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journal or publication title	Journal of Photochemistry and Photobiology B: Biology
volume	142
page range	154-168
year	2015-01-01
URL	http://hdl.handle.net/2297/40610

doi: 10.1016/j.jphotobiol.2014.12.008

**Characterization of the chemical diversity of glycosylated mycosporine-like amino acids
in the terrestrial cyanobacterium *Nostoc commune***

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Highlights:

- Glycosylated MAAs have been characterized in two genotypes of *Nostoc commune*.
- The A genotype produces β -Ara-porphyrin-334 (478 Da) with $\lambda_{\max}$ at 335 nm.
- The B genotype produces the hybrid MAA (1050 Da) with $\lambda_{\max}$ at 312 nm.

Abstract

Mycosporine-like amino acids (MAAs) are UV-absorbing pigments, and structurally unique glycosylated MAAs are found in the terrestrial cyanobacterium *Nostoc commune*. In this study, we examined two genotypes of *Nostoc commune* colonies with different water extract UV-absorption spectra. We found structurally distinct MAAs in each genotype. The water extract from genotype A showed a UV-absorbing spectrum with an absorption maximum at 335 nm. The extract contained the following compounds: 7-*O*-(β -arabinopyranosyl)-porphyrin-334 (478 Da), pentose-bound shinorine (464 Da), hexose-bound porphyrin-334 (508 Da) and porphyrin-334 (346 Da). The water extract from genotype B showed a characteristic UV-absorbing spectrum with double absorption maxima at 312 and 340 nm. The extract contained hybrid MAAs (1050 Da and 880 Da) with two distinct chromophores of 3-aminocyclohexen-1-one and 1,3-diaminocyclohexen linked to 2-*O*-(β -xylopyranosyl)- β -galactopyranoside. A novel 273-Da MAA with an absorption maximum at 310 nm was also identified in genotype B. The MAA consisted of a 3-aminocyclohexen-1-one linked to a γ -aminobutyric acid chain. These MAAs had potent radical scavenging activities *in vitro* and confirmed that the MAAs have multiple roles as a UV protectant and an antioxidant relevant to anhydrobiosis in *N. commune*. The two genotypes of *N. commune* exclusively produced their own characteristic glycosylated MAAs, which supports that MAA composition could be a chemotaxonomic marker for the classification of *N. commune*.

Keywords: anhydrobiosis; antioxidant; chemotaxonomic marker; *Nostoc commune*; UV protectant

Nostoc commune

Genotype A

M : 478
 λ_{max} : 335 nm
 ϵ : 33173 M⁻¹ cm⁻¹

Genotype B

M : 1050
 λ_{max} : 312 nm
 ϵ : 58800 M⁻¹ cm⁻¹

1. Introduction

The cyanobacterium *Nostoc commune* adapts to terrestrial environmental conditions and has a cosmopolitan distribution on the Earth [1]. In its natural habitats, *N. commune* forms visually conspicuous colonies that consist of extracellular matrix with filamentous cells embedded inside [1, 2]. *N. commune* colonies are subjected to frequent cycles of desiccation and wetting. The desiccated colonies show little to no metabolic activity, yet they retain the ability to grow for more than 100 years [3, 4]. Upon rehydration, *N. commune* cells rapidly recover respiration and photosynthesis [5-8]. This phenomenon is termed anhydrobiosis [9-12]. *N. commune* is a prokaryotic model anhydrobiote that retains oxygenic photosynthetic capabilities in vegetative cells and does not differentiate into akinetes (spores) [1, 12]. In addition to extreme desiccation tolerance, *N. commune* colonies are exposed to direct solar radiation and can tolerate UV radiation stress [13, 14]. The desiccated *N. commune* has extreme longevity [3, 4]; antioxidants protect biomolecules from oxidation and are thought to be involved in the mechanisms of anhydrobiosis in *N. commune* [15]. *N. commune* has two types of UV-absorbing pigments which are scytonemin and mycosporine-like amino acids (MAAs) [14, 16]. Both sunscreen pigments display radical scavenging activities [17-19]. Thus, these secondary metabolites are thought to play multiple roles against environmental stresses such as UV radiation and desiccation through their photoprotective and antioxidative properties [15, 20].

Mycosporine-like amino acids (MAAs) are water-soluble pigments that absorb UV radiation of 280 to 340 nm. There are structurally distinct MAAs found in taxonomically diverse organisms [21-27]. In cyanobacteria, MAAs protect the cells against solar radiation [28, 29]. The energy absorbed by MAAs is promptly dispersed into the surroundings as heat [30, 31]. Thus, MAAs do not function as accessory pigments in photosynthesis [23]. In *N. commune*, a structurally unique 1050-Da MAA with two distinct chromophores of 3-

aminocyclohexen-1-one and 1,3-diaminocyclohexen was found [17]. There is also a pentose-bound porphyrin-334 derivative (478 Da) with an absorption maximum at 335 nm [17]. We recently found the third type of *N. commune* colony with a water extract that shows a characteristic UV-absorbing spectrum with an absorption maximum at 325 nm. We identified novel glycosylated MAAs including two hexose-bound palythine-threonine derivatives with a molecular mass of 612 Da in this particular type of *N. commune* [19]. The glycosylated MAAs in *N. commune* are thought to be localized in the extracellular matrix to allow interaction with other constituents in its complex architecture [2, 32, 33]. These compounds have multiple functions including roles as UV sunscreens and radical scavengers to protect the cells in terrestrial environments [15].

In parallel with the studies of functional molecules in *N. commune* [7, 8, 17-19, 34-36], molecular taxonomical studies have revealed that *N. commune* can be classified into four genotypes based on differences in the 16S rRNA gene sequences. The sequence differences are not great enough to be recognized as distinct species. However, they are significantly different and allow the description of genotype A to D [37]. These genotypes are difficult to distinguish morphologically and there are no ecophysiological differences allowing us to separate them. Recently, the *N. commune* that produces the 612-Da MAA was identified as genotype D, and the variety of MAAs produced by genotype D *N. commune* were characterized [19]. In our previous study [17], we examined the 478-Da MAA producer and the 1050-Da MAA producer in *N. commune*. However, at that time their genotypes had not been specified. In this study, the genotypes were identified and various MAAs were purified from the 478-Da MAA producer (genotype A) and the 1050-Da MAA producer (genotype B). The chemical structures of the 478-Da MAA and the 1050-Da MAA were reexamined to determine their sugar moieties. The specific MAAs were found exclusively in their own types of *N. commune* colonies. Additionally, the MAA compositions of the laboratory culture

strains of *N. commune* were examined to confirm that each genotype produces its own characteristic MAAs in a genotype specific manner.

2. Material and Methods

2.1. Microorganisms

Colonies of *Nostoc commune* growing in the field were collected from the Kakuma Campus of Kanazawa University (the genotype A: N 36.32715, E 136.42525 and N 36.32644, E 136.42537; the genotype B: N 36.32816, E 136.42334), Ishikawa, Japan from April to November. Wet colonies naturally swelled after rain were harvested, washed with tap water to remove soil, air-dried in the laboratory, and stored at room temperature until used. The MAA was extracted with water from *N. commune* powder and then the UV–VIS absorption spectrum was measured to identify the MAA type. The genotype was characterized by PCR direct sequencing of the 1.4-kb DNA fragment containing the 16S rRNA gene as described previously [37]. The genotype A and genotype B samples of *N. commune* were separately used for MAA purification and characterization.

The laboratory strain KU002 of *N. commune* (genotype A) has been isolated and maintained at Kanazawa University since 2002 [7, 37]. In this study, another laboratory strain KU006 of *N. commune* (genotype B) was isolated from the Kakuma Campus of Kanazawa University (N 36.54388, E 136.70366) and purified by streaking on agar plates. These *N. commune* cells were cultured at 25°C under constant illumination from fluorescent lamps (15–50 $\mu\text{mol m}^{-2} \text{s}^{-1}$) on modified BG11₀ solid medium (without a nitrogen source) containing 1.5% agar supplemented with a vitamin mix at final concentrations of 1 $\mu\text{g l}^{-1}$ biotin, 2 mg l^{-1} thiamin and 1 $\mu\text{g l}^{-1}$ cyanocobalamin [38]. To remove fungus and bacterial contaminations, 25 $\mu\text{g ml}^{-1}$ of cycloheximide and 25 $\mu\text{g ml}^{-1}$ of trimethoprim were added to the medium when necessary.

2.2. MAA standards

The known MAA standards including mycosporine-glycine, palythine, shinorine, porphyra-334, and 13-*O*-(β -galactosyl)-porphyra-334 were kindly obtained from Dr. Ishihara, K. (National Research Institute of Fisheries Science, Yokohama, Japan). These compounds were used as authentic standards [39] for the comparison of retention times on HPLC analysis.

2.3. Purification of MAAs from two types of *N. commune* colonies

N. commune powder (35 g) was suspended in distilled water (1200 ml). The MAAs were extracted by stirring at room temperature for 1 h. After centrifugation at $15,240 \times g$ for 20 min at 4°C, the supernatant was vacuum-filtered with a Buchner sintered-glass filter funnel and then condensed to 300 ml with a rotary evaporator. Ethanol was added to the filtrate to yield a final concentration of 70% (v/v) ethanol. The mixture was incubated at 4°C for 1 h in the dark to precipitate the 70% ethanol-insoluble materials. After centrifugation at $15,240 \times g$ for 20 min at 4°C, the supernatant was vacuum filtered with a Buchner sintered-glass filter funnel. The filtrate was evaporated and centrifuged at $21,500 \times g$ for 10 min at 4°C. The supernatant was filtered through a 0.20- μ m syringe filter (Minisart RC 15, Sartorius Stedim, Göttingen, Germany) and injected into a HPLC system with a Hitachi L-6200 pump that was equipped with a reverse phase column (IRICA C18, 20×250 mm, Shiseido Irica Technology, Kyoto, Japan). We fractionated the water extract of *N. commune* colonies with an absorption maximum at 335 nm (genotype A). The mobile phase was 0.2% acetic acid for the initial 135 min and 100% methanol for the next 60 min. The flow rate was kept at 2 ml min⁻¹. For fractionation of the water extract from *N. commune* colonies with absorption maxima at 312 and 340 nm (genotype B) we used a mobile phase that changed stepwise from 0.2% acetic acid during the first 66 min to 5% (v/v) methanol with 0.2% (v/v) acetic acid

during the next 70 min. The mobile phase was 100% methanol during the final 35 min. The flow rate was kept at 3 ml min⁻¹. The absorbance at 330 nm (A_{330}) was monitored with a Hitachi L-4200 UV–VIS detector. The fractions with the MAAs were recovered separately, condensed with a lyophilizer and injected into an HPLC system equipped with a gel filtration column (TSKgel G2500PW, TOSOH, Tokyo, Japan). The mobile phase was water at a flow rate of 1 ml min⁻¹. The A_{330} was monitored with a Hitachi L-4200 UV–VIS detector. The MAA fractions were recovered and the final MAA products were lyophilized.

To examine the purity of the final MAA products and their retention times on HPLC we performed HPLC analysis using a reverse phase column (Inertsil ODS-3, 4.6 mm × 250 mm; GL Sciences Inc., Tokyo, Japan). The mobile phase was selected according to the targets. The methanol concentration in 0.2% (v/v) acetic acid varied from 0 to 15% (v/v). The flow rate was at 1 ml min⁻¹. The MAAs were detected by A_{330} .

To determine the extinction coefficients of the purified MAAs the diluted solutions were prepared in water and the absorbance at their absorption maxima were determined. The MAA dry weight in 1 ml of solution was measured after lyophilization.

2.4. MS analysis

MALDI-TOF MS/MS analysis was performed at the Division of Functional Genomics, Advanced Science Research Center, Kanazawa University using a tandem mass spectrometer (4800 plus MALDI TOF/TOF™ Analyzer; Applied Biosystems, Foster City, CA, USA) with 2,5-dihydroxybenzoic acid (DHB) as a matrix. The secondary mass spectrum was recorded when applicable. The expected resolution was less than ±0.2 m/z. FAB MS analysis was used to determine the accurate mass and predicted elemental composition was performed at the Research Institute for Instrumental Analysis in Kanazawa University on a mass spectrometer (JMS-SX102A) with glycerol as a matrix.

2.5. Spectroscopic methods

The UV-VIS spectra were recorded with a Hitachi U-2800 spectrophotometer. Fourier transformation infrared (FT-IR) spectra were recorded with a Nicolet NEXUS 470 FT-IR by the KBr disk method. The NMR spectra were recorded with a JEOL ECS400 spectrometer at the Research Institute for Instrumental Analysis in Kanazawa University. 3-(Trimethylsilyl)-1-propanesulfonic acid-d₆ sodium salt (TMP) was used as an internal NMR standard.

2.6. CE-MS analysis

Capillary electrophoresis mass spectrometry (CE-MS) analysis was conducted using an Agilent Technologies CE System G1600 (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent Technologies 6410 triple quad mass spectrometer (Agilent Technologies, Wilmington, DE, USA) [40]. The analytes were separated in an untreated fused-silica capillary of 50 $\mu\text{m} \times 85 \text{ cm}$ (GL Sciences, Tokyo, Japan) at a temperature of 25°C. The pH of the background electrolyte (BGE) varied in the range of 3 to 11 by changing the mix of formic acid and ammonium hydroxide. A sample solution was injected at 50 mbar for 10 s and then the BGE was injected at 50 mbar for 3 s. After each analysis, the capillary was flushed with BGE for 4 min. The power supply was operated in the constant-voltage mode at +30 kV. The scanning or selected-ion-monitoring mode was used for the detection. The electrospray ionization was conducted in the positive ion mode for all analytes. The following instrument parameters were used for ionization: gas temperature of 250°C; N₂ gas flow rate, 7 l min⁻¹, nebulizing N₂ gas pressure 10 psi at electrophoresis and 0 psi at sample injection and a capillary voltage of 4000 V. The sheath liquid was 10 mM ammonium formate/methanol (50/50, v/v) and the flow rate was maintained at 4 $\mu\text{l min}^{-1}$.

2.7. Determination of sugars by GC-MS analysis

To determine the sugars in the glycosylated MAAs, the sugars were released by acid hydrolysis. Following a reduction to alditol, the acetylated derivatives were identified by GC-MS analysis [41]. The purified glycosylated MAA (approximately 20 μ g) was hydrolyzed by 5% H_2SO_4 aq. (100 μ L) at 75°C for 8 h. After hydrolysis the pH was adjusted to 11 with solid Na_2CO_3 . Sodium borohydride solution (0.5 M in DMSO, 1 ml) was added and kept at 40°C for 90 min and then incubated at 4°C overnight to complete reduction. Glacial acetic acid (99.9%, 100 μ L), 1-methylimidazole (200 μ L) and acetic anhydride (1 ml) were added in this order at room temperature and kept at 40°C for 10 min. After acetylation we added water (2.5 ml) and the acetylated derivatives were extracted by CH_2Cl_2 (1 ml). An organic phase (lower layer) was recovered and dried with Na_2SO_4 . The CH_2Cl_2 extract was analyzed by GC-MS (GC, Agilent 6890 series equipped with DB-5ms column (0.25 mm i.d. $\times$ 30 cm; MS, Agilent 5973 series). The temperature program for the GC and MS conditions were set according to Hongbin et al. [42]. The acetylated sugar derivatives were identified according to their retention time and the fragmentation pattern of the MS spectrum.

2.8. Measurement of antioxidant capacity

The radical scavenging activity was measured with 2,2'-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a substrate in a colorimetric assay [43]. The decolorization of ABTS radical cation at A_{734} was monitored spectrophotometrically for 1 h [17]. The electron spin resonance (ESR) signals of the ABTS radical cation were recorded with a free radical monitor (JES-FR30EX, JEOL, Tokyo, Japan). Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid) and ascorbic acid were used as artificial and natural water-soluble antioxidant standards. Scytonemin is a hydrophobic UV-absorbing pigment from *N. commune* [18] and was used for a comparison.

2.9. Measurement of chlorophyll contents

Chlorophyll a (Chl a) was extracted with 100% methanol and the concentrations were determined spectrophotometrically [44]. The Chl a concentrations were calculated using the following equation (A_{750} was subtracted to correct for light scattering):

$$[\text{Chl a } (\mu\text{g ml}^{-1})] = (A_{665} - A_{750}) \times 13.9$$

2.10. MAA composition analysis

The MAA composition in the laboratory cultures of *N. commune* strain KU002 and KU006 was analyzed by HPLC. The colonies that formed on agar plates were collected and desiccated using a lyophilizer (FDU-1200, EYELA, Tokyo, Japan). The dried materials (5-50 mg) were suspended in 30% (v/v) methanol (1 ml) and the MAAs were extracted at room temperature by mixing vigorously using a tube mixer. After centrifugation at $21,500 \times g$ for 5 min, the supernatant was collected in a new tube. This extraction step was repeated three times and then the 30% methanol extract (approximately 3 ml) was lyophilized. The lyophilized powder was dissolved in 100% methanol (0.5 ml) and methanol insoluble materials were removed by centrifugation at $21,500 \times g$ for 5 min. Water was added to the supernatant to yield 20% (v/v) methanol. The UV-VIS spectrum was recorded with a Hitachi U-2800 spectrophotometer to determine the absorption maximum wavelength. Absorbance at λ_{max} (A_{max}) in a range of 300 to 400 nm was recorded and A_{400} was subtracted to correct for light scattering when necessary. The total MAA concentrations were calculated by A_{max} using an average extinction coefficient of $120 \text{ l g}^{-1} \text{ cm}^{-1}$ [28]. The MAA containing fraction was injected into a HPLC system with a Hitachi L-6000 pump that was equipped with a reverse phase column (Cholest, $4.6 \times 150 \text{ mm}$, Nacalai Tesque, Kyoto Japan). The mobile phase

was 10% (v/v) methanol with 0.1% (v/v) acetic acid. The flow rate was 0.6 ml min⁻¹. The A₃₃₀ was monitored with a UV–VIS detector (IRICA Σ873, Shiseido Irica Technology, Kyoto, Japan). The MAAs were identified according to their retention times and confirmed by co-chromatography with the purified MAAs standards. The relative amounts of total MAAs were estimated according to their peak areas on the HPLC chromatograms.

2.11. Nucleotide sequence accession number

The nucleotide sequences of the 16S rRNA genes of *N. commune* strain KU002 (genotype A) and *N. commune* strain KU006 (genotype B) have been deposited in the GenBank/DDBJ/EMBL databases with the following accession numbers: AB088375 (*N. commune* strain KU002) and AB933330 (*N. commune* strain KU006).

3. Results

3.1. UV absorption spectra of water extracts and genotypes of *N. commune*

The genotypes of starting materials for MAA purification and characterization were identified. The 1446-bp DNA fragment containing the 16S rRNA gene was obtained by PCR using a genomic DNA template from field-isolated natural colonies, of which the water extract showed a UV-absorption spectrum with a single absorption maximum at 335 nm. The nucleotide sequence showed 100% identity to the sequence of the genotype A1 of *N. commune* (accession no. AB088375). The nucleotide sequence of the 16S rRNA gene of *N. commune* that has a water extract with a characteristic UV-absorbing spectrum containing the double absorption maxima at 312 and 340 nm showed 100% identity to the sequence of the genotype B1 of *N. commune* (accession no. AB094352). These results suggest that *N. commune* colonies with a single absorption maximum at 335 nm in the water extracts are

genotype A and the other *N. commune* colonies with double absorption maxima at 312 and 340 nm in the water extracts are genotype B.

The *N. commune* strain KU002 (genotype A) and *N. commune* KU006 (genotype B) were cultured using laboratory conditions and their MAA contents were characterized. The levels of chlorophyll and MAA were similar in both laboratory strains of *N. commune* (Table 1), but the UV absorption spectra of these MAA containing extracts were different (Fig. 1). The extract of *N. commune* strain KU002 showed a single absorption maximum at 327 nm and the extract of *N. commune* strain KU006 showed a characteristic spectrum with an absorption maximum at 312 nm associated with a shoulder approximately 340 nm (Fig. 1). The *N. commune* strain KU002 contained arabinose-bound porphyrin-334 (478 Da) as a main component and its content was approximately 85% according to the estimation from the HPLC chromatogram (Fig. 1A). Pentose-bound shinorine (464 Da) was also detected in *N. commune* strain KU002 and its content was approximately 15% (Fig. 1A). Conversely, the *N. commune* strain KU006 contained the 1050-Da MAA and its estimated content was approximately 80% (Fig. 1B). The levels of MAAs detected in the two strains of *N. commune* were approximately 0.01 to 0.03% of their dry weight (Table 1). These data are in agreement with the previous reports in field-isolated natural colonies of *N. commune* that showed the amounts of MAAs are approximately 0.04% of their dry weight [36].

These results suggest that the genetically distinct strains of *N. commune* exclusively produce their own unique MAAs and that the MAA production takes place under laboratory culture conditions without supplemental UV irradiation. Stress conditions such as UV irradiation and/or desiccation may enhance the MAA production and this will be tested and reported in future studies.

3.2. Variety of MAAs in genotype A

The water extract from field-isolated natural colonies of genotype A showed a UV-absorbing spectrum with an absorption maximum at 335 nm. The extract from genotype A was examined by a preparative HPLC system and four different MAAs were identified. The MAAs were the following: 478-Da MAA, 464-Da MAA, 508-Da MAA, and porphyrin-334 with a molecular mass of 346 Da (Fig. 2). Each peak was collected and the purity was examined by an analytical HPLC system (Fig. 2BCDE). The molecular masses were determined by MALDI-TOF-MS/MS analysis.

The 478-Da MAA with an absorption maximum at 335 nm (Fig. 2I) was the main MAA from *N. commune* in genotype A and accounted for approximately 62% of the total MAAs according to the estimation from the HPLC chromatogram. The 478-Da MAA has previously been characterized as a pentose-bound porphyrin-334 derivative [17]. The purified 478-Da MAA was acid hydrolyzed, and the released pentose was determined by GC-MS analysis after derivatization as described in Materials and Methods. Penta-*O*-acetyl-arabinol (arabinose derivative) was detected (data shown in Supplement Data). This finding indicates the presence of an arabinose moiety in the 478-Da MAA. The structure was confirmed by the reexamination of the NMR spectroscopic analysis (Table 2, Fig. S1). The correlations in the HMBC spectra and vicinal coupling constant at the anomeric proton of 7.6 Hz confirm the presence of the β -form of arabinose.

The HMBC correlation of the H1' proton with C7 carbon and vice versa (Table 2, Fig. S1) indicates arabinose is linked at the C7 position of porphyrin-334. The ^{13}C chemical shift at C5' carbon appeared at 69.1 ppm, which showed a HMQC correlation with two distinct proton signals at 3.66 and 3.92 ppm (Table 2). This result indicates a pyranose-form arabinose because the methylene protons at C5' are magnetically anisotropic. The long-range coupling pattern determined by the HMBC measurements also supports the presence of arabinose in a pyranose form due to the correlation between the lower field H5' proton and

C1' carbon. Fig. 3 shows the structure of 7-*O*-(β -arabinopyranosyl)-porphyra-334 (478 Da) from the genotype A of *N. commune*.

The 464-Da MAA with an absorption maximum at 332 nm (Fig. 2F) was the second major MAA in the genotype A and it represents approximately 15% of total MAAs. MALDI-TOF MS/MS analysis was performed on the parent molecular ion fragment with m/z 465 and a fragment with m/z 333. This is identical to the molecular mass of shinorine [45, 46] detected in the second MS (Table S1). The neutral loss of 132 Da suggested the deletion of a pentose ($C_5H_8O_4$) from the fragment with m/z 465. Table S1 shows a summary of the MALDI-TOF MS/MS analysis of the 464-Da MAA and strongly suggests that the 464-Da MAA is a pentose-bound shinorine derivative.

Our prediction was confirmed by NMR spectroscopic analysis. In the ^{13}C -NMR spectrum of the 464-Da MAA, the known chemical shifts for shinorine [39, 47] were observed in the 464-Da MAA (Table S2). This finding suggests a shinorine scaffold in the 464-Da MAA. Due to the contamination of oligosaccharides from the extracellular polysaccharides it may be difficult to separate shinorine with our HPLC conditions. Therefore, it was difficult to use 2D-NMR analysis to determine the precise structure of the 464-Da MAA. We then performed CE-MS analysis on the 464-Da MAA to rule out the possibility that the oligosaccharides might be covalently bound to a shinorine scaffold. A single peak with m/z 465 was detected on the electropherogram and this confirmed its molecular mass of 464 Da (data not shown). In the 464-Da MAA, pentose is likely bound to the C7 position of shinorine via an *O*-glycoside bond similar to the 478-Da MAA as an arabinose-bound porphyra-334 derivative. There was a chemical shift at the C7 position that was downfield shifted to 77.7 ppm (Table S2).

The 508-Da MAA with an absorption maximum at 334 nm (Fig. 2H) was also purified and its estimated amount was only 3% of total MAAs. MALDI-TOF MS/MS

analysis was performed on the parent molecular ion fragment with m/z 509 and a fragment with m/z 347, which is identical to the molecular mass of porphyra-334 [45, 46] that was detected in the second MS (Table S3). The neutral loss of 162 Da suggested the deletion of a hexose ($C_6H_{10}O_5$) from the fragment with m/z 509. Table S3 shows a summary of the MALDI-TOF MS/MS analysis for the 508-Da MAA. These fragmentation patterns were similar to those from the MS analyses of known MAAs [48]. These data suggest that the 508-Da MAA is a hexose-bound porphyra-334 derivative. The NMR analysis of the 508-Da MAA from genotype A could not be performed due to the limitation of the sample purified. Therefore, we compared the retention times of the known hexose-bound porphyra-334 isoforms using an analytical HPLC. The data showed different retention times (Fig. S2) and suggested the different chemical structures were due to either variety of hexose or a different binding position of hexose to porphyra-334. In the 508-Da MAA from the genotype A of *N. commune*, hexose is likely to be bound to the C13 position of porphyra-334 via an *O*-glycoside bond. However, this tentative structure must be examined in a future study.

The 347-Da MAA with an absorption maximum at 333 nm (Fig. 2G) was purified and its estimated amount was 1% of total MAAs. Its λ_{max} , molecular mass and retention time on the HPLC chromatogram were identical to those of porphyra-334. This finding indicated that the 347-Da MAA is porphyra-334 from *N. commune*.

In genotype A of *N. commune* we found 7-*O*-(β -arabinopyranosyl)-porphyra-334 (478 Da), pentose-bound shinorine (464 Da), hexose-bound porphyra-334 (508 Da) and porphyra-334 (346 Da). Fig. 3 shows their chemical structures and one plausible biosynthetic pathway. It has been reported that 4-deoxygadusol is a common precursor for the biosynthesis of MAAs [20, 25, 49]. The 4-deoxygadusol could conjugate with a nitrogen substituent (e.g., amino acids) to generate MAAs [20, 26, 27]. The gene product of Ava-3856 from *Anabaena variabilis* can convert 4-deoxygadusol and glycine into mycosporine-glycine [49]. The

mycosporine-glycine condensation with an amino acid would be a common reaction in the generation of amino acid bi-substituted MAAs such as shinorine and porphyra-334 because most contain a glycine residue [20, 49].

In our predicted pathway for the 478-Da MAA producer of the genotype A (Fig. 4), porphyra-334 and shinorine could be generated via the addition of threonine and serine, respectively, to the mycosporine-glycine intermediate. The following glycosylation of porphyra-334 and shinorine could produce the 478-Da MAA, the 508-Da MAA and the 464-Da MAA. The glycosylation of the core ring of mycosporine-glycine may occur and there is no evidence to rule out this possibility. In the proposed biosynthetic pathway, the glycosylation used to produce the pentose-bound porphyra-334 and shinorine derivatives is a unique process in genotype A of *N. commune*. Additional molecular genetic studies to identify the genes involved in the glycosylation will elucidate the biosynthesis of the glycosylated MAAs unique in the terrestrial cyanobacterium *N. commune*.

3.3. MAAs in genotype B

The water extract from genotype B of *N. commune* showed a characteristic UV-absorbing spectrum with double absorption maxima at 312 and 340 nm. The extract was examined by a preparative HPLC system and three different MAAs including 1050-Da MAA, 880-Da MAA and 273-Da MAA were identified (Fig. 5). Each peak was collected and the purity was examined by an analytical HPLC (Fig. 5BCD). The molecular masses were determined by MALDI-TOF-MS/MS analysis.

The 1050-Da MAA has a characteristic absorption maximum at 312 nm and an associated shoulder approximately 340 nm (Fig. 5G) and was a main MAA in genotype B of *N. commune*. The 1050-Da MAA accounted for approximately 43% of the total MAAs according to the estimation from the HPLC chromatogram. The 1050-Da MAA has

previously been characterized and its unique structure consists of two distinct chromophores of 3-aminocyclohexen-1-one and 1,3-diaminocyclohexen and two pentose and hexose sugars [17]. The structure was reexamined using the purified 1050-Da MAA as described below in the text.

The 880-Da MAA has an absorption maximum at 331 nm and is associated with a shoulder at 312 nm (Fig. 5E). The 273-Da MAA has an absorption maximum at 310 nm (Fig. 5F). Both MAAs were found and purified from genotype B of *N. commune*. The contents of these MAAs varied in samples and the estimated amounts were approximately 16% (880-Da MAA) and 8% (273-Da MAA), respectively. Because there are no MAAs with a molecular mass of 880 Da nor 273 Da reported in the literature their chemical structures were further characterized in the present study.

3.3.1. 1050-Da hybrid MAA

The 1050-Da MAA is a unique hybrid MAA with double absorption maxima at 312 and 340 nm and is consistent with the presence of two 3-aminocyclohexen-1-one and a 1,3-diaminocyclohexen chromophores [17]. In the previous report, we proposed the tentative structure of the 1050-Da MAA but its details, especially the structure of the disaccharide moiety, was not characterized [17].

The mass spectra analyses and ^{13}C -NMR analysis indicated that the ornithine moiety connected two 3-aminocyclohexen-1-one and a 1,3-diaminocyclohexen chromophores to construct the scaffold of the 1050-Da MAA. In HMBC analysis, the C1 and C3 atom in the central imine-type ring showed a correlation with methylene protons (H22 and H9) at C22 and C9 carbon, respectively (Table 3, Fig. S3). Furthermore, the α -carboxyl group in ornithine appeared at 179.1 and 179.2 ppm (C13 and C26) and showed a correlation with the α -proton (H12, H25) instead of H9 and H22 (Table 3, Fig. S3). These results suggest the δ -

amino group in ornithine is linked to the central ring of 1,3-diaminocyclohexen and the α -amino group is linked to the exterior ring of 3-aminocyclohexen-1-one.

The sugar composition of the 1050-Da MAA was analyzed as described in Materials and Methods. The two monosaccharide derivatives hexa-*O*-acetylgalactitol (galactose derivative) and penta-*O*-acetylxylitol (xylose derivative) were detected (data shown in Supplement Data). These results indicate the presence of galactose and xylose moieties in the 1050-Da MAA. The coupling scheme and the details of the disaccharide structure were further characterized by the reexamination of the NMR spectroscopic analysis.

A chemical shift value of the anomer carbon of galactose (105.0 ppm) and xylose (107.6 ppm) in the 1050-Da MAA (Table 3) suggest the presence of 1-*O*-methyl- β -D-galactopyranoside (104.5 ppm) and 1-*O*-methyl- β -D-xylopyranoside (105.1 ppm) rather than 1-*O*-methyl- α -D-galactopyranoside (100.1 ppm) and 1-*O*-methyl- α -D-xylopyranoside (100.6 ppm), respectively. Furthermore, the vicinal coupling constants of the anomeric proton of galactose and xylose were 7.2 and 8.4 Hz, respectively (Table 3) and are much larger than that of a typical α -form. These results indicate that both steric structures at anomer carbons are β form. The ^{13}C chemical shift at C5'' carbon in the xylose residue appeared at 68.4 ppm and showed a HMQC correlation with two distinct proton signals at 3.25 and 3.78 ppm (Table 3). The data indicate a pyranose-form xylose because the methylene protons at C5'' are magnetically anisotropic.

In the ^{13}C -NMR spectrum, the characteristic signal appeared at 83.1 ppm and we were able to assign it to the C2' carbon in galactose (Table 3). According to the HMQC analysis, this typical peak at 83.1 ppm showed a correlation with the proton at 3.69 ppm and with both anomer carbons in the HMBC analysis. This result indicates that the carbon at 83.1 ppm must be present between two anomer carbons (C1' and C1''). The correlation signal between the anomer carbon of galactose (C1') and the methylene proton at C7 in the central imine-type

ring was observed (Table 3). The data suggest the galactose binds to the central ring at the anomer position and xylose links the C2' carbon of galactose with the chemical shift at 83.1 ppm (Fig. S3). The assignment of the signal appearing at 83.1 ppm is a key to identifying the disaccharide structure of the 1050-Da MAA. We further examined the data to confirm the coupling scheme of the disaccharide.

The DEPT 90 spectrum showed the carbon at 83.1 ppm was assigned to the methine (CH) carbon (data shown in Supplement Data), indicating that xylose does not bind at the C6' position. The ^{13}C chemical shifts of the galactose moiety in the 1050-Da MAA were compared to those of galactose derivatives and we found the 1,2-*O*-dimethyl- β -D-galactopyranoside and the β -D-galactopyranose moiety in 1-*O*-methyl-2-*O*-fucopyranosyl- β -D-galactopyranoside (Table S4). The results suggest the β -xylopyranose link is located at the C2' position of the galactose moiety.

Fig. 6 shows a plausible structure of the 1050-Da MAA. The proposed chemical structure of the 1050-Da MAA, especially the disaccharide moiety, has been updated based upon the reexamination of NMR data. We proposed that xylose binds to the C2' position of galactose as a pyranose form (Fig. 6). In our previous report we have assumed the pentose (furanose form) binds to hexose at the C6' position in the 1050-Da MAA [17].

Fig. 7 shows a plausible biosynthetic pathway for the 1050-Da MAA in the genotype B of *N. commune*. Mycosporine-ornithine is an assumed intermediate used to produce the 1050-Da MAA. It can be generated via the addition of ornithine to 4-deoxygadusol, which is a common precursor in MAA biosynthesis (Fig. 7). The symmetrical structure consists of three mycosporine rings and two ornithine molecules and can be generated via two consecutive conjugation reactions. During this reaction two mycosporine-ornithine molecules are added to a glycosylated mycosporine-amino-acid scaffold, which may also be produced via glycosylation of a mycosporine-ornithine molecule (Fig. 7). The glycosylation may occur

after assembling the core structure consisting of three rings. There is no evidence to specify when it is glycosylated and where the glycosylation reactions take place. Assuming that the glycosylation occurs at the initial step to produce a glycosylated 4-deoxygadusol scaffold, two mycosporine-ornithine molecules can successively be conjugated via imination to produce the final product of the 1050-Da MAA. In this alternative pathway, the glycosylated 4-deoxygadusol molecule will be a core ring to link with two mycosporine-ornithine molecules at their δ -amino groups. The enzymes and their responsible genes for the predicted biosynthetic pathway of the 1050-Da MAA in *N. commune* are unknown. Thus, further molecular genetic studies are required to elucidate their identity.

3.3.2. 880-Da MAA

An MAA with an absorption maximum at 331 nm associated with a shoulder at 312 nm (Fig. 5E) was purified (Fig. 5B). Its molecular mass of 880 Da and it has a double absorption maxima at 312 and 331 nm, which suggests it is a hybrid structure consisting of both 3-aminocyclohexen-1-one and 1,3-diaminocyclohexen chromophores. The absorption coefficient of the 880-Da MAA was $56.65 \text{ l g}^{-1} \text{ cm}^{-1}$ at 331 nm in water. The calculated molar absorption coefficient at 331 nm was $4.98 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

The IR spectrum of the 880-Da MAA was compared to the 1050-Da MAA (Table S5). Similar IR absorption peaks suggest that the 880-Da MAA has a similar structure to the 1050-Da MAA. The absorption peak at 1548 cm^{-1} in the 880-Da MAA indicated the presence of the conjugated imine is a characteristic structure of MAA chromophore and this corresponds to the 1541 cm^{-1} peak in the 1050-Da MAA (Table S5). The absorption peaks in the range of $1200\text{-}1300 \text{ cm}^{-1}$ and the characteristic absorption peak at 3374 cm^{-1} in the 880-Da MAA indicated the structure contains sugar(s) and several hydroxyl functional groups, respectively (Table S5).

The fragmentation pattern of the 880-Da MAA in the MALDI-TOF MS/MS analysis (Table S6) was similar to the 1050-Da MAA. The difference was the fragments resulted from the 880-Da MAA were 170 Da less than those from the 1050-Da MAA [17]. This molecular mass of 170 Da is identical to 4-deoxygadusol, which is a precursor for the biosynthesis of MAA. These results suggest that the hydrolysis of the 1050-Da MAA to remove a cyclohexenone chromophore (170 Da) could produce the 880-Da MAA (Fig. S4). Thus, the double absorption maxima of 1050-Da MAA at 312 nm associated with a shoulder approximately 340 nm (Fig. 4G) changes to the absorption maximum at 331 nm associated with a shoulder at 312 nm for the 880-Da MAA (Fig. 5E).

The MALDI-TOF MS/MS analysis of the parent molecular ion fragment with m/z 881 indicated the presence of a hexose and a pentose in its structure similar to the 1050-Da MAA (Table S6). There are also galactose and xylose moieties in the 880-Da MAA that were confirmed by sugar composition analysis (data shown in Supplement Data).

The ^{13}C -NMR spectrum of the 880-Da MAA was similar to the 1050-Da MAA (Table S7). However, the signal number of the 880-Da MAA was greater than the 1050-Da MAA. This result was inconsistent with the assumption that the 880-Da MAA is a break down compound of the 1050-Da MAA. In the spectrum of the 880-Da MAA there are multiple signals assigned to the ornithine moiety (C11, C24 and C12, C25) were recorded and they appeared close together (Table S7). This finding suggests the purified 880-Da MAA may be a mixture of isomers whose structures are different at the ornithine moiety.

Mycosporine-glycine is known to be hydrolyzed to form 4-deoxygadusol and glycine [50]. Both peripheral carbonyl-type rings in the 1050-Da MAA are thought to be susceptible to hydrolysis. If one peripheral carbonyl-type ring is removed from the 1050-Da MAA by hydrolysis (Fig. S4) then the symmetric molecule changes to asymmetric molecules with a chiral center at C5 carbon in the central ring of the 880-Da MAA (Fig. S5). Three substituent

groups on the central imine-type ring of the 880-Da MAA, labeled by X, Y and Z, have chiral centers individually (Fig. S5). To test this assumption of the 880-Da MAA, CE-MS analysis was performed on the purified 880-Da MAA sample. A symmetrical single peak of the 880-Da MAA was detected on the electropherogram when analyzed at $\leq$ pH 7 and this result confirmed its purity. At pH 11, the peak derived from the 880-Da MAA was split on the electropherogram and suggests the mixture of isomers with the identical m/z 881. The 880-Da MAA was purified using an octadecylsilyl (ODS) column and a symmetrical single peak of the purified 880-Da MAA was recorded (Fig. 5B), which suggests these isomers are difficult to separate on a regular ODS column. Thus, HPLC analysis was performed using another type of a reverse phase column with the cholesteryl-bonded silica gel (COSMOSIL Chorester, Nacalai). Consistent with the result of CE-MS analysis, two peaks with similar signal intensities were detected when the purified 880-Da MAA was analyzed using the HPLC system with the cholesteryl-bonded silica gel column (Fig. S6B). These results demonstrate that the purified 880-Da MAA contains a pair of diastereomer in equal amounts and confirms the idea that hydrolysis of the 1050-Da MAA symmetric molecule produces asymmetric molecules like the 880-Da MAA. It is unclear whether this hydrolysis of the 1050-Da MAA is enzymatic or an artificial event. In either case, this is the first report of the 880-Da MAA with both 3-aminocyclohexen-1-one and 1,3-diaminocyclohexen chromophores.

3.3.3. *Mycosporine-GABA*

The 273-Da MAA was purified from genotype B of *N. commune* (Fig. 5C) and its chemical structure was characterized. The UV absorption spectrum of the purified 273-Da MAA showed a single absorption peak at 310 nm (Fig. 5F) and its absorption coefficient was $106.05 \text{ l g}^{-1} \text{ cm}^{-1}$ at 310 nm in water. An accurate molecular mass was determined by FAB

MS to predict the elemental composition. A molecular ion fragment with m/z 274.1295 was detected and its predicted molecular formula was $C_{12}H_{20}NO_6$ within 1 ppm error. The calculated molar absorption coefficient at 310 nm was $2.89 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

The IR spectrum of the 273-Da MAA was compared with known MAAs methyl ester of mycosporine-glycine [50] and porphyra-334 [51] (Table S8). Similar IR absorption peaks were observed and this result suggests that the 273-Da MAA has a similar structure to these MAAs.

MALDI-TOF MS/MS analysis was performed on the molecular ion fragment with m/z 274 and fragments with m/z 186, m/z 137 and m/z 87 were detected (Table S9). The fragment with m/z 137 could be a divalent ion of the 273-Da MAA. The fragment with m/z 87 was thought to be a γ -aminobutyric acid chain. The fragment with m/z 186 corresponded to the remaining 3-aminocyclohexen-1-one scaffold. This fragmentation pattern of the elimination of an amino-acid side chain was common in the results of the MS analysis of the known MAAs [45, 48, 52].

Table 4 shows the summary of ^{13}C and ^1H NMR analysis of the 273-Da MAA. Signals with identical chemical shifts to mycosporine-glycine [50] were observed in the 273-Da MAA, indicating the presence of the oxocarbonyl-type chromophore (C1, 2, 3, 4, 5, 6, 7, 8). In addition, signals with identical chemical shifts to γ -aminobutyric acid (C9, 10, 11, 12) were observed. These data indicate the γ -aminobutyric acid is a side chain linked to 3-aminocyclohexen-1-one chromophore (Fig. S7). These results are consistent with the MS analysis of the 273-Da MAA. This scaffold was named as mycosporine-GABA. This is the first report of the unique mycosporine-GABA structure, which is not listed in the previously reported MAAs [21-27]. The addition of γ -aminobutyric acid can be generated via decarboxylation of glutamic acid to 4-deoxygadusol and may produce mycosporine-GABA. The identification of the mycosporine-GABA in the genotype B of *N. commune* provides one

new molecule to the characterized MAAs. However, the total amount was a small portion of all MAAs (approximately 8% or less).

3.4. Radical scavenging activity in MAAs from *N. commune*

Table 5 shows the radical scavenging activity detected in the purified 880-Da MAA and 273-Da MAA. We compared the activities in the previously reported MAAs from *N. commune*. Both showed ABTS radical scavenging activities when we monitored the decolorization of ABTS radical. During the time course experiments, the decolorization of ABTS radical increased as incubation times increased from 10 min to 2 h. These findings suggest the MAAs are slow-acting radical scavengers and may be similar to the other known MAAs from *N. commune*. The molecule used as a standard, Trolox, is known to be a fast-acting scavenger and the reaction is completed within 10 min. Based on these results, the incubation time was fixed for 1 h in the assay as described by Matsui et al. [17]. We directly monitored the decrease of ABTS radical cation by ESR and found higher activities than those measured by the colorimetry assay (Table 5). The IC₅₀ values by the colorimetry assay maybe underestimated because of changes of A₇₃₄ not due to the reaction, e.g. the increase of light scattering during the incubation for 1 h. These results indicate that the 880-Da MAA and 273-Da MAA are strong radical scavengers *in vitro* and are consistent with previous reports of MAAs from *N. commune* [17, 19].

4. Discussion

4.1. Chemical diversity of MAAs in *Nostoc commune*

Two types of *Nostoc commune* with water extracts that showed different UV-absorbing spectra were examined and several distinct MAAs were identified (Fig. 1, Fig. 2, Fig. 5). One type of *N. commune* showed a single absorption maximum at 335 nm in the water extract.

This genotype was identified as genotype A and produced 7-*O*-(β -arabinopyranosyl)-porphyr-334 (478 Da), pentose-bound shinorine (464 Da), hexose-bound porphyr-334 (508 Da) and porphyr-334 (346 Da) (Fig. 4). All of these compounds have absorption maxima approximately 330 nm (Fig. 2FGHI), indicating the presence of 1,3-diaminocyclohexen chromophore conjugated with two nitrogen substituent via C1 and 3 in their structures [27]. The other *N. commune* colonies had double absorption maxima at 312 and 340 nm in the water extract and were identified as genotype B. This genotype produced the 1050-Da, 880-Da and 273-Da MAAs (Fig. 6, Fig. S4, Fig. S7), which all have absorption maxima approximately 310 nm (Fig. 5EFG). The results indicate the presence of 3-aminocyclohexen-1-one chromophore in their structures [27]. The double absorption maxima of the purified 1050-Da MAA (Fig. 5G) and the purified 880-Da MAA (Fig. 5E) are caused by 1,3-diaminocyclohexen and 3-aminocyclohexen-1-one chromophores in their structures (Fig. 6, Fig. S5). These chromophores expand their UV-absorbing range covering UV-A/B wavelengths. The hybrid-type MAAs exclusively occur in genotype B of *N. commune* and have not been reported in any other organisms. Ecophysiological roles specific for the hybrid MAAs are unknown and microbial ecological features for the genotype B of *N. commune* remain to be studied. We have proposed the biosynthetic pathway of the 1050-Da hybrid MAA (Fig. 7) and this will be examined to identify the genes and enzymes involved by molecular biological and biochemical approaches in future studies.

The different MAA contents in these two types of *N. commune* suggest there is molecular diversity of MAAs in *N. commune* and they are genetically different to produce their type-specific MAAs. Consistent with this idea, it has been reported that the genotype D of *N. commune* type-specifically produces the 612-Da MAA, which is a glycosylated palythine-threonine derivative [19]. Four major genotypes of *N. commune* have been reported and their 16S rRNA sequences show > 96% identity and they are recognized as the same

species [37]. Thus, the diversity of these MAAs in *N. commune* are unexpectedly high. We have characterized a variety of MAAs in the genotype A and B (this study) and in genotype D [19]. The variety of the MAA contents in the genotype C of *N. commune* remains to be characterized. In order to generalize the relation between the genotypes and the MAA types, further studies on MAA contents and their biosynthetic pathways among the different genotypes of *N. commune* are ongoing and will be reported in future studies.

4.2. MAAs as potent antioxidative compounds

UV radiation causes oxidative damages in cyanobacterial cells including lipid peroxide formation, DNA strand breaks, and chlorophyll bleaching concomitant with deactivation of photosynthesis and growth inhibition [53]. MAAs are suggested to have a protective role against UV-induced oxidative stress in algae [54] and cyanobacteria [55]. It has been reported that oxocarbonyl-type MAAs such as mycosporine-glycine [56-58] and mycosporine-aurine [55] show radical scavenging activities *in vitro*. The 273-Da MAA (mycosporine-GABA) is a non-glycosylated oxocarbonyl-type MAA consisting of a cyclohexenone chromophore with a high activity (Table 5). Conversely, porphyra-334 has photoprotective properties but not antioxidant functions [30, 56]. This suggests that imine-type MAAs are not antioxidative compounds. We confirmed that no radical scavenging activity was detected in porphyra-334 using our assay system (data not shown) that routinely accesses the activities in the glycosylated MAAs.

In *N. commune*, we have demonstrated that the glycosylated MAAs show radical scavenging activities *in vitro* and suggested that the glycosylation of porphyra-334, shinorine and palythine-threonine provide the antioxidant function of these imine-type MAAs (Table 5). The hybrid-type MAAs such as the 1050-Da MAA and the 880-Da MAA have potent radical scavenging activities *in vitro* (Table 5). The presence of the cyclohexenone chromophore in

the hybrid-type MAAs suggests their radical scavenging activities. However, it remains to be elucidated whether the glycosylation enhances the activities. The energy absorbed by MAAs is dispersed as heat [30, 31] without generating reactive oxygen species (ROS). Thus, it will be interesting to determine whether UV radiation affects the radical scavenging activity of these glycosylated MAAs in *N. commune*. Future *in vitro* studies will answer this question and provide further evidence of the multifunctional abilities of these compounds.

4.3. Glycosylated MAAs and anhydrobiosis

We have isolated and characterized 10 MAAs from *N. commune* [17, 19, this study], and 8 are glycosylated. Moreover, all of the main MAA in each genotype such as 7-*O*-(β -arabinopyranosyl)-porphyrin-334 (478 Da) in the genotype A, the 1050-Da hybrid MAA in the genotype B and two hexose-bound palythine-threonine (612 Da) in the genotype D are glycosylated. The finding suggests that glycosylation could be requisite for the interaction of these MAAs within the extracellular matrix and their functions in *N. commune*. The glycosylation may stabilize these MAAs. However, this possibility must be demonstrated in future studies. The known glycosylated MAAs mycosporine-glutaminol-glucoside and mycosporine-glutamicol-glucoside have been reported in rock-inhabiting microcolonial fungi [59] and the terrestrial cyanobacteria from rock surfaces [60]. These results suggest an ecological role of glycosylated MAAs in adaptation to terrestrial environments. Using these glycosylated MAAs *N. commune* may have acquired the capabilities of surviving terrestrial environments and sustaining viability in a desiccated state. However, biochemical mechanisms for the protection of cells by these glycosylated MAAs remain to be elucidated.

Trehalose is a nonreducing disaccharide and is known to be an anti-stress molecule that protects biological membranes and proteins under various stressed conditions [61]. In *N. commune*, trehalose accumulation occurs in response to water loss during desiccation [8]. The

extracellular polysaccharides are main components of the extracellular matrix in *N. commune* [34] and play a major role in protecting cells during desiccation [7]. Glycosylated MAAs may function together with trehalose and the extracellular polysaccharides to protect cells from damage under stressed conditions. The crystal structure of palythine has demonstrated that there are twelve types of hydrogen bonds between one palythine molecule and three water molecules [62], indicating that various types of hydrogen bonds are formed not only intra- but also inter-molecularly to form a three dimensional network. The hydrogen bond network structure with water molecules is expected to contribute to the vitrification of cells in a desiccated condition [15]. In *N. commune*, MAAs are thought to be localized in the extracellular matrix and bound to extracellular polysaccharides [2] and an extracellular matrix protein called water stress protein (WspA) by physical interaction [32, 33]. The complex structures of the extracellular matrix consisted of polysaccharides, proteins, sugars and MAAs may synergistically function in vitrifying to protect cells in a desiccated condition [15]. Further studies on the glycosylated MAAs in *N. commune* will be needed to address their functions in the mechanisms of anhydrobiosis.

Acknowledgments

We thank Prof. K. Takahashi (Kanazawa Univ.) for generous assistance with IR analysis and Prof. K. Tanaka (Tokyo Institute of Technology) for suggestion to use antibiotics for the culture. This work was supported by HABA Laboratories, Inc. (research grant).

Legends to figures:

Fig. 1. Typical absorption spectra and HPLC chromatograms of the extracts of the laboratory cultures of *Nostoc commune* strain KU002 (A) and strain KU006 (B). The 30% methanol extracts showed absorption maxima at 327 nm (A) and 312 nm (B), respectively. MAAs were analyzed by HPLC with a reverse-phase column (COSMOSIL Cholesterol, 4.6 mm × 150 mm, Nacalai Tesque) using 10% methanol and 0.1% acetic acid at a flow rate of 0.6 ml min⁻¹ as the mobile phase. MAAs were detected by the A₃₃₀. *N. commune* strain KU002 (A) contained arabinose-bound porphyra-334 (478 Da) and pentose-bound shinorine (464 Da). *N. commune* strain KU006 (B) contained the hybrid MAA (1050 Da) with double absorption maxima at 312 and approximately 340 nm.

Fig. 2. HPLC chromatograms and absorption spectra of the purified MAAs from *Nostoc commune* colony with an absorption maximum at 335 nm (genotype A). (A) The crude water-soluble extract was separated by an HPLC system equipped with a reverse phase column (IRICA C18, 20 × 250 mm, Shiseido Irica Technology) as described in Materials and Methods. The purified MAAs, 464-Da MAA (B), porphyra-334 (C), 508-Da MAA (D) and 478-Da MAA (E), were analyzed by an HPLC system equipped with a reverse phase column (Inertsil ODS-3, 4.6 mm × 250 mm; GL Sciences Inc.) using 0.2% acetic acid at a flow rate of 1 ml min⁻¹ as the mobile phase and were detected by the A₃₃₀. The purified 464-Da MAA (F), porphyra-334 (G), 508-Da MAA (H) and 478-Da MAA (I) in water showed absorption maxima at 332 nm, 333 nm, 334 nm and 335 nm, respectively.

Fig. 3. The structure of the 478-Da MAA, 7-*O*-(β -arabinopyranosyl)-porphyra-334, isolated from the genotype A *Nostoc commune*. The structure of the sugar moiety of pentose in the 478-Da MAA has been reexamined and updated from our previous report [17].

Fig. 4. A plausible biosynthetic pathway for the glycosylated MAAs in the genotype A of *Nostoc commune*. The addition of arabinose to porphyra-334 produces 7-*O*-(β -arabinopyranosyl)-porphyra-334 (478 Da) as a main MAA. A hexose-bound porphyra-334 (508 Da) is also produced by the glycosylation of porphyra-334. Shinorine can be produced via conjugation of mycosporine-glycine and serine and the following glycosylation produces a pentose-bound shinorine (464 Da) as a minor pathway. The structures of the sugar moieties in the pentose-bound shinorine (464Da) and the hexose-bound porphyra-334 (508 Da) have not yet determined.

Fig. 5. HPLC chromatograms and absorption spectra of the purified MAAs from *Nostoc commune* colony with absorption maxima at 312 and 340 nm (genotype B). (A) The crude water-soluble extract was separated by an HPLC system equipped with a reverse phase column (IRICA C18, 20 $\times$ 250 mm, Shiseido Irica Technology) as described in Materials and Methods. The purified 880-Da MAA was analyzed by an HPLC system equipped with a reverse phase column (Inertsil ODS-3, 4.6 mm $\times$ 250 mm; GL Sciences Inc.) using 0.2% acetic acid at a flow rate of 1 ml min⁻¹ as the mobile phase and were detected by the A₃₃₀ (B). The 273-Da MAA (C) and 1050-Da MAA (D) were analyzed by the same HPLC system but 5% methanol with 0.2% acetic acid was used as the mobile phase. The purified 880-Da MAA (E), 273-Da MAA (F) and 1050-Da MAA (G) in water showed absorption maxima at 331 nm, 310 nm and 312 nm, respectively.

Fig. 6. The structure of the 1050-Da hybrid MAA isolated from the genotype B *Nostoc commune*. The structure of disaccharide moiety has been reexamined and updated, thus it is different from our previous report [17].

Fig. 7. A plausible biosynthetic pathway for the 1050-Da MAA in the genotype B of *Nostoc commune*. R₁ represents an amino acid, e.g., ornithine. R₂ represents the glycosyl group and 2-*O*-(β -xylopyranosyl)- β -galactopyranoside is bound at this position of the final product of the 1050-Da MAA.

Table 1. MAA contents in the laboratory cultured strains of *Nostoc commune*.

Strain	Chl a ^a	Total MAA ^b
	(mg g ⁻¹ [DW])	
KU002 (genotype A)	2.7 ± 1.0 (n = 6)	0.14 ± 0.05 ^c (n = 4)
KU006 (genotype B)	2.2 ± 0.3 (n = 6)	0.25 ± 0.08 ^d (n = 4)

Data are presented as means ± SD.

^a Chlorophyll a (Chl a) was extracted with methanol and determined spectrophotometrically [44].

^b Mycosporine-like amino acids (MAAs) were extracted with 30% methanol and determined spectrophotometrically using an absorption coefficient of 120 l g⁻¹ cm⁻¹ [28].

^c Mixture of pentose-bound porphyra-334 (85%) and pentose-bound shinorine (15%).

^d The 1050-Da MAA content was approximately 80%.

1 **Table 2.** Summary of the NMR analysis of the 478-Da MAA.

Position	¹ H [ppm]	Number of H	Multiplet	J-value [Hz]	¹³ C [ppm]	HMBC correlation
1					161.6	
2					128.4	
3					163.2	
4 ^h	2.83	1H	d	-17.4	35.9	C2, C3, C5, C6, C7
4 ^l	2.94	1H	d	-17.4		C2, C3, C5, C6
5					73.1	
6 ^h	2.83	1H	d	-17.4	36.4	C1, C2, C4, C5, C7
6 ^l	3.01	1H	d	-17.4		C1, C2, C4, C5
7 ^h	3.66	1H	d	-10.2	77.7	C4, C5, C6, C1'
7 ^l	3.90	1H	d	-10.2		C4, C5, C6, C1'
8	3.70	3H	s		62.2	C2
9	4.06	1H	s		49.5	C3, C10
10					177.7	
11	4.09	1H	d	4.2	67.3	C1, C12, C13, C14
12					178.2	
13	4.31	1H	dq	4.2, 6.0	71.0	
14	1.26	3H	d	6.0	22.2	C11, C13
1'	4.37	1H	d	7.2	106.4	C7
2'	3.61	1H	dd	7.2, 9.6	73.5	C1', C3'
3'	3.68	1H	dd	9.6, 3.6	75.0	
4'	3.95	1H	ddd	3.6, 1.2,	71.1	C3'
5' ^h	3.66	1H	dd	1.2, -13.2	69.1	C4'
5' ^l	3.92	1H	dd	2.4, -13.2		C1', C3', C4'

2 NMR spectra were recorded with a JEOL ECA600 spectrometer in D₂O as a solvent.

3 ^h : higher field signals

4 ^l : lower field signals

5

1 **Table 3.** Summary of the NMR analysis of the 1050-Da MAA.

Position	¹ H [ppm]	Number of H	Multiplet	J-value [Hz]	¹³ C [ppm]	HMBC correlation
1					162.0 [†]	
2					128.4	
3					162.3 [†]	
4 ⁿ	2.76 ^{***}	1H	d	-15.0	36.28 ^{††}	C2, C3, C5, C6
4 ⁱ	3.02	1H	d	-17.4		
5					73.5	
6 ⁿ	2.81 ^{***}	1H	d	-17.4	36.33 ^{††}	C1, C2, C4, C5
6 ⁱ	3.06	1H	d	-17.4		
7 ⁿ	3.70	1H	d	10.2	77.8	
7 ⁱ	3.91	1H	d	10.8		
8	3.65	3H	s		62.4	C2
9	3.41	2H	m		45.2	C3
10	1.68	2H	m		29.3	
11	1.98	2H	m		32.2 [‡]	
12	4.26	1H	m		61.0 ^{††}	C11, C13
13					179.1 ⁺⁺⁺	
14					162.4 [†]	
15					132.9	
16					187.1 [†]	
17 ⁿ	2.34	1H	d	-16.8	45.6 ^{†††}	C15, C16, C18, C19, C20
17 ⁱ	2.65 ^{*****}	1H	d	-17.4		
18					75.1	
19 ⁿ	2.78 ^{***}	1H	d	-16.2	35.7	C14, C15
19 ⁱ	2.85	1H	d	-17.4		
20	3.53	2H	s		70.6	C17, C18, C19
21	3.55	3H	s		62.2	C15
22	3.41	2H	m		45.2	C1
23	1.68	2H	m		29.3	
24	1.98	2H	m		32.4 [‡]	
25	4.26	1H	m		61.2 ^{††}	C24, C26
26					179.2 ⁺⁺⁺	
27					162.5 [†]	
28					132.9	
29					187.2 [†]	
30 ⁿ	2.34	1H	d	-16.8	45.7 ^{†††}	C28, C29, C31, C32, C33
30 ⁱ	2.66 ^{*****}	1H	d	-16.8		
31					75.1	
32 ⁿ	2.79 ^{***}	1H	d	-16.8	35.7	C27, C28
32 ⁱ	2.85	1H	d	-17.4		
33	3.53	2H	s		70.6	C30, C31, C32
34	3.55	3H	s		62.2	C28
1'	4.50	1H	d	7.2	105.0	C7
2'	3.69	1H	-	-	83.1	C1', C1''
3'	3.82	1H	-	-	75.7	
4'	3.93	1H	-	-	71.6	C2', C3'
5'	3.68	1H	-	-	78.1	C6'
6'	3.77	2H	-	-	63.9	
1''	4.61	1H	d	8.4	107.6	C2''
2''	3.25	1H	-	-	76.8	C1''
3''	3.41	1H	-	-	78.5	C2'', C4''
4''	3.72	1H	-	-	72.1	

5'' ⁿ	3.25	1H	-	-	68.4	C3''
5'' ^l	3.78	1H	-	-		

1

2 NMR spectra were recorded with a JEOL ECA600 spectrometer in D₂O as a solvent.

3 † †† ††† † †† ††† * ** *** **** : exchangeable signals

4 ^h: higher field signals

5 ^l: lower field signals

6 - : not analyzed due to the overlapping signals

7

1 **Table 4.** Summary of the NMR analysis of the 273-Da MAA.

Position	¹ H [ppm]	Number of H	Multiplet	J-value [Hz]	¹³ C [ppm]	COSY correlation	HMBC correlation
1					185.7		
2					131.1		
3					159.8		
4 ⁿ	2.74	1H	d	-17.4	33.7	H4 ^l	C3
4 ^l	2.86	1H	d	-17.4		H4 ⁿ	
5					73.1		
6 ⁿ	2.36	1H	d	-17.4	44.0	H6 ^l	C1, C5
6 ^l	2.64	1H	d	-17.4		H6 ⁿ	
7	3.51	2H	s		68.7		C4, C5, C6
8	3.55	3H	s		60.0		C2
9	3.39	2H	t	6.7	43.0	H10	C3
10	1.90	2H	tt	6.7, 7.1	26.2	H9, H11	C9, C11, C12
11	2.44	2H	t	7.1	32.1	H10	C9, C12
12					178.9		

2 NMR spectra were recorded with a JEOL ECS400 spectrometer in 30% methanol-d4 in D₂O
3 as a solvent.

4 ^h : higher field signals

5 ^l : lower field signals

6

Table 5. Radical scavenging activity of MAAs.^a

Assay	Colorimetry ^b	ESR ^c	References
	<i>IC₅₀ (mM)</i>		
Ascorbic acid ^d	0.28	0.16	[This study]
Trolox ^d	0.25	0.17	[This study]
Scytonemin ^e	0.57	0.036	[18]
273-Da MAA ($\lambda_{\max}$, 310 nm)	0.60	0.11	[This study]
478-Da MAA ($\lambda_{\max}$, 335 nm)	9.5	0.18	[17]
508-Da MAA ($\lambda_{\max}$, 334 nm)	58	29	[19]
612-Da MAA ($\lambda_{\max}$, 322 nm)	16	0.25	[19]
880-Da MAA ($\lambda_{\max}$, 331 nm)	0.51	0.08	[This study]
1050-Da MAA ($\lambda_{\max}$, 312 nm)	1.04	0.05	[17]

^a Radical scavenging activity was measured with ABTS radical cation as the organic radical source.

^b Decolorization of ABTS radical was monitored with a spectrophotometer for 1 h.

^c ESR signals were monitored with a free radical monitor (JEOL JES-FR30EX).

^d Ascorbic acid and Trolox were used as standards.

^e A hydrophobic UV-absorbing pigment (544 Da) isolated from *N. commune*.
IC₅₀ (50% inhibitory concentration) values are shown.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 7.

Supporting Information

Fig. S1. The chemical structure of the 478-Da MAA. Carbon numbers are shown in italic form and HMBC correlations are shown as red lines.

Fig. S2. Comparison of the glycosylated porphyrin-334 derivatives with a molecular mass of 508 Da. The retention times of these 508-Da MAAs were analyzed by an HPLC system equipped with a reverse phase column (Inertsil ODS-3, 4.6 mm × 250 mm; GL Sciences Inc., Tokyo, Japan) using 0.2% acetic acid at a flow rate of 1 ml min⁻¹ as the mobile phase. MAAs were detected by the A₃₃₀. (A) The 508-Da MAA from the genotype A *N. commune*. (B) The 508-Da MAA from *N. commune* genotype D which has been characterized as a hexose-bound porphyrin-334 at C7 position (Nazifi et al. 2013). (C) 13-*O*-(β-galactosyl)-porphyrin-334 from *Nostoc sphaericum* (generously obtained from Dr. Ishihara, K, unpublished). (D) The co-injection of these three isomers.

Fig. S3. The chemical structure of the 1050-Da MAA. Carbon numbers are shown in italic form and HMBC correlations are shown as red lines.

Fig. S4. A plausible pathway to produce the 880-Da MAA in the genotype B of *Nostoc commune*. The hydrolysis of the 1050-Da MAA and consequently the elimination of 4-deoxygadusol could produce the 880-Da MAA. R indicates 2-*O*-(β-xylopyranosyl)-β-galactopyranoside.

Fig. S5. Two possible stereoisomers for the 880-Da MAAs. Numbering of carbon atoms are the same as used in Table S7. Substituent groups to the central imine-type ring are classified

into three as shown by *X*, *Y*, and *Z*.

Fig. S6. (A) Predicted diastereomeric structure of the 880-Da MAAs. Chemical structure of the substituents expressed by *X*, *Y*, and *Z* are shown in Fig. S5. (B) A chromatogram of the 880-Da MAAs. The purified 880-Da MAAs was analyzed by HPLC with a reverse-phase column (COSMOSIL Cholester, 4.6 mm × 150 mm, Nacalai Tesque) using 0.2% acetic acid at a flow rate of 0.5 ml min⁻¹ as the mobile phase. MAAs were detected by the A₃₃₀.

Fig. S7. The structure for the 273-Da MAA with an absorption maximum at 310 nm and a molecular formula of C₁₂H₁₉NO₆. Red arrows and blue lines represent the HMBC and COSY correlations, respectively.

Table S1. Summary of MALDI-TOF MS/MS analysis of the purified 464-Da MAA.

Table S2. Summary of ¹³C-NMR analysis of the 464-Da MAA.

Table S3. Summary of MALDI-TOF MS/MS analysis of the purified 508-Da MAA.

Table S4. Comparison of the ¹³C-NMR chemical shifts of the galactose moiety of the 1050-Da MAA, 1,*n*-*O*-dimethyl-β-D-galactopyranosides and 1-*O*-methyl-*n*-*O*-β-L-fucopyranosyl-β-D-galactopyranosides.

Table S5. Comparison of IR spectra of the 880-Da MAA with the 1050-Da MAA.

Table S6. Summary of MALDI-TOF MS/MS analysis of the purified 880-Da MAA.

Table S7. ^{13}C -NMR signal comparison between the 1050-Da MAA and the 880-Da MAA.

Table S8. Comparison of IR spectra of the 273-Da MAA with known MAAs.

Table S9. Summary of MALDI-TOF MS/MS analysis of the purified 273-Da MAA.

Supplementary data

1. NMR data of the 478-Da MAA

2. NMR data of the 464-Da MAA

3. NMR data of the 1050-Da MAA

4. NMR data of the 880-Da MAA

5. GC-MS data of the determination of the sugars

6. NMR data of the 273-Da MAA

Fig. S1.

Fig. S1. The chemical structure of the 478-Da MAA. Carbon numbers are shown in italic form and HMBC correlations are shown as red lines.

Fig. S2.

Fig. S2. Comparison of the glycosylated porphyrin-334 derivatives with a molecular mass of 508 Da. The retention times of these 508-Da MAAs were analyzed by an HPLC system equipped with a reverse phase column (Inertsil ODS-3, 4.6 mm × 250 mm; GL Sciences Inc., Tokyo, Japan) using 0.2% acetic acid at a flow rate of 1 ml min⁻¹ as the mobile phase. MAAs were detected by the A₃₃₀. (A) The 508-Da MAA from the genotype *A. N. commune*. (B) The 508-Da MAA from *N. commune* genotype D which has been characterized as a hexose-bound porphyrin-334 at C7 position [Nazifi et al. 2013, ref. 19]. (C) 13-*O*-(β-galactosyl)-porphyrin-334 from *Nostoc sphaericum* (generously obtained from Dr. Ishihara, K, unpublished). (D) The co-injection of these three isomers.

Fig. S3.

Fig. S3. The chemical structure of the 1050-Da MAA. Carbon numbers are shown in italic form and HMBC correlations are shown as red lines.

Fig. S4.

Fig. S4. A plausible pathway to produce the 880-Da MAA in the genotype B of *Nostoc commune*. The hydrolysis of the 1050-Da MAA and consequently the elimination of 4-deoxygadusol could produce the 880-Da MAA. R indicates 2-*O*-(β -xylopyranosyl)- β -galactopyranoside.

Fig. S5.

Fig. S5. Two possible stereoisomers for the 880-Da MAA. Numbering of carbon atoms are the same as used in Table S7. Substituent groups to the central imine-type ring are classified into three as shown by X, Y, and Z.

Fig. S6.

Fig. S6. (A) Predicted diastereomeric structure of the 880-Da MAAs. Chemical structure of the substituents expressed by X, Y, and Z are shown in Fig. S5. (B) A chromatogram of the 880-Da MAAs. The purified 880-Da MAAs was analyzed by HPLC with a reverse-phase column (COSMOSIL Cholester, 4.6 mm $\times$ 150 mm, Nacalai Tesque) using 0.2% acetic acid at a flow rate of 0.5 ml min⁻¹ as the mobile phase. MAAs were detected by the A₃₃₀.

Fig. S7.

Fig. S7. The structure for the 273-Da MAA with an absorption maximum at 310 nm and a molecular formula of C₁₂H₁₉NO₆. Red arrows and blue lines represent the HMBC and COSY correlations, respectively.

Table S1. Summary of MALDI-TOF MS/MS analysis of the purified 464-Da MAA.

	Mass of fragment	Relative abundance	Neutral loss	Deleted fragment
MS of the purified 464-Da MAA	465.13	100		
	421.14	40	44	CO ₂
	407.12	8	58	CO ₂ + CH ₂
	377.12	21	88	2 CO ₂
	362.94	18	103	2 CO ₂ + CH ₃
	332.98	9	133	2 CO ₂ + C ₂ H ₅ O
MS ² of m/z 465	465.13			
	403.38	13	62	CO ₂ + H ₂ O
	361.38	21	104	-
	333.31	86	132	Pentose
	301.30	15	164	Pentose + CH ₃ + OH
	285.30	18	180	Pentose + OCH ₃ + OH
	257.27	13	208	Pentose + CH ₃ + OH + CO ₂
	73.13	100	392	-
	421.14			
MS ² of m/z 421	362.35	18	59	CO ₂ + CH ₃
	331.34	80	90	CO ₂ + CH ₃ + OCH ₃
	317.36	61	104	CO ₂ + CH ₃ + OCH ₃ + CH ₂
	289.32	17	132	Pentose
	287.30	29	134	-
	257.29	15	164	Pentose + CH ₃ + OH
	241.30	15	180	Pentose + OCH ₃ + OH
	73.13	100	348	-
	377.12			
MS ² of m/z 377	317.32	25	60	-
	245.29	24	132	Pentose
	243.29	31	134	-
	213.27	70	164	Pentose + CH ₃ + OH
	211.29	64	166	-
	207.27	32	170	-
	73.13	100	304	-

Table S2. Summary of ^{13}C -NMR analysis of the 464-Da MAA.

C#	464-Da MAA	Shinorine Tsuji et al. (1980)	Shinorine Oyamada et al. (2008)	478-Da MAA Matsui et al. (2011)
1	161.6 ^a	161.8	162.3	161.6
2	128.4	128.1	128.7	128.4
3	163.1 ^{a'}	162.9	163.5	163.2
4	36.5 ^b	35.8	35.8	36.4
5	73.1	73.4	74.0	73.1
6	36.1 ^{b'}	35.3	36.2	35.9
7	77.7	69.7	70.3	77.7
8	61.3	61.7	62.2	62.2
9	49.6	47.9	49.0	49.5
10	177.4 ^c	175.3	177.0	177.7
11	62.2	61.8	63.0	67.3
12	64.1	64.4	65.3	71.0
13	177.6 ^{c'}	175.3	177.0	178.2
14				22.3
Pentose		Pentose (Ara)		
1'	106.4			106.4
2'	73.7			73.5
3'	75.1			75.0
4'	72.0			71.1
5'	69.1			69.1

NMR spectra were recorded with a JEOL ECS400 spectrometer in D_2O as a solvent. Chemical shifts (ppm) for the 464-Da MAA are compared with those for shinorine [Tsuji et al. 1980, ref. 47; Oyamada et al. 2008, ref. 39] and the 478-Da arabinose-bound porphyrin-334 [Matsui et al. 2011, ref. 17; this study]. Chemical shift values denoted as x and x' can be exchangeable. Chemical shifts of shinorine reported by Oyamada et al. (2008) [ref. 39] were reanalyzed from the NMR spectrum.

Table S3. Summary of MALDI-TOF MS/MS analysis of the purified 508-Da MAA.

	Mass of fragment	Relative abundance	Neutral loss	Deleted fragment
MS of the purified 508-Da MAA	509.21	100		
	465.22	39	44	CO ₂
	451.20	7	58	CO ₂ + CH ₂
	447.20	7	62	CO ₂ + H ₂ O
	421.23	20	88	2 CO ₂
	407.17	11	102	2 CO ₂ + CH ₂
	403.21	7	106	2 CO ₂ + H ₂ O
509.21				
MS ² of m/z 509	375.22	24	134	-
	347.19	100	162	Hexose
	308.36	35	201	-
	299.21	12	210	Hexose + 2 CH ₃ + H ₂ O
	271.18	15	238	Hexose + C ₂ H ₄ O + H ₂ O + CH ₂
	225.25	17	284	-
	198.20	37	311	-
	197.21	43	312	-
465.22				
MS ² of m/z 465	405.21	33	60	-
	361.25	25	104	-
	331.22	51	134	-
	303.21	100	162	Hexose
	301.20	69	164	-
	271.20	24	194	Hexose + H ₂ O + CH ₂
	269.23	25	196	-
	255.21	26	210	Hexose + 2 CH ₃ + H ₂ O
421.23				
MS ² of m/z 421	361.21	100	60	-
	348.20	49	73	-
	287.22	16	134	-
	259.21	54	162	Hexose
	257.20	76	164	-
	227.21	24	194	Hexose + H ₂ O + CH ₂
	225.23	22	196	-
	221.18	27	200	-
	213.17	23	208	Hexose + H ₂ O + 2 CH ₂
	211.20	22	210	Hexose + H ₂ O + 2 CH ₃
	199.19	25	222	Hexose + H ₂ O + 3 CH ₂
	191.16	16	230	-
	189.15	27	232	-

Table S4. Comparison of the ^{13}C -NMR chemical shifts of the galactose moiety of the 1050-Da MAA, 1,n-*O*-dimethyl- β -D-galactopyranosides and 1-*O*-methyl-n-*O*- β -L-fucopyranosyl- β -D-galactopyranosides.

1,n- <i>O</i> -dimethyl- β -D-galactopyranoside ^{a, b}						
Methylation position	C1	C2	C3	C4	C5	C6
1 ^a	104.5	71.7	73.8	69.7	76.0	62.0
1, 2 ^b	104.2	81.1^d	73.8	69.3	75.5	61.6
1, 3 ^b	104.3	71.2	82.5^d	64.7	75.6	61.5
1, 4 ^b	104.2	70.2	72.9	79.6^d	75.8	61.1
1, 6 ^b	104.3	71.6	73.3	69.5	73.9	72.2^d
1- <i>O</i> -methyl-n- <i>O</i> -fucopyranosyl- β -D-galactopyranoside ^c						
Glycosylation	C1	C2	C3	C4	C5	C6
C2	103.6	79.4^d	72.8	69.6	76.2	62.2
C3	104.9	70.5	81.5^d	67.6	76.1	62.1
1050-Da MAA						
1,2-linkage	105.0	83.1^d	75.7	71.6	78.1	63.9

The ^{13}C -NMR chemical shifts of 1-*O*-methyl- β -D-galactopyranoside, 1,n-*O*-dimethyl- β -D-galactopyranosides and 1-*O*-methyl-n-*O*- β -L-fucopyranosyl- β -D-galactopyranosides were obtained from the references:

^a K. Bock, C. Pedersen, Carbon-13 Nuclear magnetic resonance spectroscopy of monosaccharides. Adv. Carbohydr. Chem. Biochem. 41 (1983) 27-66.

^b M. Vogtherr, T. Peters, Application of NMR based binding assays to identify key hydroxy groups for intermolecular recognition. J. Am. Chem. Soc. 122 (2000) 6093-6099.

^c A.S. Shashkov, N.E. Nifant'ev, V.Y. Amochaeva, N.K. Kochetkov, ^1H and ^{13}C NMR data for 2-*O*-, 3-*O*- and 2,3,-di-*O*-glycosylated methyl α - and β -D-glucopyranosides and β -D-galactopyranosides. Magn. Reson. Chem. 31 (1993) 599-613.

^d Chemical shift values belong to the substitution positions were shown by bold character.

Table S5. Comparison of IR spectra of the 880-Da MAA with the 1050-Da MAA.

880-Da MAA	1050-Da MAA
<i>Wavenumber (cm⁻¹)</i>	
3374	3399
2942	2929
1603	1617
1548	1541
1391	1400
1334	
1276	1275
1199	
1124, 1110	
1079, 1046	1076, 1046
980	
890	

IR spectra were recorded with a Fourier transform infrared (FT-IR) spectrometer (Nicolet NEXUS 470 FT-IR) according to the KBr disk method. The wavenumbers of absorption bands for the 880-Da MAA are compared with those for the 1050-Da MAA [Matsui et al. 2011, ref. 17].

Table S6. Summary of MALDI-TOF MS/MS analysis of the purified 880-Da MAA.

	Mass of fragment	Relative abundance	Neutral loss	Deleted fragment
MS of the purified 880-Da MAA	881.44	100		
	837.45	83	44	CO ₂
	793.45	57	88	2 CO ₂
	766.37	22	115	C ₅ H ₉ NO ₂
	722.38	23	159	C ₅ H ₉ NO ₂ + CO ₂
	596.30	53	285	C ₁₃ H ₁₉ NO ₆
	552.30	32	329	C ₁₃ H ₁₉ NO ₆ + CO ₂
	481.22	21	400	C ₅ H ₉ NO ₂ + C ₁₃ H ₁₉ NO ₆
881.44				
MS ² of m/z 881	781.38	51	100	-
	763.38	8	118	-
	749.36	18	132	Pentose
	731.37	29	150	Pentose + H ₂ O
	569.33	32	312	Pentose + H ₂ O + Hexose
	551.33	26	330	Pentose + 2 H ₂ O + Hexose
	533.32	100	348	Pentose + 3 H ₂ O + Hexose
	515.30	19	366	Pentose + 4 H ₂ O + Hexose
837.45				
MS ² of m/z 837	737.37	99	100	-
	705.38	28	132	Pentose
	687.40	14	150	Pentose + H ₂ O
	673.28	20	164	Pentose + H ₂ O + CH ₂
	525.33	26	312	Pentose + H ₂ O + Hexose
	507.34	24	330	Pentose + 2 H ₂ O + Hexose
	491.22	30	346	-
	489.31	100	348	Pentose + 3 H ₂ O + Hexose
	471.29	25	366	Pentose + 4 H ₂ O + Hexose
793.45				
MS ² of m/z 793	693.40	67	100	-
	604.36	16	189	-
	552.33	18	241	-
	499.37	9	294	Pentose + Hexose
	463.33	23	330	Pentose + 2 H ₂ O + Hexose
	447.26	13	346	-
	445.32	100	348	Pentose + 3 H ₂ O + Hexose
	428.30	30	365	Pentose + 3 H ₂ O + Hexose (C ₆ H ₁₁ O ₆)
	392.27	11	401	-
	374.26	18	419	-
	363.32	14	430	-
	356.25	24	437	-
	240.17	53	553	-
	222.16	66	571	-

208.17	37	585	-
206.16	25	587	-
192.17	18	601	-
190.17	12	603	-
72.11	95	721	-

Table S7. ^{13}C -NMR signal comparison between the 1050-Da MAA and the 880-Da MAA.

C#	1050-Da MAA	880-Da MAA
1, 3, 14, 27	162.0	161.6
	162.3	161.8
	162.4	161.9
	162.5	162.3
2	128.4	128.4
	35.7	35.3
4, 6, 19, 32	<i>35.7^a</i>	<i>35.3^a</i>
	36.3	35.6
	<i>36.3^a</i>	<i>35.6^a</i>
5	73.5	73.5
15, 28	132.9	132.9
	<i>132.9^a</i>	<i>132.9^a</i>
16, 29	187.1	187.6
	187.2	187.7
17, 30	45.6	45.9
	45.7	<i>45.9^a</i>
18, 31	75.1	75.0
	<i>75.1^a</i>	<i>75.0^a</i>
7	77.8	77.8
20, 33	70.6	70.6
	<i>70.6^a</i>	<i>70.6^a</i>
9, 22	45.2	45.1
	<i>45.2^a</i>	<i>45.1^a</i>
10, 23	29.3	29.3
	<i>29.3^a</i>	29.4
11, 24	32.2	32.3
	32.4	32.5, 32.7
12, 25	61.0	61.1
	61.2	61.5, 61.5
21, 34	62.2	61.9
	<i>62.2^a</i>	<i>61.9^a</i>
8	62.4	62.3
13, 26	179.1	178.7, 178.8
	179.2	179.0, 179.0
1'	105.0	105.0, 105.0
2'	83.1	83.0
3'	75.7	75.8, 75.8
4'	71.6	71.5
5'	78.1	78.1
6'	63.9	63.9
1''	107.6	107.5, 107.5
2''	76.8	76.9
3''	78.5	78.6
4''	72.1	72.2
5''	68.4	68.4

^a Chemical shift values in italic indicate the overlapping multiple signals.

Table S8. Comparison of IR spectra of the 273-Da MAA with known MAAs.

273-Da MAA	Methyl ester of mycosporine-glycine	Porphyra-334
<i>Wavenumber (cm⁻¹)</i>		
3375		
	3300	3300
2942		
1712	1740	
1613	1620 ^{sh}	1600
1563-1510	1570-1550	1540
1405		
1363		1380
1274		
1198		
1130, 1111		
1080, 1043		1080
978, 928		
893, 868, 835		

IR spectra were recorded with a Fourier transform infrared (FT-IR) spectrometer (Nicolet NEXUS 470 FT-IR) according to the KBr disk method. The wavenumbers of the absorption bands for the 273-Da MAA are compared to those for methyl ester of mycosporine-glycine [Ito and Hirata 1977, ref. 50] and porphyrin-334 [Torres et al. 2006, ref. 51].

Table S9. Summary of MALDI-TOF MS/MS analysis of the purified 273-Da MAA.

	Mass of fragment	Relative abundance	Neutral loss	Deleted fragment
MS of the purified 273-Da MAA	274.10	100		
MS ² of m/z 274	228.19	18	46	CH ₃ O + CH ₃
	186.16	18	88	C ₄ H ₈ O ₂
	137.10	100	137	-
	100.14	8	174	-
	87.11	10	187	C ₈ H ₁₃ NO ₄

Fig. 1H-NMR spectrum of 478-Da MAA in D₂O.

Fig. ^{13}C -NMR spectrum of 478-Da MAA in D_2O .

Fig. HMQC spectrum of 478-Da MAA in D₂O.

Fig. HMQC spectrum of 478-Da MAA in D₂O.

Fig. HMQC spectrum of 478-Da MAA in D₂O.

Fig. HMQC spectrum of 478-Da MAA in D₂O.

Fig. HMBC spectrum of 478-Da MAA in D₂O.

Fig. HMBC spectrum of 478-Da MAA in D₂O.

Fig. HMBC spectrum of 478-Da MAA in D₂O.

Fig. HMBC spectrum of 478-Da MAA in D₂O.

Peak simulation of arabinopyranose between 3.5-4.5 ppm

Frequency= 600.0 MHz

δ_1 : 4.383 ppm, $\Delta\nu$: 1.2 Hz
 δ_2 : 3.6165 ppm, $\Delta\nu$: 1.2 Hz
 δ_3 : 3.6975 ppm, $\Delta\nu$: 1.2 Hz
 δ_4 : 3.968 ppm, $\Delta\nu$: 1.2 Hz
 δ_5 : 3.676 ppm, $\Delta\nu$: 1.2 Hz
 δ_6 : 3.934 ppm, $\Delta\nu$: 1.0 Hz
 δ_7 : 3.682 ppm, $\Delta\nu$: 1.0 Hz
 δ_8 : 3.911 ppm, $\Delta\nu$: 1.0 Hz
 $J_{1,2}$ = 7.2 Hz
 $J_{1,3}$ = 0.0 Hz
 $J_{1,4}$ = 0.0 Hz
 $J_{1,5}$ = 0.0 Hz
 $J_{1,6}$ = 0.0 Hz
 $J_{1,7}$ = 0.0 Hz
 $J_{1,8}$ = 0.0 Hz
 $J_{2,3}$ = 9.6 Hz
 $J_{2,4}$ = 0.0 Hz
 $J_{2,5}$ = 0.0 Hz
 $J_{2,6}$ = 0.0 Hz
 $J_{2,7}$ = 0.0 Hz
 $J_{2,8}$ = 0.0 Hz
 $J_{3,4}$ = 3.6 Hz
 $J_{3,5}$ = 0.0 Hz
 $J_{3,6}$ = 0.0 Hz
 $J_{3,7}$ = 0.0 Hz
 $J_{3,8}$ = 0.0 Hz
 $J_{4,5}$ = 1.2 Hz
 $J_{4,6}$ = 2.4 Hz
 $J_{4,7}$ = 0.0 Hz
 $J_{4,8}$ = 0.0 Hz
 $J_{5,6}$ = -13.2 Hz
 $J_{5,7}$ = 0.0 Hz
 $J_{5,8}$ = 0.0 Hz
 $J_{6,7}$ = 0.0 Hz
 $J_{6,8}$ = 0.0 Hz
 $J_{7,8}$ = 10.2 Hz

supplemental NMR data for 464-Da MAA.

Fig. 1H-NMR spectrum of 464-Da MAA in D₂O.

Fig. 13C-NMR spectrum of 464-Da MAA in D2O.

Fig. 1H-NMR spectrum of 1050-Da MAA in D₂O.

Fig. ^{13}C -NMR spectrum of 1050-Da MAA in D_2O . Enlarged spectra are shown in the inset.

Fig. DEPT 135 (A), 90 (B), 45 (C), and ¹³C-NMR (D) spectra of 1050-Da MAA in D₂O.

Fig. Enlarged figure of DEPT 135 (A), 90 (B), 45 (C), and ^{13}C -NMR (D) spectra of 1050-Da MAA in D_2O .

Fig. Enlarged figure of DEPT 135 (A), 90 (B), 45 (C), and ¹³C-NMR (D) spectra of 1050-Da MAA in D₂O.

Fig. Enlarged figure of DEPT 135 (A), 90 (B), 45 (C), and 13C-NMR (D) spectra of 1050-Da MAA in D₂O.

Fig. HMQC spectrum of 1050-Da MAA in D₂O.

Fig. Inset-1 of HMQC spectrum of 1050-Da MAA in D₂O.

Fig. Inset-2 of HMQC spectrum of 1050-Da MAA in D₂O.

Fig. HMBC spectrum of 1050-Da MAA in D₂O.

Fig. Inset-1 of HMBC spectrum of 1050-Da MAA in D₂O.

Fig. Inset-2 of HMBC spectrum of 1050-Da MAA in D₂O.

Fig. Inset-3 of HMBC spectrum of 1050-Da MAA in D₂O.

Fig. Inset-4 of HMBC spectrum of 1050-Da MAA in D₂O.

Fig. ^1H -NMR spectrum of 880-Da MAA in D_2O (30v% CD_3OD).

Fig. ^{13}C -NMR spectrum of 880-Da MAA in D_2O (30v% CD_3OD). Enlarged spectra are shown in the inset.

Fig. DEPT 135 (A), 90 (B), and ^{13}C -NMR (C) spectra of 880-Da MAA in D₂O (30v% CD₃OD).

Fig. Enlarged figure of DEPT 135 (A), 90 (B), and ¹³C-NMR (C) spectra of 880-Da MAA in D2O (30v% CD3OD).

Fig. HMQC spectrum of 880-Da MAA in D₂O (30v% CD₃OD).

Fig. Inset-1 of HMQC spectrum of 880-Da MAA in D2O (30v% CD3OD).

Fig. Inset-2 of HMQC spectrum of 880-Da MAA in D2O (30v% CD3OD).

Fig. HMBC spectrum of 880-Da MAA in D₂O (30v% CD₃OD).

Fig. Inset-1 of HMBC spectrum of 880-Da MAA in D₂O (30v% CD₃OD).

Fig. Inset-2 of HMBC spectrum of 880-Da MAA in D₂O (30v% CD₃OD).

Fig. Inset-3 of HMBC spectrum of 880-Da MAA in D₂O (30v% CD₃OD).

Fig. Inset-4 of HMBC spectrum of 880-Da MAA in D₂O (30v% CD₃OD).

478-Da MAA

1050-Da MAA

880-Da MAA

Fig. ^1H -NMR spectrum of 273-Da MAA in D_2O (30 v% CD_3OD).

Fig. ^{13}C -NMR spectrum of 273-Da MAA in D_2O (30 v% CD_3OD).

Fig. DEPT (135) spectrum of 273-Da MAA in D2O (30 v% CD3OD).

Fig. HMQC spectrum of 273-Da MAA in D₂O (30 v% CD₃OD).

Fig. HMBC spectrum of 273-Da MAA in D₂O (30 v% CD₃OD).

Fig. Inset-2 of HMBC spectrum of 273-Da MAA in D₂O (30 v% CD₃OD).

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