Biochemistry (Moscow), Vol. 66, No. 1, 2001, pp. 72-76. Translated from Biokhimiya, Vol. 66, No. 1, 2001, pp. 92-97.

Dicarboxylic and Fatty Acid Compositions of Cyanobacteria of the Genus *Aphanizomenon*

V. M. Dembitsky[1]*, I. Shkrob[2], and J. V. Go[3]

[1]*Department of Pharmaceutical Chemistry and Natural Products, School of Pharmacy, P.O. Box 12065, The Hebrew University of Jerusalem, Jerusalem 91120, Israel; fax: +972-2675-8201; E-mail: dvalery@cc.huji.ac.il*

[2]*Division of Environmental Science, Graduate School of Applied Science, The Hebrew University of Jerusalem, Jerusalem 91904, Israel*

[3]*Group of Natural Lipids, P.O. Box 39074, The Hebrew University of Jerusalem, Jerusalem 91390, Israel*

Received April 12, 2000

Abstract—The occurrence of dioic, hydroxy, branched, and unsaturated fatty acids in cyanobacteria of the genus *Aphanizomenon* growing in different freshwater lakes has been studied. Unusual dicarboxylic (from 4.52 to 7.14%) and other fatty acids were identified by gas chromatography/mass spectrometry (GC/MS).

Key words: dicarboxylic (dioic) acids, fatty acids, *Aphanizomenon*, GC/MS

Toxic blooms of the genus *Aphanizomenon* occur in many freshwater lakes and reservoirs around the world [1]. Many toxic compounds [2-4] as well as nontoxic nitrogen-containing products [5, 6] have been isolated from the genus *Aphanizomenon*.

The major lipids of cyanobacteria consist of glyco- and sulfolipids as well as sterols, hydrocarbons, and fatty acids [7]. Studies of the hydrocarbon, lipid, main fatty acid, and carotenoid compositions of the strain *Aphanizomenon* itself have been carried out by [8-11]. We used gas chromatography/mass spectrometry for the separation and identification of low molecular weight dioic acids and fatty acids [12]. This study was performed for four strains of the *Aphanizomenon*.

This report is a part of our investigation of cyanobacteria of the genus *Aphanizomenon* [5] in the framework of a comprehensive program on the biochemistry and toxicity of freshwater cyanobacteria.

MATERIALS AND METHODS

Cyanobacterial samples. Lyophilized cells of two strains of *Aphanizomenon flos-aquae*, which were collected from cyanobacterial blooms in Klamath Lake and Upper Klamath Lake, Oregon (USA), were obtained from Prof. I. Dor (Environmental Division). *Aphanizomenon flos-aquae* strain Jaworski FBA-218 was obtained from Dr. J. Lukavsky (Czech Collection of Algae and Cyanobacteria, Trebon, Czech Republic) that was originally isolated from a toxic bloom that occurred in Queen Elizabeth reservoir in 1970 (London, United Kingdom). *Aphanizomenon ovalisporum* (Forti) was obtained from Kinneret Limnological Lab (Tiberias, Israel); it was isolated from a toxic bloom that occurred in Lake Tiberias in 1994. The two latter strains, *A. flos-aquae* (Jaworski) and *A. ovalisporum*, were cultivated in the Laboratory of Hydrobiology using BG-11 medium [13]. The cells were harvested by centrifugation, lyophilized, and stored in a deep freezer at −20°C.

Extraction of dioic and fatty acids. Cells of each strain (970 mg) were added to 100 ml of MeOH–H_2O mixture (90 : 10 v/v), and the mixture was kept at 60°C for 6 h. After cooling to room temperature, 150 ml of cold H_2O–C_5H_{12} mixture (100 : 50 v/v) was added. The layers were separated. The pentane layer was concentrated to dryness *in vacuo*. The solid was additionally extracted with 150 ml of CH_2Cl_2. It was then filtered and concentrated to dryness *in vacuo*. The pentane and dichloromethane extracts were combined and the mixture dissolved in 5 ml of MeOH. The fatty acid methylation procedure was described previously [12].

GC/MS analysis. Analysis was performed with a Hewlett-Packard 5890 gas chromatograph (Palo Alto, USA) that was modified for a glass capillary column. A HP GC/mass selective detector (5971B MSD) was used. Dicarboxylic and fatty acids were separated by gas chromatography using serially coupled capillary columns [12]:

* To whom correspondence should be addressed.

Table 1. Hydroxy, *n*-saturated, branched, and unsaturated acids (%)

Fatty acid	1	2	3	4
Total hydroxy acids	0.50	0.59	0.27	0.37
2-hydroxy-propanoic (2-OH-3:0)	0.12	0.15	n.d.	0.04
3-hydroxy-butanoic (3-OH-4:0)	0.14	0.11	0.06	n.d.
2-hydroxy-4-methyl-pentanoic (2-OH-4-Me-5:0)	0.11	0.12	0.21	0.24
2-hydroxy-3-methyl-pentanoic (2-OH-3-Me-5:0)	0.13	0.21	n.d.	0.09
n-Saturated	51.54	54.58	45.85	49.67
butanoic (4:0)	0.19	0.15	0.11	0.05
pentanoic (5:0)	0.23	0.31	0.09	0.11
hexanoic (6:0)	n.d.	0.18	0.24	0.16
heptanoic (7:0)	0.19	0.13	0.10	0.12
octanoic (8:0)	0.08	0.09	0.12	0.13
nonanoic (9:0)	0.18	0.23	0.22	0.19
decanoic (10:0)	0.19	0.11	0.21	0.29
dodecanoic (12:0)	0.44	0.26	0.31	0.18
tetradecanoic (14:0)	13.70	12.71	2.01	3.22
pentadecanoic (15:0)	3.15	1.51	0.95	0.84
hexadecanoic (16:0)	32.00	36.98	40.13	43.09
heptadecanoic (17:0)	0.68	0.18	0.22	0.31
octadecanoic (18:0)	0.51	1.74	1.14	0.98
Branched saturated	17.21	10.32	9.53	7.10
3-methyl-butanoic (3-Me-4:0)	0.21	0.25	n.d.	n.d.
2-methyl-butanoic (2-Me-4:0)	n.d.	0.08	n.d.	0.03
4-methyl-hexanoic (4-Me-6:0)	0.06	n.d.	0.09	0.12
8-methyl-decanoic (8-Me-10:0)	0.18	0.13	0.09	0.12
12-methyl-tetradecanoic (12-Me-14:0)	0.80	0.28	0.62	0.51
14-methyl-pentadecanoic (14-Me-15:0)	9.67	4.37	3.33	2.78
2-methyl-hexadecanoic (2-Me-16:0)	0.39	0.42	0.72	0.52
14-methyl-hexadecanoic (14-Me-16:0)	0.14	0.32	0.51	0.21
15-methyl-hexadecanoic (15-Me-16:0)	0.25	0.31	0.64	0.41
10-methyl-heptadecanoic (10-Me-17:0)	5.24	3.82	3.12	2.02
2-hexyl-cyclopropaneoctanoic (Cyc17:0)	0.27	0.34	0.41	0.38
Unsaturated	26.23	27.37	38.91	37.89
3-methyl-(Z)-3-pentenoic (3-Me-3-5:1)	0.09	0.03	0.11	n.d.
(Z)-9-hexadecenoic (9-16:1)	2.39	1.02	2.34	1.85
11-hexadecenoic (11-16:1)	1.24	3.19	2.14	2.55
(Z)-9-octadecenoic (9-18:1)	20.94	19.95	26.71	26.07
9,12-octadecadienoic (9,12-18:2)	0.28	0.41	4.14	5.21
10,13-octadecadienoic (10,13-18:2)	0.19	1.68	2.05	1.19
12,15-octadecadienoic (12,15-18:2)	0.16	0.34	0.61	0.43
9,12,15-octadecatrienoic (9,12,15-18:3)	0.94	0.75	0.81	0.74

Note: Here and in Table 2: 1) *Aphanizomenon flos-aquae* (Klamath Lake, USA); 2) *Aphanizomenon flos-aquae* (Upper Klamath Lake, USA); 3) *Aphanizomenon ovalisporum* (Tiberias Lake, Israel); 4) *Aphanizomenon flos-aquae* (Queen Elizabeth Reservoir, UK); n.d., not detected.

RTX-1 (Restek, USA) (30 m, ID 0.32 mm, film thickness 0.25 μm) coupled with a second capillary column RTX-1701 (Restek) (30 m, ID 0.32 mm, film thickness 0.25 μm). The GC oven program was; 40°C for 2 min, 2°C/min to 300°C, then 20 min at 300°C. Injector temperature was 180°C. The flow rate of the carrier gas (helium) was 25 cm/sec. The MS source was operated at 194°C. Electron ionization energy was 70 eV. Scan range was from 30 to 650 *m/z* at a scan rate 0.9 scan/sec. The solvent delay was 12 min. Methyl esters of dicarboxylic and fatty acids were identified by mass-spectral library search (NBS75, Wiley 138 & Wiley 275).

RESULTS AND DISCUSSION

Analysis of the total dioic and fatty acid compositions of four strains resulted in the identification of 50 different acids (Tables 1 and 2), a more complex picture than previously recognized [9].

Dioic acids. A series of fifteen dioic (dicarboxylic) acids were also identified in the genus *Aphanizomenon*, and these accounted for from 4.52 to 7.14% of the total fatty acid composition (Table 2). All of these dioic acids were also identified as dimethyl esters. An unusual finding was the identification of dioic acids such as 2(*E*)-butene-1,4-dioic, 2-hydroxy-butane-1,4-dioic, 2-methyl-butane-1,4-dioic acid, and other dioic acids. None of these dioic acids had been found previously in *Aphanizomenon* strains. The structure of all dioic acid homologs was established using GC/MS. Dimethyl esters of dioic acids have mass spectra that are different in appearance and more complex than their monocarboxylic analogs. The 70-eV mass spectra of all dioic acids were characterized by a molecular ion $[M-31]^+$ peak, which is due to the loss of the methoxyl group (H_3CO) [14-16]. The molecular ion is of low abundance and is not seen in mass spectra of all dioic acids. The figure presents representative mass spectra of six dioic acids.

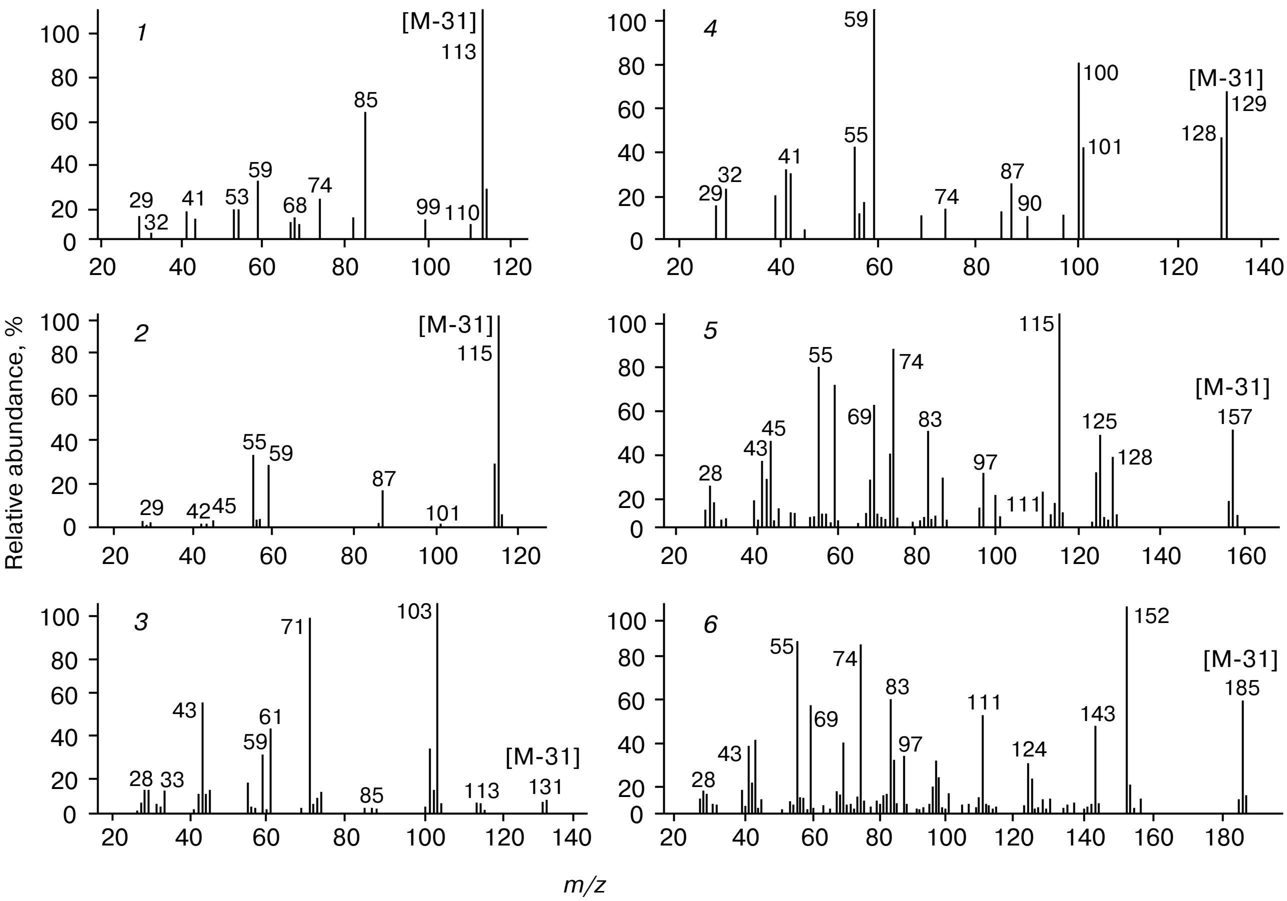
Fatty acids. Most of the fatty acids were saturated $C_{4:0}$–$C_{18:0}$ (more than 65% by weight), but unsaturated fatty acids were also identified (from 26 to 39% by weight, Table 1). Branched saturated fatty acids that were identified accounted for 7.10 to 17.21%. The predominant unsaturation (38.9% of the total monounsaturated fatty acids) was at (*Z*)-9-18:1 (26.7%). Other mono-unsaturations were observed at (*Z*)-9-16:1 and 11-16:1 (e.g., (*Z*)-9-hexadecenoic acid and 11-hexadecenoic acid). Hydroxy fatty acids were found as minor components (0.27-0.59%).

The fatty acid composition of cyanobacteria was studied first by Holton, Blecker, and Onore [17] in

Table 2. Content, structure, and abundance of major ions from dimethyl esters of dioic acids

Dioic acid	[M]	$[M-31]^+$	$[M-32]^+$	Major ions	Content, %			
	m/z, relative abundance in parentheses				1	2	3	4
Ethane-1,2-dioic	118(1)	87(1)		59 (100)	0.06	0.21	0.32	0.18
Propane-1,3-dioic	132(1)	101(100)		101(100) 59(90)	0.21	0.13	0.41	0.12
Butane-1,4-dioic	146(0.5)	115(100)	114(35)	115(100) 55(35)	0.87	1.49	1.18	2.01
2(*E*)-Butene-1,4-dioic	144(0.3)	113(100)	112(0.2)	113(100) 85(50)	0.42	0.09	0.03	0.11
2-Hydroxy-butane-1,4-dioic	162(0.5)	131(1)	130(0.8)	113(100) 71(100) 43(65)	0.33	1.31	0.38	0.21
2-Methyl-butane-1,4-dioic	160(0.1)	129(60)	128(30)	59(100) 129(60) 128(30)	0.48	0.56	0.13	0.19
Pentane-1,5-dioic	160(0.3)	129(50)	128(20)	59(100) 100(62) 129 (60)	0.12	0.09	0.14	0.35
Hexane-1,6-dioic	174(0.1)	143(30)	142(10)	59(100) 55(70) 114(60)	0.17	0.12	0.21	0.11
3-Methyl-hexane-1,6-dioic	188(0.1)	157(63)	156(10)	115(100) 157(63) 73(60)	0.07	n.d.	0.72	0.16
Heptane-1,7-dioic	188(0.1)	157(55)	156(15)	115(100) 74(90) 55(80)	0.24	0.61	0.38	0.12
3-Methyl-heptane-1,7-dioic	202(0.2)	171(69)	170(8)	69(100) 129(95) 97(90)	0.09	0.45	0.11	0.14
Octane-1,8-dioic	202(0.6)	171(55)	170(12)	55(100) 69(90) 41(85)	0.11	0.26	0.17	0.31
Nonane-1,9-dioic	216(0.2)	185(60)	184(9)	55(100) 74(80) 152(65)	1.21	1.68	0.92	0.64
Decane-1,10-dioic	230(0.2)	199(38)	198(6)	55(100) 74(80) 98(60)	0.14	0.06	0.21	0.05
Undecane-1,11-dioic	244(0.3)	213(28)	212(4)	55(100) 74(75) 139(35)	n.d.	0.08	0.13	0.12
Total dioic acids					4.52	7.14	5.44	4.82

Note: n.d., not detected.



Mass spectra of six dicarboxylic (dioic) acids identified from the genus *Aphanizomenon*: *1*) (*E*)-2-butene-1,4-dioic; *2*) butane-1,4-dioic; *3*) 2-hydroxybutane-1,4-dioic; *4*) 2-methyl-butane-1,4-dioic; *5*) heptane-1,7-dioic; *6*) nonane-1,9-dioic.

Anacystis nidulans and by Levin, Lennarz, and Bloch [18] in *Anabaena variabilis*. Major fatty acids thus far known to be present in cyanobacteria are hexadecanoic ($C_{16:0}$), (*Z*)-9-hexadecenoic ($C_{16:1}$), hexadecadienoic ($C_{16:2}$), octadecanoic ($C_{18:0}$), and (*Z*)-9-octadecenoic ($C_{18:1}$); dicarboxylic acids have not been identified from cyanobacteria [7].

Some dicarboxylic acids have potential as anti-proliferative and as general antitumor agents for primary invasive malignant melanoma [19]. Aliphatic dicarboxylic acids surprisingly afforded potent cytotoxicity, anti-neoplastic activity [20], and served lipidoic markers for identification some human and animal diseases [21, 22]. These acids are of major interest for medical specialists and biochemists.

It is known that particular groups of organisms are characterized by particular features of fatty acids [23], and these can be used as their biological markers. Natural dicarboxylic acids are major components of plant polymeric compounds such as cutin and suberin [24, 25]. They have also been found in vegetables [26] and in plant cell walls [27]; more than 26 dioic acids (C_{10}–C_{35}) were identified in spores of an ancient fern group, *Equisetum* [28]; dioic acids are also found in birch and in the inner and outer bark of spruce and pine [29, 30], in Royal Jelly [31], in marine and freshwater sediments [32, 33], and even in the Murchison meteorite [34]. However, the greatest diversity of dicarboxylic acids was found among hyperthermophilic microorganisms (this group of organisms was recently discovered by Stetter et al. [35] and also represents a unique ecological branch) and in bacteria [36-38], except for the cyanobacterial species. Our study shows that using this chromatographic assay it is possible to discover previously unknown acids in, e.g., the genus *Aphanizomenon*.

Thus, we have studied the composition of dicarboxylic and fatty acids for four of strains belonging to the genus *Aphanizomenon* using GC/MS. The results of these studies have shown for the first time the presence of fifteen dioic acids in cyanobacteria.

REFERENCES

1. Sellner, K. G. (1997) *Limnol. Oceanogr.*, **42**, 1089-1104.
2. Carmichael, W. W. (1997) *Adv. Bot. Res.*, **12**, 211-256.
3. Sivonen, K. (1996) *Phycol.*, **35** (Suppl. 6), 12-24.
4. Codd, D. A., and Poon, G. K. (1988) in *Biochemistry of the Algae and Cyanobacteria* (Rogers, L. J., and Gallon, J. R., eds.) Claderon Press, Oxford, pp. 283-296.
5. Dembitsky, V. M., Shkrob, I., and Lev, O. (2000) *J. Chem. Ecol.*, **26**, 1359-1366.
6. Slater, G. P., and Blok, V. C. (1983) *Water Sci. Technol.*, **15**, 181-190.
7. Murata, N., and Nishida, I. (1987) in *The Biochemistry of Plants. Lipids: Structure and Function* (Stumpf, P. K, ed.) Vol. 9, Academic Press, Orlando, Florida, pp. 315-347.
8. Nordback, J., Lundberg, E., and Christie, W. W. (1998) *Mar. Chem.*, **60**, 165-175.
9. Ikawa, M., Sasner, J. J., and Haney, J. F. (1994) *J. Chem. Ecol.*, **20**, 2429-2436.
10. Morris, R. J., and Brasell, S. C. (1988) *Lipids*, **23**, 256-258.
11. Hertzberg, S., and Liaaen-Jensen, S. (1966) *Phytochemistry*, **5**, 565-570.
12. Dembitsky, V. M., Shkrob, I., and Dor, I. (1999) *J. Chromatogr.*, **862**, 221-229.
13. Dor, I. (1987) *Bot. Mar.*, **30**, 507-510.
14. McLafferty, E. W., and Turecek, F. (1993) *Interpretation of Mass Spectra*, 4th Ed., University Science Books, Sausalito, California.
15. Mupphy, R. C. (1993) *Mass Spectrometry of Lipids. Handbook of Lipid Research*, Vol. 7, Plenum Press, N. Y.-London.
16. McCloskey, J. A. (1971) in *Topics in Lipid Chemistry* (Gunstone, F. D., ed.) Vol. 1, Logos Press, Ltd., London, pp. 369-440.
17. Holton, R. W., Blecker, H. H., and Onore, M. (1964) *Phytochemystry*, **3**, 595-602.
18. Levin, E., Lennarz, W. J., and Bloch, K. (1964) *Biochim. Biophys. Acta*, **84**, 471-474.
19. Breathnach, A. S. (1999) *Med. Hypothesis*, **52**, 221-226.
20. Hall, I. H., Izydore, R. A., Warren, A. E., and Barnes, C. R. (1999) *Anticancer Res.*, **19**, 205-211.
21. Ma, X., Baraona, E., Goozner, B. G., and Lieber, C. S. (1999) *Amer. J. Med.*, **106**, 70-75.
22. Martin, S. A. (1998) *J. Anim. Sci.*, **76**, 3123-3132.
23. Kerwin, J. (1994) in *Isopentenoids and Other Natural Products: Evolution and Function* (Nes, W. D., ed.) ACS Symposium, Ser. No. 562, American Chemist's Society, Washington, DC, pp. 163-201.
24. Kolattukuty, P. E. (1980) *Science*, **208**, 990.
25. Kolattukuty, P. E. (1980) in *The Biochemistry of Plants* (Stumpf, P. K., ed.) Vol. 4, Academic Press, N. Y., pp. 571-651.
26. Siddiqui, I. R. (1989) *J. Agric. Food Chem.*, **37**, 647-650.
27. Hanley, A. B., Russel, W. R., and Chesson, A. (1993) *Phytochemistry*, **33**, 957-960.
28. Rezanka, T. (1998) *Phytochemistry*, **47**, 1539-1543.
29. Ekman, R., and Eckerman, C. (1985) *Paperi ja Puu – Papper och Tra* (Finland), **65**, 255-273.
30. Ekman, R., and Reunanen, M. (1983) *Finn. Chem. Lett.*, **10**, 166-168.
31. Lercker, G., Capella, P., Conte, L. S., Ruini, F., and Giordani, G. (1981) *Lipids*, **16**, 912-919.
32. Johns, R. B., and Onder, O. M. (1975) *Geochim. Cosmochim. Acta*, **39**, 129-136.
33. Haug, P., Schnoes, H. K., and Burlingame, A. L. (1967) *Science*, **158**, 772-773.
34. Lawless, J. G., Zeutman, B., Pereira, W. E., Summons, R. E., and Duffield, A. M. (1974) *Nature*, **251**, 40-42.
35. Stetter, K. O., Fiala, G., Huber, G., Huber, R., and Segerer, A. (1990) *FEMS Microbiol. Rev.*, **75**, 117-124.
36. Freidllin, G. I. (1978) *Aliphatic Dicarboxylic Acids* [Russian translation], Nauka, Moscow.
37. Klein, R. A., Hazlewood, G. P., Kemp, P., and Dawson, R. M. C. (1979) *Biochem. J.*, **183**, 691-700.
38. Carballeira, N. M., Reyes, M., Sostre, A., Huang, H., Verhagen, M. F. J. M., and Adams, M. W. W. (1997) *J. Bacteriol.*, **179**, 2766-2768.