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Chemical constituents of the lichen *Dermatocarpon luridum* and pK_a value of isolated mycosporine

Nguyen Thi Thu Tram[1,2*], Nguyen Trong Tuan[3], Chollet-Krugler Marylène[2], Ferron Solenn[2], Boustie Joël[2]

[1]*Department of Chemistry, Faculty of Science, Can Tho University of Medicine and Pharmacy, Vietnam*

[2]*Equipe PNSCM "Produits Naturels, Synthèses et Chimie Médicinale", UMR CNRS 6226,*

Faculté des Sciences Pharmaceutiques et Biologiques, Université de Rennes 1, France

[3]*College of Natural Sciences, Cantho University*

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Abstract

A phytochemical study on the lichen *Dermatocarpon luridum* led to the isolation of four known compounds, including mycosporine glutaminol (**1**), 2-amino-3-acetylaminopropionic acid (**2**), (22*E*,24*R*)-ergosta-7,22-diene-3β,5α,6β-triol (**3**) and (2*S*,3*S*,4*R*,2'*R*)-2-(2'-hydroxytetracosanoylamino)octadecan-1,3,4-triol (**4**). Their structures were determined by extensive spectroscopic analyses including UV, IR, ESI-HRMS, 1D, 2D NMR and specific rotation as well as by comparison of the data with those in the literature. The p*K*a value in an aqueous solution of (**1**) was determined by UV-VIS spectrophotometry at 298 K. With the low p*K*a value 1.29, only the neutral form of (**1**) was present and responsible for the UV absorption in water at pH 7.

Keywords. *Dermatocarpon*, mycosporine, lichen, pK_a.

1. INTRODUCTION

Lichens are fungal and algal/cyanobacterial symbioses resulting in the production of a large number of unique secondary metabolites [1]. So far, investigation on chemical constituents of *Dermatocarpon luridum* has not been noticed well. Previous studies revealed that instead of containing common lichen subtances as phenolic compounds, dibenzofuranes, depsides, depsidones, quinones, pulvinic acid derivatives etc., lichen species belonging to genus Dermatocarpon included several sugar alcohols as mannitol, sorbitol, volemitol and arabitol [2, 3]. The first report on the occurrence of mycosporine glutaminol in the aqueous extract of *D. luridum* [4] prompted us that a phytochemical study on such lichen could be useful for chemical taxonomy. Recently, mycosporines have received much attention for their putative role in UV photoprotection. Mycosporines are small water-soluble molecules absorbing UV radiations in the wavelength range 310–320 nm, based on their common aminocyclohexenone ring linked with an amino acid or an amino alcohol. To date, more than 40 mycosporines and derivatives have been described, some bearing functional groups or being covalently linked with saccharidic units [5]. The dissociation constants of many mycosporines are not available at all. The knowledge of such data is a key parameter in absorption, distribution, metabolism, excretion and toxicity research [6]. Therefore, it is worth determining the p*K*a of (**1**) which obtains one acido-basic site to understand chemical and biochemical processes and also to confirm its presence under the acidic or the basic form in water.

2. EXPERIMENTAL

2.1. Lichen material

Dermatocarpon luridum (With.) J. R. Laundon was collected on submerged rocks in Huelgoat, Brittany, France in April 2012. The lichens were identified by Monnat Jean-Yves (Biologist, University of Bretagne Sud, France). Vocher specimen was deposited in the herbarium of Pharmacognosy and Mycology, University of Rennes 1, France with reference number JB/12/001.

2.2. General experimental procedures

Flash chromatography was performed on a SPOT Flash liquid chromatography (Armen Instrument). IR spectrum was obtained with Perkin Elmer UATR Two infrared spectrophotometer. The NMR experiments were performed on a Bruker DMX 300 and 500 spectrometer. ESI-HRMS were carried out on a MICROMASS ZabspecTOF spectrometer for electrospray ionization at the CRMPO (Centre Régional de Mesures Physiques de l'Ouest), University of Rennes 1. Uvikon 931 UV-Vis spectrophotometer with 1 cm path length cells for UV spectra.

Open column chromatography was performed on normal phase silica gel (40-63 μm, Keselgel 60, Merck 7667), reverse phase silica gel C-18 (C-18 Hydro Chromabond, Macherey-Nagel), gel Sephadex LH-20 (Sigma-Aldrich), cation exchange resin Dowex 50W-X8. TLC was performed on Kieselgel 60F254 plates (Merck) and spots were visualized under UV light or sprayed with anisaldehyde (a solution of 0.5 mL anisaldehyde in 50 mL glacial acetic acid and 1 mL 97 % sulfuric acid) then heated. Optical rotations were measured on a Perkin Elmer Model 341 polarimeter at 20 °C using thermostable optical glass cell (1 dm path length).

pH values were measured by LPH 430T pH – METER.

Solvents and chemicals: solvents for extraction and for chromatography were purchased from Carlo Erba Reactifs (Val de Reuil, France). Distilled water was obtained by an EasyPure (Barnstead, USA) water purification system. Deuterated solvents were purchased from Euriso-top (Gif-sur-Yvette, France).

2.3. Extraction and isolation

Crushed and air-dried lichen material (150 g) was macerated with 500 mL of pure water at +4 °C for 15 h. The supernatant was filtrated and the extraction was repeated until mycosporine was not detected (by TLC under UV detection at 312 nm). The combined extracts were partly concentrated under reduced pressure and then lyophilized to give the crude aqueous extract (7.5 g). The dried residue was then extracted by stirring with chloroform at room temperature, for 4 h (500 mL×3) to yield crude chloroform extract (4.5 g). The crude aqueous extract (7.5 g) was dissolved in 10 mL of water and injected on a cation exchange resin column (DOWEX 50W-X8, 80 g). After removing unwanted compounds, including sugars and polyols (fraction A1), we obtained a semi-purified aqueous extract (fraction A2, 1.5 g). Fraction A2 was subjected to flash chromatography. The stationary phase was a bare silica column (Chromabond® Flash RS 15 g SiOH Ref. 732801. Macherey-Nagel) with mobile phase A (ACN-CH_3COONH_4 50 mM 90:10, pH 5.36) and mobile phase B (ACN-H_2O-CH_3COONH_4 50 mM 50:40:10, pH 5.36). The gradient elution was: 100 % of A during 5 min, 0-100 % of B during 20 min, and 100 % of B for 15 min with the flow rate at 10 mL/min. Fractions of 10 mL were collected. Fractions 5-9 containing mycosporine were combined (fraction A2.1, 210 mg) and further purified on an open reverse phase column (C-18 Hydro Chromabond, 4.3 g, Ref. 732810, Macherey-Nagel) using water as the mobile phase with the flow rate at 1.5 mL/min to give compound **1** (m = 8.0 mg). The residue subfraction A2.2 (1.0 g) was applied to flash chromatography using column Chromabond® Flash RS 15 g SiOH Ref. 732801. Macherey-Nagel. Sample was run with a mobile phase of $CHCl_3$ and MeOH in linear gradient mode (100-0 % of $CHCl_3$ over 90 min, the flow rate at 8.0 mL/min). Fractions of 10 mL were collected. Fractions 30-65 containing a precipitate were combined and filtered to provide 90 mg of a solid. One part of this precipitate (15 mg) was subjected to column chromatography (CC) and eluted isocratically with $CHCl_3$-MeOH 6:4 to give compound **2** (7.0 mg). A part of the chloroform extract (1.0 g) was chromatographed over Sephadex LH-20 with $CHCl_3$ to yield five fractions. Fraction 3 (18 mg) was subjected to CC eluted with CH_2Cl_2-MeOH 20:1 to give compound **3** (6.0 mg). Fraction 4 was concentrated to small volume and then a white solid was precipitated from the solution. The precipitate was filtered (30 mg) and recrystallized by PE-Ac 1:1 to give compound **4** (11.0 mg).

Mycosporine glutaminol (1)

Colorless viscous liquid. R_f: 0.30 ($CHCl_3$-MeOH-H_2O, 6:4:1). UV λ_{max} 310 (H_2O, ε = 12542). IR ν_{max} cm^{-1}: 3141, 2987, 1661, 1652, 1531, 1403, 1066. ^1H NMR (500 MHz, D_2O): δ_H 2.89 (1H, *d*, *J* = 17.1 Hz, H-4a), 2.77 (1H, *d*, *J* = 17.3 Hz, H-4b), 2.67 (1H, *d*, *J* = 16.9 Hz, H-6a), 2.41 (1H, *d*, *J* = 16.9 Hz, H-6b), 3.53 (2H, *s*, H-7), 3.57 (3H, *s*, H-8), 3.73 (1H, *m*, H-9), 3.70 (1H, *dd*, *J* = 4.1, 11.6 Hz, H-10a), 3.56 (1H, *m*, H-10b), 1.88 (1H, *m*, H-11a), 1.75 (1H, *m*, H-11b), 2.29 (2H, *m*, H-12); ^{13}C NMR (125 MHz, D_2O) δ_C 185.5 (C-1), 130.0 (C-2), 158.8 (C-3), 33.4 (C-4), 72.0 (C-5), 42.6 (C-6), 67.5 (C-7), 59.1 (C-8), 55.1 (C-9), 64.2 (C-10), 27.3 (C-11), 31.4 (C-12), 181.7 (C-13); ESI-HRMS: *m/z* 303.1551 $[M+H]^+$ (calcd. for $C_{13}H_{23}N_2O_6$ 303.1550).

2-Amino-3-acetylaminopropionic acid (**2**)

White powder. R_f: 0.28 ($CHCl_3$-MeOH-H_2O, 6:4:1).

^{1}H NMR (300 MHz, D_2O) δ_H 3.91 (1H, *dd*, 3.6, 6.6, H-2), 3.81 (1H, *dd*, 3.6, 14.9, H-3a), 3.61 (1H, *dd*, 6.7, 15.0, H-3b), 2.01 (3H, *s*, H-2'); ^{13}C (75 MHz, D_2O) δ_C 172.1 (C-1), 55.1(C-2),39.8 (C-3), 175.6 (C-1'), 21.8 (C-2').ESI-HRMS: *m/z* = 169.0592 $[M+Na]^+$ (calcd. for $C_5H_{10}N_2O_3Na$ 169.0589).

(22*E*,24*R*)-Ergosta-7,22-diene-3β,5α,6β-triol (3)

White powder. M.p: 260-263 °C. R_f: 0.52 (EtOAc-CH_2Cl_2-MeOH, 15:12:3). $[\alpha]^D_{20}$: -34.0 (*c* 0.16, pyridine). ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 4.05 (1H, *m*, H-3), 2.12 (1H, *dd*, 11.4, 13.0, $H4_{ax}$), 1.80 (1H, *dd*, 5.9, 12.6, $H4_{eq}$), 3.57 (1H, *d*, 5.0, H-6), 5.30 (1H, *m*, H-7), 0.53 (3H, *s*, H-18), 1.02 (3H, *s*, H-19), 0.97 (3H, *d*, 6.6, H-21), 5.20 (1H, *dd*, 6.6, 15.2, H-22), 5.10 (1H, *dd*, 7.5, 15.2, H-23), 0.78 (3H, *d*, 6.7, H-26)**, 0.74 (3H, *d*, 6.7, H-27)**, 0.86 (3H, *d*, 6.8, H-28); ^{13}C (75 MHz, $CDCl_3$) δ_C 29.9 (C-1), 33.2 (C-2), 68.9 (C-3), 40.6 (C-4), 76.1 (C-5), 73.8 (C-6), 117.7 (C-7), 144.2 (C-8), 43.6 (C-9), 37.3 (C-10), 22.2 (C-11), 39.4 (C-12), 43.9 (C-13), 54.9 (C-14), 23.0 (C-15), 28.1 (C-16), 56.1 (C-17), 12.5 (C-18), 19.0 (C-19), 39.6 (C-20), 21.3 (C-21), 135.5 (C-22), 132.3 (C-23), 43.0 (C-24), 33 (C-25), 20.1* (C-26), 19.8* (C-27), 17.7(C-28).

,: shifts may be exchangeable*). ESI-HRMS: *m/z* = 453.3350 $[M+Na]^+$ (calcd. for $C_{28}H_{46}O_3Na$ 453.3344).

(2*S*,3*S*,4*R*,2'*R*)-2-(2'-Hydroxytetracosanoylamino)octadecan-1,3,4-triol (4)

White powder. M.p: 120-121 °C. R_f: 0.15 (Tol-EtOAc-AcOH, 70:25:5). $[\alpha]^D_{20}$: +8.0 (*c* 0.2, pyridine). IR ν_{max} (cm^{-1}): 3329 and 3203 (hydroxyl), 1620 and 1545 (amide), 723 (aliphatic). ^{1}H NMR (500 MHz, pyridine-d_5) 4.46 (1H, *dd*, 10.9, 4.9, H-1a), 4.54 (1H, *dd*, 10.5, 4.4, H-1b), 5.15 (1H, *m*, H-2), 4.39 (1H, *dd*, 6.6, 4.4, H-3), 4.32 (1H, *m*, H-4), 2.29, 1.96 (2H, *m*, H-5), 1.73 (2H, *m*, H-6), 1.28-1.44 (H-7-H-17), 0.87 (3H, *t*, 7.1, H-18), 4.65 (1H, *dd*, 7.7, 3.9, H-2'), 2.27 (2H, *m*, H-3'), 1.97 (2H, *m*, H-4'), 1.28-1.44 (H-5'-H-23'), 0.88 (3H, *t*, 7.0, H-24'), 8.61 (1H, *d*, 8.8, NH), ^{13}C (125 MHz, pyridine-d_5): 62.2 (C-1), 53.1 (C-2), 77.0 (C-3), 73.2 (C-4), 34.3(C-5), 26.0 (C-6), 23.1-32.3 (C-7-C-17), 14.4 (C-18), 175.4 (C-1'), 72.6 (C-2'), 35.9 (C-3'), 26.8 (C-4'), 23.1-32.3(C-5'-C-23'), 14.4 (C-24'). ESI-HRMS: *m/z* = 706.6324 $[M+Na]^+$ (calcd. for $C_{42}H_{85}NO_5Na$ 706.6325).

2.4. Determination of p*K*a value

The UV-Vis method was used for determination of pK_a value through measurement of absorbance of the mycosporine aqueous solution at different pH values. The pK_a value "extraction" from the pH and absorbance using a non-linear least-squares procedure with the NLREG program [13]. This regression analysis also called "curve fitting" determines the values of parameter (here pK_a and molar extinction coefficient of each form) that cause the best fit between calculated and measured absorbances. To obtain meaningful statistical parameters, six independent determinations were performed for mycosporine at 25 °C. By assimilating activity and concentration (diluted solutions) and considering the laws of matter conservation and of equilibria in water (Eqs. 1-6), Eq. 4 gives the mathematical model allowing the calculation of absorbances.

$$Ka = \frac{B\left[H_3O^+\right]}{\left[BH^+\right]} \quad (1)$$

$$B + \left[BH^+\right] = C_0 \quad (2)$$

$$A_i^\lambda = \varepsilon_{BH}^\lambda + \left[BH^+\right] + \varepsilon_B^\lambda \; B \quad (3)$$

$$A_i^\lambda = \frac{\varepsilon_{BH}^\lambda + C_0\left[H_3O^+\right]_i + \varepsilon_B^\lambda C_0 Ka}{\left[H_3O^+\right]_i + Ka} \quad (4)$$

$$\left[H_3O^+\right] = 10^{-pH} \quad (5)$$

$$Ka = 10^{-pKa} \quad (6)$$

where Ka is the acid dissociation constant of the conjugate acid BH^+; A_i^λ is the measured absorbance at pH_i and at the wavelength λ; $\varepsilon_{BH^+}{}^\lambda$ and $\varepsilon_B{}^\lambda$ are the molar extinction coefficients at the wavelength λ of each form: conjugated acid $\mathbf{BH^+}$ and base **B** respectively; C_0 is the total concentration of mycosporine in mol/L.

From Eq. 4, the regression analysis consists in calculating for each pHi, the calculated A_i^λ with arbitrary set of values (*K*a and molar absorptivities) and then to compare the calculated A_i^λ with the experimental one. The agreed values of *K*a and molar extinction coefficients are those giving the best fit between calculated and measured absorbances.

3. RESULTS AND DISCUSSION

In this study, the dried lichen material (150 g) was powdered and extracted first with water. The dried residue was next extracted with chloroform to give aqueous (7.5 g, 5.0 %) and chloroform (4.5 g, 3.0 %) extracts. From the aqueous extract, two compounds were isolated including mycosporine **1**, non-protein amino acid **2**. Two other compounds sterol **3** and

ceramide **4** were also isolated from the chloroform extract. Their structures were shown in figure 1. Non-protein amino acids now included about 250 compounds derived from the plant world and have never been described previously in lichens [7]. It has been suggested that many non-protein amino acids are toxic to the larvae of various seed-eating beetles and leaf-eating moths [8,9,10]. It would be also interesting to investigate further whether this metabolite protects lichen *D. luridum* against potential predators.

Except for compound **1**, other compounds were reported for the first time in *D. luridum*.

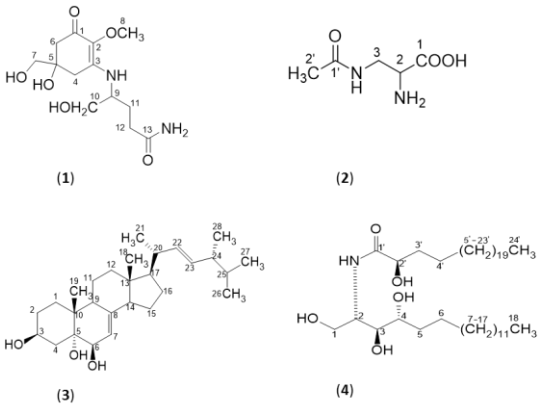

Figure 1: Structures of isolated compounds from *D. luridum*

In the previous work, the authors have shown that mycosporine **1** has a strong absorption at 310 nm in the region of UVB (290-320 nm) with high ε 12540 $M^{-1}.cm^{-1}$ suggesting the potential application as a natural photoprotectant [4]. Such compound contains one acido-basic site, however, its dissociation constant has never been published so far. In fact, the pK_a value is an important physicochemical consideration for predicting bioavailability. According to our preliminary study, no change was observed in the range of pH from 3 to 7 for mycosporine **1** (figure 2), the largest difference of absorbance occurring between pH -1 and 3 at 310 nm (figure 3). In these acidic media, the maximum wavelength λ_{max} of mycosporine showed a hypsochromic shift from 310 nm at pH 3 to 300 nm in the pH -1 solutions. The same behavior has been observed for porphyra-334 containing an aminocycloheximine ring, however, the hypsochromic shift is smaller (334 to 330 nm) than that of **1** [11]. A hypochromic shift was also observed with the increase of acidity (A = 0.37 at pH 3 and 0.28 at pH 0). From the UV spectra of mycosporine **1** in the restricted range $1.0 \leq pH \leq 3.8$ (figure 3), by comparison of molar extinction coefficients determined through the fitting process with the experimental ones, a good consistence between experimental and calculated molar extinction coefficients ε_B^{310} was obtained (table 1). Moreover, the presence of an isosbestic point at approximately 297 nm provided evidence of only one equilibrium in solution.

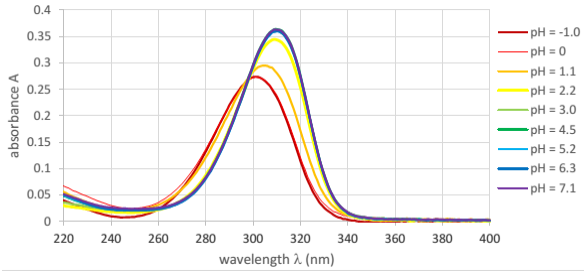

Figure 2: UV spectra of mycosporine **1** at pH range from -1 to 7 ($C = 1.56\times10^{-5}$ M)

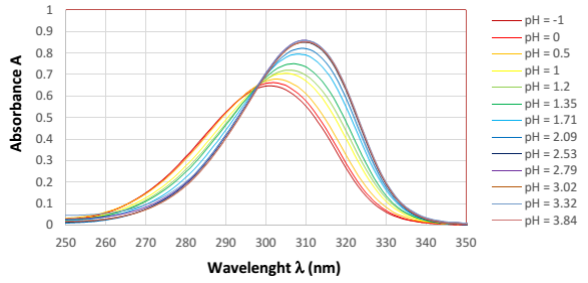

Figure 3: UV spectra of mycosporine **1** at pH range from -1 to 3.84 ($C_0 = 6.82\times10^{-5}$ M)

Table 1: pK_a and molar extinction coefficient values of mycosporines **1**

	pK_a	ε_{BH+}^{-1} ($M^{-1}.cm^{-1}$)	ε_B^{-1} ($M^{-1}.cm^{-1}$)	ε_{Bexp}^{-1} ($M^{-1}.cm^{-1}$)
Mycosporine **1**	**1.29** [1.28-1.31]	8200 [8187-8213]	12640 [12593-12691]	12540

where ε_{Bexp}^{310}: the experimental molar extinction coefficient at 310 nm; ε_{BH+}^{310} and ε_B^{310} are the calculated molar extinction coefficients at 310 nm of conjugated acid **BH**$^+$ and base **B** respectively.

The mycosporine **1** appeared to be rather a weak base or strong acid with a pK_a value very low 1.29. The conjugated acid (**BH**$^+$ form) of **1** exhibited pK_a value much weaker than the *N*-methyl-anilinium ion

(CH_3-NH_2^+-C_6H_5, pK_a = 4.85) [12]. The result can be explained by the occurrence of both a withdrawing inductive and a strong resonance effect of the cyclohexenone group which led to the global electron density decrease. The protonation of the unbounded lone pair electrons of the nitrogen atom in the **BH**$^+$ form would prevent the resonance delocalization of the molecule. So the degree of resonance delocalization is higher in the base form **B** than in the acid conjugated form **BH**$^+$ [11] and could explain the hypsochromic shift in strongly acidic solutions and the decrease of the molar extinction coefficients of **B** and **BH**$^+$ forms. The diagram of the species distribution (figure 4) allowed to conclude that only the neutral form **B** is present and is responsible for the UV absorption in water at pH 7.

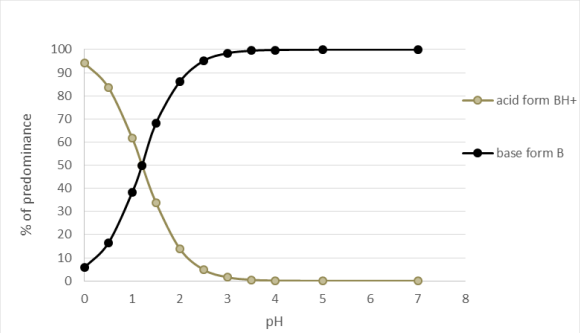

Figure 4: Distribution diagram of mycosporine **1**

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Corresponding author: **Nguyen Thi Thu Tram**
Can Tho University of Medicine and Pharmacy
No. 179, Nguyen Van Cu Str., An Khanh Ward, Ninh Kieu Dist., Can Tho City
E-mail: ntttram@ctump.edu.vn; Telephone: 0919886682