Published in final edited form as:

Chembiochem. 2011 July 25; 12(11): 1759–1766. doi:10.1002/cbic.201100062.

N-methylation of the Amide Bond by Methyltransferase Asm10 in Ansamitocin Biosynthesis

Dr. Yingying Wu^{[a],[b],†}, Qianjin Kang^{[b],†}, Dr. Guangdong Shang^[c], Peter Spiteller^[c], Brian Carroll^[c], Tin-Wein Yu^[c], Prof. Wenjin Su^[a], Dr. Linquan Bai^[b], and Prof. Heinz G. Floss^[c]

Linquan Bai: bailq@sjtu.edu.cn

^[a]State Key Laboratory of Stress Cell Biology and School of Life Sciences, Xiamen University, Xiamen 361005, China

^[b]State Key Laboratory of Microbial Metabolism, and School of Life Sciences & Biotechnology, Shanghai Jiao Tong University, Shanghai 200030, China, Fax: (+)86 21 62932418

^[c]Department of Chemistry, Box 351700, University of Washington, Seattle, WA 98195-1700, USA

Abstract

Ansamitocins are potent antitumor agents produced by *Actinosynnema pretiosum*. As deduced from their structures, an N-methylation on the amide bond is required among the various modifications. The encoded protein by *asm10* belongs to SAM-dependent methyltransferase family. Through gene inactivation and complementation, *asm10* was proved to be responsible for the N-methylation of ansamitocins. Asm10 is 33.0 kDa in size and present as monomer as determined by gel filtration. Using N-desmethyl-ansamitocin P-3 as substrate, the optimal temperature and pH were determined to be 32 °C and 10.0 respectively for Asm10 catalysis. Asm10 also showed broad substrate flexibility toward other N-desmethyl ansamycins and synthetic indolin-2-ones. Through site-directed mutagenesis, Asp154 and Leu155 of Asm10 were confirmed to be essential for its catalysis possibly thorough the binding of SAM. The characterization of this unique N-methyltransferase enriched the toolbox for engineering N-methylated derivatives from both natural and synthetic compounds, which will allow modification of known potential drugs.

Keywords

N-methylation; methyltransferase; ansamitocin; amide bond

Introduction

Ansamitocins (APs) are highly potent antitumor compounds of microbial origin, produced by *Actinosynnema pretiosum* spp. *auranticum* ATCC 31565.^[1, 2] They belong to maytansine family and play an antimitotic effect by inhibiting microtubule assembly at/or near the vinblastine-binding site.^[3–5] The severe side effects and poor efficacy of ansamitocin P-3 (Figure 1A) has prompted its use as a toxic “warhead” in immuno-toxin conjugates, and the high-affinity binding of the conjugates was observed at microtubule ends and was responsible for suppression of microtubule dynamic instability.^[6, 7] Several of these

Correspondence to: Linquan Bai, bailq@sjtu.edu.cn.

[†]These two authors contributed equally to this paper

Supporting information for this article is available on the WWW under <http://www.chembiochem.org> or from the author.

conjugates are currently in late-phase clinical trials as therapeutic agents against solid tumors, including HER2+ metastatic breast cancer, multiple myeloma and prostate cancer.^[3, 8–10] The investigations of the structure-activity relationships revealed that the ester side chain at C-3 and the free hydroxyl group at C-9 play important role in antitumor activity. Additionally, the epoxide function is thought to modify the activity, whereas *N*-methylation was found to be not essential for activity.^[11]

Deduced from their structures, at least three methylation reactions on C10, the hydroxyl group of C20, and the nitrogen of the amide bond are required for ansamitocin biosynthesis (Figure 1A). The ansamitocin biosynthetic gene clusters were cloned from a cosmid library of *A. pretiosum*, and most of the genes expected to be required for ansamitocin biosynthesis were identified (Figure 1B).^[12] Gene functions and the order of post-PKS modifications were determined by *in vivo* gene inactivation, feeding experiments with accumulated compounds, and *in vitro* enzymatic analysis with purified enzymes.^[4] Three methyltransferase genes were found in the cluster, including *asm7*, *asm10* and *asm17*. Gene *asm17* is proved to be responsible for the *O*-methylation in the biosynthesis of methoxymalonyl-ACP extender unit,^[13] whereas *asm7* was putatively assigned for the *O*-methylation at the hydroxyl group of C20.^[4] *In silico* analysis showed Asm7 belonged to the *O*-methyltransferase (*O*-MTs) family 2, and Asm17 belonged to *O*-MTs family 3. Notably, Asm10 showed very low homolog with known *N*-methyltransferases (*N*-MTs), and was found similar to some *S*-adenosylmethionine (SAM) dependent *O*-MTs. From preliminary analysis, Asm10 was suggested to be involved in the methylation of the amide bond as the final step for ansamitocin biosynthesis.

Recently, from the cultures of *A. pretiosum* on solid ISP2 medium, we had isolated a series of novel *N*-glycosylated ansamitocinosides AGP-1, AGP-2, AGP-3,^[14, 15] and accordingly identified *asm25* as the *N*-glycosyltransferase gene responsible for the macrolactam *N*-glycosylation.^[16] Therefore, both Asm10 and Asm25 are thought to modify the nitrogen of the amide bond, and catalyze the *N*-methylation and *N*-glycosylation of *N*-desmethyl-ansamitocins (PNDs), respectively.

In present work, we proved the involvement of *asm10* in the methylation of the amide bond through gene inactivation and complementation, and characterized the catalytic features of Asm10 with natural or synthetic lactams. Moreover, key amino acid residues of Asm10 possibly for SAM-binding and catalysis were identified by site-directed mutagenesis.

Results

Asm10 is involved in ansamitocin biosynthesis

To investigate whether Asm10 is involved in ansamitocin biosynthesis, gene *asm10* was inactivated through a KpnI site elimination from 674-bp to 679-bp (Figure 2A). The resulting mutant strain BLQ12 was confirmed by KpnI digestion of the *asm10* PCR products using the total genomic DNA as template. On the electrophoretic gel, BLQ12 gave the 0.90-kb expected product, whereas the wild-type showed two products, 0.20-kb and 0.70-kb (Figure 2B). LC-MS analysis revealed the production of APs was abolished in BLQ12, and instead PNDs were accumulated in BLQ12 (Figure 2C).

Further support for the involvement of Asm10 in ansamitocin biosynthesis came from complementation of the mutant BLQ12. An intact copy of *asm10* under the control of the *PermE** promoter was introduced into BLQ12 by conjugation, and the derivative was named as KW2. LC-MS analysis of the extract of KW2 showed a nearly identical chromatogram to that of the wild-type, that is, the production of APs was fully restored by expression of the cloned *asm10* in the mutant (Figure 2C).

***In vitro* N-methylation of desmethyl-ansamitocin (PND) by Asm10**

To verify that Asm10 indeed catalyzes the methylation at the macrolactam amide nitrogen of ansamitocins, the *asm10* gene was amplified by PCR from the *A. pretiosum* genome and cloned into plasmid pET28a. Asm10 was overexpressed in *E. coli* BL21(DE3)plysE as an N-terminally His₆-tagged fusion protein and purified with Ni-NTA agarose resin. The estimated molecular weight of His₆-Asm10 is 33 kDa from the SDS-PAGE (Figure 3A), which is consistent to its theoretical molecular weight of 32.97 kDa. The molecular weight of the purified Asm10 protein was further determined to be 33.0 kDa by gel filtration (Figure S4), implying its presence as a monomer.

Using PND-3 isolated from the *asm10* mutant grown on solid ISP2 medium as substrate, the enzymatic properties of Asm10 were examined. The reactions were performed with 5 μ l PND-3 (5 mM in DMSO), 3.1 μ l SAM (32 mM) in 0.1 ml basal reaction solution. The reaction product was identified as AP-3 by LC-MS compared with an authentic AP-3. Based on the reaction temperature profile from 0 to 50 °C, purified Asm10 was optimally active at 32 °C and exhibited more than 75 % of its maximal activity between 30 and 35 °C (Figure 3B). The pH profile was determined at 32 °C in 50 mM Tris-HCl buffer (pH 5.5 to 13.0), and the optimal pH is 10.0 with more than 75 % of its maximal activity exhibited in the pH range of 8.0 to 10.5 (Figure 3C). The maintenance of good activity of Asm10 under basic conditions was further proved in a linear reaction phase at pH 9, 10 or 11 (Figure 3D). Therefore, Asm10 could catalyze the methyl transfer reaction at very basic condition, indicating that it is a basophilic *N*-methyltransferase.

Initial rate of methylation was obtained with the concentration of PND-3 or SAM to be between 10 – 250 μ M. At a concentration of 100 μ M, the initial rate for Asm10 was found linearly correlated with PND-3 in the range of 10 – 60 μ M and SAM in the range of 10 – 80 μ M. For both substrates, product formation was linear for the period monitored (1 – 10 min). Assuming the enzyme preparation is 100 % active, a least-square fit to the Michaelis-Menten equation resulted in a K_m of 36.7 ± 8.98 μ M, a K_{cat} of 168.0 ± 12.95 S⁻¹, and a K_{cat}/K_m of 4.46 μ M⁻¹ S⁻¹ for PND-3. On the other hand, a K_m of 66.08 ± 8.04 μ M, a K_{cat} of 111.8 S⁻¹, and a K_{cat}/K_m of 1.69 μ M⁻¹ S⁻¹ were identified for SAM (Figure 4).

***In vitro* methylation of other ansamycins and synthetic lactams by Asm10**

To evaluate the substrate flexibility of Asm10, two ansamycins, geldanamycin and rifamycin SV, and synthetic indolin-2-one derivatives HC-14 and HC-52^[16] were used as the methyl recipients at 250 μ M in standard assays with SAM. LC-MS analysis of these assays indicated that all these four substrates were converted to the corresponding methylated products. In the LC-MS spectra, quasimolecular ion peaks were seen for geldanamycin with an m/z of 583.4 ($[M_s + Na]^+$) and for the methylated product with an m/z of 597.2 ($[M_p + Na]^+$). Similarly for rifamycin SV with an m/z of 720.4 ($[M_s + Na]^+$), the methylated product was detected with an m/z of 734.5 ($[M_p + Na]^+$). Moreover, the corresponding methylated products of HC-14 (m/z 226.2 $[M_s + Na]^+$) and HC-52 (m/z 399.4 $[M_s + H]^+$) were observed with an m/z of 240.3 ($[M_p + Na]^+$) and m/z of 413.3 ($[M_p + H]^+$), respectively (Figure 5). However, albeit all the methylated products could be detected by mass spectrometry, a HPLC peak was not found for any of these products, which made the structure elucidation through NMR nearly impossible at this stage. Except for AP-3, the structures of all the products in Figure 5 therefore were arbitrarily proposed with the methylation occurred on the nitrogen of the amide bond.

Asp154 and Leu155 are essential for the methylation by Asm10

Various studies have shown that the size of *N*-MT domains is typically 450 amino acids, while *C*-MT and *O*-MT are generally 300 amino acids.^[17] However, as a putative *N*-MT in

ansamitocin biosynthetic gene cluster, Asm10 contains only 295 amino acids. By further putative conserved domain prediction and phylogenetic analysis of the protein sequences, Asm10 was categorized to LCM (leucine carboxyl MTs) superfamily in *O*-MTs family (Figure S1). *In silico* analysis indicated that Asm10 shared the highest similarity to ML2640c from *Mycobacterium leprae*,^[18] and two amino acid residues, Asp154 and Leu155, may be the SAM binding sites in Asm10 (<http://swissmodel.expasy.org/>). Multiple alignment of the LCM methyltransferase superfamily showed these two amino acids are conserved in all the sequences (Figure S3).

The involvements of Asp154 and Leu155 in the methylation of PND-3 were studied by site-directed mutagenesis. These mutated proteins were expressed and purified (Figure S5), and their specific activities were measured using PND-3 as the substrate. Whereas the L155I mutated protein displayed a 40 % reduced methylation activity, mutated proteins (L155A, D154E or D154A) almost lost their methylation activity (Figure 6A). L155A mutated protein only retained 3.52 % methylation activity, and D154E mutated protein kept 0.82 % activity as calculated from the HPLC peaks. Further kinetic analysis of L155I mutated protein showed a K_m of $45.2 \pm 5.34 \mu\text{M}$, a K_{cat} of 111.3 S^{-1} , and a K_{cat}/K_m of $2.46 \mu\text{M}^{-1} \text{ S}^{-1}$ for PND-3, indicating a much reduced catalytic activity than the wild-type Asm10 (Figure 6B). Therefore, these two residues, Asp154 and Leu155, are essential for methylation by Asm10 possibly through SAM binding.

Discussion

The biosynthesis of ansamitocins undergoes various post-PKS modifications including the *N*-methylation, which are essential for their bioactivities.^[4] Previously *N*-glycosylation of the amide bond, catalyzed by Asm25, was proven as the last modification step among post-PKS modifications.^[16] In this work, gene deletion experiment showed that the *asm10* mutant accumulated the same products PNDs. Furthermore, *in vitro* experiments showed that Asm10 and Asm25 used the same substrates, PNDs, but produced different products, ansamitocins and ansamitocinosides, respectively.^[16] Therefore, methylation of the same amide nitrogen by Asm10 is an alternative reaction to the glycosylation catalyzed by the *N*-glycosyltransferase Asm25. During fermentation process in liquid or solid medium, *A. pretiosum* produces more ansamitocins with *N*-methyl group than ansamitocinoside with *N*-glucosyl group, indicating that Asm10 could utilize the PNDs as substrate much effectively than Asm25 does (Y Li and L Bai, unpublished).

Among the three MTs genes which were putatively assigned for the biosynthesis of ansamitocins, *asm7* and *asm17* are functionally assigned as *O*-MTs, whereas Asm10 was found similar to some *S*-adenosylmethionine (SAM) dependent *O*-MTs but to catalyze the *N*-methylation. More than 150 different members have been categorized by the Enzyme Commission, and the numbers are still rapidly increasing.^[19] However, the SAM-dependent MTs share little sequence identity, and the residues that bind the common cofactor SAM are poorly conserved.

It has been shown that MTs can utilize various amino acid residues as a general base, including tyrosine, lysine, arginine, glutamate, and histidine.^[20–23] The starting point for the SAM-dependent catalytic reaction is the formation of a Michaelis complex, in which both SAM and the methyl acceptor substrate are bound to the enzyme.^[24] For the LCM family including Asm10, the adenine base of SAM might be stabilized by hydrogen-bonding interactions with side chain of Asp154 and the nitrogen of Leu155 (Figure S3).^[18] Our results showed that the mutation of these two residues drastically reduced or abolished the methylation activity of Asm10, suggesting Asp154 to be the general base for the methylation reaction.

Usually SAM-dependent MTs don't have additional domains at the core fold, but all of them have additional domains at the N terminus.^[18] However, additional domains were found both at the N terminus and the core fold in Asm10 (Figure S2). The N-terminally additional α 1- α 3 domains might contain some residues attributed to the SAM-binding by making electrostatic interactions. On the other hand, the presence of additional α 4- α 5 domains (between β 5 and α E) and α 6- α 7 (between β 6 and β 7) could probably enlarge the cavity for substrate binding, indicating flexibility toward small or medium-size substrates. In our study, Asm10 did show substrate flexibility toward small substrates (HC-14 and HC-52) and large substrates including rifamycin, geldanamycin and desmethyl-ansamitocin (Figure 5).

Conclusion

We have showed that Asm10 is responsible for the *N*-methylation of the amide bond in ansamitocin biosynthesis, and identified it as a SAM-dependent methyltransferase in LCM family. Asm10 is probably a basophilic *N*-methyltransferase maintaining good activity at basic condition. For the first time, Asp154 and Leu155 were experimentally proved to be required for the methylation activity in LCM family.

Experimental Section

Bacterial strains and plasmids

Strains and plasmids used in this study are listed in Table 1.

Sequence analysis

DNA sequencing was performed at Shanghai Invitrogen Inc. (Shanghai, China), and multiple sequence alignment was performed with BioEdit 7.0. Phylogenetic relationship was analyzed by MEGA 3.0 software. Open-reading frames (ORFs) and ribosome binding sites (RBS) were predicted by using Frameplot 3.0.^[25] Similarity comparisons of nucleotide or amino acid sequences against public database were done by using the BLAST program on the NCBI website (<http://ncbi.nlm.nih.gov/blast>).^[26]

Inactivation of methyltransferase gene *asm10*^[16] and complementation of *asm10* mutant BLQ12 with cloned *asm10*

The *asm10* gene from the ansamitocin producer *A. pretiosum* was amplified using the following primers: asm10-F (5'-AAAGGATCCCATATGAGCCTCCCAGCGGA-3') with engineered BamHI site underlined and NdeI site in italic, and asm10-R (5'-AAAGAATTCTACCCCAGCTCGGCCTCGAACAG-3') with engineered EcoRI site underlined. PCR conditions were as follows: 5 min at 94 °C, 5 cycles of 30 s at 94 °C, 30 s at 50 °C, 45 s at 68 °C, 25 cycles of 30 s at 94 °C, 30 s at 65 °C, 45 s at 68 °C, and 5 min at 68 °C with 1U KOD plus (TOYOBO Inc.).

The PCR product was digested by NdeI and EcoRI, and cloned into plasmid pIB139 to obtain pJTU3237, which was introduced from *E. coli* into the *asm10* mutant BLQ12 through conjugation. Confirmation was carried out through plasmid rescue from the possible exconjugants. An exconjugant carrying the correct plasmid was named as KW2, and the control exconjugant carrying only the pIB139 vector was named as KW1. The wild-type strain, KW1 and KW2 were characterized by fermentation and LC-MS analysis.

Construction of the Asm10 expression plasmid and site-directed mutagenesis of Asm10

The above PCR product was digested with BamHI and EcoRI, and ligated with pET28a (Invitrogen Inc.) digested with the same enzymes to generate pJTU3239. The sequence of the cloned *asm10* was confirmed by sequencing.

Following the manufacture's instruction of the QuickChange site-directed mutagenesis kit (Stratagene Inc.), pJTU3239 was used as a template for site-directed mutagenesis to construct four mutants with individual amino acid alteration (D154A, D154E, L155I, L155A). Primers used for site-directed mutagenesis are shown in Table S1. A 50 μ l reaction mixture contained 50 ng pJTU3239, 25 ng forward primer, 25 ng reverse primer, 0.8 mM dNTPs, 5 % (v/v) DMSO, 4 mM $MgCl_2$, and 2.5 U Stratagene PFU Ultra. The amplification program used was 95 °C for 1 min, 30 cycles of 95 °C for 1 min, annealing temperature (see Table S1) for 1 min, and 68 °C for 6.5 min, and a final step at 68 °C for 7 min. The amplified products were digested with 10 U of DpnI for 1 hr at 37 °C and transformed into *E. coli* DH10B competent cells. Plasmids were analyzed by sequencing to ensure the desired mutations obtained.

Expression and purification of Asm10 and its mutated proteins

pJTU3239 and mutated plasmids were transformed into *E. coli* BL21(DE3)pLysE, and transformants were grown as seed culture in LB liquid medium supplemented with kanamycin (50 μ g/ml) and chloramphenicol (25 μ g/ml) at 37 °C and 220 rpm for 12 hrs. The seed culture was then diluted at 1:50 (v/v) with fresh LBBS medium (1 % tryptone, 0.5 % yeast extract, 1 % NaCl, 18.59 % sorbitol, 0.03 % betaine, pH 7.2, w/v). The diluted cultures were grown to $OD_{600} = 0.4\sim0.6$, then induced with 0.2~1.0 mM IPTG, and incubated further for 20 hrs at 20 °C, 220 rpm.

The cells from 200 ml culture were harvested by centrifugation (5,000 rpm, 4 °C, 15 min), resuspended in 20 ml of buffer A (50 mM Tris-HCl [pH 8.0], 300 mM NaCl), and lysed by FRENCH Press (Thermo Inc.) with two passages. After centrifugation (12,000 rpm, 4 °C, 30 min), the protein supernatant was loaded onto 2.5 ml (bed volume) of Ni-NTA agarose resin (QIAGEN Inc.) equilibrated with lysis buffer. The column was washed with 25 ml of binding buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 5 mM imidazole) and 25 ml of wash buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 60 mM imidazole). N-terminally His₆-tagged Asm10 (His₆-Asm10) was then eluted with 15 ml buffer A supplemented with 200 mM imidazole and concentrated by ultrafiltration with concomitant buffer exchange to 50 mM Tris (pH 8.0). The supernatant (about 200 μ l) was collected and used for subsequent enzymatic assays. Protein purity and molecular mass were examined by SDS-PAGE followed by Coomassie Brilliant Blue staining of the gel. For SDS-PAGE, the protein solution was mixed with Laemmli sample buffer containing β -mercaptoethanol (5 %) and incubated at 100 °C for 10 min. The protein concentration was measured to be 100 μ M against BSA concentration standards.

Gel filtration

The molecular mass of Asm10 was determined by gel filtration with a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) equilibrated with 50 mM Tris-HCl, pH 8.0 buffer using an ÄKTA fast protein liquid chromatography system (FPLC). Blue Dextran 2000 was applied to determine the void volume (V_0) and check the column packing first, then the column was calibrated with the following gel filtration molecular weight markers (Amersham Pharmacia Biotech, Uppsala, Sweden): aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa). The molecular weight of Asm10 was calculated by the calibration curve.

Characterization of Asm10

The enzymatic reaction mixture contained 1mM SAM (New England Biolabs Inc.), 0.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 250 μ M PND-3, 2 μ M Asm10 and 50 mM Tris-HCl (pH 7.0) in a total volume of 0.1 ml. The temperature optimum for Asm10 activity was examined by incubating the mixture for 1 hr at 0 °C, 10 °C, 20 °C, 30 °C, 32 °C, 35 °C, 37

°C, 40 °C, 42 °C, 45 °C, or 50 °C. The pH optimum was determined by incubating the mixture for 1 hr at 32 °C in 50 mM Tris-HCl buffer at pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, 11.5, 12.0, 12.5, or 13.0. To detect the consumption of substrate at fixed pH, reactions were stopped at 0, 5, 10, 15, 20, 30, 45, 60, 90, or 120 min at pH 9, 10, 11 respectively. All assays were performed in triplicate, and reactions with boiled Asm10 were used as the negative control. The reaction mixtures were extracted with 50 μ l *n*-butanol, and the organic phase was collected by centrifugation and used directly for LC-MS analysis.

Kinetic analysis of Asm10

Enzymatic kinetics was determined at 32 °C in 0.1 ml basal reaction solution (1 mM SAM, 0.5 mM MgCl₂, 1 mM DTT, 2 μ M Asm10 and 50 mM Tris-HCl [pH 10.0]) with various concentrations of PND-3. To determine the initial reaction rate, 10 min incubation time was chosen so that there was less than 30 % conversion of the substrate PND-3 to product. The initial reaction rate profile was constructed with PND-3 at 10, 20, 40, 60, 80, 100, 200, and 250 μ M. In addition, the enzymatic kinetics of Asm10 was further determined with SAM at 10, 20, 40, 60, 80, 100, 200, and 250 μ M in 0.1 ml basal reaction solution (1 mM PND-3, 0.5 mM MgCl₂, 1 mM DTT, 2 μ M Asm10 and 50 mM Tris-HCl [pH 10.0]). Each reaction mixture was extracted with *n*-butanol (50 μ l), and the *n*-butanol phase was collected by centrifugation and used directly for LC-MS analysis. All reactions were performed in triplicate. The software Prism 5 was used for the calculation of K_m and V_{max} .

Preparation and identification of PND-3, AP-3 and other substrates

Fermentation was carried out with 3 L solid ISP2 medium for 8 days at 30 °C. The collected culture was extracted three times with ethyl acetate-methanol-acetate (80:15:5, v/v/v) to afford a 10.0 g crude extract. The extract was fractionated with Amberlite XAD-16 eluted with a gradient of 50, 70, and 100 % ethanol. The fraction eluted with 70 % ethanol (150 mg) was further separated as previously described^[4] to afford PND-3 (15 mg). Similarly, AP-3 (10 mg) was isolated from the fermentation products of the strain *A. pretiosum* ATCC 31565. PND-3 was obtained as a white powder, C₃₁H₄₁ClN₂O₉, ESI-MS m/z : 621.3 [M + H]⁺, 643.3 [M + Na]⁺. AP-3 was obtained as a light yellow amorphous powder, C₃₂H₄₃ClN₂O₉, ESI-MS m/z : 635.5 [M + H]⁺, 657.3 [M + Na]⁺.

Geldanamycin was purchased from Beijing Rapidbio Co. Ltd., China. Rifamycin SV was bought from Shanghai WeeBeyond Scientific & Trade Co., Ltd., China. HC14 and HC52 were synthetic lactams.^[16]

Substrate specificity of Asm10

Rifamycin SV, geldanamycin and two synthetic indolin-2-one compounds HC-14 and HC-52 were tested as substrates for His₆-Asm10 in 0.1 ml reaction solution, containing 1 mM SAM, 0.5 mM MgCl₂, 1 mM DTT, 250 μ M substrate, 2 μ M Asm10 and 50 mM Tris-HCl (pH 10.0). Control reactions were set up with boiled His₆-Asm10 added. All reactions were incubated at 32 °C for 1 hr, stopped by adding 50 μ l *n*-butanol and centrifuged for 5 min at 12,000 rpm. Subsequent analysis of the supernatant was performed using LC-MS. Each assay was carried out in triplicate.

Liquid chromatography-mass spectrometry (LC-MS) analysis

LC-MS analysis was carried out using an Agilent 1100 HPLC system (Agilent Technologies). The samples were separated on an Agilent RP18 column (2.1 mm×150 mm, pore size 3.5 μ m) by using gradient elution (0–10 min 10–55 % buffer B, 10–20 min 55–75 % buffer B, 20–35 min 75–95 % buffer B, 35–45 min 95 % buffer B, 45–50 min 95–10 %

buffer B, buffer A: H₂O with 0.5 % formic acid; buffer B: methanol) with a flow rate of 0.1 ml/min. UV detection was performed at 254 nm. HPLC quantitative analysis was performed on a Waters series HPLC 2690 instrument with Waters 996 photodiode array detector and the data was analyzed with a Waters Millennium Chromatography Manager. The samples were loaded onto a Waters Xterra RP18 column (3.9 mm×150 mm, pore size 5 μm) and eluted with isocratic elution (65:35 methanol/H₂O, v/v) at a flow-rate of 200 μl/min. AP-3 was used as an external standard. The calibration curve was constructed based on peak areas of the calibrated standards within the range of 0.1~100 μg/μl for AP-3. Concentrations of all analytes were calculated from their peak areas against the calibration curve.

Acknowledgments

We would like to thank Yuemao Shen for critical reading of the manuscript and helpful discussion. This work received financial supports from the National Science Foundation of China, the Ministry of Science and Technology (973 and 863 Programs), the Ministry of Education, the Shanghai Municipal Council of Science and Technology. Work at University of Washington was supported by NIH grant CA76461.

References

1. Higashide E, Asai M, Ootsu K, Tanida S, Kozai Y, Hasegawa T, Kishi T, Sugino Y, Yoneda M. *Nature*. 1977; 270:721–722. [PubMed: 593392]
2. Tanida S, Hasegawa T, Hatano K, Higashide E, Yoneda M. *J Antibiot (Tokyo)*. 1980; 33:192–198. [PubMed: 7380728]
3. Cassady JM, Chan KK, Floss HG, Leistner E. *Chem Pharm Bull (Tokyo)*. 2004; 52:1–26. [PubMed: 14709862]
4. Spiteller P, Bai L, Shang G, Carroll BJ, Yu TW, Floss HG. *J Am Chem Soc*. 2003; 125:14236–14237. [PubMed: 14624546]
5. Xu J, Mahmud T, Floss HG. *Arch Biochem Biophys*. 2003; 411:277–288. [PubMed: 12623077]
6. Liu C, Tadayoni BM, Bourret LA, Mattocks KM, Derr SM, Widdison WC, Kedersha NL, Ariniello PD, Goldmacher VS, Lambert JM, Blattler WA, Chari RV. *Proc Natl Acad Sci USA*. 1996; 93:8618–8623. [PubMed: 8710920]
7. Hu Y, Floss HG. *J Am Chem Soc*. 2004; 126:3837–3844. [PubMed: 15038738]
8. Ikeda H, Hideshima T, Fulciniti M, Lutz RJ, Yasui H, Okawa Y, Kiziltepe T, Vallet S, Pozzi S, Santo L, Perrone G, Tai YT, Cirstea D, Raje NS, Uherek C, Dalken B, Aigner S, Osterroth F, Munshi N, Richardson P, Anderson KC. *Clin Cancer Res*. 2009; 15:4028–4037. [PubMed: 19509164]
9. Floss HG. *J Nat Prod*. 2006; 69:158–169. [PubMed: 16441092]
10. Liu Z, Floss HG, Cassady JM, Chan KK. *J Mass Spectrom*. 2005; 40:389–399. [PubMed: 15674857]
11. Liu Z, Floss HG, Cassady JM, Xiao J, Chan KK. *J Pharm Biomed Anal*. 2004; 36:815–821. [PubMed: 15533675]
12. Yu TW, Bai L, Clade D, Hoffmann D, Toelzer S, Trinh KQ, Xu J, Moss SJ, Leistner E, Floss HG. *Proc Natl Acad Sci USA*. 2002; 99:7968–7973. [PubMed: 12060743]
13. Kato Y, Bai L, Xue Q, Revill WP, Yu TW, Floss HG. *J Am Chem Soc*. 2002; 124:5268–5269. [PubMed: 11996558]
14. Lu C, Bai L, Shen Y. *J Antibiot (Tokyo)*. 2004; 57:348–350. [PubMed: 15303496]
15. Ma J, Zhao PJ, Shen YM. *Arch Pharm Res*. 2007; 30:670–673. [PubMed: 17679541]
16. Zhao P, Bai L, Ma J, Zeng Y, Li L, Zhang Y, Lu C, Dai H, Wu Z, Li Y, Wu X, Chen G, Hao X, Shen Y, Deng Z, Floss HG. *Chem Biol*. 2008; 15:863–874. [PubMed: 18721757]
17. Ansari MZ, Sharma J, Gokhale RS, Mohanty D. *BMC Bioinformatics*. 2008; 9:454. [PubMed: 18950525]
18. Grana M, Haouz A, Buschiazzo A, Miras I, Wehenkel A, Bondet V, Shepard W, Schaeffer F, Cole ST, Alzari PM. *Protein Sci*. 2007; 16:1896–1904. [PubMed: 17660248]

19. Lee BW, Sun HG, Zang T, Kim BJ, Alfaro JF, Zhou ZS. J Am Chem Soc. 2010; 132:3642–3643. [PubMed: 20196537]
20. Guo HB, Guo H. Proc Natl Acad Sci USA. 2007; 104:8797–8802. [PubMed: 17517655]
21. Frank S, Deery E, Brindley AA, Leech HK, Lawrence A, Heathcote P, Schubert HL, Brocklehurst K, Rigby SE, Warren MJ, Pickersgill RW. J Biol Chem. 2007; 282:23957–23969. [PubMed: 17567575]
22. Jois PS, Madhu N, Rao DN. Biochem J. 2008; 410:543–553. [PubMed: 17995451]
23. Youngblood B, Shieh FK, Buller F, Bullock T, Reich NO. Biochemistry. 2007; 46:8766–8775. [PubMed: 17616174]
24. Shi R, Lamb SS, Zakeri B, Proteau A, Cui Q, Sulea T, Matte A, Wright GD, Cygler M. Chem Biol. 2009; 16:401–410. [PubMed: 19389626]
25. Ishikawa J, Hotta K. FEMS Microbiol Lett. 1999; 174:251–253. [PubMed: 10339816]
26. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Nucleic Acids Res. 1997; 25:3389–3402. [PubMed: 9254694]
27. Paget MS, Chamberlin L, Atrih A, Foster SJ, Buttner MJ. J Bacteriol. 1999; 181:204–211. [PubMed: 9864331]
28. Wilkinson CJ, Hughes-Thomas ZA, Martin CJ, Bohm I, Mironenko T, Deacon M, Wheatcroft M, Wirtz G, Staunton J, Leadlay PF. J Mol Microbiol Biotechnol. 2002; 4:417–426. [PubMed: 12125822]

Figure 1.

N-desmethylansamitocins, ansamitocins and the *asm* biosynthetic gene cluster. A) Structures of *N*-desmethylansamitocins (PNDs) and ansamitocins (APs) with *N*-methyl moiety highlighted. B) The ansamitocin biosynthetic gene cluster. The *N*-methyltransferase gene *asm10* is indicated by a circle in Cluster I. AHBA, 3-amino-5-hydroxybenzoic acid; MM-ACP, methoxy-malonyl-ACP.

Figure 2. Inactivation of *asm10* and complementation of the mutant BLQ12. A) Schematic construction of the *asm10* mutant. The shuttle plasmid pJTU654 was sequentially treated by digestion with KpnI, erased with Klenow, and religated with T4 ligase. K, KpnI; B, BamHI. B) Gel electrophoresis of the PCR amplified fragments. The left gel showed the PCR products using total DNA of the wild-type or the mutant BLQ12 as templates. The right gel showed the two PCR products digested with KpnI. Whereas the PCR product of the wild-type could be cleaved into 670-bp and 210-bp fragments, the PCR product of BLQ12 kept the original size of 880 bp after KpnI digestion. C) HPLC profiles of the wild-type, *asm10* mutant BLQ12, pIB139 vector-complemented strain KW1, and *asm10*-complemented strain KW2 cultured on solid ISP2 medium. AP, ansamitocin; PND, *N*-desmethyl-ansamitocin; AGP, ansamitocinoside.

Figure 3.

Catalytic properties of Asm10. A) A representative Coomassie Brilliant Blue-stained SDS-PAGE gel loaded with molecular weight markers (lane 1), total proteins (lane 2), soluble proteins (lane 3), and purified Asm10 (lane 4). B) Temperature optimum for the *N*-methylation of PND-3 catalyzed by Asm10. C) pH optimum for the *N*-methylation of PND-3 catalyzed by Asm10. Asm10 was assayed in 50 mM Tris-HCl buffer ranging from pH 5.5 to 13.0. D) pH experiment (pH 9, 10, 11) in the linear reaction phase.

Figure 4. Kinetic characterization of Asm10. A) The initial reaction rate constructed with different concentration of PND-3. B) The initial reaction rate constructed with different concentration of SAM.

Figure 5.
 Substrate specificity of Asm10.

Figure 6.

In vitro N-methylation of PNDs by Asm10 and its mutated proteins. A) HPLC analysis of the methylation of PND-3 by Asm10 and its mutated proteins. B) Comparison of the kinetics of Asm10 and the L155I mutated protein.

Table 1

Strains and plasmids

Strains or Plasmids	Properties or products	Reference or source
Strains		
<i>Actinosynnema pretiosum</i>	wild-type, ansamitocins, ansamitocinosides	[1]
BLQ12	<i>asm10</i> with KpnI site eliminated, <i>N</i> -desmethyl-ansamitocins	[16]
KW1	BLQ12 complemented with vector pIB139	This study
KW2	BLQ12 complemented with <i>asm10</i> cloned on pIB139	This study
<i>E. coli</i>		
DH10B	F ⁻ <i>mcrA</i> Δ(<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) Φ80d <i>lacZ</i> ΔM15Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>ara</i> Δ139D(<i>ara</i> , <i>leu</i>)7697 <i>galU</i> <i>galK</i> λ ⁻ <i>rpsL</i> <i>nupG</i>	Invitrogen
BL21(DE3) plysE	F ⁻ <i>ompT</i> <i>hsdS_B</i> (<i>r_B⁻</i> <i>m_B⁻</i>) <i>gal dcm</i> (DE3) plysE (Cm ^R)	Invitrogen
ET12567 (pUZ8002)	<i>dam dcm hsdS</i> pUZ8002	[27]
XL10-Gold®	Tet ^r Δ (<i>mcrA</i>)183Δ (<i>mcrCB</i> - <i>hsdSMR</i> - <i>mrr</i>) 173 <i>endA1</i> <i>supE44</i> <i>thi-1</i> <i>recA1</i> <i>gyrA96</i> <i>relA1</i> <i>lac</i> Hte [F' <i>proAB</i> <i>lac</i> Δ <i>Z</i> ΔM15 Tn10 (Tet ^r) Amy Cam ^r]	Stratagene
Plasmids		
3C11	Cosmid containing part of the ansamitocin biosynthetic gene cluster	[12]
pBluescript KS(+)	<i>bla</i> <i>lacZ</i> orif1	Novagen
pET28a	Kan ^R P _{T7}	Novagen
pIB139	pSET152 derived vector with <i>PermE</i> * promoter	[28]
pJTU3237	<i>asm10</i> cloned into pIB139 for complementation	This study
pJTU3239	<i>asm10</i> expression plasmid derived from pET28a	This study