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Mechanistic Studies of the Biosynthesis of 2-Thiosugar: Evidence for the Formation of an Enzyme-Bound 2-Ketohexose Intermediate in BexX-Catalyzed Reaction

Eita Sasaki and Hung-wen Liu*

Division of Medicinal Chemistry, College of Pharmacy, and Department of Chemistry and Biochemistry, University of Texas at Austin, Austin, Texas 78712, United States

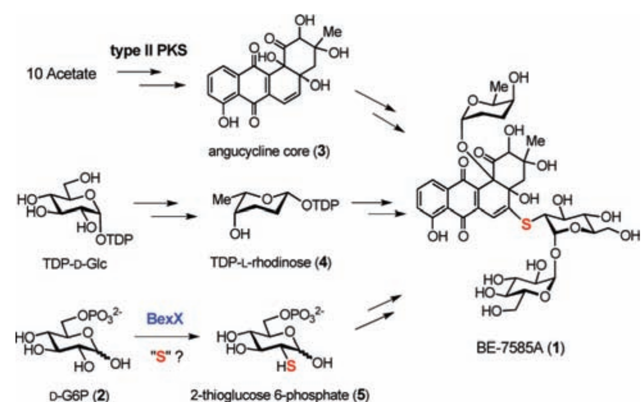
Received September 7, 2010; E-mail: h.w.liu@mail.utexas.edu

Abstract: The first mechanistic insight into 2-thiosugar production in an angucycline-type antibiotic, BE-7585A, is reported. D-Glucose 6-phosphate was identified as the substrate for the putative thiosugar biosynthetic protein, BexX, by trapping the covalently bonded enzyme−substrate intermediate. The site-specific modification at K110 residue was determined by mutagenesis studies and LC−MS/MS analysis. A key intermediate carrying a keto functionality was confirmed to exist in the enzyme−substrate complex. These results suggest that the sulfur insertion mechanism in 2-thiosugar biosynthesis shares similarities with that for thiamin biosynthesis.

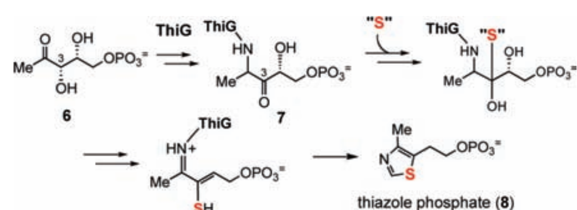
Highly modified sugars are commonly found in secondary metabolites of prokaryotes.[1,2] These sugars are vital components for the efficacy and specificity of many biologically active natural products,[2] and altering and/or exchanging these crucial sugar structures may enhance or vary the physiological characteristics of their parent molecules.[2] Exploitation of biosynthetic machineries is a powerful approach for generating new sugars[2,3] but requires a thorough understanding of the biosynthetic pathway of each target sugar, including genetic, enzymatic, and mechanistic information. Previous efforts have achieved notable advances in our understanding of the formation of deoxy-, amino-, and branched-chain sugars.[4] However, knowledge regarding the biosynthesis of thiosugars has been scarce because of the rareness of its natural occurrence. In fact, only a handful of thiosugar-containing natural products have been isolated to date.[5] Here we report the first mechanistic investigation of the biosynthesis of a 2-thiosugar found in the angucycline-type antibiotic BE-7585A (**1**)[6a] produced by the soil bacterium *Amycolatopsis orientalis* subsp. *vinearia* BA-07585. Notably, BE-7585A is one of the only two known natural products containing a 2-thiosugar moiety.[6] Our results provide strong evidence supporting the intermediacy of a 2-keto substrate−enzyme adduct and the involvement of a series of isomerization reactions as early steps in the pathway.

The biosynthetic gene cluster for **1** was recently identified by PCR-based screening of the cosmid library of *A. orientalis*, and a biosynthetic pathway for its formation was proposed (Scheme 1).[5] Along with the genes encoding type-II polyketide synthases (PKSs) and rhodinose biosynthetic enzymes, the gene *bexX*, whose translated sequence displays good similarity to thiazole synthases (ThiGs) (58% identity to ThiG from *Stigmatella aurantiaca* DW4/3−1;[7] 38% identity to ThiG from *Bacillus subtilis* subsp. *subtilis* str 168[8]) was also located in the gene cluster. Studies of thiamine biosynthesis in *B. subtilis* showed that the ThiG reaction is initiated by formation of a Schiff base between Lys96 of ThiG and the 2-keto group of 1-deoxy-D-xylulose-5-phosphate (DXP, **6**; Scheme 2).[9]

Scheme 1



Scheme 2



A sulfur carrier protein then delivers the sulfur atom to the keto moiety at C3 of the DXP−lysine−ThiG adduct (**7**).[10] Since ThiG is a key enzyme in the formation of the thiazole phosphate (**8**) of thiamin, the newly identified *bex*X gene may also play a key role in the biosynthesis of 2-thiosugar in *A. orientalis*, and the mechanism of sulfur incorporation into the 2-thiosugar in BE-7585A could resemble that of the ThiG reaction in thiamin biosynthesis.[5]

On the basis of the structure of the 2-thiosugar in BE-7585A (**1**), D-glucose 6-phosphate (D-G6P, **2**) is a postulated substrate for BexX. A possible biosynthetic pathway for 2-thiosugar is shown in Scheme 3, pathway A. An active-site lysine residue of BexX may first form a Schiff base with the linear form of **2** at C1. The resultant imine intermediate **9** facilitates abstraction of a proton at C2 to give enamine intermediate **10**. Enamine **10** is then tautomerized to C2 ketone **11**, which can be attacked by an activated sulfur donor to incorporate a sulfur atom at C2.

Alternatively, the hexose monophosphate substrate may be D-fructose 6-phosphate (D-F6P, **13**), which could form a Schiff base with the active-site lysine at C2 (**14**; Scheme 3, pathway B) to directly activate the target position, as seen in glucosamine biosynthesis.[11]

In order to verify the predicted function of BexX, the corresponding protein, BexX, was heterologously expressed in *Escheri-*

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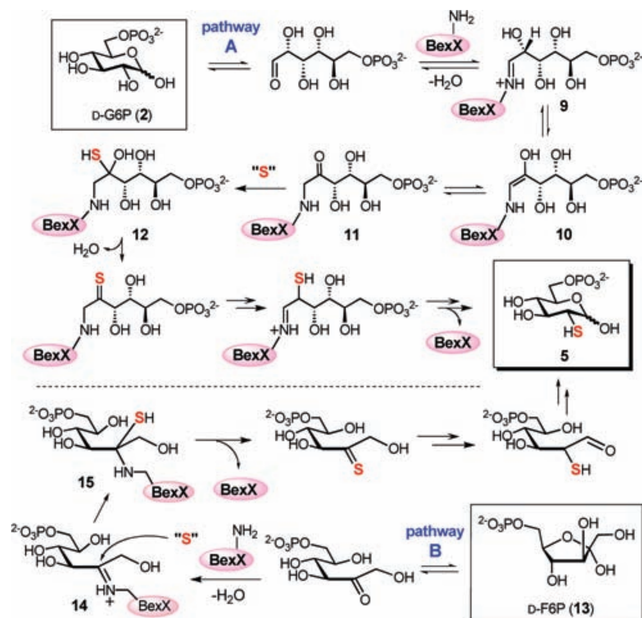
Scheme 3



chia coli and purified as a C-terminal His$_6$-tagged protein (see Figure S1 in the Supporting Information).[12] Interestingly, electrospray ionization mass spectrometry (ESI-MS) of the isolated protein displayed two peaks. The dominant peak corresponds to the expected His$_6$-tagged BexX (calcd, 28488 Da; obsd, 28485 Da), and the minor peak shows a mass increase of ~240 Da relative to the parent peak. Upon $NaBH_4$ reduction, the second peak of the reduced enzyme became more apparent (obsd, 28729 Da) (Figure 1A), and the mass increase (244 Da) is consistent with the formation of a reduced dehydration adduct between BexX and a hexose monophosphate substrate (e.g., reduced **9** or **14**). It is thus reasonable to assume that part of the purified BexX contains a trapped reaction intermediate, and the binding of the substrate−enzyme complex is likely an imine linkage, which can be stabilized by hydride reduction.

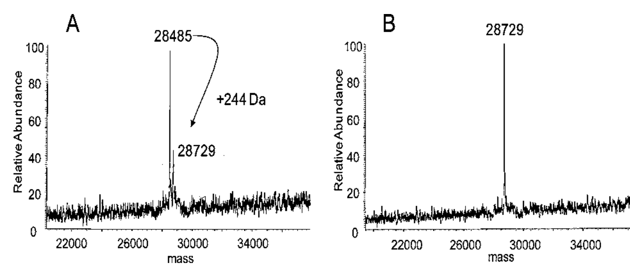


Figure 1. (A) ESI-MS of C-His$_6$-tagged BexX treated with $NaBH_4$. (B) ESI-MS of C-His$_6$-tagged BexX incubated with D-G6P (**2**) prior to $NaBH_4$ treatment. The calculated molecular weights of C-His$_6$-BexX (268 aa) and C-His$_6$-BexX-D-G6P (reduced) are 28488 and 28732 Da, respectively.

To test this hypothesis, the purified BexX was incubated with the putative substrate D-G6P (**2**) and treated with $NaBH_4$ prior to MS analysis. As shown in Figure 1B, the addition of **2** resulted in complete conversion of the parent protein peak to the enzyme−substrate-modified peak. In contrast, no change in the peak distribution was discernible when D-F6P (**13**), D-glucose, or DXP (**6**) was used instead of D-G6P (see Figure S2).[12] Thus, the observed covalent modification of BexX is clearly D-G6P-specific. These results indicate that D-G6P is the substrate for BexX and that formation of the imine intermediate **9** with an active-site lysine residue is the initial step of the BexX reaction.

Sequence alignment revealed that the catalytic lysine residue (Lys96) in ThiG of *B. subtilis* has a counterpart, Lys110, in BexX (see Figure S4).[12] To investigate whether Lys110 plays an active role in BexX catalysis, the K110A mutant was constructed, heterologously expressed in *E. coli*, and purified as a C-terminal His$_6$-tagged protein. ESI-MS of the purified mutant protein exhibited only one peak whose mass matched the calculated molecular mass of K110A (calcd, 28431 Da; obsd, 28430 Da) without any modification. No change was noted even after treatment with D-G6P and $NaBH_4$ (see Figure S3).[12] This finding strongly implicates Lys110 as the site where the adduct with D-G6P is formed.

To ensure that the modification indeed occurs at Lys110, the wild-type BexX-D-G6P complex was reduced by $NaBH_4$ and then subjected to proteolysis with trypsin. The tryptic-digested peptide fragments were analyzed by LC−MS/MS, and the peptide fragment D102−R114 [calcd, m/z 1714.89 ($[M + H]^+$); obsd, m/z 1714.92] contained a D-G6P adduct (**16**; see Figure 2 and Figure S5).[12] Further analysis of the observed b and y ions revealed the attachment of the hexose phosphate at K110 (Figure 2). These results unambiguously demonstrate that the sugar modification indeed occurs at a specific lysine residue (i.e., K110), consistent with the proposed mechanism shown in Scheme 3.

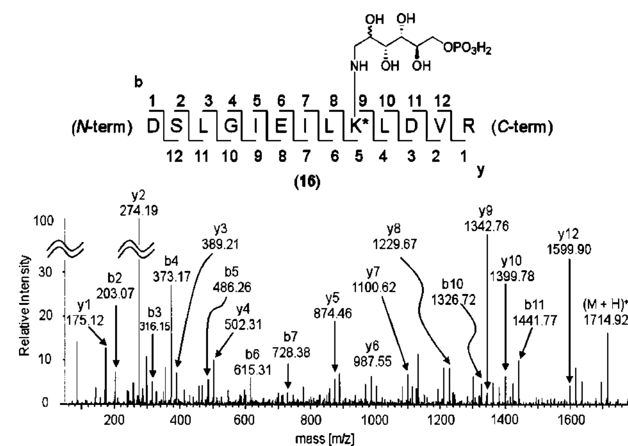


Figure 2. LC−MS/MS of the identified peptide (D102−R114) with a D-G6P coupled to the active-site lysine (K110) (**16**). The calculated m/z value for **16** was 1714.89 ($[M + H]^+$), and the observed value was 1714.92.

Interestingly, during the studies of the trypsin-digested BexX fragments, we noticed that the BexX−D-G6P adduct could be detected even without the addition of any reducing reagent ($NaBH_4$) to the reaction mixture [calcd, m/z 856.94 ($[M + 2H]^{2+}$); obsd, m/z 856.84] (Figure 3B; also see Figure S6).[12] This observation was surprising because it is unlikely that the proposed imine intermediate **9**, without prior reduction, could survive the trypsin digestion as well as the conditions used for the LC−MS/MS analysis. This prompted us to reconsider the chemical nature of the trapped intermediate in the active site of BexX. The initially formed iminium intermediate **9** is in equilibrium with **10** and **11** (Scheme 3). A likely candidate for the stable covalent BexX−D-G6P adduct is compound **11**, which is not readily hydrolyzable in solution. Accordingly, the tryptic fragment **16** detected in the LC−MS/MS analysis could result from hydride reduction of the imine moiety in **9** and/or the 2-keto group in **11** [calcd, m/z 857.95 ($[M + 2H]^{2+}$); obsd, m/z 857.84] (Figure 3A). However, without prior reduction, the modified D102−R114 fragment shown in Figure 3B likely has a structure of **17** (Scheme 4).

To test this hypothesis, the trypsin-digested BexX−D-G6P sample was treated with a carbonyl-specific labeling reagent, 2,4-dinitro-

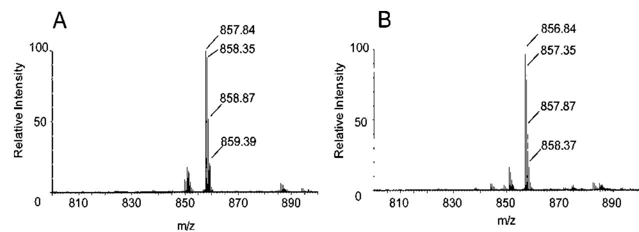
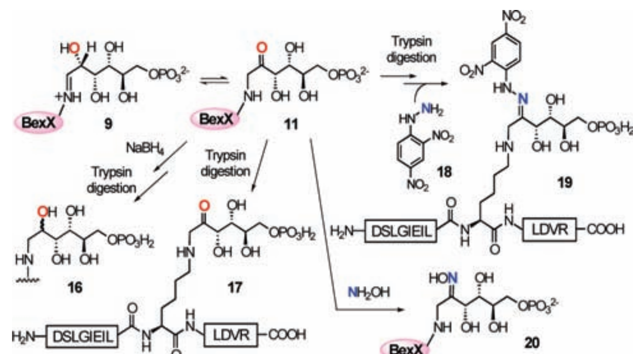


Figure 3. LC−MS of trypsin-digested BexX−D-G6P (A) with and (B) without $NaBH_4$ treatment (positive-mode analysis). The calculated *m/z* values for **16** and its unreduced form **17** are 857.95 ($[M + 2H]^{2+}$) and 856.94 ($[M + 2H]^{2+}$), respectively.

Scheme 4



phenylhydrazine (DNPH, **18**), and the resulting mixture was analyzed by LC−MS (Figure 4A). The observed *m/z* 946.96 ion ($[M + 2H]^{2+}$) is consistent with the modified peptide D102−R114 coupled with D-G6P and DNPH (**19**; Scheme 4). In a separate experiment, the undigested BexX−D-G6P complex was treated with another carbonyl-reactive reagent, NH_2OH, and analyzed by ESI-MS. The mass of the peak observed at 28742 (Figure 4B) matches the calculated molecular mass of the BexX−D-G6P−NH_2OH imine adduct (**20**; Scheme 4). These results strongly support the presence of keto intermediate **11** in the protein−substrate complex.

In summary, a key enzyme involved in the biosynthesis of 2-thiosugar in BE-7585A, BexX, was expressed and purified. Its physiological substrate was verified to be D-G6P (**2**). A stable protein−substrate adduct in the as-isolated BexX was observed by ESI-MS, and the covalent modification was demonstrated to be substrate-specific (i.e., it occurred only with D-G6P) and site-specific (at K110). Most significantly, the sugar substrate in the covalent D-G6P−enzyme adduct exists at least partially in its 2-keto form (**11**), which could be directly trapped using the carbonyl-directing reagents DNPH and NH_2OH. These results provide the first mechanistic insight into the 2-thiosugar formation reaction, which closely resembles that of the ThiG-catalyzed thiazole formation in thiamin biosynthesis.[13] The initial steps must involve several isomerization steps to generate the 2-keto intermediate (**9** → **10** → **11**), priming the sugar substrate to accept the thiol group from a nucleophilic sulfur donor. A potential sulfur carrier protein such as ThiS in thiamin biosynthesis was not found in the BE-7585A biosynthetic cluster. However, a pathway-independent sulfur carrier protein or an endogenous cystein desulfurase harboring a reactive thiocarboxylate or a cystein persulfide may serve as the sulfur donor by attacking the 2-keto intermediate **11** to introduce the sulfur atom.[14,15] *A. orientalis* genome mining is in progress in order to find this sulfur-transferring protein.

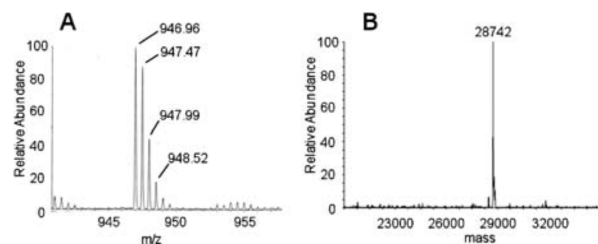


Figure 4. (A) LC−MS of the modified peptide **19** derived from trypsin-digested BexX−D-G6P after DNPH treatment. The calculated *m/z* value of **19** is 946.95 ($[M + 2H]^{2+}$). (B) ESI-MS of the C-His$_6$-BexX−D-G6P ketone intermediate trapped using NH_2OH. The calculated molecular weight of **20** is 28745 Da.

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Supporting Information Available: Experimental details, ESI-MS spectra, LC−MS spectra, and protein sequence analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) It has been demonstrated that bisulfide can be used as a substitute for the sulfur carrier protein ThiS thiocarboxylate in thiamin biosynthesis.[8] However, this is not the case for BexX, since release of the expected thiosugar product **5** from **11** was not observed in the presence of a high concentration of bisulfide (up to 20 mM).

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