Polyketide Biosynthesis

DOI: 10.1002/anie.201402623

Biosynthesis of the Structurally Unique Polycyclopropanated Polyketide–Nucleoside Hybrid Jawsamycin (FR-900848)**

*Tomoshige Hiratsuka, Hideaki Suzuki, Ryo Kariya, Takashi Seo, Atsushi Minami, and Hideaki Oikawa**

Abstract: *The biosynthetic gene cluster of antifungal agent jawsamycin (FR-900848) has been identified by heterologous expression. A series of gene inactivations and in vitro and in vivo analysis of key enzymes in the biosynthetic pathway established their functions. A novel mechanism involving a radical S-adenosyl methionine (SAM) cyclopropanase collaborating with an iterative polyketide synthase is proposed for the construction of the unique polycyclopropanated backbone. Our reconstitution system sets the stage for studying the catalytic mechanism of this intriguing contiguous cyclopropanation.*

Jawsamycin (FR-900848; **1**, Figure 1), which is produced by *Streptoverticillium fervens* HP-891, is a potent antifungal agent for various phytopathogenic fungi.[1,2] Its structure consists of 5′-amino-5′-deoxy-5,6-dihydrouridine (**2a**) and a highly unusual polycyclopropanated fatty acid. The structurally related metabolite U-106305 (Figure 1), which is an inhibitor of the cholesteryl ester transfer protein (CETP), was isolated from *Streptomyces sp.* UC 11136.[3] To date, only two polycyclopropanated metabolites have been reported. Their structures have attracted the attention of synthetic chemists, and extensive synthetic efforts toward their total synthesis have established their relative and absolute stereochemistries.[4] Intriguingly, all cyclopropane moieties of the fatty acid part in **1** and U-106305 have the same absolute stereochemistry. Initial biosynthetic studies on **1**[5] and U-106305[3] with isotopically labeled precursors suggest that the fatty acid backbones are constructed by a common polyketide pathway and that the methylene groups of the characteristic cyclopropane moieties are derived from L-methionine. The aminonucleoside unit of **1** was proposed to be derived from dihydrouridine.[5b] Later, enantioselective incorporation of putative ^{2}H-labeled diketide analogues into **1** suggested that the cyclopropane moieties are introduced in a stepwise manner during polyketide extension (Scheme 1).[6] We herein describe the identification and heterologous expression of the jawsamycin gene cluster and functional analysis of all of the biosynthetic genes. This sets the stage for in vivo and in vitro analysis of this intriguing contiguous cyclopropanation.

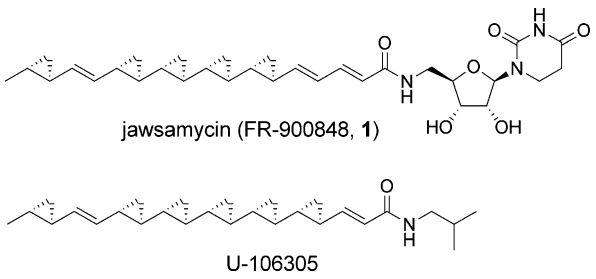


Figure 1. Structures of polycyclopropane-containing natural products.

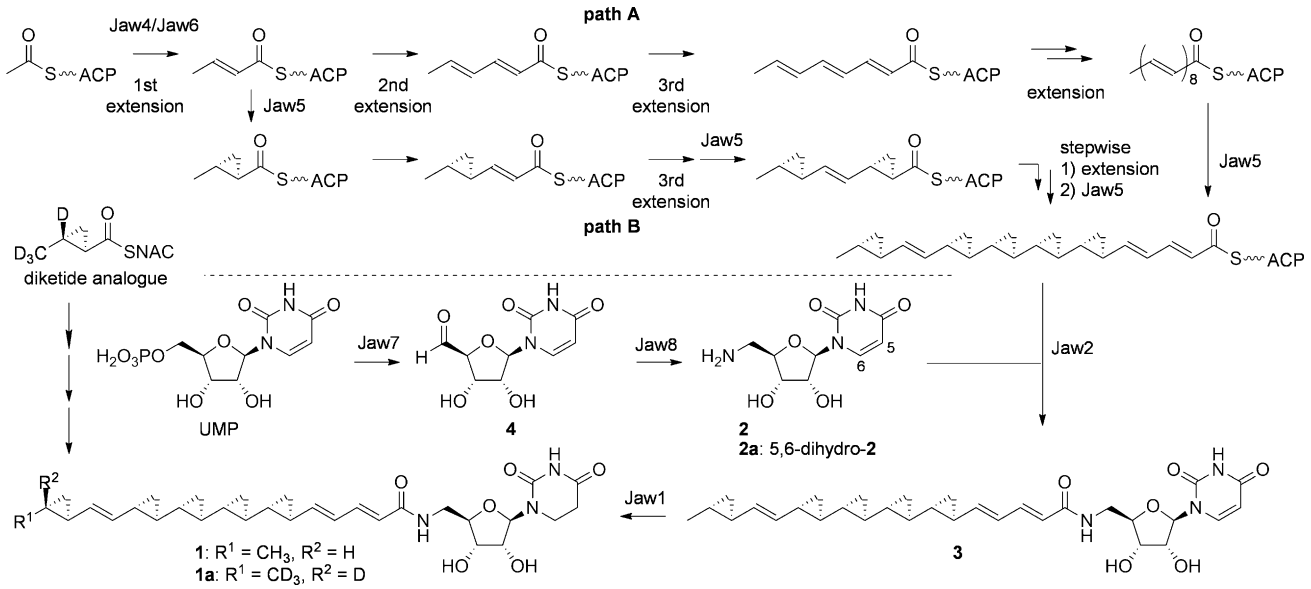
Based on feeding experiments involving putative diketide precursors with a characteristic cyclopropane moiety, we proposed the involvement of a multimodular polyketide synthase (PKS) with a plausible cyclopropanation domain.[6] Initial screening of an *S. fervens* HP-891 genomic DNA library with PCR products encoding ketosynthase (KS) domains failed to obtain PKS genes with the expected domain/module arrangement for jawsamycin biosynthesis. Next, we sequenced the genome of *S. fervens* HP-891. Bioinfomatic analysis of the draft genome sequence with the characteristic genes *lipL*[7] and *lipO*[8] for the biosynthesis of 5′-amino-5′-deoxyuridine (**2**) enabled us to identify the putative jawsamycin gene cluster, which spans approximately 14 kb on a single contig. The putative gene cluster consists of nine open reading frames (ORFs), including the ORFs *jaw3* and *jaw9*, which encode transcriptional regulator (Figure 2, Table S1 in the Supporting Information). The proteins encoded by the ORFs *jaw7* and *jaw8* exhibit 81% identity to the Fe^{II}/α-ketoglutarate-dependent dioxygenase LipL and the aminotransferase LipO, respectively, which are involved in the biosynthesis of the nucleoside antibiotic liposidomycin.[7,8] The ORF *jaw1* encodes a putative reductase for

Figure 2. Genetic organization of the jawsamycin biosynthetic gene cluster.

[*] Dr. T. Hiratsuka, H. Suzuki, R. Kariya, T. Seo, Dr. A. Minami, Prof. Dr. H. Oikawa
Division of Chemistry, Graduate School of Science
Hokkaido University, Sapporo 060-0810 (Japan)
E-mail: hoik@sci.hokudai.ac.jp

[**] This work was supported by MEXT research grant on innovative area 22108002 to H.O. We are grateful to Dr. Hideyuki Muramatsu (Astellas Pharmaceutical Co. Ltd) for supplying *Streptoverticillium fervens* HP-891 and the fermentation conditions. We also acknowledge Prof. Tetsuo Tokiwano of Akita Prefectural University for helping in the initial stage of this study, and Profs. Haruo Ikeda (Kitasato University) and Michihiko Kobayashi (University of Tsukuba) for providing vectors pKU460 and pHSA81.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201402623.



Scheme 1. Proposed biosynthetic pathway of jawsamycin (**1**).

reduction of the uracil moiety and has two domains possessing nicotinamide adenine dinucleotide (NAD) and deazaflavin cofactor binding motifs, respectively. The ORF *jaw2* encodes a protein that shows homology to the GCN5 family *N*-acetyltransferase, thus suggesting that this enzyme is responsible for condensation of the polyketide chain and **2**. Among the remaining three ORFs, *jaw4* and *jaw6*, which encode an iterative PKS containing KS-AT-DH-ACP domains and a stand-alone ketoreductase (KR), are likely responsible for constructing the polyketide backbone. The last ORF, *jaw5*, encodes a protein that shows homology to radical *S*-adenosylmethionine (SAM) enzymes with the conserved cysteine triad CX_7CX_2C motif for a [4Fe-4S] cluster binding at the N–terminus (Figure S1 in the Supporting Information).[9] Jaw5 is most likely to introduce the polycyclopropane units. Based on these annotated data, we propose the biosynthetic pathway of **1** as shown in Scheme 1.

We performed heterologous expression to verify the putative jawsamycin gene cluster. By using the *jaw8* gene as a probe, the cosmid pTH5 harboring the predicted entire *jaw* gene cluster was screened from a pOJ446-vector-based genomic DNA library. The cosmid pTH5 was introduced into the heterologous host *Streptomyces lividans* TK23 (Figure S2). By LC–MS monitoring of mycelial extract from the transformants, we detected **1** (MH^+ *m*/*z* 566 for $C_{32}H_{44}N_3O_6$), thus showing that genes located on the cosmid insert confer the ability to produce jawsamycin (Figure 3). To increase production and determine the exact boundaries of the gene cluster, an *Xho*I/*Hind*III digest of the cosmid insert covering 18 kb was subcloned into a *Streptomyces–E. coli* shuttle vector (pWHM3). The resulting construct, pJawA, was introduced into *S. lividans*, and metabolite analysis revealed that **1** was produced at wild-type levels.

To determine the exact border of the *jaw* gene cluster and to examine the function of each gene, we conducted a series of gene inactivation experiments. Through a combination of restriction-enzyme digestion and In-Fusion homologous

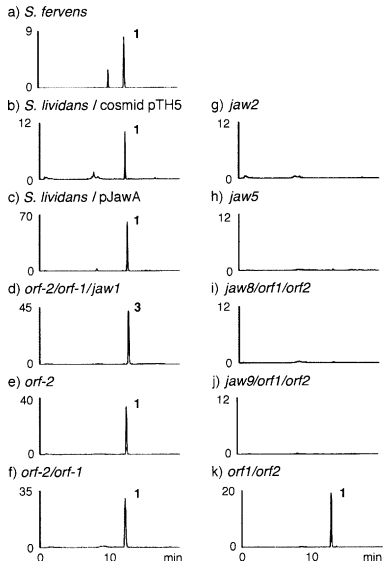


Figure 3. LC–MS analysis of metabolites produced by various bacterial strains (MS chromatogram *m*/*z* 564-566): a) *Streptoverticillium fervens* HP-891; b–k) *S. lividans* TK23 harboring cosmid pTH5, plasmid pJawA (whole gene cluster) or plasmids pJawB–I for gene inactivation.

recombination of suitable inserts, we prepared seven deletion mutants (Figure S2, Table S2 in the Supporting Information). Transformants containing pJawD and pJawH, which harbor deletions in regions upstream of *jaw1* and downstream of *jaw9*, respectively, kept ability to produce **1**, whereas deletion of *jaw2*, *jaw5*, *jaw8,* and *jaw9* completely abolished the production of **1** and none of the intermediates accumulated (Figure 3). The essential requirement of these genes was further confirmed by the production of **1** after complementation with these genes (Figure S3). As anticipated, the inactivation of *jaw1* caused accumulation of dehydrojawsamycin (**3**), the structure of which was confirmed by ESI-HR-

MS (MH^+ m/z 564.3086 for $C_{32}H_{42}N_3O_6$) and ^1H NMR spectroscopy (uridine olefinic protons at 5.62 and 7.67 ppm, $J = 11$ Hz; Figure S4, Table S3).

Jaw1 consists of two independent reductase domains. A homology search revealed that the N-terminal domain is closely related to a 7α-hydroxysteroid dehydrogenase (250 aa; 35/53% identity/similarity) and the C–terminus bears similarity to a deazaflavin-dependent nitroreductase (160 aa; 50/60% identity/similarity). In a biosynthetic study of the nucleoside antibiotic napsamycin, *npsU* was identified as the reductase gene that is responsible for the reduction of the uracil moiety but the exact timing has not been explored.[10] To determine the true substrate of Jaw1, which differs in sequence from NpsU, feeding experiments of putative precursors into deletion mutants were employed. When 5′-amino-5′-deoxyuridine (**2**) was fed to the Δ*jaw8* mutant, production of **1** was detected (Scheme 1 and Figure S5). Meanwhile, feeding **3** to the Δ*jaw2* mutant resulted the transformation of **3** into **1**. These results were further confirmed by the bioconversion of **3** into **1** when using a *S. lividans* TK23 strain in which *jaw1* was heterologously expressed from the pHSA81 vector. Based on these results, we speculated that the reduction may be catalyzed in the C-terminal domain with coenzyme F420[11] and that the N-terminal domain possibly provides the substrate binding pocket since bioinfomatic analysis suggests the loss of the essential catalytic residues in the N-terminal domain (Figure S6).[12] These results indicate that reduction of the uracil moiety occurs at the last step of biosynthesis.

To validate the functions of individual genes, Jaw7, Jaw8, and Jaw2 with appropriate tags at the N–termini were overexpressed and purified from *Escherichia coli* (Figures S7 and S8). Since Jaw7 and Jaw8 showed high homology to LipL and LipO, respectively, these enzymes could catalyze the conversion of UMP into **2**.[7,8] According to the LipL catalyzed reaction,[7] enzymatic reaction of the recombinant Jaw7 with UMP was carried out in the presence of $FeCl_2$, α-ketoglutarate, and L-ascorbate. HPLC analysis of the reaction mixtures showed a new peak that was identical to synthetic aldehyde **4**[7] (Figure S7). Aldehyde **4** was then incubated with the LipO[8] homologue Jaw8 in the presence of PLP and several amino acids. Production of **2** was confirmed by HPLC analysis through comparison with a sample of **2**[7] and L-methionine was found to be the best amino donor (Figure S7). Together with the proposed the function of Jaw1, these results showed that Jaw7 and Jaw8 are responsible for 5′-amino-5′-deoxyuridine biosynthesis.

Bioinformatic analysis of the jawsamycin gene cluster revealed that there is no common gene, such as a thioesterase, for polyketide chain release, thus suggesting that the *N*-acetyltransferase homologue Jaw2 directly catalyzes the condensation of the acyl carrier protein (ACP)-bound polyketide chain with **2**. Jaw2 showed weak homology to the GCN5 family acetyltransferase TunC, which is proposed to introduce various C7–C12 acyl chains to the core scaffold of tunicamycins.[13] To test this hypothesis, enzymatic reactions of the recombinant Jaw2 were employed with various substrate analogues. As a putative genuine substrate, ACP-bound cyclopropanated polyketide was not available so we used stearoyl coenzyme A (CoA) as an alternative. In the preliminary experiment with **2** as an amino donor, we found that Jaw2 required divalent Mg^{2+} ions, showed rather low turnover (12% conversion over 10 min when using 10 mol% of Jaw2), and catalyzed hydrolysis of the CoA ester (condensation/hydrolysis = 6.6; Figure S8). To examine the substrate selectivity, time-course analysis of the Jaw2-catalysed reaction was conducted using various acyl-CoA and amino donors. When fatty acyl-CoA esters ranging from C8 to C18 were tested, Jaw2 accepted all of the CoA esters (with a preference for C18; Figure S8) but not the corresponding *N*-acetylcysteamine (SNAC) esters (data not shown). Compared with the conversion of **2**, Jaw2 showed poor conversion of the dihydro analogue **2a**[4b] (12% conversion). Low but reproducible activity (8% conversion) was observed in the reaction of isobutylamine, a component of U-106305. These data indicate that Jaw2 accepts a broad range of fatty acyl substrates, most likely tethered to ACP, but shows relatively strict substrate specificity for amino donors.

Next, we investigated the construction of the polycyclopropanated polyketide chain through heterologous expression of *jaw456*. For reconstitution of this minimal PKS system, the PCR products for *jaw2* (0.7 kb) and *jaw456* (6.3 kb) were cloned into pHSA81 and the integration vector pKU460, respectively, to generate pHSAj2 and pKUj456. *S. lividans* TK23 was transformed with the resulting plasmids to generate the single transformant (*jaw456*) and the double transformant (*jaw2456*). In the feeding experiments with a 2:1 mixture of aminouridines **2** and **2a**, **3** and **1** were detected in the double transformant but none of jawsamycins was found in the single transformant (Figure S9). These results confirm the role of Jaw2 and provided experimental support for the hypothesis that the polyketide chain is constructed by the iterative PKS Jaw4, which contains KS-AT-DH-ACP domains, in collaboration with the highly unusual trans-KR Jaw6 and with the radical SAM enzyme Jaw5 for introducing the characteristic cyclopropane units.

Polyunsaturated chains produced by iterative PKSs are involved in the biosynthesis of myxochromides[14] and are also frequently found in the biosynthesis of enediyne antibiotics[15] and fungal highly reduced polyketides.[16] While collaboration of a trans-acting enoyl reductase (ER) is common in fungal iterative nonribosomal peptide synthetase (NRPS)/PKS-catalyzed reactions,[16b,17] to our knowledge, trans-acting KR is rare except for the multimodular PKS reaction reported recently for SIA7248 biosynthesis.[18] Although cyclopropane-containing polyketides such as ambruticin[19] and curacin[20] are known, they are constructed in a different, non-iterative manner. The putative cyclopropanase Jaw5, which sequentially introduces cyclopropane units, is thus unique in its modification of the polyketide chain.

Although C-alkylation of a polyketide chain during chain extention is relatively rare, the methylation domains in modular PKS[16a] and β-branching enzymes[21] are known to introduce extra carbon units during the chain construction of certain polyketides. The introduction of C_1 units by radical SAM enzymes is frequently involved in metabolism, such as C-methylation of RNA and other secondary metabolites.[9] However, there has been no report of a polyketide chain

being modified by radical SAM-dependent cyclopropanation. Cyclopropanation involving a radical SAM enzyme is predicted in the biosynthesis of yatakemycin.[22] The presence of an unusual trans-acting KR may be related to iterative cyclopropanation.

We speculated that cyclopropanation occurs after single-chain extension to give α,β-unsaturated polyketide chains appended to ACP in an iterative manner. This hypothesis was supported by the following evidence: 1) in-frame deletion of *jaw5* abolished the production of **1** and none of the polyunsaturated PKS product was obtained although the *N*-acyltransferase Jaw2 has rather broad substrate specificity; 2) enantioselective incorporation of ^{2}H-labeled diketide precursors possessing a cyclopropane moiety into the corresponding polyketide backbone was observed. Considering the involvement of the radical SAM enzyme Jaw5, we revised our previous mechanism[6] and propose two alternative mechanisms (Scheme S1 in the Supporting Information). As in the case of Type A radical SAM methyltransferase,[9b] the deoxyadenosyl radical removes a hydrogen from the SAM methyl group and the subsequent electron transfer from the Fe–S cluster generates a SAM ylide, which reacts with the unsaturated polyketide chain as previously proposed. Alternatively, a radical mechanism involving the SAM methyl radical instead of SAM ylide is also possible. A complex system consisting of Jaw4, Jaw6, and Jaw5 may control the timing and number of cyclopropanations to afford **1**.

In summary, we have identified the jawsamycin biosynthetic gene cluster, which consists of genes for the construction of 5′-amino-5′-deoxyuridine (**2**) and an unprecedented polycyclopropanated polyketide chain, through heterologous expression. Gene deletions and functional analysis using the recombinant enzymes established the roles of the *jaw* genes. In vivo reconstitution of a minimal PKS consisting of iterative PKS Jaw4, stand-alone ketoreductase Jaw6, and radical SAM enzyme Jaw5 confirmed reactions constructing the unusual polyketide backbone. This provides an example of a novel PKS system that lacks a KS domain and demonstrates domain-like use of trans-acting ketoreductase and radical SAM cyclopropanase. To decipher the detailed reaction mechanism of this intriguing polycyclopropanation, we are currently working on functional analysis of the minimal PKS in vivo and in vitro.

Received: February 20, 2014
Published online: April 24, 2014

Keywords: antifungal agents · biosynthesis · natural products · polycyclopropanes · polyketides

[1] Jawsamycin is a nickname of FR-900848, which was given by the researchers who developed this unusual antibiotic in Fujisawa Pharmaceuticals because the structure resembles sharks teeth.

[2] M. Yoshida, M. Ezaki, M. Hashimoto, M. Yamashita, N. Shigematsu, M. Okuhara, M. Kohsaka, K. Horikoshi, *J. Antibiot.* **1990**, *43*, 748 – 754.
[3] M. S. Kuo, R. J. Zielinski, J. I. Cialdella, C. K. Marschke, M. J. Dupuis, G. P. Li, D. A. Kloosterman, C. H. Spilman, V. P. Marshall, *J. Am. Chem. Soc.* **1995**, *117*, 10629 – 10634.
[4] a) A. G. M. Barrett, W. W. Doubleday, K. Kasdorf, G. J. Tustin, *J. Org. Chem.* **1996**, *61*, 3280 – 3288; b) J. R. Falck, B. Mekonnen, J. R. Yu, J. Y. Lai, *J. Am. Chem. Soc.* **1996**, *118*, 6096 – 6097; c) A. G. M. Barrett, D. Hamprecht, A. J. P. White, D. J. Williams, *J. Am. Chem. Soc.* **1997**, *119*, 8608 – 8615; d) J. Pietruszka, *Chem. Rev.* **2003**, *103*, 1051 – 1070.
[5] a) H. Watanabe, T. Tokiwano, H. Oikawa, *J. Antibiot.* **2006**, *59*, 607 – 610; b) H. Watanabe, T. Tokiwano, H. Oikawa, *Tetrahedron Lett.* **2006**, *47*, 1399 – 1402.
[6] T. Tokiwano, H. Watanabe, T. Seo, H. Oikawa, *Chem. Commun.* **2008**, 6016 – 6018.
[7] X. Chi, P. Pahari, K. Nonaka, S. G. Van Lanen, *J. Am. Chem. Soc.* **2011**, *133*, 14452 – 14459.
[8] Z. Yang, X. Chi, M. Funabashi, S. Baba, K. Nonaka, P. Pahari, J. Unrine, J. M. Jacobsen, G. I. Elliott, J. Rohr, S. G. Van Lanen, *J. Biol. Chem.* **2011**, *286*, 7885 – 7892.
[9] a) M. R. Challand, R. C. Driesener, P. L. Roach, *Nat. Prod. Rep.* **2011**, *28*, 1696 – 1721; b) Q. Zhang, W. A. van der Donk, W. Liu, *Acc. Chem. Res.* **2012**, *45*, 555 – 564.
[10] L. Kaysser, X. Y. Tang, E. Wemakor, K. Sedding, S. Hennig, S. Siebenberg, B. Gust, *ChemBioChem* **2011**, *12*, 477 – 487.
[11] C. Filling, K. D. Berndt, J. Benach, S. Knapp, T. Prozorovski, E. Nordling, R. Ladenstein, H. Jornvall, U. Oppermann, *J. Biol. Chem.* **2002**, *277*, 25677 – 25684.
[12] a) M. Taylor, C. Scott, G. Grogan, *Trends Biotechnol.* **2012**, *31*, 63 – 64; b) J. D. Selengut, D. H. Haft, *J. Bacteriol.* **2010**, *192*, 5788 – 5798.
[13] F. J. Wyszynski, A. R. Hesketh, M. J. Bibb, B. G. Davis, *Chem. Sci.* **2010**, *1*, 581 – 589.
[14] S. C. Wenzel, F. Gross, Y. M. Zhang, J. Fu, A. F. Stewart, R. Muller, *Chem. Biol.* **2005**, *12*, 349 – 356.
[15] a) Z. X. Liang, *Nat. Prod. Rep.* **2010**, *27*, 499 – 528; b) K. Belecki, C. A. Townsend, *J. Am. Chem. Soc.* **2013**, *135*, 14339 – 14348.
[16] a) C. Hertweck, *Angew. Chem.* **2009**, *121*, 4782 – 4811; *Angew. Chem. Int. Ed.* **2009**, *48*, 4688 – 4716; b) R. J. Cox, T. J. Simpson in *Comprehensive Natural Products II*, *Vol. 1* (Eds.: L. Mander, H.-W. Liu), Elsevier, Amsterdam, **2010**, pp. 347 – 383.
[17] D. Boettger, H. Bergmann, B. Kuehn, E. Shelest, C. Hertweck, *ChemBioChem* **2012**, *13*, 2363 – 2373.
[18] Y. Zou, H. X. Yin, D. K. Kong, Z. X. Deng, S. J. Lin, *ChemBioChem* **2013**, *14*, 679 – 683.
[19] B. Julien, Z. Q. Tian, R. Reid, C. D. Reeves, *Chem. Biol.* **2006**, *13*, 1277 – 1286.
[20] L. C. Gu, B. Wang, A. Kulkarni, T. W. Geders, R. V. Grindberg, L. Gerwick, K. Hakansson, P. Wipf, J. L. Smith, W. H. Gerwick, D. H. Sherman, *Nature* **2009**, *459*, 731 – 735.
[21] T. Bretschneider, J. B. Heim, D. Heine, R. Winkler, B. Busch, B. Kusebauch, T. Stehle, G. Zocher, C. Hertweck, *Nature* **2013**, *502*, 124 – 130.
[22] W. Huang, H. Xu, Y. Li, F. Zhang, X. Y. Chen, Q. L. He, Y. Igarashi, G. L. Tang, *J. Am. Chem. Soc.* **2012**, *134*, 8831 – 8840.

