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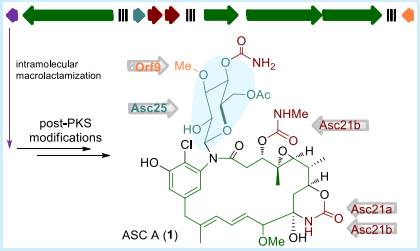
Identification of the Bacterial Maytansinoid Gene Cluster *asc* Provides Insights into the Post-PKS Modifications of Ansacarbamitocin Biosynthesis

Xiaoman Li, Xingkang Wu, and Yuemao Shen*

Key Laboratory of Chemical Biology (Ministry of Education), School of Pharmaceutical Sciences, Shandong University, No. 44 West Wenhua Road, Jinan, Shandong 250012, P. R. China

Supporting Information

ABSTRACT: A new biosynthetic gene cluster for the bacterial maytansinoids, ansacarbamitocins (ASCs), was identified in *Amycolatopsis alba* DSM 44262. The post-PKS modifications of ASCs were elucidated on the basis of bioinformatics analysis. Specific gene disruption and heterologous expression led to the isolation of seven new bacterial maytansinoids. The 3′-*O*-methyltransferase and 3-*O*-carbamyltransferase involved in bacterial maytansinoid biosynthesis were identified for the first time. The new bacterial maytansinoids **7** and **13** showed strong antitumor activities against four human cancer cell lines.



Maytansinoids are highly potent microtubule inhibitors and antitumor agents originally isolated from plants.[1,2] Despite outstanding therapeutic activity in various types of cancers,[3,4] their clinical application has been hindered by the extremely low yield from plants. Their bacterial analogues include ansamitocins and ansacarbamitocins (ASCs), namely bacterial maytansinoids. Attempts to find maytansinoid-producing microorganisms led to the isolation of ansamitocins from *Actinosynnema pretiosum* ATCC 31565,[5] *Nocardiopsis ansamitocini* EGI 80425T,[6] and maytansinoid glycosides, namely ASCs, from *Amycolatopsis* sp. CP2808.[7]

ASCs A (**1**) and A1 (**2**) were recently isolated from *Amy. alba* DSM 44262 as main metabolites.[8] The availability of genome sequence of *Amy. alba* 44262 (GenBank accession number KB913032) enabled the identification of the biosynthetic gene cluster (BGC) *asc*,[9] which showed similarity with that of the ansamitocin BGC *asm* of *Act. pretiosum* 31565.[10−15] Annotation of this putative *asc* cluster (Table S1) revealed different gene organizations from that of *asm*, especially the arrangement of these four PKS genes (Figure 1A). Sequence analysis allows all domains of the Asc PKS to be defined, suggesting the noncollinearity between the *asc* PKS genes and the biosynthetic pathway (Figure 1B). Inactivation of the *ascB* gene completely abolished the production of **1** and **2** (Figure 2Aii), confirming the involvement of *asc* BGC in the biosynthesis of ASCs in *Amy. alba* 44262.

At the left end of the *asc* gene cluster, the *asc9* gene encodes an aryl *N*-acyltransferase that shows 60% identity and 70% similarity with that of the amide synthase Asm9 encoded by the *asm* gene cluster. Knockout of the *asc9* gene completely abolished the production of ASCs (Figure 2Aiii). By contrast, knockout of the *ascL1* gene, on the left of *asc9*, did not alter the production of ASCs (Figure 2Aiv), confirming that the *asc9* gene is the left boundary of the *asc* gene cluster. On the right

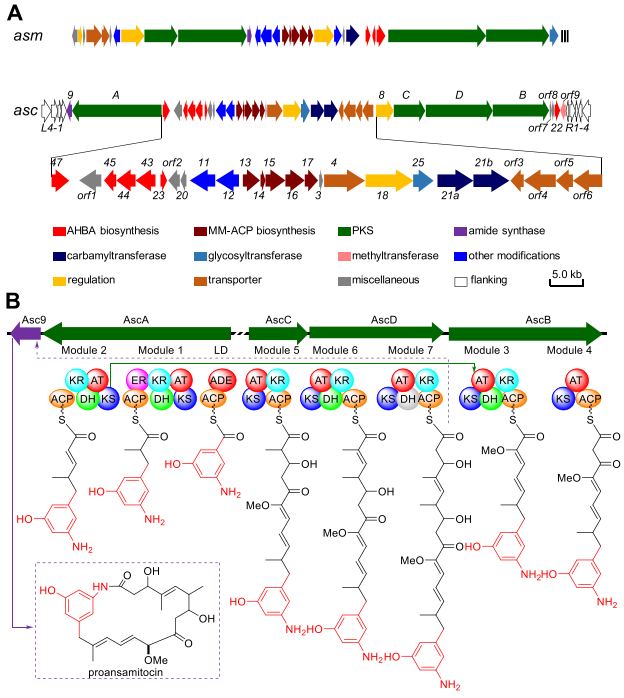


Figure 1. Biosynthesis of ansacarbamitocins. (A) BGC *asc* of the *Amy. alba* DSM 44262 strain. (B) Domain organization of the *asc* PKSs and the polyketide assembly line. ADE: adenylation domain. ACP: acyl carrier protein. KS: ketoacylsynthase. AT: acyltransferase. DH: dehydrase. ER: enoylreductase. KR: ketoreductase.

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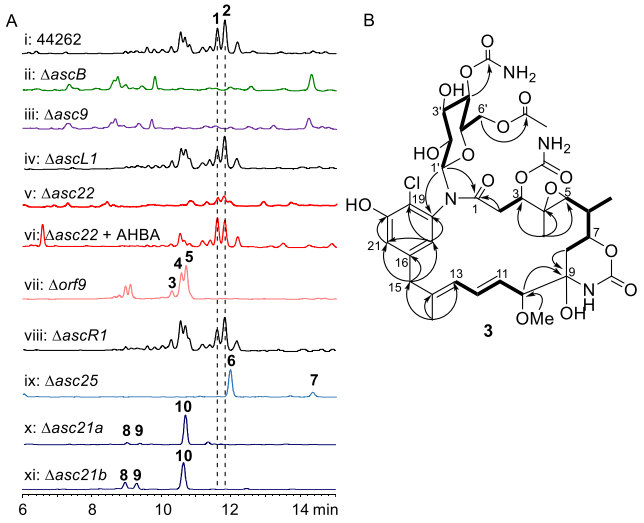

Figure 2. (A) HPLC analysis of the *Amy. alba* DSM 44262 strain (i), Δ*ascB* (ii), Δ*asc9* (iii), Δ*ascL1* (iv), Δ*asc22* (v), Δ*asc22* supplemented with AHBA (vi), Δ*orf9* (vii), Δ*ascR1* (viii), Δ*asc25* (ix), Δ*asc21a* (x), and Δ*asc21b* (xi) cultures. (B) Selected HMBC (→) and COSY (bold lines) correlations of **3**.

boundary, the 3-amino-5-hydroxybenzoic acid (AHBA) biosynthesis gene *asc22*, encoding a sugar kinase, was first inactivated. In the Δ*asc22* mutant strain, the production of ASCs was severely decreased (Figure 2Av), which was restored by supplementation with AHBA (Figure 2Avi). The fact that the Δ*asc22* mutant keeps producing ASCs though at a low level is inconsistent with the necessary role of its homologue *rifN* in AHBA biosynthesis in *Amy. mediterranei* S699.[16] Subsequently, the right boundary of the *asc* cluster was determined to be the *orf9* gene on the basis of mutagenesis and corresponding metabolite analysis (Figure 2Avii–viii). Additionally, the Δ*orf9* mutant strain produced a new compound **3** (Figure 2B), ASCs B (**4**) and B1 (**5**), confirming that *orf9* encodes the 3′-*O*-methyltransferase of bacterial maytansinoids. Overall, the *asc* cluster spans ca. 72 kb and contains an almost full complement of genes expected for the biosynthesis of ASCs, including PKS and amide synthase genes, AHBA and methoxymalonyl-ACP (MM-ACP) biosynthetic gene cassettes, regulatory and transport genes, and post-PKS modification genes.

Structural differences between ASCs from *Amy. alba* 44262 and ansamitocins from *Act. pretiosum* 31565 mainly include amide *N*-glycosylation and different 3-acyl groups. The functions of the genes in the *asc* cluster putatively involved in the amide *N*-glycosylation and 3-*O*-carbamylation were specifically investigated. Bioinformatic analysis suggests that the *asc25* gene, the homologue of *asm25*,[15] is the only candidate of the glycosyltransferase gene. The absence of the sugar moiety in compounds **6** and **7** isolated from the Δ*asc25* mutant (Figure 2Aix) demonstrated that Asc25 was involved in the amide *N*-glycosylation of ASCs. Besides, the *in vitro* assays of SUMO-tagged Asc25 demonstrated the *N*-glycosyltransferase activity (Figure S1). These results are similar to case of amide *N*-glycosylation of ansamitocins that is catalyzed by Asm25 in *Act. pretiosum* 31565.[15,17−19]

Two carbamyltransferase genes, *asc21a* and *asc21b*, were identified to be adjacent. Deletion of either *asc21a* or *asc21b* gene aborted the production of **1** and **2**. Both Δ*asc21a* and Δ*asc21b* mutants showed similar metabolite profiles (Figure 2Ax–xi) and produced two new compounds **8**/**9** and 19-chloroproansamitocin **10**, suggesting that Asc21a and Asc21b act together to carry out both the 3-*O*-carbamylation and the formation of the 7,9-oxazole ring. The possible polar effects induced by the knockout of the either *asc21a* or *asc21b* gene were ruled out on the basis of RT-PCR analysis (Figure S2).

To verify the putative 3-*O*-carbamyltransferase gene(s), *asc21a* and *asc21b* genes were individually overexpressed in HGF052+pJTU824-*asm18*,[20] a mutant of *Act. pretiosum* 31565. The main product of this mutant, *N*-desmethyl-4,5-desepoxymaytansinol **12**, was considered as a putative substrate of 3-*O*-carbamyltransferase. The overexpression of the *asc21a* gene had no influence on the metabolite profile of the HGF052+pJTU824-*asm18* strain, but the overexpression of the *asc21b* gene led to the isolation of two new compounds **13** and **14** (Figure 3Aii–iii). The isolation of **13** indicated that

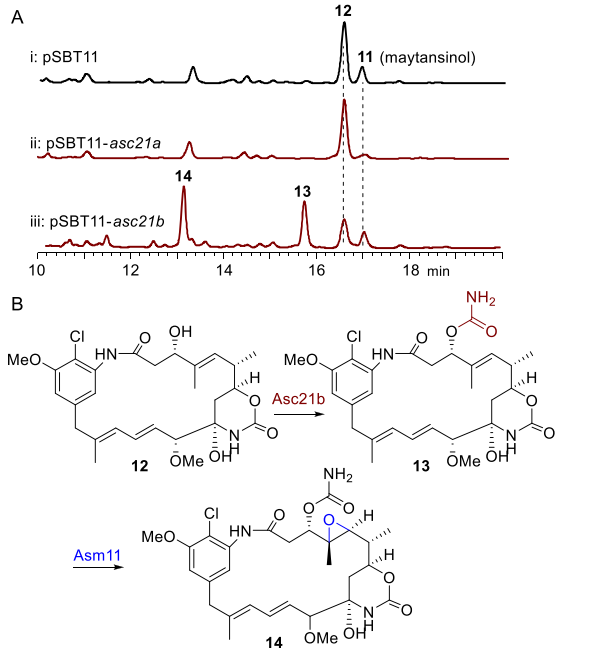

Figure 3. Heterologous expression of carbamyltransferase genes *asc21a* and *asc21b* in HGF052+pJTU824-*asm18* strain. (A) HPLC analysis of the HGF052+pJTU824-*asm18* strain carrying the empty vector pSBT11 and the carbamyltransferase genes overexpressing vectors pSBT11-*asc21a* and pSBT11-*asc21b*. (B) Proposed pathway from **12** to **14** in the HGF052+pJTU824-*asm18*+pSBT11-*asc21b* strain. The HGF052+pJTU824-*asm18* strain was constructed through overexpression of the positive transcriptional regulator gene *asm18* in the HGF052 strain (*Act. pretiosum* 31565Δ*asm19*). Ansamitocin analogs without the acyl group at C-3 produced by the HGF052+pJTU824-*asm18* strain were considered as putative substrates of the 3-*O*-carbamyltransferase.

Asc21b was capable of catalyzing 3-*O*-carbamylation of **12**, which together with *in vitro* enzymatic activity assays using MBP-tagged Asc21b established that *asc21b* encoded the 3-*O*-carbamyltransferase of bacterial maytansinoids (Figure S3). Notably, carbamyltransferases Asc21a and Asm21, highly homologous to Asc21b, yet exhibited no detectable *in vivo* enzymatic activity toward **12**. To date, Asc21b is the only 3-*O*-carbamyltransferase of bacterial maytansinoids with considerable substrate compatibility. Since carbamylations are eminent for antibiotic activity and cytotoxicity,[10] Asc21b is a potential tool for carbamyl derivatization of drug leads.

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Additionally, an endogenous epoxidase of the HGF052+pJTU824-*asm18* strain, most likely Asm11, could subsequently use **13** as the substrate to produce **14** (Figure 3B).

The antitumor activity of **6**, **7**, **13**, and **14** with the unusual carbamyl substitution at 3-OH was measured against several cancer cell lines using ansamitocin P-3 (AP-3) as a positive control (Table 1). All of the tested compounds displayed weaker cytotoxic activities compared to AP-3. In addition, both **7** and **13** showed much stronger antitumor activities than their corresponding epoxided counterparts **6** and **14**.

Table 1. *In Vitro* Antitumor Activity IC_{50} ($nmol \cdot L^{-1}$) of 6, 7, 13, 14 in Comparison to that of AP-3[a]

cell lines	6	7	13	14	AP-3
HeLa	960	2.0	6.2	2500	1.5
HCT116	450	3.6	9.3	590	0.03
MDA-MB-231	1200	10.1	12.3	260	0.6
SH-SY5Y	110	28.0	73.8	430	0.3

[a]Values shown were measured by SRB assays in parallel triplicate.

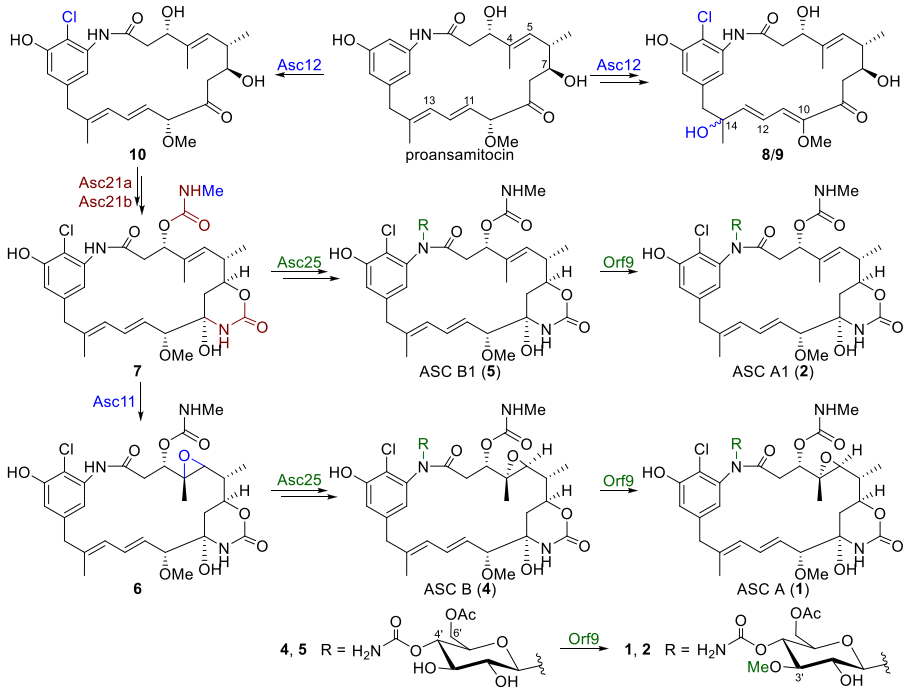
In addition to the above-mentioned 3′-*O*-methylation, *N*-glycosylation, and carbamylations, the nascent macrolactam released from the amide synthase Asc9 undergoes a series of other post-PKS modifications to form the final products ASCs, including 19-halogenation, *N*-methylation, 4,5-epoxidation, and 6′-*O*-acylation (Scheme 1). The least substituted compound isolated in this study is **10**, establishing halogenation as the first step from proansamitocin, which is presumed to be catalyzed by the putative halogenase Asc12. Then, Asc21a and Asc21b act together to catalyze the 7- and 3-*O*-carbamylation. In *Act. pretiosum* 31565, mutasynthesis of ansamitocins suggested that 7-*O*-carbamylation occurs regardless of the substituents of the aryl subunit.[17,18] The *N*-methylation of the 3-*O*-carbamyl group is proposed to occur subsequently, although the candidate gene is missing in the *asc* cluster.

The predominant accumulation of **6** in the Δ*asc25* strain suggested the epoxidation catalyzed by Asc11 precedes the amide *N*-glycosylation. Furthermore, the structural diversity of ASCs isolated from *Amy. alba* 44262[8] indicates that modifications of the glucose unit is rather promiscuous, including 3′-*O*-methylation, 4′-*O*-carbamylation, and 6′-*O*-acetylation, though the order of 4′-*O*-carbamylation and 6′-*O*-acetylation is not yet able to be defined, as the corresponding genes remain to be elucidated. The predominant accumulation of **4** and **5** in the Δ*orf9* strain establishes 3′-*O*-methylation as the terminal post-PKS step. This type of sugar-*O*-methylation was observed previously.[21−23] In *Act. pretiosum* 31565, carbamylations of the sugar moiety in *N*-ansamitocinosides are catalyzed by the dual-functional carbamyltransferase Asm21.[17,18,24,10]

Additionally, the conversion of proansamitocin to **8** and **9** involves 19-halogenation and 14-hydroxylation along with the diene shift from $\Delta^{11,13}$ to $\Delta^{10,12}$, representing a branching pathway that is exemplified in the biosynthesis of ansamitocins in *Act. pretiosum* 31565.[10,18] In the Δ*asm12*/Δ*asm2* double mutated strain of *Act. pretiosum* 31565, the 14-hydroxylation of proansamitocin occurred in the absence of the chlorine.[18] Those C-14-hydroxylated compounds were considered a result of an epoxidation reaction at C13−C14, catalyzed by a nonspecifical epoxidase.[18] Such epoxidases may exist in *Amy. alba* 44262 as well, and act prior to the halogenase Asc12 (Scheme 1).

In summary, the *asc* gene cluster of ASCs from *Amy. alba* DSM 44262 has been established and characterized on the basis of bioinformatic analysis and genetic experiments. Through *in vivo* gene inactivation, the boundaries of the *asc* cluster were defined. Subsequently, all the candidate post-PKS modification genes were identified in the *asc* cluster. Furthermore, the functions of several post-PKS genes were

Scheme 1. Post-PKS Modification Pathway of ASCs and Isolated Intermediates



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demonstrated, among which the 3-*O*-carbamyltransferase and 3′-*O*-methyltransferase genes of bacterial maytansinoids were established for the first time. Accordingly, seven new derivatives of ansamitocin were isolated and characterized. In addition, *in vitro* antitumor activity assays of 3-carbamyl analogues of ansamitocin provided novel insights into the structure−activity relationship of this family of natural products. The availability of the *asc* gene cluster and the genetic insights into the biosynthesis of bacterial maytansinoids would help to engineer more maytansinoid derivatives with improved pharmacological properties.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b01891.

Complete description of methods, additional tables, and figures, including structure elucidation and NMR data and spectra for compounds **3**−**14** (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: yshen@sdu.edu.cn.

ORCID

Yuemao Shen: 0000-0002-3881-0135

Notes

The authors declare no competing financial interest.

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