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A convenient preparative method for the 1,N^2-cyclic adducts of guanine nucleosides and nucleotides with crotonaldehyde

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Abstract—The treatment of guanine nucleosides and nucleotides with excess crotonaldehyde in pH 8.0 phosphate buffer containing an equimolar amount of L-arginine at 50°C for 2 h resulted in the selective formation of the corresponding cyclic 1,N^2-propano adducts as a mixture of its diastereomers.

Crotonaldehyde (CA) is a mutagen and carcinogen[1,2] commonly existing in the human environment as a component of mobile source emissions, tobacco smoke, and other thermal degradation products, and as a metabolite of lipid peroxidations.[3] Recently, it has been documented that, in shuttle vector plasmids propagated in human cells, CA induces a variety of mutations including dGuo–dThd transversions, dGuo–dAdo transitions, and tandem base substitutions,[4] and also that a 1,N^2-cyclic adduct (cf. **1a**) of 2′-deoxyguanosine with CA has been detected in tissues of laboratory animals in the range of three adducts/10^9 nucleotides after intake of CA via food followed by enzymatic treatment with DNase I, phosphodiesterase I, and alkaline phosphatase.[5] To study the molecular mechanisms explaining the mutagenic and carcinogenic effects of CA, a variety of the cyclic 1,N^2-propano adducts (cf. **1**) of the guanine nucleosides and nucleotides were required. However, the formation of the 1,N^2-cyclic adducts **1** in the reactions of guanosine derivatives with CA are not effective at 37°C or required severe conditions (e.g. at 90°C for 16 h),[6] for example, the treatment of 2′-deoxyguanosine with excess CA in pH 7 phosphate buffer at 37°C for 1 day affords a small amount of **1a**, together with the unstable but isolatable N^2-(3-hydroxybutylidene) dGuo adduct as a major product and trace amounts of other products such as N_7,8-cyclic adducts and the N^2-paradol dGuo adduct (Fig. 1).[6a]

We now report that the employment of L-arginine as an additive and a slightly basic buffer as the solvent in the reaction of the guanine nucleosides and nucleotides with CA

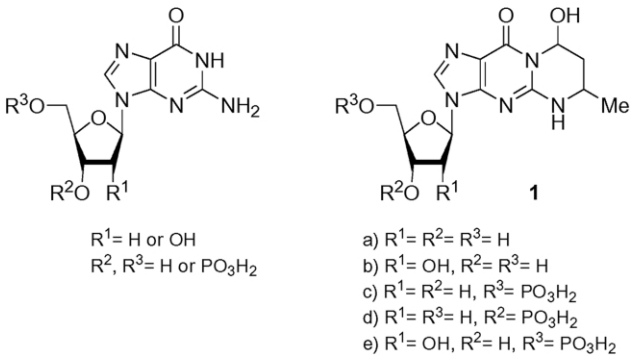

Figure 1.

are very effective for the selective formation of the 1,N^2-cyclic propano adducts (**1**).

A mixture of 2′-deoxyguanosine and excess CA was heated at 50°C for 2 h in 0.1 M phosphate buffer (pH 8.0) containing an equimolar amount of L-arginine. After adjustment to pH 5, chromatographic separation of the reaction mixture using a reversed-phase column allowed the isolation of the cyclic 1,N^2-propane dGuo adduct (**1a**) in 94% yield as a mixture of its diastereomers (ca. 1:1),[7] with recovery of the starting arginine. Analogous results were obtained using a half molar amount of L-arginine (see Fig. 2) or other amino acids such as L-lysine and L-cysteine in place of L-arginine, though the efficiency for the formation of **1a** is dependent on the nature of the functional group of the employed amino acids as shown in Table 1. On the other hand, the use of an acidic buffer as the solvent (see Section 1) or the use of acidic amino acids in place of L-arginine (see Table 1) caused a decrease in the formation of **1a** but recovery of most of the starting deoxyguanosine.

The structure of **1a** was confirmed by comparison with the

Keywords: preparative method; cyclic 1,N^2-propano guanine adduct; guanine nucleosides and nucleotides; crotonaldehyde.

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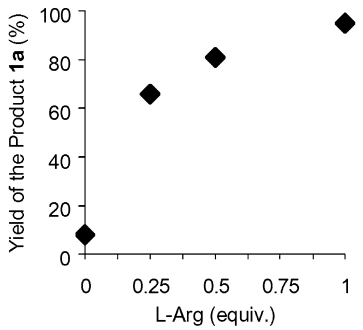


Figure 2. Concentration-dependency on L-arginine in the formation of the cyclic propano adduct **1a**.

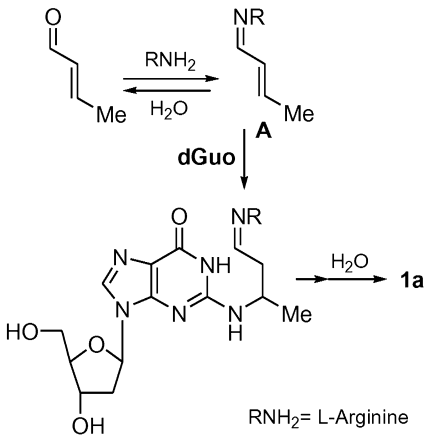
Table 1. Reactions of 2′-deoxyguanosine with crotonaldehyde in the presence of amino acids

Amino acids	1,N^2-cyclic adduct **1a** (%)	
	After 1 h	After 2 h
L-Serine	34	43
L-Tyrosine	31	43
L-Aspartic acid	7	9
L-Histidine	36	49
L-Cysteine	25	30
L-Lysine	48	59
L-Arginine	92	99

^{1}H NMR spectral data previously reported.[6b,8] For example, its ^{1}H NMR spectrum showed a broad doublet signal assignable to the Me group at δ 1.16 (3H, J=6 Hz; δ_C 20.6) ppm, and characteristic signals for the methylene group at δ 1.43 and 2.05 (each 1H, each br t and br d, J=each 12 Hz; δ_C 34.7) ppm and the aminal methine group at δ 6.03 and 6.07 (each 1/2H, each br s; δ_C 72.0) ppm. In a similar manner, the cyclic 1,N^2-propano Guo adduct (**1b**), cyclic 1,N^2-propano 5′-dGMP adduct (**1c**), cyclic 1,N^2-propano 3′-dGMP adduct (**1d**), and cyclic 1,N^2-propano 5′-GMP adduct (**1e**) were obtained in almost quantitative yields. The structural proof of the products **1b–e** is based on their spectral data (see Section 1).

The present method is based on the Michael addition of the exocyclic amino group in the guanine ring to the α,β-unsaturated aldehyde moiety in CA and subsequent intramolecular cyclization leading to the corresponding cyclic aminals **1**. These steps could be significantly accelerated by the addition of L-arginine to the medium via the transient formation of a Shiff base (**A**) of L-arginine with CA as shown in Scheme 1.[9] The transient formation of **A** during the reaction was evidenced by the ^{1}H NMR experiments carried out in a deuterated buffer (see Section 1). The employment of the basic buffer as a solvent is proper for the nucleophilic additions of the guanine moiety to the C=C and C=N bonds in the Schiff base **A**.

It should be noted that no detectable formation of any stable products was observed in the reactions of other nucleosides such as 2′-deoxyadenosine, 2′-deoxycytidine, and thymidine with CA in the presence of L-arginine under the same conditions, suggesting that the present method is, in principle, applicable to the regioselective modifications of guanine residues in oligonucleotides.



Scheme 1.

1. Experimental

All melting points are uncorrected. The ^{1}H and ^{13}C NMR spectra were obtained at 400 and 75 MHz, respectively, using deuterium oxide unless otherwise noted as the solvent. Mass spectra were determined at an ionizing voltage of 70 eV. For the thin-layer chromatographic (TLC) analyses, Merck precoated TLC plates [Merck No. 5715; silica gel 60-F_{254}; eluted with chloroform–methanol–acetic acid (40:8:1)] and Shimadzu Dual-wavelength Frying-spot Scanner CS-9000 (detected by 254 nm) were used. Column chromatographic separation was performed with Sep-Pak [Waters, Vac 35 cm^3 (10 g)] C_{18} cartridges. The product ratios of the diastereomeric products were determined by HPLC analyses carried out using a LiChroCART column (Cica-MERCK; 150-4.6) with isocratic elution by 5% CH_3CN in 10 mM sodium phosphate buffer (pH 7.0) for 5 min and then a gradient from 5 to 25% over the course of 20 min (flow rates: 1.0 mL/min; detected by 254 nm). Unless otherwise noted, the materials obtained from commercial suppliers were used without further purification.

1.1. Preparation of cyclic 1,N^2-propano adducts of guanine nucleosides and nucleotides (1a–e). General procedure

To a suspension or solution of the appropriate guanosine derivative (Sigma, >98% purity) (0.1 mmol) and L-arginine (Aldrich, >98% purity) (17.4 mg, 0.1 mmol) in 0.1 M phosphate buffer (pH 8.0) (2.0 mL) was added crotonaldehyde (CA, Kishida Chemical Co., >99% purity) (50 μL, 0.6 mmol) and then the mixture was heated at 50°C for 2 h. After being acidified to pH 5 with 1N HCl, the resulting solution was subjected to the reversed-phased column eluting with 0, 10, 20, 30, 40, and then 50% methanol–water (each 50 mL). The UV-positive fractions were collected, evaporated to dryness, and triturated with diethyl ether to obtain the desired products (**1**) as a powder in a pure state. The ^{1}H NMR spectrum of the first Ninhydrin-positive fractions eluting with water showed the recovery of a large

amount of the starting arginine [δ 1.68 (2H, m), 1.91 (2H, br t, J=6 Hz), 3.26 (2H, t, J=6 Hz), 3.77 (1H, t, J=5 Hz)].

1.1.1. 8-Hydroxy-3-(4-hydroxy-5-hydroxymethyl-tetrahydrofuran-2-yl)-6-methyl-5,6,7,8-tetrahydro-3*H*-1,3,4,5,8a-pentaaza-cyclopenta[*b*]naphthalen-9-one (cyclic 1,N^2-propano dGuo adduct) (1a).[6b,8] From fractions eluted with 20–30% methanol-containing water; 31.7 mg (94% yield; as a 23:27 mixture of its diastereomers isolatable on HPLC; retention time: 14.3 and 15.1 min); mp 155–160°C (from acetone); IR (KBr): 3394, 1686, 1571 cm^{-1}; UV (MeOH): 275 (sh), 261 nm; Mass (FAB^+): 338 $[M+H]^+$; HR-FABMS: 338.1476 (mmu: +1.1; calcd for $C_{14}H_{20}N_5O_5$); ^1H NMR δ: 1.16 (3H, d, J=6 Hz), 1.43 and 2.05 (each 1H, br t and br d, J=12 Hz), 2.34 and 2.55 (each 1H, each m), 3.63 (3H, m), 3.92 (1H, m), 4.44 (1H, m), 5.97 (1H, m), 6.03 and 6.07 (each 1/2H, each br s), 7.72 (1H, s); ^{13}C NMR δ: 20.6, 34.7, 39.4, 41.3, 62.5, 72.0 (2), 84.2, 87.8, 115.8, 137.9, 150.7, 151.7, 157.8.

1.1.2. 3-(3,4-Dihydroxy-5-hydroxymethyl-tetrahydrofuran-2-yl)-8-hydroxy-6-methyl-5,6,7,8-tetrahydro-3*H*-1,3,4,5,8a-pentaaza-cyclopenta[*b*]naphthalen-9-one (cyclic 1,N^2-propano Guo adduct) (1b). From fractions eluted with 10–20% methanol-containing water; 31.8 mg (90% yield; as a 3:2 mixture of its diastereomers isolatable on HPLC; retention time: 12.4 and 14.2 min); mp 177–179°C (from acetone); IR (KBr): 3422, 1687, 1570 cm^{-1}; UV (MeOH): 275 (sh), 261 nm; Mass (FAB^+): 354 $[M+H]^+$; HR-FABMS: 354.1406 (mmu: −0.8; calcd for $C_{14}H_{20}N_5O_6$); ^1H NMR δ: 1.12 (3H, d, J=6 Hz), 1.41 and 2.02 (each 1H, br t and br d, J=12 Hz), 3.54 (1H, m), 3.68 (2H, m) 4.03 (1H, m), 4.24 (1H, m), 4.49 and 4.55 (each 3/5 and 2/5H, each t, J=5 Hz), 5.65 (1H, m), 6.03 (1H, br s), 7.72 and 7.73 (each 2/5 and 3/5H, each s); ^{13}C NMR δ: 20.7, 34.7, 41.4, 62.3, 71.3, 72.2, 74.4, 85.9, 88.2, 116.1, 138.3, 150.9, 151.8, 158.0.

1.1.3. Phosphoric acid mono-[3-hydroxy-5-(8-hydroxy-6-methyl-9-oxo-5,7,8,9-tetrahydro-6*H*-1,3,4,5,8a-pentaaza-cyclopenta[*b*]naphthalen-3-yl)-tetrahydrofuran-2-ylmethyl] ester (cyclic 1,N^2-propano 5′-dGMP adduct) (1c). From fractions eluted with 10–20% methanol-containing water; 40.4 mg (92% yield; as a 31:19 diastereomeric mixture of its Na salt isolatable on HPLC; retention time: 12.9 and 14.0 min); IR (KBr): 3420, 1686, 1637 cm^{-1}; UV (MeOH): 275 (sh), 262 nm; Mass (FAB^+): 418 $[M+H]^+$; HR-FABMS: 418.1123 (mmu: −0.5; calcd for $C_{14}H_{20}N_5O_8P$); ^1H NMR δ: 1.22 (3H, d, J=6 Hz), 1.55 and 2.13 (each 1H, br t and br d, J=12 Hz), 2.36 and 2.66 (each 1H, each m), 3.66 (1H, m), 3.90 (2H, m), 4.09 (1H, br s), 4.61 (1H, br s), 6.17 (1H, br d, J=7 Hz), 6.19 (1H, br s), 7.91 (1H, s); ^{13}C NMR δ: 21.0, 34.8, 39.5, 41.3, 65.5, 72.1, 72.4, 84.1, 86.8, 111.6, 137.9, 151.3, 152.1, 161.0.

1.1.4. Phosphoric acid mono-[2-hydroxymethyl-5-(8-hydroxy-6-methyl-9-oxo-5,7,8,9-tetrahydro-6*H*-1,3,4,5,8a-pentaaza-cyclopenta[*b*]naphthalen-3-yl)-tetrahydrofuran-3-yl] ester (cyclic 1,N^2-propano 3′-dGMP adduct) (1d). From fractions eluted with 20–30% methanol-containing water; 40.8 mg (93% yield as a 27:23 diastereomeric mixture of its Na salt isolatable on HPLC; retention time: 10.1 and 12.5 min); IR (KBr): 3398, 1686, 1638 cm^{-1}; UV (MeOH): 275 (sh), 261 nm; Mass (FAB^+): 418 $[M+H]^+$; HR-FABMS: 418.1136 (mmu: +0.8; calcd for $C_{14}H_{20}N_5O_8P$); ^1H NMR δ: 1.21 (3H, d, J=6 Hz), 1.54 and 2.12 (each 1H, br t and br d, J=12 Hz), 2.52 and 2.70 (each 1H, each m), 3.65 (1H, m), 3.67 (2H, m), 4.13 (1H, br s), 4.78 (1H, br), 6.15 (1H, br t, J=5 Hz), 6.18 (1H, br s), 7.82 (1H, s); ^{13}C NMR δ: 22.3, 36.4, 40.3, 43.0, 64.1, 73.7, 77.5, 86.3, 88.8, 117.8, 140.2, 152.6, 153.5, 159.9.

1.1.5. Phosphoric acid mono-[3,4-dihydroxy-5-(8-hydroxy-6-methyl-9-oxo-5,7,8,9-tetrahydro-6*H*-1,3,4,5,8a-pentaaza-cyclopenta[*b*]naphthalen-3-yl)-tetrahydrofuran-2-ylmethyl] ester (cyclic 1,N^2-propano 5′-GMP adduct) (1e). From fractions eluted with 10–20% methanol-containing water; 41.4 mg (91% yield; containing a small amount of its Na salt; as a 27:73 mixture of its diastereomers isolatable on HPLC; retention time: 6.4 and 9.6 min); IR (KBr): 3412, 1686, 1637 cm^{-1}; UV (MeOH): 275 (sh), 262 nm; Mass (FAB^+): 434 $[M+H]^+$; HR-FABMS: 434.1071 (mmu: −0.6; calcd for $C_{14}H_{20}N_5O_9P$); ^1H NMR δ: 1.21 (3H, d, J=6 Hz), 1.55 and 2.13 (each 1H, br t and br d, each J=12 Hz), 3.70 (2H, m), 3.96 (1H, m), 4.19 (1H, m), 4.34 (1H, br s), 4.58 (1H, m), 5.77 (1H, d, J=6 Hz), 6.19 (1H, br s), 7.92 (1H, s).

1.2. The formation of the cyclic 1,N^2-propano adduct 1a in the reaction of 2′-deoxyguanosine with CA

pH-Dependency. CA (25 μL, 0.30 mmol) was added to a mixed solution of 0.1 M phosphate buffer (pH 5.5, 6.5, 7.5, or 8.5) (1.0 mL) containing 2′-deoxyguanosine (14.3 mg, 0.05 mmol) and L-arginine (8.7 mg, 0.05 mmol) with stirring. After continuous stirring at 50°C for 1 h, the product distributions in these reactions were estimated by TLC densitometry. The yields of the desired product **1a** were as follows: 44% (pH 5.5), 58% (pH 6.5), 94% (pH 7.5), and 95% (pH 8.5), along with recovery of the starting deoxyguanosine.

Concentration-dependency on L-arginine. CA (25 μL, 0.30 mmol) was added to the mixed solutions of 0.1 M phosphate buffer (pH 8.0) (1.0 mL) containing 2′-deoxyguanosine (14.3 mg, 0.05 mmol) and L-arginine [none, 2.2 mg (0.012 mmol), 4.4 mg (0.025 mmol), or 8.7 mg (0.05 mmol)] with stirring. After continuous stirring at 50°C for 1 h, the product distributions in these reactions were estimated by TLC densitometry. The yields of the desired product **1a** were illustrated in Fig. 2.

Concentration-dependency on CA. CA [6.2 μL (0.075 mmol), 8.3 μL (0.1 mmol), 16.6 μL (0.2 mmol), or 24.9 μL (0.30 mmol)] was added to the mixed solutions of 0.1 M phosphate buffer (pH 8.0) (1.0 mL) containing 2′-deoxyguanosine (14.3 mg, 0.05 mmol) and L-arginine (8.7 mg, 0.05 mmol) with stirring. After continuous stirring at 50°C for 1 h, the product distributions in these reactions were estimated by TLC densitometry. The yields of the desired product **1a** were as follows: 70% (1.5 equiv.), 75% (2.0 equiv.), 93% (4.0 equiv.), and 95% (6.0 equiv.), along with recovery of the starting deoxyguanosine.

Other amino acids as an additive. CA (10 μL, 0.12 mmol) was added to a mixed solution of 0.1 M phosphate buffer

(pH 8.0) (0.5 mL) containing 2′-deoxyguanosine (5.7 mg, 0.02 mmol) and an appropriate amino acid (L-serine, L-tyrosine, L-aspartic acid, L-histidine, L-L-lysine, or L-cysteine (0.02 mmol) with stirring. After continuous stirring at 50°C for 1 or 2 h, the product distributions in these reactions were estimated by TLC densitometry. The yields of the desired product **1a** were shown in Table 1.

1.3. Reactions of 2′-deoxyadenosine, adenosine, 2′-deoxycytidine, cytidine, thymidine or uridine with CA in the presence of L-arginine

To a mixed solution of an appropriate nucleoside (0.02 mmol) in 0.1 M phosphate buffer (pH 8.0) (0.5 mL) containing L-arginine (3.5 mg, 0.02 mmol), CA (10 μL, 0.12 mmol) was added and the mixture was heated at 50°C for 2 h with stirring. The TLC densitometric analyses of the reaction mixtures showed no change in the starting nucleosides. Similar results were obtained even after a prolonged reaction time (e.g. for 1 day).

1.4. ^{1}H NMR experiments on the reaction of 2′-deoxyguanosine with CA in the absence or presence of L-arginine

To a solution of 2′-deoxyguanosine (7.2 mg, 0.025 mmol) or a mixed solution of 2′-deoxyguanosine (7.2 mg, 0.025 mmol) and L-arginine (8.7 mg, 0.05 mmol) in 0.1 M deuterated phosphate buffer (pH 8.0) (1.5 mL) was added CA (12.5 μL, 0.15 mmol) and heated at 37°C in a NMR tube. These reactions were followed by ^{1}H NMR spectrometry. In the experiments using L-arginine as an additives, the transient formation of an intermediate having characteristic peaks at δ 1.99 (3H, d, J=7 Hz), 6.50 (1H, m), 7.23 (1H, m), and 7.91 (1H, d, J=8 Hz), assignable to the Schiff base of L-arginine with CA, and the smooth formation of the cyclic adduct **1a** during the reaction were observed. On the other hand, in the absence of L-arginine, the recovery of most of the starting materials, 2′-deoxyguanosine and CA was observed even after 2 days.

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7. The retention times (14.3 and 15.1 min) of the cyclic adducts **1a** on HPLC were consistent with those of the minor products[6b] obtained in the absence of L-arginine. Therefore, the methyl and hydroxyl groups on the cyclic 1,N^2-propane ring in **1a** should be in the *trans*-configuration, as reported by Chung et al.,[6b] from the steric reasons in the intramolecular cyclization process leading to the cyclic aminal.
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