Supporting Information for

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

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MATERIALS AND METHODS

Culture media were obtained from Becton Dickinson. Reagents used in this study were purchased from Sigma-Aldrich, Roche Applied Sciences, or GE Healthcare. (2-^{13}C)-L-serine, (2-^{13}C, ^{15}N)-L-serine, and (^{15}N)-L-valine were obtained from Cambridge Isotopes, while [U-^{14}C]-L-serine was purchased from PerkinElmer. *O*-Phospho-L-serine was purchased from Sigma-Aldrich. Thin layer chromatography was carried out using Whatman Partisil K6F silica gel plates. Hyperfilm MP (G.E. Healthcare) was used for autoradiography. Most NMR spectra were obtained on Bruker 500 and 800 MHz spectrometers as previously described.[1] In addition, a four-channel Varian Inova 500 MHz spectrometer with a Penta Probe (^{1}H, ^{31}P, and ^{2}H on the inner coil, ^{13}C and ^{15}N on the outer coil) was used to acquire the ^{13}C{^{1}H,^{31}P} spectrum of phosphorylated valanimycin hydrate (see below). NMR measurements of samples containing valanimycin hydrate were carried out at 4 ^{o}C in order to prevent decomposition.

Isolation of valanimycin hydrate (1) from washed cells of *S. viridifaciens vlmJ* and *vlmK* mutants: Sixty ml of valanimycin production medium[2] containing 10 μg/ml of apramycin in a 500 ml wide-mouth conical flask was inoculated with 10 μl of spores of an *S. viridifaciens vlmJ* or *vlmK* mutant[3] and incubated for 14 h at 37 ^{o}C, 250 rpm. The cells were harvested by centrifuging at 3250 x g and washed twice with phosphate buffer (50 mM Na_2HPO_4, 50 mM NaCl, pH 7.0). The pellet was suspended in 30

ml of washed cell medium (50mM Na_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.3% NaCl, pH 7.0). Valine (1 g per liter) and serine (60 mg per liter) variously labeled with stable isotopes were added to the washed cell medium as required for each experiment. When needed, 3 μCi of [U-^{14}C]-L-serine was also added. The cells were further incubated at 30 ^{o}C, 250 rpm. After 24 h of growth, the cells were removed by centrifugation, and the supernatant was adjusted to pH 3.0 with 1N HCl, saturated with NaCl, and extracted three times with 30 ml of ethyl acetate. The ethyl acetate fractions were pooled and concentrated in vacuo to about 1 ml. The crude organic extract was analyzed by tlc on silica gel using $CHCl_3$:MeOH:HOAc (5:1:0.05) as the solvent. Valanimycin hydrate (**1**) was visualized by UV light or by autoradiography. The crude valanimycin hydrate produced by administration of L-valine and L-serine labeled with combinations of carbon-13 and nitrogen-15 was characterized by NMR spectroscopy :

Table S1. NMR data obtained by incorporation of precursors into valanimycin hydrate (**1**)[a] and valanimycin[b]

entry	precursor(s)	observed NMR resonances (ppm)[c]	assignment[d]
1	(2-^{13}C, ^{15}N)-L-serine	^{13}C: 64.7 (d, $^{1}J_{CN}$ = 2.9 Hz) ^{15}N: ca. -43.0 (low S/N)	C-2 N-1'
2	(2-^{13}C)-L-serine, (^{15}N)-L-valine	^{13}C: 64.7 (d, $^{2}J_{CN}$ = 2.5 Hz) ^{15}N: -36.4 (d, $^{2}J_{CN}$ = 2.5 Hz)	C-2 N-2'
3	(2-^{13}C, ^{15}N)-L-serine, (^{15}N)-L-valine	^{13}C: 64.7 (dd, $^{1}J_{CN}$ = 2.9 Hz, $^{2}J_{CN}$ = 2.5 Hz) ^{15}N: -43.3 (dd, $^{1}J_{NN}$ = 14.4 Hz, $^{1}J_{CN}$ = 2.9 Hz) -36.4 (dd, $^{1}J_{NN}$ = 14.4 Hz, $^{2}J_{CN}$ = 2.5 Hz)	C-2 N-1' N-2'
4	(2-^{13}C, ^{15}N)-L-serine, (^{15}N)-L-valine	^{13}C: 141.6 (dd, $^{1}J_{CN}$ = 1.0 Hz, $^{2}J_{CN}$ = 3.6 Hz) ^{15}N: -48.3 (dd, $^{1}J_{NN}$ = 14.7 Hz, $^{1}J_{CN}$ = 1.0 Hz) -39.7 (dd, $^{1}J_{NN}$ = 14.7 Hz, $^{2}J_{CN}$ = 3.6 Hz)	C-2 N-1' N-2'

[a]Entries 1-3.
[b]Entry 4.
[c]Measured in $CDCl_3$. ^{13}C shifts relative to internal TMS. ^{15}N shifts relative to neat nitromethane defined as 0 ppm.
[d]Numbering as in Scheme 2 for valanimycin hydrate and correspondingly for valanimycin.

The multiple isotopomers of valanimycin studied by ^{1}H, ^{13}C, and ^{15}N NMR enable secure ^{15}N chemical shift assignments to be made, which is significant given the previously noted difficulty in unambiguously differentiating the two nitrogen sites in azoxy compounds,[4-6] particularly when their chemical shifts differ

by only a few ppm.[4,5] The ^{15}N chemical shifts in valanimycin and valanimycin hydrate are consistent with the chemical shifts for azoxy compounds reported in the literature,[5,7,8] and they differ significantly from the ^{15}N chemical shifts exhibited by azo, hydrazo, and hydrazone functionalities.

Purification of valanimycin hydrate (1) isolated from washed cells of a *vlmJ* or *vlmK* mutant: A variety of separation conditions were explored to purify **1** from the ethyl acetate extract. The best separation was obtained by HPLC using an Agilent Zorbax SB-C_{18} column (4.6 x 250 mm) with the linear gradient running from 10% acetonitrile containing 0.05% acetic acid to 70% acetonitrile containing 0.05% acetic acid over a period of 20 min. However, the presence of acetic acid caused decomposition of **1** during attempts to recover the purified compound from the solvent mixture. After additional experimentation, it was found that purification could be accomplished by using the 4.6 x 250 mm C_{18} column in combination with a linear gradient running from 10 to 70% acetonitrile in solvents that were saturated with CO_2. Using this system and a flow rate of 1 ml/min, **1** eluted with a 6 min retention time. The purified **1** was characterized by ^{1}H and ^{13}C NMR (see Table 1 in main text).

Bioconversion of 1 using cell-free extracts of *S. viridifaciens*: HPLC-purified **1** was incubated with cell-free extracts prepared from *S. viridifaciens vlmJ*, *vlmK,* and *vlmH* mutants. The cell-free extracts and the *vlmH* mutant were prepared as previously described.[9] A *vlmH* mutant was used for two reasons: 1) it is a stable mutant that is blocked at an early step in the valanimycin pathway, and 2), it does not produce valanimycin. A suspension of wild type or mutant *S. viridifaciens* spores (10 µl) was inoculated into 50 ml of valanimycin production medium[2] containing 10 µg/ml of apramycin and grown for 15 hours at 37 ^{o}C. The cells were divided into ten 5-ml portions and then centrifuged at 3,250 x g. The pellets were stored at –80^{o}C until ready to be used. The cells from 5 ml of broth were suspended in 1 ml of lysis buffer (50mM Tris, pH 7.4, 150 mM NaCl, 4 mg per ml of lysozyme, and one EDTA-free protease inhibitor mini-tablet per 10 ml) and incubated at 37^{o}C for 30 min. A cell-free extract was prepared by

centrifuging the lysed cells at 13,000 x g for 20 min at 4°C. A control incubation was carried out using **1** and no cell-free extract. The typical reaction mixture (40 µl) contained 50 mM HEPES, pH 7.2, 10 mM KCl, 5 mM ATP, 2 mM DTT, 10 mM $MgCl_2$, 1 mg BSA, 10 µl of cell-free extract, and 10 µl of purified, [^{14}C]-labeled **1** (2000 cpm). After 16 h of incubation at 30 °C, 4 µl of 100 mM o-phosphoric acid was added to bring the pH to ca. 2, followed by extraction (4 x) with 20 µl of ethyl acetate. The combined ethyl acetate extracts were concentrated in vacuo and then analyzed by tlc on silica gel using $CHCl_3$:MeOH:HOAc (5:1:0.05) as the solvent. The cell-free extracts prepared from the *vlmH* mutant, converted **1** into a radioactive, UV-absorbing compound with the same R_f as valanimycin. This compound was shown to be valanimycin by LC-MS analysis of the crude ethyl acetate extract on a Bruker MicroTOF mass spectrometer. The LC-MS separation used a 2.1 mm x 50 mm C_{18} column with a linear gradient running from 10% acetonitrile containing 0.05% formic acid to 70% acetonitrile containing 0.05% formic acid over 10 min. The flow rate was 0.2 ml/min. The valanimycin, which eluted after 2.5 min, exhibited the expected molecular mass: HRMS (ESI) m/z 173.0932 $(M+H)^+$, calculated for $C_7H_{13}N_2O_3$, 173.0926.

Additional proof for the presence of valanimycin in the crude ethyl acetate extract was obtained by ^{1}H NMR analyses at 500 and 800 MHz and by a ^{1}H-^{13}C HSQC experiment carried out at 800 MHz . The ^{1}H NMR spectrum in $CDCl_3$ exhibited signals for the vinyl hydrogens of valanimycin at 6.357 and 6.380 ppm, with the more downfield signal clearly broader at both field strengths. The ^{1}H-^{13}C HSQC experiment confirmed that these protons are correlated to a ^{13}C resonance at 122.0 ppm. A comparison of these ^{1}H resonances with the corresponding resonances in purified valanimycin revealed that the proton chemical shift positions displayed by the vinyl CH_2 group of valanimycin are highly sensitive to the concentration of valanimycin. This is illustrated in Table S2 and Figure S1. The data show that the proton chemical shifts exhibited by the vinyl CH_2 group of valanimycin move to lower field as the

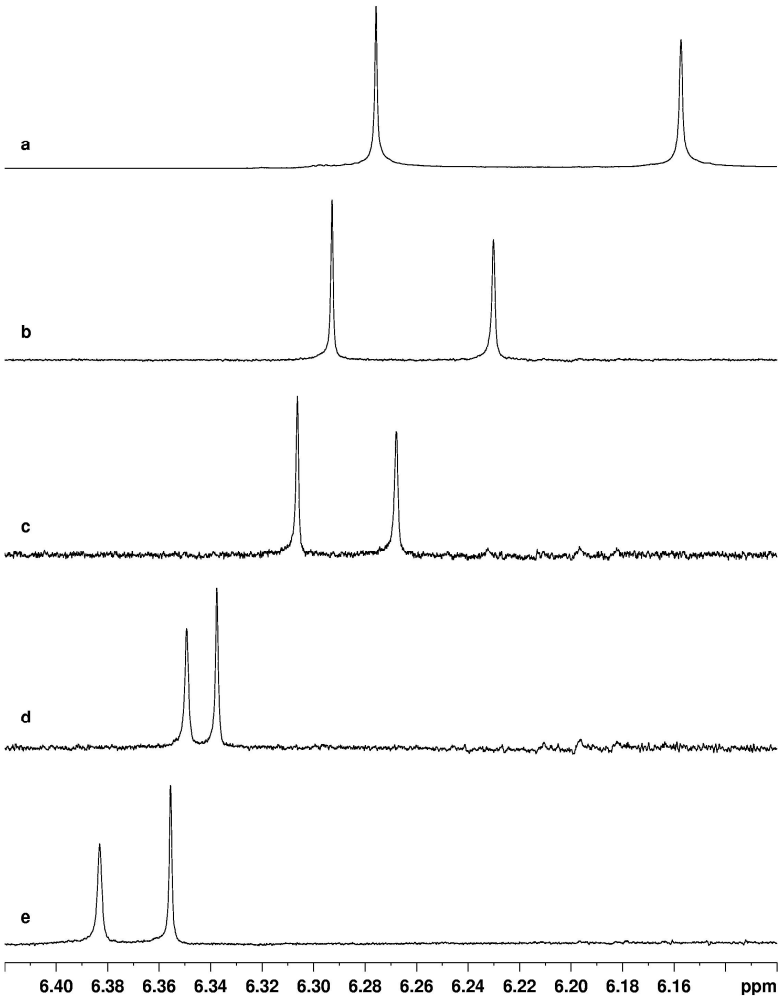
concentration of valanimycin decreases. Furthermore, the broader of the two proton resonances shifts to lower field more rapidly than the sharper proton resonance. The reasons for this behavior are not clear, but the phenomenon is probably dependent upon the degree of ionization of the valanimycin carboxyl group. Since valanimycin is unstable in undiluted form, the concentrations of valanimycin were calculated by using benzyl alcohol as an external standard.

Table S2. Concentration dependence of chemical shifts for vinyl hydrogens in valanimycin

Sample	Valanimycin concentration (μM)	^{1}H Chemical shifts (ppm)[a, b]
1	23.2	6.276 (s), 6.157 (b)
2	0.17	6.293 (s), 6.230 (b)
3	0.05	6.306 (s), 6.268 (b)
4	0.02	6.349 (b), 6.338 (s)

[a]Chemical shifts were measured in $CDCl_3$ containing ca. 2% residual ethyl acetate and are relative to internal TMS
[b]Sharper signal: (s), broader signal: (b)

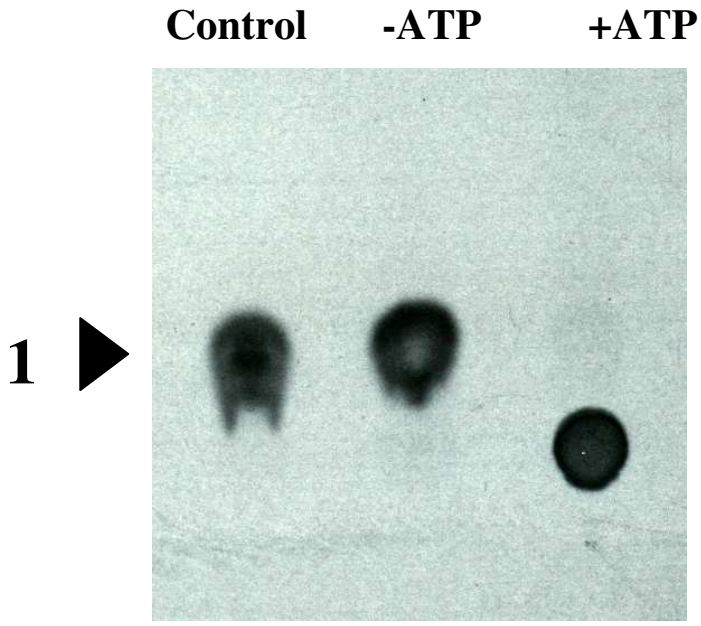
Figure S1. The effect of valanimycin concentration on the $=CH_2$ ^{1}H chemical shifts for solutions of valanimycin in $CDCl_3$ containing about 2% residual ethyl acetate. (a) Purified valanimycin, 23.2 μM. (b) Purified valanimycin, 0.17 μM. (c) Purified valanimycin, 0.05 μM. (d) Purified valanimycin, 0.02 μM. (e) Ethyl acetate extract containing valanimycin prepared by bioconversion of valanimycin hydrate incubated with cell-free extracts. All spectra were obtained at 500 MHz on a broadband inverse probe: 90° pulse, 9.36-s FID, 3-s relaxation delay, with the FID then processed with just 0.05 Hz of line broadening. The $=CH_2$ half-height linewidths consistently differ, e.g.: 0.54 Hz and 0.73 Hz in (a), 0.98 Hz and 0.57 Hz in (e). The number of scans varied from 240 in (a) to 6944 in (e). The very slight differences in the $=CH_2$ chemical shifts observed at 500 MHz (6.356 and 6.383 ppm) and at 800 MHz (6.357 and 6.380 ppm, main text) may result from slightly different probe temperatures.



ATP-dependence of bioconversion of 1 by cell-free extracts of *vlmK* mutant:

[U-^{14}C]- **1** was incubated with cell-free extracts prepared from the *vlmK* mutant in the usual manner in the presence and absence of ATP. A control incubation was also carried out in which the cell-free extract was omitted. After 16h, 10 μl of each incubation mixture was spotted directly onto a silica gel tlc plate, and the plate was developed with $CHCl_3$:MeOH:HOAc, 5:1:0.05. An autoradiograph of the developed tlc plate showed that no alteration of **1** occurred in the absence of added ATP (Figure S2).

Figure S2. Autoradiograph of thin-layer chromatogram (SiO_2, $CHCl_3$:MeOH:HOAc, 5:1:0.05) showing metabolites produced by incubation of [U-^{14}C]-labeled valanimycin hydrate (**1**) under standard conditions with a cell-free extract prepared from the *S. viridifaciens vlmK* mutant in the absence and presence of 5 mm ATP. In the absence of ATP, **1** is unchanged, while in the presence of ATP, **1** is converted into more polar compound(s) that remain at the origin of the tlc plate.



Carbon-13 NMR analysis of product formed from (2-^{13}C)-1 in cell-free extracts of the *vlmK* mutant:

A cell-free extract was prepared from the *S. viridifaciens vlmK* mutant in the usual manner and incubated with 10 µl of (2-^{13}C)-**1.** After 16 h of incubation, the mixture was passed through a spin filter with a 10 kDa molecular weight cutoff, and 1/10 volume of D_2O containing 0.075% TSP was added to the filtrate. The resulting solution was analyzed by carbon-13 NMR. To provide additional information, carbon-13 spectra of commercial *O*-phospho-L-serine were also analyzed. These experiments produced several

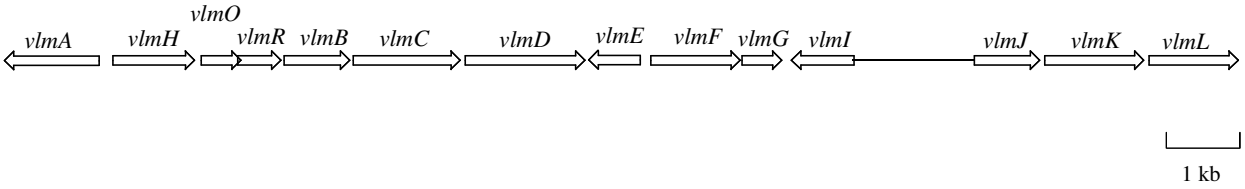
pieces of spectroscopic evidence that support the formation of phosphorylated valanimycin hydrate (**2**) in this cell-free extract:

- The incubation mixture produced from (2-^{13}C)-**1** and a cell-free extract of the *vlmK* mutant exhibits a pair of ^{13}C signals separated by 8.39 Hz and centered at 70.88 ppm in the ^{1}H-decoupled ^{13}C spectrum. This pair of signals collapses to a singlet when ^{1}H decoupling and ^{31}P decoupling are simultaneously applied.[10] Therefore, this pair of signals appears to be a doublet arising from a three-bond ^{13}C-^{31}P coupling that can be attributed to **2**. A value of 8.39 Hz for a $^{3}J_{CP}$ is consistent with a $^{3}J_{CP}$ = 8.68 Hz that was observed for C-2 of *O*-phosphoserine [$(HO)_2$-P(=O)-O-CH_2-$CH(NH_2)(COOH)$] in D_2O (C-2 centered at 56.94 ppm) and with a $^{3}J_{CP}$ = 8.27 Hz observed for C-2 of *O*-phosphoserine in a dummy reaction mixture that included the cell-free extract, but lacked (2-^{13}C)-**1** (C-2 centered at 58.11 ppm).
- The ^{13}C doublet centered at 70.88 ppm was not observed in a control incubation in which (2-^{13}C)-**1** was absent.
- The ^{13}C doublet centered at 70.88 ppm results from a methine carbon, as positive signals are observed in DEPT-135 and DEPT-90 ^{13}C experiments.
- A ^{13}C chemical shift of 70.88 ppm for the C-2 methine carbon of **2** is similar to that for the C-2 methine carbon in the valanimycin ammonia adduct (67.1 ppm).[11]
- A deshielding effect of about 14 ppm on the alpha-carbon would be expected upon converting an amine to an azoxy compound [$CH_3CH_2CH_2CH_2NH_2$ at 42.0 ppm in $CDCl_3$ (unpublished work) vs. (*Z*)-1-methyl-2-(*n*-butyl)diazene-1-oxide -CH_2-N= at 56.7 ppm in $CDCl_3$[12] or (*E*)-1-methyl-2-(*n*-butyl)diazene-1-oxide -CH_2-N= at 55.0 ppm in $CDCl_3$.[12]] In accordance with this expectation, the C-2 methine carbon of **2** (70.88 ppm) resonates 13.9 ppm downfield of the methine carbon in *O*-phosphoserine (56.94 ppm).

LC-MS evidence for the formation of 2 from 1 in cell-free extracts of the *vlmK* mutant:

A cell-free extract was prepared from the *S. viridifaciens vlmK* mutant in the usual manner and incubated with 10 µl of unlabeled **1.** After 16 h of incubation, the mixture was analyzed by high-resolution LC-MS on a Bruker MicroTOF mass spectrometer. The LC separation used a 150 x 2 mm Hilic column (Phenomenex) and a solvent system consisting of 90% acetonitrile, 10% 100 mM ammonium acetate, pH 4.0. The flow rate was 0.3 ml/min. Under these conditions, phosphorylated valanimycin hydrate (**2)** eluted after 83.6 min, and exhibited the expected molecular mass: HRMS (ESI) m/z 271.0698 $(M+H)^+$, calculated for $C_7H_{16}N_2O_7$, 271.0690.

Figure S3. The valanimycin biosynthetic gene cluster of *S. viridifaciens* MG456-hF10.



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(10) The $^{13}C\{^{1}H,^{31}P\}$ experiment used a 90° ^{13}C pulse, 0.8-s FID, and 5-s relaxation delay; 10,752 scans were obtained. The ^{13}C carrier frequency was at 63 ppm; the spectral width was 36 ppm. GARP ^{1}H decoupling was done with an rf decoupling field of 740 Hz and the ^{1}H carrier frequency at 3.74 ppm. GARP ^{31}P decoupling was done with an rf decoupling field of 1050 Hz and the ^{31}P carrier frequency at 2.5 ppm.

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