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Epoxide Hydrolase Lsd19 for Polyether Formation in the Biosynthesis of Lasalocid A: Direct Experimental Evidence on Polyene-Polyepoxide Hypothesis in Polyether Biosynthesis

Yoshihiro Shichijo,[†] Akira Migita,[†] Hiroki Oguri,*[,†] Mami Watanabe,[†] Tetsuo Tokiwano,[†,⊥] Kenji Watanabe,[‡,§] and Hideaki Oikawa*[,†]

Division of Chemistry, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan and Department of Pharmacology and Pharmaceutical Sciences, University of Southern California, Los Angeles, California 90033

Received June 4, 2008; E-mail: hoik@sci.hokudai.ac.jp; oguri@sci.hokudai.ac.jp

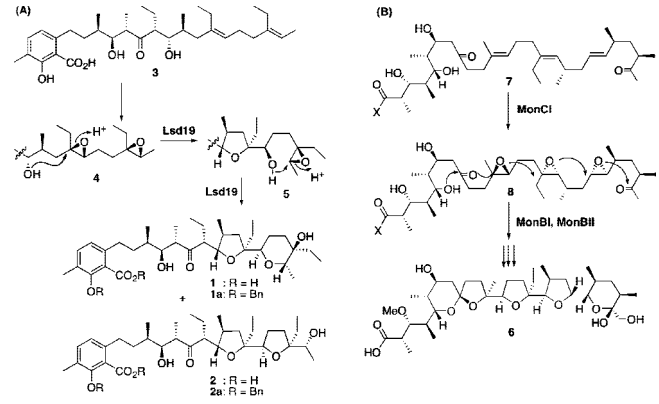
Lasalocid A[1] (**1**) isolated from *Streptomyces lasaliensis* is one of the important ionophore antibiotics among commercially available anticoccidial agents. Feeding experiments suggested that **1** is biosynthesized via a dodecaketide.[2,3] Considering coproduction of isolasalosid A (**2**) along with **1** in *S. lasaliensis*, Westley and coworkers proposed that the stereoselective epoxidation of a dodecaketide precursor prelasalocid (**3**) and the sequential ring openings of the resultant bisepoxide **4** would afford the ether ring system of **1** as shown in Scheme 1A.[4] In the biosynthetic studies on another important polyether ionophore monensin (**6**), this insightful hypothesis was supported by extensive incorporation experiments with [1-^{13}C,1-^{18}O]-short chain fatty acids and $^{18}O_2$, indicating that terminal three oxygen atoms are derived from the corresponding epoxidation of the triene precursor **7** as shown in Scheme 1B.[5] These data led to the Cane−Celmer−Westley unified hypothesis of ionophore polyether biosynthesis in actinomycetes.[6] After this proposal, several groups attempted to incorporate advanced intermediates such as **7**; however, all efforts to prove this attractive hypothesis failed.[7] In 2001, the gene cluster of monensin was indentified,[8,9] and several gene disruption experiments on epoxidase (MonCI) and hydrolases (MonBI, BII) established that these three enzymes are responsible for the conversion of all-*E* triene **7** into **6** via epoxidation and cyclization.[10,11] Yet, a detailed mechanism of the enzymatic polyether formation is still unconfirmed. Herein, we report the first enzymatic conversions of bisepoxides **4** and **11** into lasalocid skeletons.

A total DNA library screening using a probe which was prepared by PCR with degenerate primers designed for the ketosynthase domains of polyketide synthase allowed us to identify the gene cluster responsible for lasalocid biosynthesis.[12] Among the lasalocid biosynthetic genes, *lsd19*, which showed significant homology to the putative epoxide hydrolase genes *monBI* and *monBII*, was assumed to be responsible for the construction of the polyether skeleton.[11] The key gene, *lsd19*, was cloned and successfully expressed in *Escherichia coli* BL21(DE3) by the use of the expression plasmid pKW620.[13] Cell lysate containing Lsd19 expressed as an N-terminal hexa-His-tagged protein was purified using Ni-NTA column chromatography to give Lsd19 at >90% purity as measured by SDS-PAGE analysis with a yield of approximately 2 mg/L of culture.

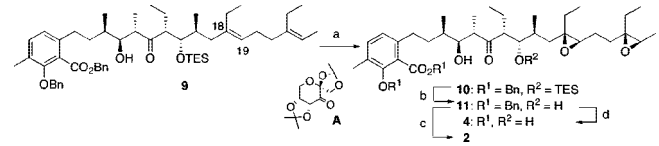
Recently, we have reported the stereocontrolled synthesis of prelasalocid (**3**), a plausible biosynthetic precursor of **1**.[14] To synthesize bisepoxyprelasalocid **4**, a proposed substrate of Lsd19, epoxidation of suitably protected prelasalocid **9** with Shi's catalyst[15] proceeded in a stereoselective manner to give **10** and its diastereomer in a 3:1 ratio as shown in Scheme 2.[16] After deprotection of the TES ether, hydrogenolysis under slightly basic conditions effected removal of the benzyl groups to provide desired **4**. However, the bisepoxide **4** is prone to undergo epoxide opening presumably due to acidic functionalities on the aromatic ring, thereby forming significant amounts of monocyclic ether **5** and isolasalosid A (**2**) having adjacent ether rings during prolonged exposure to the hydrogenolysis conditions and purification. Thus, the mixture of bisepoxides, **4** and **11**, was subjected immediately to the enzymatic reaction with Lsd19.

LC−MS analysis of bisepoxide **4** showed that the substrate contained nonenzymatic cyclization products **2** and **5**. This was confirmed by the fact that treatment of the substrate mixture with trichloroacetic acid gave 5-*exo*-tet cyclization product **2** as a single diastereomer (Figure 1A) in accordance with Baldwin's rules. On the other hand, incubation of the same substrate mixture with the

Scheme 1. Proposed Biosynthetic Pathways of Representative Polyether Antibiotics



Scheme 2[a]



[a] Reagents and conditions: (a) **A**, Oxone[R], K_2CO_3, Bu_4NHSO_4, $CH_3CN-CH_2(OCH_3)_2-H_2O$, 0 °C, 2 h, **10** (52%), diastereomer (17%); (b) TBAF (0.4 equiv), THF, rt, 30 min, **11** (24%), **10** (74%, recovery); (c) H_2, $Pd(OH)_2$, MeOH, rt, 10 min, 98%; (d) H_2, $Pd(OH)_2$, MeOH/aq. NH_4HCO_3, pH 7.8, **4** (3%), **11** (90% recovery).

[†] Hokkaido University.
[‡] University of Southern California.
[§] Current address: Division of Chemistry, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan.
[⊥] Current address: Department of Biotechnology, Faculty of Bioresource Sciences, Akita Prefectural University, Akita 010-0195, Japan.

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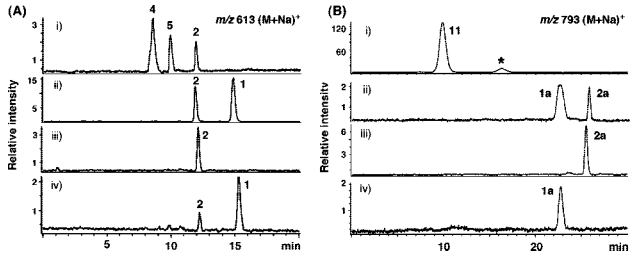

Figure 1. HPLC-MS analysis of sequential ether formation products either with acid or with Lsd19 (ESI positive mode). (A) Bisepoxyprelasalocid (**4**): (i) mixture containing substrate **4** and cyclization products **2** and **5**; (ii) authentic samples of the products **1** and **2**; (iii) reaction of **4** with trichloroacetic acid; (iv) reaction of **4** with Lsd19. (B) Bisepoxide **11**: (i) mixture containing substrate **11** and monocyclic ether (denoted by *); (ii) authentic samples of the products **1a** and **2a**; (iii) reaction with trichloroacetic acid; (iv) reaction with Lsd19. Assays and HPLC conditions are available in the Supporting Information.

purified Lsd19 predominantly afforded a 6-*endo*-tet cyclization product which was identical to lasalocid A (**1**) (Figure 1A).[17] Therefore, the enzymatic conversion of **4** into **1** unambiguously showed that bisepoxide **4** is an intermediate for lasalocid biosynthesis and that Lsd19 catalyzes sequential cyclic ether formation involving an energetically disfavored 6-*endo*-tet cyclization. This is the first example of enzymatic epoxide-opening reactions leading to a polyether natural product. In addition, detection of the monocyclic ether **5** and its conversion to **1** indicated that bicyclic ether formation occurs in a stepwise manner. To confirm the enzymatic activity of Lsd19, enzymatic reaction with the substrate analogue **11** was employed. Incubation of **11** with Lsd19 afforded 6-*endo*-tet cyclization product **1a** as a single diastereomer, while treatment of **11** with trichloroacetic acid gave 5-*exo*-tet cyclization product **2a** (Figure 1B). Thus, the experimental results clearly showed that Lsd19 is responsible for the desired polyether formation reaction to give the lasalocid skeleton.

In the biosynthesis of monensin and nanchangmycin, it has been proposed that epoxidation and cyclization occurred when intermediates were bound to a polyketide synthase (PKS).[18,19] Our experimental results indicate that in the case of lasalocid the polyether formation most likely occurs after the polyketide chain is cleaved from PKS,[12] but further study is needed to exclude the possibility that a full-length polyketide bound to ACP is a mandatory substrate.

An intramolecular ether formation of hydroxyepoxide has posed an intriguing problem in organic chemistry. In an effort to construct ladder polyethers found in marine dinoflagelate toxins, such as brevetoxin and cigatoxin, a number of synthetic protocols to overcome the energetically disfavored 6-*endo*-tet cyclization have been developed.[20,21] Recently, Jamison and his co-workers have reported that 6-*endo* cyclization proceeded nonenzymatically in an aqueous medium without any acid or base catalysis.[22] Although this proposal is attractive for synthesizing the fused cyclic ether system of the ladder polyethers, it seems unlikely to explain the universal biosynthetic mechanisms of marine polyether natural products. Alternatively, a catalytic antibody catalyzing the disfavored intramolecular cyclization of simple hydroxyepoxide has been created.[23] A structural study of this antibody provided useful information on its catalytic mechanism.[24] However, to date, little is known about the enzyme catalysis on sequential cyclic ether formation. Therefore, we believe that Lsd19 can be regarded as a model enzyme for studying multiple catalysis and regioselectivity in intramolecular cyclic ether formation. Currently, studies on substrate specificity and the reaction mechanism of Lsd19 are in progress.

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Supporting Information Available: Experimental procedures and characterization of compounds **1a**, **2**, **2a**, and **9**−**11**. This material is available free of charge via the Internet at http://pubs.ac.jp.

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