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The Characterization of Arsenosugars in Commercially Available Algal Products Including a *Nostoc* Species of Terrestrial Origin

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The arsenic species present in a range of commercially available dried-algal food products were characterized by HPLC–ICP–MS. The products of marine origin contain up to four dimethylarsinylribosides (1) in the 8–49 ppm range and some also contain dimethylarsinic acid (DMAA). These species are easily extracted and account for most of the arsenic burden. One sample of a freshwater alga *Nostoc* sp. was found to contain a lower concentration of arsenic, 3 ppm, and only 34% of this was extractable. The extract representing 1 ppm of arsenic contained one of the arsenosugars 1 found in the marine samples (93%), the rest being DMAA. This is the first report of the identification of an arsenosugar from an organism of terrestrial origin. The implications of this result in connection with the global arsenic cycle are discussed. © 1997 John Wiley & Sons, Ltd.

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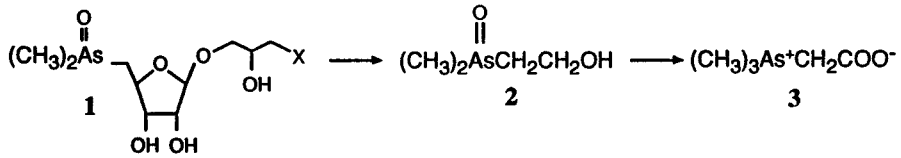
INTRODUCTION

Although the arsenic concentration in seawater is in the low parts-per-billion (1:10^9; ppb) region unusually high levels of arsenic (1–100 ppm) are found in marine animals and plants and, consequently, in food that orginates from marine sources.[1,2]

Modern studies of arsenic speciation in the marine environment have revealed a very rich chemistry, and a wide variety of compounds have been characterized.[2] Arsenosugars such as **1** are the dominant species in marine macroalgae, but in animals the most abundant arsenical is arsenobetaine (**3**), although higher concentrations of tetramethylarsonium ion can be found in some clams.[3,4] Edmonds and co-workers[5] have shown that the arsenicals in macroalgae are decomposed by anaerobic microorganisms to dimethylarsinylethanol (**2**) and, although **2** has never been detected in the environment, these workers



Scheme 1.

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suggest that **2** is a precursor to **3**. This pathway (Scheme 1) has not been directly verified even though rational steps involving oxidative methylation and reduction can be devised.

Arsenosugars have not been identified outside the marine environment apart from their presence in the urine of individuals who have ingested seafood.[6] Arsenobetaine (**3**) is also found in the urine of humans for the same reason. However, Irgolic and co-workers have recently reported[7] that **3** was present in the urine of a male subject who was exposed only to arsenic species used in the preparation of trimethylarsine, i.e. he did not eat arsenobetaine-containing food. This same group have also found **3** but not **1** in terrestrial mushrooms.[8,9] In both these instances it seems unlikely that **3** orginates from **1**.

The present paper is concerned with the characterization of the arsenic species present in commercially available algal products. Not unexpectedly, arsenosugars predominate in the products of marine origin. However we find, for the first time, an arsenosugar, but not arsenobetaine, in the terrestrial alga *Nostoc* sp., thus opening the door to the possibility that the conversion **1→3** can take place outside the marine environment.

EXPERIMENTAL

Reagents and chemicals

All chemicals used were of analytical grade unless otherwise stated and included methanol (HPLC grade, Fisher), tetraethylammonium hydroxide (20% wt, Aldrich), tetrabutylammonium hydroxide (Aldrich), malonic acid (BDH), sodium borohydride (Aldrich), hydrochloric acid (36.5%, Fisher), sulphuric acid (98%, Fisher), nitric acid (69%, sub-boiling distilled, Seastar Chemicals) and hydrogen peroxide (30%, Fisher). Deionized water with resistivity better than 1 MΩ was used for the extractions and to make up the eluent for HPLC.

Table 1 Operating parameters for ICP–MS

Forward radio-frequency	1350 W
Reflected power	<10 W
Outer (cooling) gas flow rate	13.8 l min^{-1}
Intermediate (auxiliary) gas flow rate	0.65 l min^{-1}
Nebulizer gas flow rate	1.002 l min^{-1}
Analysis mode	TRA, 1 s time slice
Quadrupole pressure	9×10^{-7} mbar
Expansion pressure	2.4 mbar

The glassware and plasticware were cleaned by soaking in 2% Extran solution overnight, rinsing with water and deionized water, then soaking in 0.1 M HNO_3 solution overnight. They were then rinsed with deionized water and air-dried.

Samples

Collection

Commercial products of *Nostoc* sp. (*Nostoc commune* var. *flagelliforme*) were purchased at food stores in Guang Zhou, China, in Richmond, BC, Canada, as well as in Honolulu, Hawaii. All products originated from China. The source of other products is given in Table 3. Oyster tissue standard reference material (SRM NIST 1566a) was purchased from the National Institute of Standards and Technology, US Department of Commerce.

Storage

Commercial products were kept in their packages away from direct sunlight in the laboratory.

Table 2 Summary of experimental conditions for HPLC

Conditions	Column	Mobile phase	Flow rate (ml min^{-1})
1(a)	Inertsil ODS (GL Sciences, Japan)	10 mM tetraethylammonium hydroxide (TEAH), 4.5 mM malonic acid, 0.1% MeOH, pH 6.8	0.8
1(b)	Inertsil ODS (GL Sciences, Japan)	10 mM tetrabutylammonium hydroxide (TBAH), 4.5 mM malonic acid, 0.1% MeOH, pH 6.8	1.0
2(a)	Inertsil 5 ODS-2 (Phenomenex)	10 mM TEAH, 4.5 mM malonic acid, 0.1% MeOH, pH 6.8	0.8
2(b)	Inertsil 5 ODS-2 (Phenomenex)	10 mM TBAH, 4.5 mM malonic acid, 0.1% MeOH, pH 6.8	1.0

Table 3 Total concentrations in the samples

Sample	Source	[As] (ppm)[a] Before extraction	[As] (ppm)[a] After extraction	Extraction efficiency (% As extracted)
Oyster tissue SRM (*Crassostrea gigas*)[b]	NIST	11.0 ± 0.2	1.29 ± 0.05	88.3
Brown algae				
Kelp powder	Eastern Canada	27.7 ± 0.08	4.0 ± 0.3	85.4
Kombu (*Laminaria groenlandica*)	BC, Canada	18.4 ± 0.4	5.1 ± 0.8	72.4
Bull kelp (*Nereocystis leutkeana*)	BC, Canada	39 ± 2	1.0 ± 0.1	97.5
Arame (*Eisenia bicyclis*)	Japan	15 ± 1	1.6 ± 0.1	89.4
Laminaria sp.	China (PRC)	49 ± 3	4.5 ± 0.2	90.9
Red algae				
Dulse (*Palmaria palmata*)	NB, Canada	7.6 ± 0.5	0.65 ± 0.09	91.4
Nori (*Porphyra tenera*)	China (PRC)	16 ± 1	0.46 ± 0.03	97.1
	Japan	7.6 ± 0.6	0.20 ± 0.09	97.4
	Taiwan (RC)	21 ± 1	0.4 ± 0.1	98.1
Blue–green algae				
Nostoc commune var. *flagelliforme* (*Nostoc flagelliforme*)	China (PRC)	2.70 ± 0.08	1.74 ± 0.07	34.2

[a] ± the standard deviation of triplicate analyses.
[b] The certified concentration of arsenic in oyster tissue SRM is 14.0 ± 1.2 ppm.

Extraction with MeOH/H_2O

As an example, the commercial *Nostoc* sp. powder (0.5–1 g dry weight) was extracted by using a procedure similar to that described by Shibata and Morita.[11] Each sample was weighed into a 15-ml or 50-ml centrifuge tube. To each tube was added 10 ml (g sample)$^{-1}$ of a methanol/water mixture (1:1, v/v). The tube was sonicated for 10 min and centrifuged for 10 min. After centrifugation, the extract was removed by means of a Pasteur pipette and placed in a round-bottom flask. The extraction procedure was repeated an additional four times for each sample. The combined extract was evaporated to dryness and dissolved in 10 ml of deionized water prior to further analysis.

Acid digestion

As an example, dry *Nostoc* (0.25 g) or the residue after H_2O/MeOH extraction (0.5 g taken originally) was placed in a 250-ml or 500-ml round-bottom flask fitted with the special condenser, Teflon capillary, four Teflon plugs and a Teflon adaptor described by Bajo *et al.*[12] To each sample, an acid mixture of $H_2SO_4/HNO_3/H_2O_2$ (1:3:3, by vol.) was added[13] and the reaction mixture was refluxed for 2 h,[14] cooled and made up to 25 ml for further analysis.

Analytical procedures

HPLC-ICP-MS

The HPLC system consisted of a Waters model 510 delivery pump, a Reodyne model 7010 injector valve with a 20 μL sample loop, and an appropriate column. The columns used include two reverse-phase C_{18} columns (GL Sciences Inertsil ODS, 250 mm × 4.6 mm; and Phenomenex Inertsil 5 ODS-2, 250 mm × 4.6 mm). A guard column packed with the same material (Supelco) preceded the analytical column. The HPLC system was connected to the ICP nebulizer via a PTFE tube (20 cm × 0.4 mm) and appropriate fittings.

A VG Plasma Quad 2 Turbo Plus inductively coupled plasma–mass spectrometer (VG Elemental, Fisons Instruments) equipped wtih an SX 300 quadrupole mass analyser, a standard ICP torch, and a de Galan V-groove nebulizer, was used as the detector. The time time-resolved analysis (TRA) mode was used to set the mass analyser to monitor the $m/z = 75$ signal peak corresponding to As^+. The TRA mode allowed the possibility of simultaneously monitoring more than one m/z value at a time. All signals were collected and the data were manipulated on a separate computer (MS Excel). A summary of the operating parameters for the ICP–MS is given in Table 1 and the columns and eluents for HPLC in Table 2.

All samples were filtered (0.45 μm) prior to injection onto the column. In general, 20 μl samples were analysed. Arsenic compounds in the samples were identified by matching the retention times of the peaks in the chromatograms with those of standards.

HG–AAS

Atomic absorption measurements were performed with a Jarrell Ash 810 atomic absorption spectrometer equiped with a hydrogen–air flame atomizer. A slit width of 1 nm and a wavelength of 193.7 nm were used. Background correction was not available. A Hewlett-Packard 3390A integrator was connected to the AA spectrometer to record signals. An open-ended T-shaped quartz absorption tube (9.5 cm × 0.8 cm i.d.) was mounted in the flame of the burner. Light from the arsenic hollow-cathode lamp (Varian) was aligned to pass through the wide end of the flame-heated quartz tube. Hydrides from the reaction of the sample with borohydride were introduced through the side arm of the quartz tube (9 cm × 0.4 cm i.d.).

The hydride generation system was the same as described previously[10] except that a commercial Nafion permeation tube (Perma Pure Inc.) fitted with a nitrogen purge (90 ml min^{-1}) was used to dry the hydrides before they reached the quartz tube.

The samples after total digestion were analysed by HG–AAS; they required dilution before analysis. The blank solution was made up from the appropriate amounts of H_2SO_4, H_2O_2 and HNO_3. Standard arsenate, As(V), solutions were also made up in a similar way. After acid digestion of the samples, the only arsenic species present is the oxidized form, As(V);[13] therefore, the use of As(V) solution for calibration was appropriate. The optimized conditions described by Le *et al.*[15] were used for the analysis. Samples were aspirated for 30 s corresponding to the uptake of 2 ml for each analysis. Triplicate analyses were performed for each sample.

RESULTS AND DISCUSSION

The arsenic concentrations in the food products and their sources are listed in Table 4. The concentrations are much as expected, with the brown algae having higher levels than the red.[2] The arsenic concentrations in brown algae from British Columbia are reported[16] to be in the range 40.8–92.4 ppm, but this seems high because the concentration of arsenic in the brown algae *Fucus* sp., widely distributed along the coast of British Columbia, varies with season and location but the average value is about 21 ppm on a dry weight basis.[14] In addition, the commercial products Kombu and Bull kelp have lower levels (Table 4).

Because the toxicity of arsenic compound is very species-dependent[1] it is important to establish the speciation of arsenicals present in arsenic-containing foodstuff. Only then can any evaluation be made regarding risk to human health.

In the case of the kelp product, the arsenic concentration is 27.7 ppm. The arsenic species that are present are easily extracted into water/methanol (85% efficiency). Chromatograms (HPLC–ICP–MS) of the kelp extracts are shown

Table 4 Relative amounts (%) of arsenic compounds in the HPLC–ICP–MS extracts

Samples	**3**	DMMA	**1a**	**1b**	**1c**	**1d**
Oyster tissue SRM	28.3	22.0	26.6	23.1	—	—
Kelp powder	—	—	35.2	3.0	16.4	45.4
Bull kelp	—	0.6	19.7	25.9	53.8	
Kombu	—	1.7	23.5	14.6	60.2	—
Laminaria sp. (China)	—	1.5	16.5	4.5	77.5	—
Arame	—	11.8	17.8	2.9	67.5	—
Dulse	—	5.8	87.8	6.4	—	—
Nori (Japan)	—	—	82.0	18.0	—	—
Nori (China)	—	—	58.1	41.9	—	—
Nori (Taiwan)	—	2.1	69.8	28.1	—	—
Nostoc sp.	—	6.4	93.6	—	—	—

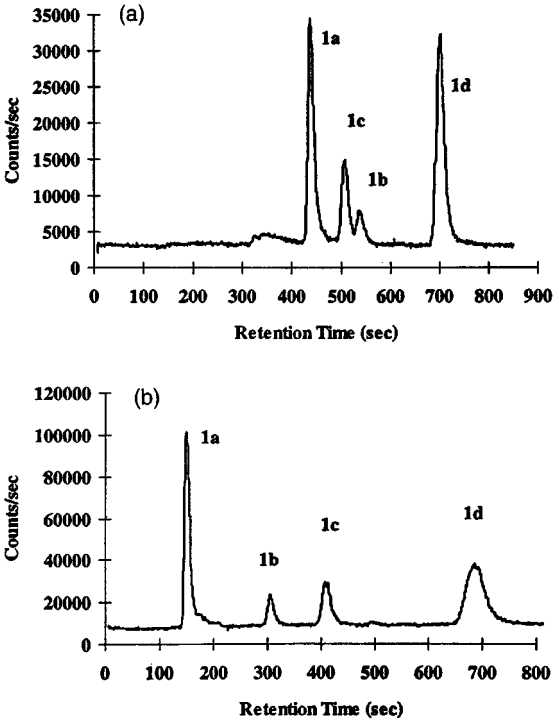

Figure 1 HPLC–ICP–MS analysis of the kelp powder (a) under conditon 1(a); (b) under condition 2(b).

in Fig. 1. It is clear that there are four arsenic compounds present and the first and third peaks can be assigned to arsenosugars **1a** and **1b**, on the basis that their retention times are identical with those of the two arsenosugars known to be present in the oyster tissue SRM.[11, 17] The extracts of the kelp and the oyster tissue were chromatographed under the four sets of conditions listed in Table 2. Examples of these are given in Fig. 1. It should be noted that the elution order of arsenosugars **1b** and **1c** switches when tetraethylammonium hydroxide (TEAH) is replaced by tetrabutylammonium hydroxide (TBAH). An advantage to be noted is that the use of the TBAH system conveniently spreads out bands and allows easy distinction between the known **1b**, and **1c**. A minor disadvantage of this combination is that arsenobetaine (**3**) and **1a** co-elute (confirmed by using standard solutions of **3**[17] even though they are separated by using the TEAH system. However, none of the samples contain **3** so this was not a problem in the present investigation.

A tentative identification of the **1c** and **1d** peaks in the kelp extract was initially made on the basis of the abundance of the arsenicals and their relative retention times.[18] The assignments were confirmed for us by Edmonds and Shibata, who ran samples of the kelp extract by using HPLC–ICP–MS (Asahipak GC-220 HQ gel-permeation column) against their standards. The relative amounts of the arsenosugars found in the kelp extract are listed in Table 4. The kelp powder did not contain detectable amounts of dimethylarsinic acid (DMAA), which is separated from the arsenosugars in the TEAH system; DMAA co-elutes with **1b** in the TBAH system.

The arsenosugar **1a** is a major component of the arsenicals found in *Hizikia fusiforme*,[19] *Sargassum thunbergii*,[20] *Sargassum lacerifolium*[21] and the kidney of the giant glam *Tridacna maxima*.[22] In the present study, the arsenosugars present in dry *Laminaria* (Kombu) from British Columbia (**1a**, **1c** and **1b**) and dry *Laminaria* sp. from China are identical, with the arsenosugar **1c** predominating; some DMAA is also present. The same three arsenosugars are found in fresh *Laminaria japonica*, Makonbu,[23] where the relative proportions of **1a**:**1c**:**1b** are 3:80:17. DMAA was not observed in this earlier investigation.[23]

The three sugars **1a**, **1c** and **1b** are also found in Bull kelp from British Columbia and Arame from Japan, although the concentration of **1b** in the latter is relatively low, and the concentration of DMAA is elevated. The hydrolysis of **1b** to **1a** has been noted,[2] and there is always the possibility that this interconversion could take place during the analytical procedure or during the preparation of products for market.

Dulse is a product from Eastern Canada. Our sample was in the form of a dried powder although it is also available unpowdered. The most significant result is the absence of **1c** and the preponderance of **1a** in the sample. In this regard this red alga, *Palmaria palmata*, is very similar to the *Porphyra* sp. used in the production of Nori. Essentially all the arsenic species are extractable from dulse and Nori, although Shibata and co-workers report lower extraction efficiencies (64–76%) for some Japanese brands.[24] Another difference is the ratio of the concentrations of **1a** and **1b** which, in the present study, we find to be 1:0.24 (average of three samples); the earlier study found 1:51 (average of four samples).[24]

The possibility of the conversion of **1b** to **1a** has been noted above although heating the alga to afford the product known as Yakinori does not appear to affect the distribution.[24] There is also

the possibility that different species of *Porphyra* are being used to prepare Nori:[24] one sample that was used in a human feeding study[6] contained only **1a**.

The arsenic concentration in the blue–green alga *Nostoc* is the lowest of those listed in Table 3, reflecting its terrestrial origin.[25] The extraction efficiency for the arsenicals in the samples (dried, but not powdered) is low, so that most of the arsenic remains with the residue. Maeda and co-workers[25,26] isolated an arsenic-resistant species of *Nostoc* and observed that arsenic accumulated in the cells. Most of this accumulation (90%), which was not extracted by MeOH/$CHCl_3$, appears to be inorganic arsenic species: the extract contains inorganic arsenic and dimethylarsenic species, but the analytical procedures that were used did not allow further identification.[25,26]

The present investigation, which used HPLC–ICP–MS to examine the extracts (Fig. 2), establishes for the first time that arsenosugar **1a** is the major soluble species present in the *Nostoc* sp. The identification was made on the basis that the arsenic species in the sample co-elutes with **1a** under all four sets of conditions outlined in Table 2. As was noted in the Introduction, there was previously no strong evidence for the formation of arsenosugars in the terrestrial environment and, indeed, many workers in the field doubted that they would be found in spite of the fact that plants contain *S*-adenosylmethionine which is believed to deliver both the methyl group and the adenosyl group to arsenic in oxidative addition reactions, the adenosyl group being the precursor to the sugar derivatives.[1,2]

The discovery of **1a** in terrestrial algae raises other important issues, such as the function of arsenosugars in living systems and whether they are the universal precursors to arsenobetain, **1→3**. The literature is essentially silent on the former question but recent studies on the marine brown alga *Fucus gardneri*[28] show a seasonal dependence of both the arsenosugar content and species distribution that may indicate some kind of function. With regard to the **1→3** conversion, the discovery of **1a** in the terrestrial environment at least makes the overall route possible.

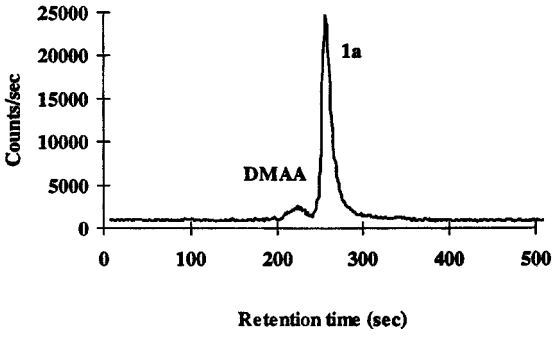

Figure 2 HPLC–ICP–MS analysis of *Nostoc* sp. under condition 2(a).

It is generally believed that arsenosugars are like arsenobetaine in that they are relatively innocuous to humans. However, it should be noted that although humans do not seem to metabolize arsenobetaine, they are able to convert arsenosugars to dimethylarsinic acid, DMAA.[6] Recent studies indicate that DMAA has the potential to be a human carcinogen.[29] It should also be noted that none of the products listed in Table 3 contains significant amounts of inorganic arsenic species, the most toxic form of the element. In contrast, the product Hijiki, made from *Hizikia fusiforme*, has ~50% of the total arsenic as arsenate, the remainder being arsenosugars.[19]

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