

## RESEARCH ARTICLE

View Article Online  
View Journal | View IssueCite this: *Org. Chem. Front.*, 2016, **3**, 1254

# Molecular engineering of thiostrepton *via* single "base"-based mutagenesis to generate side ring-derived variants†

Panpan Duan,<sup>a</sup> Qingfei Zheng,<sup>a</sup> Zhi Lin,<sup>a</sup> Shoufeng Wang,<sup>a</sup> Dandan Chen<sup>b</sup> and Wen Liu<sup>\*a,b</sup>

The quinaldic acid (QA) moiety in the side ring of thiostrepton (TSR), which can be modified regioselectively *via* precursor-directed mutational biosynthesis, was proven to be biologically relevant but tunable, affecting TSR's outstanding antibacterial activities. In this study, we sought to obtain TSR derivatives with varying amino acid residues connected to the QA moiety. The generation of these TSR derivatives relied on single "base"-based mutagenesis, and six new TSR-type compounds were obtained. Moreover, the simultaneous mutation of Ile1 and Ala2 in the TSR side ring resulted in a naturally occurring compound, siomycin (SIO), together with a new component, SIO-Dha2Ser. The anti-infection assays indicated that all of these new compounds could act as both antimicrobial agents and autophagy inducers, and these two kinds of activities can also be separated *via* regioselective modifications on the TSR side ring.

Received 5th July 2016,

Accepted 28th July 2016

DOI: 10.1039/c6qo00320f

rsc.li/frontiers-organic

Among all the ribosomally synthesized and post-translationally modified peptide (RiPP) natural products, thiostrepton (TSR) possesses the most complex structure and elusive biosynthetic pathway.<sup>1</sup> In addition, unlike most RiPPs, TSR has a side ring, of which the key building block, a quinaldic acid (QA) moiety, is biosynthesized independently of the precursor peptide.<sup>2</sup> Benefiting from this unique biosynthetic logic, precursor-directed mutational biosynthesis has been conducted to generate side ring-modified TSR derivatives with varying QA moieties *via* the exogenous chemical feeding of synthetic QA analogs into the corresponding mutant strains.<sup>3</sup> The resulting TSR variants show more potent biological activities than the parent compound and exhibit a novel dual mode of action against intracellular pathogens (*e.g.*, *Mycobacterium marinum*).<sup>4</sup> Moreover, other investigations have proven that the side ring of TSR plays significant roles in its antitumor activities and that excessive modification of the QA moiety abolishes TSR's biological activities.<sup>5</sup> Previous studies have shown that changing the amino acid residue connected to the QA moiety affects the conformation of the TSR side ring.<sup>6</sup> To regulate the chemical environment of the QA moiety and achieve rational control over

TSR's activities, in this work, we sought to replace the Ile1 of TSR with other amino acid residues (Fig. 1).

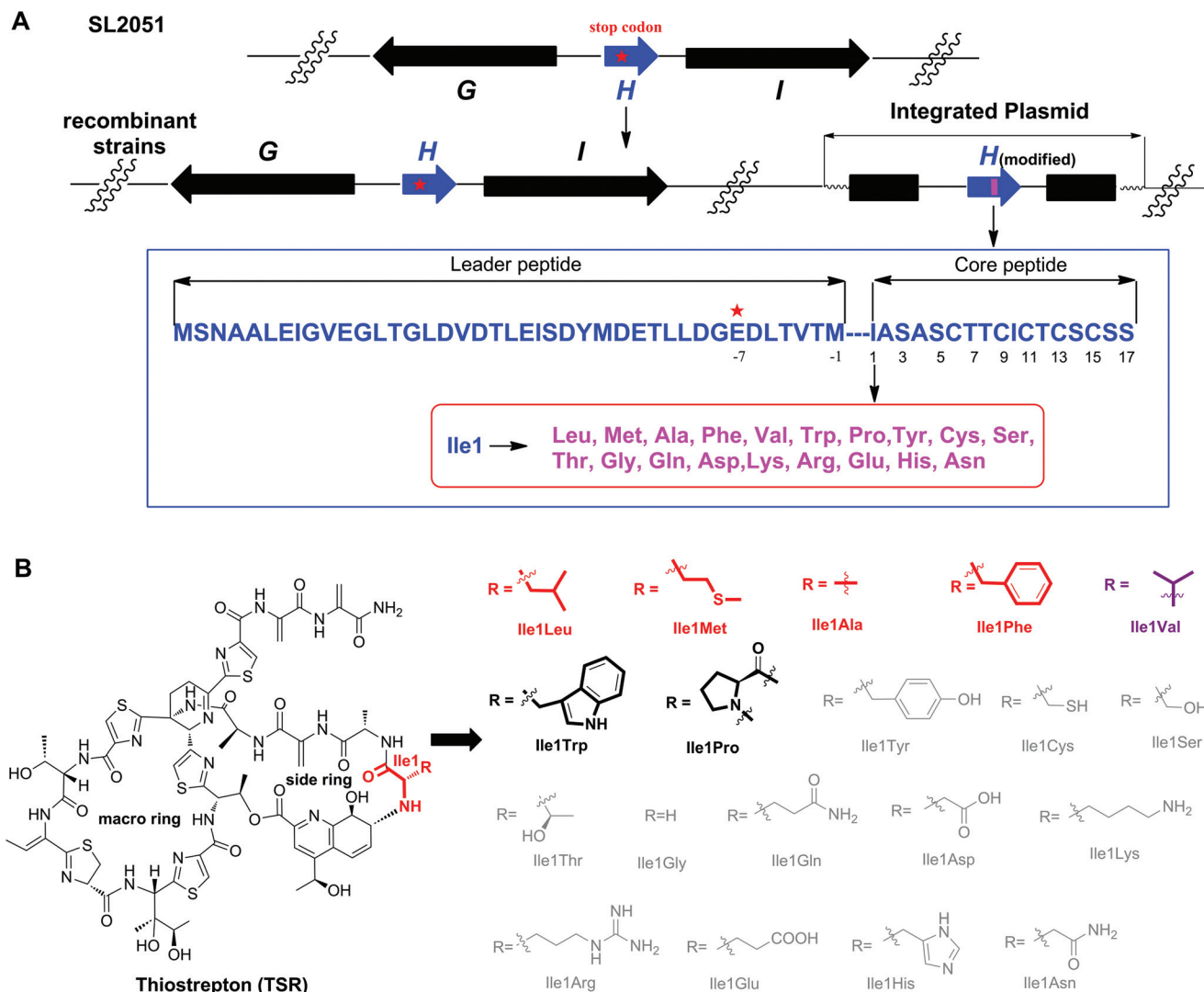
The complex structure of TSR poses great challenges for total chemical synthesis,<sup>7</sup> however the relatively low genetic complexity associated with TSR biosynthesis makes it an excellent candidate for molecular engineering *via* synthetic biology strategies.<sup>8</sup> In our previous studies, we found that the gene encoding the precursor peptide (*tsrH*) may serve as an important regulatory element in the gene cluster *tsr* and that the traditional method of gene deletion-*in trans* complementation cannot restore TSR production. Accordingly, in a previous study we developed a single "base"-based mutagenesis strategy to modify the TSR precursor peptide.<sup>9</sup> Unlike previous methods, in this strategy, the target gene is inactivated by introducing a stop codon into the gene sequence instead of an in frame-gene deletion and, thereby, not disturbing the regulatory function of the open reading frame. Thus, TSR production can be restored, and the TSR derivatives can be generated *via in trans* complementation with wild-type or modified *tsrH* genes (Fig. 1).

We first conducted saturation mutagenesis to transform Ile1 into the other 19 natural amino acids (Fig. 1). In addition to the previously reported variant TSR-Ile1Val,<sup>9</sup> high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) and ultraviolet (UV) absorption analyses revealed that six new TSR-type compounds were produced when Ile1 was replaced with Leu, Phe, Ala, Met, Trp and Pro (Fig. 2 and ESI†). The structures of TSR-Ile1Leu, TSR-Ile1Phe, TSR-Ile1Ala and TSR-Ile1Met were determined by

<sup>a</sup>State Key Laboratory of Bioorganic and Natural Products Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, 345 Lingling Road, Shanghai 200032, China. E-mail: wliu@mail.sioc.ac.cn

<sup>b</sup>Huzhou Center of Bio-Synthetic Innovation, 1366 Hongfeng Road, Huzhou 313000, China

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c6qo00320f

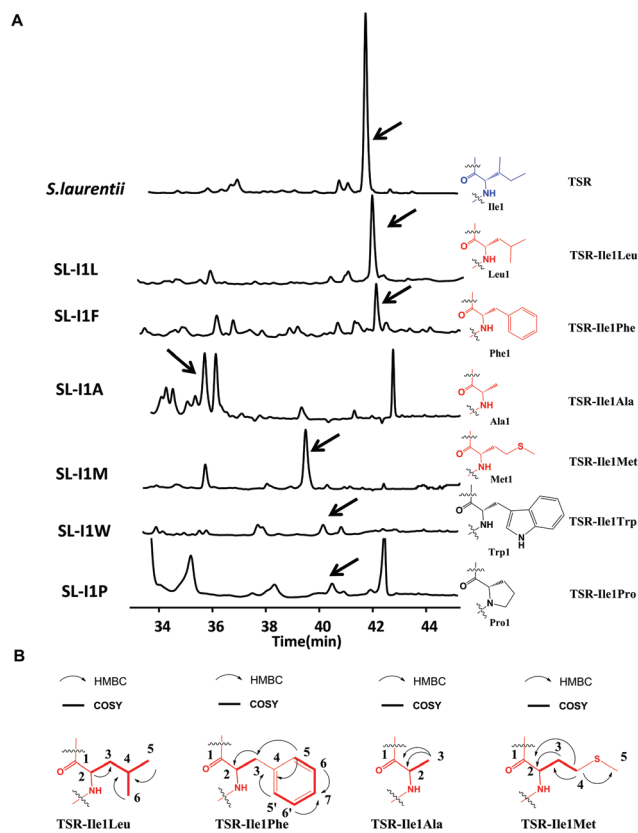


**Fig. 1** The single “base”-based mutagenesis strategy and the derivatives generated by site-directed mutagenesis of *TsrH*. (A) Genotypes of the SL2051 (by mutation of GAG for Glu-7 into the stop codon TAG) and recombinant strains with the corresponding encoded precursor peptides. The gene *tsrH* and post-translationally modified encoding genes are shown in blue and black, respectively. The position where the stop codon was introduced via an *in situ* single “base”-mutation is indicated by a red star, and the mutated position 1 is indicated by a pink bar. (B) Structures of TSR-Ile1X (X represents natural amino acids other than Ile). The amino acid residue is abbreviated using a three-letter code. The derivatives in gray are not produced. The derivatives with high yields are highlighted in red or purple (TSR-Ile1Val was reported previously), whereas those with low yields are highlighted in black.

$^1\text{H}$ ,  $^{13}\text{C}$  and two-dimensional (2D) nuclear magnetic resonance (NMR) analyses (ESI $^\dagger$ ). Overall, the spectra of TSR-Ile1Leu, TSR-Ile1Phe, TSR-Ile1Ala and TSR-Ile1Met are similar to that of the parent compound TSR (ESI $^\dagger$ ). The analysis of their 1D and 2D NMR spectra revealed that the only difference existed in residue 1. The four new TSR analogs all clearly lacked the  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR signals of Ile1 in TSR. In TSR-Ile1Leu, the establishment of correlation spectroscopy (COSY; C6, C5, C4, C3 and C2) and heteronuclear multiple bond correlation (HMBC;  $\text{H}_3\text{-6CH}_3$  to C4 and C3;  $\text{H}_3\text{-5CH}_3$  to C4 and C3; and H-2CH to C3) further supports the substitution of a *tert*-butyl group at C2 in residue 1 in place of the isobutyl group in TSR (Fig. 2). This result is consistent with residue 1 in TSR-Ile1Leu

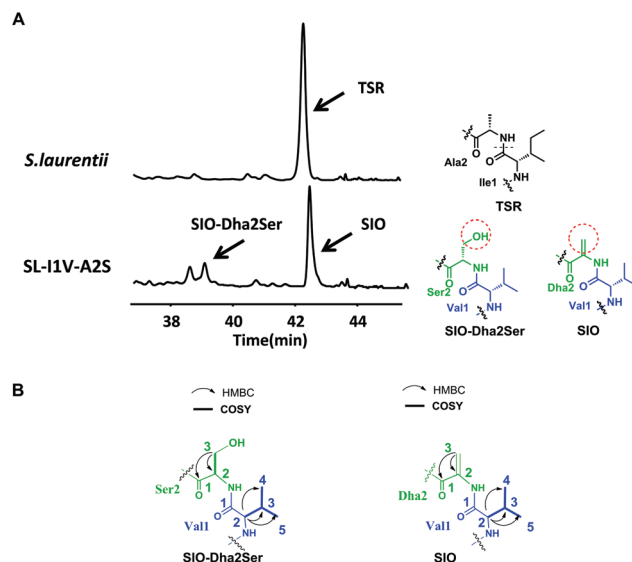
being derived from Leu (rather than Ile in TSR). The structures of TSR-Ile1Phe, TSR-Ile1Ala and TSR-Ile1Met were further verified in the same way (Fig. 2), whereas the structures of TSR-Ile1Trp and TSR-Ile1Pro were determined by UV absorption and high-resolution mass spectrometry (HRMS) because of their low yields (ESI $^\dagger$ ).

The successful engineering of TSR's Ile1 residue into six alternative amino acids suggested that this position is highly variable and encouraged us to extend the applications of the single “base”-based mutagenesis strategy. Siomycin (SIO), isolated from *Streptomyces sioyaensis*, is a naturally occurring TSR analog in which the 1st and 2nd residues in the side ring are Val1 and Dha2, respectively (rather than Ile1 and Ala2 in TSR;



**Fig. 2** Production of new Ile1-derived TSR derivatives and their structural elucidation. (A) HPLC analyses of the fermentation cultures of the wild-type TSR-producing *S. laurentii* strain and the mutant strains SL-I1L, SL-I1F, SL-I1A, SL-I1 M, SL-I1 W, and SL-I1P. The resulting TSR and its analogs are indicated by arrows. TSR is shown in blue. The derivatives with high yields are highlighted in red, whereas those with low yields are highlighted in black. (B) Selected HMBC and COSY correlations for residue 1 in the derivatives with high yields.

Fig. 3).<sup>10</sup> SIO shows more potent activities against tumor cells and plasmodia than TSR.<sup>11</sup> However, the fermentation yield of SIO is much lower than that of TSR, and the genetic manipulation and fermentation processes are more challenging for *S. sioyensis* than for the TSR-producing strain *S. laurentii*.<sup>12</sup> Thus, the simultaneous mutagenesis of TSR's Ile1 and Ala2 into Val1 and Dha2 was performed to produce SIO in the TSR-producing strain. Because the Dha2 in SIO is generated from Ser by a glutamate-dependent dehydratase during its biosynthesis,<sup>13</sup> the codons for Ser and Val were introduced into the complementation gene to replace those for Ala2 and Ile1 in *tsrH*. HPLC and LC-MS analyses revealed that SIO can thus be produced by the engineered strain SL-I1V-A2S (Fig. 3), and an unexpected new component with a molecular weight of 1665.4 (18 Da larger than that of SIO) was also found (Fig. 3). <sup>1</sup>H, <sup>13</sup>C, 2D NMR and HRMS analyses confirmed the structure of SIO and revealed that this new component is a hydroxyl SIO analog, SIO-Dha2Ser (Fig. 3 and ESI<sup>+</sup>). The unexpected production of SIO-Dha2Ser most likely occurred because the glutamate-dependent dehydratase in the TSR biosynthetic system cannot accurately recognize or completely transform Ser2 in



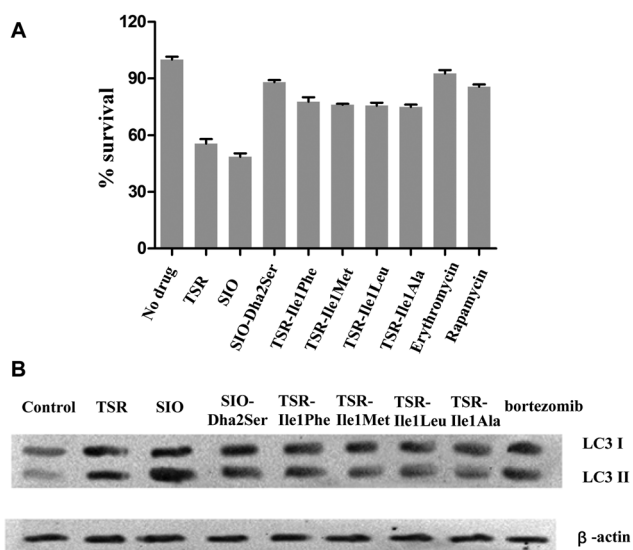
**Fig. 3** Production of SIO derivatives and their structural elucidation. (A) HPLC analyses of the fermentation cultures of the wild-type TSR-producing strain *S. laurentii* and the mutant strain SL-I1V-A2S. The resulting TSR and TSR analogs are indicated by arrows. Ile1 and Ala2 in TSR are indicated in black. Val1 in SIO and SIO-Dha2Ser is shown in blue, whereas Ser2 in SIO and Dha2 in SIO-Dha2Ser are presented in green. The differences between SIO and SIO-Dha2Ser are shown by a red dashed circle. (B) Selected HMBC and COSY correlations for residues 1 and 2 in the derivatives.

the core peptide. Meanwhile, the remarkable improvement of the SIO yield (up to  $1.3 \text{ g L}^{-1}$ ) showed that our engineering strategy may have great potential for applications in future synthetic biology research.

To examine the antibacterial activities of these newly generated TSR-type antibiotics, the corresponding bioassays were performed to determine their minimum inhibitory concentrations (MICs). The results showed that all the Ile1-mutated derivatives possess equal or reduced *in vitro* bioactivities compared with TSR, whereas the derivative SIO-Dha2Ser lost the remarkable antibacterial activity of its parent compound SIO (Table 1). Thus, the naturally selected amino acid patterns of TSR and SIO appear to be the most advantageous in terms of antibacterial effects, and the replacement of the first and second residues in their side ring with the other naturally occurring amino acids may reduce their *in vitro* antibacterial activities. In our previous work, we discovered that TSR-type antibiotics can serve as both prokaryotic ribosome inhibitors and autophagy inducers.<sup>4a</sup> Thus, we detected the *in vivo* antibacterial activities of the aforementioned engineered compounds (Fig. 4A) and their autophagy-induction properties by detecting their capacities of activating the overexpression of LC3 and the transformation of LC3-I into LC3-II (Fig. 4B). Intriguingly, the results showed that although SIO-Dha2Ser does not possess outstanding *in vitro* antibacterial activities, it is a good autophagy inducer. Separating the bioactivities of SIO (*i.e.*, ribosome inhibition and autophagy induction) by introducing a hydroxyl group onto Dha2 provides new insights

**Table 1** MICs ( $\mu\text{g mL}^{-1}$ ) of TSR and its analogs against various extracellular (a) and intracellular (b) bacterial strains. Vancomycin (VAN) and isonicotinic acid hydrazide (INH) were chosen as control drugs

	TSR	SIO	SIO-Dha2Ser	TSR-Ile1Phe	TSR-Ile1Met	TSR-Ile1Leu	TSR-Ile1Ala	VAN	INH
<i>Streptococcus pneumoniae</i> PRSP1063 <sup>a</sup>	0.001	0.001	0.5	0.125	0.25	0.125	0.5	0.25	—
<i>Streptococcus pneumoniae</i> PRSP2831 <sup>a</sup>	0.001	0.002	1	0.125	0.25	0.25	0.5	0.25	—
<i>Streptococcus pneumoniae</i> PRSP224588 <sup>a</sup>	0.008	0.004	1	0.25	1	0.5	0.25	0.25	—
<i>Staphylococcus aureus</i> MRSA-S1 <sup>a</sup>	0.032	0.032	>1	0.25	0.125	0.25	0.5	0.5	—
<i>Staphylococcus aureus</i> MRSA-SAU3 <sup>a</sup>	0.064	0.064	>1	0.25	0.125	0.25	0.25	0.5	—
<i>Staphylococcus aureus</i> MRSA-SAU5 <sup>a</sup>	0.064	0.064	>1	0.5	0.5	1	0.5	1.0	—
<i>Enterococcus faecium</i> VRE3 <sup>a</sup>	0.032	0.016	>1	0.25	1	0.25	0.5	>256	—
<i>Enterococcus faecium</i> VRE73 <sup>a</sup>	0.064	0.032	>1	0.125	0.5	0.5	0.25	256	—
<i>Enterococcus faecium</i> VRE83 <sup>a</sup>	0.064	0.064	>1	0.25	0.25	0.25	1	>256	—
<i>Mycobacterium marinum</i> <sup>b</sup>	0.5	0.5	>8	8	4	2	2	—	25



**Fig. 4** *In vivo* antibacterial activities of TSR and its analogs and their autophagy-induction properties. (A) Survival rates of pathogen colony-forming units (CFU) when the *M. marinum*-infected RAW 264.7 cells were treated with different compounds. Erythromycin (a typical ribosome inhibitor) and rapamycin (a typical autophagy inducer) were chosen as control drugs. The error bars represent the standard deviation from three different experiments. (B) LC3 conversion in the *M. marinum*-infected RAW 264.7 macrophage cell line treated with each drug (1 mM).  $\beta$ -Actin was chosen as an internal control biomarker, and a clinically used proteasome inhibitor and autophagy inducer, bortezomib, was chosen as a control drug.

into the complex structure–activity relationship (SAR) of TSR-type antibiotics.

## Conclusions

In conclusion, by utilizing a single “base”-based mutagenesis strategy, we engineered the Ile1 residue of TSR into other amino acids. The replacement of Ile1 with six different amino acids was tolerated by the TSR biosynthetic system and resulted in six new side ring-derived TSR variants. Moreover, the simultaneous mutagenesis of TSR’s Ile1 and Ala2 residues allowed SIO to be produced in the original TSR-producing

strain. An unpredicted SIO derivative, SIO-Dha2Ser, was also found in this engineered system. The detection of the *in vivo* and *in vitro* antibacterial activities of the compounds studied here facilitates the understanding of the complex SAR of TSR-type antibiotics and provides insights into their bioactivity-separation.

## Acknowledgements

This work was supported in part by grants from the NSFC (31430005 and 91413101), STCSM (13XD1404500 and 14JC1407700), “973 program” (2012CB721100), and MST (2012AA02A706) of China to Prof. Wen Liu. This research was also funded in part by NSFC (81402831) to Dr Dandan Chen. We also thank Prof. Heinz G. Floss, University of Washington, for providing the TSR-producing strain *S. laurentii* ATCC 31255.

## Notes and references

- (a) P. G. Arnison, M. J. Bibb, G. Bierbaum, A. A. Bowers, T. S. Bugni, G. Bulaj, J. A. Camarero, D. J. Campopiano, G. L. Challis, J. Clardy, P. D. Cotter, D. J. Craik, M. Dawson, E. Dittmann, S. Donadio, P. C. Dorrestein, K.-D. Entian, M. A. Fischbach, J. S. Garavelli, U. Goransson, C. W. Gruber, D. H. Haft, T. K. Hemscheidt, C. Hertweck, C. Hill, A. R. Horswill, M. Jaspars, W. L. Kelly, J. P. Klinman, O. P. Kuipers, A. J. Link, W. Liu, M. A. Marahiel, D. A. Mitchell, G. N. Moll, B. S. Moore, R. Muller, S. K. Nair, I. F. Nes, G. E. Norris, B. M. Olivera, H. Onaka, M. L. Patchett, J. Piel, M. J. T. Reaney, S. Rebuffat, R. P. Ross, H.-G. Sahl, E. W. Schmidt, M. E. Selsted, K. Severinov, B. Shen, K. Sivonen, L. Smith, T. Stein, R. D. Sussmuth, J. R. Tagg, G.-L. Tang, A. W. Truman, J. C. Vederas, C. T. Walsh, J. D. Walton, S. C. Wenzel, J. M. Willey and W. A. van der Donk, *Nat. Prod. Rep.*, 2013, **30**, 108; (b) Q. Zhang and W. Liu, *Nat. Prod. Rep.*, 2013, **30**, 218.
- (a) R. Liao, L. Duan, C. Lei, H. Pan, Y. Ding, Q. Zhang, D. Chen, B. Shen, Y. Yu and W. Liu, *Chem. Biol.*, 2009, **16**,

- 141; (b) L. Duan, S. Wang, R. Liao and W. Liu, *Chem. Biol.*, 2012, **19**, 443.
- 3 (a) S. Wang, Q. Zheng, J. Wang, Z. Zhao, Q. Li, Y. Yu, R. Wang and W. Liu, *Org. Chem. Front.*, 2015, **2**, 106; (b) S. Wang, Q. Zheng, J. Wang, D. Chen, Y. Yu and W. Liu, *Org. Chem. Front.*, 2016, **3**, 496.
- 4 (a) Q. Zheng, Q. Wang, S. Wang, J. Wu, Q. Gao and W. Liu, *Chem. Biol.*, 2015, **22**, 1002; (b) Q. Zheng and W. Liu, *Mycobact. Dis.*, 2016, **6**, 203.
- 5 N. S. Hegde, D. A. Sanders, R. Rodriguez and S. Balasubramanian, *Nat. Chem.*, 2011, **3**, 725.
- 6 F. Zhang, C. Li and W. L. Kelly, *ACS Chem. Biol.*, 2016, **11**, 415.
- 7 (a) K. C. Nicolaou, B. S. Safina, M. Zak, A. A. Estrada and S. H. Lee, *Angew. Chem., Int. Ed.*, 2004, **43**, 5087; (b) K. C. Nicolaou, M. Zak, B. S. Safina, S. H. Lee and A. A. Estrada, *Angew. Chem., Int. Ed.*, 2004, **43**, 5092.
- 8 M. A. Ortega and W. A. van der Donk, *Cell Chem. Biol.*, 2016, **23**, 31.
- 9 H. Guo, J. Wang, Y. Li, Y. Yu, Q. Zheng, J. Wu and W. Liu, *Chem. Sci.*, 2014, **5**, 240.
- 10 (a) H. Nishimura, S. Okamoto, M. Mayama, H. Ohtsuka, K. Nakajima, K. Tawara, M. Shimohira and N. Shimaoka, *J. Antibiot.*, 1961, **14**, 255; (b) K. Tori, K. Tokura, Y. Yoshimura, K. Okabe, H. Otsuka, F. Inagaki and T. Miyazawa, *J. Antibiot.*, 1979, **32**, 1072; (c) N. J. Clayden, F. Inagaki, R. J. Williams, G. A. Morris, K. Tori, K. Tokura and T. Miyazawa, *Eur. J. Biochem.*, 1982, **123**, 127.
- 11 (a) U. G. Bhat, P. A. Zipfel, D. S. Tyler and A. L. Gartel, *Cell Cycle*, 2008, **7**, 1851; (b) U. G. Bhat, M. Halasi and A. L. Gartel, *PLoS One*, 2009, **4**, e6593; (c) U. G. Bhat, M. Halasi and A. L. Gartel, *PLoS One*, 2009, **4**, e5592; (d) M. Halasi, H. Zhao, H. Dahari, U. G. Bhat, E. B. Gonzalez, A. V. Lyubimo, D. A. Tonetti and A. L. Gartel, *Cell Cycle*, 2010, **9**, 1214.
- 12 S. Yagi, S. Kitai and T. Kimura, *Appl. Microbiol.*, 1971, **22**, 153.
- 13 G. A. Hudson, Z. Zhang, J. I. Tietz, D. A. Mitchell and W. A. van der Donk, *J. Am. Chem. Soc.*, 2015, **137**, 16012.