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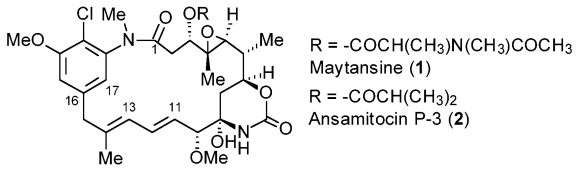
The Post-Polyketide Synthase Modification Steps in the Biosynthesis of the Antitumor Agent Ansamitocin by *Actinosynnema pretiosum*

Peter Spiteller, Linquan Bai, Guangdong Shang, Brian J. Carroll, Tin-Wein Yu,* and Heinz G. Floss*

Department of Chemistry, Box 351700, University of Washington, Seattle, Washington 98195-1700

Received August 27, 2003; E-mail: yu@u.washington.edu; floss@chem.washington.edu

The plant-derived maytansinoids (e.g., maytansine, **1**)[1] and their microbial counterparts, the ansamitocins (e.g., ansamitocin P-3 [AP-3], **2**) from *Actinosynnema pretiosum*,[2] are anticancer agents of remarkable potency, which are of current interest as "warheads" for targeted delivery by tumor-specific antibodies.[3]



Their biosynthesis, as revealed by isotopic tracer experiments[4] and by cloning and analysis of the ansamitocin (*asm*) gene cluster,[5] involves the assembly of an initial macrocylic polyketide, the hypothetical proansamitocin (**3**, Scheme 1). Proansamitocin then undergoes a series of post-PKS modifications to introduce a chlorine, two methyl groups, a cyclic carbamate, an ester side chain, and an epoxide function, to give **2**. Genes in the *asm* cluster potentially involved in these transformations have been identified on the basis of sequence homologies.[5]

To resolve which *asm* genes are responsible for particular post-PKS processing steps, in which order the reactions occur, and to isolate and elucidate the structures of intermediates, we individually inactivated each candidate gene in *A. pretiosum* ATCC 31565. The mutated genes with either a large internal deletion (*asm7*, *asm10*, *asm11*, *asm19*, *asm21*) or insertion of an apramycin resistance gene (*asm12*, *asm30*) were introduced into *A. pretiosum* and replaced the wild-type genes by sequential homologous recombinations.[5,6] The mutants were analyzed by ESIMS/MS for the production, or lack thereof, of **2** and of any novel maytansinoids. The most prominent metabolite accumulated in each mutant was isolated and characterized by MS and NMR spectroscopy (see Supporting Information).

The results, summarized in Table 1, demonstrate that many of the post-PKS gene products are not highly substrate specific. This is most evident in the mutant resulting from the *asm12* inactivation, which accumulates a spectrum of deschloro compounds that have undergone further modifications up to deschloro-AP-3. Every subsequent modification reaction can take place in the absence of the chlorine, albeit less efficiently than in its presence, leading to the accumulation of all of these compounds in the mutant, although their halogenated analogues, other than **2** and its *N*-demethyl derivative, have not been detected in the wild-type. Likewise, the mutant lacking a functional Asm21 accumulates several compounds all without the carbamoyl group, confirming that *asm21* encodes a carbamoyltransferase. As reported previously,[6] *asm19* encodes an acyltransferase which delivers the acyl group from the corresponding acyl-CoA to 3-*O* of, surprisingly, not maytansinol, but its *N*-demethyl-desepoxy derivative **6**, the compound accumulated by the Δ*asm19* mutant. Acylation, therefore, is not the terminal step in the biosynthesis. Of the two methyltransferase genes, *asm7* was shown to encode the 20-*O*-methyltransferase and *asm10* was shown to encode the *N*-methyltransferase, respectively, based on the detection of **10** as the main product in the Δ*asm7* and **11** as the main product in the Δ*asm10* mutant. As concerns the two candidate genes to encode the epoxidase, inactivation of *asm11* resulted in the accumulation of *N*-demethyl-desepoxyansamitocin P-3 (**7**), whereas the *asm30*-inactivated mutant produced **2** at wild-type levels. This identifies Asm11 as the epoxidase, whereas the function of Asm30 remains unknown.

The least substituted compounds accumulated by the *asm12* and *asm21* mutants, although not the predominant ones, are **3** and its 19-chloro derivative **4**, respectively. This establishes halogenation and carbamoylation as the first and the second step, respectively, in the post-PKS processing reactions of **2**. Likewise, the predominant accumulation of **11** in the Δ*asm10* and of **7** in the Δ*asm11* mutant, respectively, suggests *N*-methylation as the last and epoxidation as the penultimate step in the biosynthesis. The order of 20-*O*-methylation and 3-*O*-acylation is more difficult to define. The sole accumulation of **6** in the Δ*asm19* mutant suggests that *O*-methylation precedes acylation. However, the presence of unmethylated **8** among the compounds resulting from the *asm12* inactivation indicates that, at least in the absence of the chlorine, acylation can also occur prior to methylation. Nevertheless, the predominant pathway appears to proceed by *O*-methylation as the third step, followed by acylation as step 4, as shown in Scheme 1.

Only a small amount of proansamitocin is detected in the *asm12* mutant. Because the structure of this compound was of particular interest, we generated an *asm12*/Δ*asm21* double mutant. As expected, this mutant produced only **3**. The structure elucidation confirmed that, as predicted from our earlier results,[7] C-10 of **3** carries a methoxy and not a hydroxy group, and it revealed that the double bonds are shifted to the Δ11,13 positions, as in the final product, **2**.[8] Unless there is an unknown post-PKS isomerization step prior to halogenation, for which there is no plausible candidate gene, the shift of the double bonds must occur during polyketide assembly on the *asm* PKS.

The compounds accumulated by the various mutants described cannot be accommodated on a single pathway from **3** to **2**. Their diversity indicates a considerable degree of promiscuity of many, if not most, of the post-PKS modification enzymes. To further establish whether some of the compounds are dead-end shunt metabolites or whether the conversion of **3** to **2** represents a metabolic grid of multiple parallel pathways, we carried out complementation experiments in the *A. pretiosum* mutant HGF051.[5] This mutant cannot produce **2** due to a large deletion in the *asmB* gene, but has a full complement of post-PKS processing genes. Restoration of the production of **2** by supplementation with individual compounds isolated from various mutants revealed which

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Scheme 1. Post-PKS Processing Steps in the Biosynthesis of Ansamitocin P-3

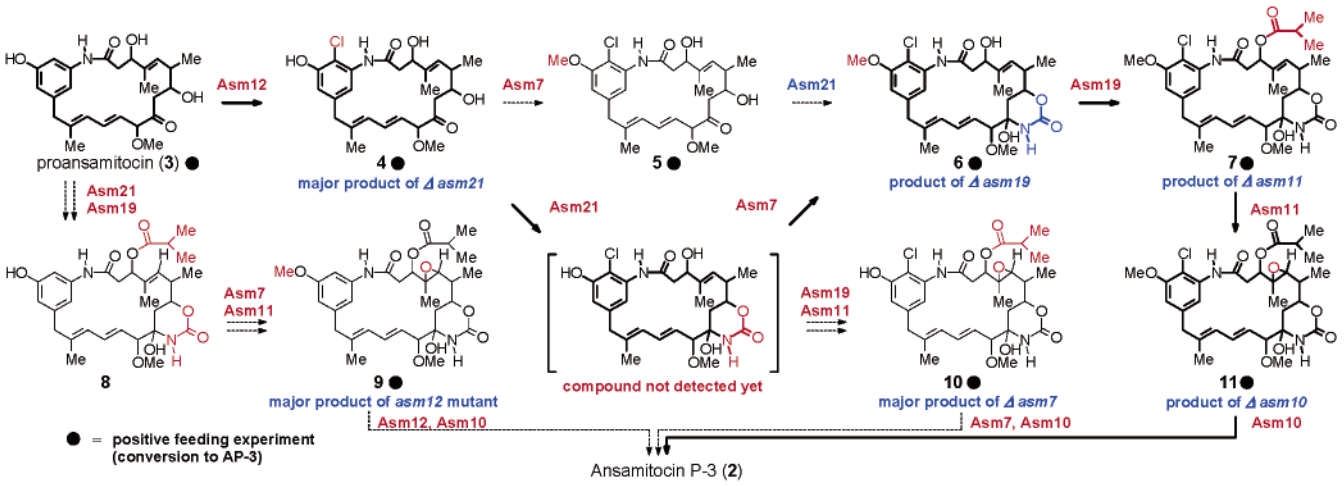

Table 1. Candidate Post-PKS Modification Genes in the Ansamitocin-Producing *A. pretiosum* Strain ATCC 31565

gene	aa	ansamitocin-related compounds in inactivated mutant	main compound	established function
asm7	348	20-*O*,*N*-didemethyl-AP-3 (**10**) (MW 606/608) 20-*O*-demethyl-AP-3 (MW 620/622)	**10**	20-*O*-methyltransferase
asm10	294	*N*-demethyl-AP-3 (**11**) (MW 620/622)	**11**	*N*-methyltransferase
asm11	480	*N*-demethyl-desepoxy-AP-3 (**7**) (MW 604/606)	**7**	4,5-epoxidase
asm12	441	proansamitocin (**3**) (MW 443) carbamoylproansamitocin (MW 486)[a] 3-*O*-isobutyryl-carbamoylproansamitocin (**8**) (MW 556)[a] 19-deschloro-*N*-demethyl-desepoxy-AP-3 (MW 570)[a] 19-deschloro-*N*-demethyl-AP-3 (**9**) (MW 586) 19-deschloro-AP-3 (MW 600)[a]	**9**	19-halogenase
asm19	378	*N*-demethyl-desepoxymaytansinol (**6**) (MW 534/536)	**6**	3-*O*-acyltransferase
asm21	668	19-chloroproansamitocin (**4**) (MW 477/479) 20-*O*-methyl-19-chloroproansamitocin (**5**) (MW 491/493)[a]	**4**	7-*O*-carbamoyltransferase
asm30	1005	AP-3 (**2**) (MW 634/636)	**2**	unknown

[a] Proposed structure (according to the mass spectral data).

of the compounds can be processed further to give **2**. As shown in Scheme 1, not only compounds thought to be intermediates on the main pathway were converted into **2**, but also some that are not on the same pathway, such as **10** and **9**. The latter result shows that chlorine can still be introduced at a very late stage. Therefore, the conversion of **3** into **2** appears to involve a metabolic grid, albeit with a predominant route of metabolic flux (bold arrows and structures in Scheme 1).

The above results open the way for selective derivatizations of the ansamitocin skeleton. In addition, the relaxed substrate specificity of most of these enzymes bodes well for the prospects of processing analogues with modified polyketide backbones.

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Supporting Information Available: Experimental methods and spectroscopic data for compounds **2**−**11** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) Normally, processing by the PKS would be expected to place the double bonds into the Δ10,12 position.

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