

Cite this: *Chem. Sci.*, 2019, 10, 4486

All publication charges for this article have been paid for by the Royal Society of Chemistry

Received 5th February 2019

Accepted 11th March 2019

DOI: 10.1039/c9sc00641a

rsc.li/chemical-science

# Biosynthetic reconstitution of deoxysugar phosphoramidate metalloprotease inhibitors using an N–P-bond-forming kinase†

Alexandra Baulig,<sup>ab</sup> Irina Helmle,<sup>a</sup> Marius Bader,<sup>ab</sup> Felix Wolf,<sup>ab</sup> Andreas Kulik,<sup>c</sup> Arwa Al-Dilaimi,<sup>d</sup> Daniel Wibberg,<sup>d</sup> Jörn Kalinowski,<sup>d</sup> Harald Gross<sup>ab</sup> and Leonard Kaysser<sup>ab</sup>

Phosphoramidon is a potent metalloprotease inhibitor and a widespread tool in cell biology research. It contains a dipeptide backbone that is uniquely linked to a 6-deoxysugar *via* a phosphoramidate bridge. Herein, we report the identification of a gene cluster for the formation of phosphoramidon and its detailed characterization. *In vitro* reconstitution of the biosynthesis established TaE as a phosphoramidate-forming kinase and TaC as the glycosyltransferase which installs the L-rhamnose moiety by phosphoester linkage.

## Introduction

Phosphoramidate and phosphonamidate moieties are common motifs in medicinal chemistry. Nucleoside phosphoramidates are developed as antiviral prodrugs with great success.<sup>1</sup> In protease inhibitors they serve as peptidomimetics replacing the carbonyl carbon in the amide bond.<sup>2</sup> They can be seen as transition-state analogues of the enzyme reaction and coordinate to the active site metal ion in metalloproteases.<sup>3</sup> In natural products, however, N–P bonds are comparably rare and little is known about their biosynthesis.<sup>4</sup> Phosphoramidon (Fig. 1; **1**) and its stereoisomer talopeptin (**2**) have been isolated from *Streptomyces* culture broths and are potent inhibitors of metalloendopeptidases including thermolysin, *Pseudomonas* elastase LasB, neutral endopeptidase and endothelin-converting enzyme.<sup>5–7</sup> Phosphoramidon was extensively investigated in animal models and pilot clinical studies for its effects on various diseases.<sup>8–10</sup> Today, this compound is still a widely used pharmacological tool in cell biological research. The inhibitory activity of **1** and **2** has been attributed to the metal-chelating properties of a phosphoramidate moiety which uniquely links a deoxysugar to the N-terminus of a L-Leu–L-Trp dipeptide backbone.<sup>11</sup> The deoxysugar consists of L-rhamnose in the case

of **1** and 6-deoxy-L-talose in the case of **2**. The two molecules belong to a small group of structurally diverse natural products with N–P bonds (Fig. 1). The biosynthesis of such compounds has drawn a lot of interest and the biosynthetic gene clusters (BGCs) were identified for microcin C7 (**3**) in *Escherichia coli*,<sup>12,13</sup>

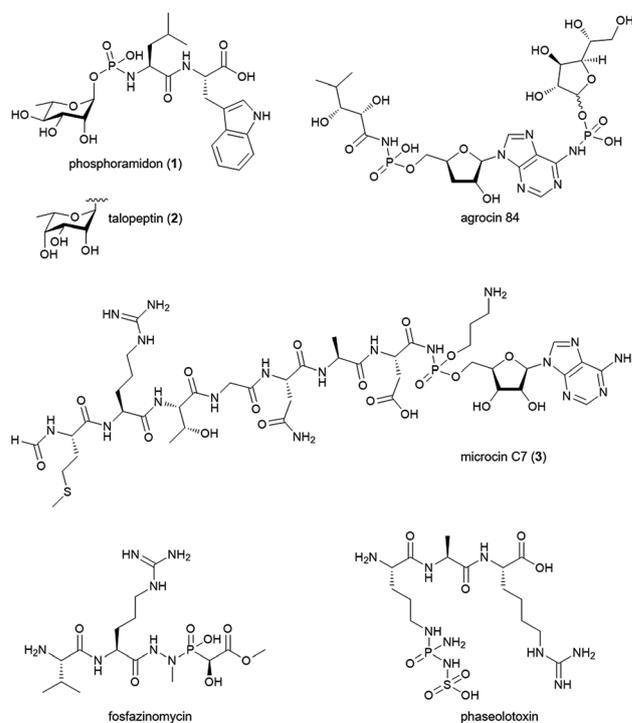


Fig. 1 Selected natural products with phosphoramidate or phosphonamidate moieties.

<sup>a</sup>Department of Pharmaceutical Biology, Pharmaceutical Institute, University of Tübingen, 72076 Tübingen, Germany. E-mail: leonard.kaysser@uni-tuebingen.de

<sup>b</sup>German Centre for Infection Research (DZIF), partner site Tübingen, 72076 Tübingen, Germany

<sup>c</sup>Interfaculty Institute for Microbiology and Infection Medicine Tübingen (IMIT), Microbiology/Biotechnology, University of Tübingen, 72076 Tübingen, Germany

<sup>d</sup>Center for Biotechnology (CeBiTec), Bielefeld University, 33615 Bielefeld, Germany

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9sc00641a



agrocin 84 in *Agrobacterium radiobacter*,<sup>14</sup> fosfazinomycin in *Streptomyces* sp. WM6372<sup>15</sup> and phaseolotoxin in *Pseudomonas syringae* pv. phaseolicola.<sup>16</sup> However, the enzymatic mechanism for the formation of the N–P bond has so far only been established for **3**. Here, the adenylyating enzyme MccB catalyzes the formation of the phosphoramidate linkage of a precursor peptide and AMP *via* the generation of a succinimide intermediate and the consumption of two molecules of ATP.<sup>17</sup> Given the structural difference to **3** and the absence of an adenylyl group we speculated about a distinct biosynthetic strategy to form the phosphoramidate bridge in **1** and **2**.

## Results and discussion

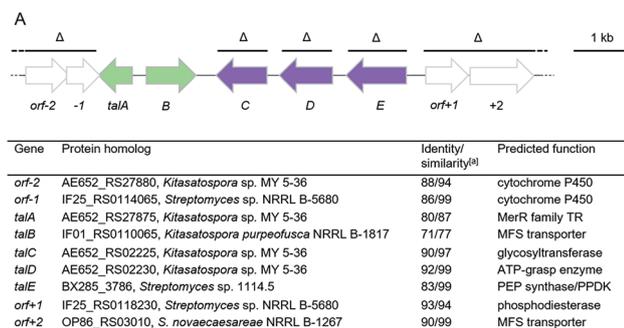
### Identification of the talopeptin biosynthetic gene cluster

To explore the genetic basis for the assembly of **1** and **2** we sequenced the genome of *Streptomyces mozunensis* MK-23, the original talopeptin producer. This approach resulted in a high quality draft genome with eight scaffolds and 7708 predicted genes. Unexpectedly, analysis of the draft genome for the presence of non-ribosomal peptide synthetase (NRPS) genes did not result in the identification of a reasonable candidate pathway. We thus used Cpz31, a rhamnosyltransferase from the caprazamycin gene cluster,<sup>18</sup> as reference to scan the genome sequence on the assumption, that a homologous enzyme could be involved in the installation of the deoxysugar in **2**. A BLAST comparison revealed one homolog, TalC, with 45% sequence identity to Cpz31. The respective gene was co-located with genes for a putative ATP-grasp enzyme (TalD)

and a putative PEP synthase/pyruvate, phosphate dikinase (TalE; Fig. 2A). Adjacent to this small operon-like structure were putative genes encoding for transport and regulatory functions. Based on these findings a three-reaction pathway to **2** (and **1**) would be conceivable (Fig. 2B). TalD would form a dipeptide (**4**) from L-Leu and L-Trp. This could serve as a precursor for TalE to install an N-terminal phosphoramidate generating **5**. The subsequent transfer of the deoxysugar could be catalyzed by TalC to afford **2**.

### Heterologous expression and gene deletion studies

To confirm the identity of the putative *tal* gene cluster, we transferred the pathway on an integrative pCC1FOS derivative into *Streptomyces* sp. MK730-62F2 for heterologous expression. This strain is known to provide activated L-rhamnose for the endogenous production of caprazamycins and the respective deoxysugar biosynthesis genes have recently been identified.<sup>18,19</sup> To our satisfaction, LC-MS analysis of culture extracts from the heterologous mutant showed the production of a single new peak with a mass, retention time and UV-spectrum identical to a commercial standard of **1** (Fig. 3, S1, S4 and S5†) but with a 0.2 min shift in retention time to **2**. This mass peak was absent in the culture extracts of the host without the *tal* cluster. This finding confirmed that we have found a BGC for the production of **1** and **2**, likely comprising the *talCDE* operon. It appears that the deoxysugar transferase TalC is flexible towards the stereo-configuration of the hydroxy group at C-4. To unambiguously determine the organization and the boundaries of the *tal* cluster we individually deleted the genes *talC*, *talD* and *talE* as well as the bordering regions *orf-7* to *orf-1* and *orf+1* to *orf+12* and expressed the mutant clusters heterologously. LC-MS analysis of culture extracts from the mutant strains showed that the biosynthesis of **1** was not affected by the  $\Delta orf-7-orf-1$  and the  $\Delta orf+1-orf+12$  deletions. In contrast, the  $\Delta talC$ ,  $\Delta talD$  and  $\Delta talE$  mutations led to an apparent loss of production (Fig. 3). This strongly indicates that *talA*, a MerR-like regulatory gene, and *talB*, encoding a putative MFS transporter, form the BGC for **1** and **2** together with the *talCDE* operon. Notably, trace amounts of **1** could still be detected in cultures of the  $\Delta talD$  mutant by MS analysis. This might be explained by the supply of **4** from standard protein degradation processes.



[a] = amino acid sequence homology [%] from blastp analysis; MFS = major facilitator superfamily; *orf* = open reading frame; PEP = phosphoenolpyruvate; PPDK = pyruvate, phosphate dikinase; S. = *Streptomyces*; sp. = species; TR = transcription regulator.

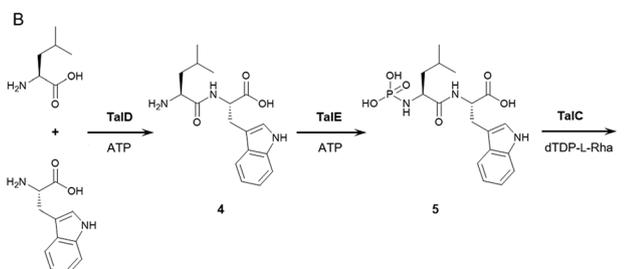


Fig. 2 Biosynthesis of **1** and **2**. (A) Organization of the talope BGC from *S. mozunensis* MK-23 and gene cluster table. Genes deleted in the course of this study are indicated ( $\Delta$ ). Purple arrows: biosynthetic genes. Green arrows: transport and regulation. (B) Proposed pathway for the biosynthesis of **1**.

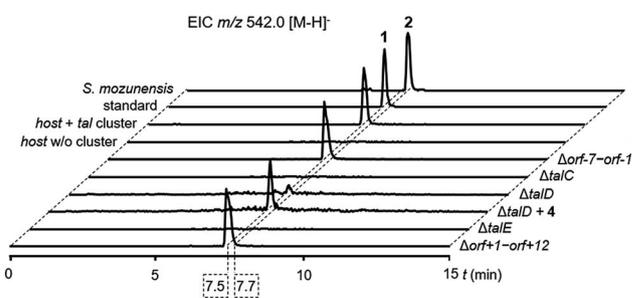


Fig. 3 LC-MS analysis of culture extracts for the production of **1** in *Streptomyces* sp. MK730-62F2/*talMB01* and **2** in *S. mozunensis* MK-23. Extracted ion chromatograms (EICs).



ATP-grasp enzymes such as TalD activate carboxylic acid substrates as acylphosphates under consumption of ATP for subsequent condensation reactions. They have received increasing attention as discrete amide ligases for their ability to form peptide bonds in natural product biosynthesis.<sup>20</sup> Our results suggest that the peptide backbone of **1** and **2** is assembled by ATP-grasp biochemistry in analogy to other small-molecule protease inhibitors *e.g.* the ketomemcins but in contrast to the class of hydroxamic acid-containing metalloprotease inhibitors which derive from NRPS systems.<sup>21–23</sup> To unambiguously assign the role of TalD in the biosynthesis of **1** and **2** we supplemented cultures of the *ΔtalD* mutant with a synthetic **4** precursor. LC-MS analysis of the culture extracts showed that the addition of the dipeptide largely restored the production of **1** in the mutant (Fig. 3). This result confirms the function of TalD as a dipeptide synthetase in the formation of **1** and **2**.

### Identification of TalE as an N-P-bond-forming kinase

Next, we wanted to explore the biosynthetic mechanism that installs the unique deoxysugar phosphoramidate moiety onto **4**. Therefore, we cloned the remaining two biosynthetic genes *talC* and *talE* for protein production in *E. coli*. The putative kinase His-TalE could be produced in yields of 2.71 mg L<sup>-1</sup>, whereas His-TalC could not be obtained as soluble protein under various conditions (Fig. S10 and S11†). TalE has homology to PEP synthases and pyruvate, phosphate dikinases (PPDKs). PPDKs catalyze the 2-step conversion of pyruvate, ATP and phosphate to phosphoenolpyruvate, AMP and P<sub>i</sub> in plants and the reversible reaction bacteria.<sup>24</sup> They use a catalytic histidine residue to generate a phosphohistidine intermediate with a high-energy N-P bond that serves as a phosphate shuttle between the two reaction centers of the enzyme.<sup>25</sup> Phosphohistidine intermediates can be found in various kinases.<sup>26</sup> Some of those enzymes *e.g.* Hpr or nucleoside diphosphate kinases, were shown to transfer the phosphoryl group onto histidine residues of other proteins to regulate their enzymatic activity.<sup>27</sup>

We thus wondered if TalE might catalyze a similar reaction using the terminal amine of a dipeptide as the acceptor substrate instead of a proteinogenic histidine. To test this hypothesis we incubated purified His-TalE with ATP and synthetic **4** for 2 hours at 30 °C. LC-MS analysis of the reaction mixture showed the presence of an additional mass peak **5** at retention time 8.4 min with *m/z* 396.1 [M - H]<sup>-</sup> (Fig. 4 and S7†). The production of this new compound was dependent on the concentration of TalE (Fig. S9†) and absent when one of the assay components was omitted (Fig. 4). HR-MS measurements of **5** indicated a molecular formula of C<sub>17</sub>H<sub>23</sub>N<sub>3</sub>O<sub>6</sub>P (*m/z*<sub>meas</sub> for [M - H]<sup>-</sup> 396.13400 *vs.* *m/z*<sub>calcd</sub> for [M - H]<sup>-</sup> 396.13300, Δ = +2.53 ppm; Fig. S2 and S3†) and thus a possible phosphoramidate as the TalE product. To unequivocally determine the structure of **5** we purified the compound for comparison of the spectral properties of the product with those of its precursor **4**. Both compounds were subjected to a series of <sup>1</sup>H/<sup>13</sup>C/<sup>15</sup>N-based 1D/2D NMR standard experiments (Fig. 4 and S12–S27†).

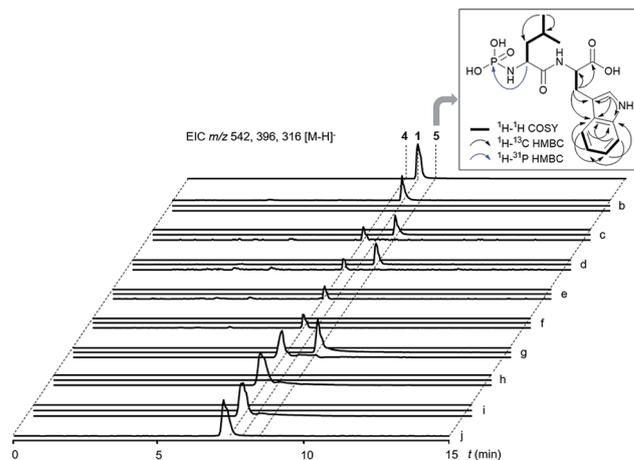


Fig. 4 LC-MS analysis of enzyme reactions. Extracted ion chromatograms (EICs) for *m/z* 542 [M - H]<sup>-</sup> (**1**), *m/z* 396 [M - H]<sup>-</sup> (**5**) and *m/z* 316 [M - H]<sup>-</sup> (**4**). (a) Commercial standard of **1**. (b) Enzymatic assay with TalC (protein extract of *ΔtalE* mutant), dTDP-L-rhamnose, TalE, **4** and ATP showed conversion of **4** to **1**. (c) Control assay of (b) without TalC using a protein extract of the *ΔtalC* mutant. (d) Control assay of (b) without dTDP-L-rhamnose. (e) Control assay: protein extract of *ΔtalC* mutant with **4**. (f) Control assay: protein extract of *ΔtalE* mutant with **4**. (g) Enzymatic assay of TalE with **4** and ATP showed conversion of **4** to **5**. (h) Control assay of (g) without TalE. (i) Control assay of (g) without ATP. (j) Control assay of (g) without **4**. Box: observed NMR correlations of **5**.

Subsequent analysis of all obtained spectra confirmed the presence of the Leu-Trp skeleton in **4** and **5**. The <sup>1</sup>H and <sup>13</sup>C NMR spectrum of **5** was highly similar to the corresponding spectra of **4**, with the exception of the resonances of Leu-α and Leu-β whose proton resonances shifted upfield, while the corresponding carbon resonances shifted downfield (Tables S1 and S2†), suggesting already that the phosphorylation had occurred at the N-terminal leucine residue of **4**. This hypothesis was further corroborated by the interpretation of a <sup>1</sup>H decoupled <sup>31</sup>P NMR spectrum, which revealed that **5** contained, in contrast to **4** a phosphorus resonance, whose shift value δ<sub>P</sub> -0.25 was indicative for a phosphoramidate group (Fig. S28 and S29†). Final evidence for the existence of a N-P bond in **5** was provided by a cross-correlation between Leu-α and P, observed in the <sup>1</sup>H-<sup>31</sup>P HMBC NMR spectrum of **5** (Fig. 4, S30†). A complementary MS/MS analysis of **5** of the [M + H]<sup>+</sup> ion at *m/z* 398 produced *b*- and *y*-series fragment ions that were also in full agreement with the proposed structure (Fig. S8†).

These results confirm that TalE catalyzes the second step in the biosynthesis of **1** and **2** using the TalD product **4** and ATP as substrates to form **5**. To the best of our knowledge, TalE represents the first enzyme in natural product biosynthesis that generates a small molecule phosphoramidate by simple phosphorylation of an amine. Very recently, Raushel and co-workers reported on a so far unprecedented enzyme-catalyzed phosphorylation of an amide to generate γ-phospho-L-glutamine.<sup>28,29</sup> The respective enzyme, Cj1418, is involved in O-methyl phosphoramidate modification of capsular polysaccharides in *Campylobacter jejuni*. Notably, Cj1418 shows conserved protein



domains typical for PEP synthases and PPKs but has no significant sequence similarity to TalE (<20% on AA level). It appears that members of this family of kinase enzymes have evolved separately in bacteria to synthesize small-molecule phosphoramidates in the primary and secondary metabolism. It is tempting to speculate that putative PPK homologs encoded in the BGCs for agrocin 84 and phaseolotoxin might have an analogous role in the respective pathways.<sup>14,16</sup>

### *In vitro* biosynthesis of phosphoramidon

We next aimed to establish **5** as a genuine intermediate in the biosynthesis of **1**. Therefore, we added dTDP-L-rhamnose and a TalC-containing protein extract to the TalE assay. After incubation for 16 h at 30 °C we analyzed the reaction mixture by LC-MS and identified a single product peak matching **1** in retention time, UV spectrum, mass and MS/MS fragmentation (Fig. 4, S1, S6 and 7†). **1** was not produced when either **5**, dTDP-L-rhamnose or TalC were absent in the assay. These results show that TalC uses **5** as an acceptor substrate to catalyze the transfer of a deoxysugar to the phosphoryl group. In general, glycosylation is a common theme in natural product biosynthesis altering target specificity and pharmacological properties of a parent scaffold.<sup>30</sup> Particularly, 6-deoxyhexoses are prevalent features in bacterial secondary metabolites. The substrate promiscuity of the respective glycosyltransferases has been extensively used to engineer the structure of bioactive small-molecules.<sup>31</sup> In most cases these enzymes build a direct O-glycosidic link to the aglycon although the formation of thioglycosides, N-glycosides and C-glycosides is also known.<sup>32–34</sup> Attachment of sugars in natural product biosynthesis *via* phosphoester linkage, however, is very rare and has only been functionally investigated for moenomycins. Here, Walker and co-workers showed that MoeGT1 transfers galacturonic acid from UDP-GalUA to a farnesyl phosphoglycerate precursor.<sup>35</sup> The enzymatic installation of an L-rhamnose moiety to the phosphoramidate of **5** thus represents a so far undescribed reaction in natural product biochemistry and adds TalE as a unique biocatalyst to the family of 6-deoxysugar transferases.

## Conclusions

In conclusion, we have identified the gene cluster for the biosynthesis of phosphoramidate metalloprotease inhibitors. Chemical complementation and *in vitro* reconstitution revealed a three-reaction pathway to form **1** and **2**, which involves exceptional biochemistry. An ATP-grasp amide ligase, a phosphoryl transferase and a deoxysugar transferase work together in the condensation of two amino acids and the subsequent linkage of a 6-deoxyhexose to the resulting dipeptide *via* a phosphoramidate bridge. A preliminary search in public genome databases found a number of homologous orphan pathways containing a *talCDE*-like putative operon (Fig. S31†). Given the possibility that the encoded ATP-grasp enzymes and glycosyltransferases may express a different substrate tolerance than TalD and TalC, respectively, the identified gene clusters could direct the synthesis of new metalloprotease inhibitors.

Additionally, both enzymes have potential as flexible biocatalysts for *in vivo* and *in vitro* engineering of bioactive small-molecules.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

We gratefully thank Hamada Saad for helpful advises, Dr Dorothee Wistuba for HR-MS support and Anika Winkler for expert assistance during DNA sequencing. This work was generously supported by the DFG (KA 3071/4-1 and GRK 1708). A. K. was supported by grants from the SFB 766.

## Notes and references

- 1 Y. Mehellou, J. Balzarini and C. McGuigan, *ChemMedChem*, 2009, **4**, 1779–1791.
- 2 J. Vagner, H. Qu and V. J. Hruby, *Curr. Opin. Chem. Biol.*, 2008, **12**, 292–296.
- 3 D. E. Tronrud, A. F. Monzingo and B. W. Matthews, *Eur. J. Biochem.*, 1986, **157**, 261–268.
- 4 A. J. Waldman, T. L. Ng, P. Wang and E. P. Balskus, *Chem. Rev.*, 2017, **117**, 5784–5863.
- 5 S. Umezawa, K. Tatsuta, O. Izawa, T. Tsuchiya and H. Umezawa, *Tetrahedron Lett.*, 1972, **13**, 97–100.
- 6 H. Suda, T. Aoyagi, T. Takeuchi and H. Umezawa, *J. Antibiot.*, 1973, **26**, 621–623.
- 7 S. Murao, M. Katsura, K.-i. Fukuhara and K. Oda, *Agric. Biol. Chem.*, 1980, **44**, 701–703.
- 8 N. Crimi, R. Polosa, G. Pulvirenti, S. Magri, G. Santonocito, G. Prosperini, C. Mastruzzo and A. Mistretta, *Thorax*, 1995, **50**, 505–510.
- 9 H. H. Kramer, K. Schmidt, S. Leis, M. Schmelz, C. Sommer and F. Birklein, *Exp. Neurol.*, 2005, **195**, 179–184.
- 10 M. P. Love, W. G. Haynes, G. A. Gray, D. J. Webb and J. J. McMurray, *Circulation*, 1996, **94**, 2131–2137.
- 11 L. H. Weaver, W. R. Kester and B. W. Matthews, *J. Mol. Biol.*, 1977, **114**, 119–132.
- 12 M. A. Novoa, L. Diaz-Guerra, J. L. San Millan and F. Moreno, *J. Bacteriol.*, 1986, **168**, 1384–1391.
- 13 J. E. Gonzalez-Pastor, J. L. San Millan, M. A. Castilla and F. Moreno, *J. Bacteriol.*, 1995, **177**, 7131–7140.
- 14 J. G. Kim, B. K. Park, S. U. Kim, D. Choi, B. H. Nahm, J. S. Moon, J. S. Reader, S. K. Farrand and I. Hwang, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 8846–8851.
- 15 J. T. Gao, K. S. Ju, X. M. Yu, J. E. Velasquez, S. Mukherjee, J. Lee, C. M. Zhao, B. S. Evans, J. R. Doroghazi, W. W. Metcalf and W. A. van der Donk, *Angew. Chem., Int. Ed.*, 2014, **53**, 1334–1337.
- 16 S. Aguilera, K. Lopez-Lopez, Y. Nieto, R. Garciduenas-Pina, G. Hernandez-Guzman, J. L. Hernandez-Flores, J. Murillo and A. Alvarez-Morales, *J. Bacteriol.*, 2007, **189**, 2834–2843.
- 17 R. F. Roush, E. M. Nolan, F. Lohr and C. T. Walsh, *J. Am. Chem. Soc.*, 2008, **130**, 3603–3609.



- 18 L. Kaysser, L. Lutsch, S. Siebenberg, E. Wemakor, B. Kammerer and B. Gust, *J. Biol. Chem.*, 2009, **284**, 14987–14996.
- 19 L. Kaysser, E. Wemakor, S. Siebenberg, J. A. Salas, J. K. Sohng, B. Kammerer and B. Gust, *Appl. Environ. Microbiol.*, 2010, **76**, 4008–4018.
- 20 Y. Ogasawara and T. Dairi, *Chem.–Eur. J.*, 2017, **23**, 10714–10724.
- 21 J. Kawata, T. Naoe, Y. Ogasawara and T. Dairi, *Angew. Chem., Int. Ed.*, 2017, **56**, 2026–2029.
- 22 F. Wolf, J. S. Bauer, T. M. Bendel, A. Kulik, J. Kalinowski, H. Gross and L. Kaysser, *Angew. Chem., Int. Ed.*, 2017, **56**, 6665–6668.
- 23 F. Leipoldt, J. Santos-Aberturas, D. P. Stegmann, F. Wolf, A. Kulik, R. Lacroix, D. Popadic, D. Keinhorster, N. Kirchner, P. Bekiesch, H. Gross, A. W. Truman and L. Kaysser, *Nat. Commun.*, 2017, **8**, 1965.
- 24 H. J. Evans and H. G. Wood, *Proc. Natl. Acad. Sci. U. S. A.*, 1968, **61**, 1448–1453.
- 25 O. Herzberg, C. C. Chen, G. Kapadia, M. McGuire, L. J. Carroll, S. J. Noh and D. Dunaway-Mariano, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 2652–2657.
- 26 S. Cheek, H. Zhang and N. V. Grishin, *J. Mol. Biol.*, 2002, **320**, 855–881.
- 27 S. R. Fuhs and T. Hunter, *Curr. Opin. Cell Biol.*, 2017, **45**, 8–16.
- 28 Z. W. Taylor, H. A. Brown, T. Narindoshvili, C. Q. Wenzel, C. M. Szymanski, H. M. Holden and F. M. Raushel, *J. Am. Chem. Soc.*, 2017, **139**, 9463–9466.
- 29 Z. W. Taylor, A. R. Chamberlain and F. M. Raushel, *Biochemistry*, 2018, **57**, 5447–5455.
- 30 S. I. Elshahawi, K. A. Shaaban, M. K. Kharel and J. S. Thorson, *Chem. Soc. Rev.*, 2015, **44**, 7591–7697.
- 31 R. W. Gantt, P. Peltier-Pain and J. S. Thorson, *Nat. Prod. Rep.*, 2011, **28**, 1811–1853.
- 32 C. Durr, D. Hoffmeister, S. E. Wohlert, K. Ichinose, M. Weber, U. Von Mulert, J. S. Thorson and A. Bechthold, *Angew. Chem., Int. Ed.*, 2004, **43**, 2962–2965.
- 33 T. J. Oman, J. M. Boettcher, H. Wang, X. N. Okalibe and W. A. van der Donk, *Nat. Chem. Biol.*, 2011, **7**, 78–80.
- 34 A. P. Salas, L. Zhu, C. Sanchez, A. F. Brana, J. Rohr, C. Mendez and J. A. Salas, *Mol. Microbiol.*, 2005, **58**, 17–27.
- 35 B. Ostash, E. H. Doud, C. Lin, I. Ostash, D. L. Perlstein, S. Fuse, M. Wolpert, D. Kahne and S. Walker, *Biochemistry*, 2009, **48**, 8830–8841.

