A broadly applicable method for extraction and characterization of mycosporines and mycosporine-like amino acids of terrestrial, marine and freshwater origin

Marc Volkmann & Anna A. Gorbushina

Geomicrobiology, Institute of Chemistry and Biology of the Marine Environment, Carl von Ossietzky University Oldenburg, Oldenburg, Germany

Correspondence: Anna A. Gorbushina, Geomicrobiology, Institute of Chemistry and Biology of the Marine Environment (ICBM), Carl von Ossietzky University Oldenburg, PO Box 2503, 26111 Oldenburg, Germany. Tel.: +49 441 798 3393; fax: +49 441 798 3384; e-mail: a.gorbushina@uni-oldenburg.de

Received 13 September 2005; revised 10 November 2005, accepted 24 November 2005. First published online February 2006.

doi:10.1111/j.1574-6968.2006.00088.x

Editor: Nina Gunde-Cimerman

Keywords
mycosporines; mycosporine-like amino acids (MAAs); microcolonial fungi; cyanobacteria; terrestrial and marine origin.

Abstract

A universal method allowing simultaneous extraction and analysis of diverse ultraviolet-B-absorbing compounds belonging to mycosporines and mycosporine-like amino acids (MAAs) is presented. Mycosporines and MAAs are found both in prokaryotes and eukaryotes and possess photoprotective properties. Our method was successfully tested by screening 31 cyanobacterial, 11 actinomycete and 45 fungal strains for their mycosporine and MAA content. The majority of the isolates tested originated from subaerial rock surfaces and were inherently protected from excessive sun irradiation. The new method includes a solid–liquid extraction procedure, followed by a reversed phase liquid chromatography/mass spectrometry. Eight different mycosporines and five MAAs were efficiently separated and identified by their retention times, absorption maxima and fragmentation patterns. Mycosporines were found both in rock-inhabiting fungi and cyanobacteria and consequently may render an ecological marker of these peculiar terrestrial environments.

Introduction

Mycosporines and mycosporine-like amino acids (MAAs) are secondary metabolites of marine and terrestrial origin. These water-soluble ultraviolet (UV) light-absorbing compounds are synthesized by a large variety of prokaryotic and eukaryotic organisms, including zooplankton and phytoplankton in marine (Karentz, 2001; Shick & Dunlap, 2002), freshwater (Sommaruga & Garcia-Pichel, 1999; Tartarotti *et al.*, 2001; Laurion *et al.*, 2002) and terrestrial (Favre-Bonvin *et al.*, 1975; Gorbushina *et al.*, 2003) environments. The first description and 'baptizing' of mycosporines refers to sporulating terrestrial fungi (Leach, 1965; Pittet *et al.*, 1983; Buscot & Bernillon, 1991; Leite & Nicholson, 1992; Whitehead *et al.*, 2001).

Mycosporines and MAAs are composed, respectively, of cyclohexenone and cycloheximine ring structures conjugated to an amino acid subunit and, in some instances, linked to various carbohydrates. Due to the conjugated double bonds in their structure they absorb radiation with a maximum between 310 and 360 nm and thus can act as photoprotective UV-B and UV-B filters in prokaryotes (Bandaranayake, 1998; Karsten *et al.*, 1998; Whitehead *et al.*, 2001). Another function of hydrophilic mycosporines is their capacity to exercise a regulatory effect on sporulation and germination in eukaryotic organisms (Leach, 1965; Leite & Nicholson, 1992). MAAs were reported to be synthesized under osmotic and UV radiation stress (Garcia-Pichel & Castenholz, 1993; Oren, 1997; Portwich & Garcia-Pichel, 1999; Libkind *et al.*, 2004), or to be constitutively present (Gorbushina *et al.*, 2003) and correlated with the taxonomic position of the organism (Garcia-Pichel & Castenholz, 1993; Garcia-Pichel *et al.*, 1998). An antioxidant role has also been assigned to mycosporines and MAAs (Dunlap & Yamamoto, 1995).

A variety of extraction and separation methods for mycosporines and MAAs has been developed and tested on a broad range of organisms (Grant *et al.*, 1985; Büdel *et al.*, 1997; Oren, 1997; Karsten *et al.*, 1998; Whitehead & Vernet, 2000; Whitehead *et al.*, 2001; Tartarotti & Sommaruga, 2002; Gorbushina *et al.*, 2003; Libkind *et al.*, 2004). A new challenge of microbial ecology, however, is shifting our attention from pure cultures to diverse natural communities. Simultaneous analysis of the whole diversity of

photoprotective compounds in a complex natural microbial community would yield an inherent characteristic of its diversity and adaptation. This, however, is possible only if the method employed extracts all possible UV light-absorbing compounds and allows for their subsequent analysis.

In the literature, a whole range of dissimilar extraction and analysis methods can be found for mycosporines and MAAs (Volkmann, 2004). The first extraction of a mycosporine was performed using absolute cold ethanol. In other extraction protocols, ethanol in concentrations from 50% (Carreto *et al*., 1990) up to 80% (Rieger & Robinson, 1997) or absolute (Leach, 1965) was used. Methanol in concentrations from 20% (Garcia-Pichel & Castenholz, 1993) up to absolute (Dunlap *et al*., 1989) provides similar results (Tartarotti & Sommaruga, 2002). Reversed phase high-performance liquid chromatography (HPLC) employing C_8-phases (Dunlap & Chalker, 1986; Carroll & Shick, 1996; Whitehead & Vernet, 2000) as well as C_{18}-phases (Arpin & Bouillant, 1981; Nakamura *et al*., 1982; Volkmann *et al*., 2003) is the most common method to separate and characterize the MAAs (Nakamura *et al*., 1982; Chioccara *et al*., 1986). Along with reversed phases, normal phases (Karentz *et al*., 1991) or phases using amino-modified silica gel (Dehorter & Bernillon, 1983; Dunlap & Chalker, 1986; Stochaj *et al*., 1994) allow for the separation and identification of both MAAs and mycosporines. Mostly binary solvent mixtures have been employed for the isocratic elution of the UV-absorbing compounds in HPLC, but there were also effective separations using gradient acetonitrile elutions (Böhm *et al*., 1995). Most common aqueous methanolic solvents (with the methanol content varying from 10% (Chioccara *et al*., 1986) to 65% Whitehead *et al*., 2001) have been shown as adequate to elute the UV-absorbing substances from reverse phase columns. A solvent mixture of 75 : 25 [water : methanol volume in volume (v/v)] with 0.1% acetic acid added has been used most commonly to separate isolated MAAs from marine organisms (Karentz *et al*., 1991; Büdel *et al*., 1997). Recently a coupling of two octadecyl silane HPLC columns with gradient elution was used to separate most of the known mycosporines and MAAs (Carreto *et al*., 2005).

This study was aimed at elaborating a universal method allowing for simultaneous extraction and analysis of mycosporines and MAAs deriving from prokaryotic and eukaryotic organisms of terrestrial, marine or freshwater origin. Except for very recent studies (Carreto *et al*., 2005) all existing methods (extraction and separation) were optimized in order to characterize the mycosporines or MAAs from single groups of organisms. Our screening was aimed at a variety of compounds present in extraordinary hostile ecological niches and therefore a broad selection of organisms from UV radiation-stressed environments was studied.

Material and methods

Organisms tested

A majority of fungal, cyanobacterial and actinomycetes strains selected for our screening originated from subaerial (atmosphere-exposed) rock surfaces – extreme desiccation- and UV radiation-stressed environments. Here organisms have to possess numerous adjustments to hostile conditions, and thus radiation protection and xerotolerance adaptations are frequent in this habitat. Another selection of related ascomycetes was isolated from hypersaline environments and included saltern-related fungi. Several species of red algae and cyanobacteria, a basidiomycete, an ascomycete and a deuteromycete were selected as known producers of MAAs and mycosporines.

Fungi are a very large and diverse group of organisms, exhibiting a remarkable diversity of mycosporines. Mycosporines and nor-mycosporines can be found both in asco- and basiodiomycetes, as well as in numerous deuteromycetes (Bouillant *et al*., 1981; Dehorter & Bernillon, 1983; Pittet *et al*., 1983; Bernillon *et al*., 1984; Buscot & Bernillon, 1991; Gorbushina *et al*., 2003; Libkind *et al*., 2004). Ecologically, mycosporine-producing fungi are found in temperate zones (saprophytic and phytopathogenic basidiomycetes, ascomycetes and deuteromycetes), as well as in ascomycetous microcolonial fungi growing on tree barks or rock surfaces in cold and hot deserts and pseudodeserts (Gorbushina *et al*., 2003; Volkmann, 2004).

The majority of fungal strains investigated belong to a group of rock-inhabiting microcolonial fungi (Ascomycetes). Microcolonial fungi, long-living modified mycelia frequently occurring on desert and pseudodesert rock surfaces, are exposed to strong UV radiation, desiccation and nutrient scarcity. This peculiar ecological group of fungi is characterized by slow-growing restricted colonies, which was why these structures were termed microcolonial fungi (MCF) (Staley *et al*., 1982). MCF can be found on most rock substrates in Mediterranean environments (Wollenzien *et al*., 1995, 1997; Sterflinger *et al*., 1999), on desert rocks (Staley *et al*., 1982) and in hypersaline waters (Gunde-Cimerman *et al*., 2000), or as plant, animal and human pathogens (de Hoog, 1993). MCF lack ascospores; all their cells grow by mitotic cell divisions and possess a thick melanized cell wall (Gorbushina *et al*., 2003, 1993). Rock-inhabiting fungi are remarkably stress tolerant (Sterflinger & Krumbein, 1995; Wollenzien *et al*., 1997) and frequently combine properties of a sclerotium (vegetative survival structure) and a growing colony (Gorbushina *et al*., 2003). This extraordinary stress tolerance is supported by a constitutive synthesis of melanins (Gorbushina *et al*., 1993) and mycosporines (Gorbushina *et al*., 2003). In our studies, MCF isolates from rock surfaces in Mediterranean and

Table 1. Diversity of the fungi screened for their mycosporine content

Name	Strain number	Source
Sarcinomyces/Mycosphaerella	A06	Turkey, Dydimae, marble
Coniosporium sp.	A10	Ukraine, Chersonesus, marble
Coniosporium sp.	A28	Ukraine, Chersonesus, marble
Coniosporium sp.	A34	Ukraine, Chersonesus, marble
Microcolonial fungus	A49	Greece, Athens, Akropolis, Parthenon, marble
Coniosporium perforans	A51	Greece, Athens, Akropolis, Parthenon, marble
Coniosporium uncinatum	A68	Greece, Athens, Akropolis, Parthenon, marble
Sarcinomyces petricola	A71	Greece, Athens, Akropolis, Parthenon, marble
Chaetothyriomycetales	A73	Greece, Athens, Akropolis, Parthenon, marble
Sarcinomyces petricola	A95	Greece, Athens, Akropolis, Parthenon, marble
Phaeococcus chersonesus	A99	Ukraine, Chersonesus, marble
Microcolonial fungus	A144	Italy, Carrara-quarry, marble
Microcolonial fungus	A145	Italy, Carrara-quarry, marble
Coniosporium-like	A148	Italy, Carrara-quarry, marble
Discosphaerina-like	AN11	Israel, Negev desert, chert (desert varnish)
Botryosphaeria-like	AN13	Israel, Negev desert, chert (desert varnish)
Coccodinium-like	AN7	Israel, Negev desert, calcareous rock
Microcolonial fungus	AN5	Israel, Negev desert, calcareous rock
Aureobasidium pullulans	B802	Slovenia, saltern, org. material in water
Trimmatostroma salinum	B734	Slovenia, saltern, org. material in water
Hortaea werneckii	B736	Slovenia, saltern, org. material in water
Phaeotheca triangularis	K34	Greece, Delos, marble
Botryosphaeria-like	DVA4	USA, desert varnish
Coniosporium apollinis	DVA7	USA, desert varnish
Cladophialophora-like	M10A	USA, silicate, desert varnish
Cladophialophora-like	M10B	USA, silicate, desert varnish
Knufia cryptophialidica	*Knufia* 1,2,3,4	Canada, Alberta, branch of an aspen
Morchella spongiola	1J8; 1J5	Germany, Jena, Kunitz
Morchella conica	21J1; 21J4; 21J3	Germany, Jena, Damenviertel
Microcolonial fungus	N26b	Namibia, Namib coastal desert, quarzite soil pebble
Microcolonial fungus	N26c	Namibia, Namib coastal desert, quarzite soil pebble
Microcolonial fungus	N26d	Namibia, Namib coastal desert, quarzite soil pebble
Microcolonial fungus	N46	Namib, Namib desert (continental part), quarzite soil pebble
Microcolonial fungus	N60	Namib, Namib desert (continental part), marble
Microcolonial fungus	N67	Namib, Namib desert (continental part), quarzite soil pebble
Microcolonial fungus	N74	Namib, Namib desert (continental part), quarzite soil pebble
Microcolonial fungus	S3	Slovenia, saltern, cement in direct contact with hypersaline water
Microcolonial fungus	S17	Slovenia, saltern, cement in direct contact with hypersaline water
Stereum hirsutum	–	Netherlands, near Wageningen, collected and identified by Thom W. Kuyper

adjacent regions (Greece, Italy, Slovenia, Turkey and Ukraine) as well as in the Negev, the Mojave and the Namib deserts were used (Table 1). Saltern isolates adapted to osmotic and UV stresses were also investigated. Fruit-body-forming ascomycetes (several strains of the genus *Morchella*), and a basidiomycete (*Stereum hirsutum*), as well as a sclerotium-forming deuteromycete *Botrytis cinerea* were examined. Altogether, 45 strains of fungi (Table 1) were checked in our study for their mycosporine and/or MAA content.

Several rock-inhabiting prokaryotes were screened for the diversity of UV light-absorbing compounds. The prokaryotes examined included strains of the rock-inhabiting actinomycete genus *Geodermatophilus*, which often form aggregates (Eppard *et al.*, 1996) morphologically similar to MCF. These strains originated from rock and soil samples collected in the Namib desert, as well as in the Amargrosa desert and other alpine and desert locations in Utah, Colorado and California (Eppard *et al.*, 1996). The spectrum of mycosporine or MAA synthesizing organisms have been enlarged with 31 marine and terrestrial cyanobacterial isolates (*Nodularia* as well as diverse *Oscillatoriaceae* including representatives of the genera *Phormidium* and *Leptolyngbya*). Also red algae (*Mastocarpus stellatus, Gelidium sesquipedale, Devalerea ramentacea* and *Porphyra umbilicalis*) have been included to ensure the suitability of our analysis method for the known mycosporines and MAA diversity.

Generally in ascomycetes and prokaryotes the whole colony was extracted. For basidiomycetes and for the ascomycetous genus *Morchella*, only fruiting bodies or fruit body initials formed in pure culture, and for deuteromycete a separate fraction of sclerotia were used for extraction.

Extraction

Prior to the extraction, 100 mg of moist biomass were placed in 2 mL sample tubes (Eppendorf, Hamburg, Germany) and lyophilized overnight using a Lyovac GT2 (Leybold-Heraeus, Köln, Germany). The lyophilized samples were then pulverized under liquid nitrogen using a pestle and mortar.

Solid–liquid extraction procedures were used to extract the UV-absorbing compounds from the samples. Of the lyophilized and pulverized sample, *c.* 20 mg was placed in a 2 mL sample tube and covered with 1 mL of 0.2% aqueous acetic acid with 0.5% methanol added (v/v). After 10 s vortexing, the samples were placed on a vertical shaker. The extraction was performed at 4 °C for 12 h. Centrifugation with a Biofuge Pico (Heraeus Instruments) at 14 000 ***g***, 5 min and filtering (0.2 μm pore size) were used to remove the particulate matter.

Separation and characterization

Two different LC systems were used to characterize the mycosporines and MAAs: (i) for screening purposes a Waters 600E HPLC (Waters, Milford, MA) system equipped with a diode array detector (Waters 996, Waters); and (ii) for LC/MS (mass spectrometry) and LC/MS/MS analyses a Thermo Separation Products HPLC System (San Jose, CA) with a UV-VIS detector and interfaced with a Thermo Finnigan LCQ ion trap mass spectrometer (San Jose, CA). Chromatographic conditions were the same on both LC systems.

A Lichrospher 100RP-18 column (5 μm, 4.6 × 220 mm, CS Chromatographie Service, Düren, Germany) equipped with a guard precolumn containing the same material was used for separation. A volume of 20 μL of the sample was injected into the column using an autosampler system. The solution primarily used for the extraction (0.2% aqueous acetic acid + 0.5% MeOH (v/v) was used as a mobile phase. The flow rate employed was 0.7 mL min^{-1}.

The diode array detector was continuously scanning from $\lambda = 250$ to 400 nm, whereas the UV detector of the LC/MS system was set to monitor at a fixed wavelength of either $\lambda = 310$ or 320 nm, depending on the screening results.

$[M+H]^+$ ions were produced using electrospray ionization (ESI). The ESI source was operated at a capillary temperature of 270 °C and a capillary voltage of 3.8 kV. N_2 was used as sheath gas (set to 80 units) and as auxiliary gas (20 units). MS/MS analyses were performed at normalized collision energy of 25%. At least 20 spectra were recorded, each with an average of three microscans at a maximum ion collection time of 50 ms. The mass spectrometer was calibrated using a mixture of caffeine, methionyl-arginyl-phenylalanyl-alanine acetate · H_2O (MRFA) and Ultramark 1621 (Sigma Chemical Co., St Louis, MO). The UV-absorbing mycosporines and MAAs were identified by their UV absorption maximum and molecular mass and/or fragmentation pattern. Some reference patterns were obtained from Whitehead *et al.* (2001).

Results and discussion

Eight different mycosporines and five MAAs were separated and identified using a Lichrospher 100-RP18 column running under isocratic aqueous conditions following a solid–liquid extraction with the same solvent used in the HPLC as a running buffer. Under the separation conditions used, all investigated mycosporines and MAAs have demonstrated different retention times. Table 2 shows information about retention times, UV absorption maxima, fragmentation patterns (main fragments are shown in bold), measured masses of quasi-molecular ions $[M+H]^+$ and calculated exact masses of all UV-absorbing compounds found in the screened organisms.

Mycosporine–glutaminol–glucoside and the corresponding free carboxylic acid mycosporine–glutami**c**ol–glucoside (note the single letter difference between these two compounds) were the main mycosporines identified in the majority of the strains of rock (*Coniosporium*, *Sarcinomyces*, *Botryosphaeria*, *Phaeoccocus*, *Discosphaerina*-like, etc.)- and plant (*Knufia*)- surface-inhabiting microcolonial fungi. Mycosporine-glutaminol-glucoside is found in terrestrial fungi and fresh water yeast of basidiomycetous affiliation (Bernillon *et al.*, 1984; Libkind *et al.*, 2004) and in microcolonial ascomycetes (Whitehead *et al.*, 2001; Gorbushina *et al.*, 2003; Volkmann *et al.*, 2003). Mycosporine-glutami**c**ol-glucoside was reported previously from ascomycetes (Pittet *et al.*, 1983), but at that time considered to be a degradation product of the amide and thus an artefact of cationic exchange chromatography. By careful analysis, however, it was convincingly demonstrated that these two glycolysed derivates of mycosporines are naturally occurring secondary metabolites of rock-inhabiting microcolonial fungi (Volkmann *et al.*, 2003). Our method allows for careful separation of both compounds and therefore proves to be a reliable instrument in mycosporines or MAAs characterization. A successful separation of four similar mycosporines from the microcolonial fungus *Sarcinomyces petricola* is shown in Fig. 1. Except for the peak with a retention time of 12.5 min, all other peaks have their absorption maximum at $\lambda = 310$ nm. The four structural related mycosporines elute after 9.5 min (mycosporine–glutaminol), 10.5 min (mycosporine–glutaminol–glucoside), 14.1 min (mycosporine

Table 2. Ultraviolet-absorbing substances found in measured samples

Substance	Retention time	λ_{max}	$[M+H]^+$	Fragmentation pattern	Mass	Organisms
Shinorine	5.5	334	333	137, 168, 185, **186**, 197, 211, 230, 241, 255*	332.12	*Nodularia spumigena* *Porphyra umbilicalis* *Mastocarpus stellatus* *Gelidium sesquipedale* *Devalerea ramentacea*
Palythine	6.1	320	245	137, 150, 155, 162, 184, **186**, 199, 209, 230*	244.11	*Porphyra umbilicalis* *Gelidium sesquipedale* *Devalerea ramentacea*
Asterina 330	6.9	330	289	137, 150, 168, **186**, 197, 199, 213, 230, 243, 273, 289*	288.13	*Gelidium sesquipedale* *Devalerea ramentacea*
Unknown	6.9	359	432	388	Unknown	All *Geodermatophilus* strains
Porphyra 334	8.4	334	365	137, 151, 168, 185, **186**, 197, 200, 243, 303*	346.14	*Nodularia spumigena* *Porphyra umbilicalis* *Gelidium sesquipedale* *Devalerea ramentacea*
Mycosporine–serinol	8.8	310	262	**244,** 216, 194	261.12	*Stereum hirsutum*
Mycosporine–glutaminol	9.5 see Fig. 1	310	303	285, 267, **235**	302.15	Microcolonial fungi *Leptolyngbya* sp. *Botrytis cinerea*
Palythinol	9.9	332	303		302.15	*Gelidium sesquipedale* *Devalerea ramentacea*
Mycosporine–glutaminol–glucoside	10.5 see Fig. 1	310	465	447, 429, **303** 285, 267, 235	464.20	Microcolonial fungi
Mycosporine–alanine	11.3	310	260	242, **171,** 147	259.11	*Oscillatoria*
Mycosporine–serine	12.0	310	276	**258,** 228, 212	275.10	*Stereum hirsutum*
Mycosporine–glutamicol	14.1 see Fig. 1	310	304	286, 268, **236**	303.13	Microcolonial fungi *Botrytis cinerea*
Mycosporine–glutamicol–glucoside	15.1 see Fig. 1	310	466	448, 430, **304** 286, 268, 236	465.18	Microcolonial fungi

*Data obtained from Whitehead *et al*. (2001). Main fragments are shown in bold.

–glutamicol) and 15.1 min (mycosporine–glutamicol–glucoside).

Glycolysed derivates of mycosporines were found quite often in the analysed samples (Table 3). The frequent appearance of these glycolysed derivates results from the fact that mycosporine glycolysates are probably the physiologically inactive storage form of mycosporines within the cells. Glycolysation protects the aglycones against hydrolases and decreases their chemical reaction activity (Pittet *et al*., 1983; Bernillon *et al*., 1984). Four glycolysed mycosporines are known so far. They have been isolated from mitotic and meiotic spores of ascomycetes or deuteromycetes of ascomycetous affiliation, e.g. from *Ascochyta fabae* (Pittet *et al*., 1983), *Ascochyta pisi*, *Septoria nodorum* (Bouillant *et al*., 1981) or *Pyronema omphaloides* (Bernillon *et al*., 1984) and *Cladosporium herbarum* (Bouillant *et al*., 1981), as well as from many different microcolonial fungi as shown herewith.

The aglycones mycosporine–glutaminol and mycosporine–glutamicol were also present in many MCFs investigated. Furthermore, mycosporine–glutaminol was detected in the extracts of sclerotia of the deuteromycete *Botrytis cinerea*, which also contained notable amounts of the corresponding carboxylic acid mycosporine–glutamicol.

In agreement with previous studies, mycosporine–serine and mycosporine–serinol were successfully extracted from fruiting bodies of *Stereum hirsutum*. In the fruiting bodies of this basidiomycete we discovered relatively high amounts ($4454\,\mu g\,g^{-1}$ dry weight) of these UV light-absorbing substances, which might be explained by the presence of numerous differentiated spores in these fructification structures. No mycosporines were found in *Morchella* cultures either due to the undeveloped status of fruit-body primordial or different species affinity of the investigated strains (Buscot & Bernillon, 1991).

Three cyanobacterial strains from Tintenstrich and similar rock-inhabiting microbial communities synthesized mycosporines. Cyanobacteria in question belonged to different genera of the same order *Oscillatoriales* (*Oscillatoria*,

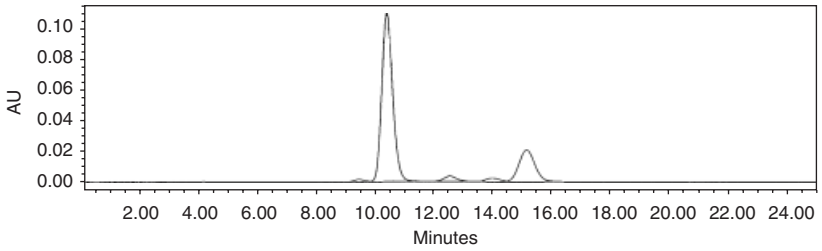

Fig. 1. Automatically scaled high-pressure liquid chromatography chromatogram (UV detector) of an extract from *Sarcinomyces petricola* (A95).

Table 3. Mycosporines identified in terrestrial samples

Sample	Myc–Gln	Myc–Gln–Glu	Myc–Glc	Myc–Glc–Glu	Myc–serine	Myc–serinol	Myc–glutamine	Myc–alanine
Botryosphaeria-like AN13	X	X	X	–	–	–	–	–
Chaetothyriomycetales A73	–	X	X	–	–	–	–	–
Cladophialophora-like M10B	–	X	–	–	–	–	–	–
Cladophialophora-like M10A	–	–	–	–	–	–	–	–
Coniosporium apollinis DVA7	–	X	–	–	–	–	–	–
Coniosporium-like A148	–	X	X	X	–	–	–	–
Coniosporium perforans A51	X	X	X	X	–	–	–	–
Coniosporium sp. A10	X	X	X	X	–	–	–	–
Coniosporium sp. A28	–	X	–	X	–	–	–	–
Coniosporium sp. A34	X	X	X	X	–	–	–	–
Coniosporium uncinatum A68	X	X	X	X	–	–	–	–
Discosphaerina like AN11	–	X	X	X	–	–	–	–
Knufia cryptophialidica (all 4 isolates)	–	X	–	X	–	–	–	–
Microcolonial fungi (AN5)	–	X	–	–	–	–	–	–
Microcolonial fungi (N26c)	X	X	X	X	–	–	–	–
Microcolonial fungi (A144)	–	X	–	X	–	–	–	–
Microcolonial fungi (A145)	X	X	X	X	–	–	–	–
Microcolonial fungi (N26b)	X	X	X	X	–	–	–	–
Microcolonial fungi (N26d)	X	X	–	X	–	–	–	–
Microcolonial fungi (N46)	–	X	–	–	–	–	–	–
Microcolonial fungi (N60)	–	X	X	X	–	–	–	–
Microcolonial fungi (N67)	–	X	–	–	–	–	–	–
Microcolonial fungi (N74)	X	X	–	–	–	–	–	–
Microcolonial fungi (S3)	X	–	–	–	–	–	X	–
Microcolonial fungi (S17)	–	X	–	X	–	–	–	–
Microcolonial fungi (A49)	X	X	–	X	–	–	–	–
Phaeococcus chersonesus A99	X	X	X	X	–	–	–	–
Phaeotheca triangularis K34	–	X	–	–	–	–	–	–
Sarcinomyces/Mycosphaerella A6	–	–	–	–	–	–	–	–
Sarcinomyces petricola A71	X	X	–	–	–	–	–	–
Sarcinomyces petricola A95	X	X	X	X	–	–	–	–
Stereum hirsutum	–	–	–	–	X	X	–	–
Botrytis cinerea	X	–	X	–	–	–	–	–
Aureobasidium pullulans B802	–	X	–	X	–	–	–	–
Hortaea werneckii B736	X	X	–	X	–	–	–	–
Trimmatostroma salinum B734	X	X	–	X	–	–	–	–
Leptolyngbya sp.	X	–	–	–	–	–	–	–
Leptolyngbya foveolarum	–	X	–	X	–	–	–	–
Oscillatoria sp. CCMEE 315	–	–	–	–	–	–	–	X

Myc–Gln, mycosporine–glutaminol; Myc–Gln–Glu, mycosporine–glutaminol–glucoside; Myc–Glc, mycosporine–glutamicol; Myc–Glc–Glu, mycosporine–glutamicol–glucoside.

Leptolyngbya sp., *Leptolyngbya foveolarum*) and came from similar subaerial habitats. Mycosporine-alanine previously described in terrestrial fungi (Leite & Nicholson, 1992) was found in *Oscillatoria* originating from quartz rock in Australia. Mycosporine–glutaminol – a mycosporine so far considered to be typical for terrestrial fungi – was discovered

in a *Leptolyngbya* sp. from granite surface in Nepal. *Leptolyngbya foveolarum* ('Tintenstrich' community, Switzerland) contained the typical terrestrial mycosporine–glutaminol–glucoside and its corresponding free carboxylic acid mycosporine–glutamicol–glucoside. Mycosporine–alanine, mycosporine–glutaminol, mycosporine–glutaminol–glucoside and mycosporine–glutamicol–glucoside were exclusively identified in terrestrial cyanobacteria from rock surfaces.

Fungi from hypersaline environments (S3, S17, *Aureobasidium pullulans*, *Hortaea werneckii*, *Trimmatostroma salinum*) that tend to microcolonial growth and are essentially adapted to osmotic and UV light stress synthesize diverse mycosporines typical for rock-inhabiting MCF (mycosporine–glutaminol, mycosporine–glutaminol–glucoside and mycosporine–glutamicol–glucoside).

Micosporine-like amino acids were observed in isolates from marine habitats with a normal salinity. In total, five different MAAs, namely shinorine, palythine, asterina 330, porphyra 334 and palythinol, were successfully separated and identified by LC/MS. All the Rhodophyta screened in the frame of this work have been previously examined for MAAs (Karsten *et al*., 1998, 1999). The results obtained by us on red algae (Table 4) were consistent with the data of Karsten *et al*. (1999). The only exception was *Devalerea ramentacea*, which in our analysis showed neither mycosporine–glycine nor palythene, which might be column dependent (Carreto *et al*., 2005; Karsten *et al*., 1999). *Nodularia spumigena* from the Baltic Sea synthesized shinorine and porphyra 334. In general, the relatively low diversity of MAAs in our screening may be correlated to the fact that MAA synthesis in cyanobacteria is inducible by light (Garcia-Pichel & Castenholz, 1993; Portwich & Garcia-Pichel, 1999), and in our studies the strains were cultivated under normal conditions and thus probably produced only constitutive secondary metabolites.

An unknown UV light-absorbing compound was revealed by our method. In five (G5, G16, G17S, G18 and G20S) of 11 samples of *Geodermatophilus* strains we found a substance with an absorption maximum of $\lambda = 359$ nm at a retention time of 6.9 min. MS investigations led to a $[M+H]^+$ with *m/z* 432. Further MS/MS investigations gave a main fragment at *m/z* 388. This substance is yet unknown, but mass spectrometric data (fragmentation pattern) point out that this substance is neither a mycosporine nor an MAA. A new compound, perhaps typical for actinobacteria, may emerge from further studies.

The application of LC/MS coupling to characterize mycosporines and MAAs, especially the ionization with an electrospray source, is relatively new and has been previously used to identify MAAs (Whitehead *et al*., 2001; Whitehead & Hedges, 2003). The identification of mycosporines from terrestrial fungi using LC/MS coupling with ESI was first applied by Volkmann *et al*. (2003). The identification of mycosporines and MAAs is frequently done by comparing the absorption maxima and retention times with those of co-chromatographed standards (Whitehead *et al*., 2001). As no mycosporine standards are commercially available, crude extracts from different organisms usually replace them. However, in natural product chemistry, these properties alone are generally considered insufficient for a secure identification of organic compounds (e.g. Carreto *et al*., 2005). Therefore, we along with other authors (Whitehead *et al*., 2001; Whitehead & Hedges, 2003) suggest using precise analytical equipment like LC/MS for accurate identification of mycosporines and MAAs, as well as for their structural characterization.

Our investigation demonstrated that mycosporines with oxy-carbonyl chromophores appear exclusively in pro- and eukaryotic microorganisms of terrestrial origin. As fungi from salterns are always associated either with organic material or inorganic support of terrestrial origin, mycosporine occurrence in them does not contradict this statement. Since in our studies cyanobacteria were not stimulated by UV illumination, this result indicates the constitutive synthesis of these compounds in some rock-inhabiting cyanobacteria. Microorganisms from subaerial rock surfaces and hypersaline environments present a selection of possible investigations objects with constitutive mycosporine synthesis, where functions and biological roles of these secondary metabolites can be further investigated. Mycosporines also appear to present a noteworthy marker of rock-inhabiting communities, and their diversity may be used for microbial ecology studies of these peculiar terrestrial environments. MAAs with imino-carbonyl chromophores were detected only in red algae and one cyanobacterium from marine environments.

The content and diversity of mycosporines and MAAs could also be used as an additional taxonomical feature for

Table 4. Identified mycosporine-like amino acids and mycosporines in marine samples

	Shinorine	Palythine	Asterina 330	Porphyra 334	Palythinol
Porphyra umbilicalis	X	X	–	X	–
Mastocarpus stellatus	X	–	–	–	–
Gelidium sesquipedale	X	X	X	X	X
Devalerea ramentacea	X	X	X	X	X
Nodularia spumigena	–	–	–	X	–

different groups of cyanobacteria (Garcia-Pichel *et al*., 1998) and microcolonial ascomycetes. This is confirmed by the uniform occurrence of identical mycosporines in different *Knufia cryptophialidica* isolates (Table 3).

The range of ecosystems in which mycosporines and MAAs may play a significant biological role is very extensive. With this new universal solid–liquid extraction procedure, using the same solvent as in HPLC, we were able to extract several different mycosporines, MAAs and other UV-absorbing water-soluble compounds of eukaryotic and prokaryotic origin. Our extraction method does not contain a re-dissolving step. This feature allows for efficient recovery and more precise quantification of analysed substances. The method suggested is applicable for quantification of such compounds in pure cultures, as well as for analysis of environmental samples (Volkmann, 2004). For its rapidity and relative simplicity, it may establish itself as a powerful tool for the characterization and distinction of materials and organisms from stressed environments.

Acknowledgements

The German Science Foundation has financially supported this work through the projects DFG Go 897/2-1 and 2-2. We thank Dr. Oliver Nixdorf (AWI) for kindly providing us with extracts of red algae. Several colleagues are thanked for supplying us with a necessary diversity of material for this investigation: Katarzyna Palinska (cyanobacterial isolates), Nina Gunde-Cimerman (B802, B734 and B736 strains), Sarah Hambleton (*Knufia* strains), Katja Sterflinger (strain K34), Francois Buscot (*Morchella* cultures) and James T. Staley (strains DVA4 and DVA7). The authors wish to thank Jürgen Rullkötter for his support of our work and an appreciated opportunity to use analytic equipment of the Organic Geochemistry laboratory (ICBM, Oldenburg). English editorial help of Randall S. Perry during the manuscript preparation is gratefully acknowledged.

References

Arpin N & Bouillant ML (1981) Light and mycosporines. *The Fungal Spore Morphogenetic Controls* (Turian G & Hohl HR, eds), pp. 435–454. Academic Press, London.

Bandaranayake WM (1998) Mycosporines: are they nature's sunscreens? *Nat Product Rep* **15**: 159–172.

Bernillon J, Bouillant M-L, Pittet J-L, Favre-Bonvin J & Arpin N (1984) Mycosporine glutamine and related mycosporines in the fungus *Pyronema omphaloides*. *Phytochemistry* **23**: 1083–1087.

Bouillant ML, Pittet JL & Bernillon J (1981) Mycosporines from *Ascochyta pisi, Cladosporium herbarum* and *Septoria nodorum*. *Phytochemistry* **20**: 2705–2707.

Böhm GA, Pfleiderer W, Böger P & Scherer S (1995) Structure of a novel oligosaccharide-mycosporine-amino acid ultraviolet A/B sunscreen pigment from the terrestrial cyanobacterium *Nostoc commune*. *J Biol Chem* **270**: 8536–8539.

Büdel B, Karsten U & Garcia-Pichel F (1997) Ultraviolet-absorbing scytonemin and mycosporine-like amino acid derivatives in exposed, rock-inhabiting cyanobacterial lichens. *Oecologia* **112**: 165–172.

Buscot F & Bernillon J (1991) Mycosporines and related compounds in field and cultured mycelial structures of *Morchella esculenta*. *Mycol Res* **95**: 752–754.

Carreto JI, Carignan MO, Daleo G & De Marco SG (1990) Occurrence of mycosporine-like amino acids in the red dinoflagellate *Alexandrium excavatum*: UV protective compounds? *J Plankton Res* **12**: 909–921.

Carreto JI, Carignan MO & Montoya NG (2005) A high-resolution reverse-phase liquid chromatography method for the analysis of mycosporine-like amino acids (MAAs) in marine organisms. *Mar Biol* **146**: 237–252.

Carroll AK & Shick JM (1996) Dietary accumulation of UV-absorbing mycosporine-like amino acids (MAAs) by the green sea urchin (*Strongylocentrotus droebachiensis*). *Mar Biol* **124**: 561–569.

Chioccara F, Zeuli L & Novellino E (1986) Occurrence of mycosporine related compounds in sea urchin eggs. *Comp Biochem Physiol B Biochem Mol Biol* **85B**: 459–461.

Dehorter B & Bernillon J (1983) Photoinduction des périthèces du *Nectria galligena*: production et activité photomorphogène des mycosporines. *Can J Botany* **61**: 1435–1442.

Dunlap WC & Chalker BE (1986) Identification and quantification of near-UV absorbing compounds (S-320) in a hermatypic scleractinian. *Coral Reefs* **5**: 155–159.

Dunlap WC, Williams DM, Chalker BE & Banaszak AT (1989) Biochemical photoadaptions in vision: UV-absorbing pigments in fish-eye tissues. *Comp Biochem Physiol B Biochem Mol Biol* **93B**: 601–607.

Dunlap WC & Yamamoto Y (1995) Small-molecule antioxidants in marine organisms: antioxidant activity of mycosporine-glycine. *Comp Biochem Physiol B Biochem Mol Biol* **112B**: 105–114.

Eppard M, Krumbein WE, Koch C, Rhiel E, Staley JT & Stackebrandt E (1996) Morphological, physiological, and molecular characterization of actinomycetes isolated from dry soil, rocks, and monument surfaces. *Arch Microbiol* **166**: 12–22.

Favre-Bonvin J, Arpin N & Brevard C (1975) Structure de la mycosporine (P310). *Can J Chem* **54**: 1105–1111.

Garcia-Pichel F & Castenholz RW (1993) Occurrence of UV-absorbing, mycosporine-like compounds among cyanobacterial isolates and an estimate of their screening capacity. *Appl Environ Microbiol* **59**: 163–169.

Garcia-Pichel F, Nübel U & Muyzer G (1998) The phylogeny of unicellular, extremely halotolerant cyanobacteria. *Arch Microbiol* **169**: 469–482.

Gorbushina AA, Krumbein WE, Hamann CH, Panina L, Soukharjevski S & Wollenzien U (1993) Role of black fungi in colour change and biodeterioration of antique marbles. *Geomicrobiol J* **11**: 205–221.

Gorbushina AA, Whitehead K, Dornieden T, Niesse A, Schulte A & Hedges JI (2003) Black fungal colonies as units of survival: hyphal mycosporines synthesized by rock-dwelling microcolonial fungi. *Can J Botany* **81**: 131–138.

Grant PT, Middletown C, Plack PA & Thomson RH (1985) The isolation of four aminocyclohexenimines (mycosporines) and a structurally related derivative of cyclohexane-1 : 3-dione (gadusol) from the brine-shrimp, *Artemia*. *Comp Biochem Physiol B Biochem Mol Biol* **80B**: 755–759.

Gunde-Cimerman N, Zalar P, de Hoog S & Plemenitas A (2000) Hypersaline waters in salterns – natural ecological niches for halophilic black yeasts. *FEMS Microbiol Ecol* **32**: 235–240.

de Hoog GS (1993) Evolution of black yeasts – possible adaptation to the human host. *Antonie Van Leeuwenhoek* **63**: 105–109.

Karentz D (2001) Chemical defences of marine organisms against solar radiation exposure: UV-absorbing mycosporine-like amino acids and scytonemin. *Marine Chemical Ecology* (McClintock JB & Baker BJ, eds), pp. 481–520. CRC Press, Boca Raton, FL, USA.

Karentz D, McEuen FS, Land MC & Dunlap WC (1991) Survey of mycosporine-like-amino acids in Antarctic marine organisms: potential protection from ultraviolet exposure. *Mar Biol* **108**: 157–166.

Karsten U, Bischof K, Hanelt D, Tüg H & Wiencke C (1999) The effect of ultraviolet radiation on photosynthesis and ultraviolet-absorbing substances in the endemic Arctic macroalga *Devalerea ramentacea* (Rhodophyta). *Physiol Planta* **105**: 58–66.

Karsten U, Sawall T, Hanelt D, Bischof K, Figueroa FL, Flores-Moya A & Wiencke C (1998) An inventory of UV-absorbing mycosporine-like amino acids in macroalgae from polar to warm-temperate regions. *Botanica Marina* **41**: 443–453.

Laurion I, Lami A & Sommaruga R (2002) Distribution of mycosporine-like amino acids and photoprotective carotenoids among freshwater phytoplankton assemblages. *Aquat Microbial Ecol* **26**: 283–294.

Leach CM (1965) Ultraviolet-absorbing substances associated with light-induced sporulation in fungi. *Can J Botany* **43**: 185–200.

Leite B & Nicholson RL (1992) Mycosporine-alanine: a self-inhibitor of germination from the conidial mucilage of *Colletotrichum graminicola*. *Exp Mycol* **16**: 76–86.

Libkind D, Perez P, Sommaruga R, del Carmen Dieguez M, Ferraro M, Brizzio S, Zagarese H & van Brook M (2004) Constitutive and UV-inducible synthesis of photoprotective compounds (carotenoids and mycosporines) by freshwater yeast. *Photochem Photobiol Sci* **3**: 281–286.

Nakamura H, Kobayashi J & Hirata Y (1982) Separation of mycosporine-like amino acids in marine organisms using reversed phased high performance liquid chromatography. *J Chromatogr* **250**: 113–118.

Oren A (1997) Mycosporine-like amino acids as osmotic solutes in a community of halophilic cyanobacteria. *Geomicrobiol J* **14**: 231–240.

Pittet JL, Bourguignon B & Arpin N (1983) Sporulation and mycosporines in the deuteromycete *Ascochyta fabae*. *Physiol Planta* **57**: 565–572.

Pittet JL, Létoublon R, Frot-Coutaz J & Arpin N (1983) Soluble uridine diphospho-D-glucose: mycosporine glucosyltransferase from spores of *Ascochyta fabae* Speg. *Planta* **159**: 159–164.

Portwich A & Garcia-Pichel F (1999) Ultraviolet and osmotic stresses induce and regulate the synthesis of mycosporines in the cyanobacterium *Chlorogloeopsis* PCC 6912. *Arch Microbiol* **172**: 187–192.

Rieger L & Robinson D (1997) Photoinduction of UV-absorbing compounds in Antarctic diatoms and *Phaeocystis antarctica*. *Mar Ecol-Progr Ser* **160**: 13–25.

Shick JM & Dunlap WC (2002) Mycosporine-like amino acids and related gadusols: biosynthesis, accumulation, and UV-protective functions in aquatic organisms. *Annu Rev Physiol* **64**: 223–262.

Sommaruga R & Garcia-Pichel F (1999) UV-absorbing compounds, in planktonic and benthic organisms from a high-mountain lake. *Archiv Hydrobiol* **144**: 225–269.

Staley JT, Palmer F & Adams JB (1982) Microcolonial fungi: common inhabitants on desert rocks? *Science* **215**: 1093–1095.

Sterflinger GS, de Hoog GS & Haase G (1999) Phylogeny and ecology of meristematic ascomycetes. *Stud Mycol* **43**: 5–22.

Sterflinger K & Krumbein WE (1995) Multiple stress factors affecting growth of rock-inhabiting black fungi. *Botanica Acta* **108**: 490–496.

Stochaj WR, Dunlap WC & Shick JM (1994) Two new UV-absorbing mycosporine-like amino acids from the sea anemone *Anthpleura elegantissima* and the effects of zooxanthellae and spectral irridiance. *Mar Biol* **118**: 149–156.

Tartarotti B, Laurion I & Sommaruga R (2001) Large variability in the concentration of mycosporine-like amino acids among zooplankton from lakes located across an altitude gradient. *Limnol Oceanogr* **46**: 1546–1552.

Tartarotti B & Sommaruga R (2002) The effect of different methanol concentrations and temperatures on the extraction of mycosporine-like-amino-acids (MAAs) in algae and zooplankton. *Archiv Hydrobiol* **154**: 691–703.

Volkmann M (2004) Analyse und Charakterisierung von Mycosporinen aus gesteinsbesiedelnden Pilze und Umweltgesteinsproben. Ph.D. Thesis, Geomikrobiologie, Carl von Ossietzky Universität, Oldenburg, p. 160.

Volkmann M, Whitehead K, Rütters H, Rullkötter J & Gorbushina AA (2003) Mycosporine-glutamicol-glucoside: a natural UV-absorbing secondary metabolite of rock-inhabiting microcolonial fungi. *Rapid Commun Mass Spectrom* **17**: 897–902.

Whitehead K, Gorbushina AA & Hedges JI (2001) Mycosporines in the environment: their analysis and implications. In: *American Chemical Society*, *Annual Meeting*, April 2001, San Diego, USA.

Whitehead K & Hedges JI (2003) Electrospray ionization tandem mass spectrometric and electron impact mass spectrometric characterization of mycosporine-like amino acids. *Rapid Commun Mass Spectrom* **17**: 2133–2138.

Whitehead K, Karentz D & Hedges JI (2001) Mycosporine-like amino acids (MAAs) in phytoplankton, a herbivorous pteropod (*Limacina helicina*) and its pteropod predator (*Clione antarctica*) in McMurdo Bay, Antarctica. *Mar Biol* **139**: 1013–1019.

Whitehead K & Vernet M (2000) Influence of mycosporine-like amino acids (MAAs) on UV absorption by particulate and dissolved organic matter in La Jolla Bay. *Limnol Oceanogr* **45**: 1788–1796.

Wollenzien U, De Hoog GS, Krumbein WE & Urzi C (1995) On the isolation of microcolonial fungi occurring on and in marble and other calcareous rocks. *Sci Total Environ* **167**: 287–294.

Wollenzien U, de Hoog GS, Krumbein W & Uijthof JMJ (1997) *Sarcinomyces petricola*, a new microcolonial fungus from marble in the Mediterranean basin. *Antonie Van Leeuwenhoek* **71**: 281–288.