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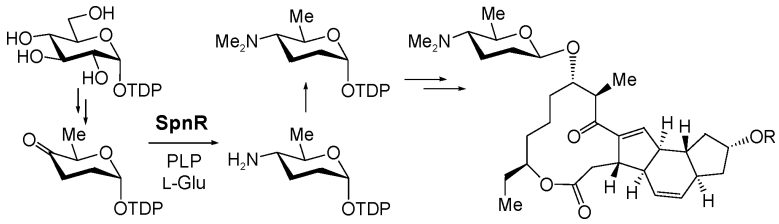

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Characterization of Protein Encoded by *spnR* from the Spinosyn Gene Cluster of *Saccharopolyspora spinosa*: Mechanistic Implications for Forosamine Biosynthesis

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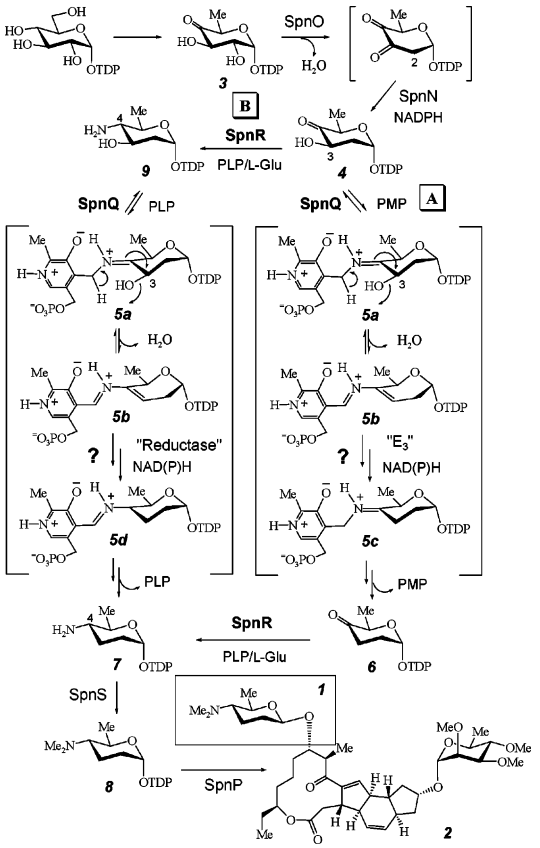
Deoxysugars are constituents of many biologically active natural products in which they are commonly the determinants of activity for the parent molecules.[1] To characterize deoxysugar biosynthetic pathways and to collect genes useful for in vivo glycodiversification of secondary metabolites,[2] we investigated the biosynthesis of D-forosamine (**1**, 4-*N*,*N*-(dimethylamino)-2,3,4,6-tetradeoxy-α-D-*threo*-hexopyranose). This unusual sugar is found in several natural products, including spiramycin,[3] a clinically useful antibiotic, and spinosyn (**2**),[4] an environmentally benign insecticide. The structure of forosamine is unique due to its highly deoxygenated nature, and its biosynthesis must include a series of C−O bond cleavage events. Early studies of spinosyn production in *Saccharopolyspora spinosa* led to the isolation of the entire biosynthetic gene cluster, in which *spnN*, *spnO*, *spnP*, *spnQ*, *spnR*, and *spnS*, were proposed to be involved in forosamine formation.[5] The tentative identification of these genes by sequence analysis allowed the assignment of potential roles for the gene products, from which two possible routes starting from **3** can be envisioned for the biosynthesis of **1** (Scheme 1). The key steps in route A involve deoxygenation at C-2 (**3** → **4**) and then at C-3 (**4** → **6**), followed by transamination at C-4 (**6** → **7**) and dimethylation of the 4-amino group to give TDP-forosamine (**8**), which is then incorporated into **2**. The reaction sequence of route B closely resembles that of route A except for the order of the C-3 deoxygenation and C-4 transamination steps (**4** → **9** → **7**), both of which are expected to be catalyzed by B_6-dependent enzymes.

The C-3 deoxygenation step is of particular interest since this transformation in the formation of other glucose-derived 3,6-dideoxysugars (such as ascarylose) requires a pair of enzymes, a B_6-dependent [2Fe−2S]-containing dehydrase (E_1), and an iron−sulfur flavoprotein reductase (E_3), acting in concert.[6,7] The typical reaction, illustrated in route A, proceeds via a dehydration/electron-transfer reduction mechanism (**5a** → **5b** → **5c**). Sequence identity (49%) between SpnQ, the gene product of *spnQ*, and E_1 of the ascarylose pathway[8] implicates SpnQ as the likely E_1-equivalent that converts **5a** → **5b** in the forosamine biosynthesis. The conservation of an "E_1-type" [2Fe−2S] binding motif[9] in SpnQ further suggests that an electron-transfer reduction is an integral part of the deoxygenation reaction in which the [2Fe−2S] center in SpnQ serves as a part of the electron-transfer conduit. However, no E_3 equivalent gene was found in the *spn* gene cluster. A generic reductase could function as an E_3 surrogate in the conversion of **4** to **6** (route A, **5b** → **5c**), following the mechanism established for ascarylose and other glucose-derived 3,6-dideoxysugars. Alternatively, the absence of an E_3 equivalent gene in the *spn* gene cluster might indicate that C-3 deoxygenation is not a typical E_1/E_3 reaction. It may instead be accomplished by the catalysis of SpnQ along with a nonspecific reductase on the aminosugar intermediate **9** to give **7** (route B, **5a** → **5b** → **5d**).

Scheme 1



As shown in Scheme 1, the substrate and the cofactor requirements for the C-3 deoxygenation by SpnQ and C-4 transamination by SpnR are clearly different depending on which route is used during forosamine production. Hence, the sequence of events in this pathway can be established by identifying the function of SpnQ. However, difficulties were encountered in our attempts to express the *spnQ* gene to directly examine the catalytic properties of purified SpnQ. To circumvent these obstacles, we investigated the substrate specificity of SpnR, using a stable TMP-phosphonate analogue of **7**. Our characterization of SpnR identifies it as an aminotransferase that catalyzes the interconversion between **6** and **7**. This result indicates that route A is preferred in the forosamine biosynthetic pathway.

To determine the function of SpnR, the *spnR* gene was amplified by the polymerase chain reaction (PCR) using genomic DNA from *S. spinosa* (NRRL18537) as template and cloned into a pET24b-(+) vector. The resulting construct, pKZR1, was used to transform the *Escherichia coli* BL21(DE3) cells. The expressed SpnR,

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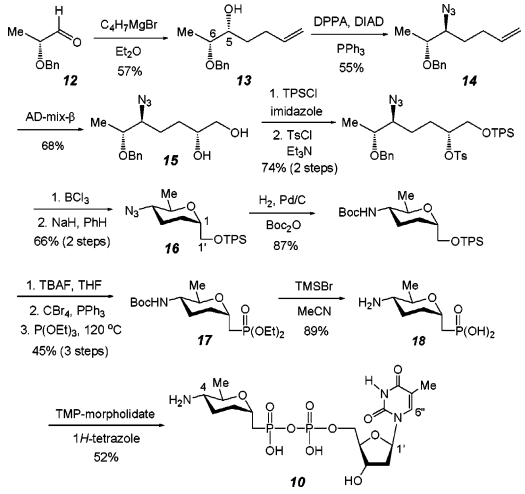
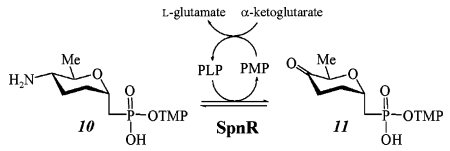
Scheme 2



Scheme 3



containing a C-terminal His_6-tag, was purified to near homogeneity by a protocol consisting of Ni−NTA affinity and Sephacryl S-200 size-exclusion chromatography. The isolated SpnR is a homodimeric protein and exhibits weak absorption at 325 and 410 nm.[10]

The predicted substrate for SpnR in route B is **4**, which could be prepared from **3** by the action of SpnO and SpnN in the presence of NADPH.[11] Preparation of the predicted substrate **6** for SpnR in route A was more challenging due to the difficulties in reconstituting SpnQ activity. Many attempts to chemically synthesize the desired compound failed due to the facile loss of the TDP substituent at C-1, a well-known property of 2-deoxyhexoses. Hence, instead of **6**, an isostere of **7** (compound **10**) in which the TMP group is joined to the hexose core through a stable C-glycosidic phosphonate linker was synthesized (Scheme 2).[12] Because enzyme-catalyzed transamination is a reversible process, if SpnR is the desired aminotransferase using **6** as the substrate, compound **10** could be converted to the corresponding keto-sugar **11** by SpnR (Scheme 3).

To determine the function and substrate specificity of SpnR, a mixture of SpnR (10 μM), **10** (1 mM), α-ketoglutarate (30 mM), and PLP (40 μM) in 100 mM potassium phosphate buffer (pH 7.5) was incubated at 24 °C. Aliquots of the incubation mixture were withdrawn at different time intervals from which a new product was detected by HPLC using an anion exchange Dionex column (4 × 250 mm).[13] A large-scale preparation was then carried out, and the isolated product was identified as **11** on the basis of ^{1}H and ^{13}C NMR analyses.[12] The conversion of **11** to **10** was also established by HPLC.[14] These results clearly demonstrated that **11**/**10**, and by inference **6**/**7**, are the substrates for SpnR. Interestingly, when the incubation was carried out using **4** as the substrate, formation of **9** was also detected.[12] However, the efficiency of **11** to **10** was much greater (more than 15-fold) than that of **4** to **9** by SpnR under the same conditions.[15] Thus, while both **4** and **11**, the isostere of **6**, could be recognized and processed by SpnR, the higher efficiency of conversion observed for **11** → **10** as compared to that for **4** → **9** suggested that **6** is more likely the physiological substrate for SpnR. Thus, SpnR can be referred to as TDP-4-keto-2,3,6-trideoxy-D-glucose 4-aminotransferase.

These results provide the first direct evidence establishing the relevance of the *spnR* gene to forosamine biosynthesis. The verification of the catalytic function of SpnR has substantiated its assigned role as an aminotransferase in the proposed biosynthetic pathway. Although a full understanding of the mechanism of the C-3 deoxygenation awaits future characterization of the SpnQ protein, our data shed light on the reaction sequence of the forosamine pathway. In particular, the substrate for SpnQ can be implicated as compound **4** on the basis of our results. Moreover, the strategy of utilizing stable TMP-phosphonate sugars in place of TDP-sugar derivatives to determine the possible functions of proteins involved in unusual sugar biosynthesis may also find general applicability for the design of probes to study related enzymes whose mechanisms involve the transformation of labile deoxysugar intermediates.

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Supporting Information Available: Synthesis of **10** and spectral data of **10** and **11** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) C-3 deoxygenation catalyzed by ColD in colitose biosynthesis is an exception in which the cofactor (PMP) is regenerated by a second function of ColD, an aminotransferring activity, instead of by the E_3-catalyzed reduction (Beyer, N.; Alam, J.; Hallis, T. M.; Guo, Z.; Liu, H.-w. *J. Am. Chem. Soc.* **2003**, *125*, 5584−5585).
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(9) E_1 and those believed to function as dehydrases have a catalytic histidine at position 220 (using the E_1 numbering system) and display an unusual, albeit well-defined iron−sulfur binding motif. Both traits are conserved in SpnQ sequence: Agnihotri, G.; Liu, Y.-n.; Paschal, B. M.; Liu, H.-w. *Biochemistry* **2004**, *43*, 14265−14274.
(10) These results suggest that isolated SpnR is predominantly an apoenzyme. Thus, PLP was included in all incubations with SpnR.
(11) Expression, purification, and characterization of SpnO and SpnN will be reported elsewhere. The spectral data of **4** are identical to those reported in the literature: Draeger, G.; Park, S.-H.; Floss, H. G. *J. Am. Chem. Soc.* **1999**, *121*, 2611−2612.
(12) See Supporting Information for details.
(13) Baseline separation of the product **11** from the substrate **10** (retention times = 31.0 and 6.7 min, respectively) was achieved using a linear gradient from 25 to 300 mM ammonium acetate (pH 7.0) over 35 min with a flow rate of 1 mL/min.
(14) A k_{cat} of 0.25 s^{-1} and a K_m of 100 μM for **11** were estimated for this reaction at room temperature. The assay mixture (100 μL) contained 1.0 μM SpnR, 250 μM PLP, 50 mM L-glutamate, 150 μM NADPH, 80 mM NH_4Cl, 0.6 U of L-glutamic dehydrogenase, and varied amounts of **11** in 50 mM potassium phosphate buffer, pH 7.5.
(15) Comparison was based on the extent of conversion measured by HPLC in each case. Determination of k_{cat} and K_m for **4** → **9** was hampered by the low conversion of this reaction.

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