meso-2,5-Dimercapto-N,N,N',N'-tetramethyladipamide: A Readily Available, Kinetically Rapid Reagent for the Reduction of Disulfides in Aqueous Solution

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meso-2,5-Dimercapto-N,N,N',N'-tetramethyladipamide (meso-DTA) reduces disulfide bonds up to 8 times faster (kinetic) than does diithiothreitol (DTT) in aqueous solution at pH 7.0. meso-DTA is easily synthesized in five steps (39% overall yield) from adipic acid. meso-DTA, which forms a cyclic disulfide, is less reducing than DTT by approximately 66 mV, but is much more reducing than mercaptoethanol.

Introduction

This paper reports the reduction of small organic disulfides and protein disulfides in water at pH 7.0 using a new reagent, meso-2,5-dimercapto-N,N,N',N'-tetramethyladipamide (meso-DTA). Disulfide-reducing reagents are used in biochemistry to inhibit the oxidation of thiol groups and to reduce disulfide groups in proteins. A useful thiol reducing reagent for disulfides should have pK<sub>a</sub> 7.0 for the SH group, a high reduction potential, ready availability, an unobjectionable odor, high solubility in water, kinetic stability at room temperature, and low toxicity.

We have previously examined N,N'-dimethyl-N,N'-bis(mercaptoacetyl)hydrazine (DMH), a reagent that reduces disulfides faster than diithiothreitol (DTT), but is more expensive to synthesize. Mercaptoethanol (ME) and diithiothreitol (DTT) are the most commonly used disulfide-reducing reagents in biochemistry. The principal advantage of ME is its low cost. ME has, however, the disadvantage of a low reduction potential and a relatively high pK<sub>a</sub>, 9.6. The primary advantage of DTT is that it is strongly reducing. DTT also has several disadvantages: oxidation of DTT by O<sub>2</sub> in the presence of transition-metal ions can generate hydrogen peroxide; it is a strong chelating agent and can sequester essential ions (especially transition metals); it is not a fast reductant (the lower pK<sub>a</sub> of the thiol groups in DTT is 9.2; thus only about 1% of DTT exists as the thiolate at pH 7.0); it is expensive. (For nomenclature, we indicate the oxidized form of a thiol, the disulfide, by the superscript "ox" and leave the reduced form as the "red" form.)

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11. The present price of DTT is $87.00/mol from Aldrich.
form, the thiol, unsuperscripted: e.g., DTT (dithiol) vs DTMH (disulfide.).

**Discussion**

The pKₐ of the first thiol in meso-DTA is 7.8 ± 0.2; therefore, at pH 7.0 approximately 15% of meso-DTA exists as the thiolate. On the basis of this pKₐ, we calculate that meso-DTA should reduce disulfides 4.4 times faster than DTT at pH 7.0. The relative rate of reduction of disulfides by meso-DTA compared to DTT is approximately 6 for small peptides and small organic disulfides (Table I), a value that is slightly above that calculated. For proteins, the relative rate of meso-DTA vs DTT to determine the values of ε' and K(ME) of meso-DTA completely reduces noncyclic disulfides (mercaptoethanol disulfide or glutathione disulfide) as determined by H NMR spectroscopy. meso-DTA only partially reduces DTMH in 50 mM phosphate buffer at pH 7.0 (Kₑ = [meso-DTA] [DTM]/[meso-DTA][DTM] = 0.010). DIT-DTA is more reducing than meso-DTA by a factor of 10 in 100 mM phosphate buffer at pH 7.0 (Kₑ = [meso-DTA] [dl-DTA]/[meso-DTA][dl-DTA] = 0.10). We used the equilibrium constant between meso-DTA and DTT to determine the values of ε' and K(ME) of meso-DTA. Some other useful physical properties of meso-DTA, DTT, and DMH are listed in Table II.

Table II. Physical Properties of DTA, DTT, and DTMH

<table>
<thead>
<tr>
<th>Physical property</th>
<th>meso-DTA</th>
<th>dl-DTA</th>
<th>DTT</th>
<th>DMH</th>
</tr>
</thead>
<tbody>
<tr>
<td>ε' MeV</td>
<td>-0.300</td>
<td>-0.328</td>
<td>-0.356</td>
<td>-0.300</td>
</tr>
<tr>
<td>pKₑ corresponds</td>
<td>7.8, 8.9</td>
<td>9.2, 10.1</td>
<td>7.6, 8.9</td>
<td></td>
</tr>
<tr>
<td>mp°C</td>
<td>118 (137)</td>
<td>42 (132)</td>
<td>38 (155)</td>
<td></td>
</tr>
<tr>
<td>kₑapp M⁻¹ s⁻¹</td>
<td>0.50</td>
<td>0.065</td>
<td>5.5</td>
<td>0.15</td>
</tr>
<tr>
<td>Kₑ M⁻¹</td>
<td>10¹</td>
<td>10⁴</td>
<td>10⁴</td>
<td>10⁴</td>
</tr>
<tr>
<td>solubility,s</td>
<td>80 (80)</td>
<td>high</td>
<td>250 (23)</td>
<td></td>
</tr>
<tr>
<td>mm²</td>
<td>weak (none)</td>
<td>weak (none)</td>
<td>weak (none)</td>
<td></td>
</tr>
</tbody>
</table>

Table I. Rates of Reduction of Disulfides with meso-DTA and DTT

<table>
<thead>
<tr>
<th>Disulfide</th>
<th>kₑapp DTA M⁻¹ s⁻¹</th>
<th>kₑapp DTT M⁻¹ s⁻¹</th>
<th>kₑapp DTA M⁻¹ s⁻¹</th>
<th>kₑapp DTT M⁻¹ s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>mercaptoethanol disulfide</td>
<td>0.50</td>
<td>0.065</td>
<td>7.7</td>
<td>1.1</td>
</tr>
<tr>
<td>glutathione disulfide</td>
<td>0.31</td>
<td>0.056</td>
<td>5.5</td>
<td>0.15</td>
</tr>
<tr>
<td>papain-S-S-Me</td>
<td>260</td>
<td>58</td>
<td>4.5</td>
<td>0.15</td>
</tr>
<tr>
<td>creatine</td>
<td>78</td>
<td>23</td>
<td>3.4</td>
<td>0.5</td>
</tr>
<tr>
<td>kinase-S-S-glutathione</td>
<td>0.34</td>
<td>0.19</td>
<td>1.8</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The rate constants are for aqueous solutions at pH 7.0 and 298 K.

5 The rate constants are for aqueous solutions at pH 7.0 and 298 K.

6 Data taken from ref 8.

7 Determined by integration of the lH NMR spectrum. meso-DTA only produces one stereoisomer, in high yield. We believe that the production of only one stereoisomer reflects isomerization under the reaction conditions. Deacylation of the thiolactate produces only one stereoisomer, meso-DTA.

8 Oxidation of the diethylamino; DTA, to the disulfide, meso-DTA, and subsequent analysis of the lH NMR coupling constants at 20 and -60°C established that the diethylamino was the meso isomer.

9 The di isomer of DTA (dl-DTA) could be isolated as a minor product by following a similar route except that the addition of thiolacetic acid

10 The rate constants are for aqueous solutions at pH 7.0 and 298 K.

11 Determined by integration of the lH NMR spectrum. meso-DTA only produces one stereoisomer, in high yield. We believe that the production of only one stereoisomer reflects isomerization under the reaction conditions. Deacylation of the thiolactate produces only one stereoisomer, meso-DTA.

12 Data taken from ref 8.


14 We also attempted the synthesis of the diethanolamine, instead of the dimethyl, derivative of DTA, but we were unable to synthesize this derivative conveniently.


17 We also attempted the synthesis of the diethanolamine, instead of the dimethyl, derivative of DTA, but we were unable to synthesize this derivative conveniently.


21 Using the Bronsted equation log k = 7.0 + 0.5 pKₐ - 0.27 pKₐ - 0.73 pKₐ and the equation k = kₑ(eq[1 + 10¹pH]), we calculate kₑapp meso-DTA/dl-DTA = 4.4.
steric interactions, because meso-DTA is a secondary thiol and DTT is a primary thiol. The difference in rates could also be due to the relative difference in hydrophobicities of the two compounds.

The equilibrium constant for reduction of ME by meso-DTA is less than that of DTT by a factor of 10², probably due to the 1,3-diaxial interaction in meso-DTA. In meso-DTA, the 1,3-diaxial interaction between the axial hydrogen and axial dimethylamido group will destabilize the cyclic disulfide (meso-DTA) relative to the noncyclic diithio (meso-DTA). This inference is supported by the fact that d,l-DTA is 10 times more reducing than meso-DTA. In DTT there are no 1,3-diaxial interactions to destabilize the oxidized form relative to the unoxidized form (DTT).

meso-DTA and DMM have similar reduction potentials and pKₐ's, but DMM reduces hindered disulfides more rapidly than does meso-DTA. This increased rate of reduction of hindered disulfides is probably due to differences in steric interactions and hydrophobicities since DMM contains a primary thiol while meso-DTA contains a secondary thiol.

meso-DTA is less soluble in water than DTT. Since nearly all applications in protein chemistry require a concentration of reducing agent less than 50 mM, the lower solubility of meso-DTA should not be disadvantageous. In fact, the lower solubility of meso-DTA in water permits its extraction from water with organic solvents.

In conclusion, none of the reagents mentioned—ME, DTT, DMM, and meso-DTA—is clearly superior as a reducing agent for biochemical applications. ME is inexpensive and commercially available, but is weakly reducing and kinetically slow. DTT is commercially available and strongly reducing, but is reasonably expensive and kinetically fast. DMM is strongly reducing and kinetically slow. DTT is commercially available and weally reducing, but is reasonably expensive and kinetically fast, but is not commercially available and is expensive to synthesize (primarily because 1,2-dimethylhydrazine, the starting material, is expensive). meso-DTA is strongly reducing, relatively inexpensive to synthesize, and kinetically fast, but is not commercially available. We believe that for most applications meso-DTA would be superior or equal to DTT.

Experimental Section

General. Starting materials were commercial products: Thionyl chloride, bromine, and thiocetic acid (Fluka); dimethylamine and adipic acid (Aldrich); papain (Boehringer Mannheim); creatine phosphokinase, deoxyribonuclease I, DNA, and N-benzyloxycarbonyl-arginine p-nitroaniline (Sigma). NMR spectra were recorded in CDCl₃. Chemical shifts are reported in δ (ppm) using CHCl₃ (7.24) as an internal standard. Elemental analyses were performed by Oneida Research Services.

di-DTA (3),16 Adipic acid (1,832 mmol, 120.0 g) and thiocyanate (2.33 mol, 170 mL) were heated at reflux for 90 min with no solvent in a three-necked 1-L flask equipped with a reflux condenser and an addition funnel. The exhaust gases from the reflux condenser were neutralized by bubbling through a 5 M NaOH solution. The 13C NMR (75 MHz) spectrum of the resulting oil showed peaks at δ 173.1, 46.2, and 23.5. Bromine (1.88 mol, 97 mL) was added over 5 h at 95 °C and the reaction mixture was kept at 95 °C for another 3 h before being cooled to 25 °C. The 1H NMR spectrum of the product showed two major components: meso- and d,l-dibromomaldehyde in a 1.6:1.0 ratio. The solution was dissolved in CHCl₃, cooled to 0 °C over 1 h, and added to a biphasic mixture of CHCl₃ (200 mL) and dimethylamine (600 mL of a 40% w/w aqueous solution) in a 3-L flask cooled by an ice/salt bath. The temperature of the reaction mixture was kept at 18 °C or less. The resulting biphasic mixture was acidified to pH 4.0 with concentrated hydrochloric acid (ca. 30 mL). The organic layer was separated and extracted with saturated aqueous sodium bicarbonate (150 mL), dried with MgSO₄, and concentrated under aspirator pressure to provide 197 g of crude mixture of meso- and di product (2:1.6:1.0 ratio). A small portion (ca. 50 mg) was separated by chromatography on silica gel (eluant: 1:1 ethyl acetate/hexane going to ethyl acetate). Major product: 1H NMR (400 MHz) δ 4.42-4.36 (m, 2 H), 3.04 (s, 3 H), 2.94 (s, 2 H), 2.29-2.30 (m, 2 H), 2.00-1.92 (m, 2 H); 13C NMR (100 MHz) δ 186.2, 42.4, 37.4, 36.2, 33.0. Minor product: 1H NMR (400 MHz) δ 4.42-4.36 (m, 2 H), 3.04 (s, 3 H), 2.92 (s, 3 H), 2.19-2.08 (m, 4 H); 13C NMR (100 MHz) δ 186.3, 42.7, 37.4, 36.2, 32.9. The crude product was divided into two portions: 97.5 g and 100 g.

The first portion was recrystallized from CH₂Cl₂ (ca. 500 mL) and ether (ca. 500 mL) to provide 74.8 g (61% overall yield) of a single diastereomer 2 (which we presume to be the meso isomer; see below). Anal. Calcd for C₁₁H₁₆N₂O₂Br₂: C, 33.54; H, 5.07; N, 7.53. Found: C, 33.52; H, 5.07; N, 7.58. The second portion was recrystallized from CH₂Cl₂ (500 mL) and ether (ca. 500 mL) to provide 27.6 g (98%) of product 3H NMR (400 MHz, δ 4.44-4.40 (m, 2 H), 3.02 (s, 3 H), 2.92 (s, 3 H), 2.30 (s, 3 H), 2.07-2.00 (m, 2 H), 1.72-1.66 (m, 2 H); 13C NMR (100 MHz) δ 194.6, 170.2, 42.2, 37.4, 36.1, 30.2, 30.1. The yellow solid and potassium carbonate (655 mmol, 90.5 g) were added to methanol (400 mL) that had been purged with argon. The mixture was stirred for 14 h under argon. CH₃CO₂H (200 mL) was added and the solution acidified to pH 2.5 with concentrated sulfuric acid (ca. 35 mL) over 1 h. The solution was partitioned between ethyl acetate (1000 mL) and water (600 mL). The layers were separated, and the water layer was extracted with ethyl acetate (2 x 300 mL). The combined organic layers were dried (MgSO₄) and concentrated under reduced pressure to provide 96.7 g of crude product, which was recrystallized from THF (ca. 100 mL) to provide 37.3 g of meso-DTA. The mother liquor was dissolved in CH₂Cl₂ (200 mL) and extracted with 200 mL of 0.2 N HCl. The organic layer was concentrated in vacuo and recrystallized from THF to provide a further 5.7 g of meso-DTA (39% total overall yield from adipic acid): 1H NMR (500 MHz) δ 3.45-3.35 (m, 2 H), 3.02 (s, 3 H), 2.92 (s, 3 H), 2.07-1.98 (m, 2 H), 1.92 (d, J = 10.8 Hz, 2 H), 1.68-1.58 (m, 2 H); 13C NMR (125 MHz) δ 172.0, 37.4, 37.2, 35.0, 34.9. Anal. Calcd for C₁₁H₁₆N₂O₂S₂: C, 45.43; H, 7.62; N, 10.59. Found: C, 45.32; H, 7.41; N, 10.39.

Oxidized meso-DTA. Ellman's reagent (2.0 g, 6.45 mmol) was added to water (75 mL) and the pH was adjusted to 7.0 with saturated aqueous NaHCO₃ solution. meso-DTA (11.0 g, 3.89 mmol) was added, and the pH of the reaction mixture was kept at 15. CH₂Cl₂ (25 mL) was added, and the layers were separated. The water layer was back-extracted with CH₂Cl₂ (2 x 50 mL). The combined organic layers were dried (MgSO₄) and concentrated at aspirator pressure to provide 961 mg (96%) of product: 1H NMR (400 MHz, 500 MHz, 125 MHz).
293 K) to 3.69 (br dd, J = 6.7, 2.2 Hz, 2 H), 3.04 (s, 6 H), 2.92 (s, 6 H), 2.81-2.74 (m, 2 H), 2.00-1.92 (m, 2 H); 13C NMR (100 MHz, 293 K) δ 169.8, 43.8 (br), 37.7, 35.9, 27.7; 1H NMR (400 MHz, 213 K) δ 4.00 (br, J = 12.0 Hz, 1 H), 3.46 (br s, 1 H), 3.06 (br s, 3 H), 3.01 (br s, 3 H), 3.09-3.00 (br, 1 H), 2.95 (br s, 3 H), 2.88 (br s, 3 H), 2.43 (br d, J = 12.3 Hz, 1 H), 2.06-1.94 (br, 2 H); 13C NMR (100 MHz, 213 K) δ 170.0, 169.5, 31.5, 37.3, 35.8, 35.4, 35.2, 29.3, 25.7. Anal. Calcd for C19H32N2O2S2: C, 45.78; H, 6.91; N, 10.68. Found: C, 45.53; H, 6.97; N, 10.54.

dl-DTA. The product of the secondary recrystallization of 2 (5.1 ratio of meso to dl, 2.50 g) was chromatographed on silica gel (ethyl acetate/hexane, 3:1, keeping to 5:1) to provide 448 mg of a 1:1 (meso/dl) mixture. This mixture was dissolved in 30 mL of methanol and cooled to 0 °C. Sodium methoxide (160 mg) and thiolacetic acid (300 μL) were added. After 15 min at 0 °C, the solution was warmed to room temperature, concentrated in vacuo, and partitioned between water (10 mL) and CH2Cl2 (20 mL). The organic layer was dried (MgSO4), concentrated in vacuo, and chromatographed on a silica gel (ethyl acetate going to ethyl acetate/methanol, 10:1) to provide 165 mg of the dl-dithiolalacetate of DTA. The dl-dithiolalacetate of DTA was dissolved in 10 mL of methanol. After addition of sodium methoxide (48 mg), the solution was stirred for 20 min, acidified with Dowex 50×8 ion-exchange resin (H+ form), filtered, and partially concentrated under aspirator pressure (2 mL). The methanolic solution was added to an aqueous solution of Eliman's reagent (120 mg, adjusted to pH 7.0 with saturated NaHCO3). After 10 min, the solution was extracted with CH2Cl2 (2 × 20 mL). The combined organics were dried (MgSO4), concentrated under aspirator pressure, and chromatographed on silica gel (ethyl acetate going to ethyl acetate/methanol, 10:1) to provide 58 mg of dl-DTA. The 1H NMR (500 MHz) δ 3.94 (br, 2 H), 3.12 (s, 6 H), 2.92 (s, 6 H), 2.43 (br d, J = 12.3 Hz, 1 H), 2.06-1.94 (br, 2 H); 13C NMR (100 MHz) δ 170.0, 169.5, 31.5, 37.3, 35.8, 35.4, 35.2, 29.3, 25.7. Anal. Calcd for C19H32N2O2S2: C, 45.78; H, 6.91; N, 10.68. Found: C, 45.53; H, 6.97; N, 10.54.

equilibrium of dl-DTA and meso-DTA. dl-DTA was prepared in 0.5 mL of buffered D2O (100 mM NaPO4, pH 7.0) and meso-DTA (6 mg in 1.5 mL of buffered D2O (100 mM NaPO4, pH 7.0)) were mixed in an NMR tube. After 4 h, a 1H NMR spectrum was obtained. The equilibrium constant between meso-DTA and dl-DTA was calculated using the integrals obtained from the 1H NMR spectrum. When meso-DTA was equilibrated with a 1.5-fold excess of mercaptoethanol disulfide (6 mM) or glutathione disulfide (6 mM), meso-DTA was oxidized completely, and no mixed disulfide or reduced meso-DTA was observed by 1H NMR spectroscopy.

kinetics of reactivation of creatine kinase-S-S-glutathione. The solution of creatine kinase-S-S-glutathione (10 μL) was diluted with deoxygenated aqueous buffer (pH 7.0, 0.1 M imidazole, 2 mM EDTA, 2.5 mM). The diluted solution was added to two flasks (1.0 mL each). DTT or meso-DTA (5 μL of a 5 mM solution in pH 6.0 aqueous imidazole buffer) was added to the flask containing enzyme (t = 0). At various times, a 50-μL aliquot was withdrawn and added to an assay solution (950 μL, pH 6.0, 0.1 M imidazole, 2 mM EDTA, 10 mM MgCl2, 2 mM ADP, 20 mM d-glucose, 2 mM NADP, 30 mM phosphocreatine, hexokinase (50 units/mL), glucose-6-phosphate dehydrogenase (35 units/mL)). The rate of increase in absorbance at 340 nm was recorded.

Kinetics of reactivation of papain S-S-Me. The papain-S-S-Me was prepared as described previously. To assay for the rate of reactivation of papain disulfide with DTT and meso-DTA, we used a procedure similar to that described in ref 8.

Registry No. 1, 124-04-9; meso-2, 137300-51-7; dl-2, 137300-52-8; meso-DTA dithioacetate, 137300-53-9; dl-DTA dithioacetate, 137300-56-2; meso-DTA, 137300-54-0; meso-DTA*, 137300-55-1; dl-DTA*, 137300-57-3; Me*, 1892-29-1; meso-2,5-dibromoadipoyl, 137300-49-3; dl-2,5-dibromoadipoyl, 137300-50-6; glutathione disulfide, 27025-41-8


(20) The procedure was analogous to that of Walters and Gilbert: Walters, D. W.; Gilbert, H. F. J. Biol. Chem. 1986, 261, 15572-15577.
