Discovery and Structure-Function Analyses of Novel Triterpenoid Synthases

All known triterpenes are generated via squalene. This is different from the biosynthesis of C10–C25 of terpenes that are formed from polyisoprenyl diphosphates. In this study, we discovered novel fungal chimeric triterpene synthases TvTS and MpMS that catalyze the generation of triterpenes via hexaprenyl diphosphate. Structure function analyses of the TvTS terpene cyclase domain and full-length MpMS with X-ray crystallography and cryo-EM analysis, respectively, revealed the intimate structural details of new enzymatic mechanism for the biosynthesis of triterpenes.

Terpenoids are the largest family of natural products [1]. More than 100,000 terpenoid compounds have been isolated so far. These compounds are used as pharmaceutical drugs, cosmetics, and fuels. The 30-carbon (C30) triterpenoids are the most important terpenoid group and are produced in all kingdoms of life, including microorganisms, plant, and human [2]. Five-carbon (C5) isoprene units of dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) are used as the precursors to form isoprenoid diphosphates in terpenoid biosynthesis. Prenyltransferases (PTs) catalyze elongation reaction of isoprene unit to generate various length of isoprenoid diphosphates, such as C10 geranyl diphosphate (GPP), C15 farnesyl diphosphate (FPP), C20 geranylgeranyl diphosphate (GGPP) and C25 geranylfarnesyl diphosphate (GFPP). Then, terpene cyclases (TCs) catalyzes the cyclization of isoprenoid diphosphates to form terpene skeletons. The different cyclization pattern of TCs generates diversity of terpenoids compounds. However, only known biosynthetic pathway for triterpenes was via a C30 squalene, which is produced by the dimerization of C15 FPP (Fig. 1). In this study, we discovered a novel biosynthetic pathway for the triterpenoids formation, and we performed the structure-function analysis of novel triterpenoid synthase family enzymes TvTS and MpMS that catalyze the production of triterpenes via hexaprenyl diphosphate (HexPP) [3].

In vivo analysis of the bifunctional chimeric terpene synthases from *Talaromyces verruculosus* TS63-918 (TvTS) and *Macrophomina phaseolina* MS619 (MpMS), which consist of PT and TC domains, indicated that these enzymes produce the triterpenes talaropentaene and macrophomene, respectively. Further *in vitro* analyses of TvTS and MpMS revealed that the PT domains of these enzymes catalyze the formation of HexPP from DMAPP and IPP, and the TC domains of TvTS and MpMS accept Hex-PP to generate talaropentaene and macrophomene, respectively.

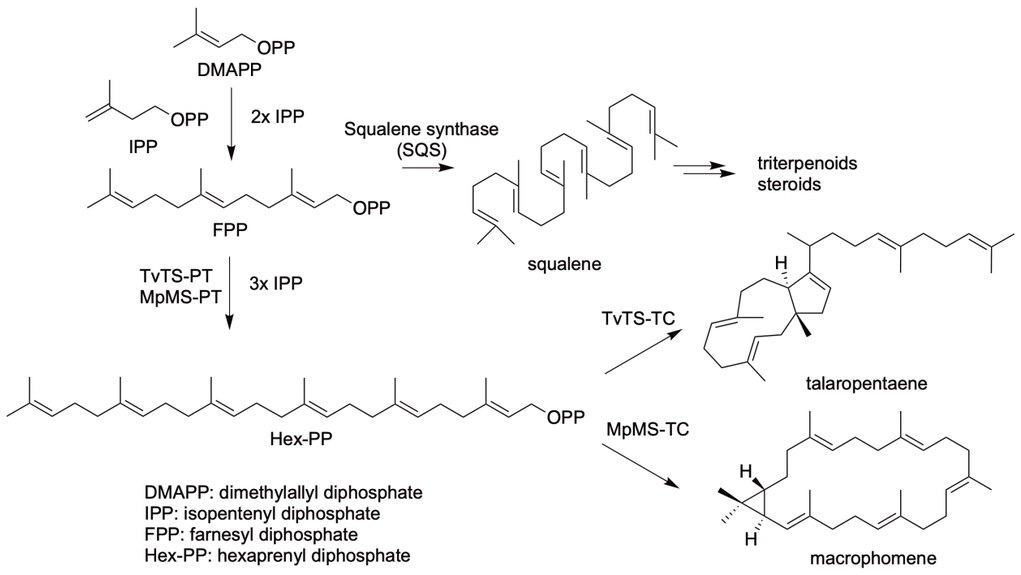

Figure 1: Biosynthesis of triterpenes. The classical pathway for triterpenes proceeds through squalene. We identified novel fungal bifunctional TSs that generate triterpenes from DMAPP and IPP via Hex-PP.

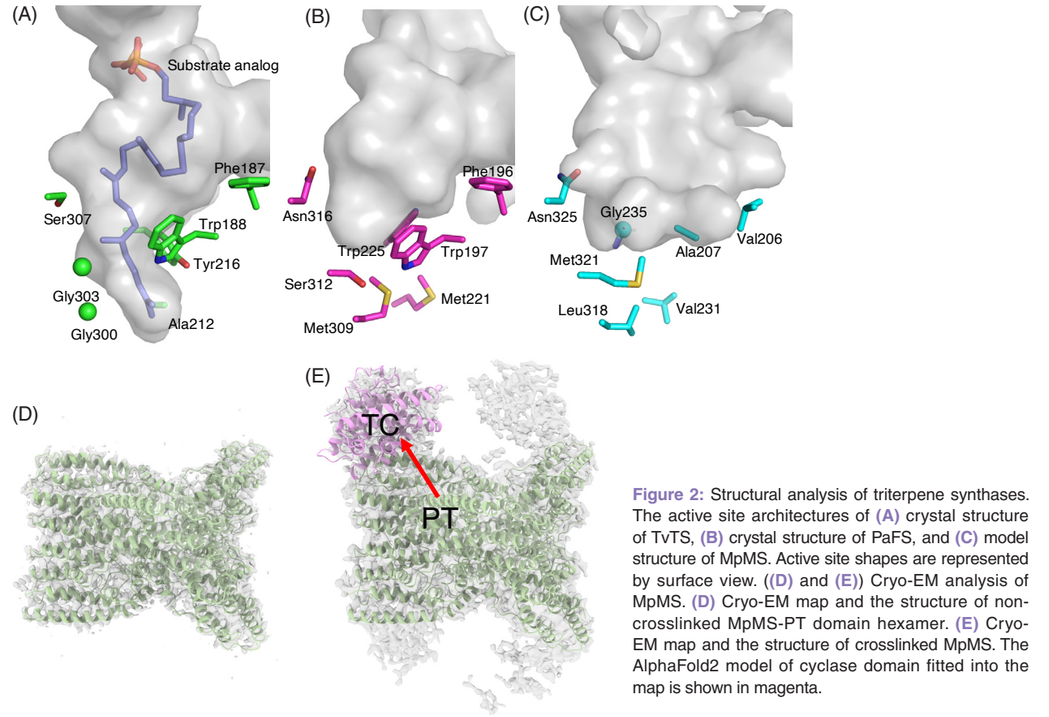

Figure 2: Structural analysis of triterpene synthases. The active site architectures of (A) crystal structure of TvTS, (B) crystal structure of PaFS, and (C) model structure of MpMS. Active site shapes are represented by surface view. ((D) and (E)) Cryo-EM analysis of MpMS. (D) Cryo-EM map and the structure of non-crosslinked MpMS-PT domain hexamer. (E) Cryo-EM map and the structure of crosslinked MpMS. The AlphaFold2 model of cyclase domain fitted into the map is shown in magenta.

To understand the molecular basis for the triterpene formation, the crystal structure of the TvTS-TC domain was solved. The X-ray diffraction data were measured at BL-1A. The overall structure of TvTS-TC is similar to those of structurally characterized terpene cyclases. The comparison of the active sites of TvTS-TC and the homologous enzyme fusicoccadiene synthase PaFS, which catalyzes the production of fusicoccadiene from C20 GGPP, indicated that some active site residues of PaFS-TC are substituted with the small amino acid residues in TvTS-TC. Accordingly, TvTS possesses a significantly large active site that can accommodate two additional isoprene units of C30 HexPP (Fig. 2(A)-(C)). The mutagenesis study of these residues in TvTS-TC revealed that the variants dramatically reduced the activity for the generation of talaropentaene. Moreover, the model structure of the MpMS-TC domain and mutagenesis study suggested that the active site of MpMS is also large enough to accept HexPP.

To obtain further structure basis for the triterpene synthase reactions, the cryo-electron microscopic analysis (cryo-EM) of MpMS was performed. We could obtain only the 3D reconstituted model of PT domain, although we analyzed the full-length MpMS (Fig. 2(D) and (E)). The overall structure of the PT domain of MpMS is quite similar to other PTs. The size of the active site of MpMS-PT is large enough to generate Hex-PP. Next, we investigated the interactions between the PT and TC domains by cross-linking of the full-length MpMS by using glutaraldehyde as crosslinker reagent. As a result of cryo-EM analysis, the additional density maps of the TC domains were observed at each vertex of the PT domain (Fig. 2(E)). These observations suggested that the cross-linking reaction fix the position of the TC domain, while the orientation of TC domain is flexible in the solution. The active sites of the PT and TC domains face each other, which may facilitate channeling of unstable intermediate HexPP from the PT domain to the TC domain.

In summary, the enzymes TvTS and MpMS, belonging to a novel family of bifunctional triterpene synthases, were identified. The structural analyses of these enzymes by X-ray crystallography and cryo-EM provided insights into the molecular basis for the mechanism of triterpenoids formation from HexPP and the channeling effect for efficient triterpenoid production [3]. These findings have contributed to our understanding of terpene biosynthesis in nature.

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