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Identification of euglenophycin – A toxin found in certain euglenoids☆

Paul V. Zimba [a,*], Peter D. Moeller [b], Kevin Beauchesne [b], Hannah E. Lane [c], Richard E. Triemer [d]

[a] National Warmwater Aquaculture Center, Agricultural Research Service, Post Office Box 38, Stoneville, MS 38701, USA
[b] NOAA/NCCOS Center for Human Health Research Hollings Marine Laboratory, 331 Fort Johnson Road, Charleston, SC 29412, USA
[c] Medical University of South Carolina, Charleston, SC 29425, USA
[d] Department of Plant Biology, Michigan State University, East Lansing, MI 48824, USA

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ABSTRACT

Currently cyanoprokaryotic algae, diatoms, haptophytes, dinoflagellates, euglenoids, and rhaphidophytes are known to produce algal toxins. A previous study by the authors reported euglenoid algae producing toxin(s) in aquaculture ponds, with confirmation based on positive fish bioassays following exposure to the isolated clonal algal cultures. Toxicity was observed in euglenoid culture isolates obtained from the pond as well as a clonal, culture collection taxon. Here we provide conclusive evidence for euglenoid toxin production, including HPLC/MS, MS/MS, and NMR analyses of a clonal (non-axenic) isolate of *Euglena sanguinea* grown in batch culture. Following wet chemical serial fractionation, toxic activity was identified in both the methanol and hexane extracts. These extracts were then purified using HPLC. Bioassay-guided HPLC fractionation of these two extracts demonstrated that a single class of toxic compounds, identical in mass and similar in molecular structure, was produced by this organism. The toxic compounds exhibited a maximal UV absorbance at 238 nm and gave diagnostic mass peaks at 306 (MH^+) and 288 (MH^+–H_2O). Unambiguous molecular structural determination was carried out by high field NMR analysis operating in 1- and 2-dimensions. Though a predominant isomer represented the bulk of the toxin, several stereo- and structural isomers were evidenced by NMR, and HPLC/MS. This compound is an alkaloid similar in structure to fire ant venom. The compound exhibits ichthyotoxic, herbicidal and anticancer activity at low ppm to ppb dosages.

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1. Introduction

The source, occurrence, and epidemiology of many freshwater, estuarine, and marine toxins produced by algae are well known (Landsberg, 2002; Chorus and Bartram, 1999; Hallegraeff, 1993; Shumway, 1990). Divisions of photosynthetic plankton known to produce toxins include the Bacillariophyceae, Dinophyceae, Haptophyceae, Raphidophyceae, as well as certain members of the Cyanoprokaryophyceae. Effects of these toxins depend upon the affected organism, as well as route, concentration, and duration of exposure.

In 2002, 2004, and 2006 we received calls from aquaculture producers and extension agents concerning the occurrence of toxic blooms in freshwater facilities. One of these cases was documented (Zimba et al., 2004): the dominant alga was a euglenoid, which was then isolated, cultured and caused fish-kills after exposure to naïve fish. The dominant alga was identified as *Euglena sanguinea* Ehrenberg. We subsequently encountered several other fish kill events where ponds were dominated by the same alga. Here we report additional confirmed fish mortality

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* Corresponding author at: Center for Coastal Studies, Texas A&M, 6300 Ocean Drive, Unit 5866, Corpus Christi, TX 78412, USA.
E-mail address: paul.zimba@tamucc.edu (P.V. Zimba).

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events as well as results from bulk culturing of this alga to produce sufficient biomass for complete chemical characterization of at least one toxin present.

2. Methods

Samples from episodic algal blooms were examined for sources of toxic algal bloom events. Water samples were examined by light microscopy (100–1000×) to identify plankton present. Potentially toxic species were isolated and grown in sterile media. Toxicity was assessed first via bioassay and then by HPLC/MS.

Unialgal isolates of *E. sanguinea* (isolated as a clonal culture from a NC fish kill event) were grown in AF6 media at 27 °C on a 14:10 light:dark photoperiod at 35 μmol photons m^{-2} s^{-1} as previously described (Zimba et al., 2004). Cell pellets were harvested from semi-continuous exponential phase cultures. Typically 35–50 L of media was harvested in each grow-out. The medium was filtered using 10 μm screening; cells were pelleted by centrifugation at 2800 RPM for 10 min from this net retained material, then immediately frozen at −80 °C. Cell pellets were examined using light microscopy and acridine orange direct microscopy (APHA, 1995) to determine culture purity and bacterial concentration.

2.1. Toxin extraction

An elutropic solvent fractionation scheme was used to extract toxin from cell biomass based on solvent defined polarity. Cell pellets were thawed in the dark, sonicated, then hexane, acetone methanol, and water were used to sequentially solubilize cellular components. Bioactivity of each fraction was assessed using GH4C1 (ATCC #CCL-82.2, Bethesda, MD) rat pituitary tissue culture cell lines as previously described for another toxin (Fairley et al., 1999). We additionally used sheepshead minnow (*Cyprinodon variegatus* Lacépède) as bioassay organisms to detect toxic cell fractions. Minnow fry were placed in 96-well plates containing 0.4 mL deionized water and 8 μL of each fraction was added to each well. Wells were monitored for 6 h to detect malaise or mortality. Samples exhibiting toxic activity were subjected to further purification using HPLC.

2.2. Toxin purification

The toxic solvent extracts were subjected to HPLC analytical fractionation. Bioassay-guided fractionation was used in all phases of separation to track sample activity as well identify cytotoxic and/or ichthyotoxic extracts. HPLC purification was carried out using a WATERS HPLC system (WATERS 2767 Sample Manager), 1525 Binary Pump, 510 pump, WATERS 2996 PDA and a WATERS ZQ Single Quadrapole Mass Detector outfitted with an active flow splitter, switching valve and using MASS LYNX software (Waters Corporation, Milford, MA). The HPLC/MS method was a water/acetonitrile (ACN) gradient with 0.2% TFA in both solvents. Extract was loaded onto a Phenomenex (Phenomenex Corporation, Torrance, CA) C18 LUNA 3 μm particle size, 250 × 4.6 mm column. The flow scheme conditions were: 1 mL/min flow rate, 90:10 Water/ACN (hold for 2 min). This was followed by a linear gradient over 20 min to 100% ACN. The 100% ACN was held for 3 min prior to re-establishment of original flow conditions. Column temperature was held at 35 °C.

After the development of LC/MS purification methodology (see above) the major toxic isomer (>80% of toxin present) was produced in sufficient quantities for analysis with a Bruker DMX 500 MHz NMR equipped with a gradient triple resonance 5 mm probe. Using a series of 1- and 2D NMR experiments (^{1}H, ^{13}C, APT: Attached Proton Test, COSY: Correlated Spectroscopy, HSQC: Homonuclear Single Quantum Coherence, HMBC: Heteronuclear Multiple Bond Correlation, NOESY: Nuclear Overhauser Effect Spectroscopy), the molecular structure of the toxin was characterized.

2.3. Growth studies of clonal euglenoid cultures

Clonal isolates of three different *E. sanguinea* strains and three closely related species were grown in AF6 media at 28 °C on a 14:10 L:D cycle. Species were chosen due to genetic evidence for similarity in ribosomal DNA (Triemer et al., 2006) and were *Euglena viridis*, *Euglena granulata*, and *Euglena splendens*. Cultures were sampled every 3–5 days for growth rate estimation; a single mid-exponential phase end point was used to determine toxicity of the other species. The goal of these experiments was to demonstrate toxin production, not to estimate maximal toxin production as might occur in late-log cultures.

2.4. Activity of toxin against other algae

Clonal isolates of five representative cyanoprokaryote, diatom, and green algae were grown in BG11 media at 28 °C on a 14:10 L:D cycle. The diatom *Gomphonema parvulum* (Kütz.) Kützing and green algae *Scenedesmus dimorphus* (Turpin) Kützing were a gift from Dr. David Czarnecki, Loras College Culture Collection, Planktothrix PCC7811 was obtained from the Pasteur Culture Collection, and *Oocystis polymorpha* Groover and Bold and *Microcystis aeruginosa* (Kütz.) Lemmermann were isolated from research ponds at the National Warmwater Aquaculture Center. At mid-exponential phase growth, 1 mL aliquots of each non-axenic culture were transferred to 96-well plates. Carrier solvent (control–0.1% MEOH) and analytically pure euglenophycin toxin was added to three or more replicate vials (at 0, 300 ppb, 3 ppm, and 30 ppm) and growth was monitored for five days. Chlorophyll was used as a biomass measure.

2.5. Activity of toxin against two colon cancer human cell lines

The response of two human cancer cell lines (HT-29, HCT116, ATCC, Manassas, VA) following exposure to analytically pure euglenophycin toxin was evaluated. Cancer cells were grown to mid-exponential growth phase. Aliquots were added to tissue culture plates with carrier (control–1.0% MeOH) and varying euglenophycin toxins (25, 50, 100 mg/L final concentration), were added with and without 5-fluorouracil. Growth was monitored by colorimetric change associated with respiration using MTS or MTT assays.