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Total Synthesis and Proof of Structure of Mycothiol Bimane

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Mycothiol (1-D-*myo*-inosityl 2-deoxy-2-(*N*-acetamido-L-cysteinamido-α-D-glucopyranoside, MSH, Figure 1) is a low-molecular weight thiol[1a−c] produced only by actinomycetes,[2] and is of significant importance in that it appears to play an analogous role to glutathione in maintaining a reducing intracellular environment in these Gram-positive bacteria.[2] Given the increasing need for new classes of antituberculars, MSH is of considerable contemporary interest because of its role in the mycobacterial detoxification pathway, which requires the enzyme mycothiol *S*-conjugate amidase (MCA).[3] In detoxification, mycothiol reacts with alkylating agents to form *S*-conjugates that are subsequently cleaved at the glycosyl amide bond by MCA. Once cleaved, the *N*-acetyl-cysteine *S*-conjugate is rapidly exported from the cell,[3] while the pseudo-disaccharide fragment is recycled for incorporation into mycothiol.[4]

In addition to MCA, a homologous deacetylase (1-D-*myo*-inosityl-2-*N*-acetyl-amido-2-deoxy-α-D-glucopyranoside deacetylase) involved in the biosynthesis of MSH in *Mycobacterium tuberculosis* was recently reported.[4] Because these mycothiol-dependent pathways are not found in eukaryotes, the enzymes involved may represent new antimycobacterial targets. Inhibition of both enzymes would result in disruption of MSH-dependent detoxification pathways at two distinct levels: biosynthesis and detoxification.

Earlier, we reported a series of new bromotyrosine-derived natural products that inhibit MCA at micromolar concentrations.[5] While comparing the structures of these and other unpublished inhibitors to those of the natural substrates (e.g., refs 3, 4, 6, 7), we became aware of discrepancies in the literature as to the absolute stereochemistry of the pseudo-disaccharide portion of MSH.[8]

MSH was first isolated as the disulfide (MSSM) from *Streptomyces* sp. AJ9463[1a] followed by isolation of the free sulfhydryl (MSH) from *M. bovis*[1b] and the bimane derivative (**1**) from *S. clavuligerus*.[1c] While Sakuda et al.[1a] proposed the absolute stereochemistry of the three components of MSH as D-glucosamine, L-cysteine, and D-*myo*-inositol, experimental evidence for the stereochemical assignments of the glucosamine and cysteine residues was not provided.[9] Moreover, in papers published by several independent groups from 1997 onward, the structure of the *myo*-inositol portion of MSH has been depicted as 1-L while being referred to as 1-D. Of key importance, this discrepancy also occurred in papers describing partial syntheses and enzyme and substrate specificity. To resolve the ambiguities surrounding the absolute stereochemistry of MSH, we describe here the total synthesis, spectral characterization and kinetic properties with MCA of mycothiol bimane (**1**). Our overall strategy is summarized in Scheme 1.

The synthesis of 6-*O*-benzyl-2,3:4,5-di-*O*-cyclohexylidene-1-D-*myo*-inositol,[10] **2**, was accomplished by following the general scheme of Mayer and Schmidt,[11] with two modifications: Following

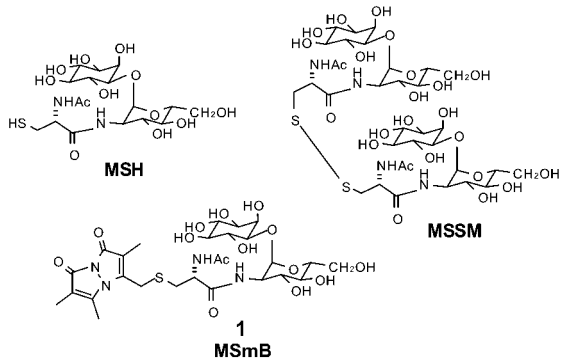
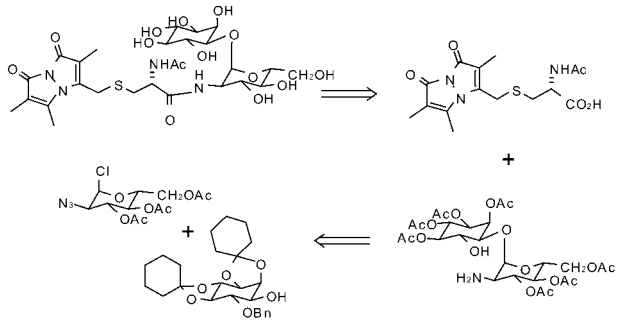


Figure 1. Mycothiol (**MSH**), the symmetric disulfide (**MSSM**) and mycothiol bimane (**MSmB, 1**).

Scheme 1



2:3,4:5-cyclohexylidene protection of D/L-*myo*-inositol, acylation with (−)-menthol chloroformate, and benzylation, the desired 1-D-*myo*-inositol fragment was obtained by recrystallization in petroleum ether.[12] Removal of the menthyl carbonate was then achieved by reduction with lithium aluminum hydride (Et_2O, 93%)[13] to give the protected inositol acceptor **2** in 26% overall yield.[14]

Glycosylation of **2** with 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-glucopyranosyl chloride[15] in the presence of silver triflate and 2,6-diisopropyl-4-methylpyridine (Scheme 2) gave a 2:1 ratio of α:*β* anomers in an approximate yield of 70%. The desired α-anomer (**3**) was isolated by column chromatography in 44% yield. Removal of the cyclohexylidine groups (CSA, ethylene glycol), followed by acetylation gave compound **4**.

Debenzylation and concomitant reduction of the azide with palladium on carbon in the presence of dilute hydrochloric acid gave amine hydrochloride **5**.

Derivatization of *N*-acetyl-L-cysteine with mono-bromobimane under basic conditions gave *N*-acetyl-L-cysteinyl monobimane **6** (Scheme 3). Coupling of amine **5** to **6** using Shioiri's reagent (diethylphosphoryl cyanide, DEPC)[16] in the presence of diisopropylethylamine gave amide **7**. Deacetylation was achieved in quantitative yields with $Mg(OMe)_2$ in methanol[17] to give the final product **1**.

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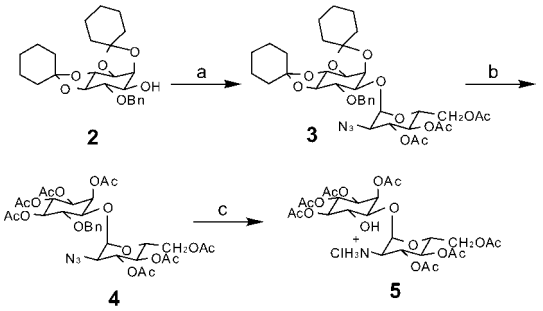
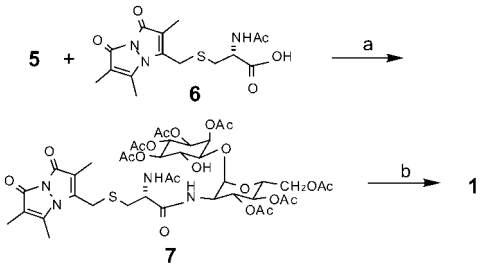
Scheme 2 [a]



[a] Reagents: (a) 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-glucopyrano-syl chloride, AgOTf, 2,6-diisopropyl-4-methyl-pyridine, CH_2Cl_2, 44%. (b) 1. ethylene glycol, (+)-camphor sulfonic acid, CH_3CN. 2. Ac_2O, pyridine 80%. (c) Pd−C, H_2, EtOAc, 86%.

Scheme 3 [a]



[a] Reagents: (a) DEPC, *i*Pr_2EtN, DMF, 31%.; (b) $Mg(OMe)_2$, MeOH (dry), 75%.

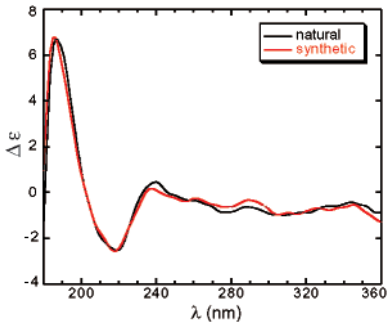


Figure 2. Molar CD of natural and synthetic MSmB (**1**). Spectra were recorded on 20 μM samples at 25 °C.

The ^{1}H and ^{13}C NMR data for synthetic mycothiol bimane (**1**) were identical to those recorded for natural material isolated from *M. smegmatis*[1c] (see Supporting Information). Moreover, the CD spectra (Figure 2) of natural and synthetic MSmB are superimposable and show negative ($\Delta\epsilon$ −2.3) and positive ($\Delta\epsilon$ +6.9) Cotton effects at 220 and 185 nm, respectively.[18]

To confirm substrate specificity of MCA, we measured in parallel the rates and extent of cleavage of the amide bond in natural and synthetic samples of MSmB by recombinant *M. tuberculosis* MCA using a fluorescence-detected HPLC assay.[3] Both samples were quantitatively cleaved within 15 min in the presence of 13 nM MCA. Kinetic studies with MCA using natural and synthetic MSmB yielded indistinguishable results with specific activities of 14 200 ± 700 nmol min^{-1} mg^{-1}, K_m values of 500 ± 30 mM, and K_{cat} values of 190 ± 30 s^{-1} (see Supporting Information). Thus, we have shown that the composition of mycothiol is 1-D-*myo*-inosityl 2-deoxy-2-(*N*-acetamido-L-cysteinamido)-α-D-glucopyranoside (**1**).

Acknowledgment. We thank Gerald Newton and Robert Fahey for natural mycothiol bimane, Noel Whitaker for mass spectrometry, Tarek Sammakia for the suggestion of recording NMR spectra on a mixed sample of natural and synthetic MSmB, and Andrew Phillips for many helpful discussions. This work was supported in part by the Intramural AIDS Targeted Antiviral Program of the Office of the Director, National Institutes of Health (C.A.B.).

Supporting Information Available: Details of enzyme kinetics; of the syntheses and analytical and spectroscopic data for all intermediates; ^{1}H and ^{13}C NMR spectra for new compounds, and for natural, synthetic and mixed samples of MSmB; and a complete table of NMR data for MSmB (**1**) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) The synthesis of a mixture of 1-L- and 1-D-*myo*-inosityl-2-amido-2-deoxy-α-D-glucopyranoside has been reported.[7] The authors used the isomeric mixture for glycosylation to give a 1:1 mixture of α- and β-linked products of the 1-D/L-*myo*-inositol mixture. After separation, the *myo*-inositol portion of the four products was assigned the 1-L- or 1-D-configuration by comparison of ^{1}H and ^{13}C NMR data with intact mycothiol, a strategy that does not permit unambiguous assignment of stereochemistry.
(9) The D configuration for *myo*-inositol was based on comparisons of the CD spectra of the 1-*O*-acetyl-2,3,4,5,6-penta-*O*-methyl derivative with those reported in the literature.[1a]
(10) The numbering scheme used throughout maintains C-1 of unsubstituted *myo*-inositol as C-1 in derivitives thereof.
(11) Mayer, T. G.; Schmidt, R. R. *Liebigs Ann./Recl.* **1997**, 859−863.
(12) We were unable to recrystallize the 1-D-*myo*-inositol fragment prior to benzylation, as reported in ref 11.
(13) Reaction time of 4 h versus 5 days at 40 °C in K_2CO_3 in methanol.[11]
(14) We note that we have completed the synthesis and characterization of D-GlcNAc-α-(1−1)-D-*myo*-inositol and D-GlcNAc-α-(1−1)-L-*myo*-inositol (unpublished data), and determined that the deacetylase enzyme used in the biosynthesis of MSH failed to deacetylate D-GlcNAc-α-(1−1)-L-*myo*-inositol, but cleaved the amide bond of GlcNAc-α-(1−1)-D-*myo*-inositol with identical kinetics as reported for the natural substrate.[4] Because this step forms a biosynthetic intermediate of mycothiol, we completed the synthesis of MSmB using the protected GlcNAc-α-(1-1)-D-*myo*-inositol derivitive.
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(18) No specific rotation of MSmB (**1**) has been reported for comparison. Our synthetic material showed a concentration-dependent sign of the specific rotation ($[\alpha]^{20}_D$ +10 (*c* 0.5 , H_2O), $[\alpha]^{20}_D$ −12 (*c* 0.1, H_2O)), leading us to record CD spectra for natural and synthetic MSmB. It is possible that the presence of the fluorescent bimane contributes to the change in sign since emission occurs through 590 nm, the wavelength of the sodium D line. (For a discussion of concentration-dependent changes in specific rotations, see Eliel, E. L. In *Stereochemistry of Organic Compounds*; Wilen, S. H., Mander, L. N., Eds.; Wiley & Sons: New York, 1994; pp 1071 ff.)

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