ORGANIC LETTERS

2011
Vol. 13, No. 7
1638–1641

Enzymatic Epoxide-Opening Cascades Catalyzed by a Pair of Epoxide Hydrolases in the Ionophore Polyether Biosynthesis

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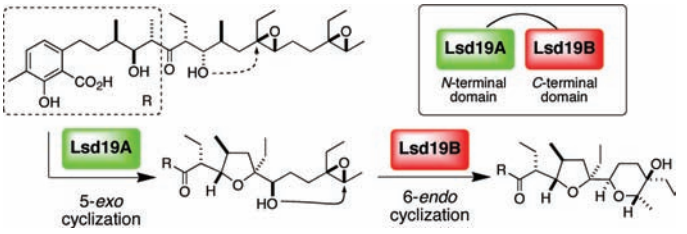
Received January 13, 2011

ABSTRACT



Our recent findings of the first epoxide hydrolase Lsd19, involved in lasalocid A biosynthesis, led us to investigate a long-standing controversial issue on the mechanism of enzymatic epoxide-opening cascades. The site-directed mutagenesis and domain dissection analysis to reveal the mechanism of the reaction catalyzed by Lsd19 is examined, especially in the role of acidic amino acid pair and catalytic domains.

Natural polyether metabolites show significant diversity in molecular skeletons found in ionophore antibiotics,[1] marine ladder toxins,[2] polyether triterpenes,[3] and annonaceous antitumor acetogenins[4] (Figure S1, Supporting Information). As represented by the Cane−Celmer−Westley unified biogenetic scheme for ionophore antibiotics[5] and the Nakanishi−Shimizu biogenetic scheme for marine ladder toxins,[6,7] it has been suggested 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. 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.[8−10] The successful biomimetic synthesis of various polyether systems supports these biosynthetic proposals. In parallel with these synthetic studies, extensive biosynthetic studies of polyethers, especially ionophore antibiotics,[11,12] have also been carried out and a number of experimental results, including identification of the gene cluster for monensin biosynthesis[13] and several gene disruption experiments,[14,15] strongly supported the polyepoxide model of ionophore polyether biosynthesis.

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10.1021/ol200100e
Published on Web 03/04/2011

Until recently, however, no experimental evidence in support of the proposals at the enzymatic level has been provided.

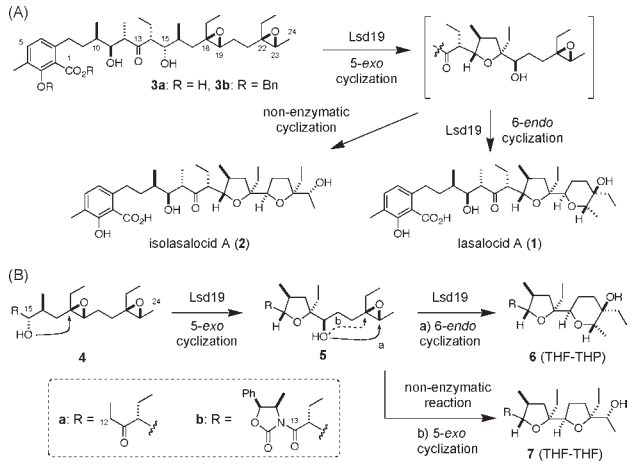

Figure 1. (A) Lsd19 catalyzing sequential epoxide-opening reaction from plausible intermediate **3** to **1**. (B) Lsd19 reaction with substrate analogs.

Smith et al. and we independently identified the gene cluster of the polyether antibiotic lasalocid A (**1**),[16,17] which has a tetrahydrofuran (THF) ring and tetrahydropyran (THP) ring interconnected via a carbon−carbon bond.[18] Furthermore, we identified the function of epoxide hydrolase (EH) Lsd19 in catalysis of the epoxide-opening cascades with a energetically favored 5-*exo* cyclization and disfavored 6-*endo* cyclization from the putative biosynthetic intermediate, bisepoxyprelasalocid (**3a**), to **1** (Figure 1A).[19] This is the first experimental evidence for the enzymatic epoxide-opening cascades. Gene disruption experiments of epoxide hydrolase also demonstrated the function as a catalysis of the epoxide-opening cascades.[16] To characterize this unique enzyme, we performed an *in vitro* study using structurally diverse bisepoxides. The results showed that Lsd19 can catalyze the epoxide-opening reaction with substrate analogs, such as benzyl-protected analog **3b**, C12−C24-bisepoxide **4a** lacking the salicylate moiety of **3a**, and C13−C24-bisepoxide **4b** in which the left-hand segment of **3a** is replaced with an oxazolidinone skeleton (Figure 1A, B).[20,21] The relaxed substrate tolerance indicates that Lsd19 provides an intriguing model system in which to investigate the mechanism of polyether formation using bisepoxide analogs. Here, we report site-directed mutagenesis and domain dissection analysis to investigate the catalytic mechanism of polyether ring formation catalyzed by Lsd19.

First, we examined the kinetic parameters of the second epoxide-opening reaction using monocyclic ether intermediates **5a** and **5b** to simplify analysis by excluding the sequential epoxide-opening reaction. The Michaelis−Menten equation was fitted to plots of initial velocity vs substrate concentration to yield $k_{cat} = 1.6 \pm 0.4\ s^{-1}$, $K_m = 88 \pm 12\ \mu M$, and $k_{cat}/K_m = 1.8 \pm 0.2 \times 10^{-2}\ s^{-1}\ \mu M^{-1}$ for **5a** and $k_{cat} = 2.1 \pm 1.1\ s^{-1}$, $K_m = 370 \pm 42\ \mu M$, and $k_{cat}/K_m = 0.57 \pm 0.2 \times 10^{-2}\ s^{-1}\ \mu M^{-1}$ for **5b**, respectively. Thus, Lsd19 is 3.2-fold more specific for **5a** than **5b**. These observations indicated that simplified analog **5a** is a more suitable substrate for Lsd19 reaction. In addition, as these results correspond approximately to our previous observation that the reactivity of epoxide-opening reactions decreased in the order **3a**, **3b**, **4a**, **4b**, and C15−C24-bisepoxide analog,[20] it is anticipated that putative biosynthetic intermediate **3a** may show the lowest K_m value among these compounds.

To date, epoxide-opening reactions are divided into two distinct types according to the reaction mechanism: stepwise hydrolysis and direct hydrolysis. In the reaction catalyzed by the former type of EHs, aspartic acid acts as a nucleophile to afford covalent enzyme−substrate complex and then hydrolysis of the intermediate results in diol formation (Figure S2A, Supporting Information).[22,23] On the other hand, in the reaction catalyzed by the latter type of EHs such as limonene 1,2-epoxide hydrolase (LEH),[24] a pair of acidic amino acid residues was revealed to play key roles in the epoxide-opening reaction with activation of the nucleophilic water and epoxide (Figure S2B, Supporting Information).[25,26] Because only the latter mechanism can be applied to epoxide-opening cascades involved in ionophore antibiotics biosynthesis, we expected Lsd19 to have a similar acidic amino acid pair for construction of the polyether system.

Recent genetic analysis of polyether antibiotics identified several putative EHs. Two fused *N*- and *C*-terminal

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domains of Lsd19 (Lsd19A: 133 aa, Lsd19B: 149 aa (Figure S3A, Supporting Information) resemble the known EH genes, such as *monBII* (140 aa) and *monBI* (144 aa) involved in monensin biosynthesis,[10] *nigBI* (145 aa), *nigBII* (155 aa) involved in nigericin biosynthesis,[27] and *tmnB* (141 aa) required for the single epoxide-opening reaction in tetronomycin biosynthesis.[28] In particular, *nanI* (313 aa) involved in nanchangmycin biosynthesis[29] shows the same fused domain organization as *lsd19*.[16,17] These observations indicate that a pair of EHs, which exists as either individual or fused subunits, is important for polyether formation. Based on these findings, multiple sequence alignment of these EHs and EH-domains was examined (Figure S3B, Supporting Information). Although key acidic residues of LEH are located at different positions to those of target EHs, we found that acidic amino acid residues, possible candidates for catalytic residues, are highly conserved among these putative polyether EHs. Therefore, these conserved acidic residues located in Lsd19A (Glu22, Asp33, Asp38, and Glu65) and Lsd19B domains (Asp154, Arg165, Glu169, Asp170, Glu181, Glu197, and Asp207) were mutated to alanine to investigate the roles for epoxide opening reaction.

We first examined the unique enzyme-required process to afford energetically disfavored 6-*endo* product **6a** (THF-THP).[30] In the enzyme reactions with cell-free extracts of each mutant and substrate analog **4a** for 60 min (Figure S4, Supporting Information), formation of **6a** was predominantly observed in the case of nine mutants, similar to the wild-type reaction. On the other hand, in the reaction with D170A and E197A mutants, production of **6a** was completely abolished and monocyclic ether **5a** was significantly accumulated (88, 82%) accompanying slow nonenzymatic formation of THF-THF product **7a** (5%, 10%). These results indicated that only D170A and E197A mutants lost the catalytic activity of the 6-*endo* cyclization reaction, while the other nine mutants retained essentially the same activity as the wild-type control.

For detailed functional analysis of D170A and E197A mutants, **4a** was incubated with purified mutants (Figure S5, S6, Supporting Information) for 5 min. LC-MS analysis of the reaction products showed a clear increase in **5a** production (43% for D170A, 52% for E197A) compared with nonenzymatic reaction (22%) (Figure 2A), but none of **6a**. Formation of **6a** was not observed even in prolonged reaction time (60 min) (Figure 2B). These results indicated that both D170A and E197A mutants had retained the 5-*exo* epoxide-opening activity but lost the second 6-*endo* epoxide-opening activity.

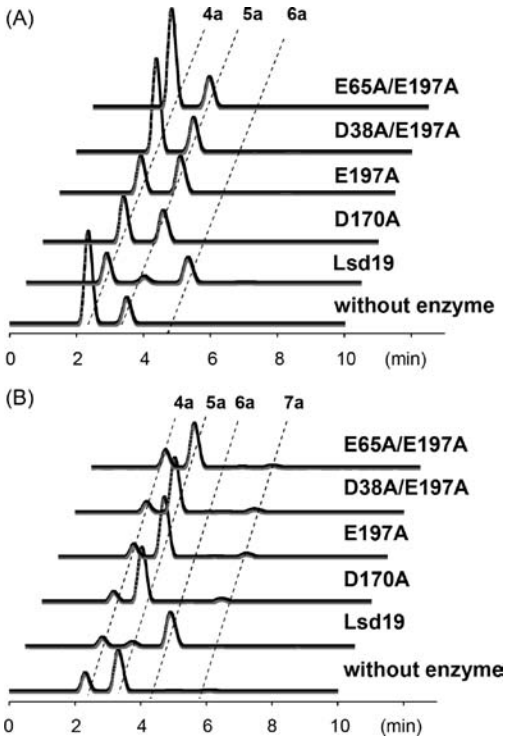

Figure 2. LC-MS profiles of the reaction products with various mutants from (A) 5 min reaction and (B) 60 min reaction.

As expected from the significant homology between Lsd19A and Lsd19B (Figure S3A, Supporting Information), the corresponding acidic amino acids to Asp170 and Glu197 are conserved in Lsd19A (Asp38 and Glu65). To focus on the first cyclization, double mutants (D38A/E197A and E65A/E197A) were constructed and the reaction time was set to 5 min. These mutants showed the same LC-MS profiles compared with control reaction without enzyme (Figure 2A, B and Figure S7, Supporting Information). Loss of the cyclization activity established that Asp38 and Glu65 located in the Lsd19A play key roles in the 5-*exo* epoxide-opening reaction.

Because each epoxide opening reactions are independently catalyzed by the individual domains (Lsd19A and Lsd19B), we predicted that discrete Lsd19A and Lsd19B may catalyze the THF and THP formation, respectively. To investigate this hypothesis, Lsd19A and Lsd19B were expressed as thioredoxin fusion proteins (Figure S5, Supporting Information), and *in vitro* analysis using equimolar amounts of each domain was carried out with **4a**. Although cyclization activity was not detected in Lsd19A reaction within the experimental error caused by competition with the nonenzymatic reaction, very low-level but reproducible formation of **6a** (25%) was observed in Lsd19B reaction (Figure S8, Supporting Information). Based on these findings and additional data with the substrate analog **4b**, it was indicated that Lsd19B catalyzes 6-*endo* cyclization of **5ab** to afford **6ab**. All our efforts to restore the enzymatic activity for sequential cyclization,

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such as cleavage of thioredoxin-tag resion and admixing of each dissected domains, were unsuccessful.

The above mutational analyses and domain dissection analysis of Lsd19 demonstrated that individual domains of Lsd19 catalyze epoxide-opening cascades independently in lasalocid biosynthesis. In addition, loss of the activity with mutants in Lsd19A (D38/E197A or E65/E197A) and Lsd19B (D170 or E197) suggested that acidic amino acid pair play critical roles in these reactions. Based on these findings, the catalytic mechanism of Lsd19 is proposed as follows (Figures 3, 4). First, Lsd19A recognizes the internal C18−C19 epoxide of the substrate and catalyzes energetically favored 5-*exo* cyclization. Either D38 or E65 directly acts as an base for deprotonation of the hydroxyl group. The other acidic amino acid may be involved in recognition and/or activation of the epoxide in a similar manner to LEH reaction. Then, energetically disfavored 6-*endo* cyclization is catalyzed by Lsd19B with another acidic amino acid pair (D170-E197) in a similar manner. The highly conserved acidic amino acid pair in various EHs involved in polyether biosynthesis (Figure S3, Supporting Information) suggests that the mechanism shown above for polyether construction is widely applicable to the biosynthesis of ionophore polyethers.

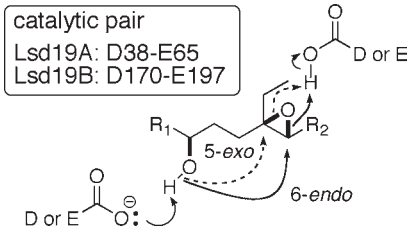

Figure 3. Catalytic mechanism of epoxide-opening reaction.

In summary, biochemical and mutational analyses of epoxide hydrolase Lsd19 significantly advance our understanding of the enzymatic epoxide-opening reaction in lasalocid A biosynthesis, especially in the catalytic mechanism including the role of each catalytic domain. The proposed mechanism catalyzed by a pair of EH may explain other ionophore polyether biosyntheses with triepoxide intermediates such as monensin and nigericin. Recent findings that most of the ionophore antibiotic gene clusters including monensin and nigericin have only two EHs or fused two EH domains suggest that a single EH or EH domain catalyzes two rounds of epoxide-opening reactions (Figure S9, Supporting Information). Furthermore, identification of the catalytic domain Lsd19B in energetically disfavored 6-*endo* cyclization set the stage to analyze an important factor to control the regioselectivity in an epoxide-opening reaction. The stereostructures of each domain are essential for this purpose. Thus, we are currently working to solve the X-ray crystal structure of Lsd19 complexed with a substrate analog.

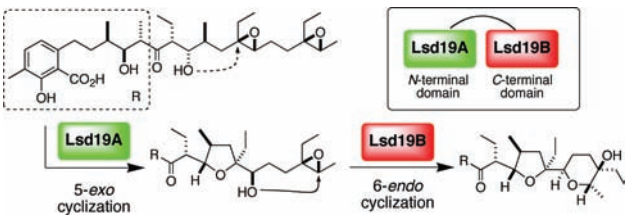

Figure 4. Epoxide-opening cascades catalyzed by Lsd19 in lasalocid A biosynthesis.

Acknowledgment. This work was supported by the Grants-in-Aid for Scientific Research [22108002 to H. Oikawa and 21710213 to A. Minami] from Japan Society for the Promotion of Science (JSPS), and partially supported by the Global COE Program (Project No. B01: Catalysis as the Basis for Innovation in Material Science) from Monbukagakusho and the Naito Foundation. We expressed sincere thanks to late Prof. C. R. Hutchinson (University of Wisconsin) for his encouragement and stimulating discussion on the lasalocid biosynthesis.

Supporting Information Available. Experimental details and LC-MS data. This material is available free of charge via the Internet at http://pubs.acs.org.