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Studies on Lipids of Crayfish, *Procambarus clarkii*. I. Furanoid Fatty Acids

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The fatty acid compositions of crayfish hepatopancreas lipids have been studied. Seven homologous unusual furanoid fatty acids, detected by gas chromatography-mass spectrometry, accounted for 18% of the cholesteryl ester fatty acids. The major component among them was 12,15-epoxy-13,14-dimethyleicosa-12,14-dienoic acid (47%), and the hitherto unknown 8,11-epoxy-9,10-dimethylhexadeca-8,10-dienoic acid was also present. These acids were concentrated in cholesteryl esters, and only trace amounts were found in triglycerides and phospholipids. The distribution of furanoid fatty acids in crayfish did not correspond to that in fish. This is the first report of fatty acids containing a furan ring in crustaceans.

Keywords——crayfish; hepatopancreas; cholesteryl ester; furanoid fatty acid; lobster

It has been established that vertebrates synthesize cholesterol from acetyl-CoA.[1] The evidence suggests that, among the invertebrates, arthropoda (represented by crustaceans and insects) cannot synthesize cholesterol.[2] Much work has been done on the biosynthesis of cholesterol and the conversion routes from phytosterols to cholesterol.[3] However, relatively little is known about the lipid composition and the fatty acid components of animals.[4]

It is desirable to compare the lipid constituents of crustaceans with those of vertebrates such as rats and fishes to identify the alterations in the lipid constituents and component acids in the presence and absence of cholesterol synthesis. This paper describes the fatty acid composition of the hepatopancreas of crayfish, *Procambarus clarkii*.[5] The analysis showed the presence of a series of unfamiliar fatty acids containing a furan ring (furanoid fatty acids) in the freshwater crayfish.

Results and Discussion

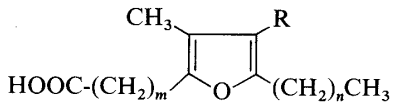
Table I shows the fatty acid composition of crayfish lipid classes. It is characterized by the presence of a series of unfamiliar fatty acids, the methyl esters of which were analyzed by gas chromatography-mass spectrometry (GC-MS). The acids were named F_0, F_1, F_2, F_3, F_4, F_5, F_6 in order of increasing retention time in gas chromatography (GC) (Fig. 1a). F_6, the major component, was isolated and examined. Mass spectral analysis of F_6 (Fig. 2a) suggested a molecular formula of $C_{23}H_{40}O_3$ (parent peak at *m/e* 364) and the structure of a furanoid fatty acid (F acid) was suggested by the presence of a specific base peak at *m/e* 179. The furan structure was supported by the ultraviolet (UV) spectrum (λ_{max} 225 nm in *n*-hexane) and the resistance to 5% Pd–charcoal catalytic hydrogenation in methanol (90% recovery after 24 h reaction). More drastic hydrogenation with PtO_2 in acetic acid gave three isomeric tetrahydrofuran derivatives. The mass spectra of F_6 (Fig. 2a) and the tetrahydrofuran derivatives (Fig. 2b) coincided well with those of the corresponding F acids reported by Glass *et al.*, who isolated them from fishes.[6] These results show that the series of fatty acids detected

TABLE I. Fatty Acid Composition[a] of Lipid Classes from Crayfish Hepatopancreas

Fatty acid	CE[b] (%) (10%)[e]	TG[c] (%) (65%)[e]	PL[d] (%) (17%)[e]
14:0	0.5	0.5	—
16:0	18.9	20.1	7.6+3.3[f]
16:1	2.1	10.1	7.6
18:0	11.3	3.7	3.1+4.5[f]
18:1	27.7	26.3	14.8
18:2	2.8	11.9	5.8
20:0	0.9	2.4	1.7
18:3+20:1	2.0	4.6	1.3
20:2	0.7	0.8	1.4
20:4	1.4	2.2	17.6
20:5	1.1	3.7	20.1
22:4	—	—	0.3
22:5	0.9	0.9	0.4
22:6	5.3	1.5	8.0
Furanoid[g]	18.3	<1	<1
Branched[h]	6.4	9.1	1.3

a) Percent by weight. *b*) Cholesteryl esters.
c) Triglycerides. *d*) Phospholipids.
e) Percent in total lipids. *f*) Dimethyl acetal, obtained from plasmalogens.
g) Furanoid fatty acids. *h*) Branched-chain fatty acids.

TABLE II. Structures of the Furanoid Fatty Acids



Compd.	*m*	*n*	R
F_0	6	4	CH_3
F_1	8	2	CH_3
F_2	8	4	H
F_3	8	4	CH_3
F_4	10	2	CH_3
F_5	10	4	H
F_6	10	4	CH_3

in crayfish hepatopancreas is a group of furan-containing fatty acids (Table II). Further, F_0, one of the series of F acids isolated from the crustancean, is a new compound not found in fishes (Table IV).[6]

Mass Spectra of Furanoid Fatty Acid Methyl Esters

The characteristic ions in the mass spectra of the F acid methyl esters (F_0–F_6) are listed in Table III and the spectrum of the new F acid methyl ester (F_0) is shown in Fig. 2c. It is well known that straight-chain saturated and mono-unsaturated fatty acid methyl esters (but not α,β-unsaturated ones) give a base peak at *m/e* 74, produced by the familiar McLafferty rearrangement.[7] On the other hand, di-unsaturated acid methyl esters such as $C_{18:2}\omega6$ and

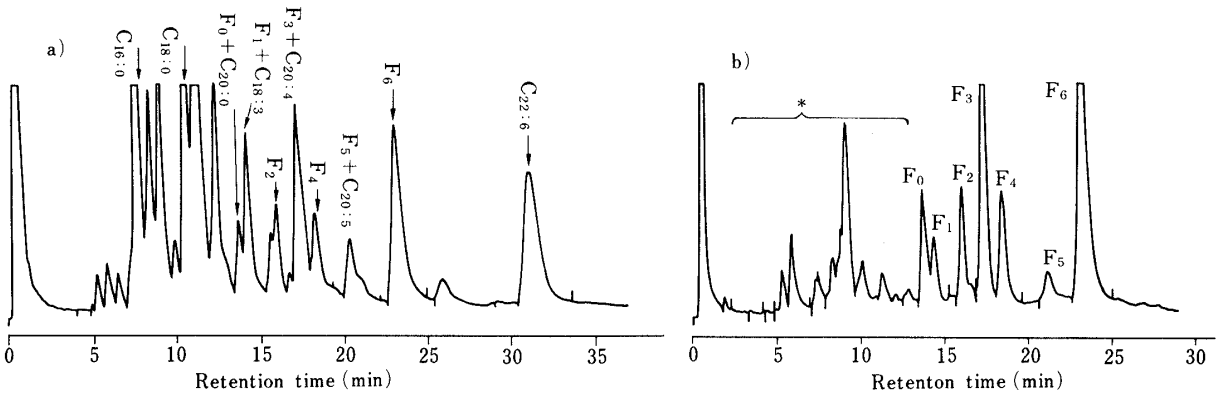


Fig. 1. a) Gas Chromatogram of Fatty Acid Methyl Esters from Cholesteryl Esters of Crayfish Hepatopancreas Lipids, b) Fatty Acid (Furanoid and Branched-chain) Methyl Esters after Urea Fractionation of Hydrogenated Fatty Acid Methyl Esters

*, Branched-chain fatty acid methyl esters, which will be dealt with in subsequent papers.

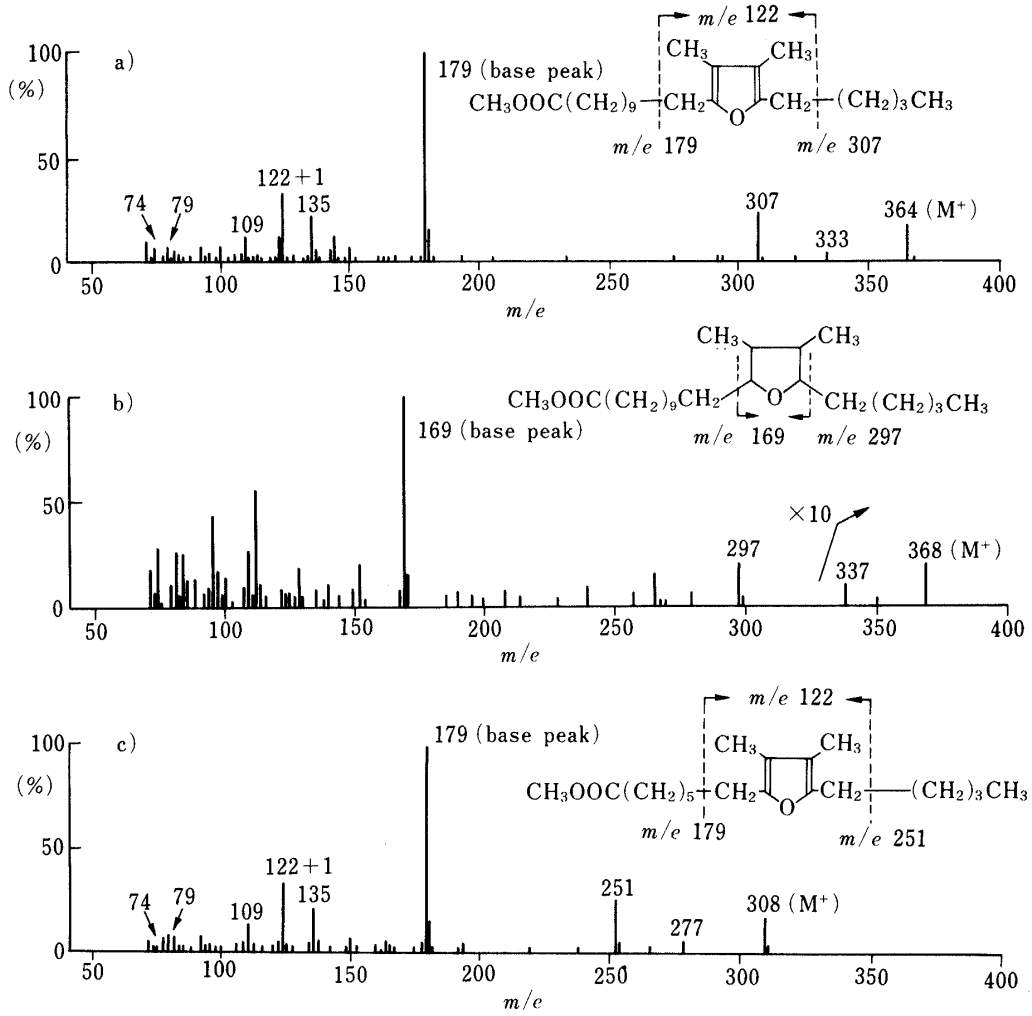


Fig. 2. a) Mass Spectra of F_6 Methyl Ester, b) Tetrahydro F_6 Methyl Ester, c) F_0 Methyl Ester

$C_{20:2}\omega6$ give a base peak at *m/e* 81, and poly-unsaturated acid methyl esters, such as $C_{18:3}\omega3$, $C_{20:3}\omega6$, $C_{20:4}\omega6$, $C_{20:5}\omega3$, $C_{22:5}\omega3$, 6 and $C_{22:6}\omega3$ have a base peak at *m/e* 79. No prominent peaks corresponding to these fragments were observed with the F acid methyl

TABLE III. Characteristic Ions in the Mass Spectra of Furanoid Fatty Acid Methyl Esters

Compd.	M^+	$M^+-C_2H_5$ or C_4H_9	Base peak	M^+-OCH_3	Furan fragment
F_0	308 (15.6)[a]	251 (24.3)	179 (100)	277 (4.3)	123 (31.0)
F_1	308 (9.7)	279 (13.6)	151 (100)	277 (3.2)	123 (5.7)
F_2	322 (8.2)	265 (3.8)	165 (100)	291 (2.4)	109 (16.1)
F_3	336 (15.0)	279 (24.2)	179 (100)	305 (3.4)	123 (30.9)
F_4	336 (9.0)	307 (12.0)	151 (100)	305 (2.1)	123 (12.0)
F_5	350 (9.1)	293 (3.3)	165 (100)	319 (2.1)	109 (15.3)
F_6	364 (16.4)	307 (22.2)	179 (100)	333 (3.0)	123 (31.1)

a) Relative abundance.

TABLE IV. Furanoid Fatty Acid Distributions in Crayfish Hepatopancreas and Fish Liver Lipids

	F acids in lipid class (%)[a]			Individual F acids (%)[b]						
	CE[c]	TG[d]	PL[e]	F_0	F_1	F_2	F_3	F_4	F_5	F_6
Crayfish	18.3	<1	<1	5.0	3.8	8.8	24.8	11.1	3.4	43.2
Brook trout[f]	10.3	—	—	—	—	—	—	10.7	24.3	65.3
Northern pike[f]	84.1	2.0	—	—	—	—	—	13.3	—	86.6
Carp[f]	50.3	0.1	—	—	—	—	—	27.8	20.0	52.0
Bull head[f]	95.8	14.1	—	—	—	2.3	—	27.6	5.3	64.8
Carpsucker[f]	86.7	12.7	1.9	—	—	17.8	—	17.8	24.0	40.0

a) Percent of total fatty acids in lipid class. b) Relative percent of furanoid acids.
c) Cholesteryl esters. d) Triglycerides. e) Phospholipids. f) Ref. 11.

esters (Fig. 2a, 2c), but allylic cleavage of the alkylcarboxyl chain at the furan ring produced the base peak (F_0, F_3, F_6: *m/e* 179. F_2, F_5: *m/e* 165. F_1, F_4: *m/e* 151). Similar allylic cleavage of the alkyl chain on the other side of the ring gave $M^+-CH_2CH_3$ from F_1 and F_4, and $M^+-(CH_2)_3CH_3$ from F_0, F_2, F_3, F_5 and F_6. The furan ring itself was represented by an *m/e* 123 peak from F_0, F_1, F_3, F_4 and F_6, and by an *m/e* 109 peak from F_2 and F_5, produced by cleavages of both allylic positions with hydrogen rearrangement.[6]

The present communication does not cover branched-chain fatty acids, which will be dealt with in subsequent papers.

Distribution of Furanoid Fatty Acids

As shown in Fig. 1a, peaks of F acid methyl esters overlapped with those of other fatty acid methyl esters. Therefore, F acid methyl esters could not be quantified exactly by GC analysis of the whole methyl esters. For this reason, quantitative analysis required the separation of F acids from other fatty acids.

Fatty acid methyl esters were hydrogenated in *n*-hexane at atmospheric pressure using Lindlar catalyst.[8] Under these conditions, the furan ring was unaffected. The normal saturated fatty acids were eliminated by urea fractionation.[9] The resulting mixture contained only furanoids and branched-chain fatty acids (Fig. 1b). The F acids were quantitated by GC, using squalane as an internal standard.[10c] Our results on the distribution of F acids are shown in Table IV together with the data for fishes.[6b]

Comparison of the Distribution of Furanoid Fatty Acids from Crayfish with Those from Fishes

A comparison of the distribution of F acids from crayfish with the results for fish liver

TABLE V. Fatty Acid Compositions[a] of Lipid Classes from Lobster (Male) Hepatopancreas

Fatty acid	CE[b] (%)	TG[c] (%)	PL[d] (%)
12:0	4.8	0.1	—
14:0	4.9	4.1	1.0
16:0	8.7	22.6	12.8+1.4[e]
16:1	16.7	8.2	9.0
18:0	2.7	7.4	9.9+4.7[e]
18:1	15.1	19.2	17.1
18:2	3.8	0.9	1.3
18:3+20:1	3.3	5.7	3.2
20:2	1.1	1.1	0.9
20:3	1.4	0.2	—
20:4	14.4	5.7	10.4
20:5	14.3	12.0	15.0
22:4	2.3	1.8	1.2
22:5	2.4	3.6	3.4
22:6	2.9	8.1	8.1

a) Percent by weight. *b*) Cholesteryl esters. *c*) Triglycerides. *d*) Phospholipids. *e*) Dimethyl acetal, obtained from plasmalogens.

(Table IV) reported by Glass *et al.*[6b] revealed the following features. 1) F acids occurred mainly as cholesteryl esters in both crayfish and fishes. 2) F_0 was a characteristic F acid of crayfish, and did not occur in fishes. 3) F_1, F_2 and F_3 in crayfish comprised about 40% of total F acids (F_3; about 25%), while in fishes, those acids were absent or minor. 4) The major component of F acids in both crayfish and fishes was F_6.

Fatty Acid Composition of the Hepatopancreas of a Marine Crustacean, Lobster

We also examined the fatty acid composition of each lipid class in the hepatopancreas of lobster (*Panulirus japonicus*, male) by GC-MS. However, as shown in Table V, F acids were not detected in the lobster lipids.

Glass *et al.* reported the presence of the F acids in the lipids of freshwater fishes,[6] and Gunstone *et al.* demonstrated the presence of these fatty acids in marine fishes.[10] Our results indicate that the F acids in crustaceans occur abundantly only in freshwater species, not in marine ones.

F acids have also been encountered in rubber latex[12] and *Exocarpus* seed oil,[13] and very recently another group of F acids has been discovered in human blood and urine (urofuran acids).[14] However, the biosynthesis and biological role of these acids remain to be elucidated.

Experimental

Animals——Crayfish, *Procambarus clarkii*, were caught in Inbanuma in Chiba prefecture near Tokyo, Japan. The animals (male), 50—70 g, were maintained in tap water at room temperature for at least two weeks, and were fed on dried sardines. Then the animals were killed and the hepatopancreas was removed.

Lipid Extraction, Lipid Fractionation and Preparation of Fatty Acid Methyl Esters——Total lipids were extracted with chloroform–methanol (2:1, v/v) by the method of Folch *et al.*[15] The lipids were fractionated into cholesteryl esters, triglycerides and phospholipids by silica gel column chromatography. The cholesteryl esters were eluted with *n*-hexane–benzene (2:1, v/v), the triglycerides with chloroform–benzene (1:1, v/v), and the phospholipids with methanol–chloroform (2:1, v/v). The fractionated lipids were transmethylated with 0.5 M sodium methoxide in methanol, or 5% hydrogen chloride in methanol.

Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS)——GC was peformed using a Hewlett Packard 5710A instrument equipped with a flame ionization detector and a 2 m × 2 mm I.D glass column

containing 5% Silar 10c on Chromosorb W-HP, programmed from 120 to 170 °C at 4 °C/min. Helium was used as the carrier gas, at a flow of 25 ml/min.

GC-MS was performed on a Hewlett Packard 5995A instrument, equipped with a 2 m × 2 mm I.D glass column containing 5% Silar 10c on Chromosorb W-HP with the same temperature programs as used for GC. All spectra were recorded at an inonization potential of 70 eV.

Catalytic Hydrogenation of Furanoid Fatty Acid (F_6)——1) F_6 (0.5 mg) was hydrogenated in methanol (2 ml) under hydrogen gas at atmospheric pressure with vigorous stirring, using 5% Pd–charcoal (0.5 mg) as a catalyst. More than 90% of the starting material (estimated by GC) remained unchanged after 24 h.

2) F_6 (0.5 mg) in acetic acid (1 ml) was hydrogenated with PtO_2 (0.5 mg) as a catalyst at atmospheric pressure for 3 h. GC-MS analysis of the resulting mixture revealed at least three peaks. Their mass fragmentation patterns were all very similar, and the parent peaks showed an increase of 4 amu. These compounds are presumably stereoisomers of tetrahydro F_6.

Catalytic Hydrogenation of Fatty Acid Methyl Esters in Cholesteryl Esters, Using Lindlar Catalyst——Whole fatty acid methyl esters (200 mg) obtained from cholesteryl esters were hydrogenated in *n*-hexane (30 ml) for 3 h at atmospheric pressure over Lindlar catalyst (30 mg).[8] GC-MS analysis showed that the furan ring was not hydrogenated under these conditions, and only unsaturated fatty acids were saturated.

Separation of F_6 from the Other Furanoids and Fatty Acids——The cholesteryl esters fraction was separated into three bands by preparative thin layer chromatography (PTLC) (20 × 20 cm, silica gel, thickness 0.5 mm, *n*-hexane–benzene = 5 : 1, v/v). The intermediate polar band was transmethylated with 0.5 M sodium methoxide in methanol, and the resulting crude fatty acid methyl esters were purified by PTLC (20 × 20 cm, silica gel, thickness 0.5 mm, *n*-hexane–benzene = 5 : 2, v/v) giving a mixture of F_6 (51%), F_5 (4%), $C_{20:4}$ (21%), $C_{20:5}$ (9%) and $C_{18:3}$ (6%), which was rechromatographed by reversed phase TLC (10 × 10 cm, RP-18, Merck Darmstadt, methanol–acetone–H_2O = 50 : 50 : 5, v/v) to afford F_6. The F_6 was found to be 96% pure by GC (the main contaminant was $C_{20:4}$).

Urea Fractionation——Urea fractionation was carried out by the method of Ackman *et al.*[9]

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