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ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · JANUARY 1988

Impact Factor: 3.53 · DOI: 10.1021/tx00001a010

Identification of Adducts Formed by Reaction of Guanine Nucleosides with Malondialdehyde and Structurally Related Aldehydes

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Received October 23, 1987

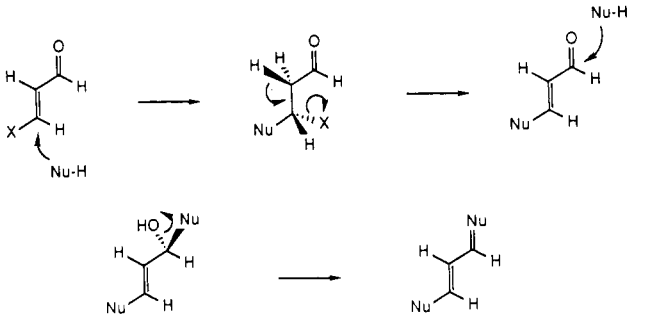
Malondialdehyde and a series of acrolein derivatives substituted in the β-position with good leaving groups react with guanine and guanine nucleosides to form two different types of adducts. The reaction with guanosine is typical. One adduct exhibits ultraviolet absorbance maxima at 253, 319, and 348 nm and is fluorescent. Its NMR spectrum exhibits three new aromatic proton resonances derived from malondialdehyde. The mass spectrum exhibits an M + 1 at 320. The spectroscopic properties are consistent with the structure, 3-β-D-*erythro*-pentofuranosylpyrimido[1,2-*a*]purin-10(3*H*)-one (PyP-ribose). The second guanosine adduct is an equal mixture of diastereomers that exhibit ultraviolet maxima at 217 and 244 nm and mirror image circular dichroism spectra. The NMR spectrum and mass spectrum (M + 1 = 392) indicate the addition of two molecules of MDA to one molecule of guanosine. Two-dimensional NMR (COSY) analysis reveals the presence of propano and enal functionalities. The spectroscopic and chemical properties suggest an oxadiazabicyclo[3.3.1]nonene structure that is confirmed by X-ray crystallography. Comparison of the deoxyguanosine adducts of malondialdehyde to those of the structurally related carbonyl compounds, methyl glyoxal and acrolein, provides a structural basis to explain the unique ability of malondialdehyde to induce frameshift mutations in bacterial mutagenesis systems.

Introduction

Malondialdehyde (MDA) is a product of polyunsaturated fatty acid oxidation that is ubiquitously distributed in the plant and animal kingdom (*1–3*). Oxygenation of arachidonic acid by PGH synthase produces bicyclic peroxide intermediates that are converted enzymatically and nonenzymatically to MDA inter alia (*4, 5*). Besides arising as a side product of the arachidonic acid cascade, MDA is the major carbonyl-containing end product of lipid peroxidation, the nonspecific oxidative degradation of unsaturated fatty acid containing lipids (*6*). Considering the abundance of MDA as a product of normal metabolism and in a variety of foodstuffs, exposure of humans to it may be considerable. MDA is mutagenic to bacteria and mammalian cells and carcinogenic to mice (*7–10*).[1]

MDA is the prototype β-dicarbonyl compound but actually exists as a tautomer, β-hydroxyacrolein in polar solvents (*11*). A priori, one expects that MDA is biologically similar to the structurally analogous molecules methyl glyoxal and acrolein (Figure 1). Superficially this appears to be the case because methyl glyoxal and acrolein are potent mutagens in bacterial systems and methyl glyoxal is carcinogenic (*12, 13*). On closer examination, the similarity breaks down. The strain specificity of mutagenesis in *Salmonella typhimurium* indicates that MDA induces frameshifts (additions and deletions) and base-pair substitutions whereas methyl glyoxal and acrolein induce only base-pair substitutions (*7, 12*). The ability of MDA to induce frameshift mutations in *Salmonella* is unexpected because most frameshift mutagens are nonpolar, aromatic molecules. In addition to the distinct types of mutations induced by MDA and related carbonyl compounds, alterations in mutagenic susceptibility caused by different host repair backgrounds suggest that adducts produced from MDA in bacterial DNA are recognized and processed differently from methyl glyoxal and acrolein adducts (*7, 12, 14*).

Scheme I. The Minimal Chemical Steps Required for Induction of Frameshift Mutations by MDA in *S. typhimurium* Strain *hisD3052*



The mutagenic potencies of a series of β-hydroxyacrolein analogues substituted with good leaving groups in place of the hydroxyl group indicate that both carbonyl equivalents must react in order for frameshift mutations to occur in *Salmonella typhimurium* strain *hisD3052* (*15*). The minimal chemical steps are outlined in Scheme I. An obvious reaction consistent with Scheme I is cross-linking of complementary strands of a double-stranded DNA molecule. MDA induces cross-linking of double-stranded DNA under nonphysiological conditions but the ability of a series of β-substituted acroleins to induce cross-links does not correlate to their ability to induce frameshift mutations (*14*). Therefore, we carried out a study of the reaction of

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[1] Spalding, J., personal communication.

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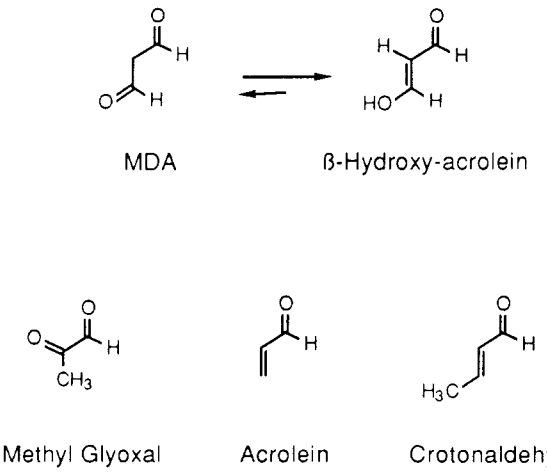


Figure 1. Malondialdehyde (MDA), β-hydroxyacrolein, methyl glyoxal, acrolein, and crotonaldehyde.

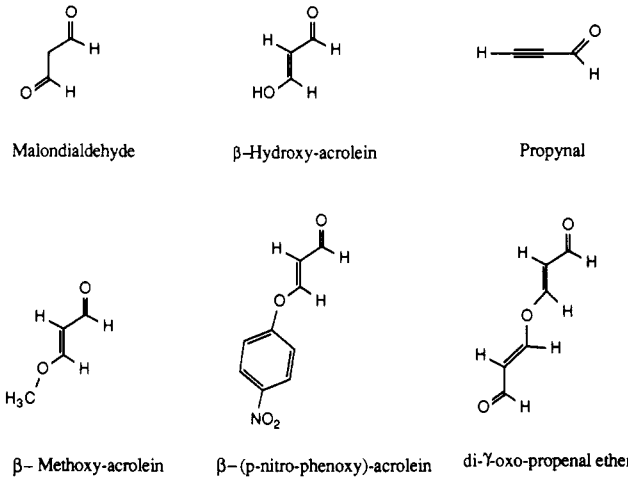


Figure 2. Molecules used in the present study to form adducts with deoxyguanosine.

the same series of compounds with deoxynucleosides in order to identify any adducts formed. The results, described herein, provide a structural basis for a hypothesis to explain the unusual pattern of mutagenicity displayed by MDA.

Materials and Methods

Deoxynucleosides were purchased from Sigma (St. Louis, MO) and Cruachem (Herndon, VA). Radiolabeled nucleosides were from Amersham. Reagent grade chemicals were from commercial chemical supply houses. HPLC solvents were purchased from Burdick and Jackson (Muskegon, MI). NMR solvents were from Aldrich Chemical (Milwaukee, WI). MDA and β-substituted acroleins were prepared as previously described (*15*). Their structures were verified and purities assessed by NMR spectroscopy.

Ultraviolet absorption spectra of synthesized compounds and adducts were recorded on a Varian/Cary 210 spectrophotometer. UV spectra of adducts eluting from HPLC columns were recorded with a Hewlett-Packard Model 1040A Diode Array Detector. NMR spectra were recorded on a General Electric QE-300 spectrometer. Mass spectra were recorded on a Finnigan 4010 spectrometer interfaced to an Incos data system. Samples were introduced via a Vestec LC-MS interface using a Beckman liquid chromatograph consisting of a Model 110B pump and a Model 420 controller. NMR and mass spectra were recorded in the Central Instrument Facility of the Wayne State University Chemistry Department. Fluorescence spectra were recorded on a Spex Fluorolog spectrophotofluorometer. Circular dichroism spectra were recorded on a Jasco Model J-40c spectrometer in the laboratory of Dr. Jules Shafer at the University of Michigan. HPLC separation of adducts was performed on a Varian Model 5000 liquid chromatograph. Radioactivity eluting from HPLC columns was detected and quantitated with Radiomatic Flo-One Model HP or IC radioactivity flow detectors using Radiomatic premixed scintillants.

The 2D COSY spectra were recorded with quadrature detection over a 2400-Hz sweep width. The COSY pulse sequence (90–t_2–90–t_1) was appended with a 200-ms delay prior to the first 90° pulse. During this delay, the decoupler was turned on and the HOD signal was saturated. A 5-ms delay was incorporated between the presaturation delay and the first 90° pulse. The recycle time was 10 ms. Data were acquired in 257 and 512 data points in the t_2 and t_1 dimensions. In the t_2 domain, 256 data points were processed; as the first data point allowed for saturation of the HOD signal. The FID's were appodized by multiplying each data point by a sine function. The data were then Fourier transformed along the t_1 and t_2 directions. To ensure a symmetrical contour plot, a symmetrization routine was applied to the spectrum.

Adducts were generated by reacting 1–4 equiv of the compound of interest with one equivalent of the nucleoside in various buffers or aqueous DMSO solutions at 37 °C. Adduct formation was monitored by HPLC of a filtered aliquot of the reaction mixture on a 4.6 × 250 mm Ultrasphere ODS II reverse-phase column (Beckman) eluted with water/acetonitrile gradients. The following gradient between H_2O (solvent A) and 50% acetonitrile/H_2O (solvent B) proved suitable for most purposes: 0–20 min, 0–10% B; 20–35 min, 10% B isochratic; 35–60 min, 10–100% B. The flow rate was 1 mL/min.

Larger amounts of adducts were prepared by reacting 0.5 M nucleoside with 1.0 M MDA or β-substituted acrolein in 0.1 N HCl for 24 h at 37 °C. The reaction mixture was extracted twice with ether and once with chloroform. The aqueous phase was lyophilized and loaded on a silica gel column. The column was eluted with chloroform/methanol (3/1) and fractions were monitored by thin-layer chromatography (silica gel; hexane/ethyl acetate, 9/1). The adducts were subjected to preparative HPLC on a 10 × 250 mm Ultrasphere ODS II column using the solvent system described above.

Guanosine adducts were depurinated by incubation in 0.1 N HCl at 90 °C for 1.5 h. Chromatographic analysis indicated the reaction was complete in this time. The guanine adducts were purified on a large scale by eluting a Sephadex LH-20 column with H_2O. Further purification was effected by reverse-phase HPLC using the conditions described above.

Results

The structures of the molecules employed in this study are displayed in Figure 2. These molecules differ in mutagenicity in *Salmonella typhimurium* as much as 200-fold; yet based on the reactions depicted in Scheme I, each compound should form the same pattern of adducts to deoxynucleoside bases. Several of the compounds were reacted with the four deoxynucleosides at 37 °C under a variety of conditions and the products analyzed by HPLC. The order of reactivity deoxyguanosine > deoxyadenosine ≫ deoxycytidine > thymidine ~ 0 was observed, which is typical of reactions of electrophiles with DNA bases. Because of the high reactivity of deoxyguanosine, we concentrated our efforts on the characterization of the guanine adducts. Many of the reactions were carried out at acidic pH, which led to substantial depurination of deoxyguanosine so initial experiments were conducted with guanosine. Preliminary experiments indicated that the extent of reaction was very low at neutral pH in aqueous buffers. Addition of DMSO which increased the solubilities of the reactants accelerated reaction as did lowering the pH. The latter effect may be due to a combination of higher solubility and enhancement of reactivity of the β-substituted acrolein by virtue of protonation of the leaving group. In the case of MDA, lowering the pH neutralizes its negative charge, which also enhances reactivity.

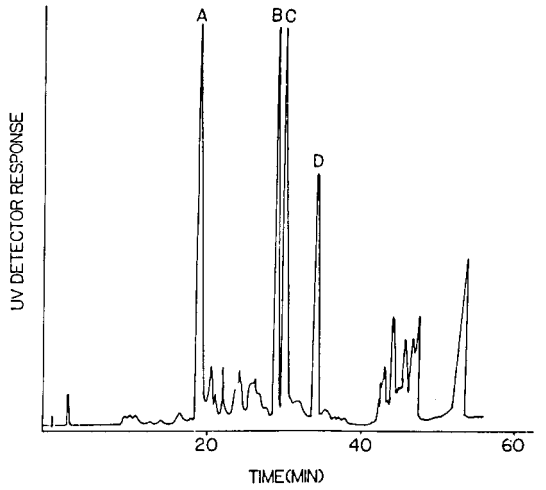


Figure 3. HPLC profile of the products of reaction of guanosine with β-(*p*-nitrophenoxy)acrolein. Conditions are described in Materials and Methods. Peak A is unreacted guanosine whereas peaks B–D are adducts.

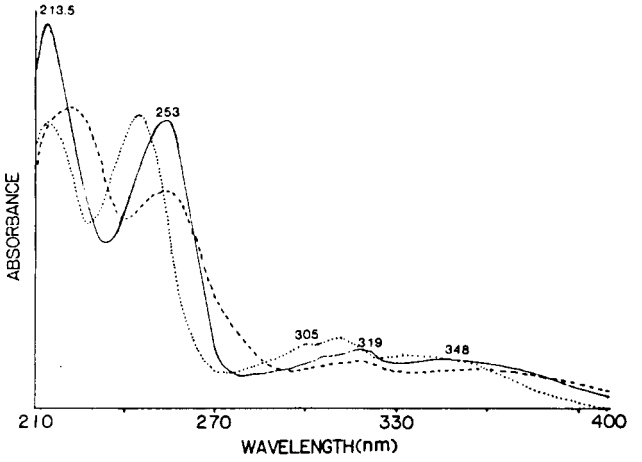


Figure 4. UV spectrum of adduct D. Spectra were recorded at (···) pH 1.0, (—) 7.0, and (- - -) 9.0.

Reaction of all the β-substituted acroleins (100 μM–500 mM) with guanosine generates the same profile of adducts. A typical chromatogram, obtained from the reaction of β-(*p*-nitrophenoxy)acrolein (PNPA), is displayed in Figure 3. Incubation of [^{3}H]guanosine with PNPA demonstrated that peak A was unreacted guanosine and peaks B–D were adducts. The remaining peaks observed in the UV profile were not radioactive and probably resulted from polymerization of the β-substituted acrolein. Propynal, the most mutagenic compound to *hisD3052* in this series, did not form adducts B–D when reacted with guanosine under purely aqueous conditions. Extensive breakdown of the nucleoside occurred as judged by elution of most of the radioactivity from [^{3}H]guanosine in the solvent front of the reversed-phase column. Small amounts of adducts were detected when reactions with low concentrations of propynal (<1 mM) were performed in ethylene glycol for short periods of time (5–10 min). Apparently, prolonged reactions with high concentrations of propynal consumes the initially formed adducts and converts them to breakdown products.

The absorption spectrum of adduct D is displayed in Figure 4. At neutral pH, maxima are observed at 253, 319, and 348 nm. Adduct D is fluorescent and exhibits an emission maximum at 512 nm when excited at 254 or 342 nm. The one- and two-dimensional NMR spectra of adduct D are shown in Figure 5. The 2-D (COSY) spectrum

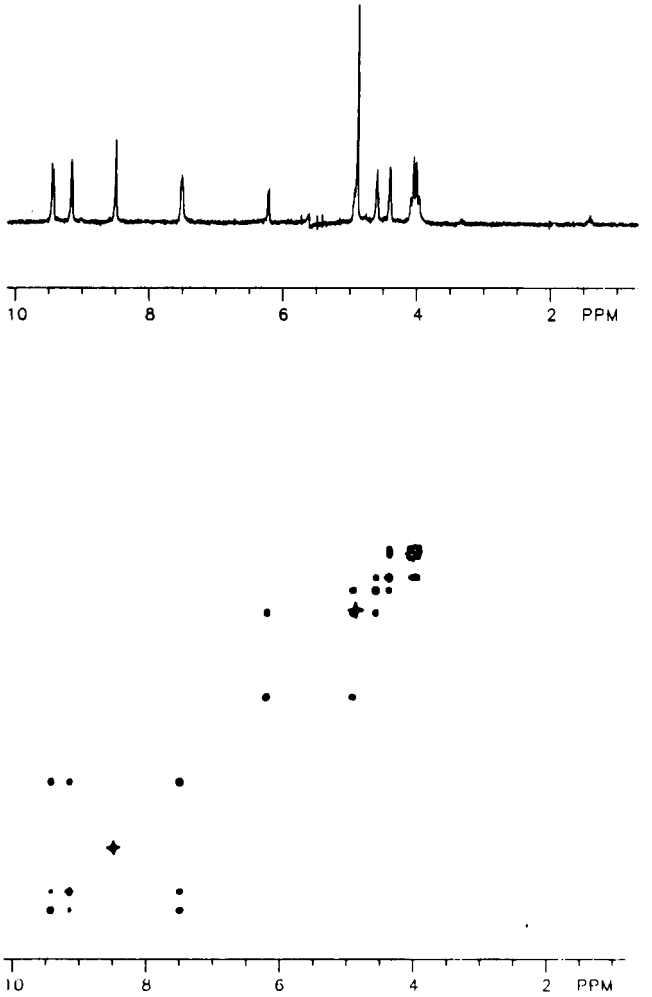


Figure 5. One- and two-dimensional (COSY) NMR spectrum of adduct D.

contains two groups of resonances. The sugar resonances appear at δ 6.5–4 in the order 1′, 2′, 3′, 4′, 5′, and 5″ (decreasing chemical shift) as indicated by the chemical shift and the coupling relationship indicated by the off-diagonal elements. The aromatic resonances of the modified base appear at δ 9.5–7.5. H-2 (H-8 of guanosine) appears as a singlet at δ 8.5, whereas H-6, H-8, and H-7 appear at δ 9.45, 9.1, and 7.5, respectively. The coupling between these protons, derived from the β-substituted acrolein, is evident in Figure 5. Finally, the mass spectrum of adduct D exhibits a molecular ion + 1 at *m/e* 320 and a base peak corresponding to base + H at *m/e* 188. The properties correspond to those previously reported for 3-β-D-*erythro*-pentofuranosylpyrimido[1,2-*a*]purin-10(3*H*)-one (PyP-ribose) (*16*).

Adducts B and C exhibited identical UV spectra (Figure 6) with maxima at 245 and 217 nm at neutral pH. The spectra in Figure 6 are similar to the spectra of 1-ethylguanosine although shifted to shorter wavelengths. Neither adduct B nor C is fluorescent but they exhibit mirror image circular dichroism spectra with maxima at 255 and 237 nm. This suggests that B and C are diastereomers related to each other as enantiomers attached to ribose. Each adduct chromatographed as a single peak when reanalyzed under conditions identical with those detailed in Figure 3. Adducts B and C were interconvertible in 0.1 N HCl at 70 °C (2 h).

The 1-D and 2-D (COSY) ^{1}H NMR spectra of a mixture of adducts B and C are displayed in Figure 7. In addition to the resonances of the ribose protons, resonances are

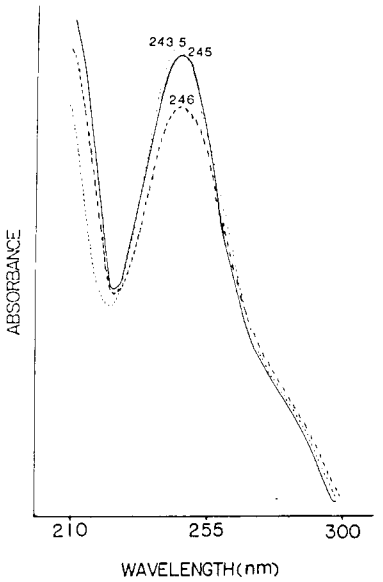


Figure 6. UV spectrum of adducts B and C. Spectra were recorded at (···) pH 1.0, (—) 7.0, and (- - -) 9.0.

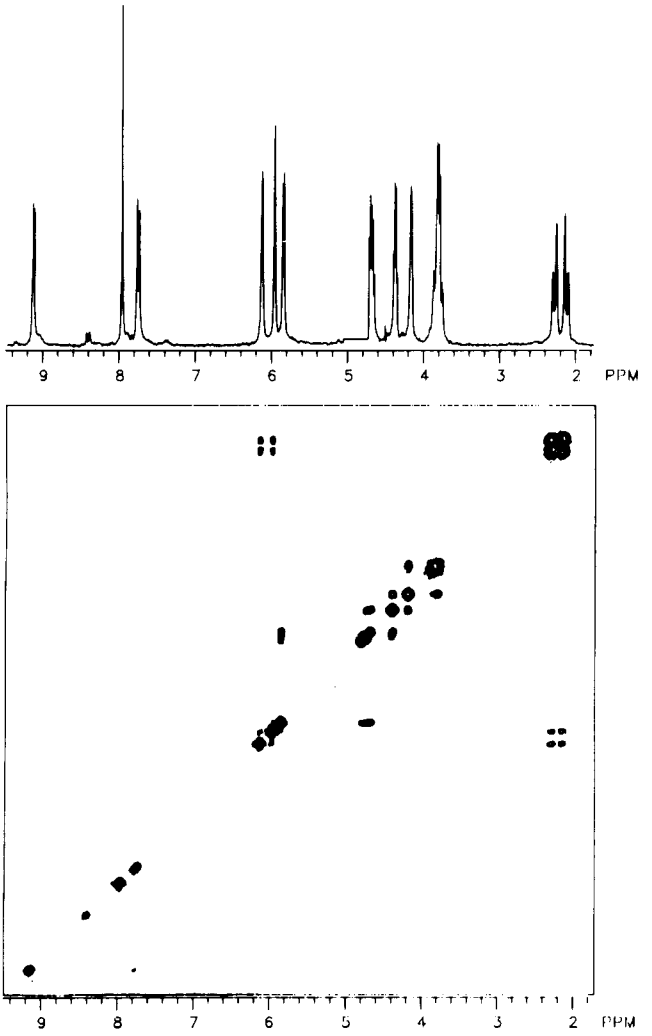


Figure 7. One- and two-dimensional (COSY) NMR spectrum of a mixture of adducts B and C. One of the off-diagonal elements connecting the resonances at δ 9.15 and 7.7 was lost during reproduction.

observed at δ 9.1, 7.7, 6.1, 5.9, and 2.2 that are attributable to the modified base. The spectrum in Figure 7 was obtained in D_2O, which removes exchangeable protons. The

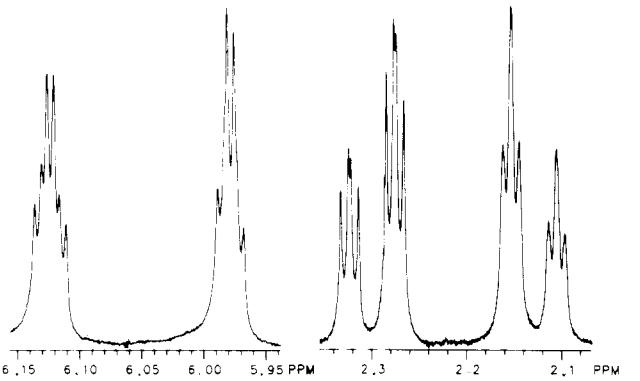


Figure 8. Expansion of the NMR signals at δ 2.3, 5.9, and 6.3 of adducts B and C.

presence of six nonexchangeable protons in the spectrum is consistent with the presence of *two* MDA units attached to the nucleoside. Scalar coupling of the six MDA-derived protons is evident from the off-diagonal elements in Figure 7. Figure 8 displays an expanded view of the resonances at δ 2.2, 5.9, and 6.1. The coupling pattern and the chemical shifts indicate that the signal at $\simeq \delta$ 2.2 arises from a methylene group that is coupled nearly equivalently to two methine groups (δ 5.9 and 6.1). The signals at δ 7.7 and 9.1 are due to a vinylic proton and an aldehydic proton, respectively, that are weakly coupled to each other. The coupling is only evident in the 2D spectrum and the appearance of both resonances as doublets in the 1D spectrum is due to the fact that it was recorded on a mixture of diastereomers. The downfield resonance of the vinylic proton suggests it is at the β-position of an α,β-unsaturated aldehyde.

The mass spectrum of a mixture of adducts B and C was obtained by LC–MS and is displayed in Figure 9. The ion at m/e 392 (M + 1) is consistent with the presence of the elements of guanosine and 2 equiv of MDA. The peak at m/e 320 represents the loss of one unit of MDA.

In order to facilitate the acquisition and interpretation of ^{13}C NMR spectra, guanosine adducts analogous to adducts B and C were subjected to acidic hydrolysis. Two peaks were detected on HPLC. One peak was fluorescent and was identified as PyP. The other had a similar UV spectrum to adducts B and C and cochromatographed with one of the products of reaction of MDA with guanine. The broad-band decoupled ^{13}C NMR spectrum of the compound derived from guanine exhibited 11 resonances (Figure 10). The unique (non-guanine) resonances are those at δ 23.08, 34.29, 75.70, 119.26, 163.44, and 187.86. A nondecoupled spectrum revealed the carbon with a chemical shift at δ 23.08 is attached to two protons and the carbon at 119.26 is attached to no protons whereas the remaining carbons are each attached to one proton. Although the carbon at 119.26 is not attached to any protons, it displays long-range coupling to three protons yielding a doublet of doublet of doublets. Selective heteronuclear decoupling revealed that the largest ^{3}H–^{13}C coupling (26 Hz) is to the aldehydic proton at δ 9.1. Nearly equivalent coupling (~6 Hz) was observed to the vinyl proton at δ 7.7 and the methine proton at δ 5.9.

The spectroscopic data indicate the presence of enal and propano functionalities derived from MDA in the guanine adduct. We were unable to define the connectivity of the functional groups from the available spectroscopic data so we crystallized the guanine adduct and subjected it to single-crystal X-ray diffraction analysis. The results of this experiment have been published and indicate the structure shown designated as Oxa in Scheme II (*17*).

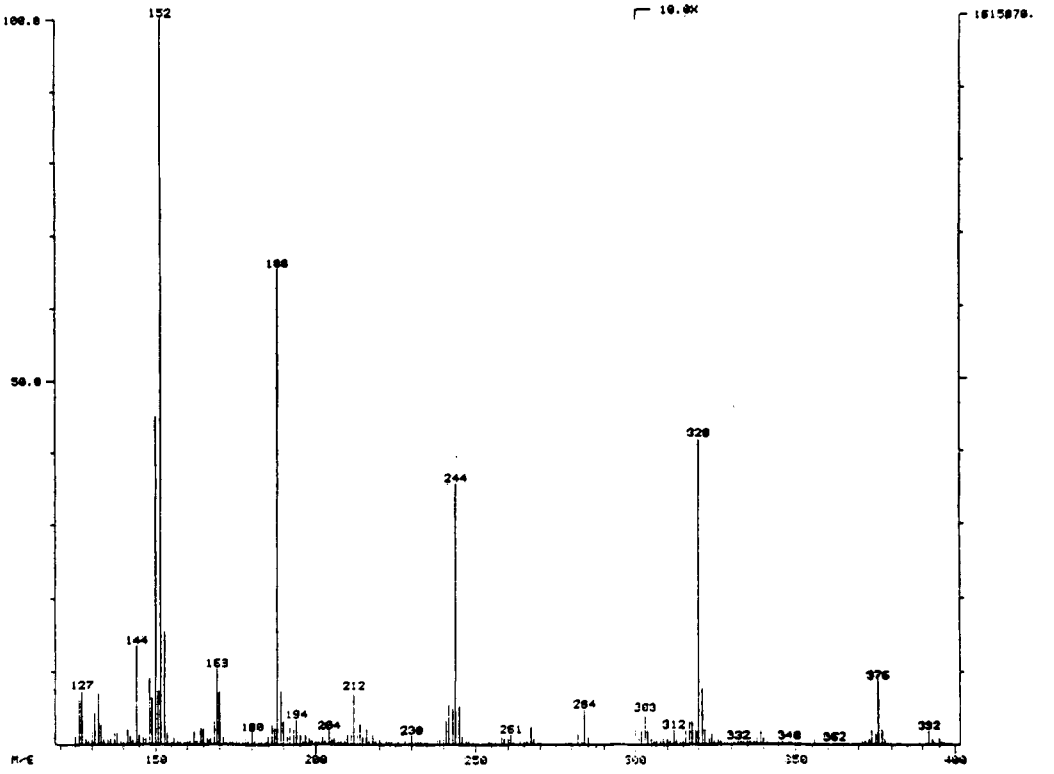


Figure 9. Mass spectrum of adducts B and C.

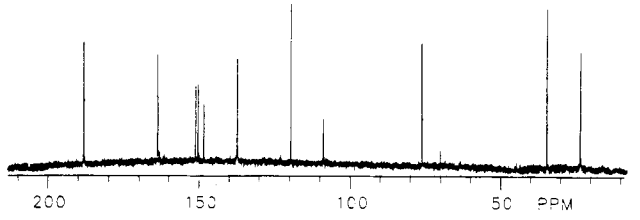


Figure 10. ^{13}C NMR of the guanine adducts produced by hydrolysis of adducts B and C.

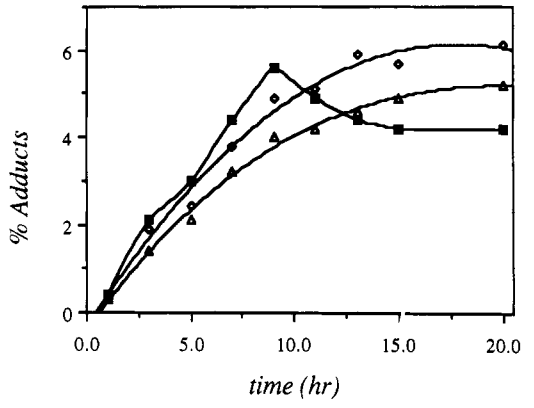
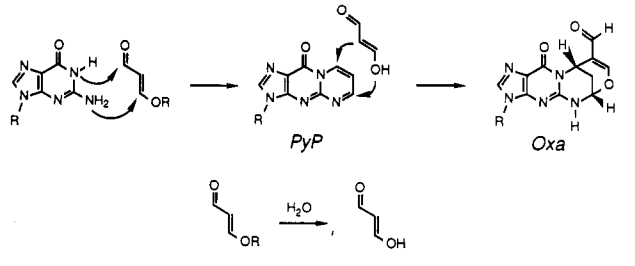


Figure 11. Time courses of formation of adducts B, C, and D by reaction of β-methoxyacrolein with guanosine: (◇) B; (△) C; (■) D.

Scheme II. Mechanism of Formation of Oxadiazabicyclo[3.3.1]nonene Adducts



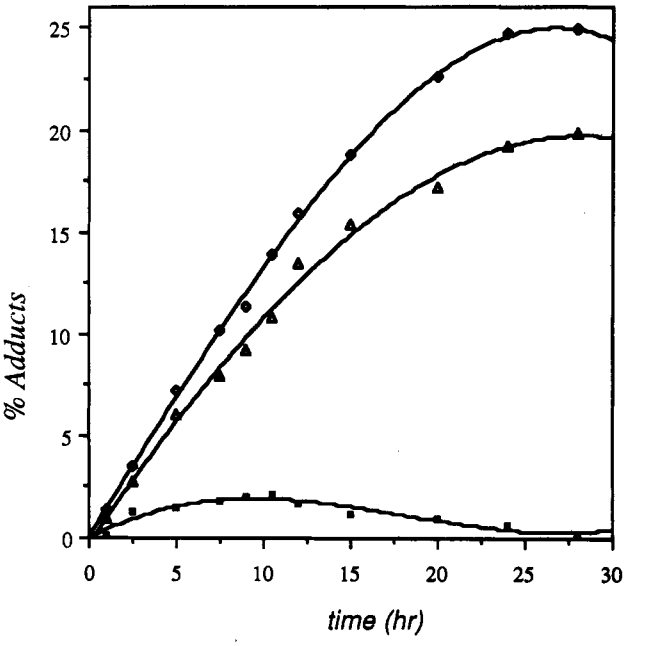


Figure 12. Time course of formation of adducts B, C, and D by reaction of bis(γ-oxopropenyl) ether with guanosine: (◇) B; (△) C; (■) D.

The rate of formation of adducts was inversely related to pH. The yield of Oxa and PyP adducts from reaction of a series of β-substituted acroleins with guanosine was twice as high at pH 1.0 than pH 2.0 and 18 times higher than at pH 4.2. Figure 11 exhibits the time course of adduct generation by β-methoxyacrolein incubated with an equimolar concentration of guanosine at 37 °C and pH 1. Approximately equal amounts of adducts were formed from all of the compounds (data not shown) except bis(γ-oxopropenyl) ether, which generated nearly three times the level of total adducts but half the amount of PyP-ribose (Figure 12). Bis(γ-oxopropenyl) ether is an anhydride of MDA and on hydrolysis generates two molecules

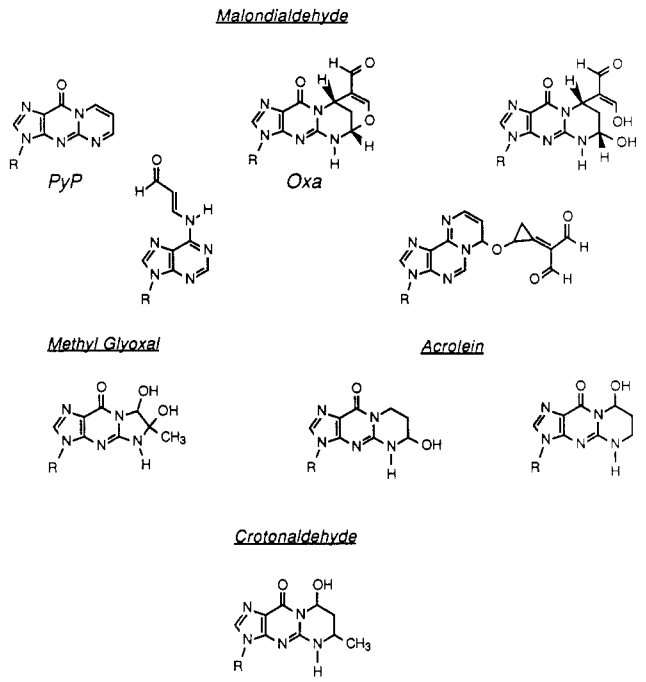


Figure 13. Structures of MDA adducts identified to date and the major adducts formed by reaction of methyl glyoxal, acrolein, and crotonaldehyde with guanine nucleosides.

of MDA. The extra equivalent of MDA can then react with PyP-ribose generating Oxa–ribose. This increases the yields of Oxa but decreases the yields of PyP. The extent of adduct formation did not correlate with the specific mutagenicities of the various compounds, which varies nearly 200-fold. All of the compounds are hydrolytically unstable and may hydrolyze to MDA, which then reacts with guanosine. This is consistent with the observation that bis(γ-oxopropenyl) ether formed higher amounts of adducts than other β-substituted acroleins did.

Discussion

The present study indicates that MDA and structurally related β-substituted acroleins react with guanine nucleosides to form two different types of adducts. The first formed adduct is PyP which has been reported as a product of reaction of MDA with guanosine and was recently detected following prolonged incubation of MDA with DNA (*18*). Pyrimidopurinones, substituted in the 7-position, were first reported as products of reaction of α-substituted malondialdehydes with guanine and guanosine (*19*). The other adduct detected in the present study is an oxadiazabicyclo[3:3:1]nonene formed by addition of a second MDA molecule to the PyP adduct (Scheme II). Formation of Oxa from β-substituted acroleins requires that one molecule of the parent compound hydrolyze to MDA which then reacts with PyP. Hydrolysis of all of the β-substituted acroleins studied here occurs rapidly enough on the time scale of adduct formation to support formation of Oxa (*15*). The formation of multimeric adducts such as Oxa is unique to MDA among all known chemical mutagens and carcinogens. This is because MDA has the ability to act as a nucleophile as well as an electrophile and thereby add to the initial product of its reaction with N^1 and N^2 of guanine and guanine nucleotides. The ability of MDA to act as a nucleophile and electrophile is also apparent from the trimeric adduct that it forms on reaction with adenine nucleotides (*20*).

Figure 13 lists the structures of the adducts identified to date that form between MDA and deoxyguanosine and deoxyadenosine. Adducts analogous to the deoxyadenosine adducts have also been reported to form in the reaction of MDA with deoxycytidine but were omitted for brevity (*20*). Included in Figure 13 is a possible structure of an open chain derivative of Oxa that may form in neutral aqueous solution.[2] Aside from PyP, none of the MDA adducts have been detected following incubation of MDA or β-substituted acroleins with intact DNA in vitro or in vivo. This may be simply a problem of insufficient sensitivity because radioactively labeled MDA of very high specific activity is as yet unavailable. Based on probability considerations alone, one might expect that the monomeric deoxyguanosine and deoxyadenosine adducts are most likely to form under in vivo conditions where MDA and β-substituted acroleins induce mutations. Multimeric adducts may only form at high concentrations of MDA that are toxic. Development of highly sensitive methodology for quantitation of individual adducts will be required to determine the profile of adducts generated under different conditions of exposure.

Also listed in Figure 13 are the structures of the major adducts formed by reaction of methyl glyoxal, acrolein, and crotonaldehyde with deoxyguanosine (*21–23*). Analogous to MDA, the major adducts from each compound are cyclic adducts formed between N^1 and N^2 of the guanine base. Attempts to explain the unique ability of MDA to induce frameshift mutations must, therefore, take into consideration other factors than the observation that MDA forms cyclic nucleic acid adducts in the base-pairing region. One obvious difference between the adducts in Figure 13 is the ability of MDA to form the PyP adduct. This adduct is fluorescent, aromatic, and hydrophobic. In contrast, all of the other adducts are nonfluorescent, puckered, and contain polar hydroxyl or carbonyl functionalities in the base-pairing region. The PyP adduct bears a striking similarity to acridine or proflavine dyes that are well-established frameshift mutagens. The ability of MDA to derivatize guanine bases to PyP may account for its ability to induce frameshift mutations in bacterial genomes. Conversely, the inability of methyl glyoxal, acrolein, and crotonaldehyde to produce analogous adducts on reaction with DNA would explain their inability to induce frameshift mutations.

Acknowledgment. This work was supported by a research grant from the National Cancer Institute (CA 22206) and partial support from the Wayne State University Molecular Biology Center. During the course of this research, L.J.M. was a recipient of an American Cancer Society Faculty Research Award (FRA 243). We are grateful to Jan Crowley for recording the mass spectra.

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