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Intriguing Substrate Tolerance of Epoxide Hydrolase Lsd19 Involved in Biosynthesis of the Ionophore Antibiotic Lasalocid A

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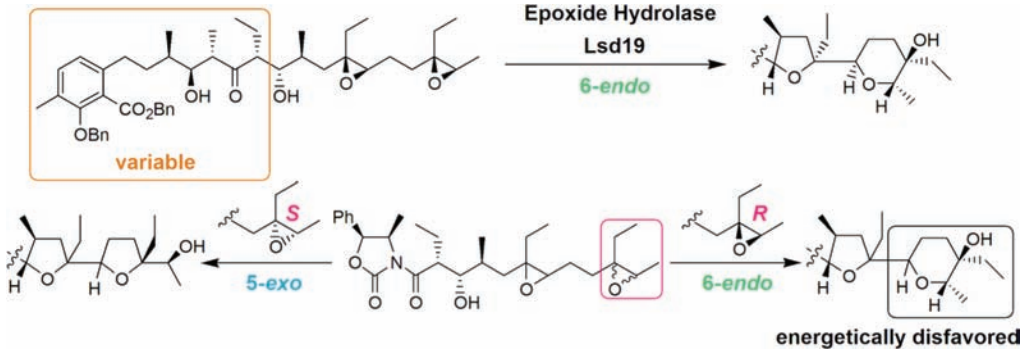
ABSTRACT



Recently, we reported that the epoxide hydrolase Lsd19, the first enzyme shown to catalyze epoxide-opening cascades, can catalyze the conversion of a putative bisepoxide intermediate to polyether antibiotic lasalocid, which involves energetically disfavored 6-*endo-tet* cyclization of the epoxy alcohol. Here, we examined the substrate tolerance of Lsd19. Lsd19 accepts various substrate analogues differing in the left segment of lasalocid and epoxide stereochemistry to afford either THF−THP or THF−THF products with excellent regioselectivity.

Naturally occurring polyether metabolites include ionophore antibiotics,[1] marine dinoflagellate toxins,[2] marine triterpenes,[3] and annonaceous antitumor acetogenins.[4] It has been proposed that these polyether skeletons are constructed by nucleophilic epoxide-opening cascades of the corresponding polyepoxides after installing a number of stereogenic centers on the simple polyolefins in enzymatic epoxidation.[5,6] This simple yet versatile strategy for the biosynthesis of complex polyether metabolites prompted chemists to synthesize ionophore antibiotics and marine dinoflagellate toxins using epoxide-opening cascades. The successful biomimetic synthesis of various polyether systems supports these biosynthetic proposals.[7] An intrinsic problem with this strategy is regioselective opening of polyepoxides. According to Baldwin's rules, the epoxide-opening reaction of hydroxyepoxides usually favors the formation of smaller cyclic ethers. Thus,

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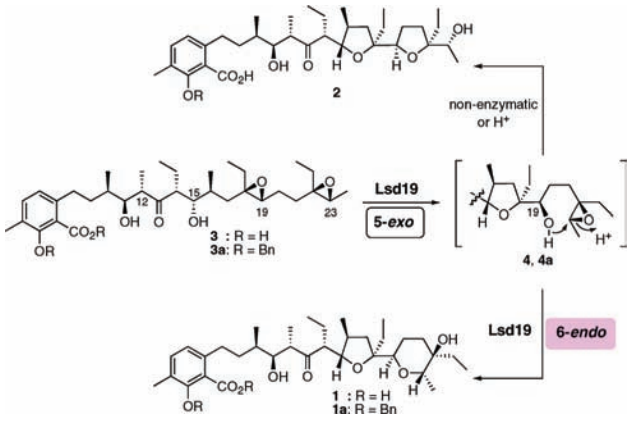
in the case of competitive formation of THF (5-*exo-tet*) and THP (6-*endo-tet*), 6-*endo* cyclization to give THP is an energetically disfavored process. Biosynthetic anti-Baldwin cyclization has been proposed to involve a specific enzyme that stabilizes the disfavored transition state.[6]

Among natural polyethers, the biosynthesis of ionophore antibiotics has been studied extensively. In 1983, Cane, Celmer, and Westley proposed a unified biogenetic scheme for ionophore antibiotics based on the results of a series of feeding experiments.[5] The CCW unified biogenesis has been supported by identification and functional analysis of biosynthetic genes.[8−11] Recently, we reported the identification of the lasalocid biosynthetic gene cluster[12] and showed that the epoxide hydrolase Lsd19 can catalyze conversion of a putative bisepoxide intermediate **3** to lasalocid A (**1**) via **4**, which involves energetically disfavored 6-*endo-tet* cyclization of the epoxyalcohol (Scheme 1).[13] On the other hand,

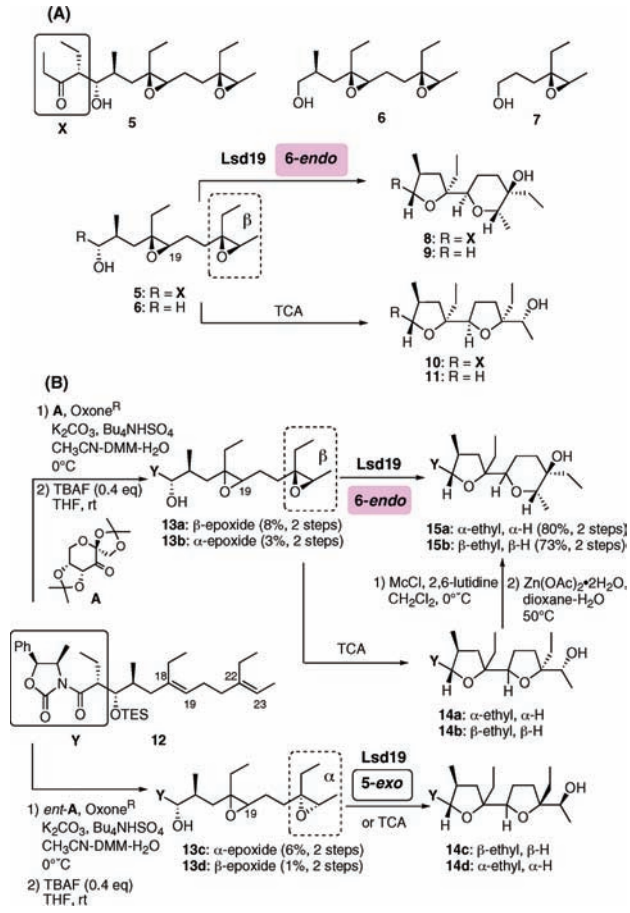
Scheme 1. Enzymatic Reaction with Epoxide Hydrolase Lsd19 Affording Polyether Antibiotic Lasalocid A (**1**)



acid treatment of **3** gave isolasalocid A (**2**) exclusively. The first direct experimental evidence sets the stage for investigating enzymatic polyether formation in vitro. In our previous study, we showed that Lsd19 is capable of converting bisbenzyl analog **3a** to **1a**.[13] This relaxed substrate specificity suggests that Lsd19 is an ideal model enzyme for mechanistic studies of the intriguing epoxide-opening cascade, using various epoxide analogues. Here, we describe the relatively broad substrate tolerance of Lsd19.

Starting from the synthetic intermediates of prelasalocid reported previously,[14] the substrate analogues **5**−**7** and **13a** were synthesized under essentially the same conditions as bisepoxides **3** and **3a** (Scheme 2).[13] First, incubation of the

Scheme 2. Enzymatic Reaction with Epoxide Hydrolase Lsd19[a]



[a] (A) Substrates with different left-hand structures. (B) Substrates with different epoxide stereochemistry.

C12−C24-bisepoxide **5** lacking a salicylate moiety with purified Lsd19 was carried out. LC−MS analysis of the reaction mixture indicated that enzymatic reaction exclusively afforded THF−THP (lasalocid-type) product **8**,[13] whereas treatment of **5** with trichloroacetic acid (TCA) gave the energetically favored THF−THF (isolasalocid-type) **10**[13] as a single product. Simple C15−C24-bisepoxide **6** and bisepoxide **13a** with an oxazolidinone were also converted into the corresponding THF−THP products **9** and **15a**, respectively. On the other hand, C19−C24-monoepoxide **7** did not give any reaction product (data not shown).

Considering the successful conversions of the truncated analogues **5** and **6** without the left segment of the putative intermediate bisepoxyprelasalocid **3**, the salicylate moiety is not essential for substrate recognition, and Lsd19 accepts

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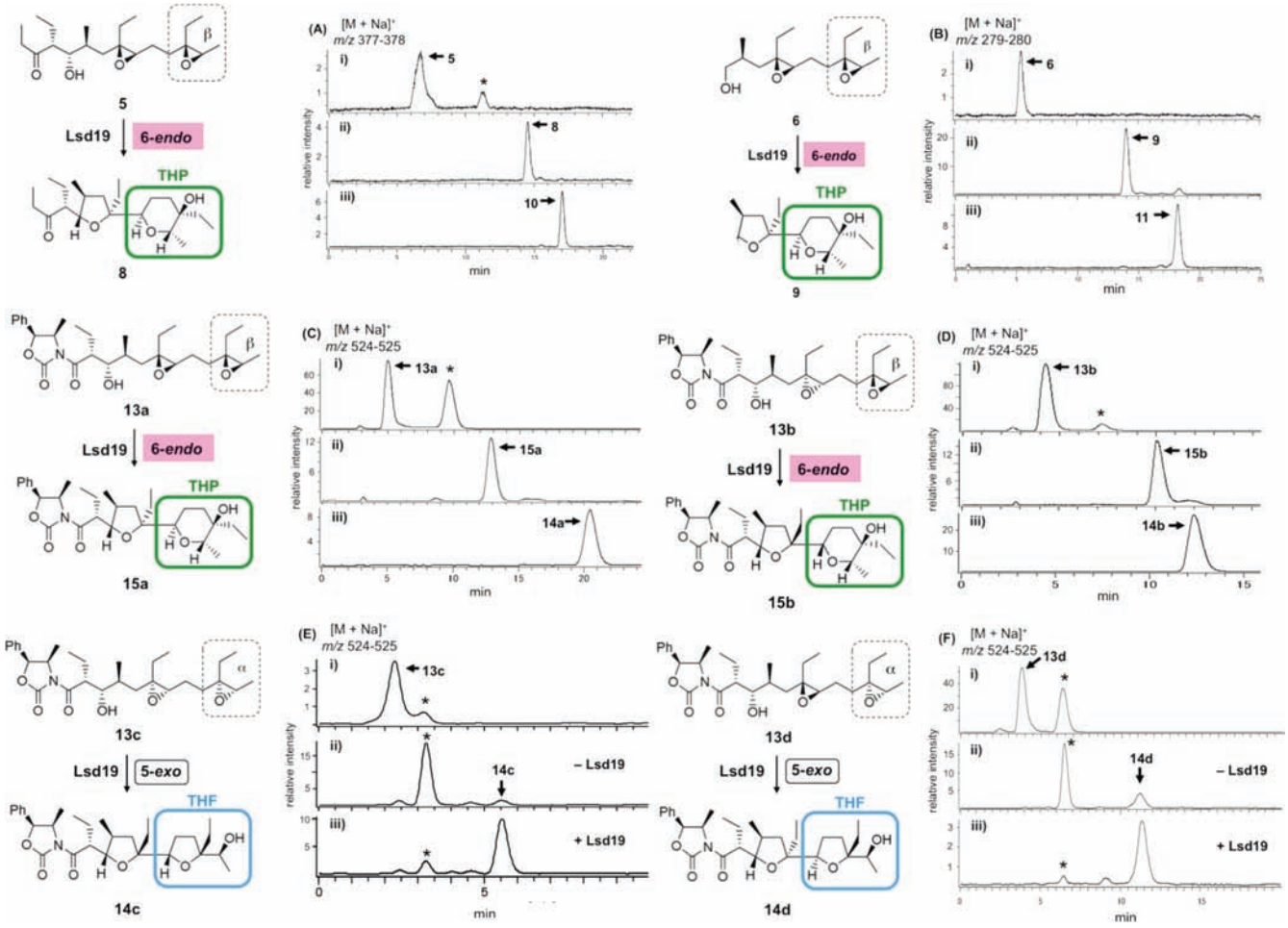

Figure 1. HPLC−MS analysis of the products in the sequential ether formation with Lsd19 (ESI positive mode). Lsd19 assays were performed in a total volume of 50 μL in Tris−HCl buffer (20 mM, pH 7.4) with 0.1 μM Lsd19 at 30 °C for 3 h including control experiments without Lsd19. The substrate concentration was fixed at 1 μM to complete the reaction. All enzymatic reactions were carried out on a preparative scale to identify major products by ^{1}H NMR measurement: (A) (i) bisepoxide **5**, (ii) reaction with Lsd19, (iii) authentic THF−THF product **10**; (B) (i) bisepoxide **6**, (ii) reaction with Lsd19, (iii) authentic THF−THF product**11**; (C) (i) bisepoxide **13a**, (ii) reaction with Lsd19, (iii) authentic THF−THF product**14a**; (D) (i) bisepoxide **13b**, (ii) reaction with Lsd19, (iii) authentic THF−THF product**14b**; (E) (i) bisepoxide **13c**, (ii) reaction without Lsd19, (iii) reaction with Lsd19; (F) (i) bisepoxide **13d**, (ii) reaction without Lsd19, (iii) reaction with Lsd19. The asterisk (*) indicates the corresponding monocyclic ether.

a broad range of substrate analogues differing in the left segment of lasalocid. Acceptance of the bisepoxide **13a** bearing an oxazolidinone at the left end supports this hypothesis.

To examine the influence of epoxide stereochemistry in the Lsd19-catalyzed reaction, epoxide diastereomers **13a**−**d** were synthesized by Shi's asymmetric epoxidation of **12**.[15] Use of D-ligand **A** gave a pair of products **13a** and **13b** as a 2.7:1 mixture, while use of enantiomeric L-ligand *ent*-**A** afforded a different set of bisepoxides, **13c** and **13d**, as a 7.5:1 mixture (Scheme 2B). Expected *exo*-cyclization products **14a**−**d** were synthesized by acid treatment of the corresponding epoxides. The corresponding *endo*-products **15a** and **15b** were synthesized by a two-step ring-expansion reaction (Scheme 2B).[16] All enzymatic reactions were nearly completed within 3 h. Using each of the four diastereomeric bisepoxides **13a**−**d**, enzymatic reactions with Lsd19 were performed (Scheme 2B). LC−MS analysis of the reaction products with bisepoxides **13a** and **13b** revealed formation of the THF−THP products **15a** and **15b**, respectively. On the other hand, the enzymatic reaction of **13c** and **13d** showed the predominant formation of THF−THF products **14c** and **14d**, respectively (Figure 1C−F).[16]

The structures of THF−THF products **14a**−**d** obtained by acid treatment of the corresponding bisepoxide diastereomers **13a**−**d** were determined by extensive NMR analysis including NOESY spectra as shown in Figure 2. In addition, the structures of THF−THP products**15a** and **15b** afforded in the Lsd19-catalyzed reaction were confirmed by com-

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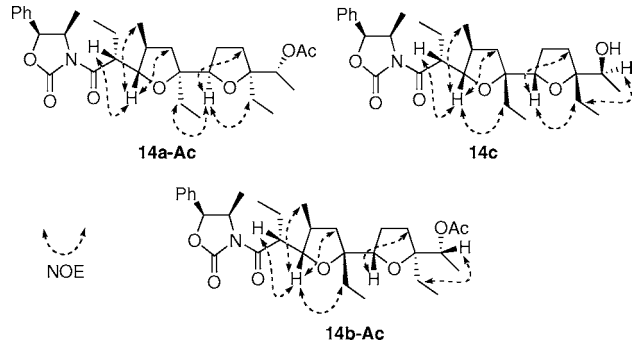

Figure 2. Selected NOE data for the cyclization products.

parison of their NMR spectra with those of the ring-expansion products[16] of **14a** and **14b**.

These experimental results showed that in the first THF formation Lsd19 accepted both 18*R*,19*R*-(natural) and 18*S*,19*S*-epoxides (unnatural) and that in the second cyclization Lsd19 switched the reaction pathways: (1) THP formation for (22*R*,23*R*)-epoxide (*β*-epoxide, natural) and (2) THF formation for (22*S*,23*S*)-epoxide (α-epoxide, unnatural). These remarkable regioselectivities provided further insight into the reaction mechanism of Lsd19-catalyzed polyether construction. As described in our paper concerning identification of the lasalocid gene cluster,[12] Lsd19 has two catalytic domains which are likely active. The role of two domains may be a key to solve these intriguing observations. We are currently working on the functional analysis of a series of Lsd19 mutants.

Synthesizing complex polyepoxide substrates and handling these rather reactive compounds are difficult and are the bottleneck of studying enzymatic epoxide-opening cascade in the natural polyether biosynthesis. In monensin biosynthesis, there are several cadidates for putative intermediates of polyether formation due to late-stage modifications such as *O*-methylation and C26 hydroxylation of polyketide backbone.[17] In addition, it was proposed that the enzyme-bound intermediate is an actual substrate for epoxidation and the subsequent cascade cyclization.[18] These factors made it difficult to perform functional analysis of the epoxide hydrolases MonBI and MonBII. Our study described in this paper indicates that truncated substrates having an epoxy alcohol moiety can be used for examining enzymatic activity due to the broad substrate specificity of epoxide hydrolases. If this is general in epoxide hydrolases in polyether antibiotic biosynthesis, use of simple substrates would significantly facilitate mechanistic study.

In the biosynthesis of monensin and nigericin, two epoxide hydrolases may be responsible for three epoxide-opening reactions because only two hydrolase genes were found in the gene clusters.[10,18] This implies that the epoxide hydrolase involved in polyether biosynthesis catalyzes more than a single reaction, and thus, small numbers of epoxide hydrolases are responsible for cascade cyclization of polyepoxide substrates. From this point of view, the substrate specificity of epoxide hydrolase is an intriguing topic for the polyether biosynthesis of not only ionophore antibiotics but also other polyether metabolites including marine ladder toxins. The study of the detailed reaction mechanism of Lsd19-catalyzed epoxide-opening reactions are currently underway in our laboratory.

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Supporting Information Available: Experimental procedures and characterization of compounds **5**−**7**, **9**, **11**, **13a**−**d**, **14a**−**d**, and **15a**,**b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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