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Lipophilic arsenic compound(s) in the liver of a tiger shark *(Galeocerdo cuvier)*

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Homogenized aliquots (100 g) of the liver (8.4 kg, 5 μg As g^{-1}) of a tiger shark (*Galeocerdo cuvier*) were extracted with chloroform/methanol, and the extracts purified by countercurrent extraction (hexane/87% ethanol), silica gel column chromatography (chloroform/methanol mixtures as mobile phases), and silica gel thin-layer chromatography (chloroform/methanol/acetic acid). The purified samples (24 mg arsenic g^{-1}) gave no ^{31}P NMR signal, but gave ^{1}H and ^{13}C NMR signals with similarities to those of dipalmitoylphosphatidic acid and salad oil and also signals indicative of the presence of methylated arsenic compounds. The sample could contain a diacyl glyceride with a methylated arsenic group.

Keywords: Arsenic, arsenolipid, shark liver, thin layer chromatography, silica gel column chromatography, NMR spectra, methylated arsenic compounds

INTRODUCTION

Recently several organic arsenic compounds were identified in marine organisms. For example, arsenobetaine $[(CH_3)_3As^+CH_2CO_2^-]$ was found in lobsters[1] and other marine organisms,[2–16] dimethyl(ribosyl)arsine oxides in brown algae,[17,18] arsenocholine $[(CH_3)_3As^+CH_2CH_2OH]$ in shrimp,[7,19] and trimethylarsine oxide $[(CH_3)_3AsO]$ in some fish.[20] These marine arsenic compounds are all water-soluble. Water-insoluble, lipophilic arsenic compounds have been described in only a few papers. In the liver of the shark *Prionace glaucus*, 60% of the arsenic was water-insoluble.[2] Lunde,[21] Wrench and coworkers,[22,23] and Benson and Nissen[24] reported that arsenic in marine algae was concentrated in phosphatides. Irgolic *et al.*,[25] Bottino *et al.*[26] and Cooney *et al.*[27] detected arsenic in lipid fractions obtained from algae. Arsenolecithins[25,26] (phosphatidylcholines in which the nitrogen atom in choline had been replaced by arsenic) and arsenic-containing neutral or zwitterionic glycolipids[23] were proposed as potential lipophilic arsenic derivatives.

We are working to identify organophilic arsenic compounds in marine animals that are at the top of the food chain. This paper describes the results of the investigations carried out on the liver of a tiger shark.

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EXPERIMENTAL

The liver (8.4 kg wet weight) of a female tiger shark (*Galeocerdo cuvier*, 234 cm long, 73 kg weight) caught offshore of Ie Island, Okinawa, Japan, was removed from the live body and frozen at −20°C.

Separation of lipids

A sample of the liver (100 g) was homogenized and the homogenate was extracted with chloroform/methanol (500 cm^3, 1:2, v/v) according to the method reported by Bligh and Dyer[28] (Fig. 1). The chloroform solutions were evaporated under reduced pressure below 40°C to produce the fractions 'Lipid 1' and 'Lipid 2'. 'Lipid 1' (4.7 kg in total) was obtained by repeated extractions (84 times). The fraction 'Lipid 1' (50 g) was partitioned between hexane (saturated with 87% ethanol) and aqueous ethanol (87% ethanol) by countercurrent extraction (Fig. 2). The two hexane fractions (I, II) and the two ethanol fractions (III, IV) were evaporated. By countercurrent extractions, repeated 94 times, 61 g of the combined fractions II and III (2600 μg arsenic) were obtained. Fractions II and III (14 g, 600 μg arsenic) were dissolved in a small volume of chloroform and placed on a column (45 cm × 4.5 cm i.d.) filled with a mixture of 4 volumes of 100-mesh silica gel (Mallinckrodt)

and 1 volume Hyflo Super-Cel (Johns–Manville Co.). Chloroform (2 dm^3), chloroform/methanol (16:1, v/v) (2 dm^3), and chloroform/methanol (1:1, v/v) (2 dm^3) were passed through the column: 78% arsenic was recovered in a 16:1 chloroform/methanol eluate (215 mg). The aliquots (170 mg) were dissolved in chloroform and placed on a silica gel/Hyflo Super-Cel column (35 cm × 3.2 cm i.d.). Chloroform (100 cm^3), chloroform/ methanol (64:1 v/v) (100 cm^3), chloroform/methanol (32:1, v/v) (150 cm^3), and chloroform/methanol (16:1, v/v) (150 cm^3) were passed through the column in sequence. The 32:1 eluate containing 69% of the arsenic was evaporated. The same procedure was repeated five times to obtain 170 mg (1500 μ g arsenic). The condensate (2 mg, 18 μg arsenic) was further purified by thin-layer chromatography (precoated Kieselgel 60, Merck, 0.2 mm) with chloroform/methanol/acetic acid (20:1:0.5, by vol.). Arsenic-containing spots were identified by X-ray fluorescence spectroscopy (9.2–11.6 keV) using a SFT-156A (Seiko Co. Ltd) coating thickness gauge.[31] The spot at R_f 0.73 that had an XRF intensity higher than 200 counts per second was scraped and extracted with chloroform/methanol (1:1, v/v) in an ultrasonic bath. An aliquot of the extract was chromatographed again on TLC (chloroform/methanol/acetic acid, 40:1:0.5) producing only one spot (R_f 0.55).

Identification of arsenic compounds and determination of arsenic

Aliquots of larger samples were digested roughly with nitric acid and then with mixed acids ($HNO_3/HClO_4/H_2SO_4$, 3:5:2.5) at 300°C for 12 h in long-necked flasks.[29] The arsenic in the digest was determined either by hydride generation–atomic absorption spectrometry using a heated quartz T-shaped tube, or in small samples by graphite furnace atomic absorption spectrometry using nickel nitrate as the matrix modifier.[30]

NMR spectra were obtained with Bruker AM-500 (^1H), JEOL GX-400 (^{13}C), or JEOL FX-100 (^{31}P)

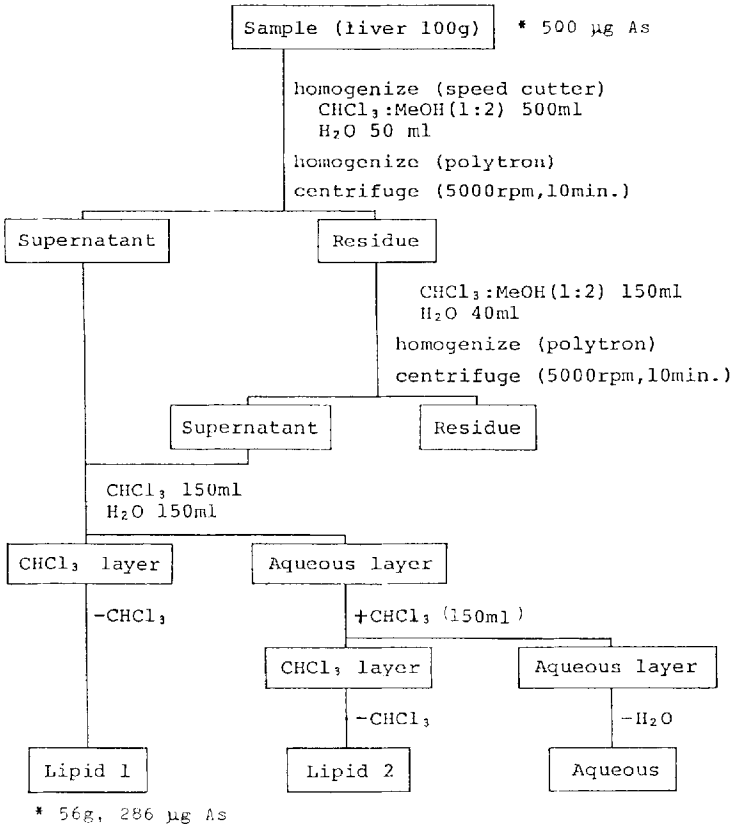

Figure 1 Fractionation of arsenic compounds in shark liver by extraction with chloroform/methanol.

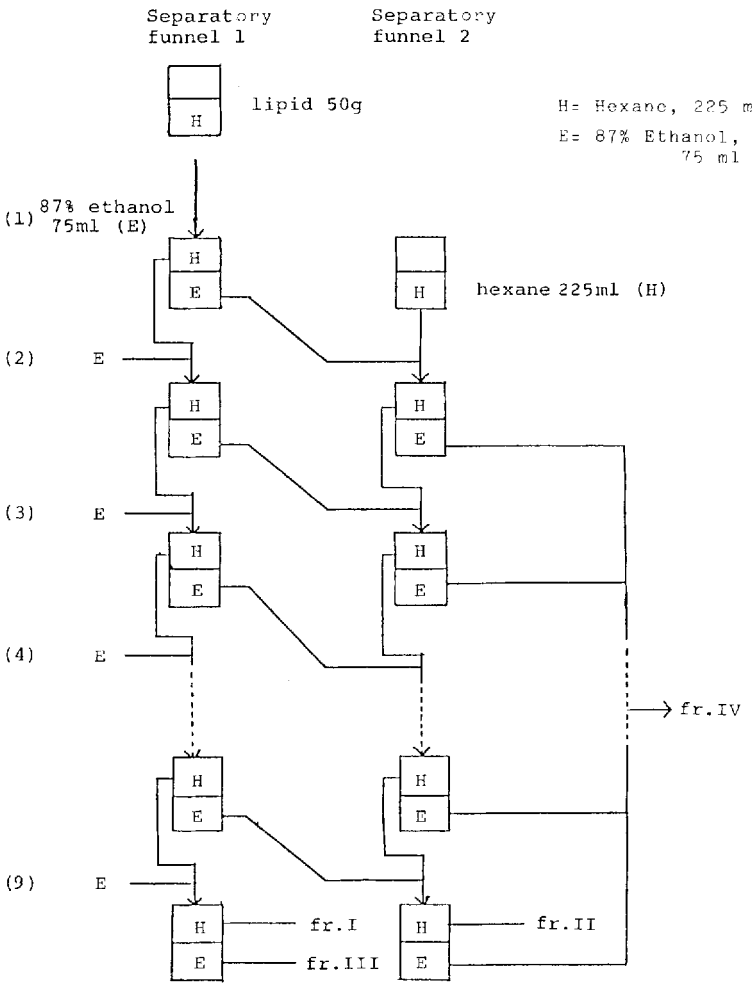

Figure 2 Countercurrent extraction of the fraction 'Lipid 1' between hexane (225 cm^3) and aqueous ethanol (87% ethanol, 75 cm^3).

NMR spectrometers. The solution for the ^{31}P NMR spectrum was prepared by dissolving 40 mg of the TLC-purified compound in 0.5 cm^3 $CDCl_3$. Methylenediphosphonic acid (3% in D_2O) was used as an external standard.

RESULTS AND DISCUSSION

The liver of the female tiger shark weighed 8.4 kg and contained 42 mg arsenic. The arsenic concentration differed over the sampling points of the liver, ranging from 0 to 24 μg arsenic g^{-1}. Extraction of a 100-g aliquot of the liver containing 500 μg arsenic with chloroform/methanol[28] (Fig. 1) produced three fractions. Fraction 'Lipid 1', weighing 56 g after evaporation and containing 286 μg arsenic, accounted for 57% of the total arsenic. The amount of arsenic in fraction 'Lipid 2' was small. The fraction 'Lipid 1' was dissolved in hexane and partitioned between hexane and 87% ethanol by countercurrent extraction (Fig. 2). All of the four fractions (I, II, III, IV) obtained contained arsenic. Fraction I had 50% and fraction IV 38% of the total arsenic in fraction 'Lipid 1'. Evaporation of the solvents from the fractions I–IV gave oily residues with the residue (49 g) from fraction I accounting for most of the material (50 g) subjected to countercurrent extraction. The arsenic concentrations were highest (~ 43 μg g^{-1}) in the residues from fractions II and III

(Table 1), although these two fractions contained only 12% of the arsenic from the fraction 'Lipid 1'.

The residue obtained by evaporation of the combined fractions II and III, containing lipids of intermediate polarity, was chromatographed on a mixture of silica gel and Hyflo Super-Cel. Sequential elution with chloroform/methanol (1:0, 16:1, 1:1) concentrated 78% of the arsenic in the chloroform/methanol 16:1 eluate (Table 2). This fraction was evaporated and the residue (0.01 g, 26 μg arsenic) was chromatographed again on silica gel/Hyflo Super-Cel with chloroform/methanol mixtures (1:0, 64:1, 32:1, 16:1) as mobile phases. The 32:1 eluate contained 69% of the arsenic. This fraction was further purified by thin-layer chromatography (silica gel) with chloroform/methanol/acetic acid (20:1:0.5). Extraction of the R_f 0.73 spot with chloroform/methanol (1:1) and evaporation of the extract produced a sample with an arsenic concentration of 24 mg g^{-1} (Table 2). This sample was used for experiments aimed at the identification of the arsenic compound present in the shark liver.

Several authors[21–27] suggested that the arsenic-containing lipids are phosphatides. Therefore the arsenic compound in the purified arsenic-containing sample obtained from shark liver was compared with egg-yolk lecithin. The arsenic compound from shark liver produced a single spot on silica gel thin-layers with chloroform/methanol/water 65:25:4 (by vol.) (R_f 0.75) and with di-isobutyl ketone/acetic acid/water 40:25:5 (by vol.) (R_f 0.70), but did not match the spot pattern of egg-yolk lecithin (Fig. 3). The R_f values of 0.75 and 0.70 for the arsenic compounds are similar to the R_f values characteristic of phosphatidic acids.[32]

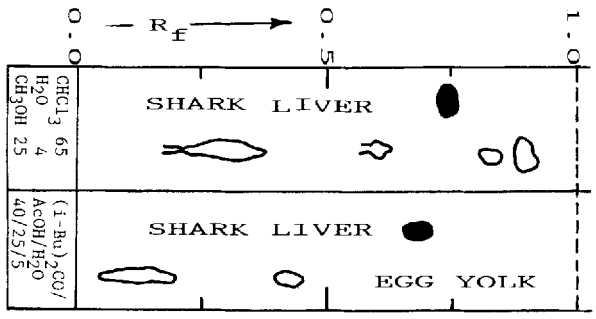

Figure 3 Thin-layer chromatograms (silica gel) of the TLC-purified, arsenic-containing extract from shark liver and of egg-yolk lecithin.

An attempt was made to obtain a ^{31}P NMR spectrum with the TLC-purified sample. The solution in $CDCl_3$ containing 40 mg of the sample (1 mg arsenic) per 0.5 cm^3 of solution did not give a ^{31}P signal. If the arsenic compound present in the sample had been an arsenic-containing phospholipid with one phosphorus atom for each arsenic atom, a ^{31}P signal of considerable intensity should have been observed.

Some signals in the ^1H NMR spectrum of the TLC-purified sample were remarkably different from the spectra of dipalmitoylphosphatidic acid and salad oil, the latter being triglycerides composed of more than 70% of C_{18}- unsaturated fatty acids. The signals from the sample (δ 1.71, 1.73, 2.38, 5.15 ppm) not coincident with the signals from the comparison compounds are marked by arrows (Fig. 4). The signals at 1.71 and 1.73 might be caused by methyl groups attached to

Table 1 Arsenic in the fractions obtained by countercurrent extraction (hexane/87% ethanol) of 50 g of 'Lipid 1'

Fraction	Mass (g)	As (μg)	[As] (μg g^{-1})	As in fraction / As in 'Lipid 1' × 100
'Lipid'	50.0	255	5.0	100
I	49.0	128	2.6	50
II	0.6	26	44.0	10
III	0.1	4	40.0	2
IV	3.8	96	25.0	38

Table 2 Arsenic in the fractions obtained by silica gel/Hyflo Super-Cel and silica gell chromatography of the residues from the combined fractions II and III (Table 1)

Fraction	Mass (mg)	As (μg)	[As] (μg g^{-1})	As in fraction / As in starting material × 100
II and III	700.0	33.0	48	100
$CHCl_3$[a]	560.0	0.0	0	0[a]
$CHCl_3$/MeOH, 16:1[a]	0.01	26.0	2600	78[a]
$CHCl_3$/MeOH, 1:1[a]	2.6	1.6	610	5[a]
$CHCl_3$/MeOH, 32:1[b]	2.0	18.0	8800	69[b]
TLC	0.6	14.0	24000	78[c]

[a] Silica gel/Hyflo Super-Cel; % arsenic based on fractions II and III. [b] Silica gel chromatography of 16:1 eluate; % arsenic based on 16:1 eluate. [c] % arsenic based on 32:1 eluate.

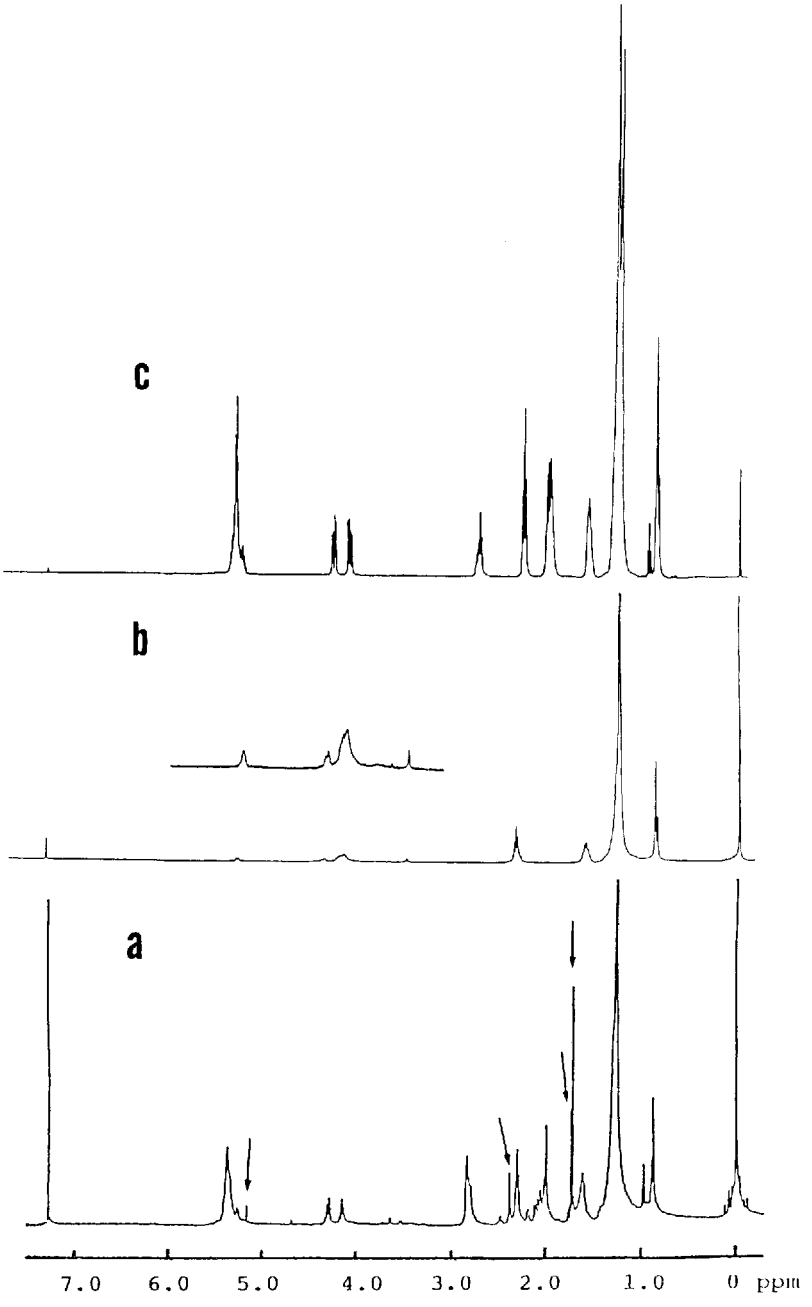

Figure 4 The ^{1}H NMR spectra ($CDCl_3$) of the TLC-purified, arsenic-containing sample from shark liver (a), of dipalmitoylphosphatidic acid (b), and of salad oil (c).

arsenic, because arsenobetaine, dimethyl(ribosyl)arsine oxides, and tetramethylarsonium iodide [$(CH_3)_4As^+I^-$] were observed to have resonances in this range (δ 1.86–1.95 ppm).[17] The corresponding ^{13}C NMR spectra are shown in Fig. 5. The TLC-purified sample did not have signals characteristic of $N-CH_3$ (55 ppm),—$As(CH_3)_3$ (8.6 ppm)[1] or ribose-carbon atoms (70–76 ppm).[17] The 14.9 ppm signal — absent in the spectra of dipalmitoylphosphatidic acid and salad oil — could come from a $(CH_3)_2As=O$ group.[17]

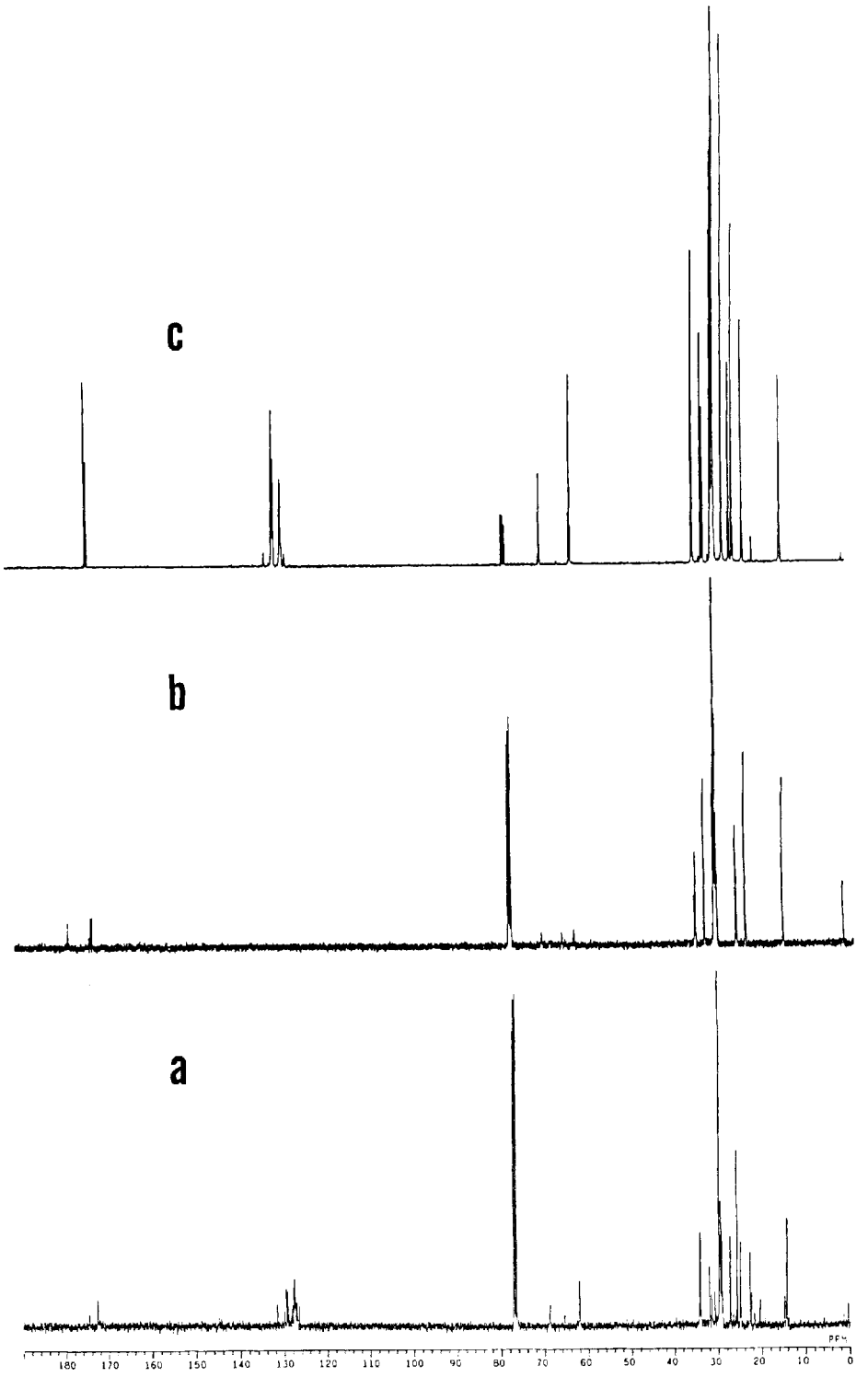

Figure 5 The ^{13}C NMR spectra ($CDCl_3$) of the TLC-purified arsenic-containing sample from shark liver (a), of dipalmitoylphosphatidic acid (b) and of salad oil (c).

CONCLUSION

The extraction of shark liver (5 μg As g^{-1}) with chloroform/methanol and the purification of the extract by countercurrent extraction (hexane/87% ethanol), column chromatography on silica gel with chloroform/methanol mixtures as mobile phases, and TLC produced a lipid sample with 24 mg arsenic g^{-1}. The lipophilic arsenic compound in this sample could not be identified by ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy. Although the ^{1}H and ^{13}C spectra of this sample were similar to the spectra of dipalmitoylphosphatidic acid and salad oil, the arsenic is very likely not associated with a phosphorus-containing lipid, because the sample did not give a ^{31}P NMR signal. If the sample had contained an arsenic-containing phospholipid with one phosphorus atom for each arsenic atom, the 24 mg arsenic g^{-1} fraction should be associated with 10 mg phosphorus g^{-1} corresponding to a phosphorus concentration of 0.3 mol kg^{-1}. At such a concentration a ^{31}P signal should have been observed. The ^{1}H and ^{13}C spectra contain signals that hint at the presence of methyl groups bonded to arsenic with

$$(CH_3)_2\ \underset{\displaystyle O}{\overset{\displaystyle \|}{As}}\ CH_2-X \qquad (X=C \text{ or } O)$$

as a most likely candidate. The arsenic compound might be a diacyl glyceride with the $(CH_3)_2As=O$ group linked to one of the glycerol carbon atoms by an arsenic–carbon (As–C) or an arsenic–sulfur–carbon (As–S–C) bond. An arsenic–oxygen–carbon (As–O–C) linkage is unlikely because of the hydrolytic instability of most esters of arsenic oxo acids. The sample is certainly not a pure arsenic-containing lipid, because in such a lipid the arsenic concentration should reach approximately 100 mg g^{-1}. The possibility might also be considered that a methylated arsenic compound (perhaps arsenocholine or arsenobetaine containing a quaternary, positively charged, arsenic atom) of lower molecular mass present in the shark liver or formed from a high-molecular-mass compound by decomposition during extraction and purification is paired with a lipophilic organic compound (e.g. a fatty acid). Such an ion-pair could behave as a lipophilic arsenic compound in the extraction and purification procedures used. The identification of the arsenic compound in the shark liver requires that extractions be carried out on a larger scale to allow the isolation of a pure arsenic compound for characterization.

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