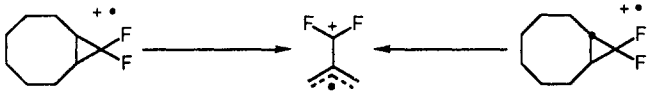
Scheme II



3 (m/e 90)

the values for *cis*-cyclooctene (8.82 eV), cyclohexene (8.95 eV), *cis*-2-butene (9.11 eV), and *trans*-2-butene (9.10 eV) are all relatively high. [2-Methylpropene (9.23 eV) appears to be an anomaly since it reacts with CF_2 in high yield;[2] however, here we suspect a markedly unsymmetric transition state due both to steric and to electronic effects.] The high reactivity of *trans*-cyclooctene is readily understood through comparison of its adiabatic ionization energy (8.53 eV[17,18]) with that of the cis isomer, which is ca. 0.3 eV higher. Thus, twisting of the π-bond pushes up the highest occupied molecular orbital (HOMO) and has the same effect as electron donation by alkyl groups, which also makes the HOMO higher in energy. This principle also appears relevant to the observation of CF_2 attack on some bicyclo[1.1.0]alkanes.[19]

Reaction with 1,2,2-trimethylbicyclo[1.1.0]butane yields a product (from concerted attack calculated to initiate at the central bond) in about 3% yield.[19] The corresponding yield from bicyclo[2.1.0]pentane is only ca. 0.5% while bicyclo[3.1.0]hexane does not react.[19] The ionization energies[15] of bicyclo[1.1.0]butane (8.70 eV), bicyclo[2.1.0]-pentane (8.7 eV), and bicyclo[3.1.0]hexane (9.16 eV) follow an expected trend. 1,2,2-Trimethylbicyclo[1.1.0]butane should have a consierably lower value by analogy with solution $E_{1/2}$ values for bicyclobutane (1.69 V), 2,2-dimethylbicyclo[1.1.0]butane (1.56 V), and 1,3-dimethylbicyclo[1.1.0]butane (1.10 V).[20]

It is worthwhile noting that while the strain energies of *cis*- and *trans*-bicyclo[6.1.0]nonanes are within 1 kcal/mol of each other, it seems possible that the fluoro substituents enhance this effect since stretching of the distal bond in *gem*-difluorocyclopropanes is associated with an increase in strain.[7,8] One small item of evidence may be seen in the comparison of the relative abundances of the *m/e* 90 ion likely to be **3** (Scheme II). In the cis isomer, the base peak is *m/e* 41 with *m/e* 90 at 94% and the parent ion at 5% (5.3% of *m/e* 90). For the trans isomer, *m/e* 90 is the base peak and the parent ion is only 2.6% of the *m/e* 90 peak. Our initial attempt to isomerize **2** to **1** at 60 °C (see ref 4) was not successful.

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Registry No. **1**, 108055-81-8; **2**, 123883-64-7; difluorocarbene, 2154-59-8; *cis*-cyclooctane, 931-87-3; *trans*-cyclooctene, 931-89-5; 7,7-difluoro-1-methylbicyclo[4.1.0]heptane, 123883-63-6.

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Nuclear Magnetic Resonance Studies of Thiol/Disulfide Chemistry. 2. Kinetics of Symmetrical Thiol/Disulfide Interchange Reactions[1]

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Introduction

The thiol group is among the most reactive groups found in biological molecules, and to a large extent the ease with which it is oxidized governs the metabolism and function of thiol-containing compounds.[2] Oxidation can occur by several mechanisms,[3,4] including thiol/disulfide interchange as described by eq 1 and 2. The net result of these two

$$RSH + R'SSR' \rightleftarrows RSSR' + R'SH \quad (1)$$

$$RSH + RSSR' \rightleftarrows RSSR + R'SH \quad (2)$$

reactions is oxidation of RSH and reduction of R'SSR', with the overall reaction proceeding through the mixed disulfide intermediate RSSR'. Thiol/disulfide interchange reactions are involved in the metabolism of endogenous thiols as well as thiol-containing drug molecules,[5-10] in the mechanism of action of penicillamine in the treatment of cystinuria,[11] and they provide a means for the reversible formation and cleavage of strong, covalent sulfur–sulfur bonds in biological molecules.[4]

Because of their importance in biological chemistry, the kinetics and mechanism of thiol/disulfide interchange reactions have been the subject of numerous studies.[12-15] Previous studies have generally been restricted to systems that form products that have some unique characteristic which can be monitored, e.g., the reaction of thiols with oxidized glutathione was monitored by an enzymatic assay for glutathione,[12b] and the reaction of a variety of thiols

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Table I. Structures, Names, and Abbreviations of Thiols and Disulfides

structure[a]	name	name of oxidized form
$^-O_2CCH(ND_2)CH_2CH_2CONDCH(CH_2S^-)CONDCH_2CO_2^-$	glutathione (GSH)	oxidized glutathione (GSSG)
$D_2NCH(CH_2S^-)CO_2^-$	cysteine (CSH)	cystine (CSSC)
$D_2NCH(CH_2CH_2S^-)CO_2^-$	homocysteine (HCSH)	homocystine (HCSSCH)
$DOCH_2CH_2S^-$	2-mercaptoethanol (MSH)	2-hydroxyethyl disulfide (MSSM)
$^-O_2CCH_2S^-$	mercaptoacetic acid (MASH)	2,2′-dithiodiacetic acid (MASSAM)
$^-O_2CCH_2CH_2S^-$	3-mercaptopropionic acid (MPSH)	3,3′-dithiodipropionic acid (MPSSPM)
$D_2NCH_2CH_2S^-$	cysteamine (CySH)	cystamine (CySSCy)

[a] Structures show the thiols in the protonation states of the major species present in D_2O solution at the pD values used in the kinetic studies.

with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) has been studied by utilizing the spectrophotometric properties of the product 5-thio-2-nitrobenzoic acid.[12a,13a,14]

The kinetics and equilibria of several thiol/disulfide interchange reactions have also been characterized by NMR spectroscopy.[12e,15] The rate of thiol/disulfide interchange is sufficiently slow on the NMR time scale that separate resonances are observed for the thiol and disulfide species in thiol/disulfide mixtures, and the rate of thiol/disulfide interchange can be determined by measuring their intensity as a function of time.[12e,15] We have recently discovered,[16] however, that the rate of thiol/disulfide interchange is sufficiently fast that it can also be measured by NMR spin-transfer methods.[17-19] In this paper, we have used spin-transfer methods to study the kinetics of symmetrical thiol/disulfide interchange reactions (eq 3) of the tripeptide glutathione and related compounds (Table I). In eq 3 the asterisk indicates the

$$R^*SH + RSSR \rightleftarrows R^*SSR + RSH \qquad (3)$$

labeling of R by the selective inversion of one of its resonances. Glutathione was chosen for this study because of its ubiquity in biological systems, where its thiol/disulfide interchange reactions are important in maintaining the thiol status of cells.[20] The other compounds were studied with the objective of determining the effect of molecular charge on thiol/disulfide interchange rates.[13c,14]

Experimental Section

Chemicals. Glutathione, oxidized glutathione, L-cysteine, L-cystine, and L-homocystine were used as received from Sigma Chemical Co. Mercaptoacetic acid, 2-hydroxyethyl disulfide, 3-mercaptopropionic acid and 3,3′-dithiodipropionic acid were obtained from Aldrich Chemical Co., and 2-mercaptoethanol was obtained from Fisher Scientific Co. A solution of 2,2′-dithiodiacetic acid was prepared by bubbling oxygen gas through a pD 9 solution of mercaptoacetic acid, and a solution of L-homocysteine was prepared by electrochemical reduction of a pD 1.0 solution of L-homocystine at a Hg pool cathode set to −1.0 V vs the saturated calomel electrode.[21] The reduction was carried out in an H-cell with the Hg pool cathode (2.3 cm^2), the reference electrode and the L-homocystine solution in one compartment, and saturated KCl solution and a platinum counterelectrode in the second compartment. The two compartments were separated by a glass frit. A Princeton Applied Research Model 174 polaragraphic analyzer was used as the voltage source. Solutions were continuously bubbled with N_2 or Ar before, during, and after electrolysis. The concentrations of the 2,2′-dithiodiacetic acid and the L-homocysteine solutions were determined by quantitative proton NMR using 3-(trimethylsilyl)-1-propanesulfonic acid (DSS) as an internal intensity standard.

Solutions used in the measurement of thiol/disulfide exchange kinetics were prepared in 99.8% D_2O, which was deoxygenated by bubbling with nitrogen before addition of the thiol and disulfide. The pD was then adjusted by addition of concentrated base (NaOH dissolved in D_2O) or strong acid (20% DCl solution). Nitrogen was bubbled through the solutions during the adjustment of pD.

pH measurements were made at 25 °C with an Orion Research Model 611 digital pH meter equipped with a Fisher Scientific Accu-pHast double junction, combination glass electrode. Fisher certified standard solutions having nominal pH values of 4.00, 7.00, and 10.00 were used for calibrating the pH meter. The exact pH of each standard solution was determined by comparison with freshly prepared NBS pH standard solutions.[22] pH meter readings were corrected for deuterium isotope effects with the relation pD = pH meter reading +0.40.[23]

NMR Measurements. ^1H and ^{13}C NMR spectra were obtained at 500 and 125 MHz, respectively, with a Varian VXR-500S spectrometer. The probe temperature was 25 °C for ^1H measurements. The temperature was higher for ^{13}C measurements due to heating from ^1H decoupling; the sample temperatures are reported with the experiimental data. To minimize sample heating, ^{13}C spectra were measured with WALTZ decoupling. ^1H chemical shifts were measured relative to the methyl resonance of *tert*-butyl alcohol and are reported relative to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). ^{13}C chemical shifts were measured relative to internal dioxane and are reported relative to tetramethylsilane. The 90° pulse was 15 μs for ^1H and 26 μs for ^{13}C.

The inversion-transfer experiments were performed with the pulse sequence:[24,25]

$$\pi/2(x) - \tau_1 - \pi/2(-x) - \tau_2 - \pi/2(x,y,-x,-y) \text{ -acquisition}$$

where τ_1 is a fixed delay equal to $1/(2\Delta\nu)$, $\Delta\nu = |\nu_A - \nu_B|$ in hertz and A and B are resonances for pairs of exchanging protons or carbons. τ_2 is a variable delay time during which transfer of inversion occurs by chemical exchange; τ_2 values ranging from 0.0001 s to 5 times the longest T_1 of the two resonances were used. Typically, 18 τ_2 values were used in each experiment. Eight transients were collected at each τ_2 value when observing ^1H, for a total experiment time of about 1 h; 100–200 transients were collected at each τ_2 value when observing ^{13}C, for a total experiment time of 10–14 h. To invert resonance B with the above pulse sequence, the transmitter was set on resonance A.

The interchange rate constant was determined from the intensity of resonance A following inversion of resonance B with equations which relate the dependence of the intensity on τ_2 to a pseudo-first-order exchange rate constant.[24] Data were fitted

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Table II. Spin-Lattice Relaxation Times for Thiols and Disulfides[a]

pD	thiol	T_1, s	disulfide	T_1, s
8.46	GSH[b]	0.87	GSSG[c]	0.43
8.93		0.84		0.39
9.42		0.82		0.42
10.41		0.79		0.42
11.40		0.80		0.42
10.60	CSH	3.16[d]	CSSC	4.19[f]
10.52		2.29[e]		1.39[g]
11.2	HCSH[h]	2.42	HCSSCH[i]	1.64
11.7		2.41		1.68
12.4		2.38		1.80
9.45	MSH[j]	6.19	MSSM[k]	2.49
10.40		4.67		2.29
11.40		4.58		2.35
10.28	MASH[l]	3.27	MASSAM	*m*
11.06		3.70		*m*
12.04		5.18		*m*
10.60	MPSH[n]	3.52	MPSSPM[o]	1.72
11.45		3.24		1.76

[a] For D_2O solutions containing only thiol or disulfide; chemical shift values which follow are approximate and, in most cases, shift with changes in pD. Uncertainties in T_1 values for GSH and GSSG are ±0.01 s; uncertainities in T_1 values for other compounds are ±0.05 s or less. [b] 0.200 M; T_1 values are for other compounds are ±0.05 s or less. [c] 0.100 M; Cys-C_α carbons, 53.4 ppm. [d] 0.200 M; $C_\alpha H$ proton, 3.3 ppm. [e] 0.300 M; C_α carbon, 59.3 ppm. [f] 0.100 M; $C_\alpha H$ protons, 3.6 ppm. [g] 0.150 M; C_α carbons, 55.7 ppm. [h] 0.240 M; C_α carbon, 56.5 ppm. [i] 0.346 M; C_α carbons, 55.9 ppm. [j] 0.196 M; β-CH_2 carbon, 27 ppm. [k] 0.138 M; β-CH_2 carbons, 40.9 ppm. [l] 0.191 M; CH_2 protons, 3.0 ppm. [m] Not determined; assumed to equal T_1 of MASH in calculation of rate constants. [n] 0.189 M; α-CH_2 carbon, 45.2 ppm. [o] 0.128 M; α-CH_2 carbons, 37.7 ppm.

to eq 4 of ref 24 using nonlinear least-squares regression analysis, which gave values for the set of parameters ϕ_1, ϕ_2, λ_1, and λ_2 (defined in ref 24). The pseudo-first-order rate constant k_{-1}, defined by eq 4, was then calculated from λ_1 and λ_2 using eq 5

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B \tag{4}$$

$$k_{-1} = \frac{\lambda_1 + \lambda_2 - 1/T_1^A - 1/T_1^B}{1 + K_e} \tag{5}$$

where T_1^A and T_1^B are spin-lattice relaxation times measured at the pH and concentrations used for the inversion-transfer experiment, and K_e = [B]/[A]. Thiol/disulfide exchange is a second-order reaction;[12b,e,13a,15a] the second-order exchange rate constant k was calculated from k_{-1} using the relationship $2k_{-1} = k$[RSH] or $k_{-1} = k$[RSSR], depending on whether the resonance for RSSR or RSH was inverted. Values used for the spin-lattice relaxation times in eq 5 were determined by the standard inversion-recovery pulse sequence using solutions containing only thiol or disulfide. Spin-lattice relaxation times are listed in Table II.

Thiol/disulfide interchange is first order in thiol and in disulfide,[12b,e,13a,15a] and the thiolate anion is the reactive form of the thiol.[12b,15a,23] To bring the rate of interchange to a time scale which can be measured by the inversion-transfer method, concentrations in the 0.1–0.2 M range were used, and solution pD was adjusted to the range where the thiol group is partly or completely deprotonated.

A typical series of inversion-transfer spectra for the GSH/GSSG system is shown in Figure 1. In this experiment the proton-decoupled resonance for the Cys-C_α carbon of GSH was inverted, and the transfer of inversion to the Cys-C_α carbon of GSSG by thiol/disulfide interchange was measured as a function of the length of the inversion-transfer time τ_2. The resonance shown in Figure 1 is for the Cys-C_α carbon of GSSG. In Figure 2, the integrated intensity of this resonance is plotted vs τ_2. The rate constant for GSH/GSSG interchange was calculated from parameters derived from a nonlinear least-squares regression analysis of the intensity vs τ_2 data as described above; the smooth curve through the points is the theoretical curve calculated with the parameters.

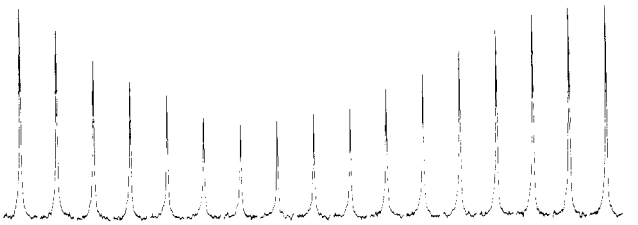

Figure 1. ^{13}C NMR spectra measured by the inversion-transfer pulse sequence for a pD 10.41 solution containing 0.20 M GSH and 0.10 M GSSG at 33 °C. The resonances shown are for the Cys-C_α carbon of GSSG; the resonance for the Cys-C_α carbon of GSH was inverted. In this experiment, τ_1 was set to 7.559×10^{-4} s and τ_2 had values of (from left to right) 0.0001, 0.01, 0.03, 0.05, 0.07, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5, 0.7, 0.9, 1.3, 2, and 3 s.

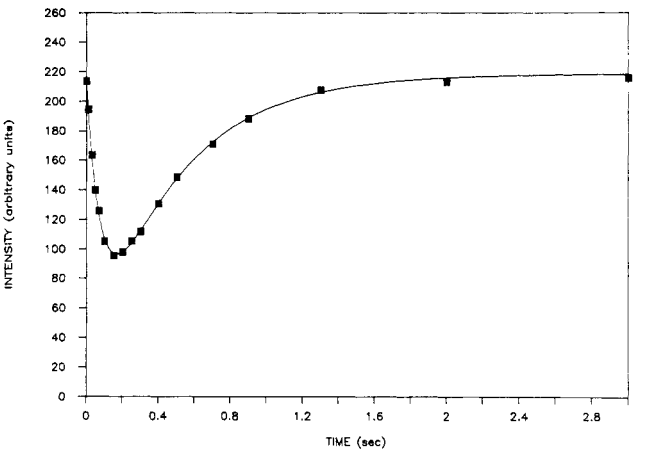

Figure 2. Integrated intensity of the resonance for the Cys-C_α carbon of GSSG as a function of the delay time τ_2; the data are from the inversion-transfer spectra in Figure 1. The smooth curve through the points is the theoretical curve calculated using the parameters obtained by nonlinear regression analysis, as described in the text.

Results and Discussion

Rate constants for symmetrical thiol/disulfide interchange for the thiol/disulfide pairs in Table I are reported in Table III. Also listed in Table III are rate constants determined in a previous study of cysteamine/cystamine exchange.[16] The activation energy for GSH/GSSG interchange at pD 10.46 was estimated to be 25 ± 2 kcal/mol from rate constants measured at several temperatures: 30.8 °C, 43 M^{-1} s^{-1}; 31.1 °C, 46 M^{-1} s^{-1}; 31.2 °C, 48 M^{-1} s^{-1}; 33.5 °C, 60 M^{-1} s^{-1}; and 36 °C, 91 M^{-1} s^{-1}. The lower temperature limit was set by sample heating due to ^1H decoupling and the upper limit was determined by decomposition of the peptides. For comparison, the activation energy for reaction of mercaptoethanol and 1,3-dithiopropan-2-ol with GSSG is 16 ± 1 kcal/mol.[12d]

The thiolate anion is the reactive form of the thiol in thiol/disulfide interchange reactions. Although rate constants for the specific reaction of the thiolate anion with disulfide, k, can be calculated from rate constants measured as a function of pH, such calculations require acid dissociation constants for the thiol groups, which have not been reported for the thiols listed in Table I in D_2O solution. However, the thiol group is essentially completely deprotonated at the highest pD values listed for each thiol/disulfide pair in Table III and thus $k \approx k_{obs}$ at these high pD values.

The relative values of the high pD rate constants for thiol/disulfide interchange are consistent with the findings of previous studies of the effect of charged substituents on the rate of thiol/disulfide interchange reactions.[13c,14]

Table III. Rate Constants for Symmetrical Thiol/Disulfide Exchange

thiol disulfide pair	pD	k, L/mol s
glutathione/oxidized glutathione	8.46	9.1[a]
	8.93	21[a]
	9.43	45[a]
	10.41	60[a]
	11.40	60[a]
cysteine/cystine	10.52	12[b]
	10.60	9.1[c]
	11.40	5.0[c]
homocysteine/homocystine	11.2	27[d]
	11.7	17[e]
	12.4	20[e]
2-mercaptoethanol/2-hydroxyethyl disulfide	9.45	4.9[f]
	10.40	27[f]
	11.40	25[f]
mercaptoacetic acid/2,2'-dithiodiacetic acid	10.28	2.6[g]
	11.06	2.5[g]
	12.04	2.6[g]
3-mercaptopropionic acid/3,3'-dithiodipropionic acid	10.60	4.2[h]
	11.45	8.9[i]
cysteamine/cystamine[j]	12.58	22[j]
	13.63	24

[a] 33 °C; 0.20 M GSH, 0.10 M GSSG; measured by doing the inversion-transfer experiment using the Cys-C_α carbon resonances of GSH and GSSG. [b] 29 °C; 0.300 M CSH, 0.150 M CSSC; C_α carbon resonances. [c] 25 °C; 0.200 M CSH, 0.100 M CSSC; $C_\alpha H$ proton resonances. [d] 38 °C; 0.362 M HCSH, 0.149 M HCSSCH; C_α carbon resonances. [e] 38 °C; 0.568 M HCSH, 0.235 M HCSSCH; C_α carbon resonances. [f] 33 °C; 0.196 M MSH, 0.138 M MSSM; β-CH_2 carbon resonances. [g] 25 °C; 0.191 M MASH, 0.148 M MASSAM; CH_2 proton resonances. [h] 30 °C; 0.189 M MPSH, 0.128 M MPSSPM; α-CH_2 carbon resonances. [i] 35 °C; 0.189 M MPSH, 0.128 M MPSSPM; α-CH_2 carbon resonances. [j] 27 °C; from ref 16.

For example, the results in Table III indicate that the rate constants for RSH/RSSR interchange are lower when R has a negative charge (e.g. CSH/CSSC, MASH/MASSAM, and MPSH/MPSSPM exchange) than when R is neutral (MSH/MSSM and CySH/CySSCy exchange) and that the rate constants increase when the number of atoms between carboxylate groups of R and the sulfur increase (e.g. HCSH/HCSSCH exchange vs CSH/CSSC exchange and MPSH/MPSSPM exchange vs MASH/MASSAM exchange). It has been found in previous studies that the rate constant for reaction of RS^- at the RS sulfur of $RSSC_6H_4NO_2$ increases as the charge on R changes from negative to zero, and it increases as the number of atoms between the negative charge on R and the sulfur increase.[13c]

In view of the effect of negative charge on the rate of thiol/disulfide interchange, the rate constants for GSH/GSSG exchange are surprisingly large (at pD 11.4, GSH has a charge of -3 and GSSG a charge of -4). However, it has been found previously that the effect of charged groups on the kinetics of thiol/disulfide interchange reactions involving cysteine residues in peptides decreases as the number of atoms separating the cysteine sulfur from positive or negative charges on adjacent residues increases.[14] In GSH and GSSG, the negative carboxylate oxygens of the glycine and glutamyl residues are seven and nine bonds removed from the sulfur atom as compared to being three, four, and five bonds removed from the sulfur in MASH, CSH, and HCSH, respectively.

The pD dependence of the observed rate constants for GSH/GSSG exchange is also consistent with an apparent absence of effects due to the charge on the glutamyl residue. Since the thiolate anion is the reactive thiol species, $k_{obs} = \alpha k$ where α is the fraction of GSH in the thiolate form and k is the rate constant for reaction of the thiolate anion of GSH with GSSG. If the rate of GSH/GSSG exchange were influenced by the protonation state of the amino group of the glutamyl residues of GSH and GSSG, and thus the charges on the glutamyl residues, the rate constant k would change over the pD region 8.46-11.40. Over this pD range, the net charges on GSH and GSSG change from -2 and -2, respectively, to -3 and -4. Substitution of the relationship $\alpha = K_{SH}/(K_{SH} + [D^+])$, where K_{SH} is the acid dissociation constant of the thiol group of GSH, into the above equation, and rearrangement gives eq 6. Using the data for GSH/GSSG in Table III, a value

$$[D^+]k_{obs} = -K_{SH}k_{obs} + kK_{SH} \quad (6)$$

of 9.24 ± 0.06 is obtained for pK_{SH} from a plot of $[D^+]k_{obs}$ vs k_{obs}. This value is in good agreement with literature values for pK_{SH} for GSH in H_2O,[26-30] which indicates that the rate constant is constant over the pD range 8.46-11.40 and does not depend on the protonation state of the glutamyl groups.

The results of this study indicate that the inversion-transfer experiment is a convenient method for measuring the rates of symmetrical thiol/disulfide interchange reactions of biological thiols. With two-dimensional exchange spectroscopy, it should be possible to also measure the rates of unsymmetrical thiol/disulfide interchange reactions for a wide range of systems under equilibrium conditions.

Registry No. GSH, 70-18-8; GSSG, 27025-41-8; CSH, 52-90-4; CSSC, 56-89-3; HCSH, 6027-13-0; HCSSCH, 462-10-2; MSH, 60-24-2; MSSM, 1892-29-1; MASH, 68-11-1; MASSAM, 505-73-7; MPSH, 107-96-0; MPSSPM, 1119-62-6; CySH, 60-23-1; CySSCy, 51-85-4.

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Sonication-Induced Reductive Decarboxylation of Thiohydroxamic Esters

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Introduction

There have been several recent reports of reductive decarboxylation of unactivated carboxylic acid derivatives by various radical methods.[1,2] One of these methods is the reductive decarboxylation of thiohydroxamic esters (mixed anhydrides).[3] This attractive method involves the

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