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Identification of Asm19 as an Acyltransferase Attaching the Biologically Essential Ester Side Chain of Ansamitocins Using *N*-Desmethyl-4,5-desepoxymaytansinol, Not Maytansinol, as Its Substrate

Steven J. Moss, Linquan Bai, Sabine Toelzer, Brian J. Carroll, Taifo Mahmud, Tin-Wein Yu,* and Heinz G. Floss*

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

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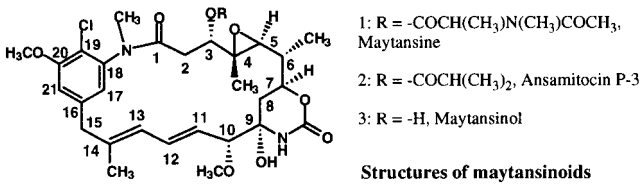
The plant-derived ansa-macrolactam maytansine (**1**)[1] and its microbial counterparts, the ansamitocins (**2**),[2] are antitumor agents of extraordinary potency. Their biosynthesis is of interest as a means of introducing structural diversity into this class of compounds. It involves the assembly of 3-amino-5-hydroxybenzoic acid (AHBA)[3] as the starter unit by addition of three acetates, three propionates, and one unusual "glycolate" extender unit into a polyketide,[4] which then undergoes further downstream processing. This knowledge allowed us to clone and sequence the genes required for ansamitocin biosynthesis, including a set of type-I polyketide synthase (PKS) genes (*asmABCD*), from the producer organism, *Actinosynnema pretiosum* ssp. *auranticum* ATCC 31565.[5]



Structures of maytansinoids

The antitumor activity of the maytansinoids is absolutely dependent on the presence of an ester side chain at position C-3;[6] although there is considerable leeway in its structure.[7] Maytansinol (**3**), a natural product both in plants[8] and in *A. pretiosum*,[9] is biologically inactive. A large number of congeners with different acyl groups have been isolated from *A. pretiosum* and the product pattern responds to the addition of specific side-chain precursors to the fermentation,[10] implying an acyltransferase of broad substrate range. A candidate gene, *asm19*, has been identified in the *asm* cluster.[5] The deduced amino acid sequence of Asm19 (378 aa) shows similarity to MdmB (36%) and to AcyA (35%), both of which are macrolide 3-*O*-acyltransferases from *Streptomyces*,[11,12] suggesting that *asm19* might code for an acyltransferase attaching the ansamitocin ester side chain.

To test this hypothesis, we constructed mutant HGF052 in which *asm19* was inactivated by an internal 549 bp in-frame deletion (Figure 1).[13] The phenotype of HGF052 was analyzed in fermentations supplemented with isobutyrate, conditions under which the wild-type organism produces almost exclusively ansamitocin P-3.[10] HGF052 no longer produced **2**, but it accumulated a new, slightly more polar compound (R_t on C_{18} 18.5 min vs 21.8 min for **2**). The mass spectral pattern identified the new compound as a maytansinoid, its mass of 534 Dalton, 30 mass units less than that for **3**, indicated that it not only lacked the ester side chain but also an additional methyl group and an oxygen. NMR analysis was

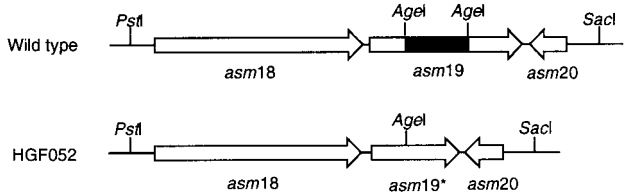


Figure 1. Genomic map of *asm18*, *asm19*, and *asm20* in *A. pretionsum* wild-type and HGF052. The black box indicates the 549-bp *AgeI* DNA fragment that is deleted in *asm19** of *A. pretiosum* HGF052.

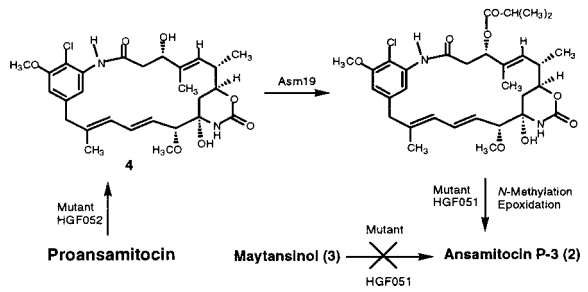


Figure 2. Reaction catalyzed by the acyltransferase Asm19, and the further steps in the biosynthetic conversion into ansamitocins.

complicated by line broadening, possibly due to the presence of two slowly interchanging conformers,[14] and complete signal assignment[17] by COSY, HSQC, and HMBC experiments required the analysis of spectra recorded at elevated temperature in two solvents, DMSO and acetone. The data revealed the presence of both methoxy groups (at δ_H 3.33 and 3.93 ppm) and the absence of the *N*-methyl group, which typically appears in maytansinoids at 3.0 ppm. Compared to those for **2**, the signals at δ_H 2.96 ppm and δ_C 60.8 and 66.7 ppm, assigned to H-5 and to C-4 and C-5, respectively, have been replaced by δ_H 5.48, δ_C 125.1 (C-5), and a very broad signal δ_C at about 114 ppm (C-4), indicating the presence of a double bond in place of the epoxide function. All the other signals are consistent with a maytansinol structure, identifying the accumulated compound as *N*-desmethyl-4,5-desepoxymaytansinol (**4**) (Figure 2).[9]

The fact that mutant HGF052 accumulates a nonesterified ansamitocin instead of **2** confirms the tentative assignment of Asm19 as the 3-*O*-acyltransferase catalyzing the attachment of the ester side chain of the ansamitocins. Since the compound accumulated is not **3**, but its *N*-desmethyldesepoxy derivative **4**, the acylation, contrary to expectation, is not the final step of the biosynthesis, but occurs prior to *N*-methylation and epoxidation. The naturally occurring maytansinol is therefore not a biosynthetic intermediate, but a shunt metabolite presumably arising from hydrolysis of 3-esterified ansamitocins. However, it is possible that

* Correspondence authors: E-mail: (T.Y.) yu@u.washington.edu, (H.G.F.) floss@chem.washington.edu.

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Asm19 can still catalyze the acylation of **3** to ansamitocins such as **2**, that is, act not only as the biosynthetic enzyme but also as a recovery enzyme which recycles the shunt metabolite **3** back into the biosynthetic manifold. Since this question is of some practical interest for the enzymatic preparation of ansamitocins with different ester side chains, we examined whether **3** can serve as a substrate for Asm19.

We carried out bioconversion experiments with a mutant, HGF051, carrying a truncated *asmB*, encoding the second *asm* PKS protein.[5] Although HGF051 cannot assemble the ansamitocin polyketide backbone and thus does not synthesize **2**, it maintains all the enzymatic machinery for the conversion of the initial polyketide, the hypothetical proansamitocin, into **2**. Fermentation of either **3** or **4** with HGF051 in the presence of isobutyrate resulted in complete bioconversion of **4** into ansamitocin P-3, but left **3** unaltered with no trace of **2** detectable. This confirms the role of maytansinol as a shunt metabolite, rather than a biosynthetic intermediate, which cannot be recycled to biologically active ansamitocins. It also reveals a high substrate specificity of this acyltransferase toward the macrolactam component, which is remarkable in view of the high promiscuity toward its acyl substrate, evident from the in vivo formation of a wide range of ansamitocins with different ester side chains.

High substrate specificity for the alcohol component combined with considerable substrate tolerance for the acid component is a property which Asm19 shares with other acyltransferases, such as MdmB[11] and AcyA.[12] Since the acylation can introduce a variety of different side chains and this step is followed by two more biosynthetic reactions, it follows that the enzymes catalyzing those, the *N*-methyltransferase and the epoxidase, must also have a relaxed specificity toward the ester side chain of their substrates, although they evidently recognize the absence of an acyl group.

To substantiate the in vivo results described here, we expressed the Asm19 protein in *Escherichia coli*. The *asm19* gene was amplified by PCR using primers which introduced suitable restriction sites, ligated into vector pRSETB (Invitrogen), and expressed in *E. coli* BL21(DE3)/pLysS (Stratagene) under the control of the T7 promoter. Incubation with the cell-free extract containing the recombinant protein and acetyl-CoA, propionyl-CoA, isobutyryl-CoA, *n*-butyryl-CoA, or isovaleryl-CoA, respectively, as cosubstrate converted **4** into the respective *O*-acyl derivative, that is, into *N*-desmethyl-4,5-desepoxy-ansamitocin P-1, P-2, P-3, P-3′, and P-4, as detected by LC−ESI-MS. To confirm these results, an identical series of incubations was conducted using a corresponding empty vector; no conversion was detected. Both alone and in competition with equimolar amounts of the other four acyl substrates, isovaleryl-CoA proved to be the best substrate, despite the fact that in the normal fermentation ansamitocin P-3 is a more prominent product than P-4. The product distribution therefore must be determined greatly by precursor availability in addition to the specificity of the enzyme. The further characterization of this enzyme will be reported in due course.

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Supporting Information Available: Stereoviews of the conformations of maytansine and compound **4**, and LC−MS tracings of the reactions of Asm19 with **4** and different acyl-CoA substrates (PDF). This material is available free of charge via the Internet at http://pbs.acs.org.

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(13) The possibility of effects of this mutation on neighboring genes was excluded by expressing *asm19* in the mutant, restoring ansamitocin production (L. Bai, unpublished result).
(14) The severe broading of some of the NMR signals of **4**, suggestive of slowly interconverting isomers or conformers is surprising, since no such phenomenon is observed with **2**, **3**, or other maytansinoids. Molecular modeling[15] indicates that the absence of the *N*-methyl group has a substantial effect on the conformation of **4** compared to the crystal structure of a derivative of **1**.[16] **4** has a trans amide bond, which is essentially coplanar with the aromatic ring, whereas in **1** (and by inference **2**) the amide bond is cis and heavily skewed relative to the aromatic ring. **4** is conformationally less rigid than **1** and has two low-energy conformations which differ in the C-1−C-9 region of the macrocylic ring, which could explain the NMR line broadening.
(15) Molecular modeling was performed using the conformational search algorithm (simulated annealing/Monte Carlo) and MMFF94 force-field implemented within the PC Spartan Pro program v.1.0.3 (Wavefunction, Inc., CA).
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(17) **4**: HR-ESI-MS: *m/z* 535.2211 ($M - H^+$), (calcd for $C_{27}H_{36}N_2O_7Cl$, −1.9 ppm error); *m/z* 552.2452 ($M - NH_4^+$), (calcd for $C_{27}H_{39}N_3O_7Cl$, −2.3 ppm error); *m/z* 557.2030 ($M - Na^+$), (calcd for $C_{27}H_{35}N_2O_7NaCl$, −0.1 ppm error); ^{1}H NMR (750 MHz, d_6-acetone, 310 K), δ_H (ppm): 1.09 (3H, d, 6.5 Hz, 6-CH_3), 1.29 (1H, t, 13.0 Hz, H-8a), 1.57 (3H, bs, 4-CH_3), 1.74 (3H, s, 14-CH_3), 1.86 (1H, bm, H-8b), 2.1 (obscured, H-2a), 2.54 (1H, m, H-6), 2.7 (obscured, H-2b), 3.27 (1H, m, H-15a), 3.33 (3H, s, 10-OCH_3), 3.47 (1H, d, 14.0 Hz, H-15b), 3.61 (1H, d, 9.0 Hz, H-10), 3.93 (3H, s, 20-OCH_3), 4.20 (2H, m, H-3, H-7), 5.48 (1H, d, 9.0 Hz, H-5), 5.60 (1H, dd, 15.5, 7.0 Hz, H-11), 6.10 (1H, d, 11.0 Hz, H-13), 6.67 (1H, dd, 15.0, 11.0 Hz, H-12), 6.70−6.85 (∼2H, broad, H-17, H-21); ^{13}C NMR (188.6 MHz, d_6-acetone, 310 K), δ_c (ppm): 14.9 (4-CH_3), 16.8 (6-CH_3), 18.0 (14-CH_3), 30.0 (obscured by solvent, C-2), 36.2 (C-8), 38.5 (C-6), 46.5 (C-15), 56.5 (10-OCH_3), 56.9 (20-OCH_3), 72.3 (C-3), 78.4 (C-7), 82.0 (C-9), 89.1 (C-10), ∼114 (very broad) (C-4), 125.1 (C-5), 127.9 (broad) (C-11, C-13) 133.9 (C-12), 136.9, 137.0, 138.3 (broad) (C-17, C-18, C-19, C-21), 139.7 (broad), 141.2 (C-14, C-16), 152.9 (carbamoyl), 156.2 (C-20), not observed (C-1).

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