DNA Guanine Adducts from 3-Methyl-1,2,3-oxadiazolinium Ions

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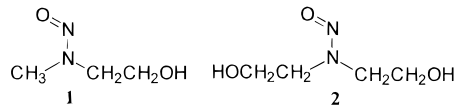
Abstract: The reaction of 3-methyl-1,2,3-oxadiazolinium tosylate **10**, a close model for a putative reactive intermediate in the carcinogenic activation of ethanol nitrosamines such as (2-hydroxyethyl)methylnitrosamine **1**, with various guanine derivatives, including acycloguanosine **12**, deoxyguanosine, deoxyguanosine monophosphate, and cyclic guanosine monophosphate, various DNA oligomers, and calf-thymus DNA has been examined to determine whether this compound methylates and hydroxyethylates guanine residues as proposed. In all of the transformations, 7-(2-(methylnitrosamino)ethyl)guanine (**14**) is the major product, following acidic hydrolysis, and exceeds the formation of 7-methylguanine by ratios ranging from 4:1 to 48:1, depending upon the guanine bearing substrate. O^6-(2-(Methylnitrosamino)ethyl)deoxyguanosine (**20**) was prepared from the Mitsunobu coupling of **1** and a protected deoxyguanosine derivative. **20** is not produced in the reaction of **10** and deoxyguanosine and decomposes to **1** and guanine upon mild acid treatment, suggesting possible neighboring group participation in its facile hydrolytic cleavage. All of the major products from the reaction of **10** and **12** have been characterized, including the direct alkylation product, 7-(2-(methylnitrosamino)ethyl)acycloguanosine (**13**), and N^2-(2-(methylnitrosamino)ethyl)guanine, which was independently synthesized. Elucidation of the reactions of DNA with **10** and other electrophiles was facilitated by the development of both partial and total enzymatic hydrolysis assays utilizing ^{32}P-5′-labeled DNA oligotetramers containing one of each base type and HPLC with radiometric detection. The partial hydrolysis assay gives information as to the type of base being modified, and the total hydrolysis assay permits a determination of the number of adducts produced for a given base. The assays permit a comparison between reactions where the same type of base adduct could be expected. Comparisons of the reactions of ethylene oxide and **10** using this methodology showed that **10** does not hydroxyethylate guanine in DNA.

Introduction

Nitrosamines, such as methylethanolnitrosamine (**1**) and *N*-nitrosodiethanolamine (**2**), derived from common commercial alkanolamines, are widespread environmental trace contaminants[1,2] exhibiting considerable carcinogenicity.[3−5] While com-



mon diakylnitrosamines appear to undergo carcinogenic activation through enzymatic α-hydroxylation as shown in Scheme 1 for dimethylnitrosamine,[6] a process which leads to DNA alkylation,[6] there are serious questions regarding the applicability of this pathway to alkanolnitrosamines such as **1** or **2**. Numerous investigators have probed the microsomal-mediated metabolic activation of *N*-nitrosodiethanolamine (**2**)[7−16] to a mutagen and have failed to observe any mutagenicity, strongly suggesting that this compound is not a substrate for the cytochrome P-450 enzymes which are responsible for the conversion of **3** to **4** (Scheme 1), for example.[7,14−16] Similar preparations have also failed to produce any evidence for metabolic conversions of **2**[14] and have led to several different hypotheses regarding the mode of activation of these compounds. At least three different biochemical activation schemes are under active consideration: (a) conversion of the β-nitrosamino alcohol into reactive aldehydes by alcohol dehydrogenase-mediated oxidation,[11−13,16−21] (b) enzymatic sulfate ester formation (sulfation) of the hydroxyl

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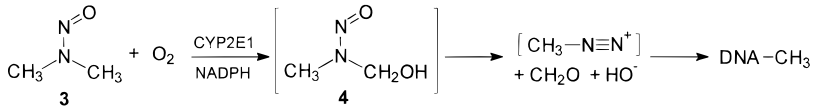
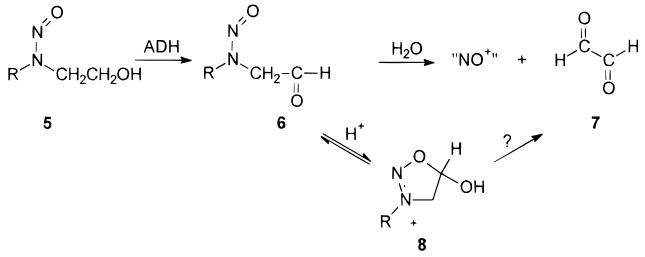
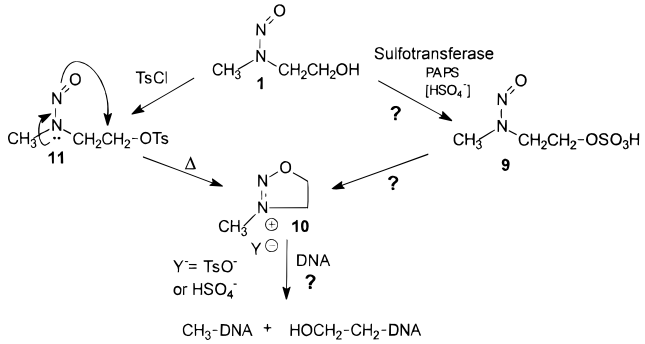
Scheme 1



Scheme 2



Scheme 3



group to generate a compound which undergoes *N*-nitroso oxygen neighboring group assisted solvolysis to generate alkylating agents,[5,21−28] and (c) chain-shortening reactions to generate methyl-substituted nitrosamines which are substrates for cytochrome P-450 enzymes.[30−34] The work presented here is principally directed at testing parts of the sulfation mode of activation which foresees the generation of a 3-methyl-1,2,3-oxadiazolinium ion **10** (Scheme 3), which then methylates or hydroxyethylates DNA.[5,21−28] We report here that these oxadiazolinium cations principally react with guanine residues in DNA through an attack of N-7 of the guanine on C-5 of the oxadiazolinium ion to produce an N-7-modified base carrying the entire nitrosamine fragment. We describe the development of a simple assay using a DNA oligotetramer which shows that guanine is the principal residue attacked by the oxadiazolinium ion and that this transformation also leads to the formation of an unstable adduct. Adduct syntheses have shown that O^6-(2-(methylnitrosamino)ethyl)guanine is unstable and hydrolyzes with possible neighboring group participation to guanine and 2-hydroxyethylmethylnitrosamine.

Alkylethanolnitrosamines **5** are known to be substrates for mammalian alcohol dehydrogenases yielding reactive α-nitrosamino aldehydes **6**[15−16,18] (Scheme 2). The aldehydes exhibit a complex chemistry,[17−21] but two important transformations connected with them involve their surprising ability to donate their nitroso group to other nitrogen compounds either forming nitrosamines,[17,18] or leading to the deamination of primary amines,[17−19,21] and their generation of glyoxal and/or glyoxal equivalents in their reactions.[17−19,21] While it is not known whether glyoxal generation is connected to their ability to enter into trans-nitrosation transformations, we have demonstrated that 2-(methylnitrosamino)ethanal **6a** (R = CH_3) cyclizes by means of neighboring group interaction between a nitroso oxygen and the carbonyl group to produce 3-methyl-5-hydroxy-1,2,3-oxadiazolinium ion (**8a**).[20] Other investigations in our laboratory have suggested that compounds such as **8** may be the progenitors of glyoxal **7** in transformations of nitrosamino aldehydes, but little is known about the chemistry of 3-alkyl-oxadiazolinium ions.

As mentioned above, another hypothesis for the activation of nitrosamino alcohols involves their sulfation at the hydroxyl group, as shown in Scheme 3, to generate the sulfate ester **9**. This ester is proposed to undergo an intramolecular nucleophilic substitution to generate the methyl oxadiazolinium sulfate **10**, which has then been proposed to be a DNA-methylating and -hydroxyethylating agent through nucleophilic attack at either the methyl group or the other carbon adjacent to the nitrogen.[5,22−28] This hypothesis was originated by C. J. Michejda and his co-workers and followed from their finding that the tosylate of methylethanolnitrosamine **11** cyclizes upon warming in methylene chloride to generate 3-methyl-1,2,3-oxadiazolinium tosylate **10** (Scheme 3).[22,23] Michejda's group also demonstrated *N*-nitroso oxygen assistance during solvolysis of this compound in acetic acid and in other solvents and demonstrated that the transformation occurred with double inversion at carbon as one would expect.[23] These neighboring group solvolysis transformations show that oxadiazolinium ions do suffer nucleophilic attack at C-5. Related oxadiazolinium ions have also been postulated as intermediates in transformations of chemotherapeutic chloroethylnitrosoureas such as carbustine[35] (bis-(2-chloroethyl)-*N*-nitrosourea). These compounds are known to introduce hydroxyethyl groups into DNA. Both Michejda's group and our group have shown that 3-alkyloxadiazolinium ions undergo nucleophilic attack at C-5.[23,29] Our work has also shown that attack occurs at the CH_3 of **10**, but to a lesser extent.[29] Numerous other transformations occur as well, and we have recently demonstrated that these compounds are also capable of oxidizing thiols to disulfides.[29]

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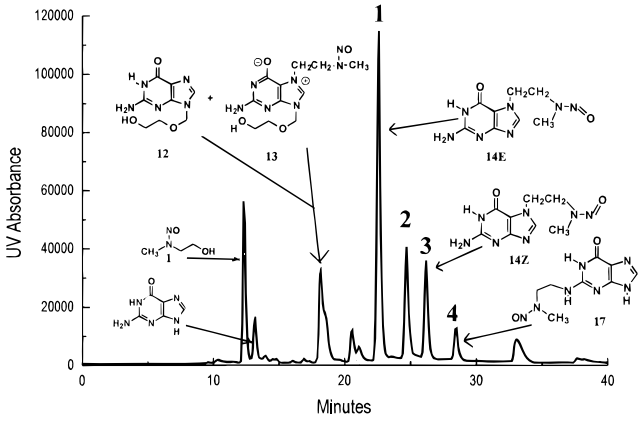

Figure 1. Reversed phase HPLC of the mixture produced from the reaction of **10** and **12** in DMF followed by acidic hydrolysis. With the exception of peaks 1 and 3, compounds were identified by spiking with authentic materials. Peak 2 is an unknown (see text).

3-Alkyloxadiazolinium ions are related to alkoxydiazenium ions (O-alkylated nitrosamines) studied some years ago by Huenig.[36] Among Huenig's findings is the important observation of the high acidity of the CH bonds adjacent to the nitrogen in these structures.[36] Indeed, we have postulated that this chemistry is responsible for the thiol oxidations which we have observed. It is obvious from these data that 1,2,3-oxadiazolinium ions can undergo a number of transformations and one cannot well anticipate how they might react with nucleotides present in DNA and RNA. This paper is directed at the elucidation of the reactions of **10** with guanine derivatives and guanine residues in DNA.

Results and Discussion

Reaction of 3-Methyl-1,2,3-oxadiazolinium Tosylate with Acycloguanosine. Because the reactivity of **10** led us to anticipate a large number of possible products from the reaction of **10** with guanine derivatives,[21] we first chose to explore the reaction of acycloguanosine **12** with **10**. Acycloguanosine is a reasonably soluble compound and has fewer potentially reactive hydroxyl groups than guanosine, or deoxyguanosine, for example. Its pseudonucleosidic linkage also permits the cleavage of the pendent group from N-9 of the purine ring through acidic hydrolysis, but is somewhat more stable than the natural nucleosides. Freshly prepared **10** was reacted with **12** in DMF at 37 °C for 3 h. The resulting product mixture was treated with aqueous hydrogen chloride to hydrolytically remove the group from N-9. Neutralization and concentration led to a mixture which was submitted to HPLC and gave the chromatogram shown in Figure 1. The chromatogram showed two major peaks in the reaction mixture (peaks 1 and 3). Preparative HPLC and fractionation led to collection of the eluent from peaks 1 and 3, respectively. These materials were stripped of solvent at room temperature, further purified by HPLC, and submitted to spectroscopic characterization.

^{1}H-NMR analysis (see Figure 2) of the material from either peak 1 or peak 3 showed that each substance contained small amounts of the other. Moreover, when these samples, generated immediately from the HPLC separation, were allowed to stand, the same product mixture spectrum was produced from each. These data suggested that the materials from peaks 1 and 3 were isomers which interconverted on standing. High-resolution mass spectrometry of the compound obtained from peak 1 exhibited a parent ion at *m/z* 237.0937, consistent with the molecular formula $C_8H_{11}N_7O_2$. The base peak in the mass spectrum is found at *m/z* 207.1022, corresponding to M − NO. These data, as well as the NMR spectral data, are consistent with a compound which contains one guanine residue and one (methylnitrosamino)ethyl fragment derived from **10**. The compounds from peaks 1 and 3 have been assigned the structure of 7-((*E*)-2-(methylnitrosamino)ethyl)guanine and 7-((*Z*)-2-(methylnitrosamino)ethyl)guanine, respectively, **14E** and **14Z** (Scheme 4). The stereoisomeric *Z* and *E* nitrosamine structures for these compounds are supported by the NMR data. CH_3 peaks for the *E* and *Z* isomers are found at δ 3.00 and 3.67, respectively. It is well-known that the nitrosamine oxygen *syn* to C or H produces significant shielding. Similarly, the methylenes attached to the nitrogen exhibit significantly different chemical shifts. Other signals in the ^{1}H spectrum are completely consistent with those expected of N-7-substituted guanine, although NMR shifts are not especially good in differentiating substitution positions in substituted guanines. It is known that *Z* and *E* isomers of nitrosamines can be separated by HPLC and that their interconversion barriers (17−21 kcal/mol) are consistent with isomerization at normal laboratory temperatures. The ^{13}C NMR spectra of the isomers are also consistent with the structural assignment (Figure 2).

The assigned position of substitution at N-7 in the guanine ring was further substantiated by ^{1}H NMR NOESY data, the UV spectra of these isomers, and the syntheses of related isomers as is described below. Assignment of the position of attachment of the nitrosamine fragment to the guanine ring is

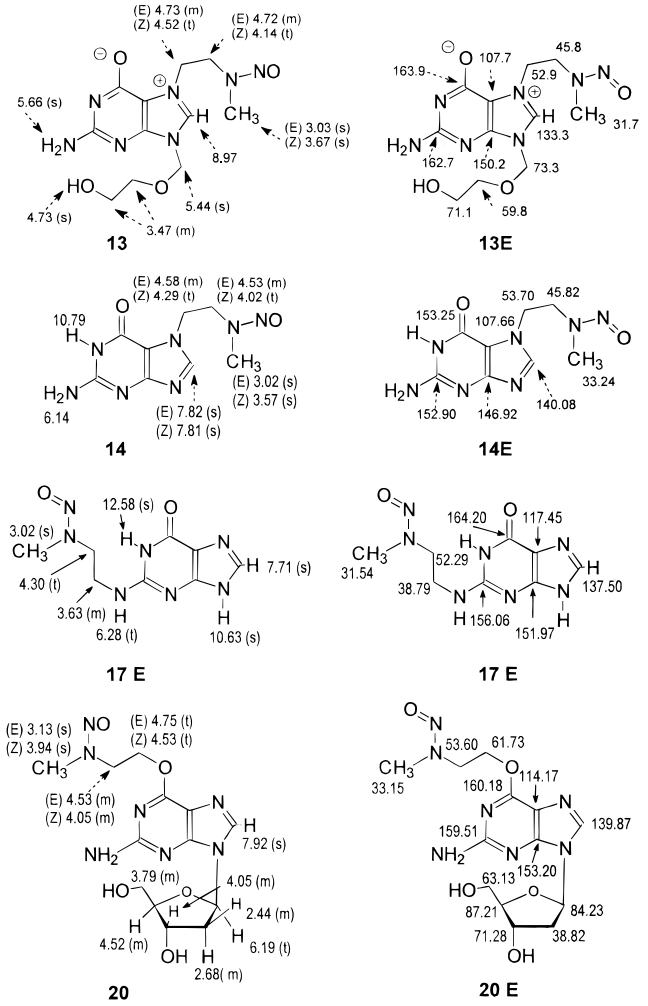

Figure 2. NMR assignments for guanine derivatives. ^{1}H data are on the left and ^{13}C are on the right. (The ^{13}C spectrum of **14** was recorded in D_2O/DCl and others as noted in the Experimental Section.)

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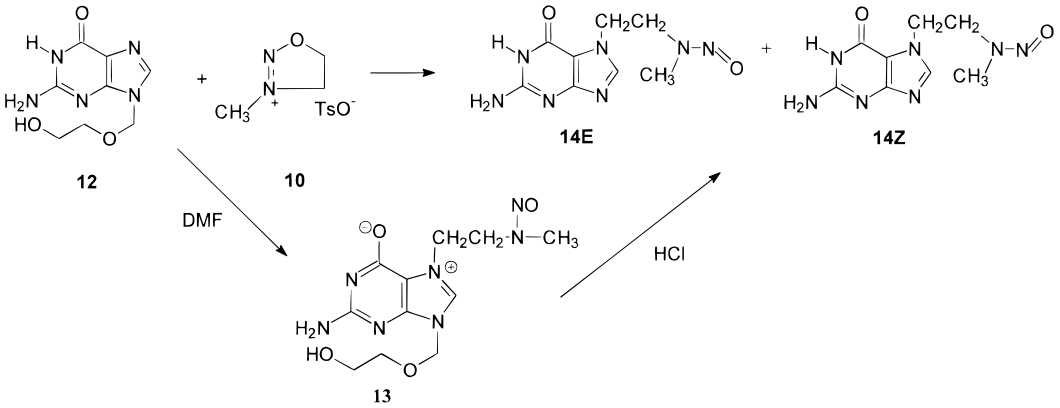
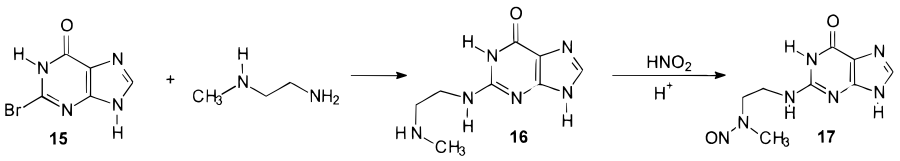
Scheme 4



Scheme 5



not straightforward using UV spectral data because the nitrosamine fragment also absorbs in UV with a maximum at approximately 240 nm and a weaker band at 360 nm. Although the UV spectra of the *Z* and *E* isomers are somewhat different, both exhibited a strong maximum between 270 and 277 nm in basic solution which was transformed into a shoulder-like maximum at pH 1 between 240 and 248 nm. 7-Ethylguanine exhibits similar UV spectral characteristics showing a maximum at 279 nm (pH 12) and 250 nm (pH 1) and a shoulder at 273 nm (pH 1).[40] Both the *E* and the *Z* isomers exhibited similar, but less pronounced, shoulder phenomena in this region. The NOESY spectra of **14E** and **14Z** showed separate but strong cross peaks between the purine C-8 H (*E* isomer, δ 7.82) and the N-bound CH_2 (*E* isomer, δ 4.58), thereby securing the positional assignment at N-7 as shown. The accumulated spectral data are completely consistent with the assigned structure **14E** and **14Z**.

The other components from the reaction of **10** and **12** are as identified in Figure 1. The material constituting peak 2 exhibited unusual behavior. Upon collection and reinjection into the chromatograph, a substance having the same retention time as **14E** was obtained. Both HPLC and NMR experiments show that the isolation of this material resulted in the isolation of **14E,** which then equilibrated with **14Z**. HPLC−MS experiments show that the material constituting peak 2 has the same nominal mass as **14E/Z** but a spectrum somewhat different from either. We speculate that this substance is a ring/chain isomer of **13** arising from the interaction of the amine N of **13** with C-8, but its behavior is sufficiently bizarre that its complete characterization will be addressed in a forthcoming publication.

Peak 4 in the chromatogram shown in Figure 1 was characterized as N^2-(2-(methylnitrosamino)ethyl)guanine **17** on the basis of MS data and its unambiguous synthesis. The guanine adduct **17** was prepared as shown in Scheme 5. 2-Bromo-6-hydroxypurine reacted smoothly with *N*-methylethylenediamine to produce N^2-(2-(methylamino)ethyl)guanine (**16**). Nitrosation of this compound under acidic conditions produced the N^2-nitrosamine adduct **17**. The NMR spectral data for **17** are completely consistent with its structural assignment, and HPLC spiking experiments showed peak 4 and **17** to be coincident.

The fact that the HPLC−MS data for peak 2 (chromatogram, Figure 1) showed it to be an isomer of **14**, as well as the known chemistry of alkylating agents toward guanine derivatives, required us to test the hypothesis that peak 2 and other unstable adducts described later could have the structure of O^6-(2-(methylnitrosamino)ethyl)guanine or a related derivative. Toward this end, considerable effort was directed at the synthesis of a suitable guanine derivative bearing this nitrosamine fragment at the O^6 position. Reaction of N^2,N^2,9-tris(trimethylsilyl)-2-amino-6-chloropurine with the sodium alkoxide of *N*-methyl-2-aminoethanol gave the protected O^6-(2-(methylamino)ethyl)guanine. The nitrosation of the amine led to an unstable compound which decomposed to give guanine and (2-hydroxylethyl)methylnitrosamine (**1**) under HPLC conditions.

An alternative synthesis utilized the protected deoxyguanosine derivative **18** (Scheme 6). Mitsunobu coupling[38] of **18** and the nitrosamino alcohol **1** resulted in the formation of the desired protected O^6-deoxyguanosine−nitrosamine adduct **19**. The protecting groups were removed to generate O^6-(2-(methylnitrosamino)ethyl)deoxyguanosine (**20**). All attempts to generate the O^6-guanine−nitrosamine adduct **21a** (Scheme 7) by acid hydrolysis of the nucleosidic linkage of **20** have failed so far. The products produced from this transformation are guanine and **1**. The mild acid hydrolysis (0.1 M HCl, 25 °C) of **20** was conducted as a function of time and followed by HPLC. Under these conditions, both the nucleosidic linkage and the nitrosamine function are cleaved from the guanine moiety. In no case was the formation of **14E** or **14Z** observed, and no peaks produced from the hydrolysis of **20** were coincident with peak 2. Moschel has reported on the facile solvolytic removal of benzyl groups from the O^6 position of guanine.[39] We believe that the nitrosamine function is being removed from the O^6 position of the guanine derivatives by a pathway involving neighboring group participation as shown in Scheme 7. This process produces the 1,2,3-oxadiazolinium ion, which suffers

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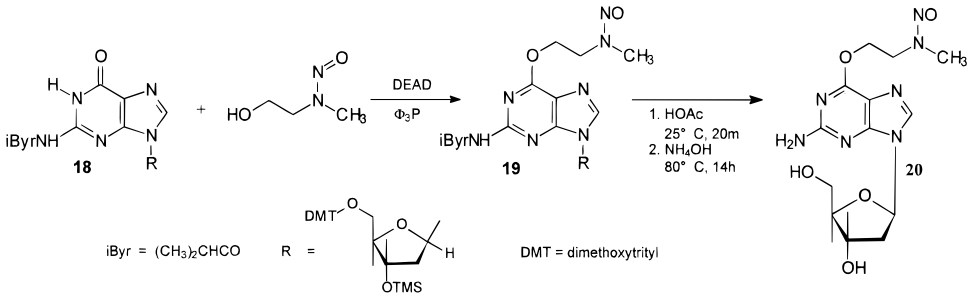
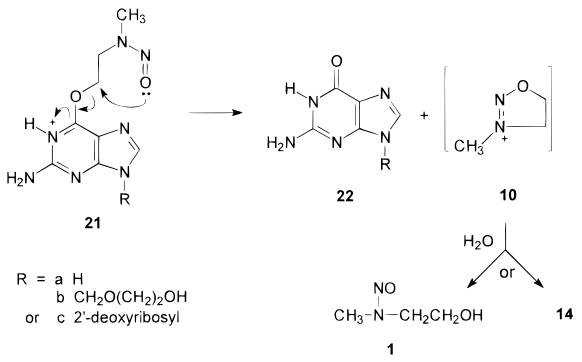
Scheme 6



Scheme 7



rapid hydrolysis to **1** under the reaction conditions, and is completely consistent with the experiments of the Michejda group,[22,23] who demonstrated neighboring group participation of the nitroso oxygen in the solvolysis of esters of **1**.

Reaction of 10 with Deoxyguanosine. In order to further determine whether peak 2 may be the O^6-G adduct **21a**, we carefully examined the reaction between **10** and deoxyguanosine (dG). Reaction of **10** and dG at 25 °C in DMF was followed as a function of time using HPLC. Within 30 min, peaks corresponding to **14Z** and **14E** were observed in the chromatogram as minor peaks. In addition to dG, two major peaks were observed which decreased in intensity over the course of 2 h as the relative amount of **14E** and **14Z** increased. Chromatographic comparison, including spiking experiments, failed to show the formation of any O^6 product **20** in the reaction mixture. Our interpretation of these experiments is that principal alkylation occurs at N-7 by the oxadiazolinium ion to produce an unstable nucleoside which undergoes spontaneous loss of the sugar moiety under the reaction conditions to produce **14**. These data are consistent with numerous varied experiments on the alkylation of guanine derivatives which demonstrate the N-7 position to be the most nucleophilic, particularly toward "S_N2" alkylating agents.[41] Only those alkylating agents which are highly reactive and exhibit considerable carbocation-like character in their transition state produce significant amounts of alkylation at the O^6 position. The oxadiazolinium ion **10** is unexpectedly selective in its ability to alkylate the N-7 position almost exclusively. These experiments further support the conclusion that the material giving rise to peak 2 in the chromatogram is not the O^6-guanine−nitrosamine adduct **21a**. It is unlikely that acycloguanosine and deoxyguanosine have significantly different reactivities toward **10**.

Characterization of the Precursor of 14E/Z. The observation of unstable peaks in our experiments with DNA oligomers, the unusual characteristics of peak 2, as well as our concern that rearrangements could be occurring during hydrolytic transformations led to a careful characterization of the structure and properties of the precursor to **14E/Z**. Employing the HPLC gradient used to produce the chromatograph shown in Figure 1, the precursor was found to have a retention time nearly the same as that of acycloguanosine. Separation was accomplished by TLC followed by HPLC. HPLC−thermospray-MS showed the precursor to have a parent ion at *m/z* 312 (M + 1). This mass value is consistent with the compound incorporating the acycloguanosine and the 2-(methylnitrosamino)ethyl fragment similar to **14** but existing in a zwitterionic form as shown. Indeed all of the spectral data (see Figure 2 for NMR assignments) are consistent with the assignment of structure **13** (Scheme 4) to this compound. The key assignment of the position of the 2-(methylnitrosamino)ethyl chain was made by NOESY which shows a cross peak between the C-8 H (δ 8.97) of the purine ring and the N-7 bound CH_2 (δ 4.73). The ^{1}H NMR shows significant downfield shift for the C-8 H of the purine ring (δ 8.97). This value is close to what is observed for the corresponding proton in 7-methyldeoxyguanosine (δ 9.2).[37] Chemical shifts for the C-8 H in the range of 7.8 are observed for guanine adducts having no substituent at N-9. The ^{1}H NMR also exhibits chemical shift differences for nitrosamine *E* and *Z* isomers, consistent with the presence of the nitrosamine fragment in the molecule. The ^{13}C NMR for this compound shows that C-5 (δ 107.6) is shifted approximately 9 ppm from its position in acycloguanosine and exhibits a chemical shift very close to what is observed for 7-methyldeoxyguanosine (δ 107.7). The C-5 carbon in O-6-substituted guanines is found in the region of δ 113.[37] The data are inconsistent with any rearrangement of **13** during the hydrolytic work of the reaction mixture. 7,9-Dimethylguanine[43] has a pK_a = 7.19, and the structural similarity between this compound and **13** and the observed mass logically lead to the conclusion that it is existing as a zwitterion as isolated.

Several experiments were conducted to determine the stability of **13** and **14** under hydrolysis conditions. Compound **13** is stable to H_2O at 75 °C for 12 h. Treatment of **13** with a 17% aqueous solution of HCl in a small amount of DMF at 70 °C for 2 h led only to the cleavage of the pseudonucleosidic linkage and the formation of the isomers **14**. No evidence was found for the cleavage of the nitrosamine fragment from N-7 with either **13** or **14** under these conditions. Both stereoisomers of **14** were stable toward either 20% aqueous HCl or 10% NaOH at 65 °C for 2 h.

Reaction of 10 with Nucleotides, Oligonucleotides, and DNA. Reaction with cdGMP. Experiments were also performed to determine whether **10** would suffer nucleophilic attack at any of its three carbon atoms by a phosphate oxygen of a nucleotide. Cyclic 3′,5′-deoxyguanosine monophosphate (cdGMP) was chosen as a model substrate for this work. Should alkylation occur at one of the phosphate oxygens to produce a phosphotriester, the product is anticipated to hydrolyze to yield

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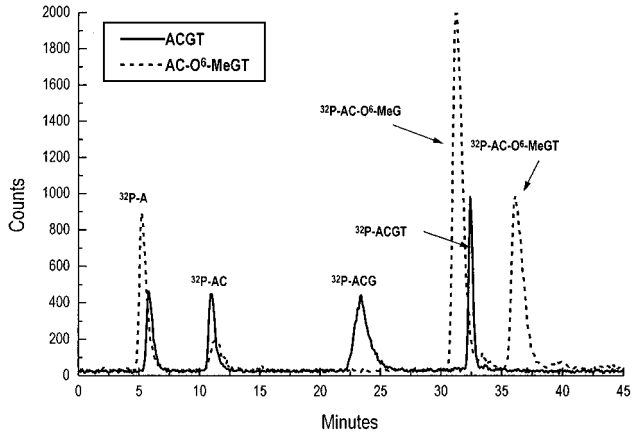

Figure 3. 3. Reversed phase, ion-paired, HPLC radiochromatogram of enzymatic partial hydrolysates of two 5′-^{32}P-labeled oligotetramers as identified. Partial hydrolysis produces three nucleotides, monomer, dimer, and trimer, in addition to the unhydrolyzed tetramer. All oligonucleotides containing the modified base shift chromatographic position permitting identification of the modified base.

two products, a phosphodiester at the 5′ position and a phosphodiester at the 3′ position. While the reaction of cdGMP with **10** gives rise to a mixture, the data presented in detail in the Experimental Section give no indication of alkylation of cdGMP at the phosphate oxygen atoms. Again, N-7 alkylation to generate **14E/Z** is the main pathway.

Development of ^{32}P Oligotetrameric Assay. It is well-known that DNA reactive alkylating agents often exhibit differing reactions and specificities with purine bases, nucleosides, nucleotides, DNA oligomers, and DNA itself. Anticipating that reactions of **10** with DNA may prove difficult to characterize, and desiring a method which could be applied to other DNA-reactive agents,[21] we sought a possible solution to this problem in the development of a simple oligotetrameric radiochemical assay. A DNA oligotetramer containing one of each base type (ACGT) was labeled with $^{32}PO_4^{3-}$ using [γ-^{32}P]-ATP and T4 polynucleotide kinase. A number of experiments employing either nuclease P_1 or a snake venom phosphodiesterase (SVP) showed that this oligotetramer could be partially hydrolyzed to give a radiochromatographically visible mixture of monomer, dimer, trimer, and tetramer. The best conditions employ the use of SVP at pH 5.3 at 0 °C for 2 min. The resulting mixture of oligomers was separated by HPLC reversed phase ion-paired chromatography utilizing a radiometric detector for the ^{32}P. The typical chromatogram shown in Figure 3 demonstrates that the retention times are proportional to the length of the oligomer.

Reaction of the labeled oligotetramer with alkylating agents produces a mixture of modified oligotetramers which can then be separated by HPLC prior to partial hydrolysis. The complete separation of all of the modified oligomers cannot always be achieved because of the numerous nucleophilic sites present. Following isolation and purification, the modified oligomer is subjected to partial enzymatic hydrolysis. The identity of the modified base can be determined by a comparison of the chromatogram of the partial hydrolysate of the modified oligotetramer with the chromatogram from the partial hydrolysate of the unmodified oligotetramer. Modification of one base results in a chromatographic shift in the retention times of all oligomers containing the modified base. This is demonstrated in Figure 3, where the chromatogram of a partial hydrolysate of 5′-labeled AGTC carrying a methyl group at the O^6 position of G is shown and compared to the partial hydrolysate of the unmodified oligomer. The modified oligotetramer was prepared using standard DNA synthesis methodology employing an *O*^{6}-methylguaninephosphoramidate. Comparison of the chromatograms in Figure 3 shows that the trimer and the tetramer carrying the methyl group at the O^6 position of guanine are shifted to longer retention times compared to the position of the unmodified oligomers carrying guanine. While this chromatographic comparison demonstrates the power of the methodology, a number of experiments using highly reactive methylating agents often presented problems related to the separation of the modified oligotetramers. Less reactive electrophiles give mixtures which can easily be separated.

Application of the Assay to the Reaction of ACGT with 10. The partial hydrolysis assay was used in concert with a complementary total hydrolysis assay and the acycloguanosine structural studies to reveal the nature of the reaction between **10** and guanine bases in DNA. This methodology, which is highly sensitive because of the use of radiotracers, showed that **10** forms an unstable adduct with guanine bases, that the major modifications are occurring at G, that there are two major G adducts in addition to the unstable one, that **10** produces a small amount of *O*^{6}-G-methylation, and that **10** does not produce *O*^{6} or N-7 hydroxyethyl adducts. The reaction of **10** with [^{32}P]-ACGT produces eight HPLC oligomeric fractions. All fractions were collected, reinjected, and submitted to the partial hydrolysis assay. The largest fraction (19.5 min) was unstable to the isolation procedure and reverted to the starting oligomer. This phenomenon was also apparent in the partial hydrolysis of this substance, which indicated that it was a G adduct. To gain a better understanding of the possible nature of the unstable adduct, we allowed **10** to compete for sites in [5′-^{32}P]GTAC. The resulting mixture of modified oligotetramers was subjected to total enzymatic hydrolysis using SVP, generating only labeled guanosine nucleotides. These experiments gave evidence for three different modified guanine nucleotides, one of which was unstable toward isolation and generated dGMP. This assay method gives an indication of the number of guanine modifications and, in principle, could be applied to the reaction of any DNA reactive compound with the choice of any base at the 5′ terminal end. The identity of the unstable adduct(s) remains unknown. The amount of fraction 5 increases with the concentration of **10**. Extensive manipulation of isolation and chromatographic conditions always led to the same result, isolation of unmodified material. While the data of the preceding section cast doubt on reactions at either O^6 or one of the phosphorus oxygens, the former type of adduct is known to be unstable and polynucleotides may exhibit different reactivity toward **10** than that observed for acycloguanosine (**12**).

Of the remaining fractions in the [^{32}P]ACGT assay, fraction 6, the next larger quantity was unreacted tetramer, followed in quantity by fraction 3 which proved to be a trimer containing a C modification. The next largest fraction (4) was modified at G. Of the minor fractions, 7 was identical in all respects with the *O*^{6}-methyl G-modified oligotetramer which we had used as a standard in this experiment as partial hydrolysis showed identical characteristics as well. It appears that a small amount of *O*^{6}-methylation by the methyloxadiazolinium ion **10** occurs.

Large-Scale Total Enzymatic Hydrolysis Assay. With these experiments as a guide, we examined the reaction of the GTAC oligotetramer with **10** on a larger scale. GTAC was mixed with a small portion of the ^{32}P-labeled GTAC as a tracer and the combination reacted with **10** at pH 7.4 in phosphate buffer for 4 h. HPLC separation of the resulting mixture gave two major fractions. Each fraction was subjected to mild thermal-acid hydrolysis to remove the modified bases (depurination), and the resulting bases were separated by HPLC. The

Table 1. Ratios of **14E/Z** and 7-Methylguanine Produced in the Reaction of **10** with Various Substrates

substrate	ratio
acycloguanosine (12)	∞
dGMP	48
$(dG)_8$	4.8
GTAC	4
DNA	6.8

data revealed the presence of 7-methylguanine, identified through a comparison of its chromatographic retention time and spiking experiments, and the nitrosamine adducts **14E** and **14Z** in a ratio of 1 (CH_3) to 4 (**14E** and **14Z**). It is important to note that the hydrolytic procedure, 1 M HCl at 60 °C for 1 h, specifically results in the hydrolysis of N-substituted guanines, and other compounds containing N^2 and O^6 modification at G would likely not be detected by this procedure. While 7-methylguanine was not a product from the reaction of **10** with acycloguanosine (followed by hydrolysis), it was observed as a very minor product in the reaction of **10** with dGMP which also gave **14** as the principal product. The relative yields of **14E/Z** and 7-methylguanine are given in Table 1 for a number of substrates. In every case **14E/Z** is the major product, but methylation becomes more important in polynucleotides, suggesting possible nucleotide catalysis of methylation.

Reaction of 10 with Double-Stranded DNA. Both N-7-methylation of guanine and the formation of **14E/Z** was observed from the reaction of **10** with calf-thymus DNA (pH 7.4, 4 h at 37 °C). The adducts were released by neutral thermal hydrolysis and were detected by HPLC. The ratio of **14E/Z** to methylation was 6.8 in this case. Thus, the methyloxadiazolinium ion does lead to the methylation of guanine in DNA as hypothesized by Michejda, but the major transformation involves the attack of the N-7 position of guanine on the 5 position of the oxadiazolinium ion as observed for acycloguanosine **12**. The larger amounts of N-7 methylation in polynucleotides may result from complexation and/or catalysis of transformations of **10** by DNA.

Tests for Other Adduct Products. Michejda and colleagues originally proposed that **10** was both a methylating and hydroxyethylating agent.[25] Indeed, the administration of ^{14}C-labeled (2-hydroxyethyl)methylnitrosamine to rats does result in both the methylation and the hydroxyethylation of guanine residues in the DNA obtained from rat liver.[26,28] On the other hand, there is no known chemistry of methyloxadiazolinium ring system to indicate that it is a progenitor of hydroxyalkyl groups. Such a possibility is, however, intuitively attractive. Nucleophilic attack at the 4 carbon of the oxadiazolinium ion could result in the breakage of C−N bond and hydroxyethylation of DNA. An analogous nucleophilic transformation at the methyl carbon could be responsible for the observed methylation of DNA. In order to determine whether even small amounts of hydroxyethylation result from the reaction of **10** with DNA, we examined the reaction of the ^{32}P-labeled oligotetramer GTAC with **10** and compared its reaction with ethylene oxide in phosphate buffer at pH 6. Both transformations were allowed to proceed for 5 h. The reaction mixtures were subjected to HPLC using a radiometric detector. Extensive reaction of the oligotetramer occurred in each case; however, the HPLC chromatograms of the modified oligotetramers showed no peaks in common. This was also true for the complete enzymatic hydrolysates of the oligotetramer. The SVP hydrolysates generated only dGMP ^{32}P-labeled guanine derivatives, and no coincident peaks were found in the two experiments. Thus, we can be reasonably sure that the methyloxadiazolinium ion **10** does not hydroxyethylate DNA. Biological experiments by Michejda and colleagues have suggested a similar conclusion.[23,25] This last experiment shows an advantage of the oligotetrameric assay procedure which we have used in other work. Various electrophilic and DNA-damaging agents, which are anticipated to give similar reaction products, easily can be compared at low concentrations, and chromatographs will show whether there are possible products in common.

Conclusions

The work described here demonstrates that the methyloxadiazolinium ion **10** reacts principally by nucleophilic attack of the N-7 nitrogen of guanine residues at the 5 carbon of **10** to give the nitrosamine adducts **14E** and **14Z**. *Ab initio* calculations of electron density in the oxadiazolinium ion ring system[27] show that C-5 and N-2 of **10** are the most electropositive atoms in the ring system. The small amount of methylation observed presumably occurs by nucleophilic attack at the methyl group attached to nitrogen. It has been speculated that removal of this methyl group can result in the generation of diazomethane, a methylating agent, through the decomposition of the resulting oxadiazoline.[27] In other work to be reported elsewhere, we have been unable to find any evidence for this pathway. Although we were able to detect a small amount of N^2-nitrosamine adduct **17** formation in the acycloguanosine experiments, we did not find it being produced in the reactions of DNA or oligomers with **10**, but its quantity could have been too low to detect.

No products resulting from the attack of the O^6 oxygen of guanine residues at position 5 of the oxadiazolinium ion were detected. The corresponding deoxyguanosine adduct was prepared, but it was found to undergo loss of the nitrosamine fragment from O^6 at a rate which exceeded hydrolysis of the nucleosidic linkage. It is probable that this process involves neighboring group participation by the nitrosamine oxygen as is observed in the formation of **10** from its tosylate.[22,23] If O^6-alkylation of G by C-5 of **10** is occurring in DNA, a pathway which our data render improbable, it is escaping our detection, and we have no evidence that it is the source of the unstable adducts. The major transformation, alkylation at N-7, is a common path for the reaction of S_N2 reactants with guanine.[41] Only much more electrophilic species show significant reaction at O^6.

Using the model O^6-G-modified ACGT oligotetramer, we were able to demonstrate, in principle, the value of the partial hydrolytic oligotetrameric assay in determining the type of base modification. In practice, this proved to be more difficult because of problems associated with the separation of the modified oligotetramers and the unpredictable behavior of purines alkylated on the imidazole nitrogen which significantly increases their susceptibility to nucleosidic hydrolysis (depurination). In combination with the total SVP-mediated hydrolytic oligotetrameric assay described above, however, these procedures were useful in demonstrating that the reaction was occurring predominantly at G and that several sites in the guanine moiety were being alkylated. The assay was also very useful in showing that no hydroxyethylation was occurring from **10** by comparing the reactions of the oligotetramer with ethylene oxide.

The results presented here show that the major transformation between **10** and guanine residues involves incorporation of the nitrosamine fragment at position 7 of the guanine. While evidence for DNA methylation by **10** is provided by the isolation of 7-methylguanine after hydrolytic workup, its quantity is always significantly less than that of **14**. It was not detected as a product in the acycloguanosine experiments, and greater quantities were found in DNA oligomers and DNA. Contrary

to the initial hypothesis,[25] **10** does not hydroxyethylate DNA. The finding that **14** predominates in all cases permits other experiments to be performed to determine whether such a process is occurring when (2-hydroxyethyl)methylnitrosamine (**1**) is administered to animals. This will allow a definitive test of the sulfation hypothesis as a mode of ethanolnitrosamine carcinogenic activation.

Experimental Section

Instrumentation. UV−vis spectra were recorded on a Perkin-Elmer 576 ST Spectrophotometer. High-pressure liquid chromatography (HPLC) was performed with a Waters chromatograph consisting of Waters Maxima 820 system controller, Waters Model 490 programmable multiwavelength detector, A-200 FLO-ONE/Beta radioactive flow detector (Radiomatic Instruments & Chemical Co., Tampa, FL), Waters Model 710B WISP autosampler, FC 203 Gilson fraction collector, and two Waters Model 510 pumps. Unless otherwise noted, three types of HPLC columns were used: a 3.9 mm × 30 cm μBondapak C18 column from Waters Inc., a 4.6 mm × 25 cm ODS Zorbax column from Liquid Chromatography Co., and a 10 mm × 25 cm Supelcosil LC-18-DB (5 micron) Semi-Prep column from Supelco Co. Proton and carbon NMR spectra were recorded on either a Nicolet NT 300 MHz, a Bruker AMX 500 MHz, a Bruker ARX 250 MHz, or a JEOL FX-90 MHz spectrometer. GC−MS spectral analyses were performed on a Hewlett-Packard 5970 mass selective detector and controlled with a Hewlett-Packard 59970 Chemstation computer. A VG-TRIO-3 thermospray HPLC/MS system was employed for LC−MS measurements. A VG ZAB high-resolution mass spectrometer was also employed with a source temperature 220 °C, 70 eV, and PFK as reference substance. High-resolution and FAB mass spectrometric measurements were obtained at either the University of Nebraska Midwest MS facility or the Washington University Mass Spectrometry Center. An Eppendorf microcentrifuge 5414 (rotational speed from 1000 to 14 000 rpm), which was situated in a 4 °C refrigerator, was used. Microscale samples were dried using a Savant Speedvac concentrator SC100.

Materials. DNA oligotetramers with different sequences such as ACGT or GTAC were made with an automatic DNA synthesizer by Dr. Forrester in the University of Missouri Molecular Biology Core facility. T4 PNK and 10× phosphorylation buffer which contains 0.5 M Tris-HCl, pH 8.0, 0.1 M $MgCl_2$, and 0.015 M spermidine were bought from USB Co. Enzymes and other biochemicals were purchased as follows: Nuclease P_1 from BRL Co.; [γ-^{32}P]ATP from Dupont; SVP and micrococcal nuclease from Worthington Biochemical Co. All other enzymes or chemicals were purchased from Sigma Chemical Co./Aldrich Chemical Co or chemical distributors. All solvents for chromatography were HPLC grade and degassed before use.

Preparation of 3-Methyl-1,2,3-oxadiazolinium Tosylate (10). The method of Koepke et al.[23] was followed. Yield: 72%. Mp: 126 °C dec (lit.[23] mp: 126 −128 °C). ^{1}H NMR ($CDCl_3$): δ 7.535 (d, 2H), 7.075 (d, 2H), 5.194 (m, 2H), 5.130 (m, 2H), 4.097 (s, 3H), 2.281 (s, 3H). ^{13}C-NMR ($CDCl_3$): δ 139.89, 129.12, 128.94, 125.51, 78.11, 60.08, 41.92, 21.20.

Reactions of Acycloguanosine 12 with 3-Methyl-1,2,3-oxadiazolinium Tosylate (10). Major Products. 9-(2-Hydroxyethoxy)methylguanine (acycloguanosine **12**, 21.6 mg, 96 μmol) was reacted with 27.1 mg of **10** (105 μmol) in 1 mL of *N*,*N*-dimethylformamide (DMF), and the reaction mixture was shaken for 3.5 h at 37 °C. The solvent was removed *in vacuo*. The residue was hydrolyzed with 200 μL of 1 M HCl at 65−70 °C for 75 min. Depending upon the goal of the experiment, various HPLC methods were employed for separation purposes. The major products **14E** and **14Z** (peaks 1 and 3) were obtained by administering the hydrolysate to a Supelcosil semiprep HPLC column with UV detection at 254 nm. The HPLC utilized two solvents, A = 0.1M HCO_2NH_4, pH 4.2, and B = methanol, and the program was as follows: (min, mL/min, % eluent A) 0, 2, 95; 25, 2, 90; 25.1, 1.5, 90; 35, 1.5, 70; 50, 2, 70. Two major peaks (peaks 1 and 3 in Figure 1) were collected from multiple HPLC injections by means of a fraction collector. Each fraction was neutralized with 10% NaOH (this is crucial), and the solvent was removed with a rotary evaporator at 30 °C with a trap of liquid nitrogen. The solid residue was added into 0.5 mL of water in a 1.5 mL microcentrifuge tube, which was centrifuged for 20 min at 4 °C. The supernatant was carefully removed. This procedure was repeated twice (in order to remove all the salt), and the white solid residue was dried *in vacuo*. The yields of peaks 1 and 3 were 3.7 and 0.74 mg, respectively. Peaks 1 and 3 were later found to be the *E* and *Z* isomers of **14**. ^{1}H NMR (DMSO-d_6, see assignments Figure 2) (*E* isomer): δ 10.79 (s, 1H), 7.82 (s, 1H), 6.14 (s, 2H), 4.58 (m, 2H), 4.53 (m, 2H), 3.02 (s, 3H); (*Z* isomer): δ 10.79 (s, 1H), 7.81 (s, 1H), 6.13 (s, 2H), 4.29 (t, 2H), 4.02 (t, 2H), 3.57 (s, 3H). A NOESY spectrum showed cross-peaks for the *E* isomer at δ 7.82 and 4.58; for the less abundant *Z* isomer cross-peaks were observed at δ 7.81 and 4.29. ^{13}C NMR (D_2O, DCl, see assignments Figure 2): δ 153.25, 152.9, 146.92, 140.08, 107.66, 53.70, 45.82, 33.24. UV characteristics of peaks 1 and 3: (peak 1) pH 12, λ_{max}= 277 nm; pH 1, λ_{max}= 240 (sh) nm; (peak 3) pH 12, λ_{max}= 270 (sh) nm; pH 1, λ_{max}= 248 nm. High-resolution MS (*m/z*, relative intensity): 237.0937 (calcd for $C_8H_{11}N_7O_2$, 237.0974) (17.2); 207.1022 (100), 190.0729 (2.9), 177.0643 (5.9), 164.0602 (72.8), 151.0477 (8.4), 87.0533 (15.9), 57.0566 (11.7), 29.9955 (26.4).

HPLC−Thermospray-MS Analysis of the Reaction Mixture. Standards of (2-hydroxyethyl)methylnitrosamine (**1**), dimethylnitrosamine, guanine, adenine, and 7-methylguanine (7-MeG) were submitted to HPLC−MS using a Synchropak reversed phase LC-18 semiprep column (10 mm × 25 cm) operating in an isocratic mode employing 95% 0.1 M ammonia acetate buffer, pH 3.9:5% MeOH at a flow rate 0.8 mL/min as an eluent. The thermospray mass spectrometer was operated with an ion energy of −25 V, capillary temperature of 320 °C, source temperature of 260 °C and 60 V. The instrument was scanned from *m/z* 50 to 400. After conditions satisfactory for the separation and detection of each known were established, a 5 μg sample of the hydrolysis residue from the reaction mixture described above was dissolved in H_2O and analyzed by HPLC−MS under the same conditions. Mass spectral and retention time comparisons with knowns permitted the identification of the compounds as indicated in Figure 1 with the exception of peaks 1−4. The material constituting peaks 1 and 3 was shown to be **14E** and **14Z** by comparison of the material isolated in the experiment above as well as their MS: Peak 1, **14E** (as BH_2), 239 (100), 209 (56), 190 (18), 166 (13), 138 (50), 119 (41); peak 2 (as BH?), 238 (20), 207 (100), 190 (82), 152 (13), 138 (15), 119 (12); peak 3, **14Z**, (as BH), 238 (19), 209 (21), 152 (100), 138 (14), 119 (18). Peak 4 contained insufficient material for MS.

Minor Products. The reaction was carried out as described above. HPLC separation (see Figure 1) was performed on a Synchropak reversed phase LC-18 semiprep column (10 mm × 25 cm). The gradient program of mobile phase was as follows: eluent A = 0.1 M HCO_2NH_4, pH 4.2, eluent B = CH_3OH (min, mL/min, % eluent A) 0, 1.5, 93; 30, 1.5, 80; 31, 1.5, 93; 46, 1.5, 93. Identity of all peaks was established by spiking experiments using authentic materials, and the identities are shown in Figure 1. Isolation of peak 1 showed that it was **14E** as indicated by its NMR (see above). Upon standing the spectrum of **14Z** grew in. Upon isolation by collection of eluent, peak 2 proved to be unstable and gave **14E** (by HPLC and NMR). Upon isolation by collection of eluent peak 3 proved to be **14Z** (see above) whose NMR spectrum was shown to convert to an equilibrium mixture of **14E** and **14Z** upon standing. Peak 4 was shown to be identical to N^2-(2-(methylnitrosamino)ethyl)guanine **17** upon isolation and through spiking experiments using two different eluent systems.

Purification and Characterization of the Intermediate Adduct 7-(2-(methylnitrosamino)ethyl)acycloguanosine (13). The reaction of **10** and **12** was conducted as described at the beginning of this section except that the residue obtained from the removal of the DMF was not subjected to hydrolysis but dissolved in CH_3OH and spotted on a TLC plate (Analtech 2 mm silica on glass, activated at 110 °C for 12 h prior to use). The TLC plate was developed with CH_3OH and compared with a standard mixture of acycloguanosine, **10**, and (2-hydroxyethyl)-methylnitrosamine (**1**) which had been applied to the same type of plate. A section of product (intermediate), having the lowest mobility, was excised from the plate and placed in a centrifuge tube. A TLC blank was generated using the same treatment. The intermediate was extracted from the silica with three portions of CH_3OH. The solvent was removed from the pooled extracts and evaporated *in vacuo* to give

a white solid residue. A sample of the dried residue was dissolved in H_2O and analyzed by HPLC using the HPLC method described for isolation and characterization of the minor products. A single sharp peak with a retention time of 20 min resulted. Material obtained in this manner was used for UV, NMR, and MS spectral characterization. The ^{1}H and ^{13}C NMR spectral assignments are given in Figure 2. J = 5.3 Hz for the pair of triplets displayed by the *Z* isomer. NOESY experiments demonstrated cross-peaks between signals at (*E* isomer) δ 8.97 and 4.73; (*Z* isomer) δ 8.97 and 4.52. UV: pH 1, λ_{max} 258 nm, sh 284 nm; pH 7 λ_{max} 230 nm, peak 288 nm; pH 13, λ_{max} 230 nm, peak 266 nm. HRFABMS: calcd for $C_{11}H_{18}N_7O_4$ 312.1420, found 312.1429.

Reaction of 2′-Deoxyguanosine with 10. 2′-Deoxyguanosine (dG) (267 mg, 1 mmol) and **10** (300 mg, 1.2 mmol) were dissolved in DMF (1 mL) in a small vial, the mixture was shaken at 37 °C for 2 h. The reaction mixture was directly injected into the HPLC, and two major peaks were found at 15.1 and 16.9 min. These peaks were found to coincide with those produced by **14E** and **14Z** under these chromatographic conditions. An analogous experiment was conducted at 25 °C. Analysis of the reaction mixture after 1 h showed peaks at 21 (dG), 21.5 (minor), and 23 (major) min. The reaction was followed for 17 h, and these latter two peaks were found to convert to those at 15.1 and 16.9 min. Collection of the final products by fraction collection showed that they were interconverting isomers **14E** and **14Z**. It is presumed that the initial peaks were due to the adducted deoxynucleoside which lost the deoxyribosyl group as time increased. Comparison of the chromatograph with *O*^{6}-(2-(methylnitrosamino)-ethyl)-2′-deoxyguanosine and spiking of this compound into the reaction mixture failed to show its presence at any time during the reaction course. A larger quantity of **14** could be obtained by diluting the DMF solution from the 37 °C run with methanol (10 mL), allowing the mixture to stand at room temperature for 2 days, and collecting the resulting fine precipitate. The mixture of **14E/Z** obtained in this way was chromatographically and spectroscopically identical to that obtained from the reaction of **10** with **12**.

Attempted Synthesis of *O*^{6}-(2-(Methylnitrosamino)ethyl)guanine (21). Method 1. (2-Hydroxyethyl)methylnitrosamine (**1**) (624 mg, 6 mmol) and dry DMSO (5 mL) were mixed in a 50 mL flask under N_2. NaH (120 mg, 5 mmol) was added to the solution. The mixture was stirred at room temperature for 4 h under N_2. 6-Chloro-2-aminopurine (100 mg, 0.6 mmol) was added, and the mixture was stirred at room temperature for 40 h. The reaction mixture was diluted with methanol (20 mL), and the unreacted starting material was filtered off. The filtrate was injected into the HPLC. The semiprep (10 × 250 mm) reverse phase column was eluted with a gradient of methanol in 100 mM aqueous NH_4OAc buffer (pH = 4.20). The fractions showing O^6 substitution (UV_{max} 286 nm, UV_{min} 250 nm) were collected (17.5 min) and neutralized immediately with dilute NaOH. Reinjection of the collected material into the HPLC showed that decomposition had occurred as the 17.5 min peak had nearly disappeared and two peaks at 5.0 (**1**) and 6.5 min (guanine) had appeared. HPLC program: eluent A = 0.1 M NH_4OAc, pH 4.2; eluent B = MeOH (min, mL/min, % eluent A) 0.0, 2.0, 95; 25.0, 2.0, 90; 25.1, 2.0, 95; 30.0, 2.0, 95.

Method 2. A stirred mixture of 6-chloro-2-aminopurine (187 mg, 1.1 mmol), $(NH_4)_2SO_4$ (45 mg), hexamethyldisilazane (5 mL), and trimethylsilyl chloride (0.5 mL) was heated in a round-bottom flask equipped with a condenser at reflux for 3 h under N_2. Volatile materials were removed from the reaction mixture by evaporation *in vacuo* with protection from moisture. In another flask NaH (100 mg, 4 mmol) was added to the mixture of 2-(*N*-methylamino)ethanol (0.5 mL, 6 mmol) and dry THF (5 mL), and the resulting mixture was stirred for 4 h. The second solution was added to the first flask, and the mixture was stirred at 80 °C for 24 h. All volatile materials were evaporated again, and isopropyl nitrite (0.89 g, 10 mmol) in dry THF (5 mL) was added to the oily residue. The solution was again stirred at room temperature for 24 h, and solvents were removed. To the flask was added dilute HCl (0.5 M, 2 mL) and stirring continued at 25 °C for 30 min. The solution was injected directly into the HPLC. The same phenomenon was found with the peak at 17.5 min, as described in method 1.

Preparation of the *N*^{2}-(2-(Methylnitrosamino)ethyl)guanine (17). *N*^{2}-(2-Methylaminoethyl)guanine (16). A 100 mL round-bottom flask equipped with a condenser was charged with water (10 mL) and 2-methoxyethanol (30 mL). 2-Bromo-6-hydroxypurine (1.6 g, 7.4 mmol), prepared according to the method of Berman et al.,[42] was dissolved in the above solvents. *N*-Methylethylenediamine was added to the clear solution. The mixture was stirred at reflux (110 °C) for 4 h. The resulting yellow suspension was cooled, and all the solvents were removed *in vacuo*. The solid residue was dissolved in hot methanol (30 mL), and recrystallization afforded 1.17 g of a light yellow solid (yield 76%), mp >300. ^{1}H-NMR (DMSO-d_6): δ 10.0 (br s, 1H), 7.82 (s, 1H), 7.72 (s, 1H), 6.89 (t, J = 5.5 Hz, 1H), 3.48 (m, J = 4.7, 5.5 Hz, 2H), 3.16 (s, 1H), 2.97 (t, J = 4.7 Hz, 2H), 2.50 (s, 3H). ^{13}C-NMR (DMSO-d_6): δ 156.42, 155.65, 152.30, 139.03, 114.50, 48.79, 38.24, 33.87. HRFABMS: calcd for $C_8H_{13}N_6O$ (M + 1) 209.1150, found 209.1153.

***N*^{2}-(2-(Methylnitrosamino)ethyl)guanine (17).** *N*^{2}-(2-(Methylamino)ethyl)guanine (312 mg, 1.5 mmol) was dissolved in HOAc (900 mg, 15 mmol) and water (5 mL). To the stirred solution was added dropwise a solution of $NaNO_2$ (310 mg, 4.5 mmol) in 2 mL of water. The mixture was stirred at room temperature for 4 h. The light yellow suspension was neutralized with saturated $NaHCO_3$. All solvents were removed *in vacuo*. The solid residue was dissolved in methanol, and the remaining solid was filtered off. The filtrate was injected into the HPLC. The semiprep (10 × 250 mm) reverse phase column was eluted with a gradient of methanol in 10 mM NH_4OAc buffer (pH = 5.10). The peaks at 25.5 and 29.0 min (*E*, *Z* isomers) were collected and the combined fractions concentrated. The fine precipitate from the concentrated solvents was carefully collected and dried. About 5 mg of a light yellow solid was obtained. UV_{max} (pH 7): 244, 282 nm. UV_{min} (pH 7): 266 nm. UV_{max} (pH 1): 250, 282 nm. UV_{min} (pH 1): 270 nm. UV_{max} (pH 12): 244, 280 nm. UV_{min} (pH 7): 264 nm. ^{1}H-NMR (DMSO-d_6): (*E*, *Z*) δ 12.58 (br s, 1H), 10.63 (s, 1H), 7.71 (s, 1H), 6.28 (t, J = 5.7 Hz, 1H), 4.30 (t, J = 5.3 Hz, 2H), 3,74 (s, 0.8 H), 3.63 (m, J = 5.4, 5.3 Hz, 2H), 3.02 (s, 2.5H). ^{13}C-NMR (DMSO-d_6): δ 164.20, 156.06, 151.97, 137.50, 117.45, 52.24, 38.29, 31.54. HRFABMS: calcd for $C_8H_{12}N_7O_2$ (M + 1) 238.1052, found 238.1044. HPLC program: eluent A = 0.01 M NH_4OAc, pH 5.10; B = CH_3OH (min, mL/min, % eluent A) 0.0, 2.5, 95; 1.0, 2.5, 95; 30.0, 2.5, 80; 35.0, 2.5, 50; 35.1, 2.5, 95; 40.0, 2.5, 95.

Synthesis of *O*^{6}-(2-(Methylnitrosamino)ethyl)-2′-deoxyguanosine (20). A procedure similar to that of Pongracz et al.[38] was followed. To the solution of 5′-*O*-(dimethoxytrityl)-*N*^{2}-isobutyryl-2′-deoxyguanosine (Sigma Chemical, 825 mg, 1.3 mmol) in 6 mL of 1,4-dioxane was added (trimethylsilyl)imidiazole (250 μL, 1.68 mmol). The mixture was stirred for 15 min, and triphenylphosphine (1.26 g, 4.8 mmol), (2-hydroxyethyl)methylnitrosamine (500 mg, 4.8 mmol), and diethyl azodicarboxylate (750 μl, 4.8 mmol) were added successively. The reaction mixture was stirred at room temperature for 1 h, and 2.5 mL of 1 M HF/pyridine solution was added. After a further 15 min of stirring, the solution was poured into 75 mL of 5% sodium bicarbonate. The solution was extracted with dichloromethane (3 × 100 mL). The combined organic extract was dried over anhydrous sodium sulfate and concentrated *in vacuo*. The oily residue was taken up in 10 mL of dichloromethane and precipitated with petroleum ether. The precipitation process was repeated three times. The pale yellow solid was purified by flash chromatography (1:1 hexane:ethyl acetate and then 20:1 chloroform:methanol). Another precipitation from dichloromethane and petroleum ether afforded 690 mg white solid product **19** (73%), mp >300 °C. ^{1}H-NMR ($CDCl_3$): (*E*, *Z*) δ 7.99 (s, 1H), 7.98 (s, 1H), 7.40−6.77 (m, 13 H), 6.59 (m, 1H), 4.91 (t, J = 5 Hz, 1.5 H), 4.75 (m, J = 5 Hz, 1.5H), 4.64 (m, J = 5 Hz, 1 Hz), 3.91 (s, 1.3H), 3.78 (d, J = 4 Hz, 1H), 3.76 (s, 6H), 3.38 (t, J = 4 Hz, 2H), 3.18 (s, 1.7H), 2.57 (m, J = 4, 5 Hz, 2H), 1.69 (m, J = 6.5 Hz, 1H), 1.19 (m, J = 6.5 Hz, 6H). ^{13}C-NMR ($CDCl_3$): δ 175.56, 159.61, 158.24, 152.73, 151.49. 144.4, 140.47, 135.56, 129.83, 127.93, 127.60, 126.64, 118.00, 112.93, 86.66, 86.18, 84.18, 72.07, 64.88, 63.96, 61.18, 54.95, 52.35, 44.36, 40.65, 40.55, 35.78, 32.58, 19.08. HRFABMS: calcd for $C_{38}H_{44}N_7O_8$ (M + 1) 726.3257, found 726.3200

5′-*O*-(Dimethoxytrityl)-*N*^{2}-isobutyryl-*O*^{6}-(2-(nitrosaminomethyl)-ethyl)-2′-deoxyguanosine (**19**) (210 mg, 0.3 mmol) was dissolved in methanol (2 mL) and concentrated ammonium hydroxide (10 mL), and the solution was transferred into a heavy-walled sealed tube and heated at 80 °C for 10 h. The mixture was cooled to room temperature, and

all solvents were removed *in vacuo*. The solid residue was dissolved in acetic acid (5 mL, 80%) and kept at room temperature for 30 min. After careful evaporation of the solvents, the residue was dissolved in methanol (5 mL) and purified by HPLC fraction collection. HPLC program: eluent A = 0.01 M NH_4OAc, pH 5.10; B = CH_3OH (min, mL/min, % eluent A) 0, 2.5, 5; 1, 2.5, 5; 30, 2.5, 20; 35, 2.5, 50; 35.1, 2.5, 5. The peaks at 21.6 and 24.5 min were collected. UV_{max} 244, 284 nm, UV_{min} 264 nm, ^{1}H-NMR (D_2O): (*E*, *Z*) δ 7.92 (s, 1H), 6.19 (t, J = 3.5 Hz, 1H), 4.75 (t, J = 5 Hz, 1.4H), 4.54 (m, J = 5 Hz, 2 H), 4.52 (m, J = 5 Hz, 1 H), 4.05 (m, J = 5 Hz, 0.4H), 3.94 (s, 1.1H), 3.79 (m, J = 4 Hz, 2H), 3.13 (s, J = 1.9 Hz), 2.71−2.39 (m, J = 3.5 Hz, 2H) ^{13}C-NMR (D_2O): δ 160.18, 159.51, 153.20, 139.87, 114.17, 87.21, 84.23, 71.28, 63.13, 61.73, 53.60, 38.87, 33.15. HRFABMS: calcd for $C_{13}H_{20}N_7O_5$ (M + 1) 354.1522, found 354.1526.

Acidic Hydrolysis of O^6-(2-(Methylnitrosamino)ethyl)-2′-deoxyguanosine (20). O^6-(2-(Methylnitrosamino)ethyl)-2′-deoxyguanosine **20** (0.34 mg, 1 μmol) was mixed with HCl (1M, 0.5 mL), and the mixture was shaken at 70 °C for 2 h. The solution was directly injected into the HPLC; only peaks for guanine and (2-hydroxyethyl)methylnitrosamine (**1**) were found. This is in agreement with the previous experiment; *i.e.*, even if the O^6-(2-(methylnitrosamino)ethyl)guanine was formed, it rapidly decomposed into guanine and **1**. In another set of experiments, 0.1 M HCl was utilized at 25 °C and the time course of the transformation was followed by HPLC. While there was an indication of peaks which may be due to **21a**, the presence of unreacted **20**, **1,** and guanine did not permit facile separation.

Reaction of dGMP with 10. 2-Deoxyguanosine monophosphate (dGMP, 3.24 mg, 9.33 μmol) was mixed with **10** (11.2 mg, 43.4 μmol) in 100 μL of 50 mM K_2HPO_4, pH 7.4. The reaction mixture in a 1.5 mL microcentrifuge tube was shaken for 4 h at room temperature and then analyzed by HPLC. The peaks were detected by UV-260 nm and collected by the fraction collector. Each peak was hydrolyzed under thermal (65 °C) acidic (4% HCl) conditions for 1.5 h. The hydrolysates were analyzed on HPLC with the UV detection at 254 nm. Two HPLC columns with different programs, A and B, were used: Program A (ODS Zorbax column) (min, mL/min, % eluent A) eluent A = 50 mM KH_2PO_4, 2 mM TBAP; Eluent B = CH_3CN; 0.0, 1.2, 98.0; 30.0, 1.2, 70.0; 40.0, 1.2, 70.0. Program B (Supelcosil semiprep column) (min, mL/min, % eluent A) eluent A = 50 mM HCO_2NH_4, pH 4.2; eluent B = CH_3 OH, 0.0, 2.0, 95.0; 15.0, 2.0, 92.0; 15.1, 1.5, 92.0; 22.0, 1.5, 85.0; 35.0, 1.5, 85.0; 38.0 2.0 70.0. The latter program provided better separation. In addition to **1** resulting from the hydrolysis of **10**, two major peaks at 11.7 and 13 min were observed. Isolation and chromatographic comparison showed that these were **14E** and **14Z**, respectively. Another minor peak at 10.5 min proved to be 7-methylguanine. The ratio of **14E** + **14Z** to 7-methylguanine was 48:1.

Reaction of cdGMP with 10. 2-Deoxyguanosine 3′,5′-cyclic monophosphate (cdGMP, 3.75 mg, 10.7 μmol) was reacted with 13.7 mg of **10** (53.2 μmol) in 250 μL of 50 mM K_2HPO_4 buffer, pH 7.4 for 4 h at room temperature. The reaction mixture was filtered through a 3 mm HPLC sintered filter, 0.45 μm, and the filtrate was applied to HPLC with the UV detection at 254 nm. A Supelcosil semiprep column was used, and the HPLC program was the same as given in the preceding experiment. The major peaks were collected from multiple HPLC injections by the fraction collector, and each was reanalyzed by a single injection. Each isolated peak was concentrated *in vacuo* and hydrolyzed under thermal (70 °C) acidic (10% HCl) conditions for 70 min. The hydrolysates were analyzed on HPLC with the UV detection at 254 nm, and each chromatogram was compared with the standard compounds. Only **14E** and **14Z** were observed to be produced. Spiking experiments with authentic 7-methylguanine failed to reveal its presence in the reaction mixture.

^{32}P Labeling of DNA Oligonucleotides with [γ-^{32}P]ATP Using T4 PNK. Small-Scale Labeling (Experiment A.1). DNA oligonucleotide (60 pmol) was added into a 1.5 mL microcentrifuge tube containing 10 μL of 10× phosphorylation buffer, and then 5 μL of [γ-^{32}P]ATP at 3000 Ci/mmol (20 pmol) was added into the reaction tube. T4 PNK was diluted to 3 units/μL with 50 mM Tris-HCl buffer, pH 8.0, just prior to the reaction. The diluted T4 PNK 6−12 units (2−4 μL) was added to the reaction tube, and autoclaved distilled water was added so that the final reaction volume was 100 μL. The labeling reaction was performed at 37 °C for 30 min and then terminated by addition of 1 μL of 0.5 M EDTA and heating at 65 °C for 5 min.

Large-Scale Labeling (Experiment A.2). DNA oligonucleotide (0.465 mg, about 0.3 μmol) was added into a 10 mL vial containing 1 mL of 10× phosphorylation buffer and 4 mL of autoclaved distilled water, and then 2.6 mg of ATP (3.3 μmol) was added into the reaction vial. The diluted T4 PNK (90 units) was added to the reaction vial (T4 PNK was diluted to 3 units/μL with 50 mM Tris-HCl buffer, pH 8.0, just prior to the reaction). The mixture was incubated in a water bath at 37 °C for 1 h, and then the reaction was terminated by addition of 3 μL of 0.5 M EDTA and heating at 65 °C for 5 min. One aliquot of ^{32}P-labeled oligonucleotide (about 20 μCi) was added into the large-scale reaction mixture before purification.

Purification of Labeled DNA Oligotetramers (Experiment A.3). The labeled DNA oligonucleotide was purified by HPLC with the radiometric detector. The column used was either a μBondapak C18 column or a ODS Zorbax column fitted with a Waters μBondapak C18 precolumn. The HPLC solvent A was 50 mM KH_2PO_4 buffer (pH 5.2) containing 2 mM TBAP. The solvent B was acetonitrile (CH_3CN). The program used on a μBondapak C18 column was started with isocratic gradient of 8% B for 8 min followed by linear gradient to 15% B over 8 min and stayed at isocratic 15% B for 26 min at a flow rate of 2.0 mL/min. With this program, the retention time of ATP was 22.5 min and that of the ^{32}P-labeled tetramer was 33.9 min. The program used on the ODS Zorbax column was started with linear gradient of 10−15% B in 5 min and stayed at isocratic 15% B for 20 min, followed by a linear gradient to 40% B in 15 min at a flow rate 2.0 mL/min. In this program, the retention time of ATP was 14.4 min and that of the ^{32}P-labeled tetramer was 25.0 min.

The ^{32}P-labeled tetramer peak was collected on the fraction collector followed by desalting on C18 Sep-Pak (Waters) cartridge. The Sep-Pak cartridge was prepared by washing sequentially with 75% acetonitrile (10 mL) and water (10 mL). The aqueous solution of the purified oligonucleotide was administered to the cartridge and washed extensively with water (10 mL). Finally, the oligonucleotide was eluted by 75% acetonitrile (4 mL) and then lyophilized to dryness by a Speedvac.

Partial Enzymatic Hydrolysis with Snake Venom Phospodiesterase (SVP). A SVP stock solution (1.52 units/μL) was prepared by dissolving 152 units of SVP into 90 μL of 50 mM Tris-HCl buffer containing 3 mM $MgCl_2$, pH 8.8 (SVP buffer), and 10 μL of BSA solution (10 μg/μL). The stock solution was kept at −28 °C (in a freezer) and diluted to 0.05 unit/μL with SVP buffer prior to use. The ^{32}P-labeled oligotetramer (about 2 pmol, 0.1 μCi) in 50 μL of SVP buffer was partially hydrolyzed with 1−2 μL of dilute SVP solution for 0.5−2 min at 0 °C (occasionally a longer time of hydrolysis was required for the modified oligonucleotides). The hydrolysis was stopped by addition of 1 μL of 0.5 M EDTA followed by heating for 4 min at 65 °C. The hydrolysate was analyzed on HPLC with the radiometric detector, using either μBondapak C18 column or the ODS Zorbax column. The same solvent program was used as in experiment A.3.

Reaction of DNA Oligotetramer with 10. ^{32}P-Labeled DNA oligotetramer ACGT (11−33 pmol, 3−30 μCi) in a 1.5 mL microcentrifuge tube was mixed with 50−200 μL of 3-methyl-1,2,3-oxadiazolinium tosylate **10** (maximum amount of **10**, was 2.6 mg) in 50 mM K_2HPO_4, pH 7.4, for 2−4 h at room temperature. The reaction mixture was directly injected onto the HPLC (the same HPLC columns and programs were used as in experiment A.3.), and the modified oligotetramers were separated. Fraction collection was performed as follows: (retention time (min), peak height (in counts)) 1 (11, 6.4); 2 (12.3, 14.6); 3 (13, 28.6); 4 (15.5, 14.7); 5 (18.7, 105); 6 (25.2, 27.1); 7 (34.6, 7.9); 8 (42.7, 6.2). Collection was followed by desalting on a C18 Sep-Pak cartridge. The solvent of each fraction was finally removed by a lyophilizer or a rotary evaporator at 6 °C under vacuum. In order to check the purity and stability, one aliquot of each isolated major adduct was reinjected into the HPLC. Each purified and dried modified fraction was subjected to partial enzymatic hydrolysis, total enzymatic hydrolysis, or acid-catalyzed hydrolysis (depurination), etc. as described in other experiments.

Total Hydrolysis of 5′-^{32}P-Labeled Oligotetramer GTAC. The 5′-^{32}P-labeled oligotetramer GTAC (approximately 10 pmol on a small scale and 10−300 nmol on a large scale, 10−30 μCi) was reacted with **10** (2.6 mg, 10 μmol in a small scale and 15 mg, 58 μmol) in 50−200

μL of 50 mM K_2HPO_4, pH 7.4, at room temperature for 4 h. The reaction mixture was directly analyzed by HPLC using the radiometric detector. The ODS Zorbax column was used, and the reaction products were eluted at a flow rate of 1.2 mL/min by 15% CH_3CN−85% 50 mM KH_2PO_4 containing 2 mM TBAP, pH 5.2, for 15 min followed by linear gradient to 32% CH_3CN in 15 min, after which the gradient was kept at isocratic 32% CH_3CN for another 10 min. Each of the modified oligonucleotides was collected from HPLC by the fraction collector and was desalted and dried *in vacuo*. The isolated, modified oligonucleotide was completely digested to 5′- monophosphate 2-deoxynucleotides with 2−4 units of SVP in SVP buffer at room temperature for 6 h. Of the four 5′-monophosphate 2-deoxynucleotides, only dGMP and the modified dGMP had ^{32}P radioactivity. The hydrolysate of ^{32}P-labeled oligotetramer was then analyzed on HPLC with the radiometric detector. The same column and solvents were used as above, but a different HPLC program was employed using a linear gradient of 2−30% CH_3CN/50 mM KH_2PO_4 containing 2 mM TBAP, pH 5.2 for 30 min at a flow rate 1.2 mL/min. The retention time of dGMP was 11.74 min.

Reactions of Ethylene Oxide with ^{32}P-Labeled Oligotetramer GTAC or dGMP. Liquid ethylene oxide was prepared by trapping gaseous ethylene oxide from a lecture bottle, by passing it through polyethylene tubing into an ice cold test tube which was fitted with a rubber stopper containing a glass tube. The ^{32}P-labeled oligotetramer GTAC (approximately 12 pmol, 2 μCi) or dGMP (20 μL of 0.46 M, 9.6 μmol) were mixed respectively with 20 μL of ethylene oxide (0.4 mmol) in a 1.5 mL microcentrifuge tube with a tight cap which contained 100 μL of 50 mM KH_2PO_4/K_2HPO_4 (1:1), pH, 6.0. The reaction mixture was shaken for 5.5 h at room temperature and then injected onto the HPLC. Both the UV detector (260 nm) and the radiometric detector (^{32}P) were used to determine the HPLC chromatogram. The same HPLC columns and programs were used as in experiment A.3 for analysis of the DNA adducts from the reaction of ^{32}P-labeled oligotetramer GTAC with ethylene oxide. The reaction mixture from dGMP with ethylene oxide was separated on a Supelcosil semiprep column. Each peak was collected by the fraction collector and its UV spectrum determined. The HPLC program is as follows: eluent A = 0.1 M NaH_2PO_4, pH 5.5; eluent B = CH_3OH, (min, mL/min, % eluent A) 0, 2.0, 95.0; 5.0, 2.0, 95.0; 20.0, 2.0, 90.0; 30.0, 2.0, 90.0; 35.0, 2.0, 65.0; 40.0, 2.0, 60.0.

Reaction of Double-Stranded Calf Thymus DNA with 10. Double-stranded calf thymus DNA (2.5 mg) was mixed with 20 mg of **10** in 0.5 mL of 50 mM K_2HPO_4 buffer, pH 7.4 at 37 °C for 4 h. The DNA was precipitated by addition of 1/10 volume of 3 M NaOAc and 2 volumes of absolute ethyl alcohol at the end of the reaction. The mixture was cooled at −78 °C for 3 h and then centrifuged for 10 min at 4 °C. The supernatant was removed, and this process was repeated one more time in order to get rid of all **10** and its decomposition products (the nitrosamines). The DNA residue was dissolved in 200 μL of water, 100 μL of which was heated at 95 °C for 35 min. The remaining DNA solution was mixed with 1 M HCl and heated at 70 °C for 45 min. The hydrolysates were then subjected to HPLC on a LC-18-DB semiprep column and monitored with a UV detector at 254 nM. The HPLC program is as follows: eluent A = 50 mM KH_2PO_4, 2 mM TBAP; eluent B = CH_3CN (min, mL/min, % eluent A) 0.0, 1.6, 98.0; 15.0, 2.0, 92.0; 15.1, 1.5, 92.0; 22.0, 1.5, 85.0; 35.0, 1.5, 85.0; ,38.0, 2.0, 70.0. Under these conditions the retention times (min) of the standards were guanine, 12.5; adenine, 20; 7-methylguanine, 21; **14E**, 27; and **14Z**, 30. Each of these compounds was identified in the hydrolysate mixture, while only guanine and adenine were observed in that of the control (DNA without **10**). The ratio of 7-methylguanine to **14** was 1:6.8.

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Supporting Information Available: ^1H and ^{13}C spectra from compounds **13**, **14**, **17**, and **20** and their precursors (16 pages). See any current masthead page for ordering and Internet access instructions.

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