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The *cis*- $\Delta^{2,3}$ -double bond of phoslactomycins is generated by a post-PKS tailoring enzyme

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Abstract

The antifungal phoslactomycins (PLM A-F), produced by *Streptomyces* sp HK803, are structurally unusual in that three of their four double bonds are in the *cis* form ($\Delta^{12,13}$, $\Delta^{14,15}$, $\Delta^{2,3}$). The PLM polyketide synthase (PKS) has the predicted dehydratase catalytic domain in modules 1,2 and 5 required for establishing two of these *cis* double bonds ($\Delta^{12,13}$, $\Delta^{14,15}$), as well as the only *trans* $\Delta^{6,7}$ double bond. By contrast, the formation of the *cis* $\Delta^{2,3}$ in the unsaturated lactone moiety of PLMs has presented an enigma because the predicted dehydratase domain in module 7 is absent. Herein, we have demonstrated that the *plmT*₂ gene product, with no homology to PKS dehydratase domains, is required for efficient formation of the *cis* $\Delta^{2,3}$ alkene. A series of new PLM products in which the C3 hydroxyl group is retained, are made in *plmT*₂ deletion mutants. In all of these cases, however, the hydroxyl group is esterified with malonic acid. These malonylated PLM products are converted to the corresponding *cis* $\Delta^{2,3}$ PLM products and acetic acid by a facile base-catalyzed decarboxylative elimination reaction. Complete or partial restoration of natural PLM production in a *plmT*₂ deletion mutant can be accomplished by plasmid based expression of *plmT*₂ or *fos ORF4* (a homologous gene from the fostriecin biosynthetic gene cluster), respectively. The data indicate that dehydratase-independent pathways also function in establishment of unsaturated 6-membered lactone moieties in other PKS pathways, and provide the first biosynthetic insights into the possible routes by which unusual malonylated polyketide products are generated.

Modular polyketide synthases (PKSs) generate a vast array of structurally diverse natural products with important biological activities.¹ They are responsible for formation of macrolides, the vast majority of which contain one or more double bonds.² The majority of other natural products made by modular PKSs similarly contain some double bonds. In the majority of cases the alkenes in the polyketide product are in the *trans* form. The formation of these double bonds has been shown to be directly attributed to the presence of a ketoreductase-dehydratase (KR-DH) didomain within the appropriate module.^{3,4} These didomains catalyze a 3-keto reduction and subsequent $\Delta^{2,3}$ elimination reaction with the PKS-tethered 3-ketoacyl polyketide intermediates.

The phoslactomycins (PLMs A-F Figure 1) and fostriecin belong to a class of phosphorylated polyketides that contain multiple double bonds in the *cis* form.^{5,6} For the PLMs there are three double bonds in the *cis* form ($\Delta^{12,13}$, $\Delta^{14,15}$, $\Delta^{2,3}$) and one in the *trans* form ($\Delta^{6,7}$).^{7,8}

The PLM biosynthetic gene cluster has been cloned and sequenced and shown to encode a modular PKS.⁹ Modules 1 and 2 contain the expected dehydratase DH-KR didomain required for formation of the conjugated diene, while module 5 contains a DH-KR domain likely responsible for formation of the *trans* $\Delta^{6,7}$ alkene. The KR-DH domains which generate a *trans* double bond do so via a D-3-hydroxyacyl intermediate (determined by the KR domain).

2-4 The L-3-hydroxyacyl product is the speculated intermediate in KR-DH domains which generate *cis* double bonds.^{3,5} A bioinformatic analyses of the KR domain in module 7 suggests it may generate a 1-3-hydroxyacyl intermediate. However, there is no cognate DH domain (Figure 1). The DH domain is also absent in the respective terminal PKS modules involved in biosynthesis of fostriecin and leptomycin,¹⁰ related natural products with an unsaturated lactone moiety. We posited that a DH-independent post-PKS enzymatic catalyzed reaction might be involved in the formation of all of these *cis* $\Delta^{2,3}$ unsaturated lactone moieties. We have identified *plmT*₂ (and its homolog ORF 4 from the fostriecin biosynthetic gene cluster) as being required for efficient formation of this alkene.

The predicted PlmT₂ peptide sequence⁹ shows up to 50% sequence similarity to a series of a family of putative NAD dependent epimerase/dehydratases. We generate NP7, a *plmT*₂ + *plmS*₂ deletion mutant. It has been shown that a *plmS*₂ deletion mutant (NP2) generates exclusively PLM B⁹ (Figure 2) and that PlmS₂ catalyzes C18-hydroxylation of PLM B (subsequent acylation then provides the final PLM products, PLM A and PLM C-F.¹¹ Thus a PLM B analog with a saturated lactone moiety and a C-3 hydroxyl substituent was one of the possible products generated by the NP7 mutant. Indeed, HPLC analyses of the fermentation broth of this mutant (Figure 2) revealed a major new and more hydrophilic PLM product, which was purified following standard protocols¹² and shown to contain the predicted saturated lactone and C-3 hydroxyl substituent. However, spectroscopic analyses (¹H- and ¹³C-NMR, and MS) revealed that the C-3 hydroxyl substituent was esterified with malonic acid. This new malonylated PLM B (M-PLM B, Figure 1), is unstable in mild basic conditions and rapidly undergoes an elimination reaction to provide PLM B and either malonic acid or acetic acid (acetic acid is observed by GC-MS analysis of the degradation products, but it remains to be determined at which stage the decarboxylation occurs). In contrast, saturated lactone compounds with a C-3 hydroxyl substituent have been generated both in vivo and in vitro by a modified form of DEBS PKS,¹³ and shown to be stable (there are no reports of formation of the corresponding unsaturated product). The low stability of M-PLM B is the most likely reason for the small levels of PLM B in the fermentation media of NP7 (Figure 2). PLM analogs or pathway intermediates in which the C-3 hydroxyl was not malonylated were not detected in NP7 fermentations, suggesting malonylation is an efficient process and may represent part of the normal biosynthetic process, facilitating the eventual formation of the unsaturated lactone. Thus one possible role for PlmT₂ is catalysis of a decarboxylative-elimination reaction with a malonylated polyketide pathway intermediate (Figure 3). Elucidation of the specific role of PlmT₂ in catalyzing formation of the unsaturated lactone remains undetermined, as does the question of which enzyme or catalytic domain is responsible for malonylation of the C-3 hydroxyl residue. Sequence analysis does not reveal any discrete AT proteins in the PLM gene cluster raising an intriguing possibility that AT in module 7 is responsible for malonylation of both the cognate ACP for the elongation process and the C-3 hydroxyl of the resulting extended PLM structure when it is attached to this ACP. We note that other polyketide natural products such as azalomycin F,¹⁴ malolactomycin A,¹⁵ shurimycins A and B¹⁶ contain malonyl esters and are almost certainly generated by modular PKSs. How these malonyl esters are formed has not been addressed, but it seems likely that there may be a similar malonylation process to that occurring in the PLM pathway. Production of M-PLM B indicates that modification steps (C-8 hydroxylation, phosphorylation of the C-9 hydroxyl and introduction of the C-25 amine functionality) of a PLM core skeleton can occur with the polyketide chain bearing a malonyl ester at the C-3 position. It has been shown that PlmS₂ catalyzes the C-18 hydroxylation of PLM B.¹⁷ To test if PlmS₂ can catalyze hydroxylation of the M-PLM B we generated an NP11 (Δ *plmT*₂) mutant in the wild strain (in which *plmS*₂ is present). In this mutant we observed formation of malonylated derivatives of PLM A, and PLM C-F (Figure 1), demonstrating that C-18 hydroxylation and subsequent O-18 esterification can occur with M-PLM B

A set of complementation experiments were carried out in the NP7 strain, using expression plasmids for *plmT*₂ (pNS5) and the *plmT*₂ homolog *fos ORF4* (pNS6). HPLC analyses (Figure 2) showed a complete restoration of PLM B with *plmT*₂ complementation (NP7/pNS5 strain), and almost complete (95% PLM B + 5% M-PLM B) for the complementation with the *fos ORF4* (NP7/pNS6 strain). These data supports the proposed role of PlmT₂ in formation of the unsaturated lactone of the PLMs and indicates that a similar process of malonylation and decarboxylative elimination may occur in the fostriecin biosynthetic process.

The unsaturated lactone of cytostatin and fostriecin plays an important role in the potent and selective activity (IC₅₀ of 1-3 nM) against protein phosphatase 2A (PP2A). This Michael acceptor is proposed to be required for formation of a covalent adduct with Cys269, unique to PP2A.¹⁸ In vitro assays have shown that various PLMs are selective but poorer inhibitors of PP2A (IC₅₀ values 3-40 μM).¹⁹ Nonetheless, PLM A targets PP2A in human fibrosarcoma cells HT1080 cells¹⁹ and Cys 269 on the catalytic residue of PP2A is essential for this interaction. Surprisingly, using a PP2A inhibitor activity studies under standard assay conditions¹⁹ we observed that the M-PLM B was slightly more effective (IC₅₀ = 12.1 ± 1.3 μM) than PLM B (IC₅₀ = 36.7 ± 4.0 μM) against PP2A (there was no decomposition of M-PLM-B to PLM B under the assay conditions). A more detailed investigation of the biological activity of the M-PLM analogs is underway.

In summary, efficient formation of unsaturated lactone in the PLMs and other related compounds has been shown to be dependent upon an enzyme such as PlmT₂, not a canonical DH domain in the modular PKS. Without PlmT₂, new PLM analogs bearing a C-3 hydroxyl group esterified with malonic acid are generated. The M-PLM B, under mild basic conditions, undergoes rapid elimination to generate the *cis* Δ^{2,3} alkene of the PLMs in a process that may reflect that catalyzed by PlmT₂. This catalytic process and formation of malonyl esters of polyketide products present new and as yet unanswered questions pertaining to processes catalyzed by modular PKSs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

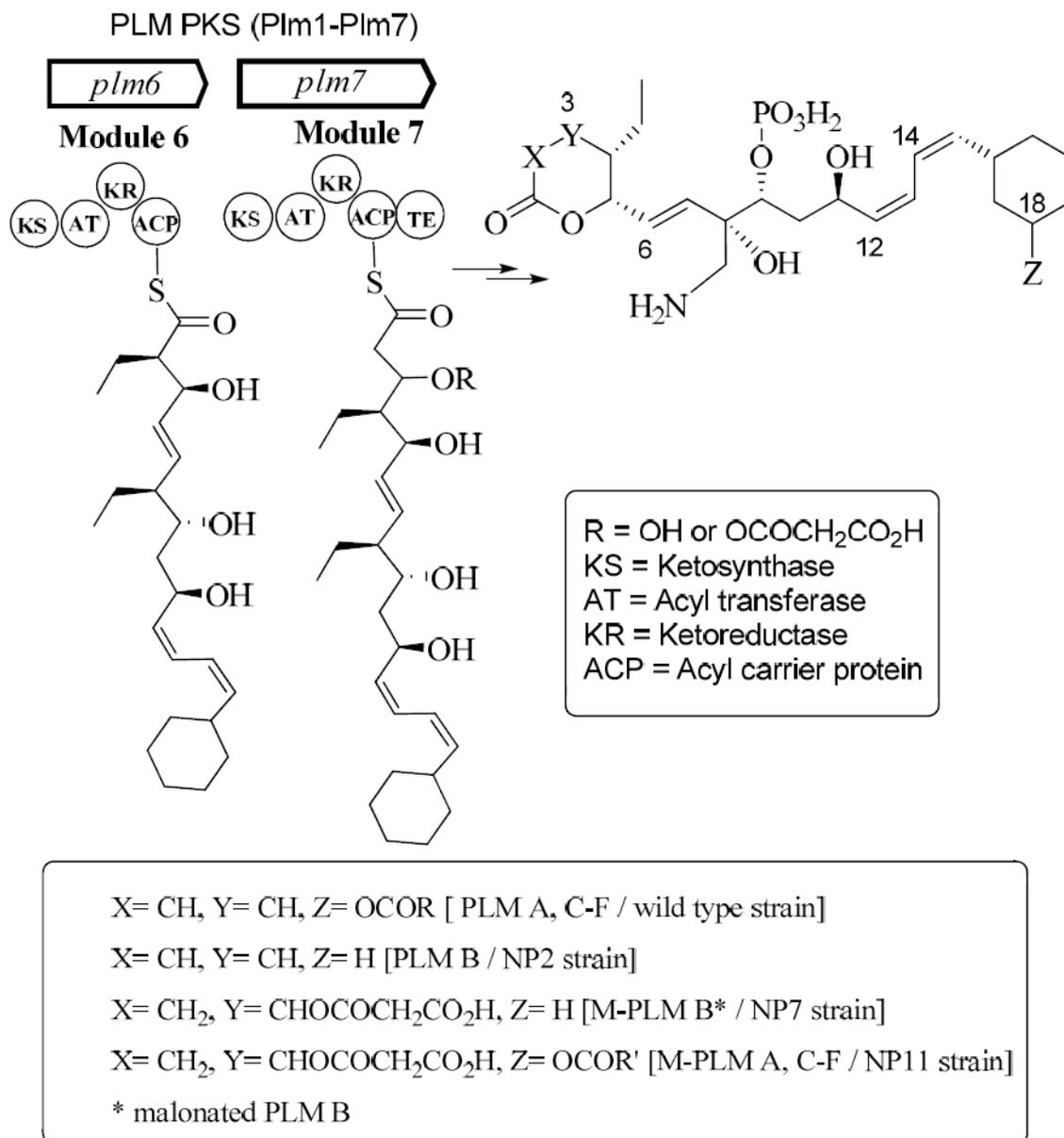
Acknowledgements

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**Figure 1.**

Proposed biosynthetic roles of module 6 and 7 of the PLM PKS and structures of PLM products made by *Streptomyces* sp. HK803 and its NP derivatives.

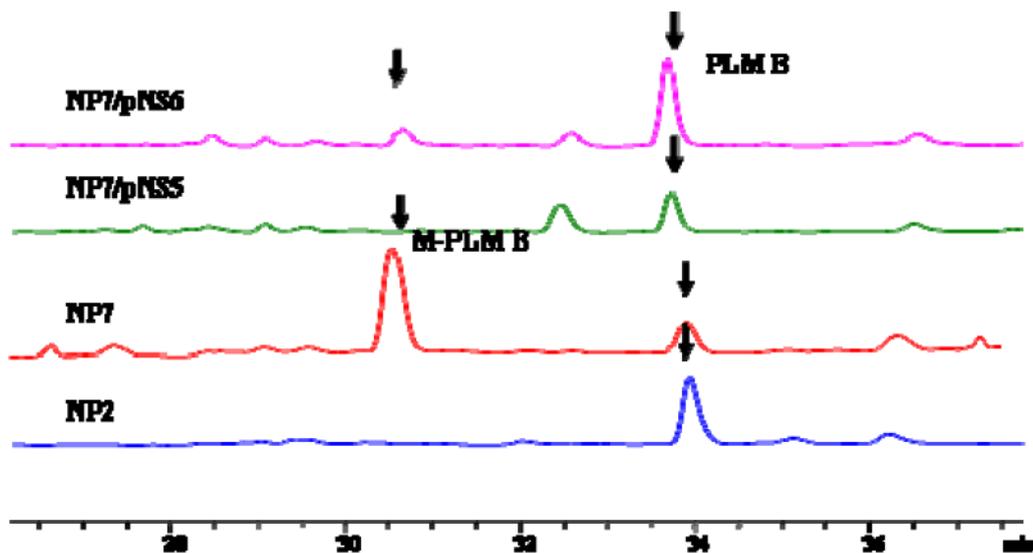


Figure 2. HPLC analyses of the fermentation broths of NP2 ($\Delta plmS_2$), NP7 ($\Delta plmT_2$ and $\Delta plmS_2$), NP7/pNS5 and NP7/pNS6 (PlmT₂ and fos ORF4 complementation plasmids, respectively).

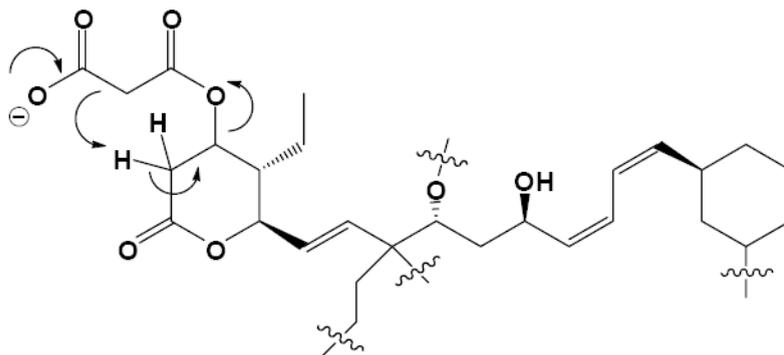


Figure 3. One possible role for PlmT₂ in catalyzing a decarboxylative elimination reaction of either M-PLM B or a pathway intermediate.