Pergamon
0305-0491(94)00062-X

Comp. Biochem. Physiol. Vol. 109B, No. 2/3, pp. 407–413, 1994
Elsevier Science Ltd
Printed in Great Britain
0305-0491/94 $7.00 + 0.00

Identification of unusual cyclopropane monounsaturated fatty acids from the deep-water lake invertebrate *Acanthogammarus grewingkii*

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In the deep-lake invertebrate, *Acanthogammarus grewingkii* a number of unusual fatty acids such as *cis*-11,12-methylene-5-eicosenoate, *cis,cis*-11,12-14,15-bis-methylene-5-eicosenoate, and their homologues, were identified. The structures were determined from spectra (^{1}HNMR, ^{13}CNMR, mass, IR) after their isolation and quantification by means of Ag^+-TLC, semipreparative RP-HPLC and capillary GC–MS. The possibility of biosynthesis and function of these unusually fatty acids is discussed, e.g. they may represent a specific feature of animals dwelling in a deep lake or may also be metabolites that are formed in cellular membranes or be of dietary origin.

Key words: Cyclopropane monounsaturated fatty acids; Deep-water lake invertebrate.

Comp. Biochem. Physiol. 109B, 407–413, 1994.

Introduction

More than 1000 species of fatty acids have been identified in nature (Gunstone, 1992), but some are very rare. Fatty acids with a cyclopropane ring occur mostly in plant oils (Sebedio and Grandgirard, 1989) and in microorganisms (Ratledge and Wilkinson, 1988), especially in mycobacteria (Qureshi *et al.*, 1978, 1980; Takayama *et al.*, 1979).

In contrast, fatty acids containing one ethylenic bond are widespread and can be detected in almost all living organisms. Those fatty acids having 16 to 22 carbon atoms in the chain are the most common, however, exceptions concerning the limits of this range have also been reported (Řezanka, 1989). Monoenoic fatty acids are synthesized by two mechanisms, aerobic and anaerobic, which have been described many times (Schweizer, 1989). A further reaction in the presence of *S*-adenosyl-methionine can give rise to acids containing the cyclopropane ring (Schweizer, 1989).

Fatty acids containing more than one cyclopropane ring in the molecule exist very rarely in nature, except in mycobacteria (Qureshi *et al.*, 1978, 1980; Takayama *et al.*, 1979). The combination of an ethylenic bond and cyclopropane ring(s) has been reported only infrequently, e.g. in mycobacteria (Takayama *et al.*, 1979), or in neohalicholactone, an unusual fatty acid metabolite from marine sponge (Kigoshi, 1991). The mycobacterial fatty acids containing these two characteristic structures were found to be much longer than the usual acids, with a chain-length of as much as 50 carbon atoms.

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Received 30 October 1993; accepted 6 April 1994.

To our knowledge, no acids having the two above structural characteristics, and whose chain length would be less than to 24 carbon atoms, have so far been detected in nature. Such a rule does not apply, however, as far as the biosynthetic intermediates of these acids are concerned. In our systematic study of deep freshwater lake fauna we were able to detect such acids in the species *Acanthogammarus grewingkii* (class Crustacea, subclass Malacostraca, superorder Peracarida, order Amphipoda).

Materials and Methods

General

The semipreparative HPLC Gradient LC System G-I (Shimadzu, Kyoto, Japan) with two LC-6A pumps (2 ml/min), an SCL-6A system controller, an SPD ultraviolet detector (210 nm), an SIL-1A sample injector and a C-R3A data processor, with an analytical column 250 × 4 mm i.d., packed with SGX C18 with 5 μm particles (Tessek, Prague, Czech Republic) was used for reversed phase-HPLC. The ^{13}CNMR (75 MHz) and ^{1}HNMR (300 MHz), spectra were measured in $CDCl_3$ by means of a Bruker AM 300 instrument. For scanning infrared spectroscopy of the methyl esters after RP-HPLC (in CCl_4) a Perkin-Elmer Model 1310 (Perkin-Elmer, Norwalk, CT, U.S.A.) infrared spectrophotometer was used. The HR-MS were determined by the Finnigan MAT 90 mass spectrometer with EI mode. All GC–MS separations were performed using a Finnigan MAT (San Jose, CA, U.S.A.) 1020 B apparatus with electron impact. Injection temperature (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) 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°C/min to 280°C, which 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 70–550. With the exception of GC–MS, which used picolinyl esters and/or methyl esters, the analytical values were measured for methyl esters.

Animal material

Acanthogammarus grewinki (from 360 m depth) specimens were collected on 21 August 1992, in Lake Baikal, Russia. The animal was classified by V. M. Dembitsky. Type specimens of these animals are on file at the Institute of Ecology of the Volga River Basin.

Extraction and isolation of acids

The total lipids were extracted by the method of Bligh and Dyer (1959). The methyl esters were prepared from the total lipids by base catalysed transesterification according to Christie (1989).

The total methyl esters were separated on 20 × 20 cm TLC plate coated with 0.5 mm layer of silica gel G with 10% $AgNO_3$ in a two-solvent system of hexane–diethylether, (80:20). After visualization with 2′,7′-dichlorofluorescein (under UV), the band corresponding to components having one double bond was scraped off and extracted by ether. The extract was dissolved in 3 ml methanol, and urea (500 mg) was then added and dissolved by warming. The solution was cooled to −15°C for 3 hr. After filtration of crystals, the filtrate was acidified and extracted with hexane to isolate the cyclopropanoic fatty acid esters. An aliquot of this extract was used for identification by GC–MS of the picolinyl esters, prepared as described by Harvey (1984). The remainder of this extract (90%) was used for the isolation of pure compounds, see below.

Separation of the individual components by HPLC

HPLC was performed with a gradient of acetonitrile–water (90:10) to pure acetonitrile during 50 min. The effluent for single peaks was collected manually. The major component esters (*cis*-13,14-methylene-5-docosenoate and *cis,cis*-13,14-16,17-bis-methylene-5-docosenoate) had retention times of 18.4 and 19.8 min, respectively, and were obtained at 93 and 95% purity (checked by GC–MS), respectively.

Structures of all eight peaks were confirmed on the basis of mass spectra of their picolinates, ^{1}H and ^{13}CNMR spectra; for ^{13}CNMR spectra, see Table 1.

Methyl cis,cis-13,14-16,17-*bis-methylene-5-docosenoate*

^{1}HNMR: $\delta -0.31$ (2H, cyclopropyl); 0.62 (6H, cyclopropyl); 0.89 (3H, CH_3 end); 1.21–1.26 (20H, methylenes); 1.63 (2H, C-3); 1.96 (2H, C-7); 2.04 (2H, C-4); 2.21 (2H, C_2); 3.64 (3H, OCH_3); 5.27 (2H, C-5, C-6). HR-MS: m/z $[M]^+$ $C_{25}H_{42}O_2$ 374.61229 (found 374.6124); $[M - CH_3O]$ $C_{24}H_{39}O$ 343.57783 (found 343.5781).

Methyl cis-13,14-*methylene-5-docosenoate*

^{1}HNMR: $\delta -0.32$ (H, cyclopropyl); 0.60 (3H, cyclopropyl); 0.88 (3H, CH_3 end); 1.21–1.26 (24H, methylenes); 1.62 (2H, C-3); 1.95 (2H, C-7); 2.05 (2H, C-4); 2.20 (2H, C-2); 3.64 (3H, OCH_3); 5.26 (2H, C-5,C-6).

HR–MS: m/z $[M]^+$ $C_{24}H_{44}O_2$ 364.61708 (found 364.6166); $[M - CH_3O]$ $C_{23}H_{41}O$ 333.58262 (found 333.5827).

The other six methyl esters had ^{1}HNMR spectra very similar to those of the two major esters. The complete structures of the esters were deduced from their ^{13}CNMR spectra, see Table 1.

GC–MS of picolinates

cis,cis-11,12-14,15-*bis-Methylene*-5-*eicosenoate*. m/z (%) 425 (12.3, $[M]^+$); 410 (1.2); 396 (3.8); 382 (5.3); 368 (9.3); 354 (4.6); 340 (0.9); 327 (8.2); 314 (6.4); 300 (5.8); 286 (1.1); 273 (10.3); 260 (4.1); 246 (3.9); 232 (5.7); 218 (8.5); 204 (9.2); 178

Table 1. ^{13}CNMR spectra of monounsaturated cyclopropanoic fatty acid methyl esters

	No. of methyl esters*							
C	1	2	3	4	5	6	7	8
1	174.3	174.3	174.2	174.0	174.1	174.1	174.0	174.1
2	34.2	34.0	34.1	34.2	34.1	34.1	34.1	34.0
3	25.1	25.2	25.1	25.2	25.1	25.1	25.1	25.1
4	28.3	28.4	28.4	28.5	28.3	28.4	28.4	28.5
5	128.7	128.8	128.7	128.7	128.8	128.8	128.9	128.8
6	130.9	130.9	130.8	130.8	130.9	130.8	130.9	130.8
7	28.5	28.6	28.5	28.5	28.4	28.6	28.5	28.5
8	29.8	29.8	29.9	29.7	29.7	29.7	29.8	29.8
9	30.4	29.5	29.5	30.4	29.3	29.4	29.4	29.5
10	28.8	29.9	29.8	28.7	29.8	29.8	29.8	29.8
11	18.3	30.2	29.8	18.2	30.3	29.7	29.7	29.7
12	18.2	28.9	29.7	18.1	28.9	29.8	29.8	29.7
13	27.7	18.3	30.2	28.9	18.2	29.8	30.2	29.7
14	18.1	18.2	28.7	30.1	18.1	30.2	28.8	29.8
15	18.2	27.7	18.3	29.8	28.8	28.8	18.2	29.7
16	28.9	18.2	18.2	29.8	30.2	18.2	18.1	30.1
17	30.3	18.1	27.6	29.7	29.8	18.1	28.7	28.9
18	32.1	28.7	18.2	32.1	29.8	28.7	30.1	18.1
19	22.6	30.2	18.1	22.4	29.7	30.2	29.7	18.0
20	14.1	32.0	28.8	14.0	32.1	32.1	29.8	28.8
21	—	22.4	30.2	—	22.4	22.3	29.5	30.3
22	—	14.0	32.3	—	14.0	14.1	32.2	32.1
23	—	—	22.5	—	—	—	22.4	22.5
24	—	—	14.1	—	—	—	14.1	14.0
Cy†	11.1	11.1	11.0	11.0	11.0	11.0	11.1	11.1
Cy‡	10.9	10.9	10.9	—	—	—	—	—

*1 *cis,cis*-11,12-14,15-bis-Methylene-5-eicosenoate,
2 *cis,cis*-13,14-16,17-bis-Methylene-5-docosenoate,
3 *cis,cis*-15,16-18,19-bis-Methylene-5-tetracosenoate,
4 *cis*-11,12-Methylene-5-eicosenoate,
5 *cis*-13,14-Methylene-5-docosenoate,
6 *cis*-16,17-Methylene-5-docosenoate,
7 *cis*-15,16-Methylene-5-tetracosenoate,
8 *cis*-18,19-Methylene-5-tetracosenoate.
†Cyclopropanoic ring near COOMe.
‡Cyclopropanoic ring farther from COOMe.

(5.1); 164 (29.3), 151 (21.3); 133 (16.7); 108 (100); 93 (64.5); 92 (82.1).

cis,cis-13,14-16,17-*bis*-*Methylene*-5-*docosenoate*. *m/z* (%) 453 (10.6, $[M]^+$); 438 (1.8); 424 (2.1); 410 (2.2); 396 (4.9); 382 (3.7); 368 (0.8); 355 (4.6); 342 (6.2); 328 (5.8); 314 (1.2); 301 (3.5); 288 (4.3); 274 (2.6); 260 (3.6); 246 (5.3); 232 (7.1); 218 (9.0); 204 (8.7); 178 (6.2); 164 (34.6); 151 (29.7); 133 (12.4); 108 (100); 93 (52.4); 92 (92.6).

cis,cis-15,16-18,19-*bis*-*Methylene*-5-*tetracosenoate*. *m/z* (%) 481 (8.2, $[M]^+$); 466 (0.9); 452 (1.1); 438 (1.4); 424 (2.7); 410 (1.9); 396 (0.5); 383 (3.0); 370 (4.4); 356 (3.7); 342 (0.9); 329 (2.7); 316 (3.2); 302 (4.2); 288 (3.7); 274 (3.6); 260 (4.2); 246 (6.0); 232 (5.6); 218 (7.8); 204 (9.1); 178 (9.2); 164 (43.1); 151 (33.1); 133 (9.7); 108 (100); 93 (48.5); 92 (86.3).

cis-11-12-*Methylene*-5-*eicosenoate*. *m/z* (%) 413 (14.1, $[M]^+$); 398 (2.4); 384 (2.7); 370 (5.2); 356 (3.9); 342 (3.8); 328 (4.1); 314 (5.6); 300 (4.7); 286 (1.2); 273 (3.1); 260 (5.2); 246 (4.7); 232 (6.3); 218 (9.8); 204 (9.8); 178 (7.5); 164 (42.1); 151 (26.2); 133 (21.5); 108 (100); 93 (72.1); 92 (91.0).

cis-13,14-*Methylene*-5-*docosenoate*. *m/z* (%) 441 (7.8, $[M]^+$); 426 (2.3); 412 (1.9); 398 (2.4); 384 (2.6); 370 (2.9); 356 (4.3); 342 (6.7); 328 (5.2); 314 (0.8); 301 (2.9); 288 (5.2); 274 (4.1); 260 (5.1); 246 (4.8); 232 (6.4); 218 (8.3); 204 (9.7); 178 (12.0); 164 (27.1), 151 (31.1); 133 (10.0); 108 (100); 93 (58.2); 92 (76.7).

cis-16,17-*Methylene*-5-*docosenoate*. *m/z* (%) 441 (8.1, $[M]^+$); 426 (1.9); 412 (2.1); 398 (2.6); 384 (3.8); 370 (3.1); 356 (0.8); 343 (2.4); 330 (4.5); 316 (3.4); 302 (4.5); 288 (6.0); 274 (5.6); 260 (7.0); 246 (6.9); 232 (7.8); 218 (9.0); 204 (10.4); 178 (11.2); 164 (19.9), 151 (16.1); 133 (9.1); 108 (100); 93 (62.1); 92 (52.8).

cis-15,16-*Methylene*-5-*tetracosenoate*. *m/z* (%) 469 (7.5, $[M]^+$); 454 (0.7); 440 (1.0); 426 (1.8); 412 (2.1); 398 (2.4); 384 (2.7); 370 (3.3); 356 (2.2); 342 (1.2); 329 (1.8); 316 (2.6); 302 (5.0); 288 (4.5); 274 (5.1); 260 (6.1); 246 (5.8); 232 (6.9); 218 (8.1); 204 (8.7); 178 (9.9); 164 (29.1), 151 (30.4); 133 (7.8); 108 (100); 93 (69.5); 92 (92.6).

cis-18,19-*Methylene*-5-*tetracosenoate*. *m/z* (%) 469 (6.2, $[M]^+$); 454 (1.0); 440 (1.1); 426 (1.6); 412 (3.9); 398 (2.1); 384 (0.7); 371 (2.1); 358 (2.8); 344 (3.0); 330 (2.9); 316 (3.2); 302 (4.6); 288 (5.1); 274 (5.6); 260 (6.2); 246 (6.4); 232 (7.7); 218 (9.3); 204 (12.3); 178 (16.7); 164 (24.6), 151 (38.0); 133 (15.1); 108 (100); 93 (60.8); 92 (98.1).

Results and Discussion

After being converted to methyl esters, the total fatty acids were initially separated by Ag^+-TLC. The band corresponding to the esters with one unsaturated bond was isolated and a crude mixture of the cyclopropane acid methyl esters was obtained by crystallization in the presence of urea since they not form urea complexes. The presence of the cyclopropane ring was confirmed by ^{1}HNMR signals in the interval from -0.31 to -0.32 ppm. The proportion of the signals at approx -0.3 ppm (H cyclopropane ring) to those at 3.63 ppm (methyl ester) was 0.85 : 3.00. These values suggested that the crude mixture could be contaminated with methyl esters that did not contain cyclopropane ring(s), or that combined structures were present. In order to examine further the structure of the compounds, the individual esters were separated using RP-HPLC, and the ^{1}H and ^{13}CNMR spectra were measured for all eight peaks exhibiting unique structural characteristics, i.e. both the cyclopropane ring and a double bond. Table 2 summarizes the relative amounts of the individual methyl esters of the acids present that contained the cyclopropane ring. The data indicate the presence of two homologous series that differed in the number of their cyclopropane rings. Table 2 also shows the ECL values that determined with reference to saturated FAME. The fractional of 0.41–0.46 and 0.42–0.47 values suggest that positional isomers of cyclopropane acids can be distinguished without problems.

Table 1, summarizing the data obtained by use of ^{13}CNMR, enables us to make conclusions with respect to the common structural characteristics of all eight esters. First, the signals at about 11 ppm confirm the presence of the cyclopropane ring(s) (Pollard, 1986). The values of the signals measured around 130 ppm correspond to the carbons 5 and 6 (Batchelor *et al.*, 1974) and provide evidence for the presence of

Table 2. Fatty acid composition from deep-lake *Acanthogammarus grewinngkii*

Fatty acid	*ECL**	%†	%‡
cis-11,12-Methylene-5-eicosenoate	21.40	7.8	0.075
cis,cis-11,12-14,15-bis-Methylene-5-eicosenoate	23.22	3.2	0.031
cis-13,14-Methylene-5-docosenoate	23.41	45.1	0.432
cis-16,17-Methylene-5-docosenoate	23.46	4.9	0.047
cis,cis-13,14-16,17-bis-Methylene-5-docosenoate	25.23	27.4	0.262
cis-15,16-Methylene-5-tetracosenoate	25.42	6.4	0.061
cis-18,19-Methylene-5-tetracosenoate	25.47	2.4	0.023
cis,cis-15,16-18,19-bis-Methylene-5-tetracosenoate	27.25	2.8	0.027

*Equivalent chain length for methyl esters on Supelcowax-10 (see Experimental).
†% of total cyclopropanoic esters.
‡% of total esters.

an ethylenic bond. Other deviations from the average value of 29.7 ppm (CH_2— "envelope" value), even though they are much smaller, confirm these structural features. In this respect, the signals around the value of 18 ppm (methine groups of the cyclopropane ring) are particularly important, as well as the steric effects resulting in the upfield and downfield shifts, e.g. the α and β effects in the neighbourhood of the double bond and the cyclopropane ring. The steric effect concerning the methylene group between two cyclopropane rings is very interesting. As already observed with a methylene group between two C=C bonds, as in this case, a greater change takes place and, therefore, the value for δ was only 27.7 ppm, compared to that of about 28.7 ppm measured for the α carbon in the ester of an acid containing only one cyclopropane ring. The ^{1}HNMR spectra were measured for all eight compounds, significant differences being observed only with respect to the homologous series (mono- and dicyclopropane). Therefore, only the ^{1}HNMR spectra of *cis,cis*-13,14-16,17-bis-methylene-5-docosenoate and *cis*-13,14-methylene-5-docosenoate are shown. In the former ^{1}HNMR spectrum, distinct signals corresponding to the hydrogen atom of the cyclopropane ring (−0.31 to −0.32 ppm) and to hydrogen (0.60 ppm) were observed. The ratio of the signal intensity at −0.31 ppm to that at 3.64 ppm (OCH_3) enabled us to determine the number of cyclopropane rings in the molecule of the fatty acid. The respective ratios for the homologous series of monounsaturated monocyclopropanoic and for the other homologous series were 0.99–1.03:3.01–3.04 and 2.01–2.03:2.97–3.00. Other signals such as 0.89 ppm (CH_3 end) or 5.27 ppm (—CH=CH—) confirmed the proposed structures.

The use of HR-MS brought confirmation of the formulae of two major esters (*cis,cis*-13,14-16,17-bis-methylene-5-docosenoate and *cis*-13,14-methylene-5-docosenoate). A further proof of the structure of all eight esters resulted from the GC–MS analysis. The total methyl esters (crude mixture, cf. Materials and Methods) were derivatized (picolinyl esters) and subsequently chromatographed on a capillary GC column. The mass spectra of all eight compounds correspond to the structures proposed. The mass spectra always contained a molecular ion and exhibited a specific scission indicating the presence of the cyclopropanoic ring(s) and the double bond. In *cis,cis*-13,14-16,17-bis-methylene-5-docosenoate, one-tenth of the base peak intensity (ion m/z 108) was $[M]^+$, whereas the $[M-1]$ ion reached a value of two-thirds of the $[M]^+$ intensity. The diagnostic ions having the m/z values of 288, 301 and 328 suggested the position of the cyclopropane ring to be at carbons 13 and 14, while the ions having m/z of 342, 355 and 382 identified the position of the other cyclopropane ring at carbons 16 and 17. In keeping with the results of Harvey (1984), ions with odd mass formed by a scission through the cyclopropane ring (i.e. ion f) as well as the ion m/z 133 were observed only in the picolinyl esters with the cyclopropane ring but not in the esters containing only double bonds. The position of the double bond is unequivocally determined by a gap of 26 amu and/or a gap of 40 amu between the respective pairs of ions m/z 204/178 and 204/164. The other two esters, *cis,cis*-

11,12-14,15-bis-methylene-5-eicosenoate and *cis,cis*-15,16-18,19-bis-methylene-5-tetracosenoate, exhibited a similar scission of picolinyl esters in the mass spectrum. In case of *cis*-13,14-methylene-5-docosenoate, the ions *m/z* 288, 301 and 328 were detected, locating the position of the cyclopropane ring at carbons 13 and 14. The double bond was situated in position 5, since ions similar to the ester of *cis,cis*-13,14-16,17-bis-methylene-5-docosenoate were detected. On the basis of all of these measurements, the structures shown in Table 2 are proposed.

We postulate that the synthesis of these compounds can be explained by taking into account known biosynthetic reactions. In the first instance of an acid with two or three double bonds one is substituted by *S*-adenosyl–methionine. In the second instance another molecule of *S*-adenosyl–methionine enters the reaction to form the second cyclopropane ring. The fact that the double bond at position 5 is not methylated in contrast to the double bonds at positions ω-6 and ω-9 (in dicyclopropyl acids) and ω-6 or ω-9 (in monocyclopropyl acids) is very interesting. Whether the substrate specificity is responsible for this reaction (*S*-adenosyl–methionine bound to the enzyme can react only with the bonds near to the CH_3 end), or whether the double bond is introduced at position δ 5 after the formation of cyclopropane acids is not clear. In two previous papers (Qureshi *et al.*, 1978; Takayama *et al.*, 1979) in which dicyclopropane acids were identified, no information on their biosynthesis was given.

As far as we know, monounsaturated cyclopropyl fatty acids identified in a deep, freshwater lake have not yet been found in nature, nor have they been synthesized in any laboratory. Several explanations may exist as to why these compounds are present in invertebrates. They may represent a specific feature of animals dwelling in a deep freshwater lake that were separated from their deep-sea relatives for millions of years. One can also hypothesize that these compounds represent metabolites that are formed and embedded into cellular membranes so that their permeability would be adapted to the freshwater/deep-water environment. The presence of the above fatty acids may also be explained by dietary origin. Cyclopropane fatty acids in a fish were attributed to bacteria in the diet (Cosper and Ackman, 1983). Which of these three hypotheses is right is difficult to say. In our opinion, it is the combination of all hypotheses that can best explain the phenomenon. In order to provide answers to this question, further experiments are being conducted in our laboratory.

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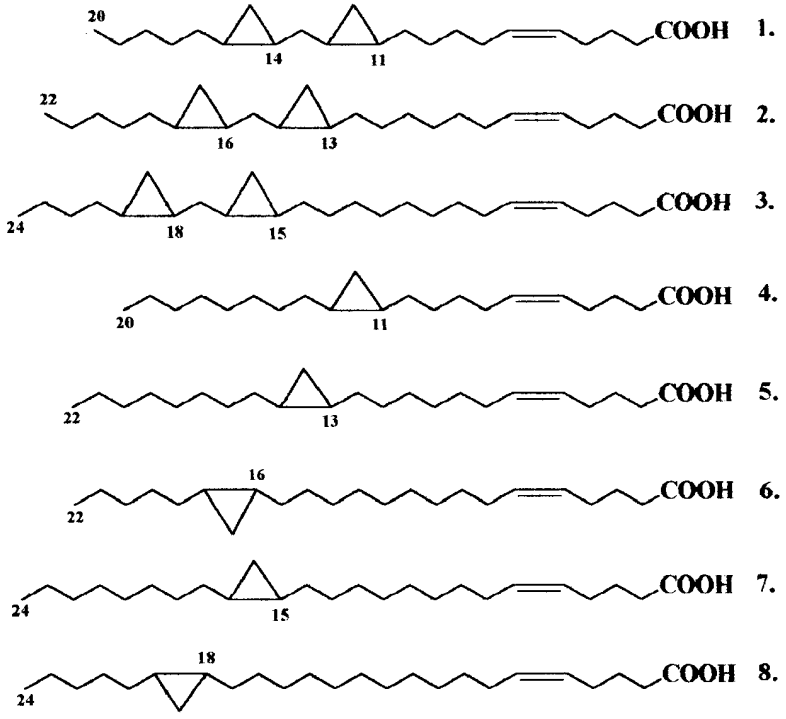

Monounsaturated cyclopropane fatty acids isolated from the deep-water amphipod crustacean *Acanthgammarus (Brachyuopus) grewingkii*