Enzymes

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Insights into Lasalocid A Ring Formation by Chemical Chain Termination In Vivo**

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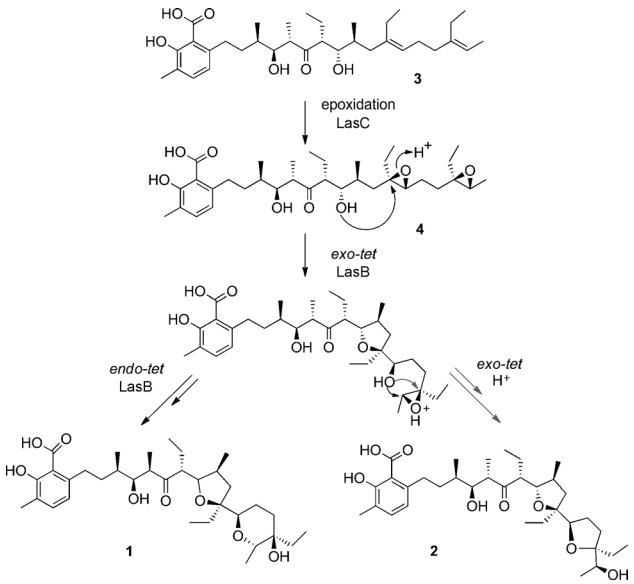
Polyether antibiotics are a unique class of compounds that are broadly used in veterinary medicine and in animal husbandry for their ability to complex inorganic cations and aid their transport across membrane barriers. They include monensin, nigericin, nanchangmycin, salinomycin, lasalocid A, tetronasin, and tetronomycin among others,[1] and all are produced by *Streptomyces* and related filamentous bacteria. Recent reports on their outstanding potency against a variety of critical infectious disease targets including protozoa, bacteria, and viruses,[2] as well as their ability to selectively kill cancer stem cells,[3] have led to a revived interest in the biosynthesis of these compounds for combinatorial chemistry purposes.

Polyether ionophores possess two or more ether rings and a terminal carboxy group, all of which serve as ligands for cation binding. Early experiments involving the feeding of labeled precursors to whole cells showed that the carbon skeleton and specific oxygen atoms were of polyketide origin, with the additional ether oxygen atoms originating from molecular oxygen. On this basis Cane, Celmer, and Westley proposed a unified mechanism for polyether biosynthesis involving the initial formation of an all-*trans* unsaturated polyketide that would undergo oxidative cyclization by stereospecific epoxidation, epoxide hydrolysis, and a cascade of nucleophilic hydroxy cyclizations.[4] This hypothesis was first validated in our laboratories, where an (*E*,*E*,*E*)-polyketide triene precursor to monensin was isolated and characterized from a blocked mutant of *Streptomyces cinnamonensis*.[5] Since then enormous progress has been made in increasing our knowledge of polyether biosynthesis through the identification and the cloning of various polyether biosynthetic clusters from actinomycetes,[6] and functional studies of epoxidases,[7] epoxide hydrolases,[8] and putative thioesterases that are responsible for polyether release from their polyketide synthases (PKSs).[9] However many details of the biosynthesis remain undefined, in particular in relation to the timing and the mechanism of ring formation.

We and others[10] have cloned and characterized the gene cluster of lasalocid A (**1**), a polyether produced by *Streptomyces lasaliensis*. Lasalocid A is widely used as a coccidiostat but it also displays antimalarial and antischistosomiasis activity.[11] Based on early feeding experiments the biosynthesis of **1** involves the formation of a dodecaketide from the decarboxylative condensation of malonate, methylmalonate, and ethylmalonate units. On this basis Westley et al. suggested that the stereoselective bis(epoxidation) of a putative dodecaketide acid precursor, prelasalocid (**3**), and a subsequent epoxide hydrolysis and cyclization cascade, would lead to **1** and its stereoisomer isolasalocid (**2**; Scheme 1).[12] We



Scheme 1. Biosynthetic pathway to lasalocid A (**1**) proposed by Westley: bis(epoxidation) of prelasalocid (**3**)[12–13] is followed by epoxide hydrolysis and cyclization controlled by the epoxide hydrolase LasB.[10a]

have recently demonstrated the role of the epoxide hydrolase LasB in directing the formation of the tetrahydrofuran (THF) and tetrahydropyran (THP) rings of lasalocid A, which is in contrast to the non-enzymatic formation of the two THF rings of isolasalocid.[10a] The Oikawa group has reported the stereoselective synthesis of prelasalocid (**3**) and its bis(epoxide) **4**, and showed that **4** can be enzymatically converted into **1**, albeit inefficiently, by the recombinant epoxide hydrolase (LasB or Lsd19).[13] More recent work on the recombinant epoxide hydrolase LasB has proven the

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Supporting information (including general methods, the construction of *S. lasaliensis* mutant strains, and LC/HRMS analysis of all the isolated derivatives of the intermediates) for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201106323.

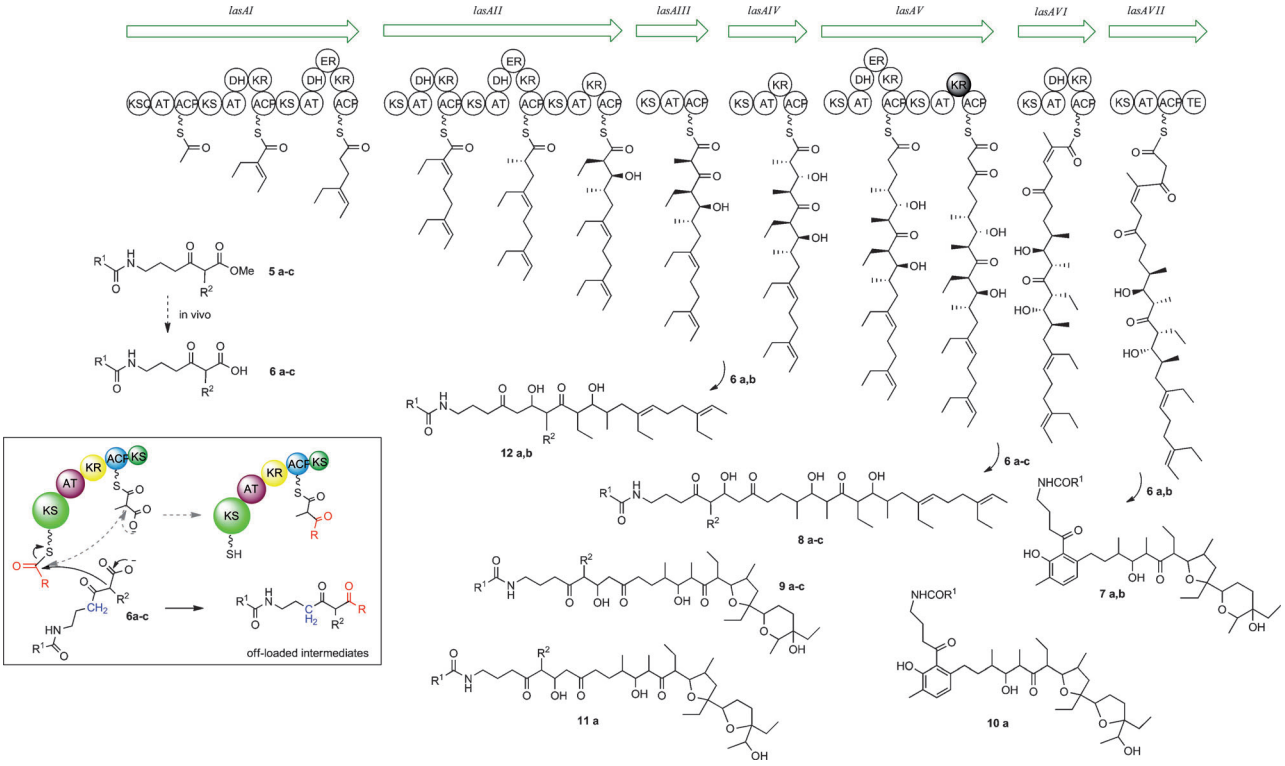
relaxed specificity of this enzyme for synthetic bis(epoxide) substrates[14] and supports a two-step mechanism for the formation of the THF–THP rings.[15] However it remains unclear whether the epoxidation and the epoxide-opening cascade, ultimately leading to the polyether, occur on a PKS-bound precursor as proposed for monensin and nanchangmycin,[6b] or after its release. We have obtained preliminary evidence that intermediates remain enzyme-bound during oxidative cyclization, in that a *S. lasaliensis* mutant bearing a deletion of the epoxidase gene *LasC* does not accumulate free prelasalocid (**3**).[10a] We wish now to establish the true nature of the substrate for the epoxidation and the subsequent steps, and thereby define the mechanism and the timing of the aromatic ring formation, a distinctive feature of lasalocid A biosynthesis, and an unusual example of an aromatic template synthesized on a modular PKS.

To gain insight into these issues we have taken advantage of a chemical strategy recently developed in our laboratories for the isolation of polyketide biosynthetic intermediates. This strategy utilizes carba(dethia) mimics of the malonyl units normally recruited for polyketide formation to intercept and off-load truncated biosynthetic species from PKSs (Scheme 2, box).[16–18]

This approach has proved successful in vitro for the isolation of intermediates from iterative[16] and modular[17] recombinant enzymes. We have recently also shown that this methodology can be employed for in vivo studies: derivatives of intermediates of erythromycin biosynthesis were conveniently isolated from the ethyl acetate extracts of the soil bacterium *Saccharopolyspora erythraea* grown in the presence of malonyl carba(dethia) *N*-acetyl cysteamine esters.[18]

We have examined wild-type *S. lasaliensis* as well as engineered mutant strains bearing a deletion in either the epoxidase *LasC* or the epoxide hydrolase *LasB* genes.[10a] In addition, we have engineered mutant strains in which selected acyl carrier protein (ACP) domains have been inactivated by point mutation (of the active serine for 4′-phosphopantetheine attachment with an inactive alanine residue) to favor release of the truncated biosynthetic species at selected stages. All these strains were grown in the presence of the carba(dethia) *N*-acetyl cysteamine esters **5a–c** (10 mM concentration) over 3–5 days. The esters **5a–c** were hydrolyzed by endogenous esterases to the active biosynthetic probes **6a–c** in situ[18] (Scheme 2).

We now report that micro LC/HRMS analyses of the ethyl acetate extracts of these bacterial cultures provide direct evidence for the off-loading of a series of intermediates from lasalocid A PKS (Scheme 2 and Table 1). In the extracts of the wild-type strain grown in the presence of the malonyl carba(dethia) substrate **6a**, derivatives of a fully cyclized dodecaketide (**7a**), a putative linear undecaketide diene (**8a**), and its oxidized counterpart **9a** were identified (Table 1, entry 1). The postulated nature of these compounds is supported by HRMS^n experiments (see the Supporting Information) and the identification of identical species from experiments that utilized both the malonyl **5a** and deuterated malonyl **5b** substrates for the ACP12 (S970A) strain (Table 1, entries 2 and 3 and Figure 1). In addition, derivative species



Scheme 2. In vivo release of derivatives of intermediates from the lasalocid A polyketide synthase by using the synthetic chain terminators **6a–c** (box),[17–18] generated in situ by the hydrolysis of the corresponding methyl esters **5a–c**[18] (Table 1). The stereochemistry of the derivatives has yet to be established.

Table 1: Isolation of derivatives of lasalocid A intermediates from *S. lasaliensis* using the synthetic probes **6a–c** (see Scheme 2).

Entry	*S.lasaliensis* strain	Substrate[a]	Putative intermediates[b]
1	WT	**5a** (R^1 = Me, R^2 = H)	**7a**,[c] **8a, 9a**
2	ACP12 (S970A)	**5a**	**7a**,[c] **8a, 9a**
3	ACP12 (S970A)	**5b** (R^1 = CD_3, R^2 = H)	**7b**,[c] **8b, 9b**
4	WT	**5c** (R^1 = Me, R^2 = Me)	**8c, 9c**
5	ΔlasB	**5a**	**8a, 10a**,[c] **11a**
6	ΔlasB-ACP12 (S970A)	**5a**	**8a, 10a**,[c] **11a**
7	ΔlasC	**5a**	**12a**[d]
8	ΔlasC	**5b**	**12b**[d]

[a] Methyl ester (10 mM). [b] As deduced by micro LC/HRMS analysis. [c] Double species (at slightly different retention times) detected, possibly arising from isomerization. [d] Detected in a minor amount.

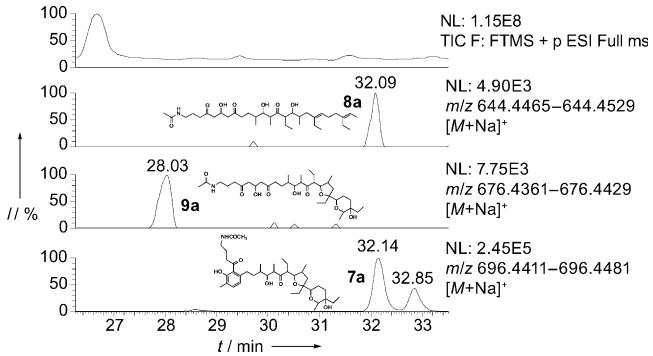


Figure 1. Micro LC/HRMS analysis (LTQ-Orbitrap) of the organic extracts of *S. lasaliensis* ACP12 (5970A) grown in the presence of **5a** (10 mM). The total ion current and the $[M+Na]^+$ extracted ion traces (5 ppm mass accuracy) for the putative intermediates **7a**, **8a**, and **9a** are shown (for $HRMS^n$ analysis of the intermediates see the Supporting Information).

bearing an extra methyl group (**8c** and **9c**) were identified from the wild-type strain grown in the presence of the methylmalonyl substrate **6c** (Table 1, entry 4). The undecaketide diene derivative **8a** was also identified in the extracts of the ΔlasB and ΔlasB-ACP12 (S970A) mutants (Table 1, entries 5 and 6), together with its oxidized counterpart **11a** and also the dodecaketide **10a**. Compounds **10a** and **11a** have been characterized as isolasalocid derivatives (featuring two THF rings) on the basis of their retention time, which differs from the corresponding lasalocid analogues **9a**,**b** and **7a**,**b** (see the Supporting Information). This finding, is fully consistent with, in the absence of LasB, the acid-catalyzed *exo*-tet cyclization of a bis(epoxide) intermediate in accordance with Baldwin's rules.[10] Surprisingly, analysis of the extracts from ΔlasC strains (Table 1, entries 7 and 8) revealed no off-loaded (near) full-length intermediates. Instead, the putative octaketide derivatives **12a**,**b**, as well as a δ-lactone resulting from the intramolecular cyclization of the ACP7-bound heptaketide were identified (see the Supporting Information).

These species were not detected in the extracts of mutant strains in which the ACP7 had been inactivated (see the Supporting Information).

The putative intermediate species identified in this study represent a novel method of sampling PKS-bound intermediates and provide novel insights into the biosynthesis of lasalocid A, in particular on the relative timing of epoxidation and aromatic ring formation, as well as on the role of the downstream epoxidase in the control of polyketide processing.

The isolation and characterization of the putative undecaketide dienes **8a–c** and their oxidized counterparts (**9a–c**; **11a** for ΔlasB mutants), as well as of the fully oxidized dodecaketide species (**7a**,**b** and **10a**) suggest that epoxidation occurs on an enzyme-bound substrate, possibly on the ACP11-bound undecaketide or on the preceding ACP10-bound decaketide. We cannot yet exclude that epoxidation might also occur on free polyketide substrates (for instance directly on the off-loaded undecaketide dienes **8a–c**). However our failure to detect off-loading of either dodecaketide diene intermediates resembling prelasalocid (**3**) or prelasalocid itself strongly argues against this hypothesis. Our results also suggest that aromatization may follow ether ring formation and occur as soon as a dodecaketide chain is formed, before chain release from the PKS.[10] The deduced retention of the 3-hydroxy group in the dienes **8a–c** suggests that the PKS dehydratase (DH) of *LasVI* is not involved in formation of the aromatic ring, which is in agreement with a previous proposal based on genetic analysis.[10] However, further work is needed to confirm the precise mechanism and timing of aromatization. Finally, the fact that all of the ΔlasC strains accumulated only derivatives of medium-length chain intermediates (**12a**,**b**) implies that the presence of the epoxidase LasC is crucial for complete assembly of the polyketide backbone, either because it stabilizes the PKS or because oxidative cyclization is a prerequisite for elongation to form full-length polyketide chains. The role of downstream enzymes in stabilizing multienzyme complexes and in constraining the catalytic specificity has been widely documented for iterative PKSs composed of discrete domains in vitro.[19] In contrast, these roles have been more difficult to establish in modular systems owing to the general inability to reconstitute entire PKS pathways in vitro. The advantage of our method lies in the direct and immediate sampling of biocatalytic intermediates in vivo. We have shown here that this can be combined with genetic manipulation to favor intermediate off-loading at selected stages of the biosynthetic pathway. It should also be possible in the future to scale-up fermentations and therefore allow confirmation of product structures using NMR spectroscopy. A current limitation, which likely precludes us from observing the full range of biosynthetic intermediates, is the inefficient hydrolysis of the methyl esters **5a–c** in vivo.[18] Work is ongoing in our laboratories to address this issue. We are currently applying chemical chain termination to the study of other polyether biosyntheses to determine the timing of ether ring formation in both PAPA and APPA systems (in which propionate–acetate–proprionate–acetate and acetate–proprionate–proprionate–acetate, respectively, are incorporated as the first four extended units),[1] and to gain valuable insights into the potential for engineering production of novel bioactive polyethers.

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