JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

COMMUNICATION

pubs.acs.org/JACS

A Natural Protecting Group Strategy To Carry an Amino Acid Starter Unit in the Biosynthesis of Macrolactam Polyketide Antibiotics

Yuji Shinohara,[†] Fumitaka Kudo,[‡] and Tadashi Eguchi*[,†]

[†]Department of Chemistry and Materials Science and [‡]Department of Chemistry, Tokyo Institute of Technology, 2-12-1 O-okayama, Meguro-ku, Tokyo 152-8551, Japan

Supporting Information

ABSTRACT: Macrolactam antibiotics are an important class of macrocyclic polyketides that contain a unique nitrogen-containing starter unit. In the present study, a set of starter biosynthetic enzymes in the macrolactam antibiotic vicenistatin was characterized. We found that the protection−deprotection strategy of the aminoacyl-ACP intermediate was critical in this system. On the basis of bioinformatics, the described pathway is also proposed as a common method for carrying amino acids in the biosynthesis of other macrolactam antibiotics.

Macrolactam polyketide antibiotics include the clinically important rifamycin, ansamitocins, and leinamycin (Figure S1 in the Supporting Information). These macrolactam antibiotics contain a unique nitrogen-containing unit at the starter position of polyketide chain elongation. Vicenistatin (Figure 1) belongs to the family of macrolactam antibiotics produced by *Streptomyces halstedii* HC34 and has a unique nitrogen-containing unit, 3-amino-2-methylpropionate (Amp), at the starter position of the polyketide.[1] We speculated that this Amp moiety would be derived from universal amino acids via a unique starter biosynthetic pathway. Our previous incorporation studies clearly showed that the Amp moiety of vicenilactam is derived from L-glutamate via (2*S*,3*S*)-3-methylaspartate (3-MeAsp) and not from (2*S*,3*R*)-3-MeAsp or Amp itself (Figure 1).[2−4]

The biosynthetic gene cluster for vicenistatin has a contiguous gene cluster consisting of glutamate mutase VinH and VinI, peptidase VinJ, acyltransferase VinK, acyl carrier protein (ACP) VinL, two ATP-dependent ligases (VinM and VinN), and pyridoxal 5′-phosphate (PLP)-dependent decarboxylase VinO, which have been proposed to be starter biosynthetic enzymes (Table S1 and Figure S2).[5] Gene inactivation for the glutamate mutase E subunit VinI encoded in the vicenistatin biosynthetic gene cluster abolished the production of vicenistatin, whereas the mutant recovered the production when 3-MeAsp was fed to the culture.[5,6] Thus, the glutamate mutase VinH/VinI appeared to convert L-glutamate to (2*S*,3*S*)-3-MeAsp, which must be carried onto polyketide synthase (PKS) by starter biosynthetic enzymes.

Among these proteins, VinJ, -K, -L, -M, and -N homologues are all conserved in structurally related macrolactam biosynthetic gene clusters, including salinilactam,[7] BE-14106,[8] and ML-449.[9] Thus, the starter biosynthesis of these macrolactam antibiotics was speculated to utilize a common strategy to carry their respective amino acids onto the corresponding PKSs. In addition, these hypothesized macrolactam starter biosynthetic genes were found in several genome sequences of actinomycetes along with PKS genes, indicating that such microorganisms potentially produce macrolactam compounds (Figure S2). This evidence prompted us to investigate the functions of enzymes in the vicenistatin biosynthetic gene cluster.

We hypothesized that the biosynthesis of the starter unit of vicenistatin is as follows. The free form of 3-MeAsp is synthesized by glutamate mutase VinH/VinI from L-glutamate. Next, one of ATP-dependent ligases (VinM or VinN) activates 3-MeAsp and ligates it to the acyl carrier protein VinL. To remove the carboxylate moiety of 3-MeAsp, the PLP-dependent decarboxylase VinO seems to catalyze the decarboxylation to afford the Amp moiety (presumably before entering into polyketide chain elongation). At this stage, another ATP-dependent ligase could capture the primary amino group of aminoacyl-ACP by the addition of an acyl group to prevent internal cyclization (especially thermodynamically stable six-membered lactam formation). Finally, the terminal acyl group could be hydrolyzed by peptidase VinJ before macrolactam formation catalyzed by the thioesterase domain of VinP4.[10] On the basis of this hypothesis (Figure 1), we carried out the enzymatic analysis of VinJ, -K, -L, -M, -N, and -O.

VinM and VinN proteins were predicted to behave as free-standing adenylation domains with two half-reactions in the nonribosomal peptide synthetase (NRPS) system. The first is the ATP-dependent activation of the carboxyl group of the amino acid to give the aminoacyl adenylate as an intermediate, which is then transferred onto the ACP (VinL) to give the aminoacyl-ACP. To detect the activity of the first half-reaction, we conducted a typical photometric assay by detecting released pyrophosphate. As a result, VinM was found to recognize small amino acids such as L-alanine, glycine, and L-serine (Figure S3). Conversely, VinN specifically recognized 3-MeAsp. Furthermore, the holo form of VinL (which was obtained by coexpression with the *sfp* gene in *Escherichia coli*[11]) was added to the VinN reaction mixture of ATP and 3-MeAsp. Liquid chromatography−electrospray ionization mass spectrometry (LC-ESI-MS) analyses of the reaction mixture clearly showed the formation of 3-MeAsp-VinL (Figure 2A). Therefore, VinN was found to catalyze the activation of 3-MeAsp as well as the transfer of 3-MeAsp onto VinL.

The formed 3-MeAsp-VinL then seemed to be recognized by the PLP-dependent decarboxylase VinO before further modification. The hypothesized substrate 3-MeAsp-VinL produced by VinN with ATP and 3-MeAsp was purified by HPLC.

Received: September 22, 2011
Published: October 19, 2011

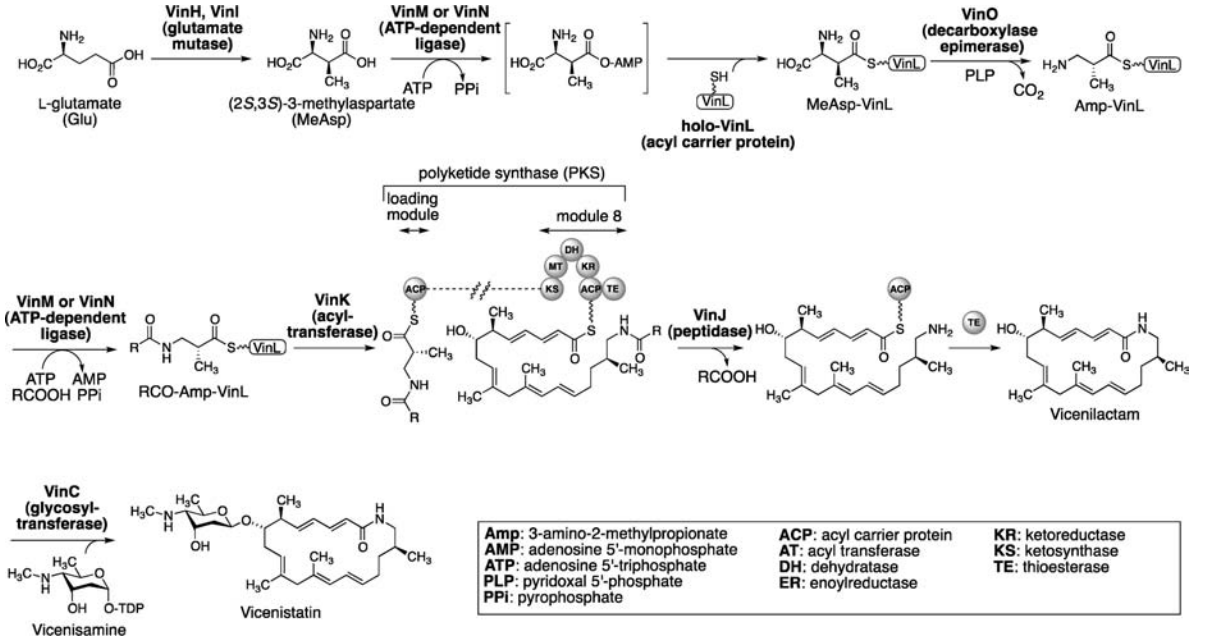

Figure 1. Biosynthetic pathway for vicenistatin. The functions of VinJ, -K, -L, -M, -N, and -O were characterized. In the newly characterized pathway, the generated Amp-VinL is acylated by the ATP-dependent ligase VinM to prevent internal cyclization, and the terminal acyl group (alanine) is removed by the peptidase VinJ before macrolactam formation. The presumable epimerization reaction at the α-position of Amp-VinL has not been confirmed.

LC-ESI-MS analyses of the VinO reaction mixture with 3-MeAsp-VinL showed the formation of decarboxylated Amp-VinL, although this peak eluted at a retention time similar to that for 3-MeAsp-VinL (Figure 2A). This PLP-dependent enzyme VinO is also proposed simultaneously to catalyze the epimerization of the C-2 position of Amp-VinL, but this was not confirmed in the present study.

On the basis of our working hypothesis, the generated Amp-VinL was then reacted with VinM, ATP, and L-alanine (the most preferable substrate of VinM). LC-ESI-MS analyses of the reaction mixture including VinM showed the signal for an alanine-attached Amp-VinL (Figure 2A). Glycine and serine were also transferred onto Amp-VinL to afford the corresponding dipeptidyl VinL (data not shown). Therefore, it was found that the ATP-dependent ligase VinM specifically captures the primary amino group of the Amp moiety in Amp-VinL. In addition, the one-pot reaction of VinN, -L, -O, and -M with substrate amino acids afforded only Ala-Amp-VinL as a reaction product, which should be selectively synthesized by these characteristic enzymes (data not shown).

The purified Ala-Amp-VinL from the one-pot reaction was then used as a substrate for acyltransferase VinK, which was speculated to catalyze the acyl transfer reaction between VinL and the VinP1-ACP domain at the loading module of PKS. In the present study, we expressed only the VinP1-ACP domain, which was indeed converted into the holo-form of ACP by Sfp. Two substrates, Ala-Amp-VinL and VinP1-ACP, were then reacted with VinK, and the reaction was analyzed by LS-ESI-MS (Figure 2C). As expected, the dipeptide transfer reaction occurred efficiently to give Ala-Amp-VinP1-ACP, which should become a starter molecule of vicenilactam PKS. Furthermore, to confirm the substrate specificity of VinK, three aminoacyl-VinLs (MeAsp-VinL, Amp-VinL, and Ala-Amp-VinL) at the same concentration were reacted with VinK and VinP1-ACP. As a result, only Ala-Amp-VinL was selectively transferred to VinP1-ACP (Figure 2C). Therefore, the substrate specificity of acyltransferase VinK against dipeptidyl-VinL appears to be crucial for entry into the vicenistatin PKS pathway.

Finally, removal of the alanyl moiety from the elongated polyketide chain seemed to be catalyzed by the peptidase VinJ before macrolactam formation by the terminal TE domain (VinP4-TE). To investigate the function of VinJ, we chemically synthesized *N*-alanyl-secovicenilactam ethyl ester (**1**) as a substrate analogue of the elongated polyketide intermediate (Figure S5). VinJ was found to hydrolyze **1** efficiently, affording secovicenilactam ethyl ester (**6**), which was then macrocyclized by VinP4-TE[10] to give vicenilactam (Figure 3). **1** itself was not macrocyclized by VinP4-TE, so the VinJ-catalyzed deprotection reaction is required for the completion of vicenilactam biosynthesis. BecP (a VinJ homologue) in BE-14106 biosynthesis was reported to catalyze the hydrolysis of L-alanine-*p*-nitroanilide (pNA), but glycine-pNA and L-proline-pNA were reported to be less-favored substrates.[8] Therefore, VinJ-type peptidases seem to catalyze the hydrolysis of N-terminal L-alaninyl substrates. Although the timing of removal of the terminal L-alanine was not revealed in the present study, removing the potential protecting group just before completion of the synthesis would seem to be a rational approach.

In this pathway, 3-MeAsp (which is generated by glutamate mutase VinH/VinI) is first activated by the ATP-dependent ligase VinN and then transferred onto ACP VinL to give 3-MeAsp-VinL, which is then decarboxylated to Amp-VinL by PLP-dependent decarboxylase VinO. Here, another ATP-dependent ligase (VinM) captures the primary amino group with small amino acids such as alanine to form dipeptidyl-VinL, which is then selectively transferred onto the loading ACP domain of PKS. In the final stage of macrolactam formation, the terminal aminoacyl group is removed by peptidase VinJ before macrolactamization by the VinP4-TE. As we observed, amino acid-carrying enzymes such as VinM and VinN are highly conserved in

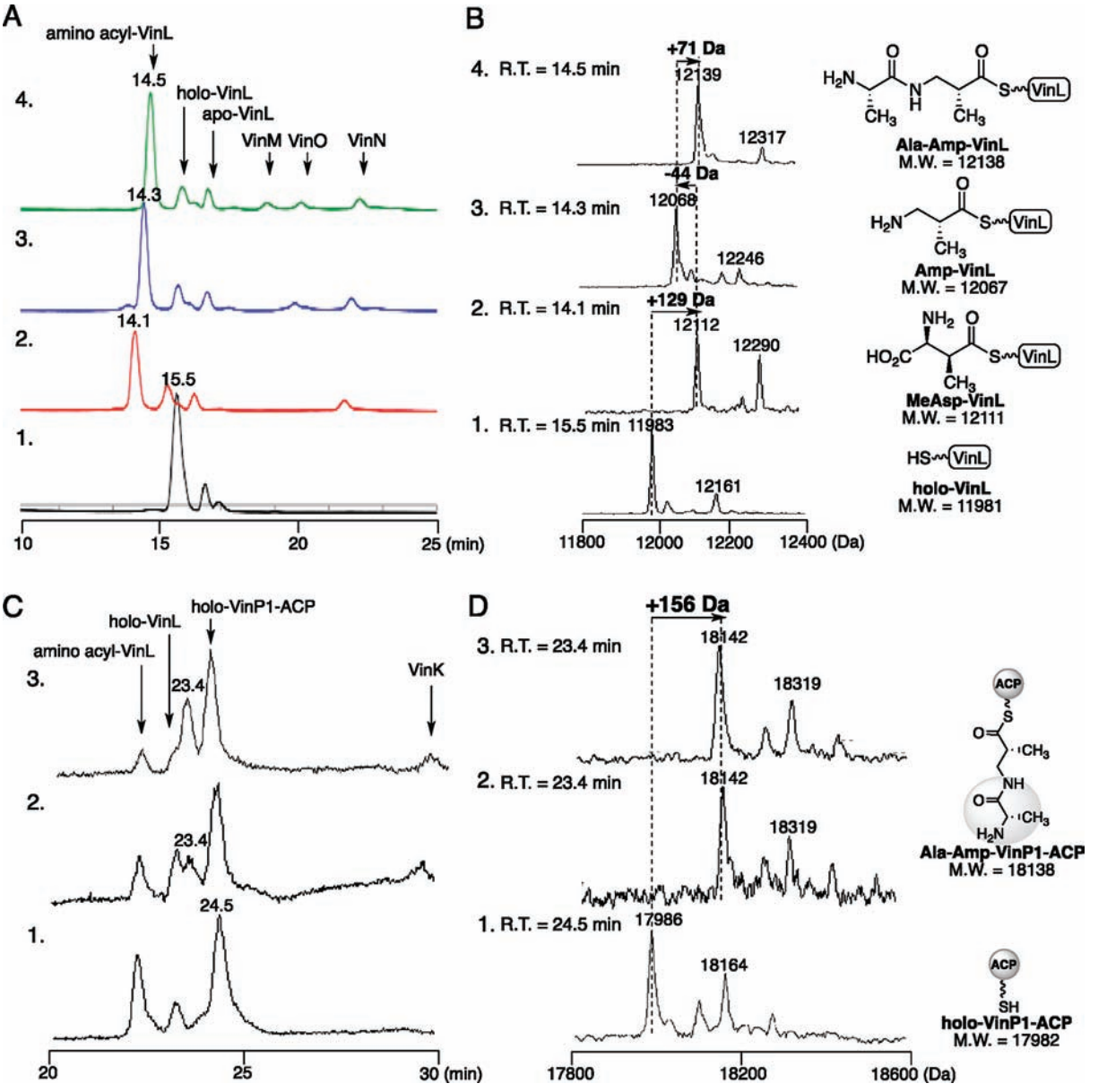

Figure 2. LC-ESI-MS analyses of aminoacyl-ACP formation. (A) HPLC traces (280 nm) of various aminoacyl-VinLs: (1) mixture of holo-VinL, ATP, and MeAsp; (2) mixture of (1) plus VinN; (3) mixture of (2) plus VinO; (4) mixture of (3) plus VinM and L-alanine. (B) ESI-MS analyses of newly generated aminoacyl-VinLs: (1) holo-VinL; (2) MeAsp-VinL; (3) Amp-VinL; (4) Ala-Amp-VinL. These mass spectra show the region from 12 000 to 12 400 Da after deconvolution. The retention time (R.T.) is shown to the left of each spectrum. (C) HPLC traces (TIC) of aminoacyl-VinP1-ACPs: (1) Ala-Amp-VinL and holo-VinP1-ACP; (2) Ala-Amp-VinL, Amp-VinL, MeAsp-VinL, holo-VinP1-ACP, and VinK; (3) Ala-Amp-VinL, holo-VinP1-ACP, and VinK. (D) ESI-MS analyses of holo-VinP1-ACP and Ala-Amp-VinP1-ACP. These mass spectra show the region from 17 800 to 18 600 Da after deconvolution. Retention times are also given.

other macrolactam biosynthetic gene clusters, including salinilactam and BE-14106 biosynthetic genes (Figure S2). Therefore, the described dipeptide strategy for carrying amino acids in the nitrogen-containing starter unit appears to be a rational approach (Figure S6).

Since two ATP-dependent ligases recognized different substrates, we closely aligned the amino acid sequences of VinM and VinN homologous proteins encoded in the macrolactam biosynthetic gene cluster (Figure S7). Structure-based models of amino acid recognition residues[12,13] are highly conserved among VinM homologues. This indicates that the VinM homologues recognize small amino acids such as alanine and glycine and capture the primary amino group of aminoacyl-ACP in each biosynthetic pathway. Conversely, VinN homologues can be classified into three groups: VinN homologues, BecJ homologues, and Strop_2775 homologues. Thus, each ATP-dependent ligase seems to recognize different amino acids in the respective biosyntheses. Consequently, the substrate specificity of VinN homologues is one of the key factors for specific starter biosynthetic pathways. Detailed studies on the recognition mechanism of VinN homologues may provide clues on how to engineer a protein that can recognize other types of amino acids to create novel macrolactam compounds.

An alternative protection−deprotection strategy is known to attach a unique amino-acid (a 4-amino-2-hydroxybutyrate moiety)

dx.doi.org/10.1021/ja208927r |J. Am. Chem. Soc. 2011, 133, 18134–18137

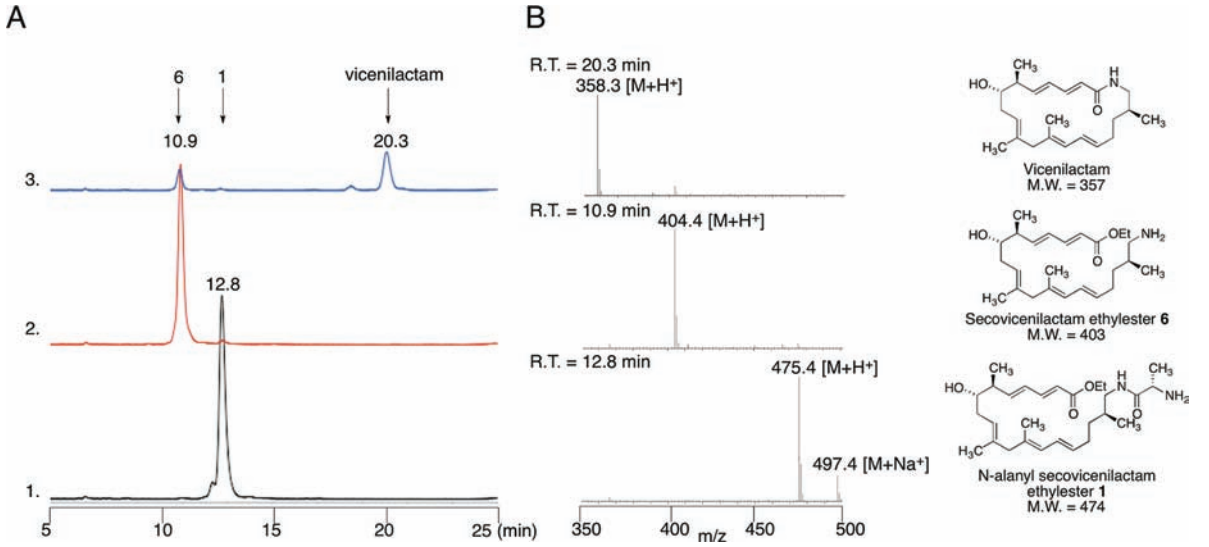

Figure 3. LC-ESI-MS analyses of the VinJ and VinP4-TE reaction. (A) HPLC analyses (254 nm) of the enzymatic reaction with VinJ and VinP4-TE: (1) **1** in Tris buffer; (2) **1** and VinJ; (3) **1**, VinJ, and VinP4-TE. (B) ESI-MS analyses of the enzymatic reaction products. Mass values for major signals are shown. In the present study, we clarified the unique starter biosynthesis of vicenistatin.

in butirosin biosynthesis, where dual-functional glutamyl ligase and glutamyl cyclotransferase are used for protection and deprotection, respectively, to avoid possible five-membered lactam formation[14,15] (Figure S8). Consequently, the formation of dipeptides to carry amino acids such as 4-aminobutyrate and 3-aminopropionate appears to be a general strategy in the biosynthesis of certain natural products, where a combination of activation enzyme (VinN, BtrJ), amino group-protecting enzyme (VinM, BtrJ), and deprotecting enzyme (VinJ, BtrG) is required. Here it should be emphasized that in macrolactam biosynthesis, a set of distinct ATP-dependent ligases are involved in the dipeptide formation.

Importantly, the findings of the present study shed light on how to engineer this unique starter biosynthesis. As a minimum, protein engineering of a VinN-type ATP-dependent ligase and a VinK-type aminoacyltransferase would be necessary to change the starter amino acid in macrolactam biosynthesis.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures, table of Vin enzymes and PCR primers, structures of related macrolactams, purification of proteins, LC-ESI-MS analyses of aminoacyl-ACPs, and synthesis of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
eguchi@cms.titech.ac.jp

■ ACKNOWLEDGMENT

We thank Professor Chaitan Khosla (Stanford University) for generously supplying us with the *sfp* expression plasmid. This work was supported in part by Grants-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

■ REFERENCES

(1) Shindo, K.; Kamishohara, M.; Odagawa, A.; Matsuoka, M.; Kawai, H. *J. Antibiot.* **1993**, *46*, 1076.
(2) Otsuka, M.; Eguchi, T.; Shindo, K.; Kakinuma, K. *Tetrahedron Lett.* **1998**, *39*, 3185.
(3) Otsuka, M.; Fujita, M.; Matsushima, Y.; Eguchi, T.; Shindo, K.; Kakinuma, K. *Tetrahedron* **2000**, *56*, 8281.
(4) Nishida, H.; Eguchi, T.; Kakinuma, K. *Tetrahedron* **2001**, *57*, 8237.
(5) Ogasawara, Y.; Katayama, K.; Minami, A.; Otsuka, M.; Eguchi, T.; Kakinuma, K. *Chem. Biol.* **2004**, *11*, 79.
(6) Ogasawara, Y.; Kakinuma, K.; Eguchi, T. *J. Antibiot.* **2005**, *58*, 468.
(7) Udwary, D. W.; Zeigler, L.; Asolkar, R. N.; Singan, V.; Lapidus, A.; Fenical, W.; Jensen, P. R.; Moore, B. S. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 10376.
(8) Jørgensen, H.; Degnes, K. F.; Sletta, H.; Fjærvik, E.; Dikiy, A.; Herfindal, L.; Bruheim, P.; Klinkenberg, G.; Bredholt, H.; Nygard, G.; Doskeland, S. O.; Ellingsen, T. E.; Zotchev, S. B. *Chem. Biol.* **2009**, *16*, 1109.
(9) Jørgensen, H.; Degnes, K. F.; Dikiy, A.; Fjærvik, E.; Klinkenberg, G.; Zotchev, S. B. *Appl. Environ. Microbiol.* **2010**, *76*, 283.
(10) Kudo, F.; Kitayama, T.; Kakinuma, K.; Eguchi, T. *Tetrahedron Lett.* **2006**, *47*, 1529.
(11) Gokhale, R. S.; Tsuji, S. Y.; Cane, D. E.; Khosla, C. *Science* **1999**, *284*, 482.
(12) Challis, G. L.; Ravel, J.; Townsend, C. A. *Chem. Biol.* **2000**, *7*, 211.
(13) Stachelhaus, T.; Mootz, H. D.; Marahiel, M. A. *Chem. Biol.* **1999**, *6*, 493.
(14) Li, Y.; Llewellyn, N. M.; Giri, R.; Huang, F.; Spencer, J. B. *Chem. Biol.* **2005**, *12*, 665.
(15) Llewellyn, N. M.; Li, Y.; Spencer, J. B. *Chem. Biol.* **2007**, *14*, 379.