7-Epicylindrospermopsin, a Toxic Minor Metabolite of the Cyanobacterium *Aphanizomenon ovalisporum* from Lake Kinneret, Israel

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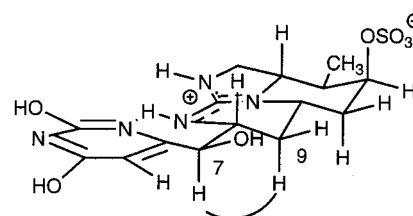
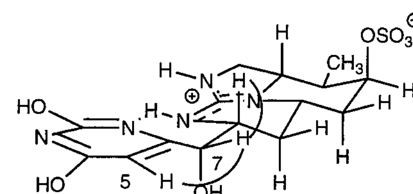
A toxic minor metabolite, 7-epicylindrospermopsin (**1**), was isolated from a culture of the cyanobacterium *Aphanizomenon ovalisporum* isolated from Lake Kinneret in Israel. Homonuclear and inverse-heteronuclear 2D NMR techniques, as well as HRMS and comparison of the NMR data with model compounds, enabled the structure determination of the new compound. Four polymethoxy-1-alkenes, **3**−**6**, were isolated from the lipophilic extract of the cyanobacterium as well.

Cylindrospermopsin is a water-soluble toxic cyanobacterial metabolite isolated from three different species of cyanobacteria: *Cylindrospermopsis raciborskii* (Woloszynska),[1] *Umezakia natans* (Watanabe),[2] and *Aphanizomenon ovalisporum* (Forti).[3,4] Cylindrospermopsin toxic effects are recognized primarily in the liver but also in the kidney, thymus, heart, and spleen.[5,6] The toxicology and biochemical action of cylindrospermopsin has not yet been fully elucidated. The seasonal occurrence of cylindrospermopsin-producing *A. ovalisporum* in Lake Kinneret imposes a serious threat to the water quality of the major reservoir of freshwater in Israel. A few years ago, in order to establish guidelines for cylindrospermopsin levels in drinking water, we initiated a survey to assess its oral acute and chronic toxicity. In the process of preparing a sufficient amount of cylindrospermopsin, we isolated a new related toxin, 7-epicylindrospermopsin (**1**). Compound **1** is the third compound with the cylindrospermopsin skeleton. The other two are cylindrospermopsin (**2**) and its nontoxic derivative deoxycylindrospermopsin, recently isolated from *C. raciborskii*.[7]

7-Epicylindrospermopsin (**1**) was isolated as a minor constituent of the hydrophilic extract of the cyanobacterium *A. ovalisporum*, while isolating cylindrospermopsin (**2**) for biological activity studies. Compound **1** had a shorter retention time on the reversed-phase HPLC column than did **2**. Its spectral data differed significantly from those of **2** in the chemical shifts of two protons and three carbon atoms (see Table 1) and in the optical rotation. The positive FABMS presented sodiated (*m*/*z* 438) and protonated (*m*/*z* 416) cluster ions, and the negative FABMS adequately presented the cluster ions at *m*/*z* 436 and 414. Positive HRFABMS measurements established the molecular formula for the sodiated cluster ion (*m*/*z* 438.1059) as $C_{15}H_{21}N_5O_7NaS$, identical to that of cylindrospermopsin (**2**). The optical rotation of **1** ($[\alpha]_D$ −20.5) is opposite in sign to that of **2** ($[\alpha]_D$ +12.5). Proton−proton connectivities from the COSY experiment (see Table 1) established, for **1**, a fragment similar to the C-7−C-15 fragment of **2**. This fragment in both compounds differed mainly in the chemical shifts of H-7 and H-8 and in the *J* value between these two protons (6.7 Hz in **1** vs 4.0 Hz in **2**, see Table 1). In accordance with the COSY conectivities, HMBC correlations (see Table 1) established, for **1**, a gross structure similar to that of **2**. Based on the assignment of the carbon atoms from the HMQC and HMBC experiments, the carbon chemical shifts, in **1** and **2**, differed most significantly for carbons 7−9. The proton and carbon NMR data implied that these differences are derived from opposite configuration of the hydroxyl at C-7. 1D NOE experiments (Figure 1) established that, while H-7 in compound **1** exhibits an NOE with H-9_{pax} (1.65 q), the same proton in compound **2** exhibits NOEs with both H-5 and H-8, further supporting the suggested structure for **1**. Recently, Snider and Xie published a synthesis of simple (demethyl, desulfate) analogues of cylindrospermopsin.[8] Comparing the NMR data of the two synthetic C-7 epimers with those of **1** and **2** revealed a good match of the chemical shifts and *J* values between the two pairs. The isomer that corresponded to compound **2** exhibits for H-7 a chemical shift of 4.70 ppm and a *J* value of 4.0 Hz, while the other isomer matches the data of compound **1**, δ_H 4.44 ppm and a *J* value of 6.8 Hz. On the basis of the arguments presented above, compound **1** was established as the C-7 epimer of compound **2**. Compound **1** shows similar potency in a mouse toxicity assay to that of **2**. The full data will be presented elsewhere.



1 7-Epicylindrospermopsin



2 Cylindrospermopsin

Figure 1. Observed NOEs of H-7 in compounds **1** and **2**.

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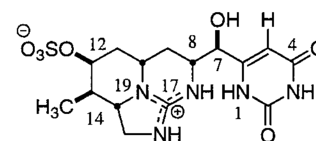
Table 1. Comparison of NMR Data of Compounds **1** and **2**[a]

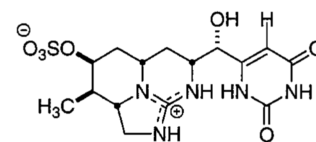
	7-epicylindrospermopsin (**1**)				cylindrospermopsin (**2**)	
position	δ_C, mult.[b]	δ_H, mult.[b], J(Hz)	H–H correlations[e]	H–C correlations[f]	δ_C, mult.[b]	δ_H, mult.[b], J(Hz)
2	155.5 s				155.5 s	
4	169.4 s			H-5	169.5 s	
5	101.9 d	5.84 s		H-7	101.8 d	5.87 s
6	158.0 s			H-5, H-7	158.3 s	
7	73.4 d	4.47 d, 6.7	H-8	H-5, H-9_{pax}	72.1 d	4.78 d, 4.0
8	54.9 d	3.80 ddd, 3.9,6.7,11.3	H-7, H-9_{peq}, H–9_{pax}	H-7, H-9_{pax}	55.4 d	3.90 ddd, 3.5,4.0,11.2
9	32.3 t	2.19 dt,[c] 13.3,3.9	H-8, H-9_{pax}, H–10	H-7, H-8, H-11_{pax}	30.3 t	2.20 dt, 13.4,3.5
		1.65 dt,[d] 13.3,11.3	H-8, H-9_{peq}, H–10			1.62 dt, 13.4,11.2
10	46.6 d	3.70 ddt, 3.7,3.9,11.3	H-9_{peq}, H-9_{pax}, H–11_{peq}, H-11_{pax}	H-9_{pax}, H-11_{pax}, H–12	46.8 d	3.70 tt, 11.2,3.5
11	37.8 t	2.48 dt,[c] 14.3,3.7	H-10, H-11_{pax}, H–12	H-9_{pax}	38.1 t	2.49 dt, 14.3,3.5
		1.55 ddd,[d] 1.8,11.3, 14.3	H-10, H-11_{peq}, H–12			1.56 dd, 14.3,11.2
12	79.8 d	4.63 brs	H-11_{peq}, H-11_{pax}, H–13	H-11_{peq}, 13–Me	79.8 d	4.64 brs
13	41.4 d	1.88 ddq, 2.0,11.1,6.7	H-12, 13-Me, H-14	H-11_{peq}, H-15_{pax}, 13–Me	41.6 d	1.89 ddq, 2.0,10.5,6.7
14	59.4 d	3.75 dt, 8.8, 11.1	H-13, H-15_{peq}, H–15_{pax}	H-12, H-13, 13-Me, H-15_{peq}	59.7 d	3.77 dt, 9.4,10.5
15	50.0 t	3.86 dd,[c] 9.2, 8.8	H-14, H-15_{pax}		50.1 t	3.89 dd, 9.9,9.4
		3.27 dd,[d] 9.2, 11.1	H-14, H-15_{peq}			3.29 dd, 9.9, 10.5
17	158.0 s			H-8, H-15_{peq}	158.0 s	
13-Me	15.3 q	1.01 d, 6.7	H-13	H-13, H-14	15.5 q	1.03 d, 6.7

[a] In D_2O, relative to TSP $\delta = 0$. [b] Multiplicity. [c] Pseudoequatorial. [d] Pseudoaxial. [e] From COSY experiment. [f] From HMBC experiment.

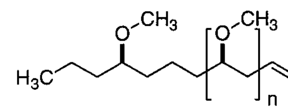
Isotactic polymethoxy-1-alkenes were first found in a field-collected sample of tolytoxin-producing *Tolypothrix conglutinata* var. *colorata* from Fanning Island.[9] A few years ago, similar and new isotactic polymethoxy-1-alkenes were isolated from several cultured, tolytoxin-producing cyanobacteria belonging to the family Scytonematacea.[10,11] In the present study, we have isolated five polymethoxy-1-alkenes from the lipophilic extract of the cultured cyanobacterium *A. ovalisporum.* Two of the compounds were identified as 4,6,8,10,12,14,16,18,22-nonamethoxy-1-pentacosene (**3**)[11] and 4,6,8,10,12,14,16,18,20,22-decamethoxy-1-heptacosene (**4**)[10] by comparison of their NMR, MS, and optical rotation data with the published values. The third compound (FAB molecular cluster ion at *m*/*z* 749) has a polymethoxy-1-alkene structure that could not be assigned due to the small amounts of material obtained from the extract. Two other fractions were obtained in the process of purification of the former compounds, and their NMR data were typical of polymethoxy-1-alkenes. Each fraction contained a mixture of two different compounds. MS analysis of these two fractions revealed them to be mixtures of **4** and 4,6,8,10,12,14,16,18,20,22,24-undecamethoxy-1-nonacosene (**5**) and of **5** and 4,6,8,10,12,14,16,18,20,22,24,26-dodecamethoxy-1-hentriacontene (**6**). An attempt to purify these two new compounds (**5** and **6**) failed due to the small amounts of material obtained, their polarity, and their structural similarity. The co-occurrence of these polymethoxy-1-alkenes with toxic polyketide metabolites of cyanobacteria is of great interest and may be used as a sign for the toxicity of the strain. We found that strains of *A. ovalisporum* that lost their capacity to produce the toxic metabolites (cylindrospermopsins) do not produce the polymethoxy-1-alkenes either.



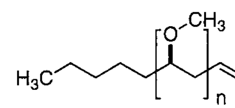
1 7-Epicylindrospermopsin



2 Cylindrospermopsin



3 n = 8



4 n = 10
5 n = 11
6 n = 12

Experimental Section

Instrumentation. LRMS and HRMS were recorded on a Fisons VG AutoSpecQ M 250 instrument. UV spectra were recorded on a Kontron 931 plus spectrophotometer. NMR spectra were recorded on a Bruker ARX-500 spectrometer at 500.136 MHz for ^{1}H and 125.76 MHz for ^{13}C. ^{1}H, ^{13}C, DEPT, COSY-45, HMQC, and HMBC spectra were recorded using standard Bruker pulse sequences. HPLC separations were performed on an ISCO HPLC system (model 2350 pump and model 2360 gradient programmer) equipped with an Applied Biosystem Inc. diode-array detector.

Culture Conditions. The cyanobacterium that bloomed in Lake Kinneret in the fall of 1994 was isolated by serial dilution of lake-water samples into BG11 medium and plating on BG11 agar.[12] The isolate was labeled ILC-164 and is currently maintained in the culture collection at Tel Aviv University. The organism was identified as *A. ovalisporum* Forti by Dr. B. Meyer of the Max Planck Institut für Limnologie, Plon, Germany, and the identification was confirmed by Prof. J. Komarek of the Institute of Botany, Trebon, Czech Republic. The cyanobacterium was cultured in 20-L glass bottles containing a BG-11 medium.[12] Cultures were illuminated continuously at an intensity of 75 μmol quanta/M^2/s from fluorescent tubes and aerated with 0.5% CO_2 in air (1 L/min) at an incubation temperature of 25 °C for 30–35 days. The cells were harvested using a continuous-flow centrifuge. Yields of lyophilized cells typically ranged from 0.15 to 0.3 g/L of culture.

Isolation Procedure. The freeze-dried cells (7 gr) were extracted with 7:3 MeOH–H_2O. The crude extract (1.5 gr) was evaporated and separated on an ODS (YMC-GEL, 120 Å, 4 ×

6.4 cm) flash column with water, 1:1 MeOH−H_2O, and MeOH (200 mL each). Fraction 1 was applied to a Sephadex G-25 column (2.5 × 30 cm) and eluted with H_2O. Eleven fractions, each of 50 mL, were collected. Fractions 9 and 10, containing cylindrospermopsin (by NMR), were combined and applied to a preparative HPLC column (YMC-Pack ODS, AQ-343-10, S-10 μm, 120 Å, 250 × 20 mm i.d.). The column was eluted with a 95:5 H_2O−MeOH solution (5 mL/min) and monitored by UV (263 nm). Semi-pure **1** (1.6 mg) was eluted from the column first (38.9 min), and semi-pure **2** (11 mg) was eluted from the column later (45.9 min). Both compounds were further purified on the same column. Semi-pure **2**, was purified using the same elution conditions to afford pure **2** (7.4 mg, 0.1% of dry cell wt). Semi-pure **1** was purified using a 98:2 H_2O−MeOH solution (5 mL/min) to afford pure **1** (0.7 mg, 0.01% of dry cell wt). The MeOH fraction from the ODS flash column (370 mg) was flash-chromatographed on a normal-phase Si-60 Si gel column (10 × 2 cm) eluted with solvent of increasing polarity. Thirteen fractions (20 mL, each)—hexane (1), 7:3 hexane−EtOAc (1), 6:4 hexane−EtOAc (8), 1:1 hexane−EtOAc (2), and MeOH (1)—were collected. The fifth fraction afforded pure **3** (6.5 mg, 0.09%, dry cell wt). The sixth fraction afforded pure **4** (14 mg, 0.2%, dry cell wt). Fractions 8 (5 mg) and 9 (4 mg) afforded mixtures of compounds **4** and **5** and compounds **5** and **6**, respectively. Fraction 10 (2 mg) contained an unidentified new compound [FABMS *m/z* 749 (MH^+), 771 (MNa^+) and 787 (MK^+)]. All polymethoxyalkenes were visualized on Si gel plates with 1% ceric sulfate−10% H_2SO_4.

7-Epicylindrospermopsin (1): white amorphous solid; $[\alpha]^{25}_D$ −20.5° (*c* 0.06, H_2O); UV (H_2O) λ_{max} (ϵ) 263 (6250) nm; for ^1H and ^{13}C NMR data, see Table 1; FABMS (positive) *m/z* (rel int) 438 (MNa^+, 95), 416 (MH^+, 50), 413 (100), 388 (80), 226 (15); FABMS (negative) *m/z* (rel intensity) 436 (MNa − H^-, 55), 414 (M − H^-, 94), 284 (85), 262 (100), 248 (60), 226 (10); HRFABMS (positive) *m/z* 438.1077 (MNa^+, calcd for $C_{15}H_{21}N_5O_7NaS$, 438.1059).

Cylindrospermopsin (2): white amorphous solid; $[\alpha]^{25}_D$ +12.5° (*c* 0.6, H_2O); UV (H_2O) λ_{max} (ϵ) 263 (6250) nm; for ^1H and ^{13}C NMR data, see Table 1; FABMS (positive) *m/z* (rel int) 438 (MNa^+, 100).

4,6,8,10,12,14,16,18,22-Nonamethoxy-1-pentacosene (3):[11] $[\alpha]^{25}_D$ +10.8° (*c* 0.07, $CHCl_3$); FABMS (positive) *m/z* 621 (MH^+), 643 (MNa^+), 659 (MK^+).

4,6,8,10,12,14,16,18,20,22-Decamethoxy-1-heptacosene (4):[10] $[\alpha]^{25}_D$ +4.1° (*c* 0.13, $CHCl_3$); FABMS (positive) *m/z* 679 (MH^+), 701 (MNa^+), 717 (MK^+).

4,6,8,10,12,14,16,18,20,22,24-Undecamethoxy-1-nonacosene (5): FABMS (positive) *m/z* 737 (MH^+), 759 (MNa^+).

4,6,8,10,12,14,16,18,20,22,24,26-Dodecamethoxy-1-hentriacontene (6): FABMS (positive) *m/z* 795 (MH^+), 817 (MNa^+).

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