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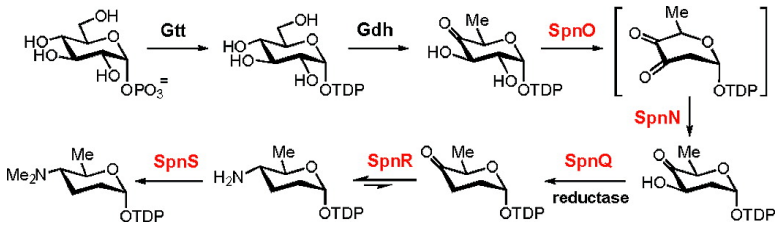

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In Vitro Characterization of the Enzymes Involved in TDP-D-Forosamine Biosynthesis in the Spinosyn Pathway of *Saccharopolyspora spinosa*

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Abstract: Forosamine (4-dimethylamino)-2,3,4,6-tetradeoxy-β-D-*threo*-hexopyranose) is a highly deoxygenated sugar component of several important natural products, including the potent yet environmentally benign insecticide spinosyns. To study D-forosamine biosynthesis, the five genes (*spnO*, *N*, *Q*, *R*, and *S*) from the spinosyn gene cluster thought to be involved in the conversion of TDP-4-keto-6-deoxy-D-glucose to TDP-D-forosamine were cloned and heterologously expressed, and the corresponding proteins were purified and their activities examined *in vitro*. Previous work demonstrated that SpnQ functions as a pyridoxamine 5′-monophosphate (PMP)-dependent 3-dehydrase which, in the presence of the cellular reductase pairs ferredoxin/ferredoxin reductase or flavodoxin/flavodoxin reductase, catalyzes C-3 deoxygenation of TDP-4-keto-2,6-dideoxy-D-glucose. It was also established that SpnR functions as a transaminase which converts the SpnQ product, TDP-4-keto-2,3,6-trideoxy-D-glucose, to TDP-4-amino-2,3,4,6-tetradeoxy-D-glucose. The results presented here provide a full account of the characterization of SpnR and SpnQ and reveal that SpnO and SpnN functions as a 2,3-dehydrase and a 3-ketoreductase, respectively. These two enzymes act sequentially to catalyze C-2 deoxygenation of TDP-4-keto-6-deoxy-D-glucose to form the SpnQ substrate, TDP-4-keto-2,6-dideoxy-D-glucose. Evidence has also been obtained to show that SpnS functions as the 4-dimethyltransferase that converts the SpnR product to TDP-D-forosamine. Thus, the biochemical functions of the five enzymes involved in TDP-D-forosamine formation have now been fully elucidated. The steady-state kinetic parameters for the SpnQ-catalyzed reaction have been determined, and the substrate specificities of SpnQ and SpnR have been explored. The implications of this work for natural product glycodiversification and comparative mechanistic analysis of SpnQ and related NDP-sugar 3-dehydrases E_1 and ColD are discussed.

Introduction

Deoxysugars are commonly found as components of bioactive natural products such as antimicrobial agents and anticancer drugs[1] and are often crucial for the activities of these compounds.[2,3] Because of the variety of deoxysugar structures found in nature and their importance for natural product bioactivity, much effort has been devoted to the elucidation of their biosynthetic pathways.[4−7] The proven feasibility of genetic and enzymatic glycodiversification strategies for generating diversity in natural product sugar structures[5−9] has underscored the importance of collecting and characterizing deoxysugar biosynthetic components for use in these endeavors. D-Forosamine (4-dimethylamino)-2,3,4,6-tetradeoxy-β-D-*threo*-hexopyranose, **1**, Scheme 1) is a highly deoxygenated sugar found in the structures of many bioactive natural products, including the insecticidal spinosyns A and D (**2**, **3**)[10] and butenylspinosyn (**4**),[11] the macrolide antibiotic spiramycin (**5**),[12] the naphthoquinone antibiotic forosaminylgriseucin A (**6**),[13] and the immunosuppressive agent dunaimycin (**7**).[14]

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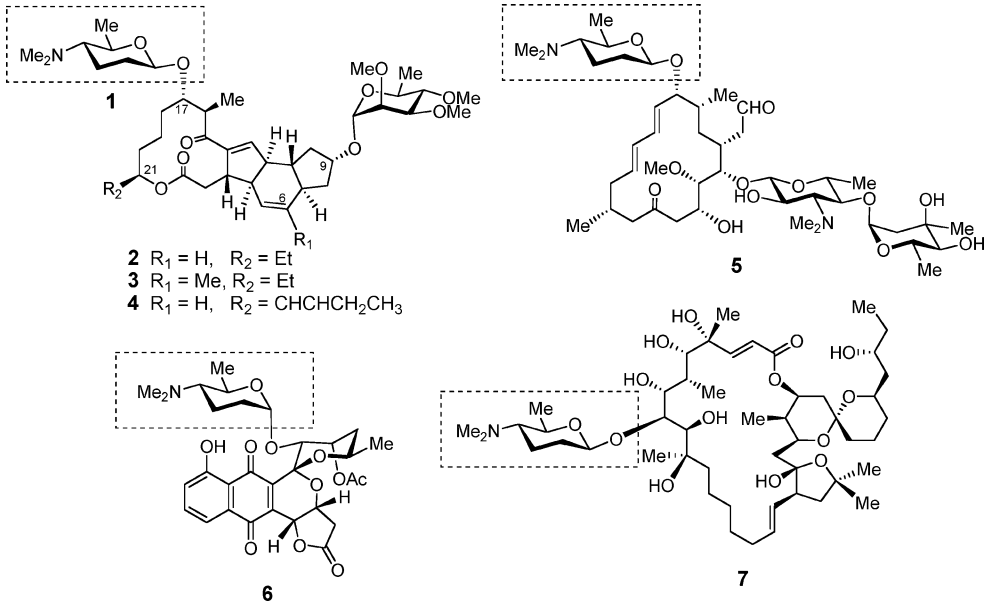
Scheme 1. Structures of Known Natural Products Containing D-forosamine Moieties



Spinosyns A and D (**2**, **3**, Scheme 1) are the major natural products made by the actinomycete *Saccharopolyspora spinosa* and are commercially marketed as the insecticide Spinosad (also known as Tracer or Naturalyte). These compounds are composed of an unusual tetracyclic macrolide and two deoxysugars, D-forosamine (**1**) and 2,3,4-tri-*O*-methyl-L-rhamnose. More than 20 minor spinosyn congeners that vary with respect to the aliphatic substituents at C-6, C-16, and C-21, and by the degree of methylation of the 2-, 3-, and 4-hydroxyl groups of the L-rhamnose moiety and of the 4-amino group of D-forosamine, are also produced by *S. spinosa*.[15] Structure−activity studies have shown that both D-forosamine and 2,3,4-tri-*O*-methyl-L-rhamnose moieties of spinosyns are critical for their insecticidal activities.[16,17]

Sequencing of the spinosyn biosynthetic gene cluster in *S. spinosa* (Figure 1) and subsequent sequence analysis combined with the results of gene disruption studies led to the proposal that five genes, *spnO*, *spnN*, *spnQ*, *spnR*, and *spnS*, are involved in the conversion of TDP-4-keto-6-deoxy-D-glucose (**10**) to TDP-D-forosamine (**16**). The *spnO* gene was predicted to encode a TDP-sugar 2,3-dehydratase and *spnN* to encode a TDP-sugar 3-ketoreductase that convert **10** to **11** and **11** to **12**, respectively. The *spnQ* gene was predicted to encode a TDP-sugar 3-dehydrase that converts **12** to **13**, while *spnR* and *spnS* were assigned as TDP-sugar aminotransferase and TDP-sugar methyltransferase converting **13** to **14** and **14** to **16**, respectively. The *spnP* product was proposed to transfer D-forosamine from **16** to the hydroxyl group at C-17 of the tetracyclic pseudoaglycones (**17**/**18**) to give **2**/**3** (Scheme 2).[10] Compound **10** is formed by thymidylylation of glucose-1-phosphate (**8**) to form TDP-D-glucose (**9**) followed by 4,6-dehydration of **9**. Normally, glucose-1-phosphate thymidylyltransferase (E_p) and TDP-D-glucose-4,6-dehydrase (E_{od}), which catalyze these reactions, are encoded by genes that are present in the glycosylated natural product gene clusters. However, the corresponding genes are absent in the spinosyn gene cluster. Instead, genes encoding these activities, designated *gtt* (*g*lucose-1-phosphate *t*hymidyl*t*ransferase) and *gdh* (TDP-D-*g*lucose-4,6-*deh*ydrase), respectively, are found elsewhere in the genome. Gtt and Gdh have been shown by gene disruption experiments to supply **10** to both spinosyn biosynthesis and cell wall polysaccharide formation in *S. spinosa*.[18]

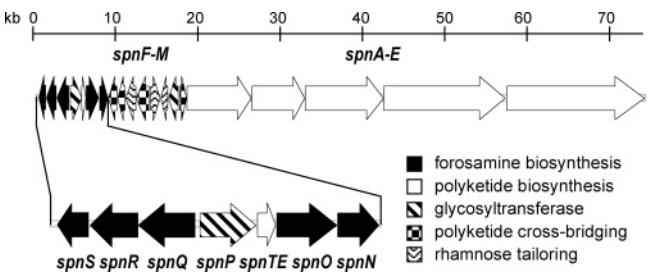


Figure 1. The spinosyn biosynthetic gene cluster of *Saccharopolyspora spinosa* showing the location and arrangement of genes encoding forosamine biosynthesis.

Among the five assigned forosamine genes, the translated *spnO* sequence is similar to several sugar 2,3-dehydratases, including TylX3 (44% identity), EvaA (45% identity), Gra27 (42% identity), and OleV (48% identity), while that of *spnN* shares significant sequence identity with 3-ketoreductases Gra26 (49% identity) and OleW (46% identity). These known 2,3-dehydratases catalyze the conversion of **10** to an unstable intermediate TDP-3,4-diketo-2,6-dideoxy-D-glucose (**11**), which is then converted to TDP-4-keto-2,6-dideoxy-D-glucose (**12**) by a 3-ketoreductase, such as Gra26 or OleW in the TDP-L-rhodinose (**19**) and TDP-L-olivose (**20**) biosynthetic pathways, respectively (Scheme 3A).[19−21] The 3-ketoreductase TylC1, which converts the TylX3 product (**11**) to TDP-4-keto-2,6-

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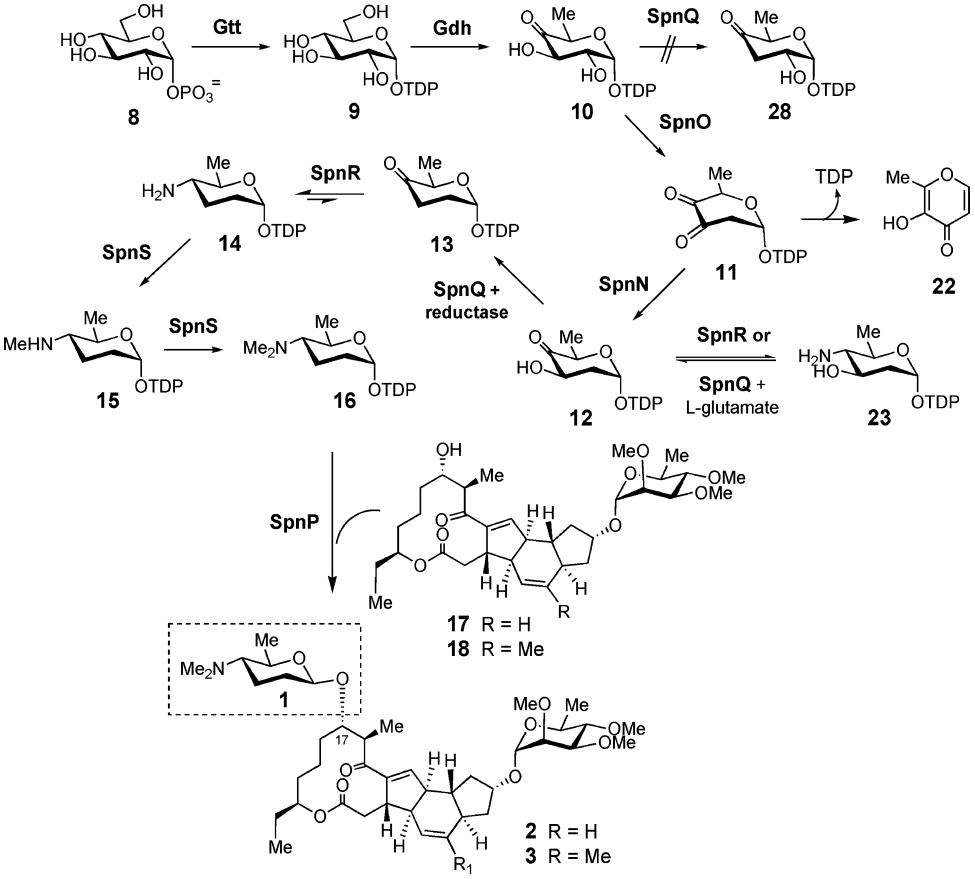
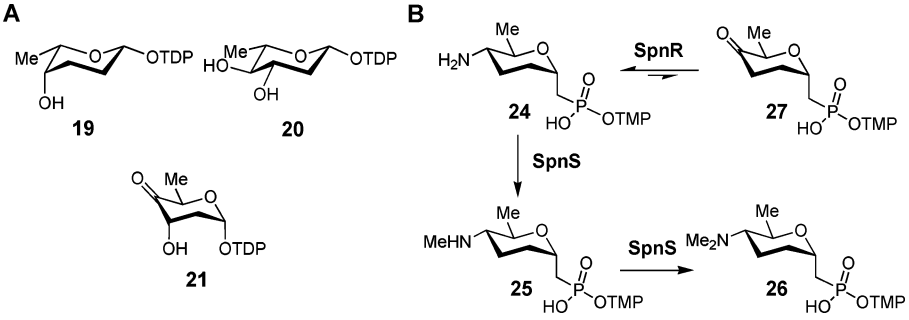
Scheme 2. The Spinosyn Biosynthetic Pathway Highlighting the Biosynthesis of TDP-D-forosamine (**16**)[a]



[a] Note that compounds **22**, **23**, and **28** are not intermediates in the native biosynthetic pathway. Gtt is the TTP-dependent glucose-1-phosphate Thymidylyltransferase converting **8** to **9**, Gdh is the Tdp− D-glucose 4,6-dehydrase converting **9** to **10**, SpnO is the TDP-4-keto-6-deoxy-D-glucose 2,3-dehydratase converting **10** to **11**, SpnN is the NAD(P)H-dependent TDP-3,4-diketo-2,6-dideoxy-D-glucose 3-ketoreductase converting **11** to **12**, SpnQ is the PMP-dependent TDP-4-keto-2,6-dideoxy-D-glucose 3-dehydrase converting **12** to **13**, SpnR is the PLP, L-glutamate-dependent TDP-4-keto-2,3,6-trideoxy-D-glucose 4-aminotransferase converting **13** to **14**, and SpnS is the SAM-dependent TDP-4-amino-2,3,6-trideoxy-D-glucose *N*,*N*-dimethyltransferase sequentially converting **14** to **15** and **15** to **16**.

Scheme 3. (A) Structures of TDP-l-rhodinose (**19**), TDP-L-olivose (**20**), and TDP-4-keto-2,6-dideoxy-D-allose (**21**). (B) Transamination and *N*-Methylation of the Isostere (**24**) of **14** by SpnR and SpnS, Respectively



dideoxy-D-allose (**21**, Scheme 3A), the C-3 epimer of **12**, is only distantly related to SpnN (14% identity). On the basis of this information, it was predicted that SpnO and SpnN catalyze the conversion of **10** to **11** and **11** to **12**, respectively. Under *in vitro* conditions when a 3-ketoreductase is not present to convert **11** to a more stable product, **11** is known to rapidly degrade to maltol (**22**) and TDP.[19,21]

SpnQ is homologous to several vitamin B_6-dependent enzymes which catalyze C-3 deoxygenation of NDP-sugar substrates, including the well-characterized CDP-4-keto-6-deoxy-D-glucose 3-dehydrase E_1[5,22] from the ascarylose biosynthetic pathway and the GDP-4-keto-6-deoxy-D-mannose 3-dehydrase ColD[23,24] from the colitose pathway (49 and 26% identity to SpnQ, respectively), both from *Yersinia pseudotuberculosis*. SpnQ also exhibits high sequence similarity to a number of putative NDP-sugar 3-dehydrases encoded in gene clusters of 2,3,6-trideoxyhexose-containing natural products, such as Gra23 from the granaticin pathway (68% identity),[25] LanQ from the landomycin pathway (67% identity),[26] and UrdQ from the

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urdamycin pathway (68% identity),[27] all of which are believed to be involved in the formation of TDP-L-rhodinose (**19**). SpnQ was therefore predicted to be a 3-dehydrase which converts **12** to TDP-4-keto-2,3,6-trideoxy-D-glucose (**13**).

SpnR is homologous to numerous NDP-sugar aminotransferases, including DesI (26% identity) and TylB (27% identity) whose functions have been established *in vitro*.[28,29] SpnR was therefore predicted to be the 4-aminotransferase which converts the SpnQ product **13** to TDP-4-amino-2,3,4,6-tetradeoxy-D-glucose (**14**). Originally, we also considered the possibility that the SpnR-catalyzed C-4 transamination might precede C-3 deoxygenation by SpnQ in the forosamine pathway. Assays of SpnR in the forward direction using both the SpnN product **12** and in the reverse direction using a more stable phosphonate mimic (**24**, Scheme 3B) of the proposed SpnR product (**14**) showed that SpnR was in fact capable of catalyzing C-4 transamination of both substrates. However, SpnR is much more efficient at transamination of **24** than **12** (more than 15-fold difference in rate), strongly suggesting that SpnQ acts first in the pathway.[30] The fact that SpnQ efficiently converts the SpnN product **12** to **13** further supports this idea.[31] We have therefore assigned SpnR the role of converting **13** to **14** in forosamine biosynthesis. SpnS is homologous to a number of NDP-aminosugar *N*,*N*-dimethyltransferases, including DesVI (46% identity) and TylM1 (40% identity) from the desosamine and mycaminose pathways, respectively, whose functions have been demonstrated *in vitro*.[32−34] SpnS is therefore predicted to catalyze the final step in forosamine biosynthesis, converting the SpnR product **14** to TDP-D-forosamine (**16**).

Herein, we report the overexpression, purification, and characterization of SpnO, SpnN, and SpnS as well as additional characterization of SpnQ and SpnR. We demonstrate via *in vitro* assays and NMR analysis of the purified SpnN product TDP-4-keto-2,6-dideoxy-D-glucose (**12**) that SpnO and SpnN are the 2,3-dehydratase and 3-ketoreductase, which together catalyze the conversion of **10** to **12**. The 4-*N*-methyl transfer activity of SpnS was also demonstrated by an *in vitro* assay using the chemically synthesized phosphonate mimic **24**, NMR characterization of the SpnS monomethylated product (**25**), and mass spectrometric characterization of the phosphonate mimic of TDP-D-forosamine (**26**) (Scheme 3B). We also assayed SpnR using its physiological substrate **13** and determined the steady-state kinetic parameters of the SpnQ-catalyzed reaction with its authentic substrate **12**. With these results, the functions of all five enzymes in the TDP-D-forosamine biosynthetic pathway have been established *in vitro*, and the catalytic properties of the enzymes have been explored. The information gained from these studies lays the foundation for *in vitro* and *in vivo* glycodiversification using components of the forosamine biosynthetic machinery, and for comparative mechanistic and structural studies of SpnQ and its 3-dehydrase homologues E_1[35] and ColD.[24]

Experimental Section

Materials. The *spnO*, *N*, *Q*, *R*, and *S* genes were amplified by polymerase chain reaction (PCR) from the genomic DNA isolated from *Saccharopolyspora spinosa* NRRL 18537, which was obtained from the Agricultural Research Service Culture Collection (Peoria, IL). *Escherichia coli* strain DH5α was purchased from Bethesda Research Laboratories (Gaithersburg, MD). Vector pET24b(+) and pET28b(+) and overexpression host *E. coli* BL21(DE3) were acquired from Novagen (Madison, WI). Enzymes used for molecular cloning experiments were products of Invitrogen (Carlsbad, CA). Ni-NTA agarose and kits for DNA gel extraction and spin miniprep were obtained from Qiagen (Valencia, CA). *Pfu* DNA polymerase and QuikChange Site-Directed Mutagenesis Kit were purchased from Stratagene (La Jolla, CA). The growth media components were acquired from Becton Dickinson (Sparks, MD). Ferredoxin and ferredoxin reductase were purchased from Sigma-Aldrich (St. Louis, MO). Unless otherwise specified, all chemicals, reagents, and enzymes were purchased from Sigma-Aldrich or Fisher Scientific (Pittsburgh, PA) and were used without further purification. Bio-gel P2 resin, Econo-Pac 10 DG desalting columns, and all reagents for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) were acquired from Bio-Rad Laboratories (Hercules, CA). Amicon YM-10 filtration products were purchased from Millipore (Billerica, MA). The CarboPac PA1 HPLC column was obtained from Dionex (Sunnyvale, CA). The Superdex 200 HR 10/30 FPLC column was the product of Pharmacia (Uppsala, Sweden). The SAX Adsorbosphere HPLC column was purchased from Alltech (Deerfield, IL). Oligonucleotide primers for cloning of all genes were prepared by Integrated DNA Technologies (Coralville, IA).

General. Isolation of *S. spinosa* genomic DNA was performed according to published procedures.[36] Protein concentrations were determined according to Bradford[37] using bovine serum albumin as the standard. The relative molecular mass and purity of enzyme samples were estimated using SDS-PAGE as described by Laemmli.[38] All reagents and solvents were purchased from commercial sources and were used without further purification unless otherwise noted. The NMR spectra were acquired on a Varian Unity 300, 400, or 500 MHz spectrometer, and chemical shifts are given relative to those for *t*-BuOH (the chemical shifts are 1.27 and 31.2 ppm for ^{1}H and ^{13}C NMR, respectively), and aqueous 85% H_3PO_4 (external, for ^{31}P NMR), with coupling constants reported in hertz (Hz). Flash chromatography was performed on Lagand Chemical silica gel (230−400 mesh) by elution with the specified solvents. Analytical thin-layer chromatography (TLC) was carried out on Polygram Sil G/UV_{254} plates (0.25 mm). TLC spots were visualized by heating plates previously stained with a solution of phosphomolybdic acid (3% in EtOH). DNA sequencing and N-terminal sequencing of purified enzymes were performed by the Core Facilities of the Institute of Cellular and Molecular Biology at the University of Texas at Austin. Chemical ionization (CI), fast-atom bombardment (FAB), and electrospray ionization (ESI) mass spectra were recorded by the MS facility in the Department of Chemistry and Biochemistry of the University of Texas at Austin. The general methods and protocols for recombinant DNA manipulations followed those described by Sambrook et al.[39] Kinetic data were analyzed by nonlinear fit using Grafit5 (Erithacus Software Ltd.).

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Gene Amplification and Cloning of *spnO, N, Q, R,* and *S*. Genes *spnO*, *spnN*, *spnQ*, *spnR*, and *spnS* were each PCR-amplified from *S. spinosa* genomic DNA using primers with engineered 5′ *Nde*I and 3′ *Xho*I (*Hin*dIII in the case of *spnO*) restriction sites. The primer used were the following, *spnO*: O-1 (5′-GGGGCCTC**CATATG**AGCAGTTCTGTCGAAGC-3′) and O-2 (5′-GAG**AAGCTT**TTATCG-CCCCAACGCCCACAAG-3′), *spnN*: N-1 (5′-GCGG**CATATG**ACCAGCTCGATGCGAA-3′) and N-2 (5′-GGAG**CTCGAG**TGTGGACCCGCA-3′), *spnQ*: Q-1 (5′-GGC**CATATG**CAGAGCCGGAAAACCAGA-3′) and Q-2 (5′-GCC**CTCGAG**CTAGGAACTCTTGGCCAC-3′), *spnR*: R-1 (5′-GCC**CATATG**ATCAACCTGCACCA-3′) and R-2 (5′-GCC**CTCGAG**CTTTCGGAGTG-3′), and *spnS*: S-1 (5′-GGG-AATTC-**CATATG**TCGCGCGTGAGCGACAC-3′) and S-2 (5′-ATCCG**CTCGAG**GTTGCGGATTCCGACGAAC-3′). Engineered restriction sites are shown in bold, start codons in bold underlined, and stop codons underlined. The PCR-amplified genes were purified, digested with the appropriate restriction enzymes, and ligated into the appropriate vector (pET24b+ for *spnN*, *spnQ*, *spnR*, and *spnS*, and pET28b+ for *spnO*) digested with the same enzymes. After transformation, positive clones were confirmed by DNA sequencing. The resulting plasmids were used to transform *E. coli* BL21(DE3) for protein overexpression. The recombinant SpnO is N-terminal His_6-tagged, while SpnN, SpnR, and SpnS are C-terminal His_6-tagged. The expressed SpnQ protein carries no tag.

Codon Changes in the *spnN* Gene. After the plasmid *spnN*/pET24b+ was obtained, the AGGAGG sequence in *spnN* encoding Arg−Arg (nucleotide 352−357) was mutated to CGTCGC, using the QuikChange Site-Directed Mutagenesis Kit according to the recommended procedures. The two complementary primers used for the mutagenesis were 5′-GGGCTGGCC**CGTCGC**AAGAACCTG-3′ and 5′-CAGGTTCTT**GCGACG**GGCCAGC-3′. The resulting plasmid was referred to as *spnN*/pET24b+-mut.

Growth of *E. coli* SpnO, SpnN, SpnQ, SpnR, and SpnS Recombinant Strains. An overnight culture of *E. coli* BL21(DE3)-*spnO*/pET28b(+) strain grown in Luria−Bertani (LB) medium containing 50 μg/mL kanamycin at 37 °C was used (1 mL each) to inoculate 6 × 1 L of the same growth medium. These cultures were incubated at 37 °C with vigorous shaking until the OD_{600} reached 0.4. Cultures were precooled to 24 °C prior to induction. Protein expression was then induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM, and the cells were allowed to grow at 24 °C for an additional 15 h. The cells were harvested by centrifugation at 4500*g* for 15 min and stored at −80 °C until lysis. The growth of *E. coli* BL21(DE3)-*spnQ*/pET24b(+) and BL21(DE3)-*spnR*/pET24b(+) strains followed an identical procedure. The growth of *E. coli* BL21(DE3)-*spnS*/pET24b(+) followed a similar procedure, except IPTG (0.1 mM) induction was carried out on cultures precooled to 30 °C after OD_{600} reaching 0.4, and cultures were grown at 30 °C for 7 h after induction. The growth of *E. coli* BL21(DE3)-*spnN*/pET24b-(+)-mut also followed a similar procedure, except IPTG (0.25 mM) induction was carried out on cultures precooled to 14 °C after OD_{600} reaching 0.6, and cultures were grown at 14 °C for 48 h after induction.

Purification of *N*-His_6-Tagged SpnO, SpnN, SpnR, SpnS, and Native SpnQ. The protocol for the purification of SpnQ was previously reported.[31] All purification steps of the His_6-tagged proteins were carried out at 4 °C. Thawed cells were first resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, 15% glycerol, pH 8.0; 2 mL/g cells). Cells were then disrupted by sonication using 5 × 1 min pulses with 1 min pauses between each pulse. The lysate was centrifuged at 30 000*g* for 25 min, and the supernatant was subjected to Ni-NTA chromatography according to the manufacturer's protocol with minor modifications. Briefly, the soluble protein fraction was incubated with washed Ni-NTA beads on a rotator at 4 °C for 1 h. Lysate and beads were then loaded onto a column, which was allowed to drain and then washed with 25 mL of each of four wash buffers (50 mM NaH_2PO_4, 300 mM NaCl, 15% glycerol, pH 8.0) containing incrementally increasing concentrations of imidazole (40, 60, 80, and 100 mM). Bound protein was eluted using 10.5 mL elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 15% glycerol, pH 8.0) and collected in 7 × 1.5 mL portions, which were pooled and dialyzed against 4 × 1 L of 20 mM Tris·HCl, pH 7.5, 15% glycerol. SpnO, SpnR, and SpnS eluted with buffer containing 250 mM imidazole, while the purest fractions of SpnN eluted with wash buffer containing 100 mM imidazole. After the Ni-NTA affinity chromatographic step, SpnR and SpnS were further purified by a Sephacryl S-200 HR column (2.5 × 100 cm) using 20 mM Tris·HCl, pH 7.5, with a flow rate of 1 mL/min. Proteins were concentrated by ultrafiltration using an Amicon concentrator with an YM-10 membrane (Amicon) and stored at −80 °C.

Preparation of Reductase Pairs Ferredoxin−Ferredoxin Reductase and Flavodoxin (FLD)-Flavodoxin Reductase (FDR). Ferredoxin and ferredoxin reductase used in these experiments were commercial products. The flavodoxin and flavodoxin reductase genes were amplified from *E. coli* genomic DNA by PCR and cloned into pET24b(+) to give proteins with C-terminal His_6 tags upon expression. The growth of *E. coli* BL21(DE3)-*fld*/pET24b(+) and *E. coli* BL21(DE3)-*fdr*/pET24b(+) and purification of the recombinant FLD and FDR followed similar procedures to those described above for forosamine biosynthetic enzymes with the following modifications. Growth media (2 × 1 L) were inoculated with overnight cultures (4 mL/L culture). The cultures were induced at OD_{600} of 0.3 with 0.1 mM IPTG and grown for 6 h at 37 °C before harvesting. All buffers for Ni-NTA chromatography contained 5 mM 2-mercaptoethanol. Dialysis buffer consisted of 50 mM Tris·HCl, pH 7.5, 15% glycerol.

Iron Quantitation of SpnQ Protein. Iron quantitation was performed according to the method of Fish.[40] Briefly, a 1 mL solution containing as-isolated SpnQ (1 mg) was treated with 0.5 mL of freshly prepared reagent A (2.25% w/v $KMnO_4$ in 6 N HCl) at 60 °C for 2 h. After the treatment was complete, 100 μL of freshly prepared reagent B (6.5 mM ferrozine, 13.1 mM neocuprine, 2 M ascorbic acid, and 5 M ammonium acetate in 25 mL ddH_2O) was added. The mixture was vigorously mixed and then incubated for 30 min at room temperature. Standards made using different concentrations of $Fe(NH_4)_2(SO_4)_2$ solution in 0.01 M HCl were treated in parallel with the protein sample, and the iron content of SpnQ was determined by comparing the absorbance of the SpnQ sample at A_{562} to the standard curve.

Estimation of the PLP Content of SpnR and the Reconstitution of SpnR with PLP. The PLP content of SpnR was estimated according to literature procedures.[41] Reconstitution of SpnR was carried out as follows. A 200 μL solution containing SpnR (120 μM), PLP (2.0 mM), DTT (1 mM), and 50 mM KH_2PO_4 buffer (pH 7.5) was incubated at room temperature for 6 h. The entire solution was then loaded onto an Econo-Pac 10 DG column. The reconstituted protein was eluted with 50 mM KH_2PO_4 buffer (pH 7.5). Fractions containing the SpnR protein were pooled and concentrated. Any residual unbound PLP was removed by repeated dilution and concentration using Centricon YM-10 microconcentrator.

Molecular Mass Determination. The native molecular masses of proteins were determined by gel filtration chromatography using an FPLC equipped with a Superdex 200 HR 10/30 column. The proteins were eluted using 50 mM NaH_2PO_4, pH 7.0, 150 mM NaCl at a flow rate of 0.8 mL/min. Calibration of the column (V_i) was achieved using protein standards (Sigma). The void volume (V_o) of the column was measured using blue dextran. The data were analyzed by the method of Andrews.[42]

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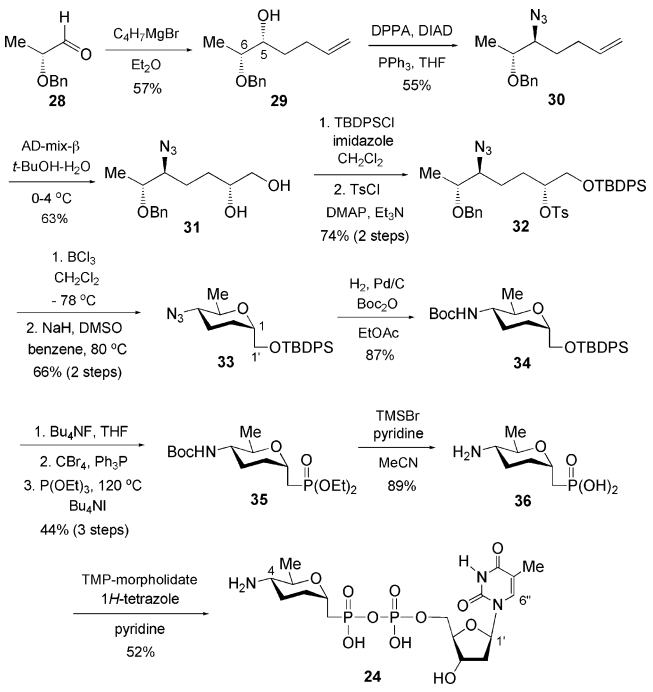
Synthesis of TDP-D-glucose (9). Compound **9** was prepared by chemical coupling of α-D-glucose-1-phosphate (**8**) and TMP morpholidate. Thymidine monophosphate (TMP) and compound **8** were purchased as sodium salts and were converted to their acidic forms using Dowex cation exchange resin. The acidic form of compound **8** was then converted to a triethylamine salt and dried by lyophilization. The acidic form of TMP was converted to TMP-morpholidate according to the procedure of Moffatt and Khorana,[43] affording the 4-morpholine *N*,*N*′-dicyclohexylcarboxamidine salt of TMP-morpholidate in 90% yield. 1*H*-tetrazole was purchased as a 3% (w/v) solution in acetonitrile. Evaporation *in vacuo* gave the solid white powder 1*H*-tetrazole. Glucose-1-phosphate triethylamine salt (540 mg, 1.5 mmol) and TMP-morpholidate (1.54 g, 2.25 mmol) were dissolved in 8 mL of anhydrous pyridine, and the solution was evaporated to dryness *in vacuo*. The process of dissolving and evaporation was repeated twice with 1*H*-tetrazole (190 mg, 2.7 mmol) added to the solution the second time. After all water had been removed in this process, the mixture was dissolved in 12 mL of anhydrous pyridine and stirred at room temperature under nitrogen for 3 days. The solvent was then removed by evaporation *in vacuo*, and the residue was resuspended in 20 mL of water and extracted with chloroform (3 × 15 mL). The aqueous portion was dried by lyophilization, and the solid residue was redissolved in 2 mL of water and applied to a Bio-Rad P2 (extra fine) column (2.5 × 120 cm). The column was run with 25 mM NH_4HCO_3 buffer at a flow rate of 0.24 mL/min, and 6 mL fractions were collected. Fractions exhibiting UV absorption at 267 nm were lyophilized, and the identity and purity of the compounds in each fraction were determined by ^{1}H and ^{31}P NMR spectroscopy. Fractions containing significant quantities of **9** were further purified by 1−2 passages through a Dowex cation exchange column using water as the solvent. These fractions were concentrated to 12 mg/mL. The fractions were neutralized with ammonium bicarbonate and lyophilized individually, and their purity was assessed by ^{1}H and ^{31}P NMR. The yield of **9** was 310 mg (>90% pure, 37% yield). ^{1}H NMR spectrum of **9** was identical to that previously reported.[44]

Enzymatic Preparation of TDP-4-keto-6-deoxy-D-glucose (10). Compound **10** was prepared from **9** by an enzymatic reaction using RfbB. Preparation of RfbB enzyme followed a previously reported procedure.[44] The reaction mixture (1.5 mL) contained **9** (30 mg, 35 mM) and RfbB (24 μM) in 20 mM Tris·HCl buffer (pH 7.5). The reaction was carried out at 37 °C for 2 h. The RfbB protein was removed with Centricon YM-10 microconcentrator (Amicon), and the filtrate containing **10** (25 mg, 78% yield, >90% pure) was used directly without further purification. ^{1}H NMR spectrum of **10** was identical to that previously reported.[46]

Enzymatic Preparation of TDP-4-keto-2,6-dideoxy-D-glucose (12). Compound **12** was prepared from **9** using RfbB, TDP-4-keto-6-deoxy-D-glucose 2,3-dehydratase (TylX3) from the tylosin biosynthetic pathway of *S. fradiae*[19] and the expressed TDP-3,4-diketo-2,6-dideoxy-D-glucose 3-ketoreductase, SpnN. The reaction mixture (1.5 mL) contained **9** (25 mg, 30 mM), RfbB (53 μM), NADPH (34 mM), TylX3 (10 μM), and SpnN (17 μM) in 50 mM Tris·HCl buffer (pH 7.5) containing 10% glycerol. The reaction was incubated at 24 °C, and its progress was monitored spectrophotometrically by following the consumption of NADPH at 340 nm. After the decrease of the absorbance at 340 nm stopped (after 7 h), the enzymes were removed with a Centricon YM-10 microconcentrator. The filtrate was applied to a Bio-Rad P2 gel filtration column (extra fine, 2.5 × 120 cm) which was then eluted with 25 mM NH_4HCO_3 at a flow rate of 0.24 mL/min. Fractions (6 mL each) were analyzed spectrophotometrically, and those displaying a single absorption maximum at 267 nm were concentrated and analyzed by NMR. Fractions containing **12** of high purity were pooled and concentrated by partial lyophilization. The concentration of **12** was determined spectrophotometrically based on a molar extinction coefficient (ε) of 9600 M^{-1} cm^{-1} at 267 nm for thymidine.[45] The yield of **12** was 10 mg (>90% pure, 38% yield). ^{1}H NMR of **12** (D_2O) δ 1.07 (3H, d, J = 6.3 Hz, 5-Me), 1.71 (1H, m, 2-H_{ax}), 1.79 (3H, s, 5″-Me), 2.01 (1H, m, 2-H_{eq}), 2.22 (2H, m, 2′-H), 3.88 (1 H, dd, J = 11.7, 5.1 Hz, 3-H), 3.90 (1H, q, J = 6.3 Hz, 5-H), 4.03 (3H, m, 4′-H, 5′-Hs), 4.48 (1H, m, 3′-H), 5.48 (1H, dd, J = 6.9, 3.3 Hz, 1-H), 6.21 (1H, t, J = 7.2 Hz, 1′-H), 7.62 (1H, s, 6″-H). ^{13}C NMR (D_2O) δ 11.6, 11.9, 36.1 (d, J = 6.3 Hz), 38.9, 68.3, 70.3, 71.2, 85.2, 85.6 (d, J = 9.3 Hz), 93.3, 94.7 (d, J = 5.5 Hz), 112.0, 137.6, 152.0, 166.9, 207.2. ^{31}P NMR (D_2O) δ −10.7 (d, J = 21.2 Hz), −12.7 (d, J = 21.2 Hz). HRMS (FAB) of **12**, calcd for $C_{16}H_{23}N_2O_{14}P_2$ $[M - H]^-$ 529.0625, found 529.0627.

Scheme 4



Chemical Synthesis of TDP-*C*-4-Amino-2,3,4,6-tetradeoxy-α-D-*erythro*-hexopyranose (24) (Scheme 4). (5*R*,6*R*)-6-Benzyloxy-5-hydroxy-L-heptene (29). To a solution of aldehyde **28** (20.0 g, 121 mmol) in diethyl ether (100 mL) at 0 °C was added dropwise over 30 min a solution of 3-butenylmagnesium bromide in dry THF (130 mL). The solution was stirred at room temperature for 3 h and then poured into saturated ammonium chloride solution (300 mL). The resulting two-phase mixture was separated. The aqueous layer was washed twice with ether, and the combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo to give an oily residue. Purification of the crude product by flash column chromatography on silica gel using 4:1 hexanes−EtOAc afforded 15.2 g (57%) of **29**, and 2.5 g (9%) of the minor diastereomer. Data for **29**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.39 (5H, m), 5.90 (1H, ddt, J = 17.1, 10.2, 6.6 Hz), 5.10 (1H, d, J = 17.1 Hz), 5.02 (1H, d, J = 10.2 Hz), 4.71 (1H, d, J = 11.4 Hz), 4.49 (1H, d, J = 11.4 Hz), 3.51 (1H, m), 3.44 (1H, dq, J = 6.3, 6.0 Hz), 2.73 (1H, d, J = 3.6 Hz), 2.31 (1H, m), 2.21 (1H, m), 1.60 (2H, m), 1.24 (3H, d, J = 6.0 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.8, 138.6, 128.7, 128.1, 128.0, 115.0, 78.6, 74.5, 71.2, 32.5, 30.2, 15.8; HRMS (CI) calcd for $C_{14}H_{21}O_2$ $[M + H]^+$ 221.1541, found 221.1531.

(5*S*,6*R*)-5-Azido-6-benzyloxy-L-heptene (30). To a solution of alcohol **29** (13.5 g, 58 mmol), triphenylphosphine (23.0 g, 87 mmol), and diisopropyl azodicarboxylate (18.0 mL, 87 mmol) in dry THF (200

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mL) was added dropwise diphenylphosphoryl azide (25.0 g, 87 mmol) at 0 °C. The resulting mixture was concentrated after stirring at room temperature for 12 h. The residue was quickly passed through a short silica gel column and washed with 5:1 hexanes−EtOAc. The eluate was concentrated under reduced pressure and purified by silica gel flash column chromatography (10:1 hexanes−EtOAc) to give 8.3 g (55%) of azide **30** as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.39 (5H, m), 5.84 (1H, m), 5.11 (1H, m), 5.07 (1H, m), 4.66 (1H, d, J = 11.7 Hz), 4.58 (1H, d, J = 11.7 Hz), 3.64 (1H, m), 3.51 (1H, m), 2.29 (1H, m), 2.18 (1H, m), 1.62 (2H, m), 1.28 (3H, d, J = 6.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.4, 137.6, 128.7, 128.0, 127.8, 115.9, 77.4, 71.1, 65.5, 30.9, 29.7, 15.4; HRMS (CI) calcd for $C_{14}H_{20}N_3O$ [M + H]$^+$ 246.1606, found 246.1615.

(2*R*,5*S*,6*R*)-5-Azido-6-benzyloxyheptane-1,2-diol (31). A suspension of compound **30** (8.9 g, 87 mmol) and AD-mix-β (45 g) in *tert*-butanol (150 mL) and water (150 mL) was vigorously stirred for 17 h at 0−4 °C. The mixture was diluted with EtOAc (200 mL) and quenched with saturated $Na_2S_2O_3$ to give a purple solution. The organic layer was separated, washed with brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was purified by silica gel flash column chromatography (hexanes−EtOAc: 2:1 to 1:1) to afford 5.5 g (63%) of the diol (**31**) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36 (5H, m), 4.63 (1H, d, J = 11.7 Hz), 4.53 (1H, d, J = 11.7 Hz), 3.63 (3H, m), 3.45 (2H, m), 3.13 (1H, m), 3.00 (1H, s), 1.71 (1H, m), 1.63 (1H, m), 1.45 (2H, m), 1.24 (3H, d, J = 6.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.4, 128.7, 128.0, 127.9, 77.5, 72.4, 71.1, 66.9, 66.5, 30.4, 26.8, 15.3; HRMS (CI) calcd for $C_{14}H_{22}N_3O_3$ [M + H]$^+$ 280.1661, found 280.1652.

(2*R*,5*S*,6*R*)-5-Azido-6-benzyloxy-L-(*tert*-butyldiphenylsilyloxy)-2-heptyl Tosylate (32). To a solution of diol **31** (5.5 g, 19.7 mmol) and *tert*-butyldimethylsilyl chloride (5.8 g, 21 mmol) in dichloromethane (150 mL) was added imidazole (1.63 g, 24 mmol) at 0 °C. After being stirred for 2 h at room temperature, the mixture was diluted with dichloromethane, washed with brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was separated by silica gel flash chromatography to give 9.7 g of the silyl ether intermediate.

Triethylamine (3.9 mL, 28 mmol) was added dropwise to a solution of the intermediate (9.7 g, 18.7 mmol) in dichloromethane (140 mL) containing tosyl chloride (4.5 g, 23 mmol) and 4-dimethylaminopyridine (100 mg). The solution was stirred at room temperature for 14 h, at which time additional tosyl chloride (2.0 g, 10 mmol) and triethylamine (3.0 mL, 21 mmol) were introduced, and the reaction was continued for another 12 h. The mixture was diluted with diethyl ether, washed sequentially with saturated sodium bicarbonate, 1 N HCl, and brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was purified by silica gel flash column chromatography (hexanes−EtOAc: 10:1 to 3:1) to give 9.8 g (74% yield, two steps) of the protected diol **32** as a yellowish oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.73 (2H, d, J = 8.4 Hz), 7.62 (4H, m), 7.39 (11H, m), 7.24 (2H, d, J = 8.4 Hz), 4.62 (1H, d, J = 11.7 Hz), 4.55 (1H, m), 4.52 (1H, d, J = 11.7 Hz), 3.67 (2H, m), 3.53 (1H, m), 3.36 (1H, dt, J = 10.2, 3.6 Hz), 2.40 (3H, s), 1.97 (1H, m), 1.72 (1H, m), 1.45 (2H, m), 1.16 (3H, d, J = 6.0 Hz), 1.06 (9H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 144.9, 138.4, 135.9, 135.8, 134.4, 133.2, 133.0, 130.1, 130.0, 128.7, 128.0, 127.9, 127.8, 127.7, 82.9, 77.4, 71.0, 66.0, 64.8, 28.7, 28.0, 27.0, 26.2, 21.9, 19.5, 15.1; HRMS (CI) calcd for $C_{37}H_{46}N_3O_5SiS$ [M + H]$^+$ 672.2927, found 672.2931.

4-Azido-1-*tert*-butyldiphenylsilyloxymethyl-1,2,3,4,6-pentadeoxy-α-D-*erythro*-hexopyranose (33). To a solution of the protected diol **32** (10.8 g, 16.0 mmol) in anhydrous dichloromethane (180 mL) was added dropwise 1.0 M BCl_3 (50 mL) in dichloromethane at −78 °C under N_2. The solution was stirred for 4 h at the same temperature, at which time TLC indicated complete consumption of the starting material. The reaction was quenched by dropwise addition of methanol (8.0 mL) and triethylamine (11.0 mL). The solution was warmed to room temperature, diluted with diethyl ether, washed with saturated sodium bicarbonate, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was flash chromatographed on silica gel (hexanes−EtOAc: 10:1 to 3:1) to give 8.5 g of the C-6 deprotected alcohol.

To a solution of the C-6 deprotected alcohol (8.4 g, 14.4 mmol) in dry benzene (300 mL) were added 95% NaH (800 mg) and dimethyl sulfoxide (2 mL). After the mixture was stirred at 80 °C for 1 h, it was cooled to room temperature and filtered through a short silica gel pad. The filtrate was concentrated in vacuo, and the residue was purified by silica gel flash column chromatography (hexanes−EtOAc: 10:1 to 4:1) to give 4.4 g (66% yield, two steps) of the *C*-glycoside **33** as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.70 (4H, m), 7.44 (6H, m), 3.90 (1H, m), 3.81 (1H, dd, J = 11.2, 5.7 Hz), 3.72 (1H, dd, J = 11.2, 6.9 Hz), 3.63 (1H, dq, J = 6.6, 6.3 Hz), 3.15 (1H, m), 1.96 (1H, m), 1.79 (2H, m), 1.68 (1H, m), 1.24 (3H, d, J = 6.3 Hz), 1.10 (9H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 135.9, 133.7, 130.0, 128.0, 71.5, 70.6, 64.4, 61.8, 27.1, 24.1, 24.0, 19.5, 18.4; HRMS (CI) calcd for $C_{23}H_{30}N_3O_2Si$ [M + H]$^+$ 408.2107, found 408.2098.

1-*tert*-Butyldiphenylsilyloxymethyl-4-*N*-carbo-*tert*-butoxyamino-1,2,3,4,6-pentadeoxy-α-D-*erythro*-hexopyranose (34). To a solution of the *C*-glycoside **33** (1.7 g, 4.0 mmol) in EtOAc (40 mL) were added Boc_2O (1.6 g, 7.2 mmol) and 10% Pd−C (350 mg) at room temperature. The suspension was flashed with H_2, and stirred under H_2 atmosphere for 5 h. The reaction mixture was then filtered through a short silica gel pad. The filtrate was concentrated in vacuo, and the residue was purified by silica gel flash column chromatography (hexanes−EtOAc: 10:1 to 5:1) to give 1.75 g (87%) of the product **34** as a viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (4H, m), 7.40 (6H, m), 4.97 (1H, d, J = 8.8 Hz), 3.77 (2H, m), 3.68 (1H, dd, J = 10.4, 5.2 Hz), 3.59 (1H, dd, J = 10.4, 6.0 Hz), 3.43 (1H, m), 1.84 (1H, m), 1.58 (3H, m), 1.44 (9H, s), 1.24 (3H, d, J = 7.2 Hz), 1.06 (9H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.5, 135.9, 133.8, 129.9, 127.9, 79.4, 73.1, 69.9, 66.4, 49.2, 28.6, 27.1, 23.5, 19.5, 17.0; HRMS (CI) calcd for $C_{28}H_{42}NO_4Si$ [M + H]$^+$ 484.2883, found 484.2867.

Diethyl (2*S*,5*S*,6*R*)-(5-*N*-Carbo-*tert*-butoxyamino-6-methyltetrahydropyranyl)-2-methanephosphonate (35). A solution of **34** (1.75 g, 3.6 mmol) and tetrabutylammonium fluoride (5 mmol) in dry THF (30 mL) was stirred at room temperature for 7 h and then concentrated in vacuo. The residue was diluted with diethyl ether, washed with water, dried over anhydrous Na_2SO_4, evaporated in vacuo, and purified by silica gel flash column chromatography (hexanes−EtOAc: 2:1 to 1:2) to give 0.82 g (92%) of the deprotected alcohol as a colorless oil.

To a solution of the deprotected alcohol (0.81 g, 3.3 mmol) and triphenylphosphine (1.73 g, 6.6 mmol) was added portionwise carbon tetrabromide (2.2 g, 6.6 mmol) at room temperature. The mixture was stirred for 11 h and then concentrated. The residue was separated by silica gel flash chromatography (hexanes−EtOAc: 10:1 to 3:1) to give 0.58 g (56%) of the corresponding bromide as a colorless oil.

A mixture of the above bromide (0.58 g, 1.8 mmol), triethyl phosphite (6.0 mL), and tetrabutylammonium iodide (50 mg) was stirred at 120 °C for 42 h under N_2, and then evaporated over an oil pump. The residue was purified by silica gel flash column chromatography (hexanes−EtOAc: 3:1 to 1:2 plus 5% ethanol) to give 0.59 g (85%) of the desired phosphonate **35** as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.04 (1H, d, J = 9.0 Hz), 4.09 (5H, m), 3.78 (1H, m), 3.42 (1H, m), 1.85−2.13 (4H, m), 1.57−1.74 (2H, m), 1.43 (9H, s), 1.29 (9H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 155.5, 79.5, 73.0, 65.3, 61.9 (d, J = 10.0 Hz), 61.8 (d, J = 10.0 Hz), 49.1, 28.6, 27.9 (d, J = 15.6 Hz), 23.9, 17.1, 16.7, 16.6; ^{31}P NMR (121 MHz, $CDCl_3$) δ 29.7; HRMS (CI) calcd for $C_{16}H_{33}NO_6P$ [M + H]$^+$ 366.2046, found 366.2052.

***C*-(4-Amino-1,2,3,4,6-pentadeoxy-α-D-*erythro*-hexopyranosyl)-methanephosphonic Acid (36).** To a solution of the phosphonate **35** (0.58 g, 1.5 mmol) and pyridine (0.8 mL) in dry acetonitrile (23 mL) was added slowly trimethylsilyl bromide (2.2 mL). The mixture was stirred for 35 h at room temperature and then concentrated in vacuo. The oily residue was treated with water (20 mL) in the presence of excess ammonium bicarbonate. The aqueous solution was extracted

with chloroform (10 mL) and then lyophilized to give the crude product as a white powder. The crude product was dissolved in water, loaded on a DOWEX 50W × 8 (H^+ form) column (1.5 × 10 cm), washed with water, and then with 1 M aqueous ammonia. The desired fractions were combined and lyophilized to give 0.32 g (89%) of the phosphonic acid product **36** as a white powder. ^{1}H NMR (300 MHz, D_2O, pD 7.5) δ 4.02 (1H, m), 3.85 (1H, dq, J = 6.3, 4.5 Hz), 3.06 (1H, m), 2.00 (1H, m), 1.78 (1H, m), 1.70 (3H, m), 1.53 (1H, m), 1.20 (3H, d, J = 6.9 Hz); ^{13}C NMR (75 MHz, D_2O, pD 7.5) δ 69.5, 67.8, 50.4, 33.8 (d, J = 128.6 Hz), 26.1 (d, J = 4.4 Hz), 21.4, 15.9; ^{31}P NMR (121 MHz, D_2O, pD 7.5) δ 19.7.

Thymidine Diphosphate *C*-4-Amino-2,3,4,6-tetradeoxy-α-D-*erythro*-hexopyranose (24). A mixture of the phosphonic acid **36** (0.32 g, 1.4 mmol) and TMP morpholidate (1.5 g, 2.1 mmol) in anhydrous pyridine (6 mL) was evaporated three times using an oil pump. To the resulting solid were added anhydrous pyridine (8 mL) and 1*H*-tetrazole (160 mg, 2.2 mmol). The mixture was stirred vigorously at room temperature for 115 h. The solvents were then removed using an oil pump, and the remaining solid was dissolved in water (~3 mL). This aqueous solution was loaded onto a Bio-Gel P2 (extra fine) column (2.0 × 120 cm) and eluted with 25 mM ammonium bicarbonate. The desired fractions, as indicated by their UV absorption at 267 nm, were pooled and lyophilized to afford 0.38 g (52%) of the isostere product **24** as a white powder. ^{1}H NMR (300 MHz, ^2H_2O, pD 7.5) δ 7.61 (1H, s, 6″-H), 6.21 (1H, dd, J = 7.2, 6.6 Hz, 1′-H), 4.48 (1H, m, 3′-H), 4.06 (4H, m, 1-H, 4′-H, 5′-H), 3.85 (1H, dq, J = 6.9, 4.5 Hz, 5-H), 3.04 (1H, m, 4-H), 2.23 (2H, m, 2′-H), 1.97 (2H, dd, J = 18.9, 6.9 Hz, CH_2−P), 1.79 (3H, s, 5″-Me), 2.02−1.48 (4H, m, 2-H, 3-H), 1.20 (3H, d, J = 6.9 Hz, 5-Me); ^{13}C NMR (75 MHz, ^2H_2O, pD 7.5) δ 166.9, 152.0, 137.6, 111.9, 85.5 (d, J = 8.7 Hz), 85.1, 71.1, 69.7, 66.9, 65.4 (d, J = 6.0 Hz), 50.4, 38.7, 33.1 (d, J = 136.4 Hz), 26.2 (d, J = 6.4 Hz), 21.5, 15.9, 11.8; ^{31}P NMR (121 MHz, ^2H_2O, pD 7.5) δ 14.4 (d, J = 26.0 Hz), −10.6 (d, J = 27.4 Hz); HRMS (FAB) calcd for $C_{17}H_{28}N_3O_{11}P_2$ $[M - H]^-$ 512.1199, found 512.1189.

HPLC Assay Methods. Unless otherwise specified, all HPLC assays were conducted using a Dionex CarboPac PA1 analytical column (4 × 250 mm) with a CarboPac PA1 guard column (4 × 50 mm). A suitable amount of reaction mixture was withdrawn at appropriate time intervals, and the reaction was quenched by 10-fold dilution with water and flash frozen in liquid nitrogen. Prior to HPLC analysis, the samples were thawed and filtered through a Microcon YM-10 membrane to remove enzyme. The filtrate was then injected into the HPLC column. The sample was eluted with a gradient of water as solvent A and 500 mM NH_4OAc (adjusted to pH 7.0 with aqueous NH_3) as solvent B. The gradient was ran from 5 to 20% B over 15 min, from 20 to 60% B over 20 min, from 60 to 100% B over 2 min, with a 3 min wash at 100% B, and from 100 to 5% B over 5 min, followed by reequilibration at 5% B for 15 min. The flow rate was 1 mL/min, and the detector was set at 267 nm.

Activity Assays for SpnO and SpnN and Coupled SpnO/SpnN Assay. A typical SpnO assay mixture (50 μL) contained **10** (30 μM), SpnO (4.2 μM), and 50 mM KH_2PO_4 buffer (pH 7.5). The reaction, carried out at 24 °C, was initiated by the addition of SpnO, and its progress was monitored by following changes in peak integrations of substrate and product by HPLC as described above. The retention times were 35.3 min for TDP-4-keto-6-deoxy-D-glucose (**10**) and 2.4 min for maltol (**22**). The activities of SpnO and SpnN were examined spectrophotometrically together at 24 °C by following the change in absorbance at 340 nm, which corresponds to NADPH consumption (ϵ_{340} = 6220 M^{-1} cm^{-1}) by SpnN. A typical assay mixture to determine SpnO and SpnN activity (100 μL) contained **9** (17 μM), NADPH (18 μM), RfbB (40 μM), SpnO (1 μM), and SpnN (2 μM) in 50 mM KH_2PO_4 buffer, pH 7.5. The reaction was initiated by the addition of SpnO. The activity of SpnN was also examined by HPLC using TylX3 as the dehydratase. A typical coupled assay mixture to determine SpnN activity (50 μL) contained **9** (0.42 mM), RfbB (40 μM), NADPH (150 μM), TylX3 (1.0 μM), SpnN (2.0 μM), and 50 mM KH_2PO_4 buffer (pH 7.5). The reaction, carried out at 24 °C, was initiated by the addition of TylX3, and was monitored by HPLC. The retention times were 34.5 min for TDP-D-glucose (**9**) and 33.4 min for TDP-4-keto-2,6-dideoxy-D-glucose (**12**).

Activity Assays for SpnQ. A typical SpnQ assay mixture (100 μL) under the "ColD-like" conditions contained **12** (1 mM), PLP (250 μM), SpnQ (10 μM), and L-glutamate (3 mM) in 50 mM KH_2PO_4 buffer (pH 7.5), and was incubated at 37 °C. The HPLC retention time was 15.8 min for TDP-4-amino-2,4,6-trideoxy-D-glucose (**23**). The efficiency of L-aspartate as the amino donor was examined by replacing L-glutamate with L-aspartate of the same concentration in the assay. A typical SpnQ assay mixture (100 μL) under the "E_1-like" conditions contained **12** (0.7 mM), PMP (250 μM), SpnQ (30 μM), and the reducing agent/system being tested (such as 0.6 mM sodium dithionite or 30 μM each of flavodoxin/flavodoxin reductase or ferredoxin/ferredoxin reductase in the presence of 0.7 mM NADPH) in 50 mM KH_2PO_4 buffer (pH 7.5) and was incubated at 24 °C. The HPLC retention time was 36.2 min for TDP-4-keto-2,3,6-trideoxy-D-glucose (**13**). SpnQ assays were conducted under aerobic conditions using as-purified enzyme except when dithionite was used as the reductant, in which case anaerobically reconstituted SpnQ was assayed under anaerobic conditions.

Isolation and Characterization of SpnQ Products 13 and 23. The reaction conditions, isolation, and characterization of the products **13** and **23** obtained from incubation of SpnQ with **12** under the "ColD-like" and the "E_1-like" conditions, respectively, were previously reported.[31]

Determination of the Kinetic Parameters for SpnQ Using 12. The steady-state kinetic parameters of the SpnQ-catalyzed reaction were determined using a continuous assay in which NADPH consumption was monitored. A series of reactions (100 μL) containing SpnQ (1.0 μM), PMP (40 μM), NADPH (150 μM), ferredoxin (10 μM), ferredoxin reductase (10 μM), and various concentrations of **12** (10−200 μM) in a total volume of 50 mM KH_2PO_4 buffer (pH 7.5) were prepared and monitored spectrophotometrically. The reactions were conducted at 24 °C, and each initial reaction rate was determined as the initial slope of the decrease in absorbance at 340 nm. The resulting data were fit to the Michaelis−Menten equation by nonlinear regression using Grafit to determine the k_{cat} and K_m values.

Activity Assays for SpnR Using 12, 13, 24, and 27. The reaction mixture (50 μL) for the SpnR assay using 4-keto substrates **12** or **27** contained substrate (1 mM), L-glutamate (10 mM), PLP (40 μM), and as-purified SpnR (10 μM) in 50 mM KH_2PO_4 buffer (pH 7.5). The HPLC retention times were 33.4 min for **12**, 15.8 min for **23**, 32.6 min for **27**, and 6.8 min for **24**. The contents of a typical reaction mixture for assaying SpnR using 4-amino substrate mimic **24** were identical to those used above, except that α-ketoglutarate (30 mM) was used in place of L-glutamate. The competence of pyruvate as the cosubstrate in the SpnR reaction using **24** as substrate was examined by replacing α-ketoglutarate with pyruvate of the same concentration in the assay. The effect of increasing PLP concentrations on the SpnR reaction using **24** as substrate was examined by maintaining the above assay conditions but varying the concentration of PLP (0, 10, 20, 40, or 80 μM). Each reaction was stopped at 3 min and analyzed by HPLC. Because of its instability, the physiological SpnR substrate **13** was generated *in situ* prior to the assay by the incubation of **12** (0.7 mM) with SpnQ (30 μM), ferredoxin/ferredoxin reductase (30 μM each), and NADPH (2 mM) in 50 mM KH_2PO_4, pH 7.5 at 24 °C for 3 h. Enzymes were removed by filtration through a Microcon YM-10 membrane. To the filtrate were added SpnR, PLP, L-glutamate, and $MgCl_2$ to give a final concentrations of 37 μM, 305 μM, 12.2 mM, and 1.22 mM, respectively. The reaction was initiated by the addition of SpnR and incubated at 24 °C for an additional 2 h. The progress of the reaction was monitored by HPLC. The HPLC retention time was 9.2 min for the SpnR product **14**.

Isolation and Characterization of the SpnR Reaction Products 23 and 27. The reaction conditions, isolation, and NMR characterization of **27** obtained from the incubation of SpnR with **24** in the presence of excess α-ketoglutarate were previously reported.[30] The isolation and mass spectrometric characterization of **23** obtained from incubation of SpnR with **12** and L-glutamate had also been reported.[30]

Determination of the K_{eq} Value for the SpnR-Catalyzed Transamination of 24. A reaction (50 μL) containing aminosugar 24 (0.6 mM), α-ketoglutarate (0.6 mM), PLP (50 μM), and as-purified SpnR (10 μM), in 50 mM KH_2PO_4, pH 7.5, was prepared and incubated at 24 °C for 12 h. Ratios of 24 and ketosugar 27 were determined based on HPLC peak integration. Values were inserted into eq 1 to calculate the equilibrium constant (K_{eq}) for the reaction.

$$K_{eq} = \frac{[\mathbf{24}]\,[\alpha\text{-KG}]}{[\mathbf{27}]\,[\text{L-glu}]} = \frac{[\mathbf{24}]\,\{[\alpha\text{-KG}]_0 - [\mathbf{27}]\}}{[\mathbf{27}]^2} \qquad (1)$$

Determination of the Kinetic Parameters for SpnR Catalysis Using 27 as Substrate. The steady-state kinetic parameters of the SpnR-catalyzed transamination of **27** were determined using a continuous L-glutamate dehydrogenase-coupled assay in which NADPH consumption was monitored.[46] Specifically, a series of reactions (100 μL) containing as-purified SpnR (1.0 μM), PLP (250 μM), L-glutamate (50 mM), NADPH (150 μM), NH_4Cl (80 mM), 0.6 U of L-glutamate dehydrogenase, and various concentrations of 27 (5−1400 μM) in 50 mM KH_2PO_4 buffer (pH 7.5) were prepared and monitored spectrophotometrically. The reactions were conducted at 24 °C, and each initial reaction rate was determined by following the initial slope of the decrease in absorbance at 340 nm. The resulting data were fit to the Michaelis−Menten equation by nonlinear regression using Grafit to determine the k_{cat} and K_m^{app} values.

Activity Assays for SpnS Using 24. A typical assay mixture (60 μL) contained substrate **24** (1 mM), *S*-adenosyl-L-methionine (SAM, 2.0 mM), $MgCl_2$ (2.0 mM), DTT (2.0 mM), SpnS (10 μM), and 50 mM KH_2PO_4 (pH 7.5). The reaction was initiated by the addition of SpnS and was carried out at 37 °C. The progress of the reaction was monitored by HPLC (Dionex CarboPac PA1 column). The HPLC retention time was 5.1 min for the SpnS monomethylated product **25**, which overlaps with an authentic sample of *S*-adenosyl-L-homocysteine (SAH). The peak of the dimethylated product **26** overlaps with those associated with SAM, SAH, and its degradation products (retention times 2.6−5.5 min) and thus could not be directly observed. The SpnS reaction was also monitored using an Adsorbosphere SAX column (5 μm, 4.6 × 250 mm) at 267 nm. A linear gradient from 0 to 20% Buffer D (500 mM KH_2PO_4 buffer, pH 3.5) in Buffer C (50 mM KH_2PO_4 buffer, pH 3.5) over 20 min gave baseline separation between substrate **24** (having a retention time of 11.0 min) and monomethylated product **25** (having a retention time of 20.2 min). Unfortunately, the peak of the dimethylated product **26** using the SAX column also overlaps with those associated with SAM, SAH, and its degradation products (retention times 2.3−4.2 min) and therefore could not be directly detected. Hence, for SpnS methylation time course experiments, the amount of **26** formation at a given time point was calculated by subtracting the integrations of the remaining substrate and the monomethylated product peaks from that of the substrate peak recorded at time zero. The effect of increasing Mg^{2+} concentrations on SpnS catalytic efficiency using **24** as substrate was examined under the above assay conditions but varying the concentration of $MgCl_2$ (100, 200, 400, and 800 μM, and 2.0 and 10 mM). Each reaction was stopped at 20 min and analyzed by HPLC (Dionex CarboPac PA1 column) to determine the extent of substrate consumption.

Isolation and Characterization of the SpnS Products 25 and 26. A preparative scale incubation was carried out in which **24** (8.5 mg, 8.25 mM), SpnS (80 μM), SAM (20 mM), $MgCl_2$ (2 mM), and DTT (2 mM) were incubated in 2 mL of 50 mM KH_2PO_4 buffer (pH 7.5). The reaction was carried out at 37 °C, and the progress of the reaction was monitored by HPLC. After 17 h, about 70% of the substrate had been converted to products as judged by substrate peak integration (30% of the substrate remained unreacted). The enzyme was removed with a Centricon YM-10 microconcentrator, and the filtrate was applied to a Bio-Rad P2 gel filtration column (extra fine, 2.5 × 120 cm) which was eluted with 25 mM NH_4HCO_3 at a flow rate of 0.24 mL/min. Fractions (6 mL each) were analyzed spectrophotometrically, and those displaying a single absorption maximum at 267 nm were concentrated and analyzed by NMR. Fractions containing **25** of high purity were pooled and concentrated by lyophilization. The concentration of **25** was determined spectrophotometrically based on a molar extinction coefficient (ε) of 9600 M^{-1} cm^{-1} at 267 nm for thymidine. The yield of **25** was 3 mg (>95% pure, 33% yield). ^{1}H NMR of **25** (D_2O) δ 1.32 (3H, d, J = 4.2 Hz, 5-Me), 1.85 (3H, s, 5″-Me), 1.44−2.16 (6H, m, 2-H, 3-H, CH_2−P), 2.24−2.34 (2H, m, 2′-H), 2.66 (3H, s, NH−Me), 3.02 (1H, m, 4-H), 4.05−4.16 (5H, m, 4′-H, 5′-H, 1-H, 5-H), 4.54 (1H, ddd, 3′-H), 6.27 (1H, t, J = 3.9 Hz, 1′-H), 7.67 (1H, s, 6″-H). ^{13}C NMR (D_2O) δ 11.9, 15.4, 19.2, 26.0, 30.9, 34.2 (d, J = 138.2), 38.8, 57.7, 65.4, 66.1, 68.9, 71.1, 85.2, 85.6, 112.2, 138.0, 152.0, 167.2. ^{31}P NMR (D_2O) δ 14.1 (d, J = 27 Hz), −10.7 (d, J = 27 Hz). HRMS (FAB) calcd for $C_{18}H_{32}N_3O_{11}P_2$ $[M + H]^+$ 528.1512, found 528.1502.

The monomethylated product **25** of the SpnS reaction isolated from the preparative scale reaction described above was used to examine the formation of the dimethylated product **26**. Assay conditions were identical to those used for the SpnS activity assays described above, except that 20 μM SpnS was used in the reaction. The reaction was carried out at 37 °C for 12 h. The enzyme was removed using a Centricon YM-10 microconcentrator, and the filtrate was subjected to Dionex-HPLC analysis. As has been mentioned before, neither **25** nor **26** could be resolved from the SAM degradation products under the HPLC conditions used. Thus, all peaks with retention times of 2.3−4.2 min were collected. The compounds were lyophilized to dryness and redissolved in water, and the resulting solution was submitted to MS analysis. ESI-MS calculated for **26**, $C_{19}H_{32}N_3O_{11}P_2$ $[M - H]^-$, was 540, and a mass of 540 was found.

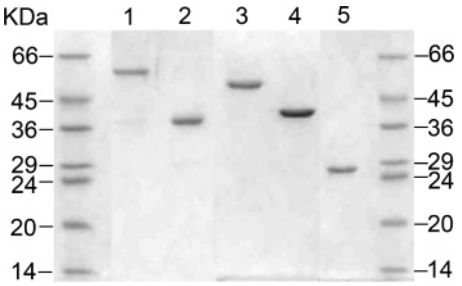

Figure 2. SDS-PAGE gels (12%) of purified SpnO (lane 1), SpnN (lane 2), SpnQ (lane 3), SpnR (lane 4), and SpnS (lane 5).

Results

Purification and Characterization of SpnO and SpnN. In order to assess the catalytic activities of the proposed TDP-4-keto-6-deoxy-D-glucose 2,3-dehydratase, SpnO, and TDP-3,4-diketo-2,6-dideoxy-D-glucose 3-ketoreductase, SpnN, several overexpression constructs of *spnO* and *spnN* were made. After testing the overexpression level and solubility of SpnO protein derived from these constructs, *spnO*/pET28b(+), which produces N-terminal His_6-tagged SpnO, was identified as the one that gives the best yield of soluble protein. However, SpnO-*N*-His_6 was prone to precipitation, and inclusion of 20% glycerol in all buffers during purification was important for minimizing precipitation. The isolated yield of SpnO-*N*-His_6 was 2 mg of pure protein (Figure 2, lane 1) per 6 L of culture after a single Ni-NTA chromatographic step. The purity was estimated to be >90%.

Expression and purification of SpnN were more problematic. After different vectors and hosts failed to yield any detectable SpnN expression, the *spnN* gene sequence was reexamined, revealing two potential problems: the start codon in the published *spnN* sequence was likely misassigned, and *spnN* contains tandem AGG (Arg) codons which are rarely found in *E. coli* genome and are known to drastically reduce protein expression level[47] and promote frameshifting.[48] Recloning of *spnN* using the alternative start codon 12 nucleotides upstream of the originally assigned start codon and site-directed mutagenesis of the rare Arg codons resulted in a significant improvement in the yield of C-terminal His_6-tagged SpnN protein, allowing the isolation of 4 mg of >90% pure SpnN-*C*-His_6 (Figure 2, lane 2) from a 6 L culture after a single Ni-NTA chromatographic step. The monomeric molecular weights of 55 kDa for SpnO and 38 kDa for SpnN estimated by SDS-PAGE are in good agreement with the values of 56 551 Da and 37 732 Da predicted from their gene sequences. Gel filtration chromatographic analysis revealed that the molecular masses for the purified SpnO-*N*-His_6 and SpnN-*C*-His_6 are 96 kDa and 38 kDa, respectively, indicating that SpnO is a homodimeric protein and SpnN is a monomeric protein. Neither protein shows absorbance above 300 nm.

Catalytic Properties of SpnO and SpnN. SpnO shares significant sequence identity with the characterized TDP-4-keto-6-deoxy-D-glucose 2,3-dehydratases, TylX3, Gra27, and OleV, which convert **10** to TDP-3,4-diketo-2,6-dideoxy-D-glucose (**11**), and was therefore proposed to play a similar role in forosamine biosynthesis. SpnN shares nearly 50% sequence identity with Gra26 and OleW, the ketoreductases shown to convert **11** to TDP-4-keto-2,6-dideoxy-D-glucose (**12**) in their respective pathways but only shares 14% sequence identity with TylC1, the reductase which converts **11** to TDP-4-keto-2,6-dideoxy-D-allose (**21**), the C-3 epimer of **12**.[19,21] SpnN was thus proposed to convert the SpnO product **11** to **12**. To verify the function of SpnO, compound **10** (Figure 3, trace b), the proposed substrate of SpnO, was chemoenzymatically synthesized and was subsequently incubated with SpnO. Previous assays using SpnO homologues demonstrated that **11** is unstable, rapidly degrading to TDP and maltol (**22**). As expected, HPLC analysis of the SpnO reaction mixture showed the formation of a new peak which has the same retention time (2.4 min) as an authenic sample of **22**.

Since reduction of **11** by SpnN will give a stable product, **12**, a tandem reaction of SpnO and SpnN was carried out to confirm the activities of SpnO as well as SpnN. Both enzymes were incubated together with TDP-D-glucose (**9**), RfbB, and either NADH or NADPH, and the reaction was monitored spectrophotometrically by following the consumption of NAD(P)H at 340 nm. In this reaction, **9** was first converted to the SpnO substrate **10** *in situ* using RfbB. A time-dependent decrease in A_{340} was observed in reactions containing SpnO, SpnN, and either NADH or NADPH, but no decrease in A_{340} was observed in control reactions lacking either SpnO or SpnN. These results support the proposed roles of SpnO and SpnN as the 2,3-dehydratase and the NAD(P)H-dependent ketoreductase

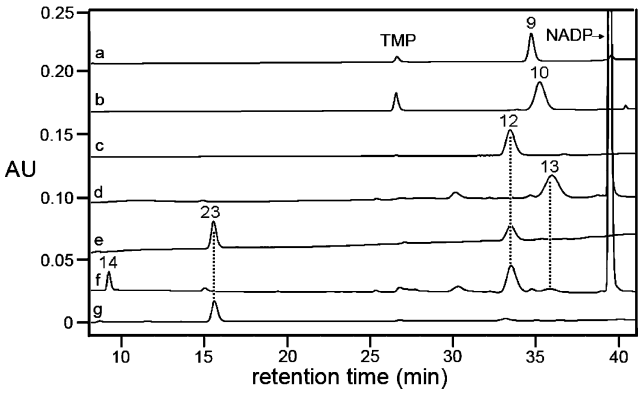

Figure 3. Enzymatic reactions of forosamine biosynthetic enzymes. (a) Purified chemically synthesized TDP-D-glucose (**9**); (b) incubation of **9** with RfbB in 20 mM Tris·HCl buffer (pH 7.5) to form TDP-4-keto-6-deoxy-D-glucose (**10**); (c) purified SpnN product TDP-4-keto-2,6-dideoxy-D-glucose (**12**) obtained by incubation of **9** (30 mM) with RfbB (53 μM), NADPH (34 mM), TylX3 (10 μM), and SpnN (17 μM) in 50 mM Tris·HCl buffer (pH 7.5), 10% glycerol; (d) incubation of **12** with SpnQ (30 μM), PMP (250 μM), ferredoxin (30 μM), ferredoxin reductase (30 μM), and NADPH (0.7 mM) in 50 mM KH_2PO_4 (pH 7.5) to form TDP-4-keto-2,3,6-trideoxy-D-glucose (**13**); (e) incubation of **12** with SpnQ (10 μM), PLP (250 μM), and L-glutamate (3 mM) in the same buffer as in d to form TDP-4-amino-2,4,6-trideoxy-D-glucose (**23**); (f) incubation of **12** under the same conditions as in d followed by removal of enzyme and incubation with SpnR (37 μM), PLP (305 μM), L-glutamate (12.2 mM), and $MgCl_2$ (1.22 mM) to form TDP-4-amino-2,3,4,6-tetradeoxy-D-glucose (**14**); (g) incubation of **12** (1 mM) with as-purified SpnR (10 μM), PLP (40 μM), and L-glutamate (10 mM) in the same buffer as in d to form **23**.

which together convert **10** to **12**. Since NADPH was consumed 2-fold faster than NADH under otherwise identical assay conditions, NADPH is likely the physiological reductant for the SpnN-catalyzed reaction. A similar preference was also found for SpnN homologues Gra26 and OleW, which use NADPH more effectively.[21]

It should be noted that SpnO is not as efficient a 2,3-dehydratase as its homologue, TylX3.[19] Therefore, in subsequent reactions TylX3 was substituted for SpnO for the preparation of **12**. The comparative catalytic efficiencies of SpnO and TylX3 were not assessed quantitatively because of the instability of the reaction product, **11**. When a coupled TylX3/SpnN reaction performed using **9**, RfbB, and NADPH was monitored by HPLC, a new peak (retention time of 33.4 min), which was presumed to be the SpnN product **12**, was detected. Figure 3, trace c, shows the HPLC chromatogram of the purified SpnN product **12** obtained from a preparative-scale incubation of **9** with RfbB, TylX3, SpnN, and NADPH. The ^{1}H NMR spectrum of the product was fully consistent with that previously reported for TDP-4-keto-2,6-dideoxy-D-glucose (**12**).[21] This data, combined with high-resolution FAB-MS of **12**, unambiguously established that SpnN is the ketoreductase responsible for the conversion of **11** to **12** in the forosamine biosynthetic pathway.

Substrate Specificity and Steady-State Kinetics of the SpnQ-Catalyzed Reaction. SpnQ was overexpressed and purified and its identity confirmed by N-terminal amino acid sequencing. SpnQ, which has a predicted molecular weight of 50 361, appeared as a 51 kDa band on SDS-PAGE (Figure 2, lane 3) and was found to be a dimer in solution (molecular mass 109.4 kDa). SpnQ was subsequently shown *in vitro* to be the 3-dehydrase responsible for conversion of the SpnN product TDP-4-keto-2,6-dideoxy-D-glucose (**12**) to TDP-4-keto-2,3,6-trideoxy-D-glucose (**13**, retention time of 36.2 min in Figure 3,

(47) Chumpolkulwong, N.; Sakamoto, K.; Hayashi, A.; Iraha, F.; Shinya, N.; Matsuda, N.; Kiga, D.; Urushibata, A.; Shirouzu, M.; Oki, K.; Kigawa, T.; Yokoyama, S. *J. Struct. Funct. Genomics* **2006**, *7*, 31−36.
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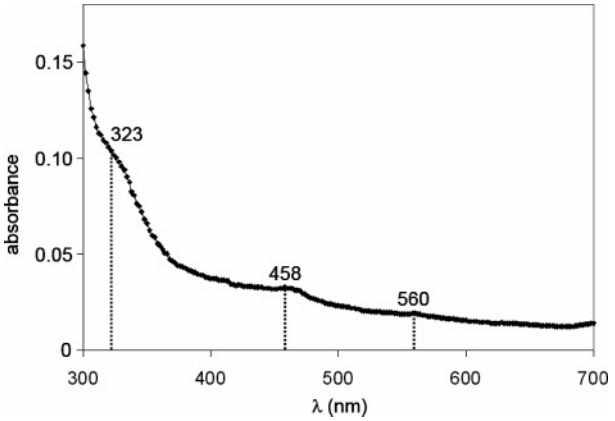

Figure 4. UV/visible spectrum of as-purified SpnQ (8.7 μM) showing characteristic [2Fe-2S] features at 323, 458, and 560 nm.

trace d) in the forosamine biosynthetic pathway.[31] SpnQ, like its homologue E_1 (49% identity), has UV/vis spectral features at 323, 458, and 560 nm and the as-purified protein contains 1.2 irons per monomer as determined by ferrozine quantitation, consistent with the enzyme having a [2Fe-2S] cluster (Figure 4).[49,51] SpnQ also requires a reductase for activity. However, no E_3 gene homologue can be found in the spinosyn gene cluster. SpnQ is therefore thought to catalyze a 3-dehydration reaction in a mechanistically similar manner to that of E_1[5,22] but employs cellular reductases ferredoxin/ferredoxin reductase or flavodoxin/flavodoxin reductase rather than a specific partner reductase, as in the E_1 case. Interestingly, in the absence of an electron source and in the presence of L-glutamate, SpnQ catalyzes a transamination reaction, converting **12** to TDP-4-amino-2,4,6-trideoxy-D-glucose (**23**, retention time of 15.8 min in Figure 3, trace e).[31] The SpnQ homologue ColD (26% identity) lacks an iron-sulfur cluster and does not require a reductase. Instead ColD catalyzes the 3-dehydration using L-glutamate to regenerate PMP.[23] In the absence of E_3 and in the presence of L-glutamate, E_1 has also been shown to carry out a 3-dehydration reaction rather than a transamination reaction,[49] making it akin to ColD in this respect. Clearly, SpnQ, E_1, and ColD each have unique catalytic properties.

To our knowledge, the TDP-4-aminosugar **23** is neither a characterized nor a predicted intermediate in any biosynthetic pathway, and therefore its preparation using SpnQ warrants optimization. It was found that L-glutamate is 20-fold more efficient than L-aspartate as an amino donor. Further analysis also showed that the reaction proceeds optimally at pH 7.5 and at 37 °C. Next, the ability of SpnQ to process an alternate substrate **10** was investigated by incubating **10** with SpnQ in the presence of ferredoxin, ferredoxin reductase, and NADPH. Unfortunately, no consumption of substrate or formation of any new products was detected by HPLC even after prolonged incubation, indicating that **10** is not a substrate for SpnQ. Finally, the steady-state kinetics parameters for SpnQ were determined using an assay similar to that previously reported for E_1/E_3[52] in which NADPH consumption was monitored spectrophotometrically in the presence of **12**, ferredoxin, and ferredoxin reductase.

Table 1. Summary of Kinetic Parameters of Reactions of Characterized PMP-Dependent 3-Dehydrases SpnQ, E_1, and ColD

enzyme	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})
SpnQ	2.6	49	0.053
E_1	3.5	44	0.079
ColD	36	49[a]	0.73[a]

[a] The K_m value for the ColD-catalyzed reaction is a K_m^{app}.

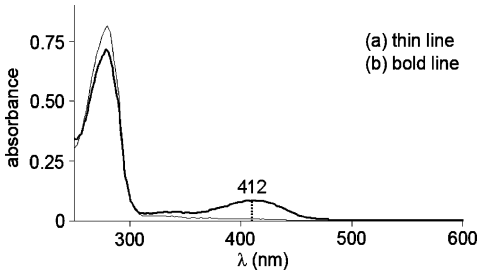

Figure 5. UV/visible spectra of (a) as-isolated SpnR (19.4 μM) and (b) SpnR reconstituted with PLP (17.0 μM). See Experimental Section for details on reconstitution.

A k_{cat} value of 2.6 min^{-1} and a K_m value of 49 μM for **12** were obtained (Table 1). These values are similar to those determined for E_1 (k_{cat} = 3.5 min^{-1}, K_m of 44 μM)[52] under similar conditions. Based on the kinetic parameters of the ColD reaction (k_{cat} = 36 min^{-1}, K_m^{app} of 49 μM),[23] the catalytic efficiency of ColD is apparently higher than those of SpnQ and E_1.

Purification and Characterization of SpnR. To assess the catalytic activity of SpnR, the proposed TDP-4-keto-2,3,6-trideoxy-D-glucose (**13**) 4-aminotransferase, *spnR*, was cloned into the pET24b(+) expression vector giving a C-terminal His_6-tagged protein upon expression in *E. coli*. The recombinant SpnR protein was purified to homogeneity after sequential Ni-NTA and Sephacryl S-200 chromatographic separations. The yield of SpnR was 80 mg of protein from 3 L of culture. A molecular mass of 43 kDa of SpnR estimated by SDS-PAGE (Figure 2, lane 4) correlates well with the predicted value of 43,356 Da calculated from the deduced amino acid sequence. A molecular mass of 85.9 kDa, as judged by gel filtration chromatography, indicates that the SpnR exists as a dimer in solution. The UV−visible spectrum of the purified SpnR showed only weak absorbance at 412 nm corresponding to less than 0.1 equiv of PLP cofactor per monomer (Figure 5, spectrum a). After reconstitution with excess PLP, SpnR was determined to have nearly 1 equiv of PLP per monomer (Figure 5, spectrum b). These results indicated that exogenous PLP is required in the assay for SpnR to be fully active.

Chemical Synthesis of the Phosphonate Mimic of SpnR Product 24. As depicted in Scheme 4, preparation of this compound started from (*R*)-2-benzoxypropionaldehyde (**28**). The key intermediate **31** was prepared in three steps, involving the diastereoselective chain extension to a (5*R*,6*R*)-diol (**29**),[53] azide displacement under modified Mitsunobu conditions to give **30**,[54] and asymmetric dihydroxylation of the terminal olefin to yield **31**.[55] After protective group manipulations, the linear azide **32**

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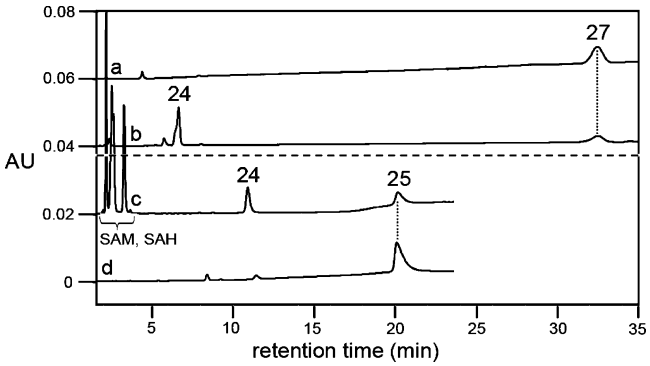

Figure 6. SpnR and SpnS reactions using phosphonate substrate mimics. Samples a and b were analyzed using a Dionex Carbopac PA1 column. Samples c and d were analyzed using the SAX Adsorbosphere column. (a) Incubation of amino phosphonate mimic **24** (1 mM) with SpnR (10 μM), α-ketoglutarate (30 mM), and PLP (40 mM) in 50 mM KH_2PO_4 buffer (pH 7.5); (b) incubation of keto phosphonate mimic **27** (1 mM) with SpnR (10 μM), L-glutamate (10 mM), and PLP (40 mM) in 50 mM KH_2PO_4 buffer (pH 7.5); (c) incubation of amino phosphonate mimic **24** (1 mM) with SpnS (10 μM), SAM (2 mM), $MgCl_2$ (2 mM), DTT (2 mM) in 50 mM KH_2PO_4 buffer (pH 7.5); (d) purified *N*-monomethylamino phosphonate mimic **25** from preparative scale SpnS reaction using substrate **24**.

was converted to the cyclic *C*-glycoside **33** by an intramolecular S_N2 substitution reaction.[56] Less than 5% cyclized product with the incorrect stereochemistry at the pseudoanomeric center was obtained, and this was easily separated from **33** by silica gel chromatography. The azide functionality was then converted to the Boc-protected amino group under reductive amidation conditions,[57] and the C−P linkage was introduced under Arbuzov conditions[58] to give phosphonate **35**. Trimethylsilyl bromide was used to remove both the phosphonate ester and the Boc protecting groups, and the resulting phosphonic acid **36** was coupled with thymidine 5′-monophosphomorpholidate in the presence of 1*H*-tetrazole to give **24**, which was purified by size-exclusion chromatography on Bio-Gel P2 extra fine resin.[59]

Catalytic Properties of SpnR. Incubation of the synthesized phosphonate **24** with SpnR in the presence of PLP and α-ketoglutarate led to the formation of the 4-keto product **27** (Figure 6, trace a), which was isolated and characterized by NMR spectroscopy and high-resolution mass spectrometry.[30] The phosphonate mimic was used because of the natural SpnR substrate **13** is unstable. Interestingly, SpnR was also able to catalyze transamination of **12**, resulting in formation of TDP-4-amino-2,4,6-trideoxy-D-glucose (**23**, Figure 3, trace g), the same compound made by SpnQ in the presence of PLP and L-glutamate. Several additional experiments were also performed to characterize the PLP-dependence and cosubstrate specificity of the SpnR-catalyzed reaction. First, replacement of the α-ketoglutarate cosubstrate with pyruvate in the transamination reaction (**24** → **27**) resulted in a 2-fold decrease in the rate of product formation, indicating that pyruvate was less efficient than α-ketoglutarate in the reaction, but could nevertheless substitute for it. To test the aminotransferase activity of SpnR in the forward direction, the ketosugar phosphonate mimic **27**, prepared using SpnR and **24**, was incubated with SpnR, PLP, and L-glutamate. HPLC analysis indicated that the conversion of **27** to **24** was almost complete within 4 h under the assay conditions (Figure 6, trace b). The ability of SpnR to catalyze transamination of its natural substrate **13** was also tested. Compound **13** was first generated from **12** using SpnQ, ferredoxin, ferredoxin reductase, and NADPH. Subsequent incubation of the filtrate from the SpnQ reaction with SpnR, PLP, and L-glutamate led to the depletion of **13** and to the formation of a new peak (retention time of 9.2 min, Figure 3, trace f), corresponding to the SpnR product TDP-4-amino-2,3,4,6-tetradeoxy-D-glucose (**14**).

Purification and Characterization of SpnS. To establish the function of SpnS as the *N*,*N*-dimethyltransferase catalyzing the final step in TDP-D-forosamine (**16**) biosynthesis, we cloned the *spnS* gene into the pET24b(+) expression vector producing a C-terminal His_6-tagged protein upon expression in *E. coli*. SpnS was purified to homogeneity by sequential Ni-NTA and Sephacryl S-200 chromatographic steps, yielding 50 mg of pure protein from a 6 L culture. The absorption spectrum of the purified protein exhibits no absorbance above 300 nm. SDS-PAGE analysis of purified SpnS (Figure 2, lane 5) shows that the protein has a molecular mass of 28 kDa, which correlates well with the predicted molecular weight of 28 730 Da deduced from the amino acid sequence. A native molecular mass of 62.1 kDa, as judged by gel filtration chromatography, indicates that SpnS exists as a dimeric protein.

Catalytic Properties of SpnS. Because of the difficulty of preparing sufficient quantities of the SpnS substrate **14** for NMR spectroscopic analysis of the expected product TDP-D-forosamine (**16**), SpnS was assayed using previously synthesized phosphonate mimic **24**. In our initial HPLC assays of SpnS with **24** and SAM, no new product peaks were detected. A survey of various assay conditions led to the discovery that Mg^{2+} was absolutely required for SpnS activity, and that 2 mM $MgCl_2$ was optimal for catalytic turnover. Under these conditions, time-dependent disappearance of substrate was observed, but the product peaks were not clearly discernible because they overlapped with those of SAM and SAH (retention times of 2.6−5.5 min). Analysis of the SpnS reaction mixture by SAX ion exchange chromatography allowed separation of the product peak (retention time of 20.2 min) from those of SAM and SAH (Figure 6, trace c). In order to characterize this compound, a preparative scale reaction using **24** was carried out (Figure 6, trace d). The new compound was purified by Biogel P2 chromatography and analyzed by ^1H, ^{13}C, and ^{31}P NMR spectroscopy and high-resolution mass spectrometry. Interestingly, the new compound was the N-monomethylated product **25** rather than the expected dimethylated product **26**.

To test the dimethyltransferase activity of SpnS, **25** was incubated with SpnS, SAM, and $MgCl_2$, and the formation of compound **26,** which coeluted from the Dionex CarboPac column with SAM (retention time of 2.6−5.5 min), was verified by mass spectrometry. Our results indicated that SpnS is indeed capable of N-methylation of **25** (Table 2). Subsequent time course study of SpnS reaction using HPLC equipped with a SAX column (Figure 7) revealed that using an initial concentration of 1 mM of **24**, the concentration of **25** reached a steady state after 1 h, while that of **24** continued to decrease for an additional 3 h. This implied that **26** was formed in a time-dependent manner from **25**. Based on the observed rate of

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Table 2. Summary of Kinetic Parameters of First and Second Methylation Reactions of Characterized TDP-sugar *N,N*-Dimethyltransferases SpnS, DesVI, and TylM1

	first methylation			second methylation		
enzyme	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($min^{-1}\,\mu M^{-1}$)	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($min^{-1}\,\mu M^{-1}$)
SpnS	1.6[a]	ND	ND	1.6[a]	ND	ND
DesVI	92.0	307.4	0.299	ND	ND	ND
TylM1	9.1	59.4	0.167	32.5	46.8	0.694

[a] The k_{cat} values for the SpnS-catalyzed reaction are k_{obs} values.

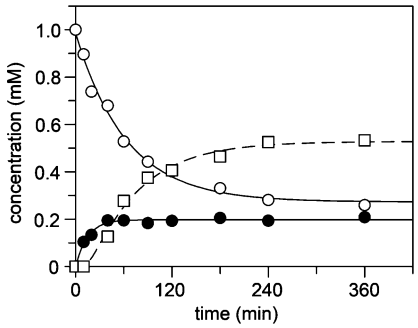

Figure 7. Time course of the SpnS (10 μM)-catalyzed reaction using **24** (1 mM) as the substrate in the presence of SAM (2 mM), $MgCl_2$ (2 mM), and DTT (2 mM) in 50 mM KH_2PO_4 (pH 7.5). Integrations of the HPLC peaks of substrate and monomethylated product **25**, and the inferred concentration of the dimethylated product **26** are plotted versus time: (○) **24**, (●) **25**, and (□) **26** (see Experimental Section for details).

substrate depletion and on the inferred rate of dimethylated product formation, k_{obs} values of 1.6 min^{-1} were calculated for both the first and second methylation reactions. These experiments provide compelling evidence that SpnS is the TDP-4-amino-2,3,4,6-tetradeoxy-D-glucose 4-*N*,*N*-methyltransferase which converts **14** to TDP-D-forosamine (**16**), and that N-methylation occurs in a stepwise manner involving release of monomethylated product from the active site, as has been observed in the characterization of the SpnS homologue TylM1.[34]

Discussion

The sugar D-forosamine is interesting because of its highly deoxygenated nature and its presence in several biologically important natural products, including the commercially available, environmentally benign insecticide spinosyns. Through studies of the biosynthesis of TDP-D-forosamine (**16**) in the spinosyn pathway of *Saccharopolyspora spinosa*, we have elucidated nature's strategy for the synthesis of this highly deoxygenated sugar. This work also provides the foundation for future exploration of the biosynthesis of other 2,3,6-trideoxy-and 2,3,4,6-tetradeoxyhexoses, which are found in many bioactive natural products.

The functional elucidation of SpnO and SpnN, and the use of SpnN to synthesize the key TDP-deoxysugar intermediate **12** are important milestones in efforts to enzymatically synthesize diverse NDP-deoxysugar donors for *in vitro* construction of new glycoforms of secondary metabolites. The results of substrate specificity studies of SpnQ and SpnR have illustrated the biosynthetic capabilities as well as the limitations of these enzymes and provided a method for preparing the non-natural TDP-sugar **23**, which may be useful in glycodiversification efforts. The *in vitro* demonstration of the 4-*N*-mono- and 4-*N*,*N*-dimethyltransferase activities of SpnS provided a synthetically useful tailoring reaction which could be applied to modify a variety of NDP-4-aminosugars.

The spinosyn pathway forosaminyltransferase SpnP has previously been shown via biotransformation experiments using an engineered *S. erythraea* host expressing *spnP*, to catalyze the attachment of the sugars L-mycarose and D-glucose to the spinosyn aglycone *in vivo*.[60] Interestingly, several naturally occurring spinosyn congeners have forosamine derivatives, including *N*-monodesmethyl-D-forosamine (spinosyns B, M, N, and R), 4-amino-2,3,4,6-tetradeoxy-D-glucose (spinosyn C), and its C-4 epimer (spinosyn G) at C-17,[15] suggesting that SpnP is also responsible for the transfer of these sugars to the spinosyn aglycone. Our finding that the N-monodesmethylated TDP-D-forosamine mimic **25** accumulates in significant quantities in the SpnS *in vitro* reaction suggests that TDP-*N*-monodesmethyl-D-forosamine (**15**, Scheme 2) may also be present in significant quantities *in vivo*. Accumulation of **15** combined with the inherent substrate flexibility of SpnP could together be responsible for the formation of spinosyns having *N*-monodesmethyl-D-forosamine. A more systematic investigation of the substrate flexibility of SpnP toward various TDP-sugars has not been carried out but is certainly warranted given the potential of using this enzyme for combinatorial biosynthesis.

The mechanism of NDP-hexose C-3 deoxygenation has been the focus of intense study over the past 30 years.[5,22] The PMP-dependent 3-dehydrase E_1 from *Y. pseudotuberculosis*, which acts on a 4-keto-6-deoxyhexose substrate, was the first characterized enzyme having this activity.[61] E_1 shares modest sequence similarity with PLP-dependent aminotransferases, indicating an evolutionary link between the two classes of enzymes.[49] However, the Schiff base-forming lysine residue conserved in vitamin B_6-dependent aminotransferases is replaced by a histidine in E_1 and its homologues. Mechanistic studies of E_1 have elucidated the details of its catalytic cycle and have made it the prototype for understanding how nature performs hexose C-3 deoxygenation. Functional elucidation and initial biochemical characterization of SpnQ illustrate that nature also employs an "E_1-like" strategy for the C-3 deoxygenation of 2,6-dideoxyhexoses, although SpnQ distinguishes itself from E_1 by using general cellular reductases rather than a conserved reductase partner (E_3) in its catalysis.

In the E_1 reaction, after initial Schiff base formation between **37** and PMP, C-4′ of the PMP−ketimine complex **38** is deprotonated, triggering the expulsion of the C-3 hydroxyl group to form the PMP-$\Delta^{3,4}$-glycoseen intermediate **39** (Scheme 5).[50] This intermediate undergoes two-electron reduction to form the Schiff base intermediate **40**, which is hydrolyzed to give product **41** and regenerate PMP (Scheme 5, Path A). Recent mechanistic[23] and structural[24] studies of the E_1 homologue ColD highlight an interesting variation on the E_1 mechanistic theme. ColD lacks electron-transfer capability but carries out C-3 deoxygenation by directly hydrolyzing the PMP-$\Delta^{3,4}$-glycoseen intermediate **39** to give enamine **42** and PLP. Enzyme-mediated tautomerization results in imine **43**, which is hydrolyzed to release the 3-deoxygenated product **41** and ammonia (Scheme 5, Path B). PMP is regenerated from PLP by a transamination reaction catalyzed by ColD using L-glutamate. SpnQ is thought to be

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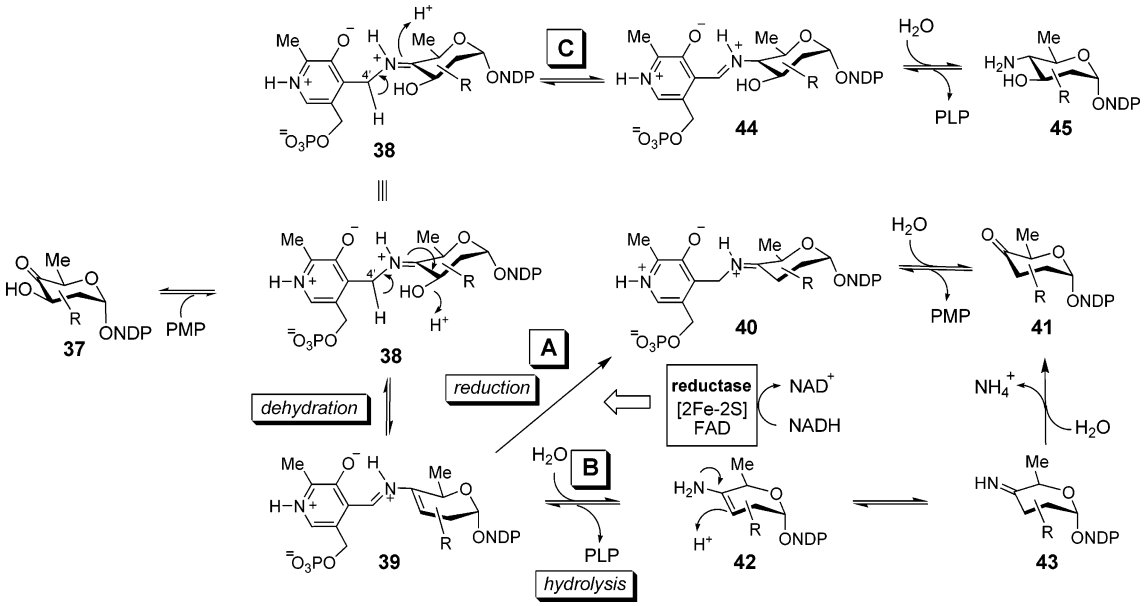
Table 3. Native Molecular Weights and Aggregation States of Spinosyn Pathway Enzymes

enzyme	SpnO	SpnN	SpnQ	SpnR	SpnS
monomer molecular weight (KDa)	55	38	51	43	28
native molecular weight (KDa)	96	38	109	86	62
native aggregation state	dimer	monomer	dimer	dimer	dimer

Scheme 5. Proposed Mechanistic Routes for PMP-Dependent 3-Dehydrases under Various Conditions[a]



[a] Path A represents the "reduction" route which occurs in the E_1 and SpnQ reactions under normal conditions. Path B represents the "hydrolysis" route undergone by ColD under normal conditions and by E_1 under "ColD-like" conditions. Path C represents the "transaminase-like" route likely undergone by SpnQ under "ColD-like" conditions.

mechanistically similar to E_1, with catalysis proceeding via Path A in the presence of an electron source. However, in the absence of an electron source and in the presence of L-glutamate, SpnQ surprisingly catalyzes transamination via Path C, producing the 3-hydroxy-4-aminosugar **45**. Under the same conditions, E_1 catalyzes a 3-dehydration reaction to give **41**, presumably via Path B.[31] Thus, although E_1, ColD, and SpnQ are homologous NDP-hexose 3-dehydrases, each enzyme has distinct biochemical properties which clearly warrant further mechanistic and structural study.

Recently, two structures of ColD from *E. coli* were solved.[24] This enzyme, like E_1, has a conserved active site His residue (His188). Interestingly, other than His188, the ColD active site is devoid of catalytic residues that are properly positioned for the various protonation and deprotonation steps required to complete the catalytic cycle. His188 is therefore proposed to catalyze essentially all acid−base chemistry during ColD catalysis: deprotonation of C-4′ of **38**, protonation of the leaving C-3 hydroxyl to generate **39**, activation of the hydrolytic water to form **42**, and stereospecific protonation of C-3 of the enamine to form **43**.[24] Although structural information on E_1 and SpnQ is not yet available, it is tempting to speculate that the divergent behaviors of E_1 and SpnQ under "ColD-like" conditions are based on subtle differences in the positioning of the catalytic residue(s) in the active sites of the two proteins. In the absence of a reductase, **38** and **39** would be in equilibrium in the active site of E_1. The catalytic residue(s) may be appropriately positioned to activate a water molecule for hydrolysis of **39** and to reprotonate at C-3 of **42** (Path B), resulting in 3-dehydration. In SpnQ, the catalytic residue(s) may be in the correct orientation to facilitate isomerization between **38** and **44**, leading to the formation of **45** after hydrolysis (Path C). Recent success in efforts to obtain a crystal structure of E_1[35] may provide further insights. With sufficient structural information in hand, it may be possible to design 3-dehydrase mutants in which aminotransferase, reductase-dependent 3-dehydrase, and reductase-independent 3-dehydrase activities are rationally interconverted.

Acknowledgment. This work was supported in part by the National Institutes of Health Grants (GM35906, 54346).

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