Published in final edited form as:

Angew Chem Int Ed Engl. 2014 October 6; 53(41): 11019–11022. doi:10.1002/anie.201405694.

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

Dr. Robin Teufel,

Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography,
University of California San Diego, La Jolla, CA 92037, USA

Dr. Leonard Kaysser,

Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography,
University of California San Diego, La Jolla, CA 92037, USA

Matthew T. Villaume,

Department of Chemistry, The Scripps Research Institute, La Jolla, CA 92037, USA

Dr. Stefan Diethelm,

Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography,
University of California San Diego, La Jolla, CA 92037, USA

Dr. Mary K. Carbullido,

Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography,
University of California San Diego, La Jolla, CA 92037, USA

Prof. Dr. Phil S. Baran, and

Department of Chemistry, The Scripps Research Institute, La Jolla, CA 92037, USA

Prof. Dr. Bradley S. Moore

Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography,
University of California San Diego, La Jolla, CA 92037, USA, Homepage: <http://scrippsscholars.ucsd.edu/bsmoore>. Skaggs School of Pharmacy and Pharmaceutical Sciences
University of California San Diego, La Jolla, CA 92093, USA

Bradley S. Moore: bsmoore@ucsd.edu

Abstract

The polycyclic merochlorin A and B are complex halogenated meroterpenoid natural products with significant antibacterial activities that are produced by the marine bacterium *Streptomyces* sp. strain CNH-189. Here we employ heterologously produced enzymes and chemical synthesis 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 that features an

**We thank Peter Bernhardt, Brendan Duggan and Stefanie Maerten for technical assistance. This research was supported by the US National Institute of Health (NIH, Grant No. R01-AI047818) and by postdoctoral fellowships to R.T. from the Deutsche Forschungsgemeinschaft (TE 931/1-1), to L.K. from the Alexander von Humboldt Foundation, and to S.D. from the Swiss National Science Foundation (SNF).

© Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Correspondence to: Bradley S. Moore, bsmoore@ucsd.edu.

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201xxxxxx>

unprecedented branched sesquiterpene moiety. 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.

Keywords

biosynthesis; enzyme catalysis; natural products; polyketides; terpenoids

Merochlorin 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 *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 pathway enzymes (see SI Figure 1) combined with chemical synthesis.

Due to 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-tetrahydroxynaphthalene (THN, **3**),^[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 that is subsequently cyclized via intramolecular Claisen and aldol condensations to **3**.^[4] Indeed, a single product was formed upon incubation of Mcl17 with malonyl-CoA, as evidenced by reversed phase (RP) HPLC. Retention time, UV-Vis spectra, and high-resolution mass spectrometry (HRMS) data fully matched the chemically synthesized **3**,^[5] thus confirming the type III PKS activity (SI Figure 2). As determined spectrophotometrically, Mcl17 catalyzed **3** formation with 2.4 ± 0.3 mU/mg (k_{cat} of 0.47 min^{-1} per malonyl-CoA). Under aerobic conditions, slow, non-enzymatic conversion of **3** to the known auto-oxidation product 2,5,7-trihydroxy-1,4-naphthoquinone (flaviolin)^[6] occurred (SI Figure 2).

In contrast to **3** formation, the biosynthesis of the isoprenic merochlorin precursor appeared more obscure. It can be envisaged, however, that it derives from an unusual 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) via conventional head-to-tail ($\text{C}(1)'$ – $\text{C}(4)$) 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, e.g., 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 C₁₀) isolated, e.g., from the eponymous lavender oil, exhibit a similar C(1')–C(2) branching as 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 (C₁₀, GPP) with DMAPP. We speculated that Mcl22 may be involved in this branching reaction due to low sequence homology to known *cis*-prenyl diphosphate synthases.^[9] Thus far, only two enzymes have been described that naturally catalyze isoprene branching, both of which are homologous to *cis*-prenyl diphosphate synthases and convert two molecules of DMAPP to lavandulyl diphosphate^[8b] (see Scheme 2 for structure) or cyclolavandulyl diphosphate,^[10] respectively.

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 (SI Figure 3). 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 MgCl₂ to the assay boosted the enzymatic activity nearly four-fold to 850 ± 20 mU/mg (*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 Mg²⁺-dependency of prenyl diphosphate synthases. The *K*_m values of Mcl22 for DMAPP and GPP were determined as 65 ± 16 and 13 ± 5 μM, respectively. Extensive NMR analyses, HRMS, and chemical synthesis confirmed the structure of **5** (see SI and Figure 1), for which we propose the name isosesquilavandulyl diphosphate. Notably, Mcl22 exhibited interesting side activities (relative activities <1% for all reactions), allowing the *in vitro* condensation of DMAPP, farnesyl diphosphate (FPP; C₁₅), or geranylgeranyl diphosphate (GGPP; C₂₀) with one molecule of DMAPP. HRMS data and retention times were consistent with the formation of the corresponding branched C₁₀, C₂₀, and C₂₅ prenyl diphosphates. Moreover, IPP was slowly condensed with GPP (see SI Figure 4).

To further investigate the Mcl22 catalytic mechanism, we used various deuterated DMAPP analogues (see ref^[12] and SI for synthesis) and GPP as substrates in enzymatic assays. Deuterium incorporation into product **5** was analyzed by HRMS. The exchange of D for H in **5** was exclusively observed when C(2)-deuterated DMAPP was proffered (see SI Figure 5). We thus propose initial C–C bond formation through nucleophilic attack of C(2) in DMAPP displacing the diphosphate leaving group at C(1) of the electrophilic primer GPP. Subsequent elimination of the C(2) 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 C(3) of DMAPP, or, alternatively, could be initiated at the same position via attack of a nucleophilic amino acid residue (e.g., a cysteine thiolate) (routes **A** and **B** in Scheme 2, respectively).

Next, we addressed the question 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 via electrophilic aromatic substitution reactions.^[13] As expected, **1** and **2** were completely absent in culture extracts of a *mcl23* mutant strain (SI Figure 3). 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 (SI Figure 6).^[14] Indeed, heterologously produced Mcl23 transformed **3** and **5** with 53 ± 3 mU/mg (k_{cat} of 1.8 min^{-1}) to a single oxygen-sensitive product in a strictly Mg^{2+} -dependent reaction (Figure 2, SI Figures 7&8).

As we were not able to enzymatically synthesize sufficient amounts for NMR analyses due to 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 α,β -unsaturated ester **7** in 91% yield. DIBAL reduction and subsequent reaction of the resulting allylic alcohol with ethylchloroformate produced carbonate **9** in 67% overall yield. Finally, carbonate **9** was coupled to **3**^[15] 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, SI Figure 8), thus confirming the Mcl23-mediated regio-selective prenylation of **3** (Scheme 2). To the best of our knowledge, Mcl23 represents the first reported aromatic prenyltransferase that utilizes a branched terpene as 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 presence of IPP, DMAPP, GPP, FPP, or GGPP (see SI Figures 9&10).

Significantly, addition of synthetic **4** to the *mcl23* mutant restored merochlorin production, thereby verifying its *in vivo* intermediacy as a shared substrate for **1** and **2** (SI Figure 11). 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 *mcl24* mutant (SI Figure 3). 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 (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 that 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, allowing for the first time the total enzymatic synthesis of meroterpenoid natural products. Astoundingly, unlike many other reconstituted 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]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. a) Kaysser L, Bernhardt P, Nam SJ, Loesgen S, Ruby JG, Skewes-Cox P, Jensen PR, Fenical W, Moore BS. *J Am Chem Soc.* 2012; 134:11988–11991. [PubMed: 22784372] b) Sakoulas G, Nam SJ, Loesgen S, Fenical W, Jensen PR, Nizet V, Hensler M. *PLoS One.* 2012; 7:e29439. [PubMed: 22279537]
2. a) Pepper HP, George JH. *Angew Chem Int Ed.* 2013; 52:12170–12173. b) Meier R, Strych S, Trauner D. *Org Lett.* 2014; 16:2634–2637. [PubMed: 24804897]
3. a) Ueda K, Kim KM, Beppu T, Horinouchi S. *J Antibiot.* 1995; 48:638–646. [PubMed: 7649862] b) Feng B, Wang X, Hauser M, Kaufmann S, Jentsch S, Haase G, Becker JM, Szaniszló PJ. *Infect Immun.* 2001; 69:1781–1794. [PubMed: 11179356] c) Kalaitzis A, Hamano Y, Nilsen G, Moore BS. *Org Lett.* 2003; 5:4449–4452. [PubMed: 14602022] d) Winter JM, Moffitt MC, Zazopoulos E, McAlpine JB, Dorrestein PC, Moore BS. *J Biol Chem.* 2007; 282:16362–16368. [PubMed: 17392281]
4. a) Funa N, Ohnishi Y, Fujii I, Shibuya M, Ebizuka Y, Horinouchi S. *Nature.* 1999; 400:897–899. [PubMed: 10476972] b) Austin B, Izumikawa M, Bowman ME, Udway DW, Ferrer JL, Moore BS, Noel JP. *J Biol Chem.* 2004; 279:45162–45174. [PubMed: 15265863]
5. 1,3,6,8-Tetrahydroxynaphthalene (3) was prepared in one step starting from chromotropic acid sodium salt according to a literature procedure: Pittelkow M, Boas U, Christensen JB. *Org Lett.* 2006; 8:5817–5820. [PubMed: 17134280]
6. Izumikawa M, Shipley PR, Hopke JN, O'Hare T, Xiang L, Noel JP, Moore BS. *J Ind Microbiol Biotechnol.* 2003; 30:510–515. [PubMed: 12905073]
7. Thulasiram HV, Erickson HK, Poulter CD. *J Am Chem Soc.* 2008; 130:1966–1971. [PubMed: 18198872]
8. a) Rivera B, Swedlund BD, King GJ, Bell RN, Hussey CE Jr, Shattuck-Eidens DM, Wrobel WM, Peiser GD, Poulter CD. *Proc Natl Acad Sci U S A.* 2001; 98:4373–4378. [PubMed: 11287653] b) Demissie A, Erland LA, Rheault MR, Mahmoud SS. *J Biol Chem.* 2013; 288:6333–6341. [PubMed: 23306202]
9. Takahashi S, Koyama T. *Chem Rec.* 2006; 6:194–205. [PubMed: 16900467]
10. Ozaki T, Zhao P, Shinada T, Nishiyama M, Kuzuyama T. *J Am Chem Soc.* 2014; 136:4837–4840. [PubMed: 24640943]
11. Zhang D, Poulter CD. *Anal Biochem.* 1993; 213:356–361. [PubMed: 8238912]
12. Thulasiram HV, Phan RM, Rivera SB, Poulter CD. *J Org Chem.* 2006; 71:1739–1741. [PubMed: 16468838]
13. a) Tello M, Kuzuyama T, Heide L, Noel JP, Richard SB. *Cell Mol Life Sci.* 2008; 65:1459–1463. [PubMed: 18322648] b) Heide L. *Curr Opin Chem Biol.* 2009; 13:171–179. [PubMed: 19299193] c) Saleh O, Haagen Y, Seeger K, Heide L. *Phytochemistry.* 2009; 70:1728–1738. [PubMed: 19559450]
14. Kuzuyama T, Noel JP, Richard SB. *Nature.* 2005; 435:983–987. [PubMed: 15959519]
15. Kimura M, Fukasaka M, Tamaru Y. *Synthesis.* 2006; 21:3611–3616.
16. Diethelm S, Teufel R, Kaysser L, Moore BS. *Angew Chem Int Ed.* 2014
17. a) Cheng Q, Xiang L, Izumikawa M, Meluzzi D, Moore BS. *Nat Chem Biol.* 2007; 3:557–558. [PubMed: 17704772] b) Sattely S, Fischbach MA, Walsh CT. *Nat Prod Rep.* 2008; 25:757–793. [PubMed: 18663394] c) Sun YH, Hahn F, Demydchuk Y, Chettle J, Tosin M, Osada H, Leadlay PF. *Nat Chem Biol.* 2010; 6:99–101. [PubMed: 20081823] d) Roberts A, Ryan KS, Moore BS,

- Gulder TA. *Top Curr Chem*. 2010; 297:149–203. [PubMed: 21495259] e) Pahari P, Kharel MK, Shepherd MD, van Lanen SG, Rohr J. *Angew Chem Int Ed*. 2012; 51:1216–1220.
18. a) Haynes W, Gao X, Tang Y, Walsh CT. *ACS Chem Biol*. 2013; 8:741–748. [PubMed: 23330675] b) Walsh CT, Haynes SW, Ames BD, Gao X, Tang Y. *ACS Chem Biol*. 2013; 8:1366–1382. [PubMed: 23659680]

Figure 1.

(A) Reversed phase HPLC analysis of the Mcl22 catalyzed reaction (UV at 214 nm). (I) Mixture of C₁₀–C₂₀ diphosphate standards containing 1 nmol of each GPP, FPP, GGPP, and chemically synthesized isosesquilavandulyl diphosphate **5**. (II) Mcl22 enzymatic assay showing the complete conversion of GPP and DMAPP to **5**. (III) Control assay with heat-inactivated Mcl22. (IV) Control assay with intact Mcl22 in presence of 10 mM EDTA. 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 MgCl₂. Both assays contained 0.62 μM Mcl22 and 2 mM of each GPP and DMAPP. Products were quantified by HPLC from three independent samples for each point of time. Error bars indicate the standard error.

Figure 2.

RP-HPLC analysis of the fully *in vitro* reconstituted merochlorin A and B biosynthetic pathways (UV at 254 nm). Assays contained Mcl17, Mcl22, Mcl23, Mcl24, GPP (0.5 mM), DMAPP (0.5 mM), malonyl-CoA (2.5 mM), H₂O₂ (1 mM), Na₃VO₄ (2 μM), MgCl₂ (5 mM), and NaCl (150 mM) unless otherwise stated. **(I)** Enzymatic formation of **1** and **2**. **(II)** Assay lacking H₂O₂ (required for Mcl24 activity). **(III)** Assay lacking Mg²⁺ (required for Mcl23 activity) and H₂O₂. **(IV)** Control assay conducted with heat-inactivated enzymes. 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 pre-merochlorin **4** was observed (oxidation products elute between 13.5 – 15.2 min as indicated by the asterisk, see also SI Figure 7).

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 in Diethelm *et al.*^[16] DMAPP = dimethylallyl diphosphate, GPP = geranyl diphosphate.

Scheme 2.
Proposed catalytic mechanisms of Mcl22 and Mcl23.

Scheme 3.

Chemical synthesis of pre-merochlorin **4**. a) CuBr•SMe₂ (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), Et₂O, −78 °C (75%); c) ethyl chloroformate (1.0 equiv), pyridine (2.0 equiv), Et₂O, 0 °C to rt, (81%); d) THN (**3**) (1.5 equiv), Pd(PPh₃)₄ (5 mol%), Et₃B (1.5 equiv), THF, 50 °C (44%).