Synthesis of Des-*myo*-Inositol Mycothiol and Demonstration of a Mycobacterial Specific Reductase Activity

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Low molecular weight thiols exist in all respiring organisms as carriers of reducing equivalents to maintain a stable intracellular redox environment. Eukaryotic and prokaryotic cells are subjected to oxidative stress from atmospheric oxygen, from basal metabolic activities, and from host−parasite interactions and are unequivocally dependent on thiols to maintain homeostasis. In addition to oxidative stress management, low molecular weight thiols have been found to be involved in critical cellular processes including DNA synthesis and formaldehyde reduction.[1,2]

With the recent increased incidence of drug-resistant pathogenic bacteria, particularly *Mycobacterium tuberculosis*,[3] an evaluation of novel metabolic pathways that have not been exploited for inhibitor development is critical. It has recently been found that mycobacteria do not contain glutathione but instead produce a unique low molecular weight thiol, 1-D-*myo*-inositol-2-(*N*-acetyl-L-cysteinyl)amino-2-deoxy-α-D-glucopyranoside, that has been given the trivial name mycothiol.[4] Chemical analysis of mycothiol shows that it is structurally unique from the more commonly occurring tripeptide-based thiols, glutathione and trypanothione, and Coenzyme A (Figure 1).[55] The unique structure of mycothiol suggests the possibility of designing inhibitors specifically against the enzymes involved in mycothiol metabolism. This work provides synthetic access to a disulfide substrate which permits mechanistic and structural studies of mycothione reductase.[5]

The partial sequence of steps involved in the biosynthesis of mycothiol in *Mycobacterium smegmatis* has been elucidated,[6] but the corresponding enzymes have not been isolated to homogeneity to provide for an enzymatic synthesis strategy. In this paper, we report the chemical synthesis of an oxidized, truncated mycothiol analogue, des-*myo*-inositol-mycothiol, which is reduced by a mycobacterial-specific reductase. In addition, we have cloned and expressed a *M. tuberculosis* gene that exhibits activity with the synthetic substrate.

The desired synthetic product is a truncated form of the authentic substrate in that it lacks the *myo*-inositol moiety. Although mycothiol is structurally unusual, containing an α-1→1 glycosidic linkage between the two monosaccharides D-glucosamine and *myo*-inositol, we posited that the des-*myo*-inositol mycothiol would afford sufficient structural specificity to detect the reductase activity. The synthesis of 2(*N*-acetyl-L-cysteinyl)-amino-2-deoxy-(α,*β*)-D-glucopyranoside, **6** (Scheme 1) proceeds by the use of α-D-glucosamine hydrochloride **1**, which is treated with base to convert it to the free amine **1a**, and coupled to *N*-α-Fmoc-*S*-acetamidomethyl-L-cysteine pentaflourophenyl ester **2** to generate the α-amino and thiol protected compound **3**. Base-catalyzed deprotection of the cysteine α-amino terminus produced the free amine **4** and dibenzofulvene. The free amine was reacted with acetic anhydride to produce the N-acetylated glycopeptide **5**. Selective N-acetylation vs sugar *O*-hydroxyl esterification was obtained by limiting the acetic anhydride and closely maintaining a pH value of 9. Oxidative cleavage of the *S*-acetamidomethyl group was carried out by treating **5** with thallium(III) trifluoroacetate, in the presence of anisole as a scavenger for the acetamidomethyl cation radical, to give the final disulfide product **6**. Unlike mycothiol, compound **6** is a mixture of α and *β* diastereomers, as evidenced by ^{1}H and ^{13}C NMR (Supporting Information).

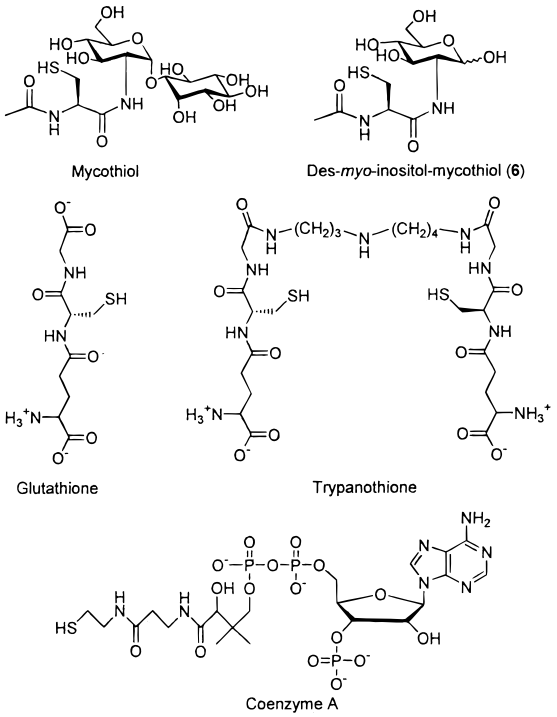

Figure 1. Structure of mycothiol and other low molecular weight thiols.

To rigorously examine the specificity of **6** toward a mycobacterial pyridine dinucleotide-dependent reductase and the level of overexpression achieved for the presumptive mycothione reductase gene, activity measurements using the homologous reductase from *Escherichia coli*, glutathione reductase, was performed. The presumptive mycothione reductase gene[7] was amplified via the polymerase chain reaction (PCR) using *M. tuberculosis* H37Rv genomic DNA and oligonucleotide primers designed from this sequence and the resulting PCR product tentatively named the *mtr* gene. An expression method for mycobacterial proteins was attempted where the *mtr* gene was subcloned into a mycobacterial expression vector, pMV261, containing a heat-shock inducible promotor and expressed in a *pyrF* deficient strain of *M. smegmatis* mc^{2}155.[8]

The mycothione reductase activity of cell-free extracts of *M. smegmatis* (wild-type and transformed) with **6** were compared to that of an *E. coli* cell lysate. The results demonstrate an NADPH-dependent reductase present in mycobacteria, but not in *E. coli*,

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(1) Fahey, R. C. Adv. *Exp. Med. Biol.* **1977**, *86A*, 1.
(2) Misset-Smits, M.; van Ophem, P. W.; Sakuda, S.; Duine, J. A. *FEBS Lett.* **1997**, *409*, 221.
(3) Bloom, B. R.; Murray, C. J. L. *Science* **1992**, *257*, 1055.
(4) Sakuda, S.; Zhou, Z. Y.; Amada, Y. *Biosci. Biotechnol. Biochem.* **1994**, *58*, 1347.
(5) Spies, H. S. C.; Steenkamp, D. J. *Eur. J. Biochem*. **1994**, *224*, 203.
(6) Bornemann, C.; Jardine, M. A.; Spies, H. S. C.; Steenkamp, D. J. *Biochem. J.* **1997**, *325*, 623.
(7) The sequence of a putative *M. tuberculosis* glutathione reductase homologue (*gor*A) has been deposited in GenBank under Accession No. AF002193.
(8) The pMV261 extrachromosomal plasmid was a gift of Dr. William R. Jacobs, Jr. (Albert Einstein College of Medicine) and the *pyrF* (encodes for OMP decarboxylase) deficient strain of *M. tuberculosis* mc^{2}155 was a gift of Dr. Thomas Shrader (Albert Einstein College of Medicine). The conditions and reagents for the PCR amplification reaction, cloning, and expression are described in Supporting Information.

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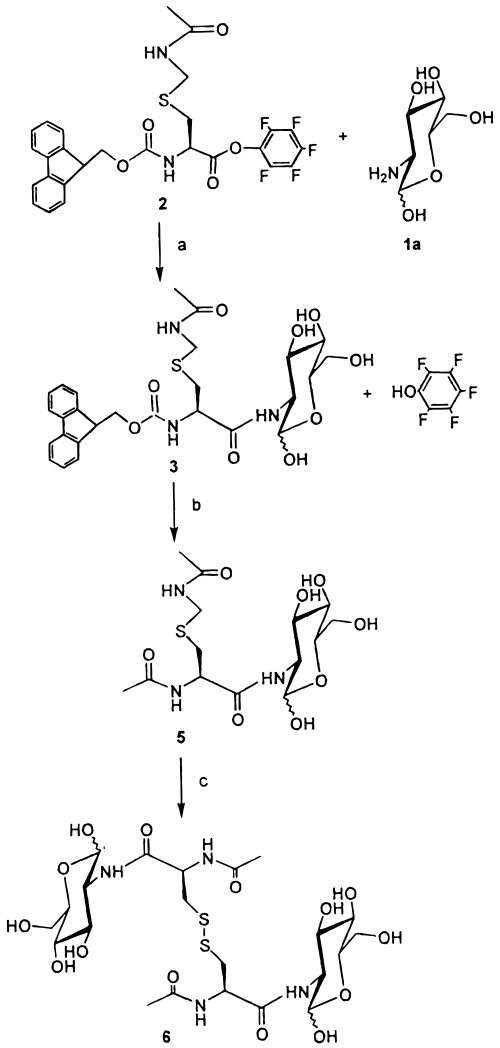
Scheme 1[a]



[a] (a) 2 equiv of hydroxybenzotriazole, 5 equiv α-D-glucosamine (free-base), DMF, room temperature, 2 h, 65%. (b) (1) 5% piperidine in DMF, room temperature 0.5 h, 77%; (2) 1.1 equiv AcO_2 over 0.5 h, 2 equiv K_2CO_3, H_2O, room temperature, 2 h, 66%. (c) 1.1 equiv $Tl(CF_3CO_2)_3$, 2 equiv anisole, TFA, 4 °C, 1 h, 28%.

that is specific for **6**. The rate and extent of reduction of **6** by a mycobacterial lysate is concentration-dependent (Figure 2a), and **6** exhibits Michaelis−Menten kinetics with a K_M value of ca. 400 μM. Analysis of the stoichiometry of the thiol product produced by the reductase using dithionitrobenzoic acid indicated the formation of 2 equiv of thiol formed per disulfide substrate **6** reduced.[9] The specific activity of the transformed *M. smegmatis* mc^2155, harboring the presumptive *M. tuberculosis* gene that encodes for mycothione reductase, is 31 nmol/min/mg, ca. 6-fold higher than that of wild-type *M. smegmatis* mc^2155.[10] The activity in *E. coli* was less than 0.65 nmol/min/mg, the lower limit for detection of the specific reduction of **6** under these assay conditions. The presence of **6** as a mixture of α and β diastereomers does not appear to affect the activity of the reductase, since the mixture was completely converted to product by MTR. The rate of reduction of glutathione disulfide by a cell-free extract of *M. smegmatis* was compared to that of *E. coli*,

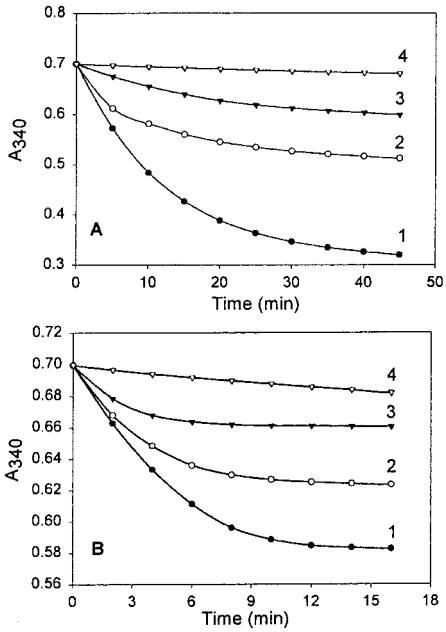


Figure 2. End-point assays of mycobacterial and *E. coli* lysates as function of [**6**] and [GSSG]. The enzymatic assays were performed in 50 mM HEPES, pH 7.6 containing 0.1 mM EDTA, 100 μM β-NADPH, and 55−75 μg total protein. (A) *M. smegmatis* lysate was used in the reactions for curves 1, 2, and 3: (1, ●) 50 μM **6**; (2, ○) 25 μM **6**; (3, ▼) 12.5 μM **6**; (4, ▽) 50 μM **6** + *E. coli* lysate. (B) *E. coli* lysate was used in the reactions for curves 1, 2, and 3: (1, ●) 20 μM GSSG; (2, ○) 10 μM GSSG; (3, ▼) 5 μM GSSG; (4, ▽) 20 μM GSSG + *M. smegmatis* lysate.

and the results indicate a specific activity of 80 nmol/min/mg for the *E. coli* lysate but less than 1.5 nmol/min/mg for *M. smegmatis* (Figure 2b). Trypanothione reductase activity was absent from both *M. smegmatis* and *E. coli* lysates, supporting the strict specificity of the mycobacterial reductase for **6**. These data indicate that the truncated disulfide substrate, lacking the *myo*-inositol moiety, provides sufficient features of the natural substrate to act as an active analogue of mycothiol. Moreover, we have cloned the *mtr* gene from *M. tuberculosis*, and expressed this enzyme in its close phylogenetic relative *M. smegmatis*.

The kinetic and mechanistic characterization of the *M. tuberculosis* mycothione reductase can now be approached, using the synthetic substrate whose synthesis is described here. These studies will provide a more detailed basis for the comparison of mycobacterial thiol metabolism to the corresponding metabolism in Gram-negative, Gram-positive, and unicellular parasites. The high degree of homology between the *M. tuberculosis* reductase and glutathione reductase (data not shown) suggests that there will be significant mechanistic and structural similarity. The long-term goal of inhibitor development will be assisted by the results of these initial studies.

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Supporting Information Available: Experimental details for the synthesis and spectral data for compounds **3**, **5**, and **6** (16 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(9) The reaction solution at end-point was filtered through a 3000 Da membrane filter (Amicon) and tested with 100 μM DTNB in 50 μM H_3BO_3, pH 9.0 (1000 mL final volume).

(10) Stover, C. K.; de la Cruz, V. F.; Fuerst, T. R.; Burlein, J. E.; Benson, L. A.; Bennett, L. T.; Bansal, G. P.; Young, J. F.; Lee, M. H.; Hatfull, G. F.; Snapper, S. B.; Barletta, R. G.; Jacobs, W. R., Jr.; Bloom, B. R. *Nature* **1991**, *351*, 456. (b) Harth, G.; Lee, B.; Horwitz, M. A. *Infect. Immun.* **1997**, *65(6)*, 2321.