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Tandem Prenyltransferases Catalyze Isoprenoid Elongation and Complexity Generation in Biosynthesis of Quinolone Alkaloids

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Abstract

Modification of natural products with prenyl groups and the ensuing oxidative transformations are important for introducing structural complexity and biological activities. Understanding the different mechanisms Nature performs prenylation can lead to new enzymatic tools. Penigequinolones (**1**) are potent insecticidal alkaloids that contain a highly modified ten-carbon prenyl group. Here we reveal an iterative prenylation mechanism for installing the ten-carbon unit using two aromatic prenyltransferases (PenI and PenG) present in the gene cluster of **1** from *Penicillium thymicola*. The initial Friedel-Crafts alkylation is catalyzed by PenI to yield the dimethylallyl quinolone **6**. The five-carbon side chain is then dehydrogenated by a Flavin-dependent monooxygenase to an aryldiene **9**, which serves as the electron-rich substrate for a second alkylation with dimethylallyl diphosphate to yield a stryrenyl product **10**. The completed, oxidized ten-carbon prenyl group is then shown to undergo further structural morphing to yield yaequinolone C **12**, the immediate precursor of **1**. Our studies therefore uncover an unprecedented prenyl chain extension mechanism in natural product biosynthesis.

An important biosynthetic transformation that generates chemical diversity in natural products is the addition of prenyl groups.[1–4] The transfer of prenyl groups, together with the subsequent modifications, significantly expand structural complexity and biological activities of all major families of natural products, including polyketides,[5–6] nonribosomal peptides[7–8] and indole alkaloids,[9–12] etc. The different prenyl precursors that are used to decorate natural products typically include dimethylallyl diphosphate (DMAPP), geranyl

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Supporting Information

Experimental details and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

diphosphate (GPP), farnesyl diphospate (FPP) or geranylgeranyl diphosphate (GGPP).[13] Formation of these building blocks are catalyzed by isoprenyl diphosphate synthases (IPPSs) through the iterative, head-to-tail addition of isopentenyl diphosphate (IPP) extender units.[14] Prenyl groups of the desired size are then transferred to an electron-rich substrate by a family of enzymes collectively known as prenyltransferases (PTases).[15] Notwithstanding this canonical model of preassembly followed by transfer of intact prenyl groups, a number of bioactive natural products contain prenyl substructures may be synthesized by unusual mechanisms. Examples include the twenty-five carbon lipid portion found in moenomycin,[16] the seven-carbon side chain found in mycophenolic acid,[17] the ten-carbon cyclolavandulyl skeleton found in lavanducyanin,[18] and the styrenyl-like framework found in fungal quinolone alkaloids represented by penigequinolone (**1**) as shown in Figure 1. Understanding new mechanisms of prenyl group transfer and modification will therefore lead to the expansion of enzymatic tools that can modify natural products with prenyl functionalities.

Penigequinolones produced by various *Penicillium* sp. and *Aspergillus* sp. are quinolone alkaloids with oxidized prenyl groups, which are crucial for the potent insecticidal activities.[19–26] The core of this family of molecules is the 6,6-bicyclic quinolone **2** that is derived from the oxidative rearrangement of methoxycyclopeptin **7**, which is the product of an nonribosomal peptide formed through the condensation between anthranilate and *O*-methyl-tyrosine.[27] C7 of the phenolic ring in **2** is proposed to undergo Friedel-Crafts geranylation to yield the proposed intermediate peniprequinolone **3** (Figure 1a).[27] Significant morphing of the geranyl unit is then proposed to take place to yield the various natural products, including penigequinolone A (**1a**) and B (**1b**) that contain the *gem*-dimethyl pyran ring;[22,26] aspoquinolone A (**4a**) and B (**4b**) that contain a fused cyclopropane-tetrahydrofuran unit;[23] and the chromene-containing yaequinolone J1 (**5a**) and J2 (**5b**).[24]

The prenyl transfer and modifications steps that transform **2** are of interest from several perspectives, including i) the quinolone scaffold as a substrate of *C*-prenyltransfer has not been reported previously, whereas prenylation of L-tyrosine, indole and polycyclic aromatics have been well-documented;[4–6,9,11–12,28] ii) all the natural products derived from the proposed intermediate **3** are heavily oxidized, including the double bond at C1′-C2′. The common oxygen atom at C3′ is proposed to derive from the epoxidation of C2′-C3′ and/or C7′-C8′ double bonds in **3** and subsequent ring-opening;[23] and iii) formation of the pyran ring in **1** as result of an unusual C-C bond rearrangement. Here we demonstrate **3**, which is the product of the proposed geranyltransfer reaction,[23,27] is in fact *not* an intermediate of the biosynthetic pathway. Instead, we show prenylation of the quinolone occurs in a unprecedented, step-wise fashion, in which two DMAPP units are iteratively added to **2** to afford an oxidized ten-carbon isoprenoid unit that is primed for subsequent rearrangement reactions. Such iterative elongation of prenyl groups has not been previously observed in natural product biosynthesis.

To understand the prenylation and subsequent modification steps, we sequenced the genome of *Penicillium thymicola* IBT5891, a producing strain of **1**.[29] Using the previously characterized cyclopenase AsqJ that converts **7** to **8** as a lead,[30] the putative *pen* cluster was

located and shown in Figure 2a. The gene cluster contains the expected nonribosomal peptide synthetase (NRPS) PenN, the AsqJ homolog PenM, and an assortment of redox and methyltransferase enzymes (Table S2). Unexpectedly, the gene cluster encodes two aromatic prenyltransferases PenG and PenI,[31] instead of the single geranyltransferase that was expected to build the proposed intermediate **3**. Genetic deletion of *penN* (Figure S1) using a split-marker approach and hygromycin as the resistance marker led to abolishment of **1** (Figure 2b, i & ii), thereby confirming the *pen* cluster is responsible for the biosynthesis of **1**.

We initially reasoned the presence of two prenyltransferases may account for the parallel modification of **2** with GPP that yields **3**, and with DMAPP that yields peniprequinolone **6**, which was identified from different *Penicillium sp*.[22,25–26] However, no trace of **6** was found in the wild type strain. Bioinformatic and phylogenetic analyses showed that i) PenI is closely related to NscD and VrtD that catalyze the Friedel-Crafts prenylation of polycyclic aromatic polyketides;[5–6] and ii) PenG clades independently from other fungal prenyltransferases and is more closely related to dimethylallyltryptophan synthases (DMATS) (Figure S2).[11–12,32] To investigate the roles of PenG and PenI, both enzymes were cloned from cDNA, expressed and purified from *Escherichia coli* to homogeneity (Figure S3). We then constructed a knockout cassette that targeted the entire region that included *penG-penI*. The mutant strain was unable to produce **1** and accumulated **2** (Figure 2b, iii), which was purified and used as substrate for biochemical assays (Table S3).

Compound **2** was first incubated with 2 mM GPP, $MgCl_2$, and either PenG or PenI. Unexpectedly, neither enzyme converted **2** to the proposed intermediate **3** (Figure 3, i & ii). In contrast, when DMAPP was used as the substrate, PenI catalyzed the near complete conversion of **2** into a new compound with mass consistent with the addition of one five-carbon unit and that of **6** (*m/z* 382 $[M\text{-}H]^-$), while PenG remained inactive (Figure 3, iii & iv). To elucidate the structure of the product, a whole-cell biotransformation using *E. coli* expressing PenI was performed followed by product extraction and purification. Complete ^1H and ^{13}C characterization confirmed the identity of the compound as **6** (Figure 3 and Table S4).[26] The *in vitro* results show that neither prenyltransferases in the *pen* cluster can function as a geranyltransferase using **2** as a substrate. Instead, PenI is a dimethylallyl transferase, while the function of PenG remained unresolved. However, these results point to the possibility that **6** is an intermediate in the biosynthetic pathway of **1** instead of **3**. Indeed, feeding **6** into the Δ*penN* blocked mutant of *P. thymicola* restored the production of **1** (Figure 2b, iv), confirming the existence of an prenyl elongation mechanism downstream in the pathway.

The head-to-tail polyprenyl elongation catalyzed by IPPS requires the terminal Δ^3 double bond present in IPP.[13] Therefore compound **6,** which contains the internal Δ^2 double bond, cannot directly attack a dimethylallyl cation intermediate to yield a ten-carbon prenyl group. With the hypothesis that PenG catalyzes the prenyl elongation step, we constructed the Δ*penG* mutant in order to identify a possible electron-rich substrate. LC-MS analysis of the extract showed the loss of **1** and the appearance of a new compound **9** (*m/z* 380 $[M\text{-}H]^-$) that has a red-shifted λ_{max} compared to **6** (from 300 nm to 337 nm) (Figure 4a, i & ii). Isolation

and structural characterization of **9** revealed the compound to be the aryldiene quinolone previously isolated as yaequinolone E (Figure 4 and Table S4).[22] Compared to **6**, **9** has undergone one dehydrogenation followed by shifting of the Δ^2 double bond to the $\Delta^{3'}$ position. To confirm that **9** is an authentic intermediate of **1**, feeding of **9** to the Δ*penN* mutant strain was performed. As expected, restoration of the biosynthesis of **1** was observed (Figure 4a, iii).

To assay the chain elongation reaction, purified **9** was incubated with PenG, DMAPP and $MgCl_2$ followed by extraction and LC-MS analysis. Near complete conversion of **9** to one major compound **10** (*m/z* 466 $[M\text{-}H]^-$), as well as other two minor compounds (*m/z* 432 $[M+H\text{-}H_2O]^+$) were observed (Figure 4b, i). In contrast, no reaction occurred when PenI was used as the prenyltransferase (Figure 4b, ii). To obtain sufficient amounts of **10** and the minor products for structural characterization, a large scale biotransformation using *S. cerevisiae* expressing PenG was performed. All three compounds were thoroughly characterized by 1D- and 2D-NMR (Table S5–S6), and are confirmed to be products containing ten-carbon prenyl groups. Compound **10** is the styrenyl quinolone containing a C3′-hydroxyl prenyl chain. Feeding of **10** to the Δ*penN* block mutant efficiently restored the biosynthesis of **1** (Figure 4, iv), confirming the compound as an on pathway intermediate of the pathway. We reasoned that **10** can be obtained from the addition of H_2O to the cationic intermediate **11** following the PenG-catalyzed chain elongation reaction (Figures 4 and 5). This mechanism is verified by performing the PenG reaction in $H_2{}^{18}O$ buffer and observing the incorporation of ^{18}O into **10** (Figure S4). The two minor compounds from the in vitro reaction are the yaequinolone J1 and J2 (**5a** and **5b**),[24] which are presumably shunt products in the biosynthesis of **1** derived from the cyclization of **11** through the phenolate oxygen (Figure 5). Interestingly, feeding of **10** to Δ*penN* led to significant accumulation of **5a** and **5b** (Figure 4, iv), suggesting that under fungal culturing conditions, the loss of H_2O by **10** to yield **11** (reverse reaction to generate the cation) may be significant. In contrast, purified **10** is stable and does not undergo spontaneous conversion to **5a** and **5b**.

Having established that the ten-carbon isoprenoid side chain in **1** is derived from the iterative addition of two DMAPP molecules, first from **2** to **6**, followed by **9** to **10**, we next aimed to identify the oxidative enzymes responsible for the conversion of **6** to **9**, as well as the downstream modification of **10**. The enzyme encoded by *penH* is a membrane-bound Flavin-dependent monooxygenase (FMO) featuring both BBE[33] and GlcD[34] conserved domains. GlcD-containing FMOs are membrane-bound respiratory D-lactate dehydrogenase, which catalyze the conversion of D-lactate to pyruvate using a covalently linked FAD as a co-factor.[34] Hence, PenH is likely a FAD-dependent dehydrogenase that may catalyze the dehydrogenation of **6** to **9.** This may proceed via base-catalyzed removal of C1′ hydrogen and capturing of the C4′ hydrogen as a hydride by the FAD cofactor (Figure 5).[35] Indeed, the Δ*penH* mutant accumulated **6** as the only product, and the biosynthesis of **1** can be restored through the feeding of **9** (Figure 4a, v & vi). Attempts to express PenH as a soluble protein from either *E. coli* or *S. cerevisiae* were not successful, thereby precluding the direct assay of this reaction using purified enzyme. Nevertheless, the identification of the role of PenH using genetic methods allowed us to map the entire pathway that converts the quinolone **2** to the first ten-carbon isoprenyl containing intermediate **10** as shown in Figure

5. The pathway starts with the canonical Friedel-Crafts alkylation of **2** with dimethylallyl cation by PenI to yield **6**, which is subjected to FAD-dependent dehydrogenation to yield the conjugated diene **9**. The $\Delta^{3'}$ double bond then serves as the site of the second alkylation with DMAPP catalyzed by PenG to yield the carbenium ion intermediate **11**, which can be attacked by H_2O to yield **10** or undergo cyclization to yield **5a** and **5b** (Figure 5).

We next targeted the identification of enzymes that modify **10** into yaequinolone C (**12**) (Figure 5), which is proposed to be the immediate precursor of **1**.[23] The conversion likely involves epoxidation of the terminal C7′–C8′ olefin in **10** to yield **13**, followed by epoxide ring opening initiated by the C3′ hydroxyl group to yield the tetrahydrofuran-containing **12**. Based on this hypothesis, we performed genetic knockout of the FMO PenE, which displays sequence homology to PaxM, a FMO catalyzing the epoxidation of the diterpene unit in the biosynthesis of fungal indole-diterpenes.[36] Inactivation of PenE led to the disappearance of **1** and the accumulation of **10**, as well as the minor metabolites **5a** and **5b** (Figure 4a, vii). This is consistent with PenE functioning as the epoxidase as shown in Figure 5. To further probe the role of PenE in the proposed morphing of **10**, we performed biotransformation experiments using *S. cerevisiae* as an expression host. When **10** was added to the yeast culture expressing PenE alone, we detected the formation of a new compound that is consistent with the molecular weight of **12 (***m/z* 482 $[M-H]^-$), as well as the formation of shunt products **5a** and **5b** (Figure S5, ii). Compound **12** was purified and characterized by NMR to be identical to yaequinolone C (Table S7).[22] The successful transformation of **10** to **12** suggests that the epoxide-opening of **13** may take place spontaneously. Interestingly, the yield of **12** was significantly elevated (~10 fold) when the biotransformation was performed in yeast host coexpressing both PenE and PenJ, a predicted cysteine hydrolase (Figure S5, iii). Increase in conversion of **10** to **12** therefore indicates the role of PenJ as an epoxide hydrolase in enhancing the rate of the 5-*exo-tet* cyclization step.

In conclusion, we identified an unprecedented mechanism of prenyl elongation in the biosynthesis of penigequinolone family of natural products. Two prenyltransferases successively transferred dimethylallyl units to the quinolone core **2** to yield **10** that contains a “pseudo-geranyl” moiety. Activation of the first dimethylallyl unit to the conjugated diene **9** is accomplished by a FAD-dependent dehydrogenation. The hydroxylated isoprenoid unit in **10** is optimally setup to undergo cyclization towards formation of the final natural products. Our work therefore reveals a new strategy employed by nature to transfer and tailor prenyl group to natural products towards generation of structural complexity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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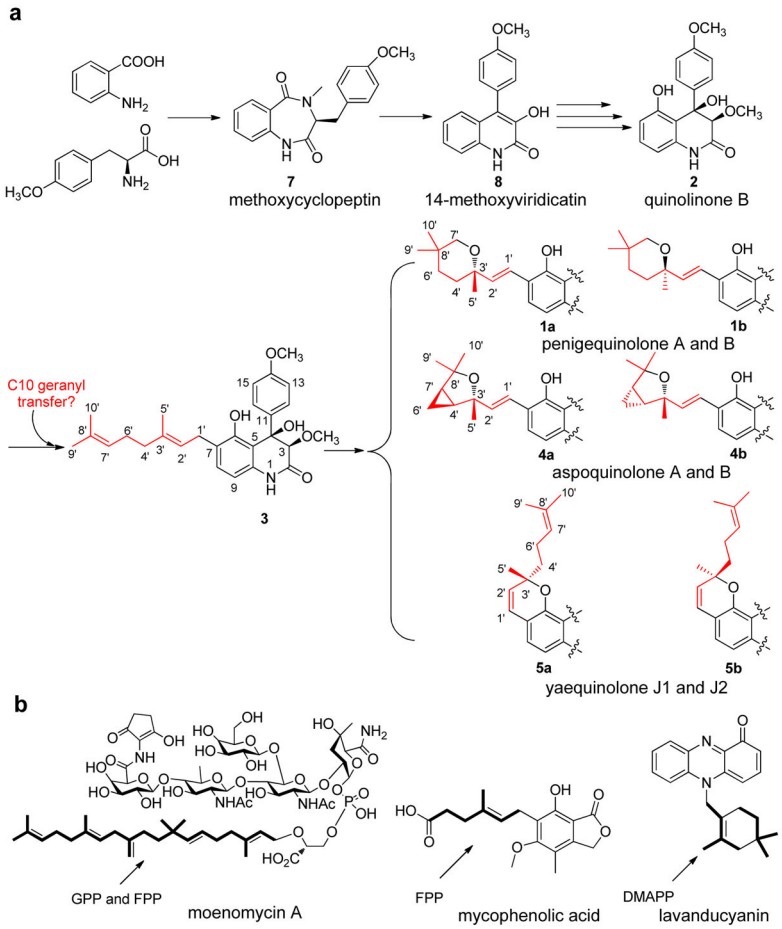

Figure 1.

Representative prenylated natural products with unusual prenyl group arrangements. **a**. Proposed biosynthetic pathway of penigequinolones family compounds. The proposed geranyl isoprenoid unit is shown in red. **b**. natural products contains unusually arranged isoprenoid groups.

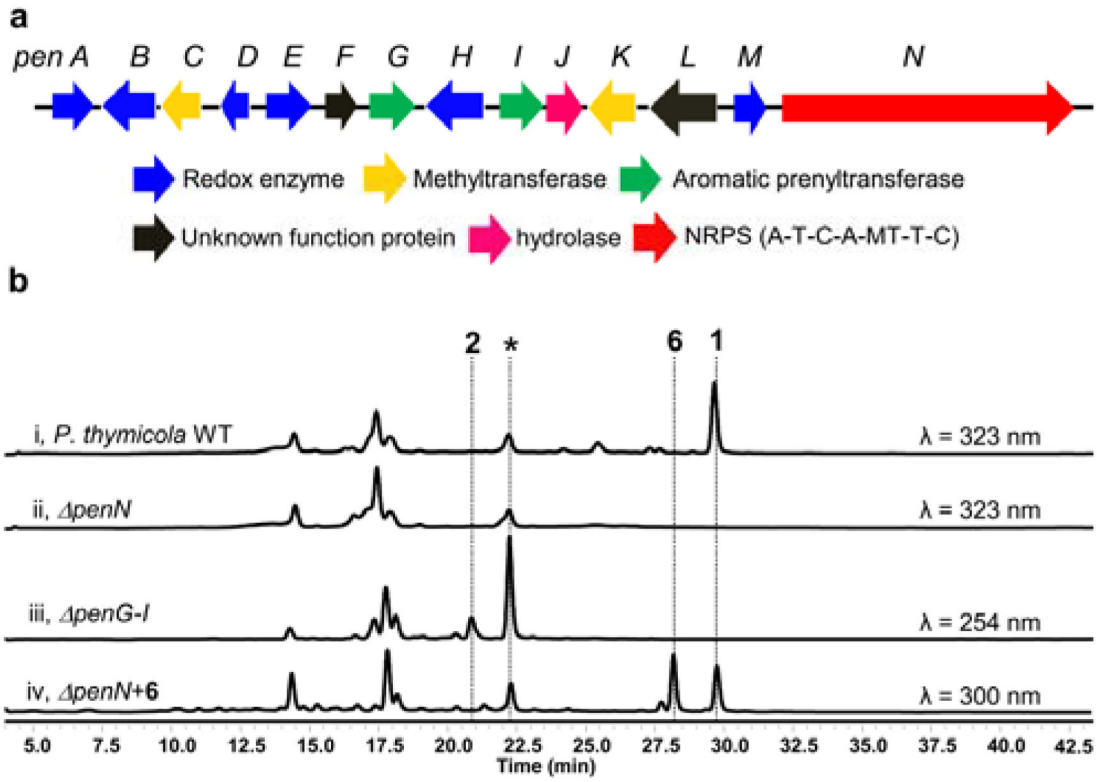

Figure 2.

Confirmation of *pen* gene cluster in *P. thymicola.* **a** Organization and proposed function of the *pen* gene cluster. **b**. LC-MS analyses of culture extracts from wild type *P. thymicola* and mutant strains. Peak labeled by asterisk is fumiquinazoline F, an unrelated metabolite.

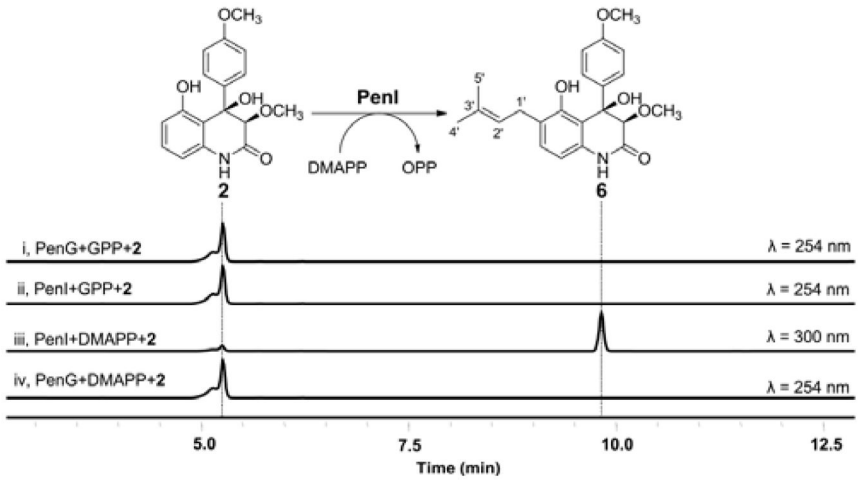

Figure 3.

In vitro biochemical analyses of PenG and PenI with **2**. All reactions performed with purified PTases in the presence of $MgCl_2$ and the substrate **2.**

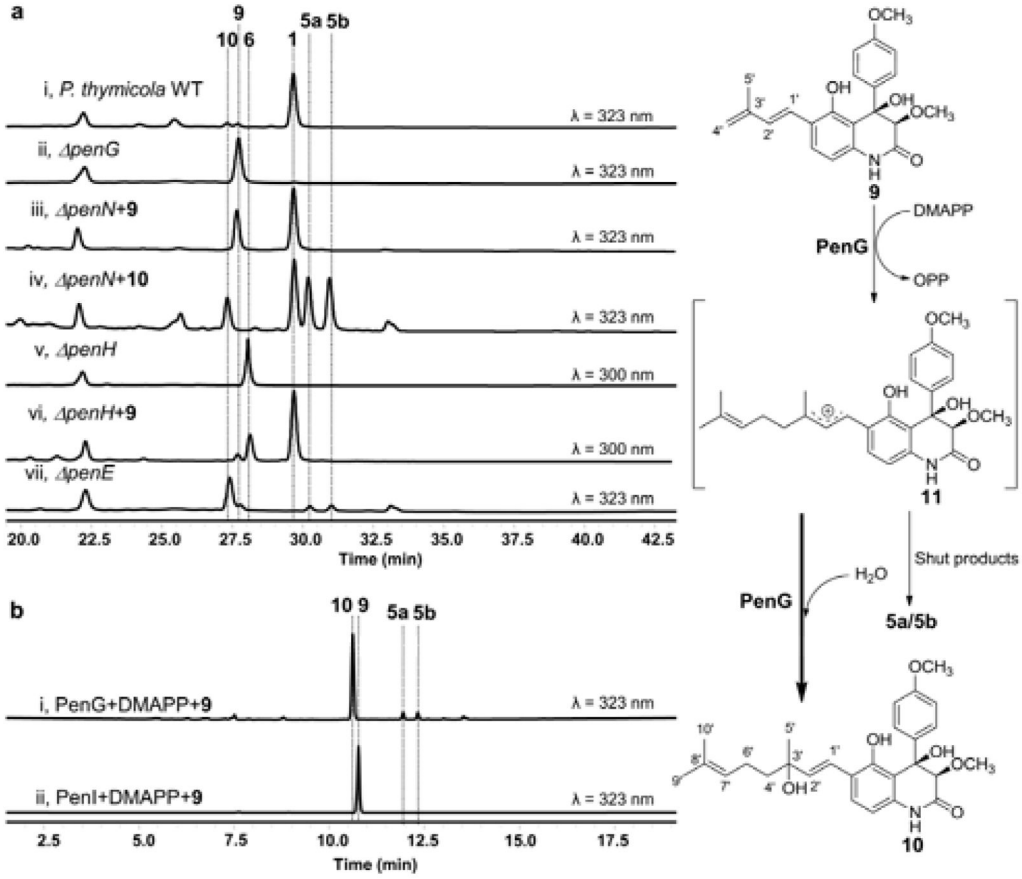

Figure 4.

Mapping the prenyl elongation step in biosynthesis of **1. a**. LC-MS analyses of culture extracts from *P. thymicola* strains. **b**. Analyses of i*n vitro* biochemical assays involving PenG or PenI with **9**.

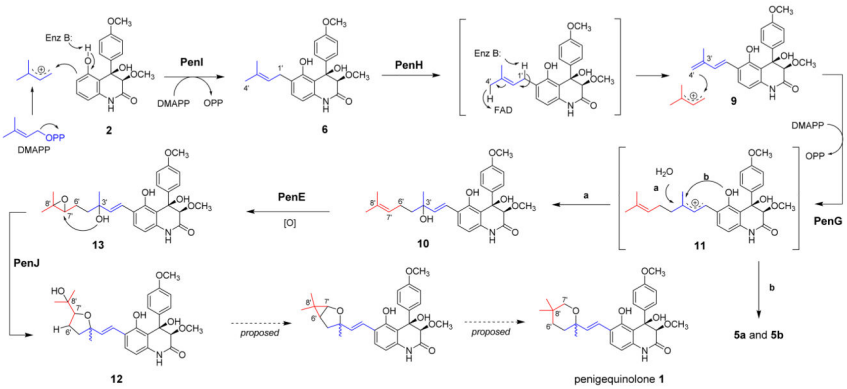

Figure 5.
Updated biosynthetic pathway of **1**.