2007

Modular Polyketide Synthases and cis Double Bond Formation: Establishment of Activated cis-3-Cyclohexylpropenoic Acid as the Diketide Intermediate in Phoslactomycin Biosynthesis

Mamoun M. Alhamadsheh
Portland State University

Nadaraj Palaniappan
Portland State University

Suparna DasChouduri
Virginia Commonwealth University

Kevin A. Reynolds
Portland State University, reynoldsk@pdx.edu

Published in final edited form as:
J Am Chem Soc. 2007 February 21; 129(7): 1910–1911. doi:10.1021/ja068818t.

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

Mamoun M. Alhamadsheh[†], **Nadaraj Palaniappan**[†], **Suparna DasChouduri**[‡], and **Kevin A. Reynolds**[†*]

†*Department of Chemistry, Portland State University, Portland, OR 97207*

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-

E-mail: reynoldsk@pdx.edu.
‡Current address: Virginia Commonwealth University.

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.

Acknowledgment

Funding for this research was generously provided by the National Institutes of Health (AI51629).

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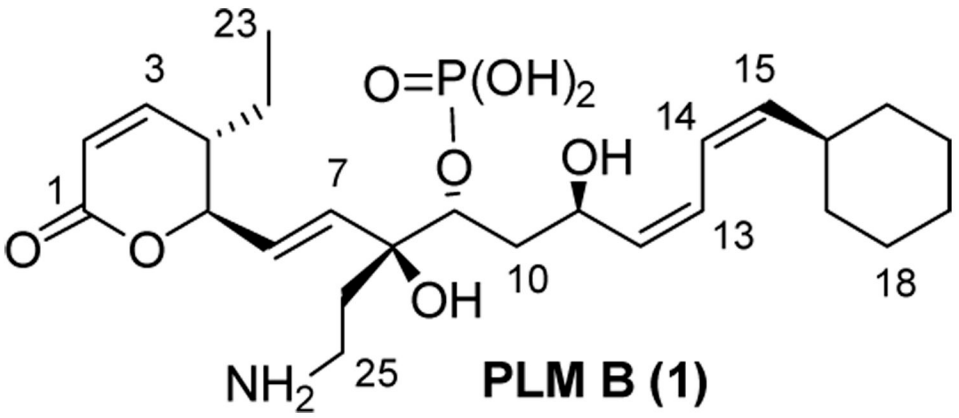

Figure 1.
Phoslactomycin B (PLM B).

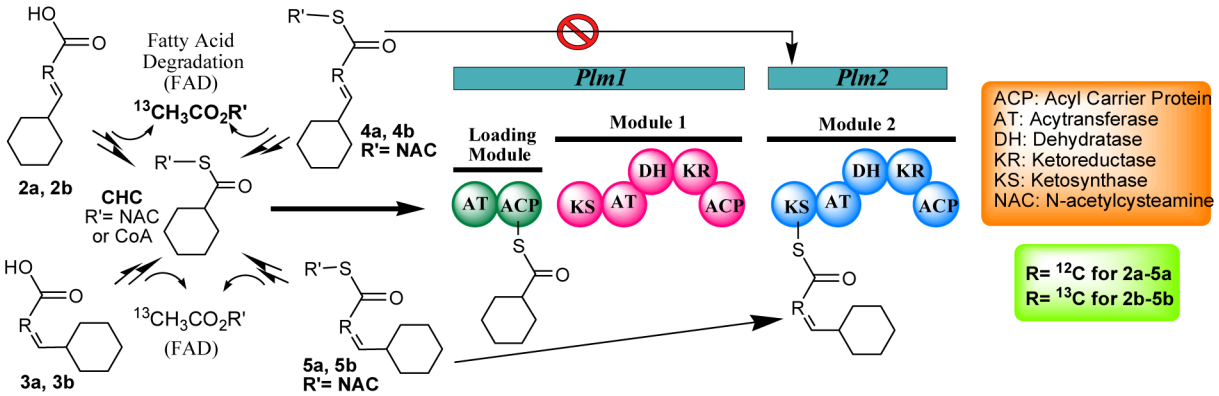

Figure 2.
Incorporation of CHC, compounds **2a**–**5a**, and **2b**–**5b** into Plm1 and Plm2 of PLM B PKS.

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