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Identification of very-long-chain polyunsaturated fatty acids from *Amphidinium carterae* by atmospheric pressure chemical ionization liquid chromatography–mass spectroscopy

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ABSTRACT

A method is described for the enrichment of very-long-chain polyunsaturated fatty acids (VLCPUFAs) from total fatty acids of *Amphidinium carterae* and their identification as picolinyl esters by means of microbore liquid chromatography–mass spectrometry with atmospheric pressure chemical ionization (LC–MS/APCI). The combination of argentation TLC and LC–MS/APCI was used to identify unusual VLCPUFAs up to hexatriacontaoctaenoic acid. Two acids, 36:7n-6 and 36:8n-3, were also synthesized to unambiguously confirm their structure. The possibilities of VLCPUFAs biosynthesis are proposed.

1. Introduction

Very-long-chain polyunsaturated fatty acids (VLCPUFAs) universally occur in autotrophic lower organisms (*e.g.* algae), in heterotrophic lower organisms (*e.g.* mycobacteria, fungi, sponges, marine invertebrates, insects, and earthworms), in higher plants, and in vertebrate tissues (fish oil, vertebrate brain, sperm, retina, etc.) (Rezanka, 1989). VLCPUFAs from different sources vary in structure and their content also varies approximately from 0.1% or less to 10% of the total fatty acids (FAs).

An interesting group of organisms in terms of VLCPUFAs composition includes about 2000 species of dinoflagellates (Dinophyta) (Taylor et al., 2003), eukaryotic unicellular organisms that can be autotrophic, heterotrophic or mixotrophic and that live in salt and fresh waters, both in the pelagic and benthic environments (Taylor, 1987). Though often harmless, dense dinoflagellate blooms can decrease light penetration in the water and inhibit growth of submerged vegetation, or cause oxygen depletion, which kills fishes and invertebrates. Some dinoflagellates also produce toxic compounds (Sournia, 1995).

Amphidinolides constitute a large family of cytotoxic macrolides produced by dinoflagellates of the genus *Amphidinium* (Kobayashi and Tsuda, 2004). *Amphidinium* species also produce long chain polyketides of more than 60 carbon atoms in length possessing polyhydroxyl groups and polyolefins (Houdai et al., 2001).

Dinoflagellates have high levels of 20:5n-3 and 22:6n-3 and many contain the unusual fatty acid 18:5n-3, which is incorporated in marine animals through the diet and also in humans through consumption of seafood. Highly unsaturated C_{28} fatty acids containing 7 and 8 methylene-interrupted double bonds were identified in small amounts in several species.

Mansour et al. (1999a) isolated from different dinoflagellates (*Prorocentrum mexicanum*, *P. micans*, *Scrippsiella* sp., *Symbiodinium microadriaticum*, *Gymnodinium* sp., *G. sanguineum*, and *Fragilidium* sp.) 28:8n-3 and 28:7n-6 acids that accounted for less than 2.3% of the total fatty acids. The main VLCPUFAs in *S. microadriaticum* was 28:7n-6; in the other species it was 28:8n-3. *Scrippsiella* sp., *Gymnodinium* sp. and *Fragilidium* sp. contained only 28:8n-3, *Gymnodinium sanguineum* contained a low proportion of 28:7n-6 in addition to 28:8n-3. Small amounts (0.9–2.2% of total FAs) of C_{28} fatty acids having seven or eight double bonds occurred in *Scrippsiella* sp., *S. microadriaticum*, *Gymnodinium* sp., *Gymnodinium sanguineum*, and *Fragilidium* sp. (Mansour et al., 1999b).

In two of 16 examined dinoflagellates, 28:7n-6 and 28:8n-3 acids were found to be associated with phospholipids (Leblond and Chapman, 2000). Hence, C_{28} fatty acids are probably located and synthesized in the cytoplasm or in an organelle other than the chloroplast, possibly with 22:6n-3 and/or 24:0 as precursors. In the heterotrophic dinoflagellate *Crypthecodinium cohnii* the 28:8n-3 acid formed about 1.2% of total FAs (Van Pelt et al.,

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1999a). In *Gymnodinium* sp., the highest production of VLCPUFAs (2.2% of total FAs) was recorded in the late logarithmic phase (Mansour et al., 2003). The phospholipid fraction from *Karenia brevis* bloom from the north-west Florida coast was found to contain small amounts of 28:8n-3 and 28:7n-6 (Leblond et al., 2003).

Five species (*Pavlova pinguis*, *Heterocapsa niei*, *Proteomonas sulcata*, *Navicula jeffreyi*, and *Thalassiosira pseudonana*) produced high proportions of PUFAs (Mansour et al., 2005). Low proportions (<1.2%) of 24:6n-3 plus trace amounts of 24:5n-6 were detected in most strains, while 28:8n-3 was found in both the dinoflagellates and in the prymnesiophyte *P. pinguis*. All non-diatomaceous species contained small amounts of 26:7n-3, the highest VLCPUFAs content being found in Dinophyceae (genera *Heterocapsa* and *Amphidinium*), which contained all five fatty acids, *i.e.* 24:5n-6, 24:6n-3, 26:7n-3, 28:7n-6, and 28:8n-3.

In eight species of ichthyotoxic marine gymnodinioid dinoflagellate (*Karenia*, *Karlodinium*, and *Takayama*) 28:7n-6 and 28:8n-3 acids were present at low levels (<1%) and were observed in all strains except *K. mikimotoi* and *T. tasmanica* (Mooney et al., 2007).

The occurrence of VLCPUFAs in diatoms was also indicated by the finding of hydrocarbons, n-C_{25} and n-C_{27} heptaenes, *e.g.* all-(*Z*) pentacosa-3,6,9,12,15,18,23-heptaene all-(*Z*) pentacosa-3,6,9,12,15,18,24-heptaene, and all-(*Z*) heptacosa-3,6,9,12,15,18,26-heptaene in the marine diatom *Rhizosolenia setigera* (Damste et al., 2000). In addition, hentriacontanonaene (n-C31:9) was identified by mass spectrometry in the non-saponifiable neutral lipid of 19 bacterial strains isolated from Antarctic sea ice (Nichols et al., 1995).

These findings show that some marine dinoflagellates and also other marine microorganisms have the ability to produce VLCPUFAs, but the amounts of precursors of these VLCPUFAs with chain lengths between 22 and 28 carbon atoms were below the detection level, with the exception of the data of Mansour et al. (2005). The function(s) of these C_{28} fatty acids as components of phospholipids in cellular membranes is currently unknown.

Based on our previous experience with the analysis and identification of VLCFA including VLCPUFA from lower organisms (Dembitsky et al., 1991; Rezanka, 1993), mostly algae (Rezanka and Podojil, 1984; Rezanka et al., 1986), we decided to analyze the fatty acids in a marine dinoflagellate.

The presence of VLCPUFAs in *Amphidinium carterae* was demonstrated by a newly developed method. Ag^+–TLC was used twice to first remove saturated FA and then to enrich the unsaturated fraction of FAs with FAs having 7–8 double bonds. This was followed by the identification of picolinyl esters of the VLCPUFAs by means of LC–MS/APCI. The present study describes also the synthesis of two polyenoic acids (36:7n-6 and 36:8n-3) for complete confirmation of the structure that have so far been neither synthesized, nor isolated from natural sources.

2. Results and discussion

The marine dinoflagellate *A. carterae* was cultured at 10 °C in seawater medium in glass Erlenmeyer flasks. Batch cultivation was carried out for 5 weeks under continuous light using cool white fluorescent lights. The cells were harvested by centrifugation. To determine the fatty acid composition of the algal sample, total lipids were extracted by the method of Folch et al. (1957). The sample of total lipid was then dried under nitrogen before transmethylation in absolute methanol containing sulphuric acid (Christie, 1982). Fatty acid methyl esters (FAMEs) were extracted and the solvent was removed under reduced pressure.

The total amount of VLCPUFAs in the dinoflagellate did not exceed several percent (up to 3%) of the total fatty acids. We therefore used for further analysis a double enrichment of PUFAs in total FAs. Methyl esters from total lipids were separated into two major fractions, one of which was composed of saturated, monoenoic and dienoic acids, the other contained from trienoic to heptaenoic acids with small quantities of octaenoic fatty acids on argentation TLC (see Fig. 1 and Table 1). The second Ag^+–TLC on

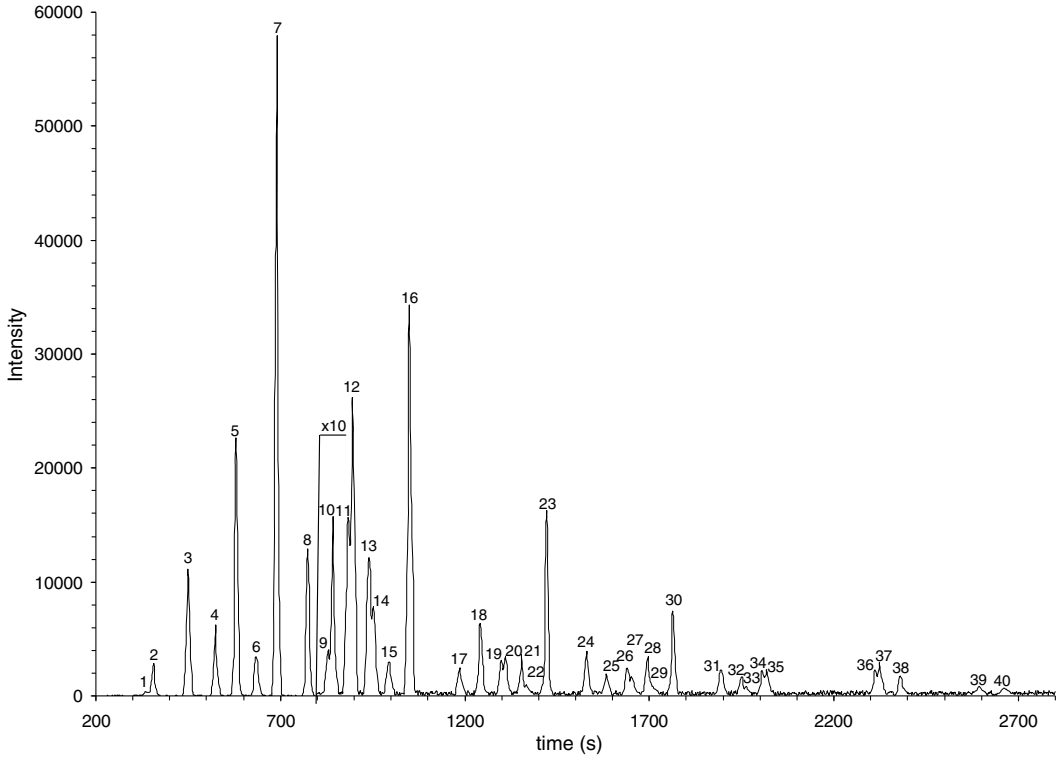


Fig. 1. LC–MS/APCI chromatogram of FAMEs from total FAs of *A. carterae* after first Ag^+–TLC; only acids with 3 and more double bonds are present. Peak identification (see Table 1).

Table 1
Total PUFAs after Ag^+–TLC; only acids with 3 and more double bonds are given

No.	PUFA	Abundance (%)
1	16:4n-1	2.04
2	18:5n-3	8.35
3	16:4n-3	0.29
4	16:3n-4	4.54
5	18:4n-3	16.46
6	20:5n-3	2.52
7	22:6n-3	42.01
8	18:3n-3	9.37
9	20:4n-3	0.30
10	18:3n-6	1.14
11	22:5n-3	1.31
12	20:4n-6	1.90
13	24:6n-3	0.88
14	22:5n-6	0.57
15	26:7n-3	0.22
16	28:8n-3	2.49
17	22:4n-3	0.21
18	24:5n-3	0.46
19	26:6n-3	0.23
20	24:5n-6	0.25
21	28:7n-3	0.26
22	26:6n-6	0.07
23	28:7n-6	1.18
24	22:3n-3	0.32
25	24:4n-3	0.14
26	26:5n-3	0.20
27	24:4n-6	0.12
28	28:6n-3	0.27
29	26:5n-6	0.06
30	28:6n-6	0.54
31	24:3n-3	0.19
32	26:4n-3	0.13
33	24:3n-6	0.03
34	28:5n-3	0.16
35	26:4n-6	0.18
36	28:4n-3	0.17
37	26:3n-6	0.24
38	28:4n-6	0.15
39	28:3n-3	0.03
40	28:3n-6	0.02

silica separated FAMEs with seven and eight double bonds that could not be further separated from each other. The main reason for this step was a vast excess of 22:6n-3 acid, for which the alga is commercially cultivated (Vazhappilly and Chen, 1998). We succeeded in reducing this excess up to 50-fold although traces of 22:6n-3 remained in the sample (see Fig. 2 and Table 2). These data imply that an increment of one or two double bonds cannot affect the behavior of 22:6n-3 in Ag^+–TLC.

Although it is commonly accepted that APCI-MS gives very little fragmentation, it is possible to obtain very rich spectra by changing the cone voltage (Rezanka, 2000). In a well-designed mass spectrometer with APCI, the abundance of a sample ion at *m/z* 200–800 decreases if $\Delta V(N-S)$ is reduced from 50 eV down to a few electronvolts. At low collision energy, the positive-ion APCI spectrum shows the $[M+H]^+$ ion practically as the only peak. Even at a moderate collision energy, where most sample ions would survive, the $[M+H]^+$ ion is split into fragments, *i.e.* M-29 $(M-Et)^+$, M-31 $(M-MeO)^+$ M-56 $(M-C_4H_9)$, and M-69 $(M-C_5H_{11})$. The largest number of fragments is observed at the highest collision energy (70 eV), especially peaks in the low *m/z* region, *i.e.* at the *m/z* 67, 79, 91, 105, 119, etc.

We achieved excellent separation of single peaks including separation of VLCPUFA. The single peaks were identified on the basis of their mass spectra. The positions of double bonds were determined on the basis of the well-known rule published by Poulos et al. (1986) "the characteristic fragment ion split from the alkyl end of FAME is produced by members of each series *i.e.* *m/z* 108 for the n-3 series, *m/z* 150 for the n-6 series and *m/z* 192 for the n-9 series". On the basis of this rule, we were able to determine the positions of double bonds in methyl esters of PUFA from the ions in their mass spectra. Unfortunately, the mass spectra do not indicate the position of the first double bond. The key fragments indicate only the fatty acid family (ω 9, 6, or 3), *i.e.* only common fatty acids with methylene-interrupted double bonds may be identified correctly (Fellenberg et al., 1987). Otherwise, derivatization is necessary as already mentioned in our preceding papers (Rezanka and Sigler, 2006, 2007). To confirm these theoretical results we used the LC–MS method with APCI for analysis of picolinyl esters.

We synthesized esters **1** and **2** to confirm the structure of both the longest fatty acids. (all-*Z*)-Hexatriaconta-12,15,18,21,24,27,30,33-octaenoic acid (**1**) and (all-*Z*)-hexatriaconta-12,15,18,21,24,27,30-heptaenoic acid (**2**) were synthesized as depicted in Fig. 3. The key reaction in both cases was oxidative degradation of acids (arachidonic and eicosapentaenoic), which led to the corresponding aldehydes (**3**) and (**4**), respectively (Bhatt et al., 1998; Flock et al., 1999). Reactions with methyl [bis(trifluoroethyl)phosphono]acetate (**5**) converted these aldehydes nearly quantitatively into the methyl esters (**7**, **8**). The two methyl esters were transformed by a sequence of reactions comprising reduction to alcohols and bromination to bromides (**10**, **11**). Alkantriynol was synthesized in the presence of cuprous salts by coupling the bis-Grignard derivatives of protected alkynol (**13**) with 1-bromo-2,5-hexadiyne (**12**) and, subsequently, this tetrahydropyrane derivative (**14**) was coupled in the presence of cuprous salts of the bis-Grignard derivative with bromides (**10**, **11**) to intermediates – alkenynoic tetrahydropyranes. The triple bonds were stereospecifically hydrogenated to *cis*-double bonds; the compounds were deprotected and oxidized to required acids, *i.e.* **1** and **2**.

The APCI mass spectra of two peaks had characteristic ions consistent with synthesized 36:7 and 36:8 picolinyl esters (see Figs. 4 and 5). In contrast to previously published spectra (Van Pelt and Brenna, 1999b; Rezanka, 2002; Rezanka and Sigler, 2006, 2007), this spectrum exhibited a peculiar feature. The pseudomolecular ion $[M+54]^+$ is conventionally taken to be mainly characteristic for PUFA, but in fact it also arises by the addition of acetonitrile from the mobile phase. We were able to identify a pseudomolecular ion arising by the double addition of acetonitrile on picolinyl esters of polyenoic fatty acids. This effect is probably due to the unusual numbers of methylene-interrupted double bonds. Nevertheless, this method was described as successful either for substances with equal length chain, but having only one double bond (Rezanka, 2002; Rezanka and Sigler, 2007), or for shorter-chain PUFAs (Lawrence and Brenna, 2006).

Picolinyl esters have prominent ions at *m/z* = 92, 108, 151 (the McLafferty ion) and 164, which are all fragments about the pyridine ring. The molecular ion is easily distinguished and it is always odd-numbered, because of the presence of the nitrogen atom, but most other ions are even-numbered. From the molecular ion, there is the loss of a methyl group, followed by a series of ions 14 amu apart for the loss of successive methylene groups. When a double bond is reached, there is a gap of 26 amu. This gap can sometimes be difficult to locate precisely, and a fragmentation at the adjacent methylene group on the carboxyl side giving a gap of 40 is often easier to locate, especially with polyenes, see below. The greater complexity of the mass spectra of PUFA picolinyl esters can make interpretation even more difficult than the interpretation of saturated FA spectra.

The gap of 26 Da between *m/z* = 582 and 556 locates the terminal double bond rather easily, and that between *m/z* = 542 and 516 locates in position 30 of the picolinyl ester **1**. This pattern is repeated up to the first double bond. Further useful features are the gaps of 40 Da for the double bond and an associated methylene

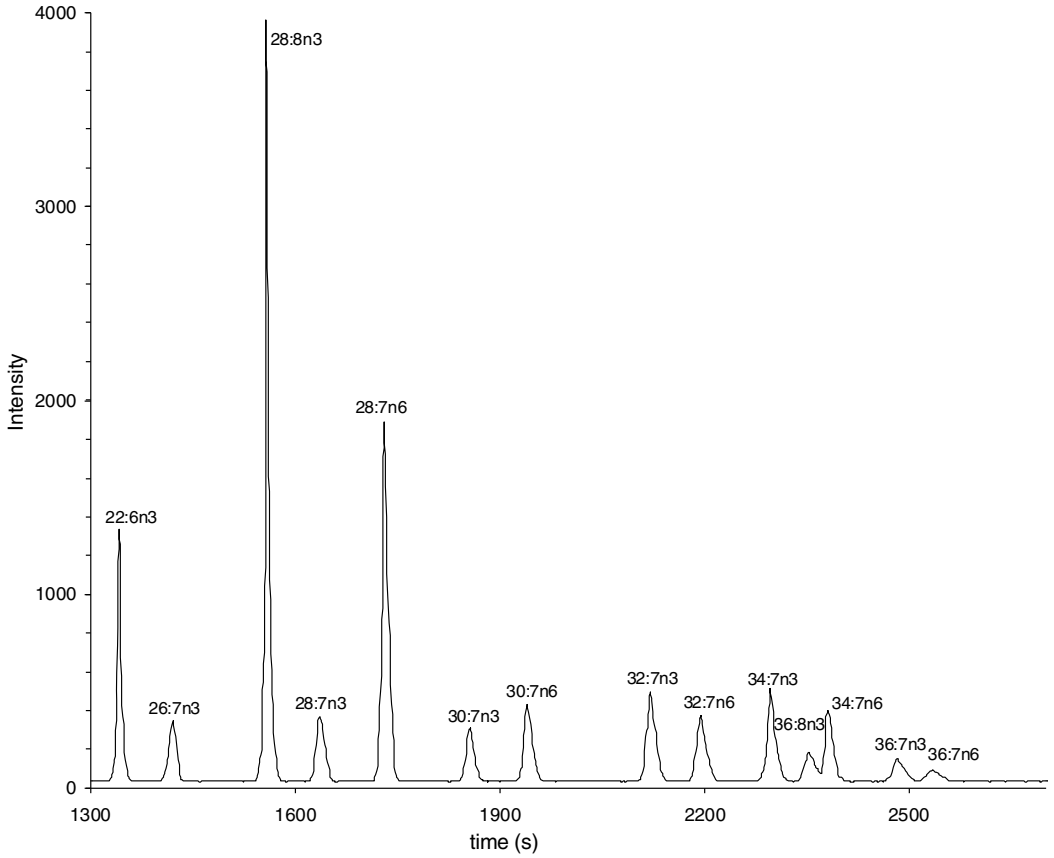


Fig. 2. LC–MS/APCI chromatogram of FAMEs of VLCPUFAs after second Ag^+–TLC; only compounds with 7 and 8 double bonds are present.

Table 2
VLCPUFAs after Ag^+–TLC; only compounds with 7 and 8 double bonds were separated

VLCPUFA[a]	Abundance (%)
22:6n-3	12.4
26:7n-3	3.2
28:8n-3	36.7
28:7n-3	3.8
28:7n-6	17.4
30:7n-3	2.9
30:7n-6	4.0
32:7n-3	4.6
32:7n-6	3.5
34:7n-3	4.8
36:8n-3	1.4
34:7n-6	3.8
36:7n-3	1.1
36:7n-6	0.4

[a] The 22:6n-3 to 28:8n-3 ratio in Table 1 and Table 2 is 50:1, which signifies a 50-fold enrichment of the oil by VLCPUFAs by means of Ag^+–TLC. The percent values refer to this fraction; relative to total FAs they should be divided by 50.

group, in the intervals (*m/z* = 276 to 316; 316 to 356; 356 to 436; 436 to 476; 476 to 516; 516 to 556; 556 to 596; see Fig. 4). The structures of other PUFAs, including the synthesized compounds, *e.g.* 36:7n-6, were identified in an analogous manner, as illustrated in Fig. 5 for 36:7n-6.

We also used other methods for the full confirmation of the above structures, *i.e.* hydrogenation of the compounds from the lower spots after Ag^+–TLC. Only peaks corresponding to saturated straight-chain FAMEs were observed by means of GC–MS. The EI mass spectra of these peaks contained fragment ions characteristic for saturated FAMEs, *i.e. m/z* 74, 87 and weak ions at $[M]^+$, together with diagnostic ions at $[M–MeO]^+$ and $[M–C_3H_7]^+$.

Further, the PUFAs were not conjugated, as shown by the UV spectra (see Section 3). The double-bond stereochemistry was established by FTIR. All double bonds were *Z* because the IR spectrum of the PUFAs exhibited absorption at 723 cm^{-1} and no absorption in the 960–980 cm^{-1} region (Doumenq et al., 1990).

Two hypotheses are generally accepted as regards the biosynthesis of VLCPUFAs (Leblond and Chapman, 2000). One of them, which is more broadly accepted, presumes that the biosynthesis of VLCPUFAs occurs by chain elongation with a concomitant desaturation of the well-known and ubiquitous C18 acids, *i.e.* linoleic (18:2n-6) and linolenic (18:3n-3) (Mansour et al., 1999a; Rezanka, 2000).

The other hypothesis presumes that chain elongation occurs only in the saturated precursor, usually stearic acid, yielding an appropriate acid, *e.g.* octacosanoic (28:0), which is then desaturated to polyenoic acids (Leblond and Chapman, 2000). The common feature of both hypotheses is that, save for a single exception (Mansour et al., 2005), no intermediates, which would be represented in the first hypothesis by, *e.g.*, 24:6n-3 or 26:5n-3 and in the second by, *e.g.*, 26:0 or 28:0, have been found.

By using double sample enrichment by Ag^+–TLC and subsequent LC–MS/APCI we succeeded in identifying more than 30 VLCPUFAs. The data in Fig. 6a and b indicate that the biosynthesis of VLCPUFAs, which considers linoleic and linolenic acids as precursors, in our strain of *A. carterae* proceeds according to the former hypothesis. Interestingly, up to C_{28} the biosynthetic pathways are complex and branched whereas for longer-chain VLCPUFAs, from C_{30} to C_{36} they feature only a single route, *i.e.* 28:7→30:7→32:7→34:7→36:7. We assume that the absence of C30–C36 PUFAs with 3–6 double bonds can be explained either as being due to their low concentration falling below the detection limit, or to their low fluidity which makes their biosynthesis undesirable for the cell.

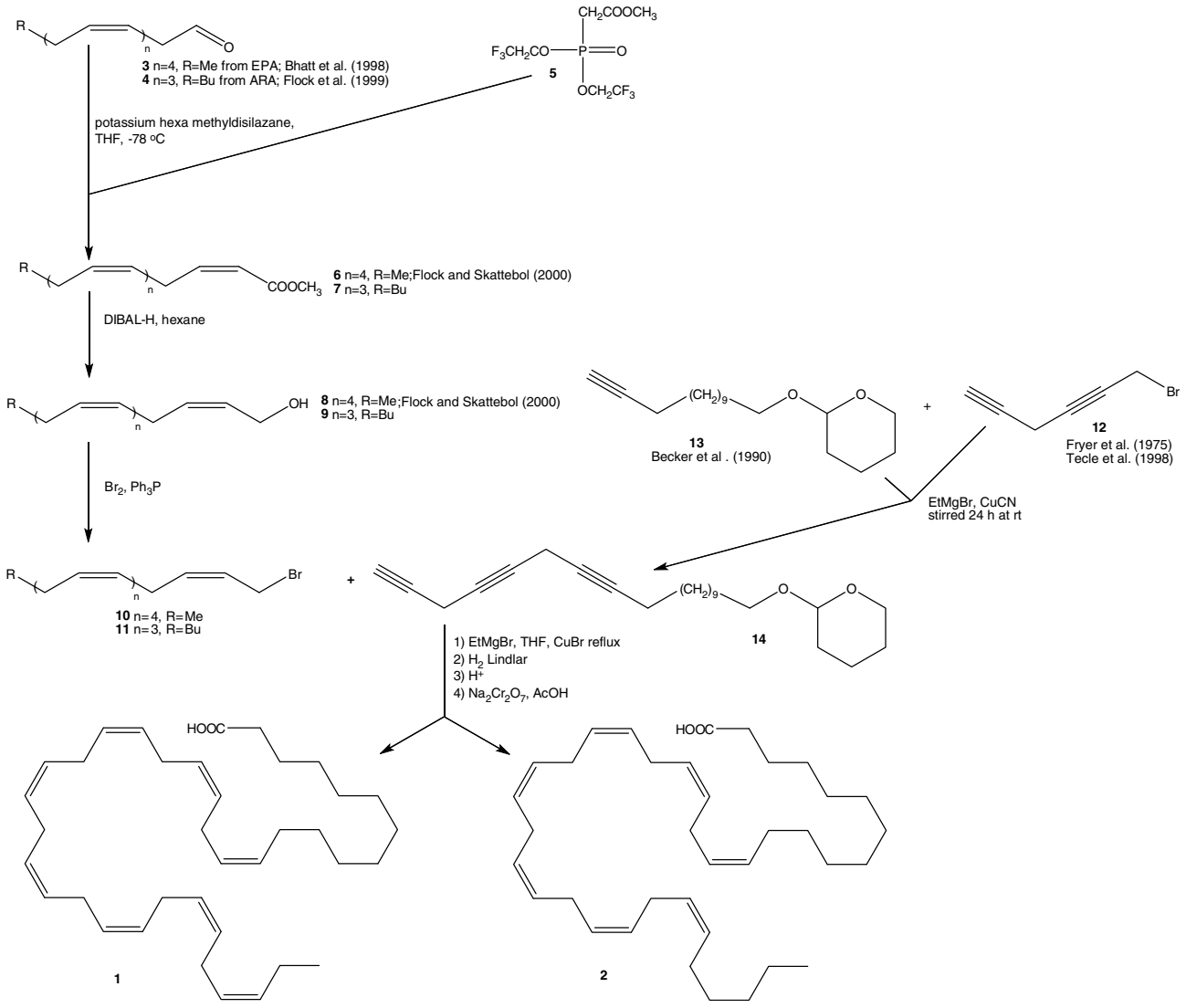


Fig. 3. Synthesis of 36:7n-6 and 36:8n-3 acids.

The combination of Ag^+–TLC and microbore LC–MS thus extends the analytical possibilities of both methods and contributes to the acquisition of new information on biological materials.

3. Experimental

3.1. Instrumental methods

UV spectra were measured by a Cary 118 (Varian) apparatus in MeOH in the range 200–350 nm. A Perkin–Elmer Model 1310 (Perkin–Elmer, Norwalk, CT) infrared spectrophotometer was used for scanning infrared spectroscopy of methyl esters as neat film. NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz (^{1}H) and 125.7 MHz (^{13}C). The fatty acids and other compounds were purchased from Sigma–Aldrich (Prague, Czech Republic). GC–MS of a FAME mixture was done on a Finnigan 1020 B in EI mode. The hydrogenated sample was injected onto a 25 m × 0.25 mm × 0.1 μm Ultra-1 capillary column (Supelco, Czech Republic) (injector and detector temperatures of 300 °C) under a temperature program of: 5 min at 50 °C, increasing at 10 °C min^{-1} to 320 °C and 15 min at 320 °C. Helium was the carrier gas at a flow of 0.52 ml min^{-1}. All spectra were scanned within the range *m/z* 50–700.

HPLC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Hewlett Packard, USA) and two Hichrom columns HIRPB-250AM 250 × 2.1 mm ID, 5 μm phase particle, in series. This setup provided us with a high-efficiency column – approximately ~26,000 plates/250 mm. A quadrupole mass spectrometer system Navigator (Finnigan MAT, San Jose, CA, USA) was used for analysis. The instrument was fitted with an atmospheric pressure chemical ionization source (vaporizer temperature 400 °C, capillary heater temperature 220 °C, corona current 5 μA, sheath gas–high-purity nitrogen, pressure 0.38 MPa, and auxiliary gas (also nitrogen) flow rate 15 ml/min. Positively charged ions with *m/z* 50–700 were scanned with a scan time of 0.5 s. The whole HPLC flow (0.37 ml/min) was introduced into the APCI source without any splitting. Fatty acid picolinyl esters were separated using a gradient solvent program with acetonitrile (ACN), dichloromethane (DCM) and propionitrile (EtCN) as follows: initial ACN/EtCN/DCM (60:30:10, vol/vol/vol); linear from 5 min to 50 min ACN/EtCN/DCM 30:40:30, vol/vol/vol); held until 60.5 min; the composition was returned to the initial conditions over 8 min. A peak threshold of 0.3% intensity was applied to the mass spectra. Data acquisition and analyses were performed using PC with MassLab 2.0 for Windows XP applications/operating software.

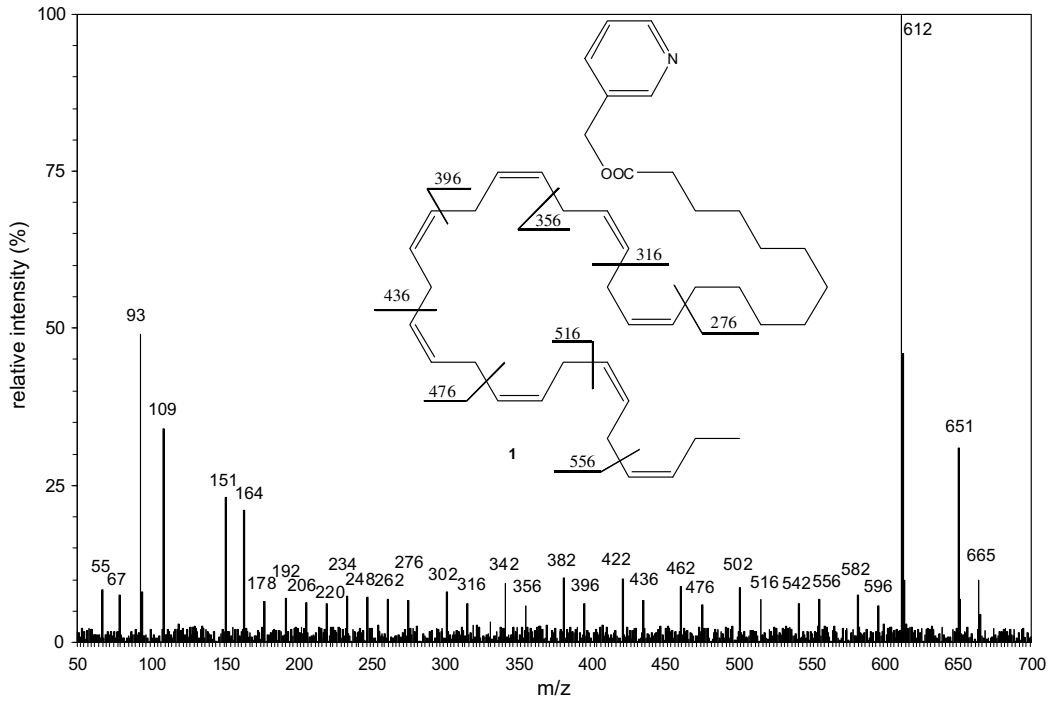


Fig. 4. Mass spectrum of picolinyl ester of 36:8n-3 acid. For explanation of values see the text.

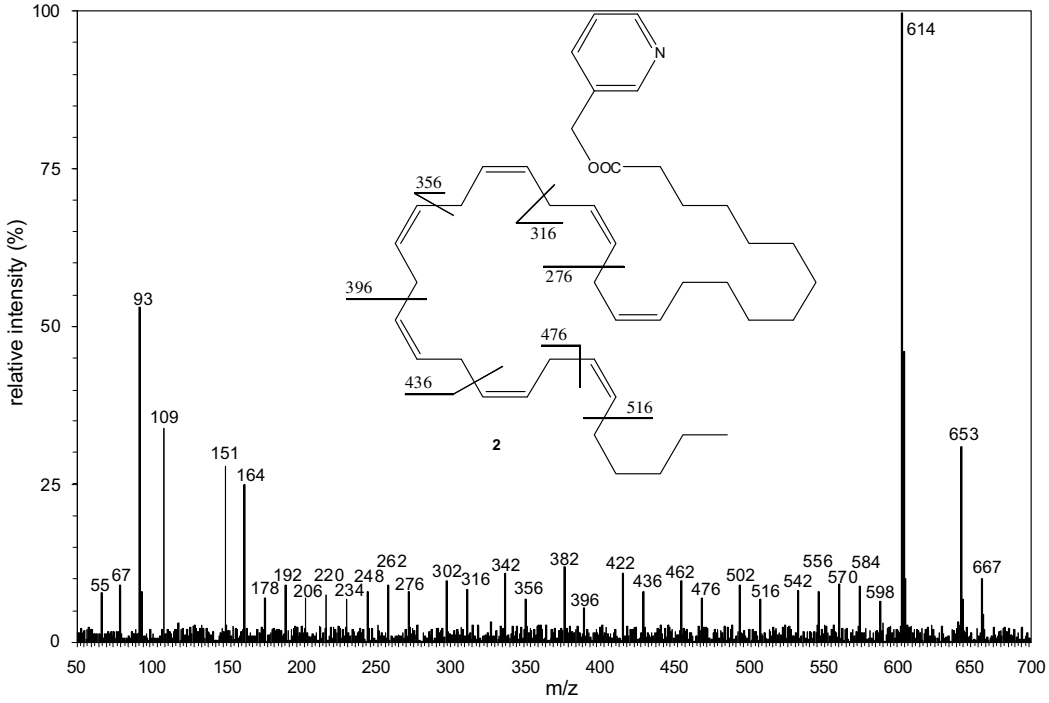


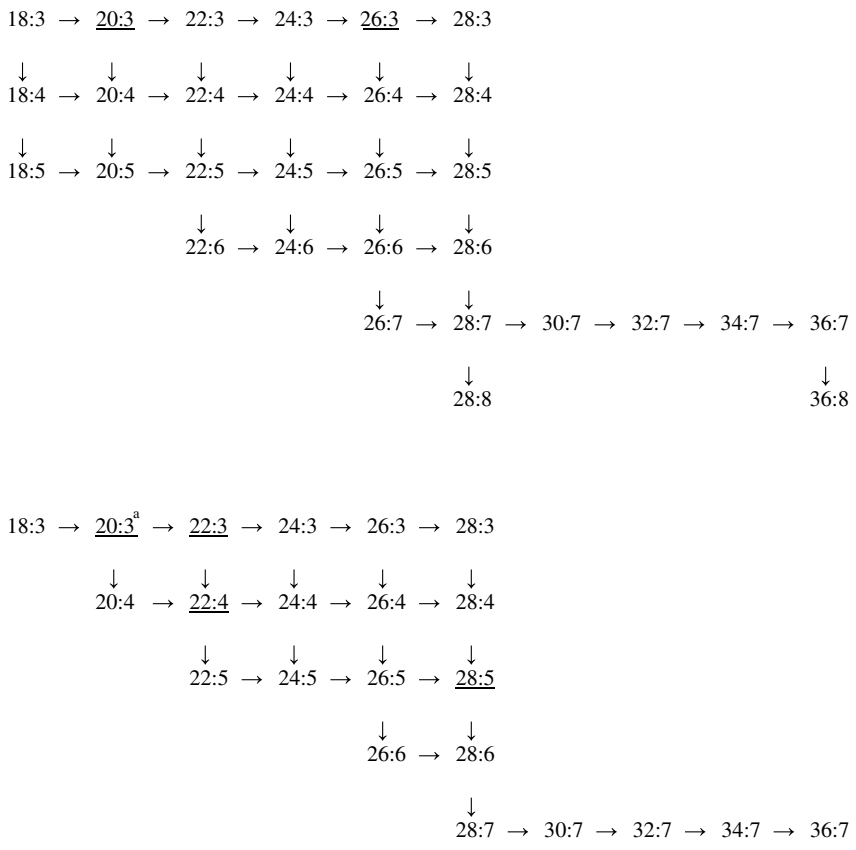
Fig. 5. Mass spectrum of picolinyl ester of 36:7n-6 acid. For explanation of values see the text.

3.2. Standards, cultivation, isolation and synthesis

Standards of fatty acids were prepared as described below. All solvents were double-distilled and degassed before use.

The strain of the dinoflagellate *A. carterae* was obtained from the Culture Collection of Algae and Protozoa in Scotland (strain CCAP 1102/3, isolated from a ditch in Essen, England). The alga was cultivated in liquid K minimum medium for marine dinoflagellates (Keller et al., 1987) at 10 °C under continuous light provided by cool white fluorescent lights. After 5 weeks, the culture was harvested by centrifugation at 1500 rpm for 10 min.

The cells of *A. carterae* (195 mg) were extracted with 3 × 30 ml of $CHCl_3$–MeOH (2:1), yielding total lipids (6.8 mg), which were dissolved in toluene (1 ml) and 1% sulfuric acid in methanol (2 ml) was added. The mixture was left overnight in a stoppered tube at 50 °C, then water (5 ml) containing sodium chloride (5%) was added and the esters were extracted with hexane (2 × 5 ml). The hexane layer was washed with water (4 ml) containing potas-



[a] Underlined acids were not identified in this study.

Fig. 6. Hypothesis of VLCPUFAs biosynthesis. (a) Biosynthesis of n-3 PUFAs. (b) Biosynthesis of n-6 PUFAs.

sium bicarbonate (2%), dried over anhydrous sodium sulfate, filtered and the solvent was removed under reduced pressure.

3.2.1. Ag^+–TLC

The FAMEs were fractionated by Ag+–TLC using petroleum ether–diethyl ether–acetic acid (90:8:2, v/v) for first separation and (70:28:2, v/v) for second separation, see above. Detection was done with 0.2% 2,7-dichlorofluorescein (in ethanol) under UV lamp at 366 nm. The zone of silica gel corresponding to PUFA methyl esters was scraped off and FAMEs were extracted from the silica gel by hexane–diethyl ether (9:1). The FAMEs were washed with saturated NaCl and 2 M NH3 to remove Ag+ and dichlorofluorescein, respectively. The structure of FAMEs was confirmed by LC–MS as described above.

3.2.2. Picolinyl esters of PUFA

A solution of potassium *tert*-butoxide in tetrahydrofuran (0.5 ml, 1.0 M) was added to nicotinyl alcohol (1 ml). After mixing, the appropriate fatty acid methyl esters (~0.5 mg) in dry dichloromethane (1 ml) were added, and the mixture was held at 40 °C for 30 min in a closed vial. After cooling to room temperature, water and hexane were added, and the organic phase was collected, dried over anhydrous sodium sulfate, and evaporated.

Hydrogenation of PUFAs was carried out in 1 ml of methanol with catalytic amounts of PtO_2. FAMEs were extracted with *n*-hexane and subjected to GC–MS.

*3.3. Methyl all-(Z)-heptadeca-2,5,8,11,14-pentaenoate (**6**)*

A solution of potassium hexamethyldisilazane (0.66 M in toluene, 3.38 ml, 2.23 mmol) was added to a mixture of methyl [bis(trifluoroethyl)phosphono]acetate (**5**, 670 mg, 2.1 mmol) and 18-crown-6 (2.5 g, 9.5 mmol) in THF (25 ml) at −78 °C, and the mixture was left stirring for 10 min. A solution of the aldehyde **3** (Bhatt et al., 1998) (414 mg, 1.90 mmol) in THF (5 ml) was then added. After 45 min at −78 °C, the mixture was allowed to attain room temperature. Saturated aqueous NH_4Cl was added and the aqueous phase extracted with ether. The combined organic extracts were washed with water, brine, and dried. The solution was concentrated under reduced pressure and the residue purified by flash chromatography (silica gel, 95:5 hexane–EtOAc) to give the ester **6** (432 mg, 82%), >97% stereochemically pure. IR ν_{max} (film) 3020, 2970, 2930, 1730, 1650, 1440, 1200, 1165 cm^{-1}; ^1H NMR δ 0.92 (*t*, *J* = 7.4 Hz, 3H), 2.05 (*m*, 2H), 2.70–2.90 (*m*, 8H), 3.67 (*s*, 3H), 5.20–5.50 (*m*, 8H), 5.75 (*m*, 1H), 6.15 (*m*, 1H); ^{13}C NMR δ 14.3 *q*, 21.7 *t*, 28.5–30.5 (4 × *t*), 51.8 *q*, 126.0–133.0 (9 × *d*), 145.4 *d*, 166.5 *s*; HREIMS *m*/*z* calcd. for 274.1933 $C_{18}H_{26}O_2$ $[M]^+$, found 274.1938.

*3.4. Methyl all-(Z)-heptadeca-2,5,8,11-tetraenoate (**7**)*

This compound was prepared from the aldehyde **4** (Flock et al., 1999) (462 mg, 2.12 mmol) and methyl [bis(trifluoroethyl)phosphono]acetate **5** (700 mg, 2.2 mmol) as described for the synthesis of the methyl ester **6**, and was obtained as yellow oil (438 mg, 75%), >98% stereochemically pure. IR ν_{max} (film) 3020, 2970, 2930, 1730, 1650, 1440, 1200, 1165 cm^{-1}; ^1H NMR δ 0.91 (*t*, *J* = 7.4 Hz, 3H), 1.25–1.35 (*m*, 6H), 2.07 (*m*, 2H), 2.65–2.90 (*m*, 6H), 3.64 (*s*, 3H), 5.20–5.50 (*m*, 6H), 5.75 (*m*, 1H), 6.15 (*m*, 1H); ^{13}C NMR δ 14.7 *q*, 22.0 *t*, 25.5–30.5 (6 × *d*), 51.8 *q*, 126.0–133.0 (7 × *d*), 145.4 *d*, 166.5 *s*; HREIMS *m*/*z* calcd. for 276.2089 $C_{18}H_{28}O_2$ $[M]^+$, found 276.2093.

3.5. all-(Z)-Heptadeca-2,5,8,11,14-pentaenol (**8**)

A solution of DIBAL-H (diisobutylaluminium hydride, 1 M cyclohexane solution, 2.7 ml, 2.7 mmol) was added to a stirred solution of the ester **6** (428 mg, 1.55 mmol) in dry hexane (20 ml) at 0 °C. After 2 h at 0 °C, HCl (1.4 M, 18 ml) was added and the aqueous phase extracted with ether. The combined organic extracts were washed with water, brine and dried (Na_2SO_4). Evaporation of solvents followed by flash chromatography (silica gel, 8:2 hexane–EtOAc) gave the alcohol **8** (337 mg, 88%). IR ν_{max} (film) 3600, 3340, 3015, 2965, 2930, 1435 cm^{-1}; ^1H NMR δ 0.90 (*t*, *J* = 7.5 Hz, 3H), 2.05 (*m*, 2H), 2.72–2.93 (*m*, 8H), 4.20 (*d*, *J* = 6.2 Hz, 2H), 5.23–5.47 (*m*, 8H), 5.48–5.67 (*m*, 2H); ^{13}C NMR δ 14.2 *q*, 20.5 *t*, 25.5–28.5 (4 × *t*), 58.4 *t*, 127.0–132.0 (10 × *d*); HREIMS *m*/*z* calcd. for 246.1984 $C_{17}H_{26}O$ $[M]^+$, found 246.1986.

3.6. all-(Z)-Heptadeca-2,5,8,11-tetraen-1-ol (**9**)

This compound was prepared from the methyl ester **7** (423 mg, 1.52 mmol) as described for the synthesis of the alcohol **8**, was obtained as yellow oil (329 mg, 86%). IR ν_{max} (film) 3600, 3350, 3020, 2960, 2930, 1440 cm^{-1}; ^1H NMR δ 0.89 (*t*, *J* = 7.4 Hz, 3H), 1.25–1.35 (*m*, 6H), 2.05 (*m*, 2H), 2.72–2.93 (*m*, 6H), 4.02 (*d*, *J* = 5.8 Hz, 2H), 5.23–5.47 (*m*, 7H), 5.48–5.67 (*m*, 1H); ^{13}C NMR δ 14.2 *q*, 22.4 *t*, 25.5–30.5 (6 × *t*), 58.4 *t*, 127.0–132.0 (8 × *d*). MS (EI): *m*/*z* 286 (M^+) (0.1%), 268, 79 (100); MS (EI): HREIMS *m*/*z* calcd. for 248.2140 $C_{17}H_{28}O$ $[M]^+$, found 248.2144. patent 4670465.

3.7. all-(Z)-1-Bromoheptadeca-2,5,8,11,14-pentaene (**10**) (Flock et al., 1999)

Bromine (211 mg, 67 μl, 1.32 mmol) was added dropwise to an ice-cooled suspension of Ph_3P (480 mg, 1.56 mmol) in acetonitrile (5 ml), followed by a solution of the alcohol **8** (322 mg, 1.3 mmol) and pyridine (2 ml) in acetonitrile (1 ml). After 1 h, hexane was added. The mixture was filtered and the filtrate concentrated under reduced pressure. The residue was passed through a short pad of silica gel which was subsequently rinsed with hexane and the bromide was obtained in 84% (341 mg) yield. IR (film): 3015, 2965, 2930, 1460, 1320, 1210, 615 cm^{-1}; ^1H NMR δ 0.91 (*t*, *J* = 7.5 Hz, 3H), 2.08 (*m*, 2H), 2.70–2.95 (*m*, 8H), 4.02 (*d*, *J* = 6.2 Hz, 2H), 5.20–5.45 (*m*, 8H), 5.48–5.65 (*m*, 2H);^{13}C NMR δ 14.2 *q*, 20.5 *t*, 25.5–28.5 (4 × *t*), 30.5 *t*, 127.0–132.0 (10 × *d*); HREIMS *m*/*z* calcd. for 308.1140 $C_{17}H_{25}{}^{35}Br$ $[M]^+$, found 308.1145; EIMS *m*/*z* 308 (100), 309 (18.7), 310 (98.6), 311 (18.3%), 312 (1.6%).

3.8. all-(Z)-1-Bromoheptadeca-2,5,8,11-tetraene (**11**)

This compound was prepared from the alcohol **9** (313 mg, 1.25 mmol) as described for the synthesis of the bromide **10**, and was obtained as yellow oil (338 mg, 86%). IR (film): 3020, 2960, 2930, 1460, 1320, 1210, 615 cm^{-1}; ^1H NMR δ 0.89 (*t*, *J* = 7.4 Hz, 3H), 1.25–1.35 (*m*, 6H), 2.05 (*m*, 2H), 2.72–2.93 (*m*, 6H), 4.02 (*d*, *J* = 5.8 Hz, 2H), 5.23-5.47 (*m*, 7H), 5.48–5.67 (*m*, 1H); ^{13}C NMR δ 14.2 *q*, 22.4 *t*, 25.5–30.5 (6 × *t*), 30.4 *t*, 127.0–132.0 (8 × *d*); HREIMS *m*/*z* calcd. for 310.1296 $C_{17}H_{27}Br$ $[M]^+$, found 310.1300; EIMS *m*/*z* 310 (100), 311 (18.4), 312 (98.5), 313 (18.2), 314 (1.5).

3.9. 2-(Nonadeca-12,15,18-triynyloxy)tetrahydro-2H-pyran (**14**)

The compound was prepared according to the modified procedures described by Fryer et al. (1975) and Kunau (1971).

To a solution of 672 mg (2.4 mmol) of **13** (prepared according to Becker et al., 1990) in 4 ml of THF at 3 °C was added dropwise a Grignard reagent prepared from ethylbromide (436 mg, 4 mmol) in THF (3 ml) and magnesium turnings (101 mg, 4.2 mmol). The mixture was stirred at room temperature for 1.5 h and 10 mg of CuCN was added. After stirring for 20 min, 1-bromo-2,5-hexadiyne (**12**) (prepared according to Tecle et al., 1998) (382 mg, 2.43 mmol) was added and rinsed in with 2 ml of THF. The mixture was then stirred for 18 h with an additional 10 mg of CuCN added after 6 h. After stirring, the mixture was poured into 5 ml of 1 M H_2SO_4 and 10 g of ice. After extraction with ether, the organic phase was concentrated *in vacuo*. The residue was dissolved in ether and the ether solution washed with water several times to remove copper, dried, and concentrated to yield brown oil (487 mg, 57%). IR (neat) 3270, 1125, 1022 cm^{-1}; ^1H NMR δ 1.21–1.53 (*m*, 24H, H-2 - H-10, H-2́- H-4́), 2.03 (*t*, 1H, *J* = 2.6, H-19), 2.10 (*dt*, 2H, 2.5, 7.4, H-11), 3.25 (*m*, 2H, H-17), 3.35 (*m*, 1H, H-1), 3.41 (*m*, 2H, H-14), 3.44 (*m*, 1H, H-1), 3.60 (*m*, 2H, H-5́), 4.33 (*dd*, 1H, *J* = 11.2, 2.1, H-1́); ^{13}C NMR δ 10.5 (C-17), 10.9 (C-14), 18.6 (C-3́), 18.7 (C-11), 25.2 (C-4́), 26.3-29.4 (C-2–C-10), 29.6 (C-2́), 62.1 (C-5́), 66.4 (C-1), 68.8 (C-19), 78.0 (C-16), 78.2 (C-15), 79.2 (C-18), 80.2 (C-13), 80.9 (C-12), 97.4 (C-1́); HREIMS *m*/*z* calcd. for 356.2715 $C_{24}H_{36}O_2$ $[M]^+$, found 356.2717.

3.10. all-(Z)-Hexatriaconta-12,15,18,21,24,27,30,33-octaenoic acid (**1**) (Heitz et al., 1989)

To an EtMgBr solution (5 ml of a 2 M solution in THF, 10.0 mmol) at 0 °C was added dropwise a solution of **14** (249 mg, 0.7 mmol) in dry THF (3 ml). The reaction mixture was warmed up to room temperature over 10 min and then heated under reflux for 10 min. After cooling at 0 °C, copper bromide (143 mg, 1 mmol) was added, followed by dropwise addition of bromide **10** (218 mg, 0.7 mmol) in dry THF (3 ml). The resulting reaction mixture was heated under reflux for 12 h, then cooled to room temperature, quenched by addition of water, and filtered. The reaction mixture was extracted with ether, and the combined organic extracts were washed with brine, dried, and concentrated. Purification of the crude product (2́-((all-*Z*)-hexatriaconta-21,24,27,30,33-pentaen-12,15,18-triynyloxy)tetrahydro-2*H*-pyran) on silica gel (hexane-diethylether 95:5) gave 205 mg (44%) of a colorless oil, HREIMS *m*/*z* calcd. for 584.4593 $C_{41}H_{60}O_2$ $[M]^+$, found 584.4600.

P-2 nickel was prepared via $NaBH_4$ reduction of $Ni(OAc)_2.4H_2O$ (147 mg, 0.4 mmol) in absolute ethanol (10 ml). The flask was purged with hydrogen, and ethylenediamine (100 μl, 90 mg, 1.5 mmol) was added followed by a derivative of pyran (199 mg, 0.34 mmol) in absolute ethanol (1 ml). Hydrogen uptake was quantitative in 4 h. The reaction mixture was filtered, diluted with water, and extracted with diethylether. The combined ether extracts were washed with water, cold 1 M HCl, brine, dried, and concentrated to give of crude product as brown colored oil. Chromatography on preparative TLC plates, using diethylether-hexane (15:85), gave 174 mg (84%) of polyene (2́-((all-*Z*)-hexatriaconta-12,15,18,21,24,27,30,33-octaenyloxy)tetrahydro-2*H*-pyran) as pale yellow colored oil. HREIMS *m*/*z* calcd. for 590.5063 $C_{41}H_{66}O_2$ $[M]^+$, found 590.5067.

A solution of polyene THP ether (169 mg, 0.29 mmol) and PPTS (pyridinium p-toluenesulfonate) (6.3 mg, 0.025 mmol) in ethanol (4 ml) was stirred at 45 °C (bath temperature) for 10 h (Miyashita et al., 1977). The solvent was evaporated *in vacuo*, and the residue was chromatographed on preparative TLC, using diethylether-hexane (1:3) to afford pure alcohol ((all-*Z*)-hexatriaconta-12,15,18,21,24,27,30,33-octaen-1-ol) (143 mg, 99%). HREIMS *m*/*z* calcd. for 506.4488 $C_{36}H_{58}O$ $[M]^+$, found 506.4488.

To a solution of polyene alcohol (137 mg, 0.27 mmol) in 10 ml acetone was added under stirring at −2 °C in 30 min a solution of $Na_2Cr_2O_7$. 2 H_2O (74 mg, 0.27 mmol) in 0.1 ml conc. sulfuric acid and 1 ml water (Kunau, 1971). The mixture was stirred for another 2 h at 0 °C and, subsequently, poured into 10 ml of ice water. After the acetone had been completely evaporated under reduced pres-

sure, the aqueous phase was thoroughly extracted with ether. The ether was washed neutral with water, dried, and evaporated, leaving a crude product. After purification by TLC on silica gel (diethylether-hexane, 1:2) the yield was 88.7 mg (63%) of acid **1** as colorless oil, purity 98% (GLC as methyl ester). IR (neat) 3550–2550 (br OH), 1710 (C=O) cm^{-1}; ^1H NMR δ 0.87 (*t*, 3H, CH_3), 1.22–1.32 (*brm*, 14H, CH_2), 1.69 (*t*, 2H, CH_2CH_2COOH), 2.09 (*m*, 2H, $=CHCH_2CH_2$), 2.02 (*m*, 2H, $CH_3CH_2CH=$), 2.35 (*t*, 2H, CH_2COOH), 2.81 (*brm*, 14H, $=CHCH_2CH=$), 5.35 (*m*, 16H, *CH*=), 10.5 (*brs*, 1H, COO*H*). ^{13}C NMR ($CDCl_3$, 125.7 MHz) δ 14.2 (*q*, C-36); 20.6 (*t*, C-35), 24.6 (*t*, C-3), 25.2–31.8 (*t*, 8 × C, C-4–C-11), 25.4–25.8 (*t*, 7× $=CH–CH_2–CH=$), 33.5 (*t*, C-2), 126.9–128.7 (*d*, 14×C), 129.1 (*d*, C-12), 131.9 (*d*, C-34), 179.6 (*s*, C-1, COOH); EIMS of methyl ester *m/z* 520 (M^+, 0.2), 488 (M-32, 1.2), 278 α-ion (5), 269 (5), 241 (6), 215 (7), 159 (15), 145 (19), 119 (36), 108 ω-ion (48), 105 (45), 91 (89), 79 (100), 67 (61), 55 (37); HREIMS *m/z* calcd. for 520.4280 $C_{36}H_{56}O_2$ $[M]^+$, found 520.4284.

3.11. all-(Z)-Hexatriaconta-12,15,18,21,24,27,30-heptaenoic acid (*2*)

This compound, prepared from the bromide (**11**) (200 mg, 0.64 mmol) and triyne **14** (228 mg, 0.64 mmol) following multistep synthesis as described for the synthesis of the acid **1**, was obtained (after purification by TLC on silica gel–diethylether–hexane, 1:2) as colorless oil (85.8 mg, 26%); purity 98% (GLC as methyl ester). IR (neat) 3550–2550 (br OH), 1710 (C=O) cm^{-1}; ^1H NMR δ 0.87 (*t*, 3H, CH_3), 1.22–1.32 (*brm*, 22H, CH_2), 1.69 (*t*, 2H, CH_2CH_2COOH), 2.09 (*m*, 2H, $=CHCH_2CH_2$), 2.35 (*t*, 2H, CH_2COOH), 2.81 (*brm*, 12H, $=CHCH_2CH=$), 5.35 (*m*, 14H, *CH*=), 10.5 (*brs*, 1H, COO*H*); ^{13}C NMR ($CDCl_3$, 125.7 MHz) δ 14.2 (*q*, C-36); 22.6 (*t*, C-35), 24.6 (*t*, C-3), 25.2-31.8 (*t*, 11 × C, C-4–C-11, C-32–C-34), 25.4–25.8 (*t*, 6× $=CH–CH_2–CH=$), 33.5 (*t*, C-2), 126.9–128.7 (*d*, 12 × C), 129.1 (*d*, C-12), 131.9 (*d*, C-31), 179.6 (*s*, C-1, COOH); EIMS of methyl ester *m/z* 522 (M^+, 0.3), 490 (M-32, 1.1), 278 α-ion (7), 269 (4), 241 (5), 215 (8), 159 (13), 150 ω-ion (22), 145 (18), 119 (32), 105 (47), 91 (85), 79 (100), 67 (64), 55 (35); HREIMS *m/z* calcd. for 522.4737 $C_{36}H_{58}O_2$ $[M]^+$, found 522.4740.

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