Minor Pyranonaphthoquinones from the Apothecia of the Lichen *Ophioparma ventosa*

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**ABSTRACT:** Four new quinonoid naphthopyranones, ophioparmin (1), 4-methoxyhaemoventosins (2a and 2b), and 4-hydroxyhaemoventosin (3), together with anhydrofusarubin lactone (4) and haemoventosin (5) were isolated from the fruiting bodies of *Ophioparma ventosa*, a crustose lichen. Their structures were determined by spectroscopic analyses, and the absolute configurations of 1 and 2 were elucidated through experimental and calculated electronic circular dichroism analyses. Compounds 1, 2, and 5 exhibited moderate to strong antioxidant activities. The main pigment haemoventosin exhibited significant cytotoxicity toward a panel of nine cell lines.

Lichens are resilient symbiotic consortia that consist of a fungus and an algal/cyanobacterial partner. As a consequence of this original lifestyle, specific secondary metabolites are produced, and the ever-expanding array of lichen metabolite bioactivities warrants further chemical investigations of these fascinating organisms. Lichens of alpine and polar habitats are of interest because their ability to withstand such stressful conditions is often based on the synthesis of protective compounds. A focused study on differentiated organs such as apothecia, which represent less than 2% of the thallus weight, is thought to not only facilitate the isolation of minor and specific compounds but also provide a better indication of their ecological significance.

As such, the crustose Alps mountain-living lichen *Ophioparma ventosa* (L.) Norman has been phytochemically investigated by several authors and reported to contain depsides (mainly divaricatic and thamnolic acids), dibenzofuran-related usnic acid, a tetrahydroxystylyc acid (ventosic acid) and some additional compounds, such as psoromic, stictic, and norstictic acids and atranorin, among others. Regarding the intense blood-red color of *O. ventosa* apothecia, Bruan and Lamvik first reported the isolation of its major pigment, haemoventosin, but they assigned an erroneous furanophthoquinone structure. The correct pyranophthoquinone structure of haemoventosin was assigned in 1995. Several authors reported on the presence of “numerous” minor pigments most likely corresponding to additional quinones without identifying any of these. Naphthoquinones are a privileged class of secondary metabolites that exhibit a wide range of biological activities, predominantly cytotoxic or antibacterial properties; however, the biological properties of haemoventosin have not yet been investigated.

The significant cytotoxic and antioxidant activities of the pigment-enriched fraction prompted an undertaking of chemical investigations of the pigments of *Ophioparma ventosa* apothecia. Herein, based on (+)-HRESIMS dereplication of this quinone-enriched fraction, the isolation and structural elucidation of four new quinonoid naphthopyranones, namely, ophioparmin (1), 4-methoxyhaemoventosins (2a and 2b), and 4-hydroxyhaemoventosin (3), are reported. Two known naphthazarinopyranones, anhydrofusarubin lactone (4) and

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(+)-haemoventosin (5), are also described, the former being new to lichens. Owing to the minute amounts of compounds isolated, only 1, 2, and 5 could be assayed for their antioxidant properties. The major pigment, 5, was tested for its in vitro inhibition of cell proliferation against a panel of nine different cell lines.

**RESULTS AND DISCUSSION**

The CH$_2$Cl$_2$ extract of the apothecia of *O. ventosa* was washed with a cyclohexane/EtOAc mixture (9/1) to afford a pigment-enriched fraction. The naphthopyranone composition of this fraction was revealed by direct infusion (+)HRESIMS analysis. Indeed, due to their strong chemical homologies, the different dyes could not be separated by HPLC. Accurate mass measurements derived from the most intense ions observed in the mass spectrum (Figure S1, Supporting Information) are collated in Table S2 (Supporting Information). All experimental values were compared with the theoretical mass calculated in an error range of ±5 ppm from candidate compositions consistent with a quinonoid naphthopyranone core encompassing C$_{12}$−20H$_{10}$−20O$_{5}$−10. Eight molecular formulas could be retrieved, among which only haemoventosin$^6$ was previously reported from *O. ventosa*.

On the basis of the significant cytotoxic and antioxidant properties of this naphthoquinone-enriched fraction, successive fractionations were performed, including silica gel chromatography and preparative TLC, affording compounds 1, 3, anhydrofusarubin lactone (4), and haemoventosin (5). A second sample of *O. ventosa* was processed, which permitted the identification of the new naphthopyranones 2a and 2b as a mixture of diastereoisomers (Figure 1).

**Table 1.** $^1$H (500 MHz) and $^{13}$C (125 MHz) NMR Data for 1−3 (CDCl$_3$)

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<thead>
<tr>
<th>position</th>
<th>$^1$C, type</th>
<th>$^1$H, mult. ($J$ in Hz)</th>
<th>$^1$C, type</th>
<th>$^1$H, mult. ($J$ in Hz)</th>
<th>$^1$C, type</th>
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<tr>
<td>3 81.7</td>
<td>5.18, dd (4.0, 2.9)</td>
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<td>5.05 dq (7.0, 1.5)</td>
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<td>1.41, d (6)</td>
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Figure 1. Structures of compounds 1−5.

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14.0, 5.5 Hz, H-11α) and 2.00 (1H, ddd, J = 14.0, 9.8, 4.0 Hz, H-11α); one aromatic methoxy singlet at δH 3.97 (3H, s, 7-OCH3); and one aliphatic methyl group at δH 1.41 (3H, d, J = 6 Hz, H-13). These data combined with the 13C NMR data obtained from HSQC and HMBC correlations were reminiscent of those of haemoventosin 5 (Table 1). The main difference was the presence of two additional coupled oxygenated methines at δH/δC 5.31/66.9 (H-4) and δH/δC 5.18/81.7 (H-3). HSQC-TOCSY correlations revealed that H-3, H-4, and H-11−H-13 belonged to the same spin system, and the sequence CH3−13/CH12/CH11−CH3/CH4 could be deduced from cross peaks in the COSY spectrum. HMBC correlations of H-4 with C-5, C-4a, and C-10a showed that C-4 was adjacent to the naphthazarin moiety. The deshielding of H-3 and H-4 and the 11 indices of hydrogen deficiency suggested the presence of a dihydronorcaro-fused α-pyrene system to fulfill the molecular formula requirements, although a shortage of material prevented the detection of the α-pyrene core.8−12 Note that the HMBC spectrum did not reveal the H-3 to C-1 cross peak characteristic of a δ-lactone ring. An overview of the spectroscopic behavior of related structures such as lasione-ctrl,8 lichenicolin A,9 pentacecilides A, B, and C13 or dihydroiscoumacarins14 revealed that none of them displayed this key correlation. This correlation is also absent from the HMBC spectrum of haemoventosin 5. In addition, the HMBC spectrum did not reveal any cross-peak between H-4 and H-12, but HMBC correlations between analogous positions in molecules displaying the same fused α-pyrene/tetrahydrofuran substructure are also often lacking (e.g., the isocoumarin series, exserolides A-E,15 and 11-hydroxymonocerin11), thus being consistent with the presence of the tetrahydrofuran ring. On the basis of the aforementioned data, the 2D structure of compound 1 was established as depicted in Figure 1. This dihydronorcaro-fused α-pyrene system has been reported for a limited number of naphthopyranones,15 for example, lasione-ctrl,8,16 and lichenicolins A and B.9 Its fusion with a naphthazarin core is described here for the first time.

Regarding the relative configuration of 1, NOESY correlations of H-11α (δH 2.00) with H-3, H-4, and CH12−13 showed that these protons and the methyl group are cofacial. The 3JHH value of 2.9 Hz is consistent with that described for related compounds having a cis junction of the tetrahydrofuran-fused α-pyrene moiety, whereas compounds having a trans junction show a lower value.10 The NOESY correlation between H-11β (δH 2.67) and H-12 confined these protons to the opposite face of the tetrahydrofuran ring (Figure 2).

The (3S, 4S, 12S) absolute configuration of 1, ophioparmin, was subsequently assigned based on a comparison between the calculated (TDDFT) and experimental electronic circular dichroism (ECD) spectra (Figure 3).

The 1H NMR spectrum of compound 2, with a molecular formula of C16H14O8, exhibited many signals analogous to that of compound 5, but rather than the diastereotropic methylene protons, the spectrum revealed the presence of a second oxygenated methine at δH 4.71 (1H, d, J = 1.5 Hz, H-4) and an aliphatic methine group at δH 3.46 (3H, s, 4-OCH3) (Table 1). Key HMBC correlations of H-4 with C-4a and C-10a and of 4-OCH3 with C-4 defined the position of this aliphatic methine group at C-4 (Figure 4). The comparison between the calculated and experimental ECD spectra facilitated the identification of 2 as (3S,4R)-4-methoxyhaemoventosin (Figure 3).

The 1H NMR spectrum of compound 2 revealed that most of the signals were duplicated to yield minor peaks that overlap more or less completely with the major ones. Two signals are notably modified: the aliphatic methyl group CH3-11, which was shifted upfield (δH 1.28 to 1.62), whereas the oxygenated methine H-3 was shielded in the minor form (δH 5.05 to 5.45) with identical coupling constants. Because the peak associated with the methine H-4 was also slightly shifted (δH 4.71 for 2a vs 4.66 for 2b), compound 2 was hypothesized to be a mixture of diastereoisomers with the major form 2a (as suggested from the ECD data of the mixture) and the minor form 2b in a 2:1 ratio. Overlapping signals arising from the common scaffold of 2a and 2b prevented the determination of13C NMR data related to the minor compound 2b. The ECD data of a diastereoisomeric mixture can still provide valuable information, particularly when the calculated ECD spectrum shows an excellent fit with the experimental spectrum of the mixture, as in Figure 3C. Because each component contributes to the overall electronic circular dichroism spectrum with a weight comparable to its molar fraction, the entire spectrum is expected to reflect that of the predominant compound.17 A proof-of-principle study established the reliability of ECD data on an epimeric mixture through the example of tetracyclins and 4-epitetracyclins.16 Regarding the identical vicinal coupling constants for H-3 and H-4 between 2a and 2b, NMR studies in related dihydro-2-pyrones indicated that small values of the vicinal coupling between H-3 and H-4 suggest a synclinal (gauche) effect but do not allow distinction between the cis (axial-equatorial) and trans (equatorial-equatorial) orientations of H-3 and H-4, only excluding a trans-diaxial arrangement of these protons.18−21 This further supported the hypothesis of two diastereoisomers, suggesting that 2b might represent a 3,4-cis isomer. Considering the identical configurations of C-3 in 1, 2a, and 5 and assuming that oxidation at C-4 occurs after cyclization during the biosynthesis process,22 2b is hypothesized to correspond to (3S,4S)-4-methoxyhaemoventosin. This hypothesis is further supported by the interpretation of the 1H NMR chemical shift differences of the oxygenated methine and the aliphatic methyl of the two isomers. Indeed, signals located in the shielding zone of the benzene ring are located in an axial or cis-configuration due to the low magnitude of the vicinal coupling constant between H-3 and H-4, the axial position of H-3 implies the equatorial position of H-4, thus confirming the 3,4-cis configuration of 2b.

Figure 2. Key COSY (bold bonds, left) and HMBC (1H → 13C) (arrows, left) and key NOESY correlations (double-headed arrows, right) of compound 1.
Compound 3 could be assigned a molecular formula of C_{15}H_{12}O_{8}, which differs from haemoventosin 5 by an additional oxygen atom. Their 1H NMR spectra exhibited close similarities, but that of 3 lacked the diastereotopic methylene signals of haemoventosin 5, which were replaced by an oxygenated methine (δH 3.93), suggesting the replacement of one diastereotopic proton by a hydroxy group (Table 1). Indeed, HMBC correlations between H-4 and C-3, C-4a, C-5, and C-10a validated the C-4 location of this hydroxy group (Figure 4), and compound 3 was thus identified as 4-hydroxyhaemoventosin.

The absolute configuration of 3 could not be established through comparison between the calculated and experimental ECD spectra, which provided no clear-cut match, irrespective of the absolute configuration used in TDDFT. The chemical shifts of the oxygenated methine and the aliphatic methyl, reminiscent of that of 2a, indicated the equatorial position of H-3. The positive Cotton effect observed at approximately 260 nm in the ECD spectrum can be correlated to the P-helicity of the heterocyclic ring (Figure 3). Collectively, these results suggested the 3S-configuration, which is shared with the metabolites described herein. Assuming the H-3α_{eq} orientation of 3, either a trans H-3α_{eq}−H-4β_{eq} or a cis H-3α_{eq}−H-4α_{ax} might account for the absence of coupling between H-3 and H-4, hence precluding the assignment of the C-4 configuration.

Compound 4 was identified as anhydrofusarubin lactone on the basis of its 1H and 13C NMR data. This naphthoquinone was previously isolated from the phytopatho-

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**Table 2. Cytotoxicity Against Cultured Cell Lines (IC_{50}) and Antioxidant Activity Results**

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<th>tested compound or fraction</th>
<th>cytotoxic activity</th>
<th>antioxidant activity</th>
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<td></td>
<td>B16</td>
<td>HaCaT</td>
</tr>
<tr>
<td>pigment fraction</td>
<td>6.30 ± 0.8 μg mL^{-1}</td>
<td>3.30 ± 0.8 μg mL^{-1}</td>
</tr>
<tr>
<td>1</td>
<td>&gt;10 μM</td>
<td>&gt;10 μM</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>&gt;500 μg mL^{-1}</td>
</tr>
<tr>
<td>5</td>
<td>2.40 ± 0.4 μM</td>
<td>14.20 ± 1.0 μM</td>
</tr>
<tr>
<td>doxorubicin</td>
<td>0.43 ± 0.13 μM</td>
<td>0.53 ± 0.07 μM</td>
</tr>
<tr>
<td>vincristine</td>
<td>0.50 ± 0.35 μM</td>
<td>0.65 ± 0.20 μM</td>
</tr>
<tr>
<td>gallic acid</td>
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<td>7.20 ± 0.3 μg mL^{-1}</td>
</tr>
<tr>
<td>ascorbic acid</td>
<td>-</td>
<td>22.10 ± 7.1 μM</td>
</tr>
</tbody>
</table>
genic fungus Fusarium solani, but this is its first report from a lichen, even though closely related molecules such as fusarubin, anhydrofusarubin lactol, and coronatoquinone are all known from lichen sources.\textsuperscript{27–29} Compound 5 was identified as (+)-haemoventosin by comparison of its \textsuperscript{1}H and \textsuperscript{13}C NMR data and optical activity with an earlier report on the structural reassessment of this compound.\textsuperscript{6}

The cytotoxicity of the pigment-enriched fraction was investigated against B16 murine melanoma and HaCaT human keratinocyte cell lines, revealing significant cytotoxic activities with respective IC\textsubscript{50} values of 6.3 ± 0.8 μg mL\textsuperscript{−1} and 3.3 ± 0.8 μg mL\textsuperscript{−1}. This semipurified extract exhibited strong antioxidant properties via an NBT test with an IC\textsubscript{50} value of 8.3 ± 1.4 μg mL\textsuperscript{−1} compared to that of ascorbic acid used as a positive control (3.8 ± 1.2 μg mL\textsuperscript{−1}). Owing to a shortage of material, only 1 and 5 could be evaluated for their cytotoxicity (Table 2). Only the latter exhibited a significant cytotoxicity, which prompted a further study of its bioactivity on seven other cell lines. Compound 5 exhibited significant cytotoxic activity toward all cell lines, with IC\textsubscript{50} values ranging from 3 to 10 μM (Figure S20, Supporting Information).

Compounds 1, 2, and 5 were tested for radical-scavenging activity against superoxide anion (O\textsubscript{2}\textsuperscript{−}), and compound 5 was also tested against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. No compounds or fractions exhibited activity during a DPPH assay (IC\textsubscript{50} > 3000 μM), which may be explained by the intramolecular hydrogen bonds between phenolic hydroxy groups and quinone moieties of all the structures described herein.\textsuperscript{30} As superoxide anion scavengers, compound 1 exhibited moderate activity, with an IC\textsubscript{50} value of 50 ± 4 μM, whereas compounds 2 and 5 both exhibited stronger activities than that of ascorbic acid used as a positive control (Table 2).

In summary, new minor quinonoid naphthopyranones ophioparmin (1), 4-methoxyhaemoventosins (2a and 2b) and 4-hydroxyhaemoventosin (3), as well as the known anhydrosusarubin lactone (4) and haemoventosin (5), were isolated from the apothecia of the lichen O. ventosa. Compounds 1, 2, and 5 exhibit moderate to strong antioxidant activities, and 5 exhibits a significant cytotoxic effect against a panel of nine cell lines. These pigments appear to be exclusively located in the fruiting bodies of the lichen, and ongoing mass spectrometric imaging should support this assumption. Reproductive structures are often reported to produce other lichen compounds than the remaining thallus\textsuperscript{31} or known to be more concentrated in phenols compared to somatic tissues.\textsuperscript{32} This is consistent with the optimal defense theory that predicts the allocation of defensive compounds to structures that are the most valuable for the fitness of the organism. Regarding the specific example of O. ventosa, the presence of such quinones might account for the atypical bacterial communities sheltered by its apothecia, selecting bacterial strains that are able to withstand such a cytotoxic mixture.\textsuperscript{33} Because these molecules concentrate at the surface of the fruiting bodies, their spreading at the surface of the spores might be beneficial to outcompete other microorganisms. Finally, these compounds might act as antioxidant and UV screening filters to protect spores during their maturation within ascii.

## EXPERIMENTAL SECTION

### General Experimental Procedures.

Optical rotations were recorded using a PerkinElmer 341 automatic polarimeter at 293 K at the sodium line; [\(\alpha\)]\textsubscript{D} are given in 10\textsuperscript{−} deg·cm\textsuperscript{−1}·g\textsuperscript{−1}. Electronic circular dichroism and corresponding UV–visible spectra were recorded using a Jasco J-815 ECD spectrometer. 1D and 2D NMR spectra were recorded in CDCl\textsubscript{3} using a 500 MHz spectrometer equipped with a TCI cryoprobe at the PRISM core facility (Rennes, France). HRMS data on the quinone-enriched fraction were measured using a Thermo Fisher Scientific Q Exactive equipped with an ESI source by direct injection at a rate of 400 nL·min\textsuperscript{−1} at the CRMPO (Centre Régional de Mesures Physiques de l’Ouest, Rennes 1), and the measurements were performed in positive-ion mode. Analytical grade solvents for extraction and chromatography were purchased from VWR International (Pennsylvania, USA). Open column chromatography was performed on silica gel (40–63 μm, Kieselgel, Merck) in normal phase and at atmospheric pressure. The preparative TLC plates used for purification were glass backed (20 × 20 cm) and normal phase silica plates (250 μm thickness) purchased from Macherey-Nagel (Düren, Germany). Solutions were applied to the preparative plate at 12 mm from the lower edge using an automatic TLC sampler III device (Camag, Muttenz, Switzerland) as streaks with a 160 mm length. The preparative TLCs were developed in dedicated solvent systems, and the plates were allowed to fully air-dry prior being rerun in the same solvent system. Migrations were thus repeated until a good separation could be achieved between the closely related naphthopyranones. Subsequently, the silica was scraped off the plate, and the band of interest was desorbed in CH\textsubscript{2}Cl\textsubscript{2}/MeOH (95/5) three times (30 min each).

### Plant Material.

Two samples of Ophioparma ventosa were harvested for this study:

- Sample 1 was harvested in Tyrol (Austria), 500 m south of Oberurgl (elevation 1800–1850 m). The lichen was collected and identified by one of the authors (J.B.) in 04/2009. A voucher specimen was deposited at the herbarium of PNNSCM laboratory with the reference JB/09/158.
- Sample 2 was harvested in Styria (Austria) at the south of the lake Großer Winterleitensee (elevation 1950–2000 m). The specimen was collected and identified by one of the authors (W.O.) in 09/2014. A voucher specimen (No. 13218a) was deposited at the herbarium GZU.

### Extraction and Purification of Compounds.

Out of 100 g of thallus, the meticulos dissection of O. ventosa’s apothecia from sample 1 yielded 1 g of apothecia that was powdered and extracted with CH\textsubscript{2}Cl\textsubscript{2} (4 × 10 mL, 4 h) to afford a total of 100 mg of extract. This raw extract was washed three times with a 9:1 mixture of cyclohexane/EtOAc to afford 40 mg of a naphthoquinone-enriched fraction. This fraction was subsequently chromatographed on silica gel using a cyclohexane/EtOAc/CH\textsubscript{2}Cl\textsubscript{2}/MeOH gradient system (1:0:0:0 to 0:0:1:1 via 0:1:0:0) to yield seven fractions (F1–F7). Fraction F7 (20 mg), which was primarily composed of naphthazarin pigments, was selected for further purification using column chromatography based on a CH\textsubscript{2}Cl\textsubscript{2}/MeOH (1:0 to 7:3), affording five subfractions (F71 to F75). Subfraction F74 (8 mg) was subjected to a preparative TLC in a toluene/EtOAc/formic acid (70/25/5) solvent system. Five consecutive runs were required to achieve a proper separation of the compounds. Three bands were desorbed from this plate, affording anhydrofusarubin lactone (4) (1 mg), haemoventosin (5) (1.8 mg), and ophioparmin (1) (0.4 mg). Subfraction F75 (6 mg) was also subjected to preparative TLC in CH\textsubscript{2}Cl\textsubscript{2}/
EtOAc/formic acid (88/10/2), and the plate was developed twice. This second plate yielded 0.4 mg of 4-hydroxyhaemoventosin (2).

Likewise, 1.1 g of apothecia could be recovered from sample 2. Apothecia were ground using a mortar and pestle prior to extracting the powdered material with CH₂Cl₂ (4 × 10 mL) at room temperature for 4 h.

The different extracts were combined to yield a total mass of 115 mg, which was subjected to silica gel column chromatography using a cyclohexane/EtOAc/MeOH gradient system (8:2:0.0 to 0.0:1:1) to yield five main fractions (F1–F5). Fraction F4 (20 mg) was separated by preparative TLC using the same protocol as described earlier for fraction F7 on the basis of its observed similarities with the latter. Owing to the higher weight of this fraction, the material was applied to three TLC plates. After five runs each, these plates afforded a total of 6 mg of haemoventosin (5) and 0.2 mg of ophioparin (1). Three successive preparative TLC runs from fraction F5 (4.5 mg) in a toluene/EtOAc/formic acid (70/25/5) solvent system afforded 0.7 mg of 4-methoxyhaemoventosin (2).

Ophioparin (1). Red amorphous powder. UV (MeCN), λ<sub>max</sub> (log ε) 233 (4.57), 285 (4.23), 501 (4.02); ECD (c = 0.9 mM, MeCN) λ<sub>max</sub> (Δε) 258 (−11.21), 311 (26.38); 1H NMR and 13C NMR data, see Table 1; (+)-HRESIMS m/z 369.05830 [M + Na]<sup>+</sup> (calcd for C₁₇H₁₄O₈Na, 369.05809).

4-Methoxyhaemoventosins (2a, 2b). Red amorphous powder. UV (MeCN), λ<sub>max</sub> (log ε) 231 (3.50), 291 (3.05), 501 (2.97); ECD (c = 0.5 mM, MeCN) λ<sub>max</sub> (Δε) 267 (2.55), 309.5 (−3.20), 374.0 (1.44); 1H NMR and 13C NMR data, see Table 1; (+)-HRESIMS m/z 357.05800 [M + Na]<sup>+</sup> (calcd for C₁₅H₁₀O₇Na, 357.05809).

4-Hydroxyhaemoventosin (3). Red amorphous powder. UV (MeCN), λ<sub>max</sub> (log ε) 228 (3.31), 289 (2.90), 490 (2.72); ECD (c = 0.5 mM, MeCN) λ<sub>max</sub> (Δε) 267.5 (3.42), 293.5 (11.17), 352 (−1.24), 441.5 (+6.24), 499.5 (−4.97); 1H NMR and 13C NMR data, see Table 1; (+)-HRESIMS m/z 343.04240 [M + Na]<sup>+</sup> (calcd for C₁₅H₁₂O₇Na, 343.04244).

Anhydrofusarubin lactone (4). Purple amorphous powder. 1H NMR (CDCl₃, 500 MHz), λ<sub>max</sub> (log ε) 14.22 (1H, s, OH-10), 12.78 (1H, s, OH-5), 6.85 (1H, s, H-4), 6.32 (1H, s, H-8), 3.97 (3H, s, OMe-7), 2.38 (3H, s, Me-11). 13C NMR (CDCl₃, 500 MHz): 184.8 (C-6), 180.5 (C-9), 162.7 (C-3), 160.8 (C-10), 160.0 (C-5), 155.2 (C-5), 141.4 (C-4a), 114.0 (C-10a), 113.4 (C-5a), 111.4 (C-8), 108.1 (9a), 98.2 (C-4), 57.0 (O-7e), 20.6 (C-11); (+)-HRESIMS m/z 325.03200 [M + Na]<sup>+</sup> (calcd for C₁₅H₁₂O₈Na, 325.03187).

Haemoventosin (5). Red bronze-shimmering amorphous powder. [α]<sub>19</sub>°D + 550 (c 0.02, CH₂Cl₂), UV (MeCN), λ<sub>max</sub> (log ε) 232 (3.40), 293 (2.97), 501 (2.88); ECD (c = 0.7 mM, MeCN) λ<sub>max</sub> (Δε) 276 (−1.76), 306.5 (20.31), 489 (4.22); 1H NMR (CDCl₃, 500 MHz): 13.70 (1H, s, OH-10), 12.25 (1H, s, OH-5), 6.23 (1H, s, H-8), 4.60 (1H, m, H-3), 3.97 (3H, s, OMe-7), 3.35 (1H, dd, J = 17.5, 3 Hz), 2.73 (1H, dd, J = 17.5, 11 Hz, H-4), 1.56 (3H, J = 8 Hz, Me-11). 13C NMR (CDCl₃, 500 MHz): 188.2 (C-6), 183.6 (C-9), 160.4 (C-7), 160.0 (C-1), 157.2 (C-10), 152.7 (C-5), 141.4 (C-4a), 122.8 (C-10a), 114.1 (C-5a), 111.1 (C-8), 110.5 (9a), 74.0 (C-3), 56.4 (Ome-7), 29.1 (C-4), 20.0 (C-11); (+)-HRESIMS m/z 327.04750 [M + Na]<sup>+</sup> (calcd for C₁₇H₁₂O₈Na, 327.04752).

### COMPUTATIONAL DETAILS

All DFT calculations were performed using the latest version of the Gaussian09 program. The B3LYP/6-311++G(2d,p) level of theory was selected to conduct the conformational analyses of the four haemoventosin derivatives (1–3, 5), taking into account the various possible enantiomeric or diastereoisomeric structures. The harmonic frequencies were computed analytically at the same level of theory to characterize the stationary points as true minima. Owing to the strong structural similarities between the various derivatives, the common scaffold has been thoroughly investigated only for the haemoventosin 5 (Figure 3), and the resulting features were transposed to structures 1–3. The naphthazarin nucleus appears as a rigid moiety with possible rotation of the C-7 methoxy group and the C-5 and C-10 hydroxy groups. The most stabilized structure involves both hydroxy groups intramolecularly hydrogen-bonded to the quinone carbonyl moieties, and the methoxy substituent repelled from the vicinal carbonyl group. This geometry represents more than 90% of the relative conformational population, irrespective of whether the solvent effects (MeCN) are considered, through Polarizable Continuum Model (PCM) calculations. The ECD spectra were computed for these structures considering various plausible configurations at C-3 and C-4. The ECD spectra were computed with the same functional and basis set as the structures, namely, B3LYP/6-311++G(2d,p), using a time-dependent density functional theory (TD-DFT) implementation. For these calculations, 50 excited states were determined, and the solvent effects were systematically included using the PCM model. Only the spectra clearly corresponding to their experimental counterparts are presented.

### Antioxidant Testing

The scavenging activity of the pigment-enriched fraction and of compound 5 on the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) was measured as described elsewhere. For the same compounds, measurement of superoxide anion scavenging activity in 96-well microplates was based on the published nonenzymatic method with some modifications. The reaction mixture in the sample wells consisted of NADH (78 μM), NBT (50 μM), PMS (10 μM), and lichen compounds (350, 116, 39, and 13 μM). The reagents were dissolved in 16 mM tris-hydrochloride buffer at pH = 8, except for all the lichen compounds, which were dissolved in DMSO. After 5 min of incubation at room temperature, the spectrophotometric measurement was performed at 560 nm against a blank sample without PMS. Ascorbic acid was used as a positive control. The percentage inhibition at steady state for each dilution was used to calculate the IC₅₀ values. This provided the amount of antioxidant required (measured as the concentration of the stock solution added to the reaction mixture) to scavenge 50% of O₂⁻. With lower values indicating more effective scavenging of O₂⁻. All tests were conducted in triplicate, and the results were averaged.

### Cell Culture and Survival Assays

The cytotoxic activity of the pigment-enriched fraction and of compound 5 was determined against B16 murine melanoma and HaCaT human keratinocyte cell lines as described elsewhere.

The in vitro inhibition of cell proliferation of 5 was determined using a panel of 6 representative cell lines, namely, Huh7D12 (differential hepatocellular carcinoma), Caco 2 (differentiating colorectal adenocarcinoma), MDA-MB-231 (breast carcinoma), HCT-116 (actively proliferating colorectal carcinoma), PC3 (prostate carcinoma), NCI-H2 (lung carcinoma), and diploid skin fibroblasts as normal cell lines for control. Cells were grown as described elsewhere. The inhibition of cell proliferation was assessed as in Coulthaly et al.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b01073.

Copies of 1D (1H, 13C) and 2D NMR and ECD spectra for compounds 1–5 as well as details regarding the cytotoxic activity of compound 5 (PDF)
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REFERENCES


The authors declare no competing financial interest.