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

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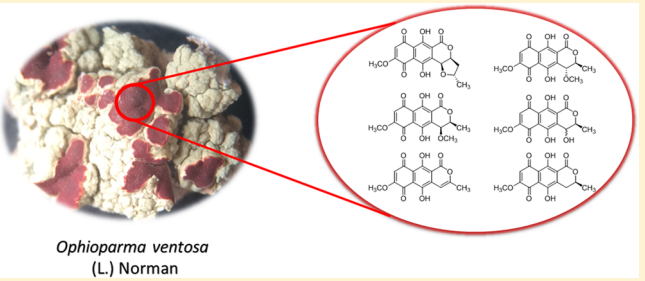
Supporting Information

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.[1] 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.[2] 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 tetrahydroxyfatty acid (ventosic acid) and some additional compounds, such as psoromic, stictic, and norstictic acids and atranorin, among others.[3,4] Regarding the intense blood-red color of *O. ventosa* apothecia, Bruun and Lamvik first reported the isolation of its major pigment, haemoventosin, but they assigned an erroneous furanonaphthoquinone structure.[5] The correct pyranonaphthoquinone structure of haemoventosin was assigned in 1995.[6] Several authors reported on the presence of "numerous" minor pigments most likely corresponding to additional quinones,[4,6] 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;[7] 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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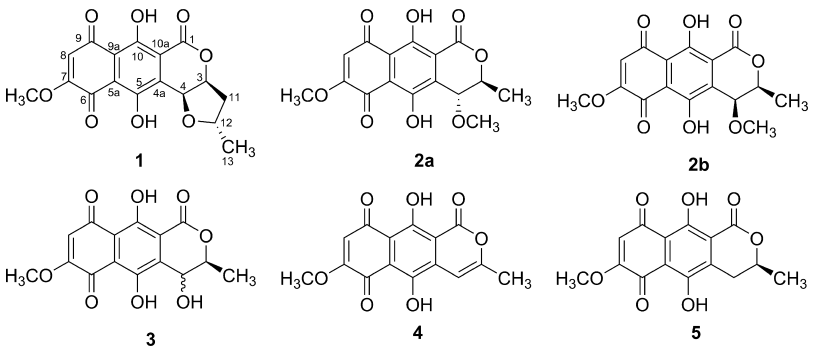

Figure 1. Structures of compounds **1**−**5**.

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data for 1−3 ($CDCl_3$)

	ophioparmin (**1**)		4-methoxyhaemoventosin (**2**)			4-hydroxyhaemoventosin (**3**)	
			major diastereoisomer (**2a**)		minor diastereoisomer (**2b**)		
position	δ_C, type	δ_H, mult. (*J* in Hz)	δ_C, type	δ_H, mult. (*J* in Hz)	δ_H, mult. (*J* in Hz)	δ_C, type	δ_H, mult. (*J* in Hz)
1	ND		159.0			157.9	
3	81.7	5.18, dd (4.0, 2.9)	76.5	5.05 dq (7.0, 1.5)	4.54, dq (6.5, 1.5)	74.2	5.14, q (6.9)
4	66.9	5.31, d (2.9)	69.8	4.71 d (1.5)	4.66, d (1.5)	39.2	3.93, s
4-OCH_3	-	-	57.5	3.46, s	3.46, s		
4a	136.7		136.8			137.9	
5	153.7		153.6			153.3	
5-OH	-	12.29	-	12.25, s	12.29, s	-	11.81, s
5a	115.4		115.4			114.4	
6	188.0		187.6			188.3	
7	160.9		160.2			160.5	
7-OCH_3	56.8	3.97, s	57.4	3.97, s	3.96, s	56.8	3.90, s
8	111.6	6.25, s	112.5	6.26, s	6.25, s	111.2	6.17, s
9	184.1		183.8			183.6	
9a	113.2		112.7			111.9	
10	156.7		156.2			156.4	
10-OH	-	13.66	-	13.51, s	13.53, s		13.52, s
10a	122.1		122.9			123.7	
11α	41.6	2.00, ddd (14.0, 9.8, 4.0)	18.0	1.28, d (7)	1.62, d (6.5)	19.6	1.35, d (6.9)
11β		2.67, dd (14.0, 5.5)					
12	76.6	4.60, m					
13	20.6	1.41, d (6)					

(+)-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_2Cl_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 $C_{12-20}H_{10-20}O_{5-10}$. 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).

Compound **1** was obtained as an amorphous red powder. The ^{13}C NMR and (+)HRESIMS data established a molecular formula of $C_{17}H_{14}O_8$, consistent with a naphthopyrone derivative, and the UV spectrum of **1** showed absorption maxima at 233, 285, and 501 nm, similar to those of haemoventosin **5**. The ^{1}H NMR and HSQC spectra revealed two hydrogen-bonded hydroxy groups at δ_H 13.66 and 12.29; one aromatic proton at δ_H 6.25 (1H, s, H-8); three oxygenated methine signals at δ_H 5.31 (1H, d, *J* = 2.9 Hz, H-4), 5.18 (1H, dd, *J* = 4.0, 2.9 Hz, H-3), and 4.60 (1H, m, H-12); two diastereotopic methylene hydrogens at δ_H 2.67 (1H, dd, *J* =

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14.0, 5.5 Hz, H-11$_\beta$) and 2.00 (1H, ddd, J = 14.0, 9.8, 4.0 Hz, H-11$_\alpha$); one aromatic methoxy singlet at δ_H 3.97 (3H, s, 7-OCH$_3$); and one aliphatic methyl group at δ_H 1.41 (3H, d, J = 6 Hz, H-13). These data combined with the ^{13}C 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 δ_H/δ_C 5.31/66.9 (H-4) and δ_H/δ_C 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 CH$_3$-13/CH-12/CH$_2$−11/CH-3/CH-4 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 dihydrofurano-fused α-pyrone system to fulfill the molecular formula requirements, although a shortage of material prevented the detection of the α-pyrone carbonyl.[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 lasionectrin;[8] lichenicolin A;[9] pentacecilides A, B, and C;[13] or dihydroisocoumarins[14] 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-pyrone/tetrahydrofuran substructure are also often lacking (e.g., the isocoumarin series, exserolides A-E,[10] and 11-hydroxymonocerin[11]), thus being consistent with the presence of the tetrahydrofuran ring. On the basis of these aforementioned data, the 2D structure of compound **1** was established as depicted in Figure 1. This dihydrofurano-fused α-pyrone system has been reported for a limited number of naphthopyranones,[15] for example, lasionectrin[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 (δ_H 2.00) with H-3, H-4, and CH$_3$-13 showed that these protons and the methyl group are cofacial. The $^3J_{3,4}$ value of 2.9 Hz is consistent with that described for related compounds having a *cis* junction of the tetrahydrofuran-fused α-pyrone moiety, whereas compounds having a *trans* junction show a lower value.[10] The NOESY correlation between H-11β (δ_H 2.67) and H-12 confined these protons to the opposite face of the tetrahydrofuran ring (Figure 2).

The (3*S*, 4*S*, 12*S*) 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).

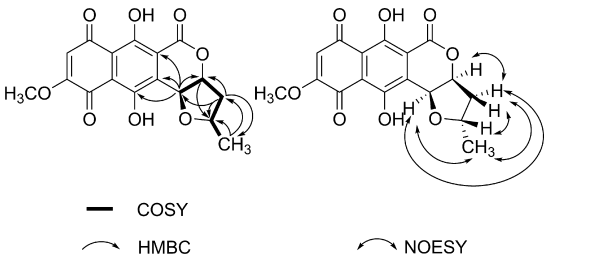

Figure 2. Key COSY (bold bonds, left) and HMBC (^{1}H → ^{13}C) (arrows, left) and key NOESY correlations (double-headed arrows, right) of compound **1**.

The ^{1}H NMR spectrum of compound **2**, with a molecular formula of $C_{16}H_{14}O_8$, exhibited many signals analogous to that of compound **5**, but rather than the diastereotopic methylene protons, the spectrum revealed the presence of a second oxygenated methine at δ_H 4.71 (1H, d, J = 1.5 Hz, H-4) and an aliphatic methoxy group at δ_H 3.46 (3H, s, 4-OCH$_3$) (Table 1). Key HMBC correlations of H-4 with C-4a and C-10a and of 4-OCH$_3$ with C-4 defined the position of this aliphatic methoxy group at C-4 (Figure 4). The comparison between the calculated and experimental ECD spectra facilitated the identification of **2** as (3*S*,4*R*)-4-methoxyhaemoventosin (Figure 3).

The ^{1}H 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 CH$_3$-11, which was shifted upfield (δ_H 1.28 to 1.62), whereas the oxygenated methine H-3 was shielded in the minor form (δ_H 5.05 to 4.54) with identical coupling constants. Because the peak associated with the methine H-4 was also slightly shifted (δ_H 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 of ^{13}C 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 tetracyclin and 4-epitetracyclin.[18] 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.[19−21] 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 (3*S*,4*S*)-4-methoxyhaemoventosin. This hypothesis is further supported by the interpretation of the ^{1}H 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 axial-like position (CH$_3$-11 in **2a** and H-3 in **2b**), whereas deshielded signals refer to moieties located in an equatorial position (CH$_3$-11 in **2b** and H-3 in **2a**), as assessed on closely related systems.[23] Given the impossibility of an axial/axial 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**.

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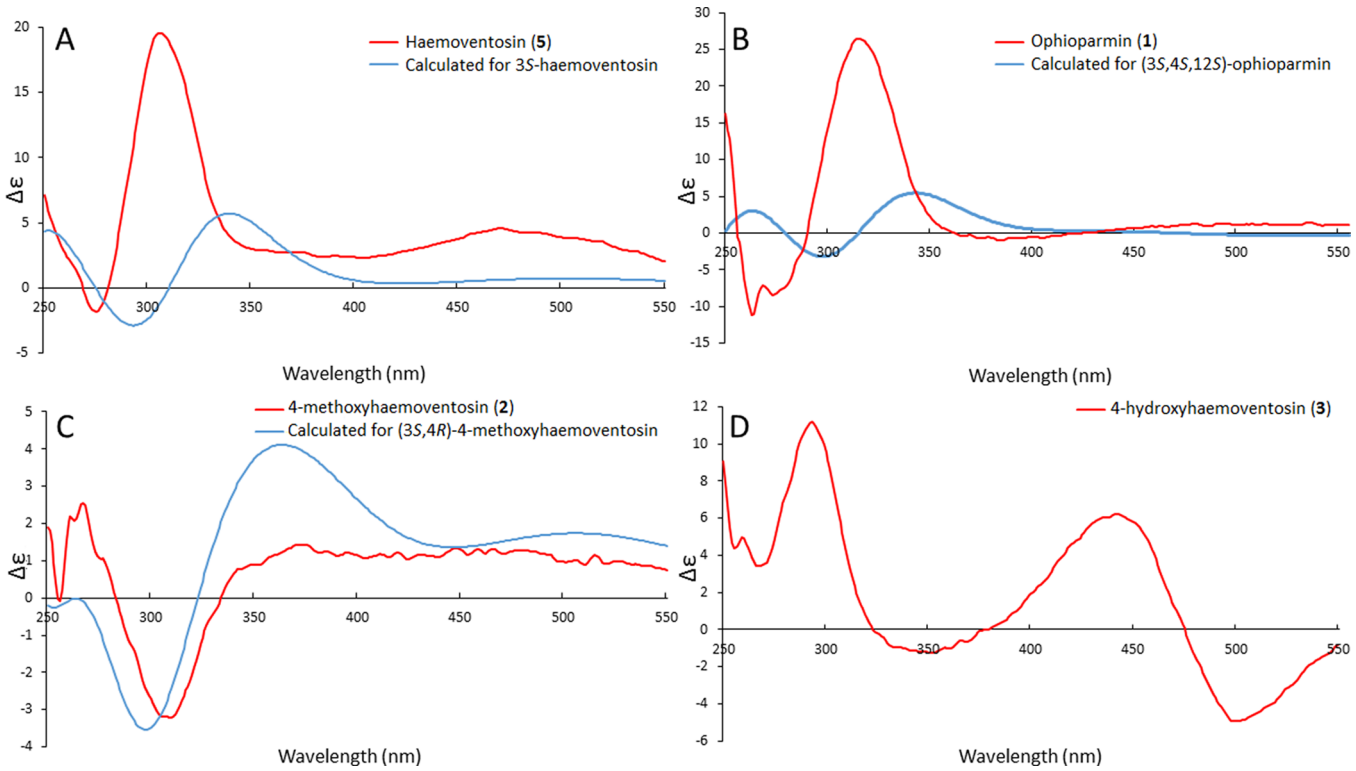

Figure 3. Comparison of the experimental ECD spectra of **1** (B), **2** (C), and **5** (A) and calculated ECD spectra for the stereoisomers shown in Figure 1. Experimental ECD spectrum of **3** (D).

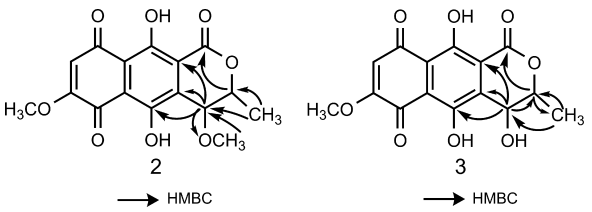

Figure 4. Diagnostic HMBC correlations ($^{1}H \rightarrow {}^{13}C$) of compounds **2** and **3**.

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 ^{1}H NMR spectra exhibited close similarities, but that of **3** lacked the diastereotopic methylene signals of haemoventosin **5**, which were replaced by an oxygenated methine (δ_H 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).[24] Collectively, these results suggested the 3*S*-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 ^{1}H and ^{13}C NMR data.[25,26] This naphthoquinone was previously isolated from the phytopatho-

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

	cytotoxic activity		antioxidant activity	
tested compound or fraction	B16	HaCaT	DPPH	NBT
pigment fraction	6.30 ± 0.8 μg mL^{-1}	3.30 ± 0.8 μg mL^{-1}	>500 μg mL^{-1}	8.30 ± 1.4 μg mL^{-1}
1	>10 μM	>10 μM	-	49.90 ± 4.0 μM
2	-	-	-	6.10 ± 1.2 μM
5	2.40 ± 0.4 μM	14.20 ± 1.0 μM	>500 μg mL^{-1}	1.90 ± 0.4 μM
doxorubicin	0.43 ± 0.13 μM	0.53 ± 0.07 μM	-	-
vincristine	0.50 ± 0.35 μM	0.65 ± 0.20 μM	-	-
gallic acid	-	-	7.20 ± 0.3 μg mL^{-1}	-
ascorbic acid	-	-	-	22.10 ± 7.1 μM

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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.[27−29] Compound **5** was identified as (+)-haemoventosin by comparison of its ^{1}H and ^{13}C NMR data and optical activity with an earlier report on the structural reassessment of this compound.[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_{50} values of 6.3 ± 0.8 μg mL^{-1} and 3.3 ± 0.8 μg mL^{-1}. This semipurified extract exhibited strong antioxidant properties via an NBT test with an IC_{50} value of 8.3 ± 1.4 μg mL^{-1} compared to that of ascorbic acid used as a positive control (3.8 ± 1.2 μg mL^{-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_{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 ($O_2^{-\bullet}$), 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_{50} **5** > 3000 μM), which may be explained by the intramolecular hydrogen bonds between phenolic hydroxy groups and quinone moieties of all the structures described herein.[30] As superoxide anion scavengers, compound **1** exhibited moderate activity, with an IC_{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 anhydrofusarubin 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[31] or known to be more concentrated in phenols compared to somatic tissues.[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.[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 asci.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were recorded using a PerkinElmer 341 automatic polarimeter at 293 K at the sodium line; $[\alpha]_D$ are given in 10^{-1} deg·cm^{-2}·g^{-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 $CDCl_3$ 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^{-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_2Cl_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 Obergurgl (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 PNSCM 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 meticulous dissection of *O. ventosa*'s apothecia from sample 1 yielded 1 g of apothecia that was powdered and extracted with CH_2Cl_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_2Cl_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_2Cl_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_2Cl_2/

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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 CH_2Cl_2 (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/CH_2Cl_2/MeOH gradient system (8:2:0:0 to 0:0:1:1) to yield five main fractions (F'1-F'5). Fraction F'4 (20 mg) was separated by preparative TLC using the same protocol as described earlier for fraction F74 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 ophioparmin (**1**). Three successive preparative TLC runs from fraction F'5 (4.5 mg) in a toluene/EtOAc/formic acid (70/25/5) solvent system afforded 0.7 mg of 4-methoxyhaemoventosin (**2**).

*Ophioparmin (**1**).* Red amorphous powder. UV (MeCN), λ_{max} (log ε) 233 (4.57), 285 (4.23), 501 (4.02); ECD (c = 0.9 mM, MeCN) λ_{max} ($\Delta\varepsilon$) 258 (−11.21), 311 (26.38); ^{1}H NMR and ^{13}C NMR data, see Table 1; (+)-HRESIMS m/z 369.05830 $[M + Na]^+$ (calcd for $C_{17}H_{14}O_8Na$, 369.05809).

*4-Methoxyhaemoventosins (**2a**, **2b**).* Red amorphous powder. UV (MeCN), λ_{max} (log ε) 231 (3.50), 291 (3.05), 501 (2.97); ECD (c = 0.5 mM, MeCN) λ_{max} ($\Delta\varepsilon$) 267 (2.55), 309.5 (−3.20), 374.0 (1.44); ^{1}H NMR and ^{13}C NMR data, see Table 1; (+)-HRESIMS m/z 357.0580 $[M + Na]^+$ (calcd for $C_{16}H_{14}O_8Na$, 357.05809).

*4-Hydroxyhaemoventosin (**3**).* Red amorphous powder. UV (MeCN), λ_{max} (log ε) 228 (3.31), 289 (2.90), 490 (2.72); ECD (c = 0.5 mM, MeCN)) λ_{max} ($\Delta\varepsilon$) 267.5 (3.42), 293.5 (11.17), 352 (−1.24), 441.5 (+6.24), 499.5 (−4.97); ^{1}H NMR and ^{13}C NMR data, see Table 1; (+)-HRESIMS m/z 343.04240 $[M + Na]^+$ (calcd for $C_{15}H_{12}O_8Na$, 343.04244).

*Anhydrofusarubin lactone (**4**).* Purple amorphous powder. ^{1}H NMR ($CDCl_3$, 500 MHz): 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). ^{13}C NMR ($CDCl_3$, 500 MHz): 184.8 (C-6), 180.5 (C-9), 162.7 (C-3), 160.8 (C-10), 160.0 (C-7), 155.2 (C-5), 141.4 (C-4a), 114.0 (C-10a), 113.4 (C-5a), 111.4 (C-8), 108.1 (C-9a), 98.2 (C-4), 57.0 (OMe-7), 20.6 (C-11); (+)-HRESIMS m/z 325.03200 $[M + Na]^+$ (calcd for $C_{15}H_{10}O_7Na$, 325.03187).

*Haemoventosin (**5**).* Red bronze-shimmering amorphous powder. $[\alpha]^{20}_D$ + 550 (c 0.02, CH_2Cl_2). UV (MeCN), λ_{max} (log ε) 232 (3.40), 293 (2.97), 501 (2.88); ECD (c = 0.7 mM, MeCN) λ_{max} ($\Delta\varepsilon$) 276 (−1.76), 306.5 (20.31), 489 (4.22); ^{1}H NMR ($CDCl_3$, 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). ^{13}C NMR ($CDCl_3$, 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 (C-9a), 74.0 (C-3), 56.4 (OMe-7), 29.1 (C-4), 20.0 (C-11); (+)-HRESIMS m/z 327.04750 $[M + Na]^+$ (calcd for $C_{15}H_{12}O_7Na$, 327.04752).

COMPUTATIONAL DETAILS

All DFT calculations were performed using the latest version of the Gaussian09 program.[34] 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.[35] 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.[36] 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 IC_{50} 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 $O_2^{-\bullet}$, with lower values indicating more effective scavenging of $O_2^{-\bullet}$. 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.[37,38]

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.[39] The inhibition of cell proliferation was assessed as in Coulibaly et al.[28]

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 (^{1}H, ^{13}C) 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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Notes

The authors declare no competing financial interest.

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