NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH Public Access
Author Manuscript
Comp Biochem Physiol C Toxicol Pharmacol. Author manuscript; available in PMC 2008 October 27.

Published in final edited form as:
Comp Biochem Physiol C Toxicol Pharmacol. 2004 December ; 139(4): 231–238. doi:10.1016/j.cca.2004.11.005.

Pharmacology and toxicology of pahayokolide A, a bioactive metabolite from a freshwater species of *Lyngbya* isolated from the Florida Everglades

John P. Berry[a,*], **Miroslav Gantar**[b], **Robert E. Gawley**[c], **Minglei Wang**[d], and **Kathleen S. Rein**[d]

[a]*Marine Biology and Fisheries, Rosenstiel School of Marine and Atmospheric Science, University of Miami, 4600 Rickenbacker Causeway, Miami, FL 33149, USA*

[b]*Department of Biological Sciences, Florida International University, Miami, FL, USA*

[c]*Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR, USA*

[d]*Department of Chemistry, Florida International University, Miami, FL, USA*

Abstract

The genus of filamentous cyanobacteria, *Lyngbya*, has been found to be a rich source of bioactive metabolites. However, identification of such compounds from *Lyngbya* has largely focused on a few marine representatives. Here, we report on the pharmacology and toxicology of pahayokolide A from a freshwater isolate, *Lyngbya* sp. strain 15−2, from the Florida Everglades. Specifically, we investigated inhibition of microbial representatives and mammalian cell lines, as well as toxicity of the compound to both invertebrate and vertebrate models. Pahayokolide A inhibited representatives of *Bacillus*, as well as the yeast, *Saccharomyces cerevisiae*. Interestingly, the compound also inhibited several representatives of green algae that were also isolated from the Everglades. Pahayokolide A was shown to inhibit a number of cancer cell lines over a range of concentrations (IC_{50} varied from 2.13 to 44.57 μM) depending on the cell-type. When tested against brine shrimp, pahayokolide was only marginally toxic at the highest concentrations tested (1 mg/mL). The compound was, however, acutely toxic to zebrafish embryos (LC_{50}=2.15 μM). Possible biomedical and environmental health aspects of the pahayokolides remain to be investigated; however, the identification of bioactive metabolites such as these demonstrates the potential of the Florida Everglades as source of new toxins and drugs.

Keywords

Algae; Cyanobacteria; Freshwater; Lyngbya; Cytotoxic; Antimicrobial; Artemia; Ichthyotoxic; Zebrafish; Everglades

1. Introduction

The bblue-green algae,Q or cyanobacteria, have received growing attention as producers of a diverse array of toxic or otherwise biologically active compounds with potential applications in biomedicine, as well as implications for environmental health (Moore, 1996; Gerwick et al., 2001; Osborne et al., 2001; Mayer and Gustafson, 2003; Shimizu, 2003). As part of ongoing research to identify and characterize bioactive metabolites from cyanobacteria, we are currently

* Corresponding author. Tel.: +1 305 284 1575; fax: +1 305 284 4571. E-mail address: jberry@miami.edu (J.P. Berry)..

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

focusing on the isolation and screening of freshwater cyanobacteria from the Florida Everglades.

The Florida Everglades is an oligotrophic marsh containing very productive microbial communities, specifically organized into either benthic or bfloating matsQ of periphyton. In particular, these microbial communities are characterized by a diversity of cyanobacteria. Of the cyanobacteria isolated from periphyton samples, one strain (15−2) of the genus *Lyngbya* has shown considerable promise as a source of bioactive metabolites. Extracts of *Lyngbya* sp. strain 15−2 were previously shown to be cytotoxic, antimicrobial and ichthyotoxic. Accordingly, bioassay-guided fractionation enabled purification of a series of cytotoxic constituents (Berry et al., in press) collectively termed the bpahayokolidesQ (named after *pahayokee*, the Miccosukee word for the Everglades). Subsequently, chemical characterization of the pahayokolides has indicated that these metabolites represent a group of previously uncharacterized compounds. Although structure elucidation is ongoing, high-resolution mass spectrometry has shown the molecular weight of the pahayokolides to be unusually large (1473 Da) when compared to metabolites previously isolated from *Lyngbya* or other cyanobacteria.

As a genus, *Lyngbya*, a filamentous cyanobacterium, has emerged as a particularly rich source of bioactive metabolites (e.g., Zhang et al., 1997; Marquez et al., 1998; Singh et al., 1999; Kan et al., 2000; Milligan et al., 2000; Mitchell et al., 2000; Tan et al., 2000, 2002, 2003; Luesch et al., 2000, 2001; Nogle et al., 2001; MacMillan and Tadeusz, 2002; Nogle and Gerwick, 2002; Williams et al., 2003). However, the majority of the investigations on biologically active compounds from *Lyngbya* have focused only on a limited number of marine species (Moore, 1996; Gerwick et al., 2001; Thacker and Paul, 2004). To our knowledge, the pahayokolides are the first bioactive metabolites to be identified from a *Lyngbya* species from the Florida Everglades.

Here, we report on the comparative toxicology and pharmacology of the major congener of the pahayokolides, pahayokolide A. Specifically, pahayokolide A was evaluated for in vitro inhibition of bacterial, fungal and microalgal representatives, as well as human cancer cell lines and for toxicity to both invertebrate and vertebrate models.

2. Materials and methods

2.1. Isolation and culture of Lyngbya

Lyngbya sp. strain 15−2 was isolated from the floating periphyton mat in the Florida Everglades. The mat sample was homogenized, diluted and filtered through 0.45-Am membrane filter (Metricel®, 47 cm diameter, Gelman® Sciences, Ann Arbor, MI), which was then placed onto BG-11 agar (Rippka et al., 1979) plate. Individual filaments were picked up and grown into a unialgal nonaxenic culture. The organism was maintained and cultured in BG-11 medium supplemented with 0.19 M Na_2CO_3 buffered with 2-morpholinoethanesulfonic acid (MES), pH 7.2.

2.2. Purification of toxin

Pahayokolide A was purified as detailed by Berry et al. (2004). Samples of biomass were harvested from cultures of *Lyngbya* 15−2 and lyophilized. The freeze-dried biomass was pulverized in liquid nitrogen and extracted for 24−48 h with 80% MeOH (bcrude extractQ). Following solvent removal, water-soluble constituents were separated by solid-phase extraction on Maxi-Cleank™ C-18 columns (Alltech, Deerfield, IL) preconditioned sequentially with MeOH and 20% MeOH (in water) and eluted sequentially with 20%, 80% and 100% MeOH. Water-soluble constituents of the 80% MeOH eluate were further separated by size-exclusion chromatography (G-10 Sephadex), solvent partitioning and either silica gel

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

column chromatography or normal-phase SPE bclean-upQ eluting sequentially with hexane, ethyl acetate, acetone and MeOH. The MeOH eluate was evaporated in vacuo to dryness and retaken in MeOH for HPLC. Finally, the pahayokolide A was purified (as the major component) from the MeOH eluate by semipreparative reversed-phase (C-18) HPLC with 70:30 20 mM ammonium acetate/acetonitrile (2.0 mL/min).

2.3. Evaluation of antimicrobial activity

Antimicrobial activity was determined by using an agar diffusion method with six bacterial, seven cyanobacterial, seven green algal and one fungal (i.e., yeast) strain (Table 1) as test organisms. Cyanobacteria and green algae were cultured in BG-11 agar medium (Sigma-Aldrich, St. Louis, MO); bacterial and yeast strains were cultured in nutrient and yeast extract/dextrose agar (Difco, Becton Dickinson, Sparks, MD), respectively. Test plates were inoculated by mixing cell suspensions in the appropriate bsoftQ agar cooled to 45 °C and pouring over prepared agar plates. The cultures were incubated in dim light (10 μE m^{-2} s^{-1}) for 24 h. Wells (6 mm diameter) were made in the agar with a sterile glass tube and filled with 30 μl of pahayokolide A at a concentration of 1 mg/ml. Plates were incubated for 2 days for bacteria and yeast or 10 days for cyanobacteria and green algae, after which the size of the inhibition zone was measured.

Minimal inhibitory concentration (MIC) was also determined for *Bacillus megaterium, B. subtilis* and *Chlamydomonas* Ev-29 as test organisms. Dilutions of pahayokolide A (prepared in MeOH) were added to wells of 96-well microtiter plates. Following evaporation of the solvent, appropriate media (i.e., nutrient broth or BG11 medium) inoculated with either the bacteria or green alga, respectively, were added to each well. Bacterial strains were grown for 18 h at 30 °C, and plates were read with a plate reader (Packard Spectra Count, Meriden, CT) at 600 nm. Algal strain, *Chlamidomonas* Ev-29, was grown in BG-11 under light intensity of 30 μE m^{-2} s^{-1}, and plates were read at 420 nm immediately after inoculation and every 24 h for 3 days. Each concentration was tested in triplicate.

2.4. Evaluation of cytotoxicity to human cancer cell lines

Cytotoxicity was evaluated for human cancer cell lines, specifically including glioblastoma (U251), lung adenocarcinoma (H460), colon adenocarcinoma (HT29), ovarian adenocarcinoma (SK-OV-3), melanoma (SK-MEL-28), renal adenocarcinoma (A498), lymphocytic leukemia (CEM) and mammary adenocarcinoma (SKBR3). All cell lines were obtained from the National Cancer Institute Division of Cancer Treatment and Diagnosis Tumor Repository (Frederick, MD), except SKBR3 that was supplied by Dr. Maria Laux of Department of Clinical Sciences at Cornell University College of Veterinary Medicine (Ithaca, NY). Cells were cultured in RPMI 1640 medium with L-glutamine (Cambrex Bio Science Walkersville, Walkersville, MD), supplemented with either 10% fetal bovine serum or newborn calf serum and antibiotics/antimycotics (Sigma-Aldrich) and subcultured twice weekly by trypsinization (except nonadherent CEM). Cytotoxicity, based on metabolic cell viability, was determined using the colorimetric indicator alamarBlue, as described (Wagner et al., 1999; Berry et al., 2002). Median inhibitory concentration (IC_{50}) values were calculated by the bleast squares methodQ for linear fits ($R^2 \geq 0.90$) between percent growth (relative to control) and log concentration of pahayokolide A.

2.5. Evaluation of brine shrimp toxicity

Toxicity to bbrine shrimpQ (*Artemia salina*), as an invertebrate model, was determined according to the method of Solis et al. (1993). Dilutions of pahayokolide A (in MeOH) or negative controls (MeOH-only) were dispensed into wells of 96-well microtiter plates, and the solvent was allowed to evaporate. Dry cysts of brine shrimp were incubated in artificial seawater at 30 °C with aeration under continuous light. After hatching, healthy nauplii were

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

collected and transferred into individual wells containing artificial seawater. After 24 h, the number of live brine shrimp was determined, and mortality rate was calculated.

2.6. Evaluation of zebrafish embryo toxicity

Toxicity of pahayokolide A to embryos of zebrafish (*Danio rerio*) as a vertebrate model was evaluated. Specifically, pahayokolide A (in MeOH) was serially diluted in bembryo rearing solutionQ (ERS) in wells of 24-well polystyrene plates. ERS is prepared fresh by diluting 25 mg of neomycin sulfate, 50 μL stock solution (35% w/v) of sodium thiosulfate, 125 μL Amquel® and 200 μL of stock solution (0.5% w/v) of methylene blue in 1 L of tap water. Zebrafish were bred once weekly, and, following successful breeding, healthy eggs with 4- to 32-cell stage [approximately ≤1 h postfertilization (hpf)] embryos were selectively collected and distributed to test plates (five eggs per well; four replicates per treatment). Mortality was assessed at 1 and 5 days postfertilization (dpf). Median lethal concentration (LC_{50}) values were estimated by the Spearman–Karber method (Hamilton et al., 1977).

3. Results

Pahayokolide A was purified from *Lyngbya* 15−2 biomass at yields of approximately 1.35% of the crude extract. Lower yields (0.44%) were obtained when cultured without Na_2CO_3 supplement. Samples of the purified compound isolated from the Na_2CO_3-supplemented cultures were used for toxicological and pharmacological evaluation.

Pahayokolide A inhibited the growth of Gram-positive bacteria, *B. megaterium* and *B. cereus*, and the yeast, *Saccharomyces cerevisiae*—as well as the green algae, *Ulothrix* Ev-17 and *Chlamydomonas* Ev-29 (Table 1). In addition, hormogonia development was inhibited within a zone around the pahayokolide A treatment in the cyanobacteria, *Nostoc* Ev-1, although growth of the organism was not otherwise affected. In the case of the green alga *Chlorococcum* 45−3, growth was partially inhibited, resulting in zone of noncomplete clearing in diffusion assays. MICs for both *B. megaterium* and *B. cereus* were estimated to be 5 μg/mL of pahayokolide A. The green alga *Chlamydomonas* Ev-29 was partially inhibited (approximately 20−30% of control) at concentrations as low as 1 μg/mL and completely inhibited at concentrations of 10 μg/mL (Fig. 1).

As shown in Table 2, pahayokolide A inhibited human cancer cell lines at a range of concentrations. The most potent inhibition was observed for lung (H460), renal (A498) and ovarian (SK-OV-3) adenocarcinoma lines with IC_{50} values of 2.13, 2.61 and 2.76 μM, respectively. A colon cancer line (HT29) was least inhibited (IC_{50}=44.57 μM), although both glioblastoma (U251) and breast cancer (SKBR3) lines also had IC_{50} values higher than 10 μM (13.33 and 16.70 μM, respectively). Moderate inhibition (IC_{50}=3.27 and 5.74 μM, respectively) was observed for a malignant melanoma (SK-MEL-28) and lymphocytic leukemia (CEM) lines.

Notable toxicity of pahayokolide A to brine shrimp was only observed at the highest concentration (1 mg/mL) tested (Table 3). At this concentration, approximately 55% mortality was observed. Only very minimal mortality (7.5% and 6.9%) was observed for smaller doses (0.1/mL and 0.01 mg/mL, respectively), whereas no mortality was observed for MeOH-only controls.

Pahayokolide A was acutely toxic to zebrafish embryos within 24 hpf, killing 100% of embryos at concentrations of 5 μg/mL or higher (Fig. 2A). Within 5 dpf, 100% mortality was observed at concentrations above 3 μg/mL (Fig. 2B). LC_{50} of pahayokolide A (and 95% confidence intervals) was estimated by the Spearman–Karber method at 2.15 (1.87−2.47) and 1.68 (1.45 −1.94) μM for 1 and 5 dpf, respectively. No specific deformities or developmental dysfunction

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

of embryos were observed, and toxicity was only observed as mortality and subsequently decomposed embryos.

4. Discussion

A number of compounds from *Lyngbya* are currently being investigated with respect to potential pharmaceutical development, as well as to human and environmental health implications of these compounds as naturally occurring toxins (Moore, 1996; Gerwick et al., 2001; Mayer and Gustafson, 2003; Shimizu, 2003; Thacker and Paul, 2004). Pharmacological activity of such *Lyngbya*-derived compounds ranges from microtubule inhibitors, such as curacin A(Gerwick et al., 1994), to potent sodium channel blockers and activators, such as kalkitoxin (Wu et al., 2000) and antillatoxin (Li et al., 2001). On the other hand, exposure to *Lyngbya* toxins have also been associated with various human health effects (Izumi and Moore, 1987; Osborne et al., 2001). In particular, dermatitis associated with exposure to *Lyngbya majuscula* has been widely documented (Izumi and Moore, 1987), and acute dermal lesions have been specifically linked to the *Lyngbya*-derived lyngbyatoxin A and debromoaplysiatoxin (Osborne et al., 2001).

In the present study, we report on pharmacological and toxicological aspects of a bioactive metabolite, pahayokolide A, purified from a freshwater isolate (15−2) of *Lyngbya* from the Florida Everglades. Although well over 100 compounds have been isolated from *L. majuscula* and other marine species of *Lyngbya* (Thacker and Paul, 2004), very few have been reported from freshwater species, and, to the authors' knowledge, this is the first such compound to be purified and characterized from an Everglades isolate. That said, a survey of microalgae (Lewis et al., 2004) in the Everglades-Florida Bay transition zone identified *Lyngbya* as one of the dominant members of the benthic and periphyton communities in this area.

Pahayokolide A is the major component purified from three apparently interconverting isomers. Chemical characterization of pahayokolide A indicates that about half of the molecular weight comprises a peptide fragment, most likely of nonribosomal biogenic origin, whereas the remainder is nonpeptide in origin. As a class of sondary metabolites, many cyanobacterial toxins are, at least partially, nonribosomal peptides (Moore, 1996; Gerwick et al., 2001; Shimizu, 2003). High-resolution electrospray ionization Fourier transform mass spectrometry of pahayokolide A using an internal standard and peak match revealed an exact mass of 1472.76716 Da for the molecular ion. ^{13}C-NMR spectroscopy, mass spectral analysis of a ^{15}N-enriched sample and elemental analysis narrowed possible molecular formulas to one: $C_{72}H_{109}N_{13}O_{20}$. This formula corresponds to an exact mass that is less than 1 ppm different from the measured mass. Further details of this analysis will be published elsewhere when the structure elucidation is complete.

Antimicrobial activity of pahayokolide A was investigated for a range of eubacteria, cyanobacteria, green algae and yeast (Table 1). Relatively little has been reported on the antimicrobial activity of *Lyngbya* toxins, although antifungal activity has been described for a few of these compounds (e.g., Milligan et al., 2000;Singh et al., 1999; see Moore, 1996). In fact, pahayokolide A was shown likewise to inhibit the yeast *S. cerevisiae* (Table 1). In terms of antibacterial activity, pahayokolide A showed specific inhibition of the two representatives of Gram-positive *Bacillus* only with estimated MICs of 5 μg/mL for both.

Perhaps even more interestingly, pahayokolide A inhibited the growth of several strains of green algae, including those of *Ulothrix, Chlorella* and *Chlamydomonas*, as well as inhibiting the development of hormogonia in a cyanobacterial strain of Nostoc, which were isolated from the Florida Everglades (Table 1; Fig. 1). These findings are intriguing as some evidence

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

(Srivastava et al., 1998,1999) has suggested that certain cyanobacterial toxins may function as allelochemicals, specifically inhibiting the growth of cooccurring cyanobacteria and other microalgae which may otherwise compete for nutrients and other resources. Although the focus of our investigation is not on the ecological roles of the pahayokolides, we have speculated that these compounds might serve to limit the growth of potentially competing microalgae. That said, it should, however, be pointed out that, although the pahayokolides are indeed somewhat water-soluble, evaluation of extracellular constituents in the aqueous culture medium did not show antimicrobial activity (data not shown), and any possible role as an allelochemical has yet to be demonstrated.

A number of bioactive compounds from *Lyngbya* are being currently investigated as potential anticancer drugs (Mayer and Gustafson, 2003). In this regard, most investigations have focused on cytotoxic metabolites, particularly those compounds that inhibit growth and viability of cancer cells. Pahayokolide A was moderately cytotoxic, inhibiting various human cancer cell lines (Table 2), as has been reported for many of the compounds isolated from *Lyngbya* (e.g., Gerwick et al., 1994; Marquez et al., 1998; Luesch et al., 2000, 2001; Tan et al., 2000, 2002, 2003; Williams et al., 2003). In fact, cytotoxicity of pahayokolide A (see Table 2) is similar in potency to that of many of the metabolites previously isolated from *Lyngbya* (Marquez et al., 1998; Luesch et al., 2000; Tan et al., 2000, 2002, 2003). Tan et al. (2002, 2003), for instance, showed that the depsipeptide guinea-mides from *L. majuscula* and the glycosidic macrolide, lyngbouilloside, from the *Lyngbya bouillonii* inhibited mouse neuroblastoma cells at IC_{50}s of approximately 15 and 17 μM, respectively. Other compounds such as the hermitamides A and B were shown to inhibit the same cell line at IC_{50}s of about 2.2 and 5.5 μM, respectively. In terms of potential application to pharmaceutical development, however, pahayokolide A is not as potent as, for example, curacin A and its derivatives which inhibits breast cancer cells in the submicromolar range (Verdier-Pinard et al., 1998) and which is currently being explored as a potential anticancer agent (Borman, 2002).

As indicated by these examples, reports of *Lyngbya* toxins are largely often limited to one or a few cell lines. In the present study, cytotoxicity was compared for a broad range of cell-types, specifically representing those employed by the National Cancer Institute (NCI) when screening for anticancer compounds (Shoemaker et al., 1988). Cytotoxicity of pahayokolide A varied considerably for different cell lines, with IC_{50}s ranging from approximately 2.13 μM for a lung adenocarcinoma line to 44.57 μM for a line of colon cancer (Table 2). Factors that determine apparent specificity of the observed cytoxicity and the possible pharmacological relevance of this remain to be investigated.

A number of *Lyngbya* metabolites have also been shown to be toxic to invertebrates (e.g., Smith, 1996; Marquez et al., 1998; Singh et al., 1999; Kan et al., 2000; Milligan et al., 2000; Tan et al., 2000). In fact, some evidence (Forsyth et al., 1990; DeMott and Moxter, 1991) suggests that cyanobacterial toxins may have an ecological role in defending against grazing by zooplankton. Most evidence (e.g., Marquez et al., 1998; Singh et al., 1999; Milligan et al., 2000; Tan et al., 2000) in this regard, however, specifically comes from utilization of the brine shrimp toxicity assay, which is used as general model of toxicity. Pahayokolide A was tested for toxicity to brine shrimp but showed only minimal activity (Table 3), with only approximately 55% mortality at the highest concentration (1 mg/mL) tested. For comparison, other *Lyngbya* toxins considered active in these assays typically show approximately 10^3- to 10^5-fold higher activity (Singh et al., 1999; Tan et al., 2000).

To assess the toxicity of pahayokolide A to vertebrates, we utilized embryos of the zebrafish (*D. rerio*). The zebrafish embryo is becoming an increasingly important model for studies in pharmacology (Parng et al., 2002) and vertebrate toxicology (Teraoka et al., 2003), as well as molecular biology (e.g., Malicki et al., 2002) and drug discovery (Moon et al., 2002).

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Pahayokolide A was toxic to zebrafish embryos in a dose-dependent manner resulting in 100% mortality at 24 hpf and 5 dpf at concentrations of 5 μg/mL and 3 μg/mL, respectively, and calculated LC_{50}s of 2.15 and 1.68 μM, respectively (Fig. 2). Although ichthyotoxicity has been utilized to assess other toxins from *Lyngbya*, and such studies report activity in a similar range of concentrations (Tan et al., 2000; Nogle et al., 2001), these studies have all focused on adult fish models rather than developing embryos. However, a limited number of investigations have similarly utilized the zebrafish embryo assay to investigate toxins from other cyanobacteria. Papendorf et al. (1997), for example, demonstrated that mueggelone and lupenyl acetate isolated from *Aphanizomenon flos-aquae* were toxic to zebrafish embryos at 10 and 100 μg/mL, respectively. Microcystin, on the other hand, was found to be toxic to zebrafish embryos at concentrations as low as 5 ng/mL; however, the observed effects only manifested as minor reductions in survival and body size of postdevelop-mental larvae but not during embryo development (Oberemm et al., 1997). In neither of these cases was exposure to toxins associated with significant mortality as observed for pahayokolide A.

Currently, the zebrafish embryo model is being utilized extensively (e.g., Oberemm et al., 1997; Papendorf et al., 1997; Chang et al., 2004; Lefebvre et al., 2004; see Teraoka et al., 2003) to identify developmental toxicity of sublethal doses of naturally occurring toxins. However, in this case, although lower concentrations of the pahayokolide A produced lower numbers of dead embryos, no specific or consistent effect on embryo development, such as morphological malformation or apparent dysfunction of developmental processes, were observed. It appears rather that toxicity of pahayokolide A is related perhaps to general cytotoxicity or other mechanisms rather than interruption of any specific developmental pathway or cell type. Indeed, comparable potency was also observed (data not shown) when newly hatched and fully developed embryos were exposed to pahayokolide A. In this case, mortality was observed at higher doses (≥5 μg/mL) within a few hours, suggesting an acute mechanism of toxicity.

As an apparently novel metabolite from a freshwater isolate of *Lyngbya*, pahayokolide A shows a broad range of biological activity. Possible pharmacological applications and environmental health aspects of the naturally occurring pahayokolides remain to be investigated; however, the identification of such molecules from the Florida Everglades underscores the potential of this rich microbial community as source of new toxins and drugs.

Acknowledgements

The authors would like to thank the National Institutes of Environmental Health Sciences (NIEHS) for financial support of this research through an NIEHS ARCH grant (S11 ES11181). We would also like to thank Drs. Jack Lay and Rohana Liyanage of the Arkansas Statewide Mass Spectrometry Facility for assistance with mass spectrometry and Patrick D.L. Gibbs for assistance with zebrafish embryo assays. This research was supported, in part, by an NIEHS Marine and Freshwater Biomedical Sciences Center Grant (ES 05705) to the University of Miami and by core facilities at the University of Arkansas supported by NIH (P20 R15569).

References

Berry JP, Laux M, Rodriguez E. Inhibition of protein tyrosine kinase and induction of apoptosis by oxindole alkaloids from *Uncaria tomentosa*. J. Med. Sci 2002;2:110–114.

Berry JP, Gantar M, Gawley RE, Rein KS. Isolation of a bioactive metabolite from a *Lyngbya* species isolated from periphyton of the Florida Everglades. Proc. Xth Intl. Conf. Harmful Algae. 2005in press

Borman S. Case study: a pharmaceutical company would never do this. Chem. Eng. News 2002;80:52.

Chang BE, Liao MH, Kuo MY, Chen CH. Developmental toxicity of arecoline, the major alkaloid in betel nuts, in zebrafish embryos. Birth Defects Res. Part A, Clin. Mol. Teratol 2004;70:28–36. [PubMed: 14745892]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

DeMott WR, Moxter F. Foraging on cyanobacteria by copepods: responses to chemical defenses and resource abundance. Ecology 1991;72:1820–1834.

Forsyth DJ, James MR, Cryer M. Alteration of seasonal and diel patterns in vertical migration of zooplankton by Anabaena and planktivorous fish. Arch. Hydrobiol 1990;117:385–404.

Gerwick WH, Proteau PJ, Nagle DG, Hamel E, Blokhin A, Slate DL. Structure of curacin A, a novel antimitotic, antiproliferative, and brine shrimp toxic natural product from the marine cyanobacterium *Lyngbya majuscula*. J. Org. Chem 1994;59:1243–1245.

Gerwick, WH.; Tan, LT.; Sitachitta, N. Nitrogen-containing metabolites from marine cyanobacteria.. In: Cordell, GA., editor. The Alkaloids. 57. Academic Press; New York: 2001. p. 75-184.

Hamilton MA, Russo RC, Thurston RV. Trimmed Spearman–Karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol 1977;11:714–719.

Izumi AK, Moore RE. Seaweed (*Lyngbya majuscula*) dermatitis. Clin. Dermatol 1987;5:92–100. [PubMed: 3311344]

Kan Y, Sakamoto B, Fujita T, Nagai H. New malyngamides from the Hawaiian cyanobacterium *Lyngbya majuscula*. J. Nat. Prod 2000;63:1599–1602. [PubMed: 11141095]

Lefebvre KA, Trainer VL, Scholz NL. Morphological abnormalities and sensorimotor deficits in larval fish exposed to dissolved saxitoxin. Aquat. Toxicol 2004;66:159–170. [PubMed: 15036871]

Lewis MA, Goodman LR, Macauley JM, Moore JC. Sediment toxicity and community composition of benthos and colonized periphyton in the Everglades-Florida Bay transitional zone. Ecotoxicology 2004;13:231–244. [PubMed: 15217247]

Li WI, Berman FW, Okino T, Yokokawa F, Shioiri T, Gerwick WH, Murray TF. Antillatoxin is a marine cyanobacterial toxin that potently activates voltage-gated sodium channels. Proc. Natl. Acad. Sci. U. S. A 2001;98:7599–7604. [PubMed: 11416227]

Luesch HT, Yoshida WY, Moore RE, Paul VJ. Isolation and structure of the cytotoxin Lyngbyabellin B and absolute configuration of Lyngbyapeptin A from the marine cyanobacterium *Lyngbya majuscula*. J. Nat. Prod 2000;63:1437–1439. [PubMed: 11076573]

Luesch H, Pangilinan R, Yoshida WY, Moore RE, Paul VJ. Pitipeptolides A and B, new cyclodepsipeptides from the marine cyanobacterium *Lyngbya majuscula*. J. Nat. Prod 2001;64:304–307. [PubMed: 11277744]

MacMillan JB, Tadeusz FM. Caylobolide A, a unique 36-membered macrolactone from a Bahamian *Lyngbya majuscula*. Org. Lett 2002;4:1535–1538. [PubMed: 11975622]

Malicki J, Jo H, Wei X, Hsiung M, Pujic Z. Analysis of gene function in the zebrafish retina. Methods 2002;28:427–438. [PubMed: 12507461]

Marquez B, Verdier-Pinard P, Hamel E, Gerwick WH. Curacin D, an antimitotic agent from the marine cyanobacterium *Lyngbya majuscula*. Phytochemistry 1998;49:2387–2389. [PubMed: 9887531]

Mayer AMS, Gustafson KR. Marine pharmacology in 2000: antitumor and cytotoxic compounds. Int. J. Cancer 2003;105:291–299. [PubMed: 12704660]

Milligan KE, Marquez BL, Williamson RT, Gerwick WH. Lyngbyabellin B, a toxic and antifungal secondary metabolite from the marine cyanobacterium *Lyngbya majuscula*. J. Nat. Prod 2000;63:1440–1443. [PubMed: 11076574]

Mitchell SS, Faulkner DJ, Rubins K, Bushman FD. Dolastatin-3 and two novel cyclic peptides from a Palauan collection of *Lyngbya majuscula*. J. Nat. Prod 2000;63:279–282. [PubMed: 10691729]

Moon H-S, Jacobson EM, Khersonsky SM, Luzung MR, Walsh DP, Xiong W, Lee JW, Parikh PB, Lam JC, Kang T-W, Rosania GR, Schier AF, Chang Y-T. A novel microtubule destabilizing entity from orthogonal synthesis of triazine library and zebrafish embryo screening. J. Am. Chem. Soc 2002;124:11608–11609. [PubMed: 12296721]

Moore RE. Cyclic peptides and depsipeptides from cyanobacteria: a review. J. Ind. Microbiol 1996;16:134–143. [PubMed: 8730577]

Nogle LM, Gerwick WH. Somocystinamide A, a novel cytotoxic disulfide dimer from a Fijian marine cyanobacterial mixed assemblage. Org. Lett 2002;4:1095–1098. [PubMed: 11922791]

Nogle LM, Okino T, Gerwick WH. Antillatoxin B, a neurotoxic lipopeptide from the marine cyanobacterium *Lyngbya majuscula*. J. Nat. Prod 2001;64:983–985. [PubMed: 11473443]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Oberemm A, Fastner J, Steinberg CEW. Effects of microcystin-LR and cyanobacterial crude extracts on embryo-larval development of zebrafish (*Danio rerio*). Water Res 1997;31:2918–2921.

Osborne NJ, Webb PM, Shaw GR. The toxins of *Lyngbya majuscula* and their human and ecological health effects. Environ. Int 2001;27:381–392. [PubMed: 11757852]

Papendorf O, Kfnig GM, Wright AD, Chorus I, Oberemm A. Mueggelone, a novel inhibitor of fish development from the fresh water cyanobacterium *Aphanizomenon flos-aquae*. J. Nat. Prod 1997;60:1298–1300. [PubMed: 9428164]

Parng C, Seng WL, Semino C, McGrath P. Zebrafish: a preclinical model for drug screening. Assay Drug Dev. Technol 2002;1:41–47. [PubMed: 15090155]

Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol 1979;111:1–61.

Shimizu Y. Microalgal metabolites. Curr. Opin. Microbiol 2003;6:236–243. [PubMed: 12831899]

Shoemaker, RH.; Monks, A.; Alley, MC. Development of human tumor cell line panels for use in disease-oriented drug screening.. In: Hall, T., editor. Prediction of Response to Cancer Chemotherapy. Allan R. Liss; New York: 1988. p. 265-286.

Singh IP, Milligan KE, Gerwick WH. Tanikolide, a toxic and antifungal lactone from the marine cyanobacterium *Lyngbya majuscula*. J. Nat. Prod 1999;62:1333–1335. [PubMed: 10514329]

Smith PT. Toxic effects of blooms of marine species of Oscillatoriales on farmed prawns (*Penaeus monodon, Penaeus japonicus*) and brine shrimp (*Artemia salina*). Toxicon 1996;34:857–869. [PubMed: 8875773]

Solis PN, Wright CW, Anderson MM, Gupta MP, Phillipson JD. A microwell cytotoxicity assay using *Artemia salina* (brine shrimp). Planta Med 1993;59:250–252. [PubMed: 8316592]

Srivastava A, Juttner F, Strasser RJ. Action of the allelochemical, fisherellin A, on photosystem II. Biochim. Biophys. Acta 1998;27(1364):326–336. [PubMed: 9630706]

Srivastava VC, Manderson GJ, Bhamidimarri R. Inhibitory metabolites production by the cyanobacterium *Fischerella muscicola*. Microbiol. Res 1999;153:309–317. [PubMed: 10052156]

Tan LT, Okino T, Gerwick WH. Hermitamides A and B, toxic malyngamide-type natural products from the marine cyanobacterium *Lyngbya majuscula*. J. Nat. Prod 2000;63:952–955. [PubMed: 10924172]

Tan LT, Marquez BL, Gerwick WH. Lyngbouilloside, a novel glycosidic macrolide from the marine cyanobacterium *Lyngbya bouillonii*. J. Nat. Prod 2002;65:925–928. [PubMed: 12088441]

Tan LT, Sitachitta N, Gerwick WH. The guineamides, novel cyclic depsipeptides from a Papua New Guinea collection of the marine cyanobacterium *Lyngbya majuscula*. J. Nat. Prod 2003;66:764–771. [PubMed: 12828459]

Teraoka H, Dong W, Hiruga T. Zebrafish as a novel experimental model for developmental toxicology. Congenit. Anom 2003;43:123–132.

Thacker RW, Paul VJ. Morphological, chemical, and genetic diversity of the tropical marine cyanobacteria Lyngbya spp. and Symploca spp. Appl. Environ. Microbiol 2004;70:3305–3312. [PubMed: 15184125]

Verdier-Pinard P, Lai J-Y, Yoo H-D, Yu J, Marquez B, Nagle DG, Nambu M, White JD, Falck JR, Gerwick WH, Day BW, Hamel E. Structure–activity analysis of the interaction of curacin A, the potent colchicine site antimitotic agent, with tubulin and effects of analogs on the growth of MCF-7 breast cancer cells. Mol. Pharmacol 1998;53:62–76. [PubMed: 9443933]

Wagner MM, Paul DC, Shih C, Jordan MA, Wilson L, Williams DC. In vitro pharmacology of cryptophycin 52 (LY355703) in human tumor cell lines. Cancer Chemother. Pharmacol 1999;43:115–125. [PubMed: 9923816]

Williams PG, Yoshida WY, Quon MK, Moore RE, Paul VJ. Ulongapeptin, a cytotoxic cyclic depsipeptide from a Palauan marine cyanobacterium *Lyngbya* sp. J. Nat. Prod 2003;66:651–654. [PubMed: 12762800]

Wu M, Okino T, Nogle LM, Marquez BL, Williamson T, Sitachitta N, Berman FW, Murray TF, McGough K, Jacobs R, Colsen K, Asano T, Yokokawa F, Shioiri T, Gerwick WH. Structure, synthesis, and biological properties of kalkitoxin, a novel neurotoxin from the marine cyanobacterium, *Lyngbya majuscula*. J. Am. Chem. Soc 2000;122:12041–12042.

Zhang LH, Longley RE, Koehn FE. Antiproliferative and immunosuppressive properties of microcolin A, a marine-derived lipopeptide. Life Sci 1997;60:751–762. [PubMed: 9064480]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

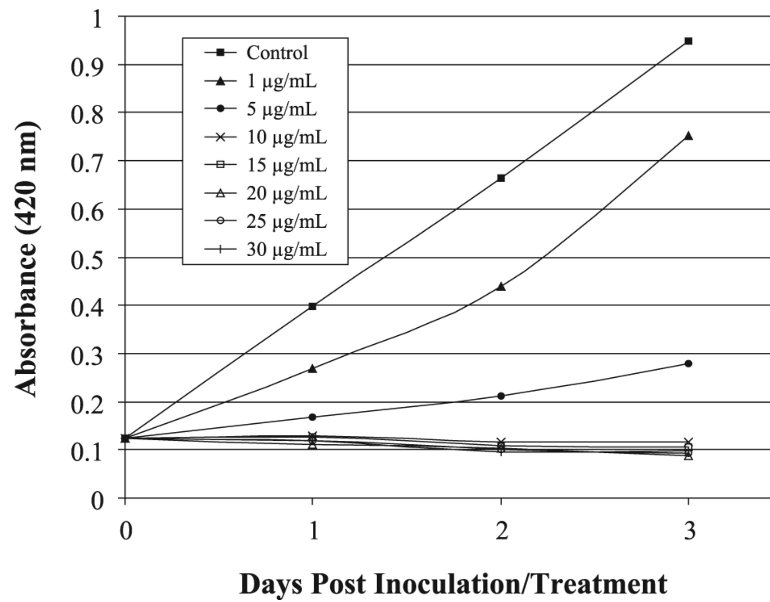

Fig. 1.

Inhibition of the green alga *Chlamydomonas* Ev-29 by pahayokolide A. *Chlamydomonas* Ev-29 grown in BG-11 medium with pahayokolide A at 1, 5, 10, 15, 20, 25 and 30 µg/mL or MeOH only (bControlQ) in 96-well plates. Growth of the alga was measured daily for 3 days by absorbance (420 nm) using a plate reader.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

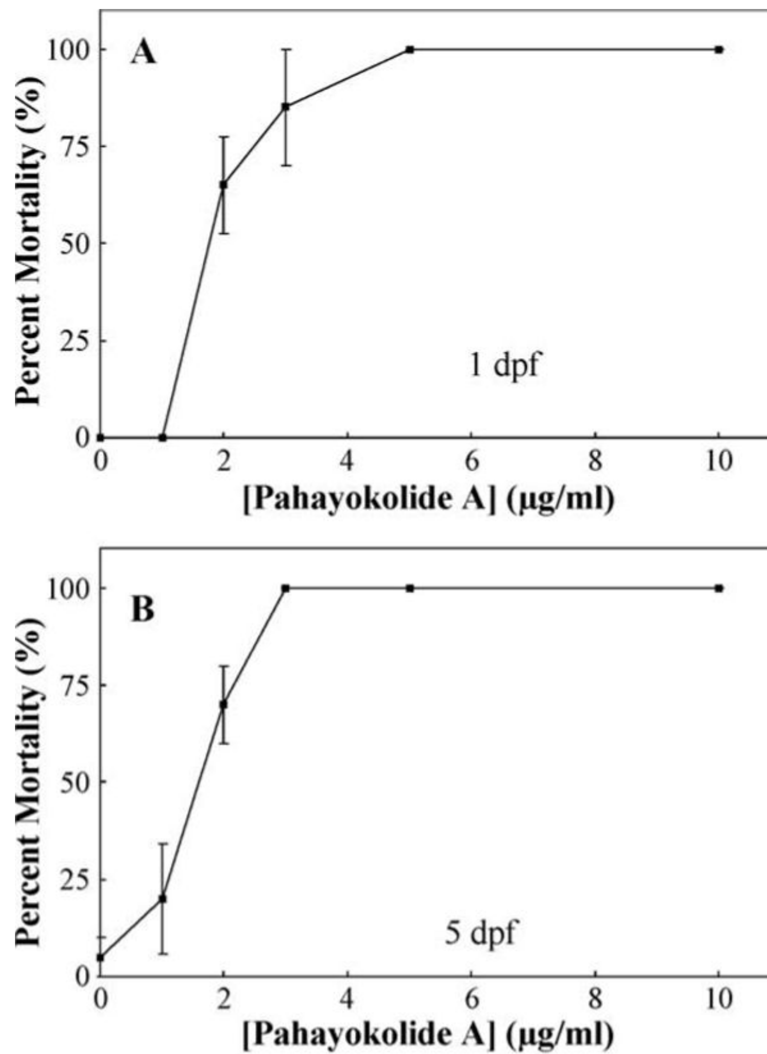

Fig. 2.

Mortality of zebrafish (*D. rerio*) embryos at 1 and 5 days postfertilization (dpf; A and B, respectively) exposed to pahayokolide A (1, 2, 3, 5 and 10 μg/mL or control (0.1% MeOH only). Embryos (4- to 32-cell stage) exposed at 0 dpf in four replicates per treatment of five embryos per replicate in 1 mL of ERS (see Materials and methods). Squares represent mean percent mortality ± S.E.M. for each group of replicates.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Table 1

Antimicrobial activity of pahayokolide A

Species/strain	Inhibition zone (mm)	MIC (μg/mL)
Bacteria		
Bacillus megaterium	32	5
B. subtilis	32	5
Pseudomonas aeruginosa	—	ND
Micrococcus luteus	—	ND
Escherichia coli	—	ND
Streptococcus epidermis	—	ND
Cyanobacteria		
Nostoc 58−2	—	ND
Nostoc 23−2	—	ND
Nostoc Ev-1	30[a]	ND
Hapalosiphon 52−1	—	ND
Oscillatoria 21−9−3	—	ND
Scytonema 26−1	—	ND
Green algae		
Ulothrix Ev-17	22	ND
Chlamydomonas Ev-29	34	10
Chlorococcum 46−4	—	ND
Chlorella 2−4	20[b]	ND
Ankistrodesmus 4−5−2	—	ND
Selanastrum 34−4	—	ND
Yeast		
Saccharomyces cerevisiae	20	ND

Zone of inhibition—diameter of a zone of inhibition around test wells in agar plates containing 30 μL of pahayokolide A (1 mg/mL). MIC—minimum inhibitory concentration in a series of dilutions of pahayokolide A at which the growth of a microbial representative is inhibited, as determined by the measure of absorbance (see Materials and methods). ND—not determined.

[a]No homogonia production in the zone was observed but no apparent growth inhibition otherwise.

[b]Not a completely clear zone of inhibition.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Table 2

Inhibition of human cancer cell lines by pahayokolide A

Cell line	Type	IC^{50}, μM (95% Confidence interval)
H460	Lung adenocarcinoma	2.13 (2.24−2.04)
A498	Renal adenocarcinoma	2.61 (2.56−2.66)
SK-OV-3	Ovarian adenocarcinoma	2.76 (2.61−2.92)
CEM	Lymphocytic leukemia	3.27 (2.96−3.63)
SK-MEL-28	Malignant melanoma	5.74 (5.43−6.07)
U251	Glioblastoma	13.33 (12.19−14.59)
SKBR3	Mammary adenocarcinoma	16.70 (14.63−19.07)
HT29	Colon adenocarcinoma, GR III	44.57 (40.00−49.67)

IC_{50}—median inhibitory concentration—concentration of pahayokolide A (in RPMI culture medium) at which inhibition of the cell viability is 50% of the untreated control, as determined colorimetrically by the AlamarBlue indicator (Berry et al., 2002). Calculated based on “least squares method” for linear relationship between percent growth and log concentration. Molarity calculated based on determined molecular mass (1472.77 amu) of pahayokolide A.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Table 3

Mortality of brine shrimp (*Artemia salina*) by pahayokolide A

Pahayokolide A	***n***	**% Mortality**
0 mg/mL	20	0
1 mg/mL	25	55
0.1 mg/mL	32	7.5
0.01 mg/mL	45	6.9

% Mortality—average percent mortality calculated for three replicates with *n* total brine shrimp nauplii.