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Mycosporine-like Amino Acids in the Zooxanthella-Ciliate Symbiosis *Maristentor dinoferus*

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Coral reef organisms living in mutualistic symbioses with phototrophic dinoflagellates are widespread in shallow UV-transparent waters. *Maristentor dinoferus* is a recently discovered species of marine benthic ciliate that hosts symbiotic dinoflagellates of the genus *Symbiodinium*. In this study, we tested this ciliate for the occurrence of mycosporine-like amino acids, a family of secondary metabolites that minimize damage from exposure to solar UV radiation by direct screening. Using high-performance liquid chromatography and liquid chromatography coupled to mass spectrometry, five mycosporine-like amino acids (shinorine, palythenic acid, palythine, mycosporine-2-glycine, and porphyra-334) were identified in aqueous methanolic extracts of the symbiosis. This is the first report of mycosporine-like amino acids in a marine ciliate.

Key words: MAAs; mixotrophic ciliates; natural sunscreen substances; reef ecosystems; secondary metabolites; solar ultraviolet radiation.

Introduction

A general characteristic of reef waters is their high transparency to solar ultraviolet (UV) radiation (Dunne and Brown 1996). Most of our knowledge about the relationship between UV radiation and mutualistic associations is based on studies of algal—invertebrate symbioses, particularly on scleractinian corals and their zooxanthellae (Shick and Dunlap 2002; Shick et al. 1996). Corals and other reef organisms have evolved chemical defenses to minimize the damaging effects of solar UV radiation (Dunlap and Chalker 1986; Shick et al. 1996). Shibata (1969) was the first to report the occurrence of UV-absorbing compounds in zooxanthellate scleractinian corals from the Great Barrier Reef. Later, modern chemical analytical methods identified the UV-absorbing compounds in several reef organisms as mycosporine-like amino acids (MAAs), a family of secondary metabolites that efficiently screen out wavelengths in the UV region, apparently without producing damaging photo-excited states (see reviews by Banaszak and Trench 2001;

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Dunlap and Shick 1998; Karentz 2001; Shick and Dunlap 2002). MAAs are intracellular water-soluble compounds with high molar extinction coefficients at absorption maxima between 309 and 360 nm.

In algal—invertebrate symbiosis, MAAs probably originate from the algae (Shick et al. 1999), but there is evidence that the host can eventually alter the composition and proportion of these compounds (Shick and Dunlap 2002). In field and laboratory studies with corals, the concentration of MAAs generally shows a positive relationship with UV exposure (Corredor et al. 2000; Shick et al. 1999). Evidence for the direct photoprotective role of MAAs in marine organisms has been recently obtained for primary producers as well as for non-symbiotic invertebrates (reviewed by Banaszak 2003) which, although unable to synthesize MAAs can obtain them through the diet (Carroll and Shick 1996; Shick et al. 2002).

In contrast to the large amount of information available for algal—invertebrate symbioses, no algal—protist symbioses have been available for comparison. Thus, when the marine algal—bearing ciliate *Maristentor dinoferus* (Heterotrichea) was described from Guam, Mariana Islands (Lobban et al. 2002; Miao et al. 2005), we took the opportunity to examine it. *M. dinoferus* is a trumpet-shaped benthic ciliate that is up to 1 mm long, forms dark clusters of individuals visible to the naked eye (Lobban et al. 2002), and can be collected in substantial quantities. *M. dinoferus* hosts 500—700 zooxanthellae of the genus *Symbiodinium* (Dinophyta) (Lobban et al. 2002), a symbiont that is also commonly found in other symbiotic reef invertebrates, particularly corals, and foraminiferans (Banaszak et al. 2000; Pochon et al. 2001).

Here, we show evidence from detailed chemical analysis by high-performance liquid chromatography (HPLC) and liquid chromatography coupled to mass spectrometry that documents the occurrence of these sunscreen compounds in *M. dinoferus*.

Results

The spectrophotometric scan of methanolic extracts of *M. dinoferus* revealed several absorption peaks with maxima at 294, 330, 339, 470, 553, 596, and 665 nm (Fig. 1). Absorption at 294 and 596 nm probably corresponds to the presence of maristentorin, which absorbs strongly in the UV range and close to the red (Mukherjee et al. in press). Absorption at 470 and 665 nm were characteristics of carotenoids and chlorophyll-*a* respectively. The broad UV-absorption peak

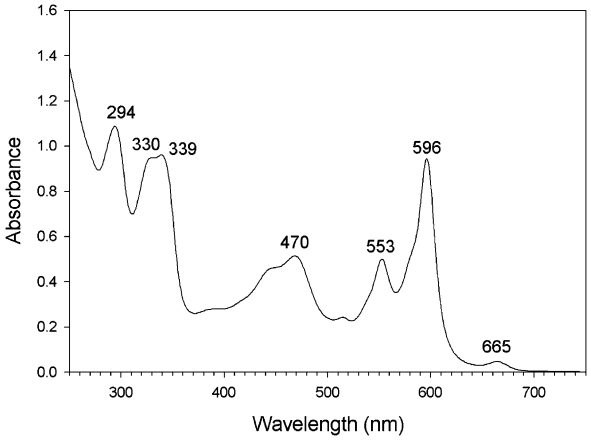


Figure 1. Absorption spectrum of an aqueous methanolic extract of *Maristentor dinoferus*. Values indicate the wavelength maximum for each peak (see text for explanations).

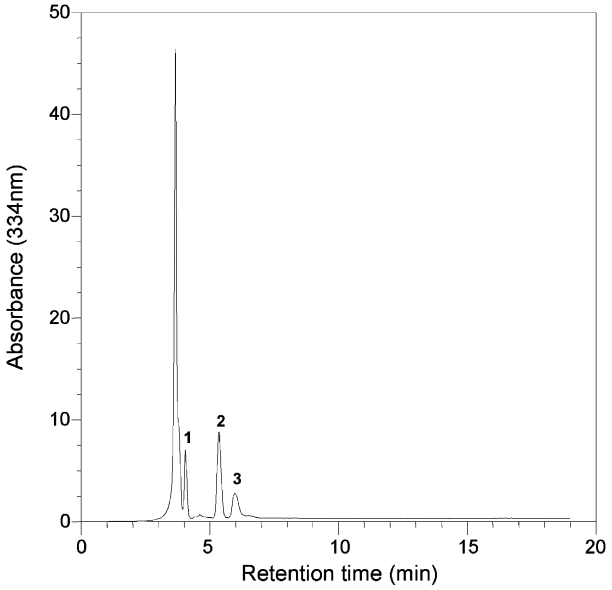


Figure 2. HPLC chromatogram (detection at 334 nm) of a methanolic extract from *Maristentor dinoferus.* Eluent was a mixture of water:methanol:acetic acid (75:25:0.1) and the flow rate 0.79 ml min^{-1}. Fractions 1, 2, and 3 correspond to shinorine, palythenic acid, and palythine respectively. Traces of porphyra-334 and mycosporine-2-glycine were also found.

between ca. 310 and 360 nm with a maximum at 330 and 339 nm is typical for MAAs (309—360 nm), but is probably influenced by the absorption of maristentorin as well (Fig. 1). Results from HPLC analyses at both laboratories confirmed the existence of three main fractions. The first fraction had a retention time (R_t) = 4.0 min and maximum absorption (λ_{max}) at 334 nm, the second eluted at R_t = 5.36 min with a λ_{max} at 337 nm, and the last fraction was observed at 5.96 min and had a λ_{max} at 320 nm (Figs 2 and 3). Based on co-chromatographic analysis with extracts from *Porphyra yezoensis*, fractions 1 and 3 were identified as shinorine and palythine respectively. Traces of another two UV-absorbing compounds were also found, but their amount was too low in the extract to resolve a clear absorption spectrum. However, based on the retention time and λ_{max}, they were putatively identified as porphyra-334 (λ_{max} 334 nm) and mycosporine-2 glycine (λ_{max} 331 nm). Liquid chromatography coupled to mass spectrometry (LC-MS) was used as an independent method to corroborate these and the other compounds, as well as to identify fraction 2. Results from LC-MS and comparison of MS/MS spectra with those previously published for MAA standards (Whitehead and Hedges 2003) confirmed all MAAs identified by HPLC and identified the second fraction as palythenic acid (Table 1).

Discussion

Maristentor dinoferus is the first ciliate known to have symbiotic *Symbiodinium* (Lobban et al. 2002), although a second zooxanthellate ciliate, *Euplotes uncinatus*, was recently discovered in the same habitat (Lobban et al. 2005). *Symbiodinium* is also common in other hosts from the same coastal area such as foraminiferans, corals, and other coelenterates (Lobban et al. 2002; Pochon et al. 2001). *Symbiodinium* species in culture have been found to synthesize several MAAs, but mycosporine-glycine, shinorine, porphyra-334, and mycosporine-2 glycine are the most common ones (Shick and Dunlap 2002). Generally, *in hospite Symbiodinium* synthesizes a more restricted suite of MAAs than do free-living representatives of dinoflagellates in culture (Shick and Dunlap 2002). In our case, the MAAs found (including those in trace amounts) resemble the

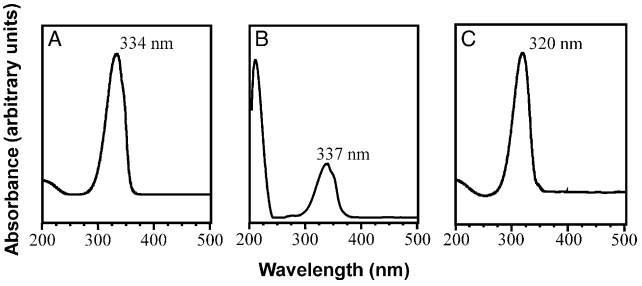
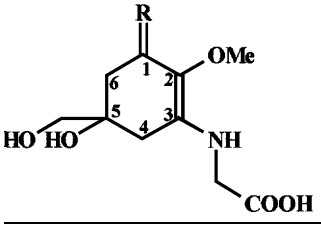


Figure 3. Spectra of the three major MAAs found in *Maristentor dinoferus*. **A**: Shinorine. **B**: Palythenic acid. **C**: Palythine.

Table 1. Mycosporine-like amino acids (MAAs) identified via LC-MS analysis are given with the measured wavelength maximum (λ_{max}, nm) and protonated mass ($[M+H]^+$). The structure for each compound is also shown.

Base structure	MAA	R	λ_{max}	$[M+H]^+$
	Palythine	NH	320	245
	Mycosporine-2-gly	$N-CH_2-CO_2H$	331	303
	Palythenic acid	$N-C(CO_2H)=CHCH_3$	337	329
	Shinorine	$N-CH(CO_2H)CH_2OH$	334	333
	Porphyra-334	$N-CH(CO_2H)CH(OH)CH_3$	334	347

classic suite of compounds seen in other dinoflagellate symbioses (Shick and Dunlap 2002), with the exception of the presence of palythenic acid, which is typically found in some ascidians and sponges (Karentz 2001). Mycosporine-glycine (λ_{max} 310 nm) was not detected in the extracts, an unusual result because this compound is structurally the simplest MAA from which other, bisubstituted MAAs probably are derived (Shick and Dunlap 2002; Portwich and Garcia-Pichel 2003). However, very low concentrations of mycosporine-glycine might have been masked by uncharacterized compounds eluting from the column at the same time.

The *Symbiodinium* endosymbionts of *M. dinoferus* have been identified using molecular methods as members of the phylotype clade C (Lobban et al. 2002). Interestingly, *Symbiodinium* phylotypes of clade C have been found to be unable to synthesize MAAs in culture (Banaszak et al. 2000). In a comparison of 27 isolates of symbiotic dinoflagellates, only those representatives of clade A were able to synthesize MAAs (Banaszak et al. 2000). Thus, the exact source of MAAs in *M. dinoferus* remains unknown until its *Symbiodinium* symbiont is obtained in culture.

In addition to MAAs, *M. dinoferus* has large quantities of maristentorin, a pigment structurally similar to stentorin and hypericin (Mukherjee et al. in press). This pigment that is present in cortical granules of the ciliate (Lobban et al. 2002; Mukherjee et al. in press) could also contribute to its UV protection. However, the biology of UV protection in *M. dinoferus* is complicated by the host's ability to redistribute both zooxanthellae and pigment granules within its giant cell (Lobban et al. 2002). For example, in bright light, the pigment granules shade the nucleus and are away from the algal cells. Thus, screening by MAAs could be especially important in the unshaded parts of the cell.

Previous surveys of MAAs in marine organisms have not included alga—bearing protists (Dunlap and Shick 1998; Karentz 2001; Karentz et al. 1991). Thus, the occurrence of MAAs in *M. dinoferus* is not only interesting because it is the first time these sunscreen compounds have been found in a marine ciliate but also because we know little about MAAs in symbioses other than in algal—invertebrate ones (Shick and Dunlap 2002; Sommaruga 2003). Mixotrophic ciliates are widespread in the open ocean and in freshwaters; however, little is known about which factors affect their dynamics and bathymetric distribution in different aquatic ecosystems (Dolan and Perez 2000; Stoecker 1991, 1998). Even less known is how these organisms cope with harmful levels of solar UV radiation typically found in transparent waters of oligotrophic areas (Dunne and Brown 1996). In oligotrophic ecosystems, inorganic nutrients needed by photoautotrophs are scarce, as are prey for heterotrophs. Symbiosis between these trophic groups is known to provide a close coupling, with inorganic nutrients passing from heterotrophs to algae and photosynthate passing from algae to heterotrophs (Muscatine 1973, 1990). Yet, although symbiosis in alga—bearing ciliates provides the advantage to resist starvation when food is scarce or not easily accessible (e.g., dominance of unsuitable sized preys), there might be additional benefits in this mutualistic association (Dolan and Perez 2000). One additional benefit that has not been considered until now could be the protection of intracellular targets such as DNA via self-shading by the symbiotic algae when ciliates are exposed to high solar UV radiation levels. Additionally, if the symbiotic algae are able to synthesize MAAs, they could significantly increase the screening factor in the UV range. Furthermore, because most MAAs have their absorption maximum in the UVA range, they will screen out wavelengths which always penetrate deeper in the water column (García-Pichel 1996). Thus, it remains to be tested whether UV-photoprotection by MAAs could be one additional benefit in *Maristentor* and other alga—ciliate symbioses.

Methods

Samples: Populations of *Maristentor dinoferus* (Heterotrichea) on the surface of the brown macroalga *Padina* sp. were collected from 3—5 m depth at Scuba Beach, on the north shore of Apra Harbor, Guam, Mariana Islands (13° 27′ N, 144° 40′ E). After these samples were placed in open dishes, *Maristentor dinoferus* left the seaweed and accumulated at the water surface. The ciliates were transferred to filters without applying vacuum by just placing the filter onto the water surface. With this procedure, these fragile organisms were adsorbed onto the filter surface without causing cell damage. The filters were then freeze-dried and sent to Innsbruck and Orono for independent HPLC analysis.

High-performance liquid chromatography: Freeze-dried samples containing an undetermined number of *Maristentor dinoferus* were extracted three times consecutively in 25% aqueous methanol

for 24 h at 4 °C followed by 2 h at 45 °C (Innsbruck) or in absolute methanol at 4 °C for 24 h (Tartarotti and Sommaruga 2002). At the beginning of the first extraction, samples were placed on ice and treated continuously with a tip sonicator for 1 min at 15 W. The extracts were then cleared by centrifugation at 16,000 *g* and stored at −80 °C. At Orono, extraction of MAAs was done similarly but in 80% aqueous methanol and at room temperature. HPLC was carried out in both laboratories using water:methanol:acetic acid (75:25:0.1) running at 0.79 ml min^{-1} for 25 min on a Phenosphere C8 column (250 × 4.6 mm, pore size 5 μm; Phenomenex, USA) protected with a RP-8 guard column (Brownlee, USA). Samples in the autosampler were kept at 15 °C, while the column was maintained at 20 °C. UV detection was done with a Dionex UVD340S diode array detector. Peak purity was checked by analysing the spectrum over the entire wavelength range. Because the results obtained with the different extraction protocols were qualitatively similar, we present only those from 25% aqueous methanol.

Liquid chromatography coupled to electrospray-ionization mass spectrometry: For LC-MS analysis, the freeze-dried extracts were sent to Seattle. The extracts were re-dissolved in 2 ml of 0.2% aqueous acetic acid with 6% methanol added (v/v) and analysed following Whitehead and Hedges (2002) with small changes as described below. The method entailed chromatographic separation using a Develosil RP8 column (150 × 2.1 mm) with a C8 guard column. The eluent consisted of (A) water:formic acid (99:1) and (B) methanol:formic acid (99:1) running at 250 μl min^{-1} in a gradient mode as follows (%B): 0 min (2%), 1 min (2%), 5 min (25%), 7 min (40%), 12 min (65%), 14 min (75%), 16 min (2%), and 24 min (2%). UV spectra (270 — 450 nm) were collected continuously. LC/MS analyses were performed using a Thermo Separation Products HPLC system (USA) with a UV—vis diode array detector and interfaced with a Thermo Finnigan LCQ ion trap mass spectrometer (San Jose, CA, USA). $[M+H]^+$ ions were produced by positive electrospray ionization (ESI). The electrospray source was operated at a capillary voltage of 4.8 kV and a capillary temperature of 335 °C. Sheath gas flow (N_2) was set to 70 units and auxiliary N_2 gas flow at 10 units. A minimum of 50 spectra were collected, each an average of 3 'microscans', with a maximum ion collection time of 50 ms. For MS—MS spectra, a collision energy of 35% was used with an isolation width of $m/z = 1$. The ion optics and trap conditions were initially optimized using the MAA palythine. The ion trap was operated at unit mass resolution and was calibrated using a standard solution prescribed by Thermo Finnigan (caffeine, MRFA and Ultramark).

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