# Lomaiviticin Biosynthesis Employs a New Strategy for Starter Unit Generation

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S Supporting Information

ABSTRACT: Lomaiviticin biosynthesis is thought to utilize a propionyl starter unit for a type II polyketide synthase (PKS). Discovery of the lomaiviticin (*lom*) biosynthetic gene cluster suggested an unusual method for starter unit generation involving a bifunctional acyltransferase/decarboxylase (AT/DC) thus far observed only in type I PKS pathways. In vitro biochemical characterization of AT/DC Lom62 confirmed its ability to generate a propionyl-acyl carrier protein (ACP), revealing a new role for this enzymatic activity within natural product biosynthesis.
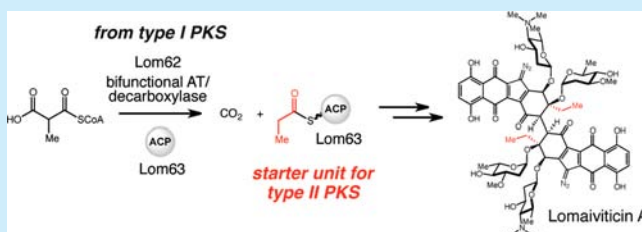
Aromatic polyketides are an important family of natural products that includes clinically used antibiotics and antitumor agents.[1] These secondary metabolites are constructed from malonate building blocks using iterative decarboxylative Claisen condensations carried out by a type II polyketide synthase (PKS). An important point for introducing structural diversity in these pathways is the initial priming of the PKS with a starter unit.[2] Understanding the logic underlying starter unit synthesis is therefore a critical component of efforts to engineer the biosynthesis of 'non-natural' analogs of aromatic polyketides.[3] Here we detail evidence supporting the involvement of a new strategy for starter unit generation in lomaiviticin biosynthesis that employs an enzyme previously associated only with type I modular PKSs.

Type II PKS systems consist of a ketosynthase-chain length factor (KSα-CLF) complex, which performs the C−C bond-forming chemistry, and an acyl carrier protein (ACP) that tethers an incoming malonyl extender unit. The acyltransferase (AT) that selects and loads malonyl-coenzyme A (CoA) is shared with fatty acid synthesis.[4] The majority of secondary metabolites made by type II PKS systems utilize an acetate starter unit generated by decarboxylation of a malonyl-ACP thioester substrate derived from malonyl-CoA.[5] Propionyl starter units are employed less frequently but are involved in the biosynthesis of the clinically important antitumor drugs doxorubicin (**1a**) and daunorubicin (**1b**) (Figure 1A).[6] To date, all characterized mechanisms for incorporating propionyl starter units into type II polyketides utilize propionyl-CoA as the source of this three carbon building block. In doxorubicin/daunorubicin biosynthesis, a separate priming KS (KSIII), homologous to the *E. coli* FabH enzyme, catalyzes an initial Claisen condensation using propionyl-CoA and ACP-bound malonyl-CoA (Figure 1B).[6] The diketide product is then transferred to the main KSα-CLF for further rounds of elongation. Other type II polyketide biosynthetic pathways
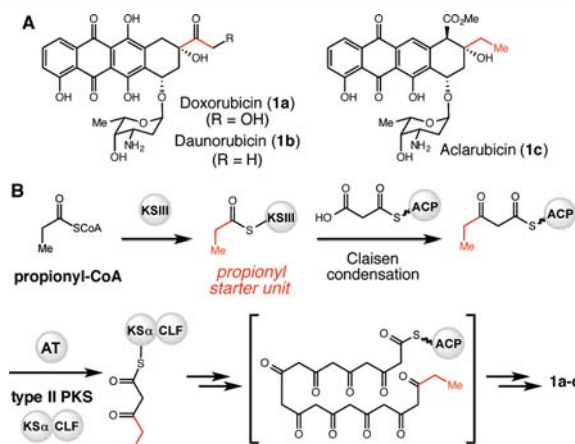

Figure 1. Propionyl starter units are employed in aromatic polyketide biosynthesis. (A) Natural products made by type II polyketide synthases (PKSs) that utilize a propionyl starter unit. (B) Characterized pathway for type II PKS priming with a propionyl starter unit.

that utilize longer alkyl chain starter units similarly employ this KSIII loading module strategy,[7] and most of the corresponding gene clusters also encode a second ACP that is dedicated to starter unit production.[7c]

The lomaiviticins (**2**) and kinamycins (**3**) are two families of diazo-containing aromatic polyketides that have attracted attention from both synthetic and biological chemists (Figure 2).[8] Though these metabolites possess considerable structural similarity, a key difference between them is the D-ring alkyl substituent, which likely arises from use of distinct type II PKS starter units. The kinamycin PKS is predicted to use a canonical
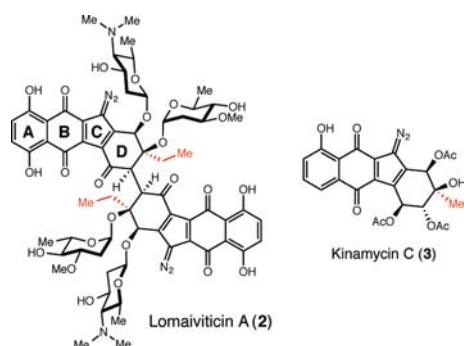
Figure 2. The lomaiviticin and kinamycin families of aromatic polyketides likely use different starter units in biosynthesis.

acetate starter unit, resulting in a D-ring methyl group. In contrast, substitution of an ethyl group at the corresponding position of the lomaiviticin scaffold suggests use of a propionyl starter unit. Recently, Moore and co-workers discovered a lomaiviticin (*lom*) biosynthetic gene cluster in the marine actinomycete *Salinispora tropica* CNB-440.[9] We have also identified a homologous biosynthetic gene cluster in the lomaiviticin producer *Salinispora pacifica* DPJ-0019.[10] Un-expectedly, neither cluster encoded an enzyme homologous to the priming KSIII typically employed in propionyl starter unit loading.[9] The absence of this enzymatic function suggested that an alternate method of starter unit synthesis could be used in lomaiviticin biosynthesis.

In our cluster annotation we identified several genes that we hypothesized could play roles in propionyl starter unit generation: a homologue of the bifunctional acyltransferase/decarboxylase (AT/DC) LnmK (*lom62*) and two ACPs (*lom60* and *lom63*). LnmK is involved in the biosynthesis of leinamycin (**3**), a natural product constructed by a type I modular PKS-nonribosomal synthetase (NRPS) assembly line (Figure 3A). Biochemical characterization of LnmK has revealed its ability to catalyze both transfer of methylmalonyl-CoA to an ACP and subsequent decarboxylation to provide a propionyl-ACP thioester.[11] This intermediate is ultimately utilized to install a β-propionyl substituent onto the polyketide backbone of leinamycin during chain elongation (Figure 3B).[12−14] Recently, the crystal structure of LnmK was solved.[15] Co-crystallization with methylmalonyl-CoA revealed putative active site residues, which led to the identification of an essential tyrosine (Tyr62) as the key functionality necessary for AT activity. Homologues of LnmK have been noted in other gene clusters encoding type I PKS-NRPS pathways, including the myxovirescin A (**5**) gene cluster.[11]

The presence of an LnmK homologue (44% amino acid identity, 60% similarity) in the *lom* gene cluster led us to hypothesize that Lom62 might catalyze an analogous reaction, transferring methylmalonyl-CoA to an ACP and then decarboxylating the resulting methylmalonyl-ACP thioester intermediate to generate a propionyl-ACP thioester (Figure 3C). Transfer of this propionyl starter unit to the KS domain of the KSα-CLF could then occur directly from the ACP or via the action of predicted AT Lom61. The presence of two ACPs in the *lom* cluster (Lom60 and Lom63) further suggests use of an alternate starter unit with one of the ACPs likely dedicated to propionyl-ACP generation. Importantly, homologues of these enzymes (Lom62 and a second ACP) are not found in the kinamycin biosynthetic gene cluster,[16] providing additional support for this hypothesis. If operative, this co-opting of type I
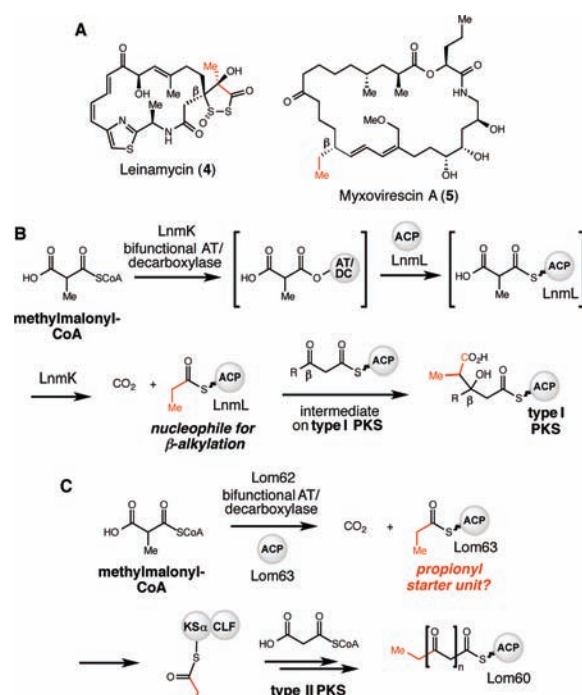

Figure 3. An enzyme involved in type I PKS biosynthesis may be utilized in lomaiviticin starter unit generation. (A) Natural products synthesized by type I PKS-nonribosomal peptide synthetase assembly lines contain β-propionyl and -ethyl branching. (B) Role of acyltransferase/decarboxylase (AT/DC) LnmK in leinamycin biosynthesis. (C) Hypothesized role of LnmK homologue Lom62 in lomaiviticin biosynthesis.

PKS tailoring enzymes would constitute a completely new mechanism for type II PKS starter unit generation. However, the dramatically different pathway contexts of Lom62 and LnmK (type II vs type I modular PKS systems) precluded the assumption that the two enzymes would exhibit the same biochemical activity.

To verify that the sequence similarity of Lom62 and LnmK was likely to reflect a shared biochemical function, we performed additional bioinformatic analyses. Alignment of the two amino acid sequences showed conservation of the essential tyrosine residue that is hypothesized to function as the nucleophile in catalysis (Figure S9, Supporting Information). Key residues that contact the CoA backbone are also conserved in Lom62. These similarities were reinforced by construction of a Lom62 homology model (Figure S11, Supporting Information). Overall, these analyses confirmed that Lom62 and LnmK might possess related functions.

We tested the hypothesis that Lom62 plays a role in propionyl starter unit generation by characterizing its activity in vitro. We cloned *lom62* and the two ACPs *lom60* and *lom63* from *S. pacifica* DPJ-0019 and expressed them individually in *E. coli* as His$_6$-tagged fusions. Analysis of LC−MS data confirmed the identities of both apo-ACPs and their ability to undergo conversion to their corresponding holo-forms in the presence of CoA and the phosphopantetheinyltransferase Sfp (Figures S2−S8, Tables S2−S4, Supporting Information).[17] We examined the activity of Lom62 toward each holo-ACP in the presence of potential substrates methylmalonyl-CoA and propionyl-CoA using an HPLC assay (Figure 4A). LC−MS analysis verified the identities of the major products (Tables S5 and S6, Supporting Information). Robust, Lom62-dependent
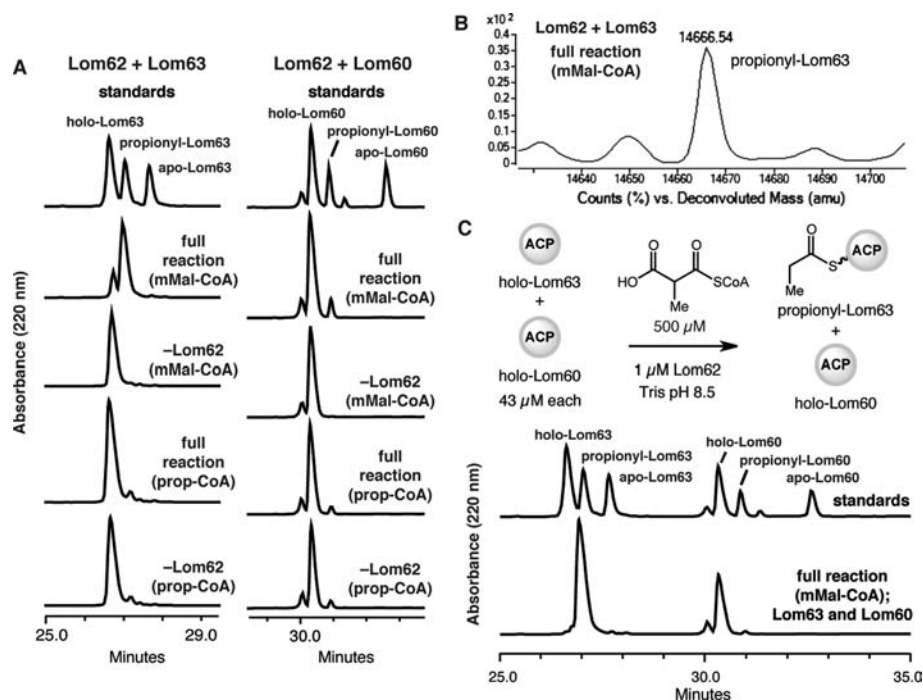
Figure 4. Lom62 generates a propionyl-ACP intermediate from methylmalonyl-CoA for potential use as starter unit in lomaiviticin biosynthesis. (A) HPLC assays of Lom62 with holo-acyl carrier proteins Lom63 and Lom60 (1 h time point) demonstrate loading and decarboxylation of methylmalonyl-CoA to generate propionyl-ACPs and a preference for Lom63. UV detection (220 nm). (B) Deconvoluted mass spectrum of the Lom62 + Lom63 mMal-CoA full reaction assay. (C) HPLC competition assay with equal amounts of ACPs (1 h time point) reveals selective generation of propionyl-Lom63. UV detection (220 nm). Abbreviations: ACP = acyl carrier protein, AT = acyltransferase, mMal-CoA = methylmalonyl-CoA, prop-CoA = propionyl-CoA.

formation of the expected propionyl-ACP thioester product was observed only in the presence of methylmalonyl-CoA and Lom63. In comparison, reactions of Lom62 with methylmalonyl-CoA and alternate ACP Lom60 were sluggish, providing <10% conversion after 1 h. Only trace amounts of propionyl-ACP product were observed when using propionyl-CoA in combination with either ACP partner, confirming that decarboxylation takes place after loading.

To further explore the specificity of Lom62 for the two ACPs, we performed a competition experiment, incubating equal concentrations (43 μM) of the two ACPs with methylmalonyl-CoA and Lom62 for 1 h (Figure 4C). Under these conditions, we observed almost exclusive formation of propionyl-Lom63 by HPLC analysis. This result suggests that LnmK preferentially interacts with only one of the two ACPs encoded in the lomaiviticin cluster. The use of a dedicated ACP for starter unit generation is also observed for other priming mechanisms that use non-acetate type II PKS starter units.[7c] Overall, these biochemical experiments support our hypothesis that Lom62 is involved in generating the propionyl starter unit required for lomaiviticin biosynthesis and reveal Lom63 as its partner ACP. Additional biochemical experiments are needed to elucidate the precise mechanism of propionyl starter unit transfer to the type II PKS.

The potential distribution of this alternate propionyl starter unit synthesis machinery across other type II PKS biosynthetic pathways was evaluated by searching for homologues of Lom62 in sequenced bacterial genomes (Table S8, Supporting Information). The majority of the most closely related enzymes were part of other *lom* clusters (32 hits). However, we found 11 Lom62/LnmK homologues that were were colocalized with genes encoding type I PKS assembly lines and other enzymes

involved in β-alkyl installation,[14] and an additional two homologues located near type II PKS biosynthetic machinery that was distinct from the *lom* gene cluster. This analysis suggests that the decarboxylative route to propionyl starter unit generation is utilized by other type II PKS systems. We hypothesize that this priming strategy could have evolved in response to differences in the availability of methylmalonyl-CoA and propionyl-CoA precursors in producing organisms.

In summary, we have discovered that a new mechanism for type II PKS propionyl starter unit generation may be operative in lomaiviticin biosynthesis. Intriguingly, this process co-opts enzymes previously associated only with type I PKS pathways. These experiments are the first step toward understanding the logic underlying this unique priming strategy, which may represent a new target for metabolic engineering efforts targeting aromatic polyketide biosynthesis.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(8) For a review of the lomaiviticin and kinamycin families of natural products see: Woo, C. M.; Herzon, S. B. *Nat. Prod. Rep.* **2012**, *29*, 87.

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(10) Details will be published elsewhere.

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