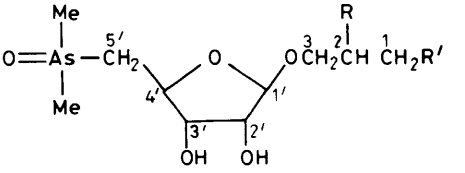
# Isolation and Identification of Arsenic-containing Ribofuranosides and Inorganic Arsenic from Japanese Edible Seaweed *Hizikia fusiforme*

**John S. Edmonds,* Masatoshi Morita, and Yasuyuki Shibata**
*National Institute for Environmental Studies, Yatabe-machi, Tsukuba, Ibaraki 305, Japan*

(2*S*)-3-[5-Deoxy-5-(dimethylarsinoyl)-β-D-ribofuranosyloxy]-2-hydroxypropyl hydrogen sulphate (**1a**), 3-[5-deoxy-5-(dimethylarsinoyl)-β-D-ribofuranosyloxy]-2-hydroxypropene-1-sulphonic acid (**1b**), 1-'glycerophosphoryl'-2-hydroxy-3-[5-deoxy-5-(dimethylarsinoyl)-β-ribofuranosyloxy]propane (**1c**), and 2-amino-3-[5-deoxy-5-(dimethylarsinoyl)-β-D-ribofuranosyloxy]propene-1-sulphonic acid (**1d**), together with inorganic arsenic have been isolated from the edible seaweed *Hizikia fusiforme*. Structures have been determined chiefly by n.m.r. spectroscopy. Compound (**1d**) has not been reported previously. Compound (**1b**) is apparently present in *Hizikia* as a pair of C-2 diastereoisomers.

Marine algae contain arsenic at concentrations of *ca.* 10 μg $g^{-1}$ (wet weight), which is some 4 000-times the concentration of arsenic in seawater.[1] In Japan algae constitute an important foodstuff and a number of studies have been undertaken to determine the chemical form of arsenic in the several species that contribute to the human diet. Of these, hijiki [*Hizikia fusiforme* (Harvey) Okamura (Phaeophyta, Sargassaceae)] has received the most attention, probably because an early report[2] that it contained inorganic arsenic generated toxicological interest.[3,4] It was subsequently reported[5,6] that aqueous extracts of hijiki contained methylarsonic and dimethylarsinic acids in addition to arsenate and arsenite. Recent publications[7–9] have reported that hijiki contains *ca.* half of its arsenic burden as inorganic arsenic, with the balance as unknown organic compounds. Some authors[2,7,10] have noted the possibility that inorganic arsenic may have been released from a larger molecule by acidic conditions generally employed for extraction or analysis in the above studies.



(**1**)

| | R | R′ |
|---|---|---|
| **a**; | OH | $OSO_3H$ |
| **b**; | OH | $SO_3H$ |
| **c**; | OH | $OPOCH_2CH(OH)CH_2OH$ (O⁻) |
| **d**; | $NH_2$ | $SO_3H$ |

## Results and Discussion

We now report that a freshly collected sample of hijiki contained *ca.* half of its arsenic load as the arsenic-containing ribofuranosides (**1a**—**d**), and half as inorganic arsenic. Compound (**1a**), the major organic arsenic compound, accounting for almost 50% of the total arsenic in hijiki, has previously been reported[11] from the kidneys of the giant clam *Tridacna maxima*; and compounds (**1b**) and (**1c**) have been isolated[12] from the brown kelp *Ecklonia radiata*. Compound (**1d**), 2-amino-3-[5-deoxy-5-(dimethylarsinoyl)-β-D-ribofuranosyloxy]-propene-1-sulphonic acid, has not been recorded previously. Compound (**1b**), 3-[5-deoxy-5-(dimethylarsinoyl)-β-D-ribofuranosyloxy]-2-hydroxypropene-1-sulphonic acid, reported[12] as occurring in *Ecklonia* as only one of the possible pair of C-2 diastereoisomers, was isolated from hijiki with both diastereoisomers apparent (see below). Together, compounds (**1b**—**d**) accounted for less than 5% of the total arsenic extracted.

Freshly collected hijiki was extracted with methanol, and arsenic compounds were isolated from the extract by gel permeation chromatography (g.p.c.), ion-exchange chromatography on DEAE Sephadex, high-pressure liquid chromatography (h.p.l.c.) and thin-layer chromatography (t.l.c.). Organic arsenic compounds were identified by $^1H$ and $^{13}C$ n.m.r. spectroscopy. $^1H$ and $^{13}C$ n.m.r. spectra revealed that compound (**1d**) was closely related to the ribofuranoside derivatives (**1a**) and (**1b**) (10 carbon atoms, 17 non-exchangeable protons) but differed in the substituents of the three-carbon ($-CH_2CHCH_2-$) side-chain. An absorbance (2 H, 8 line, AB part of an ABX system) centred at δ *ca.* 3.0 strongly suggested, by analogy with compound (**1b**), the presence of a methylene bonded to a sulphonic acid grouping. However, the position of elution of this compound from the Sephadex DEAE column (see Figure 1) indicated that it was considerably less acidic than compound (**1b**). In addition, it became apparent that the chemical shifts of all non-exchangeable protons assignable to side-chain substituents were pH dependent (Table 1). Plots of chemical shift *versus* pH for the relevant protons are shown in Figure 2. It was evident that protonation/deprotonation of the substituent at the 2-position was occurring and the $pK_a$ of 7.8, obtained by inspection of the curves, indicated the presence of an amino group.[13] The $^{13}C$ n.m.r. spectrum of compound (**1d**) was consistent with this assignment. It is interesting that the $^1H$ n.m.r. spectrum recorded at pH 3.63 showed a considerable downfield shift of the signals assigned to the methyl groups and the methylene bonded to the arsenic atom compared with spectra recorded at higher pH. Such a shift is presumably caused by protonation of the oxygen bonded to arsenic with an increase in the positive charge of the arsenic atom.

Compound (**1b**) eluted from the Sephadex DEAE column (Figure 1) and emerged from subsequent purification stages as if it were a single arsenic compound. Nevertheless, the $^1H$ n.m.r. spectrum (Table 2) indicated that two very similar compounds

* *Permanent address:* Western Australian Marine Research Laboratories, P.O. Box 20, North Beach, 6020, Western Australia.

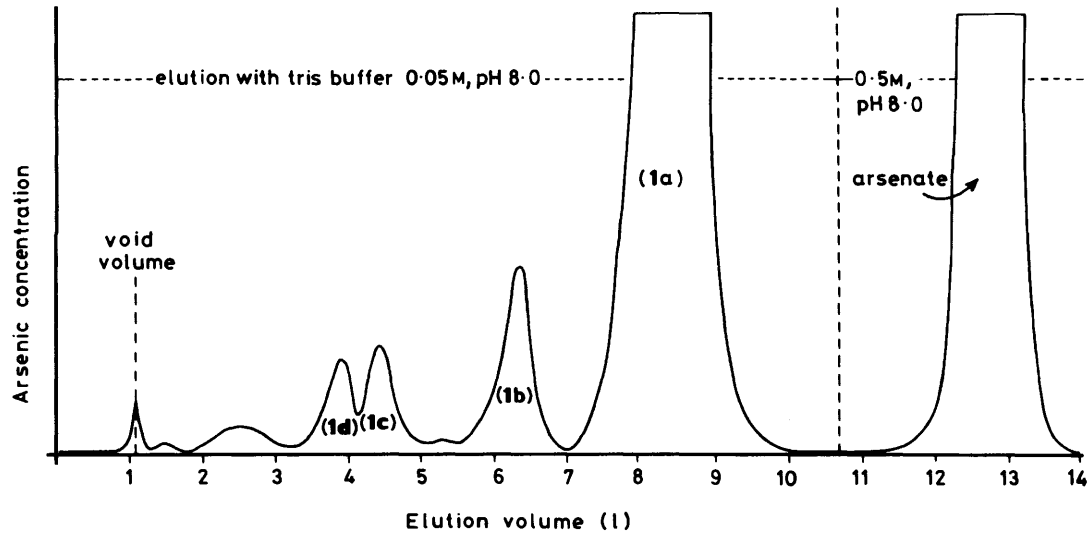



**Figure 1.** Elution of organoarsenicals (**1a**—**d**) from Sephadex DEAE

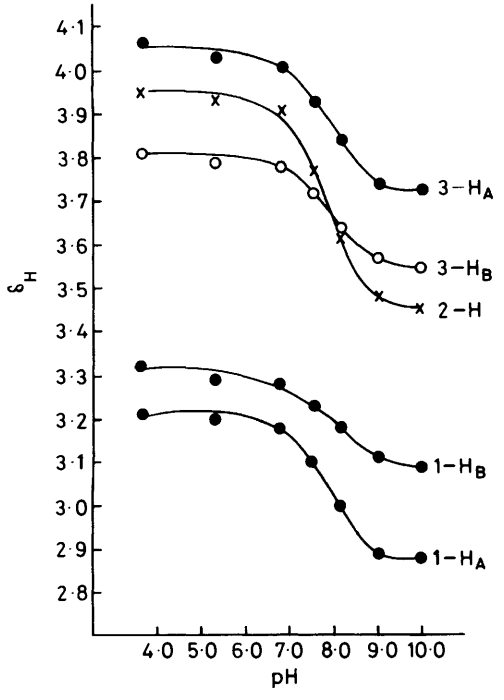



**Figure 2.** Plot of $\delta_H$ *versus* pH for aglycone protons in compound (**1d**)

were present in a ratio of *ca.* 3 to 1. The main discernible differences between the two compounds lay in the chemical shifts of methylene protons in the 3-position; *i.e.* those situated between the two asymmetric moieties of the molecule. However, small differences (*ca.* 0.01 p.p.m.), resulting in apparent duplication, were evident for all signals except those assigned to the methyl groups and those registering as broad multiplets. It was thus considered likely that compound (**1b**) was present in hijiki as both of a pair of diastereoisomers. Theoretically, the presence of the ribose moiety and the asymmetric carbon atom of the three-carbon side-chain allows the possibility of four pairs of diastereoisomers (those resulting from all possible combinations of D- or L-ribose with the side-chain adopting a 2*S* or 2*R* configuration). However, the likely biosynthetic origin of the sugar moiety of these compounds (from *S*-adenosylmethionine)[12] requires the presence of D-ribose, and the only absolute configuration available to date [compound (**1a**), when isolated from *Tridacna maxima*; β-D-*ribo*; 2*S*][11] is in accord with this view. It is thus likely that the two compounds (diastereoisomers) present in compound (**1b**) differ from one another only in the configuration at the central atom of the three-carbon side-chain. The small quantity of compound (**1b**) (*ca.* 250 μg As) isolated from hijiki precluded the possibility of further work, but a current study of another Japanese edible alga, *Laminaria japonica*, suggests that adequate quantities of this diastereoisomeric pair will become available, and this aspect will be explored more fully in a subsequent publication (Shibata *et al.*, to be published). It is interesting that no previously isolated arsenic-containing ribofuranoside (from *Ecklonia* and *Tridacna*) has been reported as other than stereochemically pure; and, indeed, the other three related compounds from hijiki show no evidence of stereochemical multiplicity. The significance of these observations to our understanding of the biosynthetic origins of the side-chain is currently unclear. Both 2-amino-3-sulphopropan-1-ol (cysteinolic acid) and 3-sulphopropane-1,2-diol have been reported to be present free in certain algal species[14] but stereochemical information on these compounds is not available. Although not enough species of algae have been examined for us to be certain, it is possible that the nature of the side-chains of the arsenic-containing ribofuranosides may have chemotaxonomic significance.

Inorganic arsenic (arsenate) in hijiki (*ca.* 50% of total arsenic) was identified by its chromatographic co-ordinates (g.p.c., h.p.l.c., and t.l.c.) and the absence of signals in $^{13}C$ and $^{1}H$ n.m.r. spectra. Arsenate was isolated primarily by buffered ion-exchange chromatography close to neutrality, and acidic conditions, employed in previous studies and giving rise to speculation[2,7,10] that the arsenate might originally be bound into larger, organic molecules, were avoided. We found no evidence to suggest that the arsenate was present in initial extracts or in purified fractions as other than free arsenate. Although arsenites, and methylarsonic and dimethylarsinic acids, have been reported[6] in hijiki extracts, we were unable to detect their presence. Dimethylarsinic acid has been reported[12] to be produced from arsenic-containing ribofuranosides at extremes of pH, and it is possible that it may have been generated by the acidic conditions employed in previous analytical procedures. The reported[6] presence of an arsenite and methylarsonic acid remains unexplained.

**Table 1.** $^1$H N.m.r. data for compound (**1d**). Recorded in $D_2O$ at 400 MHz. Chemical shifts are reported relative to internal HOD which is taken as δ 4.80 relative to external 3-(trimethylsilyl)propane-1-sulphonic acid sodium salt. *J* values in Hz

| pH | 2Me | $H_A$–C–$H_B$ 5′ | CH 4′ | CH 3′ | CH 2′ | CH 1′ | $H_A$–C–$H_B$ 1 | CH 2 | $H_A$–C–$H_B$ 3 |
|---|---|---|---|---|---|---|---|---|---|
| 3.63 | 2.03, 2.04 | δ 2.65 | 4.31m | 4.31m | 4.17 | 5.06 | 3.21, 3.32 | 3.95m | 3.81, 4.06 |
| | | 2.86 | | | | | | | |
| | | ($J_{gem}$ 15.6) | | | | | (5.3) | | (11.2) |
| | | ($J_{vic}$ 14.0, 4.7) | | | (4.4) | | (8.1, 5.9) | | (3.6, 5.3) |
| 5.30 | 1.84, 1.86 | δ 2.47 | 4.27m | 4.27m | 4.16 | 5.04 | 3.20, 3.29 | 3.93m | 3.79, 4.03 |
| | | 2.64 | | | | | | | |
| | | ($J_{gem}$ 14.0) | | | | | (14.8) | | (11.2) |
| | | ($J_{vic}$ 10.9, 3.4) | | | (4.4) | | (8.1, 5.6) | | (3.7, 5.6) |
| 6.78 | 1.84, 1.86 | δ 2.47 | 4.27m | 4.27m | 4.16 | 5.04 | 3.18, 3.28 | 3.91m | 3.78, 4.01 |
| | | 2.64 | | | | | | | |
| | | ($J_{gem}$ 14.6) | | | | | (15.4) | | (11.2) |
| | | ($J_{vic}$ 10.4, 3.7) | | | (4.7) | | (7.8, 5.6) | | (3.7, 5.6) |
| 7.50 | 1.84, 1.86 | δ 2.48 | 4.27m | 4.27m | 4.15 | 5.03 | 3.10, 3.28 | 3.77m | 3.72, 3.93 |
| | | 2.64 | | | | | | | |
| | | ($J_{gem}$ 14.6) | | | | | (15.4) | | (11.0) |
| | | ($J_{vic}$ 10.3, 3.6) | | | (4.2) | | (8.4, 5.6) | | (3.7, 5.6) |
| 8.14 | 1.85, 1.88 | δ 2.50 | 4.28m | 4.28m | 4.16 | 5.04 | 3.00, 3.18 | 3.62m | 3.64, 3.84 |
| | | 2.65 | | | | | | | |
| | | ($J_{gem}$ 14.3) | | | | | (14.8) | | (10.3) |
| | | ($J_{vic}$ 10.1, 3.4) | | | (4.4) | | (8.6, 4.7) | | (4.7, 5.6) |
| 9.00 | 1.84, 1.87 | δ 2.49 | 4.26m | 4.26m | 4.14 | 5.02 | 2.89, 3.11 | 3.48m | 3.57, 3.74 |
| | | 2.62 | | | | | | | |
| | | ($J_{gem}$ 14.2) | | | | | (14.9) | | (10.6) |
| | | ($J_{vic}$ 10.3, 3.4) | | | (4.1) | | (8.6, 4.5) | | (5.3, 6.2) |
| 9.96 | 1.84, 1.87 | δ 2.49 | 4.26m | 4.26m | 4.14 | 5.02 | 2.88, 3.09 | 3.46m | 3.55, 3.73 |
| | | 2.63 | | | | | | | |
| | | ($J_{gem}$ 14.3) | | | | | (14.9) | | (10.0) |
| | | ($J_{vic}$ 9.8, 3.6) | | | (4.4) | | (8.7, 4.4) | | (5.3, 6.8) |

**Table 2.** $^1$H N.m.r. data for compound (**1b**) (pH 7.21). Legend as for Table 1

| | 2Me | $H_A$–C–$H_B$ 5′ | CH 4′ | CH 3′ | CH 2′ | CH 1′ | $H_A$–C–$H_B$ 1 | CH 2 | $H_A$–C–$H_B$ 3 |
|---|---|---|---|---|---|---|---|---|---|
| Major | 1.88, 1.85 | δ 2.52 | 4.27m | 4.27m | 4.12 | 5.04 | 3.06, 3.16 | 4.27m | 3.66, 3.81 |
| | | 2.61 | | | | | | | |
| | | ($J_{gem}$ 14.6) | | | | | (15.0) | | (10.9) |
| | | ($J_{vic}$ 10.9, 3.9) | | | (3.4) | | (7.0, 5.6) | | (3.8, 5.7) |
| Minor | 1.88, 1.85 | δ 2.51 | 4.27m | 4.27m | 4.13 | 5.03 | 3.07, 3.15 | 4.27m | 3.57, 3.90 |
| | | 2.41 | | | | | | | |
| | | ($J_{gem}$ 14.6) | | | | | (15.0) | | (10.9) |
| | | ($J_{vic}$ 10.9, 3.9) | | | (3.4) | | (7.0, 5.5) | | (6.9, 3.4) |

## Experimental

N.m.r. spectra were recorded on a JEOL JNM GX-400 FT spectrometer at 400 MHz ($^1$H) and 100 MHz ($^{13}$C). Arsenic analyses were carried out and arsenic was located in chromatographic fractions by atomic absorption spectrophotometry using a Hitachi 180—80 instrument, or by inductively coupled argon plasma-atomic emission spectrometry using Jobin–Yvon instrumentation. 'Evaporation' refers to removal of solvent under reduced pressure at 40 °C on a rotary evaporator. Tris refers to aminotris(hydroxymethyl)methane. After ion-exchange chromatography, buffer was removed from eluted material in each case by passage through Sephadex LH-20–water or Sephadex G-15–water columns. All t.l.c. was carried out on cellulose layers (0.1 or 0.5 × 200 × 200 mm; E. Merck, Darmstadt) and butan-1-ol–acetic acid–water (60:15:25) was used for development.

*Extraction and Isolation of Arsenic Compounds from Hijiki* (Hizikia fusiforme).—*Preliminary work-up.* Hijiki was collected from near Nakaminato on the coast of Ibaraki Prefecture, Japan, on 16 July 1985. Living hijiki was removed from rocks exposed at low tide. A quantity (10.5 kg; *ca.* 0.001% As) was briefly rinsed with water to remove excess of salt, and was then extracted with methanol (2 × 20 l). The methanol extracts (containing 67.5 mg and 11.7 mg As respectively) were combined and evaporated to yield a dark syrup (280 g). This syrup was shaken with methanol (1 l) and the insoluble salty material was removed by filtration (cellulose acetate, 0.5 μm pore size) and washed with further methanol (0.5 l). The methanol extract was evaporated, the resulting syrup was dissolved in water (1 l), and the aqueous solution was extracted with diethyl ether (3 × 200 ml) to remove lipid impurities. The water was evaporated and the residue was dissolved/suspended in methanol (200 ml). The mixture was then poured into acetone (1.2 l) and kept for 1 h. The clear, dark supernatant was removed by decantation and the residue (181 g; 42 mg As) was dissolved in water (to 500 ml). This solution was then placed in 20 50-ml bottles and stored at −20 °C prior to g.p.c.

A portion (50 ml containing 18.1 g total solids) of the above solution was subjected to g.p.c. on Sephadex LH-20 (815 × 55 mm column; eluant water). The arsenic-containing fractions, eluting at 835—990 ml, were combined and evaporated to yield a pale syrup (1.8 g). This operation was repeated (19 ×) and the resulting material (18 g; 43.5 mg As) was dissolved in 0.05M-Tris

at pH 8.0 (to 110 ml), and chromatographed on a Sephadex DEAE A25 column (850 × 55 mm; equilibrated with 0.05M-Tris at pH 8.0). Initially, elution was carried out with 0.05M-Tris at pH 8.0 and arsenic compounds were eluted (see Figure 1) at 3.4—4.15 l [compound (**1d**)], 4.15—4.8 l (**1c**), 5.7—6.7 l (**1b**), and 7.3—9.6 l (**1a**). After the passage of 0.05M-Tris (10.7 l) the column was eluted with 0.5M-Tris at pH 8.0. A further arsenic compound, later shown to be an arsenate (see below), was eluted after the passage of 0.5M-Tris (1.3—3.0 l).

*Isolation of compound* (**1a**). Tris was removed from fractions containing compound (**1a**) (7.3—9.6 l see above) and the resulting colourless syrup (980 mg; 18 mg As) was subjected to g.p.c. (Sephadex G-15, 800 × 55 mm column; elution with water). A small portion was then subjected to t.l.c., and arsenic-containing material ($R_F$ 0.26) was recovered from the cellulose by extraction with water. After evaporation and final clean-up by passage through a Sephadex LH-20–water column (880 × 26 mm), the arsenic compound was obtained as a syrup (*ca.* 2 mg; *ca.* 400 μg As). It was shown by comparison of $^1H$ and $^{13}C$ n.m.r. spectra to be identical with (2*S*)-3-[5-deoxy-5-(dimethylarsinoyl)-β-D-ribofuranosyloxy]-2-hydroxypropyl hydrogen sulphate, previously isolated from *Tridacna maxima*.[11]

*Isolation of compound* (**1b**). Material eluted from the Sephadex DEAE A25 column at 5.7—6.7 l was, after removal of Tris, rechromatographed on the Sephadex G-15–water column (800 × 55 mm), subjected to t.l.c. ($R_F$ 0.22), and finally purified by passage through a DEAE-Toyopearl 650 poly(vinyl alcohol) column (330 × 16 mm; Toyosoda Co., Tokyo) equilibrated with 0.01M-Tris/0.02M-boric acid at pH 7.07. After application of the sample, the column was eluted with the equilibrating buffer (350 ml), then with 0.1M-Tris/0.2M-boric acid (120 ml), and finally the arsenical material was eluted by passage of 0.05M-Tris at pH 8.0. After removal of buffer, the arsenical material (*ca.* 250 μg As) was subjected to $^1H$ n.m.r. spectroscopy, which revealed the presence of two diastereoisomeric compounds (see Table 2 and Results and Discussion section) present in the ratio *ca.* 3 to 1. The $^1H$ n.m.r. spectrum of the major component (observed for the unseparated mixture) was identical with that of 3-[5-deoxy-5-(dimethylarsinoyl)-β-D-ribofuranosyloxy]-2-hydroxypropene-1-sulphonic acid previously isolated from *Ecklonia radiata*.[12]

*Isolation of compound* (**1c**). Material eluted from the Sephadex DEAE A25 column at 4.15—4.8 l was, after removal of Tris, subjected to g.p.c.–h.p.l.c. on an Asahipak G.S. 220 column (500 × 7.6 mm; elution with water). The $^1H$ n.m.r. spectrum of the arsenic-containing material (*ca.* 200 μg As) showed that it was identical with 1-'glycerophosphoryl'-2-hydroxy-3-[5-deoxy-5-(dimethylarsinoyl)-β-ribofuranosyl-oxy]propane previously isolated[12] from *Ecklonia radiata*.

*Isolation of compound* (**1d**). Material eluted from the Sephadex DEAE A25 column at 3.4—4.15 l was, after removal of Tris, subjected to h.p.l.c. on a Unisil Q $NH_2$ column (Gasukuro Kogyo Inc., Tokyo; 300 × 7.6 mm; elution with 15mM-sodium phosphate buffer at pH 6.8). After further g.p.c. (Sephadex G-15–water, 800 × 55 mm; elution with water) the arsenical material was purified by t.l.c. ($R_F$ 0.19). Final clean-up was effected by passage through a Sephadex LH-20–water column (800 × 55 mm) and the arsenic compound was obtained as a syrup (*ca.* 150 μg As), $\delta_H$ (400 MHz; $D_2O$) see Table 1 and Results and Discussion section; $\delta_C$ (100 MHz; $D_2O$) 109.9 [C(1′)], 79.7 [C(4′)], 78.4 [C(3′)], 77.0 [C(2′)], 69.8 [C(3)], 52.6 [C(1)], 50.5 [C(2)], 38.6 [C(5′)], and 17.2 and 16.8 [As(Me)$_2$]. From these data we assign structure (**1d**) to this compound.

*Isolation of arsenate*. A small portion of the material eluted from the Sephadex DEAE A25 column at 1.3—3.0 l (0.5M-Tris) was, after removal of buffer, subjected to g.p.c. (Sephadex LH-20–water, 880 × 26 mm) and t.l.c. The crystalline material obtained produced no signals in $^1H$ or $^{13}C$ n.m.r. spectra, and co-chromatographed with sodium arsenate in all t.l.c., g.p.c., and h.p.l.c. systems.

## Acknowledgements

We thank Mr Masayuki Kunugi for n.m.r. spectra. One of us (J. S. E.) gratefully acknowledges a Japanese Government Research Award from the Science and Technology Agency of Japan. We thank Dr Nobuo Egami, the Director of the National Institute for Environmental Studies, for permission to publish this work.

## References

1 GESAMP Working Group on Review of Potentially Harmful Substances, Hazard Evaluation for Arsenic, World Health Organisation, Geneva.
2 A. Yasui, C. Tsutsumi and S. Toda, *Agric. Biol. Chem.*, 1978, **42**, 2139.
3 T. Watanabe, T. Hirayama, T. Takahashi, T. Kokubo, and M. Ikeda, *Toxicology*, 1979, **14**, 1.
4 S. Adachi, H. Kawai, Y. Hosogai, T. Takahashi, H. Yoshimura, H. Katayama, and K. Takemoto, *J. Food Hyg. Soc. Jpn.* (*Shokuhin Eiseigaku Zasshi*), 1980, **21**, 425.
5 H. Yamauchi and Y. Yamamura, *Jpn. J. Ind. Health* (*Sangyo Igaku*), 1979, **21**, 47 (*Chem. Abstr.*, 1979, **91**, 69486j).
6 S. Tagawa, *Bull. Jpn. Soc. Sci. Fish.* (*Nippon Suisan Gakkaishi*), 1980, **46**, 1257 (*Chem. Abstr.*, 1981, **94**, 77823u).
7 A. Shinagawa, K. Shiomi, H. Yamanaka, and T. Kikuchi, *Bull. Jpn. Soc. Sci. Fish.* (*Nippon Suisan Gakkaishi*), 1983, **49**, 75 (*Chem. Abstr.*, 1983, **98**, 102160t).
8 A. Yasui, C. Tsutsumi, and S. Toda, *Agric. Biol. Chem.*, 1983, **47**, 1349.
9 J. N. C. Whyte and J. R. Englar, *Bot. Mar.*, 1983, **26**, 159.
10 S. Fukui, T. Hirayama, M. Nohara, and Y. Sakagami, *J. Hyg. Chem.* (*Eisei Kakagu*), 1982, **28**, P35.
11 J. S. Edmonds, K. A. Francesconi, P. C. Healy, and A. H. White, *J. Chem. Soc., Perkin Trans. 1*, 1982, 2989.
12 J. S. Edmonds and K. A. Francesconi, *J. Chem. Soc., Perkin Trans 1*, 1983, 2375.
13 K. Wüthrich, 'NMR in Biological Research: Peptides and Proteins,' North Holland, Amsterdam, 1976.
14 W. F. Busby, *Biochim. Biophys. Acta*, 1966, **121**, 160.

*Received* 21*st March* 1986; *Paper* 6/553