Insecticidal Compounds Against Mosquito Larvae from *Oscillatoria agardhii* Strain 27

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Received 21 April 1999; revised 1 June 1999; accepted 3 June 1999

ABSTRACT: It was found that *Oscillatoria agardhii* strain 27 produced compounds toxic against mosquito larvae (*Aedes albopictus*), therefore, these compounds were extracted and separated for insecticide development. Structural characterization of the toxic fraction by ^{1}H-NMR and GC-MS showed that these compounds are not the neurotoxins or hepatotoxins conventionally produced by *Oscillatoria*, but it contained a mixture of unsaturated fatty acids, oleic, linoleic, and γ-linolenic acids, as well as saturated fatty acids, myristic, palmitic, and stearic acids. In a bioassay developed for these hydrophobic compounds using mosquito larvae, authentic unsaturated fatty acids were shown to be toxic, whereas saturated ones were not active. The results suggested that it might be possible to use unsaturated fatty acids as environmentally safe and effective insecticides without the side effects of the chemically synthesized insecticides. Environ Toxicol 15: 114–119, 2000
Keywords: cyanobacteria; *Oscillatoria agardhii*; mosquito; unsaturated fatty acid

INTRODUCTION

Many kinds of harmful insects have caused inconvenience and diseases. For example, mosquitoes can spread malaria, filarial diseases, and dengue and yellow fevers, which are serious infections even in our modern society. Control of mosquitoes carrying infectious diseases would be much simpler and effective if the actions are directed against mosquito larvae living in puddles instead of the flying adult ones.

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Most modern insecticides are synthetic chemicals except for a group of control agents produced by *Bacillus thuringiensis* (Höfte and Whiteley, 1989). Malathion (Mulla et al., 1981), one of the commonly used synthetic insecticides, was designed to minimize the mammalian toxicity, because it would be detoxicated by a mammalian enzyme. However, it will be metabolized in insects to an active form by another enzyme. Although the risk of adverse effects of insecticides to human heath has been reduced from the early stages of insecticide development, acute poisonings still occur. Long-term health effects caused by insecticide bioaccumulation in food chains are another concern.

The problems related to the acute and chronic toxicity and environmental pollution by insecticides are not yet completely solved, although modern synthetic insecticides have been designed to show highly selective toxicity. Some approaches using natural enemies and sexual pheromones to control the harmful insects were not satisfactory due to their relatively high cost and lack of immediate effects (Coats, 1994; Copping, 1996). However, when a perfect insecticide is not available, it is still possible to apply natural products as insecticides without damaging the environment. Natural products are produced in the environment, therefore, they probably do not cause serious environmental damage. Compared to man-made pesticides, residual compounds originating from natural products should be easily detoxicated by microorganisms (Lange and Lopez, 1996). Therefore, we attempted to isolate some active compounds against mosquito larvae from cyanobacteria.

Cyanobacteria occur worldwide in eutrophic fresh and brackish waters and they sometimes cause mammalian toxicoses with a number of different toxins (Sivonen and Jones, 1999). Two main types of cyanobacterial toxins, hepatotoxins and neurotoxins, have been identified. Hepatotoxic microcystins are produced by *Microcystis*, *Anabaena*, *Nostoc*, and *Oscillatoria* (Rinehart et al., 1994). These cyclic peptides are hepatotoxic due to their selective uptake by the liver and inhibitory activity against protein phosphatases 1 and 2A (Yoshizawa et al., 1990; Honkanen et al., 1990; MacKintosh et al., 1990). They have also been identified as tumor promoters (Falconer, 1991; Nishiwaki-Matsushima et al., 1992; Ohta et al., 1994). Another group of toxins includes the neurotoxins anatoxin-a, anatoxin-a(s), saxitoxin, and related compounds (Carmichael, 1992). Neurotoxins have been isolated from a variety of species, but with unrelated structures. Generally speaking, cyanobacteria have been characterized as a rich source of bioactive compounds (Namikoshi and Rinehart, 1996), for example, antimicrobial compounds and enzyme inhibitors. Most of the bioactive molecules are cyclic peptides, while many other types are also found: linear peptides, phosphonates, macrolides, etc. (Moore et al., 1996).

In addition to the activities described above, we have recently discovered that *Oscillatoria agardhii* strain 27 could produce certain unknown active compound(s) against aquatic invertebrates, mosquito (*Aedes aegypti*), and brine shrimp (*Artemia salina*). The active compounds originating from the strain showed a high selective toxicity to some harmful insect larvae with no toxicity to mice, thus indicating the potential for being developed to insecticides. (Kiviranta and Abdel-Hameed, 1994). In this paper, we describe the isolation and structural determination of these active compounds.

MATERIALS AND METHODS

Reagents

Some organic solvents, including methanol (MeOH), ethyl acetate (EtOAc), acetone, acetonitrile (CH_3CN), and diethyl ether were of extra pure grade and distilled at atmospheric pressure before use. Tetrahydrofuran (THF), dimethyl sulfoxide (Me_2SO), ethanol (EtOH), *n*-butanol (*n*-BuOH), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), lauric acid, myristic acid, palmitic acid, stearic acid, γ-linolenic acid, linoleic acid, oleic acid, eicosapentaenoic acid, and arachidonic acid were of guaranteed grade. 2,6-Di-*tert*-butyl-*p*-cresol (BHT: butyl hydroxytoluene), (BHT: butyl hydroxytoluene), *n*-decylaldehyde, *n*-octylaldehyde, *n*-hexylaldehyde, and sorbitan monolaurate (Span 20) were of extra pure grade. These solvents and reagents were purchased from Nacalai Tesque (Kyoto, Japan). 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and polyoxyethylenesorbitan monolaurate (Tween 20) were of extra pure grade and obtained from Sigma Chemicals (St. Louis, MO, USA). Deuterated chloroform, $CDCl_3$ (isotope enrichment: 99.95%), was purchased from E. Merck (Darmstadt, Germany).

Instrumental Analysis

Proton nuclear magnetic resonance (^{1}H-NMR) spectra in $CDCl_3$ were recorded on a JNM A-600 spectrometer (JEOL, Tokyo, Japan). Gas chromatography–mass spectrometry (GC-MS) was performed using a GC-9A/GCMS-QP1000 (Shimadzu, Kyoto, Japan) under the following conditions: column: Silicone OV-101 [Gas Chrom Q, 1.1 m × 3.0 mm inner diameter (I.D.), Wako Pure Chemical], injector: CLH-702 (Shimadzu), column temperature: 130–250°C (heated 5°C/min), carrier gas: He (40 ml/min), separator temperature: 250°C, ion source temperature: 250°C, ionization mode: chemical ionization (CI) (*iso*-butane, 200 eV), mass range: m/z 60–350, interval: 3.0 s, analytical time: 1–25 min.

Mosquito

Aedes albopictus was collected in Saitama Prefecture, Japan and mosquito's eggs were stored in a refrigerator until use. They were maintained in water in a beaker at 25°C and the hatched larvae were reared until second instar. In this bioassay the second instar larvae were only used.

Bioassay

The bioassay was performed using the larvae of mosquito *Ae. Albopictus*. About 10 larvae of *Ae. albopictus* and a sample were prepared in a water solution (1 mL) containing 0.01% (v/v) Tween 20 in a glass disposable tube (75 × 10 mm I.D.) at room temperature under fluorescent lamp illumination. The following control samples were used: (a) larvae in water only and (b) larvae in water containing a surfactant. The degree of activity was judged by the percentage of living larvae (counted by the naked eye) upon exposures for up to 24 h.

Extraction

Lyophilized cells (1.3 g) of *O. agardhii* strain 27 were extracted three times (each 30 min) with MeOH (1 × 250 mL once and 2 × 125 mL) in an ultrasonicator (Branson B-1200, Yamato, Tokyo, Japan) while cooling with crushed ice. The extract was centrifuged at 3000 rpm ($900 \times g$) for 10 min and the combined supernatant was then concentrated in vacuo. to give 165.3 mg of a dark-green oily sample. The MeOH extract was partitioned between water (250 mL) and EtOAc (3 × 250 mL). The resulting EtOAc layer was evaporated to dryness and afforded a dark-brown oily residue (22.2 mg).

Separation

An ODS (octadecylsilanized) silica gel cartridge (Sep-Pak®, 0.5 g, Waters, MA, USA) was conditioned with MeOH (10 mL) followed by distilled water (10 mL). An aliquot (2.9 mg) of the EtOAc extract was adsorbed into the cartridge in a small amount of 70% MeOH/distilled water. The cartridge was rinsed with 15 mL of 70% MeOH/distilled water and eluted (each step 15 mL) with 80 and 90% MeOH/distilled water, MeOH, CH_3CN, and THF. The eluates were then evaporated to dryness as separate fractions.

Methylation

MNNG (ca. 50 mg) was added to a mixture of ether (1 mL) and 0.5 mol/l NaOH in distilled water (0.5 mL) in a small glass tube while cooling in an ice bath and stirring using a small spinning bar. An aliquot of the resulting yellow ether solution (stoichiometric excess) was individually added to each mixture of saturated fatty acids (each 2 mg) and unsaturated ones (each 2 mg) and the ether solution of the active fraction isolated from the cyanobacterium in small glass tubes while stirring with a small spinning bar at room temperature.

RESULTS

Bioassay

In a previous experiment both mosquito (*Aedes aegypti*) larvae and newborn brine shrimps (*Artemia salina*) were used for the bioassay (Kiviranta and Abdel-Hameed, 1994), but in this study, mosquito (*Aedes albopictus*) was only used because it is easily available in Japan and is taxonomically close to *Ae. aegypti* (Tanaka et al., 1979). It was very important for the bioassay to standardize the conditions to reliably evaluate the active fractions. All assays were performed using only the second instar larvae in disposable glass tubes to unify the susceptibility and to improve the reproducibility.

The MeOH extract of the lyophilized cells was first partitioned into EtOAc and water. After concentration of each layer, the EtOAc fraction showed the activity, whereas the water fraction showed no activity. This initial experiment revealed that the active compounds have a relatively high lipophilic character, and these could not likely be assayed in a purely aqueous system. Therefore, we tried to select a few organic solvents and surfactants to increase in dissolving the desired compound(s) in water. They were selected on the bases of having a high solubility for organic compounds and being easily mixed with water in arbitrary proportions. Experiments were performed to select the solvent concentrations that showed no toxicity. Acetone and Me_2SO were found to have no toxicity at concentrations less than 2.5% (v/v), while EtOH was toxic even at 2.5% (v/v). Liquid surfactants having different values for the hydrophile–lipophile balance (HLB), (Helenius and Simons, 1975) were also selected for use in the bioassay. Span 20 (HLB value: 9.0) indicated toxicity at 0.01% (v/v), while Tween 20 (HLB value: 16.7) showed no toxicity at the same concentration level. Although the powdery surfactant CHAPS was nontoxic at 0.01% (w/v), it was not effective in dissolving the active fraction. Having a low toxicity to the mosquito larvae Tween 20 was the most appropriate surfactant to promote the solubility of the active fraction, which was determined in a preliminary test using two model compounds, oleic acid and linoleic acid, as shown in Table I. Therefore, all assays were carried out with the addition of 0.01% Tween 20 in aqueous solution.

Separation

The MeOH extract showed the highest activity among the three tested extracts of the lyophilized cyanobacteria: MeOH, H_2O, and 5% acetic acid aqueous solution, which were efficient for the extraction of the hepato-

TABLE I. Comparison of activity of fatty acids in solvents with or without surfactant

No.	Sample	Solvent	Concentration (mg/mL)	Time (hr : min) 0:00	2:30	5:30	19:30
1	Control 1	H_2O	0	—	—	—	—
2	Control 2	0.01% Tween 20	0	—	—	—	—
3	Oleic acid	H_2O	1	—	—	+	+
4	Oleic acid	0.01% Tween 20	1	—	+	+	+ + ±
5	Linoleic acid	H_2O	1	—	—	—	+
6	Linoleic acid	0.01% Tween 20	1	—	+	+	+ +

+ + +: annihilation, + + ±: almost annihilation, + +: half of the larvae dead, +: some larvae dead, ±: unclear activity, —: no activity.

toxic microcystins (Harada, 1996). To eliminate impurities such as green pigments in the MeOH extract, many kinds of solvent combinations were tried for liquid–liquid partition. The desired active compounds were selectively concentrated in the EtOAc layer according to the manner shown in Fig. 1. This partition method was remarkably effective, because the activity of the EtOAc layer was significantly higher than that of another fraction, which contained most of the impurities. An aliquot of the EtOAc layer was successively separated on an ODS silica gel cartridge (Fig. 1). Only two fractions, 90% MeOH aq. and THF, among the six fractions showed activity and they were instrumentally analyzed to elucidate the structures of the active compounds.

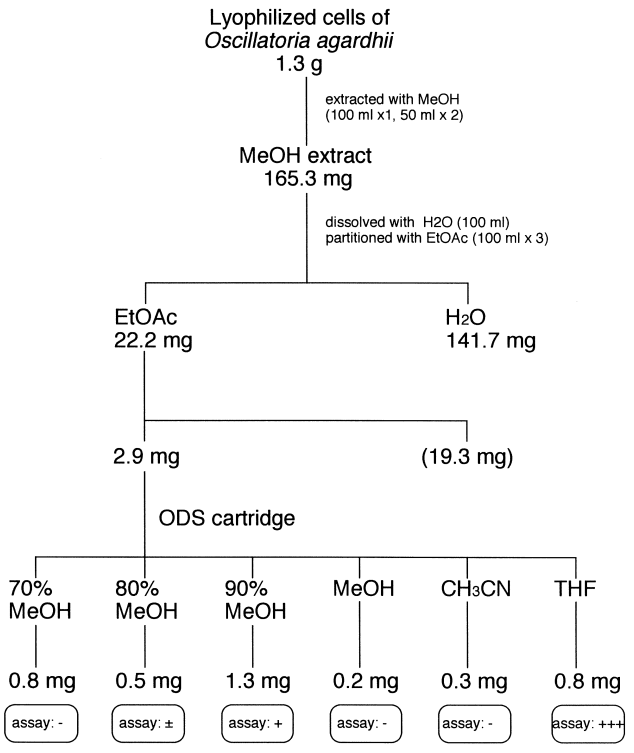

Fig. 1. Separation procedure of the active compounds from cyanobacteria.

Chemical Structures of Desired Compounds and Their Activity

The ^{1}H-NMR spectrum (Fig. 2, top) of the 90% MeOH fraction suggests that this fraction contains several unsaturated fatty acids, because the peaks at about 5.3 and 1.2 ppm are assigned to olefin and methylene protons, respectively, which are characteristic of unsaturated fatty acids with a long carbon chain. The spectrum of γ-linolenic acid (Fig. 2, bottom), as a typical

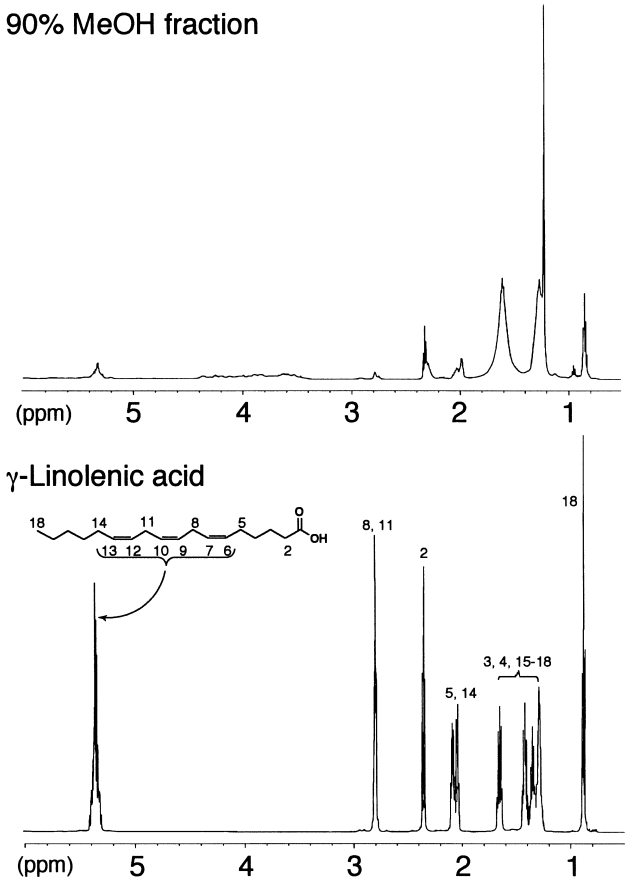

Fig. 2. Comparison of ^{1}H-NMR spectra of the 90% MeOH fraction and an unsaturated fatty acid.

example of an unsaturated fatty acid, has the corresponding signals to those of the active fraction. As it was very difficult to characterize an individual fatty acid by ^{1}H-NMR spectroscopy, the following technique was carried out to identify the fatty acids. Commercially available fatty acids were converted to their methyl esters using diazomethane and analyzed by GC-CI-MS (Fig. 3). The 90% MeOH fraction was also analyzed in the same manner and found to be a mixture of unsaturated (oleic, linoleic, and γ-linolenic acids) and saturated fatty acids (stearic, palmitic, and myristic acids), in which palmitic acid was predominantly detected. As shown in Table II, the unsaturated fatty acids have activities, whereas the saturated ones do not. Therefore, the active compounds in the active fraction were confirmed to be a mixture of unsaturated fatty acids. On the other hand, the active compound in the THF fraction was identified as BHT (butyl hydroxytoluene) by instrumental analyses and a direct comparison with an authentic sample. Actually, the authentic BHT sample also showed strong activity against the mosquito larvae. Since BHT is used as an additive against peroxide production in certain solvents such as THF, it is not derived from the cyanobacterium.

DISCUSSION

In a previous study, an active compound in the water soluble fraction from the same cyanobacterium strain was toxic to the mosquito *Aedes aegypti* and shrimp *Artemia salina* (Kiviranta et al., 1991; Kiviranta and Abdel-Hameed, 1994). In contrast with the previous results, we found that such activity is present in the nonpolar fraction in the present study. The nonpolar fraction was found to contain a mixture of unsaturated fatty acids including oleic, linoleic, and γ-linolenic acids, which are active against the mosquito larvae. The difference between the previous and present experimental results may be due to the extraction method used.

Many studies have been conducted on the biological effect of lipids including fatty acids produced by cyanobacteria. Monoglycosyl-diglyceride and diglycosyl-diglyceride from *Phormidium tenue* have autolytic properties (Murakami et al., 1991). Unsaturated fatty acids extracted from *Aphanizomenon flos-aquae* and *Microcystis aeruginosa* inhibit the growth of the green algae *Chlorella* (Ikawa et al., 1996). Furthermore, fatty acids from *Microcystis aeruginosa* had shown the potential to inhibit potently fish gill Na^+/K^+-ATPase activity (Bury et al., 1998). Morohashi et al. (1991) identified long-chain fatty acids as an inhibitor in brine shrimp, *Artemia salina*. In most cases biological activities are mainly due to unsaturated long-chain fatty acids rather than to the methylated or saturated forms (Bury et al.,

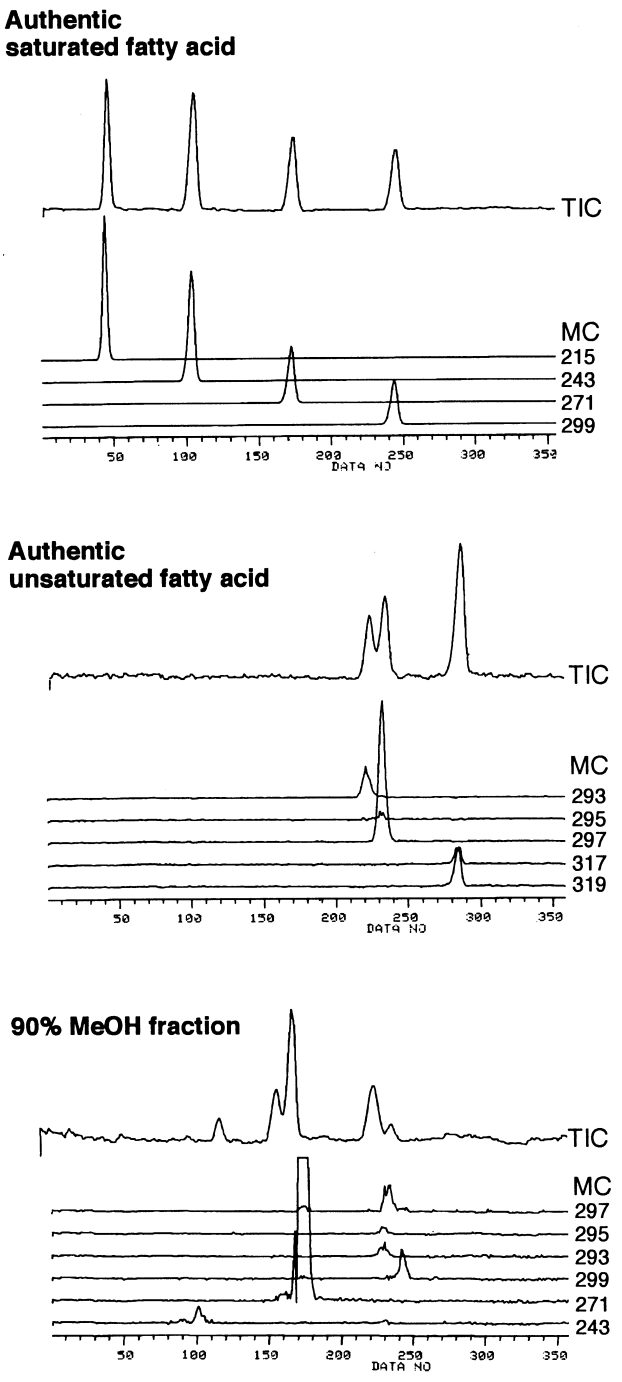

Fig. 3. GC-CI-MS chromatograms of methyl esters of authentic fatty acids and that of the active fractions. TIC: total ion chromatogram, MC: mass chromatogram, m/z value of $[M+H]^+$ of each methyl ester in MC: saturated fatty acids, m/z 215 (lauric acid); m/z 243 (myristic acid); m/z 271 (palmitic acid); m/z 299 (stearic acid), unsaturated fatty acids, m/z 293 (γ-linoleic acid); m/z 295 (linoleic acid); m/z 317 (eicosapentenoic acid); m/z 319 (arachidonic acid).

TABLE II. Comparison of activity of unsaturated fatty acids with saturated fatty acids

No.	Sample	Concentration (mg/mL)	Time 24:00
1	Palmitic acid (16:0)	1	±
2	Stearic acid (18:0)	1	—
3	Oleic acid (18:1)	1	++
4	Linoleic acid (18:2)	1	++
5	γ-Linolenic acid (18:3)	1	++±

+++: annihilation, ++±: almost annihilation, ++: half of the larvae dead, +: some larvae dead, ±: unclear activity, —: no activity.

1998; Ikawa et al., 1996; Morohashi et al. 1991). Based on the results obtained from the present and previous studies, the activity of unsaturated fatty acids to mosquito larvae may be derived from the inhibition of Na^+/K^+-ATPase. Similarly, brine shrimp, *Artemia salina*, would be affected by the unsaturated fatty acids in cyanobacteria, because it showed inhibitory behavior similar to mosquito larvae (Kiviranta and Abdel-Hameed, 1994; Morohashi et al., 1991).

Several invertebrates such as brine shrimp, mosquito, and *Daphnia* have been investigated for use in routine bioassays for cyanobacterial toxins such as microcystins and anatoxins (Kiviranta et al., 1991; Lawton et al., 1994). Of these, the brine shrimp (*Artemia salina*) has been the most popular and is available in commercial kit. In this study such invertebrates were affected by nontoxic cyanobacterium, suggesting that the observed toxicity of toxic cyanobacterium is derived not only from cyanobacterial toxins but also from unsaturated fatty acids, when a bioassay with invertebrates was used.

After the bioassay method was developed for these hydrophobic compounds, we further demonstrated that unsaturated fatty acids from cyanobacterium also have an activity against mosquito larvae. At present it is impossible to establish whether nontoxic cyanobacteria could be used as an origin for safe insecticides which should not cause serious damage to the environment. We are now focusing on another activity of the water-soluble fraction from this cyanobacterium.

We thank Dr. Jussi Meriluoto, Department of Biochemistry and Pharmacy, Äbo Akademi University, Finland, for helpful discussions.

REFERENCES

Bury, N. R.; Codd, G. A.; Wendelaar Bonga, S. E.; Flik, G. J Exp Biol 1998, 201, 81–89.

Carmichael, W. W. J Appl Bacteriol 1992, 72, 445–459.

Coats, J. R. Annu Rev Entomol 1994, 39, 489–515.

Copping, L. G. Crop Protection Agents from Nature: Natural Products and Analogues; Royal Society of Chemistry: Cambridge, UK, 1996; Introduction.

Falconer, I. R. Environ Toxicol Water Qual 1991, 6, 177–184.

Harada, K.-I. Toxic *Microcystis*; CRC Press: Boca Raton, FL, 1996; pp 103–148.

Helenius, A.; Simons, K. Biochim Biophys Acta 1975, 415, 29–79.

Höfte, H.; Whiteley, H. R. Microbiol Rev 1989, 53, 242–255.

Honkanen, R. E.; Zwiller, J.; Moore, R. E.; Daily, S. L.; Khatra, B. S.; Dukelow, M.; Boynton, A. L. J Biol Chem 1990, 265, 19410–19404.

Ikawa, M.; Haney, J. F.; Sasner, J. J Hydrobiol 1996, 331, 167–170.

Kiviranta, J.; Abdel-Hameed, A. World J Microbiol Biotech 1994, 10, 517–520.

Kiviranta, J.; Sivonen, K.; Niemelä, S. I.; Huovinen, K. Environ Toxicol Water Qual 1991, 6, 423–436.

Lange, L; Lopez, C. S. Protection Agents from Nature: Natural Products and Analogues; Royal Society of Chemistry: Cambridge, UK, 1996; Chap 1.

Lawton, L. A.; Beattie, K. A.; Hawser, S. P.; Campbell, D. L.; Codd, G. A. Detection Methods for Cyanobacterial Toxins; The Royal Society of Chemistry: Cambridge, UK, 1994; pp 111–116.

MacKintosh, C.; Beattie, K. A.; Klumpp, S.; Cohen, P.; Codd, G. A. FEBS Lett 1990, 264, 187–192.

Moore, R. E.; Corbett, T. H.; Patterson, G. M. L; Valeriote, F. A. Curr Pharm Des 1996, 2, 317–330.

Morohashi, M.; Tsuchiya, K.; Mita, T.; Kawamura, M. J Comp Physiol B 1991, 161, 69–72.

Mulla, M. S.; Mian, L. S.; Kawecki, J. A. Residue Reviews; Springer-Verlag: New York, 1981; pp 1–136.

Murakami, N.; Morimoto, T.; Imamura, H.; Ueda, T.; Nagai, S.; Sakakibara, J.; Yamada, N. Chem Pharm Bull 1991, 39, 2277–2281.

Namikoshi, M; Rinehart, K. L. J Ind Microbiol 1996, 17, 373–384.

Nishiwaki-Matsushima R.; Ohta, T.; Nishiwaki, S.; Suganuma, M.; Kohyama, K.; Ishikawa, T.; Carmichael, W. W.; Fujiki, H. J Cancer Res Clin Oncol 1992, 118, 420–424.

Ohta, T.; Sueoka, E.; Iida, N.; Komori, A.; Suganuma, M.; Nishiwaki, R.; Tatematsu, M.; Kim, S.-J.; Carmichael, W. W.; Fujiki, H. Cancer Res 1994, 54, 6402–6406.

Rinehart, K. L.; Namikoshi, M.; Choi, B. W. J Appl Phycol 1994, 6, 159–176.

Sivonen, K.; Jones, G. Toxic Cyanobacteria in Water, E & FN Spon: London, 1999; pp 41–111.

Tanaka, K; Mizusawa, K; Saugstad, E. S. Contrib of the Am Entomol Inst 1979, 16, 1–987.

Yoshizawa, S.; Matsushima, R.; Watanabe, M. F.; Harada, K.-I.; Ichihara, A.; Carmichael, W. W.; Fujiki, H. J Cancer Res Clin Oncol 1990, 116, 609–614.