Phytochemistry 70 (2009) 173–184

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Impact of the salt stress on the photosynthetic carbon flux and ^{13}C-label distribution within floridoside and digeneaside in *Solieria chordalis*

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ARTICLE INFO

Article history:
Received 16 September 2008
Received in revised form 16 December 2008

Keywords:
Solieria chordalis
Solieriaceae
Pulse-chase experiment
In vivo NMR
Cryogenic probes
Salt stress
Digeneaside
Floridoside
Isotopomers

ABSTRACT

The flux of photosynthetic carbon used in the synthesis of low-molecular weight carbohydrates (digeneaside and floridoside) was investigated by ^{13}C and ^{1}H NMR spectroscopy in samples of the red seaweed, *Solieria chordalis,* incubated at different salinities (22, 34 and 50 psu). Carbohydrates were labelled, by pulse-chase, with the stable isotope ^{13}C from $NaH^{13}CO_3$. In vivo NMR analyses carried out with a cryogenic probe optimised for ^{13}C detection were performed directly on the living algal tissues to evidence the labelling of the carbohydrates with neither preliminary extraction nor purification step(s). The isotopic enrichment of each compound was determined by high-resolution ^{1}H and ^{13}C NMR spectroscopy. These analyses evidenced different orientations of the flux of the photosynthetic carbon in the algae according to the salt stress. At normal and low salinities, the photosynthetic carbon flux was responsible of 70% and 67% of the floridoside synthetized during the pulse period, respectively, whereas it was only of 30% in the thalli exposed to the high salinity, meaning a biosynthesis of high floridoside amount from endogen source leading to the osmotic regulation. Under normal and hyper-osmotic conditions, the stock of floridoside was used for cellular needs during the chase period, whereas it was not under hypo-osmotic conditions.

The characterization of isotopomers composition of floridoside and digeneaside and the analysis of adjacent ^{13}C-labelling gives much more details on the effects of salinity on the metabolic pathways leading to the synthesis or the degradation of these molecules. High turnover of floridoside was evidenced at normal salinity during the chase period and products issued from the degradation of floridoside would not be used for the novo biosynthesis. That suggests that synthesis and degradation of floridoside may be realized in two different cellular compartments. The presence of more numerous ^{13}C–^{13}C blocks in the carbon skeleton of the molecules from the up salt stressed thalli than in those from no salt stressed algae, concomitant with a slower degree of isotopic enrichment of the molecule, provided evidence that the two metabolic pathways (endogen and photosynthetic) may not share the precursor molecules involved in the floridoside synthesis and that these two routes may be totally separate until the constitution of floridoside molecule.

1. Introduction

After a stay in the dark, algae are known to store large amounts of substrate(s) by photosynthesis in order to reconstitute their carbon pool and respond to the fluctuating conditions of their habitat (Ekman et al., 1991; Fournet, 1996). For example, in the red alga *Solieria chordalis* (Gigartinales, Solieriaceae), the flux of carbon issued from the photosynthesis is directed towards low-molecular weight carbohydrates (LMWCs) of D-galactose and D-mannose (floridoside, digeneaside), or floridean starch or cell wall polysaccharides according to the external conditions.

Floridoside (2-*O*-α-D-galactopyranosylglycerol) is considered as the main cytoplasmic carbohydrate, except in Ceramiales taxa where it is replaced by digeneaside (2-*O*-α-D-mannopyranosyl-D-glyceric acid) (Kirst, 1980; Reed, 1990). Members of the Bangiales 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) (Reed et al., 1980; Meng et al., 1987; Karsten et al., 1999). However, the recent reports of exceptions to this rule have highlighted the biochemical diversity of LMWCs in Florideophyceae (Barrow et al., 1995; Wilcox et al., 2001; Eggert et al., 2007). Further to the study by Karsten et al. (2007) only isofloridoside can be considered as a chemotaxonomic marker conversely to digeneaside. It is therefore no surprising to find digeneaside in mixture with floridoside in the red alga under study here, *S. chordalis*.

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doi:10.1016/j.phytochem.2008.12.021

Floridoside is an osmolyte (Wiencke and Läuchli, 1981; Reed, 1990) as well as a major product issued from the photosynthesis (Bean and Hassid, 1955; Macler, 1986); moreover, descriptions of metabolic route for its biosynthesis are also available (Bean and Hassid, 1955; Kauss and Schobert, 1971; Kremer and Vogl, 1975). This compound derived from the triose phosphate pathways is formed by condensation of UDP-galactose with α-glycerol phosphate and subsequent hydrolyse into galactosyl–glycerol (Macler, 1986). On the other hand, the function and biosynthesis pathway(s) of digeneaside are still unclear in seaweeds; this compound has been assumed to play a minor role in osmotic acclimation (Karsten et al., 1994) and to constitute a major photoassimilated compound in some Ceramiales species (Kremer and Vogl, 1975). According to recent investigations carried out on the Gigartinales species able to synthesise floridoside and digeneaside, their levels varie acording to the saline conditions (Broberg et al., 1998; Bondu et al., 2007).

In other respects, the tool constituted by labelling studies conducted with isotopes through pulse-chase has proven its high efficacy to elucidate the internal metabolism of organisms and get additional information about their metabolic pathways (Roscher et al., 2000). The determination of the ^{13}C isotopic enrichment of compounds by NMR spectroscopy is of great help because this technique is non invasive (Reed et al., 1995; Jones et al., 1997; Ceccaroli et al., 2003) and its sensitivity has been greatly enhanced by the recent technical developments in NMR apparatuses. Moreover, the recent availability of new cryogenic NMR probes, which have already proven their efficiency in natural abundance ^{13}C NMR analyses, has opened the way to "in vivo analyses" because of their enhanced sensitivity and efficacy. Briefly, this technique relies on the cooling of the detection head. As the signal-to-noise ratio is inversely proportional to the square root of absolute temperature, the sensitivity is significantly enhanced by cooling the radiofrequency transmitter and coils within the probe as well as the radiofrequency pre amplifier. The combination of the gains achieved by cryogenic cooling of the coils and preamplifier and by proprietary high-sensitivity hardware and software has led to a significant enhancement of the sensitivity of NMR spectroscopy accompanied with the production of well-resolved spectra (Styles et al., 1984). Among such investigations it is worth citing the degradation of aromatic compounds by the bacteria *Pseudomonas putida* (Bertini et al., 2003) and the alkaloid metabolism of the plant *Rauvolfia* sp. (Hinse et al., 2003).

All of these considerations led us to investigate the involvement of photosynthetic carbon in the synthesis of carbohydrates by samples of *S. chordalis* immersed for a short duration in seawater under different conditions of salinity to gain more insight into the impact of the osmoregulatory process upon LMWCs metabolic pathway as well as into the use of carbon atoms. To elucidate the photosynthetic flux of carbon in floridoside and digeneaside, it sounded to us worth conducting a pulse-chase experiment with labelled $NaH^{13}CO_3$ as source of carbon. The use of cryoprobe ^{13}C NMR in an in vivo analysis of seaweeds to enhance the sensitivity appeared to us as relevant and promising despite the lack, to our knowledge, of reports about such investigations in the literature. This study is completed with a determination, by high-resolution ^{1}H and ^{13}C NMR spectroscopy, of the isotopic enrichment of each labelled compound to determinate whether the orientation of the photosynthetic flux of carbon, the isotopomers composition and the ^{13}C-label distribution within the LMWCs of *S. chordalis* are affected by a saline stress.

2. Results

2.1. In vivo NMR analysis

Fig. 1 presents an in vivo ^{13}C natural abundance spectrum of a sample of *S. chordalis* thallus (bottom spectrum). Characteristic features of these ^{13}C NMR spectra are the presence of floridoside and digeneaside signals assigned by comparison with those of purified floridoside and digeneaside from *Palmaria palmata* and *Ceramium botryocarpum,* respectively; isethionic acid standard was from Sigma. These assignments were checked by 2D NMR spectroscopy (^{1}H–^{1}H COSY, ^{1}H–^{13}C HMBC, ^{1}H–^{13}C HMQC and ^{1}H–^{1}H TOCSY) carried out on an extract of *S. chordalis* (data not shown). The signals at δ_C 100.9 (C-1), 81.5 (C-2′), 73.9 (C-5), 72.2 (C-3 and C-4), 71.3 (C-2), 64.2 (C-1′), 63.8 (C-6) and 63.2 (C-3′) (^{13}C NMR spectrum) as well as the one at δ_H 5.2 (H-1) (^{1}H NMR spectrum) were attributed to floridoside (2-*O*-α-D-galactopyranosylglycerol), whereas those at δ_C 101.3 (C-1), 80.8 (C-2′), 75.8 (C-5), 73.2 (C-3), 72.9 (C-2), 69.6 (C-4), 65.9 (C-3′) and 63.8 (C-6) (^{13}C NMR spectrum) as well as the one at δ_H 4.95 (H-1) (^{1}H NMR spectrum) were assigned to digeneaside (2-*O*-α-D-mannopyranosyl-D-glyceric acid). Prior the labelling experiment, the contents of thalli in floridoside and digeneaside were, respectively, found to be 12.1 ± 0.5 and 5.3 ± 0.3 mg/g DW. However, it is worth noting the large amount of isethionic acid indicated by the signals at δ_C 55.7 and 59.5 ppm on the ^{13}C NMR spectrum as well as those at δ_H 3.2 and 4.0 ppm on the ^{1}H NMR spectrum.

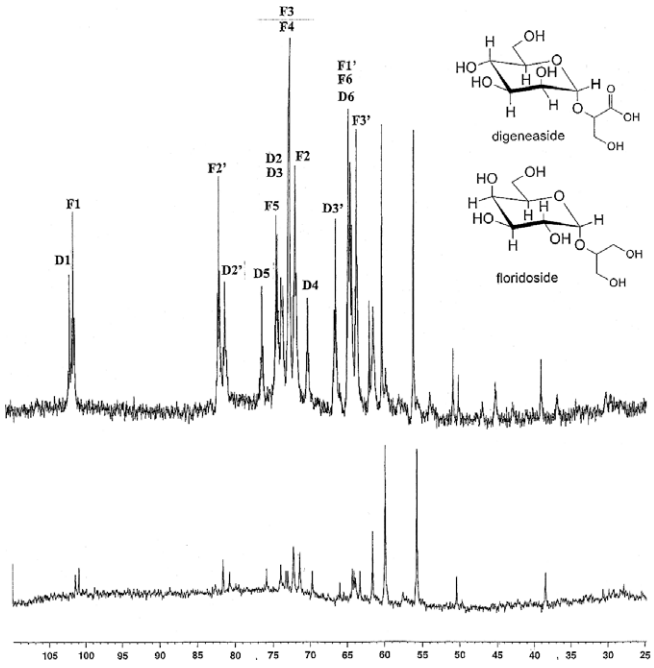

Fig. 1. In vivo ^{13}C NMR proton-decoupled spectra obtained from *S. chordalis* prior to incubation in ^{13}C-enriched medium (bottom spectrum) and after 24 h of incubation in hypo-osmotic medium containing 2.5 mM $NaH^{13}CO_3$ (top spectrum). The letters F and D on the spectra refer to the resonances of carbons of floridoside and digeneaside, respectively.

In an unlabelled molecule formed with carbon, hydrogen and oxygen atoms, the probability to have two adjacent ^{13}C (natural abundance) being only $(0.011)^2$, the ^{13}C NMR proton-decoupled pattern shows a single signal. On the contrary, in a labelled molecule with several blocks of adjacent ^{13}C atoms, the NMR spectrum displays triplet(s) or multiplet(s) (Glawischnig et al., 2002; Massou et al., 2007). The determination of enriched compounds is facilitated whenever the ^{13}C spectra are well-resolved with narrow signals allowing a clear identification of couplings. A comparison of the in vivo ^{13}C NMR spectrum from algae cultivated under hypo-osmotic conditions after the pulse period (Fig. 1, top spectrum) with the in vivo ^{13}C spectrum in natural abundance (Fig. 1, bottom spectrum) allowed us to unambiguously identify an isotopic enrichment of floridoside and digeneaside. Similar comparisons carried out on the algae cultivated at different salinities (22, 34

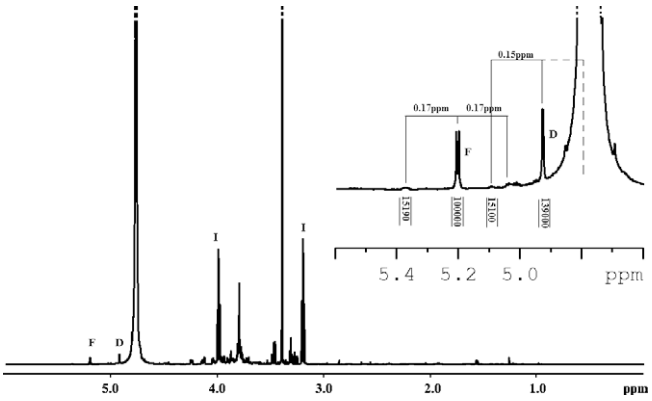

Fig. 2. ^{1}H NMR spectrum obtained from extract of *S. chordalis* incubated for 24 h under hypo-osmotic conditions in seawater enriched with 2.5 mM $NaH^{13}CO_3$ and then for 48 h in a non-enriched water. The F and D refer to the resonances of the H-1 of floridoside and digeneaside. I refers to the isethionic acid. The right picture corresponds to an enlarged view of the proton carried by the anomeric carbon of floridoside and digeneaside and shows the integrals of the ^{1}H-^{13}C satellites.

and 50 psu) after the pulse and chase periods evidenced a labelling of floridoside by ^{13}C whatever the degree of salinity and contrary to digeneaside, where such a labelling was present only in the algae cultivated under hypo-osmotic conditions (spectra no shown).

2.2. High resolution ^{1}H NMR experiment performed on the algal extracts

On a ^{1}H NMR spectrum, signals from protons appear as a more or less decoupled signal due to the ^{1}H–^{1}H homonuclear scalar couplings. The presence of the ^{13}C in a molecule induces heteronuclear scalar coupling(s), ^{1}H–^{13}C, that appear(s) as two additional patterns termed "satellites" and equally spaced upfield and downfield of the resonance of the proton carried by the ^{12}C; they are visible only on ^{1}H NMR spectra performed with no ^{13}C-decoupling. In this study, focus was on the protons carried by the anomeric carbon of floridoside and digeneaside since these signals proved to be well separated from the others. A comparison by superimposition of the ^{1}H NMR spectrum with ^{13}C-decoupling to the one with no ^{13}C-decoupling according to a technique described in Jones et al. (1997) showed that the satellite signals at δ_H 5.37 and 5.03 ppm were characteristic of the anomeric proton of the [$^{13}C_1$]-floridoside (similar observation was done in Simon-Colin et al. (2004)); the pattern at δ_H 5.1 ppm proved to correspond to the [$^{13}C_1$]-digeneaside (Fig. 2). One should note that the satellite signals due to the anomeric proton of floridoside were observed on every NMR spectrum recorded from the algae incubated at different salinities after the pulse and chase periods; on the other hand, the satellite signals due to digeneaside were observed only on the spectra recorded from the algae cultivated under hypo-osmotic conditions (data not shown). These observations were in agreement with our conclusions about our in vivo analyses. Furthermore, a comparison of the integrated areas of these ^{13}C-labelling-induced satellites against that of the main proton signal gives insight into the isotopic enrichment of carbons, which is calculated by dividing the area of the satellites by the total intensity of the resonance (Massou et al., 2007). Table 1 shows that, after the pulse period, the higher the salinity is, the less the C-1 of floridoside is enriched. It also highlights a decrease of isotopic enrichment after the chase period. On the other hand, concerning digeneaside, which was labelled only under hypo-osmotic conditions, the isotopic enrichment of its C-1 was nearly alike after the pulse and chase periods (11 against 10.8%).

Comparisons of the ^{1}H NMR spectra according to the salt conditions evidenced a relationship between the variations in the intensities of the signals corresponding to the H-1 of floridoside and digeneaside and stress by salt: floridoside showed not only a strong reduction of its H-1 signal at 22 psu, but also a very marked increase at 50 psu relative to the signal H-1 of digeneaside. The relative floridoside/digeneaside ratios (*F*/*D* ratio) calculated from the intensities of their anomeric proton signals on the ^{1}H NMR spectra were in agreement with those found from the individual quantification, by HPLC, of digeneaside and floridoside in thalli (Table 1). Under hypo-osmotic conditions, *F*/*D* ratios remained below 1 (0.508–0.739), whereas they were far much greater (3.7–4.09) under hyper-osmotic conditions. The values found in no salt stressed algae were in the range 1.47–1.96.

2.3. High resolution ^{13}C NMR analysis performed on purified floridoside and digeneaside

To assess the impact of change in salinity conditions upon floridoside and digeneaside, ^{13}C NMR spectra of labelled floridoside and labelled digeneaside purified from the thalli incubated at different salinities were recorded after the pulse and chase periods.

2.3.1. Effect of salinity on ^{13}C flux in floridoside and digeneaside

To determine the relative ^{13}C enrichment of each carbon atom of floridoside and digeneaside, the integrals of signals from a ^{13}C NMR spectrum of unlabelled compound (natural ^{13}C abundance) were compared with those of the ^{13}C NMR spectrum recorded under the same experimental conditions after labelling. Then the multiplication of the found values by the absolute ^{13}C abundance of the C-1 determined from the intensities of ^{13}C-coupled satellites in the ^{1}H NMR signals (Table 1) provided us with the absolute ^{13}C abundance of each carbon. Table 2 gives the results of the ^{13}C NMR analysis of the labelled floridoside and -digeneaside purified from the algae incubated for 24 h in a hypo-osmotic medium enriched with $NaH^{13}CO_3$ and then for 48 h in a non-enriched hypo-osmotic medium. This procedure allowed us to calculate the average of the absolute ^{13}C abundances of every carbon, which corresponds to the

Table 1
NMR pattern and isotopic enrichment of the C-1 (*via* the intensities of ^{13}C-coupled satellites in the ^{1}H NMR signals) of the floridoside and digeneaside for each experimental condition. The floridoside/digeneaside ratios (*F*/*D* ratio) have been obtained from the intensities of the H-1 on ^{1}H NMR spectra and from the quantitative analysis.

Experimental conditions		NMR data				NMR quantification	Carbohydrates quantification
Salt stress	^{13}C-labelling	C-1 NMR pattern of floridoside	Isotopic enrichment of C-1 of floridoside	C-1 NMR pattern of digeneaside	Isotopic enrichment of C-1 of digeneaside	*F*/*D* ratio	*F*/*D* ratio
Hypo-osmotic conditions	Pulse period	Triplet-like	0.233	Triplet-like	0.110	0.618	0.508
	Chase period	Triplet-like	0.152	Triplet-like	0.108	0.73	0.739
No salt stress	Pulse period	Triplet-like	0.184	Singulet	No labelling	1.48	1.47
	Chase period	Triplet-like	0.107	Singulet	No labelling	1.94	1.96
Hyper-osmotic conditions	Pulse period	Triplet-like	0.156	nd	No labelling	3.19	3.7
	Chase period	Triplet-like	0.131	nd	No labelling	4.09	4.01

Table 2
^{13}C NMR analysis of floridoside and digeneaside purified from the thalli incubated in hypo-osmotic medium after the chase period.

C atom and chemical shift	Coupling constants (Hz)	Relative ^{13}C NMR signal integrals			
		Unlabelled sample I_{ref}	Labeled sample $I*$	Ratio $I*/I_{ref}$	Absolute ^{13}C abundance
Floridoside					
C-1 (100.9 ppm)	45.53 (C-2)	1000[a]	1000[a]	1	0.152[b]
C-2 (71.3 ppm)	46.16 (C-1)	979.72	1035.59	1.06	0.161
C-3 (72.18 ppm)	nd	989.23	969.69	0.98	0.149
C-4 (72.13 ppm)	nd	1006.73	969.69	0.96	0.146
C-5 (73.9 ppm)	43.94 (C-6)	949.14	997.7	1.05	0.160
C-6 (63.8 ppm)	44.72 (C-5)	968.93	936.75	0.97	0.147
C-1′(64.2 ppm)	42.26 (C-1′)	960.69	936.75	0.98	0.149
C-2′(81.5 ppm)	42.03 (C-1′), 41.95 (C-3′)	988.65	1053.11	1.07	0.163
C-3′(63.2 ppm)	41.72 (C-2′)	950.93	934.09	0.98	0.149
Digeneaside					
C-1 (101.35 ppm)	47.23 (C-1)	1000[a]	1000[a]	1	0.108[b]
C-2 (72.9 ppm)	nd	985.37	988.87	1	0.108
C-3 (73.2 ppm)	nd	1018.54	988.87	0.97	0.105
C-4 (69.6 ppm)	39.97 (C-3)	948.06	925.64	0.98	0.106
C-5 (75.8 ppm)	41.71 (C-4)	919.57	1096.13	1.19	0.129
C-6 (63.8 ppm)	43.27 (C-5)	940.31	965.93	1.03	0.111
C-1′ (179.8 ppm)	55.31 (C-2′)	1073.56	999.91	0.93	0.100
C-2′ (80.8 ppm)	40.09 (C-3′), 54.18 (C-1′)	913.66	914.52	1	0.108
C-3′ (65.9 ppm)	40.12 (C-2′)	947.29	855.83	0.90	0.097

[a] Relative ^{13}C NMR signal integrals referenced to 1000 for the integral of the C-1 signals.
[b] Absolute ^{13}C abundance for the C-1 was obtained from the ^{13}C coupling satellites in 1H NMR signals for the H-1, these values were used to convert the $I*/I_{ref}$ ratio to absolute ^{13}C abundance for each carbon atom.

Table 3
Averaged absolute ^{13}C abundance for all carbons constituting the floridoside and digeneaside compared to the isotopic enrichment of their C-1, for each experimental conditions.

Experimental conditions		^{13}C analysis from integrals intensities			
Salt stress	^{13}C-labelling	Isotopic enrichment of C-1 of floridoside	Averaged absolute ^{13}C abundance of all carbons	Isotopic enrichment of C-1 of digeneaside	Averaged absolute ^{13}C abundance of all carbons
Hypo-osmotic conditions	Pulse period	0.233	0.246 ± 0.03	0.110	0.120 ± 0.01
	Chase period	0.152	0.153 ± 0.01	0.108	0.108 ± 0.01
No salt stress	Pulse period	0.184	0.195 ± 0.01	No labelling	
	Chase period	0.107	0.100 ± 0.01	No labelling	
Hyper-osmotic conditions	Pulse period	0.156	0.163 ± 0.01	No labelling	
	Chase period	0.131	0.139 ± 0.01	No labelling	

absolute ^{13}C abundance of the whole molecule (Table 3). One should note that a similar analysis was made for each purified labelled compound from the thalli incubated at different salinities after the pulse and chase periods (data not shown). Whatever the experimental condition (salinity, pulse and chase), the standard deviations on the calculation of absolute ^{13}C abundance in floridoside and digeneaside were always in the range 1–3%, which suggests a uniform-labelling of carbons constituting the molecules.

The main results obtained about the labelling state of floridoside and digeneaside are presented in Fig. 3 as histograms. Under hypo-osmotic conditions and after 3 days of culture in the dark, the floridoside content in the thalli was 3.86 ± 1.25 mg/g DW; it was elevated to 6.12 ± 2.43 mg/g DW after the pulse period (HPLC assay). So in 24 h, the amount of synthesised floridoside was about 2.26 mg/g DW. As the isotopic enrichment of floridoside was 0.246, the relative amount of labelled floridoside after the pulse period was therefore 1.51 ± 0.60 mg/g DW (out of the 2.26 mg/g DW generated). So 67% of the floridoside synthesised during the pulse period were labelled. During the chase period, the amount of floridoside synthesised by the algae was about 3.5 mg/g DW. At its end, the isotopic enrichment of floridoside was then 0.153, which leads to a relative amount of labelled floridoside of 1.47 ± 0.23 mg/g DW. It is worth noting that the amount of labelled floridoside remained unchanged after the chase period. Under the same conditions of culture, quantification by HPLC showed that the contents in digeneaside before and after the 24 h pulse period were 9.60 ± 1.81 and 12.04 ± 2.41 mg/g DW, respectively; about 2.44 mg/g DW digeneaside were thus generated in 24 h. As the fractional enrichment of this compound was 0.12, the relative amount of labelled digeneaside after the pulse period was 1.45 ± 0.29 mg/g DW out of the 2.44 mg/g DW generated, which means that 59% of the digeneaside synthesised over the pulse period were labelled. During the chase period, 0.97 mg/g DW of digeneaside were generated. The fractional enrichment of digeneaside after the chase period was 0.108, which corresponds to a relative amount of labelled digeneaside of 1.41 ± 0.09 mg/g DW. The amounts of labelled digeneaside in thalli before and after the chase period were similar (i.e. 1.4 mg/g DW).

Concerning the no salt stress thalli, the floridoside contents after the stay in the dark and the pulse period were 8.89 ± 1.02 and 12.32 ± 1.84 mg/g DW, respectively (HPLC assay). So in 24 h, about 3.43 mg/g DW of floridoside were generated. The fractional enrichment of this compound was 0.195. At the end of the pulse period, the relative amount of labelled floridoside was, therefore, 2.40 ± 0.36 mg/g DW out of the 3.43 mg/g DW generated: so, 70% of the floridoside synthesised during the pulse period were labelled. The algae synthesised 5.22 mg/g DW of floridoside over the chase period, and thus the fractional enrichment at the end of this period was 0.10; the relative amount of labelled floridoside in the thalli was, therefore, about 1.75 ± 0.17 mg/g DW. The amount of labelled floridoside was lower after the chase period

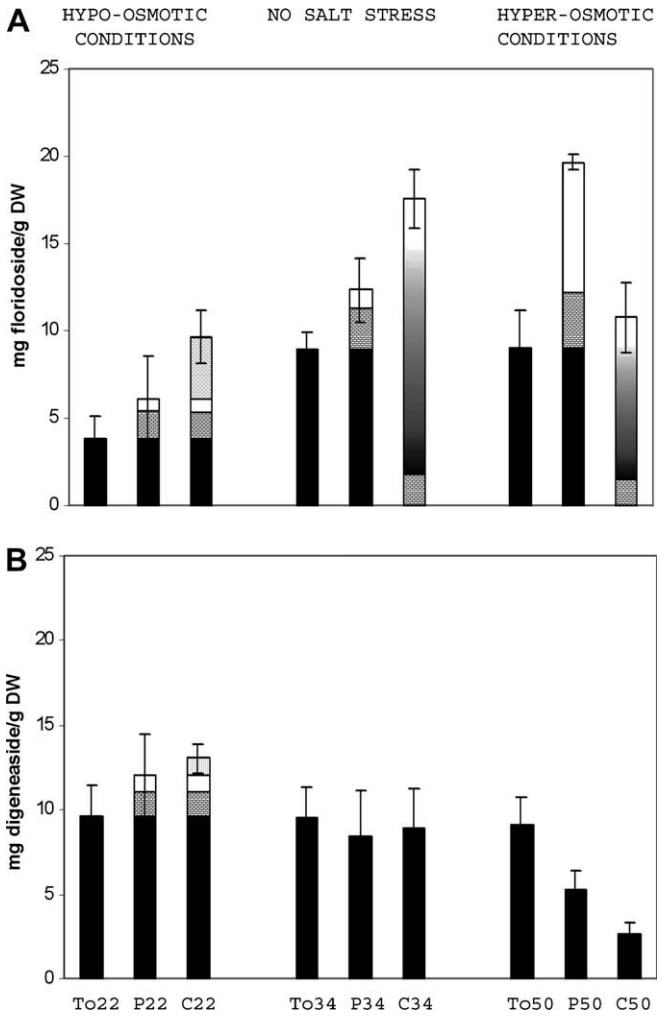

Fig. 3. Effects of stress by salt on labelled and no-labelled floridoside (A) and digeneaside (B) contents in *S. chordalis* during the pulse-chase experiment. To22, To34, To50 refer to the amounts of floridoside and digeneaside purified from thalli incubated at 22, 34 and 50 psu in salinity, respectively, after the dark treatment, P22, P34, P50, after the pulse period and C22, C34, C50, after the chase period. The black colour refers to the unlabelled carbohydrate levels present in the thalli before the start of the labelling experiment; the squared symbol and the white colour, respectively, refer to the labelled and unlabelled carbohydrate levels synthesized over the pulse period; the dotted area represents the unlabelled carbohydrate levels synthesized during the chase period. Finally, the gradation in grey colours represents the mixture of unlabelled carbohydrates synthesized during the pulse and chase periods and the one already present at the start of experiment.

than after the pulse one though the concentration of floridoside in the thallus was more elevated.

At elevated salinity (hyper-osmotic conditions), the floridoside content in thalli after 3 days in the dark was 8.99 ± 2.14 mg/g DW and rose to 19.62 ± 0.45 mg/g DW after the 24 h pulse period. So, 10.63 mg/g DW floridoside were generated in 24 h. As the isotopic enrichment was 0.163, after the pulse period the relative amount of labelled floridoside was 3.20 ± 0.07 mg/g DW out of 10.63 mg/g DW; this means that 30% of the floridoside synthesised over the pulse period were labelled. After the chase period, the finding of a decrease by 1.83 of the floridoside content in thalli indicates recycling of about 8.9 mg/g DW and mobilisation towards the synthesis of others compounds. After the chase period the fractional enrichment of floridoside was 0.139, and thus the thalli contained about 1.49 ± 0.28 mg/g DW of labelled floridoside. The percentages of labelled floridoside in the thalli were quite similar prior and after the chase period (16.3% versus 13.9%); on the other hand, the content in floridoside was decreased by 1.83.

2.3.2. Effect of salinity on the isotopomers composition of floridoside and digeneaside

The isotopomers composition of floridoside and digeneaside molecules were determined by quantitative NMR spectroscopy (Eisenreich and Bacher, 2000) displayed on each spectrum of labelled floridoside and -digeneaside purified from thalli incubated at different salinities after the pulse and chase periods. These spectra were alike and showed separate patterns corresponding to each carbon atom of the molecule; each NMR pattern presented satellite signals induced by the ^{13}C–^{13}C couplings. Fig. 4 presents the ^{13}C NMR spectra of pure labelled floridoside and -digeneaside isolated from thalli incubated for 24 h in hypo-osmotic medium enriched with $NaH^{13}CO_3$. The ^{13}C NMR pattern of carbons carrying an adjacent carbon (for example, C-1, C-6, C-1′ and C-3′ of floridoside and digeneaside) appeared as triplets (Fig. 4); the central peak corresponds to the coupling with a ^{12}C, whereas the two satellite signals correspond to the coupling with a ^{13}C. Fig. 4 also shows quintuplets identified as the characteristic pattern by NMR of a ^{13}C carbon carrying two adjacent carbons (e.g. the C-2, C-5 and C-2′ of floridoside and C-4 and C-5 of digeneaside). As these carbons carry two adjacent carbon atoms with quite similar scalar couplings constants (40–47 Hz) (Table 2), the quintuplet reveals the occurrence of four isotopomers: the central signal within the pattern reflects the coupling with two ^{12}C (–^{12}C–^{13}C–^{12}C–) and the two nearest satellite signals indicate a coupling with only one ^{13}C though two isotopomers can be formed as follows: (–^{12}C–^{13}C–^{13}C– or –^{13}C–^{13}C–^{12}C–). The two external satellite signals result from a coupling with two ^{13}C (–^{13}C–^{13}C–^{13}C–). Whenever the scalar coupling constants of adjacent carbons are very different, the separation of the two isopotomers (–^{12}C–^{13}C–^{13}C– and –^{13}C–^{13}C–^{12}C–) makes the NMR pattern appears as a heptuplet. This case-study is exemplified here with the C'_2 of digeneaside; indeed, this carbon bears an acid group and a primary alcohol one (COOH, $J_{2'/1'}$ = 54.18 Hz and CH_2OH, $J_{2'/3'}$ = 40.09 Hz) (Fig. 4B, Table 2).

The signal integrals of each specific ^{13}C satellites, in comparaison with the overall signal intensity reflect the relative amounts of each isotopomers. By multiplication of these values with the absolute ^{13}C abundance at a given molecular position, the molar contributions (mol.%) of each respective isotopomer can be calculated. The characterization of isotopomers composition for floridoside and digeneaside obtained from thalli of *S. chordalis* incubated in hypo-osmotic medium after the chase period is given as an example in Table 4. Similar quantitative analyses were carried out on the ^{13}C NMR spectra of floridoside and digeneaside from algae cultivated at different salinities (22, 34 and 50 psu) after the pulse and chase periods and the characterization of isotopomers compositions of floridoside and digeneaside under each algae culture condition were summed up in Table 5.

Based on these results, the small standard deviation calculated for each type of isotopomers of floridoside [i.e. single-($1^{13}C_1$, $2^{13}C_1$, $5^{13}C_1$, $6^{13}C_1$, $1'^{13}C_1$, $2'^{13}C_1$, $3'^{13}C_1$) or twice-($1,2^{13}C_2$, $5,6^{13}C_2$, $1',2'^{13}C_2$, $2',3'^{13}C_2$) or three-labelled isotopomers ($1,2,3^{13}C_3$, $4,5,6^{13}C_3$, $1',2',3'^{13}C_3$)] for each algae condition culture allowed us to deduce that the galactose and glycerol moieties were similarly labelled. However, it must to be notice that a higher amount of $1',2',3'^{13}C_3$ was recorded when seaweeds were incubated in hypo-osmotic medium and after the pulse period (i.e. 9.19 mol.% versus 5.62 mol.% for $1,2,3^{13}C_3$ and 6.70 mol.% for $4,5,6^{13}C_3$). The Fig. 5 shows that the higher amounts of floridoside isotopomers was obtained in algae incubated in hypo-osmotic medium after the pulse period (P22, Fig. 5), in relation with the higher absolute ^{13}C abundance found under this culture condition. In thalli exposed to salt stress (50 and 22 psu), quite similar amount of each type of isotop-

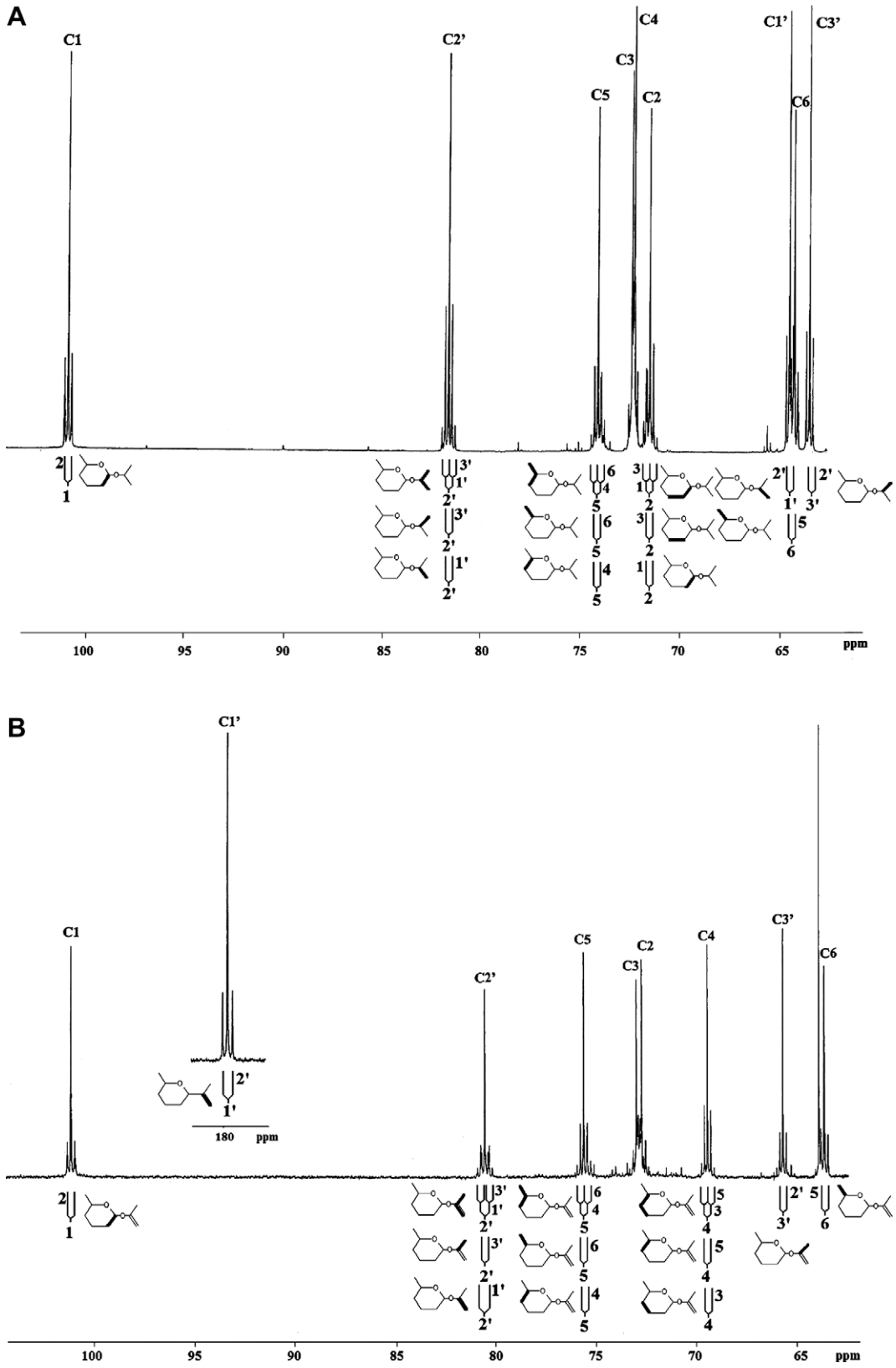

Fig. 4. ^{13}C NMR spectra of labelled floridoside (A) and labelled digeneaside (B) isolated from thalli incubated for 24 h in hypo-osmotic medium containing 2.5 mM $NaH^{13}CO_3$. ^{13}C–^{13}C coupling patterns are indicated under the spectra, multiply ^{13}C-labelling is indicated by bold line connecting ^{13}C atoms.

omers were obtained. It was not the case for the isotopomers composition of floridoside purified from no salt stress thalli where highers amounts of less-labelled isotopomers were recorded (coefficients of linear regression around −1 for P22 and P50 compared to −3 for P34). Moreover, similar amounts of each isotopomers were recorded after the pulse and chase periods in thalli

Table 4
^{13}C NMR analysis of isotopomers of floridoside and digeneaside purified from thalli of *S. chordalis* incubated in hypo-osmotic medium after the chase period.

C atom	Abs. ^{13}C abund[a]	Relative ^{13}C NMR signal intensities[b]	Isotopomers % and (mol.%)[c]
Floridoside			
C-1	0.152	$1^{13}C_1$ 100; $1,2^{13}C_2$ 89.9; $1,2,3^{13}C_3$ 74.7	$1^{13}C_1$ 37.8 (5.75); $1,2^{13}C_2$ 34.0 (2.24); $1,2,3^{13}C_3$ 28.2 (4.29)
C-2	0.161	$2^{13}C_1$ 100; $1,2^{13}C_2 + 1,2^{13}C_2$ 117.6; $1,2,3^{13}C_3$ 74.7	$2^{13}C_1$ 34.2 (5.51); $1,2^{13}C_2 + 2,3^{13}C_2$ 40.3 (6.48); $1,2,3^{13}C_3$ 25.6 (4.12)
C-3	0.149	nd	
C-4	0.146	nd	
C-5	0.160	$5^{13}C_1$ 100; $4,5^{13}C_2 + 5,6^{13}C_2$ 135; $4,5,6^{13}C_3$ 71.8	$5^{13}C_1$ 32.6 (5.22); $4,5^{13}C_2 + 5,6^{13}C_2$ 44.0 (7.04); $4,5,6^{13}C_3$ 23.4 (3.74)
C-6	0.147	$6^{13}C_1$ 100; $5,6^{13}C_2$ 96.5; $4,5,6^{13}C_3$ 71.8	$6^{13}C_1$ 37.3 (5.48); $5,6^{13}C_2$ 34.0 (5.0); $4,5,6^{13}C_3$ 26.8 (3.94)
C-1′	0.149	$1'^{13}C_1$ 100; $1',2'^{13}C_2$ 94.6; $1',2',3'^{13}C_3$ 89.1	$1'^{13}C_1$ 35.2 (5.24); $1',2'^{13}C_2$ 33.3 (4.96); $1',2',3'^{13}C_3$ 31.4 (4.68)
C-2′	0.163	$2'^{13}C_1$ 100; $1',2'^{13}C_2 + 2',3'^{13}C_2$ 147.2; $1',2',3'^{13}C_3$ 89.1	$2'^{13}C_1$ 29.7 (4.84); $1',2'^{13}C_2 + 2',3'^{13}C_2$ 43.8 (7.14); $1',2',3'^{13}C_3$ 26.5 (4.32)
C-3′	0.149	$3'^{13}C_1$ 100; $2',3'^{13}C_2$ 89.6; $1',2',3'^{13}C_3$ 89.1	$3'^{13}C_1$ 35.9 (5.35); $2',3'^{13}C_2$ 32.1 (4.78); $1',2',3'^{13}C_3$ 32.0 (4.77)
Digeneaside			
C-1	0.108	$1^{13}C_1$ 100; $1,2^{13}C_2$ 48.9	$1^{13}C_1$ 67.1 (7.25); $1,2^{13}C_2$ 32.9 (3.55)
C-2	0.108	nd	
C-3	0.105	nd	
C-4	0.106	$4^{13}C_1$ 100; $3,4^{13}C_2 + 4,5^{13}C_2$ 87.6; $3,4,5^{13}C_3$ 30.6	$4^{13}C_1$ 45.8 (4.86); $3,4^{13}C_2 + 4,5^{13}C_2$ 40.2 (4.26); $3,4,5^{13}C_3$ 14.0 (1.49)
C-5	0.129	$5^{13}C_1$ 100; $4,5^{13}C_2 + 5,6^{13}C_2$ 91.2; $4,5,6^{13}C_3$ 40.7	$5^{13}C_1$ 43.1 (5.56); $4,5^{13}C_2 + 5,6^{13}C_2$ 39.3 (5.07); $4,5,6^{13}C_3$ 17.6 (2.26)
C-6	0.111	$6^{13}C_1$ 100; $5,6^{13}C_2$ 53.5; $4,5,6^{13}C_3$ 40.7	$6^{13}C_1$ 51.5 (5.72); $5,6^{13}C_2$ 27.5 (3.06); $4,5,6^{13}C_3$ 21.0 (2.33)
C-1′	0.100	$1'^{13}C_1$ 100; $1',2'^{13}C_2$ 51.6; $1',2',3'^{13}C_3$ 34.5	$1'^{13}C_1$ 53.8 (5.38); $1',2'^{13}C_2$ 27.7 (2.77); $1',2',3'^{13}C_3$ 18.5 (1.85)
C-2′	0.108	$2'^{13}C_1$ 100; $1',2'^{13}C_2$ 41.2; $2',3'^{13}C_2$ 35.4; $1',2',3'^{13}C_3$ 34.5	$2'^{13}C_1$ 47.4 (5.12); $1',2'^{13}C_2$ 19.5 (2.11); $2',3'^{13}C_2$ 16.8 (1.81); $1',2',3'^{13}C_3$ 16.3 (1.76)
C-3′	0.097	$3'^{13}C_1$ 100; $2',3'^{13}C_2$ 46.6; $1',2',3'^{13}C_3$ 34.5	$3'^{13}C_1$ 55.2 (5.36); $2',3'^{13}C_2$ 25.7 (2.49); $1',2',3'^{13}C_3$ 19.0 (1.85)

nd: not determined because of signal overlapping.
[a] Absolute ^{13}C abundance of each carbon atom (Table 2).
[b] Relative fractions of satellite signals for given isotopomers (integrals of singulets were arbitrarily set to value of 100).
[c] Obtained by multiplication of isotopomers % with absolutes ^{13}C abundances for a given carbon atom.

Table 5
Isotopomers composition (mol.%) of floridoside and digeneaside purified from thalli of *S. chordalis* according to the culture conditions (salt stresses, pulse and chase periods).

Salt stress	Hypo-osmotic conditions		No salt stress		Hyper-osmotic conditions	
^{13}C-labelling	Pulse period	Chase period	Pulse period	Chase period	Pulse period	Chase period
Floridoside						
$1^{13}C_1$	8.9	5.75	9.43	5.56	6.30	7.48
$2^{13}C_1$	7.45	5.51	9.10	4.40	6.19	nd
$5^{13}C_1$	8.44	5.22	9.54	2.75	5.60	5.06
$6^{13}C_1$	8.93	5.48	9.75	4.33	6.53	5.91
$1'^{13}C_1$	8.73	5.24	9.38	4.53	6.22	5.42
$2'^{13}C_1$	11.31	4.84	7.54	4.62	5.23	4.89
$3'^{13}C_1$	9.32	5.35	9.43	5.06	6.79	5.68
$1,2^{13}C_2$	8.2	2.24	6.09	3.48	4.96	5.62
$5,6^{13}C_2$	8.19	5.0	6.30	2.89	5.52	5.23
$1',2'^{13}C_2$	8.37	4.96	6.09	3.04	4.38	4.02
$2',3'^{13}C_2$	9.43	4.78	6.01	3.19	5.77	4.87
$1,2,3^{13}C_3$	5.62 ± 0.51	4.21 ± 0.09	2.83 ± 0.05	1.48 ± 0.17	4.3 ± 0.04	nd
$4,5,6^{13}C_3$	6.7 ± 0.01	3.84 ± 0.10	3.2 ± 0.04	2.44 ± 0.55	3.11 ± 0.24	2.66 ± 0.21
$1',2',3'^{13}C_3$	9.19 ± 1.04	4.59 ± 0.19	3.78 ± 0.38	2.01 ± 0.10	4.42 ± 0.38	3.58 ± 0.19
Digeneaside						
$1^{13}C_1$	6.21	7.25				
$4^{13}C_1$	3.56	4.86				
$5^{13}C_1$	5.36	5.56				
$6^{13}C_1$	5.85	5.72				
$1'^{13}C_1$	nd	5.38				
$2'^{13}C_1$	nd	5.12				
$3'^{13}C_1$	7.95	5.36				
$1,2^{13}C_2$	3.96	3.55				
$5,6^{13}C_2$	4.13	3.06				
$1',2'^{13}C_2$	nd	2.44 ± 0.33				
$2',3'^{13}C_2$	4.73	2.15 ± 0.34				
$3,4,5^{13}C_3$	1.16	1.49				
$4,5,6^{13}C_3$	2.61 ± 0.12	2.30 ± 0.04				
$1',2',3'^{13}C_3$	nd	1.81 ± 0.05				

nd: not determined.

incubated in hyper-osmotic medium (Table 5, P50 and C50 in Fig. 5) whereas the recorded amounts were smaller in thalli exposed to the hypo-osmotic condition and in normal condition of salinity.

2.3.3. Effect of salinity on the labelling state of carbons adjacent to a ^{13}C in floridoside and digeneaside

The ^{13}C-label distributions within floridoside and digeneaside molecules were determined by a detailed analysis of ^{13}C patterns

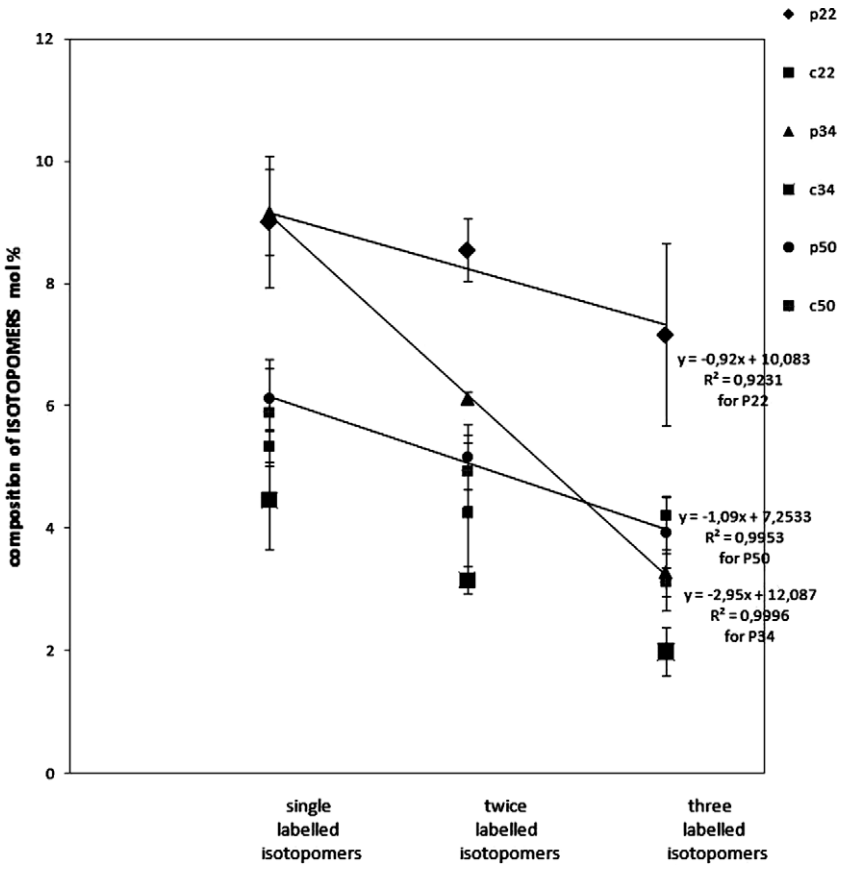

Fig. 5. Composition (mol.%) of single- ($1^{13}C_1$, $2^{13}C_1$, $5^{13}C_1$, $6^{13}C_1$, $1'^{13}C_1$, $2'^{13}C_1$, $3'^{13}C_1$) or twice- ($1,2^{13}C_2$, $5,6^{13}C_2$, $1',2'^{13}C_2$, $2',3'^{13}C_2$) or three-labelled floridoside isotopomers ($1,2,3^{13}C_3$, $4,5,6^{13}C_3$, $1',2',3'^{13}C_3$) according the culture conditions of seaweeds (P22, P34, P50 refer to the amounts of floridoside and digeneaside purified from thalli incubated at 22, 34 and 50 psu in salinity, respectively, after the pulse period and C22, C34, C50, after the chase period).

displayed on each spectrum of labelled floridoside and -digeneaside purified from thalli incubated at different salinities after the pulse and chase periods. For each ^{13}C, the probability (%) that this carbon atom was linked to one or two other ^{13}C was extracted from the integration of each satellite coupling and expressed in percentage relative to the population of molecules enriched at this position. Fig. 6 depicts the mean abundance for each type of carbons, i.e. alpha carbons such as C-1, C-6, C-1′ and C-3′ or a carbon carrying two carbons in alpha positions, e.g. C-2′, C-5 and C-2 or C-4, in the cases of floridoside (A, B, respectively) and digeneaside (C, D respectively) according the culture conditions of seaweed. About floridoside, it shows that, the percentages of isotopomers for each ^{13}C atom are alike just before and after the chase periods whatever the salinity. In other respects, Fig. 6B indicates that, in the case of a ^{13}C atom bearing two alpha carbons, the probability that one of them is ^{13}C-labelled conversely to the other is 43.2 ± 0.8% whatever the salinity. It also shows that the abundances of $-^{13}C-^{13}C-^{13}C-$ were 22.1 ± 2.6% and 25 ± 1.3%, respectively, and those of $-^{12}C-^{13}C-^{12}C-$ were 34 ± 2% and 31.4 ± 2.2%, respectively, in the molecules of floridoside from thalli kept under hyper- and hypo-osmotic conditions. These percentages have to be compared to 15.2 ± 1.3% and 42.2 ± 2.6% in floridoside from no salt stress thalli. It ensures that the distribution of ^{13}C within the molecule was therefore affected by the stress by salt: indeed, either all of the three carbon atoms were labelled or only one of them ($-^{13}C-^{13}C-^{13}C-$ and $-^{12}C-^{13}C-^{12}C-$). Furthermore, for a ^{13}C carrying one carbon in alpha, the probability to be bonded with another ^{13}C was 45.4 ± 2.6% and 48.9 ± 1% under hyper- and hypo-osmotic conditions, respectively, whereas they were only 39.2 ± 0.2% in floridoside from no salt stress thalli (Fig. 6B). So, the carbon skeleton of floridoside from salt stressed thalli was richer in $^{13}C-^{13}C$ blocks than that of floridoside from no salt stress thalli despite the difference in absolute ^{13}C abundance of the molecule. This observation was in agreement with the higher amount in single-labelled isotopomers compared to the multiply-labelled isotopomers observed in the no salt stress thalli whereas quite similar amounts for each type of isotopomers were recorded in the salt stress thalli (see Section 2.3.2.). Moreover, the standard deviation observed between results (under a given salt condition) is indicative of the variability in the labelling state of adjacent carbons. This slight standard deviation (0.3–2.6%) backs up the statement of a uniform isotopic enrichment on the floridoside molecule.

About the digeneaside purified from the thalli incubated in a hypo-osmotic medium, according to Fig. 6C and D the distribution of isopotomers looks very similar to that of floridoside from no salt stress thalli. But, the finding of a quite high variability in the labelling of adjacent carbons (0.6–4.08%) compared to that of floridoside suggests a poor uniformity of labelling in this molecule.

3. Discussion

Several labelling studies on seaweeds by NMR spectroscopy are available in the literature (Wu and Gretz, 1993; Reed et al., 1995; Simon-Colin et al., 2004). But, they focussed on the ^{13}C NMR of the algal extracts in order to highlight the isotopic enrichment of solutes. Few reports have dealt with the use of NMR spectroscopy directly on living material because a correct identification of the labelled compounds was forbidden by the poor resolution of the in vivo spectra (Ratcliffe and Shachar-Hill, 2001; Simon-Colin

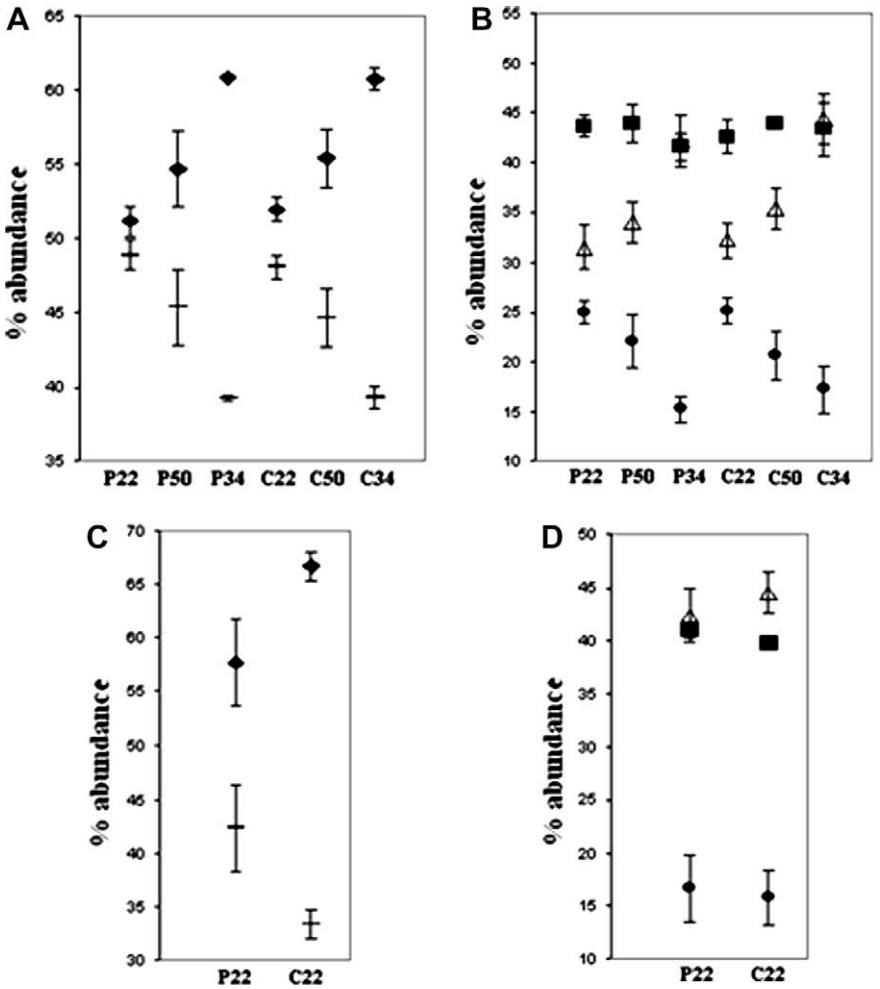

Fig. 6. Mean of probability (% abundance) that a ^{13}C atom is linked to a ^{12}C or/and ^{13}C, for carbons carrying either one adjacent carbon (A, C) or two adjacent carbons (B, D) in floridoside (A, B) and digeneaside (C, D). (♦ ^{13}C–^{12}C; – ^{13}C–^{13}C; ● ^{13}C–^{13}C–^{13}C; ■ ^{12}C–^{13}C–^{13}C; △^{12}C–^{13}C–^{12}C). These abundances are expressed in percent relative to the population of molecules labelled on each carbon. The standard deviation represents the variability in the labelling state of adjacent carbons within floridoside and digeneaside. P22, P34, P50 refer to the amounts of floridoside and digeneaside purified from thalli incubated at 22, 34 and 50 psu in salinity, respectively, after the pulse period and C22, C34, C50, after the chase period.

et al., 2004). The present assessment of the impact, in *S. chordalis*, of salt stress on the photosynthetic carbon flux relied on the use of ^{13}C-labelling through a pulse-chase experiment carried out $NaH^{13}CO_3$ and analyses by ^{13}C and ^{1}H NMR spectroscopy. It allowed us to evidence such a carbon flux and provided key information about the in vivo distribution of ^{13}C-label within floridoside and digeneaside. It also highlighted differences in the behaviour of floridoside and digeneaside by demonstrating a clear isotopic enrichment of the former at any salinity and that of digeneaside only in the algae cultivated under hypo-osmotic conditions.

This experiment showed that about 70% of floridoside synthesised during the pulse period were labelled in the no salt stressed thalli. By indicating that the carbon used for its biosynthesis is directly issued from the photosynthesis this finding is in agreement with previous reports about the red algae (Macler, 1986; Karsten et al., 1999; Simon-Colin et al., 2004). The simultaneous synthesis and degradation of floridoside evidenced by the decline of [^{13}C]-floridoside as well as the increase of floridoside content in thalli over the chase period underline the high turnover rate of this compound. The finding of similar labelling states for their adjacent carbons after the pulse and chase periods suggests that the products issued from floridoside degradation by the α-galactosidase (Yu and Pedersen, 1990; Ekman et al., 1991) would not be used for de novo biosynthesis.

That suggests that the synthesis and degradation metabolic pathways for floridoside may realized in two different cellular compartments. Besides, the transfer of carbon from floridoside to starch suggested by the elevation of about 68% of floridean starch in the thalli of *S. chordalis* over the chase period is in agreement with studies by Ekman et al. (1991) and Fournet (1996). Contrary to the continuous rise of floridoside content, the amount of digeneaside proved to remain stable (e.g. ±0.5 mg/g DW) and unlabelled; this observation suggests that the light would have no impact upon the metabolic pathway(s) leading to the synthesis and/or degradation of digeneaside conversely to the case of floridoside.

In the algae incubated under hypo-osmotic condition, the low syntheses of floridoside and digeneaside recorded after the short pulse period indicate a lack of strong simulation of the metabolic pathways to their biosynthesis. However, the small amounts of synthesised floridoside and digeneaside were both strongly labelled (67% and 59%, respectively), which is in favour of a nearly equal distribution of the flux of fixed carbon between the floridoside and digeneaside pathways. Moreover, the slightly higher (+5.4%) isotopic enrichment of floridoside within the thalli incubated in the hypo-osmotic medium compared to that in thalli incubated at normal salinity is in agreement with the finding of more multiply-labelled isotopomers and more numerous ^{13}C blocks in floridoside isotopomers (labelling of carbons adjacent to ^{13}C).

Besides, according to the very close labelling state of labelled floridoside and -digeneaside in thalli after the pulse and chase periods, no mobilisation of the stock of carbon enriched over the pulse period would occur during the chase period, and thus the enriched stock would be diluted by the non-enriched photosynthetic carbon assimilated over the chase period. That was in agreement with the results of isotopomers composition of floridoside.

In the algae incubated in the hyper-osmotic medium, only 30% of the biosynthesised floridoside were labelled after the pulse period, which means a biosynthesis of most floridoside from an endogen carbon source, but not by photosynthetic assimilation (as observed in the algae incubated in normal and hypo-osmotic media). Under hyper-osmotic conditions, the isotopic enrichment of floridoside was slightly lower than the one at normal salinity conversely to the amount of floridoside isotopomers formed with ^{13}C blocks. In this case-study, the probability to have, within floridoside, three ^{13}C-labelled carbons linked together are higher than in floridoside purified from thalli incubated at normal salinity (that exhibited higher amounts of single-labelled isotopomers than the twice and three-labelled isotopomers). It ensures that, under the hyper-osmotic condition, floridoside is synthesised from both a photoassimilated and labelled carbon and a source of non-labelled endogen carbon e.g., floridean starch or carrageenan (Ekman et al., 1991; Li et al., 2001, 2002). Our results indicate that these metabolic pathways may not share the precursor molecules leading to the synthesis of floridoside molecules (i.e. trioses phosphate) and so, these metabolic pathways may be totally separate either in space or in time. In *S. chordalis*, the carrageenan would be the only endogen carbon donor since, under hyper-osmotic conditions; its content was decreased by 45% whereas floridean starch was kept unchanged (during the pulse period). This hypothesis is supported by a recent report about ^{14}C-pulse chase experiments performed on the red microalga, *Porphyridium* sp. that provided evidence of the use of large amounts of floridoside in the synthesis of cell wall polysaccharides (Li et al., 2002). It is worth wondering about some stimulation of the reverse metabolism under hyper-osmotic conditions aimed at constituting an additional supply of carbon for floridoside synthesis. The osmotic balance within the cells would be equilibrated by such a high synthesis of floridoside; this assumption is supported by other studies about the osmoregulatory function of this compound (Reed et al., 1980; Kirst, 1980; Simon-Colin et al., 2004). The chase period was marked by a drop of floridoside concentration within thalli as previously reported in Goulard et al. (2001). The findings of very close percentages of labelling state in floridoside, similar amounts of each type of isotopomers and similar labelling states for adjacent carbons prior and after the chase period may suggest that floridoside (enriched and not) constitutes a short term reservoir of carbon for further mobilisation towards the biosynthesis of other end-products involved in a long-term osmoregulation. Moreover, the continuous reduction of digeneaside content suggests some recycling towards the synthesis of other osmolytes implicated long-term osmoregulation. To gain a better understanding of the processes it would be worth focusing on the identification of these other osmolytes.

4. Conclusion

This study relied on the use of a fast ^{13}C NMR-based method to detect the intra-cellular labelled compounds in living tissues of the seaweed, *S. chordalis*, cultivated under different conditions of salinity; among the assets of the technique, it is worth noting that the risk of introducing some artefacts through sample manipulation is greatly reduced by the elimination of extraction and extensive purification procedures prior to analysis. The carbon flux, the isotopomers composition and the ^{13}C-label distribution within floridoside and digeneaside were quantitatively analysed according to the conditions of a short stress by salt in order to better understand how *S. chordalis* adapts its metabolism to the environmental conditions. In addition, to our knowledge, this report is the first description of a ^{13}C enrichment of digeneaside evidenced from a thorough analysis of the ^{13}C NMR spectra of digeneaside and floridoside isotopomers: the couplings are explained for each atom of carbon. At last, this study also highlighted the benefit(s) of using cryogenic probes for in vivo NMR analyses of complex metabolic processes.

5. Experimental

5.1. Algal material and culture conditions

The samples of the red alga, *S. chordalis* (J. Agardh) C. Agardh (Rhodophyta), used in this study were collected at Trez Hir (Brittany, France) in September 2006 and transferred to an in-lab aquarium for keeping, till November 2006, at 12 °C under the following conditions: continuous air-flow system, a 50 μmol photons/m^2/s flux density and a 16:8 (light:dark) photoperiod. Then, some thalli were randomly removed from the aquarium and placed in three culture tanks filled with seawater at different salinities: hypo-salinity (22 psu), normal salinity (34 psu), hyper-salinity (50 psu) prepared with natural seawater at 34 psu by either dilution with distilled water (22 psu) or NaCl enrichment up to 50 psu. Prior to the pulse-chase experiment, samples of algae from every culture tank were kept 3 days in the dark at different salinities in order to lower the levels of floridean starch and floridoside in their thalli (Ekman et al., 1991; Fournet, 1996; Li et al., 2001); indeed, such a procedure prevents a dilution of ^{13}C-label by the unlabelled carbon from floridean starch and floridoside compounds present in the thalli before the labelling experiment. To measure ^{13}C assimilation by LMWCs, the water in each tank was replaced with a similar volume of water at the same salinity, but with ^{13}C-enriched sodium bicarbonate (2.5 mM $NaH^{13}CO_3$ (99% ^{13}C enrichment) purchased from Sigma–Aldrich). Then, to allow ^{13}C assimilation by thalli, the tanks were placed for 24 h under continuous light (under artificial cool-white fluorescent light at a photon flux density of 50 μmol photons/m^2/s); ^{13}C dissimilation by ^{13}C-enriched thalli was achieved by transferring them to non-enriched water (^{13}C only at natural abundance (1.1%)) for 48 h under the same conditions of light and salinity. Then, samples of thalli were immediately taken to perform in vivo ^{13}C NMR analyses. The materials were then freeze-dried and ground to powder with a ball mill to be used further for the isolation of floridoside and digeneaside.

5.2. Floridoside, digeneaside contents in S. chordalis

Pure floridoside was extracted and isolated as described by Simon-Colin et al. (2002) from the red alga, *Palmaria palmata* (Lamouroux) Silva, collected in September 2005 at Rosporden, Brittany, France.

Pure Digeneaside was extracted and isolated from the red alga, *Ceramium botryocarpum* Griffiths ex Harvey. The aqueous methanolic phase issued from the extraction with MeOH/$CHCl_3$/water (12/5/3, v/v/v) was concentrated on a rotary evaporator and freeze-dried. After dissolution in water, the crude extract was purified by ion exchange chromatography on a Cl^- form column, (1 × 8, 100–200 mesh) from Dowex-Fluka Chemika converted to OH^- form. After washing with distilled water, the column was eluted with NaOH 1 M. After neutralisation, the eluent was desalted by gel-filtration through a Sephadex G10 column (100 × 1.5 cm i.d.). Elution profiles were followed by conductimetry to

detect salt and assayed by the phenol–sulphuric method to quantify sugars.

The samples were prepared in triplicate. A dry weight of *S. chordalis* (80 mg) was extracted twice with 5 ml MeOH/$CHCl_3$/water (12/5/3, v/v/v) under magnetic stirring for 2 h. The hydro-alcoholic phase was freeze-dried and stored at 4 °C till purification by ion exchange chromatography. Then, the freeze-dried crude extracts were dissolved in 1 ml of water and applied to a two-ion-exchanger-resin system constituted of a 3 ml column of H^+ form (AG® 50W-X 8, 20–50 mesh, Bio-Rad) and a 3 ml column of Cl^- form (1 × 8, 100–200 mesh, Dowex-Fluka Chemika) converted to OH^- form. After washing with 12 ml distilled water, the neutral effluent was freeze-dried. The anion exchanger column was eluted by 10 ml of NaOH 1 M and freeze-dried. The neutral and eluent fractions were analysed by 1D ^1H NMR spectroscopy (Bruker DRX-400 spectrometer). Floridoside was contained in the neutral fraction at average yield of 85.3 ± 3.4%, whereas digeneaside was present in the eluent at 64.32 ± 6.6% as average yield.

For the quantification of floridoside, aliquots (50 µl) of neutral fraction were analysed by HPLC. The HPLC equipment consisted of an autosampler (717plus, Waters), of a pump (600E, Waters), a refractive index detector (Jasco-930, Waters) and a Suger-Pak™ column (6.5 mm ID × 300 mm L, Waters). The elution was performed at 95 °C (oven temperature), with a solution of Ca^{2+} EDTA (50 mg/l) in isocratic mode at a flow rate of 0.4 ml/min. Quantification of floridoside was achieved by comparison of peak areas against those of solutions of pure floridoside at variable concentrations in the range 0–2 mg/ml prepared in the same solvent.

The assessment of digeneaside content by HPLC was impaired by the basic pH of the eluted fraction. Indeed, the high salt content generated by the sample neutralisation is detrimental to the chromatographic separation. This led us to use the phenol-sulphuric acid method with pure digeneaside as standard (coefficient of determination R_2 = 0.9985) to determine the amount of total sugar contained in the eluted fraction, which corresponds to the amount of digeneaside.

5.3. Nuclear magnetic resonance spectroscopy

5.3.1. In vivo ^{13}C NMR analyses

About 30 mg of living samples were introduced in a 5 mm-diameter NMR tube with their culture medium. The lock signal was produced by addition of 10 µl D_2O to the NMR tube, and all analyses were performed at 25 °C. The Bruker Avance 500 spectrometer used to record in vivo ^1H-decoupled ^{13}C NMR spectra was equipped with an indirect 5 mm triple resonance cryoprobe TCI $^1H/^{13}C/^{15}N$; the standard pulse sequences are available in the Bruker software. Data were obtained by using a 30°-pulse, at 298 K, with a delay of 5 s for relaxation time and about 13,000 scans.

5.3.2. High resolution ^1H and ^{13}C NMR analysis

After dissolution of the samples under study in 700 µl of 99.97% D_2O, 1- and 2D NMR spectra were recorded at 298 K on the same apparatus as previously equipped with the same cryoprobe. The chemical shifts were expressed in δ values relative to TMS used as external reference. Double-quantum filtered ^1H–^1H correlated spectroscopy (DQF COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond coherence (HMBC) with a delay of 60 ms were performed according to standard pulse sequences to assign ^1H and ^{13}C resonances. For example, in a HMQC experiment, a set of raw data was constituted by collecting 1024 (F2) * 128 (F1) complex data points of 48 scans each, zero-filled to 1 K in the F1 dimension prior to Fourier transform with spectral widths of 17613 and 2422 Hz in the F1 and F2 dimensions, respectively, with a delay of 5 s.

The ^{13}C isotopic enrichments of floridoside and digeneaside were analysed as follows: ^{13}C NMR spectra of labelled compound and compound with natural ^{13}C abundance were recorded under the same experimental conditions. Integrals were determined for every ^{13}C NMR signal (I_{ref} for integrals in unlabelled sample and $I*$ for integrals in labelled samples, Table 2), and the signal integral for each carbon atom in the labelled compound was referenced to that of the natural abundance material ($I*/I_{ref}$ ratio, Table 2) thus affording the relative ^{13}C abundances for each position in the labelled compounds. Absolute ^{13}C abundance for the C-1 was obtained from ^{13}C coupling satellites in ^1H NMR signals for the H-1 since the coupling satellites were well separated (Table 1). These values were used to convert relative to absolute ^{13}C abundance for each other positions (% $^{13}C_{abs}$, Table 2). Moroever, the average of absolute ^{13}C abundance of all carbons corresponds to the absolute ^{13}C abundance of the whole compound (Table 3). The concentrations of labelled floridoside and labelled digeneaside in the thalli of *S. chordalis* were then calculated as follows:

$$[\text{labelled compound}] = [\text{compound in the thalli}] * \text{absolute } ^{13}C \text{ abundance of the molecule}$$

where all of the concentrations are expressed in mg/g DW.

In the ^{13}C NMR spectra of labelled samples displaying ^{13}C–^{13}C couplings, each satellite in the ^{13}C NMR pattern was integrated separately. The satellite fractions of each respective satellite pair in the total signal integral of a given carbon atom was calculated and reflected the relative amount of each isotopomer. That gave the probability that a ^{13}C atom is linked to ^{12}C or/and ^{13}C (Section 2.3.3.). By multiplication of these values with the absolute ^{13}C abundance at a given molecular position, the molar contributions (mol.%) of each respective isotopomer can be calculated (Table 4) and the isotopomers composition determined (Table 5) (Eisenreich and Bacher, 2000).

Acknowledgements

The authors would like to thank Dr. R. Pichon for the help in the analysis of NMR spectra and MP. Friocourt for English reviewing. This work was supported by a fellowship from the French Ministry of Education and Research to S.B.

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