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

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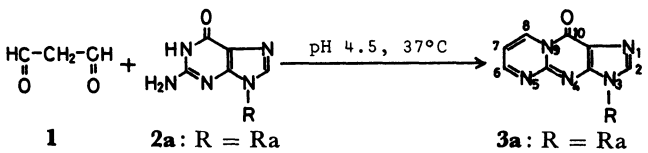
The reaction of malonaldehyde under acidic conditions (pH 4.5) with guanosine 5′-monophosphate resulted in the formation of fluorescent 3-(β-D-ribofuranosyl)pyrimido[1,2-*a*]purin-10(3*H*)-one 5′-phosphate (**3a**). This adduct was also isolated from malonaldehyde-reacted RNA. The amount of **3a** in the modified RNA was estimated to be 0.4 per cent by weight. The fluorescence spectrum of **3a** ($E_x$ max. 360 nm, $E_m$ max. 500 nm) was similar to that of guanine- and guanosine-malonaldehyde adducts which have the same type base structure. On the other hand, another type of fluorophore ($E_x$ max. 390 nm, $E_m$ max. 460 nm) was also formed in the reaction of malonaldehyde with a nucleic acid polymer. Thus, at least two different types of fluorophores are present in malonaldehyde-reacted nucleic acid.

Malonaldehyde (**1**), an end product of lipid peroxidation and a side product of prostagrandin and thromboxane biosynthesis in animal tissues,[1] is mutagenic[2–4] and carcinogenic.[4–6] These toxicological data suggest chemical interactions between **1** and various biological macromolecules. Therefore, the presence of **1** in foods[7] and in living organs[8,9] in which the lipid fraction has undergone oxidation may be of physiolosical significance. Malonaldehyde is reactive toward DNA to form fluorescent products which are accounted for by cross-linking of DNA between amino groups of the bases of DNA.[10] Recently, Nair *et al.*[11] reported novel adducts from the modification of adenine and cytosine by **1**. As previously reported,[12] the adducts formed by the reaction of **1** with guanosines are fluorescent pyrimido[1,2-*a*]-purin-10(3*H*)-one nucleosides. Malonaldehyde also reacts with DNA or RNA (presumably with guanine base), as we previously reported.[12] However, a difference was apparent between the fluorescence spectra of the modified DNA and that of the modified guanosines. An investigation regarding the reactions of **1** with guanosine 5′-monophosphate (5′-GMP) and polyguanylic acid (poly[G]) is of interest in connection with an elucidation of the effect of the phosphate group on the fluorescence (FL) spectrum.

The present paper describes the isolation and characterization of 5′-GMP-malonaldehyde and guanine-malonaldehyde adducts and the formation of a pyrimido[1,2-*a*]purine-10(3*H*)-one base by the reaction of **1** with RNA. We also describe the formation of another fluorophore which is resistant to enzymic digestion.




**1** **2a**: R = Ra **3a**: R = Ra
**2b**: R = H **3b**: R = H

Ra = β-D-ribofuranosyl 5′-monophosphate

Scheme 1.

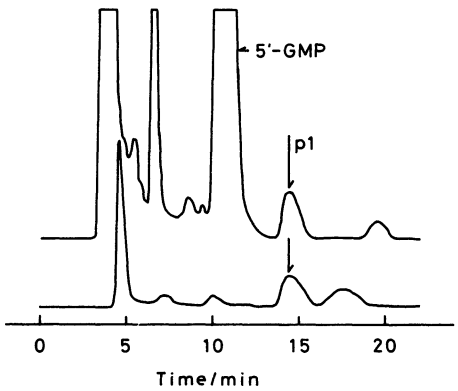



Fig. 1. HPLC chromatograms of 5′-GMP-malonaldehyde reaction mixture. Column: Partisil 10 SAX (4.6φ×250 mm). Upper: UV 254 nm, range 16, 10 μl inj, Lower: FL 365-460 nm, 50 μl inj.

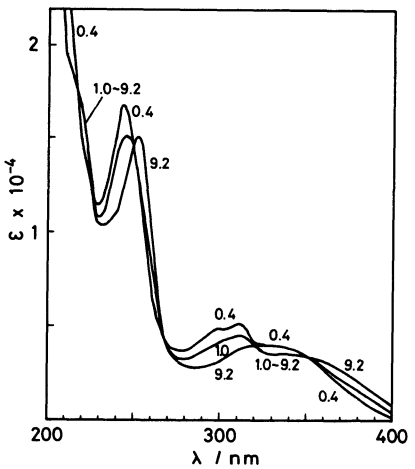



Fig. 2. UV absorption spectra of **3a** at various pH values.

## Results and Discussion

*5′-GMP-malonaldehyde Adduct.* Figure 1 shows an HPLC chromatogram of a reaction mixture of 5′-GMP (**2a**) with **1**. The fluorescence peak pl (estimated yield, 2.2%) appeared after the reaction. The

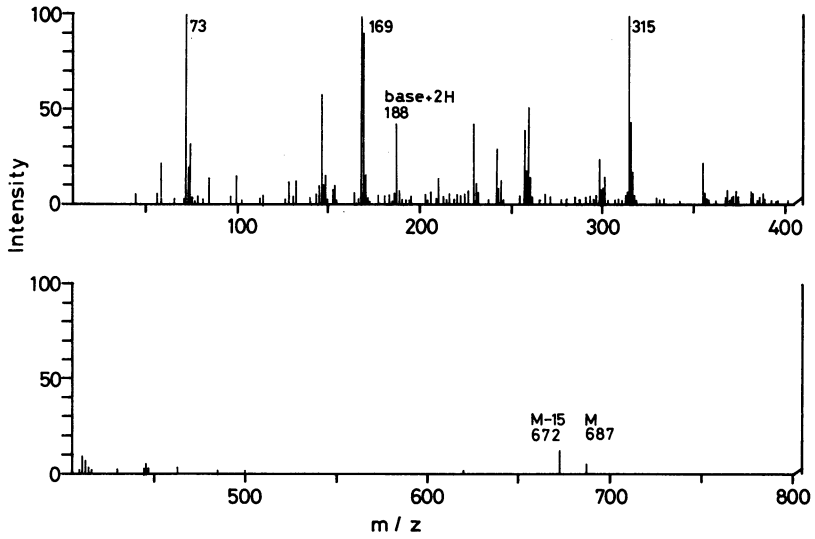



Fig. 3. EI mass spectrum of the TMS derivative of **3a**.

product, a labile light-brown solid (purity 99% as determined by HPLC analysis), was deduced to be 3-(β-D-ribofuranosyl)pyrimido[1,2-*a*]purin-10(3*H*)-one 5′-phosphate (**3a**) from the following chemical and spectroscopic evidence.

TBA (2-thiobarbituric acid)[13] and Molish tests[14] were positive. No carbonyl group (ketone or aldehyde) was detected in the 2,4-dinitrophenylhydrazine test.[14] The pH profile of the UV absorption of compound **3a** is shown in Fig. 2. The $pK_a$ value was calculated spectrophotometrically as 1.23.

The EI mass spectrum of trimethylsilylated **3a** (TMS-**3a**) showed $M^+$ at *m/z* 687 and an $(M-15)^+$ peak at *m/z* 672 (intensity ratio: *m/z* 687/ *m/z* 672=0.35)(Fig. 3). An important peak of the base was observed at *m/z* 188 $(base+2H)^+$. In the mass spectra of nucleotide-TMS, the base ion is usually accompanied by ions b+H and b+2H.[15] The ions of characteristically high abundance in the spectrum are *m/z* 315 ($HOP(OTMS)_3$) and *m/z* 169 ($C_8H_{13}O_2Si$) from ribotide. The molecular weight of TMS-**3a** was confirmed by the CI mass spectrum, which showed an MH at *m/z* 688. The molecular formula of **3a**, $C_{13}H_{14}N_5O_8P$, was established by high-resolution mass spectrometry (found: *m/z* 687.2186. Calcd for $C_{13}H_{10}N_5O_8P(TMS)_4$: M, 687.2161).

The IR (KBr) spectrum showed absorption bands at 3280 (O–H) and 1725 (C=O) $cm^{-1}$. The UV spectrum of **3a** was similar to that of pyrimido[1,2-*a*]purin-10(3*H*)-one nucleosides.[12]

A part of the proton NMR spectrum of **3a** is shown in Fig. 4. The characteristic double doublet peaks are typical AMX-type signals due to the -CH=CH-CH= group. The $^{13}C$-NMR spectrum showed the existence of 3 carbons (δ 112.3, 142.5, and 163.4 (each d)) in addition to those of **2a** (Fig. 5).

*Guanine-malonaldehyde Adduct.* The adduct was deduced to be pyrimido[1,2-*a*]purin-10(3*H*)-one (**3b**) from spectroscopic evidence (UV, IR, NMR, and MS).

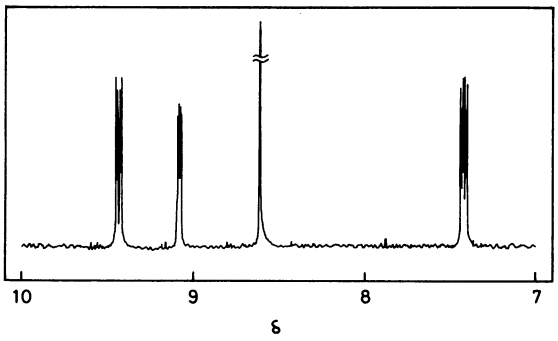



Fig. 4. $^1H$-NMR spectrum of **3a**.

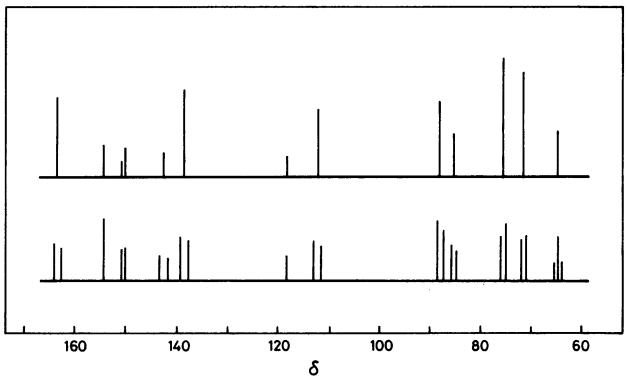



Fig. 5. $^{13}C$-NMR spectra of **3a**. Upper: Complete decoupling, Lower: Off-resonance decoupling.

Figure 6 shows an HPLC chromatogram of the extract containing **3b** (peak p2) from the precipitate. The fluorescent product, a yellow amorphous solid, was obtained in 8.4% yield after purification (purity 97% as determined by HPLC analysis). Compound **3b** showed chemical properties similar to those of **3a** in TBA[13] and the 2,4-dinitrophenylhydrazine tests.[14] The UV absorption of **3b** at various pH values is illustrated in Fig. 7. The $pK_a$ value was calculated to be 1.89.

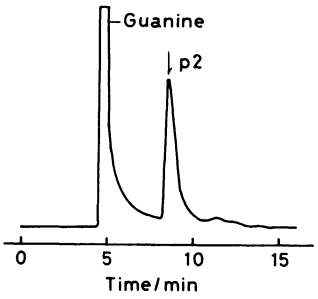



Fig. 6. HPLC chromatogram of the hot water extract from guanine-malonaldehyde reaction mixture. Column: Partisil 10 SCX (4.6$\phi$X250 mm), UV 254 nm, range 16, 10 µl inj.

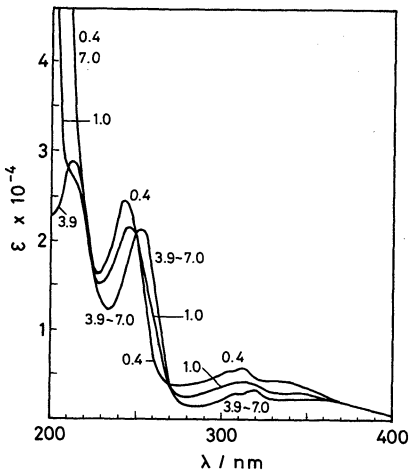



Fig. 7. UV absorption spectra of **3b** at various pH values.

*Comparison with the Fluorescence Spectra* The Fluorescence(FL) spectra of nucleic acid- and the analog-malonaldehyde adducts are summarized in Fig. 8. The FL spectra of **3a** and **3b** coincided with those of pyrimido[1,2-*a*]purin-10(3*H*)-one nucleosides. It seems that the effect of the phosphate moiety on the FL spectrum is negligible. However, the FL spectra (named Type I) of polymer-malonaldehyde adducts can be distinguished from those (named Type II) of the monomeric compounds which contain a pyrimido[1,2-*a*]purin 10(3*H*)-one base. The spectral difference between the two groups can presumably be attributed to their different structures.

*Isolation of **3a** from the Modified RNA.* Figure 9 shows an HPLC chromatogram of digested RNA which had been modified by **1**. The product in peak p3 was separated and characterized. Peak p3 gave UV and FL spectra essentially identical with those of **3a**. The absorbance ratio (A 319 nm/ A 251 nm) of the compound (0.24) was in fair agreement with that of **3a** (0.22), and the retention time of p3 on HPLC coincided with that of **3a**. TMS-treated p3 gave an EI mass spectrum similar to that of **3a**. The molecular ion $M^+$ at *m/z* 687, $(M-15)^+$ at *m/z* 672 and $(base+2H)^+$ at *m/z* 188 were observed. The product at p3 was therefore identified as compound **3a**.

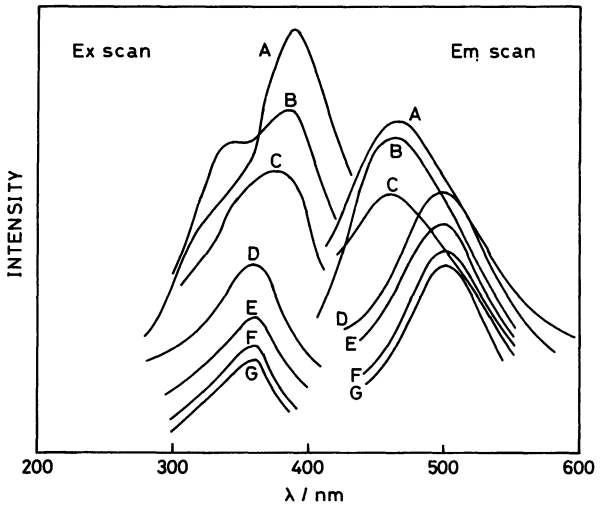



Fig. 8. Fluorescence spectra of malonaldehyde-reacted nucleic acid and analogs. Solvent: water. Substrate ($E_x$ max./nm, $E_m$ max./nm) A: DNA (390,460), B: RNA (390, 460), C: poly[G] (380, 460), D: 5′-GMP (358, 500), E: guanine (363, 500), F: guanosine (360, 500), G: 2′-deoxyguanosine (360, 500). Fluorescence emission spectra were measured by holding fluorescence excitation at 365 nm. Fluorescence excitation spectra were measured by holding fluorescence emission at 460 nm. Concentration of the solution was not normalized.

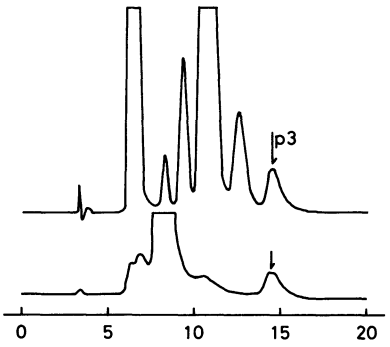



Fig. 9. HPLC chromatograms of the digested RNA solution. The sample for injection was cleaned-up on a DEAE Sephadex column. Column: Partisil 10 SAX (4.6$\phi$X250 mm). Upper: UV 254 nm, range 8, 10 µl inj., Lower: FL 365—460 nm, 50 µl inj.

The amount of **3a** formed in RNA (20 mg) modified by **1** was estimated to be 85 µg (210 nmol, 0.4 wt%) by an HPLC determination. The total amount of **1** incorporated into the RNA was at least 420 nmol as determined by the TBA method.[13] Thus, the modified RNA contains significant amounts of **1** incorporated by some other types of reaction. When the digested RNA solution was applied to a DEAE Sephadex column, a yellow fluorescent band, which contained the major fluorophore, was observed on top of the column bed. This fluorescent substance become strongly bound to the gel.

Tappel *et al.* suggested that the reaction of **1** with DNA produces interstrand crosslinking (N=CH-CH=CH-N)[10] and determined the crosslinks by a fluorescence measurement.[13] However, our result shows that at least two types of FL products (corresponding to Type I and Type II) are formed in the reaction of **1** with nucleic acid. It is necessary to elucidate the structure and properties of Type-I fluorophore.

## Experimental

*Apparatus.* Melting points are uncorrected. IR, UV, and FL spectra were recorded on Hitachi EPI-G3, Shimadzu UV 240, and Shimadzu RF-500 spectrophotometers, respectively. NMR spectra were obtained on a JEOL FX-270 spectrometer with 1,4-dioxane as an internal standard in $D_2O$ at room temperature. Mass spectra were recorded on a JEOL D-300 mass spectrometer. TLC analyses were carried out with Wako polyamide FM plates (5×10 cm). Analytical HPLC was carried out with a Shimadzu LC-2 equipped with UV (254 and 330 nm) and FL ($E_x$ 365 nm, $E_m$ 460 nm) detectors, on a Partisil 10 SAX (4.6$\phi$×250 mm) column (the mobile phase was 0.05 M $KH_2PO_4$ (1M=1 mol dm$^{-3}$) pH 3.5) and a Partisil 10 SCX (4.6$\phi$×250 mm) column (the mobile phase was 0.05 M $NH_4H_2PO_4$). The inlet of the FL detector was connected to the outlet of the UV detector.

*Determination of Malonaldehyde Incorporated into Nucleic Acid Polymers.* A 0.5 ml aliquot of the solution to be analyzed was taken, and 2 ml of 0.6% 2-thiobarbituric acid and 0.5 ml of glacial acetic acid were then added. The mixture was kept in boiling water for 30 min, and the absorbance was measured at 532 nm.

*Reaction of Malonaldehyde with 5'-GMP.* 5'-GMP·2Na (**2a**) was purchased from Yamasa Shoyu Co. Ltd., Tokyo. Malonaldehyde (**1**) was prepared by the hydrolysis of 1,1,3,3-tetraethoxypropane (4.4 g, 0.02 mol) with 0.1 M HCl (200 ml). The mixture was stirred at 40 °C for 40 min, then adjusted to pH 4.5. A solution of **1** (0.02 mol), **2a** (4.1 g, 0.01 mol) and $KH_2PO_4$ (27.2 g, 0.2 mol) in two liters of water was kept at 37 °C for 7 d, then concentrated to about 400 ml at 40 °C. Acetonitrile was then added, and the whole was kept overnight in a refrigerator (4 °C). The precipitate was collected by filtration, washed with acetonitrile and redissolved in a small amount of water. It was then subjected to DEAE Sephadex A-25 chromatography (5$\phi$×70 cm, 0.01 M $KH_2PO_4$, pH 3.5). The fluorescent fractions were collected, concentrated, and purified by Partisil 10 SAX chromatography (8$\phi$×250 mm, 0.1 M $KH_2PO_4$, pH 3.5). The fraction containing **3a** was applied to a Unisil C-18 column (8$\phi$×250 mm, water) for desalting. The product solution was evaporated to dryness *in vacuo*.

*Reaction of Malonaldehyde with Guanine.* A solution of 1,1,3,3-tetraethoxypropane (5.5 g, 25 mmol) in 25 ml of 1 M HCl was kept at 40 °C for 40 min. Subsequently, guanine (0.75 g, 5 mmol, Yamasa Shoyu Co. Ltd.) and 25 ml of 1 M HCl were added. The mixture was stirred at 40 °C for 1 h. The precipitate was collected by centrifugation (2500 min$^{-1}$, 10 min), washed with ethyl alcohol, and then centrifuged again. The guanine-malonaldehyde adduct was extracted from the precipitate with hot water (60 °C). The extract was cleaned on a polyamide column (5$\phi$×30 cm, water). The fluorescent fractions were collected and concentrated to remove sedimenting guanine. The solution was purified by Partisil 10 SCX chromatography (8$\phi$×250 mm, 0.1 M $NH_4H_2PO_4$). Finally, the eluate containing guanine-malonaldehyde adduct (**3b**) was evaporated *in vacuo*.

*Reaction of Malonaldehyde with DNA, RNA, or Poly[G].* Calf thymus DNA, baker's yeast RNA, and poly [G] were purchased from Sigma Chemical Co., St. Louis, MO. A mixture containing 10 mg of nucleic acid and 136 mg of $KH_2PO_4$ (1 mmol) in 10 ml of **1** (0.01 M) solution was maintained at 37 °C for 10 d. The solution was dialyzed against distilled water for 5 d, then evaporated *in vacuo* to give a modified nucleic acid.

*Isolation of 5'-GMP-malonaldehyde Adduct (**3a**) from the Modified RNA.* The malonaldehyde-reacted RNA (20 mg) was hydrolyzed in 10 ml of buffer solution (1 mM $KH_2PO_4$, 0.1 mM $ZnCl_2$, pH 5.3) with 1 mg of Nuclease $P_1$ from *Penicillium citrinum* (Yamasa Shoyu Co. Ltd.) at 37 °C for 2 d. The digested RNA solution was cleaned on a DEAE Sephadex A-25 column (10$\phi$×150 mm, 0.01 M $KH_2PO_4$, pH 3.5). The eluate was concentrated to 2 ml, then chromatographed on a Partisil 10 SAX column (4.6$\phi$×250 mm, 0.05 M $KH_2PO_4$, pH 3.5). The fractions of 5'-GMP-malonaldehyde adduct (**3a**) were collected, desalted and evaporated *in vacuo*.

***3a***: Mp>200 °C (dec); $R_f$=0.67 polyamide/$H_2O$, $R_f$=0.34 DEAE ppc/0.03 M $KH_2PO_4$, pH 3.5); p$K_a$ 1.23; UV ($H_2O$) 214($\varepsilon$ 22000), 251($\varepsilon$ 15000), 318($\varepsilon$ 3000), and 350 nm ($\varepsilon$ 2900); $^1$H NMR ($D_2O$) $\delta$=4.09 (2H, br., m, $C_5'$-H), 4.39(1H, br., m, C4'-H), 4.54(1H, br., dd, $C_3'$-H), 4.82(1H, br., dd, $C_2'$-H), 6.22(1H, d, $J$=5.5 Hz, $C_1'$-H), 7.39(1H, dd, $J$=3.8 and 7.1 Hz, $C_7$-H), 8.63(1H, s, $C_2$-H), 9.06(1H, dd, $J$=2.0 and 3.8 Hz, $C_6$-H), and 9.37(1H, dd, $J$=2.0 and 7.0 Hz, $C_8$-H); $^{13}$C NMR ($D_2O$) $\delta$=64.8(t), 71.5(d), 75.5(d), 85.4(d), 88.1(d), 112.3(d), 118.4(s), 138.4(d), 142.5(d), 150.1(s), 150.7(s), 154.3(s), and 163.4(d).

***3b***: Mp>270 °C (dec); $R_f$=0.62 polyamide/$H_2O$, $R_f$=0.58 CM ppc/0.025M $NH_4H_2PO_4$); p$K_a$ 1.89; UV ($H_2O$) 215($\varepsilon$ 21000), 251($\varepsilon$ 17000), 305($\varepsilon$ 3000), 316($\varepsilon$ 3500), and 350 nm ($\varepsilon$ 3000); IR (KBr) 1720 cm$^{-1}$ (C=O); MS EI(70 ev) $m/z$ 187 ($M^+$), Found: $m/z$ 187.0493. Calcd for $C_8H_5N_5O$: M, 187.0493, CI(isobutane) $m/z$ 188 ($QM^+$); $^1$H NMR($D_2O$) $\delta$=7.33(1H, dd, $J$=2.0 and 7.3 Hz, $C_7$-H), 8.33(1H. s, $C_2$-H), 8.97(1H, dd, $J$=2.0 and 4.0 Hz, $C_6$-H), and 9.21(1H, dd, $J$=2.0 and 7.1 Hz, $C_8$-H).

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