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Neurotoxic amino acids and their isomers in desert environments

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ABSTRACT

Cyanobacteria are capable of producing a wide range of bioactive compounds including highly toxic molecules which affect a variety of molecular targets. These toxins are well known from aquatic environments, and their occurrence in terrestrial environments is now gaining attention. Of the toxins produced by cyanobacteria, β-*N*-methylamino-L-alanine (BMAA) is a neurotoxic amino acid linked to human neurodegenerative diseases including Amyotrophic Lateral Sclerosis (ALS), Alzheimer's and Parkinson's disease. Two isomers of BMAA are known to be produced by cyanobacteria in axenic culture and in cyanobacterial blooms. Desert crust material was assessed for the presence of BMAA isomers. In addition to BMAA, 2,4-diaminobutyric acid (DAB) and N-(2-aminoethyl)glycine (AEG), were found to be present in desert environments. Both BMAA and DAB are known to be toxic. A preliminary assessment of AEG toxicity was performed with *Artemia salina*. At high concentrations, AEG was shown to cause mortality of *A. salina* with paralysis observed at lower concentrations. These findings show that the co-occurrence of BMAA, DAB and AEG may lead to adverse human and animal health effects and future research should consider the interaction of these three isomers for their effect on human health in arid environments.

1. Introduction

Cyanobacteria and their toxins have been found in a wide range of environments. Cyanobacteria are frequently recorded in lakes and marine environments, where they can cause issues with respect to the mortality of aquatic life and can produce toxins as well as taste and odour compounds (Codd et al., 1999, 2005). They are also found in a wide variety of terrestrial environments, including hot geothermal springs, and can grow in polar environments. They are known to inhabit terrestrial sites, where they are often found in symbiosis with lichens and are common inhabitants of well fertilised golf courses (Adams et al., 2012; Whitton, 2000). Perhaps, the largest impact of cyanobacteria in terrestrial environments is where they occur as desert crusts in a large part of the world (Belnap et al., 2003; Hu et al., 2012). The majority of these crusts are composed of *Microcoleus* and the polysaccharide tubes produced by these cyanobacteria are largely responsible for stabilising the soil as sand grains stick to the tubes producing a more stable substrate to allow plants to colonise (Hu et al., 2012).

Cyanobacteria have the potential to produce a range of highly toxic, low molecular weight compounds (Codd et al., 1999, 2005; Metcalf and Codd, 2012). The most commonly detected cyanotoxins are the microcystins and to a lesser extent, the related nodularins. These are cyclic heptapeptides and pentapeptides, respectively, and are found in cyanobacterial genera including *Microcystis*, *Anabaena* and *Planktothrix*. They are hepatotoxic and act by inhibition of protein phosphatases and phosphoprotein phosphatases (Hastie et al., 2005), and cause death by hypovolaemic shock, where the liver macrostructure breaks down and blood pools into the liver. Cyanobacteria are also capable of producing cylindrospermopsins, toxic guanidine alkaloids that are hepatotoxic and are considered to be carcinogenic (Humpage, 2008). They act by inhibition of protein translation (Froscio et al., 2001) and are commonly produced by *Cylindrospermopsis* and *Aphanizomenon* (Metcalf and Codd, 2012). Anatoxin-a is an alkaloid often produced by *Phormidium* species that can grow as mats on sediments and are commonly attractive to dogs, due to the production of taste and odour compounds such as geosmin (Codd et al., 1992; Edwards et al., 1992; Chatziefthimiou et al., 2014). Anatoxin-a acts as an acetylcholine mimic and causes death by paralysis. Anatoxin-a(*S*), although similarly named is an organophosphorous compound which acts in a manner similar to organophosphate pesticides by inhibition of acetylcholine esterases, the latter being used in their detection (Devic et al., 2002; Metcalf et al., 2012).

Although most cyanotoxins produced are sporadic and not all species or members of a genus can produce them, some toxic compounds produced by cyanobacteria are considered to be common. These include lipopolysaccharide (LPS), a characteristic of

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Gram negative bacteria (Drews and Weckesser, 1982), and the neurotoxic amino acid β-N-methylamino-L-alanine (BMAA; Cox et al., 2005) which has been linked to human neurodegenerative diseases (Cox et al., 2003; Murch et al., 2004; Pablo et al., 2009).

Analysis of cyanobacterial toxins in crust material from desert environments has found microcystins, detected by immunoassay, high performance liquid chromatography and PCR for the microcystin synthetase (*mcyD*) gene to show their presence, presumably produced by *Microcoleus*, as this organism is a member of the Oscillatoriales which contains many microcystin-producing members (Metcalf et al., 2012). Enzyme inhibition assay analysis of similar crust material from Qatar has indicated that anatoxin-a(*S*) is present in desert materials, although confirmation of the presence of this toxin is difficult. Finally, analysis of crust material has shown the presence of BMAA in desert crust material, with the potential to be produced by a range of desert cyanobacterial organisms (Cox et al., 2009).

The presence of BMAA in desert materials raises important questions concerning the risk associated with neurodegenerative disease and also shows the need to definitively identify the isomers present. Including BMAA, there are four naturally-occurring isomers known to exist. These are BMAA (Cox et al., 2005), 2,4-diaminobutyric acid (DAB; Banack et al., 2010), N-2(aminoethyl) glycine (AEG; Banack et al., 2012) and β-amino-*N*-methyl-alanine (BAMA; Jiang et al., 2012). BMAA and DAB are known neurotoxins (e.g. Purdie et al., 2009; Weiss et al., 1989), but little information is known concerning the presence and toxicity of AEG in desert environments. The purpose of this study was to assess desert cyanobacterial crust for the presence of BMAA isomers and to perform preliminary toxicity assessment of AEG with *Artemia salina* nauplii.

2. Materials and methods

2.1. Sampling of desert cyanobacteria

Cyanobacterial crust material was collected at Al Dawodyu (25°55′0.5″N, 51°9′37″E) and Al Kharrara (24°53′11.6″N, 51°16′27.4″E) in the deserts of Qatar and returned to the laboratory where it was washed and air dried. Subsamples were stored in 20% ethanol and Lugol's iodine for later microscopy to confirm the presence of cyanobacteria. Cyanobacteria were examined by light and fluorescence microscopy and cyanobacteria were confirmed to genus level according to Whitton (2002).

2.2. Extraction of BMAA isomers from crust material

The area and weight of crust material were recorded and a subsample was placed into a 3 ml glass vial for BMAA isomer extraction. To this dried material, 500 μl 6 M HCl were added and the reaction between the carbonate present within the desert material and the acid was allowed to proceed. Samples were stored at room temperature overnight. The following day, a further volume of 6 M HCl was added and any reaction between the acid and the carbonate noted. If a second reaction was observed, the samples were stored at room temperature overnight. If no reaction was observed, then the samples were hydrolysed at 110 °C for 16 h. The addition of 6 M HCl was continued until no reaction was observed between the carbonate present within the samples and the 6 M HCl prior to hydrolysis. Once all of the samples had been hydrolysed, a subsample was removed and centrifuge filtered at 12,000× *g*. The filtrate was then dried in a speedvac and stored at 4 °C until analysed.

2.3. Analysis of BMAA isomers in desert crust material

Extracts were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) and analysed by triple quadrupole LC-MS/MS (Thermo Scientific Finnigan TSQ Quantum UltraAM, San Jose, CA) after separation by a Waters Acquity- UHPLC system with a Binary Solvent Manager, Sample Manager and a Waters AccQTag Ultra column (part# 186003837, 2.1 × 100 mm) at 55 °C (Banack et al., 2010, 2011, 2012). Separation was achieved using gradient elution at 0.65 ml/min in aqueous 0.1% (v/v) formic acid (Eluent A) and 0.1% (v/v) formic acid in acetonitrile (Eluent B): 0.0 min @ 99.1% A; 0.5 min @ 99.1% A curve 6; 2 min @ 95% A curve 6; 3 min @ 95% A curve 6; 5.5 min @ 90% A curve 8; 6 min @ 15% A curve 6; 6.5 min @ 15% A curve 6; 6.6 min @ 99.1% A curve 6; 8 min @ 99.1% A curve 6. Nitrogen gas was supplied to the heated electrospray ionization (H-ESI) probe with a nebulizing pressure of 40 psi and a vaporizer temperature of 400 °C. The mass spectrometer was operated under the following conditions: the capillary temperature was set at 270 °C, capillary offset of 35, tube lens offset of 110, auxiliary gas pressure of 35, spray voltage 3500 V, source collision energy of 0 eV, and multiplier voltage of −1719 V. The second quadrupole was pressurized to 1.0 m Torr with argon. Ion *m/z* 459 was isolated in the first quadrupole filter as the precursor ion and subjected to collision induced dissociation (CID). Ion masses were analysed using selected reaction monitoring (SRM) of BMAA isomers after CID in the collision cell. Identification of BMAA isomers was based upon (a) the presence of the parent ion *m/z* 459; (b) retention time; (c) presence of collision-induced product ions (*m/z* 171 quantifier ion; *m/z* 289, *m/z* 119 qualifier ions); (d) ratios of qualifier ions relative to the quantifier ion (evaluation was based on the area calculated by Xcalibur software which was limited by the area at the specific retention time of the qualifying peaks (*m/z* 188, 214, and 258) at half peak height); (e) the presence of the qualifier ions for the different isomers (*m/z* 188 DAB, *m/z* 214 AEG, *m/z* 258 BMAA). All samples were compared with AQC-derivatised authenticated standards (AEG, TCI America # A1153, DAB (32830) and BMAA (B107), Sigma Chemical Co., St. Louis, MO).

2.4. Brine shrimp assessment of AEG toxicity

A. salina bioassays were carried out according to Metcalf et al. (2002). Brine shrimp cysts (*A. salina*) were placed in artificial seawater with aeration and illumination for 24 h to allow cysts to hatch. Hatched nauplii were transferred to fresh artificial seawater and incubated for a further 24 h with aeration and illumination. After a total of 48 h, the nauplii were diluted to a concentration of 100 organisms per ml. To the wells of a microtitre plate, 100 μl of the *A. salina* organisms were added prior to the addition of AEG. AEG (TCI America) was dissolved in artificial seawater at a concentration of 1 g per ml and diluted with artificial seawater to concentrations between 10 μg and 1 g per ml. To the wells of the microtitre plate, 100 μl of the AEG dilutions were added ($n = 3$) and the microtitre plate covered with parafilm and incubated at room temperature with illumination. Mortality and general health of the brine shrimp was assessed at 24 h intervals up to a period of 96 h.

3. Results

Analysis of desert terrestrial materials by triple quadrupole LC-MS/MS indicated that at least BMAA and 2 isomers were present, namely BMAA, DAB, AEG (Figs. 1 and 2, Table 1). The three isomers were separated by time and by the presence of qualifying ions, namely 188 (DAB), 214 (AEG) and 258 (BMAA) (Fig. 3). Using reverse phase separation, AEG eluted first (5.52 min), BMAA was next (5.57 min) and DAB was last (5.67 min). The ion ratios from *m/z* 459 to 289 and 119 were useful for identification of the isomer as they were markedly different between the BMAA isomers and the ratios were consistent when compared with authenticated standards. These ion ratios (289:119) were 15:19 (AEG), 18:34 (BMAA)