The Formation of *S*-[1-(N^2-deoxyguanosinyl)methyl]glutathione between Glutathione and DNA Induced by Formaldehyde

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Experimental Procedures

Chemicals and Enzymes. Glutathione, deoxyguanosine, calf thymus DNA, potassium phosphate, Tris-HCl, $MgCl_2$, formic acid, methanol, acetonitrile, HPLC grade water were all purchased from Sigma (St. Louis, MO). 20% formaldehyde in water was procured from Tousimis (Rockville, MD). DNase I was purchased from New England Biolabs (Ipswich, MA). Alkaline phosphatase and phosphodiesterases were ordered from Fisher Scientific (Pittsburgh, PA). All chemicals were used as received unless otherwise stated.

Instrumentation

High Performance Liquid Chromatography (HPLC). The purification of *S*-[1-(N^2-deoxyguanosinyl)methyl]glutathione was carried out on an Agilent 1200 series HPLC system equipped with a diode-array detector (Santa Clara, CA). Analytes were separated by reverse phase chromatography using a 250 mm × 2.5 mm 218MS52 analytical column from Grace Vydac (Hesperia, CA). A linear gradient was run from 2% methanol in 0.1% aqueous formic acid to 60% methanol over 15 min, at a flow rate of 200 μL/min and monitored at 254 nm. 25 μL of mixture from the small scale reaction was injected to determine retention times. *S*-[1-(N^2-deoxyguanosinyl)methyl]glutathione eluted at 11.8 min in this system. For the large scale reaction, 100 μL of reaction mixture was used for each injection to collect *S*-[1-(N^2-deoxyguanosinyl)methyl]glutathione for NMR characterization.

Liquid Chromatography-Mass Spectrometry (LC-MS). LC-MS analyses were performed on a triple quadrupole mass spectrometer TSQ-Quantum (Thermo Electron, Waltham, MA) operating in selected reaction monitoring (SRM) mode to detect and

quantify *S*-[1-(N^2-deoxyguanosinyl)methyl]glutathione. A 150 mm × 2.5 mm Hypersil Gold column (3 µm particle size) from Thermo Scientific (Pittsburgh, PA) was used. A linear gradient was run from 2% methanol in 0.1% aqueous formic acid to 60% methanol over 10 min, at 200 µL/min. The electrospray ionization (ESI) source was set as follows: spray voltage, 4.0 kV; capillary temperature, 300 °C; sheath gas pressure, 40 au; aux gas pressure, 10 au. *S*-[1-(N^2-deoxyguanosinyl)methyl]glutathione was detected at 7.10 min. The exact mass and MS/MS data were acquired on an Agilent 6500 Series Quadrupole Time-of-Flight (Q-TOF) LC/MS (Santa Clara, CA) with an ESI source. A linear gradient was run from 2% acetonitrile in 0.1% formic acid to 98% acetonitrile in 10 min at 200 µL/min. The ESI source was set as follows: gas temperature, 350 °C; drying gas, 10 L/min; Vcap, 4000 V; Nebulizer, 35 psig; fragmentor, 100 V; skimmer, 65 V. A 150 mm × 2.5 mm Hypersil Gold column (3 µm particle size) was used and *S*-[1-(N^2-deoxyguanosinyl)methyl]glutathione was detected at 4.8 min. For fragmentation of *S*-[1-(N^2-deoxyguanosinyl)methyl]glutathione, the collision energy was set at 20 V.

Quantitation of *S*-[1-(N^2-deoxyguanosinyl)methyl]glutathione from DNA Samples. The adduct was quantified on the TSQ-Quantum (Thermo Electron, Waltham, MA) using SRM mode (587m/z→308m/z). The collision energy was set at 20 V after optimization. The calibration curve for quantitation was obtained using the integrated peak area and amount of injected standard, as shown in Figure S5.

NMR. NMR spectra were recorded on a Varian INOVA 500 NMR spectrometer (Palo Alto, CA) at 500 MHz for ^{1}H and 125 MHz for ^{13}C spectra. HMBC data were acquired using the standard Varian program gHMBC, with a mixing time of 62.5 msec.

Experimental Methods. Glutathione (5 mM) was incubated with formaldehyde (2.5 mM) in 40 μL of 10 mM potassium phosphate buffer (pH=7.2) for 4 h at 37 °C. Then, 10 μL of 10 mM deoxyguanosine was added and further incubated for 8 h. The resultant reaction mixtures were either separated by reverse phase chromatography or analyzed by mass spectrometry. To prepare larger quantities of product for NMR characterization and for use as a standard, 92 mg of glutathione were treated with 100 mM formaldehyde in 3.5 mL of 100 mM potassium phosphate buffer (pH=7.2) for 4 h at 37 °C, followed by incubation with 8 mg of deoxyguanosine for 6 h at 37 °C. The resultant reaction mixture was purified by HPLC using a C18 reverse phase column. HPLC fractions corresponding to *S*-[1-(N^2-deoxyguanosinyl)methyl]glutathione were collected and dried by lyophilization, followed by ^{1}H NMR and 2D NMR analysis. To measure adduct formation between DNA and GSH, 5 mM GSH solution was first treated with different concentrations of formaldehyde (0.1, 0.5, 1, 5, 50 mM) in 100 μL of 10 mM potassium phosphate buffer (pH=7.2) for 4 h at 37 °C, followed by incubation with 100 μg of calf thymus DNA for another 12 h. The resultant modified DNA was washed with water 5 times using a Millipore Microcon YM-10 spin column. Then, DNA was treated by DNaseI (50U) for 30 min in the digestion buffer (80mM Tris-HCl 20mM $MgCl_2$ pH=7.2), followed by the addition of alkaline phosphatase (2U) and phosphodiesterases (1U) for additional 1 h. Enzymes were removed by a Millipore Microcon YM-10 spin column and the resultant solution was separated by HPLC. The fraction containing *S*-[1-(N^2-deoxyguanosinyl)methyl]glutathione was dried with speed vacuum, followed by detection with mass spectrometry.

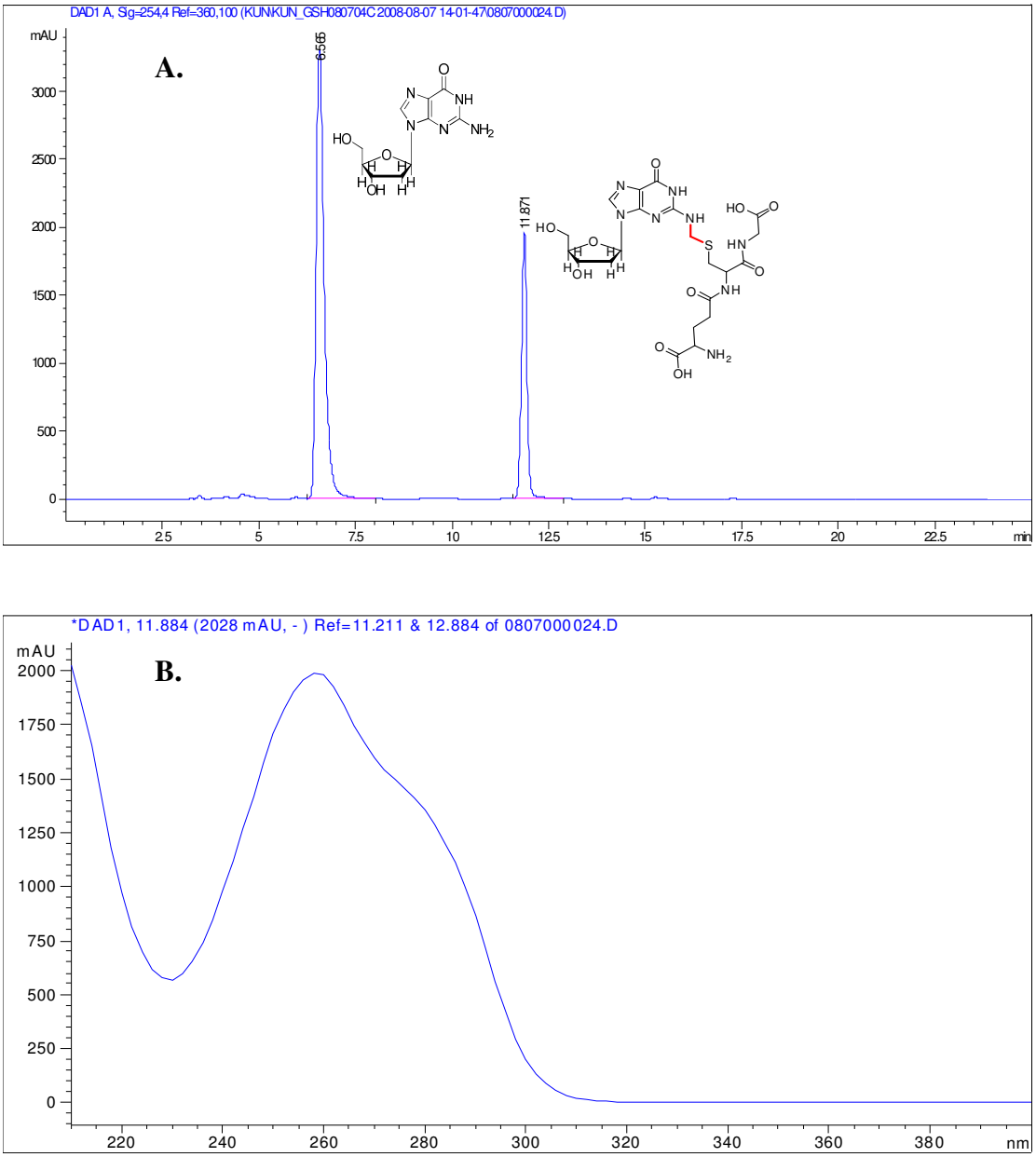

Figure S1. HPLC analysis of the products of reaction between glutathione, formaldehyde and deoxyguanosine. Panel A, chromatogram (254 nm) of the complete reaction mixture. Panel B, UV-Vis spectrum (diode array detector) of the peak at 11.9 min, identified as *S*-[1-(N^2-deoxyguanosinyl)methyl]glutathione.

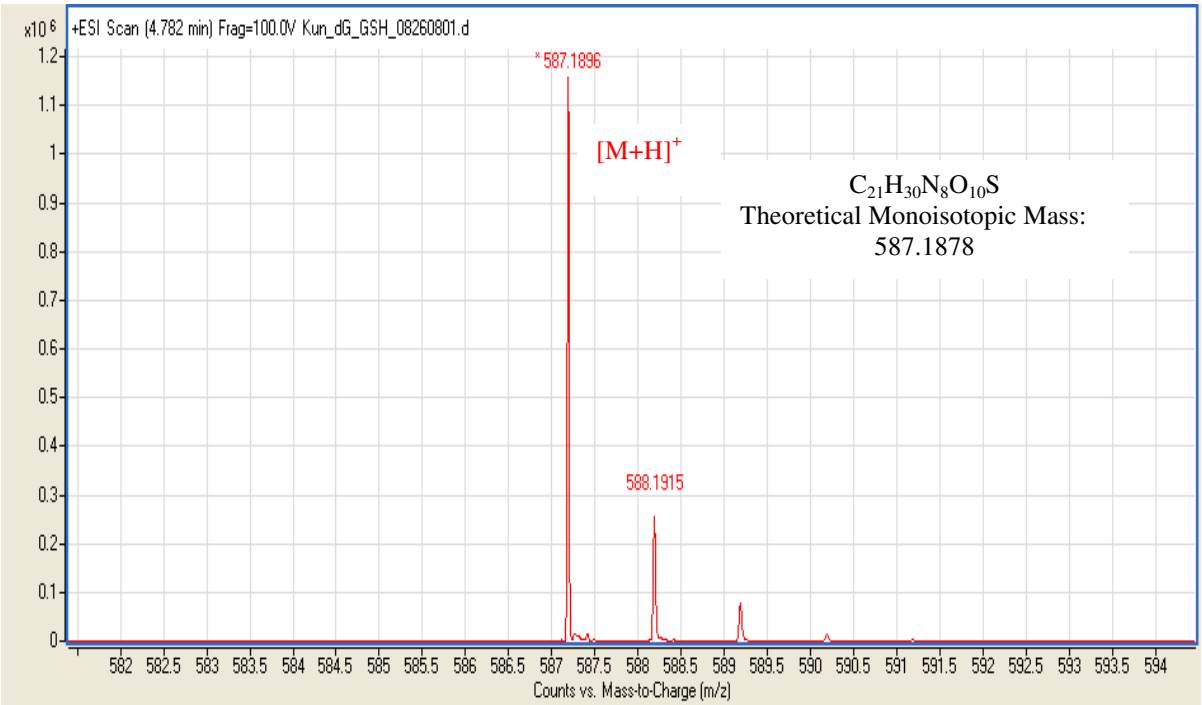

Figure S2. Exact mass of *S*-[1-(N^2-deoxyguanosinyl)methyl]glutathione.

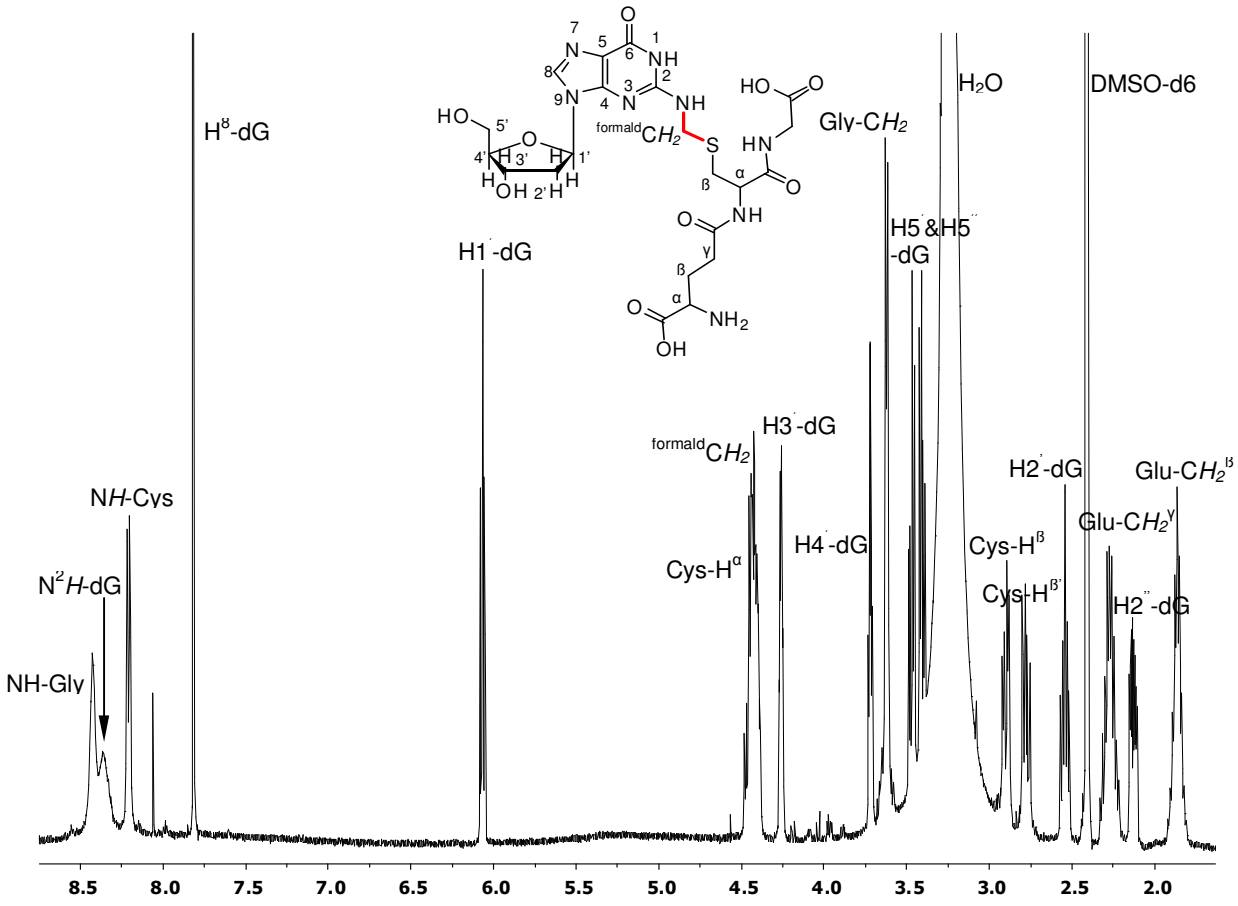

Figure S3. ^{1}H NMR spectrum [Varian INOVA 500 NMR spectrometer (Palo Alto, CA), at 500 MHz] of *S*-[1-(*N*^{2}-deoxyguanosinyl)methyl]glutathione.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.51 (bt, 1H, NH-Gly), 8.45 (bs,1H, N^{2}*H*-dG), 8.30 (bd, 1H, *J* = 8.5 Hz, N*H*-Cys), 7.90 (s, 1H, H8-dG), 6.15 (ψt, 1H, *J* ~ 7 Hz, H1′-dG), 4.49-4.4 (m, 3H, Cys-H$^{\alpha}$, ^{formald}CH_2 overlapping), 4.35 (td,1H, *J* = 6.0, 3.2, 3.0 Hz, H3′-dG), 3.81 (m, 1H, *J* = 4.9, 4.8, 3.1 Hz, H4′-dG), 3.71 (d, 2H, *J* = 6.7 Hz, Gly-CH_2), 3.56 (dd, 1H, *J* = 11.6, 4.9 Hz, H5′ or H5″-dG), 3.49 (dd, 1H, *J* = 11.6, 4.8 Hz, H5″ or H5′-dG), 2.99 (dd,1H, *J* = 14.0, 5.0 Hz, Cys-H$^{\beta}$), 2.87 (dd,1H, *J* = 14.0, 7.4 Hz, Cys-H$^{\beta'}$), 2.63 (ddd, 1H, *J* = 13.3, 7.3, 6.0 Hz, H2′-dG), 2.36 (m, 2H, Glu-CH_2^{γ}), 2.22 (ddd, 1H, *J* = 13.3, 6.3, 3.2 Hz, H2″-dG), 1.95 (m, 2H, Glu-CH_2^{β}).

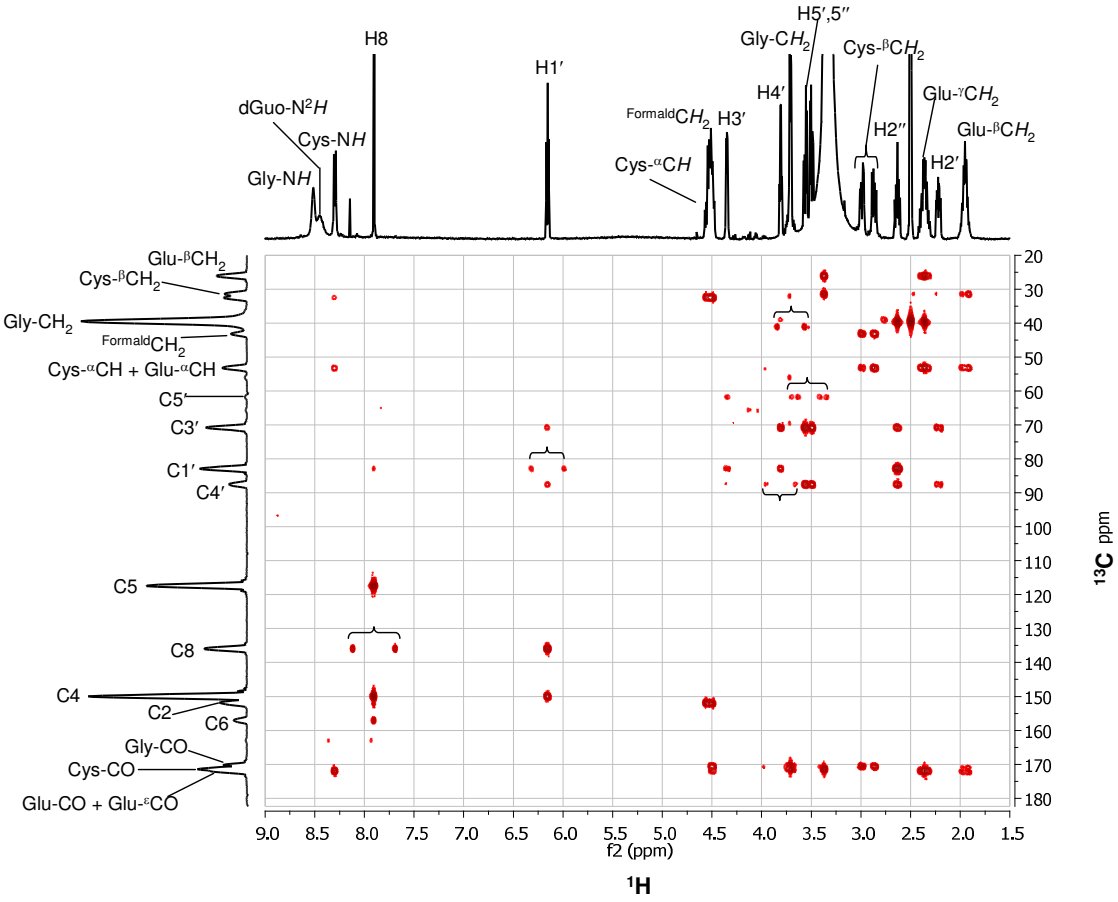

Figure S4. HMBC spectrum of *S*-[1-(*N*^{2}-deoxyguanosinyl)methyl]glutathione. Unsuppressed ^1J splittings are indicated on the spectrum by brackets.

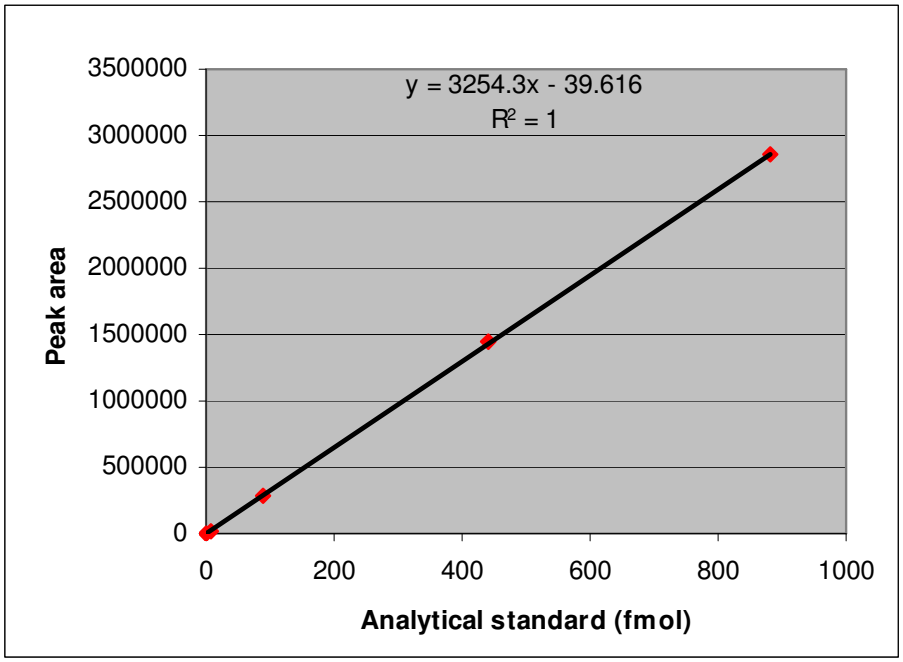

Figure S5. Calibration curve used for the quantitation of *S*-[1-(*N*^{2}-deoxyguanosinyl)methyl]glutathione from DNA. Adduct 5 isolated from large-scale reaction and quantitated by HPLC served as the standard.

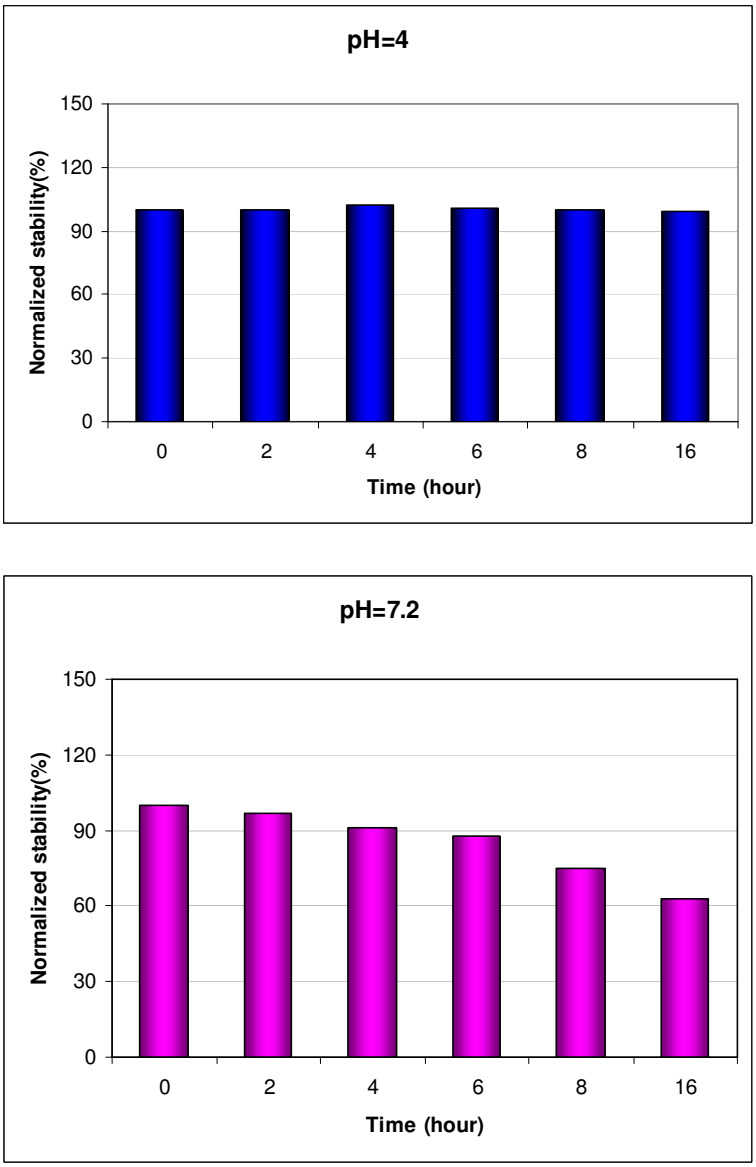

Figure S6. Stability of *S*-[1-(*N*^{2}-deoxyguanosinyl)methyl]glutathione in aqueous solutions (pH=4 and pH=7.2). Standard (100 µL, 4.14 nmol) was dissolved in 900 µL of water containing 0.1% formic acid (pH=4) or DNA digestion buffer (80mM Tris-HCl 20mM $MgCl_2$ pH=7.2). Solutions were stored at room temperature, 100 µL aliquots were analyzed at specified time points (0, 2, 4, 6, 8 and 16 h) by HPLC. Stability was calculated from the integrated peak area for each injection, relative to time = 0 h.