Structural Basis Underlying the Catalytic Mechanism of Deoxypodophyllotoxin Synthase

Deoxypodophyllotoxin synthase (DPS) catalyzes a crucial cyclization reaction to produce the fused tetracyclic structure of podophyllotoxin. This study demonstrates the catalytic characteristics of DPS from a structural perspective.

Podophyllotoxin (PPT) is a naturally occurring aryltetralin lignan and is primarily obtained through extraction from the dried roots and rhizomes of *Podophyllum* species, such as *Sinopodophyllum hexandrum* and *Podophyllum peltatum*.[1] PPT is of pharmaceutical interest because it can interfere with the association between α-tubulin and β-tubulin to disrupt microtubule assembly,[2] which leads to cell cycle arrest in the G2/M phase. Therefore, PPT is regarded as a promising compound for inhibiting abnormal cell proliferation. Furthermore, its glycoside derivatives, including etoposide and teniposide (**Fig. 1(a)**), exhibit potent anticancer activity through a distinct mode of action. Specifically, these drugs are capable of insertion into the type II topoisomerase (Top2)-mediated DNA cleavage site to interrupt the catalytic cycle of Top2,[3] which results in the accumulation of DNA double-strand breaks and subsequent apoptotic cell death. Given their outstanding antiproliferative effect, PPT derivatives have been used extensively in cancer therapy. Because PPT is a valuable starting material for the semisynthesis of anticancer drugs, its demand has increased over time. Thus, meeting the commercial need for PPT by merely obtaining PPT from natural resources is now difficult. Consequently, alternative methods—including plant cultivation, *in vitro* cell culture, total synthesis, and chemoenzymatic synthesis—have been developed to increase PPT production. Among these methods, chemoenzymatic synthesis, which is achieved using enzymes involved in the biosynthesis of PPT to overcome critical steps in compound synthesis, has emerged as an attractive approach.

Deoxypodophyllotoxin synthase (DPS) catalyzes a chemically challenging stereospecific cyclization reaction to convert yatein into deoxypodophyllotoxin in the biosynthetic pathway of PPT (**Fig. 1(b)**). On the basis of sequence similarity and the cofactors required by DPS to catalyze the reaction, DPS is classified as a member of the iron- and 2-oxoglutarate-dependent (Fe/2OG) oxygenase superfamily.[4] Although DPS has been utilized in the synthesis of deoxypodophyllotoxin analogs, how DPS recognizes its substrates and performs a stereospecific cyclization reaction remains unclear, and one of the major impediments to understanding the DPS-catalyzed mechanism is the lack of structural information about DPS. For the Fe/2OG oxygenase superfamily, no substrate-bound structural information regarding the enzymes that catalyze ring formation, especially through C–C bond formation, has been obtained.

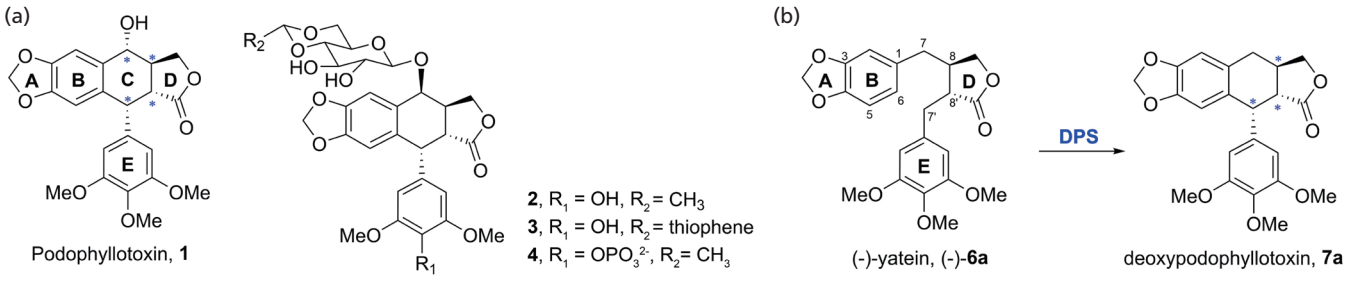

Fig. 1: (a) Structures of PPT (**1**), etoposide (**2**), teniposide (**3**), and etopophos (**4**). (b) DPS catalyzes the conversion of (–)-yatein (**6a**) into deoxypodophyllotoxin (**7a**). Asterisk (⁎) labels represent the chiral centers. [Reproduced from Ref. 5]

To investigate the detailed catalytic mechanism of DPS-catalyzed cyclization, Nei-Li Chan (National Taiwan University) and his collaborators Tun-Cheng Chien (National Taiwan Normal University) and Wei-chen Chang (North Carolina State University, USA) determined the crystal structures of DPS in multiple stages of its catalytic cycle.[5] Specifically, X-ray diffraction data were collected at **TLS 15A1** and **TPS 05A** of the NSRRC. The DPS•Fe•2OG and DPS•Fe•succinate•(±)-yatein structures were characterized at resolutions of 2.09 and 2.05 Å, respectively. Similar to other Fe/2OG oxygenases, DPS exhibits a conserved core structure featuring a double-stranded β-helix fold and an active site harboring a 2-His-1-carboxylate facial triad for iron coordination. In addition, studies have revealed that DPS exhibits certain substrate promiscuity.[6] Specifically, DPS can catalyze its native substrate, namely (–)-yatein, and substrate enantiomer, namely (+)-yatein, regardless of the opposite stereochemistry at C8 and C8′. To elucidate the structural basis of this phenomenon, Chan's team crystallized DPS in the

presence of yatein. The resultant electron density map of the DPS•Fe•succinate•(±)-yatein complex is of sufficient quality to enable the three moieties of yatein—namely the benzodioxole ring (the A- and B-rings), lactone ring (the D-ring), and phenyl ring (the E-ring)—to be placed unambiguously in the enzyme's substrate binding site. Because a racemic mixture of yatein was used in the crystallization experiments, (–)-yatein and (+)-yatein were individually tested to construct a ligand model for the substrate binding site to assess which isomer was present in the crystals. The ligand fitting results revealed that both enantiomers can be docked into the electron density map, which suggests that (–)-yatein and (+)-yatein may coexist in the crystals (**Figs. 2(a) and 2(b)**). Furthermore, the analysis of the binding mode of each enantiomer revealed that both enantiomers exhibit a U-shaped conformation, which not only places C7′ at a suitable distance for hydrogen atom transfer to activate the target C–H bond but also brings C6 close enough to C7′ for subsequent cyclization (**Figs. 2(c) and 2(d)**). This finding explains why DPS can act on both substrate enantiomers despite the difference in their stereochemistry.

Concerning the catalytic mechanism of DPS, the research team performed enzyme activity assays by employing multiple substrate analogs to explore their effects on the cyclization of yatein. In contrast to the originally proposed pathway, which involves a hydroxylated intermediate,[4] the experimental results indicated that an on-pathway benzylic carbocation was likely to trigger the C–C bond formation between C6 and C7′. After the cyclization of yatein to deoxypodophyllotoxin, a new chiral center is formed at C7′. Notably, this newly formed chiral center is always in the same stereochemical configuration, regardless of which substrate enantiomer is used in the reaction. To address the question of whether the formation of different cyclized products was possible, molecular docking was performed for analysis. Assuming that their D-rings would occupy the same site as that of yatein, four possible cyclized products were manually docked into the active site (**Figs. 2(e)–2(h)**). The results revealed that DPS imposed stereochemical constraints on the configuration of the product. Only the product whose C7′ atom is in the *R* configuration could be accommodated in the active site and therefore produced (**Figs. 2(e) and 2(f)**). Thus, because of the steric constraints imposed by DPS, cyclized products have the same configuration at the C7′ position.

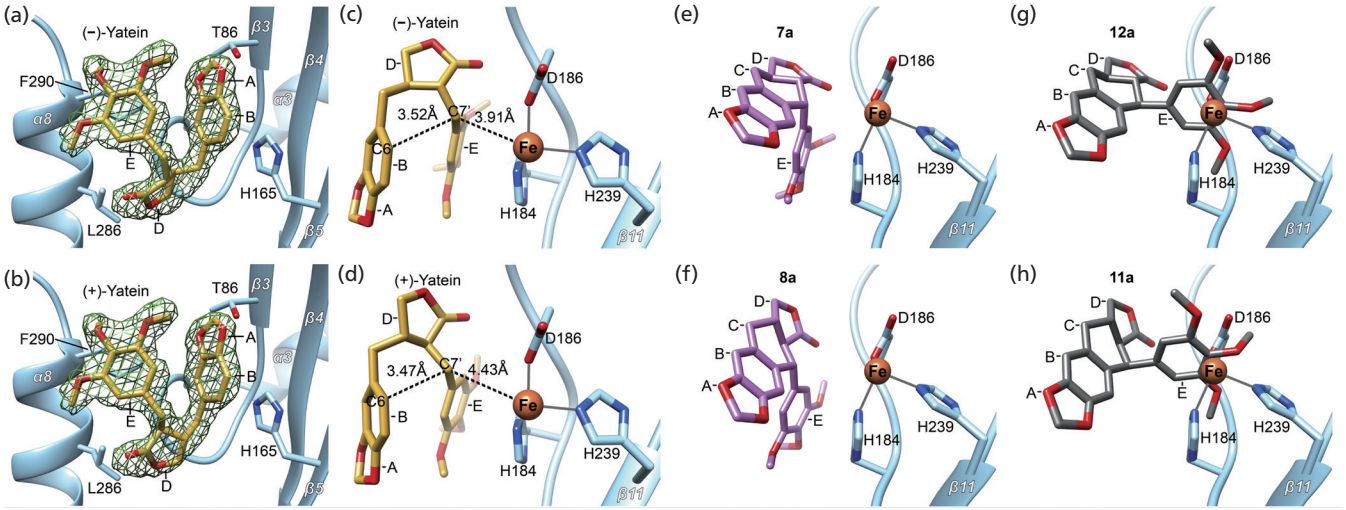

Fig. 2: (a,b) Unbiased mF_o–DF_c electron density map of the substrate contoured at 3σ (green mesh) fitted with (–)-yatein and (+)-yatein. (c,d) Distances are shown between C6–C7′ and C7′–iron in DPS-bound (–)-yatein and (+)-yatein. (e–h) Docking of four possible cyclized products into the active site. (–)-Deoxypodophyllotoxin (**7a**) and (–)-isodeoxypodophyllotoxin (**12a**) are produced after the ring closure of (–)-yatein. In the case of (+)-yatein, (+)-isodeoxypodophyllotoxin (**8a**) and (+)-deoxypodophyllotoxin (**11a**) are possible cyclized products. The black dashed lines represent distances between atoms. The coordination bonds formed by iron are represented as gray lines. [Reproduced from Ref. 5]

In summary, the unprecedented substrate-bound DPS structure not only provides a structural explanation for the substrate promiscuity of DPS but also demonstrates the effect of the steric constraints imposed by DPS to control the stereochemistry of cyclized products. The results of this study provides a basis for the future application of DPS to synthesize PPT derivatives. (Reported by Min-Hao Wu, National Taiwan University)

This report features the work of Nei-Li Chan and his collaborators published in PNAS ***119****, e2113770119 (2022).*

TPS 05A Protein Microcrystallography
TLS 15A1 Biopharmaceuticals Protein Crystallography

- XPS, Protein Crystallography
- Biological Macromolecules, Protein Structures, Life Science

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Applications of Small-Angle X-ray Scattering in Protein Folding Studies

Small-angle X-ray scattering is a powerful tool for investigating protein structures and dynamics under diverse experimental conditions. It is particularly useful in investigating conformational changes pertinent to protein folding and misfolding.

Understanding the mechanism by which polypeptide chains fold into specific three-dimensional (3D) structures to confer biological functions has been a decade-long challenge. The protein folding problem is not only fundamental to physical chemistry but also medically relevant because the failure of a protein to fold correctly or the tendency to misfold can cause debilitating diseases, such as cancer and neurodegenerative diseases. Protein folding can be considered a conformational sampling process of a funnel-like free-energy landscape, which involves all possible conformation combinations according to the degrees of freedom accessible to the polypeptide chain. Thus, the most energetically favorable state is assumed to correspond to the native state. The free-energy funnel often exhibits different degrees of roughness, or frustrations, that can trap folding intermediates in local minima. In addition to globular proteins that exhibit robust folding characteristics, a significant proportion of the proteome encodes for intrinsically disordered regions covering segments or the entirety of given proteins is becoming increasingly evident. The free-energy landscapes of these intrinsically disordered proteins (IDPs) exhibit many energetically equivalent local minima to populate an ensemble of conformations that can differ vastly. Many IDPs exhibit high aggregation propensities and self-associate into amorphous oligomers before forming highly ordered filamentous assemblies, amyloid fibrils. Environmental stress conditions, posttranslational modifications, or genetic mutations can also considerably perturb the native states of proteins such that their hydrophobic cores become more exposed, increasing their aggregation propensities. Therefore, understanding the structure-function relationships of disease-associated proteins requires in-depth understanding of the atomic structures and dynamics involved in protein folding.

Although protein crystallography has been instrumental in providing atomic insights into protein structures, it primarily addresses how the target protein folds into the defined native structure in its crystalline state. Likewise, the emergence of cryo-electron microscopy (cryo-EM) has pushed the frontier of structural biology, but conformational heterogeneity remains a limiting factor for effective single-particle reconstruction. Solution-state nuclear magnetic resonance (NMR) spectroscopy can be used to determine protein structures at atomic resolution under either native or nonnative (denaturing) conditions. NMR spectroscopy can be used to quantitatively describe protein dynamics at atomic resolution across a broad range of timescales. However, a major disadvantage of solution-state NMR spectroscopy is the size limitation of the system of interest, which is typically less than 30 kDa. Advanced NMR experiments aided by elaborately stable isotope labeling can increase size limits to the order of MDa, but such applications are exceptional.

In addition to the big three of structural biology—X-ray crystallography, NMR spectroscopy, and cryo-EM—small-angle X-ray scattering (SAXS) is exceptionally versatile in providing structural information of proteins across a broad range of spatial and temporal resolutions.[1] SAXS data can essentially be collected under any experimental conditions. Furthermore, SAXS does not have fundamental size limitations, although it is intrinsically more sensitive to larger particles. SAXS is particularly suited for protein folding studies that require the use of concentrated