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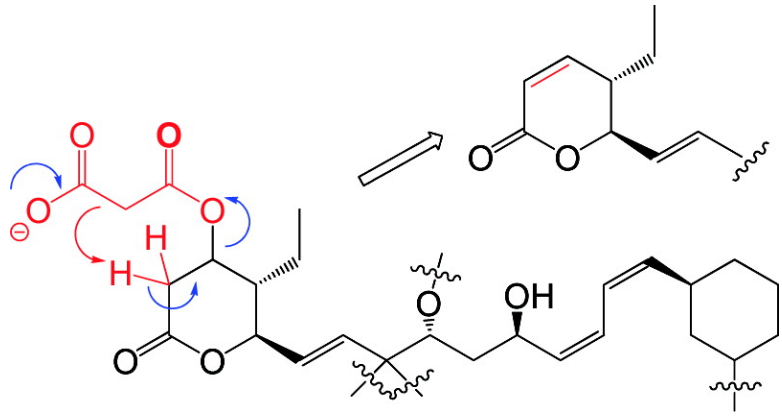
J. Am. Chem. Soc., **2008**, 130 (37), 12236-12237 • DOI: 10.1021/ja8044162 • Publication Date (Web): 21 August 2008



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Published on Web 08/21/2008

cis-$\Delta^{2,3}$-Double Bond of Phoslactomycins Is Generated by a Post-PKS Tailoring Enzyme

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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 majoritiy 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 l-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 homologue ORF 4 from the fostriecin biosynthetic gene cluster) as being required for efficient formation of this alkene.

The predicted PlmT$_2$ peptide sequence[9] 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 B[9] (Figure 2) and that PlmS$_2$ 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 analogue 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 protocols[12] and shown to contain the predicted saturated lactone and C-3 hydroxyl substituent. However, spectroscopic analyses (^{1}H and ^{13}C NMR, 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

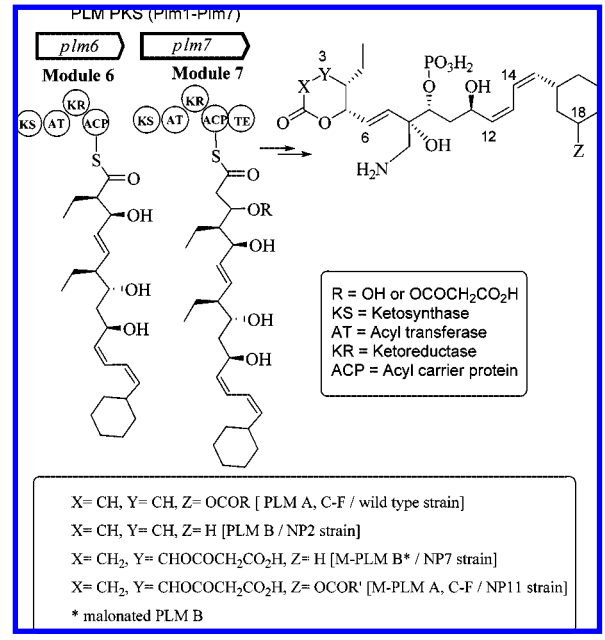

Figure 1. Proposed biosynthetic roles of modules 6 and 7 of the PLM PKS and structures of PLM products made by *Streptomyces* sp. HK803 and its NP derivatives.

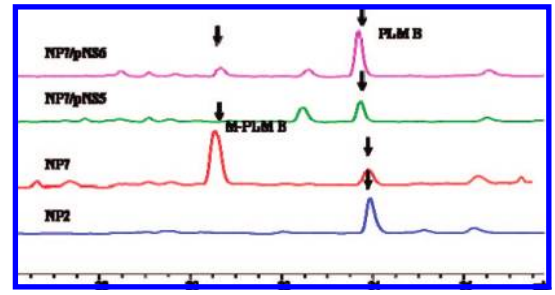

Figure 2. HPLC analyses of the fermentation broths of NP2 (Δ*plmS*$_2$), NP7 (Δ*plmT*$_2$ and Δ*plmS*$_2$), NP7/pNS5, and NP7/pNS6 (PlmT$_2$ and fos ORF4 complementation plasmids, respectively).

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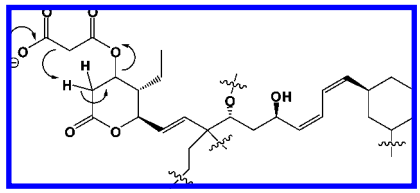

Figure 3. One possible role for $PlmT_2$ in catalyzing a decarboxylative elimination reaction of either M-PLM B or a pathway intermediate.

B is the most likely reason for the small levels of PLM B in the fermentation media of NP7 (Figure 2). PLM analogues 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 $PlmT_2$ is catalysis of a decarboxylative-elimination reaction with a malonylated polyketide pathway intermediate (Figure 3). Elucidation of the specific role of $PlmT_2$ 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] and shurimycins A and B[16] 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 $PlmS_2$ catalyzes the C-18 hydroxylation of PLM B.[17] To test if $PlmS_2$ can catalyze hydroxylation of the M-PLM B we generated an NP11 ($\Delta plmT_2$) 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 *plmT2* (pNS5) and the $plmT_2$ homologue *fos ORF4* (pNS6). HPLC analyses (Figure 2) showed a complete restoration of PLM B with $plmT_2$ complementation (NP7/pNS5 strain) and an almost complete one (95% PLM B + 5% M-PLM B) for complementation with *fos ORF4* (NP7/pNS6 strain). These data support the proposed role of $PlmT_2$ in formation of the unsaturated lactone of the PLMs and indicate 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 (IC_{50} 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 (IC_{50} values 3−40 μM).[19] Nonetheless, PLM A targets PP2A in human fibrosarcoma cells HT1080 cells,[19] and Cys 269 on the catalytic residue of PP2A is essential for this interaction. Surprisingly, using PP2A inhibitor activity studies under standard assay conditions,[19] we observed that M-PLM B was slightly more effective (IC_{50} =12.1 ± 1.3 μM) than PLM B (IC_{50} =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 analogues 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 $PlmT_2$, not a canonical DH domain in the modular PKS. Without $PlmT_2$, new PLM analogues 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* $\Delta^{2,3}$ alkene of the PLMs in a process that may reflect that catalyzed by $PlmT_2$. 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.

Acknowledgment. Funding for this research was supported by the National Institute of Health (A151629).

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

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JA8044162