Natural Products Hot Paper

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One-Pot Enzymatic Synthesis of Merochlorin A and B**

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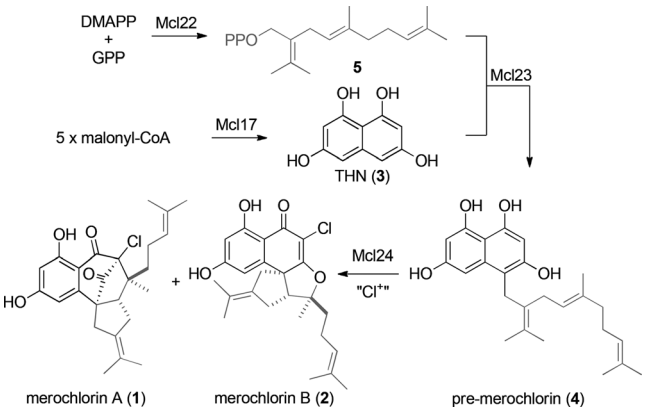
Abstract: *The polycycles merochlorin A and B are complex halogenated meroterpenoid natural products with significant antibacterial activities and are produced by the marine bacterium Streptomyces sp. strain CNH-189. Heterologously produced enzymes and chemical synthesis are employed herein to fully reconstitute the merochlorin biosynthesis in vitro. The interplay of a dedicated type III polyketide synthase, a prenyl diphosphate synthase, and an aromatic prenyltransferase allow formation of a highly unusual aromatic polyketide-terpene hybrid intermediate which features an unprecedented branched sesquiterpene moiety from isosesquilavandulyl diphosphate. As supported by in vivo experiments, this precursor is furthermore chlorinated and cyclized to merochlorin A and isomeric merochlorin B by a single vanadium-dependent haloperoxidase, thus completing the remarkably efficient pathway.*

Merochlorins A and B (**1** and **2**)[1] feature unique, stereochemically complex ring systems, namely a bicyclo-[3.2.1]octadione and a 6-5-5-fused tricycle, respectively (Scheme 1). Recently, routes for the chemical synthesis of **1** and **2** were established,[2] yet how these molecules are synthesized in nature remained largely unknown. Genetic analyses revealed that the *Streptomyces* sp. strain CNH-189 encodes the merochlorin (mcl) biosynthetic machinery within the boundaries of a 57.6 kb-sized mcl gene cluster comprising a total of 41 predicted proteins (Mcl1-Mcl41),[1a] some of which are likely not related to merochlorin biosynthesis. The current tentative biosynthetic pathway[1a] (Scheme 1) suggests the independent synthesis of the polyketidic and isoprenic fragments, followed by condensation of the two distinct units. Further modification of this shared precursor through halogenation and cyclization may then yield **1** and **2**.[1a] We set out to investigate the enzymatic synthesis of **1** and **2** in vitro using purified polyhistidyl-tagged enzymes (see Figure S1 in the Supporting Information) combined with chemical synthesis.

Because of high sequence similarity to characterized type III polyketide synthases (PKS) and gene knock-out experiments, Mcl17 was suggested to catalyze formation of 1,3,6,8-tetrahydroxynapthalene (THN; **3**; Scheme 1),[1a] a known intermediate in the biosynthesis of other streptomycete meroterpenoids as well as fungal and bacterial melanins.[3] These enzymes catalyze iterative decarboxylative condensations of malonyl coenzyme A (CoA) to produce a linear pentaketide intermediate which is subsequently cyclized by intramolecular Claisen and aldol condensations to **3**.[4] Indeed, a single product was formed upon incubation of Mcl17 with malonyl CoA, as evidenced by reverse-phase (RP) HPLC. Retention time, UV-Vis spectra, and high-resolution mass spectrometry (HRMS) data fully matched that of the chemically synthesized **3**,[5] thus confirming the type III PKS activity (see Figure S2). As determined spectrophotometrically, Mcl17 catalyzed the formation of **3** with $(2.4 \pm 0.3)\ \mathrm{mU\,mg^{-1}}$ (k_{cat} of $0.47\ \mathrm{min^{-1}}$ per malonyl-CoA). Under aerobic conditions, slow, non-enzymatic conversion of **3** into the known auto-oxidation product 2,5,7-trihydroxy-1,4-naphthoquinone (flaviolin)[6] occurred (see Figure S2).

In contrast to formation of **3**, the biosynthesis of the isoprenic merochlorin precursor appeared more obscure. It can be envisaged, however, that it derives from an unusual



Scheme 1. Reaction scheme for the biosynthetic pathway towards **1** and **2** as described in this work. Catalytic mechanisms of Mcl17, Mcl22, and Mcl23 are discussed in the text (see also Scheme 2). Details of the Mcl24-mediated reactions are reported by Diethelm et al.[16] DMAPP = dimethylallyl diphosphate, GPP = geranyl diphosphate.

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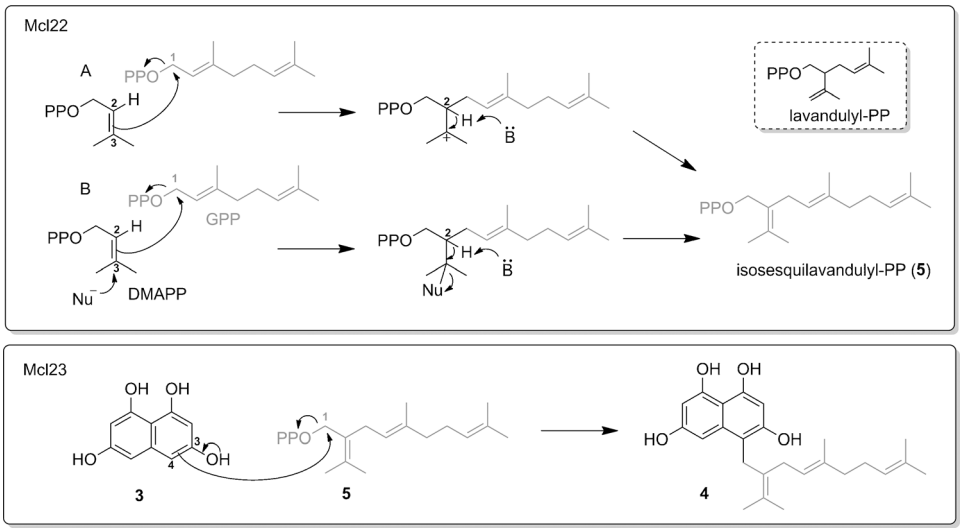


Scheme 2. Proposed catalytic mechanisms of Mcl22 and Mcl23.

branched sesquiterpene diphosphate (C_{15}, **5**).[1a] Despite the immense structural diversity of terpenes, the vast majority is assembled from isopentenyl diphosphate (C_5, IPP) and dimethylallyl diphosphate (C_5, DMAPP) by conventional head-to-tail [C(1)′–C(4)] coupling through carbocation-mediated electrophilic substitution reactions. Compounds with non-head-to-tail connectivity are synthesized by cyclopropanation, cyclobutanation, or isoprene branching reactions and comprise, for example, squalene and phytoene (sterol and carotenoid precursors), insectal pheromones, and several plant metabolites.[7,8] Among the latter, the monoterpenes lavandulol and lavandulyl acetate (both C_{10}), isolated, for example, from the eponymous lavender oil, exhibit a similar C(1′)–C(2) branching as that observed in the merochlorin terpene moieties,[8b] albeit with different double-bond arrangements (Scheme 2). Possibly, **5** arises from an exceptional head-to-torso [C(1′)–C(2)] coupling of geranyl diphosphate (C_{10}, GPP) with DMAPP. We speculated that Mcl22 may be involved in this branching reaction because of low sequence homology to known *cis*-prenyl diphosphate synthases.[9] Thus far, only two enzymes have been described which naturally catalyze isoprene branching, both of which are homologous to *cis*-prenyl diphosphate synthases and convert two molecules of DMAPP into either lavandulyl diphosphate[8b] (Scheme 2) or cyclolavandulyl diphosphate.[10]

To explore the role of *mcl22* in merochlorin biosynthesis, we deleted the gene in a heterologous *Streptomyces* host strain expressing the *mcl* gene cluster. The mutation completely abolished the production of merochlorin derivatives (see Figure S3). We then tested Mcl22 activity in enzymatic assays using GPP and DMAPP as substrates, followed by RP-HPLC analysis.[11] Gratifyingly, a single product was formed (Figure 1). Addition of $MgCl_2$ to the assay boosted the enzymatic activity nearly fourfold to $(850 \pm 20)\ mU\,mg^{-1}$ (k_{cat} of $20.3\ min^{-1}$), whereas the metal-chelating agent ethylenediaminetetraacetate (EDTA) completely abolished product formation (Figure 1). These results are in line with the common Mg^{2+} dependency of prenyl diphosphate synthases. The K_m values of Mcl22 for DMAPP and GPP were determined to be (65 ± 16) and $(13 \pm 5)\ \mu M$, respectively. Extensive NMR analyses, HRMS, and chemical synthesis confirmed the structure of **5** (see the Supporting Information and Figure 1), for which we propose the name isosesquilavandulyl diphosphate. Notably, Mcl22 exhibited interesting side activities (relative activities $< 1\%$ for all reactions), thus allowing the in vitro condensation of DMAPP, farnesyl diphosphate (FPP; C_{15}), or geranylgeranyl diphosphate (GGPP; C_{20}) with one molecule of DMAPP. HRMS data and retention times were consistent with the formation of the corresponding branched C_{10}, C_{20}, and C_{25} prenyl diphosphates. Moreover, IPP was slowly condensed with GPP (see Figure S4).

To further investigate the Mcl22 catalytic mechanism, we used various deuterated DMAPP analogues (see Ref. [12] and the Supporting Information for synthesis) and GPP as substrates in enzymatic assays. Deuterium incorporation into **5** was analyzed by HRMS. The exchange of D for H in **5** was exclusively observed when C2-deuterated DMAPP was proffered (see Figure S5). We thus propose initial C–C bond formation through nucleophilic attack by C2 in DMAPP, thus displacing the diphosphate leaving group at

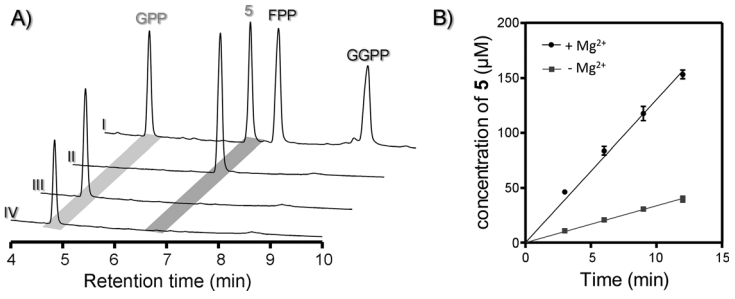


Figure 1. A) Reverse-phase HPLC analysis of the Mcl22-catalyzed reaction (UV at $\lambda = 214$ nm). Mixture of C_{10}–C_{20} diphosphate standards containing 1 nmol of each GPP, FPP, GGPP, and chemically synthesized isosesquilavandulyl diphosphate **5** (I). Mcl22 enzymatic assay showing the complete conversion of GPP and DMAPP to **5** (II). Control assay with heat-inactivated Mcl22 (III). Control assay with intact Mcl22 in presence of 10 mM EDTA (IV). Note that DMAPP did not bind to the column (elutes at < 2 min). B) Time course of the Mcl22-catalyzed formation of **5** in presence or absence of 5 mM $MgCl_2$. Both assays contained 0.62 μM Mcl22 and 2 mM of GPP and DMAPP each. Products were quantified by HPLC from three independent samples for each point of time. Error bars indicate the standard error.

C1 of the electrophilic primer GPP. Subsequent elimination of the C2 proton is then likely facilitated by a catalytic base within the Mcl22 active site (Scheme 2) and accounts for the unusual double-bond arrangement. This intriguing reaction may either involve stabilization of a transiently formed carbocation at C3 of DMAPP, or alternatively, could be initiated at the same position by attack of a nucleophilic amino acid residue (e.g., a cysteine thiolate; routes A and B in Scheme 2).

Next, we addressed the question of whether we can enzymatically assemble the two distinct building blocks **3** and **5**. Mcl23 exhibits sequence homology to aromatic ABBA prenyltransferases, which are found in bacteria and fungi and commonly attach DMAPP or GPP to phenolic substrates by electrophilic aromatic substitution reactions.[13] As expected, **1** and **2** were completely absent in culture extracts of a *mcl23* mutant strain (see Figure S3). Some members of this enzyme family require Mg^{2+} for α-phosphate binding and catalysis, whereas others may instead employ positively charged amino acid residues as catalytic surrogates.[14] Sequence alignment with the structurally elucidated NphB (formerly Orf2; 37% amino acid sequence identity, 94% coverage), revealed the presence of a conserved aspartic acid residue (D61 in Mcl23), which is typically observed in Mg^{2+}-dependent ABBA prenyltransferases (see Figure S6).[14] Indeed, heterologously produced Mcl23 transformed **3** and **5** with (53 ± 3) mU mg^{-1} (k_{cat} of 1.8 min^{-1}) into a single oxygen-sensitive product in a strictly Mg^{2+}-dependent reaction (Figure 2, and see Figures S7 and S8).

As we were not able to enzymatically synthesize sufficient amounts for NMR analyses, because of low yields and product auto-oxidation, we chemically prepared pre-merochlorin (**4**). As outlined in Scheme 3, addition of lithium dimethylcuprate (Me_2LiCu) to ethyl 2-butynoate, followed by quenching of the intermediate allenolate with geranyl bromide, delivered the

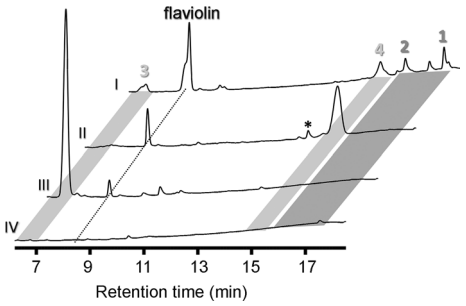
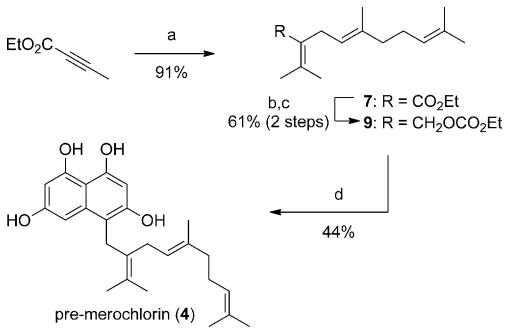


Figure 2. RP-HPLC analysis of the fully in vitro reconstituted merochlorin A and B biosynthetic pathways (UV at $\lambda = 254$ nm). Assays contained Mcl17, Mcl22, Mcl23, Mcl24, GPP (0.5 mM), DMAPP (0.5 mM), malonyl-CoA (2.5 mM), H_2O_2 (1 mM), Na_3VO_4 (2 μM), $MgCl_2$ (5 mM), and NaCl (150 mM) unless otherwise stated. Enzymatic formation of **1** and **2** (I). Assay lacking H_2O_2 (required for Mcl24 activity; II). Assay lacking Mg^{2+} (required for Mcl23 activity) and H_2O_2 (III). Control assay conducted with heat-inactivated enzymes (IV). Note that malonyl CoA, CoA (elute at < 2 min), and isoprene diphosphates (do not absorb at 254 nm) are not shown. Flaviolin arises from (non-enzymatic) oxidation of **3**. Similarly, auto-oxidation of **4** was observed (oxidation products elute between 13.5–15.2 min as indicated by the asterisk, see also Figure S7).



Scheme 3. Chemical synthesis of pre-merochlorin (**4**). a) $CuBr{\cdot}SMe_2$ (1.05 equiv), MeLi (2.0 equiv), geranyl bromide (2.0 equiv), THF, −78 °C to 0 °C (91%); b) DIBAL-H (3.0 equiv), Et_2O, −78 °C (75%); c) ethyl chloroformate (1.0 equiv), pyridine (2.0 equiv), Et_2O, 0 °C to RT, (81%); d) THN (**3**) (1.5 equiv), $[Pd(PPh_3)_4]$ (5 mol%), Et_3B (1.5 equiv), THF, 50 °C (44%). DIBAL-H = diisobutylaluminum hydride, THF = tetrahydrofuran.

α,β-unsaturated ester **7** in 91% yield. DIBAL reduction and subsequent reaction of the resulting allylic alcohol with ethylchloroformate produced the carbonate **9** in 67% overall yield. Finally, **9** was coupled to **3**[5] through palladium-catalyzed allylation in the presence of BEt_3,[15] thus completing the four-step synthesis of highly oxygen-sensitive pre-merochlorin (**4**). As expected, the enzymatically and chemically formed **4** were virtually identical based on RP-HPLC retention times, HRMS data, and characteristic UV-Vis spectra (Figure 2, and see Figure S8), thus confirming the Mcl23-mediated regioselective prenylation of **3** (Scheme 2). To the best of our knowledge, Mcl23 represents the first reported aromatic prenyltransferase which utilizes a branched terpene as a prenyl donor. Notably, Mcl23 also attached **5** to flaviolin, albeit slowly (< 1% relative activity), whereas strict substrate specificity was observed for **5**. Accordingly, no **3** prenylation was detected in the presence of IPP, DMAPP, GPP, FPP, or GGPP (see Figures S9 and S10).

Significantly, addition of synthetic **4** to the Δ*mcl23* mutant restored merochlorin production, thereby verifying its in vivo intermediacy as a shared substrate for **1** and **2** (see Figure S11). This observation raises interesting questions about how subsequent chlorination and cyclization reactions lead to the unique ring systems of the merochlorins. We previously speculated that the predicted vanadium-dependent haloperoxidase Mcl24 may be involved in the halogenation and cyclization reactions.[1a] This hypothesis was further supported by the loss of merochlorin production in a Δ*mcl24* mutant (see Figure S3). To our surprise, addition of heterologously produced Mcl24 proved sufficient for the conversion of **4** into both **1** and **2** at a rate of (17 ± 1) mU mg^{-1} (k_{cat} of 1.0 min^{-1}; Figure 2). Concurrent to this work, we report in detail how Mcl24 accomplishes this unprecedented sequence of oxidative dearomatization/terpene cyclization reactions which inspired the development of a new chemical chlorination protocol (see Ref. [16]).

In summary, we unraveled the biosynthesis of the structurally complex merochlorin A and B, thus allowing for the first time, the total enzymatic synthesis of meroterpenoid natural products. Astoundingly, unlike many other reconsti-

tuted secondary metabolic pathways,[17] merochlorin A and B biosynthesis merely involves four dedicated enzymes and the common metabolites DMAPP, GPP, and malonyl CoA. This pathway represents a new example of how structural diversity and complexity are generated in nature with a limited set of catalysts.[18]

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