# Molecular basis for two stereoselective Diels–Alderases that form enantiomeric decalin skeletons

The Diels–Alder (DA) reaction is one of the most important reactions for C–C bond formation in synthetic chemistry. This practical and very useful reaction, which forms two C–C bonds and up to four chiral centers to generate a cyclohexene from a conjugated diene and a substituted alkene, is also a key step in many natural product biosynthetic pathways. Recently, a number of enzymes catalyzing the DA reaction, the so-called Diels–Alderases (DAases), have been identified from bacterial, fungal, and plant origins [1]. It is noteworthy that, although DAases have no common structural features and are derived from distinct progenitor enzymes or proteins, such as *S*-adenosylmethionine (SAM)-dependent methyltransferase and FAD-dependent monooxygenase, they all exhibit high stereoselectivity and catalytic efficiency. However, the mechanisms underlying the extraordinary features of naturally occurring DAases, including the origin of stereoselectivity and catalytic efficiency, have remained elusive.

Fsa2 and Phm7 catalyze intramolecular DA reactions to form enantiomeric decalin scaffolds from similar linear polyenoyl tetramic acids during the biosynthesis of HIV-1 integrase inhibitors, equisetin and phomasetin, which have "enantiomeric" decalin skeletons (Fig. 1(a)). The replacement of *phm7* in a phomasetin-producing fungus with *fsa2* resulted in the production of an equisetin-type decalin scaffold, indicating that these enzymes control and determine the stereochemistry of the decalin scaffold during the biosynthesis [2]. To unveil the stereoselective DA reaction mechanisms of Fsa2 and Phm7, we first determined the crystal structures of these enzymes.

X-ray diffraction data of Fsa2 and Phm7 crystals were collected at SPring-8 **BL32XU** and **BL41XU** (Fig. 1(b)) [3]. The crystal structure of Fsa2 has a β-sandwich and a β-barrel domain at the N- and C-termini, respectively, and a large pocket is present between the two domains. Considering the substrate structure and volume, we speculated that the large cavity created by the two domains is a substrate binding site. Phm7 catalyzes intramolecular DA reactions to produce the decalin scaffold enantiomerically opposite to that of Fsa2. Despite their distinct stereoselectivity and low sequence similarity, the crystal structure of Phm7 is similar to that of Fsa2, and the shape and position of their large pockets are also similar. Unfortunately, however, the crystal structures of substrate-free Phm7 and Fsa2 did not reveal the mechanism of how they control the stereoselectivity.

To find productive binding conformations of the substrates in Fsa2 and Phm7, we carried out molecular dynamics (MD) simulations of substrate-bound enzymes by the gREST method [4], which can search possible binding poses more extensively than the conventional method. The MD simulations yield four major bound poses, including "folded" and "extended" conformations, for the substrates. The major "folded" poses of the Phm7 substrate were well defined (Fig. 2(a), left panel). The tetramic acid moiety and polyene tail of the folded poses were located at the upper front and back of the pocket, respectively, whereas a U-shaped folded alkyl chain was found at the lower side of the pocket. Representative conformations of the Phm7 substrate explain the expected phomasetin-type decalin configuration (Fig. 2(b)), indicating that the Phm7 pocket robustly regulates and stabilizes the substrate in a specific conformation. In contrast to Phm7, the pocket of Fsa2 allows various "folded" conformations of the substrate (Fig. 2(a), right panel). Nevertheless, similar tetramic acid-front and polyene-back poses were also found, which were consistent with the configuration of the equisetin-type decalin scaffold (Fig. 2(b)).

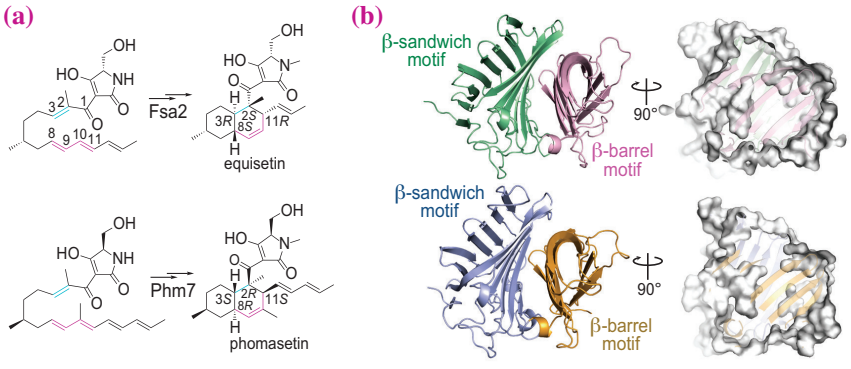


Fig. 1. **(a)** Stereoselective DA reactions catalyzed by Fsa2 and Phm7 from similar linear polyenoyl tetramic acids. **(b)** Crystal structures of Fsa2 (top) and Phm7 (bottom). Surface models show shapes of the pocket between the two domains.

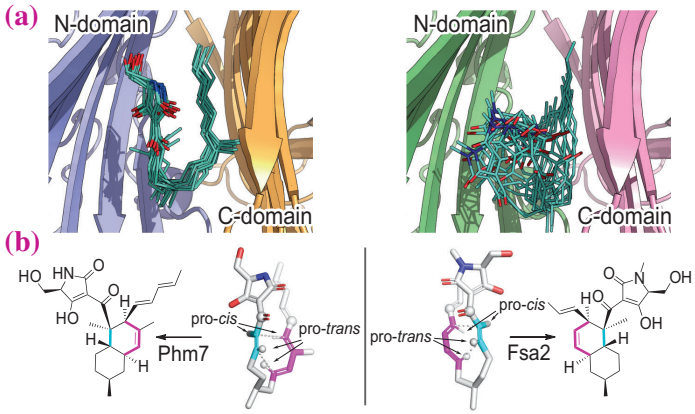


Fig. 2. Predicted binding models of Phm7 (left) and Fsa2 (right). **(a)** Collective view of 12 representative snapshots of the folded substrates in the binding pockets. **(b)** Conformations of the representative poses of the substrates and structures of the corresponding cycloadducts.

To verify the pseudo-enantiomeric substrate binding conformation and identify the amino acid residues involved in substrate binding and chemical catalysis in Phm7 and Fsa2, we carried out site-directed mutagenesis experiments of both enzymes and density functional theory calculations for Phm7. The outcomes demonstrated that the binding models predicted by the MD simulations are reliable. It was also confirmed that Phm7 tightly holds the tetramic acid moiety via multiple hydrogen bonds, and a pair of acidic and basic side chains is the chemical catalyst that activates the dienophile to promote the DA reaction. In contrast, the Fsa2 substrate undergoes a hydrogen bonding interaction with only one amino acid residue. Fsa2 likely retains the substrate affinity to the enzyme by accommodating various folded conformations and minimizing entropy loss upon binding. Moreover, there is no hydrophilic amino acid residue in proximity to the C1 carbonyl, which accelerates the reaction as a catalytic residue. Fsa2 possibly adopts a dynamic control of the reaction rate using the conformational fluctuations of the substrate.

It is likely that substrates bind to the pocket of Fsa2 and Phm7 in a similar way (tetramic acid-front and polyene-back orientation), allowing the stereoselective DA reactions to proceed. Considering that the conformation of the enzyme-bound substrate is closely correlated to the stereochemistry of the product, we propose a model of how the enzymes produce decalin scaffolds with four possible configurations via the stereoselective DA reactions (Fig. 3). The linear Fsa2 substrate is folded clockwise (Fig. 3(a)) and the Phm7 substrate counterclockwise (Fig. 3(b)) to yield (pseudo)enantiomeric conformations in the enzyme pocket. When the folded Phm7 substrate is flipped horizontally, it binds to the Phm7 pocket in the tetramic acid-front and polyene-back orientation. A key difference between the transition state structures for *trans*- and *cis*-decalin scaffolds is created by the rotation of the polyene tail at the C7–C8 bond. Thus, the *cis*-decalin forming conformation of the substrate in the enzyme pocket is predictable. More than 100 homologs of Fsa2 and Phm7 are available in the public database. A highly accurate protein structure prediction from amino acid sequences using deep learning algorithms, such as AlphaFold2, is now feasible. A combination of computational calculations, including protein structure prediction, MD-based docking simulation, and quantum chemical calculation, with biochemical experiments will provide further insight into the structure-function relationship of this stereoselective DAase family catalyzing bioactive decalin scaffolds, as well as a guiding principle to create artificial DAases.

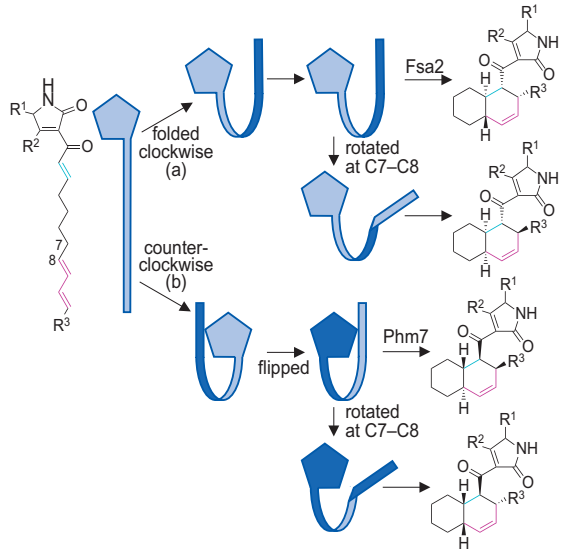


Fig. 3. Model of the enzyme-mediated formation of four diastereomeric decalin scaffolds. Linear polyenoyl tetramic acid substrates are shown schematically as a pentagon and a ribbon. Folded substrates bind to the enzymes in the tetramic acid-front and polyene-back manner.

Naoki Kato[a], Keisuke Fujiyama[b] and Shingo Nagano[c,*]

[a] Faculty of Agriculture, Setsunan University
[b] RIKEN Center for Sustainable Resource Science
[c] Department of Chemistry and Biotechnology, Tottori University

*Email: snagano@tottori-u.ac.jp

**References**

[1] B.R. Lichman *et al.*: Chem. Eur. J. **25** (2019) 6864.
[2] N. Kato *et al.*: Angew. Chem. Int. Ed. **57** (2018) 9754.
[3] *K. Fujiyama, N. Kato, S. Re, K. Kinugasa, K. Watanabe, R. Takita, T. Nogawa, T. Hino, H. Osada, Y. Sugita, S. Takahashi, S. Nagano: Angew. Chem. Int. Ed.* ***60*** *(2021) 22401.*
[4] M. Kamiya and Y. Sugita: J. Chem. Phys. **149** (2018) 072304.