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Tetrahedron
Tetrahedron 60 (2004) 4261–4264

Tetratriacontanonaenoic acid, first natural acid with nine double bonds isolated from a crustacean *Bathynella natans*

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Received 12 January 2004; revised 17 February 2004; accepted 11 March 2004

Abstract—The very-long-chain polyunsaturated fatty acid—all*Z*-4,7,10,13,16,19,22,25,28-tetratriacontanonaenoic acid was determined and identified in the freshwater crustacean species *Bathynella natans* living in caves of central Europe by means of liquid chromatography—mass spectrometry with atmospheric pressure chemical ionization. The full structure was elucidated by using extensive spectroscopic analysis (^{1}H and ^{13}C NMR, MS, IR and UV) including a chemical method. This acid was described in nature for the first time. A hypothesis is suggested for its origin and biosynthesis.

1. Introduction

As part of our scientific program, whose main goal is to discover new and unusual compounds from the flora and fauna worldwide, we have screened a number of animals collected from caves. Among these, *Bathynella natans* was chosen for detailed chemical investigation,[1,2] due to its previously discovered content of very long chain polyunsaturated fatty acids (VLCPUFA). This species belongs to the family Bathynellacea, which contains about 70 genera distributed through all geographic regions. The crustacean species belonging to the Syncacarides are characterized by a primitive organization and ancient origin. They live in alpine lakes, rivers and groundwater, i.e. in the depths of limestone caves. In 1886, the Vejdovsky discovered,[3] the first Bathynellacea, *B. natans*, in Prague. In the dry summer of 2003, when even groundwater level decreased deep below its normal level, we obtained more individuals of *B. natans*, which were then used for analyses.

VLCPUFA were recently characterized in different plants and/or animals, mainly in aquatic, e.g. Baltic herring[4] or dinoflagellates.[5] In this study, we continue our reinvestigation of the fatty acid composition of freshwater crustaceans collected in the caves near Prague.

2. Results and discussion

The total lipid fraction of *B. natans* was analyzed by two-dimensional TLC in order to determine its lipid components. One unidentified lipid, designated X, was detected. Lipid X gave a positive reaction (blue spot) with molybdate-based Zinzade's reagent and Dragendorff stain gave a dark orange spot. On the basis of this coloring, we assumed that lipid X was diacyl phosphatidyl choline (PC). This assumption was confirmed by further analysis. The APCI mass spectrum gave a pseudomolecular ion $[M+H]^+$ at *m/z* 994 as the major peak, and minor fragments at *m/z* 968, 996 and 966 components. Fragment ions derived from the peak at *m/z* 994 appeared at *m/z* 549, 729 and 812, corresponding to $[M+H-R_1CO]^+$, $[M+H-R_2CO]^+$ and $[M+H-\text{choline}]^+$. A further ion at *m/z* 184 (choline+H) confirmed the structure of lipid X as tetratriacontanonaenoyl-oleoyl phosphatidyl choline. The minor molecular species showing peaks at *m/z* 968, 996 and 966 were found to be phosphatidyl choline with acyls 16:0 or 18:0 or 16:1 and tetratriacontanonaenoyl. Lipid X was then transesterified and five FAMEs (fatty acid methyl esters) were obtained.

The FAMEs were separated according to the degree of unsaturation on plates impregnated with $AgNO_3$. The lowest spot was extracted and the mass spectrum was measured by the APCI technique, see Figure 1. The APCI mass spectrum of VLCPUFA had characteristic ions consistent with 34:9 FAME. In contrast to previously published spectra,[6,7] this spectrum exhibited a peculiar feature. The pseudomolecular ion $[M+42]^+$ is conventionally taken to be mainly characteristic for PUFA, but in fact it

Keywords: *Bathynella natans*; Freshwater crustacean; LC-MS/APCI; Tetratriacontanonaenoic acid.
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doi:10.1016/j.tet.2004.03.032

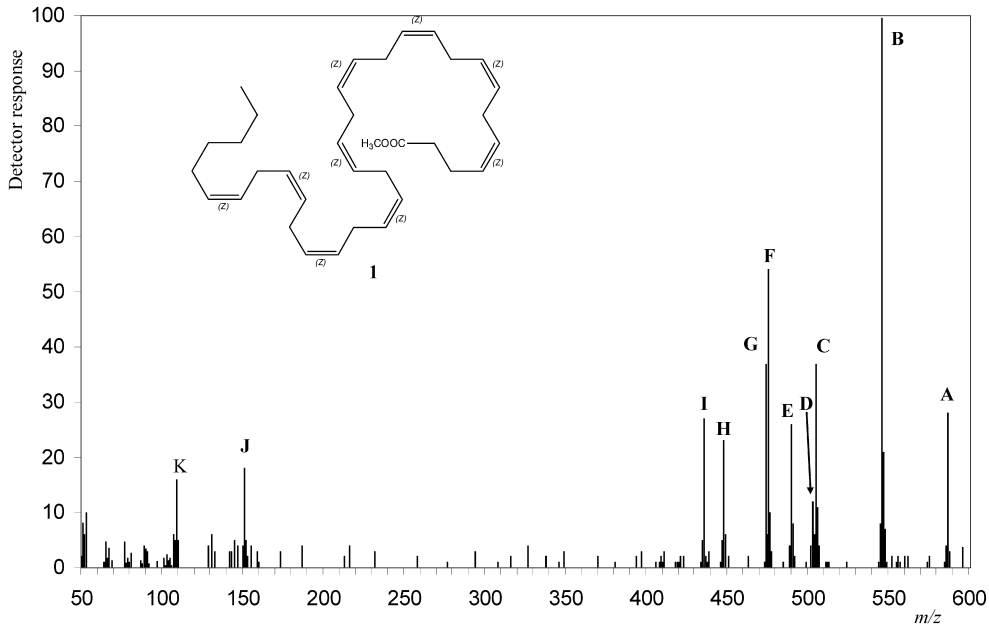

Figure 1. LC–MS/APCI of 34:9ω6 FAME from *Bathynella natans*. Ions (abundance): (A) $[M+H+2\times CH_3CN]^+$, *m/z* 587 (28); (B) $[M+H+CH_3CN]^+$, *m/z* 546 (100); (C) $[M+H]^+$, *m/z* 505 (37); (D) $[M-H[^+$, *m/z* 503 (12); (E) $[M+H-CH_3]^+$, *m/z* 490 (26); (F) $[M+H-C_2H_5]^+$, *m/z* 476 (54); (G) $[M+H-CH_3O]^+$, *m/z* 474 (37); (H) $[M+H-C_4H_9]^+$, *m/z* 448 (23); (I) $[M+H-C_5H_9]^+$, *m/z* 436 (27); (J) *m/z* 151 (18) (diagnostic ion, see text); (K) *m/z* 109 (16) (diagnostic ion, see text).

also arises by the addition of acetonitrile from the mobile phase. We were able for the first time to identify a pseudomolecular ion arising by the double addition of acetonitrile on polyenoic fatty acid. This effect is probably due to the unusual numbers of methylene-interrupted double bonds.

However, the determination of position of double bonds was unsuccessful on the basis of the cleavage of nitrogen derivatives (i.e., 4,4-dimethyloxazoline and/or picolinyl ester; data not shown). Nevertheless, this method was described as successful either for substances with equal-length chain, but having only one double bond,[6] or for shorter-chain compounds having a maximum of eight double bonds and only 28 carbon atoms.[5] In our case, this technique failed, or its results were ambiguous. Therefore, we used the method[5,6,8] based on the ratios of ions at *m/z* 109 and 151. The *m/z* 109/151 ratio is near unity and hence the presumed structure is ω-6.

We used the following methods for the full confirmation of the above structure, in particular for double bond location. After hydrogenation of the compound from the lowest spot, only one peak was seen in the gas chromatogram with an ECL value of 34:0, indicating a chain length of 34 carbon atoms without branching. The EI mass spectrum of this peak contained fragment ions characteristic for saturated FAME, i.e. *m/z* 74, 87 and weak ions at *m/z* 522 $[M]^+$, together with diagnostic ions at *m/z* 491 (M−MeO) and *m/z* 479 ($M-C_3H_7$). Further, the PUFA was 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 PUFA exhibited absorption at 723 cm^{-1} and no absorption in the 960–980 cm^{-1} region.[9] This stereochemistry was further confirmed by ^{13}C NMR, since the allylic carbons (C-6, C-9, C-12, C-15, C-18, C-21, C-24 and C-27), resonated[10] between 26 and 27 ppm. All our spectral data point to all*Z*-4,7,10,13,16,19,22,25,28-tetratriacontanonaenoic acid (**1**) as the unknown acid.

The toxicity of compound **1** was tested in the *Artemia salina* shrimp bioassay (see Table 1). Table 1 also indicates that compound **1** was active only against Gram-positive bacteria but not against Gram-negative bacteria and yeast. This compound is now studied in view of its further potential by high physiological activity.

Table 1. Bioactivities of fatty acid (**1**)

Test organism	**1**[a]
Staphylococcus aureus[b]	78.1±2.00
Bacillus subtilis[b]	95.6±2.81
Escherichia coli[b]	0.0±0.00
Saccharomyces cerevisiae[b]	0.1±0.02
Artemia salina[c,d]	1.4±0.08

[a] Presented as mean±SE (*n*=10).
[b] Sample (10 μg) was applied on 6.3 mm paper disks; values are diameters (mm) of inhibitory zones.
[c] In μg/ml (minimum lethal doses).
[d] The details are in Section 3.

The origin of **1** is most likely bacterial or algal (the diets of the crustacean) and gives rise to interesting biosynthetic considerations, in particular with regard to the order of double-bond introduction, relative to other lower organisms.[5]

Conventional theory suggests successive chain elongation and desaturation of 22:5(ω-6) as a possible mechanism for the biosynthesis of this VLCPUFA. Alternatively, 34:9(ω-6) could be formed by a different mechanism but no information is available in this respect. Further research of the pathways of polyunsaturated fatty acid biosynthesis in marine organisms is needed.

In summary, a new metabolite has been isolated from *B. natans*. The main novelty of this compound is the presence of nine non-methylene interrupted double bonds in its molecule. To our knowledge, this is the first time that a fatty acid with nine double bonds, e.g. tetratriacontanonaenoic acid, is reported as a natural or synthetic product.

3. Experimental

3.1. General

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 positive-ion MS/ESI spectrum was recorded on a VG 7070E-HF spectrometer (Micromass, Manchester, UK). The stearic, oleic, palmitic, palmitoleic, cerotic, montanic and melissic acids were purchased from Sigma-Aldrich (Prague, Czech Republic).

The LC–MS/APCI was realized as mentioned previously,[6] briefly: the HP 1090 series (HP 1090 series, Hewlett Packard, USA) was used with two columns (HIRPB-250AM 250×2.1 mm ID, 5 μm phase particle). A quadruple mass spectrometer system Navigator (Finnigan MAT, San Jose, CA, USA) was used: vaporizer temperature 400 °C, capillary heater temperature 220 °C, corona current 5 μA, sheath gas high-purity nitrogen, pressure ca. 380 kPa, and auxiliary gas (also nitrogen) flow rate 1500 ml/min. Ions with *m/z* 50–1500 were scanned with a scan time of 0.5 s, flow 0.37 ml/min. FAMEs were separated using a gradient solvent program with acetonitrile–dichloromethane (90:10) and linear from 10 to 40 min acetonitrile–dichloromethane (70:30).

GC–MS of a FAME mixture was done on a Finnigan 1020 B in EI mode. Splitless injection was 100 °C, and a fused-silica capillary column (Supelcowax 10; 60 m×0.25 mm i.d., 0.25 μm film thickness; Supelco, Prague) was used. The temperature program was as follows: 100 °C for 1 min, subsequently increasing at 20 °C/min to 180 °C and at 2 °C/min to 280 °C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm/s. All spectra were scanned within the range *m/z* 70–650.

3.2. Biological material

The specimens of *B. natans*were collected in September 2003, in caves near Srbsko near Prague at a depth of between 5 and 8 m. The crustaceans were carefully cleaned and lyophilized. A voucher specimen (TR-130903/11) is available from the first author.

3.3. Extraction and isolation

The specimens of *B. natans* (250 g), extracted with 2×150 ml of $CHCl_3$–MeOH (1:1), yielded total lipids (1630 mg). These were fractionated by preparative TLC (20 cm×20 cm) using Si gel 60, and developed by two-dimensional chromatography with chloroform–methanol–formic acid–water (65:25:9:1, v/v) and chloroform–methanol–ammonia–water (50:40:3:7, v/v). Zinzade's reagent was used to specifically identify all phospholipids, while Dragendorff's reagent was used to visualize PC. For preparative purposes, the spots were visualized by spraying with fluorescein, scraped off the silica gel plate, and extracted with chloroform–methanol (1:1). The yield of lipid X was 10.2 mg.

The fatty acyl components of the unknown PC were obtained as their methyl esters by reaction of the PC with methanolic HCl followed by column chromatography including elution with *n*-hexane–diethyl ether (9:1).

Glass plates (20×20 cm) were impregnated by silver nitrate and developed[11,12] by $CHCl_3$–MeOH (100:1) in the first, and hexane–acetone (100:3) in the second dimension. The spots were sprayed with fluorescein and detected under UV light. The appropriate spots were scraped off, and compounds **1**, **2**–**3** and **4**–**5** were extracted from the silica gel by diethyl ether. Compounds **2**–**5** were identified by GC–MS as methyl esters of 16:0, 18:0, 16:1 and 18:1 acids.

Hydrogenation of compound **1** was carried out in 1 ml of methanol with catalytic amounts of PtO_2. FAME was extracted with *n*-hexane and subjected to gas chromatography.

3.3.1. Compound 1. Methyl all*Z*-4,7,10,13,16,19,22,25,28-tetratriacontanonaenoate (**1**): Colorless oil, 4.1 mg; UV λ_{max} (MeOH) (log ε) 209 (3.36) (nm); IR (neat) ν_{max} 3500 (OH), 3010 (=CH), 2950, 2930, 2870, 1710 (C=O), 1460, 1435, 1410, 1380, 1370, 1240, 940, 720 (HC=CH, *Z*) cm^{-1}; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 5.35–5.41 (m, 18H, =C–*H*), 2.70–2.75 (m, 16H, =CH–C*H*$_2$–HC=), 2.35–2.39 (m, A_2B_2, 4H, –C*H*$_2$–C*H*$_2$–COOH), 2.00 (m, 2H, –C*H*$_2$–HC=), 1.36–1.41 (m, 6H, –C*H*$_2$–C*H*$_2$–C*H*$_2$–), 0.89 (t, 3H, C*H*$_3$–); ^{13}C NMR ($CDCl_3$, 125.7 MHz) δ 179.6 (s, C-1, *C*OOH), 131.9 (d, C-29), 126.9–128.7 (d, 16×C), 129.1 (d, C-5), 33.5 (t, C-2), 31.6 (C-32), 29.4 (t, C-31), 27.3 (t, C-30), 26.6 (t, C-4), 25.4–25.8 (t, 8× =CH–C*H*$_2$–CH=), 24.6 (t, C-3), 22.6 (t, C-33), 14.2 (q, C-34); HR-MS/ESI calcd for $C_{35}H_{52}O_2Na$, 527.3870, found *m/z* 527.3865 $[M+Na]^+$.

Methyl tetratriacontanoate: GC–MS/EI (70 eV) *m/z* $[M]^+$ 522 (38), 493 (M−C_2H_5; 14), 491 (M−MeO; 13), 479 (M−C_3H_7; 18), 199 (9), 143 (50), 87 (73), 74 (100), 69 (31), 67 (28), 55 (46).

3.4. Assay for biological activity

The sample (~0.05 mg) was dissolved in 50 μl of DMSO and added to a vial of artificial seawater (3.0 ml). Approximately 20 brine shrimp, *Artemia salina*, were added to the vial and then observed periodically over a 24 h period. A positive assay was the death of all brine shrimp. The test organisms were from the Czechoslovak Collection of Microorganisms, Brno. Antibacterial assays were carried out according to the literature.[11] The amount of the compound was 10 μg per test disk (see Table 1).

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