Mueggelone, a Novel Inhibitor of Fish Development from the Fresh Water Cyanobacterium *Aphanizomenon flos-aquae*[1]

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A novel C18 lipid, containing a 10-membered lactone, mueggelone (**1**), was isolated from a field-collected sample of *Aphanizomenon flos-aquae*, together with the known compound lupenyl acetate (**3**). Both structures were secured using extensive spectroscopic analysis (1D and 2D NMR, MS, IR). Biological activity assessment of both compounds indicated them to have significant inhibitory effects on fish embryo larval development.

Cyanobacterial blooms are a regular occurrence in many of the natural and man-made lakes and reservoirs around Berlin. In late summer 1995, a bloom-forming strain of *Aphanizomenon flos-aquae* (Nostocaceae) was dominant in the Mueggelsee. This cyanobacterium is a known producer of several neurotoxins, for example, saxitoxin[2] and neosaxitoxin. At the time of the bloom, microcystin was found in low concentrations of about 35 μg/g of dry weight of *A. flos-aquae* (as compared to 300−600 μg/g for *Microcystis aeruginosa*,[3] when it is the dominant toxic species). These traces of microcystin could be attributed to a low number of *Microcystis* spp., colonies within the *Aphanizomenon* bloom. Crude dichloromethane (CH_2Cl_2) and methanol (MeOH) extracts of the collected *A. flos-aquae* showed strong inhibitory effects at low concentrations on fish embryo larval development,[4] which were not consistent with the determined microcystin concentration and suggested the presence of other toxins in the sample. A secondary metabolite investigation of the CH_2Cl_2 extract of *A. flos-aquae* was thus undertaken to try to identify any other active or toxic principles.

Chromatographic separation of the CH_2Cl_2 extract of *A. flos-aquae* yielded two natural products, **1** and **3**. The molecular formula of **1** was deduced as $C_{18}H_{28}O_3$ by accurate mass measurement. From its ^{13}C-NMR and IR data it was evident that the molecule must contain an ester [74.9 (d), 173.5 (s) ppm, 1728 cm^{-1}], an epoxide [57.4 (d), 59.9 (d) ppm, 1253 cm^{-1}], and two carbon−carbon double bonds [122.3 (d), 128.6 (d), 132.8 (d), 135.0 (d) ppm] as functionality, indicating the molecule to be bicyclic. Based on the NMR data it was evident that the two rings must exist in the form of a lactone and an epoxide. Analysis of the EIMS, which contained characteristic signals at *m/z* 275 for $M^+ - OH$, 224 for $M^+ - OH - C_5H_9$ and 194 for $M^+ - OH - C_5H_9 - CHO$, also supported these deductions. After the association of all proton resonances with those for the directly bonded carbon atoms via a ^{1}H−^{13}C shift-correlated 2D NMR measurement (HMQC) (see Table 1), it was possible from the ^{1}H−^{1}H COSY spectrum to follow a continuous chain of coupling from H_2-6 to H_3-18, and from H_2-2 to H_2-3. HMBC correlations (see **1**) confirmed these molecular fragments of **1** and left H_2-4 and H_2-5 to be positioned by deduction. The site of ring-closure to form the 10-membered lactone was deduced from the HMBC correlation observed between H-9 and C-1, thus completing the basic structure of **1**. Within this molecule there are two double bonds and three chiral centers, which required stereochemical assignment. The $\Delta^{10,11}$ bond was assigned as *E* on the basis of $J_{10,11}$ being 15.5 Hz, while $J_{15,16} = 8.3$ Hz indicated $\Delta^{15,16}$ to be *Z*. NOE difference measurements made with **1** indicated H-12 and H-13 to be trans. Low power irradiation at δ 3.16 (H-12) causes enhancement of the resonances associated with H_2-14 [δ 2.32 (m), 2.40 (m)], and irradiation at δ 5.48 (H-11) enhances the δ 2.87 (H-13) resonance. Attempts to resolve the absolute configuration at C-9 via Mosher's method were unsuccessful as insufficient (<1 mg) amounts of the ring open (hydrolysis product) form of **1** were produced to enable Mosher's esters to be made. The absolute configuration of **1**, however, must be one of either 9*R*,12*S*,13*S*; 9*R*,12*R*,13*R*; 9*S*,12*S*,-13*S*; or 9*S*,12*R*,13*R*. Thus, in a relative sense, **1** is

Table 1. ^{1}H- (300 MHz, $CDCl_3$) and ^{13}C-NMR (75.5 MHz, $CDCl_3$) Data[a] for **1**

carbon	^{1}H	^{13}C
1		173.5 (s)[b]
2	2.20 (ddd, J = 2.3, 11.7, 15.5 Hz) 2.51 (ddd, J = 3.0, 6.4, 15.5 Hz)	35.1 (t)
3	1.54 (m), 2.04 (m)	29.9 (t)
4	1.47 (m)	27.1 (t)
5	1.38 (m), 1.53 (m)	24.2 (t)
6	1.06 (m), 1.53 (m)	23.7 (t)
7	1.29 (m),1.74 (m)	23.4 (t)
8	1.54 (m)	20.7 (d)
9	5.37 (m)	74.9 (d)
10	5.94 (dd, J = 5.3, 15.8 Hz)	132.8 (d)
11	5.48 (ddd, J = 1.1, 7.5, 15.8 Hz)	128.6 (d)
12	3.16 (dd, J = 2.3, 7.5 Hz)	57.4 (d)
13	2.87 (ddd, J = 2.3, 5.3, 5.3 Hz)	59.9 (d)
14	2.32 (m), 2.40 (m)	29.6 (t)
15	5.36 (dm, J = 8.3 Hz)	122.3 (d)
16	5.54 (dm, J = 8.3 Hz)	135.0 (d)
17	2.02 (m)	20.7 (d)
18	0.96 (t, J = 7.5 Hz)	14.2 (q)

[a] All assignments are based on extensive 1D and 2D NMR experiments, including COSY90, HMQC, and HMBC. [b] Multiplicity by DEPT, s = C, d = CH, t = CH_2, q = CH_3.

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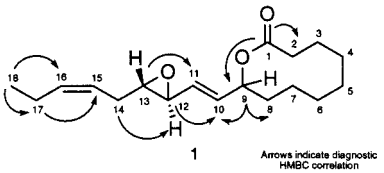
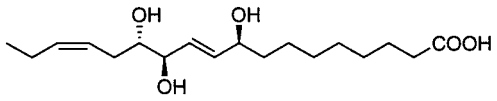
currently best described as (9ξ,10*E*,12*R**,13*R**,15*Z*)-12,-13-epoxyoctadeca-10,15-dienyl-κ-decanolactone, for which we propose the trivial name mueggelone.

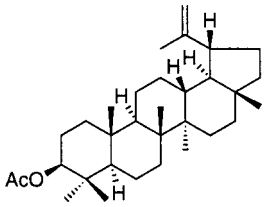
In 1982, Cardellina and Moore isolated malyngic acid (**2**) from a sample of *Lyngbya majuscula*.[5] Clearly this compound, if it has the same absolute configuration as **1**, could be either its direct precursor or one of its hydrolysis products. Together with **1**, the known triterpene, lupenyl acetate (**3**),[6] was also isolated. This is the first report of this metabolite from a cyanobacterium.



1 Arrows indicate diagnostic HMBC correlation



Malyngic acid **2**



Lupenyl acetate **3**

To see whether mueggelone (**1**) and lupenyl acetate (**3**) were possibly responsible for the originally observed arrest of fish embryo larval development they were tested for their effects toward zebra fish larvae. With mueggelone at a concentration of 10 μg/mL after 24 and 32 h, larvae showed 45% mortality. Surviving larvae demonstrated strong retardation. Survivors were retarded over several steps: after 24 and 32 h no blood circulatory system had developed, and after 3 days survivors showed edema in the heart region and thrombosis. At a concentration of 1 μg/mL mueggelone had no obvious effects on larval development.

With lupenyl acetate (**3**) at a concentration of 100 μg/mL, larvae showed development of edema in the heart region and tail bending (30%) after 3 days. After 5 days, all larvae had edema in the heart region and bent tails. At a concentration of 10 μg/mL lupenyl acetate (**3**) had no obvious effects on larval development. At both concentrations of lupenyl acetate (**3**) there was no larval mortality.

These results suggest that mueggelone (**1**), and to a lesser extent lupenyl acetate (**3**), are the likely causative agents of the originally observed fish embryo larval development retardation/mortality of the CH_2Cl_2 extract. If this deduction is correct, a probable ecological role of these compounds is in inhibition of the development of potential grazers, that is, herbivorous fish.

Both compounds **1** and **3** were tested at a concentration of 50 μg/filter disk for their antimicrobial,[7] and antialgal activities,[7] and at a concentration of 66 μg/mL for their HIV-1-reverse-transcriptase activity,[8] and at a concentration of 200 μg/mL for their tyrosin-kinase[8] activity. The results of these assays indicated both compounds to have no significant activities in the applied test systems. Currently, the antimalarial activity[9] and cytotoxicity[10] of **1** and **3** are being assessed.

Experimental Section

General Experimental Procedures. Procedures were previously published.[11]

Plant Material. The plant material of *A. flos-aquae* was collected in late summer 1995, during an algal bloom at Mueggelsee, Berlin, Germany.

Extraction and Isolation. Freeze-dried plant material (40 g) was extracted first with CH_2Cl_2 (3 L) and then with MeOH (3 L). Vacuum liquid chromatography (VLC) of the CH_2Cl_2 extract (2.68 g, 6.7%) over Si gel employing a step gradient from hexane to EtOAc to CH_3OH yielded 10 fractions, each of 90 mL. TLC and ^{1}H-NMR control of all fractions indicated fraction 3 to be of further interest on the basis of a great many lowfield resonances in its proton spectrum. HPLC purification [Si60, hexane−Me_2CO (96/4) as eluent] of this fraction yielded compounds **1** and **3**.

Mueggelone (1): clear oil (5.1 mg, 0.013%); $[\alpha]^{25}_D$ +28.3° (*c* 0.6, $CHCl_3$); IR (film) ν_{max} 2935, 1730, 1450, 1240 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; EIMS *m/z* 293 ($M^+ + 1$, <1), 292 (M^+, <1), 275 (<1), 223 (100), 224 (20), 195 (10), 194 (80); HRDCIMS (isobutane) *m/z* 293.2115 ($M^+ + 1$), (calcd for $C_{18}H_{29}O_3$ 293.2117).

Lupenyl acetate (3): white crystalline solid (1.3 mg, 0.003%); all physical and spectroscopic data in good agreement with those published by Arai et al.[5]

Fish Embryo Larval Development Test. Eggs of zebra fish (*Danio rerio*, *Brachydanio rerio* Hamilton-Buchanan) were incubated in polystyrene Petri dishes from blastula stage up to the end of the embryonic period. The test medium was changed daily. Development was monitored, and at distinct phases of development suitable endpoints were recorded (developmental stage, malformations, mortality, heart rate, motility/swimming ability, feeding growth). After termination of exposure at an age of 6 days, larvae were kept in chambered, toxin-free aquaria to monitor further development. At an age of 21 days, when larval development was complete, survival rate, body length, and weight were determined. Compounds were diluted in test medium with 0.1% Me_2CO to desired concentrations.

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References and Notes

(1) Presented at the 44th Annual Congress of the Society for Medicinal Plant Research, Prague, Czech Republic, Sept. 3−7, 1996; podium presentation 1, and at the Statusseminar, "Toxische Cyanobakterien in deutschen Gewässern-Verbreitung, Kontrollfaktoren und Ökologische Bedeutung," April 28−29, 1997, Storkow, Germany, oral presentation entitled, "Cyanobacteria: A Source of New and Biologically Active Natural Products".

(2) Sawyer, P. J.; Gentile, J. H.; Sasner, J. J. *Can. J. Microbiol.* **1968**, *14*, 1199−1204.

(3) Presented at the Statusseminar, "Toxische Cyanobakterien in deutschen Gewässern-Verbreitung, Kontrollfaktoren und Ökologische Bedeutung", April 28−29, 1997, Storkow, Germany, Ms. Jutta Fastner; oral presentation entitled, "Microcystin in 40 verschiedenen Gewässern der Bundesrepublik Deutschland".

(4) (a) Initial tests for fish embryo larval inhibitory activity indicated the CH_2Cl_2 extract to have a strong activity at a concentration of 1 mg/mL, and a weak activity at 100 mg/mL. (b) Oberemm, A.; Fastner, J.; Steinberg, C. E. W. *Water Res.*, in press.

(5) Cardellina, J. H., II; Moore, R. E. *Tetrahedron* **1980**, *36*, 993−996.

(6) Arai, Y.; Kusumoto, Y.; Nagao, M.; Shiojima, K.; Ageta, H. *Yakugaku Zasshi* **1983**, *103*, 356−359.

(7) Schulz, B.; Sucker, J.; Aust, H. J.; Krohn, K.; Ludewig, K.; Jones, P. G.; Döring, D. *Mycol. Res.* **1995**, *8*, 1007−1015.

(8) Eberle, J.; Seibl, R. *J. Virol. Methods* **1992**, *40*, 347−356.

(9) Angerhofer, C. K.; König, G. M.; Wright, A. D.; Sticher, O.; Milhous, W. K.; Cordell, G. A.; Farnsworth, N. R.; Pezzuto, J. M. In *Advances in Natural Product Chemistry*; Atta-ur-Rahman, Ed. Harwood Academic Publishers: Chur, 1992; pp 311−329.

(10) Likhitwitayawuid, K.; Angerhofer, C. K.; Ruangrungsi, N.; Cordell, G. A.; Pezzuto, J. M. *J. Nat. Prod.* **1993**, *56*, 30−38.

(11) Wright, A. D.; König, G. M.; Angerhofer, C. K.; Greenidge, P.; Linden A.; Desqueyroux-Faundez, R. *J. Nat. Prod.* **1996**, *59*, 710−716.

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