**13**). The SPDP and LC-SPDP linker arms were appended to the dialkylamine-psoralen (**9**) by using the activated N-hydroxysuccinimide esters of SPDP and LC-SPDP available from Pierce (Carlsson et al., 1978). The products from acylation, psoralens **14** and **16**, yielded the free thiol psoralen (**15**) and (**17**) after reducing the disulfide linkage with 25 mM dithiothreitol, pH 4.5 (R. Croy, personal communication).

In a series of HPLC experiments conducted by R. Croy, the ability to form a mercury-sulfur couple between the three new thiol-psoralens and Hg-dC (**7**) was studied. The thiol-psoralens (**13,15,17**), when mixed with Hg-dC (**7**), resulted in a coupled product as monitored by reversed phase HPLC and UV detection. However, before in depth studies were conducted on these molecules, R. Croy noted that the new peaks formed from reaction of the thiols with the mercurated nucleoside were not stable over time. In fact, prolonged exposure of the mercurated nucleoside products to an excess of thiol-psoralens resulted in their decomposition. This problem with reductive demercuration in the presence of excess thiols had been seen earlier with other mercurated nucleosides (Dale et al., 1975). If the coupled mercurated aptamer-thiol psoralen crosslinked DNA also underwent decomposition,

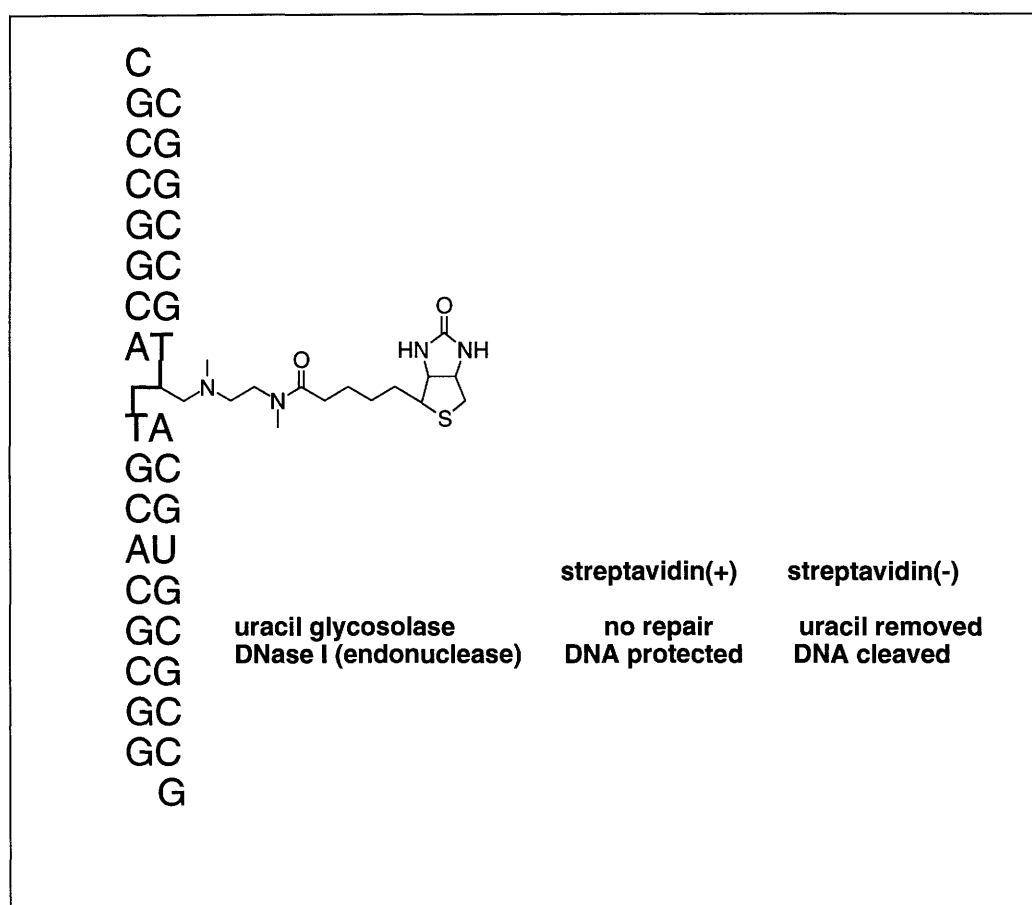
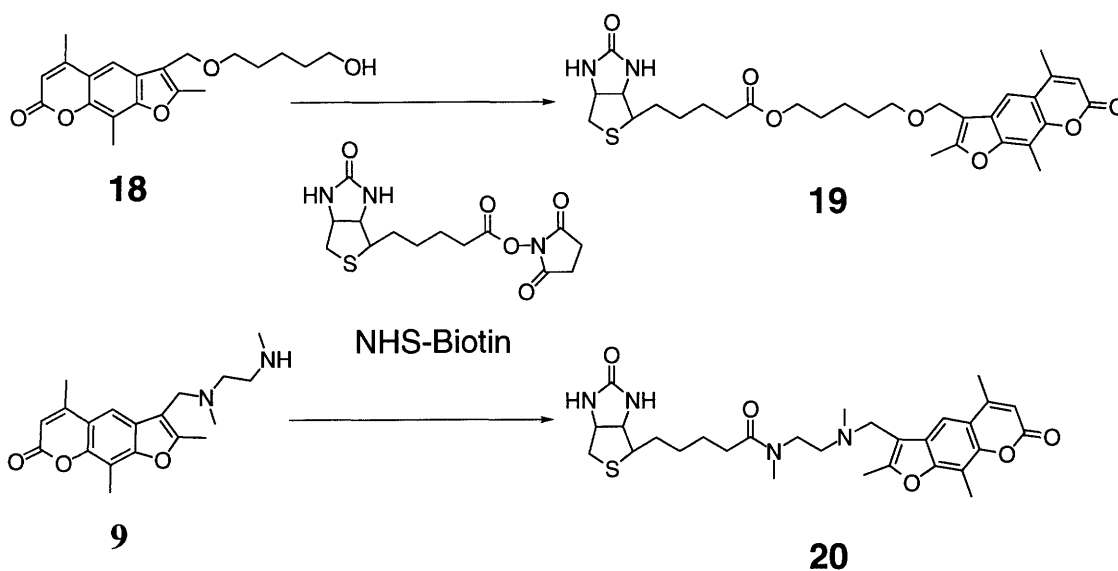
the ability to test for repair of lesion would be problematic. As a result of the poor stability of the coupling products, no further study was done with these compounds.

This failure to validate the Fatal Engineering concept with an oligonucleotide based strategy led us to reconsider our overall strategy. We decided that a simpler recognition domain was needed to validate the Fatal Engineering approach. The new strategy used biotin as the recognition domain. It was envisioned that attachment of biotin to the same trimethylpsoralen warhead would yield a compound with which to test the Fatal Engineering strategy *in vitro*.

#### **d. Psoralen Linked Biotins**

The first biotin-psoralen Fatal Engineering compound utilized a pentanediol linking arm between the trimethylpsoralen warhead and the carboxylic side chain of biotin (Figure 27). The derivatized pentanediol-psoralen (**18**) was synthesized by allowing 4'-bromomethyl-4,5',8-trimethylpsoralen (**2**) to react with 1,5-pentanediol according to a procedure by Pielec et al. (1989). The reaction of the pentanediol-psoralen (**18**) with biotin-NHS in DMF yielded our first biotin-psoralen (**19**). Owing to the poor water solubility of this molecule, reaction of biotin-psoralen (**19**) with ds DNA resulted in negligible crosslinking (Z. Chen, personal communication). Although co-solvents such as DMSO or DMF increased the solubility of the biotin-psoralen, the addition of these co-solvents resulted in increased denaturation in the target ds DNA. Consequently, insufficient crosslinked material was made for biological studies. Therefore, a new more polar psoralen-biotin was needed.

**Figure 27.** Synthesis of psoralen biotins.



The new compound was specifically designed to include a polar spacer arm. The dialkylamine-psoralen (**9**), which had previously be shown to have exceptional water solubility and reactivity with DNA (Goldenberg et al., 1988), appeared to be ideal as a warhead and linker. The biotin-linked psoralen (**20**) was prepared by allowing the dialkylamine-psoralen (**9**) to react with the biotin p-nitrophenyl ester (Figure 27). A similar synthesis was used by Saffran et al. (1988) to make the same biotin-linked psoralen in order to detect DNA concentrations by an ELISA based detection assay.

This second biotin-psoralen (**20**) was subsequently tested in a series of biological experiments for its ability to function as a potential Fatal Engineering drug. The ability to crosslink DNA was tested by exposing DNA and the biotin-psoralen to near UV light. As determined by denaturing gel electrophoresis, the extent of crosslinking was decreased by a factor of ten as compared to that seen for the underivatized psoralen, trimethylpsoralen (Z. Chen, personal communication). Accompanying this decrease in crosslinking was an increase in the amount of monoadducts formed. This change in reactivity could have resulted from the influence of the molecular structures of the derivatized psoralen on their solubilities, their photochemical reactivities, and the photodestruction of the compounds themselves (Isaacs et al., 1977). Even with this diminished yield of crosslinked DNA, adequate material was synthesized for the biological studies described below.

Through the use of denaturing gel electrophoresis, biotin-psoralen crosslinked DNA was isolated to test for the binding of streptavidin to the lesion. Of the purified psoralen-biotin crosslinked DNA, only 10-20% could bind to streptavidin as determined by an

electrophoretic mobility shift experiment (Z. Chen, personal communication). The band of retarded electrophoretic mobility corresponding to streptavidin-biotin-psoralen DNA was reversed by the addition of free biotin. This ability to titrate the binding of the streptavidin away from the DNA sample supported the role of biotin-streptavidin interactions as the reason for the band shift.

Since the psoralen damaged DNA population successfully interacted with streptavidin, this material was subsequently tested for the ability to repair DNA in the presence and absence of streptavidin. Instead of testing for the removal of the psoralen DNA lesion, the DNA was tested as a substrate for the removal of another DNA lesion incorporated into the DNA prior to crosslinking. Specifically, a 2'-deoxyuracil was incorporated into the DNA three bases away from the site of crosslinking. The removal of uracil from the DNA can be accomplished *in vitro* by uracil glycosylase, a DNA repair enzyme that protects cells from genetic instability that arises from either cytosine deamination or misincorporation of uracil from the nucleoside triphosphate pool. It was determined that the removal of the nearby uracil base from our psoralen crosslinked DNA by uracil glycosylase was inhibited only in the presence of streptavidin (Z. Chen, personal communication). This inhibition was reversed, although not completely, when free biotin was added. This experiment provided our first conclusive evidence that binding of a protein to a designed DNA lesion can inhibit DNA repair.

To examine further the shielding by streptavidin of the psoralen-biotin DNA lesion, a DNase I protection assay was done. DNase I is an endonuclease capable of digesting DNA

to mononucleoside fragments. Experiments indicate that the entire target psoralen-biotin crosslinked oligonucleotide was protected from DNase cleavage only in the presence of streptavidin (Z. Chen personal communication). In the absence of streptavidin, only a small region on the 3' side of the crosslink was resistant to DNase cleavage. This result clearly indicates that the binding of a protein to a designed DNA lesion can interfere with the function of repair enzymes.

In summary, a biotin-containing psoralen derivative (**20**) was synthesized in a simple one-step procedure from the readily available psoralen (**9**) and biotin p-nitrophenyl ester. The synthesized compound (**20**) is a bifunctional nucleic acid- and avidin-binding reagent that combines the properties of psoralen, in its specific photoreaction with DNA, and of biotin, in its strong interaction with avidin. This reagent binds covalently to DNA in a UV-dependent reaction and produces interstrand crosslinks. The biotin recognition domain of the compound (**20**) retains its affinity for avidin, even after photoaddition to DNA. Most importantly, only in the presence of streptavidin was the resulting DNA lesion able to inhibit the repair of a nearby uracil lesion or to prevent a DNA exonuclease from degrading the modified DNA.

#### **e. Psoralen Linked Estrogens**

Since the previous experiment validated *in vitro* that directed protein binding to a DNA lesion could inhibit DNA repair systems that ordinarily protect cells, we turned to the synthesis of compounds that might validate the approach *in vivo*. A successful test of the

strategy *in vivo* would have to include a protein recognition domain that would attract a biologically relevant protein. The protein we chose to target was the estrogen receptor which, as indicated earlier, is overexpressed in many breast tumors. Fortunately, a number of steroidal and non-steroidal compounds have been synthesized that show high affinity binding to the estrogen receptor protein which could serve as a protein recognition domain for our compounds (Bucourt et al., 1978; Poirier et al., 1991; Jordan et al., 1984).

Derivatives at the 17-hydroxyl of estrogen were initially viewed as the most promising candidates as the molecular magnet to attract the estrogen receptor. Three criteria were used in the selection of derivatives at this site. First, Bucourt et al. (1978) showed that estrogen derivatives with a variety of spacer arms attached to the 17 position are bound tightly by the estrogen receptor. Derivatives at the 2, 3, and 4 position of estrogen, by contrast, have greatly decreased affinity for the estrogen receptor. Second, the same workers have shown that the 17-hydroxyl linked estradiols, even when attached to a polymer resin (e.g., polyacrylamide, agarose, and polystyrene), bind the estradiol receptor. We postulated that the polymer resin to a first approximation offered a comparable steric hindrance to the estrogen receptor as would DNA in our specific example. Third, a variety of linker arms can be attached to the 17 hydroxyl via ether, ester, carbonate, or carbamate bonds.

In choosing the optimal side chain length between the psoralen warhead and the estrogen recognition domain, the work of Bucourt et al. with polymer bound estrogen indicates that a spacer chain containing more than 14 atoms is necessary for binding of the



estrogen receptor to occur. We chose a succinate-glycyl-glycyl-dialkylamine as a 14 atom bridge between the estrogen recognition domain and the psoralen warhead. By incorporating at least two amide groups, the poor water solubility encountered in our earlier compounds and in the estrogen analogs of Bucourt et al. would be avoided. We chose to use a succinate linker to the estrogen core because Bucourt et al. had already shown that succinate linked estrogens could bind with high affinity to the estrogen receptor.

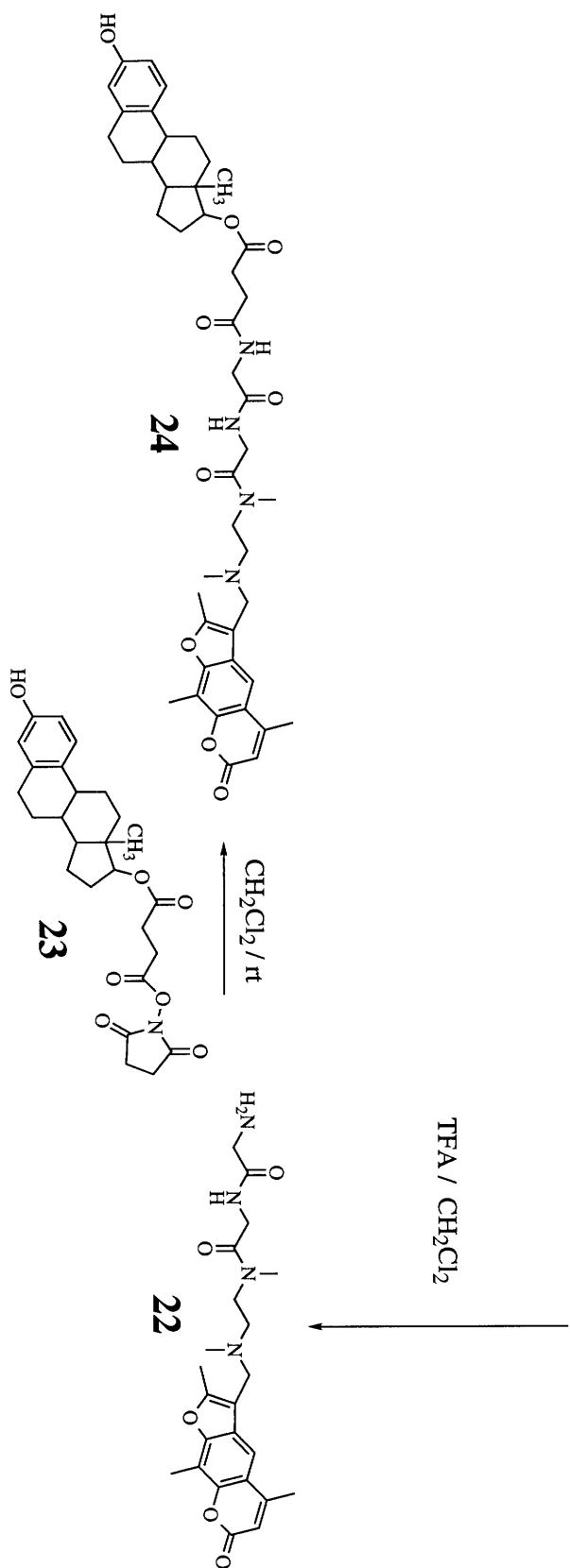
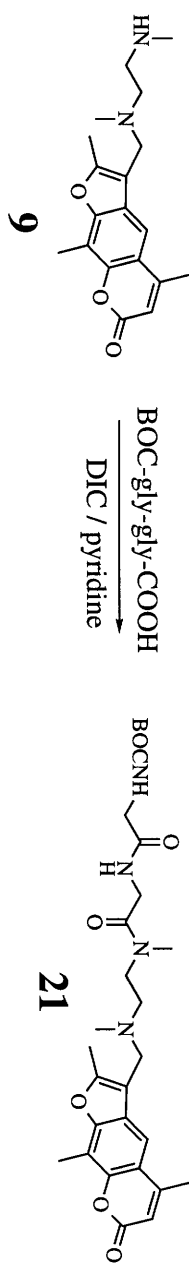
The approach used to synthesize estradiol-succinate-gly-gly-dialkylamine psoralen (**24**) was 1) to synthesize the two domains separately and 2) to couple the domains together with an amide bond (Figure 28). Based on the commercial availability of 17-hemisuccinate estrogen diol, the only domain that needed to be synthesized was the gly-gly-dialkylamine-psoralen. The necessary gly-gly-dialkylamine-psoralen could be synthesized by dipeptide coupling to the dialkylamine psoralen (**9**) used previously. The BOC-gly-gly was coupled to the dialkylamine psoralen (**9**) by *in situ* activation of the terminal carboxylic acid of the dipeptide with DIC. The BOC-gly-gly-dialkylamine-psoralen (**21**) was obtained in 77% yield after chromatography. Deprotection of the BOC group was achieved by treating the BOC-gly-gly-dialkylamine-psoralen (**21**) with TFA. The purified gly-gly-dialkylamine-psoralen (**22**) was obtained in 100% yield. The estradiol portion was coupled to the psoralen (**22**) by mixing the gly-gly-dialkylamine-psoralen (**22**) with the NHS activated succinate estradiol ester (**22**). The identity of the coupled product (**24**) was obtained by FAB-MS and NMR.

Preliminary biological testing of this compound as a candidate Fatal Engineering drug was conducted by R. Croy. A competition assay was used to determine the dissociation constant for the binding of the new drug *in vivo*. In this assay, the concentration of the psoralen-estrogen (**24**) was varied to determine the required concentration necessary to displace [<sup>3</sup>H]estradiol, the natural substrate, from the ER. The result of this assay revealed that the psoralen-estrogen (**24**) bound with comparable affinity as estradiol (R. Croy, personal communication). This result seemed fortunate, but was not expected because the addition of side chain substituents to estrogen usually decreases the binding to the estrogen receptor. This unexpected result seemed to indicate that our compound was not stable. A explanation for the possible rapid break down of psoralen-estrogen (**24**) is hydrolysis in cell extracts by steroid esterases (Katz et al., 1987). If the succinate bond were cleaved, the released estradiol would compete with equal efficiency as the tritium labeled estradiol. No studies were done to verify whether this were the problem with this compound. We chose instead to synthesize a new estrogenic compound with a linker that would not be vulnerable to enzymatic hydrolysis.

#### **f. Nitrogen Mustard Linked Estrogens**

Based on the results from the psoralen-estrogen (**24**), three changes in design were made before synthesizing the next estrogen based Fatal Engineering compound. The problems we believed needed to be overcome were: 1) the poor stability to hydrolytic cellular enzymes, 2) low water solubility, and 3) low reactivity towards DNA. The

**Figure 28.** Synthesis of estradiol-gly-gly-dialkylamine psoralen.



solutions to these problems were: 1) to change the point of attachment of the linker arm to the estrogen recognition domain, 2) to include a polar linker between the warhead and the recognition domain, and 3) to change to the warhead used in our compounds.

The first of these changes involved attaching the linker arm to the C17-position of estrogen via a stable carbon-carbon bond. The study by Bucourt et al. (1978), which earlier led us to develop the 17-succinate estrogen compound (**24**), also indicated that 17-alkyl estrogen derivatives are suitable for binding to the estrogen receptor. In determining the method of attachment to the C17 position, the work by Salman et al. (1987) indicates that an alkyne link to C17 position has greater binding affinity to the estrogen receptor than either alkane or alkene groups.

The second change involved using a different method of linking the warhead and recognition domain together. Since steroid esterases might hydrolyze our compounds again, we chose a more stable, polar linker. Cho et al. (1993) recently showed that an oligocarbamate was completely resistant to hydrolysis by proteases. This ability to resist hydrolysis by cellular proteases made carbamates superior polar linking groups as compared to amides or esters.

The third design change was to use a different DNA warhead in our compounds. Although the psoralen warhead had given us selectivity in reaction with DNA, each of our studies with a psoralen linked compound showed that the appendages on the psoralen nucleus greatly decreased the ability of the compound to react with DNA. We chose,

therefore, to utilize a different warhead that would yield more abundant DNA adducts to study. We chose chlorambucil, an aryl nitrogen mustard, as the DNA warhead (see discussion on warheads).

Having chosen the warhead and recognition domain, the only detail left to determine was the chemical composition of the linker arm. Based on the earlier finding that at least 14 atoms are needed for binding the estrogen receptor protein to polymers, we decided to keep a 14 atom linker. In determining the exact chemical composition of the linker arm, two studies by Poirier et al. (1990, 1991) indicated that five methylenes or more were necessary for optimal binding to the estrogen receptor of 17 $\alpha$ -alkynyl estrogens.

Based on all these considerations, the "ideal" molecule would include: 1) an estrogen diol recognition domain, 2) a C17 alkynyl linker between the estrogen and the linker arm, 3) at least a five methylene bridge between any other functional groups and the alkyne, 4) a non-hydrolyzable carbamate linker to the DNA warhead side chain, 5) a 14 atom long linker arm, and 6) a chlorambucil warhead. Retrosynthetically, the required components would be estrone, an eight carbon long alkyne with terminal alcohol or amine group, a phosgene equivalent and the chlorambucil amine or alcohol.

Two methods are widely used to synthesize C17 alkynyl estrogens. One method uses estrone as starting material whereby linker arms are added via a nucleophilic carbon species (i.e., grignards, alkyl lithiums, and lithium alkynes) (Miller and Christiansen, 1967; Garrouj et al., 1993). The second method begins with 17-ethynylestradiol, the main ingredient in

oral birth control pills. This method involves deprotonation of the C17-alkyne and subsequent reaction with acyl esters or alkyl iodides and bromides (Poirier et al., 1990; Poirier et al., 1991; Salman et al., 1987). The procedure chosen to link the alkynyl side chain to estrogen domain was that by Miller et al. (1967). The procedure of Miller et al. involves the alkynylation of estrone methyl ether with lithium, sodium, and potassium derivatives of 3-butyne-1-ol.

The procedure of Miller et al. appeared ideal for our study for two reasons. First, if 7-octyne-1-ol were used instead of the 3-butyne-1-ol used in the study of Miller et al., the desired five plus carbon methylene bridge between the alkyne and the next functional group would result. Second, alkynylation of estrone yielded only the  $\beta$ -ol stereoisomer. Although a mixture of stereoisomers would not be a problem for our studies, earlier results by Bucourt et al. (1978) had shown that  $\beta$ -ol alkynes bound tighter to the estrogen receptor than  $\alpha$ -ol alkynes. The only drawback of the procedure by Miller et al. was that the product from alkynylation would be an estrone methyl ether. It was postulated that the removal of the methyl protecting group from this product could be problematic. Therefore, we chose to replace the methyl ether protecting group with a benzyl ether protecting group.

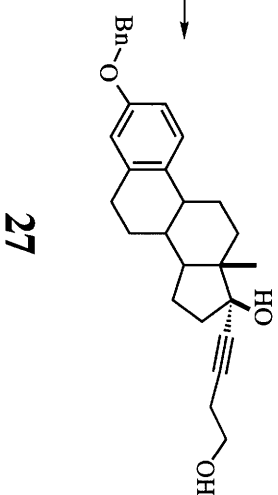
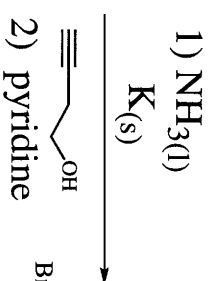
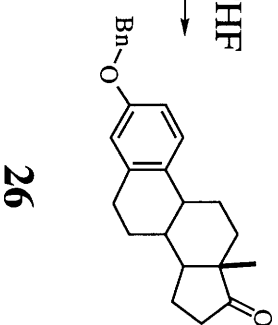
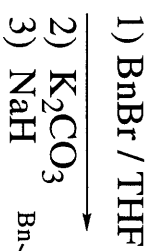
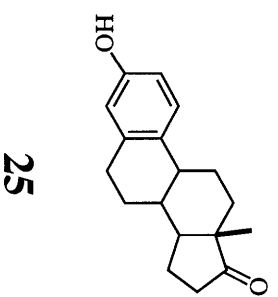
Benzyl estrone (**26**) was prepared by reaction of estrone (**25**) with benzyl bromide (210 M%) in THF with potassium carbonate (Figure 29). Even after stirring at room temperature for greater than 12 hr, the protection reaction had failed to go to completion. However, upon addition of sodium hydride, the estrone benzyl ether (**26**) rapidly formed. With the estrone benzyl ether (**26**) in hand, the alkynylation procedure was tested with 3-butyne-1-ol.

The procedure involved generating the bis-anion of the alkynyl alcohol with potassium metal in liquid ammonia followed by subsequent addition with estrone benzyl ether (**26**). The yield for this reaction was, however, only 20%. Instead of trying to optimize this reaction, we decided to try the reaction with a protected alcohol and generate the mono alkynyl anion.

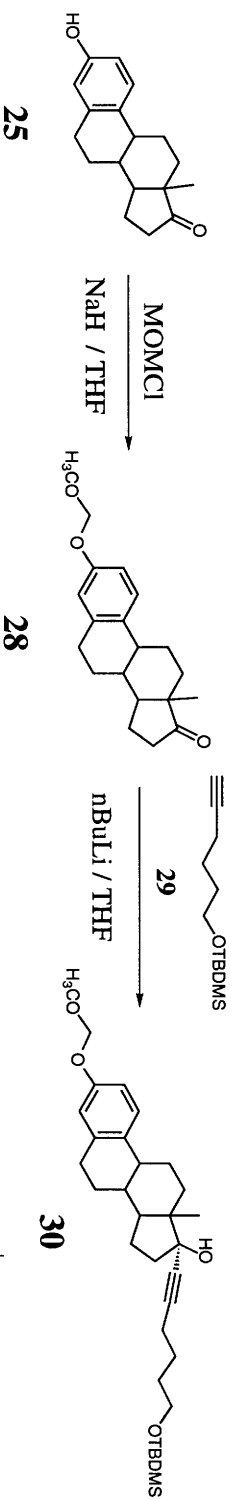
The longest commercially available terminal alkyne-1-ol is 5-hexyn-1-ol. By using this alkyne, the linker arm next to the estrogen would have the desired five carbons between the alkyne linkage to estrogen and the terminal hydroxyl. Although the total linker arm length when linked to chlorambucil would be two atoms short of the desired 14 atom linker, the availability of this alkyne-1-ol made this the compound ideal for testing the new synthetic scheme. The protecting group chosen to block the primary hydroxyl of the alkyne-1-ol was the t-butyldimethylsilyl (tBDMS). Reaction of 5-hexyn-1-ol with tBDMSCl in THF with sodium hydride yielded the 5-hexyn-1-t-butyldimethylsilyl (**29**) in 82% yield. Instead of repeating the alkynylation reaction with benzyl estrone (**26**), the reaction was done with MOM-estrone (**28**), previously synthesized in this laboratory for other studies. The alkynylation reaction was preformed by first generating the alkyne anion of compound (**29**) in THF with hexamethylphosphoramide at -78 °C with BuLi/Et<sub>2</sub>O. Subsequently, the MOM-estrone (**28**) was added and the cold bath removed to yield after 6 hr at room temperature the alkynyl-estrone (**30**). Deprotection of the silyl alcohol was accomplished by treating the alkynyl-estrone (**30**) with HF/pyridine (70% solution). Under these conditions, the MOM protecting group was completely stable. The alkynyl-estrone (**31**) was obtained after purification in 99% yield.



**Figure 29.** Alkynylation of benzyl estrone.

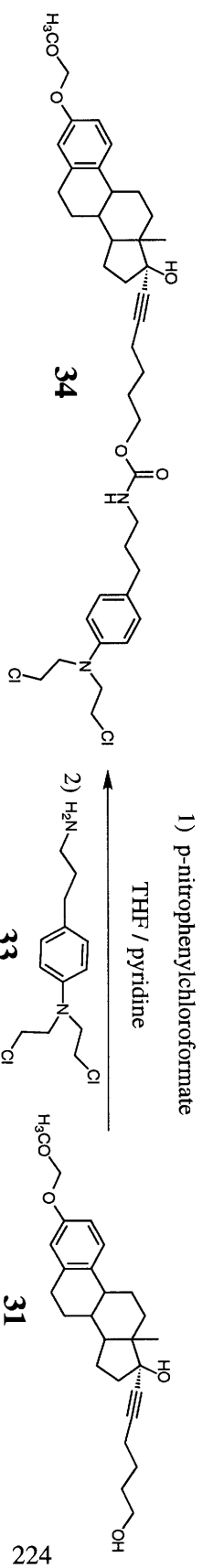


**Figure 30.** Synthesis of 12 atom linker between aryl-nitrogen-mustard and estradiol.

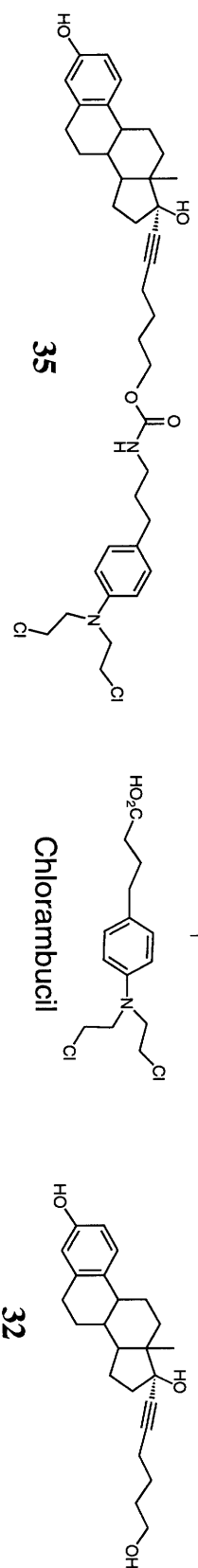


Estrone

HF / pyridine



HCl / MeOH



1) ethylchloroformate  
 2) sodium azide  
 3) benzene / reflux

HCl / MeOH

Chlorambucil

1) p-nitrophenylchloroformate

THF / pyridine

2) H<sub>2</sub>N-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-NH<sub>2</sub>

33

31

224

Based on our design criteria, in order to couple the alkynyl-estrone (**31**) with chlorambucil via a carbamate linker, chemical modification of the terminal carboxyl group of chlorambucil into an amine was required. Using a procedure by Gourdie et al. (1990), the terminal carboxyl group of chlorambucil can be changed into an amine by a Curtius reaction. The method involves ethylchloroformate activation of the carboxyl group followed by azide displacement and subsequent rearrangement at high temperatures to yield the nitrogen mustard (**33**). Subsequent coupling of the nitrogen mustard (**33**) to the alkynyl-estrone (**31**) can be done with a phosgene equivalent, p-nitrophenyl chloroformate. Procedurally, either component (amine or hydroxyl) could be activated and then allowed to react with the other component. The reaction of p-nitrophenyl chloroformate with an alcohol would yield a p-nitrophenyl carbonate, whereas the reaction of p-nitrophenyl chloroformate with an amine would yield a p-nitrophenyl carbamate. Because the reaction of the p-nitrophenyl carbonate with the amine would occur more rapidly than reaction of the p-nitrophenyl carbamate with the amine would occur more rapidly than reaction of the p-nitrophenyl carbamate with a hydroxyl group, we chose to activate the alkynyl-estrone (**31**) with p-nitrophenyl chloroformate and then couple the nitrogen mustard amine (**33**) to the p-nitrophenyl carbonate intermediate.

Activation of the alkynyl-estrone (**31**) with p-nitrophenyl chloroformate occurs at room temperature within two hours. The resulting p-nitrophenyl carbonate unfortunately hydrolyses slightly when subjected to chromatography. Consequently, the crude p-nitrophenylcarbonate isolated after aqueous workup was used without further purification.

Reaction of the isolated alkynyl-p-nitrophenyl carbonate estrone with the nitrogen mustard amine (**33**) yielded the desired estrogen-carbamate-nitrogen mustard (**34**) in approximately 70% yield. The desired deprotected Fatal Engineering compound, the estrogen-nitrogen mustard (**35**), was obtained by treating the MOM-estrogen nitrogen mustard (**34**) in methanol with  $\text{HCl}_{(\text{con})}$ . The estrogen-nitrogen mustard (**35**) could be stored as its hydrochloride salt or purified by silica gel chromatography for biological testing.

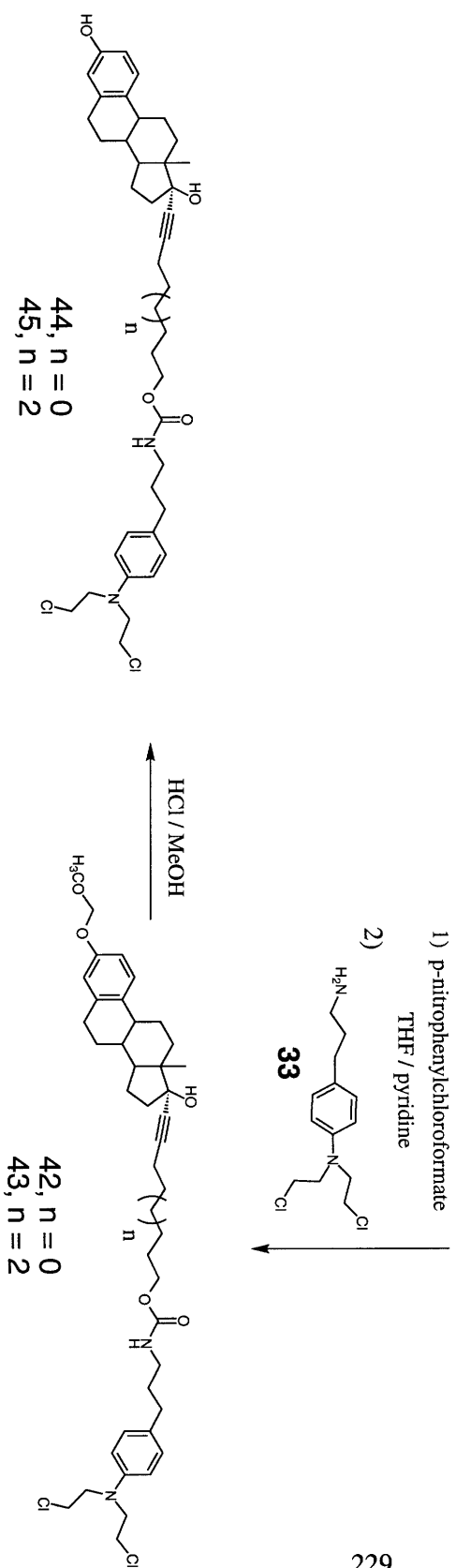
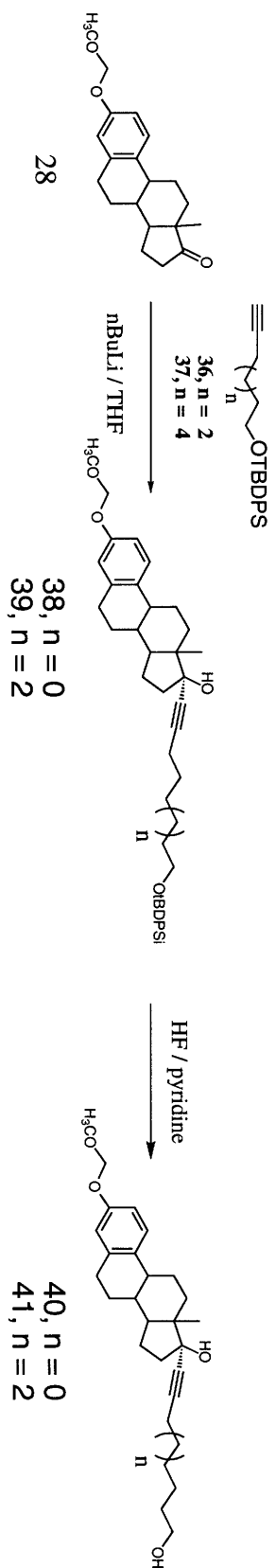
Preliminary biological evidence indicated that the estrogen-nitrogen mustard (**35**) resulted in increased toxicity in estrogen receptor positive cells as compared to estrogen receptor negative cells. Based on this result, we decided to synthesize two additional estrogen-nitrogen mustards in an attempt to increase the biological response exhibited. The approach we undertook to increase the biological activity was to vary the distance between the DNA damaging domain and the estrogen receptor. Our earlier study with the biotin-psoralen (**20**) had already indicated that decreased binding to the receptor domain occurs if the linker arm were too short for proper interaction with the targeted protein. This first estrone-chlorambucil (**35**) had twelve carbons between the C17 position on the estrogen and the phenyl group of the chlorambucil. We therefore decided to synthesize estrone-chlorambucils with 14 and 16 carbon linker arms. In order to synthesize estrogen-nitrogen mustards with longer spacer arms, two different alkynes were needed for the initial alkylation reaction. Unfortunately, the longest commercially available linear alkyne with a primary hydroxyl was the six carbon, 5-hexyn-1-ol, used for estrone-chlorambucil (**35**).

In order to synthesize the necessary alkynes (7-octyn-1-ol and 9-decyn-1-ol), six and eight carbon bromo alcohols, respectively, were allowed to react with lithium acetylide. The method of synthesis involved protection of the primary alcohols with a silyl protecting group and subsequent displacement of the primary bromides with lithium acetylide. As was done with the six carbon alkyne, the TBDMS protecting group was used. Unfortunately, without a chromophore or a method of staining to detect the reaction product, the protection reaction conditions could not be monitored. Additionally, the purification of the reaction mixture was difficult. Further, subsequent monitoring of the displacement with lithium acetylide was not possible and as a result low yields were obtained for the 7-octyn-1-TBDMS-ol and 9-decyn-1-TBDMS-ol. To allow for easier monitoring and purification, the TBDMS protecting group was replaced by t-butyldiphenylsilyl (TBDPS), which has two aromatic groups that would allow monitoring by UV absorbance. Protection of 6-bromo-1-hexanol and 8-bromo-1-octanol with TBDPS groups was done by using TBDPSCl and sodium hydride in THF. Subsequent displacement of the alkyl bromides was done with lithium acetylide, ethylene diamine complex (LAEDA) (Dejarlais et al., 1980; Brattesani et al., 1973). The necessary alkynes (**36,37**) were obtained in greater than 70% yield for two steps.

The coupling of the alkynes (**36,37**) with MOM-estrone (**28**) was done by the using the procedure developed with the 5-hexyn-1-t-butyldimethylsilyl (**29**). Unfortunately, the MOM-estrone starting material and the resulting MOM-estrone-alkyne products (**38, 39**) have almost identical retention times which made chromatographic isolation of the MOM-estrone-alkyne products problematic when incomplete conversion of the MOM-estrone (**28**)

**Figure 31.** Synthesis of 14 and 16 atom linker between aryl-nitrogen-mustard and estradiol.





occurred. As expected, treatment of the mixture of products with HF / pyridine only resulted in the removal of the TBDPS group whereby MOM-estrone-alkynes (**40**, **41**) could be easily separated from the MOM-estrone (**28**) in 60% yield for two steps. Subsequently, the same procedure involving p-nitrophenyl chloroformate was then used to couple the nitrogen mustard amine (**33**) with the alkynyl- estrogens (**40**) and (**41**). The final deprotection with HCl yielded the 14 and 16 atom estrogen-nitrogen mustards (**44,45**) desired for biological testing.

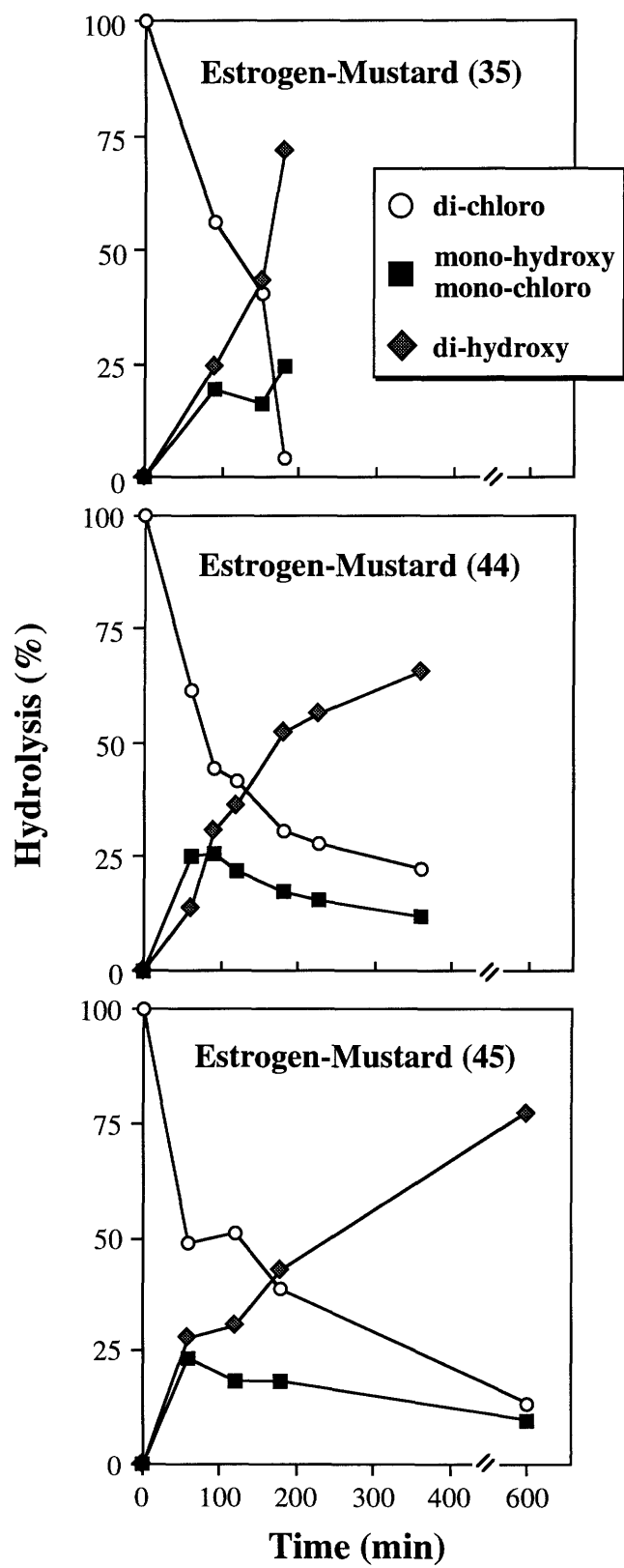
One of the first experiments conducted with the three estrogen-nitrogen mustards (**35**, **44**, **45**) was designed to determine if there were any change in the chemical reactivity of the mustard warhead as compared to chlorambucil. The reactivity of the mustard warheads was determine by examining the rate of hydrolysis with different aqueous buffers (Figure 32). Specifically, two buffers (one with and one without chloride) were examined to determine the effect of chloride concentration on the extent of hydrolysis. The extent of hydrolysis was determined by monitoring, at different time points, for the appearance of new peaks as detected by reversed phase HPLC using UV detection at 254 nm and 280 nm. With the gradient described in the Materials section, the order of elution from a C<sub>18</sub> reversed phase column of the parent estrogen-nitrogen mustards was compound **35**, compound **44**, and then compound **45**. The only difference between the three compounds was the length of their linker arms. The earliest eluting compound (**35**) had the shortest linker arm (12 atoms) and the latest eluting compound (**45**) had the longest (16 atoms). As expected the addition of methylene groups increased the relative hydrophobicity of the estrogen-nitrogen mustards. After heating (60 °C), two more polar products resulted, irrespective of the method used for

hydrolysis. Based on the retention times, the peaks were assigned as the mono-hydroxy, mono-chloro product, and the bis-hydroxy product.

We determined subsequently that the extent of hydrolysis depended on both the chloride concentration and the pH of the solution. If the pH were adjusted to less than six, the estrogen-nitrogen mustards did not undergo hydrolysis, even when heated overnight at 60 °C. When the pH was subsequently adjusted to eight, the extent of hydrolysis for the Tris-HCl buffer was only 50% after being heated overnight at 60 °C. This slow rate of hydrolysis with Tris-HCl buffer was attributed to the presence of chloride ions. As discussed earlier, the chloride ions can react with the intermediate aziridinium ion to reverse one pathway of reactivity. When we examined the extent of hydrolysis with a buffer without chloride, 10 mM sodium cacodylate pH 8.0, 50% CH<sub>3</sub>CN buffer, the extent of hydrolysis was more than 50% in less than two hours. The conclusion from these experiments was that the nitrogen mustard-estrogens (**35**, **44**, **45**) would be stable to conditions encountered *in vivo*. In fact, the high chloride concentration and lower temperature (37 °C) used for growing the estrogen receptor cells in tissue culture meant that the estrogen-nitrogen mustards would be stable for at least two hours. Consequently, the drug could slowly diffuse into the cells under study and possibly form DNA lesions.

The ability of the estrogen-nitrogen mustards (**35**, **44**, **45**) to react *in vitro* with DNA was determined by mixing the estrogen-nitrogen mustards with DNA. Reaction with DNA was conducted by using the same cacodylate buffer used in the hydrolysis experiments. In order to understand the relative reactivity of the new compounds, two other nitrogen

**Figure 32.** Hydrolysis of aryl nitrogen mustards.



mustards, mechlorethamine and chlorambucil, were studied as controls. The mechlorethamine is an alkyl mustard having only a methyl group attached to the nitrogen mustard functionality. During these preliminary biological experiments, it was determined that the estrogen-mustard (**45**) would precipitate from aqueous solution even when an organic co-solvent (DMSO or acetonitrile) was added. As a consequence, no further DNA crosslinking testing was done with this compound.

The largest extent of DNA damage was obtained by methchlorethamine and the least amount of crosslinking resulted from our nitrogen mustard-estrogens (K. Yarema, personal communication). The extent of crosslinking of chlorambucil was determined to be 20 to 100 fold lower than that caused by mechlorethamine. Crucial to our research, the extent of DNA damage by our estrogen-nitrogen mustards was 10-100 fold lower than that caused by chlorambucil. Ongoing studies are attempting to determine whether the DNA damage resulting from our compounds is due to DNA crosslinking or monoadduct formation.

The efficacy of the estrogen-nitrogen mustards (**35**, **44**, **45**) to function as selective toxins *in vivo* was tested by a growth inhibition assay using estrogen receptor positive and estrogen receptor negative cells. The experiments conducted by R. Croy tested the ability of estrogen receptor positive and estrogen receptor negative cells to grow when exposed to increasing amounts of the estrogen-nitrogen mustards. As a control compound, chlorambucil was used as a baseline for cell toxicity. As might be expected, the estrogen-nitrogen mustard (**45**), that showed greatly decreased reactivity with DNA *in vitro*, was not effective in killing cells. Although this compound did result in some cell toxicity, no

difference in cell toxicity resulted with increasing amounts of estrogen-nitrogen mustard (45). Two reasons were postulated for this result: 1) the estrogen-nitrogen mustard (45) precipitated from solution at higher concentration, and 2) the compound may be sequestered in the cell away from the DNA due to its hydrophobic nature. In either case, the estrogen-nitrogen mustard (45) apparently does not reach the cell nucleus and consequently does not form DNA lesions.

In contrast, the estrogen-nitrogen mustards (35,44) with a slightly shorter spacer arm proved to be equally toxic to estrogen receptor positive cells as chlorambucil (R. Croy, personal communication). Unfortunately, no difference was seen between the estrogen receptor positive and estrogen receptor negative cells as would have been predicted if a Fatal Engineering drug complex formed. This result was attributed to nonoptimal interaction of ER with the drug lesions. An estrogen receptor binding assay later confirmed this hypothesis. The receptor binding assay determined that the estrogen mustard drugs (35, 44) bound 100 to 1000 fold less tightly than estradiol (R. Croy, personal communication).

In summary, three trifunctional compounds were synthesized with a nitrogen mustard DNA damaging domain, an alkynyl-carbamate linker arm, and a complementary domain for the estrogen receptor. The nitrogen mustard-estrogen (45) with the longest linker arm between a DNA damaging domain and a protein recognition domain had the lowest water solubility of the three compounds. This compound showed drastically decreased ability to react with DNA *in vitro* and to induce *in vivo* toxicity experiments. In contrast, two nitrogen mustard-estrogens (35,44) with shorter linker arms had increased water solubility

and could react with DNA *in vitro* as determined by a DNA modification assay and *in vivo* as determined by a cell killing assay. In fact, preliminary results indicate that even with the attachment of the bulky side chain and protein recognition domain, the compounds were equally toxic to cells as the underivatized chlorambucil. This result contrasts with our earlier results with psoralen, where the attachment of the linker arm and recognition domain decreased reactivity with DNA greater than ten-fold. Unfortunately, no difference was determined between the targeted estrogen receptor positive and control estrogen receptor negative cells. This result was attributed to the poor binding of the estrogen receptor to the nitrogen mustard-estrogens. If the estrogen receptor protein cannot bind to the drug lesion, no difference in selectivity would consequent result. Therefore, in order to target selectively the estrogen receptor cells, the binding affinity between the nitrogen mustard-estrogen needed to be increased.

During the writing of this dissertation, the next step in the evolution of the Fatal Engineering compounds occurred. In this work, done by R. Croy with my supervision, a 2-phenylindole moiety used to in place of estradiol. 2-Phenylindoles can bind at the same site as estradiol and, in some case, bind even more tightly than estradiol. Preliminary biological tests indicate that the resultant mustard showed far greater toxicity to ER positive cells than ER negative cells (R. Croy, personal communication). Further studies are underway to determine whether this result were due to selective non-repair of the lesion in the cells overexpressing the tumor specific protein, as would be expected if the goals of the Fatal Engineering project were achieved.



## **F. Conclusions**

This chapter has described the rationale and testing of a new approach for the treatment of cancer. The new therapeutics under development in this laboratory involved coupling a DNA damaging agent to a protein recognition domain that attracts a tumor specific protein. The therapeutic efficacy of these trifunctional compounds depends on their ability to produce lethal chemical damage in cellular DNA, and on the selective blockage of repair in the presence of a tumor specific protein. The custom designed toxicity for cancer cells arises from the selective inhibition of DNA repair in the tumor as a consequence of a tumor specific protein binding to the DNA damage. The selective binding of proteins to the DNA damage in tumor cells arises either by the expression of oncogene-encoded or mutated suppressor gene-encoded proteins or by the overexpression of normal cellular proteins. Binding of the targeted protein to its recognition domain at the damaged site intentionally obstructs DNA repair enzymes. Consequently, the repair of the damaged segment of DNA would occur less efficiently and the lethal effect of the drug should be enhanced. In contrast, in the absence or with the low expression of the tumor specific protein in normal cells, repair enzymes would have free access to the damaged DNA site. Under such conditions, DNA repair could take place unobstructed and the cell would suffer far less toxicity.

Our initial objective of synthesizing compounds that were capable of both reacting with DNA and binding to a cellular protein has been accomplished. The first designed drug that

validated the Fatal Engineering concept took advantage of the high affinity of biotin for the protein streptavidin and the ability of psoralen to photo-crosslink ds DNA when irradiated with near UV light. To summarize the experiment, biotin-psoralen (**20**) was crosslinked to ds DNA by irradiation with near UV light. Subsequently, a nearby DNA lesion, uracil, was tested for repair in the presence and absence of streptavidin. In the presence of streptavidin, a protein-DNA lesion complex formed as evidenced by the inhibition of DNA repair of the nearby lesion and the diminished ability of the DNA to be degraded by an endonuclease. Although streptavidin is not expressed in mammalian cells, this system nevertheless demonstrated that proteins attracted to a DNA lesion can inhibit DNA repair.

Estrogen, a more biologically relevant recognition domain suitable for therapeutic applications, was subsequently incorporated into candidate Fatal Engineering drugs. Four compounds were synthesized and tested to various degrees in order to understand their ability to act as potential Fatal Engineering drugs: one psoralen linked estrogen and three nitrogen mustard linked estrogens. The instability of the psoralen linked estrogen, possibly due to steroid esterases, led to the development of non-hydrolyzable compounds for testing. The three nitrogen mustard-estrogens (**35,44,45**) differed only in the length of the spacer arm connecting the protein recognition domain to the DNA warhead: 12, 14 and 16 atoms, respectively. The nitrogen mustard-estrogen(**45**) with the longest linker arm had the lowest water solubility of the compounds tested. In addition, this compound showed greatly decreased DNA damaging ability *in vitro*. The nitrogen mustard-estrogens (**35** and **44**) were more water soluble and could react with DNA *in vitro*, although not as well as the

underivatized DNA warhead. Unfortunately, no difference in cell toxicity was determined between the targeted and the control cells.

Although the "magic bullet" was not developed in the studies described in detail here, the data gained from these early studies has been useful in our continuing investigation of this novel approach to treat cancer. My work has shown that one factor to incorporate into a new compound would be a protein recognition domain showing higher binding affinity to the ER. The current estrogen mustards (35, 44, 45) bind 100 times less tightly to the ER than estradiol. This binding affinity may not be strong enough to allow a stable protein-DNA lesion to form. One way to improve the binding to the ER would be to use other steroid and non-steroid analogs that have shown tighter affinity to the ER. Experiments are now underway by R. Croy, K. Yarema, and myself to manipulate the structure of the estrogen compounds to enhance the likelihood of interaction between the Fatal Engineering compounds and the estrogen receptor. Initial biological experiments with a 2-phenylindole-aryl nitrogen mustard drug indicate that the Fatal Engineering strategy may work as proposed.

## G. Experimental

### a. General Procedures and Materials

Unless otherwise specified, all chemicals were bought from Aldrich or Sigma chemical companies. N-succinimidyl 3-(2-pyridyldithio)propionate and N-succinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate were purchased from Pierce. All reactions were performed under argon atmosphere except when performed in aqueous media. Silica gel TLC plates were visualized with UV illumination followed by charring with 5% anisaldehyde in (95:5:1) ethanol-acetic acid-sulfuric acid. Silica gel (230-400 mesh) was used for normal phase chromatographic separations. All reactions were performed under an argon atmosphere except when working in aqueous media.

The purity of the thiol-mercury (**8**), psoralen-biotin products (**19**, **20**), and estrogen-aryl mustard products (**35**, **44**, **45**) were examined by reverse phase chromatography with a Beckman Ultrasphere C<sub>18</sub> column using a Beckman 114M solvent delivery system, Beckman 421A controller, and Hewlett Packard model 1040A photodiode array detector or using an Nec PC-8300 controller, Beckman Analog interface Module 406, Beckman 110B solvent Delivery Module, Nelson Analytical 760 series interface and Gateway 2000 computer. The compounds were monitored at 280 and 254 nm. The flow rate was 1 ml/min. Gradient I: a linear gradient of 0 -100% B over 50 minutes where solvent A = CH<sub>3</sub>CN/H<sub>2</sub>O (90:10) and solvent B = CH<sub>3</sub>CN. Gradient II: a linear gradient of 0-20% B over 40 min where

solvent A = acetonitrile/water (1:1) and B = acetonitrile; Gradient III: isocratic elution with 60% B where solvent A = MeOH/0.1 M NH<sub>4</sub>OAc (20: 80) and solvent B = MeOH. Gradient IV: a linear gradient of 70 -100% B over 20 min where solvent A = 0.1 M NH<sub>4</sub>OAc, 1 mM triethylamine, 1 mM hexane bisulfonic acid, 10% acetonitrile and solvent B = methanol.

GC-MS analyses were performed with a Hewlett-Packard 5971 mass spectrometer equipped with a Hewlett-Packard 5890 gas chromatograph. Fast atom bombardment (FAB-MS) was obtained with 2-nitrobenzyl alcohol as the matrix on a Finnigan 8200 mass spectrometer. High-resolution mass spectral (HRMS) data are reported in units of m/z for M<sup>+</sup> or the highest mass fragment derived from M<sup>+</sup>. <sup>1</sup>H and <sup>13</sup>C NMR were recorded on Varian VXR-300, VXR-301, or Bruker AC-250 MHz spectrometers. Chemical shifts are expressed in parts per million downfield from CDCl<sub>3</sub> (7.24 ppm or 77.00 ppm) or CD<sub>3</sub>OD (3.30 ppm and 35.00 ppm) for <sup>1</sup>H and <sup>13</sup>C, respectively. Data are reported as follows: chemical shift, integration, multiplicity, and regiochemical assignments.

## **b. Methods**

### **4'-Bromomethyl-4,5',8-trimethylpsoralen (2)**

Bromomethyl methyl ether (1.00 ml, 12.65 mmol, 14,000 M%) was added to trimethylpsoralen (**1**) (0.200 g, 0.876 mmol) that was partially dissolved in AcOH<sub>(glacial)</sub> (10 ml). After 1.5 hr at room temperature, additional bromomethyl methyl ether (1.00 ml) was

added to the clear yellow reaction. After 3 hr, a solid precipitate formed. At 4.5 hr, the precipitate was filtered and washed with AcOH<sub>(glacial)</sub> (3 x 10 ml). The filter cake was dissolved with a minimum of CH<sub>2</sub>Cl<sub>2</sub> and concentrated *in vacuo* to an off-white solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.57 (1H, s, H5), 6.25 (1H, s, H3), 4.61 (2H, s, CH<sub>2</sub>Br), 2.55 (3H, s, CH<sub>3</sub>), 2.52 (3H, s, CH<sub>3</sub>), 2.48 (3H, s, CH<sub>3</sub>).

**4'-(Hydroxyethoxymethylether)-4,5',8-trimethylpsoralen (3)**

4'-Bromomethyl-trimethylpsoralen (2) (0.36 g, 1.2 mmol) was suspended in ethylene glycol (8.00 ml) and heated (100 °C). After 2 hr, the reaction was diluted with H<sub>2</sub>O (40 ml) and CH<sub>2</sub>Cl<sub>2</sub> (40 ml). The organic layer was separated and washed with NaHCO<sub>3(sat)</sub> (75 ml) and NaCl<sub>(sat)</sub> (25 ml). The organic extract was then dried (Na<sub>2</sub>SO<sub>4</sub>), filtered, and concentrated. The 4'-(hydroxyethoxymethylether)-4,5',8-trimethylpsoralen (3) was purified by column chromatography on 15 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (96:4). to produce an off white solid (0.31 mg, 1.1 mmol, 90 % yield) (TLC rf 0.68 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 90:10) <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.58 (1H, s, H5), 6.22, (1H, s, H3), 4.68 (2H, s, OCH<sub>2</sub>Ar), 3.6-3.8 (4H, m, ethylene), 2.55 (3H, s, CH<sub>3</sub>), 2.48 (3H, s, CH<sub>3</sub>), 2.47 (3H, s, CH<sub>3</sub>).

**4'-[(Methyloxyethoxy)-O-[(2-cyanoethoxy)-N,N-diisopropylamino]-phosphoramidite]-4,5',8-trimethylpsoralen (4)**

(2-Cyanoethyl) N,N-diisopropylphosphoramidite (0.31 mg, 1.31 mmol) was added to 4'-(hydroxyethoxymethyl ether)-4,5',8-trimethylpsoralen (3) (0.31 mg, 1.19 mmol) dissolved

in THF (5 ml) and triethylamine (100  $\mu$ l). After 30 min at room temperature, the reaction was diluted with water (20 ml) and  $\text{CH}_2\text{Cl}_2$  (20 ml). The organic solution was separated and washed with  $\text{NaHCO}_{3(\text{sat})}$  (15 ml) and  $\text{NaCl}_{(\text{sat})}$  (15 ml). The organic extract was dried with  $\text{Na}_2\text{SO}_4$ , filtered, and concentrated. The 4'-[(methoxyethoxy)-O-[(2-cyanoethoxy)-N,N-diisopropylamino]-phosphoramidite]-4,5',8-trimethylpsoralen (**4**) was purified by column chromatography on 15 g silica gel eluting with  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  (96:4). Although the isolated material contained some impurities, this material was used without further purification for solid phase DNA synthesis.

#### **5'-Trimethylpsoralen-methoxyethoxy-CAGGTTGGTGTGGTTGGG (**5**)**

The oligodeoxynucleotide sequence 5'-CAG GTT GGT GTG GTT GGG-3' (10  $\mu$ mol) was synthesized on an Applied Biosystems DNA synthesizer using phosphoramidite chemistry. The psoralen phosphoramidite (**4**) was applied manually via two disposable 1 ml syringes to the resin-bound oligonucleotide. One syringe contained the phosphoramidite (**4**) (20 mg) dissolved in  $\text{CH}_3\text{CN}$  (100  $\mu$ l) while the other contained ABI tetrazole/ $\text{CH}_3\text{CN}$  solution (100  $\mu$ l). The syringes were attached to the ends of the oligonucleotide column and then passed repeatedly over the resin for 5 min. The column was placed back on the DNA synthesizer and subjected to washing and oxidation steps only. To cleave the oligonucleotide from the resin,  $\text{NH}_4\text{OH}_{(\text{con})}$  (3 x 5 ml) was passed through the oligonucleotide column. The ammonium solution containing the oligonucleotide was collected and heated overnight at 60 °C. The deprotected oligonucleotide was purified by gel electrophoresis (Z. Chen, personal communication).

**4'-((1,5-Pentanedithiol)methylether)-4,5',8-trimethylpsoralen (6)**

4'-Bromomethyl-trimethylpsoralen (**2**) was suspended in 1,5 pentanedithiol (3 ml) and heated (100 °C). After heating for 1.5 hr, the reaction was cooled and diluted with CH<sub>2</sub>Cl<sub>2</sub> (50 ml). The organic solution was washed with H<sub>2</sub>O (10 ml) and NaCl<sub>(sat)</sub> (10 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. The crude 4'-((1,5-pentanedithiol)methylether)-4,5',8-trimethylpsoralen (**6**) was purified by column chromatography on 15 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (96:4) an off white solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.52 (1H, s, H5), 6.13, (1H, s, H3), 3.72 (2H, s, H4'), 2.45 (3H, s, CH<sub>3</sub>), 2.42 (3H, s, CH<sub>3</sub>), 2.40 (3H, s, CH<sub>3</sub>), 2.42 (2H, s, SCH<sub>2</sub>), 1.6-1.4 (8H, m, CH<sub>2</sub>'s). C<sub>20</sub>H<sub>24</sub>O<sub>3</sub>S<sub>2</sub> 376.51 (calculated) GC-MS 376 [M<sup>+</sup> found], 241 [M-HS(CH<sub>2</sub>)<sub>5</sub>SCH<sub>2</sub>]<sup>+</sup>.

**5-Mercuri-2'-deoxycytidine carbonate (7)**

5-Mercuri-2'-deoxycytidine carbonate (**7**) was prepared by Z. Chen. The mercurated nucleoside was obtained by incubating 5-mercuri-2'-deoxycytidine 5'-triphosphate carbonate, tris-triethylammonium salt with calf intestine phosphatase.

**5-[4'-((1,5-Pentanedithiol)methylether)-4,5',8-trimethylpsoralen] mercuri-2'-deoxycytidine carbonate (8)**

4'-((1,5-Pentanedithiol)methylether)-4,5',8-trimethylpsoralen (**6**) dissolved in CH<sub>3</sub>CN (100 μl) was mixed with 5-mercuri-2'-deoxycytidine carbonate (**7**) dissolved in H<sub>2</sub>O (100 μl).



The reaction was monitored by reversed phase-HPLC on a C<sub>18</sub> column using gradient I.

The collected eluent for peaks 7 and 8 tested positive for mercury by atomic absorption.

**4'-[[[(Methylamino)ethyl]methylamino]methyl]-4,5',8-trimethylpsoralen (9)**

4'-Bromomethyl trimethylpsoralen (2) (0.32 mg) was suspended in N,N'-dimethylethylenediamine and heated (100 °C). After heating for 90 min, the reaction was cooled and diluted with CH<sub>2</sub>Cl<sub>2</sub> (50 ml). The organic solution was washed with H<sub>2</sub>O (10 ml) and NaCl<sub>(sat)</sub> (10 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. The 4'-[[[(methylamino)ethyl]methylamino]methyl]-4,5',8-trimethylpsoralen (9) was purified by column chromatography on 15 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (96:4) to yield an off white solid (yield not determined). (TLC rf 0.12 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 95:5) <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.63 (1H, s, H5), 6.23, (1H, s, H3), 4.06 (2H, s, NCH<sub>2</sub>Ar), 2.8-2.5 (4H, dt, NCH<sub>2</sub>CH<sub>2</sub>), 2.45 (3H, s, CH<sub>3</sub>), 2.42 (3H, s, CH<sub>3</sub>), 2.40 (3H, s, CH<sub>3</sub>), 2.35 (3H, s, NCH<sub>3</sub>), 2.23 (3H, s, NCH<sub>3</sub>). C<sub>19</sub>H<sub>24</sub>O<sub>3</sub>N<sub>2</sub> 328 (calculated) GC-MS 328 [M<sup>+</sup>], 241 [M-CH<sub>3</sub>N(CH<sub>2</sub>)<sub>2</sub>NCH<sub>2</sub>]<sup>+</sup>.

**4'-[[[(Methylamino)ethyl]methylamino]methyl]-4,5',8-trimethylpsoralen succinate (10)**

Succinic anhydride (20 mg, 0.20 mmol, 200 M%) and DMAP (4 mg, 0.03 mmol, 30 M%) was added to 4'-[[[(methylamino)ethyl]methyl]trimethylpsoralen (9) (0.034 g 0.103 mmol) dissolved in pyridine (0.5 ml). After 4 hr at room temperature, the reaction was concentrated to dryness. The 4'-[[[(methylamino)ethyl] methylamino]methyl]-4,5',8-

trimethylpsoralen succinate (**10**) was purified by column chromatography on 15 g silica gel eluting with an increasing gradient of methanol starting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (85:15) to yield an off white solid (yield not determined). (TLC rf 0.34 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 80:20) <sup>1</sup>H NMR (CDCl<sub>3</sub>)(NCH<sub>3</sub> rotamers): 7.85 (1H, s, H5), 6.18 (1H, s, H3), 4.06 (2H, s, H4'), 3.70 (2H, d, succinate CH<sub>2</sub>), 3.45, 3.18, 2.98, 2.83 (NCH<sub>3</sub> rotamers), 2.51 (3H, s, CH<sub>3</sub>), 2.50 (3H, s, CH<sub>3</sub>), 2.49 (3H, s, CH<sub>3</sub>), 2.45 (2H, d, succinate CH<sub>2</sub>).

#### **4,4'-Dimethoxytritylcysteamine (11)**

Cysteamine (0.200 g, 2.59 mmol) and 4,4'-dimethoxytritylchloride (1.02g, 3.02 mmol, 116 M%) were dissolved in pyridine (10 ml). After 6 hr at room temperature, additional 4,4'-dimethoxytritylchloride (0.50 g, 58 M%) was added. After stirring overnight, the reaction was concentrated to dryness. The residue was redissolved in CH<sub>2</sub>Cl<sub>2</sub> (50ml) and washed with H<sub>2</sub>O (50 ml), NaHCO<sub>3(sat)</sub> (15 ml), and NaCl<sub>(sat)</sub> (15 ml). The organic solution was dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and concentrated. The 4,4'-dimethoxytritylcysteamine (**11**) was purified by column chromatography on 45 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (92:8) to yield an off white solid (0.70 g, 1.90 mmol, 74 % yield). (TLC rf 0.64 CH<sub>2</sub>Cl<sub>2</sub>: MeOH 90:10) <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.2-7.4 (m, DMT), 6.75 (d, DMT), 3.72 (6H, s, DMT-OCH<sub>3</sub>), 3.4 (2H, s, NH), 2.56 (2H, t, CH<sub>2</sub>), 2.32 (2H, t, CH<sub>2</sub>).

**4'-[[[(Methylamino)ethyl]methylamino]methyl]-4,5',8-trimethylpsoralen 4,4'-dimethoxytritylcysteaminyl succinate ester (12)**

4,4'-Dimethoxytritylcysteamine (**11**) (90 mg, 0.24 mmol, 200 M%) and DIC (40  $\mu$ l, 0.26 mmol, 250 M%) was added to 4'-[[[(methylamino)ethyl]methyl]-4,5',8-trimethylpsoralen succinate (**10**) (0.103 mmol) dissolved in pyridine (4 ml). After 5 hr at room temperature, additional 4,4'-dimethoxytritylcysteamine (**11**) (40 mg, 100 M%) and DIC (40  $\mu$ l) were added. After stirring overnight, the reaction was concentrated to dryness. The residue was redissolved in CH<sub>2</sub>Cl<sub>2</sub> (50 ml) and washed with H<sub>2</sub>O (15 ml), NaHCO<sub>3(sat)</sub> (15 ml), and NaCl<sub>(sat)</sub> (15 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. The 4'-[[[(methylamino)ethyl]methylamino]methyl]-4,5',8-trimethylpsoralen 4,4'-dimethoxytritylcysteaminyl succinate ester (**12**) was purified by column chromatography on 20 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (92:8) to yield an off white solid (50 mg, 0.064 mmol, 62% yield). (TLC rf 0.64 CH<sub>2</sub>Cl<sub>2</sub>: MeOH 90:10) <sup>1</sup>H NMR (CDCl<sub>3</sub>)(two rotamers): 7.85 (1H, s, H5), 7.2-7.4 (m, DMT), 6.75 (d, DMT), 6.18 (1H, s, H3), 4.06 (2H, s, H4'), 3.72 (s, 6H, DMT-OCH<sub>3</sub>), 3.70 (2H, d, succinate CH<sub>2</sub>), 3.45, 3.18, 2.98, 2.83 (NCH<sub>3</sub> rotamers), 2.56 (t, 2H, CH<sub>2</sub>), 2.51 (3H, s, CH<sub>3</sub>), 2.50 (3H, s, CH<sub>3</sub>), 2.49 (3H, s, CH<sub>3</sub>), 2.45 (2H, d, succinate CH<sub>2</sub>), 2.32 (t, 2H, CH<sub>2</sub>).

**4'-[[[(Methylamino)ethyl]methylamino]methyl]-4,5',8-trimethylpsoralen cysteaminy succinate ester (13)**

Trichloroacetic acid/CH<sub>2</sub>Cl<sub>2</sub> (200 μl) (Applied Biosystems Oligonucleotide Reagent) was added to 4'-[[[(methylamino)ethyl]methyl]-4,5',8-trimethylpsoralen 4,4'-dimethoxytrityl cysteaminy succinate ester (12) (14 mg) dissolved in CH<sub>2</sub>Cl<sub>2</sub> (5 ml). Almost immediately, the reaction turned orange. After 30 min at room temperature, the organic solution was diluted with CH<sub>2</sub>Cl<sub>2</sub> (10 ml) and washed with H<sub>2</sub>O (15ml), NaHCO<sub>3(sat)</sub> (15ml), and NaCl<sub>(sat)</sub> (15ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. The 4'-[[[(methylamino)ethyl]methylamino]methyl]-4,5',8-trimethylpsoralen cysteaminy succinate ester (13) was purified by column chromatography on 20 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (92:8) to yield an off white solid (yield not determined). <sup>1</sup>H NMR (CDCl<sub>3</sub>)(two rotamers): 7.85 (1H, s, H5), 6.18 (1H, s, H3), 4.06 (2H, s, H4'), 3.70 (2H, d, succinate CH<sub>2</sub>), 3.45, 3.18, 2.98, 2.83 (NCH<sub>3</sub> rotamers), 2.56 (2H, t, CH<sub>2</sub>), 2.51 (3H, s, CH<sub>3</sub>), 2.50 (3H, s, CH<sub>3</sub>), 2.49 (3H, s, CH<sub>3</sub>), 2.45 (2H, d, succinate CH<sub>2</sub>), 2.32 (2H, t, CH<sub>2</sub>).

**N-(4'-[[[(Methylamino)ethyl]methylamino]methyl]-4,5',8-trimethylpsoralen) succinimidyl 3-(2-pyridyldithio)propionate (14)**

4'-[[[(Methylamino)ethyl]methyl]trimethylpsoralen (9) (0.043 g 0.131 mmol) and N-succinimidyl 3-(2-pyridyldithio)propionate (48 mg, 0.154 mmol, 120 M%) were dissolved in pyridine (2 ml). After 4 hr at room temperature, the reaction mixture was concentrated to

dryness. The N-(4'-[[[(methylamino)ethyl]methylamino]methyl]-4,5',8-trimethylpsoralen) succinimidyl 3-(2-pyridyldithio)propionate (**14**) was purified by column chromatography on 10 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (96:4). (0.046 g, 0.91 mmol, 70 % yield) (TLC rf 0.55 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 90:10) <sup>1</sup>H NMR (CDCl<sub>3</sub>): 8.40 (1H, d, pyr), 7.58 (2H, d, pyr), 7.51 (1H, s, H5), 7.05 (1H, m, pyr), 6.58 (1H, m, pyr), 6.17 (1H, s, H3), 3.5.4 (2H, t), 2.97 (2H, t), 2.86 & 2.81 (3H, s, NCH<sub>3</sub>), 2.52 (3H, s, CH<sub>3</sub>), 2.45 (3H, s, CH<sub>3</sub>), 2.42 (3H, s, CH<sub>3</sub>), 2.25 & 2.21 (3H, s, NCH<sub>3</sub>), 1.50 (4H, m, CH<sub>2</sub>-s), 1.20 (2H, m, CH<sub>2</sub>-s). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 170.42, 161.40, 159.99, 154.50, 153.24, 149.49, 148.96, 136.93, 125.62, 120.61, 119.66, 115.84, 112.58, 112.53, 111.80, 108.81, 54.44, 42.32, 42.22, 35.61, 33.71, 32.81, 19.29, 12.28, 8.40

**N-(4'-[[[(methylamino)ethyl]methylamino]methyl]-4,5',8-trimethylpsoralen) succinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate ester (**16**)**

4'-[[[(Methylamino)ethyl]methyl]trimethylpsoralen (**9**) (0.021 g 0.064 mmol) and N-succinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate (30 mg, 0.071 mmol, 110 M%) were dissolved in 2 ml pyridine. After 9 hr at room temperature, additional N-succinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate (20 mg, 0.47 mmol, 74 M%) was added. After 11 hr, the reaction was concentrated to dryness. The N-(4'-[[[(methylamino)ethyl]methylamino] methyl]-4,5',8-trimethylpsoralen) succinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate ester (**16**) was purified by column chromatography on 10 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (96:4). (TLC rf 0.55 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 90:10) <sup>1</sup>H NMR (CDCl<sub>3</sub>): 8.37 (1H, d, pyr), 7.58 (2H, d, pyr), 7.52 (1H, s, H5), 7.05 (1H, m,

pyr), 6.58 (1H, m, pyr), 6.17 (1H, s, H3), 3.6-3.4 (3H, m), 3.20 (2H, t), 3.00 (2H, t), 2.85 & 2.80 (3H, s, N-methyl amide), 2.49 (3H, s, CH<sub>3</sub>), 2.44 (3H, s, CH<sub>3</sub>), 2.40 (3H, s, CH<sub>3</sub>), 2.25 & 2.21 (3H, s, N-methyl amide), 1.50 (4H, m, CH<sub>2</sub>-s), 1.20 (2H, m, CH<sub>2</sub>-s).  
<sup>13</sup>C NMR (CDCl<sub>3</sub>): 172.37, 170.45, 161.43, 159.47, 154.42, 153.31, 149.33, 148.90, 136.92, 125.38, 120.79, 119.96, 115.75, 112.52, 112.53, 111.89, 108.78, 55.35, 54.49, 52.48, 52.43, 51.82, 51.75, 48.16, 45.76, 45.66, 42.50, 39.23, 39.03, 35.76, 34.82, 33.87, 33.17, 33.09, 32.50, 29.22, 26.60, 24.56, 24.18, 19.49, 12.46, 8.60

**4'-((1,5-Pentanediol)methylether)-4,5',8-trimethylpsoralen (18)**

4'-Bromomethyl-4,5',8-trimethylpsoralen (2) (0.21 g) was suspended in 1,5 pentanediol (6.00 ml) and heated (100 °C). After 2 hr, the reaction was allowed to cool to room temperature and diluted with H<sub>2</sub>O (40 ml) and CH<sub>2</sub>Cl<sub>2</sub> (40 ml). The organic solution was collected and washed with NaHCO<sub>3(sat)</sub> (75 ml) and NaCl<sub>(sat)</sub> (40 ml). The organic extract was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. The 4'-((1,5-pentanediol)methyl ether)-4,5',8-trimethylpsoralen (18) was purified on 15 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (96:4). <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.58 (1H, s, H5), 6.22, (1H, s, H3), 3.62 (2H, t, OCH<sub>2</sub>), 3.47 (2H, t, OCH<sub>2</sub>), 2.55 (3H, s, CH<sub>3</sub>), 2.48 (3H, s, CH<sub>3</sub>), 2.47 (3H, s, CH<sub>3</sub>), 1.6-1.4 (6H, m, 3 x CH<sub>2</sub>). C<sub>20</sub>H<sub>24</sub>O<sub>5</sub> 344.39 (calculated) GC-MS 344 (M<sup>+</sup> found)

**(d)-Biotin (4'-(1,5-pentanediol)methylether-4,5',8-trimethylpsoralen) ester (19)**

4'-((1,5-Pentanedithiol)methyl ether)-4,5',8-trimethylpsoralen (**18**) (21 mg, 0.061 mmol) and biotin 4-nitrophenyl ester (0.048 g, 0.128 mmol, 210 M%) were dissolved in 0.5 ml DMF and heated (80 °C). After 30 min, DMAP (50 mg, 0.40 mmol) was added. After stirring overnight with heat, the reaction mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> (50 ml) and washed with H<sub>2</sub>O (15 ml), NaHCO<sub>3(sat)</sub> (15 ml), and NaCl<sub>(sat)</sub> (15 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. Purification was done by reversed phase HPLC on C<sub>18</sub> column using Gradient II. <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.55 (1H, s, H5), 6.20 (1H, s, H3), 5.26 (1H, br, NH), 4.95 (1H, br, NH), 4.57 (2H, s, H4'), 4.45 (1H, m, bio), 4.26 (1H, m, bio), 3.99 (2H, t, OCH<sub>2</sub>), 3.45 (2H, t, OCH<sub>2</sub>), 3.10 (1H, m, bio), 2.87 (1H, dd, bio), 2.70 (1H, d, bio), 2.52 (3H, s, CH<sub>3</sub>), 2.46 (3H, s, CH<sub>3</sub>), 2.45 (3H, s, CH<sub>3</sub>), 2.24 (2H, t, α carbonyl), 1.61 (m, CH<sub>2</sub>), 1.40 (m, CH<sub>2</sub>), 0.80 (m, CH<sub>2</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 173.53, 161.52, 160.51, 154.72, 153.30, 130.51, 125.17, 116.16, 112.12, 111.64, 109.14, 96.14, 69.83, 64.27, 63.12, 61.89, 60.08, 55.25, 40.52, 33.81, 29.69, 29.37, 28.46, 28.30, 28.23, 24.75, 22.80, 19.34, 12.33, 8.50.

**(d)-Biotin (4'-[[[(methylamino)ethyl]methylamino]methyl]-4,5',8-trimethylpsoralen) ester (20)**

4'-(N,N'-Dimethylethylene diaminomethyl)-4,5',8-trimethylpsoralen (**9**) (0.144 g), biotin 4-nitrophenyl ester (0.047 g) and DMAP (0.028 g, 0.22 mmol) were dissolved in 2 ml DMF. After 3 hr at room temperature, DIC (200 μl) and biotin (145 mg) were added. After

stirring overnight, the reaction was diluted with  $\text{CH}_2\text{Cl}_2$  (50 ml) and washed with  $\text{H}_2\text{O}$  (15 ml),  $\text{NaHCO}_3(\text{sat})$  (15 ml), and  $\text{NaCl}(\text{sat})$  (15 ml). The organic solution was dried with  $\text{Na}_2\text{SO}_4$ , filtered, and concentrated. Purification was by reversed phase HPLC on  $\text{C}_{18}$  column using Gradient II.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): appears to be two rotamers, 7.75 & 7.71 (1H, s, H5), 6.27 & 6.26 (1H, s, H3), 4.86 (2H, s, H4'), 4.46 (1H, m, bio), 4.20 (1H, m, bio), 3.65 & 3.62 (2H, s), 3.48 (2H, m), 3.34 & 3.30 (2H, s), 2.9 (2H, m), 3.10 (1H, m, bio), 2.70 (1H, m), 2.54 (3H, s,  $\text{CH}_3$ ), 2.51 (3H, s,  $\text{CH}_3$ ), 2.46 (3H, s,  $\text{CH}_3$ ), 2.35 & 2.34 (3H, s,  $\text{CH}_3$ ), 2.20 & 2.06 (2H, t,  $\alpha$  carbonyl), 1.80-1.20 (m,  $\text{CH}_2$ ).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 173.53, 161.52, 160.51, 154.72, 153.30, 130.51, 125.17, 116.16, 112.82, 112.12, 111.64, 109.15, 96.14, 69.83, 64.27, 63.12, 61.89, 60.78, 55.25, 40.52, 33.81, 29.69, 29.37, 28.46, 28.30, 28.23, 24.75, 22.80, 19.34 ( $\text{CH}_3$ ), 12.33 ( $\text{CH}_3$ ), 8.50 ( $\text{CH}_3$ ).  $\text{C}_{29}\text{H}_{38}\text{O}_5\text{N}_4\text{S}$  554.68 (calculated), FAB found 555  $[\text{MH}]^+$

**tert-Butylcarbonate-glycine-glycyl 4'-[[[(methylamino)ethyl] methylamino]methyl]-4,5',8-trimethylpsoralen) ester (21)**

4'-(N,N'-Dimethylethylene diaminomethyl)-4,5',8-trimethylpsoralen (**9**) (0.117 g, 0.357 mmol), BOC-gly-gly (0.143 g, 0.616 mmol, 170 M%) and DIC (100  $\mu\text{l}$ , 0.643 mmol, 180 M%) were dissolved in pyridine (2 ml). After 5 hr at room temperature, the reaction was concentrated to dryness. The residue was diluted with  $\text{CH}_2\text{Cl}_2$  (20 ml) and washed with  $\text{NaHCO}_3(\text{sat})$  (10 ml) and  $\text{NaCl}(\text{sat})$  (10 ml). The tert-butyl carbonate-glycine-glycyl 4'-[[[(methylamino)ethyl] methylamino]methyl]-4,5',8-trimethylpsoralen) ester (**21**) was purified by column chromatography on 10 g of silica gel eluting with  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  (92:8)



yielding an off white solid (0.145 g, 0.276 mmol, 77% yield) (TLC rf 0.30 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 90 : 10) <sup>1</sup>H NMR (CDCl<sub>3</sub>): appears as two rotamers 7.6 (1H, br, NH), 7.5 (1H, s, H5), 7.0 (1H, br, NH), 6.2 (1H, s, H3), 5.25 (2H, s, H4'), 4.05 & 3.9 (1H, d), 3.8 (2H, t), 3.6 (m), 3.30 (2H, t), 2.82 (two singlets, NCH<sub>3</sub>), 2.60 (m, CH<sub>2</sub>-s), 2.54 (3H, s, CH<sub>3</sub>), 2.51 (3H, s, CH<sub>3</sub>), 2.46 (3H, s, CH<sub>3</sub>), 2.25 (two singlets, NCH<sub>3</sub>), 1.4 (9H, s, BOC).

**Glycine-glycyl 4'-[[[(methylamino)ethyl]methylamino]methyl]-4,5',8-trimethylpsoralen ester (22)**

BOC-gly-gly-dialkylamine psoralen (**21**) (65 mg, 0.119 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (2 ml) and treated with TFA (100 μl). Within 15 min at room temperature, the reaction was concentrated to dryness. Aqueous workup was not done because this compound was soluble in water. The glycine-glycyl 4'-[[[(methylamino)ethyl] methylamino]methyl] -4,5',8-trimethylpsoralen) ester (**22**) was purified by column chromatography on 10 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (84:16) containing trace triethylamine. (50 mg, 0.12 mmol, 100% yield) (TLC rf 0.08 CH<sub>2</sub>Cl<sub>2</sub>/MeOH 80 : 20) The glycine-glycyl 4'-[[[(methylamino)ethyl] methylamino]methyl] -4,5',8-trimethylpsoralen) ester (**22**) was additionally purified by reversed phase C<sub>18</sub> HPLC using Gradient III. <sup>1</sup>H NMR (CDCl<sub>3</sub>): appears as two rotamers 7.6 (1H, br, NH), 7.5 (1H, s, H5), 7.0 (1H, br, NH), 6.2 (1H, s, H3), 5.25 (2H, s, H4'), 4.05 & 3.9 (1H, d), 3.8 (2H, t), 3.6 (m), 3.30 (2H, t), 2.82 (two singlets, NCH<sub>3</sub>), 2.60 (m, CH<sub>2</sub>-s), 2.54 (3H, s, CH<sub>3</sub>), 2.51 (3H, s, CH<sub>3</sub>), 2.46 (3H, s, CH<sub>3</sub>), 2.25 (two singlets, NCH<sub>3</sub>).

### **17-Hemisuccinate estradiol N-hydroxysuccinimide ester (23)**

N-Hydroxysuccinimide (25 mg, 0.22 mmol, 150 M%) and DIC (30  $\mu$ l, 0.29 mmol, 210 M%) were added to 17-hemisuccinate estradiol (53 mg, 0.142 mmol) suspended in  $\text{CH}_2\text{Cl}_2$  (1ml). After 2 hr at room temperature, the reaction was diluted with  $\text{CH}_2\text{Cl}_2$  (10 ml) and washed with  $\text{NaHCO}_{3(\text{sat})}$  (10 ml) and  $\text{NaCl}_{(\text{sat})}$  (10 ml). 17-Hemisuccinate estradiol N-hydroxy succinimide ester (**23**) was purified by column chromatography on 10 g silica gel eluting with  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  (98:2) to produce an off white solid (0.054 g)  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 7.20 (1H, d, H5), 6.82 (1H, dd, H6), 6.77 (1H, d, H2), 2.87 (2H, dd), 2.5-1.8 (7H, m), 1.7-1.45 (6H, m), 0.89 (3H, s,  $\text{CH}_3$ ).

### **17-Hemisuccinate estradiol-glycine-glycyl 4'-[[[(methylamino)ethyl] methylamino]methyl]-4,5',8-trimethylpsoralen) ester (24)**

Gly-gly-dialkylamine (**22**) and NHS-succinate-estradiol (**23**) were allowed to react at room temperature for 3 hr in  $\text{CH}_2\text{Cl}_2$ . No aqueous workup was done. The crude 17-hemisuccinate estradiol-glycine-glycyl 4'-[[[(methylamino)ethyl] methylamino]methyl]-4,5',8-trimethylpsoralen) ester (**24**) was purified by column chromatography on 10 g silica gel eluting with  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  (84:16) containing trace  $\text{Et}_3\text{N}$ . (TLC rf 0.43  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  80:20)  $\text{C}_{45}\text{H}_{56}\text{O}_9\text{N}_4$  796 (calculated) FAB found  $[\text{MH}]^+ = 797$ .

### **17 $\alpha$ -1,3,5[10]-Estratriene-3 $\beta$ -[benzylether]-17 $\beta$ -diol (26)**

Estrone (**25**) (1.07 g, 3.96 mmol) was dissolved in THF (10 ml) and treated with benzyl bromide (1 ml, 8.41 mmol, 210 M%). After 2 hr, K<sub>2</sub>CO<sub>3</sub> (0.229 g) and additional benzyl bromide (1 ml, 210 M%) were added. After 12 hr at room temperature, sodium hydride (0.30 g, 1.25 mmol) was added and the reaction stirred for 30 min. The reaction was concentrated and the residue was resuspended in CH<sub>2</sub>Cl<sub>2</sub> (20 ml) and quenched slowly with H<sub>2</sub>O (20 ml). The organic solution was washed with NaHCO<sub>3(sat)</sub> (20 ml) and NaCl<sub>(sat)</sub> (20 ml), dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. 17 $\alpha$ -1,3,5[10]-Estratriene-3 $\beta$ -[benzyl ether]-17 $\beta$ -diol (**26**) was purified by column chromatography on 25 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub> to produce an off white solid (1.15 g, 3.19 mmol, 81% yield + 0.34 g mixed fractions) (TLC rf 0.54 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 98:2 ) <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.43-7.30 (5H, m, Bn), 7.18 (1H, d, H5), 6.76 (1H, dd, H6), 6.72 (1H, d, H2), 5.20 (2H, s, CH<sub>2</sub>Ar ), 2.86 (2H, m), 2.6-1.9 (m, CH<sub>2</sub>-s), 1.6-1.3 (m, CH<sub>2</sub>-s), 0.889 (3H, s, CH<sub>3</sub>)

### **17 $\alpha$ -[3'-Butynyl-1'-ol]-1,3,5[10]-estratriene-3 $\beta$ -[benzyl ether]-17 $\beta$ -diol (27)**

Potassium<sub>(s)</sub> (0.33 g, 8.46 mmol) was added in small portions to chilled (-78 C) solution of NH<sub>3(l)</sub> (15 ml) in a 3 neck 100 ml flask with chilled cold finger. Almost immediately the solution turned black/blue. After the addition of 3-butyn-1-ol (0.70 ml , 0.926 mmol), the cold bath was removed. Upon warming, the solution turns clear. After 15 min, pyridine (10 ml) and the 17 $\alpha$ -1,3,5[10]-estratriene-3 $\beta$ -[benzyl ether]-17 $\beta$ -diol (**26**) (0.76 g, 2.1 mmol) were added. After 2 hr, the reaction was concentrated. The residue was redissolved

in CH<sub>2</sub>Cl<sub>2</sub> (20ml) and quenched slowly with H<sub>2</sub>O (20ml). The organic solution was washed with NaHCO<sub>3(sat)</sub> (20ml) and NaCl<sub>(sat)</sub> (20ml), dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated (1.22 g). 17 $\alpha$ -[3'-Butynyl-1'-ol]-1,3,5[10]-estratriene-3 $\beta$ -[benzyl ether]-17 $\beta$ -diol (**27**) was purified by column chromatography on 25 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>. (0.17 g, 0.39 mmol, 20 % yield + 0.22 g mixed fractions) (TLC rf 0.08 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 98:2 ) <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.46-7.28 (5H, m, Bn), 7.16 (1H, d, H5), 6.72 (1H, dd, H6), 6.66 (1H, d, H2), 5.31 (2H, s, CH<sub>2</sub>Ar ), 3.64 (2H, t, CH<sub>2</sub>OH), 2.86 (2H, m), 2.43 (2H, t, H3'), 2.4-2.2 (m, CH<sub>2</sub>-s), 2.0-1.5 (m, CH<sub>2</sub>-s), 1.5-1.2 (m, CH<sub>2</sub>-s), 0.81 (3H, s, CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 156.01 (C1), 137.47 (C3), 136.73 (Bn), 132.54 (C4), 127.91 (Bn), 127.26 (Bn), 126.91 (Bn), 125.78 (C5), 114.25 (C2), 111.77 (C6), 84.62, 82.00, 78.98, 69.60, 60.37, 49.00, 48.72, 48.43, 48.29, 48.15, 47.86, 47.58, 47.30, 46.68, 43.28, 39.14, 38.31, 32.52, 29.42, 26.91, 26.11, 22.46, 22.36, 12.29.

#### **17 $\alpha$ -1,3,5[10]-Estratriene-3 $\beta$ -[methoxymethyl ether]-17 $\beta$ -diol (**28**)**

Chloromethylmethyl ether (2.00 ml 26.33 mmol, 140 M%) and sodium hydride (0.77 g, 32 mmol, 170 M%) were added to estrone (**25**) (5.03 g, 18.60 mmol) suspended in THF (85 ml). After 30 min at room temperature, additional sodium hydride (0.45 g, 18.75 mmol, 100 M%) was added. After 1 1/2 hr, the reaction was diluted with ethylacetate (100 ml) and washed with NaHCO<sub>3(sat)</sub> (100 ml) and NaCl<sub>(sat)</sub> (100 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. 17 $\alpha$ -1,3,5[10]-Estratriene-3 $\beta$ -[methoxymethyl ether]-17 $\beta$ -diol (**28**) was purified by column chromatography on 100 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (98:2) to yield an off white solid (4.90 g, 15.8 mmol , 85 % yield). (TLC rf

0.48 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 98:2 ) <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.20 (1H, d, H5), 6.82 (1H, dd, H6), 6.77 (1H, d, H2), 5.13 (2H, s, OCH<sub>2</sub>O), 3.46 (3H, s, OCH<sub>3</sub>), 2.87 (2H, dd, CH<sub>2</sub>), 2.5-1.8 (7H, m, CH<sub>2</sub>), 1.7-1.45 (6H, m, CH<sub>2</sub>), 0.89 (3H, s, CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 155.11 (C1), 137.76 (C3), 133.21 (C4), 126.29 (C5), 116.17 (C2), 113.79 (C6), 94.35, 55.84, 55.78, 50.31, 47.92, 43.94, 38.19, 35.79, 31.48, 29.53, 26.43, 25.80, 21.51, 13.77.

### **5-Hexyn-1-t-butyldimethylsilyl (29)**

t-Butyldimethylsilyl chloride (3.18 g, 21.2 mmol, 210 M%) and sodium hydride (0.74 g, 30.88 mmol) were added to 5-hexyn-1-ol (1.02 g, 10.19 mmol) dissolved in THF (10 ml). The reaction was quenched after 20 min with water (20 ml) and diluted with ethylacetate (50 ml). The organic solution was collected and washed with NaCl<sub>(sat)</sub> (25 ml), dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. 5-hexyn-1-t-butyldimethylsilyl (**29**) was purified by column chromatography on 25 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub> yielding a oil (1.78 g, 8.39 mmol, 82% yield). (TLC rf 0.60 4% ethylacetate/ hexane) <sup>1</sup>H NMR (CDCl<sub>3</sub>): 3.61 (2H, t, 2 x H1), 2.19 (2H,dt, 2 x H4), 1.92 (1H,t,H6), 1.7-1.5 (4H, m, 4 x CH<sub>2</sub>), 0.87 (9H, s, tBuSi), 0.028 (6H, s, MeSi). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 84.52 (C5), 68.23 (C6), 62.58 (C1), 31.81, 25.96 (SitBu), 25.69, 24.97, 18.33, 18.22, 12.52, -5.32 (SiMe).

**17-[5'-Hexynyl-1'-t-butyldimethylsilyl]-1,3,5[10]-estratriene-3 $\beta$  -[methoxymethyl ether]-17 $\beta$ -diol (30)**

5-Hexyn-1-t-butyldimethylsilyl (**29**) (0.66 g, 3.11 mmol, 210 M%) was dissolved in THF (10 ml) and hexamethylphosphoramide (0.54 ml 0.56 g, 3.10 mmol, 210 M%). The solution was chilled with a dry ice/ isopropanol bath before adding 2M BuLi (in Et<sub>2</sub>O) (1.5 ml, 3.0 mmol, 205 M%). After 10 min at -78 °C, MOM-estrone (**28**) (0.46 g, 1.46 mmol) was added and the cold bath removed. After 6 hr at room temperature, the reaction was diluted with ethylacetate (25 ml) and H<sub>2</sub>O (25 ml). The organic solution was collected and washed with NaHCO<sub>3(sat)</sub> (25 ml) and NaCl<sub>(sat)</sub> (25 ml), dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. 17 $\alpha$ -[5'-Hexynyl-1'-t-butyldimethylsilyl]-1,3,5[10]-estratriene-3 $\beta$  -[methoxymethyl ether]-17 $\beta$ -diol (**30**) was purified by column chromatography on 35 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub> increasing to CH<sub>2</sub>Cl<sub>2</sub>/MeOH (98:2). (0.40 g 0.76 mmol 52 % yield plus 0.32 g mixed fractions containing mom-estrone) (TLC rf 0.42 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 98:2 )  
<sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.12 (1H, d, H5), 7.73 (1H, dd, H6), 6.62 (1H, d, H2), 5.07 (2H, s, OCH<sub>2</sub>O), 3.56 (2H, t, OCH<sub>2</sub>), 3.40 (3H, s, OCH<sub>3</sub>), 2.74 (2H, br, CH<sub>2</sub>), 2.26 (2H, t, 2 x H4'), 2.4-1.2 (17H, m, CH<sub>2</sub>), 0.87 (9H, s, tBuSi), 0.81 (3H, s, CH<sub>3</sub>), 0.03 (6H, s, MeSi). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 154.77 (C1), 137.82 (C3), 133.72 (C4), 126.18 (C5), 116.04 (C2), 113.56 (C6), 94.30, 85.88, 83.88, 79.78, 62.57, 55.78, 49.44, 47.12, 43.71, 39.37, 39.13, 32.90, 32.01, 29.81, 27.29, 26.44, 25.96 (SitBu), 25.38, 22.82, 18.65, 18.32, 12.87, -5.20 (SiMe)

**17 $\alpha$ -[5'-Hexynyl-1'-ol]-1,3,5[10]-estratriene-3 $\beta$ -[methoxymethyl ether]-17 $\beta$ -diol (31)**

In a plastic vial, 17 $\alpha$ -[5'-hexynyl-1'-t-butyldimethylsilyl]-1,3,5[10]-estratriene-3 $\beta$ -[methoxymethyl ether]-17 $\beta$ -diol (**30**) (0.40 g, 0.76 mmol) dissolved in pyridine (5 ml) was treated with 60-70% HF/pyridine (1.0 ml). After 15 min at room temperature, additional HF/pyridine (1.0 ml) was added. After 30 min, the reaction was concentrated. The residue was redissolved in CH<sub>2</sub>Cl<sub>2</sub> (25 ml) and washed with H<sub>2</sub>O (10 ml) and NaCl<sub>(sat)</sub> (10 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. 17 $\alpha$ -[5'-hexynyl-1'-ol]-1,3,5[10]-estratriene-3 $\beta$ -[methoxymethyl ether]-17 $\beta$ -diol (**31**) was purified by column chromatography on 25 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (95:5) with increasing to CH<sub>2</sub>Cl<sub>2</sub>/MeOH (90:10). (0.31 g, 0.75 mmol, 99% yield) (TLC rf 0.40 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 95:5) <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.12 (1H, d, H5), 6.73 (1H, dd, H6), 6.62 (1H, d, H2), 5.07 (2H, s, OCH<sub>2</sub>O), 3.56 (2H, t, OCH<sub>2</sub>), 3.40 (3H, s, OCH<sub>3</sub>), 2.74 (2H, br, CH<sub>2</sub>), 2.26 (2H, t, H4'), 2.4-1.2 (17H, m, CH<sub>2</sub>) 0.81 (3H, s, CH<sub>3</sub>). <sup>13</sup>C NMR (CD<sub>3</sub>OD): 156.17(C1), 138.68 (C3), 134.71 (C4), 127.60 (C5), 117.10 (C2), 114.63 (C6), 95.37 (MOM), 86.13, 85.26, 80.36, 62.35, 56.06, 50.70, 45.07, 40.85, 40.00, 34.14, 32.76, 30.72, 28.53, 27.64, 26.44, 23.74, 19.32, 13.59.

**17 $\alpha$ -[5'-Hexynyl-1'-ol]-1,3,5[10]-estratriene-3 $\beta$ ,17 $\beta$ -diol (32)**

17 $\alpha$ -[5'-Hexynyl-1'-ol]-1,3,5[10]-estratriene-3 $\beta$ -[methoxymethyl ether]-17 $\beta$ -diol (**31**) (61 mg) was dissolved in MeOH (2ml) and treated with HCl<sub>(con)</sub> (20  $\mu$ l) at room temperature. Additional HCl<sub>(con)</sub> (20  $\mu$ l and 40  $\mu$ l, respectively) was added after 15 min and after 1 hr.

After 3 hr, the reaction mixture was concentrated. The residue was redissolved in  $\text{CH}_2\text{Cl}_2$  (25 ml) and washed with  $\text{H}_2\text{O}$  (10 ml) and  $\text{NaCl}_{(\text{sat})}$  (10 ml). The organic solution was dried with  $\text{Na}_2\text{SO}_4$ , filtered, and concentrated.  $17\alpha$ -[5'-Hexynyl-1'-ol]-1,3,5[10]-estratriene- $3\beta,17\beta$ -diol (**32**) was purified by column chromatography on 20 g silica gel eluting with  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  (90:10). (TLC rf 0.30  $\text{CH}_2\text{Cl}_2$  :  $\text{MeOH}$  95:5) (50 mg)  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 7.08 (1H, d, H5), 6.61 (1H, dd, H6), 6.55 (1H, d, H2), 3.64 (2H, t,  $\text{CH}_2\text{O}$ ), 2.73 (2H, br), 2.24 (2H, t, H4'), 2.3 -1.2 (m,  $\text{CH}_2$ -s), 0.810 (3H, s,  $\text{CH}_3$ ).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 153.50 (C1), 138.02 (C3), 132.19 (C4), 126.35 (C5), 115.23 (C2), 112.70 (C6), 85.94, 84.09, 80.00, 62.33, 53.44, 49.51, 47.20, 43.71, 39.50, 39.18, 32.97, 31.90, 29.72, 27.33, 26.54, 25.18, 22.88, 18.69, 12.94

#### **4-[p-(Bis[2-Chloroethyl]amino)-phenyl]pentyl amine (33)**

Ethylchloroformate (1.00 ml, 7.19 mmol, 110 M%) dissolved in 5 ml acetone was added to a cold solution (0 °C) of chlorambucil (2.00 g, 6.57 mmol) dissolved in acetone (5 ml) and triethyleamine (1 ml). Upon addition of the ethylchloroformate solution, a solid precipitated from solution. After 10 min, sodium azide (0.754g, 6.95 mmol, 1.06 mmol) dissolved in 5 ml water was added whereby the white suspension disappeared. After 20 min, the reaction was diluted with benzene (50 ml) and washed with ice water (100 ml) and  $\text{NaCl}_{(\text{sat})}$  (100 ml). The benzene solution was then dried with  $\text{Na}_2\text{SO}_4$ , filtered, and then heated (65 °C). After 1 hr, the reaction was concentrated and the residue treated with  $\text{HCl}_{(\text{con})}$  (25 ml). After 10 min, the reaction was concentrated to dryness. The 4-[p-(bis[2-Chloroethyl]amino)-phenyl]pentyl amine (**33**), hydrochloride salt was collected by suspending the material in



CH<sub>2</sub>Cl<sub>2</sub> and filtering the off white solid. <sup>1</sup>H NMR (D<sub>2</sub>O): 7.40 (2H, d, Ar), 7.32 (2H, d, Ar), 3.90 (4H, t, NCH<sub>2</sub>), 3.39 (4H, t, 2 x CH<sub>2</sub>Cl), 2.85 (2H, t, NCH<sub>2</sub>), 2.60 (2H, t, CH<sub>2</sub>Ar), 1.82 (2H, m, CH<sub>2</sub>). <sup>13</sup>C NMR (D<sub>2</sub>O): 146.34, 135.08, 133.09, 124.56, 61.27 (CH<sub>2</sub>Cl), 41.33 (H<sub>2</sub>NCH<sub>2</sub>), 39.71 (CH<sub>2</sub>NAr), 33.75 (CH<sub>2</sub>), 30.44 (CH<sub>2</sub>Ar),

**17 $\alpha$ -[5'-Hexynyl-1'-carbamoyl-[4-[p-(bis[2-Chloroethyl]amino)-phenyl]pentyl amine]]-1,3,5[10]-estratriene-3 $\beta$ -[methoxymethyl ether]-17 $\beta$ -diol (34)**

p-Nitrophenyl chloroformate (0.137 g, 0.68 mmol, 190 M%) was added to MOM-estrone (31) (0.15 g, 0.36 mmol) dissolved in THF (3.5 ml) and pyridine (0.5 ml). After 30 min, additional p-nitrophenyl chloroformate (0.084 g) was added. After 1.5 hr, the reaction mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> (25 ml) and washed with H<sub>2</sub>O (10 ml) and NaCl<sub>(sat)</sub> (10 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. The crude activated carbamoyl ester was used without purification in the next step. (TLC rf 0.82 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 95:5)

4-[p-(bis[2-Chloroethyl]amino)-phenyl]pentyl amine (33) (0.101 g) was added to the crude activated carbamoyl ester dissolved in (1:1) pyridine: THF (8 ml) and the mixture was then heated (T<sub>b</sub> = 60 °C). Additional amine (33) was added after 1 1/2h (0.069 g) and after 4 1/2 hr (0.102 g). After 12 hr, the reaction was diluted with ethylacetate (40 ml) and washed with NaHCO<sub>3(sat)</sub> (25 ml) and NaCl<sub>(sat)</sub> (25 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. 17 $\alpha$ -[5'-Hexynyl-1'-carbamoyl-[4-[p-(bis[2-Chloroethyl]amino)-phenyl]pentyl amine]]-1,3,5[10]-estratriene-3 $\beta$  -[methoxymethyl ether]-17 $\beta$ -diol (34) was purified by column chromatography on 25 g silica gel eluting with

hexane/ethylacetate (2:1). (170 mg, 67% yield for two steps) (TLC rf 0.90 CH<sub>2</sub>Cl<sub>2</sub>/MeOH 95:5). <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.20 (1H, d, H5), 7.04 (2H, d, H2"), 6.80 (1H, dd, H6), 6.75 (1H, d, H2), 6.63 (2H, d, H3"), 5.12 (2H, s, OCH<sub>2</sub>O), 4.56, 4.07 (2H, t, OCH<sub>2</sub>), 3.60 (8H, dd, NCH<sub>2</sub>CH<sub>2</sub>Cl), 3.45 (3H, s, OCH<sub>3</sub>), 3.13 (2H, q, NCH<sub>2</sub>), 2.83 (2H, m, CH<sub>2</sub>), 2.52 (2H, t, Ar CH<sub>2</sub>), 2.30 (2H, t, H5'), 2.4-2.1 (m, CH<sub>2</sub>-s), 2.1-1.2 (18H, m, CH<sub>2</sub>), 0.84 (3H, s, CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 156.62 (C4"), 154.98 (C1), 144.22 (C1"), 138.02 (C3), 133.86 (C4), 129.49 (C2"), 126.31 (C5), 116.22 (C2), 113.72 (C6), 112.32 (C3"), 94.45 (MOM), 85.51, 84.38, 79.86, 64.22, 55.83, 53.60 (CH<sub>2</sub>Cl), 49.48, 47.12, 43.68, 40.42 (Ar NCH<sub>2</sub>), 39.32, 39.07, 32.89, 31.74, 29.74, 28.25, 27.22, 26.37, 24.97, 22.76, 18.45, 12.77.

**17 $\alpha$ -[5'-Hexynyl-1'-carbamoyl-[4-[p-(bis[2-Chloroethyl]amino)-phenyl]pentylamine]]-1,3,5[10]-estratriene-3 $\beta$ ,17 $\beta$ -diol (35)**

Hydrochloric acid<sub>(con)</sub> (100  $\mu$ l) was added to 17 $\alpha$ -[5'-hexynyl-1'-carbamoyl-[4-[p-(bis[2-chloroethyl]amino)-phenyl]pentyl amine]]-1,3,5[10]-estratriene-3 $\beta$  -[methoxymethyl ether]-17 $\beta$ -diol (**34**) (40 mg) dissolved in MeOH (2 ml). After 1 hr at room temperature, the reaction mixture was concentrated. The residue was diluted with CH<sub>2</sub>Cl<sub>2</sub> (15 ml) and washed with H<sub>2</sub>O (10 ml) and NaCl<sub>(sat)</sub> (10 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. 17 $\alpha$ -[5'-Hexynyl-1'-carbamoyl-[4-[p-(bis[2-Chloroethyl]amino)-phenyl]pentylamine]]-1,3,5[10]-estratriene-3 $\beta$ ,17 $\beta$ -diol (**35**) was purified by column chromatography on 25 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (96:4). The purity was determined by reversed-phase HPLC on a C<sub>18</sub> column using gradient IV. (TLC

rf 0.29 CH<sub>2</sub>Cl<sub>2</sub>/MeOH 95:5 or TLC rf 0.70 ethylacetate/hexane 2:1 ) <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.15 (1H, d, H5), 7.06 (2H, d, H2"), 6.71 (2H, dd, H3"), 6.59 (1H, dd, H6), 6.53 (1H, d, H2), 5.28 (1H, br, OH), 4.59 (1H, br, OH), 4.07 (2H, t, OCH<sub>2</sub>), 3.60 (8H, dq, NCH<sub>2</sub>CH<sub>2</sub>Cl), 3.13 (2H, q, NCH<sub>2</sub>), 2.79 (2H, m), 2.51 (2H, t, Ar CH<sub>2</sub>), 2.27 (2H, t, H5'), 2.4-1.2 (H, m, CH<sub>2</sub>-s), 0.85 (3H, s, CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 156.74 (C4"), 153.48 (C1), 144.17 (C1"), 138.21 (C3), 132.48 (C4), 129.56 (C2"), 126.47 (C5), 115.27 (C2), 112.72 (C6), 112.46 (C3"), 85.61, 84.38, 79.98, 64.35, 53.69 (CH<sub>2</sub>Cl), 49.51, 47.18, 43.67, 40.51, 40.42 (ArNCH<sub>2</sub>), 39.42, 39.08, 39.00, 32.92, 31.79, 31.72, 29.64, 28.28, 27.25, 26.46, 25.01, 22.78, 18.50, 12.81, 12.50. λ<sub>max</sub> = 258 (ε = 1.6 x 10<sup>4</sup>), λ<sub>max</sub> = 254 (ε = 1.6 x 10<sup>4</sup>), λ<sub>max</sub> = 280 (ε = 3.5 x 10<sup>3</sup>). EIMS expected exact molecular weight 668.31477, high resolution result for m/z=668 (668.3154).

### 7-Octyn-1-t-butyldiphenylsilyl (36)

t-Butyldiphenylsilylchloride (22 ml, 84.60 mmol, 155 M% ) and sodium hydride (1.86 g, 77.5 mmol, 140 M%) were added to 6-bromo-1-hexanol (9.89 g, 54.40 mmol) dissolved in THF (100 ml). After 1 hr at room temperature, additional sodium hydride (0.99 g, 41.25 mmol, 75 M%) and t-butyldiphenylsilylchloride (11 ml, 42.30 mmol, 78 M%) were added. After 4 1/2 hr, the reaction was concentrated. The residue was redissolved in CH<sub>2</sub>Cl<sub>2</sub> (100 ml) and quenched slowly with H<sub>2</sub>O (50 ml). The organic solution was washed with NaHCO<sub>3(sat)</sub> (50 ml) and NaCl<sub>(sat)</sub> (50 ml), dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. 7-Octyn-1-t-butyldiphenylsilyl (36) was purified by column chromatography on 300 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub> . (Note: this solvent is too fast and does not allow separation of the

compound from tBDPSiOH. Use hexane systems as done for the 8-bromo-1-t-butyldiphenylsilyl octanol). The impure 6-bromo-1-t-butyldiphenylsilyl hexanol was used without any further purification in the next step. (TLC rf 0.60 ethylacetate/hexane 4:96 - use  $\text{H}_2\text{SO}_4$  stain).

8-Bromo-1-t-butyldiphenyl silyl octanol (9.10 g, 21.69 mmol) was added to an ice bath-cooled suspension of 90% LAEDA (3.25 g, 31.76 mmol 150 M%) dissolved in 50 ml  $\text{DMSO}_{(\text{anhydrous})}$ . After 3 hr, the reaction was diluted with  $\text{CH}_2\text{Cl}_2$  (100 ml) and washed with  $\text{H}_2\text{O}$  (50 ml),  $\text{NaHCO}_{3(\text{sat})}$  (50 ml), and  $\text{NaCl}_{(\text{sat})}$  (50 ml). The organic solution was dried with  $\text{Na}_2\text{SO}_4$ , filtered, and concentrated. 7-Octyn-1-t-butyldiphenylsilyl (**36**) was purified by column chromatography on 100 g silica gel eluting with ethylacetate/hexane (4:96). (4.61 g, 12.68 mmol, 60 % yield). (TLC rf 0.60 ethylacetate/hexane 4:96) (sulfuric acid or anisaldehyde stain).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 7.66 (4H, m, Ar), 7.39 (6H, m, Ar), 3.65 (2H, t, H1), 2.16 (2H, dt, H6), 1.93 (1H, t, H8), 1.60-1.50 (4H, m,  $\text{CH}_2$ ), 1.40-1.35 (4H, m,  $\text{CH}_2$ ), 1.04 (9H, s, tBu).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 135.56 ( $\text{C1}'$ , ArSi) 134.14 ( $\text{C2}'$ , ArSi), 129.49 ( $\text{C4}'$ , Ar Si), 127.56 ( $\text{C3}'$ , Ar Si), 84.63 (C7), 68.12 (C8), 63.83 (C1), 32.40, 28.44, 27.09, 26.87 ( $\text{C}(\text{CH}_3)_3$ ), 25.27,, 19.22, 18.35.

### 9-Decyn-1-t-butyldiphenylsilyl (**37**)

t-Butyldiphenylsilylchloride (18 ml, 69.22 mmol, 140 M%) and sodium hydride (1.73 g, 72.08 mmol, 150 M%) were added to 8-bromo-1-octanol (10.15 g ,48.53 mmol) dissolved in THF (100 ml). After 3 hr at room temperature, additional t-Butyldiphenylsilylchloride

(7.23 g, 26.39 mmol, 55 M%) and sodium hydride (1.7 g, 150 M%) were added. After stirring overnight at room temperature, the reaction was concentrated to near dryness. The residue was redissolved in  $\text{CH}_2\text{Cl}_2$  (100 ml) and washed with  $\text{H}_2\text{O}$  (100 ml),  $\text{NaHCO}_3(\text{sat})$  (100 ml), and  $\text{NaCl}(\text{sat})$  (100 ml). The organic solution was dried with  $\text{Na}_2\text{SO}_4$ , filtered, and concentrated. 9-Decyn-1-t-butylidiphenylsilyl (37) was purified by column chromatography on 300 g silica gel eluting with hexane increasing to ethylacetate/hexane (5:95). (15.08 g, 33.70 mmol, 70% yield) (TLC rf 0.58, ethylacetate/hexane 5:95 - use  $\text{H}_2\text{SO}_4$  stain).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 7.68 (4H, m, Ar), 7.40 (6H, m, Ar), 3.67 (2H, t, H1), 2.18 (2H, dt, H8), 1.94 (1H, t, H10), 1.60-1.50 (4H, m, 2 x  $\text{CH}_2$ ), 1.10-1.28 (8H, m, 4 x  $\text{CH}_2$ ), 1.06 (9H, s, tBu).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 135.47 (C1', Ar), 134.05 (C2', Ar), 129.39 (C4', Ar), 127.47 (C3', Ar), 63.95 (C1), 34.11, 32.90, 32.58, 29.24, 28.81, 28.20, 26.98 ( $\text{C}(\text{CH}_3)_3$ ), 25.76, 19.34

8-Bromo-1-t-butylidiphenyl silyl octanol (15 g, 37 mmol) was added to an ice bath cooled suspension of 90% LAEDA (5.16 g, 50.48 mmol 150 M%) dissolved in  $\text{DMSO}(\text{anhydrous})$  (50 ml). After 3 hr at room temperature, the reaction was diluted with  $\text{CH}_2\text{Cl}_2$  (300 ml) and washed with  $\text{H}_2\text{O}$  (300 ml). The organic solution was dried with  $\text{Na}_2\text{SO}_4$ , filtered, and concentrated. 9-Decyn-1-t-butylidiphenylsilyl (37) was purified by column chromatography on 150 g silica gel eluting with  $\text{CH}_2\text{Cl}_2$ . (10.0 g 25.5 mmol, 68 % yield) (TLC rf 0.60 ethylacetate/hexane 4:96)  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 7.68 (4H, m, Ar), 7.40 (6H, m, Ar), 3.67 (2H, t, H1), 2.18 (2H, dt, H8), 1.94 (1H, t, H10), 1.60-1.50 (4H, m, 2 x  $\text{CH}_2$ ), 1.40-1.28 (8H, m, 4 x  $\text{CH}_2$ ), 1.06 (9H, s, tBu).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 135.45 (C1', ArSi) 134.06 (C2',

ArSi), 129.37 (C4', ArSi), 127.46 (C3', ArSi), 84.73 (C9), 68.08 (C10), 63.99 (C1), 32.68, 29.30, 29.15, 28.78, 28.57, 26.98 (C(CH<sub>3</sub>)<sub>3</sub>), 25.81, 19.34, 18.51.

**17 $\alpha$ -[7'-Hexynyl-1'-t-butyldiphenylsilyl]-1,3,5[10]-estratriene-3 $\beta$  -[methoxymethyl ether]-17 $\beta$ -diol (38)**

n-Butyllithium (2M in Et<sub>2</sub>O) (4.0 ml, 8.0 mmol) was added to 7-octyn-1-t-butyldiphenylsilyl (36) (2.70 g, 7.43 mmol, 187 M%), dissolved in THF (25 ml) and hexamethylphosphoramide (2.86 g, 15.97 mmol), and chilled with a dry ice/ isopropanol bath. After 5 min, 17 $\alpha$ -1,3,5[10]-estratriene-3 $\beta$  -[methoxymethyl ether]-17 $\beta$ -diol (28) (1.25 g, 3.97 mmol) was added and the cold bath removed. After 5 hr, the reaction was concentrated to dryness. The residue was redissolved in CH<sub>2</sub>Cl<sub>2</sub> (100 ml) and washed with NaHCO<sub>3(sat)</sub> (50 ml) and NaCl<sub>(sat)</sub> (50 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. 17 $\alpha$ -[7'-Hexynyl-1'-t-butyldiphenylsilyl]-1,3,5[10]-estratriene-3 $\beta$  -[methoxymethyl ether]-17 $\beta$ -diol (38) was purified by column chromatography on 50 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub> increasing to CH<sub>2</sub>Cl<sub>2</sub>/MeOH (98:2). (1.61 g, 2.41 mmol, 61% yield) (TLC rf 0.68 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 98:2). <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.66 (4H, m, Ar Si), 7.38 (6H, m, Ar Si), 7.20 (1H, d, H5), 6.80 (1H, dd, H6), 6.76 (1H, d, H2), 5.28 (2H, s, OCH<sub>2</sub>O), 3.64 (2H, t, OCH<sub>2</sub>), 3.46 (3H, s, OCH<sub>3</sub>), 2.85 (2H, m, CH<sub>2</sub>), 2.22 (2H, t, H7'), 2.4-1.3 (m, CH<sub>2</sub>-s), 1.03 (9H, s, tBu), 0.85 (3H, s, CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 154.87 (C1), 137.95 (C3), 135.43 (C1', ArSi), 133.99 (C2', ArSi), 133.83 (C4), 129.37 (C4', ArSi), 127.45 (C3', ArSi), 126.30 (C5), 116.14 (C2), 113.66 (C6), 94.44, 86.33 (C8, alkyne), 83.70 (C7, alkyne), 79.97, 63.89 (C1, alkyne), 55.92, 49.52, 47.22, 43.80, 39.45, 39.20,

32.96, 32.57 (alkyne), 29.89, 28.84, 28.73 (alkyne), 27.35, 27.17 (alkyne), 26.95 (C(CH<sub>3</sub>)<sub>3</sub>), 26.52, 25.35 (alkyne), 22.89, 19.31 (alkyne), 18.81 (alkyne), 12.92.

**17 $\alpha$ -[9'-Decynyl-1'-t-butylidiphenylsilyl]-1,3,5[10]-estratriene-3 $\beta$  -[methoxymethyl ether]-17 $\beta$ -diol (39)**

n-Butyllithium (2M in Et<sub>2</sub>O) (4.0 ml, 8.0 mmol) was added to 9-decyn-1-t-butylidiphenylsilyl (37) (1.54g, 4.24 mmol, 125 M%), dissolved in THF (25 ml) and hexamethylphosphoramide (1.51 g, 8.44 mmol), and chilled with a dry ice/ isopropanol bath. After 5 min, 17 $\alpha$ -1,3,5[10]-estratriene-3 $\beta$  -[methoxymethyl ether]-17 $\beta$ -diol (28) (1.25 g, 3.97 mmol) was added and the cold bath removed. After 6 hr, the reaction was concentrated to dryness. The residue was redissolved in CH<sub>2</sub>Cl<sub>2</sub> (50 ml) and washed with NaHCO<sub>3(sat)</sub> (25 ml) and NaCl<sub>(sat)</sub> (25 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated (6.44 g crude). 17 $\alpha$ -[9'-Decynyl-1'-t-butylidiphenylsilyl]-1,3,5[10]-estratriene-3 $\beta$  -[methoxymethyl ether]-17 $\beta$ -diol (39) was purified by column chromatography on 50 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub> increasing to CH<sub>2</sub>Cl<sub>2</sub>/MeOH (98:2). (TLC rf 0.66 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 98:2). <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.65 (4H, d, ArSi), 7.37 (6H, m, ArSi), 7.18 (1H, d, H5), 6.82 (1H, dd, H6), 6.75 (1H, d, H2), 5.12 (2H, s, OCH<sub>2</sub>O), 3.61 (2H, t, OCH<sub>2</sub>), 3.45 (3H, s, OCH<sub>3</sub>), 2.81 (2H, m, CH<sub>2</sub>), 2.21 (2H, t, C8'), 2.4-1.2 (m, CH<sub>2</sub>), 1.02 (9H, s, tBu), 0.84 (3H, s, CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 155.08 (C1), 138.08 (C3), 135.57 (C1'', Ar Si), 134.22 (C2'', Ar Si), 133.99 (C4), 129.45 (C4'', Ar Si), 127.56 (C3'', Ar Si), 126.37 (C5), 116.28 (C2), 113.78 (C6), 94.53 (E), 86.43 (E), 83.76 (C9'),

77.99 (C10'), 63.98 (C1'), 55.87, 49.53, 47.20, 43.77, 39.42, 32.91, 32.57, 29.79, 29.30, 29.04, 28.84, 28.77, 27.27, 26.90, 26.44, 25.74, 22.81, 19.21, 18.77, 12.82

**17 $\alpha$ -[7'-Octynyl-1'-ol]-1,3,5[10]-estratriene-3 $\beta$ -[methoxymethyl ether]-17 $\beta$ -diol (40)**

In a plastic vial, 17 $\alpha$ -[7'-hexynyl-1'-t-butyldiphenylsilyl]-1,3,5[10]-estratriene-3 $\beta$ -[methoxymethyl ether]-17 $\beta$ -diol (**38**) (1.61 g, 2.41 mmol) dissolved in pyridine (25 ml) was treated with 60-70% HF / pyridine (2.00 ml). After 30 min at room temperature, additional HF/pyridine (5.00 ml) was added. After 1 hr, the reaction was concentrated to dryness. The residue was redissolved in CH<sub>2</sub>Cl<sub>2</sub> (100 ml) and washed with NaHCO<sub>3(sat)</sub> (50 ml) and NaCl<sub>(sat)</sub> (50 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. The 17 $\alpha$ -[7'-octynyl-1'-ol]-1,3,5[10]-estratriene-3 $\beta$ -[methoxymethyl ether]-17 $\beta$ -diol (**40**) was purified by column chromatography on 50 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (96:4) increasing to CH<sub>2</sub>Cl<sub>2</sub>/MeOH (90:10). (0.71 g, 1.51 mmol, 63 % yield). (TLC rf 0.18 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 98:2) <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.13 (1H, d, H5), 6.74 (1H, dd, H6), 6.68 (1H, d, H2), 5.06 (2H, s, OCH<sub>2</sub>O), 3.49 (2H, t, OCH<sub>2</sub>), 3.39 (3H, s, OCH<sub>3</sub>), 2.75 (2H, m, CH<sub>2</sub>), 2.17 (2H, t, H7'), 2.4-1.3 (m, CH<sub>2</sub>-s), 0.77 (3H, s, CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 154.42 (C1), 137.60 (C3), 133.59 (C4), 125.92 (C5), 115.80 (C2), 113.28 (C6), 94.03, 85.27 (C8, alkyne), 83.56 (C7, alkyne), 79.18, 61.80 (C1, alkyne), 55.47, 49.13, 46.86, 43.47, 39.13, 38.67, 32.59, 32.09 (alkyne), 29.51, 28.43, 28.33 (alkyne), 27.03, 27.03 (alkyne), 26.21, 24.97 (alkyne), 22.52, 18.41 (alkyne), 12.56.



**17 $\alpha$ -[9'-Decynyl-1'-ol]-1,3,5[10]-estratriene-3 $\beta$ -[methoxymethyl ether]-17 $\beta$ -diol (41)**

In a plastic vial, 17 $\alpha$ -[9'-decynyl-1'-t-butyldiphenylsilyl]-1,3,5[10]-estratriene-3 $\beta$ -[methoxymethyl ether]-17 $\beta$ -diol (**39**) (4.5 g, 2.41 mmol (slightly contaminated with MOM-estrone) dissolved in pyridine (75 ml) was treated with 60-70% HF/pyridine (5.00 ml). After 45 min at room temperature, an additional 5.00 ml HF was added. After 4 hr, the reaction was concentrated to dryness. The residue redissolved in CH<sub>2</sub>Cl<sub>2</sub> (100 ml) and washed with NaHCO<sub>3(sat)</sub> (50 ml) and NaCl<sub>(sat)</sub> (50 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. 17 $\alpha$ -[9'-Decynyl-1'-ol]-1,3,5[10]-estratriene-3 $\beta$ -[methoxymethyl ether]-17 $\beta$ -diol (**41**) was purified by column chromatography on 40 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (96:4) increasing to CH<sub>2</sub>Cl<sub>2</sub>/MeOH(90:10). (1.71 g, 3.46 mmol, 53 % yield). (TLC rf 0.21 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 98:8). <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.20 (1H, d, H5), 6.81 (1H, dd, H6), 6.75 (1H, d, H2), 5.28 (2H, s, OCH<sub>2</sub>O), 3.57 (2H, t, OCH<sub>2</sub>), 3.46 (3H, s, OCH<sub>3</sub>), 2.82 (2H, br, CH<sub>2</sub>), 2.20 (2H, t, C8'), 2.4-1.2 (m, CH<sub>2</sub>), 0.84 (3H, s, CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 154.84 (C1), 137.97 (C3), 133.81 (C4), 126.25 (C5), 116.17 (C2), 113.66 (C6), 94.42, 86.24 (C8, alkyne), 83.77 (C7, alkyne), 79.92, 62.93 (C1, alkyne), 55.90, 49.52, 47.20, 43.82, 39.41, 39.16, 32.94, 32.76 (alkyne), 29.86, 29.05, 28.77 (alkyne), 27.34, 26.49 (alkyne), 25.72 (alkyne), 22.85, 18.80 (alkyne), 12.91.

**17 $\alpha$ -[7'-Octynyl-1'-carbamoyl-[4-[p-(bis[2-chloroethyl]amino)-phenyl]pentyl amine]]-1,3,5[10]-estratriene-3 $\beta$ -[methoxymethyl ether]-17 $\beta$ -diol (42)**

p-Nitrophenyl chloroformate (0.20 g, 0.97 mmol, 135 M%) was added to 17 $\alpha$ -[7'-octynyl-1'-ol]-1,3,5[10]-estratriene-3 $\beta$ -[methoxymethyl ether]-17 $\beta$ -diol (**40**) (0.35 g, 0.74 mmol) dissolved in THF (2 ml) and pyridine (1 ml). After 1 1/2 hr the reaction was diluted with CH<sub>2</sub>Cl<sub>2</sub> (75 ml) and washed with H<sub>2</sub>O (2 x 25 ml) and NaCl<sub>(sat)</sub> (2 x 25 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. The crude activated carbamoyl ester was used without purification in the next step. (TLC rf 0.60 CH<sub>2</sub>Cl<sub>2</sub>/MeOH 98:2)

To the crude activated carbamoyl ester (34 mg) dissolved in pyridine (8 ml) was added the 5-[4-[N,N-bis(2-chloroethyl)amino]phenoxy]pentylamine / HCL salt (**33**) (0.101 g) and the mixture heated (60 °C). Additional amine (**33**) was added after 1 1/2 hr (0.069 g) and after 4 1/2 hr (0.102 g). After 12 hr, the reaction was diluted with ethylacetate (40 ml) and washed with NaHCO<sub>3(sat)</sub> (2 x 25 ml) and NaCl<sub>(sat)</sub> (25 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. 17 $\alpha$ -[7'-Octynyl-1'-carbamoyl-[4-[p-(bis[2-chloroethyl]amino)-phenyl]pentyl amine]]-1,3,5[10]-estratriene-3 $\beta$ -[methoxymethyl ether]-17 $\beta$ -diol (**42**) was purified by column chromatography on 50 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>. (0.248 g, 0.34 mmol, 46 % yield for two steps ) (TLC rf 0.58 CH<sub>2</sub>Cl<sub>2</sub>: MeOH 98:2). <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.2.0 (1H, d, H5), 6.80 (1H, dd, H6), 6.76 (1H, d, H2), 5.13 (2H, s, OCH<sub>2</sub>O), 4.60 (1H, br, OH), 4.03 (2H, t, OCH<sub>2</sub>), 3.60 (8H, dd, NCH<sub>2</sub>CH<sub>2</sub>Cl), 3.46 (3H, s, OCH<sub>3</sub>), 3.15 (2H, q, NCH<sub>2</sub>), 2.82 (2H, m, CH<sub>2</sub>), 2.52 (2H, t, ArCH<sub>2</sub>), 2.21 (2H, t, H5'), 2.4-1.2 (m, CH<sub>2</sub>-s ), 0.85 (3H, s, CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 156.50 (C4"),

154.71 (C1), 144.02 (C1"), 137.84 (C3), 133.71 (C4), 129.30 (C2"), 126.16 (C5), 116.02 (C2), 113.52 (C6), 111.98 (C3"), 94.27 (MOM), 85.81, 83.89, 76.58, 64.54, 55.78, 53.46 (CH<sub>2</sub>Cl), 49.39, 47.09, 43.66, 40.46 (ØNCH<sub>2</sub>), 39.31, 39.07, 32.85, 31.75, 29.76, 28.88 , 28.48, 28.30, 27.23, 26.41, 25.21, 22.78, 18.67, 12.84. EIMS C<sub>42</sub>H<sub>58</sub>N<sub>2</sub>O<sub>5</sub>Cl<sub>2</sub> exact molecular weight 740.37228, high resolution result for m/z=740 (740.3728).

**17 $\alpha$ -[9'-Decynyl-1'-carbamoyl-[4-[p-(bis[2-chloroethyl]amino)-phenyl]pentyl amine]]-1,3,5[10]-estratriene-3 $\beta$  -[methoxymethyl ether]-17 $\beta$ -diol (43)**

p-Nitrophenylchloroformate (0.697 g, 3.46 mmol, 200 M%) was added to 17 $\alpha$ -[9'-decynyl-1'-ol]-1,3,5[10]-estratriene-3 $\beta$  -[methoxymethyl ether]-17 $\beta$ -diol (**41**) (0.80 g, 1.71 mmol) dissolved in THF (4 ml) and pyridine (2 ml). After 1 1/2 hr, the reaction was concentrated to dryness. The residue was redissolved with CH<sub>2</sub>Cl<sub>2</sub> (50 ml) and washed with H<sub>2</sub>O (25 ml) and NaCl<sub>(sat)</sub> (25 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. The crude activated carbamoyl ester was used without purification in the next step. (TLC rf 0.70 CH<sub>2</sub>Cl<sub>2</sub> /MeOH 98:2)

To the crude activated carbamoyl ester dissolved in pyridine (10 ml) was added the 5-[4-[N,N-bis(2-chloroethyl)amino]phenoxy]pentylamine / HCL salt (**33**) (0.80 g). After 3 hr at 75 °C, the reaction was concentrated. The residue was redissolved with CH<sub>2</sub>Cl<sub>2</sub> (50 ml) and washed with H<sub>2</sub>O (25 ml) and NaCl<sub>(sat)</sub> (25 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. 17 $\alpha$ -[9'-Decynyl-1'-carbamoyl-[4-[p-(bis[2-chloroethyl]amino)-phenyl]pentyl amine]]-1,3,5[10]-estratriene-3 $\beta$  -[methoxymethyl ether]-

17 $\beta$ -diol (**43**) was purified by column chromatography on 50 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (98:2). (0.95 g, 1.50 mmol, 87 % yield for 2 steps) ( TLC rf 0.42 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 98:2 or 0.35 ethylacetate/hexane 1:2) <sup>1</sup>H NMR (CDCl<sub>3</sub>): <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.20 (1H, d, H5), 7.04 (2H, d, H2"), 6.80 (1H, dd, H6), 6.75 (1H, d, H2), 6.63 (2H, d, H3"), 5.12 (2H, s, OCH<sub>2</sub>O), 4.56 (1H, br, OH), 4.07 (2H, t, OCH<sub>2</sub>), 3.60 (8H, dd, NCH<sub>2</sub>CH<sub>2</sub>Cl), 3.45 (3H, s, OCH<sub>3</sub>), 3.13 (2H, q, NCH<sub>2</sub>), 2.83 (2H, m, CH<sub>2</sub>), 2.52 (2H, t, Ar CH<sub>2</sub>), 2.30 (2H, t, H5'), 2.4-1.2 (m, CH<sub>2</sub>-s), 0.84 (3H, s, CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 162.58 (C4"), 154.89 (C1), 144.16 (C1"), 137.90 (C3), 133.76 (C4), 129.99 (OCH<sub>2</sub>O), 129.31 (C2"), 125.99 (C5), 115.55 (C2), 113.64 (C6), 112.05 (C3"), 94.36 (OCH<sub>3</sub>), 86.82, 83.29, 80.35, 65.41, 55.88, 53.49 (CH<sub>2</sub>Cl), 49.38, 47.19, 43.69, 40.50 (ArNCH<sub>2</sub>), 39.02, 32.85, 31.75, 31.60, 29.76, 29.16, 28.92, 28.71, 28.66, 27.24, 26.40, 25.75, 22.79, 18.74, 12.92

**17 $\alpha$ -[7'-Octynyl-1'-carbamoyl-[4-[p-(bis[2-chloroethyl]amino)-phenyl]pentyl amine]]-1,3,5[10]-estratriene-3 $\beta$ ,17 $\beta$ -diol (**44**)**

Hydrochloric acid<sub>(con)</sub> (100  $\mu$ l ) was added to 17 $\alpha$ -[7'-octynyl-1'-carbamoyl-[4-[p-(bis[2-chloroethyl]amino)-phenyl]pentyl amine]]-1,3,5[10]-estratriene-3 $\beta$ -[methoxymethyl ether]-17 $\beta$ -diol (**42**) (0.136 g, 0.18 mmol) dissolved in MeOH (3 ml). After 1 hr at room temperature, additional HCl<sub>(con)</sub> (100  $\mu$ l ) was added. After 4 hr, the reaction was concentrated. The residue was redissolved in CH<sub>2</sub>Cl<sub>2</sub> (20 ml) and washed with NaHCO<sub>3(sat)</sub> (10 ml) and NaCl<sub>(sat)</sub> (10 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. 17 $\alpha$ -[7'-Octynyl-1'-carbamoyl-[4-[p-(bis[2-chloroethyl]amino)-phenyl]pentyl

amine]]-1,3,5[10]-estratriene-3 $\beta$ ,17 $\beta$ -diol (**44**) was purified by column chromatography on 20 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (96:4) increasing to CH<sub>2</sub>Cl<sub>2</sub>/MeOH (90:10). The purity was determined by reversed-phase HPLC on a C<sub>18</sub> column using gradient IV. (TLC rf 0.51, CH<sub>2</sub>Cl<sub>2</sub>/MeOH 98:2) <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.03 (1H, d, H5), 7.06 (2H, d, Ar), 6.64-6.55 (4H, m, Ar), 5.8 (1H, br, OH), 4.59 (1H, br, OH), 4.04 (2H, t, OCH<sub>2</sub>), 3.63 (8H, dq, NCH<sub>2</sub>CH<sub>2</sub>Cl), 3.16 (2H, q, NCH<sub>2</sub>), 2.79 (2H, m, CH<sub>2</sub>), 2.52 (2H, t, ArCH<sub>2</sub>), 2.23 (2H, t, H5'), 2.4-1.3 (m, CH<sub>2</sub>-s), 0.85 (3H, s, CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 156.88 (C4''), 153.64 (C1), 144.26 (C1''), 138.14 (C3), 132.27 (C4), 129.52 (C2''), 126.42 (C5), 115.26 (C2), 112.70 (C6), 112.14 (C3''), 86.12, 83.85, 79.99, 64.76, 53.52 (CH<sub>2</sub>Cl), 49.42, 47.15, 43.63, 40.47 (ONCH<sub>2</sub>), 39.41, 39.04, 32.83, 31.73, 29.65, 28.86, 28.47, 28.31, 27.21, 26.45, 25.19, 22.77, 18.65, 12.81, 12.44.  $\lambda_{\max}$  = 258 ( $\epsilon$  = 1.8 x 10<sup>4</sup>),  $\lambda_{\max}$  = 254 ( $\epsilon$  = 1.7 x 10<sup>4</sup>),  $\lambda_{\max}$  = 280 ( $\epsilon$  = 3.6 x 10<sup>3</sup>). EIMS C<sub>40</sub>H<sub>54</sub>N<sub>2</sub>O<sub>4</sub>Cl<sub>2</sub> exact molecular weight 696.34607, high resolution result for m/z=696 (696.3455)

**17 $\alpha$ -[9'-Decynyl-1'-carbamoyl-[4-[p-(bis[2-chloroethyl]amino)-phenyl]pentyl amine]]-1,3,5[10]-estratriene-3 $\beta$ ,17 $\beta$ -diol (**45**)**

Hydrochloric acid<sub>(con)</sub> (0.50 ml) was added to 17 $\alpha$ -[9'-decynyl-1'-carbamoyl-[4-[p-(bis[2-chloroethyl]amino)-phenyl]pentyl amine]]-1,3,5[10]-estratriene-3 $\beta$  -[methoxymethyl ether]-17 $\beta$ -diol (**43**) dissolved in MeOH (10 ml). After 1.5 hr at room temperature, additional HCl (0.25 ml) was added. After 4 hr, the reaction was concentrated to dryness. The residue redissolved in CH<sub>2</sub>Cl<sub>2</sub> (50 ml) and washed with NaHCO<sub>3(sat)</sub> (2 x 25 ml) and NaCl<sub>(sat)</sub> (2 x 25 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. 17 $\alpha$ -

[9'-Decynyl-1'-carbamoyl-[4-[p-(bis[2-chloroethyl]amino)-phenyl]pentyl amine]]-1,3,5[10]-estratriene-3 $\beta$ ,17 $\beta$ -diol (**45**) was purified by column chromatography on 50 g silica gel eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (96:4). The purity was determined by reversed-phase HPLC on a C<sub>18</sub> column using gradient IV. (TLC rf 0.64 CH<sub>2</sub>Cl<sub>2</sub> : MeOH 95:5) <sup>1</sup>H NMR (CDCl<sub>3</sub>): <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.13 (1H, d, Ar), 7.03 (2H, d, Ar), 6.70-6.50 (4H, m, Ar), 6.14 (1H, br), 4.56 (1H, br, OH), 4.75 (1H, br), 4.00 (2H, t, OCH<sub>2</sub>), 3.60 (8H, dd, NCH<sub>2</sub>CH<sub>2</sub>Cl), 3.15 (2H, q, NCH<sub>2</sub>), 2.78 (2H, m, CH<sub>2</sub>), 2.53 (2H, t, ArCH<sub>2</sub>), 2.22 (2H, t, H5'), 2.4-1.2 (m, CH<sub>2</sub>-s), 0.86 (3H, s, CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): 156.78 (C4"), 154.60 (C1), 144.11 (C1"), 137.88 (C3), 131.95 (C4), 129.37 (C2"), 126.23 (C5), 115.20 (C2), 112.68 (C6), 112.07 (C3"), 86.25, 83.69, 80.35, 65.00, 60.46, 53.54 (CH<sub>2</sub>Cl), 49.44, 47.18, 43.72, 40.50 (ONCH<sub>2</sub>), 39.46, 39.08, 32.89, 31.78, 29.76, 29.70, 29.27, 28.99, 28.73, 27.32, 26.52, 25.83, 22.82, 18.77, 12.92.  $\lambda_{\text{max}}$  = 258 ( $\epsilon$  = 2.2 x 10<sup>4</sup>),  $\lambda_{\text{max}}$  = 254 ( $\epsilon$  = 2.0 x 10<sup>4</sup>),  $\lambda_{\text{max}}$  = 280 ( $\epsilon$  = 4.0 x 10<sup>3</sup>). EIMS C<sub>42</sub>H<sub>58</sub>N<sub>2</sub>O<sub>4</sub>Cl<sub>2</sub> exact molecular weight 724.37737, high resolution result for m/z = 724 (724.3771).

#### **17 $\alpha$ -[7'-Octynyl-1'-ol]-1,3,5[10]-estratriene-3 $\beta$ ,17 $\beta$ -diol (**46**)**

17 $\alpha$ -[7'-octynyl-1'-ol]-1,3,5[10]-estratriene-3 $\beta$  -[methoxymethyl ether]-17 $\beta$ -diol (**40**) (91 mg) was dissolved in MeOH (3 ml) and treated with HCl<sub>(con)</sub> (100  $\mu$ l). After 45 min, additional HCl<sub>(con)</sub> (100  $\mu$ l) was added. After 1 1/2 hr, the reaction was concentrated. The residue was redissolved in CH<sub>2</sub>Cl<sub>2</sub> (20 ml) and washed with NaHCO<sub>3(sat)</sub> (15 ml) and NaCl<sub>(sat)</sub> (15 ml). The organic solution was dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. 17 $\alpha$ -[7'-Octynyl-1'-ol]-1,3,5[10]-estratriene-3 $\beta$ ,17 $\beta$ -diol (**46**) was purified by column

chromatography on 30 g silica gel eluting with  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  (96:4) increasing to  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  (92:8). (17 mg pure, and 20 mg mixed fractions) (TLC rf 0.31  $\text{CH}_2\text{Cl}_2$  :  $\text{MeOH}$  95:5)  $^1\text{H}$  NMR ( $\text{CDCl}_3$  /  $\text{CD}_3\text{OD}$ ): 6.97 (1H, d, H5), 6.45 (1H, dd, H6), 6.38 (1H, d, H2), 3.39 (2H, t,  $\text{CH}_2\text{O}$ ), 2.62 (2H, m,  $\text{CH}_2$ ), 2.07 (2H, t, H7'), 2.2-1.0 (m,  $\text{CH}_2$ -s), 0.673 (3H, s,  $\text{CH}_3$ ).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$  /  $\text{CD}_3\text{OD}$ ): 154.05 (C1), 137.72 (C3), 131.44 (C4), 126.05 (C5), 114.91 (C2), 112.40 (C6), 85.37 (C8, alkyne), 83.57 (C7, alkyne), 79.36, 61.86 (C1, alkyne), 46.93, 43.46, 39.30, 38.64, 32.61, 32.05 (alkyne), 29.40, 29.11, 28.43, 28.31 (alkyne), 27.09, 26.23 (alkyne), 24.93 (alkyne), 22.46, 18.35 (alkyne), 12.44.

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