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PAPER

Nucleotidylation of unsaturated carbasugar in validamycin biosynthesis†

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Validamycin A is a member of microbial-derived C_7N-aminocyclitol family of natural products that is widely used as crop protectant and the precursor of the antidiabetic drug voglibose. Its biosynthetic gene clusters have been identified in several *Streptomyces hygroscopicus* strains, and a number of genes within the clusters have been functionally analyzed. Of these genes, *valB*, which encodes a sugar nucleotidyltransferase, was found through inactivation study to be essential for validamycin biosynthesis, but its role was unclear. To characterize the role of ValB in validamycin biosynthesis, four carbasugar phosphate analogues were synthesized and tested as substrate for ValB. The results showed that ValB efficiently catalyzes the conversion of valienol 1-phosphate to its nucleotidyl diphosphate derivatives, whereas other unsaturated carbasugar phosphates were found to be not the preferred substrate. ValB requires Mg^{2+}, Mn^{2+}, or Co^{2+} for its optimal activity and uses the purine-based nucleotidyltriphosphates (ATP and GTP) more efficiently than the pyrimidine-based NTPs (CTP, dTTP, and UTP) as nucleotidyl donor. ValB represents the first member of unsaturated carbasugar nucleotidyltransferases involved in natural products biosynthesis. Its characterization not only expands our understanding of aminocyclitol-derived natural products biosynthesis, but may also facilitate the development of new tools for chemoenzymatic synthesis of carbohydrate mimetics.

Introduction

Carbohydrates are indispensable elements in living organisms, not only as molecules for energy storage but also as mediators of many complex biological processes.[1–3] Their structural diversity enables them to encode information for specific molecular transactions such as cell–cell recognition and molecular targeting.[1–3] Due to their ubiquitous involvement in many biological processes, including immune response, cancer, and inflammation, developing new carbohydrate mimetics (*e.g.*, carbasugars, aminosugars, *etc*) can provide a new roadmap for drug discovery.[4–6]

Among the naturally derived carbasugars are the C_7N-aminocyclitol family of natural products, such as the α-glucosidase inhibitor acarbose and the antifungal agent validamycin A. They have attracted attention due to their important biomedical and agricultural uses.[7] Validamycin A is a bacterial-derived crop protectant that is widely used to treat sheath blight disease in rice plants. The core aminocyclitol unit, valienamine, is used as the semi-synthetic precursor of the antidiabetic drug voglibose. Prompted by the identification of the biosynthetic gene cluster, investigations into validamycin biosynthesis have revealed the involvement of a number of unusual enzymes that exclusively recognize carbasugars (cyclitols) as substrates.[8] The pathway leads to a condensation between two cyclitol intermediates by an undetermined coupling mechanism. The lack of clear understanding of this coupling reaction has led to speculation about how the nitrogen bridge in validamycin A is formed.[8–13]

It has been speculated that the core validoxylamine unit of validamycin is formed by a condensation between a nucleotidyl unsaturated carbasugar, such as NDP-valienol (**3a**) or NDP-1-*epi*-valienol (**3b**) (Scheme 1), and an amino-bearing carbasugar.[9] **3a** and **3b** may be derived from valienol 1-phosphate (**1a**) or 1-*epi*-valienol 1-phosphate (**1b**), respectively, catalyzed by a carbasugar nucleotidyltransferase. A similar reaction has been proposed in the acarbose pathway involving 1-*epi*-valienol 1,7-diphosphate (**2b**) and NDP-1-*epi*-valienol 7-phosphate (**4b**) as the substrate and the product of the putative nucleotidyltransferase AcbR.[11,14] However, no biochemical evidence is available to support this notion.

Although sugar 1-phosphate nucleotidyltransferases have been widely studied, little is known about their involvement in carbasugar or aminocyclitol biosynthesis. The only report that directly implicated a sugar 1-phosphate nucleotidyltransferase in NDP-carbasugar formation was that of the *Escherichia coli* glucose 1-phosphate uridylyltransferase, which could catalyze the conversion of carbaglucose 1-phosphate to its UDP-product, albeit at a lower turnover rate than observed with the natural substrate, glucose 1-phosphate.[15] While it is unclear if other nucleotidyltransferases are tolerant to ring oxygen substitutions, the

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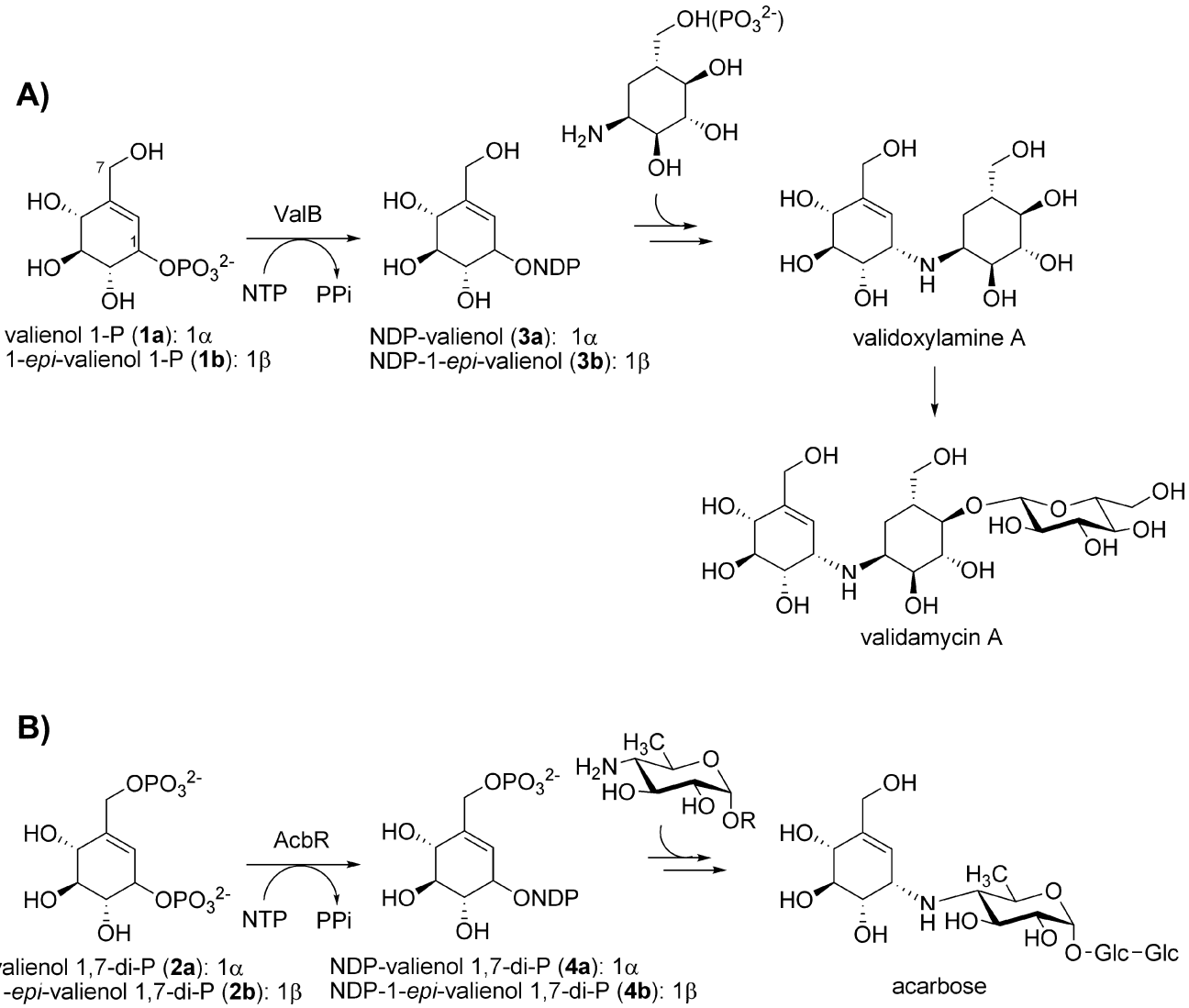


Scheme 1 Proposed biosynthetic pathways to validamycin involving either valienol 1-phosphate or 1-*epi*-valienol 1-phosphate (A) and to acarbose involving either valienol 1,7-diphosphate or 1-*epi*-valienol 1,7-diphosphate (B).

corresponding enzyme from yeast did not recognize carbaglucose 1-phosphate as substrate.[15]

A gene (*valB*) encoding a sugar 1-phosphate nucleotidyltransferase homologous to AcbR was identified in the validamycin biosynthetic gene cluster of *Streptomyces hygroscopicus* subsp. *jinggangensis* 5008.[16] While the catalytic function of ValB was unclear, its homologue (VldB, 99% identity) from another validamycin producing strain was reported to convert glucose 1-phosphate to UDP-glucose.[13] It was then postulated that VldB is responsible for the formation of pathway specific UDP-glucose, which is important for the glucosylation of validoxylamine A to validamycin A. As this result is inconsistent with the notion that ValB, and also AcbR, is involved in carbasugar nucleotidylation, we decided to investigate the actual role of ValB in validamycin biosynthesis. The present paper reports our results on the inactivation of *valB* in *S. hygroscopicus* 5008, complementation experiments, synthesis of all four possible substrates (**1a**, **1b**, valienol 1,7-diphosphate (**2a**), and **2b**) for ValB, as well as functional characterization of recombinant ValB. The results not only provide insight into the role of ValB in validamycin biosynthesis but may also facilitate the development of new tools for the production of carbohydrate mimetics.

Results

Inactivation of *valB* in *S. hygroscopicus* 5008 abolishes production of validamycin A and validoxylamine A

Whereas Kim and co-workers have demonstrated that VldB could convert glucose 1-phosphate to UDP-glucose,[13] the question remains as to what is the exact role of ValB in validamycin biosynthesis. Is ValB really responsible for the formation of UDP-glucose, the substrate for the glycosyltransferase ValG,[17] or is it involved in the formation of NDP-cyclitol, setting the stage for a subsequent coupling reaction.[9] To address this question, the *valB* gene was inactivated by replacing a 936 bp internal DNA fragment of *valB* with *oriT-aac(3)IV* in *S. hygroscopicus* 5008.

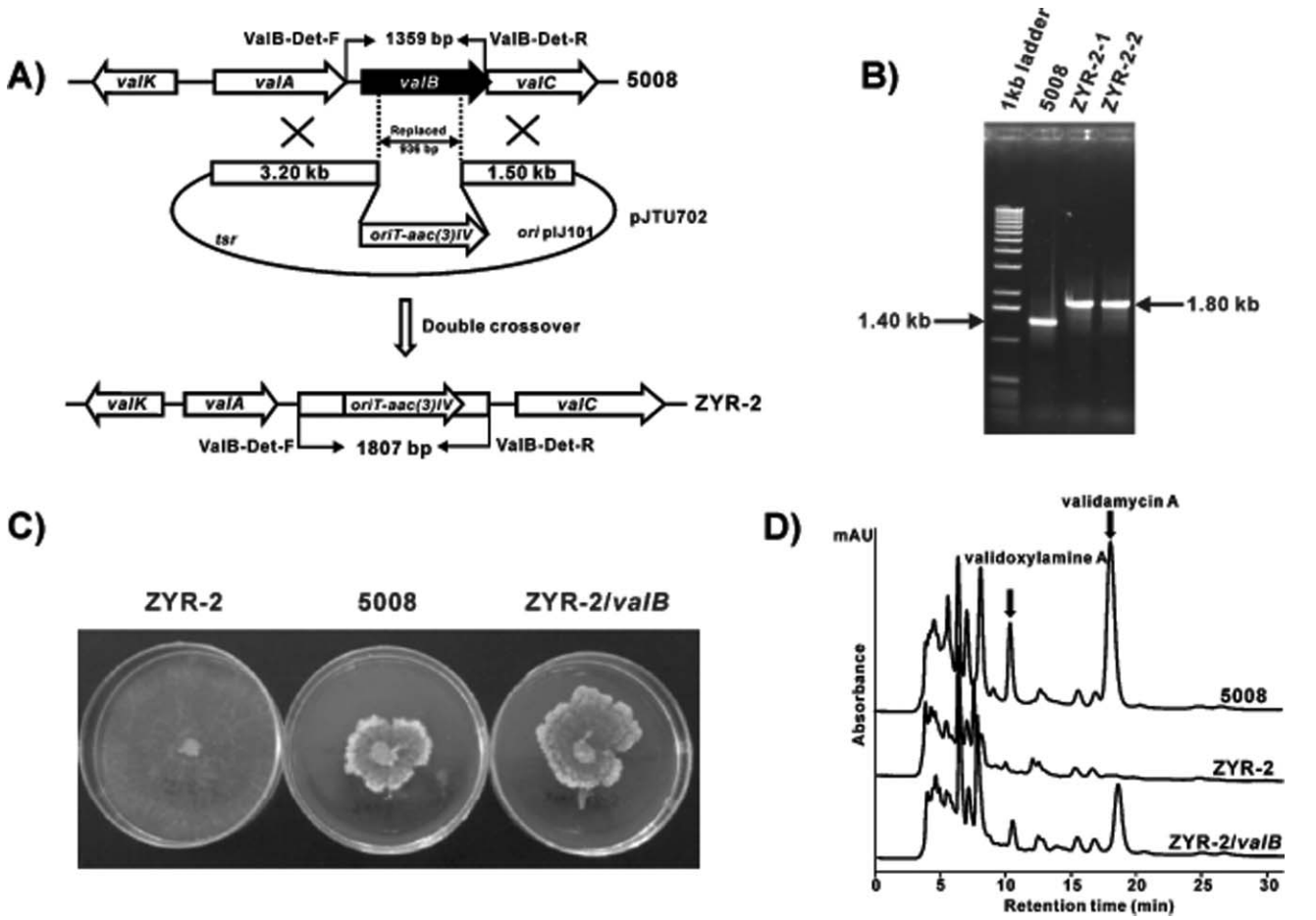


Fig. 1 Construction and analysis of *valB* mutant strains of *S. hygroscopicus* 5008. **A**, Schematic representation for the replacement of a 936 bp fragment of *valB* with *oriT-aac(3)IV*. **B**, Gel electrophoresis of PCR amplified products of the wild-type and the mutant strains. **C**, Bioassay comparison between the wild-type 5008, the *valB* mutant ZYR-2, and the *valB* mutant complemented with cloned *valB* (ZYR-2/*valB*) using *Pellicularia sasakii* as the indicator strain. Growth inhibition was determined by the appearance of abnormal branching of the mycelia and the formation of a thick border. **D**, HPLC analysis of the wild-type 5008, the *valB* mutant ZYR-2, and the *valB* mutant complemented with cloned *valB* (ZYR-2/*valB*). Validamycin A and validoxylamine A are indicated with arrows.

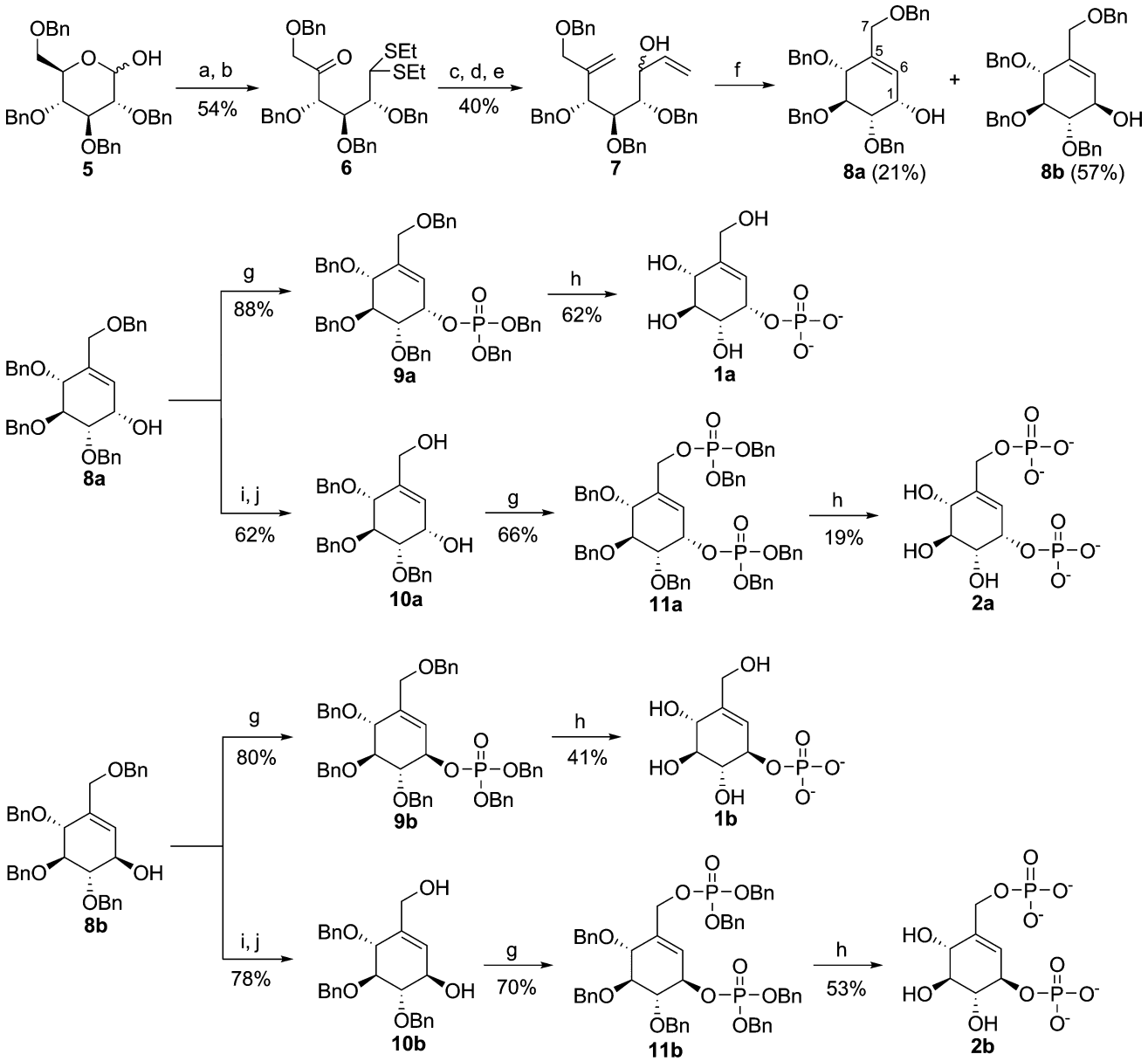
This was achieved by using a pHZ1358-derived plasmid pJTU702 in which *oriT-aac(3)IV* had been inserted between a 3.2 kb left flanking and a 1.5 kb right flanking sequence of the 936 bp DNA fragment to be replaced (Fig. 1A). The plasmid pJTU702 was introduced into the wild-type strain of *S. hygroscopicus* 5008 through conjugation[18] and two thiostrepton-sensitive apramycin-resistant transformants (ZYR-2-1 and ZYR-2-2) were obtained. Total DNA from these two mutant strains and from the wild-type strain was used as templates for PCR analysis using two primers, ValB-Det-F and ValB-Det-R. The two mutants gave a 1.8 kb PCR product, while the wild-type strain gave a 1.4 kb PCR product, which confirmed that a 936 bp DNA fragment internal to *valB* had been replaced by the 1384 bp *oriT-aac(3)IV* cassette in these mutants (Fig. 1B). Fermentation broths of the mutants were analyzed by bioassay and HPLC. No inhibition of the fungus *Pellicularia sasakii* could be detected in the bioassay (Fig. 1C) and no peak corresponding to validamycin A and validoxylamine A was detected by HPLC analysis, indicating a complete loss of production of both compounds in the *valB* mutants (Fig. 1D). The lack of validoxylamine A formation in the mutant strains indicated that ValB is involved in the formation of the core aminocyclitol unit, rather than in UDP-glucose formation.

Restoration of validamycin A and validoxylamine A production in ZYR-2 through complementation with cloned *valB*

To confirm the critical role of *valB* in validoxylamine A biosynthesis, the PCR-amplified *valB* gene was cloned onto the integrative vector pPM927 downstream of the *PermE** promoter, and the product subsequently introduced into the *valB* mutant ZYR-2-1 by conjugation. Fermentation broths of the ZYR-2 strain complemented with *valB* regained inhibitory activity in the bioassay (Fig. 1C) and HPLC analysis of the fermentation extracts clearly demonstrated that validoxylamine A and validamycin A productions were restored to about 50% of those of the wild-type strain (Fig. 1D).

Chemical synthesis of valienol phosphates

As the inactivation experiments suggested the involvement of ValB in the formation of validoxylamine A, we attempted to identify



Scheme 2 Synthesis of valienol 1-phosphate **1a**, 1-*epi*-valienol 1-phosphate **1b**, valienol 1,7-diphosphate **2a**, and 1-*epi*-valienol 1,7-diphosphate **2b**. a) EtSH, TFA; b) DMSO, Ac_2O; c) Ph_3PCH_3Br, *t*-BuOK, benzene; d) $HgCl_2$, HgO, aq. CH_3CN; e) vinylmagnesium bromide, THF, 0 °C; f) Grubbs' 2nd gen. catalyst, CH_2Cl_2, reflux; g) $iPr_2NP(OBn)_2$, 1*H*-tetrazole, CH_2Cl_2, *m*-CPBA; h) BBr_3, CH_2Cl_2; i) $ZnCl_2$, Ac_2O, AcOH; j) NaOMe, MeOH.

the substrate for this enzyme by preparing and testing a number of carbasugar compounds. Despite that numerous strategies have been applied to the synthesis of carbocyclic framework of carbasugars, both in racemic and enantiomerically pure forms,[7,19] the synthesis of unsaturated carbasugar phosphates, such as valienol phosphates and its analogues, has never been reported. In order to gain access to these unsaturated carbasugar phosphates chemical syntheses were carried out using a ring-closing metathesis strategy as shown in Scheme 2. The key intermediate tetrabenzylated diene **7** was synthesized from commercially available 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose (**5**) in five steps according to a literature procedure.[19] Ring-closing metathesis[19] of **7** mediated by Grubbs' 2nd generation catalyst[20] furnished, after column chromatography, the tetrabenzylated valienols **8a** and **8b** in 21% and 57% yields, respectively (Scheme 2). Alcohols **8a** and **8b** were then individually treated with dibenzyl diisopropyl phosphoramidite in the presence of 1*H*-tetrazole to form the phosphite intermediates, which were readily oxidized by *m*-chloroperoxybenzoic acid (*m*-CPBA) to afford phosphate compounds **9a** and **9b**, respectively.[21] Finally, debenzylation was achieved by treating the compounds with BBr_3[22] at –40 °C to give the desired products **1a** and **1b**.

The synthesis of valienol 1,7-diphosphate analogues also proceeded from compounds **8a** and **8b**. Regioselective debenzylation of **8a** and **8b** using $ZnCl_2$, Ac_2O and AcOH, followed by a deacetylation step furnished diols **10a** and **10b**, respectively.[23] The two free alcohol functionalities were then phosphorylated and the product debenzylated as described for the synthesis of **1a** and

1b to afford valienol 1,7-diphosphate (**2a**) and 1-*epi*-valienol 1,7-diphosphate (**2b**).

Preparation of recombinant ValB and initial characterization of ValB with different valienol phosphates and NTPs

With the potential substrates for the enzyme in hand, the recombinant ValB protein was then prepared by cloning the 1.1 kb *valB* gene into expression vector pRSET B (Invitrogen) and the resulting plasmid was introduced into *E. coli* BL21 Gold(DE3)/pLysS by heat-pulse transformation. Gene expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) and the recombinant protein was purified through a Ni-NTA column and dialyzed against 60 mM Tris-HCl pH 8 to give >80% pure 45 kDa protein (Fig. 2A).

Preliminary enzymatic reactions of ValB were carried out using the four different substrates (**1a**, **1b**, **2a**, and **2b**) and all five naturally occurring NTPs (ATP, GTP, CTP, dTTP, and UTP). Analysis of the products by TLC and MS revealed that only **1a** was converted to the corresponding NDP-valienols. Similar experiments with glucose 1-phosphate also gave NDP-glucose products, suggesting that ValB has somewhat relaxed substrate specificity. However, due to their instability, attempts to analyze the NDP-valienol products by HPLC or to isolate the products by chromatography were unsuccessful. We observed that during prolonged incubation and/or chromatography, the products degraded to valienol. Therefore, an alternative assay for the enzyme reaction was developed.

Coupled colorimetric assays of ValB

Because the conversion of valienol 1-phosphate and a nucleotidyl triphosphate to the corresponding NDP-valienol produces inorganic pyrophosphate (PP_i), measuring the net amount of PP_i in the reaction mixture provides an indirect measure of the NDP-valienol being produced. Therefore, a coupled colorimetric assay using EnzChek Pyrophosphate Assay Kit[24] (Molecular Probes) was used to measure the amount of PP_i in the reactions (Scheme 3). The system includes the enzymes inorganic pyrophosphatase and purine nucleoside phosphorylase (PNP), in which the former enzyme catalyzes the conversion of PP_i into two equivalents of inorganic phosphate (P_i), and the latter, in the presence of P_i, converts 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine. Enzymatic conversion of MESG results in a shift in

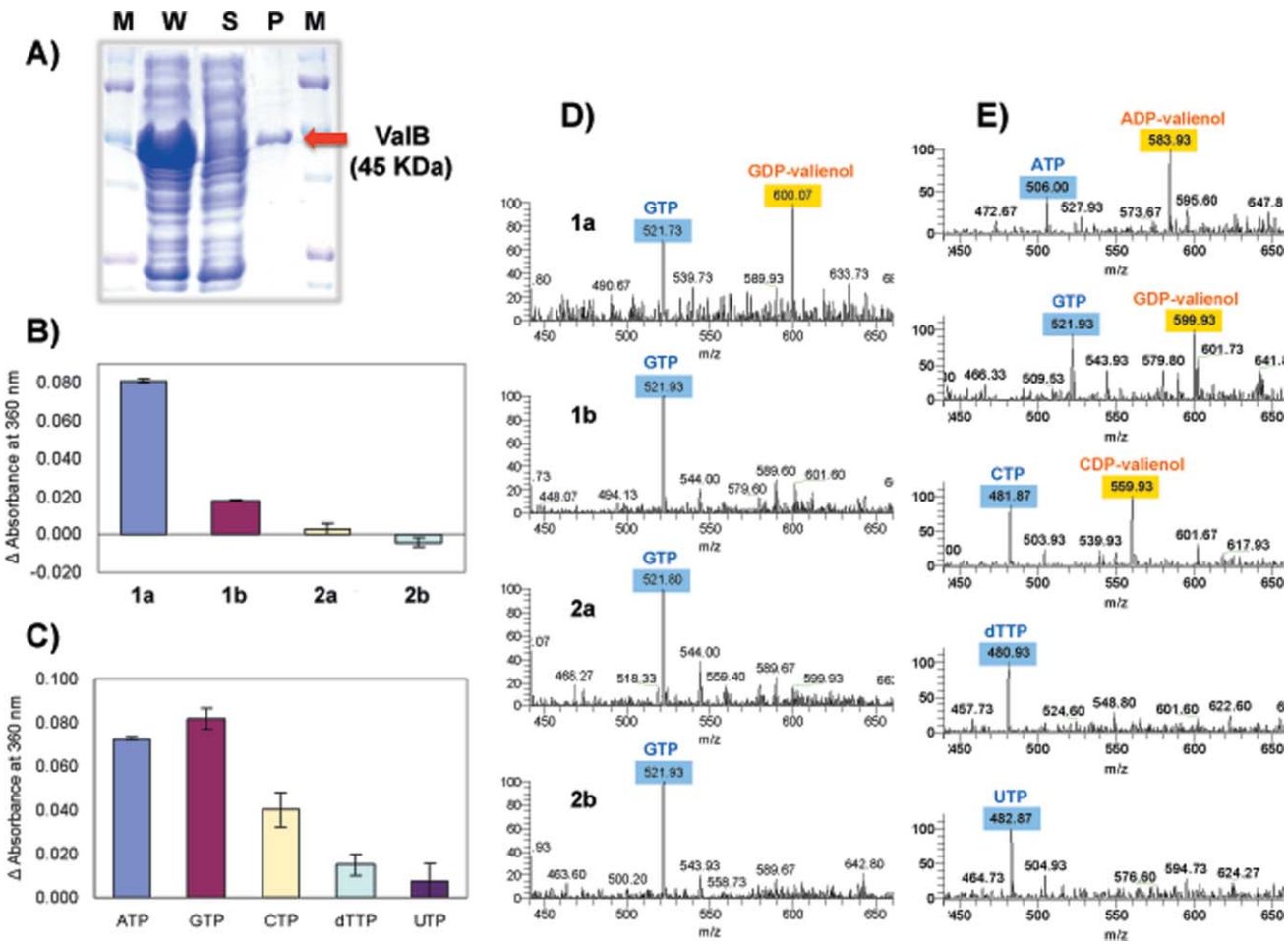
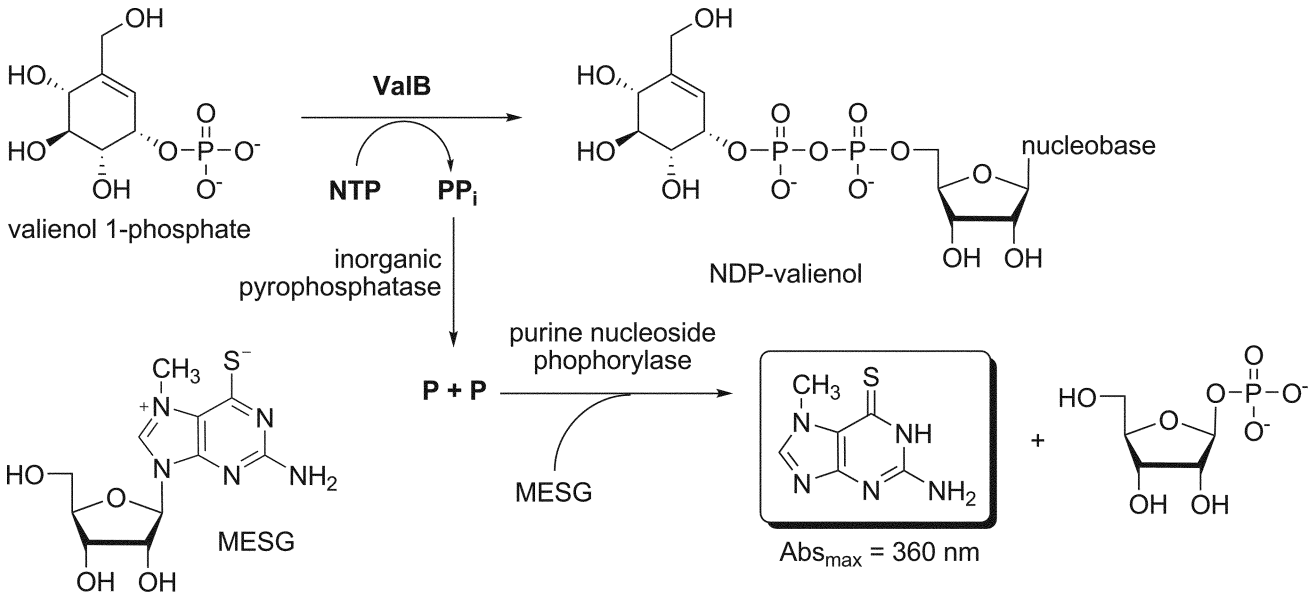


Fig. 2 Preparation and characterization of recombinant ValB. **A**, SDS PAGE Analysis of ValB (M = protein marker, W = whole cell extract, S = soluble protein, P = purified protein). **B**–**C**, Relative activity of ValB based on coupled colorimetric assays using four different valienol phosphate substrates (B) and five different NTPs (C). **D**–**E**, MS analysis of ValB using four different valienol phosphate substrates (D) and five different NTPs (E).



Scheme 3 Coupled colorimetric assays of ValB. Enzymatic conversion of MESG to ribose 1-phosphate and 2-amino-6-mecapto-7-methylpurine results in a shift in absorbance maximum from 330 nm to 360 nm.

absorbance maximum from 330 nm for the substrate to 360 nm for the product.

The substrate specificity of ValB was then reevaluated using this assay, in which **1a**, **1b**, **2a**, and **2b** were tested in the presence of GTP. After 1 h incubation, EnzChek solution was added and the reaction mixtures were incubated in a 96-well plate for 30 min at 22 °C. The change in absorbance at 360 nm was recorded in triplicate. Consistent with the preliminary results, the colorimetric assay and MS provided compelling evidence that **1a** is the preferred substrate for ValB (Fig. 2B and 2D).

Utilization of various nucleotidyltriphosphates by ValB

BLAST analysis of the amino acid sequence of ValB revealed that besides having high sequence identities with other putative carbasugar nucleotidyltransferases, AcbR (from the acarbose pathway in *Actinoplanes* sp., 64%), GagR (from the acarbose pathway in *Streptomyces glaucescens*, 67%), and SalF (from the salbostatin pathway in *Streptomyces albus*, 68%), ValB is also similar to the glucose 1-phosphate adenylyltransferases (GlgC, 27–41%). The result was inconsistent with the report by Kim and co-workers, as it indicated that VldB is a uridylyltransferase enzyme.[13] To identify the preferred nucleotidyl donor(s) in ValB reaction, the enzyme was incubated with valienol 1-phosphate in the presence of different NTPs. Colorimetric assay of the products indicated that ValB more efficiently utilized the purine-based nucleotidyltriphosphates, ATP and GTP, than pyrimidine-based NTPs as nucleotidyl donor (Fig. 2C). These results were unambiguously supported by strong signals for both ADP-valienol (*m/z* 583.93) and GDP-valienol (*m/z* 599.93) products in the mass spectra (Fig. 2E). Interestingly, ValB also used CTP as nucleotidyl donor, converting valienol 1-phosphate to CDP-valienol (*m/z* 559.93). On the other hand, only low-level activity of ValB was observed in the colorimetric assay with dTTP or UTP. However, the amounts of the products seemed to be below the MS-detectable levels.

Metal ion cofactors for ValB

Most sugar 1-phosphate nucleotidyltransferases require metal ions for their activity.[25,26] To assess the metal ion dependence of ValB activity, the reactions were carried out using EDTA-treated protein in the presence of various metal ions. However, light absorption at 360 nm by some metal ions precluded the use of the colorimetric assay for these experiments. Therefore, TLC analysis with detection under UV light at 254 nm (Fig. 3A) and MS (Fig. 3B) were used to analyze the reaction products. The results confirmed that metal ions are indeed essential for ValB activity, as reactions using EDTA-treated ValB alone did not give any product at a detectable level. Of eight metal ions tested, Mg^{2+}, Mn^{2+}, and Co^{2+} were able to rescue the activity of the EDTA-treated enzyme, whereas Fe^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+}, and Ni^{2+} were inactive (Fig. 3). The results suggest that ValB adopts a similar metal ion requirement as sugar 1-phosphate nucleotidyltransferases commonly found in biological systems.

Discussion

Sugar nucleotides are ubiquitous in Nature. They play critical roles in primary and secondary metabolism as substrates for many important enzymes, particularly the glycosyltransferases. They are normally derived from sugar 1-phosphates and NTPs catalyzed by sugar 1-phosphate nucleotidyltransferases, also referred to as sugar nucleotide pyrophosphorylases (EC 2.7.7.-). In bacteria and plants, sugar 1-phosphate nucleotidyltransferases are involved in the biosynthesis of starch, peptidoglycans, and lipopolysaccharides, and their participation in natural product biosynthesis has been fully appreciated. In addition, many genes encoding sugar 1-phosphate nucleotidyltransferases have been found in natural products biosynthetic gene clusters, including those of the C_7N-aminocyclitol natural products, such as acarbose,[11] validamycin,[16] and salbostatin.[27] However, their roles in the biosynthesis of these compounds had not been carefully studied.

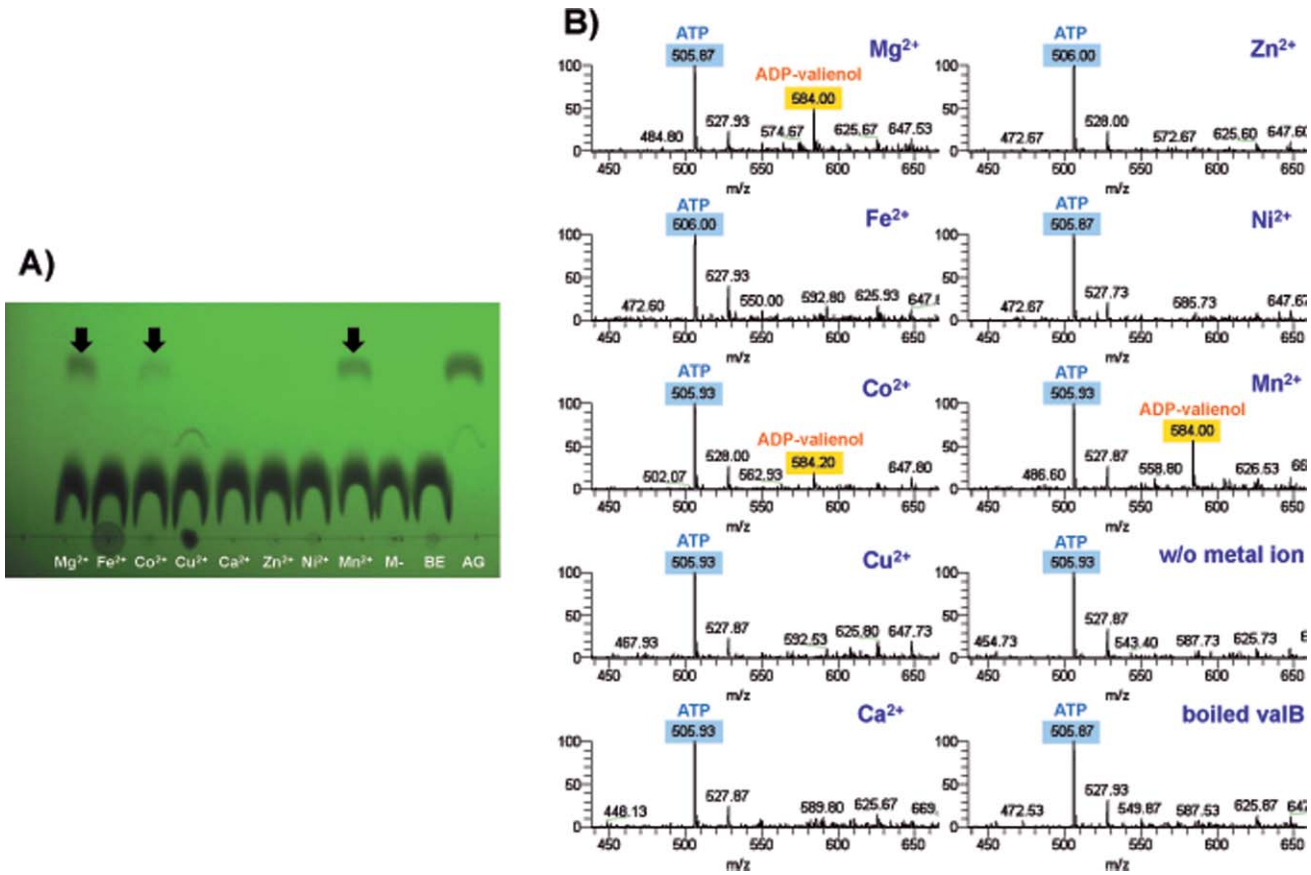


Fig. 3 Identification of metal ion cofactors for ValB. **A**, TLC analysis of ValB products with different metal ions under UV light at 254 nm (M-, with no metal ion; BE, with boiled enzyme; AG, ADP-glucose standard; thick arrows show ADP–valienol products). **B**, MS analysis of ValB products with different metal ions. All reaction mixtures contained EDTA.

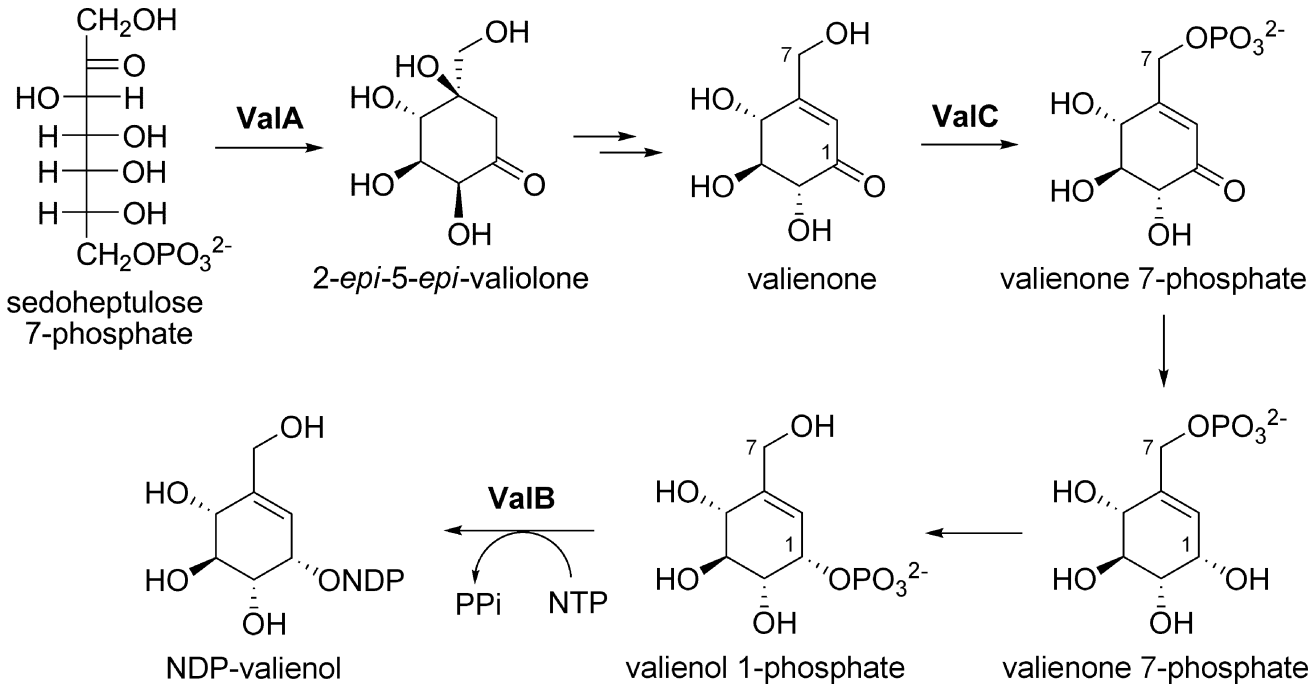
Whereas VldB, a ValB homologue (99% identity) from another validamycin producing strain, was reported to serve as a glucose 1-phosphate uridylyltransferase,[13] results from our *in vitro* and *in vivo* studies suggest otherwise. First, ValB, while able to recognize glucose 1-phosphate as substrate, efficiently converts valienol 1-phosphate to its corresponding nucleotidyl derivatives. Interestingly, the 1-*epi*- and the 1,7-diphosphate-analogues are not preferred substrates. Secondly, ValB most efficiently utilizes ATP and GTP, instead of UTP, as nucleotidyl donor. This, in fact, is consistent with the relatively high sequence identity between ValB (also VldB) and the glucose 1-phosphate adenylyltransferases (GlgC). Furthermore, inactivation of *valB* in *S. hygroscopicus* 5008 abolished the production of both validoxylamine A and validamycin A, which provided compelling evidence for the critical role of ValB in validoxylamine A biosynthesis, and not in the conversion of validoxylamine A to validamycin A. In other words, ValB is most likely responsible for the synthesis of ADP- or GDP-valienol, setting the stage for a coupling reaction leading to the bis-cyclitol core unit formation. In addition, our previous study has shown that the glycosyltransferase ValG, which catalyzes the conversion of validoxylamine A to validamycin A, most efficiently uses UDP-glucose, not ADP- or GDP-glucose, as sugar donor.[16,17] Therefore, as ValB prefers ATP and GTP as nucleotidyl donor, it is less likely that this enzyme plays a critical role in supplying UDP-glucose for ValG reaction.

Viewed together, the present study not only clarifies the catalytic function and role of ValB in validamycin A biosynthesis but also provides insight into the pathway directly upstream of valienol 1-phosphate (Scheme 4). It now can be postulated that the conversion of valienone 7-phosphate, the product of the cyclitol kinase ValC,[28] to valienol 1-phosphate would involve a stereoselective reduction of C-1 followed by phosphate transfer from C-7 to C-1. The latter step may be catalyzed by a phosphohexomutase, possibly ValO.

As a valienol 1-phosphate nucleotidyltransferase, ValB represents the first member of the unsaturated carbasugar nucleotidyltransferase family involved in natural product biosynthesis. Other members of this class of enzymes may include AcbR, GacR, and SalF.[11,14,27] Although AcbR, GacR, and SalF have been annotated as putative 1-*epi*-valienol 1,7-diphosphate adenylyltransferases, their natural substrate may very likely be the same as that of ValB. Therefore, careful characterization of those enzymes is warranted to reveal their actual role in acarbose and salbostatin biosynthesis.

Conclusion

The present study provides compelling evidence for the critical role of ValB in validamycin biosynthesis. ValB catalyzes the conversion of the unsaturated carbasugar phosphate valienol 1-phosphate to NDP-valienol. This enzyme requires Mg^{2+}, Mn^{2+}, or Co^{2+} for



Scheme 4 Biosynthetic pathway to NDP-valienol. Conversion of valienone 7-phosphate to valienol 1-phosphate requires stereoselective reduction of C-1 ketone and transfer of the phosphate group.

its activity and utilizes the purine-based nucleotidyltriphosphates ATP and GTP more efficiently than the pyrimidine-based NTPs (CTP, dTTP, and UTP) as nucleotidyl donor. ValB represents the first characterized unsaturated carbasugar nucleotidyltransferase involved in natural products biosynthesis. Members of this class of enzymes may include AcbR/GacR, and SalF from the acarbose and the salbostatin pathways, respectively.[11,14,27] Results of the present study not only expand our understanding of the biosynthesis of carbasugar-derived natural products, but may also facilitate the development of new tools for chemoenzymatic synthesis of carbohydrate mimetics.

Experimental

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 gel 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) column. Optical rotations were measured on a Jasco P1010 polarimeter (100 mm cell was used) at the sodium D line. Proton NMR spectra were recorded on Bruker 300 or 400 MHz spectrometers. Proton chemical shifts are reported in 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, br s = broad singlet, d = doublet, br d = broad doublet, t = triplet, q = quartet, m = multiplet; coupling constants are reported in Hz. Carbon NMR spectra were recorded on Bruker 300 (75 MHz) or 400 (100 MHz) spectrometers with complete proton decoupling. Carbon chemical shifts are reported in ppm (δ) relative to the residual solvent signals as the internal standard ($CDCl_3$: δ_C 77.16), or with sodium 2,2-dimethylsilapentane-5-sulfonate (DSS) (δ 0.0) as an external standard. Phosphorus NMR spectra were recorded on a Bruker 300 (112 MHz) spectrometer with complete proton decoupling. Phosphorus chemical shifts are reported in 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 Waters/Micromass LCT spectrometer. Ion-exchange column chromatography was carried out on Dowex 1×8 (Aldrich) and size exclusion chromatography was done on Sephadex LH-20 (Pharmacia). Restriction enzymes, T_4 DNA ligase and Taq polymerase were purchased from various companies (New England Biolabs, Takara, MBI Fermentas, and Toyobo). Synthesis of oligonucleotide primers and DNA sequencing of PCR products were performed by Shanghai Sangon and Invitrogen Co., Ltd. Gel Recovery Kit (Tiangen) was used for DNA purification from agarose gels. TSBY liquid medium (per litre: TSB 30 g, yeast extract 10 g, sucrose 103 g [pH 7.2]) was used for the growth of mycelia and the isolation of total DNA. SFM medium (2% agar, 2% mannitol, 2% soybean powder [pH 7.2]) was used for sporulation and conjugation. YMG liquid medium (0.4% yeast extract, 1% malt extract, 0.4% glucose [pH 7.3]) was used for the fermentation of *S. hygroscopicus* 5008 and its derivatives. For *Streptomyces*, apramycin and thiostrepton were used at 30 μg ml^{-1} and 25 μg ml^{-1}, respectively, both in SFM agar and in liquid media. *E. coli* strains were cultured as described elsewhere.[29]

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table S1.† *S. hygroscopicus* 5008, the wild-type producer of

validamycin, was used for validamycin isolation, bioassay, and generation of mutant strains. *E. coli* DH10B was used as the cloning host, and *E. coli* BW25113 was used as the host for λ-Red-mediated recombination.[30] pMD-T vector (Takara) was used for the cloning of PCR amplified fragments. pHZ1358 (an *E. coli* and *Streptomyces* shuttle vector) was used for gene replacement in 5008.[31] pJTU968[32] was used as a transitional cloning vector. pPM927 was used for mutant complementation.[33]

Construction of pJTU702 for targeted replacement of a 936 bp DNA fragment internal to *valB*

A 5.8 kb BamHI fragment from pHZ2229 was cloned into the BamHI site of pHZ1358 to generate pJTU700. Using the gel-purified 1384-bp EcoRI/HindIII fragment from pIJ773 as template,[30] a 1.4 kb disruption cassette was obtained by PCR amplification with the primers ValB-PCR-F (5′-ATGGGGCCGCTGGGACGCGGCAGGCTCAAGCCGCTG-GTGattccggggatccgtcgacc-3′, nucleotides of pIJ773 are in lowercase) and ValB-PCR-R (5′- GATGGCCGACGCCAGGT-GTGTTCCGTCCGGTACCTGGGCtgtaggctggagctgcttc -3′, nucleotides of pIJ773 are in lowercase). Amplification was performed with Taq DNA polymerase in a 50 μl reaction with 100 ng template DNA, 10 mM dNTPs, 50 pmol each primer, and 5% DMSO in a thermocycler (BioRad). After initial denaturation at 94 °C for 2 min, 10 cycles were performed with denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 90 s, followed by 15 cycles with the annealing temperature increased to 55 °C. A last elongation step was done at 72 °C for 5 min. This fragment was used to replace the 936 bp DNA internal to *valB* in pJTU700 through the λ-Red-mediated recombination. The resulting plasmid pJTU702 was used for targeted replacement of a 936 bp DNA fragment internal to *valB* with the 1.4 kb *oriT-aac(3)IV* cassette in the wild-type strain 5008. Oligonucleotide primers ValB-Det-F (5′-TGCTCCACCTGCCTGTA-3′) and ValB-Det-R (5′-CACCTCTTCCCGCTCC-3′) were used for the confirmation of the *valB* mutant. PCR amplification was performed with Taq DNA polymerase under similar conditions. The PCR product was analyzed by gel electrophoresis and enzymatic digestion.

Construction of pJTU918 for the complementation of ZYR-2 with full-length cloned *valB*

A 1.1-kb NdeI/EcoRI fragment from the expression plasmid pJTU707 harboring a full-length *valB* was ligated to the NdeI/EcoRI-digested pJTU968 to give pJTU915. Then the 1.4 kb MfeI/EcoRI fragment, containing *PermE** promoter and *valB*, was cleaved from pJTU915 and ligated into EcoRI-digested pPM927 to give pJTU918. The plasmid was introduced into ZYR-2 through conjugation as previously described.[18] The thiostrepton-resistant exconjugants were selected.

HPLC analysis of validamycin A

For HPLC analysis, the strains were cultured in 50 mL YMG liquid medium in 250 mL baffled flasks at 37 °C and 220 rpm for 6 days. The fermentation broth was acidified with oxalic acid and applied to a Dowex 50 W column (25 mL) as previously reported.[34] The extracts were loaded onto Agilent ZORBAX SB-C18 column (5 μm, 4.6 × 250 mm) for HPLC analysis (Waters 2690). The mobile phase (5 mM sodium phosphate buffer–methanol, 98 : 2, v/v) was applied with the flow rate of 0.6 mL min^{-1} at room temperature. The eluate was monitored at 210 nm with Waters 996 photodiode array detector and the data was analyzed with a Waters Millennium Chromatography Manager.

Synthesis of valienol 1-phosphate, 1-*epi*-valienol 1-phosphate, valienol 1,7-diphosphate, and 1-*epi*-valienol 1,7-diphosphate

Dibenzyl (1*S*,4*R*,5*S*,6*R*)-4,5,6-tris(benzyloxy)-3-[(benzyloxy)-methyl]cyclohex-2-en-1-yl phosphate (9a). A solution of **8a** (740 mg, 1.38 mmol) in CH_2Cl_2 (5 mL) was added to a solution of dibenzyl *N*,*N*-diisopropylphosphoramidite (944 mg, 2.76 mmol) and 1*H*-tetrazole (290 mg, 4.14 mmol) in CH_2Cl_2 (25 mL), which had been stirred for 40 min at room temperature under Ar, and the reaction mixture was further stirred for 4 h at room temperature. The reaction mixture was cooled to −78 °C and *m*-CPBA (595 mg, 3.45 mmol) was added. After the cooling bath was removed, stirring of the reaction mixture was continued for an additional 40 min at room temperature. The solvent was removed using a rotary evaporator, and the residue was redissolved in EtOAc (150 mL). The organic layer was washed with 10% aqueous Na_2SO_3 (100 mL), saturated aqueous $NaHCO_3$ (100 mL), and brine (100 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced vacuum. Column chromatography (silica gel, Hex : EtOAc = 2 : 1) yielded the title compound (963 mg, 88%); $[\alpha]_D^{23}$ +43.2 (*c* 1.0 in $CHCl_3$); δ_H(300 MHz; $CDCl_3$) 3.62 (1 H, ddd, *J* 2.1, 3.3, 9.8), 3.92 (1 H, d, *J* 13.3), 4.12 (1 H, dd, *J* 7.4, 9.7), 4.18 (2 H, bd, *J* 11.2), 4.46 (2 H, dd, *J* 11.9, 14.6), 4.64–4.68 (1 H, m), 4.70 (2 H, d, *J* 11.3), 4.83 (1 H, d, *J* 11.0), 4.88 (1 H, d, *J* 11.5), 4.96 (1 H, d, *J* 11.0), 4.99–5.11 (4 H, m), 5.18–5.23 (1 H, m), 5.89 (1 H, dd, *J* 1.1, 5.7), 7.23–7.41 (30 H, m); δ_C(75 MHz; $CDCl_3$) 69.23 (d, $J_{c\text{-}p}$ 4.8), 69.30 (d, $J_{c\text{-}p}$ 4.8), 70.0, 71.2 (d, $J_{c\text{-}p}$ 5.9), 72.4, 72.9, 74.6, 75.2, 78.3 (d, $J_{c\text{-}p}$ 5.1), 79.8, 80.3, 121.3 (d, $J_{c\text{-}p}$ 2.8), 127.74, 127.76, 127.79, 127.82, 127.85, 128.0, 128.31, 128.34, 128.46, 128.49, 128.55, 128.63, 136.1 (d, $J_{c\text{-}p}$ 7.4), 136.2 (d, $J_{c\text{-}p}$ 7.7), 138.01, 138.05, 138.5, 138.7, 143.1; *m/z* (ESI) 819.3073 ([M + Na]$^+$. $C_{49}H_{49}O_8PNa$ requires 819.3063).

Dibenzyl (1*R*,4*R*,5*S*,6*R*)-4,5,6-tris(benzyloxy)-3-[(benzyloxy)-methyl]cyclohex-2-en-1-yl phosphate (9b). In a manner analogous to the preparation of **9a** from **8a**, **8b** gave **9b** in 80% yield. $[\alpha]_D^{24}$ −42.9 (*c* 1.0 in $CHCl_3$); δ_H(300 MHz; $CDCl_3$) 3.77–3.80 (2 H, m), 3.88 (1 H, d, *J* 12.4), 4.20 (1 H, d, *J* 12.5), 4.30 (1 H, d, *J* 5.3), 4.46 (2 H, dd, *J* 4.9, 11.5), 4.71 (1 H, d, *J* 10.8), 4.79 (1 H, d, *J* 11.1), 4.83 (2 H, d, *J* 10.8), 4.88 (1 H, d, *J* 11.0), 4.95 (1 H, d, *J* 10.8), 4.97–5.03 (4 H, m), 5.07 (1 H, m), 7.20–7.35 (30 H, m); δ_C(75 MHz; $CDCl_3$) 69.36 (d, $J_{c\text{-}p}$ 5.7), 69.45 (d, $J_{c\text{-}p}$ 5.7), 72.6, 75.1, 75.7, 78.87 (d, $J_{c\text{-}p}$ 6.3), 79.7, 82.67 (d, $J_{c\text{-}p}$ 6.5), 83.95 (d, $J_{c\text{-}p}$ 1.7), 124.1, 127.7, 127.8, 127.9, 127.9, 128.0, 128.1, 128.4, 128.5, 128.6, 128.7, 135.88 (d, $J_{c\text{-}p}$ 2.0), 135.97 (d, $J_{c\text{-}p}$ 2.1), 138.1, 138.37, 138.41, 138.44; *m/z* (ESI) 819.3120 ([M + Na]$^+$. $C_{49}H_{49}O_8PNa$ requires 819.3063).

Valienol 1-phosphate (1a). BBr_3 (195 μL of 1.0 M solution in CH_2Cl_2, 0.195 mmol) was added to a solution of **9a** (21 mg, 0.0263 mmol) in CH_2Cl_2, and the reaction mixture was stirred for 45 min at −35 °C. Water (0.7 mL) and MeOH (0.7 mL) were

added to the reaction mixture at the same temperature, and the pH of the solution was adjusted to weak basic using saturated aqueous $NaHCO_3$ solution. The reaction mixture was diluted with H_2O (3 mL) and CH_2Cl_2 (3 mL), and the aqueous layer was further extracted with CH_2Cl_2 (3 mL). The aqueous layer was subjected to a Dowex 1 × 8 anion-exchange column (Cl^-, 1.5 × 7.5 cm), washed with H_2O (30 mL), and the product was eluted with a NaCl gradient (30 mL of 0.1 M, 0.2 M, 0.3 M, and 0.4 M solution). The product fractions were pooled and lyophilized. The resulting white solid was dissolved in H_2O and desalted by passing it through a Sephadex LH-20 column using H_2O as an eluent. The product fractions were combined and lyophilized to give the title compound (4.9 mg, 62%); $[\alpha]_D^{20}$ +62.3 (*c* 0.13 in H_2O); δ_H(300 MHz; D_2O) 3.56 (1 H, dd, *J* 3.8, 9.9), 3.80 (1 H, dd, *J* 7.8, 10.5), 4.02 (1 H, d, *J* 8.1), 4.12 (1 H, d, *J* 14.4), 4.20 (1 H, d, *J* 13.8), 4.66 (1 H, m), 5.91 (1 H, d, *J* 4.5); δ_C(75 MHz; D_2O; DSS) 64.0, 72.2 (d, $J_{c\text{-}p}$ 4.9), 73.3 (d, $J_{c\text{-}p}$ 4.1), 74.8, 75.8, 124.6 (d, $J_{c\text{-}p}$ 2.5), 144.7; δ_P(122 MHz; D_2O; H_3PO_4) 0.27; *m/z* (ESI) 255.0264 ($[M - H]^-$. $C_7H_{12}O_8P$ requires 255.0270).

1-*epi*-Valienol 1-phosphate (1b). In a manner analogous to the preparation of **1a** from **9a**, **9b** gave **1b** in 41% yield. $[\alpha]_D^{20}$ −50.4 (*c* 1.0 in H_2O); δ_H(300 MHz; D_2O) 3.55–3.60 (2 H, m), 4.08 (1 H, d, *J* 14.1), 4.17–4.21 (2 H, m), 4.59 (1 H, m), 5.70 (1 H, s); δ_C(75 MHz; D_2O; DSS) 63.8, 74.4, 77.1 (d, $J_{c\text{-}p}$ 4.0), 78.1, 78.3 (d, $J_{c\text{-}p}$ 4.9), 126.6 (d, $J_{c\text{-}p}$ 3.6), 141.5; δ_P(122 MHz; D_2O; H_3PO_4) 2.85; *m/z* (ESI) 255.0268 ($[M - H]^-$. $C_7H_{12}O_8P$ requires 255.0270).

(1*S*,4*R*,5*S*,6*S*)-4,5,6-Tris(benzyloxy)-3-(hydroxymethyl)cyclohex-2-en-1-ol (10a). $ZnCl_2$ (240 mg, 1.84 mmol) was added to a solution of **8a** (99 mg, 0.184 mmol) in Ac_2O–AcOH (2 : 1; 2 mL), and the reaction mixture was stirred for 2 h at room temperature. The reaction mixture was diluted with water (5 mL) and EtOAc (10 mL), and the organic layer further washed with water (5 mL) and saturated aqueous Na_2CO_3 solution (3 × 5 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced vacuum to give a crude sample. The crude sample was dissolved in MeOH (2.5 mL) and NaOMe (0.8 mL of 30% solution in MeOH) was added to the reaction mixture. The reaction mixture was stirred for 4 h at room temperature and quenched with water (2 mL) and saturated aqueous NH_4Cl (10 mL). The reaction mixture was diluted with EtOAc (5 mL), and the organic layer further washed with water (5 mL) and brine (5 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced vacuum. Column chromatography (silica gel, Hex : EtOAc = 1 : 2) yielded the title compound (51 mg, 62%); $[\alpha]_D^{24}$ +2.3 (*c* 0.23 in $CHCl_3$); δ_H(300 MHz; $CDCl_3$) 1.73 (1 H, t, *J* 6.3), 2.58 (1 H, d, *J* 3.3), 3.60 (1 H, dd, *J* 3.9, 9.2), 4.05–4.13 (3 H, m), 4.16 (1 H, d, *J* 6.9), 4.30 (1 H, q, *J* 4.2), 4.67 (1 H, d, *J* 11.4), 4.69 (1 H, d, *J* 11.7), 4.75 (1 H, d, *J* 11.1), 4.80 (1 H, d, *J* 11.4), 4.83 (1 H, d, *J* 11.1), 4.92 (1 h, d, *J* 11.1 Hz), 5.85 (1 H, dd, *J* 1.5, 4.8), 7.29–7.39 (15 H, m); δ_C(75 MHz; $CDCl_3$) 64.0, 65.2, 73.1, 74.2, 74.9, 79.1, 79.28, 79.33, 124.1, 127.90, 128.1, 128.2, 128.6, 128.70, 128.72, 138.1, 138.3, 138.6, 141.7; *m/z* (ESI) 469.1978 ($[M + Na]^+$. $C_{28}H_{30}O_5Na$ requires 469.1991).

Dibenzyl (1*S*,4*R*,5*S*,6*R*)-4,5,6-tris(benzyloxy)-3-(([bis(benzoxy)-phosphoryl]oxy)methyl)cyclohex-2-en-1-yl phosphate (11a). A solution of **10a** (41 mg, 0.92 mmol) in CH_2Cl_2 (2 mL) was added to a solution of dibenzyl *N*,*N*-diisopropylphosphoramidite (125 mg, 0.367 mmol) and 1*H*-tetrazole (45 mg, 0.643 mmol) in CH_2Cl_2 (4 mL), which had been stirred for 40 min at room temperature under Ar, and the reaction mixture was further stirred for 4 h at room temperature. The reaction mixture was cooled to −78 °C and *m*-CPBA (79 mg, 0.456 mmol) was added. After the cooling bath was removed, stirring of the reaction mixture was continued for an additional 40 min at room temperature. The solvent was removed using a rotary evaporator, and the residue was redissolved in EtOAc (15 mL). The organic layer was washed with 10% aqueous Na_2SO_3 (10 mL), saturated aqueous $NaHCO_3$ (10 mL), and brine (10 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced vacuum. After two successive column chromatography (silica gel, Hex : EtOAc = 1 : 2 and toluene–EtOAc = 3 : 1) the title compound was obtained (58.1 mg, 66%); $[\alpha]_D^{23}$ +26.0 (*c* 1.0 in $CHCl_3$); δ_H(300 MHz; $CDCl_3$) 3.47–3.52 (1 H, m), 3.99–4.07 (2 H, m), 4.40 (1 h, dd, *J* 6.3, 12.6), 4.53–4.61 (3 H, m), 4.64 (1 H, d, *J* 11.1), 4.77 (1 H, d, *J* 10.8), 4.82 (1 H, d, *J* 11.7), 4.91 (1 H, d, *J* 11.1), 4.94–5.04 (8 H, m), 5.04–5.09 (1 H, m), 5.74 (1 h, d, *J* 5.4), 7.20–7.39 (35 H, m); δ_C(75 MHz; $CDCl_3$) 67.0 (d, $J_{c\text{-}p}$ 5.3), 69.30 (d, $J_{c\text{-}p}$ 5.8), 69.36 (d, $J_{c\text{-}p}$ 6.1), 69.51 (d, $J_{c\text{-}p}$ 5.6), 69.56 (d, $J_{c\text{-}p}$ 5.5), 70.8 (d, $J_{c\text{-}p}$ 6.2), 72.5, 74.7, 75.1, 78.1 (d, $J_{c\text{-}p}$ 4.8), 78.8, 80.0, 122.5, 127.75, 127.79, 128.0, 128.06, 128.11, 128.3, 128.4, 128.49, 128.53, 128.58, 128.69, 128.76, 135.79, 135.88, 135.94, 136.04, 136.10, 136.20, 138.0, 138.2, 138.6, 140.5, 140.6; *m/z* (ESI) 989.3180 ($[M + Na]^+$. $C_{56}H_{56}O_{11}P_2Na$ requires 989.3196).

(1*R*,4*R*,5*S*,6*S*)-4,5,6-Tris(benzyloxy)-3-(hydroxymethyl)cyclohex-2-en-1-ol (10b). In a manner analogous to the preparation of **10a** from **8a**, **8b** gave **10b** in 78% yield. $[\alpha]_D^{20}$ −77.6 (*c* 1.0 in $CHCl_3$); δ_H(300 MHz; $CDCl_3$) 1.90 (1 H, t, *J* 6.3), 2.14 (1 H, d, *J* 4.2), 3.55 (1 H, dd, *J* 7.2, 9.9), 3.86 (1 H, dd, *J* 7.2, 9.9), 4.08 (2 H, d, *J* 5.7), 4.33 (2 h, m), 4.72 (1 H, d, *J* 11.1), 4.73 (1 H, d, *J* 11.4), 4.82 (1 H, d, *J* 11.1), 4.88 (1 H, d, *J* 11.1), 4.98 (2 H, d, *J* 11.1), 5.67 (1 H, s), 7.28–7.38 (15 H, m); δ_C(75 MHz; $CDCl_3$) 64.0, 71.5, 74.8, 75.2, 75.3, 80.5, 83.8, 84.2, 126.7, 127.97, 128.04, 128.13, 128.16, 128.19, 128.3, 128.67, 128.75, 128.83, 138.1, 138.29, 138.34, 138.5; *m/z* (ESI) 469.2010 ($[M + Na]^+$. $C_{28}H_{30}O_5Na$ requires 469.1991).

Dibenzyl (1*R*,4*R*,5*S*,6*R*)-4,5,6-tris(benzyloxy)-3-(([bis(benzyloxy)phosphoryl]oxy)methyl)cyclohex-2-en-1-yl phosphate (11b). In a manner analogous to the preparation of **11a** from **10a**, **10b** gave **11b** in 70% yield. $[\alpha]_D^{23}$ −33.0 (*c* 1.0 in $CHCl_3$); δ_H(300 MHz; $CDCl_3$) 3.65–3.78 (2 H, m), 4.21 (1 H, bd, *J* 7.2), 4.41 (1 H, dd, *J* 5.9, 12.6), 4.56 (1 H, d, *J* 8.4), 4.62 (1 H, d, *J* 10.8), 4.73 (1 H, d, *J* 11.1), 4.78 (1 H, d, *J* 11.1), 4.79 (1 H, d, *J* 11.4), 4.83 (1 H, d, *J* 11.4), 4.92 (2 H, d, *J* 12.0), 4.97–5.03 (8 H, m), 7.19–7.33 (35 H, m); δ_C(75 MHz; $CDCl_3$) 69.35, 69.49, 69.53, 69.57, 69.61, 75.2, 75.5, 75.6, 78.4 (d, $J_{c\text{-}p}$ 6.3), 78.7, 82.4 (d, $J_{c\text{-}p}$ 6.4), 83.9, 125.3, 127.7, 127.86, 127.94, 127.96, 128.05, 128.06, 128.11, 128.45, 128.56, 128.59, 128.67, 128.72, 128.77, 135.8, 135.9, 136.4, 136.5, 138.1, 138.27, 138.31; *m/z* (ESI) 989.3163 ($[M + Na]^+$. $C_{56}H_{56}O_{11}P_2Na$ requires 989.3196).

Valienol 1,7-diphosphate (2a). BBr_3 (437 μL of 1.0 M solution in CH_2Cl_2, 0.437 mmol) was added to a solution of **11a** (47 mg, 0.049 mmol) in CH_2Cl_2, and the reaction mixture was stirred for 90 min at −50 °C. Water (1.0 mL) was added to the reaction mixture at the same temperature, and the pH of the solution was adjusted to weak basic using saturated aqueous $NaHCO_3$ solution. The reaction mixture was diluted with H_2O (5 mL) and CH_2Cl_2

(5 mL), and the aqueous layer was further extracted with CH_2Cl_2 (5 mL). The aqueous layer was subjected to Dowex 1 × 8 anion-exchange column (Cl^-, 2.5 × 8.0 cm), washed with H_2O (80 mL), and the product was eluted with a NaCl gradient (80 mL of 0.1 M, 0.2 M, 0.3 M, and 0.4 M solution). The product fractions were pooled and lyophilized. The resulting white solid was dissolved in H_2O and desalted by passing it through a Sephadex LH-20 column using H_2O as an eluent. The product fractions were combined and lyophilized to give the title compound (3.1 mg, 19%); $[\alpha]_D^{25}$ +57.3 (*c* 0.12 in H_2O); δ_H(300 MHz; D_2O) 3.76 (1 H, bd, *J* 10.8), 3.89–3.96 (1 H, m), 4.21 (1 H, d, *J* 7.8), 4.49 (1 H, dd, *J* 12.9), 4.63 (1 H, dd, *J* 13.2, 10.2), 4.85 (1 H, m), 6.12 (1 H, d, *J* 4.2); δ_C(75 MHz; D_2O; DSS) 67.5 (d, $J_{c\text{-}p}$ 4.7), 72.8 (d, $J_{c\text{-}p}$ 6.2), 73.1 (d, $J_{c\text{-}p}$ 5.5), 74.4, 75.3, 125.3, 143.0 (d, $J_{c\text{-}p}$ 6.4); δ_P(122 MHz; D_2O; H_3PO_4) 1.22, 1.23; *m/z* (ESI) 334.9946 ([M – H]$^-$. $C_7H_{13}O_{11}P_2$ requires 334.9933).

1-*epi*-Valienol 1,7-diphosphate (2b). In a manner analogous to the preparation of **2a** from **11a**, **11b** gave **2b** in 53% yield. $[\alpha]_D^{25}$ –39.1 (*c* 0.07 in H_2O); δ_H(300 MHz; D_2O) 3.60–3.72 (2 H, m), 4.38 (1 H, dd, *J* 6.6, 13.8), 4.51–4.57 (1 H, m), 4.67 (1 H, m), 5.86 (1 H, s); δ_C(75 MHz; D_2O; DSS) δ 67.2 (d, $J_{c\text{-}p}$ 4.6), 74.0, 77.3 (d, $J_{c\text{-}p}$ 3.8), 77.9, 78.3 (d, $J_{c\text{-}p}$ 5.0), 128.1 (d, $J_{c\text{-}p}$ 4.4), 138.9 (d, $J_{c\text{-}p}$ 6.3); δ_P(122 MHz; D_2O; H_3PO_4) 0.96, 1.38; *m/z* (ESI) 334.9928 ([M – H]$^-$. $C_7H_{13}O_{11}P_2$ requires 334.9933).

Cloning and expression of the *valB* gene in *E. coli*. The 1.1-kb *valB* gene was amplified by PCR (primers: ValB-F, 5′-GGATCCACATATGGACGGAGTGCGTGCC-3′, ValB-R, 5′-GAATTCACAGCGCCACCTCTTCCCGCTC-3′) and ligated to pMD T vector (Takara) and sequenced. The confirmed gene was cut out with BamHI and EcoRI and inserted into pRSET B expression vector (Invitrogen). The *valB* plasmid DNA was introduced into *E. coli* BL21 Gold(DE3)/pLysS by heat-pulse transformation. The mixture was then plated onto LB agar plates containing ampicillin (100 μg mL^{-1}) and chloramphenicol (25 μg mL^{-1}), and incubated at 37 °C for overnight. Single colonies were transferred into test tubes containing LB medium with the above antibiotics and incubated on a rotary shaker (220 rpm) at 37 °C for overnight. Seed cultures in LB medium (10 mL) containing the antibiotics were inoculated with 100 μL of the test tube culture and were grown at 37 °C for overnight. The production cultures in LBBS medium (50 mL) containing the antibiotics were inoculated with seed cultures (5 mL), and were incubated at 37 °C to an OD600 of 0.60. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.2 mM) was then added, and the incubation was continued at 28 °C to an OD600 of 1.04. The cells were harvested by centrifugation at 5000 rpm for 15 min and stored frozen at –80 °C until further use.

Preparation of cell-free extracts and purification of His_6-tagged ValB. Cells were thawed and resuspended in disruption buffer (20 mM Tris buffer pH 8, 300 mM NaCl, 10 mM imidazole). The suspension was sonicated three times for 20 s each, and cell debris was removed by centrifugation at 14,000 rpm for 10 min. The protein solution was applied to a Ni-NTA column and centrifuged at 5000 rpm for 7 s. The column was washed three times with washing buffer (20 mM Tris-HCl pH 8, 300 mM NaCl, 10 mM imidazole). The ValB protein was eluted with elution buffer (20 mM Tris-HCl pH 8, 300 mM NaCl, 200 mM imidazole) and dialyzed for 14 h against 1 litre of dialysis buffer (60 mM Tris-HCl pH 8, 10 mM $MgCl_2$, and 0.05 mM DTT).

Enzymatic assays. The ValB enzymatic reaction was assayed by measurement of inorganic pyrophosphate (PP_i) production, which was detected by the coupled colorimetric assay using the EnzChek Pyrophosphate Assay Kit (Molecular Probes). The purified ValB protein solution (20 μL, 0.35 mg mL^{-1}) was incubated with different valienol phosphates (0.2 mM), NTPs (0.2 mM), $MgSO_4$ (0.2 μL), and Tris·HCl pH 7.5 buffer (25 μL) in a 50 μL total volume for 30 min. ValB enzymatic reaction solution was added to the EnzChek solution (150 μL) in a 96-well plate. The plate was then immediately placed into a spectrophotometric microplate reader and incubated for 30 min at 22 °C. The change in absorbance at 360 nm was obtained in triplicate after subtracting the background absorbance, and P_i contamination in valienol phosphates and NTPs.

Enzymatic assays for electrospray mass spectroscopy and TLC analyses were carried out using ten times concentrations of substrates and metal ions. The mixtures were incubated for 1 h at 30 °C and the products were checked by TLC (254 nm) and mass spectroscopy (ESI, negative ion mode).

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