Received: July 30, 2014 | Accepted: August 4, 2014 | Web Released: August 8, 2014 CL-140721

Analysis of Enantiofacial Selective Epoxidation Catalyzed by Flavin-containing Monooxygenase Lsd18 Involved in Ionophore Polyether Lasalocid Biosynthesis

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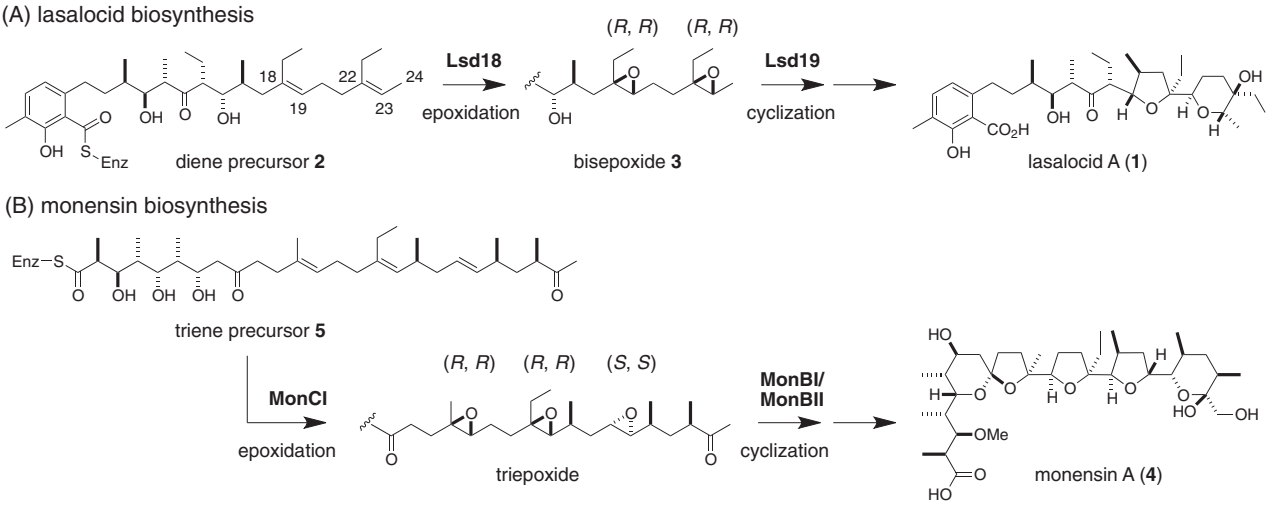
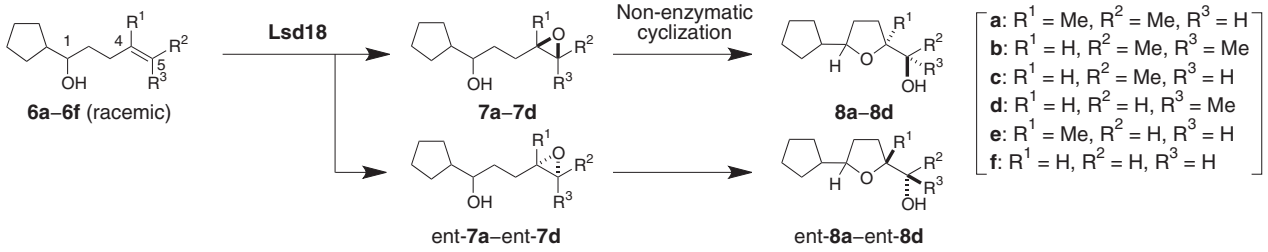
Enzymatic epoxidation represents a key biosynthetic transformation in the construction of polyether skeletons. A single flavin-containing monooxygenase, Lsd18, is involved in ionophore polyether lasalocid biosynthesis and participates in the enantioselective epoxidations of the diene precursor. Biotransformation studies utilizing structurally simplified monoolefin analogs with different substitution patterns revealed important structural requirements for the enantiofacial selectivity of Lsd18-catalyzed epoxidations. These results enabled us to propose a substrate binding model of Lsd18, which was applied to the biosynthesis of other polyethers.

Polyethers such as ionophore polyethers, ladder polyethers, and oxasqualenoids constitute a structurally unique class of natural products that contain the polycyclic polyether skeleton. The structural diversity of the polyether skeleton is primarily dictated by the number of ether rings and the manner in which the ring closure occurs. The unique core structure has attracted many chemists, and extensive biosynthetic studies concerning the polyether skeleton have been reported. Following the initial proposal of the unified biosynthetic model in 1983,[1] Leadlay's group identified two key enzymes, epoxidase and epoxide hydrolase, responsible for polyether formation.[2] In 2008, an in vitro enzymatic transformation was reported for the skeletal construction of lasalocid A (**1**) catalyzed by epoxide hydrolase Las19.[3] Additionally, the functions of key enzymes, epoxidase Lsd18[4] and Lsd19,[5–7] and a pair of epoxide hydrolases MonBI/MonBII[8,9] involved in the biosynthesis of **1** and monensin A (**4**) (Scheme 1) were also elucidated.[10] Lsd18 is a flavoprotein that contains tightly bound flavin adenine dinucleotide and catalyzes two rounds of epoxidations on the olefin moieties of diene precursor **2** to afford the corresponding bisepoxide **3** (Scheme 1A). A homologous epoxidase is found in other ionophore polyether biosynthetic gene clusters, suggesting that a single epoxidase catalyzes multiple rounds of enantioselective epoxidations in biosyntheses (Scheme 1B).[2,11] Intriguingly, some epoxidases catalyze the epoxidation of polyene precursors in a unique and enantioselective manner; for example, in monensin biosynthesis, a single epoxidase, MonCI, installs (*R*,*R*)- and (*S*,*S*)-epoxides on the internal and terminal olefin moieties, respectively.[1] Enantioselective epoxidation is an intriguing topic in polyether construction, because it defines the stereochemistry of polyethers. However, difficulties regarding the preparation of functionally active epoxidases and putative polyene precursors have been a bottleneck in determining the functions of the epoxidases.

A previous study[10] and Leadlay's observations[12] showed that polyether formation occurs during chain elongation by polyketide synthase (PKS). In lasalocid biosynthesis, the linear polyketide chain bound to acyl carrier protein enter the active site of epoxidase Lsd18 from the diene terminal. Recently, a biotransformation system was established to investigate the function of Lsd18 by utilizing an Lsd18-overexpressed host, and it was found that simple monoolefin **6a** with a sterically demanding moiety such as a cyclopentyl group was accepted by Lsd18 as an alternative substrate.[4] The highly stereoselective epoxidation of **6a**, which mimics the C19–C24 portion of **2**, provides an opportunity to investigate important structural motifs in the enzymatic enantiofacial epoxidation of structurally simplified analogs. In this study, several substrate analogs, **6b–6f**, were designed with different substitution patterns on the olefin moiety



Scheme 1. Enzymatic polyether construction of (A) lasalocid and (B) monensin.



Scheme 2. Epoxidation of tested substrates with Lsd18-overexpressed *Rhodococcus erythropolis*.

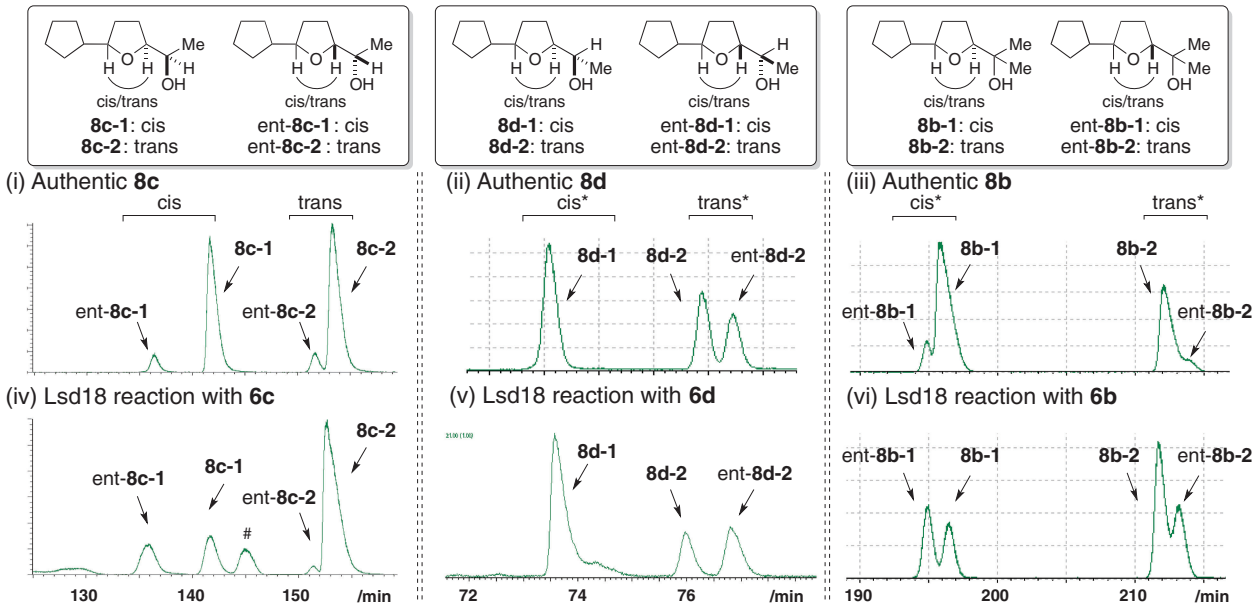


Figure 1. GC-MS profiles of authentic samples (i) **8c**, (ii) **8d**, and (iii) **8b**, and Lsd18-catalyzed reaction products with (iv) **6c**, (v) **6d**, and (vi) **6b**. *: exchangeable. #: impurity.

in order to examine the substrate specificity and enantiofacial selectivity in Lsd18 epoxidation (Scheme 2).

Simple olefins **6b** and **6c** were synthesized from the corresponding γ,δ-unsaturated ester (Johnson orthoester Claisen rearrangement product) according to a previous report[4] (Scheme S1) or from the ester with a terminal alkyne[13] (Scheme S2). To obtain authentic samples, epoxidation of **6b**–**6d** with *m*CPBA was carried out. The resulting epoxides were prone to epoxide-opening reactions, thereby yielding cyclization products **8b**–**8d** (Schemes S1 and S2). Hardly separable C6 diastereomers of **8c-1** and **8c-2** were isolated by HPLC following their conversion into the corresponding (*R*)-2-methoxy-2-(1-naphthyl)propionate (MNPA) esters, whose structures were determined by extensive NMR analysis. Asymmetric epoxidation using Shi's catalyst[14] yielded the enantiomerically enriched cyclization products. Using the synthetic standards, we established the appropriate separation conditions of three sets of stereoisomers **8b**–**8d** by GC-MS using a chiral capillary column. Chiral GC analysis of the asymmetric epoxidation products revealed enantiomerically enriched peaks, thus enabling the assignment of the absolute configurations, as shown in Figure 1. The oxymethine stereochemistry of substrate olefins **6b** and **6c** did not affect the enantioselectivity of asymmetric epoxidations, with the exception of *Z*-olefin **6d**.

Synthetic analogs **6a**–**6f** were separately incubated with Lsd18-overexpressed *Rhodococcus erythropolis* in the reaction buffer.[4] GC-MS analysis of the extracts confirmed the production of **8b**–**8d** and ent-**8b**–ent-**8d** from **6b**–**6d** (Figure S2). On the other hand, no new peak was detected in the reactions with **6e** and **6f**. Long incubation times led to decreased conversions, possibly due to the degradation of the substrates and products by the host strain. The conversion efficiency decreased in the following order: trisubstituted **8a** {40% (15 min)}, **8b** (71%), disubstituted **8d** (32%), and **8c** (22%) (Table 1). The enantiofacial selectivity of nearly all the substrates suggested a *re*-face attack at the C4 position. In the reactions with very low enantioselectivity (**8b-1**: 14% ee, **8d-2**: 7% ee), reversed enantioselectivity (*si*-face attack)

Table 1. Summary of Lsd18-catalyzed epoxidation exchangeable

	Conversion /%		Relative conversion /%	ee/% ee (epoxide)
8a	40 (15 min)	**8a-1** (cis)	49	99 (4*R*,5*R*)
		8a-2 (trans)	51	87 (4*R*,5*R*)
8b	71 (2 h)	**8b-1** (cis)[a]	38	14 (4*S*)
		8b-2 (trans)[a]	62	27 (4*R*)
8c	22 (2 h)	**8c-1** (cis)	33	4 (4*R*,5*R*)
		8c-2 (trans)	67	94 (4*R*,5*R*)
8d	32 (2 h)	**8d-1** (cis)[a]	61	76 (4*R*,5*S*)
		8d-2 (trans)[a]	39	7 (4*S*,5*R*)

[a]Exchangeable.

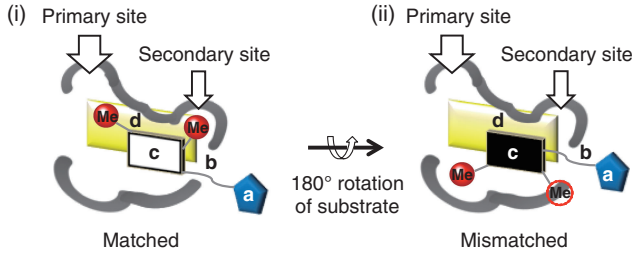


Figure 2. Substrate binding model of **8a** in Lsd18-catalyzed epoxidation: **a**: cyclopentyl moiety, **b**: flexible linker, **c**: olefin plane, **d**: flavin wall.

was observed. These results indicated that Lsd18 binds to the substrates and exposes the olefin plane to the 4-hydroperoxyflavin moiety, an oxidation agent in the enzymatic epoxidation. Substrates harboring C5 methyl groups were converted to the corresponding epoxides, while no conversion was detected in the reactions with **6e** and **6f**, suggesting that substituents in the C5 position are crucial for substrate binding and enantiofacial selectivity. With regard to the conversion, 5,5-dimethyl-substituted olefin **6b** resulted in a better yield than 5-methyl-substituted olefins **6c** and **6d**. On the other hand, the observed selectivity in the reactions with **6c** and **6d** was better than that with **6b**, indicating that the substitution pattern played a role in the enantiofacial selectivity. The higher enantiofacial selectivity and conversion of **6a** than that of **6c**, in addition to the lack of conversion with gem-disubstituted **6e**, indicated that C4-methyl substitution may assist in fixing the olefin plane, but is not a primary determinant in Lsd18 recognition. Based on these results, a substrate binding model of Lsd18 was proposed. Lsd18 has primary and secondary recognition sites for C5 and C4 methyl groups, respectively, to fix the olefin plane of **6a** (Figure 2(i)). An internal rotation of the olefin plane by 180° cannot occur, partly due to the steric hindrance between the C4 methyl group and Lsd18 (Figure 2(ii)). In the case of **6b**, both orientations can be accepted by Lsd18 with similar efficiencies because of the lack of the C4 methyl group. Additionally, this resulted in a good conversion, but low enantioselectivity. The difference in enantioselectivity in the reactions with **6c** and **6d** was probably due to the C1 hydroxy group. In order to predict the enantioselectivity of the substrates, the binding model should be fine-tuned by incorporating the crystal structure data.

The proposed binding model can explain the enantiofacial selectivity of diene precursor **2** in lasalocid biosynthesis (Figure 1). The trisubstituted *E*-olefin moiety is a common structural motif found in most biosynthetic polyene precursors of ionophore polyethers. Intriguingly, in most cases, the epoxidation occurs via the *re*-face to afford (*R*,*R*)-epoxides. Thus, the proposed model can be applied to the epoxidation of trisubstituted *E*-olefins. In some cases, the installation of (*S*,*S*)-epoxides on disubstituted *E*-olefins also occurs in the biosynthesis of several ionophore polyethers, including monensin (Scheme 1B). In the original prediction, the substitution pattern (tri- vs. disubstitution) was deemed to be important in defining the enantiofacial selectivity. However, changing the substitution pattern from trisubstituted **6a** to disubstituted **6c** did not result in the switched enantiofacial selectivity. One of the possible reasons behind this observation was that the simplified monoolefin analogs did not have sufficiently large substituents in the C5 position to mimic the terminus of triene precursor **5**.

The epoxidation of **6b**, which has the same partial structure as that of the biosynthetic intermediates of oxasqualenoids, suggested that Lsd18 epoxidizes trisubstituted olefin that resembles the isoprene unit. The reaction with (*R*)-linalool was previously reported to afford the corresponding epoxides in a nearly 1:1 mixture of the diastereomers.[4] The epoxidation of substrate[15] mimicking the partial structure of oxasqualenoid afforded a pair of diastereomers in equal amounts (Figure S3), suggesting that Lsd18 epoxidizes isoprene-type olefins in a nonenantiofacially selective manner, unlike polyketide polyenes.

In summary, Lsd18-catalyzed epoxidation was examined with six differently substituted olefins to evaluate the structural requirements of Lsd18 substrate recognition. The substitution pattern on the olefin moiety significantly affected the conversion and enantiofacial selectivity. Based on the results, a substrate binding model of epoxidase that explains the enantiofacial selectivity of various olefins was proposed. The model can be applied to most enantioselective enzymatic epoxidations of PKS-derived *E*-olefins in polyether biosynthesis.

This work was supported by a MEXT research grant on innovative area No. 22108002 to H. Oikawa and No. 26750361 to A. Minami.

Supporting Information is available electronically on J-STAGE.

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