Carbohydrate Research, 161 (1987) 171–180
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

CHEMICAL CHARACTERIZATION OF FLORIDOSIDES FROM *Porphyra perforata*

JIANXIN MENG, KARL-GUNNAR ROSELL*, AND LALIT M. SRIVASTAVA

Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia V5A 1S6 (Canada)

(Received May 5th, 1986; accepted for publication in revised form, September 12th, 1986)

ABSTRACT

The chemical structures and configurations of floridoside [α-D-galactopyranosyl-(1→2)-glycerol] and isofloridoside, "D" form [α-D-galactopyranosyl-(1→1)-D-glycerol] and "L" form [α-D-galactopyranosyl-(1→1)-L-glycerol], obtained by extraction of the red alga *Porphyra perforata*, were studied by using nuclear magnetic resonance spectroscopy (n.m.r.) and gas–liquid chromatography–mass spectrometry (g.l.c.–m.s.). Assignments of the signals in the n.m.r. spectra were made by consideration of the previously assigned signals of related compounds and by proton-decoupling experiments. Both the D and the L form of isofloridoside, in addition to floridoside, were found in *P. perforata*. Separation and identification of the D and L forms of isofloridoside was achieved by g.l.c.–m.s., which provides a simple way in which to quantify the three floridosides present in red algae. N.m.r. spectroscopy (^{1}H- and ^{13}C-) can also be utilized for quantitative purposes. The ratio of the D to the L form of isofloridoside differed in the various samples analyzed.

INTRODUCTION

Floridoside [α-D-galactopyranosyl-(1→2)-glycerol] and isofloridoside, "D" form [α-D-galactopyranosyl-(1→1)-D-glycerol] and "L" form [α-D-galactopyranosyl-(1→1)-L-glycerol], are the major, soluble, low-molecular-weight carbohydrates in *Porphyra*[1,2]. In this genus, isofloridoside amounts to between 2.5 and 10.8% of the dry weight, and floridoside accounts for 0.8 to 6.1%[3]. It is well established that, in both floridoside and isofloridoside, the galactose is in the D configuration[4,5].

There is some disagreement about the form in which isofloridoside occurs naturally in *Porphyra*. Wickberg[5] described isofloridoside as an isomorphous D and L mixture; this was confirmed by Craigie *et al.*[1]. In contrast, Peat and Rees[6], and Su and Hassid[7] reported that isofloridoside occurred as the pure D form. This difference was attributed to different *Porphyra* species being analyzed[7].

*Present address: B. C. Research, Chemistry Technology Division, 3650 Wesbrook Mall, Vancouver, B.C. V6S 2L2, Canada.

0008-6215/87/$ 03.50

The structure of floridoside has been confirmed by gas–liquid chromatography–mass spectrometry (g.l.c.–m.s.)[2,8] and by ^{1}H-nuclear magnetic resonance (^{1}H-n.m.r.)[9] and ^{13}C-n.m.r. spectroscopy[10], but few data have been published with regard to the structure of isofloridoside. No satisfactory method for the separation of the D and L forms of isofloridoside has been reported. Although g.l.c.–m.s. analysis of per(trimethylsilyl)ated isofloridoside has been published[8] and Beier *et al.*[11] assigned part of the ^{13}C-n.m.r. spectrum of isofloridoside, they did not distinguish between the D and L forms of isofloridoside.

In the present study, the D and L forms of isofloridoside and floridoside were separated, and their structures were analyzed by ^{1}H-n.m.r. and ^{13}C-n.m.r. spectroscopy and g.l.c.–m.s.

EXPERIMENTAL

Materials. — *Porphyra perforata* J. Ag. was collected at various places along the coastline of Vancouver, B.C., Canada. One sample was taken from Friday Harbor, Washington, U.S.A. The material was cleaned of visible epiphytes and epifauna, and extracted immediately with 80% ethanol. The extract was evaporated to dryness in a rotary evaporator at a temperature below 40°. The pigments and lipids were removed by extraction with chloroform. The residue was dissolved in water, and the solution desalted either by passing through columns (1.2 × 20 cm) of Dowex 1 (OH^-) and Dowex 50 (H^+) ion-exchange resin or by adding an equal amount of these two resins to the sample solution while keeping the pH at 7. The neutral fraction was evaporated, dissolved in absolute ethanol, and concentrated to a thick syrup. This material was used for separation of floridoside and isofloridoside by paper chromatography.

Paper chromatography. — Whatman No. 1 and 3 papers were used for descending paper chromatography (p.c.) using the solvent system ethyl acetate–pyridine–water (8:2:1) and developed for 48 h (ref. 12). Floridoside and isofloridoside were detected by the periodate–benzidine reagent[13]. The R_f values were similar to those reported[12].

Acetylation of floridosides. — The samples and standards (5 mg) were each acetylated with 1:1 pyridine–acetic anhydride (1 mL) for 1 h at 100°, evaporated to dryness, and the residue dissolved in a small volume of ethyl acetate and analyzed by g.l.c.–m.s.

(Trimethylsilyl)ation of floridosides. — The dried samples (5 mg) were treated with TRI-SIL "Z" (1 mL; Pierce Chemical Co., Rockford, IL, U.S.A.) for 1 h at 60–70°, with occasional sonication to enhance dissolution of the samples. The resulting Me_3Si derivatives were analyzed by g.l.c.–m.s.

Methylation of floridosides. — The floridosides (5 mg) were methylated with methyl iodide in the presence of methylsulfinyl anion according to the method of Hakomori[14]. The methylated products were isolated by partitioning between water and chloroform. The chloroform layer was concentrated to a small volume, and

analyzed by g.l.c.–m.s. For methylation analysis, the permethylated floridosides were treated with 90% formic acid (2 mL) for 2 h at 100°, and the solutions were cooled and evaporated to dryness. The residue was further hydrolyzed with 0.5M trifluoroacetic acid (2 mL) in an ampoule for 16 h at 100°. The resulting, partially methylated monosaccharides were analyzed as their alditol acetates, which were obtained by reduction with sodium borohydride and acetylation.

Gas–liquid chromatography–mass spectrometry. — G.l.c. was performed in a Hewlett–Packard 5790A instrument equipped with a flame-ionization detector and connected to a model 3390A electronic integrator. A fused-silica capillary column (SE-30, 12 m × 0.2 mm) was employed, with helium as the carrier gas. Combined g.l.c.–m.s. was carried out in a Hewlett–Packard 5985B GC/MS/DS apparatus, using the same column and an ionization potential of 70 eV.

Nuclear magnetic resonance spectroscopy. — N.m.r. spectra were recorded at a probe temperature of 21° with a Bruker WM-400 instrument operated in the pulsed, Fourier-transform mode, with complete proton decoupling for ^{13}-C-n.m.r. spectra. The chemical shifts are reported in parts per million (p.p.m.) and are related to internal acetone (δ 31.4 for ^{13}C-n.m.r. spectra, and 2.20 for ^{1}H-n.m.r. spectra). The samples used for ^{1}H-n.m.r. spectroscopy were dissolved in 99.7% D_2O and lyophilized three times, and examined in the same solvent.

Infrared spectroscopy. — The samples were prepared as KBr discs, and spectra were recorded with a Perkin–Elmer double-bond spectrometer Model 599B at room temperature.

RESULTS AND DISCUSSION

Extraction of *Porphyra perforata* with 80% ethanol, removal of pigments and lipids, and desalting by passing through anion- and cation-exchange columns resulted in a neutral fraction suitable for analysis. From this fraction, it was possible to obtain, by precipitation with acetone, a compound in a yield of 0.5%. This compound was recrystallized five times from 1:1 ethanol–methanol, and characterized as one form of isofloridoside by g.l.c.–m.s. and n.m.r. spectroscopy. The melting point of this compound was 130.0–130.5° and its infrared spectrum was identical with that published[5] for the L form of isofloridoside.

G.l.c. separation of floridoside from isofloridoside was achieved by using the per(trimethylsilyl)ated derivatives. However, in spite of using an SE-30 fused-silica, capillary column and a variety of temperature programs, the D and L forms of isofloridoside could not be resolved one from the other (see also, refs. 2 and 8). Separation of the permethylated floridosides gave essentially similar results as the Me_3Si derivatives. The retention times of the permethylated derivatives were: floridoside, 3.06 min, and isofloridoside, 3.27 min, at an isothermal temperature of 190°. Peracetylation, in contrast, enabled the complete separation of the two isomers of isofloridoside. At 230°, the relative retention times were D-mannitol (internal standard), 1.00 (1.88 min); D form, 3.07; and L form, 3.17. This is consis-

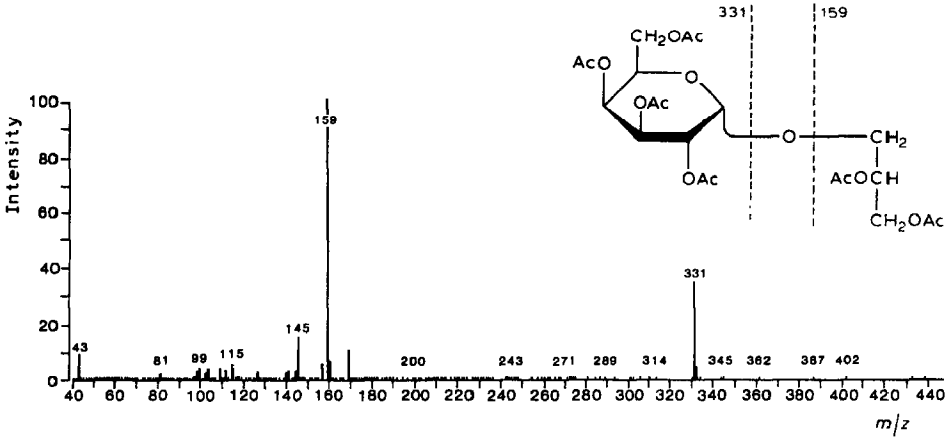

Fig. 1. Mass spectrum of per-*O*-acetylated L form of isofloridoside.

tent with the reported separation of derivatized diastereoisomeric glycosides on a g.l.c. nonchiral-phase, capillary column, which has been accomplished in the determination of D and L sugars[15,16]. Under the same conditions, floridoside has the same retention time as the L form of isofloridoside. By using a temperature program from 100° to 250° at the rate of 2°/min, floridoside can be partially separated from the L form of isofloridoside.

Hence, for routine analysis, a combination of acetylation and either (trimethylsilyl)ation or methylation can be used to quantify the floridoside and the two enantiomers of isofloridoside. This method avoids the time-consuming separation of isofloridoside from floridoside by p.c.

The mass spectrum of per(trimethylsilyl)ated floridosides showed the following major fragments (relative intensities in parentheses): floridoside, *m/z* 361 (8), 337 (26), 217 (20), 204 (100), 147 (10), and 103 (9); isofloridoside, *m/z* 361 (28), 337 (18), 217 (26), 204 (100), 147 (15), and 103 (13). No peak was observed at *m/z* >491. That the intensity ratios of these sugars at *m/z* 204 and 217 are ⩾1 indicates that the D-galactosyl groups of these glycosides are all in pyranoid forms[17].

G.l.c.–m.s. analysis of permethylated floridoside and isofloridoside gave a similar fragmentation-pattern. The following floridoside fragments are listed by using the terminology of Kochetkov and Chizov[18]: *m/z* 278 $(M-CH_3-CH_2OCH_3)^+$, 219 (aA1; 7), 187 (aA2; 41), 163 (abJ1; 100), 155 (aA3; low intensity), 103 (bA1; 14), 101 (aF1; 35), 88 (aH1; 62), 71 (aF2; 13), and 45 (15); and isofloridoside: *m/z* 278, 219 (low intensity), 187 (22), 163 (100), 103 (19), 101 (46), 88 (82), 75 (9), 71 (14), and 45 (13). The most abundant peak, *m/z* 163, derives from glycerol and part of the D-galactose, which confirms the glycosidic linkage of D-galactose to glycerol[19]. Hydrolysis of the per-*O*-methylated isofloridosides and floridoside yielded 2,3,4,6-tetra-*O*-methyl-D-galactopyranose, as indicated by g.l.c.–m.s., a result which is also consistent with the proposed structures.

The mass spectrum of the acetylated L form of isofloridoside is presented in Fig. 1; it shows two major fragments, at *m/z* 159 (100) and 331 (35), which arise from the cleavage of the glycosidic linkage, and correspond to the glycerol and galactose moieties, respectively. Most of the other fragments derive from the tetra-*O*-acetylpyranosyl ring. Floridoside and the D form of isofloridoside have spectra similar to that of the L form of isofloridoside. The most obvious difference is the intensity at *m/z* 331, which is 75, 41, and 35%, respectively. Hydrolysis of the floridosides yielded D-galactose and glycerol in equimolar amounts, as indicated by g.l.c.–m.s.

G.l.c.–m.s. alone is inadequate in providing unambiguous information on the type of glycosidic linkage, although differences in intensity were observed for certain ion peaks; *e.g.*, for acetylated derivatives at *m/z* 331, which may help to distinguish floridoside and isofloridoside. It is well known, however, that intensity can be different on different instruments; even use of the same instrument at different times will give slightly different intensities due to slight changes in conditions; this can be seen by comparison of reported mass data for floridoside or isofloridoside[8–10].

The 400-MHz, ^{1}H-n.m.r. spectrum of the purified L form of isofloridoside is presented in Fig. 2. The galactose H-1 signals showed a doublet at δ 4.93 ($J_{1,2}$ 3.7

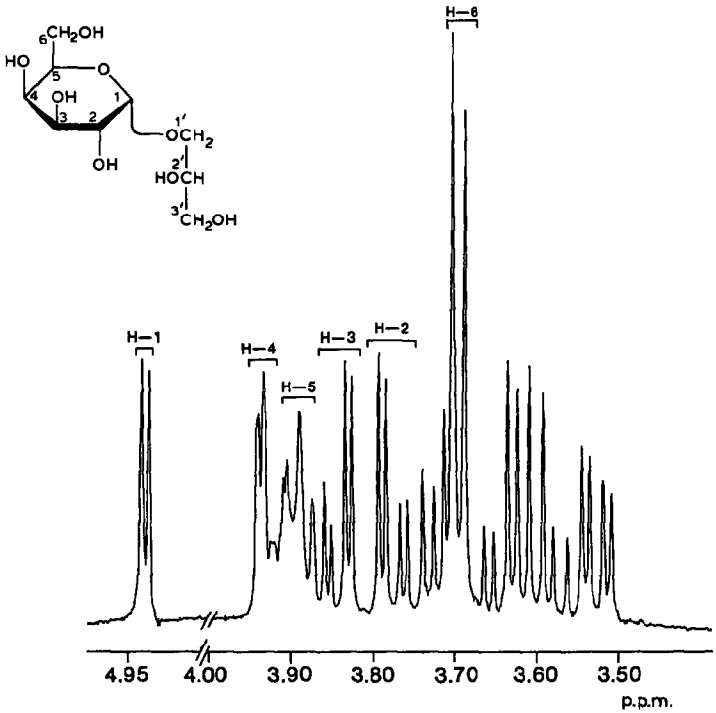

Fig. 2. 400-MHz, ^{1}H-n.m.r. spectrum of L form of isofloridoside in D_2O, recorded at 21°. Chemical-shift values are presented relative to internal acetone (δ 2.20).

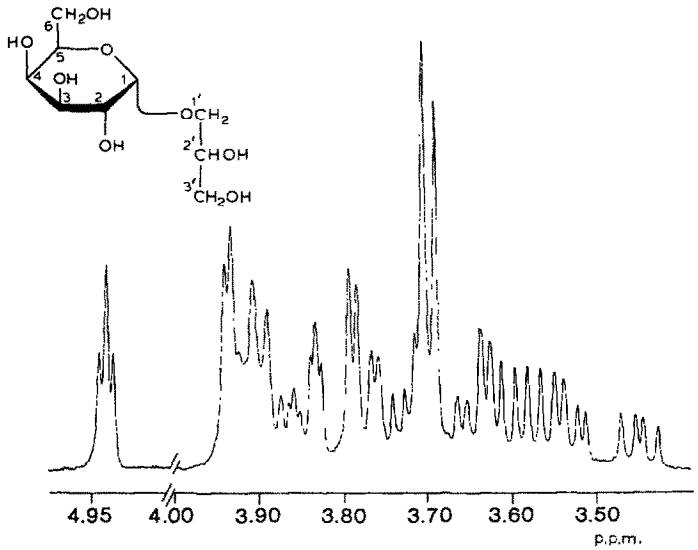

Fig. 3. 400-MHz, ^{1}H-n.m.r. spectrum of isofloridoside (a mixture of the D and L forms) in D_2O, recorded at 21°. Chemical-shift values are presented relative to internal acetone (δ 2.20).

Hz), indicating a glycosidic linkage between the D-galactopyranosyl group and glycerol. The H-2, H-3, H-4, and H-5 resonances of galactose have been assigned on the basis of selective, spin-decoupling experiments. The coupling constants of this compound were: $J_{1,2}$ 3.7, $J_{2,3}$ 10.3, and $J_{3,4}$ 3.2 Hz. A slight overlap between the H-4 and H-5 signals rendered the determination of the coupling constant between these nuclei difficult, and therefore this problem was not pursued. Our ^{1}H-n.m.r. spectrum of floridoside (data not shown) is very similar to the reported 500-MHz, ^{1}H-n.m.r. spectrum[19], and the assignments of the protons, based on the decoupling experiment, are consistent with those published[19].

The ^{1}H-n.m.r. spectrum of isofloridoside (see Fig. 3), isolated from a neutral fraction of *P. perforata* by p.c., showed two isomers in almost equal amounts, with two partially superimposed doublets at 4.93 and 4.94 p.p.m., which are in the anomeric region. Because the L form of isofloridoside shows a doublet only at 4.93 p.p.m., the resonance at 4.94 p.p.m. belongs to the D form of isofloridoside. Furthermore, there is an additional multiplet, in the region of 3.42–3.47 p.p.m., corresponding to a single proton on C-2′, which is characteristic of the D form. These facts can be utilized for quantitative purposes. Since the chemical shift of the anomeric proton of floridoside is 5.14 p.p.m., which is well separated from those of the D and L forms of isofloridoside (4.93 and 4.94 p.p.m.), all three floridosides can be quantitatively determined by ^{1}H-n.m.r. spectroscopy.

The ^{13}C-n.m.r. spectrum of the neutral fraction of *P. perforata* is shown in Fig. 4. The anomeric signals at 99.2, 99.6, and 99.9 p.p.m. indicate the presence of three sugars. By comparison with standards, these three sugars were identified as

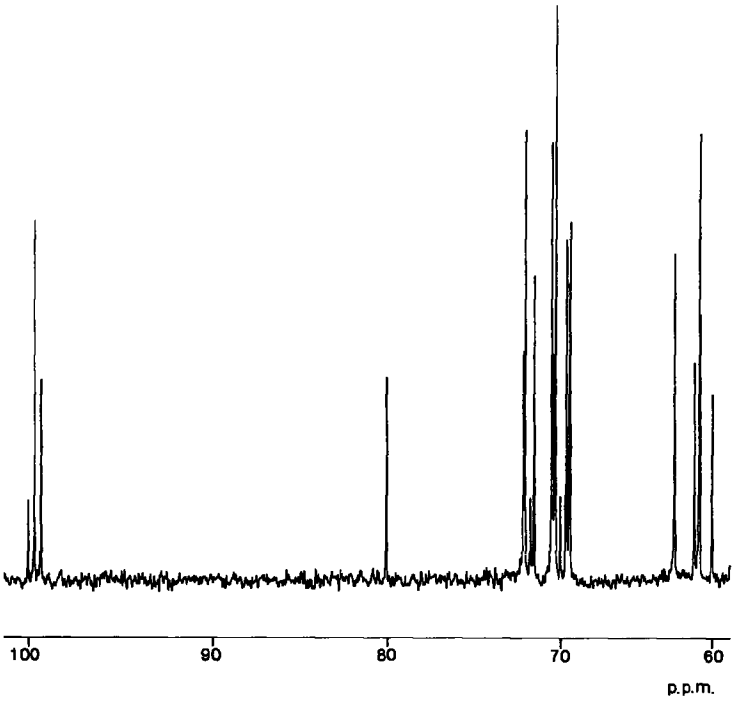

Fig. 4. 400-MHz, ^{13}C-n.m.r. spectrum of floridoside and isofloridoside (D and L forms) in D_2O, recorded at 21°. Chemical-shift values are relative to internal acetone (δ 31.4).

floridoside (99.2 p.p.m.), the D form of isofloridoside (99.9 p.p.m.), and the L form of isofloridoside (99.6 p.p.m.). The ^{13}C-n.m.r. chemical shifts and the assignments of floridoside and the D and L forms of isofloridoside are presented in Table I. Comparison of these data with each other, as well as with those of model compounds, namely, α-D-galactopyranose, methyl α-D-galactopyranoside, and glycerol, makes complete assignments possible. The α configuration of the D-galactosyl groups in the three floridosides is evident from the resonances of the anomeric carbon atoms in the region of 99–100 p.p.m., which was statistically established[11] as the α configuration region for D-galactopyranosides. ^{13}C-N.m.r. chemical shifts of floridoside and the D and L forms of isofloridoside showed that most carbon atoms in the D-galactopyranosyl groups (except the anomeric carbon atoms) had almost identical chemical-shift values, except for a minor difference in the C-5 signal (see Table I), which resonated 0.7–0.8 p.p.m. downfield compared to the C-5 signal of α-D-galactopyranose. This displacement can be attributed to the nature of the aglycon, as no similar displacement occurs in the C-5 signal of methyl α-D-galactopyranoside. The biggest difference between the spectra of the D and L forms of isofloridoside is the 0.3 p.p.m. downfield shift for the C-2′ signal of the D form. The fact that, in the spectrum of floridoside, three free hydroxylated carbon signals (at 61.5, 62.3, and 62.5 p.p.m.) appear indicates that both hydroxymethyl

TABLE I

CARBON-13 CHEMICAL SHIFTS OF FLORIDOSIDES AND RELEVANT COMPOUNDS

Compounds[a]	*Chemical shifts in p.p.m.*								
	Galactosyl group						*Glycerol residue*		
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1'*	*C-2'*	*C-3'*
α-D-Gal*p*-(1→2)-glycerol (floridoside)	99.2	69.6	70.5[b]	70.4[b]	72.2	62.3[c]	62,5[c]	79.9	61.5[c]
α-D-Gal*p*-(1→1)-D-glycerol (D form of isofloridoside)	99.9	69.6	70.6[b]	70.4[b]	72.1	62.3[c]	71.8	70.1	63.7[c]
α-D-Gal*p*-(1→1)-L-glycerol (L form of isofloridoside)	99.6	69.8	70.6[b]	70.4[b]	72.1	62.3[c]	71.6	69.8	63.7[c]
Methyl α-D-Gal*p*[d]	100.1	69.2	70.5	70.2	71.6	62.2			
α-D-Galactopyranose[d]	93.2	69.4	70.2	70.3	71.4	62.2			
Glycerol[e]							63.8	73.3	63.8

[a]Gal*p* = galactopyranosyl or galactopyranoside. [b]Assignments may be reversed. [c]Assignments may be interchanged. [d]Taken from ref. 20. [e]Taken from ref. 21.

groups of the glycerol residue must be unsubstituted and primary, implying that the linkage occurs at C-2′ (79.9 p.p.m.). This is in contrast to the spectra of the D and L forms of isofloridoside, which show only two free hydroxylated carbon signals (at 62.3 and 63.7 p.p.m., respectively), suggesting that the linkage occurs at C-1′. Compared to glycerol, the large downfield shifts for C-2′ in floridoside (6.6 p.p.m.) and for C-1′ in the D and L forms of isofloridoside (8.0 and 7.8 p.p.m.) also support these assumptions.

^{13}C-N.m.r. spectroscopy proved to be the most useful method in the present analyses. Most carbon atoms in the three isomers could be readily distinguished. For floridoside, our assignment coincides with that of van der Kaaden *et al.*[10]. There is a 0.1 p.p.m. difference between our chemical-shift data for the D form of isofloridoside and those of Beier *et al.*[11], except at C-5, where the shift difference was 0.5 p.p.m. Since they did not publish the whole assignment for isofloridoside, and did not distinguish its D and L forms, it is difficult to compare their data with those reported here. Their C-3 and C-4 signals were not resolved, but a 0.2 p.p.m. difference was observed. It is also possible that there are some temperature-related variations which might cause the differences.

In preliminary experiments, we noted that the ratios of the D and L forms of isofloridoside in *P. perforata* change from time to time, *e.g.*, the sample collected at Stanley Park, Vancouver, on Nov. 20, 1984, had a D to L ratio of almost 1:1, whereas, for a sample collected at the same place on May 20, 1985, the ratio was 0.01:1. Thus, the contradictory reports[1,5–7] about isofloridoside forms could be explained by different proportions of D and L forms in the analyzed materials collected at different times and locations.

Many studies have dealt with the possible physiological function of floridosides, but they did not distinguish between the D and L forms of isofloridoside, and, sometimes, not even between floridoside and isofloridoside. Floridoside is well known as a photosynthetic product, and has an important physiological function as an osmotic regulator. Isofloridoside, on the other hand, does not appear to be a photosynthetic product or an osmotic regulator[1,2], and there is little published information on the biosynthetic precursors and the physiological significance of isofloridoside, even though it sometimes exists in much larger amounts than floridoside[3].

ACKNOWLEDGMENTS

The authors are greatly indebted to Dr. J. S. Craigie for supplying the standard floridoside and isofloridoside. They thank Drs. G. G. S. Dutton and A. Tracey for critically reviewing the manuscript, Dr. M. Amat for assistance in collecting seaweed, Mr. G. Owen for providing the g.l.c.–mass spectra, and Mrs. M. Tracey for recording the n.m.r. spectra. This research was supported by Grant A2905 from the Natural Sciences and Engineering Research Council of Canada (to L.M.S.), and J.M. thanks the Chinese Academy of Sciences for financial support.

REFERENCES

1 J. S. CRAIGIE, J. MCLACHLAN, AND R. D. TOCHER, *Can. J. Bot.*, 46 (1968) 605–611.
2 R. H. REED, J. C. COLLINS, AND G. RUSSELL, *J. Exp. Bot.*, 31 (1980) 1539–1554.
3 J. MCLACHLAN, J. S. CRAIGIE, AND L. C.-M. CHEN, *Proc. Int. Seaweed Symp.*, 7 (1972) 473–476.
4 E. W. PUTMAN AND W. Z. HASSID, *J. Am. Chem. Soc.*, 76 (1954) 2221–2223.
5 B. WICKBERG, *Acta Chem. Scand.*, 12 (1958) 1187–1201.
6 S. PEAT AND D. A. REES, *Biochem. J.*, 79 (1961) 7–12.
7 J.-C. SU AND W. Z. HASSID, *Biochemistry*, 1 (1962) 468–474.
8 H. NAGASHIMA AND I. FUKUDA, *Phytochemistry*, 22 (1983) 1949–1951.
9 R. T. APLIN, L. J. DURHAM, Y. KANAZAWA, AND S. SAFE, *J. Chem. Soc., C*, (1967) 1346–1347.
10 A. VAN DER KAADEN, J. I. M. VAN DOORN-VAN WAKERREN, J. P. KAMERLING, J. F. G. VLIEGENTHART, AND R. H. TIESJEMA, *Eur. J. Biochem.*, 141 (1984) 513–519.
11 R. C. BEIER, B. P. MUNDY, AND G. A. STROBEL, *Can. J. Chem.*, 58 (1980) 2800–2804.
12 H. NAGASHIMA AND I. FUKUDA, *Phytochemistry*, 20 (1981) 439–442.
13 H. T. GORDON, W. THORNBURG, AND L. N. WERUM, *Anal. Chem.*, 28 (1956) 849–855.
14 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
15 G. J. GERWIG, J. P. KAMERLING, AND J. F. G. VLIEGENTHART, *Carbohydr. Res.*, 62 (1978) 349–357.
16 K. LEONTEIN, B. LINDBERG, AND J. LÖNNGREN, *Carbohydr. Res.*, 62 (1978) 359–362.
17 J. P. KAMERLING, J. F. G. VLIEGENTHART, J. VINK, AND J. J. DE RIDDER, *Tetrahedron*, 27 (1971) 4275–4288.
18 N. K. KOCHETKOV AND O. S. CHIZOV, *Adv. Carbohydr. Chem.*, 21 (1966) 39–93.
19 J. LÖNNGREN AND S. SVENSSON, *Adv. Carbohydr. Chem. Biochem.*, 29 (1975) 41–106.
20 K. BOCK AND H. THØGERSEN, *Annu. Rep. NMR Spectrosc.*, 13 (1982) 1–57.
21 W. R. DE BOER, F. J. KRUYSSEN, J. T. M. WOUTERS, AND C. KRUK, *Eur. J. Biochem.*, 62 (1976) 1–6.