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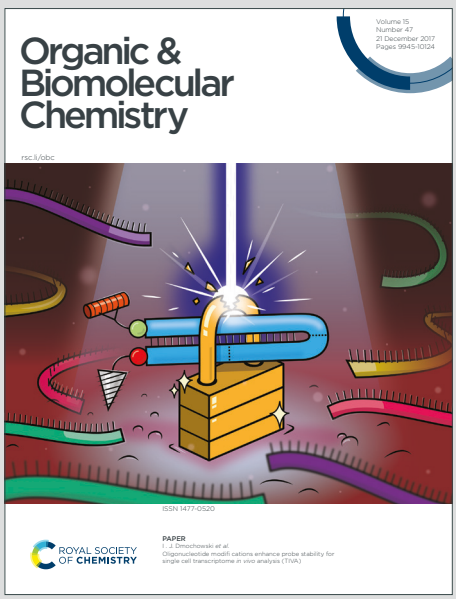

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Guanidyl modification of the 1-azabicyclo[3.1.0]hexane ring in ficellomycin essential for its biological activity

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Abstract

The 1-azabicyclo[3.1.0]hexane ring is a key moiety in the natural products for biological activities against bacteria, fungi, and tumor through DNA alkylation. Ficellomycin is a dipeptide that consists of L-valine and a non-proteinogenic amino acid with the 1-azabicyclo[3.1.0]hexane ring structure. Although the biosynthetic gene cluster of ficellomycin has been identified, the biosynthetic pathway currently remains unclear. We herein report the final stage of ficellomycin biosynthesis involving ring modifications and successive dipeptide formation. After the ring is formed, the hydroxy group of the ring is converted into the guanidyl unit by three enzymes, which includes an aminotransferase with a novel inter ω-ω amino-transferring activity. In the last step, the resulting 1-azabicyclo[3.1.0]hexane ring-containing amino acid is connected with L-valine by an amino acid ligase to yield ficellomycin. The present study revealed a new machinery that expands the structural and biological diversities of natural products.

Introduction

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Ficellomycin isolated from *Streptomyces ficellus*[1] is a dipeptide consisting of L-valine and the non-proteinogenic amino acid with a 1-azabicyclo[3.1.0]hexane ring moiety (azabicyclo hexane ring)[2]. Ficellomycin exhibits the antibacterial activity, for which the azabicyclo hexane ring is a necessary skeleton[3]. We previously reported the novel azabicyclo hexane ring-containing dipeptide, vazabitide A, from *Streptomyces* sp. SANK 60404[4]. Although the structure of vazabitide A is similar to that of ficellomycin, vazabitide A did not exhibit any antibacterial activity. The only structural difference between ficellomycin and vazabitide A was the guanidyl group and hydroxy group modifying the azabicyclo hexane ring (Fig. 1a). The difference in the structure and activity of the related compounds indicates that the modification of the azabicyclo hexane ring is important for the biological activity. The guanidyl group is used in a pharmaceutical field because it facilitates the membrane permeability of active coumpounds[5] and interaction with high molecular-weight proteins, such as protease[6] and ion channels[7]. Although the biosynthetic cluster of ficellomycin had already been reported[8], the biosynthetic pathway including the guanidyl modification mechanism of the azabicyclo hexane ring currently remains unclear.

We recently demonstrated that the azabicyclo hexane ring in vazabitide A is formed from (2*S*,6*R*)-diamino-(5*R*,7)-dihydroxy-heptanoic acid (DADH)[4]. DADH is a non-proteinogenic amino acid that is biosynthesized using amino-group carrier protein (AmCP)[9]. Since the structures of ficellomycin and vazabitide A are similar to each other, ficellomycin is probably biosynthesized from DADH. In fact, all DADH biosynthetic gene homologs are conserved in the *fic* cluster (Fig. 1b). We hypothesized that the four genes specifically present in the *fic* cluster were the candidates for the enzyme catalyzing the last steps of the ficellomycin biosynthesis. Through structural analyses of the compounds accumulated in the knockout mutants of these candidate genes coupled with *in vitro* enzymatic analyses, we revealed that three out of the four enzymes are responsible for the formation of the guanidylated azabicyclo hexane ring-containing amino acid and the remaining enzyme plays a role in dipeptide formation by ligating L-

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valine with the guanidylated azabicyclo hexane ring-containing amino acid to yield ficellomycin.

We herein report the final stage of ficellomycin biosynthesis (Fig. 1c).

Results & discussion

Involvement of *fic22* and *fic16* in ficellomycin biosynthesis

To verify that ficellomycin is biosynthesized using AmCP, we constructed an in-frame knockout mutant of *fic22* encoding AmCP. As expected, the Δ*fic22* mutant of *S. ficellus* JCM4946 did not produce ficellomycin (Fig. 2a), suggesting that ficellomycin is synthesized using AmCP with DADH as an intermediate. The *fic* cluster contains many genes similar to those in the vazabitide A biosynthetic gene cluster. Among the genes in the *fic* cluster, the genes present only in the *fic* cluster were the probable candidates for guanidyl group modification because this group is unique to ficellomycin. Based on this information, we listed four genes, *fic13* (dehydrogenase), *fic15* (amino acid ligase), *fic16* (ornithine aminotransferase) and *fic36* (amidinotransferase), and performed the in-frame deletion of *fic16*. When compounds accumulated in its culture broth were analyzed by LC-MS, the Δ*fic16* mutation was found to abolish the production of ficellomycin, but induce the accumulation of two putative biosynthetic intermediates, **1** and **2**, with *m/z* of 173.09 $[M+H]^+$ and 171.08 $[M+H]^+$, respectively, in the culture broth (Fig. 2b-d).

We purified **2** from the broth of Δ*fic16* and fed **2** into the culture of the Δ*fic22* mutant that lacks the ability to produce ficellomycin. The feeding of **2** restored ficellomycin production by the mutant (Fig. 2e). This result indicated that **2** is a biosynthetic intermediate of ficellomycin and produced via AmCP. We then examined the chemical structure of **2** by NMR as the dinitrophenyl (DNP) unit-conjugated derivative using 2,4-dinitrofluorobenzene (DNFB) to increase its hydrophobicity[10]. **2** was elucidated as 2-amino-2-(4-oxo-1-azabicyclo[3.1.0]hexan-2-yl)acetic acid (Figure S1-6). NOESY spectrum revealed that the relative stereochemistry was in accordance with that of ficellomycin[11] (Figure S1 and S7). We attempted to isolate and purify **1**; however, it was unsuccessful because its DNP derivative still showed high hydrophilicity. Nevertheless, MS/MS analysis supported information on the proposal structure of **1** with the hydroxy group at the C4 position (Figure S8).

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In vitro reconstitution of the guanidyl unit formation

To clarify the functions of Fic13, Fic16, and Fic36 in the guanidyl group modification, we prepared three enzymes, each as an N-terminal $(His)_8$-tagged protein, using recombinant *Escherichia coli* cells (Figure S9) and performed an *in vitro* assay. Fic13 is annotated as a dehydrogenase[12]. Therefore, we expected Fic13 to oxidize **1** to **2**. When Fic13 was incubated with **1** in the presence of 2 mM $NAD(P)^+$ at 30˚C for 2 h, the generation of **2** along with consumption of **1** was observed in the LC-MS analysis of the reaction mixture. This analysis also revealed that Fic13 exhibited a preference for NAD^+ to $NADP^+$ as a coenzyme (Figure S9). Thus, Fic13 was shown to function as a dehydrogenase that converts **1** into **2** in an NAD^+-dependent manner.

An *in vitro* assay of Fic16, annotated as an ornithine aminotransferase (OAT), was then conducted. OAT transfers the α-amino group of glutamate to glutamate-ϒ-semialdehyde and generates 2-oxoglutarate and ornithine[13] (Fig. 3a). We firstly hypothesized that Fic16 transferred the α-amino group of glutamate to **2** in order to produce **3**. However, when Fic16 was incubated with **2** and 0.5 mM glutamate at 30˚C for 2 h, no consumption of **2** was observed. We examined several amino acids as a potential amino donor for the reaction and found that Fic16 consumed **2** only in the presence of ornithine (Fig. 3b). When Fic16 was incubated with asparagine, aspartate, glutamine, and lysine, the consumption of **2** was not observed (Fig S11). We also attempted to detect the putative intermediate **3** in the reaction mixture; however, **3** was not detected, suggesting that **3** was unstable. Since ornithine has two amino groups in its structure, we tried to identify which amino group (α or δ) of ornithine Fic16 transferred to **2**. When the ω-amino group of ornithine is transferred, ornithine must be converted into glutamate-ϒ-semialdehyde, which is spontaneously cyclized to 1-pyrroline-5-carboxylic acid (P5C) (Fig. 3c). P5C can be converted to proline by reduction. We added $NaBH_4$ (final 20 mM) to the Fic16 reaction mixture and derivatized the product with DNFB. LC-MS analysis revealed that proline-DNP was produced in the presence of Fic16 and $NaBH_4$ (Fig. 3d). Based on these results, we concluded that Fic16 is the enzyme that converts **2** into **3** and the first ω-ω specific aminotransferase that transfers the ω-amino group of ornithine to the ω position of the substrate.

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Fic36, annotated as an amidinotransferase, shows 68% identity with StrB1 in amino acid sequence[14]. StrB1 transfers the amidino group of arginine to the amino group of the substrate. We hypothesized that Fic36 catalyzes a similar reaction and converts **3** into **4**, which has the guanidyl group on the azabicyclo hexane ring. Since we were not able to isolate **3**, which may have been due to its instability, we examined the function of Fic36 in a coupling assay with Fic16 using **2** as the substrate. Fic16 and Fic36 was incubated with **2**, 0.5 mM ornithine, and 5 mM arginine at 30˚C for 2 h. After the reaction, we derivatized the reaction product with DNFB at 60˚C for 1 h. An LC-MS analysis detected the compound with of *m/z* 380.13 $[M+H]^+$, which corresponded to that of **4**-DNP (Figure S12). MS/MS analysis supported information on the **4**-DNP structure with the guanidyl unit at the C4 position and DNP unit at the α-amino group (Figure S13). These results show that Fic36 converts **3** into **4**, resulting in the guanidyl group modification of the azabicyclo hexane ring.

In vitro reconstitution of ficellomycin production

Ficellomycin is a dipeptide composed of L-valine and **4**. In the biosynthesis of vazabitide A, a similar dipeptide composed of L-valine and the azabicyclo hexane ring-containing amino acid with the hydroxy group, the two amino acids are ligated by a non-ribosomal peptide synthase (NRPS)[15], Vzb15. However, a gene homologous to *vzb15* was not found in the *fic* cluster, suggesting that the bond is formed by a different mechanism without using NRPS. As described above, four genes, *fic13*, *fic15*, *fic16*, and *fic36*, are specifically present in the *fic* cluster. Among these, *fic15* encodes a putative ATP-grasp superfamily protein, some of which exhibit amino acid ligase activity[16]. Therefore, we hypothesized that Fic15 ligates L-valine with **4** to yield ficellomycin.

To clarify this hypothesis, we conducted a gene knockout experiment of *fic15*. The knockout mutant lost the ability to produce ficellomycin (Figure S14). This result demonstrated that *fic15* is involved in the biosynthesis of ficellomycin. We then performed an *in vitro* assay on Fic15 in a coupled reaction with Fic13, Fic16, and Fic36 using **1** as the initial substrate. When the reaction product was derivatized with DNFB and analyzed by LC-MS,

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ficellomycin-DNP was detected in the reaction mixture containing all the necessary enzymes and compounds (Fig. 4).

Conclusions

We demonstrated that the *amcp* gene was necessary for the biosynthesis of ficellomycin by gene deletion experiments. This has become the second example that directly demonstrated the involvement of AmCP in secondary metabolite production in *Streptomyces*[4]. Ficellomycin is probably synthesized from DADH as a key intermediate, which is biosynthesized using the AmCP-mediated machinery. DADH must be converted into the azabicyclo hexane-ring containing amino acid **1**, and **1** is further converted into ficellomycin by the four enzymes, Fic13, Fic16, Fic36, and Fic15. Among them, we assume that Fic16 and Fic15 are key enzymes specifying the ficellomycin biosynthesis. Fic16 is an aminotransferase that converts **2** to **3** using ornithine as the amino donor. Aminotransferases can be divided into the following three types according to the donor/acceptor relationship of the amino groups; (i) α-α type, (ii) α-ω type, and (iii) amine-ketone type[17]. Fic16 transferred the ω-amino group of ornithine to the ω position of the substrate. Thus, Fic16 is the first specific ω-ω type aminotransferase that transfers the ω-amino group of an amino donor to the keto group at the ω position of an amino acceptor. Further studies are required to elucidate the underlying mechanisms in more detail through crystal structure analysis of Fic16.

Although ficellomycin and vazabitide A are both dipeptides composed of an azabicyclo hexane ring-containing amino acid and L-valine, their peptide bonds are formed via different mechanisms. While the peptide bond in ficellomycin is formed by Fic15 annotated as an amino acid ligase, the corresponding dipeptide formation is presumably catalysed by Vzb7 (NRPS) in vazabitide A biosynthesis. We recently provided another example of an amino acid ligase being involved in the biosynthesis of azabicyclo hexane ring-containing natural product, s56-p1[18], in which an amino acid ligase is proposed to ligate glycine with an azabicyclo hexane ring-containing amino acid. An azabicyclo hexane ring-containing amino acid appears to be a more versatile substrate of

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both amino acid ligase and NRPS than originally expected. Interestingly, the relative stereochemistry of the azabicyclo ring in ficellomycin is different from that of vazabitide A. In each biosynthesis, the enzyme responsible for the peptide bond formation is different as mentioned above. We speculate that C domain of the Vzb7-like protein encoded in the ficellomycin biosynthetic gene cluster is not able to recognize **1** as a substrate due to the different stereochemistry. Structural similarity and resemblance of biosynthetic pathway between ficellomycin and vazabitide A suggests that these natural products share biosynthetic origin. It is intriguing how each stereochemistry had been diverged from the common biosynthetic pathway. Bioinformatics study is revealing that many gene clusters contain the DADH biosynthetic gene homologs in *Streptomyces*[4]. Therefore, discovery of unexploited azabicyclo hexane ring-containing natural products based on their structures and functions will be expected, which will expand the structural and biological diversities of these natural products to new medicines.

Experimental

Bacterial strains and plasmids.

The bacterial strains and plasmids used in the present study are listed in Table S1.

Biochemicals and enzymes for genetic manipulations were purchased from TaKaRa Bio (Otsu, Japan), TOYOBO (Osaka, Japan), and Bio-Rad Laboratories (Hercules, CA). Eluent additives in liquid chromatography were purchased from Sigma-Aldrich (St. Louis, MO). Oligonucleotides used for genetic manipulations (Table S3) were purchased from Operon Biotechnologies (Tokyo, Japan) or FASMAC (Kanagawa, Japan). 2,4-Dinitrofluorobenzene (DNFB) was purchased from Tokyo Chemical Industry (Tokyo, Japan). All other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan), Kanto Chemicals (Tokyo, Japan), and Nacalai Tesque (Kyoto, Japan) unless otherwise noted.

Gene deletion experiment of *fic15*, *fic16*, and *fic22.*

Streptomyces ficellus mutants each lacking the *fic15*, *fic16*, or *fic22*

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gene, were constructed. The plasmid for the in-frame knockout of each gene was constructed as follows. The first polymerase chain reactions (PCR) were performed with the primers Δ*fic16*UpFw and Δ*fic16*UpRv to amplify the upstream region (2 kb) of the *fic16* gene. The second PCR was performed with the primers Δ*fic16*DnFw and Δ*fic16*DnRv to amplify the downstream region (2 kb) of the *fic16* gene. The first and second amplified fragments were digested with *Xba*I/*Eco*RI and *Eco*RI/*Hin*dIII, respectively, and cloned separately into pBlueScript II KS(+) (Stratagene, San Diego, CA) for sequence verification. Two fragments with the correct sequences were cloned into the *Hin*dIII site of the pUC118-*aac(3)*IV vector. The resulting plasmid, pΔ*fic16*, was introduced into *S. ficellus* to knockout the *fic16* gene. The polyethylene-glycol-assisted protoplast transformation of *S. ficellus* JCM 4946 was performed as described previously[19]. Colonies that grew on R2YE[19] supplemented with 25 μg/ml apramycin were selected, and the transformant was cultured in TSB[19] medium for 2 d. Protoplasts were prepared in YEME[19] medium and regenerated on R2YE plates. Apramycin-sensitive colonies from the regenerated colonies were selected. Knockout was confirmed by PCR using Δ*fic16*check-Fw and Δ*fic16*check-Rv. The resulting knockout mutant was named *S. ficellus* Δ*fic16*. The *S. ficellus* mutant lacking *fic15* or *fic22* was constructed in a similar manner using two sets of primers.

Gene complementation of *fic15*, *fic16*, and *fic22* in *S. ficellus* Δ*fic15*, Δ*fic16*, and Δ*fic22*.

The genetically complemented strains of *S. ficellus* Δ*fic15*, Δ*fic16*, and *S. ficellus* Δ*fic22* were constructed as follows. Each gene was amplified by PCR from the genome of *S. ficellus* using the primers listed in Table S1. Primers were designed to amplify DNA regions, each containing the *fic15*, *fic16*, or *fic22* gene, with the upstream region including its ribosomal binding site. The DNA fragment containing the target gene was cloned downstream of the *lacZ* promoter in pSE101[20] to give pSE101_*fic15*, pSE101-*fic16*, and pSE101-*fic22*. *S. ficellus* Δ*fic15*, Δ*fic16*, and Δ*fic22* were transformed with pSE101-*fic15*, pSE101-*fic16*, and pSE101-*fic22*, respectively, according to a previously reported protocol[19].

LC-MS analysis of the metabolites of the *fic* gene knockout mutants and

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genetically complemented strains.

The wild-type *S. ficellus* JCM 4946 and *fic* gene knockout mutants were inoculated into 10 mL TSB medium (30 g L^{-1} tryptic soya broth) and cultured with shaking (300 rpm) at 30°C for 3 d. The preculture (1 mL) was transferred into 100 mL of ficellomycin production medium A (4.0% starch, 2.5% pharmamedia, 1.5% glucose, 2.0% molasses, and 0.8% $CaCO_3$)[1] with 1 g of XAD™7HP and further cultured with shaking (180 rpm) at 27°C for an additional 2 or 3 d. The culture broth was centrifuged at 20,630 × *g* for 10 min and the resultant supernatant was diluted 10-fold with methanol. The diluted sample was further centrifuged at 20,630 × *g* for 10 min. The supernatant was applied to a high-resolution Triple TOF® 5600 system (SCIEX, Tokyo, Japan) equipped with a UFLC Nexera system (Shimadzu, Kyoto, Japan). Samples were separated by the ACQUITY UPLC® BEH HILIC column, 130 Å, 1.7 µm, 2.1 × 50 mm (Waters). The LC conditions were as follows: mobile phase A, H_2O + 0.1% formic acid; mobile phase B, acetonitrile + 0.1% formic acid; 95–40% B over 5 min, 40% B for 5 min, and then 95% B for 5 min, at a flow rate of 0.4 ml min^{-1}. MS analyses were simultaneously performed using electrospray ionization in a positive mode. MS/MS analyses were performed with a collision energy of 35 eV.

Purification of 2 from the culture broth of *S. ficellus* Δ*fic16*.

A single colony of *S. ficellus* Δ*fic16* was inoculated into 10 mL TSB medium (30 g L^{-1} tryptic soya broth) and incubated with shaking (300 rpm) at 30°C for 3 d. The preculture (1 mL) was inoculated into 100 mL of ficellomycin production medium B, modified medium A lacking molasses, with 1 g of XAD and the incubated with shaking (180 rpm) at 27°C for 3 d. The culture broth was centrifuged at 2,500 × *g* for 10 min and the supernatant was collected. The supernatant was subjected to an activated charcoal column. The flow-through and water-washed fractions were combined. After the pH of the fractions was adjusted to approximately 10 by the addition of 1 N NaOH, they were subjected to the anion exchange column AG 1-X8 (Bio-Rad) in bicarbonate forms. The adsorbed compounds were eluted with 0, 0.05, and 0.1 M of NaCl and fractions containing **2** were combined and concentrated.

Feeding experiment of 2 into the culture of *S. ficellus* Δ*fic22*.

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S. ficellus Δ*fic22* was cultured in a same manner as described above. The solution (0.1 mL) containing **2** or deionized water was added to 10 ml of K media[21] with 1 g of XAD and cells were cultured at 30˚C for 2 d. The supernatant of the culture broth was diluted 10-fold with methanol and centrifuged at 20,630 × *g* for 10 min. The supernatant of the culture broth was diluted with appropriate volumes of methanol and subjected to LC-MS using the same analytical conditions described above.

Structural elucidation of 2 isolated from *S. ficellus* Δ*fic16*.

2, which was partially purified in the same manner described above, was derivatized with DNFB. An equal volume of methanol with 5% DNFB, 0.5 × volumes of DMSO, and 0.2 × volumes of 1 N NaOH were mixed, and the resultant mixture was incubated at 60˚C for 1 h. The mixture was subjected to vacuum distillation to remove methanol, and then applied to Purif-aios (SHOKO Science, Kanagawa, Japan). The evaporated sample was separated by the ODS column (50 μm, 60 mL) (SHOKO Science). The LC conditions used were as follows: mobile phase A, H_2O; mobile phase B, isopropanol; 2% B in 1 min, 2–50% B over 20 min, at a flow rate of 20 ml min^{-1}. Column elutes were monitored at 341 and 254 nm. Fractions containing **2** modified with DNP molecules (**2**-DNP) were combined. The resultant sample was subjected to separation by the JASCO Extrema HPLC system (JASCO, Tokyo, Japan) equipped with CAPCELL PAK MGII (10 mm × 250 mm) (Shiseido, Tokyo, Japan). The LC conditions for the preparation of **2**-DNP were as follows: mobile phase A, H_2O; mobile phase B, methanol; 30% B, at a flow rate of 5 ml min^{-1}. UV absorbance at 341 and 254 nm was monitored. Fractions containing **2**-DNP were combined. After the removal of acetonitrile or methanol *in vacuo*, **2**-DNP of 6.7 mg was purified as the yellow powder from 7.5 L of the culture broth.

NMR and optical rotation measurement.

The NMR spectra of **2**-DNP were obtained using the ECA-600 spectrometer (JEOL, Tokyo, Japan). ^{1}H NMR spectra and all 2D spectra were acquired in 600 MHz, and ^{13}C NMR spectra were acquired in 150 MHz. **2**-DNP were dissolved in *d6*-DMSO and filled into a 5-mm NMR tube. All experiments were performed at an ambient temperature.

Optical rotation was measured on a JASCO P-2100 polarimeter

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(JASCO, Tokyo, Japan).

Preparation of Fic13, Fic16, Fic36, and Fic15 from recombinant *E. coli*.

A DNA fragment, carrying the *fic13*, *fic16*, *fic36,* or *fic15* gene, was amplified by PCR with a single set of the primers Fic_Fw/Fic_Rv using the genome DNA of *S. ficellus* as the template. The amplified fragment was inserted into pBluescript II KS(+) using the *Bam*HI and *Hin*dIII sites. The inserted fragment was further subcloned into pHIS8[22] using the same restriction sites to yield pHis8_Fic for the production of Fic enzymes with the $(His)_8$ tag at the N terminus. The resulting plasmid was introduced into *E. coli* BL21-CodonPlus(DE3)-RIL. Recombinant *E. coli* cells were grown in 200 ml of 2 × YT media (1.6% Bacto Tryptone, 1.0% Bacto Yeast Extract, and 0.5% NaCl) supplemented with 50 μg/ml of kanamycin (Km) and 30 μg/ml of chloramphenicol (Cm) at 37 ˚C for 3 h. Isopropyl β-$_D$-1-thiogalactopyranoside (IPTG) was then added to give a final concentration of 0.1 mM for induction and cells were further cultured at 25˚C overnight. Cells were harvested by centrifugation and washed with buffer A (20 mM Tris-HCl, pH 8.0, 150 mM NaCl). The resultant cells were disrupted using an ultrasonic homogenizer and debris was removed by centrifugation at 4˚C (34,700 × *g*, 20 min). The supernatant was passed through a Minisart syringe filter (Sartorius, Göttingen, Germany) and applied to a Ni-NTA His Bind® Resin (EMD Millipore, Billerica, MA) column. The column was washed with buffer B (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 20 mM imidazole) and bound proteins were eluted by buffer C (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 500 mM imidazole). The eluate was concentrated using the VIVASPIN 10,000 MWCO PES membrane (Sartorius). The concentration of the protein solution was measured using Bio-Rad Protein Assay Dye Regent Concentrate (Bio-Rad).

Activity assay of Fic13.

The reaction mixture containing 25 mM Tris-HCl, pH 8.0, 0.2 mM NAD(P)H, an appropriate amount of **1,** and 20 μg of Fic13 was prepared in a total volume of 100 μl. The reaction mixture was incubated at 30˚C for 2 h and reactions were quenched by adding 400 μl of methanol. The resultant mixtures were centrifuged at 20,630 × *g* for 10 min. The supernatant was applied to a high-resolution Triple TOF® 5600 system equipped with a

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UFLC Nexera system. Samples were separated by the ACQUITY UPLC® BEH HILIC column, 130 Å, 1.7 μm, 2.1 × 50 mm. LC conditions were the same as those described above.

Activity assay of Fic16.

The reaction mixture containing 50 mM HEPES-NaOH, pH 8.0, 0.02 mM PLP, 0.5 mM amino acids (ornithine, glutamate, glutamine, arginine, lysine, or asparagine), an appropriate amount of **2,** and 50 μg of Fic16 was prepared in a total volume of 100 μl. The reaction mixture was incubated at 30˚C for 2 h and reactions were quenched by adding 400 μl of methanol. The resultant mixtures were centrifuged at 20,630 × *g* for 10 min. A decrease in **2** was analyzed by LC-MS as described in the previous section.

Conversion of glutamate-γ-semialdehyde into proline to confirm the Fic16 reaction.

The reaction mixture of Fic16 incubated at 30˚C for 2 h as described above was treated with 20 mM $NaBH_4$ and incubated at 30 ˚C for 10 min. A portion of the supernatant (50 μl) was treated with DNFB as described above and proline-DNP formation was analyzed by LC-MS. Samples were separated by the CAPCELL PAK C18 IF 2.0 × 50 mm (Shiseido). The LC conditions used were as follows: mobile phase A, H_2O + 0.1% formic acid; mobile phase B, acetonitrile + 0.1% formic acid; 2–98% B over 5 min, 98% B for 5 min, and then 2% B for 5 min, at a flow rate of 0.4 ml min^{-1}. MS analyses were simultaneously performed using electrospray ionization in a positive mode.

Assay for Fic36 activity through coupled reactions with Fic16.

The reaction mixture containing 50 mM Tris-HCl, pH 8.0, 0.02 mM PLP, 0.5 mM ornithine, 5 mM arginine, an appropriate amount of **2**, 20 μg of Fic16, and 150 μg of Fic36 was prepared in a total volume of 100 μl. The reaction mixture was incubated at 30 ˚C for 2 h. The reaction mixture was centrifuged at 20,630 × *g* for 10 min. A portion of the supernatant (50 μl) was treated with DNFB and the formation of **4**-DNP was analyzed by LC-MS as described above. MS/MS analyses were performed with a collision energy of 45 eV.

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Ficellomycin synthesis from 1 using Fic13, Fic16, Fic36, and Fic15.

The reaction mixture containing 50 mM Tris-HCl, pH 8.0, 0.2 mM NAD^+, 0.02 mM PLP, 5 mM ATP, 5 mM $MgSO_4$, 0.5 mM ornithine, 5 mM arginine, 0.5 mM valine, an appropriate amount of **1**, 20 μg of Fic13, 40 μg of Fic16, 40 μg of Fic36, and 70 μg of Fic15 was prepared in a total volume of 100 μl. The reaction mixture was incubated at 30˚C for 2 h and then centrifuged at 20,630 × *g* for 10 min. A portion of the supernatant (50 μl) was treated DNFB as described above and ficellomycin synthesis was analyzed by LC-MS in the same manner.

Conflicts of interest

There are no conflicts to declare.

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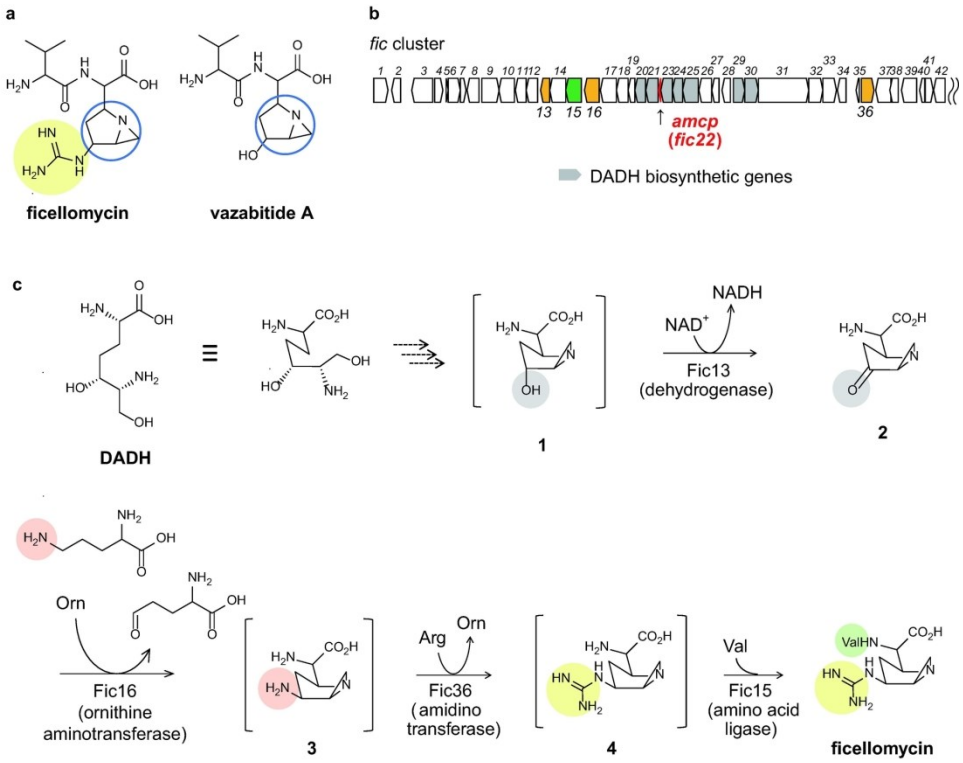

Fig. 1. (a) Chemical structures of ficellomycin and vazabitide A. The 1-azabicyclo[3.1.0]hexane rings are circled by blue ring and the guanidyl unit in ficellomycin is shown in yellow. (b) The *fic* gene cluster. The DADH biosynthetic gene homologs are colored in gray and the *amcp* gene is shown in red. Four candidate genes responsible for the ring modification and dipeptide formation are highlighted in orange and green. (c) The final four reactions of the ficellomycin biosynthetic pathway identified in the present study. The proposed chemical structures of the intermediates are shown with the relative chemistry in brackets.

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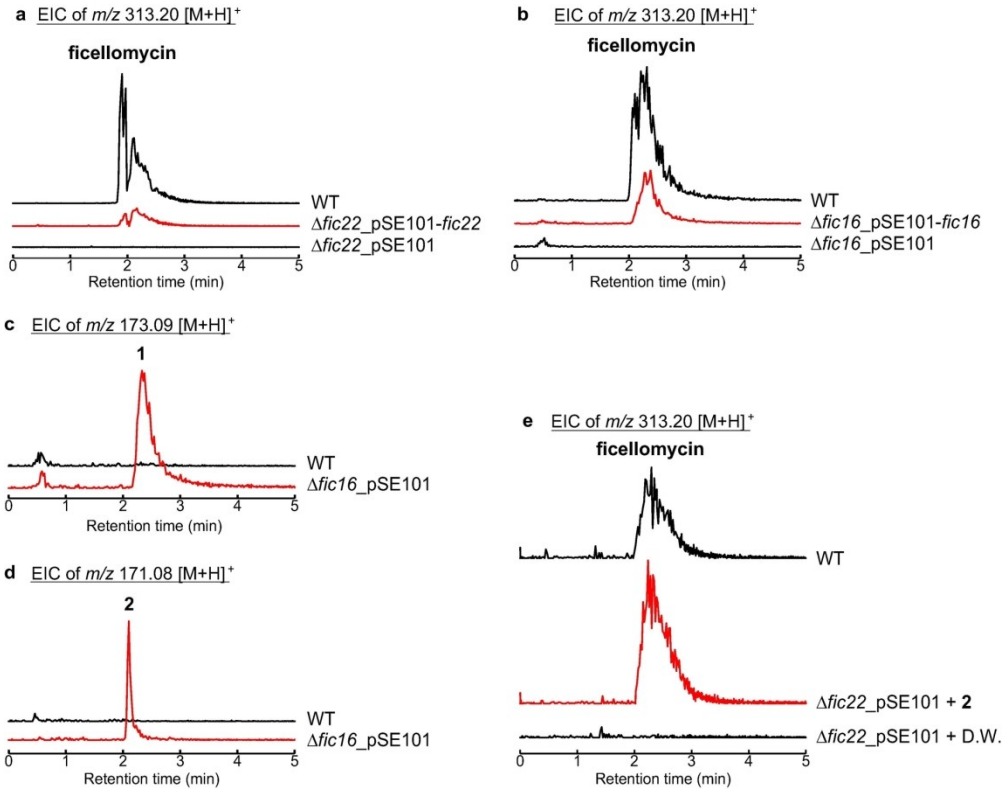

Fig. 2. Metabolite analysis of *S. ficellus* mutants and genetically complemented strains. (a), (b) Extracted ion count (EIC) chromatograms for ficellomycin (*m/z* 313.20 $[M + H]^+$) obtained via an LC-MS analysis of the culture extracts from *S. ficellus* mutants and genetically complemented strains. (c) EIC chromatograms for **1** and **2** (*m/z* 173.09 $[M + H]^+$ and *m/z* 171.08 $[M + H]^+$) obtained via an LC-MS analysis of the culture extracts from Δ*fic16*. (d) Feeding experiment of **2** into *S. ficellus* Δ*fic22*_pSE101. EIC chromatograms for ficellomycin (*m/z* 313.20 $[M + H]^+$) are shown.

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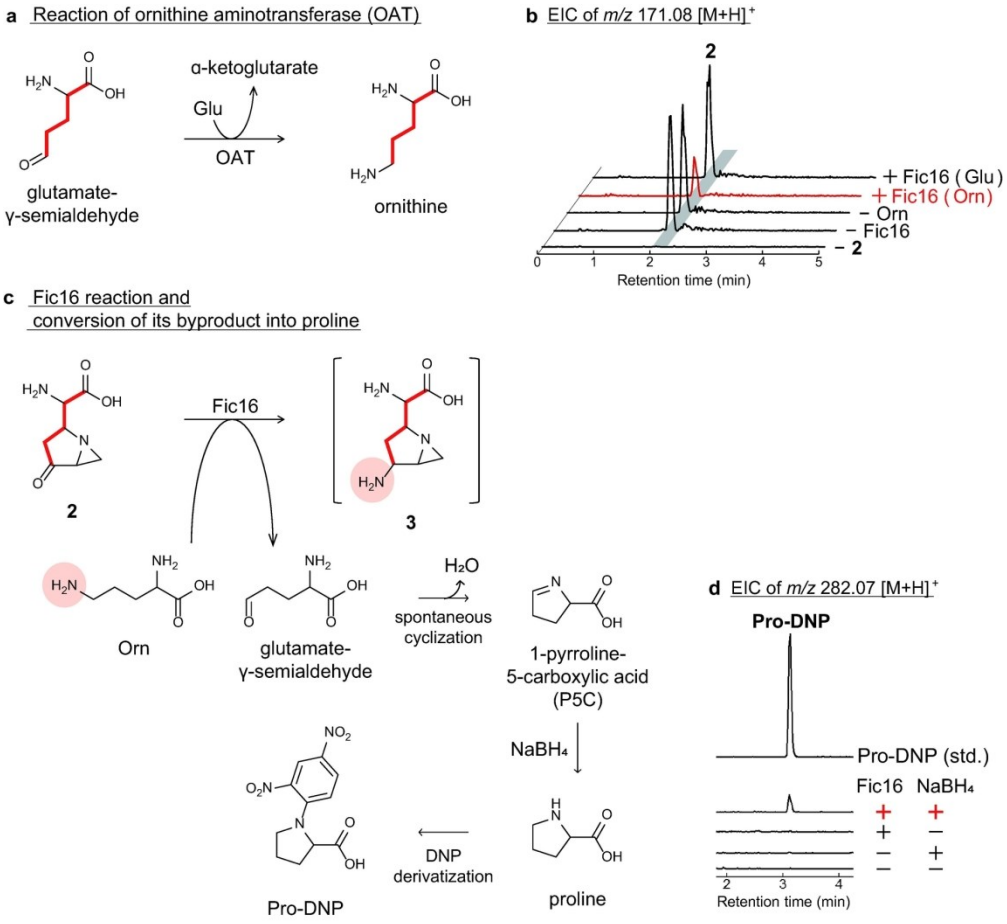

Fig. 3. *In vitro* assay of Fic16. (a) Reaction of ornithine aminotransferase. (b) LC-MS analysis of the *in vitro* reaction mixture of Fic16. Extracted ion count (EIC) chromatograms for **2** (*m/z* 171.09 $[M+H]^+$). (c) Fic16 reaction and a scheme to convert P5C into proline-DNP. (d) Detection of proline generated by the spontaneous cyclization of glutamate-γ-semialdehyde followed by reduction with $NaBH_4$. EIC chromatogram for proline-DNP (*m/z* 282.07 $[M+H]^+$) in LC-MS.

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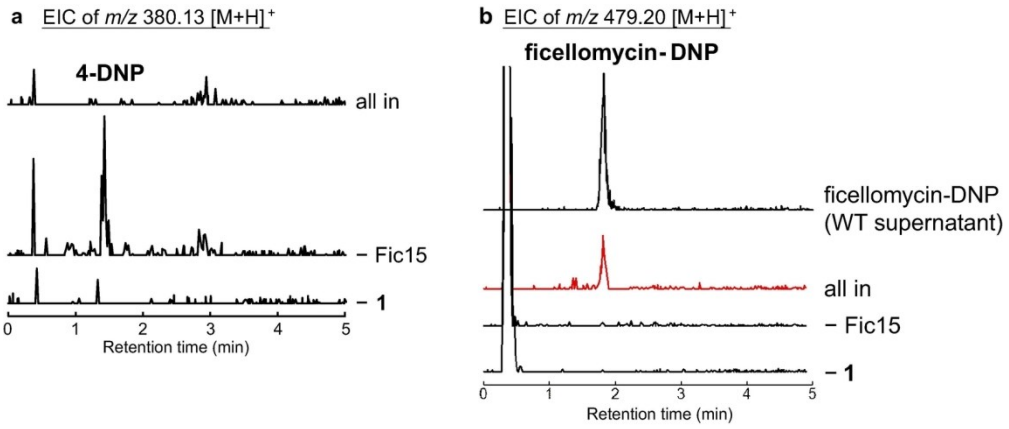

Fig. 4. LC-MS analysis of the *in vitro* reaction mixture of Fic13, Fic16, Fic36, and Fic15. (a) EIC chromatograms for **4**-DNP (*m/z* 380.13 $[M + H]^+$). (b) EIC chromatograms for ficellomycin-DNP (*m/z* 479.20 $[M + H]^+$).

147x62mm (300 x 300 DPI)