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Purification and structural characterization of caged DNA oligonucleotides

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PURIFICATION AND STRUCTURAL CHARACTERIZATION OF
CAGED DNA OLIGONUCLEOTIDES

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Brendan M. McAdams
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Abstract

"Cage" molecules reversibly block the bioactivity of a target substrate molecule by a photolyzable covalent bond formed at a functional site of the target molecule. The attachment of cage molecules to DNA oligodeoxynucleotides (ODNs) to transiently block bioactivity, and site-specific restoration of bioactivity using targeted light exposure, would enable a new method of control for use in gene therapy, molecular/DNA computing, molecular biology, and drug delivery. The reaction of the cage molecule 1-(4,5-dimethoxy-2-nitrophenyl)diazoethane (DMNPE) with DNA ODNs in an batch reaction yields a mixture of products with varying degrees of caging. Purification and verification of the hypothesized site of DMNPE attachment are necessary for future applications of this technology to control DNA bioactivity with light. Size exclusion chromatography, high pressure liquid chromatography (HPLC), polyacrylamide gel electrophoresis (PAGE), and nuclear magnetic resonance (NMR) were performed on caged DNA samples.

Alternatives to manganese dioxide (MnO_2) as a DMNPE activator were investigated because MnO_2 was found to interfere with NMR. Nickel peroxide (NiO_2) was found to be an effective alternative. Increased caging was found to correspond with a broadening and small upfield shifts of 1-D ^{31}P NMR resonances. 2-D heteronuclear multiple bond correlation (HMBC) NMR experiments successfully matched previous characterizations of the DMNPE site of attachment on caged ATP, and show crosspeaks between the ribose ring and phosphate moiety of ATP and DNA structures, but did not show a crosspeak between the DMNPE benzyl proton and DNA phosphate moiety. This may be due to bond angle or relaxation effects of the cage adduct. Because no phosphate

attachment was discovered, base alkylation was evaluated by reaction of deoxynucleosides and DNA dimers with DMNPE. 2′-deoxynucleosides showed no caging under similar reaction conditions (pH 5.5). DNA dimers dTpT and dApA in those reaction conditions showed a caged product on thin layer chromatography plates, and dGpG and dCpC results also suggested some minimal product formation. Thus, the initial hypothesized site of DMNPE attachment at the phosphate backbone was retained. These results demonstrate useful techniques for future efforts in purification and characterization of caged nucleic acid species.

Chapter 1. Introduction and Background

Overview

The photolabile protecting group 1-(4,5-dimethoxy-2-nitrophenyl)diazoethane (DMNPE) is known to attach to nucleic acids, forming a "caged DNA" complex which is biologically inert with respect to hybridization and transcription until exposed to light (Monroe, McQuain et al. 1999; Ghosn, Haselton et al. 2005). This technique can be used to control the spatial and temporal activity of nucleic acids. Efforts in molecular and cell biology, gene therapy, genomics and proteomics, and even DNA computing would be furthered by the introduction of a tool for the spatial and temporal control of DNA hybridization and transcription in both space and time. In order to build upon this DNA caging technology, the caging and uncaging reaction must be optimized and the structure of the caged nucleic acid must be unambiguously determined. In this study the cage molecule DMNPE was used together with a 20-mer oligodeoxynucleotide (ODN) as a model cage-DNA complex. Structural characterization of the DMNPE-DNA complex will be used to further kinetic studies and the investigation of other cage and nucleotide groups.

The two principal thrusts of this project were to purify the DMNPE-caged ODNs and to determine their exact chemical structure. It is necessary to purify the caged DNA because in the current reaction protocol, adduction of ODN occurs during a batch reaction with most likely a range of products comprising different degrees of cage adduction per DNA strand. A uniform caged DNA product must be purified to ensure uniform bioactivity control and uncaging kinetics. It is also important to characterize the

structure of caged DNA because which functional group are caged affects both the bioactivity and uncaging kinetics of caged DNA.

The structure of caged DNA will direct future steps in photoprotection of nucleic acid species, determining which possibilities of functional protection and bioactivity blockade are feasible to pursue. Caging at sites other than the phosphate backbone may enable specialized blocking of bioactivity, and different caged ODN half-life under UV irradiation (Abramova, Leonetti et al. 2000). The DMNPE attachment site(s) will also indicate which variables may be optimized to improve the efficiency of the caging reaction and purification. While plasmids, oligonucleotides, RNA and other nucleic acid species have been photoprotected with various cage molecules, none of the adducted structures have been confirmed using analytical techniques. Here a short oligonucleotide was used for a model complex of DMNPE-adducted DNA that shows altered absorbance, gel mobility, and hybridization activity from assays previously established. Nuclear magnetic resonance (NMR,) mass spectrometry, and chromatographic assays of caged ODNs and their substituents combine for a structural characterization of this complex. Development of these assays lend to future work to characterize nucleic acids adducted with cage compounds other than DMNPE.

In this study, the methods tested for the purification of the cage reaction products included reverse-phase high pressure liquid chromatography (RP-HPLC) and size exclusion columns. The structure of caged DNA was investigated with 1D and 2D ^{31}P NMR spectroscopy, and the alkylation of sites other than the phosphate backbone was investigated using deoxynucleosides as reagents in caging reactions. This introduction includes a brief review of these topics relevant to this study.

Cage Molecules

"Cage" molecules, also called photolabile protecting groups (Pelliccioli and Wirz 2002), form a photolyzable covalent bond to a functional group of a target molecule. The adduction to the target molecule's functional group blocks one or more of the target's bioactivities. Upon exposure to a light source of specific wavelength, the cage molecule photocleaves, restoring the original bioactivity of the target molecule. The term "caged," in this context, is descriptive of the photo-activation property (Kaplan et al. 1978) and

DMNPE-Caged ATP
(biologically inert)

bioactive ATP

released cage
(DMNAP)

Figure 1. The bioactivity of ATP is restored upon photolysis of DMNPE-caged ATP
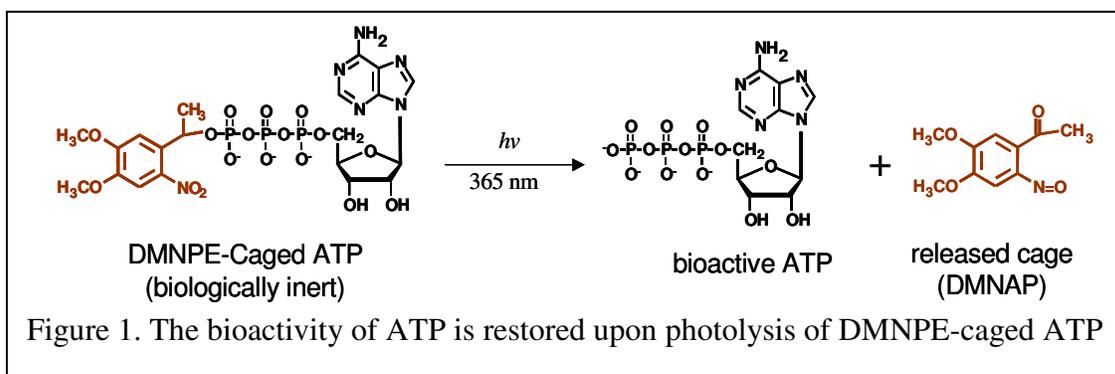
does not refer to physical trapping of the inactivated substance within a crystal lattice or shell. A classic example is DMNPE-caged ATP (Figure 1). In kinetic studies, caged ATP is introduced into cells, but the caged ATP is not bioactive. A rapid increase in the concentration of bioactive ATP is achieved by a pulse of light which releases the caging group from the g-phosphate and restores the caged ATP to its native bioactive state.

Photolabile "cage" protecting groups have been used in studies of molecular kinetics (Hess and Grewer 1998; Meldrum, Chittock et al. 1998; Scheidig, Burmester et al. 1998), the functionality of peptides (Sreekumar, Ikebe et al. 1998), enzymes, proteins, hormones (Allan, Ward et al. 1998), neurotransmitters (Wilcox, Viola et al. 1990; Gee, Carpenter et al. 1998), fluorescence dampening (Mitchison, Sawin et al. 1998), and gene expression (Monroe, McQuain et al. 1999; Ando, Furuta et al. 2001). Most are benzyl

ring derivatives, with diazonium or bromine as the leaving group in the caging reaction. Many analogues have been synthesized, to change the rate or efficiency of the caging reaction or the photorelease, to decrease the bioactivity of the photolyzed benzyl molecule (Givens, Weber et al. 1998), or to tailor the caging group to bond to a specific target (Mitchison, Sawin et al. 1998).

The ability of DMNPE to photocleave is a result of an interaction between the 2-nitro moiety and the nearby ethyl ether which occurs upon exposure to irradiation of a given wavelength. Upon excitation of the 2-nitro moiety an *aci*-nitro intermediate structure is formed bridging the ethyl and the nitro moiety. This unstable structure changes to a 2-nitrosoacetophenone leaving group (Walker, Reid et al. 1988; Givens and Kueper 1993; Pelliccioli and Wirz 2002). In the case of DMNPE-caged DNA, 365nm UVA irradiation causes the photolabile adduct to dissociate, thus leaving the DNA in its original bioactive form.

Purification of DMNPE-ODN Complexes

DNA is typically caged with DMNPE in a batch-style process that results in products with different degrees of adduction, and also possibly of different adduction sites, both of which affect the level of bioactivity for the caged DNA. Non-adducted or insufficiently adducted oligonucleotides within the heterogeneous batch would create 'leak' activity in the uninduced state. Purification of the caged products before introduction into biological system will more completely block bioactivity of the DNA, and assist in determination of light doses necessary for deprotection.

Size Exclusion Chromatography

An excess of the DMNPE cage adduct is used in DNA caging reactions to ensure sufficient caging of the final products to block bioactivity. These excess cage molecules may react with downstream characterization assays or applications in cells or other biological systems. Because of this, the excess cage molecules which are left in the reaction solution must be removed before any other steps are taken with the caged DNA.

Size exclusion chromatography separates compounds based on significant differences in size. A gel bed of porous beads of a specified size allow smaller molecules to enter into the beads, while molecules larger than the bead pores pass through the 'void volume' between the beads, eluting earlier than smaller compounds. Examples of porous media used for size exclusion are cross-linked dextrans such as sephadex or sepharose. Size exclusion chromatography has been used as a preparative purification step for guanine-alkylated DNA (Salvati, Moran et al. 1992). This technique is explored as a first step purification of the caging reaction, removing excess cage molecules from the caged DNA solution based on their size differences and also allowing solvent exchange if desired. The molecular weight of the 20-mer oligo used in this study is 6,063 while the calculated MW of the DMNPE moiety is 193. Size exclusion chromatography purification should be sufficient to resolve caged DNA from the reaction contaminants.

High Pressure Liquid Chromatography

High pressure liquid chromatography (HPLC) purification is a standard step in DNA oligonucleotide synthesis (Vydac 1998). A shallow reverse-phase HPLC gradient of acetonitrile in water has been used to purify DNA tetramers (Gill, Min et al. 1993) and dodecamers (Baruah and Bierbach 2004) with aromatic adducts. Both anion-exchange

and reverse-phase columns have been used to separate the complete ODN sequence from crude synthesis materials and failed (incomplete) sequences. The dimethoxytrityl group used to protect the 5' DNA end during synthesis is known to effect the elution time of the ODN, as its hydrophobic nature leads to a longer retention time in RP-HPLC than de-tritylated DNA. Due to the hydrophobic nature of the DMNPE molecule, a similar effect to that of the DMT group on the DNA retention time might be expected. A reverse-phase HPLC method could separate caged from non-caged DNA based on a different relative affinity of the DNA for the mobile phase over the stationary phase by the number of cage molecules attached, with heavily caged DNA eluting later than lightly caged or native DNA.

Cage Molecule Attachment Sites

DMNPE has been shown to react with the gamma-phosphate of ATP and the sulfur of ATP(γ, S) (Walker, Reid et al. 1988). DMNPE cages molecule analogues such as 1-(2-nitrophenyl)-diazoethane (NPE) have been shown in messenger functionality studies to react with the P-O- group of phosphatidic acid (Williger, Reich et al. 1995), which is similar to the phosphate backbone structure of DNA. However, DMNPE and NPE have also been shown to react with carboxylic acids to create esters (Wilcox, Viola et al. 1990; Allan, Ward et al. 1998; Gee, Carpenter et al. 1998; Mitchison, Sawin et al. 1998), and NPE has been used to cage fluoresceins at keto-enol resonance sites (Mitchison, Sawin et al. 1998). It is reasonable to propose that any functional group with a relatively acidic proton might be caged. The internal phosphate of dTpT and the 3' phosphate of thymidine phosphoramidite synthones have been caged using o-nitrobenzyl alcohol and o-nitroveratryl alcohol cage molecules (Abramova, Leonetti et al. 2000).

Diazoalkanes react most rapidly with acidic functional groups such as carboxylic acids (March 1992). The mechanism requires protonation of the diazoalkane to give a diazonium ion, followed by nucleophilic displacement of nitrogen (N_2) (Singer 1975; Singer 1986; Lee, Aarhus et al. 1997). N-alkyl-N-nitrosoureas, precursors to diazoalkanes, alkylate oligonucleotides lacking terminal phosphates at both nucleophilic sites of the bases as well as the phosphodiester backbone (Lawley 1984). The relative reactivity of the base and phosphodiester group was reported to be pH dependent for the reaction of poly(U) (Friedman, Mahapatra et al. 1965; Kusmierek and Singer 1976). At lower pH, a greater proportion of phosphotriester is observed. DNA has also been shown to undergo alkylation at the amino positions when reacted with diazoalkanes (Kriek and Emmelot 1964). Thus although there is good evidence to support the initial hypothesis that the caging agent is bound to the phosphate backbone, DMNPE attachment to the bases or sugar rings of the DNA has not been ruled out.

Nuclear Magnetic Resonance Verification of Caged Species

The attachment sites of many adducts of short DNA ODNs have been definitively confirmed through nuclear magnetic resonance (NMR) structural analysis (Chandrasekaran, Kusuma et al. 1986; Harding, Harden et al. 1993; Fan, Ohms et al. 1999). However, most NMR characterization of DNA-ligand complexes deals with DNA intercalating compounds, which differ in their DNA interactions with respect to DMNPE. In addition, the vast majority of literature on NMR of DNA deals with short double stranded DNA, usually Drew-Dickerson dodecamer-like chimeric ODNs (Privé, Yanagi et al. 1991), while the 20-mer ODN used in this study is non-self complementary and is of necessity single stranded because cage adducts prevent duplex formation. For the 20-

mer ODN used in this study a full characterization would be necessary in order to detect any preference for which backbone phosphates are caged. However this is not ostensibly feasible because the phosphates all have very similar and overlapping chemical shifts (δ) due to the dynamic backbone structure. The non-modified 20-mer ODN used in this study has yet to be fully assigned by NMR.

The assignment of DMNPE and the assignments of analogs have been published both as their hydrazone precursors and as adducts on various target molecules (Walker, Reid et al. 1988; Wilcox, Viola et al. 1990; Cohen, Stoddard et al. 1997; Yamaguchi, Tsuda et al. 1998). The majority of spectral characterization has been by 1-dimensional proton NMR. For a range of analogs, the benzyl proton in a caged-target complex has a resonance ranging from 4.9 to 6.0.

1-Dimensional ^{31}P NMR has been used to characterize DMNPE-caged forms of NAADP (Lee, Aarhus et al. 1997), and various caged dinucleotides (eg. TpT) (Abramova, Leonetti et al. 2000). In caged NTPs with modified groups on the end (-S or -NH3 replacing the -O), the particular phosphate that is caged (α, β, or γ) can be determined due to a significant downfield shift (Walker, Reid et al. 1988). An upfield shift was observed when the 2'-phosphate of NAADP was caged (Lee, Aarhus et al. 1997). For a phophodiester, a downfield shift can be expected if cage attachment causes a narrowing of the O-P-O bond angle

Figure 2: The hypothetical adduction site of DMNPE on the phosphate backbone of DNA.
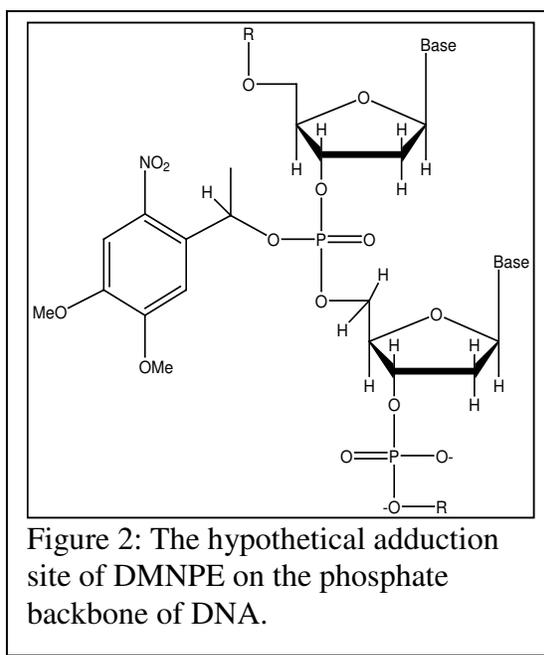
(Gorenstein 1994). In a number of studies the ^{1}H-^{31}P J coupling constant (Hz measurement of the split of an NMR peak, caused by a coupled nucleus) between the benzyl proton of the cage group and a phosphate moiety target ranged from 7 to 9.5 (Walker, Reid et al. 1989; Williger, Reich et al. 1995). However, while 1-D ^{31}P NMR has been used to characterize caged nucleotides that show notable downfield shifts when caged (Walker, Reid et al. 1988), single-stranded DNA ODNs characteristically display ranges of resonances (Braddock, Baber et al. 2002; Znosko, Barnes et al. 2003) in solvents such as DMSO that are also known to shift the peaks of the ^{31}P spectrum (Gorenstein 1994). These facts may make shifts due to DMNPE attachment difficult to discern. Therefore 2-D ^{31}P-^{1}H NMR experiments such as Heteronuclear Multiple Bond Correlation (HMBC) were used instead of 1-D NMR to precisely detect phosphotriesters in adducted nucleotides and oligonucleotides. An HMBC protocol has previously been used for the detection of the 3-J through-bond couplings for P–H3' and P–H4'/H5' (Sklenar, Miyashiro et al. 1986; Quin and Verkade 1994). This experiment should detect an extra 3-J H-C-O-P coupling between the DMNPE and phosphate groups (illustrated in Figure 2). In setting up this experiment it is necessary to closely estimate the coupling constants for the coupling of interest. One study found significant differences in the P-31 – H-1 coupling constants between different nucleotides for a 5-mer DNA strand (Searle and Lane 1992). The H-C-O-P, 3J coupling constants in native DNA are controlled by the dihedral bond angles in the DNA backbone. The relation between this coupling constant and the dihedral angle is often plotted as a Karplus curve with the equation (Wemmer 2000):

$$^3J(\mathbf{HCOP}) = 15.3 \cos^2 \Phi - 6.1 \cos \Phi + 1.6$$

Chapter 2. Purification and Characterization of Caged DNA Oligonucleotides

Introduction

This effort sought to control the bioactivity of short DNA oligodeoxynucleotides (ODNs) with light-sensitive "cage" compounds. ODNs are a common component in molecular biology, nanotechnology, and biosensor assays that also are used as drugs in genetic therapies. The ability to regulate the bioactivity of these ODNs spatially and temporally will improve control and targeting of therapeutic and molecular assays. Adduction with photolabile cage molecules such as 1-(4,5-dimethoxy-2-nitrophenyl)ethyl ester (DMNPE) have been shown to block hybridization of ODNs until exposed to light. Because the adduction of DNA with these compounds is currently performed in a batch-style reaction, the actual adduction site could vary and has not been verified. Purification and characterization is necessary to understand the mechanism of this control strategy.

The reaction of DMNPE with DNA ODNs in a batch reaction yields a mixture of products with varying degrees of caging. Before eventual large-scale production of caged ODNs can be viable, it will be necessary to streamline purification of the batch reaction mixture. Experimented have been performed with several techniques to purify batch reactions of caged ODNs, including HPLC and size-exclusion columns.

Strategies for the confirmation of chemical structure of DMNPE attachment consist primarily of NMR techniques. ^{31}P-^{1}H heteronuclear multiple bond correlation (HMBC) data show crosspeaks between the backbone phosphates and the 3'-5' protons on the ribose rings of non-modified DNA. This NMR technique should also show a correlating crosspeak between the phosphate and an adducted ethyl ether from the cage

10

molecule, if present. It was found that the MnO_2 used to activate the DMNPE was carried through filtration procedures and interfered with collection of NMR data. Two other oxidizers, Magtrieve™ (CrO_2) and NiO_2, were evaluated for their interference with NMR signals and their ability to activate DMNPE. Ko et al. (Ko and Kim 1999) have used Magtrieve™ (CrO_2) to oxidize benzophenone hydrazone to diphenyldiazomethane. NiO_2 was tested because it is known to be a non-paramagnetic oxidizer, and thus should give minimal interference in NMR.

The possibility of caging at sites other than the phosphate backbone has also been explored. Alternative sites of cage adduction may enable specialized blocking of bioactivity, and different caged ODN half-life under UV irradiation. To test whether DMNPE might adduct moieties on the nitrogenous bases or the sugar ring of DNA, DMNPE was reacted with the four 2'-deoxynucleosides adenosine, guanosine, thymidine, and cytidine, and DNA dimers dApA, dCpC, dGpG, and dTpT at pH 5-6 similar to the conditions of the ODN reaction. TLC data for nucleosides show regions with retention factor (R_f) values that are identical to the reaction starting materials, indicating no alkylation of the ribose ring or nitrogenous base moieties, however TLC data for dimers do show evidence of product formation. These purification and characterization techniques may serve as a platform to examine other caged nucleic acid species that will enable the specific control of DNA and RNA with light.

Materials and Methods

Oxidation of the DMNPE Hydrazone

Before NMR spectra of caged DNA could be collected, an alternative to the oxidizing reagent used during the preparation of DMNPE needed to be determined. 1-

(4,5-dimethoxy-2-nitrophenyl)ethyl hydrazone, the hydrazone precursor to DMNPE, is activated by oxidation of the hydrazone to a diazoethane (shown in Figure 3). MnO_2 is the recommended oxidizer used in this scheme, however trace amounts of oxidizer may pass through standard filtration techniques and be carried through to the reaction containing DNA. Trace MnO_2 interferes with collection of NMR data. Weakness of the ^{31}P NMR signal from initial caged DNA samples was attributed to

Figure 3: Oxidation of the DMNPE hydrazone to its active diazoethane form used to cage DNA.
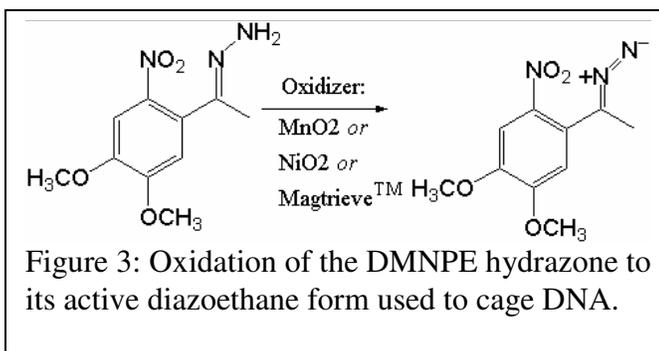
paramagnetic interference from trace MnO_2 contaminant from this DMNPE activation step. Elimination of MnO_2 from the caging reaction will facilitate NMR analysis of caged effector molecules in general.

To test the relative attenuation of NMR signal by trace amounts of oxidizers, 50 mg of NiO_2 and MnO_2 were each solvated separately in 1 ml DMSO, agitated 20 min. and filtered through a syringe packed with diatomaceous earth and glass wool, as indicated in Molecular Probes' DMNPE Generation Kit (MP 02516). 50 mg Magtrieve™ in 1 ml of DMSO was agitated 20 min., centrifuged briefly and decanted under a magnet, as described by Lee at al. (Lee, 1997). 200 μl from the filtrates were added to 200 μl solutions of potassium phosphate (KH_2PO_4). Final KH_2PO_4 concentration of all samples was 13.11 mM. The samples were scanned in a 400MHz NMR spectrometer and 1D ^{31}P spectra were collected.

To determine whether NiO_2 and/or CrO_2 would oxidize the DMNPE hydrazone precursor to activated DMNPE, 5 mg of oxidizers CrO_2, NiO_2, and MnO_2 (for a positive

control) were added to solutions of 149 μM hydrazone in 150 μl acetonitrile (ACN) and agitated for 20 min. NiO_2, MnO_2, and CrO_2 were filtered as described above. Hydrazone precursor was used as a negative control. UV absorbance spectrophotometry with an ACN blank was used to detect a characteristic shift in absorbance indicative of DMNPE activation.

Typical Protocol for ODN Caging with DMNPE

Unless otherwise specified, reagents were purchased from Sigma-Aldrich (St. Louis, MO). DNA ODNs were synthesized by Integrated DNA Technologies, Inc. or Alpha DNA, Inc. in HPLC-purified form. The 1-(4,5-dimethoxy-2-nitrophenyl) diazoethane Generation Kit (Molecular Probes, Eugene, OR) specifies a protocol in which 25 mg of 1-(4,5-dimethoxy)-2-nitroacetophenone hydrazone and 100 mg of oxidizer MnO_2 are gently agitated in 1 ml of dimethylsulfoxide (DMSO), N,N-dimethylformamide (DMF), or chloroform at 25 °C for 20 min. MnO_2 is removed from the activated 1-(4,5-dimethoxy-2-nitrophenyl) diazoethane (DMNPE) by filtering the solution through 100 mg of Celite^{TM} supported by glass wool in a 1 cc tuberculin syringe. The solution of the activated diazoethane cage is then added to the target molecule. In final form, the protocol was modified as follows: 5 mg of DMNPE hydrazone was solvated in a polar aprotic solvent (DMSO or acetonitrile as noted per sample) and agitated with ~50 mg NiO_2 oxidizer for 20 min. Filtration of active cage was performed as per the protocol. 150 μl aliquots of the filtrate were added to 20-mer DNA ODN (5'-GCCCAAGCTGGCATCCGTCA-3') solution every half hour until all filtrate solution had been added. Solution was agitated overnight at 4 °C.

Purification of a Batch Caging Reaction by Size Exclusion Chromatography

Typically, the DMNPE-DNA ODN solution was added from the reaction directly to a Sephadex® size exclusion column (NAP™-25 column, Amersham Biosciences). The sample solution was allowed to settle into the bed of the column, and water was added in 1 ml increments, at a rate of 1 ml/min, for 20 minutes. Aliquots were collected at half-minute intervals to 7 minutes and in 1-minute intervals thereafter. Eluates were analyzed by UV absorbance spectrophotometry and the relative concentrations of DNA and DMNPE were calculated for each sample. A ε_{355} of 4512 was used to determine the DMNPE concentration. A DMNPE $\varepsilon_{260} = 3064$ was used to calculate DMNPE contribution to the absorbance at 260nm. The calculated A_{260} was subtracted from the A_{260} of the sample and the concentration for DNA was calculated from the remaining A_{260}. The "% caging" of a caged DNA sample was calculated as follows:

$$100*[DMNPE] / ([DNA]*19)$$

This expresses the degree of caging in terms of percentage of the 19 possible phosphate caging sites that would be changed to phosphotriesters. The ε_{355} and ε_{260} of phosphate-bound DMNPE were calculated from dilutions of caged H_3PO_4 by Richard Blidner (unpublished work).

An experiment was also performed using size exclusion chromatography to determine whether unactivated DMNPE hydrazone might react with DNA, which would possibly lead to a greater variety of cage adduction sites. For this experiment, 15ug of ODN (4.95 uM) and DMNPE hydrazone precursor (1.14 mM) were agitated overnight in 500 μl of 50% acetonitrile, 50% water. The reaction solution was then added to a size

exclusion column and eluted with HPLC grade water. Aliquots were collected at 1 minute intervals and UV absorbance spectra of the eluents were analyzed.

Photolysis of Caged Samples

The light source used for photolysis of caged samples was a mercury arc lamp (B100-AP, UVP, Inc.) with a characteristic output at 365 nm with a fluence rate of 4.68 mW/cm^2. The typical UV exposure time of 40 min. is equivalent to 11.2 J/cm^2. Samples were flashed in 0.2 ml eppendorf microtubes, which showed negligible absorbance at UVA wavelengths.

High Pressure Liquid Chromatography

After reaction with DMNPE and size exclusion filtration as described above, two eluates of DMNPE-caged DNA with estimated DMNPE:phosphate moiety ratios of 0.22:1 and 0.47:1 were compared to native (non-caged) DNA control with HPLC. The samples were injected into a reverse-phase HPLC system (DX 500 HPLC system, Dionex) with a C-4 column (TP214™, Vydac). Samples were run in a ramped 37 minute gradient of 0-100% acetonitrile in 0.1% TEAA buffer and monitored using absorbance detection at 260 nm, followed by a second aliquot of each sample while collecting absorbance detection data at 355 nm. Aliquots of these samples were then exposed to a 365nm light source (B-100AP, UVP), for 40 minutes. These samples were run with the same gradient, and 260 and 355 nm absorbance data were collected.

To determine whether the HPLC elution profile of caged oligonucleotides would be variably effected by other exposure times to ultraviolet light, 100 μl aliquots of caged DNA from a single size exclusion column eluate were placed under the UV lamp for

periods of 2, 5, 10, 20, or 60 minutes. These were run with the same HPLC method used for the caged samples above. Absorbance data was collected at 260 nm.

Polyacrylamide Gel Electrophoresis (PAGE) of Caged DNA Samples

Samples from the two size exclusion column eluates specified above, with 0.22:1 and 0.47:1 DMNPE to DNA phosphates, were run on 15% acrylamide non-denaturing PAGE gels in TBE buffer for 100 minutes at 60 V/cm on a BioRad EZ gel electrophoresis assembly. Other samples run on the gel include a native and UV-exposed aliquots of the caged DNA samples. The gel was then gently agitated for 40 minutes in SYBR-Gold DNA stain and visualized with a BioRad gel box at 320 nm excitation.

NMR Investigation of DMNPE Adduction Site

A Bruker DPX 400 MHz spectrometer was used for 1-D ^{31}P and 2-D ^{1}H-^{31}P HMBC NMR experiments that were performed on native and DMNPE-caged ATP and ODN before and after 40 minutes of exposure to UV light. The two size exclusion column eluates described above of DMNPE-caged DNA with estimated DMNPE:phosphate moiety ratios of 0.22:1 and 0.47:1 were compared to native DNA in NMR. 1-D ^{31}P proton-decoupled NMR scans were performed on a Bruker ARX 300 MHz spectrometer at 298 K, in water with 10% D_2O. A 2-D ^{31}P-^{1}H HMBC experiment was performed on native and caged DNA in 80% DMSO, 20% D2O using a Bruker DPX 400 MHz spectrometer at 298 K. XWIN-NMR (Bruker) and MestReC (MestreLab Research) were used for analysis of NMR spectra.

Investigation of DMNPE Adduction at Non-Phosphatidic Sites

To test whether DMNPE might adduct moieties on the nitrogenous bases or the sugar ring of DNA, 25 µl of DMNPE cage in DMSO solution (21.5 mM) was added to 25

ul, 10 mM BisTris buffered (pH 5.5) solutions of 10 mM 2'-deoxynuclesides adenosine, guanosine, thymidine, and cytidine. The formation of cage product from DNA dimers containing a phosphate site was also analyzed. To 250 μl of dApA (10 mM), dGpG (min. 3.9 mM), dCpC (18 mM), and dTpT (25 mM) dinucleotides were added 200 μl activated DMNPE (21.5 mM). The solutions were reacted overnight and analyzed for the formation of new products by thin layer chromatography (TLC) using a solvent system of 40% MeOH in water. The samples analyzed with TLC included a DMNPE control, a photoexposed DMNPE control, a 'native' nucleoside or dinucleotide control, the caging reaction solution, and caging reaction solution after UV exposure. For the photoexposed caging reaction solutions and photoexposed DMNPE control, aliquots of the solutions were flashed for 40 minutes under 365 nm UV irradiation. The TLC R_f band regions were visualized with 254 nm and 365 nm UV light.

Figure 4. Absorbance spectra of DMNPE hydrazone solutions exposed to different oxidizers.
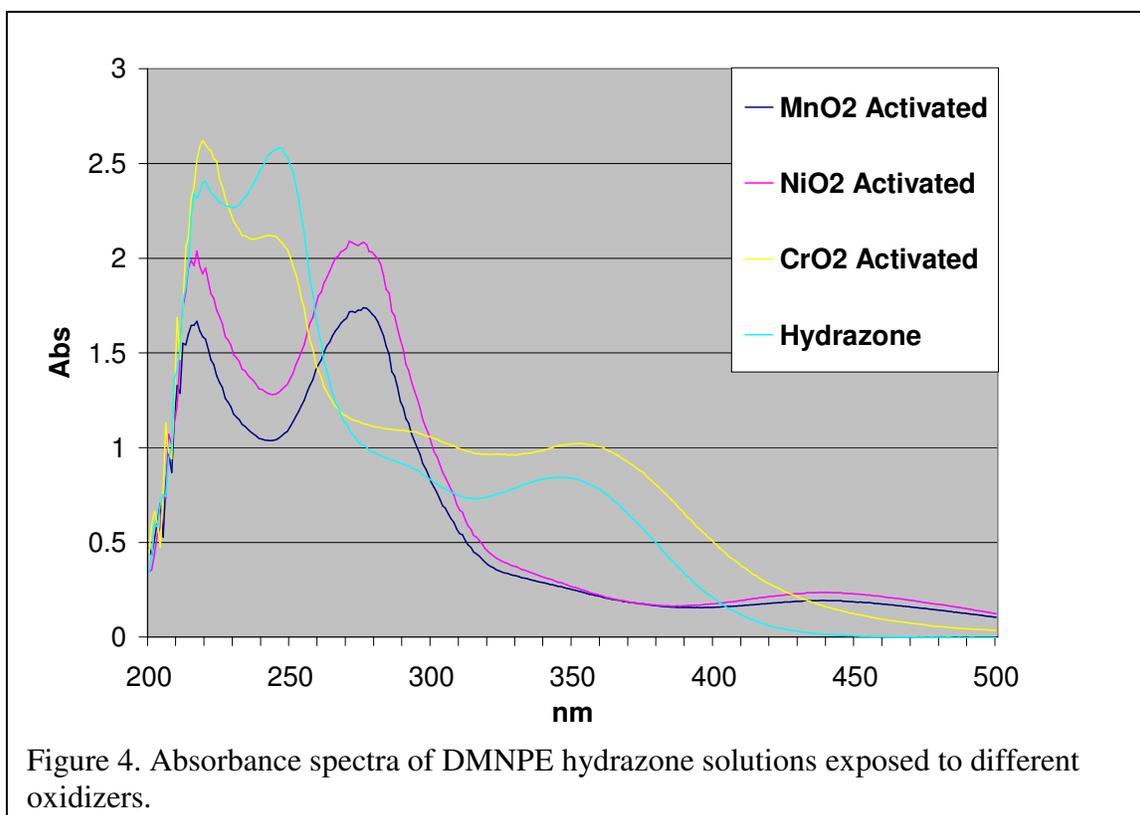
Results and Discussion

Oxidation of DMNPE Hydrazone and Oxidizer Effects on NMR Signal Strength

Neither nickel peroxide nor Magtrieve™ showed attenuation of the ^{31}P NMR signal of potassium phosphate compared to the control solution containing no oxidizer. Signal-to-noise ratios are summarized in Table 1. NMR spectra of the control, CrO_2-contaminated, and MnO_2-contaminated solutions are shown in Figure 5. From these data, nickel peroxide and Magtrieve™ appeared to be compatible for NMR studies. However in UV absorbance spectrometry only the spectrum of NiO_2-exposed DMNPE hydrazone solution matches that of the MnO_2-activated DMNPE, indicating that oxidation by MnO_2 and NiO_2 yield identical products. The spectrum of the CrO_2 (Magtrieve™)-exposed solution does not show a similar degree of activation. Acetonitrile solution was found to show a greater difference than other solvents between spectra of the

Table 1. Signal-to-noise ratios of NMR signal from KH_2PO_4, showing severity of attenuation from trace MnO_2 oxidizer contamination.

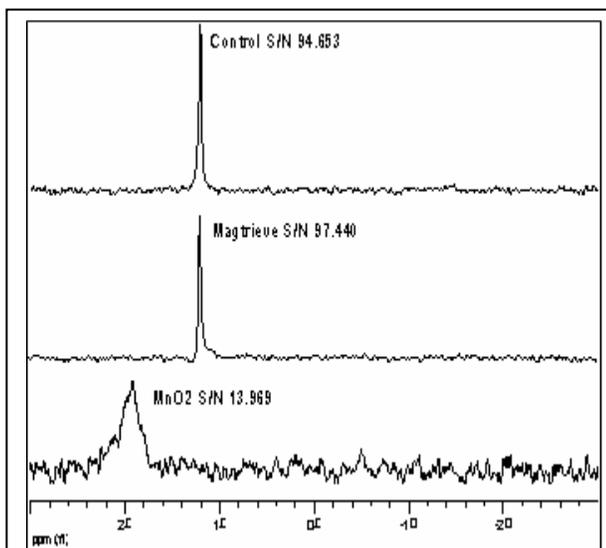| Sample	Filtration	Signal to Noise
Control	No Filtration	94.653
CrO_2 "Magtrieve™"	Magnet	97.440
MnO_2	Syringe	13.969
NiO_2	Syringe	95.278

Figure 5. NMR of KH_2PO_4 solutions. Top trace shows a control; middle trace shows effect of CrO_2 contamination; Bottom trace shows effect of MnO_2 contamination

hydrazone precursor and activated DMNPE as seen in Figure 4. Since NiO_2 does not

attenuate the NMR signal, and also oxidizes the hydrazone precursor to activated DMNPE, it was determined to be an effective alternative to MnO_2 and was subsequently used for the preparation of DMNPE-caged ODNs intended for NMR characterization.

Purification of Batch Reaction by Size Exclusion Chromatography

Shown in Figure 6 are the typical UV absorbance spectra of caged DNA and excess DMNPE cage after a typical caging reaction. These characteristic spectra are used together with polyacrylamide gel electro-phoresis (PAGE) determine which eluents from the size exclusion column contain caged DNA and which contain excess unattached cage. Diluted aliquots of SE eluents containing caged DNA are analyzed by UV spectrophoto-metry to determine the percent caging. The ratio of
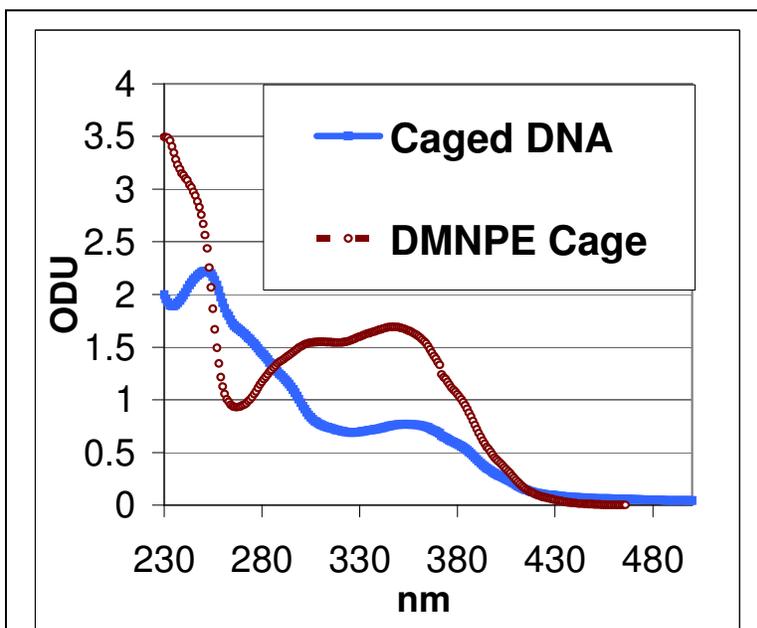

Figure 6. Chracteristic absorbtion spectra of DMNPE-caged DNA and of DMNPE alone.
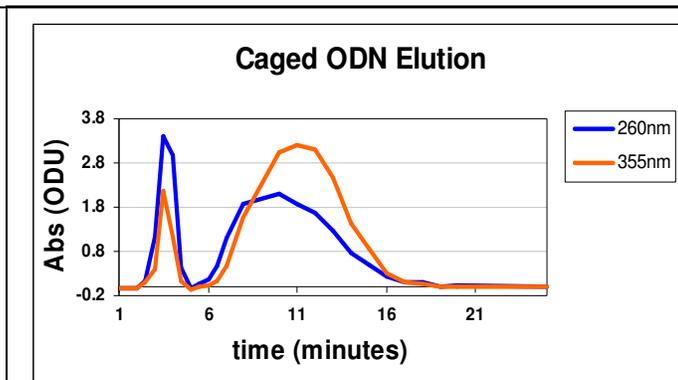

Figure 7: UV absorbance at 260 and 355 nm of the 1-minute aliquots collected from a typical SE column purification of a DNA caging reaction.
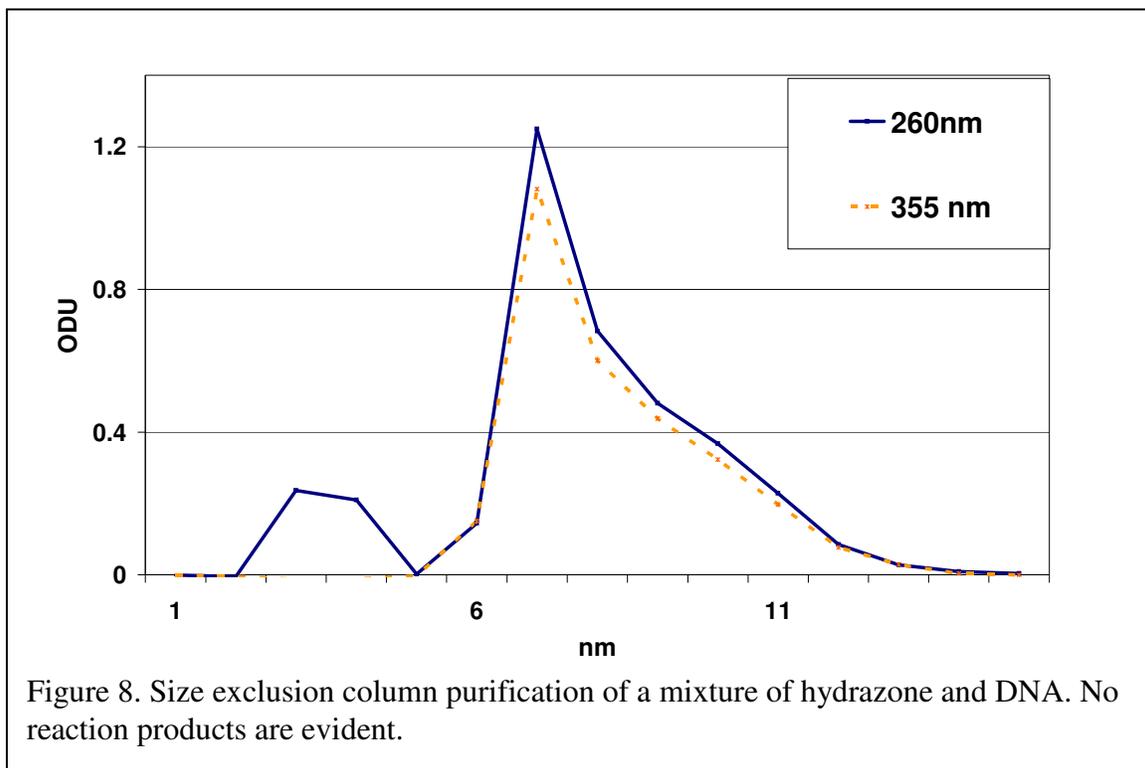
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355 nm to 260 nm absorbance is used to estimate the degree of caging for each caged DNA eluent.

Typical results of size exclusion column purification are shown in Figure 7. Native DNA has a characteristic peak absorbance at 260 nm. DMNPE shows absorbance peaks at both 355 nm and 260 nm. The first eluates from the column (eluting at between 2.5-4.5 minutes), with a high 260 nm absorbance and a lower 355 nm absorbance, were determined by their characteristic spectra and PAGE to be caged DNA. Absorbance baseline was regained at 5 minutes before a second eluate (5.5-18 minutes) was detected, with absorbance spectra characteristic of excess DMNPE, and samples of which showed no staining with PAGE and was hypothesized to be free DMNPE or caged water (DMNPE-OH). This shows that the progress of unattached DMNPE cage molecules with a molecular weight of 221 (or 210 for caged water) through the column was retarded by porous silica beads, while the DNA oligonucleotides with MW 6,063, and larger with cage attached, eluted with the void volume. This technique shows separation of caged DNA from unattached cage, and though some separation of the DNA by number of cage molecules attached is seen (as estimated by the absorbance at 355 nm relative to that at 260 nm), it is not resolved enough to separate products of DNA with only n DMNPE adducts attached per eluate.

The eluents of the DMNPE hydrazone precursor and DNA reaction mixture show no 355 absorbance for the DNA eluates (shown in Figure 8). This suggests that the hydrazone did not react with the DNA. Baseline is achieved between the DNA eluate and the DMNPE hydrazone eluate, which shows a high 355 absorbance.

Native, Caged, and Caged-irradiated DNA Elution Profiles in HPLC

Figure 9 shows the elution profiles of native and 23.5% caged (i.e. 23.5:1 cage:phosphate ratio) before and after UV exposure. Note the presence of multiple contributing peaks in the caged sample, of which fewer are evident in the flashed sample. Figure 10 shows the same for the 51.2% caged DNA. The later eluting peaks (15-20 minutes) appear at a higher intensity in the 51.2% caged sample. Fewer contributing peaks are apparent when compared to the 23.5% caged ODN. There is also less absorbance within the base of the native peak. Both the 23.5% and 51.2% caged samples show a partial return to the elution profile of the native DNA after exposure to UV light. The 355nm absorbance spectra for these samples (shown in Figure 11) show a higher

Figure 8. Size exclusion column purification of a mixture of hydrazone and DNA. No reaction products are evident.

355nm absorbance for the 51.2% caged DNA, as expected. The desired resolution of separation of native from caged DNA elution times was not achieved. The elution peaks

Figure 9 (Left). 260 mn elution profile of 23.5% caged DNA before and after exposure to UV irradiation.

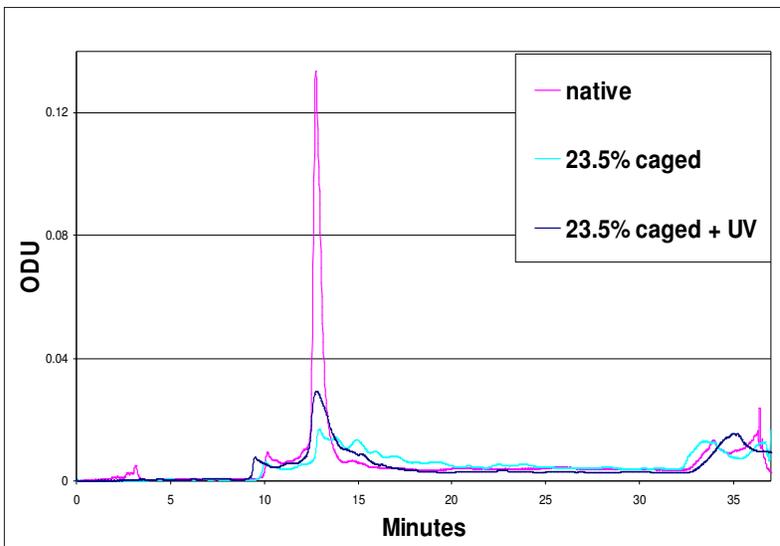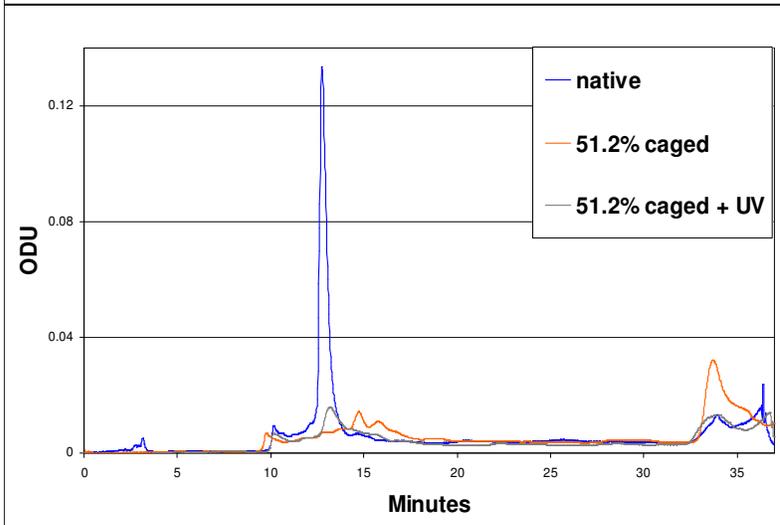

Figure 10 (Left). 260 nm elution profile of 51.2% caged DNA before and after UV irradiation.
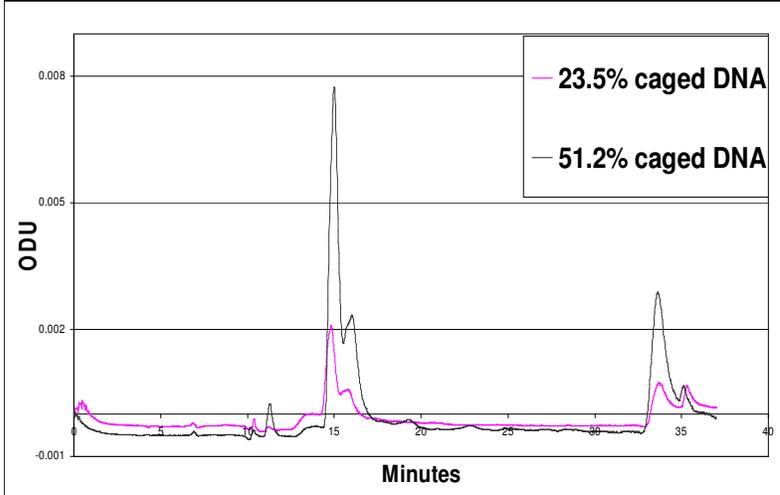

Figure 11 (Left). 355 nm elution profile of 23.5% and 51.2% caged DNA.

of native and caged DNA partially overlap, and the caged DNA elution profiles, though showing several contributing peaks, do not have baseline resolution or sufficient

resolution to fractionate purified, uniform products at the end of the column.

Figure 12 shows HPLC retention time and absorbance data for native (non-caged) 20mer ODN and each of the irradiated DMNPE-caged ODN samples. As UV exposure time increased, caged DNA registered a retention and absorbance profile nearer to that of the native sample. The trailing shoulder of the peak in caged samples is reduced with UV irradiation, indicating a return to the native DNA elution profile and structure. Once baseline is achieved between samples with different degrees of caging, the number of cage molecules adducted before and after UV exposure may be calculated from the peak widths and heights relative to the area under the curve.
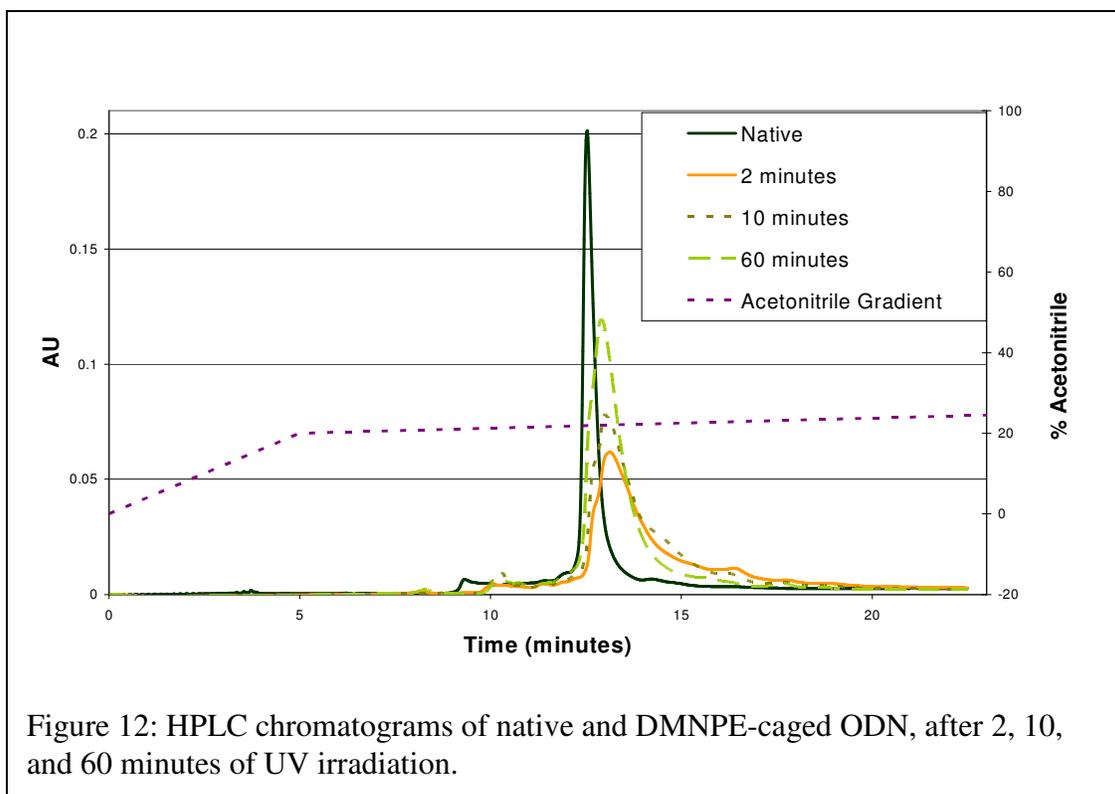

Figure 12: HPLC chromatograms of native and DMNPE-caged ODN, after 2, 10, and 60 minutes of UV irradiation.

Polyacrylamide Gel Electrophoresis (PAGE) of Caged DNA Samples

Electrophoretic mobility and affinity for DNA stain was characterized for the 23.5% and 51.2% DMNPE-caged ODN. The lanes of the gel in Figure 13 contain
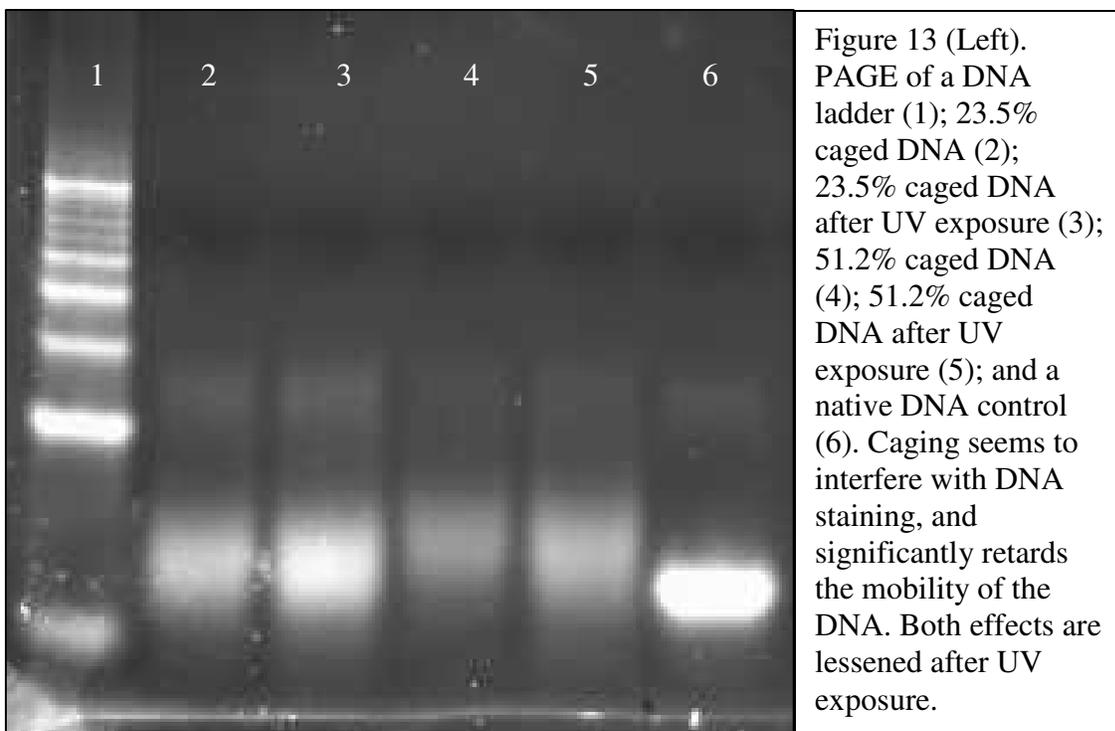
Figure 13 (Left). PAGE of a DNA ladder (1); 23.5% caged DNA (2); 23.5% caged DNA after UV exposure (3); 51.2% caged DNA (4); 51.2% caged DNA after UV exposure (5); and a native DNA control (6). Caging seems to interfere with DNA staining, and significantly retards the mobility of the DNA. Both effects are lessened after UV exposure.

samples of 1. DNA ladder; 2. 23.5% caged DNA; 3. 23.5% caged DNA after 40 minutes exposure to UV light; 4. 51.2% caged DNA; 5. 51.2% caged DNA after 40 minutes of exposure to UV light; 5. a native 20-mer control. In this gel, 23.5% and 51.2% caged DNA samples exhibit a mobility shift and a decrease in fluorescence intensity, both of which are partially restored after exposure to 40 minutes of 365 nm UV irradiation. The alterations in staining intensity between caged and native ODNs suggest that attachment of the DMNPE may also block some reported base-associated labeling of the SYBR-Gold nucleic acid stain used to visualize these ODNs in gels.

NMR Structural Characterization

The crosspeaks between the 4', 5' protons and the α phosphorous moiety are apparent in the HMBC of native and caged ATP (Figures 14 and 15). In the native interferogram, crosspeaks are seen between the α phosphate appearing at -11.5 ppm (f1)
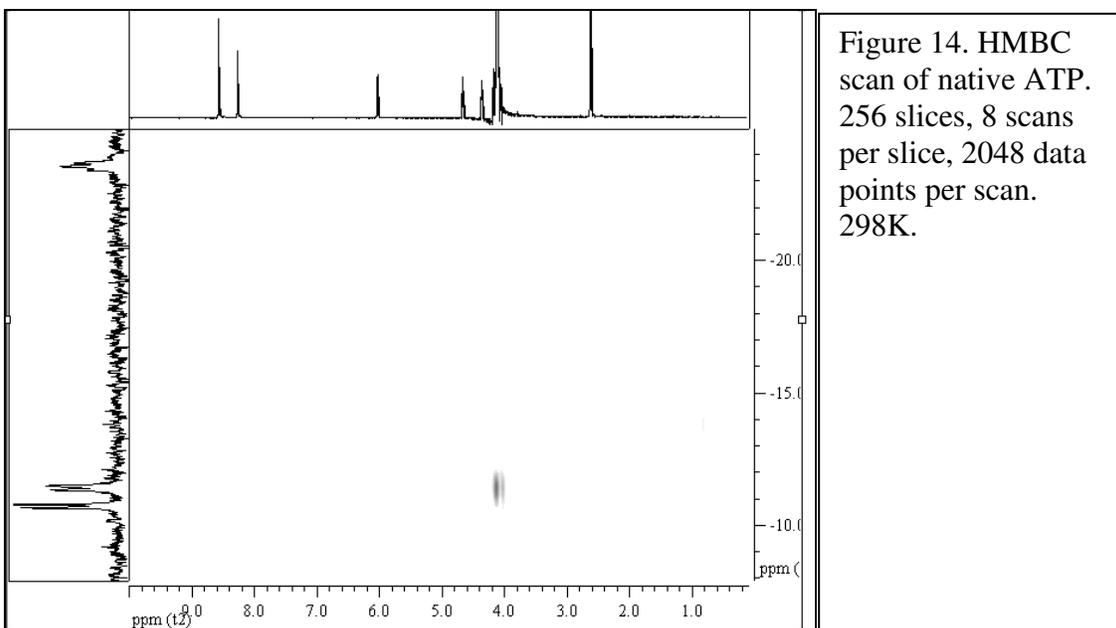
Figure 14. HMBC scan of native ATP. 256 slices, 8 scans per slice, 2048 data points per scan. 298K.
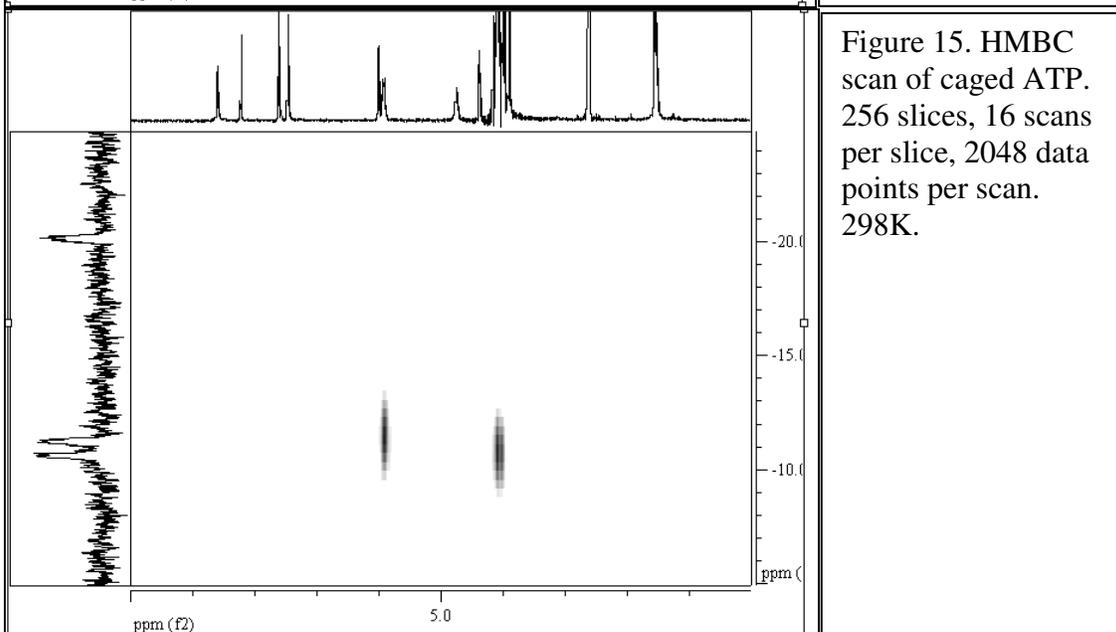
Figure 15. HMBC scan of caged ATP. 256 slices, 16 scans per slice, 2048 data points per scan. 298K.

and the 4',5' protons on the ribose ring, which are lost in the HDO solvent peak at 4.1 ppm. In the HMBC of caged ATP, the α phosphate appears at -10.6 ppm An extra crosspeak is seen between the γ phosphate (-11.2 ppm) and the benzyl proton of the cage molecule DMNPE at 5.9 ppm in the ^{1}H spectrum (f2). In native 1-D spectra, the γ phosphate triplet and α phosphate doublet are well resolved, however in caged spectra these fine structures are not resolved. This is most likely due to the shortened free

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induction decay (FID) T1 relaxation time of this sample of caged ATP, which was about

100 ms, as contrasted to the native FID which lasted well over 250 ms. This may be due

to the attachment of the DMNPE itself, but may also be the effect of contaminating

oxidizer in the sample solution.

Figure 16: Native, 23.5% caged, and 51.2% caged ODNs (top to bottom). Spreading of ^{31}P chemical shifts correlates with increased caging.

Figure 17: 27% caged ODN before (top) and after (bottom) 60 minutes photolysis.
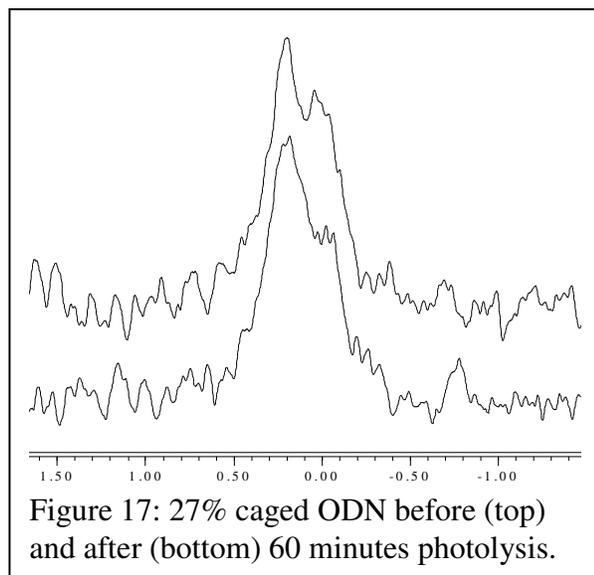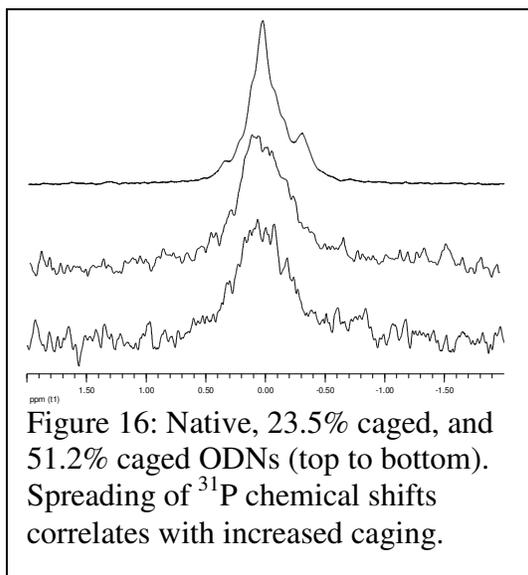
The ^{31}P NMR spectra of caged DNA ODNs compared to native ODN (Figure 16)

and caged ODN after exposure to UV light (Figure 17) show the difficulty of drawing

conclusions based on the 1-D ^{31}P spectra alone. Figure 16 does show increased number of

chemical shifts as the degree of cage adduction increases. However the small changes in

[31]P shifts with caging of 20-mer ODNs may be a result of backbone torsion due to cage

adduction elsewhere on the oligonucleotide rather than cage adduction at the phosphate

backbone, or may even be the effect of remaining NiO_2 oxidizer contamination. Variation

between similar or even identical ODN samples is often as great as that seen here

between native and caged ODN. Figure 17 also shows the similarity of the caged ODN

spectra before and after photoexposure. The ODN ^{31}P resonance changes little with

caging and photorelease of the DNA, in contrast to ATP and duplex DNA with

attachment of adducts at the phosphate sites. This is because the ODN backbone is highly mobile in solution, leading to an averaging of the ³¹P resonance. To overcome these obstacles, a two-dimensional experiment to show a crosspeak between ³¹P and any nearby protons was attempted next.

The 2-D ³¹P-¹H HMBC experiment on native DNA (Figure 18) successfully showed cross-correlation between the backbone phosphates and the H3' and H4'/H5' regions of the ribose rings. However, the HMBC of caged DNA (Figure 19) does not show a new crosspeak between the benzyl proton of DMNPE and the phosphorous resonances. This does not however definitively rule out the phosphate as the site of cage attachment. It is possible that the cage molecule is attached at an angle that would prevent a crosspeak from appearing due to an extremely low ^{3}J coupling constant. According to
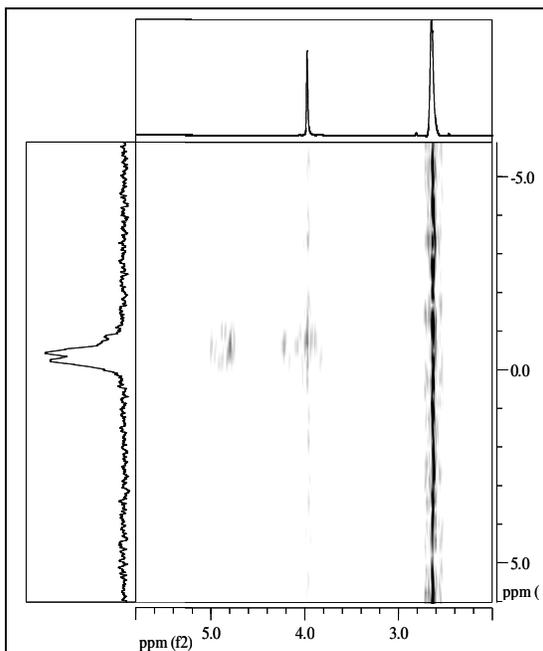

Figure 17: HMBC (NMR) of native 20-mer DNA ODN showing the 3-J crosspeaks of the phosphate backbone (f1 axis) and the H3', H4'/5' regions of the ribose ring (f2 axis). 128 slices, 64 scans per slice, 1024 data points per scan, 298K.
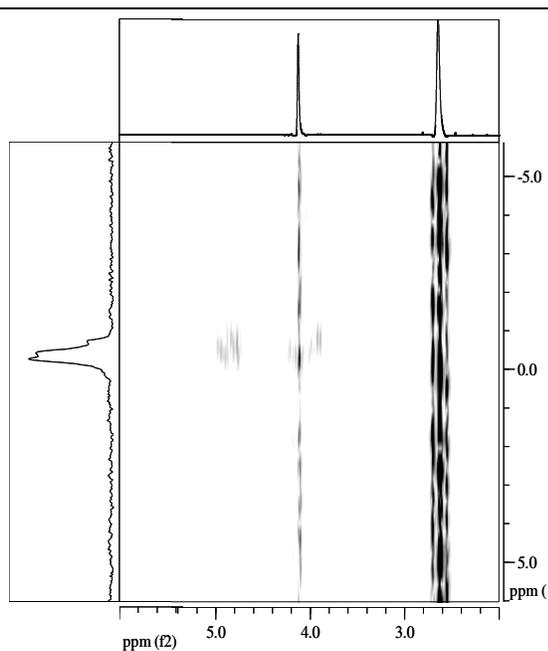
Figure 18: HMBC of DMNPE-caged DNA ODN. No new crosspeak is visible. 128 slices, 16 scans per slice, 1024 data points per scan, 298K.

the HCOP Karplus curve, a backbone dihedral bond angle Φ with a value between 80 and 120 would have a ^3J coupling constant of about 2 Hz or smaller, which may not be strong enough to allow a crosspeak to build up before relaxation effects predominate.

Investigation of DMNPE Adduction at Non-Phosphatidic Sites

RF regions were visualized with both 365 nm light and 254 nm light. Nucleoside and dinucleotide control samples were only visible under 254 nm irradiation. Shadows seen under the 365 nm source were indicative of the presence of DMNPE cage. Thin-layer chromatography results of DMNPE caging reactions of nucleosides were similar for all four nucleosides, as shown in Table 2. The regions in bold font absorbed at 365 nm. No R_f bands appear in the '+ Cage' and 'After UV' samples which are not accounted for in the nucleoside and cage control samples. Thus no new products are evident. Note that the photoexposed cage control for the nucleosides was run six times. On two of the TLC plates, two regions were seen. For the other four only a low or high R_f region was evident. The plates were collectively interpreted as showing two regions. The difference is most likely due to combination of variation in sample present, as TLC does not allow fine control of amount of sample loaded, and regions may have been present but too faint to see and mark.

Table 2: R_f values in TLC of 2'-deoxynucleoside caging reactions. R_f (n, range)			
	Nucleoside Control	+ Cage	After UV
2'-deoxyadenosine	0.81 (3, +/-0.05)	0.81 (3, +/-0.05); **0.67** (3, +/-0.06)	0.83 (4, +/-0.00); **0.80** (4, +/-0.02); **0.61** (4, +/-0.02)
2'-deoxyguanosine	0.91 (3, +/-0.01)	0.92 (3, +/-0.01); **0.71** (3, +/-0.02)	0.92 (3, +/-0.02); **0.88** (3, +/-0.02); **0.63** (3, +/-0.03)
2'-deoxycytidine	0.78 (3, +/-0.11)	0.78 (3, +/-0.11); **0.64** (3, +/-0.10)	0.79 (3, +/-0.011); **0.73** (3, +/-0.07); **0.56** (3, +/-0.08)
2'-deoxythymidine	0.91 (3, +/-0.02)	0.91 (3, +/-0.02); **0.64** (3, +/-0.00)	0.90 (3, +/-0.03); **0.84** (3, +/-0.04); **0.60** (3, +/-0.01)
DMNPE Control		**0.66** (5, +/-0.07)	**0.73** (4, +/-0.06); **0.56** (4, +/-0.08) (see text)

Table 3 shows TLC data of DNA dimers. Formation of a new product in the dTpT solution is clearly evident. dGpG and dCpC do not show product formation in the table, however when more sample was loaded onto TLC plates, dGpG showed a new 365 nm-absorbing region with R_f value 0.67, and dCpC showed two new 365 nm-absorbing regions with R_f values 0.63 and 0.49. Two AA dimer controls showed a second R_f region for the native dinucleotide, R_f 0.71 and 0.72. The 40% MeOH in water solvent system was not ideal for the AA dinucleotide, often leaving streaks or smears of sample on the

Table 3: R_f values in TLC of DNA dinucloetide caging reactions. R_f (*n*, range)			
	Dimer Control	+ Cage	After UV
dApA	0.80 (4, +/-0.03)	0.80 (4, +/-0.03); **0.72** (4, +/-0.07); **0.45** (3, +/-0.06)	0.80 (4, +/-0.03); **0.61** (4, +/-0.03)
dGpG	0.95 (3, +/-0.08)	0.95 (3, +/-0.08); **0.83** (3, +/-0.08)	0.95 (3, +/-0.08); **0.74** (3, +/-0.07)
dCpC	0.85 (3, +/-0.03)	0.85 (3, +/-0.03); **0.82** (3, +/-0.16)	0.85 (3, +/-0.03); **0.72** (3, +/-0.16)
dTpT	0.92 (5, +/-0.05)	0.92 (5, +/-0.05); **0.86** (5, +/-0.06); **0.71** (5, +/-0.04)	0.92 (5, +/-0.05); **0.86** (5, +/-0.06); **0.70** (5, +/-0.05)
DMNPE		**0.78** (10, +/-0.20)	**0.65** (7, +/-0.07)

TLC plates. The dApA dinucleotide did show new regions when more sample was loaded, and the overall results suggest formation of caged product, however the results were variable. While one TLC plate showed no regions in the caging reaction solution that weren't accounted for by the nucleotide and DMNPE controls, the most heavily loaded plate showed a extra 365 nm-absorbing regions at R_f 0.64 and 0.28, as well as retaining a second region (260 nm-absorbing only, R_f 0.72) for native nucleotide that had appeared in the nucleotide control lane. Only the consistently appearing regions are shown in the table.

Conclusions and Future Recommendations

Here is shown the first effort to purify and determine the structure of DMNPE-caged DNA ODNs. Size exclusion was successful as a first step purification method to remove excess cage molecules from the caged ODN complexes in the batch reaction. The data support that a range of products are formed with a varying ratio of adducted cages to ODNs. These different products could have different properties with respect to their bioactivity and will vary in the required intensities or durations of light needed to photoactivate them. This method of purification of caged DNA to remove unattached cage in solution via size exclusion chromatography will facilitate further analytical characterization.

The restoration of DMNPE-caged DNA to its native counterpart in the 260 nm absorbance and retention time of HPLC samples showed light-reversible adduction consistent with previous findings. Although baseline resolution was not achieved between lightly and heavily caged DNA, HPLC results are promising and warrant further study. At the point where the native and caged DNA elute in HPLC, the solution percent acetonitrile is practically isocratic rather than a gradient. At such a shallow gradient it is hard to say whether the ODNs elute at one particular solution percent acetonitrile rather than another. Variation in the percent acetonitrile throughout the column may occur due to diffusion, eddies and stagnant areas in the column, and while normally these variations would be too small to be significant, one can't distinguish the effects of one percentage of acetonitrile versus another with reliability. An isocratic elution method with 20% ACN might yield better resolution between the caged and native DNA peaks. Since the C-4 column used in these experiments was not optimized for the separation of DNA, a C-18

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column made specifically for an n, n-1, etc. DNA separation and purification would expectedly result in better resolution as well. Other solvent systems could also be tried. Some research groups have successfully separated adduct-modified oligos and nucleosides with gradients of methanol (Mae, Margulis et al. 1992; Barry, Day et al. 2005).

NMR attenuation signals and absorbance scans showed that nickel peroxide (NiO_2) is an effective alternative to manganese dioxide MnO_2 as an oxidizer for the DMNPE cage hydrazone in preparation of caged products intended for NMR characterization. The use of an alternate oxidizer was a necessary step toward enabling structural analysis of DMNPE-caged substrates by NMR. ^{31}P NMR analysis has neither conclusively confirmed nor ruled out the phosphate backbone as the primary site of cage attachment, however TLC data of pH 5.5 caging reactions with 2'-deoxynucleosides showed an absence of base caging, while DNA dimers do show formation of a new product, suggesting that the initial hypothetical attachment site of DMNPE on the phosphate backbone of DNA is correct.

Future caging reactions with nucleosides under varying reaction conditions will give data on the effect of pH on reaction product formation, and whether the formation of certain caged products can be controlled through pH. Future HMBC and 1-D NMR experiments on caged and flashed DNA may give further insight into the primary sites of cage adduction. Other future structural characterization assays, such as Fourier-transform infrared spectroscopy (FTIR), may avoid the difficulties associated with NMR characterization while still providing moiety-specific information for DMNPE alkylation of DNA ODNs. Another possibility is to cage dimers or to degrade caged ODNs with

enzymatic digestion, and analyze these simpler products with MS. The results of these purification and characterization experiments will be used to guide future research into the production of caged DNA and other nucleic acid species.

References

Abramova, T. V., J. P. Leonetti, V. V. Vlassov and B. Lebleu (2000). "Synthesis and properties of photolabile (caged) phosphotriester derivatives of dinucleoside phosphates." Russian Journal of Bioorganic Chemistry (Translation of Bioorganicheskaya Khimiya) **26**(3): 174-182.

Allan, A. C., J. L. Ward, M. H. Beale and A. J. Trewavas (1998). Caged plant growth regulators.

Ando, H., T. Furuta, R. Y. Tsien and H. Okamoto (2001). "Photo-mediated gene activation using caged RNA/DNA in zebrafish embryos." Nature Genetics **28**(4): 317-325.

Barry, C. G., C. S. Day and U. Bierbach (2005). "Duplex-Promoted Platination of Adenine-N3 in the Minor
Groove of DNA: Challenging a Longstanding Bioinorganic
Paradigm." Journal of the American Chemical Society **127**(4): 1160-1169.

Baruah, H. and U. Bierbach (2004). "Biophysical characterization and molecular modeling of the coordinativeintercalative DNA monoadduct of a platinum-acridinylthiourea agent in a site-specifically modified dodecamer." J Biol Inorg Chem **9**: 335-344.

Braddock, D. T., J. L. Baber, D. Levens and G. M. Clore (2002). "Molecular basis of sequence-specific single-stranded DNA recognition by KH domains: solution structure of a complex between hnRNP K KH3 and single-stranded DNA." The EMBO Journal **21**(13): 3476-3485.

Chandrasekaran, S., S. Kusuma, D. W. Boykin and W. D. Wilson (1986). "A new approach utilizing high-resolution proton NMR in structural analysis of intercalation complexes of natural DNA." Magnetic Resonance in Chemistry **24**(7): 630-7.

Cohen, B. E., B. L. Stoddard and D. E. Koshland, Jr. (1997). "Caged NADP and NAD. Synthesis and Characterization of Functionally Distinct Caged Compounds." Biochemistry **36**(29): 9035-9044.

Fan, J.-Y., S. J. Ohms, M. Boyd and W. A. Denny (1999). "DNA Adducts of 9-Anilinoacridine Mustards: Characterization by NMR." Chemical Research in Toxicology **12**(12): 1166-1172.

Friedman, O. M., G. N. Mahapatra, B. Dash and R. Stevenson (1965). "Studies on the action of diazomethane on deoxyribonucleic acid. The action of diazomethane on deoxyribonucleosides." Biochimica et Biophysica Acta **103**(2): 286-97.

Gee, K. R., B. K. Carpenter and G. P. Hess (1998). Synthesis, photochemistry, and biological characterization of photolabile protecting groups for carboxylic acids and neurotransmitters.

Ghosn, B., F. R. Haselton and W. T. Monroe (2005). "Light-based Control of DNA Hybridization with Photocleavable Adducts." Photochemistry and Photobiology **Submitted**.

Gill, R. D., Z. Min, C. Cortez, R. G. Harvey, E. L. Loechler and J. DiGiovanni (1993). "Construct ion of Escherichia coli Vectors Containing Deoxyadenosine and Deoxyguanosine Adducts from (+)-anti-Dibenz[a,j]anthracene Diol Epoxide at a Defined Site." Chem. Res. Toxicol. **6**: 681-689.

Givens, R. S. and L. W. Kueper, III (1993). "Photochemistry of phosphate esters." Chemical Reviews (Washington, DC, United States) **93**(1): 55-66.

Givens, R. S., J. F. W. Weber, A. H. Jung and C.-H. Park (1998). New photoprotecting groups: desyl and p-hydroxyphenacyl phosphate and carboxylate esters.

Gorenstein, D. G. (1994). 31-P NMR of Nucleic Acids. Phosphorus-31 NMR spectral properties in compound characterization and structural analysis. L. D. Quin and J. G. Verkade. New York, VCH.

Gorenstein, D. G. (1994). "Conformation and Dynamics of DNA and Protein-DNA Complexes by ^{31}P NMR." Chemical Reviews **94**(5): 1315-1338.

Harding, M. M., G. J. Harden and L. D. Field (1993). "A 31P NMR study of the interaction of the antitumor active metallocene Cp2MoCl2 with calf thymus DNA." FEBS Letters **322**(3): 291-4.

Hess, G. P. and C. Grewer (1998). Development and application of caged ligands for neurotransmitter receptors in transient kinetic and neuronal circuit mapping studies.

Ko, K.-Y. and J.-Y. Kim (1999). "Generation of Dipheyldiazomethane by Oxidation of Benzophenone Hydrazone with Magtrieve[TM]." <u>Bulletin of the Korean Chemical Society</u> **20**(7): 771-2.

Kriek, E. and P. Emmelot (1964). "Methylation of Deoxyribonucleic Acid by Diazomethane." <u>Biochimica et Biophysica Acta</u> **91**: 59-66.

Kusmierek, J. T. and B. Singer (1976). "Sites of alkylation of poly(U) by agents of varying carcinogenicity and stability of products." <u>Biochimica et Biophysica Acta</u> **442**(3): 420-31.

Lawley, P. D. (1984). <u>Carcinogenesis by Alkylating Agents</u>. Washington, DC, American Chemical Society.

Lee, H. C., R. Aarhus, K. R. Gee and T. Kestner (1997). "Caged nicotinic acid adenine dinucleotide phosphate. Synthesis and use." <u>Journal of Biological Chemistry</u> **272**(7): 4172-4178.

Mae, B., L. A. Margulis, B. Li, V. Ibanez, H. Lee, R. G. Harvey and N. E. Geacintov (1992). "Direct Synthesis and Identification of Benzo[alpyrene Diol Epoxide-Deoxyguanosine Binding Sites in Modified Oligodeoxynucleotides." <u>Chem. Res. Toxicol.</u> **5**(6): 773-778.

March, J. (1992). <u>Advanced Organic Chemistry</u>. NY, J. Wiley & Sons.

Meldrum, R. A., K. S. Chittock and C. W. Wharton (1998). <u>Use of caged compounds in studies of the kinetics of DNA repair.</u>

Mitchison, T. J., K. E. Sawin, J. A. Theriot, T. K. Gee and A. Mallavarapu (1998). <u>Caged fluorescent probes.</u>

Monroe, W. T., M. M. McQuain, M. S. Chang, J. S. Alexander and F. R. Haselton (1999). "Targeting expression with light using caged DNA." <u>Journal of biological chemistry</u> **274**(30): 20895-900.

Pelliccioli, A. P. and J. Wirz (2002). "Photoremovable protecting groups: reaction mechanisms and applications." <u>Photochemical & photobiological sciences: Official journal of the European Photochemistry Association and the European Society for Photobiology</u> **1**(7): 441-58.

Privé, G. G., K. Yanagi and R. E. Dickerson (1991). "Structure of the B-DNA decamer C-C-A-A-C-G-T-T-G-G and comparison with isomorphous decamers C-C-A-A-G-A-T-T-G-G and C-C-A-G-G-C-C-T-G-G." Journal of Molecular Biology **217**(1): 177-199.

Quin, L. D. and J. G. Verkade, Eds. (1994). Phosphorus-31 NMR spectral properties in compound characterization and structural analysis. Methods in stereochemical analysis. New York, VCH.

Salvati, M. E., E. J. Moran and R. W. Armstrong (1992). "Simplified method for the isolation of thermally labile drug-DNA adducts: characterization of chlorambucil and carzinophilin/azinomycin B alkylation products." Tetrahedron Letters **33**(26): 3711-14.

Scheidig, A. J., C. Burmester and R. S. Goody (1998). Use of caged nucleotides to characterize unstable intermediates by x-ray crystallography.

Searle, M. S. and A. M. Lane (1992). "31P NMR investigation of the backbone conformation and dynamics of the hexamer duplex d(5 -GCATGC)2 in its complex with the antibiotic nogalamycin." FEBS Letters **297**(3): 292-296.

Singer, B. (1975). "Methylation and ethylation of uridylic acid and thymidylic acid. Reactivity of the ring and phosphate as a function of pH and alkyl group." Biochemistry **14**(20): 4353-7.

Singer, B. (1986). "O-alkyl pyrimidines in mutagenesis and carcinogenesis: occurrence and significance." Cancer Res **46**(10): 4879-85.

Sklenar, V., H. Miyashiro, G. Zon, H. T. Miles and A. Bax (1986). "Assignments of the ^{31}P and ^{1}H resonances in oligonucleotides by two-dimensional NMR spectroscopy." FEBS Letters **208**(1): 94-98.

Sreekumar, R., M. Ikebe and F. S. Fay (1998). Biologically active peptides caged on tyrosine.

Vydac (1998). Oligonucleotide Purification and Analysis by Anion-Exchange and Reversed-Phase Chromatography. Hesperia, CA, The Separations Group.

Walker, J. W., G. P. Reid, J. A. McCray and D. R. Trentham (1988). "Photolabile 1-(2-nitrophenyl)ethyl phosphate esters of adenine nucleotide analogs. Synthesis and mechanism of photolysis." Journal of the American Chemical Society **110**(21): 7170-7.

Walker, J. W., G. P. Reid and D. R. Trentham (1989). "Synthesis and properties of caged nucleotides." Methods in Enzymology **172**(Biomembranes, Pt. S): 288-301.

Wemmer, D. E. (2000). Structure and Dynamics by NMR. Nucleic Acids: Structures, Properties, and Function. V. A. Bloomfield, D. M. Crothers and J. Ignacio Tinoco. Sausalito, CA, University Science Books**:** 111-163.

Wilcox, M., R. W. Viola, K. W. Johnson, A. P. Billington, B. K. Carpenter, J. A. McCray, A. P. Guzikowski and G. P. Hess (1990). "Synthesis of photolabile precursors of amino acid neurotransmitters." Journal of Organic Chemistry **55**(5): 1585-9.

Wilcox, M., R. W. Viola, K. W. Johnson, A. P. Billington, B. K. Carpenter, J. A. McCray, A. P. Guzikowski and G. P. Hess (1990). "Synthesis of photolabile precursors of amino acid neurotransmitters." Journal of Organic Chemistry **55**(5): 1585-9.

Williger, B. T., R. Reich, M. Neeman, T. Bercovici and M. Liscovitch (1995). "Release of gelatinase A (matrix metalloproteinase 2) induced by photolysis of caged phosphatidic acid in HT 1080 metastatic fibrosarcoma cells." Journal of Biological Chemistry **270**(50): 29656-9.

Yamaguchi, K., Y. Tsuda, T.-a. Shimakage and A. Kusumi (1998). "Syntheses of phospholipids containing 2-nitrobenzyl ester moieties at the terminals of alkyl chains and properties of photodegradable liposomes from the lipids." Bulletin of the Chemical Society of Japan **71**(8): 1923-1929.

Znosko, B. M., T. W. Barnes, III, T. R. Krugh and D. H. Turner (2003). "NMR Studies of DNA Single Strands and DNA:RNA Hybrids with and without 1-Propynylation at C5 of Oligopyrimidines." Journal of the American Chemical Society **125**(20): 6090-6097.

Vita

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