Injury-Induced Biosynthesis of Methyl-Branched Polyene Pigments in a White-Rotting Basidiomycete

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Supporting Information

ABSTRACT: A stereaceous basidiomycete was investigated with regard to its capacity to produce yellow pigments after physical injury of the mycelium. Two pigments were isolated from mycelial extracts, and their structures were elucidated by ESI-MS and one- and two-dimensional NMR methods. The structures were identified as the previously undescribed polyenes (3Z,5E,7E,9E,11E,13Z,15E,17E)-18-methyl-19-oxoicosa-3,5,7,9,11,13,15,17-octanoic acid (1) and (3E,5Z,7E,9E,11E,13E,15Z,17E,19E)-20-methyl-21-oxodocos-3,5,7,9,11,13,15,17,19-nonanoic acid (2). Stable-isotope feeding with [1-13C]acetate and L-[methyl-13C3]methionine demonstrated a polyketide backbone and that the introduction of the sole methyl branch is most likely S-adenosyl-L-methionine-dependent. Dose-dependent inhibition of Drosophila melanogaster larval development was observed with both polyenes in concentrations between 12.5 and 100 μM. GI50 values for 1 and 2 against HUVEC (K-562 cells) were 71.6 and 17.4 μM (15.4 and 1.1 μM), respectively, whereas CC50 values for HeLa cells were virtually identical (44.1 and 45.1 μM).

RESULTS AND DISCUSSION

Taxonomic Placement of BY1. The fungus BY1 was isolated from dead aspen wood that showed a white rot type of wood decay, and a pure axenic culture was obtained. As fruiting bodies were not available we were unable to assign a taxonomic position based on morphological characteristics. Therefore, we amplified and sequenced the internal transcribed spacer (ITS) region. A 665 bp amplicon (GenBank accession number KC514809) was used to query the NCBI database, which returned sequences with 92%, 91%, and 90% identical nucleotides respectively from Acanthophysium lividocaeruleum (GenBank accession numbers AB873012 and AB873013, and accession number KC514809). Sequencing of a 1750 bp portion of the large ribosomal subunit gene (accession number AB873012 and AB873013).
number KM101461) confirmed the relationship of BY1 with the genus *Stereum*, as sequences with 97% identical nucleotides from *Stereum* spp., *FCUG2671*, *Stereum subtomentosum*, and *Xylobolus frustulatus* (accession numbers AF506483, AF506482, and AF506491, respectively) were returned when searching the NCBI sequence database. We therefore have assigned BY1 to the Stereaceae family within the order Russulales, a well-known group of organisms following a white-rotting lifestyle.

**Isolation and Structural Elucidation of Polyene Pigments.** Under standard laboratory conditions, BY1 developed a white, undifferentiated mycelium when grown on solid complex media such as yeast-malt-glucose medium. However, after being physically injured, e.g., by incision with a scalpel, the mycelium turned yellow 3 to 4 days postwounding. This pigmentation developed around at the edges of the injured site (Figure 1) and remained virtually unchanged for weeks. HPLC-DAD analysis of crude acetone extracts of BY1 mycelia, which had been injured prior to extraction, indicated the presence of two major compounds, 1 and 2. Their UV absorption maxima at $\lambda = 416$ and 425 nm, respectively (Figures S1 and S2), suggested that the metabolites might indeed contribute to the observed yellow coloration.

**Table 1. NMR Spectroscopic Data for Polyenes 1 and 2**

<table>
<thead>
<tr>
<th>pos.</th>
<th>$\delta_C$, type</th>
<th>$\delta_H$ (J in Hz)</th>
<th>NOESY</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>172.3, C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>33.5, CH$_2$</td>
<td>3.19, dd (7.5, 1.9)</td>
<td>(3), 5</td>
</tr>
<tr>
<td>3</td>
<td>125.5, CH</td>
<td>5.64, dt (10.7, 7.5)</td>
<td>(2), 4</td>
</tr>
<tr>
<td>4</td>
<td>131.4, CH</td>
<td>6.16, ddd (12.8, 10.7, 1.9)</td>
<td>3, 6</td>
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<tr>
<td>5</td>
<td>129.8, CH</td>
<td>6.58, t (12.8)</td>
<td>2, 6</td>
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<tr>
<td>6</td>
<td>134.8, CH</td>
<td>6.38, m</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>135.7, CH</td>
<td>6.40, ddd (13.7, 11.0)</td>
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<tr>
<td>8</td>
<td>129.6, CH</td>
<td>6.85, dd (13.7, 10.6)</td>
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</tr>
<tr>
<td>9</td>
<td>132.2, CH</td>
<td>6.13, dd (13.7, 10.6)</td>
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<td>11</td>
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<td>129.9, CH</td>
<td>6.88, dd (14.6, 10.6)</td>
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<td>13</td>
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<td>14</td>
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<td>7.21, dd (14.5, 11.5)</td>
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<tr>
<td>16</td>
<td>130.3, CH</td>
<td>6.67, dd (14.5, 11.4)</td>
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<tr>
<td>18</td>
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<td>19</td>
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<td>25.7, CH$_3$</td>
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<tr>
<td>27</td>
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<td>n.d.</td>
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For spectra see Figures S5, S6, and S9–S14. *Not assigned due to signal overlap.*
bands between 2000 and 1600 cm\(^{-1}\) supported the presence of olefinic groups, whereas alkyne or allene bands could not be detected. Furthermore, strong bending vibrations at 969 and 739 cm\(^{-1}\) suggested that I harbors both E- and Z-configured carbon–carbon double bonds. Subsequent inspection of the \(^{13}\)C NMR spectrum (Figure S11) confirmed the conclusions that had been drawn from the interpretation of the IR data. Two signals, which were observed at \(\delta_C 197.8\) and 172.2 ppm, were fully consistent with a ketone and a carboxylic acid. Since 16 further carbon signals could be ascribed to sp\(^2\)-hybridized atoms on the basis of their chemical shifts (Table 1), the degrees of unsaturation in 1 are due to eight carbon–carbon double bonds and the two aforementioned carbonyl moieties. Only three carbon atoms of 1 are located in the aliphatic region of the \(^{13}\)C NMR spectrum. The corresponding signals were identified as two methyl and a methylene group via a DEPT135 experiment. Afterward, all proton signals were attributed to their directly attached carbon atoms by heteronuclear single-quantum coherence (HSQC). Proton–proton correlation spectroscopy (COSY) in combination with homonuclear J-resolved \(^1\)H NMR spectroscopy (Figures S7 and S8) and first-order multiplet analysis revealed a continuous spin system in 1 that comprised all proton groups except for the methyl signals at \(\delta_H 2.26\) and 1.86 ppm. Heteronuclear long-range correlations from H-2 and H-3 to C-1 established the position of the carboxylic acid group. Likewise, the quaternary C-18 could be placed next to CH-17, thereby defining the location of the last carbon–carbon double bond. On the basis of the previous assumptions, the latter had to be conjugated with the remaining ketone and, indeed, H-17 was clearly correlated with C-19 in the heteronuclear multiple-bond correlation (HMBC) spectrum. Long-range correlations from H-20 to C-19 and from H\(_{21}\) to C-18 concluded the determination of the planar structure of 1. The configuration of the double bonds was deduced from the respective \(^3\)J values and NOEs (Figure S13), respectively. On the basis of the above data, we identified compound 1 as (3Z,5E,7E,9E,11E,13Z,15E,17E)-18-methyl-19-oxoicosa-3,5,7,9,11,13,15,17-octaeanoic acid (Chart 1).

Physical and spectroscopic data of 2 were similar to 1. However, a mass increase of 26 Da was suggestive of an additional carbon–carbon double bond (Figure S4). The extension of the conjugated olefinic system was reflected by the bathochromically shifted absorbance maximum of 2 (\(\lambda = 425\) nm versus 416 nm in 1, Figure S2). IR data confirmed the presence of a carboxylic acid and a ketone in conjugation to at least two double bonds. Bands found in the IR spectra of 1 at 969 and 739 cm\(^{-1}\) suggested that the structure of 2 includes both E- and Z-configured carbon–carbon double bonds. 1D and 2D NMR data (Figures S9, S10, and S14) confirmed these findings. Assignment of proton signals and their multiplicity was accomplished in acetone-\(d_6\). Twenty signals in the \(^{13}\)C NMR spectrum could be attributed to sp\(^2\)-hybridized atoms (Table 1; Figure S12). Interpretation of their chemical shifts indicated the presence of nine carbon–carbon double bonds, a carboxylic acid, and a ketone function. 2D NMR spectra showed the same connectivity as found for 1, except for the already anticipated extension of the olefinic chain. The two cis-configured double bonds in 2 were deduced from \(^3\)J values and NOEs, respectively (Figure S14). Therefore, the structure of 2 was determined as (3E,5Z,7E,9E,11E,13E,15Z,17E,19E)-20-methyl-21-oxocosa-3,5,7,9,11,13,15,17,19-nonaenoic acid (Chart 1).

Among the known fungal metabolites with similar structural features are piptoporic acid (Chart 1) and related compounds isolated from the basidiomycete Piptoporus australiensis, and laetiporic acid A (Chart 1) and derivatives, which are the yellow pigments of the “chicken of the woods” fungus Laetiporus sulphureus. While the double bonds of piptoporic acid are all-trans configured, the major isomers of laetiporic acid A and 2-dehydro-3-deoxylaetiporic acid A show two and three cis-configured double bonds, respectively. Compound 1 and piptoporic acid share the same sum formula. However, NMR data proved that these molecules are dissimilar in regard to the double-bond placement, as piptoporic acid features one double bond in conjugation with the carboxylic acid moiety that is separated from the polyene chromophore by a saturated methylene group. This results in distinct chemical shifts of the

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- **Bold chemical bonds in 1** represent acetate units as deduced from \([1-{^{13}\text{C}}]\text{acetate}\) labeling pattern; the asterisk shows \(^{13}\text{C}\) incorporation after feeding \([1-{^{13}\text{C}}\text{methyl}]\text{methionine}\) to cultures of BY1. Relevant HMBC (solid arrows) and NOE correlations (dashed arrows) are shown.
To determine the biosynthetic origin of BY1 polyenes, 1 was purified from cultures containing stable-isotope-labeled substrates. Feeding of both [1-13C]acetate and L-[methyl-13C]-methionine resulted in incorporation and increased 13C NMR signal intensity of individual carbon atoms (Figures S15 and S16). When 1 was isolated from cultures fed with L-[methyl-13C]methionine, only the signal corresponding to the methyl group residing at C-21 was enhanced in the 13C NMR spectrum (Figure S16). In contrast, the signal pattern resulting from [1-13C]acetate feeding showed an increase for every other carbon atom of 1 (Figure S15), beginning with the carboxylic acid functionality (C-1, Chart 1) and extending throughout the entire linear main chain. These results prove a polyketidic biosynthesis, in which the identified formal acetate units correspond to the canonical building blocks acetyl-CoA (starter unit) and malonyl-CoA (extender unit). Moreover, the increased signal intensity of C-21 shows the S-adenosyl-L-methionine (SAM)-dependent introduction of this methyl group, most likely by C-methyltransferase activity, which is frequently integral to fungal reducing polyketide synthases.13,14 Intriguingly, both 1 and 2 exclusively feature βγ-positioned double bonds within the formal acetate units.

Following the biosynthetic logic of polyketide synthesis, shifted double bonds represent a rare exception to the standard pattern of polyketide natural products where double bonds are found between the formal acetate units (i.e., αβ- or βγ-positioned to the acetate carbonyl function). Beyond the laetiporic acid/piptoporic acid family of basidiomycete compounds, precedent for shifted polyketide double bonds also stems from research on the antitumor compound ansamitocin, which is produced by Actinosynnema pretiosum. Taft et al. (2009) proposed a mechanism in which a vinylogous syn-elimination by a dehydratase domain in the third module of the asm polyketide synthase mediates the double-bond migration.15 Specifically, a tetraketide intermediate oriented in a specific, required conformation within the catalytic center prevents the canonical αβ-syn-elimination. A second well-documented example of shifted polyketide double bonds pertains to the formation of the shifted diene system in rhizoxin D1, a biosynthetic intermediate en route to rhizoxin, which is made by the endosymbiotic bacterium Burkholderia rhizoxinica.16 The double bonds introduced at positions 9 and 11 are shifted sequentially by two different dehydratase domains. Mechanistically, the first double-bond migration takes place analogously to the ansamitocin mode, whereas the subsequent second shift is most likely carried out by a dedicated module intrinsic to the rhizoxin polyketide synthase.

Anti-insect Activity of Polyenes 1 and 2. As polyene production was induced by physically injured mycelium (Figure 1), we hypothesized that 1 and 2 might be bioactive molecules and play a role in fungal defense, e.g., against fungivores. A report on a possible role of piptoporic acid to protect Piptopus australiensis carphores from flies feeding on fruiting bodies8 prompted us to perform bioactivity tests on the insect model Drosophila melanogaster. Further support for our hypothesis comes from earlier studies that showed that certain fungal pigments reduce the pupation rate of D. melanogaster.17 However, this study did not include polyene structures. To expose all stages of the D. melanogaster life cycle to polyenes 1 and 2, adult female flies were kept on either untreated or polyene-amended medium. After 7 days, the number of pupating larvae was determined and the pupation number was determined relative to controls on polyene-free medium (Figure 2). Both 1 and 2 impacted the development of D. melanogaster, although they differed in their respective efficacy. While 1 did not reduce the number of pupating larvae at 12.5 μM, a clear dose-dependent inhibition at concentrations up to 100 μM was observed (5 versus 18 in controls). When exposed to 12.5 μM 2, only a minimum number of pupating larvae were observed (two animals in one of the vessels). At higher doses, pupation was not observed at all.

Figure 2. Relative pupation rate of Drosophila melanogaster progeny with respect to concentrations of polyenes 1 and 2 in the medium. Control pupation levels were determined on unmodified medium and normalized to 100%.
observed (Figure S17), which is highly suggestive of a pigment uptake. Second, insects, and specifically D. melanogaster, evaluate food sources primarily based on intricate olfactory cues, 21 which would favor volatiles serving as repellent compounds. This was shown for naphthalenes produced by the endophytic fungus Muscodor vitigenus, which repel the wheat stem sawfly, Cephus cinctus. 22 Neither 1 nor 2 is volatile. However, they may represent components of a multicomponent repellent/protection system.

Bioactivity against Human Cell Lines. We investigated the effect of 1 and 2 on HUVEC (human umbilical vein epithelial cells) and K-562 leukemia cells in antiproliferative assays. GI50 values for 1 and 2 were 71.6 and 17.4 μM against HUVEC and 15.4 and 1.1 μM against K-562 cells (Figures S18 and S19). The pronounced difference in GI50 values between HUVEC and K-562 cell lines suggests a stronger effect on faster dividing cells, and the latter value is comparable with those of clinically used drugs that were tested in parallel in the same assay. For irinotecan, tamoxifen, and idarubicin, GI50 values of clinically used drugs that were tested in parallel in the same dividing cells, and the latter value is comparable with those of (CC50 = 44.1 and 45.1 μM).

Both polyene pigments exerted equal cytotoxicity on HeLa cells (CC50 = 44.1 and 45.1 μM for 1 and 2, respectively, Figure S20).

Research on the structurally related falcenosones A and B regarding their inhibitory effect on the HL60 leukemia cell line and other cancer cell lines in vitro and in vivo suggests that the 1-methyl-2-oxo-1-propylidene terminus, in conjunction with a conjugated olefinic system, favors cytotoxic effects on mammalian cell lines. 23, 24 This structural feature is shared by all members of the laetiporic/piptoporic acid family of natural products. However, other structural elements are required for activity, and our results point to chain length being relevant for cytotoxic properties, as the chain-extended polyene 2 is more active than 1.

Basidiomycete polyenes have been credited with protecting mycelium from fungivory; however they have not been described in the context of an inducible defense. The polyenes 1 and 2 are novel members of the laetiporic acid/piptoporic acid family of basidiomycete pigments, which is diversified by the new compounds in that all double bonds are shifted. Our results point to chain length being relevant for activity, and our results point to chain length being relevant for cytotoxic properties, as the chain-extended polyene 2 is more active than 1.

Experimental Section

General Experimental Procedures. To record UV-vis spectra, compounds were dissolved in methanol. Spectra were acquired on a ScanDrop instrument (Analytik Jena) using Winaspect software (version 2.4). IR spectra were measured on a Jasco FT/IR 4100 instrument. 1D and 2D NMR spectra of polyenes 1 and 2 were recorded in THF-d8 and acetone-d6 respectively, at 300 K, and chemical shifts were referenced relative to internal residual non-deuterated solvent traces (THF: δH 1.72 ppm, δC 25.3 ppm; acetone: δH 2.04 ppm, δC 29.8 ppm). NMR spectra were recorded on Bruker Avance III spectrometers. HRRESIMS data were generated on an Exactive Orbitrap instrument (Thermo Scientific) using the direct injection port. For preparative HPLC an Agilent 1260 series instrument equipped with a Phenomenex Luna C18 column (250 × 21.2 mm, 10 μm particle size) was used. Analytical HPLC was performed on an Agilent 1200 instrument fitted with a Zorbax Eclipse XDB C18 column (150 × 4.6 mm, 3.5 μm particle size). All chromatograms were recorded at λ = 420 nm; the respective diode array detectors covered the wavelength range λ = 190–600 nm. Chemicals, solvents, stable-isotope-labeled compounds, and media components were from Cytoscreen, Deutero, Euroisotop, Roth, Sigma-Aldrich, and VWR. Oligonucleotide primers were synthesized by Eurofins MWG Operon; DNA sequencing (Sanger) was performed by GenScript.

Fungal Strain and Culture Conditions. The BY1 isolate was cultured from aspen wood located at the University of Minnesota Cloquet Forestry Station located in northern Minnesota, and isolation procedures were previously reported. 25 The culture has been deposited at the Jena Microbial Resource Collection (JMRC) under the registration number SF011241. The fungus was maintained and cultured on YM agar (4 g/L d-glucose, 4 g/L yeast extract, 10 g/L malt extract, 18 g/L agar). Growth was at room temperature in the dark, for approximately 2 weeks. To induce pigment formation, the mycelium was injured using a scalpel and incubated for another 3–4 d, followed by harvest of the mycelium for compound extraction. Stable isotope feeding experiments were carried out essentially as described above, but with a total culture volume of 2 L (100 Petri dishes). The 13C-label was supplied by adding either filter-sterilized sodium [1-13C]acetate (12.2 mM final) or L-[methyl-13C]methionine (3.35 mM final) to YM medium prior to pouring the Petri dishes. To extract genomic DNA from mycelium, BY1 was grown in liquid YM medium as a stationary culture for 2 weeks at room temperature in the dark.

Polyyne Isolation and Analysis. Polyene isolation from mycelium was carried out on ice and, whenever possible, protected from light. Pigment-producing mycelium was frozen at –20 °C, crushed, and immediately exhaustively extracted by vigorous shaking with acetone. The resulting organic phase was passed through a cellulose filter and subsequently transferred into brown glass flasks. The acetone was evaporated in a rotary evaporator at room temperature. The impure and water-insoluble compounds precipitated in residual water and were collected by centrifugation for 5 min at 3220g at 4 °C. After decanting the water, the samples were stored at –80 °C and dissolved in methanol for chromatographic purification. Pure compounds were obtained by preparative HPLC running a water/acetonitrile (ACN) solvent system. The gradient began at 60% ACN and was linearly increased to 100% ACN over 20 min at a flow rate of 21 mL/min. Fractions containing single compounds were pooled. The acetonitrile was evaporated and the polyenes were precipitated in the aqueous phase and collected as described above.

Bioactivity Assays. HUVEC and K-562 cell lines were used in antiproliferation assays, and HeLa cells were used to determine...
cytotoxic activity, according to described protocols.\textsuperscript{29} Substances tested were dissolved in dimethyl sulfoxide, which was also used as control. Inhibitory activity against \textit{D. melanogaster} was essentially determined as described.\textsuperscript{33} Three female \textit{D. melanogaster} WT specimens (\textit{Canton-S} strain, adult stage, 6 days of age) were transferred into plastic vessels, each containing 20 mL of nutrition medium (118 g/L treacle, 11 g/L brewer’s yeast, 4.1 g/L agar, 95 g/L maize meal, 2.4 g/L propionic acid, 3.3 g/L napipin 30\%) either with or without compounds 1 and 2 (12.5, 25, 50, and 100 μM; added prior to solidification of the medium; all experimental setups as biological triplicate). All vessels were kept at 25 °C in a moist atmosphere. Fly development was examined under a dissecting microscope to assess development and to quantitate \textit{Drosophila} progeny. After 7 d, the number of larvae in the puparium stage was determined and pupation rate was calculated relative to untreated controls.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
 & HUVEC & K-562 & HeLa \\
\hline
\textsuperscript{a}GI\textsubscript{50} (μM) & 71.6 & 15.4 & 44.1 \\
\textsuperscript{b}CC\textsubscript{50} (μM) & 17.4 & 1.1 & 45.1 \\
\hline
\end{tabular}
\caption{Biological Activity of Polyenes 1 and 2 against Human Cell Lines}
\end{table}

\textsuperscript{a}GI\textsubscript{50} (growth inhibition): concentration that causes 50\% of maximum inhibition of cell proliferation. \textsuperscript{b}CC\textsubscript{50} (cytotoxicity concentration): concentration that causes cell death of 50\% of host cells.

\section{REFERENCES}

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\section{ASSOCIATED CONTENT}

\section{Supporting Information}

UV–vis, 1D and 2D NMR spectra, a photograph of larval coloration, and bioactivity data of 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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\textbf{Notes}

The authors declare no competing financial interest.

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