VIP Biosynthesis Very Important Paper

International Edition: DOI: 10.1002/anie.201508576
German Edition: DOI: 10.1002/ange.201508576

A Peptidyl-Transesterifying Type I Thioesterase in Salinamide Biosynthesis

*Lauren Ray, Kazuya Yamanaka, and Bradley S. Moore**

Abstract: *Salinamide A belongs to a rare class of bicyclic depsipeptide antibiotics in which the installation of a (4-methylhexa-2,4-dienoyl)glycine handle across a hexadepsipeptide core contributes to its chemical complexity and biological properties. Herein, we report the genetic and biochemical basis for salinamide construction in the marine bacterium Streptomyces sp. CNB-091, which involves a novel intermolecular transesterification reaction catalyzed by a type I thioesterase. Heterologous expression studies revealed the central role of the nonribosomal peptide synthetase Sln9 in constructing and installing the distinctive acylglycine "basket handle" of salinamide. Biochemical characterization of the Sln9 thioesterase domain established that transesterification of the serine residue of desmethylsalinamide E with acylated glycyl thioesters yields desmethylsalinamide C.*

Non-ribosomal peptides (NRPs) have long been recognized as a highly diverse and important class of natural products, both in terms of structure and function.[1] Their construction by large, multifunctional assembly line proteins involves ATP-dependent activation and transfer of amino acid precursors to arrayed carrier protein (CP) domains. Once the mature peptide intermediate has been assembled by the nonribosomal peptide synthetase (NRPS), a myriad of protein off-loading reactions await to yield the released peptide product.[2] The most common terminating reaction is catalyzed by thioesterase (TE) proteins, which typically occupy the terminal domain of the NRPS and function to release the products as linear or macrocyclic peptides.[3] Herein, we report a new role for a terminating TE that involves an intermolecular transesterification reaction associated with the construction of the salinamides, a family of unusual bicyclic depsipeptides that exhibit anti-inflammatory and antibacterial properties.

The first in this class of depsipeptides to be isolated from the marine actinomycete *Streptomyces* sp. CNB-091 were salinamides A (**1**) and B (**2**).[4] This series of NRPs has since been expanded through the isolation and characterization of various derivatives to include salinamides C (**3**), D (**4**), E (**5**), and F (**6**).[5] All salinamides share a common depsipeptide core that comprises a β-hydroxy acid moiety derived from isobutyrate and propionate and six amino acid residues, namely L-serine, L-phenylalanine, *para*-hydroxy-L-phenylglycine (*p*-HPG), L-threonine, D-threonine, and D-isoleucine (or D-valine in the case of **4**). The simplest of the salinamide derivatives, **5**, is methylated at the *p*-HPG position. The remaining salinamide derivatives are all decorated with a (4-methylhexa-2,4,-dienoyl)glycine moiety tethered to the serine hydroxy group (Ser-OH) via an ester bond. Within **1**, **2**, **4**, and **6**, this extended serine side chain is cyclized onto the phenoxy group of the *p*-HPG residue (*p*-HPG-OH) to create a "basket handle" across the cyclic core. Probing the metabolic origins of the carbon residues within the salinamides previously revealed that the Ser-modified acylglycine side chain is derived from the condensation of 2-methylbutyrate and malonate (Figure 1).[6]

The structures of this series of salinamide derivatives and isotope incorporation data suggest the involvement of a novel biosynthetic pathway in which a mature cyclic depsipeptide is processed by the addition of a (4-methylhexa-2,4,-dienoyl)-glycine unit to the Ser-OH moiety. Subsequent oxidative cyclization of this side chain onto *p*-HPG-OH would yield the bicyclic depsipeptide **1**. This biosynthetic scenario suggested the possibility of new peptide side chain modification enzymology involving a CP-bound substrate. Precedent examples are nonexistent. Thiotemplated assembly line syntheses of ester linkages are known to involve NRPS condensation (C) domain reactions, such as for the didemnins and hectochlorins, in which hydroxy acid rather than amino acid building blocks are incorporated into NRP products.[7] That scenario, however, does not fit salinamide assembly in which the ester is derived in a very different manner involving a side-chain hydroxy group. Standalone C domains are rather known from polyketide biosynthesis to attach carboxylic acid substrates to ketide hydroxy groups as in C-1027 and fumonisin biosynthesis.[8] Although this mechanism is plausible for salinamide esterification, we show a new role for a terminal TE domain in catalyzing a transesterification reaction.

To understand the mechanism responsible for the installation of the salinamide handle, we obtained a draft genome sequence of *Streptomyces* sp. CNB-091. The high quality of

[*] Dr. L. Ray, Dr. K. Yamanaka, Prof. Dr. B. S. Moore
Center for Marine Biotechnology and Biomedicine
Scripps Institution of Oceanography
University of California, San Diego
La Jolla, CA 92037 (USA)
E-mail: bsmoore@ucsd.edu
Homepage: http://scrippsscholars.ucsd.edu/bsmoore
Dr. K. Yamanaka
Yokohama Research Center, JNC Corporation
Yokohama 236-8605 (Japan)
Prof. Dr. B. S. Moore
Skaggs School of Pharmacy and Pharmaceutical Sciences
University of California, San Diego
La Jolla, CA 92037 (USA)

Supporting information and ORCID(s) from the author(s) for this article are available on the WWW under http://dx.doi.org/10.1002/anie.201508576.

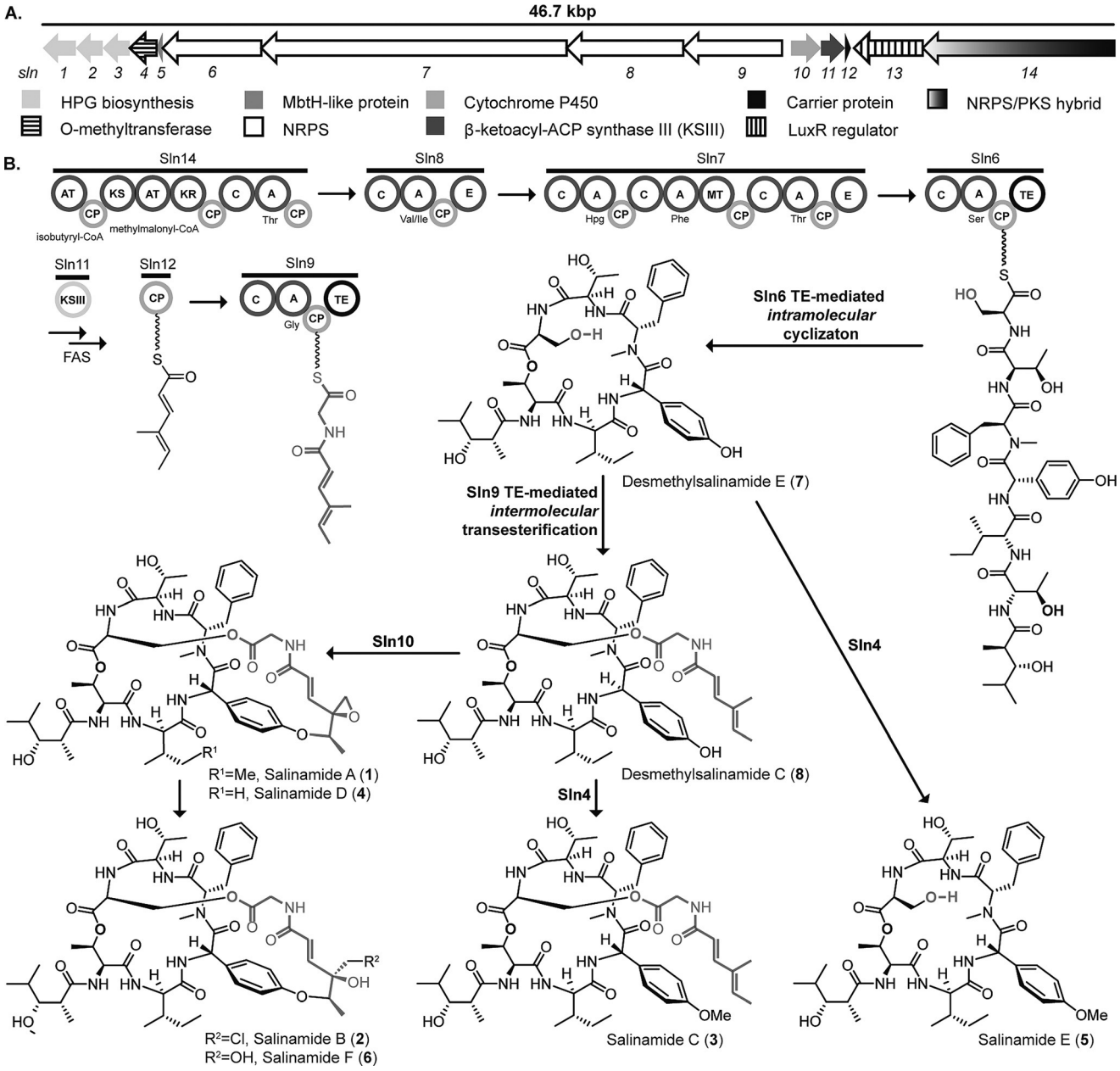

Figure 1. A) Gene organization of the salinamide (*sln*) biosynthetic gene cluster from *Streptomyces* sp. CNB-091 (see the Supporting Information for the proposed function assignments of the coding sequences). B) Proposed biosynthesis of the salinamides. The A domain of Sln8 is able to accept isoleucine and valine; consequently, two different salinamide series are biosynthesized. All Ile derivatives, **1**–**3** and **5**–**8**, are illustrated within this figure; however, **4** is the only Val derivative shown. A: adenylation; AT: acyltransferase; C: condensation; CP: carrier protein; E: epimerization; FAS: fatty acid synthase; *p*-HPG: hydroxyphenylglycine; KR: ketoreductase; KS: ketosynthase; MT: methyltransferase; TE: thioesterase.

this draft sequence resulted in the 8.2 Mbp genome being split across 50 scaffolds, with a GC content of 71.6%. Subsequent bioinformatic analysis of this draft genome unveiled the full biosynthetic potential of *Streptomyces* sp. CNB-091.[9] A vast array of putative secondary-metabolite gene clusters were illuminated, with a total of 38 clusters encoding the synthesis of polyketides, NRPs, terpenes, and ribosomally synthesized and post-translationally modified peptide natural products. Intriguingly, the salinamides are, to date, the only class of molecules reported from *Streptomyces* sp. CNB-091, thus indicating that *Streptomyces* sp. CNB-091 is a relatively untapped resource with regards to its potential to produce diverse natural products.

From the 38 biosynthetic gene clusters, we identified a single candidate cluster for salinamide biosynthesis. This 47.6 kbp, 14 coding sequence polyketide synthase (PKS)/NRPS hybrid gene cluster was designated the *sln* locus (Figure 1 A). The *sln* cluster contains four genes that encode NRPS proteins, one that encodes a PKS/NRPS hybrid, and genes encoding enzymes that are responsible for precursor

biosynthesis and post-NRPS tailoring enzymes. Significantly, the domain structure and module organization of the encoded PKS/NRPS megasynthetase was consistent with the structures of the salinamides in which two amino acid residues are D-configured (Val/Ile-2 and Thr-5) and another (Phe-4) is methylated.[10] Moreover, genes supporting the biosynthesis of the non-proteinogenic amino acid residue *p*-HPG were subclustered in a gene cassette (*sln1-3*).[11] One salient feature of the *sln* cluster is the presence of two genes, *sln6* and *sln9*, that both encode tetradomain NRPS modules containing a C-terminal TE domain. This finding led to the proposal that one of these type I TE domains plays a central role in the addition of the (4-methylhexa-2,4,-dienoyl)glycine moiety onto the Ser-OH group, rather than this being the product of C domain chemistry (Figure 1B).

To validate that this putative biosynthetic gene cluster is responsible for the production of the salinamides, we directly captured the *sln* locus from gDNA by yeast-based transformation-associated recombination (TAR) in the multipurpose expression plasmid pCAP01.[12] Following capture, the *sln* cluster was integrated into the genome of the model actinomycete *Streptomyces coelicolor* M1146 for heterologous expression.[13] Fermentation of this genome-minimized strain revealed the production of the full suite of salinamides, confirming that the *sln* locus is responsible for salinamide biosynthesis (Figure 2).

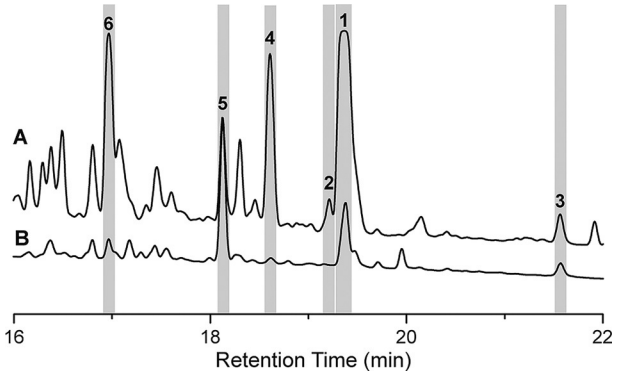

Figure 2. HPLC analysis of salinamide production in native *S.* sp. CNB-091 (A) and the heterologous host *S. coelicolor* M1146 into which the pCAP*sln* vector had been integrated (B). The UV absorption at 210 nm was monitored, and the peaks corresponding to salinamide A–F (**1**–**6**) are highlighted.

Successful capture and heterologous expression of the salinamide gene cluster provided a mechanism for interrogating the in vivo biosynthesis of salinamides. We deleted several *sln* genes by λ-Red-mediated recombination and analyzed chemical extracts of the mutants by HPLC-MS. First, we focused on the biosynthesis of the salinamide depsipeptide core by the canonical PKS/NRPS hybrid. This was probed through the deletion of *sln6*, which encodes the putative final module in this assembly line, terminating with the cyclizing TE. As anticipated, this mutation completely abolished salinamide production (Figure S1C). A loss of salinamide production was also observed upon deletion of the LuxR regulatory protein (Figure S1B). We then turned out attention to the tailoring genes within the pathway. Deletion of the *O*-methyltransferase (MT) *sln4* eliminated **3** and **5** with concomitant production of **1** and a new desmethylsalinamide, the postulated intermediate **7**, which was subsequently isolated and characterized by HR-MS and NMR spectroscopy (Figure S1F). The production of **1** was uniquely abolished by the deletion of the cytochrome P450 (CYP450) encoding *sln10* gene, as all other salinamides, including **3** and **5**, were maintained in the mutant (Figure S1E). This observation strongly suggests that CYP450 is responsible for catalyzing the closure of the salinamide A "basket handle" between *p*-HPG and the side-chain diene. Furthermore, these results suggest that **3** and **5** are end-of-pathway products rather than intermediates towards the more chemically elaborate **1** (Figure 1B).

We next focused on the biosynthesis of the N-acylated glycine moiety and the mode of its transfer to the Ser-OH group in **1** and **3**. Previously, we showed that the acyl diene fragment originates from the condensation of isoleucine-derived tiglic acid and acetate,[6] which is consistent with the activity of FabH-based enzymology. We thus deleted the FabH homologue *sln11*. Whilst the level of production of **5** was maintained, the production of all other salinamide derivatives was significantly reduced, suggesting genetic complementation by a host FabH gene (Figure S1D). Inactivation of the Sln9 NRPS gene resulted in the elimination of **1** and **3** while maintaining the production of **5** and **7** (Figure S1G). Based on these outcomes, we surmise that the tetradomain Sln9 activates glycine, condenses it with the Sln11 product 4-methyl-2,4-hexadienoyl-*S*-Sln12, and, involving unprecedented TE domain chemistry, transfers the acylglycine substrate to the Ser-OH residue in **7** to yield **8**. This proposed activity would signal a departure from the NRPS assembly line paradigm in which TEs normally catalyze offloading hydrolytic or intramolecular reactions, much like that observed with the C-terminal TE domain of Sln6.[14]

To explore this proposal, we biochemically characterized the Sln9 TE domain. We isolated the presumed substrate **7** from fermentation broths of the Δ*sln4* MT mutant and synthesized two acylglycine–CP substrate mimics, namely *N*-acetylcysteamine thioester **9** and *N*-pantatheinylcysteamine **10** (Figure 3A).[15] We also cloned, expressed, and purified the recombinant hexahistadyl-tagged Sln9 TE domain (Figure S17). Indeed, as hypothesized, upon incubation of the His_6-tagged Sln9 TE with **7** and the acylglycine–CP substrate mimics, **9** or **10**, we were able to observe the transfer of the acylglycine moiety onto Ser-OH (Figure 3B). This result confirms that the Sln9 TE represents the first example of an intermolecular-acting TE domain within a bacterial NRPS. This observation increases the scope of TE domain chemistry to include the modification of NRP side chains through the transesterification of peptidyl substrates.

In conclusion, we have successfully characterized the biosynthetic logic of salinamide assembly. Direct capture of the salinamide biosynthetic gene cluster by TAR cloning facilitated heterologous expression studies, which allowed for

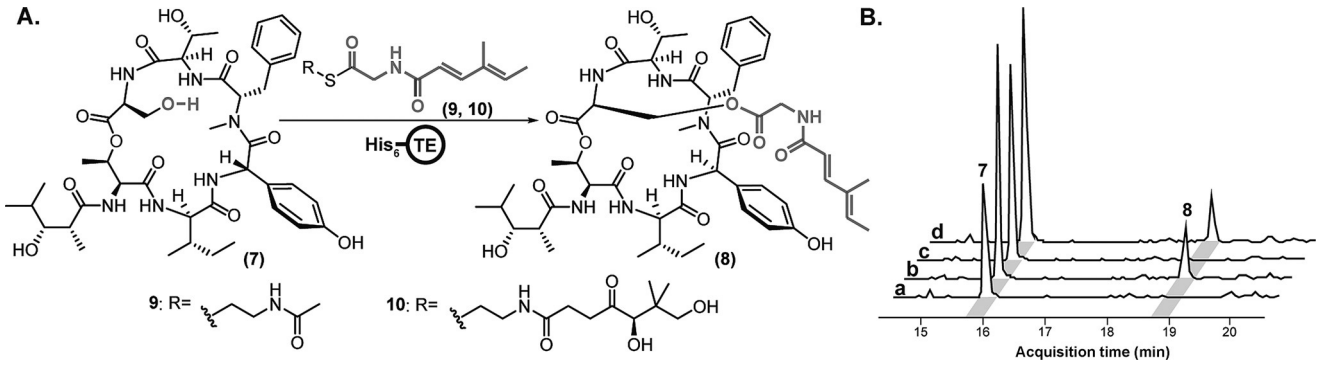

Figure 3. Biochemical characterization of the Sln9 type I TE domain with **7** as a substrate; **5** was also accepted as a substrate by Sln9 TE (see Figure S19). A) Intermolecular transesterification reaction catalyzed by the Sln9 TE domain. The synthesis of substrates **9** and **10** and the preparation of recombinant hexahistidine-tagged Sln9 TE are described in the Supporting Information. B) HR-LCMS analysis of the Sln9 TE reaction, all traces are extracted ion chromatograms (EICs) for *m/z* 841.43 ($[M+H]^+$ of **7**) and 1006.51 ($[M+H]^+$ of **8**). a) Control assay with a heat-inactivated His_6-tagged Sln9 TE domain using **9** as a substrate. b) His_6-tagged Sln9 TE domain enzymatic assay showing the conversion of **7** into **8** using substrate **9**. c) Control assay with a heat-inactivated His_6-tagged Sln9 TE domain using **10** as a substrate. d) His_6-tagged Sln9 TE domain enzymatic assay showing the conversion of **7** into **8** using substrate **10**.

the genetic interrogation of the *sln* gene locus. Significantly, this work uncovered the first example of an intermolecular-acting thioesterase domain within a bacterial NRPS to mediate the transesterification of a CP-bound substrate to a cyclic depsipeptide. Investigations to probe the substrate specificity of this TE are currently ongoing along with efforts to understand the structural features that enable the controlled acylation of peptide substrates.

Acknowledgements

We thank our UCSD colleagues B. M. Duggan for assistance with NMR data acquisition, W. Fenical and P. R. Jensen for *Streptomyces* sp. CNB-091, and V. Agarwal, S. M. Mantovani, and S. Diethelm for helpful discussions. Funding was generously provided by the NIH (R01-GM085770 and R01-AI047818).

Keywords: biosynthesis · intermolecular transesterification · peptides · salinamide · thioesterase

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 364–367
Angew. Chem. **2016**, *128*, 372–375

[1] S. A. Sieber, M. A. Marahiel, *Chem. Rev.* **2005**, *105*, 715–738.
[2] M. A. Fischbach, C. T. Walsh, *Chem. Rev.* **2006**, *106*, 3468–3496.
[3] M. E. Horsman, T. P. Hari, C. N. Boddy, *Nat. Prod. Rep.* **2015**, DOI: 10.1039/C4NP00158F.
[4] a) J. A. Trischman, D. M. Tapiolas, P. R. Jensen, R. Dwight, W. Fenical, T. C. McKee, C. M. Ireland, T. J. Stout, J. Clardy, *J. Am. Chem. Soc.* **1994**, *116*, 757–758; b) S. C. Miao, M. R. Anstee, K. LaMarco, J. Matthew, L. H. T. Huang, M. M. Brasseur, *J. Nat. Prod.* **1997**, *60*, 858–861; c) D. Degen, Y. Feng, Y. Zhang, K. Y. Ebright, Y. W. Ebright, M. Gigliotti, H. Vahedian-Movahed, S. Mandal, M. Talaue, N. Connell, E. Arnold, W. Fenical, R. H. Ebright, *eLife* **2014**, *3*, e02451.
[5] a) B. S. Moore, J. A. Trischman, D. Seng, D. Kho, P. R. Jensen, W. Fenical, *J. Org. Chem.* **1999**, *64*, 1145–1150; b) H. M. Hassan, D. Degen, K. H. Jang, R. H. Ebright, W. Fenical, *J. Antibiot.* **2015**, *68*, 206–209.
[6] B. S. Moore, D. Seng, *Tetrahedron Lett.* **1998**, *39*, 3915–3918.
[7] a) Y. Xu, R. D. Kersten, S. J. Nam, L. Lu, A. M. Al-Suwailem, H. Zheng, W. Fenical, P. C. Dorrestein, B. S. Moore, P. Y. Qian, *J. Am. Chem. Soc.* **2012**, *134*, 8625–8632; b) A. V. Ramaswamy, C. M. Sorrels, W. H. Gerwick, *J. Nat. Prod.* **2007**, *70*, 1977–1986.
[8] a) K. Zaleta-Rivera, C. P. Xu, F. G. Yu, R. A. E. Butchko, R. H. Proctor, M. E. Hidalgo-Lara, A. Raza, P. H. Dussault, L. C. Du, *Biochemistry* **2006**, *45*, 2561–2569; b) S. Lin, S. G. Van Lanen, B. Shen, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 4183–4188.
[9] a) N. Ziemert, S. Podell, K. Penn, J. H. Badger, E. Allen, P. R. Jensen, *PLoS One* **2012**, *7*, e34064; b) T. Weber, K. Blin, S. Duddela, D. Krug, H. U. Kim, R. Bruccoleri, S. Y. Lee, M. A. Fischbach, R. Mueller, W. Wohlleben, R. Breitling, E. Takano, M. H. Medema, *Nucleic Acids Res.* **2015**, *43*, W237–W243.
[10] a) T. Stachelhaus, H. D. Mootz, M. A. Marahiel, *Chem. Biol.* **1999**, *6*, 493–505; b) G. L. Challis, J. Ravel, C. A. Townsend, *Chem. Biol.* **2000**, *7*, 211–224.
[11] B. K. Hubbard, M. G. Thomas, C. T. Walsh, *Chem. Biol.* **2000**, *7*, 931–942.
[12] K. Yamanaka, K. A. Reynolds, R. D. Kersten, K. S. Ryan, D. J. Gonzalez, V. Nizet, P. C. Dorrestein, B. S. Moore, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 1957–1962.
[13] J. P. Gomez-Escribano, M. J. Bibb, *Microb. Biotechnol.* **2011**, *4*, 207–215.
[14] a) More recently, the breadth of reactions catalyzed by TEs has expanded significantly to include dehydration for *cis* double bond formation in FR901464 biosynthesis and epimerization in nocardicin biosynthesis; however, these are all intramolecular reactions; b) N. M. Gaudelli, C. A. Townsend, *Nat. Chem. Biol.* **2014**, *10*, 251–258; c) H.-Y. He, M.-C. Tang, F. Zhang, G.-L. Tang, *J. Am. Chem. Soc.* **2014**, *136*, 4488–4491.
[15] V. Agarwal, S. Diethelm, L. Ray, N. Garg, T. Awakawa, P. C. Dorrestein, B. S. Moore, *Org. Lett.* **2015**, *17*, 4452–4455.

Received: September 13, 2015
Published online: November 10, 2015

