Reaction of Malonaldehyde with Nucleic Acid. I. Formation of Fluorescent Pyrimido[1,2-*a*]purin-10(3*H*)-one Nucleosides

Hiroshi SETO,* Taisuke OKUDA,† Tomoyuki TAKESUE,† and Tadashi IKEMURA†
Tokyo Metropolitan Research Laboratory of Public Health, Shinjuku-ku, Tokyo 160
†*College of Science and Technology, Nihon University, Chiyoda-ku, Tokyo 101*
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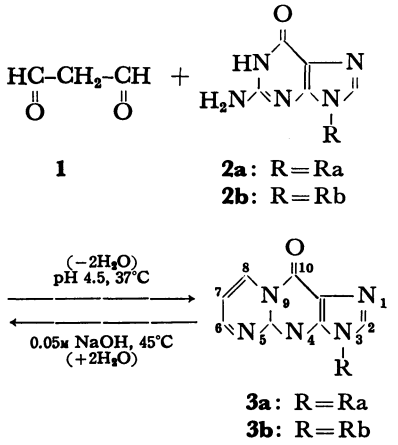
The reactions of malonaldehyde with guanosine and 2′-deoxyguanosine proceed slowly under acidic conditions to give new pyrimidopurine nucleosides, **3a** and **3b**, respectively. These compounds emit strong yellow fluorescence and are hydrolyzed by NaOH into malonaldehyde and guanosine from **3a** (2′-deoxyguanosine from **3b**). From chemical and spectroscopic evidence, these compounds were deduced to be 3-β-D-ribofuranosylpyrimido[1,2-*a*]-purin-10(3*H*)-one (**3a**) and 3-(2-deoxy-β-D-*erythro*-pentofuranosyl)pyrimido[1,2-*a*]purin-10(3*H*)-one (**3b**). No products were detected in the reaction mixture of malonaldehyde with 1-methylguanosine, adenosine, cytidine, uridine, thymidine, 2′-deoxyadenosine, or 2′-deoxycytidine.

Malonaldehyde (**1**) has been found to be one of the decomposition products of many oxidized lipid materials.[1,2] The presence of **1** in foods[3–5] and in living organs[6,7] in which the lipid fraction has undergone oxidation may be of physiological significance.

It has been reported that **1** initiates skin carcinogenesis in mice[8] and that it has mutagenic activity.[9,10] DNA is modified by **1** under acidic conditions, and becomes fluorescent.[11] The compound **1** presumably cross-links the amino groups of DNA in solution, probably through the formation of Schiff bases.[11] Brooks *et al.*[12] suggested that **1** is associated preferentially with the guanine and cytosine moieties of the DNA core. However, the mechanism of interaction between DNA and **1** still remains obscure.

When we attempted the reaction of several nucleosides with **1**, guanosine (**2a**) and 2′-deoxyguanosine (**2b**) yielded new orange-yellow fluorescent compounds, **3a** and **3b**, respectively. Lee *et al.*[13] had reported a similar fluorescent compound as a simple guanosine-malonaldehyde adduct. We previously reported a preliminary form[14] and proposed structure for the modified guanosine. In this paper, we describe the formation of the compounds in detail, and present evidence supporting the proposed structures. The physiological significance of these results is discussed.



1 **2a**: R=Ra **2b**: R=Rb

3a: R=Ra **3b**: R=Rb

Ra=β-D-ribofuranosyl,
Rb=2-deoxy-β-D-*erythro*-pentofuranosyl

Scheme 1.

Results and Discussion

Product Analysis of Nucleoside-malonaldehyde Reaction Mixture by HPLC. Some HPLC chromatograms are shown in Fig. 1, together with the control. Fluorescent products (p1 and p2) appeared on the reaction of **1** with **2a** and **2b**, respectively. Peaks in the fluorescence (FL) chromatograms appeared later than in the UV traces because of the capacity of the connecting tube, but the FL chromatograms shown in the figure have been corrected for this effect.

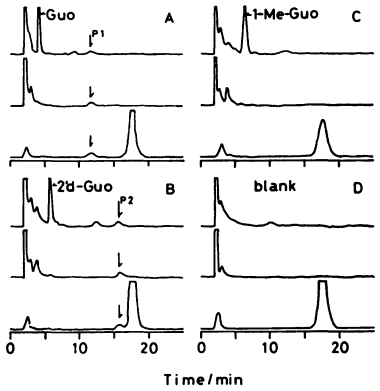


Fig. 1. HPLC chromatograms of nucleoside-malonaldehyde reaction mixtures. (upper): UV 254 nm, range 16, 10 μl inj., (middle): UV 330 nm, range 8, 50 μl inj., (bottom): FL 365—460 nm, 50 μl inj., A time lag of each FL chromatogram is excluded.

DNA was allowed to react with **1** to form fluorescent products with max. emission at 460 nm when excited at 390 nm, and a new absorption peak at 325 nm was also formed.[11] The present products, peaks p1 and p2, showed the same spectral characteristics. However, peaks having these spectral characteristics were not detected in the reaction mixtures of **1** with other nucleosides: 1-methylguanosine, adenosine, cytidine, uridine, thymidine, 2′-deoxyadenosine, and 2′-deoxycytidine. From these findings, it seems that the sugar moiety is not modified by **1**, since **3a** and **3b** have different sugar moieties. Usually, under the chromatographic conditions used, a 2′-deoxyribonucleoside has a longer retention time than the corresponding ribonucleoside.

Our results suggest that one or more sites on the guanine moiety of DNA is reactive to **1**.

Structure of the Modified Nucleoside Formed by Guanosine-malonaldehyde Reaction. Compound **3a** (peak p1) was isolated as a yellow solid (34 mg), mp 195 °C (decomp), by repeated liquid chromatography. The purity of **3a** was 99.7% as determined by HPLC analysis.

Compound **3a** was hygroscopic and highly soluble in water and in dimethyl sulfoxide (DMSO). **3a** was partially hydrolyzed by 0.05 M NaOH (1 M=1 mol dm^{-3}) into **2a** and **1**. The Molish test for sugar was positive. No carbonyl group of ketone and/or aldehyde was detected in the 2,4-dinitrophenylhydrazine test. On the other hand, the TBA (2-thiobarbituric acid) test, which is used for the detection of malonaldehyde and its precursor or adduct, was positive. The pH profile of UV absorption of compound **3a** is shown in Fig. 2. The pK_a value was calculated spectrophotometrically as 1.03.

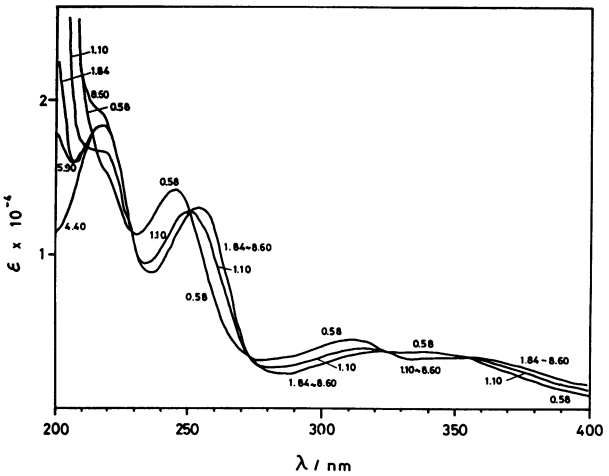


Fig. 2. UV absorption spectra of compound **3a** at various pH.

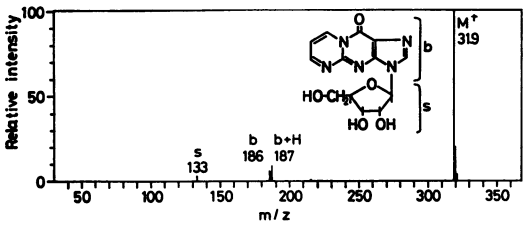


Fig. 3. FD mass spectrum of compound **3a**.

The FD mass spectrum of **3a** indicated *m/z* values of 319 (M), 187 (base+H) and 133 (ribose) (Fig. 3). The EI and CI mass spectra were obtained after trimethylsilylation of **3a**. The molecular ion peak of the trimethylsilyl (TMS) derivative was observed at an *m/z* value of 535 (*m/z* was 536 for CI with methane). Therefore, the number of substitutable active hydrogens by TMS is three. The molecular formula of **3a**, $C_{13}H_{13}N_5O_5$, was established by high resolution mass spectrometry (Found: *m/z* 535.2099. Calcd for $C_{13}H_{10}N_5O_5(TMS)_3$: M, 535.2102).

The IR(KBr) and UV(H_2O) spectra showed absorption bands at 3350 (O–H), 1723 (C=O) and 1630

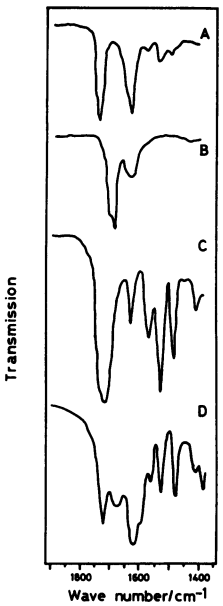


Fig. 4. IR spectra of compound **3a** and **2a**. (A), (B): **3a** and **2a** in acetonitrile, (C), (D): **3a** and **2a** in solid phase.

(C=C, C=N) cm^{-1} and 215 (ε 18600), 251 (ε 13200), 308 (ε 2700), 319 (ε 3140), and 348 (ε 2750) nm, respectively. There were serious differencies between **3a** and **2a** in respect to the absorption in the C=O stretching region. When **3a** was examined in dilute solution (acetonitrile), the relative strength of C=O to C=C and C=N absorption (Fig. 4A) was considerably weaker than that of **2a** (Fig. 4B). On the other hand, in the solid state, the C=O absorption of **2a** was influenced by intermolecular hydrogen bonding, and a complex spectrum was observed (Fig. 4D). In the FL spectrum, the E_x and E_m maxima were 360 and 500 nm, respectively.

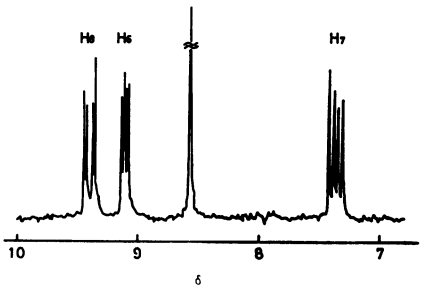


Fig. 5. Proton-NMR spectrum of compound **3a**.

The ^{1}H-NMR spectrum of **3a** (Fig. 5) exhibited typical AMX-type signals due to the –CH=CH–CH= group (δ 7.33 (J=3.9 and 6.8 Hz), 9.08 (J=2.0 and 3.4 Hz) and 9.38 (J=2.0 and 7.3 Hz), each 1H, dd). The signal of the exocyclic amino group of **2a** could no longer be seen. There were no changes in the signals of C_2 methine and ribose protons. The ^{13}C-NMR spectrum showed the existence of three carbons (δ 110.5, 141.0 and 161.8 (each d)) in addition to those of **2a**. The singlet signal of C_{10} was shifted to higher magnetic field in comparison with the original compound **2a**. It

Table 1. Assignment of ^1H chemical shifts of the nucleosides in DMSO-d_6

		3a		3b
C_2–H	8.54	1H(s)	8.47	1H(s)
C_6–H	9.08	1H(dd, J=2.0 and 3.4)	9.05	1H(dd, J=2.2and 3.7)
C_7–H	7.33	1H(dd, J=3.9 and 6.8)	7.30	1H(dd, J=3.9and 7.3)
C_8–H	9.38	1H(dd, J=2.0 and 7.3)	9.35	1H (dd, J=2.2 and 7.1)
$C_{1'}$–H	6.02	1H (d, J=5.9)	6.46	1H (t, J=6.8)
$C_{2'}$–H	4.60	1H (brds., dd)	*	
$C_{3'}$–H	4.20	1H (brds., dd)	4.46	1H (brds., m)
$C_{4'}$–H	4.01	1H (brds., m)	3.94	1H (brds., m)
$C_{5'}$–H	3.65	2H (brds., m)	3.59	2H (brds., m)

s: Singlet, d: doublet, dd: double doublet, t : triplet, m : multiplet, brds: broads, J values are in Hz, *:$C_{2'}$–H gave a overlap with the solvent (δ 2.4—2.6).

seems noteworthy that 1-methylguanosine was unreactive to malonaldehyde (**1**), as shown in Fig. 1C.

These results led us to conclude that the structure of the strongly fluorescent compound is 3-β-D-ribofuranosylpyrimido[1,2-*a*]purin-10(3*H*)-one.

The fluorescent nucleoside (peak p2) from **2b** showed similar properties. Spectroscopic evidence led us to assign the structure 3-(2-deoxy-β-D-*erythro*-pentofuranosyl)pyrimido[1,2-*a*]purin-10(3*H*)-one. The base moieties of these modified nucleosides were the same.

The yields of products were only 1.4% from **2a** and 1.8% from **2b**.

The products formed by reactions of amino compounds[15] or protein[16,17] with **1** were found to be positive in the TBA test. Aldehyde **1** reacted with lysine residues of casein (37 °C for 24 h).[18] The reaction proceeded in two steps: the formation of enamine structure (O=CHCH=CHNHR) and then the formation of aminoiminopropene structure (RN=CHCH=CHNHR) with the characteristic fluorescence spectra.[18] Our results suggest a similar reaction mechanism in the present case. The formation of pyrimidopurine is attributed to an ionic reaction of nucleophilic nitrogen atoms at the 1 and N_2 positions of guanine with carbonium ions of malonaldehyde. The reaction, involving cyclization, is very slow, though some substituted malonaldehydes were reported to react readily with guanine.[19]

The biological significance of our results is not clear at present. Reiss *et al.*[11] reported that the formation of fluorescent products correlates linearly with loss of DNA template activity. It can be assumed that the modified guanine base is unable to form a base-pair with cytosine of DNA. Thus, the decrease of the template activity can probably be attributed to the modification of guanine by **1**. Moreover, such modification is likely to be a major cause of cancer and mutation initiated by **1**. Structural determination of the fluorescent products derived from DNA modified by **1** is in progress.

Experimental

Apparatus. Melting points are uncorrected. IR, UV and FL spectra were recorded on Hitachi EPI-G3, Union Giken SM-401 and Shimadzu RF-500 spectrophotometers, respectively. NMR spectra were obtained on JEOL FX-100 and FX-270 spectrometers with TMS as an internal standard in DMSO-d_6 at room temperature. Mass spectra were recorded on JEOL JMS D-300 (EI, CI and high resolution) and 01SG-2 (FD) mass spectrometers. TLC analyses were carried out with WAKO polyamide FM plates (5 cm × 10 cm). Analytical HPLC was carried out with a Shimadzu LC-2 machine equipped with UV (254 and 330 nm) and FL (E_x 365 nm, E_m 460 nm) detectors, on a Unisil C-18, 5 μm (4 mmϕ × 25 cm) column (the mobile phase was 5% acetonitrile–water). The inlet of the FL detector was connected to the outlet of the UV detector.

Reactions of Malonaldehyde with Nucleosides. All reagents were purchased from commercial sources, and the purity of the nucleosides was checked by HPLC. Aldehyde **1** was prepared by the hydrolysis of 1,1,3,3-tetraethoxypropane. A mixture (pH 4.5) of 0.1 M malonaldehyde (1 ml), 0.01 M nucleosides (1 ml), and 0.1 M dipotassium hydrogenphosphate (8 ml) was heated at 37 °C for 10 d. The mixture was analyzed by HPLC. No growth of microorganisms was apparent during the reaction.

Isolation of the Fluorescent Nucleoside from Guanosine-malonaldehyde Reaction Mixture. Aldehyde **1** was obtained by the hydrolysis of 1,1,3,3-tetraethoxypropane (2.2 g, 0.01 mol) with 0.1 M HCl (100 ml). The mixture was heated at 40 °C for 40 min, then adjusted to pH 4.5. A mixture of **1** (0.01 mol), **2a** (700 mg, 2.5 mmol) and dipotassium hydrogenphosphate (13.6 g, 0.1 mol) in one liter of water was heated at 37 °C for 10 d. The mixture was concentrated to about 100 ml at 40 °C. Acetonitrile (600 ml) was added to the residue. The mixture was stored overnight in a refrigerator (4 °C), then the precipitate was filtered off and washed with 90% acetonitrile. The filtrate was concentrated to 50 ml, then stored overnight at 4 °C. The precipitate was filtered off. The filtrate was cleaned-up by polyamide (Woelm) column chromatography (50 mmϕ × 30 cm) with water as the eluent. The eluate was concentrated to 50 ml. The solution was chromatographed on a Lichroprep RP-18 (Merck, 22 mmϕ × 30 cm) column with 7% acetonitrile as the eluent. The fraction including **3a** was concentrated to 50 ml. The chromatography was repeated twice. Subsequently, the solution was further chromatographed on a Lichroprep RP-18 column (10 mmϕ × 25 cm) with 5% acetonitrile as the eluent. The chromatography was repeated 3 times. All operations of the synthesis and purification procedure described above were repeated 6 times. Guanosine (**2a**) used, therefore, amounted to 4.2 g (14.8 mmol). The final eluate was collected after each synthesis. The pooled eluates were dried and concentrated *in vacuo*.

The fluorescent product **3b** from the 2′-deoxyguanosine-malonaldehyde reaction was synthesized and isolated in a similar manner, but on a smaller scale: 2′-deoxyguanosine (**2b**) used totalled 1.85 g (6.9 mmol).

Trimethylsilylation. To prepare the TMS derivative, acetonitrile (100 μl) and *N,N*-bis(trimethylsilyl)trifluoroacet-

amide containing 1% trimethylchlorosilane (150 μl) were added to the dried nucleoside (100 μg). Then the mixture was heated at 75 °C for 3 h (1.5 h for the 2'-deoxy deriv.) in a reaction vial.

3a: Mp 195 °C (decomp), R_f 0.74 ppc/H_2O, 0.86 polyamide/H_2O, pK_a 1.03, ^{13}C-NMR(DMSO-d_6) 61.3(t), 70.0(d), 74.0(d), 85.7(d), 87.4(d), 110.5(d), 117.9(s), 137.4(d), 141.0(d) 149.0(s), 149.7(s), 152.3(s), 161.8(d).

3b: Mp 145 °C (decomp), R_f 0.80 ppc/H_2O, 0.74 polyamide/H_2O, pK_a 1.05, UV (H_2O) 214 (ε 23700), 253 (ε 16400), 308 (ε 3500), 320 (ε 3800), 350 nm (ε 3200), FL Ex max. 360 nm, Em max. 500 nm, IR(KBr) 3320 (O–H), 1723 (C=O), 1630 cm^{-1} (C=C, C=N), MS FD 303 (M^+), 187 (base+H), 186(base), 117(s), EI(TMS deriv.) 447(M^+), 187(base+H), CI(TMS deriv. isobutane) 448(QM^+), 188(base+2H), ^{13}C-NMR(DMSO-d_6) 61.8(t), 70.9(d), 83.8(d), 88.1(d), 110.7(d), 118.0(s), 137.4(d), 140.9(d), 149.1(s), 149.5(s), 152.5(s), 161.9(d). The $C_{2'}$ signal overlapped with the solvent signal.

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References

1) R. O. Sinnhuber, T. G. Yu, and C. T. Yu, *Food Res.* **23**, 624 (1958).
2) B. G. Tarladgis and B. M. Watts, *J. Am. Oil Chemists' Soc.*, **37**, 403 (1960).
3) B. G. Tarladgis, B. M. Watts, and M. T. Younathan, *J. Am. Oil Chemists' Soc.*, **37**, 44 (1960).
4) V. C. Witte, G. F. Kranse, and M. E. Bailey, *J. Food Sci.*, **35**, 582 (1970).
5) K. S. Rhee, *J. Food Sci.*, **43**, 1776 (1978).
6) K. Yagi, *Biochem. Med.*, **15**, 212 (1976).
7) D. H. Boehme, R. Kosecki, S. Carson, F. Stern, and N. Narks, *Brain Res.*, **136**, 11 (1977).
8) R. J. Shamberger, T. L. Andreone, and C. E. Willis, *J. Natl. Cancer Inst.*, **53**, 1771 (1974).
9) F. H. Mukai and B. D. Goldstein, *Science* **191**, 868 (1976).
10) R. J. Shamberger, C. L. Corlett, K. D. Beaman, and B. L. Kasten, *Mutat. Res.* **66**, 349 (1979).
11) U. Reiss, A. L. Tappel, and K. S. Chio, *Biochem. Biophys. Res. Commun.*, **48**, 921 (1972).
12) B. R. Brooks and O. L. Klamerth, *Eur. J. Biochem.*, **5**, 178 (1968).
13) Y. Lee, I. Park, and Y. Y. Lee, *Proc. Coll. Nat. Sci.* Sec. 3 (Seoul Natl. Univ.) **2**, 73 (1977); *Chem. Abstr.*, **89**, 142024x (1978).
14) H. Seto, T. Hashimoto, T. Okuda, T. Takesue, and T. Ikemura, *Chem. Lett.*, **1981**, 707.
15) K. Kikugawa, K. Tsukuda, and T. Kurechi, *Chem. Pharm. Bull.*, **28**, 3323 (1980).
16) T. W. Kwon, D. B. Menzel, and H. S. Olcott, *J. Food Sci.*, **30**, 808 (1965).
17) D. L. Crawford, T. C. Yu, and R. O. Sinnbuber, *J. Food Sci.*, **32**, 332 (1967).
18) H. Chiba, H. Doi, M. Yoshikawa, and E. Sugimoto, *Agric. Biol. Chem.*, **40**, 1001 (1976).
19) R. C. Moschel and N. J. Leonard, *J. Org. Chem.*, **41**, 294 (1976).