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REVIEW

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Genetic manipulation of the biosynthetic process leading to phoslactomycins, potent protein phosphatase 2A inhibitors

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Abstract Phoslactomycins (PLMs) represent an unusual structural class of natural products secreted by various streptomycetes, containing an α,β-unsaturated δ-lactone, an amino group, phosphate ester, conjugated diene and a cyclohexane ring. Phosphazomycins, phospholines and leustroducsins contain the same structural moieties, varying only in the acyl substituent at the C-18 hydroxyl position. These compounds possess either antifungal or antitumor activities or both. The antitumor activity of the PLM class of compounds has been attributed to a potent and selective inhibition of protein phosphatase 2A (PP2A). The cysteine-269 residue of PP2Ac-subunit has been shown to be the site of covalent modification by PLMs. In this article, we review previous work on the isolation, structure elucidation and biological activities of PLMs and related compounds and current status of our work on both PLM stability and genetic manipulation of the biosynthetic process. Our work has shown that PLM B is surprisingly stable in solution, with a pH optimum of 6. Preliminary biosynthetic studies utilizing isotopically labeled shikimic acid and cyclohexanecarboxylic acid (CHC) suggested PLM B to be a polyketide-type antibiotic synthesized using CHC as a starter unit. Using a gene (*chcA*) from a set of CHC-CoA biosynthesis genes from *Streptomyces collinus* as a probe, a 75 kb region of 29 ORFs encoding PLM biosynthesis was located in the genome of *Streptomyces* sp. strain HK803. Analysis and subsequent manipulation of $plmS_2$ and $plmR_2$ in the gene cluster has allowed for rational engineering of a strain that produces only one PLM analog, PLM B, at ninefold higher titers than the wild type strain. A strain producing PLM G (the penultimate intermediate in PLMs biosynthesis) has also been generated. Current work is aimed at selective in vitro acylation of PLM G with various carboxylic acids and a precursor-directed biosynthesis in a *chcA* deletion mutant with the aim of generating novel PLM analogs.

Keywords Phoslactomycin · Protein phosphatase 2A inhibitor · Cyclohexanecarboxylic acid · Modular polyketide synthase

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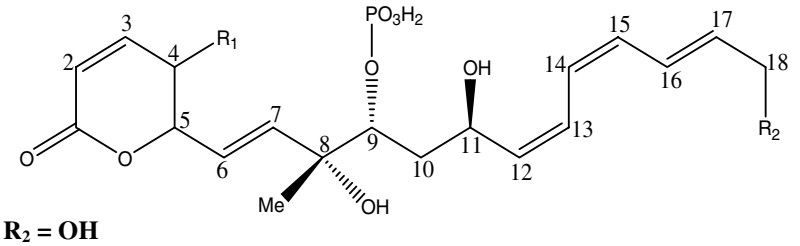
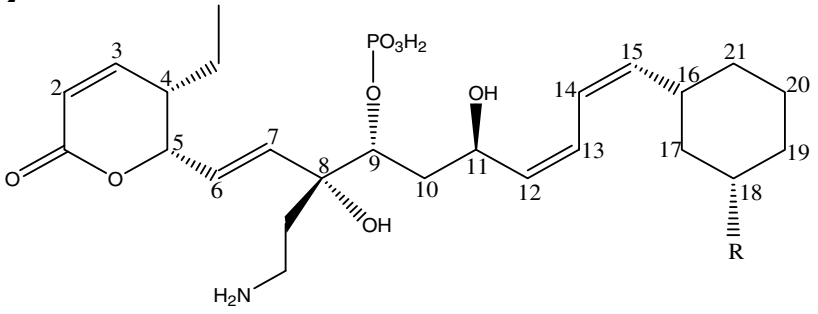
Introduction

Polyketides representing a large family of complex natural products built from simple carboxylic acid residues, by several bacteria and fungi, find wide ranging applications as antibiotics, immunosuppressants, antitumor agents and antiparasitic agents [13]. These important medicinal and agrochemical properties have led to significant interest in deducing, analyzing and manipulating the biosynthetic processes which produce these compounds. In recent years, particular attention has been placed on identifying the clustered biosynthetic genes and the subsequent application of this knowledge to either improve the fermentation process or access new natural product structures [36]. In this article, we review previous work on the isolation, structure elucidation and biological activities of phoslactomycins (PLMs) and related compounds (Fig. 1) and the current status of our work on genetic manipulation of the biosynthetic process.

Phoslactomycins represent an unusual structural class of compounds produced by various streptomycetes, containing an α,β-unsaturated δ-lactone, an amino group, a phosphate ester, a conjugated diene and a cyclohexane ring. These natural products possess either antifungal or antitumor activities or both [8, 16, 29, 43]. Phosphazomycins or phospholines and leustroducsins (LSNs) contain the same structural moieties [8, 16, 30, 43] and, in fact, all of these compounds vary only in the



Fostriecin R_1 = H, R_2 = OH
PD113, 270 R_1 = R_2 = H
PD113, 271 R_1 = R_2 = OH



Phoslactomycins (PLMs) and Leustroducsins (LSNs)

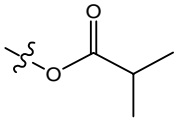
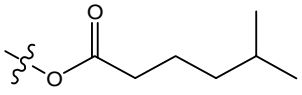
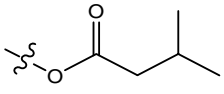
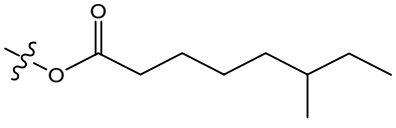
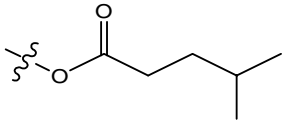
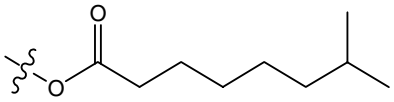
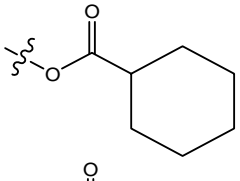
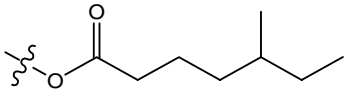
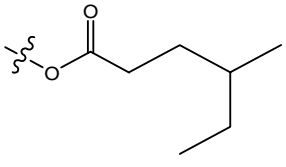
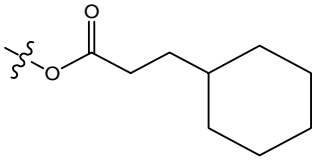
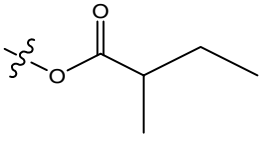
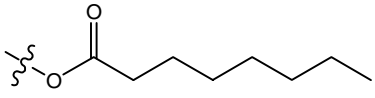
Name of compound	R	Name of compound	R
Phospholine/ PLM B	**H**	PLM G/ LSN H	**OH**
PLM A		LSN A	
PLM C/ Phosphazomycin C_1		LSN B	
PLM D		LSN C	
PLM E		I-h	
PLM F		I-i	
Phosphazomycin C_2		I-j	

Fig. 1 Structures of fostriecin, phoslactomycins (PLMs), and leustroducsins (LSNs). Various R groups distinguishing the analogs are also shown

C-18 acyl substituent (the hydroxyl group can be esterified with a wide array of carboxylic acids ranging in length from 4–9 carbons) [9, 17]. Fostriecin and the structurally related PD 113,270 and PD 113,271, are three related natural products produced by *Streptomyces pulveraceus* subsp. *fostreus* ATCC 31906 [41, 45]. Their structures differ from PLMs mainly by the presence of a methyl group at the C-8 position in place of an aminoethyl moiety and a hydroxymethyltrienyl group instead of a cyclohexyldienyl group (Fig. 1). Fostriecin has efficacious in vivo antitumor activity but has no significant antimicrobial activity [45].

Isolation, physico-chemical properties and structure elucidation of PLMs and LSNs

In 1985, Uramoto et al. reported an antibiotic complex from *Streptomyces* sp. No. HK-803 composed of at least four active components (phosphazomycin A through D), active against phytopathogenic fungi [46]. Phosphazomycin A, one of the main active components, was purified by preparative HPLC and was further characterized by UV and IR spectroscopy and NMR. A tentative molecular formula of $C_{37–38}H_{56–60}O_{12–13}NP$ was proposed for phosphazomycin A. Later, they reported the structure elucidation of phosphazomycin C—another active component [43]. Although purified phosphazomycin C gave a single peak in HPLC analysis, NMR spectroscopy revealed it to be a mixture of two components C_1 and C_2. Phosphazomycin C_1 is structurally identical to PLM C (Fig. 1).

In 1989, PLMs A through F were isolated from *S. nigrescens* SC-273 (the same PLMs/phosphazomycins have been subsequently shown to be produced by *Streptomyces* sp. No. HK-803 [8]) as new antifungal antibiotics containing α,β-unsaturated δ-lactone, an amino group, a phosphate ester, conjugated diene and cyclohexane ring moieties [8, 9]. These analogs were purified by butanol extraction, gel filtration and reverse phase chromatography as a colorless powder, soluble mainly in methanol and sparingly soluble in water. Initial elucidation of their functional groups came from the positive reaction to ninhydrin and ammonium molybdate perchloric acid detecting primary amino group and a phosphate group, respectively. UV and IR spectroscopy revealed an α,β-unsaturated δ-lactone in their structure. High-resolution FAB-MS, ^{1}H and ^{13}C NMR were used to determine the molecular weight and molecular formulae. Each compound had the same antimicrobial spectrum despite the difference in their chemical structure.

Phospholine, an amphoteric compound, was isolated as an antitumor antibiotic from the fermentation broth of *S. hygroscopicus* [29]. It was shown to possess a δ-lactone and a phosphoric acid as functional groups by FAB-MS and NMR [30]. Phospholine is structurally identical to PLM B.

In 1993, three metabolites that induce the production of colony stimulating factors (CSFs) in vitro by bone marrow stromal cells were isolated from the culture broth of *S. platensis* SANK 60191 [16]. These metabolites were called LSNs (A through C). Their physicochemical properties and structure elucidation revealed that they belong to the PLM group of antibiotics [17]. LSN A has the same molecular formula and molecular weight as that of PLM F whereas LSN B and LSN C possess the same molecular formula ($C_{34}H_{56}NO_{10}P$; MW 669) but different acyl chains at the C-18 position (Fig. 1). The production of these various PLMs/LSNs in the fermentations presents clear challenge in terms of obtaining purified material for further study. Hence, Shibata et al. prepared LSN H—a derivative prepared by removing acyl moieties from the cyclohexane ring by treating the mixture of LSNs with porcine liver esterase [37]. LSN H is structurally identical to PLM G (Fig. 1). The authors further reported the absolute configuration of LSNs to be 4*S*, 5*S*, 8*R*, 9*R*, 11*R*, 16*R*, 18*S* using a modified Mosher method [38]. PLMs presumably possess the same absolute configuration. A convergent total synthesis of LSN B was successfully carried out by Shimada et al. in 2003 with the long-term objective that a series of additional studies on LSNs would lead to the development of a new class of potent and safe thrombopoietic agents [39].

Biological activity of the PLM and related compounds

The first report on antifungal activity of the PLM class of compounds came in 1985 wherein phosphazomycin A through D were shown to possess antifungal activity against phytopathogenic fungi [46]. Phosphazomycin A, purified by preparative HPLC, was shown to induce fungal mycelial swelling leading to growth inhibition. Purified phosphazomycin C was also shown to exhibit strong antifungal activity by a conventional agar dilution method [43]. Later PLMs A through F, differing only at the C-18 substituent, were all shown to possess same antifungal spectrum, thereby demonstrating that this moiety does not play a critical role for this biological activity [8].

Phospholine (PLM B) was reported as a novel antitumor antibiotic from *S. hygroscopicus* with the purified compound exhibiting in vitro toxicity against L1210, P388 and El-4 cell lines [29]. The first report on CSF-inducing activity by members of the PLM class appeared in 1993 [16]. LSNs from *S. platensis* SANK 60191 were shown to induce in vitro the production of CSFs by the stromal cell line KM-102. Four other structurally related compounds, PLM F and compound I-h, I-i and I-j (Fig. 1) produced by strain SANK 60191 were also shown to induce the production of both G- and GM-CSF by stromal cell line KM-102. The LSNs were shown to possess both antifungal and cytotoxic activities, similar to those of PLMs, consistent with their close

structural relationship [17]. LSN B induced thrombocytosis in mice on intraperitoneal administration [19]. Augmentation of host resistance against bacterial infection by treatment with LSN B was also shown in mice [18]. LSN H, a derivative enzymatically prepared by removing acyl moieties from the CHC ring of LSNs, was shown to cause thrombocytosis comparable to that of LSN B in mice. However, LSN H was found to be 100 times less effective than LSN B in vitro [37]. The dephosphorylated derivative of LSN H showed no significant thrombopoetic activity, indicating the phosphoric acid moiety to be essential for its activity. Recently, Shimada et al. have reviewed the biological properties and pharmacological activities of these low molecular weight thrombopoetic agents [40].

It has been shown in vitro that various PLMs are potent and highly selective inhibitors of protein phosphatase 2A (PP2A) with IC_{50} values ranging from 3.7 to 5.8 μM (PLMs are poor PP1 inhibitors with IC_{50} > 1 mM) [47]. It would appear that this activity is the basis for much of PLMs biological activity. Neutral killer (NK) cells are known to be critical for both innate and adoptive immunity against tumor development and selective augmentation of NK cells could suppress tumor metastasis. PLM A and LSN H were shown to augment NK cell activity in mice [14] and LSN H was shown to inhibit lung metastasis, supporting a role for PP2A inhibition in NK cell function. Most recently, Teruya et al. used a chemical genetic approach to describe a direct interaction between the PP2A catalytic subunit (PP2Ac) and its inhibitor, PLM A [42]. PP2A exists as a variety of trimers consisting of A, B and C subunits in mammalian cells, with the C subunit being the enzymatically active component [2]. PLM A was biotinylated and used as a probe to demonstrate that it directly binds to the PP2Ac in cells. Cysteine-269 residue of PP2Ac was shown to be the target of PLM A by systematic mutagenesis of PP2Ac [42].

Fostriecin, a structurally related novel phosphate ester is active in vitro against leukemia (L1210, IC_{50} = 0.46 μM), lung, breast and ovarian cancer, and also exhibits efficacious in vivo antitumor activity [20, 21]. Initially its activity was attributed to a direct, albeit weak ($IC_{50,}$ 140 mM) inhibition of the enzyme topoisomerase II [12]. However, recent studies have shown that fostriecin inhibits the mitotic entry checkpoint through the much more potent and selective inhibition of PP2A and PP4 (IC_{50} 1.5 and 3.0 nM, respectively) [1, 22]. The chemistry and biological activity of fostriecin has recently been reviewed [22].

The unique and selective biological activity of this class of natural products that target protein phosphatases has attracted considerable interest in recent years, particularly as an opportunity to generate new antiviral and anticancer agents. Phase I clinical trials of fostriecin were reportedly halted due to inherent drug instability and unpredictable purity in the clinical supply of the natural products [22]. In an attempt to address these limitations and further develop this class of novel antitumor agents, no less than six total syntheses of fostriecin were developed over a short period of 2 years [1, 6, 7, 26, 33, 50]. A total synthesis of LSN B has also been accomplished [39].

A complementary approach to these synthetic approaches is a genetic approach in which the biosynthetic process in the producing microorganism is manipulated. We have used PLM biosynthesis as the starting point for this work and have shown that PLM is surprisingly stable at neutral pH and that we can use genetic methods to maximize PLM production from fermentation processes. Subsequent work has set the stage for generation of new PLM analogs. Progress in all of these areas is reviewed.

PLM B stability studies

The kinetics of PLM B degradation was studied in the pH range of 2–10 at both 30 and 50°C by HPLC analysis [5]. The rate of reaction for the decomposition of PLM B was determined by regression analysis and the resulting k_{OBS} values for degradation of were used to construct a pH–rate constant profile (Fig. 2). A U-shaped profile was observed, suggesting PLM B can undergo both acid- and base-catalyzed decomposition (Fig. 2). The decomposition at a given pH could be described by the equation $k_{OBS} = k_H \times 10^{-pH} + k_{OH} \times 10^{pH-14}$, where k_H and k_{OH} are the rate constants in acidic and basic conditions (e.g. k_H = 45 ± 7 M^{-1} h^{-1}; k_{OH} = 448 ± 73 M^{-1} h^{-1} at 50°C). In the pH range 5–7, PLM-B exhibited relatively slow degradation, and at pH 6 it appears to have maximal solution stability with k_{OBS} = 0.0004 h^{-1} (50°C; $t_{1/2}$ = 72 days); approximately three orders of magnitude slower than at either pH 2.0 or 10.0.

Phoslactomycins B was found to decompose into three different products under basic as well as acidic aqueous conditions as evidenced by HPLC chromatograms [5]. Under alkaline conditions, ring opening of the

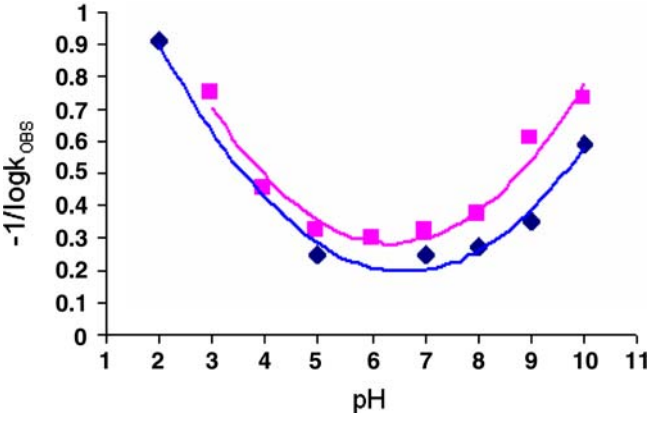


Fig. 2 Plot showing the rate constant–pH profile for the degradation of PLM B at 30°C (*filled diamond*) and 50°C (*filled square*). k_{OBS} at any given pH is the sum of $k_H \times 10^{-pH} + k_{OH} \times 10^{pH-14}$ (where k_H and k_{OH} are the rate constants in acidic and basic conditions) at that pH

unsaturated lactone of PLM B (**1**) gives **2** as the major product (Fig. 3), as confirmed by MS and ^{1}H-NMR analyses. The two other products **3** and **4** involve an initial addition reaction across the α,β-double bond to produce an intermediate product with subsequent lactone hydrolysis and/or methanolysis step. The two-step reaction for the formation of products **3** and **4** is slower than the direct one-step hydrolysis for the formation of **2**.

All three acidic degradation products showed a decrease of 18 Da (m/z 496 $[M + H]^+$) in mass but with different retention times consistent with formation of dehydration products. HSQC analysis revealed that a dehydration step provided a C_9–C_{11} phosphorinane derivative of PLM B (**5**) as one of the major products (Fig. 3). Compound **5** differs from a C-8/C-9 cyclic phosphorinane previously obtained by treatment of phospholine with dicyclohexylcarbodiimide [30]. A similar C-8/C-9 phosphorinane derivative of fostriecin has also been reported [3]. The remaining acid degradation products were shown to be a mixture of various dehydration products containing an additional double bond in the central core of the PLM B carbon skeleton. None of the PLM B degradation products exhibited significant antifungal activity, demonstrating that an intact lactone ring, the phosphate group and the C-8 hydroxyl group, all contribute to its antifungal activity. These groups have been shown to be important for the selective protein phosphatase activity of fostriecin [3]. The apparent stability of PLM B provides an exciting base for the continued development of this class of compounds for pharmaceutical applications.

Preliminary biosynthetic studies

Phoslactomycins B was proposed to be a polyketide-type antibiotic that uses cyclohexanecarboxylic acid (CHC) as starter unit [35]. Previous biosynthetic studies of ansatrienin A [4, 27] and ω-cyclohexyl fatty acids [28] have all revealed that the CHC moiety was biosynthesized from shikimic acid. A pathway involving a specific sequence of dehydrations and double bond reductions has been determined (Fig. 4). It was proposed that a similar pathway might also provide the CHC starter unit for PLM B biosynthesis [35]. An incorporation study with [2-^{13}C]shikimic acid revealed that it was efficiently incorporated into C-21 of PLM B [35], consistent with proposed pathway (Fig. 4). The other PLMs were similarly labeled suggesting that they were also generated from a CHC starter unit and that PLM B might be an intermediate in the pathway to production of PLM A and PLM C-F.

This initial experiment did not rule out alternative possibilities in which these PLMs are generated by using a *cis*-3-hydroxycyclohexanecarboxylic acid. Such a starter unit might originate by hydroxylation of CHC or

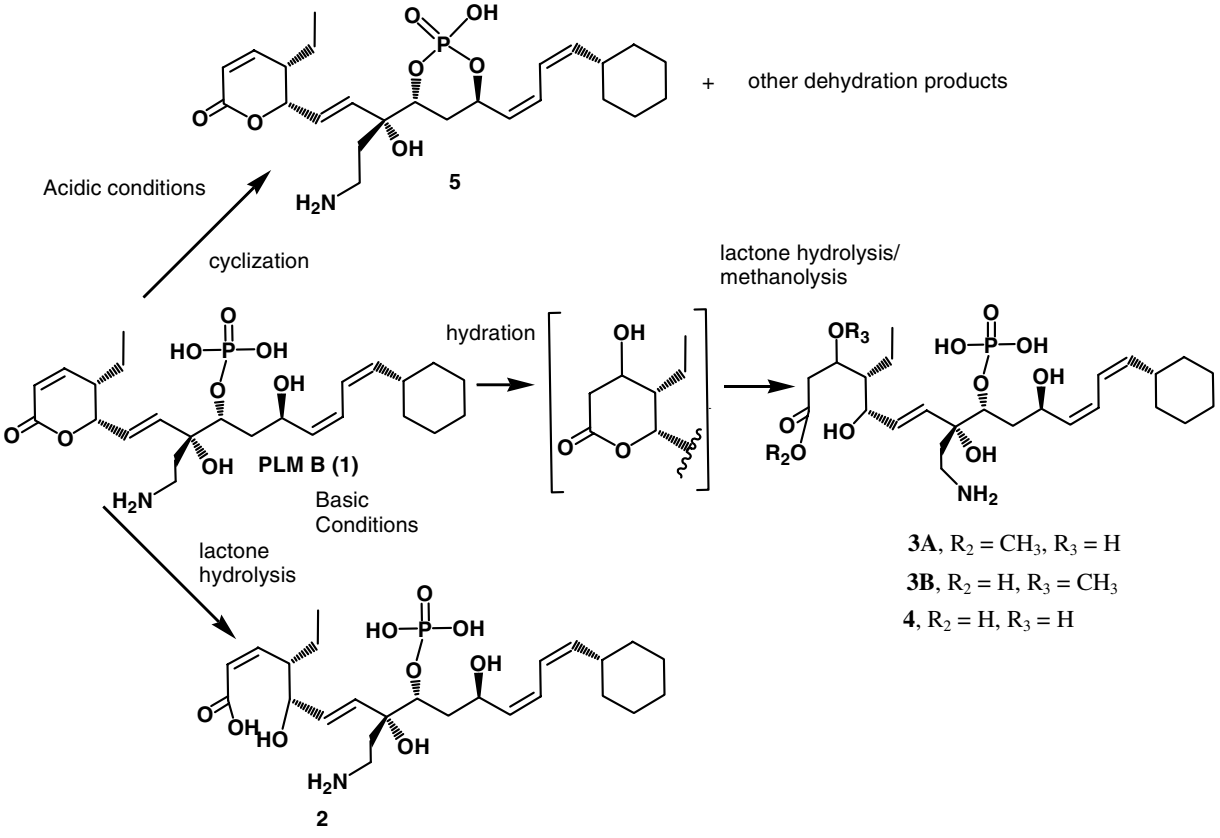


Fig. 3 A proposed degradation mechanism for PLM B in acidic and basic aqueous medium

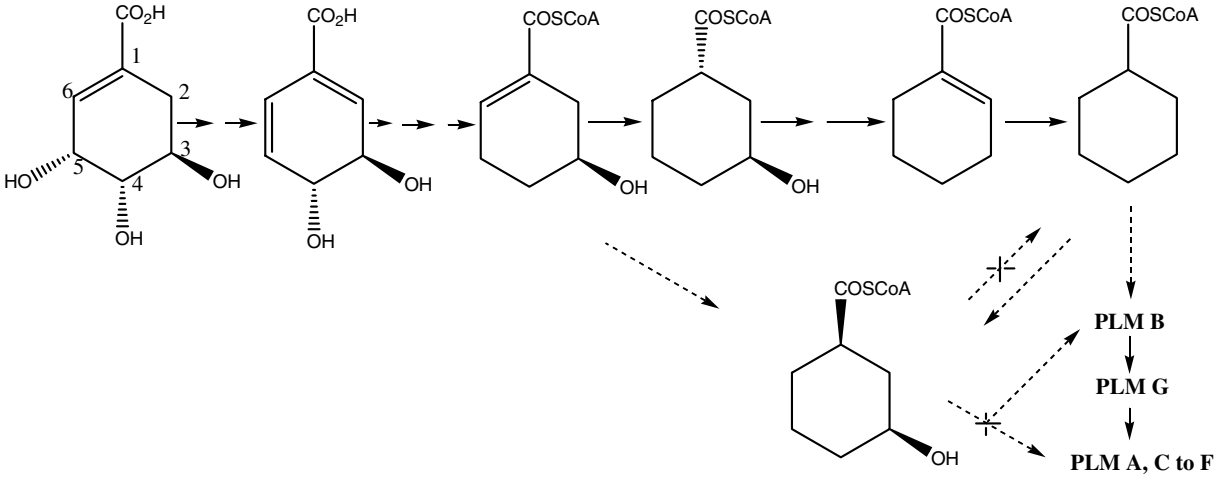


Fig. 4 Proposed pathway from shikimic acid to a cyclohexanecarboxylic acid derivative used to initiate PLM biosynthesis

from a branch point in the CHC pathway (shikimic acid provided the dihydroxycyclohexanecarboxylic acid starter units for FK-520 [34, 48], and rapamycin [23, 24] biosynthesis, by pathways similar but distinct to that used to generate to CHC). Feeding experiments with *cis*- and *trans*-3-hydroxy [7-^{13}C]cyclohexanecarboxylic acid did not result in production of labeled PLMs. This observation and an additional set of PLM labeling studies carried out using [7-^{13}C]- and [$^{2}H_{11}$]cyclohexanecarboxylic acid were all consistent with the proposed pathway (Fig. 4) in which CHC primes biosynthesis of PLM B which is further elaborated to other PLMs [31].

Identification and analysis of the PLM biosynthetic gene cluster

Based on the results of incorporation studies with labeled shikimic acid and CHC, the presence of a set of genes encoding enzymes for catalyzing biosynthesis of the CHC-CoA starter moiety in the PLM biosynthetic gene cluster was anticipated. A set of CHC-CoA biosynthetic genes, including the *chcA* gene, has previously been identified in *S. collinus* [49]. This *chcA* was used as a probe to identify the cosmid clone 3A11 from a cosmid genomic DNA library of *Streptomyces* sp. HK-803. This cosmid clone contained part of the PLM biosynthetic gene cluster and the entire set of CHC-CoA biosynthetic genes. Further screening yielded two overlapping cosmid clones, namely 10B4 and 3E5, which covered the entire PLM gene cluster. These three cosmids covering 97 kb of the *Streptomyces* sp. HK-803 genome were sequenced and analyzed. A 75 kb region encoding 29 ORFs belonging to PLM biosynthesis was located [31]. The sequence analysis of PLM biosynthetic gene cluster revealed the existence of a complete set of highly conserved CHC-CoA biosynthetic genes namely, *plmJK*, *plmL*, *chcA*, *plmM* as well as *plmI* (encoding a 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase) responsible for formation of the CHC-CoA starter unit (Fig. 5a). As in *S. collinus*, the *chcB* gene encoding the $\Delta^{2,3}$-enoyl CoA isomerase putatively responsible for catalyzing the penultimate step in the CHC-CoA pathway does not appear to be located close to the other CHC-CoA biosynthetic genes, or within the antibiotic biosynthetic gene cluster [32]. Six large ORFs encoding the core PLM PKS were identified and designated as *plm*1 through *plm7* based on their predicted modular role in PLM biosynthesis (Fig. 5b). The *plm*1 gene product consists of an initiation domain, presumably responsible for loading the CHC-CoA, and the first extension module 1, while Plm2–3 has two modules catalyzing the predicted second and third extension steps. The remaining PKS ORFs, *plm*4–*plm*7, each encode PKS monomodules. Plm7 has a thioesterase (TE) domain, consistent with a role in catalyzing the last extension step and subsequent release of the polyketide chain by formation of the α, β-unsaturated δ-lactone (formation of a six-membered lactone differs to many TE domains for type I PKSs studied to date, which catalyze formation of large 12–14 macrolactone structures under natural conditions) [25, 44]. Two of the PKS polypeptides Plm4 and Plm6 contain the same catalytic domains, precluding unambiguous assignment in this process. One of the many interesting features of the PLM PKS cluster is that it contains only three dehydratase domains, yet produces a compound with three *Z* and one *E* double bonds (Plm7 does not contain the predicted dehydratase domain).

The PLM gene cluster also contains *plm8*, which is predicted to encode a separate type II TE protein (these are known to play an important role in the editing and proofreading of PKSs [15]) and a crotonyl CoA reductase ($plmT_7$), presumably required for providing the ethylmalonyl CoA extender unit used in the fourth and sixth extension steps. The $plmT_5$ gene product has some sequence identity with homoserine kinases and is likely responsible for phosphorylation of the C-9 hydroxyl group and providing biological activity to PLM. Analysis of the gene cluster also predicts an oxidoreductase

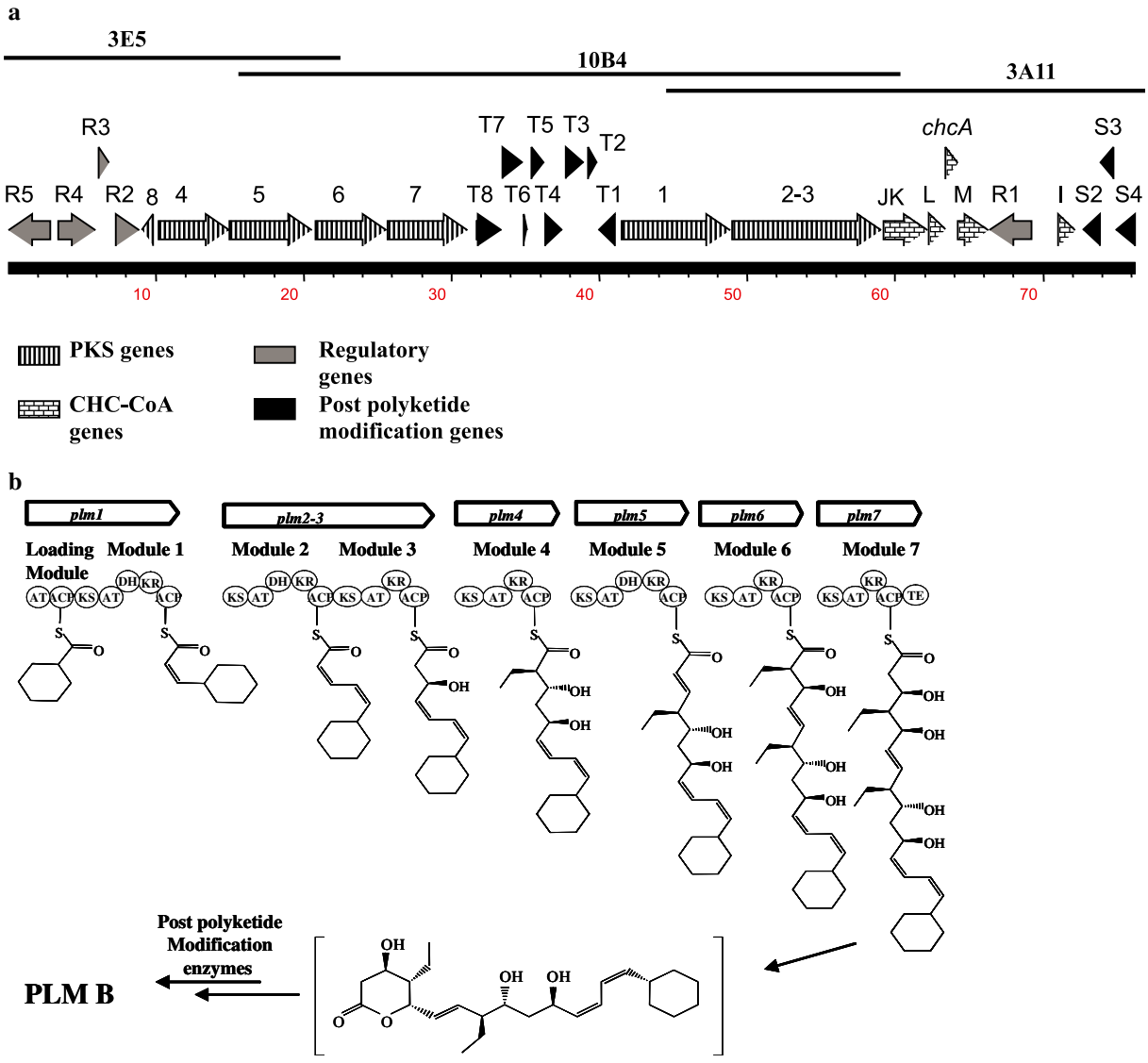


Fig. 5 **a** The PLM biosynthetic gene cluster. The sequence of the entire gene cluster and the proposed function for individual ORFs has been deposited in GenBank under accession number AY354515. **b** Proposed role for the six polypetides of the PLM PKS in PLM B biosynthesis. The structure of the PKS bound intermediates and the product released from the PKS (*in parentheses*) are hypothetical

(*plmT*$_8$) and an aminotransferase (*plmT*$_1$), likely to be involved in the replacement of a hydroxyl group of a C-8 hydroxyethyl side chain with an amine. The gene cluster also contains numerous other genes involved in regulation and resistance.

Genetic manipulation of PLM biosynthetic process

Generation and analysis of the *plmS*$_2$ mutant

Analysis of the PLM biosynthetic gene cluster revealed two ORFs, *plmT*$_4$ and *plmS*$_2$, both encoding proteins with high sequence similarity to each other as well as to cytochrome P-450 monooxygenases (CYPs) from several microorganisms, including those involved in modification of streptomycetes natural products [51]. These two proteins were predicted to be involved in the three post-PKS hydroxylation steps (C-18, C-8 and C-8 ethyl side-chain) [31]. Application of PCR targeting technology in wild type *Streptomyces* sp. HK803 allowed rapid gene replacement of *plmS*$_2$ with apramycin resistance gene [12]. The resulting NP1 mutant produced only PLM-B and at sixfold higher levels than the wild type strain (which produces PLM A through F) (Fig. 6). The exclusive production of PLM B by NP1 mutant clearly suggests a PLM biosynthetic pathway in which the final two steps are a PlmS$_2$-catalyzed C-18 hydroxylation of PLM B followed by esterification [31]. Hydroxylation at C-8 and the C-8 ethyl side chain are most likely catalyzed by the second CYP, encoded by *plmT*$_4$. A mutant strain, NP2 was generated from NP1 mutant of *Streptomyces* sp.

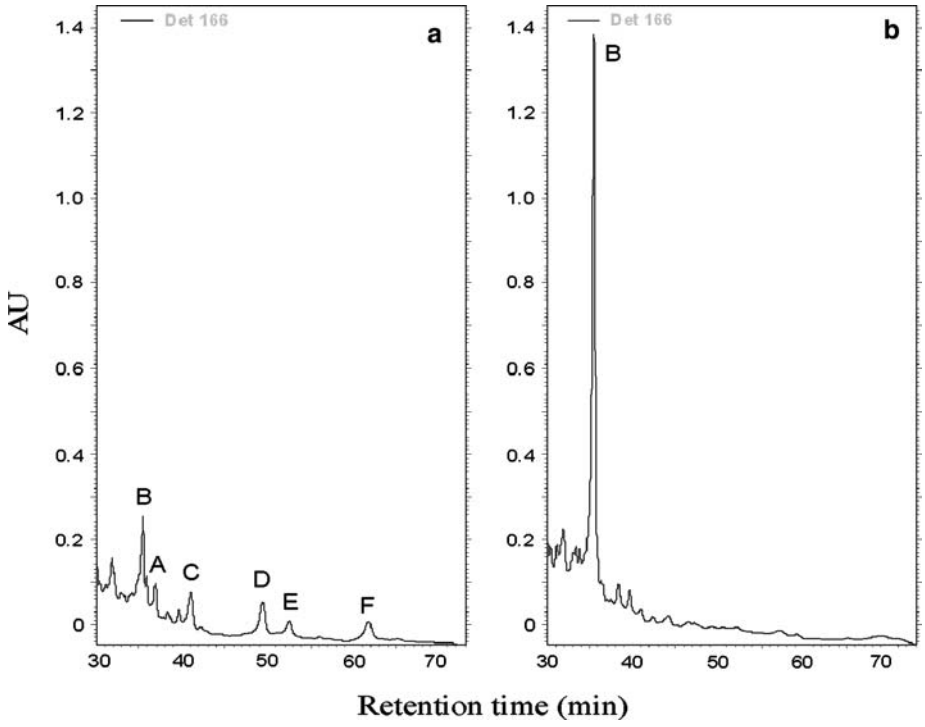


Fig. 6 HPLC chromatograms revealing production of PLMs by the (a) wild type and (b) NP1 mutant of *Streptomyces* sp. strain HK803; *AU* absorbance units at 235 nm

HK803 by deletion of the apramycin resistance gene (Table 1). This mutant produces PLM B exclusively and is serving as a basis for further genetic manipulation of the PLM biosynthetic process.

Complementation of the NP2 mutant

A plasmid pMSG1 with the *plm*S_2 under the control of an *ermE** promoter was constructed and used to complement the NP2 mutant strain. The resulting NP2/pMSG1 strain produced PLM A and PLM C through F [10]. No PLM B was observed in the NP2/pMSG1 in contrast to *Streptomyces* sp. HK803 or the NP1 and NP2 derivative. This observation is attributed to higher levels of $PlmS_2$ than in the wild type strain. The integrative plasmid used in this experiment is a pSET152 derivative and represents a convenient vector to deliver genes into the *Streptomyces* sp. HK803 or mutant strains for stable expression, and has become an important tool for subsequent genetic studies probing the role of individual *plm* genes in the biosynthetic process.

In vitro characterization of $PlmS_2$

Much of the promise for combinatorial biosynthetic approaches to drug discovery and development hinge on the modifying enzymes such as oxygenases and group transferases, which are crucial for the addition of important functional groups to polyketide skeletons. These enzymes are key to the structural diversity and biological activity of this class of natural products. The enzymes that modify the PLM core structure are thus of significant interest and an area of ongoing study. The first enzyme to be studied has been $PlmS_2$. The *plm*S_2 ORF was over-expressed as an N-terminal polyhistidine tagged protein in *S. coelicolor* M511 [10]. The affinity purified $PlmS_2$ was shown to carry out a C-18 hydroxylation of PLM B generating PLM G (Fig. 7), the proposed final common intermediate in the PLM

Table 1 Derivatives of *Streptomyces* sp. strain HK803 used for manipulation of PLM biosynthesis

Strain/derivatives	Description
Streptomyces sp. strain HK803	Wild type; produces PLMs A through F
NP1	*plm*S_2 deletion mutant: Ap^r : produces only PLM B
NP2	*plm*S_2 deletion mutant: Ap^s : produces only PLM B
NP2/ pMSG1	Produces PLM A and PLM C through F (no PLM B)
NP8	*plm*S_2–*plm*S_3 deletion mutant: Ap^r: produces only PLM B
NP8/ pMSG1	Produces only PLM G
NP2/ pMSG2	Produces ~ 1.5 times PLM B than the NP2 mutant
NP3	*chcA* deletion mutant: Ap^r: produces PLM B only when grown in the presence of CHC

Ap^r apramycin resistance gene, Ap^s apramycin sensitive, *pMSG1* and *pMSG2* pSET152 derivatives with *plm*S_2 and *plm*R_2 under control of the *ermE** promoter, respectively

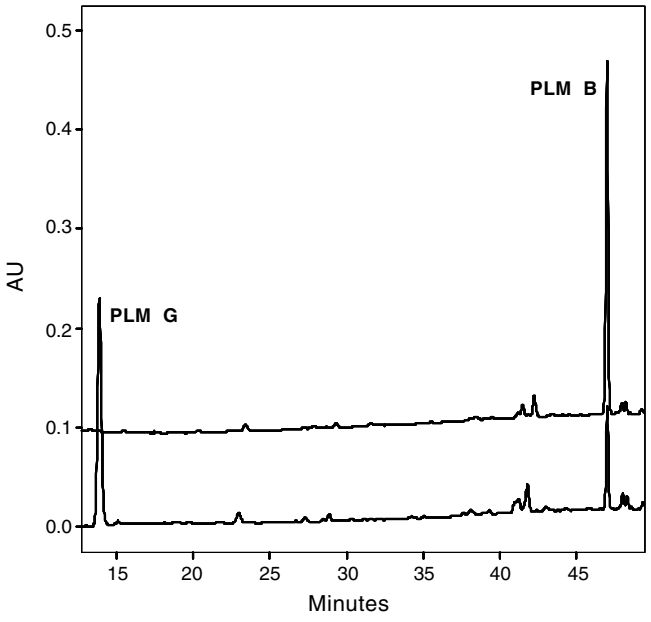


Fig. 7 HPLC chromatogram revealing in vitro conversion of PLM B to PLM G by $PlmS_2$. *AU* absorbance units at 235 nm

biosynthetic pathway. PLM G was observed to have less antifungal activity than PLM A–F, and is not observed in fermentations of either the wild type strain or NP2/pMSG1.

The $PlmS_2$ was highly specific for PLM B. Acid or base treatment of PLM B generates a range of related compounds, including products in which the lactone ring was hydrolyzed or the C-9 phosphate ester was converted to C-9/C-11 phosphorinane (Fig. 3). These compounds were not substrates for $PlmS_2$ [10]. The substrate specificity of native form of $PlmS_2$ likely will prelude the most facile direct application in generating the unnatural or hybrid natural products.

Generation of the *PlmS*$_3$ mutant and implication of a role as the acyl transferase

A gene encoding an acyl tranferase is expected to be responsible for the esterification of PLM G with various carboxylic acids to yield different analogs namely, PLM A and PLM C-F. Presumably, this enzyme can tolerate a broad range of substrates and plays a key role in determining the types of PLMs or LSNs made by a particular bacterial strain. An ORF adjacent to *plmS*$_2$ encodes a protein $PlmS_3$ which exhibits a very limited amino acid sequence similarity to other acyl transferases involved in modification of polyketide skeletons, and appears to be responsible for this step. A derivative strain, NP8 was generated from *Streptomyces* sp. HK803 in which both the adjacent *plmS*$_2$ and *plmS*$_3$ genes were replaced with apramycin resistance gene (Table 1). The NP8 mutant produced only PLM B, consistent with the proposal that $PlmS_2$ and $PlmS_3$ are required for subsequent steps in the pathway. Additional evidence for the role of $PlmS_3$ in esterification of PLM G was obtained by carrying out a complementation experiment using pMSG1 (a recombinant conjugative plasmid for expression of the *plmS*$_2$ gene) in the NP8 mutant. The resulting NP8/pMSG1 generated only PLM G (unpublished data). This observation clearly suggested that the *plmS*$_3$ gene product functions as an acyl transferase and catalyzes the final step in PLM biosynthesis. Biochemical characterization of $PlmS_3$ is being currently pursued in our laboratory with the objective of selective in vitro acylation of PLM G with various carboxylic acids.

Amplification of regulatory gene, *plmR*$_2$

Analysis of the regulatory genes in the antibiotic biosynthetic gene clusters provides crucial insight into the mechanisms of regulation, and opportunities to use genetic methods to enhance production titers (an issue of particular importance for commercial natural products). Analysis of the PLM biosynthetic gene cluster reveals five regulatory genes, labeled *plmR*$_1$ to *plmR*$_5$ (Fig. 5a). All of these genes are predicted to play a role in governing the expression of the PLM biosynthetic gene cluster and the levels of PLM production. Of these, *plmR*$_2$ showed significant homology with other *Streptomyces* antibiotic regulatory protein (SARP) family genes encoding a putative transcriptional activator. Increased expression of PLM B in the NP2 mutant was accomplished by the introduction of pMSG2, pSET152 derivative with the *plmR*$_2$ gene under control of the constitutive *ermE** promoter. In liquid culture, the resulting NP2/pMSG2 derivative generated about 50% more PLM B (117 ± 24 μg/ml) than the NP2 mutant (73 ± 18 μg/ml). Thus, by means of two different approaches, namely pathway engineering (generating the NP2 mutant) and over-expression of regulatory protein via pMSG2, we have achieved overall ninefold higher titers of PLM B than the wild type strain. The difference in PLM B production levels of the NP2/pMSG2 and NP2 in solid phase are currently under evaluation. Under solid-phase fermentation conditions, supplementation of the SY agar (sporulation medium) with CHC has consistently shown increased levels of PLM B production for both NP2 and NP2/pMSG2 strains. It seems likely from these preliminary analyses that manipulation of the fermentation media and conditions will lead to additional increases in PLM B titers.

Generation of *chcA* mutant and restoration of PLM B production

Preliminary biosynthetic studies with isotopically labeled shikimic acid, and presence of a complete set of highly conserved CHC-CoA biosynthesis genes in the PLM biosynthetic gene cluster prompted us to explore

the possibility of generating a wide range of novel PLM analogs by a mutational biosynthetic process. Previously, 1-cyclohexenylcarbonyl CoA reductase (ChcA) from *S. collinus* has been shown to catalyze in vitro reductive steps in the CHC-CoA biosynthesis pathway [49]. CHC-CoA biosynthesis in the PLM B producing NP2 mutant was blocked by replacing *chcA* with apramycin resistance gene with the help of PCR targeting technology mentioned above. The newly generated NP3 mutant lacked the ability to produce PLM B (Fig. 8, trace 1). However, supplementation of CHC (10 mg per 70 ml of production medium) restored PLM B production in *chcA*-deleted mutant under both liquid (Fig. 8, trace 2) and solid fermentation conditions. Studies on precursor-directed biosynthesis of novel PLM analogs with wide range of C-15 substituents, by means of feeding different carboxylic acids to NP3 mutant is currently underway in our laboratory.

Concluding comments

The work described herein represents the first report on the cloning of biosynthetic gene cluster for a potent and selective PP2A inhibitor. Analysis and subsequent manipulation of two genes namely $plmS_2$ and $plmR_2$ from the PLM gene cluster has allowed for the rational engineering of a strain that produces only one analog, PLM B, at significantly higher levels (ninefold higher) than the wild type strain. Some of the key drawbacks to the continued development of this class of naturally occurring compounds, including low fermentation titers and resolution of the various analogs produced in the fermentation (both contributing to concerns about drug purity), have now been overcome. Our work has also shown that the PLMs have significant solution stability.

Current efforts are now focused on the generation of novel PLM analogs with improved biological activity. One of the key structural differences between PLMs and fostriecin is the C-8 ethylamine substituent, which may contribute to less potent activity [47]. We have generated a *Streptomyces* sp. HK803 mutant in which $plmT_1$ which is predicted to encode an aminotransferase responsible for introducing this group has been deleted. Analyses of the products made by this mutant are ongoing. A combinatorial biosynthetic approach in which components of different natural product biosynthetic processes are combined, should allow for the generation of a novel hybrid structure bearing features of both fostriecin and PLM and is also underway. A third approach involves chemical modification of the amino group (such a modification of PLM A has been recently shown to lead to a 30-fold increase in PP2A inhibitory activity of PLM A [42]). In vitro acylation of PLM G with various carboxylic acids and precursor-directed biosynthesis in *chcA* deletion mutant of *Streptomyces* sp. HK 803 by feeding different carboxylic acids represent additional approaches which can be combined with these other approaches to further diversify the library of PLM B analogs that can be generated and evaluated. Results for these studies will be described in due course.

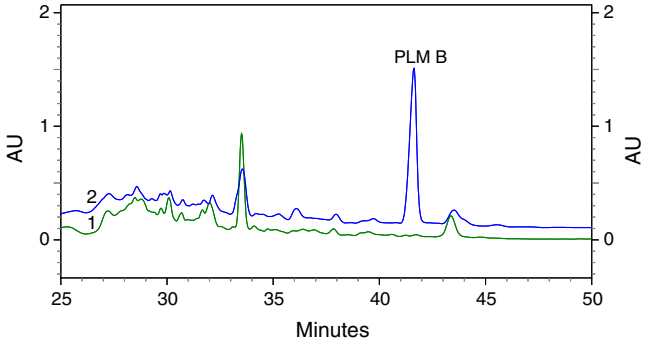


Fig. 8 Restoration of PLM B production by feeding cyclohexanecarboxylic acid (CHC) to fermentations of the NP3 mutant. HPLC C-18 column chromatogram of an Amberlite XAD 4 extract of fermentation broth of the NP3 mutant with (*line 2*) and without (*line 1*) CHC supplementation

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