Biosynthesis of mycothiol: elucidation of the sequence of steps in *Mycobacterium smegmatis*

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Several members of the *Actinomycetales*, including the medically important mycobacteria, produce 1-D-*myo*-inosityl-2-(*N*-acetyl-L-cysteinyl)amino-2-deoxy- α -D-glucopyranoside (trivial name mycothiol) as their principal low-molecular-mass thiol. The pseudo-disaccharide component of mycothiol, 1-D-*myo*-inosityl-2-amino-2-deoxy- α -D-glucopyranoside (α -D-GI), was synthesized by ligation of 1-D,L-2,3,4,5,6-penta-*O*-acetyl-*myo*-inositol to 3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,4-dinitrophenylamino)- α -D-glucopyranosyl bromide to give, in the first instance, an isomeric mixture of α - and β -linked pseudo-disaccharides. The α -coupled D,D and D,L isomers, α -D-GI and α -L-GI respectively, were purified from the mixture by TLC, followed by removal of the protecting groups. A cell-free extract of *Mycobacterium*

smegmatis catalysed the ligation of cysteine, acetate and α -D-GI in the presence of ATP and Mg^{2+} to form mycothiol, as judged by HPLC. When no acetate was added to the incubation mixture, an additional thiol accumulated. In the presence of [^{14}C]acetate no radiolabel was recovered in this species, but only in mycothiol. The additional thiol was isolated as the bimane derivative, and 1H and 1H - 1H COSY NMR spectra confirmed its identity as desacetylmycothiol. A more complete conversion of desacetylmycothiol into mycothiol was achieved in the presence of acetyl-S-CoA. These results indicate that the biosynthesis of mycothiol proceeds by the sequential addition of cysteine and acetate to α -D-GI. The inositol moiety appears to be an important determinant of specificity, since α -L-GI was poorly utilized.

INTRODUCTION

All organisms capable of growth in an aerobic environment produce one or more low-molecular-mass thiols which are thought to be important for the maintenance of a reducing intracellular milieu. Studies on the distribution of these compounds in prokaryotes have brought to light fundamental differences between Gram-negative and Gram-positive bacteria [1,2]. As in the vast majority of eukaryotes, glutathione is the principal antioxidant thiol in Gram-negative bacteria. Fahey and co-workers [2,3] proposed that the need to incorporate cysteine into the form of this proteolytically stable tripeptide stemmed from the rapid rate of autooxidation of free cysteine, which generates peroxide as a harmful product.

Considerable evidence has accumulated that Gram-positive bacteria lack glutathione and, instead, produce alternative thiols. A number of actinomycetes produce 1-D-*myo*-inosityl-2-(*N*-acetyl-L-cysteinyl)amino-2-deoxy- α -D-glucopyranoside (Scheme 1), to which we have assigned the trivial name mycothiol [4–6]. Mycothiol is maintained in the reduced form by an NADPH-dependent mycothiol disulphide reductase [4]. Evidence has been presented that mycothiol is even less prone to metal-ion-catalysed autooxidation than is glutathione [5].

Interest in mycothiol derives from several considerations. The role of glutathione in bacteria is mainly a protective one, and mutants defective in its synthesis show significantly enhanced sensitivity to oxidative stress and alkylating reagents [7]. Studies on the enteric bacteria have demonstrated the presence of an *oxyR* gene which regulates the expression of several gene products in response to oxidative stress induced by peroxide [8,9]. Enzymes

involved in the metabolism of glutathione, particularly glutathione reductase, are among the gene products that are elevated.

Recent reports indicate that members of the *Mycobacterium tuberculosis* complex differ from the enteric bacteria in their response to reactive oxygen and nitrogen intermediates [10,11]. As a result of multiple mutations, the *oxyR* gene in *Mycobacterium*

Scheme 1 Structure of the bimane derivative of mycothiol

Abbreviations used: α -D-GI, 1-D-*myo*-inosityl-2-amino-2-deoxy- α -D-glucopyranoside; α -L-GI, 1-L-*myo*-inosityl-2-amino-2-deoxy- α -D-glucopyranoside; DMSM, 2,5-dimethoxystilbene-4'-maleimide; DTT, dithiothreitol; Et₂N(MalNPh)CMe, 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin.

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bacterium tuberculosis is inactive, and exposure to hydrogen peroxide does not elicit the response seen in the enterobacteria. However, the loss of a functional *katG* gene product during the development of isoniazid resistance, which would deprive the organism of a part of its antioxidant defence, is compensated for by an increased expression of alkylhydroperoxidase [12]. Since mycothiol is the major low-molecular-mass thiol of the mycobacteria, its functions are presumably analogous to those of glutathione, and enzymes involved in the metabolism of mycothiol could well play a role in these adaptive responses. The production and utilization of mycothiol are pathogen-specific processes that can potentially be exploited as drug targets. It is important to establish whether mycothiol is essential for the survival *in vivo* of pathogenic members of the *Actinomycetales*. There is, moreover, evidence that the disulphide reductases play a role in the reductive activation of prodrugs [13,14]. Mycothiol disulphide reductase is the most recently discovered of these enzymes.

In the present study we show that the pseudo-disaccharide 1-D-*myo*-inosityl-2-amino-2-deoxy- α -D-glucopyranoside (α -D-GI) is part of the biosynthetic pathway leading to mycothiol, which is formed by the sequential addition of cysteine and then acetate to α -D-GI.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma Chemical Co., Fluka or Aldrich, and were of the highest purity available. L-[³⁵S]Cysteine and sodium [2-¹⁴C]acetate were from Amersham, and were used within one half-life. 2,5-Dimethoxystilbene-4'-maleimide (DMSM) and 7-diethylamino-3-(4'-maleimidyl-phenyl)-4-methylcoumarin [Et₃N(MalNPh)CMe] were from Molecular Probes (Eugene, OR, U.S.A.).

Chemical synthesis of the isomers of α -GI

The pseudo-disaccharide moiety of mycothiol consists of D-glucosamine glycosidically coupled (α 1-1') to D-*myo*-inositol [4]. Such a coupling can be achieved synthetically, using an appropriately protected glycosyl donor and a *myo*-inositol acceptor. This synthetic route (Scheme 2) necessitated the selective protection of *myo*-inositol in such a manner that it renders the 1'-OH group available for glycosidic coupling. Commercially available *myo*-inositol (**1**; Scheme 2) was converted into 1,2:4,5-dicyclohexylidene-*myo*-inositol (**2**) (26%) [15]. The protected *myo*-inositol (**2**) was subsequently regioselectively benzylated to give the 3-benzylated product (**3**) (60%) [16]. Acid hydrolysis secured the removal of the cyclohexylidene groups to give the monobenzylated *myo*-inositol (**4**), which was subsequently acetylated to give 1-D,L-*O*-benzyl-2,3,4,5,6-penta-*O*-acetyl-*myo*-inositol (**5**). Reduction of the benzyl group gave 1-D,L-hydroxy-2,3,4,5,6-penta-*O*-acetyl-*myo*-inositol (**6**). Koenigs-Knorr coupling of product (**6**) with 3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,4-dinitrophenylamino)- α -D-glucopyranosyl bromide (**7**) [17] gave an isomeric mixture of α - and β -linked disaccharides (α/β , 1:1, w/w) (60%) (**8-11**). The α -coupled D,D (**8**) and D,L (**9**) isomers were successfully separated using TLC, whereas the β -coupled products were difficult to separate. Treatment of the α -coupled glycosides (**8**) and (**9**) with an anion-exchange resin (OH) gave the required products α -D-GI (**12**) and 1-L-*myo*-inosityl-2-amino-2-deoxy- α -D-glucopyranoside (α -L-GI) (**13**) respectively. Assignment of the stereochemistry of these compounds was based on their relationship with mycothiol, as determined by one- and

two-dimensional ¹H and ¹³C NMR spectroscopy. The total synthesis of mycothiol and the corresponding NMR data will be dealt with in detail in a separate paper.

Scheme 2 Chemical synthesis of the isomers of α -GI

See the text for details. Bn, benzyl. Agents used: i, 1-ethoxycyclohexene, p-toluenesulphonic acid and dimethylformamide; ii, NaH, BnBr and toluene; iii, 80% (v/v) acetic acid; iv, acetic pyridine; v, H₂ and Pd; vi, silver trifluoromethanesulphonate, 2,6-di-*tert*-butylpyridine and dichloromethane.

Bacterial culture

Stock cultures of *Mycobacterium smegmatis* A.T.C.C. 19420 were kindly provided by Mr. C. Snijman (South African Medical Research Council, Pretoria, South Africa), and were maintained on Löwenstein–Jensen slants. Liquid cultures were grown on modified Middlebrook medium consisting of 4.7 g/l dehydrated Bacto Middlebrook 7H9 broth (Difco), 0.5 g/l Tween-80 and 50 ml/l glycerol. Cultures were shaken in Erlenmeyer flasks at 200 rev./min at 37 °C. For primary cultures, a lump of bacteria was transferred from stock culture to 50 ml of liquid medium and grown for up to 1 week. For secondary cultures, 5 ml of densely grown primary culture was inoculated into 250 ml of medium and incubated overnight. The purity of the cultures was checked routinely with the Ziehl–Neelsen staining method.

Protein extraction

The bacteria from 0.5–1.0 litre of liquid culture were harvested in the exponential phase of growth and washed once with 50 mM sodium phosphate, pH 7.5, containing 1 mM dithiothreitol (DTT) (buffer A). The wet cells were suspended in 2 parts (v/w) of a mixture of acetone and buffer A in the proportions 7:100 (v/v) which contained 35 μ M each of the protease inhibitors PMSF, L-1-*p*-tosylamino-2-phenylethylchloromethane and tosyl-L-lysylchloromethane. The cells were disrupted by sonication for 15 min, and the crude homogenate was clarified by centrifugation for 60 min at 100 000 *g*. A clear golden-beige supernatant was obtained with some turbidity in the upper part. This supernatant was filtered and fractionated with ammonium sulphate. The fraction that was precipitated between 30 and 50 % saturation with $(\text{NH}_4)_2\text{SO}_4$ was redissolved in buffer A and dialysed for 3 h against a 1000-fold volume of buffer A, with one change of buffer after 90 min. This fraction contained enzymes that catalysed the formation of mycothiol from α -D-GI.

For initial experiments, cell breakage was carried out in 50 mM Na-Pipes, pH 7.5, containing 1 mM DTT and 17.5 μ M of the protease inhibitors, and the crude homogenate was clarified at low speed (15 min at 11 000 *g*). The turbid beige supernatant was assayed.

Enzyme assays

Assay mixtures for the detection of ligase activities contained 8 μ mol of sodium phosphate, pH 7.5, 160 nmol of DTT, 49 nmol of L-[35 S]cysteine (specific radioactivity 1.9 GBq/nmol), 12 nmol of α -D-GI, 200 nmol of ATP, 400 nmol of magnesium acetate and 0.3–1.5 mg of protein in a final volume of 0.2 ml. DTT was included to reduce the cystine present in the radiolabelled cysteine during a 30 min preincubation period, following which the reaction was started by addition of the enzyme protein. After a 15 min incubation at 30 °C, reactions were stopped by the addition of 0.1 ml of 0.75 M HClO_4 , and the mixtures were stored at –20 °C until analysed.

The reaction mixtures were thawed and clarified by centrifugation. A 50 μ l aliquot was neutralized with 25 μ l of 0.4 M Tris and treated for 5 min with 50 μ l of 2.5 mM DMSM in acetonitrile. An 80 μ l portion of the derivatized material was applied to a 250 mm $\times$ 4.6 mm Phenyl-Vydac HPLC column (Vydac 219TP54) which had been equilibrated with solvent A (700 ml of water, 300 ml of acetonitrile and 1 ml of trifluoroacetic acid) at a flow rate of 0.8 ml/min. The column was eluted for 11 min with solvent A, then for 2 min with a linear gradient to 100 % solvent B (acetonitrile) and for 5 min at 100 % solvent B, followed by a return to initial conditions. Fractions (1 min) were collected from 2 to 20 min and analysed by liquid scintillation counting. Product

formation was calculated as the percentage of the total radioactivity eluted that was recovered in the product fractions. The specific radioactivity of cysteine-DMSM in the eluate was estimated by comparison of the fluorescence peak area with that of a known amount of the DMSM derivative of cysteine, which had been quantified by the method of Grasetti and Murray [18].

Enzymic formation of 1-*D*-myo-inositol-2-(L-cysteinyl)amino-2-deoxy- α -D-glucopyranoside (desacetylmycothiol) and mycothiol for structural analysis

For the enzymic synthesis of desacetylmycothiol, the reaction mixture contained 0.3 mmol of Na-Pipes, pH 7.5, 12 μ mol of DTT, 6 μ mol of L-cysteine, 1 μ Ci of L-[35 S]cysteine, 3 μ mol of α -D-GI, 12 μ mol of ATP, 24 μ mol of MgCl_2 and 31 mg of enzyme preparation in a final volume of 6 ml. The mixture was pre-incubated for 30 min at 30 °C to reduce any cystine that might have been present, and the reaction was started by addition of the enzyme. The reaction was terminated after 90 min by the addition of 3 ml of 0.75 M HClO_4 . The pH of the sample was adjusted to 8.0 by the addition of solid K_2CO_3 . Thiols were then derivatized for 60 min at 40 °C with a 1.5-fold excess of bromobimane added in 0.1 vol. of acetonitrile. The derivatized sample was subjected to reverse-phase chromatography on a Vydac 218TP1022 preparative HPLC column equilibrated with solvent C (0.1 % trifluoroacetic acid) at a flow rate of 4 ml/min. After injection, the column was eluted for 10 min with 5 % solvent B (acetonitrile), then for 40 min with a linear gradient to 20 % solvent B, then for 5 min to 100 % B, followed by a return to initial conditions. After isolation, the bimane derivative of the major radiolabelled product was lyophilized.

The reaction mixture and conditions for the enzymic synthesis of mycothiol were identical to those described for its desacetyl derivative, except for the following modifications: Mg^{2+} was included as the acetate, the buffer was 0.13 mmol of sodium phosphate, pH 7.5, 48 mg of protein was used, and the mixture contained in addition 3 μ mol of acetyl-S-CoA. After termination of the reaction with perchloric acid, the product was first chromatographed as the free thiol, then derivatized with bromobimane and rechromatographed as the bimane according to a previously described protocol [4]. A total of 1.2 μ mol of synthetic mycothiol was isolated as the bimane and quantified by measuring the absorbance spectrum of the bimane moiety ($\epsilon = 5.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 387 nm).

In both cases, the residue from lyophilization of the bimane derivative was redissolved and lyophilized twice from $^2\text{H}_2\text{O}$. ^1H NMR spectroscopy was performed as described [19].

RESULTS AND DISCUSSION

Mycothiol formation in cell-free extracts of *M. smegmatis*

A crude undialysed cell-free extract of *M. smegmatis* catalysed the conversion of α -D-GI into a thiol that co-eluted with mycothiol as the $\text{Et}_3\text{N}(\text{MalNPh})\text{CMe}$ derivative. The reaction was dependent on ATP and α -D-GI. The omission of acetate from the assay mixture had only a minor effect on product formation (Figure 1). The experiment shown in Figure 1 was conducted using a concentration of α -D-GI that was saturating, and was allowed to proceed until depletion of the available cysteine. In most experiments, however, a much lower, unsaturating, concentration of 60 μ M α -D-GI was used in order to conserve this substrate.

The ligase activities responsible for the formation of mycothiol were recovered in the 100 000 *g* supernatant after centrifugation and, therefore, reside in the cytoplasmic fraction. The activities

Figure 1 Mycothiol formation by a crude, undialysed extract of *Mycobacterium smegmatis*

Assay mixtures contained 10 μ mol of Na-Pipes, pH 7.5, 1 μ mol of $MgCl_2$ and 1.5 μ Ci of [^{35}S]cysteine (3.33 mCi/mmol). When present, other components were: 0.5 μ mol of ATP, 0.5 μ mol of sodium acetate and 0.2 μ mol of α -D-GI. Reactions were started by the addition of 80 μ l of undialysed low-speed supernatant containing 1.84 mg of protein to obtain a final volume of 0.2 ml. After incubation for 2 h at 30 °C, the reaction was terminated by addition of $HClO_4$. Thiols were analysed as described in the Materials and methods section, except that $Et_2N(MaInPh)CMe$ was used instead of DMSM and the column was eluted as described in [19]. The traces represent elution profiles for assay mixtures that contained all components (solid line), no acetate (·-·-·), no ATP (· · · · ·) or no α -D-GI (---). Scintillation counting of the fractions showed that radiolabel was incorporated mainly into a species co-eluting with mycothiol.

were concentrated and partially purified by ammonium sulphate fractionation as described in the Materials and methods section. The preparation obtained after dialysis was used for further experiments. At the low concentration of the pseudo-disaccharide used, the rate of mycothiol formation was not linear with time,

Figure 2 Thiol species obtained as the DMSM derivatives in assays performed at low acetate concentrations

The assay and conditions for reverse-phase chromatography were as described in the Materials and methods section, but variable acetate concentrations and an incubation time of 30 min were used. Magnesium acetate was replaced by $MgCl_2$. The elution profiles shown are for samples to which either no acetate had been added (lower trace) or sodium acetate had been added at 0.4 μ mol per assay (upper trace). Note that the upper trace is displaced towards the right.

Figure 3 Effect of acetate concentration on the recovery of radiolabel in fractions 1 and 2 of Figure 2

Experimental conditions were as described in the legend to Figure 2. The symbols represent desacetylmycothiol (fraction 1; ▼), mycothiol (fraction 2; ▲) and their sum (○).

as may be expected. A 2-fold increase in the concentration of ATP did not alter the rate of product formation, indicating that the concentration of ATP used was saturating.

Order in which acetate and α -D-GI are ligated to cysteine

The question of whether *N*-acetylcysteine could serve as a substrate for the formation of mycothiol could not be addressed directly, since it was rapidly converted into cysteine by the relatively crude preparations. Therefore the dependence of mycothiol formation on acetate concentration was investigated. The HPLC method described in the Materials and methods section was modified to achieve adequate resolution of the DMSM derivatives of mycothiol and cysteine. As shown in Figure 2, two products with retention times of about 7 min and 9 min were obtained at low acetate concentrations. Fraction 2 co-eluted with mycothiol at 9 min and was poorly separated from a small amount of material that was not labelled with [^{35}S]cysteine. The contribution of this material did not change with the time of incubation or with the concentration of α -D-GI. Whereas the sum of the radioactivity recovered in the two fractions was invariant with acetate concentration (Figure 3), their relative contributions varied. When no acetate was included in the assay, 65 % of product radioactivity was recovered in the 7 min peak (fraction 1) during a 30 min incubation period; this de-yclined to 18 % in the presence of 4 mM acetate (Figure 3). When the incubation period was only 15 min, the corresponding values were 75 % and 38 % of product recovered in fraction 1. These results suggest that the product recovered in fraction 1 is a precursor, most probably desacetylmycothiol, rather than a degradation product of mycothiol. A higher proportion of radiolabel was also recovered in fraction 1 at higher α -D-GI concentrations, which would have had the effect of increasing the rate of formation of the precursor. Further experiments were undertaken to define the conditions required for the formation of the two products more unambiguously. Prolonged dialysis of the ammonium sulphate fraction (24 h, with several buffer changes) failed to eliminate the formation of mycothiol completely. It is possible that acetate is formed from cysteine during the assay, since a loss of radiolabel derived from [^{35}S]cysteine was observed. Up to 25 % of the radiolabel was converted into hydrogen sulphide, reaching equilibrium within 1 h, and was then not recovered in the acid stop mixtures. Such decomposition of cysteine, which leads to the formation of acetate via pyruvate,

Figure 4 Distribution of reaction products between fractions 1 and 2 at elevated concentrations of the reactants

Reaction mixtures were as described in the Materials and methods section, but contained 0.1 μmol of α -D-Gl, 0.2 μmol of cysteine (specific radioactivity 0.5 Gbq/mmol), 0.16 μmol of DTT, 0.8 μmol of Mg^{2+} , either as the acetate (**A** and **B**) or the chloride (**C**), and 1.6 mg of enzyme protein in a total volume of 0.2 ml. Reaction mixtures represented in (**B**) contained in addition 0.1 μmol of acetyl-S-CoA. Aliquots were withdrawn at the indicated times. The symbols represent desacetylmicothiol (fraction 1; ▼), mycothiol (fraction 2; ▲) and their sum (○).

could be due to known reaction sequences involving enzymes such as cysteine desulphydrase that occur in micro-organisms [20,21].

Additional evidence for the identity of fraction 1 as desacetylmicothiol was obtained by performing the incubation in the presence of [2- ^{14}C]acetate. As expected, radiolabelled acetate was incorporated into fraction 2 (mycothiol), but not into fraction 1. When higher concentrations of all reactants (other than the ammonium sulphate fraction and the buffer) were used in the assays, the conversion of desacetylmicothiol into mycothiol was incomplete even in the presence of an excess of acetate (Figure 4). Instead a requirement for acetyl-S-CoA for the formation of mycothiol became evident (Figure 4B). The incorporation of [^{14}C]acetate into mycothiol probably proceeded through the intermediate ATP-dependent formation of catalytic amounts of acetyl-S-CoA by reactions that have been well demonstrated in microbial extracts [22].

Figure 5 Separation of the products of the enzymic reaction as the bimeane derivatives

Assay conditions were as described in the Materials and methods section, but the incubation time was 2 h. Samples were neutralized with K_2CO_3 and derivatized by incubation with 0.1 vol. of 50 mM bromobimane in acetonitrile for 40 min at 40 °C. Derivatized thiols were separated on a C_{18} reverse-phase column (Vydac 218TP54) at a flow rate of 0.8 ml/min. The column was eluted for 5 min with 10% solvent B, for 15 min with a linear gradient to 25% B, for 2 min with a linear gradient to 100% B, and for 5 min at 100% B, followed by a return to initial conditions (A, 0.1% trifluoroacetic acid; B, acetonitrile). Fractions of 1 min were collected and radioactivity was determined by scintillation counting. (**A**) Elution profile in the absence of α -D-Gl in the assay mixture; (**B**) elution profile obtained with the complete assay mixture; (**C**) profile when acetate was omitted. Mycothiol co-eluted at 11.5 min together with an unknown radiolabelled species which was also present in the absence of α -D-Gl. The species corresponding to fraction 1 in Figure 2 was eluted at 6 min, and is most clearly shown in (**C**).

More definitive evidence for the identities of fractions 1 and 2 was obtained by NMR spectroscopy of the bimeane derivatives. The bimeane derivatives of thiols present in the enzymic reaction mixtures were separated by C_{18} reverse-phase HPLC (Figure 5). The thiol species corresponding to fraction 1 in Figure 2 was eluted in this system at 6 min, cysteine at 8 min and mycothiol at 11.5 min. Conditions could, therefore, be established for the isolation of the thiol component present in fraction 1 by chromatography on a Vydac 218TP1022 preparative column. ^1H NMR spectroscopy confirmed the presence of resonances attributable to the inositol and glucosamine moieties, while the resonances centred around δ 2.922 p.p.m. showed a splitting pattern characteristic of the AB part of the ABX spin system expected for the β protons of amino acids (Figure 6). Resonances due to the α proton were hidden under those due to the pseudo-disaccharide moiety, but were readily identified in a ^1H - ^1H COSY spectrum (Figure 7) as being centred around δ 3.647 p.p.m. The ^1H NMR spectrum (Figure 6) of the putative precursor was, therefore, very similar to that reported earlier for mycothiol, but differed mainly due to the absence of the methyl group attributable to the *N*-acetylcysteine moiety and the upfield shift observed for the α proton due to the absence of the *N*-acetyl group (Table 1). This confirmed the assignment of fraction 1 as desacetylmicothiol. The thiol component giving rise to fraction 2 was isolated as the bimeane derivative using a two-stage protocol, as outlined in the Materials and methods section, and its ^1H NMR spectrum was found to be identical to that of mycothiol

Figure 6 ^1H NMR spectrum (300 MHz) of the bimane derivative of the intermediate (fraction 1) produced *in vitro* at low acetate concentrations

Sodium 4,4-dimethyl-4-silapentane-1-sulphonate at δ 0.0 p.p.m. was used as external reference. The analysis was performed on 0.48 μmol of the bimane derivative, as estimated from the absorbance of the bimane moiety ($\epsilon = 5.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). The solvent was $^2\text{H}_2\text{O}$ and the experiment was conducted at 25 $^\circ\text{C}$.

Figure 7 ^1H – ^1H COSY spectrum of the bimane derivative of the intermediate produced *in vitro* at low acetate concentrations

The experimental conditions were as described in the legend to Figure 6.

(Table 1 and [4]). This result demonstrates conclusively that α -D-GI is on the biosynthetic pathway leading to mycothiol.

Specificity of α -D-GI–cysteine ligase

The dependence of product formation on the concentrations of the two isomers of α -GI is shown in Figure 8. Product formation was calculated as the sum of radiolabel recovered in fractions 1 and 2 (Figure 2) and reflects the rate of catalysis by the first enzyme, GI–cysteine ligase. For the α -D- isomer, $V_{\text{max}}(\text{app})$ was $0.92 \pm 0.02 \text{ nmol/min per mg}$ and $K_{\text{m}}(\text{app})$ was $140 \pm 9 \mu\text{M}$,

Table 1 ^1H (300 MHz) spectral data of the bimane derivatives of mycothiol and desacetylmicothiol

Spectra were recorded at 25 $^\circ\text{C}$ in $^2\text{H}_2\text{O}$. Chemical shifts were measured relative to sodium 4,4-dimethyl-4-silapentane-1-sulphonate at δ 0.0 p.p.m. as external reference.

H	δ (p.p.m.)	
	Desacetylmycothiol bimane	Mycothiol bimane
Glucosamine*		
1	5.108 (d, 3.6)	5.093 (d, 3.7)
2	3.929 (dd, 10.7/3.6)	3.937 (dd, 10.8/3.7)
3	3.804 (dd, 10.7/8.9)	3.787 (dd, 10.8/8.9)
4	3.462 (dd, 9.7/8.9)	3.452 (dd, 9.9/8.9)
Inositol		
1'	3.542 (dd, 10.0/2.8)	3.549 (dd, 10.1/2.8)
2'	4.176 (t, 2.8)	4.162 (t, 2.8)
3'	3.493 (dd, 10.0/2.8)	3.496 (d, 9.9/2.8)
4'	3.601 (dd, 10.0/9.2)	3.603 (dd, 9.9/9.2)
5'	3.247 (t, 9.3)	3.259 (t, 9.2)
6'	3.738 (dd, 10.0/9.5)	3.736 (dd, 10.1/9.2)
N-Acetylcysteine		
α	3.647†	4.542 (dd, 8.4/5.3)
β_A	2.963 (dd, 13.4/5.9)	3.118 (dd, 13.9/5.3)
β_B	2.881 (dd, 13.4/5.6)	2.938 (dd, 13.9/8.4)
CH_3 (E)	—	2.011 (s)
Bimane		
D_A	3.888 (bs)	3.929 (d, 15.0)
D_B	3.888 (bs)	3.847 (d, 15.0)
CH_3 (A)	1.857 (s)	1.850 (s)
CH_3 (B)	1.774 (q, 0.8)	1.776 (q, 0.9)
CH_3 (C)	2.437 (q, 0.8)	2.433 (q, 0.9)

* Resonances for the three protons on carbons 5 and 6 were not assigned due to substantial overlap of lines.

† Obtained from the ^1H – ^1H COSY spectrum.

Figure 8 Dependence of the rate of product formation on the concentration of the two isomers of $\alpha\text{-GI}$

Assays were conducted as described in the Materials and methods section, but at variable concentrations of $\alpha\text{-D-GI}$ (●) or $\alpha\text{-L-GI}$ (■). The data were fitted to a hyperbola using Sigma Plot software.

Scheme 3 Proposed sequence of steps in the biosynthesis of mycothiol

while the corresponding values for the $\alpha\text{-L}$ -isomer were 0.51 ± 0.22 nmol/min per mg and 2.69 ± 1.34 mM respectively. This finding indicates a pronounced selectivity for the natural

substrate, particularly with regard to the configuration of the inositol moiety, as compared with the $\alpha\text{-L}$ - isomer.

The results reported here indicate that the biosynthesis of mycothiol proceeds by the sequential addition of glucosamine, L-cysteine and acetate to inositol, as shown in Scheme 3. The biosynthesis of $\alpha\text{-D-GI}$ has not yet been characterized, but it is evident that the final two steps, involving the addition of cysteine and acetate to the pseudo-disaccharide, take place in the cytoplasm. In the biosynthesis of glycosylphosphatidylinositol anchors in eukaryotes, glucosamine is ligated to phosphatidylinositol using UDP-*N*-acetylglucosamine [23]. The acetyl group of the *N*-acetylglucosamine moiety is subsequently removed by an acetylase. More than one enzymic reaction may also be involved in the formation of $\alpha\text{-D-GI}$ in the actinomycetes, although, of course, the position of substitution at inositol in $\alpha\text{-D-GI}$ is not the same as in the glycosylphosphatidylinositol anchors.

The present study paves the way for the characterization of the newly discovered enzyme activities and for the design of potential inhibitors of the biosynthesis of mycothiol, which in turn could be of value in studies of its function. Strategies for the design of ligase inhibitors were evolved by Meister and his colleagues [24] and were further elaborated in studies of D-Ala-D-Ala ligase [25], an enzyme involved in peptidoglycan biosynthesis.

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