

## Mechanism of diterpene cyclization in cyclooctatine biosynthesis

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### Introduction

Cyclooctatin is a potent inhibitor of lysophospholipase, which catalyzes the hydrolysis of the fatty acid ester bonds of lysophospholipids. This inhibitor was isolated from the broth of *Streptomyces melanosporofaciens* MI614-43F2 while screening for lead compounds for the development of anti-inflammatory drugs targeting lysophospholipase.

Cyclooctatin has a unique tricyclic diterpene skeleton characterized by a 5-8-5 fused ring system (Fig. 1A). In the biosynthesis of cyclooctatin, the biosynthetic gene product CotB1 synthesizes geranylgeranyl diphosphate (GGDP) from isopentenyl diphosphate and dimethylallyl diphosphate, and then CotB2 catalyzes the stereospecific cyclization of GGDP to give cycloocta-9-en-7-ol (Fig. 1B). Next, the cytochrome P450 enzymes, CotB3 and CotB4, catalyze the stereospecific hydroxylation to yield the final product, cyclooctatin. Although cyclization is a key reaction in cyclooctatin biosynthesis, mechanism of formation of stereo- and regio specific ring is unclear since no 3D-structural information has been available to date. To clarify the cyclization mechanism of CotB2, we determined the crystal structure of apo-form of CotB, and the complex with a substrate analog, geranylgeranylthiodiphosphate (GGSP).

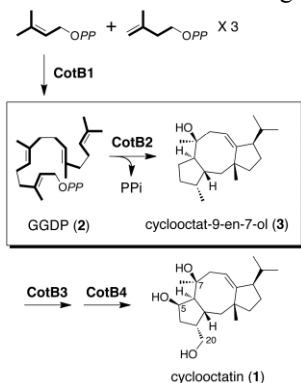


Fig. 1 Biosynthetic pathway of cyclooctatin

### Materials and Methods

**Crystallization** - Crystal of apo CotB2 (apo type II) was obtained by the hanging-drop vapor-diffusion method. The reservoir solution contained 0.1 M HEPES-NaOH (pH7.0-8.5) and 2.2 M ammonium formate. Crystal of CotB2 complexed with GGSP/Mg<sup>2+</sup> (GGSP/Mg<sup>2+</sup> type I) is obtained with reservoir solution which contains 20 % PEG6000, 100 mM Bicine-NaOH (pH9.0), 5 mM MgSO<sub>4</sub>, and 1 mM GGSP.

**Data collection and processing** -The X-ray diffraction data were collected using the beamline NW-12, 5A at PF. The image sets were integrated and scaled using HKL2000.

### Results and Discussion

**Active site structure** - The crystal structure of apo form of CotB2 was determined at 1.8 Å resolution by means of MR method with the model of apo form with 2.4 Å resolution as a search model. Apo CotB2 is composed of a terpene cyclase fold (Gly9-Asn292), forming 13 α-helices. The electron density of N-terminal and C-terminal residues of CotB2 (Met1-Ala8 and Lys293-Gln307) was not observed, indicating that this region is disordered in the crystal. In apo form, DDMD motif and NSE/DTE motif conserved among terpene cyclase family are located at the putative active site. In the GGSP/Mg<sup>2+</sup> form, GGSP is bound at the pocket of the enzyme with a unique S-shaped conformation (Fig. 2B). Mg<sup>2+</sup> ion was also bound near diphosphate group of GGSP. Asn220, Ser224, and Glu228 from NSE/DTE motif binds with Mg<sup>2+</sup>. The diphosphate group of GGSP was recognized by Arg117 and bound with Mg<sup>2+</sup>. Asp111 from DDMD motif forms Arg294 from 'basic motif' to close the active site. Newly defined Tyr295 forms diphosphate group of GGSP. C20 carbon chain was surrounded by many aromatic and hydrophobic residues and several Asn residues. To elucidate the reaction mechanism, the mutational analysis of the residues which form active site is now on going.

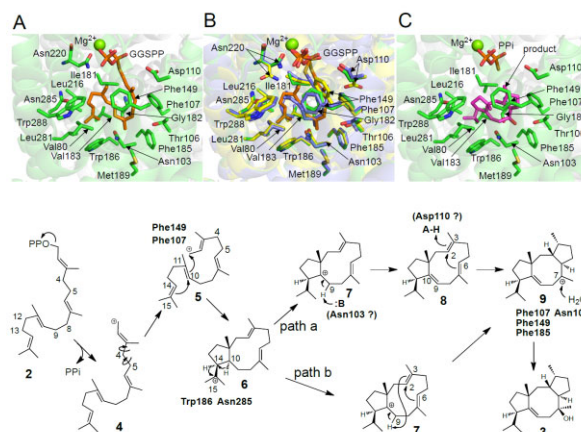


Fig. 2 Active site structures of CotB2. Apo form (A), GGSP/Mg<sup>2+</sup> bound form (B), and the model docked with the product (C). Proposed reaction mechanism (D).

1) T. Tomita *et al.* in preparation.

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