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Remarkable synergistic effect between MonBI and MonBII on epoxide opening reaction in ionophore polyether monensin biosynthesis

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ABSTRACT

Enzymatic epoxide-opening cascade is one of the key biosynthetic processes for constructing structurally diverse polyethers. Here we report the first in vitro analysis of the cyclization catalyzed by two epoxide hydrolases, MonBI and MonBII involved in monensin biosynthesis, using simple epoxy-alcohols and the unprecedented synergistic effect on the epoxide-opening activity between these epoxide hydrolases.

Ionophore polyethers,[1] one of the major groups of natural polyketides, have an unique polyether skeleton composed of small numbers of tetrahydrofuran (THF) and tetrahydropyran (THP) rings connected via a carbon–carbon bond. Although the number of ether rings resulting from different cyclization modes is reflected the structural diversity of this type of natural product, as represented by the Cane–Celmer–Westley unified biosynthetic scheme,[2] it is suggested that polyether skeletons are constructed by a common strategy involving nucleophilic epoxide-opening cascades of the polyepoxides after enzymatic asymmetric epoxidation of the linear polyolefins. This simple yet versatile strategy for the biosynthesis of complex polyether metabolites prompted chemists to synthesize ionophore antibiotics using epoxide-opening cascades.[3,4] The successful biomimetic synthesis of those polyether systems supports the biosynthetic proposal.

To investigate the detailed biosynthesis for polyether construction, four polyether biosynthetic gene clusters involved in monensin (**1**) (*mon*),[5] nanchangmycin (*nan*),[6] nigericin (*nig*),[7] and lasalocid (*lsd*, *las*) biosynthesis[8] and the gene cluster involved in tetronomycin (*tet*) biosynthesis,[9] a related natural product, have so far been characterized and it revealed that the putative key genes for polyether construction, the epoxidase gene and epoxide hydrolase gene, are conserved in these biosynthetic gene clusters. Gene disruption experiments of the epoxidase gene (*monCI*) and epoxide hydrolase gene (*monBI* and *monBII*) in monensin biosynthesis demonstrated that, as expected in the Cane–Celmer–Westley model, epoxidation of the (*E*,*E*,*E*)-triene precursor and epoxide-opening reaction of the corresponding epoxide is catalyzed by MonCI, and MonBI and/or MonBII, respectively.[10,11] However, the detailed catalytic mechanism of epoxidase and epoxide hydrolase (EH) at the enzymatic level remained unclear.

Recently, we have conducted functional analysis of epoxide hydrolase Lsd19 involved in lasalocid A biosynthesis as a model system of enzymatic epoxide-opening cascades. In 2008, we identified the function of Lsd19 in catalysis of the epoxide-opening reactions with an energetically favored 5-*exo* cyclization and an energetically disfavored 6-*endo* cyclization to afford a polyether skeleton of lasalocid from the putative biosynthetic intermediate, bisepoxyprelasalocid.[12] Following this first in vitro characterization, we investigated substrate specificity of Lsd19 using structurally diverse bisepoxides.[13] As reported for limonene epoxide hydrolase and other EHs,[14] Lsd19 also accepted various structurally simplified bisepoxides. In addition, specificity toward the stereochemistry of bisepoxide moiety revealed that the second epoxide-opening reaction can be altered by the stereochemistry at the terminal epoxide. The relaxed substrate tolerance of Lsd19 allowed us to investigate the detailed catalytic mechanism using these substrate analogs. A site-directed mutagenesis study using these simplified analogs revealed the conserved acidic amino acid pair, located at the *N*- and *C*-terminal domain, as key catalytic residues for the epoxide-opening reaction.[15] Further domain dissection analysis clearly showed that the epoxide-opening cascades involving 5-*exo* and 6-*endo* cyclization are separately catalyzed by two catalytic domains, named Lsd19A (*N*-terminal region) and Lsd19B (*C*-terminal region). These results demonstrated a simple

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strategy for polyether construction in which EH domain independently catalyzes the single epoxide-opening reaction in a stepwise manner. However, in monensin biosynthesis, the hypothesis that a single EH domain/subunit catalyzes a single epoxide-opening reaction cannot be applied because the number of domains/subunits (MonBI and MonBII) is not the same as that of the epoxide-opening reactions (triepoxide intermediate). This probably reflects previous gene disruption experiments of MonBI and/or MonBII, which did not yield the expected partially cyclized intermediates but essentially the same products. We herein report the enzymatic epoxide-opening reaction with MonBI and MonBII using structurally simple substrate analogs and the unprecedented synergistic effect of MonBI and MonBII.

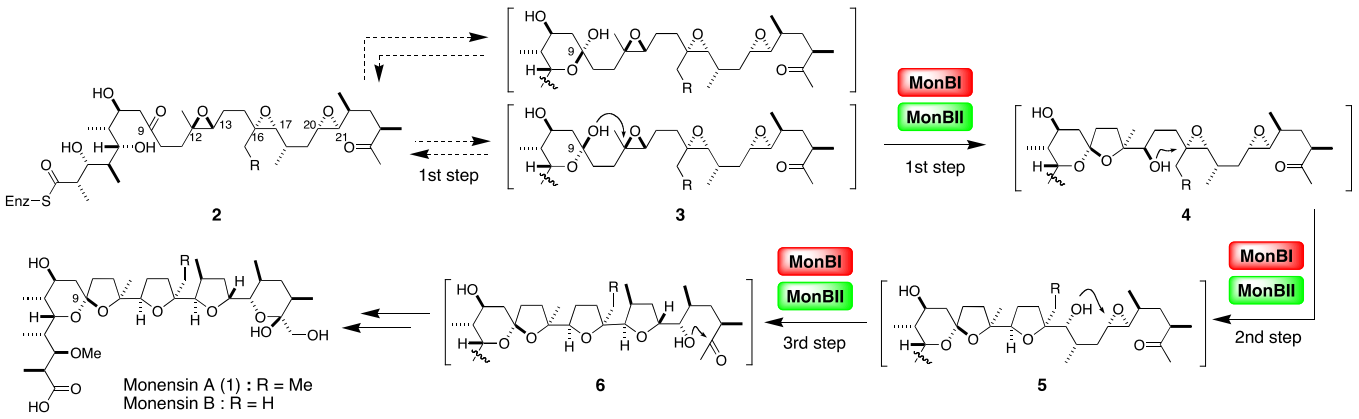
Epoxide-opening cascades in monensin biosynthesis are proposed as follows (Scheme 1): (1) EH(s) selects a single hemiacetal from the equilibrium mixture which undergoes 5-*exo* cyclization to give the spiroketal intermediate **4**; (2) EH(s) catalyzes the second and third 5-*exo* cyclization to afford the cyclic ether intermediates **5** and **6**. Given the broad substrate tolerance of Lsd19 including epoxide stereochemistry, we designed a simplified epoxy-alcohol (**8a**–**d**) to examine the enzymatic activity of MonBI and MonBII (Scheme 2). The model substrate **8** is an analog of the epoxy-alcohol part at C12–C19 which may also mimic the C8–C13 and C16–C21 part when a series of acyl groups ranging from C2 to C10 chain length is employed.

Monoepoxy-alcohols (**8a**–**d**) were synthesized as described in Scheme 2. It commenced with the protection of commercially available (*S*)-methyl 3-hydroxy-2-methylpropanoate as silyl ether followed by reduction with DIBAL to afford **11**. After Swern oxidation, aldehyde was treated with vinyl lithium reagent to give the allyl alcohol **12** as a diastereomeric mixture. Synthesis of the desired *E*-trisubstituted olefin **13** was achieved by the ortho ester Claisen rearrangement.[16] Treatment of methyl ester **13** with MeMgBr and subsequent removal of silyl group afforded the common diol intermediate **14**. Finally, condensations of various acyl groups followed by Shi's asymmetric epoxidation[17] afforded a series of the desired monoepoxy-alcohol (**8a**–**d**). The diastereoselectivity of **8a**–**d** was determined as 10:1 to 20:1 (*R*/*S* stereochemistry) based on ^{1}H NMR analysis.

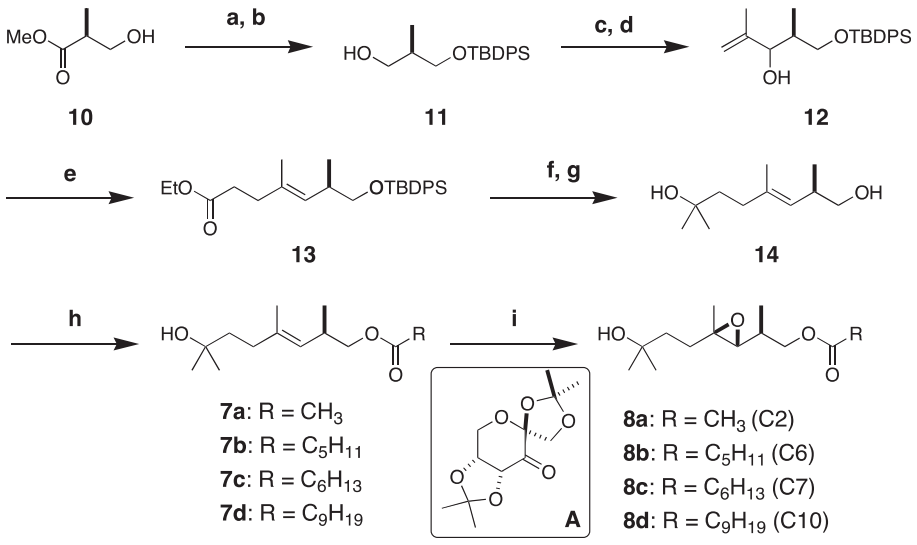
A synthetic *monBI* gene with codons optimized for expression in *Escherichia coli*[18] and *monBII* gene were separately cloned and expressed in *E. coli* BL21-Gold (DE3) as an N-terminal His_6-tagged protein and individual recombinant proteins were purified by Ni-NTA column chromatography to a state of homogeneity as judged by SDS–PAGE (Fig. S1).[19] In addition, as reported in our previous paper, alignment of MonBI, MonBII, and Lsd19 revealed that the acidic amino acid pair corresponding to the catalytic residue for the Lsd19 reaction (Lsd19A; D38-E65, Lsd19B; D170-E197) is conserved in MonBI (D37 and E64) and MonBII (D38 and E65). Therefore, alanine mutants were also constructed and purified by the same procedure for wild type enzymes (Fig. S1).[19]

In our study with Lsd19, we found that various epoxy-alcohols were prone to cyclization affording a 5-*exo* cyclization product according to Baldwin's rule.[20] Therefore, prior to the detailed functional analysis of MonBI and MonBII, the feasibility of 5-*exo* cyclization of synthetic monoepoxy-alcohol (**8a**–**d**) was examined in several pH ranges. LC–MS analysis of the reaction product revealed that, in all cases, no cyclized product (**9a**–**d**) was detected in the pH range of 6.5–8.0 even after 6 h incubation at 37 °C. This facilitated the distinction of MonBI and/or MonBII catalyzing 5-*exo* cyclization from the nonenzymatic reaction. Based on this result, the following reaction conditions for the functional analysis of MonBI and MonBII were established: 20 mM MOPS (pH 7.0), 10% glycerol, 8 μM monoepoxy-alcohol, 8 μM MonBI, and/or MonBII.[21] Then, monoepoxy-alcohols (**8a**–**d**) were separately subjected to the MonBI or MonBII reaction, and subsequent LC–MS analysis revealed a new peak only in the MonBII reaction (Figs. 1, S2). Since the retention time of the product is identical to those of authentic samples **9a**–**d** prepared by the acid treatment, the MonBII reaction product was confirmed as a 5-*exo* cyclization product (THF-product). C10 analog represented the highest activity in the MonBII reaction (C2: 0%, C6: 12%, C7: 13%, C10: 30%) (Fig. S2C) In contrast, MonBII mutant (BII-D38A and BII-E65A) showed no catalytic activity toward the C10 analog. More interestingly, the epoxide opening activity was dramatically enhanced with the addition of MonBI to the MonBII reaction mixture (Fig. 1). In this MonBII//MonBI system (described in the order of EH//partner EH), C6 analog showed the highest activity among those tested analogs (C2: 2%, C6: 95%, C7: 91%, C10: 90%) (Figs. 1d, S2). This enhancement of cyclization activity was also observed using MonBI-E64A as a partner EH (Fig. 1e). In addition, the activity gradually increased depending on the MonBI-E64A concentration (Fig. S3) Thus the observed intriguing synergistic effect on the epoxide-opening reaction catalyzed by MonBII in the presence of partner EH prompted us to examine the MonBI catalyzing reaction again in the presence of MonBII-D38A mutant. As expected, enhancement of MonBI catalytic activity was observed in this MonBI//MonBII-D38A system (C2: 0%, C6: 22%, C7: 19% and C10: 9%) (Figs. 1f, S2). In contrast, a small but detectable amount of THF-product was observed in the MonBI-E64A//MonBII-D38A system using C6 analog (4%) (Fig. 1g).

The discovery of specific thioesterases catalyzing hydrolysis of several PKS-bound polyether intermediates in the biosynthesis of monensin and nanchangmycin showed that actual substrates



Scheme 1. Enzymatic epoxide-opening cascades in monensin biosynthesis.



Scheme 2. Reagents and conditions: (a) TBDPSCl, imidazole, THF, quant.; (b) DIBAL, Et_2O, 96%; (c) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2; (d) 2-bromo propene, *t*-BuLi, Et_2O, two steps 60%; (e) $CH_3CH(OMe)_3$, 140 °C, 70%; (f) MeMgBr, THF, 63%; (g) TBAF, THF, 83%; (h) fatty acid, DCC, DMAP, CH_2Cl_2, 70–99%; (i) **A**, Oxone^R, K_2CO_3, Bu_4NHSO_4, CH_3CN–$CH_2(OMe)_2$–H_2O, 0 °C, 51–69%.

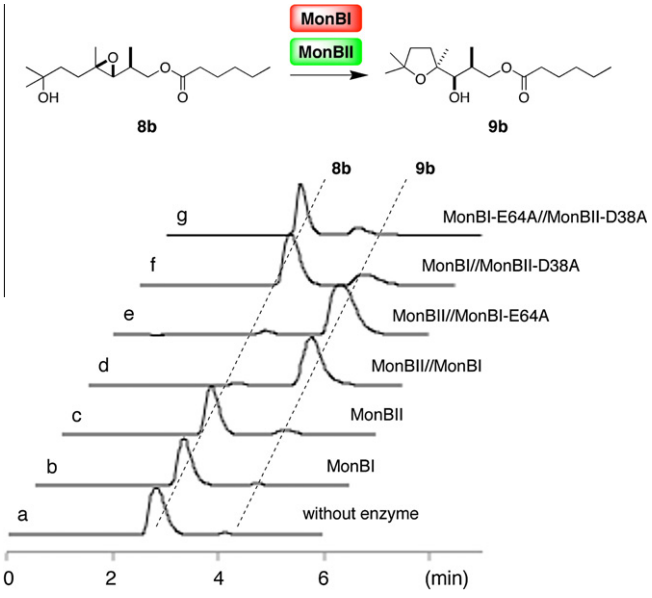


Figure 1. LC–MS profiles of the reaction products with (a) control experiment, (b) MonBI reaction, (c) MonBII reaction, (d) MonBII//MonBI reaction, (e) MonBII//MonBI-E64A reaction, (f) MonBI//MonBII-D38A reaction, (g) MonBI-E64A//MonBII-D38A reaction.

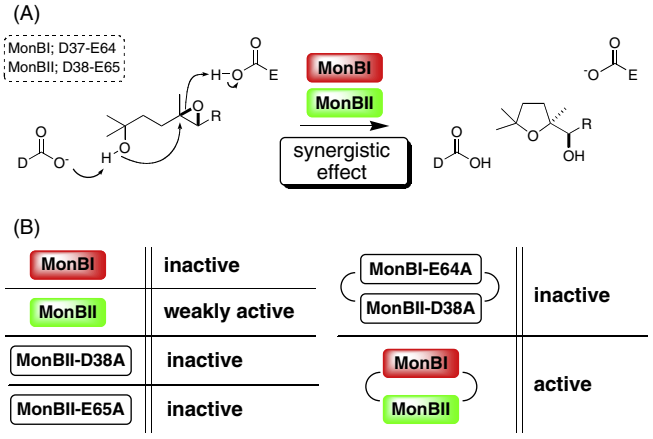


Figure 2. (A) Epoxide opening cascades catalyzed by MonBI and MonBII. (B) Summary of synergistic effect.

of EHs are PKS-bound polyepoxides, which are structurally complex and thus difficult to synthesize.[22] The in vitro analyses of MonBI and MonBII described above are the first direct evidence that both act as an epoxide hydrolase to catalyze 5-*exo* cyclization. This clearly indicates that the use of simple epoxy-alcohol analogs offers a practical approach to the analysis of EHs involved in ionophore polyether biosynthesis. Loss of the cyclization activity in MonBII-D38A, MonBII-E65A, and MonBI-E64A//MonBII-D38A reactions indicates that each acidic amino acid pair is the catalytic residue for the epoxide-opening reaction (Fig. 2). This is in accordance with our previous results on Lsd19 and reveals that the highly conserved motifs are key catalytic residues of various EHs found in the polyether biosynthetic gene cluster. Clear preference of MonBII toward C6–C10 analogs showed that MonBII recognizes acyl chain moiety with medium chain length as an imitator of the putative intermediates **3** and/or **4** in the active site. This hypothesis is supported by the structural model of MonBII based on the Lsd19 crystal structure that MonBII has an active site to accept a relatively long polyketide chain compared with Lsd19A (unpublished result).

In the previous gene disruption experiments on either *monBI* or *monBII* or both, all mutants afforded mixtures of the same intermediates including 9-epimonensin instead of natural-type polyether product. The intriguing synergistic effect between MonBI and MonBII for their cyclization activity might explain the puzzling observation as described above because disruption of each gene causes overall decrease of epoxide opening activity (Fig. 2). The fact that both *monBI* and *monBII* homolog are highly conserved either as a separated EH system (NigBI//NigBII) in a nigericin biosynthetic gene cluster or a fused EH system [Lsd19 (Lsd19A//Lsd19B) and NanI (NanIA//NanIB)] in lasalocid, and a nanchangmycin biosynthetic gene cluster implies that the synergistic effect is a common feature of the enzymatic epoxide-opening cascades in polyether biosynthesis. Although DNA methyltransferase shows a similar synergistic function,[23] to our knowledge, the effect on epoxide-opening activity is unprecedented and probably a characteristic for the biosynthetic construction of the polyether system.

In summary, we successfully observed enzymatic activities of the epoxide hydrolase MonBI and MonBII in monensin biosynthesis using simple monoepoxy-alcohol as an imitator of structurally complex intermediate. Furthermore, the unexpected synergistic effect found in this study advances our understanding of the enzymatic epoxide-opening cascades for polyether construction in the biosynthesis of ionophore antibiotics. Our approach using simple substrate analogs is proven to be effective for the functional analysis of EHs because the bottleneck of the biochemical analysis of EHs exists in the synthesis of the putative polyepoxide substrates. To examine the cooperative effect of polyether EHs, we are currently studying further various combinations of other EHs such as Lsd19 and its equivalents.

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Supplementary data

Supplementary data (LC–MS profiles) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2011.07.145.

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