Reprinted from The Journal of Organic Chemistry, 1993, 58.

Synthesis of Dithiols as Reducing Agents for Disulfides in Neutral Aqueous Solution and Comparison of Reduction Potentials

Guy V. Lamoureux and George M. Whitesides*

Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138

Received August 25, 1992

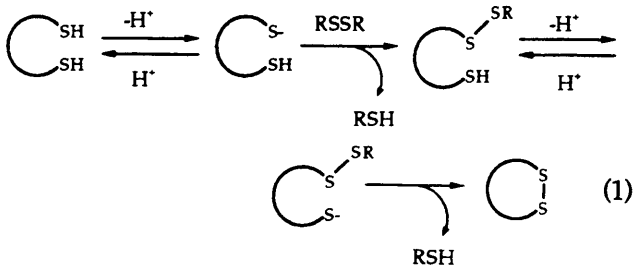
Several dithiols have been prepared that are useful for the reduction of disulfides in aqueous solution. The reduction potential of these dithiols have been determined from the measurement of the equilibrium constant of thiol/disulfide interchange with oxidized dithiothreitol using a ^{1}H NMR assay. The values of pK_a of some of the dithiols were measured to estimate their rate of reduction of disulfides. Bis(2-mercaptoethyl) sulfone (**2**), *N,N'*-dimethyl-*N,N'*-bis(mercaptoacetyl)hydrazine (**5**), and *meso*-2,5-dimercapto-*N,N,N',N'*-tetramethyladipamide (**6**) are especially interesting as alternatives to dithiothreitol for the reduction of disulfides.

Introduction

The oxidation state of sulfhydryl groups influences the structure and activity of many biological systems; the thiol/disulfide interchange reaction is important in determining this state.[1,2] For example, thiol/disulfide interchange is important in the folding of proteins.[3] Many enzymes require a cysteine in their active sites for catalysis: the thiol proteases,[4] enolase,[5] β-ketoacylthiolase,[6] and thioredoxin[7] are rendered inactive by oxidative conversion of the reactive thiol group to a disulfide. Thiol/trisulfide interchange has been implicated as the "triggering event" in the cleavage of DNA by calichemicin and esperamicin.[8]

In an effort to develop reagents useful in controlling thiol/disulfide interchange in aqueous solutions, we have previously examined a number of dithiols for their usefulness as reducing agents for disulfides.[9,10] Although several other reagents are already available for this reaction,[11] there is still room for improvement. Dithiothreitol (DTT, Cleland's reagent) is one reagent that is widely used for reduction of a disulfide bond.[12] It is a strong reductant, but expensive. It is also kinetically slow at pH = 7. Mercaptoethanol (ME) is inexpensive, but it is a weak and slow reducing agent; formation of mixed disulfides with ME is common.

We wished to design a reagent that would have properties superior to the compounds presently in use for the reduction of disulfide bonds. The practical properties that are important in the design of dithiols for the efficient reduction of acyclic disulfides are high solubility in aqueous solutions, low cost, low odor, and low toxicity.[13] We were especially interested in the rate of reduction and the redox potential. A high rate of reduction depends largely on a correct choice of pK_a.[14] The initial steps in disulfide reduction are the deprotonation of the dithiol and S_N2 reaction involving the thiolate anion (eq 1); the nucleophilic



(1)

attack of a *thiol* on a disulfide has not been observed.[15] The maximum observed rate of thiol/disulfide interchange in aqueous solution is usually achieved when the pK_a of the thiol is approximately equal to the pH of the solution (p$K_a \approx$ pH ≈ 7 for a neutral solution).[14] The rate of thiol/disulfide interchange in aqueous solution can be estimated from the pK_a of the attacking thiolate, the pK_a of the leaving group on the disulfide, and the pK_a of the nucleofugic sulfur of the disulfide. For the reduction of simple disulfides in pH = 7 aqueous solution, dithiols with values of pK_a in the range 7.8–8.0 are predicted to have apparent rate constants (k_{app}) that are 3.5–5.0 times faster than DTT (pK_1 = 9.2).[14] In actual practice, rates of reduction that are ca. 5–7 times faster than DTT have been observed.[10]

In addition to a fast rate of reduction, a useful dithiol should be strongly reducing in order to reduce disulfides quantitatively and to maintain thiols in the reduced state without the inconvenience of mixed disulfides. Since DTT

(1) Jocelyn, P. C. *Biochemistry of the SH Group*; Academic: London, 1972.
(2) Gilbert, H. F. *Adv. Enzymol.* **1990**, *63*, 69–172.
(3) The role of sulfur in protein folding has been reviewed: Kim, P. S.; Baldwin, R. L. *Annu. Rev. Biochem.* **1990**, *59*, 631–660.
(4) Glazer, A. N.; Smith, E. L. *The Enzymes Vol. III*, 3rd ed.; Boyer, P. D., Ed.; Academic: New York, 1971; pp 501–546.
(5) Weiss, P. M.; Boerner, R. J.; Cleland, W. W. *J. Am. Chem. Soc.* **1987**, *109*, 7201–7202.
(6) Thompson, S.; Mayerl, F.; Peoples, O. P.; Masamune, S.; Sinskey, A. J.; Walsh, C. T. *Biochemistry* **1989**, *28*, 5735–5742. Masamune, S.; Walsh, C. T.; Sinskey, A. J.; Peoples, O. P. *Pure Appl. Chem.* **1989**, *61*, 303–312. Masamune, S.; Palmer, M. A. J.; Gamboni, R.; Thompson, S.; Davis, J. T.; Williams, S. F.; Peoples, O. P.; Sinskey, A. J.; Walsh, C. T. *J. Am. Chem. Soc.* **1989**, *111*, 1879–1881.
(7) Holmgren, A. *Annu. Rev. Biochem.* **1985**, *54*, 237–271.
(8) Golik, J.; Clardy, J.; Dubay, G.; Groenewold, G.; Kawaguchi, H.; Konishi, M.; Krishnan, B.; Ohkuma, H.; Saitoh, K.; Doyle, T. W. *J. Am. Chem. Soc.* **1987**, *109*, 3461–3462. Golik, J.; Dubay, G.; Groenewold, G.; Kawaguchi, H.; Konishi, M.; Krishnan, B.; Ohkuma, H.; Saitoh, K.; Doyle, T. W. *J. Am. Chem. Soc.* **1987**, *109*, 3462–3464. Lee, M. D.; Dunne, T. S.; Siegel, M. M.; Chang, C. C.; Morton, G. O.; Borders, D. B. *J. Am. Chem. Soc.* **1987**, *109*, 3464–3466. Lee, M. D.; Dunne, T. S.; Chang, C. C.; Ellestad, G. A.; Siegel, M. M.; Morton, G. O.; McGahren, W. J.; Borders, D. B. *J. Am. Chem. Soc.* **1987**, *109*, 3466–3468.
(9) Singh, R.; Whitesides, G. M. *J. Org. Chem.* **1991**, *56*, 2332–2337.
(10) Lees, W. J.; Singh, R.; Whitesides, G. M. *J. Org. Chem.* **1991**, *56*, 7328–7331.
(11) Jocelyn, P. C. *Methods Enzymol.* **1987**, *143*, 246–256.
(12) Cleland, W. W. *Biochemistry* **1964**, *3*, 480–482.
(13) Whitesides, G. M.; Lilburn, J. E.; Szajewski, R. P. *J. Org. Chem.* **1977**, *42*, 332–338.
(14) A Brønsted correlation has been developed to predict thiolate nucleophilicity at a given pH from pK_a data. See: Szajewski, R. P.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 2011–2026.
(15) Singh, R.; Whitesides, G. M. *The Chemistry of the Thiol Group, Supplement*; Patai, S., Ed.; John Wiley: London, in press.

0022-3263/93/1958-0633$04.00/0

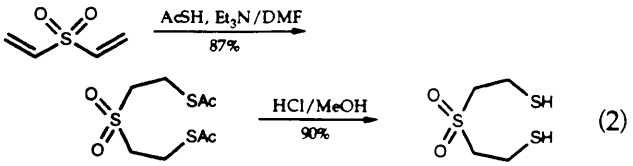
is one of the most strongly reducing dithiols, the larger the equilibrium constant for thiol/disulfide exchange is between a dithiol and DTT^{ox}, the stronger the reductant. We have used this equilibrium to evaluate several dithiols in terms of their reduction strength.

Results and Discussion

Central to our design of dithiols is the incorporation of electron-withdrawing groups to lower the pK_a of the sulfhydryl group and increase the rate of reductions involving them. The normal range of values of pK_a for an alkanethiol is 9–10. In general, aromatic thiols and thiols α or β to electron-withdrawing groups have significantly lower values ($pK_a \approx 7$–9).[16] We selected dithiols with β-mercaptosulfonyl (**2**), *N*-(mercaptomethyl)amide (**3**, **11**, **12**), or α-thioglycolamide (**5**, **8**, **10**) groups as promising candidates. These compounds, and their reduction properties compared to other dithiols, are listed in Table I. The determination of the data in Table I is described in the Experimental Section.

We have expressed the thermodynamic reducing ability of dithiols by several means: the equilibrium constant (K_{eq}) of the dithiol versus a standard dithiol (DTT), the electrochemical half-cell potential ($\epsilon°$),[17] and the thermodynamic "effective concentration" (EC)—the comparison between the equilibrium constant of an intermolecular process (in this case, formation of ME^{ox}) and that of an intramolecular ring formation.[18] Each of these comparisons is useful in different situations. The equilibrium EC is perhaps the most easily interpreted indicator of thermodynamic reactivity; a large value of EC indicates that the sulfhydryl groups are properly poised for disulfide formation and that the disulfide product is relatively free of strain. Dithiols arranged to form cyclic (5–8 membered), monomeric disulfides are likely to possess high reduction potentials.

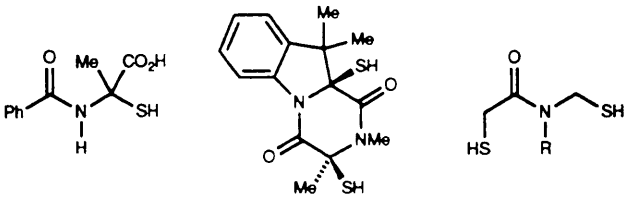
Bis(2-mercaptoethyl) sulfone (**2**) was synthesized in two steps from divinyl sulfone (eq 2). Divinyl sulfone acts as a bifunctional Michael acceptor, and the sulfone group is inert to reduction by thiols.[19] The sulfone moiety is also useful in conveying water solubility, electron withdrawal, and overall stability to this system. The seven-membered ring provides an unstrained disulfide bond;[20] the value of EC for **2** suggests, however, that its ring closure is less favorable overall than that for DTT. Overall, compound **2** should be a useful reducing agent. Since it has a low pK_a ($pK_1 = 7.9$),[21] at pH = 7 approximately 11% is present as thiolate monoanion.[22] Aqueous solutions of this dithiol (50 mM in pD = 7 or 10 D_2O buffer; 100 mM in phosphate, 25 °C) showed no signs of decomposition over several days when protected from atmospheric oxygen.



(2)

2

Compounds **3**, **11**, and **12** all contain the *N*-(mercaptomethyl) functionality.[23] One of our chief concerns was the stability of dithiols containing this functional group, but we concluded that this functionality would be stable to the conditions of thiol/disulfide exchange. The synthesis of 2-benzamido-2-mercaptopropanoic acid (**13**) demonstrated that these compounds are surprisingly stable to atmospheric oxygen, methanolic HCl, and dilute aqueous NaOH.[24] The synthesis of epidithiopiperazinedione analogs, such as **14**, indicated that *N*-(mercaptomethyl) groups owe their stability to the acylation on nitrogen.[25] A series of dithiols (**15**, R = H, Me, *i*-Pr), containing both the α-thioglycolamide and the *N*-(mercaptomethyl) functional groups, had been investigated previously by Gronowitz.[26] The stability of **15** demonstrates that substitution at the α-carbon is not required.



13 **14** **15**

The synthesis of this class of compounds proceeds via the easily prepared *N*-(hydroxymethyl)- and *N*-(chloromethyl)amides; the synthesis of **3** provides an example (eq 3).[27] The *N*-(mercaptomethyl) functionality was stable to acidic conditions and pH = 7 buffer but decomposed

(16) Kreevoy, M. M.; Harper, E. T.; Duvall, R. E.; Wilgus, H. S.; Ditsch, L. T. *J. Am. Chem. Soc.* **1960**, *82*, 4899–4902.

(17) Clarke, W. M. *Oxidation-Reduction Potentials of Organic Systems*; Williams and Wilkins: Baltimore, 1960; p 484.

(18) Creighton, T. E. *Biopolymers* **1983**, *22*, 49–58. Lin, T.-Y.; Kim, P. S. *Biochemistry* **1989**, *28*, 5282–5287.

(19) Zahn, H.; Lumper, L. *Hoppe-Seyler's Z. Physiol. Chem.* **1968**, *349*, 485–488.

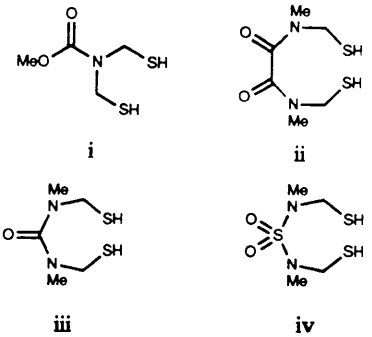
(20) Molecular mechanics (MM2 on MacroModel, V3.0, 1990) calculates a C–S–S–C dihedral angle of 83° in the minimized sulfone disulfide.

(21) Sulfur in high oxidation states is a potent electron-withdrawing species: The pK_a of α-(methylsulfonyl)acetic acid is 2.36 whereas α-(methylthio)acetic acid has a pK_a of 3.72. Also, the pK_a of β-mercaptoethyl sulfonate (9.1) is significantly lower than that of β-mercaptopropionate (10.3). See: Danehy, J. P.; Noel, C. J. *J. Am. Chem. Soc.* **1960**, *82*, 2511–2515.

(22) The percentage monothiolate is calculated by eq i

$$\% \text{ monothiolate} = 100/(10^{(pK_1-pH)} + 1 + 10^{(pH-pK_2)}) \quad \text{(i)}$$

(23) We also attempted the synthesis of the following dithiols containing the *N*-(mercaptomethyl) functional group. Polymerization (i) or decomposition (ii, iii, iv) occurred during formation or oxidation of these dithiols.



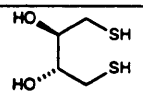
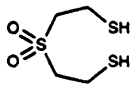
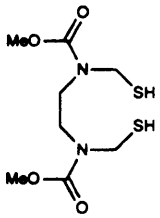
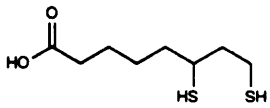
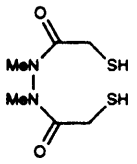
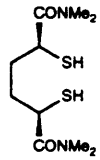
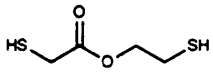
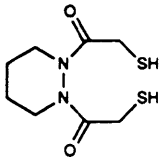
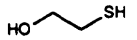
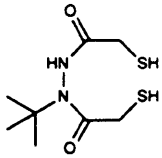
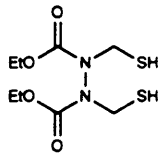
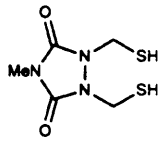
i ii iii iv

(24) Pojer, P. M.; Rae, I. D. *Tetrahedron Lett.* **1971**, 3077–3079. Pojer, P. M.; Rae, I. D. *Aust. J. Chem.* **1972**, *25*, 1737–1749.

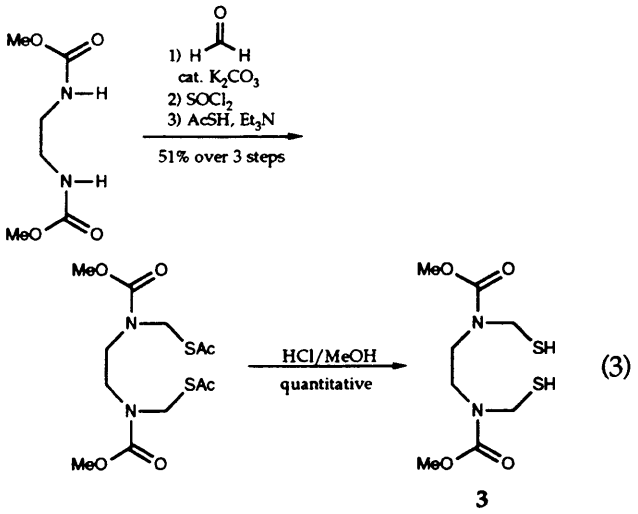
(25) Ottenheijm, H. C.; Spande, T. F.; Witkop, B. *J. Am. Chem. Soc.* **1973**, *95*, 1989–1996. Ottenheijm, H. C.; Herscheid, J. D.; Kerkhoff, G. P.; Spande, T. F. *J. Org. Chem.* **1976**, *41*, 3433–3438.

(26) Cynkier, I.; Gronowitz, S.; Hope, H.; Lidert, Z. *J. Org. Chem.* **1979**, *44*, 4699–4701. Gronowitz, S.; Lidert, Z. *Synthesis* **1979**, 810. Gronowitz, S.; Lidert, Z. *Chem. Scr.* **1980**, *16*, 97–101. Gronowitz, S.; Lidert, Z. *Chem. Scr.* **1980**, *16*, 102–107.

Table I. Comparison of Dithiols

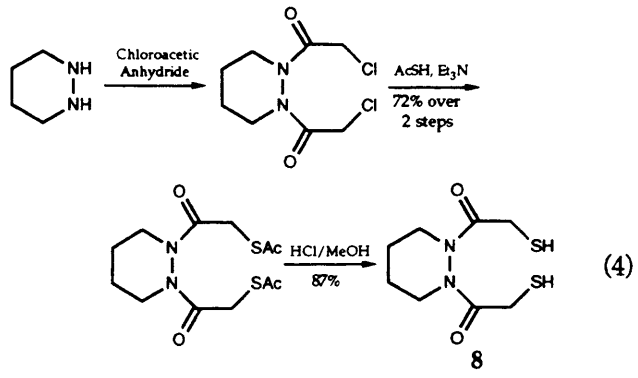
compd	Structure	K_{eq}[a]	ϵ° (V)[b]	EC (M)[c]	pK_a[d]	ref
1		1.0	−0.33	180	9.2, 10.1	*f*
2		0.35	−0.31	63	7.9, 9.0	*g*
3		8.1×10^{-2}	−0.30	15	7.0, 8.7	*g*
4		4.7×10^{-2}	−0.29	8.6	4.9, 10.7, 10.7	*h*
5		1.4×10^{-2}	−0.27	2.5	7.6, 8.9	*i*
6		1.0×10^{-2}	−0.27	1.8	7.8, 8.9	*j*
7		7.8×10^{-3}	−0.27	1.4	7.9, 9.9	*g*
8		6.6×10^{-3}	−0.26	1.2	*e*	*g*
9		5.6×10^{-3} M	−0.26		9.6	*k*
10		2.3×10^{-3}	−0.25	0.41	*e*	*g*
11		1.7×10^{-3}	−0.25	0.31	*e*	*g*
12		1.0×10^{-3}	−0.24	0.18	*e*	*g*

[a] The equilibrium constants are for the reduction of DTT^{ox}; all values are determined by ^{1}H NMR spectroscopy in buffered D_2O or D_2O/CD_3OD solutions. Accuracy is ±10%. [b] All ϵ° values are calculated based on comparison of equilibrium constants with that of lipoic acid (ϵ° = −0.288 V). These values of ϵ° are relative to the standard hydrogen electrode (SHE) at pH 7.0 and 25 °C. [c] Equilibrium effective concentrations (EC) are equilibrium constants relative to that of oxidized mercaptoethanol (ME^{ox}). [d] The values of pK_a are measured in aqueous solutions at 25 °C and are accurate to ±0.2 pK_a unit. [e] The values of pK_a of these compounds were not measured; by analogy to the other values measured in the table, α-thioglycolamides should have values of p$K_a \approx 7.8$ and *N*-(mercaptomethyl) compounds should have a p$K_a \approx 7.0$. [f] Cleland, W. W. *Biochemistry* **1964**, *3*, 480–482. [g] This compound was synthesized and characterized in this work. [h] Schmidt, U.; Grafen, P.; Altland, K.; Goedde, H. W. *Adv. Enzymol.* **1969**, *32*, 423–469. [i] Singh, R.; Whitesides, G. M. *J. Org. Chem.* **1991**, *56*, 2332–2337. [j] Lees, W. J.; Singh, R.; Whitesides, G. M. *J. Org. Chem.* **1991**, *56*, 7328–7331. [k] Jocelyn, P. C. *Methods Enzymol.* **1987**, *143*, 246–256.



rapidly when the pH was raised above 10. This instability precludes the use of these reagents for general purposes, but their ease of synthesis allows many variations to be made and tested. The only member of this group that has a useful combination of reducing strength and rate of reduction is compound **3**.

The last class of dithiols we examined contains the α-thioglycolamide functionality. This functional group has a low value of pK_a, and the carbonyl group is expected to enhance disulfide formation. Macrocyclic disulfides containing amide linkages are formed from their corresponding dithiols in higher yield than their carbocyclic analogs.[28] Their ease of cyclization has been ascribed to the restricted conformation of the acyclic precursor as well as lack of crowding and strain in the disulfide. We focused our attention on dithiols based on diacylhydrazines: these compounds form eight-membered rings readily on oxidation.[29] Equation 4 shows a representative synthesis (that of **8**). Compounds **5**,[9] **8**, and **10** are easy to prepare but are more expensive than the other reagents, mainly due to the expense of the alkylhydrazine. They are, however, rapid reducing agents, and this property is their major advantage over ME or DTT.

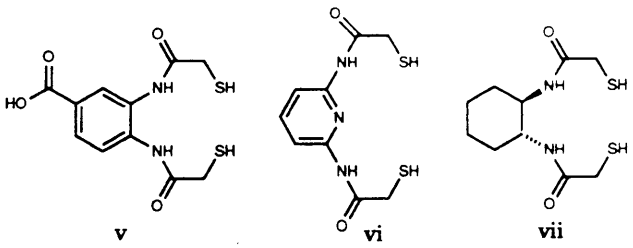


Related to the diacylhydrazine compounds are the adipamide **6**[10] and the ester **7**. These compounds are easily prepared and may be advantageous when reagents of a faster rate and a larger redox potential than ME are required.[30]

From a practical view, a fast or strong reducing agent will not be used if it does not have other desirable properties. Table II illustrates some of the characteristics of the best reducing agents in Table I. In general, a crystalline dithiol with low odor and high aqueous solubility is most easily handled. Disulfides that are crystalline and have low solubility can be useful if one wishes to extract the reagent away from the aqueous solution after the reduction. From Table II, compounds **1**, **2**, **5**, and **6** seem to have the greatest practical advantages except in cost.

Conclusions

Although the thiol groups in several of the compounds in Table I are significantly more acidic that those in DTT (and these compounds are, thus, expected to be faster reducing agents than DTT at pH 7–8), none exceeds the thermodynamic strength of DTT in disulfide reduction.[31] We are still trying to develop a compound that is simultaneously more reducing than DTT, a faster reducing agent, water soluble, and easily prepared. It is encouraging that constraining the sulfhydryl groups by their attachment to a cyclohexyl ring (**16**) increases the EC by a factor of 8.3 compared to DTT.[32] Hence, we continue to believe that it is possible in principle to find a reducing agent that is both stronger and faster than DTT. We believe that even the dithiols with the highest reduction potentials develop significant strain upon oxidation to the disulfide. For 1,2-dithianes, where the C–S–S–C angle is held to 60° rather than the preferred 90°, a relative torsional strain of 1.5–2.2 kcal/mol has been calculated by molecular mechanics (MM2).[32] This estimate agrees with the calculated difference in energy for dimethyl disulfide with the C–S–S–C angle constrained to 90° and 60° (≈2.0 kcal/mol using a 6-31G* basis set).[33] From these data, it appears that enthalpy affects the reduction potential to a small degree.[34,35] The entropic gain in conformational freedom seen with medium-membered rings may also lower the reducing strength. For example, the EC of DTT is much greater than compounds **2**, **3**, or **5**, whose oxidized products should be relatively unstrained.



(27) For reviews on reagents for α-amidoalkylation, see: Zaugg, H. E.; Martin, W. B. *Organic Reactions*; John Wiley: New York, 1965; Vol. 14, Chapter 2. Zaugg, H. E. *Synthesis*, **1984**, 181–212.

(28) Owen, T. C.; Fayadh, J. M. *J. Org. Chem.* **1970**, *35*, 3198–3199. Owen, T. C.; Fayadh, J. M.; Chen, J. S. *J. Org. Chem.* **1973**, *38*, 937–939.

(29) The parent system, perhydro-1,2,5,6-dithiadiazocine-4,7-dione, has been synthesized previously: Mahmood, A. A.; Fayadh, J. M. *Egypt. J. Chem.* **1976**, *19*, 539–541.

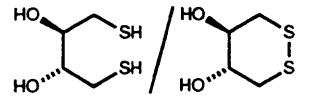
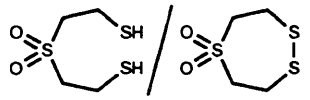
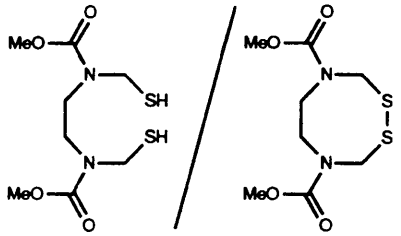
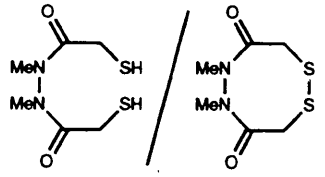
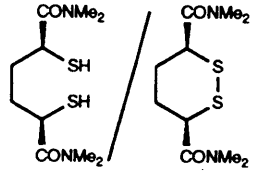
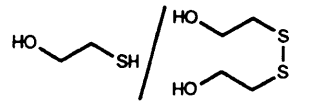
(30) The following compounds were also synthesized by a similar sequence of reactions as the diacylhydrazines; their reduction potentials versus DTT^{ox} were too low to be measured by our NMR technique (K_{eq}(dithiol vs DTT^{ox}) < 10^{-4}).

(31) It is difficult to predict what the maximum EC is possible for thiol/disulfide exchange. Although values of EC for simple ring closures may exceed 10^6 M, the values for the most strongly reducing dithiols examined so far are EC ≈ 200 M. For reviews on EC, see: Page, M. I. *Angew. Chem., Int. Ed. Engl.* **1977**, *16*, 449–459. Page, M. I. *Chem. Soc. Rev.* **1973**, *2*, 295–323.

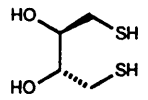
(32) Burns, J. A.; Whitesides, G. M. *J. Am. Chem. Soc.* **1990**, *112*, 6296–6303.

(33) Aida, M.; Nagata, C. *Theor. Chim. Acta* **1986**, *70*, 73–80.

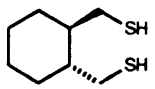
Table II. Properties of Various Dithiols and Their Corresponding Disulfides

dithiol/disulfide	mp (°C)	odor	solubility[a] (mM)	cost/mmol
	42/132	weak/none	>200/>200	$0.90[b]
	57/137	weak/none	140/30	c
	oil/oil	weak/none	100/45	c
	38/155	weak/none	250/23	c
	118/137	weak/none	80/80	$10[d]
	oil/25	strong/none	>200/>200	$0.01[b]

[a] Solubilities were determined in D_2O buffer (pD = 7.0, 50 mM in phosphate, 25 °C). [b] Cost based on recent prices of 100-g quantities from Aldrich Chemical Co. [c] This compound is not commercially available. [d] Cost based on recent prices of 5-g quantities from United States Biochemical.



1
EC = 180 M



16
EC = 1500 M

In conclusion, the combination of low pK_a, large EC, favorable physical properties, and potential for a low-cost synthesis of compounds **2**, **5**, and **6** make these reagents attractive for the reduction of disulfides in chemical or biochemical systems.

Experimental Section

General Methods. Melting points are uncorrected. Infrared spectra were recorded as thin films unless otherwise indicated. ^1H NMR chemical shift data are reported relative to $CHCl_3$ (7.26 ppm). Peaks are assigned by multiplicity, coupling constant(s) in hertz, and integration. Ambiguous assignments were resolved on the basis of 1D and 2D decoupling experiments. ^{13}C NMR chemical shift data are reported relative to the center $CDCl_3$ peak (77.0 ppm). Analytical thin-layer chromatography (TLC) was developed by staining with I_2 or a 10 mM solution of Ellman's reagent in DMF. Flash chromatography was performed according to the procedure of Still[36] on EM Reagents silica gel 60 (230–400 mesh). The conditions used are described as (column diameter × column length, solvent used). Unless otherwise noted, non-aqueous reactions were carried out in flame-dried glassware under a dry nitrogen or argon atmosphere. Anhydrous Na_2SO_4 was used to dry organic solutions. When necessary, solvents and reagents were purified prior to use. Starting materials were commercial products and were used without purification: Thionyl chloride, chloroacetic anhydride, thiolacetic acid (Fluka); 1,2-dicarbethoxyhydrazine, 4-methylurazole, divinyl sulfone, methyl chloroformate, chloroacetic chloride, Ellman's reagent, DTT, DTT^{ox} (Aldrich) and $SOCl_2$, formaldehyde, K_2CO_3 (Mallinkrodt).

General Experimental for Thiol/Disulfide Interchange. The dithiol (0.05 mol) was dissolved in deoxygenated CD_3OD (0.5 mL). The NMR spectrum of this solution was recorded at 25 °C. A solution of DTT^{ox} (0.05 mol) in deoxygenated D_2O buffer (pD = 7.0,[37] 100 mM in phosphate, 0.5 mL) was added. The solution was mixed, and the NMR spectrum of the mixture was recorded. The sample was stored under a positive pressure of N_2 during a 48-h period, at the end of which the NMR spectrum of the mixture was recorded to determine the equilibrium ratio of the species in solution.

Determination of K_{eq}. The equilibrium constant for thiol/

(34) If a dithiol could have the same spatial proximity of the sulfhydryl groups as DTT, but could oxidize to a strain-free disulfide (using $\Delta\Delta H = 2 \times 10^3$ cal/mol as the energy difference between DTT and an unstrained disulfide, C–S–S–C = 90°), the EC would increase by approximately a factor of 30 (eq ii)

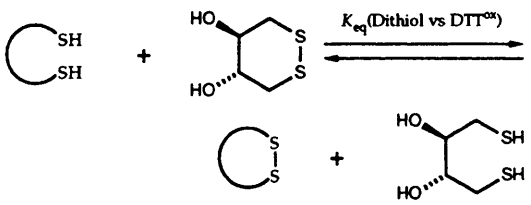
$$EC_{unstrained}/EC_{DTT} = e^{\Delta\Delta H/RT} = 30 \quad \text{(ii)}$$

(35) Enthalpic considerations are important (and may outweigh entropic gains) when increased strain on the rest of the molecule is produced on oxidation to a cyclic disulfide; compare the reduction potentials of compounds **5** and **8** and also **11** and **12**.

(36) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923–2925.

(37) The pD was determined by a pH meter and a correction factor: pD = pH + 0.4. Thus, for pD = 7.0, the D_2O was buffered to pH = 6.6. See: Glasoe, P. K.; Long, F. A. *J. Phys. Chem.* **1960**, *64*, 188–191.

disulfide exchange between a dithiol and DTT was determined by integration of the signals at 3.2–2.8 ppm (SCH_2, DTT^{ox}) and 2.65 ppm (SCH_2, DTT^{red}) in the ^1H NMR spectrum. In some cases where peaks overlapped, the signal at 3.55 ppm (CHOH, DTT^{ox}) was used instead of the signals at 3.2–2.8 ppm, and a factor of 2 was incorporated into the calculations. Since equimolar amounts of dithiol and DTT^{ox} were used, the equilibrium constant is equal to the ratio of DTT^{red} to DTT^{ox} (as measured by integration) squared (eqs 5 and 6).



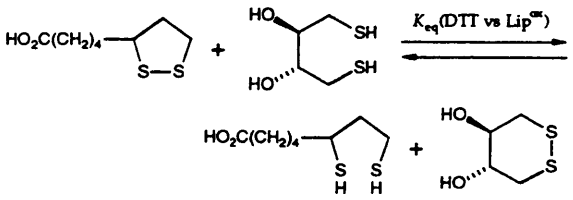
(5)

$$K_{eq}(\text{dithiol vs } DTT^{ox}) = \frac{[\text{disulfide}][DTT^{red}]}{[\text{dithiol}][DTT^{ox}]} = \left(\frac{[DTT^{red}]}{[DTT^{ox}]}\right)^2 \quad (6)$$

The reverse reaction, the thiol/disulfide exchange between equimolar disulfide and DTT^{red}, was run as a control to establish that each system had reached equilibrium (except in the case of dithiol **10**, where the disulfide could not be isolated from oxidation of the dithiol). In all cases, these values (eq 7) agreed within 10% of the K_{eq} in the forward direction.

$$K_{eq}^{rev} = \frac{[\text{dithiol}][DTT^{ox}]}{[\text{disulfide}][DTT^{red}]} = \left(\frac{[DTT^{ox}]}{[DTT^{red}]}\right)^2 \quad (7)$$

Calculation of ϵ°. The reduction potential of the dithiols was calculated relative to the standard hydrogen electrode (SHE) by comparison with the known reduction potential of lipoic acid ($\epsilon^\circ_{Lip} = -0.288$ V).[38] Using a value of 21 for K_{eq}(DTT vs Lip^{ox}) (eqs 8 and 9)[39] and the Nernst equation (eq 10), a value of −0.33 V was determined for the reduction potential of DTT (ϵ°_{DTT}) (eq 11). The reduction potentials of the other dithiols were determined relative to DTT using this calculated value and the measured equilibrium constants (eq 12).



(8)

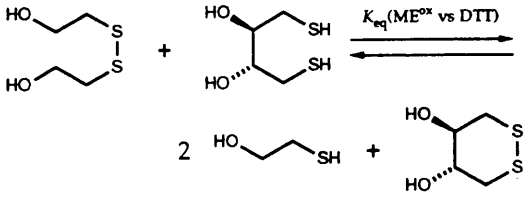
$$K_{eq}(\text{DTT vs } Lip^{ox}) = \frac{[DTT^{ox}][Lip^{red}]}{[DTT^{red}][Lip^{ox}]} = 21 \quad (9)$$

$$\Delta\epsilon^\circ = -\left(\frac{RT}{nF}\right)(\ln K_{eq}) = -\left(\frac{(8.314)(298)}{(2)(9.648 \times 10^4)}\right)(\ln K_{eq}) \quad (10)$$

$$\epsilon^\circ_{DTT} = \epsilon^\circ_{Lip} + \Delta\epsilon^\circ = -0.29\text{ V} + -0.04\text{ V} = -0.33\text{ V} \quad (11)$$

$$\epsilon^\circ_{dithiol} = \Delta\epsilon^\circ_{DTT} + \Delta\epsilon^\circ = -0.33\text{ V} + \Delta\epsilon^\circ \quad (12)$$

Calculation of EC. The equilibrium constant between ME^{ox} and DTT (eqs 13 and 14) is a measure of the effective concentration of DTT, EC(DTT). To convert from the equilibrium constant of a dithiol versus DTT^{ox} to that of ME^{ox}, one needs an accurate value of EC(DTT). A value of ≈200 M has been determined for the equilibrium between DTT and glutathione (a monothiol).[40,41] We have independently determined a value for EC(DTT) of 180 M.[42] The product of EC(DTT) and K_{eq}(dithiol vs DTT^{ox}) provides the EC of the dithiol (eq 15).



(13)

$$K_{eq}(ME^{ox} \text{ vs DTT}) = \frac{[DTT^{ox}][ME^{red}]^2}{[DTT^{red}][ME^{ox}]} = \text{EC(DTT)} \quad (14)$$

$$\text{EC} = K_{eq}(\text{dithiol vs } DTT^{ox})K_{eq}(ME^{ox} \text{ vs DTT}) \quad (15)$$

Determination of pK_a.[43] The absorbance at 238 nm (λ_{max} of a thiolate, RS^-) of the dithiols (100 μL of an 8 mM methanolic solution) in various aqueous buffers (3 mL, 50 mM: 2,2-dimethylsuccinate, pH 6.0, 6.3, 6.7; Tris, pH 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2, 8.4, 8.6, 8.8; glycine, pH 9.0, 9.5, 10.0) was measured in a quartz cell. The observed absorbance was plotted against pH. To find pK_1 and pK_2 values that best fit the data, this experimental curve was then compared with a family of curves derived from the following formula (ϵ is the molar absorption coefficient, C is the total concentration of thiol thiolate species):

$$A = \frac{\epsilon C\{1 + 2(10^{pH-pK_2})\}}{(10^{(pK_1-pH)} + 1 + 10^{(pH-pK_2)})} \quad (16)$$

Determination of Solubilities in D_2O buffer. A saturated solution of a dithiol (or disulfide) was prepared in 0.5 mL of deoxygenated D_2O buffer (pD = 7.50 mM in phosphate, 10 mM of dioxane as internal standard). The solubility of the dithiol (or disulfide) was determined by integration of the ^1H NMR spectrum and by applying an appropriate molar correction.

Bis(2-mercaptoethyl) Sulfone (2). Divinyl sulfone (1.0 mL, 10 mmol) was added dropwise to a solution of thiolacetic acid (1.4 mL, 20 mmol) and Et_3N (2.8 mL, 20 mmol) in anhyd DMF (10 mL) cooled to 0 °C. The resulting orange solution was slowly warmed to room temperature and was stirred for 36 h under Ar. The solvent was removed in vacuo, and the brown residue was filtered through a plug of silica gel (50% ethyl acetate/hexane as eluant). A light-brown solid was isolated. This material was recrystallized from CCl_4 to provide white crystals of the bis(thiolacetate) product (2.35 g, 87%), which was pure by spectroscopic (NMR) analysis: mp 82–83 °C; IR (thin film) 2997, 1679, 1422, 1284, 1268, 1230, 1151, 1109, 929, 514 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 3.27 (m, 8 H, $SCH_2CH_2SO_2$), 2.37 (s, 6 H, $COCH_3$) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 194.8, 52.7, 30.5, 21.8 ppm; TLC R_f = 0.25 (50% ethyl acetate/hexane); MS (Pos. CI with NH_3) calcd for $C_8H_{14}O_4S_3$ m/e 270, found m/e 288 (M + 18). Anal. Calcd for $C_8H_{14}O_4S_3$: C, 35.54; H, 5.22. Found: C, 35.36; H, 5.13.

The bis(thiolacetate) (0.612 g, 2.26 mmol) was dissolved in 1.2 M HCl/MeOH (50 mL), and the clear solution was left at 23 °C for 48 h. The solvent was removed in vacuo to yield a light-yellow solid (0.42 g, 100%) which was pure by spectroscopic (NMR) analysis. An analytical sample was prepared by recrystallization from hexane to provide pure product (0.38 g, 90%) as white, fluffy crystals: mp 57–58 °C; IR (thin film) 2995, 2567, 1306, 1248, 1124, 729, 502 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 3.33 (m, 4 H, $SCH_2CH_2SO_2$), 3.00 (m, 4 H, $SCH_2CH_2SO_2$), 1.80 (t, J = 8.5 Hz, 2 H, SH) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 57.2, 16.8 ppm; MS (Pos. CI with NH_3) calcd for $C_4H_{10}O_2S_3$ m/e 186, found m/e 204 (M + 18). Anal. Calcd for $C_4H_{10}O_2S_3$: C, 25.79; H, 5.41. Found: C, 25.67; H, 5.32.

(38) Massey, V. *Biochem. Biophys. Acta* **1960**, *37*, 314–322.
(39) Lees, W. J.; Whitesides, G. W. Unpublished results.
(40) Chau, M.-H.; Nelson, J. W. *FEBS Lett.* **1991**, *291*, 296–298.
(41) Rothwarf, D. M.; Scheraga, H. A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 7944–7948.
(42) Lees, W. J.; Whitesides, G. W. Unpublished results.
(43) Benesch, R. E.; Benesch, R. *J. Am. Chem. Soc.* **1955**, *77*, 5877–5881.

This dithiol has also been made on a larger scale without chromatography. A solution of divinyl sulfone (17 mL, 0.15 mol) in anhyd DMF (100 mL) was added dropwise to a solution of thiolacetic acid (22 mL, 0.30 mol) and Et_3N (42 mL, 0.30 mol) in anhyd DMF (500 mL) cooled to 0 °C. The resulting orange solution was slowly warmed to room temperature and was stirred for 16 h under Ar. The solvent was removed in vacuo (high vacuum) to yield 47 g of a brown solid. This residue is taken to the next step without purification. The bis(thiolacetate) was dissolved in 1.2 M HCl/MeOH (500 mL), and the orange solution was left at 30 °C for 18 h. The solvent was removed in vacuo to leave a brown solid. This residue was recrystallized from deoxygenated, distilled H_2O (with the addition of decolorizing charcoal to remove impurities) to provide the dithiol **2** (20 g, 72%) as white crystals (mp 57–58 °C) with identical properties as the previous material.

Bis(2-mercaptoethyl) Sulfone Disulfide. Dithiol **2** (0.42 g, 2.26 mmol) was dissolved in DMF (50 mL), and Ellman's reagent (0.90 g, 2.27 mmol) was added. The dark orange solution was left at 23 °C for 48 h and then the solvent was removed in vacuo. The residue was purified by flash chromatography (3 × 16 cm, 50% ethyl acetate/hexane) to yield 0.23 g (56%) of the disulfide product as a white powder. An analytical sample was prepared by recrystallization from hexane to provide pure product as white, fluffy crystals: mp 137–139 °C; IR (thin film) 2980, 2911, 2567, 1395, 1328, 1278, 1197, 1152, 1127, 1108, 902, 854, 827, 689 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 3.63 (m, 4 H, $SCH_2CH_2SO_2$), 3.04 (m, 4 H, $SCH_2CH_2SO_2$) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 56.1, 29.6 ppm; MS (Pos. EI) calcd for $C_4H_8O_2S_3$ *m/e* 184, found *m/e* 184. Anal. Calcd for $C_4H_8O_2S_3$: C, 26.07; H, 4.38. Found: C, 26.13; H, 4.30.

***N,N'*-Dicarbomethoxy-*N,N'*-bis(mercaptomethyl)ethylenediamine (3).** A biphasic mixture of ethylenediamine (6.7 mL, 100 mmol) and K_2CO_3 (27.6 g, 200 mmol) in H_2O (100 mL) and CH_2Cl_2 (100 mL) was cooled to 0 °C. Methyl chloroformate (20 mL, 250 mmol) was added dropwise to the vigorously stirring mixture. The white slurry was slowly warmed to room temperature and stirred for 2 h. The mixture was filtered, and the precipitate was washed with 50 mL of distilled H_2O and 50 mL of CH_2Cl_2. The dicarbomethoxy product (16.6 g) was isolated as a white solid. The filtrate and washings were extracted with additional CH_2Cl_2, and the combined organic layers were dried, filtered, and concentrated. The product (0.20 g, total = 16.8 g, 95%) was isolated as a white powder. Product obtained by both isolation procedures was pure by spectroscopic (NMR) analysis: mp 130–131 °C; IR (thin film) 3338, 2947, 1692, 1667, 1548, 1452, 1324, 1283, 1233, 1151, 1013, 781 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.17 (br s, 2 H, NH), 3.66 (s, 6 H, OCH_3), 3.30 (s, 4 H, NCH_2CH_2N) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 157.5, 52.2, 41.2 ppm; TLC R_f = 0.32 (20% ethyl acetate/hexane). Anal. Calcd for $C_6H_{12}N_2O_4$: C, 40.91; H, 6.87; N, 15.90. Found: C, 41.04; H, 6.80; N, 15.86.

To a solution of the dicarbomethoxy compound (16.6 g, 94 mmol) in distilled H_2O (50 mL) was added K_2CO_3 (1.0 g) and 30% aqueous formaldehyde (20 mL, 250 mmol). The clear solution was heated to 50 °C for 0.5 h. The resulting mixture was concentrated in vacuo and was further dried by addition of toluene and removing the toluene/water azeotrope in vacuo. The residue (containing the bis(hydroxymethyl) compound) was used immediately; toluene (50 mL) was added to the unpurified residue, and to this slurry was added $SOCl_2$ (17 mL, 250 mmol). The clear solution was left at 23 °C for 30 min, and the mixture was concentrated in vacuo. The bis(chloromethyl) compound produced was used immediately; this unpurified residue was dissolved in freshly distilled CH_2Cl_2 (100 mL), and the clear solution was cooled to 0 °C under N_2. Thiolacetic acid (20 mL, 280 mmol) was added, followed by Et_3N (39 mL, 280 mmol). The resulting white slurry was slowly warmed to room temperature and was stirred for 48 h. The reaction was quenched by the addition of saturated aqueous $NaHCO_3$ (50 mL), and the layers were separated. The aqueous layer was washed with additional CH_2Cl_2, and the combined organic layers were dried, filtered, and concentrated. The yellow residue was purified by flash chromatography (7 × 18 cm, 40% ethyl acetate/hexane). A white solid was isolated which was recrystallized from hexane to provide white crystals of the bis((acetylthio)methyl) product (17.0 g, 51.4%) which was pure by spectroscopic (NMR) analysis: mp 56–58 °C; IR (thin film) 2947, 1704, 1697, 1472, 1399, 1235, 1131, 959, 772 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 4.80 (br s, 2 H, NCH_2S), 3.70 (s, 6 H, OCH_3), 3.42 (br s, 4 H, NCH_2CH_2N), 2.34 (s, 6 H, $SCOCH_3$) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 195.6, 195.1, 156.5, 155.9, 53.1, 47.8, 47.3, 46.9, 46.7, 45.6, 45.3, 44.9, 44.6, 30.6, 30.4 ppm; TLC R_f = 0.40 (50% ethyl acetate/hexane); MS (Pos. CI with isobutane) calcd for $C_{12}H_{20}N_2O_6S_2$ *m/e* 352, found *m/e* 353 (M + 1). Anal. Calcd for $C_{12}H_{20}N_2O_6S_2$: C, 40.90; H, 5.72; N, 7.95. Found: C, 40.99; H, 5.67; N, 7.99.

The bis((acetylthio)methyl) compound (176 mg, 0.50 mmol) was dissolved in 1.2 M HCl/MeOH (20 mL) at 0 °C. The clear solution was slowly warmed to 23 °C and was left for 20 h. The solvent was removed in vacuo, and the dithiol **3** (135 mg, 100%) was isolated as a clear oil which was pure by spectroscopic (NMR) analysis: IR (thin film) 2955, 2560, 1701, 1472, 1443, 1401, 1362, 1234, 1133, 1110, 960, 770 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 4.6–4.4 (several m, 2 H, NCH_2S), 3.72 (br s, 6 H, OCH_3), 3.6–3.4 (several m, 4 H, NCH_2CH_2N), 2.4–2.1 (m, 2 H, SH) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 155.6, 53.1, 49.3, 48.9, 48.6, 48.3, 48.0, 47.6, 47.4, 45.2, 44.8, 44.6, 44.5, 44.2, 44.0, 30.6 ppm.

***N,N'*-Dicarbomethoxy-*N,N'*-bis(mercaptomethyl)ethylenediamine Disulfide.** The disulfide was made in two steps from the corresponding bis(thiolacetate). The bis(thiolacetate) (352 mg, 1.0 mmol) was dissolved in 5 mL of 1.2 M HCl/MeOH at 0 °C. The clear solution was slowly warmed to 23 °C, and the mixture was left at room temperature for 20 h. This solution containing the dithiol **3** was added dropwise to a solution of DDQ (454 mg, 2.0 mmol) in dioxane (50 mL) cooled to 0 °C.[44] The yellow solution was slowly warmed to 23 °C, and the mixture was left at room temperature for 16 h. The solvent was removed in vacuo, and the residue was partitioned between 50% ethyl acetate/hexane and saturated aqueous $NaHCO_3$. The layers were separated, and the aqueous layer was washed with additional 50% ethyl acetate/hexane. The combined organic layers were dried, filtered, and concentrated to yield a orange oil. Purification by flash chromatography (3 × 16 cm, 10% ethyl acetate/CH_2Cl_2) provided 157 mg (59%) of the disulfide product as a clear oil, which was pure by spectroscopic (NMR) analysis: IR (thin film) 2953, 1704, 1465, 1436, 1403, 1225, 1100, 981, 763 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 4.5 (br s, 2 H, NCH_2S), 3.77–3.74 (m, 6 H, OCH_3), 3.6 (br s, 4 H, NCH_2CH_2N) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.1, 53.2, 53.1, 53.0, 48.1 ppm; TLC R_f = 0.40 (*tert*-butyl methyl ether); MS (Pos. CI with isobutane) calcd for $C_8H_{14}N_2O_4S_2$ *m/e* 266, found *m/e* 267 (M + 1).

2-Mercaptoethyl Thioglycolate (7). This compound was prepared by the method of Shaked et al.[45] To a solution of methyl thioglycolate (0.84 g, 9.4 mmol) in benzene (30 mL) was added *p*-toluenesulfonic acid (10 mg), followed by mercaptoethanol (0.90 mL, 13 mmol). The mixture was refluxed for 4 days, and the benzene was removed by distillation. The residue was purified by vacuum distillation (80 °C at 100 mTorr) to yield 0.56 g (40%) of dithiol **7** as a clear oil with a strong odor of thiol: IR (thin film) 2948, 2565, 1736, 1456, 1417, 1378, 1302, 1275, 1156, 1028, 979 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 4.22 (t, *J* = 6.6 Hz, 2 H, $OCOCH_2$), 3.25 (d, *J* = 8.4 Hz, 2 H, SCH_2CO), 2.73 (m, 2 H, OCH_2CH_2S), 2.00 (t, *J* = 8.4 Hz, 1 H, $HSCH_2CO$), 1.51 (t, *J* = 8.5 Hz, 2 H, SH) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.4, 65.5, 26.2, 23.0 ppm; HRMS (Pos. CI with NH_3) calcd for $C_4H_8O_2S_2$ *m/e* 151.9966, found *m/e* 170.0315 (M + 18.0349).

***N,N'*-Bis(2-mercaptoacetyl)hexahydropyridazine (8).** In a 250-mL round-bottomed flask were mixed hexahydropyridazine monohydrochloride[46] (1.2 g, 10 mmol) and chloroacetic anhydride (9.4 g, 55 mmol). The flask was connected to a bubbler, and the mixture was heated to 85 °C. The clear melt was stirred vigorously for 0.5 h, and then the flask was cooled to room temperature. The residue was poured onto ice, and CH_2Cl_2 was added to form a biphasic mixture. A 1 M solution of NaOH was added dropwise

(44) Other oxidation procedures gave the seven-membered cyclic sulfide as the major product. DDQ is particularly useful for the oxidation of base-sensitive dithiols or for the formation of strained disulfides. See: Murdock, K. C. *J. Med. Chem.* **1974**, *17*, 827–835.

(45) Shaked, Z.; Szajewski, R. P.; Whitesides, G. M. *Biochemistry* **1980**, *19*, 4156–4166.

(46) Hunter, W. T. U.S. Pat. 2841584; *Chemical Abstracts* **1962**, *56*, 2460f.

until the aqueous layer was neutral. The layers were separated, and the organic layer was dried, filtered, and concentrated to yield 2.6 g of the bis(chloroacetyl)hydrazine as a light-yellow solid. This material was used in the next step without purification, but an analytical sample was prepared by recrystallization from MeOH to provide pure product as white crystals:[47] mp 122–124 °C; IR (thin film) 2953, 2861, 1688, 1448, 1387, 1241, 1190, 1011, 778 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.60 (m, 2 H, NCH), 4.15 (m, 4 H, $COCH_2Cl$), 4.00–2.8 (several m, 2 H, NCH), 1.75 (m, 4 H, NCH_2CH_2) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.1, 45.9, 40.3, 23.0 ppm; MS (Pos. EI) calcd for $C_8H_{12}N_2O_2Cl_2$ *m/e* 238, found *m/e* 238.

To a solution of the bis(chloroacetyl)hydrazine (theoretical = 10 mmol) in CH_2Cl_2 (250 mL) cooled to 0 °C was added thiolacetic acid (1.8 mL, 25 mmol), followed by Et_3N (4.2 mL, 30 mmol). The clear yellow solution was slowly warmed to 23 °C, and the mixture was stirred for 5 h. The reaction was quenched with saturated aqueous $NaHCO_3$, and the layers were separated. The aqueous layer was washed with CH_2Cl_2, and the combined organic layers were dried, filtered, and concentrated to yield a yellow oil. Purification by flash chromatography (3 × 16 cm, 50% ethyl acetate/hexane) yielded 2.3 g (72%) of the bis(thiolacetate) as a clear oil which was pure by spectroscopic (NMR) analysis: IR (thin film) 2928, 1682, 1375, 1135, 1000, 961 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.60 (m, 2 H, NCH), 3.85 (m, 4 H, $COCH_2S$), 3.6–2.8 (several m, 2 H, NCH), 2.39 (four s, 6 H, $COCH_3$), 1.74 (m, 4 H, NCH_2CH_2) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 194.4, 193.8, 170.1, 169.6, 45.3, 39.0, 32.2, 31.6, 30.1, 30.0 ppm; MS (Pos. CI with NH_3) calcd for $C_{12}H_{18}N_2O_4S_2$ *m/e* 318, found *m/e* 336 (M + 18).

The bis(thiolacetate) (0.45 g, 1.4 mmol) was dissolved in 1.2 M HCl/MeOH (50 mL). The clear solution was left at 23 °C for 20 h. The solvent was removed in vacuo to provide the dithiol **8** (0.29 g, 90%) as a clear oil: IR (thin film) 2945, 2555, 1669, 1447, 1387, 1299, 1279, 1188, 1012 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.6–4.0 (several m, 2 H, NCH), 3.45 (m, 4 H, $COCH_2S$), 3.0–2.7 (several m, 2 H, NCH), 2.2–2.0 (m, 2 H, SH), 1.9–1.4 (m, 4 H, NCH_2CH_2) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 45.4, 24.9, 23.2 ppm; HRMS (Pos. EI) calcd for $C_8H_{14}N_2O_2S_2$ *m/e* 234.0497, found *m/e* 234.0499.

***N,N'*-Bis(2-mercaptoacetyl)hexahydropyridazine Disulfide.** Dithiol **8** (0.29 g, 1.2 mmol) was dissolved in a mixture of ethyl acetate (50 mL) and saturated aqueous $NaHCO_3$ (20 mL). The biphasic mixture was cooled to 0 °C, and a 0.1 M solution of I_2 in ethyl acetate was added dropwise to the cold mixture. After the reaction retains a brown color, aqueous $Na_2S_2O_3$ (1 M) was added to remove the excess I_2. The layers were separated, and the aqueous layer was washed with CH_2Cl_2. The combined organic layers were dried, filtered, and concentrated to yield a yellow oil. Purification by flash chromatography (3 × 15 cm, 50% ethyl acetate/hexane) provided the disulfide product (27 mg, 9%) as a white solid which was pure by spectroscopic (NMR) analysis: mp 185–187 °C; IR (thin film) 3003, 2958, 1658, 1447, 1401, 1278, 1264, 1011, 912 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.63 (d, J = 12.8 Hz, 2 H, NCH), 3.8–3.5 (AB quartet, J = 13.2 Hz, 4 H, $COCH_2S$), 2.73 (m, 2 H, NCH), 1.78 (m, 4 H, NCH_2CH_2) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 45.7, 41.4, 23.5 ppm; TLC R_f = 0.20 (30% ethyl acetate/hexane); MS (Pos. EI) calcd for $C_8H_{12}N_2O_2S_2$ *m/e* 232, found *m/e* 232.

***N,N'*-Bis(2-mercaptoacetyl)-*N*-*tert*-butylhydrazine (10).** In a 500-mL round-bottomed flask were mixed *tert*-butylhydrazine monohydrochloride (9.5 g, 76 mmol) and chloroacetic anhydride (72 g, 0.42 mol). The flask was connected to a bubbler, and the mixture was heated to 85 °C. The clear melt was stirred vigorously for 0.5 h, and then the flask was cooled to room temperature. The residue was poured onto ice, and CH_2Cl_2 was added to form a biphasic mixture. A 1 M solution of NaOH was added dropwise until the aqueous layer was neutral. The layers were separated, and the organic layer was dried, filtered, and concentrated to yield 16 g (87%) of the bis(chloroacetyl)hydrazine as a light-yellow solid which was pure by spectroscopic (NMR) analysis. An analytical sample was prepared by recrystallization from *tert*-butyl methyl ether to provide pure product as white crystals: mp 138–140 °C; IR (thin film) 3207, 2979, 1704, 1652, 1532, 1387, 1363, 1236 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.71 (br s, 1 H, NH), 4.15 (s, 2 H, $COCH_2Cl$), 4.00 (AB quartet, J = 12 and 82 Hz, 2 H, $COCH_2Cl$), 1.45 (s, 9 H, $(CH_3)_3C$) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 167.6, 165.6, 62.4, 43.5, 40.9, 27.5 ppm; TLC R_f = 0.50 (*tert*-butyl methyl ether). Anal. Calcd for $C_8H_{18}N_2O_2Cl_2$: C, 39.85; H, 5.85; N, 11.62. Found: C, 39.88; H, 5.80; N, 11.70.

To a solution of the bis(chloroacetyl)hydrazine (16 g, 66 mmol) in CH_2Cl_2 (500 mL) cooled to 0 °C was added thiolacetic acid (15 mL, 0.20 mol), followed by Et_3N (26 mL, 0.19 mol). The clear yellow solution was slowly warmed to 23 °C, and the mixture was stirred for 3 h. The reaction was quenched with saturated aqueous $NaHCO_3$, and the layers were separated. The aqueous layer was washed with CH_2Cl_2, and the combined organic layers were dried, filtered, and concentrated to yield a yellow oil. Purification by flash chromatography (5 × 15 cm, ethyl acetate) yielded 20 g (95%) of the bis(thiolacetate) as a white solid. An analytical sample was prepared by recrystallization from CCl_4 to provide pure product as white crystals: mp 76–77 °C; IR (thin film) 3289, 2982, 2925, 1696, 1654, 1362, 1215, 1133, 961, 624 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.37 (br s, 1 H, NH), 4.00 and 3.35 (AB quartet, J = 15 and 250 Hz, 2 H, $COCH_2Cl$), 3.59 (AB quartet, J = 14.5 and 29 Hz, 2 H, $COCH_2Cl$), 2.46 (s, 3 H, $SCOCH_3$), 2.36 (s, 3 H, $SCOCH_3$), 1.38 (s, 9 H, $(CH_3)_3C$) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 197.0, 195.4, 169.2, 167.7, 62.1, 33.3, 31.1, 30.2, 30.1, 27.5 ppm; MS (Pos. CI with NH_3) calcd for $C_{12}H_{20}N_2O_4S_2$ *m/e* 320, found *m/e* 338 (M + 18). Anal. Calcd for $C_{12}H_{20}N_2O_4S_2$: C, 44.98; H, 6.29; N, 8.74. Found: C, 44.98; H, 6.34; N, 8.65.

The bis(thiolacetate) (0.16 g, 0.50 mmol) was dissolved in 1.2 M HCl/MeOH (50 mL) at 0 °C. The clear solution was slowly warmed to 23 °C, and the mixture was left at room temperature for 20 h. The solvent was removed in vacuo to provide the dithiol **10** as a white solid residue: IR (thin film) 3253, 2981, 2933, 2556, 1693, 1686, 1520, 1364, 1312, 1226, 1203, 1136 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.37 (br s, 1 H, NH), 3.7–3.2 (several m, 4 H, $COCH_2Cl$), 2.1–1.8 (several m, 2 H, SH), 1.44 (s, 9 H, $(CH_3)_3C$) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.6, 168.8, 61.8, 31.4, 29.1, 28.3, 27.6, 27.5, 26.3, 24.7 ppm; MS (Pos. CI with NH_3) calcd for $C_8H_{16}N_2O_2S_2$ *m/e* 236, found *m/e* 254 (M + 18).

***N,N'*-Dicarbethoxy-*N,N'*-bis(mercaptomethyl)hydrazine (11).** To a solution of 1,2-dicarbethoxyhydrazine (17.6 g, 0.1 mol) in 50 mL of distilled H_2O was added 30% aqueous formaldehyde (20 mL, 0.25 mol) and K_2CO_3 (1 g). The mixture was heated at 50 °C for 12 h. At this time, the clear solution was cooled to room temperature and filtered through a plug of silica gel. The silica was washed several times with ethyl acetate, and the filtrate was concentrated to a clear oil. The bis(hydroxymethyl) product (20.2 g, 85%) was pure by spectroscopic (NMR) analysis: IR (thin film) 3428 (br), 2984, 2560, 1724, 1470, 1413, 1383, 1288, 1254, 1032, 733, cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.5–4.5 (several bm, 4 H, NCH_2O), 4.22 (m, 4 H, OCH_2Me), 4.0–3.6 (br s, 2 H, OH), 1.35–1.20 (m, 6 H, OCH_2CH_3) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 155.0, 154.7, 154.3, 87.9, 74.1, 74.0, 73.4, 73.0, 63.2, 63.0, 62.8, 62.7, 62.2, 14.2, 14.1 ppm; TLC R_f = 0.20 (50% ethyl acetate/hexane). Anal. Calcd for $C_8H_{16}N_2O_6$: C, 40.68; H, 6.83; N, 11.86. Found: C, 40.59; H, 6.79; N, 11.96.

To a solution of the bis(hydroxymethyl) compound (3.28 g, 13.9 mmol) in 50 mL of toluene was added $SOCl_2$ (2.5 mL, 35 mmol). After 15 min at 23 °C, the mixture was concentrated under high vacuum. The residue was dissolved in 100 mL of dry CH_2Cl_2, and the clear solution was cooled to 0 °C under an atmosphere of argon. Thiolacetic acid (2.5 mL, 35 mmol) was added, followed by Et_3N (4.9 mL, 35 mmol). The resulting cloudy white slurry was slowly warmed to 23 °C and was stirred for 8 h. The reaction was quenched by the addition of saturated aqueous $NaHCO_3$, and the layers were separated. The aqueous layer was washed with additional CH_2Cl_2, and the combined organic layers were dried, filtered, and concentrated. The yellow oily residue was purified by flash chromatography (5 × 16 cm, 20% ethyl acetate/hexane) to yield bis(thiolacetate) product (2.0 g, 41%) as a clear oil which was pure by spectroscopic (NMR) analysis: IR (thin film) 3000, 1717, 1420, 1376, 1218, 1130, 624 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.1–4.85 (m, 4 H, NCH_2S), 4.15 (s, 4 H, OCH_2Me), 2.34 (s, 6 H, $COCH_3$), 1.3–1.2 (m, 6 H,

(47) *N,N'*-Bis(2-chloroacetyl)hexahydropyridazine has also been prepared by an alternate route. See: Groszkowski, S.; Wrona, J.; Szuflet, W. *Rocz. Chem.* **1973**, *47*, 1551–1553.

OCH_2CH_3) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 194.5, 193.9, 193.5, 154.6, 154.3, 154.0, 153.7, 63.1, 63.0, 62.7, 49.7, 49.0, 48.9, 30.4, 30.3, 30.2, 14.2, 14.1 ppm; TLC R_f = 0.25 (20% ethyl acetate/hexane); MS (Pos. CI with NH_3) calcd for $C_{12}H_{20}O_6N_2S_2$ *m/e* 352, found *m/e* 370 (M + 18). Anal. Calcd for $C_{12}H_{20}N_2O_6S_2$: C, 40.90; H, 5.72; N, 7.95. Found: C, 41.03; H, 5.79; N, 8.09.

To the bis(thiolacetate) (100 mg, 0.284 mmol) was added 5 mL of a 1.2 M solution of HCl/MeOH. The clear solution was left at 23 °C for 20 h, and the solvent was removed in vacuo. The unpurified residue of dithiol 11 (75 mg, 100%) was pure by spectroscopic (NMR) analysis: IR (thin film) 2982, 2562, 1718, 1424, 1380, 1339, 1275, 1228, 1055, 877, 773, 758 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.2–4.9 (m, 4 H, NCH_2S), 4.4–4.1 (m, 4 H, OCH_2Me), 2.65–2.45 (m, 2 H, SH), 1.4–1.2 (m, 6 H, OCH_2CH_3) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 154.1, 63.2, 62.8, 47.6, 47.4, 47.1, 47.0, 14.4 ppm. Anal. Calcd for $C_8H_{16}N_2O_4S_2$: C, 35.81; H, 6.01; N, 10.44. Found: C, 36.41; H, 5.41; N, 10.08.

***N,N'*-Dicarbethoxy-*N,N'*-bis(mercaptomethyl)hydrazine Disulfide.** To the dithiol 11 (74 mg, 0.28 mmol) was added a 10 mM solution of Ellman's reagent in DMF (31 mL, 0.31 mmol). The dark orange solution was left at 23 °C until the solution turned a light yellow (12 h). The solvent was removed in vacuo, and the residue was purified by flash chromatography (1 × 16 cm, 20% ethyl acetate/hexane). The disulfide product (62 mg, 84%) was isolated as a white solid which was pure by spectroscopic (NMR) analysis: mp 54–56 °C; IR (thin film) 2980, 1717, 1416, 1378, 1341, 1247, 1220, 1198, 1043, 1020 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.3–5.0 (m, 2 H, NCH_2S), 4.7–4.35 (m, 2 H, NCH_2S), 4.35–4.15 (m, 4 H, OCH_2Me), 1.4–1.2 (m, 6 H, OCH_2CH_3) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 153.7, 63.4, 63.2, 52.0, 51.4, 50.1, 49.6, 14.5 ppm; TLC R_f = 0.32 (20% ethyl acetate/hexane). Anal. Calcd for $C_8H_{14}N_2O_4S_2$: C, 36.08; H, 5.30; N, 10.52. Found: C, 36.12; H, 5.34; N, 10.48.

1,2-Bis(mercaptomethyl)-4-methylurazole (12). To 4-methylurazole (5.2 g, 45 mmol) in 5 mL of distilled H_2O was added 30% aqueous formaldehyde (8.0 mL, 0.10 mol) and K_2CO_3 (0.1 g). The mixture was heated to 50 °C for 1 h. The clear solution was cooled to 0 °C, and a white precipitate formed. The mixture was filtered, and the filtrate was extracted with ethyl acetate. The organic layers were dried, filtered, and concentrated, and the residue was combined with the precipitate isolated from the aqueous layer. The bis(hydroxymethyl) product was isolated as a white solid (6.4 g, 80%), which was pure by spectroscopic (NMR) analysis: mp 142–144 °C; IR (thin film) 3282, 1695, 1687, 1490, 1457, 1396, 1043, 758, 669, 630 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.14 (d, *J* = 8.0 Hz, 4 H, NCH_2O), 3.11 (s, 3 H, NCH_3), 3.08 (t, *J* = 7.9 Hz, 2 H, OH) ppm; ^{13}C NMR (CD_3OD, 100 MHz) δ 156.8, 68.8, 25.5 ppm; TLC R_f = 0.30 (10% MeOH/ethyl acetate). Anal. Calcd for $C_5H_9N_3O_4$: C, 34.29; H, 5.18; N, 23.99. Found: C, 34.26; H, 5.38; N, 23.91.

To a slurry of the bis(hydroxymethyl) compound (5.3, 0.03 mmol) in toluene (50 mL) was added $SOCl_2$ (5.5 mL, 0.08 mmol). The clear solution was left at 23 °C for 35 min, and then the mixture was concentrated in vacuo. The resulting white solid (containing the bis(chloromethyl) compound) was used immediately; this unpurified residue was dissolved in freshly distilled CH_2Cl_2 (100 mL), and the clear solution was cooled to 0 °C under N_2. Thiolacetic acid (4.8 mL, 66 mmol) was added, followed by Et_3N (9.5 mL, 67 mmol). The resulting white slurry was slowly warmed to room temperature and was stirred for 48 h. The reaction was quenched by the addition of saturated aqueous $NaHCO_3$ (50 mL), and the layers were separated. The aqueous layer was washed with additional CH_2Cl_2, and the combined organic layers were dried, filtered, and concentrated. The residue was purified by flash chromatography (5 × 18 cm, 30% ethyl acetate/hexane). A white solid was isolated which was recrystallized from ethyl acetate to provide the bis(thiolacetate) product (6.8 g, 78%), which was pure by spectroscopic (NMR) analysis: mp 132–134 °C; IR (thin film) 3000, 1786, 1721, 1702, 1468, 1398, 1227, 1129, 952, 771, 622, 590 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.15 (s, 4 H, NCH_2S), 3.05 (s, 3 H, NCH_3), 2.39 (s, 6 H, $COCH_3$) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 193.8, 155.5, 44.4, 30.7, 25.7 ppm; TLC R_f = 0.20 (30% ethyl acetate/hexane). Anal. Calcd for $C_9H_{13}N_3O_4S_2$: C, 37.10; H, 4.50; N, 14.42. Found: C, 37.13; H, 4.56; N, 14.46.

The bis(thiolacetate) compound (57 mg, 0.20 mmol) was dissolved in 1.2 M HCl/MeOH (10 mL), and the clear solution was left at 23 °C for 20 h. The solvent was removed in vacuo, and the dithiol 12 (41 mg, 100%) was isolated as a white solid which was pure by spectroscopic (NMR) analysis: mp 78–79 °C; IR (thin film) 3019, 2954, 2554, 2361, 1777, 1710, 1471, 1398, 1284, 1225, 1059, 764, 689, 585 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 4.61 (d, *J* = 8.7 Hz, 4 H, NCH_2S), 3.08 (s, 3 H, NCH_3), 2.24 (t, *J* = 8.7 Hz, 2 H, SH) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 155.2, 41.9, 25.7 ppm. Anal. Calcd for $C_5H_9N_3O_2S_2$: C, 28.98; H, 4.38; N, 20.27. Found: C, 29.02; H, 4.32; N, 20.29.

1,2-Bis(mercaptomethyl)-4-methylurazole Disulfide. Dithiol 12 (41 mg, 0.20 mmol) was dissolved in DMF (10 mL), and Ellman's reagent (80 mg, 0.20 mmol) was added. The dark orange solution was left at 23 °C for 20 h (until solution turns clear). Silica gel (100 mg) was added, and the solvent was removed in vacuo. The resulting free-flowing powder was placed on top of a flash column (1 × 17 cm, CH_2Cl_2 then 5% ethyl acetate/CH_2Cl_2). The disulfide product (24 mg, 58%) was isolated as a white solid which was pure by spectroscopic (NMR) analysis: mp 140–142 °C; IR (thin film) 2998, 1772, 1704, 1472, 1403, 1278, 1229, 1051, 898, 786, 736 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 4.76 (s, 4 H, NCH_2S), 3.09 (s, 3 H, NCH_3) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 153.0, 51.3, 25.2 ppm; TLC R_f = 0.20 (30% ethyl acetate/hexane); MS (Pos. EI) calcd for $C_5H_7N_3O_2S_2$ *m/e* 205, found *m/e* 205. Anal. Calcd for $C_5H_7N_3O_2S_2$: C, 29.26; H, 3.44; N, 20.47. Found: C, 29.32; H, 3.53; N, 20.43.

Acknowledgment. This research was supported by National Institute of Health Grant GM 30367 and in part by the Office of Naval Research and the Defense Advanced Research Projects Agency. NMR instrumentation was supported by National Science Foundation Grant CHE-84-10774. Mass spectra were obtained by Dr. A. Tyler. The Harvard University Chemistry Department Mass Spectrometry Facility was supported by National Science Foundation Grant CHE-90-20043 and National Institute of Health Grant 1 S10 RR06716-01. We wish to thank Dr. R. Singh and W. J. Lees for helpful discussions and advice.

Supplementary Material Available: Experimental details for the preparation of compounds not included in Table I (11 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.