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A Potent, Versatile Disulfide-Reducing Agent from Aspartic Acid

John C. Lukesh, III,[†] Michael J. Palte,[‡,§] and Ronald T. Raines*[,†,∥]

[†]Department of Chemistry, [‡]Medical Scientist Training Program, [§]Molecular & Cellular Pharmacology Graduate Training Program, and [∥]Department of Biochemistry, University of Wisconsin−Madison, Madison, Wisconsin 53706, United States

Supporting Information

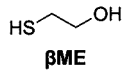
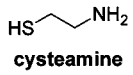
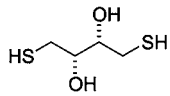
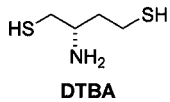
ABSTRACT: Dithiothreitol (DTT) is the standard reagent for reducing disulfide bonds between and within biological molecules. At neutral pH, however, >99% of DTT thiol groups are protonated and thus unreactive. Herein, we report on (2*S*)-2-amino-1,4-dimercaptobutane (dithiobutylamine or DTBA), a dithiol that can be synthesized from L-aspartic acid in a few high-yielding steps that are amenable to a large-scale process. DTBA has thiol pK_a values that are ∼1 unit lower than those of DTT and forms a disulfide with a similar $E^{\circ\prime}$ value. DTBA reduces disulfide bonds in both small molecules and proteins faster than does DTT. The amino group of DTBA enables its isolation by cation-exchange and facilitates its conjugation. These attributes indicate that DTBA is a superior reagent for reducing disulfide bonds in aqueous solution.

Approximately 20% of human proteins are predicted to contain disulfide bonds between cysteine residues.[1] Small-molecule thiols can reduce these (and other) disulfide bonds, thereby modulating biomolecular function.[2] The reaction mechanism involves thiol−disulfide interchange initiated by a thiolate.[3] The ensuing mixed disulfide can become trapped if the reagent is a monothiol, such as β-mercaptoethanol (βME).[4] To overcome this problem, Cleland developed racemic (2*S*,3*S*)-1,4-dimercaptobutane-2,3-diol (dithiothreitol or DTT; Table 1), a dithiol that resolves a mixed disulfide by forming a six-membered ring.[2a,5] DTT is a potent reducing agent ($E^{\circ\prime}$ = −0.327 V),[2g] and has been the preferred reagent for the quantitative reduction of disulfide bonds for decades, despite its high cost.[6,7]

At physiological pH, DTT is a sluggish reducing agent. The reactivity of a dithiol is governed by the lower of its two thiol pK_a values.[2,3] With its lower thiol pK_a value being 9.2 (Table 1), <1% of DTT resides in a reactive thiolate form at pH 7.0.[8]

We sought to develop a nonracemic dithiol with low thiol pK_a and disulfide $E^{\circ\prime}$. Moreover, we sought a reagent that could be accessed in high yield from an inexpensive source. We envisioned that (2*S*)-2-amino-1,4-dimercaptobutane (dithiobutylamine or DTBA; Table 1) could fulfill our physicochemical criteria, and be synthesized from L-aspartic acid, which is an abundant amino acid.[9,10] We accessed DTBA via the two routes depicted in Scheme 1. A five-step route commenced with the esterification of the amino acid and protection of its amino group. Reduction with lithium aluminum hydride yielded a diol, which was subjected to Mitsunobu conditions to install the requisite sulfur functionality.[11] Deprotection gave DTBA as its HCl salt in 99% purity and an overall yield of 60%. A six-step route that avoids generation of triphenylphosphine oxide, a recalcitrant byproduct of the Mitsunobu reaction,[11] provided DTBA·HCl in an overall yield of 56%. In both routes, the product of every step is a white solid.

Table 1. Physical Properties of Disulfide-Reducing Agents

	Thiol pK_a	Disulfide Reduction Potential ($E^{\circ\prime}$)
 βME	9.61 [a]	−0.196 V [b]
 cysteamine	8.37 [c]	−0.203 V [b]
 DTT (racemate)	9.2 (10.1) [d]	−0.327 V [e]
 DTBA	8.2 ± 0.2 (9.3 ± 0.1) [f]	(−0.317 ± 0.002) V [f]

[a]Value is from ref 12. [b]Values are from ref 3f. [c]Value is from ref 13. [d]Values are from ref 3a. [e]Value is from ref 14. [f]Values are the mean ± SE from this work.

DTBA has desirable physicochemical attributes. Its HCl salt is a nearly odorless white solid with high solubility in water. Using a pH-titration monitored by ultraviolet spectroscopy,[15] we determined the thiol pK_a values of DTBA to be 8.2 ± 0.2 and 9.3 ± 0.1 (Figure S1; Table 1).[16] These values are ∼1 unit lower than those of DTT. This difference is comparable to that between cysteamine and βME, and likely results from the strong Coulombic and inductive effects of the protonated amino group. By equilibrating reduced DTBA with oxidized DTT and using HPLC to quantify reduced and oxidized species, we found the reduction potential of oxidized DTBA to be $E^{\circ\prime}$ = (−0.317 ± 0.002) V (Figure S2; Table 1). This $E^{\circ\prime}$ value is slightly less than that of DTT, consistent with more acidic thiols forming less stable disulfide bonds[17] and with the preorganization of DTT for disulfide-bond formation by its hydroxyl groups, which can form an intramolecular hydrogen bond and manifest a gauche effect.

DTBA is an efficacious reducing agent for disulfide bonds in small molecules. We found that DTBA reduces the disulfide bond in oxidized βME 3.5-fold faster than does DTT at pH 7.0,

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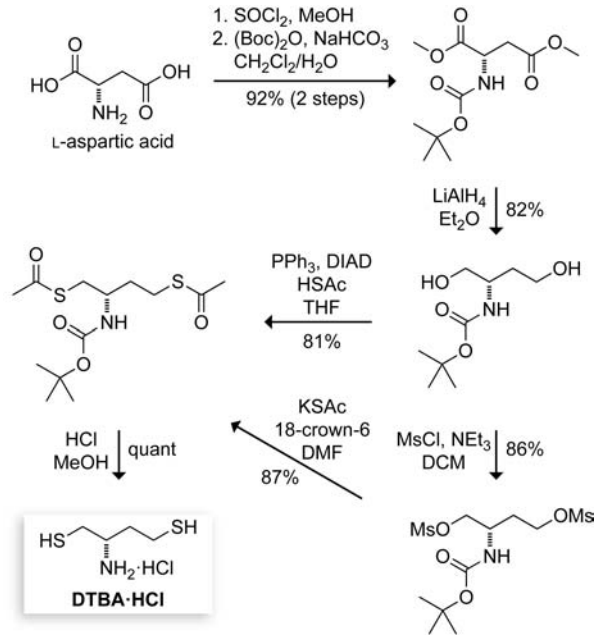
Scheme 1



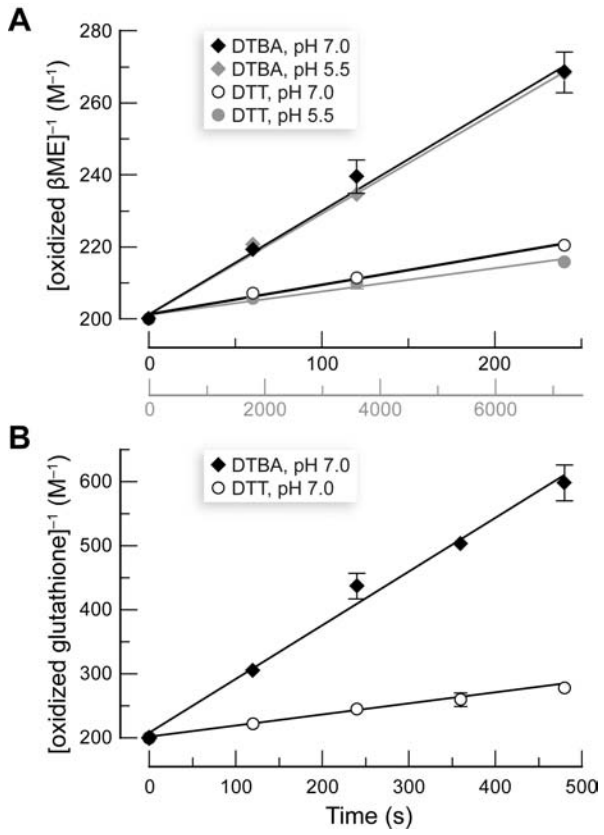


Figure 1. Time-course for the reduction of a mixed disulfide in small molecules by DTBA and DTT in 50 mM potassium phosphate buffer. (A) Reduction of oxidized βME; $k_{obs}^{DTBA}/k_{obs}^{DTT}$ = 3.5 at pH 7.0; $k_{obs}^{DTBA}/k_{obs}^{DTT}$ = 4.4 at pH 5.5. (B) Reduction of oxidized L-glutathione; $k_{obs}^{DTBA}/k_{obs}^{DTT}$ = 5.2 at pH 7.0.

and 4.4-fold faster at pH 5.5 (Figure 1A). These rate accelerations are commensurate with the lower thiol pK_a of DTBA. At pH 7.0, DTBA reduces oxidized L-glutathione 5.2-fold more rapidly than does DTT (Figure 1B). As oxidized L-glutathione has a net charge of −2 near neutral pH, a favorable Coulombic interaction could contribute to this higher rate acceleration.

DTBA is also an efficacious reducing agent for disulfide bonds in proteins. A cysteine residue resides within the active site of papain (Cys25) and near that of creatine kinase (Cys283). Forming a mixed disulfide with those cysteine residues is known to eliminate their enzymatic activities.[2c,18] These two enzymes differ, however, in the electrostatic environment of their active sites. The active site of papain is hydrophobic like its substrates, though there is an anionic region nearby (Figure 2A).[19] In contrast, the active site of

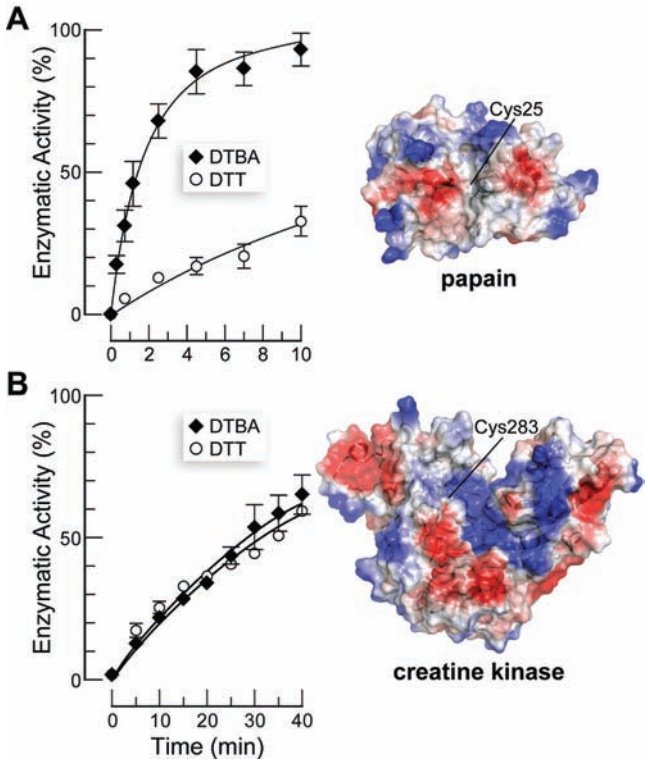


Figure 2. Time-course for the reduction of a mixed disulfide in enzymic active sites by DTBA and DTT in 0.10 M imidazole−HCl buffer, pH 7.0, containing EDTA (2 mM). (A) Reduction of papain-Cys35−S−S−CH_3; $k_{obs}^{DTBA}/k_{obs}^{DTT}$ = 14. (B) Reduction of creatine kinase-Cys283−S−S−L-glutathione; $k_{obs}^{DTBA}/k_{obs}^{DTT}$ = 1.1. Insets: Electrostatic potential maps with red = anionic and blue = cationic, as generated by the program PyMOL (Schrödinger, Portland, OR) using PDB entries 1ppn[19b] and 2crk.[20]

creatine kinase is cationic, complementary to its anionic substrates (Figure 2B).[20,21a−c] We found that DTBA reduces a disulfide bond in the hydrophobic/anionic active site of papain 14-fold faster than does DTT (Figure 2A). In contrast, the two reagents reduce a disulfide bond near the cationic active site of creatine kinase at a similar rate.

The amino group of DTBA confers additional benefits. For example, a disulfide-reducing agent that can be readily isolated, regenerated, and reused incurs less cost and generates less waste.[22] Moreover, extraneous disulfide bonds absorb light at 280 nm, which can confound standard measurements of protein concentration.[23] We reasoned that DTBA could be isolated by its adsorption to a cation-exchange resin. Indeed, >99% of DTBA (but <1% of DTT) was removed from a sodium phosphate buffer, pH 8.0, upon addition of Dowex 50

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resin (see: Supporting Information). We also note that the amino group of DTBA enables its covalent attachment to a soluble molecule, resin, or surface by simple reactions, such as reductive amination (which preserves the cationic charge) or N-acylation. We conclude that the attributes of DTBA could enable it to supplant DTT as the preferred reagent for reducing disulfide bonds in biomolecules.

■ ASSOCIATED CONTENT

Supporting Information

Experimental protocols and analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

rtraines@wisc.edu

Notes

The authors declare no competing financial interest.

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(6) The current price of DTT is 10^2-fold greater per thiol group than that of βME (Sigma−Aldrich, St. Louis, MO).

(7) Tris(2-carboxyethyl)phosphine (TCEP) is more potent than DTT at reducing disulfide bonds between small molecules[2e] but not within proteins.[2f]

(8) Another commercial dithiol, bis(2-mercaptoethyl) sulfone (BMS), has low thiol pK_a values of 7.9 ± 0.2 and 9.0 ± 0.2.[2g] Upon oxidation, however, BMS forms a seven-membered ring with $E^{o\prime}$ = (−0.291 ± 0.002) V (Figure S3), making BMS a less potent reducing agent than DTT.

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(16) The amino group of DTBA is assumed to be cationic throughout its pH-titration, as cysteamine has an amino pK_a of 10.44.[13]

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