Metabolites from the Lichen *Ochrolechia parella* Growing under Two Different Heliotropic Conditions[⊥]

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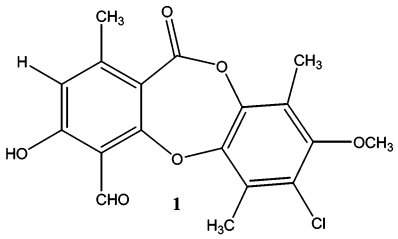
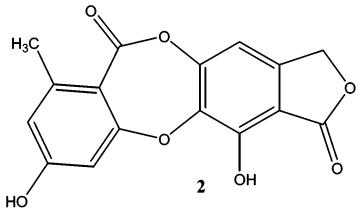
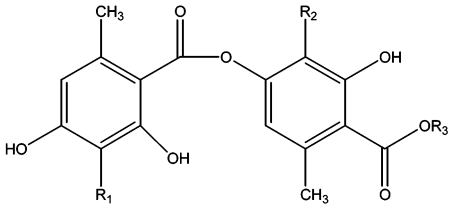
A new chloro-depsidone (**1**) and five known compounds, variolaric acid (**2**), lecanoric acid (**3**), α-alectoronic acid (**4**), atranorin (**5**), and ergosterol peroxide (**6**), have been isolated from the lichen *Ochrolechia parella*. The structure of compound **1** was elucidated by spectroscopic analysis. Additionally, the tautomeric equilibrium of compound **4** was investigated. In the present study, two specimens of this lichen, growing under different light conditions, were analyzed. The major compound in both samples was found to be **2**, but the amount of this metabolite was significantly higher in the shaded specimen (0.76% w/w). The new compound parellin (**1**) predominated in the specimen grown under shady conditions, while atranorin (**5**) was found only in the sunlit specimen. The cytotoxic activities of **2**, **4**, and **6** against B16 melanoma cells were evaluated.

Ochrolechia parella (L.) A. Massal. (Pertusariaceae) is a common lichen found on siliceous rocks, walls, or trees. In Brittany, France, this gray to buff-gray crustose lichen with flesh to white apothecia surrounded by thick margins is found particularly on the seashore. Up to the end of the last century, *O. parella* was collected to produce the purple dye "parelle".[1] A depsidone, variolaric acid,[2] and a depside, lecanoric acid,[3] have previously been reported as major components of this lichen. Additional metabolites such as gyrophoric acid have been characterized in a large taxonomic survey of the genus *Ochrolechia* in Europe.[4] Herein, we describe the isolation and structural elucidation of a new depsidone, parellin (**1**), and report four constituents of previously known structures (**2**−**6**) as well as the cytotoxic activities of **2**, **4**, and **6** against B16 melanoma cells. Variations were observed in the secondary metabolites produced when two specimens of this species, growing on the same substrate but under shaded or sunlight conditions, were investigated separately. Three successive extractions with solvents of increasing polarity (*n*-heptane, CH_2Cl_2, THF) were performed on these two samples. Whereas the extraction percentages of the CH_2Cl_2 extracts for the shaded lichen and the sunlit one were similar (0.7% and 0.5% w/w, respectively), notable differences have been observed for the *n*-heptane extracts (0.13% and 0.08% w/w, respectively) and especially for the THF extracts (5.54% and 2.7% w/w, respectively).

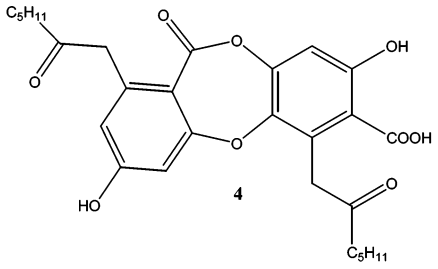
Isolation and purification of compounds from *O. parella* were performed using combinations of two column chromatographic techniques (Sephadex LH-20, silica gel). After chromatography on a silica gel column (see Experimental Section), the *n*-heptane extract of the shaded sample afforded compound **1** (1.5 mg), whereas the sunlit sample yielded compound **5** (2.9 mg). This qualitative difference was confirmed by comparing the TLC profiles of the two extracts. Compound **1** appeared as a prominent bright white-blue spot in the shaded sample. The widely distributed depside atranorin (**5**) was identified on the basis of spectroscopic data,[5] but was only found in the sunlit lichen sample. Indeed, a previous study has reported the increased accumulation of **5** in *Cladonia* species under UV irradiation.[6] Ergosterol peroxide (**6**) (1.6 mg) and the less common α-alectoronic acid (**4**) (80 mg) were obtained from both samples following silica gel column chromatography of the CH_2Cl_2 extract. The latter is a depsidone for which the ^1H NMR spectrum showed signals characteristic for two *β*-keto alkyl C-7

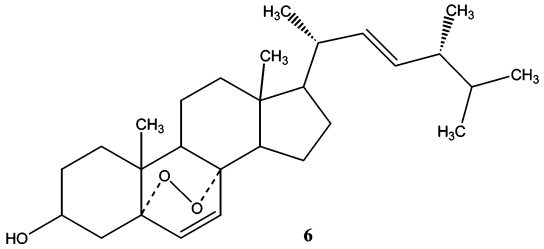






3: $R_1 = R_2 = R_3 = H$
5: $R_1 = CHO$; $R_2 = R_3 = CH_3$





[⊥] Dedicated to the memory of Prof. Pierre Potier of the Institut de Chimie des Substances Naturelles, Gif-sur-Yvette.

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Table 1. ^{13}C and ^{1}H NMR Data of Compounds **1** (DMSO-d_6) and **4a** and **4b** (CD_6CO)

	1		**4a**		**4b**	
position	δ_C	δ_H (mult., *J*, Hz)	δ_C	δ_H (mult., *J*, Hz)	δ_C	δ_H (mult., *J*, Hz)
1	112.6		113.6		113.6	
2	*a*		162.5		162.5	
3	111.0		106.4	6.89 (s)	106.3	6.89 (s)
4	165.5		162.9		162.5	
5	117.8	6.71 (s)	117.7	6.74 (s)	117.6	6.74 (s)
6	154.2		142.6		142.6	
7	*a*		162.7		162.7	
8	22.5	2.54 (s)				
9	195.0	10.71 (s)				
1′	125.5[b]		106.0		106.0	
2′	152.6		160.5		160.3	
3′	123.3		107.7	6.81 (s)	107.6	6.81 (s)
4′	141.8		151.2		151.2	
5′	145.9		141.0		141.0	
6′	126.6[b]		132.1		132.1	
7′	10.4	2.33 (s)	169.6		169.5	
8′	60.5	3.79 (s)				
9′	14.1	2.45 (s)				
1″			47.8	4.09 (AB, 18)	47.8	4.09 (AB, 18)
2″			206.6		206.6	
3″			42.7	2.59 (t, 7.5)	42.7	2.59 (t, 7.5)
4″			23.9	1.52 (m)	23.9	1.52 (m)
5″			32.0	1.24−1.31 (m)	32.0	1.24−1.31 (m)
6″			23.4	1.24−1.37 (m)	23.4	1.24−1.37 (m)
7″			14.5	0.89 (t, 7)	14.5	0.89 (t, 7)
1‴			31.0	3.73 (H_β, AB, 17) 3.39 (H_α, AB, 17)	31.0	3.73 (H_β, AB, 17) 3.39 (H_α, AB, 17)
2‴			207.1		106.5	
3‴			41.3	2.08−2.10 (m)	41.3	2.08−2.10 (m)
4‴			24.2	1.62 (m)	24.2	1.62 (m)
5‴			32.6	1.33−1.37 (m)	32.6	1.33−1.37 (m)
6‴			23.4	1.24−1.37 (m)	23.4	1.24−1.37 (m)
7‴			14.6	0.93	14.6	0.93
OH-4				10.36		10.36
OH-2′				11.39		11.39
OH-2‴						7.15
OH		12.18 (s)				

[a] Not determined. [b] Signals may be transposed.

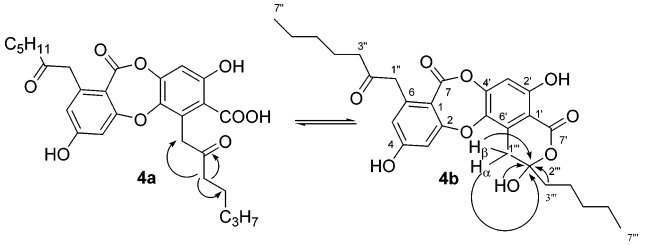

Figure 1. Ring−chain tautomerism of α-alectoronic acid (−50 °C) and selected HMBC correlations (H → C).

side chains (δ value from 0.89 to 4.09). The presence of a sharp 2H methylene singlet at δ 4.09 and very broad 2H methylene singlets at higher field (δ 3.39, 3.73) indicated that this compound exhibited ring−chain tautomerism. The pseudoacid tautomer with two cyclized forms has been reported previously by a NMR experiment at −50 °C.[7] In a similar experiment, we confirmed the equilibrium depicted in Figure 1 by the ^{13}C NMR signals of C-2‴ at 207.1 and 106.5 ppm, respectively (Table 1). Distinct ^{13}C NMR signals such as C-4 at 162.9 (**4a**) and 162.5 (**4b**) ppm (Table 1) indicated that the ketone form, **4a**, is predominant.

Compound **2** was found to be present in the two THF extracts and readily obtained by precipitation with THF from the shaded exposed lichen, where it was the major compound present (0.76% w/w of the dried lichen material). This depsidone was also present in small amounts in the sample exposed to sunlight (0.03% w/w) and was identified as the known compound variolaric acid (**2**) by comparison with published spectroscopic data.[8−10] Lecanoric acid (**3**) (3.5 mg) was also isolated from the THF extract, and its NMR data were in full agreement with the literature.[11] By TLC and

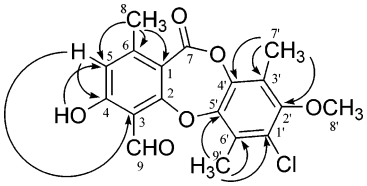

Figure 2. Selected HMBC correlations (H → C) of parellin (**1**).

Table 2. Cytotoxic Activities of Compounds **2**, **4**, and **6** against B16 Murine Melanoma Cells

	variolaric acid (**2**)	α-alectoronic acid (**4**)	ergosterol peroxide (**6**)	doxorubicin (control)	cisplatin (control)
IC_{50} (μM)	38.7 ± 2.0	10.3 ± 3.2	77.9 ± 14.4	0.1 ± 0.04	30.3 ± 17.1

passage over a Sephadex LH-20 column using methanol as eluent, this depside was converted progressively into two aromatic compounds: methyl orsellinate and orsellinic acid.[12,13] This alcoholysis has also been reported for atranorin[13] but was not observed here in the *n*-heptane extract of *O. parella*. Gyrophoric acid, a tridepside, was detected by TLC in THF extracts as previously described.[4] Except for ergosterol peroxide (**6**) and α-alectoronic acid (**4**), all of these compounds are biogenetically related to orcinol (lecanoric acid) or β-orcinol[14] metabolism, including the new chlorinated compound **1**, isolated from the shaded sample. It is interesting to note that atranorin (**5**) and parellin (**1**), respectively, are structurally related, suggesting that they are derived from the same biosynthetic pathway from haemmatomic acid.[15]

Compound **1** appeared as white-yellow needles and gave a molecular formula of $C_{18}H_{15}ClO_6$ as determined by its HREIMS. The ^{1}H NMR spectrum (Table 1) indicated the presence of signals for one aromatic proton at δ 6.71, three methyls at δ 2.33, 2.45, and 2.54, one methoxy at δ 3.79, a phenolic hydroxy at δ 12.18, and an aldehyde group at δ 10.71. The complete structure was established using HMBC correlations (Figure 2) with the following connectivities: methyl protons (δ 2.54) with C-1 (δ 112.6), C-5 (δ 117.8), and C-6 (δ 154.2); methyl protons (δ 2.33) with C-3′ (δ 123.3), C-4′ (δ 141.8), and C-2′ (δ 152.6); and methyl protons (δ 2.45) with C-1′ (δ 125.5), C-5′ (δ 145.9), and C-6′ (δ 126.6). The *O*-methyl protons correlated with C-2′ (δ 152.6), and the hydroxy phenolic proton (δ 12.18) correlated with C-4 (δ 165.5) and C-3 (δ 111.0) (Figure 2). The strong downfield signal for the hydroxy group strongly suggests a chelation by the neighboring aldehyde carbonyl. These data (Table 1) were used to define compound **1** (parellin), a new chloro-depsidone structurally close to pannarin (the correct structure of which was determined by Elix and collaborators[16] in 1975). Due to the low concentrations of **1**, signals due to C-2 and C-7 were not observed.

The cytotoxic activities of variolaric acid (**2**), α-alectoronic acid (**4**), and ergosterol peroxide (**6**) were evaluated against the B16 murine melanoma cell line using the MTT assay. These compounds were considerably less effective than doxorubicin (used as a positive control) (see Table 2). However, variolaric acid (**2**) (IC_{50} = 38.7 μM) and alectoronic acid (**4**) (IC_{50} = 10.3 μM), the most active isolated compounds, showed in turn an equal and 2-fold higher IC_{50} value than that observed for cisplatin (IC_{50} = 30.3 μM), another positive control substance.

Experimental Section

General Experimental Procedures. Melting points were measured on a hot-stage Kofler apparatus. UV spectra were performed on a UVIKON 931 spectrophotometer. FTIR spectra were run on a Perkin-Elmer 16 PC spectrometer. ^{1}H NMR and ^{13}C NMR data were recorded at 500 and 125 MHz, respectively, on a Bruker DMX 500 WB NMR spectrometer or at 270 and 67.5 MHz, respectively, on a JEOL GSX 270 WB, using $CDCl_3$, DMSO-d_6, and acetone-d_6 (and TMS as internal standard). COSY and CH experiments were recorded on the JEOL GSX 270 WB instrument. High-resolution mass spectrometric (HRMS)

measurements for exact mass determination were performed on a Varian-MAT 311 mass spectrometer at the Centre Régional de Mesures Physiques de l'Ouest. Chromatographic separation was performed using column chromatography on silica gel (35−70 μm).

Plant Material. *Ochrolechia parella* (L.) A. Massal. thalli were collected on the rocks of the sea coast near Dinard, Ille et Vilaine, France, in July 2005. One batch was collected on the rocks strongly exposed to sunlight and the second batch on shaded rocks. Voucher specimens have been deposited at the Herbarium of Pharmacognosy and Mycology, Rennes, France (REN-ABB), with the reference number JB/05/e28. The identification of the lichen material was confirmed by Dr. J. C. Massé as *O. parella* and supported by thalline chemical tests (cortex: C−, UV−, disk: C+ red, KC+ red, UV+ white).

Extraction and Isolation. The samples of *O. parella* (40 g for the shaded sample and 60 g for the sunlit sample) were first homogenized and then successively extracted with *n*-heptane, CH_2Cl_2, THF, and MeOH using a Soxhlet apparatus. The *n*-heptane extracts were chromatographed separately on a silica gel column with a gradient solvent system of *n*-heptane−AcOEt (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 2:8, 0:10), 50 mL of each solvent, to yield 50 fractions (Fa1−Fa51). Fractions 18−20 afforded pure compound **1** (1.5 mg) in the shaded sample, and workup of fractions F20−28 led to purification of compound **5** (2.9 mg) in the sunlit sample. The silica gel column chromatography of the CH_2Cl_2 extract with a gradient solvent system of *n*-heptane−AcOEt (8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 0:10), 100 mL of each solvent, yielded 50 further fractions (Fb1−Fb50). Fraction Fb16 contained compound **6** (1.6 mg) and fractions Fb17−Fb19, compound **4** (80 mg). After cooling the THF extract and gentle evaporation of the solvent, 300 mg and 22 mg of compound **2** were obtained, respectively, from the shaded and the sunlit samples by filtration of each mixture. The THF filtrates were combined and chromatographed on a silica gel column with a gradient solvent system of *n*-heptane−AcOEt (7:3, 6:4, 5:5, 4:6, 2:8, 0:10) and AcOEt−MeOH successively, to separate 50 additional fractions. Compound **3** (1.5 mg) was obtained from fractions Fc13 to Fc19.

Parellin (1): yellow needles (CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 208 (3.67), 245 (3.37), 302 (3.00) nm; ^{1}H and ^{13}C NMR (DMSO-d_6, 270 MHz) data, see Table 1; EIMS *m*/*z* 362 $[M]^+$ (10) 327 $[M - Cl]^+$ (18); HREIMS *m*/*z* 362.0550 $[M]^+$ (calcd for $C_{18}H_{15}O_6Cl$ 362.0557); R_f = 0.68 (toluene−EtOAc−formic acid, 6:3:0.3).

Variolaric acid (2): white powder (DMSO); mp 230 °C dec; UV (MeOH) λ_{max} (log ϵ) 226 (4.46), 249 (4.12), 333 (3.37) nm; IR (KBr disk) ν_{max} 3410, 3060, 1748, 1727, 1626, 1577 cm^{-1}; ^{1}H NMR (DMSO-d_6, 270 MHz) and ^{13}C NMR (DMSO-d_6, 67.5 MHz) data comparable to published values;[8,9] EIMS *m*/*z* 314/315/316 $[M]^+$ (100/20/5); HREIMS *m*/*z* 314.0430 $[M]^+$ (calcd for $C_{16}H_{10}O_7$ 314.0427); R_f = 0.68 (*n*-heptane−Et_2O−formic acid, 5:4:1).

Lecanoric acid (3): white powder (acetone); ^{1}H NMR (acetone-d_6, 270 MHz) comparable to published values;[11] EIMS *m*/*z* 168/169/170 $[M - C_8H_6O_3]^+$ (15/5/1); HRESIMS *m*/*z* 168.0411 (calcd for $C_8H_8O_4$ 168.04226); R_f = 0.37 (toluene−EtOAc−formic acid, 7:2:0.5).

α-Alectoronic acid (4): green-white needles (acetone); mp 188 °C; UV (MeOH) λ_{max} (log ϵ) 211 (4.66), 270 (4.39) nm; IR (KBr disk) ν_{max} 3190, 2927, 2868, 1725, 1706, 1674, 1613 cm^{-1}; ^{1}H and ^{13}C NMR (DMSO-d_6, 270 MHz) data, see Table 1; EIMS *m*/*z* 494/495/496 $[M - H_2O]^+$ (100/30/7), 468/469/470 $[M - CO_2]^+$ (10/7/3), 370/371/372 $[M - CO_2 - C_6H_{10}O]^+$ (40/11/7); HREIMS *m*/*z* 494.1913 $[M - H_2O]^+$ (calcd for $C_{28}H_{30}O_8$ 494.1941); R_f = 0.51 (toluene−EtOAc−formic acid, 7:2:0.5).

Atranorin (5): yellow needles (CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 270 MHz) data comparable to published values; [5] EIMS *m*/*z* (%) 374/375/376 $[M]^+$ (8/4/1), 196/197/198 $[M - C_9H_6O_4]^+$ (70/15/1); HREIMS *m*/*z* 374.1001 (calcd for $C_{19}H_{18}O_8$ 374.1002); R_f = 0.57 (toluene−acetic acid, 9:1).

Ergosterol peroxide (6): white oil (CH_2Cl_2); ^{1}H NMR (acetone-d_6, 270 MHz) data comparable to published values;[17] EIMS *m*/*z* (%) 396/397/398 $[M - O_2]^+$ (55/25/5); HREIMS *m*/*z* 396.3398 (calcd for $C_{28}H_{44}O$ 396.3392); R_f = 0.53 (toluene−EtOAc−acetic acid, 6:4:1).

Cytotoxicity Bioassay. Cell Culture. The murine cancer cell line B16-F1 (melanoma; ATCC CRL-6323) was used. The cells were maintained as previously described except the use of RMPI as medium culture with 5% FBS instead of 10% FBS.[18] Diluents (300 mM) of test compounds were prepared in dimethyl sulfoxide and added to each well 1 day after seeding. The amount of DMSO was adjusted to give a final concentration lower than 0.1%.

Cytotoxic Assay. Each experiment was repeated at least three times, and three different wells were used for each concentration. Cytotoxic activity was determined on B16 cells seeded at 20 000 cells/mL at day 0. Compounds were serially diluted in RPMI 1640 at day 1 in a 96-well plate, with concentrations ranging from 2.5 to 300 μM. Incubation was performed at 37 °C in an atmosphere of 10% CO_2. After 48 h of incubation, corresponding to day 3, compounds were added a second time. After a new 48 h incubation period, cell growth and viability were measured at day 5, using a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, as previously described.[19]

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Supporting Information Available: NMR spectra of α-alectoronic acid, available free of charge via the Internet at http://pubs.acs.org.

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