Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 68 (2007) 2493–2497
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Insecticidal activity of 12-*epi*-hapalindole J isonitrile

Paul G. Becher [a,*], Simone Keller [b], Günther Jung [c], Roderich D. Süssmuth [b], Friedrich Jüttner [a]

[a] *Limnological Station, Institute of Plant Biology, University of Zürich, Seestrasse 187, 8802 Kilchberg, Switzerland*
[b] *Institute of Chemistry / Biological Chemistry, Technische Universität Berlin, Strasse des 17. Juni 135, 10623 Berlin, Germany*
[c] *Institute of Organic Chemistry, Eberhard-Karls-University of Tübingen, Auf der Morgenstelle 18, 72076 Tübingen, Germany*

Received 20 January 2007; received in revised form 15 June 2007; accepted 18 June 2007
Available online 7 August 2007

Abstract

12-*epi*-Hapalindole J isonitrile (**1**) and three previously described hapalindoles, 12-*epi*-hapalindole C isonitrile (**2**), hapalindole L (**3**) and 12-*epi*-hapalindole E isonitrile (**4**) were isolated and identified as insecticidal alkaloids of the biofilm-forming freshwater cyanobacterium *Fischerella* ATCC 43239 (Stigonematales). The structures of the purified compounds were elucidated by ESI–FTICR–MS, GC–EI–MS and various 2D NMR experiments. At 26 μM hapalindole **1** killed 100% of the larvae of the dipteran *Chironomus riparius* within 48 h. Insecticidal activities were also found at similar concentration for the hapalindoles **2–4**. The bioactivity of hapalindoles demonstrates that cyanobacterial biofilms can be considered as promising sources of insecticidal metabolites which might be useful for the biocontrol of dipterans.

Keywords: *Fischerella* sp.; Cyanobacteria; *Chironomus riparius*; Bioassay-guided isolation; Insecticidal activity; Indole alkaloid; Hapalindole

1. Introduction

Cyanobacteria produce numerous secondary metabolites exhibiting diverse bioactivities (Burja et al., 2001; Wiegand and Pflugmacher, 2005). These comprise inhibitory properties against microorganisms (bacteria, cyanobacteria, algae, viruses, fungi), and toxicity to invertebrates (crustaceans, bivalves) and vertebrates (fish, birds, mammals). Many of these activities are of allelochemical character. They strengthen cyanobacterial strains in their competition for nutrients, space and light, and protect them from viral, bacterial and fungal pathogens, as well as from grazing animals. Few studies have dealt with the insecticidal activities of cyanobacteria (Kiviranta and Abdel-Hameed, 1994), cyanobacterial extracts (Kiviranta and Abdel-Hameed, 1994; Kiviranta et al., 1993; Harada et al., 2000; Biondi et al., 2004) and metabolites of cyanobacteria (Kiviranta et al., 1992, 1993; Harada et al., 2000; Turell and Middlebrook, 1988; Delaney and Wilkins, 1995). Strict bioassay-guided isolations of insecticides from cyanobacteria are rare (Harada et al., 2000; Kiviranta et al., 1992). This low number of studies is surprising, as cyanobacteria can often be found in photoautotrophic biofilms that are a major diet for many juvenile aquatic insects (Brock, 1960; Robles and Cubit, 1981; Lamberti and Resh, 1983). In contrast, numerous insecticidal compounds were isolated from terrestrial plants which suffer from grazing pests, and several of these phytochemicals (pyrethrum and neem) are already in commercial use for pest control (Isman, 2006; Hedin et al., 1997).

When we studied biofilm-forming cyanobacteria for their resistance against grazing insects (Becher and Jüttner, 2005), *Fischerella* ATCC 43239 showed strong acute insecticidal activity in the toxicity assay of *Chironomus*

* Corresponding author. Present address: Institute of Biology, University of Neuchâtel, Rue Emile-Argand 11, Case postale 158, 2009 Neuchâtel, Switzerland. Tel.: +41 32 718 30 82/86; fax: +41 32 718 30 01.
E-mail address: paul.becher@unine.ch (P.G. Becher).

0031-9422/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2007.06.024

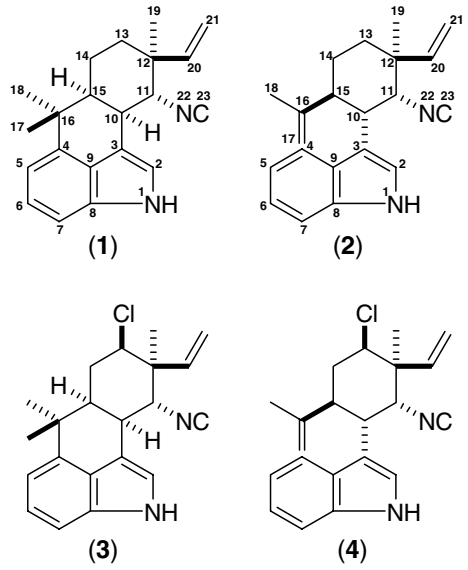

Fig. 1. Constitutional formulae of hapalindoles including the configurational assignment of stereocenters from *Fischerella* ATCC 43239.

larvae, which was attributed to a fraction consisting of four indole derivatives. These have now been characterised as members of a class of cyanobacterial indole alkaloids called hapalindoles. One of these hapalindoles was determined to be the new compound 12-*epi*-hapalindole J isonitrile (**1**), whereas the others (**2**–**4**) have been described before from strains of the cyanobacterial genera *Fischerella* and *Hapalosiphon* (Fig. 1) (Schwartz et al., 1987, 1990; Stratmann et al., 1994; Klein et al., 1995; Doan et al., 2000, 2001; Moore et al., 1987). The insecticidal activity observed for these compounds reflects the high potential of bioactive secondary metabolites in cyanobacteria and represents a new feature of cyanobacterial indole alkaloids.

2. Results and discussion

The detection and isolation of the cyanobacterial insecticides was performed by a bioassay-guided purification procedure. Axenic biofilms of *Fischerella* ATCC 43239 showed acute insecticidal activity when applied to larvae of chironomids (Becher and Jüttner, 2005). The insecticidal activity was extracted with 60% aqueous CH_3CN and yielded a fraction of four hapalindoles which were only poorly separated on a C18-HPLC column. However, nearly baseline separation of these compounds was achieved by application of a cyanopropyl column. The absorption and fluorescence maxima of the purified compounds indicated the presence of indole moieties (Hansen-Møller, 1992; Mills and Finlay, 1991). ESI–MS and GC–EI–MS revealed two isobaric compounds with the molecular mass of 304 Da and two isobaric compounds with molecular masses of 338/340 Da, the latter indicating a characteristic isotopic pattern for the presence of one chlorine atom in the molecules. GC–MS spectra of an extract obtained from a ^{15}N-labelled culture showed for the molecular ions a shift of 2 amu to higher molecular masses and proved the presence of two nitrogen atoms in each of the four hapalindoles **1**–**4**.

The molecular formula of **1** was determined as $C_{21}H_{24}N_2$ based on high-resolution ESI–FTICR mass spectra. 2D NMR experiments assigned the structures of the four insecticidal compounds. All four isolates were determined as indole alkaloids belonging to the group of hapalindoles.

The NMR spectroscopic data obtained from the compounds **2**–**4** were identical to the data for 12-*epi*-hapalindole C isonitrile (Stratmann et al., 1994), hapalindole L (Moore et al., 1987) and 12-*epi*-hapalindole E isonitrile (Schwartz et al., 1987, 1990), respectively.

The ^1H and ^{13}C NMR spectral data of **1** in $CDCl_3$ and DMSO-d_6 are shown in Table 1. The structure deduced from the 2D NMR experiments COSY, TOCSY, HMQC, and HMBC was found to be similar to hapalindole J, H, and U, except for chemical shift differences especially in the cyclohexane ring (C-10 to C-15). Coupling constants of **1** are similar to those described for hapalindole L (**3**) (Moore et al., 1987). The relative stereochemistry was deduced from NOE experiments. As described for hapalindole A, J, M, N, O and P (Moore et al., 1987), and observed in our experiments for **3**, irradiation of C-17-H induced NOEs in the C-5-H, C-14-H_{eq}, and C-15-H, and

Table 1
NMR (500 MHz) data for 12-*epi*-hapalindole J isonitrile (**1**)

Position	In $CDCl_3$		In DMSO-d_6	
	^1H NMR δ	^{13}C NMR δ	^1H NMR δ (J in Hz)	^{13}C NMR δ
1	7.98	–	10.70 (*br*)	–
2	6.77	120.1	7.10 (*br*)	120.7
3	–	109.5	–	108.9
4	–	138.6	–	137.7
5	6.95	113.8	6.77 (*d*, $J = 7.0$)	111.9
6	7.158	123.1	6.96 (*dd*, $J = 7.0$, 8.0)	121.5
7	7.159	108.2	7.07 (*d*, $J = 8.0$)	107.9
8	–	133.3	–	133.3
9	–	123.3	–	124.3
10	3.83	37,5	3.67 (*br, d*, $J = 4.2$)	36.8
11	4.21	63.2	4.64 (*br*)	61.7
12	–	38.4	–	38.5
13ax	1.68	31.5	1.60 (*td*, $J = 3.2$, 12.8, 13.9)	29.9
13eq	1.56		1.49 (*br, dt*, $J = 3.2$, 3.9, 13.9)	
14ax	1.07	25.3	0.91 (n.d.)	19.3
14eq	1.50		1.48 (*m*, $J = 3.2$, 3.7, 3.9, 14.1)	
15	2.11	43.7	1.92 (*dt*, $J = 3.7$, 4.2, 12.2)	43.2
16	–	37.7	–	37.6
17	1.50	24.9	1.42 (*s*)	24.3
18	1.22	31.7	1.10 (*s*)	31.4
19	1.23	27.9	1.11 (*s*)	28.2
20	5.57	143.7	5.66 (*dd*, $J = 10.8$, 17.9)	143.1
21*E*	4.61	111.3	4.45 (*d*, $J = 10.8$)	109.7
21*Z*	4.75		4.58 (*d*, $J = 17.9$)	
23	–	n.d.	–	156.0

n.d., not determined.

irradiation of the C-18-H revealed NOEs in the C-10-H and C-15-H. Upon irradiation of the methyl group at C-12, NOEs were obtained in the C-11-H, C-13-H_{ax}, C-20-H and C-21-H_Z, as described for **3** (Moore et al., 1987) suggesting that compound **1** has the same relative stereochemistry as **3**.

We designated compound **1** analogously to its epimer hapalindole J (Park et al., 1992; Muratake and Natsume, 1990), which differs only in the configuration at the C-12. Whereas **1** is a new metabolite, the hapalindoles **2**–**4** have been reported from *Fischerella* and *Hapalosiphon* before.

The hapalindoles **1**–**4** killed all larvae of *Chironomus riparius* in the highest concentration (which was 37 μM for **2** and 26 μM for the hapalindoles **1**, **3** and **4**). At concentrations <2.6 μM, the 48 h-mortality for hapalindoles **3** and **4** was not different from the controls (0–20%). Hapalindole **1** and **2** showed increased mortality at 2.6 μM (60%) for **1**, and 3.7 μM (50%) for **2**, respectively. Hapalindole **1** was also tested on the freshwater crustacean *Thamnocephalus platyurus* and killed all animals at 2.6 and 26 μM during 48 h. No mortality was found at 0.026 μM and for the control. The limited amount of pure compounds did not allow the precise determination of LC_{50} values. The 48-h LC_{50} for *C. riparius* of 281–450 mg/l described for Margosan-O®, a commercial neem-based formulation of a natural insecticide (Scott and Kaushik, 1998) illustrates the high potential of hapalindoles for pest control. Insecticidal activity has not yet been described for any hapalindole. However, for the hapalindoles **2**–**4,** bioactivities were reported to be directed against microorganisms (antibacterial, anticyanobacterial, antigreenalgal, antimycotic) and reflect the high ecological significance of these compounds as allelochemicals (Moore et al., 1987; Stratmann et al., 1994; Doan et al., 2000). A moderate inhibitory activity against bacterial RNA polymerase was shown for compound **4**, but other still unknown molecular targets of hapalindoles are very likely (Doan et al., 2001).

The bioactivity of cyanobacteria against insects is not surprising as the presence of insect larvae, which belong to the most efficient grazers of freshwater cyanobacterial biofilms, is one of the most serious threats for them and suggests adaptations by development of potent defence agents with insecticidal features. The synthesis of insecticidal compounds in biofilm-forming cyanobacteria is of high importance as many dipterans which are known as pests and vectors of serious diseases, like malaria and yellow fever, recruit from freshwater ecosystems in which biofilms of cyanobacteria are common and used as food and habitat (Ali, 1990; Rejmankova et al., 1996; Thiery et al., 1991). Because of numerous problems caused by synthetic pesticides, the development of biogenic pesticides with reduced risk potential is desirable (Isman, 2006). The described insecticidal activity of hapalindoles from *Fischerella* ATCC 43239 demonstrates that cyanobacteria are a yet underestimated source of potent natural insecticides.

3. Experimental

3.1. General experimental procedures

Chemicals and solvents were purchased from Fluka and Sigma–Aldrich, Switzerland. Separation of the hapalindoles was performed on a HPLC (LC-10AVP, Shimadzu, Reinach, Switzerland) equipped with a column oven (30 °C), photodiode array detector, an auto-injector and auto-sampler. Absorption spectra were recorded on a spectrophotometer (Varian, Cary 3, Steinhausen, Switzerland) and fluorescence spectra on a fluorescence spectro-photometer (F-2000, Hitachi, Switzerland). NMR experiments were measured on a Bruker (Karlsruhe, Germany) DRX500 NMR 500 MHz spectrometer (operating temperature 298 K) equipped with a 5 mm diameter broad band inverse probehead with *z* gradients using 3.0 mm tubes in association with a Bruker Biospin MATCH™. Spectra were recorded in and referenced to chloroform-d_1 (7.25 ppm; 77.0 ppm) and DMSO-d_6 (2.49 ppm; 39.5 ppm). 2D COSY, NOESY, HMQC and HMBC experiments were carried out with Bruker software (XWinNMR 3.2). Mass spectra were recorded on an electrospray mass spectrometer (LCQ Duo mass spectrometer, Thermoquest Finnigan, Waltham, USA) and a combined gas chromatograph mass spectrometer (GC–MS, GC 8000 Top, MD 800, CE Instruments, Milan, Italy/Fisons Instruments, Manchester, England) equipped with a capillary column (DB-1, 30 m, 0.32 i.d., film thickness 0.25 μm; J&W Scientific, Folsom, USA). ESI–FT–ICR-mass spectra were recorded on an APEX II FTICR mass spectrometer (4.7T, Bruker-Daltonics, Bremen, Germany).

3.2. Culture of Fischerella ATCC 43239

Fischerella ATCC 43239 was obtained from the American Type Culture Collection (ATCC), Rockville, USA. Mass cultivation of *Fischerella* ATCC 43239 was performed in 5-l glass tube reactors aerated with CO_2-enriched air (0.1% v/v). The culture conditions for large scale batch cultures have been published (Jüttner and Wessel, 2003). The cyanobacterial biomass was harvested by centrifugation or by use of gauze (polyester, 21 μm mesh width; Sefar, Rüschlikon, Switzerland). ^{15}N-labelled biomass was obtained by replacing ^{14}N-nitrate of the mineral medium by ^{15}N-labelled nitrate (98 atom%, Cambridge Isotope Laboratories, Inc., Andover, USA).

3.3. Extraction and isolation of hapalindoles

For extraction of *Fischerella* ATCC 43239, fresh or lyophilised biomass was used. The biomass was extracted with 60% (v/v) aqueous CH_3CN for 24 h at 4 °C. The solvents of the extracts obtained were evaporated on a centrifugal vacuum concentrator (Speed Vac Plus, Savant, Runcorn, UK) or a rotary evaporator at 40 °C. The residue was dissolved in 1 ml 95% (v/v) aqueous CH_3CN (1 ml was used

per 1 g of fresh biomass) and filtered through 0.45-μm syringe filters (Semadeni, Ostermundingen, Switzerland). Volumes of 300–500 μl of the extract (equivalent to 300–500 mg fresh biomass) were separated on a preparative reversed-phase column (C18 Grom-Sil 120 ODS-4 HE, 10 × 250 mm, 5 μm, Grom, Rottenburg-Hailfingen, Germany) using 95% (v/v) aqueous CH_3CN as the solvent (2 ml min^{-1} flow rate). The hapalindole containing insecticidal fraction could readily be detected by the strong UV absorption peaks at about 220 and 280 nm. The major insecticidal fraction eluted at 10.8–12.1 min. This fraction was collected and the solvent evaporated. The residue was dissolved in MeOH and deionised H_2O was added to a final concentration of 70% (v/v) aqueous MeOH for further HPLC separations. Pure compounds were obtained on a preparative cyanopropyl column (CN Grom-Sil 120 Cyano-3 CP, 8 × 250 mm, 5 μm, Grom, Rottenburg-Hailfingen, Germany) using a gradient of 70–88% (v/v) aqueous MeOH in 12 min and the flow rate of 2 ml min^{-1}. The procedure allowed the separation of four hapalindoles. The compounds eluted at R_t 9.8 min (**2**), R_t 10.3 min (**1**), R_t 11.2 min (**4**) and R_t 11.7 min (**3**). To obtain spectral purities >95% (absorption at 281 nm), each compound was rechromatographed on this column several times. The described isolation procedures finally yielded 1.0–1.4 mg of the purified compounds **1–4** from 40 g fresh wt biomass.

12-*epi*-Hapalindole J isonitrile **(1):** colourless amorphous deposit; UV (MeOH) λ_{max} 221, 281 nm; ^1H NMR and ^{13}C NMR (500 MHz, $CDCl_3$ and DMSO-d_6) see Table 1; EIMS 70 eV *m/z* (rel. int.) 304 $[M]^+$ (52), 289 (100), 168 (71), 167 (35), 181 (35), 182 (34), 207 (25), 180 (23), 154 (22), 290 (21); HR–ESI–FTICR–MS *m/z* $[M+H]^+$ 305.2013 (calc. for $C_{21}H_{25}N_2$ *m/z* 305.2012, relative mass error $\Delta_m = 0.27$ ppm).

3.4. Acute toxicity assay

Larvae of the midge *C. riparius* (Meigen) used for the acute insect toxicity assays were cultivated in the laboratory under defined conditions (OECD, 2001). Quartz sand (Saint-Gobain Weber GmbH, Amstetten, Germany) was used as the sediment in the culture vessels. Kieselguhr which is also reported to be used as the sediment showed a very compact consistency and proved to be suboptimal for culturing of *C. riparius*. The toxicity assays were performed in 24-well tissue culture plates. Each concentration of the hapalindoles **1–4** was tested in one well with ten *Chironomus* larvae. For the assay, different molarities (0.037, 0.37, 3.7 and 37 μM for hapalindole **2** and 0.026, 0.26, 2.6 and 26 μM for hapalindoles **1**, **3** and **4**) of the purified compounds dissolved in aqueous MeOH were added to the wells and the solvent removed under a stream of N_2. Before adding 10 larvae to each well, the residues were dissolved in 5–10 μl MeOH and diluted with 0.5 ml moderately hard standard synthetic mineral water (Weber, 1993). The final concentration of <2% MeOH did no harm to the larvae in control experiments. The observation time was 48 h. Larvae were considered dead when a reaction to a mechanical stimulus and the motion of inner organs could no longer be observed under a stereomicroscope (MZ FLIII, Leica, Wetzlar, Germany).

Hapalindole **1** was also tested at the same molarities on the freshwater crustacean *Thamnocephalus platyurus* (Thamnotoxkit F; G. Persoone, State University of Ghent, Belgium) using instar II–III larvae hatched from cysts. The testing procedure was similar as described for *C. riparius* except of the number of test animals (12–15 per concentration) and the test medium (standard synthetic mineral water diluted with deionised water (1:8 v/v); final concentration of <1% MeOH).

Acknowledgements

P.G.B. gratefully acknowledges the financial support by the National Science Foundation, Bern, and Hydrobiologie Limnologie Stiftung, Zürich. We thank E. Loher, H. Lüthi-Schaller and A. Mechsner for excellent technical assistance and Graeme Nicholson for performing ESI–FTICR mass spectrometry. The work of R.D.S was supported by an Emmy-Noether fellowship of the Deutsche Forschungsgemeinschaft (DFG SU 239-2/2) and Schering AG.

References

Ali, A., 1990. Seasonal changes of larval food and feeding of *Chironomus crassicaudatus* (Diptera: Chironomidae) in a subtropical lake. J. Am. Mosq. Control. Assoc. 6, 84–88.

Becher, P.G., Jüttner, F., 2005. Insecticidal compounds of the biofilm-forming cyanobacterium *Fischerella* sp. (ATCC 43239). Environ. Toxicol. 20, 363–372.

Biondi, N., Piccardi, R., Margheri, M.C., Rodolfi, L., Smith, G.D., Tredici, M.R., 2004. Evaluation of *Nostoc* strain ATCC 53789 as a potential source of natural pesticides. Appl. Environ. Microbiol. 70, 3313–3320.

Brock, E.M., 1960. Mutualism between the midge *Cricotopus* and the alga *Nostoc*. Ecology 41, 474–483.

Burja, A.M., Banaigs, B., Abou-Mansour, E., Burgess, J.G., Wright, P.C., 2001. Marine cyanobacteria – a prolific source of natural products. Tetrahedron 57, 9347–9377.

Delaney, J.M., Wilkins, R.M., 1995. Toxicity of microcystin-LR, isolated from *Microcystis aeruginosa*, against various insect species. Toxicon 33, 771–778.

Doan, N.T., Rickards, R.W., Rothschild, J.M., Smith, G.D., 2000. Allelopathic actions of the alkaloid 12-*epi*-hapalindole E isonitrile and calothrixin A from cyanobacteria of the genera *Fischerella* and *Calothrix*. J. Appl. Phycol. 12, 409–416.

Doan, N.T., Stewart, P.R., Smith, G.D., 2001. Inhibition of bacterial RNA polymerase by the cyanobacterial metabolites 12-*epi*-hapalindole E isonitrile and calothrixin A. FEMS Microbiol. Lett. 196, 135–139.

Hansen-Møller, J., 1992. Determination of indolic compounds in pig back fat by solid-phase extraction and gradient high-performance liquid chromatography with special emphasis on the boar taint compound skatole. J. Chromatogr. A 624, 479–490.

Harada, K.I., Suomalainen, M., Uchida, H., Masui, H., Ohmura, K., Kiviranta, J., Niku-Paavola, M.-L., Ikemoto, T., 2000. Insecticidal compounds against mosquito larvae from *Oscillatoria agardhii* strain 27. Environ. Toxicol. 15, 114–119.

Hedin, P.A., Hollingworth, R.M., Masler, E.P., Miyamoto, J., Thompson, D.G., 1997. Phytochemicals for Pest Control. In: Am. Chem. Soc. Symp. Ser., vol. 658. American Chemical Society, Washington, DC.

Isman, M.B., 2006. The role of botanical insecticides, deterrents and repellents in modern agriculture and an increasingly regulated world. Ann. Rev. Entomol. 51, 45–66.

Jüttner, F., Wessel, H.P., 2003. Isolation of di(hydroxymethyl)dihydroxypyrrolidine from the cyanobacterial genus *Cylindrospermum* that effectively inhibits digestive glucosidases of aquatic insects and crustacean grazers. J. Phycol. 39, 26–32.

Kiviranta, J., Namikoshi, M., Sivonen, K., Evans, W.R., Carmichael, W.W., Rinehart, K.L., 1992. Structure determination and toxicity of a new microcystin from *Microcystis aeruginosa* strain 205. Toxicon 30, 1093–1098.

Kiviranta, J., Abdel-Hameed, A., Sivonen, K., Niemelä, S.I., Carlberg, G., 1993. Toxicity of cyanobacteria to mosquito larvae: screening of active compounds. Environ. Toxicol. Water Qual. 8, 63–71.

Kiviranta, J., Abdel-Hameed, A., 1994. Toxicity of the blue-green alga *Oscillatoria agardhii* to the mosquito *Aedes aegypti* and the shrimp *Artemia salina*. World J. Microbiol. Biotechnol. 10, 517–520.

Klein, D., Daloze, D., Braekman, J.C., Hoffmann, L., Demoulin, V., 1995. New hapalindoles from the cyanophyte *Hapalosiphon laingii*. J. Nat. Prod. 58, 1781–1785.

Lamberti, G.A., Resh, V.H., 1983. Stream periphyton and insect herbivores: an experimental study of grazing by a caddisfly population. Ecology 64, 1124–1135.

Mills, M.H., Finlay, D.C., 1991. Determination of melatonin and monoamines in rat pineal using reversed-phase ion-interaction chromatography with fluorescence detection. J. Chromatogr. Biomed. Appl. 564, 93–102.

Moore, R.E., Cheuk, C., Yang, X.Q.G., Patterson, G.M.L., Bonjouklian, R., Smitka, T.A., Mynderse, J.S., Foster, R.S., Jones, N.D., Swartzendruber, J.K., Deeter, J.B., 1987. Hapalindoles, antibacterial and antimycotic alkaloids from the cyanophyte *Hapalosiphon fontinalis*. J. Org. Chem. 52, 1036–1043.

Muratake, H., Natsume, M., 1990. Synthetic studies of marine alkaloids hapalindoles. Part 1. Total synthesis of (+/−)-hapalindoles J and M. Tetrahedron 46, 6331–6342.

OECD, 2001. Sediment-water chironomid toxicity test using spiked water. Proposal for a new guideline 219. OECD Guidelines for the testing of chemicals.

Park, A., Moore, R.E., Patterson, G.M.L., 1992. Fischerindole L, a new isonitrile from the terrestrial blue-green alga *Fischerella muscicola*. Tetrahedron Lett. 33, 3257–3260.

Rejmankova, E., Roberts, D.R., Manguin, S., Pope, K.O., Komárek, J., Post, R.A., 1996. *Anopheles albimanus* (Diptera: Culicidae) and cyanobacteria: an example of larval habitat selection. Environ. Entomol. 25, 1058–1067.

Robles, C.D., Cubit, J., 1981. Influence of biotic factors in an upper intertidal community – Dipteran larvae grazing on algae. Ecology 62, 1536–1547.

Schwartz, R.E., Hirsch, C.F., Springer, J.P., Pettibone, D.J., Zink, D.L., 1987. Unusual cyclopropane-containing hapalindolinones from a cultured cyanobacterium. J. Org. Chem. 52, 3704–3706.

Schwartz, R.E., Hirsch, C.F., Sesin, D.F., Flor, J.E., Chartrain, M., Fromtling, R.E., Harris, G.H., Salvatore, M.J., Liesch, J.M., Yudin, K., 1990. Pharmaceuticals from cultured algae. J. Ind. Microbiol. Biotechnol. 5, 113–124.

Scott, I.M., Kaushik, N.K., 1998. The toxicity of Margosan-O, a product of neem seeds, to selected target and nontarget aquatic invertebrates. Arch. Environ. Contam. Toxicol. 35, 426–431.

Stratmann, K., Moore, R.E., Bonjouklian, R., Deeter, J.B., Patterson, G.M.L., Shaffer, S., Smith, C.D., Smitka, T.A., 1994. Welwitindolinones, unusual alkaloids from the blue-green algae *Hapalosiphon welwitschii* and *Westiella intricata*. Relationship to fischerindoles and hapalindoles. J. Am. Chem. Soc. 116, 9935–9942.

Thiery, I., Nicolas, L., Rippka, R., Tandeau de Marsac, N., 1991. Selection of cyanobacteria isolated from mosquito breeding sites as a potential food source for mosquito larvae. Appl. Environ. Microbiol. 57, 1354–1359.

Turell, M.J., Middlebrook, J.L., 1988. Mosquito inoculation: an alternative bioassay for toxins. Toxicon 26, 1089–1094.

Weber, C.I., 1993. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. EPA/600/4-90/027F. Environmental Monitoring Systems Laboratory, Office of Research and Development, US Environmental Protection Agency, Cincinnati, OH. <http://www.epa.gov/waterscience/WET/atx.pdf> (accessed 17.12.2006.).

Wiegand, C., Pflugmacher, S., 2005. Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. Toxicol. Appl. Pharmacol. 203, 201–218.