CHIRALITY 23:487–494 (2011)

Absolute Configurations and Stability of Cyclic Guanosine Mono-adducts with Glyoxal and Methylglyoxal

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ABSTRACT Glyoxal and methylglyoxal are two endogenous and mutagenic 1,2-dicarbonyl compounds, which can readily form adducts with guanosine. The molecular structures of cyclic guanosine-glyoxal (G-g) and guanosine-methylglyoxal (G-mg) mono-adducts have been extensively studied before. However, diastereoisomers of these adducts have not yet been studied in detail. In this work, one pair of G-g and two pairs of G-mg diastereoisomers were baseline separated by reverse phase HPLC, whose structures were identified as the previously reported cyclic forms, and their absolute configurations were determined by circular dichroism, the octant rule, and molecular modeling. According to the HPLC elution order, configurations of two G-g (as well as *trans* G-mg) were (6R,7R) and (6S,7S), respectively. Meanwhile, the stability of each isomer in neutral solution was also investigated, which revealed the stability order G-g > *cis* G-mg > *trans* G-mg and also indicated distinct transformation processes for different G-mg configurations. *Trans* G-mg only racemized between each other, while *cis* G-mg transformed to both cis and trans forms. Different intermediates in the racemization processes were proposed to explain the observations. These results may shed light on further understanding the roles of these two small molecules in mutagenesis. *Chirality 23:487–494, 2011.*

KEY WORDS: glyoxal/methylglyoxal; guanosine adduct; circular dichroism; octant rule; diastereoisomer; racemization

INTRODUCTION

Glyoxal and methylglyoxal are two widespread 1,2-dicarbonyl compounds which are toxic and mutagenic in mammalian cells.[1,2] Both of them can be formed endogenously from a multitude of physiological processes, such as lipid peroxidation,[3] sugar or DNA autoxidation,[4,5] and DNA damage.[6] Glyoxal can also be formed from the metabolites of some carcinogens (e.g., nitrosamines, N-nitrosomorpholine, or 2-nitroimidazole[7–9]) in vivo, which was found to induce DNA single-strand breaks through the formation of 1,N^2-glyoxal-deoxyguanosine adduct.[7,10,11] Previous studies revealed that glyoxal and methylglyoxal had the most mutagenic activities to *S. typhimurium* strain TA100 among nine α-dicarbonyl compounds.[12] Many data also showed that accumulation of these compounds in mammalian cells could cause unscheduled DNA synthesis, DNA single strand breaks[13,14] and inhibition to DNA replication.[15] Gene mutations were greatly enhanced by introduction of glyoxal or methylglyoxal and the main mutation type was base pair substitution on G:C site.[16–18]

As DNA adduct formation with glyoxal and methylglyoxal changes base pairing and results in gene mutation, it is highly significant and necessary to study the reactions and the adduct structures, ideally to have their absolute configurations. The structures of guanosine-glyoxal (G-g) and guanosine-methylglyoxal (G-mg) mono-adducts have been extensively investigated using nuclear magnetic resonance spectroscopy. G-g was confirmed to be a cyclic adduct 3-(β-D-erythro-pentofuranosyl)-5,6,7-trihydro-6,7-dihyroxy-imidazo[1,2-a]purine-9-one,[7,11,19–25] and a more stable compound transformed from G-g, named N^2-(carboxymethyl)-2′-deoxyguanosine, was recently found.[26] While G-mg was identified as 3-(β-D-erythro-pentofuranosyl)-5,7-dihydro-6,7-dihyroxy-6-methyl-imidazo[1,2-a]purine-9-one in the initial reaction[21,22,27,28] and then transformed to N^2-(1-carboxylethyl)-2′-deoxyguanosine after a longer incubation time.[29,30] The detailed mechanisms for the formation of these open chain products, and particularly their possible transformation processes from the cyclic adducts are still far from being clear. Although the molecular structures of cyclic G-g and G-mg have been reported repeatedly, diastereoisomers present in the product[19] mixture have not yet been investigated in detail. Cyclic G-g adducts

Additional Supporting Information may be found in the online version of this article.

Abbreviations: CD, circular dichroism; CE, Cotton effect; G-g, guanosine-glyoxal mono-adduct; G-mg, guanosine-methylglyoxal mono-adduct; HPLC, high performance liquid chromatography; MSn, multistage mass spectrometry; NMR, nuclear magnetic resonance; RSD, relative standard deviation.

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Contract grant sponsor: National Natural Science Foundation of China, Contract grant numbers: 20575006 and 20727002

Received for publication 20 June 2009; Accepted 14 April 2010

DOI: 10.1002/chir.20875

Published online 15 April 2011 in Wiley Online Library (wileyonlinelibrary.com).

are typically a pair of diastereoisomers where the 6- and 7-hydroxyl groups are at transpositions,[19,20] though a small amount of cis-adducts has once been found at hydrolytic equilibrium conditions.[7] Nonetheless, little effort has been so far attempted to completely separate them and to study the absolute configuration and stability of each individual cyclic G-g diastereoisomer. In addition, the cyclic G-mg adducts had long been expected to have two pairs of diastereoisomers, but only one guanosine mono-adduct could be detected when human lymphocytes were treated with methylglyoxal in vitro.[27] This result suggests that there may be significant differences among the four cyclic G-mg diastereoisomers whose chemical stability and transformation might be important factors.

Circular dichroism (CD) spectroscopy is a powerful method for the analysis of chiral compounds in solution. Configurations of stereoisomers can be obtained by using the octant rule which was originally proposed by Moffitt, et al. as a semiempirical theory,[31] which was testified to be very useful in various ketones in particular with the help of molecular modeling.[32,33] As both G-g and G-mg have new chiral centers close to the carbonyl chromophore on a rigid ring, the octant rule is applicable in elucidation of the absolute configurations from their CD spectra.

Here we report detailed studies of cyclic G-g and G-mg adducts, using reverse phase HPLC, LC/MS^n, NMR, CD methods, and molecular modeling. The purpose of this work is to obtain further information of these cyclic mono-adducts, i.e., the absolute configuration for each diastereoisomer, its stability and racemization at neutral condition. The detailed information of cyclic guanosine mono-adducts with glyoxal and methylglyoxal can be very useful to further understand the roles of these two endogenous small molecules in biological processes.

MATERIALS AND METHODS

Guanosine, glyoxal aqueous solution (40%) and methylglyoxal aqueous solution (40%) were purchased from Sigma-Aldrich (St. Louis, MO). Glyoxal and methylglyoxal have been found to be mutagenic in bacteria and mammalian cells. Caution should therefore be exercised in the handling of the compounds. HPLC grade acetonitrile (ACN) was obtained from Fisher Scientific (Fair Lawn, NJ). All the other analytical reagents were procured from Beijing Reagent Company (Beijing, China) and used without further purification.

A Thermo Finnigan LCQ Advantage MAX ion trap mass spectrometer (San Jose, CA) was used in conjunction with an HPLC system which had a build-in variable wavelength UV detector (VWD) and the wavelength was set at 254 nm for all the LC/UV/MS experiments. The HPLC system and all the columns used were from Agilent Technologies (Santa Clara, CA). A ZORBAX Eclipse XDB-C18 column (2.1 $\times$ 150 mm, 3.5 μm particle size) was used for LC/MS analysis. A ZORBAX Eclipse XDB-C18 column (4.6 $\times$ 150 mm, 5 μm particle size) was used for general reaction mixture analysis and a ZORBAX 300SB-C18 column (9.4 $\times$ 250 mm, 5 μm particle size) was used for semipreparation. Two elution gradients were used for separating different products. They are conveniently designated as: gradient **1**: eluting isocratically for 12 min with ACN and H_2O (1/99, v/v), gradient **2**: eluting isocratically for 25 min with ACN and H_2O (5/95, v/v). All LC/UV/MS experiments were done with the flow rate of 0.2 mL/min, spray voltage 4.5 kV, sheath gas flow rate 24 units, auxiliary gas flow rate 5 units, and capillary temperature 275°C. Helium was the buffer gas and was used as the collision partner in the collision induced dissociation (CID) experiments.

Preparation of Cyclic G-g and G-mg

Guanosine (5.6 mg) suspension in 1 mL of 50 mM sodium phosphate buffer (pH 7.4) was incubated with 23 μL glyoxal or 31 μL methylglyoxal in a water bath at 37°C. After 2 h, guanosine was almost used up in both reaction solutions. Glyoxal containing mixture was separated by HPLC with gradient **1** at a flow rate of 1 mL/min. Methylglyoxal containing mixture was separated with gradient **2** at a flow rate of 0.5 mL/min.

For ^{1}H NMR analysis, scale-up preparations of G-g and G-mg were taken under the above conditions with 10 times of the reactants' concentrations. Products were separated by semipreparative HPLC at a flow rate of 2.5 mL/min. Every HPLC fraction of interest was collected and lyophilized by using a Christ Alpha1-4 lyophilizer (Christ AG, Germany). A Varian 300M Mercury Plus NMR spectrometer (Chicago, USA) was used to record ^{1}H NMR data of samples in d^6-Me_2SO.

CD Analysis of G-g/G-mg Diastereoisomers

Fractions for each G-g (or G-mg) product collected in HPLC (solution pH = 6.5) was analyzed by CD immediately. The measurements were carried out on a Jasco J-810 circular dichroism spectrometer (Jasco UK, Great Dunmow, Essex, UK) with a 0.2 cm path cell at room temperature. Each of the CD spectra was the average result of three scans from 350 to 200 nm at 0.1 nm intervals with a 1 nm slit width.

Energy Calculation and Conformation Optimization

All the adducts' conformations were optimized and their internal energies were calculated by Gaussian 03W software[34] using B3LYP/6-31G(d) basis set. To simplify the calculation, the ribose group of guanosine was substituted by a hydrogen atom. Such treatment did not affect the adduct conformation significantly, nor would it change interpretation for the major CD absorptions (e.g., ~280 nm for the carbonyl group) of these adducts. Additionally, the conformation of stereo-selectively synthesized analog, deoxyguanosine-crotonaldehyde mono-adduct (dG-c),[35] was similarly optimized.

Stability and Transformation Studies for Cyclic G-g and G-mg Diastereoisomers

HPLC fraction for each G-g (or G-mg) product was collected (solution pH = 6.5) and immediately incubated at 37°C water bath for certain hours, during which aliquots were taken out periodically and analyzed using LC/UV/MS. The decomposition and transformation products of those diastereoisomers were identified by both retention times and MS^n spectra. The rate constants of the racemization reactions for G-g were calculated from time-dependent changes in the chromatographic peak areas, and the relative standard deviations (RSD) were obtained from three individual experiments.

RESULTS AND DISCUSSION

Separation and Identification of Cyclic G-g and G-mg Diastereoisomers

With optimized HPLC conditions, two G-g products with almost equal amount were baseline separated (Fig. 1a) and four G-mg products were also well separated (Fig. 1b). The chromatograms and elution requirement revealed that these adducts were quite hydrophilic, so it was easy to overload the reverse phase HPLC column. However,

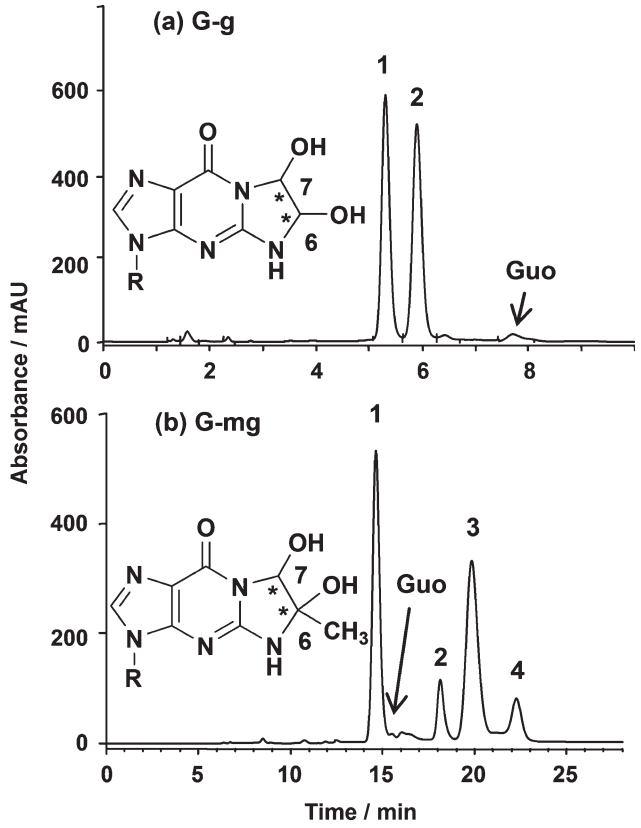

Fig. 1. HPLC chromatograms of (a) guanosine-glyoxal and (b) guanosine-methylglyoxal reaction mixtures. **1** and **2** in (a) stand for the two G-g diastereoisomers, while **1**, **2**, **3**, and **4** in (b) represent the four G-mg diastereoisomers. Structures of G-g and G-mg are shown as insets and the newly formed chiral carbons are indicated by asterisks.

the optimized HPLC methods could be used to desalt for on-line LC-MS analysis of these adducts.

LC-MS^n (n = 1–5) experiments suggested that two G-g adducts had identical fragmentation pattern (data not shown), and four G-mg also showed almost the same product ion spectra, except their MS^3 spectra (Fig. S1). These results indicated that the molecular structures of two G-g or four G-mg were the same, but the configurations of newly formed chiral carbons differentiated the adducts in chromatography.

Because of complete separation, each adduct could be analyzed by ^{1}H NMR spectrometry with little interference from isomeric impurities. However, such interference is inevitable because of the stability and racemization of these diastereoisomers (*vide infra*). The two G-g diastereoisomers had almost the same ^{1}H NMR chemical shifts as the reported results[7,19] [Table S1 in supporting information (SI)], which suggested that G-g was formed as the previously determined cyclic structure with the two hydroxyl groups at trans positions (the inset in Fig. 1a). Little G-g cis adduct,[7] G-g carboxyl containing adduct or G-2g bis adduct[26] was observed under this condition. Almost identical ^{1}H NMR chemical shifts for four G-mg were also observed, suggesting the cyclic structure (Table S2 in SI) with two hydroxyl groups at both trans and cis positions, which could account for the four diastereoisomers (Fig. 1b). Under our experimental conditions, all G-mg can further react with another methylglyoxal and form bis-adducts after 48 h incubation, which is outside the scope of this article and is now being investigated in our laboratory.

Elucidation of Configurations of Cyclic G-g and G-mg Diastereoisomers

All of the six adducts (two G-g and four G-mg) clearly showed Cotton effect (CE) around 280 nm in the CD analysis (see Fig. 2). G-g **1** and **2** showed mostly overlapping curves except exhibiting opposite signals from 260 to 300 nm, which strongly confirmed the enantiotropy in these two G-g adducts. G-mg adducts exhibited similar CD behaviors. Considering the area of each G-mg peak in the chromatogram (Fig. 1b), i.e., the concentration of the collected fraction, and its corresponding absorption intensity

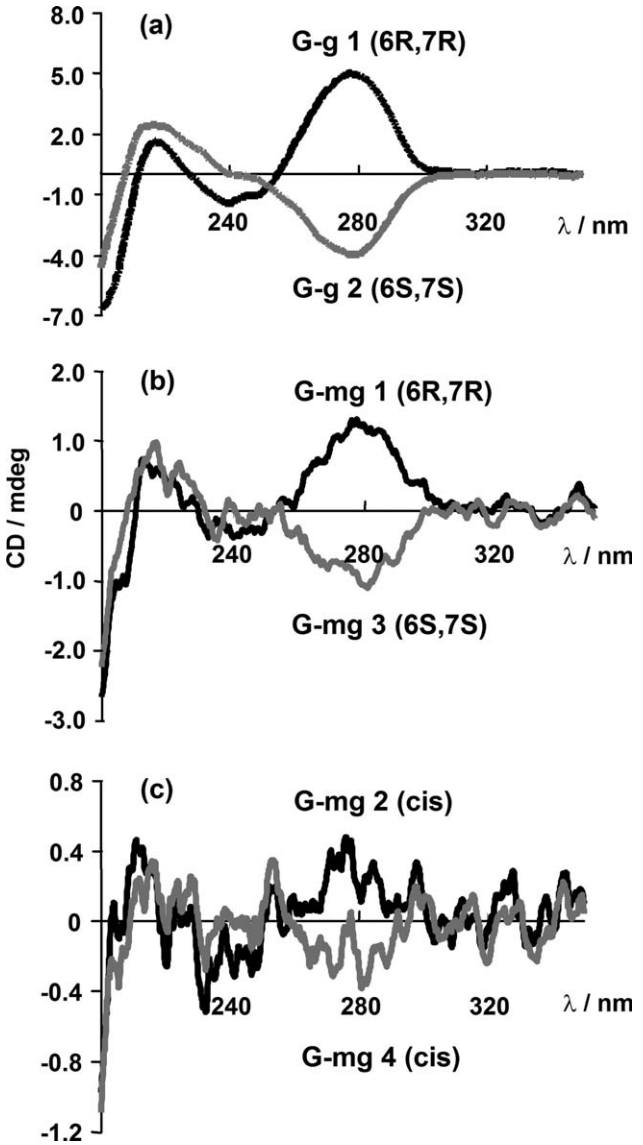

Fig. 2. CD spectra of (a) G-g **1** and G-g **2**, (b) G-mg **1** and G-mg **3**, (c) G-mg **2** and G-mg **4**.

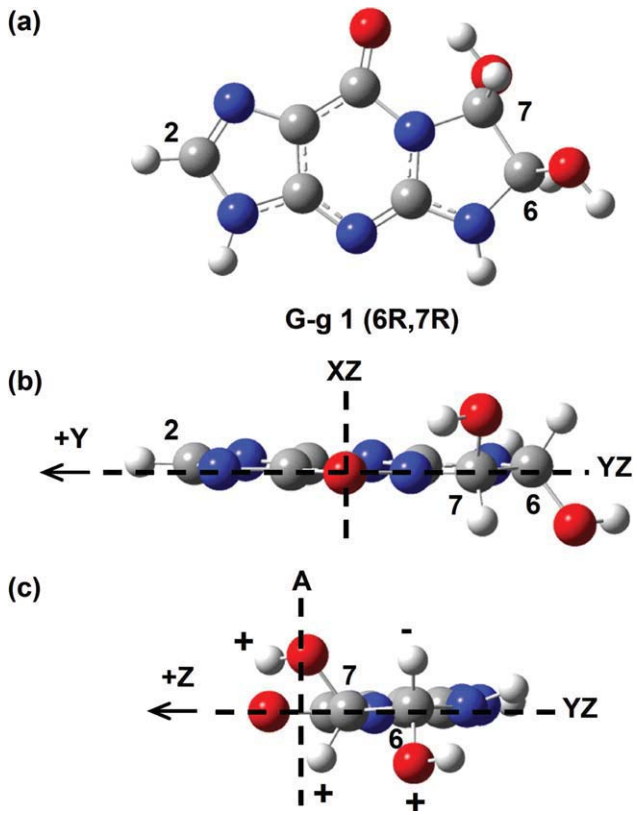

Fig. 3. Optimized G-g (6R,7R) conformation (a) and its projections in the octant coordinates (b and c). Note that the nodal surface A is the plane of the paper in b and it dissects at the middle of the carbonyl double bond. The CE signs due to the main perturbers are indicated. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in the CD spectra (Figs. 2b and 2c), one can easily conclude that G-mg **1** and **3** are a pair of diastereoisomers while G-mg **2** and **4** are the other pair.

Further determination of their absolute configurations is facilitated by molecular modeling and the classic octant rule[31,36] in CD spectroscopy. Take *trans* G-g (6R,7R) as an example. Figure 3b clearly shows that all perturbers at C-6 and C-7 are in the right octants. Further seen from the negative Y axis in the octant coordinate (Fig. 3c), the hydrogen atom of OH-7 is in the upper front octant, which gives a positive CE contribution, while the oxygen atom is just cross the nodal surface A, exhibiting no CE; the H-7 is in the lower back octant, though it is close to the nodal surface A, still giving a positive CE contribution. OH-6 and H-6 are distant from the carbonyl group and they are in the lower and upper back octant, respectively. Their additive CE contributions should be slightly positive as OH group is larger. Taking these together, G-g (6R, 7R) should show a positive CD signal around 280 nm which is observed for G-g **1**. Similarly, G-g **2** can be assigned with absolute configuration of (6S, 7S) (Fig. S2 in SI).

Our stability and racemization studies suggest that one pair of G-mg (G-mg **2** and **4**) are more stable than the other pair (G-mg **1** and **3**) under the same conditions (vide infra). Calculations by Gaussian software also reveal that internal energies for *cis* G-mg are indeed more stable with 15.8 kJ/mol lower in energy than trans pair (Table S3 in SI). The typical value of O—H—O hydrogen bond is 21 kJ/mol,[37] therefore, the additional stabilization in cis adducts might be from intramolecular hydrogen bonding between the neighboring OH groups. Thus, as G-g diastereoisomers, the absolute configurations of *trans* G-mg **1** and **3** can be identified as (6R, 7R) and (6S, 7S), respectively, but that of *cis* G-mg cannot be unambiguously determined with the present data (Fig. S2 in SI). The above configuration assignments for G-g and *trans* G-mg diastereoisomers can be further justified by using analog compounds with known absolute configurations. For example, the deoxyguanosine-crotonaldehyde mono-adducts (dG-c) stereo-selectively synthesized by Harris and coworkers[35] have similar structures with G-g and G-mg. Their CD observations are in very good agreement with what we predict for dG-c using the octant rule (Figs. S3 and S4, in SI), which is a convincing proof that the classic octant rule can be used to determine the absolute configurations for this type of compounds.

The reaction of 2′-deoxyguanosine with glyoxal and methylglyoxal were also studied in our lab. The HPLC trace of dG-g (dG-mg) was quite comparable with that of G-g (G-mg). Additionally, LC-MS^n (n = 2–5) spectra of dG-g (dG-mg), which corresponded to the CID results on parent ion after loss of the deoxyribose, were identical with the corresponding data of G-g (G-mg) (data not shown). Therefore, the conclusion for guanosine adducts may also be applicable for the corresponding deoxyguanosine adducts.

Stability and Racemization Studies of Cyclic G-g and G-mg Diastereoisomers

Each diastereoisomer in G-g pair was found to transform to the other one when it was incubated in an almost neutral solution at 37°C. LC/UV/MS results showed that G-g **1** and **2** transformed to each other at almost equal rates with little decomposition. After 45 h, the transformation process seemed to reach a steady state where the amount of G-g **1** and G-g **2** were almost equal, no matter starting with G-g **1** or **2** (see Fig. 4). Assuming no decomposition occurred in the first 10 h, we obtained the rate constants of this racemization for G-g **1** and G-g **2** to be 0.045 h^{-1} (RSD = 10%) and 0.044 h^{-1} (RSD = 10%), respectively (see the supporting information for details).

In comparison with G-g, the racemization of G-mg isomers was much more complicated under the same conditions (see Fig. 5). The *trans* pair G-mg **1** and **3** quickly decomposed to the reactants and also underwent racemization between each other in the first incubation hour. After 6 h, the amount ratio of G-mg **1**/G-mg **3** reached about 1:1 (no matter starting with G-mg **1** or **3**). In contrast, the transformations of cis isomers (G-mg **2** and **4**) occurred not only between each other, but also to the trans pair. Except to the transformed G-mg and decomposition product guanosine, the LC/UV/MS traces only showed another minor product (Fig. 5, indicated by an asterisk) with a molecular weight of 313. Its quantity increased steadily during the incubation process. It might be a degradation product whose identity and formation mechanism

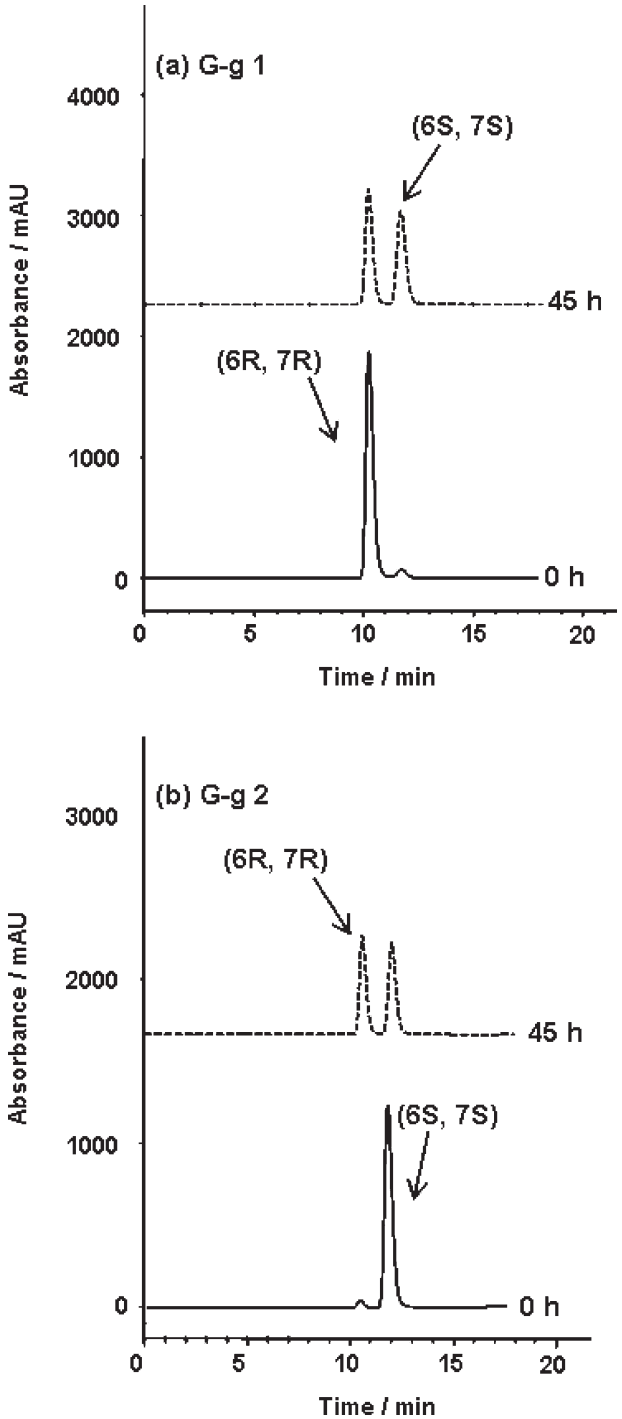

Fig. 4. Chromatograms of (a) G-g **1** and (b) G-g **2** at 0 and 45 h of the incubation clearly indicate the transformation. The integration curves that are used to calculate the transformation rate constants are presented in the Supporting Information.

are not yet clear. The relative amounts of G-mg transformation products are summarized in Table 1. The large deviations observed are mostly due to the decomposition reaction, though it is not surprise to see smaller relative standard deviations for the most abundant peaks. As there was no significant difference between the chromatograms of the 6-h and the 24-h incubation mixtures, the G-mg transformation was believed to reach its equilibrium in the first 6 h. Compared with the behavior of G-g isomers, decomposition was the main reaction channel for G-mg. Cis isomers (G-mg **2** and **4**) behaved more stable than the trans pair (G-mg **1** and **3**), and could generate trans structures through racemization. Meanwhile, the trans isomers, just like the G-g pair, only underwent racemization (except some unknown side reactions) between each other even in prolonged 24-h incubation.

There were some pH-dependent studies on the stability of G-g adducts in the previous reports. For example, Shapiro[38] and Montoya[39] independently revealed that cyclic G-g was more stable in acidic solution, while Loeppky[7] suggested that the equilibrium constant for dG-g dissociation decreases with increasing pH due to the formation of an unknown complex between dG-g and hydroxyl anion. However, we here aimed to follow the transformation reactions of cyclic G-g and G-mg mainly at close to neutral condition, expecting to study the transformation behaviors between various isomers in a pseudo physiological environment. Comparing the stability results of G-g and G-mg, one could conclude that G-mg was less stable than G-g in a neutral solution, which was in agreement with the previous report that G-mg had a smaller formation constant than G-g under neutral condition.[39]

The obviously different racemization behaviors for cis and trans isomers in an almost neutral solution suggest different mechanisms for these mono-adduct transformation. The simple dissociation/reassociation model of a reversible formation of the hemiaminal link cannot explain the difference. We hypothesized that the distinct behaviors between cis and trans adducts might be due to their different dehydrolyzed intermediates. In G-g, G-mg **1** and **3**, the OH-6, and OH-7 groups are at trans positions. During the incubation, these isomers may experience an epoxy intermediate structure with the loss of a H_2O molecule via SN_2 mechanism in the slightly acidic solution. When another H_2O attacks C-6 or C-7, because of the spatial hindrance, it could only attack from the back of the epoxy structure, which results in the reformation of two transpositioned hydroxyl groups (Fig. 6a). For G-mg **2** and **4**, however, the intermediate structure after loss of a H_2O molecule might be planar at the enol segment. Another H_2O can freely attack C-6 or C-7 from both sides of the planar segment and consequently to form trans and cis structures (Fig. 6b). Different dissociation patterns for *trans* and *cis* G-mg were observed in LC-MS^3 analysis. The two pairs of G-mg gave two different MS^3 spectra under the same CID conditions (Fig. S1, in SI), where relative intensity of the daughter ion $[M\text{-}H_2O]^+$ was about two times higher for *cis* G-mg than *trans* one, suggesting the former ion is more stable than the later. In general consideration, an enol structure in a partial aromatic system could be more stable than an epoxy ion structure. Though there were no such intermediates directly detected in the incubation solution, the observed intrinsic discrepancies of *trans* and *cis* G-mg supported this proposal to a large extent.

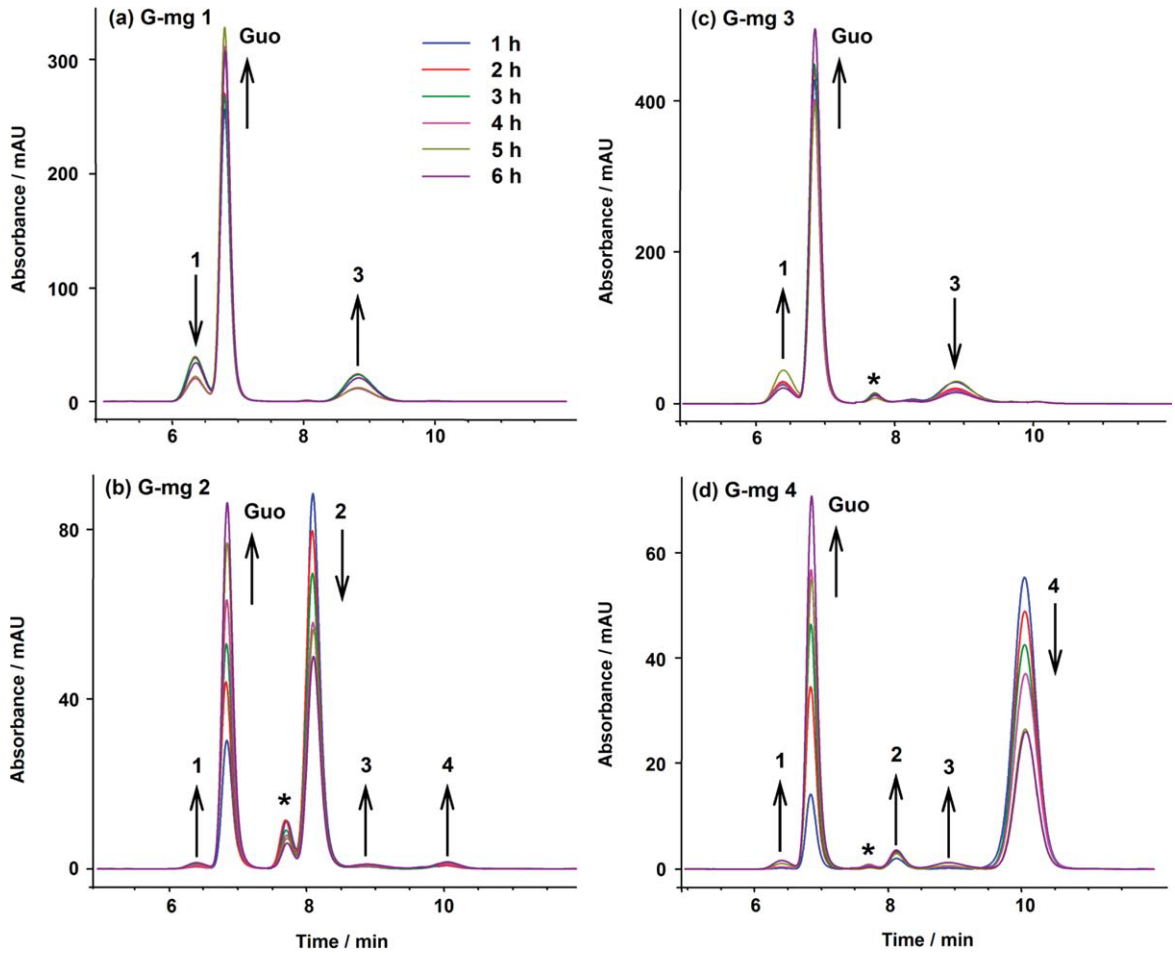

Fig. 5. HPLC studies of the transformation from (a) G-mg **1**, (b) G-mg **2**, (c) G-mg **3** and (d) G-mg **4**. The sign of ↓ points to the initial compound which decomposes and/or transforms into the other structures which are indicated by ↑ during the incubation time. Unidentified products are indicated by asterisks. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CONCLUSIONS

Cyclic guanosine mono-adducts with glyoxal and methylglyoxal were studied in detail, particularly with respect to their absolute configurations and transformation characteristics under nearly neutral conditions. Under the experimental conditions, these mono-adducts had the following stability order: G-g > *cis* G-mg > *trans* G-mg. *Cis* G-mg was further found to have different racemization behavior from that of *trans* G-mg, and mechanistic pathways involving different intermediates for the racemization processes were proposed to explain the observations. As guanosine residue is the hot spot in DNA adduct formation with methylglyoxal, these results may shed light on the possible involvement of different types of G-mg adducts in mutagenesis.

SURPPORTING INFORMATION

The supporting information includes: LC-MS^n (n = 1–5) spectra of G-mg, ^{1}H NMR data and Gaussian calculations of G-g and G-mg diastereoisomers, CD responses of G-g,

TABLE 1. Relative amounts of transformation products from four G-mg isomers in a weak acidic solution (pH = 6.5)[a]

Transformation starting from	Transformation products				
	Guanosine	G-mg 1	G-mg 2	G-mg 3	G-mg 4
G-mg 1	78.4 (10.9)	11.6 (5.8)	N/A	10.0 (5.1)	N/A
G-mg 2	37.0 (16.5)	0.5 (0.8)	58.5 (16.6)	0.3 (0.6)	3.7 (1.3)
G-mg 3	83.9 (10.0)	7.1 (4.4)	N/A	9.0 (5.6)	N/A
G-mg 4	29.8 (20.3)	0.9 (1.1)	5.5 (2.1)	0.8 (1.3)	63.0 (20.1)

N/A stands for "none of this product being observed in HPLC."
[a]The incubation time is 6 h and the temperature is 37°C. Values in the table are average percentages of the peak areas of the transformation products with respect to that of all the five compounds detected in HPLC. Values in the parentheses are the standard deviations of 3 independent experiments.

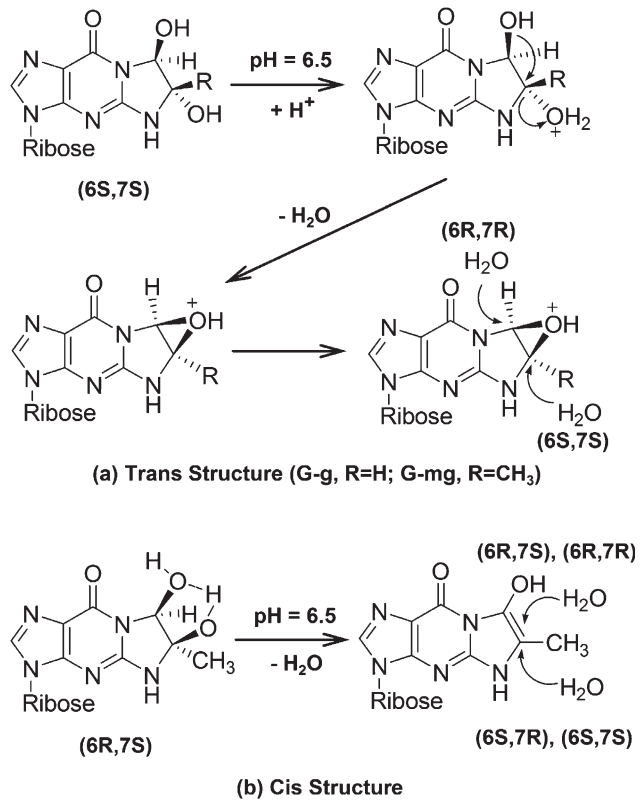

Fig. 6. Possible mechanisms for the racemization of G-g and G-mg. Trans (a) and cis (b) structures have different reaction pathways.

G-mg, dG-c diastereoisomers and their structure projections in the octant coordinates, calculation of the racemization rate constants of cyclic G-g adducts.

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