Stictic Acid Derivatives from the Lichen *Usnea articulata* and Their Antioxidant Activities

Françoise Lohézic-Le Dévéhat,*[,†] Sophie Tomasi,[†] John A. Elix,[‡] Aurélie Bernard,[†] Isabelle Rouaud,[†] Philippe Uriac,[†] and Joël Boustie[†]

Institut de Chimie de Rennes, EA 4090 "Substances Lichéniques et Photoprotection", Faculté de Pharmacie, Université de Rennes, 2 Avenue du Professeur Léon Bernard, 35043 Rennes Cedex, France, and Department of Chemistry, The Faculties, Australian National University, Canberra, ACT 0200, Australia

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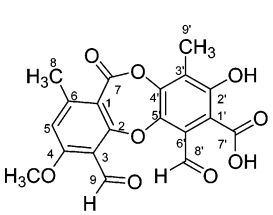
Two new β-orcinol depsidones, **1** and **2**, together with 13 known compounds were isolated from the lichen *Usnea articulata*. The structures of **1** and **2** were elucidated by spectroscopic analyses and those of known compounds by comparison of their spectroscopic data with literature values or by direct comparison with authentic standards. Compounds **1**, **2**, and **5** exhibited moderate antiradical activity in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The depsidones **4** and **5** showed better superoxide anion scavenging activity (IC_{50} = 566 and 580 μM, respectively) than quercetin (IC_{50} = 754 μM).

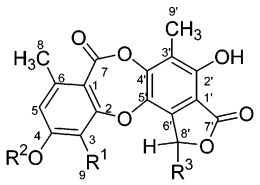
Lichens, symbiotic organisms consisting of fungi and algae, are distributed worldwide and can survive under various harsh environmental conditions (e.g., at high altitude, direct sunlight). They are also resistant to UV irradiation due to the production of a large number of unique secondary metabolites (the colorless depsides, depsidones, lactones, and dibenzofuran derivatives, etc.)[1] and pigments (anthraquinones, usnic acid derivatives, xanthones), which can act as filters as well as antioxidants.[2] As part of our ongoing study of photoprotective and antioxidant compounds from lichens, a phytochemical investigation was conducted on a fruticose lichen, *Usnea articulata* (L.) Hoffm. (Parmeliaceae), from Indonesia. The older portions of the pendent thallus are characterized by conspicuous articulations forming sausage-like segments. Previous studies on the chemical constituents of *U. articulata* reported the presence of usnic acid,[3] barbatic acid,[4] atranorin,[5] methyl orcinolcarboxylate,[5] and ergosterol peroxide.[4] The major depsidones from *U. articulata* harvested in Australia[6] and in Europe[7,8] were fumarprotocetraric acid and protocetraric acid. However, several chemical races have been described in the *U. articulata* complex,[9] and to our knowledge there have been no recent reports on the constituents of Asian specimens.[10]

Our present study led to the isolation or identification of nine compounds belonging to the stictic acid chemosyndrome:[11] two new depsidones (**1**, **2**) in addition to stictic acid (**3**),[12] norstictic acid (**4**),[12] peristictic acid,[11] cryptostictic acid,[13] menegazziaic acid,[14] constictic acid,[14] 3-*O*-methylconsalazinic acid,[14] fumarprotocetraric acid (**5**),[15] barbatic acid,[4] atranorin,[5] methyl β-orcinolcarboxylate,[5] usnic acid,[3] and ergosterol peroxide.[4] Antiradical activities using the DPPH and superoxide scavenging assays were reported for some of the compounds isolated.

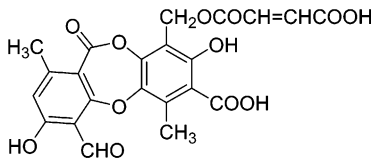
Compound **1** was assigned the molecular formula $C_{19}H_{14}O_8$, as indicated by HREIMS, identical to that of stictic acid (**3**). The ^{1}H NMR spectrum exhibited signals corresponding to two aromatic methyl groups (δ 2.14, 2.49), one methoxy group (δ 3.90), one aromatic proton (δ 7.05), and two aldehydic protons (δ 10.15, 10.24). The latter protons were correlated with C-9 (δ 187.6) and C-7′ (δ 191.7), respectively, on the basis of the HMQC experiment. The presence of two *C*OO (ν 1738, 1731 cm^{-1}) and two aldehyde functions (1681, 1697 cm^{-1}) was confirmed by IR, and the absence of a band at 1744 cm^{-1} confirmed the lack of a butyrolactone ring.[16] The NMR features of **1** were similar to those of stictic acid (**3**) except for replacement of the butyrolactone unit possessing a hydroxyl group (δ 8.20) and a lactol proton (δ 6.62) by an aldehyde (δ 10.24) and a carboxylic acid group. The assignment of CHO-7′ was established by a weak correlation with CHO-9 observed in the NOESY spectrum. From the above data, and the fact that **1** was converted into **3** under acidic conditions (TFA), **1** was considered to be the open-ring form of stictic acid. To our knowledge this is the first time that the open form of a lactonic depsidone has been described as a natural product. The stability of **1** is thought to be a consequence of intramolecular hydrogen bonding.[17]



1



2 R^1 =CH_2OH, R^2 = CH_3, R^3 = H
3 R^1 =CHO, R^2 = CH_3, R^3 = OH
4 R^1 =CHO, R^2 = H, R^3 = OH



5 fumarprotocetraric acid

The molecular formula of compound **2** was established as $C_{19}H_{16}O_8$ by HREIMS data. IR absorptions at 1746, 1736, and 1731 cm^{-1} confirmed the presence of two carbonyl groups and a butyrolactone ring. The ^{1}H NMR spectrum was very similar to that of stictic acid (**3**), but lacked an aldehydic proton (δ 10.46) and exhibited signals corresponding to a CH_2O group (δ 4.59) and an OH group (δ 5.01). The methylene protons (δ 5.66) correlated with C-8′ (δ 66.9) in the HMQC experiment, suggesting that **2** lacked the OH group at C-8′ as compared to **3**. The quaternary carbon corresponding to the carbonyl group at C-7 was not observed, probably due to a very long relaxation delay. This is the first reported isolation of cryptostictinolide (**2**).[13,18]

Three known depsidones, stictic acid (**3**), norstictic acid (**4**), and fumarprotocetraric acid (**5**), the depside barbatic acid, the dibenzofuran derivative usnic acid, and a terpenoid, ergosterol peroxide, were isolated and identified by comparison of the observed

* To whom correspondence should be addressed. Phone: + 33 (0) 2 23 23 48 16. Fax: + 33 (0) 2 23 23 47 04. E-mail: francoise.le-devehat@univ-rennes1.fr.
† Université de Rennes.
‡ Australian National University.

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spectroscopic data with literature values.[3,4,12,15] The known compounds were identified by direct comparison with authentic samples purchased from Sigma or provided by J. Elix, using HPLC−DAD and TLC techniques in appropriate solvent systems.[5,11,13,14]

Although lichens have long been known to contain bioactive compounds, few attempts have been made to screen them. Most publications describe the antioxidant activities of crude lichen extracts,[19−24] but those using pure compounds reported better lipid peroxidation protection activity for depsidones rather than for depsides.[25−28] The radical-scavenging effect of antioxidants on DPPH is a simple and reliable method to quantify the hydrogen-donating potency of chemicals. Lichen compounds do not seem to have labile hydrogen atoms. In fact, the most active compounds were depsidones possessing no butyrolactone ring (**1**, **5**) and compound **2**, exhibiting 30%, 20%, and 18% inhibition at 3000 μM, respectively. Kumar and collaborators previously reported low activity for atranorin, methyl β-orcinolcarboxylate, and usnic acid.[29] Concerning the superoxide-scavenging effect, the depsidones **4** and **5** were the most active [IC_{50} 580 and 566 μM, respectively], activities higher than that of the well-known flavonoid quercetin (IC_{50} 754 μM). Both depsidones possess a 4-OH and 3-CHO, but other similarly substituted compounds should be tested to determine whether these substituents are associated with the activity. Given the common occurrence of depsidones in lichens and their potent superoxide-scavenger activity, it is likely that they contribute to the antioxidant defenses of these organisms.[2,30] Thus, lichens are an interesting source for new antioxidants, compounds that could also act as photoprotectors by limiting the deleterious effects of UV light via ROS formation.[31]

Experimental Section

General Experimental Procedures. Melting points were measured on a hot-stage Kofler apparatus. UV spectra were performed on a UVIKON 931 spectrophotometer. Optical rotations were measured with a Perkin-Elmer 341 polarimeter. Infrared spectra were recorded on a Perkin-Elmer model 16PC IR spectrometer using KBr disks. NMR spectra were recorded on a Bruker DMX 500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C) or on a JEOL JSX 270 WB spectrometer at 270 MHz (^{1}H) and 67.5 MHz (^{13}C), respectively. High-resolution mass spectrometric (HRMS) measurements were recorded using a Varian MAT 311 mass spectrometer (70 eV) at the Centre Régional de Mesures Physiques de l'Ouest. HPLC was done on a Kontron 325 using a reversed-phase C_{18} Hypersil column (250 × 4.6 mm, 5 μM) or a semipreparative CN BDS-Hypersil column (250 × 10 mm, 5 μM) and using a diode-array detector (Kontron, 530A). Column chromatography was performed using silica gel 60 (Merck TA467667, 40−63 μM) and Sephadex LH-20 (BioChemika Fluka, 84952). Circular centrifugal chromatography (CCC) was accomplished using a Chromatotron 8924. Preparative TLC was conducted on silica plates (Macherey-Nagel SIL-G100, 20 × 20, 809061). TLC plates (Merck silica gel 60F_{254}) were eluted using four solvent systems: toluene−dioxane−acetic acid (180:45:5), *n*-hexane−*tert*-butyl methyl ether−formic acid (140:72:18), toluene−acetic acid (170:30), toluene−EtOAc−formic acid (139:83:8).[32−34] Visualization of plates was carried out under UV light (254 and 365 nm) and using anisaldehyde−H_2SO_4 and *o*-dianisidine reagents. Quercetin (Q-0125), 1,1-diphenyl-2-picrylhydrazyl (DPPH, Fluka 43180), nitro blue tetrazolium (NBT, N-6876), NADH (N-8129), phenazine methosulfate (Fluka 68600), atranorin (A-6652), and methyl β-orcinolcarboxylate (SO83837) were purchased from Sigma (St. Louis, MO).

Lichen Material. *Usnea articulata* (L.) Hoffm. (Parmeliaceae) was collected by Dr. Amri Bakhtiar in Sukabumi, East Sumatra, Indonesia, at an altitude of 1500 m, on various trees in February 2003. After cleaning and identification by P. Clerc (Geneva, Switzerland), a voucher specimen (reference JB/06/e91) was deposited in the herbarium of Pharmacognosy and Mycology, Rennes, France.

Extraction and Isolation. The air-dried lichen thalli (210 g) were ground and successively extracted with 200 mL of *n*-hexane, diethyl ether, acetone, CH_3OH, and H_2O using a hot Soxhlet process. Pure yellow (+)-usnic acid (1.06 g) precipitated from the *n*-hexane extract (1.32 g). Silica gel column chromatography (25 g) of the supernatant fraction (260 mg) by gradient elution using *n*-hexane−CH_3OH (1:1 to 0:1) through EtOAc produced four subfractions: A (124.7 mg), B (48.2 mg), C (31.3 mg), and D (57.0 mg). Fraction B, eluted with *n*-hexane−EtOAc (1:1), was further purified by CCC using *n*-hexane−diethyl ether (1:4, 100 mL as a mobile phase) to yield ergosterol peroxide (5 mg, recrystallized from CH_3OH). Fraction D, eluted with EtOAc−CH_3OH gradient (0:1 to 1:1), was fractionated by CCC, and fraction 4, which eluted with CH_3OH−acetone (1:1), was further purified on a Sephadex LH-20 column (130 g) with acetone followed by HPLC (C_{18}, CH_3OH-H_2O, 8:2, flow rate, 1 mL/min) to yield barbatic acid (15 mg). After two elutions with *n*-hexane−EtOAc (3:2) on a silica gel column (3 g) and further on silica gel with *n*-hexane−CH_2Cl_2 (1:1), fraction A (1.5 mg) afforded atranorin and methyl β-orcinolcarboxylate, identified by HPLC (C_{18}, CH_3OH-H_2O, 8:2, flow rate, 1 mL/min) using authentic samples purchased from Sigma. The diethyl ether supernatant fraction (1.1 g) was separated on a silica gel column using $CHCl_3$−MeOH (1:0 to 0:1) followed by preparative TLC, developed with toluene−EtOAc−HCOOH (139:83:8), and afforded seven bands, two of which yielded further quantities of barbatic acid and usnic acid and four other compounds: **2** (11.5 mg), **3** (7 mg), **4** (5 mg), and **5** (1 mg). The precipitate from the acetone extract (1.89 g) was purified by CCC using $CHCl_3$−MeOH (9:1 to 0:1, 300 mL for each fraction) to give four subfractions. Fraction 2 afforded compound **1** (40 mg), purified further using gradient semipreparative HPLC (CN, 3 mL/min, acetonitrile−H_2O, 1:9, to 100% acetonitrile). Known compounds were identified by comparison with authentic samples.

Compound 1: white, amorphous powder; mp 270−272 °C (dec); UV (MeOH) λ_{max} (log ϵ) 210 (4.54), 266sh, 312 (3.71) nm; IR (KBr disk) ν_{max} 2877 broad, 1738, 1731, 1697, 1681 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 10.24 (1H, s, H-7′), 10.15 (1H, s, H-9), 7.05 (1H, s, H-5), 3.90 (3H, s, H-10), 2.49 (3H, s, H-8), 2.14 (3H, s, H-9′); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 191.7 (CHO, C-8′), 187.6 (CHO, C-9), 170.6 (COOH, C-7′), 164.7 (C, C-2), 162.6 (C, C-4), 162.1 (COO, C-7), 159.5 (C, C-2′), 151.1 (C, C-6), 144.8 (C, C-4′), 137.4 (C, C-6′), 132.1 (C, C-5′), 118.8 (C, C-3′), 114.9 (C, C-3), 114.7 (C, C-1′), 113.7 (C, C-1), 112.9 (CH, C-5), 57.2 (OCH_3, C-10), 22.0 (CH_3, C-8), 9.7 (CH_3, C-9′); ESIMS *m*/*z* 385 [M − H]$^-$; HRESIMS *m*/*z* 385.0558 (calcd for $C_{19}H_{13}O_9$ 385.0559).

Cryptostictinolide (2): white, amorphous powder; mp 275−276 °C (dec); IR (KBr disk) ν_{max} 3424, 3292, 2948, 1746, 1736, 1731 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 10.05 (1H, br s, OH-2′), 6.95 (1H, s, H-5), 5.66 (2H, s, H-8′), 4.59 (2H, s, H-9), 3.87 (3H, s, H-10), 2.45 (3H, s, H-8), 2.18 (3H, s, H-9′); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 169.0 (COO, C-7′), 162.0 (C, C-4), 159.2 (C, C-2), 152.6 (C, C-2′), 148.4 (C, C-4′), 144.8 (C, C-6), 136.8 (C, C-6′), 136.6 (C, C-5′), 118.6 (C, C-3′), 118.4 (C, C-3), 113.2 (C, C-1), 112.0 (CH, C-5), 108.5 (C, C-1′), 66.9 (CH_2, C-8′), 56.7 (OCH_3, C-10), 51.4 (CH_2OH, C-9), 21.4 (CH_3, C-8), 9.9 (CH_3, C-9′); EIMS *m*/*z* 372/373/374 [M]$^{+\bullet}$ (100/14/1), [M − H_2O]$^{+\bullet}$ 354/355/356 (77/14/1); HREIMS *m*/*z* 372.0832 [M]$^{+\bullet}$ (calcd for $C_{19}H_{16}O_8$ 372.0845).

Stictic acid (3): white, amorphous powder; ^{1}H NMR (DMSO-d_6, 500 MHz) data comparable to published data;[12] ESIMS *m*/*z* 385 [M − H]$^-$; HRESIMS *m*/*z* 385.0553 (calcd for $C_{19}H_{13}O_9$ 385.0559).

Norstictic acid (**4**): white, amorphous powder; ^{1}H NMR (DMSO-d_6, 500 MHz) data comparable to published data;[12] ESIMS *m*/*z* 372 [M]$^{+\bullet}$; HRESIMS *m*/*z* 372.0505 (calcd for $C_{18}H_{12}O_9$ 372.0481).

Fumarprotocetraric acid (5): white, amorphous powder; ^{1}H NMR (DMSO-d_6, 500 MHz) data comparable to published data;[15] ESIMS *m*/*z* 471 [M − $^\bullet$H]$^+$; HRESIMS *m*/*z* 471.0577 (calcd for $C_{22}H_{15}O_{12}$ 471.0563).

Barbatic acid: white, amorphous powder; ^{1}H NMR and ^{13}C NMR (DMSO-d_6, 500 MHz) data in accordance with published data;[4] ESIMS *m*/*z* 359 [M − $^\bullet$H]$^+$; HRESIMS *m*/*z* 359.1134 (calcd for $C_{19}H_{19}O_7$ 359.1131).

(+) Usnic acid: yellow needles ($CHCl_3$); $[\alpha]^{20}_D$ +494 (*c* 0.4, $CHCl_3$); ^{1}H NMR and ^{13}C NMR ($CDCl_3$-d_6, 500 MHz) data comparable to published data;[3] LSIMS *m*/*z* 345 [M + H]$^+$; HRLSIMS *m*/*z* 345.0967 (calcd for $C_{18}H_{17}O_7$ 345.0974).

Ergosterol peroxide: colorless needles (Me_2CO); ^{1}H NMR ($CDCl_3$-d_6, 500 MHz) data comparable to published data;[4] ESIMS *m*/*z* 451 [M + Na]$^+$; HRESIMS *m*/*z* 451.3187 (calcd for $C_{28}H_{44}O_3Na$ 451.3188).

Antioxidant Assays. Extracted compounds (**1**, **2**, **4**, barbatic acid, (+)-usnic acid, and ergosterol peroxide) together with **5** isolated from

the lichen *Cladonia convoluta*,[15] atranorin, and methyl β-orcinolcarboxylate were evaluated for antioxidant activity.

DPPH Assay. Scavenging activity of the lichen compounds on the 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) was measured using the Matsukawa[35] method with some modifications. A reaction mixture containing 100 μL of DPPH (0.5 mM) in MeOH and 10 μL of the lichen compound in DMSO diluted to give final concentrations of 3000, 1500, 750, 375, and 187.5 μM was placed in each microplate well. Quercetin was used as a control on each plate. All tests were done in triplicate and the results averaged. The percentage inhibition at steady state for each dilution was used to determine graphically the IC_{50} values.

$O_2^{-\bullet}$ Scavenging Activity. Measurement of superoxide anion scavenging activity in 96-well microplates was based on the nonenzymatic method described by Valentao[36] with some modifications. The reaction mixture in the sample wells consisted of NADH (720 μM), NBT (360 μM), PMS (30 μM), and lichen compounds (3000, 1500, 750, 375 μM). The reagents were dissolved in 0.1 M phosphate buffer $K_2HPO_4-KH_2PO_4$, at pH 7.4, except for all the lichen compounds, which were dissolved in DMSO. After 2 min of incubation at room temperature, the reaction was stopped by adding 0.1 M HCl and the microplates were centrifuged for 2 min at 3000 tr/min. To avoid the variability in absorbance readings due to the water insolubility of the diformazan salts, the supernatants were removed and the crystals of final product of NBT reduction were dissolved in 200 μL of DMSO. Spectrophotometric measurement was performed at 560 nm against blank samples without PMS. Quercetin was used as positive control. The percentage inhibition at steady state for each dilution was used to calculate the IC_{50} values. This gave 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 done in triplicate and the results averaged.

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Supporting Information Available: ^{1}H NMR and ^{13}C NMR spectra of compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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