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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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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, has shown this KR generates the D-3-hydroxy product.[6] A bioinformatic analysis of other cryptic KR−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)

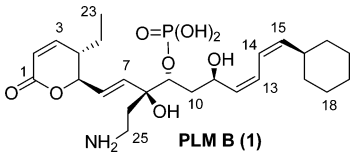


***Figure 1.*** Phoslactomycin B (PLM B).

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 analogues **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 Supporting Information] (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

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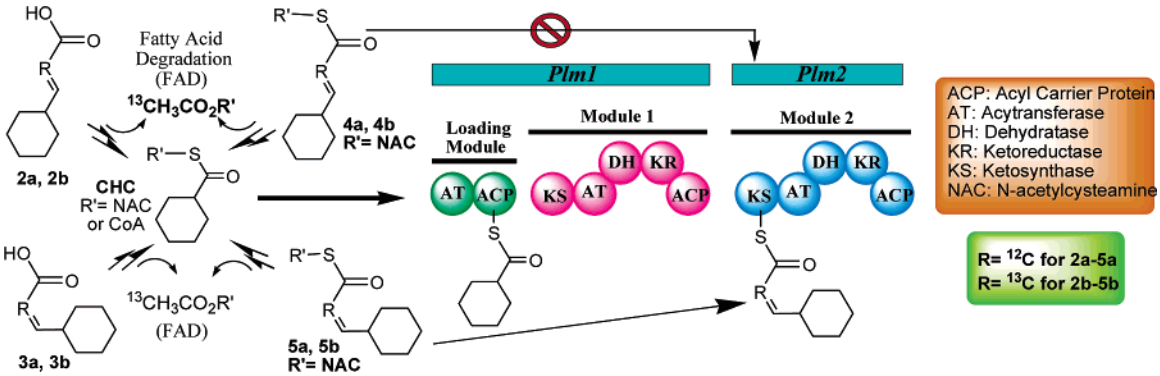


***Figure 2.*** Incorporation of CHC, compounds **2a**−**5a**, and **2b**−**5b** into PLM1 and PLM2 of PLM B PKS.

***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 that **4a** contained trace levels of **5a** (<1%).

***Table 2.*** Percent 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 that **4b** contained trace levels of **5b** (<1%). ND: No PLM B production was detected.

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 (Tables 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 analogue 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.

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**Supporting Information Available:** Experimental procedures and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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