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Coral Reefs

Identification and quantitation of near-UV absorbing compounds (S-320) in a hermatypic scleractinian*

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Abstract. Reef-building corals from shallow waters are known to contain a suite of water soluble compounds (collectively named S-320) which strongly absorb near-UV light. Compounds of this type have now been isolated from the Pacific staghorn coral *Acropora formosa* and identified as a series of mycosporine-like amino acids including mycosporine-Gly ($\lambda_{max}=310$ nm), palythine ($\lambda_{max}=320$ nm) and palythinol ($\lambda_{max}=332$ nm). These compounds were separated and quantified by high-performance liquid chromatography. Serial extraction efficiencies were calculated using a simple formula which is derived herein. For 12-cm long coral branches collected from a depth of 3 m at Rib Reef, Great Barrier Reef, Australia (146° 53′E, 18° 29′S) the average concentrations of mycosporine-Gly, palythine, and palythinol were 37.8, 56.4 and 0.895 nmol per mg coral protein, respectively. The coral samples can be stored at −20 °C for at least 144 days without degradation of the mycosporine-like amino acids.

Introduction

Shallow-water reef-building corals are normally exposed to high levels of near-UV light (285–350 nm). This is due not only to the general UV transparency of tropical ocean waters (Jerlov 1950; 1976; Smith and Baker 1979) but also to the relative thinness of the earth's equatorial ozone layer (Green et al. 1974; Baker et al. 1980). Near-UV light can inhibit algal photosynthesis (Lorenzen 1979; Caldwell 1981) and kill reef invertebrates (Worrest et al. 1978; Jokiel 1980). The biologically damaging effects of near-UV light have been reviewed by Worrest (1982) and by Smith and Baker (1982).

Reef-building corals contain large populations of the endosymbiotic dinoflagellate *Symbiodinium microadriaticum*, and the coral polyps are relatively transparent which permits the transmission of the photosynthetically active radiation required for algal photosynthesis. This animal-algal association would thus appear to be particularly vulnerable to the biologically damaging effects of near-UV light. The abundance of scleractinian corals on tropical reefs, however, suggests that they may have some mechanism for protection from near-UV light.

Shibata (1969) demonstrated that aqueous extracts of five *Acropora* spp. and one *Pocillopora* sp. contain large amounts of a material, named S-320, having a broad absorbance maximum at approximately 320 nm. The exact absorbance maximum varied amongst the samples from 315 to 323 nm suggesting the presence of a group of spectrally similar compounds. Maragos (1972) demonstrated that colonies of *Porites lobata* collected from different depths have concentrations of S-320 which are directly proportional to ambient light intensity. This correlation may explain why corals collected from depths of 1–2 m are more resistant to artificial near-UV exposure than are corals of the same species growing at depths of 5–6 m (Siebeck 1981).

Jokiel and York (1982) demonstrated that when screened from environmental near-UV light *Pocillopora damicornis* grows faster and contains less S-320 than when exposed to normal irradiance. This suggests that S-320 is synthesized in response to near-UV light and not in response to decreasing temperature, increasing pressure, or any other bathymetrically distributed environmental parameter. These results also suggest that near-UV is an important ecological parameter which influences the physiology and growth of corals under normal environmental conditions on coral reefs.

One objective of our current research has been to identify the chemical composition of S-320 in order to enable quantitative studies on the bathymetric distribution of near-UV absorbing compounds in reef-building corals. Our preliminary HPLC analyses had indicated that S-320 consisted of at least three polar, UV-absorbing compounds which could be isolated as clear, soft glasses. However, structural determination by normal methods

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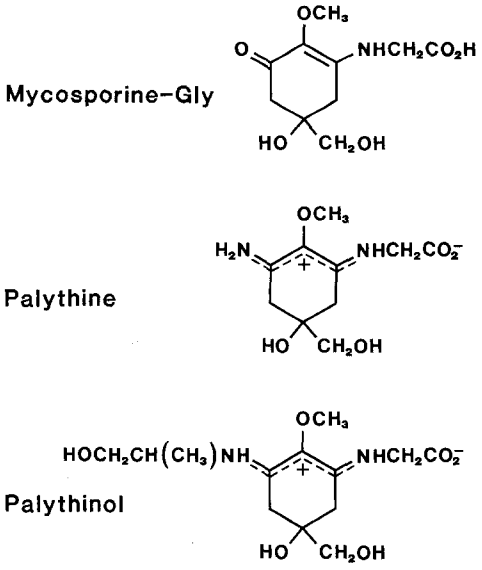

Fig. 1. Mycosporine-like amino acids isolated from both the zoanthidian *Palythoa tuberculosa* and the Pacific staghorn coral *Acropora formosa*

was not possible due to sample contamination by a clear, viscous, oily material which we were unable to remove. We have therefore identified the constituents of S-320 by reference to authenticated compounds isolated from the zoanthidian *Palythoa tuberculosa.*

This communication reports that in the Pacific staghorn coral *Acropora formosa* S-320 consists of a series of mycosporine-like amino acids including: (1) mycosporine-Gly (λ_{max} = 310 nm), (2) palythine (λ_{max} = 320 nm) and (3) palythinol (λ_{max} = 332 nm) (Fig. 1). In addition it presents several systems for the quantitation of mycosporine-like amino acids by high performance liquid chromatography. It evaluates the efficiency with which these compounds can be extracted from a reef-building coral, and documents the chemical stability of these compounds during storage. This communication thus provides information and techniques which are necessary for further quantitative study of coral UV-photobiology and photoadaptation.

Materials and methods

Collection and maintenance of specimens. The scleractinian coral *Acropora formosa* and the zoanthidian *Palythoa tuberculosa* weew collected at depths from 1–3 m from the southern side of Myrmidon Reef, Great Barrier Reef, Australia (147°, 23′E:18° 16′s) and immediately frozen at −20 °C. For the determination of storage stability, 12-cm long branches of *A. formosa* were collected from a depth of 3 m at Rib Reef (146° 53′E:18° 29′S) and transported in aquaria with flowing seawater to the laboratory on shore.

Isolation of mycosporine-like amino acids from P. tuberculosa. Freeze dried colonies of *P. tuberculosa* (100 g) were pulverised in a blender and extracted with 80% aqueous methanol. The methanol was evaporated under reduced pressure and the precipitated pigments were discarded. The resulting extract was washed with hexane (3 × 100 ml), decolorised with activated charcoal (1 g), and lyophilized. The solid residue was treated with methanol (3 × 10 ml) and insoluble material was discarded. The methanol was evaporated and the residue redissolved in water (10 ml). Remaining apolar contaminants were removed on a Waters C-18 Sep-Pak cartridge. Palythinol was separated by preparative reverse-phase chromatography on a Whatman 10 μ Partisil ODS-3 column (22.1 × 500 mm) with 10% aqueous methanol as the mobile phase. The fraction consisting of mycosporine-Gly and palythine was separated using a mobile phase consisting of acetic acid, methanol, and water (1:10:89). Elution was monitored by UV-absorption at 313 nm.

The identities of these purified compounds were confirmed by UV- and mass-spectrometry: mycosporine-Gly, UV (H_2O) λ_{max} = 310 nm, EI-MS m/z 227 (M^+-H_2O), CI^+-MS m/z 246 (MH^+); palythine, UV (H_2O) λ_{max} = 320 nm, EI-MS m/z 226 (M^+-H_2O), CI^+-MS m/z 245 (MH^+); and palythinol, UV (H_2O) λ_{max} = 332 nm, EI-MS m/z 284 (M^+-H_2O), CI^+-MS m/z 303 (MH^+).

Identification of S-320 from A. formosa. Using techniques similar to those applied to *P. tuberculosa*, three near-UV absorbing compounds were isolated from *A. formosa.* The UV-light absorption spectrum was recorded for aqueous solutions of each compound. Using a variety of columns and mobile phases (Table 1), each compound was co-chromatographed with the authenticated samples of mycosporine-like amino acids isolated from *P. tuberculosa.*

Quantitative analysis. Coral samples were extracted three times for 5 min with 20 ml of 20% tetrahydrofuran (THF) and 80% methanol under sonication in an ice bath. This solvent system was chosen because it efficiently extracts both mycosporine-like amino acids and photosynthetic pigments. It is also compatable with HPLC analysis of photosynthetic pigments (Chalker and Dunlap 1982). ten ml subsamples were diluted with two ml of distilled water and filtered through two Waters C-18 Sep-Paks connected in series to remove the apolar photosynthetic pigments. The Sep-Paks were washed with 10 ml of distilled water, the aqueous elutants were combined, and the organic solvents were removed by rotary evaporation. The aqueous residues were then passed through two additional Sep-Paks, which were subsequently washed with 10 ml of distilled water. The combined aqueous solutions were lyophilized and resuspended in 2 ml of the mobile phase prior to quantitation by isocratic HPLC.

Quantitative isocratic HPLC analysis is possible on any of the pairs of columns and mobile phases described in Table 1. Most commonly,

Table 1. Columns and mobile phases used in the co-chromatography of mycosporine-like amino acids isolated from *P. tuberculosa* and *A. formosa:* (1) mycosporine-Gly, (2) palythine, and (3) palythinol

Column	Mobile phase (v:v)	Flow rate (ml/min)	Retention time (min)		
			(1)	(2)	(3)
Whatman, 10 μ Partisil, ODS-3 (22.1 mm ID × 50 cm)	1% acetic acid 10% ethanol 89% water	5.0	36.50	25.00	27.91
Whatman, 10 μ Partisil, ODS-3 (22.1 mm ID × 50 cm)	10% methanol 90% water	5.0	24.43	27.73	32.53
Brownlee, Spheri-5, RP-8 (4.6 mm ID × 25 cm), with RP-8 guard (4.6 mm ID × 5 cm)	0.1% acetic acid 10% methanol 89.9% water	0.7	4.45	9.71	13.18
Brownlee, Spheri-5, Amino (4.6 mm ID × 25 cm)	5 mM aqueous phosphate buffer	0.4	9.75	6.01	7.82

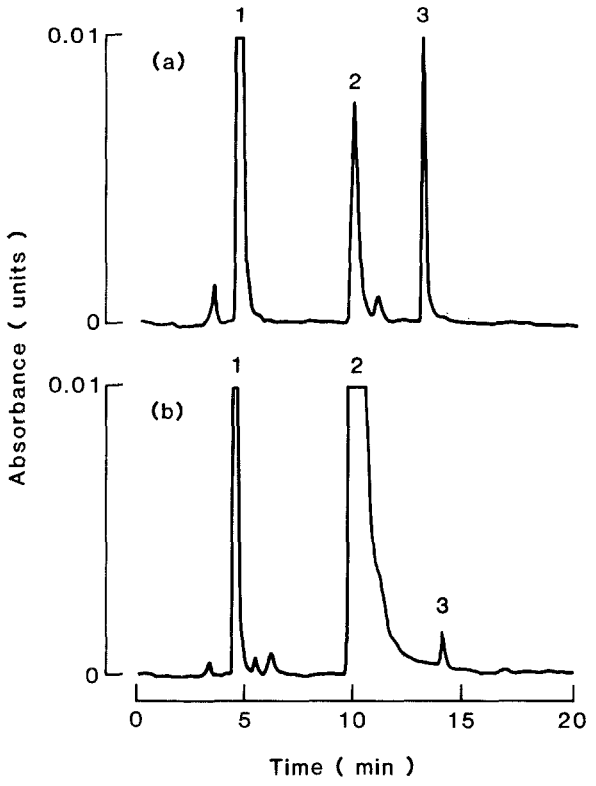

Fig. 2 a, b. High-performance liquid chromatographic separation of polar near-UV absorbing compounds isolated from **a** *P. tuberculosa* and **b** *A. formosa* including the mycosporine-like amino acids (1) mycosporine-Gly, (2) palythine, and (3) palythinol. Brownlee, Spheri-5, RP-8 column (4.6 mm ID × 25 cm) with RP-8 guard (4.6 mm ID × 5 cm); mobile phase, 0.1% aqueous acetic acid and 10% methanol (v:v), flow rate, 0.7 ml/min; detection by absorption at 313 nm

10–40 µl samples were injected onto a Brownlee RP-8 column protected with an RP-8 guard. The mobile phase was 0.1% acetic acid and 10% methanol at a flow rate of 0.7 ml/min. Detection was by UV-light absorption at 313 nm. Typical chromatograms for extracts from *P. tuberculosa* and *A. formosa* are illustrated in Fig. 2.

Standard solutions of authentic mycosporine-like amino acids (isolated by preparative reverse phase HPLC as previously described) were prepared for chromatographic peak area standardisation. The aqueous standards were quantified by UV spectrophotometry on a Varian model 635 scanning spectrophotometer using the published extinction coefficients at the wavelengths of maximum absorbance: mycosporine-Gly methyl ester ($\varepsilon_{310}=28100$) (Ito and Hirata 1977) palythine ($\varepsilon_{320}=36200$) (Takano et al. 1978a), and palythinol ($\varepsilon_{332}=43500$) (Takano et al. 1978b). Since mycosporine-Gly is unstable, Takano et al. (1978a) were able to measure the extinction coefficient only for the stable methyl ester. We assume that the extinction coefficients for mycosporine-Gly and its methyl ester are the same because this methylation does not alter the chromophore.

Extraction efficiency. The extraction efficiency must be known in order to determine the concentration of mycosporine-like amino acids within the coral tissues. A 12-cm coral branch was extracted 5 times for 5 min with 20 ml portions of 20% THF and 80% methanol under sonication in an ice bath. The concentrations of mycosporine-like amino acids in each extract were determined as previously described.

For all three mycosporine-like amino acids the extraction efficiencies were calculated using an expansion of the method of Chalker and Dunlap (1982). The absorbance (A_N) at a given wavelength for a compound in solution following multiple extractions is described by the equation,

$$\ln A_N = aN + b \quad (1)$$

Table 2. Stability of mycosporine-like amino acids within branches of *A. formosa* during storage at −20 °C (mean ± 1 S.D., $n=4$)

Storage time	Concentrations of mycosporine-like amino acids (nmol/mg protein)		
(days)	Mycosporine-Gly	Palthine	Palythinol
0	37.8±2.63	56.4± 2.35	0.895±0.113
12	39.6±4.75	54.4±14.5	0.860±0.293
19	34.4±6.89	49.3±13.1	0.806±0.231
47	41.0±7.08	53.7± 7.43	0.716±0.268
82	28.8±2.84	38.1± 4.55	0.787±0.135
144	40.1±2.78	58.5± 8.76	0.954±0.249

where N is the number of a specific extraction, and a and b are constants. The total absorbance (A_t) is given by the equation,

$$A_t = \sum_{N=1}^{\infty} \exp(aN+b) = \exp(a+b)/(1-\exp(a)). \quad (2)$$

The extraction coefficient (E_1) is defined as the fraction of the total pigment removed during the initial extraction or the fraction of the remaining pigment removed during each subsequent extraction under a given set of experimental conditions. The extraction coefficient is calculated from the equation,

$$E_1 = 1 - \exp(a). \quad (3)$$

The extinction efficiency or the fraction of total material removed after a given number of repetative extractions (E_N) is given by the equation,

$$E_N = 1 - \exp(aN). \quad (4)$$

Storage. Forty 12 cm long branches of *A. formosa* were transported by ship in flowing seawater aquaria from Rib Reef to the laboratory on shore. Four branches were extracted immediately, and the rest were frozen at −20 °C. At intervals thereafter (Table 2), quadruplicate replicates were sampled for near-UV absorbing compounds as previously described.

Results

Identification of near-UV absorbing compounds

Three compounds, tentatively identified as mycosporine-Gly, palythine, and palythinol, were isolated from *A. formosa* as clear glasses. The wavelengths of maximum absorbance for aqueous solutions were 310, 320 and 332 nm. These values corresponded exactly with those obtained experimentally for mycosporine-like amino acids isolated from *P. tuberculosa* and with published values (Ito and Hirata 1977; Takano et al. 1978a, b). On all isocratic HPLC systems, these compounds co-chromatographed exactly with authentic compounds from *P. tuberculosa* (Fig. 2); however, they were not sufficiently pure to attempt structural confirmation by mass spectrometry.

Extraction efficiencies

The amounts of mycosporine-like amino acids obtained from repetitive extractions of a 6 cm tip of *A. formosa* with 20% tetrahydrofuran in methanol (v:v) are shown in Fig. 3. When the three sets of points are fitted using the method of least squares and Eq. (1), the constants for mycosporine-Gly are $a=-0.830$, $b=6.51$ and $r^2=0.985$;

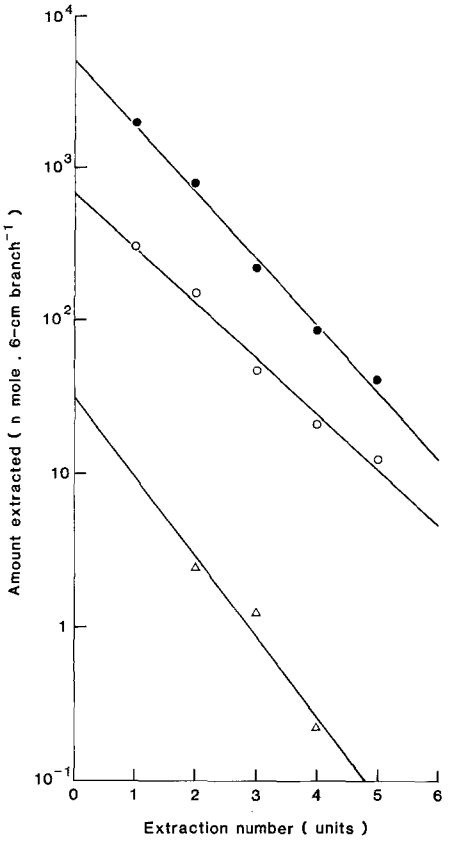

Fig. 3. Extraction of the mycosporine-like amino acids mycosporine-Gly (open circles), palythine (closed circles), and palythinol (open triangles) from a 12 cm branch of *A. formosa*. The solvent is 20% tetrahydrofuran and 80% methanol

the constants for palythine were a = −1.00, b = 8.55 and $r^2 = 0.992$; and the constants for palythinol are a = −1.19, b = 3.44 and $r^2 = 0.929$. The extraction coefficients (E_1) are 0.564, 0.632 and 0.695, respectively. The extraction efficiencies after three extractions are 0.917, 0.950 and 0.972 respectively.

Storage

The concentrations of mycosporine-Gly, palythine and palythinol within 12 cm branches of *A. formosa* after storage at −20 °C are shown in Table 2. These data were examined by ANOVA. For each compound mean concentrations were not significantly different (F-test, $P > 0.05$). Thus these compounds were all stable in the coral specimens for at least 144 days when frozen at −20 °C.

Discussion

Studies of coral UV-photobiology have been necessarily restricted because the chemical composition of the near-UV absorbing compounds (S-320) has been unknown, and quantitation has been limited to the measurement of the absorbance of the 320 nm spectral peak above an unidentified background. These limitations would vanish if the constituents of S-320 could be identified. We had hoped to isolate the constituents of S-320 by high performance liquid chromatography and to identify those compounds by normal chemical procedure such as NMR and mass spectroscopy. Such isolation has been impossible to date because all the compounds we isolated from corals were contaminated by large concentrations of a clear, viscous, oily material. This difficulty was circumvented when it was found that S-320 consisted of compounds isolated previously from other species and identified.

Our preliminary HPLC analysis had indicated that S-320 consisted of at least three polar, UV-absorbing compounds which decomposed in aqueous solutions to yield phenolic compounds. Two groups of compounds with these properties are the mycosporines and the mycosporine-like amino acids. Mycosporines are fungal metabolites which were first isolated by Favre-Bonvin et al. (1976). Reviews of their chemical structures and biological distributions are given by Arpin et al. (1979) and Arpin and Bouillant (1981). The mycosporine-like amino acids were first isolated from the zoanthidian *Palythoa tuberculosa* (Ito and Hirata 1977; Takano et al. 1978 a, b; Hirata et al. 1979), and later identified in algae, sponges, molluscs, echinoderms, and tunicates (Tsujino et al. 1978; Takano et al. 1979; Nakamura et al. 1981, 1982). Hermatypic scleractinians had not previously been tested for the presence of mycosporine-like amino acids.

We isolated the mycosporine-like amino acids mycosporine-Gly, palythine, and palythinol from the zoanthidian *Palythoa tuberculosa*, and confirmed the chemical structures of these compounds by mass spectroscopy. These were the authenticated compounds against which the compounds isolated from corals were subsequently compared.

S-320 from the hermatypic scleractinian *A. formosa* was separated by HPLC into three near-UV absorbing compounds. The first had an absorption maximum in water at 310 nm and co-chromatographed with mycosporine-Gly, the second had an absorption maximum at 320 nm and co-chromatographed with palythine, and the third had an absorption maximum at 332 nm and co-chromatographed with palythinol. Therefore we concluded that the near-UV absorbing compounds isolated from these two species were identical.

The analysis of corals for mycosporine-like amino acids required relatively simple procedures for preservation, extraction, separation and quantitation. When samples of *A. formosa* were frozen at −20 °C these compounds were stable for at least 144 days. This finding was in marked contrast to the known instability of these compounds, particularly mycosporine-Gly (Ito and Hirata 1977) in solution. This was convenient because material could be collected in the field and transported without decomposition to a laboratory on shore for later analysis. Mycosporine-like amino acids could be readily extracted in solutions of tetrahydrofuran-methanol, ethanol-water, or methanol-water; and extraction efficiencies after three extractions typically exceeded 90%. These compounds could then be quickly separated and quantified by isocratic HPLC with UV detection.

Now that the composition of S-320 in *A. formosa* is known and suitable analytical techniques have been developed, further investigations are possible. The phylogenetic and bathymetric distributions of the mycosporine-like amino acids are of particular interest. If the endosymbiotic alga *Symbiodinium microadriaticum* is associated with the biosynthesis of mycosporine-like amino acids, then the presence or absence of this alga may profoundly influence the distribution of reef invertebrates even beyond the nutritional influence of algal photosynthesis.

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