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Reconsidering the chemical nature of strand breaks derived from abasic sites in cellular DNA: evidence for 3'-glutathionylation

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Abstract

The hydrolytic loss of coding bases from cellular DNA is a common and unavoidable reaction. The resulting abasic sites can undergo β-elimination of the 3'-phosphoryl group to generate a strand break with an electrophilic α,β-unsaturated aldehyde residue on the 3'-terminus. The work reported here provides evidence that the thiol residue of the cellular tripeptide glutathione rapidly adds to the alkenal group on the 3'-terminus of an AP-derived strand break. The resulting glutathionylated adduct is the only major cleavage product observed when β-elimination occurs at an AP site in the presence of glutathione. Formation of the glutathionylated cleavage product is reversible but, in the presence of physiological concentrations of glutathione, the adduct persists for days. Biochemical experiments provided evidence that the 3'-phosphodiesterase activity of the enzyme apurinic/apyrimidinic endonuclease (APE1) can remove the glutathionylated sugar remnant from an AP-derived strand break to generate the 3'OH residue required for repair via base excision or single-strand break repair pathways. The results suggest that a previously unrecognized 3'glutathionylated sugar remnant – and *not* the canonical α,β-unsaturated aldehyde end group –

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Supporting Information

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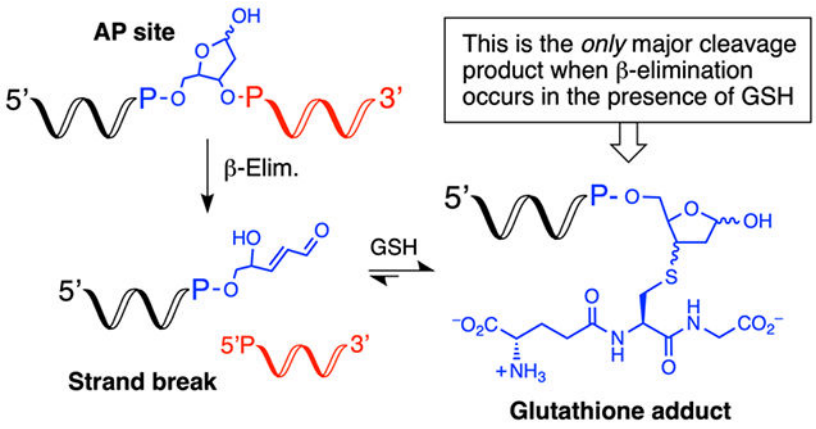
Treatment of AP-containing duplex with GSH, ESI-TOF-MS analysis of 3'GS-ddR adduct in DNA, NMR spectra of the nucleosidic GS-ddR adduct, gel electrophoretic analyses of the stability of the 3'GS-ddR adduct under various conditions, trimming of the 3'dR end group from a DNA strand break by APE1, and treatment of the 3'GS-ddR cleavage product with Fpg.

The authors declare no conflicts of interest

may be the true strand cleavage product arising from β-elimination at an abasic site in cellular DNA. This work introduces the 3'glutathionylated cleavage product as the major blocking group that must be trimmed to enable repair of abasic site-derived strand breaks by the base excision repair or single-strand break repair pathways.

Graphical Abstract



INTRODUCTION

The sequence of nucleobases in DNA provides the genetic information that guides the operation of living organisms.[1–2] Stability of the genetic material is critical for life, yet cellular DNA is constantly subject to unavoidable chemical modification involving reactions with water, reactive oxygen species, and various electrophilic metabolites.[3–5] Cellular repair systems evolved to correct the resulting DNA damage,[6] but some lesions inevitably evade repair, with important biological consequences including mutation, cancer, aging, and neurodegeneration.[7–11]

There are many different types of endogenous DNA damage,[3–5, 12–13] but single-strand breaks are among the most common unavoidable lesions.[14–16] Unrepaired strand breaks are cytotoxic because they can lead to double-strand breaks and replication fork collapse in dividing cells.[17–20] The biological significance of single-strand breaks is highlighted by the fact that hereditary defects in proteins that repair these lesions cause various neurodegenerative diseases.[14, 16, 21–27]

A variety of processes can give rise to DNA strand breaks including the attack of radicals on the deoxyribose-phosphate backbone,[28–29] the excision of misincorporated ribonucleotides by RNase H2,[30–31] and stalled topoisomerase-DNA complexes.[32] Abasic (apurinic/apyrimidinic, AP) sites arising from spontaneous[33] or enzyme-catalyzed[34–35] hydrolysis of the glycosidic bonds in DNA are another source of strand breaks in cells (Scheme 1). The acidic character of the α-protons[36] in the ring-opened aldehyde form of the AP site[37] enables spontaneous strand cleavage via β-elimination of the 3'-phosphate residue (Scheme 1).[38–40] Low molecular weight cellular polyamines[41–42] and amine residues of DNA-binding and DNA-repair proteins can catalyze strand scission at AP sites.[34, 43–55]

This process proceeds via a covalent mechanism involving conversion of the AP aldehyde to an iminium ion,[44, 47, 56–60] in which the α-protons are more acidic than those of the corresponding aldehyde.[61–64]

Earlier biochemical studies showed that spontaneous and enzyme-catalyzed β-elimination at an AP site in DNA initially generates a strand break with 5'-phosphoryl (5'P) and 3'-*trans*-phospho-α,β-unsaturated aldehyde (3'*trans*-PUA) end groups at the nick (Scheme 1).[45–46, 65] As an aside, the 3'-sugar remnant generated by β-elimination is often incorrectly drawn as the *cis*-isomer.[4, 34, 66–67] The early results led to the widespread belief that the 3'*trans*-PUA strand cleavage product must be present in cellular DNA. For example, most reviews of base excision repair (BER) describe the 3'*trans*-PUA end group as the intermediate that must be "cleaned" from the 3'-end of an AP-derived strand break to enable repair synthesis.[34, 68] However, two facts suggest that 3'*trans*-PUA may not be the ultimate cleavage product resulting from β-elimination at AP sites in cellular DNA. First, cells contain high concentrations (0.5-10 mM) of the thiol-containing tripeptide, glutathione (GSH)[69–70] and, second, conjugate addition of GSH to α,β-unsaturated aldehydes under physiological conditions is kinetically and thermodynamically favorable.[71–74] Together, these facts suggested to us that *the true product generated by β-elimination at an AP site in cellular DNA may be a strand break with a 3'-glutathionylated sugar remnant* rather than the canonical 3'*trans*-PUA cleavage product (Scheme 1).

In the work reported here, we characterized the formation and properties of a previously uncharacterized glutathionylated DNA-cleavage product generated when β-elimination at an AP site occurs in the presence of the biological thiol, GSH. We provide evidence that conjugate addition of GSH to the initial *trans*-α,β-unsaturated aldehyde cleavage product (3'trans-PUA) generates the 3-glutathionyl-2,3-dideoxyribose end group (3'GS-ddR, Scheme 1) rapidly and in high yield. In fact, this 3'glutathionylated end group is the only major product observed when cleavage of an AP site occurs in the presence of GSH. Formation of the 3'GS-ddR strand cleavage product is reversible but, in the presence of physiological concentrations of GSH, the adduct is stable for days. Our work suggests that the previously unrecognized 3'gluathionylated cleavage product may be an important blocking group that must be trimmed to enable repair of AP-derived strand breaks via the base excision repair (BER) or single-strand break repair (SSBR) pathways. Along these lines, we present the results of biochemical experiments showing that the 3'-phosphodiesterase activity of the enzyme apurinic/apyrimidinic endonuclease (APE1) can remove the 3'GS-ddR adduct to provide the 3'OH end group required for repair synthesis in BER or SSBR.

EXPERIMENTAL PROCEDURES

Material and Methods.

Oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, IA), Eurofins Genomics (Louisville, KY) and Sigma-Aldrich (St. Louis, MO). Uracil DNA glycosylase (UDG), human apurinic/apyrimidinic endonuclease (APE1), endonuclease III (Endo III, Nth), formamidopyrimidine DNA glycosylase (Fpg) were purchased from New England Biolabs (Ipswich, MA). Acrylamide/bis-acrylamide 19:1 (40% solution,

electrophoresis grade) was purchased from Fisher Scientific (Waltham, MA). Glutathione, sodium borohydride, buffers, DTT and other chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions of spermine and glutathione were neutralized before use. Deuterated DMSO-d_6 was purchased from Cambridge Isotope Laboratories. The ^{1}H NMR spectra were obtained using 600 MHz spectrometer, while ^{13}C NMR spectra was obtained on the same instrument at 151 MHz. The chemical shift values (δ) are reported in ppm versus residual DMSO (δ = 2.50 ppm and 39.51 ppm for ^{1}H and ^{13}C NMR, respectively). The ^{1}H spectra are reported as follows δ (multiplicity, coupling constant *J*, number of protons). The pH of buffers was adjusted to the reported values at 24 °C. DMBAA (dimethylbutylammonium acetate) solutions used in the ESI-MS experiments was prepared as follows: a stock solution of *N*,*N*-dimethylbutyl amine (7.125 M) was diluted to 100 mM with water and adjusted to pH 7.1 with glacial acetic acid.

Generation of 3’GS-ddR cleavage product by spermine-mediated cleavage of an AP site in the presence of GSH.

The 2’-deoxyuridine (dU)-containing oligonucleotide labeled with a 1,1′-diethyl-2,2′-dicarbocyanine (Cy5) fluorophore on the 5’-end was annealed to its unlabeled complementary strand (1 equiv) by heating to 95 °C for 5 min in HEPES buffer pH 7.4 (100 mM containing 200 mM NaCl), followed by cooling slowly to room temperature. The dU-containing duplex (1 nmol) was incubated for 2 h at 37 °C with uracil DNA glycosylase (UDG, 0.8 unit/μL, final concentration) in HEPES buffer (100 mM, pH to 7.4) containing NaCl (200 mM). The DNA was ethanol precipitated[75] and redissolved in HEPES buffer (100 mM, pH 7.4) containing NaCl (200 mM), GSH (5 mM), and spermine (5 mM), followed by incubation for 1 h at 37 °C. Samples were ethanol precipitated before gel electrophoretic analysis then redissolved in formamide loading buffer, loaded onto a 0.4 mm thick, denaturing 20% polyacrylamide gel (containing 7 M urea), and electrophoresed for 15 h at 500 V. (Interestingly, we’ve noticed that the 3’GS-ddR and 3’transPUA cleavage products may not be well resolved on a 20% polyacrylamide gel containing only 4 M urea). The labeled products resolved by electrophoresis were quantitatively visualized by fluorescence imaging.

Generation of 3’GS-ddR cleavage product by heat-induced cleavage of an AP site in the presence of GSH.

The AP-containing duplex in HEPES buffer (100 mM, pH 7.4 containing 200 mM NaCl) was heated at 85 °C for 30 min. In the absence of GSH, this generates a mixture of 3’*trans*-PUA and 3’-phosphoryl cleavage products. In the presence of GSH (5 mM), this process generated a mixture of the 3’GS-ddR cleavage product and intact AP-containing oligodeoxynucleotide.

Time course experiments measuring the stability of the 3’GS-ddR end group in DNA under various conditions.

The 3’GS-ddR cleavage product was prepared by treatment of the AP-containing duplex with spermine as described above. The DNA was ethanol precipitated and redissolved in the desired buffer. Aliquots of the reaction mixture (5 μL) were removed at prescribed time points and stored at −20 °C prior to gel electrophoretic analysis as described above.

Removal of 3’GS-ddR end group by APE1.

The 3’GS-ddR cleavage product was prepared by treatment of the AP-containing duplex with spermine as described above. The DNA was ethanol precipitated and redissolved in Tris-acetate buffer (pH 7.9, 20 mM) containing potassium acetate (50 mM), magnesium acetate (10 mM), DTT (1 mM) and APE1 (0.8 unit/μL, 26 nM, final concentration). Aliquots (5 μL) of the reaction mixture were removed at prescribed time points and stored at −20 °C prior to gel electrophoretic analysis as described above. The 3’dR cleavage generated by the AP-lyase action of Endo III[54, 76–77] was employed as a “canonical” 3’-blocking group for comparison. Toward this end, the AP-containing duplex was treated with Endo III (0.5 unit/μL, 28 nM, final concentration) in a buffer composed of Tris-HCl (20 mM, pH 8) EDTA (1 mM), and DTT (1 mM) for 2 h at 37 °C. The DNA was then ethanol precipitated and redissolved in the appropriate buffer for the subsequent APE1 trimming experiments shown in the Supporting Information.

Treatment of 3’GS-ddR cleavage product with Fpg.

The GS-ddR cleavage product was incubated at 37 °C with formamidopyrimidine DNA glycosylase (Fpg, 1.5 unit/μL, 2.6 μM) in Tris-HCl buffer (40 mM, pH 7.4) containing $MgCl_2$ (10 mM) and BSA (0.2 μg/μL). Aliquots were removed at prescribed times and stored frozen at −20 °C until gel electrophoretic analysis as described above.

Synthesis of N^5-((2R)-1-((carboxymethyl)amino)-1-oxo-3-(((2R)-1,2,5-trihydroxypentan-3-yl)thio)propan-2-yl)-L-glutamine (3).

The compound (*S,E*)-4,5-dihydroxypent-2-enal **1** (25 mg, 0.22 mmol) prepared as described in our previous work[78] was dissolved in water (4 mL) containing potassium carbonate (71 mg, 0.52 mmol) and glutathione (79 mg, 26 mmol). The reaction mixture was stirred for 8 h, followed by addition of sodium borohydride (40 mg, 1.1 mmol) and stirring for an additional 2 h at 24 °C. The product was purified by preparative HPLC using a C18 column (250 mm, 5 μm, 10 mm) eluted with acetonitrile-water (2% acetonitrile for 6 min, followed by 15% acetonitrile from 6 to 15 min, and 90% acetonitrile for 4 min at a flow rate of 4 mL/min). Lyophilization of the collected material afforded **3** (60.4 mg, 66% yield) as a white solid: ^{1}H NMR (600 MHz, DMSO) δ (diastereomers) 8.28 (td, *J* = 5.3, 2.0 Hz, 2H), 4.42 (dddd, *J* = 15.8, 9.6, 8.4, 4.7 Hz, 1H), 3.86 (t, *J* = 6.4 Hz, 1H), 3.81 – 3.67 (m, 2H), 3.60 – 3.52 (m, 2H), 3.52 – 3.33 (m, 3H), 3.00 – 2.77 (m, 2H), 2.74 – 2.61 (m, 1H), 2.42 – 2.27 (m, 2H), 2.09 – 1.93 (m, 2H), 1.91 – 1.75 (m, 1H), 1.71 – 1.36 (m, 1H). ^{13}C NMR (151 MHz, DMSO) δ (diastereomers) 171.2, 170.9, 170.8, 170.7, 74.5 (73.7), 63.4, 58.7 (58.5), 53.0 (52.7), 51.9, 45.9 (45.4), 40.8, 35.7 (33.3), 33.5 (33.0), 30.8, 26.1. HRMS (ESI, $[M+H]^+$) *m/z* calcd for $C_{15}H_{28}N_3O_9S$: 426.1541; found 426.1537.

ESI-QTOF-LC-MS analysis of AP-derived cleavage products.

Samples for mass spectrometric analysis were prepared using 5 nmol of the AP-containing oligonucleotide. The glutathionylated cleavage product was generated by spermine-mediated cleavage of the AP-containing oligonucleotide in the presence of GSH as described above. LC-MS data were acquired on an Agilent Technologies 6520A Accurate Mass QTOF. Samples were analyzed according to the protocol of Studzinska and Buszewski, with

slight modifications as outlined.[79] Sample was injected onto a C8 trap column (Michrom Bioresources Captrap) at a flow rate of 5 μL/min of 10 mM DMBAA pH 7.1 over 4 min. and separated by isocratic elution (either 80% or 42.5% methanol, 15 mM DMBAA, pH 7.1) at a flow rate of 0.4 μL/min on a 10 cm × 75 μm C8 analytical column (fused silica packed with Michrom Bioresources C8, 3.5 μm particles). Following the 4-min sample loading to trap column, separation on the trap/analytical columns continued for 16 min, under isocratic elution conditions. Total run time was 20 min. Mass spectra were acquired using the following parameters: negative-ion mode; VCap 2500 V; mass range 290-3200 m/z; 0.63 spectra/second; fragmentor at 300 V (250 V for IDT oligo); internal MS recalibration was achieved using the K/Na adducted Hexakis 1221 Chip Cube High Mass Reference compound (m/z 1279.99). Samples were loaded in sequence as follows: blank (10 mM DMBAA), sample, and blank. Multiply-charged DNA peaks were deconvoluted using the maximum entropy algorithm in Qualitative Analysis software (version B.07.00 Agilent Technologies) with the following parameters: adduct = proton-loss; m/z range = 600-1500 m/z; mass range = expected mass ±2 kDa; peak height to calculate mass = 25%. The m/z values reported are neutral deconvoluted masses.

LC-MS/MS/MS Analysis of the 3'GS-ddR Strand Cleavage Product.

A 30-μL solution containing oligodeoxynucleotides (200 pmol), sodium acetate (30 mM, pH 5.6), $ZnCl_2$ (10 mM) and nuclease P1 (1 unit), was incubated at 37 °C overnight. To the mixture were subsequently added calf intestinal phosphatase (1 unit), phosphodiesterase I (0.01 unit), 4 μL of Tris-HCl (0.5 M, pH 8.9), and water to make the total volume of the solution 40 μL. The digestion mixture was incubated at 37 °C for 2 h. The enzymes in the digestion mixture were subsequently removed by chloroform extraction. The resulting aqueous layer was dried, reconstituted in doubly distilled water to give a solution of approximately 1 pmol/μL, and subjected to LC-MS/MS/MS analysis.

A 0.5 × 250 mm Zorbax SBC18 column (particle size, 5 μm, Agilent) was used for the separation of the nucleoside mixture arising from the above-mentioned enzymatic digestion, and the flow rate was 8.0 μL/min, which was delivered by an Agilent 1200 capillary HPLC pump (Agilent Technologies). A solution of 2 mM ammonium bicarbonate (pH 7.0) in water (solution A) and methanol (solution B) were used as mobile phases, and a gradient of 30 min 0-50% B was employed for the separation. The effluent from the LC column was directed to an LTQ linear ion-trap mass spectrometer (Thermo Fisher Scientific), which was set up in the positive-ion mode for monitoring the fragmentation of singly protonated ($[M+H]^+$) ion of the glutathione-conjugated sugar remnant in the positive-ion mode. We also acquired the MS/MS/MS for the further fragmentations of the $[M - H_2O + H]^+$ ion (*m/z* 406) observed in MS/MS for the $[M + H]^+$ ion (*m/z* 424) of the unreduced crosslink remnant, and the fragment ion arising from the neutral loss of a glutamic acid moiety (*m/z* 297) observed in MS/MS for the $[M + H]^+$ ion (*m/z* 426) of the reduced crosslink remnant.

RESULTS AND DISCUSSION

Identification of a Novel DNA-Cleavage Product Arising from β-Elimination at an Abasic Site in the Presence of the Biological Thiol Glutathione.

We generated a 35-nucleotide DNA duplex containing a single AP site at a defined location by treatment of the corresponding 2'-deoxyuridine-containing duplex with the enzyme uracil DNA glycosylase (UDG).[80–83] The 2'-deoxyuridine-containing strand and the resulting AP-containing strand in the duplex were labeled on the 5'-end with a fluorescent Cy5 group to enable quantitative detection of the products generated under various conditions.[84] The labeled products were resolved by electrophoresis on a denaturing 20% polyacrylamide gel and visualized by fluorescence imaging. Successful installation of the AP site in the duplex was confirmed by treatment of the duplex with NaOH (200 mM, 90 °C, 5 min) to induce cleavage of the AP-containing strand, with corresponding generation of fast-migrating DNA fragments bearing 3'P and 3'*trans*-PUA end groups via sequential α,β-and γ,δ-elimination reactions (Figure 1, lane 2).[54]

In experiments designed to examine the formation of glutathionylated cleavage products, we induced cleavage of the AP-containing duplex using either heat (85 °C, 30 min) or spermine (5 mM, 37 °C, 1 h) in pH 7.4 HEPES buffer (100 mM) containing NaCl (200 mM). It may be important to note that spermine is a biologically-relevant polyamine present at a concentration of 0.5-5 mM in cells.[98–99] Cationic polyamines are associated with chromatin in the nucleus.[99,85] In the absence of GSH, these cleavage conditions generated mixtures of the fast-migrating 3'P and 3'*trans*-PUA fragments (Figure 1, lanes 3 and 5).[42, 53–54, 86–87] Importantly, a different product was generated when cleavage of the AP site was carried out in the presence of a physiologically relevant[69–70] concentration of GSH (5 mM, Figure 1, lanes 4 and 6).

We suspected that the new product resulted from conjugate addition of GSH to the α,β-unsaturated aldehyde sugar remnant on the 3'-terminus of the AP-derived strand break (Scheme 1). This type of thiol addition can be termed a 1,4-addition or thia-Michael-type reaction. Previous work provided evidence that thiols can add to the 3'*trans*-PUA group to generate cleavage products with altered gel mobility.[53, 88] However, in earlier studies, the structure and properties of these products were not well characterized[53–54, 65, 88] and, to the best of our knowledge, the reaction of glutathione with AP-derived strand cleavage products has not been reported previously. The glutathionylated cleavage product (3'GS-ddR) generated in our experiments migrated more slowly than the 3'*trans*-PUA product in the denaturing gel, due to the large size of the GSH-tripeptide appended to the 3'-end of the cleavage fragment (Figure 1, lanes 4 and 6).

When spermine-catalyzed cleavage of the AP-containing duplex was conducted in the presence of GSH (5 mM), the 3'GS-ddR cleavage product completely supplanted the 3'*trans*-PUA cleavage product (compare lanes 5 and 6 in Figure 1). Addition of GSH to the 3'*trans*-PUA cleavage product was fast, giving complete conversion to the 3'GS-ddR product in less than 5 min at 37 °C in pH 7.4 buffer (Figure S2). Capture of the *trans*-α,β-unsaturated aldehyde sugar remnant by GSH prevented δ-elimination of the sugar remnant to give the 3'P product (compare lanes 5 and 6 in Figure 1).

We employed nanospray LC-ESI-TOF-MS to further characterize the product formed by spermine-induced cleavage of the AP site in the presence of GSH at 37 °C. To facilitate mass spectrometric characterization we employed a smaller, unlabeled 25 nucleotide AP-containing duplex (Figure S3). The major signals observed in the mass spectrum (Figure S3) were consistent with that expected for the 3'GS-ddR and 3'P cleavage products (shown in Scheme 1).

Taken together, the results described above provided evidence for a previously uncharacterized, glutathionylated DNA cleavage product generated when β-elimination at an AP site takes place in the presence of the biological thiol GSH. The evidence indicates that conjugate addition of GSH to the initial α,β-unsaturated aldehyde group generated on the 3'-terminus of an AP-derived strand break produces the 3'GS-ddR adduct rapidly and in high yield.

Characterization of the 3'GS-ddR Strand Cleavage Product Using LC-MS/MS/MS.

We employed LC-MS/MS/MS analysis to further characterize the 3'GS-ddR cleavage product generated in duplex DNA. The 35-nucleotide duplex containing the 3'GS-ddR cleavage product was generated by treatment of the AP-containing duplex with spermine in the presence of GSH and the resulting DNA digested using a three-enzyme cocktail consisting of nuclease P1, alkaline phosphatase, and phosphodiesterase I. Selected-ion chromatograms from the LC-MS/MS/MS analysis of the digests were obtained using previously reported conditions.[89–91] We observed an early-eluting peak displaying the *m/z* 424➔406➔308 transitions corresponding to the neutral loss of water and 2-deoxyribose from the expected GS-ddR adduct (Figure 2, Scheme 2). Further cleavage of the *m/z* 308 ion produced characteristic[92] glutathione fragments at *m/z* 179 and 162 (inset, Figure 2).

We then used chemical synthesis to prepare a standard corresponding to the anticipated structure of the GS-ddR cleavage product detected in the LC-MS/MS/MS analysis of the DNA digest described above. The authentic standard was prepared by reaction of GSH with (*S,E*)-4,5-dihydroxypent-2-enal (**1**) in water (Scheme 3). The NMR and high-resolution mass spectral analysis of the resulting product were consistent with the nucleosidic GS-ddR product **2** (Scheme 3). The absence of alkene and aldehyde resonances in the proton NMR matched our expectation that conjugate addition of GSH would give a product with the sugar residue predominantly in ring-closed forms.[73] However, the NMR spectra were too complex for detailed assignment of all resonances because the material exists as a mixture of up to eight isomers due to R/S stereocenters at C1 and C3 and equilibrating pyranose and furanose forms of the sugar residue. Fortunately, the material could be characterized following treatment with $NaBH_4$.[65, 73] Hydride reduction of the ring-opened aldehyde to the corresponding alcohol dramatically simplified the NMR spectra due to eradication of the stereocenter at C1 and the associated possibility for pyranose and furanose isomers.[73, 93–94] The 1D-NMR, 2D-NMR, and high-resolution mass spectral analyses of the reduced product were consistent with a diastereomeric mixture of the glutathione conjugate **3** (Scheme 3, Table S1 and Figure S4).

We found that the LC-MS/MS/MS properties of the synthetic standard **2** mirrored those of the actual glutathionylated cleavage product formed in duplex DNA (Figure 2).

Overall, the LC-MS/MS analyses provided additional evidence for the structure of the 3'-glutathionylated cleavage product arising from β-elimination at an AP site in the presence of GSH and provide a method that can be applied to the detection of the 3'GS-ddR cleavage product in cellular DNA.

Chemical Stability of the 3'GS-ddR Adduct.

Chemical precedents indicate that conjugate addition of thiols to α,β-unsaturated ketones and aldehydes can be a reversible reaction.[54, 71, 87, 95–97] Therefore, it was important to determine the inherent chemical stability of the 3'GS-ddR lesion in DNA. We generated the 3'GS-ddR cleavage product by treatment of the 35 nucleotide AP-containing duplex with spermine in the presence of GSH, followed by ethanol precipitation of the DNA. The DNA was redissolved in pH 7.4 HEPES buffer (100 mM) containing 200 mM NaCl (no GSH) and the stability of the 3'GS-ddR cleavage product monitored over the course of 48 h at 37 °C using gel electrophoretic analysis (Figure 3). Under these conditions, the 3'GS-ddR cleavage product displayed considerable stability, disappearing with a half-life of approximately 13 h. The data indicated that the 3'GS-ddR group decomposes via β-elimination of GSH (a retro-thia-Michael reaction) to regenerate the 3'*trans*-PUA end group, followed by γ,δ-elimination of the unsaturated sugar remnant to give the 3'P end group. The stability of the 3'GS-ddR end group was similar in neutral Tris buffer (pH 7.4, 10 mM) containing NaCl (100 mM), decomposing with a half-life of approximately 16 h (Figure S5). On the other hand, in basic Tris buffer (pH 8.0, 10 mM, containing 100 mM NaCl) the stability of the 3'GS-ddR end group was substantially decreased, with a half-life for elimination of approximately 4 h (Figure S5). This result is consistent with literature indicating that the rates of retro-thia-Michael reactions are higher under basic conditions.[95–96]

The 3'GS-ddR adduct was significantly less stable in the presence of a physiological concentration[98–99] of spermine (1 mM), disappearing with a half-life of about 6 h (Figures 3B and S6). Again, the gel electrophoretic evidence indicates that the 3'GS-ddR group decomposes via sequential β- and δ-elimination reactions to generate the 3'*trans*-PUA and 3'P cleavage products, respectively. Spermine catalyzes these elimination reactions via the formation of iminium ion intermediates that increase the acidity of the α- and γ-protons (Scheme 4).[42, 54, 57, 61–64, 87]

We found that the 3'GS-ddR end group was dramatically stabilized by the inclusion of GSH in the assay buffers. In a pH 7.4 buffer containing 5 mM GSH, 75% of the 3'GS-ddR cleavage product remained intact after 2.5 days (60 h, Figures 3B and S7) whereas, in the absence of GSH, the 3'GS-ddR group had completely reverted to 3'*trans*-PUA after 2 days. Similarly, inclusion of GSH in the assay buffer stabilized the 3'GS-ddR end group against spermine-catalyzed elimination, with more than 75% of the 3'GS-ddR cleavage product remaining intact after 2.5 days (60 h), compared to a half-life of 6 h in the presence of spermine, but without GSH present (Figures 3B and S8).

GSH shifts the equilibria shown in Scheme 4 toward the GSH-added structures. The resulting decrease in the equilibrium levels of the 3'*trans*-PUA β-elimination product, in turn, depresses the rate of δ-elimination leading to the 3'P product. The cell nucleus is rich in amines[48, 98–99] and GSH.[100] Our results demonstrating the stability of the 3'GS-ddR

adduct in the presence of both GSH and spermine suggest that the glutathionylated cleavage product may persist in DNA for extended periods of time under physiological conditions.

The 3’-Phosphodiesterase Activity of APE1 Trims the 3’GS-ddR End Group from a DNA Strand Break.

Sugar remnants on the ends of DNA strand breaks must be “trimmed” or “cleaned” to enable repair via the BER or SSBR pathways.[14–15, 34, 68] End-cleaning reactions ultimately must generate a 3’OH group that serves as a substrate for a gap-filling repair synthesis by polymerases such as pol β, δ, or ε.[14–15, 68, 101] A number of different enzymes have the capacity to remove repair-blocking groups on the 3’-terminus of strand breaks.[14–15, 68, 101] For example, APE1 and APE2 may play major roles in 3’-end cleaning.[102–108]

We found that APE1 removes the 3’GS-ddR end group from a strand break in duplex DNA with a half-life of approximately 40 min (at an enzyme concentration of 26 nM, Figure 4). The gel mobility of the resulting product was consistent with generation of the 3’OH end group via the 3’-phosphodiesterase activity of the enzyme. At longer incubation times, a product one nucleotide shorter was generated by the 3’-exonuclease activity of the enzyme (this product is marked “exo” on Figure 4).[102, 109–111]

For comparison, we determined the ability of APE1 to remove of the 3’-sugar remnant produced by the AP lyase action of Endo III.[54, 76–77, 112] We found that APE1 removes this canonical 3’-blocking group with a half-life of approximately 2 h (at an enzyme concentration of 26 nM, Figure S10). Overall, the results show that APE1 can trim the 3’GS-ddR group from a strand break in duplex DNA and that the rate of this process is comparable to that observed for the removal of a 3’-blocking characterized previously.[54, 66, 76]

The Lyase Activity of the Base Excision Repair Enzyme Fpg Does Not Remove the 3’GS-ddR End Group.

Active site amine residues in some DNA glycosylases such as NEIL1, NEIL2 and Fpg have the capacity to catalyze sequential β- and δ-elimination reactions on AP sites in DNA.[52, 113–116] These β- and δ-lyase reactions are chemically analogous to the spermine-catalyzed β- and δ-elimination reactions shown in Scheme 2. The δ-lyase activity of these enzymes eliminates the 3’*trans*-PUA sugar remnant from DNA, leaving a 3’-phosphoryl group that is subsequently trimmed by polynucleotide kinase phosphatase (PNPK) in eukaryotes, Xth/exonuclease III in bacteria, or ZDP 3’-exonuclease in Arabidopsis to generate the 3’OH terminus required for repair synthesis.[66, 117–119] This enzymatic repair sequence evades the requirement for DNA incision by APE and, accordingly, has been termed APE-independent BER.[66]

Here we examined whether the lyase activity of the base excision repair glycosylase Fpg has the capacity to catalyze removal of the 3’GS-ddR end group from a DNA strand break. We found that Fpg fails to remove the 3’GS-ddR end group from DNA (Figure S11). A control reaction showed that the lyase activity of enzyme was active under the assay conditions, as the AP-containing duplex was cleanly converted to the expected 3’P cleavage product.

DISCUSSION AND CONCLUSIONS

AP sites are abundant lesions in cellular DNA[67, 120] as a result of spontaneous[33] and enzyme-catalyzed[34–35] depurination and depyrimidination. In the cellular environment, AP sites in DNA have the potential to generate strand breaks with an electrophilic *trans*-α,β-unsaturated aldehyde sugar remnant on the 3'-terminus (3'*trans*-PUA, Scheme 1).[34, 41–48, 50–51, 53, 65, 70, 98] The work reported here was inspired by our suspicion that the 3'*trans*-PUA cleavage product is not likely to persist in the cellular environment. This expectation was founded in precedents from chemical toxicology and drug metabolism showing that GSH readily undergoes conjugate 1,4-addition to low molecular weight α,β-unsaturated aldehydes such as 4-hydroxy-2(*E*)-nonenal, crotonaldehyde, and acrolein.[71–74] Similarly, thiols add to the α,β-unsaturated butenolide generated by elimination of the 3'-phosphate from 2-ribonolactone lesions in DNA.[121] We believed that the same type of reaction should be expected for the α,β-unsaturated aldehyde group on the 3'-end of an AP-derived DNA strand break. Indeed, Bailly and Verly reported in 1988 that various thiols add to AP-derived strand cleavage products.[53] Although reactions with GSH were not part of their work, Bailly and Verly further suggested that the addition of biological thiols to AP-derived cleavage products might influence cellular DNA repair processes.[53] Surprisingly, these observations seem to have been completely overlooked in the ensuing 30+ years. Instead, the 3'PUA cleavage product has been shown in myriad articles and reviews describing cellular generation, cleavage, and repair of AP sites, without recognition that cellular thiols might react with the α,β-unsaturated aldehyde residue in this product.[4, 34, 67]

Our results provide evidence that GSH reacts rapidly and completely with the α,β-unsaturated aldehyde residue on the 3'-terminus of an AP-derived strand break. Our data is consistent with the fast rates and favorable equilibrium constants measured previously for the conjugate addition of GSH to low molecular weight α,β-unsaturated aldehydes such as 4-hydroxy-2(*E*)-nonenal in neutral aqueous buffers.[71] In fact, the glutathionylated cleavage product 3'GS-ddR is the *only* major cleavage product observed when β-elimination at an AP site occurs in the presence of GSH. Formation of the glutathionylated cleavage product is reversible but, in the presence of physiological concentrations of GSH, the adduct persists for days. Our results strongly suggest that the ultimate product generated by β-elimination at a DNA AP site in the cellular environment may be a strand break with a 3'-glutathionylated sugar remnant rather than the canonical 3'*trans*-PUA cleavage product (Scheme 1). This further suggests that the glutathionylated 3'-blocking group should be included in BER and SSBR pathways depicting the repair of strand breaks derived from β-elimination at AP site in cellular DNA.

It will be interesting to directly assess the presence of glutathionylated AP-derived strand breaks in cellular DNA. The LC-MS/MS/MS method reported here provides a platform for such experiments. Carell and coworkers previously reported the use of LC-MS/MS methods to detect the 3'*trans*-PUA cleavage product at levels of 1.7 lesions per 10^6 nucleotides in the DNA of cultured human stem cells, but the possibility of a glutathionylated PUA adduct was not considered in their work.[67] Given that various DNA-binding and DNA-damaging agents can readily access their target sites in nucleosomal DNA and chromatin,[122] it seems likely that GSH will be able to react with the 3'PUA cleavage product in cellular DNA. With

regard to analytical strategies aimed at detection of the 3'GS-ddR adduct, our work (Figure S5) suggests that it may be critical to stabilize the lesion via borohydride reduction or oxime derivatization in order to prevent its reversion to the 3'PUA product in the pH 8 Tris buffers that are commonly used[123] for cell lysis and DNA extraction. We will report the results of such analytical studies in due course.

The GSH reaction described here could have a functional role in mitigating the toxicity of AP-derived strand breaks. This is analogous to the detoxification of genotoxic, low molecular weight α,β-unsaturated aldehydes such as acrolein and 4-hydroxy-2(*E*)-nonenal by conjugate addition of glutathione.[72–73, 124–128] The α,β-unsaturated aldehyde generated at AP-derived strand breaks is highly electrophilic and can generate DNA-DNA interstrand cross-links via conjugate addition of nucleobases on the opposing strand.[57, 76] Conjugate addition of GSH to the α,β-unsaturated aldehyde residue in the 3'*trans*-PUA end group prevents this type of reaction, thus mitigating the toxicities associated with the formation of difficult-to-repair interstrand DNA cross-links. There are a handful of examples where GSH quenches other electrophilic intermediates generated within DNA.[129–131]

Finally, the chemical stability of the 3'GS-ddR cleavage product under physiological conditions makes it interesting to consider how this previously unidentified 3'-blocking group might be removed by cellular enzymes to enable repair of AP-derived single-strand breaks. We found that APE1 cleans the 3'GS-ddR from the 3'-end of an AP-derived strand break in duplex DNA to generate the 3'OH group required for repair synthesis by DNA polymerases. The glutathionylated end group is substantially larger than other 3'-blocking groups for which trimming by APE1 has been characterized, but a recent crystal structure of the enzyme removing a 3'-phosphoglycolate group from a DNA strand break suggests that the GSH tripeptide likely can extend from the active site without encountering steric hindrance.[103]

It is important to recognize that proteins other than APE1 have the potential to remove the 3'GS-ddR blocking group from a DNA strand break, including APE2,[106] MRE11,[132] XPF-ERCC1/RECQ1,[133–135] and TDP1.[136] TDP1 is a good candidate for trimming the GSH tripeptide from the 3'-end of a strand break, as this enzyme has the capacity to remove peptide adducts derived from stalled topoisomerase (TOP1) complexes attached to the 3'-terminus of a DNA strand break. Indeed, experiments in human cells suggest that TDP1 plays a role in the repair of DNA strand breaks derived from β-elimination at AP sites.[137] Similarly, biochemical experiments have shown that the endonuclease activity of XPF-ERCC1 endonuclease can trim a tyrosine-DNA adduct from the 3'-end of a DNA strand break.[138] It is possible that other proteins involved in the resolution of DNA-protein cross-links could contribute to the repair of the DNA-peptide linkage in the 3'GS-ddR lesion.[139]

In future studies designed to investigate the repair of AP-derived strand breaks, it will be important consider that the true product generated by β-elimination at an AP site in cellular DNA may be a 3'-glutathionylated strand break, rather than the canonical 3'*trans*-PUA cleavage product. It remains uncertain whether formation of the GS-ddR adduct may enhance or impede the rate at which AP-derived strand breaks are repaired, relative to the

3’PUA end group. Further study will be required to determine which repair proteins are most important in trimming the glutathionylated sugar remnant from the 3’-terminus of an AP-derived strand break to enable repair synthesis via the BER and SSBR pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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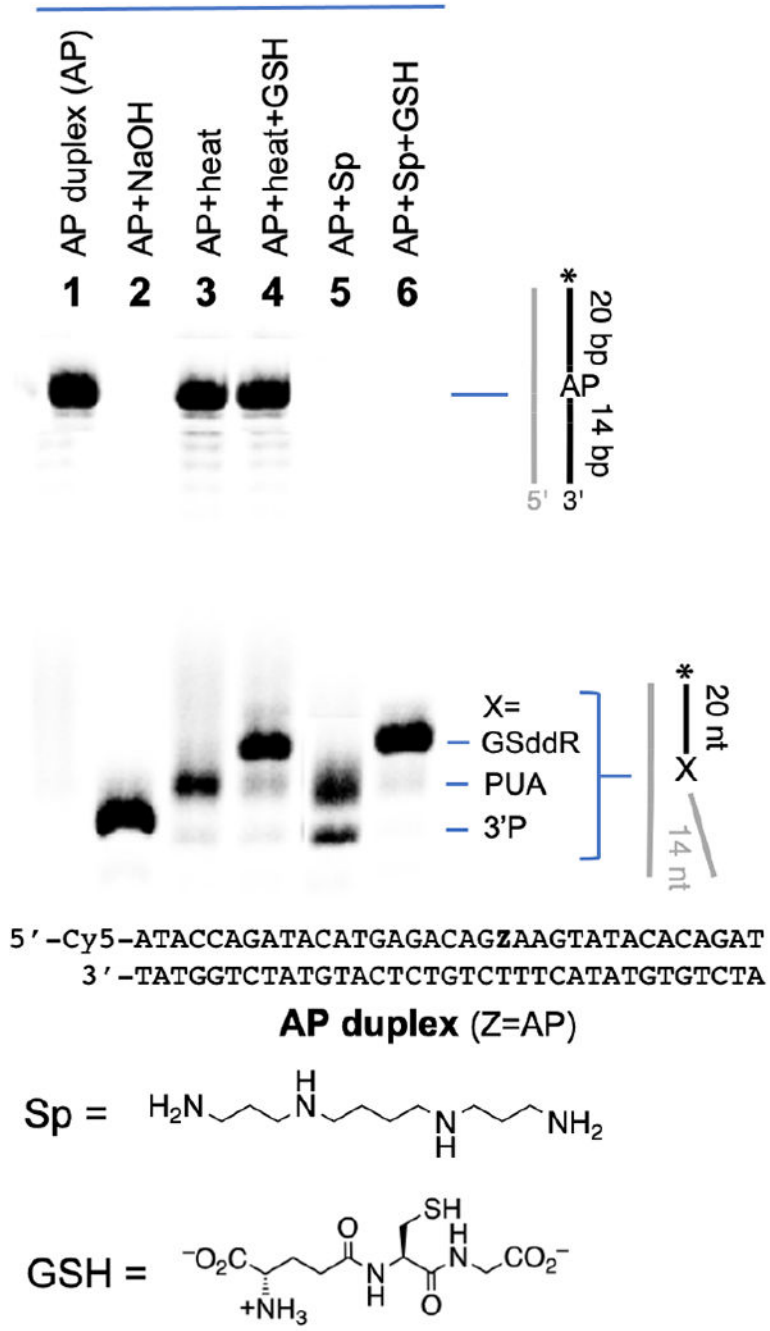


Figure 1. Gel electrophoretic evidence for generation of a novel glutathione-DNA adduct arising from β-elimination at an AP site in the presence of GSH.

These experiments employed the 5'-Cy5-labeled DNA duplex shown in the Figure. Labeled DNA fragments were resolved by denaturing 20% polyacrylamide gel electrophoresis and visualized by fluorescence imaging. Lane 1: the AP-containing duplex. Lane 2: Treatment of the AP-containing duplex with NaOH (200 mM, 90 °C, 5 min) generated the 3'P cleavage product. Lane 3: heat treatment of the AP-containing duplex (85 °C, 30 min, in HEPES buffer (100 mM, pH 7.4) containing 200 mM NaCl) generated a mixture of the uncleaved AP-containing DNA oligomer and the 3'*trans*-PUA cleavage product. Lane 4:

heat treatment of the AP-containing duplex (85 °C, 30 min, in 100 mM HEPES buffer pH 7.4, containing 200 mM NaCl) in the presence of GSH (5 mM) gave a mixture of the uncleaved AP-containing DNA oligomer and the 3'GS-ddR cleavage product, with only traces of an "apparent" 3'*trans*-PUA cleavage product remaining. In fact, we suspect that this unreacted material may be a small amount of the 3'dR product that is a known side product resulting from thermolysis of AP-containing DNA.[86] Lane 5: treatment of the AP-containing duplex with spermine (5 mM) in HEPES buffer (100 mM, pH 7.4) containing NaCl (200 mM) at 37 °C for 1 h generated a mixture of the 3'*trans*-PUA and 3'P cleavage products. Lane 6: treatment of the AP-containing duplex with spermine (Sp, 5 mM) and GSH (5 mM) in HEPES buffer (100 mM, pH 7.4) containing NaCl (200 mM) at 37 °C for 1 h generated 3'GS-ddR cleavage product accompanied by a trace of the 3'*trans*-PUA cleavage product (note: the 3'P product is not generated in the presence of GSH). A control experiment showed that treatment of the AP-containing duplex with GSH alone, in the absence of heat or spermine, did not result in the generation of significant amounts of cleavage products (Figure S1).

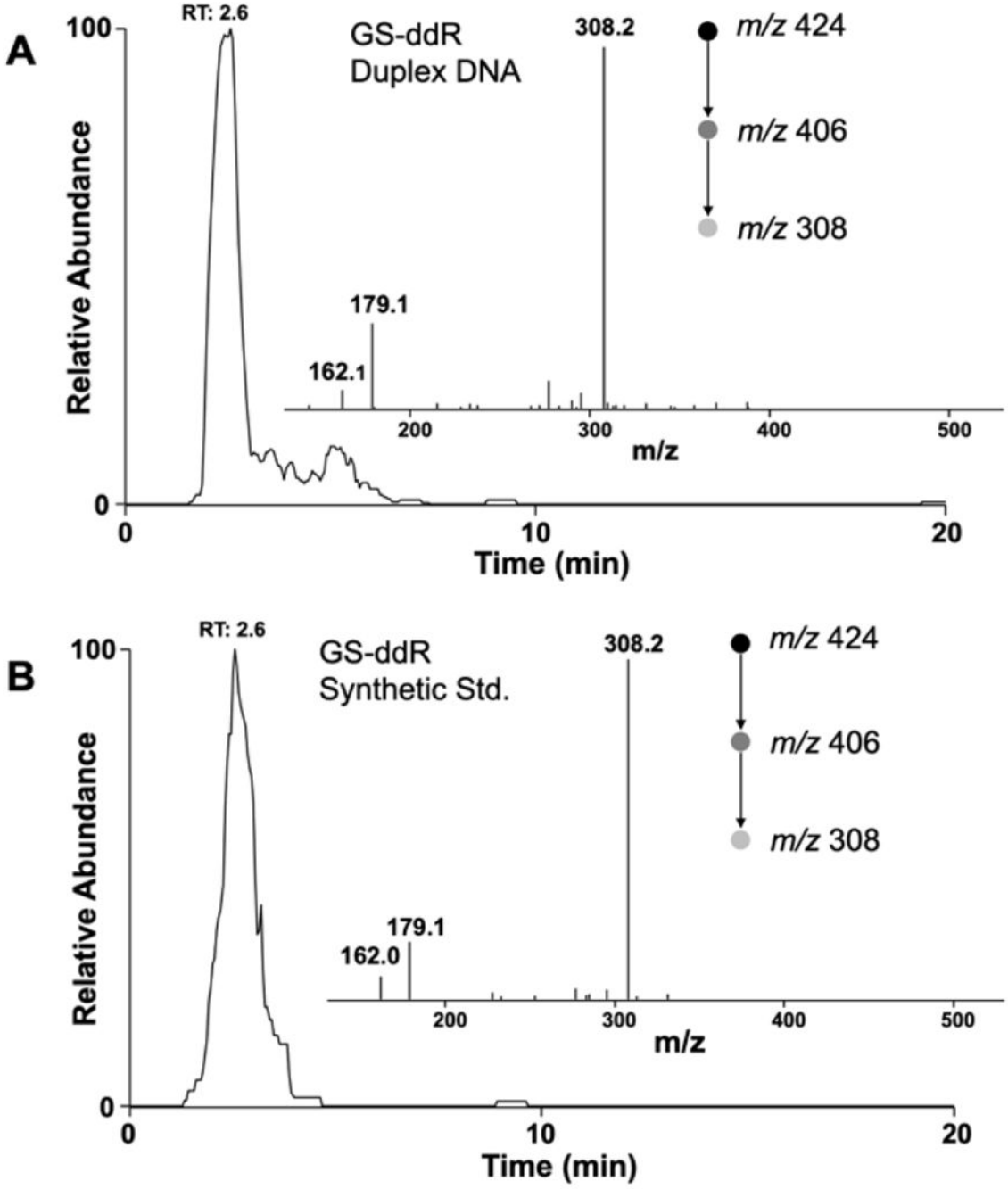


Figure 2. LC-MS/MS/MS analysis of the 3'GS-ddR cleavage product.

Panel A: Selected-ion chromatogram monitoring the *m/z* 424➔406➔308 transition in the digest of a DNA duplex prepared by spermine-mediated strand cleavage of an AP-containing duplex in the presence of GSH. Panel B: Selected-ion chromatogram monitoring the *m/z* 424➔406➔308 transition of the synthetic standard of the GS-ddR adduct **(2)** prepared as shown in Scheme 3.

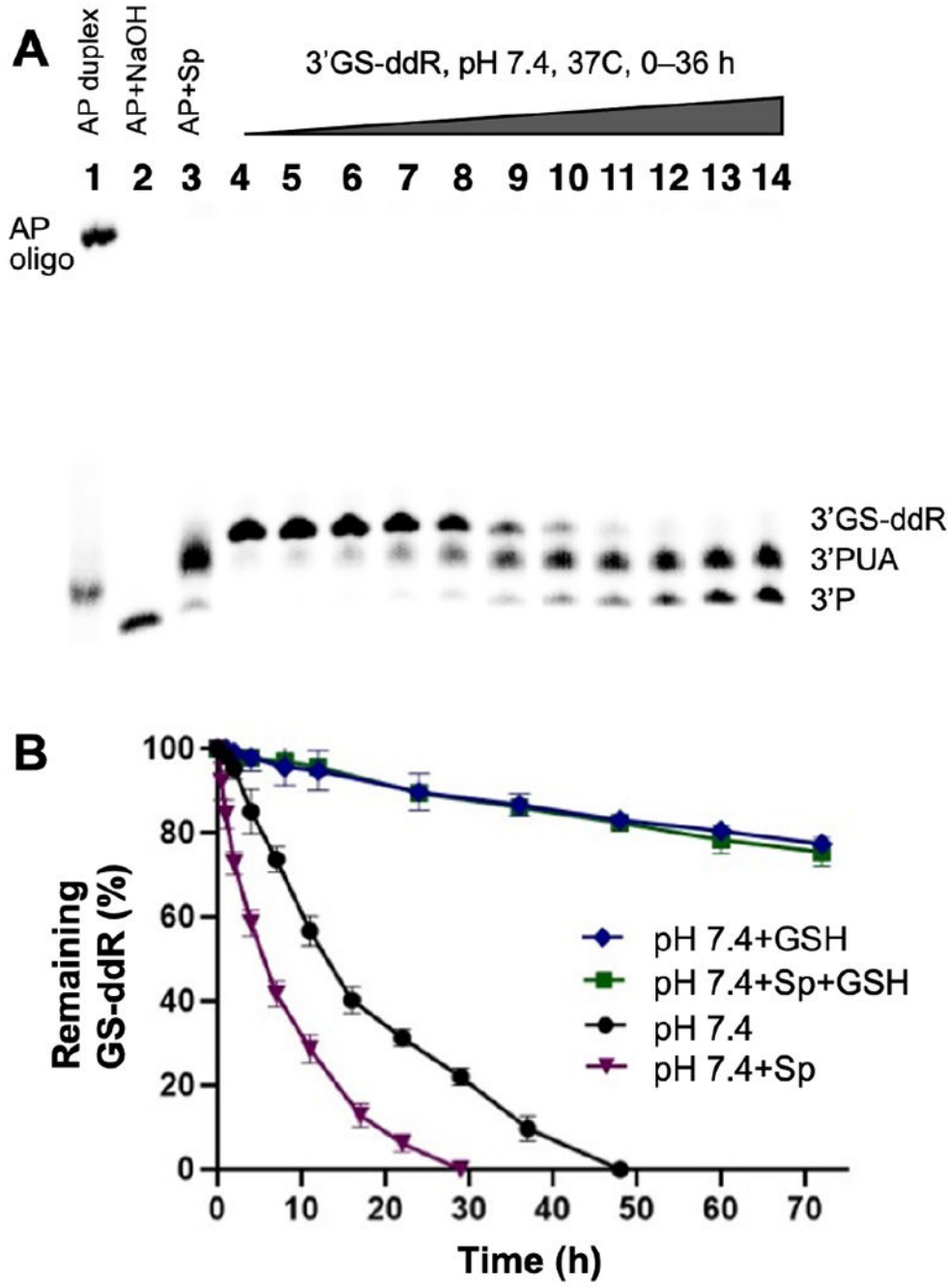


Figure 3. The 3'GS-ddR cleavage product is stable in pH 7.4 buffer.

Labeled DNA fragments were resolved by denaturing 20% polyacrylamide gel electrophoresis and visualized by fluorescence imaging. The 3'GS-ddR cleavage product was generated by treatment of the AP-containing duplex with spermine (5 mM) and GSH (5 mM) in HEPES buffer (pH 7.4, 100 mM) containing NaCl (200 mM) for 1 h at 37 °C. The DNA was isolated by ethanol precipitation, redissolved in HEPES buffer (100 mM, pH 7.4) containing 200 mM NaCl (no spermine) and the stability of the 3'GS-ddR product analyzed by gel electrophoresis. Panel A. Lane 1: the AP-containing duplex containing a

small amount of the fast-migrating 3'*trans*-PUA cleavage product. Lane 2: Treatment of the AP-containing duplex with NaOH (200 mM, 90 °C, 5 min) generated the 3'P cleavage product. Lane 3: treatment of the AP-containing duplex with spermine (5 mM) in HEPES buffer (100 mM, pH 7.4) containing 200 mM NaCl generated a mixture of the 3'*trans*-PUA and 3'P cleavage products. Lanes 4-14: incubation of the 3'GS-ddR in HEPES buffer (100 mM, pH 7.4) containing 200 mM NaCl for 0 h, 1 h, 2 h, 4 h, 7 h, 11 h, 16 h, 22 h, 29 h, 37 h, 48 h. The 3'GS-ddR cleavage product, under these conditions, decomposes slowly ($t_{1/2}$ = 13 h) via β-elimination to generate the 3'*trans*-PUA product that, in turn, undergoes δ-elimination to generate the 3'P product. Panel B. The plot shows the remaining fraction of 3'GS-ddR in the absence of spermine or GSH, in the presence of GSH, in the presence of spermine, and in the presence of spermine and GSH.

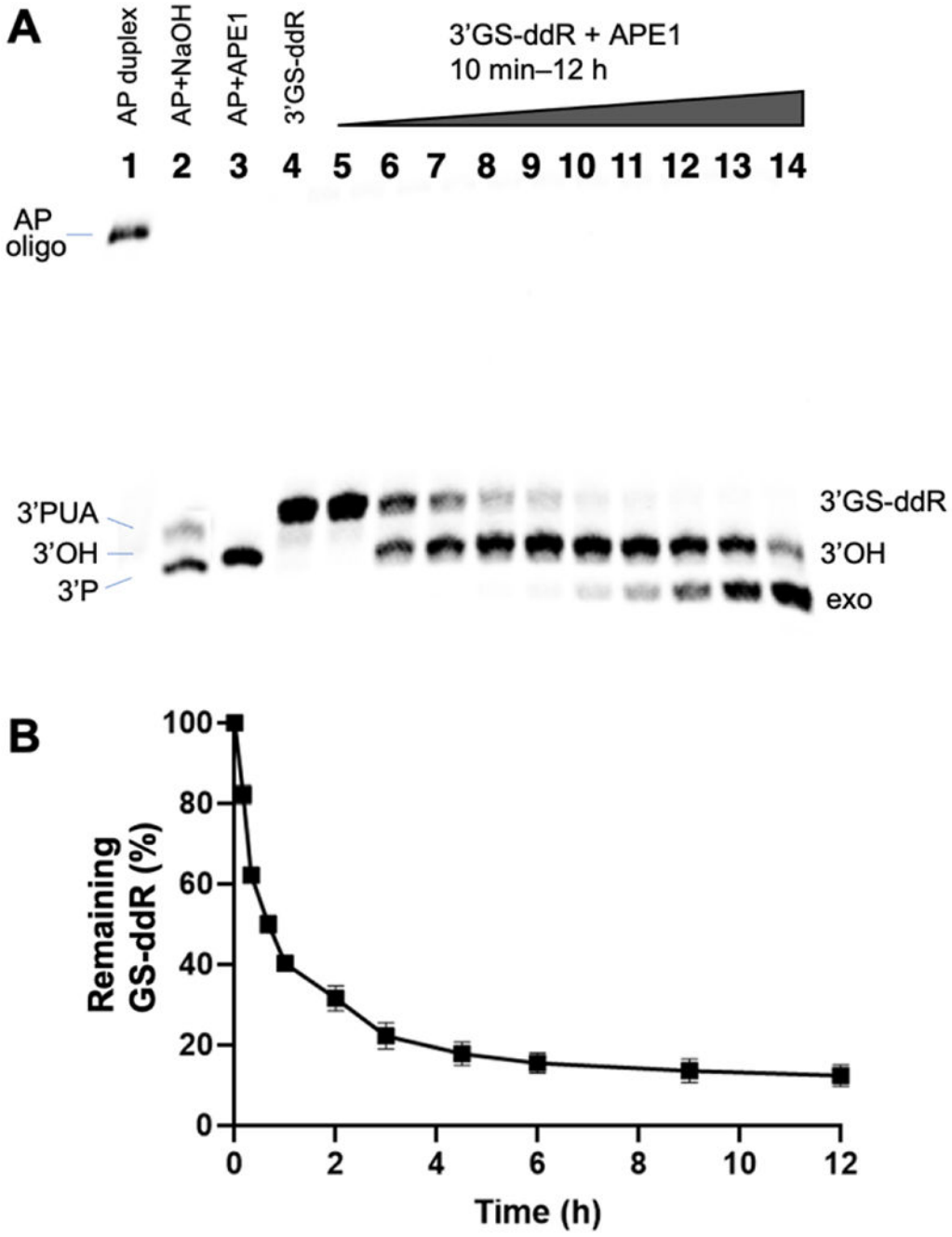
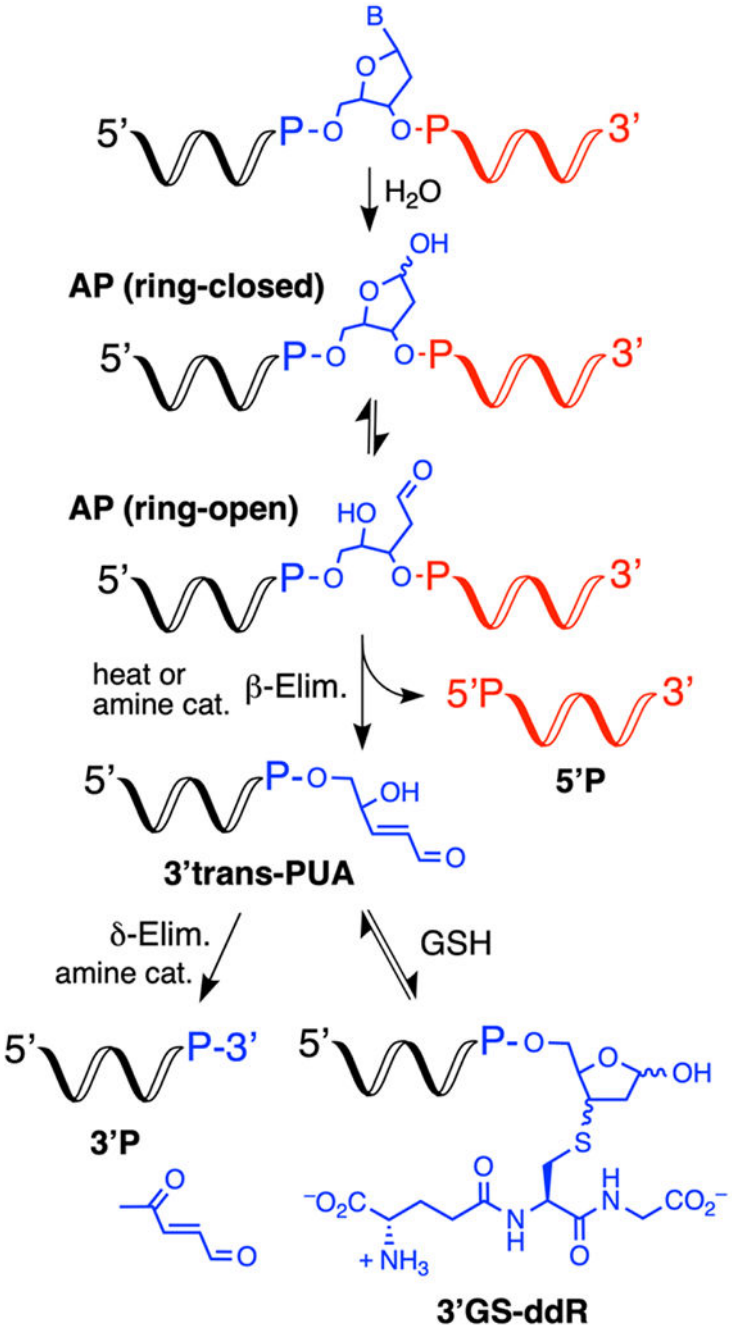


Figure 4. The 3'GS-ddR end product can be trimmed by the 3'-phosphodiesterase activity of APE1.

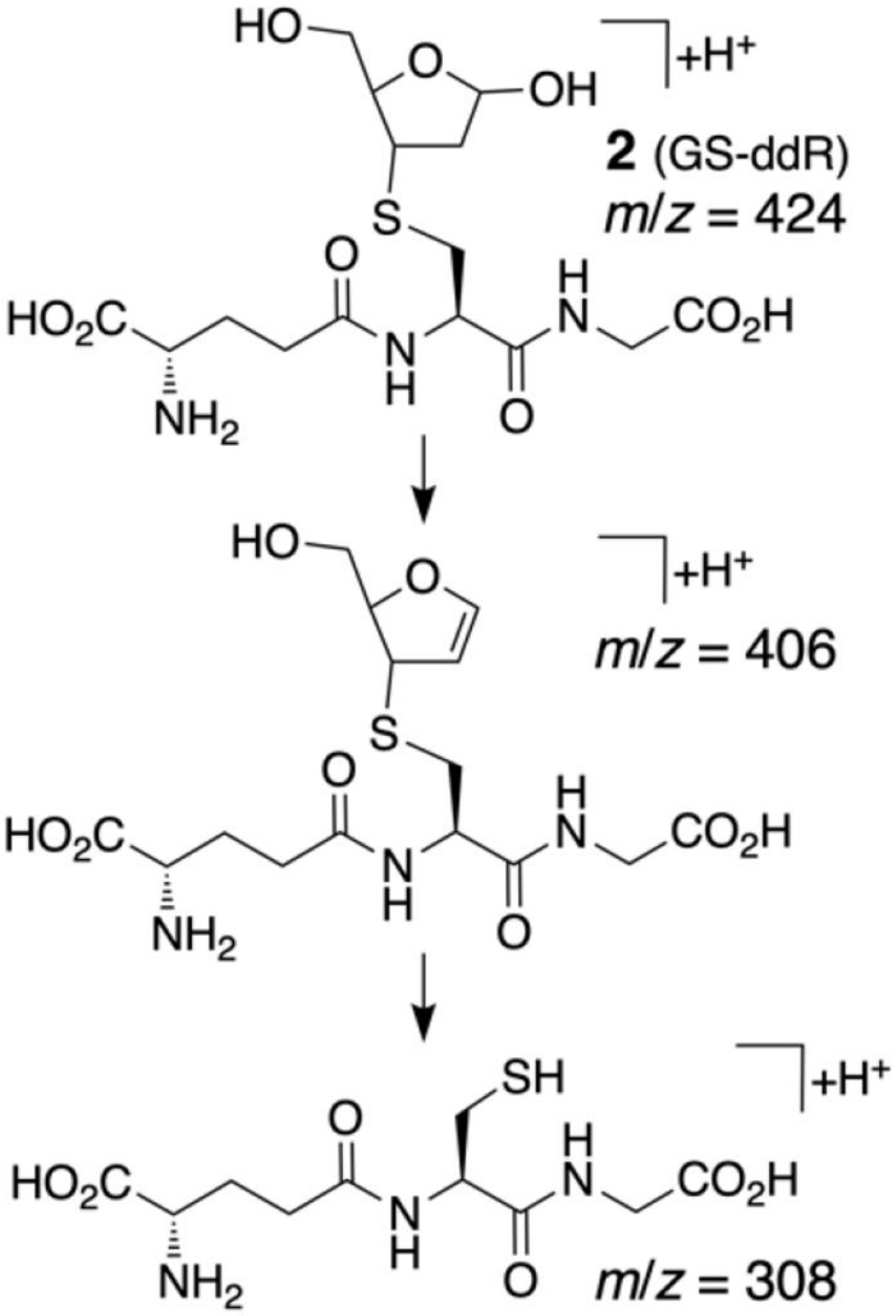
These experiments employed the 5'-Cy5-labeled duplex shown in Figure 1. Labeled DNA fragments were resolved by denaturing 20% polyacrylamide gel electrophoresis and visualized by fluorescence imaging. Panel A. Lane 1: the AP-containing duplex. Lane 2: Treatment of the AP-containing duplex with NaOH (200 mM, 37 °C, 20 min) generated a mixture of the 3'trans-PUA and the 3'P cleavage products. Lane 3: size marker for the 3'OH cleavage product generated by the action of APE1 on the AP-containing duplex. The AP-containing duplex was incubated with APE1 (0.8 unit/μL, 26 nM) in Tris-acetate (20 mM,

pH 7.9) containing magnesium acetate (10 mM), potassium acetate (50 mM), glutathione (5 mM), and dithiothreitol (1 mM) at 37 °C for 2 h to give the 3'OH cleavage product. Lane 4: a no-enzyme control in which the 3'GS-ddR cleavage product was incubated for 2 h in the APE1 assay buffer (Figure S9, provides a 12 h timecourse showing stability of the 3'GS-ddR end group in the assay buffer, without enzyme). Lanes 5-14: The 3'GS-ddR cleavage product was incubated at 37 °C with APE1 (0.8 unit/μL, 26 nM) in Tris-acetate (20 mM, pH 7.9) containing magnesium acetate (10 mM), potassium acetate (50 mM), glutathione (5 mM), and dithiothreitol (1 mM) and aliquots were removed at 10 min, 20 min, 40 min, 1 h, 2 h, 3 h, 4.5 h, 6 h, 9 h, 12 h, and 15 h and frozen until gel electrophoretic analysis. The 3'-phosphodiesterase activity of the APE1 removes the 3'GS-ddR end group with a half-life of approximately 40 min to give the 3'OH product. Subsequent 3'-exonuclease activity of APE1 generates the product that is shorter by one nucleotide, labeled "exo" on the gel image. Panel B. Shows a plot of remaining 3'GS-ddR end group as a function of time measured from the gel electrophoretic analysis.

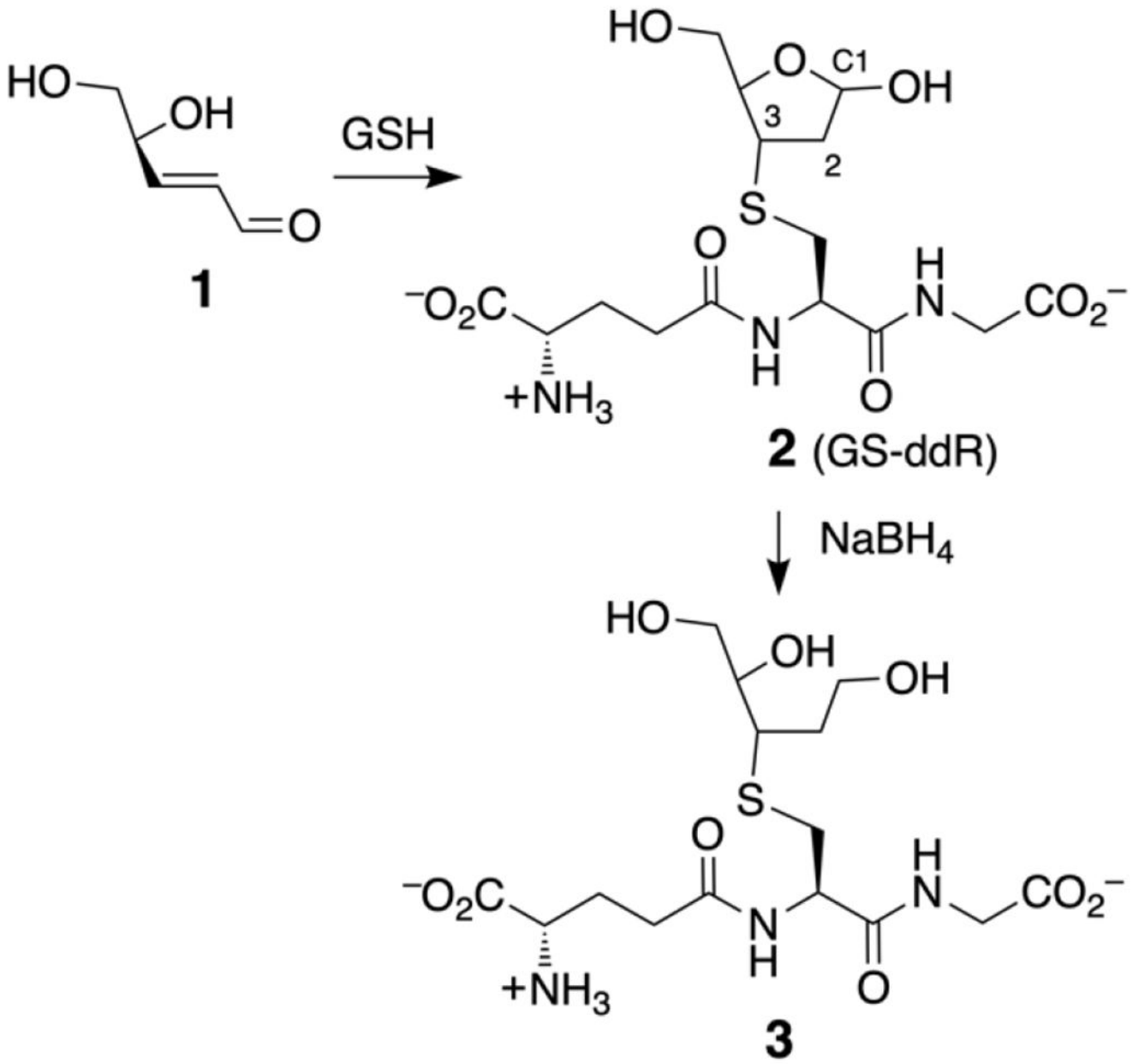


Scheme 1. Strand cleavage involving β-elimination at an AP site in DNA in the presence of the biological thiol glutathione generates a glutathione adduct via conjugate addition of glutathione to the initial 3'*trans*-PUA cleavage product.

The resulting 3'GS-ddR adduct is referred to as a singular species in the text but, in fact, has the potential to exist as four distinct diastereomers. The work described here does not define the stereoisomeric nature of the adduct. In this Scheme, the black and red ribbons represent DNA strands and P represents a phosphodiester linkage or a terminal phosphoryl group.

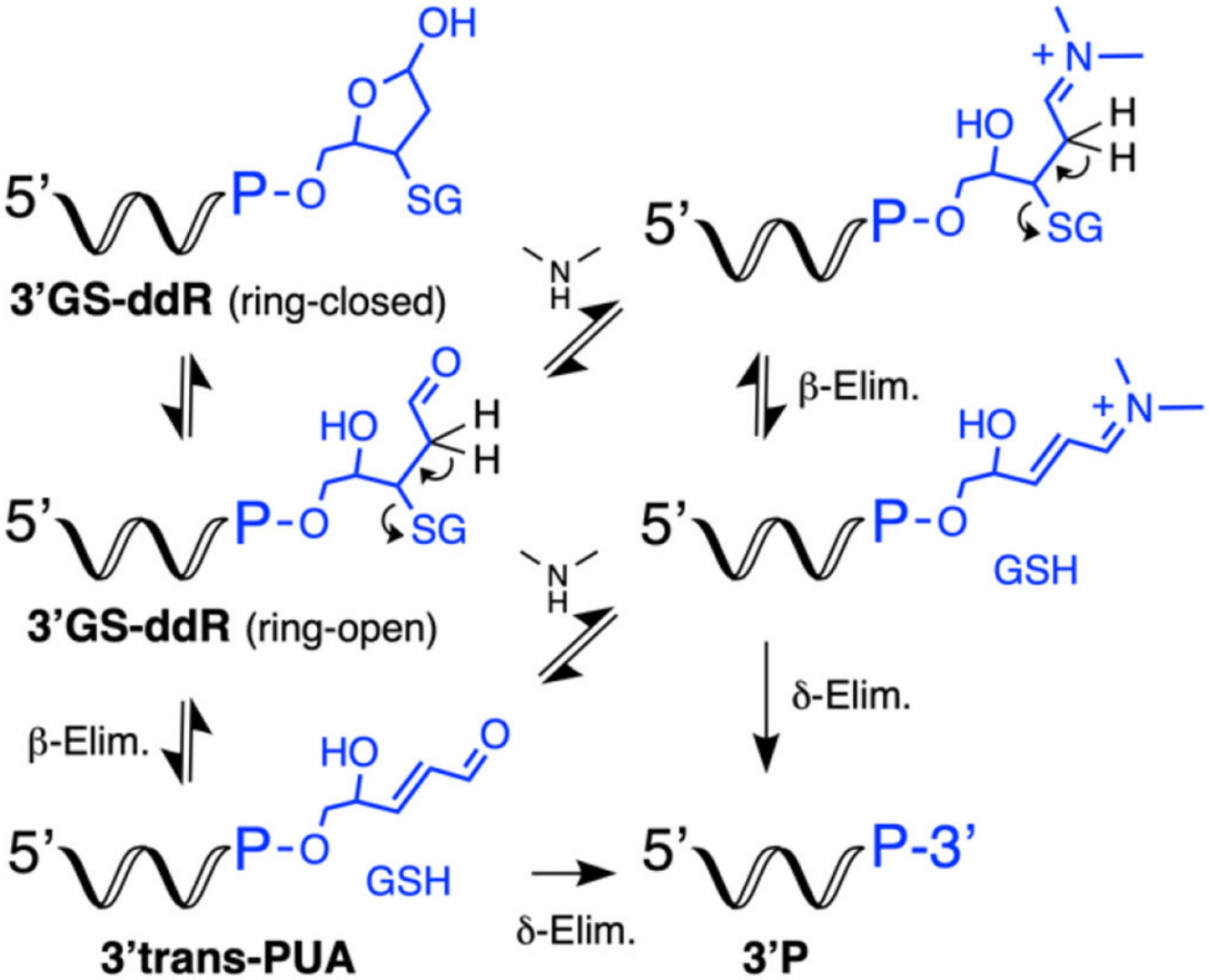


Scheme 2.
Fragmentation of GS-ddR adduct in LC-MS/MS/MS experiments.



Scheme 3.

Chemical synthesis of a structurally-defined standard corresponding to the GS-ddR adduct detected in the LC-MS/MS analysis of the DNA digest (Figure 2A). The sugar residue in 2 is shown in the furanose form, but exists as an equilibrating mixture of furanose and pyranose isomers.[93–94]



Scheme 4. The 3'GS-ddR adduct can decompose via β-elimination of glutathione to regenerate 3'*trans*-PUA that, in turn, undergoes δ-elimination to give the 3'P end product.

The β-elimination reaction is slow in the absence of an amine catalyst, occurring with a $t_{1/2}$ of 14 h (pH 7.4, 37 °C). For brevity, spermine is abbreviated as a simple dialkylamine and glutathione is abbreviated as GSH (the actual structures of spermine and glutathione are shown in Figure 1). The elimination of GSH is catalyzed by spermine via formation of iminium ion intermediates, in which the α- and γ-protons have increased acidity compared to the parent α,β-unsaturated aldehyde of the 3'*trans*-PUA end group. The amine-catalyzed process occurs with a half-life of 4 h (pH 7.4, 37 °C, 1 mM spermine). The black ribbons represent DNA strands and P represents a phosphodiester linkage or a terminal phosphoryl group.