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Identification of a Set of Genes Involved in the Formation of the Substrate for the Incorporation of the Unusual "Glycolate" Chain Extension Unit in Ansamitocin Biosynthesis

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The ansamitocins,[1] exemplified by ansamitocin P-3 (Figure 1),[2] are members of the maytansinoids, a family of extraordinarily potent antitumor agents isolated from higher plants as well as microorganisms.[3] The biosynthesis of the ansamitocins in the producing microorganism, the actinomycete *Actinosynnema pretiosum*, involves the assembly of a macrocyclic lactam from an aromatic starter unit, 3-amino-5-hydroxybenzoic acid (AHBA),[4] by seven chain extension steps on a type I modular polyketide synthase (PKS). Three of the chain extension reactions incorporate propionate, and three incorporate acetate units, that is, two use methylmalonyl-CoA and malonyl-CoA, respectively, as substrate and the third one incorporates an unusual oxygenated two-carbon unit sometimes called a "glycolate" unit.[5] Such "glycolate" units are also found in other macrolactam or macrolide antibiotics, such as geldanamycin,[6] leucomycin,[7] FK520 and FK506,[8] and soraphen.[9] In most cases, as in ansamitocin, the α-oxygen of this unusual extender unit is methylated, although there are some exceptions, such as the aflastatins[10] and zwittermicin.[11] Isotopic tracer experiments[5−10,12] have shown that these "glycolate" units are not derived from acetate, but probably arise from carbohydrate metabolism.

Analogy with the other chain extension reactions suggests that the substrate for the incorporation of a "glycolate" unit should be 2-hydroxymalonyl-CoA or 2-methoxymalonyl-CoA. To test this notion we synthesized ^{13}C-labeled 2-hydroxy- and 2-methoxymalonyl-*N*-acetylcysteamine (SNAC) thioester as cell-permeable analogues of the corresponding CoA thioesters acceptable to type I PKSs in vivo[13] and in vitro[14] (Scheme 1). Feeding of 2-methoxymalonyl-SNAC (>98% ^{13}C, 150 mg/L) to eight 100-mL cultures of *A. pretiosum* ATCC 31565 in the presence of the side-chain precursor isobutyrate gave 9 mg of purified ansamitocin P-3 which by NMR and mass spectrometry showed no isotope enrichment over natural abundance. In a second experiment, 2-hydroxymalonyl-SNAC (89% ^{13}C, 50 mg/L) and 2-methoxymalonyl-SNAC (50 mg/L) were each fed to two 50-mL cultures. ES-MS analysis of the resulting ansamitocin P-3 again showed no enrichment. We therefore conclude that the third chain extension step in ansamitocin biosynthesis does not use the CoA thioester of hydroxymalonate or methoxymalonate as substrate, although it may involve a different thioester of one of these acyl groups.

Recently, we have cloned and sequenced a gene cluster from *A. pretiosum* which encodes all the biosynthetic machinery necessary for the formation of ansamitocin.[15] A set of five genes, *asm13−17*, which appear to form a transcription operon, were considered to be involved in the formation of either the ansamitocin ester side chain or the substrate for the unusual chain extension step.

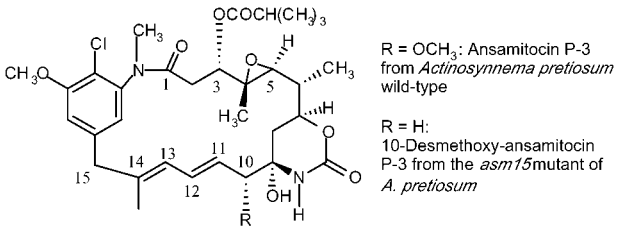
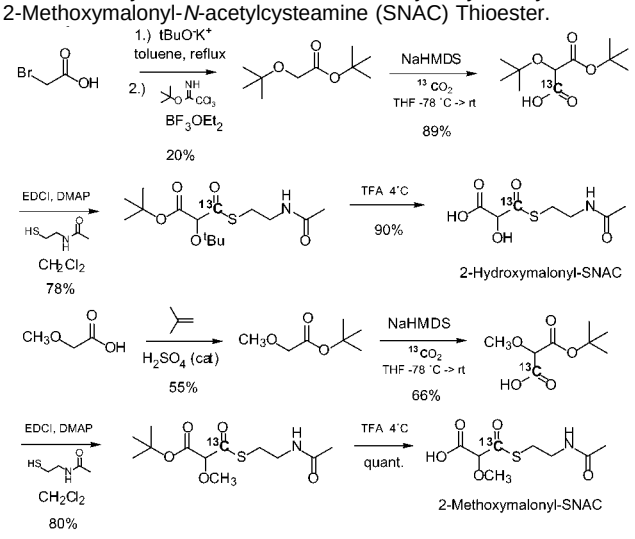


Figure 1. Structures of ansamitocins.

Scheme 1. Synthesis of 1-^{13}C-Labeled 2-Hydroxymalonyl- and 2-Methoxymalonyl-*N*-acetylcysteamine (SNAC) Thioester.



Comparison with the sequences of putative or proven biosynthetic gene clusters of three other antibiotics containing "glycolate" extender units[9a,11,16] revealed sets of homologous genes (Figure 2). Notably, each contained a small open reading frame (ORF) predicted to encode an acyl or peptidyl carrier protein (ACP) which could serve as an alternate carrier of the hydroxy- or methoxymalonate moiety after activation by transfer of the pantetheinyl moiety of CoA.[17] To determine whether this ACP is involved in ansamitocin formation, we constructed a mutant of *A. pretiosum* in which the *asm14* gene was inactivated by inserting a 100-bp DNA fragment into the central coding region. The mutant failed to produce ansamitocin or accumulate any new compounds. This demonstrates that *asm14* is indeed essential for ansamitocin formation.

The *asm13−17* subcluster and a related subcluster, *fkbG-K*, from the FK520 biosynthetic gene cluster of *Streptomyces hygroscopicus*[16] both contain two dehydrogenase genes, one (*asm13*/*fkbK*) homologous to β-hydroxyacyl-CoA dehydrogenases and the other

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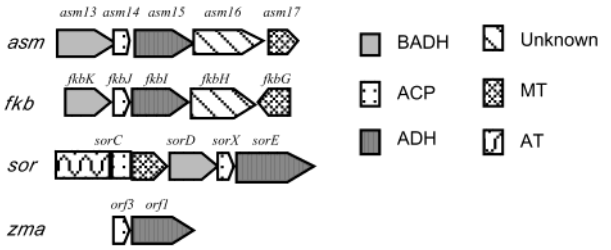

Figure 2. Organization of genes putatively involved in the formation of the novel hydroxylated 2-carbon extender unit in four biosynthetic gene clusters: *asm* (AF453501), ansamitocin from *A. pretiosum*; *fkb* (AF235504), FK520 from *S. hygroscopicus*; *sor* (U24241), soraphen from *Polyangium cellulosum*; *zma* (AF155831), zwittermicin from *Bacillus cereus*. The abbreviations of gene or domain homologues are as follows: BADH, 3-hydroxyacyl-CoA dehydrogenase; ACP, acyl or peptidyl carrier protein; ADH, acyl-CoA dehydrogenase; MT, *O*-methyltransferase; AT, acyltransferase. Note that the *zma* cluster has only been partially sequenced.

(*asm15*/*fkbI*) to acyl-CoA dehydrogenases, as well as a methyltransferase gene (*asm17*/*fkbG*) and an ORF (*asm16*/*fkbH*) of unknown function (Figure 2). This led to the suggestion that these five genes control the formation of 2-methoxymalonyl-ACP, the proposed substrate for the "glycolate" extender unit, from glycolytic intermediates via an ACP-bound glycerate.[16] To probe the role of these genes, we inactivated *asm15* in the *A. pretiosum* genome by inserting an apramycin resistance gene, *aac(3)IV*. The mutant did not produce any ansamitocin P-3, even when supplemented with isobutyrate, indicating that *asm15* is required for formation of the ansamitocin backbone rather than the ester side chain. Feeding with 2-hydroxymalonyl-SNAC or 2-methoxymalonyl-SNAC did not restore ansamitocin formation. Analysis of the fermentation of the *asm15* mutant revealed the presence of a small amount of a new compound, not detected in the wild-type fermentation, which was shown to have the structure of 10-desmethoxy-ansamitocin P-3 (Figure 1).[18,19] Evidently, this compound results from the, albeit inefficient, incorporation of a malonate unit in the absence of the substrate for the "glycolate"extender unit. This clearly demonstrates that *asm15*, and probably others of the *asm13*−*17* genes, must be involved in the synthesis of the unusual "glycolate" unit and its delivery to the PKS. Interestingly, no 10-desmethoxy-ansamitocin P-3 was detected in the fermentation of the *asm14* mutant. This suggests that the ACP encoded by *asm14* is required for the aberrant incorporation of a malonate unit in the third chain extension step, for example, to maintain the PKS in a functionally competent conformation or to actually deliver the malonate to the AT3 on the PKS.

The above results indicate that the substrate for the unusual chain extension reaction incorporating a "glycolate" unit into ansamitocin and other antibiotics is most likely either 2-hydroxy- or 2-methoxymalonyl-ACP rather than the corresponding CoA thioester. The *asm13*−*17* operon is probably responsible for the formation of this substrate from an unidentified intermediate of carbohydrate metabolism. It is noteworthy that the homologues of *asm16*/*fkbH* (unknown) and *asm17*/*fkbG* (methyltransferase) are not present in the putative hydroxy/methoxymalonate subcluster of the soraphen biosynthetic gene cluster. Instead, a gene, *sorC*, encoding a three-domain protein encompassing an acyltransferase, an ACP, and a methyltransferase, has been identified immediately upstream of *sorD* (Figure 2). Such AT/ACP pairs combined with other genes have recently been shown to be involved in the activation and modification of amino acids,[20,21] suggesting that *sorC* may be involved in activating and methylating a precursor of the hydroxy/methoxymalonate moiety, which is then converted into methoxymalonyl-ACP by the action of the *sorDEX* gene products. This suggests that *asm16* may play a role in activating a precursor molecule, perhaps loading an acyl compound onto the ACP encoded by *asm14* for ansamitocin biosynthesis. It also raises the possibility that O-methylation catalyzed by Asm17 may occur early in the elaboration of this unusual chain extension substrate.

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Supporting Information Available: Experimental procedures for the synthesis of 2-hydroxymalonyl-SNAC and 2-methoxymalonyl-SNAC and description of the construction of the *asm14* and *asm15* mutants of *A. pretiosum* (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(18) 10-Desmethoxy-ansamitocin: ^{1}H NMR (499 MHz, CD_3OD), δ (ppm) 0.90 (3H, s, 4-CH_3), 1.22 (3H, d, 2′-CH_3), 1.23 (3H, d, 6.5 Hz, 2′-CH_3), 1.41 (3H, bs, 6-CH_3), 1.52 (1H, bdd, 13.0 Hz, H-8b), 1.55 (1H, m, H-6), 1.70 (3H, bs, 14-CH_3), 1.71 (1H, dd, 14.0, 3.0 Hz, H-8a), 2.15 (1H, m, 14.0, 2.0 Hz, H-2b), 2.20 (1H, m, 11.0 Hz, H-10b), 2.65 (1H, m, 14.0 Hz, H-2a), 2.68 (1H, m, 8.5, 4.0 Hz, H-10a), 2.74 (1H, m, H-2′), 2.82 (1H, bd, 9.5 Hz, H-5), 3.14 (3H, s, NCH_3), 3.3 (1H, obsc., H-15b), 3.5 (1H, obsc., H-15a), 3.98 (3H, s, OCH_3), 4.19 (1H, bt, 11.0 Hz, H-7), 4.70 (1H, dd, 12.0, 2.5 Hz, H-3), 5.67 (1H, ddd, 15.0, 10.5, 4.5 Hz, H-11), 6.18 (1H, bd, 10.5 Hz, H-13), 6.40 (1H, dd, 15.0, 11.0 Hz, H-12), 6.97 (1H, s, H-21), 7.14 (1H, s, H-17). ^{13}C NMR (125.7 MHz, CD_3OD), δ (ppm) 12.8 (4-CH_3), 15.0 (6-CH_3), 15.9 (14-CH_3), 18.6 (2′-CH_3), 20.9 (2′-CH_3), 33.9 (C-3), 35.1 (C-2′), 36.3 (N-CH_3), 39.5 (c-8), 39.8 (C-6), 47.0 (C-10), 47.5 (C-15), 57.4 (O-CH_3), 62.1 (C-4), 68.2 (C-5), 76.3 (C-7), 78.1 (C-3), 81.2 (C-9), 115.0 (C-21), 120.0 (C-19), 123.1 (C-17), 126.8 (C-13), 128.1 (C-11), 132.8 (C-12), 138.6 (C-14), 143. 2 (C-18), 143.3 (C-16), 155.7 (OCONH), 157.8 (C-20), 171.6 (C-1), 177.9 (C-1′).
(19) An unusual feature of the ansamitocin structure is the location of the double bonds at Δ11, 12, and Δ13, 14 instead of Δ10, 11, and Δ12, 14 where normal PKS processing would place them. This implies that a double bond shift must occur during the biosynthesis. The structure of the product from the *asm15* mutant indicates that this double bond shift is not dependent on the presence of the 10-methoxy group
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