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Bifunctional Acyltransferase/Decarboxylase LnmK as the Missing Link for β-Alkylation in Polyketide Biosynthesis

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Both α- and β-alkylations contribute to the vast structural diversity displayed by polyketide natural products (Figure 1A).[1] While the α-alkyl branches are typically derived from extender units, the choice of which is dictated by the acyltransferase (AT) domain of modular polyketide synthases (PKSs),[1] the β-alkyl branches often result from the activities of hydroxymethylglutaryl-CoA (HMG-CoA) synthase homologues (HCSs).[2] For a β-methyl branch, HCS catalyzes condensation of acetyl-*S*-acyl carrier protein (acetyl-*S*-ACP) with the β-carbonyl group of the PKS−ACP-tethered growing polyketide intermediate to afford an HMG-*S*-ACP intermediate, which is subsequently dehydrated and decarboxylated by two enoyl-CoA hydratase homologues (ECH1 and ECH2) sequentially to afford a β-methylated intermediate in either olefinic form (Figure 1B). This pathway has been experimentally confirmed in the biosynthesis of bacillaene (**1**),[3] curacin (**2**),[4] and myxovirescin A (also known as TA) (**3**),[5] and a dedicated set of three proteins—an ACP, an AT, and a ketosynthase homologue (KS)—that derives acetyl-*S*-ACP from malonyl-CoA for this pathway has been identified (Figure 1C).[2−5]

A parallel pathway replacing acetyl-*S*-ACP with propionyl-*S*-ACP could be envisaged for introduction of a β-ethyl branch, and this proposal has been supported for **3** using chemoenzymatically prepared propionyl-*S*-ACP as a substrate (Figure 1B).[5b] However, counterparts for propionyl-*S*-ACP biosynthesis from methylmalonyl-CoA, such as the AT and KS enzymes required to generate acetyl-*S*-ACP from malonyl-CoA, are absent from gene clusters known to encode biosynthesis of polyketides with β-ethyl branches (Table 1); the origin of propionyl-*S*-ACP remains unknown.[2−5]

Leinamycin (Lnm, **4**), a potent antitumor antibiotic, possesses a β-branched C3 unit that is part of its unique five-membered 1,3-dioxo-1,2-dithiolane moiety. We have previously cloned, sequenced, and characterized the *lnm* biosynthetic gene cluster from *Streptomyces atroolivaceus* S-140.[6] Close examination of the *lnm* cluster revealed a subset of four genes—*lnmL*, *lnmM*, *lnmF*, and *lnmK*—encoding an ACP (LnmL), an HCS (LnmM), an ECH1 (LnmF), and a protein of unknown function (LnmK). Counterparts of LnmL, LnmM, and LnmF are present in biosynthetic clusters of polyketides with both β-methyl and β-ethyl branches,[2−6] supporting the proposal that the C3 β-branch in **4** is likely installed by LnmL/LnmM/LnmF in a mechanistic analogy to the β-methyl branch in **1**, **2** and **3**. Homologues of LnmK, however, can only be found in gene clusters encoding the biosynthesis of ethyl-branch-bearing polyketides, suggesting LnmK as a candidate for propionyl-*S*-ACP biosynthesis (Table 1, Figure 1C). Here we report the characterization of LnmK as a bifunctional acyltransferase/decarboxylase (AT/DC) that derives propionyl-*S*-ACP from methylmalonyl-CoA.

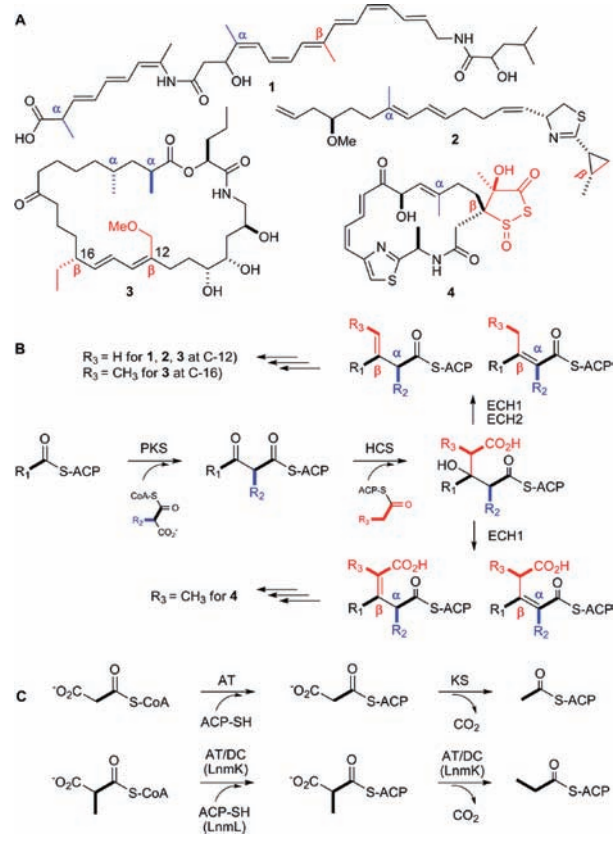

Figure 1. (A) Selected polyketides bacillaene (**1**), curacin (**2**), myxovirescin A (**3**), and leinamycin (**4**) with (blue) α- or (red) β-alkyl branches. (B) Unified pathway for β-alkylation utilizing both acetyl-*S*-ACP and propionyl-*S*-ACP as substrates. (C) Distinct pathways for acetyl-*S*-ACP and propionyl-*S*-ACP biosynthesis.

Table 1. Enzymes That Generate Acetyl-*S*-ACP and Propionyl-*S*-ACP and Incorporate Them into Polyketides with a β-Alkyl Branch (Methyl for **1**, **2**, and **3** at C-12 or Ethyl for **3** at C-16 and Propionyl for **4**)[a]

compd	AT/DC	AT	KS	ACP	HCS	ECH1	ECH2
1	−	PksC	PksF	AcpK	PksG	PksH	PksI
2	−	−	CurC	CurB	CurD	CurE	CurF
3 (C-12)	−	TaV	TaK	TaB	TaC	TaX	TaY
3 (C-16)	TaD	−	−	TaE	TaF	TaX	TaY
4	LnmK	−	−	LnmL	LnmM	LnmF	−

[a] See refs 2−6.

Hence, LnmK represents a new family of AT/DC enzymes supplying a key substrate for β-alkylation in polyketide biosynthesis.

We first overproduced both LnmL and LnmK in *Escherichia coli* BL21(DE3) and purified them to near homogeneity (Figure

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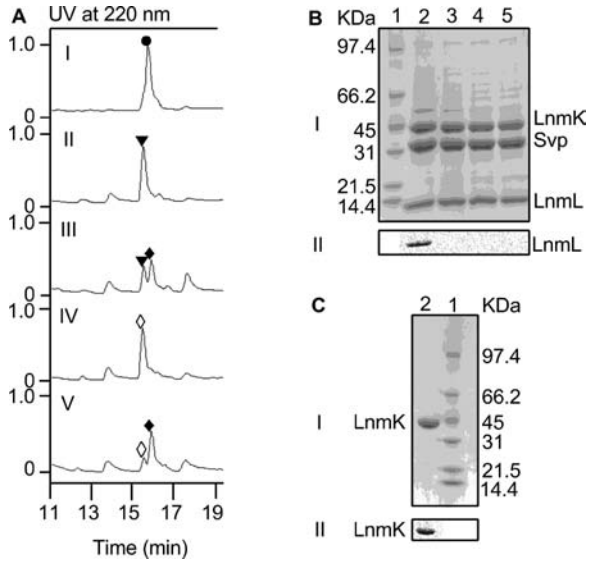

Figure 2. (A) HPLC analysis of LnmK-catalyzed formation of propionyl-*S*-LnmL: (I) apo-LnmL (●); (II) holo-LnmL (▼); (III) holo-LnmL and propionyl-*S*-LnmL (◆); (IV) methylmalonyl-*S*-LnmL (◇); (V) propionyl-*S*-LnmL. (B) LnmK-catalyzed loading of acyl-CoA's to holo-LnmL and (C) LnmK-catalyzed self-acylation as judged by (I) 4−15% SDS-PAGE and (II) autoradiogram: lane 1, molecular weight standards; lane 2, [1,3-$^{14}C_2$]methylmalonyl-CoA; lane 3, [1,3-$^{14}C_2$]malonyl-CoA; lane 4, [1-^{14}C]propionyl-CoA; lane 5, [1-^{14}C]acetyl-CoA.

S1 in the Supporting Information). The purified LnmL was eluted as a single peak upon HPLC analysis (Figure 2A, panel I) and confirmed to be in its apo form by electrospray ionization mass spectrometry (ESI-MS) analysis (Table S1). In vitro phosphopantetheinylation was carried out by incubating apo-LnmL with CoA in the presence of the known promiscuous phosphopantetheinyl-transferase Svp,[7] and the resultant holo-LnmL was confirmed by HPLC (Figure 2A, panel II) and ESI-MS (Table S1) analyses.

We then established that LnmK is a bifunctional AT/DC catalyzing the formation of propionyl-*S*-LnmL. Holo-LnmL was incubated with [1-^{14}C]acetyl-, [1-^{14}C]propionyl-, [1,3-$^{14}C_2$]malonyl-, or [1,3-$^{14}C_2$]-methylmalonyl-CoA in the presence of LnmK, and the reaction mixtures were subjected to SDS-PAGE and phosphorimaging. LnmK specifically and efficiently catalyzed the loading of methylmalonyl-CoA to holo-LnmL, and no loading was observed with the other acyl-CoA's tested (Figure 2B). To verify the molecular identity of the acyl-*S*-LnmL species, the reaction was repeated with cold methylmalonyl-CoA, and the resultant product was subjected to HPLC and ESI-MS analyses. A distinct new product was formed (Figure 2A, panel III), which ESI-MS analysis remarkably identified as propionyl-*S*-LnmL (Table S1); LnmK apparently acts as bifunctional AT/DC, catalyzing both methylmalonyl transfer to form the methylmalonyl-*S*-LnmL intermediate and its subsequent decarboxylation to yield propionyl-*S*-LnmL (Figure 1C).

We finally determined the precise timing of the acyl transfer and decarboxylation events catalyzed by LnmK. The fact that LnmK cannot decarboxylate methylmalonyl-CoA and loads only methylmalonyl-CoA (but not propionyl-CoA) to holo-LnmL indicates that decarboxylation most likely occurs on methylmalonyl-*S*-LnmL. To directly verify this mechanism, we prepared methylmalonyl-*S*-LnmL via in vitro phosphopantetheinylation by incubating apo-LnmL with methylmalonyl-CoA in the presence of Svp.[7] Methylmalonyl-*S*-LnmL formation was monitored by HPLC (Figure 2A, panel IV) and confirmed by ESI-MS (Table S1) analyses. Incubation of methylmalonyl-*S*-LnmL with LnmK allowed us to investigate LnmK's DC activity directly. LnmK catalyzes specific and efficient decarboxylation of methylmalonyl-*S*-LnmL to yield propionyl-*S*-LnmL, whose identity was confirmed by HPLC (Figure 2A, panel V) and ESI-MS (Table S1) analyses. Taken together, these results unambiguously establish that LnmK first transfers methylmalonyl from methylmalonyl-CoA to holo-LnmL to form methylmalonyl-*S*-LnmL and then decarboxylates the latter to form propionyl-*S*-LnmL (Figure 1C).

LnmK homologues are known but to date have all been annotated as hypothetical proteins (Figure S2).[2−5] We now propose that LnmK represents a new family of AT/DC enzymes supplying substrates for β-alkylation in polyketide biosynthesis. To further probe the catalytic mechanism of this newly discovered family of AT/DC enzymes, LnmK was incubated with [1,3-$^{14}C_2$]methylmalonyl-CoA in the absence of holo-LnmL, and the reaction mixtures were subjected to SDS-PAGE and phosphorimaging. Specific and efficient loading of [1,3-$^{14}C_2$]methylmalonyl-CoA onto LnmK was observed (Figure 2C), indicative of a transient acyl-LnmK intermediate in LnmK catalysis. This is reminiscent of ATs with Ser at their active sites,[8] although no conserved AT or DC active-site motif is apparent in LnmK (Figure S2).

In summary, LnmK has been characterized as a bifunctional AT/DC that catalyzes the formation of propionyl-*S*-ACP from methylmalonyl-CoA, accounting for the missing link of the β-ethyl or propionyl branch in polyketide biosynthesis. LnmK therefore could be exploited by combinatorial biosynthesis methods to engineer novel polyketides, especially those with β-alkyl branches. LnmK also represents an emerging family of novel AT/DC enzymes.

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Supporting Information Available: Full experimental details, Figures S1 and S2, and Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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