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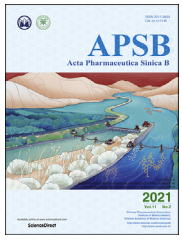

ORIGINAL ARTICLE

A four-protein metabolon assembled by a small peptide protein creates the pentacyclic carbonate ring of aldgamycins

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Abstract Organic carbonates (OCs) are a class of compounds featured by a carbonyl flanked by two alkoxy/aryloxy groups. They exist in either linear or cyclic forms, of which the majority encountered in nature adopt a pentacyclic structure. However, the enzymatic basis for pentacyclic carbonate ring formation remains elusive. Here, we reported that a four-protein metabolon (AlmUII–UV) assembled by a small peptide protein (AlmUV) appends a reactive *N*-hydroxylcarbamoyl moiety to the decarboxylated aldgamycins followed by a non-enzymatic condensation to give the pentacyclic carbonate ring. Our results have documented an unprecedent mechanism for carbonate formation.

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1. Introduction

Organic carbonates (OCs) are a class of compounds characterized by a carbonyl flanked by two alkoxy/aryloxy groups. They have been widely applied in modern organic synthesis as versatile heterocyclic substrates for stereo- and enantio-selective C−N, C−O, C−C, C−S and C−B bonds formation[1−3]. On the other hand, study on natural OCs grows slowly in terms of occurrence, synthesis, and biogenesis. Up to date, only about 60 natural OCs have been discovered from nature[2,4−15]. According to whether the carbonate is cyclized or not, they can be divided into two major groups: linear carbonate and cyclic carbonate, and the latter is more popular in nature (Fig. 1)[2]. Aldgamycin E is the earliest natural OC discovered from a variant strain of *Streptomyces lavendulae* in 1965[16], which possesses a rare branched-chain sugar D-aldgarose appended with a pentacyclic carbonate ring. Since then, several biologically active carbonates including NCS Chrom A[17], cytochalasin E[18], hololeucin[19], and miniolutelide A[20] were isolated (Fig. 1). Because of their potential association with biological activity, biosynthesis of carbonates has attracted much interest. As early as 1970, Schmid and Grisebach[21] performed an isotope labelling experiment and showed that the pentacyclic carbonate ring of aldgamycin E comes from sodium bicarbonate. Later, Hensens and co-workers[22] used a similar approach and demonstrated that the ^{14}C-labeled sodium bicarbonate also becomes incorporated into the carbonate ring of NCS Chrom A. In 2014, Hu and co-workers[23] identified a Baeyer-Villiger monooxygenase CcsB, which catalysed the successive insertion of two oxygen atoms into a polyketide chain to form an in-line carbonate moiety in cytochalasin E. However, the molecular mechanism of the formation of pentacyclic carbonate ring, which occurs most frequently in nature, remains unclear.

We have previously isolated a marine-derived *Streptomyces* sp. HK-2006-1, which is capable of producing two kinds of macrolide antibiotics: aldgamycins and chalcomycins (Fig. 2A)[5]. Based on gene deletion experiments, we have demonstrated that aldgamycins and chalcomycins are biosynthesized from a same gene cluster (*alm* gene cluster) and characterized their biosynthetic pathway[4,24], but the genes responsible for the pentacyclic carbonate ring formation in aldgamycins are not elucidated. In the *alm* gene cluster, there are five genes (*almUI−UV*) remained to be characterized (Supporting Information Fig. S1). We thus speculated that they might be involved in the carbonate ring formation. Here, we reported an unprecedent mechanism responsible for the pentacyclic carbonate ring formation, in which a four-protein metabolon assembled by a small peptide protein transfers a reactive *N*-hydroxylcarbamoyl moiety to the decarboxylated aldgamycins followed by spontaneous condensation to form the pentacyclic carbonate ring.

2. Results and discussion

2.1. *Identification of the genes involved in carbonate ring formation*

To determine whether these five uncharacterized genes (*almUI−UV*) in *alm* gene cluster are involved in carbonate ring formation, we individually disrupted each of these five genes using an in-frame deletion approach. Inactivation of each target gene of mutants was confirmed by genome-typing PCR (Supporting Information Fig. S2). When *almUI*, a hypothetical *O*-methyltransferase, was deleted (Supporting Information Fig. S3), no effect was observed on the production of aldgamycin P (**5**) and

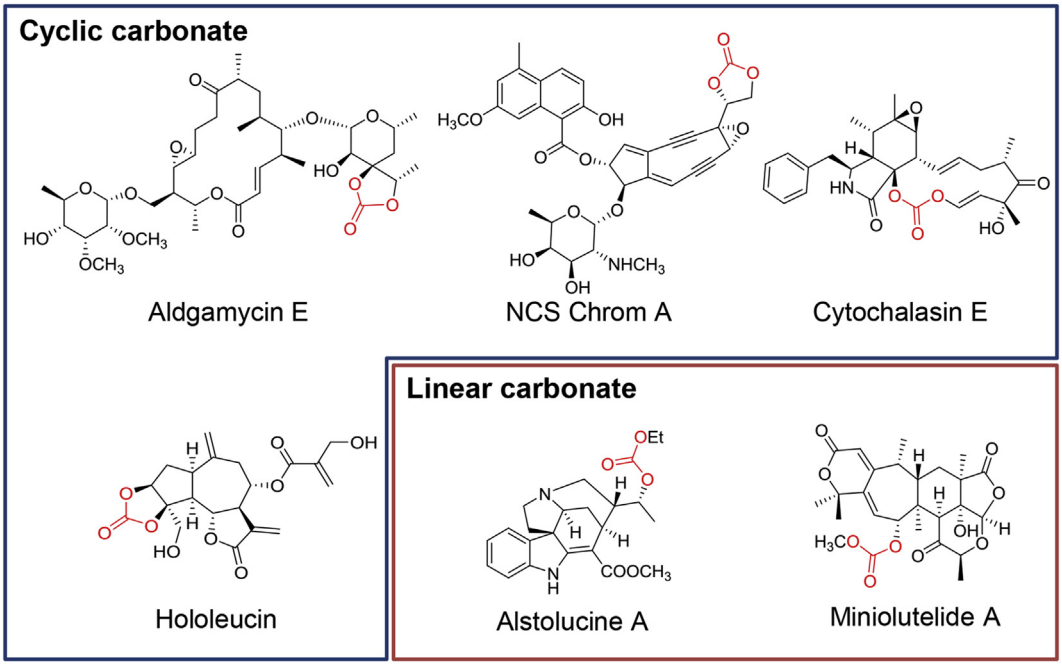


Figure 1 Typical natural organic carbonates.

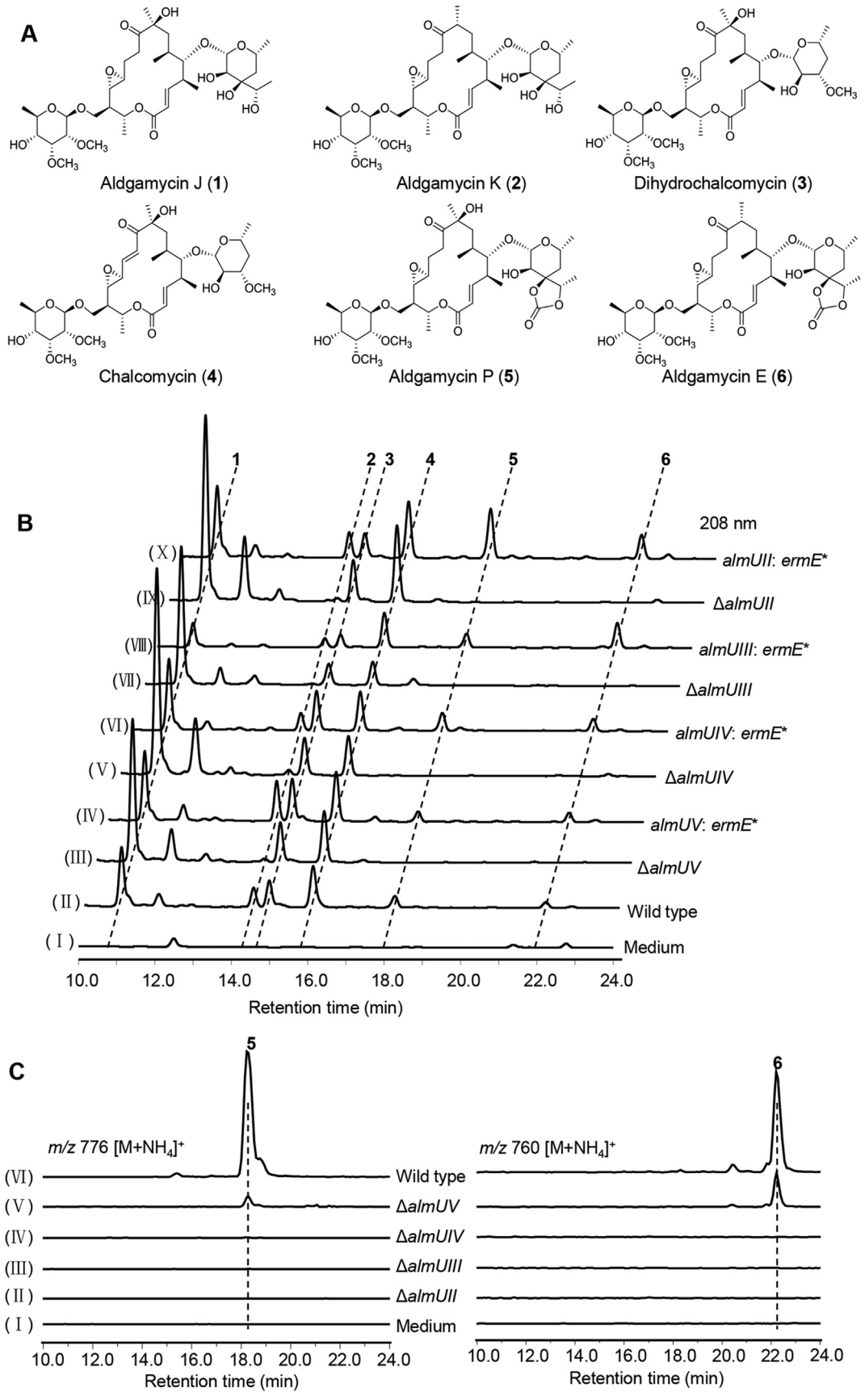


Figure 2 Determination of the genes involved in biosynthesis of the pentacyclic carbonate in aldgamycins. (A) Structures isolated from *Streptomyces* sp. HK-2006-1. (B) Metabolite analysis of *almUII−UV* deletion and complementation mutants. (C) EIC analysis of *almUII−UV* deletion mutants.

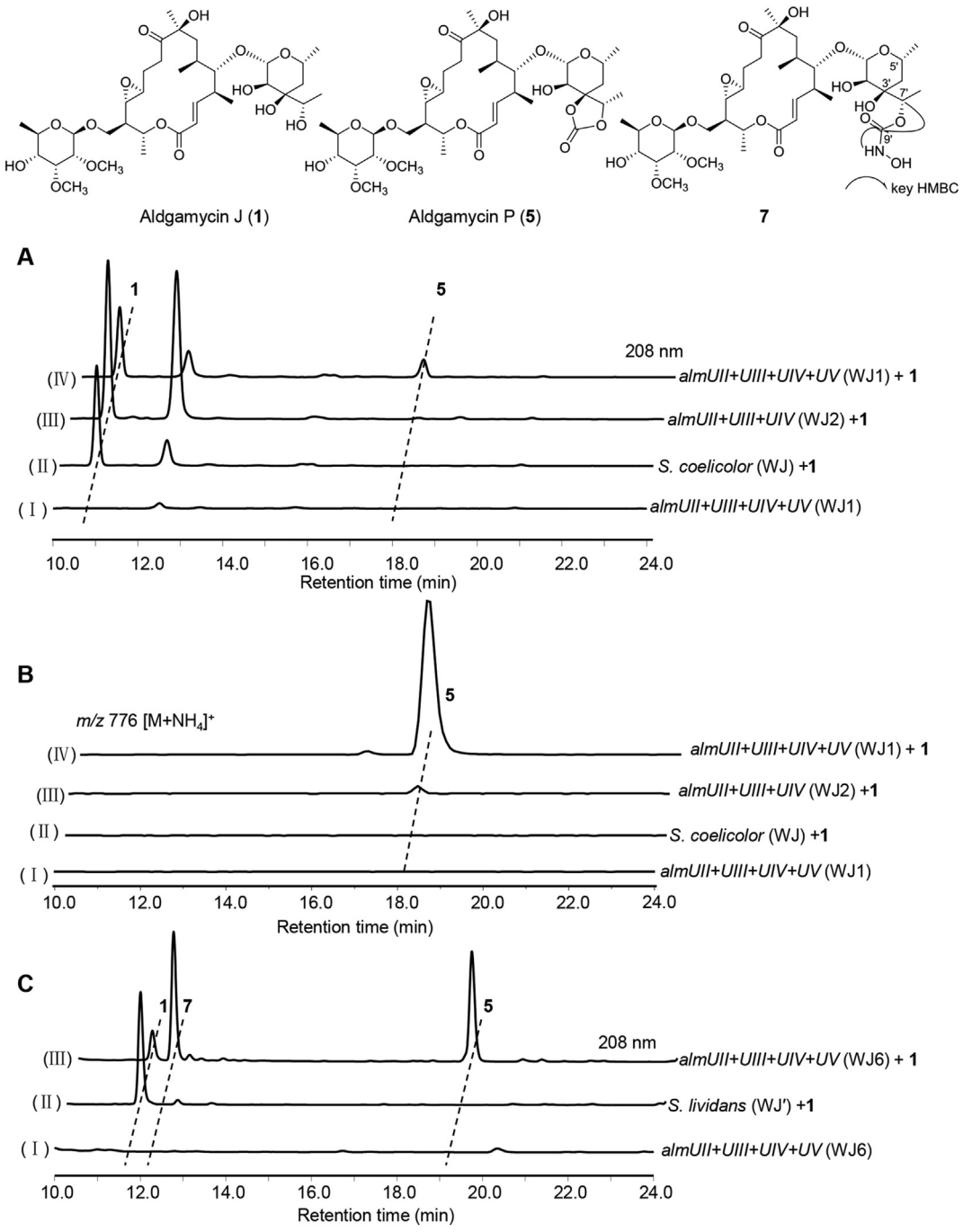


Figure 3 Heterologous expression of genes involved in the biosynthesis of pentacyclic carbonate. HPLC analysis (A) and EIC analysis (B) by heterologous expression of *almUII−UV* in *S. coelicolor* using pSET152. (C) HPLC analysis by heterologous expression of *almUII−UV* in *S. lividans* using pTNT.

aldgamycin E (**6**), thus excluding its involvement. Disrupting each of the other four genes (*almUII–UV*) abolished the production of **5** and **6** and led to the accumulation of the decarboxylated product aldgamycin J (**1**, Fig. 2B). Moreover, the production of **5** and **6** were restored by re-expression of *almUII−UV* back to the relevant gene knockout strains (Fig. 2B). These observations suggested that *almUII−UV* are involved in the carbonate ring formation. Further LC–MS analysis showed that trace amount of **5** and **6** could be detected in *ΔalmUV* strain, which did not occur in the *almUII*, *almUIII* and *almUIV* knockout strains (Fig. 2C). This finding indicated that AlmUV is not involved in direct synthesis of pentacyclic carbonate, but may be important for maintaining the activity of AlmUII, AlmUIII and AlmUIV at high level.

To determine whether these four genes are sufficient to catalyse the carbonate ring formation, *almUII−UV* were co-expressed into *Streptomyces coelicolor* to obtain strain WJ1. Feeding of WJ1 with **1** led to the production of **5**, but not the wild-type strain (Fig. 3A). This clearly indicates that the four genes are enough to append the pentacyclic carbonate ring of aldgamycins. But no transformations of **1** were detected in the absence of any of

almUII, *almUIII* and *almUIV* (Supporting Information Fig. S4) except that trace amount of **5** was detected in WJ2 lacking *almUV* (Fig. 3B and Fig. S4). These results further confirmed that AlmUII, AlmUIII and AlmUIV are essential for pentacyclic carbonate formation whereas AlmUV is not directly involved in enzymatic synthesis.

2.2. *Identification of the key intermediate during carbonate ring formation*

Though we have identified the genes responsible for carbonate ring formation, the individual function of each gene is unclear because no intermediates were detected when omitting any of the four genes in either gene knockout or heterologous expression experiments. This raises a possibility that the proteins encoded by these four genes might form a complex to execute their functions. To test this hypothesis, co-immunoprecipitation experiments[25] were used to investigate the protein−protein interaction. Since the gene expression in pSET152/*S. coelicolor* system is too low to detect the protein interaction, a hyper-expression system, pTNT/*Streptomyces lividans*, was used. *almUII−UV* were cloned into the pTNT vector and then transferred into *S. lividans* to obtain transformant strain WJ6. SDS-PAGE analysis confirmed that the expression of AlmUII in WJ6 was significantly increased compared to WJ1 (Supporting Information Fig. S5). To test whether these genes were actively expressed in WJ6, feeding of WJ6 with **1** was carried out. Consistent with the results from pSET152/*S. coelicolor* system, conversion of **1** to **5** was also observed, indicating the active expression of these genes in pTNT/*S. lividans* system (Fig. 3C). In addition, a new major product **7** was observed (Fig. 3C). LC–MS analysis showed that it has a molecular weight of 791 (*m/z* 792 $[M+H]^+$), which is 59 Da larger than **1**. Isolation and NMR analysis (Supporting Information Table S8 and Fig. S7) revealed that it shows a nearly identical structural feature to that of **1**, except the observation of an additional carbonyl carbon at δ_C 159.0 and a –NH– at δ_H 7.50 (1H, s). HMBC correlation from –NH– [δ_H 7.50 (1H, s)] to C-9′ (δ_C 159.0) indicates the connection of –NH– to C-9′. In addition, HMBC correlation from δ_H 4.94 (1H, q, J = 6.5 Hz, H-7′) to C-9′ (δ_C 159.0) suggests the connection of C-9′ to 7′-OH. Since there is still 17 Da (OH) left, a −OH group was then assigned to link to nitrogen of the –(C=O)NH– moiety. Base on these results, **7** is determined to be compound **1** bearing an *N*-hydroxylcarbamoyl group at 7′-OH. This inference was supported by the reported NMR data of *N*-hydroxylcarbamoyl moiety[26,27].

Isolation of **7** makes us wonder if it is an intermediate en route to **5**. To verify this, *in vitro* enzymatic experiment was carried out. Compound **7** was incubated with the crude extract of WJ6 or boiled extract. As a result, **7** was completely converted into **5** within half an hour. However, no difference was observed between the crude extract and the boiled extract (Supporting Information Fig. S8), suggesting that transformation from **7** to **5** is a non-enzymatic step and **7** is the actual enzymatic product of the four genes. The reason why we failed to isolate compound **7** from pSET152/*S. coelicolor* expression system may be due to the low expression of genes, so that the production rate of compound **7** is less than its consumption rate.

2.3. *Protein−protein interaction between AlmUII−UV*

The above results have shown that the four genes can be abundantly and actively expressed in pTNT/*S. lividans* system. This makes us want to investigate the individual function of AlmUII−UV using this new system. When **1** was fed to the AlmUII−UV separately expressing strains (WJ7−WJ10), respectively, no transformation product was detected (Supporting Information Fig. S9), confirming that each gene expressed separately has no function. We also carried out *in vitro* enzymatic assay using cell crude extracts. When compound **1** was incubated with cell lysate of the four-gene expressing strain WJ11, trace amount of **5** was detected by LC–MS analysis, but not with the cell lysate mixture of the AlmUII−UV separately expressing strains WJ7−WJ10 (Supporting Information Fig. S10). These results indicated that simultaneous expression of the four genes is essential for carbonate ring formation, further suggesting that the four proteins are likely to form a complex to execute their functions.

Since specific antibodies against AlmUII−UV are not available, we then expressed them as tagged proteins for co-immunoprecipitation assay. *almUII*, *almUIII*, *almUIV* and *almUV* were constructed into pTNT with His-tag, c-Myc-tag, Flag-tag, and HA-tag, respectively. Transformation of this plasmid to *S. lividans* generated strain WJ11. Feeding of WJ11 with **1** suggested that these tags have no significant effects on protein activity (Supporting Information Fig. S11). Then, co-immunoprecipitation from the protein extract of WJ11 or *S. lividans* using anti-Flag antibody (M2) against Flag-tagged AlmUIV was performed (Fig. 4A−D). Though a faint band with similar size to Flag-tagged AlmUIV was detected in IgG control lane, significant enrichment of Flag-tagged AlmUIV by M2 antibody from WJ11 (M2-IP) but not the *S. lividans* was observed (Fig. 4A). This confirmed the specificity of the immunoprecipitation. Subsequent immunoblotting the precipitated samples with anti-His antibody showed a clear band of the predicted molecular mass of His-tagged AlmUII in M2-IP samples from WJ11, but not from *S. lividans* and IgG precipitated samples (Fig. 4B). Similar results were obtained from the immunoblotting with anti-c-Myc for c-Myc-tagged AlmUIII (Fig. 4C) and anti-HA for HA-tagged AlmUV (Fig. 4D). These data clearly demonstrated that AlmUII, AlmUIII, AlmUIV and AlmUV form a protein complex inside the cell.

2.4. *The small peptide protein AlmUV is crucial for protein complex formation*

Since gene knockout and heterologous expression experiments have shown that AlmUV is not directly involved in enzymatic synthesis, we wondered whether it plays a role in the protein complex formation. We thus constructed a three-gene expression strain WJ12 without HA-tagged AlmUV. Feeding of WJ12 with **1** confirmed that these three genes are actively expressed (Supporting Information Fig. S12). We then investigated the effects of AlmUV by comparison of protein complex formation between WJ12 and WJ11. As shown in Fig. 4E−G, the expression of AlmUII, AlmUIII and AlmUIV were not obviously affected in the absence of AlmUV (input), but the protein complex formation observed in WJ11 (M2-IP) was hardly detected in WJ12 (M2-IP, Fig. 4F and G). These data suggest that AlmUV is crucial for protein complex formation. Interestingly, we found that the precipitation of Flag-tagged AlmUIV by M2 antibody from WJ12 was significantly reduced compared to that from WJ11 though the expression of Flag-tagged AlmUIV was similar in both strains (Fig. 4E). This might be caused by a change in the complex

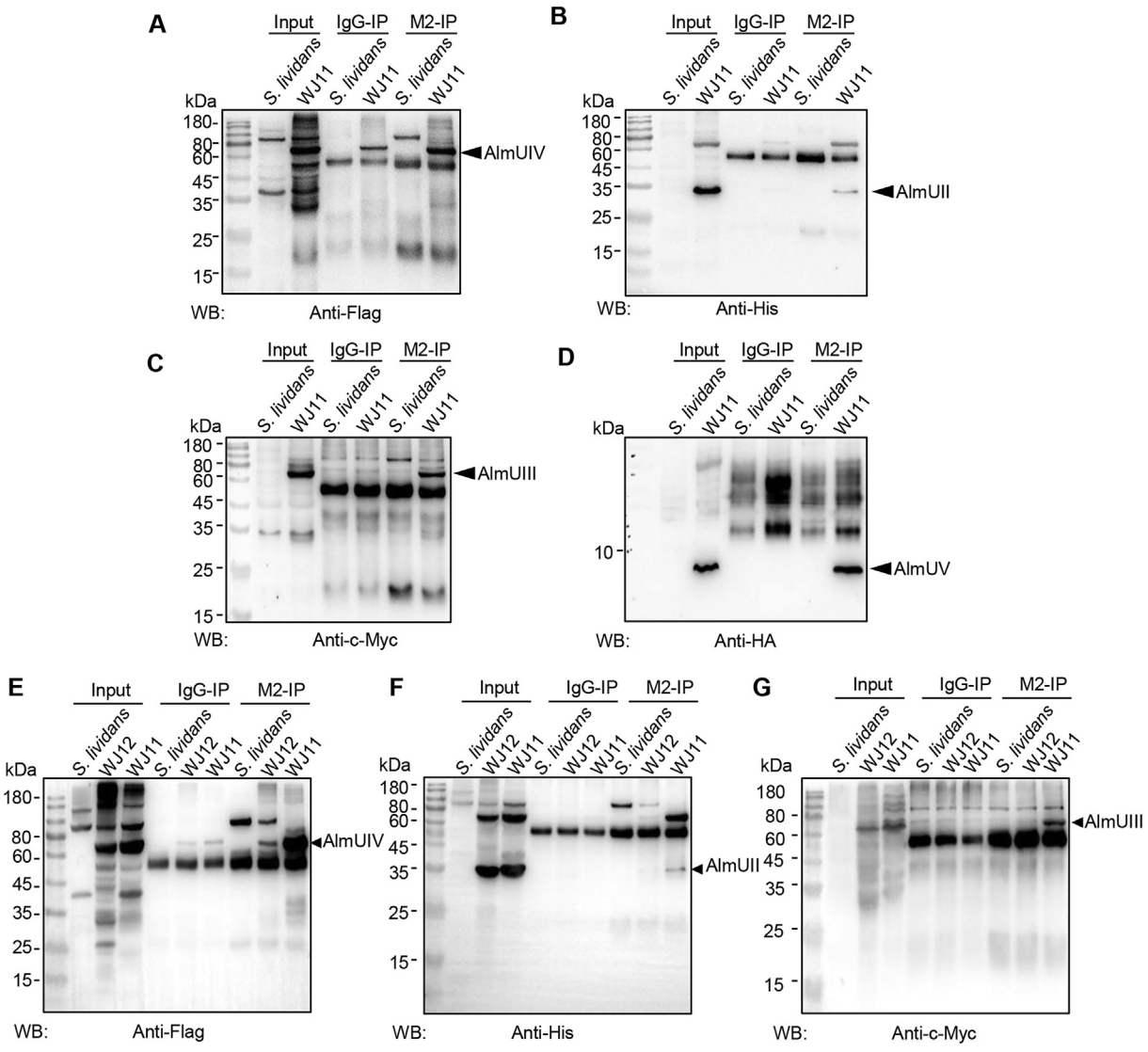
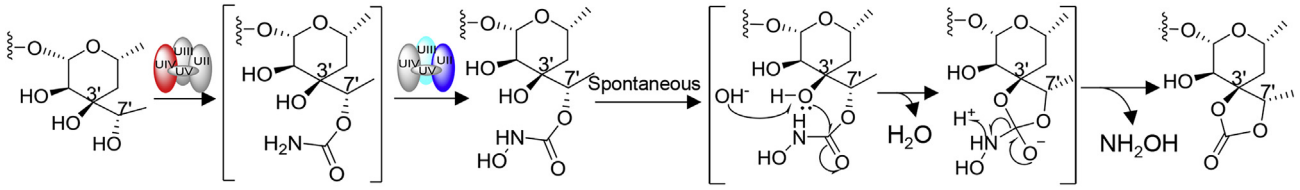


Figure 4 Co-immunoprecipitation analysis of AlmUII−UV in WJ11 (A−D) and WJ12 (E−G). Total protein extracts from WJ11 or WJ12 were immunoprecipitated with anti-flag antibody (M2) or IgG. *S. lividans* harboring empty pTNT was used as negative control.

formation in the absence of AlmUV, which prevents the Flag-tag from being normally exposed.

Based on the above results, we have disclosed the enzymatic basis for forming the pentacyclic carbonate ring of aldgamycins. This transformation is mediated by a four-protein metabolon (AlmUII−UV). Among the four-protein complex, AlmUIV shows 38% identity to NovN, an *O*-carbamoyltransferase involved in novobiocin biosynthesis[28]. Carbamoyltransferase uses carbamoyl phosphate as a donor that is synthesized from ammonia and bicarbonate by carbamoyl phosphate synthetase[29,30]. This is consistent with the previous isotope labelling experiments that the ^{14}C-labeled sodium bicarbonate is incorporated into the carbonate ring of aldgamycin E. Based on these, we therefore propose that biosynthesis of carbonate ring begins with the transfer a carbamoyl group to the *C*7′-OH of decarboxylated aldgamycins by AlmUIV (Scheme 1). Subsequent *N*-hydroxylation is likely to be achieved by two hypothetical proteins AlmUII and AlmUIII. This is supported by observation of a heme oxygenase domain at the C-terminal of AlmUIII[31]. AlmUV is a small peptide protein only possessing 52 amino acids, which is not thought as a functional gene at the beginning. In the present study, we have shown that though this small peptide protein is not directly involved in enzymatic synthesis, it is important for the carbonate ring formation, which is due to its role in protein complex assembly.

Protein rarely works alone, two or more proteins bind together to mediate a wide range of cellular processes such as protein modification, signal transduction, protein trafficking, and structural folding. However, protein complexes are not common in biosynthesis of small molecules. A few examples include pyruvate dehydrogenase multienzymes complex[32], pyridoxal 5-phosphate (PLP) synthase complex[33], and the multienzyme polyketide synthases (PKSs) and nonribosomal polypeptide synthetases (NRPSs)[34−36]. Additional cases involve binding of coenzyme to the zymogen to form the active enzyme[37]. Recently, Pierrel and co-workers[38] identified that a seven-protein metabolon catalyses the last six sequential reactions of the ubiquinone biosynthetic pathway in *Escherichia coli*[38]. Among this protein complex, UbiK is a 96-residue small protein belonging to the BMFP (*Brucella* membrane fusogenic protein), which is supposed to act as an assembly factor[39]. In this study, we have identified a new protein



Scheme 1 Proposed mechanism for carbonate ring formation in aldgamycins.

assembly factor AlmUV, which is also a small peptide protein containing only 52 amino acids but shows no homology to UbiK. A homology search of AlmUII, AlmUIII, AlmUIV, and AlmUV using MultiGeneBlast[40] at the NCBI database revealed that these genes usually forms a cluster and widely distributed in actinomyces genomes, suggesting that the formation of pentacyclic carbonate ring mediated by the four-protein metabolon is a common process in actinomyces (Supporting Information Fig. S13).

3. Conclusions

In this study, we have identified an unprecedented process for the pentacyclic carbonate ring formation in macrolide antibiotics aldgamycins, which begins with appending a reactive *N*-hydroxylcarbamoyl moiety to a hydroxyl group of the decarboxylated macrolide followed by a non-enzymatic condensation. This process is accomplished by a four-protein complex (AlmUII−UV) assembled by a small peptide protein.

4. Experimental

4.1. General materials

Primer synthesis and DNA sequencing were performed by Sangon Biotech Co., Ltd. (Shanghai, China). Plasmid purification kits and agarose gel DNA extraction kits were purchased from Sangon Biotech Co., Ltd. PCR was carried out using a Mastercycler nexus gradient (Eppendorf, Hamburg, Germany) with KOD FX DNA polymerase (Toyobo, Osaka, Japan) or Primer STAR HS DNA polymerase (TaKaRa Bio Inc., Dalian, China) or LA Taq (TaKaRa Bio Inc.). In-Fusion® HD Cloning Kit and T4 DNA polymerase were purchased from TaKaRa Bio Inc. Other DNA modification reagents were purchased from Thermo Fisher Scientific Inc. (Shenzhen, China). Protein A/G agarose beads and normal mouse IgG was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-Flag, anti-HA, anti-His, and anti-c-Myc mouse monoclonal antibodies were purchased from TransGen Biotech (Beijing, China). HRP-conjugated goat anti-mouse IgG (h+l) was purchased from FDbio Science Biotech Co., Ltd. (Guangzhou, China). 10% SDS-PAGE and Tricine-SDS-PAGE solution were purchased from FDbio Science Biotech Co., Ltd. PVDF membranes (#IPVH00010, pore size 0.45 μm; #10600001, pore size 0.2 μm) were purchased from Merck Millipore Co. (Billerica, MA, USA). HPLC grade acetonitrile (CH_3CN) was purchased from Oceanpak Alexative Chemical Co., Ltd. (Gothenburg, Sweden). Analytical-grade petroleum ether and ethyl acetate (EtOAc) were purchased from Fine Chemical Co., Ltd. (Tianjin, China). All chemical reagent used in this study are analytical-grade, purchased from commercial resources.

The HR-ESI-MS data were obtained on a Micromass Q-TOF mass spectrometer (Waters Corporation, Milford, MA, USA). 1D and 2D NMR spectra were obtained with Bruker AV 600 spectrometers (Bruker BioSpin Group, Faellanden, Switzerland) using the solvent signals ($CDCl_3$: δ_H 7.26/δ_C 77.0) as internal standards.

High-performance liquid chromatography (HPLC) and liquid chromatography−mass spectrometer (LC−MS) were carried out on an Ultimate 3000 HPLC system (Dionex, Germering, Germany) and an amaZon SL ion trap mass spectrometer (Bruker Daltonics Inc., Billerica, Boston, MA, USA) using electrospray ionization with a Cosmosil 5C18-MS-II column (250 mm × 4.6 mm, 5 μm; Nacalai Tesque, Inc., Kyoto, Japan) and a YMC-24 pack ODS-A column (250 mm × 4.6 mm, 5 μm; YMC Co., Ltd., Kyoto, Japan). Elution was subjected to a linear gradient [H_2O (A) and CH_3CN (B); 1 mL/min; 30%−65% B (0−25 min), 65%−100% B (25−30 min), 100% B (10 min)]. Absorbance was recorded at 208 nm.

The semi-preparative HPLC was performed on an Ultimate 3000 HPLC system (Dionex) using a YMC-Pack ODS-A column (250 mm × 10.0 mm, 5 μm; YMC Co., Ltd.). Column chromatography was performed with silica gel (200−300 mesh, Haiyang Chemical Co., Ltd., Qingdao, China) and ODS (50 μm, YMC Co., Ltd., Tokyo, Japan).

4.2. Strains and culture conditions

All strains used in this study are listed in Supporting Information Table S1. *E. coli* DH5α (general cloning) and *E. coli* ET12567 (pUZ8002, donor for intergeneric conjugation) were cultured in lysogeny broth (LB). *Streptomyces* sp. HK-2006-1 (producer of aldgamycins) or its derivatives, and *S. coelicolor* (host for heterologous expression) were grown on ISP-2 medium (0.4% yeast extract, 1% malt extract, 0.4% glucose, pH 7.2, 2% agar) at 28 °C for sporulation and cultured in fermentation medium (2% soluble starch, 0.5% glucose, 1.5% soybean powder, 0.5% tryptone, 0.5% yeast extract, 1.8% sea salt, pH 7.2, 0.4% $CaCO_3$) for production of secondary metabolites. *S. lividans* (host for heterologous hyperexpression) was grown on YMS medium (0.4% yeast extract, 1% malt extract, 0.4% soluble starch, pH 7.4, 2% agar, 10 mmol/L $MgCl_2$, 10 mmol/L $CaCl_2$) at 30 °C for sporulation. The transformants of *S. lividans* were cultured in 10 mL TSB medium (3% tryptic soy broth) at 30 °C for 1 day as seed broth, and then the seed broth was transferred into YEME medium (0.3% yeast extract, 0.3% malt extract, 0.5% bacto peptone, 0.5% glucose, 34% sucrose, 2% agar, pH 7.2, 5 mmol/L $MgCl_2$) for four more days, the expression of exogenous genes were induced by thiostrepton at 30 °C.

4.3. Gene inactivation and complementation

Plasmid constructions for gene inactivation or complementation are in the Supporting Information Table S2. Introduction of plasmid DNA into *Streptomyces* sp. HK-2006-1 was carried out by *E. coli*−*Streptomyces* sp. HK-2006-1 conjugation according to the procedure described previously[41]. For the gene disruption mutants,

exconjugant colonies were streaked onto ISP-2 agar medium containing apramycin (Am, 50 μg/mL) and incubated at 37 °C for 2−3 days to generate the single-crossover mutants. For in-frame deletion, the single-crossover mutant selected on ISP-2 agar medium containing 50 μg/mL Am was transferred into liquid fermentation medium without Am and incubated at 28 °C for 10−15 days. Colonies that were Am-sensitive at 37 °C were identified as double-cross mutants, and successful inactivation of target genes was confirmed by PCR with gene-specific primers (Supporting Information Table S6 and Fig. S2). For gene complementation, each gene was transferred into the mutant strain in which the corresponding gene had been deleted and expressed in *trans* under control of the *ermE** promoter. All mutant strains were fermented and analyzed for production of aldgamycins by HPLC (control: wild-type *Streptomyces* sp. HK-2006-1). All of the mutants and complemented strains used in this report are listed in Table S1.

4.4. Preparation protoplast of S. lividans

The spore suspension of *S. lividans* was inoculated in Bennett-maltose medium (0.1% yeast extract, 0.1% meat extract, 0.2% NZ amine A, 1% maltose, pH 7.2, 2% agar) at 30 °C for 5 days, approximately 2 cm^2 of the sporulated agar of *S. lividans* was cut, and incubated into 100 mL YEME medium with 1% glycine, and then incubated at 30 °C and 220 rpm (CRYSTAL IS-RDS4 incubator shaker, Addison, TX, USA) for 40 h. Mycelia were collected and washed twice with 10 mL 0.35 mol/L sucrose buffer. Cell walls were digested using 20% lysozyme in 10 mL P buffer (25 mmol/L TES, 0.368% $CaCl_2$, 0.005% KH_2PO_4, 0.3 mol/L sucrose, 9.9 mmol/L $MgCl_2$, 1.42 mmol/L K_2SO_4 and 0.198% (*v*/*v*) trace element (0.004% $ZnCl_2$, 0.02% $FeCl_2$, 0.001% $CuCl_2$, 0.001% $MnCl_2$, 0.001% $Na_2B_4O_7$ and 0.001% $(NH_4)_6Mo_7O_{24}$) mixed gently and incubated at 27 °C for 40 min (mixed every 10 min). After removed residues by filtration, protoplasts were centrifuged at 2800 rpm (TOMY CAX-371, Tokyo, Japan) for 10 min at 4 °C and washed with P buffer twice. Protoplast was resuspended in 1 mL P buffer and separated into 50 μL each, stored at −80 °C.

4.5. Protoplast transformation of S. lividans

Then the 1−5 μg plasmid DNA was added into 50 μL protoplasts solution and mixed by pipette immediately, then which was added 200 μL PEG-buffer and mixed by pipette, then spread it to R5 medium (10.3% sucrose, 1% glucose, 0.025% K_2SO_4, 1.012% $MgCl_2 \cdot 6H_2O$, 0.5% yeast extract, 0.01% casamino acid, 25 mmol/L TES, 0.3% L-proline, 0.2% (*v*/*v*) trace element (0.004% $ZnCl_2$, 0.02% $FeCl_2$, 0.001% $CuCl_2$, 0.001% $MnCl_2$, 0.001% $Na_2B_4O_7$ and 0.001% $(NH_4)_6Mo_7O_{24}$), 0.005% KH_2PO_4, 20 mmol/L $CaCl_2$, 7 mmol/L NaOH and 2% agar, pH 7.2) and incubated overnight at 30 °C. 1 mL water containing 400 μL kanamycin (50 mg/mL) was overlaid on the plate, which was then incubated at 30 °C for 3−5 days, then replicated colonies to Bennett-maltose medium (50 μg/mL kanamycin) to select kanamycin-resistant colonies. All of the transformants of *S. lividans* used in this report are listed in Table S1.

4.6. Analysis of secondary metabolites of Streptomyces sp. HK-2006-1 and its mutant strains

The mutant and wild-type strains were inoculated in 50 mL fermentation medium in a 250 mL flask incubated at 28 °C for 7−8 days with shaking (220 rpm, CRYSTAL IS-RDS4 incubator shaker). Supernatant of culture was extracted with EtOAc, and then the organic phase was evaporated to dryness, and dissolved in 2 mL methanol for HPLC and LC–MS analysis.

4.7. Feeding experiments using S. lividans and S. coelicolor transformants

The transformants of *S. lividans* were cultured in 10 mL TSB medium containing 5 μg/mL kanamycin at 30 °C and 220 rpm (CRYSTAL IS-RDS4 incubator shaker) for 2 days. 2 mL of the resulting culture broth was added into 65 mL of the YEME medium containing with 5 μg/mL kanamycin for scale-up. After cultivation for 1 day at 30 °C and 220 rpm (CRYSTAL IS-RDS4 incubator shaker), the culture medium was supplemented with 2 mg of **1** dissolved in 1 mL bacteria free water and 5 μg/mL of thiostrepton, cultivated for another 3 days. The 65 mL of culture was centrifuged at 6000×*g* for 5 min to separate the mycelium, and the supernatant was extracted with an equal volume of EtOAc. The extracts were evaporated to dryness, and dissolved in 2 mL methanol for HPLC and LC–MS analysis.

Introduction of plasmid DNA into *S. coelicolor* was carried out by *E. coli*−*S. coelicolor* conjugation according to the procedure described previously[41]. The transformants of *S. coelicolor* were inoculated into 50 mL fermentation medium in a 250 mL flask with shaking at 28 °C and 220 rpm (CRYSTAL IS-RDS4 incubator shaker) for 3 days. Then, 0.2 mL of the preculture medium was seeded into another 50 mL fresh fermentation medium incubated. After cultivation for 3 days, the culture medium was supplemented with 2 mg of **1** dissolved in 1 mL bacteria free water and cultivated for another 3−4 days. The culture supernatant was extracted with an equal volume of EtOAc and the dried extract was dissolved in 2 mL methanol for HPLC and LC–MS analysis.

4.8. Western blot analysis

To prepare the crude protein extract, the mycelium was harvested by centrifugation at 4 °C (6000×*g*, 10 min), and resuspended in lysis buffer contained 50 mmol/L Tris-HCl pH 8.0, 300 mmol/L NaCl, 10% glycerol, followed by disrupted ultrasonication on ice. The fragmentized liquid was centrifuged under 7000×*g* at 4 °C, and the supernatant was collect for further experiments.

Equal amounts of total protein (~20 μg/lane) was separated on 10% SDS-PAGE or Tricine-SDS-PAGE (FDbio Science Biotech Co., Ltd.), then transferred to PVDF membranes (Millipore, Billerica, MA, USA, #IPVH00010, pore size 0.45 μm; #10600001, pore size 0.2 μm), blocked with 5% nonfat dry milk, probed with primary antibodies overnight at 4 °C, incubated with HRP-conjugated secondary antibodies for 1 h at room temperature, and imaged by the Tanon 5200 chemiluminescence imaging system (BioTanon, Shanghai, China).

4.9. Co-immunoprecipitation analysis

After preparation of protein extracts, 20 μg of protein extract was used as input, two equal amount samples (~2 mg) were transferred into new 1.5 mL Eppendorf tubes (Hamburg, Germany) on ice respectively, and incubated with 20 μL protein A/G agarose beads (Santa Cruz Biotechnology) at 4 °C for 30 min, and then centrifuged at 9000 rpm (TOMY CAX-371) at 4 °C for 1 min. The remaining supernatant samples were added 1 μg of normal control

IgG (Santa Cruz Biotechnology) or anti-Flag mouse monoclonal antibody (TransGen Biotech) respectively, followed by incubation overnight at 4 °C. Thereafter, 50 μL pre-treated protein A/G agarose beads were added to each of two tubes, followed by incubation 4 h at 4 °C. After centrifugation, beads were washed with phosphate buffer solution (PBS) for three times, and the harvested beads were resuspended in the 40 μL 2 × loading buffer, heated at 95 °C for 5 min. The resulting samples were performed regular Western blot analysis.

4.10. *In vitro enzymatic assay using cell lysates*

In vitro enzymatic assays using cell lysate of WJ6 with 7 were carried out with a reaction mixture (100 μL) containing Tris-HCl (50 mmol/L, pH 8.0), ATP (5 mmol/L), compound **7** (0.25 mmol/L), NaCl (5 mmol/L) and $MgCl_2$ (5 mmol/L). The reaction was initiated by addition of WJ6 cell lysate (200 μg). For *in vitro* enzymatic assay using cell lysate of WJ11, or the cell lysate mixture of WJ7−WJ10, a reaction mixture (250 μL) containing Tris-HCl (50 mmol/L, pH 8.0), ATP (5 mmol/L), compound **1** (0.25 mmol/L), NaCl (5 mmol/L) and $MgCl_2$ (5 mmol/L) was prepared. The reaction was initiated by addition of cell lysate of WJ11 (180 μg), or the mixture of the cell lysates of *almUII−UV* separately expressing strains WJ7−WJ10 [180 μg (1-fold), 360 μg (2-fold), and 720 μg (4-fold)]. All these reactions were carried out at 30 °C, with shaking at 220 rpm (CRYSTAL IS-RDS4 incubator shaker). The boiled crude protein extract was used as negative control. The reaction solution was then extracted with EtOAc thrice. The organic extract was evaporated to dryness, and submitted for LC–MS analysis.

4.11. *Isolation of* **7**

Cell-free supernatant (∼1.2 L) from a culture of WJ6 fed with 50 mg compound **1** was extracted with equal volume of EtOAc thrice, the organic solvent was combined and evaporated to dryness under vacuum to afford a crude extract (856 mg). The crude extract was subjected to silica gel column chromatography (18 cm × 2.4 cm), and eluate with petroleum ether/acetone (300:100, 200:100, 200:200, *v*/*v*), yield 7 fractions. Fraction 4 (44.9 mg) was further purified by HPLC with a YMC-24 pack ODS-A column (250 mm × 4.6 mm, 5 μm; YMC Co., Ltd.), with isocratic elution by CH_3CN (28%, *v*/*v*) in water (flow rate 1 mL/min) to yield **7** (t_R: 27.8 min, 1.5 mg). **7** could only be isolated under the protection of ice bath and freeze drying.

4.12. *Structure characterization*

Compound **7**: white amorphous powder, ESI-MS (positive) *m*/*z* 792.41 $[M+H]^+$; HR-ESI-MS (positive) *m*/*z* 792.4037 $[M+H]^+$ (Calcd. for $C_{37}H_{62}NO_{17}$ 792.4018), Supporting Information Fig. S6; the NMR data, see Table S8; the NMR spectra, see Fig. S7.

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Author contributions

Qiaozhen Wang, Xiaolong Tang and Ping Dai carried out the research work. And Qiaozhen Wang and Xiaolong Tang drafted the manuscript. Chuanxi Wang and Weiyang Zhang helped to acquire the NMR data. Guodong Chen helped to analyze and interpretate the NMR data. Kui Hong isolated the strain *Streptomyces* sp. HK-2006-1. Xinsheng Yao, Hao Gao and Dan Hu designed this study and revised manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2020.07.015.

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