J. Phycol. **39,** 26–32 (2003)

ISOLATION OF DI(HYDROXYMETHYL)DIHYDROXYPYRROLIDINE FROM THE CYANOBACTERIAL GENUS *CYLINDROSPERMUM* THAT EFFECTIVELY INHIBITS DIGESTIVE GLUCOSIDASES OF AQUATIC INSECTS AND CRUSTACEAN GRAZERS[1]

Friedrich Jüttner[2]

Institute of Plant Biology/Limnology, University of Zürich, Seestrasse 187, CH-8802 Kilchberg, Switzerland

and

Hans Peter Wessel

F. Hoffmann-La Roche Ltd., Pharma Research Basel, Discovery Chemistry, CH-4070 Basel, Switzerland

An effective glucosidase inhibitor was isolated from the cyanobacterial genus *Cylindrospermum.* Its chemical structure was determined by MS and NMR spectrometry to be di(hydroxymethyl)dihydroxypyrrolidine (DMDP; 2(*R*),5(*R*)-*bis*-(hydroxymethyl)-3(*R*), 4(*R*)-dihydroxypyrrolidine). Its identity was established by comparison with an authentic compound. All five species of *Cylindrospermum* investigated synthesized this compound but accumulated it to a different extent intracellularly. Particularly active producers were the axenic *C. licheniforme* (22 pmol·nmol chl a^{-1}) and a monoxenic unknown species of *Cylindrospermum* that contained the maximum amount (159 pmol·nmol chl a^{-1}). The major part of DMDP was found to be extracellular for all species investigated. The isolated compound inhibited digestive α- and β-glucosidases isolated from crustacean zooplankton (IC_{50} 19 and 49 nM, respectively). The bacterial 1-deoxynojirimycin, which was used as a well-studied reference glucosidase inhibitor, was less inhibitory (IC_{50} 520 and 2190, respectively). Digestive enzymes of macrozoobenthos (chironomids, trichoptera, and ephemeroptera) were less sensitive to DMDP. The insect digestive β-glucosidase was more effectively inhibited than the α-glucosidase. Beside others, the ecological function of the glucosidase inhibitor may be the reduction of the digestibility of the cyanobacterium for grazers.

Key index words: **aquatic insect larvae; crustaceans; *Cylindrospermum*; digestive enzyme; di(hydroxymethyl) dihydroxypyrrolidine; enzyme inhibitor; α-glucosidase; β-glucosidase; 2(*R*),5(*R*)-*bis*-(hydroxymethyl)-3(*R*), 4(*R*)-dihydroxypyrrolidine**

Abbreviations: **DMDP, di(hydroxymethyl)dihydroxypyrrolidine; DNJ, 1-deoxynojirimycin; LC_{50}, lethal concentration**

Extensive research on secondary metabolites in cyanobacteria in the past decade has succeeded in the discovery of an array of compounds with new basic structures. A biological activity or ecological value could not yet be shown for some of these metabolites (Fujii et al. 2000, Golakoti et al. 2000), whereas other metabolites have turned out to be extremely toxic to animals (Nagle and Paul 1999, Faulkner 2000) or inhibitory to competitors, such as other photoautotrophic cyanobacteria and algae (Smith and Doan 1999). By synthesizing complex compounds that affect animals, cyanobacteria gain the advantage of reduced grazing pressure. The determination of lethal concentration value of a compound has been accepted as a general measure of toxicity for different organisms. These values indicate if the amount of a toxin in a food organism is sufficiently high to cause a relevant effect on the grazers. For other metabolites recently isolated from cyanobacteria, target enzymes have been found that were selectively and effectively inhibited. Particularly widespread were protease inhibitors isolated from planktonic cyanobacteria of the genera *Microcystis* (Banker and Carmeli 1999, Ishida et al. 2000, Reshef and Carmeli 2001) and *Planktothrix* (Sano and Kaya 1996, Shin et al. 1996a,b) and the benthic mat-forming cyanobacteria *Plectonema* (Matsuda et al. 1996) and *Microchaete* (Bonjouklian et al. 1996). Typically, these metabolites are cyclic peptides or depsipeptides and are effective against various digestive proteases. Compounds have been found that inhibited serine proteases, cysteine proteases, aspartate proteases, and metalloproteases. Although not studied, the ecological role of these compounds is most likely the reduction of digestibility of the cyanobacterial food for the grazers (Ryan 1981). By inhibiting the catalytic activity of digestive proteases in saliva and the digestive tract, these metabolites reduce the nutrient value of ingested biomass. Beside proteins, carbohydrates are also important components of the cyanobacteria that serve as an energy source for grazers. From studies of higher plants, it is well known that certain compounds inhibit digestive glucosidases of insects, thus making the food less digestible (Fellows et al. 1989). Pentagalloylglucose, which has the property of an α-glucosidase inhibitor, has previously been described in *Spirogyra varians* (Cannell et al. 1988), but other compounds with that feature have not yet been observed in benthic and pelagic freshwater organisms.

[1]Received 5 March 2002. Accepted 2 October 2002.

[2]Author for correspondence: e-mail juttner@limnol.unizh.ch.

A search for glucosidase inhibitors in natural littoral epilithic biofilms (Corves and Jüttner 2000) and cyanobacterial biofilms (Jüttner and Wu 2000) succeeded in the detection of such activities. When several biofilm forming cyanobacteria were screened, we found *Cylindrospermum*, which forms biofilms on wet soil and can be abundant in stagnant waters (Frémy 1929), to contain strong inhibitory activity against glucosidases (Corves 1999). We isolated the compound responsible and determined its structure and inhibitory activity against model glucosidases and digestive glucosidases from several different pelagic crustacean grazers and aquatic insect larvae, which were dominant species of the macrozoobenthos.

MATERIALS AND METHODS

Origin and cultivation of cyanobacteria. An axenic culture of *Cylindrospermum licheniforme* Kützing ATCC 29412 was kindly supplied by P. Wolk (Michigan State University, East Lansing, MI, USA). The other strains were monoxenic; *C. alatosporum* F. E. Fritsch SAG 43.79, *C. muscicola* Kützing SAG 44.79, and *C. stagnale* (Kütz.) Born. et Flah. SAG 45.79 were from the Sammlung von Algenkulturen Göttingen (SAG), Germany, and *Cylindrospermum* sp. CCAP 1415/1 was from the Culture Collection of Algae and Protozoa, Ambleside, United Kingdom. The cyanobacteria were cultivated as standing cultures in 300-mL Erlenmeyer flasks that were supplied with 120 mL of suspension and closed with a stopper of cellulose (Steristoppers, H. Herenz, Hamburg, Germany). The mineral medium, which was optimized for cyanobacteria, has previously been published (Jüttner et al. 1983). Continuous light of 5 μmol photons·m^{-2}·s^{-1} was provided by fluorescent tubes, and the temperature was held at 20° C. Large-scale batch cultures (4.6 l suspension) of *C. licheniforme* were grown in round-bottomed glass tube reactors (7.2 cm i.d., 145 cm length) that were continuously stirred with a magnetic bar. Air enriched with 0.2% CO_2 was continuously introduced to the bottom through a central glass tube and sterilized by passing through a cotton filter. The growth temperature was 28° C, and light from two vertically arranged fluorescent tubes provided 15 μmol photons·m^{-2}·s^{-1}. The cyanobacteria were harvested after 4 weeks by a continuous flowthrough centrifuge (Westfalia Separator AG, Oelde, Germany). The dense cyanobacterial suspension obtained was further concentrated by centrifugation in a Sorvall RC 5C Plus (GSA rotor, Dupont, Newtown CT, USA). The biomass yield was approximately 100 g wet weight from six tube reactors and was stored at −20° C in a freezer.

Extraction and purification. Purification of the bioactive compound followed, in part, the procedure given by Paek et al. (1997). The frozen biomass (100 g wet weight) was extracted with 200 mL 60% aqueous methanol and centrifuged. Methanol was removed under vacuum from the supernatant in a rotary evaporator, and the aqueous portion was brought to dryness in a centrifugal vacuum concentrator (Speed Vac Plus, Savant, Runcorn, England). The residue was taken up in 20 mL water and, in nine portions, was subjected to cation exchange resin-filled columns (each 1 g of BioRad 50 × 8, NH_4^+ form, 100–200 mesh), which were previously conditioned by extensive washings with 0.1 M HCl, 0.5 M NH_4OH, and water. The inhibitor sorbed to the column was eluted with 9 mL 0.5 M NH_4OH. The elute was dried in a centrifugal vacuum concentrator and further purified by two subsequent HPLC separations. The first separation was performed on a reversed-phase column (ODS-AQ-303, 250 × 4.6 mm, S-5 μm, YMC, Wilmington, NC, USA). A gradient was used that began with 0.05 M ammonium acetate and ended after 12 min with 50% methanol. The flow rate was 1.0 mL·min^{-1}. The bioactive compound that eluted as a major peak at R_t = 3.0 min was detected by absorption measurement at 220 nm and collected. The drying process that was subsequently applied in the centrifugal vacuum concentrator was sufficiently effective to remove most of the ammonium acetate. The residue was taken up in water and subjected to an HPLC separation on an amino column (Pack-NH_2, 250 × 4.6 mm, S-5 μm, 120A YMC). The separation was performed under isocratic conditions using 50% aqueous acetonitrile as the solvent. The absorbance was read at 195 nm, and the flow rate was 1 mL·min^{-1}. All the inhibitor activity was contained in the peak eluting at 5.45 min. The peak was collected and the fraction dried in a centrifugal vacuum concentrator to give a honey-yellow residue. The residue was dissolved in 3 mL of water and passed through three small columns of activated charcoal (150 mg activated charcoal, Merck, Darmstadt, Germany) in parallel and eluted with 2 mL of water. Charcoal particles of the elute were removed with a syringe filter, and the solution was dried in a centrifugal vacuum concentrator. The residue was a colorless glassy matter; the yield of the purified compound obtained from 100 g wet weight biomass was 5 mg.

Derivatization, mass spectrometry (MS), and NMR spectrometry. DMDP was trifluoroacetylated to give the *pentakis*-trifluoroacetate by incubation with trifluoroactic acid anhydride for 60 min at 45° C. The pentasilylated compound was obtained after reaction of the substance with 100 μL *N,O-bis*-(trimethylsilyl)-trifluoroacetamide in 100 μL pyridine for 30 min at 80° C. Both derivatives were separated on a capillary column (DB 1301, 30 m, 0.32 mm i.d., 0.25 μm film thickness) with an initial helium gas flow of 1.6 mL·min^{-1}. The head pressure was 50 kPa, and the gas flow of the split was 15 mL·min^{-1}. The temperature program was 1 min isotherm at 100° C and 10° C·min^{-1} to 250° C. The *pentakis*-trifluoroacetylated DMDP (Fig.1) eluted at R_t 8.85 min and exhibited an electron impact mass spectrum *m/z* 69 (100%, CF_3), *m/z* 97 (11%, CF_3CO), *m/z* 176 (9%), *m/z* 288 (88%, M-3(CF_3COOH)-CH_2+H), *m/z* 302 (24%, M-3(CF_3COOH)+H), *m/z* 416 (1%, M-2(CF_3COOH)+H), *m/z* 516 (2%, M-CF_3COOCH_2), *m/z* 530 (8%, M-CF_3COO), *m/z* 624 (4%, M-F), and *m/z* 643 (M^+, not detectable). The *pentakis*-trimethylsilyl derivative eluted at R_t 10.99 min and showed an electron impact mass spectrum of *m/z* 73 (100%), *m/z* 103 (6%), *m/z* 147 (11%), *m/z* 420 (25%, M-Si$(CH_3)_3OCH_2$), *m/z* 508 (0.3 M-CH_3), and *m/z* 523 (M^+, not detectable). Mass spectra in the positive electron impact mode were obtained on a combined gas chromatograph-mass spectrometer (Fison MD 800, Fisions Instruments, Manchester, UK). Electrospray mass spectra of the compound and its derivatives were measured on an LC-MS (Finnigan LC Qduo, ion trap, ThermoQuest LC/MS Division, San Jose, CA, USA) in the positive mode with an atmospheric pressure ionization source. Solvent was 50% aqueous methanol with 1% acetic acid. The application was performed by infusion with a syringe pump (Bruker, Karlsruhe, Germany) (10 μL·min^{-1}).

^{1}H-NMR spectra were recorded on a Bruker AM 400 spectrometer with Aspect 3000; chemical shifts are given in ppm relative to tetramethylsilane. For the isolated compound, the following was found: (D_2O, 400 MHz) δ 3.86 (m_c, 2H, H-3, H-4), 3.74 (dd, 2H, $J_{1a,1b} = J_{6a,6b}$ = 11.7 Hz, $J_{1a,2} = J_{6a,5}$ = 4.3 Hz, H-1a, H-6a), 3.65 (dd, 2H, $J_{1b,2} = J_{6b,5}$ = 6.3 Hz, H-1b, H-6b), 3.06 (m_c, 2H, H-2, H-5); (CD_3OD, 400 MHz) δ 3.78 (m_c, 2H, H-3, H-4), 3.69 (dd, 2H, $J_{1a,1b} = J_{6a,6b}$ = 11.2 Hz, $J_{1a,2} = J_{6a,5}$ = 4 Hz, H-1a, H-6a), 3.58 (dd, 2H, $J_{1b,2} = J_{6b,5}$ = 6.1 Hz, H-1b, H-6b), 3.01 (m_c, 2H, H–2, H-5).

Quantitative determination of DMDP. DMDP was quantitatively determined in 3-month-old standing cultures of different species of *Cylindrospermum.* Three standing cultures were combined, and 400 mL of cell suspension was separated by centrifugation into about 4 mg biomass (wet weight) and medium. One third of the cyanobacteria was extracted with 20 mL of 50% aqueous methanol for 10 min in an ultrasonic water bath under ice cooling. 1-Deoxynojirimycin (DNJ) was added as an internal standard (50 μL; 1 μg·mL^{-1} 50% aqueous methanol). The extraction was repeated twice with 10 mL of solvent. The extracts were combined and dried in a rotary evaporator at 40° C. The residue was taken up in a minimum amount of 50% aqueous methanol, transferred into a vial, and dried in a gas stream of nitrogen. After derivatization with 200 μL trifluoroactic acid anhydride for 1 h at 45° C in a heating block, the residual re-

agent was removed in a nitrogen gas stream. The residue was taken up in 200 μL *tert.*butylmethylether and subjected within a few hours to analysis by GC-MS. At this time scale the trifluoroactic acid derivatives were stable. The fragment ion *m/z* 288 was extracted from the full spectra and used for quantifying DMDP and DNJ. The areas of the peaks were calibrated with weight reference compounds.

To determine DMDP in the growth medium, the supernatant was passed through a glass fiber filter (GF 6, Schleicher & Schuell, Dassel, Germany). The filtrate (120 mL) was supplemented with the internal standard, reduced to dryness in a rotary evaporator, and extracted three times with 1 mL of 50% MeOH. The other steps of the determination were the same as for the cell extracts.

Determination of chl a. The cyanobacterial biomass was extracted with methanol in the cold and the chl *a* absorption was measured at 665 nm using the molar extinction coefficient given by Seely and Jensen (1965).

Determination of glucosidase inhibition. Glucosidase activities were determined using 4-methylumbelliferyl glucopyranosides (Hoppe 1983) as the substrates essentially as described recently (Corves and Jüttner 2000). All glassware was autoclaved. The buffer for enzyme analysis (0.1 M K_3PO_4/3.2 mM $MgCl_2$, pH 6.8 with HCl) was made from sterile water and filtered through 0.2-μm membranes (Nuclepore, Whatman, Clifton, NJ, USA) before use. The stop buffer contained 0.1 M glycine and 0.1 M NaCl and was adjusted to pH 10.0 with 1 M NaOH. α-Glucosidase (maltase from yeast) exhibited an activity of about 65 $U \cdot mg^{-1}$ and β-glucosidase (from almonds) of about 6 $U \cdot mg^{-1}$. The final concentrations of the enzymes in the assays were 0.1 $U \cdot mL^{-1}$ and 0.08 $U \cdot mL^{-1}$, respectively. 4-Methylumbelliferyl-α-D-glucopyranoside and 4-methylumbelliferyl-β-D-glucopyranoside were added to give a final concentration of 10 μM. Both derivatives were first dissolved in 2-methoxyethanol before dilution with sterile water. The assay consisted of 600 μL water or aqueous inhibitor solution, 100 μL enzyme buffer at pH 6.8, and 100 μL enzyme solution. To start the reaction, 100 μL substrate solution was added and thoroughly mixed. For the determination of t_0, 100 μL were removed and transferred into a cuvette with 0.5 mL stop buffer. After dilution with 3.0 mL water, the fluorescence emission at 446 nm (excitation wavelength 368) was measured on a fluorometer (Hitachi F-2000 with a xenon lamp L 2175-01, Hamamatsu Photonics, Harnamatsu City, Japan). The total reaction and percentage inhibition was determined after 30 min in the same way at 23° C. Typical fluorescence of the uninhibited reactions at the start was 20 to 60 arbitrary units and after a 30-min reaction time 2000 to 4000 arbitrary units. The IC_{50}, using logarithmically scaled inhibitor concentrations, was calculated with the program Prism 2.0 (GraphPed Software, Inc., San Diego, CA, USA).

Glucosidases from aquatic insect larvae. Insect larvae were collected from epilithic biofilms of the littoral zone of Lake Zürich and epilithic diatom biofilms of the Sihl stream near Lake Zürich. About 5 of the large larvae and 20 of the small larvae were ground in buffer and centrifuged, and the supernatants were used for enzyme assays without further purification.

Acute grazer toxicity. Acute 24-h grazer toxicity was determined with *Thamnocephalus platyurus* Packard as described in Blom et al. (2001).

Chemicals. DMDP was purchased from Sigma (Buchs, Switzerland) (no. H 9632, (2*R*,5*R*)-*bis*(hydroxymethyl)-(3*R*,4*R*)-dihydroxypyrrolidine) as a reference compound. The 4-methylumbelliferyl derivatized sugars, α-glucosidase (no. 63412) and β-glucosidase (no. 49290) were from Fluka (Buchs, Switzerland) and DNJ hydrochloride was from Calbiochem (San Diego, CA, USA).

RESULTS

When *C. licheniforme* was tested for inhibitory activity against α- and β-glucosidases, high activity was found in both the medium and cell extract. Because it is much easier to extract biomass rather than media, cells were used both to isolate the responsible compound in an assay-guided isolation procedure and to determine its chemical structure. The cells were extracted once with aqueous methanol or ethanol that removed nearly quantitatively the inhibitory activity from the cells. Because most of the activity was removed by this treatment, further extraction was not performed so as to minimize the amount of inactive contaminants. Passage through a cation exchange resin, on which the inhibitory activity was retained and could be eluted with ammonia, yielded a glassy product. The purified compound was obtained by two consecutive HPLC separations, one on a reversed-phase C18 column and one on an amino column. The compound was detectable at a short wavelength (195 nm) that restricted the choice of solvents. The separations were accompanied by fractionations. Before performing activity assays against glucosidases, each fraction was normalized so that it represented the same amount of biomass. A single compound that contributed to all of the inhibitory activity of *C. licheniforme* was found in the cellular extract. Other derivatives of that compound were not observed.

Electrospray MS in the positive mode of the purified compound showed a quasi-molecular ion at *m/z* 164 ($[M+H]^+$) and a *tetrakis*-(trimethylsilyl) derivative at *m/z* 452 ($[M+H]^+$). The uneven molecular mass (M = 163) suggested the presence of a nitrogen atom and the formation of a *tetrakis*-(trimethylsilyl) derivative the presence of four hydroxyl groups (i.e. an elemental composition of $C_6H_{12}O_4N$).

The ^{1}H-NMR spectra in deuterated water and methanol indicated that the compound had C_2 or C_{2v} symmetry. Compounds compatible with these requirements are *bis*-(hydroxymethyl)-dihydroxypyrrolidines. A comparison of the spectra with published (Asano et al. 1995) ^{1}H-NMR data of *bis*-(hydroxymethyl)-dihydroxypyrrolidines indicated (maximal deviation −0.02 ppm, −0.1 Hz) that the isolated compound is 2(*R*),5(*R*)-*bis*-

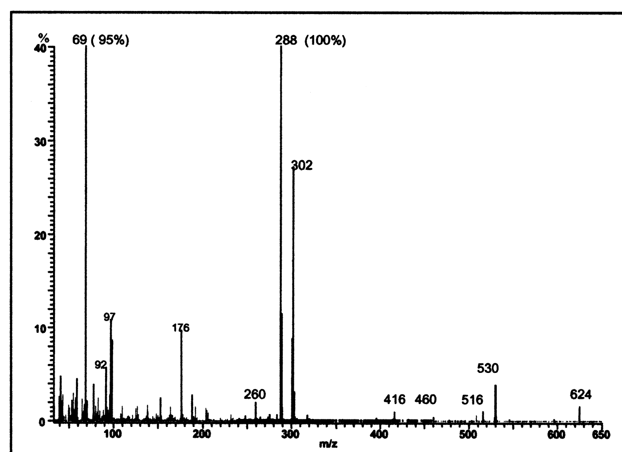

FIG. 1. Electron impact mass spectrum of trifluoroacetylated DMDP isolated from *Cylindrospermum licheniforme.* The molecular ion *m/z* 643 (M^+) is not visible.

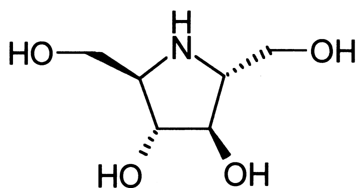

FIG. 2. Chemical structure of DMDP.

(hydroxymethyl)-3(*R*),4(*R*) dihydroxypyrrolidine (Fig. 2), also referred to as DMDP (*D*i(hydroxy*m*ethyl)*d*ihydroxy*p*yrrolidine).

The stereochemistry of this compound was confirmed by comparison of the ^{1}H-NMR spectra with those of an authentic commercial sample. This identity with a DMDP reference compound was further supported by the *pentakis*-trifluoroacetylated (Fig. 1) and *pentakis*-(trimethylsilyl) derivatives. The derivatives of both compounds exhibited the same electron impact fragmentation patterns and retention times by GC-MS. This assumption was further corroborated by the specific inhibitory activities against the model enzymes from almonds and yeast that were of the same size in the isolated and the authentic compound (Table 1).

The cellular content of DMDP was determined for five standing (unshaken) batch cultures of different species of *Cylindrospermum.* The axenic culture of *C. licheniforme* and an undetermined strain of *Cylindrospermum* exhibited high concentrations of DMDP (Table 2), whereas the biomass of the other three strains investigated also contained this molecule but at lower concentrations. However, normalized to chl *a* concentrations in the culture, the extracellular amount of DMDP surpassed the intracellular in all strains. The measured extracellular amounts of the nonaxenic strains are minimum values because partial degradation of this compound by the bacterial contaminants cannot be ruled out.

DMDP was active against both α-glucosidase and β-glucosidase, isolated from yeast and almonds, respectively (Table 1). Because it was not clear whether the inhibitor was also active for digestive enzymes of aquatic invertebrates, against which these inhibitors may be directed, it was also tested against digestive glucosidases of aquatic grazers. Zooplankton were harvested from the pelagial of Lake Zürich; larvae of chironomids and *Tinodes waeneri* L. (trichoptera) were collected from biofilms of the littoral zone of Lake Zürich and larvae of *Serratella ignita* Popa and *Ecdyonurus dispar* Curtis (ephemeroptera) from diatom biofilms that had developed on cobbles in a stream nearby. The crude enzyme extracts obtained from these animals were used to determine the inhibitory activity of DMDP and DNJ as a well-known reference inhibitor (Table 3). Both inhibitors were active against the digestive enzymes of the zooplankton and showed higher activity against α-glucosidase than β-glucosidase on a molar basis. As compared with DNJ, equimolar

TABLE 1. Comparison of the IC_{50} (nM) of authentic DMDP and the isolated compound from *Cylindrospermum licheniforme* against α-glucosidase and β-glucosidase from yeast and almonds, respectively.

Enzyme	IC_{50} Isolated compound	DMDP
α-Glucosidase	84 ± 6.8	77 ± 2.0
β-Glucosidase	201 ± 6.4	185 ± 7.8

Values are means ± SD (n = 3).

concentrations of DMDP were 27 and 44 times more active against α-glucosidase and β-glucosidase, respectively. The digestive enzymes isolated from the insect larvae were less sensitive to DMDP. In addition, for all insect glucosidases, higher inhibitory activity was found against β-glucosidase than α-glucosidase. The bacterial DNJ had a reverse effect and was more inhibitory for α-glucosidase than for β-glucosidase from insects. However, both glucosidases of *Tinodes waeneri* exhibited similar sensitivity against DMDP. In a 24-h acute grazer toxicity assay against the crustacean *Thamnocephalus platyurus*, DMDP was not toxic up to a concentration of 100 μM.

DISCUSSION

DMDP is the first glucosidase inhibitor described for cyanobacteria. In its basic structure it resembles imino sugars isolated from bacteria (Gräfe 1992) and higher plants. The compound was first isolated from the plant *Derris elliptica* (Welter et al. 1976, Fleet and Smith 1985) and was later found in several other higher plants that were poisonous to livestock or caused digestive disturbances if eaten by livestock (Fellows et al. 1989). DMDP is the first secondary metabolite of cyanobacteria that was also found in higher plants. The relatively few biosynthesis steps necessary to create this compound from fructose (i.e. transamination, oxidation, and cyclization reactions) make it likely that this imino sugar developed independently as a grazer defense molecule in both groups of organisms (Piepersberg 1997).

Extensive studies have shown that this compound effectively inhibits various glycosidases. Its ecological function has been considered in reducing the nutritional value of the food (Kite et al. 1997) by inhibiting digestive enzymes in the saliva and gut of the grazers. The strong inhibition of digestive glucosidases was previously observed for the enzymes of Lepidoptera, Diptera, Coleoptera, and other orders of insects (Scofield et al. 1995). We also found this inhibition in insect larvae belonging to the macrozoobenthos that graze heavily on epilithic photoautotrophic biofilms of the littoral zone of Lake Zürich and a nearby stream. Insect larvae are active grazers in these habitats, as are snails, harpactoid crustaceans, and worms. The energy balance of grazers is assumed to be negatively influenced by digestive inhibitors. An exploratory calculation suggests DMDP may occur in ecological relevant

TABLE 2. DMDP concentration of the biomass and in the growth medium of 3-month-old standing (unshaken) batch cultures of different species of *Cylindrospermum.*

	Biomass			Medium		
Strain	pmol·nmol^{-1}[a]	pmol·mg^{-1}[b]	SD[c]	pmol·nmol^{-1}[d]	pmol·mL^{-1}[e]	SD[c]
C. alatosporum	1.4	0.5	±22	42	303	±28
C. licheniforme	22	58	±9	68	603	±25
C. muscicola	1.1	0.2	±71	—	—	
C. stagnale	0.9	0.6	±63	118	528	±12
Cylindrospermum sp.	159	88	±6	2080	11,300	±26

[a] Concentration of DMDP in the biomass as pmol DMDP per nmol chl *a*.
[b] Concentration of DMDP in the biomass as pmol DMDP per mg wet weight.
[c] Values are means ± SD for three separate determinations of three combined standing cultures.
[d] Concentration of DMDP in the medium given as pmol per nmol cellular chl *a*.
[e] Concentration of DMDP in the medium given as pmol per mL.

concentrations in the gut of grazers. When 5% of the ingested food is *Cylindrospermum*, this concentration would be sufficient to cause a 50% inhibition of the most resistant glucosidase found in Ephemeridae. A *Cylindrospermum* food ratio of 1:4000 would be sufficient to cause the same effect in the more sensitive crustaceans.

In addition, glucosidase inhibitors may favor the survival of cyanobacterial cells during passage through the digestive system of grazers because the glucosidase inhibitors help to maintain the integrity of the mucilage that shields the cells. Experiments with *Acanthodiaptomus* have shown that cyanobacterial cells are rapidly digested when the mucilage has been removed but remain unchanged when the residence time in the gut is not sufficient to allow hydrolysis of the mucilage (Goarant et al. 1994).

Cylindrospermum frequently forms biofilms that are exposed to grazing. Although more than 80% of the synthesized DMDP in a culture was found in the medium, the cellular concentrations were high because the volume of the biomass represented only a very small part of the total volume of the culture. The distribution of DMDP between the cellular and extracellular compartments found in the cultures should only be used as a rough estimate because numerous environmental factors influence the DMDP distribution, as previously shown for an allelochemical produced by the planktonic cyanobacterium *Trichormus doliolum* (von Elert and Jüttner 1997). Under natural conditions, the concentrations of DMDP in the intercellular space of biofilms are strongly influenced by the exchange of water. This exchange is dependent on the rigidity of the biofilm, the turbulence of the supernatant water, and the activity of animals. Situations favoring both much lower or much higher concentrations may also occur.

The digestive enzymes of the zooplankton were relatively sensitive to DMDP, whereas those of benthic insect larvae required higher concentrations before becoming inhibited. A 50% inhibition of digestive α-glucosidase from *Seratella* was only achieved through the application of a much higher concentration of DMDP—215 times higher than that required to induce inhibition in the crustacean grazers. This large difference in digestive enzyme sensitivity between zooplankton grazers and benthic insect larvae likely indicates that resistance against such compounds has not evolved in pelagic crustaceans. The investigation of different insect larvae further revealed that the resistance to such molecules among different insect groups have developed to different extents.

By applying electrophysiological methods and behavioral observations, DMDP has been shown to be an effective deterrent to grazing by lepidopterans (Simmonds et al. 1990). The responses to DMDP were dose independent. The experiments conducted with aquatic insect larvae and crustaceans are not suitable

TABLE 3. IC_{50} (nM) of DMDP isolated from *Cylindrospermum licheniforme* and DNJ for digestive α-glucosidase (α-Gluc) and β-glucosidase (β-Gluc) isolated from different grazers.

	IC_{50} (nM)			
	DMDP		DNJ	
Grazer	α-Gluc	β-Gluc	α-Gluc	β-Gluc
Crustacean zooplankton	19 ± 15.9	49 ± 4.2	520 ± 0.8	2190 ± 6.0
Chironomids	2220 ± 9.8	210 ± 10.5	610 ± 5.4	37,500 ± 3.2
Trichoptera				
Tinodes waeneri	230 ± 3.0	150 ± 4.4	2180 ± 4.0	3240 ± 2.2
Ephemerida				
Serratella ignita	4090 ± 4.9	320 ± 5.8	52 ± 7.3	6140 ± 2.8
Ecdyonurus dispar	1230 ± 3.8	120 ± 4.7	280 ± 11.7	3990 ± 1.6

Values are means ± SD (n = 3).

to give any information on the existence of such an effect, although grazing deterrent activity has been reported for cyanobacteria. *Eudiaptomus* ingested fragmented filaments of *Planktothrix rubescens* only after extraction with methanol, thus making the existence of such compounds likely (Kurmayer and Jüttner 1999). Grazing deterrent activities were also described for *Microcystis* (Rohrlack et al. 1999).

In addition, toxicity against coleopterans (Evans et al. 1985) and nematodes (Birch et al. 1993) has been reported for DMDP. Although we did not find acute toxicity against *Thamnocephalus platyurus,* we have not ruled out the possibility of chronic toxicity, inhibition of embryogenesis, and differential sensitivity among grazers. Possible target sites of DMDP may be glycoprotein processing, because DMDP has been shown to be a powerful inhibitor of glucosidase I (Elbein 1991). However, it is not known to what extent the strongly hydrophilic DMDP is taken up and accumulated in the cells of grazers.

DMDP may also have protective functions against the action of microorganisms for *Cylindrospermum.* Of primary importance to biofilm and pelagic food organisms is the maintenance of the mucilage integrity as a protective barrier. Attack of the mucilage is expected by bacteria that use the mucilage matrix as a nutrient and by pathogenic bacteria that may use both α- and β-glucosidases to hydrolyze the sugar bonds for penetration, in analogy to the better studied systems in humans. β-Glucosidic linkages are dominant in the walls of akinetes of *C. licheniforme* (Cardemil and Wolk 1979); DMDP, which is particularly inhibitory to β-glucosidases, would protect the integrity of the cell walls. The high extracellular glycosidase activities observed in photoautotrophic biofilms (Romaní and Sabater 2000) support the view that such an attack is common in this habitat. Any impregnation of the mucilage with glucosidase inhibitors that are extremely hydrophilic will unavoidably cause a continuous loss of these compounds into the surrounding water. Because the mucilage is an extracellular compartment, we do not expect there the strict regulation of uptake and release of compounds, as is observed for live cells. This would explain the appreciable amounts of DMDP found in the media of the cultures.

Although it is not known to what extent the different target sites in competitors and grazers are inhibited by DMDP, the general importance of glucosidase inhibitors has been shown by the wide distribution of this phenomenon in aquatic ecosystems. Investigation of subtropical cyanobacterial biofilms (Jüttner and Wu 2000) and of algal biofilms of the littoral zone of Lake Zürich demonstrates a high frequency of occurrence of such inhibitors, but the responsible compounds have not yet been determined in these cases (Corves and Jüttner 2000). The detection of DMDP should help to study this phenomenon in future on a more molecular basis.

We are grateful for the technical assistance of Eugen Loher, Helga Lüthi, and Angela Mechsner and of Odile Roger (Roche) for literature work. NMR spectra were recorded by Dr. W. Arnold, F.Hoffmann-La Roche Ltd. Dr. H. Vicentini, Fisch- und Gewässerökologie, Zürich, is thanked for collecting and identifying the insect larvae. This study was supported in part by the National Science Foundation, Bern, Switzerland.

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