Natural Products

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Elucidating the Biosynthetic Pathway for Vibralactone: A Pancreatic Lipase Inhibitor with a Fused Bicyclic β-Lactone**

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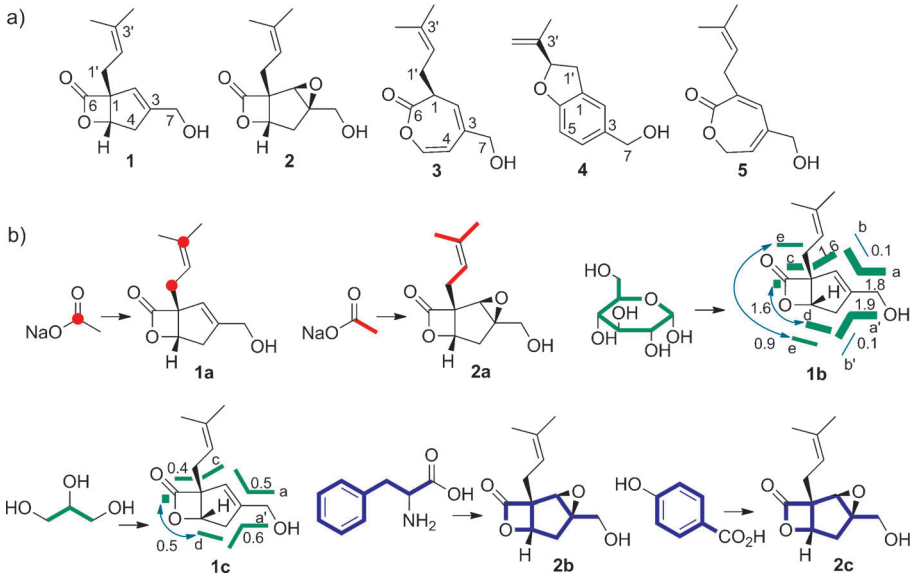
Vibralactone (**1**, Scheme 1 a), a rare fused bicyclic β-lactone from the basidiomycete fungus *Boreostereum vibrans* (syn. *Stereum vibrans*), was found to inhibit pancreatic lipases highly relevant to fat absorption.[1] Inspired by its clinical potential for the treatment of obesity, Zhou and Snider developed an elegant 10-step chemical route to the total synthesis of (±)-vibralactone and extended the work to (−)-vibralactone and (−)-vibralactone C.[2] Recent investigations established that the unusual fused β-lactone bicycle system of vibralactone may account for binding of both types of caseinolytic peptidases vital for bacterial virulence.[3] While vibralactone seems structurally simple for its small size, contradicting biosynthetic origins, including sesquiterpenoid and polyketide, of this bicyclic β-lactone have been proposed.[4] Furthermore, very little has been done on the biosynthetic mechanisms for natural products isolated from basidiomycete fungi in general.[5] We therefore set out to investigate the biosynthesis of vibralactone by ^{13}C-labeling and metabolite profiling studies, in addition to biochemical evidence. The results unequivocally show that the bicyclic lactone core of vibralactone is derived neither from a seemingly plausible polyketide nor a sesquiterpenoid origin, but instead a shikimate pathway. Additionally, VibPT is an aromatic prenyltransferase (PTase) potentially implicated in the biosynthesis of vibralactone. Our findings provide the first insight into a novel route for β-lactone formation in mushrooms.



Scheme 1. a) Vibralactone (**1**) and related compounds from the fungus *B. vibrans*. b) Labeling patterns from feedings of ^{13}C-labeled precursors. Bold lines indicate ^{13}C-labeled isotopologue groups with directly adjacent ^{13}C atoms. Numbers represent the molar abundances of multiply ^{13}C-labeled isotopologues. Arrows indicate ^{13}C-isotopologue groups with two directly adjacent ^{13}C atoms and one ^{13}C atom in the same molecule detected by long-range coupling in the ^{13}C NMR spectra (see the Supporting Information for details).

Based on the six-carbon lactone skeleton and the dimethylallyl side chain of vibralactone (**1**), we initially speculated that a polyketide pathway might be responsible for the assembly of the lactone core with subsequent attachment of a dimethylallyl moiety to eventually afford **1**. To test this idea, sodium [1-^{13}C]acetate was fed, in the beginning, to the culture of *B. vibrans*. We used a multiple-feeding procedure by adding five aliquots (5 × 100 mg) of each precursor at days 12, 14, 16, 18, and 20 after inoculation. The culture was harvested after an additional two days of incubation. The labeled products (titer 10–110 mg L^{-1}) were isolated by the method described in the Supporting Information (SI-1). The signal structures of the ^{13}C signals in the labeled **1** or **2** were analyzed according to

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Werner et al.,[6] and further confirmed by two-dimensional INADEQUATE experiments. The relative ^{13}C abundance of individual carbon atoms was calculated by comparison of integrals of the ^{13}C signal for the ^{13}C-labeled and unlabeled compounds. The absolute ^{13}C abundance was determined by quantitative NMR spectroscopy by analyzing ^{13}C-coupled satellite signals in the ^{1}H NMR spectra.[6]

To our surprise, no label from [1-^{13}C]acetate was evident by ^{13}C NMR spectroscopy in any of the bicyclic lactone carbon atoms in **1a** (Scheme 1b). In contrast, a high level of labeling was observed at the dimethylallyl carbon atoms C1′ and C3′ with a relative abundance of 14.9% and 23.9%, respectively (see SI-2 in the Supporting Information). Feeding of sodium [1,2-^{13}C]acetate resulted in **2a** with intact incorporation of the acetate units at C1′/C2′ ($J = 45.8$ Hz), C3′/C4′ ($J = 41.8$ Hz), and C3′/C5′ ($J = 42.3$ Hz) with 4.9–6.5% abundances (see SI-3 in the Supporting Information), thus leading directly to the evidence for the origin of the dimethylallyl moiety through mevalonate as anticipated. Likewise, no clear abundance of the bicyclic carbon atoms could be discerned. This labeling pattern highlighted the sharp distinction between the biosynthetic origins of each moiety and ruled out the possibility of a polyketide pathway which correlates to the bicyclic carbon backbone of **1**. Consequently, we hypothesized lysine to be the putative precursor because lysine has six carbon atoms matching perfectly with the carbon numbers of the bicyclic core structure of **1**. Nevertheless, no carbon atoms in **1** or its congeners were clearly labeled on feeding with [1,2-^{13}C]lysine. We next administered the general precursor (uniformly labeled) [U-^{13}C]glucose to *B. vibrans*. This resulted in all the carbon atoms being labeled in **1b** and **2** with 3.8–11% abundances (see SI-4 in the Supporting Information). As shown in Scheme 1b, $^{13}C_3$-isotopologue groups with three contiguous ^{13}C atoms were observed in **1b** in addition to $^{13}C_4$ isotopologues. Strong correlations were found between C7 and C3, and C3 and C2 in the INADEQUATE NMR spectra. This observation, in combination with the feeding pattern from ^{13}C acetate, suggested that molecules earlier than acetyl-CoA in the glycolytic pathway must be the carbon units from which the in vivo biosynthetic precursor pools are formed for generating the bicyclic carbon skeleton of **1**. The presence of $^{13}C_3$ isotopologues such as C2-C3-C7, and C4-C3-C7 presumably indicates their common origin from a three-carbon unit. This proposition was further supported by the identical $^{13}C_3$-isotopologue patterns in **1c** from feedings of [U-^{13}C]glycerol, as shown in Scheme 1b and SI-5 in the Supporting Information.

The three-carbon unit of phosphoenolpyruvate is combined with the four-carbon unit of erythrose 4-phosphate from the pentose phosphate cycle to form shikimate, a fundamental precursor for aryl compounds biosynthesis. Although the shikimate pathway is relatively less common in fungi than in green plants, the basidiomycetes produced a number of benzofurans which originate from shikimate pathway as confirmed by ^{14}C-labeling studies in *Stereum subpileaturn*.[7] Besides, in our previous work a prenylated aryl compound (**4**, Scheme 1a) was isolated as a minor metabolite from culture broths of the fungus *B. vibrans*, which is the producer of **1** and taxonomically the same as *Stereum* genus.[1] Accordingly, we hypothesized that a shikimate-derived aryl molecule might be the precursor for developing the bicyclic skeleton of **1** despite its apparently non-aryl structure. To test this hypothesis, [U-^{13}C]DL-phenylalanine (Phe) was fed and assimilated consequently to produce **2b** showing strong abundance (16–18%; see SI-6 in the Supporting Information) and coupling at C1/C2/C3/C4/C5/C6/C7 ($J_{1\text{-}2} = 29.3$ Hz, $J_{2\text{-}3} = 41.9$ Hz, $J_{4\text{-}3} = 42.5$ Hz, $J_{4\text{-}5} = 38.5$ Hz, $J_{5\text{-}1} = 29.3$ Hz, $J_{6\text{-}1} = 45.2$ Hz, $J_{7\text{-}3} = 48.5$ Hz), and very weak (ca. 2%) labeling at the dimethylallyl carbon atoms. Acetyl-CoA originating from degradation of Phe appears to be a rational source for the weak incorporation of the label at the dimethylallyl moiety. Significant correlations of C6/C1/C2/C3(/C7)/C4/C5 were observed in the INADEQUATE spectrum (SI-6), thus indicating a seven-carbon unit originating from the benzenoid ring of Phe. This observation was well corroborated by the feeding of [U-^{13}C]4-hydroxybenzoate, thus giving rise to the labeled product **2c** with distinctly high levels (43.4%; see SI-7a in the Supporting Information) of intact incorporation of benzoate at C1/C2/C3/C4/C5/C6/C7. Strong correlations exist between all seven adjacent carbon atoms of the bicyclic skeleton, as shown in the INADEQUATE spectrum of **2c** (SI-7a). Using LC/MS with selected ion monitoring, specific quasimolecular ions at *m/z* 254 $[M+7+Na]^+$ and *m/z* 264 $[M+7+CH_3OH+H]^+$ for **2c** were distinctively recognized along with the normal **2** at *m/z* 247 $[M+Na]^+$ and *m/z* 257 $[M+CH_3OH+H]^+$, all corresponding to the same retention time (see SI-7b in the Supporting Information). The above labeling patterns confirmed that the bicyclic lactone core of **1** is derived from an aryl ring that both Phe and shikimate routes may contribute to. 4-Hydroxybenzoate can be considered as a candidate precursor, which might be formed by two independent pathways in this fungus: from chorismate by the chorismate lyase reaction as normally known in bacteria and fungi, and from Phe as validated by this research. The Phe route leading to a C_6C_1 unit like 4-hydroxybenzoate occurs widely in plants by way of Phe→cinnamate→4-coumarate, with subsequent cleavage of two carbon atoms from the 4-coumarate side chain. Whereas in a different way, the basidiomycete fungi proceed as follows: Phe→cinnamate→benzoate→4-hydroxybenzoate, as confirmed from feedings of labeled L-Phe in *Bjerkandera adusta* and *Phanerochaete chrysosporium* where a ubiquitous lignin peroxidase can form 4-hydroxybenzoate from benzoate.[8] Although the conversion of Phe into tyrosine by phenylalanine hydroxylase has been well established in animals, available evidence proved the absence of this route in the basidiomycete fungi such as *Stereum sanguinolentun* and *Polyporus nidulans*.[9] Nevertheless, L-tyrosine can produce 4-hydroxybenzoate through 4-coumaroyl-CoA as verified in yeast.

To probe the details hidden in the dramatic changes from an aryl ring to the fused bicyclic β-lactone, we attempted a time-course metabolite profiling approach. The culture broths on days 10, 14, 18, and 22 (final) were analyzed by LC/MS, and the results showed remarkable differences in product profiles (Figure 1, and see SI-8 in the Supporting Information). The broths on day 10 contained a very small amount of 1,5-secovibralactone (**3**) and a barely detectable amount of **1**.

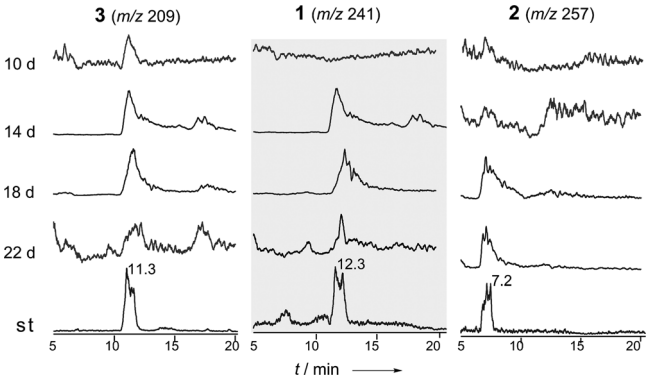


Figure 1. LC/MS metabolite profiling of the time-course cultures. The authentic standards (st) show quasimolecular ions $[M+H]^+$ at m/z 209 for **3**, $[M+CH_3OH+H]^+$ at m/z 241 for **1**, and $[M+CH_3OH+H]^+$ at m/z 257 for **2**.

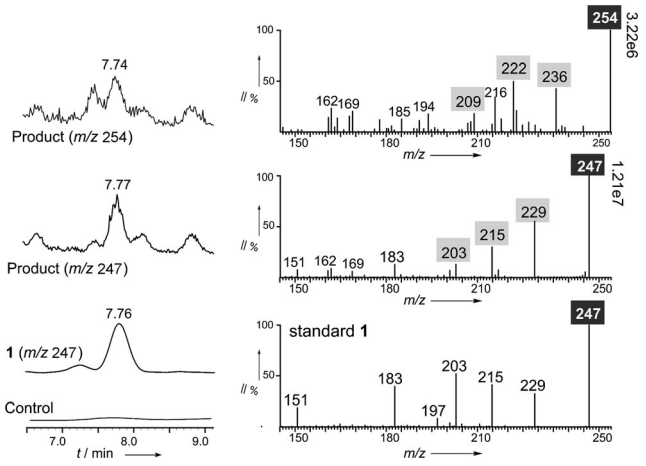
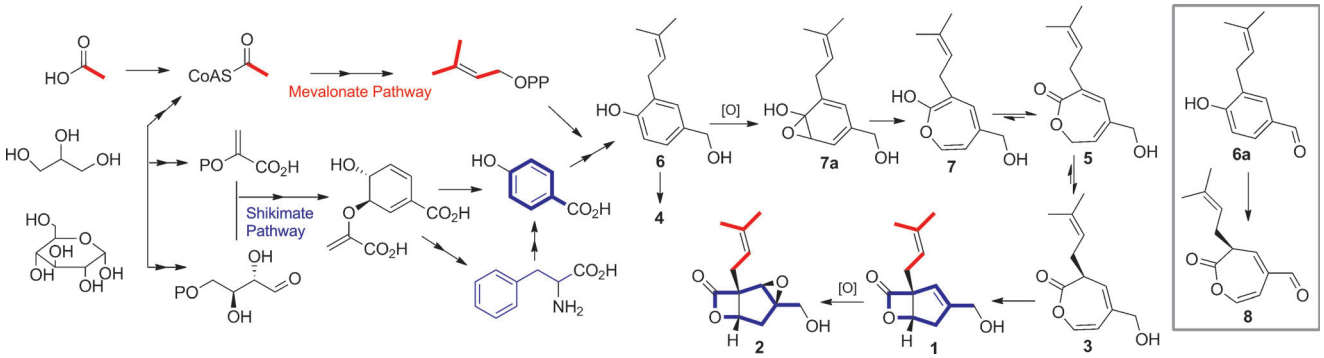


Figure 2. The cell-free enzymatic conversion of **3** (normal and labeled) to **1** as confirmed by triple quadrupole LC/MS/MS.

The highest production of **3** was observed on days 14–18, during which **1** started to form. From days 18 to 22, the amount of compound **3** declined eventually to a barely detectable level, while the amounts of **1** and **2** concomitantly increased. The results suggest that **1** is likely to be derived by cyclic rearrangement from **3**, which may involve a novel ring rearrangement mechanism. Interestingly, the new compound **5** (Scheme 1a and see SI-9a in the Supporting Information), an isomer of **3**, was identified as a minor metabolite from *B. vibrans* in the feeding of [U-^{13}C]4-hydroxybenzoate. As confirmed by NMR analyses, **5** can afford **3**, and vice versa. From the same feeding, labeled [$^{13}C_{1-7}$]**3** was also purified with approximately 30% ^{13}C abundance (see SI-9b in the Supporting Information). As a substrate, [$^{13}C_{1-7}$]**3** would give the labeled [$^{13}C_{1-7}$]**1** which is highly specific to detection for enzyme assays where formation of **1** from **3** can be biochemically verified. We performed the in vitro enzymatic conversion of **3** into **1** using a cell-free enzyme preparation from mycelia of 20 days. When incubated with [$^{13}C_{1-7}$]**3**, the crude enzyme eventually transformed **3** into **1** as a significant product identified by LC/MS/MS. Compared to the boiled enzyme control, the quasimolecular ions $[M+7+K]^+$ at m/z 254 for the labeled [$^{13}C_{1-7}$]**1** and $[M+K]^+$ at m/z 247 for normal **1** were both observed exclusively in the enzymatic product, all corresponding to the same retention time and mass of the authentic standard **1** (normal) showing the quasimolecular ion $[M+K]^+$ at m/z 247 (Figure 2). As expected, higher yield of the normal **1** (1.21e7, the base peak) was observed in the enzymatic product since the substrate is mainly composed of the normal **3** (SI-9b). The collision induced MS/MS analysis of the parent ion $[M+K]^+$ at m/z 247 produced the dominant fragment ions at m/z 229 for the loss of H_2O, at m/z 215 for the loss of H_2O and CH_2, at m/z 203 for the loss of CH_2 and CHOH (probably at C7; see SI-10 in the Supporting Information), and is in good agreement with those of the standard **1**. Specifically, the parent ion $[M+7+K]^+$ at m/z 254 showed +7 fragmentation patterns with dominant ions at m/z 236 (from 229 + 7) and m/z 222 (from 215 + 7), in addition to +6 fragment ion at m/z 209 (from 203 + 6). The results additionally confirm the enzymatic production of **1** from **3**.

Next, we speculated that an aryl compound, such as **6** (Scheme 2), is most likely to be the early intermediate which would give the seven-membered ring oxepin **7** by epoxidation and rearrangement. To test this hypothesis, we synthesized **6** starting from 3,3-dimethylallyl bromide and 4-hydroxybenzaldehyde with subsequent reduction (see SI-11 in the Supporting Information). Using **6** as a reference, we searched through all the time-course cultures under LC/MS but failed to detect the intermediate **6**. The reason for this is probably owing to the transient nature of **6** on the metabolic pathway of



Scheme 2. Proposed biosynthesis of vibralactone (**1**)

this fungus. However, the in vivo transformation of **6a** to the new compound **8** (Scheme 2 and see SI-12 in the Supporting Information) was observed within 24 hours after feeding the 14 day mycelia with **6a**. Therefore, it is possible that an oxidative expansion on the aryl ring can take place after prenylation. To accommodate all of these observations, we have proposed the following mechanism to account for this unusual pathway as shown in Scheme 2. By a unique prenyltransferase, the dimethylallyl moiety from a common mevalonate pathway might be attached to the aryl ring moiety that both shikimate and phenylalanine routes may contribute to, thus leading to the likely intermediate **6**. The prenylation at an aryl ring could be closely analogous to that proposed for benzofuran biosynthesis in basidiomycete fungus *Heterobasidion occidentale* and root cultures of *Tagetes patula*, respectively.[10] Presumably **6** is required to undergo an epoxidation to **7a** and subsequent rearrangement to the oxepin intermediate **7**, which would rapidly tautomerize to the seven-membered ring oxepinone **5** and then isomerize to **3**. The oxidation mechanism could potentially resemble that of the final steps in the biosynthesis of aflatoxin B1 in *Aspergillus*, where a ring expansion of a phenol to a lactone is mediated by a cytochrome P450.[11] Eventually an intramolecular cyclization of **3** can afford **1**, followed by an additional epoxidation to **2**. This unusual cyclization can be rationalized by formulating a base-catalyzed deprotonation at C1 followed by formation of an enolate which can then attack the ring enolate to form the β-lactone system and protonation of the neighboring carbon atom. It is also possible to assume an initial H-abstraction from the sp3-CH ring atom, followed by Cope rearrangement as proposed by Schwarzer et al.[12] Alternatively, a single-electron oxidation of **3** at C1 may be considered, followed by radical cyclization, which is strongly favored by recent studies.[13]

Finally, we attempted to identify the prenyltransferase (PTase) gene involved in vibralactone biosynthesis. From the genomic DNA of *B. vibrans* we constructed a genomic library followed by a probe hybridization for screening of candidate fosmids. Random sequencing followed by bioinformatics analysis enabled our discovery of a fosmid of about 30 kb which harbors a putative aromatic PTase gene (*vibPT*) and a putative cytochrome P450 monooxygenase (CYP) gene (*vibP450*) nearby. The deduced amino acid sequence of *vibPT* is 90% identical to its homologue ShPT recently found in the genome of *Stereum hirsutum* (see SI-13 in the Supporting Information), and shared 14–20% pairwise identity with the verified aromatic PTases in ascomycete fungi such as indole PTases (FgaPT2, SirD) and polycyclic PTases (VrtC),[14–16] and grouped together with the putative aromatic PTases from basidiomycete fungi (Figure 3A, shaded region). The crude recombinant VibPT was shown to catalyze the formation of **6** from 4-hydroxybenzyl alcohol and dimethylallyl diphosphate (Figure 3B and see SI-13). Furthermore, based on the RNA-Seq the *vibPT* transcripts were significantly accumulated on days 14 and 20, about 4–5-fold greater than that of day 7. The relatively more abundant levels of *vibPT* expression on days 14 and 20 correlated well with the remarkably higher production of **3**, **1**, and **2** during the same time (Figure 1). Thus, VibPT can be considered as a potential enzyme to catalyze the transfer of a dimethylallyl group to the aryl ring moiety, and most likely dedicated to the vibralactone biosynthesis.

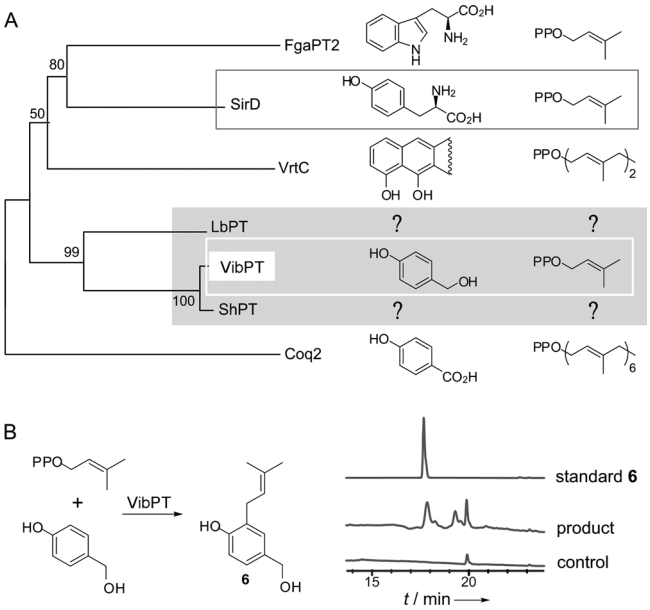


Figure 3. A) Phylogenetic connection of VibPT and other fungal aromatic PTases. The tree scale was modified to adapt for the chemical structures (see SI-13 in the Supporting Information). Shown in parallel are the corresponding substrates which each enzyme prefers. B) HPLC analysis of products formed by the crude recombinant VibPT and the empty pET control.

In summary, we have established the biosynthetic origin of vibralactone and obtained evidence for its biosynthetic pathway which includes several very interesting reactions which may involve unusual enzymes. The bicyclic lactone core of vibralactone could be attributed to an intramolecular cyclization of the seven-membered ring intermediate, which is likely to arise from an oxidative expansion of the aryl ring moiety that is derived from phenylalanine and shikimate pathways. Vibralactone has a unique chemical structure and potent biological activity, and is isolated from a basidiomycete fungus. All of these attributes make vibralactone a worthy target for biosynthetic investigation. The establishment of its biosynthetic pathway will be valuable for future elucidation of the molecular mechanism for its biosynthesis.

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