Natural Products

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Chemical and Chemoenzymatic Syntheses of Bacillithiol: A Unique Low-Molecular-Weight Thiol amongst Low G + C Gram-Positive Bacteria**

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In eukaryotes and Gram-negative bacteria, the cysteinyl tripeptide glutathione (GSH, Figure 1) is the predominant low-molecular-weight thiol. It plays a critical role in maintaining an intracellular reducing environment and serves many other important metabolic functions.[1] For instance, the reversible formation of GS-*S*-protein disulfides (glutathionylation) is an important post-translational modification for regulating protein function and protecting exposed cysteine residues from irreversible oxidative damage.[2] Glutathione-*S*-transferases also mediate xenobiotic detoxification by *S* conjugation with GSH. Most Gram-positive bacteria lack GSH, but instead produce other, distinctly different low-molecular-weight thiols. Gram-positive high G + C content actinobacteria produce mycothiol (MSH, Figure 1), which serves analogous functions to GSH.[3] Low G + C Gram-positive bacteria (Firmicutes) produce neither GSH nor MSH and until recently the identity of their major, cysteine-derived, low-molecular-weight thiol has been elusive. In 2007, an unknown 398 Da thiol was observed in *Bacillus anthra*cis cell extracts[4] and in *Bacillus subtilis* as a mixed disulfide with the redox controlled *ohr* regulator protein (OhrR).[5] The same thiol was subsequently isolated by treating *Deinococcus radiodurans* cell extracts with monobromobimane (mBBr) from which the structure of bacillithiol (BSH, **1**) was then elucidated as its corresponding fluorescently labeled *S*-bimane (mB) derivative BSmB (**3**, Figure 1).[6]

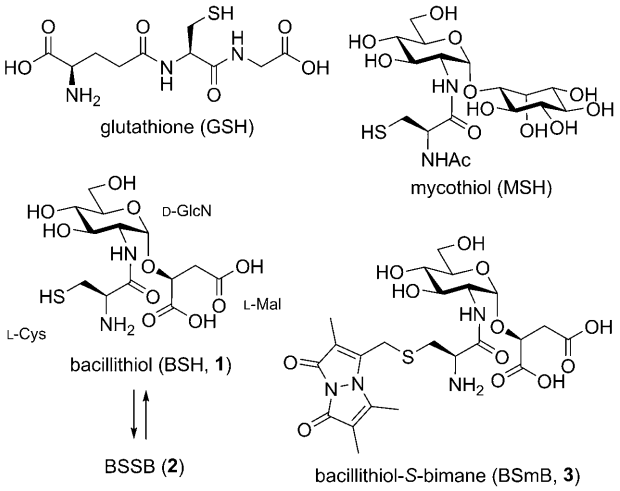

Figure 1. Structures of bacillithiol (BSH, **1**, with its subunits indicated, and its conversion into its disulfide BSSB, **2**), bimane derivative (BSmB, **3**), and related redox thiols GSH and MSH.

BSH (**1**) occurs in several clinically important pathogens including *B. anthra*cis, *Bacillus cereus*, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, and *Streptococcus agalactiae*, which do not produce GSH or MSH.[6] Evidence is now emerging suggesting that BSH (**1**) plays an important role in metabolic regulation including sporulation,[7,8] acid and salt resistance, and detoxification of electrophilic xenobiotics such as fosfomycin.[7,9] Thiol-*S*-transferase fosfomycin resistance genes (*fosB*) have been detected in many BSH-producing bacteria.[10] FosB is mechanistically related to the glutathione-*S*-transferase, FosA, which catalyzes the *S* conjugation of GSH with the epoxide motif of fosfomycin.[11] However, cysteine and GSH are extremely poor substrates for FosB.[12,13] BSH deficient mutant strains of *B. subtilis*[7] and *B. anthra*cis[9] exhibit markedly increased sensitivity to fosfomycin. This implies that FosB is a bacillithiol-*S*-transferase. Additionally, several bacillithiol disulfide (BSSB, Figure 1) reductases have been proposed,[7] that could help maintain intracellular BSH:BSSB redox ratios (ca. 200:1),[6] but further analyses of these requires access to the substrate BSSB (**2**).

Recent studies have shown that the biosynthesis of BSH (**1**), is initiated by a retaining glycosyltransferase (BshA) that catalyses the glycosylation of L-malic acid with UDP-GlcNAc to afford D-GlcNAc-L-Mal (**4**, Figure 2).[7,9] An *N*-acetyl hydrolase (BshB) then liberates the free amine D-GlcN-L-

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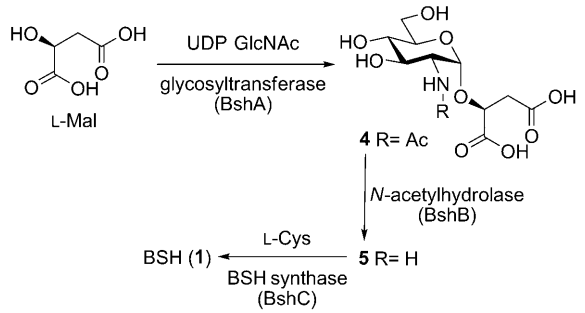


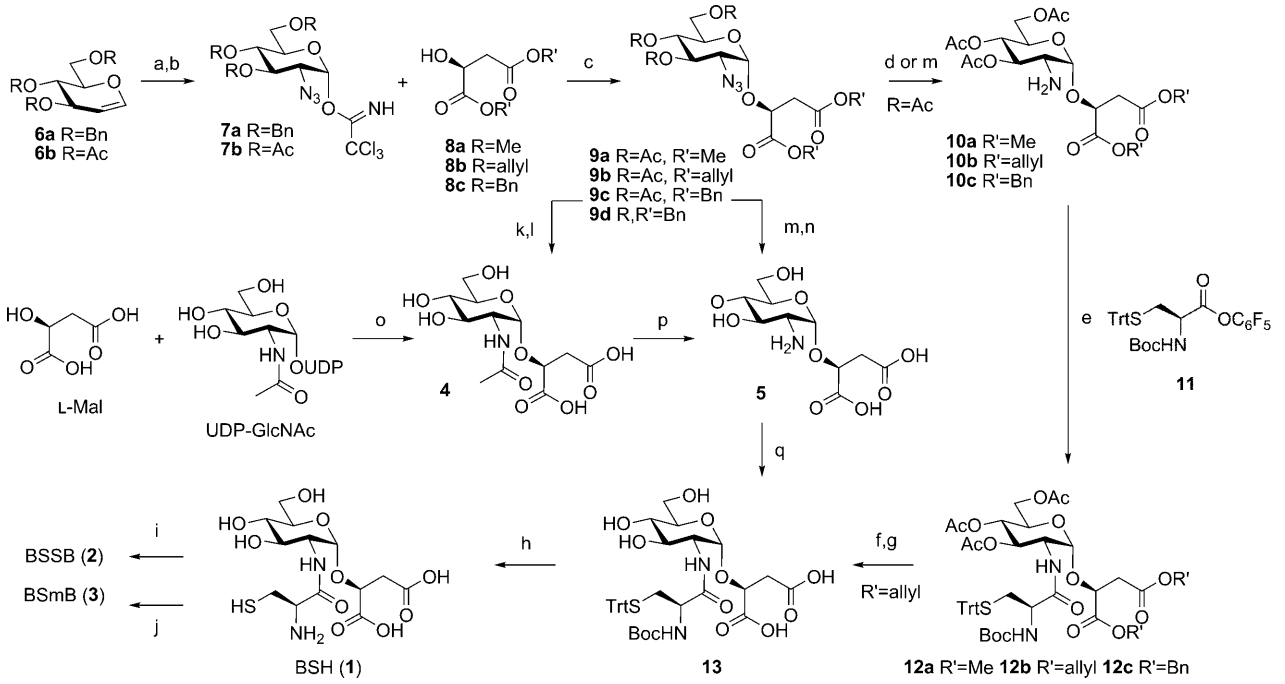
Figure 2. Biosynthetic pathway to bacillithiol (BSH, **1**).

Mal (**5**).[7,9] Gene knockout studies in *B. subtilis* have identified a bacillithiol synthase (BshC),[7] which mediates the condensation of D-GlcN-L-Mal (**5**) with L-cysteine to deliver BSH (**1**), but the limited availability of **5**, has limited studies of this enzyme.

To date, BSH (**1**) has only been isolated as BSmB (**3**) from *D. radiodurans* in a yield of approximately 50 $\mu g L^{-1}$ of cell culture.[6] A synthetic route to BSH (**1**), as well as its biosynthetic precursors **4** and **5**, is clearly needed to further study its biosynthesis and to reveal its metabolic functions. Towards this goal, we report complementary chemical and chemoenzymatic methods for the synthesis of BSH (**1**), its biosynthetic intermediates D-GlcNAc-L-Mal (**4**) and D-GlcN-L-Mal (**5**, Figure 2), and its symmetrical disulfide BSSB (**2**, Figure 1).

Initial efforts focused on the per-benzylated glycoside **9d** (Scheme 1). Me_3SiOTf promoted coupling of per-benzylated trichloroacetimidate donor **7a**[14] with dibenzylmalate **8c** provided glycoside **9d** as an anomeric mixture. However, the desired α-glycoside could not be separated by chromatography. Considering the difficulties encountered with debenzylation of fully protected BSH precursors (see below) this route was pursued no further.[14]

Under the same coupling conditions, per-acetylated donor **7b**[15] delivered α-glycosides **9a–9c** from the corresponding dimethyl-, diallyl-, or dibenzyl-L-malate esters, **8a–8c**, in good yields and with excellent anomeric selectivity (α:β ratios in most cases greater than 95:5). Anomerically pure samples of **9a–9c** were readily obtained by recrystallization or flash chromatography. Reduction of the azide moiety in **9a–9c** afforded the free amines **10a–10c** and subsequent coupling with cysteinyl pentafluorophenyl ester **11** provided the fully protected BSH derivatives **12a–12c**. Deprotection strategies for each of **12a–12c** were explored as follows. Attempts to



Scheme 1. Synthesis of BSH (**1**) along with its conversion into BSSB (**2**) and fluorescently labeled BSmB (**3**) is achieved by a route that also provides access to biosynthetic precursors **4** and **5**. Reagents and conditions: a) CAN (2.6 equiv), NaN_3 (2.0 equiv), CH_3CN, then H_2O; b) NaH, Cl_3CCN, THF, 0°C, **7a** 24% and **7b** 67% over two steps; c) Me_3SiOTf (0.5 equiv), CH_2Cl_2, −30°→−10°C, 3–5 h, **9a** 72% (only α), **9b** 84% (α:β 95:5), **9c** 90% (α:β 96:4), **9d** 37% (α:β 31:69); d) Ph_3P (3 equiv), THF, H_2O (8:1), room temperature, 15 h; e) **11** (1.2 equiv), HOBT (2 equiv), Et_3N (2 equiv), DMF, room temperature, 6–18 h, **12a** 66%, **12b** 35%, **12c** 37% over two steps; f) $[Pd(PPh_3)_4]$ (10 mol%), imidazole (10 equiv), THF, room temperature, 14 h; g) NaOMe, MeOH, 0°C, 75 min, 68% over two steps; h) TFA, Et_3SiH, CH_2Cl_2 (80:2:20), 0°C, 20 min, 99%; i) aq NH_4HCO_3, 1 h, 99%; j) mBBr, $NaHCO_3$, CH_3CN, H_2O, 2 h, 90%; k) Ac_2O, Pd/C, Et_3SiH, EtOAc, room temperature, 12 h; l) NaOMe, MeOH, 0°C, 50% over two steps; m) Pd/C, H_2 (50 psi), EtOAc, aq HCl; n) NaOMe, MeOH, 0°C, 48% over two steps; o) BshA, L-malate, UDP-GlcNAc, 0.1 M NaCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 25 mM HEPES pH 7.5, 20 h 37°C, 93%; p) BshB, pH 7, 23 h 37°C, 90%; q) **5**, Boc-Cys(Trt)CO_2H, HATU, $EtNiPr_2$, DMF, 15%. Abbreviations: DMF = *N*,*N*-dimethylformamide, HATU = 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOBT = 1-hydroxybenzotriazole, UDP = uridine 5′-diphosphate, TFA = trifluoroacetic acid, Boc = *tert*-butyloxycarbonyl, HEPES (buffer: 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid), CAN = cerium(IV) ammonium nitrate, OTf = triflate (CF_3SO_3).

remove the *O*-acetate and methyl ester protecting groups from **12a** under a variety of conditions (i.e., $Ba(OH)_2$, LiOH, KOH, Et_3N) proved problematic owing to cleavage of the malate aglycone (see Table 6 in the Supporting Information). Attempts to cleanly remove the benzyl esters from **12c** under a various reducing conditions were also unsuccessful (Table 7 in the Supporting Information).

A successful route to BSH (**1**) was finally established via **12b** (Scheme 1) whose allyl esters were readily removed using catalytic $[Pd(PPh_3)_4]$ in the presence of excess imidazole.[16] Subsequent removal of the acetate protecting groups under mild Zemplén conditions provided **13**. Unlike the hydrolysis of **12a**, this two-step process avoided loss of the malate aglycone. The acid-labile protecting groups on the cysteine moiety of **13** enabled the thiol to be unveiled in the final step under non-basic conditions preventing purification complications that would otherwise be anticipated due to partial oxidation of BSH (**1**) to BSSB (**2**).[17] Using this sequence, BSH (**1**) was obtained from **12b** in 67% yield over three steps. When dissolved in aqueous NH_4HCO_3,[18] BSH (**1**) was readily oxidized to provide BSSB (**2**) in quantitative yield. A sample of BSH (**1**) was converted to its *S*-bimane derivative BSmB (**3**) for comparison with an authentic sample isolated from *D. radiodurans* cell lysates.[6] A combination of comparative NMR spectroscopy, circular dichroism (CD), and HPLC analyses of synthetic and natural BSmB (**3**) were in agreement, thereby confirming the structure of BSH (**1**).

This synthesis also provided access to the biosynthetic intermediates D-GlcNAc-L-Mal (**4**) and D-GlcN-L-Mal (**5**), which were effectively prepared from **9c** (Scheme 1). An alternative chemoenzymatic route to BSH (**1**) was also developed (Scheme 1). Incubation of recombinant BshA (*B. subtilis*) with stoichiometric ratios of commercially available L-Mal and UDP-GlcNAc afforded D-GlcNAc-L-Mal (**4**) in excellent yield after purification by HPLC. Recombinant BshB (*B. anthra*cis)[9] was then used to *N*-deacetylate **4** delivering D-GlcN-L-Mal (**5**). Enzymatically synthesized D-GlcN-L-Mal (**5**) was further elaborated to intercept our total synthetic route to BSH (**1**). Chemical coupling of **5** with Boc-Cys(Trt)-OH provided pure **13** after HPLC purification, which was spectroscopically identical to that prepared through the total synthetic route.

While this chemoenzymatic route requires an expensive building block, UDP-GlcNAc, its short and stereospecific nature makes it attractive for use in laboratories equipped for enzymatic methods. Furthermore, it is quite likely that combined multi-step enzymatic strategies[19] may also allow the production of **4** or **5** in a single step from GlcNAc, thereby further optimizing the procedure. Once the function of the final biosynthetic enzyme (BshC) has been proven it should be possible to enzymatically prepare BSH using BshA-C. Access to synthetic BSH (**1**) has now enabled us to enzymatically verify that FosB functions as a bacillithiol-*S*-transferase in the detoxification of fosfomycin (Figure 3a). Before BSH (**1**) was discovered,[6] L-cysteine was reported to be a weak substrate with genomically encoded FosB from *B. subtilis*.[12] Using the homologous *S. aureus* FosB (59% sequence identity), we have now compared the relative substrate activities of physiologically relevant concentrations of L-cysteine and synthetic BSH (**1**) in FosB-catalyzed *S* conjugation with fosfomycin. The results clearly demonstrate that BSH (**1**) is the preferred thiol substrate (Figure 3b) for FosB, which has long been known to confer fosfomycin resistance in many clinically important Staphylococci.[10]

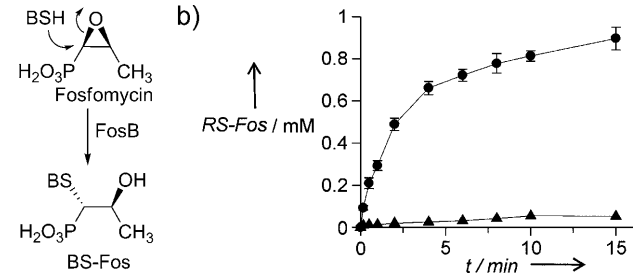


Figure 3. a) Mechanism of FosB-catalyzed inactivation of fosfomycin; b) time course for SaFosB-catalyzed fosfomycin-*S*-conjugate formation. Reagents and conditions: 20 mM HEPES (pH 7), 1 mM $MgCl_2$, 2 mM fosfomycin, 500 nM FosB, and 1 mM BSH (●) or 1 mM Cys (▲), 22 °C.

Herein, we have reported the first preparations and spectroscopic characterization of BSH (**1**) as its native free thiol and further confirmed the structure of BSH (**1**) and BSmB (**3**) as originally assigned.[6] Access to BSH (**1**), BSSB (**2**), as well as the biosynthetic intermediates D-GlcNAc-L-Mal (**4**) and D-GlcN-L-Mal (**5**) will now enable the detailed elucidation of the biosynthesis and metabolic functions of this unique biothiol amongst numerous bacteria of medical and industrial significance.

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