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Note

Complete ^{1}H and ^{13}C NMR assignment of digeneaside, a low-molecular-mass carbohydrate produced by red seaweeds

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Abstract—Digeneaside (α-D-mannopyranosyl-(1→2)-D-glycerate) was extracted from the red algae, *Bostrychia binderii*, and purified by adsorption and gel-filtration chromatography. HPLC and ESIMS techniques were used to follow purification steps and characterize digeneaside. NMR spectroscopy experiments (1D ^{1}H, ^{13}C, DEPT and 2D HMQC, COSY and TOCSY) were used to fully assign the ^{1}H and ^{13}C spectra.

Keywords: Digeneaside; *Bostrychia binderii*; Low-molecular-mass carbohydrates; NMR; ESIMS; HPLC

Red seaweeds biosynthesize different types of low-molecular-mass carbohydrates (LMMCs) and hexitols, such as digeneaside, floridoside, D- and L-isofloridoside, glucitol, galactitol and mannitol.[1] These compounds are thought to function as osmolytes and compatible solutes, with stabilizing effects on enzymes, membranes and structural macromolecules under hypersaline concentrations.[2–4] The first report of digeneaside (α-D-mannopyranosyl-(1→2)-D-glycerate) was published by Colin and Augier in 1939.[5] This glycoside was first isolated from the red seaweed, *Polysiphonia fastigiata* (Rhodophyceae), and had its molecular structure established through methylation techniques by Bouveng et al.[6] Digeneaside has been considered as the main photosynthetic product of the species belonging to the order Ceramiales.[7–9] These authors have suggested that the presence of digeneaside could be used as a chemotaxonomic marker for the Ceramiales. However, other authors have only detected the presence of floridoside (α-D-galactopyranosyl-(1→2)-glycerol) in species of this order.[10,11] GC is the routine technique used for detection of floridoside and hexitols, but it is inadequate for analysis of the acidic LMMC digeneaside. For this reason many of the studies involving LMMCs, especially digeneaside, use NMR spectroscopy.[12–15] Despite this, some NMR assignments are still ambiguous and need further refinement. Recently Simon-Colin et al.[16] published the complete ^{1}H and ^{13}C NMR assignment of floridoside. In our study we report the equivalent NMR data for digeneaside.

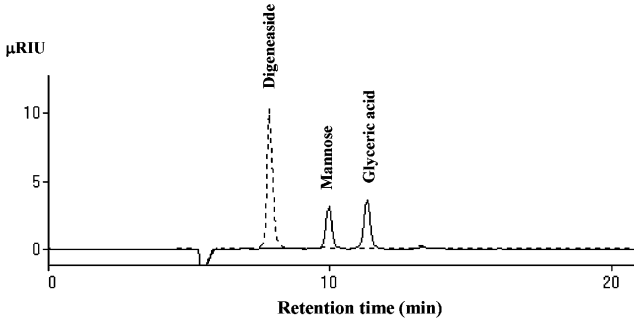

Figure 1. HPLC chromatogram of digeneaside (dotted line) and of its hydrolysis products (solid line).

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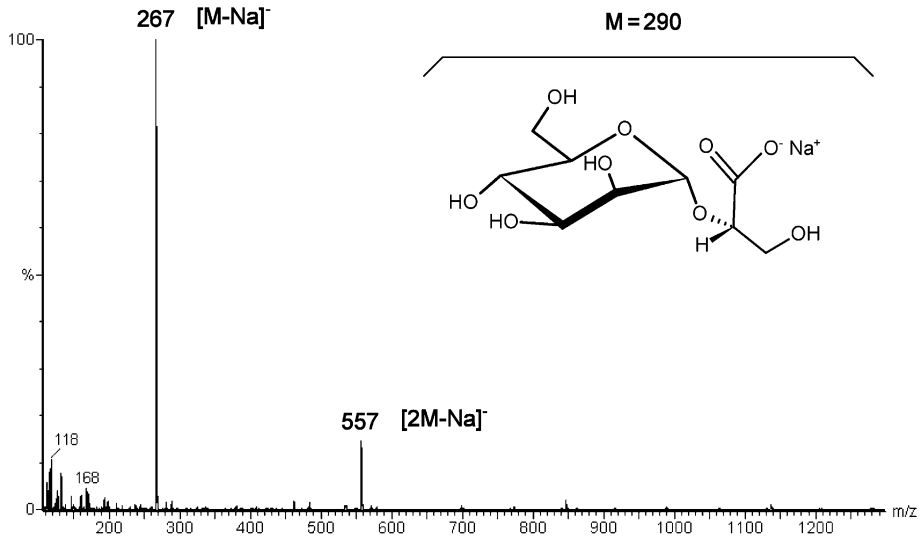

Figure 2. ESI-MS spectrum of digeneaside (MW = 290, sodium salt).

Digeneaside was isolated from the red seaweed, *Bostrychia binderii* Harvey, by ethanolic extraction. Purification by adsorption and gel-filtration chromatography led to a pure sample of sodium digeneaside as shown by HPLC and ESIMS analyses. An HPLC chromatogram of the digeneaside sample showed only one peak (Fig. 1), and after total acid hydrolysis (see Experimental), its products were identified as mannose and glyceric acid (Fig. 1). ESIMS analysis of the sodium salt of digeneaside (MW = 290) in the negative-ion mode, produced a main ion with *m*/*z* 267 corresponding to $[M-Na]^-$ and a low-intensity ion with *m*/*z* 557, attributed to $[2M-Na]^-$ (Fig. 2).

The ^{13}C NMR spectrum of the sodium salt of digeneaside showed eight intense signals in the region between 100 and 60 ppm, one of those corresponding to the anomeric carbon of Man*p* units (98.5 ppm), and a low-intensity carboxyl signal at 176.9 ppm (Fig. 3). The ^{13}C NMR DEPT spectrum showed two negative signals at 61.0 and 63.0 ppm, corresponding to two unsubstituted primary carbons (CH_2OH) (data not shown). For complete ^1H and ^{13}C NMR assignments, digeneaside was submitted to 2D ^1H, ^1H COSY, TOCSY and ^1H, ^{13}C HMQC analyses. From the HMQC experiment (Fig. 4), it was possible to correlate the C-1 (98.5 ppm) with its geminal proton (H-1 at 4.88 ppm, d, $J_{H1,H2}$ 1.8 Hz, $J_{H1,C1}$ 170.0 Hz). Having located H-1 of α-D-Man*p*, H-2 (4.08 ppm), H-3 (3.93) and H-4 (3.67 ppm) resonances were assigned from the COSY spectrum (Fig. 5). The TOCSY experiment (data not shown) confirms the previous assignments. COSY and TOCSY spectra showed two other correlations at 4.20/3.83 and 4.20/3.76 ppm from a different spin system and therefore were assigned to the sodium glycerate moiety. HMQC

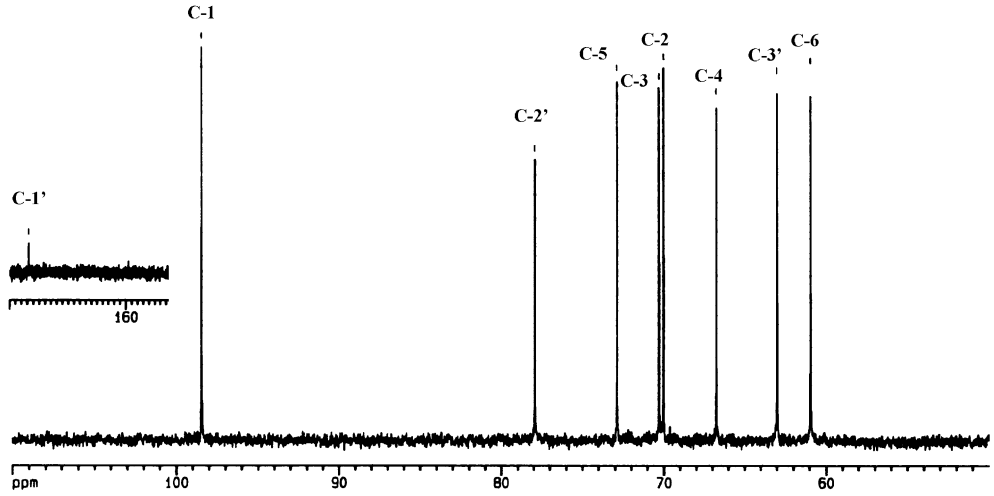

Figure 3. ^{13}C NMR spectrum (100.63 MHz) in D_2O of digeneaside. The inset shows the carboxyl signal (C-1′).

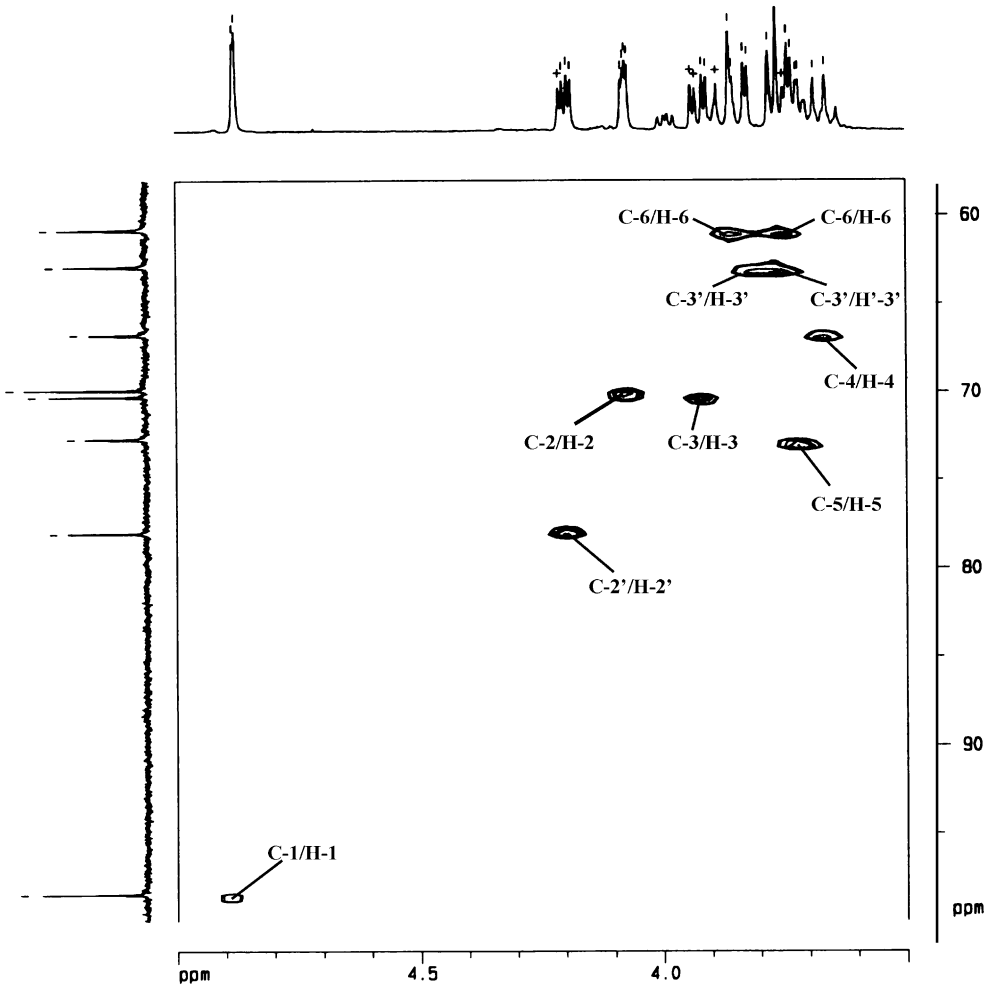

Figure 4. $^{13}C/^{1}H$ 2D HMQC NMR spectrum in D_2O of digeneaside.

correlations at 3.83/63.0 and 3.76/63.0 ppm (Fig. 4) indicated that the ^{13}C resonance at 63.0 ppm (primary carbon) corresponded to C-3′ of glycerate. Therefore the ^{1}H signals at 3.83 and 3.76 ppm were assigned to H-3′ and H′-3′, respectively, and the one at 4.20 ppm to H-2′. The C-5 signal of the Man*p* unit was assigned with the assistance of the Me α-D-Man*p* chemical shifts previously described.[17] Using the HMQC technique it was possible to correlate each ^{1}H with its directly attached ^{13}C (Fig. 4). The complete assignment of sodium digeneaside is shown in Table 1.

Karsten et al.[13] reported the first complete characterization of digeneaside by modern NMR methods. While they assigned C-6 of α-D-Man*p* units at lower field than C-3′ of glycerate (63.9 and 61.8 ppm, respectively), it is obvious from the 2D NMR correlations shown in our report that these assignments can be interchanged.

Complete and unambiguous NMR assignment of the different LMMCs is a necessary step to allow the use of this powerful tool in physiological and biochemical studies of red seaweeds. We are using NMR spectroscopy to identify the presence of these molecules in red algae to determine their potential as chemotaxonomic markers.

1. Experimental

1.1. Materials and methods

The red seaweed *B. binderii* was collected at Bombinhas, Santa Catarina State, Brazil. The fresh material was washed with distilled water and dried in the shade. The dry alga was milled until it became a fine powder. It was then transferred to an oven at 60 °C and dried to a constant mass.

1.2. Digeneaside extraction and purification

The algal material (dried and milled) was submitted to ethanolic extraction (EtOH 70% v/v) at 70 °C for 3 h. The material was then centrifuged, and the supernatant was concentrated on a rotary evaporator. The crude extract was submitted to adsorption chromatography

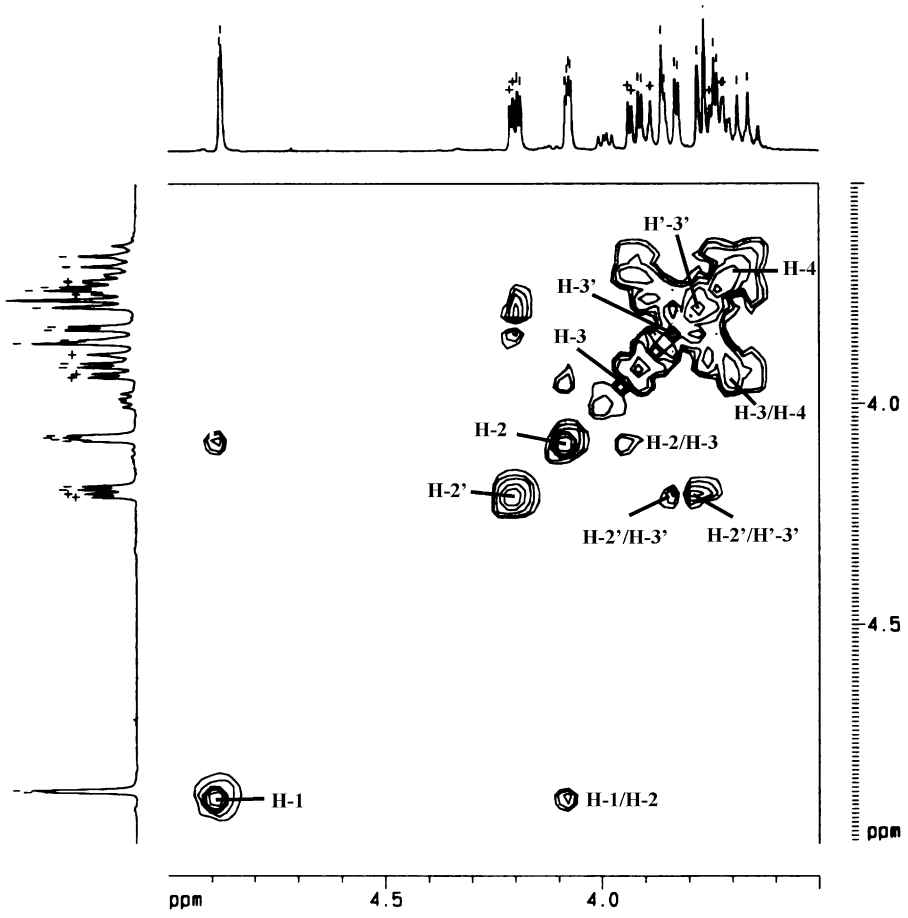

Figure 5. $^{1}H/^{1}H$ 2D COSY NMR spectrum in D_2O of digeneaside.

Table 1. ^{13}C and ^{1}H NMR assignments (ppm) of digeneaside[a]

Atoms	1	2	3	4	5	6	1′	2′	3′
^{13}C	98.5	70.0	70.3	66.8	72.9	61.0	176.9	78.0	63.0
$^{1}J_{H,C}$ (Hz)	170.0	149.5	140.6	148.2	143.9	142.3	—	146.8	145.4
^{1}H	4.88	4.08	3.93	3.67	3.74	3.89, 3.77	—	4.20	3.83, 3.76
$^{3}J_{H,H}$ (Hz)	1.8	3.5	9.3	9.5	ND[b]	ND[b]	—	3.0, 6.8	ND[b]

[a] The primed atoms refer to the glyceric acid moiety.
[b] ND = not determined.

using a 1:1 charcoal–Celite column (65 × 30 mm i.d.). NMR analysis of the aqueous eluent showed the presence of digeneaside as the main component. This fraction was purified by gel-filtration chromatography using a BioGel P-2 column (100 × 1.5 cm i.d.) eluted with *mQ* water at a flow rate of 0.2 mL min^{-1}. A main symmetric peak was eluted and characterized as digeneaside by NMR spectroscopy. Elution profiles of both the adsorption and gel-filtration chromatography were determined by the phenol–sulfuric method for sugar detection,[18] and the silver nitrate test for detection of chloride salts.[19] Sodium digeneaside was obtained after eluting the purified digeneaside sample through a DOWEX 50 × 8 resin column (sodium form), $[\alpha]_D^{21}$ +105.5 (*c* 0.3, H_2O), lit.[6] $[\alpha]_D$ +106, lit.[20] $[\alpha]_D^{25}$ +106, lit.[21] $[\alpha]_D^{25}$ +110.

1.3. High-performance liquid chromatography (HPLC)

HPLC analysis of the sodium digeneaside sample showed only one peak (t_R 7.85 min), which was collected using a fraction collector (Shimadzu), evaporated to dryness in a Speed Vac and submitted to hydrolysis with TFA (2 M) for 4 h at 100 °C. After evaporation of the TFA, HPLC analysis of the hydrolysis products showed

the presence of two peaks with retention times identical to those of mannose (t_R 9.89 min) and glyceric acid standards (t_R 11.25 min) (Fig. 1).

HPLC analyses were performed with a Shimadzu system equipped with a refractive index detector (Shimadzu 10A) at 40 °C. Carbohydrates were analyzed using an Aminex® (BioRad, Inc.) ion-exclusion column (HPX 87-H, 300 × 7.8 mm) after passing through a Micro-Guard (BioRad, Inc.) cartridge filled with the same material as the main column. Samples (20 μL) were injected into the column using a manual injection valve and eluted with a degassed solution of sulfuric acid (8 mmol) in *mQ* water at a flow rate of 0.6 mL min^{-1}.

1.4. Determination of the absolute configuration of mannose and glyceric acid

Sodium digeneaside was hydrolyzed (2 M TFA at 100 °C for 4 h). After evaporation of the acid, the hydrolyzate was carefully neutralized with ammonium hydroxide, applied to an Amberlite IRA-93 column (carbonate form, 40 × 1.5 mm i.d.) and eluted with water. The eluant was concentrated, passed through a column of BioGel P-2 and freeze-dried, yielding D-mannose, $[\alpha]_D^{21}$ +15.5 (*c* 0.5, H_2O), in agreement with an authentic standard of D-mannose. Elution of the Amberlite column with 0.05 M ammonium carbonate, followed by repeated evaporation of the eluant with water and elution through a DOWEX 50 × 8 resin column (calcium form) rendered D-glycerate (calcium salt), $[\alpha]_D^{21}$ +14.5 (*c* 0.4, H_2O), in agreement with an authentic standard of D-glycerate (calcium salt).

Optical rotations were measured on a Rudolph Autopol III automatic polarimeter using a 1.0-dm cell with a solute concentration *c* (g/100 mL).

1.5. Electrospray ionization-mass spectrometry (ESIMS)

ESIMS experiments were recorded on a Micromass Quattro LC electrospray mass spectrometer. The sample was diluted in deionized water (1 mg mL^{-1}) for analysis. Acetonitrile–water (80:20) was used as mobile phase at a flow rate of 0.1 mL min^{-1} controlled by a Shimadzu LC pump. The mass spectrum was obtained in negative-ion mode by direct injection of the sample. ESI was carried out using a capillary with an inner diameter of 0.1 mm. The tip was held at 4.00 kV in a negative-ion detection mode. Nebulization was assisted by N_2 gas (99.8%) at a flow rate of 10 L min^{-1}, and the spray chamber was held at 300 °C. The ion optics zone was optimized for a maximal ion transmission. The best signal was obtained when a declustering potential (fragmentor voltage) of 100 and 1.6 V for ion energy was set for detection. Data were acquired across a mass range of *m*/*z* 100–1300 using a conventional quadrupole mass detector with a cycle time of 3 s.

1.6. Nuclear magnetic resonance (NMR)

NMR experiments were performed in a Bruker Avance DXR 400 spectrometer using a 5-mm inverse-probe at 40 °C. The sample was deuterium exchanged by successive lyophilization steps in D_2O (99.9%) and then dissolved in 0.5 mL of D_2O (10 mg mL^{-1}). Chemical shifts are reported in ppm relative to an internal acetone standard at 2.224 and 30.2 ppm for ^1H and ^{13}C NMR spectra, respectively. 1D ^1H, coupled and decoupled ^{13}C, DEPT and 2D ^1H, ^1H COSY, TOCSY and ^1H, ^{13}C HMQC spectra were obtained at a base frequency of 100.63 MHz for ^{13}C and 400 MHz for ^1H nuclei. For ^{13}C NMR spectroscopy, the pulse sequence used a delay (D1) and acquisition time (AQ) of 0.10 and 0.59 s, respectively, a spectral width of 31.0 kHz, 32 K data points, 90° pulse (7.1 μs) and 2000–10,000 scans. A ^{13}C NMR DEPT spectrum was obtained at $\theta z = 135°$ where *C*H and *C*H_3 signals appear in the positive phase, and *C*H_2 appears in the negative phase. For the ^1H NMR experiment, D1 = 1.0 s, AQ = 2.0 s, spectral width of 8.0 kHz, 32 K data points, 90° pulse (7.1 μs) and 32 scans were used. The DO*H* signal was suppressed by low-power irradiation during relaxation. 2D ^1H, ^1H COSY, TOCSY and ^1H, ^{13}C HMQC spectra were carried out using the pulse programs supplied with the apparatus.

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