31.5, 27.5, 25.1, 22.6, 22.5, 21.2, 13.9; IR 2933, 2867, 1764, 1684, 1452, 1245, 1184, 1025 $cm^{-1}$; exact mass calcd for $C_{12}H_{18}O_2$ $(M)^+$ 194.1305, found 194.1322.

**Acknowledgment.** We express our graditude to Dr. Martha Bruch for the 2D NMR spectra and help with their assignments. We would like to thank the donors of the Petroleum Research Fund, administered by the American Chemical Society, and the Univeristy of Delaware Research Foundation for support of this research.

**Supplementary Material Available:** $^1H$ and $^{13}C$ NMR spectra for all compounds (55 pages). Ordering information is given on any current masthead page.

# A Reagent for Reduction of Disulfide Bonds in Proteins That Reduces Disulfide Bonds Faster Than Does Dithiothreitol

Rajeeva Singh and George M. Whitesides*

*Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138*

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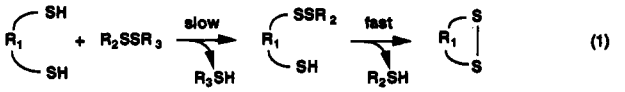
We have synthesized a new reagent—*N,N'*-dimethyl-*N,N'*-bis(mercaptoacetyl)hydrazine (DMH)—for the reduction of disulfide bonds in proteins. DMH reduces disulfide bonds 7 times faster than does dithiothreitol (DTT) in water at pH 7. DMH reduces mixed disulfides of cysteine proteases (papain and ficin) especially rapdily (30 times faster than DTT). DMH ($\epsilon^\circ$ = -0.300 V) reduces noncyclic disulfides completely, although it is less strongly reducing than DTT ($\epsilon^\circ$ = -0.356 V).

## Introduction

This report describes the synthesis of *N,N'*-dimethyl-*N,N'*-bis(mercaptoacetyl)hydrazine (DMH) and the use of this reagent for reduction of disulfide bonds in water at pH ~7. Disulfide-reducing reagents are used in biochemistry for a number of purposes, especially in reduction of cystine groups in proteins and in maintaining essential thiol groups in reduced state.[1-3] The requirements for an optimal reducing reagent for cystine groups in proteins are as follows: (i) a reduction potential higher than the cystine group(s) to be reduced; (ii) a $pK_a$ for thiol groups close to the pH of the solution in which the protein is to be manipulated; (iii) convenient physical properties (a crystalline solid with low odor and adequate solubility in water).[4,5]

Thiol-disulfide interchange involves the nucleophilic attack of thiolate anion along the S-S bond axis of the disulfide.[5-9] The $pK_a$ of simple alkanethiols in water is ~9-10; for these thiols, only a small fraction (1-0.1%) is present as thiolate at pH 7. The apparent rate of thiol-disulfide interchange is maximum when the $pK_a$ of the thiol is close to the pH of the solution.[5,6] $\alpha,\omega$-Dithiols are more strongly reducing than monothiols, because the intramolecular reaction (the second step in eq 1) is faster




(1)

than the corresponding intermolecular reaction for monothiols. Among $\alpha,\omega$-dithiols, the dithiols that form cyclic six-membered disulfide are the most reducing, reflecting a balance between the thermodynamic stability of the CSSC dihedral angle and the entropy of formation of the ring from the acyclic reduced form.[6,10,11]

Dithiothreitol (DTT)[4] and $\beta$-mercaptoethanol (ME) are the favorite disulfide-reducing reagents in biochemistry.[1] DTT reduces noncyclic disulfides to thiols completely, and intermediate mixed disulfides are absent in any significant concentration (eq 1).[4] DTT has, however, some shortcomings: the value of its first thiol $pK_a$ is 9.2,[6] and it is therefore relatively slow as a reducing reagent at pH ~7. It is expensive.[12] Its ability to chelate metals and generate $H_2O_2$ on exposure to air can cause problems.[13] Because mercaptoethanol is inexpensive, it can be used in large amounts (0.1-0.7 M) in biochemical manipulations (for example, in conjunction with SDS gel electrophoresis).[1] The $pK_a$ of mercaptoethanol is 9.6.[6] The disadvantages of mercaptoethanol are that it is a weak reducing reagent and is foul-smelling. Because it is weakly reducing, it often generates complex reaction mixtures containing mixed disulfides.[6]

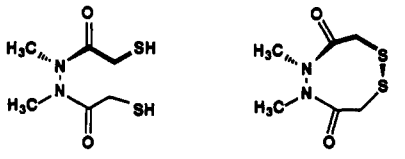




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**Table I. Physical Characteristics of DMH, DTT, and ME**

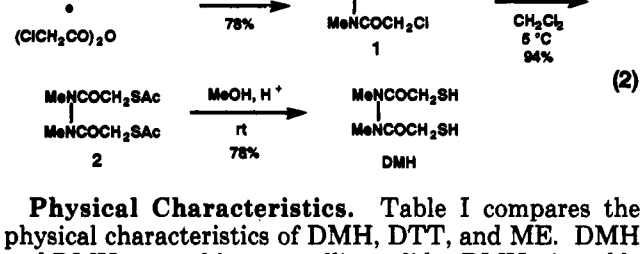
| | DMH | | DTT | | ME | |
|---|---|---|---|---|---|---|
| | red | ox | red | ox | red | ox |
| mp (°C) | 38 | 155 | 42 | 132 | | |
| $pK_a$ | 7.6, 8.9 | | 9.2, 10.1 | | 9.6 | |
| $\epsilon^o$ (V) | −0.300 | | −0.356 | | −0.209 | |
| $K^a$ | 1200 M | | $10^5$ M | | 1 | |
| $k^{app\,b}$ ($M^{-1}$ $s^{-1}$) | 67 | | 10 | | | |
| odor | weak | no | weak | no | strong | no |
| solubility[c] (mM) | 250 | 23 | | | | |

[a] The values of the equilibrium constant ($K$) are for the reduction of $ME^{ox}$ by DMH and by DTT (see Table II). [b] The apparent rate constants ($k^{app}$) are for the reduction of the mixed disulfide of creatine phosphokinase and glutathione (see Table III). [c] The solubilities were determined in $D_2O$ buffer (pD 7, 50 mM in phosphate, 25 °C). ME and DTT are very soluble in water.

We designed the new reagent DMH for use in reduction of S–S bonds in water at pH ~7 with the following expectations: (i) The first $pK_a$ of its thiol groups would be ~7, and the rate at which the thiol would act as a nucleophile in thiol–disulfide interchange would be rapid. (ii) The steric bulk of the methyl groups on adjacent nitrogen atoms would force the two amide moieties into approximately perpendicular planes and thereby allow the formation of cyclic eight-membered disulfide having a thermodynamically stable CSSC dihedral angle of ~90°. (iii) The rigidity of the two sterically constrained amide units would limit the degrees of rotational freedom that would have to be frozen on going from dithiol to cyclic disulfide, and thus give a high reduction potential. We also anticipated (although we have not so far demonstrated experimentally) that the substituents at nitrogen could be changed to make the reagent more water soluble and to prepare chiral or polymer-linked reagents.

## Results and Discussion

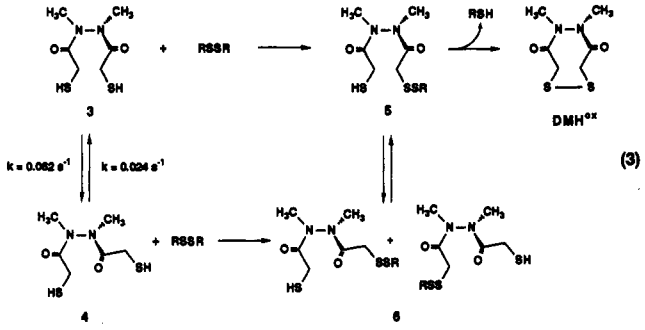
**Synthesis.** The synthesis of DMH from *N,N'*-dimethylhydrazine dihydrochloride is straightforward (eq 2). The steps involve commercially available reagents, mild reaction conditions, easy separations, and good yields. The most expensive reagent in the synthesis is *N,N'*-dimethylhydrazine dihydrochloride.[14]




(2)

**Physical Characteristics.** Table I compares the physical characteristics of DMH, DTT, and ME. DMH and $DMH^{ox}$ are white, crystalline solids. $DMH^{ox}$ is stable to heat and does not polymerize on heating at 155 °C for 20 min, in contrast to strained disulfides which polymerize easily on heating and some of which are polymeric at room temperature.[15] $DMH^{ox}$ shows no UV absorption in 320–400-nm region, in contrast to the strained cyclic five-membered disulfides ($\lambda_{max}$ = 330 nm).[16] We infer that $DMH^{ox}$ is relatively unstrained. The aqueous solubility for $DMH^{ox}$ is lower than for $DTT^{ox}$ and for $ME^{ox}$; the low solubility of $DMH^{ox}$ may be useful for its convenient extraction from the reaction mixture of a water-soluble disulfide and DMH.

We infer that DMH exists in two conformations from its $^1H$ NMR spectrum. The assignments of the two conformations—symmetrical (**3**, eq 3) and unsymmetrical (**4**, eq 3)—are described in the Experimental Section. In




(3)

the reduction of disulfides (RSSR) by DMH, the intermediate (mixed disulfide of DMH and RSH) can cyclize directly to form $DMH^{ox}$ for the symmetrical conformation (**5**) only. The intermediate mixed disulfide from the unsymmetrical conformation (**6**) must isomerize to the symmetrical form (**5**) before it can cyclize to form $DMH^{ox}$. Using dynamic NMR, we have calculated that the value of the intramolecular rate constant for the interchange of the unsymmetrical to symmetrical form at 25 °C is 0.024 $s^{-1}$ (see the Experimental Section). At 10 mM concentration of RSSR, the pseudounimolecular intermolecular rate constant for the conversion of **4** to **6** is calculated to be ~0.005 $s^{-1}$.[17] The rate-determining step in the reduction of disulfides by DMH in dilute solutions is therefore the intermolecular thiol–disulfide interchange, and not the intramolecular isomerization of the unsymmetrical mixed disulfide (**6**) into the symmetrical form (**5**).

**Equilibrium Constants for Thiol–Disulfide Interchange of DMH with Cyclic and Noncyclic Disulfides.** Table II lists the equilibrium constants for thiol–disulfide interchange reactions between DMH and several disulfides. DMH reduces noncyclic disulfides completely; DMH oxidizes completely to $DMH^{ox}$ on reaction with excess dithiodiglycolic acid, and no mixed disulfide is seen (Figure 1). The reduction potential of DMH is comparable to that for dihydrolipoic acid, although it is less than that for dithiothreitol. DMH is more reducing than 1,6-hexanedithiol probably because fewer degrees of rotational freedom are lost on going from the dithiol to the cyclic disulfide.

**Kinetics of Reduction of Disulfides by DMH.** DMH reduces disulfides faster than does DTT in water at pH 7 (Table III). The rate of reduction of $ME^{ox}$ by DMH is 7-fold higher than that by DTT. The factor of 7 is approximately that expected from the Brønsted equation because the $pK_a$ of thiol in DMH is lower than that in DTT.[18] As model protein disulfides, we prepared mixed disulfides of enzymes that are active in thiol form.[19] We

(14) The cost of 1,2-dimethylhydrazine dihydrochloride is $4/g (FW 133).

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(17) The apparent second-order rate constant for the reaction of DMH and $ME^{ox}$ in $D_2O$ at pD 7 is 0.5 $M^{-1}$ $s^{-1}$. Assuming that the concentration of the disulfide ($ME^{ox}$) is 0.01 M, the pseudounimolecular rate constant for the conversion of **4** to **6** is calculated to be 0.005 $s^{-1}$ (=0.5 $M^{-1}$ $s^{-1}$ × 0.01 M); for a smaller concentration (<0.01 M) of the disulfide, the rate constant would be smaller than 0.005 $s^{-1}$.

(18) Using the Brønsted equation $\log k = 7.0 + 0.5pK_a^{nuc} - 0.27pK_a^c - 0.73pK_a^{lg}$ and the equation $k = k^{app}(1 + 10^{pK_a - pH})$,[6] we derive $\log k^{app}_{DMH} - \log k^{app}_{DTT} = 0.5(pK_a^{DMH} - pK_a^{DTT}) - \log(1 + 10^{pK_a^{DMH} - pH}) + \log(1 + 10^{pK_a^{DTT} - pH})$. Using $pK_a^{DMH} = 7.6$, $pK_a^{DTT} = 9.2$, and pH = 7.0, we get $k^{app}_{DMH}/k^{app}_{DTT} = 5.1$.

**Table II. Equilibrium Constants of Thiol–Disulfide Interchange of DMH with Cyclic and Noncyclic Disulfides in Water at pH 7**

| disulfide | ring size | $K_{eq}$ | $\epsilon^\circ$ (V)[a] |
|---|---|---|---|
| bis(2-hydroxyethyl) disulfide ($ME^{ox}$) | | 1200 M[b] | −0.209 |
| 1,2-dithiocane | 8 | 10[c] | −0.269 |
| lipoic acid | 5 | 0.18[c] | −0.320 |
| *trans*-4,5-dihydroxy-1,2-dithiane ($DTT^{ox}$) | 6 | 0.014[c] | −0.356 |

[a] All values of $\epsilon^\circ$ are relative to that of mercaptoethanol (−0.209 V) as reference.[10] [b] $K_{eq}$ = ([RSH]$^2$[$DMH^{ox}$])/([RSSR][DMH]). This value of $K_{eq}$ is estimated from $\epsilon^\circ$ for DMH = −0.300 V. [c] $K_{eq}$ = ([HSRSH][$DMH^{ox}$])/([$\overline{SRS}$][DMH]). All values of $K_{eq}$ were determined by $^1$H NMR spectroscopy. The $K_{eq}$ for lipoic acid was also determined by UV spectroscopy. These values of $K_{eq}$ are unitless. 1,2-Dithiocane is $\overline{S(CH_2)_6S}$.

**Table III. Comparison of Rate Constants for Reduction of Disulfides by DMH and DTT in Water at pH 7**

| disulfide | $k^{app}$ ($M^{-1}$ $s^{-1}$)[a,b] DMH | DTT | $k_{DMH}/k_{DTT}$ |
|---|---|---|---|
| $HOCH_2CH_2$-S-S-$CH_2CH_2OH$ ($ME^{ox}$) | 0.52 | 0.075 | 7 |
| creatine kinase-S-S-glutathione | 67 | 10 | 7 |
| papain-S-S-$CH_3$ | 1400 | 44 | 30 |
| ficin-S-S-$CH_3$ | 690 | 21 | 30 |
| deoxyribonuclease I | 1.3 | 0.29 | 4.5 |

[a] The rate constants are for 25 °C. [b] The error in apparent rate constant ($k^{app}$) is ±10%; the values are an average of two experiments.

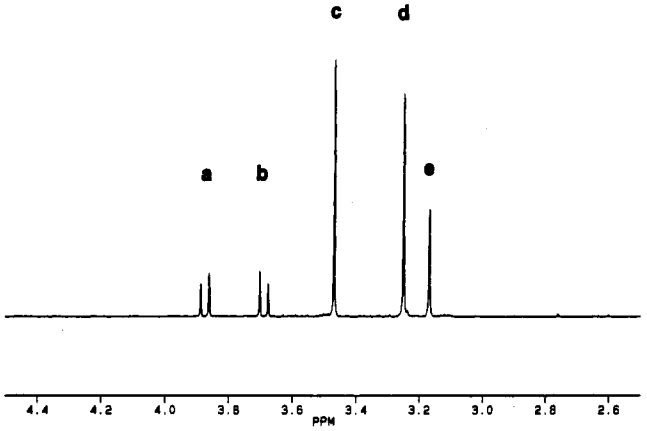


**Figure 1.** $^1$H NMR spectrum (500 MHz) of a mixture initially containing DMH (8.5 mM) and excess dithiodiglycolic acid ($^-OOCCH_2SSCH_2COO^-$, 20 mM) in $D_2O$ buffer at pD 7. Peak assignments: (a, b) $CH_2$ ($DMH^{ox}$); (d) $CH_3$ ($DMH^{ox}$); (c) $CH_2$ ($^-OOCCH_2SSCH_2COO^-$); (e) $CH_2$ ($^-OOCCH_2SH$).

studied the rates of reduction of the mixed disulfides by DMH and by DTT from the regeneration of the enzymatic activity.[7] The rate of regeneration of the active thiol group of creatine kinase (from creatine kinase-S–S-glutathione) by DMH is also faster by a factor of 7 than that by DTT (Figure 2). DMH regenerates the active thiol groups of papain and ficin (by reduction of papain-S–S-$CH_3$ and ficin-S–S-$CH_3$) faster than does DTT by a factor of 30 (Figure 2). The rate of reduction of papain-S–S-$CH_3$ and ficin-S–S-$CH_3$ by DMH is anomalously high relative to that by DTT and may result from the higher affinity of DMH to the enzymes.

We studied the loss of activity of deoxyribonuclease I (DNaseI) by cleavage of its native disulfide bonds.[7,20,21]

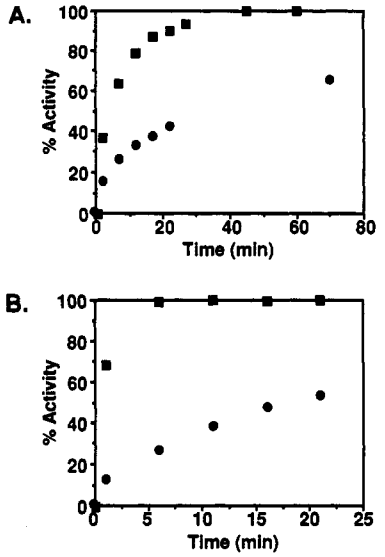


**Figure 2.** Regeneration of essential thiol groups in enzymes containing mixed disulfides: (A) reduction of creatine kinase-S–S-glutathione by DMH (■) and by DTT (●); (B) reduction of ficin-S–S-$CH_3$ by DMH (■) and by DTT (●).

DMH inactivates DNaseI 4.5 times faster than does DTT. The cleavage of the disulfide bond may not be the only rate-determining step in the inactivation of DNaseI, because the conformational changes after the cleavage of the disulfide bond may also contribute to the overall rate of loss of activity.

## Conclusions

DMH reduces disulfide bonds in water at pH 7 faster than does DTT. Although DMH is a weaker reducing agent than is DTT, it reduces noncyclic disulfides completely. Like DTT it does not give mixed disulfides. The synthesis of DMH is straightforward from *N,N'*-dimethylhydrazine dihydrochloride. The most expensive reagent in the synthesis of DMH is *N,N'*-dimethylhydrazine. The availability of a cheaper and improved synthesis of *N,N'*-dialkylhydrazine would reduce significantly the overall cost of DMH.[22]

DMH would be useful in biochemical manipulations at pH ≤7 when the rate of reduction is important: Its rate of reaction is approximately 7 times faster than DTT and mercaptoethanol. DMH may be especially useful in regeneration of activities of cysteine proteases: The thiol groups of papain and ficin are regenerated from mixed disulfides 30 times faster by DMH than by DTT. DMH is less reducing than DTT and may not completely reduce some cyclic disulfides. The low solubility of $DMH^{ox}$ may be useful for convenient extraction with ethyl acetate in the reduction of ionic nonprotein disulfides.

## Experimental Section

**General Procedures.** *N,N'*-Dimethylhydrazine dihydrochloride, dithiothreitol, *trans*-4,5-dihydroxy-1,2-dithiane ($DTT^{ox}$), lipoic acid, methylmethanethiosulfonate, and hexane-1,6-dithiol

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(21) Lysozyme did not lose any activity over 3 h on incubation with 10 mM DMH or DTT.

(22) The traditional synthesis of *N,N'*-dimethylhydrazine involves benzoylation of hydrazine, followed by methylation and acidic hydrolysis: Hatt, H. H. *Organic Syntheses*; Wiley: New York, 1943; Collect. Vol. 2 pp 208–211. A more recent and possibly cheaper synthesis is by electrochemical reduction of nitromethane: Iversen, P. E. *Chem. Ber.* **1971**, *104*, 2195–2198.

were purchased from Aldrich. Chloroacetic anhydride and thiolacetic acid were purchased from Fluka. Papain (EC 3.4.4.10, papaya latex) was purchased from Boehringer Mannheim. Creatine kinase (EC 2.7.3.2, rabbit muscle), ficin (EC 3.4.4.12, fig latex), deoxyribonuclease I (EC 3.1.21.1, bovine pancreas), DNA (calf thymus, highly polymerized), and *N*-benzoyl-L-arginyl-*p*-nitroanilide were purchased from Sigma.

***N,N'*-Dimethyl-*N,N'*-bis(chloroacetyl)hydrazine (1).**[23] To a 500-mL round-bottomed flask equipped with a stir bar were added, *N,N'*-dimethylhydrazine dihydrochloride (10.15 g, 76.3 mmol) and chloroacetic anhydride (71.8 g, 420 mmol). The flask was stoppered with a rubber septum, and a needle connected to a bubbler was pierced through the septum. *Caution: N,N'-Dimethylhydrazine dihydrochloride is a carcinogen, and this reaction should be done in a well-ventillated hood.* The flask was heated to 85 °C in 20 min, and the flask was kept at 85 °C for 20 min. The molten reaction mixture became clear. The solution was cooled to room temperature and poured dropwise into water (1.2 L) in an ice bath. Methylene chloride (400 mL) was added to the aqueous suspension. Sodium hydroxide (40% aqueous solution) was added dropwise to the water/methylene chloride suspension in an ice bath with stirring until the pH of the aqueous layer was 7. The methylene chloride layer was separated, washed with brine solution (100 mL), dried ($MgSO_4$), and concentrated at reduced pressure to yield a white solid (14.0 g, 86%). The crude white solid was recrystallized with ethyl acetate to yield a white crystalline solid (12.7 g, 78%): mp 117–118 °C; $^1H$ NMR ($CDCl_3$) (two conformers were seen in 1.4:1 ratio) $\delta$ 4.11 (s, 4 H, $CH_2Cl$), 3.24 (s, 6 H, $CH_3N$) (major conformation), 4.02 (m, $CH_2Cl$), 3.43 (s, $CH_3N$), 3.13 (s, $CH_3N$) (minor conformation). Anal. Calcd for $C_6H_{10}Cl_2N_2O_2$: C, 33.82; H, 4.73; N, 13.15. Found: C, 34.02; H, 4.77; N, 13.15.

***N,N'*-Dimethyl-*N,N'*-bis[(acetylthio)acetyl]hydrazine (2).** To a solution of *N,N'*-dimethyl-*N,N'*-bis(chloroacetyl)hydrazine (1.30 g, 6.10 mmol) in methylene chloride (100 mL) in an ice bath was added thiolacetic acid (1.30 mL, 18.2 mmol) followed by triethylamine (2.40 mL, 17.3 mmol). The solution was stirred in ice bath for 30 min, washed with water (100 mL), with cold 10% aqueous $NaHCO_3$ solution, and finally with brine solution (100 mL), dried ($MgSO_4$), and concentrated at reduced pressure to yield a pale viscous liquid (2.06 g). The crude oil was purified by flash chromatography on a silica gel column with ethyl acetate to yield a pale liquid ($R_f$ 0.48) that solidified at room temperature (1.68 g, 94%): mp 74 °C; $^1H$ NMR ($CDCl_3$, 400 MHz) (two conformers were seen in 2.6:1 ratio) $\delta$ 3.95, 3.91, 3.74, 3.70 (AB quartet, $J$ = 16.3 Hz, 4 H, $CH_2$), 3.26 (s, 6 H, $CH_3N$), 2.39 (s, 6 H, $CH_3CO$) (major conformer); 3.47 (s, $CH_3N$), 3.09 (s, $CH_3N$), 2.42 (s, $CH_3CO$), 2.36 (s, $CH_3CO$) (minor conformer). Anal. Calcd for $C_{10}H_{16}N_2O_4S_2$: C, 41.08; H, 5.52; N, 9.58. Found: C, 41.30; H, 5.54; N, 9.47.

***N,N'*-Dimethyl-*N,N'*-bis(mercaptoacetyl)hydrazine (DMH).** To a solution of *N,N'*-dimethyl-*N,N'*-bis[(acetylthio)-acetyl]hydrazine (1.00 g, 3.42 mmol) in methanol (20 mL) was added concentrated HCl (0.2 mL of a 37 wt % aqueous solution), and the solution was stirred at room temperature for 80 h. The reaction mixture was concentrated at reduced pressure and in vacuo to yield a colorless liquid (0.73 g). For further purification, the crude oil was dissolved in methanol (6 mL), and the solution was cooled in a dry ice/acetone bath; the supernatant was decanted, and the white precipitate was dried in vacuo (0.56 g, 78%): mp 37–38 °C. Anal. Calcd for $C_6H_{12}N_2O_2S_2$: C, 34.60; H, 5.81; N, 13.45. Found: C, 34.15; H, 5.98; N, 13.28.

**DMH$^{ox}$.** To a solution of crude *N,N'*-dimethyl-*N,N'*-bis-(mercaptoacetyl)hydrazine (1.08 g, 5.2 mmol) in ethyl acetate (150 mL) in an ice bath was added cold $KHCO_3$ solution (50 mL of a 10% aqueous solution). A solution of iodine (1.32 g, 5.2 mmol) in ethyl acetate (50 mL) was added dropwise with stirring until the reaction mixture turned brown. An aqueous solution of sodium thiosulfate was added to quench the excess iodine. The ethyl acetate layer was separated, and the aqueous layer was extracted once more with ethyl acetate (50 mL). The combined ethyl acetate extract was dried ($Na_2SO_4$) and concentrated at reduced pressure to yield a white crystalline solid (0.89 g, 83%). The crude product was recrystallized with ethyl acetate: mp 155 °C; $^1H$ NMR ($CD_3CN$, 300 MHz) $\delta$ 3.76, 3.72, 3.54, 3.50 (AB quartet, $J$ = 12.8 Hz, 4 H), 3.12 (s, 6 H). Anal. Calcd for $C_6H_{10}N_2O_2S_2$: C, 34.94; H, 4.89; N, 13.58. Found: C, 34.83; H, 4.81; N, 13.41.

**Papain-S–S-$CH_3$.** Papain-S–S-$CH_3$ was prepared by a modification of the reported procedure of Shaked et al.[7] Methylmethanethiosulfonate (5.0 $\mu$L, $4.9 \times 10^{-5}$ mol) was dissolved in 15 mL of aqueous buffer (0.1 M in phosphate, 2 mM in EDTA, pH 7) to prepare a stock solution (3.2 mM). To 1.5 mL of the stock solution of methylmethanethiosulfonate was added KCl (0.011 g, 0.15 mmol); the solution was deoxygenated by bubbling argon through it for 15 min, and papain (0.5 mL of a suspension purchased from Boehringer Mannheim, ~5 mg of protein, MW 21 000, ~150 U) was added. The solution was kept at room temperature for 12 h under argon. The excess methylmethanethiosulfonate was removed by size-exclusion chromatography on a Sephadex G-25 column with aqueous buffer (0.1 M in phosphate, 0.1 M in KCl, 0.5 mM in EDTA, pH 7.0). The concentration of Papain-S–S-$CH_3$ in the chromatographed fraction was $2 \times 10^{-5}$ M using $\epsilon^{1\%}_{278} = 25$.

**Ficin-S–S-$CH_3$.** From a solution of methylmethanethiosulfonate (4 $\mu$L, 39 $\mu$mol) in 3.5 mL of deoxygenated aqueous buffer (pH 7, 0.1 M in phosphate, 2 mM in EDTA), a 1.75-mL aliquot was added to ficin solution (0.25 mL of a suspension in 2 M NaCl, 0.03 M in cysteine, pH 5, ~6 mg of protein, MW 26 000), and the mixture was stirred under argon for 2 h. The solution was concentrated and was washed with aqueous buffer ($8 \times 0.5$ mL, pH 7, 0.1 M in phosphate, 2 mM in EDTA) by centrifugation over a Centricon microconcentrator (Amicon, MW cutoff 10 000) to remove excess methylmethanethiosulfonate. The final volume of ficin-S–S-$CH_3$ solution was 0.35 mL.

**Creatine Kinase-S–S-Glutathione.**[24] In a Centricon microconcentrator (Amicon, MW cutoff 10 000) was taken creatine kinase (1 mg of lyophilized powder from rabbit muscle, MW 81 000) in 1 mL of aqueous buffer (pH 8, 0.1 M in Tris, 2 mM in EDTA), and oxidized glutathione (0.0092 g, 15 $\mu$mol) was added. The solution was ~12 $\mu$M in creatine kinase and ~15 mM in oxidized glutathione. The solution was kept at room temperature for 2 h, centrifuged, and washed with aqueous buffer ($5 \times 0.5$ mL, pH 8, 0.1 M in Tris, 2 mM in EDTA) in order to remove excess oxidized glutathione.

**General Methods for Deoxygenation of Aqueous Reaction Mixtures in Kinetic and Equilibrium Studies.** All flasks and NMR tubes were stoppered with rubber septa and were purged with argon. All transfers were done with use of gas-tight syringes. The $D_2O$ buffer (pD 7.0, 50 mM in phosphate),[25] or DMSO-$d_6$, was deoxygenated by bubbling argon through it for 1 h.

**Kinetics of Reduction of Papain-S–S-$CH_3$ (or Ficin-S–S-$CH_3$) by DMH and by DTT.** Papain-S–S-$CH_3$ (0.26 mL of the chromatographed fraction) was diluted with 2.34 mL of deoxygenated aqueous buffer (pH 7, 0.1 M in imidazole, 2 mM in EDTA), and the enzyme solution was added to two flasks (1.2 mL in each). DTT or DMH (6 $\mu$L of a 5 mM solution) was added to the flask containing the enzyme solution, and the stopwatch was started. The initial concentrations in the reaction mixture were $[\text{papain-S-S-CH}_3]_{init} = 2 \times 10^{-6}$ M and $[DTT]_{init} = 2.5 \times 10^{-5}$ M. From the reaction mixture, aliquots (0.2 mL) were withdrawn at various time intervals and were added to a cuvette containing the substrate solution (0.8 mL of a 1.25 mM solution of *N*-benzoyl-L-arginyl-*p*-nitroanilide in aqueous buffer, pH 6, 0.1 M in imidazole, 2 mM in EDTA), and the rate of change of absorbance at 410 nm was recorded.[26] The values of concentrations in the cuvette were [papain-SH] + [papain-S–S-$CH_3$] = 0.4 $\mu$M and [*N*-benzoyl-L-arginyl-*p*-nitroanilide] = 1.0 mM. DTT

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was present in excess over papain-S-S-$CH_3$ in the regeneration mixture, and the concentration of DTT was therefore assumed to be constant during the reaction. The apparent rate ($k^{app}$) was calculated from the slope for the plot of $-\ln \{([papain]_{total} - [papain]_{SH})/[papain]_{total}\}$ vs time, for which the slope = $k^{app}[DTT]$. The activation curves were biphasic, and the plots for DMH and DTT were compared after the initial burst, i.e., for activities greater than ~15 % of the total activity. At the end of experiment, the thiol contents of DTT and DMH stock solutions were verified by Ellman's assay.[27] Ficin was assayed with the same substrate as above by a similar procedure. The enzyme solution of ficin-S-S-$CH_3$ was prepared by dissolving 0.05 mL of the stock solution of ficin-S-S-$CH_3$ in 2.55 mL of deoxygenated buffer (pH 7, 0.1 M in imidazole, 2 mM in EDTA).

**Kinetics of Reduction of Creatine Kinase-S-S-Glutathione by DMH and by DTT.** The solution of the mixed disulfide of creatine kinase and glutathione (10 μL) was diluted with 2.5 mL of deoxygenated aqueous buffer (pH 7, 0.1 M in imidazole, 2 mM in EDTA), and the diluted solution was added to two flasks (1.0 mL in each). To the flask containing the enzyme solution was added DTT or DMH (5 μL of a 5 mM solution in pH 6 aqueous imidazole buffer), and the stopwatch was started. From the reaction mixture, aliquots (50 μL) were withdrawn and were added to 950 μL of assay solution (pH 6, 0.1 M in imidazole, 2 mM in EDTA), and the rate of increase in absorbance at 340 nm was recorded. The assay solution contained $Mg^{2+}$ (10 mM), ADP (2 mM), D-glucose (20 mM), $NADP^+$ (2 mM), phosphocreatine (30 mM), hexokinase (50 U), and glucose-6-phosphate dehydrogenase (35 U).[28] The apparent rate constant was determined in the same manner as described for papain.

**Kinetics of Inactivation of Deoxyribonuclease I (DNaseI) by DMH and by DTT.** The assay was similar to that described by Price et al.[20] The solution of DNA in acetate buffer containing $Mg^{2+}$ was prepared according to the Sigma protocol.

**Kinetics of Reduction of Bis(2-hydroxyethyl) Disulfide ($ME^{ox}$) by DMH and DTT by $^1H$ NMR Spectroscopy.** Stock solutions (20 mM) of DMH (0.0040 g in 0.96 mL of $D_2O$ buffer), DTT (0.0046 g in 1.50 mL of $D_2O$ buffer), and bis(2-hydroxyethyl) disulfide (0.0060 g in 1.94 mL of $D_2O$ buffer) were prepared in $D_2O$ buffer (pD 7.0, 50 mM in phosphate). In a NMR tube containing $ME^{ox}$ (0.25 mL) was added DMH or DTT (0.25 mL), and the stopwatch was started. The reaction was quenched after 2 min (or 4 min) by addition of DCl (30 μL of a 12 wt % solution in $D_2O$). The extent of reaction was determined from integration of the $^1H$ NMR peak areas. The initial values of concentration in the reaction mixture were $[DMH] = [ME^{ox}] = c_{init} = 10$ mM. The apparent rate constant ($k^{app}$) was calculated by $k^{app} = [(1/c_{final}) - (1/c_{init})]/t$.

**Determination of the Equilibrium Constant of DMH with $DTT^{ox}$ by $^1H$ NMR Spectroscopy.** To a flask containing $DTT^{ox}$ (0.0017 g, 11 μmol) was added a stock solution of DMH (1.0 mL of a 11 mM solution in $D_2O$ buffer) that had been estimated previously by Ellman's assay. The equilibrium mixture was transferred to an NMR tube with a cannula under argon. The top of the NMR tube was sealed with paraffin wax, and the $^1H$ NMR spectra were recorded after 29 h. The amounts of DMH and $DMH^{ox}$ at equilibrium were estimated by integration of the areas of $CH_3N$ peaks, and the amounts of DTT and $DTT^{ox}$ at equilibrium were estimated by integration of the areas of $CH_2S$ peaks. A similar procedure was used for determining the equilibrium constant of DMH with lipoic acid.

**Determination of the Equilibrium Constant of DMH with Lipoic Acid by UV Spectroscopy.** A solution of lipoic acid (0.0024 g, $1.2 \times 10^{-5}$ mol) in 3 mL of deoxygenated aqueous phosphate buffer (pH 7.0, 2 mM in EDTA, 0.1 M in phosphate) was added to a flask containing DMH (0.0030 g, $1.4 \times 10^{-5}$ mol). The equilibrium mixture was transferred to a cuvette, and the absorbance at 330 nm was recorded. Extinction coefficient $\epsilon_{330}$ = 147 $M^{-1}$ $cm^{-1}$ was used for calculating the concentration of the cyclic five-membered lipoic acid at equilibrium.[6]

**Determination of the Equilibrium Constant of $DMH^{ox}$ with Hexane-1,6-dithiol by $^1H$ NMR Spectroscopy.** In a flask containing $DMH^{ox}$ (0.0052 g, $2.5 \times 10^{-5}$ mol) and hexane-1,6-dithiol (0.0038 g, $2.5 \times 10^{-5}$ mol) was added deoxygenated DMSO-$d_6$ (0.5 mL). In another flask containing potassium *tert*-butoxide (0.0159 g, 0.142 mmol) was added DMSO-$d_6$ (0.45 mL) to prepare a stock solution of potassium *tert*-butoxide (0.315 M). To an NMR tube were added DMSO-$d_6$ (0.25 mL), the solution of $DMH^{ox}$ and hexane-1,6-dithiol in DMSO-$d_6$ (0.25 mL, $1.25 \times 10^{-5}$ mol of each), and the stock solution of potassium *tert*-butoxide in DMSO-$d_6$ (3 μL of a 0.315 M solution, $1 \times 10^{-6}$ mol). The top of the NMR tube was sealed with paraffin wax, and the $^1H$ NMR spectra were recorded after 30 min and 2 h. The values of initial concentration in the NMR tube were $[DMH^{ox}] = 25$ mM, [hexane-1,6-dithiol] = 25 mM, and [potassium *tert*-butoxide] = 2 mM. The equilibrium concentrations were determined by integration of the $^1H$ NMR peak areas. The same value of $K_{eq}$ (=10) was obtained from spectra recorded after 30 min and after 2 h.

**Determination of the Rate of Conformational Interchange of DMH in Water by Dynamic $^1H$ NMR Spectroscopy.** To an NMR tube containing DMH (0.0022 g, $1.1 \times 10^{-5}$ mol) was added deoxygenated $D_2O$ buffer (0.5 mL), and the top of the NMR tube was sealed with Apiezon W cement. The $^1H$ NMR spectra (500 and 250 MHz) of the DMH solution (21 mM) were recorded at various temperatures in the range 45–73 °C. The NMR temperature values were calibrated against a sample of ethylene glycol.[29] The $^1H$ NMR spectra were simulated with the program DNMR4[30] on a VAX 8600 for various rate constants ($k$) and were visually compared with the experimental spectra for the best fit. Only two conformations of DMH were observed in the $^1H$ NMR spectrum: a symmetrical conformation (**3**) (*E,E*) showed one $CH_3N$ peak at 3.23 ppm; an unsymmetrical conformation (**4**) (*E,Z*) showed two $CH_3N$ peaks at 3.43 and 3.10 ppm (eq 3). The symmetrical conformation was presumed to be **3** because the chemical shift of the $CH_3N$ peak (3.23 ppm) was similar to that of the $CH_3N$ peak (3.25 ppm) of $DMH^{ox}$. Another conformation (*Z,Z*), possible for DMH, was not observed.[31] For DNMR simulation, the population ratio of **3** to **4** was 0.275:0.725, $T_2^*$ = 0.126 s (500 MHz), and $T_2^*$ = 0.160 s (250 MHz).[32] The plot of log $k$ vs $1/T$ was a straight line ($R^2$ = 0.996 for seven points). The extrapolated values of rate constant at 25 °C were 0.062 $s^{-1}$ for **3** going to **4** and 0.024 $s^{-1}$ for **4** going to **3**. Similar values for rate constants of conformational interchange were observed for *N,N'*-dimethyl-*N,N'*-bis(5-hydroxy-3-thiapentanoyl)hydrazide ($(HOCH_2CH_2SCH_2CONMe)_2$). We infer that the rate constants for the isomerization of **5** and **6** (eq 3) would be similar to those for the isomerization of **3** and **4**. The population ratio **3**:**4** in $CD_3CN$ was 0.45:0.55. The symmetrical form (**3**) has a lower dipole moment than the unsymmetrical form (**4**) and should be more favored in a solvent of low dielectric constant than in a solvent of higher dielectric constant.

**Determination of the $pK_a$ of DMH.** The spectroscopic method of Benesch and Benesch[33] was used for determining the $pK_a$ of the thiol groups in DMH. The absorbance due to thiolate for solutions of DMH (0.26 mM) in various buffers in the pH range 5–10 was measured at 238 nm. The dissociation curves of the two thiols overlap; the values of first and second $pK_a$ are estimated as 7.6 and 8.9, respectively.

**Determination of the Solubilities of DMH and $DMH^{ox}$ in $D_2O$ Buffer.** A saturated solution of DMH (or $DMH^{ox}$) was

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prepared in 1 mL of deoxygenated $D_2O$ buffer (pD 7, 50 mM in phosphate as buffer, 20 mM in 2,3-butynediol as $^1H$ NMR standard). The solubility of DMH (or $DMH^{ox}$) was determined by integration of the $^1H$ NMR peak areas of $CH_3N$ peaks for DMH (or $DMH^{ox}$) and of $CH_2OH$ peaks for 2,3-butynediol ($HOCH_2C{-}CCH_2OH$).

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**Registry No.** 1, 89580-95-0; 2, 131760-66-2; DMH, 131760-67-3; $DMH^{ox}$, 131760-68-4; DTT, 27565-41-9; $DTT^{ox}$, 86023-22-5; $ME^{ox}$, 1892-29-1; DNaseI, 9003-98-9; MeNHNHMe·2HCl, 306-37-6; $(ClCH_2CO)_2O$, 541-88-8; AcSH, 507-09-5; $HS(CH_2)_6SH$, 1191-43-1; lipoic acid, 62-46-4.

# Clavudiol A and Clavirolide A, Two Marine Dolabellane Diterpenes from the Soft Coral *Clavularia viridis*

Jingyu Su,* Yongli Zhong, and Kailiang Shi

*Department of Chemistry, Zhongshan University, Guangzhou, People's Republic of China*

Qi Cheng and John K. Snyder*

*Department of Chemistry, Boston University, 590 Commonwealth Ave., Boston, Massachusetts 02215*

Shengzhi Hu and Youqing Huang

*Department of Chemistry, Xiamen University, Xiamen, Fuzhou, People's Republic of China*

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The structures of two new marine diterpenes, clavudiol A (1) and clavirolide A (2), isolated from the Pacific soft coral *Clavularia viridis* collected off the Xisha Islands in the South China Sea are reported. Particularly valuable in the structure assignments of these two natural products was the two-dimensional NMR FLOCK sequence for detection of long range heteronuclear ($^{13}C$,$^1H$) couplings, and the use of the QUANTA/CHARMm molecular modeling program to support the NMR conformational analysis. The absolute stereochemistry of 1 and 2 were established by circular dichroism, and the structure of 1 (relative stereochemistry) ultimately confirmed by X-ray crystallography. Both are members of the dolabellane class of diterpenes.

The Pacific soft coral *Clavularia viridis* has proven to be a rich source of intriguing natural products with structural types including the well-known antitumor[1] eicosanoids[2] such as the clavulones[3] and chlorovulone.[4] These compounds, most notably the clavulones, have been the targets of several synthetic efforts beginning with Corey's initial report in 1984.[5] Cytotoxic steroids[6] and diterpenoids of the dolabellane class[7] have also been found in *C. viridis*, with the latter class of diterpenes also reported from an unidentified species of *Clavularia*.[8] Related species of *Clavularia* are also a source of terpenoids including the biosynthetically related neodolabellanes and dolastannes.[9]

**Table I. NOE and Long-Range Scalar Coupling Connectivities for Clavudiol A (1)[a]**

| $^1H$ NOE's[b] | $^1H$–$^1H$ long-range coupling[c] | $^{13}C$–$^1H$ long-range coupling[d] |
|---|---|---|
| H7–H3a,H6a,H9b | H2b–H15 | H10–C12 (FLOCK) |
| H9a–H9b | H7–H17 | H15–C2,C11,C14 (FLOCK) |
| H10–H9a,H15,H17 | H16a–H3a | H16a–C4,C5 (FLOCK) |
| | H16b–H5a | H16b–C3,C4 (FLOCK) |
| H14a–H14b | H14b–H15 | H17–C7,C8 (FLOCK) |
| H16a–H3b | | H19–C20 (FLOCK) |
| H16b–H5a | | H20–C19 (FLOCK) |
| H17–H9a,H10 | | H9b–C7 (SINEPT) |
| | | H19/H20–C12 (SINEPT) |
| | | H7–C9,C17 (SINEPT) |

[a] Spectra recorded in $CDCl_3$ unless otherwise noted. [b] From 2D-NOE spectrum. [c] From long-range COSY spectrum, $\Delta$ = 300 ms. [d] From 2D FLOCK and selective INEPT (SINEPT).

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