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Communication

Molecular Structure and Dynamic Properties of a Sulfonamide Derivative of Glutathione That Is Produced Under Conditions of Oxidative Stress by Hypochlorous Acid

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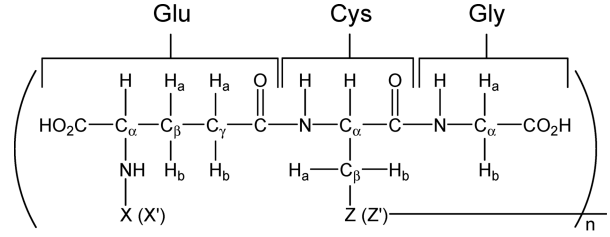
Reduced glutathione (GSH) is a cornerstone of the antioxidant stratagem for eukaryotes and some prokaryotes. Hypochlorous acid (HOCl), which is produced by neutrophilic myeloperoxidase, reacts rapidly with excess GSH to yield mainly oxidized glutathione (GSSG). GSSG can be further oxidized to give first *N*-chloro derivatives and, later, higher oxidation states at the S centers. Under certain conditions, another major species that is observed during the oxidation of GSH by HOCl (and a minor species for other oxidants) exhibits a molecular mass that is 30 mass units heavier than GSH. This GSH+2O-2H species, which has been employed as a biomarker for oxidative stress, has been previously proposed to be a sulfonamide. Employing NMR spectroscopy and mass spectrometry, we demonstrate that the GSH+2O-2H species is indeed a nine-membered cyclic sulfonamide. Alternative formulations, including six-membered 1,2,5-oxathiazine heterocycles, have been ruled out. Remarkably, the sulfonamide exists as a 2:1 equilibrium mixture of two diastereomers. Isotope tracer studies have demonstrated that it is the Glu C_α center that has undergone racemization. It is proposed that the racemization takes place via an acyclic imine−sulfinic acid intermediate. The glutathione sulfonamides are stable products of GSH that have been detected in physiological systems. Elucidation of the structures of the glutathione sulfonamides provides further impetus to explore their potential as biomarkers of hypochlorous acid formation.

Introduction

The tripeptide glutathione (GSH) is the principal intracellular nonprotein thiol in eukaryotes (*1*) and some prokaryotes (*2, 3*), and as such, it serves to maintain intracellular redox states. GSH acts as a substrate for various enzymes involved in redox regulation (*4*), and it can also be oxidized directly by various reactive oxygen species (ROS) (*5*). There is mounting evidence that two-electron oxidants, such as the neutrophil-derived hypochlorous acid (HOCl), may play significant roles in inflammatory diseases (*6*). Mechanistic investigations of the rates of reaction of HOCl with physiological constituents that have been studied thus far reveal that the sulfur-containing compounds cysteine (CySH) (*7–11*), methionine (Met) (*9, 10*), and thiocyanate (SCN^-) (*12, 13*) are most reactive.

Because of its possible connection to inflammatory diseases, there is considerable interest in identifying biomarkers for oxidative stress by HOCl, and GSH-derived products are potential sources for these biomarkers. A number of products of the reaction of GSH and HOCl have been identified, including those of Scheme 1. Of course, oxidized derivatives of GSH that are reversibly reduced or that are of transitory nature cannot serve as useful biomarkers. For the derivatives of Scheme 1, only GSSG has been generally known to be reversibly reduced (e.g., by GSH reductase), but with the discovery of sulfinate

Scheme 1. Oxidation Derivatives of GSH



	n	X	X'	Z	Z'
GSH	1	H		SH	
GSOH	1	H		SOH	
GSO_2H	1	H		SO_2H	
GSO_3H	1	H		SO_3H	
GSSG	2	H	H	S	S
GS(=O)SG	2	H	H	SO	S
$GS(=O)_2SG$	2	H	H	SO_2	S
NCG	2	H	Cl	S	S
NDG	2	Cl	Cl	S	S

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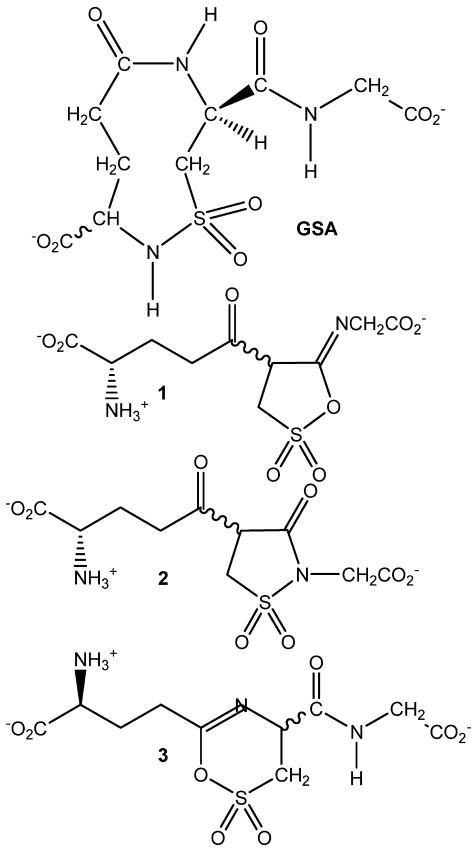
Scheme 2. Alternative Structure for the GSH+2O-2H Species



(*14*) and sulfonate (*15*) reductases, it is conceivable that GSO_2H and GSO_3H are also reduced in vivo. The ester derivatives of GSH are also unstable, and they are known to spontaneously react to give mixtures of GSH, GSSG, GSO_2H, and GSO_3H, depending on the reaction conditions. In addition to the sulfur center, the amine moiety of the Glu residue of GSH derivatives can also be oxidized by hypohalous acids to initially produce the haloamines [for example, GSSG gives *N*-chloro and *N*,*N*′-dichloro derivatives (*16*)]. However, the haloamines are also transitory reactive intermediates (*16*), and as such, they are not useful biomarkers.

In addition to the fully characterized GSH derivatives of Scheme 1, two novel structurally uncharacterized oxidation products have been identified that exhibit nominal molecular masses that are two mass units lighter (GSH-2H) and 30 mass units heavier (GSH+2O-2H) than GSH (*17*). The latter derivative is the focus of this paper. On the basis of the mass of the parent peak and the fact that the GSH+2O-2H species did not react with *o*-phthalaldehyde or ninhydrin (suggesting that it did not contain an amino group), it has been proposed that the GSH+2O-2H species contains a covalent sulfonamide bond between the cysteinyl sulfur and the amino group of the γ-glutamyl residue of GSH (GSA). GSA is produced by the MPO system (*18*), it has been detected in neutrophils and endothelial cells that have been treated with HOCl (*19, 20*), and it has been observed in PMA-stimulated neutrophils (*21*). Apart from the original characterization of GSA (*17*), no structural studies have been performed. Because the proposed structure contains an unusual nine-membered (mesocyclic) ring (*22*), strained aliphatic sulfonamide groups with reactive neighboring groups are generally unstable in an aqueous environment (*23–27*), and alternative structures are consistent with the observed mass (Scheme 2), we sought to confirm the structure of GSA using NMR. Complementary MS data are also presented. The proposed sulfonamide structure has been established by the present study, but we surprisingly find that it exists in two diastereomeric forms that interconvert vis-à-vis epimerization of the Glu C_α stereocenter.

Materials and Methods

Reagents. All chemicals were ACS certified grade or better. GSH was from Sigma-Aldrich (St. Louis, MO). Acetonitrile was from Mallinckrodt Baker (Paris, KY). NaOCl was from Sarah Lee (Auckland, NZ), and its concentration was determined spectrophotometrically (pH 12, $\epsilon_{292} = 350\ M^{-1}\ cm^{-1}$). Deuterium oxide (99.9%) and labeled GSH ([glycine 1,2-$^{13}C_2$,^{15}N]GSH) were from Cambridge Isotope Laboratories (Andover, MA). Water was doubly distilled in glass. The buffer solutions were prepared from K_3PO_4, and the pH/pD was adjusted with NaOH or NaOD (prepared by the careful addition of Na sand to D_2O under an atmosphere of Ar. Caution: Hydrogen gas evolves and can be ignited by the hot Na if O_2 is not excluded.

pH/pD Measurements. The [OH^-] for the unbuffered solutions was determined by acid−base titration against standardized HCl solutions. The [H^+] of the buffered solutions was determined with an Orion Ion Analyzer EA920 using an Ag/AgCl combination pH electrode. pD measurements in D_2O were made using the same pH electrode by adding 0.4 units to the measurement (*28*).

Synthesis of GSA. GSA was synthesized by the reaction of GSH with an equimolar amount of HOCl. HOCl (5 mL of a 100 mM solution in 100 mM phosphate buffer, pH 7.4) was added to 5 mL of a stirred solution of 100 mM GSH in water at room temperature. The equimolar ratio of thiol and oxidant was chosen because it gave an acceptable yield of GSA without forming excessive amounts of GSO_3H or GSO_2SG. More GSA could be formed with the addition of greater amounts of HOCl, but the reaction mixture became discolored and led to an impure preparation of the sulfonamide. GSA was formed immediately upon mixing, and it could be subsequently separated on a Phenomenex Jupiter semipreparative C18 column (4.6 mm × 250 mm) using an isocratic elution (50 mM formic acid, pH 2.4) with UV detection at 222 nm. GSA eluted at 8 min, baseline resolved from the other components of the reaction mixture. The GSA was manually collected and then freeze-dried to give a fluffy off-white powder that was stored at −20 °C until use. Importantly, lower yields of GSA were formed when turbulent mixing (e.g., stopped-flow) conditions were applied. To generate GSA containing an isotopically labeled glycine residue, commercially available [glycine 1,2-$^{13}C_2$,^{15}N]GSH was oxidized with an equal volume of an equimolar amount of HOCl. The conditions for isolation were the same as those that were used for unlabeled GSA. Once formed, GSA was stable in water or 10 mM phosphate buffer (pH 7.4) for at least 6 months at −20 °C.

NMR Studies. ^1H (499.883 MHz), ^2H (76.728 MHz), ^{13}C (125.708 MHz), and ^{15}N (50.657 MHz) NMR spectra were recorded with a Varian VNMRS 500 spectrometer at the indicated temperatures (±0.5) °C using the 1D presaturation gradient-selected COSY, gradient-selected absolute value HMBC, gradient-selected $^1H-^{13}C$ HSQC, and $^1H-^{15}N$ HSQC gradient sensitivity enhanced for ^{15}N pulse sequences as supplied by the Varian Biopack software. The ^1H chemical shifts (ppm) were referenced to sodium 2,2-dimethyl-2-silapentane-5-sulfonate

Table 1. ^{1}H NMR Chemical Shifts (ppm) of Reduced GSH, Oxidized Glutathione (GSSG), Glutathione Sulfonate (GSO_3^-), and the Major and Minor Diastereomers of Gluathione Sulfonamide (GSA) at pH 7.4, 22 °C, and 500 MHz

	GSH	GSSG	GSO_3^-	GSA (major)	GSA (minor)
Gly α-H_a	3.78	3.78	3.73	3.75	3.76
Gly α-H_b	3.79	3.78	3.76	3.87	3.88
Gly NH	8.29	8.33	8.29	8.45	8.67
Cys α-H	4.58	4.80	4.84	4.75[a]	4.73[a]
Cys β-H_a	2.94	2.97	3.28	3.45	3.32
Cys β-H_b	2.99	3.31	3.47	3.58	3.61
Cys NH	8.51	8.61	8.51	9.68[b]	9.67[b]
Glu α-H	3.79	3.78	3.77	4.53	4.51
Glu β-H_a	2.17	2.16	2.14	2.17	2.20
Glu β-H_b	2.17	2.16	2.20	2.61	2.62
Glu γ-H_a	2.54	2.51	2.52	3.03	3.08
Glu γ-H_b	2.56	2.57	2.56	3.05	3.14

[a] Under the water peak. [b] Measured at 2 °C.

Table 2. ^{1}H NMR Coupling Constants (Hz) of Reduced GSH, GSSG, GSO_3^-, and the Major and Minor Diastereomers of GSA at pH 7.4, 22 °C, and 500 MHz

	GSH	GSSG	GSO_3^-	GSA major	GSA minor
$^3J_{Glyα-Ha,Glyα-Hb}$				17.2	17.1
$^3J_{Cysα-H,Cysβ-Ha}$	5.2	4.5	10.0	6.7	5.4
$^3J_{Cysα-H,Cysβ-Hb}$	7.1	9.7	3.3	8.5	10.2
$^2J_{Cysβ-Ha,Cysβ-Hb}$	14.1	14.4	14.6	14.8	14.8
$^3J_{Gluα-H,Gluβ-Ha,b}$	6.3	6.4	6.3	8.7	5.5
$^3J_{Gluβ-Ha,Gluγ-Ha,b}$	7.7	7.6	7.8	6.7	9.5
$^2J_{Gluγ-Ha,Gluγ-Hb}$	14.7	15.5	16.0	12.0[a]	12.0[a]
$^3J_{GluNH,Gluα-H}$	5.4	5.7[a]	5.5	4.2[a]	4.2[a]
$^3J_{CysNH,Cysα-H}$		7.6[a]	7.5		

[a] At 2 °C.

(DSS,[1] $\delta = 0.015$ ppm). The ^{13}C chemical shifts (ppm) were referenced to internal 1,4-dioxane (dioxane, $\delta = 66.0$ ppm). The ^{15}N chemical shifts (ppm) were referenced to external ^{15}N-labeled benzamide ($\delta = 110.0$ ppm).

MS Studies. High-resolution electrospray ionization mass spectra (HRESIMS) were recorded on a Micromass LCT spectrometer using a probe voltage of 3200 V, an operating temperature of 150 °C, and a source temperature of 80 °C. Samples of 100 μM GSA were prepared in 50:50 acetonitrile/water and infused into the instrument at a flow rate of 20 μL/min. Leucine enkephalin was used as the lock mass internal standard. A mass (m/z) of 338.0656 was observed for GSA, which differs from the calculated mass of GSA by only −0.6 ppm. As a difference of <5 ppm is the accepted criterion for confirmation of a molecular formula, the elemental composition of GSA is unequivocally GSH+2O-2H.

Both isotopically labeled and naturally abundant GSA (both 100 μM in 1:1 methanol/water supplemented with 1% acetic acid) were monitored by direct infusion positive electrospray ionization mass spectrometry using a Thermo Finnigan LCQ Deca XP Plus (San Jose, CA) instrument. Fragmentation spectra were generated by collision-induced dissociation of the respective $[M + H]^+$ ions.

Isotope Tracer Studies. GSA was synthesized in H_2O using the aforementioned procedure. The sample was subsequently lyophilized and redissolved in a pH 7.4 buffer containing a 1:1 mixture of H_2O and D_2O. A ^{1}H NMR spectrum of the sample yielded an integration of 0.5 H (relative to other assigned resonances) that was assigned to Glu α-H.

[1] Abbreviations: DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; iP, inorganic phosphate ($H_3PO_4 + H_2PO_4^- + HPO_4^{2-} + PO_4^{3-}$).

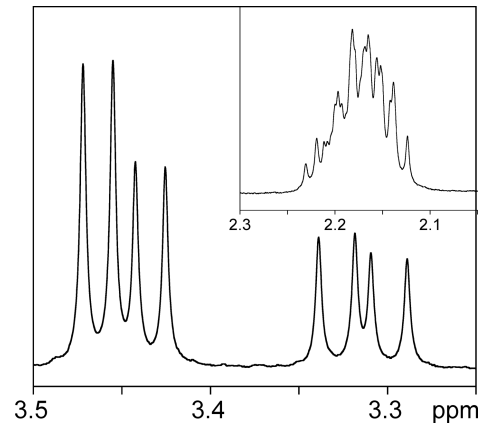


Figure 1. ^{1}H NMR resonances for Cys β-H_a of the major (3.45 ppm) and minor (3.30 ppm) diastereomers of GSA at 22 °C and pH 7.4. Inset: Overlapping ^{1}H NMR resonances of Glu β-$H_{a,b}$ of the major (2.17 ppm) and minor (2.20 ppm) diasteromers.

Table 3. ^{13}C and ^{15}N NMR Chemical Shifts (ppm) of Reduced GSH, GSSG, GSO_3^-, and the Major and Minor Diastereomers of GSA at pH 7.4, 22 °C, and 500 MHz

	GSH	GSSG	GSO_3^-	GSA[a] major	GSA[a] minor
Gly COO	175.8	176.4	176.4	175.6	173.2
Gly α-C	42.9	43.7	43.8	43.0	43.0
Cys CON	171.2	172.0	171.4	168.2	169.0
Cys α-C	55.2	52.8	50.0	54.1	54.2
Cys β-C	25.1	38.8	50.9	50.5	50.7
Glu COO	173.5	174.2	174.3	176.5	177.0
Glu α-C	53.7	54.4	54.4	63.4	62.4
Glu β-C	25.7	26.4	26.2	24.9	24.7
Glu γ-C	30.8	31.5	31.5	30.2	27.9
Glu CON	174.5	175.2	174.9	169.3	171.4
Gly N		118.5		118.1	118.4
Cys N		125.2		109.4	

[a] At 2 °C.

Molecular Modeling. Chem3D Ultra 8.0.3 (CambridgeSoft, Cambridge, MA) was employed to model the molecular structures of the two diastereomers of GSA. Geometry optimization was achieved using the MM2 force field. Essentially, the same molecular structures were obtained when the PM3 semiempirical molecular orbital method was employed during optimization.

Results

Assignment of the NMR Spectrum of GSA. The ^{1}H NMR chemical shifts were assigned on the basis of correlations in the COSY spectra and by comparison with the spectra of similar compounds (Tables 1 and 2). The NMR spectra evidenced the presence of two isomers of GSA. The relationships between the observed chemical shifts and the coupling constants for the isomers of GSA demonstrate that the two isomers are diastereomers. The ratio of the major and minor diastereomers of GSA is under thermodynamic (equilibrium) control, and under the conditions of our measurements (pH 7.4 and 2−60 °C, not all data shown), the ratio remained approximately 2:1 (Figure 1). The ^{13}C NMR chemical shifts (Table 3) were assigned with the aid of HSQC and ^{13}C−^{1}H HMBC spectra. The ^{15}N chemical shifts of GSA were assigned using a ^{15}N−^{1}H HMBC spectrum. 2D EXSY spectra were collected up to a temperature of 48 °C. Weak negative cross-peaks that correspond to nuclear Overhauser effect (NOE) coupling and very weak positive cross-peaks that result from zero-quantum coherence (zero-quantum-filtered COSY) were observed in the EXSY, but no strong

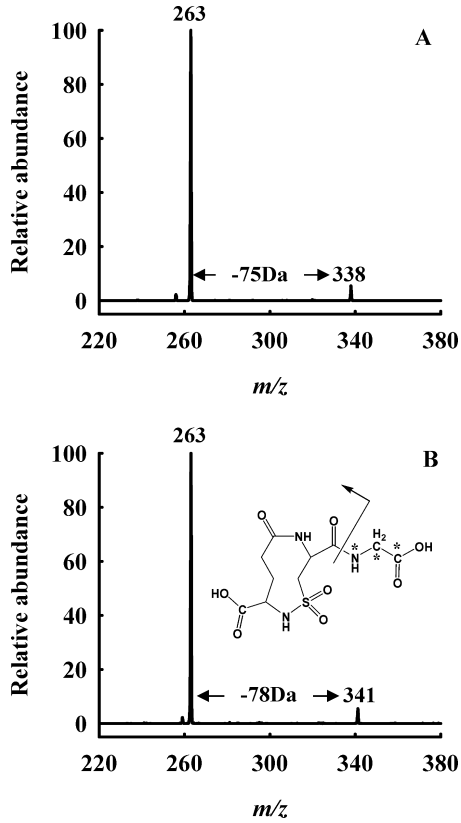


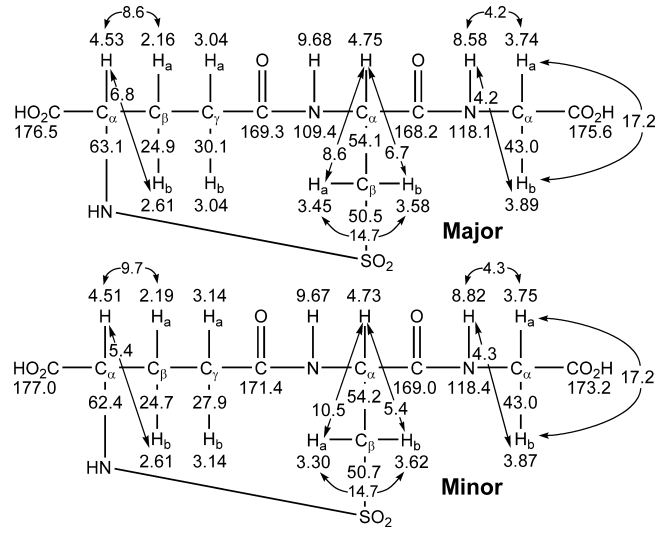
Figure 2. Collision-induced dissociation of naturally abundant and glycine-labeled GSA. Naturally abundant GSA (A) and glycine-labeled GSA (B) were infused (5 μL/min) at a concentration of 100 μM in 1:1 MeOH/H_2O supplemented with 0.1% formic acid into an ion trap mass spectrometer and monitored by positive ESI. The $[M + H]^+$ ion of each species was isolated and fragmented using collision-induced dissociation. The resulting fragmentation spectra were monitored between *m/z* 220 and 380. The proposed structure of glutathione sulfonamide is given (the ionizing proton is not shown), and the arrow designates a bond cleavage site when undergoing fragmentation. Stars associated with the GSA structure designate isotopically labeled atoms (^{13}C or ^{15}N).

positive off-diagonal peaks were observed that could be attributed to chemical exchange.

Results of the NMR Isotope Tracer Studies. When GSA was synthesized in H_2O and NMR spectra were recorded in H_2O (with a small amount of D_2O added to facilitate frequency locking and shimming), the proton NMR resonances for Glu α-H that correspond to the two diastereomers of GSA were observed (Figure 1), and they integrate properly with respect to the other NMR resonances that were assigned to the two diastereomers. When a solution of fully protonated GSA in H_2O was diluted with D_2O, the integrations of Glu α-H resonances were found to be proportional to the H_2O/D_2O ratio.

Results of the MS Studies. The exact mass of GSA confirmed its formulation as GSH+2O-2H. Electrospray MS of GSA gave a $[M + H]^+$ ion with *m/z* 338, which was fragmented by collision-induced dissociation to a predominant fragment ion with *m/z* 263 (Figure 2). When [glycine 1,2-$^{13}C_2$,^{15}N]GSH was treated with HOCl, a corresponding GSA species was formed with the incorporation of labeled glycine. MS showed the nominal mass of this species to be the expected three mass units heavier than the naturally abundant form. The fragmentation pattern of labeled GSA was identical to that observed for unlabeled GSA, which indicated that the glycinyl portion was lost as a neutral fragment and implied that it was not incorporated into the heterocyclic ring structure of GSA.

Scheme 3. Assignments of the ^1H (with Coupling Constants), ^{13}C, and ^{15}N NMR Spectra of the Major and Minor Diastereomers of GSA at pH 7.4 and 2 °C



Discussion

Assignment of the NMR Spectra of GSA. The ^1H, ^{13}C, and ^{15}N NMR spectra of GSH and GSSG have been previously assigned using NMR methods that were similar to those that we have employed in the present study (*29–31*). Because it is well-known that the NMR spectra of derivatives of GSH are very sensitive to pH and other solvent influences (*32*), for the purpose of making direct comparisons, we measured the spectra of GSH and GSSG under precisely the same conditions that were used to measure the spectra of GSA that are reported herein. In addition, we measured the NMR spectra of GSO_3^- (since the oxidation state at the S-center is the same for GSA and GSO_3^-). Examination of the ^1H NMR chemical shift of Table 1 suggests only that the Cys β protons are sensitive to functionalization of the S-center. The corresponding Cys β resonances of GSA suggest that there has indeed been such a functionalization. Proton coupling constants are sensitive to the conformations of organic molecules (the Karplus equation) (*33*), and conformationally constrained molecules sometimes exhibit unusual $^1H-^1H$ coupling constants. The most striking feature of the NMR spectra of GSA is the existence of two sets of resonances in an approximately 2:1 ratio at pH 7.4 and 20 °C. The isomeric ratio did not vary significantly between 2 and 60 °C (pH not corrected of the temperature change). The coupling constants of Table 2, particularly those that are associated with the CH_2-CH_2 moiety of the Glu residue, suggest constrained and somewhat different conformations for the major and minor isomers of GSA. Most of the ^{13}C nuclei exhibit similar chemical shifts (Table 3). However, the Cys β-C appears to be diagnostic of the oxidation state of the S-center (*34*), and the ^{13}C chemical shifts of both isomers of GSA are similar to those of GSO_3^-. We note that the chemical shifts for Glu α-C of both diastereomers of GSA (ca. 64 ppm) suggest functionalization as they are markedly different from the Glu α-C chemical shifts of the other derivatives of GSH (ca. 54 ppm). An examination of the alternate formulations of GSA in Scheme 2 suggests that the nine-membered sulfonamide is most consistent with the NMR spectra. The five-membered 1,2-oxathiolane (Scheme 2, **1**) is not favored, because the spectra of the Gly residue of both isomers of GSA are similar to those of the other GSH derivatives and the Gly NH resonance is observed. Furthermore, one would expect Cys α-H to be acid-labile, which it apparently is not.

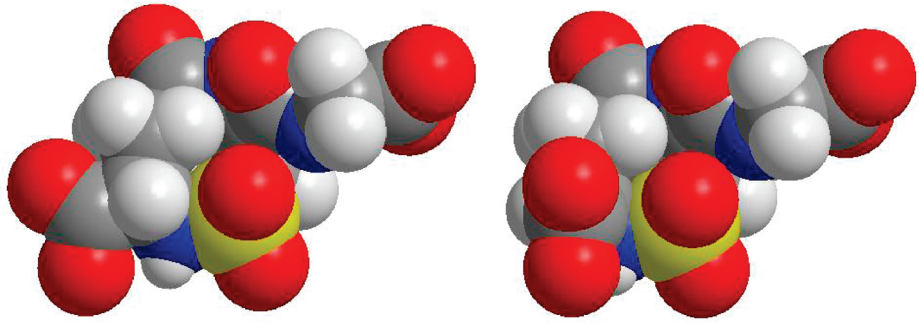
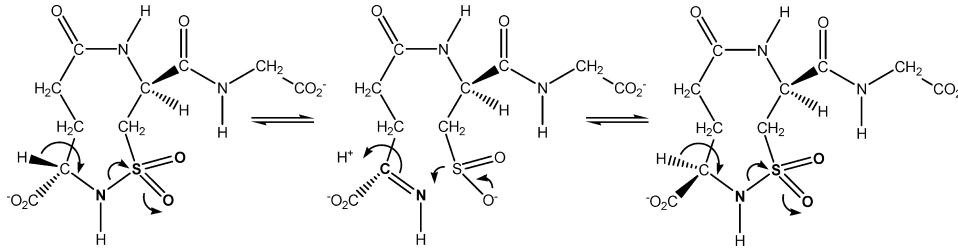


Figure 3. Space-filling models of the two diastereomers of GSA as computed by MM2.

Scheme 4. Proposed Mechanism for the Isomerization of GSA Vis-à-Vis an Imine-Sulfinate



The five-membered isothiazolidine structure (Scheme 2, **2**) is not favored for the arguments made for **1** concerning the Gly resonances. The six-membered 1,2,5-oxathiazine structure (Scheme 2, **3**) is ruled out because the Cys NH proton is clearly present in both isomers. In contrast to structures of **1**−**3**, the structure of the sulfonamide GSA is consistent with all of the available spectroscopic data (Scheme 3).

Molecular Structure of GSA. The NMR spectra of GSA demonstrate that it is a diastereomeric mixture of two nine-membered sulfonamides. Deuterium tracer studies have proven that it is the Glu α-C that has undergone racemization. The assigned structure is consistent with the observed lack of amine functionality. Furthermore, the MS fragmentation patterns would be difficult to reconcile for the alternative structures of Scheme 2. Given our preconception that the mesocyclic structure would be unfavorable, we have modeled the structures of the two diastereomers of GSA computationally. The MM2 force field-minimized structures of the two diastereomers are illustrated in Figure 3 as space-filling models. The sulfonamide moiety is located at the bottom, and the Glu α-C center is on the left of each structure. There are two interesting observations. First, the nine-membered rings appear to adopt compact structures with little conformation freedom but are nonetheless sterically unhindered. Second, epimerization of the Glu α-C center does not have a marked effect on the conformation of the rest of the molecule. Accordingly, the NMR spectra of the two diastereomers are very comparable (Scheme 3). The similarity of the conformations also explains why we were unable to make an absolute assignment of the stereochemistries using NOEs, as the internuclear distances between the proton centers are comparable in the two structures. The total energy of the two diastereomeric structures of Figure 3 (as computed by MM2) favors the structure of the left, but we are not sufficiently confident in the computed energies to assign that structure to the major diastereomer as there could be additional factors (e.g., solvent or bonding effects) that influence the observed equilibrium.

Interconversion of the Diastereomers of GSA. 2D EXSY NMR spectra (at temperatures up to 48 °C) do not show off-diagonal peaks that are assigned to chemical exchange, so the interconversion of the two diastereomers of GSA is slow on the spin-relaxation (second) time scale. However, the two diastereomers do interconvert, as indicated by the deuterium tracer studies. These studies also prove that it is Glu α-C that is undergoing racemization (Scheme 4). This observation is consistent with our preconception that the N−SO_2 bond of GSA should be relatively unstable. We note that pyridoxal-dependent amino acid racemases employ an imine intermediate to epimerize the α-center of amino acids (*35*).

Conclusion

GSA, a stable product formed when GSH is oxidized by neutrophil-derived HOCl, has been structurally characterized as a nine-membered cyclic sulfonamide. Because little GSA is formed in the presence of physiologically relevant concentrations of other oxidants, it is potentially useful as a biomarker for detecting production of HOCl in vivo. GSA levels may reflect the localized inflammatory status due to neutrophil oxidant production. The scene is now set to attempt to detect this species in clinical samples from inflammatory conditions.

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