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Analyses of photoprotective compounds in red algae from the Brazilian coast

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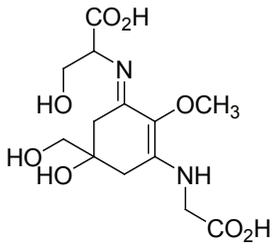
Abstract: Qualitative and quantitative studies of mycosporine-like amino acids (MAAs) in three species of the genus *Gracilaria* Greville (*G. birdiae, G. domingensis* and *G. tenuistipitata*) were performed. A simple and efficient extraction procedure based on ethanol was described. HPLC, UV and mass spectrometry experiments revealed different profiles between extracts obtained from one species cultivated in the laboratory (*G. tenuistipitata*) and two species collected in their natural environment (*G. birdiae* and *G. domingensis*). The levels detected in the latter two species were approximately 150 times higher than in the species cultivated *in vitro*. This study revealed that *G. birdiae* and *G. domingensis* present a potential source for economical exploration of MAAs.

Introduction

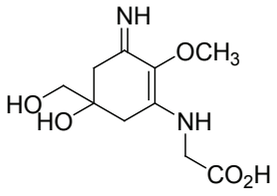
Solar ultraviolet radiation (UVR) exerts deleterious effects on aquatic and terrestrial ecosystems (Häder et al., 1998; Häder & Sinha, 2005). Since many organisms are light-dependent, they cannot avoid UVR exposure. In order to attenuate the toxic UVR effects, photosynthetic organisms have developed several defense mechanisms. Vertical movement within the water column (Smith et al., 1992), DNA repair by photoreactivation and excision (Britt, 1995) and accumulation of antioxidant compounds (Dunlap & Yamamoto, 1995; Ehling-Schulz et al., 1997) are some examples of these methods. Another important defense mechanism, developed by cyanobacteria and algae, is the synthesis and accumulation of mycosporine-like amino acids (MAAs), which strongly absorb in the UVR range (Shick & Dunlap, 2002).

The MAAs are water-soluble compounds present intracellularly in many marine and freshwater organisms (Dunlap & Shick, 1998; Gröniger et al., 2000, Sinha et al., 2007). They are characterized by a cyclohexenone or cyclohexenimine chromophore conjugated with a nitrogen substituent of an amino acid, amino alcohol or amino group, having absorption maxima ranging from 310 to 360 nm (Nakamura & Kobayashi, 1982). Over 25 mycosporines and MAAs have been characterized in fungi and aquatic organisms and this number is increasing with the application of more sensitive methodologies such as mass spectrometry (Volkmann et al., 2006; Carignan et al., 2009).

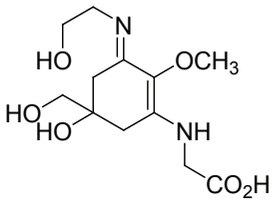
The biosynthesis of MAA has been reported to occur in bacteria, cyanobacteria and algae. Other aquatic organisms acquire them through diet transfer and also by symbiotic or bacterial association (Shick et al., 1992; Stochaj et al., 1994; Carroll & Shick, 1996). Biosynthesis of MAAs is not yet fully understood. It was hypothesized that MAA synthesis derives from a branch of the shikimate pathway, which is responsible for the synthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan. This hypothesis was tested with the use of radioactive labeling of shikimic acid intermediates in fungus (Favre-Bonvin et al., 1987) and inhibition of the pathway with glyphosate in coral (Shick et al., 1999). However, a recent study contradicted this theory by showing that an enzyme



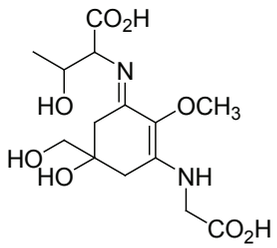
shinorine (**1**)



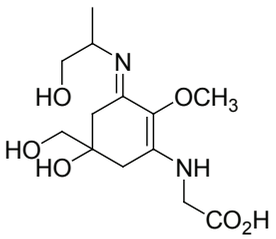
palythine (**2**)



asterina-330 (**3**)



porphyra-334 (**4**)



palythinol (**5**)

involved in the synthesis of the MAA shinorine (**1**) utilizes a pentose phosphate pathway intermediate instead of the shikimate pathway intermediate (Balksus & Walsh, 2010).

The photoprotective function of MAAs in marine organisms can be inferred from their efficiency of absorbing ultraviolet A (320-400 nm) and ultraviolet B (280-320 nm) radiation due to their high molar absorption coefficients and frequent observations correlating higher MAAs concentrations with higher levels of UVR (Klisch & Häder, 2000; Sinha et al., 2000; Arróniz-Crespo et al., 2005). Besides their function as photoprotective compounds, it has been suggested that MAAs also have antioxidant activity (Dunlap & Yamamoto, 1995), osmotic functions (Oren, 1997) and a regulatory role in reproduction (Bandaranayake & Des Rocher, 1999).

Their photoprotective properties have been already explored in commercial formulations. For instance, Helioguard 365® is a mixture of MAAs used in skin care products. They also have been considered as photostabilizers for plastics and dyes and are the subject of several patents because of their commercial relevance (Miyamoto et al., 2009; Aguilera et al., 2009; Schmid et al., 2004). Despite their economical importance, no synthetic routes to prepare MAAs are available; therefore, they are obtained exclusively from natural sources.

Given their expressive economic exploration, the Rhodophyta (red algae) are promising sources of secondary metabolites, including MAAs (Takamatsu et al., 2003; Cardozo et al., 2007; Blunt et al., 2010; Machado et al., 2010; Lhullier et al., 2010). In fact, some reports describe that MAAs are found in the Rhodophyta in higher concentrations and greater diversity when compared with Phaeophyta and Chlorophyta (Karsten et al., 1998a,b). Moreover, the amount of MAAs in the tropical Rhodophyta seems to be higher than in temperate organisms, possibly reflecting acclimatization to the stronger solar radiation typical of lower latitudes (Karsten et al., 1998a). Among the red algae, a genus of great economical interest is *Gracilaria* Greville, known for producing high-quality agar. The widespread use of agar in the food, pharmaceutical and cosmetic industries promotes their worldwide cultivation, including in Asia, Southern Africa and South America. These cultivations are also being made in association with animal aquacultures (Marinho-Soriano et al., 2009; Salles et al., 2010) in order to attenuate the culture's impact on coastal waters, since the release of large amounts of nutrients into aquatic ecosystems can lead to eutrophication. The production of a secondary crop (algae) in an integrated cultivation system could improve water quality and provide extra income through exploration of agar and MAAs.

The present work reports the characterization and MAAs composition of two economically important macroalgae of the genus *Gracilaria* cultivated in the northeast of Brazil. The MAAs profile is compared with *G. tenuistipitata*, a model red macroalgae routinely cultivated since 1993 in the laboratory. To our knowledge, this is the first report of the detection of MAAs in *Gracilaria* species grown on the Brazilian coast.

Materials and Methods

Chemicals

All solvents used were HPLC grade (Tedia, São Paulo, Brazil and J. T. Baker, Phillipsburg, USA). The remaining chemicals were from Sigma-Aldrich (St. Louis, USA). Ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, USA).

Organisms

Gracilaria birdiae Plastino *et* Oliveira and *Gracilaria domingensis* (Kützing) Sonder ex Dickie were collected from Praia do Cotovelo (05°56'S - 035°09'W), located on the south coast of Rio Grande do Norte State, Brazil. The samples were kept at -80 °C until MAAs extraction.

Gracilaria tenuistipitata var. *liui* Zhang *et* Xia (strain 63) from Haikou, China, was collected and decontaminated by E. C. Oliveira in 1993 and deposited in the germoplasm bank at the Institute of Biosciences of the University of São Paulo (Lourenço & Vieira, 2004). The cultures were maintained at 20 °C under constant air bubbling in 2 L-flasks containing von Stosch medium (Edwards, 1970) under a 12 h photoperiod (cool white fluorescent lights; 90 µmol photons $m^{-2}s^{-1}$). Samples were frozen in liquid nitrogen and kept at -80 °C until MAAs extraction.

Sample preparation

Frozen algal samples were homogenized in a blender with liquid nitrogen and extracted at 4 °C for 24 h with organic solvent and water. Different extraction solvent compositions were evaluated: 20% methanol, 50% methanol, 20% ethanol and 50% ethanol (v/v in water). The extracts were then filtered and the algal debris reextracted as described previously. This procedure was repeated until spectrophotometric analysis (scan range 250-400 nm) revealed the absence of MAAs in the extracts. The combined extracts were centrifuged (10000 rpm/10 min) and evaporated in a speed-vacuum (SpeedVac, Savant, Farmingdale, USA). Dried extracts were solubilized in 0.1% formic acid and analyzed by HPLC with photodiode array (DAD) and mass spectrometry detection.

Chromatographic and mass spectrometric conditions

Qualitative analyses of MAAs were performed using a Shimadzu Prominence liquid chromatographer with photodiode array (Shimadzu Co., Kyoto, Japan) and ion trap mass spectrometer detectors (Esquire HCT, Bruker Daltonics, Billerica, USA) equipped with an electrospray source. Separation was performed using two columns in tandem: Synergi Polar-RP (Phenomenex, 250 x 4.6 mm, 4 µm) and Synergi Fusion RP (Phenomenex, 250 x 4.6 mm, 4 µm) at 30 °C. The solvent system was an aqueous solution of 0.1% pH 3.14 ammonium formate in pump A and methanol in pump B. The selected conditions started with 0% of solvent B during the first 10 min; then a linear increase from 0 to 50% of B between 10 and 20 min; maintaining 50% of B for 10 min, with a total run of 40 min. The flow rate was 1 mL min^{-1} and spectrophotometric detection was performed at 330 nm. Multi-stage mass analyses on the ion trap instrument were achieved in the positive ion mode using nitrogen as the nebulising (10 psi) and drying (4 L min^{-1}, 300 °C) gas and helium as the buffer gas (4 x 10^{-6} mbar). Capillary high voltage was set to 3500 V. To avoid space-charge effects, the smart ion charge control (ICC) was set to the arbitrary value of 100,000 with a maximum accumulation time of 100 ms. Isolation width was 2.0 Da and a collision energy of 30% was used for tandem mass spectrometry (MS^2 and MS^3) experiments. Five spectra were averaged for each data point. High-resolution mass analyses of the extracts were obtained using a quadrupole orthogonal time-of-flight mass spectrometer (Q-TOF, Micromass/ Waters, Manchester, UK). The needle voltage was set at 3.3 kV and the cone voltage at 35 V. Nitrogen was used as nebulising gas. Argon was used as the collision gas with the collision energy set at 4 eV.

Quantitative analyses of the extracts were performed according to the previously published HPLC method (Carreto et. al., 2005).

Results and Discussion

In this study, the effect of different organic solvents was evaluated to achieve the most efficient extraction of MAAs from three species of the genus *Gracilaria* Greville (*G. birdiae, G. domingensis* and *G. tenuistipitata*). Analysis by HPLC with DAD detection revealed that the extraction with 20% methanol was the most efficient and with 50% ethanol was the least efficient among the tested combinations (data not shown). These findings are in agreement with a previous report describing higher yields of MAAs when 25% methanol was used instead of 100% methanol (Tartarotti & Sommaruga, 2002). Although, higher yields can be obtained with 20% methanol, we found that 20% ethanol is an effective alternative. Since ethanol is less expensive and less toxic than methanol, it could make extraction on large scale more feasible.

The HPLC-DAD chromatograms of the ethanolic extracts from the three species of red macroalgae are shown in Figure 1. The identity of each peak was inferred from UV spectra and from $ESI\text{-}MS^n$

analysis by comparison with the fragmentation patterns previously described (Cardozo et al., 2006; Cardozo et al., 2008) (Table 1). In addition, the identity of each MAA was confirmed by accurate-mass measurement after isolation of the peaks (Table 2). Five MAAs were identified in the three macroalgae tested: shinorine (**1**), palythine (**2**), asterina-330 (**3**), porphyra-334 (**4**) and palythinol (**5**).

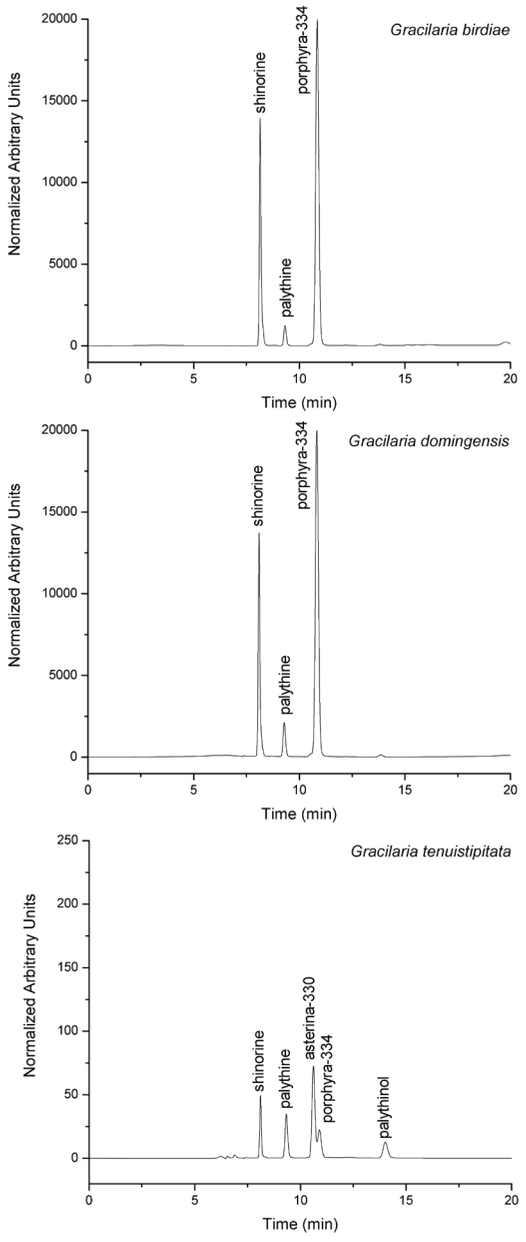


Figure 1. HPLC-DAD (330 nm) chromatograms of mycosporine-like amino acids detected in three species of *Gracilaria* Greville. *G. birdiae* and *G. domingensis* were collected from the sea and *G. tenuistipitata* was cultivated in the laboratory.

Quantitative analyses of MAAs were performed on the ethanolic extracts as previously described (Carreto et al., 2005). Identification of MAAs was inferred by their absorption maxima, retention time and by co-chromatography with authentic standards. Two distinct patterns of MAAs distribution were observed in three *Gracilaria* species (Table 3). The species cultivated *in vitro* (*G. tenuistipitata*) presented similar levels of shinorine (**1**), palythine (**2**), asterina-330 (**3**), porphyra-334 (**4**) and palythinol (**5**). Asterina-330 was the most abundant MAA, corresponding to 40% of the total MAAs identified. In the species collected from the natural environment (*G. birdiae* and *G. domingensis*) only shinorine (**1**), palythine (**2**) and porphyra-334 (**4**) were detected at levels above the lower limit of quantitation. Porphyra-334 (**4**) was the most abundant MAA in *G. birdiae* and *G. domingensis,* corresponding to approximately 70% of the total MAAs. Interestingly, not only the MAAs composition, but also the concentration differ substantially (Table 3). The concentration of MAAs was 40 to 700 times higher in *G. birdiae* and *G. domingensis* than in *G. tenuistipitata*. This expressive difference should be studied further, but it is possibly related to the higher exposure to solar UVR in the species collected from the sea compared to that cultivated *in vitro*. In fact, the amount of MAAs is higher in the tropical Rhodophyta than in those from temperate regions, which might be explained by the acclimatization to the more intense solar radiation typical of lower latitudes (Karsten et al., 1998a).

The ethanolic extract of *G. birdiae* and *G. domingensis* resulted in a yield of 1.5%, of which 2.7% (*G. birdiae*) and 2.9% (*G. domingensis*) corresponded to MAAs content. This good yield reflects the high amounts of these compounds found in these species cultivated at latitudes with high incidence of UVR. Therefore, *G. birdiae* and *G. domingensis* are potential sources of MAAs for economical exploration.

Conclusion

MAAs are important photoprotective compounds that can be obtained from two species of *Gracilaria* cultivated in northeastern coast of Brazil in good yields via simple extraction procedure. The association of algae cultivation with animal aquacultures has already been implemented and has positive effects on the protection from eutrophication phenomena. The presence of high levels of MAAs in algae, as described in this study, offers a new source of income for communities that depend on aquaculture for their subsistence.

Table 1. Identification of mycosporine-like amino acids by chromatography, spectrophotometry and mass spectrometry.

Compound	TR (min)	λ_{max} (nm)	$[M+H]^+$	Fragments
Shinorine (**1**)	8.1	332	333	186, 230, 274, 303 318
Palythine (**2**)	9.3	319	245	186, 197, 209, 230
Asterina-330	10.6	333	289	186, 209, 230, 274
Porphyra-334 (**4**)	10.9	333	347	186, 227, 288, 303, 332
Palythinol (**5**)	14.1	331	303	186, 230, 244, 288

Table 2. Accurate-mass measurements of isolated mycosporine-like amino acids.

Protonated molecule $[M+H]^+$	Observed *m/z* value	Calculated *m/z* value	Error (ppm)	Formula
Shinorine (**1**)	333.1304	333.1298	1.8	$C_{13}H_{21}N_2O_8$
Palythine (**2**)	245.1146	245.1138	3.2	$C_{10}H_{17}N_2O_5$
Asterina-330 (**3**)	289.1395	289.1400	-1.0	$C_{12}H_{21}N_2O_6$
Porphyra-334 (**4**)	347.1433	347.1449	-4.0	$C_{14}H_{23}N_2O_8$
Palythinol (**5**)	303.1559	303.1556	1.0	$C_{13}H_{23}N_2O_6$

Table 3. Distribution and content of mycosporine-like amino acids in three species of *Gracilaria* Greville.

Compound	Content of MAA (mg g extract^{-1})		
	G. birdiae	*G. domingensis*	*G. tenuistipitata*
Shinorine (**1**)	6.5	7.2	0.0264
Palythine (**2**)	2.4	1.9	0.0454
Asterina-330 (**3**)	nd	nd	0.0728
Porphyra-334 (**4**)	18.3	20.0	0.0278
Palythinol (**5**)	nd	nd	0.0197
Total	27.2	29.1	0.1921

MAA: Mycosporine-like amino acids. The absence of a MAA is indicated as not detected (nd).

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