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7-Deoxy-desulfo-cylindrospermopsin and 7-deoxy-desulfo-12-acetylcylindrospermopsin: Two new cylindrospermopsin analogs isolated from a Thai strain of *Cylindrospermopsis raciborskii*

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ABSTRACT

As environmental changes such as eutrophication lead to increased size and frequency of cyanobacterial blooms, research into the toxins produced by these blooms becomes increasingly important. One of the common toxins produced by cyanobacterial blooms is cylindrospermopsin (**1**), a potent inhibitor of protein synthesis. To date, only two additional analogs of cylindrospermopsin have been isolated, namely 7-epicylindrospermopsin (**2**) and 7-deoxy-cylindrospermopsin (**3**). This report details the isolation and structure determination of an additional two new analogs, 7-deoxy-desulfo-cylindrospermopsin (**4**) and 7-deoxy-desulfo-12-acetylcylindrospermopsin (**5**). These are the first new analogs of cylindrospermopsin to be reported in over a decade. Based on their structural features, it is likely that these new analogs also possess the harmful biological activities displayed by the rest of the cylindrospermopsin family.

1. Introduction

Cyanobacterial harmful algal blooms (CHABs) pose serious concerns for human and animal health as well as severe economic concerns for surrounding communities as fisheries and recreational waterways are closed during bloom events (Fristachi and Sinclair, 2008; Dodds et al., 2009). Because of the serious effects of these blooms, a major research focus in recent years has centered on understanding the toxic nature of these blooms. Extensive chemical investigation over the last several decades has identified several main families of freshwater CHAB toxins including microcystins, anatoxins, saxitoxins, lyngbyatoxins, nodularins and cylindrospermopsins. Some of these toxin families contain a large number of analogs such as the microcystin family with over 60 reported congeners while others such as the cylindrospermopsin family contain only 3 previously reported structures (Fig. 1) (Norris et al., 1999; Banker et al., 2000; Lifshits and Carmeli, 2012).

Cylindrospermopsins are produced primarily by cyanobacteria of the genera *Cylindrospermopsis* but can also be found in several additional genera including *Aphanizomenon*, *Lyngbya*, *Raphidiopsis* and *Umezakia* (Ohtani and Moore,1992; Banker et al., 2000; Li et al., 2001; Seifert et al., 2007). The most common producer of cylindrospermopsin is *Cylindrospermopsis raciborskii*, once thought to be only present in tropical environments, can now be found in many sub-tropical and temperate locations (Dyble et al., 2002). It appears to be especially invasive in the midwest, southeast and southwest regions of the United States (Calandrino and Paerl, 2011). A recent survey of reservoirs in North Carolina found *C. raciborskii* to be present in 100% of the water samples at one of the highest abundance with 10^4 cells/mL on average (Touchette et al., 2007).

Cylindrospermopsins are important to CHAB research because of their potent toxicity as well as potential chronic effects at exposure levels below the toxicity threshold. While a thorough investigation into the mechanism of toxicity of cylindrospermopsin is far from complete, initial molecular studies indicate that it inhibits protein synthesis, glutathione synthesis and pyrimidine synthesis, as well as causing DNA strand breakage, hepatotoxicity, and changes in the levels cholesterol and phospholipids in red blood cells (Carson, 2000; Norris et al., 2001, 2002; Froscio et al., 2001, 2003; Griffiths and Saker, 2003; Reisner et al., 2004; Beyer et al., 2009). At a more clinical level, cylindrospermopsin toxicity can lead to short term effects including GI and liver inflammation as well as hemorrhages, dermatitis and pneumonia while longer

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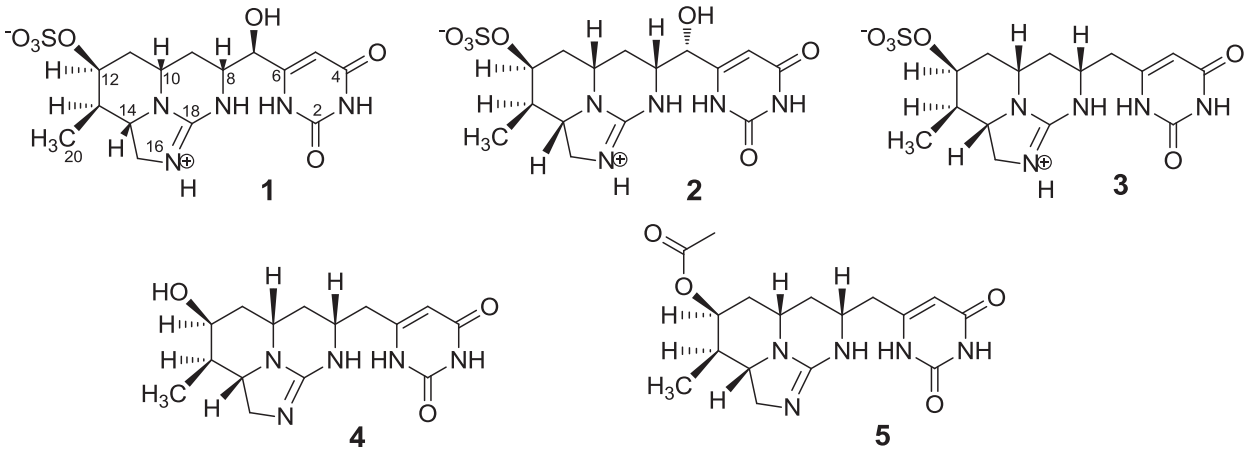

Fig. 1. Three known ((**1**)–(**3**)) and two new ((**4**) and (**5**)) cylindropermopsins: (**1**) cylindrospermopsin; (**2**) 7-epi-cylindrospermopsin; (**3**) 7-deoxy-cylindrospermopsin; (**4**) 7-deoxy-desulfo-cylindrospermopsin; (**5**) 7-deoxy-desulfo-12-acetylcylindrospermopsin.

term exposure to cylindrospermopsin has been linked to anorexia and liver failure (Falconer and Humpage, 2006).

In order to fully assess the potential health risks of a bloom, it is critical to know the identities of all the toxic components. To this end, our group has undertaken extensive chemical analysis of several known CHAB species. Our recent investigations into a strain of *Cylindrospermopsis raciborskii* isolated off the coast of New Zealand revealed the presence of not only cylindrospermopsin, but two new analogs (**4**, **5**). Cylindrospermopsin (**1**) is a water-soluble zwitterionic compound consisting of a tricyclic guanidine group attached to a hydroxymethyl uracil (Ohtani and Moore, 1992). Interestingly, only two additional analogs, 7-epi-cylindrospermopsin (**2**) and 7-deoxy-cylindrospermopsin (**3**) had previously been described (Fig. 1) (Norris et al., 1999; Banker et al., 2000).

2. Material and methods

2.1. General experimental methods

All reagents used throughout this study were HPLC Grade unless otherwise specified and were purchased through Fisher Scientific (Fairlawn, NJ, USA) and Honeywell International, Inc. (Muskegon, MI, USA). High performance liquid chromatography (HPLC) was performed using two Waters Corp Model 515 HPLC pumps controlled by an automated gradient controller, a Model 2487 Dual Wavelength Detector, and a Kipp and Zonen Servogor 102 chart recorder. Electrospray ionization mass spectrometry (ESI-MS) was performed using a Waters ZQ 2000 Mass Spectrometer tandem a Hewlett-Packard Series 1100 HPLC system (LC–MS) equipped with four pumps, a degasser, an autosampler, column warmer, and diode array detector. UV data was acquired on a Molecular Devices Flexstation 3 and analyzed with Software Pro version 5.2. Nuclear magnetic resonance (NMR) analyses were performed using a Bruker® Avance 1 500 MHz system run by TopSpin version 2.0. All NMR samples were suspended in solvents from Cambridge Isotope Laboratories, Inc., Andover, MA, USA. HRMS spectra were obtained from the University of Illinois Mass Spectroscopy Facility using a Waters Q-TOF Ultima ESI-MS.

2.2. Cyanobacterial culturing and harvesting

Freshwater cultures of *Cylindrospermopsis raciborskii* were grown in 3 L Fernbach flasks containing 1 L of Bold 3 N medium (The following was added to enough ultra-filtered H_2O to form 1 L of medium:6.0 mL $NaNO_3$ (Stock solution was 125 g/L), 2.0 mL $CaCl_2{\cdot}2\ H_2O$ (Stock solution was 5 g/400 mL), 2.0 mL $MgSO_4{\cdot}7\ H_2O$ (Stock solution was 15 g/400 mL), 2.0 mL K_2HPO_4 (Stock solution was 15 g/400 mL), 2.0 mL KH_2PO_4 (Stock solution was 35 g/400 mL), 2.0 mL NaCl (Stock solution was 5 g/400 mL), 0.15 mL vitamin B_{12} (Stock solution was 3 mg/L), 2.0 mL bold 3 N trace metal solution (premade; see below); trace metals: 400 mg EDTA was added to 190 mL ultra-filtered H_2O and was allowed to fully dissolve. The following ingredients were added to this EDTA solution: 58.2 mg $FeCl_3{\cdot}6\ H_2O$, 24.6 mg $MnCl_2{\cdot}4\ H_2O$, 10 mL. The following ingredients were added to 100 mL of ultra-filtered H_2O: 30 mg $ZnCl_2$, 12 mg $CoCl_2{\cdot}6\ H_2O$, 24 mg $Na_2MoO_4{\cdot}2\ H_2O$) and 250 mL of inoculum. Growth was then scaled up using Nalgene® polycarbonate carboys (10 L) containing 7 L of culture medium and 750 mL of inoculum. The cultures were provided with 14 h of light and 10 h of dark at 24 °C in a temperature-controlled culture chamber. Flasks were swirled daily to aerate and redistribute nutrients and cells, and the cultures were harvested by vacuum filtration approximately four weeks after being inoculated. Cells were collected on gF/A (1.6 μm) glass fiber filter papers (VWR International, LLC brand, 15 cm diameter) and then wrapped in aluminum foil and stored at −20 °C prior to chemical extraction. The filtrate from each harvest was collected and stored at room temperature in 4 L amber bottles. One milliliter of HPLC grade chloroform was added to each bottle to prevent the growth of contaminating organisms in the spent culture medium during storage.

2.3. Isolation of cylindrospermopsin (*1*)

Cylindrospermopsin isolation procedures were adapted from two studies (Norris et al., 2001; Metcalf et al., 2002) that outlined the use of graphitized carbon to adsorb cylindrospermopsin during the solid phase extraction of spent *Cylindrospermopsis raciborskii* culture medium. A 40 g cartridge was packed with 40 g of Bakerbond® C_{18} resin. A second 4 g cartridge was packed with 3.5 g of graphitized carbon (SPE Bulk Sorbent Carbograph, Alltech, State College, PA, USA). The graphitized carbon cartridge was attached in series to the end of the C_{18} cartridge and vacuum adapters were used to adjust the mouth of a sidearm vacuum flask (4 L) to make a seal that would accommodate the graphitized carbon cartridge. The packed cartridges were activated with 100% MeOH and conditioned 5% aq. MeOH. The spent culture medium was then passed through the system to extract cylindrospermopsin. The cartridges were then separated and the graphitized carbon cartridge was eluted with 5% formic acid in MeOH. The C_{18} cartridge was eluted with 5% aq. MeOH (500 mL) which was dried and weighed, followed by 100% MeOH (250 mL) and acetone