

# Mycosporine-Like Compounds in Chlorolichens: Isolation from *Dermatocarpon luridum* and *Dermatocarpon miniatum*, and their Photoprotective Properties

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## Abstract

Two mycosporines were isolated for the first time in two chlorolichen species, *Dermatocarpon luridum* and *Dermatocarpon miniatum*. Mycosporine glutaminol (**1**) and mycosporine glutamicol (**2**) were isolated along with the ethyl ester of mycosporine glutamicol (**3**), which was formed during the purification process. Aqueous extracts and pure mycosporines were then investigated for their antioxidant activities and photoprotective properties along with their photostability and photocytotoxicity. Semi-purified mycosporine fractions were much more antioxidant than lichen aqueous crude extracts. Compound **3** ( $IC_{50} = 4.00 \mu\text{g/mL}$ ) was found to be as active as quercetin ( $IC_{50} = 6.75 \mu\text{g/mL}$ ), while the two genuine mycosporines **1** and **2** exhibited moderate activity. The three mycosporines were found to be stable until 5J/m<sup>2</sup> UVA and UVB radiations whereas Trolox, used as a positive control, was degraded up to 10% and 19%, respectively. Moreover, these mycosporines and semi-purified extracts did not induce any phototoxicity on HaCaT cells exposed to UVA radiations.

## Key words

*Dermatocarpon luridum* · *Dermatocarpon miniatum* · Verrucariaceae · lichen · mycosporine-like amino acids (MAAs) · photoprotection

## Abbreviations

|       |   |
|-------|---|
| DL1:  | crude aqueous extract of <i>D. luridum</i>          |
| DL2:  | semi-purified aqueous extract of <i>D. luridum</i>  |
| DM1:  | crude aqueous extract <i>D. miniatum</i>            |
| DM2:  | semi-purified aqueous extract of <i>D. miniatum</i> |
| MAAs: | mycosporine-like amino acids                        |
| PDA:  | photodiode array                                    |
| PIF:  | photo-irritancy factors                             |

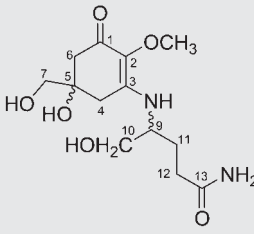
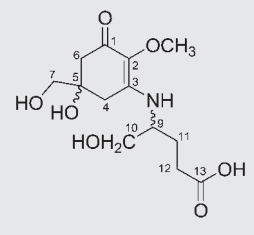
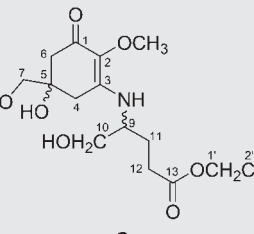
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Mycosporine-like compounds comprising mycosporines and MAAs have received much attention for their putative role in UV photoprotection as antioxidants and osmoprotectants [1–4]. Mycosporine-like compounds are small water-soluble molecules composed of cyclohexenone (oxo-mycosporine) or cyclohexenimine (imino-mycosporine) ring structures conjugated to an ami-

no acid or an amino alcohol subunit. They are characterized by a unique strong peak in the wavelength range of 310–365 nm ( $\epsilon = 28\,000\text{--}50\,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ). About 40 mycosporines and derivatives have been described so far, and some of them are substituted with functional groups or are covalently bound to saccharidic units [5–8]. Such UV-absorbing compounds are distributed in taxonomically diverse organisms of terrestrial, marine, or freshwater origin [9]. More recently, the distribution of mycosporine-like compounds has been reported in cyanolichens (symbiotic organisms resulting from an association between a fungus and a cyanobacterium) [10,11]. Although chlorolichens correspond to 90% of lichen species, mycosporine-like compounds have not yet been described in chlorolichens in which cyanobacteria are replaced by green algae. Their secondary metabolite profile is generally characterized by a variety of specific phenolic compounds, and most of them have UV absorbing properties [2,12]. A previous phytochemical screening revealed that chlorolichens belonging to the *Dermatocarpon* genus (Verrucariaceae) did not contain the usual lichen substances such as depsides, depsidones, dibenzofurans, xanthonones, and anthraquinones, which are considered photoprotectants [13–16]. In the present study, we have investigated two *Dermatocarpon* species for their photoprotective compounds: *Dermatocarpon luridum* and *Dermatocarpon miniatum* containing *Diplosphaera chodatii* (Trebouxiophyceae) photobionts [17]. A phytochemical study led to the isolation and identification of three mycosporines. The aqueous extracts and purified mycosporines were tested for their antioxidant activities, photostability, and photocytotoxicity.

To investigate *D. luridum* and *D. miniatum* for mycosporine-like compounds, a protocol previously described [10] was used to extract lichen material with water (+4 °C) followed by semi-purification on cation exchange resin chromatography (see Supporting Information). The HPLC-DAD-MS<sup>2</sup> analysis of DL2 and DM2 revealed the presence of three mycosporines, **1**, **2**, and **3**, with an absorption maximum at 310 nm (Table 1). These compounds were then isolated from an aqueous extract of *D. luridum* (5 mg, 6.5 mg, and 6 mg, respectively) and their structures were determined by ESI<sup>+</sup>-HRMS, NMR, and IR spectra (Figs. 1.15–3.85, Supporting Information). Spectroscopic analysis and MS data comparison of compounds **1** and **2** with the literature [6] led to the identification of mycosporine glutaminol (**1**) ( $m/z$  [M + H]<sup>+</sup> 303) and mycosporine glutamicol (**2**) ( $m/z$  [M + H]<sup>+</sup> 304). As lichens are symbiotic organisms, involvement of their respective partners in the biogenesis of mycosporines should be elucidated. Until now, cyanobacteria were supposed to produce mycosporine-like compounds in cyanolichens [10]. Thus far, mycosporines **1** and **2** and their glycolysed derivatives have been described in various terrestrial ascomycetous fungi [18–21]. In addition, compound **1** has also been detected in the terrestrial cyanobacteria *Leptolyngbya sp.* [6] and compound **2** in the cyanolichen *Degelia plumbea* [10]. As no green algae are described to date to synthesize these mycosporines, the fungal partner may also be involved in the biosynthesis of mycosporines **1** and **2**, especially since mycosporine described in lichens have the characteristic carbonyl moiety of fungal mycosporines. Compound **3** ( $m/z$  [M + H]<sup>+</sup> 332) did not correspond to any previously described mycosporine and ESI<sup>+</sup>-HRMS revealed a C<sub>15</sub>H<sub>25</sub>NO<sub>7</sub> molecular formula differing from mycosporine glutamicol by  $m/z$  28 (Fig. 3.25, Supporting Information). Its IR spectrum showed a large band in the 3200–3650 cm<sup>-1</sup> range indicating the presence of hydroxyl and amino groups, two strong bands at 1665 and 1712 cm<sup>-1</sup> corresponding to an  $\alpha,\beta$ -unsaturated ketone group and a carbonyl ester group,

**Table 1** Physicochemical properties and mass fragmentation pattern of mycosporines **1**, **2**, and **3**.

| Structure  | $\lambda_{\max}$<br>(nm) | $R_{t_a}$<br>(min) | $R_{t_b}$<br>(min) | $m/z$<br>[M + H] <sup>+</sup> | ESI-MS fragmentation<br>pattern   | ESI-HRMS<br>[M + H] <sup>+</sup><br>(0 ppm)  | Molecular<br>formula  |
|--|--------------------------|--------------------|--------------------|-------------------------------|---|--|---|
|   | 310                      | 2.95               | 4.87               | 303                           | 235 [M + H-68] <sup>+</sup> ,<br>267 [M + H-2H2O] <sup>+</sup> ,<br>285 [M + H-H2O] <sup>+</sup>  | 303.1551<br>[M + H] <sup>+</sup><br>(0 ppm)  | C <sub>13</sub> H <sub>22</sub> N <sub>2</sub> O <sub>6</sub> |
|   | 310                      | 4.73               | 5.62               | 304                           | 236 [M + H-68] <sup>+</sup> ,<br>258 [M + H-H2O-CO] <sup>+</sup> ,<br>268 [M + H-2H2O] <sup>+</sup> ,<br>286 [M + H-H2O] <sup>+</sup>     | 326.1198<br>[M + Na] <sup>+</sup><br>(5 ppm) | C <sub>13</sub> H <sub>21</sub> NO <sub>7</sub>               |
|  | 310                      | 15.75              | 1.91               | 332                           | 218 [M + H-C2H6O-68] <sup>+</sup> ,<br>264 [M + H-68] <sup>+</sup> ,<br>286 [M + H-H2O-CO] <sup>+</sup> ,<br>314 [M + H-H2O] <sup>+</sup> | 354.1522<br>[M + Na] <sup>+</sup><br>(0 ppm) | C <sub>15</sub> H <sub>25</sub> NO <sub>7</sub>               |

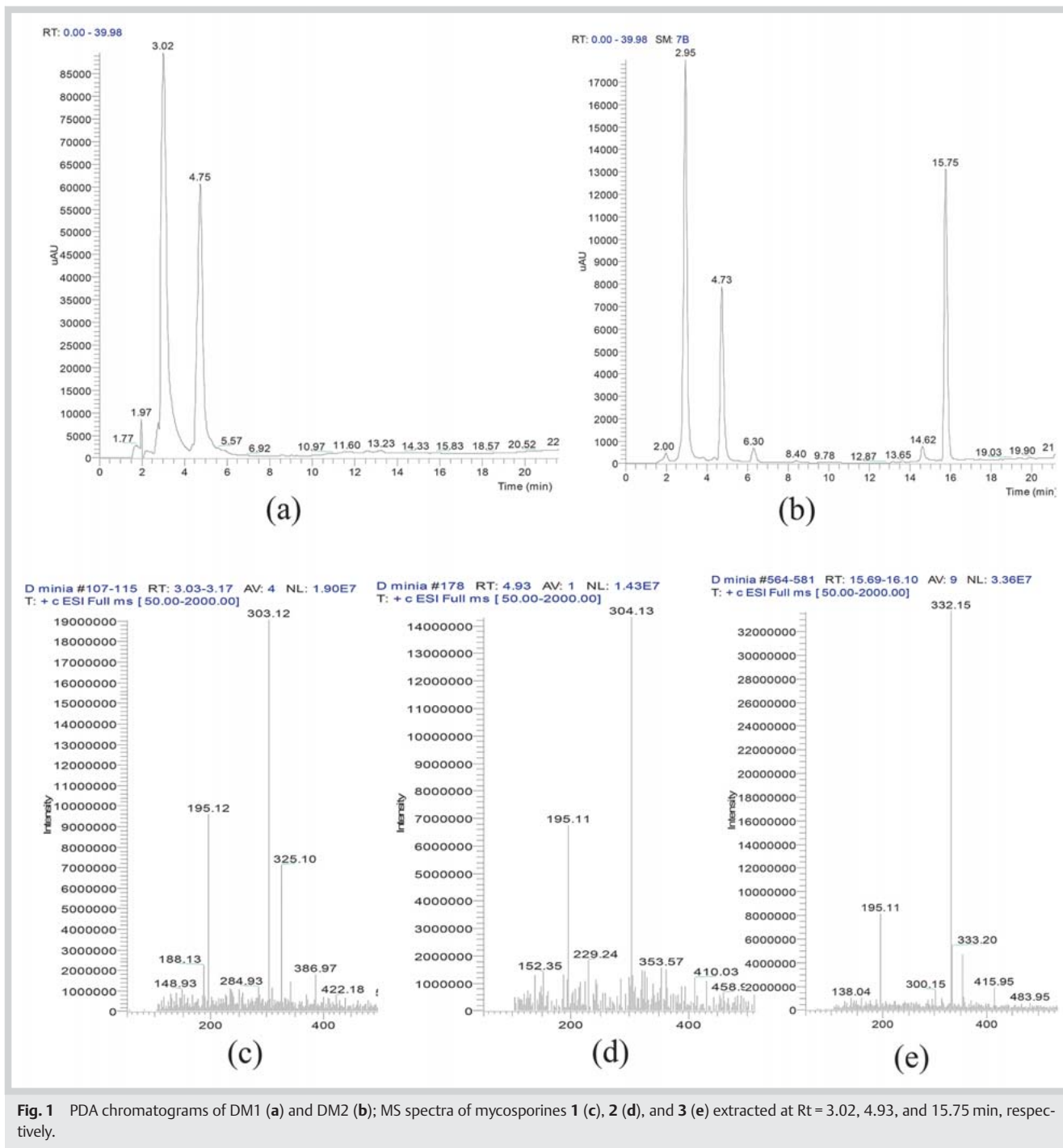
$R_{t_a}$ : retention time on HPLC-DAD-MS<sup>2</sup> using a Zorbax Eclipse XDB-C18 (3.5  $\mu$ m, 150  $\times$  2.1 mm) column;  $R_{t_b}$ : retention time on HPLC-DAD using a Kinetex HILIC 100  $\text{\AA}$  (2.6  $\mu$ m, 100  $\times$  4.60 mm) column

respectively (Fig. 3.15, Supporting Information). <sup>1</sup>H, <sup>13</sup>C-NMR, COSY, and HMBC correlations were in full agreement with the structure of **3** (Figs. 3.35–3.85, Supporting Information). Compound **3** was supposed to be formed by the esterification between mycosporine glutamicol and ethanol, containing some acidic traces, during NaCl removal in the semi-purification step. Indeed, many MAAs can be converted to their methyl esters by treatment with HCl-methanol [1]. Comparison of the crude and the semi-purified aqueous extracts using HPLC-DAD-MS<sup>2</sup> analysis confirmed that compound **3** ( $R_t = 15.75$  min) was a by-product as only found in the semi-purified extract (Fig. 1).

Regarding the strong absorption of the genuine mycosporines **1** and **2** at 310 nm in the UVA–UVB overlap region (molar extinction coefficients 12542, 17248 M<sup>-1</sup>.cm<sup>-1</sup>, respectively) (Fig. 2), we assume that mycosporines **1** and **2** may be partly involved in the photoprotection of these lichens [2]. Therefore, we investigated some biological activities related to UV filter requirements including the ethyl derivative **3**, which exhibited a higher molar extinction coefficient 21 295 M<sup>-1</sup>.cm<sup>-1</sup>. Until now, most mycosporine-like compounds tested for their antioxidant activities are either pure imino-mycosporines or a mixture of several imino-mycosporines [7, 22–26]. Thus, oxo-mycosporines are less reported, and only mycosporine glycine and mycosporine glutaminol glucoside have been tested so far [23, 27–29]. So, the antioxidant properties using DPPH and NBT assays of the pure oxo-mycospor-

ines **1**, **2**, and **3** along with aqueous lichen extracts which contain **1** and **2** were evaluated (Table 2). DL2 and DM2 were slightly more active than their respective crude extracts DL1 and DM1, while the three mycosporines displayed weak electron transfer activity, as shown in the DPPH assay. At 80  $\mu$ g/mL, the semi-purified extracts DL2 and DM2 with a higher mycosporine concentration (data not shown) scavenged, respectively, 2-fold and 4-fold more superoxide anion than the crude extracts DL1 and DM1. Likewise, DM2 (IC<sub>50</sub> = 5.20  $\pm$  2.20  $\mu$ g/mL) was more active than DL2 (IC<sub>50</sub> = 11.00  $\pm$  5.00  $\mu$ g/mL), which appeared also to be correlated with the mycosporine concentration. However, the activity of the individual mycosporines **1** and **2** were found to be moderate (no IC<sub>50</sub> could be determined). The esterification of the mycosporine glutamicol carboxyl group resulted in a significant superoxide anion scavenging activity increase with a better activity (IC<sub>50</sub> = 4.00  $\mu$ g/mL) of compound **3** compared to the standard quercetin (IC<sub>50</sub> = 6.75  $\mu$ g/mL).

Looking for UV protectants, photostability under UVA and UVB, and low toxicity on cells before and after UVA irradiation are prerequisites. Aqueous solutions of the DM2 and the three mycosporines were irradiated under six UV doses covering the UVA and UVB range. By-products of the positive control Trolox appeared at 0.5 J/m<sup>2</sup> exposures, and a 10% and 19% degradation rate was observed at 5 J/m<sup>2</sup> exposures to UVA and UVB, respectively. Concerning DM2 and the three mycosporines, no degradation oc-



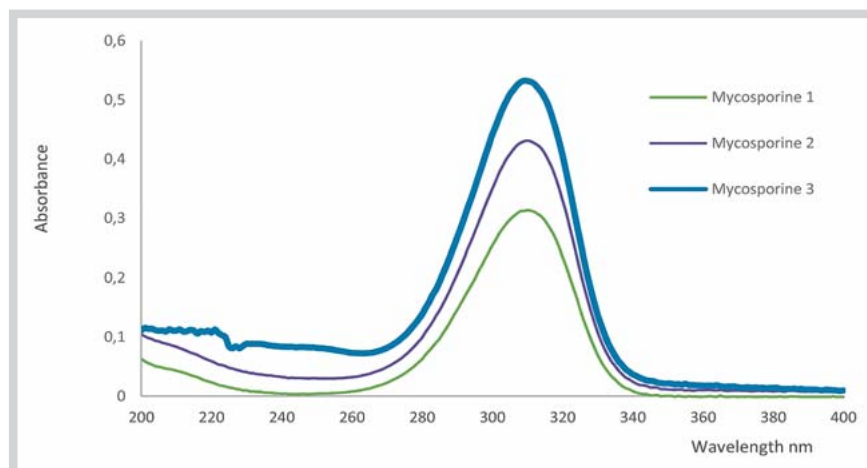
**Fig. 1** PDA chromatograms of DM1 (a) and DM2 (b); MS spectra of mycosporines 1 (c), 2 (d), and 3 (e) extracted at Rt = 3.02, 4.93, and 15.75 min, respectively.

occurred even at 5 J/m<sup>2</sup> (Fig. 5S, Supporting Information). These results confirmed the absence of photoproducts observed by Moliné et al. when mycosporine-glutaminol-glucoside was subjected to UVB irradiation [29].

According to clinical and experimental studies, noncytotoxic doses of many chemicals could, however, induce phototoxic responses when exposed to non-phototoxic doses of UV radiation [30,31]. In this way, the PIF values of the mycosporines and extracts were determined and compared to the positive chlorpromazine control highlighting a phototoxic effect with a PIF > 5 (Table 3). Except a slight PIF = 1.1 calculated for compound 2, no cytotoxicity with or without UVA irradiation could be observed.

served for the other tested compounds and extracts. These PIF values << 5 suggest that these lichen extracts and mycosporines are non-phototoxic.

In regards to their good photostability, non-photocytotoxicity, and their antioxidant activities, mycosporines and the semi-purified aqueous extracts appear to be considered as eligible candidates for photoprotection and further developed with regard to Helioguard<sup>®</sup>.



**Fig. 2** UV absorption spectra of mycosporines **1**, **2**, and **3** in water at  $C = 2.5 \times 10^{-5}$  M.

| Extract/mycosporine | % Inhibition |             |
|---------------------|--------------|-------------|
|                     | DPPH method  | NBT method  |
| DL1                 | 5.2 ± 2.0    | 44.6 ± 15.7 |
| DL2                 | 18.4 ± 3.9   | 80.2 ± 9.8  |
| DM1                 | 13.6 ± 5.7   | 21.5 ± 12.3 |
| DM2                 | 24.8 ± 6.2   | 86.4 ± 2.5  |
| (1)                 | 1.9 ± 2.3    | 42.1 ± 2.2  |
| (2)                 | 0.5 ± 0.8    | 44.6 ± 5.8  |
| (3)                 | 30.4 ± 3.3   | 85.4 ± 1.4  |
| Quercetin           | 100.0 ± 0.1  | 100.0 ± 0.1 |

DL1: crude aqueous extract, DL2: semi-purified aqueous extract of *D. luridum*; DM1: crude aqueous extract, DM2: semi-purified aqueous extract of *D. miniatum*. (1): Mycosporine glutaminol; (2): mycosporine glutamicol; (3): ethyl ester of mycosporine glutamicol

**Table 2** Antioxidant activity of the aqueous extracts and mycosporines **1**, **2**, and **3** through DPPH free radical scavenging activity and superoxide anion scavenging ability (NBT method). Activity is expressed as % inhibition at the maximum dose (400 and 80 µg/mL, respectively).

| Extract/mycosporine         | Photocytotoxic activities on HaCaT cells |                  | Photo-irritancy factor (PIF) |
|-----------------------------|--|------------------|------------------------------|
|                             | IC <sub>50</sub> ± SD (µg/mL)            |                  |                              |
|                             | Without irradiation                      | With irradiation |                              |
| DL1                         | > 40                                     | > 40             | *1 <sup>b</sup>              |
| DL2                         | > 40                                     | > 40             | *1 <sup>b</sup>              |
| DM1                         | > 40                                     | > 40             | *1 <sup>b</sup>              |
| DM2                         | > 40                                     | > 40             | *1 <sup>b</sup>              |
| (1)                         | > 40                                     | > 40             | *1 <sup>b</sup>              |
| (2)                         | 30.0 ± 6.0                               | 27.0 ± 4.0       | 1.1 <sup>c</sup>             |
| (3)                         | > 40                                     | > 40             | *1 <sup>b</sup>              |
| Chlorpromazine <sup>a</sup> | 22.0 ± 0.5                               | 3.5 ± 0.0        | 6.3 <sup>c</sup>             |

DL1: crude aqueous extract, DL2: semi-purified aqueous extract of *D. luridum*; DM1: crude aqueous extract, DM2: semi-purified aqueous extract of *D. miniatum*. (1): Mycosporine glutaminol; (2): mycosporine glutamicol; (3): ethyl ester of mycosporine glutamicol. <sup>a</sup>Photocytotoxic positive control; <sup>b</sup>formal PIF =  $C_{\max}(-UV)/C_{\max}(+UV)$ ; <sup>c</sup>formal PIF =  $IC_{50}(-UV)/IC_{50}(+UV)$

**Table 3** Cytotoxic and photocytotoxic activities of the aqueous extracts and mycosporines.

## Materials and Methods

### Lichen material

*D. luridum* (With.) J.R. Laundon was collected in Huelgoat, Brittany, France (April 2012). *D. miniatum* (L.) W. Mann was collected in Moëlan-sur-Mer, Brittany, France (September 2013). The lichens were identified by Jean-Yves Monnat (biologist, University of Bretagne Sud, France). Voucher specimens were deposited in the herbarium of Pharmacognosy and Mycology, University of Rennes 1, France with reference numbers JB/12/001 and JB/13/004, respectively.

### Extraction and isolation of mycosporines

Lichen material was macerated with pure water at +4 °C to give a crude aqueous extract. The extract was purified by the cation exchange resin Dowex to remove sugars and polyols. Next, a purification protocol including bare silica-HILIC flash chromatography, open reverse-phase column chromatography, and semi-preparative HPLC-DAD was applied to the semi-purified extract to isolate compounds **1**, **2**, and **3**. The detailed extraction and isolation of the three compounds as well as ESI-HRMS, IR, and NMR data of the compounds are available as Supporting Information



## Supporting information

The general experimental procedures, optimized extraction and purification method followed by HPLC-DAD-MS<sup>2</sup> analysis, extraction and isolation of mycosporines as well as the copies of their HRMS, IR, <sup>1</sup>H, <sup>13</sup>C, 2D NMR spectra, antioxidant assays, photostability under UVA and UVB, and cytotoxic and photocytotoxic activities are available as Supporting Information.

## Acknowledgements

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## Conflict of Interest

There is no conflict of interest among all authors.

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