Eur. J. Biochem. **271**, 780–784 (2004) © FEBS 2004 doi:10.1111/j.1432-1033.2004.03981.x

A new UV-B absorbing mycosporine with photo protective activity from the lichenized ascomycete *Collema cristatum*

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A novel photo protective mycosporine was isolated from the lichenized ascomycete *Collema cristatum*. Biological activity was measured in terms of protection against UV-B induced membrane destruction and pyrimidine dimer formation in cultured human keratinocytes, and prevention of UV-B induced erythema. It was found that the pure isolated compound prevented UV-B induced cell destruction in a dose-dependent manner, that the compound partially prevented pyrimidine dimer formation and completely prevented UV-B induced erythema when applied to the skin prior to irradiation.

Keywords: lichenized ascomycete; *Collema cristatum*; mycosporine; photo protective activity; UV-B.

Lichens are symbiotic organisms of fungi, algae and/or cyanobacteria that synthesize numerous secondary metabolites, which comprise aliphatic, aromatic, terpenoic and halogenated compounds. Several species absorb strongly in the UV-B range [1,2,4]. Lichens and their metabolites have been demonstrated to possess numerous biological activities, including: antiviral, antibacterial, antitumor, antiallergic, antiherbivore and enzyme inhibitory activity. Some active lichen substances are used in the pharmaceutical industry (V. M. Dembitsky, unpublished observation, [4]).

The sun radiation reaching the earth spans from the shortwaved UV (UV-C), which is absorbed in the ozone layer, through the UV-B (280–315 nm), the UV-A (315–400 nm) and the visible range (400–800 nm), to the infrared. The shorter the wavelength, the more energetic and potentially harmful is the radiation. UV-B radiation was recognized long ago as the cause for skin erythema (sunburn), and accumulated exposure results in DNA damage and immunosuppression, eventually leading to skin cancer [5–7]. Most commercial sunscreens are designed to prevent sunburn on the assumption that this activity will also prevent skin cancer [8–10]. However, the dramatic increase in skin cancer incidence rates demonstrates the inadequacy of traditional sun protecting agents, and emphasizes the urgent need to look for new, alternative molecules.

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Abbreviations: MED, minimal erythema dose; MAA, mycosporine-like amino acid.

(Received 16 November 2003, revised 23 December 2003, accepted 6 January 2004)

Such compounds may be found in certain ancient photosynthetic organisms. As ancient plants depend on solar irradiation as their primary source of energy, but at the same time must provide means that can counteract the damaging effects of UV-B irradiation on proteins, DNA and other biologically active molecules, they have developed various protective mechanisms that enable continuous survival under direct and UV radiation [11–13]. In addition to DNA repair mechanisms such as photoreactivation and excision repair, accumulation of carotenoids, detoxifying enzymes, radical quenchers and antioxidants, these organisms can also synthesize highly effective UV absorbing pigments such as scytonemin, mycosporine and MAAs (mycosporine-like amino acids) [11].

Herein, we report our demonstration of the photo protective properties of a mycosporine, isolated from the lichenized ascomycete *Collema cristatum*. This mycosporine, which we have named Collemin A, has not been described previously.

Materials and methods

Lichen samples

The lichenized ascomycete *Collema cristatum* (L) F.H. Wigg. (Jelly lichen, Collemataceae Family) was collected in January 2001 from sun exposed rock surfaces around Jerusalem at about 700 meters above sea level. It was identified by M. Temina (Biodiversity and Biotechnology Center of Cryptogamic Plants and Fungi, Institute of Evolution, University of Haifa, Israel) and has its voucher, HAI-031511, deposited in the Herbarium of the Institute of Evolution.

Isolation and cultivation of mycobionts

Mycobionts from *Collema cristatum* were obtained from the spores discharged from the apothecia of a thallus, and were

cultivated in test tubes containing 20.0 g malt extract (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), 4 g yeast extract (Becton, Dickinson and Company), 100 g sucrose, 15 g agar, H_2O, pH = 7.0, at 20 °C in the dark. After cultivation for three months, the colonies and slants were harvested. Cultivation of the mycobionts isolated from *Collema cristatum* and used in our study, was carried out by I. Dor (Division of Environment Science, The Fredy and Nadine Herrmann Graduate School of Applied Sciences, The Hebrew University of Jerusalem, Israel).

Extraction and isolation

Extraction of the lichen body and/or cultivated mycobiont was carried out by a mixture of methanol/water (90 : 10, v/v), and initial column chromatography was performed on silica gel by isocratic elution with 60% EtOH – 40% MeOH, to yield 50 mg of a pale yellow powder. Purity of the isolated compound was confirmed by HPLC analysis, which showed a single sharp peak with a retention time of 14.1 min. The analysis was carried out on a reverse-phase column RP-18 (Waters Corp.) using gradient elution with a flow rate of 1 $mL{\cdot}s^{-1}$ ranging from 90% of 0.05% acetic acid in water and 10% acetonitrile, to 10% of 0.05% acetic acid in water, to 90% acetonitrile [6], and revealed a new, highly polar, water soluble compound that absorbs strongly in the UV-B region.

Cell cultures

A human keratinocyte cell line, HaCaT, was grown at 37 °C in 5% CO_2 in DMEM (Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal bovine serum, L-glutamine 2 mM and antibiotics (100 U penicillin per mL and 100 mg streptomycin per mL). Cells were grown to 80% confluence in 9 cm culture dishes, overlaid with fresh $NaCl/P_i$ and placed under a UV-B light source. The cell line originated from the laboratory of N. Fusenig, Heidelberg, Germany. Only early passages (< 50) were used for the experiments.

UV irradiation

UV irradiation was performed with a bank of four FS40 fluorescent lamps that emit UV-B at wavelengths between 280 and 315 nm with a peak at 313 nm. Light intensity was determined using a Waldmann UV radiometer (Herbert Waldmann GBH, Schwenningen, Germany). HaCaT cells were irradiated at an irradiance of 3.4 $mW{\cdot}s^{-1}$ with a dose of 200 $mJ{\cdot}cm^{-2}$ (cell death) or 60 $mJ{\cdot}cm^{-2}$ (DNA damage). The test materials (300 $\mu L{\cdot}100\ cm^{-2}$) were spread on a quartz plate placed on the top of the Petri dish, through which the cells were irradiated. To guarantee even distribution of the test materials, the solutions were contained inside a rubber ring glued to the quartz plate. Keratinocytes were harvested by trypsination either immediately (pyrimidine dimers) or 24 h (cell survival) after irradiation. The *in vivo* biological activity was assayed by the ability to prevent UV induced erythema of human skin. After informed consent and approval from the Ethical Committee on Experiments on Humans (Helsinki Committee), the pure compound was diluted 1 : 10 in olive oil and applied to the inside forearm of a volunteer at a concentration of 6 $\mu g{\cdot}cm^{-2}$. Olive oil without the isolated compound served as control. Fifteen minutes after application, four minimal erythema doses (MED; 360 $mJ{\cdot}cm^{-2}$) of UV-B irradiation were delivered to the treated areas, and the resulting erythema was evaluated after 24 h as described previously [15].

Estimation of cell survival

Cell survival was measured 24 h after irradiation using the trypan blue exclusion assay. Cells were harvested using 0.25% trypsin and 0.05% EDTA in PUCK's saline (Biological Industries, Beit-Haemek, Israel), washed with $NaCl/P_i$ and counted in 0.2% trypan blue solution (Biological Industries). Viability was calculated as the average percent of trypan blue negative cells in five fields of a standard hemocytometer.

Pyrimidine dimers

The DNA was extracted immediately after irradiation using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Pyrimidine dimers were determined by ELISA technique [14]. In brief, 5 μg per well of denatured DNA was applied, in triplicates, into poly L-lysine (Sigma) precoated ELISA plates, washed five times with $NaCl/P_i$ and blocked with 2% fetal bovine serum in $NaCl/P_i$. As first antibody, an anti-(thymidine dimer H3 clone 4F6) Ig (Affiteck, Oslo, Norway) diluted 1 : 1000 in 2% fetal bovine serum in $NaCl/P_i$, was used. As the secondary antibody, a biotin-SP conjugated goat anti-mouse Ig diluted 1 : 50 000 was used, followed by peroxidase conjugated strepavidin (Jackson, West Grove, PA, USA) diluted 1 : 10 000. The peroxidase reaction was performed using 0.4 $mg{\cdot}mL^{-1}$ *o*-phenylenediamine (Sigma) in the presence of 0.02% H_2O_2, and color intensity was measured by spectrophotometry at 492 nm.

Isolation of Collemin A

HPLC analysis was carried out on a Waters 600 instrument, using a RP-18 column (symmetry 4.6 × 250 mm) connected to a symmetry guard column, outfitted with Waters 996 photodiode array detector. Infrared spectra were recorded on a Perkin-Elmer 2000 Fourier transformed infrared instrument. High-resolution mass spectrometry analysis was made on a Q-TOF-micro-LC mass spectrometer (MicroMass, Manchester, UK) in Bar-Ilan University. One dimentional and two dimentional NMR spectra were measured on a Bruker 400 MHz spectrometer (Bruker BioSpin Corp., MA, USA). The ^1H NMR chemical shifts (referenced to CD_3OD observed at 3.30 p.p.m.) were assigned using a combination of data from correlation spectroscopy (COSY) and heteronuclear multiple quantum correlation (HMQC) experiments [16].

Results and discussions

Structure of Collemin A

Collemin A (Fig. 1) has a molecular formula of $C_{19}H_{32}N_2O_{13}$ (496 MW), which was determined by high-

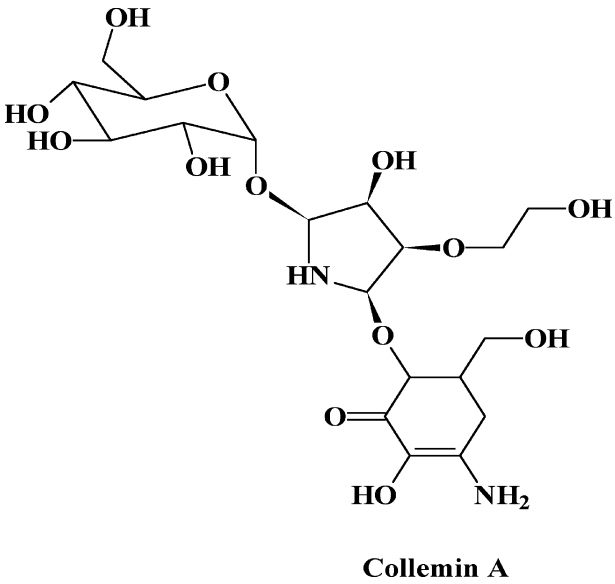

Fig. 1. Chemical structure of Collemin A.

resolution fast-atom-bombardment MS (HRFABMS) in conjunction with ^{1}H and ^{13}C NMR data. The structure was determined by two dimentional NMR experiments, including correlation spectroscopy (COSY), distortionless enhancement by polarization transfer (DEPT), heteronuclear single quantum correlation (HSQC), hetero nuclear multiple bond correlation spectroscopy (HMBC) and nuclear Overhauser effect spectroscopy (NOESY) as reported by us recently [16]. Additional physical and spectroscopic data for Collemin A is reported here for the first time: IR (KBr Pellets): $\nu = 3382$ cm^{-1} (OH), 2936 cm^{-1} (NH_2), 1653 cm^{-1} (CO). UV-VIS (MeOH): λ_{max} (ε) = 311 nm (34 000 M^{-1}·cm^{-1}, Fig. 1); MS: 497 [MH^+], 340 [M-$C_7O_3NH_{10}$], 435 [M-O-CH_2-CH_2OH], 519 [M-Na]$^+$. UV spectra of lichen and mycobiont have the same absorption maximum, at 311 nm (Fig. 2). According to this, a molar extinction coefficient (ε) of 34 000 M^{-1}·cm^{-1} was determined. The high ε-value gives the isolated compound a huge advantage over common commercial sunscreens by having a great potential to protect from UV-B radiation. Collemin A is a new mycosporine, which incorporates a novel pyrrolidine ring. The UV-B absorbance ability of this compound is probably of fungal origin. Isolation of the dominant fungus (one out of four) from the lichen, gave a UV spectrum with a similar λ_{max} when compared to the lichen itself (λ_{max} = 311 nm).

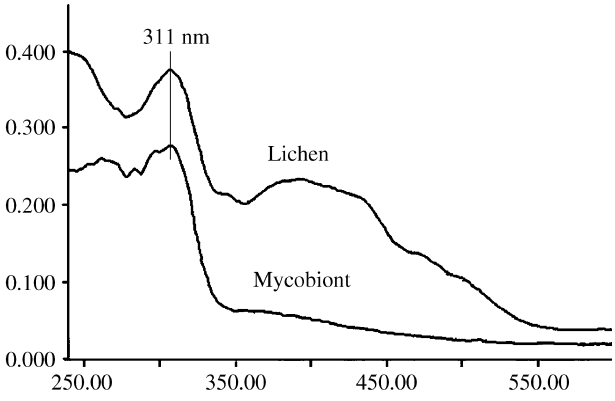

Fig. 2. Comparative UV spectra of the fungus (mycobiont) and lichen *Collema cristatum*. The mycobiont has a maximum at 311 nm.

Biological activity of Collemin A

To test for photo protective activity, cell survival as estimated by the trypan blue exclusion assay was used as a screening test. This test is simple to perform and is essentially a measure of UV-B induced membrane destruction. As can be seen in Fig. 3, the pure compound provided protection against UV-B damage in a dose-related manner.

DNA is the primary cutaneous target of UV radiation. Upon exposure of DNA to wavelengths approaching its absorbance maximum, pyrimidine dimers are formed by covalent interaction of two adjacent pyrimidines in the same polynucleotide chain [17]. To study the ability of the isolated compound to prevent pyrimidine dimer formation, HaCaT keratinocytes were irradiated through a quartz plate on which the compound was spread, cells were harvested immediately following irradiation and analysed for pyrimidine dimer formation. Non-irradiated cells and cells irradiated through a naked quartz plate served as controls. As can be seen in Fig. 4, the number of pyrimidine dimers in cells irradiated through the isolated compound were much fewer than were the numbers found in irradiated nonprotected cells, in fact approaching the numbers in nonirradiated cultures.

The *in vivo* biological activity of the isolated compound was assayed by the ability to prevent UV induced erythema (suburn) of human skin. The pure compound was applied to the inside forearm of a volunteer 30 min prior to four MED of UV-B irradiation (Fig. 5). A commercial sunscreen

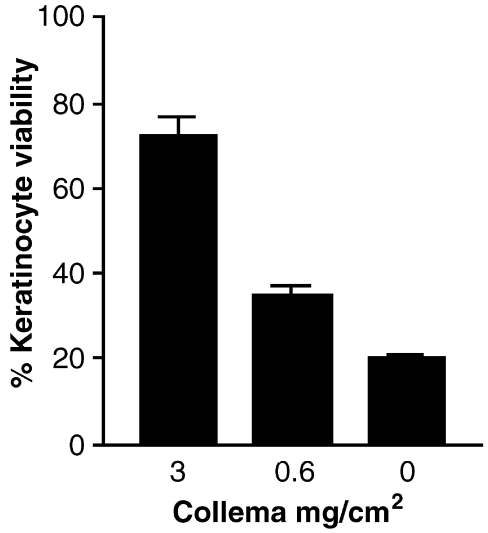

Fig. 3. Effect of the pure compound isolated from *Collema cristatum* on keratinocyte survival after UV-B exposure. HaCaT cells were irradiated with UV-B (200 mJ·cm^{-2} delivered at an irradiance of 3.4 mW·s^{-1}) through a quartz plate on which the pure compound was placed. Cell survival was estimated by trypan blue exclusion. The *Collema* compound provided photoprotection in a dose-dependent manner.

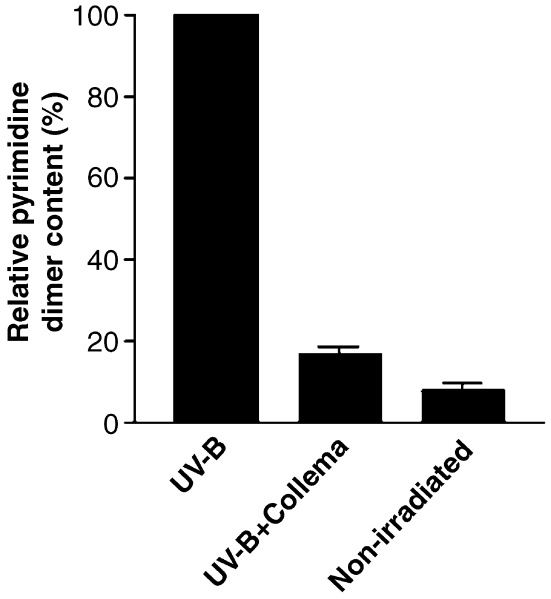

Fig. 4. Prevention of UV-B induced pyrimidine dimer formation by the *Collema* compound. HaCaT cells were irradiated with UV-B (60 mJ·cm^{-2} delivered at an irradiance of 3.4 mW·s^{-1}) through a quartz plate on which the pure compound was placed at a concentration of 6 μg·cm^{-2}. Nonirradiated cells and cells irradiated through a naked quartz plate served as controls. Pyrimidine dimers were measured immediately following UV-B irradiation by the ELISA technique. The pyrimidine dimer content is presented as percentages of the content in unprotected UV-B radiated cells.

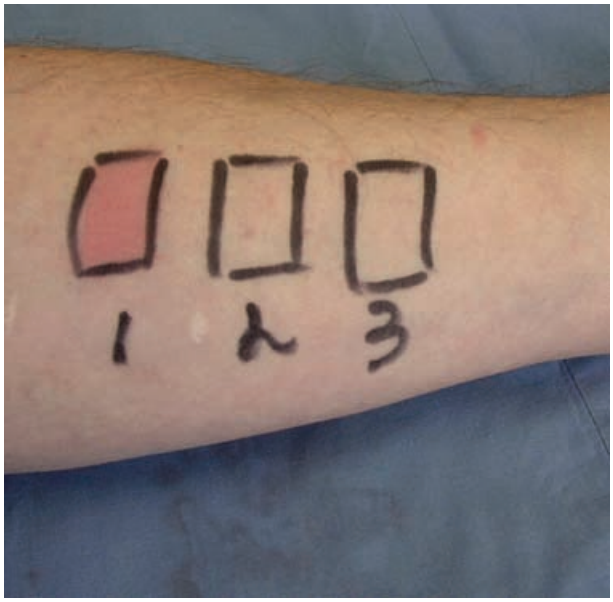

Fig. 5. Prevention of UV-B induced erythema on human skin. The pure compound was diluted 1 : 10 in olive oil and applied to the inside forearm of a volunteer at a concentration of 6 μg·cm^{-2}. Fifteen minutes after application, four MEDs (360 mJ·cm^{-2}) of UV-B irradiation were delivered to the treated areas and the resulting erythema was evaluated after 24 h. A commercial octylmethoxycinnamate containing sunscreen (SPF 12) served as a control. 1, No protection (olive oil); 2, commercial sunscreen; 3, pure compound.

containing octylmethoxycinnamate with a sun protecting factor (SPF) of 12 served as control. The isolated compound totally prevented UV-B induced erythema.

Sunburn and DNA damage are the results of excessive UV-B exposure and are considered central events in the initiation and promotion of skin cancer [18]. The biological significance of such DNA lesions depends on the capacity of the cell to repair the damage before it can be incorporated permanently into the genome. Typically, DNA damage is repaired at a relative high rate in human cells by various repair mechanisms including photoreactivation, nucleotide excision and global repair [19,20]. The ability of the isolated compound to block pyrimidine dimer formation points to a potential role for such compounds in the prevention of UV-induced skin cancer.

The defence mechanisms developed by ancient photosynthetic organisms such as lichens, fungi, cyanobacteria, corals and other marine organisms are much more advanced than those of mammals because photosynthetic organisms depend on solar irradiation as their primary source of energy, and at the same time must provide a mechanism that can counteract the damaging effects of UV-B irradiation on proteins, DNA and other biologically active molecules. These photosynthetic organisms have developed biosynthetic pathways for the synthesis of UV absorbing compounds that enable them to survive direct and intense UV radiation [1,11,21]. Such compounds include scytonemin, mycosporine and MAAs.

Scytonemin is lipid soluble and has a prominent absorption maximum in the near ultraviolet region of the UV-A spectrum with a long tail extending into the infrared region. The two other UV absorbing compounds contain one of two different ring units: an aminocyclohexenone or an aminocyclohexenimine. Mycosporines are fungal metabolites with UV absorption at 310–320 nm, and can be considered to be Schiff bases (enamino ketones), which possess a common cyclohexenone ring system linked by an amino acid or an amino-alcohol [11]. In contrast, MAAs are UV absorbing metabolites of algae that contain an aminocyclohexenimine ring system, with UV absorption maxima between 310 and 360 nm. To date, 17 different aminocyclohexenimines and 15 aminocyclohexenones have been identified from marine and terrestrial organisms [11].

The occurrence of high concentrations of mycosporines and MAAs in organisms exposed to intense solar radiation has been described to provide protection as a UV-absorbing/screening compound [22–24]. They have been reported to prevent three out of ten photons from hitting cytoplasmic targets in cyanobacteria. Cells with high concentrations of MAAs are approximately 25% more resistant to UV radiation centered at 320 nm than those with no or low concentrations of MAAs [25]. MAAs have been shown to protect against UV-B induced damage of motility and swimming velocity in a dinoflagellate *Gyrodinium dorsum* [26]. Similarly, MAAs have been reported to protect against photo induced inhibition of photosynthesis in another dinoflagellate, *Gymnodinium sanguineum* [27]. It is evident from the present investigation that the studied cyanobacteria are able to increase their MAA content in response to UV-B radiation and thus may be able to adapt to daily fluctuations in solar radiation impinging on their natural environment. Action spectra for the induction of

MAAs in lichen's cyanobacteria also support this hypothesis.

In summary, we have described the structure and the photo protective qualities of a new mycosporine, Collemin A, isolated from the lichenized ascomycete, *Collema cristatum*.

References

1. Torres, A., Dor, I., Rotem, J., Srebnik, M. & Dembitsky, V.M. (2003) Characterization of surface *n*-alkanes and fatty acids of the epiphytic lichen *Xanthoria parietina*, its photobiont a green alga *Trebuxia* sp. and its mycobiont, from the Jerusalem hills. *Eur. J. Biochem.* **270**, 2120–2125.
2. Dembitsky, V.M. (1992) Lipids of lichens. *Prog. Lipid Res.* **31**, 373–397.
3. Reference withdrawn.
4. Muller, K. (2001) Pharmaceutically relevant metabolites from lichens. *Appl. Microbiol. Biotechnol.* **56**, 9–16.
5. Setlow, R.B. (2002) Shedding light on proteins, nucleic acids, cells, humans and fish. *Mutat. Res.* **511**, 1–14.
6. Berneburg, M. & Krutmann, J. (2000) Photoimmunology, DNA repair and photocarcinogenesis. *J. Photochem. Photobiol. B, Biol.* **54**, 87–93.
7. Slaper, H., Velders, G.J.M. & Matthijsen, J. (1998) Ozone depletion and skin cancer incidence: a source risk approach. *J. Hazard. Mater.* **61**, 77–84.
8. Wang, R.-F., Zeng, G., Johnston, S.F., Voo, K. & Ying, H. (2002) Cell-mediated immune responses in melanoma: implications for immunotherapy. *Crit. Rev. Oncol. Hematol.* **43**, 1–11.
9. Kawada, M., Kawatsu, M., Masuda, T., Ohba, S., Amemiya, M., Kohama, T., Ishizuka, M. & Takeuchi, T. (2003) Specific inhibitors of protein phosphatase 2A inhibit tumor metastasis through augmentation of natural killer cells. *Int. Immunopharmacol.* **3**, 179–188.
10. Das, P.K., van den Wijngaard, R.M.J.G.J., Wankowicz-Kalinska, A. & Le Poole, I.C. (2001) A symbiotic concept of autoimmunity and tumour immunity: lessons from vitiligo. *Trends Immunol.* **22**, 130–136.
11. Bandaranayake, W.M. (1998) Mycosporines: are they nature's sunscreens? *Nat. Prod. Report* **15**, 159–172.
12. De Nys, R. & Steinberg, P.D. (2002) Linking marine biology and biotechnology. *Curr. Opin. Biotechnol.* **13**, 244–248.
13. Sinha, R.P., Ambasht, N.K., Sinha, J.P., Klisch, M. & Häder, D.-P. (2003) UV-B-induced synthesis of mycosporine-like amino acids in three strains of *Nodularia* (cyanobacteria). *J. Photochem. Photobiol. B, Biol.* **71**, 51–58.
14. Mori, T., Nakane, M., Hattori, T., Matsunagam, T., Ihara, M. & Nikaido, O. (1993) Simultaneous establishment of monoclonal-antibodies specific for either cyclobutane pyrimidine dimer or (6–4) photoproduct from the same mouse immunized with Ultraviolet-irradiated DNA. *Photochem. Photobiol.* **54**, 225–232.
15. Enk, D.C., Hochberg, M., Torres, A., Dembitsky, V.M. & Srebnik, M. (2004) Photoprotection by *Cichorium endivia* extracts: prevention of UVB induced erythema, pyrimidine dimer formation and IL-6 expression. *Skin Pharmacol. Appl. Skin Physiol.* **17**, 42–48.
16. Enk, D.C., Hochberg, M., Torres, A., Lev, O., Dor, I., Dembitsky, V.M. & Srebnik, M. (2002) Natural UV Filters Derived from Pigments of Lichens. Patent Application IL02/00725.
17. Han, M.J. & Chang, J.Y. (2000) Polynucleotide analogues. *Adv. Polymer Sci.* **153**, 1–36.
18. Kaina, B. (2003) DNA damage-triggered apoptosis: critical role of DNA repair, double-strand breaks, cell proliferation and signaling. *Biochem. Pharmacol.* **66**, 1547–1554.
19. Dlugosz, A., Merlino, G. & Yuspa, S.H. (2002) Progress in cutaneous cancer research. *J. Investig. Dermatol. Symp. Proc.* **7**, 17–26.
20. Sancar, A. (1994) Mechanisms of DNA excision repair. *Science* **266**, 1954–1956.
21. Budel, 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.
22. Garcia-Pichel, F. & Castenholz, R.W. (1993) Occurrence of UV-absorbing, mycosporine-like compounds among cyanobacterial isolates and an estimate of their screening capacity. *Appl. Environ. Microbiol.* **59**, 163–169.
23. Ehling-Schulz, M. & Scherer, S. (1999) UV protection in cyanobacteria. *Eur. J. Phycol.* **34**, 329–338.
24. Sinha, R.P., Klisch, M., Helbling, E.W. & Häder, D.-P. (2001) Induction of mycosporine-like amino acids (MAAs) in cyanobacteria by solar ultraviolet radiation. *J. Photochem. Photobiol. B, Biol.* **60**, 129–135.
25. Garcia-Pichel, F., Wingard, C.W. & Castenholz, R.W. (1993) Evidence regarding the UV sunscreen role of a mycosporine-like compound in the cyanobacterium *Gloeocapsa* sp. *Appl. Environ. Microbiol.* **59**, 170–176.
26. Klisch, M., Sinha, R.P., Richter, P.R. & Häder, D.-P. (2001) Mycosporine-like amino acids (MAAs) protect against UV-B induced damage in *Gyrodinium dorsum* Kofoid. *J. Plant Physiol.* **158**, 1449–1454.
27. Neale, P.J., Banaszak, A.T. & Jarriel, C.R. (1998) Ultraviolet sunscreens in *Gymnodinium sanguineum* (Dinophyceae): mycosporine-like amino acids protect against inhibition of photosynthesis. *J. Phycol.* **34**, 928–938.