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The α -Ketoglutarate/Fe(II)-dependent Dioxygenase VldW is Responsible for the Formation of Validamycin B

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Validamycin A (**1**), an antifungal agent used widely as a crop protectant, is the main component of the validamycin complex produced by *Streptomyces hygroscopicus* subsp. *limoneus*.^[1, 2] The antifungal activity of **1** has been attributed to its core structure, validoxylamine A (**4**), which consists of two pseudosugar units, valienamine (**7**) and validamine (**8**) (Scheme 1). Commercially available validamycin usually contains ~60% validamycin A (**1**), ~15% validamycin B (**2**), and other minor analogues. In contrast to **1**, the hydroxylated analogue **2** is significantly less active against fungal pathogens. Therefore, it is desirable to abolish the production of **2** while increasing the yield of **1**. On the other hand, validamycin G (**3**), another hydroxylated analogue of **1**, has great potential to be used as a direct source of valioline (**10**), the precursor of the antidiabetic drug voglibose (**11**). However, the production yield of **3** by *S. hygroscopicus* subsp. *limoneus* is extremely low (0.008% of the crude validamycins),^[3] making it impractical to produce voglibose from this natural product. Efforts to control or improve the production of these hydroxylated validamycins have been hampered by the lack of knowledge of their biosynthesis.

While the biosynthesis of **1** has been studied extensively, the modes of formation of the hydroxylated validamycins were not clearly understood. Early speculations suggested that the formation of **2** and **3** may involve hydroxylation of early cyclitol intermediates in the pathway.^[4] However, no experimental data were available to support that notion. The identification of the biosynthetic gene clusters of validamycin in several strains of *S. hygroscopicus*, e.g., *S. hygroscopicus* subsp. *jinggangensis* 5008 and *S. hygroscopicus* subsp. *limoneus* KCCM 11405 (IFO 12704),^[5, 6] however, provides new opportunities to investigate the modes of formation of these compounds. Direct comparison of the former (the *val* cluster) and the latter (the *vld* cluster) has shown that both clusters share similar sets of genes necessary for the biosynthesis of validamycin A (Figure S1).^[7] However, no candidate genes for the formation of **2** and **3** were identified. To this end, we first investigated two genes within the *val* cluster (*valE* and *valJ*) from *S. hygroscopicus* subsp. *jinggangensis* 5008 that may be involved in the formation of hydroxylated validamycins. ValE and ValJ are homologous enzymes (67% identity) that show high identity to α -ketoglutarate/Fe(II)-dependent dioxygenases, non-heme enzymes that catalyze a variety of oxidative transformations. This family of enzymes catalyze a diverse array of

biotransformations in primary and secondary metabolism, including many bioactive natural products such as penicillin,^[8] clavulanic acid,^[9, 10] viomycin,^[11, 12] morphine,^[13] and flavonoids.^[14] Both ValE and ValJ contain a highly conserved Fe(II) binding HXD/E...H triad motif (Figure S2). However, attempts to express *valJ* in *Escherichia coli* did not yield soluble protein, whereas overexpression of *valE* gave a moderately soluble recombinant protein. However, no catalytic activity of ValE was observed when **1** and **4** were used as substrate (data not shown). Interestingly, while there are two α -ketoglutarate/Fe(II)-dependent dioxygenase genes in the *val* cluster, only one homologous gene, *vldW*, is present in the *vld* cluster. Multiple amino acid sequence alignment of VldW, ValE, and ValJ revealed that the N-terminal sequence of VldW is highly similar to that of ValJ and the C-terminal sequence is more similar to that of ValE (Figure S2), suggesting that VldW may be a hybrid protein originated from ValE and ValJ. Inactivation of *vldW* in *S. hygroscopicus* subsp. *limoneus* has been reported to have no effects in the production of **1**.^[6] However, the study did not show whether this inactivation had any effects to the production of hydroxylated validamycins.

To examine if VldW is involved in the formation of hydroxylated validamycins, we cloned the gene from the chromosome of *S. hygroscopicus* subsp. *limoneus* and the product was inserted into an expression vector pRSET B to give pTMS005. The plasmid was then used to transform *E. coli* BL21(DE3)pLysS. The expression of the gene was induced by isopropyl- β -D-thiogalactopyranoside (IPTG) to give a 43.4 kDa soluble His₆-tagged protein (Figure S3A). Incubation of the enzyme with validamycin A in the presence of α -ketoglutarate and Fe(NH₄)₂(SO₄)₂ showed that the enzyme can catalyze the conversion of **1** (m/z 498 [M+H]⁺) to its hydroxylated product (m/z 514 [M+H]⁺) (Figure S3C–S3E). TLC analysis showed that the product has an R_f value comparable to that of **2** (Figure S3B). However, the results cannot rule out **3** as a possible product, as, due to the lack of an authentic sample, no direct comparison could be made with the latter compound.

To determine the chemical structure of the VldW product, we scaled up the enzymatic reaction and purified the product using ion-exchange resin [DOWEX 1 (OH[−] form)] and gel filtration (Sephadex LH-20) column chromatography. ¹H and ¹³C NMR spectra of the pure compound showed signals identical to those of the authentic **2**, suggesting that VldW is indeed a validamycin B synthase (Figures S4 and S5). In addition, the C-6' methylene protons of the substrate **1** [δ_H 1.39 ppm (brt) and 2.01 ppm (brd)] (Figure S4C) are missing in the VldW product (Figure S4B), indicating that the hydroxylation occurs at the C-6' position. Moreover, DEPT-135 experiments with the product showed that it has only three methylene carbons at around 60 ppm (Figure S5B), which are attributed to the primary alcohol carbons C-7, C-7', and C-6''. All together the data provided convincing evidence that the product of VldW is **2** and there is no indication that **3** is coproduced during the biotransformation. Because there is only one α -ketoglutarate/Fe(II)-dependent dioxygenase gene present in the *vld* cluster in *S. hygroscopicus* subsp. *limoneus*, we speculate that **3** is a shunt product of an unspecific cellular dioxygenase or cytochrome P450 monooxygenase enzyme. In addition, its low yield production may be due to an unfavorable hydroxylation of the less reactive C-5' position.

Previously, it was proposed that the formation of **2** and **3** might involve earlier hydroxylated cyclitol intermediates, e.g., hydroxyvalidamine (**9**) or valioline (**10**). However, no experimental evidence was available to suggest that the hydroxylation occurs early in the pathway. To determine the timing of the hydroxylation reaction and the substrate specificity of VldW, we tested validamine (**8**), validamine 7-phosphate (**12**), validoxylamine A 7'-phosphate (**13**), **4**, and **1** as substrates (Scheme 1). Compounds **8**, **12**, **13**, and **4** were prepared by chemical transformations as reported previously.^[15] Whereas the involvement

of **8** in validamycin biosynthesis is still obscure, **12**, **13**, and **4** have recently been biochemically demonstrated to be involved in **1** biosynthesis.^[15]

As shown in Figure 1, among the compounds tested using cell-free extracts of *E. coli* harboring *vldW*, **1** appears to be the true substrate for VldW. Parallel experiments using cell-free extract of *E. coli* harboring empty vector (pRSET B) did not give any product (Figure S6). VldW is also able to convert **4** (m/z 336 [M+H]⁺) to its hydroxylated derivative (m/z 352 [M+H]⁺) (Figure 1D), albeit much less efficiently than the conversion of **1** to **2** (Figure 1E). It is most likely that **4** is not the natural substrate for VldW. No products were observed when **8**, **12**, or **13** were used as substrate (Figures 1A, 1B, 1C). The results confirm that **2** is derived from **1** and the hydroxylation reaction occurs late in the pathway.

Whereas VldW is a relatively stable enzyme, its catalytic activity is affected by Ni²⁺ ions. A significant reduction of activity was observed when the protein was purified using Ni-NTA column. However, the activity can be restored by dialysis of the protein in a buffer solution containing 0.1 mM EDTA, followed by the addition of 0.2 mM of Fe²⁺ to the protein solution. To determine the optimal conditions for enzyme catalysis, four different buffers (HEPES buffer pH 7.5, MOPS buffer pH 7.5, Tris-HCl buffer pH 7.5, potassium phosphate buffer pH 7.5) were used to incubate VldW and **1**. The results showed that VldW is most active in potassium phosphate buffer (Figure S7A). The hydroxylation of **1** by VldW was found to have a pH optimum at approximately 7.2 (Figure S7B). The kinetics values were determined by using a succinyl-CoA synthetase (SCS), pyruvate kinase (PK) and lactate dehydrogenase (LDH) coupled enzyme assay (Scheme 2).^[16] Oxidation of NADH to NAD⁺ was monitored at 340 nm in 96-well plates using a spectrophotometric microplate reader. The apparent kinetic parameters, obtained from Hanes-Woolf plots, were K_m of 303 ± 36 μ M and 19 ± 3.5 μ M for **1** and α -ketoglutarate, respectively, and a K_{cat} of 0.97 ± 0.14 min⁻¹ (Figure 2).

The present study demonstrated that validamycin B (**2**) is derived from validamycin A (**1**) by the action of VldW, an α -ketoglutarate/Fe(II)-dependent dioxygenase that regioselectively hydroxylates the C-6' position of **1**. The result suggests that inactivation of the *vldW* gene in the producing strains may abolish the production of **2** and **5**, which in turn may lead to an increased overall production of the important crop protectant validamycin A.

Experimental Section

Detailed experimental methods can be found in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Mass spectral analyses of VldW reactions with various substrates. (A) with validamine, (B) with validamine 7-phosphate, (C) with validoxylamine A 7'-phosphate, (D) with validoxylamine A, and (E) with validamycin A.

Figure 2. Kinetic parameters of VldW. (A) Michaelis-Menten curve for validamycin A; (B) Michaelis-Menten curve for α-ketoglutarate.

Scheme 1.

Chemical structures of the validamycins and related compounds.

Scheme 2.

Biochemical characterization of VldW. PEP, phosphoenolpyruvate.