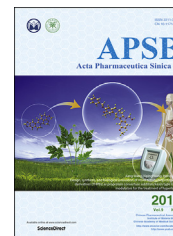




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SHORT COMMUNICATION

Immunosuppressant mycophenolic acid biosynthesis employs a new globin-like enzyme for prenyl side chain cleavage



Xiwei Chen^a, Lu Wang^b, Jinmei Zhang^a, Tao Jiang^a, Changhua Hu^a,
Dehai Li^{b,c,*}, Yi Zou^{a,d,*}

^aCollege of Pharmaceutical Sciences, Southwest University, Chongqing 400715, China

^bKey Laboratory of Marine Drugs, Chinese Ministry of Education, School of Medicine and Pharmacy, Ocean University of China, Qingdao 266003, China

^cLaboratory for Marine Drugs and Bioproducts of Qingdao, National Laboratory for Marine Science and Technology, Qingdao 266237, China

^dBiological Science Research Center, Southwest University, Chongqing 400715, China

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Abstract Mycophenolic acid (MPA, **1**) and its derivatives are first-line immunosuppressants used in organ transplantation and for treating autoimmune diseases. Despite chemical synthetic achievements, the biosynthetic formation of a seven-carbon carboxylic acid pharmacophore side chain of **1**, especially the processes involving the cleavage of the prenyl side chain between DHMP (**4**) and DMMPA (**5**), remains unknown. In this work, we identified a membrane-bound prenyltransferase, PgMpaA, that transfers FPP to **4** to yield FDHMP (**6**). Compound **6** undergoes the first cleavage step *via* a new globin-like enzyme PgMpaB to form a cryptic intermediate **12**. Heterologous expression of *PgMpa* genes in *Aspergillus nidulans* demonstrates that the second cleavage step (from **12** to **5**) of **1** is a *PgMpa* cluster-independent process *in vivo*. Our results, especially the discovery of the broad tolerance of substrates recognized by PgMpaB, set up a strategy for the formation of “pseudo-isopentenyl” natural products using fungal globin-like enzymes.

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*Corresponding authors.

E-mail addresses: dehaili@ouc.edu.cn (Dehai Li), zouyi31@swu.edu.cn (Yi Zou).

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1. Introduction

Fungal polyketide-terpenoid hybrid natural products (PK-TPs) belong to the largest class of meroterpenoids exhibiting a wide range of biological activities¹. PK-TPs typically consist of a cyclic aromatic backbone featuring a prenyl side chain attached *via* a C–O or C–C bond linkage^{2,3}. The prenyl side chains are diverse partially due to different biogenetic origins and include dimethylallyl diphosphate (DMAPP, C5), geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15), and geranylgeranyl diphosphate (GGPP, C20)^{2,3}. In the development of complex structural scaffolds, after transferring the prenyl chain to the polyketide core, a flavin-dependent oxidase, a membrane-bound terpene cyclase and other redox enzymes usually catalyze tandem epoxidation, cyclization and oxidation reactions^{4–10}. During the course of these reactions, the linear prenyl side chain can undergo changes in C–C connectivity to form mono- or polycyclic chiral centers, whereas the carbons are highly oxidized (Scheme 1A). Mycophenolic acid (MPA, **1**) featuring a seven-carbon carboxylic acid pharmacophore, is putatively derived from an intact C15 prenyl moiety by a cleavage reaction rather than through classical epoxidation and cyclization reactions and represents a unique example of fungal PK-TPs (Scheme 1A)^{2,3}.

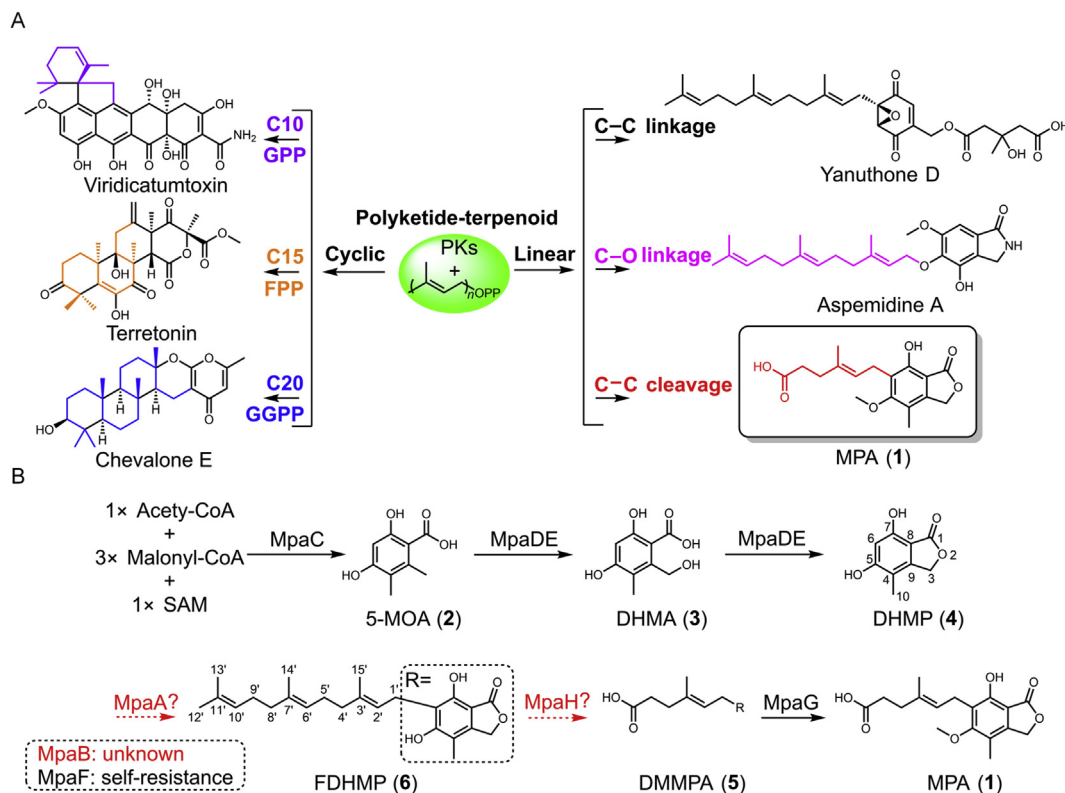
As a highly potential inosine-5'-monophosphate dehydrogenase (IMPDH) inhibitor, MPA (**1**) was isolated from *Penicillium brevicompactum* in 1893. MPA and its derivatives are first-line immunosuppressants used in organ transplantations and treating autoimmune diseases^{11–14}. However, the genetic and enzymatic mechanisms for **1** have lagged far behind its discovery, synthesis, and medicinal applications, especially with respect to the installation and cleavage of

prenyl side chain and associated responsible enzymes. Previous biosynthetic studies have suggested several mechanisms and enzymes (Scheme 1B): (i) a nonreducing polyketide synthase (NRPKS) MpaC catalyzes the formation of 5-methylorsellinic acid (5-MOA, **2**) from one acetyl-CoA unit, three malonyl-CoA units and one *S*-adenosyl-L-methionine (SAM) unit^{15,16}; (ii) an unusual cytochrome P450 and hydrolase fused enzyme MpaDE catalyzes the hydroxylation of **2** to form 4,6-dihydroxy-2-(hydroxymethyl)-3-methylbenzoic acid (DHMB, **3**) and subsequently catalyzes intramolecular dehydration to produce 5,7-dihydroxy-4-methylphthalide (DHMP, **4**)¹⁷; (iii) MpaG catalyzes the last step of a C5 O-methylation of demethylmycophenolic acid (DMMPA, **5**) to yield the final product **1**¹⁸; and (iv) MpaF, an IMPDH-like protein, plays a critical role in self-resistance toward **1**¹⁹. Although gene silencing studies have indicated that three additional genes (*MpaA*, *MpaB* and *MpaH*) are involved in **1** production²⁰, the actual functions and roles of these genes and conversion details between **4** and **5** remain unresolved.

Here we identified a membrane-bound prenyltransferase PgMpaA catalyzing the regioselective C6 farnesylation of **4** to yield FDHMP (**6**), the resulting C15 prenyl side chain of which was undergone the first pivotal cleavage step by a new globin-like enzyme PgMpaB. Subsequent heterologous expression of *PgMpa* genes in *Aspergillus nidulans* demonstrated that the second cleavage step of **1** was a *PgMpa* cluster-independent process *in vivo*.

2. Results and discussions

In our continuous studies on discovering enzymes for the cleavage of prenyl chains in natural product biosyntheses^{21,22}, we have focused on **1** using an MPA high-producing strain *Penicillium*



Scheme 1 Biosynthetic diversity of fungal polyketide-terpenoid hybrid natural products. (A) The various modifications of prenyl side chain. (B) Previously proposed biosynthetic pathway of MPA (**1**).

griseofulvum (Supporting Information Fig. S1)^{23,24}. The strain provides a convenient platform for analyzing accumulated intermediates produced by gene mutants. One *PgMpa* gene cluster was computationally identified; this cluster showed high similarity/identity and conserved gene organization to the *Pbm* gene cluster from *P. brevicompactum* (Fig. 1A). Deletion of *PgMpaC* (SI) led to the complete abolishment of **1** production (Fig. 1B, i and ii; Supporting Information Fig. S2B), confirming a clear relationship of the *PgMpa* cluster with **1** biosynthesis in *P. griseofulvum*. To identify the three genes *PgMpaA*, *PgMpaB* and *PgMpaH* functionally responsible for the seven-carbon carboxylic acid side-chain formation, we first examined *PgMpaA*. *PgMpaA* catalyzes Friedel–Crafts farnesylation to yield 6-farnesyl-5,7-dihydroxy-4-methylphtalide (FDHMP, **6**) using **4** as the substrate. The Δ *PgMpaA* mutant strain was unable to produce **1** and instead, accumulated **4** (Fig. 1B iii), which was purified and confirmed by NMR characterization (Supporting Information Table S3, and Figs. S17 and S18) and used as a substrate for biochemical assays.

PgMpaA is a fungal membrane-bound prenyltransferase containing six transmembrane regions (Supporting Information Fig. S3). Phylogenetic tree analyses (Supporting Information Fig. S4) showed that *PgMpaA* was closest to Pyr6, the farnesyltransferase from the pyripropene A biosynthetic gene cluster²⁵. Compound **4** was incubated with 0.2 mmol/L FPP, 10 mmol/L Mg²⁺, and microsome fractions of *S. cerevisiae*BJ5464-*NpgA::PgMpaA* (*BJ-PgMpaA*, SI). Expectedly, there was a nearly ~25% conversion of **4** into a new compound in 3 h with mass (m/z 383 [M–H][–]). This was consistent with farnesyl-**4**, whereas the *BJ* control remained inactive (Fig. 1C, i and ii). When

DMAPP, GPP or GGPP were used, one new compound consistent with the mass of geranyl-**4** (m/z 315 [M–H][–]) was detected by LC–MS, albeit with a lower turnover (Supporting Information Fig. S5). A competition and time-course assay of *BJ-PgMpaA* microsome in the presence of equimolar amounts of GPP and FPP (SI) confirmed that *PgMpaA* preferentially used FPP as the donor for the prenylation of **4** (Supporting Information Fig. S6). Indeed, under a sustainable supply of isoprene intermediate metabolic pools *in vivo*, the whole-cell biotransformation using *BJ-PgMpaA* with **4** only produced farnesyl-**4** (Supporting Information Fig. S7). After product extraction and purification, NMR characterization confirmed the compound as **6** (Supporting Information Table S4 and Figs. S19–S22). Feeding **6** into the Δ *PgMpaC* mutant restored the production of **1**, which demonstrated the role of **6** as a key precursor (Supporting Information Fig. S8). The *in vivo* and *in vitro* results indicated (i) *PgMpaA* exhibited broad substrate tolerance toward FPP and GPP. However, *PgMpaA* specifically catalyzed the regioselective C6 farnesylation of **4** to **6**. (ii) The seven-carbon carboxylic acid side chain of **1** comes from an intact FPP (C15) moiety of **6**. The unusual cleavage mechanism existed downstream in the pathway.

We next identified enzymes responsible for this cleavage step. The ideal candidate was *PgMpaH* based on bioinformatic analyses, which showed that *PgMpaH* contained an α/β -hydrolase fold and had very weak amino acid sequence similarity/identity with 1-*H*-3-hydroxy-4-oxoquinoline 2,4-dioxygenase (HOD) and 1-*H*-3-hydroxy-4-oxoquinoline 2,4-dioxygenase (QDO)²⁶. These two enzymes are cofactor-independent dioxygenases involved in the cleavage of the C–C double bond in *N*-heteroaromatic compounds²⁶. To clarify the function of *PgMpaH*, it was inactivated in *P. griseofulvum*. Unexpectedly, the Δ *PgMpaH* mutant retained ~50% ability to produce **1** and two new compounds with m/z 291 [M–H][–] (**7**) and m/z 293 [M–H][–] (**8**) (Fig. 1B iv). Purification and structural characterization (Supporting Information Tables S5 and 6 and Figs. S23–S28) indicated that **7** and **8** were the C5- and C6-chain analogues of **5** (C7-chain), respectively (Fig. 1B). They are probable abnormal cleavage products of **6** *in vivo* but not the precursors of **1**. Indeed, feeding **7** and **8** into the Δ *PgMpaC* mutant did not restore **1** production (Fig. S8). These results excluded the direct role of *PgMpaH* taking part in the breakdown of the C15-chain of **6** *in vivo*. However, the data suggested *PgMpaH* may be functional as an assistant enzyme to ensure a correct and efficient whole cleavage process.

Gene *PgMpaH* was excluded from the candidate genes for C–C bond cleavage prompting the investigation of the function of *PgMpaB*. NCBI blast or Pfam domain analyses showed that *PgMpaB* was an unknown function protein. A potential conserved three-dimensional structure using Phyre2 analysis showed that *PgMpaB* possibly belonged to the globin superfamily protein (Supporting Information Fig. S9). *PgMpaB* showed 41%/22% amino acid similarity/identity with the bacterial globin-like enzyme latex-clearing protein (Lcp_{k30}) from *Streptomyces* sp.^{27,28}. Phylogenetic tree analyses of *PgMpaB* with other globin-like enzymes, such as bacterial Lcp_{k30}, rubber oxygenase (RoxA²⁹, from *Xanthomonas* sp.) and fungal flavohemoglobin (Fhb³⁰, from *Aspergillus* sp.), showed that *PgMpaB* was clustered into an independent clade (Supporting Information Fig. S10). Lcp_{k30} and RoxA catalyzed the oxidative cleavage of poly (*cis*-1,4-isoprene) during rubber degradation. This reaction was similar to the cleavage of the prenyl side chain in **1** biosynthesis.

To uncover its function, gene *PgMpaB* was inactivated in *P. griseofulvum*, and the metabolites of the Δ *PgMpaB* mutant were

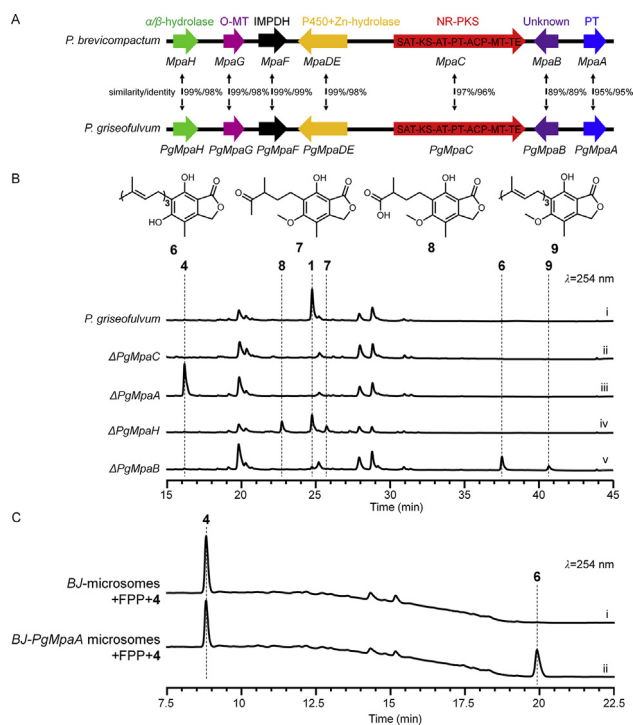


Figure 1 Confirmation of the *PgMpa* gene cluster. (A) Organization and proposed function of *PgMpa* A–H. (B) LC–MS analyses of culture extracts from *P. griseofulvum* wild type and mutants. (C) *In vitro* biochemical assays of *BJ-PgMpaA* or *BJ* control microsome with FPP.

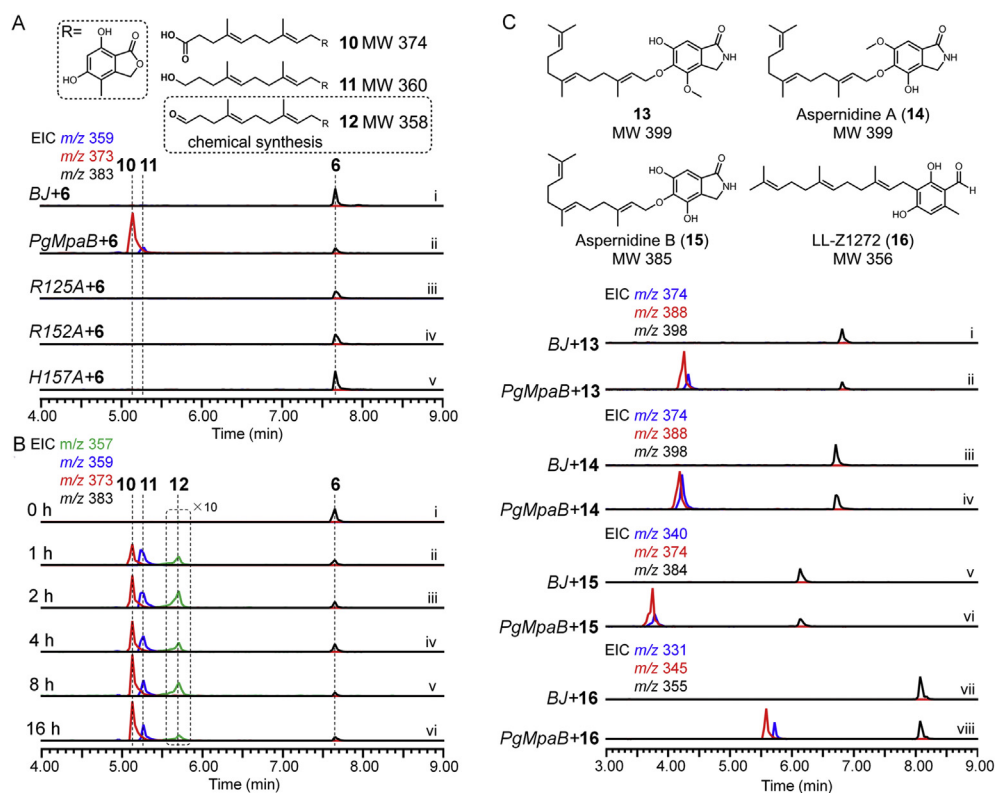


Figure 2 *In vitro* biochemical assays of the PgMpaB cell-free extracts with different substrates. (A) Identification of the key amino acid residues for PgMpaB activity. (B) Time-course analyses of the PgMpaB-catalyzed cleavage reaction. (C) The substrate tolerance of PgMpaB. The extracted ion chromatograms (EIC) under negative ionization are shown.

analyzed by LC–MS. This revealed that the production of **1** dramatically decreased ($\sim 98\%$) in the $\Delta PgMpaB$ mutant, indicating that *PgMpaB* played an important role for **1** biosynthesis (Fig. 1B v). The trace yield of **1** retained in the $\Delta PgMpaB$ mutant suggested the existence of minor complementary activity for PgMpaB in *P. griseofulvum*. Indeed, two other *PgMpaB* homologues (*PgMpaB-scaffold3* and *PgMpaB-scaffold17*, Supporting Information Fig. S11) existed in the genome of *P. griseofulvum*, whereas the *PgMpaB-scaffold3* was actively transcribed (Fig. S11). In addition to the **6**, a minor new compound **9** with m/z 397 $[M-H]^-$ was also detected in the $\Delta PgMpaB$ mutant, whose structure was determined to be 5-*O*-methyl-**6** (Fig. 1B) by NMR analyses (Supporting Information Table S7 and Figs. S29–S33). Compound **9** was the shunt methyl-product of **6**, possibly catalyzed by PgMpaG, because its broad substrate tolerance has been previously demonstrated¹⁸. The successful bioconversion of **6** to **9** in the *BJ-PgMpaG* strain (Supporting Information Fig. S12) also confirmed that PgMpaG was responsible for this step.

The accumulation of **6** in the $\Delta PgMpaB$ mutant suggested PgMpaB may be the real cleavage enzyme. Due to the insoluble expression of PgMpaB in *Escherichia coli*, the intron-free *PgMpaB* was introduced into *BJ* (*BJ-PgMpaB*) under control of the ADH2 promoter. When **6** was incubated with the cell-free extract of *BJ-PgMpaB*, two new compounds with m/z 373 $[M-H]^-$ (**10**) and m/z 359 $[M-H]^-$ (**11**) were detected (Fig. 2A). Purified **10** and **11** from large-scale conversion assays, followed by structural NMR characterization, indicated that **10** and **11** were the twelve-carbon carboxylic acid and hydroxylated side-chain analogues of **6**, respectively (Fig. 2A, Supporting Information Tables S8 and S9, and Figs. S34–43). Compounds **10** and **11**

were likely the spontaneous oxidized and reduced products of the corresponding aldehyde precursor **12** during the formation of complex cell-free extracts, respectively (Fig. 2A). We proposed that compound **12** was the real product from the PgMpaB-catalyzed cleavage of the terminal olefin (Δ^{10}) of **6**, which was detected in the time-course analyses (Fig. 2B). We chemically semisynthesized **12** using **10** as the starting material (SI) and confirmed **12** indeed as the precursor of **1** due to its ability to restore **1** production in the $\Delta PgMpaC$ mutant (Fig. S8). Bioinformatic analyses of PgMpaB and its homologues from the NCBI database showed that these fungal globin-like proteins were rich in arginine, glutamic acid, lysine and histidine (Supporting Information Fig. S13). Mutation results confirmed that (i)

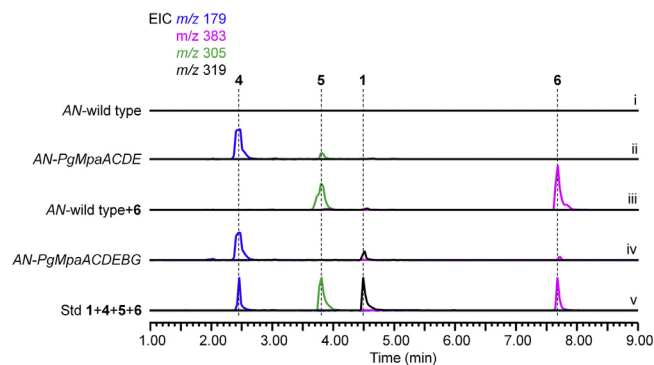
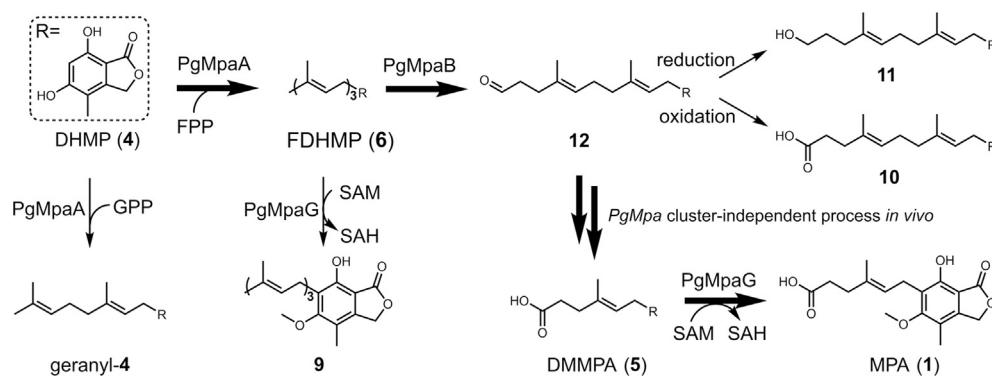


Figure 3 Reconstruction of **1** pathway in *A. nidulans* confirms two cleavage stages of the C15 prenyl side chain in **1** biosynthesis. The extracted ion chromatograms (EIC) under negative ionization are shown.



Scheme 2 The updated biosynthetic pathway and confirmed key intermediates of **1**. Bold and plain arrows indicate the on-pathway and shunt pathway of **1**, respectively.

Arg125, Arg152 and His157 were essential for the activity of PgMpaB (Fig. 2A); (ii) Arg62, Arg99, Glu135, Arg145 and Glu245 mutants significantly decreased the activity of PgMpaB (Supporting Information Fig. S14); (iii) Lys254 and His275 had no effect on the activity of PgMpaB (Fig. S14). Further investigations of the substrate tolerance showed that PgMpaB had the ability to catalyze the cleavage of various *O*- and *C*-farnesyl side-chain analogues of **1** (Fig. 2C), which indicated future applications of PgMpaB as a biocatalyst for C–C bond cleavage.

The production of **12** but not **5** in a PgMpaB-catalyzed reaction as well as the feeding of **12** to restore the production of **1** indicated (i) PgMpaB exclusively catalyzed the regioselective double bond ($\Delta^{10'}$) cleavage and that (ii) the mature process of C7 side chain of **1** should be performed by two cleavage steps (from **6** to **12**, and from **12** to **5**). The latter step (from **12** to **5**) was likely an independent process *in vivo* not related with the *PgMpa* cluster. To prove this hypothesis, we introduced the *PgMpa* genes into the heterologous host *A. nidulans*, the strain that could not produce **1**³¹. Bioinformatic analyses showed that two PgMpaB homologues (AN10293.4 and AN1187.2) existed in the genome of *A. nidulans*, showing 73%/54% and 73%/57% (similarity/identity) to PgMpaB, respectively (Supporting Information Fig. S15). Compared with PgMpaB, these two proteins had the same identical active amino acid residues (Fig. S15), indicating that they had the ability to complement the activity of PgMpaB. Indeed, biochemical assays showed that these two proteins (AN10293.4 and AN1187.2) could catalyze the C–C bond cleavage of **6** to form **12** (Supporting Information Fig. S16). Upon the coexpression of *PgMpaACDE* in *A. nidulans* (*AN-PgMpaACDE*, SI), besides **4** and **6**, one compound with *m/z* 305 [M–H][–] was detected (Fig. 3, i and ii), which was further isolated and identified as **5** by NMR analyses (Supporting Information Table S10 and Figs. S44–S47). Further feeding of **6** to *A. nidulans* showed that **5** was detected (Fig. 3 iii). These results unambiguously confirmed that the latter cleavage step of the farnesyl side chain in **1** biosynthesis was a *PgMpa* cluster-independent process (Scheme 2). We finally reconstructed the whole pathway of **1** in *A. nidulans* by the coexpression of *AN-PgMpaABCDEFG* (Fig. 3 iv). Overlapping in time frame with the work presented here, a preprint manuscript reported that the β -oxidation of primary metabolism was proposedly involved in the biosynthesis of **1**³². However, the *in vitro* biochemical details required further investigation.

3. Conclusions

In this study, the missing step of the biosynthetic pathway of the immunosuppressive drug mycophenolic acid (**1**) was identified

from *P. griseofulvum*. A membrane-bound prenyltransferase PgMpaA transferred an isopentenyl unit to the polyketide backbone **4** to yield **6** containing the C15 farnesyl moiety, which subsequently underwent the first cleavage step by a new globin-like enzyme (PgMpaB) to form cryptic intermediate **12**. Heterologous expression of the *PgMpa* gene cluster in *A. nidulans* indicated that the second cleavage step of **1** was a *PgMpa* cluster-independent process *in vivo*. Our results, especially the discovery of the broad tolerance of substrate recognized by PgMpaB, set up a strategy for the formation of “pseudo-isopentenyl” natural products using fungal globin-like enzymes.

Acknowledgments

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Appendix A. Supporting information

Supporting data to this article can be found online at <https://doi.org/10.1016/j.apsb.2019.06.009>.

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