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Sequential Enzymatic Epoxidation Involved in Polyether Lasalocid Biosynthesis

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Supporting Information

ABSTRACT: Enantioselective epoxidation followed by regioselective epoxide opening reaction are the key processes in construction of the polyether skeleton. Recent genetic analysis of ionophore polyether biosynthetic gene clusters suggested that flavin-containing monooxygenases (FMOs) could be involved in the oxidation steps. In vivo and in vitro analyses of Lsd18, an FMO involved in the biosynthesis of polyether lasalocid, using simple olefin or truncated diene of a putative substrate as substrate mimics demonstrated that enantioselective epoxidation affords natural type mono- or bis-epoxide in a stepwise manner. These findings allow us to figure out enzymatic polyether construction in lasalocid biosynthesis.

Ionophore polyethers, a structurally unique group of natural polyketides, has a polycyclic ether skeleton with multiple stereocenters.[1] Structural diversity of these natural products is derived from combination of the number and size of ether rings. In 1983, Cane, Celmer, and Westley proposed a unified biosynthetic model (CCW model) for polyether construction in which epoxidation of the linear polyene intermediate in a stereoselective manner followed by regioselective cascade cyclization provided these polyethers.[2]

After the pioneering work by Leadlay and co-workers on identification of monensin biosynthetic gene cluster,[3] a series of gene inactivation experiments established the involvement of polyene and polyepoxide intermediates in polyether construction.[4,5] Thus, the CCW model consisting of epoxidation and epoxide-opening reaction was firmly established. Recently, the second step of polyether formation has been studied extensively by our group. We found that recombinant epoxide hydrolase Lsd19 catalyzes energetically disfavored conversion of the chemically synthesized putative precursor, bisepoxide of prelasalocid (**2**), to lasalocid (**1**) in a highly regioselective manner and that this epoxide hydrolase shows promiscuous substrate specificity (Figure 1A).[6−8] More recently, we proposed a detailed reaction mechanism based on biochemical analysis and solved the structure of Lsd19 complexed with a substrate analogue.[9,10] This allowed us to propose the mechanism of anti-Baldwin cyclization catalyzed by Lsd19. Applying this established procedure, we also characterized the function of epoxide hydrolases involved in monensin biosynthesis.[11] In contrast

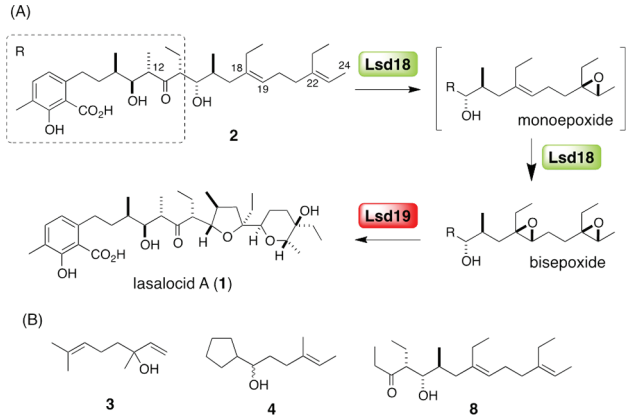

Figure 1. (A) Enzymatic polyether formation catalyzed by Lsd18 and Lsd19 in lasalocid biosynthesis. (B) Compounds tested as substrate mimic for Lsd18 reaction.

to the second step of polyether formation, initial sequential epoxidation, which is an important step for introducing multiple chiral centers, has not been investigated in vitro and its detailed mechanism has not yet been determined. In this paper, we describe the enzymatic epoxidation with Lsd18, a flavin-containing monooxygenase (FMO), involved in lasalocid biosynthesis (Figure 1A).

FMO catalyzes various reactions, including epoxidation in which FMOs utilize 4a-hydroperoxyflavin as an electrophile.[12] As this oxidant is prepared from reduced flavin and molecular oxygen, reduction of an oxidized flavin is one of the key processes in FMO-catalyzed reactions. For this purpose, flavin reductases, such as SsuE[13] and Fre,[14] are frequently used as alternatives to the native reductase. To detect enzymatic activeity of Lsd18 in a simple system, we conducted biotransformation studies using a *Rhodococcus* host, which exhibits resistance toward organic solvents and is therefore an industrially important genus of actinomycetes.[15]

The *lsd18* gene was cloned into pTipQC2[16] and expressed in *Rhodococcus erythropolis* L-88. As expected, Lsd18 expression was observed upon addition of thiostrepton (Figure S1). On the basis of the results of a previous biotransformation study

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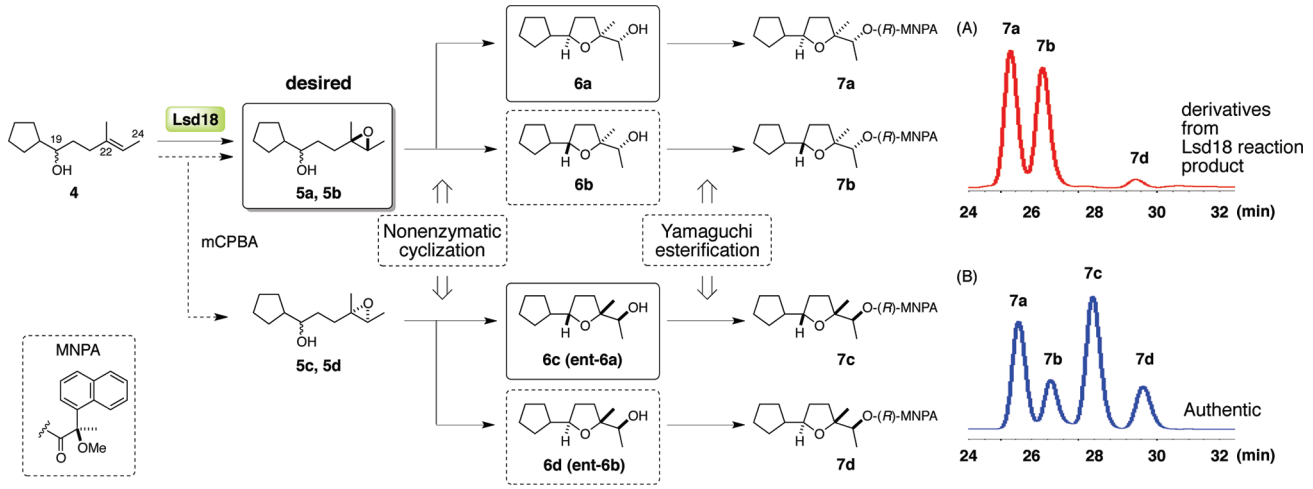

Figure 2. HPLC profiles of the MNPA derivatives. (A) Derivatives from Lsd18 catalyzed reaction product **5**, (B) mixture of four possible derivatives (**7a**–**7d**). To simplify the discussion on Lsd18 catalyzed epoxidations, we used the same numbering system of substrate analogues as that of prelasalocid **2**.

with polyether-producing organisms and *Streptomyces* host with a putative epoxidase gene,[17] we initially tested (±)-linalool **3** as a substrate in our biotransformation system (Figure 1B). GC–MS analysis of the extracts from whole-cell reaction mixture revealed 65% conversion to linalool oxide (syn/anti = 1/1) after 49 h of incubation (Figure S2). Formation of linalool oxide only in the induced cells indicated that trisubstituted olefin was epoxidized and spontaneous cyclization occurred, strongly suggesting that Lsd18 epoxidized **3**. Efficient conversion in this system allowed simple screening of substrate analogues (Figure S3). Therefore, we employed the reaction with a simple substrate **4** possessing terminal trisubstituted olefin moiety mimicking the C19–C24 part of **2** (Figure 1B). LC–MS analysis revealed that **4** was epoxidized to afford **5** and **6** after 60 min of incubation. To determine the stereoselectivity in this epoxidation, reaction products were esterified with (*R*)-2-methoxy-2-(1-naphthyl) propionate (MNPA) and the resultant esters **7** were analyzed by HPLC.[18] The results showed two major peaks corresponding to authentic diastereomers of **7a** and **7b** and one trace peak corresponding to **7d**, indicating that Lsd18-catalyzed epoxidation proceeded in a highly stereocontrolled manner {**6a**, 99% ee; **6b**, 87% ee}. Determined absolute stereochemistry of **5a** and **5b** are identical to those of the bisepoxide of **2** (Figure 2). Formation of **7d**, an unnatural (22*S*, 23*S*)-epoxide, suggests that C19 stereochemistry of **4** affected stereoselectivity of Lsd18-catalyzed epoxidation.

We next examined bioconversion of C12–C24 diene **8**, which has a structure identical to that of the C12–C24 moiety of **2** (Figure 1B).[7,19] Time course analysis showed rapid consumption of **8** and spontaneous production of hydroxy-ethers (**11**, **12**) via mono- and bis-epoxide (Figure S4). After 6 h of incubation, **8** was converted to monoepoxide **9** (10%), monocyclic ether **11** (43%), and isolasalocid ketone **12** (47%) (Figure 3A). Identification of **12** as a single diastereomer indicated that Lsd18 catalyzed epoxidation in a highly enantioselective manner. In addition, acid treatment of the reaction mixture predominantly gave diol **S3** instead of tetrahydrofuran **S5** (Figure S5), suggesting that initial epoxidation occurred at the terminal olefin.

To determine the cofactor requirements and detailed reaction mechanism, enzymatic reaction with recombinant Lsd18 was

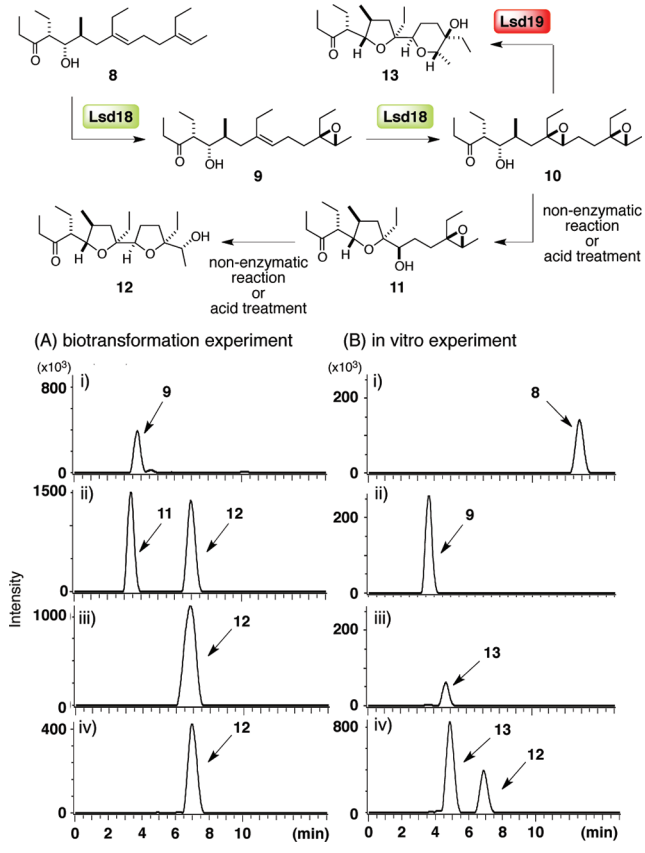

Figure 3. (A) Biotransformation experiment with **8** in Lsd18 transformant, (i) mass chromatogram (*m*/*z* 361), (ii) mass chromatogram (*m*/*z* 377) of the reaction products, (iii) mass chromatogram (*m*/*z* 377) after acid treatment, (iv) authentic **12**. (B) In vitro experiment with **8** in co-incubation of Lsd18 and Lsd19, (i) mass chromatogram (*m*/*z* 345), (ii) mass chromatogram (*m*/*z* 361), (iii) mass chromatogram (*m*/*z* 377) of the reaction products, (iv) authentic **12** and **13**.

examined. Lsd18 was expressed as an N-terminal His_6-tagged protein, and purified by Ni-NTA column chromatography (Figure S6). Solutions of the purified Lsd18 were yellow color and showed characteristic UV–visible spectrum of flavoprotein at 362 and 451 nm (Figure S7). Denaturation of Lsd18 with methanol followed

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by reverse-phase HPLC analysis showed a major peak identical to FAD, indicating that FAD exists as a tightly bound form and the molar ratio of Lsd18 and FAD was estimated as 1/0.82 (Figure S8).

Then, epoxidation of **8** with Lsd18 was carried out in the presence of NAD(P)H, FAD, and flavin reductase under various conditions (Figures S9, S10). In the time course experiments, Lsd18 reaction with NADH afforded monoepoxide **9** in higher conversion rate than that of NADPH (Figure S10 (D) vs (E), Figure S11). The preference of Lsd18 to NADH was also supported by rapid decrease of NADH compared with NADPH in monitoring of absorbance at 340 nm (Figure S12 (A) vs (B)). Exogenous FAD was not necessary when using freshly prepared Lsd18, but addition of FAD was effective in maintaining the epoxidation activity (Figure S10 (F) vs (G) vs (A)). These results indicate that epoxidase Lsd18 can reduce FAD using NADH. However, addition of flavin reductase Fre to the reaction mixture increased production of **9** (1.5 times increase) (Figure S10 (A) vs (H)). In the presence of Fre, complete consumption of NADH (within 6 min) was observed (Figure S12 (C)), indicating the formation of reduced FAD although it is not completely correlated with epoxidation. The results shown above suggest that a temporary increase of the reduced FAD in the reaction mixture with Fre caused its saturation in the active site of Lsd18 and this allows effective turnover compared with the reaction without Fre. However, the effect of Fre for the epoxidation reaction was limited because rapid consumption of NADH simply increased concomitant decomposition of direct reactive species, 4a-hydroperoxyflavin, derived from the reduced FAD. Applying the established conditions in sequential reaction of Lsd18 and Lsd19 with **8**, two products were detected on LC−MS analysis—the expected product **9** (52%) and lasalocid ketone **13** (13%), which was identical to the authentic sample (Figure 3B, Figure S13).

Motif analysis of Lsd18 with Pfam[20] revealed that it has the FAD binding-3 motif similar to other FMOs, such as *p*-hydroxybenzoate hydroxylase and TetX.[21,22] The biochemical properties of Lsd18 also corresponded to these enzymes. Therefore, Lsd18 could be classified into subclass A catalyzing two half reactions in the single polypeptide:[12] (1) reductive half-reaction using NAD(P)H and oxidized FAD to give reduced FAD and (2) oxidative half-reaction using the reduced FAD and molecular oxygen to afford 4a-hydroperoxyflavin followed by epoxidation of the substrate (Figure 4). A similar epoxidation

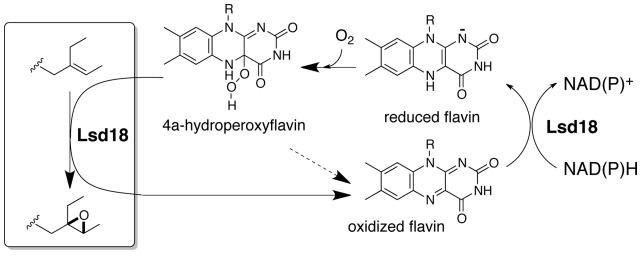

Figure 4. Reaction mechanism of Lsd18 catalyzed epoxidation.

mechanism may be applied to the biosynthesis of other polyethers, because two sequence motifs for FAD binding, GxGxxG and GD,[23,24] are conserved in FMOs involved in polyether biosynthesis (Figure S14). On the other hand, epoxidases, squalene epoxidase and zeaxanthin epoxidase, belonging to subclass A have already been reported. In contrast to Lsd18, these enzymes require NADPH-cytochrome P450-reductase and ferredoxin-like reductives for their activity, respectively.[25,26] In addition, Lsd18 epoxidizes both terminal and internal olefin but the other two FMOs epoxidize terminal olefin only in polyene precursor. The differences between these epoxidases with regard to function are intriguing (Figure S15).

Recently, artificial cleavage with the malonyl CoA analogue from the linear polyketide intermediates having different chain lengths appended to lasalocid polyketide synthase (PKS) has been reported.[27] Extensive analysis of the cleavage products indicated that polyene precursors bound to PKS are epoxidized and cyclized to form a polyether skeleton. Preparation of these intermediate appended to PKS and its in vitro experiment would be difficult. However, based on our experimental results regarding Lsd18 and Lsd19, in vitro analysis using model substrates would be useful for investigating the intriguing epoxidation and epoxide-opening cascade. In addition, this finding also suggests that only a small number of epoxidases and epoxide hydrolases are required to install multiple chiral centers in enzymatic synthesis of the polyether skeleton. As installation of multiple chiral centers requires multistep transformations in total synthesis of natural polyethers, including monensin[28] and brevetoxin,[29] enzymatic construction of the polyether skeleton is very attractive. To verify this hypothesis, we are currently working on analysis of a much more complex system.

In conclusion, we established an in vivo rapid screening system for substrates of epoxidation using *Rhodococcus* host. This enabled us to find epoxidase activity for Lsd18 against a simple olefin or truncated diene substrates as substrate mimic. Sequential epoxidations of the diene and predominant formation of a product with the correct absolute configurations strongly support the role of Lsd18 in lasalocid biosynthesis.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Dutton, C. J.; Banks, B. J.; Cooper, C. B. *Nat. Prod. Rep.* **1995**, *12*, 165−181.

(2) Cane, D. E.; Celmer, W. D.; Westley, J. W. *J. Am. Chem. Soc.* **1983**, *105*, 3594−3600.

(3) Oliynyk, M.; Stark, C. B. W.; Bhatt, A.; Jones, M. A.; Hughes-Thomas, Z. A.; Wilkinson, C.; Oliynyk, Z.; Demydchuk, Y.; Staunton, J.; Leadlay, P. F. *Mol. Microbiol.* **2003**, *49*, 1179−1190.

(4) Bhatt, A.; Stark, C. B. W.; Harvey, B. M.; Gallimore, A. R.; Demydchuk, Y. A.; Spencer, J. B.; Staunton, J.; Leadlay, P. F. *Angew. Chem., Int. Ed.* **2005**, *44*, 7075−7078.

(5) Gallimore, A. R.; Stark, C. B. W.; Bhatt, A.; Harvey, B. M.; Demydchuk, Y.; Bolanos-Garcia, V.; Fowler, D. J.; Staunton, J.; Leadlay, P. F.; Spencer, J, B. *Chem. Biol.* **2006**, *13*, 453−460.

(6) (a) Migita, A.; Watanabe, M.; Hirose, Y.; Watanabe, K.; Tokiwano, T.; Kinashi, H.; Oikawa, H. *Biosci. Biotechnol. Biochem.* **2009**, *73*, 169−176. (b) Smith, L.; Hong, H.; Spencer, J, B.; Leadlay, P, F. *ChemBioChem* **2008**, *9*, 2967−2975.

(7) Shichijo, Y.; Migita, A.; Oguri, H.; Watanabe, M.; Tokiwano, T.; Watanabe, K.; Oikawa, H. *J. Am. Chem. Soc.* **2008**, *130*, 12230−12231.
(8) Matsuura, Y.; Shichijo, Y.; Minami, A.; Migita, A.; Oguri, H.; Watanabe, M.; Tokiwano, T.; Watanabe, K.; Oikawa, H. *Org. Lett.* **2010**, *12*, 2226−2229.
(9) Minami, A.; Migita, A.; Inada, D.; Hotta, K.; Watanabe, K.; Oguri, H.; Oikawa, H. *Org. Lett.* **2011**, *13*, 1638−1641.
(10) Hotta, K.; Chen, X.; Paton, R. S.; Minami, A.; Li, H.; Swaminathan, K.; Mathews, I.; Watanabe, K.; Oikawa, H.; Houk, K. N.; Kim., C.-Y. *Nature* **2012**, *483*, 355−358.
(11) Sato, K.; Minami, A.; Ose, T.; Oguri, H.; Oikawa, H. *Tetrahedron Lett.* **2011**, *52*, 5277−5280.
(12) van Berkel, W. J. H.; Kamerbeek, N. M.; Fraaije, M. W. *J. Biotechnol.* **2006**, *124*, 670−689.
(13) Eichhorn, E.; van der Ploeg, J. R.; Leisinger, T. *J. Biol. Chem.* **1999**, *274*, 26639−26646.
(14) Spyrou, G.; Haggard-Ljungquist, E.; Krook, M.; Jornvall, H.; Nilsson, E.; Reichard, P. *J. Bacteriol.* **1991**, *173*, 3673−3679.
(15) Komeda, H.; Hori, Y.; Kobayashi, M.; Shimizu, S. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 10572−10577.
(16) Nakashima, N.; Tamura, T. *Appl. Environ. Microbiol.* **2004**, *70*, 5557−5568.
(17) Liu, T; Cane, D. E.; Deng, Z. Complex Enzymes in Microbial Natural Product Biosynthesis, Part B: Polyketides, Aminocoumarins and Carbohydrates. In *Methods in Enzymology*; Academic Press: London, U.K., 2009; Vol. *459*, pp 187−214.
(18) (a) In our experience, MNPA is more suitable chiral auxially for chromatographic separation than MTPA. (b) Harada, N. *Chirality* **2008**, *20*, 691−723.
(19) Migita, A.; Shichijo, Y.; Oguri, H.; Watanabe, M.; Tokiwano, T.; Oikawa, H. *Tetrahedron Lett.* **2008**, *49*, 1021−1025.
(20) Finn, R. D.; Mistry, J.; Tate, J.; Coggill, P.; Heger, A.; Pollington, J. E.; Gavin, O. L.; Gunasekaran, P.; Ceric, G.; Forslund, K.; Holm, L.; Sonnhammer, E. L. L.; Eddy, S. R.; Bateman, A. *Nucleic Acid Res.* **2010**, *38*, D211−D222.
(21) van Berkel, W. J. H.; Eppink, M. H. M.; Schreuder, H. A. *Protein Sci.* **1994**, *3*, 2245−2253.
(22) Volkers, G.; Palm, G. J.; Weiss, M. S.; Wright, G. D.; Hinrichs, W. *FEBS Lett.* **2011**, *585*, 1061−1066.
(23) Wierenga, R. K.; Terpstra, P.; Hol, W. G. J. *J. Mol. Biol.* **1986**, *187*, 101−107.
(24) Eggink, G.; Engel, H.; Vriend, G.; Terpstra, P.; Witholt, B. *J. Mol. Biol.* **1990**, *212*, 135−142.
(25) Laden, B. P.; Tang, Y.; Porter, T. D. *Arch. Biochem. Biophys.* **2000**, *374*, 381−388.
(26) Hieber, A. D.; Bugos, R. C.; Yamamoto, H. Y. *Biochim. Biophys. Acta* **2000**, *1482*, 84−91.
(27) Tosin, M.; Smith, L.; Leadlay, P. F. *Angew. Chem., Int. Ed.* **2011**, *50*, 11930−11933.
(28) Faul, M. M.; Huff, B. E. *Chem. Rev.* **2000**, *100*, 2407−2473.
(29) Nicolaou, K. C.; Frederick, M. O.; Aversa, R. J. *Angew. Chem., Int. Ed.* **2008**, *47*, 7182−7225.