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Synthesis of Bacillithiol and the Catalytic Selectivity of FosB-Type Fosfomycin Resistance Proteins

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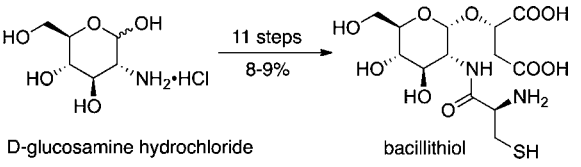
ABSTRACT



Bacillithiol (BSH) has been prepared on the gram scale from the inexpensive starting material, D-glucosamine hydrochloride, in 11 steps and 8−9% overall yield. The BSH was used to survey the substrate and metal-ion selectivity of FosB enzymes from four Gram-positive microorganisms associated with the deactivation of the antibiotic fosfomycin. The *in vitro* results indicate that the preferred thiol substrate and metal ion for the FosB from *Staphylococcus aureus* are BSH and Ni(II), respectively. However, the metal-ion selectivity is less distinct with FosB from *Bacillus subtilis*, *Bacillus anthracis*, or *Bacillus cereus*.

Low-molecular-weight thiols are critical to living systems for maintaining a reducing environment in the cytosol and preventing oxidation of cysteine residues in proteins. Eukaryotes typically utilize glutathione for these purposes while most Gram-positive bacteria and Archae lack glutathione and instead utilize novel cysteine derived thiols.[1] For example, the *N*-acetyl cysteine derivative mycothiol (MSH, Figure 1) is found in most actinomycetes including mycobacteria and streptomycetes.[2] In 2009 a structurally related thiol, bacillithiol (BSH, Figure 1), was detected in Gram-positive bacteria, including *Bacilli* and *Staphylococcus aureus* and then isolated from *Deinococcus radiodurans* and characterized as its S-bimane derivative (BSmB, Figure 1).[3,4] Subsequently, a biosynthetic pathway for BSH was proposed and the first chemical synthesis was reported.[5,6]

Although the functions of BSH in biology are not fully understood, recent reports suggest that it may be the preferred substrate for the FosB-type fosfomycin resistance proteins.[5,6] FosB is a metallo-enzyme, found in Gram-positive microorganisms, that catalyzes the general reaction illustrated in Scheme 1 and confers resistance to the antibiotic, fosfomycin.[7] Previous work in our laboratory suggested that the preferred thiol substrate and divalent metal ion might be L-Cys and Mg^{2+}, respectively.[7] The discovery of BSH and the availability in our lab of FosB enzymes from several Gram-positive microorganisms prompted us to initiate an investigation of their substrate and metal-ion selectivity. In this report we describe a complete chemical synthesis of BSH from inexpensive

(1) (a) Fahey, R. C. *Annu. Rev. Microbiol.* **2001**, *55*, 333. (b) Masip, L.; Veeravalli, K.; Georgioui, G. *Antioxid. Redox Sign.* **2006**, *8*, 753.
(2) Jothivasan, V. K.; Hamilton, C. J. *Nat. Prod. Rep.* **2008**, *25*, 1091.
(3) Newton, G. L.; Rawat, M.; La Clair, J. J.; Jothivasan, V. K.; Budiarto, T.; Hamilton, C. J.; Claiborne, A.; Helmann, J. D.; Fahey, R. C. *Nat. Chem. Biol.* **2009**, *5*, 625.
(4) Review: Helmann, J. D. *Antioxid. Redox Sign.* **2011**, *15*, 123.
(5) Biosynthesis studies: (a) Gaballa, A.; Newton, G. L.; Antelmann, H.; Parsonage, D.; Upton, H.; Rawat, M.; Claiborne, A.; Fahey, R. C.; Helmann, J. D. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 6482. (b) Parsonage, D.; Newton, G. L.; Holder, R. C.; Wallace, B. D.; Paige, C.; Hamilton, C. J.; Dos Santos, P. C.; Redinbo, M. R.; Reid, S. D.; Claiborne, A. *Biochemistry* **2010**, *49*, 8398. (c) Upton, H.; Newton, G. L.; Gushiken, M.; Lo, K.; Holden, D.; Fahey, R. C.; Rawat, M. *FEBS Lett.* **2012**, *586*, 1004.
(6) Sharma, S. V.; Jothivasan, V. K.; Newton, G. L.; Upton, H.; Wakabayashi, J. I.; Kane, M. G.; Roberts, A. A.; Rawat, M.; La Clair, J. J.; Hamilton, C. J. *Angew. Chem., Int. Ed.* **2011**, *50*, 7101.
(7) (a) Cao, M.; Bernat, B. A.; Wang, Z. P.; Armstrong, R. N.; Helmann, J. D. *J. Bacteriol.* **2001**, *183*, 2380. (b) Rigsby, R. E.; Fillgrove, K. L.; Beihoffer, L.; Armstrong, R. N. *Methods Enzymol.* **2005**, *401*, 367.

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starting materials, a new continuous ^{31}P NMR assay of FosB enzymes, and its use to further elucidate the substrate and metal-ion selectivity of FosB enzymes from four Gram-positive microorganisms.

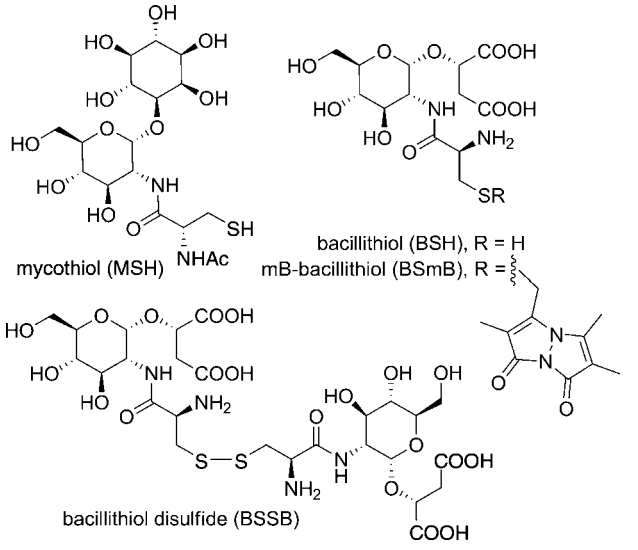
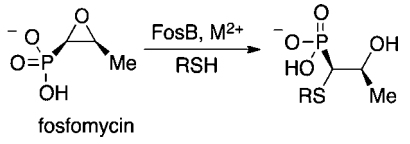


Figure 1. Mycothiol, bacillithiol, S-bimane bacillithiol, and bacillithiol disulfide.

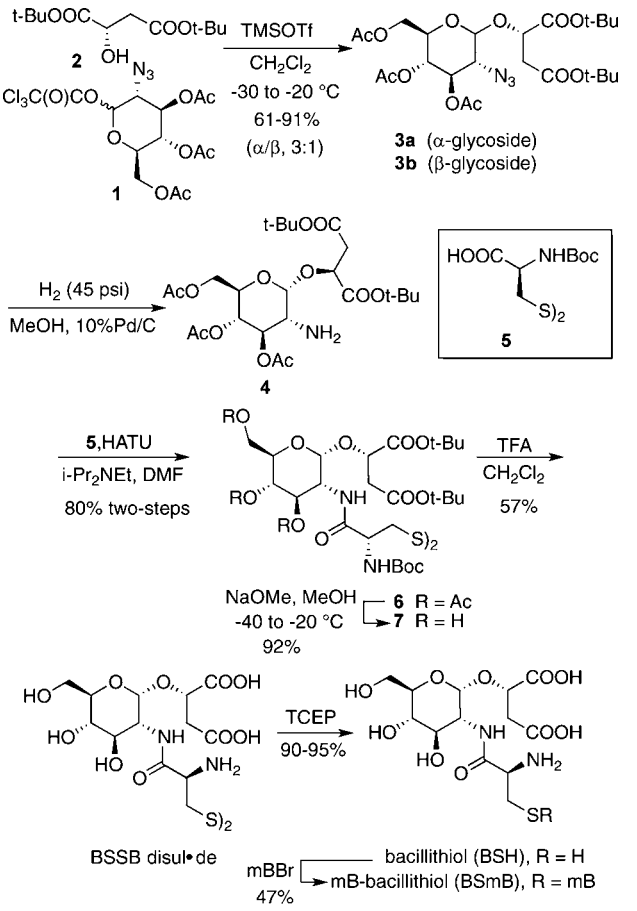
Scheme 1



Our synthetic strategy (Scheme 2) targeted the disulfide BSSB and the use of D-glucosamine as a starting point. While our synthetic studies were underway, Hamilton and co-workers[6] reported the chemical synthesis of bacillithiol starting with trichloroacetimidate **1** prepared by the oxidative azidation (CAN, NaN_3)[8] of D-glucal. We also started from trichloroacetimidate, **1**, but in our case derived **1** from D-glucosamine by way of a known[9] diazo transfer-acetylation reaction (TfN_3 then Ac_2O) followed by selective removal of the C1 acetate (NH_2NH_2, MeOH)[10] and trichloroacetimidate formation (Cl_3CCN, NaH).[11] Coupling of trichloroacetimidate **1** with di-*tert*-butylmalate **2**[12] under

(8) Lemieux, R. U.; Racliffe, R. M. *Can. J. Chem.* **1979**, *57*, 1244.
(9) (a) Nyffeler, P. T.; Liang, C. H.; Koeller, K. M.; Wong, C. H. *J. Am. Chem. Soc.* **2002**, *124*, 10773. (b) Alper, P. B.; Hung, S. C.; Wong, C. H. *Tetrahedron Lett.* **1996**, *37*, 6029.
(10) Exoffier, G.; Gagnaiere, D.; Utille, J.-P. *Carbohydr. Res.* **1975**, *39*, 368.
(11) (a) Lee, S.; Rosazza, J. P. N. *Org. Lett.* **2004**, *6*, 365. (b) Rele, S. M.; Iyer, S. S.; Chaikof, E. L. *Tetrahedron Lett.* **2007**, *48*, 5055.
(12) (a) Rotzoll, N.; Dunkel, A.; Hofmann, T. *J. Agric. Food Chem.* **2005**, *53*, 4149. (b) Allais, F.; Martinet, S.; Ducrot, P.-H. *Synthesis* **2009**, 3571.

Scheme 2. Synthesis of Bacillithiol



TMSOTf activation afforded **3** in yields ranging from 61 to 91% (3:1 α/β), with highest yields observed on a 10 g reaction scale. Alpha (**3a**) and beta (**3b**) glycosides are separable by chromatography leading to an isolated yield of 52% for the desired **3a**. Reduction of azide **3a** was first examined using polymer-supported triphenylphosphine followed by treatment of the crude product with 4 N HCl-dioxane leading to isolation of the corresponding hydrochloride salt (**4**•HCl) albeit in low yield (ca. 30%). It was subsequently determined that hydrogenation (45 psi) of azide **3a** over palladium–carbon in methanol afforded crude amine (**4**) of sufficient purity to be condensed directly with commercially available Boc protected cysteine disulfide (**5**) to afford disulfide **6** in 80% yield (two steps). Bacillithiol disulfide was derived from **6** by a two-step deprotection sequence starting with removal of acetyl groups (NaOMe, MeOH, −40 to −20 °C) to provide disulfide **7** in 92% yield. Next, treatment of **7** with a 50% TFA–dichloromethane solution served to remove remaining acid labile protecting groups and following elution through a strong cation exchange column with 5% acetic acid–methanol yielded BSSB disulfide as its ammonium acetate salt (BSSB•2HOAc).

Access to BSH allowed us to screen several FosB enzymes from *Staphylococcus aureus* ($FosB^{SA}$), *Bacillus*

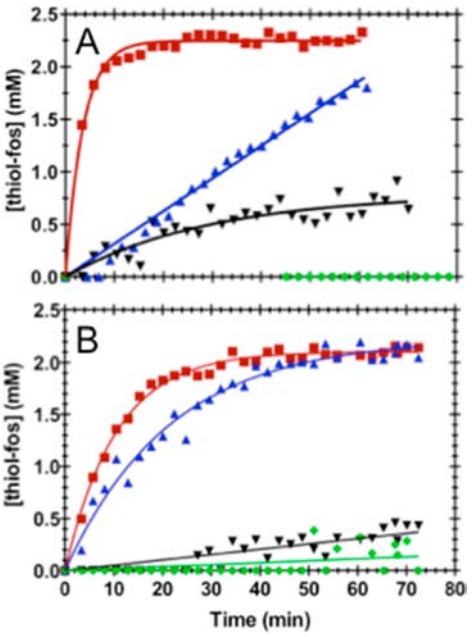


Figure 2. Time course of the FosB catalyzed-addition of BSH or L-Cys to fosfomycin in the presence of Mg^{2+} or Ni(II). Results for $FosB^{SA}$ and $FosB^{BS}$ are shown in panels A and B, respectively. Reactions were run at 25 °C in 20 mM HEPES (pH 7.0) with 4 mM fosfomycin and 0.5 μM enzyme in the presence of (■) 2 mM BSH and 10 μM Ni(II), (▲) 2 mM BSH and 1 mM Mg^{2+}, (▼) 2 mM L-Cys and 10 μM Ni(II), or (◆) 2 mM L-Cys and 1 mM Mg^{2+}.

cereus ($FosB^{BC}$), *Bacillus anthracis* ($FosB^{BA}$), and *Bacillus subtilis* ($FosB^{BS}$). Time courses for the reactions were followed by integrated peak intensities in a continuous ^{31}P NMR assay, which is quite convenient with diamagnetic metals. The assay results for the $FosB^{SA}$ and $FosB^{BC}$ enzymes with combinations of Ni(II), Mg^{2+}, L-Cys, and BSH are illustrated in Figure 2. The results indicate that there is a very clear preference of the $FosB^{SA}$ enzyme for BSH and Ni(II). We detected no activity with L-Cys in the presence of Mg^{2+}. The enzymes from bacilli also show a substantial preference for BSH but exhibit far less discrimination between the two metals (Figure 2B) and Figure S2 (Supporting Information). In other experiments we have found Zn(II) is an excellent inhibitor of the FosB enzymes (data not shown).

Although the continuous ^{31}P NMR assay is quick and convenient, it is not suitable with paramagnetic metals or for performing detailed steady-state kinetics. Nevertheless, approximate minimum turnover numbers (k_{cat}) can be estimated from the initial rates of the reactions. The apparent k_{cat} for $FosB^{SA}$ with BSH and Ni(II) is ~30 s^{-1} but with Mg^{2+} drops to ~1 s^{-1}. The $FosB^{SA}$ activity in the presence of Ni(II) also drops to ~0.9 s^{-1} when L-Cys is the substrate. The results with Mg^{2+} are consistent with previous observations.[6] The apparent k_{cat} values for $FosB^{BS}$ with BSH and Ni(II) or Mg^{2+} are ~6 and 3 s^{-1}, respectively. With L-Cys, the k_{cat} values are much lower, on the order of 0.05 to 0.2 s^{-1}.

In summary, BSH is clearly the preferred *in vitro* thiol substrate for FosB enzymes from several Gram-positive microorganisms. However, the metal-ion preferences of the enzymes are not so clear and bear further investigation particularly with respect to what the preferred metal is *in vivo*. It is interesting to note that the preferred metal in the reaction catalyzed by the anthrax enzyme ($FosB^{BA}$) is Mg^{2+} (Figure S2A, Supporting Information). The availability of synthetic BSH opens the door to more detailed kinetic and metal-ion preference studies of the enzymes as well as structural investigation of the FosB•BSH and FosB•product complexes.

By way of the described 10-step reaction sequence, we have prepared over 1 g of BSSB•2HOAc. Reduction of BSSB disulfide to afford BSH is easily accomplished by stirring with [tris(2-carboxyethyl)phosphine] (TCEP) reducing gel in water. The bromobimane derivative (BSmB) was prepared, and its properties were compared favorably by LC-MS and NMR analysis to those of an authentic sample of BSmB.[3]

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Supporting Information Available. Experimental procedures and full spectroscopic data for all new compounds; enzyme expression, purification, and assays. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.