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Phytochemical and cytotoxic investigations of the lichen *Stereocaulon evolutum* Graewe

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Abstract

Lichens are fungal and algal/cyanobacterial symbioses resulting in the production of specific metabolites with a great variety of effects such as antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic and antiproliferative. As a part of searching cytotoxic compounds from lichens, a phytochemical investigation was conducted on a squamulose lichen, *Stereocaulon evolutum* Graewe, collected in France. Five known compounds including atranorin (**1**), atranol (**2**), lobaric acid (**3**), ursolic acid (**4**), and methyl 3'-methyllecanorate (**5**) were isolated for the first time from such lichen. Their structures were elucidated by ESI-HR-MS, NMR spectral analysis and compared with literature data. Cytotoxicity screening of five isolated compounds were evaluated on seven cell lines including HuH7, CaCo-2, MDA-MB-231, HCT116, PC3, NCI-H727 and HaCaT. Compound (**5**) was found high cytotoxicity effect on all investigated cell lines while other compounds showed non-cytotoxic.

Keywords. Lichen, *Stereocaulon*, atranorin, cytotoxicity.

1. INTRODUCTION

Lichens are stable, consistent and identifiable mutualistic associations between algae and/or cyanobacteria and fungi [1]. About 1035 secondary metabolites have been isolated from the 18500 lichen species described to date, and many more compounds remain to be characterized. Most of these compounds are polyketides, polyphenols, quinones or terpenoids, presumably of fungal origin and their biological activities remain largely underexplored [2, 3]. However, a few have been shown to have antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic activities [3]. *Stereocaulon* is a widely distributed worldwide lichen genus with around 130 species but approximate 40 species were investigated phytochemistry due to their difficult identification. Among 75 isolated substances from *Stereocaulon*, atranorin is a constant compound as well as the common occurrence of three depsidones including lobaric acid, stictic acid and norstictic acid is useful for taxonomy in such genus [4]. Various isolated lichen compounds often demonstrate significant inhibitory activity against several cancer cell lines at very low concentrations.

Although lichens are a source for excellent anticancer active compounds, only a small number have been tested for their biological significance [5]. In this paper, as a part of our searching cytotoxic compounds from lichens, a phytochemical investigation was conducted on a squamulose lichen, *Stereocaulon evolutum* Graewe, collected in France. Atranorin (**1**), a major compound, along with four other compounds atranol (**2**), lobaric acid (**3**), ursolic acid (**4**) and methyl 3'-methyllecanorate (**5**) were isolated. Their cytotoxic activity was evaluated on seven cell lines HuH7, CaCo-2, MDA-MB-231, HCT116, PC3, NCI-H727 and HaCaT.

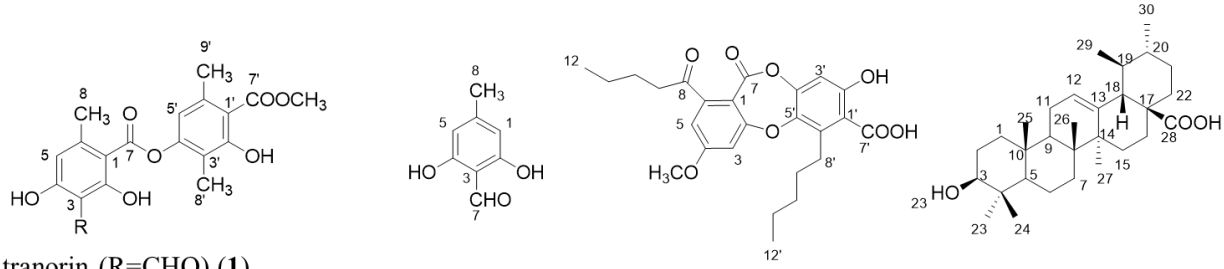

Figure 1: Structures of compounds isolated from the lichen *S. evolutum*

2. MATERIALS AND METHODS

2.1. General procedures

All solvents for chromatography were purchased from Sigma-Aldrich (France). Thin-layer chromatography (TLC) was carried out on silica gel plates (Merck silica gel 60F254) with the A (toluene:dioxane:acetic acid 180:45:5), B (*n*-hexane:diethyl ether:formic acid 130:80:20), and C (toluene:acetic acid 170:30) standard solvent systems for the identification of substances from lichens [6]. The spots were first visualized under UV light and then after spraying with anisaldehyde–H_2SO_4 reagent. Circular chromatography and column chromatography were carried out on silica gel (40-63 µm, Kieselgel 60, Merck, 7667). Flash column chromatography was performed on a SPOT Flash liquid chromatography (Armen Instrument), using RP-18 columns (Chromabond, Merck). The NMR experiments were performed on a Bruker DMX 300 and 500 spectrometers with deuterated solvents. High-resolution mass spectrometry (HR-MS) measurements were carried out to determine exact mass, on a Bruker Maxis 4G, MicrO-Tof Q 2, a Thermo-fisher Q-Exactive or a Waters Q-Tof 2 mass spectrometer for chemical ionization. Melting points were measured on a Kofler LEICA VMHB.

2.2. Lichen materials

Stereocaulon evolutum Graewe was collected in Bretagne, France, on November 2011. The scientific name was identified by PhD Lohézic-Le Dévéhat Françoise, Faculty of Pharmacy, University of Rennes 1, France. A voucher specimen (JB/10/121) was deposited in the herbarium of Pharmacognosy and Mycology, University of Rennes 1, France.

2.3. Cytotoxicity assay

The cell lines were obtained from the ECACC collection. Cells were grown according to ECACC recommendations. The toxicity test of the compounds on these cells was as follows: 2.103 cells/well for HCT116 cell line or 4.103 cells/well for the other cell lines were seeded in 96 well plates. After 24 h of seeding, cells were exposed to the compounds at concentration 10 µM. After 48h of treatment, the cells were washed in PBS and fixed in ethanol/acetic acid (90:5 *v:v*) for 20 min. Then, the nuclei were stained with Hoechst 3342 (Sigma). Image acquisition and analysis was performed using a Cellomics ArrayScan VTI/HCS Reader (Thermo Scientific).

2.4. Extraction and isolation

Air-dried crushed thalli of the lichen *S. evolutum* Graewe (300 g) were successively extracted with *n*-hexane, acetone and tetrahydrofuran, by maceration 24 h at room temperature (3 times x 2 L). A pure colorless compound **1** (6.2 g) was precipitated from the *n*-hexane and acetone extracts by evaporating off the solvents at room temperature. The *n*-hexane filtrate (1.5 g) was fractionated on successive silica gel columns and then circular chromatography using *n*-hexane:dichloromethane 3:7 to afford compound **4** (9.0 mg). The acetone filtrate (13.4 g) was subjected to silica gel flash column chromatography (Chromabond® flash, RS 40 C18) using a water:acetonitrile gradient system (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 0:1, each 225 mL) to give seven fractions Ac1-Ac7. A precipitate in fraction Ac6 was filtrated and recrystallization in acetone to yield compound **3** (1.2 g). Fraction Ac7 was purified by a silica gel column chromatography using *n*-hexane:ethyl acetate 8:2 to give compound **2** (10.0 mg) and compound **5** (21 mg).

Atranorin (**1**): colorless powder; R_f = 0.78 (B), R_f = 0.70 (C); M.p 196-197 °C; ^{1}H NMR (DMSO-d_6, 500 MHz): δ_H 10.52 (1H, *s*, 2-O**H**), 10.21 (1H, *s*, 3-C**H**O), 6.41 (1H, *s*, H-5), 2.35 (3H, *s*, H-8), 6.65 (1H, *s*, H-5'), 2.04 (3H, *s*, H-8'), 2.39 (3H, *s*, H-9'), 3.88 (3H, *s*, 7'-OC**H**$_3$); ^{13}C NMR (DMSO-d_6, 125 MHz) δ_C: 107.9 (C-1), 163.6 (C-2), 109.0 (C-3),

161.7 (C-4), 115.2 (C-5), 151.4 (C-6), 164.5 (C-7), 193.8 (C-8), 20.1 (C-9), 110.6 (C-1'), 157.4 (C-2'), 116.3 (C-3'), 148.8 (C-4'), 115.7 (C-5'), 136.5 (C-6'), 169.7 (C-7'), 9.3 (C-8'), 21.1 (C-9'), 52.3 (7'-O**C**H_3); ESI-HRMS *m/z* 397.0899 $[M+Na]^+$ (calcd. for $C_{19}H_{18}O_8$).

Atranol (**2**): yellow needle (in $CHCl_3$); R_f = 0,70 (C); M.p 124 °C; ^{1}H NMR (acetone-d_6, 500 MHz): δ_H 10.69 (1H, *s*, 2-O**H**), 10.69 (1H, *s*, 4-O**H**), 10.27 (1H, *s*, H-7), 6.26 (1H, *s*, H-1), 6.26 (1H, *s*, H-5), 2.23 (3H, *s*, H-8); ^{13}C NMR (acetone-d_6, 125 MHz) δ_C: 108.4 (C-1), 163.0 (C-2), 109.2 (C-3), 163.0 (C-4), 108.4 (C-5), 151.5 (C-6), 194.1 (C-7), 22.2 (C-8); ESI-HRMS *m/z* 175.0373 $[M+Na]^+$ (calcd. for $C_8H_8O_3$).

Lobaric acid (**3**): white crystal (in acetone); R_f = 0.46 (B), R_f = 0.41 (C); M.p 196-197 °C; ^{1}H NMR (acetone-d_6, 500 MHz): δ_H 7.00 (1H, *d*, 2.2, H-3), 7.00 (1H, *d*, 2.2, H-5), 6.74 (1H, *s*, H-3'), 3.98 (3H, *s*, 4-OC**H**$_3$), 3.30 (2H, *t*, 5.5, H-8'), 2.85 (2H, *t*, 7.7, H-9), 1.63 (2H, *m*, H-10), 1.63 (2H, *m*, H-9'), 1.51 (2H, *m*, H-10'), 1.39 (2H, *m*, H-11), 1.39 (2H, *m*, H-11'), 0.91 (2H, *m*, H-12), 0.91 (2H, *m*, H-12'); ^{13}C NMR (acetone-d_6, 125 MHz) δ_C: 112.9 (C-1), 163.9 (C-2), 106.9 (C-3), 165.5 (C-4), 112.2 (C-5), 150.2 (C-6), 161.0 (C-7), 203.4 (C-8), 42.3 (C-9), 26.7 (C-10), 23.2 (C-11), 14.2 (C-12), 112.2 (C-1'), 162.5 (C-2'), 107.8 (C-3'), 148.9 (C-4'), 142.3 (C-5'), 139.9 (C-6'), 172.4 (C-7'), 28.8 (C-8'), 32.0 (C-9'), 33.0 (C-10'), 22.8 (C-11'), 14.4 (C-12'), 56.9 (4-O**C**H_3); ESI-HRMS *m/z* 455.1713 $[M-H]^-$ (calcd. for $C_{25}H_{28}O_8$).

Ursolic acid (**4**): white solid; R_f = 0.50 (B), R_f = 0.49 (C); M.p 290-292 °C; ^{1}H NMR (CD_3OD, 300 MHz): δ_H 5.23 (1H, t, 3.5, H-12), 3.16 (1H, dd, 6.0, 6.0, H-3), 2.22 (1H, bd, H-18), 1.12, 0.98, 0.96, 0.90, 0.87, 0.85, 0.78 (21H, 7× s, 7×-Me). ^{13}C NMR (CD_3OD, 75 MHz) δ_C: 39.8 (C-1), 27.9 (C-2), 79.7 (C-3), 40.4 (C-4), 56.7 (C-5), 19.5 (C-6), 34.3 (C-7), 40.8 (C-8), 48.1 (C-9), 38.1 (C-10), 24.1 (C-11), 126.8 (C-12), 139.6 (C-13), 43.2 (C-14), 28.7 (C-15), 25.3 (C-16), 48.1 (C-17), 54.4 (C-18), 40.4 (C-19), 40.0 (C-20), 31.8 (C-21), 38.1 (C-22), 28.7 (C-23), 16.4 (C-24), 16.0 (C-25), 17.6 (C-26), 24.3 (C-27), 181.7 (C-28), 17.8 (C-29), 21.6 (C-30); ESI-HRMS *m/z* 479.3499 $[M+Na]^+$ (calcd. for $C_{30}H_{48}O_3$).

Methyl 3'-methyllecanorate (**5**): white crystal (in acetone); R_f = 0.55 (A), R_f = 0.40 (C); M.p 126-128 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ_H 11.43 (1H, *s*, 2-O**H**), 11.96 (1H, *s*, 2'-O**H**), 6.53 (1H, *s*, H-5'), 6.63 (1H, *d*, 2.58, H-3), 6.63 (1H, *d*, 2.58, H-5), 5.47 (1H, *s*, 4-O**H**), 3.99 (3H, *s*, 7-OC**H**$_3$) 2.65 (3H, *s*, H-8), 2.54 (3H, *s*, H-9'), 2.10 (3H, *s*, H-8'); ^{13}C NMR ($CDCl_3$, 75 MHz) δ_C: 104.5 (C-1), 166.3 (C-2), 101.5 (C-3), 161.1 (C-4), 111.9 (C-5), 144.4 (C-6), 169.7 (C-7), 24.6 (C-8), 109.9 (C-1'), 162.8 (C-2'), 116.8 (C-3'), 152.3 (C-4'), 116.2 (C-5'), 139.7 (C-6'), 172.2 (C-7'), 9.3 (C-8'), 24.0 (C-9'), 52.3 (7'-O**C**H_3); ESI-HRMS *m/z* 345.0979 $[M-H]^-$ (calcd. for $C_{18}H_{18}O_7$).

3. RESULTS AND DISCUSSION

The five known compounds atranorin (**1**, 6.2 g), atranol (**2**, 10 mg), lobaric acid (**3**, 1.2 g), ursolic acid (**4**, 9.0 mg) and methyl 3'-methyllecanorate (**5**, 21 mg) were identified by direct comparison of their physical and spectral data in the literature [6, 7]. Although the compounds have previously been described in some others species of genus *Stereocaulon* [4], here, they were reported for the first time in *Stereocaulon evolutum* (figure 1).

The cytotoxic activity of the five isolated compounds was performed on seven cell lines at concentration of 10 µM. The results showed that no cytotoxicity was observed in almost investigated compounds, except **5**. In fact, the percentage of cell viability was over 80 % for **1**, **2**, **3** and **4** while this value of **5** is 52 %, 30 %, 66 %, 17 %, 35 %, 69 % and 27 % on HuH7, Caco-2, MDA-MB231, HCT116, PC3, NCI-H727 and HaCaT, respectively (table 1). Compound **5** displayed higher toxicity than atranorin (**1**) which possesses an aldehyde functional group on C-3 which is well known responsible to apoptosis [8]. It is worth nothing that the screening results were not opposite to previous reports [4] since the investigated compounds showed cytotoxic activities in dose much higher than 10 µM.

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Table 1: Cytotoxicity of compounds **1**, **2**, **3**, **4**, and **5** against seven cell lines at C = 10 µM

Cell lines / **Compounds**	**Cell viability (%)**						
	HuH7	Caco-2	MDA-MB231	HCT116	PC3	NCI-H727	HaCaT
Atranorin (1)	98	109	85	107	101	82	97
Atranol (2)	94	125	100	103	103	91	102
Lobaric acid (3)	96	123	87	102	88	93	92
Ursolic acid (4)	104	111	106	122	105	108	115
Methyl 3'-methyl lecanorate (5)	**52**	**30**	**66**	**17**	**35**	**69**	**27**

Note: Huh7 (human hepato cellular carcinoma), CaCo-2 (human colorectal adenocarcinoma), MDA-MB-231 (human breast cancer), HCT116 (human colon cancer), PC3 (human prostate cancer), NCI-H727 (human lung cancer), HaCaT (human immortalized keratinocytes).

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