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Biosynthesis of the 4-Methyloxazoline-Containing Nonribosomal Peptides, JBIR-34 and -35, in *Streptomyces* sp. Sp080513GE-23

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SUMMARY

JBIR-34 and -35 produced by *Streptomyces* sp. Sp080513GE-23 are nonribosomal peptides that possess an unusual 4-methyloxazoline moiety. Through draft genome sequencing, cosmid cloning, and gene disruption, the JBIR-34 and -35 biosynthesis gene cluster (*fmo* cluster) was identified; it encodes 20 proteins including five nonribosomal peptide synthetases (NRPSs). Disruption of one of these NRPS genes (*fmoA3*) resulted in no JBIR-34 and -35 production and accumulation of 6-chloro-4-hydroxyindole-3-carboxylic acid. Stable isotope-feeding experiments indicated that the methyl group of the methyloxazoline ring is derived from alanine rather than methionine. A recombinant FmoH protein, a glycine/serine hydroxymethyltransferase homolog, catalyzed conversion of α-methyl-L-serine into D-alanine (the reverse reaction of α-methyl-L-serine synthesis catalyzed by FmoH in vivo). Taken together, we concluded that α-methyl-L-serine synthesized from D-alanine is incorporated into JBIR-34 and -35 to form the 4-methyloxazoline moiety. We also propose the biosynthesis pathway of JBIR-34 and -35.

INTRODUCTION

JBIR-34 and -35 (**1** and **2**) produced by *Streptomyces* sp. Sp080513GE-23 are small chlorinated nonribosomal peptides (NRPs) having a weak radical scavenging activity (Figure 1A; Motohashi et al., 2010). These compounds possess a characteristic 4-methyloxazoline moiety, which is rarely found in natural products. BE-32030 derivatives, which are inhibitors of cancer proliferation, are the only compounds that possess this moiety other than JBIR-34 and -35 (Figure 1B; Tsukamoto et al., 1997). Almost all of the methyloxazoline moieties observed in natural products are 5-methyloxazoline, which is derived from the proteinogenic amino acid, threonine. In contrast, the 4-methyloxazoline moieties are probably derived from the nonproteinogenic amino acid, α-methylserine, or produced by methylation of oxazoline synthesized from serine. The similar five-membered ring, 4-methylthiazoline, which is found in the structures of yersiniabactin (Figure 1C), hoiamides, largazoles, and thiazohalostatin, is synthesized via α-methylation of cysteine during peptide elongation (Patel et al., 2003; Choi et al., 2010; Ungermannova et al., 2012; Shindo et al., 1993). To date, however, there is no biosynthetic study on the origin of 4-methyloxazoline.

NRPs, some of which have valuable bioactivities, are produced by a wide variety of microorganisms. Pharmaceutically important antibiotics including vancomycin, daptomycin, and beta-lactam antibiotics belong to this class, which emphasizes the importance of NRPs in the industry (Martin, 2000; Walsh, 2004). They also attract interest because combinatorial biosynthesis can be applied for production of novel NRPs (Walsh, 2004). Typical nonribosomal peptide synthetases (NRPSs) show modular structure, and each module is responsible for the condensation of one amino acid. One module is usually composed of three domains, condensation (C) domain, adenylation (A) domain, and thiolation (T) domain. The T domain is a carrier protein containing a serine residue that is phosphopantetheinylated; an amino acid or a growing peptide is covalently attached to the phosphopantetheine arm via a thioester bond. The A domain is responsible for amino acid selection and catalyzes formation of a thioester bond between the phosphopantetheine arm of the T domain and an amino acid substrate by consuming ATP. The C domain is responsible for the peptide bond formation between an amino acid attached to one T domain and the growing peptide (or an amino acid in the initial condensation) attached to another T domain. Sometimes, a module contains an accessory enzyme domain such as methyltransferase (MT) domain and epimerization (E) domain to modify the growing peptide or amino acid before condensation. In some cases, the C domain is substituted by the heterocyclization (CYC) domain, catalyzing formation of the oxazoline or thiazoline ring. In the end, the synthesized peptide is cleaved off from the enzyme by the thioesterase (TE) domain, which catalyzes simple hydrolysis or macrocycle formation. This modular structure enabled engineering of NRPSs to produce various peptides by substituting A domains, which are responsible for amino acid selection. Nguyen et al. (2006) successfully produced daptomycin analogs

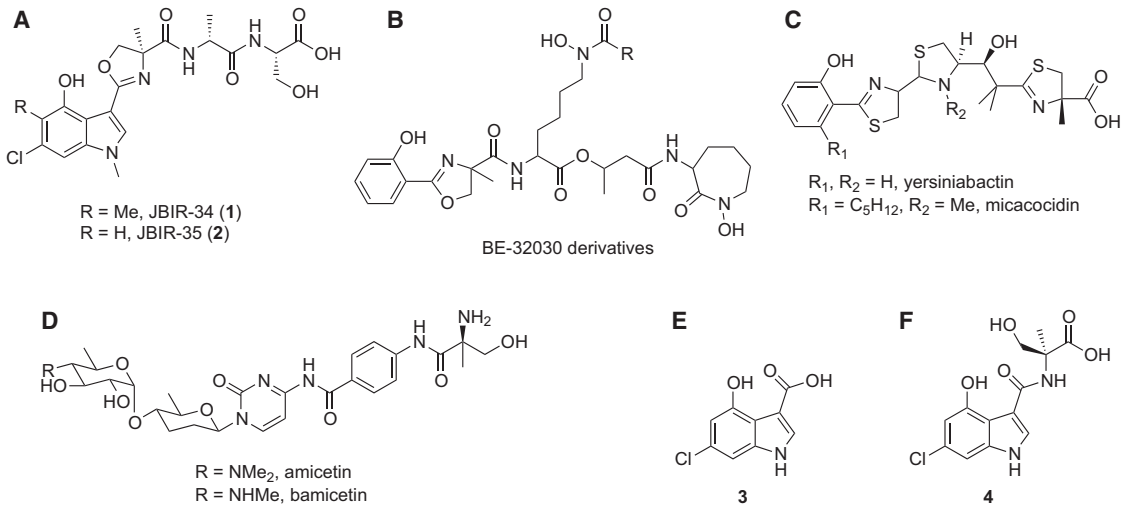

Figure 1. Structures of the Compounds

(A and B) JBIR-34, -35 (A), and BE-32030 derivatives (B) possess a distinct 4-methyloxazoline moiety.

(C) Yersiniabactin and micacocidin possess a 4-methylthiazoline moiety, which is derived from cysteine via methylation by a SAM-dependent methyltransferase.

(D) Amicetin possesses an α-methyl-L-serine moiety attached to the aromatic amine group.

(E and F) 6-Chloro-4-hydroxyindole-3-carboxylic acid (E) and *N*-(6-chloro-4-hydroxyindole-3-carboxyl)-α-methyl-L-serine (F) were isolated from *Streptomyces* sp. Sp080513GE-23 in this study and are intermediates of JBIR-34 and -35 biosynthesis.

by engineering the daptomycin-biosynthesis NRPSs. A fascinating feature of NRPSs is their ability to use nonproteinogenic amino acids (Walsh et al., 2013). For instance, hydroxyphenylglycine, ornithine, and alkylproline moieties are observed in vancomycin, daptomycin, and sibiromycin, respectively (Recktenwald et al., 2002; Miao et al., 2005; Li et al., 2009). Incorporation of nonproteinogenic amino acids is important for their structural diversity and bioactivity. Therefore, exploring the enzymatic chemistry of the A domain for incorporation of novel nonproteinogenic amino acids could enhance the structural diversity of NRPs because it would be available for combinatorial biosynthesis.

Glycine/serine hydroxymethyltransferase is a pyridoxal-5′-phosphate (PLP)-dependent enzyme and catalyzes the formation of serine from glycine using 5,10-methylenetetrahydrofolate (mTHF) as a hydroxymethyl group donor. It is also predicted to be responsible for biosynthesis of nonproteinogenic amino acids in secondary metabolite biosynthesis; AmiS in amicetin biosynthesis by *Streptomyces vinaceusdrappus* NRRL 2363 has been hypothesized to catalyze α-methylserine synthesis using alanine instead of glycine as a substrate (Zhang et al., 2012). In addition to AmiS, there are two examples of enzymes that catalyze the conversion between D-alanine and α-methyl-L-serine. These enzymes were purified from *Paracoccus* sp. AJ110402 (BAG31000) and *Aminobacter* sp. (BAG31001) and characterized in vitro (Nozaki et al., 2008, 2009). A neighbor of glycine/serine hydroxymethyltransferase, threonine aldolase, has recently been attracting attention as an enzyme for synthesizing nonproteinogenic amino acids. Barnard-Britson et al. (2012) have shown that LipK is a transaldolase that synthesizes the β-hydroxy-α-amino acid, 5′-*C*-glycyluridine, in A-90289 biosynthesis. Glycine/serine hydroxymethyltransferase and threonine aldolase are promising biocatalysts for forming various β-hydroxy-α-amino acids (Schirch and Gross, 1968; Gutierrez et al., 2008), because these reactions are stereospecific.

In this study, we analyzed the biosynthesis of JBIR-34 and -35 by identifying their biosynthesis gene cluster, disrupting an NRPS gene, stable isotope-feeding experiments, and in vitro enzyme assays. We mainly focused on the biosynthesis origin of an intriguing 4-methyloxazoline ring and revealed that it is synthesized from α-methyl-L-serine. This key intermediate is synthesized from D-alanine by FmoH, which is a glycine/serine hydroxymethyltransferase homolog. Taken together with several other results, we predicted the entire biosynthesis pathway of JBIR-34 and -35.

RESULTS

Search for JBIR-34 and -35 Biosynthesis Intermediates

To obtain insight into the biosynthesis pathway of JBIR-34 and -35, we attempted to isolate JBIR-34 and -35 derivatives from the culture broth of the producer strain. We assumed that the chlorine group of these compounds is incorporated at an early stage of biosynthesis and searched for compounds containing a chlorine group using liquid chromatography-mass spectrometry (LC-MS) analysis. Compounds containing a chlorine group show a characteristic isotope pattern, which enables us to search for chlorine-containing compounds only with LC-MS. As a result, we succeeded in the detection of two compounds (compounds **3** and **4**), which showed $[M + H]^+$ monoisotopic ions at *m/z* 212 and 313, respectively. These compounds were purified and their structures were analyzed with ^1H nuclear magnetic resonance (NMR), ^{13}C NMR, correlated spectroscopy (COSY), heteronuclear multiple-quantum correlation (HMQC), and heteronuclear multiple bond correlation (HMBC). Compounds **3** and **4** were identified as 6-chloro-4-hydroxyindole-3-carboxylic acid (Figure 1E; Figure S1A available online) and *N*-(6-chloro-4-hydroxyindole-3-carboxyl)-α-methyl-L-serine (Figures 1F and S1B), respectively. The stereochemistry of α-methyl-L-serine was

determined by Marfey's method (Figure S1C; Bhushan and Brückner, 2004). Isolation of these chlorinated compounds supported our hypothesis that chlorination of the indole moiety occurs at an early stage of the biosynthesis. These compounds seem to be intermediates released from NRPS during the peptide synthesis. The isolation of compound **4**, which seems to be an intermediate before heterocyclization to form the 4-methyloxazoline moiety, suggests that the 4-methyloxazoline moiety is synthesized via α-methyl-L-serine, although it was not clear whether α-methyl-L-serine was used as a substrate of NRPS or synthesized by methylation of L-serine during peptide elongation.

Cloning and Sequencing of JBIR-34 and -35 Biosynthesis Gene Cluster

First, we performed a draft genome sequencing of *Streptomyces* sp. Sp080513GE-23. However, the quality was not good enough to reveal the entire sequence of the putative JBIR-34 and -35 biosynthesis gene cluster, which was later named *fmo* (four methyl oxazoline) cluster. Second, to clone and determine the accurate nucleotide sequence of the *fmo* gene cluster, we constructed a cosmid library of the genomic DNA of *Streptomyces* sp. Sp080513GE-23 using pTOYAMAcos (Onaka et al., 2003). Using a flavin-dependent halogenase gene (*fmoD*), which was discovered by the draft genome sequence, as a probe for colony hybridization, we obtained a cosmid (pTOYAMAcos cl. 15) carrying part of the *fmo* gene cluster. Next, we obtained another cosmid (pTOYAMAcos C56) carrying the remaining part of the *fmo* gene cluster by colony hybridization using an NRPS gene (*fmoA5*) as a probe, which is included in the insert fragment of pTOYAMAcos cl.15. Then, we determined the nucleotide sequence of the inserts of these two cosmids, to reveal the entire sequence of the *fmo* gene cluster (Table 1 and Figure 2). To prove by heterologous expression that the *fmo* gene cluster is responsible for the biosynthesis of JBIR-34 and -35, we constructed a cosmid (pTJ34-3) carrying the entire *fmo* gene cluster (see Supplemental Experimental Information). However, we could not observe heterologous production of JBIR-34 or -35 by *Streptomyces lividans* or *Streptomyces albus* harboring pTJ34-3. Therefore, we disrupted *fmoA3* encoding an NRPS in *Streptomyces* sp. Sp080513GE-23 (Figure S2). The Δ*fmoA3* mutant did not produce both JBIR-34 and -35 (Figure 3), which clearly showed that this gene cluster is responsible for their biosynthesis. Furthermore, the Δ*fmoA3* mutant produced a larger amount of compound **3** (approximately 0.3 mg/l) than the wild-type strain (approximately 0.1 mg/l), which suggested that this compound was the priming unit for the NRP synthesis. The wild-type strain produced approximately 7 and 2 mg/l of JBIR-34 and -35, respectively, and the amount of compound **3** produced by the Δ*fmoA3* mutant was therefore much lower than expected. This result may be attributed to the product inhibition of the enzyme involved in the biosynthesis of compound **3**. The Δ*fmoA3* mutant showed normal growth and morphological development on ISP2 agar (data not shown).

In Silico Analysis of NRPSs and Related Enzymes

To predict the biosynthesis pathway of JBIR-34 and -35, proteins encoded by the *fmo* cluster were analyzed in silico. FmoA1 is composed of an A-T didomain. Using NRPSpredictor2 (http://nrps.informatik.uni-tuebingen.de/Controller?cmd=SubmitJob; Röttig et al., 2011), the A domain was predicted to recognize tryptophan. Importantly, FmoA1 showed significant similarity to several didomain NRPSs, such as SimH (36% identity) from the simocyclinone biosynthesis gene cluster (Galm et al., 2002), NovH (35%) from the novobiocin biosynthesis gene cluster (Steffensky et al., 2000; Chen and Walsh, 2001), and NikP1 (32%) from the nikkomycin biosynthesis gene cluster (Chen et al., 2002). These NRPSs were predicted or proved to be responsible for β-hydroxylation of tyrosine or histidine in collaboration with a cytochrome P450, which is encoded by a gene adjacent to the NRPS genes. Furthermore, the *fmoA1* gene is located adjacent to *fmoC* that encodes a putative cytochrome P450 showing similarity to NikQ (39%), SimI (37%), and NovI (33%), which were predicted or proved to catalyze β-hydroxylation of amino acids attached to T domain. Thus, FmoA1 and FmoC are likely to be responsible for β-hydroxylation of tryptophan or its derivative. The resulting β-hydroxyamino acid can be cleaved to give an aldehyde and glycine by a threonine aldolase-catalyzed *retro*-aldol reaction (di Salvo et al., 2014). The resulting aldehyde can be further oxidized to a carboxylic acid by an aldehyde dehydrogenase (Kostichka et al., 2001). FmoM and FmoI, which are threonine aldolase and aldehyde dehydrogenase homologs, respectively, appeared to be responsible for these reactions, and it was therefore highly likely that FmoA1 was involved in the biosynthesis of compound **3** (6-chloro-4-hydroxyindole-3-carboxylic acid), which is the putative priming unit for the NRP synthesis.

FmoA2 is composed of A, T, and CYC domains followed by an MT domain. FmoA3 possesses CYC, A, and T domains. Because these enzymes possess a CYC domain, they are likely to be responsible for the formation of the methyloxazoline group. However, the A domains of FmoA2 and FmoA3 were predicted by the NRPSpredictor2 to recognize alanine and cysteine, respectively. According to the domain structure of FmoA2 and FmoA3, we postulated two possible biosynthesis pathways for 4-methyloxazoline. In the first hypothesis, *N*-(6-chloro-4-hydroxyindole-3-carboxyl)-L-serinyl-T domain is synthesized from 6-chloro-4-hydroxyindole-3-carboxylic acid (**3**) and L-serine by FmoA2 and FmoA3. This compound is then methylated by the MT domain of FmoA2 to synthesize *N*-(6-chloro-4-hydroxyindole-3-carboxyl)-α-methyl-L-serinyl-T domain, which is further heterocyclized by the CYC domains of FmoA2 and/or FmoA3 to form the 4-methyloxazoline moiety. This pathway is similar to that for 4-methylthiazoline formation in the biosynthesis of yersiniabactin (Patel et al., 2003). In the second hypothesis, *N*-(6-chloro-4-hydroxyindole-3-carboxyl)-α-methyl-L-serinyl-T domain is synthesized directly from 6-chloro-4-hydroxyindole-3-carboxylic acid (**3**) and α-methyl-L-serine by FmoA2 and FmoA3. α-Methyl-L-serine may be synthesized from alanine by FmoH, a homolog of AmiS (58.2%), BAG31000 (41.3%), and BAG31001 (41.3%; Zhang et al., 2012; Nozaki et al., 2008, 2009).

FmoA4 is composed of C, A, T, and MT domains. This A domain was predicted by the NRPSpredictor2 to incorporate isoleucine, but this prediction is not consistent with the structures of JBIR-34 and -35. Because FmoA4 contains neither CYC domain nor TE domain, it is not likely to be involved in oxazoline ring formation or the last peptide bond formation.

Table 1. Biosynthesis Gene Cluster of JBIR-34 and -35

Open Reading Frame	No. of Amino Acids	Proposed Function	Protein Homolgy	Amino Acid Identity (%)
orf1	368	metalloprotease	metalloprotease [*Streptomyces mobaraensis* NBRC 13819] ZP_23070617.1	142/371 (38)
fmoR1	289	SARP-family transcriptional regulator	SARP-family transcriptional regulator [*Amycolatopsis mediterranei* U32] YP_003765343	102/241 (42)
fmoA1	588	NRPS (A-T)	SimH [*Streptomyces antibioticus*] AF321122_3	208/583 (36)
fmoB	246	thioesterase	AnaA [*Anabaena* sp. 37] AEQ38173.1	78/240 (33)
fmoC	398	cytochrome P450 monooxygenase	NikQ [*Streptomyces tendae*] CAC11139.1	156/400 (39)
fmoD	550	tryptophan 6-halogenase	KtzQ [*Kutzneria* sp. 744] ABV56597.1	307/526 (58)
fmoE	177	flavin reductase	KtzS [*Kutzneria* sp. 744] ABV56599.1	74/157 (47)
fmoA2	1,496	NRPS (A-T-CYC-MT)	NRPS [*Kribbella flavida* DSM 17836] YP_003381984.1	477/1085 (44)
fmoF	218	hypothetical protein	hypothetical protein WSS_A10807 [*Rhodococcus opacus* M213] ZP_14478590.1	95/206 (46)
fmoG	423	integral membrane ion antiporter	ForY [*Micromonospora olivasterospora*] CAF31532.1	199/406 (49)
fmoH	407	alanine/α-methylserine hydroxymethyltransferase	glycine/serine hydroxymethyltransferase [*Streptomyces hygroscopicus* subsp. jinggangensis 5008] YP_006242991.1	235/402 (58)
fmoA3	1,127	NRPS (CYC-A-T)	CurF [*Lyngbya majuscula*] AAT70101.1	428/1101 (39)
fmoI	490	aldehyde dehydrogenase	betaine-aldehyde dehydrogenase [*Thermobifida fusca* YX] YP_289834.1	251/465 (54)
fmoJ	246	esterase	esterase [*Saccharopolyspora erythraea* NRRL 2338] YP_001105518.1	126/248 (51)
fmoR2	323	transcriptional regulator	helix-turn-helix, type 11 [*Micromonospora* sp. L5] YP_004081693.1	163/316 (52)
fmoA4	1,459	NRPS (C-A-T-MT)	NRPS [*Myxococcus stipitatus* DSM 14675] YP_007359089.1	448/1077 (42)
fmoK	487	transmembrane transporter	transmembrane efflux protein [*Streptosporangium roseum* DSM 43021] YP_003342489.1	219/473 (46)
fmoA5	1,868	NRPS (C-T-TE-TE-A-T)	NRPS [*Streptomyces fungicidicus*] ABD65960.1	363/804 (45)
fmoL	390	hypothetical protein	hypothetical protein [*Streptomyces tsukubaensis*] WP_006346030.1	157/338 (46)
fmoM	364	L-threonine aldolase	threonine aldolase [*Streptosporangium roseum* DSM 43021] YP_003344742.1	164/350 (47)
fmoR3	306	LysR-family transcriptional regulator	LysR-family transcriptional regulator [*Streptomyces* sp. SirexAA-E] YP_004802185.1	171/293 (58)
orf2	148	hypothetical protein	hypothetical protein [*Streptomyces bottropensis*] WP_005481797.1	62/119 (52)
orf3	140	endoribonuclease L-PSP family protein	endoribonuclease L-PSP [*Streptomyces ipomoeae*] WP_009312170.1	114/129 (88)
orf4	386	acyl-CoA dehydrogenase	acyl-CoA dehydrogenase [*Streptomyces zinciresistens*] WP_007496998.1	326/385 (85)

Therefore, we assumed that FmoA4 catalyzes condensation of alanine (a probable amino acid for the second elongation step). The MT domain of FmoA4 may be involved in methylation of the indole ring.

FmoA5 has the domain structure of C-T-TE-TE-A-T. This A domain was predicted by the NRPSpredictor2 to incorporate serine or threonine. Because FmoA5 possesses TE domains, this enzyme seems to catalyze the formation of the last peptide

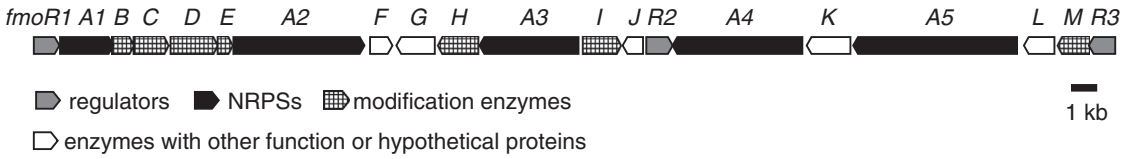

Figure 2. Biosynthesis Gene Cluster of JBIR-34 and -35

bond in the JBIR-34 and -35 biosynthesis. This is consistent with the NRPSpredictor2 prediction; according to the structures of JBIR-34 and -35, serine should be the last amino acid to be incorporated. The two TE domains were subjected to phylogenetic analysis with various type I and type II TE domains, according to the procedure reported by Buntin et al. (2010), in an attempt to predict their functions (Figure S3). Type I TE is generally responsible for the release of a fully elongated peptide from the enzyme, whereas type II TE is involved in the "proofreading" of the peptide during the elongation process, i.e., the cleavage of any mistakenly loaded acyl moieties from the T domain (Koglin et al., 2008). The phylogenetic tree showed that the TE domain closer to the N terminus belonged to a clade composed of type I TE domains from the NRPSs involved in the biosynthesis of albicidin and thailandamide (Royer et al., 2004; Ishida et al., 2010), whereas the TE domain closer to the C terminus belonged to a clade which mainly contained type II TEs. This result suggested that these two TEs performed different functions in the biosynthesis of JBIR-34 and -35. The observation that FmoA5 possessed a putative type II TE domain in *cis* was particularly interesting, because most type II TEs exist in *trans* (Koglin et al., 2008). FmoA5 also possessed two T domains, which both had a conserved Ser residue that was capable of binding to phosphopantetheine. It was difficult to predict which T domain was involved in the peptide elongation process.

As described above, the prediction of the substrate specificity of more than half of the A domains did not match with the structures of JBIR-34 and -35. These A domains may select unusual amino acids, which would make it difficult for the NRPSpredictor2 to predict the genuine amino acid substrate.

The C domains of FmoA4 and FmoA5 were analyzed by phylogenetic analysis according to the procedure reported by Rausch et al. (2007). We found that both of them belong to the $^{L}C_{L}$ domain, which catalyzes peptide bond formation between two L-amino acids (data not shown).

Feeding of [Methyl-^{13}C]-Methionine and [3-^{13}C]-Alanine

To deduce the biosynthetic origin of the methyl group of the methyloxazoline moiety, we carried out feeding experiments using a stable isotope. Prior to the feeding experiments, we analyzed JBIR-34 with high-resolution tandem mass spectrometry (HR-MS/MS) and observed three major fragments, A (m/z = 348.1106 $[M + H]^+$), B (m/z = 277.0735 $[M + H]^+$), and C (m/z = 222.0315 $[M + H]^+$; Figure S4A). Importantly, each fragment showed an ion that was 2 Da larger than the corresponding monoisotopic ion (the difference came from ^{37}Cl), demonstrating that each fragment contained chloride. Based on this result, we determined the molecular formulas of the fragments: A, $C_{17}H_{19}ClN_3O_3^+$; B, $C_{14}H_{14}ClN_2O_2^+$; and C, $C_{11}H_9ClNO_2^+$. Furthermore, taking the structure of JBIR-34 into consideration, we concluded that fragment A is generated by *a* type cleavage of the amide bond between serine and alanine (Figure S4B). Similarly, fragment B is generated by *a* type cleavage of the amide bond between alanine and methyloxazoline (Figure S4B). Fragment C is derived from the hydrolysis of the methyloxazoline moiety followed by *b* type cleavage of the peptide bond (Figure S4B). We also observed a similar fragmentation pattern of JBIR-35 (Figures S4C and S4D). Thus, we concluded that incorporation of ^{13}C into the methyloxazoline moiety could be detected by comparing the isotopic composition of fragments B and C.

We initially expected that the methyl group of the methyloxazoline moiety would be synthesized by a SAM-dependent methyltransferase (for example, the MT domain of FmoA2), because the methylthiazoline moiety in yersiniabactin has been predicted to be synthesized via methylation of cysteine catalyzed by the MT domain of NRPS (Patel et al., 2003). To test this, [methyl-^{13}C]-methionine was added to the medium and incubated with *Streptomyces* sp. Sp080513GE-23. The resulting JBIR-34 was analyzed by HR-MS/MS to examine the incorporation of ^{13}C. The m/z value of the parent ion $[M + H]^+$ of JBIR-34 increased from 481.1473 to 483.1546, suggesting that two ^{13}C atoms were incorporated into the peptide (Figure S4E). The MS data suggested that almost all of the JBIR-34 had been labeled with two ^{13}C atoms. Furthermore, the m/z values of the A, B, and C fragments increased by 2, which was consistent with the incorporation of two ^{13}C atoms. In contrast to our expectation, this result indicated that the two methyl groups of the indole chromophore came from methionine, and that the methyl group of the 4-methyloxazoline ring was not derived from methionine (Figure S4E).

Next, we hypothesized that the methyloxazoline moiety may be formed by heterocyclization of α-methyl-L-serine, which could be synthesized from D-alanine by FmoH, a homolog of AmiS in the amicetin biosynthesis (Zhang et al., 2012). To test our second hypothesis, we fed [3-^{13}C]-L-alanine, which seems to be easily converted to [3-^{13}C]-D-alanine in vivo, to *Streptomyces* sp. Sp080513GE-23. The produced JBIR-34 was analyzed by HR-MS/MS to examine the incorporation of ^{13}C. The parent ion of JBIR-34 showed several peaks in addition to the native peaks; there were two strong peaks corresponding to two ions that were 1 and 2 Da larger than the monoisotopic parent ion (Figure S4F). This result suggested that two alanine molecules could be incorporated into JBIR-34. Analysis by mass spectroscopy revealed that approximately 46% and 39% of the JBIR-34 had been labeled with one and two ^{13}C atoms, respectively. Fragment A also showed a similar isotopic composition to the parent ion (Figure S4F). In contrast, fragment B showed a different isotopic composition from the parent ion and fragment A; approximately 58% and 6% of JBIR-34 were

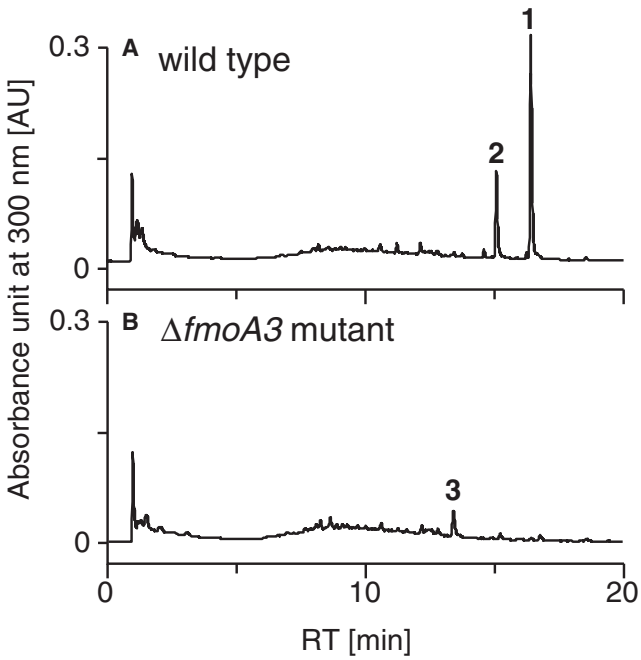

Figure 3. LC-MS Analysis of the Compounds

LC-MS analysis of the compounds produced by *Streptomyces* sp. Sp080513GE-23 wild-type (A) and Δ*fmoA3* mutant (B). The Δ*fmoA3* mutant did not produce JBIR-34 (**1**) or -35 (**2**). However, it produced larger amounts of compound **3** than the wild-type strain. The production of **3** and **4** by the wild-type strain could not be detected in these conditions, because the yields of these compounds were very low.

labeled with one ^{13}C and two ^{13}C, respectively. The most abundant ion of fragment B was 1 Da larger than the monoisotopic ion and the ratio of the 2 Da-larger ion to the 1 Da-larger ion was much lower than that in the parent ion or fragment A (Figure S4F), suggesting that only one alanine molecule was incorporated into fragment B. Furthermore, the ratio of isotopes observed in fragment C was similar to the ratio observed in JBIR-34 obtained from medium without [3-^{13}C]-L-alanine, indicating that no alanine was incorporated into fragment C. Taken together, we concluded that [3-^{13}C]-L-alanine was incorporated into the methyloxazoline moiety. In addition, [3-^{13}C]-L-alanine was similarly incorporated into compound **4** (data not shown). These results clearly support our second hypothesis.

In Vitro Analysis of FmoH

To investigate the activity of FmoH, we prepared a recombinant FmoH protein using the pET system in *Escherichia coli*. A histidine tag was fused to the N terminus of the recombinant protein, and it was purified by Ni^{2+} affinity chromatography (Figure 4A). The purified FmoH did not show any UV absorbance above 300 nm (data not shown), which indicated that FmoH, in contrast to typical PLP-dependent enzymes, was not copurified with PLP (Schirch and Gross, 1968; Nozaki et al., 2008). We assumed that FmoH would catalyze the formation of α-methyl-serine from alanine coupled with the conversion of mTHF to tetrahydrofolate (THF). However, we analyzed the reverse reaction, because mTHF was not commercially available. FmoH was incubated with α-methyl-L-serine or α-methyl-D-serine and THF. The products were derivatized using N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-leucineamide (L-FDLA) and analyzed with LC-MS according to the method described previously (Motohashi et al., 2010; Bhushan and Brückner, 2004). We observed the conversion of α-methyl-L-serine to D-alanine (Figure S5D); however, α-methyl-D-serine was not converted to alanine (Figure S5C). This result suggests that FmoH is capable of synthesizing α-methyl-L-serine from D-alanine by transferring a hydroxymethyl group from mTHF. Based on this result, we analyzed the FmoH-catalyzed reaction in vitro using 9-fluorenylmethyloxycarbonyl chloride instead of L-FDLA to derivatize the reaction products. Derivatization with 9-fluorenylmethyloxycarbonyl chloride allowed for the quantification to be conducted with a higher level of sensitivity. Removal of THF from the FmoH reaction mixture revealed that the production of D-alanine had not occurred, which indicated that THF was essential for the reaction (Figure 4B). Furthermore, the removal of PLP from the reaction mixture led to a 20% reduction in the rate of the reaction (Figure 4B). This result suggested that some of the recombinant FmoH proteins were retaining PLP, even after the purification process. The substrate specificity of FmoH was then analyzed using α-methyl-L-serine, α-methyl-D-serine, L-serine, and D-serine as substrates (Figures 4C and 4D). Among these substrates, FmoH exhibited a preference for α-methyl-L-serine, which was converted to alanine with a high level of efficiency. Alanine was also synthesized from α-methyl-D-serine by FmoH, but the rate of the reaction was very low. Furthermore, FmoH catalyzed the conversion of L-serine to glycine, but the rate of the reaction was also very low (Figures 4C and 4D). The k_{cat} and K_m values for the synthesis of D-alanine from α-methyl-L-serine were determined to be 0.40 ± 0.02 min^{-1} and 105 ± 9 μM, respectively (Figure 4E). The K_m value was lower than that reported for the synthesis of glycine (900 μM) from L-serine using the glycine/serine hydroxymethyltransferase from *Bacillus stearothermophilus* (Pai et al., 2009), which suggested that α-methyl-L-serine is the physiological product of the in vivo FmoH reaction. Taken together, these characteristic properties of recombinant FmoH strongly supported our hypothesis that FmoH is the α-methyl-L-serine synthase involved in the biosynthesis of JBIR-34 and -35.

Substrate Specificities of A Domains in the *fmo* Cluster

To obtain further insight into the biosynthesis of JBIR-34 and -35, we attempted to analyze the substrate specificity of the A domains of FmoA proteins. The adenylating activity of each FmoA protein was analyzed by a previously described procedure with some modifications (McQuade et al., 2009; Kadi and Challis, 2009). In this assay, pyrophosphate formed with acyl-AMP was hydrolyzed to phosphate by pyrophosphatase. The resultant phosphate was quantified using a malachite green phosphate assay kit (BioAssay Systems). The full-length recombinant proteins of each FmoA were prepared for this analysis. Although the recombinant FmoA1 efficiently catalyzed the adenylation of tryptophan and 6-chlorotryptophan, it showed a clear preference for 6-chlorotryptophan over tryptophan (Figure 5A). This result indicates that halogenation of the indole ring occurs at an early stage of the biosynthesis. The recombinant FmoA2 seemed to specifically accept

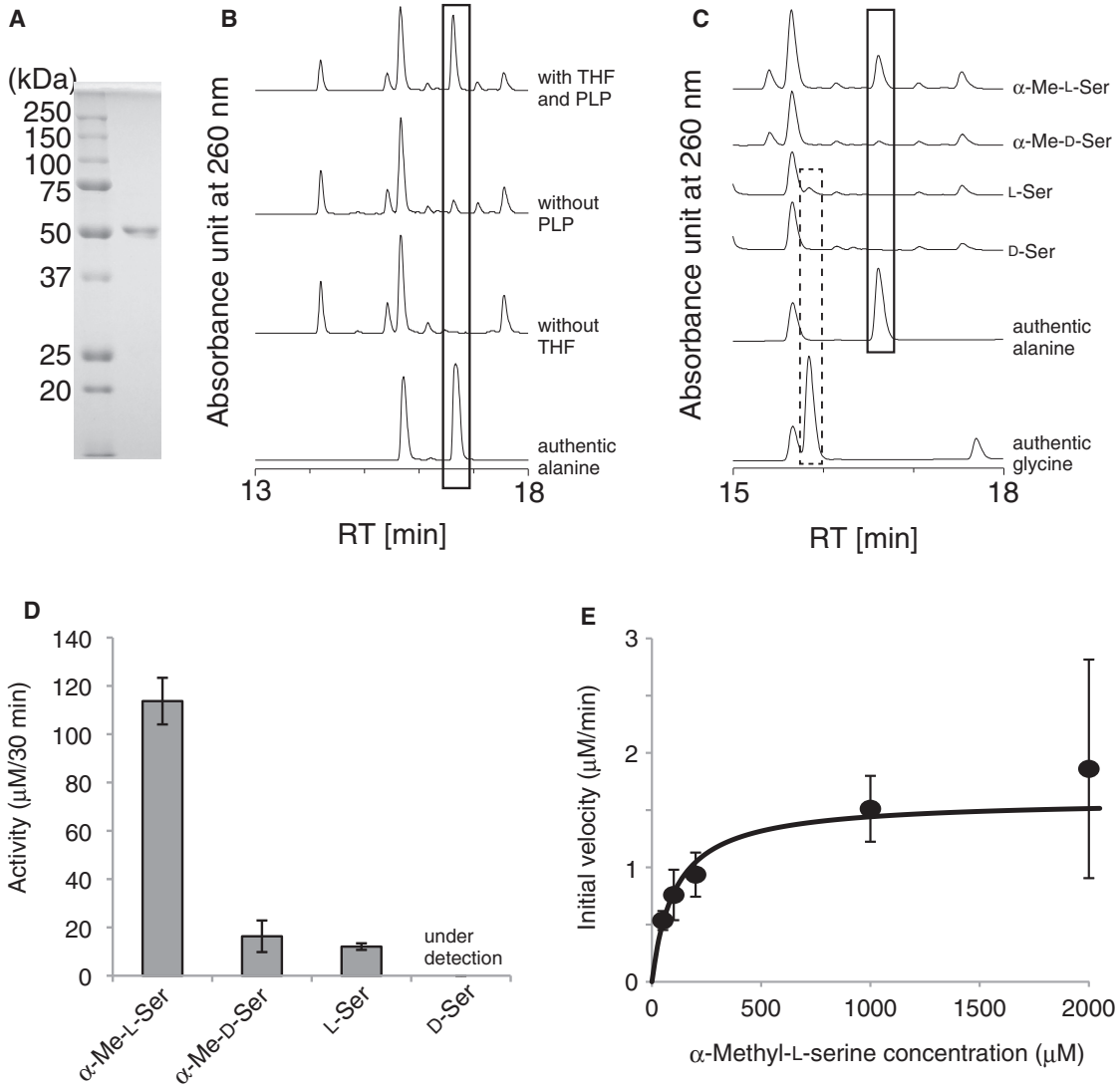

Figure 4. In Vitro Analysis of FmoH

(A) SDS-PAGE analysis of purified FmoH.

(B) Cofactor dependence of the reaction catalyzed by FmoH. FmoH required THF for the synthesis of D-alanine. The addition of PLP enhanced the reaction, which supported the observation that FmoH was not copurified with PLP. The box indicates the derivatized alanine.

(C) LC-ESI MS analysis of the products synthesized by FmoH after derivatization. Significant quantities of alanine were synthesized when FmoH was incubated with α-methyl-L-serine. In contrast, the incubation of FmoH with α-methyl-D-serine and L-serine resulted in the synthesis of trace amounts of alanine and glycine, respectively. The boxes drawn with solid and broken lines indicate the derivatized alanine and glycine compounds, respectively.

(D) The activity of FmoH in the synthesis of alanine or glycine from different substrates. Error bars represent SEM (n = 3).

(E) Kinetic analysis of FmoH using α-methyl-L-serine as a substrate. Error bars represent SEM (n = 3).

6-chloro-4-hydroxyindole-3-carboxylic acid (**3**; Figure 5B). This result is consistent with the previous observation that compound **3** accumulated in the Δ*fmoA3* mutant. The recombinant FmoA3 showed significant adenylating activity when it was incubated with α-methyl-L-serine, suggesting that α-methyl-L-serine is the physiological substrate of the A domain of FmoA3 (Figure 5C). This result supports our hypothesis that 4-methyloxazoline is synthesized from α-methyl-L-serine. Recombinant FmoA4 preferred D-alanine as a substrate, and this result was consistent with the structures of JBIR-34 and -35. Although the substrate specificity of FmoA5 appeared to be relatively broad, it showed a significant preference for L-serine. This result was also consistent with the structures of JBIR-34 and -35.

DISCUSSION

According to our results, we propose that JBIR-34 and -35 are synthesized as follows (Figure 6). First, tryptophan is halogenated by the activity of FmoD. FmoE, a flavin reductase, probably stimulates the halogenation by reducing the flavin cofactor, which is essential for halogenation. The obtained

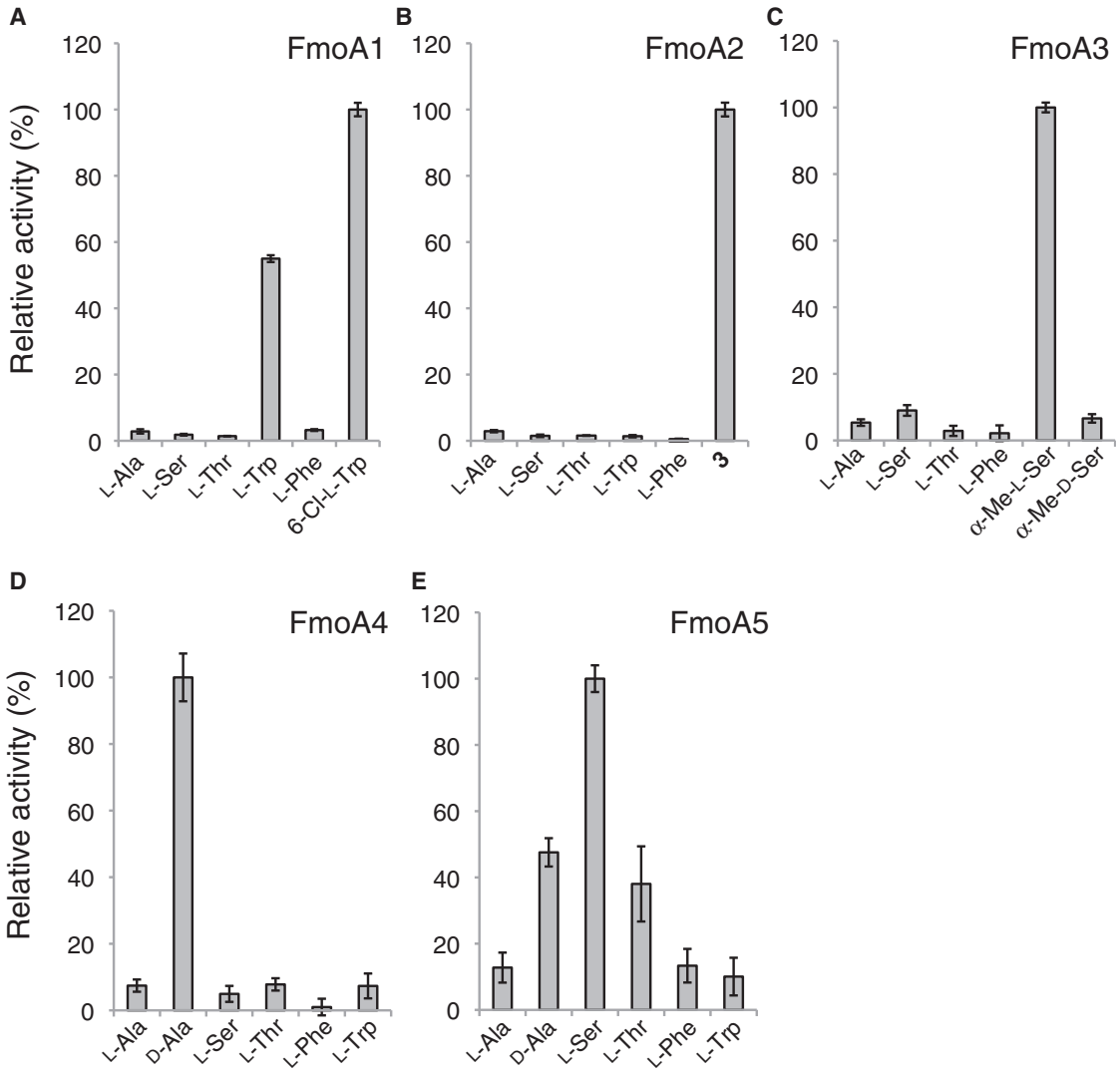

Figure 5. Amino Acid Selectivity of the FmoA Proteins

(A–E) The relative adenylation activity was estimated by quantifying phosphate using the malachite green phosphate assay kit. Phosphate was produced from pyrophosphate, which was produced during the adenylation of amino acids (or compound **3**). Error bars represent SEM (n = 3).

(A) The FmoA1 preferred 6-chloro-L-tryptophan to other amino acids.

(B) The FmoA2 preferred 6-chloro-4-hydroxyindole-3-carboxylic acid (**3**).

(C) FmoA3 preferred α-methyl-L-serine.

(D) FmoA4 preferred D-alanine.

(E) FmoA5 preferred L-serine.

6-chlorotryptophan is then activated by FmoA1 and covalently binds with the active site of the T domain of FmoA1 via a thioester bond. The 6-chlorotryptophan attached to FmoA1 is oxidized to β-hydroxyamino acid by the activity of FmoC, a heme-iron-dependent monooxygenase. Based on our present results, we could not completely clarify at which stage the hydroxylation of the indole ring occurs. We assumed that FmoC is a dual function P450, which catalyzes hydroxylation of the indole ring in addition to β-hydroxylation. As there are several examples of P450 that catalyzes hydroxylation at different positions (Podust and Sherman, 2012), this hypothesis is not astounding. A dihydroxy intermediate is then released from the T domain by the activity of FmoB. The released intermediate is then converted to 6-chloro-4-hydroxyindole-3-carboxylic acid (**3**) by *retro*-aldol reaction catalyzed by FmoM and oxidation catalyzed by FmoI. In parallel, α-methyl-L-serine is synthesized from D-alanine by the activity of FmoH. FmoA2, FmoA3, FmoA4, and FmoA5 assemble 6-chloro-4-hydroxyindole-3-carboxylic acid with α-methyl-L-serine, D-alanine, and L-serine to form a linear peptide. 4-Methyloxazoline formation occurs during the peptide elongation, when the intermediate is attached to FmoA3. The methylations of the indole ring are probably catalyzed by the MT domains of FmoA2 and FmoA4 during the peptide elongation. The D-alanine used for the synthesis of the α-methyl-L-serine and the

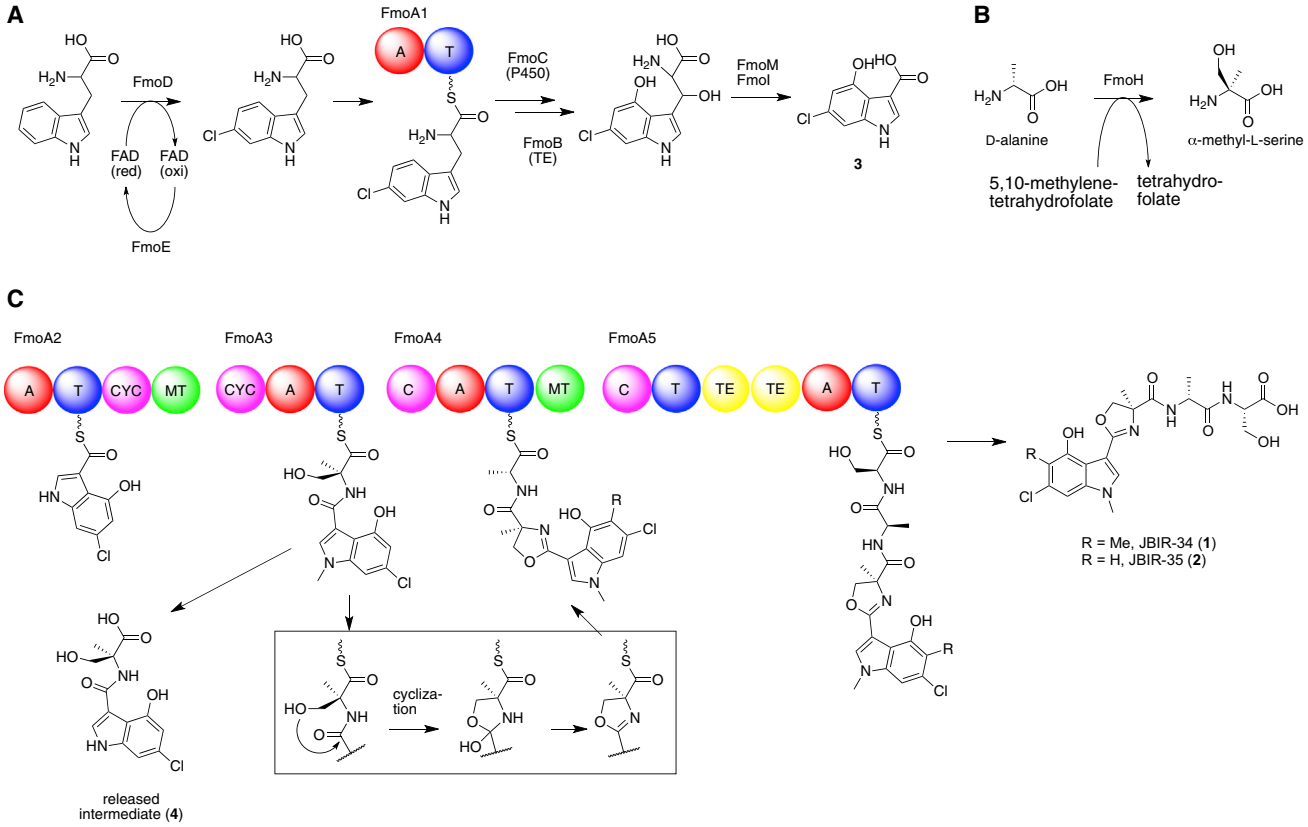

Figure 6. Proposed Biosynthesis Pathway of JBIR-34 and -35

First, 6-chloro-4-hydroxyindole-3-carboxylic acid (**3**) is synthesized from tryptophan (A) and α-methyl-L-serine is synthesized from D-alanine (B). Compound **3** is assembled with α-methyl-L-serine, D-alanine, and L-serine by four NRPSs, FmoA2, FmoA3, FmoA4, and FmoA5, resulting in JBIR-34 and -35 (C). The methylation of the indole moiety is probably catalyzed by the MT domains of FmoA2 and FmoA4.

elongation of the peptide was presumably synthesized by the alanine racemase involved in the biosynthesis of peptidoglycan (Radkov and Moe, 2014). Only one alanine racemase gene was found in the draft genome sequence of *Streptomyces* sp. Sp080513GE-23, and the product of this gene showed a high level of sequence homology to the alanine racemase from *Streptomyces coelicolor* A3(2) (82% identity). It is highly likely that the enzyme encoded by this gene would be responsible for the biosynthesis of D-alanine in *Streptomyces* sp. Sp080513GE-23.

The most important enzyme for 4-methyloxazoline moiety biosynthesis is FmoH, which catalyzes the formation of α-methyl-L-serine from D-alanine and mTHF. To date, there are only a few natural products that are composed of α-methylserine. Amicetin isolated from *S. vinaceusdrappus* NRRL 2363, citreamicins from *Micromonospora citrea* (Carter et al., 1990), conagenin from *Streptomyces roseosporus* (Yamashita et al., 1991), neocitreamicins from *Nocardia* (Peoples et al., 2008), oxamicetin (Konishi et al., 1973) from *Arthrobacter oxamicetus* sp. nov., piperazimycin from marine-derived *Streptomyces* (Miller et al., 2007), JBIR-111 from *Streptomyces sp*. RM72 (Hosoya et al., 2012), and JBIR-39 from *Streptomyces* sp. Sp080513SC-24 (Kozone et al., 2011) are almost all natural products reported to contain an α-methylserine moiety. FmoH homologs are probably involved in the biosynthesis of these compounds. Indeed, the biosynthesis gene cluster of amicetin has an FmoH homolog and this enzyme has been predicted to be responsible for α-methyl-L-serine synthesis (Zhang et al., 2012). However, the activity of this enzyme was not biochemically elucidated. Our in vitro analysis of FmoH is a characterization of α-methyl-L-serine synthase that correlates with NRP biosynthesis.

To date, there are only a few examples of A domains that are predicted to recognize α-methyl-L-serine. Therefore, there is still not enough information to deduce the selectivity of these A domains. Further examples of A domains recognizing α-methyl-L-serine and structural elucidation of the domains are required to reveal the amino acid residues that are responsible for the recognition of α-methyl-L-serine. By BLAST search using FmoH as a query, we discovered several FmoH homologs that are encoded adjacent to the NRPS genes in *Azospirillum* sp. B510, *Streptomyces hygroscopicus* subsp. jinggangensis 5008, *Streptomyces griseus* subsp. griseus NBRC 13350, *Bacillus amyloliquefaciens* DSM 7, and *Pseudomonas* sp. GM17. We built a phylogenetic tree using the A domains of FmoH-related NRPSs and typical NRPSs, such as the NRPS responsible for daptomycin biosynthesis, and found that these FmoH-related NRPSs, except for NRPSs from *S. hygroscopicus* subsp. jinggangensis 5008, have one A domain that falls into the same clade as the A domains from FmoA3 and AmiT, which is the

NRPS responsible for amicetin biosynthesis (Figure S6). Although these strains are not known to produce peptides containing α-methyl-L-serine, this bioinformatic analysis suggests that these strains may be potential producers of such peptides, and that NRPs containing α-methyl-L-serine may be produced by a broader range of organisms than expected. The FmoH-based genome mining may result in the discovery of novel compounds containing an α-methyl-L-serine moiety.

SIGNIFICANCE

In this study, we identified the biosynthetic origin of the unusual 4-methyloxazoline moiety of JBIR-34 and -35 as α-methyl-L-serine. We showed that the A domain of FmoA3 recognizes α-methyl-L-serine. Because the ability of NRPSs to utilize nonproteinogenic amino acids is important for the structural diversity of NRPs, this study will open the way for synthesizing novel NRPs by genetic engineering of NRPSs. We also revealed that FmoH, a glycine/serine hydroxymethyltransferase homolog, catalyzed the conversion between D-alanine and α-methyl-L-serine, indicating that FmoH functions as an α-methyl-L-serine synthase. Our in vitro analysis of FmoH is a characterization of α-methyl-L-serine synthase that correlates with NRP biosynthesis. Identification of the α-methyl-L-serine-recognizing A domain and α-methyl-L-serine synthase will facilitate genome mining of novel NRPs.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of Compounds 3 and 4

Streptomyces sp. Sp080513GE-23 was inoculated into 100 ml of ISP-2 medium (see Supplemental Experimental Procedures) and incubated at 30°C for 2 days. A portion (20 ml) of the culture was transferred into 1 l of ISP-2 medium and further incubated at 30°C for 5 days. Then, 20 g of HP20 was added to the culture and stirred at room temperature for 2 hr. The mixture of cells and HP20 was harvested by centrifugation, and then extracted with methanol. The extract was evaporated to dryness. The residual materials were applied to medium pressure liquid chromatography (Purif-Compact A, Shoko Scientific) equipped with an ODS column (Purif-Pack ODS, SIZE60, Shoko Scientific). Compounds were eluted using linear gradient from solvent A (10% methanol containing 0.1% formic acid) to solvent B (methanol containing 0.1% formic acid). The fractions containing compounds **3** and **4** were evaporated to dryness, and compounds **3** and **4** in the residual material were further purified by high performance liquid chromatography equipped with COSMOSIL C_{18}-AR-II (10 mm × 250 mm). The compounds were eluted with a linear gradient of 50%–100% methanol containing 0.1% formic acid, resulting in 1.1 mg of compound **3** and 0.6 mg of compound **4**. The ^{1}H NMR, ^{13}C NMR, COSY, HMQC, and HMBC spectra of compound **3** were recorded in dimethyl sulfoxide (DMSO)-d_6 on a JNM-A500 NMR System (JEOL, Tokyo, Japan). The ^{1}H NMR, ^{13}C NMR, COSY, HMQC, and HMBC spectra of compound **4** were recorded in DMSO-d_6 on a Varian 600 NB CL NMR System (Varian).

Construction of the *Streptomyces* sp. Sp080513GE-23 Δ*fmoA3* Mutant

A PCR targeting system using the cosmid pTJ34-3 carrying the gene cluster was used to construct the deletion mutant. The pKGLP2 vector carrying a beta-glucuronidase as a selection marker (Myronovskyi et al., 2011) was used. The details are described in Supplemental Experimental Procedures.

Production, Extraction, and LC-ESI MS Analysis of JBIR-34 and -35

Streptomyces sp. Sp080513GE-23 or the Δ*fmoA3* mutant was inoculated into 10 ml ISP-2 medium and incubated at 30°C for 2 days. Two and a half milliliters of the culture were transferred to fresh 100 ml ISP-2 medium and further incubated at 30°C for 5 days. The supernatant was extracted with ethyl acetate three times and further extracted with butanol twice. The butanol layer was evaporated to dryness and the residual material was dissolved in 200 μl of DMSO for liquid chromatography-electron spray ionization mass spectrometry (LC-ESI MS) analysis. LC-ESI MS was carried out using Agilent 1100 series (Agilent Technologies) and the high-capacity trap plus system (Bruker Daltonics) equipped with a MonoBis column (2 × 50 mm; Kyoto Monotech). The compounds were eluted with a linear gradient using water and acetonitrile containing 0.1% formic acid as the mobile phase. The amounts of JBIR-34 and -35 and compound **3** were estimated by integrating the corresponding peak areas in the chromatogram (absorbance unit at 300 nm) and comparing these values to those of the purified samples.

Feeding Experiments Using [Methyl-^{13}C]-L-Methionine and [3-^{13}C]-L-Alanine

[Methyl-^{13}C]-L-methionine and [3-^{13}C]-L-alanine were purchased from ISOTEC and Cambridge Isotope Laboratories, respectively. *Streptomyces* sp. Sp080513GE-23 was inoculated into 10 ml ISP-2 medium and incubated at 30°C for 2 days. Fifty microliters of the culture were transferred to fresh 5 ml ISP-2 medium containing 2 g/l of [methyl-^{13}C]-L-methionine or [3-^{13}C]-L-alanine and further incubated at 30°C for 5 days. The supernatant was extracted with ethyl acetate three times and further extracted with butanol twice. The butanol layer was evaporated to dryness and the residual material was dissolved in 200 μl of DMSO for LC-ESI MS analysis. LC-ESI MS was carried out as described previously. HR-MS/MS analysis was carried out using Waters SYNAPT G2 (Waters).

Expression and Purification of FmoH

pET28a-*fmoH* was constructed by cloning the *fmoH* gene amplified by PCR into the NdeI and HindIII sites of pET28a. From the *E. coli* BL21(DE3) harboring pET28a-*fmoH*, FmoH fused N-terminally with a histidine tag was purified as described in Supplemental Experimental Procedures.

In Vitro Assay of FmoH

The standard reaction mixture (100 μl) contained 2 μM of FmoH, 1 mM substrate, 0.5 mM THF, 10 μM PLP, and 50 mM potassium phosphate (pH 7.5). After incubation at 30°C for 1 hr, the reaction was quenched by adding 100 μl of methanol (for FDLA derivatization) or acetonitrile (for 9-fluorenylmethyloxycarbonyl derivatization). After derivatization was performed as described in the Supplemental Experimental Procedures, the products were analyzed with LC-ESI MS equipped with a MonoBis column (2 × 50 mm; Kyoto Monotech). Compounds were eluted with a linear gradient using water and acetonitrile containing 0.1% formic acid as the mobile phase. For the analysis of the kinetic parameters, the reaction mixture (50 μl) containing 4 μM of FmoH, different concentrations of α-methyl-L-serine (varied in the range of 50–2,000 μM), 0.5 mM THF, 20 μM PLP, and 50 mM potassium phosphate (pH 7.5) was incubated at 30°C for 5 min. The reaction was quenched by the addition of 100 μl of acetonitrile. The 9-fluorenylmethyloxycarbonyl derivatives were then analyzed with LC-ESI MS, and the yields were estimated by comparing the peak areas with those of the authentic standards. The kinetic parameters were calculated by fitting the substrate concentration-initial velocity plot to the equitation $V_{max}*[S]/(K_m+[S])$.

Expression and Purification of FmoA Proteins

The DNA fragments encoding whole *fmoA1*, *fmoA2*, *fmoA3*, *fmoA4*, and *fmoA5* were amplified by PCR and cloned into pColdI (Takara), resulting in pColdI-*fmoA1*, *2*, *3*, *4*, and *5*. From the *E. coli* BL21(DE3) harboring one of these plasmids, each FmoA fused N-terminally with a histidine tag was purified as described in the Supplemental Experimental Procedures.

In Vitro Analysis of Adenylating Activity

Each FmoA protein (1 μM) was incubated with 5 mM possible substrates in 80 μl of buffer containing 150 mM hydroxylamine, 1 mM dithiothreitol, 0.4 U/ml pyrophosphatase (Sigma), 0.5 mM ATP, 10 mM $MgCl_2$, and 50 mM Tris-HCl buffer (pH 7.5). Hydroxylamine solution was prepared according to the method reported by Kadi and Challis (2009). The reaction mixture was preincubated for 2 min, and the reaction was initiated by adding ATP. After 30-min incubation, the reaction was quenched by adding 20 μl of working

reagent of malachite green phosphate assay kit (BioAssay Systems). After a 1.5-hr incubation at room temperature, absorption at 620 nm (A_{620}) was measured. We subtracted the A_{620} value of the reaction mixture with no substrate from the A_{620} value of a reaction mixture containing the substrate to estimate the relative adenylation activity.

ACCESSION NUMBERS

The DNA Data Bank of Japan accession number for the nucleotide sequence of the *fmo* gene cluster reported in this paper is AB902962.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.06.004.

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REFERENCES

Barnard-Britson, S., Chi, X., Nonaka, K., Spork, A.P., Tibrewal, N., Goswami, A., Pahari, P., Ducho, C., Rohr, J., and Van Lanen, S.G. (2012). Amalgamation of nucleosides and amino acids in antibiotic biosynthesis: discovery of an L-threonine:uridine-5′-aldehyde transaldolase. J. Am. Chem. Soc. *134*, 18514–18517.

Bhushan, R., and Brückner, H. (2004). Marfey's reagent for chiral amino acid analysis: a review. Amino Acids *27*, 231–247.

Buntin, K., Weissman, K.J., and Müller, R. (2010). An unusual thioesterase promotes isochromanone ring formation in ajudazol biosynthesis. ChemBioChem *11*, 1137–1146.

Carter, G.T., Nietsche, J.A., Williams, D.R., and Borders, D.B. (1990). Citreamicins, novel antibiotics from *Micromonospora citrea*: isolation, characterization, and structure determination. J. Antibiot. *43*, 504–512.

Chen, H., and Walsh, C.T. (2001). Coumarin formation in novobiocin biosynthesis: beta-hydroxylation of the aminoacyl enzyme tyrosyl-S-NovH by a cytochrome P450 NovI. Chem. Biol. *8*, 301–312.

Chen, H., Hubbard, B.K., O'Connor, S.E., and Walsh, C.T. (2002). Formation of beta-hydroxy histidine in the biosynthesis of nikkomycin antibiotics. Chem. Biol. *9*, 103–112.

Choi, H., Pereira, A.R., Cao, Z., Shuman, C.F., Engene, N., Byrum, T., Matainaho, T., Murray, T.F., Mangoni, A., and Gerwick, W.H. (2010). The hoiamides, structurally intriguing neurotoxic lipopeptides from Papua New Guinea marine cyanobacteria. J. Nat. Prod. *73*, 1411–1421.

di Salvo, M.L., Remesh, S.G., Vivoli, M., Ghatge, M.S., Paiardini, A., D'Aguanno, S., Safo, M.K., and Contestabile, R. (2014). On the catalytic mechanism and stereospecificity of *Escherichia coli* L-threonine aldolase. FEBS J. *281*, 129–145.

Galm, U., Schimana, J., Fiedler, H.-P., Schmidt, J., Li, S.-M., and Heide, L. (2002). Cloning and analysis of the simocyclinone biosynthetic gene cluster of *Streptomyces antibioticus* Tü 6040. Arch. Microbiol. *178*, 102–114.

Gutierrez, M.L., Garrabou, X., Agosta, E., Servi, S., Parella, T., Joglar, J., and Clapés, P. (2008). Serine hydroxymethyl transferase from *Streptococcus thermophilus* and L-threonine aldolase from *Escherichia coli* as stereocomplementary biocatalysts for the synthesis of beta-hydroxy-alpha, omega-diamino acid derivatives. Chemistry *14*, 4647–4656.

Hosoya, T., Hirokawa, T., Takagi, M., and Shin-ya, K. (2012). Trichostatin analogues JBIR-109, JBIR-110, and JBIR-111 from the marine sponge-derived *Streptomyces* sp. RM72. J. Nat. Prod. *75*, 285–289.

Ishida, K., Lincke, T., Behnken, S., and Hertweck, C. (2010). Induced biosynthesis of cryptic polyketide metabolites in a *Burkholderia thailandensis* quorum sensing mutant. J. Am. Chem. Soc. *132*, 13966–13968.

Kadi, N., and Challis, G.L. (2009). Chapter 17. Siderophore biosynthesis a substrate specificity assay for nonribosomal peptide synthetase-independent siderophore synthetases involving trapping of acyl-adenylate intermediates with hydroxylamine. Methods Enzymol. *458*, 431–457.

Koglin, A., Löhr, F., Bernhard, F., Rogov, V.V., Frueh, D.P., Strieter, E.R., Mofid, M.R., Güntert, P., Wagner, G., Walsh, C.T., et al. (2008). Structural basis for the selectivity of the external thioesterase of the surfactin synthetase. Nature *454*, 907–911.

Konishi, M., Kimeda, M., Tsukiura, H., Yamamoto, H., and Hoshiya, T. (1973). Oxamicetin, a new antibiotic of bacterial origin. I. Production, isolation and properties. J. Antibiot. *26*, 752–756.

Kostichka, K., Thomas, S.M., Gibson, K.J., Nagarajan, V., and Cheng, Q. (2001). Cloning and characterization of a gene cluster for cyclododecanone oxidation in *Rhodococcus ruber* SC1. J. Bacteriol. *183*, 6478–6486.

Kozone, I., Izumikawa, M., Motohashi, K., Nagai, A., Yoshida, M., Doi, T., Takagi, N., and Shin-ya, K. (2011). Isolation of new hexapeptides-JBIR-39 and JBIR-40 from a marine sponge-derived *Streptomyces* sp. Sp080513SC-24. J. Marine Sci. Res. Develop. *1*, 1000101.

Li, W., Khullar, A., Chou, S., Sacramo, A., and Gerratana, B. (2009). Biosynthesis of sibiromycin, a potent antitumor antibiotic. Appl. Environ. Microbiol. *75*, 2869–2878.

Martin, J.F. (2000). Alpha-aminoadipyl-cysteinyl-valine synthetases in beta-lactam producing organisms. From Abraham's discoveries to novel concepts of non-ribosomal peptide synthesis. J. Antibiot. *53*, 1008–1021.

McQuade, T.J., Shallop, A.D., Sheoran, A., Delproposto, J.E., Tsodikov, O.V., and Garneau-Tsodikova, S. (2009). A nonradioactive high-throughput assay for screening and characterization of adenylation domains for nonribosomal peptide combinatorial biosynthesis. Anal. Biochem. *386*, 244–250.

Miao, V., Coëffet-Legal, M.F., Brian, P., Brost, R., Penn, J., Whiting, A., Martin, S., Ford, R., Parr, I., Bouchard, M., et al. (2005). Daptomycin biosynthesis in *Streptomyces roseosporus*: cloning and analysis of the gene cluster and revision of peptide stereochemistry. Microbiology *151*, 1507–1523.

Miller, E.D., Kauffman, C.A., Jensen, P.R., and Fenical, W. (2007). Piperazimycins: cytotoxic hexadepsipeptides from a marine-derived bacterium of the genus *Streptomyces*. J. Org. Chem. *72*, 323–330.

Motohashi, K., Takagi, M., and Shin-Ya, K. (2010). Tetrapeptides possessing a unique skeleton, JBIR-34 and JBIR-35, isolated from a sponge-derived actinomycete, *Streptomyces* sp. Sp080513GE-23. J. Nat. Prod. *73*, 226–228.

Myronovskyi, M., Welle, E., Fedorenko, V., and Luzhetskyy, A. (2011). Beta-glucuronidase as a sensitive and versatile reporter in actinomycetes. Appl. Environ. Microbiol. *77*, 5370–5383.

Nguyen, K.T., Ritz, D., Gu, J.Q., Alexander, D., Chu, M., Miao, V., Brian, P., and Baltz, R.H. (2006). Combinatorial biosynthesis of novel antibiotics related to daptomycin. Proc. Natl. Acad. Sci. USA *103*, 17462–17467.

Nozaki, H., Kuroda, S., Watanabe, K., and Yokozeki, K. (2008). Cloning of the gene encoding alpha-methylserine hydroxymethyltransferase from *Aminobacter* sp. AJ110403 and *Ensifer* sp. AJ110404 and characterization of the recombinant enzyme. Biosci. Biotechnol. Biochem. *72*, 3002–3005.

Nozaki, H., Kuroda, S., and Watanabe, K. (2009). Screening of microorganisms producing α-methylserine hydroxymethyltransferase, purification of the enzyme, gene cloning, and application to the enzymatic synthesis of α-methyl-L-serine. J. Mol. Catal., B Enzym. *56*, 221–226.

Onaka, H., Taniguchi, S., Ikeda, H., Igarashi, Y., and Furumai, T. (2003). pTOYAMAcos, pTYM18, and pTYM19, actinomycete-*Escherichia coli* integrating vectors for heterologous gene expression. J. Antibiot. *56*, 950–956.

Pai, V.R., Rajaram, V., Bisht, S., Bhavani, B.S., Rao, N.A., Murthy, M.R., and Savithri, H.S. (2009). Structural and functional studies of *Bacillus stearothermophilus* serine hydroxymethyltransferase: the role of Asn(341), Tyr(60) and Phe(351) in tetrahydrofolate binding. Biochem. J. *418*, 635–642.

Patel, H.M., Tao, J., and Walsh, C.T. (2003). Epimerization of an L-cysteinyl to a D-cysteinyl residue during thiazoline ring formation in siderophore chain elongation by pyochelin synthetase from *Pseudomonas aeruginosa*. Biochemistry *42*, 10514–10527.

Peoples, A.J., Zhang, Q., Millett, W.P., Rothfeder, M.T., Pescatore, B.C., Madden, A.A., Ling, L.L., and Moore, C.M. (2008). Neocitreamicins I and II, novel antibiotics with activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci*. J. Antibiot. *61*, 457–463.

Podust, L.M., and Sherman, D.H. (2012). Diversity of P450 enzymes in the biosynthesis of natural products. Nat. Prod. Rep. *29*, 1251–1266.

Radkov, A.D., and Moe, L.A. (2014). Bacterial synthesis of D-amino acids. Appl. Microbiol. Biotechnol. *98*, 5363–5374.

Rausch, C., Hoof, I., Weber, T., Wohlleben, W., and Huson, D.H. (2007). Phylogenetic analysis of condensation domains in NRPS sheds light on their functional evolution. BMC Evol. Biol. *7*, 78.

Recktenwald, J., Shawky, R., Puk, O., Pfennig, F., Keller, U., Wohlleben, W., and Pelzer, S. (2002). Nonribosomal biosynthesis of vancomycin-type antibiotics: a heptapeptide backbone and eight peptide synthetase modules. Microbiology *148*, 1105–1118.

Röttig, M., Medema, M.H., Blin, K., Weber, T., Rausch, C., and Kohlbacher, O. (2011). NRPSpredictor2—a web server for predicting NRPS adenylation domain specificity. Nucleic Acids Res. *39*, W362–W367.

Royer, M., Costet, L., Vivien, E., Bes, M., Cousin, A., Damais, A., Pieretti, I., Savin, A., Megessier, S., Viard, M., et al. (2004). Albicidin pathotoxin produced by *Xanthomonas albilineans* is encoded by three large PKS and NRPS genes present in a gene cluster also containing several putative modifying, regulatory, and resistance genes. Mol. Plant Microbe Interact. *17*, 414–427.

Schirch, L., and Gross, T. (1968). Serine transhydroxymethylase. Identification as the threonine and allothreonine aldolases. J. Biol. Chem. *243*, 5651–5655.

Shindo, K., Yamagishi, Y., and Kawai, H. (1993). Thiazohalostatin, a new cytoprotective substance produced by Actinomadura. II. Physico-chemical properties and structure determination. J. Antibiot. *46*, 1638–1642.

Steffensky, M., Mühlenweg, A., Wang, Z.X., Li, S.M., and Heide, L. (2000). Identification of the novobiocin biosynthetic gene cluster of *Streptomyces spheroides* NCIB 11891. Antimicrob. Agents Chemother. *44*, 1214–1222.

Tsukamoto, M., Murooka, K., Nakajima, S., Abe, S., Suzuki, H., Hirano, K., Kondo, H., Kojiri, K., and Suda, H. (1997). BE-32030 A, B, C, D and E, new antitumor substances produced by *Nocardia* sp. A32030. J. Antibiot. *50*, 815–821.

Ungermannova, D., Parker, S.J., Nasveschuk, C.G., Wang, W., Quade, B., Zhang, G., Kuchta, R.D., Phillips, A.J., and Liu, X. (2012). Largazole and its derivatives selectively inhibit ubiquitin activating enzyme (e1). PLoS One *7*, e29208.

Walsh, C.T. (2004). Polyketide and nonribosomal peptide antibiotics: modularity and versatility. Science *303*, 1805–1810.

Walsh, C.T., O'Brien, R.V., and Khosla, C. (2013). Nonproteinogenic amino acid building blocks for nonribosomal peptide and hybrid polyketide scaffolds. Angew. Chem. Int. Ed. Engl. *52*, 7098–7124.

Yamashita, T., Iijima, M., Nakamura, H., Isshiki, K., Naganawa, H., Hattori, S., Hamada, M., Ishizuka, M., Takeuchi, T., and Iitaka, Y. (1991). Conagenin, a low molecular weight immunomodulator produced by *Streptomyces roseosporus*. J. Antibiot. *44*, 557–559.

Zhang, G., Zhang, H., Li, S., Xiao, J., Zhang, G., Zhu, Y., Niu, S., Ju, J., and Zhang, C. (2012). Characterization of the amicetin biosynthesis gene cluster from *Streptomyces vinaceusdrappus* NRRL 2363 implicates two alternative strategies for amide bond formation. Appl. Environ. Microbiol. *78*, 2393–2401.

