Identification of an Ethenoformyl Adduct Formed in the Reaction of the Potent Bacterial Mutagen 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone with Guanosine

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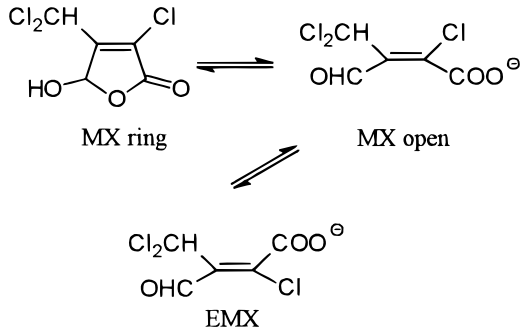
3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) is a potent direct-acting bacterial mutagen and a rodent carcinogen occurring in chlorine-disinfected drinking water. In this study, we have reacted MX with guanosine, cytidine, thymidine, and calf thymus DNA in aqueous solutions. HPLC analyses of the reaction mixture of MX with guanosine showed that one main product peak was formed. In the reactions of MX with cytidine or thymidine, no product peaks representing base-modified nucleosides could be observed. The product from the MX guanosine reaction mixture was isolated by preparative chromatography on reversed phase C18 columns, and its structure was determined by UV absorbance, ^{1}H and ^{13}C NMR spectroscopy, and mass spectrometry. The product was identified as 3-(β-D-ribofuranosyl)-7-formylimidazo[1,2-*a*]purin-9(4*H*)-one (ϵfGuo), and the yield for the reaction carried out at pH 7.4 and 37 °C was about 0.3 mol %. The adduct could not be observed at the detection limit of five adducts per 10^7 bases in the hydrolysate of the calf thymus DNA reacted with MX. However, this failure does not rule out the possibility that lower amounts of the adduct might be involved in the observed mispairing (adenine incorporated opposite an adducted guanine base) caused by MX in the *Salmonella typhimurium* strain TA100.

Introduction

3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX)[1] (Chart 1) is one of the most potent direct-acting mutagens ever tested in the Ames mutagenicity assay (*1*−*3*). MX is formed during chlorine disinfection of drinking water as a consequence of the reaction of chlorine with natural humic substances in the raw water (*4*−*6*). MX is present in chlorinated drinking waters at very low concentrations (a few nanograms per liter to 60 ng/L), but due to its extreme mutagenicity, it has been estimated to account for a large part (30−50%) of the Ames mutagenicity in drinking water (*7*−*13*). Besides the mutagenicity in the Ames assay, MX has also been reported to cause damage to the genetic material in several in vitro and in vivo assays (*14*−*21*). In a very recent work by Komulainen et al. (*22*), it was found that MX is a potent multisite carcinogen in rats.

Chart 1



MX occurs mainly in its cyclic furanone form in acidic water solutions, but undergoes a tautomeric transition to the dissociated acyclic form in the pH range of 4−6 (Chart 1). Under neutral conditions, MX exists primarily in the open acyclic form (*3*). In water solutions, MX undergoes isomerization to its geometric isomer, (*E*)-2-chloro-3-(dichloromethyl)-4-oxobutenoic acid (E-MX) (*23*).

Many studies have shown that reactive electrophilic compounds react with the base units of DNA and form covalent adducts. The base modifications can lead to gene mutations during the replication of DNA and contribute to the initiation of cancer (*24*, *25*). Previous work in our laboratory has shown that mucochloric acid (MCA) and 3-chloro-4-methyl-5-hydroxy-2-(5*H*)-furanone (MCF), chlorinated hydroxyfuranones with genotoxic properties and structures similar to those of MX, react with nucleosides and DNA and form adducts (*26*−*31*). Recently, we

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[1] Abbreviations: MX, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone; E-MX, (*E*)-2-chloro-3-(dichloromethyl)-4-oxobutenoic acid; CMCF, 3-chloro-4-(chloromethyl)-5-hydroxy-2(5*H*)-furanone; MCF, 3-chloro-4-methyl-5-hydroxy-2(5*H*)-furanone; MCA, 3,4-dichloro-5-hydroxy-2(5*H*)-furanone, mucochloric acid; BMA, bromomalonaldehyde; ϵfGuo, 3-(β-D-ribofuranosyl)-7-formylimidazo[1,2-*a*]purin-9(4*H*)-one; ϵfdGuo, 3-(2′-deoxy-β-D-ribofuranosyl)-7-formylimidazo[1,2-*a*]purin-9(4*H*)-one; ϵfG, 7-formylimidazo[1,2-*a*]purin-9(4*H*)-one; 5-Me-ϵfGuo, 3-(β-D-ribofuranosyl)-7-formyl-5-methylimidazo[1,2-*a*]purin-9-one; ϵGuo, 3-(β-D-ribofuranosyl)imidazo[1,2-*a*]purin-9(5*H*)-one (1,N^2-ethenoguanosine); DMF, *N*,*N*-dimethylformamide; PEG, poly(ethylene glycol); NOE, nuclear Overhauser effect; COSY, correlation spectroscopy (H−H); COLOC, C−H shift correlation NMR spectroscopy via long-range coupling.

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reported that MX reacts with 2′-deoxyadenosine and DNA, and forms propenal and fluorescent propenoformyl derivatives (*31*, *32*).

The mutational spectrum of MX in *Salmonella typhimurium* strain TA100 has shown that MX induces mainly GC → TA transversions (*33*). This mutational specificity indicates that MX modifies the guanine base, and the mutation results from misincorporation of adenine opposite the modified guanine base. The structural identification of products from the reactions of MX with nucleosides may contribute to a better understanding of the molecular mechanisms of MX-induced mutagenicity and carcinogenicity. The purpose of this study was to examine whether MX forms adducts in reactions with guanosine, cytidine, or thymidine and to determine the chemical structures of produced adducts. The aim was also to determine whether such adducts are formed in calf thymus DNA incubated with MX. The work resulted in the identification of a stable adduct produced in the reaction of MX with guanosine. The new guanosine derivative consists of an etheno bridge to which a formyl group is attached.

Materials and Methods

Caution: *MX has been found to be a rodent carcinogen and is one of the strongest known direct-acting mutagens tested in the Ames mutagenicity assay with S. typhimurium (TA100). Therefore, caution should be exercised in the handling and the disposal of the compound.*

Chemicals. Guanosine, cytidine, thymidine, 2′-deoxyguanosine, calf thymus DNA, DNase from bovine pancreas, nuclease P1 from *Penicillium citrinum*, alkaline phosphatase from bovine intestinal mucosa, and acid phosphatase from white potato were obtained from Sigma Chemical Co. (St Louis, MO). 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) was synthesized according to the method of Franzén and Kronberg (*34*). The purity of MX was at least 98%, as estimated by ^{1}H NMR and GC. 1,N^2-Ethenoguanosine was prepared by reacting mucochloric acid with guanosine as described by Kronberg et al. (*26*). Bromomalonaldehyde was prepared according to the method of Trofimenko (*35*).

Chromatographic Methods. Analytical HPLC was performed on a Kontron Instruments liquid chromatographic system consisting of a model 322 pump, a 440 diode-array detector (UV), and a KromaSystem 2000 data handling program (Kontron Instruments S. P. A., Milan, Italy). The reaction mixtures were chromatographed on a 5 μm, 4 mm × 125 mm reversed phase C18 analytical column (Spherisorb ODS2, Hewlett-Packard, Espoo/Esbo, Finland). The column was eluted isocratically with 0.01 M phosphate buffer (pH 7.1) for 5 min and then with a gradient from 0 to 30% acetonitrile over the course of 25 min at a flow rate of 1 mL/min. The compounds were isolated from the reaction mixtures by column chromatography on a 4 cm × 4 cm column of preparative C18 bonded silica grade (40 μm, Bondesil, Analytichem International, Harbor City, CA). The compounds were further purified on a semipreparative 8 μm, 10 mm × 250 mm (Hyperprep ODS, Hypersil, Krotek, Tampere/Tammerfors, Finland) reversed phase C18 column. The column was coupled to a Shimadzu HPLC system which consisted of two Shimadzu LC-9A pumps and a variable-wavelength Shimadzu SPD-6A UV spectrophotometric detector.

Spectroscopic and Spectrometric Methods. The ^{1}H and ^{13}C NMR spectra were recorded at 30 °C on a JEOL JNM-A500 Fourier transform NMR spectrometer at 500 and 125 MHz, respectively. The samples were dissolved in Me_2SO-d_6, and TMS was used as an internal standard. The ^{1}H NMR signal assignments were based on chemical shifts and H−H and C−H correlation data. The determination of the shifts and the coupling constants of the multiplets of the proton signals in the ribose and deoxyribose units were based on a first-order approach and are given with an accuracy of ±0.3 Hz. Assignment of carbon signals was based on chemical shifts, C−H correlations, and carbon−proton couplings.

The electrospray ionization mass spectra were recorded on a Fisons ZabSpec-oaTOF instrument (Manchester, U.K.). Ionization was carried out using nitrogen as both the nebulizing and bath gas. A potential of 8.0 kV was applied to the ESI needle. The temperature of the pepperpot counter electrode was 90 °C. The samples were introduced by loop injection at a flow rate of 20 μL/min (80/20/1 H_2O/CH_3CN/acetic acid). PEG 200 was used as standard for the exact mass determinations. The mass spectrometer had a resolution of 7000.

The UV spectra of the compounds were recorded with the diode-array detector as the peaks eluted from the HPLC column. A Shimadzu UV-160 spectrophotometer was used for the determination of the molar extinction coefficient (ϵ).

Preparation of 3-(β-D-Ribofuranosyl)-7-formylimidazo-[1,2-*a*]purin-9(4*H*)-one (ϵfGuo). MX (1 g, 4.6 mmol) was reacted with guanosine (651 mg, 2.3 mmol) in 400 mL of 0.5 M phosphate buffer solution (pH 8.2). The pH of the reaction mixture was checked daily and readjusted with 1 M NaOH when necessary. The reaction was performed at 37 °C for 7 days and was followed by HPLC analyses on the C18 analytical column. The reaction mixture was filtered and then passed through the preparative C18 column. The column was first eluted with 200 mL of H_2O and then with 100 mL batches of 5, 10, and 20% acetonitrile solutions in water. Fractions of 30 mL were collected. The compound ϵfGuo eluted from the column with the 5% acetonitrile eluent. The fractions containing the product were combined and concentrated by rotary evaporation to about 25 mL. Further purification of the compound was carried out using the semipreparative column. The column was eluted isocratically with 5% acetonitrile in 0.01 M phosphate buffer solution (pH 7.1) for 2 min and then with a gradient from 5 to 30% acetonitrile over the course of 28 min at a flow rate of 4 mL/min. The collected fraction was then desalted using the preparative C18 column. The desalted solution was evaporated to dryness, and the residue was subjected to spectroscopic and spectrometric studies. The isolated amount of the compound was 3.0 mg.

The isolated compound had the following spectral characteristics: UV spectrum [HPLC eluent, 10% acetonitrile in 0.01 M phosphate buffer (pH 7.1)] UV_{max} 232 and 360 nm (ϵ = 18 500 M^{-1} cm^{-1}), UV_{min} 312 nm, with a shoulder between 248 and 265 nm. The positive ion electrospray mass spectrum showed the following ions *m*/*z* (relative abundance, formation): 336 (100, MH^+), 204 (26, MH^+ − ribosyl + H). High-resolution mass spectrometry gave a protonated molecular formula of $C_{13}H_{14}N_5O_6$ (MH^+ 336.0944, calcd 336.0944).

The ^{1}H and ^{13}C NMR spectroscopic data are presented in Table 1.

Preparation of 3-(2′-Deoxy-β-D-ribofuranosyl)-7-formylimidazo[1,2-*a*]purin-9(4*H*)-one (ϵfdGuo). The 2′-deoxyguanosine derivative was prepared in exactly the same way as the guanosine derivative. The isolated 2′-deoxy derivative was obtained in about the same yield as ϵfGuo, and it had the following spectral characteristics: UV spectrum [HPLC eluent, 11% acetonitrile in 0.01 M phosphate buffer (pH 7.1)] UV_{max} 232 and 360 nm, UV_{min} 312 nm, with a shoulder between 248 and 265 nm. The positive ion electrospray mass spectrum showed the following ions *m*/*z* (relative abundance, formation): 320 (27, MH^+), 204 (8, MH^+ − deoxyribosyl + H). High-resolution mass spectrometry gave a protonated molecular formula of $C_{13}H_{14}N_5O_5$ (MH^+ 320.0990, calcd 320.0995). The ^{1}H NMR spectral data were as follows: δ 10.61 (s, 1H, C*H*O), 7.99 (s, 1H, H-6), 7.97 (s, 1H, H-2), 6.29 (dd, 1H, H-1′, *J* = 8.3, 5.9 Hz), 5.38 (dd, 1H, 5′-O*H*, *J* = 7.1, 4.7 Hz), 5.25 (d, 1H, 3′-O*H*, *J* = 4.0 Hz), 4.40 (m, 1H, H-3′), 3.87 (dt, 1H, H-4′, *J* = 4.1, 2.5 Hz), 3.63 (m, 1H, H-5′), 3.53 (ddd, 1H, H-5″, *J* = 11.9, 7.2, 4.1 Hz), 2.71 (ddd, 1H, H-2′, *J* = 13.5, 8.0, 5.3 Hz), 2.20 (ddd, 1H, H-2″, *J* = 13.1, 6.0, 2.7 Hz). The ^{13}C NMR spectral data were as follows: δ 178.3 (*C*HO), 155.0 (C-9), 154.5 (C-4a), 149.3 (C-

Table 1. ^{1}H and ^{13}C Chemical Shifts (δ)[a] and Spin−Spin Coupling Constants, $J_{H,H}$ and $J_{C,H}$ (Hertz), of Protons and Carbons in ϵfGuo

proton	δ	multiplicity	$J_{H,H}$	carbon	δ	multiplicity	$^1J_{C,H}$	$^{>1}J_{C,H}$
H-2 (1H)	7.97	s		C-2	137.3	dd	211.6	3.9
H-6 (1H)	8.00	d	0.5	C-6	140.2	dd	185.7	4.1
C*H*O (1H)	10.62	d	0.5	*C*HO	178.3	d	180.0	
				C-3a	149.4	dd		4.7, 3.9
				C-4a	154.4	dd		14.4, 0.8
				C-7	127.0	dd		20.1, 14.0
				C-9	155.0	d		0.8
				C-9a	116.3	d		10.9
H-1′ (1H)	5.81	d	6.3	C-1′	87.8	d	164.0	
H-2′ (1H)	4.65	dd	6.3, 5.0	C-2′	72.8	d	147.9	
H-3′ (1H)	4.14	dd	5.0, 2.9	C-3′	70.7	d	148.5	
H-4′ (1H)	3.95	q	3.2	C-4′	85.6	d	147.5	
H-5′ (1H)	3.68	dd	12.2, 3.4	C-5′	61.8	t	140.7	
H-5″ (1H)	3.55	dd	12.1, 3.2					
5′-O*H* (1H)	5.62	br						
2′-O*H* (1H)	5.38	br						
3′-O*H* (1H)	5.08	br						

[a] Relative to TMS.

3a), 140.3 (C-6), 136.7 (C-2), 126.9 (C-7), 116.1 (C-9a), 87.7 (C-4′), 83.6 (C-1′), 71.1 (C-3′), 62.1 (C-5′), 39.3 (C-2′).

Cleavage of the 2′-Deoxyribose Unit from ϵfdGuo by Acid Hydrolysis. Pure ϵfdGuo (1 mg) was dissolved in 4 mL of 0.1 M HCl and the mixture heated at 60 °C. The HPLC analysis of the mixture showed that the cleavage of the 2′-deoxyribose moiety was complete after 35 min. The new compound, ϵfG, eluted 2 min earlier than ϵfdGuo. The compound was isolated and purified using the semipreparative column. The following spectral characteristics were recorded: UV spectrum [HPLC eluent, 9% acetonitrile in 0.01 M phosphate buffer (pH 7.1)] UV_{max} 224, 252, and 360 nm, UV_{min} 240 and 308 nm. The positive ion electrospray mass spectrum showed the protonated molecular ion at 204, with a relative abundance of 100%. High-resolution mass spectrometry gave a protonated molecular formula of $C_8H_6N_5O_2$ (MH^+ 204.0528, calcd 204.0521). The ^{1}H NMR spectral data were as follows: δ 10.59 (s, 1H, C*H*O), 7.99 (s, 1H, H-6), 7.70 (s, 1H, H-2). (The assignment of H-6 was based on selective decoupling of the formyl proton that led to an increase in intensity of the signal at $\delta = 7.99$ ppm due to NOE enhancement.)

Small Scale Reactions of MX with Guanosine, Cytidine, and Thymidine. MX (9.9 mg, 0.046 mmol) was reacted with 0.023 mmol of guanosine, cytidine, and thymidine in 5 mL of 0.5 M phosphate buffer solutions at pH 9, 8.2, 7.4, 6, and 4.6. The reaction mixtures were incubated at 37 °C. The pHs of the reaction mixtures were checked daily and readjusted with 1 M NaOH if necessary. The reaction mixtures were analyzed by HPLC using the C18 analytical column.

Methylation of ϵfGuo [Preparation of 3-(β-D-Ribofuranosyl)-7-formyl-5-methylimidazo[1,2-*a*]purin-9-one (5-Me-ϵfGuo)]. Triethylamine (60 mg, 0.59 mmol) and methyl iodide (64 mg, 0.45 mmol) were added to a solution of ϵfGuo (2.7 mg, 0.008 mmol) in 8 mL of DMF. The solution was stirred for 7 h at 37 °C, and the reaction was followed by HPLC. After the reaction was complete, DMF was removed by rotary evaporation and the residue was dissolved in 3 mL of water. The product was then purified on the semipreparative HPLC column. The column was eluted isocratically with 5% acetonitrile in water for 2 min and then with a gradient from 5 to 30% acetonitrile over the course of 28 min at a flow rate of 4 mL/min. The collected fraction was rotary evaporated to dryness, and the residue was analyzed by ^{1}H NMR and electrospray MS. The isolated compound 5-Me-ϵfGuo had the following spectral characteristics: UV spectrum [HPLC eluent, 12% acetonitrile in 0.01 M phosphate buffer (pH 7.1)] UV_{max} 224 and 332 nm, UV_{min} 270 nm; MS ESI *m*/*z* (relative abundance, formation) 350 (56, MH^+), 218 (100, MH^+ − ribosyl + H). High-resolution mass spectrometry gave a protonated molecular formula of $C_{14}H_{16}N_5O_6$ (MH^+ 350.1109, calcd 350.1100): ^{1}H NMR δ 10.78 (s, 1H, C*H*O), 8.48 (s, 1H, H-6), 8.28 (s, 1H, H-2), 5.90 (d, 1H, H-1′, $J = 6.1$ Hz), 4.58 (dd, 1H, H-2′, $J = 5.8, 5.0$ Hz), 4.16 (dd, 1H, H-3′, $J = 4.9, 3.4$ Hz), 3.95 (q, 1H, H-4′, $J = 3.2$ Hz), 3.74 (s, 3H, CH_3), 3.66 (dd, 1H, H-5′, $J = 11.7, 4.4$ Hz), 3.57 (dd, 1H, H-5″, $J = 11.9, 4.1$ Hz).

Reaction of Bromomalonaldehyde with Guanosine. Bromomalonaldehyde (5.3 mg, 0.035 mmol) was reacted with guanosine (5 mg, 0.018 mmol) in 3 mL of 0.5 M phosphate buffer solutions at pH 9, 8.2, 7.4, 6, and 4.6. The reaction mixtures were stirred at 37 °C. The pHs of the reaction mixtures were checked daily, and readjusted with 1 M NaOH if necessary. The reaction mixtures were analyzed by HPLC using the C18 analytical column.

Determination of Product Yields. Quantitative ^{1}H NMR analysis, using 1,1,1-trichloroethane as an internal standard, was performed on aliquots of the ϵfGuo and ϵGuo adducts. HPLC standard solutions were prepared by taking an exact volume of the NMR samples and diluting it with an appropriate volume of water. The quantitative determination of the amount of ϵfGuo and ϵGuo in the reaction mixtures was made by comparing the peak area of the compound in question in the standard solution with the peak area of the compounds in the reaction mixtures. The ϵfGuo and ϵGuo derivatives were quantified using UV detection at 360 and 230 nm, respectively. The molar yields were calculated from the original amount of guanosine in the reaction mixtures.

Reactions of MX with Calf Thymus DNA. MX (50 mg) was reacted with double-strand calf thymus DNA (10 mg) in 10 mL of 0.5 M phosphate buffer at pH 7.4. The mixture was stirred and incubated at 37 °C for 4 days. During the first 12 h of reaction and then twice a day, the pH of the incubation mixture was monitored and readjusted if necessary. The modified DNA was recovered by precipitation with cold ethanol. To 1 volume of the incubation mixture were added 0.1 volume of 5 M NaCl and 2 volumes of cold 96% ethanol. This mixture was centrifugated (10 min, 3000 rpm), and the supernatant was collected. The supernatant was concentrated about 40 times and analyzed by HPLC to search for modified purines and pyrimidines. The precipitated DNA was washed with 1 volume of 70% ethanol and then redissolved in 1 volume of water. This precipitation/washing procedure was performed (at least twice) until there was no more unreacted MX left in the supernatant (controlled by HPLC analyses). The enzymatic hydrolysis of the DNA was carried out following the procedure described by Martin et al. (*36*). Briefly, the modified DNA was dissolved (1 mg/mL) in 0.1 M phosphate buffer (pH 7.4) containing 5 mM $MgCl_2$. DNase I (dissolved at 10 mg of DNase/mL in 0.9% NaCl) was added to obtain a level of 0.1 mg of DNase/mL. The mixture was incubated and stirred for 3 h at 37 °C. Nuclease P_1 (dissolved at 0.5 mg of nuclease P_1/mL in 1 mM $ZnCl_2$) was added to obtain 20 μg of nuclease/mL as the final concentration. Finally, alkaline phosphatase (87 units/mL in water) and acid

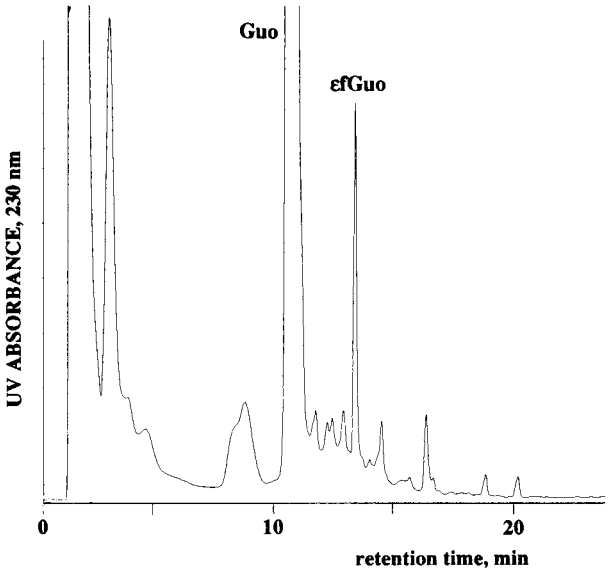


Figure 1. C18 column HPLC separation of the reaction mixture of MX and guanosine held at 37 °C and pH 8.2 for 6 days. For analysis conditions, see Materials and Methods.

phosphatase (20 units/mL in water) were added to give final concentrations of 0.5 and 0.3 unit/mL, respectively. The mixture was incubated and stirred at 37 °C for 18 h. The mixture of the hydrolyzed DNA was rotary evaporated to near dryness. The residue was washed three times with 3 mL of 1/1 ethanol/methanol. The washes were combined, and insoluble particles were removed by centrifugation (20 min, 3000 rpm). Finally, the solution was evaporated to near dryness; an appropriate amount of water (250 μL for 10 mg of DNA) was added, and 50−100 μL of the solution was injected on the analytical HPLC column.

Blanks and HPLC Checks. A blank sample was prepared by allowing calf thymus DNA to stand in an aqueous solution at pH 7.4 and 37 °C for 2 days. The precipitation, the enzymatic hydrolysis, and the HPLC sample preparation were then carried out exactly as described above.

Results and Discussion

The small scale reactions of MX with guanosine, cytidine, and thymidine were analyzed by HPLC on the analytical column. No product peaks representing base-modified nucleosides were observed in the reactions of MX with thymidine or cytidine. In the reaction of MX with guanosine, one main product peak with a retention time longer than that of guanosine was formed (Figure 1). The compound marked εfGuo eluted from the C18 analytical column at 13.5 min. The product was formed at all the studied pH conditions, but in very low yields at pH 4.6 and 6. The product was obtained in higher yields in the reactions carried out under neutral or slightly basic pH conditions. The yield was about 0.3% at pH 7.4 and about 0.9% at pH 8.2 and 9 (Figure 2).

To be able to characterize the product by spectroscopic and spectrometric methods, we performed a large scale reaction and isolated the product from the reaction mixture by preparative C18 column chromatography and HPLC.

The UV spectrum of εfGuo showed an absorption maximum at 360 nm (Figure 3). This bathochromic shift compared to 1,N^2-ethenoguanosine (UV_{max} = 284 nm) (*26*) is most likely caused by the extended conjugation in the compound because of the formyl group.

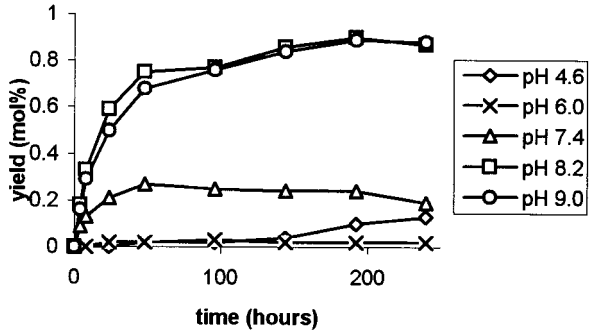


Figure 2. Formation of εfGuo under various pH conditions at 37 °C.

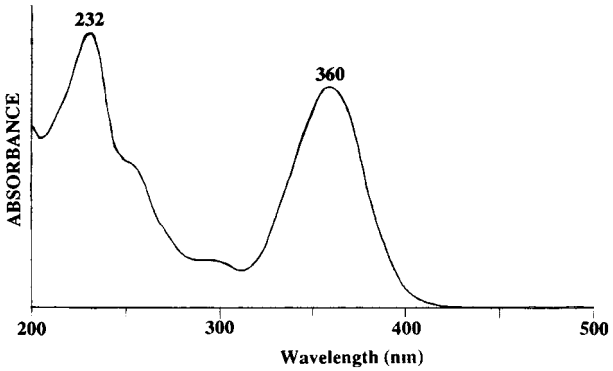


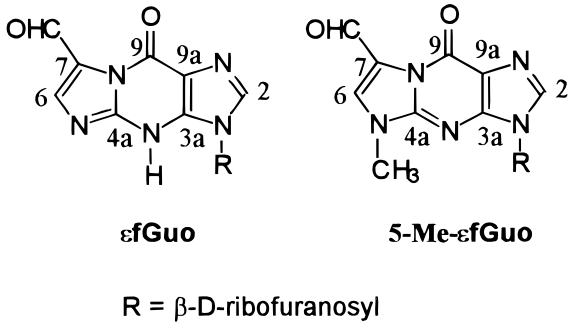
Figure 3. UV absorbance spectrum of εfGuo. The UV spectrum was recorded with the diode-array detector as the compound eluted from the HPLC column.

In the positive ion electrospray mass spectrum of the product, the protonated molecular ion was the most abundant ion, and it was observed at *m*/*z* 336. A second major fragment peak corresponding to the loss of the ribosyl unit was present at *m*/*z* 204 at a relative abundance of 26%.

The ^{1}H NMR spectrum displayed, besides the signals of the protons of the ribose moiety which were assigned using H−H correlation data, one-proton signals at δ = 10.62, 8, and 7.97 ppm (Table 1). The signal at δ = 10.62 ppm was assigned to the formyl proton on the basis of the downfield chemical shift and the one-bond C−H correlation with the carbon signal at δ = 178.3 ppm. The signal at δ = 8 ppm was assigned to the olefinic proton, H-6, and it displayed a long-range H−H coupling to the formyl proton. The proton at position 2 in the purine ring appeared at δ = 7.97 ppm. The signal showed a H−H correlation (COSY) with the H-1′ signal in the ribose moiety at δ = 5.81 ppm.

The ^{13}C NMR spectrum showed three carbon signals in addition to the 10 signals arising from the guanosine unit (Table 1). The signal observed at δ = 178.3 ppm displayed a strong one-bond C−H coupling and was assigned to the formyl carbon. The assignment of the signal at δ = 127 ppm to C-7 was confirmed by its strong two-bond C−H coupling (^2J = 20.1 Hz) to the formyl proton (*37*). The signal was further split into a doublet of doublets due to coupling to H-6 (^2J = 14 Hz) as confirmed by selective decoupling at δ = 8 ppm. The signal at δ = 140.2 ppm was assigned to C-6 on the basis of the strong one-bond C−H coupling (^1J = 185.7 Hz) to H-6 and a weak three-bond coupling to the formyl proton (^3J = 4.1 Hz). The signal of C-6 also showed C−H correlation with the formyl proton (COLOC). The signal at δ = 137.3 ppm was assigned to C-2 on the basis of the

Chart 2



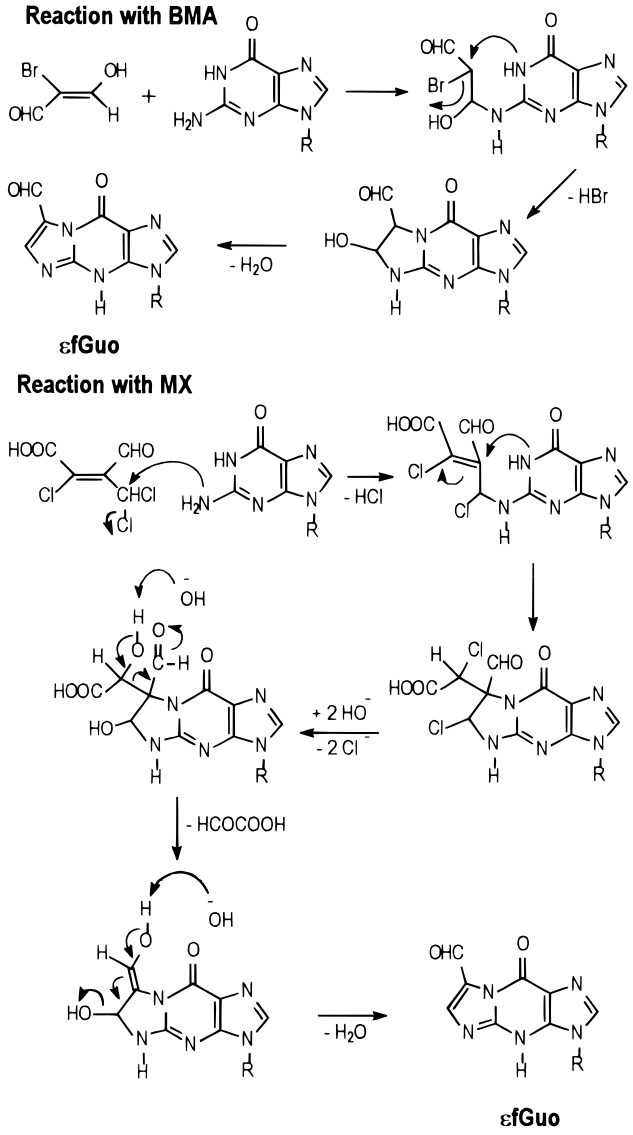
strong one-bond coupling (^1J = 211.6 Hz) to the H-2 proton. The carbon C-3a resonance signal appeared at δ = 149.4 ppm. It showed C−H correlation with the signal of H-2. The signal at δ = 154.4 ppm was assigned to C-4a, and it was split into a doublet due to a three-bond coupling of 14.4 Hz to H-6 as confirmed by selective decoupling of H-6. Due to this strong coupling, we assume that a double bond is placed between C-4a and N-5. The carbon resonance signal of C-9 appeared at δ = 155 ppm as a weakly coupled (J = 0.8 Hz) doublet. The signal at δ = 116.3 ppm was assigned to C-9a on the basis of the chemical shift and the three-bond coupling (^3J = 10.9 Hz) to H-2. Further, the corresponding C−H correlation was observed (COLOC).

To obtain additional support for the assignment of the position of the formyl group in the etheno bridge (C-6 or C-7), we methylated ϵfGuo with methyl iodide and analyzed the product with ^{1}H NMR spectroscopy. In the ^{1}H NMR spectrum, the methyl protons appeared at δ = 3.74 ppm and showed a NOE and a long-range H−H correlation with the H-6 proton at δ = 8.48 ppm. No NOE or long-range correlations between the formyl proton and the methyl protons were observed. Therefore, we conclude that ϵfGuo was methylated at N-5 and the formyl group must be bound to C-7 (Chart 2). The location of the methyl group at N-5 is also reflected in the downfield shifts of the H-6 (0.48 ppm) and H-2 (0.31 ppm) proton resonance signals and, in the UV spectrum, in a significant blue shift of the long-wavelength maximum from 360 to 332 nm. These spectral changes are most likely due to the difference in the conjugation of the double bonds in the two compounds.

The NMR and the UV spectroscopic and the mass spectrometric data are consistent with the structure of ϵfGuo presented in Chart 2.

Previously, Nair et al. (*38*) prepared ethenoformyl derivatives of adenosine and cytidine by reacting the nucleosides with bromomalonaldehyde. In the present work, we studied the reaction of bromomalonaldehyde with guanosine and searched for ϵfGuo. The HPLC analyses of the reaction mixtures showed that one main product with a retention time of 14.5 min was formed. The UV spectrum of the product displayed absorption maxima at 224 and 284 nm and was identical with the spectrum of 1,N^2-ethenoguanosine (*26*). In the chromatograms, a small peak at exactly the same retention time as ϵfGuo was also present. When we spiked the reaction mixture with pure standards of ϵGuo and ϵfGuo, we found that the compounds in the reaction mixture coeluted with the standards and gave sharp peaks. Since the UV spectra of the products were also identical with the spectra of ϵGuo and ϵfGuo, we concluded that ϵGuo and

Chart 3



ϵfGuo were formed in the reaction of bromomalonaldehyde with guanosine. The compounds were formed under all the studied pH conditions. The optimal pH for the formation of ϵGuo was 7.4, and at this pH, the yield was about 3% after a reaction time of 7 days. ϵfGuo was formed under all the studied pH conditions in about 0.1% yields. The formation of ϵfGuo may be explained through an initial formation of a carbinolamine (Chart 3). Following nucleophilic displacement of bromide by N-1 of guanosine and dehydration, ϵfGuo is obtained. In the reaction of MX with guanosine, we propose a mechanism in which the dichloromethyl carbon atom of MX is initially attacked by the exocyclic amino group of guanosine and HCl is displaced (Chart 3). Next, an ethano bridge is formed by attack of the endocyclic nitrogen on the double bond. The chlorine atoms are substituted by hydroxyl groups; glyoxylic acid is cleaved from the intermediate through a retrograde aldol reaction (*39*), and the adduct is obtained by dehydration of the enol.

Reaction of MX with Calf Thymus DNA. It has been reported that the mutational specificity of MX could be explained by the formation of guanosine adducts or by the occurrence of apurinic sites (*33*, *40*, *41*). To determine whether ϵfdGuo is formed in DNA, we incu-

bated DNA with MX and subsequently carried out a search for the adduct in the DNA hydrolysate. The chromatograms obtained by HPLC analyses of the hydrolysate did not show any peak which could represent ϵfdGuo. Apurinic sites may occur as a consequence of adduct formation followed by breakage of the glycosidic bond and release of the adducted base from DNA. We prepared ethenoformylguanine (ϵfG) by hydrolysis of ϵfdGuo and used the compound as a standard to search for the adducted base in the supernatant, collected during precipitation of the incubated calf thymus DNA. The outcome of the HPLC analysis was that the modified guanine base unit could not be detected in the supernatant. These results show that, if the ethenoformyl unit is incorporated into the guanine base of DNA, the ethenoformyl guanine derivative is formed in amounts lower than the detection limit of our HPLC analytical method. The detection limit of our HPLC analytical method was about five adducts per 10^7 bases.

Very recently, Franzén et al. (*42*) identified a MX−guanosine adduct, named 10-formyl-1,N^2-benzoquinone propenoguanosine. We have repeated the experiment according to the procedure described by Franzén et al., but by using 98% pure MX, we could not detect the claimed adduct in our HPLC chromatograms.

The mutational spectrum of MX in *S. typhimurium* strain TA100 has shown that MX causes primarily GC → TA transversions at the second position of the target sequence CCC in the *hisG46* allele (*33*). These mutational events suggest a mechanism in which MX forms an adduct with guanine or via depurination of the guanine base. In a study by Marsteinstredet et al. (*40*), it was demonstrated by a DNA sequence analysis of a MX-treated DNA fragment, that MX reacts preferentially with the guanine base in vitro to produce chemically stable DNA adducts. The results of the current study demonstrate that MX modifies guanosine by forming a stable, cyclic 1,N^2-ethenoformylguanosine adduct. Since the N-1 and N^2 positions of guanine are involved in base pairing, it seems possible that the adduct might be involved in the genotoxic effects of MX. However, this theory would be strengthened if the ϵfdGuo adduct could be detected in calf thymus DNA. Therefore, further work is needed to lower the detection limit of the analysis of the adduct in DNA to establish whether ϵfdGuo is formed at all in DNA.

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Supporting Information Available: NMR spectra and electrospray mass spectra of ϵfGuo and the UV spectrum of ϵfG (5 pages). Ordering information is given on any current masthead page.

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