# PRODUCTION OF SUNSCREEN MYCOSPORINE-LIKE AMINO ACIDS (MAAs) FROM EGYPTIAN ISOLATE OF *NOSTOC COMMUNE* USING UV RADIATION

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*Abstract.* Continuous depletion of the stratospheric ozone layer, mainly due to anthropogenically released atmospheric pollutants such as chlorofluoro carbons (CFCs) has resulted in an increase in Ultraviolet-B (UV-B; 280–315 nm) radiation on the Earth's surface which inhibits photochemical and photobiological processes in cyanobacteria. However, these organisms have developed several lines of defense mechanisms such as screening to counteract the damaging effects of UVR. The cyanobacterium used in this study was *Nostoc commune*. It was isolated from Egyptian rice fields. The organisms were grown in 1 L glass flasks containing 500 mL of BG11 medium. A locally manufactured ultraviolet source was used for exposure experiments. It consisted of mercury (Hg) arc lamp (HBO, Philips, 200 W/2) with a continuous spectrum of wavelengths from 220 nm to 900 nm. A coloured cut-off glass filter was used [WG280 nm (2mm thick), with a dimension of 100 mm × 100 mm]. MAAs were extracted using 100% HPLC grade methanol and separated through high-performance liquid chromatography. Absorption spectroscopic analyses of the methanolic extracts of samples revealed a typical MAA peak at 335 nm in *Nostoc commune*. The high performance liquid chromatographic (HPLC) analysis of water-soluble compounds revealed the biosynthesis of only one type of MAA, shinorine (retention time = 2.25 min and absorption maximum at 334 nm). MAA content was highly increased by UV exposure. 16 h exposure of UV-light induced the largest amount of MAA for a sample covered with WG280 nm cut-off filter as compared to UV non-exposed sample as control. Liquid chromatography/mass spectrometry (LC/MS) analysis of MAA reveals the molecular weight of shinorine (m/z = 333.1 Da). *Nostoc commune* was able to synthesize MAA in response to UV radiation. It was found to possess a very effective mechanism for adapting to deleterious doses of UV-B radiation. MAA formation was photo-induced and the final MAA concentration was controlled by irradiance and duration of exposure. The present strain could act as a model organism for studying the biosynthetic route of MAAs in cyanobacteria and could be used for the industrial production of MAAs.

*Keywords*: high performance liquid chromatography (HPLC), liquid chromatography/mass spectrometry (LC/MS), mycosporine-like amino acids (MAAs), *Nostoc commune*, ultraviolet radiation (UVR).

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## INTRODUCTION

Light is one of the most important factors determining the growth of photosynthetic organisms in their natural habitats. Since, biologically effective doses of UV-B radiation can penetrate deep into ecologically significant depths in natural waters, the observed increases in surface UV-B radiation may adversely affect the productivity of aquatic organisms. UV-B has the potential to cause a wide range of effects, including alteration in the structure of proteins, DNA and other biologically relevant molecules, chronic depression of key physiological processes and acute physiological stress leading to either reduction in growth and cell division rates or death of the organism. Studies have shown a wide variation in tolerance to UV-B among species and taxonomic groups [18].

The anthropogenically released atmospheric pollutants, such as chlorofluorocarbons (CFCs), chlorocarbons (CCs), and organobromides (OBs), have resulted in the continued depletion of the stratospheric ozone layer and subsequent increase in the ultraviolet radiation (UVR; 280–400 nm) impinging onto the Earth's surface [13].

Cyanobacteria (also known as blue green algae) are a morphologically diverse group of photosynthetic gram-negative prokaryotes, that were the dominant form of life on earth for more than 1.5 billion years. These organisms have the ability to live in extreme conditions such as habitats with high temperatures and salinities. All cyanobacteria are photoautotrophic organisms, yet many can grow heterotrophically, using light for energy and organic compounds as a carbon source [22].

Recently, cyanobacteria have become an attractive source of several innovative classes of secondary metabolites that can be used in agriculture, industry, and especially in pharmaceuticals and biomedical research [10]. Many cyanobacteria can fix atmospheric $N_2$ into ammonia ($NH_3$) in the presence of nitrogenase enzyme and produce $H_2$ as a byproduct that can be used as a renewable and environmental friendly source of energy carrier [1].

In spite of adverse effects of solar UVR, cyanobacteria are not defenseless and have developed various strategies, such as the formation of antioxidants or efficient DNA repair mechanisms to counteract the damaging effects of UVR. A different but not less interesting property of these microorganisms is their capacity of overcoming the toxicity of UVR by means of UV-absorbing/screening compounds, such as MAAs and scytonemin [11].

In the 1970s various substances were isolated and characterized which had a maximum absorption in the UV range. These substances were related to the mycosporines found in terrestrial fungi [5] and named mycosporine-like amino acids (MAAs).

MAAs are small secondary metabolites produced by organisms that live in environments with high volumes of sunlight, usually marine environments. So far there are up to 20 known MAAs identified. MAAs are water soluble substances with 310–362 nm absorption maxima having a molecular weight of between 244 and 334 g $mol^{-1}$ and a high molar absorptivity ($\varepsilon$ = 28100–50000 $mol^{-1}$ $cm^{-1}$) [7].

MAAs are composed of cyclohexenone or cyclohexenimine chromophores conjugated with the nitrogen substituent of an amino acid or its imino alcohol. The absorption characteristic of these molecules is dependent on a system of double bonds in the core ring structure, which is altered through the conjugated amines. Monosubstituted MAAs (oxo MAAs; *e.g.* mycosporine glycine) with a cyclohexenone core have their absorption maximum in the UV-B, while bisubstituted MAAs (imino MAAs; *e.g*, shinorine) with a cyclohexenine core have their absorption maximum in the UV-A (Table 1) [12].

*Table 1*

MAAs found in cyanobacteria with their corresponding absorption maxima, extinction coefficient and molecular structure [19]

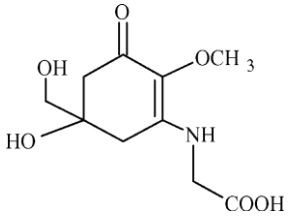
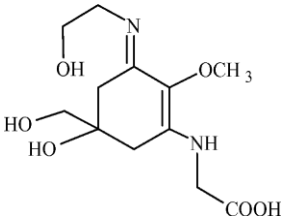
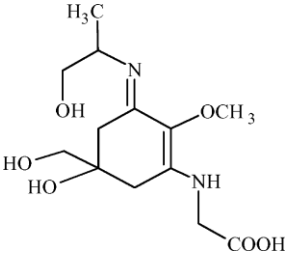
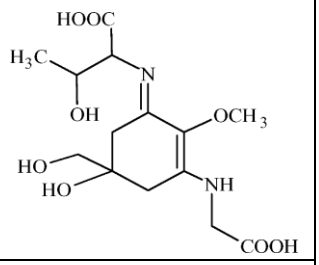
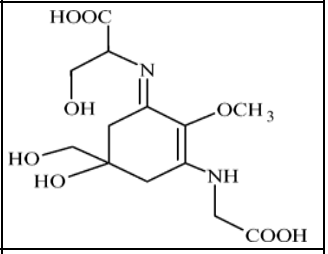
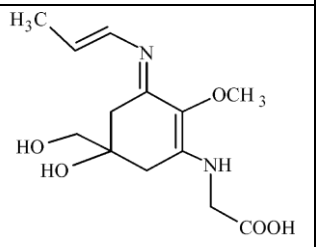
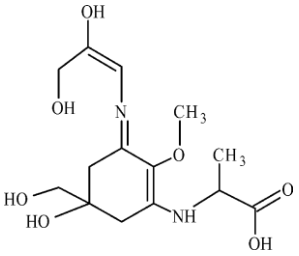
| **MAAs** | **$\lambda_{max}$ (nm)** | **$\varepsilon$ ($M^{-1}$ $cm^{-1}$)** | **Molecular structure** |
|---|---|---|---|
| Mycosporine-glycine | 310 | 28100 |  |
| Asterina-330 | 330 | 43500 |  |
| Palythinol | 332 | 43500 |  |

*Table 1*

(continued)

| MAAs | $\lambda_{max}$ (nm) | ε ($M^{-1}$ $cm^{-1}$) | Molecular structure |
|---|---|---|---|
| Porphyra-334 | 334 | 42300 |  |
| Shinorine | 334 | 44700 |  |
| Palythene | 360 | 50000 |  |
| Euhalothece-362 | 362 | – |  |

MAAs protect the cells by absorbing highly energetic UVR and then dissipating this energy in the form of harmless heat radiation to their surroundings. MAAs can also act as strong antioxidants to avoid the damaging effects of UVR [3].

Sunscreen (also commonly known as sun block or sun cream) is a lotion, spray, gel or other topical product that absorbs or reflects some of the sun's UV radiation on the skin exposed to sunlight. Sunscreens used for the protection of human skin against the harmful effects of solar radiation must contain certain amounts of UV-absorbing substances [15]. Those can be organic UV-absorbing molecules such as, cinnamic acid derivatives, benzophenones, triazines, benzotriazoles, p-aminobenzoic acid esters, or methoxy dibenzoylmethane derivatives [8]. On the other hand, inorganic particulate UV filters that reflect, scatter, and absorb UV light such as titanium dioxide and zinc oxide are also used in sunscreens [23]. For use in sunscreen formulations, titanium dioxide particles are coated, *e.g.*, with aluminum oxide in order to avoid photocatalysis. Recently, another concept of filters for sunscreen formulations was introduced by employing organic particulate UV absorbers [8].

The aim of the present study was to screen the mycosporine-like amino acids (MAAs) from cyanobacterial strain (*Nostoc commune*), observe their synthesis under various stress conditions and characterize their different types using spectrophotometric and chromatographic techniques.

## MATERIALS AND METHODS

### ORGANISM AND CULTURE CONDITIONS

The nitrogen-fixing cyanobacterium, *Nostoc commune*, was isolated from Egyptian rice fields. Cultures were grown in 1L glass flasks containing 500 mL of BG11 medium [14] at a temperature of 20 ± 2 °C and continuous white fluorescent light of 12 ± 2 $Wm^{-2}$. The organism was grown for a period of 6–12 days.

### EXPOSURE OF *NOSTOC COMMUNE* CELLS TO SIMULATED SOLAR RADIATION

The studied samples were divided to exposed samples and UV non-exposed sample as control each containing 50–60 mL. The culture (50–60 mL volume) was exposed to artificial UV radiation in open Petri dish glass (100 mm in diameter) that was covered on top with WG280 nm cut-off filter and placed in a temperature-control (35 $^{o}$C) at intensity adjusted to 0.9 $Wm^{-2}$. The WG280 nm cut-off filter served to eliminate the UV-C irradiation. Open glass Petri dish containing culture of *Nostoc commune* was placed on a rotary shaker (magnetic stirrer) to ensure

uniform exposure (avoid self-shading). *Nostoc* cells were exposed for different times (4, 8 and 16 h) under WG280 nm cut-off filter.

### EXTRACTION AND PARTIAL PURIFICATION OF MAAs

Cells were harvested (10–20 mL) by centrifugation at 4000 rpm for 10 min and MAAs extracted in 4 mL of 100% HPLC grade methanol overnight at 4 °C in a refrigerator. After extraction aliquots were centrifuged at 6000 rpm for 10 min (to separate the cells from the supernatant) and supernatants were subjected to the spectroscopic analysis between 300 and 700 nm in a double beam spectrophotometer JASCO V-630 (JASCO, Japan). After scanning the methanolic extracts (to determine the MAAs content), the supernatants were evaporated to dryness at 45 °C in a rotary evaporator and the extracts were re-dissolved in 1 mL double distilled water. A few drops of chloroform were added to this solution and mixed using a vortex shaker. The water phase was transferred carefully after centrifugation into new Eppendorf tubes to remove contaminant photosynthetic pigments. Finally, the samples were filtered through 0.2 μm pore-sized micro-centrifuge filters for partial purification and subsequently subjected to the HPLC analysis [16].

### HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS OF MAAs

Further analysis and purification of partially purified MAAs was performed using an HPLC system Agilent 1100 series (Agilent, Germany) equipped with a Licrospher RP 18 column and guard (5 μm packing; 250 × 4 mm I.D.). The samples (50 μl) were injected into the HPLC column through a Waters 717 plus auto-sampler. The wavelength for detection of MAAs was 330 nm. The mobile phase was 0.02% acetic acid (v/v) in double distilled water, run isocratically at a flow rate of 1.0 mL/min [16].

## RESULTS

The effect of UV radiation on cyanobacterial strain (*Nostoc commune*) was studied using WG280 nm cut-off filter for different time durations (4, 8 and 16 h) at an intensity of 0.9 $Wm^{-2}$. Control samples were not subject to any UV radiation. All samples were temperature-controlled at 35 $^{o}$C.

In order to study all of the components extracted from *Nostoc commune*, we performed UV-VIS absorption spectroscopy on the samples covered with WG280 nm cut-off filter at (4, 8 and 16 h) of exposure and for the control samples as well. The control consists in samples for each time of exposure, grown in the same condition, but non-exposed sample to UV radiation. Figure 1 shows that the plots of absorbance *versus* wavelength in the range between 300–700 nm for the absorption spectra of the methanolic extracts of cyanobacteria (*Nostoc commune*) reveal five major peaks at 335 (MAA-UV-absorbing compounds), 436 and 666 (chlorophyll a), 474 (carotenoids) and 618 (phycobiliproteins) nm.

The broad UV-absorption peak between 310 and 360 nm with a maximum at 335 is typical for MAA (310–362 nm). The induction of MAA using WG280 nm cut-off filter is evident to gradually increase compared to control with exposure time from 4 h to 16 h (Fig. 1).

MAAs extracted from *Nostoc commune* was purified using HPLC and identified according to their retention times and absorption spectra. HPLC analyses of the samples were performed to find out whether the absorbing compound in the UV range is a single MAA or a mixture of more than one MAAs.

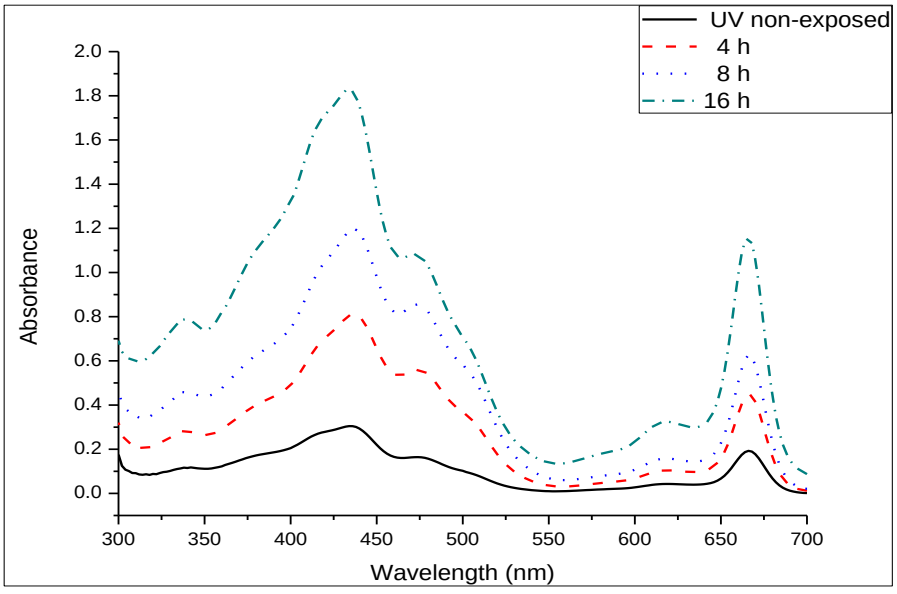



Fig. 1. Absorption spectra of methanolic extract of *Nostoc commune* culture after different durations of exposure to artificial UV radiation in combination with WG280 cut-off filter. Five main absorption peaks can be identified at: 335 nm (MAA), 436 nm (Chl a), 474 nm (carotenoids), 618 nm (phycocyanin) and 666 nm (Chl a).

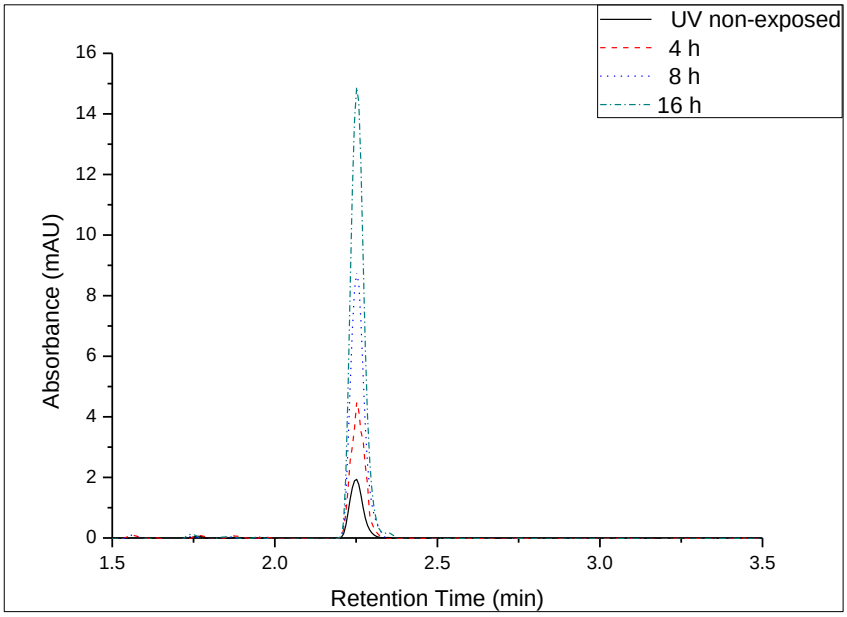



Fig. 2. HPLC chromatogram shows variation of absorbance of MAA (at retention time = 2.25 min) produced from *Nostoc commune* (WG280 nm cut-off filter) at different exposure periods compared to control sample.

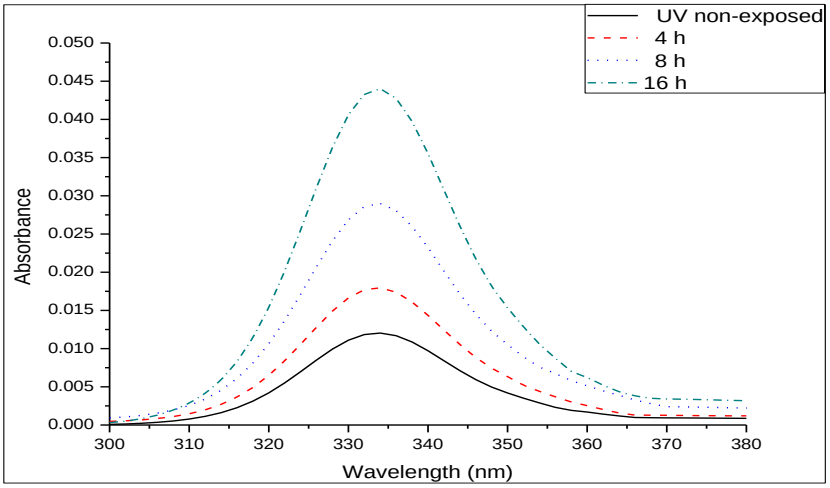



Fig. 3. Absorption spectra of *Nostoc commune* after different durations of exposure with artificial UV radiation in combination with WG280 nm cut-off filter.

HPLC chromatograms of the samples revealed the existence of a single MAA (retention time 2.25 min) in *Nostoc commune* (Fig. 2). This compound was found to be considerably induced in the samples which were covered by WG280 nm cut-off filter indicating that UV radiation plays an important role in the induction process of MAA in *Nostoc commune*.

After purification of samples using HPLC technique, absorption spectroscopic analyses of the HPLC samples revealed that the MAA in *Nostoc commune* had an absorption maximum at 334 nm. The absorption spectra of *Nostoc commune* following different durations of exposure under simulated solar radiation in combination with cut-off filter of WG280 nm are presented in Figure 3.

After 16 h of exposure under the solar lamp, the cells, which were covered by a WG280 nm cut-off filter, had produced larger concentration of MAA (according to peak area quantified from spectroscopic analyses) in comparison with UV non-exposed sample (Fig. 3).

Based on the retention time and $\lambda_{max}$, the MAA extracted from *Nostoc commune* was identified as shinorine ($\lambda_{max}$ = 334 nm).

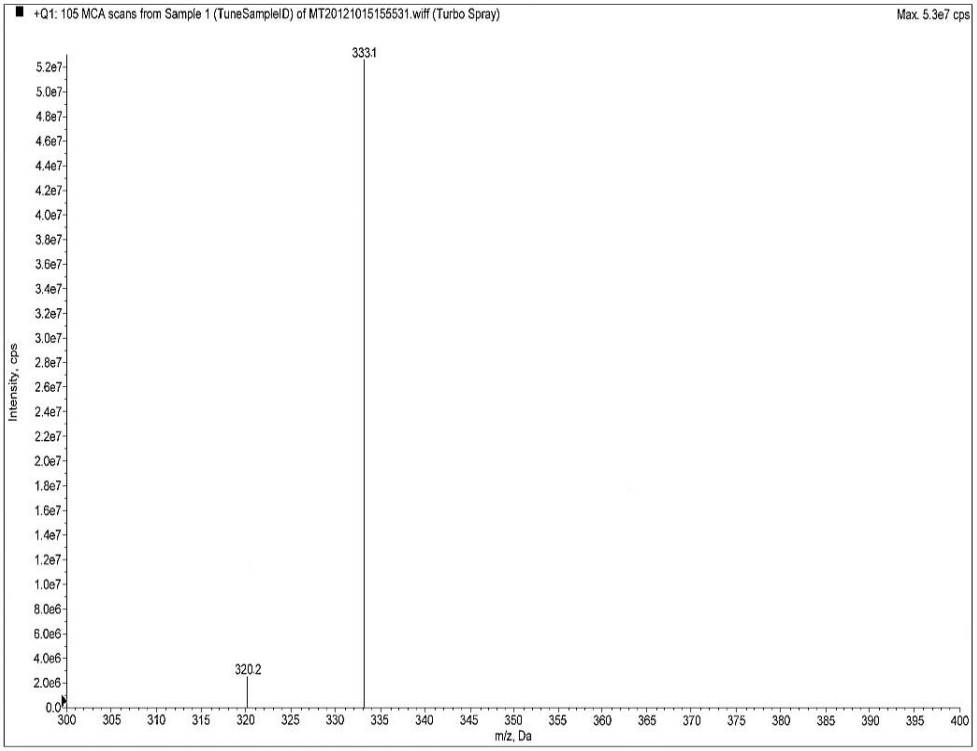



Fig. 4. Mass spectrometric analysis of MAA after 16 h of exposure using WG280nm cut-off filter reveals m/z = 333.1 Da.

Liquid Chromatography/Mass Spectrometry (LC/MS) was used to check the identification of the isolated MAA as well as to obtain the mass to charge ratio of the protonated molecule. Mass spectrometric analysis was performed by LC/MS from 300 Da to 400 Da, with ion extractions at a specific m/z in the positive ion mode.

The output of the mass spectrometer from Figure 4 shows a plot of relative intensity *versus* the mass-to-charge ratio (m/z). The most intense peak in the spectrum is termed the base peak and the other is reported relative to its intensity. The peaks themselves are typically very sharp, and are often simply represented as vertical lines.

The HPLC and LC/MS analysis of MAA revealed a molecular weight of shinorine (m/z = 333.1 Da).

## DISCUSSION

The main aim of this study was to investigate the ability of this Egyptian isolate of cyanobacterium (*Nostoc commune*) to produce UV-screening MAAs that might be helpful to perform ecological functions in harsh conditions by absorbing the lethal doses of UV-A and UV-B radiations.

Irradiation of *Nostoc* cultures to different doses of UV radiation at different wavelengths (λ) using cut-off filter showed the presence of water soluble, UV-absorbing substance (MAA). Light had been found to be essential for MAA synthesis. The presence of high concentrations of MAAs in cells is supposed to provide protection by absorbing lethal doses of UV radiation [20]. In this study it was found that *Nostoc commune* synthesized a single MAA, i.e., shinorine, which can be induced by photosynthetic active radiation (PAR) and UVR.

Nowadays MAAs are getting much consideration as these compounds are not only present in cyanobacteria, but they have also been reported in macroalgae, phytoplankton and various animals [21]. UV radiation causes oxidative damage in cyanobacterial cells, including lipid peroxide formation, DNA strand breaks, and chlorophyll bleaching concomitant with deactivation of photosynthesis and growth inhibition. MAAs are suggested to have a protective role against UV induced oxidative stress in algae and cyanobacteria [6].

Portwich and Garcia-Pichel have reported that MAA synthesis can be induced by salt stress without PAR or UV radiation [9]. Therefore, they distinguish between salt-dependent biochemical and light-dependent photosensory induction of MAA synthesis.

Earlier studies showed that cyanobacteria increase their MAA content in response to UV radiation and were able to adapt to a changing daily solar radiation in their natural habitat [17]. The induction of MAA (shinorine) by UVB radiation in our experiment clearly indicated that this organism had a strong protective

mechanism against UVR that it faced in its habitats. The presence of MAA might help *Nostoc commune* to cope with oxidative stress under intense solar radiation.

The exact location of MAAs is not known in most cyanobacteria, but in *Nostoc commune* it is extracellular and linked to oligosaccharides in the sheath [2]. These glycosylated MAAs are the only known example of MAAs that are actively excreted and accumulated extracellularly and, therefore, act as true screening compounds [4].

Based on the presented results, we can conclude that the Egyptian isolate of *Nostoc commune* produced MAA (shinorine, 333.1 Da, $\lambda_{max}$ = 334 nm) under UV stress. The present strain can act as a model organism for studying the biosynthetic route of MAA in cyanobacteria and could also be used in industrial and medical applications.

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