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NOTE

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Involvement of Glutamate Mutase in the Biosynthesis of the Unique Starter Unit of the Macrolactam Polyketide Antibiotic Vicenistatin

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Abstract The macrolactam antibiotic vicenistatin, produced in *Streptomyces halstedii* HC34, is biosynthesized by the polyketide pathway, using a unique 3-methylaspartate-derived molecule as starter unit. The *vinI* gene in the vicenistatin biosynthetic gene cluster encoding glutamate mutase, which rearranges glutamate to 3-methylaspartate, was disrupted. The *vinI* disruption completely abolished the production of vicenistatin, while the disruptant recovered the production of vicenistatin when 3-methylaspartate was added to the culture. These results indicate that *vinI* is essential for the 3-methylaspartate formation in the vicenistatin biosynthesis. Furthermore, the mutant accumulated new vicenistatin derivatives (desmethylvicenistatins), which lacked a methyl group in the starter unit. The desmethylvicenistatins were shown by feeding experiments to be derived from aspartate instead of 3-methylaspartate as the starter unit. These results indicate that the vicenistatin polyketide synthase can accept alternative starter units toward the production of novel polyketides.

Keywords vicenistatin, polyketide, macrolactam, antitumor antibiotic, glutamate mutase, gene disruption

The antitumor antibiotic vicenistatin (**1**), isolated from *Streptomyces halstedii* HC34, is a 20-membered macrolactam aglycone, vicenilactam, with an aminosugar, vicenisamine (Fig. 1) [1]. The characteristic vicenilactam appears to be constructed by a polyketide synthase (PKS) from a unique amino acid starter unit. Feeding experiments using labeled precursors have clearly shown that the crucial starter unit of vicenilactam biosynthesis is derived from glutamate *via* (2*S*,3*S*)-3-methylaspartate [2~4]. It was, thus, proposed that glutamate mutase must be involved in the formation of 3-methylaspartate (Fig. 2). Glutamate mutase is known to consist of two subunits (S and E subunits) and catalyzes the reversible rearrangement of

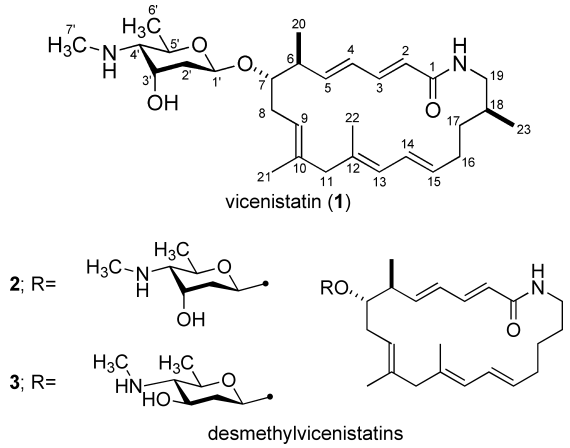

Fig. 1 Structures of vicenistatin and desmethylvicenistatins.

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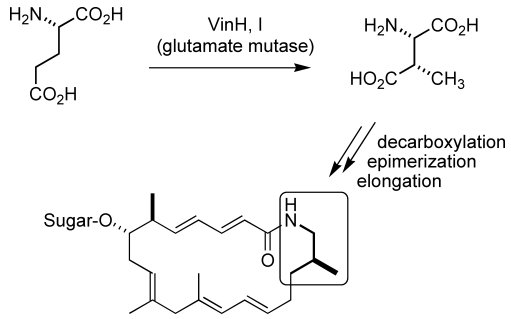

Fig. 2 Involvement of glutamate mutase in the starter unit biosynthesis.

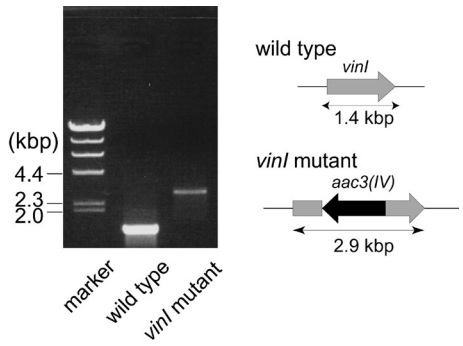

Fig. 3 Electrophoresis of PCR products from wild type and *vinI* mutant with primers designed for the two sides of the *vinI* gene.

glutamate to (2*S*,3*S*)-3-methylaspartate in the mesaconate pathway for glutamate fermentation in various species of Clostridia [5].

More recently, the vicenistatin biosynthetic (*vin*) gene cluster was cloned, sequenced, and analyzed [6]. Homology search analysis showed that VinH and VinI encoded in the *vin* cluster were homologous to the S and E subunits of glutamate mutase, respectively. Thus, it was reasonably explained on the genetic level that glutamate mutase would be involved in the 3-methylaspartate formation in the biosynthesis of vicenistatin. In this paper, we describe the functional confirmation by gene disruption of the *vinI* gene encoding a putative glutamate mutase.

The *vinI* gene was disrupted by insertional inactivation. A 5.6 kbp *SalI* fragment around the *vinI* region was subcloned into *E. coli–Streptomyces* shuttle vector pWHM3 (containing the thiostrepton resistance gene) [7], and the apramycin resistant gene cassette, *aac3*(*IV*) [8], was inserted between two *SphI* sites in the *vinI* coding region. The resulting plasmid pWHM-dGM was introduced into protoplasts of *S. halstedii*. For homologous recombination, the appropriate transformant was sporulated on R5 agar plates for 20 days. The resulting spores were separated into single clones, and the colonies, which showed a phenotype of thiostrepton-sensitive and apramycin-resistant, were picked up. The expected *vinI* gene disruption was confirmed by PCR using primers designed for the two sides of the *vinI* gene. The size of the PCR product from the disruptant genome was 1.5 kbp larger (size of the apramycin resistant gene cassette) than that of the wild type as shown in Fig. 3.

The mutant and wild type of *S. halstedii* were cultured, and the products was extracted with acetone and ethyl acetate as previously described [1]. The resulting extracts were analyzed by HPLC with a Senshu Pak ODS column (PEGASIL ODS 4.6 mm, 250 mm) at 40°C with a solvent of MeOH : water : TFA (70 : 30 : 0.1) at a flow rate of 1 ml/minute, and the elution was monitored at 254 nm. As expected, the resulting *vinI* disruptant did not produce vicenistatin at all, while the wild-type of *S. halstedii* produced vicenistatin under the same conditions (Fig. 4A, 4C). On the other hand, by feeding of 3-methylaspartate to the culture of the *vinI* mutant, the vicenistatin production

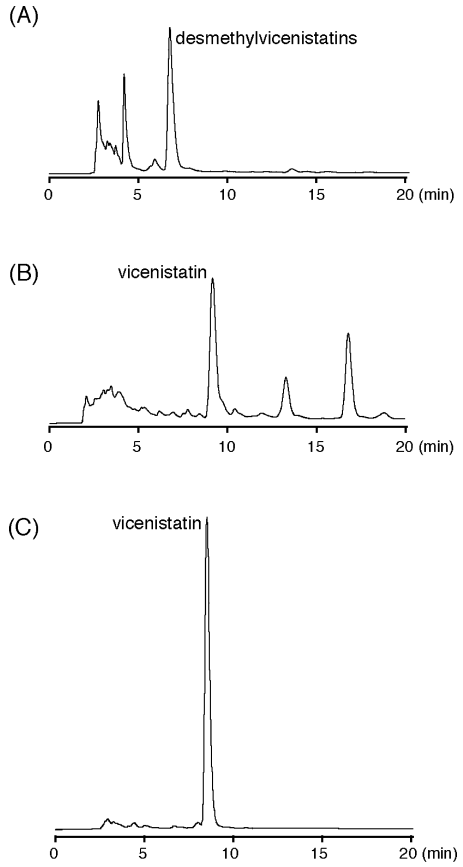

Fig. 4 HPLC profiles of products from (A) *vinI* mutant, (B) *vinI* mutant supplemented with 3-methylaspartate, and (C) wild-type.

Table 1 125 MHz ^{13}C-NMR and 500 MHz ^{1}H-NMR spectral data of vicenistatin (**1**), 18-desmethylvicenistatin (**2**) and 3′-epi-18-desmethylvicenistatin (**3**) in CD_3OD

Position	Vicenistatin		18-Desmethylvicenistatin		3′-epi-18-Desmethylvicenistatin	
	δ_C	δ_H (multiplicity, *J* in Hz)	δ_C	δ_H (multiplicity, *J* in Hz)	δ_C	δ_H (multiplicity, *J* in Hz)
1	168.7	—	168.8	—	168.8	—
2	124.1	5.86 (d, 15.1)	124.4	5.86 (d, 15.0)	124.4	5.86 (d, 15.0)
3	141.8	7.03 (dd, 11.0, 15.1)	141.4	7.01 (dd, 11.1, 15.0)	141.4	7.01 (dd, 11.1, 15.0)
4	129.3	6.16 (dd, 11.0, 15.1)	130.0	6.14 (dd, 11.1, 15.0)	130.0	6.14 (dd, 11.1, 15.0)
5	144.6	5.82 (dd, 9.4, 15.1)	143.8	5.80*	143.7	5.80*
6	46.7	2.29 (m)	46.0	2.30*	45.9	2.30*
7	87.3	3.24 (dt, 1.4, 8.8)	86.9	3.37 (t, 7.8)	86.9	3.37 (t, 7.8)
8	36.7	2.66 (dd, 7.2, 14.3), 2.15 (*)	35.9	2.57*, 2.28*	35.8	2.57*, 2.28*
9	122.7	4.98 (t, 7.4)	122.2	5.05 (t, 7.0)	122.1	5.05 (t, 7.0)
10	135.8	—	135.8	—	135.8	—
11	50.3	2.53 (d, 4.6), 2.61 (d, 4.6)	50.5	2.53 (d, 6.8), 2.58*	50.5	2.53 (d, 6.8), 2.58*
12	134.6	—	134.7	—	134.7	—
13	128.3	5.65 (d, 10.9)	128.0	5.67 (d, 10.9)	128.0	5.67 (d, 10.9)
14	128.8	6.24 (dd, 10.9, 14.8)	128.9	6.25 (dd, 10.9, 14.9)	128.9	6.25 (dd, 10.9, 14.9)
15	132.9	5.45 (ddd, 5.4, 8.8, 14.8)	132.7	5.47 (dt, 11.2, 14.9)	132.7	5.47 (dt, 11.2, 14.9)
16	28.5	2.00 (m), 2.14 (m*)	28.2**	2.12*	28.5**	2.12*
17	33.7	1.28 (m*), 1.49 (m)	26.5**	1.38*, 1.60*	26.5**	1.38*, 1.60*
18	33.9	1.76 (m)	27.1**	1.57*	27.1**	1.57*
19	44.3	3.47 (dd, 10.1, 13.4), 2.89 (dd, 4.7, 13.5)	41.4	3.71*, 2.94 (m)	41.4	3.71*, 2.94 (m)
20	18.9	1.07 (d, 6.6)	18.6	1.08 (d, 6.6)	18.6	1.08 (d, 6.6)
21	17.7	1.55 (s)	17.4	1.50 (s)	17.4	1.50 (s)
22	17.2	1.71 (s)	17.2	1.70 (s)	17.2	1.70 (s)
23	18.3	0.94 (d, 6.9)	—	—	—	—
1′	101.3	4.90 (dd, 2.0, 9.8)	101.2	4.97 (dd, 1.4, 9.6)	102.5	4.67 (dd, 1.4, 9.7)
2′	39.7	2.06 (ddd, 2.0, 3.2, 13.3), 1.61 (ddd, 2.8, 9.8, 13.3)	38.6	2.12*, 1.73*	39.3	2.27*, 1.60*
3′	64.6	4.20 (dt, 3.2, 2.9)	62.7	4.36 (br)	63.2	3.91 (dt, 4.9, 10.6)
4′	65.4	2.12 (dd, 2.9, 9.8)	66.3	2.90 (dd, 2.6, 9.8)	67.1	2.69 (t, 10.1)
5′	71.0	3.68 (dq, 9.8, 6.3)	67.9	4.01 (dq, 9.8, 6.2)	68.3	3.69*
6′	19.4	1.26 (d, 6.3)	19.0	1.33 (d, 6.2)	18.6	1.38 (d, 6.0)
7′	33.9	2.38 (s)	31.1**	2.57 (s)	31.1**	2.74 (s)

*: Obscured by overlapping, **: Assignment may be interchanged

was restored (Fig. 4B). These results clearly prove that *vinI* is involved in the 3-methylaspartate formation in vicenistatin biosynthesis. This is the first evidence of involvement of the *vin* genes in the vicenistatin biosynthesis on the genetic level.

Interestingly, instead of vicenistatin a new peak with a retention time of 7 minutes appeared in the culture of the *vinI* mutant (Fig. 4A). This peak was purified by silica gel chromatography ($CHCl_3$: MeOH = 10 : 1) and by preparative HPLC (same conditions as described above), and analyzed by MS, ^{1}H-NMR and ^{13}C-NMR including H-H-COSY. The positive FAB mass spectrum showed a pseudo molecular ion at *m/z* 487 $[M+H]^+$, 14 mass units less than that of vicenistatin. However, paired signals (1 : 1) were observed in the ^{1}H- and ^{13}C-NMR spectra. Since the mixture (**2** and **3**) was inseparable under several conditions, the structures were determined without further separation. The ^{1}H and ^{13}C-NMR data of **2** and **3** are summarized in Table 1. Signals for the aglycone parts of the two compounds were almost identical to those of vicenistatin, although the signal for H-23 (0.94 ppm) of the starter methyl group of vicenistatin was absent. Thus, the

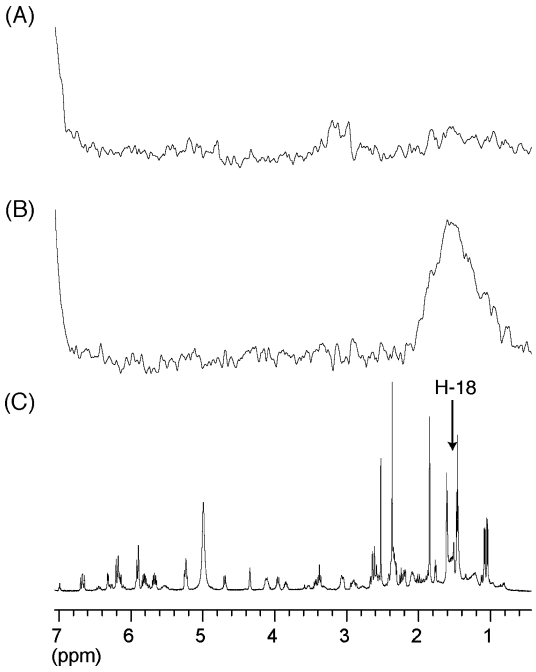

Fig. 5 ^{2}H-NMR spectra (60 MHz, pyridine) of desmethylvicenistatins produced by feeding (A) *β*-[2,2-$^{2}H_2$]alanine and (B) DL-[2,3,3-$^{2}H_3$]aspartate. (C) ^{1}H-NMR spectrum (500 MHz, pyridine-d_5) of desmethylvicenistatins.

desmethylvicenilactam skeleton was deduced for the new compounds. A significant difference between these two compounds was found in the sugar moiety. One of the sugar moieties was determined to be vicenisamine by comparison with the signals of vicenistatin. The ^{1}H-spin network and coupling constants (J=10 Hz) between 3′-H and 4′-H indicated that the other was a C-3 epimer of vicenisamine, 3-epivicenisamine. Thus, **2** and **3** were determined to be 18-desmethylvicenistatin and 3′-epi-18-desmethylvicenistatin, respectively. The production of 3′-epi-18-desmethylvicenistatin was unexpected, because 3′-epivicenisaminide has not been isolated in the wild type of *S. halstedii*. Although it is not clear at the moment the reason why the *vinI* mutant produces 3'-epivicenisaminide, manipulation of the *vinI* gene may affect sugar biosynthesis.

Since comparable amounts of desmethylvicenistatins **2** and **3** were not found in the culture of the wild-type and the *vinI* mutant supplied with 3-methylaspartate, **2** and **3** might be produced only in the absence of 3-methylaspartate. It was speculated that aspartate, instead of 3-methylaspartate, was converted to *β*-alanine, which became a starter unit for the desmethylvicenilactams. To clarify this, feeding experiments with DL-[2,3,3-$^{2}H_3$]aspartate and *β*-[2,2-$^{2}H_2$]alanine were carried out. Labeled aspartate [9] and *β*-alanine [10] were prepared by published methods. The results show that the deuterium of labeled aspartate was incorporated into H-18 of desmethylvicenistatins, but no incorporation was observed in the case of *β*-alanine (Fig. 5). The similar phenomenon was observed in previous feeding experiments, in which 3-methylaspartate was incorporated into vicenistatin, but its decarboxylated product aminomethylpropionate was not. As we proposed previously [4], it is hypothesized that a more complicated pathway between 3-methylaspartate/aspartate and aminomethylpropionate/*β*-alanine could exist beyond just a decarboxylation prior to the PKS reaction.

In summary, the glutamate mutase *vinI* mutant of *S. halstedii* was successfully constructed and it was clearly proven that *vinI* is essential for 3-methylaspartate formation in vicenistatin biosynthesis. In addition, desmethylvicenistatins were isolated from the *vinI* mutant. These results show that the vicenistatin biosynthetic enzymes, including the PKS, are able to accept a starter unit besides 3-methylaspartate. The precise functional analysis of the biosynthetic enzymes will provide opportunities to produce new vicenistatin derivatives.

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