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Note

In vivo conversion of 6-*O*-sulfo-L-galactopyranosyl residues into 3,6-anhydro-L-galactopyranosyl residues in *Gracilaria chilensis* Bird, McLachlan *et* Oliveira

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Abstract

The agar polysaccharides of the red seaweed *Gracilaria chilensis* Bird, McLachlan *et* Oliveira were labelled with the stable isotope ^{13}C by pulse feeding $NaH^{13}CO_3$ to samples of this alga acclimatised to indoor culture. ^{13}C NMR spectroscopy of the labelled polysaccharides extracted from these samples has been used to demonstrate conversion in vivo of 6-*O*-sulfo-L-galactopyranosyl residues into 3,6-anhydro-L-galactopyranosyl residues. The 6-*O*-sulfo-L-galactopyranosyl residues can be linked to either 6-*O*-methyl-D-galactopyranosyl residues or D-galactopyranosyl residues. © 1996 Elsevier Science Ltd.

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A generally accepted model for agar polysaccharide biosynthesis involves the following sequence: (*a*) formation of chains of alternating D-galactopyranosyl and L-galactopyranosyl residues in the Golgi apparatus, (*b*) sulfation at O-6 of the L-galactopyranosyl residues in these chains at an early stage in the biosynthesis, and (*c*) conversion into 3,6-anhydro-L-galactopyranosyl residues at a later stage [1–6]. The enzymes involved are thought to be sulfotransferases and sulfohydrolases [2–6]. Rees [4] extracted an enzyme from *Porphyra umbilicalis* and carried out step (*c*) in vitro, but there is little detailed knowledge about these processes and the in vivo conversion of 6-*O*-sulfo-L-galactopyranosyl residues into 3,6-anhydro-L-galactopyranosyl residues has not been demonstrated.

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In a previous paper [7], we described our use of a ^{13}C-labelling and GC–MS analysis technique to examine the biosynthesis of the four major galactopyranosyl residues present in the agar polysaccharides of *G. chilensis*, namely, D-galactopyranosyl (G), 6-*O*-methyl-D-galactopyranosyl (G6M), 6-*O*-sulfo-L-galactopyranosyl (L6S), and 3,6-anhydro-L-galactopyranosyl (LA) residues. However, using this technique, the biosynthesis of L6S residues and their subsequent reactions could only be examined indirectly, for example, by subtracting data for untreated algal samples from data for base-treated samples, so the errors in the data derived for these residues were large.

In this note we describe the use of ^{13}C labelling and ^{13}C NMR spectroscopy to monitor the formation of L6S residues and follow their conversion into LA residues. The same technique was also used to examine the level of 6-*O*-methylation in the D-galactopyranosyl residues linked to the L6S residues, in order to provide confirmatory evidence that methylation occurs early in the biosynthetic pathway in this alga [7]. It should be noted that proportions estimated below for the labelled samples apply predominantly to newly formed agar-type material, while for the control sample they apply to the whole of the material.

A ^{13}C-labelling pulse/chase experiment was conducted with *G. chilensis* (see Experimental). ^{13}C NMR spectra of labelled agar polysaccharides extracted from algal samples taken at the end of the 32-h ^{13}C-labelling pulse (S2) and at the end of the 6-day dark chase (S5) are shown in Figs. 1 and 2, respectively. The spectrum of non-labelled agar polysaccharides extracted from a control sample taken prior to the labelling pulse (S1) is shown in Fig. 3. Only weak signals assigned to L6S residues occur in the spectrum of the control sample (Fig. 3), which is primarily that of LA residues linked to a mixture of G and G6M residues. In contrast, signals for L6S residues predominate in the spectrum of the sample taken at the end of the labelling pulse (S2, Fig. 1) and the signals assigned to LA residues are weak. (Enrichment with ^{13}C carbon in L6S residues is estimated to be about sevenfold.) The signals for LA residues predominate again in the spectrum of the sample taken at the end of the 6-day dark chase (S5, Fig. 2), but the signals for L6S residues are still considerably stronger than those in the spectrum of the control sample. In the spectrum of the sample taken 1 day into the dark chase (S3, not shown), the signals for the two types of residue are of approximately equal strength, while the signals for the LA residues predominate in the spectrum of the sample taken at 4 days (S4, not shown). The proportion of L6S residues in each of the samples was estimated from the integrated C-1 signal intensities for the L6S and LA residues. Values of 24% (S1, control), 69% (S2, end of labelling pulse), 57% (S3, 1-day dark chase), 42% (S4, 4-day dark chase), and 37% (S5, 6-day dark chase) were obtained. This series of spectra clearly shows the formation of L6S residues during the labelling pulse and their subsequent conversion into LA residues during the dark chase.

A difference spectrum representing only the precursor material (Fig. 4), that is, polymer sequences comprised of alternating L6S residues and their associated partially 6-*O*-methylated D-galactopyranosyl residues, was obtained by subtracting the spectrum of the control sample (Fig. 3) from that of the sample taken at the end of the labelling pulse (Fig. 1), the spectra being scaled to nullify the signal for C-1 of the LA residues. Signals for C-5 and C-6 of G6M residues are present at 73.7 and 71.9 ppm, respectively, their positions little affected by changing the adjacent residue from LA to L6S. The

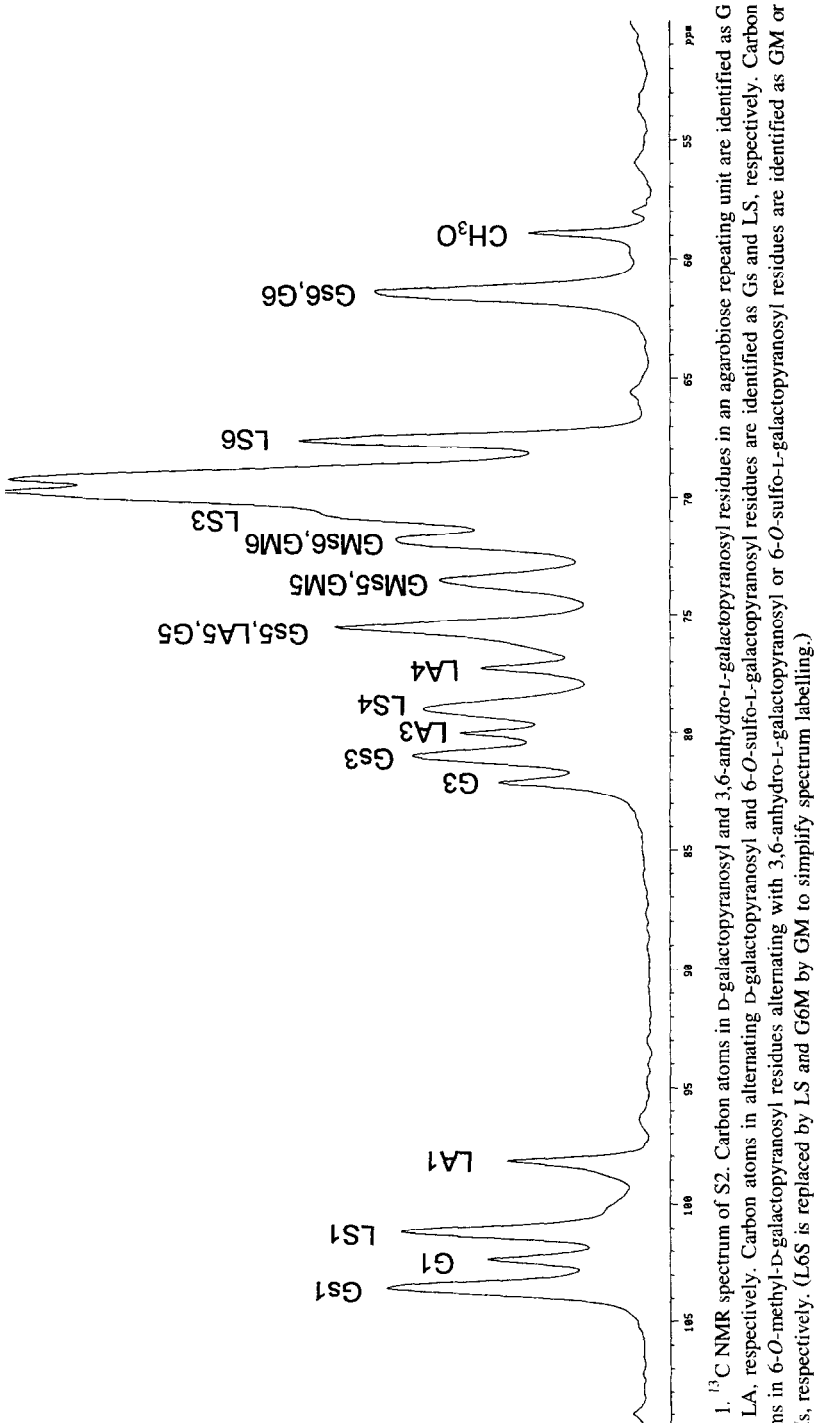

Fig. 1. ^{13}C NMR spectrum of S2. Carbon atoms in D-galactopyranosyl and 3,6-anhydro-L-galactopyranosyl residues in an agarobiose repeating unit are identified as G and LA, respectively. Carbon atoms in alternating D-galactopyranosyl and 6-*O*-sulfo-L-galactopyranosyl residues are identified as Gs and LS, respectively. Carbon atoms in 6-*O*-methyl-D-galactopyranosyl residues alternating with 3,6-anhydro-L-galactopyranosyl or 6-*O*-sulfo-L-galactopyranosyl residues are identified as GM or GMs, respectively. (L6S is replaced by LS and G6M by GM to simplify spectrum labelling.)

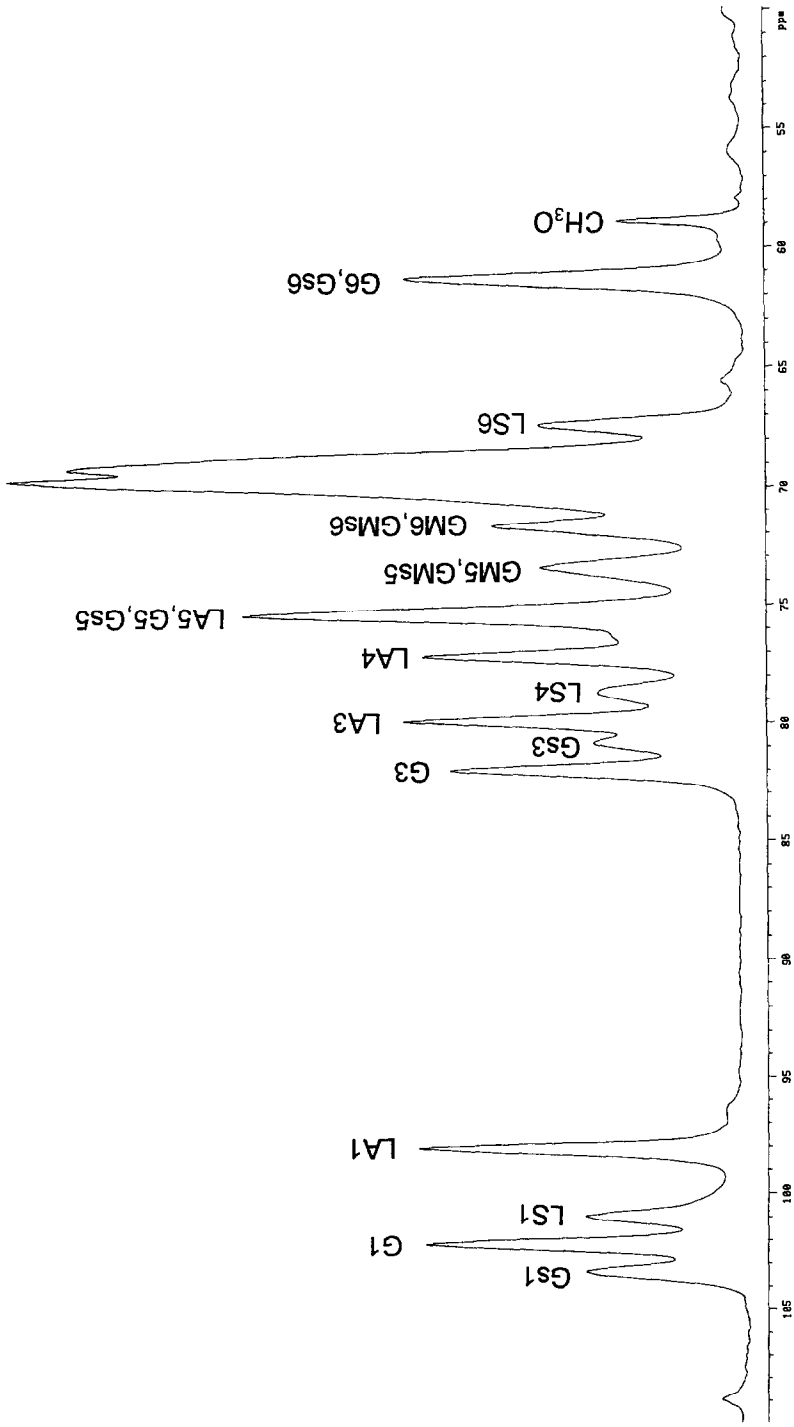

Fig. 2. ^{13}C NMR spectrum of S5. (Symbols as for Fig. 1.)

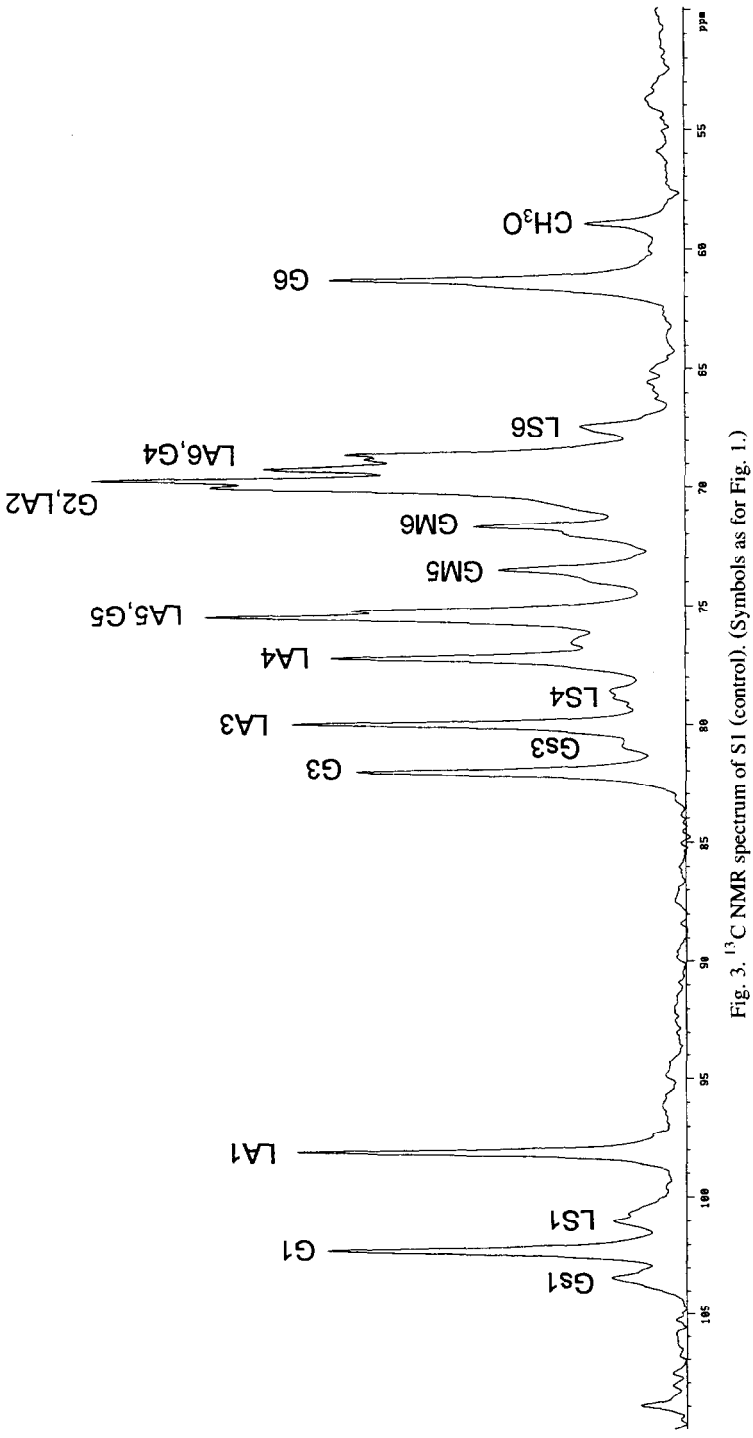

Fig. 3. ^{13}C NMR spectrum of S1 (control). (Symbols as for Fig. 1.)

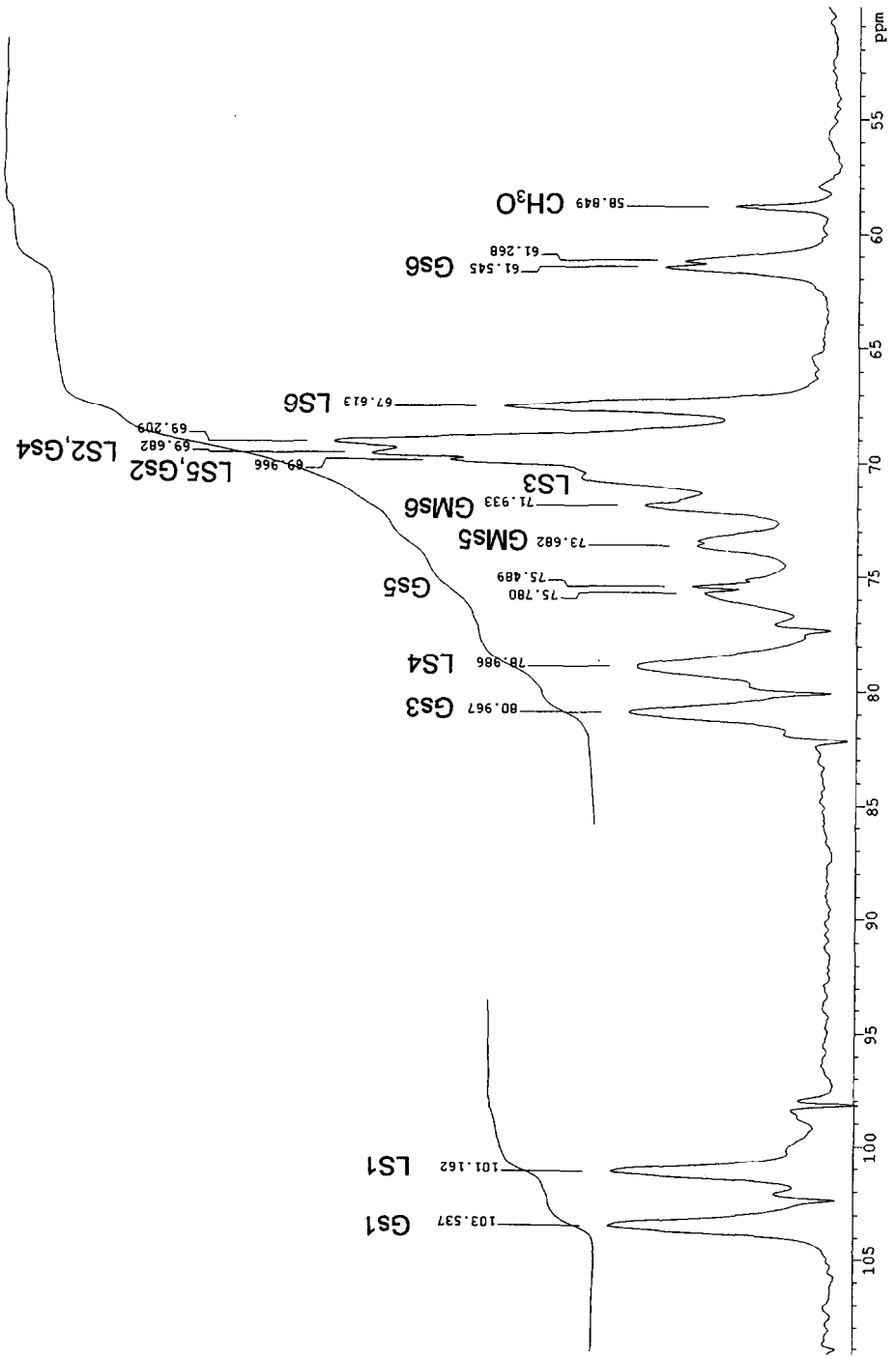

Fig. 4. Spectrum of precursor residues = spectrum of S2 − spectrum of S1 (signal LA1 nullified).

presence of signals for G6M residues in Fig. 4 clearly shows that L6S residues are linked to G6M residues as well as to G residues. The proportion of G6M residues can be estimated by comparing the integrated intensities of the C-6 or C-5 signals given by G6M residues with that of the C-6 signal given by G residues. The latter comparison is more accurate, in that the C-5 signal of G6M residues overlaps less with other signals, but the molar value obtained is less accurate, as the comparison is between different types of carbon atom, methine and methylene, which have different relaxation properties. However, the values obtained for the spectrum shown in Fig. 4, and throughout the spectra for the labelled samples, indicated that the proportion of methylated D-galactopyranosyl residues in each of the labelled samples was similar to that in the control sample. Therefore, the proportion of methylated D-galactopyranosyl residues which are linked to L6S residues in newly biosynthesised material is at least equal to that linked to LA residues in the control sample. This is in accordance with our previous finding that methylation is an early process in the biosynthesis of *G. chilensis* agar polysaccharides [7].

1. Conclusions

The in vivo conversion of 6-*O*-sulfo-L-galactopyranosyl residues into 3,6-anhydro-L-galactopyranosyl residues during agar polysaccharide biosynthesis has been demonstrated. The 6-*O*-sulfo-L-galactopyranosyl residues are linked to 6-*O*-methyl-D-galactopyranosyl residues as well as to D-galactopyranosyl residues, which supports an earlier finding that methylation is an early process in this alga.

2. Experimental

Materials.—*Gracilaria chilensis* Bird, McLachlan *et* Oliveira was collected from Scorching Bay, Wellington, New Zealand and cultured indoors at NIWA Aquaculture Research Centre, Mahanga Bay, Wellington, as described previously [7]. An herbarium sample taken from this site is deposited with the Museum of New Zealand (WELT A 4437).

^{13}C-Labelling.—A pulse/chase labelling experiment was carried out using the method described previously [7]. Acclimatised alga (14.6 g = one sample of 5 g and four samples of 2.4 g) was suspended in a flask of filtered, natural seawater, which was slowly aerated with compressed air. The flask was shielded from light for 4 days to reduce floridean starch and floridoside levels in the alga. The medium was then replaced with filtered, TRIS-buffered (pH 7.5–8.0, 0.02 M) seawater, and $NaH^{13}CO_3$ (MSD Isotopes, Merck Frosst, Canada, 99 atom %, 3.7 mM) was added as described previously. The system was closed and irradiated with 60 μmol m^{-2} s^{-1} photosynthetically active radiation for 32 h. Agitation of the medium was provided by magnetic stirring. The medium was then replaced with fresh, slowly aerated seawater, and the flask was shielded from light for 6 days. Samples were removed for analysis at the end of the prelabelling dark treatment (S1, 6.6 g), at the end of the labelling pulse (S2, 3.5 g), and

after 1, 4, and 6 days of dark chase (S3–S5, 3.6–3.9 g). The samples were washed in distilled water to remove floridoside [7] and dried in an evacuated desiccator, giving 540 mg of S1 and 290–310 mg of S2–S5. The dry samples were then divided into fine and coarse material by separating lateral filaments from axial filaments. Each sample of labelled fine material (ca. 220 mg) was extracted twice with 20 mL of phosphate buffer (pH 7, 0.01 M) at 120 °C for 30 min (S1, 400 mg, was extracted with 35 mL). The combined extracts were treated with amyloglucosidase (1.5–1.8 mg) overnight in order to hydrolyse floridean starch, heated to boiling to destroy the enzyme, dialysed against distilled water, and lyophilised to give 132 mg of polysaccharide from the control sample and 73–88 mg from the labelled samples. Yields were 33–39% by weight of the dry washed weed.

^{13}C NMR spectra.—Spectra were recorded in 10-mm tubes at 90 °C using a Varian Unity 500 spectrometer (125 MHz, 1.092 s acquisition time, 0.4 s delay time, 90° pulse width). The samples S1 (127 mg), S2 (83 mg), S3 (73 mg), S4 (74 mg), and S5 (76 mg) were dissolved in D_2O (2.6 mL) containing Me_2SO as internal reference (39.47 ppm). Spectra were recorded after 128, 320, 640, 1280, 2432, and 3648 transients in order to check sample stability with respect to loss of sulfate half-ester groups or the 3,6-anhydride bridge. (The signal/noise ratio at 128 transients was good for all samples except S1.) No change in the relative intensities of the C-1 signals or any other signals was detected. Signal assignments are as described by Lahaye et al. [8]. Integrated signal intensities were determined digitally and by cutting and weighing.

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