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Unexpected Metabolic Versatility in a Combined Fungal Fomannoxin/Vibralactone Biosynthesis

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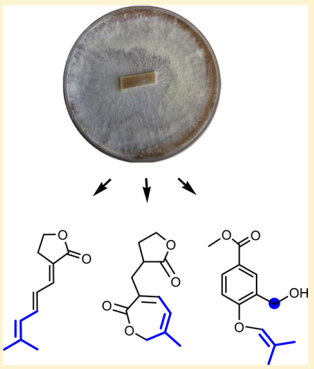
Supporting Information

ABSTRACT: The secondary metabolome of an undescribed stereaceous basidiomycete (BY1) was investigated for bioactive compounds. Along with a known fomannoxin derivative and two known vibralactones, we here describe three new compounds of these natural product families, whose structures were elucidated using 1D and 2D NMR spectroscopy and high-resolution mass spectrometry. The new compound vibralactone S (**4**) shows a 3,6-substituted oxepin-2(7*H*)-one ring system, which is unprecedented for the vibralactone/fomannoxin class of compounds. Stable isotope labeling established a biosynthetic route that is dissimilar to the two published cascades of oxepinone formation. Another new compound, the antifungal methyl *seco*-fomannoxinate (**6**), features a 2-methylprop-1-enyl ether moiety, which is only rarely observed with natural products. The structure of **6** was confirmed by total synthesis. ^{13}C-labeling experiments revealed that the unusual 2-methylprop-1-enyl ether residue derives from an isoprene unit. The diversity of BY1's combined fomannoxin/vibralactone metabolism is remarkable in that these compound families, although biosynthetically related, usually occur in different organisms.



The order Russulales represents an evolutionary lineage of the basidiomycetes that includes saprotrophic, symbiotic, and parasitic species. As white-rotting lignocellulose degraders or heterotrophic partners in mycorrhizal tree-fungus symbioses, they play a critical role in global carbon cycling and the healthy functioning of forest ecosystems. The Russulales also comprise the *Heterobasidion annosum* species complex, i.e., detrimental forest pathogens that negatively impact conifer forests in the northern hemisphere, as they cause a root and butt rot.[1,2] Given the ecological relevance of *Heterobasidion* and the Russulales in general, the secondary metabolism of these fungi has extensively been investigated and revealed intriguing compounds, such as the vibralactones **1** and **2** from *Boreostereum vibrans* (Figure 1).[3,4] Very recently, vibralactone oximes have been described from the same fungus.[5] For molecules of this class of compounds, various bioactivities have been recognized, among them inhibition of pancreatic lipase[3] as well as modest inhibition of mammalian 11β-hydroxysteroid dehydrogenases.[4] Also, **1** covalently binds and, thus, inhibits both caseinolytic proteases ClpP1 and ClpP2 of the human pathogenic bacterium *Listeria monocytogenes*.[6]

As a severe phytopathogen, *H. annosum* (*sensu lato*) received particular interest regarding identification of its small-molecule toxins, among them sesquiterpenes, such as the fomannosins[7] and the fomajorins[8] along with other highly functionalized and rearranged Δ^6-protoilludene-derived products.[9] Besides sesquiterpenes, *Heterobasidion* produces fomannoxins, such as **5** (Figure 1). These are phytotoxic benzofurans and their derivatives[10−13] that support the infection process, as they are present in infected conifer heartwood.[14] Fomannoxin production was also elicited through coculturing of *H. annosum* with spruce cells *in vitro*.[11] A hybrid biosynthetic route is taken to elaborate the fomannoxins, as the shikimic acid pathway supplies the aromatic portion to which is then added a prenyl moiety.[15] We recently investigated the metabolism of basidiomycete BY1 that was isolated from dead aspen wood showing signs of initial white rot.[16] Although the taxonomy of this fungus has not fully been clarified, DNA sequencing of the internal transcribed spacer (ITS) region of its genome suggested an evolutionary relationship to the Russulales and, more specifically, to the family Stereaceae. Initially, the rationale behind our research into the BY1 secondary metabolome was more profound insight in its antifungal and antilarval effects. The latter could be assigned to methyl-branched polyene polyketides. However, neither does this class

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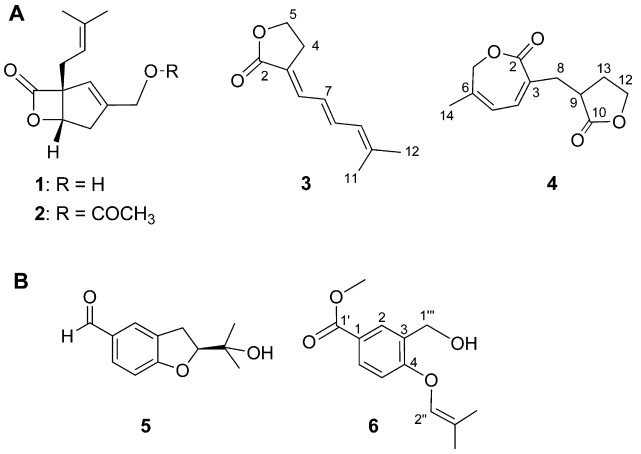

Figure 1. Chemical structures of small molecules isolated from the basidiomycete BY1: (A) vibralactone (**1**), vibralactone acetate (**2**), vibralactone R (**3**), and vibralactone S (**4**); (B) 5-formyl-2-(isopropyl-1′-ol)-2,3-dihydrobenzofuran (**5**) and methyl *seco*-fomannoxinate (**6**).

of compounds allow chemotaxonomic inferences, nor did the polyenes exert antifungal effects.

We here describe reinvestigation of this fungus's secondary metabolome (i) to support the taxonomic placement on the grounds of natural product biosyntheses and (ii) to identify the antifungal principle of BY1's mycelial extracts. Along with the three known compounds vibralactone (**1**), vibralactone acetate (**2**), and 5-formyl-2-(isopropyl-1′-ol)-2,3-dihydrobenzofuran (**5**), three new fomannoxin- and vibralactone-type natural products are described (Figure 1), whose structures were elucidated by interpretation of 1D and 2D NMR data and high-resolution mass spectrometry. The newly described molecules include the γ-lactone vibralactone R (**3**), the 2(7*H*)-oxepinone vibralactone S (**4**), which features an unprecedented substitution pattern in this family of compounds, and the antifungal methyl *seco*-fomannoxinate (**6**). The production of vibralactones and fomannoxins by the same species in conjunction with the above newly described derivatives underscores an unusually diversified metabolism.

RESULTS AND DISCUSSION

Isolation and Structure Elucidation of Natural Products. Ethyl acetate extracts of three-week-old BY1 cultures displayed antifungal activity in the agar diffusion assay. Bioactivity-guided chromatographic fractionation led to the isolation of various compounds, among them vibralactone **1** and vibralactone acetate **2**, which were identified by comparison of their spectroscopic data with literature values.[3,17] The capacity of BY1 to produce vibralactones that were previously isolated from *Boreostereum* and *Stereum* species underscores its phylogenetic relationship within the Stereaceae family of basidiomycetes.[3,18] This taxonomic assignment was already suggested from an analysis of the ITS region of BY1.[16] Two further metabolites with antifungal properties, now referred to as vibralactones R (**3**) and S (**4**) (Figure 1), were identified.

HRESIMS of **3** indicated a molecular formula of $C_{11}H_{14}O_2$, which corresponds to five degrees of unsaturation. A strong absorption band at 1730 cm^{-1} in the IR spectrum suggested the presence of an ester or aldehyde moiety. The latter possibility was ruled out after inspecting the ^{13}C NMR spectrum, as the only carbonyl resonance was observed at 171.4 ppm (Table 1, Figures S2 and S4). Six additional carbon atoms (C-3, C-6, C-7, C-8, C-9, C-10) are sp^2-hybridized according to their chemical shifts. Thus, compound **3** must feature three carbon−carbon double bonds and one ring structure to comply with the required degrees of unsaturation. The signals in the ^1H NMR spectrum (Figure S1) were attributed to their directly attached carbon atoms by heteronuclear single-quantum coherence (HSQC, Figure S6). Proton−proton correlation spectroscopy (COSY, Figure S5) revealed two discrete spin systems. The first spin system included four olefinic protons (H-6, H-7, H-8, H-9), while the second spin system was composed of two methylene groups, i.e., CH_2-4 and CH_2-5. The two spin systems

Table 1. NMR Spectroscopic Data for Vibralactone R (3), Vibralactone S (4), and Methyl *seco*-Fomannoxinate (6)[a]

3			4			6		
no.	δ_C, mult	δ_H, M (*J* in Hz)	no.	δ_C, mult	δ_H, M (*J* in Hz)	no.	δ_C, mult	δ_H, M (*J* in Hz)
1			1			1	122.6, C	
2	171.4, C		2	168.9, C		2	128.3, CH	8.06, d (2.0)
3	123.6, C		3	132.9, C		3	130.9, C	
4	25.2, CH_2	2.96, dt (7.5, 2.1)	4	135.5, CH[b]	6.66, d (6.0)	4	157.4, C	
5	65.3, CH_2	4.34, t (7.5)	5	126.9, CH	6.22, dd (6.0, 1.5)	5	112.4, CH	7.07, d (8.6)
6	135.2, CH	7.07, dt (11.7, 2.1)	6	143.2, C[b]		6	129.5, CH	7.84, dd (8.6, 2.0)
7	125.7, CH	6.27, dd (14.5, 11.7)	7	66.3, CH_2	(a) 4.44, d (12.7)	1′	166.0, C	
					(b) 4.37, d (12.7)			
8	138.2, CH[b]	7.02, dd (14.5, 11.7)	8	35.1, CH_2	(a) 2.86, m	3′	51.9, CH_3	3.83, s
					(b) 2.46, m			
9	125.4, CH	6.05, d (11.7)	9	38.3, CH	2.84, m	2″	134.1, CH	6.54, s
10	142.1, C[b]		10	178.3, C		3″	118.6, C[b]	
11	18.6, CH_3	1.84, s	11			4″	19.0, CH_3	1.69, s
12	26.1, CH_3	1.84, s	12	66.3, CH_2	(a) 4.30, dt (8.8, 3.0)	5″	15.1, CH_3	1.66, s
					(b) 4.15, dt (8.8, 6.7)			
			13	28.0, CH_2	(a) 2.31, dddd (12.3, 8.6, 6.7, 3.0)	1‴	57.3, CH_2[b]	4.58, d (5.7)
					(b) 1.90, m			
			14	21.9, CH_3	2.02, s	1‴-OH		5.29, t (5.7)

[a]DMSO-d_6, compound **3**: 500 MHz for ^1H NMR, 125 MHz for ^{13}C NMR; compounds **4** and **6**: 600 MHz for ^1H NMR, 150 MHz for ^{13}C NMR. [b]Signal increase upon [1-^{13}C]acetate addition to the culture.

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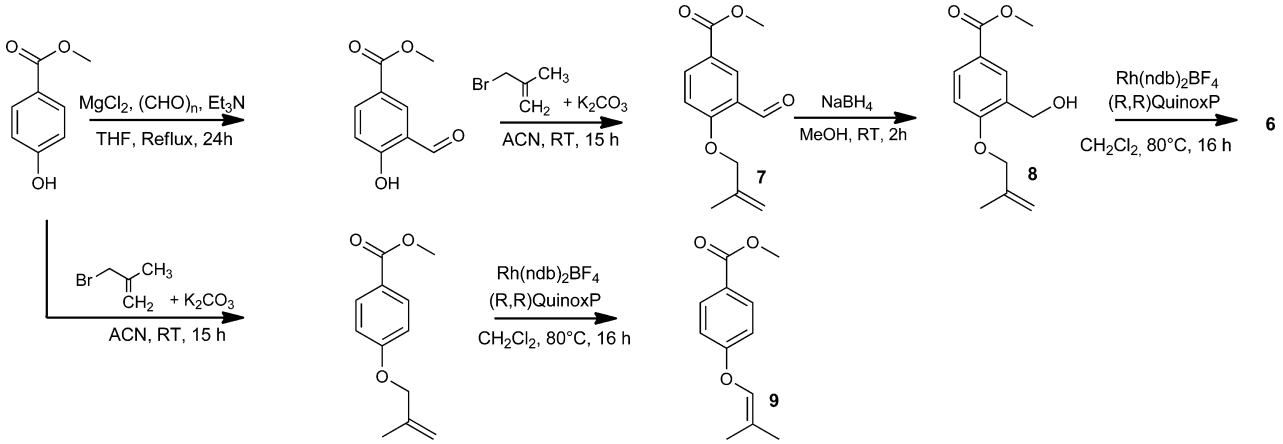
Scheme 1. Synthetic Route toward Methyl *seco*-Fomannoxinate (6) and Respective Intermediates/Analogues 7−9



could be connected via C-3 through heteronuclear multiple-bond correlation (HMBC, Figure S7) spectroscopy. $^{1}H,^{13}C$ long-range interactions from H-4, H-5, and H-6 to C-2 established the γ-lactone moiety of **3**. Furthermore, HMBC data allowed a linkage of the two remaining methyl groups CH_3-11 and CH_3-12 via C-10 with the rest of the molecule. The positions of CH_3-11 and CH_3-12 were assigned according to literature data.[3] The *E* configuration of the $\Delta^{3,6}$ double bond was deduced from a strong NOE between H_2-4 and H-7 (Figure S8). A large coupling constant of 14.5 Hz between H-7 and H-8 indicated an *E*-configured double bond $\Delta^{7,8}$.

HRESIMS data of **4** (*m*/*z* 223.0967 $[M + H]^+$) suggested a sum formula of $C_{12}H_{14}O_4$ and six double-bond equivalents. The optical rotation value of **4** corroborated a biosynthetic origin. The IR spectrum of **4** displayed a strong band at 1733 cm^{-1}, corresponding to a carbonyl functionality. Since no absorption bands associated with O−H bond stretching were observed, **4** does not contain carboxylic acid moieties. In the ^{13}C NMR spectrum (Figure S10), only 11 discrete resonances were resolved. Two ester functions ($\delta_{C\text{-}2}$ = 168.9 and $\delta_{C\text{-}10}$ = 178.3 ppm) as well as two carbon−carbon double bonds ($\delta_{C\text{-}3}$ = 132.9 ppm, $\delta_{C\text{-}4}$ = 135.5 ppm, $\delta_{C\text{-}5}$ = 126.9 ppm, and $\delta_{C\text{-}6}$ = 143.2 ppm) were readily identified. Subsequently, ^{1}H NMR, DEPT135, and HSQC experiments (spectra in Figures S9, S12, and S14) allowed the assignment of two methylenoxy groups (CH_2-7 and CH_2-12) that exhibited overlapping chemical shifts (δ_C = 66.3 ppm), thus providing an explanation for the apparently missing resonance. The remaining signals in the ^{13}C NMR spectrum were attributed to a methine (CH-9), a methyl (CH_3-14), and two methylene groups (CH_2-8 and CH_2-13). The chemical shifts of CH_3-14 were suggestive of a methyl group connected to an sp^2-hybridized carbon. This assumption was confirmed in the HMBC (Figure S15) spectrum, which indicated C-6 as the directly bonded carbon atom. On the basis of further $^{1}H,^{13}C$ long-range interactions as well as spin coupling between H-4 and H-5, a 3-substituted 6-methyloxepin-2-(7*H*)-one partial structure was elucidated. COSY data (Figure S13) combined with an interpretation of the HMBC spectrum then led to the identification of a (2-oxotetrahydrofuran-3-yl)methylene moiety, which was finally linked to the oxepin-2-(7*H*)-one ring due to long-range interactions from H-8 to C-2, C-3, and C-4. Compound **4** was hence identified as 6-methyl-3-((2-oxotetrahydrofuran-3-yl)methyl)oxepin-2(7*H*)-one, for which we propose the name vibralactone S.

Compound **5** of the basidiomycete BY1 was identified as (*S*)-5-formyl-2-(isopropyl-1′-ol)-2,3-dihydrobenzofuran,[19] based on polarimetric analyses, HRESIMS, and ^{1}H and ^{13}C NMR data. The spectroscopic data of another compound (**6**), however, did not match any previously isolated fomannoxin or vibralactone. The empirical formula of **6** was assigned to be $C_{13}H_{16}O_4$ by HRESIMS, which corresponds to six degrees of unsaturation. Strong stretching vibrations at 3410 and 1713 cm^{-1} indicated the presence of hydroxy and carbonyl moieties, respectively. Inspection of the ^{1}H NMR and COSY spectra (Figures S16 and S20) revealed two discrete spin systems. Three resonances in the aromatic region occur as an isolated AMX spectrum (δ_A 7.07, δ_M 7.84, δ_X 8.06, J_{AM} = 8.6 Hz, J_{MX} = 2.0 Hz), characteristic of a 1,2,4-trisubstituted benzene derivative. This assumption is in agreement with ^{13}C, DEPT 135, HSQC, and HMBC data (Figures S17, S19, S21, and S22, respectively), which identified six ^{13}C resonances as constituents of the postulated benzene moiety (Table 1). The second spin system corresponded to a hydroxymethylene residue, which could be connected to the aromatic core of **6** due to long-range interactions from H_2-1‴ to C-2, C-3, and C-4 and from H-2 to C-1‴. HMBC data also allowed for the identification of the two remaining substituents, that is, a methyl ester group at C-1 and a (2-methylprop-1-en-1-yl)oxy group at C-4. Hereafter, this compound is referred to as methyl *seco*-fomannoxinate. A (2-methylprop-1-en-1-yl)oxy group is rarely observed in natural products but represented by 4-(1-hydroxyethyl)-1-*O*-(2-methylprop-1-en-1-yl)salicylaldehyde, which had been reported from *Osteospermum muricatum* (Compositae, sunflower family).[20]

Synthesis of Methyl *seco*-Fomannoxinate. To confirm the structural proposal and to also secure a sufficient supply for biological testing, compound **6**, now referred to as methyl *seco*-fomannoxinate, was synthesized (Scheme 1). 4-Hydroxybenzoic acid methyl ester was regioselectively formylated at C-3, following a published procedure.[21] In our hands, the strategy by Ma et al.,[22] to directly install an isobut-1-enyl group at the phenolic OH via a nucleophilic substitution with 1-bromo-2-methylprop-1-ene, repeatedly failed to yield the desired product. Attempts to introduce the isobut-1-enyl group as a triflate, as proposed,[23] did not prove successful either, perhaps due to the phenolic group being deactivated by the *ortho*-positioned aldehyde functionality. A successful alternative included a two-step approach: *O*-alkylation of the phenol with 3-bromo-2-methylprop-1-ene followed by a subsequent isomerization of the double bond resulted in the desired vinyl

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Table 2. NMR Spectroscopic Data for Synthetic Products 7–9[a]

	7		8		9	
no.	δ_C, mult	δ_H, M (J in Hz)	δ_C, mult	δ_H, M (J in Hz)	δ_C, mult	δ_H, M (J in Hz)
1	123.0, C		122.6, C		123.6, C	
2	130.5, CH	8.48, d (1.8)	130.0, CH	7.99, d (2.2)	131.6, CH	7.97, d (8.8)
3	124.6, C		129.3, C		115.1, CH	6.96, d (8.8)
4	163.9, C		160.0, C		161.4, C	
5	112.7, CH_2	7.00, d (8.7)	110.8, CH	6.86, d (8.6)	115.1, CH	6.96, d (8.8)
6	137.0, CH	8.19, dd (8.7, 1.8)	131.0, CH	7.94, dd (8.6, 2.2)	131.6, CH	7.97, d (8.8)
1′	166.0, C		166.8, C		166.8, q	
3′	52.1, CH_3	3.85, s	51.9, CH_3	3.85, s	51.9, CH_3	3.88, s
2″	72.4, CH_2	4.60, s	71.8, CH_2	4.50, s	134.1, CH	6.23, m
3″	139.3, C		139.9, C		119.7, C	
4″	113.8, CH_2	(a) 5.11, s	113.2, CH2	(a) 5.07, s	15.3, CH_3	1.70, s
		(b) 5.05, s		(b) 5.00, s		
5″	19.3, CH_3	1.85, s	19.4, CH_3	1.80, s	19.5, CH_3	1.70, s
1‴	188.7, CH	10.50, s	61.5, CH_2	4.70, s		

[a]Chloroform-d_1, 500 MHz for ^{1}H NMR, 125 MHz for ^{13}C NMR.

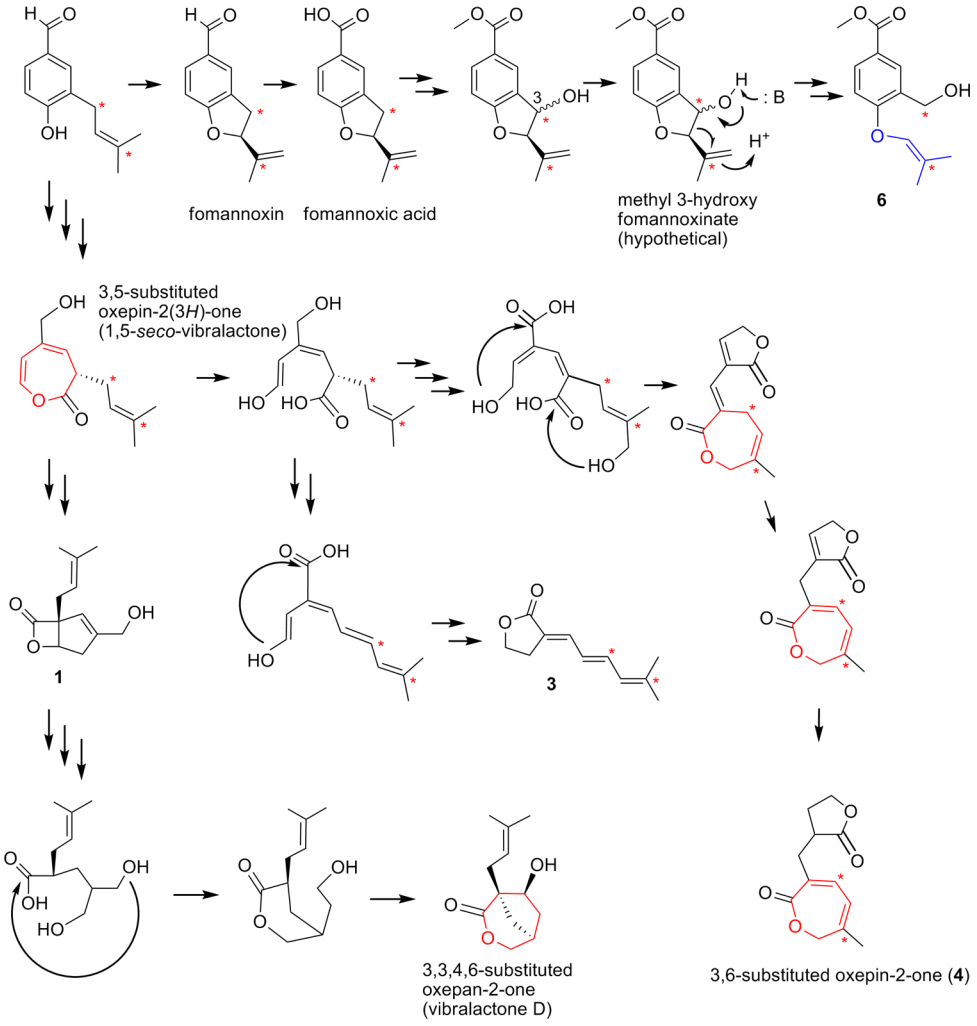

Figure 2. Proposed and simplified routes of the combined vibralactone and fomannoxin biosynthesis. Vibralactone, vibralactone D, and fomannoxin formation according to published data.[15,28] Asterisks indicate ^{13}C-enriched carbons after adding [1-^{13}C]acetate to the medium. The 2-methylprop-1-enyl ether moiety of methyl *seco*-fomannoxinate (**6**) is highlighted in blue,; oxepanone/oxepinone rings are highlighted in red.

ether. *O*-Alkylation proved efficient. Since the leaving group of the alkylating agent was attached to an sp^3-configured carbon atom, the first step (nucleophilic substitution) could be performed smoothly according to established procedures,[24] with virtually quantitative turnover of educts, leading to intermediate **7** (^{1}H and ^{13}C NMR data in Table 2, NMR spectra in Figures S23–S28). Reduction of the aldehyde with methanolic $NaBH_4$ yielded compound **8** in near-quantitative

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yield (^{1}H and ^{13}C NMR data in Table 2, NMR spectra in Figures S29−S34). Its conversion into the corresponding vinyl ether followed the procedure of Okamoto et al.,[24] who describe a rhodium-catalyzed route for dihydrobenzofuran derivatives through an olefin isomerization and enantioselective intramolecular Alder-ene reaction cascade. The combination of $Rh(ndb)_2BF_4$ and (*R*,*R*)QuinoxP in anhydrous $(CH_2Cl)_2$ as catalyst and ligand performed only modestly with regard to the intramolecular Alder-ene reaction, but supported reasonable turnover in the olefin isomerization. Consequently, this procedure was modified, and CH_2Cl_2 was used as solvent, which led to near-quantitative conversion of **8** into **6**. Reduction of the aldehyde function prior to isomerization proved crucial, as **7** did not isomerize into the respective vinyl ether. ^{1}H and ^{13}C NMR spectra of the final synthetic product were identical to those of **6**, isolated from the fungal source. Analogously, **9** was synthesized from 4-hydroxybenzoic acid methyl ester without an initial formylation step (Scheme 1, ^{1}H and ^{13}C NMR data in Table 2, NMR spectra in Figures S35−S40).

Biosynthetic Studies. The new γ-lactone **3** was also isolated from cultures of BY1 grown on [1-^{13}C]sodium acetate-enriched medium. ^{13}C NMR data revealed a selective increase of the signals at δ_C = 138.2 ppm and δ_C = 142.1 ppm (Figure S3). These signals correspond to C-8 and C-10, respectively (Figure 1 and Table 1). Fungi synthesize γ,γ-dimethylallyldiphosphate (DMAPP) exclusively from acetyl-CoA via the mevalonate pathway.[25] Consequently, upon routing [1-^{13}C]-acetate into this metabolism, C-1 and C-3 of DMAPP will carry the stable isotope label, which correspond to carbon atoms C-8 and C-10 in **3**. As a common structural feature, **3** shares the γ-lactone moiety with vibralactones G−J, L, and M. These compounds presumably derive from 1,5-*seco*-vibralactone by ester hydrolysis, decarboxylation in the case of vibralactones G and H, and re-esterification as principal steps.[26,27] Our results are perfectly compatible with this hypothesis, confirm this biosynthetic route and also emphasize the central role of 1,5-*seco*-vibralactone as an intermediate toward bicyclic β- and γ-lactones.

Compound **4** represents a new 2(7*H*)-oxepinone that results from BY1's highly diverse fomannoxin/vibralactone metabolism. Precedence for seven-membered, oxygen-containing ring systems in the vibralactone family stems, for example, from 1,5-*seco*-vibralactone (Figure 2), which is the 3,5-branched immediate precursor to vibralactone.[28] Vibralactone D (Figure 2) features a 2-oxepane moiety and is considered a follow-up product of vibralactone that underwent β-lactone cleavage, reduction of the cyclopentene, and re-esterification.[4] Collectively, these data support two routes of heterocycle formation, which both maintain the prenyl group as an exocyclic substituent. Intriguingly, **4** has a γ-lactone substituent and possesses a 3,6-substituted oxepin-2(7*H*)-one core, which is incompatible with the above routes that result in different substitution patterns. This finding implies a third, independent biogenesis for seven-membered vibralactone-type heterocycles in the basidiomycete BY1, which was investigated by [1-^{13}C]-acetate stable isotope labeling. The ^{13}C NMR spectra of **4** showed two selectively enriched signals (δ_C = 135.5 ppm and δ_C = 143.2 ppm, respectively, corresponding to C-4 and C-6 (Figure S11)). This result demonstrates that the exocyclic prenyl group, likely of the intermediate 1,5-*seco*-vibralactone, has been integrated into a seven-membered heterocycle to yield the branched vibralactone ε-lactone ring system of **4**. We propose that 1,5-*seco*-vibralactone as the above-mentioned central intermediate undergoes hydrolysis of its oxepin-2(7*H*)-one ring as well as hydroxylation at the distal position of the isoprene unit, followed by dual intermolecular esterification (via C-7 and C-2 and via C-12 and C-10) as well as double-bond migration and reduction of the γ-lactone (Figure 2).

Compound **6** appeared structurally somewhat related to the fomannoxins despite the absence of a benzofurane core and an overall ambiguous biosynthetic origin. Fomannoxin-type isobut-1-enyl ethers have never been described. Analogously to the assumed route for the above-mentioned *Osteospermum* salicylaldehyde derivative,[20] we hypothesized that the biosynthesis of **6** may proceed from fomannoxic acid with the pathway being extended to the (as yet undescribed) 3-hydroxyfomannoxic acid methyl ester. Subsequently, it undergoes cleavage of the dihydrofuran ring, followed by the reduction of the aldehyde (Figure 2). As described above, fungi produce DMAPP exclusively via the mevalonate pathway. The benzylic carbon atom found in *ortho*-position to the phenolic group (C-1‴) as well as the sp^2-hybridized carbon atom of the isobut-1-enyl moiety (C-3″) of **6** would therefore derive from C-1 and C-3 of DMAPP, respectively. Hence, feeding of [1-^{13}C]acetate should lead to enriched ^{13}C isotopic abundance at these positions. To test the hypothesis, **6** was purified from BY1 cultures grown in the presence of 24.4 mM sodium [1-^{13}C]acetate. The ^{13}C spectrum of **6** showed selective enrichment of the signals at δ_C = 57.3 ppm and δ_C = 118.6 ppm (Figure S18), which correspond to positions C1‴ and C3″, respectively (Table 1). These findings verify our hypothesis that both carbon atoms originate from an isoprene unit whose transfer *ortho* to the alcohol functionality of 4-hydroxybenzaldehyde or 4-hydroxybenzoic acid initiates fomannoxin biosynthesis.[15,28] Our results also verify the fragmentation step as the key biosynthetic event toward **6** and make the postulated route for the *Osteospermum* salicylaldehyde isobut-1-enyl ether plausible.[20] In return, this raises the question if this compound is truly a plant metabolite or perhaps the product of an endophytic fungus.

Antifungal Bioactivity. The pure compounds **1**−**9** were tested for antifungal activity against the human pathogenic fungi *Candida albicans*, *Aspergillus fumigatus*, and *Arthroderma benhamiae*, as well as against *Penicillium notatum*. *C. albicans* was not inhibited by any compound. Likewise, **1**−**4** did not inhibit *A. fumigatus*. However, inhibition was detected for *A. benhamiae* (MIC of 12.5 μg/mL for **3**, 50 μg/mL for **1** and **2**, Table 3) and *P. notatum* (MIC: 25 μg/mL). **6** was particularly active against *A. benhamiae* (MIC: 6.25 μg/mL). The synthetic analogues **7**−**9** were included in the test as well (MIC: 6.25 μg/mL for **7** and 25 μg/mL for **8**, respectively, but 100 μg/mL for **9**). As *A. fumigatus* was less inhibited, or not at all, divergent structural features may be required for activity against this species, or differences exist with regard to transport into or out of the cell.

None of the vibralactones **1**−**4** were active against *C. albicans* and *A. fumigatus* at concentrations of ≤100 μg/mL (Table 3). However, **1**−**3** inhibited *P. notatum* at 25 μg/mL. A more heterogeneous situation was observed with *A. benhamiae*, as this pathogen was not inhibited by the oxepinone **4**, but clearly by **3** (MIC 12.5 μg/mL), whereas vibralactones **1** and **2** showed MIC values of 50 μg/mL. The fomannoxins are typical metabolites for *Heterobasidion* (Bondarzewiaceae), while the vibralactones are found in *Stereum*[x] and *Boreostereum* species

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Table 3. Antifungal Activity of Described and New Natural Products 1−6 and Synthetic Compounds 7−9 (MIC in μg/mL)

compound	*Aspergillus fumigatus*	*Penicillium notatum*	*Arthroderma benhamiae*
Vibralactones			
1	>100	25	50
2	>100	25	50
3	>100	25	12.5
4	>100	25	>100
Methyl *seco*-Fomannoxinate and Its Synthetic Analogues			
6	50	25	6.25
7	50	25	6.25
8	>100	25	25
9	>100	25	100

(Stereaceae). Both fungal families belong to the Russulales.[29] Given that members of these two natural product classes, known from phylogenetically distinct families, occur side by side in the BY1 fungus, its metabolism is intriguing and strongly underscores its relationship to the Russulales from the chemotaxonomic perspective. This relationship was previously based solely on genetic evidence.[16] However, the ecological relevance of BY's simultaneous and greatly diversified fomannoxin/vibralactone metabolism remains shrouded. Fomannoxin is known to exert phytotoxic effects, with the 2-isopropenyl side chain being relevant for phytotoxicity.[30] Activity against *E. coli* and, to a lesser degree, against the basidiomycete *Radulomyces confluens* was found as well.[10,11] The antifungal activity of vibralactone was established during this study. BY1 is a fast-growing pioneer colonist that causes a white rot of woody biomass. Given their antifungal activity, these metabolites may confer a selective advantage for the fungus that helps it thwart off and compete with other fast-growing, ubiquitous soil and plant surface inhabitants, such as *Penicillium* species, although phytotoxic fomannoxins may not be primarily required to secure growth in its habitat. BY1 combines (i) the ability to colonize wood in an early stage of decay and (ii) the capacity to break down lignin with (iii) a metabolism that produces a diversity of bioactive small molecules. Therefore, this fungus may represent a future model organism for technologies such as biopulping and preprocessing of lignocellulosic material for biobased ethanol production.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotation was measured on a Jasco P-1020 polarimeter, with methanol (MeOH) as solvent, in a 1 mL cell (path length d = 5.0 cm, λ = 589 nm, T = 20 °C). UV−vis spectra were recorded on a ScanDrop instrument (Analytik Jena) using Winaspect software. Samples were dissolved in MeOH. IR spectra were recorded on a Jasco FT/IR 4100 instrument. 1D and 2D NMR spectra of compounds **1**−**8** were recorded in DMSO-d_6 and chloroform-d_1, respectively, at 300 K. Chemical shifts were referenced relative to internal residual nondeuterated solvent traces (DMSO-d_5: δ_H 2.50 ppm; δ_C 39.5 ppm, chloroform δ_H 7.24 ppm; δ_C 77.2 ppm). NMR spectra were recorded on Bruker Avance III 500 and 600 MHz spectrometers. Preparative HPLC was accomplished on an Agilent 1260 instrument equipped with a Phenomenex Luna C_{18} column (250 × 21.2 mm, 10 μm particle size). Routine mass spectrometry was performed on an Agilent 1260 chromatograph with a Zorbax Eclipse XDB C_{18} column (150 × 4.6 mm, 3.5 μm particle size), coupled to an Agilent 6130 mass detector using electrospray ionization in positive and negative mode and applying the gradient described below. HRESIMS data were recorded on an Exactive Orbitrap instrument (Thermo Scientific) using the direct injection port. Analytical and semipreparative HPLC was performed on an Agilent 1200 instrument fitted with Zorbax Eclipse XDB C_{18} columns (5 μm particle size, 150 × 4.6 mm or 250 × 9.6 mm, respectively). Chromatograms were recorded at λ = 254 and 280 nm; the respective diode array detectors covered the wavelength range of λ = 190−600 nm. Chemicals, solvents, and media components were purchased from Deutero, Roth, Sigma-Aldrich, and VWR, and sodium [1-^{13}C]acetate was from Cortecnet.

Microbiological Methods. The fungus BY1[16] was routinely maintained on solid HA medium (4 g/L D-glucose, 4 g/L yeast extract, 10 g/L malt extract, 18 g/L agar, pH = 6.5). For metabolite production, modified liquid HA medium (1 L per 4 L penicillin flask) was used (D-glucose and yeast extract increased to 10 g/L each) and cultivated as stationary culture for 21 days at room temperature in the dark or until HPLC analysis showed secondary metabolite production. Stable isotope labeling was carried out by adding filter-sterilized sodium [1-^{13}C]sodium acetate (2 g/L, 24.4 mM final) upon inoculation.

Compound Isolation and Chromatography. The culture broth was separated by filtration from the mycelia and subsequently extracted three times with equal volumes of ethyl acetate. The organic phase was filtered, and the solvent was removed under reduced pressure using a rotary evaporator. The resulting crude extract was dissolved in 25% MeOH in water (v/v) and prefractionated on Waters Sep-Pak C_{18} cartridges (5 g), conditioned equally prior to sample loading. A step gradient of 25, 50, 75, and 100% MeOH was used for elution. The respective fractions were subjected to preparative chromatography running a linear water/acetonitrile (ACN) gradient from 15% to 100% ACN within 18 min at a flow rate of 20 mL/min. Final purification was accomplished through semipreparative chromatography applying an isocratic flow of 4 mL/min. The water/ACN ratio was chosen based on the polarity of the compounds to be separated and ranged between 25% and 50% ACN. Analytical HPLC of the crude extract, fractions, and pure substances was run using the following water/ACN gradient (1 mL/min): 10% ACN, held constant for 10 min, increase to 90% within 22 min, increase to 100% ACN within 1 min.

Synthesis of ***6****.* The synthesis of 3-formyl-4-hydroxybenzoic acid methyl ester from commercially available 4-hydroxybenzoic acid methyl ester followed a published protocol with 81% yield after workup.[21] The *O*-alkylation of phenolic groups to yield **7** was carried out as described.[24] A 0.5 mmol amount of phenol was mixed with 0.6 mmol of 3-bromo-2-methylprop-1-ene and 1 mmol of potassium carbonate in 3 mL of ACN. The reaction was stirred for 15 h at room temperature and then stopped by adding an equal volume of water. Products were extracted with ethyl acetate, the organic phases were combined, and volatiles were removed under reduced pressure in a rotary evaporator. Subsequent purification accomplished by silica gel column chromatography was performed as described above, but using a step gradient of ethyl acetate/cyclohexane (9:1, 8:2, 7:3, v/v) as mobile phase. A near-quantitative turnover (99% yield) of the phenol educt into 2-methylprop-1-en-3-yl ether **7** was observed. The aldehyde of **7** was then reduced with sodium borohydride in MeOH at room temperature. After 2 h, the reaction had proceeded quantitively to yield **8**. Isomerization of the allylic ether into the respective vinylic ether of **6** was completed following a published procedure[24] in modified form: **8** was mixed in a glass vial with 0.1 molar equiv of $Rh(ndb)_2BF_4$ and (*R*,*R*)QuinoxP in anhydrous CH_2Cl_2. The reaction was sealed and heated to 80 °C for 16 h with constant stirring. The reaction was cooled to room temperature, quenched by addition of water, and subsequently extracted with an equal volume of ethyl acetate. Volatile compounds of the combined ethyl acetate phases were removed, and traces of remaining catalysts were removed during HPLC as described above to yield product **6** (86% yield after chromatography). An analogous synthesis route (except initial formylation) was taken to synthesize **9** (Scheme 1).

Bioactivity Tests. The pure compounds (Table 3) were dissolved in MeOH (1 mg/mL) for antimicrobial tests. Minimal inhibitory

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concentration (MIC) values against *Candida albicans*, *Aspergillus nidulans*, *Aspergillus flavus*, and *Penicillium notatum* were determined with the broth dilution assay according to a published procedure.[31]

*Vibralactone R (**3**):* colorless oil; UV (MeOH) λ_{max} 325 (log ε = 3.98) nm; $IR_{(neat)}$ 1730 (s), 1648 (w), 1190 (s), 1083 (s) cm^{-1}; ^1H and ^{13}C NMR data (DMSO-d_6, 500 and 125 MHz, respectively), see Table 1; HRESIMS *m*/*z* 179.1068 $[M + H]^+$ calcd for $C_{11}H_{15}O_2$ 179.1067.

*Vibralactone S (**4**):* colorless oil; $[\alpha]^{20}_D$ +8 (*c* 0.1, MeOH); UV (MeOH) λ_{max} 275 (log ε = 3.38) nm; $IR_{(neat)}$ 1733 (s), 1652 (w), 1194 (s) cm^{-1}; ^1H and ^{13}C NMR data (DMSO-d_6, 600 and 150 MHz, respectively), see Table 1; HRESIMS *m*/*z* 223.0967 $[M + H]^+$, calcd for $C_{12}H_{15}O_4$ 223.0965.

*Methyl seco-fomannoxinate (**6**):* colorless oil; UV (MeOH) λ_{max} 263 (log ε = 4.62) nm; $IR_{(neat)}$ 3410 (b), 1713 (s), 1606 (s), 1388 (s), 1248 (s), 1132 (s), 767 (s) cm^{-1}; ^1H and ^{13}C NMR data (DMSO-d_6, 600 and 150 MHz, respectively), see Table 1; HRESIMS *m*/*z* 237.1124 $[M + H]^+$, calcd for $C_{13}H_{17}O_4$ 237.1121.

*Methyl 4-(2-methylallyloxy)-3-formylbenzoate (**7**):* colorless oil; UV (MeOH) λ_{max} 233 (log ε = 4.59) and 256 (log ε = 4.52) nm; $IR_{(neat)}$ 1715 (s), 1686 (s), 1607 (s), 1124 (s), 766 (s) cm^{-1}; ^1H and ^{13}C NMR data ($CDCl_3$, 500 and 125 MHz, respectively), see Table 2; HRESIMS *m*/*z* 235.0962 $[M + H]^+$, calcd for $C_{13}H_{15}O_4$ 235.0965.

*Methyl 4-(2-methylallyloxy)-3-(hydroxymethyl)benzoate (**8**):* colorless oil; UV (MeOH) λ_{max} 258 (log ε = 4.45) nm; $IR_{(neat)}$ 3429 (b), 1712 (s), 1606 (s), 1249 (s), 1127 (s), 1132 (s) cm^{-1}; ^1H and ^{13}C NMR data ($CDCl_3$, 500 and 125 MHz, respectively), see Table 2; HRESIMS *m*/*z* 235.0977 $[M - H]^-$, calcd for $C_{13}H_{15}O_4$ 235.0976.

*Methyl 4-(2-methylprop-1-enyloxy)benzoate (**9**):* colorless oil; UV (MeOH) λ_{max} 255 (log ε = 3.16) nm; $IR_{(neat)}$ 1716 (s), 1603 (s), 1244 (s), 1109 (s), 769 (s) cm^{-1}; ^1H and ^{13}C NMR data ($CDCl_3$, 500 and 125 MHz, respectively), see Table 2; HRESIMS *m*/*z* 207.1019 $[M + H]^+$, calcd for $C_{12}H_{15}O_3$ 207.1016.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00147.

1D and 2D NMR spectra of new natural products and synthetic compounds (PDF)

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Notes

The authors declare no competing financial interest.

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