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# Arsenic-containing ribofuranosides and dimethylarsinic acid in green seaweed, *Codium fragile*

Kazuo Jin,*† Takaaki Hayashi,† Yasuyuki Shibata‡ and Masatoshi Morita‡
† Hokkaido Institute of Public Health, North 19, West 12, Kitaku, Sapporo 060, Japan, and ‡ National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305, Japan

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**Three water-soluble arsenic compounds were isolated from the green seaweed *Codium fragile*. These compounds were identified as 1-glycerophosphoryl-2-hydroxy-3-[5′-deoxy-5′-(dimethylarsinoyl)-β-ribofuranosyloxy]propane (1a), 1′-(1,2-dihydroxypropyl)-5′-deoxy-5′-(dimethylarsinoyl)-β-ribofuranoside (1b), and dimethylarsinic acid ($(CH_3)_2AsOOH$). The structures of these compounds were ascertained by $^1H$ NMR spectroscopy. Compounds 1a and 1b accounted for 60 % and dimethylarsinic acid for 5% of the water-soluble arsenic.**

**Keywords: Arsenic, seaweed, *Codium fragile*, arsenic-containing ribofuranosides, dimethylarsinic acid**

## INTRODUCTION

Many authors have shown that marine algae contain arsenic at substantial concentrations, typically in the range of 1–100 mg $kg^{-1}$.[1–7] However, structural information on arsenic compounds in algae is very limited. Several authors reported[8–11] during the early stages of speciation studies the presence of dimethylarsinic acid, methylarsonic acid and inorganic arsenite and arsenate in some algae. However, the major water-soluble arsenic compounds in many algae (except in Sargassaceae) are known to be organic arsenic derivatives more complex than simple methylated arsenic acids.[7,12–15]

Arsenic-containing ribofuranosides (ACRs, dimethylribosylarsine oxides) (Fig. 1) were first identified by Edmonds and Francesconi[16,17] in *Ecklonia radiata* and in the giant clam *Tridacna maxima* as the major water-soluble arsenic compounds. Since then, ACRs have been found in the Japanese edible seaweeds *Laminaria japonica*[18] and *Hizikia fusiforme*.[19] Preferences for certain R groups in ACRs (Fig. 1) by certain species have become apparent. Although ACRs were suggested to be ubiquitous in algae,[20] only a few brown algae were examined. Structural information about the arsenic compounds in green and red algae, which generally do not accumulate arsenic to the same extent as brown algae,[1–7] is not available. This paper reports the purification and identification of two ACRs and dimethylarsinic acid present in the green alga, *Codium fragile* (Siphonales) (MIRU in Japanese).

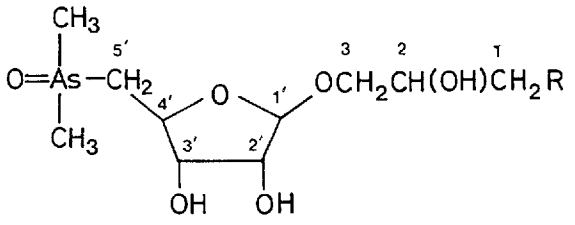
*Author to whom correspondence should be addressed.

## EXPERIMENTAL

Total arsenic was determined by graphite furnace atomic absorption spectrometry (Hitachi Zeeman 170-70) after digestion of the samples with $HNO_3$/$HClO_4$/$H_2SO_4$.[21] Aliquots of chromatographic fractions were not digested but directly injected into the graphite furnace. $^1H$ NMR spectra were measured on a JEOL JNM GX-400 FT spectrometer at 400 MHz at 20°C in $D_2O$ with sodium 2,2-dimethyl-2-silapentanesulfonate as an internal or external standard. 'Evaporation' refers to removal of solvent under reduced pressure at 40°C on a rotary evaporator. Buffer salts were removed from ion-exchange chromatographic fractions by passage through a Sephadex G-15 column (2.6 cm × 90 cm) with water as a mobile phase.

### Extraction and purification of arsenic compounds

The seaweed was collected at the Wakkanai coast of Hokkaido, Japan, on 1 August 1986. Living *C. fragile* were removed from the rocks. The sample (12 kg wet weight; *ca* 7 mg As) was briefly rinsed with tap water and then cut into small pieces in an electric blender (0.5 kg at a time) in the presence of methanol



1a: R = $OP(O)(O^-)OCH_2CH(OH)CH_2OH$

1b: R = OH

**Figure 1** Structures of dimethyl(ribosyl)arsine oxides.

(10 $dm^3$). The mixture was kept for two days at room temperature. The supernatant was obtained by filtration and the extraction repeated two more times (12 $dm^3$ methanol each). The methanol extracts were combined and evaporated to yield a dark syrup (330 g; 6.0 mg arsenic). This syrup was dissolved in water (3 $dm^3$) and the solution successively extracted with diethyl ether (3 $dm^3$) and ethyl acetate (3 $dm^3$). The diethyl ether layer (0.94 mg arsenic) and the ethyl acetate layer (0.02 mg arsenic) were not further examined. The aqueous layer was evaporated to yield a solid gum (300 g; 4.95 mg arsenic). This material was extracted twice with methanol (300 $cm^3$ each). The methanol phase was filtered and the filtrate evaporated to a brown gum (63 g; 4.0 mg arsenic). The gum was dissolved in water (final volume 325 $cm^3$). Seven portions of this solution (40 $cm^3$ each) were chromatographed on a Sephadex LH-20 column (5 cm × 85 cm) with water as the mobile phase. The arsenic compounds eluted at 760–900 $cm^3$ (fraction I), 925–1070 $cm^3$ (fraction II), and 1095–1165 $cm^3$ (fraction III). After pooling and evaporation, the combined fractions I produced 2.6 g residue (0.5 mg arsenic), fractions II 8.6 g residue (2.0 mg arsenic), and fractions III 12.9 g residue (1.0 mg arsenic).

## Isolation of 1-glycerophosphoryl-2-hydroxy-3-[5′-deoxy-5′-dimethylarsinoyl)-β-ribofuranosyloxy]propane (1a)

The residue from the combined fractions I was dissolved in a mixture of water (6 $cm^3$) and 0.05 mol $dm^{-3}$ aqueous Tris buffer (pH 8.0, 4 $cm^3$) (Fig. 2A). This solution was placed on a DEAE-Sephadex A-25 column (2.6 cm × 90 cm; equlibrated with pH 8.0 Tris buffer). Isocratic elution with the same buffer produced arsenic-containing bands at 320–380 $cm^3$ (fraction I-1) and 440–1650 $cm^3$ (fraction I-2). Fraction I-1 (*ca* 60 μg arsenic) was not further purified. Fraction I-2 (*ca* 400 μg arsenic) was further fractionated on a Sephadex G-15 column with water as a mobile phase. Fraction I-2a (310 μg arsenic) left the column before fraction I-2b (90 μg arsenic). The center of fraction I-2a (120 mg; 260 μg arsenic) was collected and chromatographed twice on a DEAE-Toyopearl 650 M column (1.6 cm × 27 cm; Toyosoda Co., Tokyo) with 0.1 mol $dm^{-3}$ Tris/0.2 mol $dm^{-3}$ boric acid (pH 7.0; 360 $cm^3$), 0.01 mol $dm^{-3}$ Tris/0.02 mol $dm^{-3}$ boric acid (pH 7.0; 78 $cm^3$), and 0.05 mol $dm^{-3}$ Tris buffer (pH 8.0) as the mobile phases. The pH 8 Tris buffer eluted the arsenic compound. This fraction was passed through a Sephadex G-15 column for final clean-up. The $^1H$ NMR spectrum of the arsenic-containing material (*ca* 140 μg arsenic) showed it to be identical with 1-glycerophosporyl-2-hydroxy-3-[5′-(dimethylarsinoyl)-β-ribofuranosyloxy]propane (**1a**) previously isolated from *E. radiata*[17] and *L. japonica*.[18]

## Isolation of 1′-(1,2-dihydroxypropyl)-5′-deoxy-5′-(dimethylarsinoyl)-β-ribofuranoside (1b)

Fraction II from the Sephadex LH-20 column was dissolved in water (40 $cm^3$), and Tris buffer (0.05 mol $dm^{-3}$, pH 8.0, 10 $cm^3$) was added. The solution was placed on a DEAE-Sephadex A-25 column (5 cm × 85 cm; equilibrated with 0.05 mol $dm^{-3}$ Tris buffer, pH 8.0) (Fig. 2B). The elution was isocratic with the same buffer. Arsenic-containing fractions were collected at 1.15–1.37 $dm^3$ (fraction II-1; *ca* 1.7 mg arsenic), 3.4–4.6 $dm^3$ (fraction II-2; *ca* 50 μg arsenic) and 5.5–6.8 $dm^3$ (fraction II-3; *ca* 100 μg arsenic). Fraction II-1 was evaporated and buffer was removed by passage through a Sephadex G-15 column. The arsenic fraction (0.86 g) was dissolved in water (2 $cm^3$) and mixed with 0.05 mol $dm^{-3}$, pH 4.0, acetate buffer (2 $cm^3$). This solution was placed on a CM-Sephadex C-25 column (2.6 cm × 42 cm; equilibrated with 0.05 mol $dm^{-3}$ acetate buffer; elution with the same buffer) (Fig. 3). The arsenic fraction (1.5 mg arsenic) was chromatographed twice on a DEAE-Toyopearl column (1.6 cm × 27 cm; eluted with 0.01 mol $dm^{-3}$ Tris/0.02 mol $dm^{-3}$ boric acid, pH 7.0). The arsenic compound was finally purified by passage through a Sephadex G-15 column. The $^1H$ NMR spectrum of the arsenic compound (1.1 mg arsenic) showed it to be identical with 1′(1,2-dihydroxypropyl)-5′-deoxy-5′-(dimethylarsinoyl)-β-ribofuranoside (**1b**) previously isolated from *E. radiata*[16] and *L. japonica*[18]

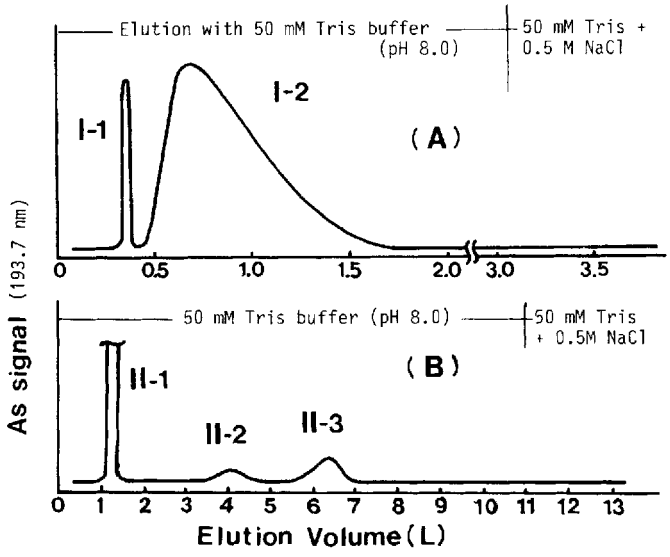



**Figure 2** Elution profiles of arsenic compounds from DEAE-Sephadex A-25 columns: (A) fraction I; column size, 2.6 cm × 60 cm; (B) fraction II; column size, 5.0 cm × 85 cm.

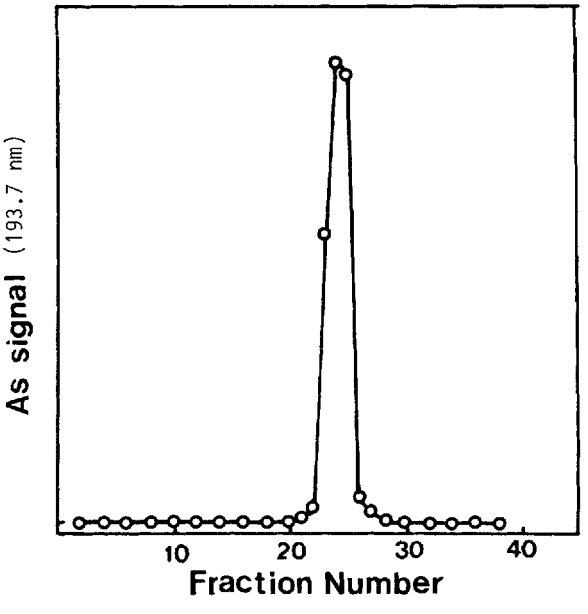



**Figure 3** Elution profile of arsenic compound from CM Sephadex C-25 column: fraction II-1; column size, 2.6 cm × 42 cm, fractions of 19 $cm^3$ were collected at a flow rate of 19 $cm^3\ h^{-1}$.

### Isolation of dimethylarsinic acid

Fraction II-3 (100 μg arsenic) from the DEAE-Sephadex chromatography was placed on a DEAE-Toyopearl column (1.5 cm × 27 cm) after removal of buffer salts. The arsenic compound was not retained when 0.1 mol $dm^{-3}$ Tris/0.2 mol $dm^{-3}$ boric acid (pH 7.0) or 0.05 mol $dm^{-3}$ Tris buffer (pH8.0) were used as mobile phases. However, the arsenic compound was retained when the residue from Fraction II-3 was dissolved in 0.01 mol $dm^{-3}$ Tris/0.02 mol $dm^{-3}$ boric acid (pH 7.0, 1.0 $cm^3$) and this solution placed on the column. The Tris/boric acid buffer, pH 7.0 (75 $cm^3$) and 0.05 mol $dm^{-3}$ Tris buffer, pH 8.0, served, in sequence, as mobile phases. The Tris buffer eluted the arsenic compound. The arsenic-containing fraction was evaporated and finally passed through a Sephadex G-15 column. The $^1H$ NMR spectrum of this compound (50 μg arsenic) was identical with that of dimethylarsinic acid (Fig. 4). This compound had retention times identical with those of dimethylarsinic acid in HPLC inductively coupled plasma–atomic emission ([ICP–AE] detection[22]) on a Asahipack GS-220 column (Asahi Kasei Kogyo Co., Tokyo, Japan; 7.6 mm × 500 mm; elution with 0.05 mol $dm^{-3}$ phosphate buffer, pH 6.8) and on a Nucleosil 5SB column (Nagel, Düren, FRG; 4.6 mm × 250 mm).

## RESULTS AND DISCUSSION

### Fractionation of arsenic compounds

The extraction and purification of arsenic compounds from *C. fragile* were carried out according to methods

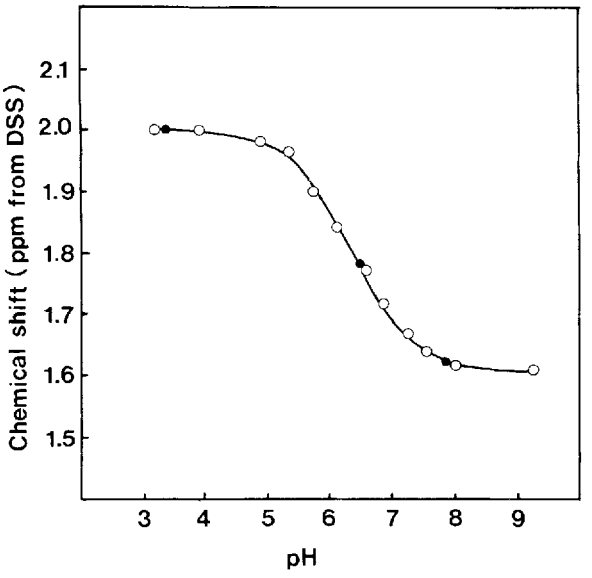



**Figure 4** pH titration profile of $^1$H NMR signal of arsenic compound purified from fraction II-3: (•) synthetic dimethylarsinic acid. DSS, sodium 2,2-dimethyl-2-silapentanesulfate. Chemical shifts are reported relative to internal HDO which is taken as $\delta$ 4.80 relative to DSS.

reported previously.[17–19] The methanol extract was chromatographed on a Sephadex LH-20 column. Three arsenic-containing fractions (I–III) were obtained. Chromatography of fraction I on a DEAE-Sephadex A-25 column produced two arsenic-containing bands and chromatography of fraction II three bands (Fig. 2). Fraction I-2 (Fig. 2A), containing most of the arsenic that was present in fraction I, was further separated by passage through a Sephadex G-15 column into fractions I-2a and I-2b. The major arsenic compound in fraction II left the DEAE-Sephadex column with the solvent front (fraction II-1). The two minor compounds (II-2 and II-3) with anionic character had elution volumes of 4 and 6 dm$^3$, respectively (Fig. 2B).

## Purification of the arsenic compounds

The arsenic compounds in fractions I-1 and I-2b had identical chromatographic behavior as did those in fractions II-1 and II-3, respectively. The amount of arsenic in fraction II-2 was too small for isolation. Fraction III from the Sephadex LH-20 column contained a large amount of salty impurity and was not further purified. The fractions I-2a, II-1, and II-3 were further purified by ion-exchange chromatography (DEAE and CM columns) and by gel chromatography with Sephadex G-15.

The arsenic compound in fraction II-1, which had no charge at neutral pH, had a retention volume of 475 cm$^3$ on a CM-Sephadex C-25 column with a pH 4.0 acetate buffer as mobile phase (Fig. 3). This compound was also retained on a DEAE-Toyopearl column with a borate-containing buffer at pH 8.0. The arsenic compound in fraction I-2a was retained firmly on the DEAE-Toyopearl column under the same conditions and was eluted with pH 8.0 Tris buffer. The similarity of the chromatographic behavior of the arsenic compounds in fractions I-2a and II-1 to the chromatographic behavior of previously isolated ACRs[18] suggested that these two compounds are ACR derivatives. These compounds were further purified by chromatography on the DEAE-Toyopearl column. The arsenic compound in fraction II-3 was retained only weakly on the DEAE-Toyopearl column.

## Identification of the arsenic compounds

The arsenic compounds were identified by comparing their $^1$H NMR spectra with previously reported spectra.[17,18] The comparison revealed that the two arsenic compounds obtained from fractions I-2a and II-1 contain a 5-deoxy-5-(dimethylarsinoyl)-$\beta$-ribofuranoside moiety. The characteristic $^1$H signals are: methyl protons $\delta$1.85 and 1.87 (6H); methylene protons 2.75 (2H, 8 lines, AB part of ABX system); protons in the ribose ring 5.0 (1H, s, 1′), 4.3 (1H, m, 4′), 4.2 (1H, m, 3′), and 4.1 (1H, d, 2′).

The arsenic compound from fractions I-2a had ten non-exchangeable protons in addition to the protons in the ribofuranoside moiety and the protons associated with arsenic. By comparing the $^1$H NMR spectrum of this compound with the spectra of the compounds previously isolated from *E. radiata*[17] and *L. japonica*,[18] the fraction I-2a compound was identified as compound **1a** (Fig. 1). Assignable proton signals (pH 6.5) were $\delta$ 4.03 (1H, m; 2-H), 3.79 (1H, dd, $J$ = 10.2 and 6.0 Hz; 3*a*-H), 3.68 (H, dd, $J$ = 11.8 and 4.0 Hz; 3″*a*-H), 3.63 (1H, dd, $J$ = 10.2 and 3.5 Hz; 3*b*-H), and 3.61 (1H, dd, $J$ = 11.8 and 6.0 Hz; 3″*b*-H). The signals at $\delta$ 3.8–3.95 (5H; 1,1″ and 2″-positions) were identical with those previously reported.[18]

The arsenic compound from fraction II-2 was similarly assigned structure **1b** (Fig. 1).[17,18] The proton signals (pH 6.8) of the side-chain were located at $\delta$ 3.90 (1H, m; 2-H), 3.75 (1H, dd, $J$ = 10.5 and 6.3 Hz; 3*a*-H), 3.64 (1H, dd, $J$ = 11.6 and 4.9 Hz; 1*a*-H), 3.60 (1H, dd, $J$ = 10.5 and 3.8 Hz; 3*b*-H), and 3.57 (1H, dd, $J$ = 11.6 and 6.4 Hz; 1*b*-H).

The $^1$H NMR spectrum of the arsenic compound

from fraction II-3 consisted of one singlet with a pH-dependent chemical shift (Fig. 4). The $pK_a$ of 6.30 and the HPLC properties identified this compound as dimethylarsinic acid. The arsenic compound from fraction I-2b, similarly identified as dimethylarsinic acid, contained traces of impurities.

The arsenic concentration in the *C. fragile* sample was rather low at 0.58 $\mu g\ g^{-1}$ (wet weight). However, we succeeded in isolating dimethylarsinic acid and two ACRs (compounds **1a** and **1b**) as the major water-soluble arsenic species. These compounds account for 5% (dimethylarsinic acid), 10% (**1a**), and 50% (**1b**) of the extracted arsenic.

Compounds **1a** and **1b** were found previously in brown seaweeds, *E. radiata*[16,17] *L. japonica*[18] and *H. fusiforme*.[19] In *E. radiata* and *L. japonica*, belonging to the order Laminariales, the most abundant ACR contains a sulfonyl group (R = $SO_3^-$ in Fig. 1). In *H. fusiforme* (order Fucales) the most abundant ACR has a sulfate group (R = $OSO_3^-$ in Fig. 1). *C. fragile* does not contain these sulfonate and sulfate groups. The fact that compound **1a** has been identified in all algal species studied is noteworthy, because this compound is considered to be a key intermediate between water-soluble arsenic compounds and arsenolipids.[20]

It was observed that ACRs decompose to dimethylarsinic acid at extremes of pH.[16] Therefore, dimethylarsinic acid reported to be present in algal extracts[9–11] could be a product of the decomposition of ACRs during work-up. The dimethylarsinic acid found in *C. fragile* is probably not a decomposition product, because the ACRs were never in contact with strong acids or a high-pH medium.

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