JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

COMMUNICATION

pubs.acs.org/JACS

Thiostrepton Maturation Involving a Deesterification−Amidation Way To Process the C-Terminally Methylated Peptide Backbone

Rijing Liao and Wen Liu*

State Key Laboratory of Bioorganic and Natural Products Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, 345 Lingling Road, Shanghai 200032, China

S *Supporting Information*

ABSTRACT: Thiopeptides are a class of clinically interesting and highly modified peptide antibiotics. Their biosyntheses share a common paradigm for characteristic core formation but differ in tailoring to afford individual members. Herein we report an unusual deesterification−amidation process in thiostrepton maturation to furnish the terminal amide moiety. TsrB, serving as a carboxylesterase, catalyzes the hydrolysis of the methyl ester intermediate to provide the carboxylate intermediate, which can be converted to the amide product by an amidotransferase, TsrC. These findings revealed a C-terminal methylation of the precursor peptide, which is cryptic in thiostrepton biosynthesis but potentially common in the formation of its homologous series of thiopeptides that vary in the C-terminal form as methyl ester, carboxylate, or amide.

Thiopeptides are a growing class of sulfur-rich, highly modified peptide antibiotics possessing a characteristic macrocyclic core that consists of a six-membered nitrogen ring central to multiple thiazoles and dehydroamino acids.[1] Many members of this family are active against various drug-resistant bacterial pathogens, motivating the interest in new analogue development to overcome their physical drawbacks for clinical use (e.g., poor water solubility). For thiopeptide framework formation, the newly established biosynthetic pathways are remarkably concise,[2] showing conserved post-translational modifications on a ribosomally synthesized precursor peptide. The reactions include cyclodehydrations and subsequent dehydrogenations to form aromatic thiazoles, dehydrations to generate dehydroamino acids, and an intramolecular cyclization to afford the nitrogen heterocycle. However, thiopeptide biosynthesis involves a number of pathway-specific enzymes for converting the similar framework into individual bioactive members,[2] which differ in decoration of the core system, substitution of the central heterocycle domain, installation of the side ring system, and C-terminal functionalization of the extended side chain.

Thiostrepton (**1**; Figure 1), first isolated in 1954,[3] has often been known as the parent compound of a thiopeptide family that currently contains over 80 entities, historically because of its imposing architecture,[4] phenomenal bioactivities,[1,5] and unusual mode of action for medicinal use.[6] **1** falls into a series of thiopeptides including siomycins, thiopeptins, Sch 18640, and Sch 40832 (Figure S1 in the Supporting Information)[1a] that

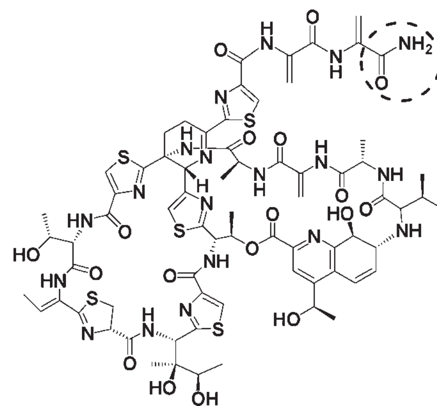

Figure 1. Structure of thiostrepton (**1**). The dashed circle indicates the C-terminal amide moiety.

feature a (dehydro)piperidine central domain and a side ring system containing a quinaldic moiety. Starting with the characterization of a pathway-specific gene *tsrB* in the biosynthesis of **1** (Figure 2A),[2b] we herein have exploited the chemistry for generality and variation of the members of the **1** series with respect to the extended side chain that appends different functionalities. We annotated TsrB as a carboxylesterase acting on a methyl ester intermediate to generate a carboxylate product, conversion of which to **1** in an amide form is subsequently catalyzed by TsrC, an amidotransferase. These findings uncovered an unusual deesterification−amidation process for maturating thiostrepton and indicated a C-terminal methylation of the precursor peptide that is cryptic in biosynthesis of **1** but potentially common in the formation of its homologous series of thiopeptides.

TsrB (named TsrS in ref 2c) belongs to an α/β hydrolase superfamily (Figure S2).[2b,c] To determine its role in biosynthesis of **1**, we first inactivated *tsrB* in the producing strain *Streptomyces laurentii*[7] by in-frame deletion (to exclude the polar effects on downstream gene expression). The resulting mutant strain SL1051 completely lost the ability to produce **1** (Figure 3, trace II); however, it produced a distinct compound [$C_{73}H_{86}N_{18}O_{19}S_5Na$ (**2**); HR-MALDI-MS m/z: calcd 1701.4812, found 1701.4843] displaying UV−vis absorptions (at λ_{max} = 198, 250, and 294 nm; Figure S3) quite similar to those of the parent compound **1**. For structural elucidation, purified **2** was subjected to comparative NMR spectroscopic analysis with **1**. In spite of

Received: December 10, 2010
Published: February 16, 2011

ACS Publications © 2011 American Chemical Society

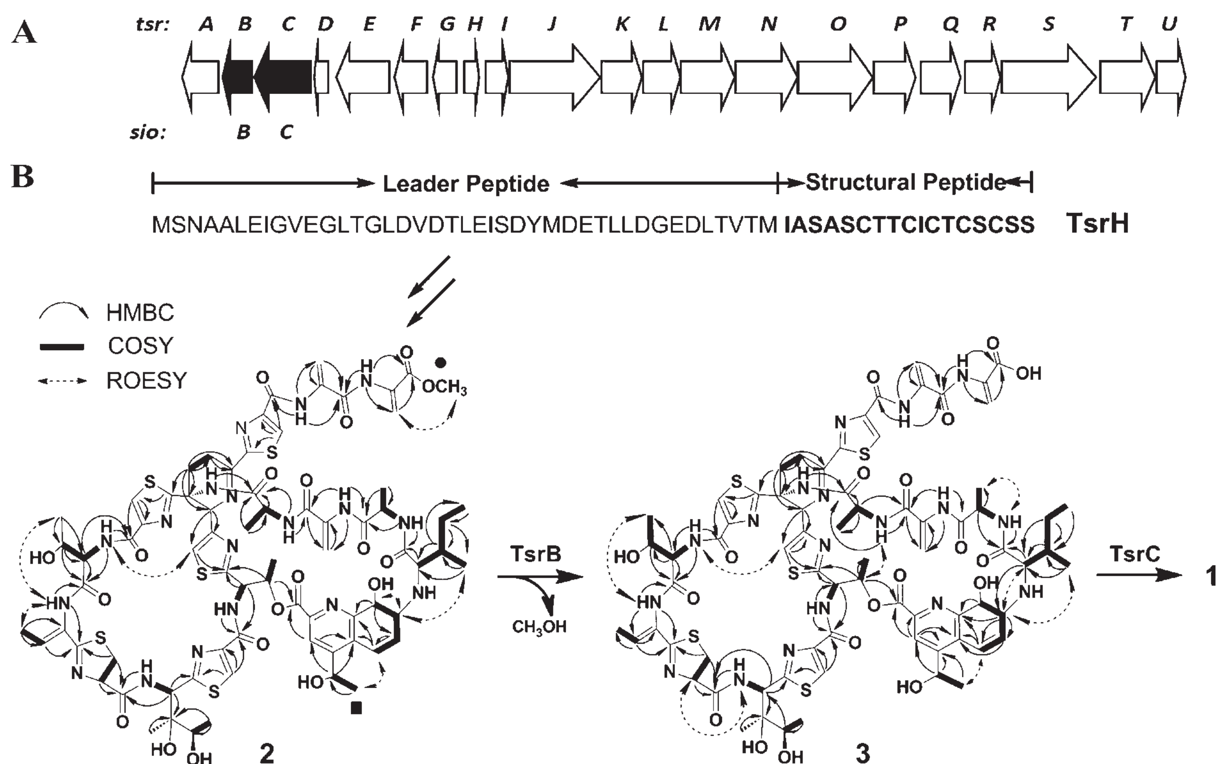

Figure 2. Gene cluster and biosynthetic pathway for maturation of **1**. (A) Organization of the *tsr* biosynthetic genes. The tailoring genes *tsrB* and *tsrC* (along with their counterparts *sioB* and *sioC* in siomycin biosynthesis) are labeled in black. (B) Deesterification−amidation of the methyl ester intermediate **2** to afford **1**. ^{1}H−^{1}H COSY, ROESY, and HMBC correlations for the structural elucidation of compounds **2** and **3** in this study are shown. The solid dot (●) indicates the methoxy carbon atom at the C-terminus. The solid square (■) indicates C12′ of the quinaldic acid moiety.

the overall similarity in the spectra (Figures S4 and S5 and Table S2), the distinct signals showed their only difference to be in the C-terminal functional group of the peptidyl side chain. For compound **2**, the ^{1}H NMR spectrum showed a nitrogen proton at δ 8.53, representing an upfield shift of 0.49 ppm from that at δ 9.02 for **1**, and an apparent singlet of methyl protons at δ 3.87 (Figure S5A). These indicated the presence of a C-terminal methyl ester, which was further supported by a key ROESY correlation between the methyl and olefin (at δ 6.01) protons (Figure S5F). Together with its ^{13}C NMR, ^{1}H−^{1}H COSY, HSQC, and HMBC spectra, these data established that **2** is a new thiostrepton intermediate bearing a methyl ester functionality at the C-terminus (Figure 2B), strongly supporting the conclusion that TsrB acts at the late-modification stage on the peptide backbone for maturation of **1**.

To probe the origin of the methyl ester in **2**, we fed L-[CH_3-^{13}C]methionine into the culture broth of SL1051. ^{13}C NMR analysis of the resulting **2** revealed two enhanced resonances at δ 53.31 (~2% ^{13}C enrichment) and 23.17 (~3.7% ^{13}C enrichment) that were correlated with the methoxy carbon atom at the C-terminus of the peptide backbone and C12′ of the quinaldic acid moiety, respectively (Figures 2B and 4). Thus, the methionine-based substrate (*S*)-adenosylmethionine (SAM) may serve as the methyl donor in both cases. Similar labeling studies of the final product **1** were carried out by Floss and coworkers,[8] showing that only carbon atom C12′ was labeled. Apparently, the C-terminal methylation of the peptide backbone outlined in **2** is cryptic in biosynthesis of **1** (Figure 2B), though the timing of the action awaits further investigations. It can be suspected that formation of the methyl ester is important to dehydration of the C-terminal Ser residue of the precursor peptide TsrH by lowering the pK_a value of the α hydrogen to facilitate water elimination.

We then overproduced TsrB in a 6-His-tagged form and purified it from *Escherichia coli* B12(DE3) to homogeneity for an in vitro assay of the activity (Figure S7A). In the presence of TsrB, conversion of **2** efficiently took place to give a distinct product (**3**) (Figure 3, trace VI), whereas no change of **2** was found in the control reaction using inactivated TrsB as the catalyst (Figure 3, trace VII). The molecular formula $C_{72}H_{84}N_{18}O_{19}S_5Na$ for **3** was established by HR-MALDI-MS (*m*/*z* for $[M + Na]^+$ ion: found 1687.4671, calcd 1687.4656), suggesting that this product differs from substrate **2** by possessing a terminally free carboxylic group (Figure 2B).

Compound **3** was previously proposed by Kelly and coworkers[2c] to be an intermediate in biosynthesis of **1** on the basis of product examination (by HPLC−MS) of the mutant strain generated by inactivation of *tsrC* (also named *tsrT* in ref 2c). TsrC encodes an asparagine synthetase-like amidotransferase that is assumed to be responsible for the last biosynthetic step to form a carboxyl-terminal amide to give **1**.[2b,c] However, this intermediate has not been fully elucidated by spectroscopic analysis. To ascertain the function of TsrC and the above TsrB-catalyzed reaction, we carried out in-frame deletion of *tsrC* and isolated the intermediate accumulated in the corresponding mutant stain SL1052 for structure determination (Figure 3, trace IV). Upon extensive 1D and 2D NMR spectroscopic analysis (Figure S6 and Table S3), the resulting Δ*tsrC* intermediate was characterized as a thiostrepon analogue featuring a carboxylate-terminated side chain (Figure 2B), and its identity to the product

3 of TsrB was validated upon their same HPLC retention time, UV−vis absorption spectrum, and molecular weight as determined by HR-MALDI-MS. These studies unambiguously characterized TsrB as a carboxylesterase that acts on the methyl ester intermediate **2** by releasing the free carboxylic acid, affording the intermediate **3** (along with the byproduct methanol), which can be further amidated by the amidotransferase TsrC to give the final product, thiostrepton (**1**) (Figure 2B).

Determination of the substrate and product of TsrB allowed subsequent evaluation of the TsrB-catalyzed reaction in vitro. Conversion of **2** to **3** required no cofactor. Addition of EDTA did not significantly affect the enzymatic activity, indicative of the metal-ion independence of TsrB. The steady-state kinetic parameters for this reaction were measured at the optimized pH 7.5 (Figure S7B,C), and a K_m value of 1.7 ± 0.6 μM for **2** and a k_{cat} value of 13.9 ± 3.5 $\min^{-1}$ were obtained. Consistent with the sequence homology to various lipases/esterases with a typical catalytic triad comprising Ser, Asp/Glu, and His (Figure S2), TsrB may utilize the residue Ser109 (within the conserved motif Gly-X-Ser-X-Gly) as a nucleophile participating in the hydrolysis of the methyl ester group of **2** to produce **3**.

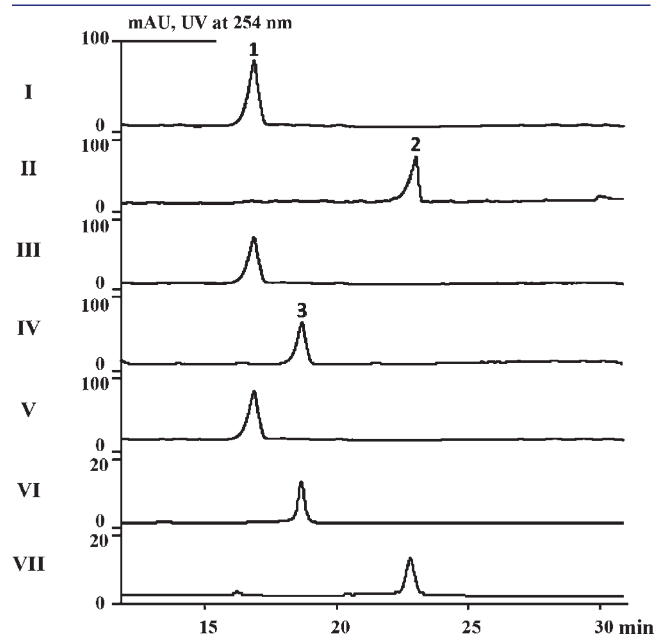

Figure 3. HPLC analysis for product examination. (I−V) In vivo thiopeptide production in *S. laurentii* strains, including the wild type (I), *tsrB* mutant SL1051 (II), SL1053 derived from SL1051 by expression of *sioB* (III), *tsrC* mutant SL1052 (IV), and SL1054 derived from SL1053 by expression of *sioC* (V). (VI, VII) In vitro conversions catalyzed by active TsrB (VI) and TsrB inactivated by heating (VII).

The unusual deesterification−amidation process is specific for biosynthesis of **1**-series thiopeptides. Among available biosynthetic pathways of thiopeptides,[2] the only counterparts of TsrB and TsrC found were SioB (80% identity to TsrB) and SioC (82% identity to TsrC) in siomycin biosynthesis (Figure 2A).[2b] To validate their functional identities, we carried out heterologous complementation of *sioB* and *sioC* to their corresponding gene mutant, **1**-nonproducing strains. While introduction of *sioB* to SL1051 (Δ*tsrB*) generated the recombinant strain SL1053, transfer of *sioC* into SL1052 (Δ*tsrC*) gave SL1054. Remarkably, in both SL1053 and SL1054, **1** production was restored (Figure 3, traces III and V), showing yields comparable to that of the wild-type strain. This supported the conclusion that the process can be general for maturation of **1**-series thiopeptides from similar methyl ester intermediates. Either deesterification or amidation can be insufficient in or omitted from the biosynthetic pathway (e.g., for the methyl ester Sch 40832, siomycin C, or thiopeptin A and for the carboxylate thiopeptin B, respectively), leading to the generation of individual thiopeptides varying in the C-terminal form as methyl ester, carboxylate, or amide (Figure S1).[1a]

Finally, we estimated the effect of C-terminal diversity of the peptide backbone on the physical properties and biological activity of thiopeptides (Table 1). Newly obtained compounds **2** and **3** were subjected to comparative analysis with the parent compound **1**. Compound **2** was poor in water solubility (18−27-fold lower than compounds **1** and **3**), consistent with the fact that methyl esters are less polar than carboxylates or amides. For in vitro susceptibility testing, whereas the caboxylate form **3** showed a minimum inhibitory concentration (MIC) of 0.125 μg/mL (8-fold lower than the amide form **1** in bioactivity), the methyl ester form **2** displayed remarkable antibacterial activity against the test strain *Bacillus subtilis* (with a MIC at 0.002 μg/mL, 8-fold higher than **1** in bioactivity). Notably, the change of this substitution significantly affects the bioactivity of thiopeptides, indicating the importance of the C-terminal functional group to the action of thiopeptides.

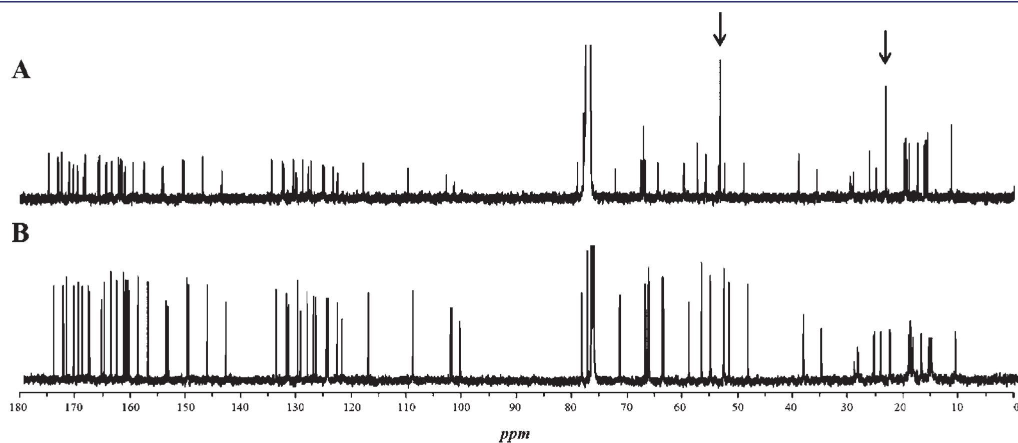

Figure 4. ^{13}C NMR spectra of compound **2** isolated from SL1051 (A) with and (B) without feeding of L-[CH_3-^{13}C]methionine. The arrows indicate the enhanced signals at δ 53.31 and 23.17 after feeding.

Table 1. Minimum Inhibitory Concentration (MIC) and Water Solubility of Thiopeptides in This Study

compound	MIC[a,b]	water solubility[a]
1	0.016	0.91
2	0.002	0.05
3	0.125	1.37

[a] Values in μg/mL. [b] For MIC measurements, the test organism *B. subtilis* SIPI-JD1001 (deposited at the Shanghai Institute of Pharmaceutical Industry) was used.

In summary, we have uncovered a paradigm for thiostrepton maturation via an unusual deesterification−amidation process that requires the TsrB and TsrC functions to afford the carboxyl-terminal amide moiety (Figure 2B). TsrB, serving as a carboxylesterase, catalyzes the hydrolysis of the methyl ester intermediate **2** to provide the carboxylate intermediate **3**, which can be turned over by TsrC, an ATP-dependent amidotransferase, for terminal amidation to afford the amide product **1**. The terminal amide has often been found in other series of thiopeptides, such as the monocyclic member GE2270A and indole-containing bicyclic members nosiheptide and nocathiacins.[2d,f,g] However, these thiopeptides employ a distinct way (given that TsrB and TsrC are absent in their biosynthetic pathways) that involves processing the precursor peptides featuring a Ser residue extension, where Ser can be removed except the nitrogen atom as the source of the terminal amide.[9] In nosiheptide biosynthesis, we recently reported a novel protein NosA that acts on an intermediate bearing a bisdehydroalanine tail and catalyzes an enamide dealkylation to eliminate the acrylate unit originating from the extended Ser.[10] The methyl ester-based tailoring is potentially common for biosynthesis of **1**-series thiopeptides. Though the C-terminal methylation of the peptide backbone is cryptic in biosynthesis of **1**, variation in deesterification or amidation may lead to generation of individual members of this series of thiopeptides with different terminal forms. This can be applicable to combinatorial biosynthesis, complementing recent advances in sequence permutation of the precursor peptide for structural diversity in developing new bioactive thiopeptide agents.[11]

ASSOCIATED CONTENT

Supporting Information. Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author
wliu@mail.sioc.ac.cn

ACKNOWLEDGMENT

We thank Prof. Heinz G. Floss (University of Washington) for providing *S. laurentii* ATCC 31255 and for his pioneering works on thiopeptide biosynthesis. This work was supported in part by grants from the National Natural Science Foundation (20832009, 30525001, and 20921091), the Ministry of Science and Technology (2009ZX09501-008), the "973 Program" (2010CB833200), the Chinese Academy of Sciences (KJCX2-YW-H08 and KSCX2-YW-G-06), and the Science and Technology Commission of Shanghai Municipality (09QH1402700) of China.

REFERENCES

(1) (a) Bagley, M. C.; Dale, J. W.; Merritt, E. A.; Xiong, X. *Chem. Rev.* **2005**, *105*, 685–714. (b) Hughes, R. A.; Moody, C. J. *Angew. Chem., Int. Ed.* **2007**, *46*, 7930–7954. (c) Nicolaou, K. C.; Chen, J. S.; Edmonds, D. J.; Estrada, A. A. *Angew. Chem., Int. Ed.* **2009**, *48*, 660–719.

(2) (a) Brown, L. C. W.; Acker, M. G.; Clardy, J.; Walsh, C. T.; Fischbach, M. A. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 2549–2553. (b) Liao, R.; Duan, L.; Lei, C.; Pan, H.; Ding, Y.; Zhang, Q.; Chen, D.; Shen, B.; Yu, Y.; Liu, W. *Chem. Biol.* **2009**, *16*, 141–147. (c) Kelly, W. L.; Pan, L.; Li, C. *J. Am. Chem. Soc.* **2009**, *131*, 4327–4334. (d) Morris, R. P.; Leeds, J. A.; Naegeli, H. U.; Oberer, L.; Memmert, K.; Weber, E.; LaMarche, M. J.; Parker, C. N.; Burrer, N.; Esterow, S.; Hein, A. E.; Schmitt, E. K.; Krastel, P. *J. Am. Chem. Soc.* **2009**, *131*, 5946–5955. (e) Yu, Y.; Duan, L.; Zhang, Q.; Liao, R.; Ding, Y.; Pan, H.; Wendt-Pienkowski, E.; Tang, G.; Shen, B.; Liu, W. *ACS Chem. Biol.* **2009**, *4*, 855–864. (f) Wang, J.; Yu, Y.; Tang, K.; Liu, W.; He, X.; Huang, X.; Deng, Z. *Appl. Environ. Microbiol.* **2010**, *76*, 2335–2344. (g) Ding, Y.; Yu, Y.; Pan, H.; Guo, H.; Li, Y.; Liu, W. *Mol. BioSyst.* **2010**, *6*, 1180–1185.

(3) (a) Pagano, J. F.; Weinstein, M. J.; Stout, H. A.; Donovick, R. *Antibiot. Annu.* **1955−1956**, 554–559. (b) Vandeputte, J.; Dutcher, J. D. *Antibiot. Annu.* **1955−1956**, 560–561. (c) Steinberg, B. A.; Jambor, W. P.; Suydam, L. O. *Antibiot. Annu.* **1955−1956**, 562–565.

(4) (a) Anderson, B.; Hodgkin, D. C.; Viswamitra, M. A. *Nature* **1970**, *225*, 233–235. (b) Hensens, O. D.; Albers-Schönberg, G. *J. Antibiot.* **1983**, *36*, 799–813. (c) Hensens, O. D.; Albers-Schönberg, G. *J. Antibiot.* **1983**, *36*, 814–831. (d) Hensens, O. D.; Albers-Schönberg, G. *J. Antibiot.* **1983**, *36*, 832–845. (e) Mocek, U.; Beale, J. M.; Floss, H. G. *J. Antibiot.* **1989**, *42*, 1649–1652.

(5) (a) Jones, W. F.; Finland, M. *Antibiot. Chemother.* **1958**, *8*, 387–391. (b) Kutscher, A. H.; Seguin, L.; Rankow, R. M.; Piro, J. D. *Antibiot. Chemother.* **1958**, *8*, 576–583. (c) Clough, B.; Rangachari, K.; Strath, M.; Preiser, P. R.; Wilson, R. J. M. *Protist* **1999**, *150*, 189–195. (d) McConkey, G. A.; Rogers, M. J.; McCutchan, T. F. *J. Biol. Chem.* **1997**, *272*, 2046–2049.

(6) (a) Porse, B. T.; Leviev, I.; Mankin, A. S.; Garrett, R. A. *J. Mol. Biol.* **1998**, *276*, 391–404. (b) Harms, J. M.; Wilson, D. N.; Schluenzen, F.; Connell, S. R.; Stachelhaus, T.; Zaborowska, Z.; Spahn, C. M. T.; Fucini, P. *Mol. Cell* **2008**, *30*, 26–38.

(7) Trejo, W. H.; Dean, L. D.; Pluscec, J.; Meyers, E.; Brown, W. E. *J. Antibiot.* **1977**, *30*, 639–643.

(8) (a) Zhou, P.; O'Hagan, D.; Mocek, U.; Zeng, Z.; Yuen, L. D.; Frenzel, T.; Unkefer, C. J.; Beale, J. M.; Floss, H. G. *J. Am. Chem. Soc.* **1989**, *111*, 7274–7276. (b) Frenzel, T.; Zhou, P.; Floss, H. G. *Arch. Biochem. Biophys.* **1990**, *278*, 35–40. (c) Mocek, U.; Zeng, Z.; O'Hagan, D.; Zhou, P.; Fan, L. D. G.; Beale, J. M.; Floss, H. G. *J. Am. Chem. Soc.* **1993**, *115*, 7992–8001. (d) Priestley, N. D.; Smith, T. M.; Shipley, P. R.; Floss, H. G. *Bioorg. Med. Chem.* **1996**, *4*, 1135–1147.

(9) Mocek, U.; Knaggs, A. R.; Tsuchiya, R.; Nguyen, T.; Beale, J. M.; Floss, H. G. *J. Am. Chem. Soc.* **1993**, *115*, 7557–7568.

(10) Yu, Y.; Guo., H.; Zhang, Q.; Duan, L.; Ding, Y.; Liao, R.; Lei, C.; Shen, B.; Liu, W. *J. Am. Chem. Soc.* **2010**, *132*, 16324–16326.

(11) (a) Acker, M. G.; Bowers, A. A.; Walsh, C. T. *J. Am. Chem. Soc.* **2009**, *131*, 17563–17565. (b) Bowers, A. A.; Acker, M. G.; Koglin, A.; Walsh, C. T. *J. Am. Chem. Soc.* **2010**, *132*, 7519–7527.