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Pseudoglycosyltransferase Catalyzes Nonglycosidic C−N Coupling in Validamycin A Biosynthesis

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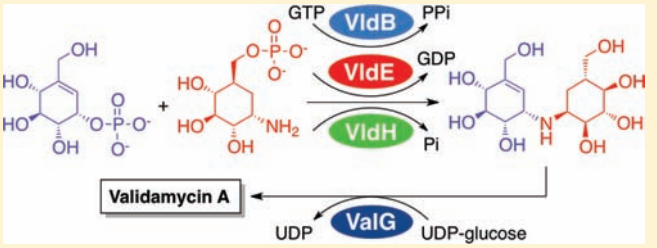
Supporting Information

ABSTRACT: Glycosyltransferases are ubiquitous in nature. They catalyze a glycosidic bond formation between sugar donors and sugar or nonsugar acceptors to produce oligo/polysaccharides, glycoproteins, glycolipids, glycosylated natural products, and other sugar-containing entities. However, a trehalose 6-phosphate synthase-like protein has been found to catalyze an unprecedented nonglycosidic C−N bond formation in the biosynthesis of the aminocyclitol antibiotic validamycin A. This dedicated 'pseudoglycosyltransferase' catalyzes a condensation between GDP-valienol and validamine 7-phosphate to give validoxylamine A 7′-phosphate with net retention of the 'anomeric' configuration of the donor cyclitol in the product. The enzyme operates in sequence with a phosphatase, which dephosphorylates validoxylamine A 7′-phosphate to validoxylamine A.



INTRODUCTION

Glycosyltransferases (EC 2.4.x.y) are among the most abundant enzymes in nature and play a central role in structural and physiological aspects of living organisms. They catalyze the transfer of a sugar moiety from an activated donor sugar onto sugar or nonsugar acceptors, resulting in sugar-containing products, such as oligo/polysaccharides, glycoproteins, glycolipids, glycosylated natural products, and many others. To date, there are more than 44 000 genes which encode proteins across the 92 known families of glycosyltransferases listed in the NCBI database, and this number is fast growing.[1] However, only a fraction of them have actually been functionally characterized.

Whereas all glycosyltransferases characterized to date catalyze glycosylation reactions, some glycosyltransferase-like proteins have recently been implicated in nonglycosidic C−N bond formations in natural products biosyntheses. Examples include ValL/VldE, trehalose 6-phosphate synthase-like proteins involved in the biosynthesis of the crop protectant validamycin A (**1**) (Figure 1) in several *Streptomyces hygroscopicus* sp.,[2,3] and AcbS/GacS, glycosyltransferase-like enzymes proposed to be involved in the biosynthesis of the antidiabetic drug acarbose (**2**) in *Actinoplanes* sp. SE50/110 and *Streptomyces glaucescens*.[4,5]

Validamycin A (**1**) is a bacterial-derived natural product that consists of an aminocyclitol moiety, validoxylamine A, and glucose. The validoxylamine A core structure is built of an unsaturated cyclitol and a saturated cyclitol, which are connected through a shared single nitrogen bridge. Previous investigations using isotopically labeled precursors and biochemical experiments demonstrated that these cyclitol units are derived from sedoheptulose 7-phosphate via a set of cyclic intermediates,[6−9] and further downstream the pathway requires a coupling reaction between the two cyclitols. However, the biochemical mechanism underlying this coupling reaction had been poorly understood, stimulating speculations around how the nitrogen bridge in this compound is formed.[2,3,6] Here, we describe the first biochemical evidence of an unprecedented nonglycosidic C−N coupling in validamycin biosynthesis catalyzed by a trehalose 6-phosphate synthase-like enzyme, VldE. This dedicated 'pseudoglycosyltransferase' recognizes GDP-valienol and validamine 7-phosphate as substrates and operates in sequence with a phosphatase (VldH), which dephosphorylates the VldE product validoxylamine A 7′-phosphate to validoxylamine A, a process that is similar to that of trehalose biosynthesis by trehalose 6-phosphate synthase and trehalose 6-phosphate phosphatase.

RESULTS

Validation of VldB as a Cyclitol Nucleotidyltransferase. Biosynthetic gene clusters of validamycin have been identified in a number of *Streptomyces* strains, for example, *S. hygroscopicus* subsp. *jinggangensis* 5008 and *S. hygroscopicus* subsp. *limoneus* KCCM 11405 (IFO 12704).[2,3] Direct comparison of the former (the *val* cluster) and the latter (the *vld* cluster) has shown that both clusters contain almost identical sets of genes necessary for the biosynthesis of validamycin A (Figure 2A),[10] suggesting that both producers may be closely related. As part of our investigation on validamycin biosynthesis in *S. hygroscopicus* 5008, we recently established the role of ValB, a cyclitol nucleotidyltransferase that catalyzes the conversion of valienol 1-phosphate to GDP-valienol.[11] However, its homologue (VldB) from *S. hygroscopicus* subsp. *limoneus* KCCM 11405 was reported as a glucose

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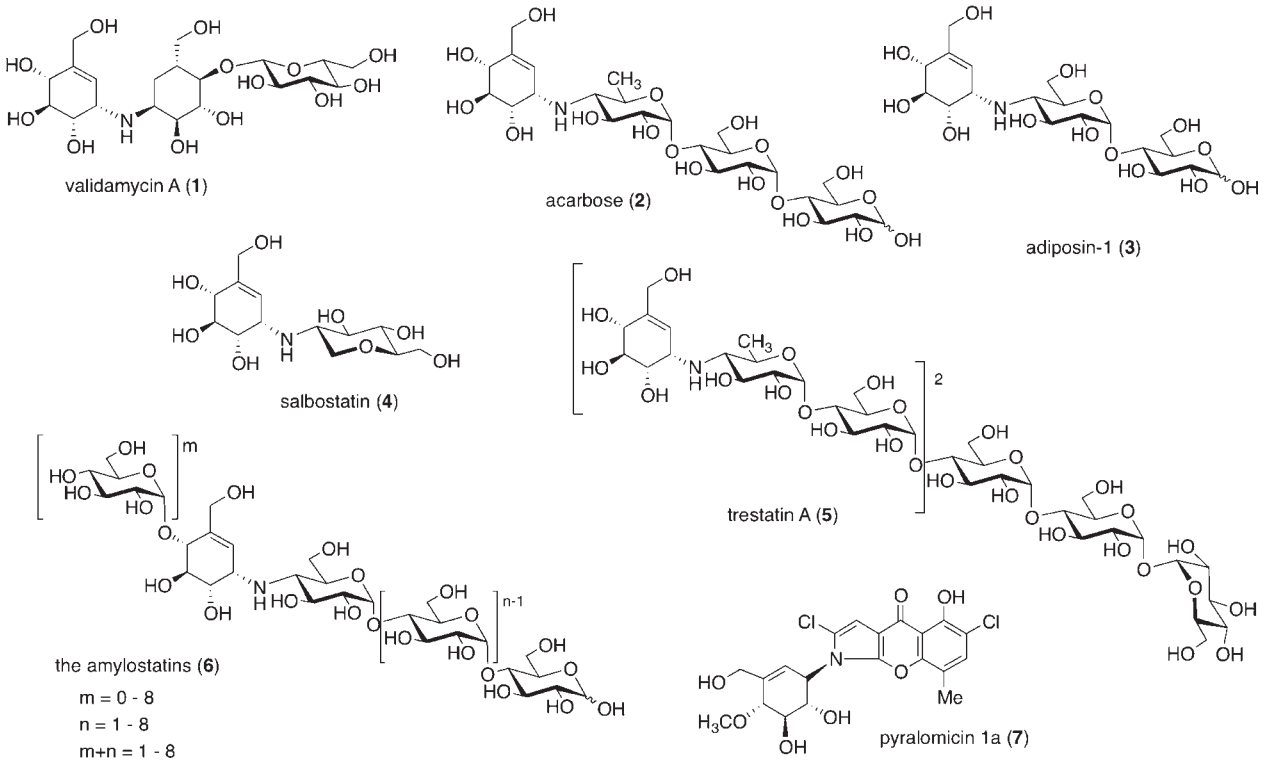

Figure 1. Chemical structures of validamycin A, acarbose, and related natural products.

1-phosphate uridylyltransferase that catalyzes the formation of UDP-glucose.[12] To validate the function of VldB as a cyclitol nucleotidyltransferase, the recombinant protein was tested using synthetic valienol 1-phosphate[11] as substrate in the presence of all five naturally occurring NTPs (ATP, GTP, CTP, dTTP, and UTP). The results showed that VldB, in reactions containing 1 mM Mg^{2+}, effectively catalyzes the conversion of valienol 1-phosphate to GDP-valienol and less effectively to ADP-valienol or other NDP derivatives (Figure 2C). However, interestingly, in reactions containing 10 mM Mg^{2+}, the enzyme more effectively used ATP as nucleotidyl donor than GTP or other NTPs (Figure 2C). Although the reason for this nucleotidyl donor preference switch is unclear, the results confirm our hypothesis that VldB is a cyclitol nucleotidyltransferase that plays a critical role in supplying NDP-valienol, most likely GDP-valienol, in validamycin biosynthesis. Therefore, the formation of validoxylamine A may involve a coupling reaction between GDP-valienol and an amino-bearing cyclitol counterpart.

Syntheses of Validamine (16) and Validamine 7-Phosphate (21). To explore the above-proposed coupling reaction, we synthesized two potential aminocyclitol substrates, validamine (**16**) and validamine 7-phosphate (**21**). The two compounds were prepared from validoxylamine A (**8**) as shown in Scheme 1. Thus, complete benzylation of validoxylamine A using BnBr and NaH furnished perbenzylated validoxylamine A (**9**), which was then treated with NBS to give a mixture of four products, tetrabenzylvalienone (**10**), tetrabenzylvalidone (**11**), tetrabenzylvalidamine (**12**), and tetrabenzylvalienamine (**13**). The benzylated validamine and valienamine products were chromatographically separated from their keto-analogues, and the mixture was subjected to Cbz-protection to give 1-Cbz-2,3,4,7-tetrabenzylvalienamine (**14**) and 1-Cbz-2,3,4,7-tetrabenzylvalidamine (**15**). The compounds were then chromatographically separated, and complete deprotection of **15** by hydrogenolysis afforded validamine (**16**) in a quantitative yield.

For the synthesis of validamine 7-phosphate (**21**), **15** was regioselectively debenzylated and acetylated using $ZnCl_2$, Ac_2O, and AcOH, followed by a deacetylation step to furnish a free alcohol product, 1-Cbz-2,3,4-tribenzylvalidamine (**19**). The alcohol **19** was then treated with dibenzyl diisopropyl phosphoramidite in the presence of 1*H*-tetrazole to form the phosphite intermediate, which was readily oxidized by *m*-chloroperoxybenzoic acid (*m*-CPBA) to afford a phosphate product (**20**). Finally, debenzylation was achieved by hydrogenolysis with Pd/C to give the desired product validamine 7-phosphate (**21**).

Characterization of the Glycosyltransferase-like Protein VldE. As validoxylamine A is structurally similar to trehalose, the trehalose 6-phosphate synthase-like protein VldE was suspected to catalyze this coupling reaction. Trehalose 6-phosphate synthases (EC 2.4.1.15) catalyze the synthesis of trehalose 6-phosphate from UDP-glucose and glucose 6-phosphate with net retention of the anomeric configuration of the donor sugar in the product (Scheme S1). The notion that its homologue is involved in validoxylamine formation is intriguing, but raises fundamental questions as to how a glycosyltransferase-like enzyme catalyzes a condensation of two nonsugar molecules. To gain insight into the catalytic function of VldE and its involvement in the coupling reaction, we cloned and heterologously expressed the gene in *E. coli* and characterized its biochemical activity. As the potential donor substrate GDP-valienol is relatively unstable, the compound was prepared *in situ* using VldB, valienol 1-phosphate, and GTP. In addition to these components, recombinant VldE and the synthetic amino-bearing cyclitol, either validamine or validamine 7-phosphate, were included in the reaction mixture (Scheme 2). The results showed that only the incubation containing GDP-valienol and validamine

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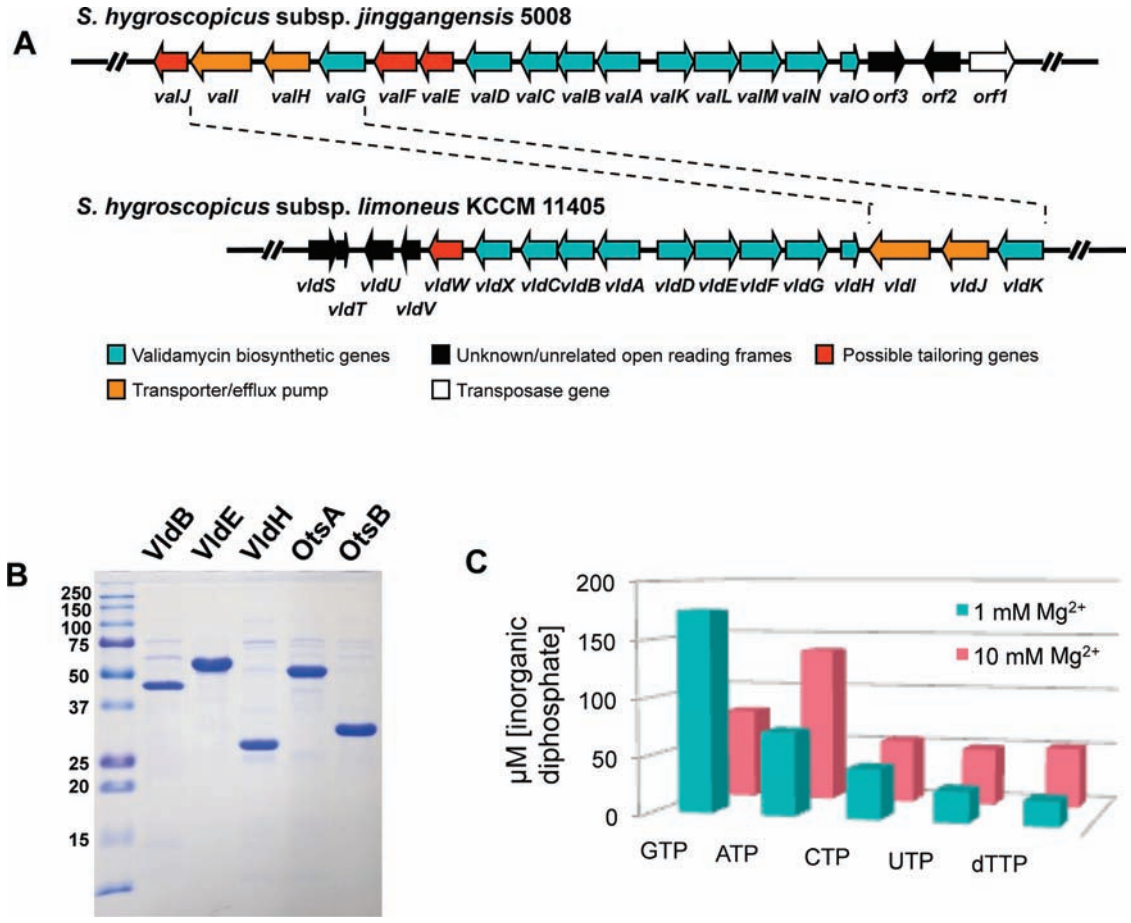


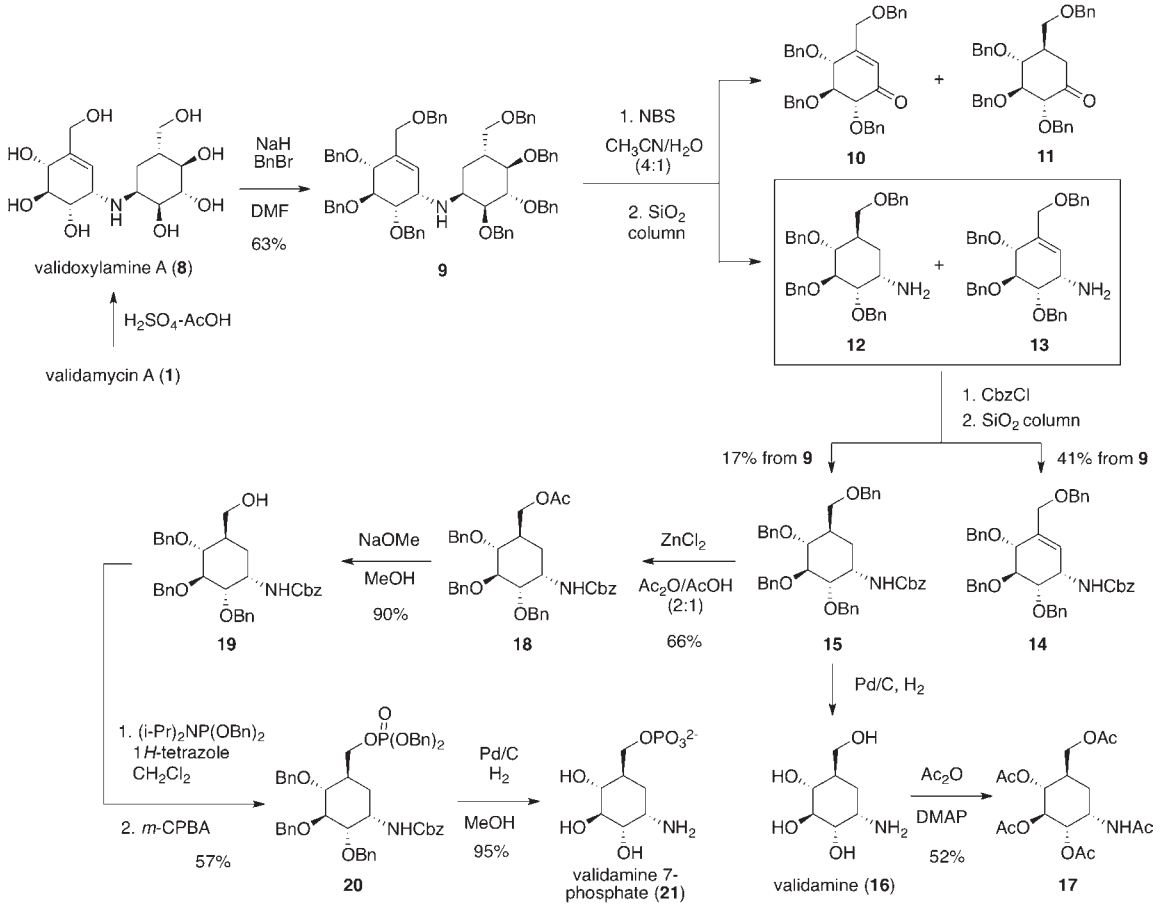
Figure 2. Biosynthetic gene clusters for validamycin A and characterization of VldB. (A) Genetic organizations of the validamycin biosynthetic gene clusters from *S. hygroscopicus* 5008 and *S. hygroscopicus* subsp. *limoneus*; (B) SDS−PAGE of recombinant VldB, VldE, VldH, OtsA (*Escherichia coli* trehalose 6-phosphate synthase), and OtsB (*E. coli* trehalose 6-phosphate phosphatase) purified by Ni-NTA affinity chromatography; (C) relative specificity for nucleotidyl donors of VldB in the presence of 1 mM and 10 mM Mg^{2+}.

7-phosphate gave a product (m/z 416 $[M + H]^+$), which is consistent with the molecular mass of the expected product validoxylamine A 7′-phosphate (Figure 3G, and Figures S2 and S3). MS/MS analysis of the product gave fragment ions consistent with those observed in authentic validoxylamine A, except that most of the fragment ions of validoxylamine A 7′-phosphate are 80 amu higher than those of validoxylamine A, indicating the presence of a phosphate moiety in the former compound (Figure 3L). HR-ESTOFMS analysis of the product revealed a molecular formula of $C_{14}H_{26}NO_{11}P$, consistent with that of validoxylamine A 7′-phosphate. Reactions containing validamine did not give any corresponding product, suggesting that VldE does not recognize an unphosphorylated substrate (Figure S3).

Synthesis of Validoxylamine A 7′-Phosphate (26) and Confirmation of the VldE Product. To further confirm the product of VldE, we synthesized validoxylamine A 7′-phosphate (**26**) and compared the VldE product with the synthetic compound. The synthesis was carried out using validoxylamine A (**8**), which was prepared from acid hydrolysis of validamycin A (**1**), as starting material. Validoxylamine A 7′-phosphate (**26**) was previously synthesized by Davies and co-workers in 10 steps starting from **8**.[13] However, using a modified procedure, we were able to obtain **26** from **8** in 5 steps (Scheme 3). Thus, **8** was treated with benzaldehyde dimethyl acetal in the presence of *p*-TsOH to selectively protect the 4′- and 7′-hydroxy groups. The product was then perbenzylated with benzyl bromide and NaH to give **28**. Compound **28** was treated with DIBAL-H for selective acetal opening to give **29**. The free alcohol functionality was then phosphorylated as described for the synthesis of validamine 7-phosphate (**21**) and the product was debenzylated using BBr_3 at −40 °C to give validoxylamine A 7′-phosphate (**26**). This compound was used as reference for the assay of VldE catalysis. HPLC, MS, and MS/MS analyses of the VldE product gave chromatographic and spectral data identical to those of the synthetic compound, confirming its identity as validoxylamine A 7′-phosphate (**26**).

Kinetic Properties of VldE. The kinetic properties of VldE were determined using a pyruvate kinase/lactate dehydrogenase (PK/LDH) coupled colorimetric assay (Scheme 2).[14] Reactions were initiated by addition of a saturating amount of either GDP-valienol (**25**) or validamine 7-phosphate (**21**), and the oxidation of NADH to NAD^+, which is stoichiometrically equivalent to the production of GDP, was measured by the decrease in A_{340}. Compound **25** was obtained from valienol 1-phosphate (**24**) and GTP catalyzed by VldB and the concentration was determined using a coupled colorimetric assay containing inorganic pyrophosphatase and purine nucleoside phosphorylase (PNP) [EnzChek Pyrophosphate Assay Kit[15] (Molecular Probes)].[11] For the kinetic studies, a protein concentration of 1−3 μM, a pH of 7.5, and a reaction time of 1−3 min, which showed linear decrease of

Scheme 1. Synthesis of Validamine (16) and Validamine 7-Phosphate (21)



absorbance, were chosen. The apparent kinetic values, obtained from Hanes-Woolf plots, were K_m 308.0 ± 34.5 μM and k_{cat} 7.0 ± 0.4 min^{-1} for **21** and K_m 59.7 ± 11.9 μM and k_{cat} 7.0 ± 0.7 min^{-1} for **25** (Figure 4).

VldE Is a Dedicated Pseudoglycosyltransferase. Whereas VldE is genetically similar to trehalose 6-phosphate synthases, it does not have any trehalose 6-phosphate synthase activity. Incubation of the enzyme with UDP- or GDP-glucose and glucose 6-phosphate did not give any product (Figure S5). The same result was observed when only one of the cyclitol derivatives was replaced by its glucose analogue (Figure S6). These results suggest that VldE is a dedicated pseudoglycosyltransferase that only recognizes GDP-valienol and validamine 7-phosphate as substrates, and not a trehalose 6-phosphate synthase with relaxed substrate specificity.

The *E. coli* Trehalose 6-Phosphate Synthase OtsA Does Not Recognize GDP-Valienol and Validamine 7-Phosphate as Substrates. To investigate if a trehalose 6-phosphate synthase can alternatively catalyze the formation of validoxylamine A 7′-phosphate, we cloned and expressed in *E. coli* the trehalose 6-phosphate synthase gene *otsA* and characterized its catalytic function. While the recombinant OtsA can catalyze the formation of trehalose 6-phosphate, the enzyme was not able to catalyze the formation of validoxylamine A 7′-phosphate (Figure S7). Replacements of GDP-valienol with UDP-/GDP-glucose or validamine 7-phosphate with glucose 6-phosphate did not give any hybrid products (Figure S8), indicating that OtsA does not recognize any activated cyclitols as substrate.

VldE Is Not a Validoxylamine A Glycosyltransferase. Interestingly, VldE has previously been reported as a glycosyltransferase that catalyzes the glucosylation of validoxylamine A to give validamycin A.[16] However, a wealth of evidence is available to conclude that another glycosyltransferase, ValG (VldK), is responsible for this conversion.[2,17] Nevertheless, we explored the reported glycosyltransferase activity of VldE by incubating the enzyme with validoxylamine A and UDP-glucose and analyzing the product by ESIMS. No validamycin A could be identified in this reaction (Figure S9). The results confirmed the distinct role of VldE as a dedicated validoxylamine A 7′-phosphate synthase and not a validoxylamine A glycosyltransferase.

The Role of the Phosphatase VldH. The production of validoxylamine A 7′-phosphate by VldE suggests that a dephosphorylation reaction is necessary downstream in the pathway to provide a phosphate-free substrate for the glycosyltransferase ValG (VldK).[2,17] The only candidate phosphatase gene within the cluster is *vldH*, which encodes a protein that has high identity with proteins from the haloacid dehalogenase-like superfamily, which includes L-2-haloacid dehalogenase, epoxide hydrolase, phosphoserine phosphatase, phosphomannomutase, phosphoglycolate phosphatase, P-type ATPase, and many others.[18] Interestingly, the G + C content of the *vldH* gene is only 60%, relatively low compared to that of other genes in the cluster (68−72%).

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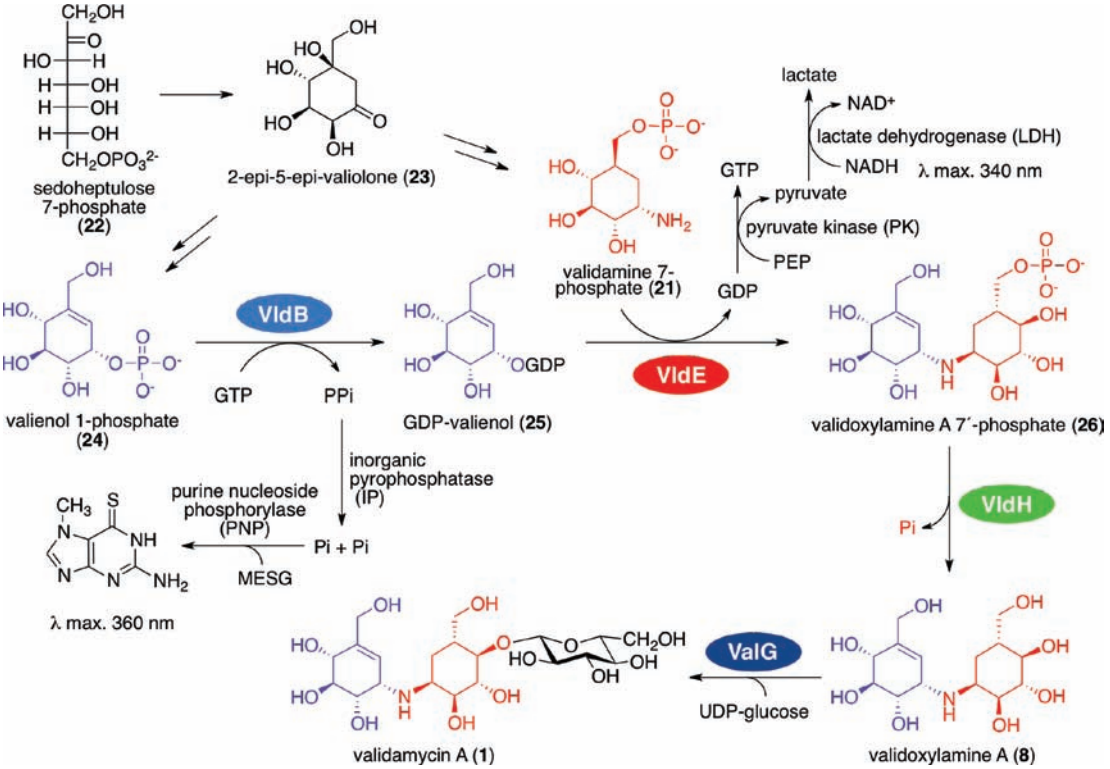
Scheme 2. Biosynthetic Pathway to Validamycin A and Characterization of VldB, VldE, and VldH



To investigate the function of VldH as a phosphatase, the recombinant protein was produced (Figure 2B) and co-incubated with VldB and VldE in the presence of valienol 1-phosphate, GTP, and validamine 7-phosphate (Scheme 2). The results showed that addition of VldH to the mixture resulted in a phosphate-free product, validoxylamine A (m/z 336 $[M + H]^+$) (Figure 3D,I). In addition, MS/MS analysis of the product gave fragment ions identical to those of authentic validoxylamine A (Figure 3N,O), providing compelling evidence for the critical roles of VldB, VldE, and VldH in the formation of validoxylamine A. However, in contrast to VldE, VldH appears to have broader substrate permissiveness, as it could also convert trehalose 6-phosphate to trehalose (Figure S10). On the other hand, the trehalose 6-phosphate phosphatase OtsB, which was recombinantly produced in *E. coli* (Figure 2B), does not dephosphorylate validoxylamine A 7′-phosphate (Figure S4), suggesting that the role of VldH in validamycin A biosynthesis cannot be assumed by OtsB.

DISCUSSION

The biochemical analysis of VldB, VldE, and VldH and the establishment of their roles in validoxylamine A biosynthesis have opened up a new dimension in our understanding of pseudoglycoside biosynthesis. Particularly, the involvement of VldE and VldH, which resemble OtsA and OtsB, in validoxylamine A formation is evolutionarily and mechanistically intriguing. VldE might have evolved from an ancestral trehalose 6-phosphate synthase to the extent that it no longer recognizes activated sugars as substrates, but still retains some of the catalytic properties, including the net retention of the anomeric configuration of the product.

In addition, the use of the aminocyclitol unit as pseudosugar acceptor by VldE to form *N*-pseudoglycosyl linkage is a significant departure from the OtsA catalyzed reaction. In comparison to the *O*-glycosyltransferases such as OtsA, glycogen synthase, cellulose synthase, and others, *N*-glycosyltransferase enzymes are less abundant, but play significant roles, in biological systems. Among the known *N*-glycosyltransferases are the eukaryotic oligosaccharyltransferase (OST), which catalyzes asparagine-linked glycosylation of proteins, and UGT72B1, a bifunctional *N*- and *O*-glycosyltransferase that is involved in xenobiotic metabolism in plants.[19,20] Other *N*-glycosyltransferases that are involved in natural products biosynthesis include the indolocarbazole *N*-glycosyltransferases RebG and StaG and the ansamitocin *N*-glycosyltransferase Asm25.[21−23] However, most, if not all, of these enzymes catalyze the formation of glycosidic bonds with inversion of configuration at the anomeric center of the sugar donors.

Whereas inverting glycosyltransferases generally operate through a direct displacement, S_N2-like mechanism, retaining glycosyltransferases are believed to function via either a two-step double-displacement mechanism or an internal return (S_Ni)-like mechanism, which is also known as a $D_N{}^*A_N$ss ion pair mechanism.[24] Structural studies of OtsA suggest that its activity is likely to occur via the latter mechanism.[13] As VldE shares a high number of conserved active site residues with OtsA, they may have similar catalytic mechanisms. However, the question remains how a glycosyltransferase-like enzyme catalyzes a condensation of two nonglycosyl units with retention of stereochemistry. Whereas the detailed mechanism underlying this unique capability is yet to be determined, we believe that the olefinic moiety of GDP-valienol plays a critical role in facilitating the coupling

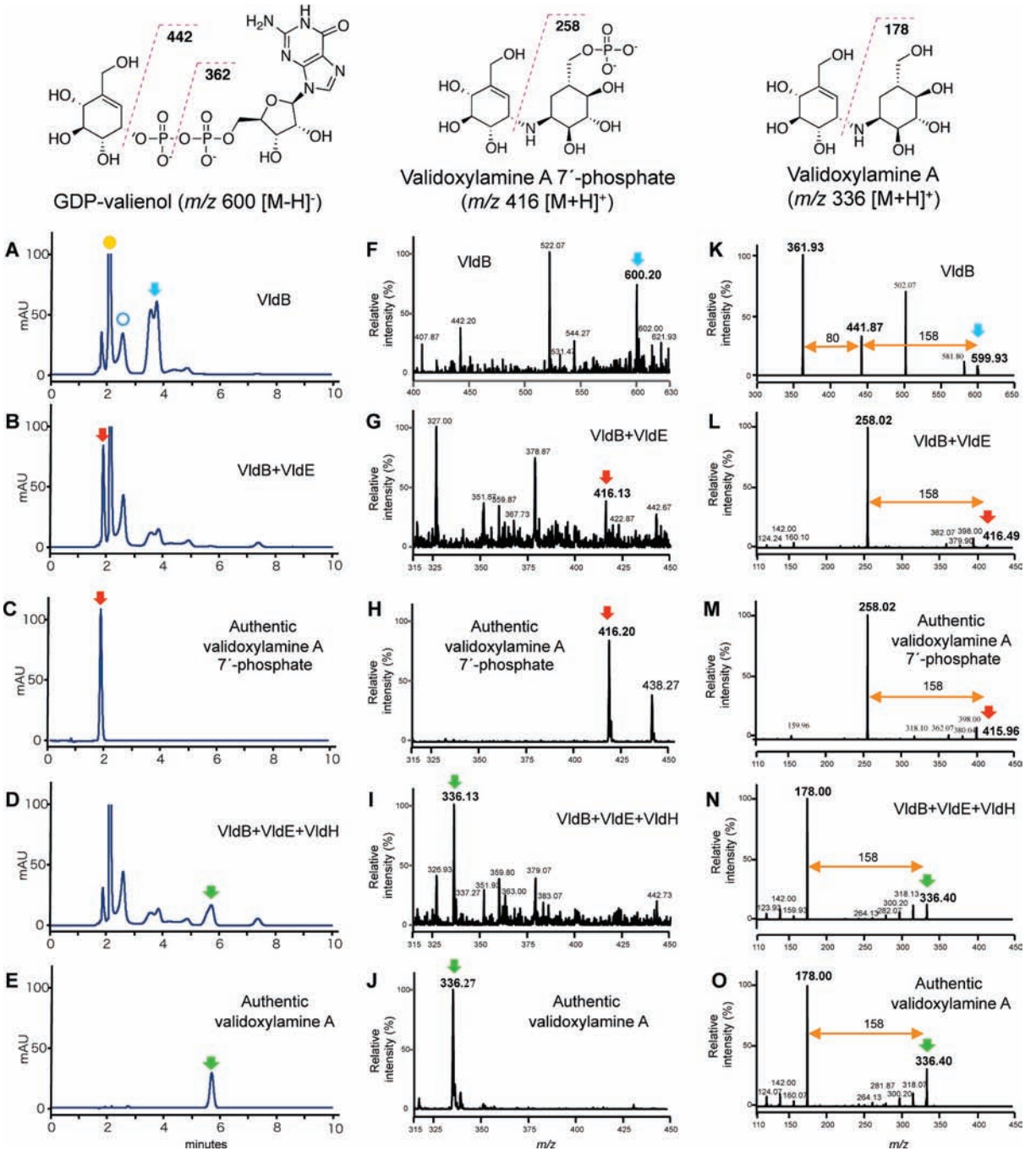
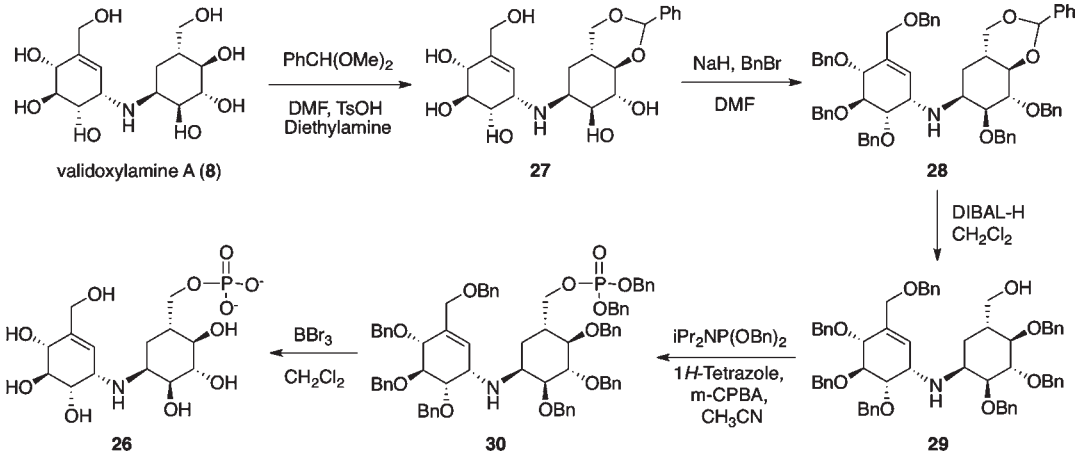


Figure 3. HPLC and ESI-MS analyses of VldB, VldE, and VldH reaction products. (A) HPLC traces of reaction mixture containing VldB, valienol 1-phosphate, and GTP. GDP-valienol (cyan block arrow) eluted in two tautomeric forms; (B) HPLC trace of reaction mixture containing VldB, VldE, valienol 1-phosphate, GTP, and validamine 7-phosphate; (C) HPLC trace of authentic (synthetically prepared) validoxylamine A 7′-phosphate; (D) HPLC trace of reaction mixture containing VldB, VldE, VldH, valienol 1-phosphate, GTP, and validamine 7-phosphate; (E) HPLC trace of authentic validoxylamine A; (F) (−)ESIMS of reaction mixture containing VldB, valienol 1-phosphate, and GTP; (G) (+)ESIMS of reaction mixture containing VldB, VldE, valienol 1-phosphate, GTP, and validamine 7-phosphate; (H) (+)ESIMS of authentic validoxylamine A 7′-phosphate; (I) (+)ESIMS of reaction mixture containing VldB, VldE, VldH, valienol 1-phosphate, GTP, and validamine 7-phosphate; (J) (+)ESIMS of authentic validoxylamine A; (K) ESI-MS/MS of GDP-valienol (*m*/*z* 600, cyan block arrow) from panel F; (L) ESI-MS/MS of validoxylamine A 7′-phosphate (*m*/*z* 416, red block arrow) from panel G; (M) ESI-MS/MS of authentic validoxylamine A 7′-phosphate; (N) ESI-MS/MS of validoxylamine A (*m*/*z* 336, green block arrow) from panel I; (O) ESI-MS/MS of authentic validoxylamine A. Yellow circle, GTP; open circle, GDP.

reaction. This is somewhat analogous to the involvement of the allylic moiety in farnesyl diphosphate (FPP) synthase-catalyzed reactions (Figure 5).[25,26] In both VldE and FPP synthase, and also in glycosyltransferases, a nucleophilic substitution reaction takes place at a carbon center with a diphosphate or nucleotidyl diphosphate acting as leaving group. Therefore, it may be proposed that at the enzyme active site the π-electrons of the unsaturated cyclitol resonate to form stabilized short-lived ion

Scheme 3. Synthesis of Validoxylamine A 7′-Phosphate (26)



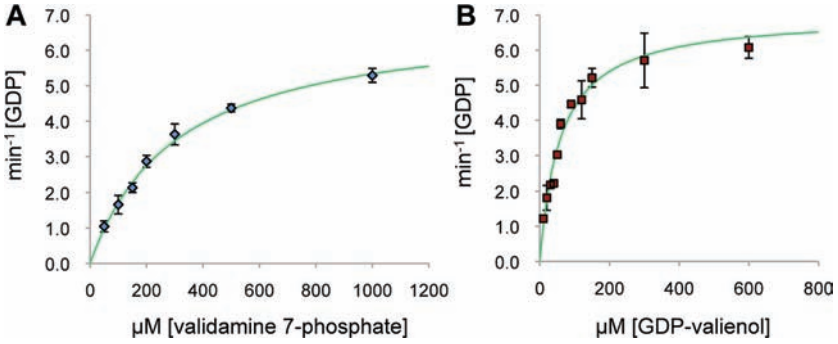


Figure 4. Kinetic studies of VldE. (A) Michaelis−Menten curve for validamine 7-phosphate; (B) Michaelis−Menten curve for GDP-valienol.

pair intermediates similar to the oxocarbenium-ion like transition states and the diphosphate group acts as a base to deprotonate the ammonium ($-NH_3^+$) group of validamine 7-phosphate. The primary amine then attacks the 'pseudoanomeric' carbon, leading to a product with retained configuration.

Adoption of the S_Ni-like mechanism in VldE catalysis, however, may have a consequence, as the ion pair intermediate could collapse to give back starting material, which may affect the rate constant of the enzyme.[24] In fact, we observed a rather low turnover rate of VldE as evidenced from the MS data and its kinetic values (k_{cat}/K_m 0.023 $min^{-1}\cdot\mu M^{-1}$ for validamine 7-phosphate and k_{cat}/K_m 0.12 $min^{-1}\cdot\mu M^{-1}$ for GDP-valienol). However, this observation might be circumstantial; therefore, a more detailed study is necessary to determine the catalytic mechanism of this unusual enzyme. Nevertheless, the results of the present study suggest that certain glycosyltransferase-like enzymes are capable of catalyzing coupling reactions between nonglycosidic allylic cation-forming donors and amino-bearing acceptors. Some, if not all, of them are similar to members of certain groups of glycosyltransferases, for example, VldE to trehalose 6-phosphate synthases (GT20 family) and AcbS/GacS to glycogen synthases (GT5 family),[4,5] suggesting that this poorly recognized class of enzymes may have evolved from or shared ancestral traits with known glycosyltransferases. Similar glycosyltransferase-like enzymes may be involved in the biosynthesis of other natural products, for example, the adiposins (**3**), salbostatin (**4**), the trestatins (**5**), the amylostatins (**6**), and the pyralomicins (**7**) (Figure 1).[27] Therefore, the results have not only laid a foundation for understanding and discovering new classes of pseudoglycosyltransferases, but may also facilitate the development of new tools for generating carbohydrate mimetics and redesigning glycoconjugates and glycosylated natural products.

EXPERIMENTAL SECTION

General. All chemical reactions were performed under an argon or nitrogen atmosphere employing oven-dried glassware. Analytical thin-layer chromatography (TLC) was performed using silica plates (60 Å) with a fluorescent indicator (254 nm), which were visualized with a UV lamp and ceric ammonium molybdate (CAM) solution. Chromatographic purification of products was performed on silica gel (60 Å, 72−230 mesh). Optical rotations were measured on a Jasco P1010 polarimeter (100 mm cells were used) at the sodium D line. Proton NMR spectra were recorded on Bruker 300 or 400 MHz spectrometers. Proton chemical shifts are reported in parts per million (ppm) (δ) relative to the residual solvent signals as the internal standard ($CDCl_3$: δ_H 7.26; D_2O: δ_H 4.79). Multiplicities in the ^1H NMR spectra are described as follows: s = singlet, bs = broad singlet, d = doublet, bd = broad doublet, t = triplet, bt = broad triplet, q = quartet, m = multiplet; coupling constants are reported in hertz (Hz). Carbon NMR spectra were recorded on a Bruker 300 (75 MHz) spectrometer with complete proton decoupling. Carbon chemical shifts are reported in parts per million (ppm) (δ) relative to the residual solvent signal as the internal standard ($CDCl_3$: δ_C 77.16), or with sodium 2,2-dimethylsilapentane-5-

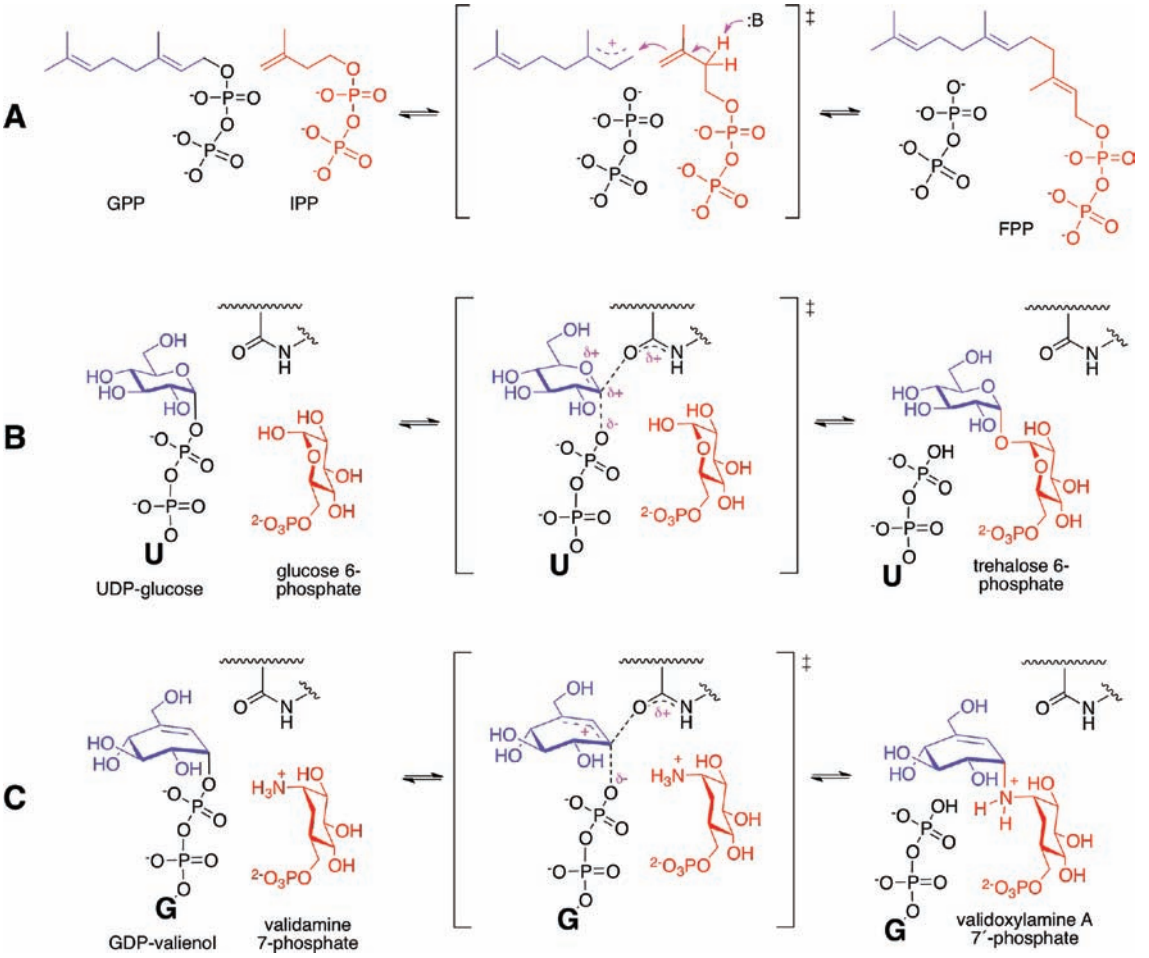


Figure 5. Proposed catalytic mechanism for VldE and its similarity to that of farnesyl diphosphate (FPP) synthase and trehalose 6-phosphate synthase (OtsA). (A) Catalytic mechanism of FPP synthases; (B) proposed S_Ni-like mechanism for OtsA; (C) proposed catalytic mechanism of VldE adopting those of FPP synthase and OtsA.

sulfonate (DSS) (δ 0.0) as an external standard. Phosphorus NMR spectra were recorded on a Bruker 400 (122 MHz) spectrometer with complete proton decoupling. Phosphorus chemical shifts are reported in parts per million (ppm) (δ) relative to an 85% H_3PO_4 (δ 0.0) external standard. Low-resolution electrospray ionization (ESI) mass spectra were recorded on a ThermoFinnigan liquid chromatograph-ion trap mass spectrometer, and high-resolution electrospray mass spectra were recorded on a Waters/Micromass LCT spectrometer. Size exclusion chromatography was done on Sephadex LH-20 (Pharmacia).

Chemical Synthesis of Validamine (16) and Validamine 7-Phosphate (21). *Benzyl ((1S,2S,3S,4R,5R)-2,3,4-Tris(benzyloxy)-5-((benzyloxy)methyl)cyclohexyl)-carbamate (**15**).* To a solution of (1*S*,4*R*,5*S*,6*S*)-4,5,6-tris(benzyloxy)-3-((benzyloxy)methyl)-*N*-((1*S*,2*S*,3*S*,4*R*,5*R*)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)-cyclohexyl)-cyclohex-2-enamine (**9**)[28] (1.77 g, 1.676 mmol) in CH_3CN/H_2O (4:1; 40 mL) was added NBS (447 mg, 2.51 mmol), and the reaction mixture was stirred for 15 h at room temperature (rt). The reaction mixture was diluted with water (150 mL) and EtOAc (150 mL), and the aqueous layer extracted with EtOAc (150 mL). The combined organic layer was washed with brine (150 mL), dried over $MgSO_4$, filtered and concentrated under vacuum to give crude product. Column chromatography (silica gel, Hex/EtOAc = 3:1 and then EtOAc/MeOH = 4:1) of the product yielded a mixture of amine products (626 mg).

Benzyl chloroformate (439 mg, 2.57 mmol; 50% solution in toluene) in $CHCl_3$ (8 mL) was slowly added to a solution of the above amine products (626 mg) and $NaHCO_3$ (491 mg, 5.85 mmol) in MeOH (16 mL). The reaction mixture was stirred for 15 h at rt and diluted with EtOAc (150 mL) and water (150 mL). The organic layer was washed with brine (150 mL), dried over $MgSO_4$, filtered and concentrated under vacuum. Column chromatography (silica gel, Hex/EtOAc = 5:1 and then toluene/EtOAc = 10:1) yielded **15** (8.7 mg, 17% from **9**); $[\alpha]^{20}_D$= +47.1° (*c* 0.72, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.25−7.42 (m, 25H), 5.17 (d, *J* = 1.5, 2H), 5.12 (m, 1H), 4.98 (d, *J* = 10.5 Hz, 1H), 4.92 (d, *J* = 10.8 Hz, 1H), 4.82 (d, *J* = 10.8 Hz, 1H), 4.75 (d, *J* = 11.4 Hz, 1H), 4.60 (d, *J* = 11.7 Hz, 1H), 4.59 (d, *J* = 10.8 Hz, 1H), 4.48 (s, 2H), 3.55−3.66 (m, 3H), 3.48 (dd, *J* = 9.0, 2.4 Hz, 1H), 2.30 (bd, *J* = 14.7 Hz, 1H), 1.95 (m, 1H), 1.62 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 156.5, 138.94, 138.85, 138.6, 138.0, 136.6, 128.6, 128.5, 128.4, 128.19, 128.15, 128.08, 127.97, 127.96, 127.8, 127.6, 129.6, 83.8, 80.9, 80.3, 75.8, 75.3, 73.2, 71.7, 69.8, 66.8, 48.1, 37.5, 28.7. HRMS (ESI) *m*/*z* 672.3338 (calcd for $C_{43}H_{46}NO_6$ $[M + H]^+$: 672.3325).

*Validamine (**6**).* A mixture of **15** (15 mg, 22.33 μmol) and 10% Pd/C (15.0 mg) in MeOH (1.8 mL) and EtOAc (1.2 mL) containing 12 M HCl (30 μL) was hydrogenated under atmospheric pressure for 20 h. The reaction mixture was filtered through Celite and a cellulose syringe filter, washed with MeOH, and concentrated. The crude product was further purified with silica gel column chromatography ($CHCl_3$/MeOH/25% NH_4OH = 2:3:1) to give the pure product (3.7 mg, 95%).

To compare its physicochemical properties with those reported in the literature,[29,30] the product (1.0 mg, 5.6 μmol) was acetylated using Ac_2O

(0.12 mL) in the presence of DMAP (1.4 mg, 11.3 μmol) in pyridine (0.8 mL) and the product was purified by silica gel column chromatography ($CHCl_3$/MeOH = 15:1) to give pentaacetylvalidamine (**17**) (1.1 mg, 52%). The physicochemical data for the product are in agreement with those reported in the literature.[29,30] $[\alpha]^{19}_D$= +59.1° (*c* 0.19, $CHCl_3$) (lit.[30] $[\alpha]^{20}_D$= +60.2° (*c* 0.6, $CHCl_3$)); ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.55 (m, 1H), 5.21 (dd, *J* = 9.9, 10.2 Hz, 1H), 4.94−5.02 (m, 2H), 4.53 (m, 1H), 4.13 (dd, *J* = 4.5, 11.4 Hz, 1H), 3.90 (dd, *J* = 3.6, 11.1 Hz, 1H), 2.14−2.24 (m, 2H), 2.06 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H) (×2), 1.60−1.71 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.9, 170.53, 170.47, 170.0, 169.7, 71.7, 71.5, 63.3, 47.0, 35.3, 23.7, 20.88, 20.82, 20.76 (×3).

*((1R,2R,3S,4S,5S)-2,3,4-Tris(benzyloxy)-5-(((benzyloxy)carbonyl)-amino)-cyclohexyl)methyl Acetate (**18**).* To a solution of **15** (135 mg, 0.20 mmol) in Ac_2O/AcOH (2:1; 2 mL) was added $ZnCl_2$ (328 mg, 2.4 mmol), and the reaction mixture was stirred for 5 h at rt. The reaction mixture was diluted with water (100 mL) and EtOAc (100 mL), and the organic layer washed with sat. aq. Na_2CO_3 (3 × 50 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure to give a crude product. Column chromatography (silica gel, Hex/EtOAc = 3:1) of the product yielded **18** (82 mg, 66%); $[\alpha]^{20}_D$= +29.3° (*c* 1.19, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.27−7.38 (m, 20H), 5.14 (s, 2H), 5.10 (m, 1H), 4.95 (d, *J* = 10.8 Hz, 1H), 4.90 (d, *J* = 10.8 Hz, 1H), 4.78 (d, *J* = 10.5 Hz, 1H), 4.71 (d, *J* = 11.4 Hz, 1H), 4.56 (d, *J* = 11.4 Hz, 2H), 4.23−4.28 (m, 2H), 4.13 (dd, *J* = 11.1, 2.7 Hz, 1H), 3.57−3.68 (m, 2H), 3.38 (dd, *J* = 8.1, 10.5 Hz, 1H), 2.26 (bd, *J* = 14.1 Hz, 1H), 2.03 (m, 1H), 2.00 (s, 3H), 1.37 (bt, *J* = 14.7 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.9, 156.5, 138.71, 138.33, 137.83, 136.5, 128.7, 128.54, 128.49, 128.18, 128.15, 128.09, 128.0, 127.95, 127.87, 127.7, 83.6, 80.8, 79.9, 75.8, 75.2, 71.7, 64.4, 66.9, 47.8, 36.2, 28.4, 20.9. HRMS (ESI) *m*/*z* 624.2982 (calcd for $C_{38}H_{42}NO_7$ $[M + H]^+$: 624.2961).

*Benzyl ((1S,2S,3S,4R,5R)-2,3,4-Tris(benzyloxy)-5-(hydroxymethyl)-cyclohexyl)carbamate (**19**).* To a solution of **18** (82 mg, 0.13 mmol) in MeOH (6.0 mL) was added 30% NaOMe in MeOH (2.4 mL) via syringe at 0 °C, and the reaction mixture was stirred for 4 h at rt. The reaction was quenched by addition of water (3.0 mL) and sat. aq. NH_4Cl (5.0 mL), and diluted with water (50 mL) and EtOAc (50 mL). The organic layer was washed with brine (50 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure to give a crude product. Column chromatography of the product (silica gel, Hex/EtOAc = 1:1) yielded **19** (68 mg, 90%); $[\alpha]^{20}_D$= +59.3° (*c* 1.44, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.31−7.37 (m, 20H), 5.08−5.18 (m, 3H), 4.96 (d, *J* = 10.8 Hz, 1H), 4.94 (d, *J* = 11.1 Hz, 1H), 4.78 (d, *J* = 10.8 Hz, 1H), 4.68 (dd, *J* = 8.7, 11.1 Hz, 2H), 4.55 (d, *J* = 11.4 Hz, 1H), 4.23 (bs, 1H), 3.55−3.72 (m, 4H), 3.41 (bt, *J* = 8.7 Hz, 1H), 2.17 (bd, *J* = 14.4 Hz, 1H), 1.85 (m, 1H), 1.31 (td, *J* = 13.7, 2.7 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 156.5, 138.8, 138.4, 137.9, 136.5, 128.7, 128.55, 128.50, 128.3, 128.2, 128.11, 128.05, 128.02, 127.9, 127.7, 83.8, 81.6, 81.0, 75.8, 75.1, 71.7, 66.9, 64.3, 47.9, 38.6, 28.2. HRMS (ESI) *m*/*z* 582.2853 (calcd for $C_{36}H_{40}NO_6$ $[M + H]^+$: 582.2856).

*Benzyl ((1S,2S,3S,4R,5R)-2,3,4-Tris(benzyloxy)-5-(((bis(benzyloxy)-phosphoryl)oxy)methyl)cyclohexyl)carbamate (**20**).* To a solution of **19** (42 mg, 72.7 μmol) and 1*H*-tetrazole (12.6 mg, 0.18 mmol) in CH_2Cl_2 (2 mL) was added dibenzyl *N*,*N*-diisopropylphosphoramidite (50 mg, 0.144 mmol) in CH_2Cl_2 (1 mL) at rt under Ar, and the reaction mixture was stirred for 1 h at rt. The reaction mixture was cooled to 0 °C and *m*-CPBA (35.6 mg (70%), 0.144 mmol) was added. After 10 min stirring at 0 °C, the reaction mixture was diluted with EtOAc (25 mL) and washed with $NaHCO_3$ (2 × 25 mL) and brine (3 × 25 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. Column chromatography of the product (silica gel, Hex/EtOAc = 3:2) yielded **20** (41 mg, 68%); $[\alpha]^{19}_D$= +27.1° (*c* 0.72, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.19−7.37 (m, 30H), 5.10 (s, 2H), 5.02 (d, *J* = 9.0 Hz, 5H), 4.90 (d, *J* = 10.8 Hz, 1H), 4.82 (d, *J* = 10.8 Hz, 1H), 4.73 (d, *J* = 10.8 Hz, 1H), 4.67 (d, *J* = 11.7 Hz, 1H), 4.51−4.56 (m, 2H), 4.25−4.31 (m, 1H), 4.13 (bs, 1H), 3.95−3.99 (m, 1H), 3.45−3.58 (m, 2H), 3.29 (bt, *J* = 8.4 Hz, 1H), 2.15 (d, *J* = 14.4 Hz, 1H), 1.86 (m, 1H), 1.27 (bt, *J* = 13.8, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 156.5, 138.8, 138.5, 137.9, 136.5, 136.03 (d, $J_{c\text{-}p}$ = 1.8 Hz), 135.95 (d, $J_{c\text{-}p}$ = 2.0 Hz), 128.75, 128.71, 128.59, 128.53, 128.49, 128.3, 128.22, 128.12, 128.08, 128.02, 127.9, 127.8, 127.7, 83.5, 80.5, 79.3, 75.8, 75.4, 71.7, 69.53 (d, $J_{c\text{-}p}$ = 1.9 Hz), 69.46 (d, $J_{c\text{-}p}$ = 1.9 Hz), 67.5 (d, $J_{c\text{-}p}$ = 5.85 Hz), 66.9, 47.9, 37.4 (d, $J_{c\text{-}p}$ = 8.76 Hz), 27.8. HRMS (ESI) *m*/*z* 842.3435 (calcd for $C_{50}H_{52}NO_9$ $[M + H]^+$: 842.3458).

*Validamine 7-Phosphate (**21**).* A mixture of **20** (10 mg, 11.9 μmol) and 10% Pd/C (5.0 mg) in MeOH (0.6 mL) and EtOAc (0.4 mL) containing 12 M HCl (10 μL) was hydrogenated under atmospheric pressure for 20 h. The reaction mixture was filtered through Celite and a cellulose syringe filter, washed with MeOH and concentrated. The crude product was further purified with Sephadex LH-20 to give the pure product **21** (2.1 mg, 70%); $[\alpha]^{23}_D$= +46.7° (*c* 0.13, H_2O); ^{1}H NMR (300 MHz, D_2O): δ 4.0−4.02 (m, 2H), 3.79−3.86 (m, 2H), 3.57 (t, *J* = 9.3 Hz, 1H), 3.46 (t, *J* = 9.0 Hz, 1H), 1.82−1.90 (m, 2H); ^{13}C NMR (75 MHz, D_2O; DSS): δ 76.3, 74.2, 72.7, 67.5 (d, $J_{c\text{-}p}$ = 4.5 Hz), 54.0, 39.8 (d, $J_{c\text{-}p}$ = 7.5 Hz), 28.6; ^{31}P NMR (122 MHz, D_2O; H_3PO_4): δ 1.70. HRMS (ESI) *m*/*z* 256.0576 (calcd for $C_7H_{15}NO_7P$ $[M - H]^-$: 256.0586).

Chemical Synthesis of Validoxylamine A 7′-Phosphate (26). *(4aR,6S,7S,8S,8aR)-7,8-Bis(benzyloxy)-2-phenyl-N-((1S,4R,5S,6S)-4,5,6-tris(benzyloxy)-3-((benzyloxy)methyl)cyclohex-2-en-1-yl)hexahydro-4H-benzo[d][1,3]dioxin-6-amine (**28**).* To a solution of validoxylamine A (**8**) (320 mg, 0.95 mmol) in 5 mL dry DMF, benzaldehyde dimethyl acetal (189 mg, 1.24 mmol), and *p*-TsOH (181 mg, 0.95 mmol) were added and the mixture was stirred at 60 °C for 3 h. The reaction was quenched with diethylamine, and the pH was adjusted to 8. To the same pot, NaH (275 mg, 11.5 mmol, 12 equiv) was added at 0 °C and stirred for 30 min. The mixture was brought to rt and benzyl bromide (1.4 mL, 11.5 mmol, 12 equiv) was added and the mixture was stirred for 3 h. The reaction was quenched with sat. aq. NH_4Cl and extracted with EtOAc (3 × 5 mL). The EtOAc fractions were combined and washed with brine (3 × 5 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was subjected to column chromatography (silica gel, hexane/EtOAc = 7:1) to afford **28** (420 mg, 2 steps, 46%). ^{1}H NMR (300 MHz, $CDCl_3$): 7.13−7.54 (m), 5.92 (brs, 1H), 5.56 (s, 1H, HCPh), 4.89 (d, *J* = 11 Hz, 1H), 4.34−4.80 (m, 16H), 4.21 (d, *J* = 12 Hz, 1H), 3.81−4.14 (m, 6H), 3.62 (m, 1H), 3.51 (bt, *J* = 9 Hz), 3.31 (m, 3H), 2.54 (m, 1H), 1.88 (m, 1H), 1.63 (bd, *J* = 14 Hz, 1H), 1.54 (S, 1H), 0.823 (t, *J* = 14 Hz); ^{13}C NMR (300 MHz, $CDCl_3$); 139,1, 138.8, 138.4, 135.25, 127.3 - 128.1, 126.9, 126.2, 101.1, 84.6, 82.8, 82.3, 81.16, 81.16, 80.2, 76.6, 75.4, 74.6, 74.6, 73.3, 72.7, 72.3, 71.4, 71.3, 52.9, 51.4, 31.8, 28.7, 26.7. Electrospray MS: *m*/*z* 964.40 $[M + H]^+$. HRMS (ESI) *m*/*z* 964.4743 (calcd for $C_{63}H_{66}NO_8$ $[M + H]^+$: 964.4788).

*((1R,2R,3S,4S,5S)-2,3,4-Tris(benzyloxy)-5-(((1S,4R,5S,6S)-4,5,6-tris-(benzyloxy)-3-((benzyloxy)methyl)cyclohex-2-en-1-yl)amino)cyclohexyl)-methanol (**29**).* To a solution of **28** (200 mg, 0.21 mmol) in CH_2Cl_2 (4 mL) DIBAL-H (1.0 M in hexane, 311 μL, 0.311 mmol) was added at 0 °C. After stirring at rt for 3 h, more DIBAL-H (150 μL) was added. The reaction mixture was stirred for 3.5 h and quenched by adding few drops of saturated solution of potassium sodium tartrate at 0 °C. The mixture was brought to rt with continuous addition of potassium sodium tartrate (5 mL). The mixture was diluted with EtOAc and vigorously stirred for 2.5 h. The aqueous phase was extracted three times with EtOAc. The organic fraction was washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was subjected to column chromatography (silica gel, hexane/EtOAc = 3:1) to afford **29** (150 mg, 74.9%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): 7.25 (m), 5.87 (brs, 1H), 4.90 (bt, *J* = 11 Hz, 2H), 4.58−4.87 (m, 13H), 4.22 (d, *J* = 12 Hz, 1H), 3.84−4.09 (m, 4H), 3.60 (m, 1H),

3.51 (m, 1H), 3.34 (m, 3H), 2.27 (m, 1H) 1.74 (brd, J = 14 Hz), 1.52 (s), 1.03 (t, J = 12 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): 139.0, 138.9, 138.7, 135.6, 128.0−128.9, 127.9, 127.8, 102.2, 100.6, 83.9, 83.8, 83.5, 78.2, 78.1, 75.9, 75.2, 74.1, 73.8, 72.9, 72.3, 65.3, 51.4, 51.2, 37.7, 28.1, 21.2. Electrospray MS: m/z 966.47 $[M+H]^+$. HRMS (ESI) m/z 966.4984 (calcd for $C_{63}H_{68}NO_8$ $[M + H]^+$: 966.4945).

*Dibenzyl (((1R,2R,3S,4S,5S)-2,3,4-Tris(benzyloxy)-5-(((1S,4R,5S,6S)-4,5,6-tris(benzyloxy)-3-((benzyloxy)methyl)cyclohex-2-en-1-yl)amino)-cyclohexyl)methyl) Phosphate (**30**).* A solution of **29** (70 mg, 0.0725 mmol) in CH_2Cl_2 (2 mL) was added to a mixture of dibenzyl *N*,*N*-diisopropylphosphoramidite (37.5 mg, 0.108 mmol) and 1*H*−tetrazole (15.2 mg, 0.21 mmol) in CH_2Cl_2 (2 mL), which had been stirred for 40 min, at rt and the reaction mixture was further stirred for 3 h. The reaction mixture was cooled to 0 °C and *m*-CPBA (12.5 mg, 0.0725 mmol) was added. After the cooling bath was removed, the reaction mixture was stirred for an additional 30 min at rt. The solvent was evapored *in vacuo*, and the residue was redissolved in EtOAc (5 mL). The EtOAc solution was washed with 10% aqueous Na_2SO_3 (5 mL), saturated aqueous $NaHCO_3$ (5 mL) and brine (5 mL), dried over anhydrous $MgSO_4$, filtered and concentrated in a rotary evaporator. The product was purified using column chromatography (silica gel, toluene−EtOAc = 3: 1) to afford **30** (65 mg, 73%). ^{1}H NMR (300 MHz, $CDCl_3$): 7.25 (m), 5.92 (br s, 1H), 4.96 (d, J = 9 Hz, 4H), 4.85 (t, J = 10 Hz, 2H), 4.38 - 4.75 (m, 14H), 4.22 (d, J = 12 Hz, 2H), 4.02 (br d, J = 4 Hz, 1H), 3.88 (m, 3H), 3.71 (m, 1H), 3.55 (m, 1H), 3.38 (dd, J = 9 Hz, J = 4 Hz, 1H), 3.26 (m, 3H), 2.28 (m, 1H), 1.67 (m, 1H), 1.12 (t, J = 15 Hz); ^{13}C NMR (300 MHz, CDCl3) 138.6−139.4, 126.4−128.9, 83.6, 83.2, 80.3, 77.9, 77.6, 77.4, 76.8, 75.7, 75.3, 74.2, 73.6, 72.6, 72.5, 71.1, 69.5, 69.4, 68.3, 51.5, 51.3, 36.7, 36.6, 27.9, 20.7. Electrospray MS: m/z 1226.53 $[M+H]^+$. HRMS (ESI) m/z 1226.5573 (calcd for $C_{77}H_{81}NO_{11}P$ $[M + H]^+$: 1226.5542).

*Validoxylamine A 7′-Phosphate (**26**).* BBr_3 (417 μL of 1.0 M solution in CH_2Cl_2, 0.417 mmol) was added to a solution of **30** (28.4 mg, 0.023 mmol) in CH_2Cl_2, and the reaction mixture was stirred for 60 min at −40 °C. The mixture was then placed in an ice bath and several drops of saturated aqueous $NaHCO_3$ solution were slowly added until no more effervescence was observed. The mixture was then partitioned between H_2O (1 mL) and CH_2Cl_2 (1 mL). The aqueous fraction was collected and dried in a SpeedVac rotator and the residue was dissolved in H_2O (500 μL). The solution (500 μL) was subjected to Biogel P-2 Gel fine column chromatography (10 cm ×0.5 cm) and eluted with H_2O. Fractions containing the product were pooled, dried, and purified on Sephadex LH-20 (75 cm ×1 cm) to afford **26** (4.5 mg, 46%). ^{1}H NMR (300 MHz, D_2O): 5.98 (br s, 1H), 4.33 (s, 2H), 4.22 (br d, J = 4.5 Hz, 2H), 4.13 (m, 1H), 4.04 (br s, 2H), 3.90 (m, 3H), 3.69 (t, J = 8 Hz, 1H), 3.51 (t, J = 9 Hz, 1H), 2.31 (brd, J = 14 Hz), 2.03 (m, 1H), 1.86 (t, J = 14 Hz); ^{13}C NMR (300 MHz, D_2O): 114.9, 73.7, 71.9, 71.5, 70.7, 70.4, 66.5, 64.7, 61.2, 58.1, 56.9, 37.4, 24.5, 20.9. Electrospray MS: m/z 416.20 $[M + H]^+$. HRMS (ESI) m/z 416.1320 (calcd for $C_{14}H_{27}NO_{11}P$ $[M + H]^+$: 416.1322).

VldB, VldE, and VldH Expression Plasmid Construction. Genomic DNA of *S. hygroscopicus* subsp. *limoneus* was prepared by a general procedure.[31] The *vldB*, *vldE*, and *vldH* genes were amplified by PCR from the genomic DNA using the following primer pairs: 5′-TGG GGC TCG AGG CAT ATG GAC GGA GTG CGT-3′ and 5′-TTC GAA CTC GAG CAG CGC CAC-3′ for *vldB*, 5′-AAG ATC TCG AGA CAT ATG ACC GGA TCT GAG-3′ and 5′-TCA GAA TTC TCA GAG GTC TGC-3′ for *vldE*, 5′-GGT GAC TCG AGA CAT ATG TAC AAG GTT GCA-3′ and 5′-TGA GAA TTC TCA GGA AGG ACC AAT ATG CGG-3′ for *vldH* (*Xho*I, *Nde*I, and *Eco*RI sites are underlined). The *vldB* PCR fragment was digested with *Nde*I and *Xho*I and ligated into pET20b (Novagen) to give pET20b-vldB. The *vldE* and *vldH* PCR fragments were digested with *Xho*I and *Eco*RI and ligated into pRSET-B (Invitrogen) to give pRSET-B-vldE and pRSET-B-vldH. The DNA sequences of *vldB*, *vldE*, and *vldH* were confirmed by the Center for Genome Research and Biocomputing (CGRB) at Oregon State University.

OtsA and OtsB Expression Plasmid Construction. The *otsA* and *otsB* genes from *E. coli* MG1655 were amplified by PCR from plasmid pTrc99A-otsAB (a gift from Dr. C-J. Kim) using the following primer pairs: 5′-AGG CTC GAG GCA TAT GAG TCG TTT AGT CGT-3′ and 5′-GAC GAA TTC TCA CGC AAG CTT TGG AAA GGT-3′ for *otsA*, 5′-GAC CTC GAG ACA TAT GAC AGA ACC GTT AAC-3′ and 5′-GCA GAA TTC TCA GAT ACT ACG ACT AAA CGA-3′ for *otsB* (*Xho*I and *Eco*RI sites are underlined). The *otsA* and *otsB* PCR fragments were digested with *Xho*I and *Eco*RI and ligated into pRSET-B (Invitrogen) to give pRSET-B-otsA and pRSET-B-otsB. DNA sequences of *otsA* and *otsB* were confirmed by CGRB.

VldB, VldE, and VldH Production and Purification. pET20-*vldB*, pRSETB-*vldE* and pRSETB-*vldH* were used to transform *E. coli* BL21(DE3) pLysS. Transformants were grown overnight at 37 °C on LB agar plate containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol. A single colony was inoculated into 3 mL of LB medium and cultured at 37 °C for 6 h and then 1 mL of seed culture was transferred into 100 mL of LB medium in a 500 mL flask and grown at 28 °C until OD_{600} reached 0.6. Then, the temperature was reduced to 18 °C, and after 2 h of adaptation, 0.1 mM IPTG was added to induce the C-terminal hexahistidine-tagged VldB and the N-terminal hexahistidine-tagged VldE and VldH proteins. After further growth for 14 h, the cells were harvested by centrifugation (5000 rpm, 10 min, 4 °C) and stored at −80 °C until used. Cell pellets from 50 mL of culture were washed with 1 mL of binding (B) buffer (40 mM HEPES (pH 8.0), 300 mM NaCl, 10% glycerol, and 10 mM imidazole) and centrifuged (6000 rpm, 3 min, 4 °C). Then, 1 mL of B buffer was added and the mixtures were sonicated (8 W, 15 s, 4 times). After centrifugation (14 500 rpm, 20 min, 4 °C), 0.8 mL of the supernatants was each mixed with 0.2 mL of B buffer-equilibrated Ni-NTA resin (QIAGEN) and incubated for 1 h at 4 °C. After incubation, the mixtures were centrifuged (4000 rpm, 3 min, 4 °C) and the supernatants were discarded. One milliliter of washing (W) buffer (40 mM HEPES pH 8.0, 300 mM NaCl, 10% glycerol, and 20 mM imidazole in the case of VldE and VldH or 100 mM imidazole in the case of VldB) was added and the mixture centrifuged (4000 rpm, 3 min, 4 °C). This washing step was repeated three times. Subsequently, 0.5 mL of elution (E) buffer (40 mM HEPES pH 8.0, containing 300 mM NaCl, 10% glycerol, and 500 mM imidazole) was added to elute the desired proteins. This elution step was repeated twice. Eluted VldB, VldE, and VldH were dialyzed against 1 L of dialysis buffer (10 mM Tris-HCl pH 7.5, 0.1 mM DTT, 1 mM $MgCl_2$) 3 times for 3 h each. Purified proteins were analyzed by SDS−PAGE and concentrated by ultrafiltration using Amicon YM-10 (Millipore). Protein concentration was determined using the Bradford assay (BIO-RAD) with BSA as standard.

Coupled VldB/VldE Assay with ATP and GTP. To determine whether ATP or GTP is the natural nucleotidyl donor in the coupled VldB/VldE reaction, VldB (1 μM) and VldE (3 μM) were incubated with 4 mM valienol 1-phosphate, 4 mM validamine 7-phosphate, 6 mM of either ATP or GTP, and 2 mM $MgCl_2$ in 60 mM Tris-HCl, pH 7.5. Reaction mixtures were incubated at 30 °C for 3 h. Then, 2 vol of MeOH were added and mixed. The mixture was centrifuged (14 500 rpm, 5 min, 4 °C) and the supernatant was subjected to MS analysis. The results revealed that GTP is used more effectively in the VldB/VldE coupled assay than ATP.

Combined VldB, VldE, and VldH Assay. Typical reaction condition for activity assays were 60 mM Tris-HCl, pH 7.5, 4 mM valienol 1-phosphate; 6 mM NTPs; 2 mM $MgCl_2$; 1 μM VldB; 4 mM validamine 7-phosphate; 3 μM VldE; and 3 μM VldH. Reaction mixtures were incubated at 30 °C for 3 h. For enzyme inactivation, VldB, VldE, and VldH were heated at 100 °C for 5 min.

HPLC Analysis of VldB, VldE, and VldH Products. For HPLC analysis, the reaction was quenched by adding 2 vol of MeOH and centrifuged, and the supernatant was analyzed by HPLC (Shimadzu SPD-20A system) with a Gemini C18 column (4.6 × 150 mm, 5 μm,

Phenomenex) using 0.5% MeOH in H_2O containing 10 mM NH_4HCO_3 as eluent at a flow rate of 1 mL/min. Elution profiles were monitored at 210 nm.

High-Resolution Mass Spectral Analysis of VldB/VldE and VldB/VldE/VldH Products. HR-ESTOFMS of the VldB/VldE product: m/z 416.1315 (calcd for $C_{14}H_{27}NO_{11}P$ $[M + H]^+$: 416.1322). HR-ESTOFMS of the VldB/VldE/VldH product: m/z 336.1657 (calcd for $C_{14}H_{26}NO_8$ $[M + H]^+$: 336.1658).

Preparation of GDP-Valienol for Kinetic Studies. A reaction mixture (100 μL) containing 60 mM Tris-HCl, pH 7.5; 2 mM $MgCl_2$; 5 mM valienol 1-phosphate; 7.5 mM GTP; and 3 μM VldB was incubated at 30 °C for 2 h and VldB was removed by ultrafiltration (YM-10, Amicon). GDP-valienol formed in the reaction mixture was measured by quantification of the generated inorganic diphosphate generated using the EnzChek pyrophosphate assay kit (E-6645, Invitrogen). Reaction mixtures lacking VldB were used as negative control (blank).

K_m and k_{cat} Determination for VldE. K_m and k_{cat} values for VldE were determined by using the phosphopyruvate kinase (PK) and lactate dehydrogenase (LDH) coupled assay (Sigma-Aldrich). The reaction mixture (100 μL) contained 50 mM Tris-HCl, pH 7.5; 5 mM $MgCl_2$; 1.0−3.0 μM VldE; 1.5 mM phosphoenolpyruvate; 0.5 mM NADH; 37 U of PK; and 46 U of LDH, in addition to 0, 10, 20, 30, 40, 50, 60, 90, 120, 150, 300, 600 μM GDP-valienol and 1 mM validamine 7-phosphate for the determination of the value for GDP-valienol, and 0, 50, 100, 150, 200, 300, 500, 1000 μM validamine 7-phosphate and 300 μM GDP-valienol for the determination of the value for validamine 7-phosphate. Oxidation of NADH to NAD^+ was monitored in 96-well plates using a spectrophotometric microplate reader at 340 nm. Reaction mixtures lacking VldE were used as negative control (blank). The data were collected in duplicate (for GDP-valienol) and triplicate (for validamine 7-phosphate). Hanes-Woolf plot was used for K_m and k_{cat} value determination.

Assay of VldE for Trehalose 6-Phosphate Synthase Activity. To determine if VldE has trehalose 6-phosphate synthase activity, the enzyme (6 μM) was incubated with 1 mM donor substrate (either GDP-valienol, UDP-glucose, or GDP-glucose), 1 mM acceptor substrate (either validamine 7-phosphate or glucose 6-phosphate), 2 mM $MgCl_2$, 16 μM VldH or OtsB in 50 mM Tris-HCl (pH 7.5). After a 3-h incubation at 30 °C, 2 vol of MeOH were added and mixed. The mixture was centrifuged (14 500 rpm, 5 min, 4 °C) and the supernatant was subjected to MS analysis. The results revealed that VldE does not recognize UDP-glucose, GDP-glucose, or glucose 6-phosphate as substrates.

OtsA and OtsB Production and Purification. The productions and purifications of N-terminal hexahistidine-tagged OtsA and OtsB were carried out by the same procedures as used for VldE and VldH.

Assay of OtsA for Validoxylamine A 7′-Phosphate Synthase Activity. To determine if OtsA has validoxylamine A 7′-phosphate synthase activity, the enzyme (6 μM) was incubated with 1 mM donor substrate (either GDP-valienol or UDP-glucose), 1 mM acceptor substrate (either validamine 7-phosphate or glucose 6-phosphate), 5 mM $MgCl_2$, 16 μM OtsB or VldH in 50 mM Tris-HCl (pH 7.5). After 3 h incubation at 30 °C, 2 vol of MeOH were added and mixed. The mixture was centrifuged (14 500 rpm, 5 min, 4 °C) and the supernatant was subjected to MS analysis. The results revealed that OtsA does not recognize GDP-valienol and validamine 7-phosphate as substrates.

Characterization of OtsB and VldH as Trehalose 6-Phosphate Phosphatases. To determine if VldH has trehalose 6-phosphate phosphatase activity, the enzyme (8 μM) was incubated with 5 mM trehalose 6-phosphate, and 10 mM $MgCl_2$ in 50 mM Tris-HCl (pH 7.5). OtsB was used as positive control. After 1.5 h incubation at 30 °C, 2 vol of MeOH were added and mixed. The mixture was centrifuged (14 500 rpm, 5 min, 4 °C) and the supernatant was subjected to MS analysis. The results revealed that VldH was able to catalyze dephosphorylation of trehalose 6-phosphate to give trehalose.

Assay of VldE for Validoxylamine A Glycosyltransferase Activity. To determine if VldE can catalyze the glycosylation of validoxylamine A to validamycin A, the enzyme (50 μM) was incubated with 1 mM validoxylamine A, 1 mM UDP-glucose, 10 mM $MgCl_2$, and 10 mM NH_4Cl in 50 mM Tris-HCl (pH 8.0). After 3 h incubation at 30 °C, 2 vol of MeOH were added and mixed. The mixture was centrifuged (14 500 rpm, 5 min, 4 °C) and the supernatant was subjected to MS analysis. The results show that VldE is not a validoxylamine A glycosyltransferase.

■ ASSOCIATED CONTENT

Supporting Information. Comparison of catalytic activities of trehalose 6-phosphate synthase and VldE (Scheme S1), complete HPLC analysis of VldB, VldE, and VldH (Figure S1), MS analysis of VldB, VldE, and VldH (Figures S2−S10), amino acid alignment of trehalose 6-phosphate synthase and VldE (Figure S11), ^{1}H-, ^{13}C-, and ^{31}P NMR data for synthetic compounds generated in this study (Figures S12−S30). This material is available free of charge via the Internet at http://pubs.acs.org.

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