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NMR Detection and Study of Hydrolysis of HNO-Derived Sulfinamides

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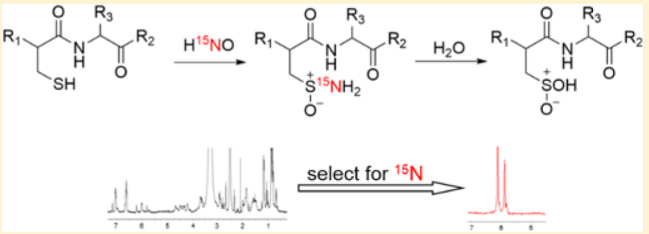
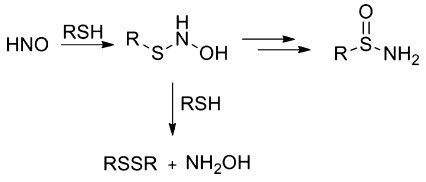
Supporting Information

ABSTRACT: Nitroxyl (HNO), a potential heart failure therapeutic, is known to post-translationally modify cysteine residues. Among reactive nitrogen oxide species, the modification of cysteine residues to sulfinamides [$RS(O)NH_2$] is unique to HNO. We have applied ^{15}N-edited ^{1}H NMR techniques to detect the HNO-induced thiol to sulfinamide modification in several small organic molecules, peptides, and the cysteine protease, papain. Relevant reactions of sulfinamides involve reduction to free thiols in the presence of excess thiol and hydrolysis to form sulfinic acids [RS(O)OH]. We have investigated sulfinamide hydrolysis at physiological pH and temperature. Studies with papain and a related model peptide containing the active site thiol suggest that sulfinamide hydrolysis can be enhanced in a protein environment. These findings are also supported by modeling studies. In addition, analysis of peptide sulfinamides at various pH values suggests that hydrolysis becomes more facile under acidic conditions.



Cysteine residues are known to be targets of reactive nitrogen oxide species (RNOS).[1−3] Nitroxyl (HNO), the protonated, one-electron reduced form of NO, has been shown to have potential uses in the treatments of heart failure, alcoholism, vascular dysfunction, and cancer.[2,4−6] HNO is very reactive with thiols, forming sulfinamides or disulfides depending on the concentration of thiol (Scheme 1).[7,8] Sulfinamide formation dominates at low thiol concentrations, whereas disulfide and hydroxylamine become the major end products in the presence of excess thiol.

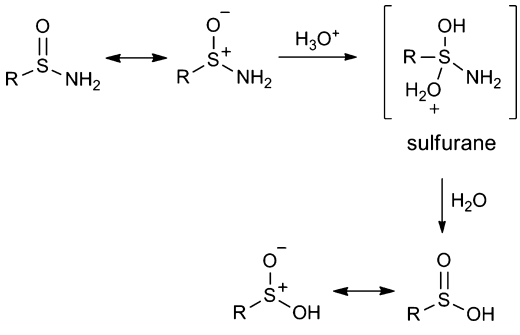
Scheme 1. Reaction of HNO with Thiols



HNO-induced effects have been detected on several cysteine-containing proteins.[9−23] In many of these proteins, the observed effects are attributed to the formation of a sulfinamide. Apart from these HNO studies, administration of sulfonamide antibiotics and arylamines has been shown to result in the formation of N-substituted sulfinamides in proteins.[24,25] To understand the pharmacological effects of HNO more completely, the reactivity of sulfinamides under biologically relevant conditions requires further investigation.

At physiological pH and temperature, the major reactions of sulfinamides involve the reduction to free thiols in the presence of excess thiol and hydrolysis to form sulfinic acids.[26,27] There are very few studies on the hydrolysis of sulfinamides, the majority of which have been carried out under aqueous, acidic conditions.[28−30] The mechanism is thought to involve a sulfurane intermediate (Scheme 2).[30] Studies conducted with

Scheme 2. Hydrolysis of Sulfinamides



several N-substituted alkyl sulfinamides indicate that aliphatic secondary sulfinamides are relatively stable at basic pH, but hydrolysis can proceed readily at pH 3.[17,28] Also, tertiary sulfinamides have been found to be more reactive than secondary sulfinamides.[28] Although the hydrolysis of HNO-derived sulfinamides has been observed,[17,26,27] the reaction has not been studied in detail under physiological conditions. In

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recent studies, sulfinic acid modifications of thiols were seen upon exposure of HNO-treated samples to proteomic analysis or upon storage.[17,26] Moreover, we recently reported the slow conversion of primary sulfinamides to sulfinic acids under physiological conditions as observed by ESI-MS.[27]

Isotope-edited ^{1}H NMR techniques are generally used for investigating interactions in macromolecular complexes such as ligand−protein, protein−protein, or protein−nucleic acid interactions.[31−33] In this method, one or more components of the complex are labeled with NMR active isotopes and the selective detection of protons bonded to the isotope-labeled atom is achieved.[31] Sulfamate groups on glycosaminoglycans and a DNA−peptide adduct were recently characterized using an analogous NMR method.[34,35] Herein, we are pleased to report the application of ^{15}N-edited NMR spectroscopy to detect and study the hydrolysis of sulfinamides in several small organic molecules, peptides, and the cysteine protease, papain.

EXPERIMENTAL METHODS

Reagents. Glutathione (GSH), papain, N_α-Benzoyl-L-arginine 4-nitroanilide hydrochloride (L-BAPNA), β-mercaptoethanol (BME), *tert*-butanesulfinamide (racemic), *N*-ethylmaleimide (NEM), and dithiothreitol (DTT) were of the highest purity available and purchased from Sigma (St. Louis, MO). 2-Phenylethanethiol and 2-(bromoethyl)benzene were purchased from Acros. HPLC grade acetonitrile (ACN) was purchased from Thermo Fisher Scientific (Rockford, IL). Dimethyl sulfoxide-d_6 (DMSO-d_6), ^{15}N-labeled hydroxylamine hydrochloride and ^{15}N-labeled benzamide were purchased from Cambridge Isotope Laboratories (Andover, MA). The syntheses of 2-phenylethanesulfinamide, the HNO-donor, Angeli's salt ($Na_2N_2O_3$, AS), and the NO-donor (DEA/NO) were carried out as previously described.[27,36−38] The ^{15}N-labeled HNO donor, *N*-hydroxy-2-(methylsulfonyl)-benzenesulfonamide[39] (^{15}N-2-MSPA), was a generous gift from Cardioxyl Pharmaceuticals. Milli-Q water was used for all purifications and experiments.

Peptide Synthesis and Purification. Synthetic peptides VYPCLA and AGSCWA were synthesized on a Symphony Quartet peptide synthesizer (Protein Technologies Inc., Tucson, AZ) following Fmoc solid-phase peptide synthesis methods.[40] The crude product was dissolved in 0.1% trifluoroacetic acid (TFA) and purified by HPLC (Waters HPLC equipped with Delta 600 pump system and dual wavelength absorbance detector) on an Apollo C_{18} reverse-phase column using a linear gradient of 5−75% ACN with 0.1% TFA over 50 min at room temperature. Peptide fractions were identified by electrospray ionization mass spectrometry (ESI-MS). Pure fractions were pooled and lyophilized, and the purified product was quantified based on the absorbance at 280 nm (ε_{280} = 1490 M^{-1} cm^{-1}).[41] In all cases, peptides were stored at −80 °C in lyophilized form until use.

Synthesis of ^{15}N-labeled Angeli's Salt (^{15}N-AS). Synthesis of ^{15}N-labeled AS, which produces ^{15}N-labeled HNO ($H^{15}NO$) and unlabeled nitrite (NO_2^-), was carried out based on a literature procedure by reacting $^{15}NH_2OH$ and isoamyl nitrate in basic methanol.[42] The purity of ^{15}N-AS was confirmed by comparison of the extinction coefficient at 248 nm in 0.01 M NaOH with the literature value (ε_{248} = 8300 M^{-1} cm^{-1}).[37]

Formation of Sulfinamides by Reaction with HNO. Organic thiols or thiol-containing peptides were dissolved in 10 mM sodium phosphate buffer with 50 μM of the metal chelator, diethylenetriamine pentaacetic acid (DTPA), at pH 7.4 to have a final concentration of 0.1 or 0.2 mM and used immediately. Stock solutions of AS or ^{15}N-AS were prepared in 0.01 M NaOH, kept on ice, and used within 15 min of preparation. Stock solutions of ^{15}N-labeled *N*-hydroxy-2-(methylsulfonyl)benzenesulfonamide (2-MSPA) were prepared in ACN and used within 15 min of preparation. The organic molecules or peptides were incubated with 1 mM AS or ^{15}N-AS at 37 °C for 30 min in a block heater. The samples were then aliquoted, flash-frozen, and lyophilized. Following lyophilization, the samples were redissolved in DMSO-d_6 for immediate NMR analysis.

For experiments conducted at varied pH's, glutathione sulfinamide was formed by incubating GSH with 1 mM ^{15}N-2-MSPA (rather than with AS) at 37 °C for 30 min in pH 7.4 phosphate buffer due to higher sulfinamide yield obtained. The samples were prepared for NMR analysis as described above.

Incubations with DEA/NO. The VYPCLA peptide was dissolved in 10 mM sodium phosphate buffer with 50 μM DTPA, at pH 7.4 to have a final concentration of 0.1 mM and was used immediately. Stock solutions of DEA/NO were prepared in 0.01 M NaOH, kept on ice, and were used within 15 min of preparation. The peptide was incubated with 0.5 mM DEA/NO at 37 °C for 30 min in a block heater. (Note that DEA/NO was administered at half the concentration of HNO donors used to account for 2 equiv of NO production from 1 equiv of DEA/NO.) The samples were then prepared as described above for NMR analysis.

Preparation of NMR Samples for Synthetic Sulfinamides. Commercially available *tert*-butanesulfinamide and synthetic 2-phenylethanesulfinamide were dissolved in DMSO-d_6 to have a final concentration of 100 and 10 mM. The samples were analyzed by NMR without any further treatment.

HPLC Analyses of 2-Phenylethanesulfinamide. All the analyses were performed on an Apollo C_{18} reverse-phase column connected to the HPLC system described above. A linear gradient of 35−80% ACN with 0.1% TFA over 35 min was employed at room temperature. The compounds were followed at 220 nm, and peaks were assigned based on coinjection with authentic samples.

Activation of Papain. A 0.2 mM papain solution was prepared by dissolving the lyophilized enzyme in 10 mM sodium phosphate buffer with 50 μM DTPA at pH 7.4. The papain sample was activated by treatment with 1 mM DTT for 30 min at room temperature to reconvert its single free cysteine to the sulfhydryl form. The activated enzyme was then desalted with Zeba spin desalting columns to remove excess DTT.

Generation of Sulfinamide-Modified Papain. The activated papain in 10 mM sodium phosphate buffer with 50 μM DTPA at pH 7.4 was immediately incubated with 1 mM ^{15}N-AS at 37 °C for 30 min to form the ^{15}N-labeled sulfinamide as described above.

Papain Activity Assay. An aliquot from the 0.2 mM papain (with or without prior AS treatment) in 10 mM sodium phosphate buffer with 50 μM DTPA at pH 7.4 was diluted into buffer to have a final papain concentration of 8.5 μM. The activity of papain was analyzed by a 20 min spectrophotometric assay using L-BAPNA as the substrate.[43]

ESI-MS Analyses of Papain. An aliquot from the 0.2 mM papain (with or without prior AS treatment) in 10 mM sodium phosphate buffer with 50 μM DTPA at pH 7.4 was lyophilized and purified by HPLC on a Viva C_4 reverse-phase analytical

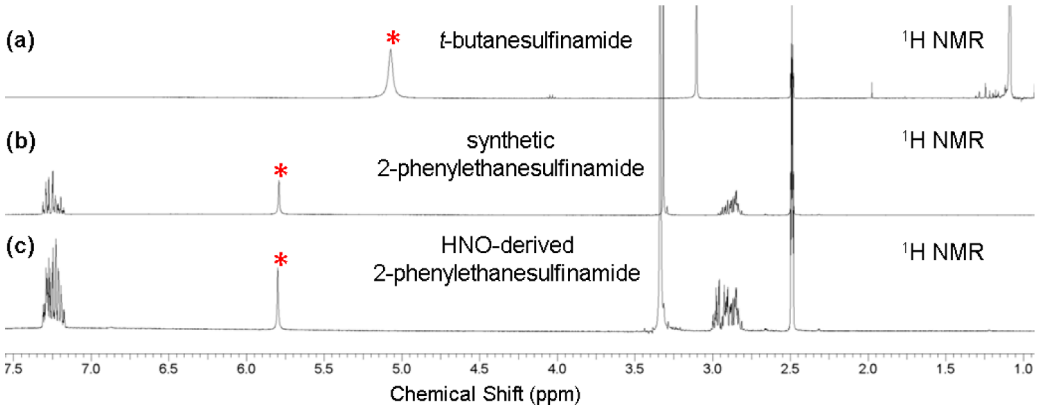

Figure 1. ^{1}H NMR spectra of (a) *tert*-butanesulfinamide, (b) synthetic, and (c) HNO-derived 2-phenylethanesulfinamide collected in DMSO-d_6 at 30 °C. The HNO-derived sulfinamide was formed by treating 2-phenylethanethiol (0.1 mM) with AS (1 mM) in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min. An asterisk indicates the sulfinamide NH signals.

column using a linear gradient of 5−95% ACN with 0.1% TFA over 80 min at room temperature.[44] The samples were then analyzed on a Thermo Finnigan LCQ Deca Ion Trap Mass Spectrometer fitted with an electrospray ionization source, operating in the positive ion mode. In all experiments, the samples were introduced to the instrument at a rate of 10 μL/min using a syringe pump via a silica capillary line. The heated capillary temperature was 250 °C and the spray voltage was 5 kV. Data analysis and deconvolution were carried out using Bioworks 3.2 software.

As a control, an aliquot from the papain sample was incubated with 2 mM NEM (with or without prior AS treatment) in buffer at 37 °C for 1.5 h. The samples were purified and analyzed by ESI-MS as described above.

Incubations of Peptides and Proteins in Buffer. Aliquots of the sulfinamide-containing samples (in 10 mM sodium phosphate buffer with 50 μM DTPA, at pH 7.4) were incubated at 37 °C. Individual aliquots were removed from the samples for analysis at certain time intervals, flash-frozen, lyophilized, and prepared for immediate NMR analysis as described above. In all cases, 1 mM ^{15}N-labeled benzamide was added to the NMR sample as an internal standard. In some cases, VYPCLA sulfinamide was incubated in the presence of 50 mM BME (as indicated), and the samples were prepared as described above for NMR analysis.

Incubations of Peptides at Varied pH. Aliquots of the sulfinamide-containing samples (in 10 mM sodium phosphate buffer with 212 μM DTPA at pH 7.4) were adjusted to the desired pH by adding 10% H_3PO_4 or 1 M NaOH while stirring. The samples were incubated at 37 °C. Individual aliquots were removed from the samples for analysis at certain time intervals, readjusted to pH 7.4, flash-frozen, lyophilized, and prepared for immediate NMR analysis as described above. In all cases, 1 mM ^{15}N-labeled benzamide was added to the NMR sample as an internal standard.

Potentiometric Determination of *tert*-Butanesulfinamide pK_a. The potentiometric titration was adapted from a literature procedure.[45] Briefly, commercially available *tert*-butanesulfinamide was dissolved in 0.05 M KCl to have a concentration of 2 mM. The solution was titrated with either 20 mM NaOH or 20 mM HCl prepared in 0.05 M KCl at room temperature. The changes in pH were determined by using a general purpose combination pH probe with a built-in temperature sensor connected to a pH meter.

NMR Analyses. All ^{1}H NMR and ^{15}N-edited ^{1}H 1D-NMR analyses were carried out on a Bruker Avance 400 MHz FT-NMR spectrometer at 303 K. In some cases, spectra were collected at temperatures from 303 to 343 K (as indicated). ^{15}N-edited ^{1}H 1D-NMR spectra were acquired using the HSQC pulse sequence for selection. 2D-ROESY and COSY spectra of the VYPCLA sulfinamide were obtained on a Bruker Avance 600 MHz FT-NMR spectrometer. All chemical shifts are reported in parts per million (ppm) relative to residual DMSO (2.49 ppm for ^{1}H).

Active Site Modeling. A Python (v2.7) script was written using the PyRosetta v3.4.0 r53345 libraries.[46] Atom type parameters were added to the Rosetta database for the sp^3-hybridized NH_2 group of sulfinamide. Rosetta residue type parameter files were added for cysteine sulfinate and each cysteine sulfinamide stereoisomer. Ideal bond lengths and angles were taken from PDB ID 1ACD and several references.[47,48] The Dunbrack rotamer library[49] was used to select rotamers for each standard amino acid residue side chain. Ten cycles of side-chain packing followed by steepest descent minimization were performed for each protein variant examined using the standard "score12" scoring function. Atom type and residue type parameters, as well as the Python script used, can be found in the Supporting Information. Structures generated by Rosetta were output as pdb files and viewed in DS Visualizer.

■ RESULTS AND DISCUSSION

Because protein structure and function can be altered significantly due to post-translational modifications of cysteine residues, the biological effects of HNO are often attributed to its reactivity with thiols.[6,50−53] Sulfinamides are one of the major thiol modifications observed upon exposure to HNO.[7,8] Although thiols are known to react with other RNOS, sulfinamide formation is considered to be unique to HNO.[54] Despite being a well-established modification, very few studies on the reactivity of HNO-derived sulfinamides have been reported.

To detect and study the reactivity of sulfinamides in a facile manner, we have applied ^{15}N-edited NMR techniques, which are commonly used to study ligand−protein interactions.[31−33] These techniques are on the basis of distinguishing between protons bonded to isotope-labeled and unlabeled nuclei.[31−33] Although direct ^{15}N NMR detection is a well-known technique,

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unfortunately, it suffers from low sensitivity.[55−57] To determine the potential applicability of direct ^{15}N NMR spectroscopy to the study of sulfinamides, we explored its detection limit by employing ^{15}N-labeled urea. These preliminary studies have shown that although 1 M ^{15}N-labeled urea can be observed in less than 30 min, the addition of relaxation agents[57] and an extended number of scans are needed to observe 10−100 mM ^{15}N-labeled urea. These results suggest that ^{15}N NMR spectroscopy would not be convenient for the detection of HNO-derived sulfinamides, which are expected to be formed in much lower concentrations. Apart from retaining the sensitivity of ^{1}H NMR, the major advantage of ^{15}N-edited NMR procedures is the ability to provide simpler and more easily interpretable spectra, which becomes especially important for the detection of protein modifications.

Detection of Synthetic and HNO-Derived Small Organic Molecule Sulfinamides by ^{1}H NMR. We initially investigated the conditions necessary to detect sulfinamides by ^{1}H NMR to identify characteristic chemical shifts for the sulfinamide functional group. For this purpose, commercially available *tert*-butanesulfinamide was employed. Although we attempted NMR analysis directly in buffer using water suppression techniques, the sulfinamide NH signal resonates too closely with the water signal, precluding its detection. Among the NMR solvents employed ($CDCl_3$, ACN-d_3, DMSO-d_6, and THF-d_8), DMSO-d_6 provided the best results in terms of sample solubility and the clean observation of sulfinamide chemical shifts (5.1−6.3 ppm) (Figure 1). Also, the lack of exchangeable protons in DMSO-d_6 avoided the possible exchange between the sulfinamide NH's and deuterated solvent, allowing for better detection and accurate quantification. Similar results were obtained with synthetic 2-phenylethanesulfinamide, confirming the characteristic chemical shift region for the sulfinamide functional group and the general applicability of the method to other sulfinamides (Figure 1). Therefore, we carried out all reactions in phosphate buffer and then transferred the samples into DMSO-d_6 (via lyophilization) for NMR analyses.

To determine if the above conditions can be applied to HNO-derived sulfinamides, we treated 2-phenylethanethiol with AS in buffer to form the corresponding 2-phenylethanesulfinamide. Similar to our previous ESI-MS results,[27] a thiol to HNO donor ratio of 1:5 or 1:10 provided sufficient amount of 2-phenylethanesulfinamide. HPLC and ESI-MS analysis confirmed that the reaction of 2-phenylethanethiol with HNO produces the corresponding sulfinamide with a small amount of disulfide (Supporting Information). The ^{1}H NMR analysis was carried out following lyophilization of the sulfinamide from buffer and redissolving it in DMSO-d_6. As expected, the characteristic sulfinamide NH signal was observed at the same chemical shift for the synthetic and HNO-derived 2-phenylethanesulfinamides (5.8 ppm) (Figure 1b,c).

Because hydroxylamine is obtained as a byproduct in the case of HNO-induced disulfide formation (Scheme 1), we carried out control experiments with hydroxylamine exposed to the same sample preparation procedures or directly dissolved in the NMR solvent. These results did not show any peaks in the chemical shift region assigned to the sulfinamide NH's (data not shown).

^{15}N-Edited ^{1}H 1D NMR to Detect ^{15}N-Labeled Small Organic Molecule Sulfinamides. As mentioned previously, the use of isotope-edited methods simplifies the NMR spectra of complex samples by allowing the selective detection of protons bonded to the isotope-labeled atom. The labeling of sulfinamides with ^{15}N was achieved by incubating thiols with ^{15}N-AS, which decomposes to produce ^{15}N-labeled HNO ($H^{15}NO$) and unlabeled NO_2^-. As seen in Figure 2a, treatment

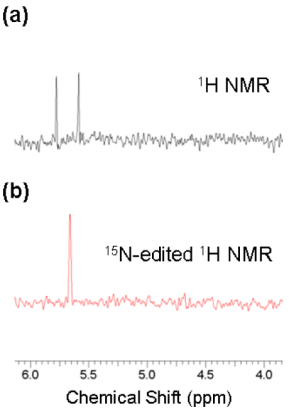

Figure 2. Selected region of ^{1}H NMR spectrum showing (a) sulfinamide ^{15}NH signals following the treatment of β-mercaptoethanol (0.1 mM) with ^{15}N-AS (1 mM) in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min and (b) the corresponding region of the ^{15}N-edited ^{1}H 1D-NMR decoupled spectrum acquired using the HSQC pulse sequence for selection. The spectra were collected in DMSO-d_6 at 30 °C.

of β-mercaptoethanol (BME) with $H^{15}NO$ resulted in the formation of the corresponding ^{15}N-labeled sulfinamide (BME-S(O)$^{15}NH_2$) with a ^{1}H−^{15}N coupling constant (J_{NH}) of 76 Hz. Upon application of an isotope filter to select for ^{15}N, only the sulfinamide ^{15}NH signal is observed in the decoupled ^{15}N-edited spectrum (Figure 2b).

Detection of HNO-Derived Sulfinamides in Peptides. Given the results described above, the hexapeptide, VYPCLA, was employed to test our detection method on a more complex system. In our previous work, the sulfinamide yield of this peptide was determined to be greater than 90% upon incubation with 10 times excess AS.[27] As seen in Figure 3a−e, the sulfinamide NH peaks (5.8−6.3 ppm) are observed only after HNO treatment of the thiol-containing peptide. Control experiments conducted with the unmodified peptide or following treatment with DEA/NO, did not result in any sulfinamide NH signals (Figure 3a,e).

Unlike the signals for small organic molecule sulfinamides, two different signals are observed for the VYPCLA-generated sulfinamide (Figure 3b−d). This result can be explained based on the known chirality of the sulfur atom in sulfinamides.[58,59] Upon sulfinamide formation, the stereochemistry at sulfur can be either R or S. In a small organic molecule sulfinamide, where the sulfur is the only chiral center, the resulting compound is racemic, and therefore, shows only one NMR signal. However, in the case of an L-peptide, the resulting sample is composed of two diastereomers (more specifically epimers) with the L-peptide containing either an (*R*)- or (*S*)-sulfinamide, consistent with the observed two NMR signals.

We have also conducted two-dimensional rotating-frame nuclear Overhauser effect correlation spectroscopy (2D-ROESY) and correlation spectroscopy (COSY) experiments on the HNO-derived VYPCLA sulfinamide. These data indicate the presence of two distinct sulfinamide species with a single NH_2 signal at 5.97 and 6.07 ppm (Figure 4). Moreover, the 5.97 ppm peak shows ROE cross peaks with the cysteine β- and

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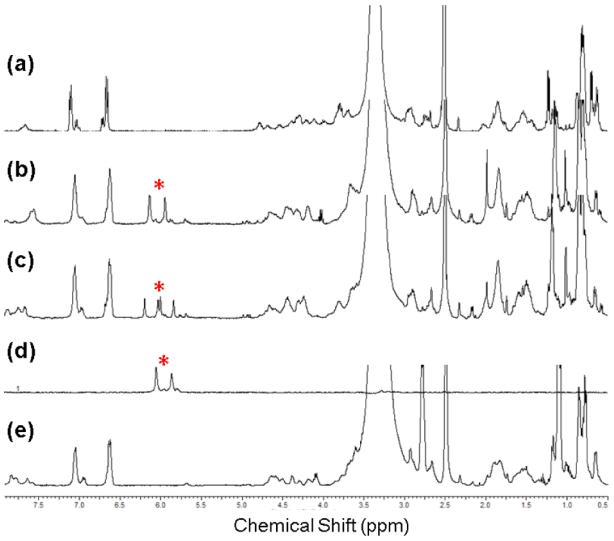

Figure 3. NMR spectra observed for VYPCLA (0.1 mM) (a) untreated or treated with (b) 1 mM AS (c, d), 1 mM ^{15}N-AS, and (e) 0.5 mM DEA/NO in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min. Spectra (a–c) and (e) are ^{1}H NMR spectra and (d) is an ^{15}N-edited ^{1}H 1D-NMR spectrum, which uses the HSQC pulse sequence for selection. The spectra were collected in DMSO-d_6 at 30 °C. An asterisk indicates the sulfinamide NH and ^{15}NH signals.

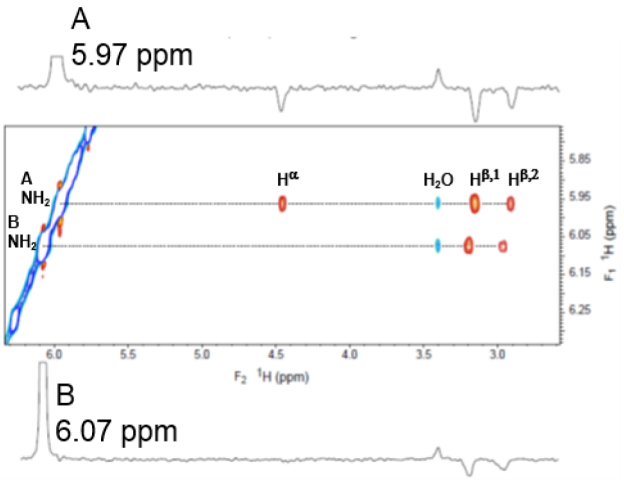

Figure 4. Selected region of 2D-ROESY spectrum showing ROE cross peaks involving sulfinamide NH_2 signals of VYPCLA (0.1 mM) treated with 1 mM AS in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min. The spectra were collected in DMSO-d_6. (A) and (B) indicate the 5.97 and 6.07 ppm signals, respectively.

α-H's, whereas the downfield species shows ROE cross peaks only with the cysteine β-H's (Figure 4). These results are consistent with the presence of two diastereomers. Comparison of the cysteine β-H's also reveals a 0.2 ppm downfield shift between the unmodified and sulfinamide modified-VYPCLA, further confirming that the HNO-derived modification is on the cysteine residue (Supporting Information).

The presence of two peaks could potentially be due to restricted rotation of the sulfinamide group causing the NH's to be magnetically nonequivalent. However, the discrepancy between small organic molecule and peptide sulfinamides, as well as the low rotational barrier (ca. 8.5 kcal/mol) suggested for the sulfinamide S–N bond, make restricted rotation unlikely.[60] Nevertheless, to eliminate this possibility, we performed NMR experiments in which the same NMR sample was analyzed at temperatures from 30 to 70 °C (Supporting Information). These data show that two sulfinamide peaks can be observed even at 70 °C, suggesting that the two peaks are not due to restricted rotation around the S–N bond.

As seen in Figure 5, results similar to those observed with VYPCLA are obtained upon treatment of glutathione with

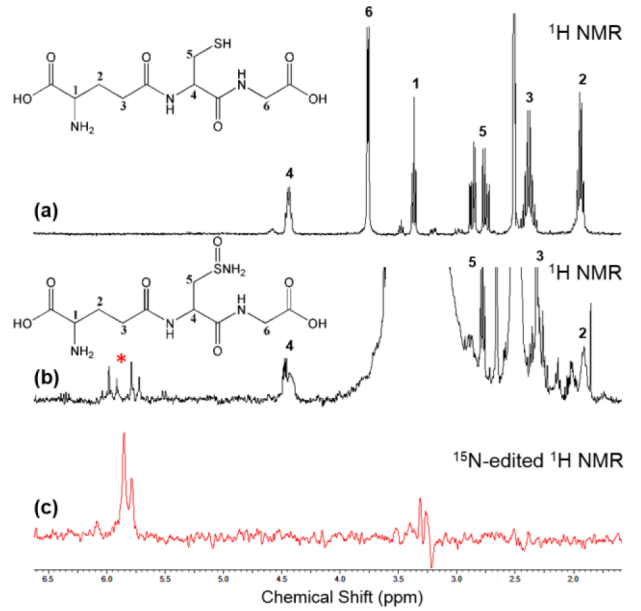

Figure 5. ^{1}H NMR spectrum of (a) unmodified GSH or (b) the glutathione sulfinamide formed by treating GSH (0.2 mM) with 1 mM ^{15}N-AS in 2 mM phosphate buffer with 12.5 μM DTPA (pH 6) at 37 °C for 30 min. (c) ^{15}N-edited ^{1}H 1D-NMR spectrum of the glutathione sulfinamide acquired using the HSQC pulse sequence for selection. The samples were analyzed in DMSO-d_6 at 55 °C. An asterisk indicates the sulfinamide ^{15}NH signals.

H^{15}NO to produce the glutathione sulfinamide. Moreover, for both peptides, unequal amounts of the two diastereomers were obtained, indicating that one diastereomer has lower energy and/or a lower kinetic barrier to formation. The ^{1}H–^{15}N coupling was determined to be 76 Hz, as observed for all the peptide sulfinamides detected.

Detection of an HNO-Derived Sulfinamide in Papain. The 23.4 kDa cysteine protease, papain, is one of the several enzymes known to be inhibited by HNO.[43] It has significant similarities to important mammalian proteases such as cathepsins and calpains.[61] The active site of papain contains a catalytic triad, which involves Cys25, His159, and Asn175.[62] In its active form, Cys25 exists as a thiolate and constitutes the only free thiol in the papain structure.[62,63] The catalytic mechanism of papain involves nucleophilic attack of the Cys25 thiolate to form an acylenzyme intermediate followed by a deacylation step.[62,64] The roles of His159 include participation in a thiolate/imidazolium ion pair to maintain the thiolate anion and also participation as a general acid catalyst during acylation and as a general base catalyst during deacylation.[62,63]

Although the formation of a sulfinamide has been proposed for papain after HNO treatment, to the best of our knowledge, it has not been observed directly. Therefore, we applied the ^{15}N-edited ^{1}H 1D NMR method to characterize the HNO-derived modification of papain. Figure 6 demonstrates that

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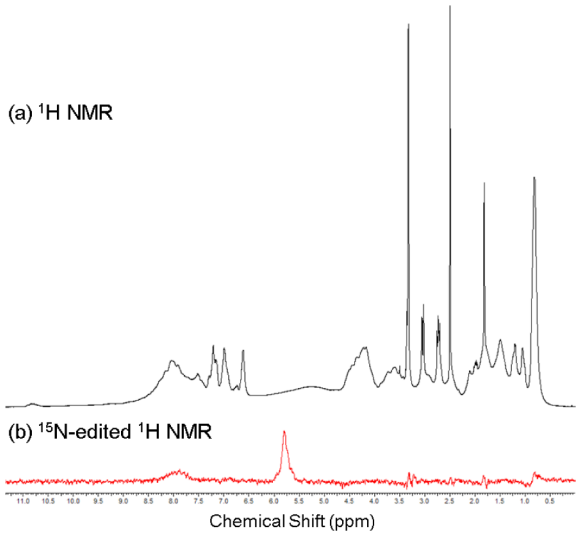

Figure 6. (a) ^{1}H NMR spectrum of sulfinamide modified-papain formed by treating papain (0.2 mM) with 1 mM ^{15}N-AS in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min. (b) ^{15}N-edited ^{1}H NMR spectrum of sulfinamide modified-papain acquired using the HSQC pulse sequence for selection. The spectra were collected in DMSO-d_6 at 30 °C. The relatively small, broad peak observed at approximately 7.9 ppm in Figure 6b corresponds to the natural abundance ^{15}NH of backbone NH's.

although the sulfinamide NH signal could not be unambiguously detected by ^{1}H NMR spectroscopy due to the interference of other signals and an inherently weak signal, it can be observed clearly in the ^{15}N-edited NMR spectrum (at 5.8 ppm), demonstrating that this technique is a convenient method to study HNO-derived sulfinamides in proteins.

Note that only one sulfinamide signal is detected, potentially due to the large peak width (>90 Hz) associated with this protein sulfinamide. However, the preferential formation of one diastereomer in the papain active site is certainly possible. Indeed, steepest descent minimizations performed with the Rosetta modeling suite suggests that the (*R*)-sulfinamide papain variant is slightly more stable than the *S* sulfinamide variant (−308.05 ± 0.08 Rosetta energy units versus −307.1 ± 0.2 REU, respectively).

The formation of the sulfinamide modification in papain was also confirmed by ESI-MS (Supporting Information). Moreover, the inhibition of activated papain by AS under the conditions described above was confirmed by papain activity assays. As has been observed previously,[43] no significant papain activity could be detected after AS treatment, consistent with sulfinamide formation (Supporting Information).

Reduction of Peptide Sulfinamides. Previously we had used ESI-MS to demonstrate that approximately 60% of the VYPCLA sulfinamide can be reverted back to free thiol upon 26 h-incubation with excess thiol at physiological pH and temperature. To determine if the ^{15}N-edited ^{1}H NMR method can be applied to study sulfinamide reactivity, we repeated the above experiment by incubating VYPCLA sulfinamide with BME and employing ^{15}N-labeled benzamide as the internal standard during NMR analysis. The NMR data, which indicates 57% sulfinamide reduction, is in very good agreement with the previous ESI-MS results (Supporting Information).

Hydrolysis of Peptide Sulfinamides. Sulfinamides are known to undergo hydrolysis to form sulfinic acids, especially under acidic conditions.[17,26,28−30] We have previously observed the slow conversion of primary sulfinamides to their corresponding sulfinic acids[27] and have now explored this reactivity using the ^{15}N-edited ^{1}H NMR method.

The extent of sulfinamide hydrolysis was investigated by incubating the HNO-derived VYPCLA sulfinamide under physiological conditions and analyzing aliquots at different time intervals. Following 28 h of incubation, a 13% decrease in the sulfinamide peak was observed with respect to the initial (Figure 7), confirming that slow hydrolysis of sulfinamides

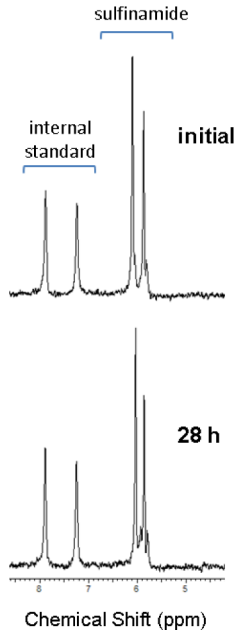

Figure 7. Representative ^{15}N-edited ^{1}H NMR spectra showing the hydrolysis of HNO-derived VYPCLA sulfinamide. VYPCLA (0.1 mM) was treated with 1 mM ^{15}N-AS in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min to form the corresponding sulfinamide. The sample was incubated in buffer at 37 °C for 28 h resulting in a 13% decrease in the sulfinamide peak with respect to its initial intensity. ^{15}N-labeled benzamide was added as an internal standard before NMR analysis. ^{15}N-edited ^{1}H NMR spectra were acquired using the HSQC pulse sequence for selection. The spectra were collected in DMSO-d_6 at 30 °C.

takes place under physiological conditions.[26−28] Moreover, these findings are consistent with our previous ESI-MS results obtained for a similar hexapeptide (VYPCGA).[27] Although the observation of the corresponding sulfinic acids was not possible with this NMR method, our previous studies involving HPLC and fluorescence analyses of synthetic 2-phenylethanesulfinamide and ESI-MS analysis of HNO-derived VYPCGA sulfinamide have shown that sulfinic acids and ammonia are the only products formed under these conditions.[27] Upon 1 week of incubation under similar conditions, very slow sulfinamide hydrolysis (50%) was observed (data not shown). (No peptide bond cleavage was observed when the incubations were carried out at physiological temperature.) Almost complete sulfinamide hydrolysis (94%) was achieved upon boiling the sample for 5 days (Supporting Information). The hydrolysis of glutathione sulfinamide was found to be 28% after 28 h of incubation at physiological pH and temperature, indicating this peptide is more prone to hydrolysis compared with the VYPCLA sulfinamide. Moreover, similar results were obtained when the glutathione sulfinamide was formed using 2-

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MSPA as the HNO donor. Unlike AS, this donor does not produce NO_2^- as a byproduct, indicating that sulfinamide formation is independent of NO_2^-.

We have explored the effect of pH on the hydrolysis of primary sulfinamides by incubating peptide sulfinamides at pH 5−9. Upon 26 h of incubation of the VYPCLA sulfinamide, we observed 33, 16, 13, and 14% sulfinamide hydrolysis at pH 5, 6, 7.4, and 9, respectively. Similarly, 46 and 24% hydrolysis is detected upon incubation of the glutathione sulfinamide at pH 5 and 7.4 for 26 h, respectively. These results indicate that sulfinamide hydrolysis is more facile at acidic pH values, presumably due to protonation of the sulfinamide (Scheme 2). Potentiometric titration studies of the primary sulfinamide, *tert*-butanesulfinamide, indicate that its pK_a is ∼3.9 (Supporting Information), consistent with the above results and the reported value of 3−3.5 for secondary sulfinamides.[65]

Hydrolysis of Sulfinamide-Modified Papain and Comparison with Its Model Peptide. Tertiary structure and local environment are known to have significant effects on reactivity.[66−68] To determine the extent of sulfinamide hydrolysis in a protein environment, we investigated the reactivity of this modification in ^{15}N-AS-treated papain. Interestingly, 54% of the sulfinamide was hydrolyzed after 28 h of incubation under physiological conditions (Figure 8),

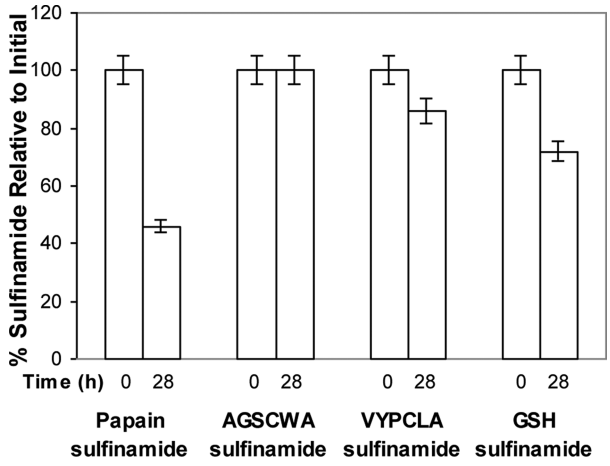

Figure 8. Hydrolysis of HNO-derived sulfinamides in papain, AGSCWA, VYPCLA, and glutathione (GSH). Papain (0.2 mM), AGSCWA (0.1 mM), VYPCLA (0.1 mM), and GSH (0.2 mM) were treated with 1 mM ^{15}N-AS in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min to form the corresponding sulfinamides. The samples were incubated in buffer at 37 °C for 28 h. The amounts of sulfinamide were normalized with respect to that detected in the initial peptide samples (SEM ± 5%, n = 3).

which points to the reaction being significantly faster in the papain active site compared to the peptides tested (i.e., VYPCLA (13%) and GSH (28%)). Moreover, activity studies conducted with AS-treated papain revealed no significant change in papain activity after 28 h of incubation, supporting the formation of a sulfinic acid (data not shown).

The active site thiol is contained in the sequence GSCWA (residues 23−27) of papain.[69] Because variations in the peptide sequence might cause changes in reactivity, a model peptide (AGSCWA) of the papain active site was synthesized. Upon analyzing the extent of hydrolysis in this H^{15}NO-treated hexapeptide, no significant sulfinamide hydrolysis was observed after 28 h (Figure 8). These results indicate that the hydrolysis reaction is significantly enhanced by the papain environment rather than as a result of the specific peptide sequence.

We used Rosetta to assist in formulating a hypothesis for the mechanism of sulfinamide hydrolysis within the papain active site environment. The crystal structure of papain (PDB ID 9PAP), which contains a sulfonate modification on Cys25, was used as a template for generating PDB files for the wild-type (WT), (*R*)-sulfinamide, (*S*)-sulfinamide, and sulfinate (hydrolysis product) papain variants. The WT papain structure and the three variants were minimized, and the resulting structures were examined. In the case of all three variants, the oxygen atom bound to sulfur was placed in the oxyanion hole (Gln19) for the standard papain reaction (Figure 9). This is similar to the orientation of the sulfonate oxygen in the 9PAP structure.

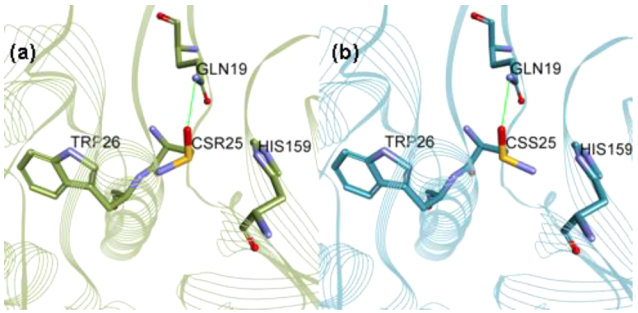

Figure 9. Active site of sulfinamide-modified papain involving (a) (*R*)- and (b) (*S*)-sulfinamide. Models were generated using Rosetta software.

The first step in sulfinamide hydrolysis is generally thought to be protonation of the sulfinamide oxygen. In the *S* sulfinamide structure (Figure 9b), the sulfinamide NH_2 can hydrogen bond with His159; in the (*R*)-sulfinamide structure (Figure 9a), the NH_2 sits at the positively polarized end of an α helix and hydrogen bonds with the backbone of Trp26. Only in the (*R*)-sulfinamide structure is the catalytic His159 poised to function as a general base for deprotonation of water, just as it does in the standard papain reaction. In our hydrolysis product model, Nδ_1 of His159 is within hydrogen bonding distance of the second oxygen bound to sulfur. It is also worth noting that the Rosetta score for the product (−311.7 ± 4 REU) is lower than either the (*R*)- or (*S*)-sulfinamide (−308.05 ± 0.08 and −307.1 ± 0.2 REU, respectively). Thus, we propose the following mechanism, shown in Scheme 3. The sulfinamide is activated by the partial positive character of the enzymatic oxyanion hole and the positive dipole of the α helix and the backbone amide of Trp26. As in the papain enzymatic reaction, His159 deprotonates a water molecule for nucleophilic attack. After formation of the sulfurane, solvent-assisted proton transfer occurs, followed by loss of free ammonia to give the sulfinic acid.

CONCLUSIONS

Using the ^{15}N-edited NMR method, we have been able to detect primary sulfinamides in organic molecules, peptides, and proteins. Moreover, analysis of the peptide sulfinamides indicates the generation of peptide diastereomers upon sulfinamide formation. The capability to distinguish between stereoisomers makes this a beneficial method for sulfinamide detection. Furthermore, this technique could also be used for

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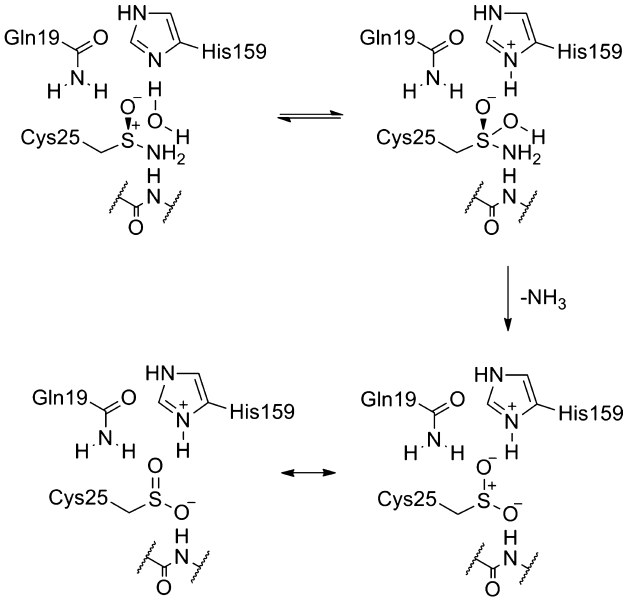
Scheme 3. Hydrolysis of Sulfinamide in Papain



the detection of N-substituted sulfinamides provided that there is a proton bonded to the labeled nucleus.

We have extended our previous work[27] on sulfinamide reactivity to investigate further sulfinamide hydrolysis at physiological pH and temperature. Our results with peptide sulfinamides are consistent with our initial findings, indicating that despite being a slow reaction, hydrolysis is viable under physiological conditions. Although the number of cases examined to date is limited, the hydrolysis of peptide sulfinamides seems to be affected minimally by peptide sequence (e.g., AGSCWA versus VYPCLA versus GSH) but substantially more by protein environment (e.g., papain); further investigations are required. In addition, the hydrolysis reaction is facilitated at lower pH values similar to that observed for secondary sulfinamides and consistent with the generally accepted mechanism.[17,26,28−30] Recent studies point to the potential signaling role of sulfinic acid formation in controlling protein function.[70−72] Moreover, the reduction of sulfinic acids to thiols by an ATP-dependent process is well-established for the peroxiredoxin system.[73−75] The hydrolysis of HNO-derived sulfinamides to sulfinic acids might represent another pathway to sulfinic acid formation in proteins.

Papain, whose activity depends on a catalytic triad of Cys25, His159, and Asn175, is one of the most studied cysteine proteases.[62] Our NMR experiments demonstrate sulfinamide formation upon HNO treatment, which correlates well with previous predictions that the observed inhibition of papain is due to the modification of its single free cysteine residue by HNO.[43] Further analysis has shown that sulfinamide hydrolysis is significantly more efficient in the papain active site compared to small organic molecules[27] and related peptide systems. These findings are also supported by computational studies, which indicate that hydrolysis can be enhanced in papain active site due to activation of the sulfinamide and facilitation of nucleophilic attack.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR spectra for VYPCLA sulfinamide at various temperatures, titration curve for *tert*-butanesulfinamide, additional information about 2-phenylethanesulfinamide, papain, and the reactivity of VYPCLA sulfinamide, Python script, atom type, and residue type parameters for active site modeling. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): J.P.T. is a co-founder, stockholder, and serves on the Scientific Advisory Board of Cardioxyl Pharmaceuticals.

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■ ABBREVIATIONS

ACN, acetonitrile; AS, Angeli's salt; ^{15}N-AS, ^{15}N-labeled Angeli's salt; BME, β-mercaptoethanol; BME-S(O)NH$_2$, β-mercaptoethanol-derived sulfinamide; COSY, correlation spectroscopy; DEA/NO, 1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate; DMSO, dimethyl sulfoxide; DTPA, diethylenetriamine pentaacetic acid; DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; FT-NMR, Fourier-transform nuclear magnetic resonance; GSH, glutathione; GS(O)NH$_2$, glutathione-derived sulfinamide; HPLC, high pressure liquid chromatography; HSQC, heteronuclear single quantum correlation; L-BAPNA, N$_\alpha$-benzoyl-L-arginine 4-nitroanilide hydrochloride; 2-MSPA, *N*-hydroxy-2-(methylsulfonyl)-benzenesulfonamide; NEM, *N*-ethylmaleimide; NMR, nuclear magnetic resonance; PE-S(O)NH$_2$, 2-phenylethanesulfinamide; PDB, Protein Data Bank; REU, Rosetta energy units; RNOS, reactive nitrogen oxide species; ROESY, rotating frame nuclear Overhauser effect spectroscopy; TFA, trifluoroacetic acid; THF, tetrahydrofuran; WT, wild-type

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