Reaction of Mucochloric and Mucobromic Acids with Adenosine and Cytidine: Formation of Chloro- and Bromopropenal Derivatives

Leif Kronberg,* Deanne Asplund, Jukka Mäki, and Rainer Sjöholm

Department of Organic Chemistry, Åbo Akademi University, Akademigatan 1, FIN-20500 Turku/Åbo, Finland

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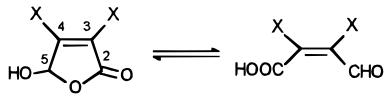
Mucochloric (MCA) and mucobromic acid (MBA)—bacterial mutagens and water disinfection byproducts—were reacted with adenosine, cytidine, and guanosine in *N*,*N*-dimethylformamide (DMF). In the MCA reaction with adenosine and cytidine and in the MBA reaction with adenosine one major product was formed. In the reactions of MBA with cytidine and in the reactions of MCA and MBA with guanosine only trace levels of products could be detected, and these were not further characterized. The products from the adenosine and cytidine reactions were isolated by preparative chromatography on octadecylsilane columns and structurally characterized by UV absorbance, ^{1}H and ^{13}C NMR spectroscopy, and mass spectrometry. The products were identified as 3-(N^6-adenosinyl)-2-chloro-2-propenal (**M_{Cl}A**), 3-(N^6-adenosinyl)-2-bromo-2-propenal (**M_{Br}A**), and 3-(N^4-cytidinyl)-2-chloro-2-propenal (**M_{Cl}C**). The yields of **M_{Cl}A**, **M_{Br}A**, and **M_{Cl}C** were 19, 4, and 7 mol %, respectively. These halopropenal derivatives were formed also in reactions carried out in aqueous solutions at pH 7.4 and 37 °C at low yields, about 5×10^{-3}%. The mechanism of formation of the halopropenal derivatives and of the previously identified etheno and ethenocarbaldehyde derivatives was elucidated by reacting ^{13}C-3 labeled MCA with adenosine in DMF and in water. The location of the labeled carbon in the products was determined from the ^{13}C NMR spectra. It was concluded that the halopropenal derivatives were formed by mechanisms that differ completely from the one responsible for the formation of the propenal adducts (M_1A and M_1C) previously reported to be formed in reactions of malonaldehyde with adenosine and cytidine.

Introduction

Mucochloric acid (MCA;[1] Scheme 1) is one of several chlorohydroxyfuranones identified in chlorine disinfected drinking water (*1*). The chlorohydroxyfuranones are direct acting mutagens in *Salmonella typhimurium* and account for part of the mutagenicity of chlorinated drinking water (*1*−*4*). They are formed in the water treatment plant by reaction of chlorine with residual naturally occurring macromolecular organic matter, i.e., humic substances (*1*, *5*−*7*).

Mucobromic acid (MBA; Scheme 1), the brominated analogue of MCA, has not been identified in drinking water, but the compound is believed to be present in water, and especially in water derived from surface water with a high content of bromide. During water treatment, bromide is oxidized by chlorine and hypobromous acid is formed which in turn reacts to form brominated organic compounds (*7*, *8*). In fact, several brominated compounds have been identified in drinking water, e.g., brominated trihalomethanes, acetonitriles, and acetic acids (*9*−*11*). MBA has been reported to induce about 5 rev/nmol in Ames tester strain TA100 (*12*).

Scheme 1



MCA, X = Cl
MBA, X = Br

It is generally considered that the base units of DNA are the receptors of genotoxic compounds, and numerous studies have shown that genotoxic compounds may bind covalently to the purine and pyrimidine units (*13*). In order to elucidate whether MCA could react with the base units of DNA, our laboratory has been engaged in the study of products formed in aqueous reactions of MCA with adenosine, cytidine, and guanosine. These studies have resulted in the identification of etheno adducts of adenosine (ϵA), cytidine (ϵC), and guanosine (ϵG), of ethenocarbaldehyde (ϵcA), and of "dimeric" (ϵoA,A and ϵcA,A) adducts of adenosine (*14*−*16*).

In the current study, we have carried out MCA reactions with nucleosides in *N*,*N*-dimethylformamide, and we now report on the formation and identification of previously unkown reaction products, namely, chloropropenal and bromopropenal derivatives of adenosine and a chloropropenal derivative of cytidine (Chart 1). These adducts are halogenated analogues to the previously known propenal adducts derived from malonaldehyde (Chart 1) (*17*−*19*). Also, we report on the detection of trace amounts of these novel adducts in aqueous reaction mixtures held at pH 7.4 and 6.0 for 5 days.

* Author for correspondence. E-mail: leif.kronberg@abo.fi; phone: +358-2-2654186; FAX: +358-2-2654866.

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[1] Abbreviations: MCA, 3,4-dichloro-5-hydroxy-2(5*H*)-furanone; MBA, 3,4-dibromo-5-hydroxy-2(5*H*)-furanone; CAA, chloroacetaldehyde; BAA, bromoacetaldehyde; DMF, *N*,*N*-dimethylformamide; ϵA, 1,N^6-ethenoadenosine; ϵcA,3-(β-D-ribofuranosyl)-7-formylimidazo[2,1-*i*]purine; ϵoA, A,3-(β-D-ribofuranosyl)-7-oxalo-8-[9′-(β-D-ribofuranosyl)-N^6-adenosinyl]-imidazo[2,1-*i*]purine; ϵcA, A,3-(β-D-ribofuranosyl)-7-formyl-8-[9′-(β-D-ribofuranosyl)-N^6-adenosinyl]imidazo[2,1-*i*]purine; ϵC, 3,N^4-ethenocytidine; ϵG, 1,N^2-ethenoguanosine.

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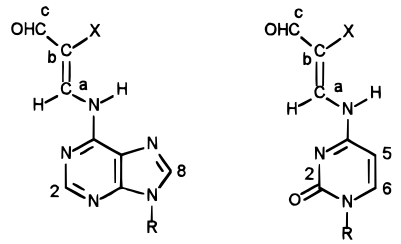
Chart 1



$M_{Cl}A$, X = Cl $M_{Cl}C$, X = Cl
$M_{Br}A$, X = Br M_IC, X = H
M_IA, X = H

R = β-D-ribofuranosyl

Materials and Methods

Caution: *Mucochloric acid and mucobromic acid have tested positive in the Ames mutagenicity assay with S. typhimurium (TA100) without metabolic activation. Therefore, caution should be exercised in the handling and disposal of the compounds.*

HPLC analyses were performed with a system consisting of two Shimadzu LC-9A pumps and a variable wavelength Shimadzu SPD-6A UV spectrophotometric detector (Shimadzu Europe, Germany). In addition, analyses were carried out on a Hewlett-Packard 1090 liquid chromatograph equipped with a diode-array detector (Hewlett-Packard, Esbo/Espoo, Finland). The separations were done on a 5 μm, 4- × 125-mm C18 analytical column (Spherisorb ODS2, Hewlett-Packard, Esbo/Espoo, Finland). The column was eluted isocratically for 5 min with 5% acetonitrile in 0.01 M potassium dihydrogen phosphate (pH 4.6) and then with a gradient from 5% to 30% in 25 min at a flow rate of 1 mL/min. Preparative isolation of the products was carried out by column chromatography on a 2.5- × 10-cm column of preparative C18 bonded silica grade (40 μm, Bondesil, Analytichem International, Harbor City, CA). 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 determination of the shifts and the coupling constants of the multiplets of the proton signals in the ribose units of **$M_{Cl}A$** and **$M_{Br}A$** were based on a first order approach. In the spectrum of **$M_{Cl}C$**, the strong interproton couplings and the small shift differences resulted in higher order coupling patterns, and therefore spectral parameters were calculated using the PERCH program.[2] However, the differences between the shifts and the couplings obtained by a first order treatment of data and the calculated ones were <0.01 ppm and 0.5 Hz, respectively. Assignment of carbon signals was based on chemical shifts and carbon−proton couplings. The assignment of H-2 and H-8 in the adenine moiety was made by recording selectively proton-decoupled ^{13}C NMR spectra. The direct inlet chemical ionization (DCI) mass spectra were recorded on a VG 7070E mass spectrometer. Methane was used as ionization gas. The UV spectra of the isolated products were recorded with a Shimadzu UV-160 spectrophotometer (Shimadzu Europa, Germany). ^{13}C-3 labeled MCA was synthesized and purified according to the method of Franzén and Kronberg (*20*).

Preparation of 3-(N^6-Adenosinyl)-2-chloro-2-propenal ($M_{Cl}A$), 3-(N^6-Adenosinyl)-2-bromo-2-propenal ($M_{Br}A$), and 3-(N^4-Cytidinyl)-2-chloro-2-propenal ($M_{Cl}C$). Adenosine (159 mg, 0.59 mmol) was reacted with MCA (200 mg, 1.19 mmol) and with MBA (323 mg, 1.26 mmol) in 8 mL of DMF for 3 days at 37 °C. In exactly the same way, MCA, mixed with the ^{13}C-3 labeled MCA (15 mol %), was allowed to react with adenosine. Cytidine (143 mg, 0.59 mmol) was reacted with MCA (200 mg, 1.19 mmol) in 20 mL of DMF for 5 days at 37 °C. When the reactions were completed, the solvent was removed by rotary evaporation at 50 °C and the residue was dissolved in a few milliliters of water. The filtered solution was passed through the preparative C18 column. The column was equilibrated with water and eluted batchwise with 0%, 5%, 10%, and 15% acetonitrile in water; the volume of each batch was 100 mL. The products of the adenosine reactions were found in fractions of 15% acetonitrile, and the cytidine reaction product eluted with 10% acetonitrile. The fractions containing the products were rotary evaporated to dryness, and the residue was crystallized from water.

The UV spectral properties in water of the isolated compounds were as follows: **$M_{Cl}A$**, UV_{max} 330 nm (ϵ 5.0 × 10^4 M^{-1} cm^{-1}), UV_{min} 278 nm; **$M_{Br}A$**, UV_{max} 330 nm (ϵ 5.2 × 10^4 M^{-1} cm^{-1}), UV_{min} 279 nm; **$M_{Cl}C$**, UV_{max} 330 nm (ϵ 5.2 × 10^4 M^{-1} cm^{-1}), UV_{min} 259 nm.

The ^{1}H and ^{13}C NMR spectroscopic data of the compounds are presented in Table 1 and 2, respectively, and the main fragment ions in the mass spectra are compiled in Table 3. High resolution mass spectrometry gave the molecular formulas of **$M_{Cl}A$** as $C_{13}H_{14}N_5O_5Cl_1$ (M^+ 355.0684, calcd 355.06835), of **$M_{Cl}C$** as $C_{12}H_{14}N_3O_6Cl_1$ ($M^+ - H_2O$ 313.0463, calcd 313.04655), and of **$M_{Br}A$** as $C_{13}H_{14}N_5O_5Br_1$ (M^+ 399.0176, calcd 399.01783).

The reactions of cytidine with MBA and of guanosine with MBA and MCA were performed exactly in the same way as the MCA reaction with cytidine. HPLC analyses of these reaction mixtures showed that only trace levels of products were formed, and the reactions were not further studied.

Small Scale Aqueous Reactions of Adenosine with MCA and MBA, and of Cytidine with MCA. Adenosine (12 mg, 0.045 mmol) was reacted with MCA (15 mg, 0.09 mmol) and MBA (23 mg, 0.09 mmol), and cytidine (11 mg, 0.045 mmol) was reacted with MCA (0.09 mmol) in 2 mL of 0.5 M phosphate buffer at pH 7.4 and pH 6.0 at 37 °C. Following 5 days of reaction, the formation of products were determined by HPLC analyses of aliquots of the reaction mixtures.

Aqueous Reaction of ^{13}C-3 Labeled MCA with Adenosine. Adenosine (85 mg, 0.32 mmol) and MCA mixed with ^{13}C-3 labeled MCA (in total 102 mg, 0.61 mmol, of which 13 mol % was ^{13}C-3 labeled MCA) was dissolved in 0.5 M phosphate buffer adjusted to pH 6.0. The reaction mixture was heated to 90 °C and held at this temperature for 12 h. The solution was then filtered, and ϵA and ϵcA were isolated by use of the preparative C18 column according to the same procedure described for isolation of **$M_{Cl}A$**, **$M_{Br}A$**, and **$M_{Cl}C$**. Upon evaporation of the fractions containing ϵA and ϵcA, the compounds crystallized. Recrystallization was performed from warm water.

Reaction of Bromomalonaldehyde with Adenosine in DMF. Adenosine (200 mg, 0,80 mmol) was reacted with bromomalonaldehyde (480 mg, 3.2 mmol) in 2 mL of DMF at room temparature for 4.5 h. The reaction mixture was diluted with 40 mL of water, filtered, and passed through the preparative C18 column. The coulmn was eluted batchwise with 0%, 5%, 10%, 15%, and 20% of acetonitrile in water, 100 mL each batch. The fractions containing pure ϵA and pure ϵcA were evaporated to dryness, and the compounds were crystallized from warm water. The yield of ϵA was about 5% and of ϵcA about 20%. Bromomalonaldehyde was prepared by the method of Trofimenko (*21*).

Determination of Product Yields. Quantitative ^{1}H NMR analysis, using 1,1,1-trichloroethane as an internal standard, was performed on aliquots of the adducts. Then standard solutions were prepared for HPLC analysis by taking exact volumes of the NMR samples and diluting them with appropriate volumes of water. The quantitative determination of adducts in the reaction mixtures was made by comparing the peak area of the adducts at 330 nm in the standard solutions with the area of the adduct peaks in the reaction mixtures. The molar yields were calculated from the original amount of adenosine or cytidine in the reaction mixtures.

Results and Discussion

Previous reports from this laboratory on the reactions of MCA and various nucleosides have dealt with the

[2] PERCH Software, University of Kuopio, Kuopio, Finland.

Table 1. ^{1}H Chemical Shifts and Spin−Spin Coupling Constants, $J_{H,H}$, of Protons in $M_{Cl}A$, $M_{Br}A$, and $M_{Cl}C$

	$M_{Cl}A$		$M_{Br}A$			$M_{Cl}C$	
proton[a]	δ	J (Hz)	δ	J (Hz)	proton[a]	δ	J (Hz)
H-2	8.80 s		8.81 s		H-5	6.47 d	7.3
H-8	8.65 s		8.66 s		H-6	8.37 d	7.3
N*H*	10.53 d	11.6	10.05 br, s		N*H*	10.57 d	11.7
H-a	9.25 d	11.6	9.37 br, s		H-a	8.65 d	11.7
C*H*O	9.42 s		9.34 br, s		C*H*O	9.43 s	
H-1′	6.03 d	5.5	6.07 d	5.5	H-1′	5.80 d	3.4
H-2′	4.62 dd	5.0; 5.5	4.66 t	5.5	H-2′	3.99 dd	3.4; 4.9
H-3′	4.20 dd	5.0; 4.0	4.25 dd	5.5; 4.0	H-3′	3.97 dd	4.9; 6.2
H-4′	3.99 q	4.0	4.05 q	4.0	H-4′	3.92 ddd	6.2; 3.2; 2.9
H-5′	3.71 dd	12.0; 4.0	3.76 dd	12.0; 4.0	H-5′	3.74 dd	12.3; 2.9
	3.59 dd	12.0; 4.0	3.64 dd	12.0; 4.0		3.60 dd	12.3; 3.2
O*H*-2	5.52 br		5.52 br		O*H*-2	5.47 br	
O*H*-3	5.22 br		5.22 br		O*H*-3	5.03 br	
O*H*-5	5.13 br		5.13 br		O*H*-5	5.16 br	

[a] H-1′−H-5′ and O*H*-2−O*H*-5 = protons and hydroxyl groups in the ribosyl unit. The determination of the shifts and coupling constants in the ribosyl units of the adenosine derivatives was based on a first order approach. The spectral parameters of **$M_{Cl}C$** were calculated using the PERCH program.[2]

Table 2. ^{13}C Chemical Shifts and Spin−Spin Coupling Constants, $J_{C,H}$, of Carbons in $M_{Cl}A$, $M_{Br}A$, and $M_{Cl}C$

	$M_{Cl}A$			$M_{Br}A$			$M_{Cl}C$		
carbon[a]	δ	multiplicity	J_{H-C}	δ	multiplicity	J_{H-C}	δ	multiplicity	J_{H-C}
C-2	151.83	d	205.3	151.84	d	205.6	154.08	d	5.7
C-4	151.79	ddd	11.9; 5.5; 2.7	151.64	ddd	11.9; 5.1; 2.8	160.55	d	8.8
C-5	121.14	d	11.9	121.06	dd	11.6; 1.3	94.78	dd	177.3; 3.2
C-6	148.83	dd	11.9; 3.7	148.50	dd	11.4; 3.6	144.80	d	183.6
C-8	143.12	dd	215.4; 4.5	143.22	dd	215.7; 4.1			
C-a	144.32	d	175.1	146.18	d	175.6	141.88	d	176.4
C-b	113.30	dd	41.3; 10.1	105.12	dd	42.9; 9.6	114.02	dd	41.4;10.4
C*H*O	184.03	dd	180.5; 4.5	184.39	dd	180.5; 5.2	184.71	dd	182.6; 4.7
C-1′	87.83	d	165.9	87.95	d	166.8	89.98	d	172.3
C-2′	73.75	d	148.4	73.87	d	148.7	74.42	d	150.8
C-3′	70.19	d	148.5	70.21	d	148.2	68.65	d	146.6
C-4′	85.69	d	147.5	85.72	d	149.2	84.06	d	147.4
C-5′	61.17	t	140.7	61.17	t	140.3	59.87	t	140.9

[a] C-1′−C-5′ = carbons in the ribosyl unit. For the ribosyl carbons only one-bond couplings are given.

Table 3. Selected Fragment Ions from the Mass Spectra of 3-(N^6-Adenosinyl)-2-chloropropenal ($M_{Cl}A$), 3-(N^6-Adenosinyl)-2-bromopropenal ($M_{Br}A$), and 3-(N^4-Cytidinyl)-2-chloropropenal ($M_{Cl}C$)

	m/*z* (relative intensity)					
	$M_{Cl}A$		$M_{Br}A$		$M_{Cl}C$	
formation[a]						
$[M + H]^+$	356/358	(50/17)	400/402	(76/72)	332/334	(3/1)
$[M - X]^+$	320	(8)	320	(23)	296	(3)
$[M - \text{ribose} + CHOH]^+$	252/254	(10/3)	296/298	(21/19)		
$[M - \text{ribose} + 2H]^+$	224/226	(48/16)	268/270	(81/77)		
$[M - C_3H_2OX]^+$	266	(36)				
$[\text{adenosine} + H]^+$	268	(28)				
$[M - \text{ribose} - X + H]^+$	188	(19)	188	(100)		
$[\text{adenine} + H]^+$	136	(100)	136	(20)		
miscellaneous					314/316[b]	(6/2)
					182/184[c]	(100/35)

[a] X = halogen. [b] Loss of water from $(M + H)^+$. [c] Proposed formation of the fragment ion is presented in Scheme 2.

structural identification of products formed in aqueous solutions (*14*−*16*). In the current study, we have reacted MCA and its brominated analogue MBA with adenosine, cytidine, and guanosine in DMF. HPLC analysis of the reaction mixtures revealed that in the reaction with adenosine both compounds yielded one single major product. On the other hand, in reaction with cytidine only MCA yielded a distinct product peak whereas no clearly observable products were formed in the MBA reaction. In reactions with guanosine, both compounds formed only trace levels of product, and the guanosine reaction was not studied further. Investigation of the chromatograms obtained from the aqueous reactions of MCA and MBA with adenosine, and MCA with cytidine, showed that in each reaction a small peak was present at the same retention time as the new adenosine and cytidine adducts. Spiking the aqueous reaction mixtures with the products isolated from the DMF reactions showed that the products coeluted from the HPLC column (Figure 1). Since also the UV spectra of the peaks at matching retention times were in all features identical, we concluded that the same products were formed in reactions carried out in aqueous solutions and in DMF.

The unkown products were isolated on the preparative C18 column. The structures of the isolated compounds were assigned as **$M_{Cl}A$**, **$M_{Br}A$**, and **$M_{Cl}C$** on the basis of data obtained by UV and NMR spectroscopy, and mass spectrometry (Chart 1).

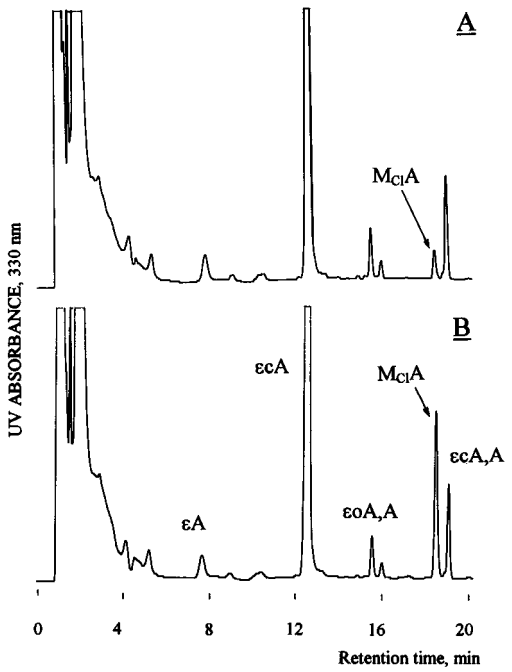


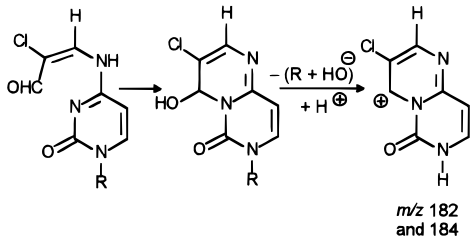
Figure 1. (A) C18 column HPLC separation of the reaction mixture of MCA and adenosine held at 37 °C and pH 6.0 for 5 days. (B) The same reaction mixture but spiked with pure $\mathbf{M_{Cl}A}$. Abbreviations: see footnote 1.

The UV spectra of $\mathbf{M_{Cl}A}$, $\mathbf{M_{Br}A}$, and $\mathbf{M_{Cl}C}$ were very similar to each other and to those of the previously identified propenal adducts of adenosine (M_1A) and cytidine (M_1C) and to the recently identified oxobutenoic adduct of adenosine (*17*−*19*, *22*). The compounds exhibited a UV maximum at 330 nm. This slight red shift in comparison to M_1A (UV_{max} 320 nm) and M_1C (UV_{max} 323) may be explained by resonance interactions of the unshared chlorine and bromine electrons with the conjugated aldehyde function (*23*).

The ^{1}H NMR spectra of $\mathbf{M_{Cl}A}$, $\mathbf{M_{Br}A}$, and $\mathbf{M_{Cl}C}$ displayed five signals, besides the ribose protons (Table 1). The singlet signals of $\mathbf{M_{Cl}A}$ at $\delta = 8.80$ and 8.65 ppm and of $\mathbf{M_{Br}A}$ at $\delta = 8.81$ and 8.66 ppm were attributed to the purine moiety protons H-2 and H-8, respectively. In $\mathbf{M_{Cl}C}$, the proton signals of the pyrimidine moiety were observed as coupled doublets at $\delta = 6.47$ and 8.37 ppm and were assigned to H-5 and H-6, respectively. The aldehyde proton signals at $\delta = 9.42$ ppm in $\mathbf{M_{Cl}A}$, at $\delta =$ 9.34 ppm in $\mathbf{M_{Br}A}$, and at $\delta = 9.43$ in $\mathbf{M_{Cl}C}$ were unsplit, showing absence of a proton at C-b. In M_1A and M_1C the aldehyde signal was reported to be a doublet due to coupling to the proton at C-b (*16*, *18*). The resonance signal at $\delta > 10$ ppm in the compounds was attributed to the NH proton. The signal appeared as a doublet in the spectra of chloropropenal derivatives due to coupling to the H-a protons at $\delta = 9.25$ and 8.65 ppm in $\mathbf{M_{Cl}A}$ and $\mathbf{M_{Cl}C}$, respectively. The NH signal of $\mathbf{M_{Br}A}$ was strongly broadened, and for this reason no couplings to the H-a proton could be observed.

The ^{13}C NMR spectra of $\mathbf{M_{Cl}A}$, $\mathbf{M_{Br}A}$, and $\mathbf{M_{Cl}C}$ exhibited three resonance signals in addition to those arising from the base and ribose units (Table 2). The signals observed at $\delta \approx 184$ ppm were assigned to the formyl carbon in the respective compounds. The signals appeared as doublets of doublets in the coupled ^{13}C NMR spectra due to one-bond C−H couplings and long-range C−H couplings to the H-a protons. The values of the long-range coupling constants ranged from 4.5 to 5.2 Hz and indicated a *cis* arrangement of the formyl group and the H-a proton (as depicted in Chart 1) (*24*). The resonance signals of C-b were observed at $\delta = 113.29$, 105.12, and 114.02 ppm in $\mathbf{M_{Cl}A}$, $\mathbf{M_{Br}A}$, and $\mathbf{M_{Cl}C}$, respectively. The C-b signals were splitted in doublets of doublets due to two-bond couplings to the formyl protons and to the H-a protons. The lack of one-bond couplings of the C-b signals and the large value of the coupling to the formyl protons ($J = 41-43$ Hz) showed that the halogen atoms were bound at the C-b carbon (*23*). The signals of C-a appeared as one-bond doublets at $\delta = 144.32$ ppm in $\mathbf{M_{Cl}A}$, at $\delta = 146.18$ ppm in $\mathbf{M_{Br}A}$, and at $\delta = 141.88$ ppm in $\mathbf{M_{Cl}C}$.

Scheme 2



In the DCI mass spectrum of $\mathbf{M_{Cl}A}$ and $\mathbf{M_{Br}A}$ the protonated molecular ion peaks were observed at *m*/*z* 356 and 400, respectively (Table 3). The intensity ratio of the $MH^+/MH^+ + 2$ isotope peaks was 3:1 in $\mathbf{M_{Cl}A}$ and 1:1 in $\mathbf{M_{Br}A}$, showing the presence of one chlorine and one bromine atom in the respective compounds. Major fragment ions of the compounds corresponded to loss of the halogen, part of the sugar unit, the sugar unit, and loss of the sugar unit together with the halogen. A fragment formed by the loss of the 2-chloropropenal unit was found in the spectrum of $\mathbf{M_{Cl}A}$ (*m*/*z* 266).

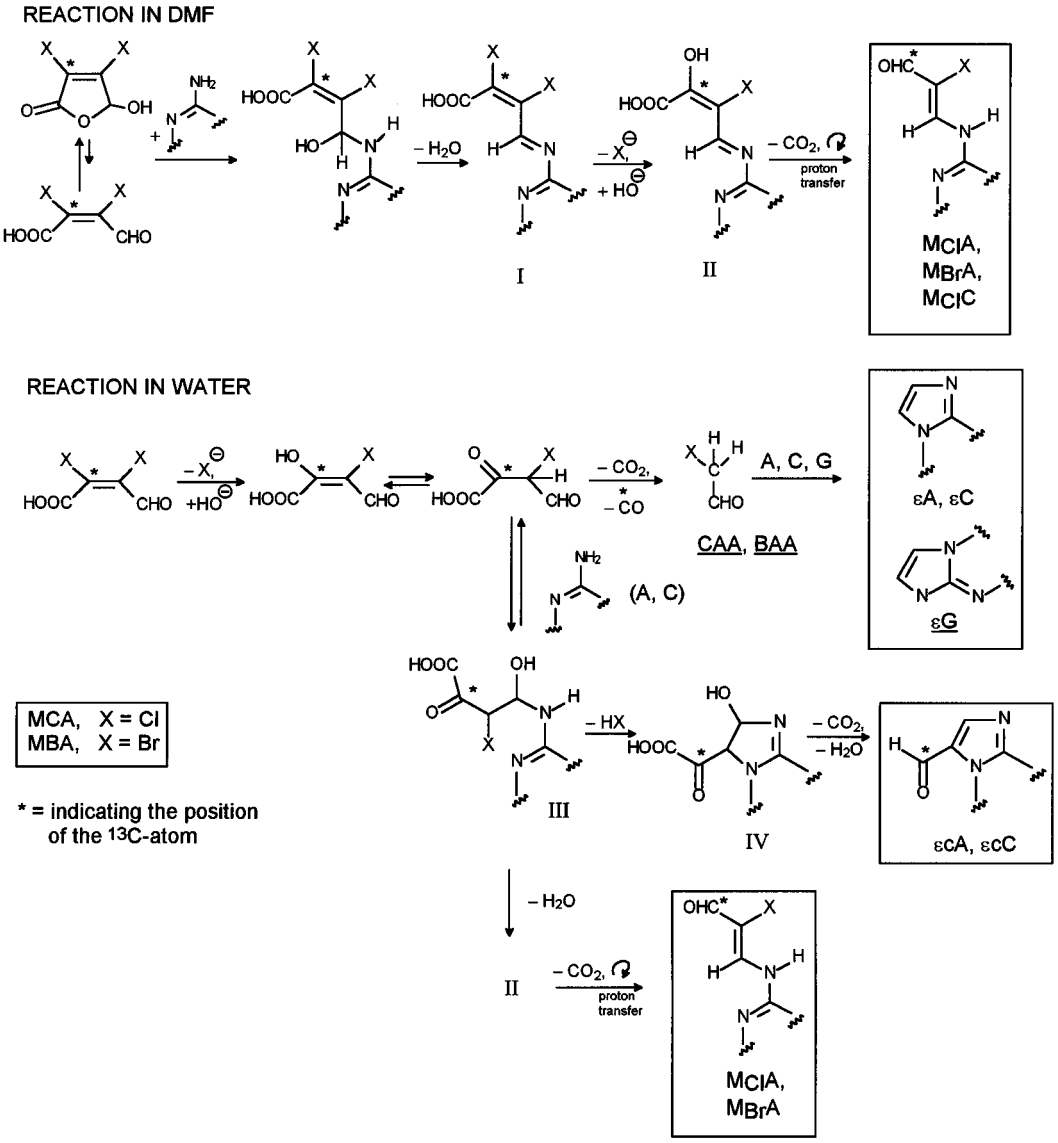
In the CI mass spectrum of $\mathbf{M_{Cl}C}$ the protonated molecular ion peaks were observed at *m*/*z* 332 and 334 at a relative intensity of 3 and 1 (Table 3). Also, weak fragment peaks were observed at *m*/*z* 314/316 and *m*/*z* 296 which corresponded to the loss of water and hydrochloride from the protonated molecular ion. The formation of the most abundant ions, *m*/z 182 and 184 (ratio 3:1), cannot be explained by simple cleavages of bonds in $\mathbf{M_{Cl}C}$. We propose that in the ion source of the mass spectrometer $\mathbf{M_{Cl}C}$ undergoes a cyclization and a new condensed pyrimidine ring is formed (Scheme 2). Subsequent loss of the hydroxyl group and the sugar unit from the pyrimidopyrimidone structure will yield a fragment with mass units of 182 and 184. In the $\mathbf{M_{Cl}C}$ mass spectrum, these ion peaks were the most abundant ones and the only peaks occurring at a relative intensity higher than 10%.

The NMR and the UV spectroscopic and the mass spectrometric data are consistent with the structures of $\mathbf{M_{Cl}A}$, $\mathbf{M_{Br}A}$, and $\mathbf{M_{Cl}C}$ presented in Chart 1.

In DMF, the yields of $\mathbf{M_{Cl}A}$, $\mathbf{M_{Br}A}$, and $\mathbf{M_{Cl}C}$ were 18.5%, 4.4%, and 7.0%, respectively. Following 5 days of reaction in aqueous solutions at pH 6.0 and 7.4, the yield of $\mathbf{M_{Cl}A}$ and $\mathbf{M_{Cl}C}$ was about 5×10^{-3}% at both pH conditions, while the yield of $\mathbf{M_{Br}A}$ was about 20×10^{-3}% at pH 6.0 and 5×10^{-3}% at pH 7.4.

For the purpose of determining the mechanism of formation of the halopropenal derivatives, we reacted ^{13}C-3 labeled MCA with adenosine and subsequently observed the labeling pattern of the isolated product by ^{13}C NMR spectroscopy. In the ^{13}C NMR spectrum of $\mathbf{M_{Cl}A}$, only the signal due to the aldehyde carbon of the

Scheme 3



chloropropenal unit (δ = 184.03 ppm) was significantly more intensive than those due to any other carbons. In the ^{1}H NMR spectrum the aldehyde proton signal (δ = 9.42 ppm) was found to be strongly split (J = 180.5 Hz) due to the attachment of the proton to the ^{13}C.

On the basis of these observations, we suggest that the mechanism for the formation of the halopropenal derivatives is as depicted in Scheme 3. It is known that MCA and MBA exist in two tautomeric forms, the ring form and the open chain form (Scheme 1) (*25*). In organic solvents, the compounds adopt primarily the ring form, but trace amounts of the open chain form may be present and the exposed aldehyde group should react readily with the exocyclic amino group of adenosine and cytidine. Initially, a carbinolamine will be formed which subsequently will be dehydrated and the Schiff base (**I**) will be obtained as an intermediate. Due to the extended resonance in **I**, the halogen α to the carboxyl group should be readily replaced by a hydroxyl group and the intermediate **II** would be formed. The halopropenal derivatives will be obtained following decarboxylation of **II** and conversion of the enol to the keto form.

We have also reacted ^{13}C-labeled MCA with adenosine in aqueous solutions, isolated the previously identified products, ϵA and ϵcA (*13, 14*), and observed the labeling pattern of the products by ^{13}C NMR spectroscopy. It was found that, in the spectrum of ϵcA, the only carbon labeled was the carbon in the formyl group. In the spectrum of ϵA, none of the carbon resonance peaks were markedly more intensive than the other, and thus the etheno bridge carbons were unlabeled. We have previously suggested that MCA is broken down to chloromalonaldehyde and that the ethenocarbaldehyde and etheno derivatives of nucleosides are formed by reaction of chloromalonaldehyde (*14*). If this would be the case, we would find two labeled carbons in ϵcA, namely, the carbon in the formyl group and the etheno carbon β to the formyl group, and in ϵA the carbon bound to the exocyclic nitrogen (N^6) would have been labeled (*26, 27*). Since these labeling patterns were not observed, we have to revise our previous proposal for the formation of the ethenocarbaldehyde and etheno derivatives.

It has been shown that the halogen β to the aldehyde group of the open form of mucohalic acids is easily replaced by a hydroxyl group and mucoxyhalic acids are formed (*28*). These acids may be further broken down to chloroacetaldehyde (CAA) and bromoacetaldehyde (BAA) through loss of the oxalo group with the labeled carbon atom (Scheme 3). The breakdown could proceed *via* the halogenated malonaldehydes which in turn could

be decarbonylated in the same way as malonaldehyde (*29*). Finally, the haloacetaldehydes react with nucleosides and form etheno derivatives according to known mechanisms (*26, 27*). The etheno derivatives are the main products of MCA and MBA reactions with adenosine, cytidine, and guanosine, and the only products identified hitherto in reactions with guanosine (*14*).

The ethenocarbaldehyde derivatives may be formed from the mucoxyhalic acids through attack on the aldehyde group by the exocyclic amino group of adenosine and cytidine. In the resulting carbinolamine **III** (Scheme 3), the halogen is displaced by a conjugate attack *via* the endocyclic nitrogen in adenine (N-1) and in cytosine (N-3), and a dihydroimidazole ring is formed (**IV**). Following dehydration and a decarboxylation, the ethenocarbaldehyde derivatives are obtained.

In water, the halopropenal derivatives are most likely formed from **III**. Dehydration of **III** yields the Shiff base **II** which subsequently produces $\mathbf{M_{Cl}A}$, $\mathbf{M_{Cl}C}$, and $\mathbf{M_{Br}A}$ (Scheme 3). The above presented reaction mechanisms explain the labeling patterns of the products observed by ^{13}C NMR spectroscopy. However, it has to be pointed out that further work is needed to finally establish the course of formation of nucleoside derivatives from mucohalic acids.

When bromomalonaldehyde was reacted with adenosine in DMF, ϵA and ϵcA, but not $\mathbf{M_{Br}A}$, were obtained. It is likely, that the first step in production of ϵcA is formation of a carbinolamine similar to **III** (Scheme 3), with the exception that the oxalo group is replaced by a formyl group. In the following reaction step, the carbinolamine undergoes an intramolecular displacement of bromine, and in the final step, the dihydroimidazole ring loses water and produces ϵcA. If the carbinolamine would have lost water immediately after its formation, $\mathbf{M_{Br}A}$ would have been obtained. The formation of ϵA could be due to production of bromoacetaldehyde by bond cleavage in the carbinolamine (*30*) or by direct breakdown of bromomalonaldehyde to bromoacetaldehyde (*29*).

The compounds $\mathbf{M_{Cl}A}$, $\mathbf{M_{Cl}C}$, and $\mathbf{M_{Br}A}$ represent chlorinated and brominated derivatives of the propenal adducts previously shown to be formed in reactions of malonaldehyde and adenosine (M_1A) and cytidine (M_1C) (Chart 1) (*17*−*19*). However, the mechanism for the formation of the halopropenal and propenal derivatives is not the same; the propenal derivatives are formed from malonaldehyde while the halogenated propenal derivatives are formed from mucoxyhalic acids.

The halogenated propenal derivatives are formed in detectable amounts in water at pH 7.4 and 37 °C. It is not known whether they are formed in reaction with DNA, but if they are formed, some conclusions can be drawn on their impact on MCA mutation from the mutational specificities of MCA in *S. typhimurium* (*31*). The main mutational events of MCA were reported to be C→T transitions. These transitions were explained as a consequence of formation of ethenoguanosine and ethenocytidine adducts. However, it cannot be excluded that also $\mathbf{M_{Cl}C}$ could account for at least a part of the C→T transitions. If this is the case, it seems likely that also $\mathbf{M_{Cl}A}$ would be a premutagenic lesion which would cause A→G transitions. However, Knasmüller et al. (*31*) could not find A→G transitions in the MCA mutation spectrum. It might be difficult to explain why only one of the structurally related halopropenal adducts ($\mathbf{M_{Cl}C}$) would be formed in DNA and would induce transition and not the other one ($\mathbf{M_{Cl}A}$). Thus, the conclusion is that it is unlikely that the halopropenal adducts are of importance for the mutagenicity of MCA. Recently, Marnett (*32*) reported that malonaldehyde induced mainly three types of base-pair substitutions (in the *lacZ*α gene of a recombinant bacteriophage M13), namely, G→T, C→T, and A→G. Marnett suggested that the C→T and A→G transitions could be due to formation of M_1C and M_1A, respectively, and further that these adducts could be very efficient premutagenic lesions. These contradictory ideas about the role of the propenal adducts on the mutational specificity of MCA and malonaldehyde may be explained by the fact that MCA has the potential while malonaldehyde lacks the potential to produce etheno adducts. Also, the extent at which propenal adducts are formed in DNA should have an impact on their role as mediators of mutagenicity. At present, nothing is known about DNA adducts of mucohalic acids, and as far as we know, propenal adducts have not been identified in DNA reacted with malonaldehyde.

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