Reactive Sulfur Species: Hydrolysis of Hypothiocyanite to Give Thiocarbamate-S-oxide

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SUPPORTING INFORMATION

EXPERIMENTAL

Reagents. All chemicals were A.C.S. certified grade or better. Water was doubly-distilled in glass. Solutions of NaOH, mostly free of CO_2 contamination, were quantified by titration with potassium hydrogen phthalate or standardized HCl or $HClO_4$ solutions using phenolphthalein as an indicator. HCl and $HClO_4$ were standardized against bicarbonate. The buffer solutions were prepared from the solids K_3PO_4, $NaH_2PO_4 \cdot H_2O$, Na_2HPO_4 and $Na_3PO_4 \cdot 12\ H_2O$, the ionic strength was adjusted with $NaClO_4$, and the pH/pD was adjusted with NaOH, NaOD, $HClO_4$ or DCl. L-Cystine, L-cysteine, L-cysteic acid monohydrate, L-cysteinesulfinic acid monohydrate, peracetic acid, deuterium chloride (35 wt % solution in D_2O), NaOD (40 wt % solution in D_2O), $NaClO_4$, and K_3PO_4 were used as received from Sigma-Aldrich. $NaH_2PO_4 \cdot H_2O$, Na_2HPO_4 and $Na_3PO_4 \cdot 12H_2O$ was used as received from Mallinckrodt. The monosodium salt of 4-[(2,4-dihydroxyphenyl)azo]benzenesulfonic acid (Tropaeolin O) was received from Allied Chemical and Dye Corporation (N.Y. USA). Deuterium oxide (99.9%) was obtained from Cambridge Isotope Laboratories. Stock solutions of NaOCl were prepared by sparging Cl_2 into a 0.3 M solution of NaOH. The sparging was stopped when the [OCl^-] achieved ca. 100 mM, as determined spectrophotometrically ($\varepsilon\ (OCl^-)_{292\ nm}$ = 350 $M^{-1}cm^{-1}$). Solutions of NaOBr were prepared by adding Br_2 to ice cold solutions of NaOH.[1] Solutions of OBr^- were standardized spectrophotometrically at 329 nm (ε_{329} = 332 $M^{-1}cm^{-1}$). The solutions of OBr^- were used within two hours of the preparations to minimize errors due to decomposition. The synthesis of cysteine thiosulfinate ester, CyS(=O)SCy, and cysteine thiosulfonate ester, $CyS(=O)_2SCy$, were accomplished using a published procedure.[2]

Synthesis of Ammonium Thiocarbamate. Ammonium thiocarbamate was prepared following a standard procedure.[3] A stirred solution containing 25 mL of ethyl acetate and 4.2 mL of ethyl alcohol was bubbled with anhydrous ammonia gas for 12 minutes at -10 ^oC. This was followed by the addition of carbonyl sulfide at – 5 ^oC over a six-minute period. Then the mixture was kept in water-ice bath for an additional thirty minutes. The white solid was obtained by filtration, washed with 12 mL of diethyl ether and air-dried for 30 minutes at room temperature. ^{13}C NMR (0.10M NaOD, 300 MHz) δ 187.39. UV-vis (0.1 M NaOH), ε_{224} = 11, 535 $M^{-1}\ cm^{-1}$.

Methods of Generating Thiocarbamate-S-oxide. Essentially two independent procedures were employed to synthesize thiocarbamate-S-oxide, hydrolysis of $OSCN^-$ at pH 13 and oxidation of thiocarbamate with HOCl. All three known procedures of synthesizing $OSCN^-$ produced thiocarbamate S-oxide upon hydrolysis at pH 13.

Hydrolysis of $OSCN^-$ produced by oxidation of SCN^- with HOCl. $OSCN^-$ was generated at pH 13 by the reaction of OX^- (X = Cl or Br) and SCN^- in the presence of a sufficiently high excess of SCN^- (typically 200-400 fold excess over OX^-) to avoid overoxidation.[4, 5] Turbulent mixing of the reagents was necessary to insure homogeneity of the reaction mixtures in the time-frame of the chemical reaction, and this was achieved by employing a hand mixer comprised of two Hamilton syringes and a T-mixer. Failure to quickly mix solutions of HOX and SCN^- resulted in locally high concentrations of OX^- and hence overoxidation. For the NMR experiments, a 10-fold excess of SCN^- was employed,

which results in > 50% yield of $OSCN^-$ (as determined by UV-vis spectroscopy at λ = 376 nm). The over-oxidation product(s) were not observed by NMR and they did not appear to affect the lifetime of the ^{13}C and ^{15}N NMR spectra of 190 or $OSCN^-$.

Hydrolysis of $OSCN^-$ produced from $(SCN)_2$. $(SCN)_2$ was generated by the heterogeneous reaction of $Pb(SCN)_2$ with Br_2 in CCl_4. $Pb(SCN)_2$ was synthesized by mixing ice-cold aqueous solutions of $Pb(NO_3)_2$ and NaSCN. The resulting precipitate was filtered, washed with ice-cold water, and dried. The concentration of Br_2 in water-saturated CCl_4 was determined spectrophotometrically (ε_{400nm} = 160 $M^{-1}cm^{-1}$). Excess $Pb(SCN)_2$ was added to the CCl_4 solution of Br_2 and the resulting slurry was vortexed until the solution became colorless. The Pb salts were removed by centrifuging and decanting. The concentration of $(SCN)_2$ in CCl_4 was determined spectrophotometrically (ε_{296nm} = 140 $M^{-1}cm^{-1}$). An aliquot of the CCl_4 solution of $(SCN)_2$ was added to 0.1 M aqueous NaOH with vigorous stirring (the volume of the aqueous phase was at least 10 times the volume of the CCl_4 phase). The two phase solution was vortexed for one minute, then the emulsion was separated into two phases by centrifugation (the top layer is the aqueous phase).

Hydrolysis of $OSCN^-$ produced from LPO. $OSCN^-$ was generated by the LPO catalyzed oxidation of SCN^- with H_2O_2 at pH 7.2. The appropriate amounts of SCN^- and LPO were incubated in a 0.1 M iP buffer at 20 ^oC. The reaction was initiated by the addition of H_2O_2. 2.5 minutes after the addition of the H_2O_2 aliquot a pH jump to pH 13 was applied by mixing the reaction mixture (in a 1:1 ratio using a hand mixer comprised of two Hamilton syringes and a T-mixer) with 0.317 M NaOH solution.

Oxidation of thiocarbamate with HOCl. Thiocarbamate-S-oxide was generated from the oxidation of thiocarbamate by HOCl in 0.10 M NaOH. The yield of thiocarbamate-S-oxide was about 50 % if HOCl was mixed with 2 molar equivalent of thiocarbamate. To avoid overoxidation and obtain 100 % yield of thiocarbamate-S-oxide, at least 100-fold excess of thiocarbamate is needed (and the solution was vortexed during mixing). Figure S7 shows that the UV-vis spectra of thiocarbamate-S-oxide that was generated from the oxidation of thiocarbamate (80 μM) by HOCl (30 μM) in 0.10 M NaOH. Its UV-vis spectra have characteristic absorbance at 250 nm, consistent with that of thiocarbamate-S-oxide that was generated from the hydrolysis of $OSCN^-$,as shown in Figure S1.

Decomposition of Thiocarbamate-S-oxide at Neutral pH. LPO (0.80 μM) was employed to catalyze the oxidation of SCN^- (8.0 mM) by 8.0 mM H_2O_2 at pH 7.0, with a 50% yield. Thiocarbamate-S-oxide (0.10 mM) was produced after 150-minute of hydrolysis of $OSCN^-$ (0.20 mM) at pH 13, then the solution was mixed 1:1 with 200 mM phosphate buffer (I = 1.0 M, $NaClO_4$) to give pH 6.64. The kinetics of decomposition of thiocarbamate-S-oxide was measured with a HP 8452 diode-array spectrometer.

pH/pD Measurements. The $[OH^-]$ for the unbuffered solutions were determined by acid-base titration against standardized HCl or standardized $HClO_4$ solutions. The $[H^+]$ of the buffered solutions were determined with an Orion Ion Analyzer EA920 using an Ag/AgCl combination pH electrode. The ionic strength was kept constant at 1.0 M for

most solutions ($NaClO_4$ + NaOH/iP). To obtain the $[H^+]$ or $[OH^-]$ of the buffered solutions from the measured pH values, all pH measurements were corrected for the "Irving factor" and the ionic product of water (pK_w) that were measured by titration of a 1.0 M $NaClO_4$ solution by a standardized 0.1 M NaOH (in 1.0 M $NaClO_4$) solution. pD measurements in D_2O were made using the same pH electrode by adding 0.4 units to the measurement.

NMR Studies. *^{1}H NMR Measurements.* ^{1}H NMR spectra were recorded with a Varian XL-300 spectrometer at 20 (±0.5) ^oC. Deuterated buffers were prepared from D_2O solutions of anhydrous K_3PO_4 by adding DCl, by dilution of a 40 wt % NaOD solution with D_2O or by dilution of a 35 wt % DCl solution with D_2O. The chemical shifts (ppm) were referenced to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, δ = 0.015 ppm). *^{13}C NMR Measurements.* The ^{13}C NMR measurements were made with a Varian Inova 400 MHz NMR spectrometer at 100.57 MHz using a Varian 4-nuclei switchable 5 mm probe while employing D_2O as a frequency lock. Chemical shifts were referenced to internal dioxane (δ = 66.6 ppm). *^{15}N NMR Measurements.* The ^{15}N NMR measurements were made with a Varian Inova 600 MHz NMR spectrometer at 60.79 MHz using a Varian broadband 5 mm probe while employing D_2O as a frequency lock. Chemical shifts were referenced to external NO_3^- (0.0 ppm).

Ion Chromatography. Ion chromatography was performed on a Dionex ICS-3000 with 100 mM of NaOH as eluents. For the anions that were measured using an integrated amperometry detector, the experimental conditions were: eluents gradient, 5 — 10 min, 5 mM; 10 — 25 min, 60 mM. Column: Ionpac AS16. Sample holder tray temperature: 10 ^oC; Detection compartment temperature: 30 ^oC. Volume of the injection loop: 10 μL. For the anions that were detected by conductivity mode, the experimental conditions were set as: eluents gradient, 5 — 16 min, 12 mM NaOH; 10 — 25 min, 60 mM NaOH. Column: Ionpac®AS18. Suppressor type: ASRS_4mm. Sample holder tray temperature: 10 ^oC; Column temperature: 30 ^oC. Detection compartment temperature: 30 ^oC. Cell-heat temperature: 35 ^oC. Volume of the injection loop: 10 μL. The anions $OSCN^-$, $H_2NC(=O)SO^-$, CN^-, SCN^- and HS^- were measured using an integrated amperometry detector. The ions SO_3^{2-}, SO_4^{2-}, $S_2O_3^{2-}$, OCN^-, Cl^-, Br^- and other halogen derivatives were measured by a conductivity detector. Six standard solutions with different concentrations (in the desired concentration range) were used to develop the calibration curves. Each solution was injected 6 times to ensure reproducibility. All data points (6x6) were used in the development of the standard curves.

UV/vis Spectroscopy. Electronic spectra were measured using a HP 8452A diode array spectrophotometer using quartz cells with calibrated 1 mm, 2 mm and 1 cm path lengths at 20 ^oC, or the monochromator of the HI-TECH SF-61 DX2 stopped-flow instrument with a Xe arc lamp at 18 ^oC.

Kinetic Hydrolysis of $OSCN^-$. The kinetics of hydrolysis of $OSCN^-$ was studied in the range of 0.025 to 0.55 M OH^-. The hydrolysis of $OSCN^-$ follows pseudo first-order kinetic dependence on $OSCN^-$, and the first-order rate constant is proportional to the

concentration of hydroxide, as shown in Figure S5, with a rate constant of $(4.24 \pm 0.05) \times 10^{-3}$ M^{-1} s^{-1}.

Acid Dissociation Constant of Thiocarbamate-S-oxide. Thiocarbamate-S-oxide generated from the extraction of $(SCN)_2$ in 0.10 M NaOH. The UV-vis spectra of carbamothioperoxoic acid at various pH were obtained by mixing thiocarbamate-S-oxide (0.46 mM) with the corresponding buffer solutions with the ratio of 1:1 (i.e., [thiocarbamate-S-oxide] = 0.23 mM after mixing). The absorbance (250 nm) are shown in Table 1. Plot of absorbance at 250 nm versus $[H^+]$ is shown in Figure S8. The parameters (ε_{250}(HA), ε_{250}(A) and K_a) were obtained by fitting the experimental results with the following equation:

$$A = \frac{\varepsilon_{HA}[H^+] + \varepsilon_A K_a}{[H^+] + K_a}[C]_{tot}$$

Where ε_{250}(HA) = (214 ± 36) M^{-1} cm^{-1}; ε_{250}(A) = 3381 M^{-1} cm^{-1}; $K_a = (2.10 \pm 0.13) \times 10^{-7}$ M. Based on the above experimental and fitting results, the pK_a of carbamothioperoxoic acid (I = 1.0 M) was calculated to be 6.68(3).

Reaction of Thiocarbamate S-oxide with Cysteine. Thiocarbamate-S-oxide (0.62 mM) was generated from the oxidation of thiocarbamate (2.5 mM) by hypochlorite (1.25 mM) in 0.10 mM NaOH (yield of thiocarbamate-S-oxide: ≈ 50%). Part of the kinetic trace in the oxidation of cysteine (25.0 mM) by thiocarbamate-S-oxide (0.31 mM) in 0.10 M NaOH is shown in Figure S9. It demonstrates that an intermediate product was formed with a rate constant of $(1.40 \pm 0.09) \times 10^{-3}$ s^{-1}, which then reacts slowly with cysteine to form cystine and thiocarbamate (Refer to the ^{1}H-NMR spectra). The identification of the product of the reaction of thiocarbamate-S-oxide with excess cysteine in 0.10 M NaOH were made by ^{1}H-NMR spectroscopy. Figure S11 shows the ^{1}H-NMR spectrum for the reaction between cysteine (20 mM) and thiocarbamate-S-oxide (2.5 mM) / thiocarbamate (5.0 mM) in 0.10 M NaOH after 50 minutes of reaction. The appearance of two new peaks (3.60 (dd, J = 9.3, 3.9; 2H); 2.60, (dd, J = 13.2, 9.3; 2H); the other peak overlap with cysteine) confirms the formation of an intermediate product which is assigned as the thiocarbamate/cysteine mixed didulfide. The intermediate then slowly reacts with the excess cysteine to form cystine and thiocarbamate, as shown in Figure S12. Similar results were obtained when thiocarbamate-S-oxide was generated by the hydrolysis of $OSCN^-$ (Figure S10).

Quantification of $[OH^-]$ in the Hydrolysis of $OSCN^-$. The concentration of OH^- during the hydrolysis of $OSCN^-$ was determined by using Tropaeolin O as indicator. The UV-vis absorbance of Tropaeolin O at 500 nm is proportional to its concentration in a very narrow pH range (Figure S6 illustrates the calibration curve of Tropaeolin O for $0.50 \leq [OH^-] \leq 8.0$ mM). Since Tropaeolin O is photosensitive, the change in $[OH^-]$ during the hydrolysis of $OSCN^-$ could not be obtained in real time. Instead, $OSCN^-$ (2.5 mM) in 5.0 mM NaOH was prepared from the oxidation of SCN^- (1.0 M) by OCl^- (5.0 mM) in 0.10 M NaOH. A pH-jump to 5.0 mM NaOH was achieved by dilution of the $OSCN^-$ solution. The $[OH^-]$ was obtained by UV-vis spectroscopy every hour during the hydrolysis of $OSCN^-$ by adding Tropaeolin O (25 μM) to an aliquot of the reaction mixture. The results

indicate that the [OH^-] did not change during the hydrolysis of $OSCN^-$ in 5.0 mM NaOH (over a period of 10 h).

Qualitative Analysis of S_8. Qualitative analysis of the S_8 the precipitate that forms upon the hydrolysis of thiocarbamate-S-oxide was carried out by mass spectrometry (VG ZAB SE two sector mass spectrometer, using EI/CI ionization source) by direct infusion. The molecular mass, fragmentation and isotope patterns allowed us to identify the yellow precipitate as cyclooctasulfur.

Quantitative Analysis of S_8. $OSCN^-$ was generated by the reaction of SCN^- (515 mM) and OCl^- (2.48 mM) at pH = 13 and T = 20 ^oC using turbulent mixing conditions. Based on preliminary experiments under these conditions, no overoxidation could be observed. The reaction mixture was incubated in the dark for 3 hours (the time that is required for the hydrolysis reaction of $OSCN^-$ to be completed). The incubation was followed by a pH-jump to 6.98 by a 1:1 mixing with a iP = 0.1M, [HCl] = 0.1M buffer. The reaction mixture was incubated for an additional 1 hour after the pH jump in order for the decomposition of thiocarbamate-S-oxide to be completed. The yellow precipitate was extracted into $CHCl_3$ (the volume of the $CHCl_3$ phase was at least 5 times the volume of the aqueous phase). The two phases were separated and the sulfur concentration of the $CHCl_3$ phase was determined by UV-vis spectroscopy using ε_{280} = 811 $M^{-1}cm^{-1}$ (that was determined in a separate experiment by dissolving different amounts of the authentic sample of S_8 in $CHCl_3$). Note that the electronic spectrum of the precipitate from the reaction mixture was similar to the electronic spectrum of the authentic sample (in $CHCl_3$). The stoichiometry of the reaction is found to be: 1 $^-OSC(=O)NH_2$ → 1 S. The experiment was repeated 5 times and similar results were obtained.

Kinetic Data Analysis. Polychromatic data were analyzed using SPECFIT/32 (Spectrum Software Associates), a multivariate data analysis program. The concentration dependencies of the pseudo-first-order rate constants were obtained by linear least-squares fits of the data with KaleidaGraph 3.6 (Synergy Software).

1. Troy, R. C.; Margerum, D. W., *Inorg. Chem.* **1991,** 30, 3538-43.
2. Nagy, P.; Ashby, M. T., *Chem. Res. Toxicol.* **2007,** 20, 79-87.
3. Mueller-Litz, W.; Thomzik, D., *J. Praktische Chemie (Leipzig)* **1977,** 319, 677-81.
4. Nagy, P.; Alguindigue, S. S.; Ashby, M. T., *Biochemistry* **2006,** 45, 12610-12616.
5. Ashby, M. T.; Carlson, A. C.; Scott, M. J., *J. Am. Chem. Soc.* **2004,** 126, 15976-15977.

Table S1. Absorbance of thiocarbamate-S-oxide (233 μM and I = 1.0) vs. pH at 250 nm.

	$[NaClO_4]$, M	[buffer], M	pH	Abs, 250 nm
#1	0.85	[acetate] = 0.10	4.12	0.03638
#2	0.85	[acetate] = 0.10	4.61	0.04726
#3	0.85	[acetate] = 0.10	5.13	0.06943
#4	0.85	[acetate] = 0.10	5.48	0.10239
#5	0.71	[phosphate] = 0.10	6.09	0.23203
#6	0.69	[phosphate] = 0.10	6.46	0.34335
#7	0.68	[phosphate] = 0.10	6.67	0.40273
#8	0.67	[phosphate] = 0.10	6.90	0.50029
#9	0.66	[phosphate] = 0.10	7.31	0.64613
#10	0.85	[tris] = 0.10	7.90	0.74242
#11	0.85	[tris] = 0.10	8.40	0.77168
#12	0.80	[carbonate] = 0.10	9.52	0.79232
#13	0.76	[carbonate] = 0.10	9.83	0.81443

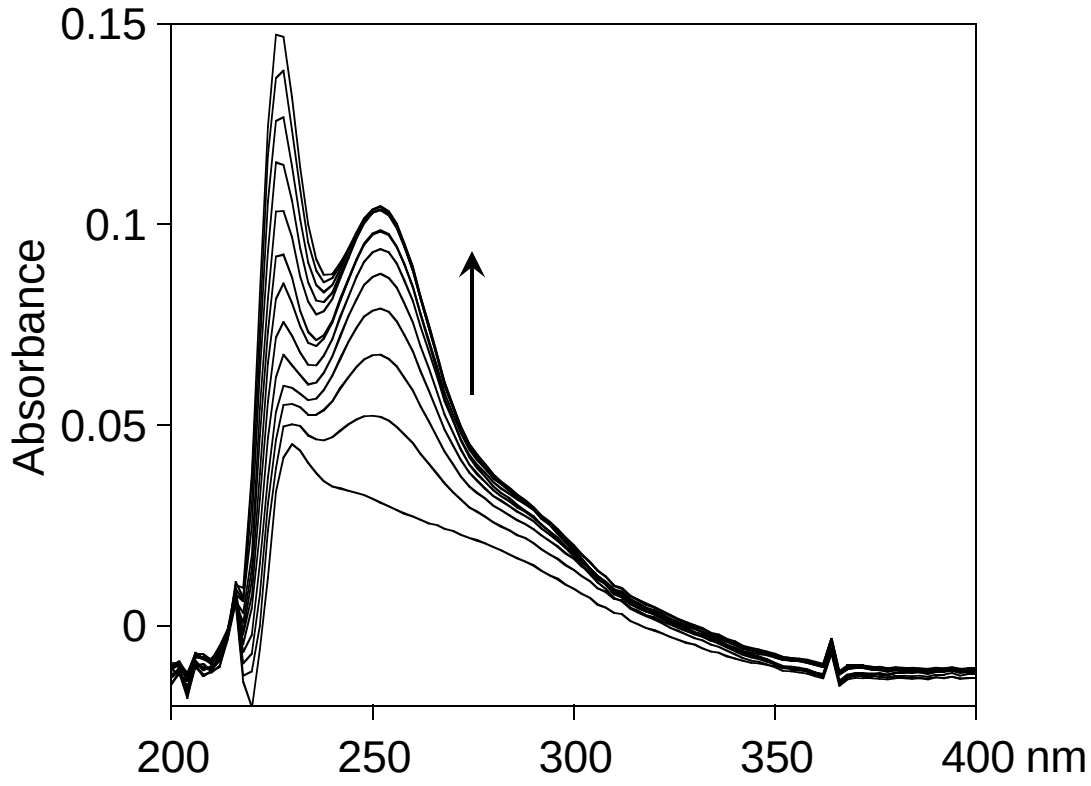

Figure S1. Time resolved UV spectra of the hydrolysis of $OSCN^-$ to give $H_2NC(=O)SO^-$ at pH 13 for low $[OSCN^-]_0$. The spectra are 150 s apart. $OSCN^-$ was generated in the reaction of 49.8 μM OCl^- and 550 μM SCN^- at pH = 13. The spectrum of the excess SCN^- and NaOH were subtracted from each spectrum. Conditions: $[OCl^-]_0$ = 49.8 μM, $[SCN^-]_0$ = 550 μM, pH =13, T = 20 ^oC.

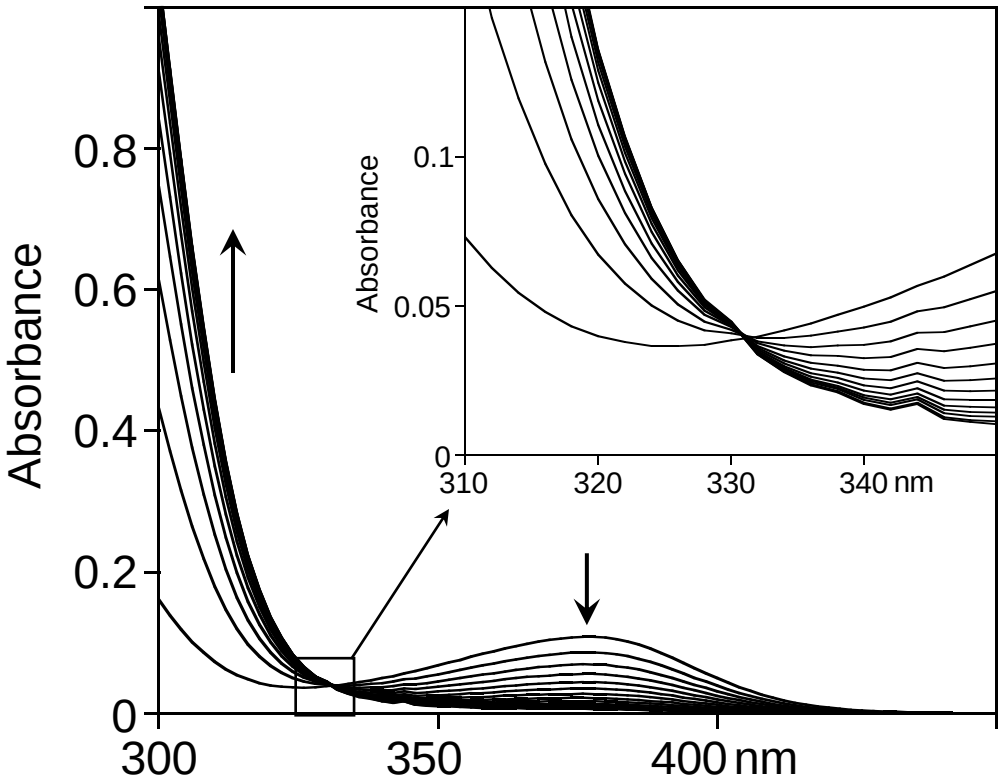

Figure S2. Time resolved UV spectra of the hydrolysis of $OSCN^-$ to give $H_2NC(=O)SO^-$ at pH 13 for high $[OSCN^-]_0$. The spectra are 213 s apart. $OSCN^-$ was generated in the reaction of 10 mM OCl^- and 100 mM SCN^- at pH = 13. Conditions: $[OCl^-]_0$ = 10 mM, $[SCN^-]_0$ = 100 mM, pH =13, T = 20 ^oC. Notice the isosbestic point (inset).

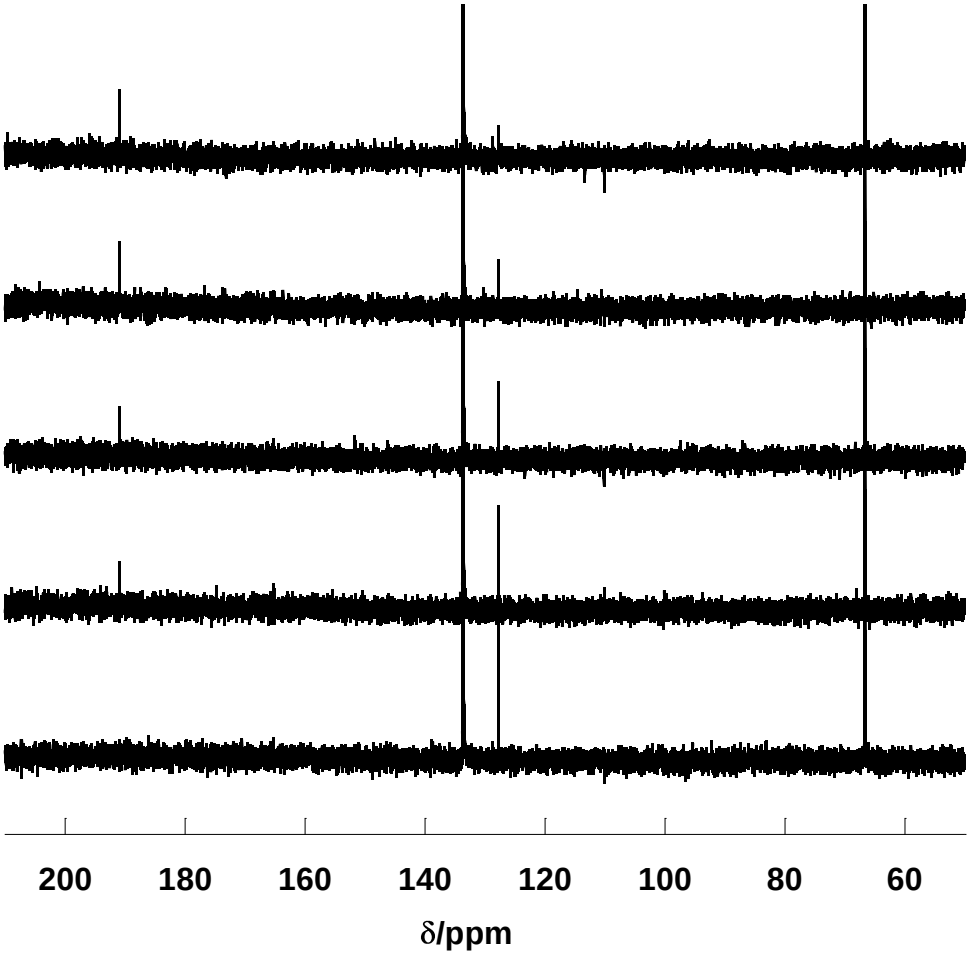

Figure S3. Time resolved ^{13}C NMR spectra (from bottom to top) of the $OSCN^-$ → 190 reaction. $OSCN^-$ was generated by the oxidation of 100 mM SCN^- with 10 mM OCl^- in 0.10 N NaOH. Conditions: $[OCl^-]_0$ = 10 mM, $[S^{13}CN^-]_0$ = 100 mM, pH =13, T = 20 ^oC, dead time (between mixing and starting the data collection): 1 min 21 s, spectra are 26 min and 32 s apart, nt = 128, d1 = 5s. Peak assignments: δ (Dioxane internal standard = 66.6 ppm), δ($OSCN^-$) = 127.8 ppm, δ(SCN^-) = 136.5 ppm, δ($^-OSC(=O)NH_2$) = 190.9 ppm.

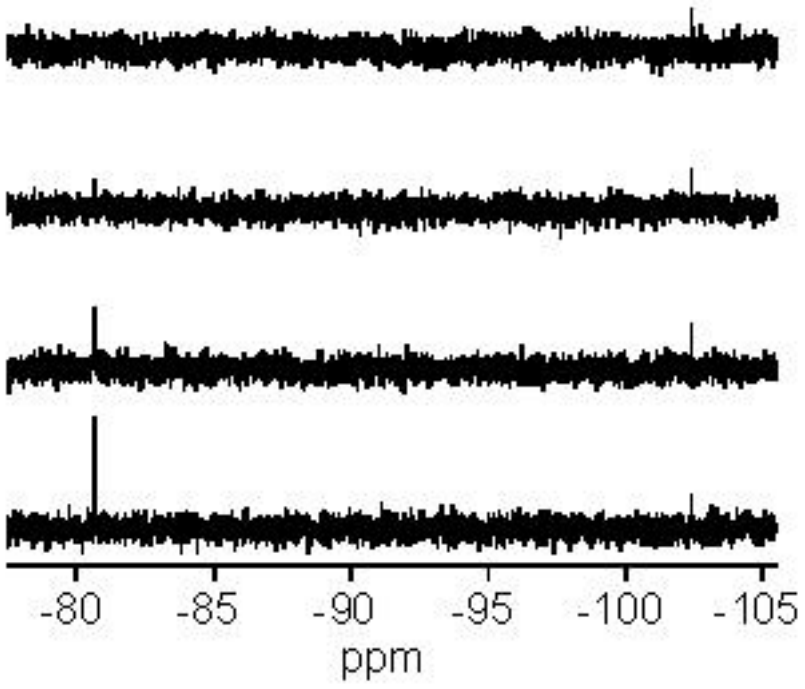

Figure S4. Time resolved ^{15}N NMR spectra (from bottom to top) of the $OSCN^- \rightarrow 190$ reaction. $OSCN^-$ was generated by the reaction of OCl^- with SCN^-. Conditions: $[OCl^-]_0$ = 20 mM, $[S^{15}CN^-]_0$ = 200 mM, pH =13, T = 20 ^{0}C, dead time (between mixing and starting the data collection) = 53 s, spectra are 35 min and 15 s apart, nt = 64, d1 = 25s, at = 8, sw = from -295.38 to 34.35 ppm, fn (zero filling) = 524 288. Peak assignments: $\delta(OSCN^-)$ = -80.6 ppm, $\delta(^-OSC(=O)NH_2)$ = -101.6 ppm.

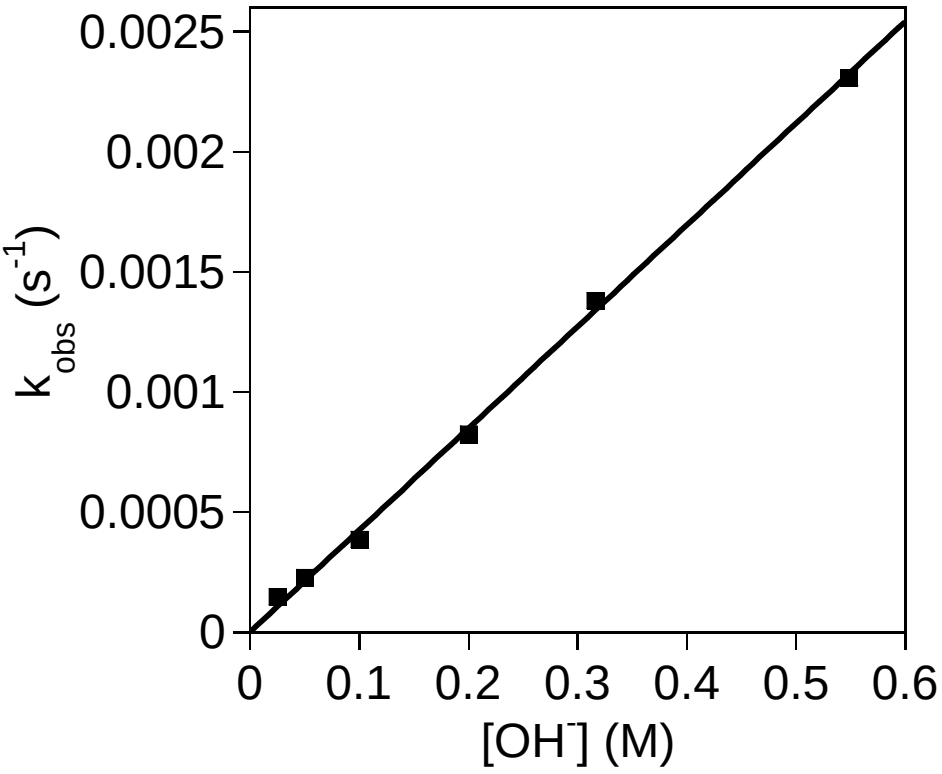

Figure S5. Plot of k_{obs} vs $[OH^-]_0$ for the hydrolysis of $OSCN^-$, in the presence of 0.50 M SCN^-, and at I = 1.0 M ($NaClO_4$). Slope: (4.24 ± 0.05) × 10^{-3} M^{-1} s^{-1}.

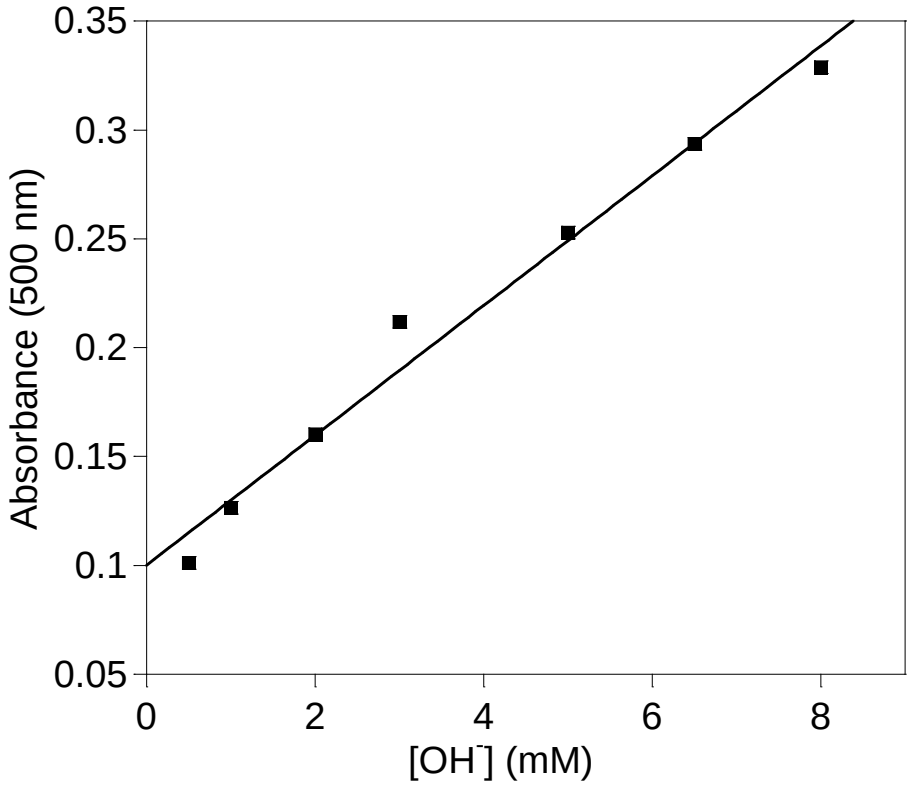

Figure S6. Plot of absorbance of 25 μM Tropaeolin O at 500 nm vs [OH^-].

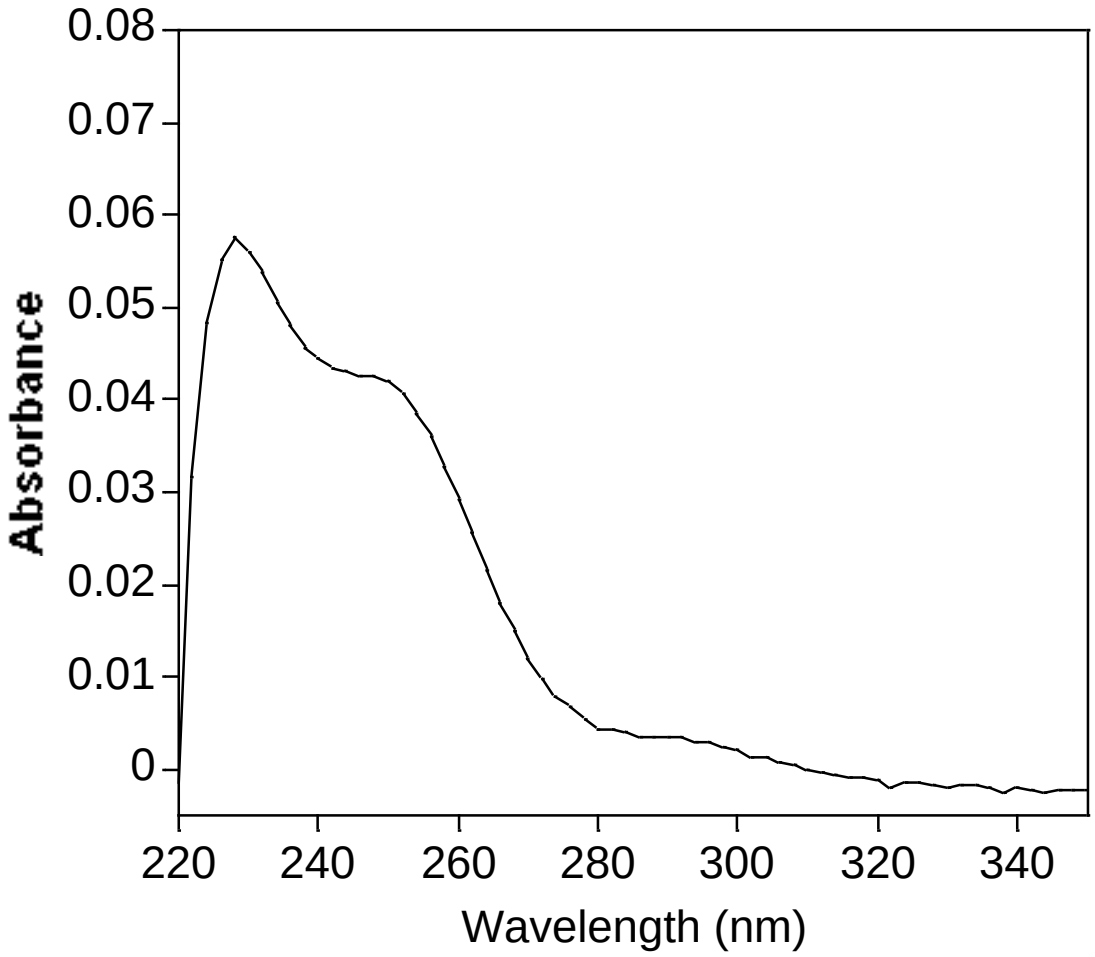

Figure S7. UV-vis spectrum of thiocarbamate-S-oxide from the reaction of thiocarbamate (80 μM) and OCl^- (30 μM) in 0.10 M NaOH.

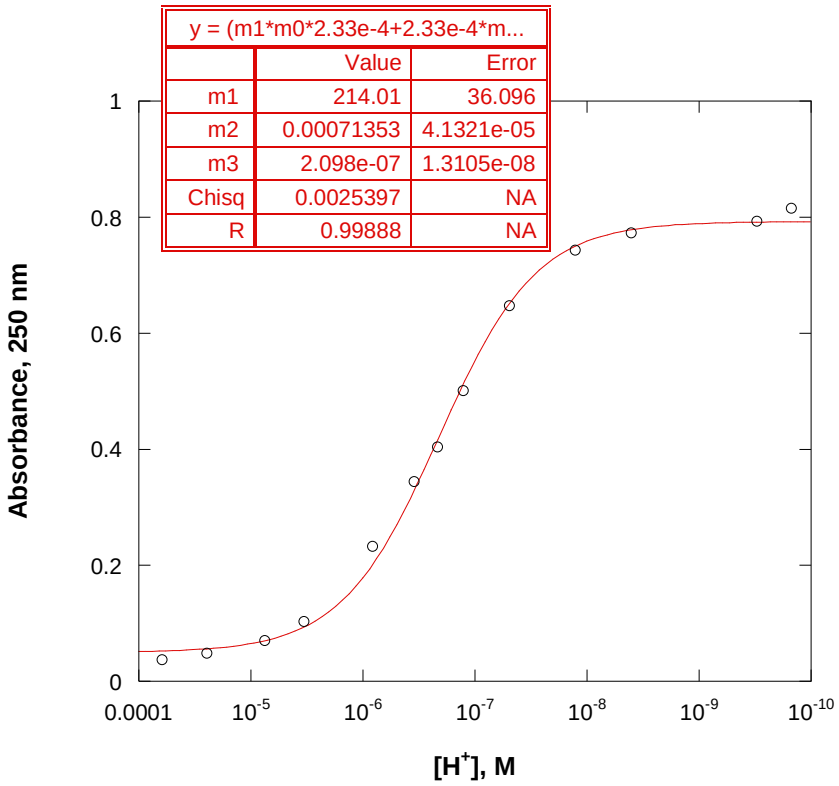

Figure S8. Absorbance of carbamothioperoxoic acid at 250 nm vs $[H^+]$ (I = 1.0 M, $NaClO_4$).

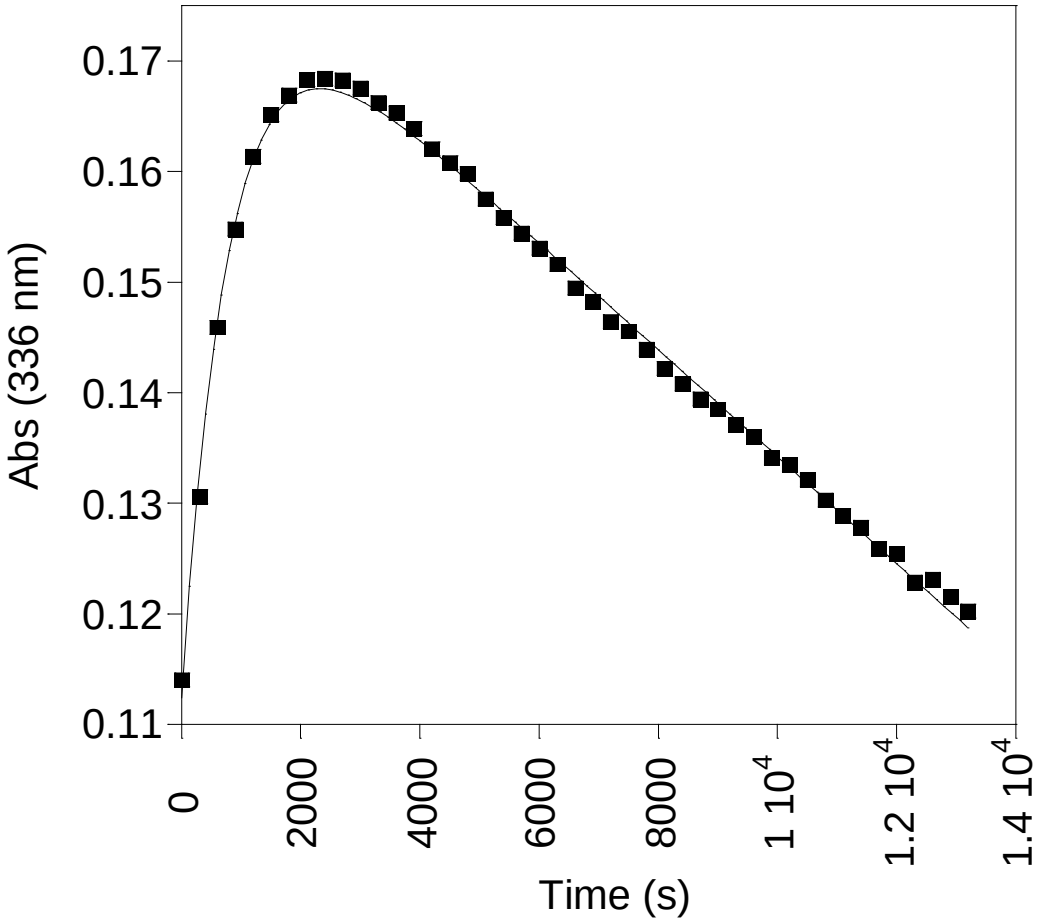

Figure S9 Kinetic trace for the reaction between cysteine (50 mM) and $^{-}OSC(=O)NH_2$ (0.61 mM) / $^{-}SC(=O)NH_2$ (1.25 mM) in 0.10 M NaOH.

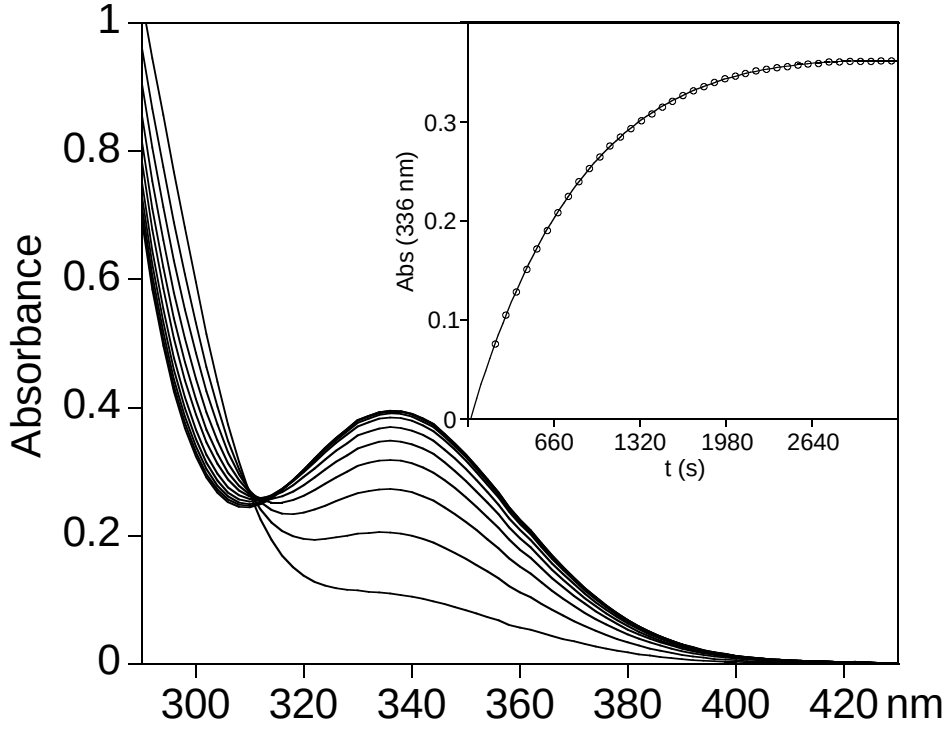

Figure S10. Time resolved spectra of the reaction of $^{-}OSC(=O)NH_2$ (generated by the hydrolysis of $OSCN^{-}$) and cysteine at pH = 13. Spectra are 80 s apart. The pseudo first order rate constant was computed to be: $k_{obs} = 6.7 \times 10^{-4}$ s^{-1} using SVD analysis. Inset: Kinetic trace recorded at $\lambda = 336$ nm together with an exponential fit. Conditions: $[^{-}OSC(=O)NH_2]_0 = 10$ mM, $[\text{cysteine}]_0 = 100$ mM, pH =13, T = 20 ^{o}C.

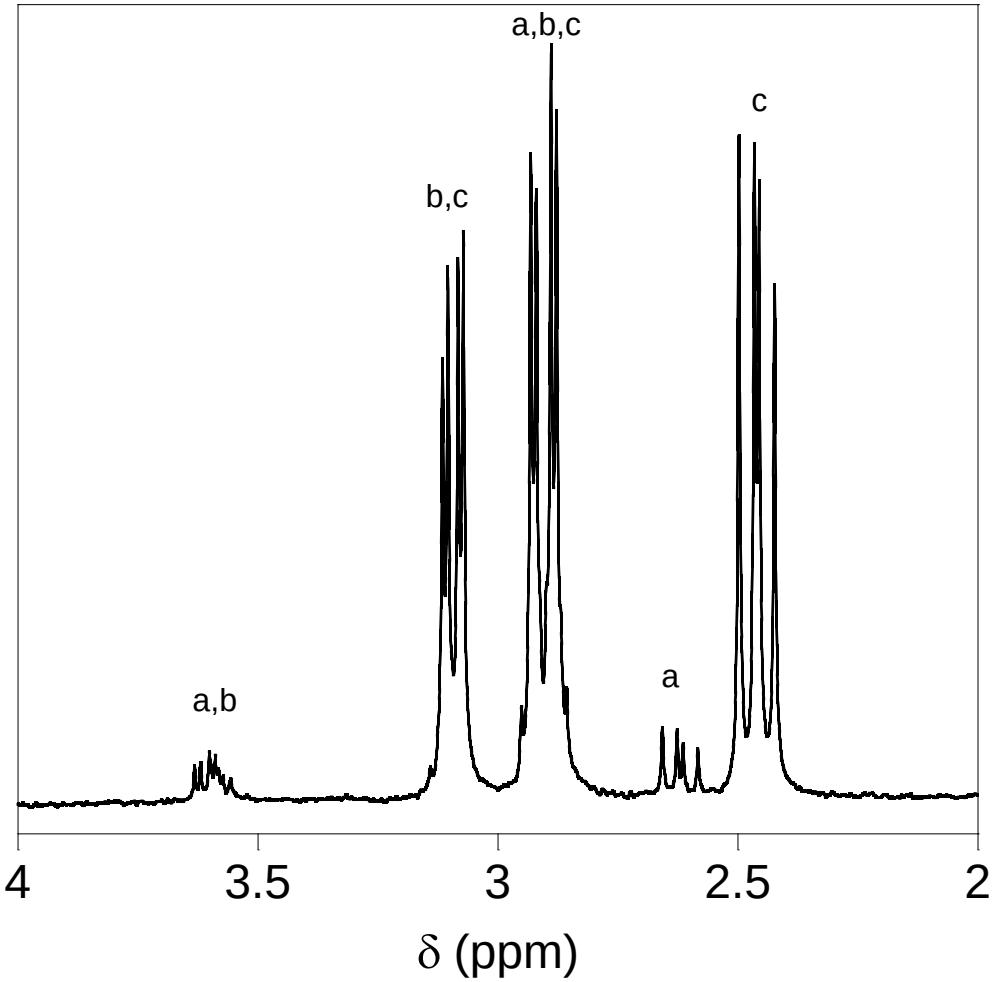

Figure S11. ^{1}H-NMR spectrum for the reaction between 20 mM cysteine and 2.5 mM thiocarbamate-S-oxide /5.0 mM thiocarbamate (TCM) at pH 13.0, with DSS as standard (after 50 minutes of reaction). Peak assignments: $H_2NC(=O)SSCy$ (doublet of doublets) = a, CySSCy (doublet of doublets) = b, CySH (doublet of doublets) = c.

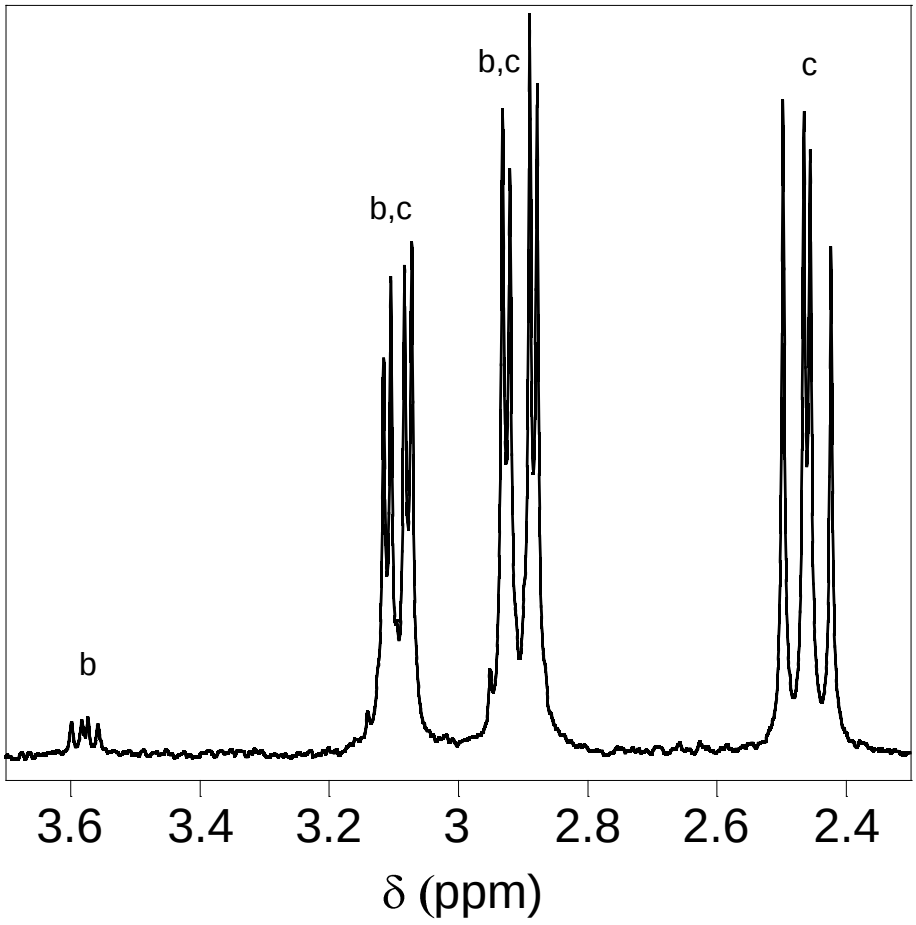

Figure S12. ^{1}H-NMR spectrum for the reaction between 20 mM cysteine and 2.5 mM thiocarbamate-S-oxide/5.0 mM thiocarbamate (TCM) at pH 13.0, with DSS as standard (after 24 hours of reaction). Peak assignments: CySSCy (doublet of doublets) = b, CySH (doublet of doublets) = c.