JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
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Tandem Prenyltransferases Catalyze Isoprenoid Elongation and Complexity Generation in Biosynthesis of Quinolone Alkaloids

Yi Zou,[†] Zhajun Zhan,[†,§] Dehai Li,[†,∥] Mancheng Tang,[†] Ralph A. Cacho,[†] Kenji Watanabe,[⊥] and Yi Tang*[,†,‡]

[†]Department of Chemical and Biomolecular Engineering and [‡]Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90095, United States

[§]College of Pharmaceutical Science, Zhejiang University of Technology, Hangzhou 310014, P. R. China

[∥]Key Laboratory of Marine Drugs, Chinese Ministry of Education, School of Medicine and Pharmacy, Ocean University of China, Qingdao 266003, P. R. China

[⊥]Department of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422-8526, Japan

Supporting Information

ABSTRACT: Modification of natural products with prenyl groups and the ensuing oxidative transformations are important for introducing structural complexity and biological activities. Penigequinolones (**1**) are potent insecticidal alkaloids that contain a highly modified 10-carbon prenyl group. Here we reveal an iterative prenylation mechanism for installing the 10-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 dimethylallyl quinolone **6**. The five-carbon side chain is then dehydrogenated by a flavin-dependent monooxygenase to give aryl diene **9**, which serves as the electron-rich substrate for a second alkylation with dimethylallyl diphosphate to yield stryrenyl product **10**. The completed, oxidized 10-carbon prenyl group then undergoes further structural morphing to yield yaequinolone C (**12**), the immediate precursor of **1**. Our studies have therefore uncovered 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 the structural complexity and biological activities of all major families of natural products, including polyketides,[5,6] nonribosomal peptides,[7,8] indole alkaloids,[9−12] etc. The different prenyl precursors that are used to decorate natural products typically include dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP).[13] The formation of these building blocks is 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 containing prenyl substructures may be synthesized by unusual mechanisms. Examples include the 25-carbon lipid portion found in moenomycin,[16] the seven-carbon side chain found in mycophenolic acid,[17] the 10-carbon cyclolavandulyl skeleton found in lavanducyanin,[18] and the styrenyl-like framework found in fungal quinolone alkaloids represented by penigequinolones (**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* spp. and *Aspergillus* spp. 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** derived from oxidative rearrangement of methoxycyclopeptin (**7**), which is the product of an nonribosomal peptide formed by condensation of anthranilate and *O*-methyltyrosine.[27] C7 of the phenolic ring in **2** is proposed to undergo Friedel−Crafts geranylation to yield the proposed intermediate peniprequinolone **3** (Figure 1b).[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**) containing the *gem*-dimethylpyran ring,[22,26] aspoquinolone A (**4a**) and B (**4b**) containing a fused cyclopropane−tetrahydrofuran unit,[23] and the chromene-containing yaequinolone J1 (**5a**) and J2 (**5b**).[24]

The prenyl transfer and modification steps that transform **2** are of interest from several perspectives: (i) The quinolone scaffold as a substrate of *C*-prenyltransferase 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 of the natural products derived from the proposed intermediate **3** are more oxidized, including the double bond at C1′−C2′. The common oxygen atom at C3′ has been proposed to derive from the epoxidation of C2′−C3′ and/or C7′−C8′ double bonds in **3** and subsequent ring opening.[23] (iii) The pyran ring in **1** forms as a result of an unusual C−C bond rearrangement. Here we demonstrate that prenylation of the

Received: March 23, 2015
Published: April 10, 2015

DOI: 10.1021/jacs.5b03022
J. Am. Chem. Soc. 2015, 137, 4980−4983

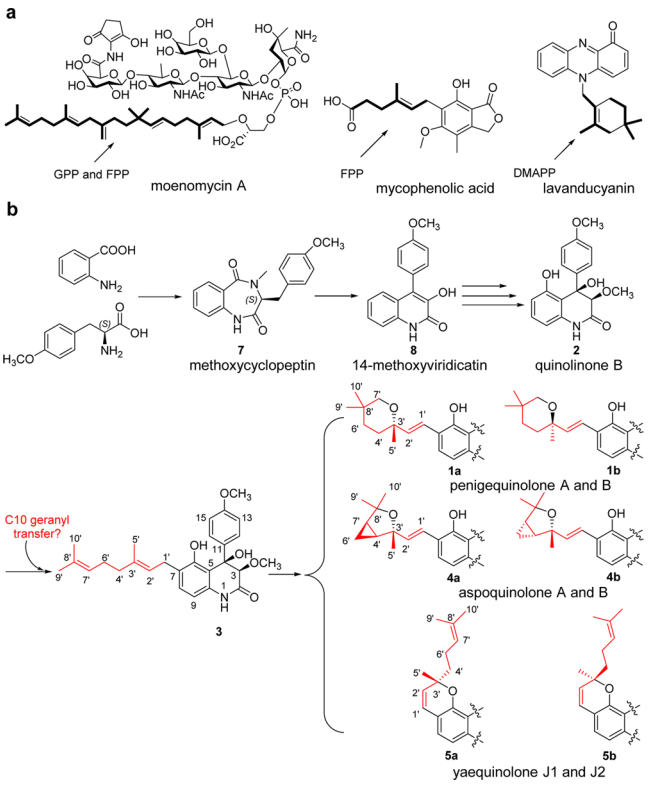

Figure 1. Representative prenylated natural products with unusual prenyl group structures. (a) Natural products containing unusually structured isoprenoid groups. (b) Penigequinolones and the proposed biosynthetic pathway, with the proposed geranyl isoprenoid unit shown in red.

quinolone occurs in a unprecedented stepwise fashion in which two DMAPP units are iteratively added to **2** to afford an oxidized 10-carbon isoprenoid unit that is primed for subsequent rearrangement reactions.

To understand the prenylation and subsequent modification steps, we sequenced the genome of *Penicillium thymicola* IBT5891, a producing strain of **1**.[29] With the previously characterized cyclopenase AsqJ that converts **7** to **8** as a lead,[30] the putative *pen* cluster was located and is shown in Figure 2a.

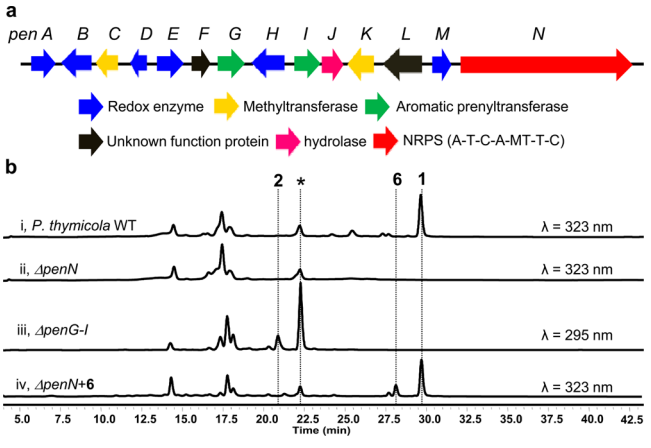

Figure 2. Confirmation of the *pen* gene cluster in *P. thymicola*. (a) Organization and proposed function of the *pen* gene cluster. (b) LC–MS analyses of culture extracts from wild-type and mutant. The peak labeled with an asterisk is fumiquinazoline F, an unrelated metabolite.

The gene cluster contains the expected nonribosomal peptide synthetase (NRPS) PenN, the AsqJ homologue PenM, and an assortment of redox and methyltransferase enzymes (Table S2 in the Supporting Information (SI)). 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 in the SI) led to abolishment of **1** (Figure 2b, i and ii), thereby confirming that the *pen* cluster is responsible for the biosynthesis of **1**.

We initially reasoned that the presence of two prenyltransferases may account for the parallel modification of **2** with GPP to yield **3** and with DMAPP to yield peniprequinolone **6**, which was identified from different *Penicillium* spp.[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, which catalyze the Friedel–Crafts prenylation of polycyclic aromatic polyketides,[5,6] and (ii) PenG is more closely related to dimethylallyltryptophan synthases (DMATS) (Figure S2).[11,12,32] To investigate the roles of PenG and PenI, both enzymes were expressed from *Escherichia coli* and purified to homogeneity (Figure S3). We then constructed a knockout cassette targeting 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 a 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 and ii). In contrast,

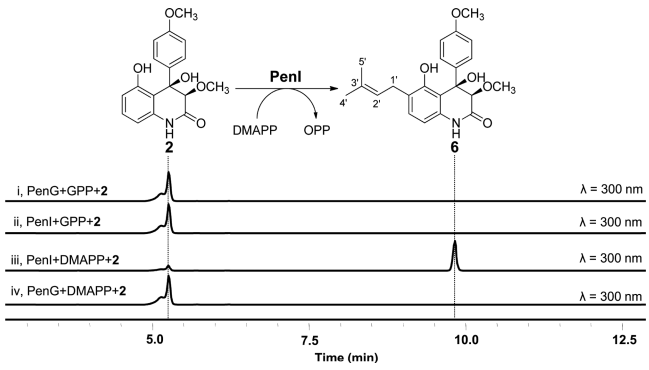

Figure 3. In vitro biochemical analyses of PenG and PenI with **2**.

when DMAPP was used as the substrate, PenI catalyzed the nearly 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 $[M-H]^-$), while PenG remained inactive (Figure 3, iii and 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 NMR characterization confirmed the identity of the compound as **6** (Figure 3 and Table S4).[26] The in vitro results show that neither prenyltransferase can function as a geranyltransferase. 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* mutant of *P. thymicola* restored the production of **1** (Figure 2b, iv), confirming the existence of a prenyl elongation mechanism downstream in the pathway.

DOI: 10.1021/jacs.5b03022
J. Am. Chem. Soc. 2015, 137, 4980–4983

The head-to-tail polyprenyl elongation catalyzed by IPPS requires the terminal Δ^3 double bond present in IPP.[13] Therefore, **6**, which contains the internal $\Delta^{2'}$ double bond, cannot directly attack a dimethylallyl cation intermediate to yield a 10-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 $[M - H]^-$), that has a red-shifted λ_{max} compared with **6** (from 300 to 337 nm) (Figure 4a, i and ii). Isolation and structural characterization of **9** revealed

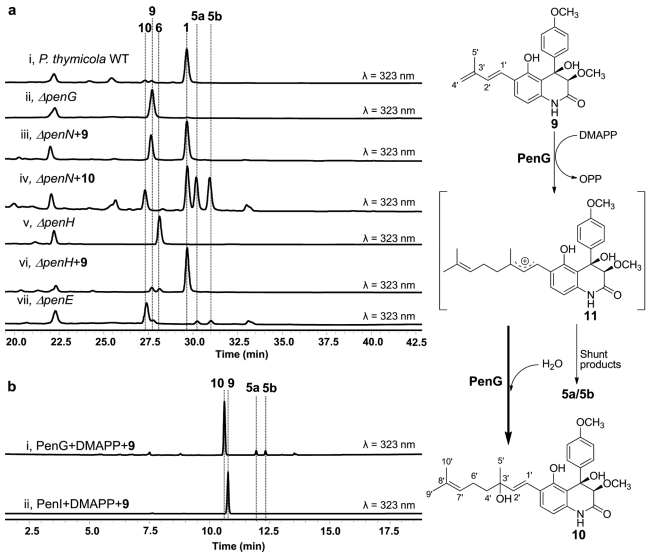

Figure 4. Mapping of the prenyl elongation step in the biosynthesis of **1**. (a) LC−MS analyses of culture extracts from *P. thymicola* strains. (b) Analyses of in vitro biochemical assays involving PenG or PenI with **9**.

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

To assay the chain elongation reaction, **9** was incubated with PenG, DMAPP, and $MgCl_2$, followed by LC−MS analysis. Nearly complete conversion of **9** to one major compound **10** (*m*/*z* 466 $[M - H]^-$) and two minor compounds (*m*/*z* 432 $[M + H - H_2O]^+$) was observed (Figure 4b, i). In contrast, no reaction occurred when PenI was used as the prenyltransferase (Figure 4b, ii). A large-scale biotransformation using *Saccharomyces cerevisiae* expressing PenG was performed. All three compounds were confirmed to be products containing 10-carbon prenyl groups (Tables S5 and S6). Compound **10** is the styrenyl quinolone containing a C3′-hydroxyprenyl chain. Feeding of **10** to the Δ*penN* mutant efficiently restored **1** (Figure 4a, iv), confirming the compound to be an on-pathway intermediate. 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 was 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 yaequinolone J1 (**5a**) and J2 (**5b**),[24] which are presumably shunt products in the biosynthesis of **1** derived from cyclization of **11** through the phenolic oxygen (Figure 5). Interestingly, feeding of **10** to Δ*penN* led to significant accumulation of **5a** and **5b** (Figure 4a, 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.

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. 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 the C1′ hydrogen and capture 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,

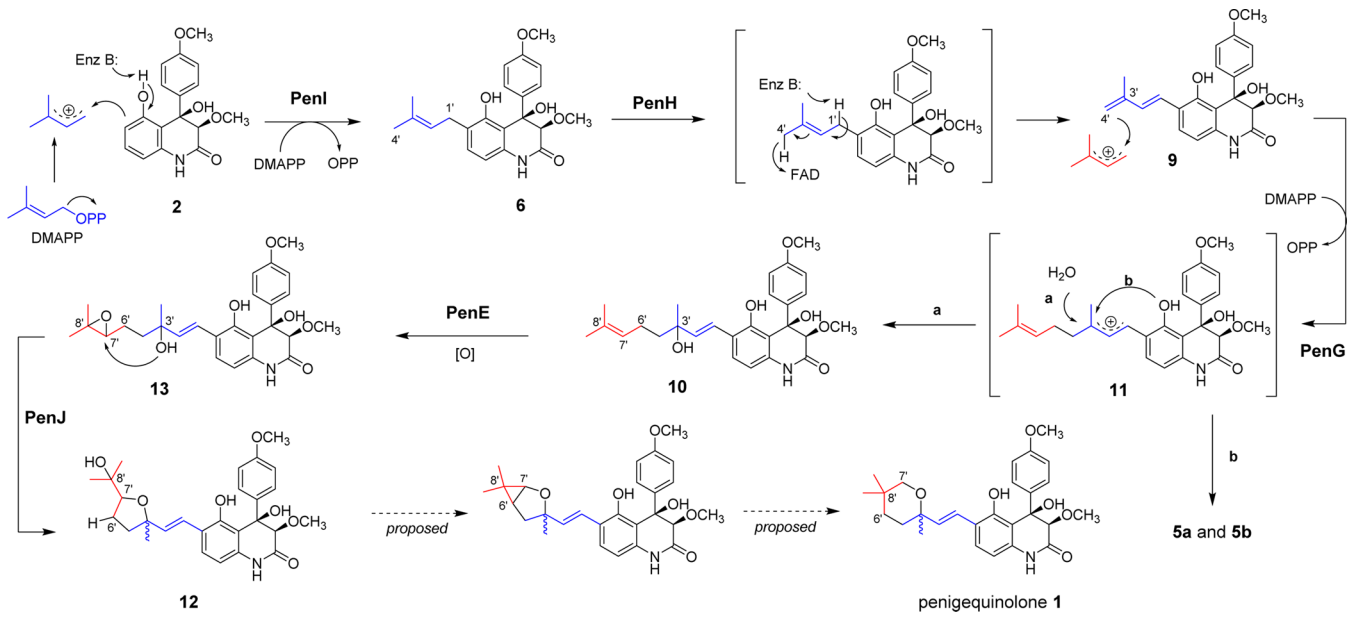

Figure 5. Updated biosynthetic pathway of **1**.

DOI: 10.1021/jacs.5b03022
J. Am. Chem. Soc. 2015, 137, 4980−4983

and the biosynthesis of **1** can be restored through the feeding of **9** (Figure 4a, v and 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. The entire pathway that converts quinolone **2** into **10** is 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 conjugated diene **9**. The $\Delta^{3'}$ double bond then serves as the site of the second alkylation with DMAPP catalyzed by PenG to yield 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**. On the basis of this hypothesis, we performed a genetic knockout of the FMO PenE, which displays sequence homology to PaxM, an FMO catalyzing the epoxidation of the diterpene unit in the biosynthesis of fungal indolediterpenes.[36] Indeed, 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). We then 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 **12** (Table S7)[22] along with the shunt products **5a** and **5b** (Figure S5, ii). The successful transformation of **10** to **12** suggests that the epoxide ring opening of **13** may take place spontaneously. 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). The 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 have identified an unprecedented mechanism of prenyl elongation in the biosynthesis of the penigequinolone family of natural products. Two prenyltransferases successively transfer dimethylallyl units to the quinolone core **2** to yield **10** containing 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 set up to undergo cyclization toward the formation of the final natural products. Our work therefore reveals a new strategy employed by nature to transfer and tailor prenyl groups to natural products toward the generation of structural complexity.

■ ASSOCIATED CONTENT

Supporting Information

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

■ AUTHOR INFORMATION

Corresponding Author

*yitang@ucla.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by NIH Grants 1R56AI101141 and 1DP1GM106413 to Y.T. NMR instrumentation was supported by NSF Equipment Grant CHE-1048804. Z.Z. and D.L. were supported by fellowships from the China Scholarship Council.

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DOI: 10.1021/jacs.5b03022
J. Am. Chem. Soc. 2015, 137, 4980−4983