Biosynthesis

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A Monooxygenase from *Boreostereum vibrans* Catalyzes Oxidative Decarboxylation in a Divergent Vibralactone Biosynthesis Pathway

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Abstract: *The oxidative decarboxylation of prenyl 4-hydroxybenzoate to prenylhydroquinone has been frequently proposed for the biosynthesis of prenylated (hydro)quinone derivates (sometimes meroterpenoids), yet no corresponding genes or enzymes have so far been reported. A FAD-binding monooxygenase (VibMO1) was identified that converts prenyl 4-hydroxybenzoate into prenylhydroquinone and is likely involved in the biosynthesis of vibralactones and other meroterpenoids in the basidiomycete Boreostereum vibrans. Feeding of 3-allyl-4-hydroxybenzylalcohol, an analogue of the vibralactone pathway intermediate 3-prenyl-4-hydroxybenzylalcohol, generated 20 analogues with different scaffolds. This demonstrated divergent pathways to skeletally distinct compounds initiating from a single precursor, thus providing the first insight into a novel biosynthetic pathway for 3-substituted γ-butyrolactones from a shikimate origin.*

Natural products with architecturally distinct scaffolds remain attractive for the discovery of potential drugs and biological probes.[1] To collect new compounds with privileged skeletons, however, is becoming more challenging. Through various combinations of chemical and biosynthetic approaches,[2] large numbers of structurally interesting and biologically active natural product analogues have been generated to focus on a common scaffold in each case.[3] On the other side, biosynthetic machineries usually accommodate divergent pathways to produce a dazzling array of architecturally distinct skeletons from only a handful of basic building blocks. For example, terpenoids, which comprise the largest group of natural products, can ultimately be made up from a C_5 isoprene unit. Terpene biosynthesis thus provides a prominent example of nature's strategy for combinatorial synthesis and diversity.[4] This strategy has inspired the chemical synthesis of compounds with unprecedented numbers of scaffold variations.[5] Further insight into how structural diversity is expanded from relatively few fundamental blocks is therefore necessary to promote the development of creative approaches for the skeletal diversification of compounds. With this aim in mind, our attention was captured by the basidiomycete fungus *Boreostereum vibrans* (*syn. Stereum vibrans*), since more than thirty metabolites with eight distinct scaffolds (see Section S1 in the Supporting Information) were identified in our ongoing chemistry research on *B. vibrans*. These compounds share common C_5 prenyl marks, thus suggesting that they may also share an early intermediate. Based on our recent elucidation of the biosynthetic pathway for vibralactone (**10**),[6] we speculate that 3-prenyl-4-hydroxybenzylalcohol (**1**) may lead to different vibralactones via a divergent biosynthetic pathway, and an enzyme catalyzing oxidative decarboxylation of 3-prenyl 4-hydroxybenzoate (**3**) to prenylhydroquinone (**4**) can be expected (Figure 1). Conversions similar to **3**→**4** have been proposed to occur in the biosynthesis of shikonin[7] from the medicinal plant *Lithospermum erythrorhizon* and meroterpenoids from the basidiomycete fungi,[8] yet no corresponding genes or enzymes have so far been reported. Still unknown are the enzymes responsible for the successive decarboxylation and hydroxylation of 5-methoxy-4-hydroxy-3-hexaprenyl benzoate, an intermediate in yeast coenzyme Q biosynthesis.[9] Given that the enzyme for this conversion has been overlooked, and since only a few of the biosynthetic genes and enzymes in basidiomycetes have been functionally characterized,[10] we set out to identify the relevant enzyme first.

We began with MNX1, a fungal NAD(P)H-dependent flavoenzyme identified in the pathogenic yeast *Candida parapsilosis* to specifically convert 4-hydroxybenzoate (4-HBA) into 1,4-dihydroxybenzene (hydroquinone) and probably involved in lignin degradation.[11] By using MNX1 as a probe, a homologue with 42% identity, designated *VibMO1*, was found in our *B. vibrans* genome draft assembly to encode a putative FAD-binding monooxygenase and aromatic-ring hydroxylase (see Section S2.1 in the Supporting Information). Furthermore, the formation of **4a** (an analogue of **4**) from **3a** (an analogue of **3**) was evident in the fungal cell-free system (see Section S2.2), thus indicating that the relevant enzyme is rather active. In an attempt to identify VibMO1, gene constructs were subcloned into a pET expression vector to achieve higher expression levels in *E. coli*. When the *E. coli* cell lysate as crude recombinant enzyme was incubated with **3**, **3a**, or 4-HBA, the production of **4**, **4a**, or hydroquinone was observed, respectively. The purified recombinant VibMO1 was further confirmed to be active for the above substrates; the heat-denatured enzyme control yielded no detectable

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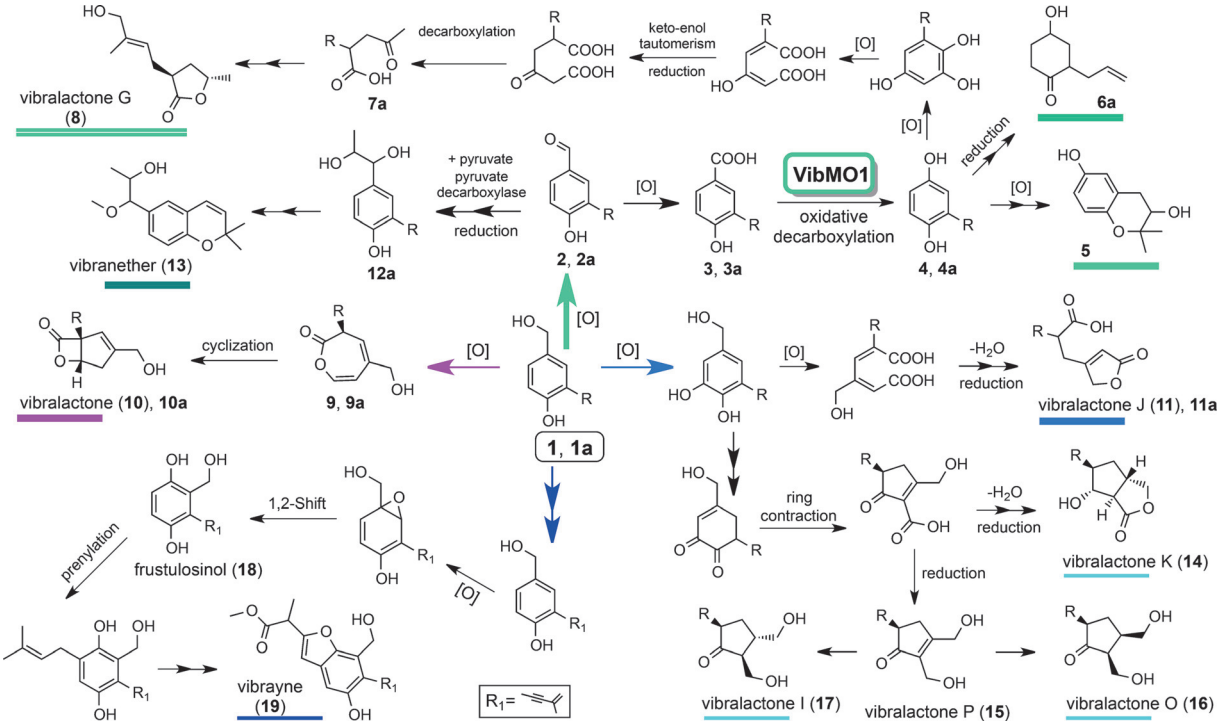

Figure 1. Proposed divergent pathways for vibralactones and other meroterpenoids in normal and **1a** feeding broths of *B. vibrans*. R = allyl for analogues with compound designations containing **a**; R = prenyl for natural products. All numbered compounds were purified from the cultures, apart from **1**–**4** and **1a**, which were synthesized and thoroughly characterized by NMR (see Section S4).

products (Figure 2A and Section S2.3). VibMO1 did not show activity with salicylate, which is in agreement with its low similarity (21%) to shyA, a verified salicylate hydroxylase from the ascomycete fungus *Epichloë festucae*.[12] We next screened for sequences similar to VibMO1 in the published genomes of fungi known to produce prenylated quinones and hydroquinones. As shown in the phylogenetic tree (Figure 2B, see Section S2.1), VibMO1 clustered with putative enzymes of those producers and closely related to MNX1. In contrast, VibMO1 is quite distant from Coq6 and shyA, which indicates stringent substrate specificity, hence leading to distinct enzymatic pathways. Based on RNA-seq analyses, the *VibMO1* transcripts significantly accumulated in the mycelia on days 14 and 20. Interestingly, the *B. vibrans* native metabolites **4** and **8** were obviously present on days 16 and 22, as evidenced from a time-course metabolite LC/MS profiling (see Section S3).The oxidative cyclization of **4** to **5** is common, and **4** would give **8** through a unique pathway (Figure 1). Oxidative decarboxylation by the enzyme VibMO1 can afford **4** from **3**, which is structurally similar to **1**. LC/MS analyses of the fungal native metabolites confirmed the presence of **1**, the aryl ring of which is known to originate from a shikimate pathway.[6b] VibMO1, catalyzing the oxidative decarboxylation of 3-prenyl 4-hydroxybenzoate (**3**) to prenylhydroquinone (**4**), can thus be considered as the enzyme most likely to be involved in the biosynthesis of

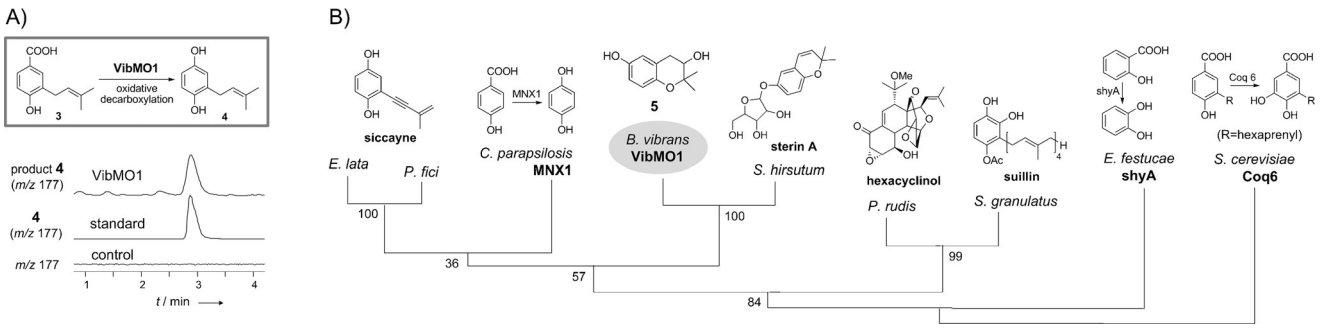

Figure 2. A) LC/MS analysis of the VibMO1 activity by single-ion monitoring of $[M-H]^-$ at $m/z = 177$ for the enzymatic product **4**. B) Phylogenetic connection of VibMO1 (GenBank: KU668560) and its fungal homologues. The tree scale was modified to accommodate the chemical structures (see Section S2). Shown at the top of the tree are the corresponding prenyl(hydro)quinone derivates or the verified enzymatic reactions of each fungus.

vibralactones (**8**) and other meroterpenoids (**5**) in *B. vibrans* fungus (see Section S1). Our research gives rise to a shikimate origin for the nonterpenoid moieties, despite the fact that most prenylated quinones and hydroquinones are confirmed to have mixed polyketide/terpenoid origin.[13] The VibMO1 catalysis mechanism could potentially resemble the oxidative hydroxylation/decarboxylation of 4-HBA by MNX1 in *C. parapsilosis* yeast.[11b]

Next, we investigated whether **1** can form different vibralactones through divergent pathways, based on the traditional platform of precursor-directed biosynthesis.[3a,c,e,14] Since an allyl C_3 mark is extremely rare in natural products from higher fungi, compound **1a**, an analogue of **1** that contains an allyl rather than a prenyl moiety, was synthesized and fed (2 mM) to a culture (500 mL) of *B. vibrans* on day 12 after inoculation. The culture broth was harvested after an additional ten days of incubation to provide crude extracts for LC/MS analyses. While vibralactones **9** and **10** were abundant in both normal and feeding broths, signals corresponding to the quasimolecular ions $[M+Na]^+$ at m/z 203 for putative analogues **9a** and **10a** were exclusively revealed in the **1a** feeding broth (see Section S3.2 in the Supporting Information). Scale-up feedings and systematic purification afforded a collection of thirteen new compounds and seven compounds known solely from synthetic chemistry, all arising from **1a**. Their structures were elucidated on the basis of extensive spectroscopic analysis (ESI-MS, HR-ESI-MS, and 1D and 2D NMR) and by comparison of their NMR data with those reported in the literature (see Section S1, S4).

Furthermore, divergent pathways in *B. vibrans* were clarified through evidence of analogous intermediates. As shown in Figure 1, the production of **2a**–**6a** in the feedings reveals reliable oxidative decarboxylation of **3a** to **4a** by VibMO1 in vivo, and suggests a reasonable route of **1a**→**2a**→**3a**→**4a**→**6a**. Likewise, the conversion of **3** into **4** is required for **1** to give **5** and **8**. The next three steps from **4** toward **8** may be closely analogous to those proposed for lignin degradation in yeast[11c] and aculins biosynthesis in *Aspergillus aculeatus*.[15] It is reasonable that subsequent decarboxylation to **7a**, the lactonization of which would eventually form an analogue of **8**, takes place. 3-substituted γ-butyrolactones such as **8** found in natural products display various biological activities,[16] which has prompted a number of research efforts towards their chemical synthesis.[17] Compared to its streptomycete congeners, blastmycinone and butenolide, which are formed through the polyketide/peptide hybrid antimycin biosynthetic pathway,[3f,18] the 3-substituted γ-butyrolactone **8** is distinct in starting from an aryl ring of shikimate origin. The pathway to **8** with key intermediacy from analogues **4a** and **7a** is unusual and the first time such a pathway has been described in natural product chemistry.

The vibralactone (**10**) pathway starting from **1** was further verified by the formation of **9a** and **10a**, since **1a** is transformed along the native pathway by analogous reactions to those for **1**. Vibralactone J (**11**) and its analogues are 4-substituted 2(5*H*)-furanones, which are of interest both in natural product chemistry[19] and synthetic chemistry.[20] Most of them are believed to originate from a terpene biosynthesis pathway.[21] Analogues **11a** and **11a$_1$** were obtained from **1a** in this study, which strongly suggests a shikimate origin for the featured moieties of these metabolites in *B. vibrans*. The pathway to **11** (Figure 1) may share a process with that of veratryl alcohol cleavage during lignin degradation in the *Phanerochaete chrysosporium* fungus.[22] Similar transformations have been proposed to occur in the synthesis of the fungal polyketides biosynthesis of patulin[23] and aculins.[15] Finally, by analogy, we propose the pathway for **13** to initiate with C_2 extension of **2a** by a pyruvate decarboxylase, followed by reduction (Figure 1). Similar pathways have been identified in basidiomycetes for the biosynthesis of chloroarylpropane diols and fomannoxin derivates.[24] Although our collection lacked analogues of **14**–**17**, signals corresponding to the putative ions were detected in the **1a** feeding broth (see Section S3.2). In addition, the dimeric analogues **20a**–**23a** were evaluated for inhibition of platelet aggregation (see Section S5).

This study affords in vivo and in vitro verification of the oxidative decarboxylation of 4-hydroxybenzoates. This led to the discovery and first genetic and biochemical characterization of a FAD-binding monooxygenase (VibMO1) that converts prenyl 4-hydroxybenzoate into prenylhydroquinone and is likely involved in the biosynthesis of vibralactones and other meroterpenoids in *B. vibrans*. This identification of VibMO1 will also be informative for the determination of enzymes essential for similar conversion steps in the biosynthesis of yeast coenzyme Q and plant prenyl(hydro)quinone derivates, where the oxidative decarboxylation of (poly)-prenyl hydroxybenzoates to their hydroquinones is understood to occur yet no corresponding genes or enzymes have so far been described. This study also demonstrates divergent biosynthetic pathways toward distinct scaffolds from a single precursor. To create skeletal diversification of compounds, further insight into nature's strategy for expanding structural diversity is needed.

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