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Research Article

Synthesis of 3,7,8-$^{15}N_3$-N^1-(β-D-*erythro*-pentofuranosyl)-5-guanidinohydantoin

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Summary

3,7,8-$^{15}N_3$-N^1-(β-D-*erythro*-pentofuranosyl)-5-guanidinohydantoin was synthesized from the oxidation of 1,7,NH_2-$^{15}N_3$-8-oxo-7,8-dihydro-2′-deoxyguanosine with 2 equivalents of Ir(IV) in pH 4.5 potassium phosphate buffer. The synthesis of 1,7,NH_2-$^{15}N_3$-8-oxo-7,8-dihydro-2′-deoxyguanosine started with bromination of 1,7,NH_2-$^{15}N_3$-2′-deoxyguanosine. The resulting 1,7,NH_2-$^{15}N_3$-8-bromo-7,8-dihydro-2′-deoxyguanosine reacted with sodium benzyloxide to afford 1,7,NH_2-$^{15}N_3$-8-benzyloxy-7,8-dihydro-2′-deoxyguanosine. Subsequent catalytic transfer hydrogenation of 1,7,NH_2-$^{15}N_3$-8-benzyloxy-7,8-dihydro-2′-deoxyguanosine with cyclohexene and 10% Pd/C yielded 1,7,NH_2-$^{15}N_3$-8-oxo-7,8-dihydro-2′-deoxyguanosine. Purification of 3,7,8-$^{15}N_3$-N^1-(β-D-*erythro*-pentofuranosyl)-5-guanidinohydantoin was first carried out on a C18 column and the product was further purified on a graphite column. ESI-MS was used to confirm the identity and to determine the isotopic purity of all the labeled compounds. The isotopic purity of 3,7,8-$^{15}N_3$-N^1-(β-D-*erythro*-pentofuranosyl)-5-guanidinohydantoin was 99.4 atom% based on LC-MS measurements. Copyright © 2003 John Wiley & Sons, Ltd.

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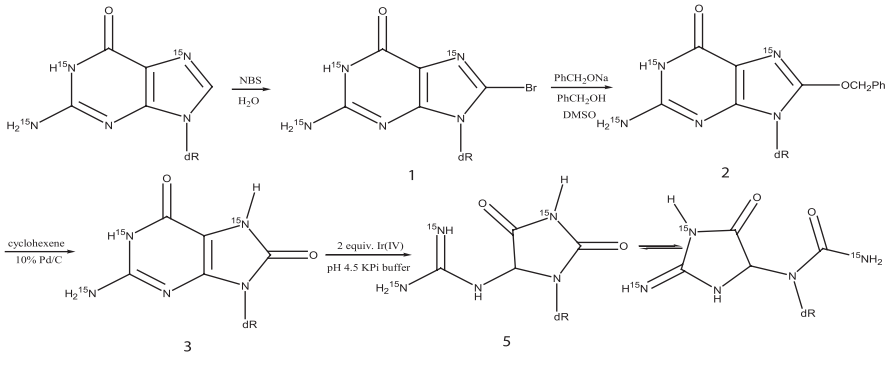
Introduction

Nitric oxide ($^{\bullet}NO$) is a physiological mediator of many cellular processes,[1] but has deleterious effects if overproduced.[2] During chronic inflammation, upregulation of inducible nitric oxide synthase (iNOS) leads to high $^{\bullet}NO$ fluxes, which can react with superoxide ($O_2^{\bullet -}$) to form peroxynitrite($ONOO^-$).[3] Peroxynitrite, a very reactive species, decomposes to nitrogen dioxide radical ($^{\bullet}NO_2$) and carbonate radical anion ($CO_3^{\bullet -}$) in the presence of CO_2. Both $^{\bullet}NO_2$ and $CO_3^{\bullet -}$ radicals are oxidizing agents. $^{\bullet}NO_2$ is a nitrating agent as well. Therefore, peroxynitrite induces both oxidative and nitrative damage to a wide variety of biological components, including lipids, proteins and DNA. The damage of DNA by peroxynitrite has received intense attention because it is potentially toxic or mutagenic, thereby contributing to tissue damage and increased cancer risk. Guanine, with the lowest redox potential of four DNA bases,[4] is highly susceptible to oxidation by peroxynitrite.[5,6] To date, a series of products from the oxidation of 2′-deoxyguanosine(dGuo) by peroxynitrite have been identified. The major products include 8-oxo-7,8-dihydroguanosine(8-oxo-dGuo),[7] spiroiminodihydantoin,[8] guanidine-hydantoin,[9] oxazolone,[6] and nitroimidazole.[10] 8-Oxo-dGuo is reported to be at least 1000-fold more reactive toward peroxynitrite than dGuo and can be further oxidized to various products.[11] Several research groups demonstrated that guanidinohydantoin was produced from the oxidation of 8-oxo-dGuo or dGuo in duplex DNA.[12–15] We are particularly interested in the nature of DNA damage induced by peroxynitrite and its genotoxic consequences. We have identified and measured several peroxynitrite-induced lesions in DNA.[16] Because of the very low levels of these lesions in DNA (about 1 lesion in 10^7 DNA bases), isotope dilution mass spectrometry was used to ensure reliable measurements using isotope-labeled internal standards of the lesions of interest. Here, we report the synthesis of 3,7,8-$^{15}N_3$-N^1-(β-D-*erythro*-pentofuranosyl)-5-guanidinohydantoin as an internal standard for the quantitation of peroxynitrite induced N^1-(β-D-*erythro*-pentofuranosyl)-5-guanidinohydantoin in DNA.

Results and discussion

3,7,8-$^{15}N_3$-N^1-(β-D-*erythro*-pentofuranosyl)-5-guanidinohydantoin (**5**) was prepared according to the Scheme. The synthesis of 1,7,NH_2-$^{15}N_3$-8-oxo-7,8-dihydro-2′-deoxyguanosine (**3**) was based on a published method[17] with several modifications to improve the yield. The first modification was to use N-bromosuccinimide (NBS),[18] instead of Br_2/H_2O, as a brominating agent to avoid deglycosylation of 1,7,NH_2-$^{15}N_3$-8-bromo-2′-deoxyguanosine (**1**) under the acidic conditions inherent in the Br_2/H_2O bromination. The second modification was introduced in the synthesis of 1,7,NH_2-$^{15}N_3$-8-benzyloxy-7,8-dihydro-2′-deoxyguanosine (**2**). Anhydrous benzyl alcohol and sodium hydride were used to prepare sodium benzyloxide under N_2 protection. Anhydrous DMSO was used to dissolve **1**. The air and moisture-free reaction conditions greatly improved the yield of **2**. The third modification was to not isolate **2** to avoid its loss during the purification. Instead the entire mixture containing **2** was catalytically hydrogenated to afford **3** at 70% yield from **1**.

Scheme Synthesis of 5



Scheme 1.

The synthesis of **5** basically followed the procedure reported by Luo *et al*. for the preparation of N^1-(2′,3′,5′-tri-O-acetyl-β-D-*erythro*-pentanosyl)-5-guanidinohydantoin (**6**).[9] The oxidation of **3** was carried out in pH 4.5 potassium phosphate buffer to minimize the formation of spiroiminodihydantoin. Two equivalents of Ir(IV) was added in 4 equal portions every 5 min to avoid over-oxidation of **5**. N^1-(β-D-*erythro*-pentofuranosyl)-5-guanidinohydantoin (**4**, Gh) is known to undergo pH

and temperature-dependent isomerization to iminoallantoin (Ia).[9] The dynamic isomerization between Gh and Ia, and their highly hydrophilic nature (in contrast to **6**), made the purification of Gh/Ia a challenging process. The Gh/Ia mixture of **4** (or **5**) was first purified on a C18 column with 0.1% TFA in water as the eluent. At this pH, both Gh and Ia are positively charged and may form ion-pairs with trifluoroacetate anions. The Gh/Ia mixture was eluted as two peaks at 8.6 and 9.1 min. Further purification to obtain salt-free Gh/Ia mixture was carried out on a graphite column, which elutes inorganic salts with the solvent front while retaining polar organic compounds efficiently. On this column Gh/Ia also were eluted at 12.6 and 13.3 min. The HPLC retention times of **4** and **5** were identical.

The identity of each labeled compound was confirmed by comparing its UV spectra and HPLC retention times with those of the corresponding unlabeled compound. Electrospray ionization-mass spectrometric analyses of the labeled compounds and their unlabeled counterparts were also carried out. The ESI-MS of **4** and **5** are shown in Figure 1 (A and B). For each compound the protonated molecular ion ($[M+H]^+$) was observed along with a major fragment ($[B+H_2]^+$) corresponding to the loss of the 2′-deoxyribose moiety. The ESI-MS data of **1**, **2**, **3**, **5** and their unlabeled counterparts are listed in Table 1.

Figure ESI-MS of **4** (A) and **5** (B)

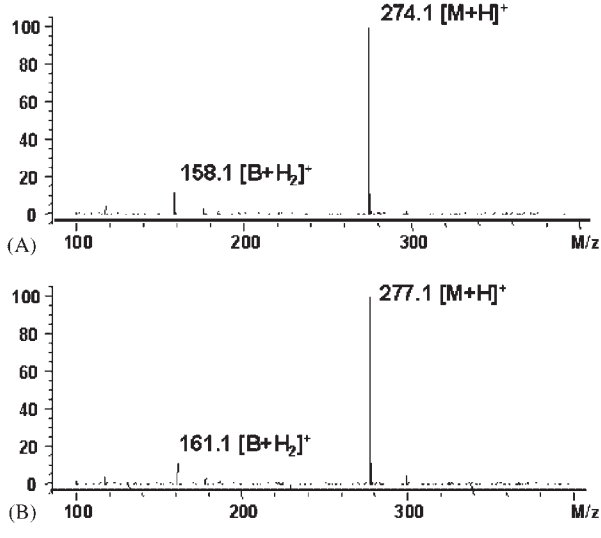


Figure 1.

Table 1. ESI-MS and isotopic purity of 1, 2, 3, 5 and ESI-MS of the corresponding unlabeled compounds

Labeled	$[M+H]^+$	$[B+H_2]^+$	Isotopic Purity (at%)	Unlabeled	$[M+H]^+$	$[B+H_2]^+$
1	349.1	233.0	98.8	8-Br-dGuo	346.1	230.0
2	377.2	211.2	99.8	8-benzyloxy-dGuo	374.2	208.2
3	287.2	171.1	99.9	8-oxo-dGuo	284.2	168.2
5	277.1	161.1	99.4	**4**	274.1	158.1

The isotopic purity of each labeled compound was determined by LC-ESI-MS measurements. The ratio between fragments $[M+H]^+$ and $[M+H\text{-}3]^+$ was measured and calculated. The isotopic purity of each labeled compound is also listed in Table 1.

Experimental

All reagents were of commercial quality. 8-Oxo-7,8-dihydro-2′-deoxyguanosine was purchased from Sigma (St. Louis, MO). Bromine, sodium hydride, anhydrous benzyl alcohol, anhydrous DMSO, cyclohexene, 10% Pd/C were purchased from Aldrich (Milwaukee, WI). Glacial acetic acid and acetonitrile (HPLC grade) were purchased from EM Science (Darmstadt, Germany). Sodium hexachloroiridate (IV) was purchased from Alfa Aesar® (Ward Hill, MA). Water was deionized and distilled from $KMnO_4$. Centrifugation was carried on an Eppendorf centrifuge 5415D. Freeze-drying was carried out on a Savant Speed Vac® SC110. Vortexing was carried out on an S|P® Vortex Mixer. Analytical HPLC analyses were conducted on either a Supelco SUPELCOSIL™ LC-18-DB column (250 × 2.1 mm, 5 μm), an Agilent Zorbax Eclipse XDB C18 column (250 × 4.6 mm, 5 μm), or a Thermo Hypersil-Keystone Hypercarb column (3.0 × 150 mm, 5 μm). Preparative HPLC was carried on an Alltech Nucleosil C18 column (250 × 10 mm, 5 μm). LC-MS analyses were carried out on an Agilent 1100 Series LC/MSD with quaternary pumps, a degasser, an auto-injector, a temperature controller, a diode array detector, and a single-quadruple mass-selective detector. Typical LC-MS analysis conditions were: LC: 0.1% formic acid in H_2O and CH_3CN as mobile phase at 0.2 ml/min; MS: nebulizer gas pressure at 45 psig, drying gas at 11.0 l/min and 300°C, quad temperature at 100°C, and fragmentor voltage at 100 V. UV spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer.

*1,7,NH_2-$^{15}N_3$-8-Bromo-2′-deoxy-guanosine (**1**)*

The synthesis of **1** was a modification of Gannett and Sura's method.[18] 1,7,NH_2-$^{15}N_3$-2′-deoxyguanosine (a gift from R. Jones, 6.8 mg, 0.025 mmol)[19] was suspended in 250 µl of water in a 1.5-ml Eppendorf tube and the resulting mixture was cooled in an ice bath. To the vigorously stirred mixture a total of 265 µl of N-bromosuccinimide (6.7 mg, 0.038 mmol) aqueous suspension was added in 13 portions (20 µl every 3 min). **1** precipitated. The Eppendorf tube was centrifuged at a speed of 13 000g for 1 min at 4°C, the supernatant was removed and the remaining solids were washed and centrifuged with ice-water (3×150 µl) and cold acetone (4×90 µl). The collected solids were dried *in vacuo* overnight to afford 3.0 mg of dry **1**. The supernatants from each centrifugation were pooled. The remaining starting material and **1** in the supernatant were purified by preparative HPLC to afford 2.6 mg of **1** and 2.0 mg of 1,7,NH_2-$^{15}N_3$-2′-deoxyguanosine (HPLC conditions: acetonitrile and water at 3.0 ml/min; acetonitrile increased from 5% to 25% in 20 min; 1,7,NH_2-$^{15}N_3$-2′-deoxyguanosine retention time: 8.5 min; **1** retention time: 12.7 min). The purified **1** was 5.6 mg (90%, based on the consumed 1,7,NH_2-$^{15}N_3$-2′-deoxyguanosine) (ESI-MS of **1**: 349.1 $[M+H]^+$ and 351.1 $[M+H+2]^+$ (1:1 peak ratio); 233.1 and 235.1 $[B+H_2]^+$ and $[B+H_2+2]^+$ (1:1 peak ratio)).

*1,7,NH_2-$^{15}N_3$-8-Benzyloxy-2′-deoxyguanosine (**2**)*

The synthesis of **2** is a modification of a published method.[20] Sodium hydride (4.6 mg, 0.19 mmol) was dissolved in 225 µl of anhydrous benzyl alcohol under N_2 protection. The mixture was stirred at room temperature for 30 min to afford a homogeneous solution. **1** (2.5 mg, 7.2 µmol) was dissolved in 200 µl of anhydrous DMSO. The resulting solution was added to the sodium benzyloxide/benzyl alcohol solution and heated at 88°C for 5 h as the color gradually changed to red. After cooling to room temperature, 10.4 µl of glacial acetic acid was added, leading to formation of a precipitate. To the resulting mixture 20 ml of ethyl ether was added. The mixture was vigorously vortexed and centrifuged. The solids were collected and washed with ethyl ether (3×2 ml) and dried in air. A portion of the solids (0.5 mg) was dissolved in 1 ml of ethanol. An aliquot of the solution was analyzed by LC-MS on the LC-18-DB column (HPLC conditions: acetonitrile and 0.1% formic acid in water at 0.2 ml/min; acetonitrile increased from 20% to

40% in 20 min; **2** retention time: 9.1 min; ESI-MS: 374.2 $[M+H]^+$ and 258.2 $[B+H_2]^+$).

*1,7,NH_2-$^{15}N_3$-8-Oxo-7,8-dihydro-2′-deoxyguanosine (**3**)*

The solids (200 mg) containing **2** from the above reaction were dissolved in 6 ml of ethanol and 0.5 ml of water. To the solution 1.34 ml of cyclohexene and 32 mg of 10% Pd/C were added. The mixture was heated at 80°C for 1 h. The catalyst was filtered off and washed with 10 ml of ethanol and water (1:1 v/v). Solvents of the filtrate were evaporated *in vacuo*. The residue was redissolved in 3.0 ml of water and purified by preparative HPLC (HPLC conditions: acetonitrile and water at 3.0 ml/min; 20% acetonitrile for the first 6 min, 50% acetonitrile from 7 to 10 min; **3** retention time: 3.5 min; **2** retention time: 7.1 min) to afford 1.4 mg of **3** (70%). The ESI-MS of **3**: 287.2 $[M+H]^+$ and 171.1 $[B+H_2]^+$.

*N^1-(β-D-erythro-pentofuranosyl)-5-guanidinohydantoin (**4**)*

The synthesis of **4** followed Luo's procedure.[9] A 500 µl aqueous solution of unlabeled 8-oxodGuo (2.34 mM, 1.2 µmol) was mixed with 500 µl of potassium phosphate buffer (150 mM, pH 4.5). To the resulting solution a total of 80 µl of sodium hexachloroiridate(IV) aqueous solution (29.07 mM, 2.4 µmol) was added in 4 portions (20 µl every 5 min). After the addition of Ir(IV) the resulting solution was stirred at room temperature for 15 min. **4** was first purified on the Zorbax Eclipse XDB C18 column (HPLC conditions: 100% 0.1% TFA in water at 0.4 ml/min for 10 min; **4** retention time at 8.8 and 9.2 min) and further purified on the graphite column (HPLC conditions: 0.1% TFA in water and acetonitrile at 0.4 ml/min; acetonitrile increased from 0% to 9% in 15 min; **4** retention time at 12.6 and 13.3 min) to afford 0.72 µmol of pure **4** (60%) (determined by the UV absorbance at 220 nm).[9]

*3,7,8-$^{15}N_3$-N^1-(β-D-erythro-pentofuranosyl)-5-guanidinohydantoin (**5**)*

The synthesis of **5** was similar to that of **4** except that 50 µl aqueous solution of **3** (1.66 mM, 0.083 µmol) and 40 µl of Ir(IV) aqueous solution (4.14 mM, 0.16 µmol) were used to afford 0.043 µmol of pure **5** (52%) (determined by the UV absorbance at 220 nm).[9]

Conclusion

1,7,NH_2-$^{15}N_3$-8-Oxo-7,8-dihydro-2′-deoxyguanosine was synthesized from 1,7,NH_2-$^{15}N_3$-2′-deoxyguanosine with an overall yield of 63%. 3,7,8-$^{15}N_3$-N^1-(β-D-*erythro*-pentofuranosyl)-5-guanidinohydantoin and N^1-(β-D-*erythro*-pentofuranosyl)-5-guani-dinohydantoin were synthesized with yields of 52% and 60%, respectively, from the corresponding 8-oxo-7,8-dihydro-2′-deoxyguanosine. The high isotopic purity of 3,7,8-$^{15}N_3$-N^1-(β-D-*erythro*-pentofuranosyl)-5-guanidinohydantoin makes it an excellent internal standard in the measurement of N^1-(β-D-*erythro*-pentofuranosyl)-5-guanidinohydantoin in peroxynitrite oxidized DNA using isotope-dilution mass spectrometry.

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