Elucidating the mechanism of chain termination switching in the picromycin/methymycin polyketide synthase

Li Tang, Hong Fu, Melanie C Betlach and Robert McDaniel

Background: A single modular polyketide synthase (PKS) gene cluster is responsible for production of both the 14-membered macrolide antibiotic picromycin and the 12-membered macrolide antibiotic methymycin in *Streptomyces venezuelae*. Building on the success of the heterologous expression system engineered using the erythromycin PKS, we have constructed an analogous system for the picromycin/methymycin PKS. Through heterologous expression and construction of a hybrid PKS, we have examined the contributions that the PKS, its internal thioesterase domain (pikTE) and the Pik TEII thioesterase domain make in termination and cyclization of the two polyketide intermediates.

Results: The picromycin/methymycin PKS genes were functionally expressed in the heterologous host *Streptomyces lividans*, resulting in production of both narbonolide and 10-deoxymethynolide (the precursors of picromycin and methymycin, respectively). Co-expression with the Pik TEII thioesterase led to increased production levels, but did not change the ratio of the two compounds produced, leaving the function of this protein largely unknown. Fusion of the PKS thioesterase domain (pikTE) to 6-deoxyerythronolide B synthase (DEBS) resulted in formation of only 14-membered macrolactones.

Conclusions: These experiments demonstrate that the PKS alone is capable of catalyzing the synthesis of both 14- and 12-membered macrolactones and favor a model by which different macrolactone rings result from a combination of the arrangement between the module 5 and module 6 subunits in the picromycin PKS complex and the selectivity of the pikTE domain.

Address: KOSAN Biosciences, Inc., 3832 Bay Center Place, Hayward, CA 94545, USA.

Correspondence: Robert McDaniel
E-mail: mcdaniel@kosan.com

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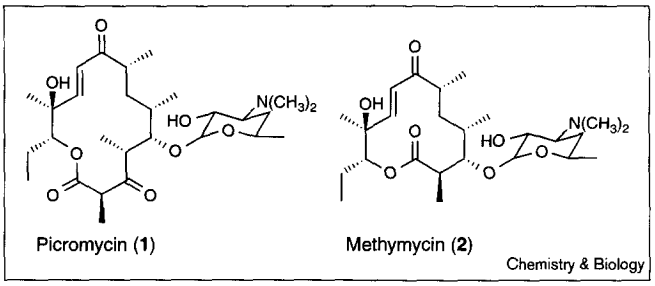
Introduction

Modular polyketide synthases (PKSs) are responsible for producing a large number of 12-, 14- and 16-membered macrolide antibiotics including methymycin, erythromycin and tylosin. These large, multifunctional enzymes (>300,000 kDa) catalyze the biosynthesis of polyketide macrolactones through multistep pathways involving decarboxylative condensations between acyl thioesters followed by cycles of varying β-carbon processing [1]. The ability to control aspects of polyketide biosynthesis, such as monomer selection, degree of β-carbon processing and stereoselectivity, by genetic manipulation of PKSs has stimulated great interest in the combinatorial engineering of novel antibiotics [2,3]. Picromycin (**1**; Figure 1) is of particular interest for such use because of its close structural relationship to ketolide compounds (e.g. HMR 3004), a new class of semisynthetic macrolides with activity against pathogens resistant to erythromycin [4]. Genetic systems that allow rapid engineering of the picromycin PKS would therefore be valuable for creating novel ketolide analogs for pharmaceutical applications.

A pivotal development for modular PKS engineering was the heterologous expression of the erythromycin PKS (6-deoxyerythronolide B synthase, DEBS) in *Streptomyces coelicolor* [5,6]. The advantages to this plasmid-based genetic system for DEBS were that it overcame the tedious and limited techniques for manipulating the natural DEBS host organism, *Saccharopolyspora erythaea*, it allowed more facile construction of mutant PKSs, and it reduced the complexity of PKS property analysis by providing a 'clean' host background. This system also expedited the construction of the first combinatorial modular polyketide library in *Streptomyces* [7]. Despite the success of this system, as well as the increased number of modular PKS gene clusters currently available, DEBS remained the only modular PKS reported to be expressed in a heterologous organism.

The picromycin gene cluster from *Streptomyces venezuelae* was recently cloned and sequenced by Sherman and coworkers [8] and in our own laboratories ([9]; M.C.B., unpublished observations). *S. venezuelae* is unique among macrolide-producing organisms because it produces primary polyketides with two different ring sizes. In addition to the 14-membered macrolide picromycin (**1**), the 12-membered macrolide methymycin (**2**; Figure 1) is also produced. Based on the structural similarities between

Figure 1



Structures of picromycin and methymycin.

picromycin and methymycin, it was speculated that methymycin would result from premature cyclization of a hexaketide intermediate in the picromycin pathway (Figure 2). Gene-disruption experiments have since confirmed that the same PKS is indeed responsible for producing the polyketide precursors to both compounds [8]. Although the mechanism for this dual polyketide chain-length specificity is not currently understood, analysis of the gene cluster has generated some hypotheses. Xue *et al.* [8] postulated that a separate external thioesterase (Pik TEII, Figure 3) located immediately downstream of the PKS might play a role in chain-length control. However, homologs of this enzyme are found in other macrolide gene clusters, such as erythromycin and tylosin, that do not produce analogs with alternative ring sizes [10,11]. Another possible explanation is that the covalent uncoupling of the two terminal modules (modules 5 and 6) could result in subunit complexes that promote cyclization of either intermediate. This mechanism was suggested to explain the premature release of triketide products from a trimodular DEBS system *in vitro* [12].

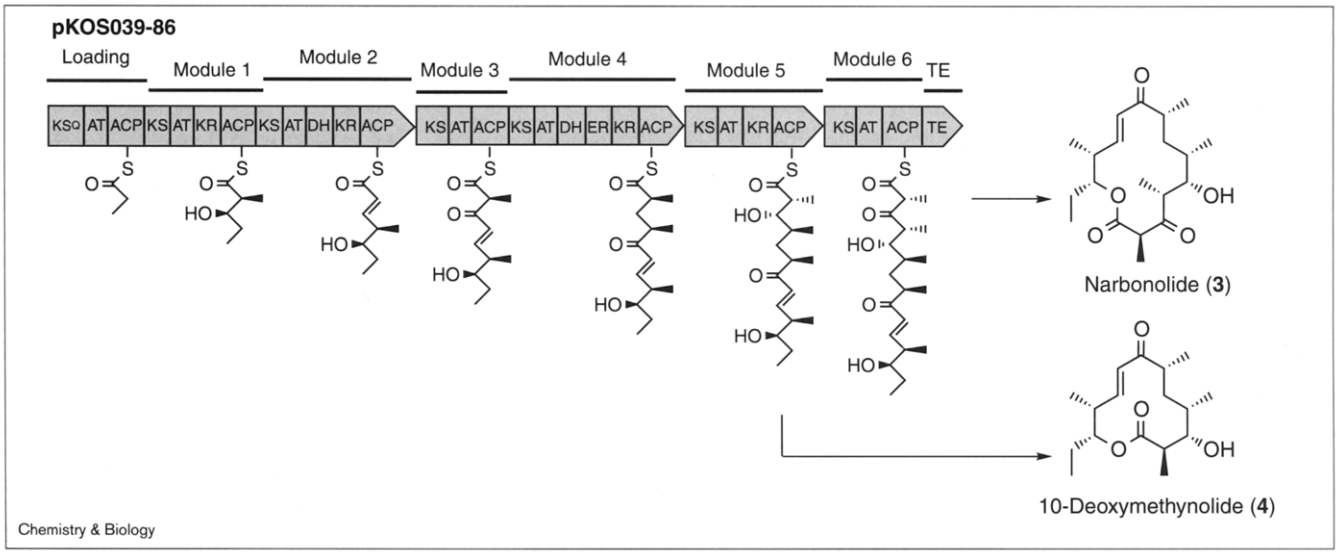
We have developed a plasmid-based expression system for the picromycin PKS as a complementary system to DEBS for studying macrolide PKSs. Heterologous expression of the complete picromycin PKS in *Streptomyces lividans* allowed us to examine the inherent ring-size specificity of the picromycin PKS and the influence that various PKS-associated thioesterases have in the formation of polyketide structures.

Results and discussion

Construction of a picromycin PKS expression system

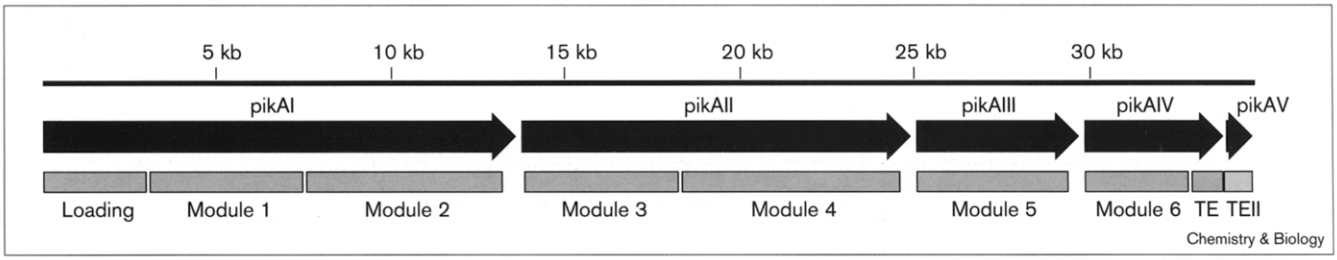
The vector used for expression of the picromycin PKS is analogous to that developed for DEBS [5]. The three DEBS open reading frames (ORFs) in pCK7 [5] were replaced with all four picromycin PKS ORFs to generate pKOS039-86. Like pCK7, pKOS039-86 is a shuttle vector that permits rapid manipulation in *Escherichia coli* and expression in *Streptomyces*. The host strain selected for this

Figure 2



Modular arrangement of the picromycin/methymycin PKS. The picromycin PKS consists of six modules, a loading domain and a thioesterase (TE) domain on four separate polypeptides. Each module possesses a ketosynthase (KS), acyltransferase (AT) and acyl carrier protein (ACP) domain, which together catalyze addition of each extender unit. Narbonolide (**3**), the polyketide precursor to picromycin (**1**), is derived from six propionates (loading, modules 1,3,4,5,6) and one acetate (module 2). Modules 1, 5 and 6 contain a ketoreductase (KR) domain for β-keto processing, modules 3 and 6 have no functional β-keto processing domains, module 2 contains KR and dehydratase (DH) domains, and module 4 contains KR, DH and enoyl reductase (ER) domains. 10-Deoxymethynolide (**4**) results from premature cyclization of the hexaketide intermediate from module 5. KS^{Q}, domain homologous to KS but which contains an active-site cysteine to glutamine mutation. The entire PKS gene cluster was subcloned to create expression plasmid pKOS039-86.

Figure 3



Organization of the picromycin/methymycin PKS genes and thioesterase domains. The external thioesterase, Pik TEII, is encoded as a separate polypeptide. The function of this class of thioesterase protein found in macrolide gene clusters is unknown.

study was *S. lividans* K4–114 [13], which contains a deletion of the entire actinorhodin (*act*) polyketide gene cluster and produces no known macrolides. This strain has a high transformation efficiency and a relatively low restriction barrier, eliminating the need to first pass the DNA through methylase-deficient strains, as is required for most *Streptomyces* species.

The picromycin PKS produces 12- and 14-membered macrolactones

Transformation of *S. lividans* K4–114 with plasmid pKOS039–86 resulted in a strain that produced two compounds in similar yield (~5–10 mg/l each). Analysis of fermentation extracts using liquid chromatography/mass spectrometry (LC/MS) and ^{1}H nuclear magnetic resonance (NMR) spectroscopy of the purified compounds established their identities as narbonolide (**3**) [14] and 10-deoxymethynolide (**4**) [15], the respective 14- and 12-membered polyketide precursors of picromycin (**1**) and methymycin (**2**; Figure 2).

The production of narbonolide in *S. lividans* represents the second reported expression of an entire modular polyketide pathway in a heterologous host. The combined yields of the two polyketides are similar to those obtained with expression of DEBS from pCK7 [5]. Furthermore, the ~1:1 ratio of narbonolide (**3**) to 10-deoxymethynolide (**4**) produced suggests that the picromycin PKS itself possesses an inherent ability to produce both 12- and 14-membered macrolactones without the requirement of additional activities unique to *S. venezuelae*. Although we cannot rule out the existence of a complementary enzyme present in *S. lividans* that provides this function, it would be unusual to find such a specific enzyme in an organism that does not produce any macrolide.

Co-expression with the Pik TEII thioesterase.

The possible role of the Pik TEII enzyme in picromycin/methymycin biosynthesis was examined by co-expressing this enzyme with the picromycin PKS. The *pikAV* gene encoding Pik TEII (Figure 3) was cloned into a *Streptomyces* genome-integrating vector under control of the same promoter (P*actI*) [5,6] that drives expression of the PKS. Transformation of *S. lividans* K4-114 with this vector resulted in *S. lividans* K39-18.

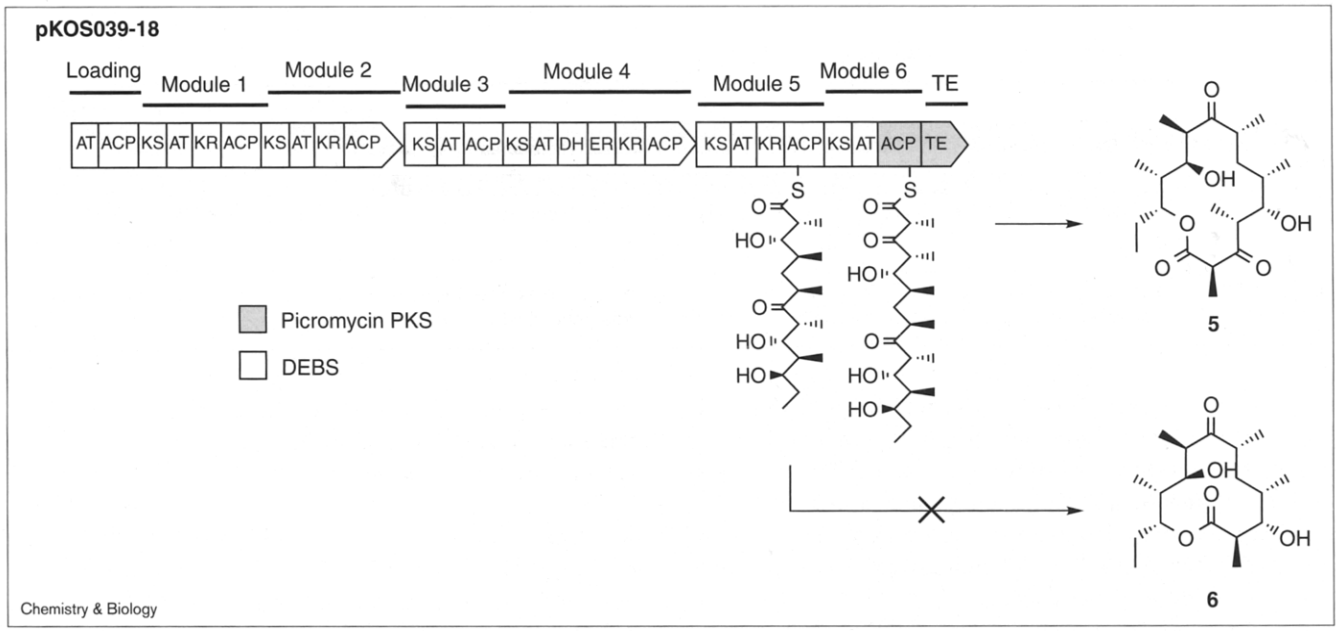
A comparison of strains K39-18/pKOS039-86 and K4-114/pKOS039-86 grown under identical conditions indicated that the strain containing Pik TEII produced 4–7 times more total polyketide. This indicates that the enzyme is functional in this strain and is consistent with the observation by Xue *et al.* [8] who report that yields fall to below 5% for both picromycin and methymycin when *picAV* is disrupted in *S. venezuelae*. However, the production levels of narbonolide (**3**) and 10-deoxymethynolide (**4**) from K39-18/pKOS039-86 increased by the same relative amounts, indicating that Pik TEII does not appear to influence the ratio of 12- to 14-membered lactone ring formation. The effect of Pik TEII on 6-deoxyerythronolide B (6-dEB) production by DEBS was also tested by co-expression. *S. lividans* K39-18 transformed with pCK7 failed to produce any 12-membered macrolide and had little or no effect on production levels of 6-dEB.

At present it remains unclear what function Pik TEII or its homologs provide during polyketide biosynthesis. Similar observations have prompted others [16,17] to propose that such thioesterase domains might serve as 'editing' enzymes that release aberrant thioesters from the PKS blocking polyketide synthesis. If so, the above result suggests that these enzymes may have some specificity towards their cognate PKS complexes in order to provide optimal activity.

Specificity of the pikTE domain

We examined whether the thioesterase domain encoded by the PKS (pikTE) could promote formation of 12-membered lactones in the context of a different PKS. A construct was generated, pKOS039-18, in which pikTE was fused with DEBS in place of its native thioesterase domain (Figure 4). In order to allow the pikTE to distinguish between substrates most closely resembling those generated by the picromycin PKS, the fusion junction was chosen between eryAT6 and picACP6 in order to eliminate

Figure 4



DEBS/pikTE hybrid PKS and polyketide product. Replacement of the DEBS TE domain with the pikTE domain leads to production of the expected 14-membered macrolide, but no 12-membered macrolides are generated. pKOS039-18 is the plasmid construct that expresses the hybrid PKS.

ketoreductase activity in module 6. This results in a mutant PKS that presents the pikTE with a β-keto heptaketide intermediate and a β-(*S*)-hydroxy hexaketide intermediate to cyclize, as in narbonolide or 10-deoxymethynolide biosynthesis. Analysis of the compounds generated by this construct indicated the production of the 14-membered ketolide 3,6-dideoxy-3-oxo-erythronolide B (**5**) [7], but the predicted 12-membered macrolactone, (8*R*,9*S*)-8,9-dihydro-8-methyl-9-hydroxy-10-deoxymethynolide — the product of a pentamodular DEBS system (**6**) [18] — was not detected (Figure 4). The pikTE domain, therefore, is incapable of forcing premature cyclization of the hexaketide intermediate generated by DEBS.

The cumulative results reported here indicate that the thioesterases found in the *pik* gene cluster do not determine the length of the polyketide chain and lend support to the notion that protein interactions between the module 5 and module 6 + TE subunits of the picromycin PKS play a role in formation of the two macrolides. This is consistent with recent *in vitro* studies by Gokhale *et al.* [19] using the DEBS thioesterase domain. They demonstrated that a DEBS module 3 + TE subunit could catalyze triketide cyclization from DEBS1 with tenfold higher turnover when compared to the DEBS TE domain alone. This suggested that interactions between DEBS1 and module 3 established a route for premature cyclization. If so, our results provide evidence that the decision by the pikTE domain to cyclize either a hexaketide or heptaketide intermediate is mediated by the formation of protein complexes between modules 5 and 6 of DEBS or the picromycin PKS. Together these studies support a general mechanistic model for polyketide chain termination in which interactions between modules or subunits in combination with the selectivity of thioesterase domains control the point of chain termination.

Utility of picromycin PKS/DEBS hybrid engineering

The above example illustrates how related modular polyketide gene clusters can be combined to gain insights into the basis of particular enzyme functions or specificities. The similarities between the picromycin and erythromycin pathways make them a very compatible system for hybrid engineering. The expression tool described here will facilitate the study of these pathways where differences occur or the pathways diverge. In the example above, it demonstrates utility for the 'fine tuning' of novel polyketide engineering. Compound **5** was originally generated by deletion of the KR6 domain in DEBS in an attempt to engineer a 3-ketolide-producing PKS [7]. Although the desired molecule was made, purification from this strain was hampered by the unexpected presence of 2-desmethyl ketolides that could not be easily separated. Extracts from *S. lividans* K4-114/pKOS039-18, however, do not contain the 2-desmethyl compounds, greatly simplifying purification.

Significance

The heterologous expression system used in this study was originally developed with the erythromycin polyketide

synthase (PKS). It has been a valuable tool for examining properties of modular PKSs because it allows rapid genetic engineering and expression of mutant PKSs. Despite the number of modular gene cluster sequences that have been reported since this development, no other examples of heterologous expression of modular PKSs have been reported. We have functionally expressed the complete picromycin PKS in the polyketide 'clean' host *S. lividans* K4-114, demonstrating the universal applicability of this system for modular PKS engineering. We have shown that the picromycin PKS alone is capable of producing both 12- and 14-membered macrolides without a requirement for any other activities. The unique dual specificity of the picromycin PKS allowed us to probe the mechanism by which different polyketide chain lengths can be synthesized by a single modular PKS. Experiments with the pikTE domain and the Pik TEII thioesterase domain suggest that subunit interactions between PKS modules appear to be the underlying determinant for this switch rather than the selectivity of thioesterase domains. We also confirm that TEII proteins, which are present in all macrolide gene clusters, may be important for optimal turnover of PKSs.

Materials and methods

Bacterial strains and culture conditions

DNA manipulations were performed in *E. coli* XL1 Blue (Stratagene) using standard culture conditions. *S. lividans* K4-114 [13] or *S. lividans* K39-18 (reported here) were used as hosts for expression of engineered PKSs. *S. lividans* K39-18 is derived from *S. lividans* K4-114 and contains an integrated copy of plasmid pKOS39-44 (see below). *S. lividans* transformants were grown on R2YE [20] agar plates with appropriate antibiotic selection. Liquid R2YE + 2 μg/ml thiostrepton was used for production of polyketides.

Manipulation of DNA and organisms

Manipulation and transformation of DNA in *E. coli* was performed using standard procedures [21]. The polymerase chain reaction (PCR) was performed using Pfu polymerase (Stratagene) under conditions recommended by the manufacturer. *S. lividans* protoplasts were transformed using the standard procedure [20]. Transformants with PKS expression plasmids were selected using 1 ml of a 1 mg/ml thiostrepton overlay when using *S. lividans* K4-114 and 1 mg/ml thiostrepton + 2 mg/ml apramycin overlay when using *S. lividans* K39-18.

Construction of PKS expression plasmids pKOS039-18 and pKOS039-86

The picromycin PKS expression plasmid, pKOS039-86, is a derivative of pCK7 in which the DEBS genes encoded between the *Nde*I site and *Eco*RI site have been replaced by the *picAI-IV* genes using conventional multistep cloning procedures. There is also an additional kanamycin antibiotic resistance marker cloned into the *Hind*III site of pCK7.

The pikACP6-TE domain used in construction of plasmid pKOS039-18 was PCR amplified using the following oligonucleotides (cloning sites in italics); forward 5′-TTT*ATGCAT*CCCGCGGGTCCCGGCGAG-3′ and reverse 5′-TCA*GAATTC*TGTCGGTCACTTGCCCGC-3′.

Expression plasmid pKOS39-18 was constructed by replacing the eryKR6 + ACP6 + TE domains of DEBS with the picACP6-TE *Nsi*I–*Eco*RI fragment in a pCK7 derivative containing an engineered *Pst*I site at the end of eryAT6 boundary [7].

Construction of the pikTEII integrating vector

The *pik*AV gene was PCR amplified from cosmid pKOS023–27 [9] using the following oligonucleotide primers (engineered cloning sites in italics): forward 5′-GT*CATATG*CTGGCTCGACGCCATCGAGG-GCA-3′ and reverse 5′-CC*TCTAG*AGGTCGTCGGTCACCGTGG-GTTCTGCCA-3′.

Plasmid pKOS39-44 contains the *Nde*I–*Xba*I fragment of the cloned *pik*AV gene inserted into the *Nde*I/*Spe*I sites of pKOS010-157 (R. Zierman and M.C.B., unpublished observations), a pSET152 [22] based integrating vector. This results in a vector which contains the same 2.8 kb *Hind*III–*Nde*I fragment from pCK7 [5], containing the *act*II-4 transcriptional activator and P*act*I promoter, upstream of *pik*AV.

Analysis of polyketide-producing cultures

Samples of fermentation broth (0.25–1 ml) were loaded onto to a (0.46 × 1 cm) C-18 precolumn, washed with approximately 5 volumes of water and eluted directly onto a (0.46 × 14 cm) C-18 high performance liquid chromatography (HPLC) column with a 0–100% acetonitrile in water gradient. Detection was performed with atmospheric pressure chemical ionization (APCI) mass spectrometry (LC/MS) for component analysis and light scattering detection (ELSD) for quantitation. Authentic samples of narbonolide (**3**) and 10-deoxymethynolide (**4**) were prepared as described below. A previously purified sample of 3,6-dideoxy-3-oxo-erythronolide B (**5**) [7] was also used for comparison. The mass and retention time of identified compounds matched those of the standards.

*Purification and characterization of narbonolide (**3**) and 10-deoxymethynolide (**4**)*

A 2 l shake flask culture of *S. lividans* K4-114/pKOS039-86 was grown for 7 days at 30°C. The mycelia was filtered and the aqueous layer was extracted with 2 × 2 l ethyl acetate. The organic layers were combined, dried over $MgSO_4$, filtered and evaporated to dryness. Polyketides were separated from the crude extract using silica gel chromatography (1:4 to 1:2 ethyl acetate:hexane gradient) to give a 10 mg mixture of narbonolide and 10-deoxymethynolide, as indicated by LC/MS and ^{1}H NMR. Purification of these two compounds was achieved by HPLC on a C-18 reverse phase column (20-80% acetonitrile in water over 45 min). This procedure yielded ~5 mg each of narbonolide (**3**) and 10-deoxymethynolide (**4**). The ^{1}-H NMR (300 MHz) spectra of both compounds were identical to those previously reported [14,15].

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