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Characterization of inthomycin biosynthetic gene cluster revealing new insights into carboxamide formation

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[ABSTRACT] Inthomycins are polyketide antibiotics which contain a terminal carboxamide group and a triene chain. Inthomycin B (**1**) and its two new analogues **2** and **3** were isolated from the crude extract of *Streptomyces pactum* L8. Identification of the gene cluster for inthomycin biosynthesis as well as the ^{15}N-labeled glycine incorporation into inthomycins are described. Combined with the gene deletion of the rare P450 domain in the NRPS module, a formation mechanism of carboxamide moiety in inthomycins was proposed *via* an oxidative release of the assembly chain assisted by the P450 domain.

[KEY WORDS] Inthomycins; Biosynthesis; Gene cluster; Carboxamide; P450
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Introduction

Inthomycins are polyketide antibiotics that were produced by *Streptomyces* sp. OM-5714 [1], *Streptomyces* sp. (strain Gö 2) [2] and *Streptomyces avermitilis* KA-320 [3]. Represented by members of inthomycins A (also named phthoxazolin A [4]), B, and C, as well as phthoxazolins [5] B, C and D, compounds in this family are structurally characterized by a mono-substituted oxazole ring, a configuration-diversified triene moiety, and a terminal carboxamide group. As reported, this small family of natural products display promising biological activities including specific inhibition of cellulose biosynthesis [1], as well as antifungal [4], anticancer [6, 7], and herbicidal [4, 8] activities. Due to their interesting profile of biological activities, the chemical synthesis of inthomycins has attracted much attention, and been completed in succession [9-15].

When it came to the biosynthesis of inthomycins, the biosynthetic gene cluster (BGC) was firstly reported through comparative genomic analysis and gene disruption of a discrete acyltransferase (AT) PtxA [3]. Coincidentally, inthomycins structurally represent nearly one half of architecture in oxazolomycin family [16]. Accordingly, the initial biosynthetic pathway of inthomycin is similar to that of oxazolomycin [17]. The shared peptide-polyketide chain in their structures is initiated with a formyl glycine moiety and elongated by hybrid non-ribosomal peptide synthetase (NRPS)-polyketide synthases (PKSs), and the corresponding PKSs are defined as AT-less type I PKSs [3, 17]. Dissimilarly, the release of a full-length hybrid peptide-polyketide product was proposed by a condensation (C) domain [17]. However, the length of actual carbon chain in inthomycins is much shorter than that of the predicated one based on the gene cluster sequence.

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[Δ]These authors contributed equally to this work.
These authors have no conflict of interest to declare.
This Paper is Dedicated to Professor TU You-You, the 2015 Nobel Prize Laureate of Physiology or Medicine on the Occasion of Her 90th Birthday.

Consequently, a NRPS-PKS chain-tailored process is hypothesized for the inthomycins biosynthesis, in which the detailed mechanism remains unknown to date. In this paper, we report the isolation and characterization of inthomycin B (**1**) and two new analogues **2** and **3** from the fermentation broth of *Streptomyces pactum* L8 as well as cloning of inthomycins BGC to elucidate the pathway and mechanism of carboxamide moiety formation in inthomycins.

Results

Isolation and structure identification of inthomycin B (1) and its analogues 2 and 3

Previously, we reported the strain *Streptomyces pactum* L8 is a producer of inthomycins [18]. This strain was fermented in five different media (MD, M15, MO, MP, and MQ medium, Table S1) to optimize fermentation conditions of a high yield inthomycins. As a result, MD medium fermentation condition of *S. pactum* L8 was selected for an inthomycin biosynthesis project. Two new inthomycin analogues **2** and **3** were obtained together with the improved yield of inthomycin B (**1**) from *S. pactum* L8 (Fig. 1).

Compound **2** was obtained as colorless oil from MeOH. HRESI-MS analysis (*m/z* 308.1498 $[M + H]^+$, Calcd. for 308.1492) of **2** revealed its molecular formula as $C_{16}H_{21}NO_5$, corresponding to seven degrees of unsaturation. With the comparison of ^1H and ^{13}C NMR data between **1** and **2** (Table 1), the existence of two proton resonances at δ_H 8.08 (1H, s) and 6.89 (1H, s), and three carbon chemical shifts at δ_C 152.7, 151.9 and 124.1 of **2** suggested the existence of a monosubstituted oxazole ring. ^1H-^1H COSY correlations (Fig. 1) of H_2-10/H-9/H-8/H-7/H-6/H-5 showed the existence of a six-carbon chain linked to C-11, which was identified by the HMBC correlations from H_2-10 to C-12, H-9 to C-11. Further

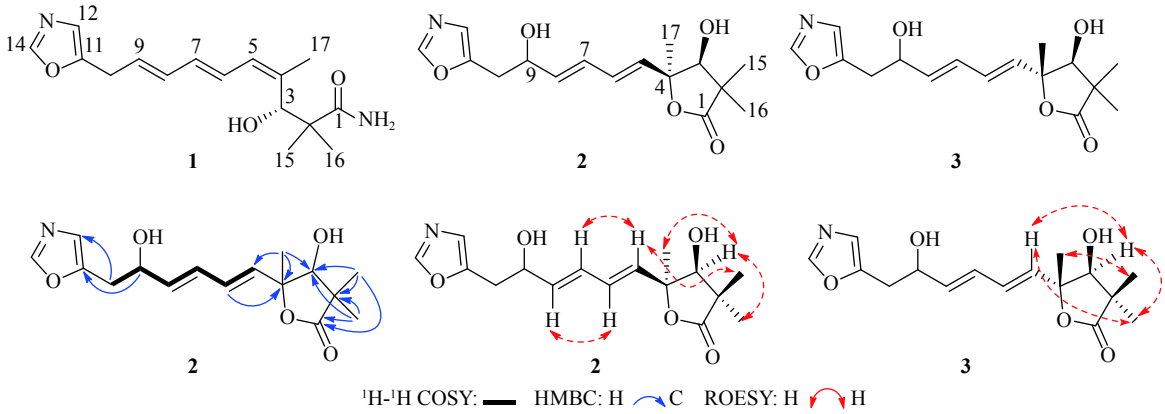

Fig. 1 Structures of compounds 1–3 and Key 2D NMR correlations of 2 and 3

Table 1 ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for compounds 1–3 in CD_3OD (*J* in Hz)

Position	1		2		3	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		183.5		182.8		183.5
2		46.8		44.9		45.9
3	4.68 s	76.0	3.94 s	83.7	3.93 s	81.5
4		139.5		86.2		88.0
5	6.02 d (11.3)	131.1	6.01 d (14.9)	134.9	5.89 d (14.5)	138.4
6	6.56 dd (14.0, 11.7)	129.4	6.23 dd (15.0, 10.6)	129.8	6.24 [a]	129.3
7	6.16 dd (14.0, 10.9)	133.1	6.27 dd (14.6, 10.6)	131.4	6.25 [a]	130.9
8	6.26 dd (14.4, 10.9)	134.9	5.76 dd (14.6, 6.5)	136.8	5.79 dd (14.5, 6.3)	137.4
9	5.77 dt (14.4, 7.2)	128.4	4.42 m	71.3	4.42 m	71.2
10	3.52 d (6.7)	29.6	2.91 m	34.5	2.91 m	34.5
11		153.0		151.9		151.8
12	6.86 s	122.8	6.89 s	124.1	6.90 s	124.1
14	8.10 s	153.3	8.08 s	152.7	8.09 s	152.7
15	1.05 s	22.1	1.04 s	19.9	1.18 s	20.5
16	1.26 s	26.4	1.20 s	25.4	1.21 s	26.3
17	1.80 s	20.0	1.48 s	27.5	1.42 s	22.2

[a] Overlapped signals

HMBC correlations from H_3-15 to C-1, C-2, C-3, H_3-16 to C-1, C-2, C-3 and H_3-17 to C-3, C-4, C-5 indicated that C-1 to C-4 chain was linked to C-5, two methyl groups were located at C-2 and one methyl group was located at C-4. What's more, the last two degrees of unsaturation implied the existence of another ring (a *δ*-lactone ring). Thus, the planar structure of compound **2** was established. The geometry of the diene was determined as 5*E* and 7*E* by the large vicinal coupling constants ($J_{H\text{-}5/H\text{-}6}$ = 15.0 Hz, $J_{H\text{-}7/H\text{-}8}$ = 14.6 Hz), as well as the ROESY correlations of H-5/H-7 and H-6/H-8. And the key ROESY correlations (H_3-16/H-3, H_3-17/H-3, H_3-17/H_3-16, and H_3-15/H-5, Fig. 1) indicated H-3, Me-16, and Me-17 to be cofacial in **2**. Hence, the structure of **2** was identified as shown in Fig. 1.

Compound **3** share the same molecular formula of $C_{16}H_{21}NO_5$ with that of **2** based on its HRESI-MS data (*m/z* 308.1496 $[M + H]^+$, Calcd. for 308.1492). The ^1H and ^{13}C NMR data of **3** was similar to those of **2** (Table 1), expect that the chemical shifts of the *δ*-lactone moiety shifted a bit. Therefore, **3** was supposed to be a stereoisomer of **2**. Analogously, key ROESY correlations of H_3-16/H-3, H-3/H-5, H_3-16/H-5, and H_3-17/H_3-15 (Fig. 1) indicated OH-3, Me-15, and Me-17 to be cofacial in **3**. Thus, the structure of **3** was established as a C-4 epimer of **2**.

Identification of the inthomycin gene cluster

In order to find the inthomycin (*int*) gene cluster, the genome of *S. pactum* L8 was sequenced, and the genomic sequence was scanned with the amino acid sequence of OzmP (ABS90477), which was inferred to be involved in the formation of oxazole ring in oxazolomycins biosynthetic pathway [17]. In all *S. pactum* L8 protein sequences, IntD showed 76.3% identity to OzmP, and there are six desirable genes encoding PKSs or NRPSs near the gene *IntD*. The arrangement of these PKSs or NRPSs is similar to that of *ptx* gene cluster, with IntD showing 91.1% identity to PtxD [3]. In order to determine indispensable genes involved in inthomycin biosynthesis, the hypothetical related genes and nearby genes were disrupted by gene inactivation experiments.

The SuperCos 1 genomic libraries of *S. pactum* L8 were constructed and screened to obtain the targeted cosmids containing the hypothetical genes. As a result, four SuperCos 1 cosmids (8C6, 14D12, 15C8 and 16G1, Fig. 2) were obtained. These cosmids comprised all hypothetical genes and other adjacent genes. Eight genes from *IntA* to *IntH* were inactivated by PCR targeting strategy [19]. Later, HPLC analysis of the fermentation extracts of the gene knockout mutants (*S. pactum* L8 Δ*IntA*−Δ*IntH*) showed that inthomycins production was abolished in all the constructed eight mutants, and no other similar metabolites can be detected (Fig. S1). Therefore, these eight genes (*IntA*−*H*) were identified as inthomycin biosynthesis related genes. Deletion of the adjacent genes [*orf* (*−18*) to *orf* (*+3*)] did not abolish the production of inthomycins, leading to the assignment of these *orf* (*−18*) to *orf* (*+3*) genes outside of the gene cluster. In summary, the *int* gene cluster contains eight genes (Fig. 2), encoding six PKSs, NRPSs or hybrid PKS-NRPSs (IntB, C, E, F, G and H), one discrete acyltransferase protein (IntA) and one hypothetic protein (IntD).

The proposed PKSs, NRPSs or hybrid PKS-NRPSs mediated assembled line in inthomycin biosynthesis

Based on the sequence analysis of PKSs, NRPSs and hybrid PKS-NRPSs in *int* gene cluster and the previously reported route [3], a possible inthomycin biosynthetic pathway was proposed as shown in Fig. 3. There were a loading module and 13 modules in the *int* cluster, including 10 PKS modules and 4 NRPS modules (loading module, modules 6, 12 and 13). These 10 PKS modules functioned as incorporations and modifications of eight two-carbon units in the inthomycin assembly chain, due to the missing of ketosynthase (KS) conserved catalytic sites in modules 9 and 11. Using antiSMASH 4.0 [20], the adenylation (A) domain in the loading module, modules 6 and 12 were predicted to activate glycine, glycine and serine, respectively, which were incorporated into the assembly chain by condensation (C) domain. No amino acid was clearly predicted to be activated by the A domain in module 13 through antiSMASH 4.0 [20], LSI based A-domain function predictor [21], NRPSsp [22] and PKS/NRPS analysis web-site [23]. Thus, the activated substrate of last extender unit of *int* gene cluster was not clear (Fig. 3).

The second C domain of module 13 might function similar to heterocyclase (Cy) domain, which was reported to create the heterocycle and make the extender chain drop from

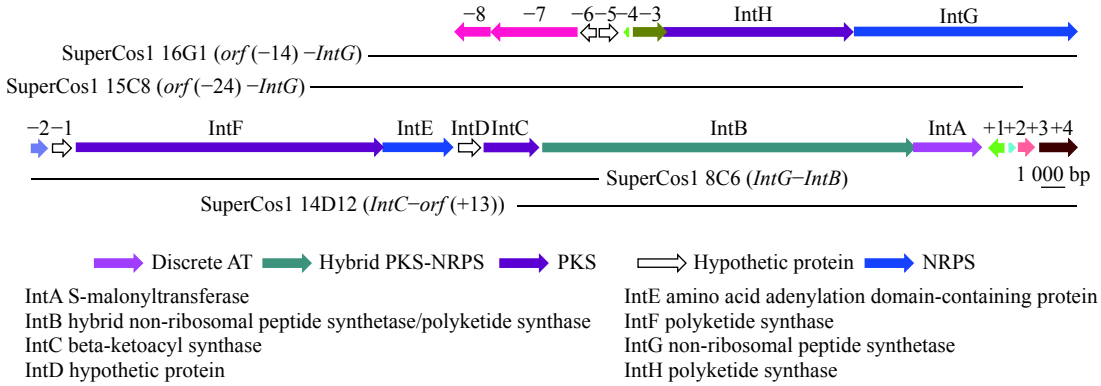

Fig. 2 The covering sequences of four target SuperCos1 cosmids

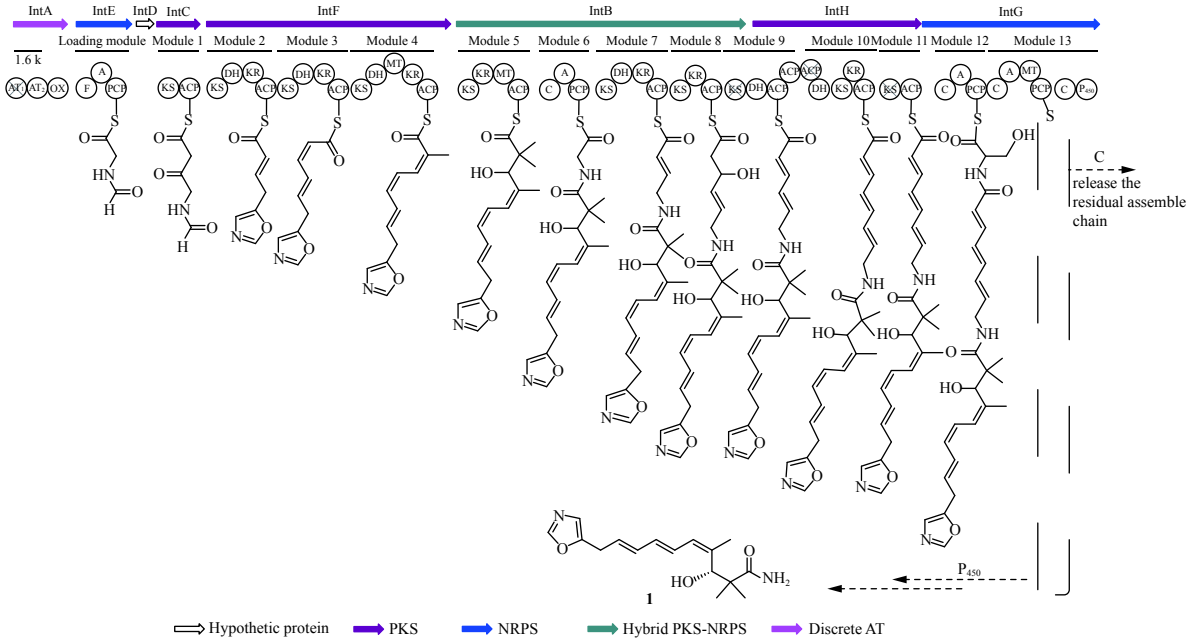

Fig. 3 Proposed assembled line in biosynthetic pathway of inthomycins

the module [17]. The conserved motif of routine C domain was HHXXXDG and the His residue is indispensable for the condensation function [17]. However, the corresponding motif of the second C domain in the module 13 was PWXXXDG, making it lose the routine C domain function. The function of motif PWXXXDG might be similar to that of the conserved motif DXXXXD in Cy domain, in which the two aspartate residues played structural roles instead of catalytic ones [24-25]. There was a possibility that the second C domain might be responsible for the removal of the assembly chain, leading to the regeneration of the whole assembly line.

The carboxamide formation mechanism of inthomycins

As the carbon chain length in inthomycins is much shorter than that in the predicated inthomycin assembly chain (Fig. 3), two possible chain-tailored pathways to form inthomycin from the long assembly chain were proposed (Fig. 4). In pathway A, the assembly chain was hydrolyzed to form the acid-form inthomycin analogue (**4**), which could be transformed into the final product **1** by an amidotransferase. In pathway B, a direct oxidative dealkylation reaction in the assembly chain led to the release of final product **1**. To verify pathway A, all protein sequences near the *int* gene cluster were analyzed to search an encoding amidotransferase gene. As a result, no amidotransferase gene was found. However, the direct difference of pathway A and B is the origin of the *N* atom in carboxamide group (Fig. 4). In pathway B, the *N* atom originates from glycine, which is incorporated into assembly chain by A domain in module 6. In view of this point, ^{15}N-glycine was fed into the fermentation medium of *S.*

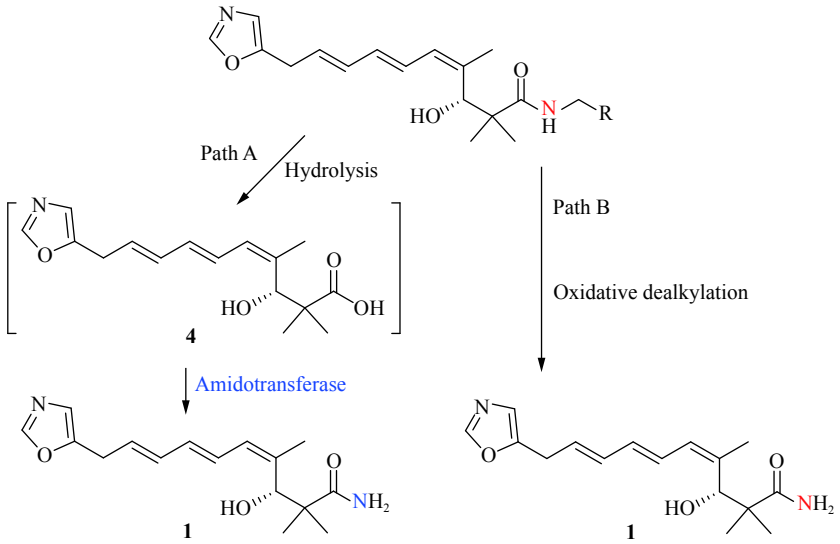

Fig. 4 Two possible pathways for carboxamide formation in inthomycins

pactum L8 wild type strain. The ^{15}N-glycine feeding experiment showed that inthomycin was labeled with two ^{15}N atoms according to its ESI-MS spectral data (Fig. S3), indicating that two *N* atoms in inthomycin all originated from glycine. Therefore, pathway B shown in Fig. 4 might be the probable route to form **1** *in vivo*.

Besides, there was a rare P450 domain in the NRPS module 13. It was reported that the middle position of the assembly chain could be oxidized by P450 when the assembly chain was linked to the NRPS module [26-28]. Thus, an oxidation process catalyzed by this P450 domain at *α* methylene carbon of amide was proposed when the assembly chain was still linked to the NRPS module in IntG (Fig. 5). In order to make sure whether the P450 domain was involved in the biosynthesis of inthomycin, the gene of P450 domain was deleted in the *S. pactum* L8 strain. The related knockout mutations abolished the production of inthomycins and didn't accumulate any other similar metabolites (Fig. S4). Therefore, as shown in Fig. 5, the P450 domain might participate in an oxidation reaction of the long assembly chain [29]. The *α* carbon of amide (the methylene one) in the assembly chain was speculated to be oxidized and hydrolyzed subsequently to release an aldehyde group and afford inthomycins products finally. Then the second C domain in module 13 might participate in the removal of the residual assembly chain, facilitating the whole assembly line be regenerated. However, except for the major oxidation in the *α* methylene carbon of amide assisted by P450 domain, a minor *N*-oxide by-product, which could result in the formation of two isolated new inthomycin analogues **2** and **3** [30], also might be yielded.

Discussion

In summary, inthomycin B (**1**) and two new analogues **2** and **3** were isolated from *S. pactum* L8, and the gene cluster for inthomycin biosynthesis was identified by gene inactivation experiments. Combined with a careful analysis of *int* gene cluster and ^{15}N-glycine feeding experiment, as well as the deletion characterization of rare P450 domain in the NRPS module, a formation mechanism of terminal carboxamide moiety in inthomycins was proposed *via* an oxidation reaction of the assembly chain assisted by the P450 domain.

Terminal carboxamides exist widely in nature and their biosynthesis have been studied extensively. Three main biosynthetic routes were provided to form terminal carboxamides (Fig. S5) [31, 32]. Route A: a direct amidation of a terminal acid group catalyzed by amidotransferase *via* an activated carboxylate intermediate, such as the formation of thiotetroamide C and myxochelin B [31, 33]. Route B: an oxidative cleavage of *C*-terminal glycine by a peptidylglycine *α*-amidating monooxygenase or a flavin-dependent monooxygenase, such as the formation of zwittermicin A [34]. Route C: an enamine dealkylation of *C*-terminal serine extended precursor peptide, such as the formation of nosiheptide [32]. In inthomycins, the formation process of the terminal carboxamide is similar to route B. However, the oxidation process usually happens in the adjacent glycine extender module in route B. In inthomycins, a rare long-distance oxidation of the assembly chain catalyzed by P450 domain led to the release of inthomycins.

With the release of inthomycin from the assembly chain,

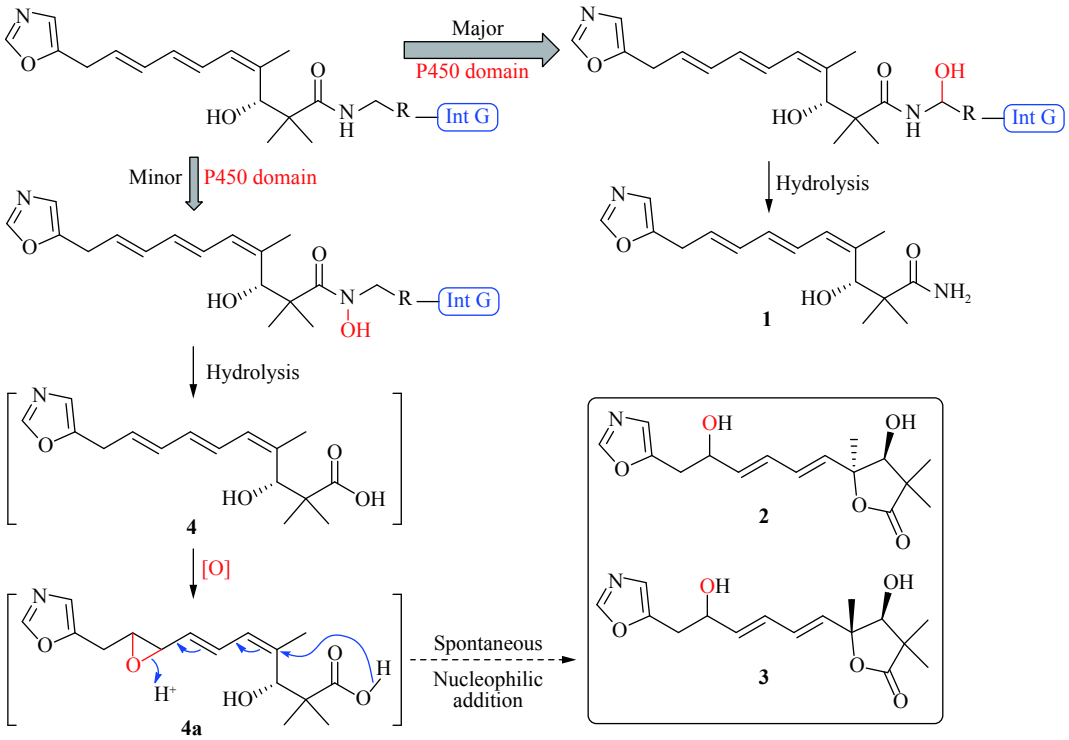

Fig. 5 The proposed biosynthetic pathways of compounds 1–3

the residual assembly chain catalyzed by modules 7 to 13 just act as a transition intermediate in the biosynthetic process of inthomycins. In colibactins, the transition intermediate was reported to act as a protective group to avoid host strain being damaged by colibactins [35]. For strain *S. pactum* L8, the actual function of the residual assembly chain is worthy of further study.

Experimental

General experimental procedures

NMR spectra were recorded in CD_3OD using a Bruker AVANCE III-600 spectrometer (Bruker Corpration, Germany) with TMS as an internal standard. ESI-MS and HRESI-MS data were obtained using an Agilent G6230 UPLC/Q-TOF mass spectrometer (Agilent Technologies Inc., United States). Column chromatography (CC) was performed using silica gel (200−300 mesh, Qingdao Marine Chemical Inc., China) and Sephadex LH-20 (18−111 μm, Pharmacia Biotech Ltd., Sweden). Semipreparative HPLC was conducted on a Hitachi Chromaster system (Hitachi Ltd., Japan) equipped with a DAD detector, a YMC-Triart C_{18} column (250 mm × 10 mm i.d., 5 μm, YMC Ltd., Japan), a flow rate of 3.0 mL·min^{-1}, and a column temperature at 28 °C. And all HPLC analyses were performed in the same equipment with an YMC-Triart C_{18} column (250 mm × 4.6 mm i.d., 5 μm) at a flow rate of 1.0 mL·min^{-1}, and a 28 min gradient mobile phase (0.0−20.0 min, 10% MeOH in H_2O to 100% MeOH; 20.0−24.0 min, 100% MeOH; 24.1−28.0 min, 10% MeOH in H_2O, 0.1 % acetic acid was added to each phase).

Strain material and fermentation medium

Streptomyces pactum L8, a strain isolated from the soil sample collected in Qinghaihu (Qinghai Province, China), was identified and provided by Prof. XUE Quan-Hong in North West Agriculture and Forestry University (Yangling, China).

Fermentation, extraction and isolation

Strain *S. pactum* L8 was grown on MS agar plates (soybean meal 20 g, yeast extract 4 g, and agar 20 g in 1 L water, pH 7.2) for 5 days at 30 °C. The mycelium was inoculated into sterile seed medium (TSB medium: tryptone soy broth, 30 g·L^{-1}) and was cultivated for 24 h at 28 °C on a rotary shaker (200 r·min^{-1}). Then, the cultivated seed medium was transferred into the MD fermentation medium in a proportion of 5%, the resulting fermentation medium was cultivated on a rotary shaker (200 r·min^{-1}) at 28 °C for 5 days.

The supernatant of the fermentation broth (40 L) was extracted with EtOAc (20 L × 5 times). The organic phase was concentrated in vacuo at 37 °C to yield an oily crude extract. The mycelium was extracted with MeOH/H_2O (9 : 1) and the extract was concentrated in vacuo, then the residual aqueous concentrate was extracted with EtOAc in equal volume. The combined extract 6.0 g was subjected to silica gel (60 g, 200−300 mesh) by column chromatography with a successive elution of petroleum ether−EtOAc (10 : 0, 10 : 1, 2 : 1, 1 : 1, 0 : 1, *V/V*) to give five fractions (Fr.A–Fr.E). Fr.D fraction was subjected to Sephadex LH-20 eluting with MeOH to yield fractions (Fr.D.1−4). Fr.D.2 was purified by semipreparative HPLC to yield compounds **1** (50.0 mg), **2** (1.3 mg), **3** (2.8 mg).

Strain genome sequencing

The genome sequencing of *S. pactum* L8 was performed with the Illumina Genome Analyzer (Illumina, San Diego, CA) by BGI (BGI-Shenzhen, China). The genomic DNA isolated from *S. pactum* L8 was used to construct small (200−500 bp) and large (2−10 kb) random sequencing libraries. The reads were filtered and assembled into 156 contigs using SOAPdenovo (http://soap.genomics.org.cn/). Then the paired-end information was used to join the 156 contigs into 95 scaffolds. Putative protein-coding sequences were predicted using the GLIMMER program (V3.02) and the annotation was accomplished by BlastP analysis of sequences in the Cog, Kegg and SwissProt databases.

Genomic library construction, mutants construction and fermentation

The SuperCos 1 genomic libraries were constructed according to the standard SuperCos 1 cosmid vector kit (Stratagene, catalog #251301) manual. About 2000 *E. coli* clones were picked and stored at −80 °C. The target SuperCos 1 cosmids were screened by PCR with specific primers as shown in Table S3. The mutant strains were constructed according to the PCR targeting strategy [19]. The target cosmids were transformed into the *E. coli* BW25113/pIJ790 for electroporation with resistance cassette which contained an apramycin resistance gene *aac(3)IV* and two 39 bp homologous arms. The resulting cosmids were transformed into the non-methylating *E. coli* ET12567/ pUZ8002, which can transfer the non-methylated cosmids into *S. pactum* L8 (wild type) by intergeneric conjugation. Then double cross-over exconjugants were screened on the MS plate containing apramycin (Aladdin Reagent Co., Ltd., Shanghai, China) with and without kanamycin (Aladdin Reagent Co., Ltd., Shanghai, China). The genome of kanamycin^S and apramycin^R strains were extracted and analyzed by PCR with specific primers in Table S3. Double cross-over strains gave a single PCR band with the target size and the wild-type strain gave a band at original length (Fig. S6). Then these mutants were fermented three times in the 250 mL baffle Erlenmeyer flasks containing 50 mL MD medium with apramycin at a concentration of 50 μg·mL^{-1}. The supernatants were extracted with equal volume EtOAc, and the yielded crude extracts were analyzed by HPLC.

Feeding the S. pactum L8 (wild type) with ^{15}N-glycine

The optimal feeding conditions was selected according to the growth curve of inthomycins (Fig. S1). Strain *S. pactum* L8 (wild type) was grown in seed medium for 24 h, then about 2.5 mL seed culture (5% inoculums) was inoculated into 50 mL production medium [Modified medium MD, the concentration of nitrogen sources (tryptone and yeast extract) both decreased to 1.0 g·L^{-1}] at 28 °C. After 24 h incubation, ^{15}N-glycine (25 mg) (Cambridge Isotope Labotories Inc.,

USA)in 500 μL ddH_2O was added into the production culture and fermented for additional 4 days. The yielded extracts were analyzed by HPLC, and targeted inthomycins were submitted to an ESI-MS measurement.

Supporting Information

Supporting information of this paper can be requested by sending E-mails to the corresponding authors.

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