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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 C14–C15 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.
DH domains which generate trans-double bonds infer 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. 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. 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 (C12–C13 and C14–C15 of PLM B, respectively). 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). 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 and NRPS modules, as well as trans to cis double bond isomerization in retinoid cycle have been reported). Alternatively, these KR-DH didomains 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 ΔchcA 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-acetylcyesteine (SNAC) thioester (4a and 5a, Figure 2) forms and added to separate fermentations of this ΔchcA 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 C14–C15 double bond in the cis configuration, as confirmed by 1H 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 [2-13C] labeled analogs 2b–5b (Figure 2) to the ΔchcA 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 13C-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 C14–C15 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**

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**References**

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 ΔchcA and Δplm1 mutants.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>ΔchcA mutant</th>
<th>Δplm1 mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CHC</td>
<td>68 ± 3.9</td>
<td>0</td>
</tr>
<tr>
<td>2a</td>
<td>100 ± 7</td>
<td>0</td>
</tr>
<tr>
<td>3a</td>
<td>50 ± 3</td>
<td>0</td>
</tr>
<tr>
<td>4a</td>
<td>98 ± 3.6</td>
<td>~ 0.5%</td>
</tr>
<tr>
<td>5a</td>
<td>72 ± 7</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^a\) 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 $\Delta$chcA and $\Delta$plm1 mutants.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$\Delta$chcA mutant</th>
<th>$\Delta$plm1 mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>CHC</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>2b</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>3b</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>4b</td>
<td>0</td>
<td>99%&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5b</td>
<td>$\approx$ 20%</td>
<td>99%&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>LC-MS analysis demonstrated 4b contained trace levels of 5b (<1%).

ND: No PLM B production was detected