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# Formation of adducts in the reaction of glyoxal with 2′-deoxyguanosine and with calf thymus DNA

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**Abstract**

The reactions of glyoxal with 2′-deoxyguanosine and calf thymus single- and double-stranded DNA in aqueous buffered solutions at physiological conditions resulted in the formation of two previously undetected adducts in addition to the known reaction product 3-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-5,6,7-trihydro-6,7-dihydroxyimidazo[1,2-*a*]purine-9-one (Gx-dG). The adducts were isolated and purified by reversed-phase liquid chromatography and structurally characterised by UV absorbance, mass spectrometry, $^{1}$H and $^{13}$C NMR spectroscopy. The hitherto unknown adducts were identified as: 5-carboxymethyl-3-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-5,6,7-trihydro-6,7-dihydroxyimidazo[1,2-*a*]purine-9-one ($Gx_2$-dG) and $N^2$-(carboxymethyl)-9-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-purin-6(9*H*)-one ($Gx_1$-dG). Both adducts were shown to arise from Gx-dG. Gx-dG and $Gx_2$-dG were found to be unstable and partly transformed to $Gx_1$-dG, which is a stable adduct and seems to be the end-product of the glyoxal reaction with 2′-deoxyguanosine. All adducts formed in the reaction of glyoxal with 2′-deoxyguanosine were observed in calf thymus DNA. Also in DNA, $Gx_1$-dG was the only stable adduct. The transformation of Gx-dG to $Gx_1$-dG seemed to take place in single-stranded DNA and therefore, $Gx_1$-dG may be a potentially reliable biomarker for glyoxal exposure and may be involved in the genotoxic properties of the compound.


*Keywords:* Glyoxal; 2′-Deoxyguanosine adducts; DNA modifications; Endogenous mutagens; Structural characterisation

## 1. Introduction

DNA damage plays a significant role in mutagenesis and carcinogenesis. Chemical events leading to DNA damage include reactions of the base units with agents of endogenous and exogenous origin. The types of damage arising from cellular processes such as metabolism and lipid peroxidation are identical or very similar to lesions induced by exposure to environmental agents [1]. A detailed knowledge of the types of DNA damage is essential for an understanding of the mechanisms of the endogenous agents' interaction and their influence on the induction of cancer or other diseases.

One of endogenous as well as exogenous DNA-damaging agent is glyoxal. The aldehyde is a widely used industrial chemical, but is also found in foods, beverages and cigarette smoke [2,3]. Endogenously, glyoxal is formed in reaction of amino groups in proteins with reducing sugars [4–6], during sugar autoxidation [7], peroxidation of unsaturated fatty acids [8,9], DNA oxidation [10,11], and by metabolism of a number of nitrosamines [12,13]. Glyoxal has been shown to be mutagenic in bacteria and mammalian cells [14–16]. The majority of glyoxal-induced mutations were single-base substitutions, mainly G:C → T:A transversions, but deletions and frameshift mutations were also observed [14].

As a highly reactive α-dicarbonyl, glyoxal modifies proteins [17,18] and DNA bases [19–24]. The reactions of glyoxal with DNA and nucleosides occur preferentially with

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the guanine moiety to form diastereomers of a tricyclic compound in which the third five-membered ring is fused between *N*-1 and $N^2$ of the guanine unit and bears a pair of vicinal hydroxyl groups [22,24] (Scheme 1, Gx-dG). Glyoxal forms adducts also with 2′-deoxyadenosine, 2′-deoxycytidine and cytidine [22,24]. Recently, structures of coupling products of nucleosides with Gx-dG were elucidated [25]. Although the glyoxal-induced DNA modifications have been extensively studied, Gx-dG is the only known adduct produced in reaction of glyoxal with 2′-deoxyguanosine at physiological conditions.

In the present study, the reaction of glyoxal with 2′-deoxyguanosine was examined in detail and the work resulted in the identification of two previously uncharacterised products formed in this reaction. The paper deals with the structural characterisation of these products and determination of their formation in calf thymus DNA incubated with glyoxal.

## 2. Materials and methods

### 2.1. Materials

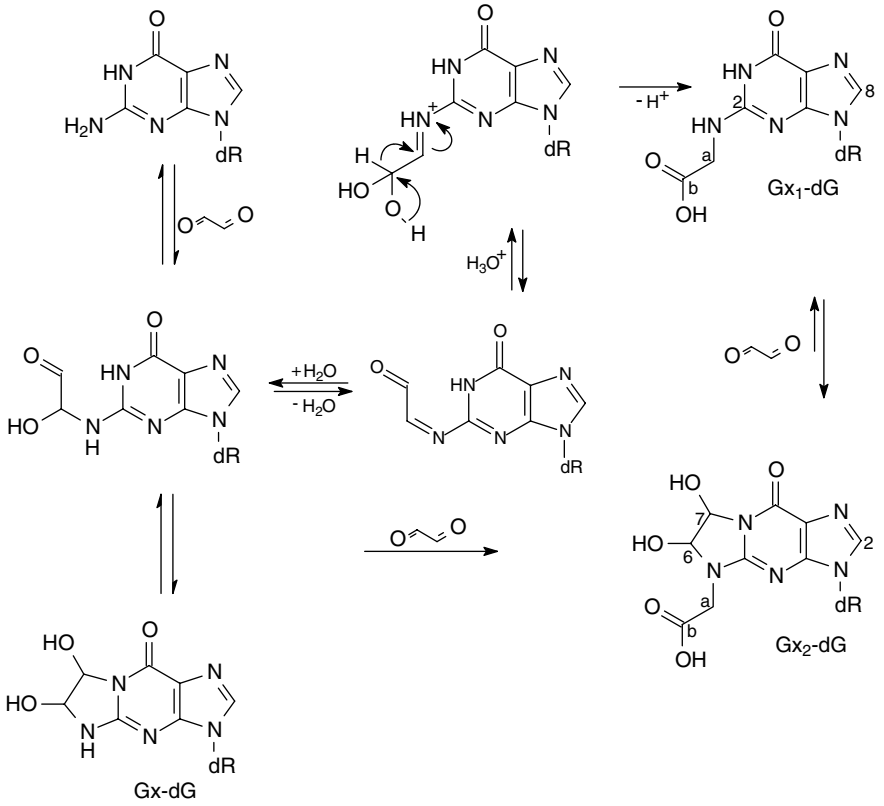
2′-Deoxyguanosine, glyoxal (40% solution in water), calf thymus DNA (Type I: sodium salt, highly polymerised), Bis–tris buffer (bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane hydrochloride), nuclease P1 from *Penicillium citrinum*, alkaline phosphatase (*Escherichia coli* Type III), acid phosphatase (wheat germ Type I) and acetonitrile (gradient grade for chromatography) were purchased from Sigma–Aldrich Company.

### 2.2. Chromatographic methods

HPLC analyses were performed on an Agilent 1100 Series liquid chromatographic system consisting of a model G1312A binary pump, a G1379A vacuum degasser, a G1313A autosampler, a G1315B diode-array detector (UV), a G1321A fluorescence detector and Agilent ChemStation data handling program. The reaction mixtures were chromatographed on a 5-μm, 4 × 125 mm reversed-phase C18 analytical column (Hypersil BDS-C18). 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 in 25 min at a flow rate of 1.5 mL/min. Preparative isolation of the products was performed on a semipreparative 5 μm, 19 × 100 mm (XTerra RP18) reversed-phase C18 column. The column was coupled to the Agilent 1100 Series HPLC system.

### 2.3. Spectrometric and spectroscopic methods

LC-ESI-MS (liquid chromatography/electrospray ionisation mass spectrometry) analyses were performed on an Agilent 1100 Series LC/MSD Trap SL instrument equipped with an electrospray source and operated in the




Scheme 1. Formation of the glyoxal–2′-deoxyguanosine adducts.

positive ion mode. Ionisation was carried out using nitrogen as both nebuliser gas (40 psi) and drying gas (12 L/min) heated to 350 °C. The capillary exit offset had a value of 115.3 V and skim 1 voltage was set at 40 V. The maximum ion accumulation time was 2.00 ms and the target value was 20.000. $Gx_1$-dG and $Gx_2$-dG were introduced through the LC system using a reversed-phase C18 analytical column (5-μm, 4 × 125 mm, Zorbax Hypersil BDS) eluted isocratically for 3 min with 1% acetonitrile in 2% aqueous solution of acetic acid and then with a gradient from 1% to 40% acetonitrile in 17 min at a flow rate of 0.5 mL/min. Gx-dG was analysed using a reversed-phase C18 analytical column (5-μm, 4 × 125 mm, Zorbax CN). The column was eluted isocratically for 5 min with 0.01 M ammonium acetate (pH 7) and then with a gradient from 0% to 40% acetonitrile in 25 min at a flow rate of 0.5 mL/min.

The $^1$H NMR spectra were recorded on a Varian and Bruker UltraShield™ 600 (Bruker, Germany), NMR spectrometers at 300 and 600 MHz, respectively. The samples were dissolved in $Me_2SO$-$d_6$, and the solvent was used as an internal reference standard. The $^1$H NMR signal assignments were based on chemical shifts and $^1$H–$^1$H and $^{13}$C–$^1$H correlation data. The assignment of carbon signals was based on chemical shifts and $^{13}$C–$^1$H correlations. All chemical shifts are reported in ppm.

The UV spectra of the isolated compounds were recorded with the diode-array detector as the peaks eluted from the HPLC column.

Analyses of DNA adducts by LC-ESI-MS/MS (liquid chromatography/electrospray ionisation tandem mass spectrometry). A Micro LC triple-quadrupole mass spectrometer equipped with an electrospray interface was used with a source block temperature of 120 °C and a desolvation temperature of 325 °C. Nitrogen was used as the desolvation gas (700 L/h) and a cone gas (33 L/h), while argon was used as the collision gas at a collision cell pressure of $6.1 \times 10^3$ mbar. Positive ions were acquired in multiple reaction monitoring (MRM) mode with a dwell time of 0.2 s and interchannel delay of 0.05 s. The LC separations were performed on an Agilent 1100 system consisting of a binary pump, a vacuum degasser, an autosampler and a thermostated column oven. The DNA samples were chromatographed using the same conditions as previously described for LC-ESI-MS methods.

### 2.4. Reaction of 2′-deoxyguanosine with glyoxal: preparation of 5-carboxymethyl-3-(2′-deoxy-β-D-erythro-pentofuranosyl)-5,6,7-trihydro-6,7-dihydroxyimidazo[1,2-a]purine-9-one ($Gx_2$-dG) and 3-(2′-deoxy-β-D-erythro-pentofuranosyl)-5,6,7-trihydro-6,7-dihydroxyimidazo[1,2-a]purine-9-one (Gx-dG)

2′-Deoxyguanosine (0.186 g, 0.7 mmol) was suspended in 100 mL of a 0.5 M phosphate buffer solution at pH 7.4. For the purpose of increasing the solubility of the nucleoside 30 mL of water was added to the phosphate buffer solution. Glyoxal (40% solution in water, 2.2 mL, 1.1 g, 19 mmol) was then added to the solution of 2′-deoxyguanosine. The mixture was stirred at 37 °C for 7 days. The progress of the reaction was followed by LC-DAD analyses on the C18 analytical column. After 7 days the reaction was stopped and the reaction mixture was concentrated by rotary evaporation to about 10 mL. Separation and purification of the products were carried out using the semipreparative C18 column. The column was eluted with a gradient consisting of 0.005 M ammonium bicarbonate (pH adjusted to 7.7 with 1 M NaOH), and acetonitrile starting from 5% acetonitrile and ending after 30 min at 40% acetonitrile at the flow rate of 4 mL/min. The fractions containing the pure compounds were evaporated to dryness and dried using argon. The residues were subjected to spectroscopic and spectrometric studies. The isolated amounts of the adducts were 9 mg for $Gx_2$-dG and 100 mg for Gx-dG.

$Gx_2$-dG had the following spectral characteristic: $UV_{max}$: 284 and 252 nm, $UV_{min}$: 272 and 226 nm (HPLC eluent, 0.01 M phosphate buffer pH 7.1).

In the positive ion electrospray mass spectrum of $Gx_2$-dG the following ions were observed (*m*/*z*, relative abundance, formation): 384 (25%, $MH^+$), $MS^2$ of 384, 286 (100%, $MH^+$−deoxyribosyl + H).

The $^1$H and $^{13}$C NMR spectroscopic data of $Gx_2$-dG are presented in Table 1.

Gx-dG had the following spectral characteristic: $UV_{max}$: 280 and 250 nm, $UV_{min}$: 270 and 224 nm (HPLC eluent, approximately 1.7% acetonitrile in 0.01 M phosphate buffer pH 7.1).

In the positive ion electrospray mass spectrum of Gx-dG the following ions were observed (*m*/*z*, relative abundance, formation): 326 (20%, $MH^+$), $MS^2$ of 326, 210 (100%, $MH^+$−deoxyribosyl + H).

The $^1$H NMR spectroscopic data of Gx-dG were as follows: 8.82 (s, 1H, N*H*), 7.96 (s, 1H, H-2), 7.22 (d, O*H*-7, $J = 6.5$ Hz), 6.48 (d, 1H, O*H*-6, $J = 7.3$ Hz), 6.12 (t, 1H, H-1′, $J = 6.7$ Hz), 5.47 (d, 1H, H-7, $J = 3.7$ Hz), 5.29 (d, 1H, O*H*-3′, $J = 2.8$ Hz), 4.95 (t, 1H, O*H*-5′, $J = 5.4$ Hz), 4.86 (d, 1H, H-6, $J = 4.9$ Hz), 4.34 (dd, 1H, H-3′, $J = 5.1$; 2.9 Hz), 3.83 (m, 1H, H-4′), 3.56 (dd, 1H, H-5′, $J = -11.5$; 4.6 Hz), 3.50 (dd, 1H, H-5″, $J = -11.5$; 4.6 Hz), 2.53 (ddd, 1H, H-2″, $J = -13.2$; 7.4; 5.9 Hz), 2.23 (ddd, 1H, H-2′, $J = -13.2$; 6.2; 3.1 Hz).

### 2.5. Preparation of $N^2$-(carboxymethyl)-9-(2′-deoxy-β-D-erythro-pentofuranosyl)-purin-6(9H)-one ($Gx_1$-dG)

Gx-dG (0.136 g, 0.42 mmol) was dissolved in 35 mL of a 0.5 M phosphate buffer solution at pH 7.4. The solution was stirred and kept at 37 °C. The incubation was allowed to proceed for 6 days and was monitored by HPLC analyses on the C18 analytical column. After 6 days, the reaction mixture was concentrated by rotary evaporation and the adduct was subsequently isolated and purified by use of the semipreparative C18 column. The column was eluted

Table 1
$^1$H and $^{13}$C chemical shifts ($\delta$) and spin–spin coupling constants, $J_{H,H}$ of protons, and long-range C–H correlations (HMBC) in $Gx_2$-dG

| Proton | $\delta$ (ppm) | Multiplicity | $J_{H,H}$ (Hz) | Carbon | $\delta$ (ppm) | HMBC |
|---|---|---|---|---|---|---|
| H-2 (1H) | 7.96 | s | | C-2[a] | 135.97, 135.93 | H-1′ |
| O*H*-7 (1H) | 7.28 | s | | | | |
| O*H*-6 (1H) | 6.85 | s | | | | |
| H-7 (1H)[a] | 5.380, 5.378 | s | | C-7 | 82.39 | H-6 |
| H-6 (1H) | 4.93 | s | | C-6 | 88.28 | H-7, H-$a_1$, H-$a_2$ |
| H-$a_1$ (1H) | 4.08 | dd | 16.9, 4.4 | C-a | 48.29 | H-6 (weak) |
| H-$a_2$ (1H) | 3.76 | dd | 16.9, 7.9 | | | |
| | | | | C-b | 171.67 | H-$a_1$, H-$a_2$ |
| | | | | C-3a[a] | 150.58, 150.55 | H-1′, H-2 |
| | | | | C-9a[a] | 117.61, 117.56 | H-2 |
| | | | | C-4a | 153.31 | H-$a_1$, H-$a_2$, H-6, H-7, H-2 |
| | | | | C-9 | 155.02 | H-2, H-6 and H-7 (weak) |
| H-1′ (1H) | 6.15 | t | 6.9 | C-1′ | 82.80 | H-2, H-3′, H-4′, H-2″ |
| H-3′ (1H) | 4.35 | dd | 5.1, 2.9 | C-3′[a] | 70.86, 70.90 | H-1′, H-4′, H-2′, H-2″, H-5′ |
| H-4′ (1H) | 3.82 | ddd | 9.3, 4.6, 2.7 | C-4′ | 87.66 | H-2′, H-2″, H-5′, H-5″ |
| H-5′ (1H) | 3.56 | dd | −11.5, 4.6 | C-5′ | 61.71 | H-3′ |
| H-5″ (1H)[a] | 3.491, 3.459 | dd | −11.5, 4.6 | | | |
| H-2″ (1H)[a] | 2.592, 2.548 | ddd | −13.3, 7.3, 6.0 | | | |
| H-2′ (1H)[a] | 2.219, 2.236 | ddd | −13.3, 6.2, 3.0 | C-2′ | 39.62 | |
| O*H*-3′ (1H) | 5.33 | | | | | |
| O*H*-5′ (1H) | 4.92 | | | | | |

[a] Separate shifts due to the presence of two diastereomers.

isocratically with 2% acetonitrile in water for 5 min, and then with a gradient from 2% to 40% over the course of 30 min at the flow rate of 4 mL/min. The collected fractions were combined and evaporated to dryness. The obtained residue was dried by use of argon and then subjected to spectroscopic and spectrometric studies. The isolated amount of the compound was 6 mg.

$Gx_1$-dG had the following spectral characteristic: $UV_{max}$: 282 and 254 nm, $UV_{min}$: 272 and 226 nm (HPLC eluent, 0.01 M phosphate buffer pH 7.1).

In the positive ion electrospray mass spectrum of $Gx_1$-dG the following ions were observed (*m/z*, relative abundance, formation): 326 (15%, $MH^+$), $MS^2$ of 326, 210 (100%, $MH^+$−deoxyribosyl + H).

The $^1$H and $^{13}$C NMR spectroscopic data of $Gx_1$-dG are presented in Table 2.

### 2.6. Small-scale reaction of glyoxal with 2′-deoxyguanosine

2′-Deoxyguanosine (5.4 mg, 0.02 mmol) was allowed to react separately with 0.15 mL (75 mg, 1.3 mmol) and 0.008 mL (4 mg, 0.06 mmol) of glyoxal, respectively, in 3 mL of a 0.5 M phosphate buffer solution at pH 7.4. The reactions were performed at 37 °C, and the progress of the reactions was followed by LC-DAD analyses of aliquots of the reaction mixtures.

### 2.7. Incubation of Gx-dG

Gx-dG (13 mg, 0.04 mmol) was dissolved in 3 and in 3.3 mL of 0.5 M phosphate buffer solution at pH 7.4, and was incubated at 37 °C with glyoxal (40% solution in water, 0.3 mL, 150 mg, 2.5 mmol), and in the absence of glyoxal, respectively. The reactions were allowed to proceed for 4 days and were monitored by LC-DAD analyses. The analytical C18 column was eluted isocratically for 5 min with 0.01 M aqueous solution of ammonium bicarbonate (pH adjusted to 7.7), and then with a gradient from 0% to 30% acetonitrile in 25 min at a flow rate of 1.5 mL/min.

### 2.8. Reactions of glyoxal with calf thymus DNA and quantification of DNA adducts

Single-stranded (ss) DNA was prepared by heating the solution of double-stranded (ds) DNA at 100 °C for 15 min followed by its rapid cooling on ice [26]. Glyoxal (30 mg, 0.52 mmol) was allowed to react with ss DNA (2.5 mg) and ds DNA (2.5 mg), in 2.0 mL of 0.1 M phosphate buffer (pH 7.4). Two reactions of ss DNA and two of ds DNA with glyoxal were performed. The reaction mixtures were stirred at 37 °C for 7 days. The DNA was precipitated by adding of 5 M NaCl (0.5 mL), cold ethanol (7.5 mL), and cooling the solutions at −20 °C. The mixtures were centrifuged (10 min at 3000 rpm), and the supernatants were collected. The DNA recovered from each reaction was washed first with cold 70% ethanol (2.5 mL), then with cold ethanol (2.5 mL) before to be dissolved in $H_2O$ (2.5 mL). The DNA was reprecipitated from the solutions by addition of cold ethanol (7.5 mL) and cooling to −20 °C, and then recovered by centrifuging (15 min at 3000 rpm).

The DNA recovered from one of the reactions of ss DNA and one of the reactions of ds DNA with glyoxal was then dissolved in 2 mL of 100 mM Bis–tris buffer (pH 6.5), containing 2 mM $MgCl_2$. The DNA was enzymatically hydrolysed by adding Nuclease P1 (dissolved at

Table 2
$^1$H and $^{13}$C chemical shifts ($\delta$) and spin–spin coupling constants, $J_{H,H}$ of protons, and long-range C–H correlations (HMBC) in $Gx_1$-dG

| Proton | $\delta$ (ppm) | Multiplicity | $J_{H,H}$ (Hz) | Carbon | $\delta$ (ppm) | HMBC |
|---|---|---|---|---|---|---|
| H-8 (1H) | 7.86 | s | | C-8 | 135.66 | H-1′ |
| $N^2$-*H* (1H) | 6.89 | t | 3.9 | | | |
| H-a (2H) | 3.63 | d | 3.9 | C-a | 44.78 | $N^2$-*H* |
| | | | | C-b | 170.52 | H-a, $N^2$-*H* |
| | | | | C-6 | 156.64 | H-8 (weak) |
| | | | | C-2 | 151.94 | H-a, $N^2$-*H* |
| | | | | C-4 | 150.36 | H-8, H-1′, $N^2$-*H* |
| | | | | C-5 | 116.73 | H-8 |
| H-1′ (1H) | 6,14 | dd | 7.4, 6.5 | C-1′ | 82.91 | H-8, H-3′, H-4′, H-2′′ |
| H-3′ (1H) | 4.37 | dt | 5.7, 2.9 | C-3′ | 70.87 | H-1′, H-4′, H-2′, H-2′′, H-5′ |
| H-4′ (1H) | 3.81 | dt | 4.9, 2.9 | C-4′ | 87.52 | H-1′, H-3′, H-2′, H-2′′, H-5′ |
| H-5′ (1H) | 3.57 | dd | −11.6, 5.1 | C-5′ | 61.84 | H-3′ |
| H-5′′ (1H) | 3.49 | dd | −11.6, 5.1 | | | |
| H-2′′ (1H) | 2.65 | ddd | −13.4, 7.6, 5.9 | | | |
| H-2′ (1H) | 2.19 | ddd | −13.4, 6.3, 3.1 | C-2′ | 38.42 | |
| O*H*-3′ (1H) | 5.35 | | | | | |
| O*H*-5′ (1H) | 4.85 | | | | | |

a concentration of 1 mg/mL in 1 mM $ZnCl_2$) to obtain a final concentration of 50 U/mL. The mixtures were incubated and stirred at 37 °C for 4 h. Then bacterial alkaline phosphatase and wheat germ acid phosphatase (the latter dissolved in Bis–tris–$MgCl_2$ buffer at a concentration of 10 mg/mL) were added to give the final concentration of 6 and 0.4 U/mL, respectively. The mixtures were incubated and stirred at 37 °C for 18 h. The enzyme digest mixtures were loaded onto a Centricon YM-3 filter prerinsed with water and centrifuged at 3000 rpm. The ultrafiltrates were recovered, concentrated by rotary evaporation at 37 °C to near dryness and reconstituted in 300 μL of a solution of methanol (40%) in water. The resulting solutions were analysed by LC-ESI-MS/MS.

The DNA recovered from the other two reactions was dissolved in 2 mL of 0.5 M phosphate buffer (pH 7.4) and stirred at 37 °C for 6 days. Then the DNA was precipitated, washed and enzymatically hydrolysed according to procedure describing above. The final solutions were analysed by LC-ESI-MS/MS.

The DNA adducts were quantified by using LC-ESI tandem quadrupole MS in MRM mode. The quantification was done with the calibration curves obtained from standard solutions of the pure 2′-deoxyguanosine adducts.

## 3. Results and discussion

### 3.1. Reactions of glyoxal with 2′-deoxyguanosine

Glyoxal was reacted with 2′-deoxyguanosine at pH 7.4 and 37 °C and the progress of the reaction was monitored by LC-DAD analyses (Fig. 1). The partly unresolved product peaks Gx-dG and $Gx_2$-dG were isolated from the reaction mixture by semipreparative chromatography and the pure compounds were subjected to spectroscopic and spectrometric analyses. On the basis of UV, MS and NMR spectra the peaks marked Gx-dG were identified as diastereomers of the previously known adduct, 3-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-5,6,7-trihydro-6,7-dihydroxyimidazo[1,2-*a*]purine-9-one (Scheme 1), while the peaks marked $Gx_2$-dG were identified as diastereomers of 5-carboxymethyl-3-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-5,6,7-trihy-6,7-dihydroxyimidazo[1,2-*a*]purine-9-one (Scheme 1 and Table 1). In the $^1$H NMR spectrum of $Gx_2$-dG (Table 1), the one-proton signals at $\delta = 4.93$ and 5.38 ppm were assigned to the protons in a new, five-membered ring of the adduct, H-6 and H-7, respectively. The assignment was based on the long-range C–H correlation spectrum in which the proton at $\delta = 4.93$ ppm showed a weak correlation to the carbon at $\delta = 48.29$ ppm (C-a), while this correlation was not observed from the proton at $\delta = 5.38$ ppm. Moreover, the carbon at $\delta = 88.28$ ppm (C-6) was connected to the proton at $\delta = 4.93$ ppm and displayed a very strong correlation to the protons at $\delta = 4.08$ and 3.76 ppm whose signals were assigned to the two geminal

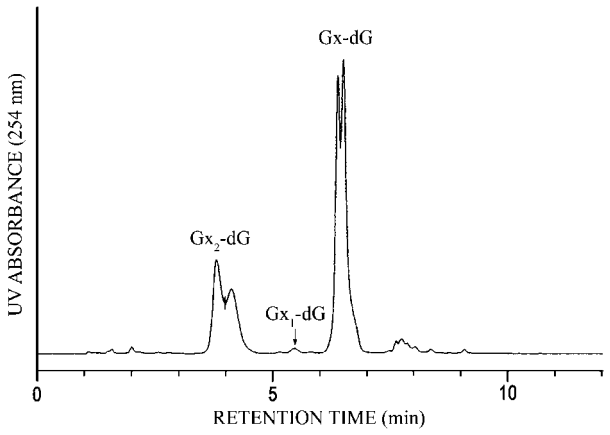



Fig. 1. C18 analytical column LC-DAD chromatogram of the reaction mixture of glyoxal (0.06 mmol) with 2′-deoxyguanosine (0.02 mmol) in 0.5 M phosphate buffer (pH 7.4) held at 37 °C for 7 days. For analysis conditions, see Section 2.2. in Section 2.

protons of a methylene group (H-$a_1$ and H-$a_2$). The carbon at $\delta = 82.39$ ppm (C-7) connected to the proton at $\delta = 5.38$ ppm did not show such a correlation. The assignment of the signals at $\delta = 4.08$ and 3.76 ppm to the protons of the methylene group was based on the chemical shift and the one bond C–H correlation with the carbon at $\delta = 48.29$ ppm.

The spectral data of the isolated compounds are in all essential features identical with those previously reported for Gx-dG and $Gx_2$-G (5-carboxymethyl-3-(β-D-*erythro*-pentofuranosyl)-5,6,7-trihydro-6,7-dihydroxyimidazo[1,2-*a*]purine-9-one) [25,27]. In the work of Ruohola et al. [27], $Gx_2$-G was found to be formed in reaction of bromomalonaldehyde with guanosine at basic conditions and elevated temperatures and was thought to arise by transformation of bromomalonaldehyde to glyoxal at these reaction conditions.

Since it was found that the peaks of $Gx_2$-dG appeared after the reaction time of two hours while Gx-dG was detectable almost immediately and that prolonged reaction resulted in the increase in the yield of $Gx_2$-dG, while at the same time the yield of Gx-dG declined, it was suggested that Gx-dG was a precursor for $Gx_2$-dG. To further clarify the origin of $Gx_2$-dG, experiments were performed where pure Gx-dG was dissolved in 0.5 M phosphate buffer solution (pH 7.4) and stored at 37 °C in the presence and absence of glyoxal. The LC-DAD analyses of the solutions showed that $Gx_2$-dG was formed in both solutions, in conjunction with a compound marked $Gx_1$-dG (Scheme 1). The peak of $Gx_1$-dG was also found in the chromatogram of the reaction mixture of glyoxal and 2′-deoxyguanosine, but the compound was formed in only trace amounts (Fig. 1). When the incubation of Gx-dG was performed in the presence of glyoxal, $Gx_2$-dG was formed almost immediately, while $Gx_1$-dG was detected only after 1 day of storage. On the other hand, in the solution incubated in the absence of glyoxal, $Gx_1$-dG was formed almost instantly, while $Gx_2$-dG could be detected only after 1 day (Figs. 2 and 3). Prolonged incubations led to further increase in the yields of $Gx_1$-dG and $Gx_2$-dG and a decline in the amount of Gx-dG. These observations show that both $Gx_1$-dG and $Gx_2$-dG are formed from Gx-dG. The yield of $Gx_1$-dG was markedly higher in the solution stored in the absence of glyoxal while $Gx_2$-dG was formed in bigger amounts when the incubation was performed in the presence of the aldehyde. It has been previously shown [22] and was also observed during the incubation experiments that the adduct Gx-dG decomposes back to 2′-deoxyguanosine and glyoxal. The glyoxal delivered may react with undecomposed Gx-dG producing $Gx_2$-dG.

For the purpose of determining the structure of $Gx_1$-dG, the compound was isolated by semipreparative HPLC from a phosphate buffer (pH 7.4) solution of Gx-dG stored at 37 °C for 6 days. On the basis of data collected from UV and NMR spectroscopy, and electrospray mass spectrometry, the structure of the compound was assigned as $N^2$-(carboxymethyl)-9-(2′-deoxy-β-D-*ery-*

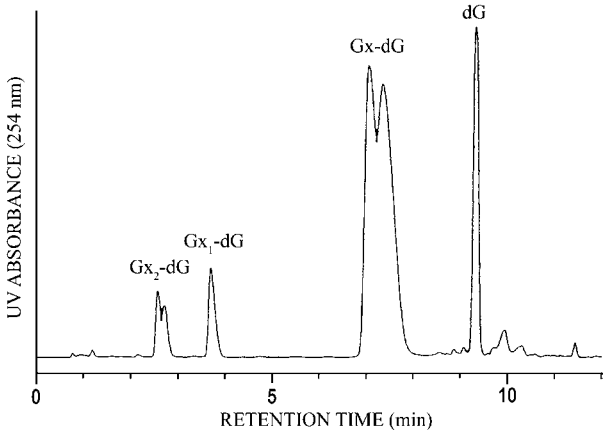



Fig. 2. C18 analytical column LC-DAD chromatogram of the solution of Gx-dG in phosphate buffer (pH 7.4) stirred at 37 °C for 3 days without addition of glyoxal. For analysis conditions, see Section 2.7. in Section 2.

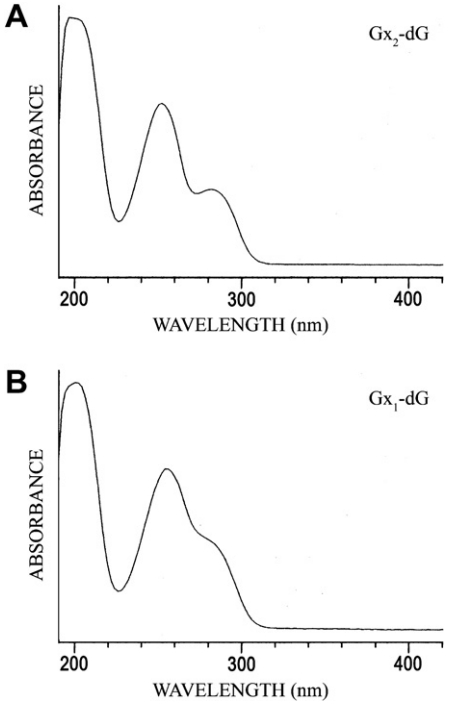



Fig. 3. UV absorbance spectra of $Gx_2$-dG (A), and $Gx_1$-dG (B). The spectra were recorded with the diode-array detector as the compounds eluted from the LC column.

*thro*-pentofuranosyl)-purin-6(9*H*)-one (Table 2). In the $^1$H NMR spectrum of $Gx_1$-dG (Table 2), the two-proton signal at $\delta = 3.63$ ppm was assigned to the two H-a protons. The assignment was based on the chemical shift and on the one bond C–H correlation with the carbon at $\delta = 44.78$ ppm. This two-proton signal appeared as a doublet ($J = 3.9$ Hz) due to the three-bond coupling to the $N^2$-H proton. Consequently the signal assigned to the $N^2$-H proton ($\delta = 6.89$ ppm) was split into a triplet because of the coupling to the methylene protons. In the COSY spectrum a very strong correlation from the $N^2$-H proton to the H-a protons was observed. The carbon signal at $\delta = 170.52$ ppm was

assigned to the C-b carbon on the basis of the chemical shift and on the lack of one bond correlation with any proton. The C-b carbon displayed C–H long-range correlation to the H-a and $N^2$-H protons. In the HMBC spectrum the C-a carbon showed correlation to the $N^2$-H proton. The correlations between the C-2 and the methylene protons, and the proton bound to the exocyclic nitrogen atom were also observed.

### 3.2. Mechanism for the formation of $Gx_1$-dG and $Gx_2$-dG

As already discussed Gx-dG was found to be a precursor for $Gx_1$-dG and $Gx_2$-dG (Scheme 1). The ring opened form of Gx-dG may decompose back to glyoxal and 2′-deoxyguanosine, or may be transformed to an imine from which $Gx_1$-dG is afforded through addition of $H_3O^+$ and hydride ion migration (a pinacol rearrangement) in conjunction with the loss of a proton.

$Gx_2$-dG may be formed by attack of glyoxal on Gx-dG, but it was found that the adduct was also formed when a solution of pure $Gx_1$-dG and glyoxal was stored at pH 7.4 and 37 °C. The transformation of $Gx_1$-dG to $Gx_2$-dG was quantitative and complete after 15 h of storage. Also the reversible albeit slow reaction was observed; $Gx_2$-dG could be transformed to $Gx_1$-dG. $Gx_1$-dG was the only compound that was found to be stable at neutral condition indicating that the adduct is the end product of the reaction of glyoxal with 2′-deoxyguanosine.

### 3.3. Reactions of glyoxal with calf thymus DNA

Glyoxal was allowed to react with both single- and double-stranded calf thymus DNA. After DNA precipitation and washing with 70% ethanol and then with 99% ethanol, one of the ss and one of the ds DNA samples were enzymatically hydrolysed to 2′-deoxyribonucleosides, while the other DNA samples were redissolved in phosphate buffer and the solutions were stored for an additional time of 7 days. Thereafter, the DNA was subjected to enzymatic hydrolysis. The analysis of the DNA hydrolysates was performed by LC-ESI-MS/MS. The adducts were identified in the hydrolysates by positive ion electrospray MS/MS spectra and coelution with the 2′-deoxyguanosine standards. The ion peaks monitored were the protonated molecular ions and the fragment peaks obtained by cleavage of the deoxyribosyl moiety (*m*/*z* 116) from the parent ions (Table 3).

Gx-dG was found to be the major glyoxal adduct in DNA and was found in the hydrolysates at nmol/mg DNA levels (Table 4), while $Gx_1$-dG and $Gx_2$-dG were detected in the hydrolysates at pmol/mg DNA levels.

In the hydrolysate of the DNA that was redissolved in phosphate buffer following incubation with glyoxal and precipitation, a marked drop in the amount of the Gx-dG adduct was observed. In ds DNA the amount of the adduct was about 7-fold and in ss DNA it was almost 40-fold lower than in the DNA hydrolysed after the incubation with glyoxal. Also a drop in the yield of $Gx_2$-dG was noted in the DNA stored for 7 days in the absence of glyoxal, although the drop was not as drastic as for Gx-dG. On the other hand, the amount of the adduct $Gx_1$-dG in ds DNA remained the same in both DNA samples and in ss DNA an almost 2-fold increase in the adduct level could be noticed. These observations can be explained by the instability of the Gx-dG and the $Gx_2$-dG adducts which afford mainly 2′-deoxyguanosine but in part also the transformation product $Gx_1$-dG. The results of the DNA incubation experiments indicate that $Gx_1$-dG is stable in DNA and may be the end product of glyoxal reaction with the guanine base in DNA.

## 4. Conclusions

Studies on structural characterisation of nucleoside adducts of mutagenic compounds are essential for elucidating the possible interactions of chemicals with DNA and

Table 3
Retention times and the recorded transitions of nucleoside adducts analysed in the DNA hydrolysates

| Adduct | Retention time (min) | Transition | Mode of formation | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|---|---|
| $Gx_1$-dG | 10.5 | 326 → 210 | $MH^+$-dR+H | 18 | 14 |
| $Gx_2$-dG | 9.8 | 384 → 268 | $MH^+$-dR+H | 18 | 15 |
| Gx-dG | 11.3 | 326 → 210 | $MH^+$-dR+H | 17 | 13 |

Table 4
The Levels of Nucleoside Adducts Detected in the DNA Hydrolysates

| Adduct | ds DNA incubated with Gx | ss DNA incubated with Gx | ds DNA redissolved in phosphate buffer | ss DNA redissolved in phosphate buffer |
|---|---|---|---|---|
| $Gx_1$-dG | 2.3 pmol/mg DNA<br>1.7 adducts/$10^6$ nucl. | 6.4 pmol/mg DNA<br>1.9 adducts/$10^6$ nucl. | 2.1 pmol/mg DNA<br>0.6 adducts/$10^6$ nucl. | 11.4 pmol/mg DNA<br>3.5 adducts/$10^6$ nucl. |
| $Gx_2$-dG | 4.4 pmol/mg DNA<br>3.2 adducts/$10^6$ nucl. | 66 pmol/mg DNA<br>18 adducts/$10^6$ nucl. | 2.5 pmol/mg DNA<br>0.8 adducts/$10^6$ nucl. | 19 nmol/mg DNA<br>6 adducts/$10^6$ nucl. |
| Gx-dG | 7.7 nmol/mg DNA<br>290 adducts/$10^5$ nucl. | 92 nmol/mg DNA<br>2500 adducts/$10^5$ nucl. | 1.2 nmol/mg DNA<br>37 adducts/$10^5$ nucl. | 2.4 nmol/mg DNA<br>76 adducts/$10^5$ nucl. |

for clarifying the mechanism responsible for mutagenic properties of the chemicals.

In the present study, we identified and determined the structures of two adducts being products of the reaction of 2′-deoxyguanosine with glyoxal carried out at physiological conditions. The adducts, $Gx_1$-dG and $Gx_2$-dG appeared after the reaction time of 1 day and 2 h, respectively, and were shown to arise from the major glyoxal–2′-deoxyguanosine adduct, Gx-dG. The adducts Gx-dG and $Gx_2$-dG were found to be unstable upon storage at neutral conditions and converted in part to $Gx_1$-dG which in turn was found to be stable. All three adducts were detected in the hydrolysates of calf thymus DNA incubated with glyoxal. Also in DNA, Gx-dG and $Gx_2$-dG where not stable, while $Gx_1$-dG was stable and even a slight increase of the amount of $Gx_1$-dG could be noted upon prolonged storage of the DNA.

$Gx_2$-dG is a 2:1 adduct that consists of two units derived from glyoxal and therefore the biological significance of the adduct seems to be questionable. The results of this study indicate however that $Gx_1$-dG is the end product of reaction of glyoxal with the guanine moiety and thus may be of importance for the mutagenic properties of glyoxal. The adduct could also be a potentially reliable biomarker for glyoxal exposure.

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Financial support from the State Committee for Scientific Research (Grant No. 3 T09A 035 26) (D. Pluskota-Karwatka), and from the Graduate School of Organic Chemistry and Chemical Biology at Åbo Akademi University (A.J. Pawłowicz) is acknowledged.

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