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Note

Separation of floridoside and isofloridosides by HPLC and complete ^{1}H and ^{13}C NMR spectral assignments for D-isofloridoside

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Abstract—Isofloridosides (1-*O*-α-D-galactopyranosylglycerol) and floridoside (2-*O*-α-D-galactopyranosylglycerol) were extracted from the red alga *Porphyra umbilicalis* (Linné) Kützing (Bangiales, Rhodophyta). Their separation was achieved by HPLC (NH_2 P50 column) after successive purification of the crude extract by ion-exchange chromatography and HPLC (Sugar-Pak TM1 column). 1D and 2D NMR spectroscopy experiments allowed to completely assign the ^{1}H and ^{13}C spectra of D-isofloridoside.

Keywords: *Porphyra umbilicalis*; Isofloridoside; NMR; Floridoside

Floridoside (2-*O*-α-D-galactopyranosylglycerol) is considered to be the main photosynthetic reserve product and the main organic solute involved in the osmotic adaptation in most red algae[1,2] except Ceramiales for which digeneaside (2-*O*-α-D-mannopyranosyl-D-glyceric acid)[3,4] seems to be involved. Members of the Bangiales order such as Porphyra species also contain isofloridoside under two enantiomeric forms: D-isofloridoside (1-*O*-α-D-galactopyranosyl-D-glycerol) and L-isofloridoside (1-*O*-α-D-galactopyranosyl-L-glycerol).[5] A number of reports have dealt with the quantitative assessment of these galactosides in Porphyra species according to environmental parameters,[6] seasonal harvesting,[7,8] and experimental osmotic acclimatization.[7] In all of these studies, quantification was performed by GC–MS using derivatives and spectroscopy.

The red seaweed studied in this paper (*Porphyra umbilicalis*) was previously analyzed by Karsten et al.[9] In that paper they identified (using ^{13}C NMR) and quantified (by GLC) floridoside and D- and L-isofloridoside from *P. umbilicalis*. The present report aims at developing an HPLC methodology to separate floridoside and isofloridosides. This furthermore allowed to completely assign and correct NMR spectra for these isomers, which complete the NMR assignments for the glycopyranosylglycerol series including floridoside and digeneaside already reported by Simon-Colin et al.[10] and Ascencio et al.,[11] respectively.

Hydro-alcoholic extraction of *P. umbilicalis* algae followed by successive purification of the low molecular weight carbohydrate components over two ion-exchange resins and a Sugar-Pak column led to an extract containing mostly floridoside and isofloridoside. This first HPLC step is necessary to improve the purity of the isofloridoside fraction obtained thereafter. Further HPLC step on an NH_2 column, led to separate floridoside (t_R 18.32 min) (Fig. 1), and a mixed fraction containing D- and L-isofloridoside (t_R 20.94 min) (Fig. 1). ESIMS analysis of the isofloridoside fraction in the positive-ion mode, produced a main ion at *m*/*z* 292.9 $[M+K]^+$ and a low intensity ion at *m*/*z* 277 $[M+Na]^+$.

The ^{1}H NMR spectrum of isofloridoside is shown in Figure 2. It can be noticed on this spectrum that the doublet of the anomeric proton at 4.93 ppm presents a

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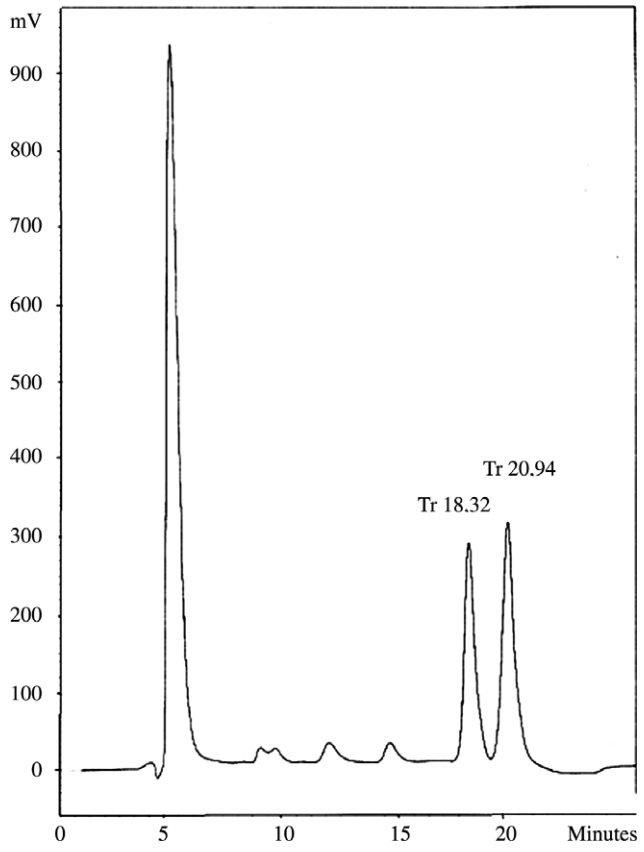

Figure 1. HPLC profile of a mixture of isofloridosides and floridoside purified on a NH_2-P50 Shodex (Asahipak). Floridoside and the mixture of isofloridosides are eluted at 18.32 min and 20.94 min, respectively, with 4:1 ACN–0.01 M KH_2PO4 at 0.8 mL min^{-1} and 40 °C.

small shoulder, characteristic of the mixture of L and D-isofloridosides. The ^1H NMR spectrum presented also a multiplet in the region 3.42–3.47 ppm, attributed to D-isofloridoside according to Meng et al.[5] These authors compared the ^1H NMR spectrum of pure L-isofloridoside (characterized by GC–MS and FTIR analysis) and the mixture of L- and D-forms. They noticed that this multiplet could be seen only in the spectrum of the mixture and assigned it to D-isofloridoside. It is possible to estimate the D/L epimeric ratio in the isofloridoside purified in this work, considering the integral value of the multiplet H′-1 belonging to D-isofloridoside and the anomeric proton of the mixture of isofloridosides. The calculated D/L epimeric ratio of isofloridoside is then 4:1. Moreover, a pollution by floridoside, seen at 5.09 ppm, is estimated as 6.5%.

The 1D and 2D NMR data let to assign the NMR signals of the major component D-isofloridoside. The ^{13}C and ^1H resonances of D-isofloridoside, assigned using COSY (Fig. S1, see Supplementary data), HMQC (Fig. S2, see Supplementary data), HMBC (Fig. S3, see Supplementary data), TOCSY (Fig. S4, see Supplementary data) and *J*-modulated spin echo experiments, are reported in Table 1. Our results allow to conclude that the multiplet at 3.41–3.44 ppm, corresponds to one of two non equivalent protons H′-1, and not to H′-2 as previously reported by Meng et al.[5] In addition, the ^{13}C NMR assignment reported here for D-isofloridoside show a shift difference of 1.6 ppm as compared to lit.[5] probably due to some variation of pH and calibration methods.

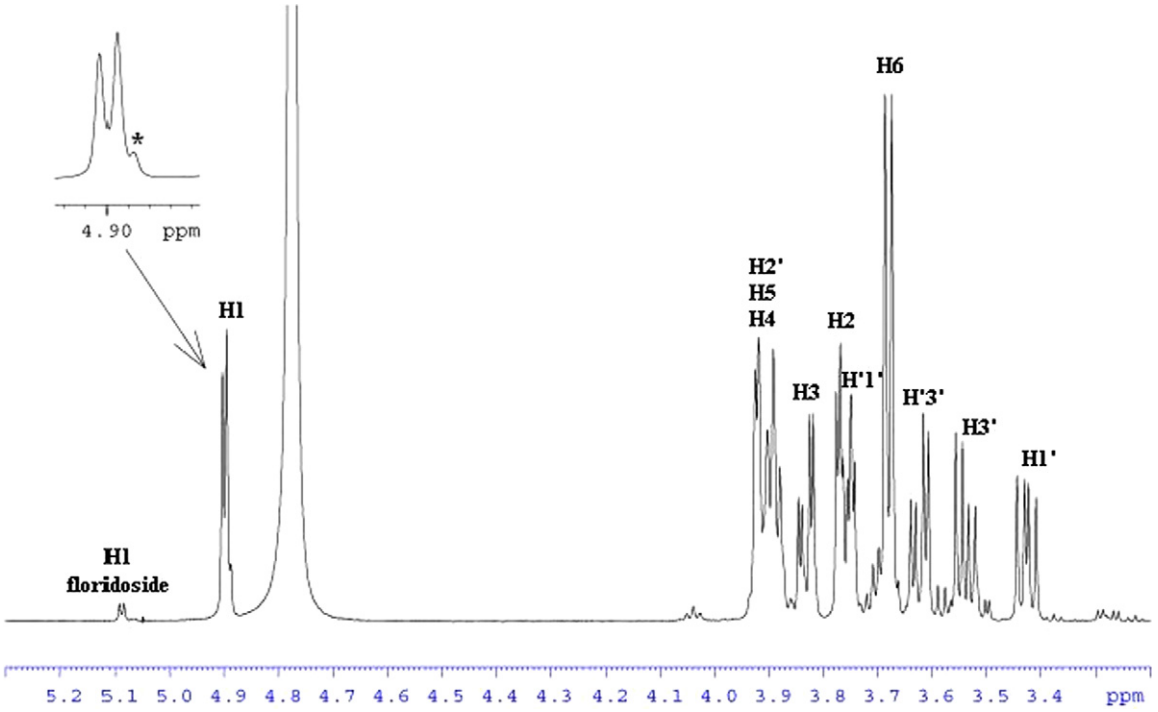

Figure 2. ^1H NMR spectrum in D_2O of D-isofloridoside (∗: ^1H L-isofloridoside).

Table 1. ^{13}C and ^{1}H NMR assignments (ppm) of D-isofloridoside (the primed atoms refer to the glycerol moiety)

Atoms	1	2	3	4	5	6	1′	2′	3′
^{13}C	101.5	71.22	72.2	72	73.7	63.95	71.6	73.43	65.25
^{1}H	4.9	3.78	3.82	3.92	3.88	3.68	3.42–3.75	3.88	3.52–3.62

1. Experimental

1.1. Material

P. umbilicalis was collected in the upper intertidal zone at Treiz Hir, in the bay of Brest, France, in February 2006.

1.2. Extraction and purification of isofloridoside

The fresh material (200 g) was briefly washed with water and extracted immediately with 12:5:3 MeOH–$CHCl_3$–water (500 mL) for 2 h with magnetic stirring. The hydroalcoholic phase was concentrated in a rotary evaporator. The crude extract (5.8 g) was purified successively on two ions exchange-resin columns involving first AG® 50W-X8, 20–50 mesh, Bio-Rad, (H^+ form, 20 mL) then 1 X8, 100–200 mesh, Dowex-Fluka Chemika, (Cl^- form, 20 mL), converted to the OH^- form. This system was washed with distilled water. The neutral effluent was concentrated under diminished pressure and freeze-dried. The resulting powder (1.5 g) contained essentially isofloridoside and floridoside. Only 150 mg was passed 10 times on a Sugar-Pak TM1 (Waters) column (6.5 mm ID * 300 mm L), placed in an oven at 95 °C in isocratic mode at a flow rate of 0.3 mL/min with water as eluent. The detection system was a refractive index detector (Jasco-930). The major peak at 19.78 min was collected and freeze-dried (104 mg). It was shown to contain a 0.7:1 mixture of isofloridoside and floridoside (^{1}H NMR). It was applied six times on an NH_2-P50 Shodex (Asahipak) column (4.6 mm ID * 250 mm L) thermostated at 40 °C with 4:1 MeCN–0.01 M KH_2PO_4 as eluent (isocratic mode at a flow rate of 0.8 mL min^{-1}) resulting in the successive elution of floridoside (41.2 mg) and isofloridosides (mixture of L- and D-forms, 31.1 mg); $[\alpha]_D^{20}$ +38.5 (*c* 1.4, water).

1.3. NMR spectroscopy

One and two dimensional NMR spectra were recorded at 25 °C on a Bruker Avance 500 spectrometer equipped with an indirect 5 mm triple resonance probehead TXI $^{1}H/^{31}P/^{13}C$ using standard pulse sequences available in the Bruker software. The samples were dissolved in 99.97% D_2O (700 μL). Chemical shifts were expressed in ppm relative to TMS as external reference. Double-quantum filtered ^{1}H–^{1}H correlated spectroscopy (DQF COSY), HMQC and HMBC experiments were performed with a delay of 60 ms according to standard pulse sequences in order to assign ^{1}H and ^{13}C resonances. For example, in a HMQC experiment the raw data set consisted of collecting 1024 (F2) * 128 (F1) complex data point of 48 scans each, zero filled to 1 K in the F1 dimension prior to Fourier transform with a spectral width of 17,613 and 2422 Hz in the F1 and F2 dimensions, respectively, with a relaxation delay of 2 s. 2D TOCSY experiments were performed with a mixing time of 100 ms on a Bruker DRX500 equipped with an indirect 5 mm triple resonance probehead TBI $^{1}H/(BB)/^{13}C$ at 25 °C.

1.4. Optical rotation

It was measured on a polarimeter JASCO P-1010 using a 1.0-dm cell at 589 nm (at room temperature).

1.5. Mass spectrometry

ESIMS experiments were recorded on a Micromass Quattro Ultima electrospray mass spectrometer. The sample was diluted in deionized water (10 mg L^{-1}) for analysis. The mass spectrum was obtained in positive-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 3.50 kV in a positive-ion detection mode. Nebulization was assisted by N_2 gas (99.8%) at a flow rate of 800 L h^{-1}, and the spray chamber was held at 120 °C. Data were acquired across a mass range of *m*/*z* 50–500 using a triple quadrupole mass detector with a cycle time of 0.4 s.

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Supplementary data

Supplementary data (i.e., Figs. S1–S4) associated with this article can be found, in the online version, at doi:10.1016/j.carres.2007.07.021.

References

1. Reed, R. H. *Br. Phycol. J.* **1985**, *20*, 211–218.
2. Karsten, U.; Barrow, K. D.; Mostaert, A. S.; King, R. J. *Estuar. Coast. Shelf Sci.* **1995**, *40*, 239–247.
3. Kirst, G. O. *Phytochemistry* **1980**, *19*, 1107–1110.
4. Reed, R. H. In *Biology of the Red Algae*; Cole, K. M., Sheath, R. G., Eds.; Cambridge University Press: Cambridge, 1990; pp 147–170.
5. Meng, J.; Rosell, K. G.; Srivastava, L. M. *Carbohydr. Res.* **1987**, *161*, 171–180.
6. Karsten, U. *New. Phytol.* **1999**, *143*, 561–571.
7. Karsten, U.; Barrow, K. D.; King, R. J. *Plant Physiol.* **1993**, *103*, 485–491.
8. Karsten, U.; West, J. A. *J. Exp. Mar. Biol. Ecol.* **2000**, *254*, 221–234.
9. Karsten, U.; West, J. A.; Zuccarello, G. C.; Nixdorf, O.; Barrow, K. D.; King, R. J. *J. Phycol.* **1999**, *35*, 967–976.
10. Simon-Colin, C.; Kervarec, N.; Pichon, R.; Deslandes, E. *Carbohydr. Res.* **2002**, *337*, 279–280.
11. Ascencio, S. D.; Orsato, A.; Franca, R. A.; Duarte, M. E. R.; Noseda, M. D. *Carbohydr. Res.* **2006**, *341*, 677–682.