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The occurrence and structural identification of long-chain unsaturated ketones in the deep-lake invertebrate *Acanthogammarus grewingkii*

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Twenty-two long-chain saturated and unsaturated methyl-alkylketones were identified in the deep-lake invertebrate *Acanthogammarus grewingkii* from Lake Baikal, Russia. The ketones were in the range of C_{33} to C_{40} and ranged from saturated to tetraenoic alkyl chains; all double bonds are *cis*. The majority of the ketones were isolated and identified by means of TLC, reversed-phase HPLC, MS, ^{1}H-NMR and ^{13}C-NMR. The possible routes of biosynthesis and the function of their unusual ketones are discussed.

Key words: Long chain ketones; Invertebrates; *Acanthogammarus grewingkii*; GC–MS; HPLC.

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Introduction

Saturated methyl (more rarely ethyl) ketones occur in nature in peat (Lehtonen and Ketola, 1990) and in higher plants. C_{26}–C_{31} ketones with 98% of C_{29} were identified in seed pods of cabbage (Richter and Krain, 1981).

Long-chain unsaturated methyl and ethyl ketones are rarer but, in spite of that, are found in sea sediments of different ages (Conte *et al.*, 1992). They get to the sediments from plankton that, after having died, sink to the bottom. The first identified source of these compounds was the omnipresent marine coccolithophorid *Emiliania huxleyi* (Volkman *et al.*, 1980). These ketones are also used as markers of the coccolithophorid input into the sediments (Volkman *et al.*, 1979). Except for the above-mentioned occurrence in *E. huxleyi*, they are not frequently encountered among the marine algae of the class Prymnesiophyceae (Marlowe *et al.*, 1984a). In di- and trienoic ketones, double bonds are in the positions ω-15, ω-22, and ω-29 (De Leeuw *et al.*, 1980), always in the *trans*-configuration. In tetraenoic ketones, the additional double bond is in the position ω-8 (Marlowe *et al.*, 1984b). Only one paper reports the presence of methyl and ethyl ketones with 37–40 carbon atoms and two to four double bonds in lacustrine sediments (Cranwell, 1985). In this case, the biological source could not be reliably determined. The author, nevertheless, expressed the opinion that, by analogy with the marine source, it was Chrysophyta algae.

The presence of similar compounds (i.e. saturated, monoenoic and dienoic methyl ketones) in the skin of the snake *Boiga irregularis* is also very interesting (Murata *et al.*, 1991). One of the double bonds (whose configuration was always *cis*, in contrast to the sediments and *E. huxleyi*) was always in the position ω-9 and the other in the position 6 or 8.

In the course of a systematic investigation of the fresh-water-lake flora and fauna of Lake Baikal, we discovered saturated and unsaturated methyl ketones having a chain length of C_{33}–C_{40} in the deep water invertebrate *Acanthogammarus grewinkii*.

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Materials and Methods

Extraction and isolation of ketones

The total lipids from whole animals, extracted according to Bligh and Dyer (1959), were dissolved in a mixture of methanol and water (5:1) containing KOH (pH 12, 24 hr at room temperature). The unsaponifiable portion was extracted with a mixture of hexane–diethyl ether (9:1) and then separated by TLC (Silicagel H, 20 × 20 cm and layer thickness 0.25 mm, hexane–diethyl ether–acetic acid, 89:10:1). R_f of the methyl ketones (3.4% of total lipids) had the value of 0.45. The obtained mixture of methyl ketones was further separated by using RP–HPLC. A gradient of acetonitrile–methyl-*t*-butyl–ether (70:30–10:90) was used for elution within 30 min. The individual peaks were collected manually. The major peak (C_{37}) was further separated by using preparative Ag^+-TLC (10% $AgNO_3$ impregnation, 20 × 20 cm and layer thickness 0.25 mm). Double development was used: first, by a mixture of hexane–diethyl ether (9:1) to reach the height of 20 cm and then, after drying by a mixture of hexane–diethyl ether (3:1) to reach the height of 11 cm. After detection with dichloro-fluorescein, the ketones were extracted from the individual bands by mixture hexane–diethyl ether (1:3) (R_f of $C_{37:0}$–0.75; R_f of $C_{37:1}$–0.6; R_f of $C_{37:2}$–0.5; R_f of $C_{37:3}$–0.35; R_f of $C_{37:4}$–0.25).

General experimental procedures

The semipreparative Gradient LC System G-I (Shimadzu, Kyoto, Japan) with two LC-6A pumps (2 ml/min), SCL-6A system controller, SPD ultraviolet detector (280 nm), SIL-1A sample injector and C-R3A data processor, with an analytical column, packed with SGX C18 phase (250 × 4 mm ID, 5 μm particle size) (Tessek, Prague, Czech Republic) was used for RP-HPLC. The ^{13}C-NMR (75 MHz) and ^{3}H-NMR (300 MHz), spectra were measured using a Bruker AM 300 instrument (Karlsruhe, Germany) in $CDCl_3$. For scanning infra-red spectroscopy of the ketones in CCl_4 after RP-HPLC a Perkin–Elmer Model 1310 (Perkin-Elmer, Norwalk, CT) infra-red spectrophotometer was used. All GC–MS separations of the oxazolines and long chain (greater than C_{30}) fatty acid methyl esters were performed using an electron impact Finnigan MAT (San Jose, CA) 1020 B mass spectrometer. The injection temperature (on-column) was 100°C and a fused-silica capillary column from Supelco (SPB^{tm}-1) (30 m × 0.20 mm ID, 0.20 μm film thickness) was used. The temperature programme was as follows: 100°C for 1 min, then increased at 20°C/min to 180°C and at 2.5°C/min to 320°C, where it was maintained for 1 min. The carrier gas was hydrogen at the linear velocity of 60 cm/sec. All mass spectra were scanned within the range m/z 100–650. Dimethyl esters and short chain methyl esters were identified under similar conditions as oxazolines, the only difference being the column temperature, which was programmed from 100 to 230°C at the rate of 5°C/min.

Identification of double bond position

The $C_{37:1, 37:2, 37:3, 3:4}$ ketones (after Ag^+-TLC) were split by a mixture of permanganate–periodate (Kates, 1988). The reaction mixture was extracted by diethyl ether and the free mono- and dicarboxylic acids were transformed into methyl esters by using a solution of diazomethane in diethyl ether (Kates, 1988) and identified by GC–MS.

The total ketones after TLC were split by the halogenform reaction (Kates, 1988). The acids obtained were transformed into oxazolines (Zhang *et al.*, 1988) and subsequently identified by GC–MS.

Results and Discussion

The preliminary analysis of the mixture of the total unsaponifiable fraction suggested the presence of compounds containing 35–39 carbon atoms. On the basis of the in MS fragmentation pattern, unsaturated long chain ketones, an oxo group in position 2 could be expected. The isolation methods we used were chosen and slightly modified in keeping with the relevant literary data (De Leeuw *et al.*, 1980). At first, a fraction of methyl ketones was isolated from the total unsaponifiable portion by using TLC. In order to define the position and configuration of the double bonds, it was necessary to isolate the individual compounds. Previous experience (Řezanka, 1990) showed the separation of compounds containing a long aliphatic chain by RP-HPLC to be very good, and the total methyl ketones were therefore subjected to reverse-phase chromatography. In spite of optimizing the mobile phase, we could separate the compounds only according to the number of carbon atoms. However, some indications of a separation according to the number of double bonds could be observed in the case of the major peak of C_{37}.

For complete separation of the individual compounds Ag^+-TLC was used. Five different bands (0–4 double bonds) were obtained by double development. The spectra of the individual C_{37} ketones were measured (IR, ^{1}H-NMR, ^{13}C-NMR, and MS) and the ketones were also chemically split.

By using IR we found that the double bonds of the individual C_{37} ketones were in the *cis*

position. No characteristic band was found in the interval of 990–900 cm^{-1} (i.e. *trans* double bond) but, significantly, a value of 719 cm^{-1} corresponding to the stretch of *cis* CH=CH was measured. Other absorption values corresponding to the individual structural elements were also detected in the IR spectrum (C=O, C=C, CH_2).

In the ^{1}H-NMR and ^{13}C-NMR spectra, characteristic signals fully confirming the proposed structures (Table 1) were observed in the ketones $C_{37:0}$, $C_{37:1}$, $C_{37:2}$, $C_{37:3}$, and $C_{37:4}$. ^{1}H-NMR spectra of the $C_{37:1}$–$C_{37:4}$ ketones clearly show the presence of a methyl ketone (2.12 s), a terminal methyl group (0.87, t), olefinic protons (5.33–5.35, m), allylic methylene protons (1.95–1.97, m), and of a great number of aliphatic methylene groups (1.24–1.25, br s).

^{13}C-NMR spectra also confirmed structural elements proposed above, especially the signals of the keto group (215.73), ω-terminal methyl (14.20), ω-1 and ω-2 methylene groups (22.71 and 31.94, respectively) and the methylene groups in the neighbourhood of both a keto group and a double bond (43.41 and 27.31, respectively). The number of double bonds is determined by the signals in the neighbourhood of 130 ppm, e.g. eight signals in the interval of 128.97–129.88 were found for $C_{37:4}$.

The values of the chemical transition of the allyl methylene that in *cis* C=C showed a signal at 27.31 ppm in ^{13}C-NMR brought additional evidence of the *cis* C=C configuration. In the case of trans C=C, the allyl methylene group has a transition of 32.7 ppm (Murata *et al.*, 1991) but this value was not found.

The methyl ketones first separated by TLC were also very well resolved on a nonpolar capillary column, i.e. the base line value was reached between the individual maxima. The exceptions were the couples $C_{37:3}/C_{37:4}$ and $C_{39:3}/C_{39:4}$. The separation of these couples was very difficult; also in previous work (Cranwell, 1985) these polyenoic ketones could not be completely resolved.

Distinct molecular ions (M^+) were observed in mass spectra of all the ketones (Table 2). Other ions occur in the region M^+, see ions M-15 (M-CH_3), M-18 (M-H_2O), M-43 (M-CH_3CO), and M-58 (McLafferty rearrangement). These ions are weak (about 10% of the base peak) in all spectra, reflecting in part the low sensitivity of the quadrupole mass spectrometer to ions of high mass to charge ratio. Each spectrum exhibits an exponential type of the intensity decrease, which is characteristic of unbranched chains. The other ions in MS of long-chain ketones were identified only in the region of low masses (m/z <150); they

Table 1. ^{1}H-NMR and ^{13}C-NMR chemical shift of C_{37} ketones

	$C_{37:0}$		$C_{37:1}$		$C_{37:2}$		$C_{37:3}$		$C_{37:4}$	
	^{1}H-NMR*	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR
—CH_2—CH_3	0.87 t 3	14.21	0.87 t 3	14.19	0.87 t 3	14.20	0.87 t 3	14.19	0.87 t 3	14.20
—CH_2—CH—	—	22.70	—	22.71	—	22.70	—	22.72	—	22.71
—CH_2—CH—CO—	1.60 m 2	23.82	1.60 m 2	23.84	1.61 m 2	23.84	1.61 m 2	23.83	1.61 m 2	23.84
—CH_2—CH =	—	—	1.97 m 4	27.31	1.96 m 8	27.32	1.96 m 12	27.31	1.95 m 16	27.31
—CH_2—	1.24 brs 64	29.7	1.24 brs 56	29.7	1.24 brs 48	29.7	1.25 brs 40	29.7	1.25 brs 32	29.7
—CH_2—CH—CH—	—	31.92	—	31.94	—	31.93	—	31.94	—	31.94
—CH_2—CO—	2.39	43.52	2.39 t 2	43.50	2.38 t 2	43.52	2.37 t 2	43.51	2.37 t 2	43.52
—CH =	—	—	5.33 m 2	128.8–	5.33 m 4	128.9–	5.34 m 6	129.0	5.35 m 8	129.0–
				128.9		129.4		129.6		129.9
—CH_2—CO—	2.12 s 3	29.4	2.12 s 3	29.4	2.12 s 3	29.4	2.12 s 3	29.4	2.12 s 3	29.4
>C = O	—	215.7	—	215.7	—	215.7	—	215.7	—	215.7

*The value after letter = number of hydrogen atoms.

Table 2. Composition of ketones from *Acanthogammarus grewinkii*

Ketones	%	ECL*	M†	M-18	M-43	M-58
33:0	1.28	33.00	478 (7)	460 (14)	435 (3)	420 (8)†
25 34:1	2.12	33.79	490 (5)	472 (15)	447 (2)	432 (7)
34:0	1.17	34.00	492 (7)	474 (13)	449 (2)	434 (6)
6,26-35:2	2.51	34.40	502 (4)	484 (17)	459 (2)	444 (10)
26-35:1	3.02	34.80	504 (5)	486 (14)	461 (3)	446 (9)
35:0	2.17	35.00	506 (4)	488 (11)	463 (2)	448 (7)
6,10,27-36:3	3.49	35.31	514 (2)	496 (23)	471 (3)	456 (12)
6,27 36:2	4.01	35.40	516 (3)	498 (20)	473 (3)	458 (9)
27 36:1	2.28	35.81	518 (4)	500 (13)	475 (2)	460 (8)
36:0	0.30	36.00	520 (5)	502 (10)	477 (1)	462 (6)
6,10,14,28-37:4	7.02	36.22	526 (2)	508 (21)	483 (2)	468 (11)
6,10,28 37:3	16.55	36.32	528 (3)	510 (19)	485 (2)	470 (10)
6,28-37:2	19.05	36.41	530 (4)	512 (16)	487 (3)	472 (8)
28 37:1	10.42	36.81	532 (4)	514 (12)	489 (2)	474 (7)
37:0	5.46	37.00	534 (5)	516 (9)	491 (1)	476 (5)
6,10,29-38:3	1.78	37.34	542 (1)	524 (17)	499 (3)	484 (8)
6,29-38:2	1.11	37.43	544 (1)	526 (15)	501 (3)	486 (7)
29-38:1	0.56	37.82	546 (2)	528 (10)	503 (2)	488 (5)
6,10,14,30 39:4	6.91	38.25	554 (1)	536 (19)	511 (3)	496 (8)
6,10,30-39:3	4.62	38.35	556 (1)	538 (17)	513 (3)	498 (6)
6,30-39:2	3.28	38.47	558 (2)	540 (13)	515 (2)	500 (5)
6,31 40:2	0.89	39.49	572 (1)	554 (11)	529 (3)	514 (4)

*Equivalent chain length.
†Values in parentheses = percentual intensity of ions (base peak has 100%).

did not provide any information on the structure of the corresponding ketones. For this reason, suitable derivatives had to be prepared (cf. below).

To determine the position of the double bond(s) in the ketones, the haloform reaction was used which is very mild and does not attack the double bonds. During this reaction the chain of methyl ketones is shortened by one carbon atom to produce the corresponding fatty acid and trihalogen methane (from the methyl group). The positions of double bonds in VLCFA are routinely identified as nitrogen derivatives. The currently used pyrrolidides and picolinyl esters, however, increase the elution temperature by 50°C (Christie *et al.*, 1986). The most suitable derivatives used are oxazolines, whose elution temperature increases, in comparison with methyl esters, only by 10°C (Yu *et al.*, 1988). By using on-column injection, together with a suitable temperature gradient (including the use of hydrogen as the carrier gas), the individual oxazolines could be well separated (Fig. 1).

The separation of all peaks with the exception of the couples 36:4/36:3 and 38:4/38:3 was very good, without overlap of peaks. Individual peaks and the positions of the double bonds were identified on the basis of the splitting of oxazolines by MS (Table 3). The table includes only the ions that contributed to the description of the structure of the compounds tested. The base peak for all oxazolines was due to an ion having an m/z of 113.

As can be seen in the mass spectrum (Fig. 1), the identification was more complicated in the range of higher masses, mainly due to a low abundance of these ions. The fatty acid oxazoline 36:4 can be mentioned as an example (Fig. 1). M^+ having m/z of 581 can be well distinguished, similarly the values belonging to the ions after the methylene groups were split off. The locations of double bonds are indicated by the presence of a 12 a.m.u. gap between two neighbouring homolog ion peaks in the mass spectra. An empirical rule is formulated as follows: *if a mass separation of* 12 *instead of regular* 14 *a.m.u. observed between two adjacent homologous fragments containing* n-1 *and* n *carbon atoms of the original acid moiety, a double bond exists between carbon* n *and* $n + 1$ *in the chain* (Zhang *et al.*, 1988). The first interruption of the monotone succession represents the ions having m/z of 454 and 442, that formed a gap of 12 a.m.u. The gaps of 26, respective 40 a.m.u. between the ions having m/z of 442 and 468, respective between 468 and 428, is also pronounced. In case of these relatively high m/z values, the ion abundance was very low, making the identification of the double bond positions rather difficult. The identification of the positions of the other three double bonds was much simpler. Again, the ions belonging to the gap of 40 a.m.u. were more abundant than those of the gap of 26 a.m.u. (cf. Fig. 1 and Table 3).

Another method we used to confirm the positions of the double bonds was the splitting of the individual C_{37} ketones by a mixture of periodate–permanganate leading to the formation of monocarboxylic and dicarboxylic acids. After the GC–MS identification of $C_{37:4}$, nonanoic acid was found as well as a mixture

Table 3. Fatty acid composition after haloform reaction

Fatty acid oxazolines	ECL*	M⁺†	Important structural ions† Δ^5	Δ^9	Δ^{13}	ω-9
32:0	32.00	533 (12)‡	—	—	—	—
24–33:1	32.78	545 (9)		—	—	392 (8)–406 (5)–432 (10)
33:0	33.00	547 (11)	—	—	—	—
5,25–34:2	33.67	557 (8)	126 (49)–140 (35) 166 (24)	—	—	404 (8)–418 (4)–444 (9)
25–34:1	33.78	559 (9)	—	—	—	406 (7)–420 (5)–446 (8)
34:0	34.00	561 (11)	—	—	—	—
5,9,26–35:3	34.60	569 (6)	126 (42)–140 (33) 166 (25)	180 (16)–194 (12)–220 (15)	—	416 (5)–430 (3)–456 (6)
5,26–35:2	34.68	571 (7)	126 (40) 140 (32)–166 (23)	—		418 (5)–432 (3)–458 (6)
26 35:1	34.79	573 (7)	—	—	—	420 (6) 434 (4)–460 (8)
35:0	35.00	575 (10)	—	—	—	—
5,9,13,27–36:4	35.57	581 (5)	126 (37)–140 (29)–166 (31)	180 (12)–194 (8)–220 (11)	234 (7)–248 (3)–274 (5)	428 (4)–442 (2)–468 (3)
5,9,27–36:3	35.61	583 (6)	126 (42)–140 (30)–166 (23)	180 (13) 194 (10)–220 (14)	—	430 (4) 444 (2)–470 (4)
5,27–36:2	35.69	585 (8)	126 (44) 140 (28) 166 (20)	—	—	432 (5)–446 (1)–472 (3)
27–36:1	35.79	587 (8)		—	—	434 (4)–448 (1)–474 (3)
36:0	36.00	589 (9)	—		—	—
5,9,28–37:3	36.61	597 (4)	126 (32) 140 (26) 166 (19)	180 (10)–194 (6)–220 (9)	—	444 (4)–458 (2)–484 (5)
5,28–37:2	36.70	599 (7)	126 (33)–140 (26)–166 (20)	—	—	446 (5)–460 (2) 486 (4)
28 37:1	36.80	601 (9)	—		—	448 (4)–462 (1)–488 (4)
5,9,13,29–38:4	37.58	609 (3)	126 (30) 140 (25)–166 (18)	180 (8)–194 (6)–220 (9)	234 (6) 248 (3)–274 (4)	456 (3)–470 (1)–496 (3)
5,9,29–38:3	37.61	611 (4)	126 (29)–140 (25)–166 (19)	180 (7)–194 (4) 220 (5)	—	458 (3)–472 (1) 498 (2)
5,29 38:2	37.70	613 (6)	126 (27)–140 (22)–166 (18)	—	—	460 (2)–474 (1)–500 (3)
5,30–39:2	37.71	627 (6)	126 (23) 140 (17)–166 (13)	—	—	474 (2)–488 (1)–514 (2)

*For fatty acid methyl esters.
†For fatty acid oxazolines.
‡Values in parentheses = percentual intensity of ions (base peak has 100%).

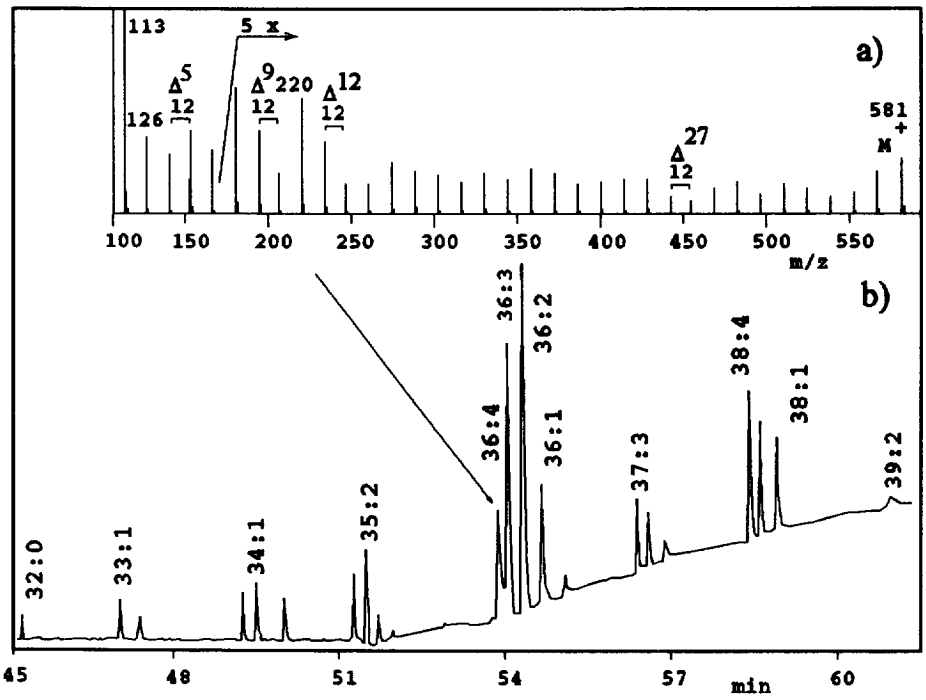

Fig. 1. GC MS of fatty acid oxazolines obtained after haloform reaction. Definite interpretation for Fig. 1(a) is easily obtained based on the 12 a.m.u. gap approach as shown below:

Δ^5	Δ^9	Δ^{12}	Δ^{27}
140–152	194–246	234–246	442–454

of dicarboxylic acids (succinic, glutaric and tetradecanedioic acids). These observations showed that the double bonds were located in the positions 6, 10, 14 and 28 (i.e. ω-9). In the ketones $C_{37:3}$ and $C_{37:2}$, the respective double bond positions were 6, 10, 28 and 6, 28. In the case of a monoenoic C_{37} ketone, the double bond was found to be only in position 28. With other ketones (C_{34}, C_{35}, C_{36}, C_{38}, C_{39}, C_{40}), the products of the haloform reaction and the identification of the formed products by GC–MS were only used for detection of the position of the double bond(s). In all cases, the positions of the double bonds were analogous, as mentioned above.

The presence of very long-chain polyunsaturated ketones in the deep-water invertebrate *A. grewinkii* is rather remarkable. We think that the ketones, which amount to 1% of the total lipids, can originate either in the food (plankton, algae, residues of dead animals) or can be synthesized in the organism itself. The hypothesis of an external source is supported by the similarity to the coccolithoporid *E. huxleyi* where long-chain ketones containing double bonds in *trans* configuration were found, e.g. the ketone $C_{38:3}$ with the positions of double bonds 9, 16 and 23. In the ketones described in this work, however, the double bonds with *cis* configuration were detected in different positions. We therefore think that the ketones analyzed in our work originate from other sources than those providing the ketones for the lacustrine sediments (Cranwell, 1985) or are produced by the organism itself.

The most plausible hypothesis is to postulate the incorporation of VLCFA into *A. grewinkii* via food uptake. At that stage the double bond may already be present in the molecule at the position ω-9. Its origin could be oleic acid as a precursor. In the metabolic reactions of the invertebrate, other double bonds seem to be introduced, e.g. in the positions 5, 9, and 13. The similarity of the C=C positions with those of the VLCFA double bonds in sponges is rather striking. It could mean that *A. grewinkii* possesses the same desaturase or elongase as the fresh water sponge.

Another biosynthetic step is the transformation of the acid to the ketone. Volkman *et al.* (1980) express the opinion that ketones are formed from hydrocarbons although the corresponding intermediate alcohol has never been found. The authors also did not find a direct correlation between the contents of alkenes and ketones. We assume that the ketones are synthesized directly by the reaction of VLCFA with

malonate and by subsequent decarboxylation of the β-keto acid. These ketones have the keto group in position 2. This hypothesis is strongly supported by the position of double bonds in the ketones. We do not think that these long-chain ketones have a function of any importance for *A. grewinkii*. They have not been detected in related species living close to the water surface. It is possible that their presence is related to the depth (about 400 m) and the resulting hydrostatic pressure in which these invertebrates live.

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