Nostocyclamide: A New Macrocyclic, Thiazole-Containing Allelochemical from *Nostoc* sp. 31 (Cyanobacteria)

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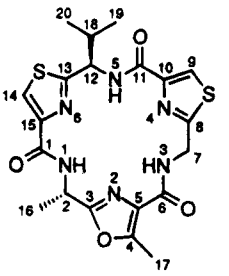
A new anticyanobacterial and antialgal secondary metabolite, nostocyclamide, that is also toxic against *Brachionus calyciflorus* has been isolated as a major component from the dinitrogen-fixing cyanobacterium *Nostoc* 31. Extensive ^1H and ^{13}C NMR and X-ray analyses proved this compound to be a cyclic peptide which has not previously been observed in other microorganisms. The planar macrocyclic structure exhibits substituted oxazole and thiazole residues that are linked by three peptide units.

The genus *Nostoc* is represented by filamentous cyanobacteria that can express heterocysts for dinitrogen fixation. *Nostoc* species are terrestrial and benthic microorganisms which often form extended mucilaginous layers on the soil and in the aquatic environment on stones and mud. Several secondary metabolites with new structures have been isolated from these organisms. In nature these components may be directed against phototrophic competitors (cyanobacteria and algae) and grazers. The pharmacological value of these substances is that they have been shown to exhibit antiviral and antitumor properties, and *Nostoc*, as cyanobacteria in general, have gained much interest as a natural source of such compounds.

Indolo[2,3-*a*]carbazoles were isolated from *Nostoc sphaericum* and exhibited moderate antiviral activity against herpes simplex virus type 2 and weak, nonselective cytotoxicity.[1] *Nostoc linckia* was used as the source of several paracyclophanes and showed moderate cytotoxicity against KB and LoVo tumor cell lines.[2] The basic structure, nostocyclophane C, was accompanied by the *O*-methyl derivative and the mono- and bis-*β*-glucosides.[3] An unclassified *Nostoc* species, isolated from a bloom of *Aphanizomenon* in a Finnish lake, contained seven different hepatotoxic cyclic peptides of the basic structure of microcystins.[4–6] By using the development of fertilized sea urchin eggs as a guide, the cytotoxic compound nostodione A was isolated from naturally occurring layers of *Nostoc commune*.[7] Several other strains of *Nostoc* have been shown to exhibit antibiotic,[8] antifungal,[9] algicidal,[10] and antiviral[11] activity, but the compounds responsible for these effects have not been determined. Potent antitumor depsipeptides have recently been isolated from *Nostoc* GSV 224.[12]

Chart 1



In an extended survey, Flores and Wolk[13] tested 65 filamentous cyanobacteria for the production of antibiotics. One of these strains, *Nostoc* sp. 31, that was inhibitory against most cyanobacterial strains tested is now studied in more detail, and the result is reported in this contribution. We purified the major anticyanobacterial allelochemical by using a bioassay-guided isolation procedure and determined it to be a novel macrocyclic compound consisting of an 18-membered ring derived from two thiazole and one oxazole moieties alternating with amide groups (Chart 1).

Spectral Data. Nostocyclamide is a lipophilic compound, mp 255.8–256.9 °C dec, which is readily soluble in dichloromethane and chloroform, less soluble in methanol, and insoluble in water. The UV spectrum (CH_3OH) exhibits strong absorption maxima at 202 (log ϵ = 4.49) and 223 nm (log ϵ = 4.40). The IR spectrum ($CHCl_3$) displays bands at 3397 (m, N–H), 3007 (m, =C–H), 2967 (w) and 2928 (w, C–H), 1666 (s), 1642 (m), 1543 (s), 1521 (m), and 1497 (m) cm^{-1}. The EI MS shows the molecular ion at *m*/*z* 474 (5) and characteristic fragment ions

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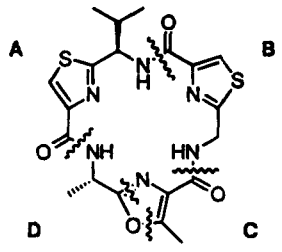
Chart 2



(relative intensity) at m/z 431 (100; $M^+ - C_3H_7$), 276 (80), 222 (7), 141 (10), 136 (24), and 43 (18).

An ^{15}N-labeling experiment was carried out to determine the number of nitrogen atoms. The atom percent labeling of the ^{15}N labeled compound (2 mg was obtained from 2 g dry weight of *Nostoc*) was 13% for the four-times, 24% for the five-times, and 41% for the six-times labeled species, which suggested the presence of six nitrogen atoms. The molecular ion was used for the calculation according to Biemann,[14] and it was observed that the molecular ion shifted from m/z 474 to 480, while the fragment ions shifted from m/z 431 to 437, m/z 276 to 279, m/z 222 to 224, m/z 141 to 143, m/z 139 to 141, and m/z 136 to 137.

Hydrolysis of nostocyclamide (MeOH/3 M HCl, 48 h at 110 °C) provided several methyl esters which were analyzed by TSMS without previous separation. The ions obtained were m/z 104, $(C_4H_{10}NO_2)^+$, [D = Ala]H^+; m/z 173 $(C_6H_9N_2O_2S)^+$, [B]H^+; m/z 215 $(C_9H_{15}N_2O_2S)^+$, [A]H^+; m/z 286 $(C_{12}H_{20}N_3O_3S)^+$, [A + D]H^+; m/z 354 $(C_{14}H_{18}N_4O_3S_2)^+$, [A + B]$^+$; m/z 426 $(C_{17}H_{24}N_5O_4S_2)^+$, [A + B + D − H]$^+$ representing the different amino acids and di- and tripeptides, respectively (Chart 2).

Upon hydrolysis and subsequent derivatization with TFAA/CH_2Cl_2 (1/1, v/v) for 1 h at 80 °C, several compounds could be separated on a 30 m DB-1 fused silica capillary column (1 min at 70 °C, 70−270 °C, 10 °C/min) that exhibited molecular ions at m/z 199 (D in Chart 2 = Ala), 268 (B), 280 (C + D), 310 (A), and 381 (A + D).

For further characterization of the compound, we have carried out extensive NMR studies. The 1D ^1H and ^{13}C and the 2D $^1H,^1H$ and $^1H,^{13}C$ correlated spectra were obtained on a 600 MHz spectrometer. For further details, see the Experimental Section. The proton spectra obtained in CD_3OD and CD_3SOCD_3 solutions exhibit signals for the following groups: 3 CH_3(CH), 1 CH_3(C), 1 CH_AH_B, 2 CH(CH_3), 1 CH(CH), 2 =CH, and 3 NH signals (2 doublets, 1 triplet) in the 8.3−8.6 ppm region, which are exchangeable for deuterium (Table 1). Hence, the compound contains 22 hydrogen atoms. The carbon-13 spectra obtained in the same solvents are very similar and, together with the information from a DEPT spectrum, exhibit the presence of 20 carbon signals which can be assigned to 4 CH_3, 1 CH_2, 5 CH, and 10 nonprotonated carbon atoms (Table 1). Thus, the compound has a $C_{20}H_{22}$ skeleton.

A detailed inspection of these 1D spectra reveals the presence of an isopropyl group, two isolated olefinic protons, and 10 nonprotonated sp^2 C atoms in the 130−170 ppm range. Further information about the structural subunits was derived from homonuclear 2D correlation experiments (double-quantum-filtered COSY and TOCSY) which link the 3 NH resonances to 4 corresponding CH signals, revealing the presence of two NH−CH and one NH−CH_2 systems. The spectra illustrate that one CH is substituted by the isopropyl group, the other by a methyl group. The unknown compound, therefore, contains the structural elements of the amino acids glycine (CH_2NH), valine (($CH_3)_2CHCHNH$), and alanine (CH_3CHNH).

Table 1. ^1H and ^{13}C Chemical Shifts (δ/ppm), $^1H,^1H$ Coupling Constants (J/Hz), and Long-Range C,H Correlations[a]

	δ(H)	δ(C)	J(H,H)	correlation C → H
C(1)		159.63 (s)[b]		N(1)H
C(2)H	5.126 (dq)	44.47 (d)	5.0; 6.7	N(1)H; C(16)H
C(3)		161.53 (s)		N(1)H; C(16)H; C(2)H
C(4)		153.09 (s)		C(17)H
C(5)		127.80 (s)		N(3)H; C(17)H
C(6)		157.83 (s)		C(7)H^A; C(7)H^B; N(3)H
C(7)H^A	5.012 (dd)	40.64 (t)	17.8; 5.0	
C(7)H^B	4.740 (dd)		17.8; 3.2	
C(9)H[c]	8.397 (s)	125.78 (d)		
C(10)		147.94 (s)		C(9)H
C(11)		159.20 (s)		C(12)H; N(5)H
C(12)H	5.660 (dd)	55.07 (d)	9.1; 3.9	C(19)H; C(20)H
C(13)		168.85 (s)		N(5)H; C(18)H; C(12)H
C(14)H[c]	8.395 (s)	125.04 (d)		
C(15)		147.65 (s)		C(14)H
C(16)H_3	1.575 (d)	19.47 (q)	6.7	C(2)H
C(17)H_3	2.501 (s)	11.16 (q)		
C(18)H	2.242 (m)	35.69 (d)		C(12)H; C(19)H; C(20)H
C(19)H_3[c]	0.812 (d)	18.57 (q)	6.9	C(12)H; C(20)H
C(20)H_3[c]	0.858 (d)	16.95 (q)	6.7	C(12)H; C(19)H
N(1)H	8.558 (d)		5.0	
N(3)H	8.376 (dd)		4.1; 4.0	
N(5)H	8.460 (d)		9.0	

[a] Correlations were obtained by HMBC using a delay corresponding to ^nJ(C,H) = 8 Hz and, therefore, are not complete. [b] Refers to one-bond C,H coupling multiplicity. [c] Arbitrary assignments.

Heteronuclear 2D correlation experiments (one-bond C,H HMQC and long-range C,H HMBC) were utilized to assign the sp^3 carbon resonances and to obtain structural information about the 10 nonprotonated carbons. The resonances of C(4), C(6), C(8), C(11), and C(13) can be assigned on the basis of their chemical shifts and long-range correlations to protons already identified (see Table 1). The nature of the remaining five nonprotonated carbon atoms was recognized by using the X-ray structure together with the long-range C,H correlation data. These atoms were the amide carbonyl C(1) and the heterocyclic ring carbon atoms C(3), C(5), C(10), and C(15).

X-ray Structure. It was difficult to obtain high-quality crystals for the analysis; therefore the estimated standard deviations of the atomic parameters are slightly larger than normal. However, the conformation of the molecule is clearly defined. A view of the nostocyclamide molecule is shown in Figure 1.[15] The crystals are enantiomerically pure, and an attempt was made to determine the absolute configuration. The enantiopole parameter[16] was refined and yielded a value of −0.03(15) which indicates that the configuration of the molecule depicted in the diagrams and defined by the atomic coordinates most likely represents the true absolute configuration, although the estimated standard deviation on this parameter is quite high. The configuration at atoms C(2) and C(12) is therefore *S* and *R*, respectively (Figure 1).

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Table 2. Selected Torsion Angles (deg) for Nostocyclamide

S(1)–C(8)–N(4)–C(10)	−0.3(4)	N(4)–C(10)–C(11)–N(5)	7.5(5)
S(1)–C(8)–C(7)–N(3)	−174.7(3)	N(5)–C(12)–C(13)–N(6)	13.8(5)
S(1)–C(9)–C(10)–N(4)	−0.5(5)	N(6)–C(13)–S(2)–C(14)	1.7(3)
S(1)–C(9)–C(10)–C(11)	179.5(3)	C(1)–N(1)–C(2)–C(3)	−171.2(3)
S(2)–C(13)–N(6)–C(15)	−1.6(4)	C(1)–C(15)–N(6)–C(13)	−179.8(3)
S(2)–C(13)–C(12)–N(5)	−169.0(3)	C(2)–N(1)–C(1)–C(15)	−175.9(3)
S(2)–C(14)–C(15)–N(6)	0.7(5)	C(2)–C(3)–O(2)–C(4)	−179.0(3)
S(2)–C(14)–C(15)–C(1)	−178.9(3)	C(2)–C(3)–N(2)–C(5)	178.7(4)
O(1)–C(1)–N(1)–C(2)	5.9(6)	C(3)–O(2)–C(4)–C(5)	0.3(4)
O(1)–C(1)–C(15)–N(6)	170.4(4)	C(3)–N(2)–C(5)–C(4)	0.3(4)
O(2)–C(3)–N(2)–C(5)	0.0(4)	C(3)–N(2)–C(5)–C(6)	−178.7(4)
O(2)–C(3)–C(2)–N(1)	164.2(3)	C(5)–C(6)–N(3)–C(7)	178.8(4)
O(2)–C(4)–C(5)–N(2)	−0.4(4)	C(6)–N(3)–C(7)–C(8)	−164.8(4)
O(2)–C(4)–C(5)–C(6)	178.5(4)	C(7)–C(8)–S(1)–C(9)	177.4(4)
O(3)–C(6)–N(3)–C(7)	−1.0(7)	C(7)–C(8)–N(4)–C(10)	−177.6(4)
O(3)–C(6)–C(5)–N(2)	175.5(4)	C(8)–S(1)–C(9)–C(10)	0.3(3)
O(4)–C(11)–N(5)–C(12)	−3.9(6)	C(8)–N(4)–C(10)–C(9)	0.5(5)
O(4)–C(11)–C(10)–N(4)	−173.9(4)	C(8)–N(4)–C(10)–C(11)	−179.5(3)
N(1)–C(1)–C(15)–N(6)	−7.8(5)	C(10)–C(11)–N(5)–C(12)	174.6(3)
N(1)–C(2)–C(3)–N(2)	−14.5(5)	C(11)–N(5)–C(12)–C(13)	161.3(3)
N(2)–C(3)–O(2)–C(4)	−0.2(4)	C(12)–C(13)–S(2)–C(14)	−175.8(3)
N(2)–C(5)–C(6)–N(3)	−4.3(5)	C(12)–C(13)–N(6)–C(15)	175.8(3)
N(3)–C(7)–C(8)–N(4)	2.3(6)	C(13)–S(2)–C(14)–C(15)	−1.2(3)
N(4)–C(8)–S(1)–C(9)	0.0(3)	C(13)–N(6)–C(15)–C(14)	0.6(5)

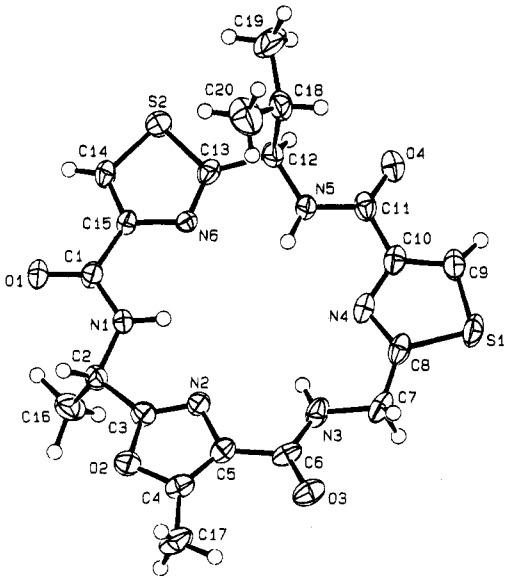

Figure 1. Thermal ellipsoid plot (50% probability ellipsoids) of the nostocyclamide molecule.

Table 3. Crystallographic Data for Nostocyclamide

empirical formula	$C_{20}H_{22}N_6O_4S_2 \cdot {}^1/_2CH_3OH$
formula weight	490.57
crystal color, habit	colorless, prism
crystal dimensions (mm)	0.18 × 0.20 × 0.43
temperature (K)	173 (1)
crystal system	orthorhombic
space group	$P2_12_12_1$ (no. 19)
a (Å)	13.263(3)
b (Å)	13.835(7)
c (Å)	12.384(6)
V (Å^3)	2273(2)
Z	4
F(000)	1028
D_{calc} (g cm^{-3})	1.434
μ(Mo $K\alpha$) (mm^{-1})	0.278
$2\theta_{(max)}$ (deg)	55
no. of measured reflns	6914
no. of unique reflns	5186
no. of observations [$I > 3\sigma(I)$]	3303
no. of parameters	377
R	0.0442
R_w	0.0373
goodness of fit	1.611
final Δ_{max}/σ	0.007
$\Delta\rho$ (max; min) [e Å^{-3}]	0.48; −0.33

The crystals contain solvent of crystallization, which is probably methanol, although disorder within the solvent region makes it difficult to be absolutely certain about the composition of the solvent. The electron density distribution in the region of the solvent molecule shows that the solvent is composed of two disordered light atoms. The solvent site is also only partially occupied such that the ratio of solvent molecules to nostocyclamide molecules in the crystal is 1:2.

The bond lengths and angles within the amide groups and the 5-membered rings are all normal. The estimated standard deviations in the bond lengths and angles are in the range 0.004–0.007 Å and 0.2–0.4°, respectively. Each of the 5-membered rings is planar, with the maximum deviation of any atom from its ring plane being 0.014(4) Å for C(13). The entire nostocyclamide molecule also forms a relatively planar system. The angles between the planes of the three 5-membered rings are all less than 11°, and excluding C(16) and the *i*-Pr group, the mean deviation of all of the atoms of the molecule from the mean plane is 0.14 Å. Selected torsion angles for the molecule are given in Table 2. There are no intramolecular hydrogen bonds in the molecule, and it does not form any intermolecular hydrogen bonds with neighboring nostocyclamide molecules. The solvent does not lie near the center of the nostocyclamide ring and is therefore not coordinated by the toxin; however, solvent molecules lie within 2.9–3.0 Å of each amide O atom, which could be an indication of possible intermolecular hydrogen bonding interactions between the nostocyclamide molecule and surrounding solvent molecules.

The geometry of the nostocyclamide molecule makes it a potential porphyrin-like ligand. The distances between diametrically opposed N atoms are in the range 5.2–5.6 Å, and a cavity of this size could easily accommodate small cations such as Li^+, Na^+, and Mg^{2+}, as well as a range of transition metal cations, although many of these cations are too small to be simultaneously coordinated by all of the donor N atoms of the nostocyclamide ring. The cavity is too small to allow coordination to K^+ or Ca^{2+} ions unless the molecule distorted significantly from planarity during coordination.

The structures of several macrocyclic thiazole-containing amides are known; these being the bicyclic compound, nosiheptide,[17] cyclo[L-Pro-L-Leu-L-Val-(Gly)Thz-(Gly)-Thz],[18] ascidiacyclamide,[19] patellamide D,[20] and tawicyclamide.[21] While the first two compounds have complex or irregular sequences of groups, each of the latter three compounds has a 24-membered macrocyclic ring made up of four heterocyclic 5-membered rings and intervening amide linkages. In each case these rings possess a saddle-shaped conformation. Nostocyclamide is only an 18-membered macrocycle, having one less heterocyclic 5-membered ring and amide linkage, and as such, the geometrical constraints within the molecule apparently permit it to adopt an almost planar conformation which has hitherto not been observed in this class of compounds.

Bioassays. Nostocyclamide was very growth inhibitory against cyanobacteria (*Anabaena* P-9, *Anabaena* PCC 7120, *Synechococcus* PCC 6911 and *Synechocystis* PCC 6308), diatoms (*Navicula minima*), and chlorophyceae (*Nannochloris coccoides* SAG 251-1). In the overlayer test systems, it behaved as a cytotoxic rather than a cytostatic agent against the indicator strains. The compound did not show antifungal activity when tested against *Saccharomyces cerevisiae*. In liquid cultures, nostocyclamide was inhibitory to the growth of *Anabaena* P-9 at a concentration of 0.1 μM (1 week incubation in the light at 25 °C). Considerable toxicity was observed (LC_{50} 12 μM) in a freshwater rotifer (*Brachionus calyciflorus*) bioassay. Details of the bioassays and the target site of the compound will be presented in a separate paper.

Biosynthetic Aspects. Several thiazole-containing cytotoxic compounds, such as dolastatin 3, ulithiacyclamides, and patellamides, have been isolated from sea hares and tunicates,[22] although these compounds actually may come from the photosynthetic active prokaryotic symbionts of these marine animals. However, in free living cyanobacteria, thiazole-containing secondary metabolites have, so far, only been found in *Scytonema mirabile*.[23] Mirabazole A, which was isolated from this cyanobacterium, contains a 2,4-connected thiazole which is linearly arranged with thiazolidine moieties. Other thiazole-containing compounds observed in *Scytonema* proved to be artifacts obtained by oxidation of the thiazolidine rings of the mirabazoles and tantazoles. Although the formation of mirabazoles and tantazoles requires extensive structural modifications when amino acids are assumed to be precursors of these molecules, nostocyclamide can easily be deduced from a cyclic hexapeptide which is composed of the proteinaceous amino acids Ala, Val, Gly, Thr, and possibly two Cys molecules. The peptide bonds have, however, undergone severe modifications in order to create the thiazole and oxazole structural elements. The reaction of the carbonyl group with the thiol group of Cys has been shown to occur during the biosynthesis of thiazole rings in the antibiotic bleomycin[24] by using ^{14}C-labeled amino acids.

Experimental Section

General. Low-resolution mass spectra (EI mode) were obtained on a GC–MS. The fused silica capillary column employed was a 10 m SIM-DIST (0.32 i.d., Chrompack), the temperature program 1 min at 200 °C, 10 °C min^{-1} up to 300 °C and 10 min isothermal at 300 °C. Helium served as the carrier gas (90 kPa head pressure). The retention time of nostocyclamide was 8.2 min. Thermospray mass spectra were run on a thermospray mass spectrometer by injection of 50 μL samples (flow rate 1 mL min^{-1}, MeOH/0.1 M ammonium acetate, 1:1, v/v; vaporizer tip temperature at 215 °C; block temperature at 300 °C).

The ^1H and ^{13}C NMR spectra were measured at 600 and 150 MHz, respectively, and at 300 K. A solution of 8 mg of the natural product in 0.4 mL of DMSO-d_6 was used. The 2D spectra were obtained with standard pulse sequences, i.e., double-quantum-filtered phase sensitive COSY, TOCSY, phase sensitive HMQC, ROESY, and HMBC. All experiments were carried out with a 5 mm INVERSE probehead except the ^{13}C measurements for which a standard 5 mm broad-band probe was used.

Sources and Cultivation of Cyanobacteria. Axenic *Anabaena* P-9 and *Nostoc* sp. 31 were obtained from P. Wolk, Michigan State University, East Lansing. *Synechococcus* 6911, *Synechocystis* 6308, and *Anabaena* 7120 were from the Pasteur Culture Collection, Paris. *Nannochloris coccoides* SAG 251-1 was from the Sammlung von Algenkulturen, Göttingen, Germany. The cyanobacteria were grown under photoautotrophic conditions on a cyanobacterial medium[25] in 6-L glass tubes as described previously.[26] *Nostoc* cells were harvested in a continuous flow-through centrifuge. The yield of lyophilized cells averaged 0.4 g/L of culture suspension. ^{15}N-labeled substances were obtained with culture media in which the naturally labeled nitrate was replaced by ^{15}N-labeled nitrate (99% purity, 20 mmol).

Isolation and Purification. Cyanobacterial biomass (25 g wet weight) was extracted 3 times with 50 mL of ethanol (each time treated for 15 min with a sonifier tip in the intermittent mode) and the ethanolic solution twice with 75 mL of *tert*-butyl methyl ether. Water was added in quantities to obtain good separation of the phases. The ether phases were combined, dried over anhydrous Na_2SO_4, and brought to dryness with a rotary evaporator. The residue was dissolved in 10 mL of 90% ethanol and passed through a cartridge packed with 2 g of C18 reversed phase sorbent (Mega Bond Elut, Varian). The eluate and washings (5 mL of 90% ethanol) were evaporated, and the residue was dissolved in 2 mL of methanol and subjected to preparative HPLC-separation using a LC8 column (250 × 10 mm, 5 μm Nucleosil, Macherey & Nagel, Düren, Germany) and a H_2O/MeOH gradient (12 min from 50:50 (v/v) to 0:100 (v/v); 10 min MeOH) with a flow rate of 3 mL min^{-1}. The absorption was monitored at 265 and 450 nm to trace the active compound and contaminating pigments, respectively. The major allelopathic activity was found at a retention time of 15.8 min. The active fraction was further purified by preparative TLC on 1 mm silica gel plates (60 F_{254}, Merck, Darmstadt) using $CHCl_3$/MeOH (40:1, v/v) as the solvent. The strips containing nostocyclamide (R_f 0.36), detected by their blue fluorescence under UV light (254 nm excitation), were scraped off and eluted with dichloromethane/diethyl ether (1/1, v/v) to give pure nostocyclamide. Blue spots were obtained when nostocyclamide was treated with anisal-

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dehyde spray reagent and white spots upon treatment with iodine reagent.[27] The average yield of nostocyclamide was 1 mg/g cyanobacterial dry weight.

X-ray Crystal-Structure Determination. Crystals of nostocyclamide, obtained from methanol, were used for a low-temperature X-ray structure determination. All measurements were made on a Rigaku AFC5R diffractometer using graphite-monochromated Mo Kα radiation (λ = 0.710 69 Å) and a 12 kW rotating anode generator. Most of the data collection and refinement parameters are given in Table 3.[28] The unit cell constants were obtained from a least-squares refinement of the setting angles of 22 carefully centered reflections in the range $9° < 2\theta < 26°$. The $\omega/2\theta$ scan mode was employed for data collection, where the ω scan width was $(1.37 + 0.35 \tan \theta)°$ and the ω scan speed was 8° min^{-1}. The weaker reflections [$I < 10\sigma(I)$] were rescanned up to a maximum of 4 scans and the counts were accumulated. The intensities of reflections having indices $+h+k+l$, together with their Friedel opposites, were recorded. The intensities of three standard reflections were measured after every 150 reflections and remained stable throughout the data collection. The intensities were corrected for Lorentz and polarization effects, but not for absorption. The space group was determined from the systematic absences. Equivalent reflections, other than Friedel pairs, were merged.

The structure was solved by direct methods using SHELXS86,[29] which revealed the positions of all non-hydrogen atoms. The crystals contain disordered solvent of crystallization, which is probably methanol. The solvent site is also only partially occupied such that the ratio of solvent molecules to toxin molecules in the crystal is 1:2. The non-hydrogen atoms were refined anisotropically. All of the H atoms of the nostocyclamide molecule, except those bonded to the methyl C atoms of the *i*-Pr group, were placed in the positions indicated by a difference electron density map, and their positions were allowed to refine. The remaining H atoms were fixed in geometrically calculated positions with a C−H distance of 0.95 Å. Individual isotropic temperature factors were refined for all of the H atoms. The H atoms associated with the disordered solvent molecule were not included in the model. Refinement of the structure was carried out on F using full-matrix least-squares procedures, which minimized the function $\Sigma w(|F_o| - |F_c|)^2$, where $w = [\sigma^2(F_o) + (0.005F_o)^2]^{-1}$. The analysis of $\Sigma w(|F_o| - |F_c|)^2$ showed no unusual trends. A correction for secondary extinction was not applied. The absolute configuration of the structure was determined using the method of Flack.[16] Refinement of the structure and the enantiopole parameter (x) with the CRYSTALS program[30] converged to give $x = -0.03(15)$ which suggests that the enantiomorph whose coordinates were used in the refinement is the correct one.

Neutral atom scattering factors and anomalous dispersion coefficients for non-hydrogen atoms were taken from Wilson.[31] The scattering factors for H-atoms were taken from Stewart *et al.*[32] All calculations, except those for the absolute configuration determination, were performed using the TEXSAN crystallographic software package.[33]

Agar Diffusion Assay. The anticyanobacterial activity was determined by an agar diffusion assay, essentially as described by Flores and Wolk.[13] The ethanolic samples (5−50 μL) were diluted with an equal volume of sterile water and spread in spots of 4 mm diameter on Petri dishes containing a cyanophyceen medium solidified with 1% agar. The drops were dried in a stream of sterile air and overlaid with 10 mL of a suspension of an indicator strain (*Anabaena variabilis* P-9, *Anabaena* PCC 7120, *Synechococcus* PCC 6911, *Synechocystis* PCC 6308) in 1% agar (Difco Noble). Inhibitory activities became evident by clearing zones formed around the spots after 4−10 days of incubation in the light of a fluorescent tube (Osram L36w/41 Lumilux Interna) at a distance of 57 cm corresponding to 32 μmol quanta cm^{-2} s^{-1} at the surface of the Petri dish. The inhibitory activities were quantitatively determined by serial dilutions and by the diameter of the clearing zones. Controls were performed with 50% aqueous ethanol. Overlayers with diatoms (*Navicula minima*, isolated from a brook nearby) consisted of a diatom medium[34] solidified with 1% of agar. Chlorophyceans were tested as described previously.[26]

***Brachionus* Bioassay.** The *Brachionus calyciflorus* bioassay was performed by using the method of Janssen et al.[35] with slight modification applying the ROTOXKIT F (Creasel, Deinze/Belgium). Each well in a sample plate was filled with 300 μL of artificial lakewater and 10 individuals of *Brachionus*. The wells were supplied with 3 μL of an ethanolic solution of nostocyclamide in a dilution series (six replicates). The final concentrations were between 2 and 100 μM. The incubation was performed in the dark at 25 °C for 24 h.

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