Published in final edited form as:

Org Lett. 2012 October 19; 14(20): 5207–5209. doi:10.1021/ol302327t.

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

Alexander P. Lamers, Mary E. Keithly, Kwangho Kim, Paul D. Cook, Donald F. Stec, Kelly M. Hines, Gary A. Sulikowski, and Richard N. Armstrong

Departments of Chemistry and Biochemistry, Vanderbilt University, Vanderbilt Institute of Chemical Biology, Nashville, TN 37235, USA

Gary A. Sulikowski: gary.a.sulikowski@vanderbilt.edu; Richard N. Armstrong: r.armstrong@vanderbilt.edu

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 *Bacillus* 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 Mg²⁺, respectively.⁷ The discovery of BSH and the availability in our lab of FosB enzymes from several Gram-positive microorganisms

Correspondence to: Gary A. Sulikowski, gary.a.sulikowski@vanderbilt.edu; Richard N. Armstrong, r.armstrong@vanderbilt.edu.

Supporting Information **Available**: Experimental procedures and full spectroscopic data for all new compounds, enzyme expression and purification and assays (19 pages). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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 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.

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 coworkers⁶ reported the chemical synthesis of bacillithiol starting 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 known⁹ 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 TMSOTf activation afforded **3** in yields ranging from 61 to 91% (3:1 α/β), with highest yields observed on a 10 gram 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 4N 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 **3b** 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 thru a strong cation exchange column with 5% acetic acid-methanol yielded BSSB disulfide as its ammonium acetate salt ($\text{BSSB}\cdot 2\text{HOAc}$).

Access to BSH allowed us to screen several FosB enzymes from *Staphylococcus aureus* (FosB^{SA}), *Bacillus 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 nor 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 $\sim 30\text{ s}^{-1}$ but with Mg^{2+} drops to $\sim 1\text{ s}^{-1}$. The FosB^{SA} activity in the presence of Ni(II) also drops to $\sim 0.9\text{ s}^{-1}$ 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 $\sim 6\text{ s}^{-1}$ 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 Mg²⁺ (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 one gram 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 compared favorably by LC-MS and NMR analysis to an authentic sample of BSmB.³

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the National Institutes of Health R01 GM030910, F32 GM093507, T32 ES007028 and P30 ES000267 and the Vanderbilt Institute of Chemical Biology. We gratefully acknowledge Professor Robert Fahey (UC-San Diego) for a sample of mB-bacillithiol.

References

1. (a) Fahey RC. *Ann Rev Microbiol.* 2001; 55:333. [PubMed: 11544359] (b) Masip L, Veeravalli K, Georgioui G. *Antioxid Redox Sign.* 2006; 8:753.
2. Jothivasan VK, Hamilton CJ. *Nat Prod Rep.* 2008; 25:1091. [PubMed: 19030604]
3. Newton GL, Rawat M, La Clair JJ, Jothivasan VK, Budiarto T, Hamilton CJ, Claiborne A, Helmann JD, Fahey RC. *Nat Chem Biol.* 2009; 5:625. [PubMed: 19578333]
4. Review: Helmann JD. *Antioxid Redox Sign.* 2011; 15:123.
5. Biosynthesis studies: Gaballa A, Newton GL, Antelmann H, Parsonage D, Upton H, Rawat M, Claiborne A, Fahey RC, Helmann JD. *Proc Nat Sci USA.* 2010; 107:6482. (b) Parsonage D, Newton GL, Holder RC, Wallace BD, Paige C, Hamilton CJ, Dos Santos PC, Redinbo MR, Reid SD, Claiborne A. *Biochemistry.* 2010; 49:8398. [PubMed: 20799687] (c) Upton H, Newton GL, Gushiken M, Lo K, Holden D, Fahey RC, Rawat M. *Febs Lett.* 2012; 586:1004. [PubMed: 22569254]
6. Sharma SV, Jothivasan VK, Newton GL, Upton H, Wakabayashi JJ, Kane MG, Roberts AA, Rawat M, La Clair JJ, Hamilton CJ. *Angew Chem Int Edit.* 2011; 50:7101.
7. (a) Cao M, Bernat BA, Wang ZP, Armstrong RN, Helmann JD. *J Bacteriol.* 2001; 183:2380. [PubMed: 11244082] (b) Rigsby RE, Fillgrove KL, Beihoffer L, Armstrong RN. *Methods Enzymol.* 2005; 401:367. [PubMed: 16399398]
8. Lemieux RU, Racliffe RM. *Can J Chem.* 1979; 57:1244.
9. (a) Nyffeler PT, Liang CH, Koeller KM, Wong CH. *J Am Chem Soc.* 2002; 124:10773. [PubMed: 12207533] (b) Alper PB, Hung SC, Wong CH. *Tetrahedron Lett.* 1996; 37:6029.
10. Exoffier G, Gagnaiere D, Utille JP. *Carbohydr Res.* 1975; 39:368.
11. (a) Lee S, Rosazza JPN. *Org Lett.* 2004; 6:365. [PubMed: 14748594] (b) Rele SM, Iyer SS, Chaikof EL. *Tetrahedron Lett.* 2007; 48:5055. [PubMed: 19847317]
12. (a) Rotzoll N, Dunkel A, Hofmann T. *J Agric Food Chem.* 2005; 53:4149. [PubMed: 15884853] (b) Allais F, Martinet S, Ducrot PH. *Synthesis.* 2009:3571.

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

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+} .

Scheme 1.

Scheme 2.
Synthesis of bacillithiol.