Biosynthesis

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Mechanistic Insights into Polycycle Formation by Reductive Cyclization in Ikarugamycin Biosynthesis**

*Guangtao Zhang, Wenjun Zhang, Qingbo Zhang, Ting Shi, Liang Ma, Yiguang Zhu, Sumei Li, Haibo Zhang, Yi-Lei Zhao, Rong Shi, and Changsheng Zhang**

Abstract: *Ikarugamycin is a member of the polycyclic tetramate macrolactams (PTMs) family of natural products with diverse biological activities. The biochemical mechanisms for the formation of polycyclic ring systems in PTMs remain elusive. The enzymatic mechanism of constructing an inner five-membered ring in ikarugamycin is reported. A three-gene-cassette ikaABC from the marine-derived Streptomyces sp. ZJ306 is sufficient for conferring ikarugamycin production in a heterologous host. IkaC catalyzes a reductive cyclization reaction to form the inner five-membered ring by a Michael addition-like reaction. This study provides the first biochemical evidence for polycycle formation in PTMs and suggests a reductive cyclization strategy which may be potentially applicable in general to the corresponding ring formation in other PTMs.*

Polycyclic tetramate macrolactams (PTMs), a widely encountered family of natural products, feature a macrocyclic lactam with an embedded tetramic acid ring. PTMs display further structural diversity by fusing the macrolactam ring with a different set of carbocyclic rings (Scheme 1), such as a 5-6-5 ring system in ikarugamycin (**1**),[1] clifednamides,[2] a 5-5-6 ring system in the heat-stable antifungal factor (HSAF),[3] discodermide,[4] and frontalamides,[5] a 5-5 ring system in alteramide,[6] cylindramide,[7] and a 5-4-6 ring system in compound d (see Scheme 1) characterized from a recent study by activating a cryptic PTM biosynthetic gene cluster.[8] PTMs exhibit a wide range of biological activities such as antifungal, antibiotic, and antitumor properties.[1–7] Ikarugamycin was isolated mostly from *Streptomyces* strains.[1,2,9] In addition to potent antiprotozoal, antiulcer, antibacterial, antiviral, cytotoxic, and apoptosis-inducing activities,[1,10] **1** has also been reported to inhibit oxidized low-density lipoprotein-induced uptake and the clathrin-dependent endocytosis,[10] making it a promising drug lead.

Although the biosynthesis of **1** was proposed shortly after its discovery to originate from one ornithine and two 12-carbon chains,[9d] the biosynthetic locus of **1** has not been reported yet.[11] Recently, the complete biosynthetic gene clusters for PTM family members HSAF,[3,12] frontalamides,[5] and compound d[8] have been elucidated, and a genome mining analysis has revealed the presence of a number of similar PTM gene clusters in diverse bacteria.[5] It has been proposed that the PTM family shares a common biosynthetic pathway involving an unusual hybrid polyketide synthase/nonribosomal peptide synthetase (PKS/NRPS),[5] in which the PKS portion is iteratively used to generate two separate polyketide chains. This arrangement resembles the eukaryotic hybrid enzyme employed in fungal biosynthesis of tenellin.[13] A subsequent in vitro study has confirmed that the single HSAF NRPS module can accept two acyl chains to make two amide bonds with the α- and the δ-amino groups of L-ornithine, respectively, forming the tetramate ring by a Dieckmann-type reaction.[12] In addition, the unusual dual activities of both a protease and a peptide ligase for the HSAF thioesterase (TE) domain,[14] and the tailoring enzymes for hydroxylations of both frontalamides and HSAF have been described.[5,15] However, it remains unclear how the polycycles in PTMs are formed after release of the nascent product from the PKS/NRPS assembly line. In this study, we report the identification of a three-gene cassette (*ikaABC*) from the marine-derived *Streptomyces* sp. ZJ306, which is sufficient for conferring the production of **1** in a heterologous host. The alcohol dehydrogenase IkaC is

[*] Dr. G. Zhang,[+] Dr. W. Zhang,[+] Dr. Q. Zhang, L. Ma, Dr. Y. Zhu, Dr. S. Li, Dr. H. Zhang, Prof. Dr. C. Zhang
Key Laboratory of Tropical Marine Bio-resources and Ecology
RNAM Center for Marine Microbiology
Guangdong Key Laboratory of Marine Materia Medica
South China Sea Institute of Oceanology
Chinese Academy of Sciences
164 West Xingang Road, Guangzhou 510301 (China)
E-mail: czhang2006@gmail.com
T. Shi, Prof. Dr. Y.-L. Zhao
State Key Laboratory of Microbial Metabolism and
School of Life Sciences and Biotechnology
Shanghai Jiao Tong University, Shanghai 200240 (China)
Prof. Dr. R. Shi
Département de biochimie, de microbiologie et
de bio-informatique
PROTEO et IBIS, Université Laval, Québec, G1V 0A6 (Canada)

[+] These authors contributed equally to this work.

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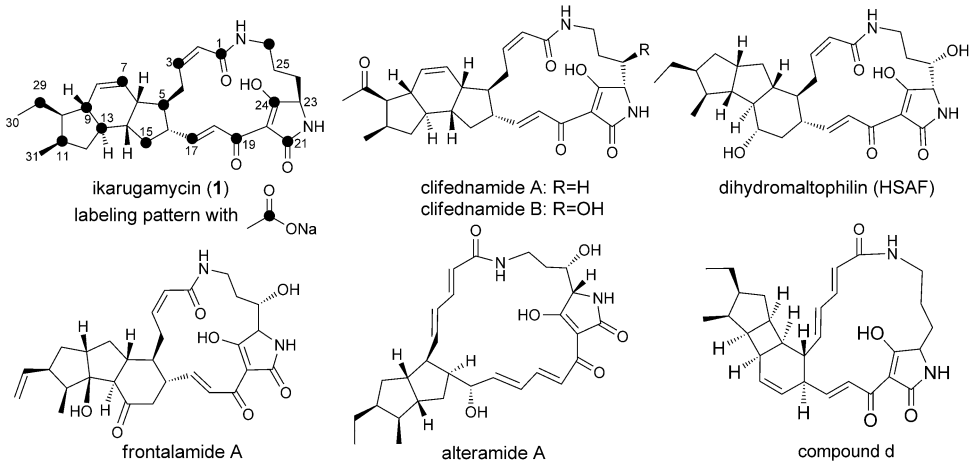

Scheme 1. Chemical structures for representative PTMs. The labeling pattern of ikarugamycin (**1**) isolated from feeding experiments with sodium [1-^{13}C]acetate was also shown.

demonstrated to catalyze an unusual reductive cyclization reaction to form the inner five-membered ring in **1**.

The strain *Streptomyces* sp. ZJ306 (GenBank accession number KF954540 for its 16S rRNA gene) was isolated from a sediment sample collected near the Pearl River estuary of China. Chemical investigations on cultures of *Streptomyces* sp. ZJ306 led to the isolation of **1** as the main product, which was confirmed by comparison of MS and NMR spectroscopic data with those reported for ikarugamycin (see Table S1 and Figure S1 in the Supporting Information).[16] To verify the previous proposal that the biosynthesis of **1** was derived from a PKS/NRPS pathway,[9d] feeding experiments were performed in *Streptomyces* sp. ZJ306 using sodium [1-^{13}C]acetate. Consistent with the predicted PKS origin, high levels of enrichments were observed at C-1, C-3, C-5, C-7, C-9, C-29, C-11, C-13, C-15, C-17, C-19, and C-21 (Scheme 1, Figure S2). Additionally, the enrichments at C-24 and C-27 were consistent with the incorporation of an L-ornithine unit derived from L-glutamate.[17]

The biosynthetic gene cluster of **1** was identified from *Streptomyces* sp. ZJ306 by a PCR approach with degenerate primers (Table S2) targeting the conserved PKS/NRPS genes for PTM biosynthesis (Figure S3). Shot-gun sequencing of a positive cosmid pCSG3500 (Table S3, Figure S4) yielded 36,760 bp of DNA sequence (GenBank accession number KF954512), comprising 25 open reading frames (orfs) (Figure 1, Table S4). Bioinformatics analyses revealed that only three proteins encoded by *ikaABC* showed high sequence similarity to related enzymes in other PTM pathways. IkaA exhibits 66% sequence identity to the hybrid PKS/NRPS in HSAF biosynthesis in *Lysobacter enzymogenes* C3,[3] and shares the same catalytic domain order (KS-AT-DH-KR-ACP-C-A-PCP-TE) as those putative hybrid PKS/NRPSs involved in other PTM biosynthetic pathways,[5] containing an adenylation (A) domain with a conserved ornithine binding motif (DVGEIGSIDK).[18] IkaB contains a Rossmann-fold NAD(P)H binding domain at the N-terminus and displays similarity to the phytoene desaturase family enzymes, such as OX3 and OX2 in the HSAF pathway,[12] and FtdC in the frontalamide pathway.[5] IkaC is predicted as an alcohol dehydrogenase and shares a high similarity to OX4,[12] FtdE and related enzymes in other PTM pathways.[5] Surprisingly, the genes flanking *ikaABC* encode functions that appear not to be relevant to **1** biosynthesis (Table S4). In fact, all of the Δ*orf1*-Δ*orf5* mutants (Figure S5) were still able to produce **1** (Figure S6), indicating that the *orf1-orf5* genes are not essential for **1** biosynthesis. In contrast, the Δ*ikaA* mutant completely lost the ability to produce **1** and related products (Figure 2A, traces i and ii). In the Δ*ikaB* mutant, a new product **2** was produced (Figure 2A, trace iii), which showed an *m*/*z* of 475.2587 ($[M+H]^+$, $C_{29}H_{35}N_2O_4$, calcd 475.2597) in high-resolution electrospray ionization mass spectrometry (HRESIMS) analysis (Figure S7). However, compound **2** was found to be very unstable and was sensitive to light and heat during our attempts to isolate it for structure characterization. On the basis of the HRMS/MS analysis (Figure S7) and the UV spectrum of compound **2** (Figure S8), its structure was tentatively assigned as shown in Figure 2B. The Δ*ikaC* mutant also produced a new product **3** (Figure 2A, trace iv) with an *m*/*z* of 477.2768 ($[M+H]^+$, $C_{29}H_{37}N_2O_4$, calcd 477.2753) (Figure S9), and a UV spectrum distinct from those of **1** and **2** (Figure S8). Compound **3** was isolated from a 20 L-scale fermentation of the Δ*ikaC* mutant and its structure was established by detailed analyses and comparisons of the ^{1}H and ^{13}C NMR spectra of **3** (Table S1, Figure S9) and **1**, confirming that **3** differed from **1** by the absence of the inner five-membered ring (Figure 2B).

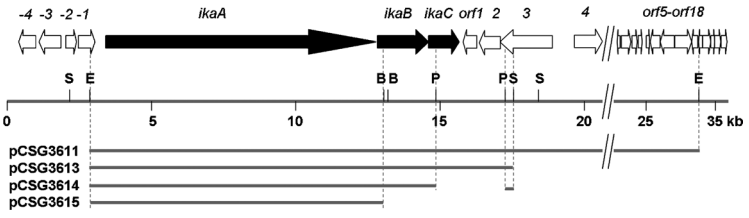

Figure 1. Organization of the ikarugamycin (**1**) gene cluster and construction of expression plasmids. Restriction sites: S, *Sac*I; E, *Eco*RI; B, *Bgl*II; P, *Pvu*I.

To further confirm the boundary of the biosynthetic gene cluster of **1**, heterologous expression studies were carried out by constructing four pSET152-based expression plasmids pCSG3611, pCSG3613–3615 (Figure 1, Table S3). Introduction of pCSG3611 (covering the DNA region between *ikaA* and *orf13*) into *S. lividans* TK64 led to the production of **1** and **3** (Figure 2A, trace v). This

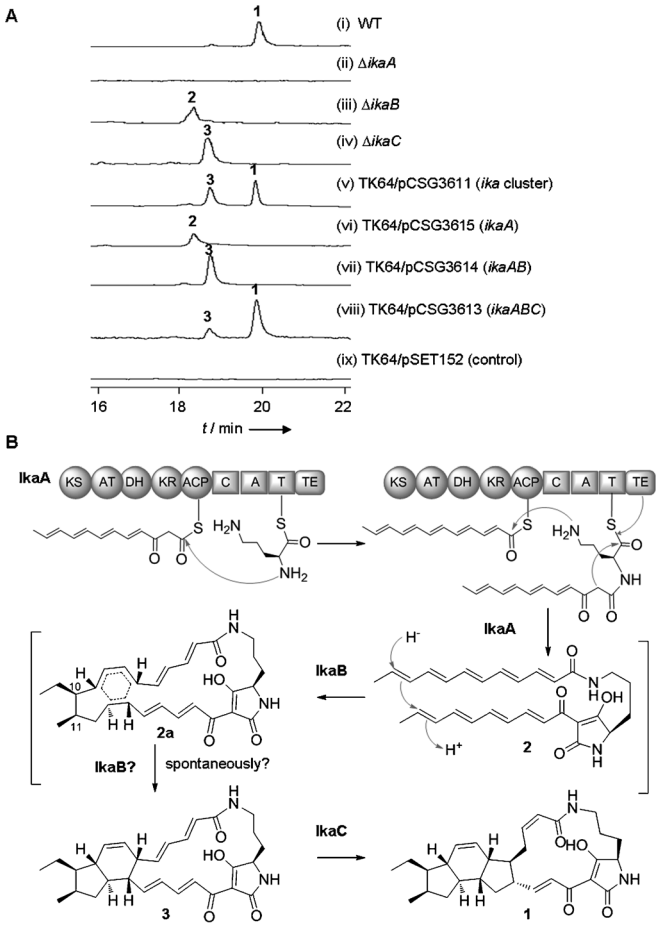

Figure 2. Characterization of the ikarugamycin gene cluster and the proposed biosynthetic pathway. A) HPLC analysis of metabolite profile for i) *Streptomyces* sp. ZJ306, ii) Δ*ikaA* mutant, iii) Δ*ikaB* mutant, iv) Δ*ikaC* mutant, v) *S. lividans* TK64/pCSG3611, vi) *S. lividans* TK64/pCSG3615, vii) *S. lividans* TK64/pCSG3614, viii) *S. lividans* TK64/pCSG3613, and ix) *S. lividans* TK64/pSET152. B) The proposed biosynthetic pathway for **1**. KS (keto synthase), AT (acyltransferase), DH (dehydratase), KR (ketoreductase), ACP (acyl-carrier protein), C (condensation), A (adenylation), T (thiolation), and TE (thioesterase).

observation suggested that the four genes *orf(−1)-orf(−4)* upstream of the *ikaA* gene (Figure 1) were not essential for the biosynthesis of **1**. A minor amount of **2** was detected in *S. lividans* TK64/pCSG3615 (harboring only intact *ikaA*) (Figure 2 A, trace vi). Compound **3** and a minor amount of **2** were produced in *S. lividans* TK64/pCSG3614 (harboring intact *ikaAB*) (Figure 2 A, trace vii), while both **1** and **3** were produced in *S. lividans* TK64/pCSG3613 (covering *ikaABC*, *orf1* and *orf2*) (Figure 1; Figure 2 A, trace viii). In contrast, no **1**-related products were detected in the control strain *S. lividans* TK64/pSET152 (Figure 2 A, trace ix). These observations, in combination with gene-inactivation results of *orf1* and *orf2*, led to the conclusion that the minimal three-gene cassette *ikaABC* is sufficient for the production of **1** in a heterologous host *S. lividans* TK64. Furthermore, the timing of the enzymatic consequences was also established: 1) first, IkaA is responsible for the synthesis of **2**; 2) subsequently, compound **2** is converted to **3** by IkaB; and 3) finally, IkaC catalyzes the conversion of **3** to **1**.

To confirm the function of IkaC, soluble N-His_6-fused IkaC protein was produced in *E. coli* BL21(DE3) harboring the pET28a-based expression plasmid pCSG3621 (Table S3), and was purified to near homogeneity via Ni-NTA chromatography (Figure S10). Incubation of **3** with IkaC in the presence of NADPH yielded a product that displayed the same retention time and molecular mass as those of authentic **1** (Figure 3 A, traces i and ii), while **3** remained unchanged when either IkaC or NADPH was omitted in control assays (Figure 3 A, traces iii and iv). When NADPH was replaced by NADH, the conversion of **3** to **1** was also observed (Figure 3 A, trace v).

To explore the mechanism of IkaC-catalyzed ring closure reaction, IkaC assays were coupled with the glucose dehydrogenase from *Bacillus megaterium* DSM 2894 (BmGDH) to provide (*S*)-[4-^{2}H]NADPH,[19] or with the glucose dehydrogenase from *Thermoplasma acidophilum* ATCC 25905 (TaGDH) to provide (*R*)-[4-^{2}H]NADPH,[20] respectively, using D-[1-^{2}H]glucose as the deuterium donor.[21] The IkaC/GDH coupled assays were then analyzed by LC-HRMS (Figure S11). A fragment ion at *m*/*z* 479.2895 corresponding to **1** was observed when using (*S*)-[4-^{2}H]NADPH as the cofactor (Figure 3 Bi). In contrast, a 1 Da-shifted fragment ion at *m*/*z* 480.2981 (Figure 3 Bii) was observed when using (*R*)-[4-^{2}H]NADPH as the cofactor, indicating the incorporation of one equivalent of ^{2}H into the product **1a** (Figure 3 C). To locate the exact position of deuterium incorporation in **1a**, we performed a large scale (50 mL) reaction by incubating **3**, $NADP^+$, and D-[1-^{2}H]glucose with purified IkaC and TaGDH. The reaction product **1a** was isolated and analyzed by ^{1}H NMR (Table S5, Figure S12). Careful comparison of ^{1}H-^{1}H COSY data of **1** and **1a** clearly showed that the correlation between H-16 and H-15a in **1** was not observed in **1a**, while the correlations between H-16 and H-15b, between H-4a and H-4b were observed in both **1** and **1a** (Figure S13). These observations confirmed the deuterium incorporation at C-15 in **1a**. The key NOESY correlation between H-15b and H-5 assigned the stereochemistry at C-15 of **1a** as 15*R* (Figure S14). Next, IkaC assay was performed in deuterium oxide (^2H_2O) in the presence of NADPH. LC-HRMS analysis showed a reaction product with a 1 Da-shifted fragment ion at *m*/*z* 480.2977 (**1b**; Figure 3 B iii), indicating one equivalent of ^{2}H from ^2H_2O was incorporated. When IkaC assay was done in ^2H_2O with (*R*)-[4-^{2}H]NADPH, in situ generated by TaGDH, a 2 Da-shifted fragment ion at *m*/*z* 481.3034 (**1c**) was observed (Figure 3 Biv), consistent with the incorporation of two equivalents of ^{2}H, one from (*R*)-[4-^{2}H]NADPH and another from ^2H_2O.

On the basis of these observations, we propose that IkaC catalyzes a nucleophilic conjugate addition resembling Michael addition (Figure 3 C). The *pro*-R hydride of NADPH was utilized to attack the electron-deficient C-15 of the C-15/C-16 alkene bond conjugated with the C-19 carbonyl group, which concomitantly induced the C–C bond formation between C-16 and C-5, with the electrons being pushed through conjugation to the electron negative oxygen atom of the C-1 carbonyl to generate the transient intermediate **3a**. It should be noted that the $E\Delta^{2,3}$ bond ($J_{2,3}$ 14.5 Hz) in the enzyme substrate **3** became the $Z\Delta^{2,3}$ bond

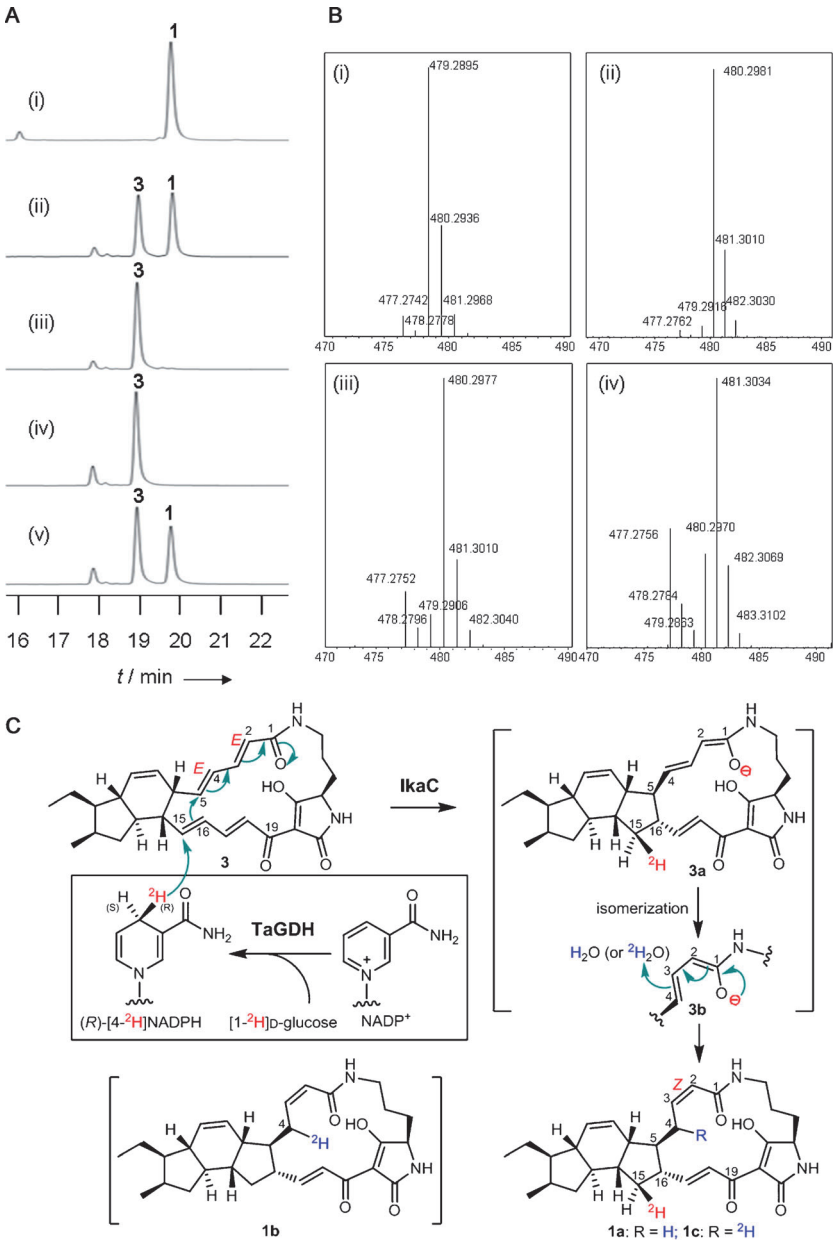

Figure 3. Biochemical characterization of IkaC and the proposed IkaC mechanism. A) HPLC analysis of IkaC reactions. i) The standard ikarugamycin (**1**); ii) **3**, IkaC and NADPH; iii) **3**, heat-denatured IkaC and NADPH; iv) **3** and IkaC, v) **3**, IkaC, and NADH. B) LC-HRMS analysis of reaction products of IkaC/GDH coupled assays. i) The product **1** from assays in normal water containing **3**, IkaC, BmGDH, $NADP^+$, and D-[1-^{2}H]glucose; ii) the product **1a** from assays in normal water containing **3**, IkaC, TaGDH, $NADP^+$, and D-[1-^{2}H]glucose; iii) the product **1b** from assays in ^2H_2O containing **3**, IkaC, and NADPH; iv) the product **1c** from assays in ^2H_2O containing **3**, IkaC, TaGDH, $NADP^+$, and D-[1-^{2}H]glucose. See also Figure S11 for full spectra of HRMS analysis for compounds **1**, **1a**–**c**. C) Schematic representation of a proposed reaction mechanism of IkaC catalysis.

($J_{2,3}$ 10.5 Hz) in the enzyme product **1** (Table S1). Given that the **1** isomer with an $E\Delta^{2,3}$ bond was calculated to be more stable than **1** (with a $Z\Delta^{2,3}$ bond) by about 5 kcal mol^{-1} (Figure S15), we hypothesized that the isomerization of the $E\Delta^{2,3}$ in the substrate **3** to the $Z\Delta^{2,3}$ in the product **1** was probably attributed to the IkaC-assisted rotation of the transiently generated single C-2/C-3 bond in **3a** leading to **3b** (Figure 3C). Subsequently, a water molecule was deprotonated by the conjugated C-3/C-4 alkene of the transient intermediate **3b** to recreate the C-1 carbonyl group and the $\Delta^{2,3}$ bond. On the chemical basis of this reaction mechanism, we propose that the ^{2}H from ^2H_2O was probably incorporated at C-4 in **1b** and **1c** (Figure 3C), although lacking experimental verification.

Based on our experimental data, an ikarugamycin biosynthetic pathway was proposed (Figure 2B). The production of compound **2** from both Δ*ikaB* mutant and *S. lividans* expressing only *ikaA* confirmed the previously-proposed iterative use of the PKS/NRPS enzyme in PTM biosynthesis,[5,12] which was also consistent with ^{13}C-labelling pattern in **1** (Scheme 1). Dissection and reconstitution of **1** biosynthesis by heterologous expression studies not only identified a minimal three-gene cassette *ikaABC* capable of conferring the heterologous production of **1**, but also established the function and reaction timing of IkaABC (Figure 2). IkaA synthesizes two slightly different 12-carbon polyketide chains, which are condensed respectively with the α- and the δ-amino groups of L-ornithine loaded on the T-domain of the NRPS module in IkaA. After an intramolecular Dieckmann-type reaction leading to the tetramate ring formation, the product **2** is released from the PKS/NRPS enzyme by the TE domain. We propose that the phytoene desaturase family enzyme IkaB catalyzes the C–C bond formation between C-10 and C-11 in a similar manner as IkaC catalysis, to generate an intermediate **2a** (Figure 2B) that can undergo a Diels–Alder reaction to form the cyclohexene ring in **3**, by a spontaneous or IkaB-catalyzed transannular [4+2] cycloaddition. Finally, the IkaC-catalyzed cyclization completes the biosynthesis of the pentacyclic core of **1**.

Although a similar 5-6-5 ring system is found in the insecticide spinosyn A, the inner five-membered ring was catalyzed by a previously unannotated protein SpnL, through a transannular cyclization reaction in a manner consistent with a Rauhut–Currier reaction.[22] Unlike the cofactor-free SpnL catalysis, IkaC catalyzes a NAD(P)H-dependent reductive cyclization reaction to form the inner five-membered ring in **1**, by a Michael addition-like [1+6] addition reaction (Figure 3C). Recently, a similar NAD(P)H-dependent reductive cyclization reaction, catalyzed by a progesterone-5β-reductase-like iridoid synthase, was discovered to be an alternative route for the biosynthesis of cyclic terpenes.[23] However, the iridoid synthase appeared to

catalyze a 1,4-addition reduction, generating an enol intermediate that can undergo either a Diels–Alder or a Michael addition reaction.[23] Given the ubiquitous presence of IkaC homologues in PTM gene clusters (Figure S16), the same strategy of IkaC-catalysis might be generally applied in the formation of different kinds of ring systems in various PTMs (Scheme 1). In support of this proposal, alteramide A (Scheme 1) has been isolated from either *OX2* or *OX4* (*ikaC* homologues) gene-disruption mutants,[24] indicating that one of OX2 or OX4 might catalyze a reductive cyclization to convert alteramide A to HSAF.

Taken together, our results provide the first biochemical evidence for reductive cyclization reactions in polycycle formation in PTMs. Further studies on the crystal structure of IkaC in complex with natural substrates would lead to a more detailed understanding of this unusual enzyme. Also, this study suggests that the same reductive cyclization strategy as IkaC catalysis may be generally applicable in the formation of polycyclic rings in other PTMs.

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