Analysis of Specific Mutants in the Lasalocid Gene Cluster: Evidence for Enzymatic Catalysis of a Disfavoured Polyether Ring Closure

ARTICLE *in* CHEMBIOCHEM · DECEMBER 2008

Impact Factor: 3.09 · DOI: 10.1002/cbic.200800585 · Source: PubMed

DOI: 10.1002/cbic.200800585

Analysis of Specific Mutants in the Lasalocid Gene Cluster: Evidence for Enzymatic Catalysis of a Disfavoured Polyether Ring Closure

Luke Smith,[a] Hui Hong,[a] Jonathan B. Spencer†,[b] and Peter F. Leadlay*[a]

Lasalocid is a highly atypical polyether ionophoric antibiotic, firstly because it contains a type of aromatic ring normally associated with fungal polyketides, and secondly because the formation of its tetrahydropyran ring appears to contravene Baldwin's rules, which predict the kinetically preferred routes for cyclisation reactions in organic chemistry. The lasalocid biosynthetic gene cluster has been cloned from Streptomyces lasaliensis*, and the* las *locus (73 533 bp) was found to contain seven modular polyketide synthase (PKS) genes, including all the activities necessary for the synthesis of the aromatic moiety. Specific deletion from the gene cluster of the flanking* lasC *gene, which is predicted to encode a flavin-linked epoxidase, abolished production both of lasalocid and of the minor cometabolite iso-lasalocid without leading to accumulation of an identifiable intermediate; this suggests that oxidative cyclisation to form the polyether rings takes place on the PKS before release of the full-length polyketide product. Meanwhile, a mutant in which the adjacent epoxide hydrolase* lasB *had been deleted produced iso-lasalocid only. Iso-lasalocid differs from lasalocid in the replacement of the tetrahydropyran ring by a tetrohydrofuran ring and represents the kinetically favoured product of cyclisation. The LasB epoxide hydrolase is therefore directly implicated in control of the stereochemical course of polyether ring formation during lasalocid biosynthesis.*

Introduction

Polyether ionophores are abundant and diverse polyketide natural products, isolated mainly from *Streptomyces* and allied Gram-positive filamentous bacteria.[1,2] They exert their antibiotic action by transporting specific cations across bacterial membranes, leading to collapse in membrane potential and cell death.[1] They have been widely used in animal husbandry[1,2] and have also shown activity against drug-resistant strains of the malarial parasite *Plasmodium falciparum*,[3,4] although their clinical use remains limited by their toxicity. Their structures are characterised by large numbers of chiral centres, some of which are established during polyketide chain synthesis on a giant modular polyketide synthase (PKS), and the remainder during the stereospecific oxidative cyclisation of the initially formed chain to form the characteristic polyether rings.[5] Despite recent advances, the molecular basis for this exquisite stereospecificity, and the exact sequence of enzymatic steps, remain unclear. Even less is known of the biosynthesis by marine dinoflagellates of notorious polyether toxins such as brevetoxin and maitotoxin,[6] easily the most structurally complex and poisonous of polyketide natural products. An added incentive to study terrestrial polyether ionophore biosynthesis is the possibility of using information gleaned from these simpler systems to guide the search for the marine toxin genes.[7]

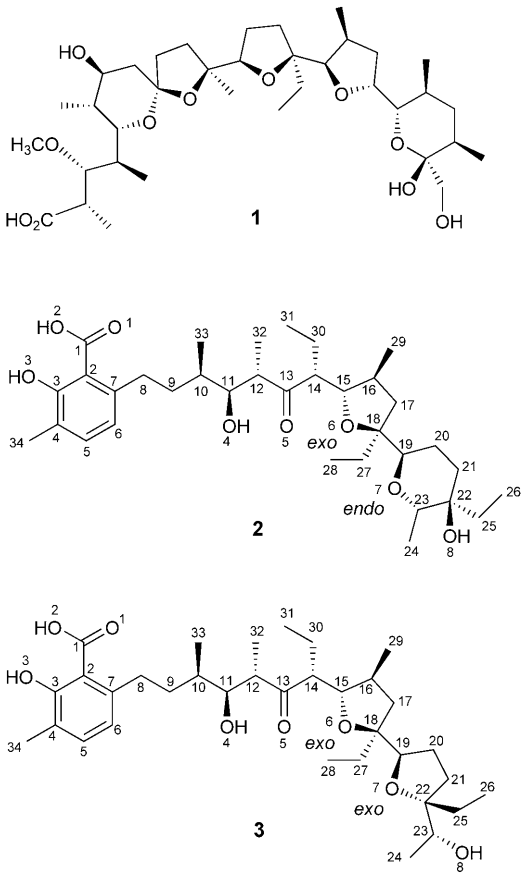
The best-understood polyether biosynthetic pathway is that for monensin A (**1**, Scheme 1) in *Streptomyces cinnamonensis*.[8] On the basis of studies with isotopically labelled precursors, a biosynthetic scheme has been proposed[9,10] involving formation of an all-*E* polyketide triene, followed by stereospecific epoxidation to a triepoxide and subsequent ring opening. This hypothesis has received support from subsequent genetic studies on the biosynthesis of monensin[11,12] and related ionophoric polyethers.[13–15] From analysis of specific *mon* mutants, it appears that the polyketide chain, which contains three *E* carbon–carbon double bonds,[16] is transferred to a separate acyl carrier protein (ACP) for oxidative processing, first by a single flavin-linked epoxidase (MonCI) to form a triepoxide,[11,12] and then by a novel class of epoxide hydrolase (MonBI, MonBII),[17] which catalyses the opening of the epoxide rings with concomitant formation of the polyether rings. MonB-like proteins are found in all ionophoric polyether gene clusters examined so far,[12–15,18] and these proteins are predicted to have protein folds essentially identical to that of an authentic terpene epoxide hydrolase.[19] Release of the fully formed monensin by a novel thioesterase (MonCII) appears to be the final step.[18] Analogous thioesterase enzymes appear to operate in the nigericin[14] and nanchangmycin pathways.[13,20] The kinetically favoured product of acid-catalysed conversion of the putative triepoxide intermediate into a polyether is predicted by Baldwin's rules[21] to be monensin, formed by consecutive

[a] *L. Smith, Dr. H. Hong, Prof. P. F. Leadlay*
Department of Biochemistry, University of Cambridge
Tennis Court Road, Cambridge CB2 1GA (UK)
Fax: (+44) 1223-766002
E-mail: pfl10@mole.bio.cam.ac.uk

[b] *Dr. J. B. Spencer*
Department of Chemistry, University of Cambridge
Lensfield Road, Cambridge CB2 1EW (UK)

[†] *Deceased 6th April, 2008.*

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.200800585.



Scheme 1. Structures of monensin (**1**), lasalocid (**2**) and iso-lasalocid (**3**). Polyether rings arising from either *exo-tet* (kinetically favoured) ring closure or *endo-tet* (disfavoured) ring closure, according to Baldwin's rules,[6] are labelled accordingly.

exo-tet ring closures to give tetrahydrofuran rings, rather than by the alternative, less favoured *endo-tet* ring closures that would afford tetrahydropyran rings.

In contrast, the biosynthetic route to the marine polyether toxins clearly involves a very large number of *endo-tet* ring closures, which form characteristic and striking "ladder" structures.[22] It is of great interest to determine whether MonB-like enzymes control this stereochemical outcome.[6,23] Unfortunately, the genetic investigation of marine polyether biosynthesis remains extremely challenging, so this mechanistic link has yet to be made. However, as previously pointed out,[6] the terminal tetrahydropyran rings of certain terrestrial ionophoric polyethers, including narasin, salinomycin and lasalocid, are clearly formed by a 6-*endo-tet* process, in apparent contravention of Baldwin's rules.[6] Their biosynthesis therefore offers a useful model system to study the same type of ring closure as is thought to occur in the marine polyethers. In the case of lasalocid (**2**, Scheme 1) from *Streptomyces lasaliensis*, a minor component of the wild-type fermentation has already been characterised as iso-lasalocid (**3**, Scheme 1),[24] the kinetically favoured product according to Baldwin's rules. The study of oxidative cyclisation in lasalocid biosynthesis therefore offers an excellent opportunity to study *exo-tet* and *endo-tet* ring closures in the same pathway (Scheme 1). Oikawa and his colleagues have recently accomplished the chemical synthesis of a structural analogue of the presumed diepoxide intermediate in lasalocid biosynthesis.[25] The diepoxide is converted to iso-lasalocid with dilute acid, and (predominantly) to lasalocid in the presence of a recombinant MonB-like protein, for which they have cloned the gene from *S. lasaliensis*.[26] Here, we present our independent sequencing and analysis of the entire lasalocid gene cluster from *S. lasaliensis*, together with the results of specific mutation of each of the presumed epoxidase (*lasC*) and epoxide hydrolase (*lasB*) genes. The findings point towards oxidative cyclisation taking place on enzyme-bound intermediates, and show that a functional *lasB* gene is essential in vivo in order to channel biosynthesis towards lasalocid rather than towards iso-lasalocid. Our analysis also provides the first evidence that formation of a benzenoid aromatic ring might be catalysed by a modular PKS.

Results and Discussion

Cloning, sequence analysis and organisation of the *las* gene cluster from *Streptomyces lasaliensis*

The strategy used to clone the lasalocid gene cluster was to screen a cosmid library of genomic DNA from the lasalocid-producing strain with a hybridisation probe consisting of 1.2 kbp of DNA encoding part of the ketosynthase (KS) domain of module 2 of the erythromycin-producing PKS. Since KS domains are the most highly conserved domains in canonical modular PKSs, this probe would be expected to detect all polyketide biosynthetic gene clusters containing such multienzymes. Positive colonies were end-sequenced, and colonies in which the ends showed PKS-encoding DNA were subjected to restriction mapping. Initial inspection of the deduced sequence from one region, to which several overlapping cosmids mapped, suggested that it might contain the *las* gene cluster. Further cosmids were identified by chromosome walking. The overlapping cosmids 4B12, U12G, S3F and M7G and subclones of them were shotgun sequenced, to give a total of about 110 kbp of DNA sequence. Computer-assisted analysis and comparison with genes in public databases revealed 53 open reading frames (orfs) including four pseudogenes. Of these, 16 could plausibly be assigned roles in lasalocid biosynthesis on the basis of database comparisons. The positions of the most relevant orfs are shown in Figure 1, and the putative functions of the orfs predicted to be involved in lasalocid biosynthesis are summarised in Table 1; the Supporting Information summarises the putative function of all flanking orfs. The complete sequence data have been deposited in the EMBL/GenBank database under the accession number AB193609.

Biosynthesis of the polyketide backbone of lasalocid

Seven large adjacent genes in the *las* cluster—*lasAI*–*lasAVII*—encode the modular PKS that assembles the polyketide chain of lasalocid (Figure 1). Detailed analysis of the constituent enzymatic domains (Table 1) confirmed the presence of a

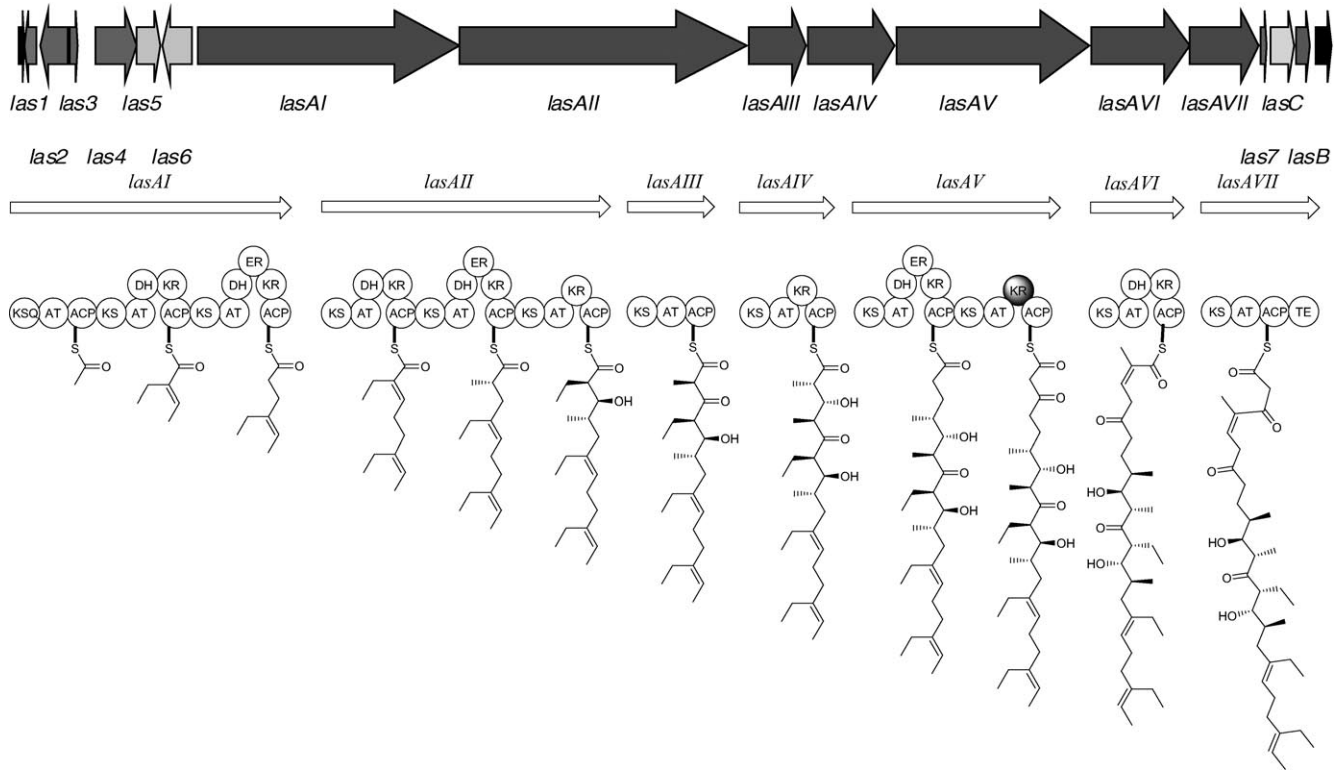


Figure 1. Genetic organisation of the lasalocid biosynthetic gene cluster and polyketide chain extension on the lasalocid PKS. The proposed functions of the gene products are summarised in Table 1. KS: ketosynthase; AT: acyl transferase; DH: dehydratase; ER: enoyl reductase; KR: ketoreductase; TE: thioesterase. The inactive KR of module 9 is not shown. KSQ[26] denotes a decarboxylase in the loading module.

decarboxylative loading module containing a so-called KSQ[27] domain, as well as of 11 extension modules. In almost all other polyether clusters that have been sequenced, a gene (*ksX*) has been found whose product catalyses polyketide chain transfer to a discrete ACP, where oxidative cyclisation then takes place.[18] Surprisingly, the final multienzyme in the *las* PKS assembly line has an integral C-terminal thioesterase, which is likely to catalyse direct chain release, as in macrolide PKSs.[28] The predicted linear polyketide triene product based on examination of the individual modules in the PKS is exactly that required to give rise to lasalocid, assuming the normal colinear functioning of a canonical modular PKS.[28] In particular, all the ring carbons of the aromatic ring appear to be provided by the action of the modular PKS, the first time that a modular PKS has been shown to be involved in synthesis of this moiety. We propose that aromatisation occurs as soon as the full-length PKS-bound intermediate is formed, as shown in Scheme 2, although no functionality on the PKS has yet been identified as involved in catalysis of this transformation. A clear precedent for this transformation is provided by the analogous aromatisation step in the biosynthesis of the fungal macrolactone zearalenone.[29] The double bond produced by module 10 is shown as *Z* in Figures 1 and Scheme 2, as this geometry is required for cyclisation to occur. However, the PKS-catalysed dehydration would normally be expected to give rise to a double bond with an *E* configuration. Either the *Z* double bond is formed directly, or the initially formed *E* double bond undergoes subsequent isomerisation. Inspection of the specificity motifs of the acyltransferase (AT) domains of each module[30–32] (Scheme 2) showed the pattern expected for incorporation of four propionate units and four acetate units onto an acetate starter unit.[9,33,34] The three AT domains predicted to incorporate ethylmalonate shared a specificity motif that differed from those reported for malonate and methylmalonate.

Analysis of the ketoreductase (KR) domain sequences revealed that KR domain of module 9 has a significant deletion in the sequence and also lacks an essential active site tyrosine residue[35] (Figure 1), which is consistent with the production of a keto group at this point in chain synthesis. Certain sequence motifs at KR active sites[35,36] of modular PKSs have been shown to correlate with the stereochemical course of reduction, and the KR domain of *las* PKS module 5 differs in these sequence motifs from those at the active sites of the other seven active KR domains (Figure 2), in full agreement with the alcohol configurations in the product being as shown in Figure 1. Similarly, the configuration shown in Figure 1 for the methyl branch in the fully reduced extension unit inserted by module 4, and required for lasalocid, is that anticipated from the presence of a key tyrosine residue in the active site.[37]

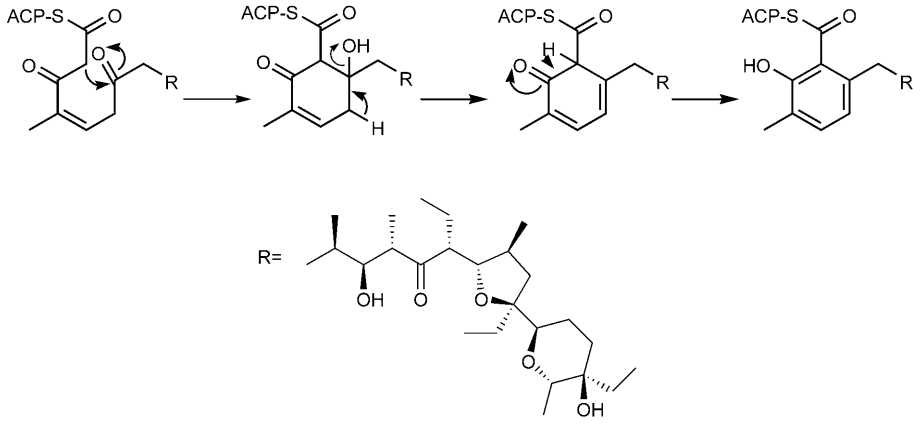
Genes involved in regulation, export, provision of precursors for Las biosynthesis, and in oxidative cyclisation

Flanking the PKS genes are several genes that, from their sequences, are predicted to encode important ancillary proteins

Table 1. Deduced function of genes in the lasalocid cluster.

ORF[a]	Size[b]	Homologue and origin	Identity/ similarity [%]	Proposed function
las1	249	dbj|BAC68117.1| (PteH) *Streptomyces avermitilis* MA-4680	59/71	type II thioesterase
las2	518	gb|AAO65793.1|AF440781_12 (MonT) *Streptomyces cinnamonensis*	54/72	resistance protein
las3	161	ref|NP_627826.1| *Streptomyces coelicolor* A3(2)	60/77	transcription regulator
las4	793	ref|NP_824078.1| *Streptomyces avermitilis* MA-4680	42/54	LuxR family regulator
las5	454	gb|AAQ84149.1| (PlmT7) *Streptomyces* sp. HK803	79/89	ethylmalonyl CoA synthase
las6	573	gb|AAQ84148.1| (PlmT8) *Streptomyces* sp. HK803	62/75	3-hydroxybutyryl CoA dehydrogenase
lasAI	4986	gb|AAZ77693.1| (ChlA1) *Streptomyces antibioticus*	53/63	polyketide synthase KS, AT, ACP, KS, AT DH, KR, ACP, KS, AT, DH, ER, KR, ACP
lasAII	5469	gb|AAX98191.1| (*orf16*) *Streptomyces aizunensis*	52/65	polyketide synthase KS, AT, DH, KR, ACP, KS, AT, DH, ER, KR, ACP, KS, AT, KR, ACP
lasAIII	1108	gb|AAZ94389.1| *Streptomyces neyagawaensis*	61/71	polyketide synthase KS, AT, ACP
lasAIV	1647	gb|AAX98190.1| (*orf15*) *Streptomyces aizunensis*	56/70	polyketide synthase KS, AT, KR, ACP
lasAV	3679	gb|AAX98191.1| (*orf16*) *Streptomyces aizunensis*	56/68	polyketide synthase KS, AT, DH, ER, KR, ACP, KS, AT, ACP
lasAVI	1877	gb|AAZ94389.1| *Streptomyces neyagawaensis*	55/67	polyketide synthase KS, AT, DH, KR, ACP
lasAVII	1311	gb|ABB52545.1| *Streptomyces* sp. KCTC 0041BP	48/59	polyketide synthase KS, AT, ACP, TE
las7	122	ref|YP_001108251.1| *Saccharopolyspora erythraea* NRRL 2338	38/56	putative regulator
lasC	472	dbj|BAE93732.1| (TmnC) *Streptomyces* sp. NRRL 11266	49/64	epoxidase
lasB	282	gb|ABC84468.1| (NigBI) *Streptomyces violaceusniger*	47/62	epoxide hydrolase

[a] A complete table of orfs is provided in the Supporting Information; [b] numbers refer to amino acid residues.



Scheme 2. Proposed mechanism of aromatisation to form lasalocid.

for lasalocid biosynthesis. Bioinformatic analyses suggest that the left-hand boundary of the cluster is defined by a gene encoding an IS10-like transposase (*orf6*) and three pseudogenes (*orf7–orf9*), adjacent to *las1*, which encodes a discrete thioesterase (TEII) highly typical of modular PKS-containing gene clusters and which is thought to play an activating (but not essential) role by hydrolysing mis-acylated PKS active sites.[38] There follow genes for a putative transporter (Las2) and for two putative transcriptional regulators (Las3 and Las4, the latter belonging to the LuxR family).[39] Adjacent to this lie a pair of genes predicted to encode crotonyl-CoA reductase and 3-hydroxyacyl CoA dehydrogenase, which are both likely to be involved in the provision of ethylmalonyl CoA, the source of the unusual butyrate units for the PKS. On the right-hand flank of the *las* cluster is found a small gene of unknown function (*las7*), possibly involved in regulation. Adjacent to *las7* are two genes that, from comparisons with previously analysed polyether clusters, are highly likely to encode i) a flavin-linked epoxidase (LasC) responsible for initiating oxidative cyclisation to form the presumed diepoxide intermediate, and ii) a single ring opening epoxide hydrolase (LasB). LasB resembles NanI in the nanchangmycin cluster[13] and appears to represent a head-to-tail fusion of the putative epoxide hydrolases MonBII and MonBI in the monensin cluster, and of NigBII and NigBI in the

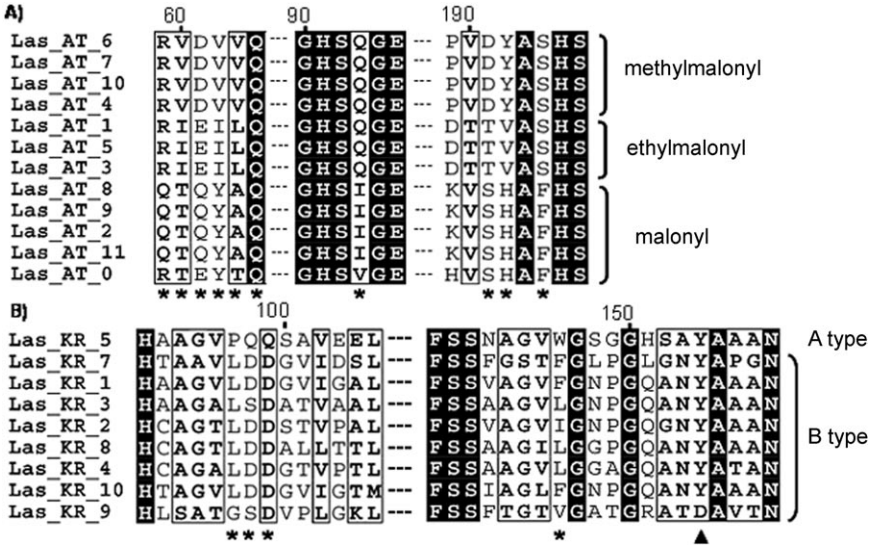


Figure 2. Multiple sequence alignment of the AT and KR domains from the lasalocid PKS. A) Alignment of specificity motifs from AT domains of the lasalocid PKS. Asterisks mark the conserved regions that determine substrate specificity. The substrate specificity is indicated on the right. B) Multiple alignment of specificity motifs of KR domains of lasalocid PKS. Asterisks indicate conserved amino acids that determine stereochemistry. The arrow indicates the active site tyrosine.

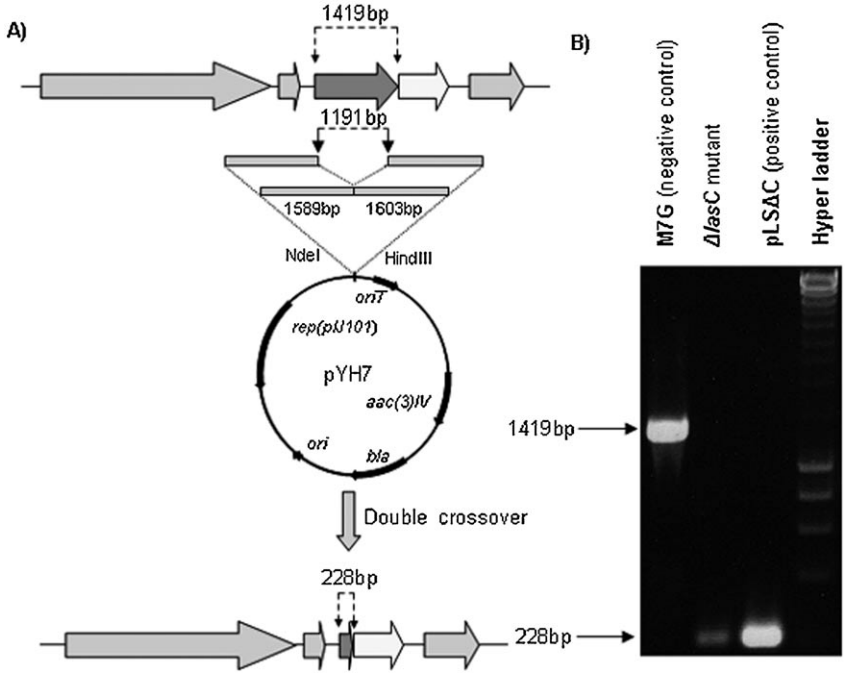


Figure 3. In-frame deletion of *lasC*. A) Schematic representation of the in-frame deletion in *lasC* through the use of shuttle vector pYH7. The numbers 1419, 1191 and 228, represent the expected size of the PCR product amplified from wild-type *S. lasaliensis*, the size of the internal deletion, and the expected size of the PCR product amplified from the *ΔlasC* mutant, respectively. The sizes of the flanking regions used to obtain homologous recombination between construct and chromosome are indicated. B) Confirmation of *ΔlasC* by PCR screening. Cosmid M7G served as a negative control and plasmid pLSΔC served as a positive control.

nigericin cluster,[14] respectively. None of the orfs beyond *lasB* appears to be involved in the biosynthetic pathway, although this remains to be confirmed by detailed mutational analysis.

Deletion of the *lasC* and *lasB* genes

An in-frame deletion of the *lasC* gene of *S. lasaliensis* was produced as described in the Experimental Section and illustrated in Figure 3. The mutant was grown in lasalocid production medium, and extracts were analysed by LC-MS and compared to those from wild-type *S. lasaliensis*. The LC-MS profile differed from that of the wild type in that no trace either of lasalocid or iso-lasalocid was found; this confirms that the *lasC* gene, which encodes a flavin-linked epoxidase, is essential for lasalocid production. Neither was there any evidence for the production of a "pre-lasalocid" diene nor any other lasalocid-related metabolite (see the Supporting Information).

To confirm that the loss of lasalocid production was caused by specific loss of the *lasC* gene, the mutant was complemented *in trans* by a plasmid-borne copy of *lasC* introduced under its native promoter (see the Supporting Information). The resulting recombinant *S. lasaliensis* strain was found by LC-MS analysis to produce both lasalocid (**2**) and iso-lasalocid (**3**), in the same ratio as in the wild type but in reduced yields (~25%). Although admittedly negative evidence, taken together these results strongly argue against the release from the PKS, during lasalocid biosynthesis, of "pre-lasalocid diene" as a free intermediate that is subsequently acted upon by LasC (epoxidase) and then LasB (epoxide hydrolase)—a mechanism recently proposed on the basis of model experiments with synthetic **5** and purified LasB.[26] Rather, we propose that the PKS-bound diene is the substrate for LasB and LasC. The exact timing of the formation of the aromatic ring (Scheme 2) cannot be deduced from the results of these experiments, but it is reasonable to assume that this also occurs before chain release, and probably before formation of the diepoxide.

An in-frame deletion was created in the *lasB* gene, as described in the Experimental Section. When this mutant was fermented in lasalocid production medium and extracts were analysed by LC-MS, the profile differed from that of the wild type in that no trace of lasalocid was found, and iso-lasalocid was the exclusive product (Figure 4), at about the same levels as produced by the wild type. To confirm that the loss of lasalocid production was caused by specific loss of the *lasB* gene, the mutant was complemented in trans by a plasmid-borne copy of *lasB* introduced under its native promoter (see the Supporting Information). The resulting recombinant *S. lasaliensis* strain was found by LC-MS analysis to produce iso-lasalocid (**3**) in a yield comparable to that of the wild type, together with lasalocid (**2**) at about 35% of the typical wild-type yield. If LasB controls the conversion of the PKS-bound diepoxide intermediate into a polyether, then in the *lasB* deletion mutant this intermediate should accumulate. The metabolites found in fermentation extracts should therefore represent the products of the chemically favoured process of epoxide ring opening in this intermediate. The observation that iso-lasalocid (**3**) is the sole product of a *lasB* mutant can be explained as the result of a kinetically favoured nonenzymatic 6-*exo-tet* cyclisation of both polyether rings in conformity with Baldwin's rules.

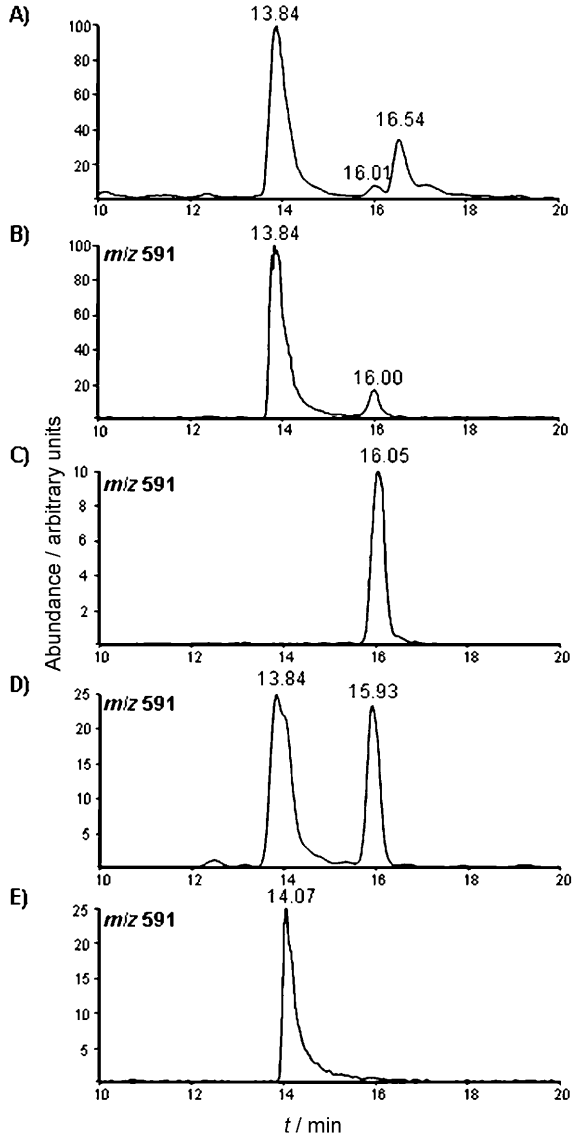
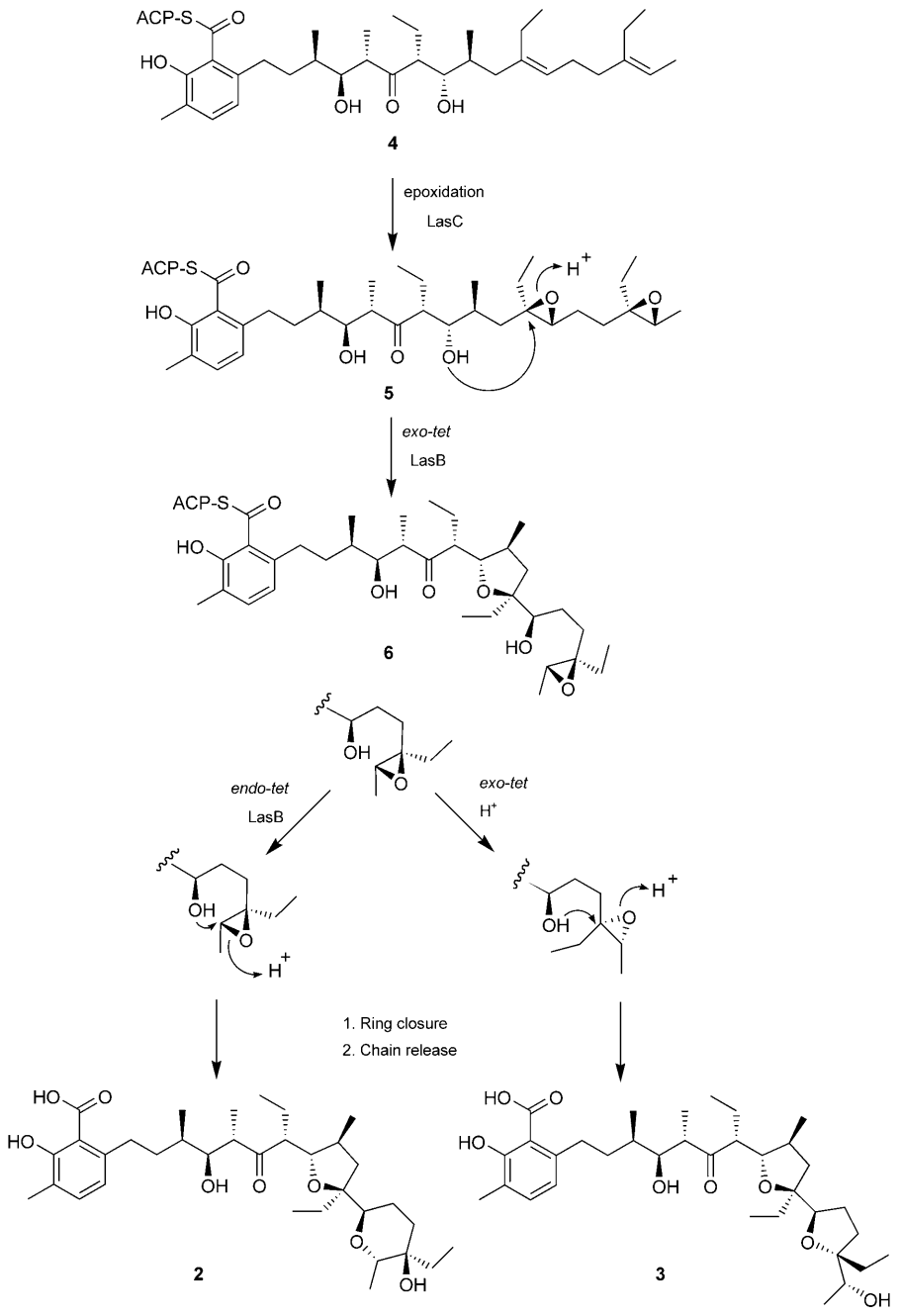


Figure 4. HPLC analysis of lasalocid and iso-lasalocid from *S. lasaliensis* strains. A) Wild-type *S. lasaliensis* (total ion current). The peak at 16.54 corresponds to a mixture of lasalocid B, lasalocid C and lasalocid D (*m/z* 605 $[M+H]^+$), minor products formed through the incorporation of an additional butyrate extender unit in place of propionate.[47] B) Wild-type *S. lasaliensis* (selection for *m/z* 591 $[M+H]^+$). C) *S. lasaliensis ΔlasB* mutant (*m/z* 591 $[M+H]^+$). D) *S. lasaliensis ΔlasB* complemented with expression plasmid pSETlasBNP (*m/z* 591 $[M+H]^+$). E) Lasalocid A standard (*m/z* 591 $[M+H]^+$).

Scheme 3 shows the proposed mechanism for formation of lasalocid and iso-lasalocid, taking these findings into account. LasC epoxidises each of the *E* double bonds in the PKS-bound diene **4**, from the same face of the double bond, to form the diepoxide **5**. The hydroxy group at C-15 opens the first epoxide to form a tetrahydrofuran ring, by nucleophilic attack at the more hindered carbon, and with inversion of configuration at this centre. This preference can be understood on the basis of Baldwin's rules, in that the alternative attack at the less hindered carbon would be a kinetically disfavoured 6-*endo-tet* cyclisation. It remains an open question as to whether enzymatic catalysis by LasB is actually required for this step, although it seems likely. That the ring closures in polyether formation occur stepwise in a definite order is fully consistent with our previous results obtained on the MonB-catalysed formation of monensins in *S. cinnamonensis*.[17] It is also supported by the recent results of Oikawa and colleagues,[26] who have shown that a synthetic compound resembling **6**, in which the first polyether ring has been formed, is chemically competent to be converted into iso-lasalocid, and can act as a substrate for recombinant LasB in vitro. Intermediate **6** partitions differently in the wild type and the *lasB* mutant. In the wild type, the presence of LasB clearly changes the stereochemical course of polyether ring formation, channelling the intermediate **6** to form lasalocid (**2**) as the major product, despite the fact that in chemical terms this is predicted to be a kinetically disfavoured cyclisation pathway. In the mutant, the formation of the second ring appears to be exclusively by the kinetically favoured pathway.

Conclusions

The analysis of the biosynthetic gene cluster for lasalocid has provided the first evidence of a modular PKS catalysing the formation of an aromatic moiety. The lack of a detectable intermediate from the *lasC* mutant provides evidence that oxidative cyclisation occurs while the polyketide chain is enzyme-bound, as in other polyether biosynthetic pathways. Importantly, we have obtained direct evidence for the role of LasB in oxidative cyclisation. The finding that LasB can dictate, and accelerate, a ring-formation pathway regarded as kinetically disfavoured offers an intriguing parallel to the more complex assembly process that leads to the giant polyether ladder structures of marine toxins. Jamison and colleagues have already elegantly demonstrated in synthetic models[23] that the latter process, which involves 6-*endo-tet* ring closures, becomes the kinetically



Scheme 3. Proposed mechanism for oxidative cyclisation of a PKS-bound diene intermediate to produce either lasalocid or iso-lasalocid. In the wild-type strain formation of **2** *predominates*, *whereas in a ΔlasB* mutant only **3** is formed.

favoured one when carried out in water rather than in organic solvent, if there is a single tetrahydropyran ring already in place to act as a template. It may be that when an epoxide is bound at the epoxide hydrolase active site this places equivalent constraints on the mechanistic pathway. The successful creation of a catalytic antibody that favours a kinetically disfavoured cyclisation reaction provides added support for this idea.[40] Certainly, the results reported here encourage the view that equivalent enzymes to LasC and LasB might also be involved in the formation of marine ladder toxins.[6,23]

Experimental Section

Bacterial strains, plasmids and DNA manipulation: *S. lasaliensis* NRRL3382 was the lasalocid-producing strain originally described.[9] It was maintained on modified A medium plates (10 g wheat starch, 2.5 g corn steep powder, 3 g yeast extract, 3 g $CaCO_3$, 100 mg $FeSO_4 \cdot 7H_2O$, 20 g agar, 1 L H_2O). For isolation of DNA it was grown for 2 days at 30 °C with shaking at 200 rpm in M79 liquid medium (10 g glucose, 10 g peptone, 2 g yeast extract, 6 g NaCl, 10 g casein hydrolysate, 1 L H_2O). For lasalocid production, it was grown for 7–10 days at 30 °C with shaking at 200 rpm, in LOSP medium (10 g lard oil, 10 g split peas, 10 g dextrin, 4 g yeast extract, 2 g K_2HPO_4, 1 L H_2O). *E. coli* strains DH10B and XL1-Blue MR were used for routine cloning and for cosmid library construction, respectively, and strain ET12567 (also containing the helper plasmid pUZ8002) was used in the conjugation of *Streptomyces* strains. *E. coli* strains were cultured in 2TY (16 g tryptone, 10 g yeast extract, 5 g NaCl, 1 L H_2O) medium at 37 °C with the appropriate antibiotic selection at a final concentration: carbenicillin (100 μg mL^{-1}), hygromycin B (100 μg mL^{-1}), apramycin (50 μg mL^{-1}), chloramphenicol (25 μg mL^{-1}) or nalidixic acid (25 μg mL^{-1}). Plasmid pYH7[41,42] was used for gene deletions in *S. lasaliensis*, and plasmid pHSG397[43] for subcloning fragments for DNA sequencing. DNA manipulations were performed by using standard procedures for *E. coli*[44] and *Streptomyces*.[45]

Construction and screening of the genomic cosmid library: A genomic library of *S. lasaliensis* was constructed in the cosmid vector SuperCos 1 (Stratagene). The genomic DNA was partially digested with BamHI, dephosphorylated with shrimp alkaline phosphatase and ligated to the prepared vector without fractionation. The Gigapack III XL packaging extract (Stratagene) was used for library construction by following the manufacturer's instructions. About 2000 colonies were screened by colony-hybridisation with digoxigenin-labelled PKS-probe, which is a 1.2 kbp conserved fragment of the erythromycin KS2 domain. Positive colonies were analysed by restriction enzyme digestion. Further details of the cloning procedure are given in the Supporting Information.

Sequencing and annotation of the lasalocid biosynthetic gene cluster: The overlapping cosmids 4B12, U12G, S3F and MK7G were

sequenced by shotgun sequencing of a subclone library, consisting of 2.0–7.0 kbp fragments (obtained through partial digestion with Sau3AI) cloned in pHSG397. DNA sequencing was carried out with an ABI Prism 3700 DNA Analyzer automated sequencer (Applied Biosystems). The raw sequence data were processed and assembled with the *phred/phrap/consed* software package (http://www.phrap.org). Open reading frames were detected with fgenesB.[46] Annotation was performed through database comparison with the BLAST search tools on the server of the National Center for Biotechnology Information, Bethesda, Maryland (http://www.ncbi.nlm.nih.gov).

Gene disruption and complementation: The constructs for gene disruption and complementation used in this study are summarised in the Supporting Information. The constructs were introduced into *S. lasaliensis* by conjugation with donor strain ET12657/pUZ8002 on modified A medium plates (20 mL). After incubation at 30 °C for 18 h, exconjugants were selected by the addition of apramycin (1 mg) and nalidixic acid (500 μg) in water (1 mL) to each plate. Single colonies from the plate were plated out on modified A medium without apramycin. To screen the resulting double-crossover mutants, single colonies were patched both onto modified A medium plates containing apramycin (50 $\mu g\,mL^{-1}$) and onto plates without apramycin. Candidate clones with the correct phenotype (Apr^S) were used for further verification by PCR and sequencing (see the Supporting Information).

For complementation of in-frame mutant strain Δ*lasC*, a pSET152-derived construct pSETlasCNP was used; this carries the complete *lasC* gene with its natural promoter (see the Supporting Information). The complementation plasmid was introduced into Δ*lasC* by conjugation, and exconjugants were selected with apramycin. Resistant colonies were confirmed by PCR and sequencing. A similar procedure was used for complementation of the Δ*lasB* mutant with the construct pSETlasCNP, housing the *lasB* gene with its own promoter (see the Supporting Information).

Production, isolation and analysis of lasalocid: To assess lasalocid production by *S. lasaliensis* (wild type) and its mutants, a seed culture containing M79 medium (50 mL) was inoculated and grown as described above. After 2–3 days, the culture was used to inoculate LOSP production medium (8 L). After a further 7–10 days the culture was extracted with an equal volume of ethyl acetate. The extracts were dried by removal of the solvent under reduced pressure, and the remaining oil was dissolved in methanol. The bulk of the oil was then removed by filtration at −20 °C. The methanol was removed under reduced pressure, and the extract was further purified, first with a silica column (15 cm × 3 cm) with elution with a dichloromethane/ethyl acetate gradient, followed by chromatography by preparative HPLC (Gilson) on a Luna 10 C18 (10 μm, 250 × 21 mm) reversed-phase column (Phenomenex) with elution with a gradient of 70–100% acetonitrile containing formic acid (0.1%) over 30 min. NMR spectroscopy was carried out with a Bruker DRX 500 MHz Cryoprobe spectrometer; samples were dissolved in $CDCl_3$.

Online liquid chromatography-mass spectrometry (LC-MS), LC-MS/MS and LC-MS^n analyses were carried out with an LTQ mass spectrometer (Thermo Finnigan) by positive-mode electrospray ionisation. The LTQ was coupled to an HP 1200 LC (Agilent) fitted with a Prodigy C18 reversed-phase column (5 μm, 4.6 × 250 mm, Phenomenex) equilibrated with formic acid (0.1%) in acetonitrile (85%). Samples were eluted with a gradient of 85–100% acetonitrile over 20 min. The mass spectrometer was set to full scan (from *m/z* 200 to 2000), MS/MS and MS^3 modes with normalised collision energy of 35%.

Acknowledgements

This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) through a project grant (P.F.L. and J.B.S.) and a PhD studentship (L.S.) We are grateful to Prof. J. C. Vederas for an authentic sample of lasalocid and to Qingzhi Fan and Markiyan Samborskyy for helpful advice.

Keywords: antibiotics · Baldwin's rules · biosynthesis · ionophores · polyketides

[1] J. W. Westley in *Antibiotics IV. Biosynthesis* (Ed.: J. W. Corcoran), Springer, New York, **1981**, pp. 41–73.
[2] C. J. Dutton, B. J. Banks, C. B. Cooper, *Nat. Prod. Rep.* **1995**, *12*, 165–181.
[3] C. Gumila, M. L. Anseklin, A. M. Delort, G. Jeminet, H. J. Vial, *Antimicrob. Agents Chemother.* **1997**, *41*, 523–529.
[4] K. Otoguro, A. Kohana, C. Manabe, A. Ishiyama, H. Ui, K. Shiomi, H. Yamada, S. Omura, *J. Antibiot. (Tokyo)* **2001**, *54*, 658–663.
[5] B. J. Rawlings, *Nat. Prod. Rep.* **2001**, *18*, 231–281.
[6] A. R. Gallimore, J. B. Spencer, *Angew. Chem.* **2006**, *118*, 4514–4521.
[7] A. M. Hill, *Polyketide Biosynthesis, Polyethers, Wiley Encyclopaedia of Chemical Biology*, **2008**; DOI: 10.1002/9780470048672.wecb460.
[8] A. Agtarap, J. W. Chamberlin, M. Pinkerton, L. Steinrau, *J. Am. Chem. Soc.* **1967**, *89*, 5737–5748.
[9] J. W. Westley, R. H. Evans, G. Harvey, R. G. Pitcher, D. L. Pruess, *J. Antibiot. (Tokyo)* **1974**, *27*, 288–297.
[10] D. E. Cane, W. B. Celmer, J. W. Westley, *J. Am. Chem. Soc.* **1983**, *105*, 3594–3600.
[11] P. F. Leadlay, J. Staunton, M. Oliynyk, C. Bisang, J. Cortés, E. Frost, Z. A. Hughes-Thomas, M. A. Jones, S. G. Kendrew, P. F. Long, H. A. I. McArthur, E. L. McCormick, Z. Oliynyk, C. B. W. Stark, C. J. Wilkinson, *J. Ind. Microbiol. Biotechnol.* **2001**, *27*, 360–367.
[12] M. Oliynyk, C. B. W. Stark, A. Bhatt, M. A. Jones, Z. A. Hughes-Thomas, C. J. Wilkinson, Z. Oliynyk, Y. Demydchuk, J. Staunton, P. F. Leadlay, *Mol. Microbiol.* **2003**, *49*, 1179–1190.
[13] Y. Sun, X. Zhou, H. Dong, G. Tu, M. Wang, B. Wang, Z. Deng, *Chem. Biol.* **2003**, *10*, 431–441.
[14] B. M. Harvey, T. Mironenko, Y. Sun, H. Hong, Z. Deng, P. F. Leadlay, K. J. Weissman, S. F. Haydock, *Chem. Biol.* **2007**, *14*, 703–714.
[15] Y. Demydchuk, Y. Sun, H. Hong, J. Staunton, J. B. Spencer, P. F. Leadlay, *ChemBioChem* **2008**, *9*, 1136–1145.
[16] A. Bhatt, C. B. W. Stark, B. M. Harvey, A. R. Gallimore, Y. Demydchuk, J. Staunton, J. B. Spencer, P. F. Leadlay, *Angew. Chem.* **2005**, *117*, 7237–7240; *Angew. Chem. Int. Ed.* **2005**, *44*, 7075–7078.
[17] A. R. Gallimore, C. B. W. Stark, A. Bhatt, B. M. Harvey, Y. Demydchuk, V. Bolanos-Garcia, D. J. Fowler, J. Staunton, P. F. Leadlay, J. B. Spencer, *Chem. Biol.* **2006**, *13*, 453–460.
[18] B. M. Harvey, H. Hong, M. A. Jones, Z. A. Hughes-Thomas, R. A. Goss, M. L. Heathcote, V. M. Bolanos-Garcia, W. Kroutil, J. Staunton, P. F. Leadlay, J. B. Spencer, *ChemBioChem* **2006**, *7*, 1435–1442.
[19] M. Arand, B. M. Hallberg, J. Zou, T. Bergfors, F. Oesch, M. J. van der Werf, J. A. M. de Bont, T. A. Jones, S. L. Mowbray, *EMBO. J.* **2003**, *22*, 2583–2592.
[20] T. Liu, X. Lin, X. Zhou, Z. Deng, D. E. Cane, *Chem. Biol.* **2008**, *15*, 449–458.
[21] J. E. Baldwin, *J. Chem. Soc. Chem. Commun.* **1976**, 734–736.
[22] T. Yasumoto, M. Murata, *Chem. Rev.* **1993**, *93*, 1897–1909.
[23] I. Vilotijevic, T. F. Jamison, *Science* **2007**, *317*, 1189–1192.
[24] J. W. Westley, J. F. Blount, R. H. Evans, Jr., A. Stempel, J. Berger, *J. Antibiot.* **1974**, *27*, 597–604.
[25] A. Migita, Y. Shichijo, H. Oguri, M. Watanabe, T. Tokiwano, H. Oikawa, *Tetrahedron Lett.* **2008**, *49*, 1021–1025.

[26] Y. Shichijo, A. Migita, H. Oguri, M. Watanabe, T. Tokiwano, K. Watanabe, H. Oikawa, *J. Am. Chem. Soc.* **2008**, *130*, 12230–12231.
[27] C. Bisang, P. F. Long, J. Cortés, J. Westcott, J. Crosby, A. L. Matharu, R. J. Cox, T. J. Simpson, J. Staunton, P. F. Leadlay, *Nature* **1999**, *401*, 502–505.
[28] J. Staunton, K. J. Weissman, *Nat. Prod. Rep.* **2001**, *18*, 380–416.
[29] H. Zhou, J. Zhan, K. Watanabe, X. Xie, Y. Tang, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 6249–6254.
[30] S. F. Haydock, J. F. Aparicio, I. Molnár, T. Schwecke, L. E. Khaw, A. König, A. F. Marsden, I. S. Galloway, J. Staunton, P. F. Leadlay, *FEBS Lett.* **1995**, *374*, 246–248.
[31] J. Lau, H. Fu, D. E. Cane, C. Khosla, *Biochemistry* **1999**, *38*, 1643–1651.
[32] S. J. Kakavas, L. Katz, D. Stassi, *J. Bacteriol.* **1997**, *179*, 7515–7522.
[33] M. M. Sherman, S. Yue, C. R. Hutchinson, *J. Antibiot. (Tokyo)* **1986**, *34*, 1135–1143.
[34] C. R. Hutchinson, M. M. Sherman, A. G. McInnes, J. A. Walter, J. C. Vederas, *J. Am. Chem. Soc.* **1981**, *103*, 5956–5959.
[35] R. Reid, M. Piagentini, E. Rodriguez, G. Ashley, N. Viswanathan, J. Carney, D. Santi, C. R. Hutchinson, R. McDaniel, *Biochemistry* **2003**, *42*, 72–79.
[36] P. Caffrey, *ChemBioChem* **2003**, *4*, 654–657.
[37] D. H. Kwan, Y. Sun, F. Schulz, P. F. Leadlay, *Chem. Biol.* **2008**, *15*, 1–10.
[38] M. L. Heathcote, J. Staunton, P. F. Leadlay, *Chem. Biol.* **2001**, *8*, 207–220.
[39] A. De Schrijver, R. De Mot, *Microbiology* **1999**, *145*, 1287–1288.
[40] K. D. Janda, C. G. Shevlin, R. A. Lerner, *Science* **1993**, *259*, 490–493.
[41] Y. Sun, H. Hong, M. Samborskyy, T. Mironenko, P. F. Leadlay, S. F. Haydock, *Microbiology* **2006**, *152*, 3507–3515.
[42] Y. Sun, X. Zhou, J. Liu, K. Bao, G. Zhang, G. Tu, T. Kieser, Z. Deng, *Microbiology* **2002**, *148*, 361–371.
[43] T. Hashimoto-Gotoh, T. Mizuno, Y. Ogasahara, M. Nakagawa, *Gene* **1995**, *152*, 271–275.
[44] J. Sambrook, D. W. Russell, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, **2001**.
[45] T. Kieser, M. J. Bibb, M. J. Buttner, K. F. Chater, D. A. Hopwood, *Practical Streptomyces Genetics*, John Innes Foundation, Norwich, **2000**.
[46] A. V. Lukashin, M. Borodovsky, *Nucleic Acids Res.* **1998**, *26*, 1107–1115.
[47] J. W. Westley, W. Benz, J. Donahue, R. H. Evans, Jr., C. G. Scott, A. Stempel, J. Berger, *J. Antibiot. (Tokyo)* **1974**, *27*, 744–753.

Received: August 30, 2008
Published online on November 24, 2008

