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Occurrence of New Polyenoic Very Long Chain Acyl Residues in Lipids from *Acanthamoeba castellanii*

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Summary. The cellular fatty acid composition of *Acanthamoeba castellanii*, a unicellular bacteriovorous organism, was reinvestigated. Lipids from amoebae grown axenically in proteose peptone-yeast extract-glucose medium were extracted with chloroform–methanol and separated by silicic acid column chromatography into non-polar and polar fractions. The fatty acid composition of the lipids and the double-bond position of the unsaturated acids have been determined by capillary gas chromatography-mass spectrometry (GC-MS) of their corresponding methyl esters, 2-alkenyl-4,4-dimethyloxazoline (DMOX) derivatives and dimethyldisulfide (DMDS) adducts. Evidence is given that lipids from *A. castellanii* in addition to the three already identified saturated straight chain fatty acids: tetradecanoic (C14:0), hexadecanoic (C16:0), octadecanoic (C18:0), and six preponderant unsaturated fatty acids: hexadecenoic (C16:1 Δ^{7}), octadecenoic (C18:1 Δ^{9}), octadecadienoic (C18:2 $\Delta^{9,12}$), eicosadienoic (C20:2 $\Delta^{11,14}$), eicosatrienoic (C20:3 $\Delta^{8,11,14}$), and eicosatetraenoic (C20:4 $\Delta^{5,8,11,14}$), contain additionally four very long chain unsaturated fatty acids: octacosenoic (C28:1 Δ^{21}), octacosadienoic (C28:2 $\Delta^{5,21}$), triacontadienoic (30:2 $\Delta^{21,24}$), and triacontatrienoic (C30:3 $\Delta^{5,21,24}$) previously unreported in lipids of *A. castellanii*. These new long chain fatty acids account for approximately 25% of total fatty acids. To our knowledge, this is the first report of very long chain polyenoic fatty acids present in lipids extracted from *A. castellanii* cells.

Key words: *Acanthamoeba castellanii*; endoparasites, long chain polyenoic fatty acids, double bond position, dimethyloxazoline derivatives, dimetyldisulfide adducts, non- methylene interrupted fatty acids.

INTRODUCTION

Small free-living amoebae of the genus *Acanthamoeba* are among the most prevalent protozoa distributed in soil, fresh or brackish water and air. Some species of *Acanthamoeba* are amphizoic, capable of existing as free-living or as parasites causing keratitis or meningoencephalitis in an immunocompromised person (Martinez and Visvesvara 1997, Schuster 2002, Marciano-Cabral and Cabral 2003). The life cycle of these naked unicellular animal organisms consists of two stages, one of which is active feeding and dividing by binary fusion trophozoites and the other stage is dormant cysts. Locomotion involves formation of a hyaline pseudopodium. In natural environment amoebae appear to be entirely dependent on endocytic processes to fulfil their nutritional requirements. The trophozoites feed mainly on

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bacteria, algae and yeast in the environment but in laboratory conditions they can exist axenically on proteose peptone, yeast extract, glucose or on chemically defined liquid media taken up through pinocytosis. It is well established that amoebae are the most important predators of bacteria in their environment. Grazing by amoebae has a significant effect on the control of a bacterial population, hence the predator – prey relationship has an influence on soil fertility and on natural purification of polluted water from bacteria that are pathogenic for humans. Grazing on bacteria has also a major evolutionary consequence, allowing some species of bacteria to survive and adapt to live within amoebae. Feeding on microorganisms by phagocytosis and digestion of the prey takes place within phagosomes, where living organisms are killed and digested by lysosomal enzymes; however, the process of intracellular digestion is often insufficient to eliminate some kind of ingested bacteria and viruses. Approximately 20% of *Acanthamoeba* spp. isolates recovered from clinical and environmental sources were found to harbour bacterial endocytobionts (Fritsche *et al.* 1999). Some of the many kinds of the hitherto recognised bacteria living within amoebae cells are pathogens for humans (Barker and Brown 1994, Greub and Raoult 2004, Molmeret *et al.* 2005, Michel *et al.* 2005, Corsaro and Greub 2006, Roault *et al.* 2007). *Legionella lytica*, previously described as *Sarcobium lyticum* was the first pathogen demonstrated to multiply intracellularly and cause a fatal infection of amoebae (Drożański 1956, 1991; Drożański and Chmielewski 1979; Springer *et al.* 1992; Hookey *et al.* 1996). However, the risk for humans connected with the presence of pathogens within amoebae had been disregarded until Rowbotham (1980) showed that *A. polyphaga* might serve as a natural host for *Legionella pneumophila*. Currently, there are over 50 species of *Legionella* recognised which are protozoonotic (Euzeby 2008). Dozens of other microorganisms, some of which are uncultivable on artificial media, require amoebae for growth (Marciano-Cabral and Cabral 2003, Greub and Raoult 2004, Corsaro and Greub 2006, Raoult *et al.* 2007). Detection and identification of endocytobionts, scattered between hosts' organelles and uncultivable outside a living cell by conventional methods, is difficult if not impossible, hence more sophisticated molecular and chemical procedures are a pre-requisite. Fatty acids have frequently been used as chemotaxonomic markers for identification of clinically important bacteria. From our previous studies on fatty acids extracted from *L. lytica*, proliferating within *A. castellanii* (Palusińska-Szysz *et al.* 2001) and in consistency with the recent results on the fatty acids from phospholipids of the bacterium propagated on artificial medium (Palusińska-Szysz *et al.* 2008), it appears that the bacterium harvested from the amoebae contained two fatty acids (eicosadienoic and eicosatrienoic) of the host origin and it additionally contained two other very long chain polyenoic fatty acids (triacontadienoic and triacontatrienoic), not recognised in lipids of *A. castellanii* as yet. The work reported in this paper is considered to be the reinvestigation of fatty acid composition of lipids from *A. castellanii* cells free of intracellular bacteria. It is our hope that the detailed knowledge on fatty acid composition of the host cell will be useful in discriminating between self and non-self; therefore, it should be a useful alternative to morphological and genetic methodologies for the identification of pathogens when within the host cell.

MATERIALS AND METHODS

Organism, medium, and culture conditions

Acanthamoeba castellanii, strain ATCC 3034 was originally obtained from Dr W. Balamuth (Department of Zoology, University of California). The endocytobiont free amoeba was grown axenically in 300 ml Erlenmayer flasks containing 100 ml of proteose peptone-yeast extract-glucose (PYG) medium, pH 6.6. The chemical composition of PYG medium was essentially the same as that described by Band (1959) and consisted of 15 g proteose peptone 3 (Difco), 5 g yeast extract (Difco), 10 g glucose, 120 mg NaCl, 3 mg $MgCl_2$ $6H_20$, 3 mg $CaCl_2$, 3 mg $FeSO_4$, 142 mg Na_2HPO_4, 136 mg KH_2PO_4, and water to amount to 1l. The flasks were inoculated with a 3-day-old amoeba culture to obtain an initial population of approximately 5×10^3 organisms/ml. The culture was incubated on a rotary shaker with an acentric rotation of 3 cm (120 rev/min) at 28°C. The number of cells was determined using a Büchner hemocytometer. Amoebae from the early stationary phase of growth were harvested by centrifugation at 300 x g for 10 min., washed in amoeba saline prepared after Band (1959) and lyophilised.

Lipid analysis

Extraction of lipids

Lipids were extracted from the dried cells by the method of Bligh and Dyer (1959), at room temperature on a magnetic stirrer. The insoluble material was removed by centrifugation at 4000 × g for 30 min. The residue was re-extracted overnight with chloroform-methanol 2:1. The combined extracts were evaporated to dryness under a stream of nitrogen, re-suspended in chloroform-methanol and washed with 0.75 % KCl to remove water-soluble components. The washed lipid extract, after evaporation, was suspended in a small volume of chloroform and stored at 4°C under nitrogen.

Column chromatography

The total extractable lipid was separated on a silicic acid column into neutral and polar fractions (Kunsman 1970). The silicic acid (1.3 g; 100 mesh, Aldrich Chemical Co.), after activation at 110ºC overnight, was suspended in 2 ml of chloroform, poured into a 0.6 cm (inner diameter) column and allowed to settle by gravity. The column was washed with 10 ml of chloroform, and a 0.6 cm layer of glass beads (120/200 mesh, BDH Chemicals LTD) was placed on top of the column to prevent disruption of the surface. 20 mg of lipid dissolved in 1 ml of chloroform was applied to a pre-washed silicic column. Neutral lipids were eluted with 10 ml aliquots of chloroform, 5 ml chloroform-acetone (1:1), and 5 ml of acetone. Polar lipids were eluted with 10 ml chloroform-methanol (8:2) and 10 ml of chloroform-methanol (1:9) v/v.

Fatty acid analysis

A. Preparation of fatty acid methyl esters

Fatty acids were released by heating a dried aliquot of lipid classes with 0.8 M NaOH in 50% methanol for 1 h at 80ºC in nitrogen-filled sealed glass tubes. The free fatty acids extracted with $CHCl_3$ from acidified samples were then converted to fatty acid methyl esters by heating in 0.5 M HCl in anhydrous methanol at 80ºC for 60 min. The solution was then cooled to room temperature and evaporated. Fatty acid methyl esters were recovered by extraction with chloroform/water and analysed by gas-liquid chromatography.

B. Hydrogenation

Unsaturated fatty acid methyl esters were hydrogenated by exposure to hydrogen in the presence of 10% palladium on charcoal as follows: the methyl ester sample was evaporated to dryness under N_2, re-dissolved in ethanol (0.5 ml), and hydrogenated for 2 h at room temperature. The solution was filtered to remove the catalyst, and the required saturated esters were taken up in hexane (1 ml) for GLC analysis.

C. Determination of double band position in new very long chain polyenoic fatty acids

c1. Dimethyloxazoline (DMOX) derivativatives

The 2-amino-2-methyl-1-propanol (300 µl) was added to the fraction of free fatty acids (up to 10 mg) and the mixture was heated at 180°C for two hours in nitrogen atmosphere (Dobson and Christie 1996). After cooling, the DMOX derivatives were dissolved in hexane (2 ml) and washed twice with distilled water. The hexane layer was then passed through a drying column of glass wool and Na_2SO_4. After the solvent had been evaporated, the dry residue was dissolved in chloroform and analysed by the GC/MS method.

c2. Formation of dimethyl-disulfide (DMDS) adducts

The fatty acid methyl esters (10 mg) were dissolved in dimethyl-disulfide (1 ml) and a solution of iodine (100 ml) in diethyl ether (60 mg/ml) was added; the mixture was incubated at 50°C for 48 h (Pope *et al.* 1997). After cooling, J_2 was reduced by adding a solution of $Na_2S_2O_3$ (5% in water) to quench the reaction. The derivatives were extracted three times with n-hexane. Combined organic phases were joined, dried on $MgSO_4$ and concentrated under a gentle flux of nitrogen before analysis by GC/MS.

Gas-liquid chromatography - mass spectrometry

Fatty acid methyl esters were analysed using a Hewlett-Packard gas chromatograph (model HP 5890A) equipped with a capillary column (HP-5MS, 30 m × 0.25 mm internal diameter) and connected to a mass selective detector (MSD model HP5971). The carrier gas was helium (at the flow rate of = 0.7 ml/min.) and the temperature programme was initially 150°C for 5 min. then raised to 310ºC at a ramp of 5°C $min.^{-1}$, with 10 min. final time (Injector temperature was 250ºC and detector temperature was 310°C). For separation of DMDS adducts of fatty acid methyl esters, the initial temperature was 200°C for 5 min. then raised to 310°C at a ramp of 5°C $min.^{-1}$, with 45 min. final time.

Saturated and unsaturated fatty acids in the form of their methyl esters were identified by comparing their retention times with those of the standards, when available, and by comparing m/z values of ions on individual mass spectra with calculated values for corresponding $[M]^+$ or $[M-32]^+$ ions. The relative content (%) of each fatty acid was calculated from the ratio of the area of its peak to the total area of all peaks. For determination of fatty acid content, tricosanoate (C23:0) methyl ester was used as an internal standard added to the test suspensions before derivatisation.

D. Determination of fatty acids composition in the growth medium

The ingredients of PYG medium: proteose peptone (10 g) and yeast extract (5 g) in the amounts required to prepare one liter of the growth medium were used to determine the composition of fatty acids in the nutrients for amoebae. The lipophilic constituents of proteose peptone and yeast extract were extracted by the method of Bligh and Dyer (1959). Fatty acid methyl esters of the extracted lipids were prepared and analyzed as described above.

RESULTS AND DISCUSSION

Identification of fatty acids

The gas-liquid chromatograms of the fatty acid methyl esters extracted from the *A. castellanii* lipid are shown in Fig. 1 and quantitified as presented in Table 1. Fifteen main peaks were identified with the aid of reference substances and mass spectrometry as methyl esters of four straight chain saturated fatty acids: tetradecanoate, hexadecanoate, octadecanoate, and the novel one octacosenoate, three monounsaturated acids: hexadecenoate, octadecenoate octacosenoate; and eight polyenoic acids: octadecadienoate (C18:2), eicosadienoate (C20:2), eicosatrienoate (C20:3), eicosatetraenoate (C20:4), eicosapentaenoate (C20:5) octacosadienoate (C28:2), triacontadienoate (C30:2), and triacontatrienoate (C30:3).

Unsaturated fatty acids were further characterised by (GC-MS) spectrometry of the product obtained be-

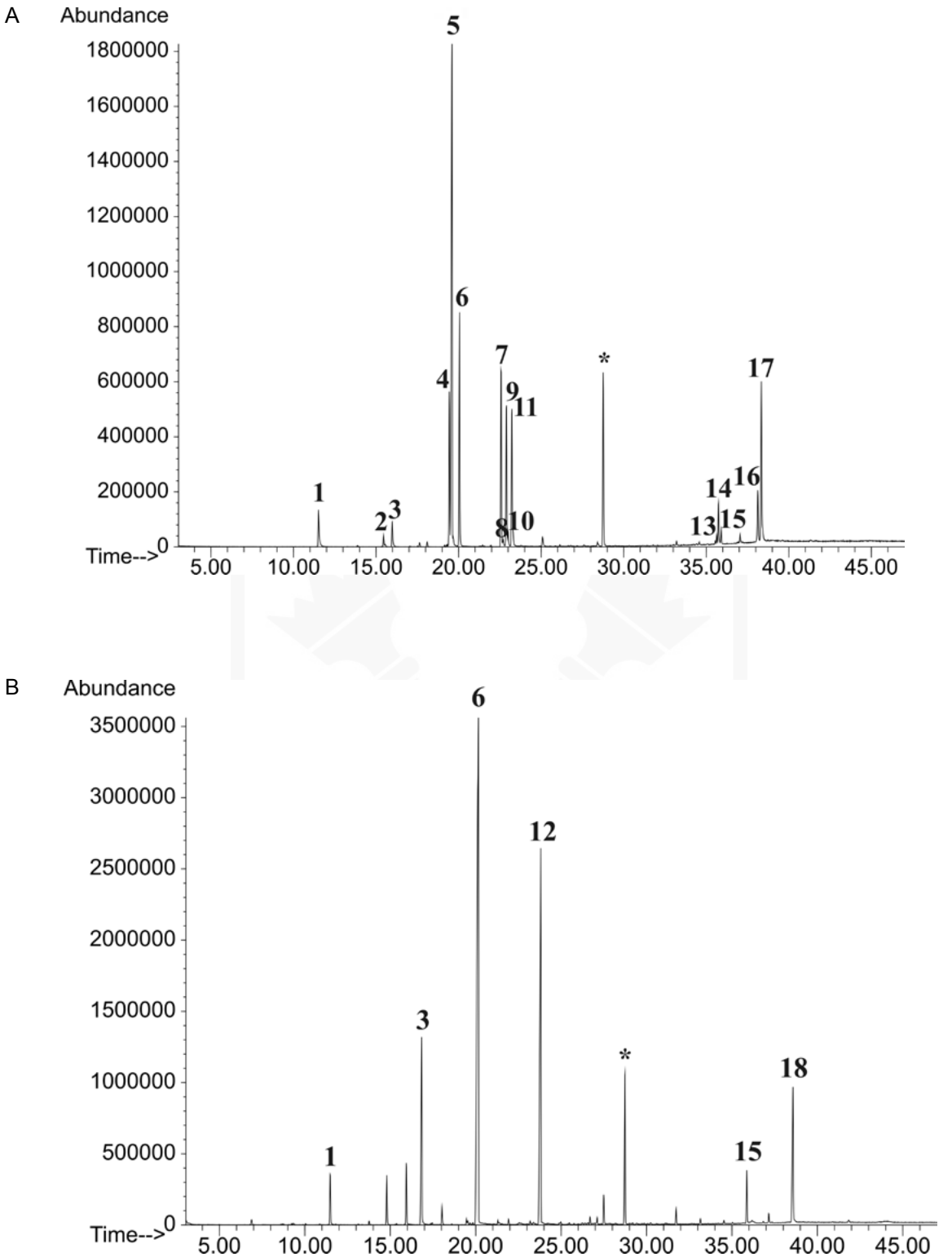

Fig. 1. Gas-liquid chromatograms of methyl esters of the fatty acids from the *A. castellanii* lipid. **A** – before hydrogenation; **B** – after hydrogenation. Number marked peaks denote methyl derivatives: **1** – mirystate; **2** – hexadecenoate; **3** – palmitate; **4** – octadecadienoate; **5** – octadecenoate; **6** – stearate; **7** – eicosatetraenoate; **8** – eicosapentaenoate; **9** – eicosatrienoate; **10** – eicosadienoate; **11** – eicosadienoate; **12** – eicosanoate; ***** – tricosanoate, internal standard; **13** – octacosadienoate; **14** – octacosenoate; **15** – octacosanoate; **16** – triacontatrienoate; **17** – triacontadienoate; **18** – triacontanoate.

fore and after hydrogenation. Hydrogenation shifted fatty acids under peak 2, peaks 4–5 as well as peaks 13–14 to new positions already occupied by methyl esters of hexadecanoic, octadecanoic and octacosanoic acids respectively, hence the original peaks of these three saturated acids increased in size (Fig. 1B and Table 1 column B and C). Two groups of peaks: the first group composed of 20-carbon chain length acids (Fig. 1A, peaks 7 to 10) and the second group of peaks comprising thirty carbon chain acids (Fig. 1A, peaks 16 and 17) disappeared after hydrogenation but two new peaks appeared identical in the position of the original methyl esters of eicosanoic and triacontanoic acids (Fig. 1B peaks 12 and 18), respectively. The novel, very long, previously unreported acids are localised in two clusters. One cluster is represented by three 28 carbon chain length acids: octacosanoate, octacosenoate and octacosadienoate. The latter acid was detected in investigal amounts. The second cluster of peaks comprises two 30-carbon chain length acids: triacontadienoate and triacontatrienoate both present in an abundant amount. Altogether these new long chain fatty acids constituted approximately 25% of the total acids extracted with chloroform-methanol from *A. castellanii* lipids.

Electron-impact mass spectra of the new fatty acid methyl esters after hydrogenation showed very intensive ion at m/z 74 and ion at m/z 87 characteristic for saturated fatty acids. These two ions as well as a molecular ion $[M]^+$ and ions at m/z $[M-31]^+$ and $[M-43]^+$ showed decreasing intensity with the number of olefinic bonds present in the methyl esters of C28 and C30 fatty acids. Their quasi-absence on the spectrum after hydrogenation suggests that the structure is polyunsaturated. Fragments in the low m/z range indicate one, two or three double bonds on the alkyl chain and the spectrum

Table 1. The fatty acid composition of extractable cellular lipids of *A. castellanii.*

Peak number	Retention time (min.)	Molecular ion $[M^+]$	Fatty acid methyl ester	Concentration (µg/mg)	
			A	B	C
1	11.5	242	14:0	5.8	4.9
2	15.4	268	16:1	11.8	0
3	15.9	270	16:0	8.1	18.9
4	19.4	294	18:2	29.4	0
5	19.6	296	18:1	117.9	0
6	20.0	298	18:0	47.3	190.3
7	22.5	318	20:4	44.1	0
8	22.7	316	20:5	3.2	0
9	22.9	320	20:3	36.8	0
10	22.9	322	20:2	trace	0
11	23.2	322	20:2	33.3	0
12	23.3	326	20:0	0	115.3
13	35.6	434	28:2	0.6	0
14	35.7	436	28:1	3.8	0
15	35.9	438	28:0	2.5	6.0
16	38.1	460	30:3	6.0	0
17	38.3	464	30:2	13.8	0
18	38.5	466	30:0	0	18.3

Explanation: condition of chromatography as in Fig. 1.
A – the number before the colon refers to the number of carbon atoms, the number after the colon is the number of double bonds.
B – before hydrogenation.
C – after hydrogenation.

showed no evidence for the presence of any hydroxyl group, intra-chain cycle or for an oxo group.

Location of double bonds position of new unsaturated fatty acids

By using GC-MS analysis of DMOX derivatives, the unsaturated fatty acids were identified as presented in Table 3. In the spectrum of each derivative, beside the intense molecular ion, three types of ions were found: a) the MacLafferty rearrangement product at m/z 113 characteristic for 2-unsubstituted series of fatty acids, b) the ion due to cyclization-displacement at m/z 126, c) a series of homologous ions differing by 14 atomic mass units (amu) from the $[M-15]^+$ ion to m/z 126 ion interrupted by an interval of 12 amu, flanked by two higher homologous peaks forming a number of easily recognisable markers and separated by 40 amu in the spectrum profile arising from allylic cleavage.

The representative chromatogram of DMOX derivative of 30 carbon chain length acid is shown in Fig 2. In the spectrum, beside the intense molecular ion at m/z 499, a double bond on carbon five from the functionalised side was recognised by a gap of 12 amu between m/z 140 and m/z 152. This was confirmed by the presence of the strong odd mass ion at m/z 153 and the prominent gap of 40 amu between the ions at m/z 126 and m/z 166. The position of the other two double bonds interrupted by a single methylene group was localised by increment of 12 amu between the ions at m/z 362–374 and the ions at m/z 402–414 surrounded by gaps of 40 amu at m/z 348–388 and m/z 388–428, respectively. In the spectrum of the second 30 carbon chain length acid, the molecular ion was located at m/z 501 and the positions of the double bonds were assigned by the gaps of 12 amu between m/z 364–376 and m/z 404–416 on C21 and C24. GC-MS analysis of DMOX derivative of octacosadienoic acid indicated the molecular ion at m/z 473 and located a double bond in position Δ^5 whereas the location of the second double bond of this acid as well as the location of a double bound of octacosenoic acid could not be unambiguously resolved by spectrum interpretation, hence this was only supposed to be on C7 from the methyl terminal group. The position of the original double bond of octacosenoic acid was verified by comparative GC-MS analysis of DMDS adduct (Fig. 3). The fragment suites produced by the cleavage of the carbon-carbon bond between two methylthio substituents yielded intense peaks at m/z 145 at the methyl side and at m/z 385 of the functionalized side;

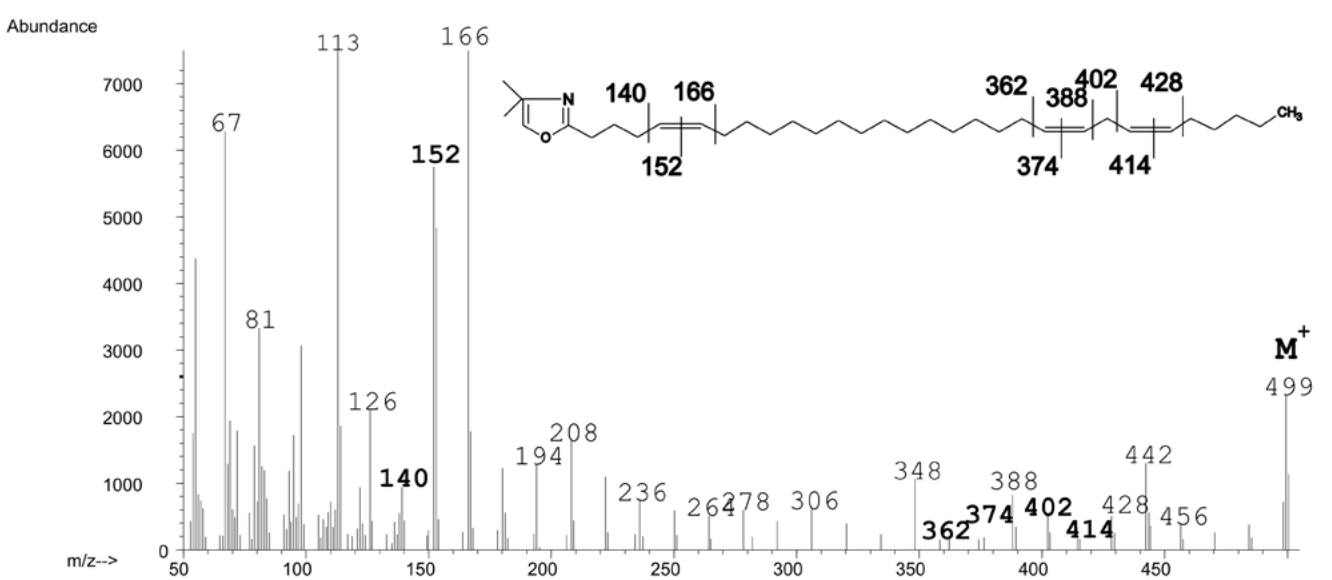

Fig. 2. Mass-spectrum of the oxazoline derivative of triacontatrienoate (30:3 $\Delta^{5,21,24}$) obtained from *A. castellanii* lipids. Values m/z for characteristic for double bonds ions are in bold.

this together with the molecular ion at m/z 530 definitely locates the double bond of octacosenoic acid at C7 from the methyl terminal group. Although the fatty acid composition of *A. castellanii* has been examined in several laboratories, the occurrence of non methylene interrupted very long chain fatty acids are described here for the first time. The fact that fatty acids of this kind in amoebae lipids have not been described previously can be explained by analytical reasons.

Fatty acid composition of neutral and polar fractions

As shown in Table 2, the same fatty acids were found in the neutral lipid preparation as well as in phospholipid fractions, although quantitatively, they occurred in different proportions. In all of the tested fractions, the sum of saturated fatty acids: tetradecanoic, hexadecanoic, octadecanoic together with the new straight chain 28 carbon chain length octacosanoic acid, did not exceed 20% in the fraction of neutral lipid and constituted only 12–16% in phospholipids fractions. Octadecanoic acid was the most abundant one but octacosanoic acid was detected in investigial amounts. The 28 carbon straight fatty acid found in lipids extracted with chloroform-methanol is one of the thirty fatty acids recognised in lipophosphonoglycan of *A. castellanii*. It deserves emphasis that *A. castellanii,* apart from fatty acids bound to lipids soluble in chloroform-methanol, additionally contain about thirty different fatty acids bound to lipophosphonoglycan. This unusual macromolecule accounts for 1/3 of the mass of the plasma membrane and contains a large spectrum of long chain fatty acids which comprise: a) saturated normal and branched (C16:0 to C28:0 – 11 FAs), b) normal and branched α-hydroxy (C20:0 to C28:0 – 8 FAs), c) α-hydroxy-3-methyl (C22:0 to C28:0 – 5 FAs), d) branched α-hydroxy-3-methyl (C20:0 to C28:0 – 4 FAs) but only two monounsaturated fatty acids: octadecenoic and eicosenoic and none of polyunsaturated acids. Studies carried out in our laboratory on fatty acid composition of the remainders of amoeba cells left after exhaustive extraction of lipids with chloroform-methanol yielded fatty acid composition similar to that found in lipophosphonoglycan (data not published, Dearborn and Korn 1974). Only two saturated fatty acids: hexadecanoic and octacosanoic and two monounsaturated fatty acids: octadecenoic and eicosenoic were shared between lipophosphonoglycan and lipids found in the remainder of *A. castellanii* cell.

Of the ten unsaturated fatty acids described in this paper, six, including: hexadecenoic, octadecenoic, octadecadienoic, eicosadienoic, eicosatrienoic, and eicosatetranoic had been recognised more than 45 years ago by Korn (Korn 1963a). In these GC/MS studies, owing

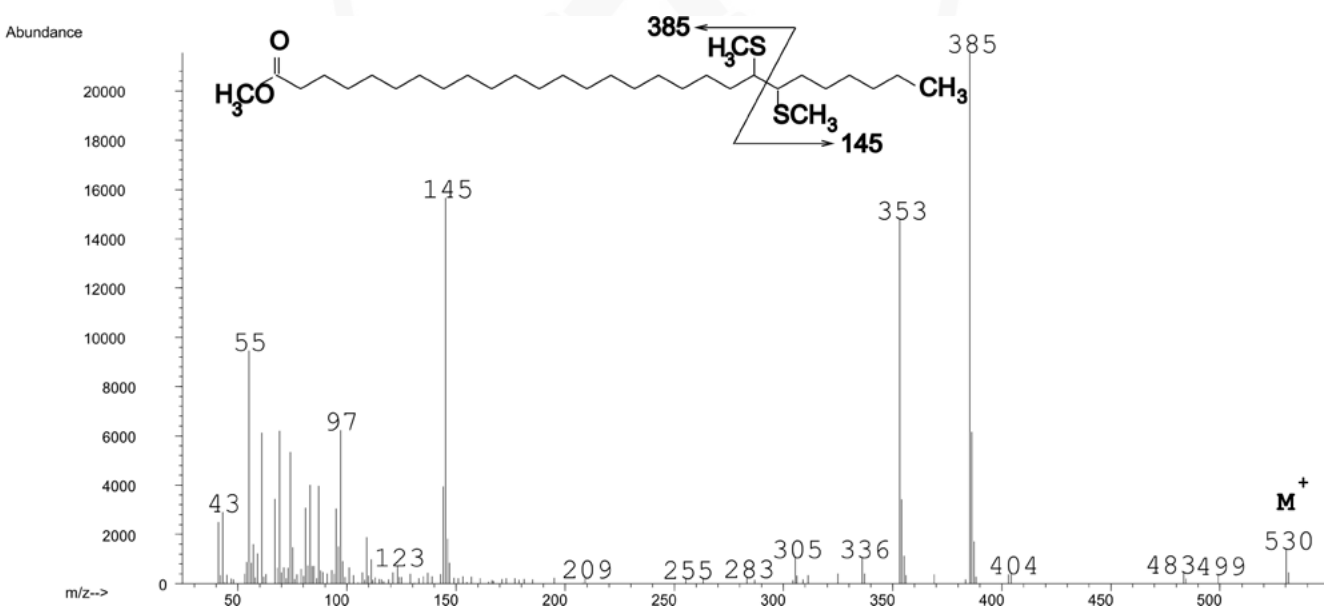

Fig. 3. Mass-spectrum of the DMDS adduct fatty acid methyl ester of 28:1 Δ^{21} derived from *A. castellanii* lipids.

Table 2. Fatty acid composition of lipids from *A. castellanii* and ingredients of the medium.

Fatty acid	Relative area (%)				
	A	B_1	B_2	C	D
Saturated					
14:0	3	1	2	1	2
16:0	6	3	4	30	43
17:0	0	0	0	0.9	trace
18:0	10	6	10	15	37
20:0	0	0	0	0.7	0
22:0	0	0	0	1	0
24:0	0	0	0	2	trace
27:0	0	0	0	2	0
28:0	trace	trace	trace	0	0
Monounsaturated					
16:1	0.3	0.2	0.4	1	7
17:1	0	0	0	0	2
18:1	24	23	25	22	7
20:1	trace	trace	0.4	0	0
22:1	0	0	0	3	0
Polyunsaturated					
18:2	6	5	6	17	1
20:4	9	15	11	4	0
20:5	0.6	1	0.8	0	0
20:3	11	12	25	trace	trace
20:2	trace	trace	trace	0	0
20:2	5	7	7	trace	trace
Long chain unsaturated					
28:2	trace	0.4	trace	0	0
28:1	0.7	0.9	0.3	0	0
30:3	10	10	3	0	0
30:2	14	15	5	0	0

A – fraction of neutral lipid.

B_1 – low polar phospholipids

B_2 – polar phospholipids.

C – proteose peptone.

D – yeast extract.

to the high elution temperature of 310°C and the final time of 10 min applied in gas liquid chromatography, we were able to obtain additionally four novel well-separated peaks of very long chain unsaturated non methylene interrupted acids: octacosenoic (C28:1 Δ^{21}), octacosadienoic (C28:2 $\Delta^{5,21}$), triacontadienoic (C30:2 $\Delta^{21,24}$), and triacontatrienoic (C30:3 $\Delta^{5,21,24}$). These new long chain unsaturated fatty acids were recognised also in lipids of *A. castellanii* grown in monoxenic culture on expense of *Enterobacter aerogenes* as a sole source of food. Noteworthy is the fact that the bacterium as well as ingredients of the PYG medium: proteose peptone and yeast extract are devoid of this kind of long chain acids (Table 2, column C and D) (Whittaker 2007). Ac-

Table 3. Characteristic ions (m/z) observed in mass spectra of 4,4-dimethyloxazoline derivatives of unsaturated fatty acids isolated from *A. castellanii*.

Fatty acid	Base peak	Molecular ion $[M]^+$	Characteristic 12 unit gaps*
16:1 Δ^7	113	307	168/180
18:2 $\Delta^{9,12}$	113	333	196/208; 236/248
18:1 Δ^9	113	335	196/208
20:4 $\Delta^{5,8,11,14}$	113	357	140/152; 180/192; 220/232; 260/272
20:3 $\Delta^{8,11,14}$	113	359	182/194; 222/234; 262/274
20:2 $\Delta^{11,14}$	113	361	224/236; 264/276
28:2 $\Delta^{5,21}$	113	473	140/152; 362/374
28:1 Δ^{21}	113	475	364/376
30:3 $\Delta^{5,21,24}$	113	499	140/152; 362/374; 402/414
30:2 $\Delta^{21,24}$	113	501	364/376; 404/416

* The values presented, different for 12 mass units, indicate ion pairs formed by cleavage of carbon-carbon linkages before and in position of double bonds.

cording to Korn (1964) and Jones *et al.* (1991, 1993) *A. castellanii,* unlike higher animals, is not dependent on exogenous precursors but is capable of forming de novo essential FAs of the n-6 family. In nature, according to the authors' knowledge, mainly marine sponge contains a high level of long chain polyenoic fatty acids (Barnathan *et al.* 1996, Litchfield 1979).

Approximately 80% of FAs in phospholipids and neutral lipid fractions are unsaturated (Ulsamer *et al.* 1969). A striking feature of the unsaturated fatty acids content in lipids of *A. castellanii* is the presence of relatively large amounts of octadecanoic acid ca. 25 to 50% of the total acids (this paper and Korn 1963; Jones *et al.* 1991, 1993; Avery *et al.* 1995; Barker *et al.* 1993; Rutter *et al.* 2002; Vandenesch *et al.* 1990).

Increased unsaturation of fatty acids is the usual response of poikilotherm to lowered environmental temperature (Jones *et al.* 1993; Rutter *et al.* 2002; Avery *et al.* 1994, 1995; Ulsamer *et al.* 1969). The role of the very long polyunsaturated FAs in the regulation of fluidity of the plasma membrane and phagocytic activity needs to be explained.

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