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Synthesis of deuterium and C-13 labelled ethyl glycolate and their subsequent use in the synthesis of labelled analogues of the DNA adduct O^6-carboxymethyl-2'-deoxyguanosine

Sharon A. Moore[1*] and David E. G. Shuker[2]

[1]School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool L3 3AF, UK

[2]Department of Chemistry and Analytical Sciences, Open University, Milton Keynes MK7 6AA, UK

Email: s.a.moore@ljmu.ac.uk; Tel: +44 151 231 2049; Fax: +44 151 231 2170

Short title Deuterium and C-13 labelled O^6-carboxymethyl-2'-deoxyguanosine

Abstract

The adduct O^6-carboxymethyl-2'-deoxyguanosine (O^6CMdG) is of importance as it has been previously linked to a high red meat diet in humans and, as yet, a LC-MS method has not been developed due to lack of appropriate standards. The synthesis of the deuterated and C-13 analogues required the use of [$^2H_2$]- and [$^{13}C_2$]ethyl glycolate to label the carboxymethyl moiety of O^6CMdG. [$^2H_2$]Ethyl glycolate was synthesised via acid hydrolysis of ethyl diazoacetate using deuterated solvents (59% yield), whilst [$^{13}C_2$]ethyl glycolate was synthesised from [$^{13}C_2$]glycine in a three step procedure (35% yield). The labelled ethyl glycolates were then used to synthesise [$^2H_2$]- and [$^{13}C_2$]O^6CMdG for future use as internal standards in the LC-MS analysis of biological samples.

Keywords stable labelled synthesis, C-13, H-2, O^6-carboxymethyl-2'-deoxyguanosine, O^6CMdG, ethyl glycolate, LC-MS

Introduction

Cancer is a disease that afflicts approximately one-third of the global population at some point in their lifetimes. Consequently, there is a vast quantity of research into the mechanisms and prevention of cancer, and differences have been found according to geographical regions which has been linked to a numbers of factors including diet[1]. Mutagenic and carcinogenic compounds arise from many exogenous and endogenous routes, including diet, and may result in chemical modifications to the DNA structure, i.e. DNA adducts. One such adduct is O^6-carboxymethyl- 2'-deoxyguanosine (O^6CMdG) that has been linked to nitrosated amines from red meat diets[2]. Previous research has examined this adduct using an immunoslot blot assay which is very sensitive but only a single adduct can be analysed at a time and difficulties can occur due to cross-reactivity of the antibodies[3, 4]. A limited supply of a polyclonal antibody does exist for O^6CMdG but attempts to produce a monoclonal antibody have been unsuccessful and a different technique will therefore be needed in the future. There has been considerable progress in the area of DNA adduct analysis by LC-MS and good sensitivity can be achieved[(e.g.[5])]. Hence, we pursued the use of LC-MS as a more selective, and potentially sensitive, technique to analyse DNA adducts in biological samples. The synthesis of unlabelled O^6CMdG, **1** (Scheme 1), has been reported by other researchers[6] where methyl glycolate was used to furnish the carboxymethyl group at the O6 position of 2'-deoxyguanosine. However, we utilized ethyl glycolate due to the availability of starting materials to insert the labelled atoms for [$^2H_2$]- and [$^{13}C_2$]O^6CMdG, **2** and **3**. Hence this research initially focussed on the synthesis of [$^2H_2$]- and [$^{13}C_2$]ethyl glycolate (**4** and **5** (Scheme 2)) and subsequently that of **2** and **3**.

[Insert Scheme 1]

[Insert Scheme 2]

Experimental

General

All chemicals were purchased from Sigma-Aldrich (Dorset, UK) and used without further purification. Solvents were purchased from Fisher (Loughborough, UK). Organic extracts were dried over $MgSO_4$. NMR data where obtained on Bruker Avance 300 spectrometer at 300.1 MHz (^1H) or 75.5 MHz (^{13}C). Chemical shifts were determined relative to the residual solvent peak and reported as parts per million (ppm) and coupling constants in Hz. Reactions and purifications were monitored by HPLC on a Waters Alliance system equipped with a Waters 996 photodiode array detector with a narrow-bore Hypersil BDS C18 column (3 µm, 100 × 2.1 mm), flow rate 0.2 ml/min, MeOH/0.02 M ammonium acetate (pH 5.4) 80:20. ESI-MS spectra were recorded on VG Quattro mass spectrometer with a narrow-bore Hypersil BDS C18 column (3 µm, 100 × 2.1 mm), flow rate 0.2 ml/min and 0.1% formic acid/methanol gradient.

Glycine ethyl ester hydrochloride

Glycine (0.5 g, 6.66 mmol) was suspended in ethanol (15 cm^3) and acetyl chloride (1.5 cm^3, 21.12 mmol), and heated at 85° C for 30 mins under N_2. The solvents were removed to give a white powder (1.01 g, >100% yield). NMR (D_2O) δ_H ppm 1.19 (3H, t, CH_3, J = 7.14), 3.81 (2H, s, N-CH_2), 4.20 (2H, q, O-CH_2, J = 7.14); δ_C ppm 14.03 (CH_3), 41.05 (N-C), 64.16 (O-CH_2), 169.00 (C=O)

[$^{13}C_2$]Glycine ethyl ester hydrochloride

[$^{13}C_2$]Glycine (0.5 g, 6.49 mmol) was reacted as above to give a white powder (0.93 g, >100% yield). NMR (D_2O) δ_H ppm 1.17 (3H, t, CH_3, J = 7.14), 3.79 (2H, dd, N-CH_2, J^{CH} = 145.64, 6.32), 4.19 (2H, dq, O-CH_2, J^{HH} = 7.14, J^{CH} 2.94); δ_C ppm 14.02 (CH_3), 41.02 (N-C, d, J = 62.40), 64.15 (O-CH_2), 169.00 (C=O, d, J = 62.40)

Ethyl diazoacetate

Glycine ethyl ester.HCl (0.99 g, 7.09 mmol) was dissolved in water (1.8 cm^3) and dichloromethane (4 cm^3), stirred and cooled to -10° C. Aq. sodium nitrite (4.43 M, 1.8 cm^3) was added to the cooled solution, then 5% H_2SO_4 (0.65 cm^3) was added dropwise and the solution stirred for 10 mins. The organic layer was poured into 5% $NaCO_3$ at 0° C. The aqueous layer was extracted with dichloromethane and the organic extract combined with the organic/$NaCO_3$ solutions. The combined solution was shaken thoroughly and the pH checked to ensure that it was alkaline. The solvents were removed to give 0.66 g of a pale yellow oil (5.78 mmol, 82% yield). NMR ($CDCl_3$) δ_H ppm 1.21 (3H, t, CH_3, J = 7.14 Hz), 4.15 (2H, q, CH_2, J = 7.14), 4.67 (1H, CH); δ_C ppm 14.43 (CH_3), 46.11 (CH), 60.84 (CH_2), 166.86 (C=O)

[$^{13}C_2$]Ethyl diazoacetate

[$^{13}C_2$]Glycine ethyl ester.HCl (0.80 g, 5.65 mmol) was reacted as described above to give a pale yellow oil (0.36 g, 55% yield). NMR ($CDCl_3$) δ_H ppm 1.21 (3H, t, CH_3, J = 7.14), 4.15 (2H, dq, CH_2, J^{HH} = 7.14, J^{CH} = 3.30), 4.75 (1H, d, CH, J^{CH} = 259.05); δ_C ppm 14.41 (CH_3), 46.12 (CH, d, J = 96.84), 60.85 (CH_2), 166.87 (C=O, d, J = 96.84)

Ethyl glycolate

Ethyl diazoacetate (0.40 g, 3.51 mmol) was dissolved in dichloromethane (6 cm^3) and 1% aqueous acetic acid (6 cm^3) added and stirred for 72 h in the dark. The aqueous layer was extracted with dichloromethane, and the organic extracts combined with the organic layer, washed with water, dried and the solvents removed to give a pale yellow oil (0.24 g, 66% yield). NMR ($CDCl_3$) δ_H ppm 1.23 (3H, t, CH_3, J = 7.14 Hz), 4.08 (2H, s, CH_2OH), 4.20 (2H, q, CH_2CH_3, J = 7.14); δ_C ppm 14.21 (CH_3), 53.32 (CH_2CH_3), 61.64 (CH_2OH), 173.42 (C=O)

[$^2H_2$]Ethyl glycolate (4)

Ethyl diazoacetate (10 cm^3, 96.41 mmol) was reacted as above substituting D_2O for H_2O, and *d*-acetic acid for acetic acid to give a pale yellow oil (6.05 g, 59% yield). NMR ($CDCl_3$) δ_H ppm 1.28 (3H, t, CH_3, J = 5.36), 4.24 (2H, q, CH_2, J = 5.36)

[$^{13}C_2$]Ethyl glycolate (5)

[$^{13}C_2$]Ethyl diazoacetate (0.40 g, 3.49 mmol) was reacted as for ethyl glycolate to give a pale yellow oil (0.24 g, 66% yield). NMR ($CDCl_3$) δ_H ppm 1.26 (3H, t, CH_3, J = 7.14), 4.08 (2H, dd, CH_2OH, J^{CH} = 145.56, 5.13), 4.15 (2H, dq, J^{HH} = 7.14, J^{CH} = 3.30); δ_C ppm 14.13 (CH_3), 53.40 (CH_2CH_3), 60.53 (CH_2OH, d, J = 58.93), 173.32 (C=O, d, J = 58.93)

O^6-[1-azonia-bicyclo[2.2.2]octane]-3’,5’-bis-*O*-(isobutyryloxy)-2’-deoxyguanosine

Step (i): 2’-deoxyguanosine (1.05g, 3.74 mmol) was dried twice from toluene (20 cm^3) and dissolved in dry tetrohydrofuran (20 cm^3) with 4-(dimethylamino)pyridine (DMAP) (0.024 g, 0.2 mmol) and *iso*-butyric anhydride (2 cm^3, 12.13 mmol) and stirred under N_2 for 16 h. $NaHCO_3$ (aq) was added to give pH 8 and stirred for 2 h. The solvent was removed and the precipitate collected, washed (H_2O) and dried to give a white powder (1.30 g, 85% yield). Step (ii): The product of (i) (1.00 g, 2.45 mmol) was dissolved in dry dichloromethane (30 cm^3) with DMAP (0.03 g, 0.25 mmol) and methanesulfonyl chloride (1.00 g, 8.73 mmol), triethylamine (3 cm^3) was added dropwise and stirred for 3 h. Purification was carried out on silica (chloroform/methanol, 97:3) to give a yellow oil (1.86 g, 98% yield). Step (iii): The product of (ii) was dissolved in dry dichloromethane (30 cm^3) with quinuclidine HCl (1.0 g, 6.77 mmol) and dry triethylamine (3 cm^3) and stirred for 2 h. TLC showed a single blue spot on the baseline (methanol/dichloromethane, 10:90). The solvent was removed and the product used in further reactions without purification.

O^6-Carboxymethyl-2’-deoxyguanosine. Na (1)

Step (iv): The product from (iii) was dissolved in dry dichloromethane (20 cm^3). Methyl glycolate (0.5 cm^3, 6.48 mmol) and 1,8-Diazabicyclo[5.4.0]undec-7-ene (0.5 cm^3, 3.35 mmol) were added and stirred for 16 h. The solvents were removed and the resulting oil purified on silica (methanol/dichloromethane, 5:95) to give a yellow oil (1.60 g). Step (v): the oil was dissolved in methanol (20cm^3) with triethylamine (1 cm^3) and stirred for 72 h. The solvents were removed to give a yellow oil (1.67 g) which was purified on a Supelclean Envi-18 6 ml column (Supelco, USA) pre-conditioned with methanol (2 cm^3), then H_2O (2 cm^3). The sample was applied to the column in H_2O (0.5 cm^3), eluted, washed with H_2O (2 cm^3), then 5% methanol / 95% H_2O (2 cm^3). The pure fractions were lyophilised to give a slightly yellow powder (0.075 g). Step (vi): the powder was dissolved in 0.1 M NaOH (4.4 cm^3) and stirred for 4 h, then lyophilised to give **1** as a white powder (0.080 g, 6.2% overall yield). NMR (DMSO) δ_H 2.21 (1H, m, 2’-H), 2.59 (1H, m, 2’-H), 3.53 (2H, m, 5’-H), 3.83 (1H, m, 4’-H), 4.37 (1H, m, 3’-H), 4.58 (2H, s, CH_2), 5.17 (1H, br, 5’-OH), 5.44 (1H, br, 3’-OH), 6.19 (1H, t, 1’-H, J = 6.27), 6.22 (2H, s, NH_2), 8.06 (1H, s, 8-H); δ_C ppm 39.49 (2’-C), 61.64 (5’-C), 64.86 (CH_2), 70.69 (3’-C), 82.70 (1’-C), 87.57 (4’-C), 114.37 (5-C), 137.23 (8-C), 153.45 (6-C), 159.60 (2-C), 160.74 (4-C), 170.40 (COOH); M^- 324

$[^2H_2]O^6$-Carboxymethyl-2'-deoxyguanosine.Na (2)

Steps (iv-vi) were performed as for **1** substituting **4** (1 cm^3, 10.37 mmol) for methyl glycolate, and D_2O for H_2O to give **2** as a white powder (83.4 mg, 6.4% overall yield). NMR (DMSO) δ_H ppm 2.27 (1H, m, 2'-H), 2.65 (1H, m, 2'-H), 3.60 (2H, m, 5'-H), 3.89 (1H, m, 4'-H), 4.43 (1H, m, 3'-H), 5.19 (1H, br, 5'-OH), 5.45 (1H, br, 3'-OH), 6.27 (1H, t, 1'-H, *J* = 6.87), 6.34 (2H, s, NH_2), 8.14 (1H, s, 8-H); δ_C ppm 35.42 (2'-C), 61.99 (5'-C), 68.41 (CD_2), 70.85 (3'-C), 83.40 (1'-C), 87.82 (4'-C), 114.39 (5-C), 133.95 (8-C), 140.22 (6-C), 143.54 (2-C), 160.15 (4-C), 175.65 (COOH); M^- 326

$[^{13}C_2]O^6$-Carboxymethyl-2'-deoxyguanosine.Na (3)

Steps (iv-vi) were performed as for **1** substituting **5** (0.2 cm^3, 2.07 mmol) for methyl glycolate to give **3** as a white powder (3.5 mg, 0.52% overall yield). NMR (DMSO) δ_H ppm 2.59 (2H, m, 2'-H), 3.57 (2H, m, 5'-H), 3.83 (1H, m, 4'-H), 4.37 (1H, m, 3'-H), 4.53 (1H, dd, CH_2, *J* = 3.83, 145.49), 5.07 (1H, br, 5'-OH), 5.30 (1H, br, 3'-OH), 6.18 (1H, t, 1'-H, *J* = 6.86), 6.30 (2H, s, NH_2), 8.04 (1H, s, 8-H); δ_C ppm 36.90 (2'-C), 61.76 (5'-C), 64.98 (CH_2, *J* = 54.19), 70.76 (3'-C), 82.75 (1'-C), 87.55 (4'-C), 114.43 (5-C), 137.07 (8-C), 153.42 (6-C), 159.57 (2-C), 160.95 (4-C), 169.39 (COOH, *J* = 54.19); M^- 326

Results and discussion

The synthesis of **1** was carried out with methyl glycolate and gave a lower overall yield (6.2%) than previously reported[6] despite several attempts to improve yields at each stage. We then investigated the hydrolysis of ethyl diazoacetate to give ethyl glycolate in both aqueous and deuterated solvents (Scheme 2, step (iii)). As discussed later, the C-13 labelled ethyl glycolate was subsequently required; hence the procedure was extended to give ethyl glycolate from glycine (Scheme 2). Kresge and Popik had shown that the hydrolysis of diazoketones took place in both water and deuterium oxide to give the corresponding alcohol[7]. Whilst they found that the reactions took place at different rates according to the structure of the diazoketone, single products were acquired in high yield. We found that the hydrolysis of ethyl diazoacetate proceeded well under both conditions and with no substantial difference in the yields. The NMR for the unlabelled compound showed a singlet for the CH_2-OH protons at 4.1 ppm that was not present in **4** confirming that the diazo group was converted to the alcohol with 100% incorporation of deuterium at the α-carbon. Subsequently **4** was used to give **2** (Scheme 1) in a low overall yield (6.4%) which was comparable with **1**. Deuterium-proton exchange was evident in **2** as the NMR revealed protons at the CH_2 position (17%) of the carboxymethyl group which had not been present in **4**. This was improved by performing the final step in D_2O (11%) but it is not known whether the exchange occurred during the synthesis or purification of **2**. However, the NMR spectra of intermediates suggest that some exchange was occurring at each stage. Furthermore, the use of the **2** in LC-MS revealed additional deuterium-proton exchange resulting in peaks in the mass spectrum corresponding to single and di-deuterated compounds. Hence, a C-13 labelled O^6CMdG standard (**3**) was required for use as a stable LC-MS standard.

$[^{13}C_2]$Glycine was converted to the ethyl ester by reaction with acyl chloride rather than an acid/ethanol reflux which had given lower yields in initial attempts (data not shown). The conversion to $[^{13}C_2]$ethyl diazoaceate was performed by a known procedure[8], and converted to **5** in the same manner as for **4**. The major difference in the NMR, between the labelled and unlabelled compounds, was either the lack of a peak or the very large coupling constants observed between protons and the C-13 atoms in the labelled compounds which enabled unambiguous assignment of the NMR spectra. The C-13 labelled compounds had peaks for the α-H that showed splitting due to coupling to the two C-13 atoms with the exception of $[^{13}C_2]$ethyl diazoacetate. This compound had broader peaks than the other compounds, possibly due to the diazo group charge distribution, which did not allow observation of the splitting due to coupling with the carbonyl C-13 as well as the α-C. The synthesis of **3** was then performed in the same manner as for **2**, and the double splitting pattern of the α-H was observed. However, for **3**, the limiting factor was the quantity of **5** that was available due to the high cost of the material. Hence, yields for intermediates of Scheme 1 (steps (iv-vi)) of **3** were expected to be much lower and the purification was more difficult due to the high quantity of unreacted precursors. Nevertheless, sufficient pure material was obtained (0.52% yield) for use as an internal standard and the problem of deuterium-proton exchange had been overcome.

Conclusion

Both of the labelled ethyl glycolates, **4** and **5,** were obtained in high yield. However, when **4** was used to synthesise **2**, some deuterium-proton exchange was observed. This was exacerbated when LC-MS analysis was attempted. The synthesis of **5** did not pose any major problems but this compound then became the limiting factor in the synthesis of **3**. In terms of cost, **2** was the preferred option but proved to be problematic for LC-MS analysis. Thus, **5**, the C-13 labelled analogue of O^6CMdG was obtained in sufficient yield for future analyses of biological samples by LC-MS.

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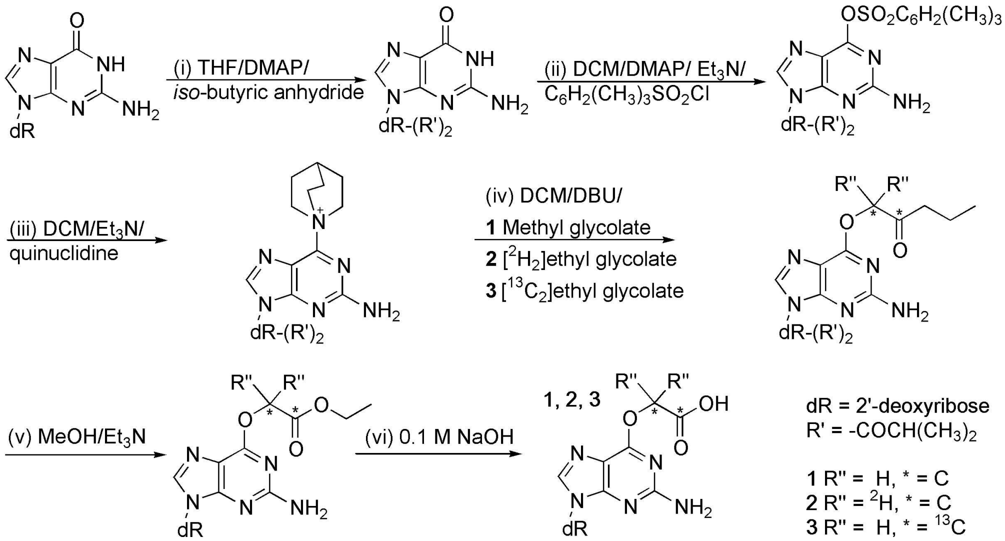
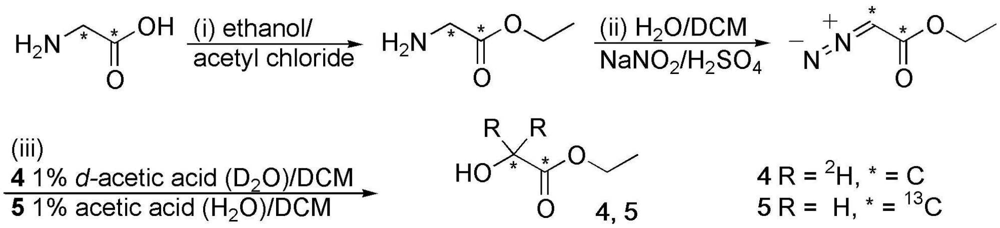
Scheme Legends

Scheme 1 Synthesis of O^6CMdG (**1**), [$^2H_2$]O^6CMdG (**2**) and [$^{13}C_2$]O^6CMdG (**3**)

Scheme 2 Synthesis of [$^2H_2$]- and [$^{13}C_2$]ethyl glycolate (**4** and **5)**



Scheme 1 Synthesis of O^{6}CMdG (**1**), [$^2H_2$]O^{6}CMdG (**2**) and [$^{13}C_2$]O^{6}CMdG (**3**)
204x108mm (300 x 300 DPI)



Scheme 2 Synthesis of [$^2H_2$]- and [$^{13}C_2$]ethyl glycolate (**4** and **5**)
167x36mm (300 x 300 DPI)