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Modular polyketide synthases and *cis*-double bond formation: Establishment of activated *cis*-3-cyclohexylpropenoic acid as the diketide intermediate in phoslactomycin biosynthesis

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Abstract

The majority of modular polyketide synthase (PKS) systems which generate unsaturated products do so with *trans* double bonds. Phoslactomycin B (PLM B) presents a class of antitumor and antiviral natural polyketide products that have unique structural features, including a linear unsaturated backbone with one *trans* and three *cis* double bonds. There is substantial evidence that *trans* double bonds are established by ketoreductase-dehydratase (KR-DH) didomains within a PKS module. In cases where modules containing these didomains appear to generate product containing a *cis* double bond there is no experimental evidence to determine if they do so directly, or if they also form a *trans* double bond with a subsequent isomerization step. A critical step in addressing this issue is establishing the stereochemistry of the polyketide intermediate which passes to the subsequent module. Herein, we demonstrate through a series of experiments that an activated *cis*-3-cyclohexylpropenoic acid is the diketide intermediate which passes from module 1 to module 2 of the PLM PKS. The *trans* isomer of the diketide intermediate could not be processed directly into PLM B by module 2, but could be converted to PLM B by degradation to cyclohexanecarboxylic acid and elongation by the entire PLM PKS. These observations indicate not only that module 1 with a DH-KR didomain is responsible for establishing C_{14}–C_{15} *cis* double bond of PLM B, but that the subsequent modules of the PKS clearly discriminate between the *cis* and *trans*-diketide intermediate and do not contain domains capable of catalyzing double bond isomerization.

Phoslactomycins (PLMs), exemplified by PLM B (Figure 1), are a unique class of antitumor, antiviral, and antifungal polyketide natural products.[1,2] The antitumor activity of PLMs is attributed to a potent and selective inhibition of protein Ser/Thr phosphatase 2A (PP2A).[3] The PLM biosynthetic gene cluster from *Streptomyces* sp. HK803 has been cloned and sequenced.[4] The PLM polyketide synthase (PKS) is a modular PKS comprised of a loading domain and seven extension modules which are responsible for the synthesis of a unique linear unsaturated polyketide structure containing three *cis* (*Z*) and one *trans* (*E*) double bonds.

Modular PKSs which generate unsaturated products typically do so using *trans* double bonds.[5] These double bonds are established by ketoreductase-dehydratase (KR-DH) domains which sequentially carry out ketoreduction and dehydration steps on the 3-ketoacyl-ACP products of the KS domains. The dehydration step makes the stereochemical course of the KR-catalyzed step cryptic. Recently in vitro work using a DH-inactivated module 2 of the pikromycin PKS, which establishes the single *trans* double bond of pikromycin and methymycin, have shown this KR generates the D-3-hydroxy product.[6] A bioinformatic analysis of other cryptic KR-

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DH domains which generate *trans*-double bonds infers a D- hydroxyl configuration (this analysis is based on an established correlation of diagnostic residues in KR primary sequences and their known stereochemical products).[5,7]

Polyketide products containing *cis* double bonds are rare and appear to arise through a variety of mechanisms.[8] In many cases such as modules 7 of PLM and module 4 of the epothilone PKS the required DH activity is absent from the module.[4,9] Modules 1 and 2 of the PLM PKS are intriguing because they have combined KR-DH didomains which appear to establish two conjugated *cis* double bonds (C_{12}–C_{13} and C_{14}–C_{15} of PLM B, respectively).[4] Bioinformatic analysis of the primary sequence of these KR domains does not clearly predict a D-hydroxyl configuration (which evidence indicates precedes *trans* double bond formation) or L-hydroxy configuration (which has been speculated might precede *cis* double bond formation).[7] Thus in each case the combined activity of these KR-DH didomains might establish a *trans* double bond with a subsequent isomerization step to a *cis* double bond (epimerization domains, in both PKS[10] and NRPS[11] modules, as well as *trans* to *cis* double bond isomerization in retinoid cycle[12] have been reported). Alternatively, these KR-DH domains might establish the *cis* double bond directly.

In this work, we have distinguished between these two possibilities by determining the stereochemistry of the polyketide intermediate which is transferred from module 1 to module 2. PLM1 contains a loading domain and the first extension module of the PKS and is predicted to generate either *cis* or *trans* 3-cyclohexylpropenoic acid (Figure 2) from an activated cyclohexanecarboxylic acid (CHC) starter unit. We generated a Δ*chcA* mutant (NP3), blocked in biosynthesis of the starter unit, and demonstrated that it only produces PLM B when grown in the presence of CHC (Table 1). The *trans* and *cis* diketide products of PLM1 were synthesized in both the acid (**2a** and **3a**, Figure 2) and N-acetylcysteamine (SNAC) thioester (**4a** and **5a**, Figure 2) forms and added to separate fermentations of this Δ*chcA* mutant. Surprisingly, compounds **2a**–**5a** all restored PLM B production. PLM B production levels were the highest for the *trans*-products (**2a** and **4a**) and were 40% higher than that observed with either CHC supplementation or the *cis*-SNAC (**5a**) (Table 1). The lowest level of PLM B production was observed with the *cis*-acid (**3a**). Interestingly, the PLM B isolated from feeding *trans*-acid **2a** had the C_{14}–C_{15} double bond in the *cis* configuration, as confirmed by ^{1}H NMR and NOESY experiments. This initial result suggested that the *trans*-diketide intermediate might be the preferred substrate for PLM2, with a subsequent *trans* to *cis* isomerization step.

Alternatively, the *trans*-compounds might be converted efficiently to the activated CHC starter unit by fatty acid degradation and subsequently elongated by the entire PLM PKS (in this way the *trans* double bond would be lost through degradation and reintroduced as a *cis* double bond by PLM1) (Figure 2). To distinguish between these two hypotheses we synthesized and fed the [2-^{13}C] labeled analogs **2b**–**5b** (Figure 2) to the Δ*chcA* mutant. Mass spectroscopy revealed that isotopic enrichment over natural abundance for the PLM B product was only observed with the *cis*-SNAC **5b** (20% isotope enrichment, Table 2). These data showed that both *cis*- and *trans*-compounds undergo degradation to form the activated CHC starter unit, and that this is the primary route for PLM B production in these experiments. Furthermore, the experiments established that only *cis* SNAC (**5a**,**5b**) could prime PLM2 directly. The *cis*-acid (**3a**,**3b**) which gives the lowest levels of PLM B restoration levels can be transported into the mutant and degraded to the activated CHC (at about 50% the efficiency of the corresponding *trans*-diketides) but cannot be activated intact such that it can prime PLM2.

A consistent and predictable set of results was obtained by generation and analysis of a *plm1* deletion mutant [NP9, see supplementation data] (Figure 2). PLM B production was abrogated in this mutant and was only significantly restored by growth in the presence of the *cis*-SNAC compounds **5a** and its ^{13}C-labeled counterpart **5b** (Table 1). In the case of **5b** the PLM B now

contained the same level of isotopic enrichment (>99%) as the diketide substrate (Table 2). No restoration of PLM B was seen with *cis* or *trans* acids (**2a**,**2b**,**3a**,**3b**) and low levels of PLM B were observed with the trans SNAC diketides (**4a**,**4b**) and correlated with LC-MS detection of trace levels of the corresponding *cis*-SNAC diketides (**5a**,**5b**) in these samples (Table 1 and 2).

These observations unequivocally demonstrate that only the SNAC derivative of the *cis*-diketide can prime PLM2 directly and that all other diketides give rise to PLM B production only through degradation to an activated CHC and elongation using PLM1. The product of PLM1 must therefore be the *cis*-3-cyclohexylpropenoic acid. These experiments also demonstrate that the PLM biosynthetic process cannot process the *trans*-diketide intermediate either into PLM B (ruling out an isomerization domain in the subsequent PKS modules) or a PLM analog with *trans* C_{14}–C_{15} double bond. This last observation indicates significant challenges to successful alteration of the stereochemistry of unsaturated polyketide products through either directed biosynthesis or KR-DH didomain switches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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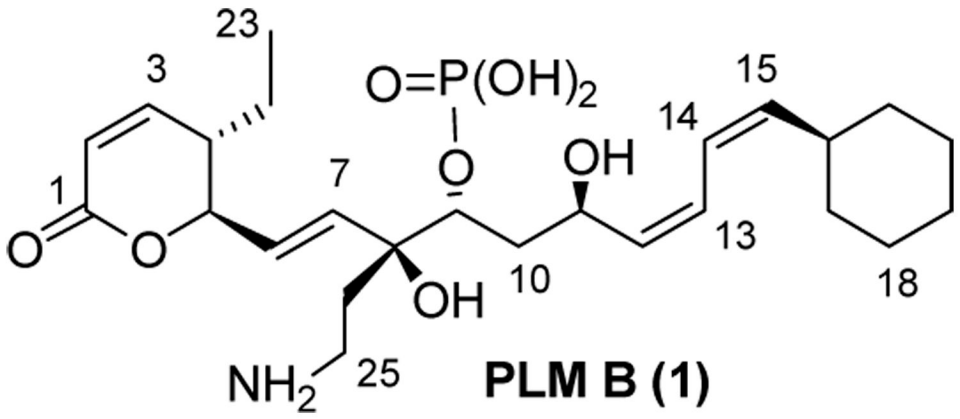

Figure 1.
Phoslactomycin B (PLM B).

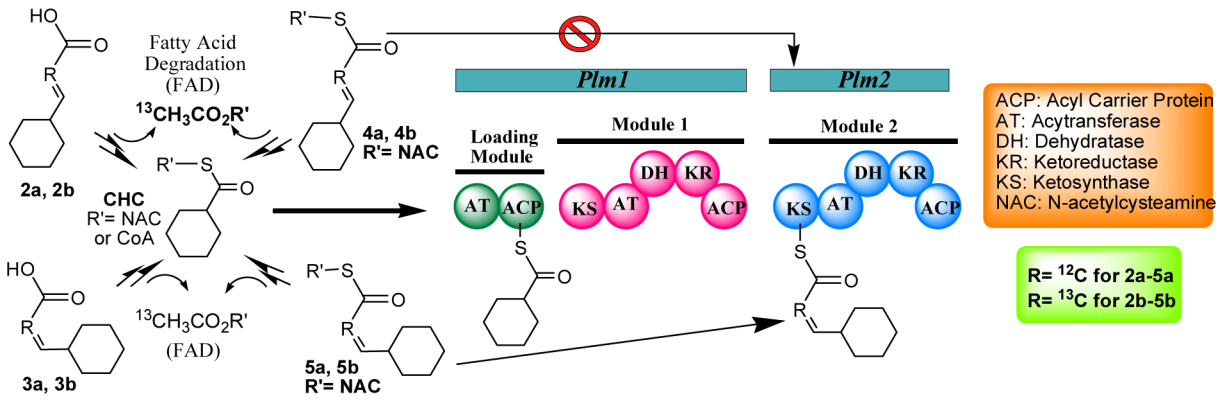

Figure 2.
Incorporation of CHC, compounds **2a**–**5a**, and **2b**–**5b** into Plm1 and Plm2 of PLM B PKS.

Table 1

Relative % of PLM B production by feeding CHC and compounds **2a**–**5a** to Δ*chcA* and Δ*plm1* mutants.

Substrate	**Δ*chcA* mutant**	**Δ*plm1* mutant**
control	0	0
CHC	68 ± 3.9	0
2a	100 ± 7	0
3a	50 ± 3	0
4a	98 ± 6	~ 0.5[a]
5a	72 ± 7	100

[a] LC-MS analysis demonstrated **4a** contained trace levels of **5a** (<1%).

Table 2

% of ^{13}C isotope enrichment in produced PLM B generated by feeding CHC and compounds **2b**–**5b** to Δ*chcA* and Δ*plm1* mutants.

Substrate	Δ*chcA* mutant	Δ*plm1* mutant
control	0	ND
CHC	0	ND
2b	0	ND
3b	0	ND
4b	0	99%[a]
5b	~ 20%	99%

[a] LC-MS analysis demonstrated **4b** contained trace levels of **5b** (<1%).

ND: No PLM B production was detected