Identification of Fluorescent 2′-Deoxyadenosine Adducts Formed in Reactions of Conjugates of Malonaldehyde and Acetaldehyde, and of Malonaldehyde and Formaldehyde

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2′-Deoxyadenosine was reacted with malonaldehyde in the presence of formaldehyde or acetaldehyde. The reactions were carried out at 37 °C in aqueous solution at acidic conditions. The reaction mixtures were analyzed by HPLC. In both reactions, two major products were formed. The reaction products were isolated and purified by C18 chromatography, and their structures were characterized by UV absorbance, fluorescence emission, ^{1}H and ^{13}C NMR spectroscopy, and mass spectrometry. The reaction products isolated from the mixture containing formaldehyde, malonaldehyde, and deoxyadenosine were identified as 3-(2′-deoxy-β-D-ribofuranosyl)-7*H*-8-formyl[2,1-*i*]pyrimidopurine (M_1FA-dA) and 9-(2′-deoxy-β-D-ribofuranosyl)-6-(3,5-diformyl-1,4-dihydro-1-pyridyl)purine (M_2FA-dA). In the reaction mixture consisting of acetaldehyde, malonaldehyde, and deoxyadenosine, the identities of the products were determined to be 3-(2′-deoxy-β-D-ribofuranosyl)-7-methyl-8-formyl[2,1-*i*]pyrimidopurine (M_1AA-dA) and 9-(2′-deoxy-β-D-ribofuranosyl)-6-(3,5-diformyl-4-methyl-1,4-dihydro-1-pyridyl)purine (M_2AA-dA). The yields of the compounds were 1.8 and 0.7% for M_1FA-dA and M_2FA-dA, respectively, and 6.8 and 10% for M_1AA-dA and M_2AA-dA, respectively. All compounds exhibited marked fluorescent properties. These findings show that in addition to direct reaction of a specific aldehyde with 2′-deoxyadenosine, aldehyde conjugates also may react with the base. Although three of the adducts (M_1FA-dA, M_2FA-dA, and M_1AA-dA) could not be detected in reactions carried out under neutral conditions, the possibility that trace amounts of the adducts may be formed under physiological conditions cannot be ruled out. Therefore, conjugate adducts must be considered in work that aims at clarifying the mechanism of aldehyde genotoxicity.

Introduction

Aldehydes are ubiquitously distributed in the human environment. Malonaldehyde is present as a lipid metabolite in human and animal tissues (*1*, *2*) and can occur at high levels in rancid foods. Formaldehyde is a potent irritating and sensitizing compound widely used in industry and can be released from several manufactured items such as resins, building materials, paints, disinfectants, carpets, or personal care products (*3*, *4*). Acetaldehyde is used in the manufacturing of acetic acid, perfumes, and flavors, but the main human exposure to acetaldehyde is through alcohol consumption. Malonaldehyde is mutagenic in bacteria and mammalian cells (*5*−*7*). Formaldehyde and acetaldehyde induce genotoxic effects in various biological assays (*8*−*21*).

Malonaldehyde has been shown to be carcinogenic in rats (*22*), and consequently, the compound is classified as possibly carcinogenic to animals (group 3) by the International Agency for Research on Cancer (*23*). Formaldehyde was listed in group 2A, i.e., carcinogenic to animals and probably carcinogenic to humans (*24*). Acetaldehyde was classified in group 2B, i.e., carcinogenic to animals and possibly carcinogenic to humans (*23*). Chemical mutagens and carcinogens generally react with DNA base units and form stable adducts. The formation of exocyclic adducts in DNA is, along with the formation of DNA−protein cross-links, one of the most probable early types of damage in the carcinogenicity process. Aldehydes such as malonaldehyde, acrolein, or crotonaldehyde are known to form stable DNA adducts (*25*, *30*). Formaldehyde was shown to produce stable adducts in reaction with guanosine, adenosine, or cytidine (*31*, *32*). In reactions with nucleosides, acetaldehyde was shown to produce unstable Schiff bases that can be stabilized by reduction (*33*, *34*). At present, the early events leading to the carcinogenicity of malonaldehyde, formaldehyde, and acetaldehyde in animals are not clearly identified.

Recently, it was reported that acetaldehyde forms a stable fluorescent protein adduct in the liver of ethanol-fed rats (*35*). The adduct was produced through the initial condensation of one molecule of acetaldehyde with two molecules of malonaldehyde. We have previously shown that this malonaldehyde−aldehyde conjugate also reacts with deoxyadenosine, forming a strongly fluorescent dihydropyridine adduct (*36*).

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In the current work, we have reacted 2′-deoxyadenosine with malonaldehyde in the presence of acetaldehyde and in the presence of formaldehyde. The aim of the present study is twofold: to find out whether the malonaldehyde−acetaldehyde condensation pattern is observed when acetaldehyde is replaced with formaldehyde and to determine whether other products are formed following other condensation patterns.

Materials and Methods

Chemicals. Malonaldehyde was prepared by acid hydrolysis of 1,1,3,3-tetraethoxypropane (TEP) in 0.1 mL of HCl for 30 min at 40 °C. 2′-Deoxyadenosine and 1,1,3,3-tetraethoxypropane (97% pure) were purchased from Sigma Chemical Co. (St. Louis, MO). Formaldehyde (37% solution stabilized with 10−15% methanol) and acetaldehyde (>99% pure) were obtained from J. T. Baker B. V. (Deventer, Holland). The water was Aqua Sterilisata from Pharmacia AB (Stockholm, Sweden). Acetonitrile was gradient grade for chromatography from Merck (Darmstadt, Germany). 1,1,1-Trichloroethane was obtained from Fluka Chemie AG (Buchs, Switzerland).

Chromatographic Methods. HPLC analyses were performed on a Kontron Instruments liquid chromatographic system consisting of a model 322 pump, a 440 diode array detector (UV), a Jasco FP-920 fluorescence detector, and the 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 (Hypersil BDS-C18, Hewlett-Packard, Espoo/Esbo, Finland). The column was eluted isocratically for 5 min with 0.01 M phosphate buffer (pH 7.1) and then with a gradient from 0 to 30% acetonitrile over the course of 25 min at a flow rate of 1 mL/min. Preparative isolation of the products was performed 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 products 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 (Shimadzu Europe).

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 (JEOL). 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 determinations of the shifts and the coupling constants of the multiplets of the proton signals in the deoxyribose unit 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 isolated compound was introduced by loop injection at a flow rate of 20 μL/min (80/20/1 H_2O/CH_3CN/acetic acid mixture). PEG[1] 200 was used as the standard for the exact mass determination. The mass spectrometer was working at a resolution of 7000.

The UV spectra of the isolated compounds were recorded with the diode array detector as the peak eluted from the HPLC column. A Shimadzu UV-160 spectrophotometer (Shimadzu Europa) was used to determine the molar extinction coefficient (ϵ). The fluorescence spectra were recorded with a Hitachi F-2000 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan).

Preparation of 3-(2′-Deoxy-β-D-ribofuranosyl)-7*H*-8-formyl[2,1-*i*]pyrimidopurine (M_1FA-dA) and 9-(2′-Deoxy-β-D-ribofuranosyl)-6-(3,5-diformyl-1,4-dihydro-1-pyridyl)-purine (M_2FA-dA). Malonaldehyde hydrolyzed from TEP (4 g, 18,2 mmol) and formaldehyde (2.04 g, 68 mmol) were reacted with 2′-deoxyadenosine (1 g, 4 mmol) in 250 mL of 0.5 M phosphate buffer solutions at pH 3.5. The reaction was performed at 37 °C. M_1FA-dA was isolated from a reaction stopped after 24 h, and M_2FA-dA was isolated from a reaction stopped after 3 days. The progress of the reaction was followed by HPLC analyses on the C18 analytical column. After the reactions were stopped, the reaction mixtures were filtered and finally passed through the preparative C18 column. The column was eluted with 200 mL of water and then with 100 mL of 5, 10, 15, 20, 25, 30, and 35% acetonitrile solutions in water. Fractions (30 mL) were collected. The M_1FA-dA eluted from the column with the 5 to 15% acetonitrile washes, and the M_2FA-dA eluted with the 25% wash. The fractions containing each product were combined and concentrated by rotary evaporation to about 20 mL. Both compounds were further purified by using the semipreparative HPLC column with a flow rate of 4 mL/min; for M_1FA-dA the column was eluted with a gradient from 10 to 30% acetonitrile in 0.01 M phosphate buffer (pH 7.1) over the course of 9 min and for M_2FA-dA with a gradient from 20 to 40% acetonitrile in water over the course of 28 min. The collected M_1FA-dA fractions were desalted by use of the preparative C18 column. The solutions containing the pure compounds were then rotary evaporated to dryness, and the residues (a yellow-green powder for M_1FA-dA and a slightly yellow powder for M_2FA-dA) were subjected to spectroscopic and spectrometric studies. The isolated amount of compound was 6 mg for M_1FA-dA and 2 mg for M_2FA-dA.

The isolated compounds had the following spectral characteristics: UV spectrum for M_1FA-dA, UV_{max} 212, 240, 408 nm (ϵ = 23 300 M^{-1} cm^{-1}), UV_{min} 284 nm (HPLC eluent, 10% acetonitrile in 0.01 M phosphate buffer at pH 7.1); UV spectrum for M_2FA-dA, UV_{max} 320 nm (ϵ = 20 200 M^{-1} cm^{-1}), 224 nm, UV_{min} 273 nm (HPLC eluent, 23% acetonitrile in 0.01 M phosphate buffer at pH 7.1); fluorescence spectrum (H_2O) for M_1FA-dA, $\lambda_{ex,max}$ 379 nm, $\lambda_{em,max}$ 462 nm; fluorescence spectrum (H_2O) for M_2FA-dA, $\lambda_{ex,max}$ 320 nm, $\lambda_{em,max}$ 449 nm.

In the positive ion electrospray mass spectra, the following ions were observed (*m*/*z*, relative abundance, formation): M_1FA-dA 318 (35, MH^+), 202 (100, MH^+ − deoxyribosyl + H), high-resolution mass spectrometry gave the protonated molecular formula as $C_{14}H_{16}N_5O_4$ (MH^+ 318.1179, calcd 318.1202); M_2FA-dA 372 (82%, MH^+), 256 (76%, MH^+ − deoxyribosyl + H^+), high-resolution mass spectrometry gave the protonated molecular formula as $C_{17}H_{18}N_5O_5$ (MH^+ 372.1290, calcd 372.1307).

The ^{1}H NMR spectroscopic data of M_1FA-dA were as follows: δ 9.28 (t, 1 H, *CH*O, J = 0.4 Hz), 8.49 (m, 1 H, H-2), 8.30 (m, 1 H, H-5), 7.50 (t, 1 H, H-9, J = 0.6 Hz), 5.00, 5.01 (m, 2 H, H-7a, H-7b, J = 14.4 Hz), 6.32 (dd, 1 H, H-1′, J = 7.0, 6.5 Hz), 4.40 (ddd, 1 H, H-3′, J = 5.9, 3.7, 3.1 Hz), 3.87 (dt, 1 H, H-4′, J = 4.6, 3.1 Hz), 3.59 (dd, 1 H, H-5′, J = 11.8, 4.6 Hz), 3.52 (dd, 1 H, H-5″, J = 11.8, 4.6 Hz), 2.64 (ddd, 1 H, H-2′, J = 13.3, 7.2, 6.1 Hz), 2.33 (ddd, 1 H, H-2″, J = 13.3, 6.3, 3.5 Hz). The NMR data for M_1FA-dA were described in detail in the work of Munter et al. (*37*).

The ^{1}H and ^{13}C NMR spectroscopic data of M_2FA-dA are presented in Table 1.

[1] Abbreviations: M_1FA-dA, 3-(2′-deoxy-β-D-ribofuranosyl)-7*H*-8-formyl[2,1-*i*]pyrimidopurines; M_1AA-dA, 3-(2′-deoxy-β-D-ribofuranosyl)-7-methyl-8-formyl[2,1-*i*]pyrimidopurine; M_2FA-dA, 9-(2′-deoxyribosyl)-6-(3,5-diformyl-1,4-dihydro-1-pyridyl)purine; M_2AA-dA, 9-(2′-deoxyribosyl)-6-(3,5-diformyl-4-methyl-1,4-dihydro-1-pyridyl)purine; COLOC, C−H shift correlation NMR spectroscopy via long-range coupling; COSY, correlation spectroscopy (H−H); CHSHF, C−H shift correlation NMR spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; PEG, poly(ethylene glycol).

Table 1. ^{1}H and ^{13}C Chemical Shifts (δ),[a] Spin−Spin Coupling Constants, and $J_{H,H}$ Values of Protons in M_2FA-dA

proton	δ (ppm)	multiplicity	$J_{H,H}$ (Hz)	carbon	δ (ppm)
1,4-dihydropyridine unit					
H-2/H-6 (2H)	9.10	s		C-2/C-6	141.8
				C-3/C-5	121.2
C*H*O (2H)	9.63	s		*C*HO	190.9
H-4 (2H)	3.03	br		C-4	17.3
purine unit					
H-2p (1H)	8.78	s		C-2p	151.2
				C-4p	152.4
				C-5p	121.4
				C-6p	146.2
H-8p (1H)	8.89	s		C-8p	143.5
2′-deoxyribosyl unit					
H-1′ (1H)	6.52	t	6.6	C-1′	84.0
H-2′ (1H)	2.76	dt	13.4, 6.3	C-2′	39.5
H-2″ (1H)	2.40	ddd	13.4, 6.3, 3.7		
H-3′ (1H)	4.46	m		C-3′	70.4
H-4′ (1H)	3.92	dt	4.3, 3.7	C-4′	88.1
H-5′ (1H)	3.64	m		C-5′	61.3
H-5″ (1H)	3.56	m			
O*H* (1H)	5.38	br			
O*H* (1H)	5.01	br			

[a] Relative to TMS.

Preparation of 3-(2′-Deoxy-β-D-ribofuranosyl)-7-methyl-8-formyl[2,1-*i*]pyrimidopurine (M_1AA-dA) and 9-(2′-Deoxy-β-D-ribofuranosyl)-6-(3,5-diformyl-4-methyl-1,4-dihydro-1-pyridyl)purine (M_2AA-dA). Malonaldehyde hydrolyzed from TEP (4 g, 18,2 mmol) and acetaldehyde (0.8 g, 18.2 mmol) were reacted with 2′-deoxyadenosine (1 g, 4 mmol) in 250 mL of 0.5 M phosphate buffer solutions at pH 4.6. The reaction was carried out at 37 °C, and the progress of the reaction was followed by HPLC analyses on the C18 analytical column. After 7 days, the reaction was stopped and the reaction mixture was filtered and finally passed through the preparative C18 column. The column was eluted with 200 mL of water and then with 100 mL of 5, 10, 15, 20, 25, 30, and 35% acetonitrile solutions in water. Fractions (30 mL) were collected. The M_1AA-dA eluted from the column with the 10 and 15% acetonitrile washes, and the M_2-AA-dA eluted with the 25 and 30% washes. The fractions containing the product were combined and concentrated by rotary evaporation to about 20 mL. Further purification was carried out by using the semipreparative HPLC column as follows: for M_1AA-dA, the column was eluted with a gradient from 11 to 35% acetonitrile in 0.01 M phosphate buffer (pH 7.1) over the course of 10 min at a flow rate of 4 mL/min; for M_2-AA-dA, the elution gradient was from 17 to 30% acetonitrile in 0.01 M phosphate buffer at pH 7.1 over the course of 15 min at a flow rate of 4 mL/min. The fractions containing each pure compound were combined and then desalted by use of the preparative C18 column. The desalted solutions were rotary evaporated to dryness. The residues (a yellow-green powder for M_1AA-dA and a slightly yellow powder for M_2AA-dA) were subjected to spectroscopic and spectrometric studies. The isolated amounts of M_1AA-dA and M_2AA-dA were 37 and 52 mg, respectively.

The isolated compounds had the following spectral characteristics: UV spectrum for M_1AA-dA, UV_{max} 208, 240, 400 nm (ϵ = 20 900 M^{-1} cm^{-1}), UV_{min} 232, 288 nm (HPLC eluent, 10% acetonitrile in 0.01 M phosphate buffer at pH 7.1); UV spectrum for M_2AA-dA, UV_{max} 320 nm (ϵ = 46 500 M^{-1} cm^{-1}), 224 nm, UV_{min} 272, 208 nm, shoulder between 348 and 378 nm (HPLC eluent, 25% acetonitrile in 0.01 M phosphate buffer at pH 7.1); fluorescence spectrum (H_2O) for M_1AA-dA, $\lambda_{ex,max}$ 408 nm, $\lambda_{em,max}$ 456 nm; and fluorescence spectrum (H_2O) for M_2AA-dA $\lambda_{ex,max}$ 319 nm, $\lambda_{em,max}$ 444 nm.

In the positive ion electrospray mass spectra, the following ions were observed (*m*/*z*, relative abundance, formation): M_1-AA-dA 332 (47, MH^+), 216 (100, MH^+ − deoxyribosyl + H), high-resolution mass spectrometry gave the protonated molecular formula as $C_{15}H_{18}N_5O_4$ (MH^+ 332.1326, calcd 332.1359); M_2AA-dA 386 (100, MH^+), 270 (54, MH^+ − deoxyribosyl + H^+), high-resolution mass spectrometry gave the protonated molecular formula as $C_{18}H_{20}N_5O_5$ (MH^+ 386.1460, calcd 386.1464).

The ^{1}H NMR spectroscopic data of M_2AA-dA were as follows: δ 9.59 (s, 2 H, *CH*O), 9.06 (s, 2 H, H-2/H-6), 8.89 (s, 1 H, H-8p), 8.78 (s, 1 H, H-2p), 3.70 (q, 1 H, H-4, J = 6.7 Hz), 1.08 (d, 3 H, C*H*$_3$, J = 6.7 Hz), 6.51 (t, 1 H, H-1′, J = 6.5 Hz), 4.46 (dt, 1 H, H-3′, J = 6.5, 4.0 Hz), 3.92 (dt, 1 H, H-4′, J = 4.5, 4.0 Hz), 3.64 (dd, 1 H, H-5′, J = 11.5, 4.5 Hz), 3.56 (dd, 1 H, H-5″, J = 11.5, 4.5 Hz), 2.75 (dt, 1 H, H-2′, J = 13.5, 6.5 Hz), 2.40 (ddd, 1 H, H-2″, J = 13.5, 6.5, 4.0 Hz), 5.37 (br, 1 H, O*H*), 5.02 (br, 1 H, O*H*). The detailed NMR data for M_2AA-dA were reported in a previous study (*36*).

The ^{1}H and ^{13}C NMR spectroscopic data of M_1AA-dA are presented in Table 2.

Small-Scale Reactions. 2′-Deoxyadenosine (10 mg, 0.04 mmol) and malonaldehyde hydrolyzed from TEP (40 mg, 0.18 mmol) were mixed with formaldehyde (20 mg, 0.67 mmol) or with acetaldehyde (8 mg, 0.18 mmol) in 3 mL of 0.5 M phosphate buffer at pH 3.5 (with formaldehyde) or 4.6 (with acetaldehyde). The appropriate aldehyde (formaldehyde or acetaldehyde) was added to malonaldehyde, and this mixture was then added to the solution of 2′-deoxyadenosine. The reactions were performed at 37 °C, and the progress of the reaction was followed daily by HPLC analyses of aliquots of the reaction mixtures.

Table 2. ^{1}H and^{13}C Chemical Shifts (δ),[a] Spin−Spin Coupling Constants, and $J_{H,H}$ Values of Protons in M_1AA-dA

proton	δ	multiplicity	$J_{H,H}$ (Hz)	carbon[b]	δ	multiplicity	$^1J_{C,H}$ (Hz)	$^{>1}J_{C,H}$ (Hz)
H-2 (1H)	8.50	s		C-2	141.5	dd	215.8	4.1
				C-3a	145.9	dddd		13.3, 7.7, 5.0, 2.1
H-5 (1H)	8.54	s		C-5	147.3	dd	211.7	3.2
H-7 (1H)	5.54	dq	6.4, 1.7	C-7	50.9	dm	148.0	
				C-8	119.1	dm		24.3
H-9 (1H)	7.53	d	0.8	C-9	154.6	dtd	176.9	3.4, 1.4
				C-10a	147.2	m		
				C-10b	124.0	dd		11.5, 4.6
C*H*O (1H)	9.27	s		*C*HO	187.4	dddd	171.8	5.5, 5.3, 1.9
C*H*$_3$ (3H)	1.30	dd	6.5, 2.8	*C*H$_3$	23.8	qd	128.5	2.3
2′-deoxyribosyl unit								
H-1′ (1H)	6.32	dt	6.6, 1.6	C-1′	83.7	d	166.8	
H-2′ (1H)	2.65	ddd	6.8, 6.5, 6.3	C-2′	39.7	t	134.3	
H-2″ (1H)	2.33							
H-3′ (1H)	4.40	ddd	3.3, 3.1, 2.7	C-3′	70.5	d	148.4	
H-4′ (1H)	3.87	m		C-4′	88.0	d	148.0	
H-5′ (1H)	3.60	m		C-5′	61.4	td	140.2	
OH	4.98	br						
OH	5.34	br						

[a] Relative to TMS. [b] The signals of the sugar carbons were further split into complex multiplets.

Determination of Product Yields. Quantitative ^{1}H NMR analysis, using 1,1,1-trichloroethane as an internal standard, was performed on an aliquot of M_2FA-dA and M_1AA-dA. Then a HPLC standard solution was prepared by taking an exact volume of the NMR sample and diluting it with an appropriate volume of water. The quantitative determination of the amount of M_2FA-dA and M_1AA-dA in the reaction mixtures was made by comparing the chromatographic peak area (recorded at the wavelength corresponding to the maximum UV absorbance) of the compound in the HPLC standard solution with the peak area of the compound in the reaction mixtures. The molar yield was calculated from the original amount of 2′-deoxyadenosine in the reaction mixtures. Quantitative NMR was not performed for M_1FA-dA and M_2AA-dA because their extinction coefficients were determined in previous studies (refs *37* and *36*, respectively).

Results and Discussion

HPLC analyses of the reaction mixture that contained formaldehyde, malonaldehyde, and 2′-deoxyadenosine (formaldehyde reaction) showed the formation of three major product peaks. The product peaks are marked M_1-FA-Ade, M_1FA-dA, and M_2FA-dA, respectively, in Figure 1A. Also in the HPLC chromatogram of the reaction mixture containing acetaldehyde, malonaldehyde, and 2′-deoxyadenosine (acetaldehyde reaction), three major product peaks were observed. These product peaks are marked M_1AA-Ade, M_1AA-dA, and M_2AA-dA, respectively, in Figure 1B. Large-scale reactions were performed to isolate sufficient amounts of the products for structural determination by spectrometric and spectroscopic methods.

On the basis of UV, fluorescence and NMR spectroscopy, and electrospray mass spectrometry, the products of the formaldehyde reaction were identified as 8-formyl-[2,1-*i*]-7*H*-pyrimidopurine (M_1FA-Ade), 3-(2′-deoxy-β-D-ribofuranosyl)-7*H*-8-formyl[2,1-*i*]pyrimidopurine (M_1FA-dA), and 9-(2′-deoxyribosyl)-6-(3,5-diformyl-1,4-dihydro-1-pyridyl)purine (M_2FA-dA).

The compound M_1FA-Ade exhibited UV and fluorescence spectra identical to those of the compound M_1FA-dA. In the ESI mass spectrum, the compound generated a molecular ion at 202 *m*/*z*, corresponding to the aglycone of M_1FA-dA. Further, when a solution of M_1FA-Ade and hydrolyzed M_1FA-dA (30 min at 40 °C in 0.1 M HCl) was analyzed by HPLC, the only peak that was detected was the one representing M_1FA-Ade.

The UV, fluorescence, ^{1}H NMR, and mass spectra of M_1FA-dA were identical to those recorded for the compound previously reported to be formed in reaction of the hydroxyfuranones 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) or 3-chloro-4-(chloromethyl)-5-hydroxy-2(5*H*)-furanone (CMCF), and named pfA-dR (*37*, *38*). Thus, the compound M_1FA-dA is identical to the compound formed by the hydroxyfuranones. However, the adduct is not produced in the formaldehyde reaction by the same mechanism as in the reaction of the hydroxyfuranones (see the later discussion).

The compound M_2FA-dA exhibited a UV absorption maximum at 320 nm (Figure 2) and displayed a fluorescence spectrum with an emission maximum at 449 nm (Figure 3). These features are very similar to those of the compound M_2AA-dA formed in the acetaldehyde reaction and characterized previously (*36*). The ^{1}H NMR spectrum of M_2FA-dA displayed, besides the signals from the protons of the deoxyribose moiety, one-proton singlets

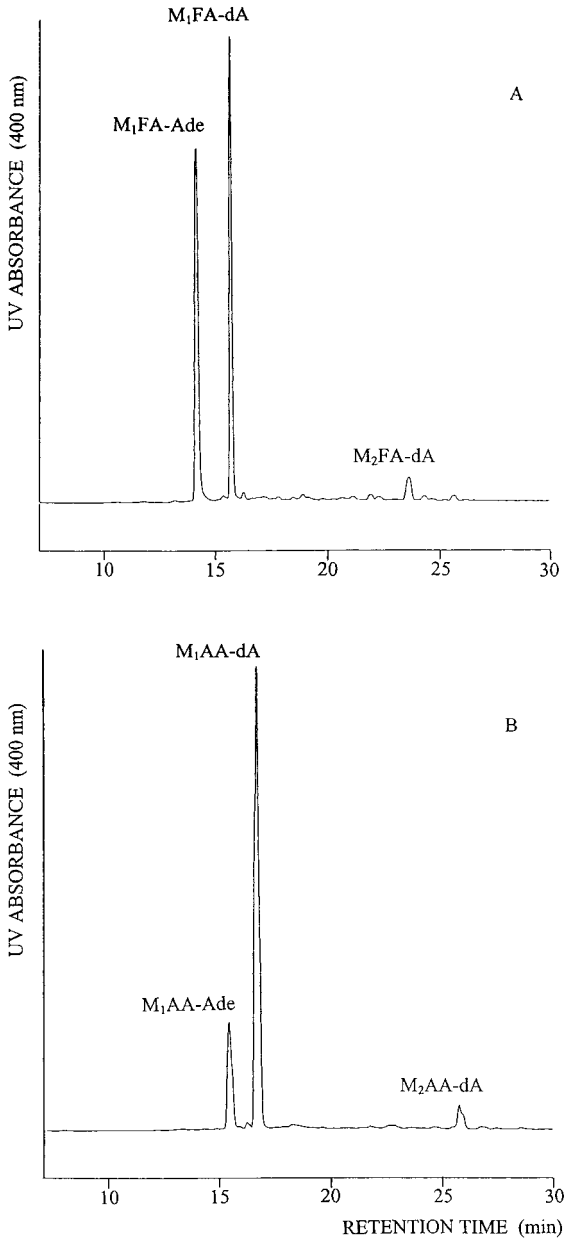


Figure 1. C18 analytical column HPLC chromatogram of the reaction mixture of 2′-deoxyadenosine with malonaldehyde and formaldehyde (pH 3.5, 3 days) (A) or acetaldehyde (B) (pH 4.5, 7 days) held at 37 °C. For analysis conditions, see Materials and Methods.

at δ 8.89 and 8.78 ppm and two-proton singlets at δ 9.63, 9.10, and 3.03 ppm (Table 1). This set of signals is almost identical to the ones observed in the ^{1}H NMR spectrum of M_2AA-dA (*36*). The only marked differences are that M_2FA-dA displays no signal at δ 1.08 ppm (signal which had been assigned to the methyl protons in M_2AA-dA; see Scheme 1) and that the signal at δ 3.03 ppm is now a two-proton singlet (whereas the corresponding signal was a one-proton quartet in M_2AA-dA). In analogy with the NMR data of M_2AA-dA, the signals at δ 8.89 and 8.78 ppm were assigned to H-8p and H-2p, respectively, and the signals at δ 9.63 and 9.10 ppm were attributed to the aldehyde proton and H-2/H-6 (equivalent protons), respectively.

The ^{13}C NMR spectrum of M_2FA-dA showed, besides the signals from the purine and the deoxyribose moieties, carbon signals at δ 190.9, 141.8, 121.2, and 17.3 ppm

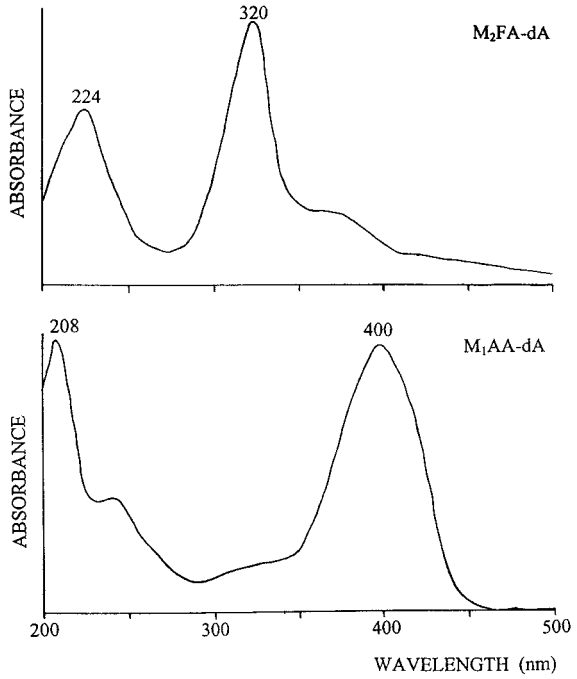


Figure 2. UV absorbance spectra of M_2FA-dA and M_1AA-dA. The UV spectra were recorded with the diode array detector as the compounds eluted from the column.

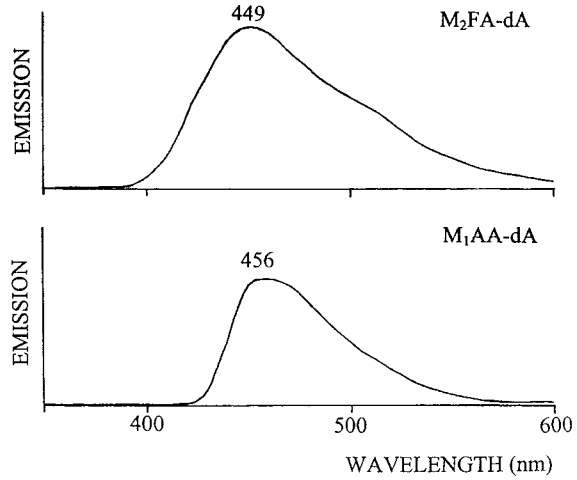
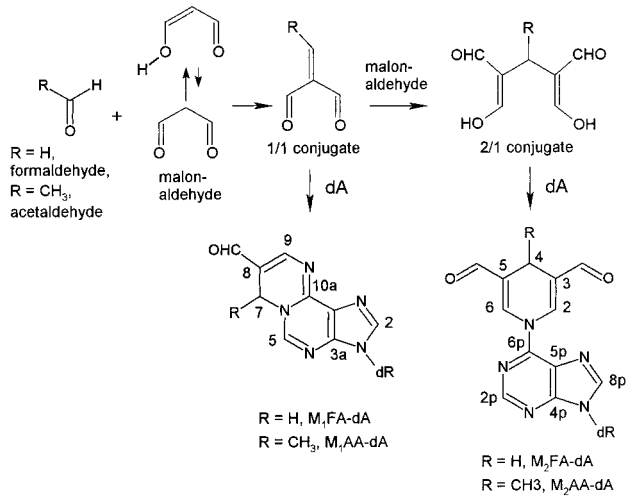


Figure 3. Fluorescence emission spectra of M_2FA-dA (λ_{ex} = 320 nm) and M_1AA-dA (λ_{ex} = 408 nm).

(Table 1). In analogy with the NMR spectra published for M_2AA-dA (*36*), the signal at δ 190.9 ppm was assigned to the formyl carbons and the signals at δ 141.8 and 121.2 ppm were assigned to C-2/C-6 and C-3/C-5, respectively. The signal at δ 17.3 ppm was assigned to the methylene carbon, C-4. The NMR, UV and fluorescence spectroscopic, and the mass spectrometric data were consistent with the structure of M_2FA-dA presented in Scheme 1.

In the acetaldehyde reaction, the products were identified as 8-formyl-7-methyl[2,1-*i*]pyrimidopurine (M_1AA-Ade), 3-(2′-deoxy-β-D-ribofuranosyl)-7-methyl-8-formyl-[2,1-*i*]pyrimidopurine (M_1AA-dA), and 9-(2′-deoxyribosyl)-6-(3,5-diformyl-4-methyl-1,4-dihydro-1-pyridyl)purine (M_2AA-dA). The compounds M_1AA-Ade and M_1AA-dA exhibited identical UV and fluorescence spectra. The mass unit difference of the molecular ions was 116 units, which corresponds to the loss of the deoxyribose unit. Finally, it was found that hydrolysis of M_1AA-dA yielded a compound with exactly the same spectroscopic, spectrometric, and chromatographic characteristics as M_1AA-Ade. Collectively, these findings show that M_1AA-Ade represents the aglycone of M_1AA-dA.

Scheme 1



The compound M_1AA-dA and the compound M_1FA-dA from the formaldehyde reaction exhibited very similar UV spectra. The UV absorption maximum of M_1AA-dA was found at 400 nm (Figure 2), a slightly shorter wavelength (8 nm) than that for M_1FA-dA (*37*, *38*). The fluorescence spectrum of M_1AA-dA exhibited an emission maximum at 456 nm (Figure 3), while the emission maximum was 462 nm for M_1FA-dA (*37*, *38*). The ^{1}H NMR spectrum of M_1AA-dA exhibited, besides the signals from the protons of the deoxyribose moiety, one-proton signals at δ 9.27, 8.54, 8.50, 7.53, and 5.54 ppm and a three-proton signal at δ 1.30 ppm (Table 2). The main difference in the resonance signals of M_1FA-dA and M_1-AA-dA is that M_1FA-dA displayed a two-proton signal at δ 5.005 ppm, whereas M_1AA-dA exhibited a one-proton signal at δ 5.54 ppm and a three-proton signal at δ 1.30 ppm. The signal at δ 1.30 ppm was assigned to three methyl protons on the basis of the chemical shift and of the C−H connectivity to the carbon signal at δ 23.8 ppm. The signal at δ 5.54 ppm was attributed to the methine proton, H-7 (Scheme 1). The proton signal appeared as a quartet and showed a H−H (COSY) correlation with the methyl protons at δ 1.30 ppm. The assignment of the signal at δ 8.54 ppm to H-5 was done on the basis of the chemical shift and the NOE interaction between H-7 and H-5 (two-dimensional NOESY experiment). On the basis of chemical shifts and in analogy with the ^{1}H NMR data of M_1FA-dA, we assigned the signal at δ 7.53 ppm to H-9. The signal at δ 9.27 ppm was attributed to the aldehyde proton on the basis of the downfield chemical shift and the one-bond C−H shift correlation with the carbon signal at δ 187.4 ppm. A two-dimensional NOESY experiment confirmed NOE interactions between the aldehyde proton and H-9 and between the methyl protons and H-5. The signal at δ 8.50 ppm was then assigned to H-2, and it exhibited NOE interactions with protons H-1′, H-2′, and H-3′ in the deoxyribose moiety. The signal H-9 appeared as a doublet because of the coupling (J = 0.8 Hz) to the aldehyde proton (this coupling is also observed in the H−H correlation spectrum), and H-7 was split into a doublet and a quartet due to the couplings to H-5 and to the methyl protons, respectively. The methyl protons gave rise to a doublet of doublets because of their coupling to H-7 and H-5.

The ^{13}C NMR spectrum of M_1AA-dA exhibited, besides the signals from the purine and the deoxyribose moieties, carbon signals at δ 187.4, 154.6, 147.3, 147.2, 145.9, 141.5, 124.0, 119.1, 50.9, and 23.8 ppm (Table 2). This set of signals is quite similar to the ^{13}C NMR data from M_1FA-dA except that it displays one additional carbon. The additional carbon signal, observed at δ 23.8 ppm, was attributed to the methyl carbon on the basis of the upfield chemical shift and the C−H correlation with the methyl protons (CHSHF experiments). The CHSHF correlation data allowed the assignment of the signals at δ 154.6, 147.3, and 141.5 ppm to the three olefinic methine carbons (C-9, C-5, and C-2, respectively). The signal at δ 50.9 ppm was attributed to the methine carbon C-7, and the signal at δ 187.4 ppm was assigned to the aldehyde carbon. The signal at δ 147.2 ppm was attributed to C-10a on the basis of chemical shifts and of C−H connectivities (COLOC) with the signals of H-9 and H-5. The assignment of the signal at δ 145.9 ppm to C-3a was based on the chemical shift and on the C−H correlation between C-3a and H-5. Further, C−H correlation data (COLOC experiment) displayed correlations between the aldehyde carbon and H-9, between C-10a and H-2, and between C-8 and H-9, the methyl protons, and the aldehyde protons and between C-7 and H-9, the methyl protons, and the aldehyde protons. The NMR, UV and fluorescence spectroscopic, and mass spectrometric data were consistent with the structure of M_1AA-dA presented in Scheme 1.

The work on the structural characterization of M_2AA-dA has been presented in a previous paper from our laboratory (*36*).

The yields of the adducts from the formaldehyde reaction were found to be 1.8 and 0.7% for M_1FA-dA and M_2FA-dA, respectively, when reacted at pH 3.5. The yields of the adducts derived from acetaldehyde were 6.8 and 10% for M_1AA-dA and M_2AA-dA, respectively, in reactions performed at pH 4.6. The yields decreased markedly under higher-pH conditions, and with the exception of M_2AA-dA (*36*), the compounds could not be detected by the UV detector in reactions performed under neutral conditions.

Gómez-Sánchez et al. described the formation of the 2/1 conjugate (Scheme 1) from malonaldehyde and acetaldehyde and found that the conjugate readily reacts with amines (*39*). We previously reported that the 2/1 conjugate forms with deoxyadenosine a strongly fluorescent methyl-substituted dihydropyridine adduct (M_2AA-dA, Scheme 1) (*36*). Moreover, it was shown that the analogous dihydropyridine protein adduct is formed in the presence of malonaldehyde and acetaldehyde (*35*, *40*). In the current work, we demonstrate that when acetaldehyde is replaced with formaldehyde the unsubstituted dihydropyridine adduct is obtained (M_2FA-dA). Recently, Kearley et al. reported that the 1/1 malonaldehyde−acetaldehyde conjugates form nonfluorescent protein adducts (*41*). Our work shows that the 1/1 conjugates of malonaldehyde and acetaldehyde, or malonaldehyde and formaldehyde, also react with the base unit of adenosine. In these reactions, fluorescent adducts with a substituted propeno bridge at N-1 and N^6 of deoxyadenosine are formed (M_1AA-dA and M_1FA-dA). The formation of the adducts may be explained by an initial attack of the endocyclic nitrogen (N-1) of adenosine on the β-carbon of the conjugates. Ring closure takes place through attack of the exocyclic amino group on one of the aldehyde carbons in the intermediates, and finally, the substituted propeno adducts are obtained by loss of water and a proton. The fluorescence intensities of the propeno adducts are much weaker than those of the dihydropyridine adducts.

The mechanism we suggest for the formation of M_1-AA-dA, M_1FA-dA, and M_2AA-dA implies that the conjugates are formed in the first step and in the second step the conjugates react with adenosine. However, a mechanism that is based on sequential addition of the aldehydes to amino groups has been suggested in the literature (*42*, *43*). At present, there is no conclusive data in favor of either mechanism.

Malonaldehyde is the main byproduct of lipid peroxidation and is therefore ubiquitous in human tissues. Acetaldehyde and formaldehyde occur in human tissues following alcohol intake and occupational or environmental exposures. Malonaldehyde and acetaldehyde or formaldehyde may thus be present at the same time in the same biological tissues, and adduct formation with DNA is possible. However, since the deoxyadenosine adducts were mainly formed under slightly acidic conditions, it is not known to what extent the adducts are formed in biological fluids.

In conclusion, the current study has shown that malonaldehyde in the presence of acetaldehyde or formaldehyde reacts with 2′-deoxyadenosine to produce stable fluorescent adducts. We have identified and determined the structure of two novel adducts: M_1AA-dA was produced in the reaction of 2′-deoxyadenosine with malonaldehyde and acetaldehyde, and M_2FA-dA was formed in the reaction of 2′-deoxyadenosine with malonaldehyde and formaldehyde. In addition, we found that the adduct M_1FA-dA, previously identified in the reaction of 2′-deoxyadenosine with the chlorohydroxyfuranone MX or CMCF (*37*, *38*), is also one of the main products in the reaction of the base with malonaldehyde and formaldehyde. The marked fluorescent properties of the adducts may have interesting biological or pharmaceutical applications. The reactions described in the present paper must be taken into account in works that aim at elucidating the mechanism behind the genotoxicity of aldehydes.

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