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The presence of gigartinine in a New Zealand *Gracilaria* species

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Abstract

Gigartinine, 5-(3-amidinoureido)-2-aminovaleric acid, serves as a chemotaxonomic marker to distinguish two species of *Gracilaria* with very similar morphologies. Gigartinine was identified by ^{13}C-NMR spectroscopy and amino acid analysis of a cold-water extract from *Gracilaria* sp. nov., collected from a sheltered harbour locality at Blockhouse Bay, Auckland, New Zealand. Levels of this amino acid, naturally ca. 5% by dry weight of seaweed, were able to be depleted and then restored during a nitrogen pulsing experiment. In contrast, native and pulsed samples of *Gracilaria chilensis* from Point Arthur, Wellington showed no extractable gigartinine. Although these two species are unable to be distinguished in the field by morphological characteristics, they can be separated by the presence or absence of gigartinine.

Introduction

The amino acid gigartinine, 5-(3-amidinoureido)-2-aminovaleric acid (Figure 1), was first isolated from *Gymnogongrus flabelliformis* (Rhodophyta) (Ito et al., 1966) and has since been found in many red seaweeds (Ito et al., 1967). It is commonly thought to be a nitrogen reservoir for the plant and seasonal studies suggest that it allows for fast spring growth, as conditions become favourable (Laycock & Craigie, 1977).

The presence of gigartinine in a sample of *Gracilaria* sp. nov. from Blockhouse Bay, Manukau Harbour, Auckland, New Zealand, was detected in a cold-water extract by the characteristic downfield absorption of the ureido and guanadino carbon atoms in the ^{13}C-NMR spectrum of the extract. Subsequent examination of cold-water extracts of samples of *G. chilensis* from Point Arthur, Wellington Harbour, New Zealand, failed to detect the presence of gigartinine. The aim of this study was first to confirm the presence of gigartinine in the *Gracilaria* sample from Blockhouse Bay, Manukau Harbour, and second to examine its role by conducting a nitrogen pulsing experiment. A similar experiment was conducted with samples of *G. chilensis* from Point Arthur, Wellington Harbour.

Materials and methods

Collection of Gracilaria *samples*

Samples of *Gracilaria* were collected from Blockhouse Bay, Manukau Harbour, Auckland and Point Arthur, Wellington Harbour in May 1999 (late Autumn). Voucher specimens were placed in the herbarium at the Museum of New Zealand, Te Papa Tongawera. WELT numbers were A22664 and A22657 for the Blockhouse Bay and Point Arthur samples respectively. The Blockhouse Bay (36° 51′S; 174° 42′E) alga was fine in form and growing on cobbles in the low intertidal zone of a protected shore. The Point Arthur (41° 18′S; 174° 50′E) alga was coarser and growing on rocks in the low intertidal and upper subtidal zones of an exposed beach, partly

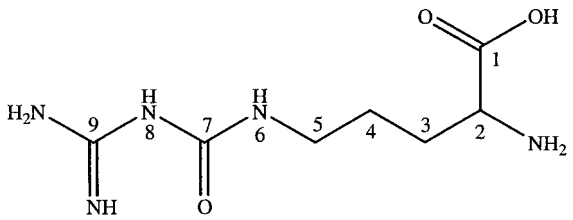

Figure 1. Gigartinine.

sheltered by a low reef. The samples were stored in plastic bags with paper towels soaked in seawater and refrigerated until the experiments began.

Nitrogen pulsing experiments

Gracilaria thalli from Blockhouse Bay and Point Arthur were briefly rinsed in filtered seawater (Whatman GF/B) and any epiphytes were removed. A number were frozen and retained as the native sample. The remainder (200 g) were placed in a glass tank (600 × 300 × 300 mm) containing filtered seawater (8 L, pH 7) and held at ambient temperature (14–17 °C) under low light conditions (i.e. shielded from direct laboratory lighting) for 7 days. The tank was swirled gently each day and the pH and temperature were recorded. After 7 days, half of the plants were removed and frozen for analysis as the acclimatised sample. The remaining half were placed in fresh seawater enriched with ammonium chloride (200 μM) and disodium hydrogen orthophosphate (50 μM) (Pickering et al., 1993). After 24 h under the low light conditions described above, the nitrogen pulsed plants were harvested and frozen for analysis. Crude extracts of the samples were prepared as described below.

Isolation and characterisation of gigartinine

Air-dried *Gracilaria* (1.7 kg) from Blockhouse Bay was soaked in water (10 L) for 2 days. The resulting extract was filtered through glass wool and gravity fed through a Dowex 50W-X8 column. The column was then washed with water, NH_4OH (1M, 2x) and finally NaOH (2M). Fractions containing gigartinine, as detected by TLC spots on cellulose plates giving a positive Sakaguchi reaction (Ito et al., 1966), were combined to yield the crude extract (100 mg). The R*f* (*n*-BuOH:HOAc:H_2O, 2:1:1) of the extract was compared with that of a sample of authentic gigartinine nitrate. ^{13}C-NMR spectroscopic examination (Laycock & Craigie, 1977) revealed the presence of essentially pure gigartinine.

Preparation of crude extracts from native, acclimatised and pulsed algal samples

Samples of fresh plant material (30 g) or the equivalent of dried material (~3 g) were cut into 5 cm lengths and stirred in distilled water (200 mL) for 24 h. After pressure filtration through GF/D then GF/B glass fibre filters, the extracts were lyophylised to give cream to pink solids in yields of 30–36% of the air-dried weed by weight.

NMR spectra

Samples of the extracts (100 mg) were dissolved in D_2O (0.8 mL) and placed in 5 mm (o.d.) tubes. The disodium salt of EDTA (ethylenediaminetetra-acetic acid) (15 mg) was added to complex any paramagnetic ions present (Karsten et al., 1994) and one drop of acetone was added as internal reference (33.2 ppm). Proton decoupled ^{13}C-NMR spectra were recorded at 30 °C on a Bruker Avance 300 spectrometer (75 MHz, 0.865 s acquisition time, 0.5 s delay time, 30° pulse width, 11000 transients).

Amino acid analyses

The crude extracts were analysed by HPLC, after conversion to phenylisothiocyanate (PITC) derivatives, as described by Hubbard (1995 and 1996) and Hubbard & McHugh (1996). A gigartinine nitrate standard was used to identify gigartinine in the traces and determine its concentration in the samples. The analyses were conducted at the Protein Microchemistry Facility, Department of Biochemistry, University of Otago.

Constituent sugar analyses

The constituent neutral sugars of the extracts were determined by reductive hydrolysis, acetylation and gas chromatography of the resulting alditol acetate derivatives (Stevenson & Furneaux, 1991). The alditol acetates were analyzed on a 15 m × 0.25 mm (i.d.) Supelco SP^{TM} 2330 fused silica capillary column at 215 °C with flame ionization detection. Relative response factors were determined using synthesized alditol acetate standards.

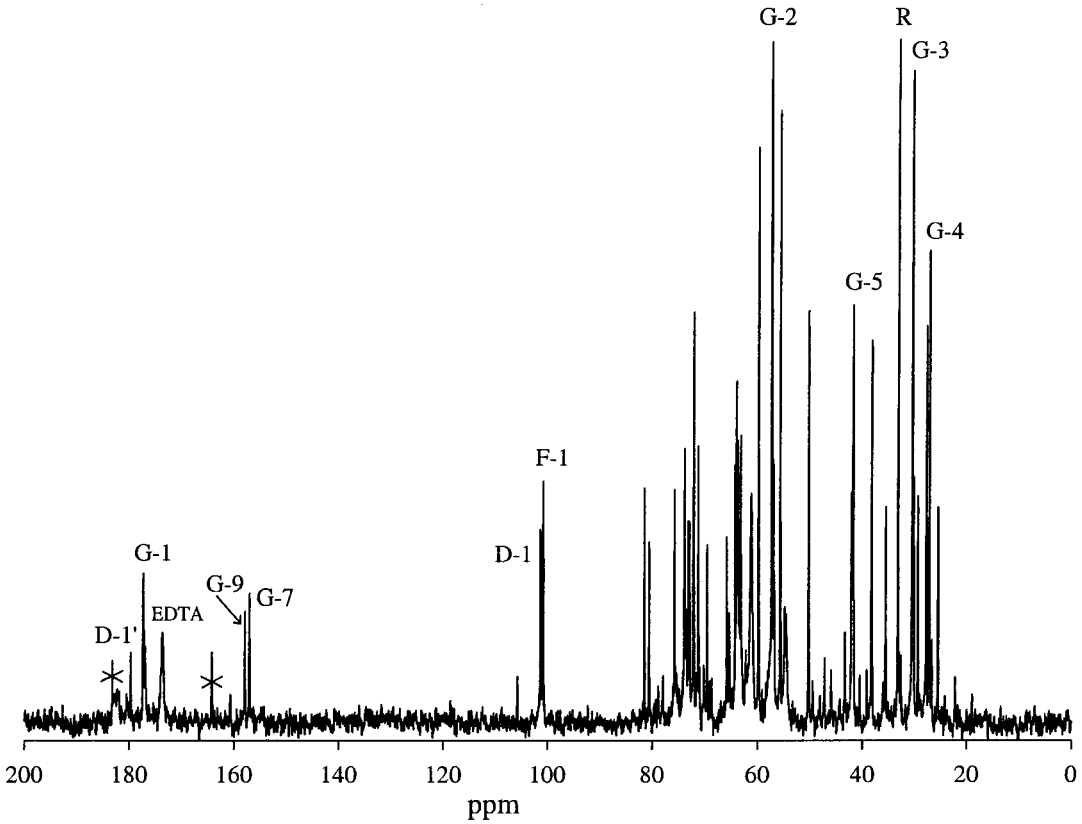

Figure 2. ^{13}C-NMR spectrum of the extract of nitrogen pulsed Blockhouse Bay *Gracilaria* sp. nov.digeneas (+ EDTA). Signals for gigartinine (G), floridoside (F), digeneaside (D) and added acetone (R) are indicated.

Results

NMR spectra

The presence of gigartinine in the cold-water extracts of native and nitrogen pulsed Blockhouse Bay algal samples was confirmed by NMR spectroscopy (Figure 2), all spectra showing signals assignable to gigartinine (Laycock & Craigie, 1977) and in particular, the presence of the characteristic resonances of the ureido and guanadino carbon atoms at 157.1 and 157.9 ppm, respectively. (Signals in our spectra were shifted downfield 2.3 ppm from the positions reported due to the different reference systems; acetone compared with TMS in CCl_4.) The gigartinine carboxyl resonance at 177.3 ppm was often not visible in the absence of the EDTA salt. Other signals in the NMR spectra have been assigned to floridoside and digeneaside (Karsten et al., 1999). No gigartinine was detected in the NMR spectra of the Port Arthur algal extracts.

Amino acid analyses

The level of gigartinine in native, acclimatised and pulsed Blockhouse Bay samples was determined by amino acid analyses of the algal extracts. The cold-water extract of the native alga contained 14.6% gigartinine by weight (ca. 4.8% of the algal dry weight), the extract of the acclimatised sample 0.03% and the extract of the pulsed sample 12.8% (Figure 3). Therefore after 7 days acclimatisation, the alga had almost completely utilised its gigartinine, but the gigartinine was restored to levels comparable to those in the native samples after the nitrogen pulse, which accords with it being a nitrogen reservoir in the algal population. In contrast, the Port Arthur sample contained at best only a trace of gigartinine throughout the pulsing experiment.

Other components

A new peak appeared in the amino acid analysis trace of the extract of the acclimatised Blockhouse Bay alga

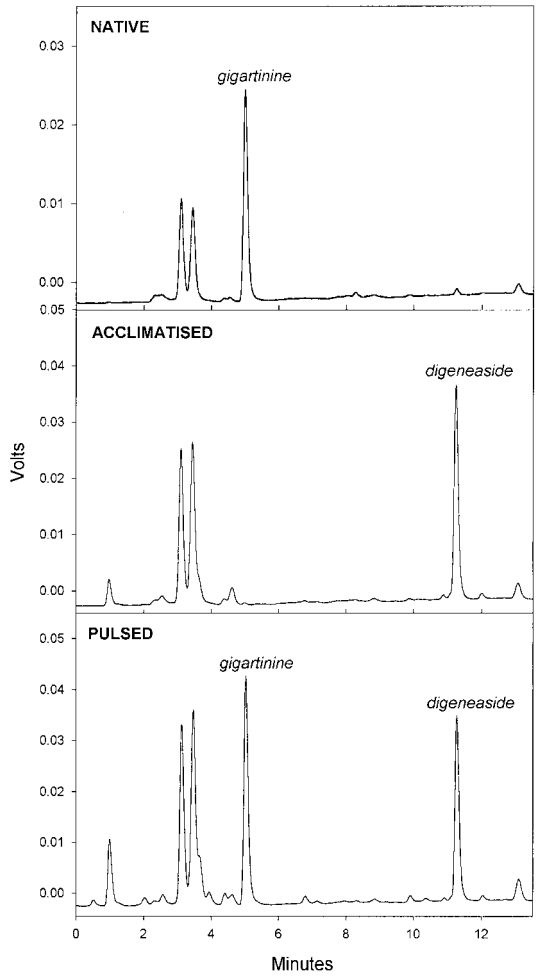

Figure 3. Amino acid analyses (HPLC traces of PITC derivatives) of extracts from native, acclimatised and nitrogen pulsed samples of Blockhouse Bay *Gracilaria* sp. nov.

(Figure 3) and it remained at a similar level ($\sim$ 10%) in the extract of the pulsed sample. New peaks also appeared in the ^{13}C-NMR spectra (Figure 2) and the compound was identified as digeneaside [2-*O*-α-D-mannopyranosyl-glycerate] by the presence of resonances assignable to the anomeric and carboxyl carbons of this compound at 101.3 ppm and 179.7 ppm, respectively (Karsten et al., 1999). Constituent sugar analyses were used to monitor the change in the ratio of floridoside [2-*O*-α-D-galactopyranosyl-glycerol] to digeneaside during the course of the experiment. A decrease in the ratio of galactose: mannose from 7.2:1 in the extract of the native alga to 2.3:1 and 1.9:1 in the extracts of the acclimatised and pulsed Blockhouse Bay samples respectively, corresponded with the changes in signal intensity for these low molecular weight carbohydrates in the ^{13}C-NMR spectrum. Digeneaside was also present in the extracts of acclimatised and pulsed samples of the Point Arthur alga. Other peaks seen in the amino acid analysis traces of the Blockhouse Bay samples and the native Point Arthur sample remained relatively constant throughout the pulsing experiment and were not identified.

Discussion

Analysis of the cold-water extracts of algal samples from Blockhouse Bay and Point Arthur has clearly shown that gigartinine is present in the former and absent in the latter and the nitrogen pulsing experiments conducted with both alga have clearly shown that gigartinine is a storage compound for one population and not the other. By depleting stored nitrogen by nutrient limitation under identical conditions and then restoring nutrient access with a nitrogen pulse, differences in nitrogen metabolism and storage between the samples could be assessed, undistorted by other variables.

Recently, DNA-ITS RFLP patterns have shown that a sample of *Gracilaria* collected from the Blockhouse Bay site in Manukau Harbour was distinct from samples of *G. chilensis* collected from another site in New Zealand and from sites in Chile (Candia et al., 1999) and therefore apparently another species. The presence of gigartinine as a nitrogen reservoir in this population raises the possibility of its existence in crude extracts being used as a chemotaxonomic marker for the presence of this undescribed species of *Gracilaria* in other New Zealand populations. The results of a survey of New Zealand *G. chilensis* populations for the presence of gigartinine will be published in a subsequent paper.

Other compounds present in the extracts were common to both the Blockhouse Bay and Point Arthur samples and therefore of no chemotaxonomic significance.

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