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Arsenolipids

Arsenic-Containing Long-Chain Fatty Acids in Cod-Liver Oil: A Result of Biosynthetic Infidelity?**

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Cod-liver oil, like other oils derived from marine fish, contains appreciable concentrations of lipid-soluble arsenic compounds.[1] Despite extensive studies on arsenic in marine samples,[2] the structures of these arsenolipids have remained unknown. We now report the isolation of six arsenolipids from cod-liver oil, and their identification as a series of novel arsenic-containing long-chain fatty acids (Figure 1). These arsenic compounds occur in cod liver alongside the usual long-chain fatty acids. Their presence raises questions about the fidelity of biosynthetic reactions and the consequences of consumption of arsenic-containing analogues of such important dietary components.

Crude cod-liver oil (containing 5 μg $As\,g^{-1}$) was partitioned between hexane and aqueous methanol, and the polar phase subjected to preparative chromatography with size-exclusion and anion-exchange media to yield a fraction enriched in polar arsenolipids. Analysis of this fraction by HPLC–inductively coupled plasma mass spectrometry (ICP-MS) revealed the presence of at least 15 arsenolipids (Figure 2). Further investigation of the fraction with HPLC–electrospray ionization MS (ESI-MS), under conditions that provided simultaneous detection of elemental arsenic and molecular masses,[3] showed that six of the major arsenicals (**A**–**F** in Figure 2) had the following molecular masses: **A** 334, **B** 362, **C** 390, **D** 418, **E** 388, and **F** 436. The mass spectral data for four of these compounds (**A**–**D**) were consistent with the presence of a homologous series of arsenic-containing saturated fatty acids of the type $(CH_3)_2As(O)$-$(CH_2)_nCOOH$ (n = 12, 14, 16, and 18) with a dimethylarsinoyl group, $(CH_3)_2As(O)$-, replacing the methyl group in myristic, palmitic, stearic, and arachidic acids.

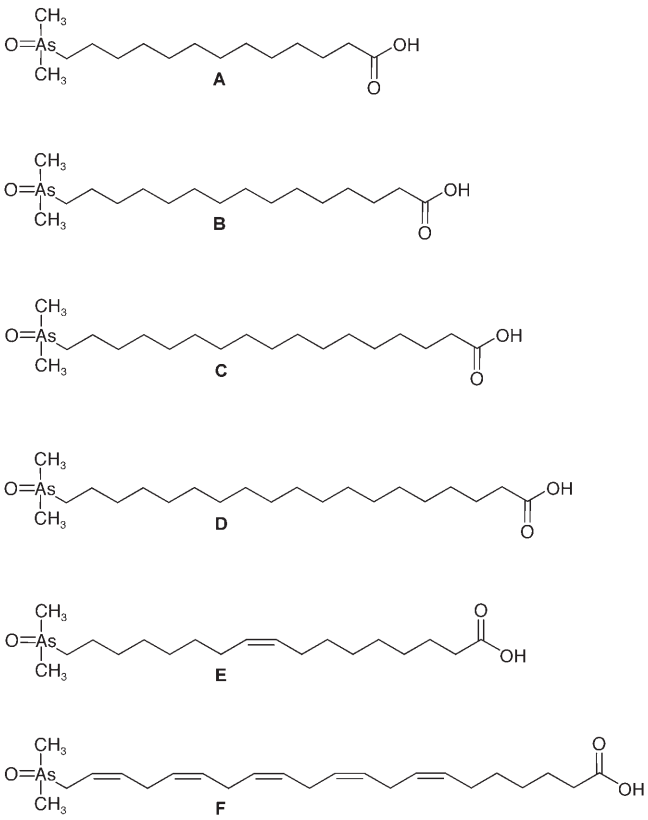

Figure 1. Arsenic-containing fatty acids identified in cod-liver oil. The letters **A**–**F** refer to chromatographic peaks in Figure 2. The position and geometry of the double bonds in compounds **E** and **F** were not determined.

High-resolution MS (HRMS) was then performed on the isolated compounds corresponding to HPLC peaks **A**–**F**. The experimental values closely matched (Δm = 0.6–4.5 ppm; see the Supporting Information) the calculated values for the four arsenic-containing saturated fatty acids (Figure 1, **A**–**D**), thereby supporting the proposed structures. We synthesized the palmitic homologue (n = 14) of the series; the synthesized arsenic-containing fatty acid displayed chromatographic and mass spectral behavior identical with those of the natural product. In addition to the four saturated fatty acids, peaks **E** and **F** could be assigned to unsaturated fatty acids, one with a single double bond (m/z calcd for $C_{19}H_{37}AsO_3$: 389.2032 $[M+H]^+$; found: 389.2019; Δm = 3.3 ppm) and the other with five double bonds (m/z calcd for $C_{23}H_{37}AsO_3$:

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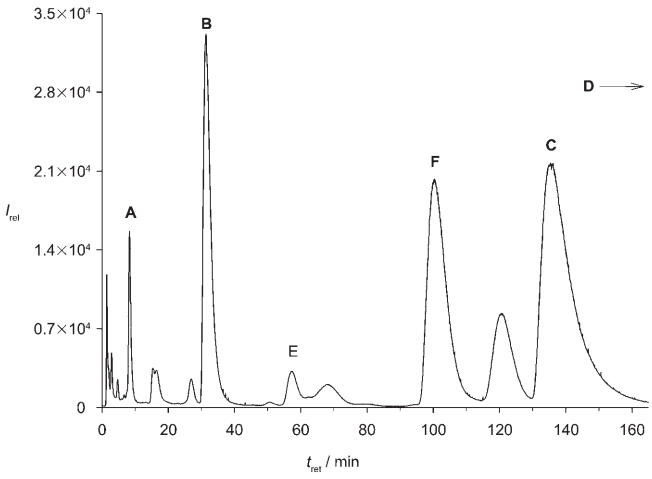

Figure 2. Reversed-phase HPLC of polar arsenolipids concentrated from cod-liver oil; each peak represents an arsenolipid. Compounds **A**–**F** were further investigated by HPLC–ESI-MS. Compound **D** eluted from the HPLC column with increasing methanol concentration in the mobile phase.

437.2032 $[M+H]^+$; found: 437.2028; Δm = 0.9 ppm). Although the positions of the double bonds in the two unsaturated fatty acids cannot presently be assigned, they are likely to be analogous to those of the unsaturated fatty acids oleic acid (C18:1 *n*-9) and 7,10,13,16,19-docosapentaenoic acid (C22:5 *n*-3) commonly found in cod-liver oil.[4]

The arsenic-containing fatty acids identified in the current study account for about 20% of the total arsenolipid content of cod-liver oil. This value is similar to the contribution of free normal fatty acids to the total lipid content of cod-liver oil.[4] Another 20% of the total arsenic was shown to also comprise polar compounds by its partitioning into the aqueous methanol fraction; these compounds, however, were non-acidic as they were not retained on the anion-exchange medium. The bulk of the arsenic (60% of the total) comprised nonpolar (hexane-soluble) arsenic; we have so far been unable to further characterize this arsenic.

The sensitivity and selectivity of ICP-MS allows the detection by HPLC–ICP-MS of arsenic compounds that are present in extracts at very low concentrations. For example, we estimate that compound A, $(CH_3)_2As(O)\text{-}(CH_2)_{12}COOH$, is present in cod-liver oil at a concentration of less than 0.02 μg As g^{-1}. The isolation techniques, particularly anion-exchange chromatography, used to generate the fraction that yielded the HPLC–ICP-MS trace shown in Figure 2 suggest that all 15 or so compounds revealed are closely related to each other, namely, that all are dimethylarsinoyl carboxylic acids.

How and why do such compounds occur, and do they have any toxicological significance? Is the synthesis of the group of compounds as a whole part of a detoxification process that "locks up" potentially toxic arsenic (the lack of biosynthetic specificity being unimportant), or does the production of the entire group of compounds result from a lack of fidelity in the biosynthetic mechanisms for the generation of the essential fatty acids that these arsenic compounds mimic? At present this question is unresolved, although the number of compounds revealed in the trace shown in Figure 2, and their approximate reflection of the essential fatty acids found in cod-liver oil,[4] suggests the latter may be the case.

The nature of the arsenical precursor that becomes ensnared in fatty acid synthesis is far from clear. The alkane backbones of fatty acids are elongated by the addition of two-carbon units derived from acetyl coenzyme A.[5] If these arsinoyl carboxylic acids are lengthened in the same way, what might the starting compound be? The common arsenical metabolite dimethylarsinic acid (cacodylic acid, $(CH_3)_2As(O)OH$) is unlikely to enter directly into the biosynthetic process and would, in any case, by the addition of two-carbon units, yield compounds with the equivalent of an odd number of carbon atoms, whereas all compounds identified here contain the equivalent of an even number. The same is true of dimethylarsinoylacetic acid,[6] $(CH_3)_2As(O)\text{-}CH_2COOH$; the equivalent of an odd number of carbon atoms would result. A more likely starting point is dimethylarsinoylpropionic acid, $(CH_3)_2As(O)\text{-}CH_2CH_2COOH$, possibly resulting from an "arsenylation" by dimethylarsinous acid of oxaloacetate.[7,8] Dimethylarsinoylpropionic acid[6] and its trimethylarsonio analogue,[8,9] the betaine $(CH_3)_3As^+CH_2CH_2COO^-$, are well-characterized water-soluble components of marine samples, which adds some weight to this possibility.

The toxicological relevance of the arsenicals in cod-liver oil remains to be evaluated. Arsenic, a well-known toxic element and a proven human carcinogen, is currently a major human health concern because it occurs in high concentrations, predominantly as arsenate of geological origin, in some sources of drinking water.[10] The human metabolism of arsenate results in the excretion of mainly dimethylarsinic acid in the urine; the methylation processes and toxic intermediates, such as dimethylarsinous acid, generated thereby might be implicated in arsenic's mode of toxic action.[11] Humans also excrete mainly dimethylarsinic acid when they ingest cod-liver oil.[12] Beta-oxidation of the dimethylarsinoyl carboxylic acids with the equivalent of an even number of carbon atoms, as reported here, might indeed yield dimethylarsinic acid. Sequential removal of two-carbon units would lead to $(CH_3)_2As^+(OH)COO^-$, which would most likely spontaneously decarboxylate to yield dimethylarsinous acid; oxidation would then give dimethylarsinic acid. Thus, although the arsenic-containing fatty acids in cod-liver oil could be converted into dimethylarsinic acid without a methylation step, and hence their toxicology might be quite different from that of arsenate, they could nevertheless produce the same toxic species, dimethylarsinous acid, en route to dimethylarsinic acid.

In summary, we have reported the presence of a novel group of arsenic-containing long-chain fatty acids as natural constituents of cod-liver oil. In future work, we will investigate the presence of these and related arsenolipids in other marine samples, and delineate their possible biological and toxicological significance.

Experimental Section

Concentration of arsenolipids: Crude cod-liver oil (3.3 kg containing ca. 0.0005% As) was partitioned batchwise between hexane and water/methanol (1:9, v/v). Evaporation of the aqueous methanol layer yielded a fraction concentrated in arsenic (25.7 g; 0.022% As). Arsenic was determined in the fractions by ICP-MS after acid mineralization of a portion of the fractions.

Preparative size-exclusion chromatography and anion-exchange chromatography were performed on a portion of the aqueous methanol-soluble fraction with Sephadex LH-20 + methanol and DEAE Sephadex A-25 + methanol/chloroform/aqueous sodium acetate (60:30:8, v/v/v), respectively. Chromatography was performed with increasing concentrations of sodium acetate (from 0.02 to 1.0 M), and the arsenic was monitored in the fractions by graphite-furnace atomic absorption spectrometry. A portion (50%) of the arsenic eluted from the anion-exchange column in the neutral/basic fraction, whereas the remaining 50% was shown to be acidic by its retention on the column and subsequent elution as a single peak. The acetate buffer was removed (Sephadex LH-20 + methanol) to yield the acidic arsenolipid fraction (5.8 mg, ca. 3.5% As), which was used for HPLC–MS measurements.

HPLC–ICP-MS: A Waters Atlantis dC_{18} reversed-phase HPLC column (1.0 × 150 mm) was used at 30 °C with methanol/20 mM ammonium acetate, pH 6 (1:1, v/v) as mobile phase at a flow rate of 100 μL min^{-1}. The injection volume was 2 μL and contained about 0.2 μg As. Detection was by an Agilent 7500ce ICP-MS instrument measuring As^+ at *m*/*z* 75.

HPLC–ESI-MS: The HPLC conditions were the same as above, but with 5 mM ammonium acetate as buffer. Chromatography was performed with gradient elution: 0–10 min, 50% MeOH; 10–30 min increasing to 90% MeOH; 30–50 min, 90% MeOH. The injection volume was 2 μL containing about 0.2 μg As. Simultaneous detection of arsenic (selected ion monitoring (SIM), *m*/*z* 75) and arsenolipids (SIM at fixed *m*/*z*, and scan from *m*/*z* 250 to 500) was by ESI-MS (Agilent single quadrupole) in positive-ion mode with variable fragmentor voltages.[3] In a repeat chromatographic run, the eluent from the column was collected in 1-min (100-μL) fractions, each of which was evaporated before analysis by HRMS.

HRMS: Accurate masses were obtained either by matrix-assisted laser desorption/ionization (MALDI) Fourier-transform ion cyclotron resonance MS using an IonSpec mass spectrometer equipped with a 4.7-T magnet, or by nano-ESI using an MDX/Sciex Q-Star Pulsar (quadrupole time-of-flight) mass spectrometer.

For the MALDI measurements (performed on compounds **A**–**C** and **F**), evaporated fractions from HPLC were redissolved in methanol (ca. 200 μL), and nanoliter amounts were deposited on a matrix of crystallized 3,5-dihydroxybenzoic acid. Spectra were obtained from five to ten laser shots and recalibrated using a matrix ion (*m*/*z* 273) as an internal standard. All the MALDI spectra (see the Supporting Information) showed, besides the ions from the protonated arsenolipid, ions arising from a sodiated species. Three of the four spectra also showed ions resulting from loss of water from the protonated species. The mass resolution (full width at half maximum height) for the protonated species of the four compounds ranged from 17000 to 28700.

For the electrospray measurements (performed on compounds **D** and **E**), the fractions were redissolved in either methanol or methanol/5 mM ammonium acetate (1:1, v/v; ca. 200 μL). In each case, spectra were recorded immediately after the instrument had been externally calibrated with the two cluster ions from a NaI solution closest to the relevant mass (that is, *m*/*z* 322 and 472). Both of the electrospray spectra showed sodiated species in addition to the protonated arsenolipid. The mass resolution (full width at half maximum height) for the protonated species was 11500 for compound **D**, and 8800 for compound **E**.

Synthesis of 15-dimethylarsinoylpentadecanoic acid: Methyl 15-hydroxypentadecanoate, synthesized from ω-pentadecalactone according to the method of Hostetler et al.,[13] was converted to the triflate by the method of Klotz and Schmidt.[14] The triflate was treated with sodium dimethylarsenide,[15] and the product was base-hydrolyzed to yield 15-dimethylarsinoylpentadecanoic acid.

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