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Reactivity of C-Terminal Cysteines with HNO

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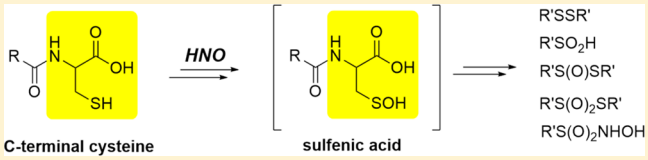
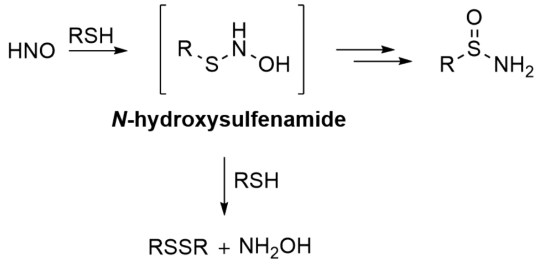
Supporting Information

ABSTRACT: Nitroxyl (HNO), a potential heart failure therapeutic, is known to target cysteine residues to form sulfinamides and/or disulfides. Because HNO-derived modifications may depend on their local environment, we have investigated the reactivity of HNO with cysteine derivatives and C-terminal cysteine-containing peptides at physiological pH and temperature. Our findings indicate that the nature of HNO-derived modifications of C-terminal cysteines is affected by the C-terminal carboxylate. Apart from the lack of sulfinamide formation, these studies have revealed the presence of new products, a sulfohydroxamic acid derivative ($RS(O)_2NHOH$) and a thiosulfonate ($RS(O)_2SR$), presumably produced under our experimental conditions via the intermediacy of a cyclic structure that is hydrolyzed to give a sulfenic acid (RSOH). Moreover, these modifications are formed independent of oxygen.



Cysteine residues are known to form several oxidative modifications in the presence of reactive oxygen or nitrogen species.[1−4] These post-translational modifications, including disulfide bonds, sulfinamides, and sulfenic, sulfinic, and sulfonic acids, can affect protein structure and function with potential biological impacts.[1,5,6] Apart from the crucial roles of disulfide bonds, recent studies have emphasized the importance of sulfenic and sulfinic acids in redox catalysis and cell signaling.[3,7−9] Nitroxyl (HNO), the protonated, one-electron reduced form of NO, is a potential therapeutic for several conditions including heart failure, alcoholism, vascular dysfunction, and cancer.[4,10−12] Because of the highly thiophilic nature of HNO, the majority of its biological effects have been attributed to its reactivity with cysteines.[4,12,13] As shown in Scheme 1, HNO reacts with thiols to form an *N*-hydroxysulfenamide intermediate. This unstable species has been reported to form a sulfinamide or a disulfide depending on the concentration of thiol.[14,15] At low thiol concentrations, the *N*-hydroxysulfenamide rearranges to produce a sulfinamide, presumably via a reaction pathway that involves the dehydration of the protonated *N*-hydroxysulfenamide to form an alkyliminosulfonium intermediate ($RS^+{=}NH$) followed by reaction with water, as suggested by computational studies.[16] At high thiol concentrations, the formation of disulfide and hydroxylamine is favored.

Scheme 1. Reaction of HNO with Thiols



To gain insight into the pharmacological effects of HNO, we and others have investigated the production of HNO-derived thiol modifications and their consequences on protein function.[17−35] Previous studies on sulfinamide reactivity indicate that reduction to free thiols in the presence of excess thiol and hydrolysis to form sulfinic acids constitute the major reactions of sulfinamides at physiological pH and temperature.[22−24] We have also shown that sulfinamide hydrolysis can be facilitated in the active site of a cysteine protease, indicating that HNO-derived modifications can have different reactivities and consequently different biological impacts depending on their local environment.[23] In addition, recent reviews have highlighted the importance of microenvironment on the reaction of HNO with thiols.[36,37]

With the aim of learning more about the factors that affect HNO-derived thiol modifications, we have further examined the reactivity of HNO with cysteine derivatives and cysteine-containing peptides. These efforts have led to the observation that, regardless of the conditions employed, sulfinamide formation does not occur on a C-terminal cysteine under physiological conditions. Mechanistic investigations have pointed to the intermediacy of a cyclic structure, **1**, which can be hydrolyzed to form a sulfenic acid **2** (Scheme 2).

EXPERIMENTAL METHODS

Reagents. L-Cysteine, *N*-acetyl-L-cysteine (NAC), *N*-acetylcysteine methyl ester (NAC-E), L-cysteine ethyl ester, D-penicillamine, L-cystine, L-cysteinesulfinic acid monohydrate, sodium methanesulfonate, *S*-methylmethanethiosulfonate, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and dithiothreitol

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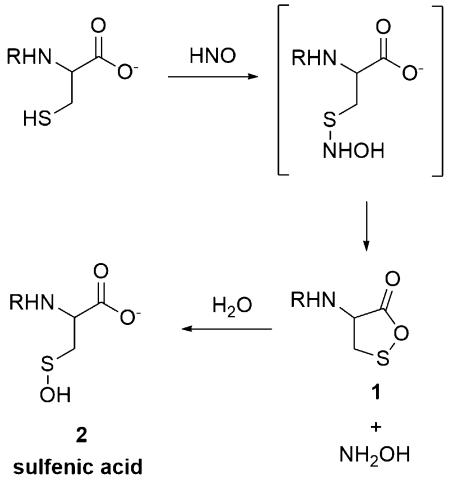
Scheme 2. Reaction of HNO with C-Terminal Cysteines



(DTT) were of the highest purity available and purchased from Sigma (St. Louis, MO). Dimethyl disulfide, sodium methanesulfinate, and dimedone were purchased from Acros. 2-(Hydroxymethyl)benzeneboronic acid hemiester was purchased from Alfa Aesar (Ward Hill, MA). Peracetic acid (35%) was purchased from FMC Corporation (Philadelphia, PA). HPLC and MS grade acetonitrile (ACN) were purchased from Thermo Fisher Scientific (Rockford, IL). Dimethyl sulfoxide-d_6 (DMSO-d_6), D_2O, and ^{15}N-labeled hydroxylamine hydrochloride were purchased from Cambridge Isotope Laboratories (Andover, MA). The syntheses of HNO donors, Angeli's salt ($Na_2N_2O_3$, AS),[38] ^{15}N-labeled Angeli's salt (^{15}N-AS),[39] and 5-(*N*-hydroxylamine)-5-(acetyl-*O*-methoxyoxime)-*N*,*N*-dimethylbarbituric acid (HABA)[40] were carried out as previously described. The HNO donor, *N*-hydroxy-2-(methylsulfonyl)benzenesulfonamide (2-MSPA),[41] the ^{15}N-labeled HNO donor, ^{15}N-hydroxy-2-(methylsulfonyl)-benzenesulfonamide (^{15}N-2-MSPA),[41] and the donor byproduct, 2-(methylsulfonyl)benzenesulfinic acid (2-MSSA), were a generous gift from Cardioxyl Pharmaceuticals. Methanesulfohydroxamic acid ($CH_3S(O)_2NHOH$) was synthesized following a literature procedure.[42,43] The synthesis of cysteine thiosulfinate was carried out following a literature procedure, and the purity of the product was confirmed by electrospray ionization mass spectrometry (ESI-MS).[44,45] Milli-Q water was used for all purifications and experiments.

Peptide Synthesis and Purification. Synthetic peptides AFAAAC, AAAAAC, CL, and LC were synthesized on a Symphony Quartet peptide synthesizer (Protein Technologies Inc., Tucson, AZ) following Fmoc solid-phase peptide synthesis methods.[46] The crude product was dissolved in 0.1% trifluoroacetic acid (TFA) and purified by HPLC (Waters HPLC equipped with a Delta 600 pump system and a 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. Synthesis of AFAAAC hydroxamic acid was carried out by employing hydroxylamine Wang resin, and the crude product was purified by HPLC in the absence of TFA. In all cases, peptide fractions were identified by ESI-MS. Pure fractions were pooled and lyophilized, and the purified product was quantified on the basis of the absorbance at 257 nm (ε_{257} = 195 M^{-1} cm^{-1}) or a DTNB assay.[47,48] Peptides were stored at −80 °C in lyophilized form until use.

AFAAAC methyl ester was synthesized following literature procedures.[49] Briefly, AFAAAC was dissolved in 0.1 M acetyl chloride in methanol to a final concentration of 2 mM and stirred at room temperature for 25 h. The solvent was then removed under vacuum at room temperature in a Savant Speedvac apparatus, and the peptide was stored at −80 °C until use. The complete conversion of the peptide C-terminal carboxylic acid to the corresponding methyl ester was confirmed by ESI-MS.

Incubation of Thiols with HNO. The incubations were carried out as previously described.[23,24] Cysteine derivatives or cysteine-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 a final concentration of 0.1 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 2-MSPA, ^{15}N-2-MSPA, or HABA were prepared in ACN and used within 15 min of preparation. Stock solutions of the donor byproducts, NO_2^- and 2-MSSA, were dissolved in 0.01 M NaOH and ACN/H_2O (1:1, v/v), respectively. The cysteine derivatives or cysteine-containing peptides were incubated with 1 mM AS, ^{15}N-AS, 2-MSPA, ^{15}N-2-MSPA, or HABA (as indicated) at 37 °C for 30 min in a block heater. In all cases, the final volume of ACN did not exceed 1% of the total. As controls, incubations were also carried out with donor byproducts NO_2^- and 2-MSSA under the same conditions. The samples were then prepared for either ESI-MS or NMR analysis.

For immediate ESI-MS analysis, the peptides were purified and desalted with C_{18} PepClean spin columns and then diluted into 70% ACN with 0.1% TFA as described previously.[24] For NMR analysis, the samples were flash-frozen and lyophilized. Following lyophilization, the samples were redissolved in DMSO-d_6 for NMR analysis as described previously.[23]

Incubation of Thiols with HNO under Anaerobic Conditions. Cysteine-containing peptides were dissolved in pH 7.4 phosphate buffer as described above. The peptide solutions were purged with argon for 30 min prior to incubation with HNO donors. A 0.1 M stock solution of 2-MSPA was prepared in ACN and purged with argon for 10 min. The argon-purged 2-MSPA solution was introduced via an airtight syringe to a final concentration of 1 mM, and the samples were incubated at 37 °C for 30 min under continuous argon purging. Following incubation, the samples were purged for an additional 30 min at room temperature, opened to air, and prepared for immediate ESI-MS analysis as described above. Also, control samples were prepared under the same conditions in the absence of 2-MSPA.

Incubation of MethylMethanethiosulfonate with Hydroxylamine. Stock solutions of methanesulfinate ($CH_3SO_2^-$), methylmethanethiosulfonate ($CH_3S(O)_2SCH_3$), and hydroxylamine were prepared in pH 7.4 phosphate buffer and used within 15 min of preparation. Each oxidized sulfur compound was diluted into 250 mM phosphate buffer with 50 μM DTPA containing 10% D_2O to a final concentration of 1 mM. The samples were incubated at 37 °C for 30 min in the presence or absence of 1 mM hydroxylamine (as indicated). The samples were then immediately analyzed by ^1H NMR spectroscopy.

Incubation of Methanesulfinate with HNO Donors. Stock solutions of methanesulfinate, AS, 2-MSPA, and ^{15}N-2-MSPA were prepared as described above. Methanesulfinate was

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diluted into 250 mM phosphate buffer with 50 μM DTPA containing 10% D_2O to a final concentration of 1 mM. The samples were incubated at 37 °C for 30 min in the presence or absence of 10 mM AS or 2-MSPA (as indicated). The samples were then immediately analyzed by ^{1}H NMR spectroscopy.

In some cases, methanesulfinate was diluted into 10 mM phosphate buffer with 50 μM DTPA to a final concentration of 0.1 mM, and the samples were incubated at 37 °C for 30 min in the presence of ^{15}N-2-MSPA (as indicated). The samples were then flash-frozen and lyophilized. Following lyophilization, the samples were redissolved in DMSO-d_6 for NMR analysis as described above.

Incubation of HNO Donors with Dimedone and Benzoxaborole. Stock solution of ^{15}N-2-MSPA was prepared as described above. Stock solutions of dimedone were prepared in DMSO. Dimedone was diluted into 10 mM phosphate buffer with 50 μM DTPA at pH 7.4 to a final concentration of 0.5 mM. The sample was then incubated with ^{15}N-2-MSPA (1 mM) at 37 °C for 30 min. Following lyophilization, the samples were analyzed by NMR spectroscopy.

Stock solution of AS and 2-MSPA were prepared as described above. HNO donors (9 mM) were incubated with benzoxaborole (2-(hydroxymethyl)benzeneboronic acid hemi-ester) (9 mM) in 250 mM phosphate buffer with 50 μM DTPA at pH 7.4 containing 10% D_2O at 37 °C for 30 min. As a control, benzoxaborole (9 mM) was incubated in the absence of HNO donors. The samples were immediately analyzed by ^{11}B NMR spectroscopy.

DTNB Assay for Quantification of Free Thiol. Stock solutions of DTT (1 M) were freshly prepared in water and used within 15 min of preparation. AFAAAC was incubated with the HNO donor, 2-MSPA, or its byproduct as described above. The samples were then incubated with 10 mM DTT at 25 °C for 20 min. Aliquots were taken from each sample at various stages, and the peptides were desalted with C_{18} PepClean spin columns to remove excess DTT. The solvent was then removed under vacuum at room temperature, and the residue was redissolved in 10 mM phosphate buffer with 50 μM DTPA at pH 8. The free sulfhydryl content was immediately determined by DTNB titration.[48] The results were normalized with respect to the amount of free thiol in the initial sample. Control samples treated with donor byproduct were also analyzed under the same conditions.

NMR Analyses. All NMR analyses were conducted on a Bruker Avance-400 FT-NMR spectrometer operating at 400 and 128 MHz for ^{1}H and ^{11}B, respectively. ^{1}H NMR and ^{15}N-edited ^{1}H 1D-NMR analyses were carried out in DMSO-d_6 at 303 K. In some cases, ^{1}H NMR spectra were collected in pH 7.4 phosphate buffer with 10% D_2O at 298 K (as indicated). Solvent was suppressed by using 1 s presaturation pulse. ^{15}N-edited ^{1}H 1D-NMR spectra were acquired using the heteronuclear single quantum correlation (HSQC) pulse sequence for selection. Chemical shifts are reported in parts per million (ppm) relative to residual DMSO (2.49 ppm for ^{1}H) or residual H_2O (4.7 ppm for ^{1}H). All ^{11}B NMR experiments were carried out in pH 7.4 phosphate buffer with 10% D_2O at 295 K. Samples were analyzed in quartz NMR tubes, and chemical shifts were referenced to $Et_2O{\cdot}BF_3$ (41.6 ppm for ^{11}B) in $CDCl_3$.

Computational Analyses. Calculations were performed using Spartan'14.[50] Following a conformation search on *N*-acetylcysteine sulfohydroxamic acid using molecular mechanics, the geometry minimum in DMSO was determined by density functional theory (DFT) at the B3LYP/6-31G* level with the SM8 solvation model.[51]

Mass Spectrometric Analyses. ESI-MS analysis was carried out on a Thermo Finnigan LCQ deca ion trap mass spectrometer fitted with an electrospray ionization source, operating in the positive ion mode with an accuracy of ca. 0.1 m/z. In all experiments, 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.

Nitrous Oxide Quantification by Headspace Gas Chromatography (GC). To determine the reactivity of dimedone with HNO, its dimerization product, nitrous oxide (N_2O), was analyzed by gas chromatography. Experiments were performed on a Varian CP-3800 instrument equipped with a 1041 manual injector, electron capture detector, and a Restek ShinCarbon ST 80/100 molecular sieve-packed column. Grade 5.0 nitrogen was used as both the carrier (8 mL/min) and the makeup (22 mL/min) gas. The injector oven and the detector oven were kept at 200 and 300 °C, respectively. All N_2O analyses were performed with the column oven held constant at 150 °C. Stock solutions of AS, 2-MSPA, and dimedone were prepared as described above. HNO donors were diluted into argon-saturated, pH 7.4 phosphate buffer with 50 μM DTPA to a final concentration of 0.1 mM. The samples were incubated for 3 h at 37 °C in the presence or absence of dimedone (0.5 mM) to ensure complete decomposition and equilibration of N_2O with the headspace. All gas injections were made using a 100 μL gastight syringe with a sample lock.

RESULTS AND DISCUSSION

Thiols are considered to be one of the major targets of HNO in biological systems.[12,13,36,52] Consistent with the complicated chemistry of sulfur, the nature and fate of HNO-derived modifications seem to be affected by factors including thiol concentration and the identity of nearby residues.[14−16,23,24,31,36] To gain more insight into the influence of local environment on HNO reactivity, we have explored how the position of the cysteine residue affects reactivity. For this purpose, we have investigated the formation of HNO-derived sulfinamides in cysteine, cysteine derivatives, and cysteine-containing peptides.

Effect of C-Terminus on HNO-Derived Sulfinamides in Cysteine Derivatives. Our previous studies have shown that several small organic molecule thiols and peptides containing internal cysteine residues can be modified to the corresponding sulfinamides by employing a thiol-to-HNO donor ratio of 1:5 or 1:10 under physiological conditions.[24] We treated *N*-acetyl-L-cysteine (NAC) with the H^{15}NO donor, ^{15}N-2-MSPA, under the same conditions and analyzed the resulting products with our ^{15}N-edited NMR sulfinamide detection method.[23] This method involves the application of an isotope filter for ^{15}N to achieve selective detection of protons attached to the ^{15}N nuclei, providing simplified NMR spectra.[23,53] Surprisingly, no peaks were detected in the characteristic sulfinamide region (5.5−6.5 ppm) (Figure 1a), but a new peak was observed at 9.53 ppm. A similar spectrum was obtained upon treatment of NAC with ^{15}N-AS (Supporting Information). Although a previous study suggests the formation of *N*-acetylcysteinesulfinamide from the reaction of NAC and HNO, the product was reported to be unstable and could not be structurally characterized.[54]

To determine if the carboxylic acid of the C-terminus affects sulfinamide formation, we treated *N*-acetyl-L-cysteine methyl

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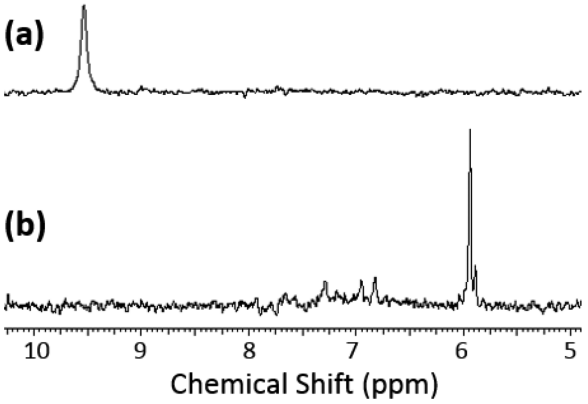

Figure 1. Selected region of ^{15}N-edited ^{1}H NMR spectrum (in DMSO-d_6 at 30 °C) showing ^{15}NH signals following the treatment of (a) NAC (0.1 mM) and (b) NAC-E (0.1 mM) with ^{15}N-2-MSPA (1 mM) in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min.

ester (NAC-E), in which the free carboxylic acid is replaced with an ester, with H^{15}NO. As shown in Figure 1b, the corresponding ^{15}N-labeled sulfinamide signals are now observed. Consistent with our previous studies, two characteristic sulfinamide peaks are detected at 5.89 and 5.94 ppm because of the formation of diastereomers.[23] Similar results were obtained when HABA was used as the HNO donor (Supporting Information). Control experiments carried out with NAC and NAC-E in the presence of donor byproducts or hydroxylamine did not result in any ^{15}N-edited peaks (data not shown), indicating that these observations (Figure 1) are due to HNO reactivity.

Effect of N-Terminus on HNO-Derived Sulfinamides in Cysteine Derivatives. To determine if the free amino group of the N-terminus has a similar effect on the production of an HNO-derived sulfinamide, we conducted experiments with L-cysteine and L-cysteine ethyl ester, both possessing a free N-terminus. Consistent with the above results, sulfinamide signals (5.76 and 5.90 ppm) were detected with the L-cysteine ethyl ester but not with L-cysteine, indicating that the presence of a free N-terminus does not affect formation of the HNO-derived sulfinamide (Figure 2a,b). Similarly, no sulfinamide was observed with D-penicillamine, which contains a free C-terminus, upon treatment with ^{15}N-2-MSPA (data not shown).

Effect of C-Terminus on HNO-Derived Sulfinamides in Peptides. Given the above results, we next investigated the reactivity of HNO with cysteines in the dipeptide system, LC and CL. No sulfinamide peaks were observed upon analysis of H^{15}NO-treated LC by ^{15}N-edited NMR spectroscopy (Supporting Information). However, treatment of the N-terminal cysteine-containing peptide, CL, with ^{15}N-AS produced the corresponding sulfinamide (Supporting Information). Consistent with these observations, reaction of the hexapeptide AAAAAC with 2-MSPA also did not generate a sulfinamide (data not shown). These results suggest that cysteine derivatives and C-terminal cysteine-containing peptides react similarly with HNO, presumably because of the presence of a free carboxylate group.

HNO-Derived Modifications of C-Terminal Cysteines. Because sulfinamides are not observed with C-terminal cysteines, we explored the identity of HNO-induced modifications in these systems using the hexapeptide AFAAAC. To confirm the reactivity of HNO with this peptide and its

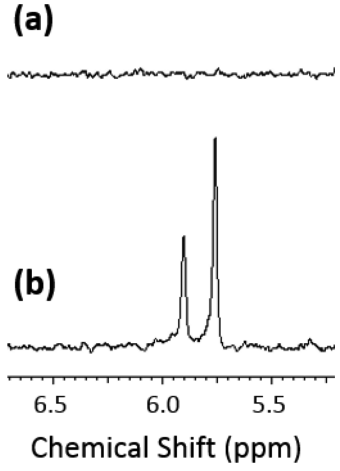

Figure 2. Selected region of ^{15}N-edited ^{1}H NMR spectrum (in DMSO-d_6 at 30 °C) showing ^{15}NH signals following the treatment of (a) L-cysteine (0.1 mM) and (b) L-cysteine ethyl ester (0.1 mM) with ^{15}N-2-MSPA (1 mM) in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min.

methyl ester derivative, we initially applied our ^{15}N-edited NMR method (Figure 3a,b). As expected, characteristic

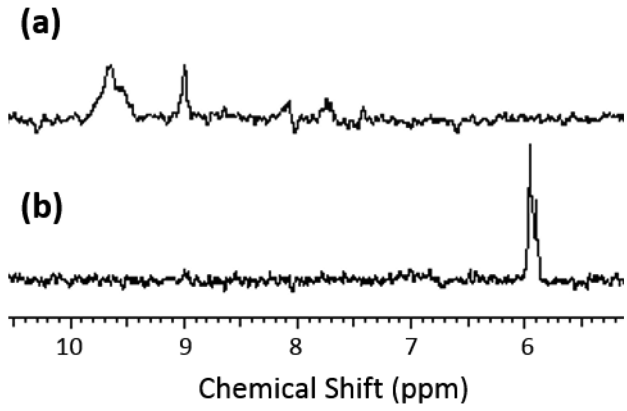

Figure 3. Selected region of ^{15}N-edited ^{1}H NMR spectrum (in DMSO-d_6 at 30 °C) showing ^{15}NH signals following the treatment of (a) AFAAAC (0.1 mM) and (b) AFAAAC methyl ester (0.1 mM) with ^{15}N-2-MSPA (1 mM) in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min. In spectrum a, the peak at 9.00 ppm corresponds to the ^{15}NH signal of ^{15}N-2-MSPA, which was spiked into the sample.

sulfinamide signals at 5.90 and 5.95 ppm were detected only for the AFAAAC methyl ester (Figure 3b). Similar to our results with H^{15}NO-treated NAC (Figure 1a), a new peak at 9.65 ppm was observed in the ^{15}N-edited NMR spectrum of H^{15}NO-treated AFAAAC (Figure 3a). As indicated previously, the ^{15}N-edited NMR method detects only protons bonded to a ^{15}N nucleus.[23,53] To test if the 9.65 ppm peak was due to residual ^{15}N-2-MSPA, we spiked the sample with the standard compound. As seen in Figure 3a, an NMR signal for this compound appears at 9.00 ppm. Additionally, no signal was detected upon exposure of ^{15}N-2-MSPA to the same incubation and sample preparation conditions in the absence of thiol (data not shown).

The reactivity of HNO with AFAAAC was further investigated by ESI-MS (Figure 4a–c). As shown in Figure 4b, a signal 32 Da heavier than AFAAAC disulfide **8** (m/z 1135

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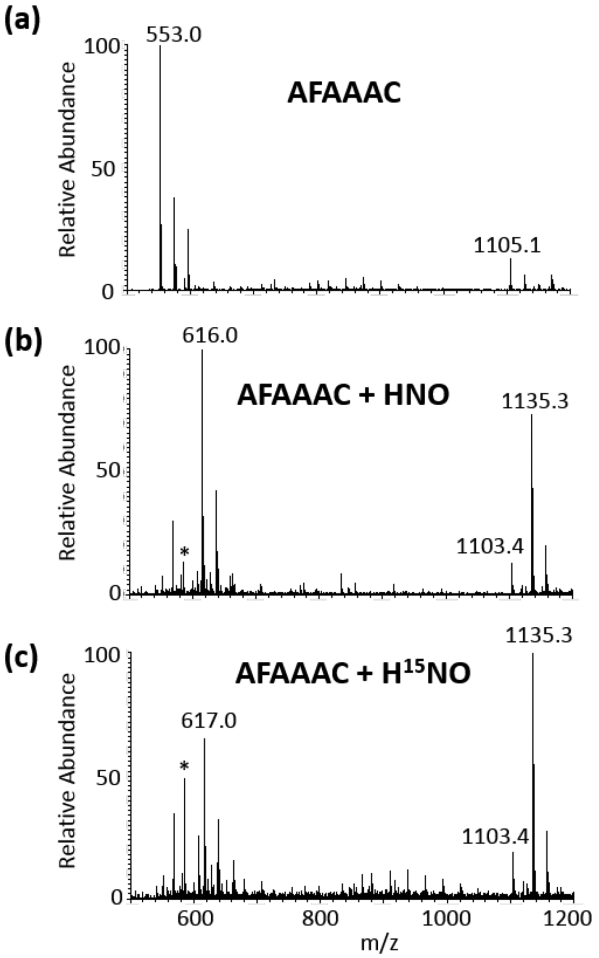
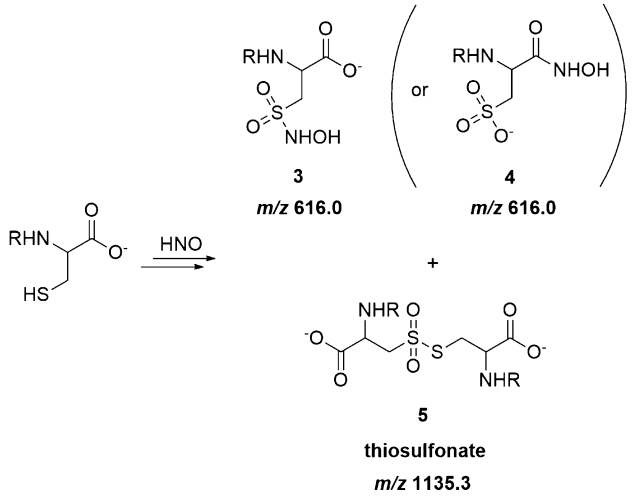


Figure 4. Selected region of ESI-MS spectrum showing AFAAAC (0.1 mM) (a) untreated or treated with (b) 2-MSPA (1 mM) or (c) ^{15}N-2-MSPA in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min. Spectra show the peaks for unmodified peptide (m/z 553.0 ± 0.1), sulfinic acid **6** (m/z 585.0 ± 0.1, indicated by an asterisk (*)), sulfohydroxamic acid **3** or hydroxamic acid derivative **4** (m/z 616.0 ± 0.1), disulfide **8** (m/z 1103.3 ± 0.1), dimeric adduct (m/z 1105.1 ± 0.1) (commonly observed by mass spectrometry), and thiosulfonate **5** (m/z 1135.3 ± 0.1).

vs 1103), which could correspond to peptide thiosulfonate **5** ($RS(O)_2SR$) (Scheme 3), is observed as well as less intense signals assigned to sulfinic acid **6** (RSO_2H, m/z 585) and AFAAAC disulfide **8** (m/z 1103). We hypothesize that thiosulfonate **5** is formed from a sulfenic acid-derived thiosulfinate **9** (see below). Moreover, another intense signal 63 Da heavier than the unmodified peptide (m/z 616 vs 553) is also detected (Figure 4b). Two possibilities for this latter signal are a peptide sulfohydroxamic acid **3** or hydroxamic acid derivative **4**, both of which have the same exact mass (Scheme 3). Because the formation of either **3** or **4** requires the addition of a nitrogen-containing species, we conducted an analogous experiment with the ^{15}N-labeled HNO donor, ^{15}N-2-MSPA. The +63 Da signal (m/z 616) is now replaced by a +64 Da signal (m/z 617) (Figure 4c). Notably, no other species were labeled with ^{15}N. Similar results were obtained with AS (data not shown), further indicating that these observations are independent of the HNO donor used.

Because the identity of the +63 Da peak could not be determined unambiguously from the ESI-MS data alone, we

Scheme 3. Thiosulfonate and Sulfohydroxamic/Hydroxamic Acid Species



examined the AFAAAC peptide containing a hydroxamic acid group at the C-terminus by ^{1}H NMR spectroscopy. In contrast to the HNO-treated AFAAAC sample, this peptide did not show any peaks at 9.65 ppm upon NMR analysis (data not shown), suggesting that the 9.65 ppm peak does not correspond to **4** and is likely due to sulfohydroxamic acid **3** (see Scheme 6 and text below for a detailed discussion).

HNO-Derived Modifications of C-Terminal Cysteines under Anaerobic Conditions. HNO is known to react with molecular oxygen to form an as yet unidentified HNO−O_2 adduct that can react as an oxidant.[55,56] To understand whether the observed reactivity with C-terminal cysteines is due to HNO or the HNO−O_2 adduct, we incubated the AFAAAC peptide with 2-MSPA under argon-saturated conditions. As expected, control samples exposed to the same sample preparation procedures in the absence of 2-MSPA remained unchanged (Figure 5a). For HNO-treated samples, identical products were obtained under air or argon-saturated conditions (Figures 4b and 5b), indicating that the observed modifications are independent of oxygen.

Formation of Thiosulfonate. To gain further evidence for the production of a thiosulfonate **5**, we also examined the HNO-treated AFAAAC samples by a standard DTNB assay. As expected, no free thiol is detected following HNO treatment. Subsequent incubation with excess DTT results in the recovery of ca. 65% of the total thiol content (data not shown), suggesting the presence of reducible (65%) and not-readily reducible (35%) species.

In addition, reaction of HNO-treated AFAAAC with thiophenol was examined by ESI-MS. Consistent with the known reactivity of thiosulfonate **5** with thiols (Scheme 4),[57] the m/z 1135 signal completely disappeared following a short (20 min) incubation with thiol at room temperature. In addition to the peptide sulfinic acid **6** and mixed disulfide **7** (expected from thiol reduction of the thiosulfonate), sulfohydroxamic acid **3** is still observed at m/z 616 under these conditions (Figure 6). No significant changes were detected in the ESI-MS spectrum following a similar, short incubation in the absence of thiol. On the basis of these results, sulfohydroxamic acid **3** (and peptide sulfinic acid **6** formed from the reduction of peptide thiosulfonate **5**) may correspond to the nonreducible species observed in the above DTNB assay.

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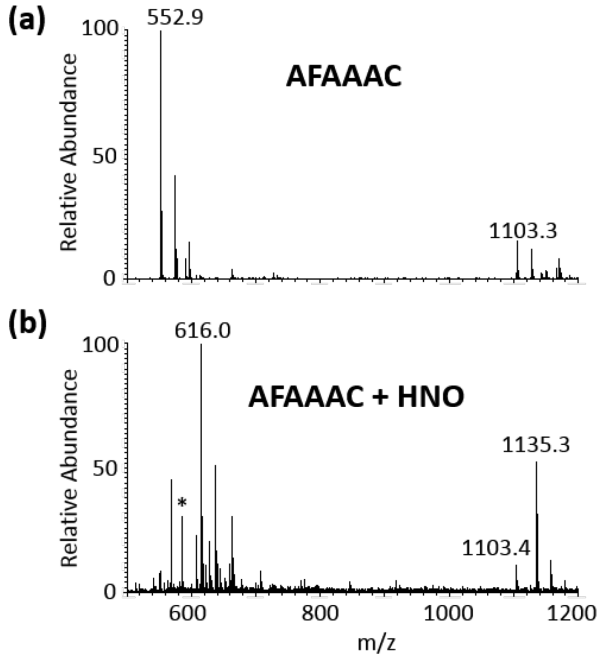
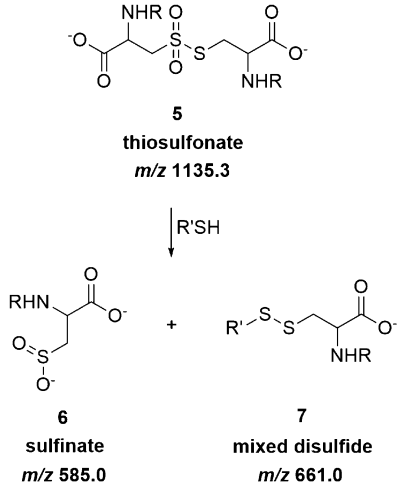


Figure 5. Selected region of ESI-MS spectrum showing AFAAAC (0.1 mM) (a) untreated or treated with (b) 2-MSPA (1 mM) in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min under argon-saturated conditions. Spectra show the peaks for unmodified peptide (m/z 553.0 ± 0.1), sulfinic acid **6** (m/z 585.0 ± 0.1, indicated by an asterisk (*)), sulfohydroxamic derivative **3** (m/z 616.0 ± 0.1), disulfide **8** (m/z 1103.3 ± 0.1), dimeric adduct (m/z 1105.1 ± 0.1) (commonly observed by mass spectrometry), and thiosulfonate **5** (m/z 1135.3 ± 0.1).

Scheme 4. Reduction of Thiosulfonates



Cysteine thiosulfonate is known to be hydrolyzed to form the corresponding sulfinic acid and disulfide (Scheme 5).[57] To test this reactivity, we incubated HNO-treated AFAAAC samples in buffer under physiological conditions for 22 h. The peptide thiosulfonate **5** signal at m/z 1135 disappeared completely, and the signals for sulfinic acid **6** and disulfide **8** are observed (Figure 7a,b), again consistent with thiosulfonate chemistry.

Intermediacy of Sulfenic Acid. The formation of thiosulfonate **5** from the reaction of HNO with C-terminal cysteines could be explained by the intermediacy of a sulfenic acid **2** (RSOH) (Scheme 6). As mentioned previously, reaction

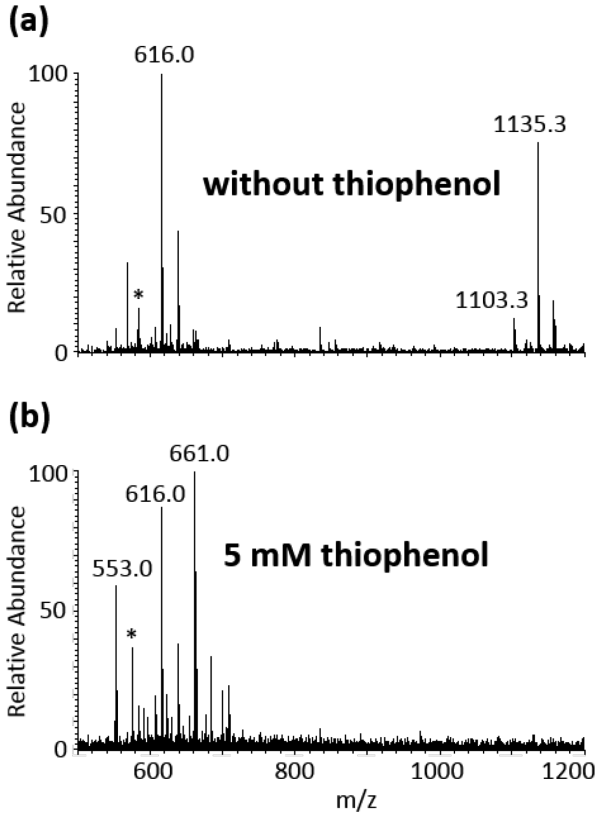
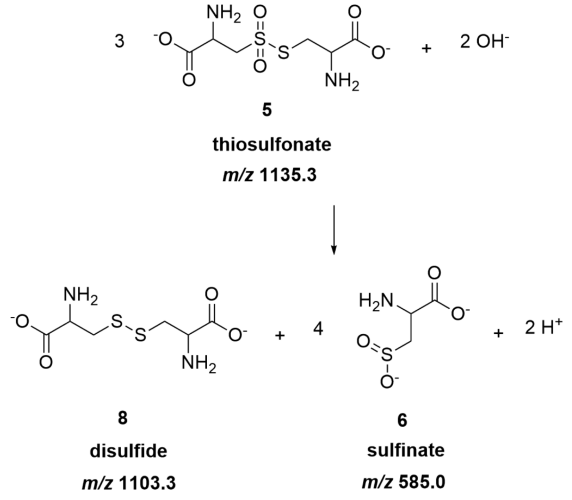


Figure 6. Selected region of ESI-MS spectrum showing AFAAAC (0.1 mM) treated with 2-MSPA (1 mM) in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min under argon-saturated conditions. The samples were incubated for an additional 20 min at room temperature (a) without or (b) with 5 mM thiophenol. Spectra show peaks for the unmodified peptide (m/z 553.0 ± 0.1), sulfinic acid **6** (m/z 585.0 ± 0.1, indicated by an asterisk (*)), sulfohydroxamic derivative **3** (m/z 616.0 ± 0.1), disulfide **8** (m/z 1103.3 ± 0.1), mixed disulfide **7** (m/z 661.0 ± 0.1), and thiosulfonate **5** (m/z 1135.3 ± 0.1).

Scheme 5. Hydrolysis of Cysteine Thiosulfonate



of HNO with thiols results in the formation of an *N*-hydroxysulfenamide intermediate (Scheme 1). We hypothesize that in the case of a C-terminal cysteine this unstable species can react with the carboxylic acid group to form a cyclic intermediate, **1** (Schemes 2 and 6), whose generation has been proposed for the bromine-dependent oxidation of cystine to

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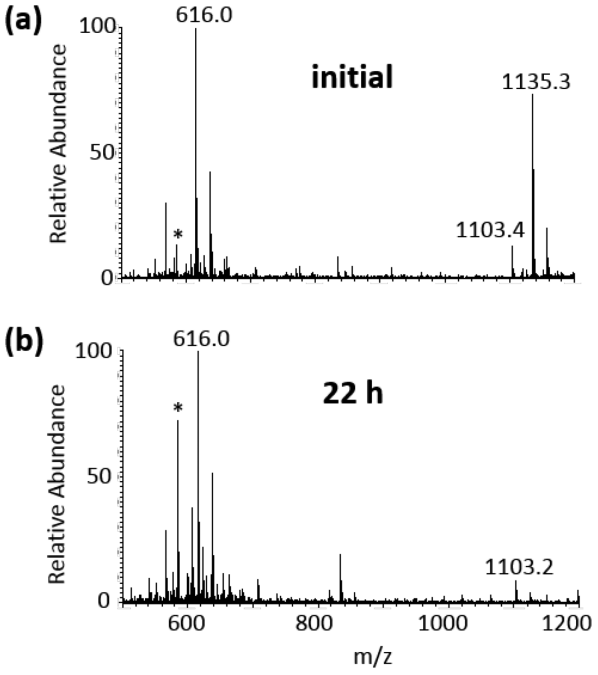


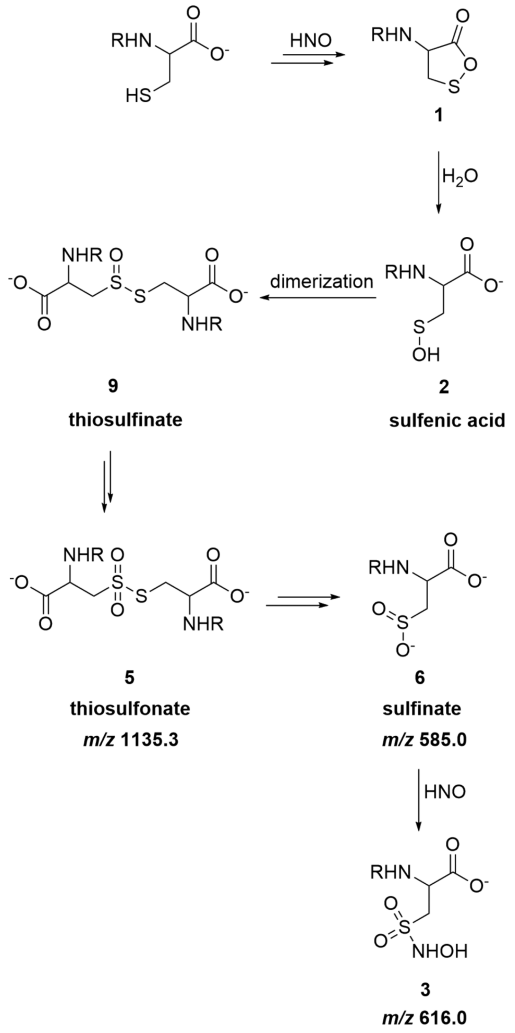
Figure 7. Selected region of ESI-MS spectrum showing AFAAAC (0.1 mM) treated with 2-MSPA (1 mM) in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min. The samples were incubated at physiological pH and temperature for 22 h. Spectra show the peaks for sulfinic acid **6** (m/z 585.0 ± 0.1, indicated by an asterisk (*)), sulfohydroxamic derivative **3** (m/z 616.0 ± 0.1), disulfide **8** (m/z 1103.3 ± 0.1), and thiosulfonate **5** (m/z 1135.3 ± 0.1) at (a) initial time and (b) after 22 h.

cysteic acid.[58] Subsequent hydrolysis of **1** would produce a sulfenic acid, **2**.[59] In the absence of excess thiol or stabilizing factors such as sterics, aromaticity, hydrogen bonding, or protein environment, sulfenic acids **2** are known to dimerize rapidly to form thiosulfinates **9** (Scheme 6).[60−62] Recent studies indicate that at pH < 10 cysteine thiosulfinates are converted to cysteine thiosulfonates.[57] Moreover, cysteine thiosulfonates ($RS(O)_2SR$) have been reported to be more stable at pH < 7 and consequently become the predominant species at lower pH values.[57] Presumably, once peptide sulfenic acid **2** is formed, under our experimental conditions, it is converted to corresponding thiosulfinate **9** (RS(O)SR), which is subsequently hydrolyzed to thiosulfonate **5**. The resulting peptide thiosulfonate **5** ($RS(O)_2SR$) is expected to be stabilized by the acidic sample preparation conditions employed for ESI-MS, consistent with its observation.

To explore other potential pathways for thiosulfonate formation, we conducted several control experiments involving oxidized derivatives of methanethiol or cysteine as model systems. Incubation of cysteinesulfinic acid, methanesulfinic acid, or dimethyl disulfide under physiologically relevant conditions did not generate the corresponding thiosulfonates ($RS(O)_2SR$) (data not shown), indicating that simple oxidation is not the origin of this species. Similarly, no reaction was observed upon incubation of dimethyl disulfide with methanesulfinic acid or L-cystine with L-cysteinesulfinic acid (data not shown). These results are consistent with the formation of thiosulfonate via the intermediacy of a sulfenic acid.

Because of the reactive nature of sulfenic acids, they are generally detected by employing dimedone-based traps.[63−66] Also, recent studies point to the use of benzoxaboroles as

Scheme 6. Formation of the Observed Products



alternative sulfenic acid traps.[67] The use of either class of sulfenic acid trap, however, was not feasible because of the reactivity of dimedone-based traps with HNO, as determined by headspace GC and ^{15}N-edited NMR analyses, and the direct reactivity of benzoxaborole with HNO donors (Supporting Information). Moreover, cysteinethiosulfinate and methylmethanethiosulfonate were observed to react with dimedone, consistent with recent reports (Supporting Information).[68]

Formation of Sulfohydroxamic Acid. As seen in Schemes 1 and 2, hydroxylamine (NH_2OH) is expected to be released upon reaction of HNO with excess thiol. Because hydroxylamine might play a role in the generation of sulfohydroxamic acid **3**, we looked into its reactivity in buffer with methanesulfinate ($CH_3SO_2^-$) and methylmethanethiosulfonate ($CH_3S(O)_2SCH_3$) by ^{1}H NMR. Although a reaction was observed between hydroxylamine and methylmethanethiosulfonate, the product does not correspond to methanesulfohydroxamic acid ($CH_3S(O)_2NHOH$) (Supporting Information), suggesting that the formation of **3** proceeds via a different pathway. No products were formed upon incubation of methanesulfinate with hydroxylamine (data not shown).

Previous kinetic studies indicate that sulfinates (RSO_2^-) can trap HNO, producing sulfohydroxamic acids.[69,70] To test the feasibility of this reaction under the conditions of our experiments, we incubated methanesulfinate with the HNO

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donors, 2-MSPA and AS (Figure 8a,b and Supporting Information, respectively). In both cases, the product was

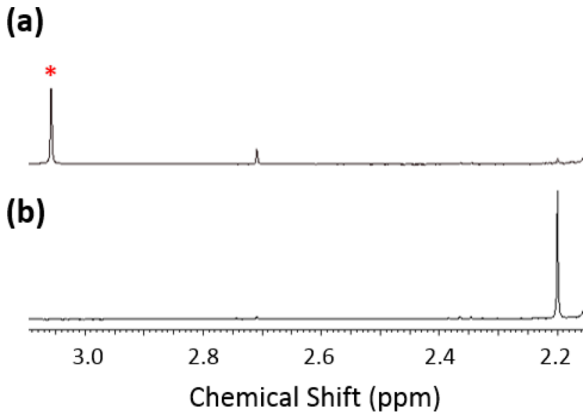

Figure 8. Selected region of ^{1}H NMR spectrum showing methanesulfinate (1 mM) incubated in 250 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min (a) with or (b) without 2-MSPA (10 mM). The peak at 2.18 ppm corresponds to methanesulfinate. An asterisk indicates the product (methanesulfohydroxamic acid, 3.06 ppm) formed because of the reaction of methanesulfinate and HNO. The small peak at 2.71 ppm corresponds to methanesulfonate (commonly observed due to oxidation of methanesulfinate). The spectra were collected in buffer/D_2O (90:10) at 25 °C.

determined to be methanesulfohydroxamic acid ($CH_3S(O)_2NHOH$) via comparison with an independently prepared authentic standard. Moreover, the corresponding ^{15}NH peak, similar to the peak (ca. 9.6 ppm) detected with HNO-treated C-terminal cysteines (Figures 1 and 3), is observed upon treatment of methanesulfinate with ^{15}N-2-MSPA (Supporting Information). The downfield shift observed for the NAC (or peptide) sulfohydroxamic acid (ca. 9.6 ppm) compared with methanesulfohydroxamic acid (ca. 9 ppm) is presumably due to hydrogen bonding between the sulfohydroxamic acid ^{15}NH and C-terminal carboxylate. Indeed, such an interaction is suggested by computational studies (Supporting Information).[71−73] Because sulfinates **6** are formed upon reduction or hydrolysis of thiosulfonates **5** (Schemes 4 and 5), these results represent a potential pathway to sulfohydroxamic acid **3** (Scheme 6). Consistent with these results, treatment of methylmethanethiosulfonate with either 2-MSPA or AS under the same conditions resulted in the formation of methanesulfohydroxamic acid, as expected (data not shown).

CONCLUSIONS

We have demonstrated that the C-terminal carboxylate influences the nature of HNO-derived modifications of C-terminal cysteines. Unlike the standard end products (sulfinamide and disulfide), the major products are now a thiosulfonate and a sulfohydroxamic acid derivative under the conditions of our experiments. Moreover, the formation of these new products takes place independent of oxygen, presumably via the intermediacy of a cyclic structure followed by formation of a sulfenic acid (Schemes 2 and 6). To the best of our knowledge, this is the first report of HNO-derived thiol modifications involving a sulfenic acid intermediate. Introduction of a C-terminal ester replaces this reactivity with the well-known sulfinamide generation, emphasizing the importance of local environment on HNO-induced modifications. Further investigations are required to determine if similar effects are also produced by adjacent side chain carboxylates (e.g., aspartate or glutamate).

Proteomics studies conducted with cultured mammalian cells suggest that less than 10% of total protein cysteines are involved in disulfide bond formation, indicating the high availability of cysteine residues for modification.[74] Although the in vivo relevance of our results is yet to be determined, a simple database search reveals several important human proteins possessing C-terminal cysteine residues and unmodified carboxylates such as guanosine monophosphate reductase-2 (GMPR2), dihydropyrimidine dehydrogenase (DPD), elongin C, and sterol 27-hydroxylase (see the Supporting Information for a more complete list). Considering the central role of sulfenic acids in cell signaling and redox regulation, which may modulate transcription factors, channel activity, and also affect phosphorylation, SUMOylation, and ubiquitination levels in cells,[7,75,76] these findings may point to new biological and pharmacological properties of HNO.

ASSOCIATED CONTENT

Supporting Information

NMR spectra for HNO-treated LC and CL; headspace GC chromatogram of HNO donors treated with dimedone; ^{11}B NMR spectra of benzoxaborole treated with HNO donors; computational data for NAC sulfohydroxamic acid; additional information about NAC, AFAAAC, and the reactivity of methanesulfinate, cysteine thiosulfinate, and methylmethanethiosulfonate; and list of some C-terminal cysteine-containing proteins with free carboxylate. 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 USED

ACN, acetonitrile; AS, Angeli's salt; ^{15}N-AS, ^{15}N-labeled Angeli's salt; HABA, 5-(*N*-hydroxylamine)-5-(acetyl-*O*-methoxyoxime)-*N*,*N*-dimethylbarbituric acid; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTPA, diethylenetriamine pentaacetic acid; DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; GC, gas chromatography; HPLC, high-pressure liquid chromatography; HSQC, heteronuclear single quantum correlation; 2-MSPA, *N*-hydroxy-2-(methylsulfonyl)benzenesulfonamide; ^{15}N-2-MSPA, ^{15}*N*-hydroxy-2-(methylsulfonyl)benzenesulfonamide; 2-MSSA, 2-(methylsulfonyl)benzenesulfinic acid; NAC, *N*-acetyl-L-cys-

teine; NAC-E, *N*-acetylcysteine methyl ester; NMR, nuclear magnetic resonance; TFA, trifluoroacetic acid

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