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Rapid Communication

Arsenosugar phospholipids and arsenic hydrocarbons in two species of brown macroalgae

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Environmental context. Although organoarsenic compounds occur in marine organisms at high concentrations, the origin and role of these compounds is unknown. Arsenic-containing lipids (arsenolipids) are newly discovered compounds in fish. We identify a range of arsenolipids in algae and propose that algae are the origin of these unusual arsenic compounds in marine ecosystems.

Abstract. Fourteen arsenolipids, including 11 new compounds, were identified and quantified in two species of brown algae, Wakame (*Undaria pinnatifida*) and Hijiki (*Hizikia fusiformis*), by high resolution mass spectrometry, high performance liquid chromatography–mass spectrometry and gas chromatography–mass spectrometry. Both algal species contained arsenosugar-phospholipids as the major type of arsenolipid, and arsenic-hydrocarbons were also significant components, particularly in Hijiki. The origin of the various arsenolipids, and the possible significance of their relative quantities, is briefly discussed.

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Arsenic-containing organic compounds are abundant in marine ecosystems where they are thought to play a pivotal role in the cycling and detoxification of potentially toxic inorganic arsenic (arsenate) present in seawater.[1] Although most of the arsenic compounds identified so far have been water-soluble species, the early work on arsenic marine chemistry focussed on lipid-soluble compounds, so called arsenolipids.[2–4] Identification of these arsenolipids proved difficult, however, and it was not until 1988 that an arsenolipid was first rigorously characterised and identified as an arsenosugar-containing phospholipid[5] (see Table 1, compound As-PL958).

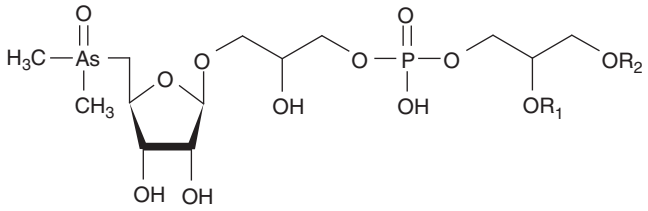
Subsequently, the range of naturally occurring arsenolipids has been extended with the discovery of arsenic-containing fatty acids in fish oils,[6] and arsenic-containing hydrocarbons in fish oils,[7] fish liver,[8] sashimi tuna[9] and fish meal.[10] The origin of these compounds was presumed to be algae. We report the arsenolipid profiles of two species of brown algae, determined mainly by high performance liquid chromatography–mass spectrometry (HPLC-MS), and we briefly discuss the possible biosynthetic origin of these unusual compounds.

Samples of Wakame (*Undaria pinnatifida*, 40 ± 3 μg As g^{-1} dry mass) and Hijiki (*Hizikia fusiformis*, 113 ± 5 μg As g^{-1} dry mass),[A] obtained from a Japanese commercial source, were extracted with a mixture of chloroform and methanol using a modification[B] of the classical procedure of Bligh and Dyer.[11] The lipid fraction containing 6.7 % (Wakame) and 1.6 % (Hijiki)

[A]*Determination of arsenic contents.* Total arsenic analyses were performed on portions of the dry powders, extracts or combined fractions from the silica columns by ICP-MS (Agilent 7500ce) in helium collision cell mode following a microwave-assisted acid mineralisation step. The method was validated by analysis of reference material NIES No. 9 Sargasso (certified As content 115 ± 5 μg As g^{-1}); we obtained 116 ± 2 μg As g^{-1} ($n = 3$).

[B]*Extraction and purification of arsenolipids from algae.* Extraction of arsenolipids from algae was performed in triplicate from two species of algae (Wakame and Hijiki). Following is a typical procedure and outcome for these extractions. A portion (1.013 g dry mass containing 40 μg of As) of Wakame was extracted with a mixture of chloroform/methanol (2 : 1 v/v; 25 g) in a mechanical shaker overnight. The mixture was centrifuged, and the supernatant was washed with bicarbonate solution (1 % w/v; 2 × 20 mL) to remove the water-soluble arsenic compounds. The chloroform layer was separated and evaporated to dryness to yield a residue (crude As-lipid fraction, 175 mg containing 2.7 μg of As), which was re-dissolved in a mixture of chloroform/acetone (1 : 1 v/v, 1 mL). A portion (500 μL) of this solution was applied to a 'plug' of silica (conditioned with chloroform/acetone, 1 : 1 containing 1 % formic acid), packed into a Pasteur pipette. The silica was washed with the conditioning solvent mixture (5 × 1 mL), and then methanol (3 × 1 mL) and finally methanol containing 1 % aqueous ammonia (10 × 1 mL). Arsenic was located in the fractions by using graphite furnace atomic absorption spectrometry. Five of the methanol–ammonia fractions containing >85 % of the total arsenic applied to the column were combined and evaporated to dryness (purified As-lipid fraction, 8.8 mg containing 1.2 μg of As). This material was re-dissolved in methanol (200 μL) and, together with the crude As-lipid fraction, analysed by HPLC-ICPMS and HPLC-ESMS.

Table 1. Arsenosugar-phospholipids in algae identified by high resolution mass spectrometry (for spectra, see Supplementary material)
The positions of R_1 and R_2 relative to each other are arbitrary; the proposed position of the double bond is based on the natural occurrence of unsaturated fatty acid groups. Compounds are coded to designate that they are arsenosugar-phospholipids (As-PL) with the stated nominal molecular mass. Compounds are listed in order of increasing molecular mass; the order of high performance liquid chromatography elution differs at times from the molecular mass order because of the increased polarity resulting from the inclusion of a double bond. Δm is the mass difference between calculated and found molecular masses



Compound code	R_1, R_2	Molecular formula	$[M + H]^+_{found}$	$[M + H]^+_{calc}$	Δm
As-PL930	$-C(O)(CH_2)_{12}CH_3$ $-C(O)(CH_2)_{14}CH_3$	$C_{43}H_{85}O_{14}PAs$	931.4883	931.4887	−0.46
As-PL944	$-C(O)(CH_2)_{12}CH_3$ $-C(O)(CH_2)_{15}CH_3$	$C_{44}H_{87}O_{14}PAs$	945.5036	945.5032	0.49
As-PL956	$-C(O)(CH_2)_{12}CH_3$ $-C(O)(CH_2)_7CH=CH(CH_2)_5CH_3$	$C_{45}H_{87}O_{14}PAs$	957.5048	957.5044	0.43
As-PL958[A]	$-C(O)(CH_2)_{14}CH_3$ $-C(O)(CH_2)_{14}CH_3$	$C_{45}H_{89}O_{14}PAs$	959.5195	959.5200	−0.52
As-PL982	$-C(O)(CH_2)_7CH=CH(CH_2)_5CH_3$ $-C(O)(CH_2)_7CH=CH(CH_2)_5CH_3$	$C_{47}H_{89}O_{14}PAs$	983.5192	983.5200	−0.90
As-PL984	$-C(O)(CH_2)_7CH=CH(CH_2)_7CH_3$ $-C(O)(CH_2)_{16}CH_3$	$C_{47}H_{91}O_{14}PAs$	985.5345	985.5357	−1.22
As-PL986	$-C(O)(CH_2)_{14}CH_3$ $-C(O)(CH_2)_{16}CH_3$	$C_{47}H_{93}O_{14}PAs$	987.5506	987.5513	−0.80
As-PL1012	$-C(O)(CH_2)_{16}CH_3$ $-C(O)(CH_2)_7CH=CH(CH_2)_5CH_3$	$C_{49}H_{95}O_{14}PAs$	1013.5663	1013.5670	−0.72
As-PL1014	$-C(O)(CH_2)_{16}CH_3$ $-C(O)(CH_2)_{16}CH_3$	$C_{49}H_{97}O_{14}PAs$	1015.5822	1015.5826	−0.44
As-PL1042	$-C(O)(CH_2)_{16}CH_3$ $-C(O)(CH_2)_{18}CH_3$	$C_{51}H_{101}O_{14}PAs$	1043.6136	1043.6139	−0.30
As-PL1070	$-C(O)(CH_2)_{18}CH_3$ $-C(O)(CH_2)_{18}CH_3$	$C_{53}H_{105}O_{14}PAs$	1071.6431	1071.6452	−1.97

[A]As-PL958 is the arsenosugar-phospholipid first reported in Wakame by Morita and Shibata.[5]

of the initial total arsenic was passed through a small plug of silica, which removed 90 % of the sample mass with little loss of arsenic. Both the pre- and post-silica fractions were then analysed by reversed-phase HPLC with simultaneous detection of arsenic and organo-arsenic compounds by using inductively coupled plasma mass spectrometry (ICPMS) and electrospray mass spectrometry (ESMS) respectively.[C] The clean-up step with silica was essential to obtain good ESMS data. ICPMS, a more robust detection method, was capable of measuring the arsenolipids in both pre- and post-silica fractions, and provided data showing that the pattern of arsenolipids was essentially unchanged by the silica clean-up step.

HPLC-MS revealed the presence of at least 10 arsenic-containing peaks or bands (Fig. 1). The peaks eluted at ~23 to 25 min from the HPLC column were shown to contain two known arsenic-hydrocarbons (Fig. 2, As-HC332 and As-HC360) by accurate mass measurements and gas chromatography (GC)-MS analysis (see Supplementary material, Fig. S1).[12] In addition, a new arsenic-hydrocarbon (retention time (RT) 26.5 min; Fig. 2, As-HC388) was identified in Wakame by accurate mass measurements and GC-MS. A major peak in the HPLC chromatogram (RT = 27.5 min) for both Wakame and Hijiki had a protonated molecular mass of 959, which matched that for the arsenosugar-phospholipid previously characterised by Morita and Shibata.[5] In addition, there was a series of peaks before and after the peak with *m/z* 959 separated by 28, 26 or 24 mass units, which indicated the presence of a homologous series of saturated and unsaturated arsenosugar-phospholipids (Fig. 1). These assignments were strongly supported by accurate mass

[C]*HPLC-ICPMS and -ESMS analyses.* Separation of the lipid-soluble arsenic species was performed under reversed-phase HPLC conditions by using a Zorbax Eclipse XDB-C8 column (4.6 × 150 mm; 5-μm particle size; Agilent Technologies, Waldbronn, Germany) and a mobile phase comprising acetic acid (10 mmol L^{-1} at pH 6.0, adjusted with aqueous ammonia) and methanol under the following gradient elution conditions: 0–25 min, 50–95 % methanol; 25–40 min, 95 % methanol. The column effluent was split whereby 10 % was directed to the ICPMS and 90 % to the ESMS using an Agilent G1968D active splitter and introducing a sheath flow of 0.2 mL min^{-1} (5 % methanol and 0.1 % formic acid). To prevent deposition of carbon on the interface cones of the ICPMS, an optional gas (1 % oxygen in argon) was introduced. ESMS data were obtained by selected ion monitoring (SIM) in positive ion mode at a fragmentor voltage of 150 V.

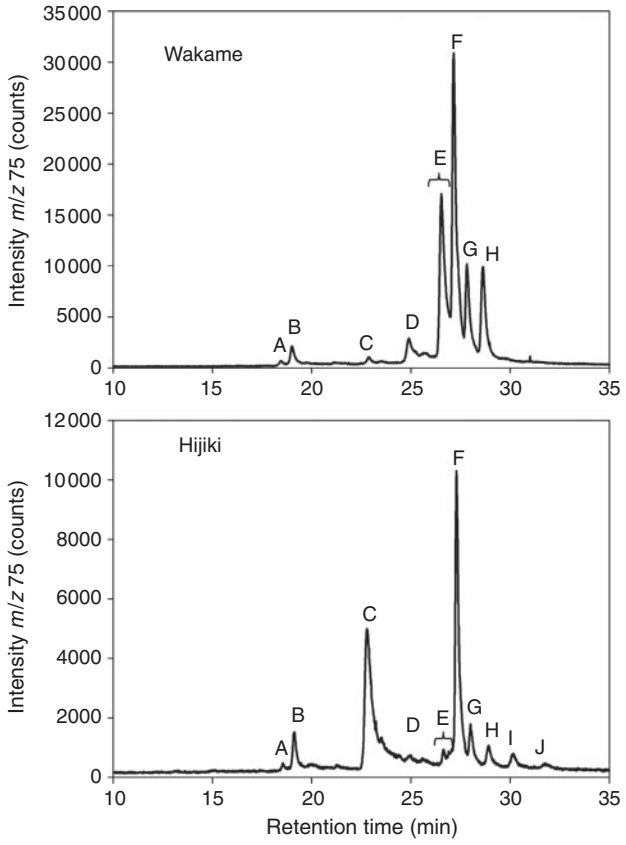
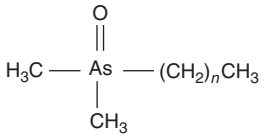


Fig. 1. High performance liquid chromatography (HPLC)–inductively coupled plasma mass spectrometry of lipid extracts (post-silica clean-up) from Wakame and Hijiki.[C] A small peak (polar arsenic) was always present at the column void volume (~2 min) resulting from partial degradation of the arsenolipids during sample handling.



Arsenic-hydrocarbons
As-HC332 (n = 14, molecular mass 332)
As-HC360 (n = 16, molecular mass 360)
As-HC388 (n = 18, molecular mass 388)

Fig. 2. Arsenic-hydrocarbons in Wakame and Hijiki; As-HC332 & As-HC360, previously identified in fish oil[7] and sashimi tuna,[9] were present in both Wakame and Hijiki. The homologue As-HC388 is first reported here where it was shown to be present in Wakame (but not Hijiki). As-HC388 was identified by high resolution mass spectrometry {$[M+H]^+_{calc.}$ 389.2759, $[M+H]^+_{found}$ 389.2755, Δm 1.04} with supporting tandem mass spectrometry and gas chromatography–mass spectrometry data (see Supplementary material).

measurements[D] performed on 11 of the compounds, which agreed in every case to within 2 ppm of the calculated molecular masses (Table 1). The possible presence in algae of a range of arsenosugar-phospholipids differing only in their fatty acid content had been predicted in 1988 by Morita and Shibata[5]; our data confirm their astute early prediction.

Quantification of the major arsenic compounds in the two algal species was achieved by HPLC-ICPMS using a modification of reported methods (Table 2).[13,14] Although both species contained arsenosugar-phospholipid As-PL958 as a major compound, Wakame also contained significant quantities of other arsenosugar-phospholipids, whereas Hijiki contained a higher proportion of arsenic-hydrocarbons. The possible significance of these differences awaits studies with more samples of these two algae and other algal species from various locations. Such studies would be best carried out with samples collected directly from the natural environment, rather than with commercial products, thereby excluding the possibility of artefact formation.

The presence of both arsenic-hydrocarbons and arsenosugar-phospholipids in algae raises questions as to how these unusual compounds are synthesised, and what their possible roles in algae might be. The early experiments of Benson and co-workers provide some information on the biochemical processes involved.[3] By using radiolabelled arsenic and monitoring products by radiochromatography, they showed that unicellular algae take up arsenate from seawater and convert it into lipid- and water-soluble arsenic species. By selective use of enzymes (e.g. phospholipases A and D), they also demonstrated that the lipid-soluble arsenic comprised three types of lipids: phospholipids, lysophospholipids and an unknown lipid class. Although the water-soluble products from the enzyme treatments were incorrectly assigned at that time, they were later correctly ascribed to arsenosugars.[1,15–17] Our definitive results on the structures of the major arsenolipids in algae are consistent with the earlier observations. Collectively, the data suggest that the arsenosugar-phospholipids might be the target arsenic species synthesised by algae, and that the arsenosugars are merely degradation products of compounds excess to the algae's requirements.

The third, unknown, class of lipid reported by Benson and co-workers[3] did not degrade with the enzymes they tested. Possibly, that group of compounds comprised the arsenic-hydrocarbons found in our study. These compounds are likely to have a biosynthetic pathway quite different from that of the arsenosugar-phospholipids, although their properties might be similar since they both contain polar head groups and long non-polar tails. We speculate that both these types of arsenolipids could be used by algae in membrane chemistry.

Possibly, arsenolipids impart a particular useful property to cell membranes not attainable from normal membrane lipids. In this regard, the recent work by Van Mooy et al.[18] is relevant because they reported that algae can utilise S and N in place of P to form membrane lipids. In that case, however, the driving force was considered to be the need for algae to conserve phosphate for use in other essential cell biochemistry. Thus, the relative quantities of arsenosugar-phospholipids and arsenic-hydrocarbons in the algae might reflect phosphate levels in water or the relative phosphate requirements of algal species; i.e. when phosphate levels are limiting, arsenic-hydrocarbons are preferentially synthesised by algae for membrane chemistry. We are currently investigating these biological questions,

[D] *Accurate mass measurements*. In a separate HPLC run, the effluent was split with 10% going to the ICPMS and the rest collected in 100- or 200-μL fractions. Accurate mass measurements were made on these fractions by direct infusion (5 μL min^{-1}) with a LTQ Orbitrap XL (Thermo Scientific), equipped with a heated electrospray ion source. The system was operated in Fourier transform mass spectrometry (FTMS) high resolution positive scan mode (60 000 resolution) under the following conditions: source voltage, 5.01 kV; vaporiser temperature, 36 °C; sheath gas flow, 20.08 arb; auxiliary gas, 9.98 arb; capillary voltage, 16.02–36 V; capillary temperature, 275 °C; tube lens voltage, 69–124 V.

Table 2. Quantification of arsenolipids in two species of brown algae

Data represent the mean ± s.d. of three analyses each involving extraction, clean-up and high performance liquid chromatography (HPLC)–inductively coupled plasma mass spectrometry. The percentage lipid values are based on the total As applied to the HPLC column (post-silica fraction); column recoveries ranged from ~70 to 110 %

Peak or band number (Fig. 1)	Assigned structure (Table 1)	Wakame		Hijiki	
		Concentration ($\mu g\ As\ kg^{-1}$)	Percentage of lipid As	Concentration ($\mu g\ As\ kg^{-1}$)	Percentage of lipid As
A	U1	10 ± 1	0.68 ± 0.08	9 ± 1	0.70 ± 0.08
B	U2	46 ± 8	3.0 ± 0.9	53 ± 1	3.9 ± 0.2
C	As-HC332	22 ± 3	1.4 ± 0.3	309 ± 16	22.9 ± 0.2
D	As-HC360	82 ± 37	6 ± 3	35 ± 9	2.6 ± 0.6
E	As-HC388	406 ± 121	27 ± 11	22 ± 2	1.7 ± 0.1
	As-PL930				
	As-PL944	Not quantified	–	Not quantified	–
	As-PL956	Not quantified	–	Not quantified	–
	As-PL982	Not quantified	–	Not quantified	–
F	As-PL958	426 ± 136	28 ± 12	251 ± 39	18 ± 2
	As-PL988	144 ± 52	10 ± 4	85 ± 3	6.3 ± 0.4
G	As-PL956	200 ± 74	13 ± 6	58 ± 9	4.2 ± 0.6
	As-PL1012				
H	As-PL1014	226 ± 66	15 ± 6	48 ± 12	3.6 ± 0.8
I	As-PL1042	27 ± 11	1.8 ± 0.5	32 ± 10	2.4 ± 0.8
J	As-PL1070	Not quantified	–	21 ± 3	1.6 ± 0.1

building on the preliminary quantitative results reported here for different types of arsenolipids in algae.

Supplementary material

The following information is available as Supplementary material:

- GC/MS of arsenic-hydrocarbons in Wakame and Hijiki.
- High resolution mass spectra of arsenic-hydrocarbons found in Wakame or Hijiki.
- High resolution mass spectra of arsenosugar-phospholipids found in Hijiki.

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