Natural Product Synthesis

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A Multitasking Vanadium-Dependent Chloroperoxidase as an Inspiration for the Chemical Synthesis of the Merochlorins**

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Abstract: *The vanadium-dependent chloroperoxidase Mcl24 was discovered to mediate a complex series of unprecedented transformations in the biosynthesis of the merochlorin meroterpenoid antibiotics. In particular, a site-selective naphthol chlorination is followed by an oxidative dearomatization/terpene cyclization sequence to build up the stereochemically complex carbon framework of the merochlorins in one step. Inspired by the enzyme reactivity, a chemical chlorination protocol paralleling the biocatalytic process was developed. These chemical studies led to the identification of previously overlooked merochlorin natural products.*

In the past decades, marine microorganisms have emerged as valuable sources for structurally diverse secondary metabolites.[1] In this context, the isolation and characterization of natural products from marine bacteria incorporating unusual carbon skeletons has been of particular interest. Merochlorins A (**1**) and B (**2**; see Scheme 1) were recently isolated from marine *Streptomyces* sp. CNH-189, thus constituting a new class of structurally unprecedented meroterpenoid secondary metabolites.[2,3] The merochlorins are composed of a tetrahydroxynaphthalene fragment decorated with a rare terpenoid appendage. Furthermore, chlorination at C11/C12 adds to the structural complexity of these compounds. These unusual structural features along with the antimicrobial activity of the merochlorins attracted the interest of the synthetic community, thus culminating in elegant total syntheses of both merochlorins A and B.[4] To shed light on the biosynthetic origin of these natural products, our group recently identified the gene cluster associated with the production of the merochlorins.[3] The genomic data suggested a biosynthetic pathway starting with the prenylation of 1,3,6,8-tetrahydroxynaphthalene (THN; **3**) to produce the triene **4** (pre-merochlorin) as a key intermediate (Scheme 1). In the preceding publication, we reported on the investigation of these early steps in the merochlorin biosynthesis, thus leading to the identification of a highly unusual pathway for the synthesis and transfer of the isosesquilavandulyl terpene unit **5** onto THN (**3**).[5]

We herein report the biochemical characterization of Mcl24, a multitasking vanadium-dependent chloroperoxidase involved in the late stage of merochlorin biosynthesis (Scheme 1). The enzyme was found to mediate a complex series of transformations, including a site-selective naphthol chlorination, followed by a sequence of oxidative dearomatization/terpene cyclization. Furthermore, the enzyme's reactivity served as a blueprint for the development of a parallel chemical chlorination protocol, thereby enabling the oxidative dearomatization/cyclization of the enzyme substrate and leading to the production of various merochlorin analogues. Besides the identification of previously overlooked natural merochlorin isomers, the chemical transformation suggests a mechanistic rational for the enzymatic reaction cascade. Most importantly, the applied concept showcases the yet largely unexplored potential of unusual biosynthesis pathways to serve as an inspiration for the development of novel synthetic reagent systems based on biosynthetic logic.[6]

The identification of the prenylated naphthalene derivative **4** as a key intermediate towards merochlorins A and B[5] suggested an oxidative cyclization event to account for the formation of the polycyclic core structure of both natural products. Surprisingly, although numerous secondary metabolites have been suggested to arise from oxidative cyclization involving an aromatic core, no enzymatic system capable of

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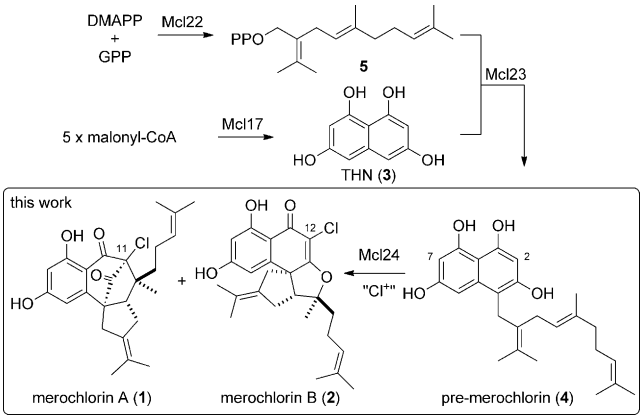


Scheme 1. Biosynthetic pathway towards merochlorins A (**1**) and B (**2**). DMAPP = dimethylallyl diphosphate, GPP = geranyl diphosphate.

mediating such a transformation has been characterized to date.[7] In the merochlorin gene cluster we identified a number of oxidative enzymes, which were possible candidates to promote such a reaction, including an iron-sulfur cluster containing protein.[3] Interestingly though, deletion of the gene *mcl30*, annotated to code for this protein, did not affect merochlorin production in the mutant strain. Moreover, the gene cluster contained the vanadium-dependent haloperoxidase (VHPO) Mcl24, which we suspected was likely to be responsible for introduction of the chlorine substituent. This enzyme particularly attracted our interest, as VHPO's represent a class of biocatalysts still poorly characterized in the context of complex molecule biosynthesis.[8,9] At the outset of our studies directed towards investigation of the final steps in the merochlorin biosynthesis, the nature of the oxidative cyclization event and the exact role of the haloperoxidase were largely unclear.

We commenced our efforts with the heterologous expression of octahistidyl-tagged Mcl24 in *Escherichia coli* to address the proposed C2 chlorination of **4**.[5] Accordingly, purified Mcl24 was subjected to a standard VHPO enzyme assay (pH 6.0 MES buffer, 50 mM KCl, 1 mM H_2O_2, 100 μM Na_3VO_4) using chemically synthesized **4**[10] as the putative enzyme substrate. To our surprise, analysis of the Mcl24 reaction suggested the direct formation of the natural products **1** and **2** by the chloroperoxidase enzyme, as indicated by reverse-phase HPLC retention time as well as UV and MS spectral data of the product peaks (Figure 1 A). Isolation of the major reaction components and ^{1}H NMR analysis confirmed the identity of the products formed, by comparison with spectral data of the isolated natural products.[3] The unexpected finding that Mcl24 is able to catalyze

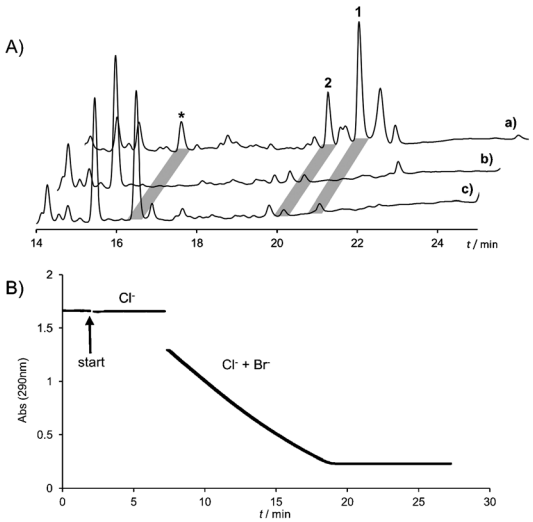


Figure 1. Reactivity of Mcl24. A) Reverse-phase HPLC chromatogram (UV at $\lambda = 254$ nm) of the reaction of Mcl24 with **4**: Mcl24 (50 μg mL^{-1}), KCl (50 mM), Na_3VO_4 (100 μM), H_2O_2 (1 mM), **4** (100 μmol), pH 6.0 MES buffer (50 mM); (a). * = non-enzymatic oxidized degradation product. Negative control: no substrate **4** (b). Negative control: no enzyme (c). B) Spectrophotometric MCD assay for Mcl24. The reaction was initiated by addition of H_2O_2. As no decrease in absorbance was observed in the presence of chloride, KBr was added to the mixture after 7 min.

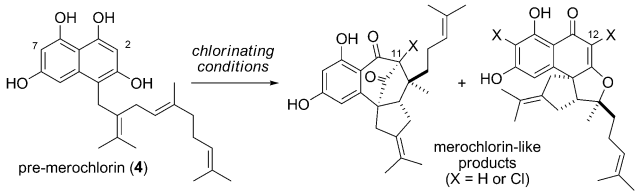
multiple transformations, including a site-selective chlorination at C2 along with a highly unusual oxidative cyclization event, raised questions about the exact functional role of the enzyme in this reaction cascade. To gain further insight, we next tested various substrate analogues closely resembling the physiological substrate **4**, as well as the simple terpene alcohols geraniol and nerolidol. Surprisingly, none of these alternative substrates were accepted by the enzyme (see the Supporting Information for details). We further subjected Mcl24 to a monochlorodimedone (MCD) assay, a standard protocol to assess the activity of halonium-generating halogenating enzymes.[11] As indicated in Figure 1 B, we found that Mcl24 is not able to oxidize MCD under standard assay conditions in the presence of chloride only.

However, when KBr was added to the reaction mixture, rapid disappearance of the MCD signal was observed. We recently reported a similar observation with the vanadium-dependent chloroperoxidase NapH1, which is involved in a highly selective chloroether formation in the biosynthesis of the napyradiomycins.[9c] As such, these results raise questions about the feasibility of the MCD assay to properly reflect the natural role of halogenating enzymes.

On the basis of these initial observations, we hypothesized that an active chloronium species could trigger an oxidative dearomatization of the highly electron-rich naphthol moiety in **4**. The thus generated reactive intermediate would then initiate a terpene cyclization to arrive at the polycyclic framework of the merochlorins. To gain further insight into the details of such an enzyme-catalyzed oxidative naphthol dearomatization/cyclization cascade, we opted for the development of a parallel chemical protocol inducing oxidative cyclization of **4** through a chlorination event. Although oxidative dearomatization/cyclization cascades initiated by a halogenating species are, to our knowledge, unprecedented in the chemical literature,[12] we were curious, if chemical chlorination conditions could be identified that would enable generating cyclized products starting with **4**. Accordingly, various chlorination conditions were evaluated towards this end as outlined in Table 1 (see the Supporting Information for further details). In particular, we sought protocols mediating selective *ortho*-chlorination of phenol and naphthol derivatives, thus paralleling the chlorination reactivity of Mcl24.[13] 2,3,4,5,6,6-Hexachloro-2,4-cyclohexadien-1-one has been reported to effect such transformations in the presence of DMF as a cosolvent. Subjecting **4** to these reaction conditions led to complete decomposition of the starting material (entry 1).[14] Interestingly though, addition of 5 equivalents of triethylamine to this reaction effected formation of trace quantities of a set of products with very similar retention times on reverse-phase HPLC as well as identical UV and MS spectra to that of the enzymatic products **1** and **2** (entry 2).

Following this initial result, we set out to further study the oxidative cyclization of **4**. A number of standard chlorinating reagents failed to give any of the previously observed products (Table 1, entries 3–6). Interestingly, the use of hypochlorous acid, the generally postulated reactive species of chloroperoxidases, only led to decomposition of **4** (entry 7).[15] At this point we began to suspect that the amine additive in the previously successful reaction condi-

Table 1: Chemical chlorination of **4**.



Entry[a]	Chlorinating agent	Additive	Solvent	Yield [%][b]
1	HCDO	DMF (100 equiv)	CCl_4	–
2	HCDO	NEt_3 (5 equiv)	CCl_4	< 5
3	NCS	–	CH_2Cl_2	–
4	TCCA	–	CH_2Cl_2	–
5	*t*BuOCl	–	CH_2Cl_2	–
6	$SOCl_2$	*i*Pr_2NH (2 equiv)	toluene	–
7	HOCl	–	acetone	–
8[c]	NCS	*i*Pr_2NH (2 equiv)	CH_2Cl_2	30
9	*t*$BuNCl_2$	–	CCl_4	ca. 5
10[d,e]	NCS	NaH (2 equiv)	CH_2Cl_2	22

[a] Reaction conditions: substrate (5 mg, 12.6 μmol), chlorinating agent (2.0 equiv), additive, solvent (1 mL), 25 °C, 2 h. [b] Combined yield of product mixture after silica gel purification. [c] At 0 °C. [d] At −78 °C. [e] Only the nonchlorinated isomers **6** and **8** produced. DMF = *N*,*N*-dimethylformamide, HCDO = 2,3,4,5,6,6-hexachloro-2,4-cyclohexadien-1-one, NCS = *N*-chlorosuccinimide, TCCA = trichloroisocyanuric acid.

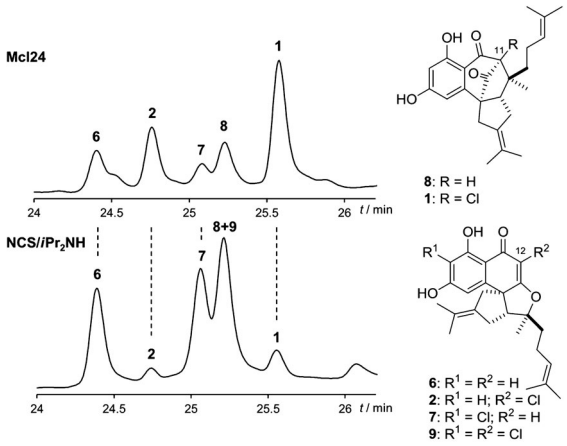


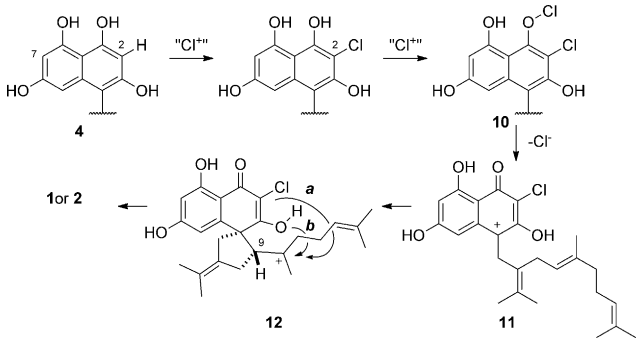
Figure 2. HR-LCMS comparison (UV at $\lambda = 254$ nm shown) of the Mcl24 reaction (top) with the NCS/*i*Pr_2NH reaction (bottom) of **4**.

tions might play a key role in the reaction, possibly leading to the formation of a reactive chloramine intermediate.[16] Accordingly, **4** was subjected to *N*-chlorosuccinimide in the presence of 2 equivalents of *i*Pr_2NH.[17] To our delight, formation of the previously observed product mixture ensued in a total yield of 30% (entry 8). Moreover, the use of independently prepared *N*,*N*-dichloro-*tert*-butylamine produced the cyclized products, albeit in lower yield (entry 9).[18] Finally, the use of stronger bases was tested. Addition of sodium hydride led equally to the formation of the characteristic product pattern in 22% yield when conducting the reaction at −78 °C (entry 10).

With an efficient protocol for chemical oxidative cyclization of **4** in hand, we turned to a closer investigation of the individual components observed in the product mixture. As shown in Figure 2, HR-LCMS comparison of the enzymatic Mcl24 reaction with the chemical oxidation of **4** using NCS/*i*Pr_2NH indicated a markedly different product distribution pattern. Upon preparative HPLC purification of both the enzymatic and the chemical reaction mixtures, the individual products were characterized by detailed two-dimensional NMR and MS analysis. As depicted in Figure 2, a full spectrum of differently chlorinated merochlorin A and B analogues were identified. The NCS/*i*Pr_2NH reaction thereby predominantly produced deschloro-merochlorin B (**6**), isochloro-merochlorin B (**7**), deschloro-merochlorin A (**8**), and minor amounts of dichloro-merochlorin B (**9**; coelutes with **8**). In contrast, the enzymatic reaction preferentially provides the previously described merochlorins A (**1**) and B (**2**), along with only minor amounts of isomers **6**, **7**, and **8**. Reinvestigation of the natural merochlorin producer strain *Streptomyces* sp. CNH-189 revealed that all of the observed merochlorin isomers were in fact produced by this organism, albeit in only minor amounts (see the Supporting Information for details).

The inverted selectivity profiles of the chemical and the enzymatic oxidative cyclization of **4** suggested implications on the reaction path Mcl24 follows. Remarkably, the enzyme seems to control both, the site-selectivity of halogenation as well as the timing of the chlorination reaction in respect to the oxidative cyclization step. In contrast to the NCS/*i*Pr_2NH reaction, Mcl24 preferentially chlorinates at C2 in **4** prior to cyclization. On the other hand, treatment of **4** with NCS/*i*Pr_2NH leads to an initial oxidative cyclization followed by optional chlorination at C7. In fact, conducting the chemical chlorination at low temperature (−78 °C) prevents this chlorination step, thus leading to the exclusive production of the isomers **6** and **8** (Table 1, entry 10 and the Supporting Information). The origin of the distinct reactivity profile of the enzymatic reaction remains unclear at this point.

The studies undertaken towards chemical oxidative cyclization of **4** led us to propose a mechanistic rationale for merochlorin production. As mentioned before, we surmise that an in situ generated chloramine intermediate is the active oxidant for the chemical transformation. Interestingly, a ε-lysine chloramine intermediate is postulated for the rebeccamyin flavin-dependent chlorinase RebH.[19] The chlorinated amino acid side chain was proposed to transfer its halogen atom in a highly selective manner to the enzyme substrate. It seems attractive to invoke a similar intermediate with a chlorinated amino acid side chain to account for the unusual selectivity pattern observed for Mcl24. We further hypothesize that the active chloronium species initiates the enzymatic reaction by selective C2 chlorination of **4** (Scheme 2).[20] A second chlorination step would then likely produce the aromatic hypochlorite species **10**, and upon loss of chloride would give the benzylic carbocation **11**.[21] The proposed formation of **10** is also consistent with the observation that a NaH/NCS reagent combination can promote cyclization of **4** (Table 1, entry 10). Finally, a cation-induced terpene cyclization would explain formation of the merochlorins via the intermediate **12**.



Scheme 2. Proposed mechanism for the oxidative dearomatization/cyclization towards merochlorin A (path a) and B (path b).

In conclusion, the characterization of the vanadium-dependent chloroperoxidase Mcl24 led to the discovery of an unprecedented enzymatic reaction cascade. Mcl24 was found to catalyze a site-selective naphthol chlorination prior to the initiation of an oxidative dearomatization event which further triggers a terpene cylization. We were able to shed light on the characteristics of the enzymatic reaction by the development of a synthetic chlorination protocol mimicking Mcl24 reactivity. In addition, this novel reagent system enabled the total synthesis of previously unnoticed merochlorin natural products in only five steps. The applied concept stresses the potential of unusual biosynthetic transformations to serve as an inspiration for the development of synthetic reagent systems.

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