J|A|C|S COMMUNICATIONS

Published on Web 06/23/2009

Identification, Characterization, and Bioconversion of a New Intermediate in Valanimycin Biosynthesis

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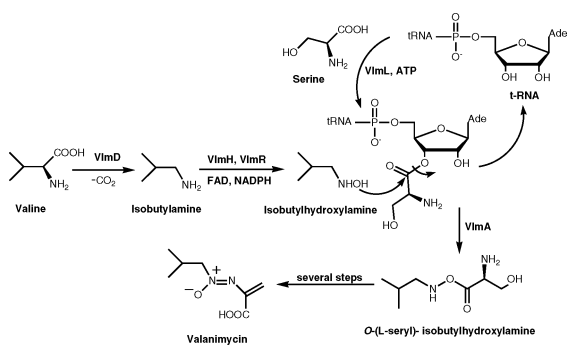
The antibiotic valanimycin is a naturally occurring azoxy compound isolated from the fermentation broth of *Streptomyces viridifaciens* MG456-hF10.[1] Enzymatic and genetic investigations have led to the cloning of the valanimycin gene cluster, which was found to contain 14 genes (Figure S3 in the Supporting Information).[2] The functions of seven of these genes have now been established.[2,3] VlmF, which is a member of the major facilitator family of transport proteins, confers valanimycin resistance. VlmD, VlmH, and VlmR catalyze the conversion of L-valine into isobutylhydroxylamine, while VlmL catalyzes the formation of L-seryl-tRNA from L-serine. Recently, VlmA has been shown to catalyze the transfer of L-serine from L-seryl-tRNA to isobutylhydroxylamine to produce *O*-(L-seryl)isobutylhydroxylamine. Finally, VlmI has been found to be a *Streptomyces* antibiotic regulatory protein (SARP) that is a positive regulator of valanimycin biosynthesis.[2d] These studies and the results from precursor incorporation experiments[4] have allowed the formulation of the biosynthetic pathway for valanimycin shown in Scheme 1. The remaining uncertainties in the valanimycin pathway involve the steps required to convert *O*-(L-seryl)isobutylhydroxylamine into valanimycin. Sequence analysis of the valanimycin biosynthetic genes provides relatively few clues to the nature of the later stages of the pathway. Two exceptions are provided by the *vlmJ* and *vlmK* genes. The translation product of *vlmJ* exhibits similarity to diacylglycerol kinases, while the translation product of *vlmK* exhibits a weak similarity to the MmgE/PrpD superfamily of proteins. This superfamily includes 2-methylcitrate dehydratase, an enzyme required for propionate catabolism. In this communication, we provide evidence supporting the hypothesis that *vlmJ* and *vlmK* play a role in the final stages of valanimycin biosynthesis.

Scheme 1



Previous investigations have shown that washed cells of *S. viridifaciens* efficiently incorporate labeled serine into valanimycin.[4b] Accordingly, L-[U-^{14}C]serine was administered to washed cells of *vlmJ* and *vlmK* mutants of *S. viridifaciens* along with L-valine to stimulate valanimycin production.[5] After 24 h, the supernatants were acidified to pH 3, saturated with sodium chloride, and extracted with ethyl acetate. TLC analysis of the concentrated ethyl acetate extracts revealed the presence of an unknown metabolite, **1**, that could be visualized by autoradiography (Figure 1A). When a similar experiment was carried out with a *vlmH* mutant,[3c] which cannot form isobutylhydroxylamine, the metabolite was absent.[6] This suggested that the metabolite was related to the valanimycin pathway. Preliminary characterization of this metabolite was carried out by NMR analysis of the crude compound produced by administration of L-valine and L-serine labeled with combinations of ^{13}C and ^{15}N to washed cells (entries 1−3 of Table S1 in the Supporting Information). In each of these experiments, a ^{13}C resonance was observed at 64.7 ppm with multiplicities arising from coupling to ^{15}N. A DEPT NMR experiment confirmed that this resonance was due to a CH moiety. The ^{15}N chemical shift data observed for the metabolite were consistent with the presence of an azoxy group.[7] Additional support for the presence of an azoxy group in the unknown species was provided by comparison with the NMR data for valanimycin biosynthesized from L-(^{15}N)valine and L-(2-^{13}C,^{15}N)serine (Table S1, entry 4).

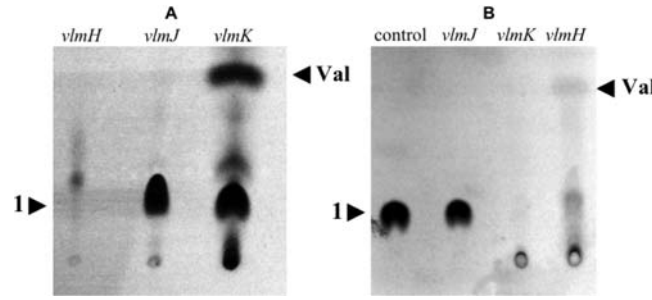


Figure 1. (A) TLC analysis of extractable metabolites produced by washed cells of *S. viridifaciens vlmH*, *vlmJ*, and *vlmK* mutants after administration of L-serine and L-valine. (B) TLC analysis of extractable metabolites produced by incubation of **1** with cell-free extracts of *vlmJ*, *vlmK*, and *vlmH* mutants. Val = valanimycin. See the Supporting Information for details.

Since preliminary analysis suggested that the unknown metabolite was probably on the valanimycin pathway, the compound was purified by preparative reversed-phase HPLC. Detailed NMR analyses of the purified metabolite using ^{1}H, ^{13}C, ^{1}H−^{1}H COSY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC experiments unequivocally demonstrated that compound **1** corresponds to valanimycin hydrate (Scheme 2, Table 1). Additional support for the assigned structure was provided by high-resolution mass spectrometry, which showed a peak at *m*/*z* 191.1023 ([M + H]$^{+}$; calcd for $C_7H_{15}N_2O_4$, 191.1032).

Once the structure of the metabolite produced by the *S. viridifaciens vlmJ* and *vlmK* mutants had been determined, experi-

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10.1021/ja901243p CCC: $40.75

Table 1. NMR Assignments (ppm) for Valanimycin Hydrate (**1**)[a]

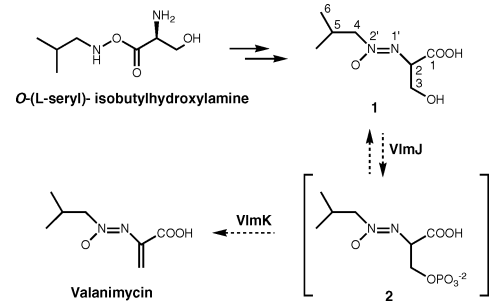
position	^{1}H	^{13}C
1	N/A	171.337
2	4.656 (1H, dd, $^3J_{HH} \approx 4.9$ Hz, $^3J_{HH} \approx 4.9$ Hz)	64.580
3	4.114 (1H, dd, $^2J_{HH} = 11.5$ Hz, $^3J_{HH} \approx 5.0$ Hz)	61.855
	4.095 (1H, dd, $^2J_{HH} = 11.5$ Hz, $^3J_{HH} \approx 5.0$ Hz)	
4	4.104 (1H, dd, $^2J_{HH} = 11.4$ Hz, $^3J_{HH} = 7.2$ Hz)	76.875
	4.134 (1H, dd, $^2J_{HH} = 11.4$ Hz, $^3J_{HH} = 7.8$ Hz	
5	2.479 (1H, nominal nonet, $^3J_{HH} \approx 6.9$ Hz)	27.965
6	1.021 (3H, d, $^3J_{HH} = 6.72$ Hz)	19.638[b]
	1.034 (3H, d, $^3J_{HH} = 6.72$ Hz)	19.485[b]

[a] Measured in $CDCl_3$ with shifts defined relative to TMS. [b] The specific pairwise correlations were not established.

ments were conducted to determine whether **1** is a viable intermediate in valanimycin biosynthesis. HPLC-purified, radiolabeled **1** was incubated with cell-free extracts prepared from an *S. viridifaciens vlmH* mutant. After 16 h, the incubation mixture was acidified and extracted with ethyl acetate, and the concentrated extract was analyzed by TLC on silica gel for the presence of valanimycin. Visualization of the thin-layer chromatogram by autoradiography showed the presence of a compound with the same R_f as authentic valanimycin (Figure 1B). A cell-free extract prepared from an *S. viridifaciens vlmI* mutant failed to catalyze the conversion of radiolabeled **1** into valanimycin. This observation shows that the transformation of **1** into valanimycin in the cell-free extracts requires the presence of valanimycin biosynthetic enzymes. The conversion of **1** into valanimycin by cell-free extracts of the *vlmH* mutant was confirmed by NMR and mass spectral analysis. Attempts to purify the valanimycin from the incubation mixture by standard methods failed because of the low concentration of valanimycin produced in the extract. However, ^{1}H NMR analysis of the unpurified valanimycin showed the presence of signals at 6.357 and 6.380 ppm assignable to the vinyl hydrogen atoms of valanimycin,[8] and an ^{1}H−^{13}C HSQC experiment on the same sample showed that both of these vinyl hydrogens correlate to a ^{13}C resonance at 122.0 ppm, a value that is close to the ^{13}C resonance position for the vinyl CH_2 group of purified valanimycin (120.0 ppm). LC−MS analysis of the crude valanimycin showed the presence of a compound with the same retention time and exact molecular mass as valanimycin: *m/z* 173.0932 ([M + H]$^+$; calcd for $C_7H_{13}N_2O_3$, 173.0926). We therefore conclude that **1** is an intermediate in valanimycin biosynthesis.

Additional insight into the nature of the dehydration reaction was obtained by experiments with cell-free extracts prepared from *vlmJ* and *vlmK* mutants of *S. viridifaciens*. The cell-free extract of the *vlmJ* mutant was unable to convert **1** into valanimycin, suggesting that *vlmJ* is required for the dehydration reaction (Figure 1B). On the other hand, the cell-free extract of a *vlmK* mutant, in which VlmJ was active, appeared to convert **1** into a water-soluble compound that could not be extracted into ethyl acetate. This conversion was dependent upon the addition of ATP to the cell-free extract (Figure S2 in the Supporting Information). Furthermore, high-resolution LC−MS analysis of the crude product formed from **1** and ATP in cell-free extracts of the *vlmK* mutant revealed the presence of a compound whose exact mass corresponds to that of **2**, thereby supporting the hypothesis that VlmJ catalyzes the conversion of **1** to **2** shown in Scheme 2 (see the Supporting Information). ^{13}C NMR analysis of the crude product formed from (2-^{13}C)-**1** and ATP in a *vlmK* cell-free extract also provided additional evidence for the formation of **2** (see the Supporting Information). The excretion of **1** by the *vlmK* mutant can be explained by dephosphorylation of **2** or by feedback inhibition of VlmJ when **2** accumulates in vivo. Precedent for the type of elimination reaction shown in Scheme 2 is found in the mechanism for dehydration of serine residues during lantibiotic biosynthesis.[9]

Scheme 2



Acknowledgment. We thank the National Institutes of Health (GM053818) and the Robert A. Welch Foundation (C-0729) for financial support. We thank Dr. Tan Guo for mass spectral data.

Supporting Information Available: Experimental procedures for the purification of compound **1**, NMR and mass spectral characterization of **1**, bioconversion of **1** in cell-free extracts, and LC−MS and NMR evidence for the formation of **2** from **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA901243P