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Desulfation of sulfated galactans with chlorotrimethylsilane. Characterization of β-carrageenan by ^{1}H NMR spectroscopy

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Abstract—A desulfation method using chlorotrimethylsilane for treatment of pyridinium salts of sulfated galactans was developed. It proved to be appropriate for desulfation of polysaccharides of both agar and carrageenan families. In order to evaluate its efficiency in presence of the maximum content of 3,6-anhydrogalactose, it was applied to commercial κ-carrageenan, leading to obtention of a product mainly composed by β-carrageenan. Best experimental conditions for achieving desulfation of κ-carrageenan—in terms of low sulfate content, high recovery and low degradation of the product—were found. In addition, the complete assignment of the ^{1}H NMR spectrum of β-carrageenan was achieved by means of 1D and 2D NMR techniques.

Keywords: Sulfated galactans; Desulfation method; Agarans; κ-Carrageenan; β-Carrageenan

1. Introduction

The major, matrix-phase polysaccharides extracted from marine red macroalgae are sulfated galactans. These galactans essentially consist of linear chains of alternating 3-linked β-D-galactopyranosyl and 4-linked α-galactopyranosyl units, which are classified either as carrageenans if the 4-linked residue is in the D configuration or agarans if it is in the L configuration. This regular backbone is usually masked by different *O*-linked groups, particularly methyl ether, sulfate ester, pyruvate acetal or β-D-xylopyranosyl residues and also some of the α-galactopyranosyl units may occur in the 3,6-anhydro form.[1,2]

Structural analysis of red seaweed galactans usually requires determination of the sulfated positions on the backbone. This can be deduced by comparison of methylation analysis between native and desulfated polysaccharides. Thus, an accurate structural elucidation implies the application of a desulfation method that leads to sulfate removal without modifying the underlying polysaccharide. Several nonselective desulfation methods have been previously described, namely: acid desulfation with methanolic hydrogen chloride,[3,4] solvolytic desulfation in Me_2SO_4–water—methanol—pyridine mixtures[5–8] and desulfation with pyromellitic acid and an amphoteric nonhydrogen-bonding sulfate acceptor like As_2O_3.[9] In addition, a selective desulfation method for primary alcohols using the silylating reagents *N*,*O*-bis(trimethylsilyl)acetamide (BTSA) and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BTSTFA), was reported in 1992 by Takano et al.[10] A comparison between desulfation rate constants for methyl α-D-galactoside 3-sulfate and methyl α-D-galactoside 6-sulfate, relative to the solvolytic reaction in pyridine, led to the classification of various silylating reagents in three groups: (a) inefficient for desulfation, (b) selective towards desulfation of the 6-position and (c) nonselective but still active desulfating agents.[11] Even though the efficiency of the compounds included in group (c) was lower than that one exhibited by the selective agents,

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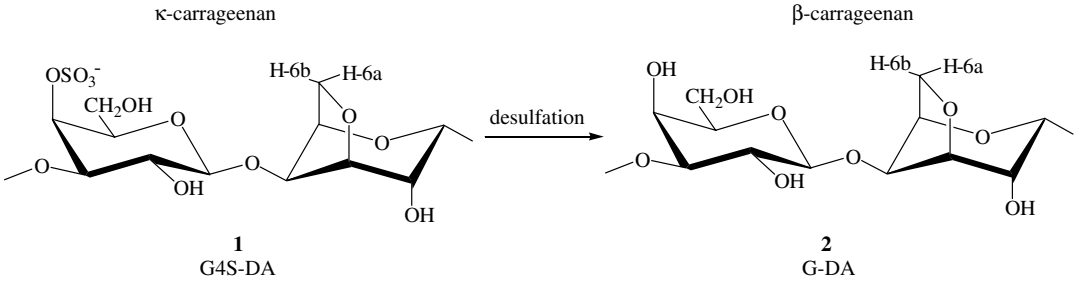


Scheme 1.

they could be considered as potentially useful for desulfation of primary and secondary hydroxyl groups. Among these reagents, chlorotrimethylsilane (CTMS) was one of the most promising compounds. Its evaluation as a nonselective desulfating agent for red-algal sulfated galactans is included herein.

The development of suitable desulfation conditions, using CTMS, for agarans from *Georgiella confluens* (Reinsch) Kylin made possible its structural elucidation and revealed an unusual substitution pattern: sulfation mainly at the 3-position of the α-L-galactose units and the presence of xylose side chains at the 4-position of the β-D-galactose residues.[12]

κ-Carrageenan (Scheme 1), constituted by a repeating disaccharidic sequence of →3)-β-D-Gal*p* 4-sulfate-(1→4)-3,6-anhydro-α-D-Gal*p*-(1→(G4S-DA as proposed by Knutsen et al.[1]), was considered as an interesting starting material for testing reaction conditions in the presence of the maximum content of labile 3,6-anhydrogalactose residues. It was also intended to make the complete assignment of the ^{1}H NMR spectrum of β-carrageenan—obtained by desulfation of the κ-polysaccharide—with only three of its 12 signals assigned in the literature.[13]

2. Results and discussion

2.1. Desulfation of galactans from *G. confluens*

Due to the simultaneous presence of xylose side chains and sulfate esters on the naturally methylated agaran backbone of the polysaccharides isolated from *G. confluens*, it was necessary to carry out desulfation–ethylation/desulfation–methylation analyses in order to deduce the substitution pattern of the backbone. Desulfation by acid methanolysis may give deep depolymerization of galactans with labile 3,6-anhydrogalactose units[2] and thus precluded its application on the samples. Therefore, the first approach was solvolytic desulfation, which involves heating the pyridinium salt of the sulfated polysaccharide in dimethyl sulfoxide–pyridine in the presence of methanol and/or water,[5–8] but no significant elimination of sulfate was observed in the several conditions assayed. Treatment with pyromellitic acid[9] was not tested.

Desulfation was achieved by reaction with CTMS in anhydrous pyridine. Experimental conditions were determined as follows: taking into account that the rate constants for CTMS were lower than those registered for BTSA, the native polysaccharide CP was treated with CTMS in anhydrous pyridine, trying to keep high concentrations of sulfated polysaccharide and CTMS. The first assay was made with a CTMS–sulfate molar ratio of 90:1 in a 1% (w/v) polysaccharide solution, giving a product with only an 8% desulfation (Table 1, line 1). An increase in time, temperature and CTMS concentration (Table 1, line 2) gave a product with a recovery of 57% (see footnote *a* in Table 2) and 52% desulfation. Heating for only 8 h and slightly reducing the CTMS concentration (Table 1, line 3) increased percentages of recovery and desulfation to 78% and 60%, respectively. In order to check reproducibility, this reaction was repeated (Table 1, line 4). This product was combined with that described in line 3 and named

Table 1. Desulfation conditions assayed on CP and F2′ using CTMS

	Sample	CTMS–sulfate molar ratio	Temp (°C)	Time (h)	Sulfate (% SO_3Na)	Desulfation (%)
1	CP	90:1	95	4.5	11.6	8
2	CP	470:1	(i) 100	11		
			(ii) 100→40[a]	16	6.1	52
3	CP	390:1[b]	100	8	5.0	60
4	CP	380:1[b]	100	8	4.5	64
5	F2′	470:1	100	8	4.6	64

[a] Temperature: 100 °C for 11 h, then from 100 to 40 °C in 16 h.
[b] Mixture of both products gave rise to CPDes.

Table 2. Desulfation conditions assayed on κ-carrageenan (sulfate content, molecular weight and percentage of product recovery)

Prod.	CTMS–sulfate molar ratio	Temp (°C)	Time (h)	R^a (%)	MW[b]	Sulfate[c] (% SO_3Na)	G4S:G molar ratio	
							ICCD[d]	C-1 DAG[e]
0	Native carrageenan	—	—	—	82,800	20.0[f]	99:1	97:3
1	0	100	3	91	4900	18.1	84:16	92:8
2	0	100	8	66	2700	18.8	89:11	90:10
3	200:1	100	3	65	1500	5.5	18:82	8:92
4	100:1	100	3	82	2600	7.0	24:76	10:90
5	50:1	100	3	96	6700	5.0	16:84	13:87
6	10:1	100	3	90	7300	12.1	47:53	14:86
7	4:1	100	3	40	2800	16.3	72:28	79:21
8	1:1	100	3	46	2200	16.5	73:27	78:22
9	10:1	60	3	68	2800	16.5	73:27	75:25
10	50:1	100	6	87	5800	5.3	18:82	11:89
11	200:1	100	8	64	n.d.	6.9[g]	24:76	4:96

[a]Percentage of recovery: corresponding to carbohydrate percentage, corrected by sulfate content (measured by ion chromatography) of the starting material and the product: $R = \frac{m_r \times (1-S_r)}{m_0 \times (1-S_0)} \times 100$ where: m_r, recovered mass; S_r, sulfate content of the recovered product; m_0, initial mass; S_0, initial sulfate content of the polysaccharide.

[b]Determined by quantification of terminal reducing ends.

[c]Determined by ion chromatography with conductimetric detection (ICCD).

[d]Determined from the sulfate content measured by ion chromatography.

[e]Determined by ratios of ^{13}C NMR intensities corresponding to DAG anomeric signals.

[f]Sulfate content determined by turbidimetric method: 22.2% SO_3Na.

[g]Sulfate content determined by turbidimetric method: 11.3% SO_3Na.

CPDes. Additionally, F2′, the main fraction isolated from CP, was treated with CTMS in a CTMS–sulfate molar ratio of 470:1, heating at 100 °C for 8 h, to give F2′ Des in 60% yield with 64% desulfation. Both desulfated samples, CPDes and F2′Des, had monosaccharide compositions similar to the original samples and were subjected to structural analyses (methylation; ethylation; reductive amination of hydrolyzed, methylated polysaccharides with a chiral amine; NMR spectroscopy).[12] Desulfation was a key step in the structural elucidation of the polysaccharides isolated from *G. confluens* and led to the first report on an unusual substitution pattern for sulfated galactans: highly methylated agarans, with low 3,6-anhydrogalactose content, bearing xylose single stubs linked at the 4-position of β-D-galactose and sulfated mainly at the 3-position of α-L-galactose, with a lesser amount of sulfation at the 4-position of β-D-galactose.

2.2. Desulfation of κ-carrageenan

Due to its high content in labile 3,6-anhydrogalactose residues, κ-carrageenan (carrageenan type III, from *Eucheuma cottonii*, Sigma) was the chosen polysaccharide for evaluating desulfation with CTMS. With the aim of finding the best reaction conditions, pyridinium salts of κ-carrageenan were treated with several CTMS–sulfate molar ratios and different reaction times, at 100 and 60 °C (Table 2). In a first approach, composition and sulfate content of the products were studied by ^{13}C NMR spectroscopy. In all cases, (a) spectra included only signals derived from κ-[14] and β-[15]carrageenan; and (b) the integration ratios for the anomeric resonances of α- and β-units were 1:1, indicating that the alternating structure of the backbone remained intact. A quantitative estimation of the galactose 4-sulfate–galactose (G4S:G) ratios was made, based on the anomeric signal intensities of 3,6-anhydrogalactose residues in κ- and β-carrageenan (at 95.0 and 94.7 ppm, respectively) (Table 2).

Products **1** and **2** were obtained under solvolytic conditions (heating in pyridine, without CTMS), to confirm that desulfation was only due to the presence of silylating reagent.

The resonance intensities for κ-carrageenan in the ^{13}C NMR spectra for products **3**, **4**, **10** and **11** were almost indistinguishable from the baseline, while reactions **5** and **6** gave peaks that were scarcely detectable. Thus, at first sight, complete removal of sulfate groups could be inferred. Spectra of reactions of **7** and **8** showed the simultaneous presence of β- and κ-diads, prevailing those from κ. A similar situation was observed when conditions for reaction **6** (with the minimum time and CTMS–sulfate molar ratio giving prevalence of desulfated polysaccharide at 100 °C) were applied at 60 °C (reaction of **9**).

Thus, the analysis of ^{13}C NMR spectra led to the conclusion that almost complete desulfation of κ-carrageenan could be achieved using a minimum CTMS–sulfate ratio of 10:1, heating at 100 °C for 3 h. Under these conditions, polysaccharide degradation would not be significant since terminal residues were not observed in the spectrum.

However, a more detailed analysis of the products (measuring sulfate content, percentage of recovery and

estimating the average molecular weights, Table 2) revealed that:

(i) Desulfation reaction reached a constant level within 3 h (products **5** and **10**).
(ii) The content of minor components was underestimated by spectroscopic quantification, as compared to the values obtained from quantification by ion chromatography with conductimetric detection.
(iii) Figure 1 shows desulfation (as G residues, from Table 2) versus CTMS–sulfate molar ratio. For chromatographic values, the saturation level of the curve is reached at a 16:1 ratio; while for spectroscopic values, the saturation level is achieved at 11:1. Both results are consistent with the ratio of 15:1 reported for desulfation of methyl α-D-galactopyranoside 6-sulfate with BTSA.[10] Furthermore, the general agreement between chromatographic and spectroscopic results proves that the spectroscopic approach is a valid estimation with an associated error, depending on the signal-to-noise ratio of the spectrum.
(iv) Methylation analysis of products **3** and **10** gave two alternative G4S:G ratios (both of 10:90, calculated from 2,6-di-*O*-methyl:2,4,6-tri-*O*-methylgalactose molar ratios). These values were closer to those obtained by ^{13}C NMR intensities; however, it should be kept in mind that methylation implies an additional chemical treatment under vigorous conditions, and thus subsequent degradation of the polysaccharide, may occur (as evidenced by the methylation yield, see Experimental section). Then, methylation might involve polysaccharide degradation and selective losses during the complete derivatization procedure, giving results that might not represent the original material. Therefore, the actual polysaccharide sulfate content should be some value between those ones determined by ion chromatography and NMR spectroscopy or methylation analysis. The results obtained by ion chromatography should be considered as maximal values, since some noncovalently linked sulfate may remain in the desulfated polysaccharide and would be quantified by this technique. On the other hand, NMR integrations could underestimate minor sulfated components giving the opposite deviation.
(v) The molecular weights of control samples (**1** and **2**) are markedly reduced. Even though at first sight all the molecular weights included in Table 2 seem to indicate similar depolymerization levels, a plot (for reactions at 100 °C for 3 h) of molecular weight versus CTMS–sulfate molar ratio gives a regular curve with a maximum at 7300 Da (Fig. 2). Considering that the average molecular weight of sample **1** was 4900 Da, the addition of silylating reagent in low concentration promoted the cleavage of glycosidic linkages. When this concentration was raised—increasing silylation and desulfation degrees—degradation was inhibited, and the average molecular weight was higher, even when compared with the control sample.

At low CTMS concentrations, the cleavage of glycosidic bonds prevails. As total CTMS–hydroxyl molar ratio is increased, depolymerization could be sterically hindered by the increase in silylation degree. The initial protecting effect observed could be attributed to: (a) the simultaneous achievement of a silylation threshold (product **6** would be a partially silylated, desulfated polysaccharide), and (b) the absence of high concentration of CTMS or byproducts derived from desulfation, that might improve depolymerization. In this way, products **5** and **10**, obtained with the same reagent concentrations and temperature but with different reaction times, have similar sulfate contents (5.0% and 5.3% SO_3Na) and molecular weights (6700 and 5800 Da). It implies that a longer exposure of a desulfated, partially silylated carrageenan would not significantly improve degradation. On the other hand, product **9** was subjected to the same time and concentration

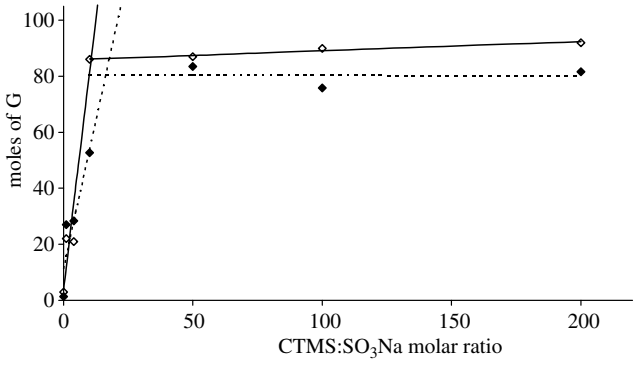


Figure 1. Desulfation of κ-carrageenan at 100 °C for 3 h (products **3**–**8**): chromatographic values (◆), spectroscopic values (◇).

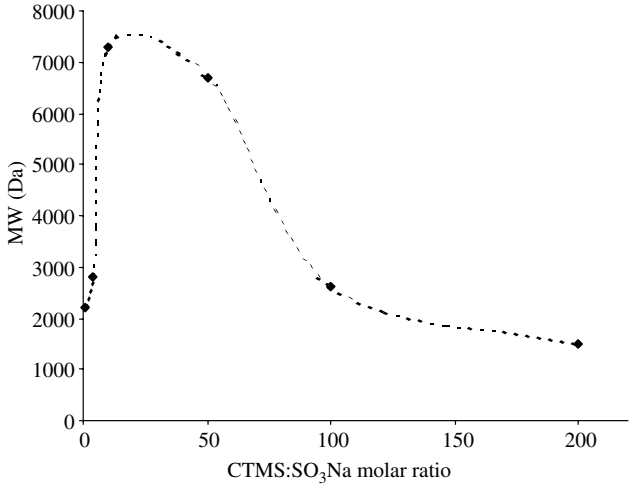


Figure 2. Variation of molecular weight by treatment with CTMS at 100 °C for 3 h (products **3**–**8**).

conditions as product **6**, but the molecular weight of the product at 60 °C (2800 Da) was lower than that at 100 °C (7300 Da). This fact could indicate that a slower silylation process with CTMS might allow glycosidic degradation of the sulfated polysaccharide without reaching the substitution degree that induces protection.

In conclusion, the best conditions found for desulfation of κ-carrageenan—in terms of higher desulfation with lower depolymerization and better product recovery—are treatment at 100 °C for 3 h with a CTMS–sulfate molar ratio of 50:1.

2.3. Characterization of β-carrageenan by NMR spectroscopy

Characterization of β-carrageenan was made by one- and two-dimensional NMR spectroscopy, including $^{1}H,^{1}H$ COSY, $^{13}C,^{1}H$ HETCOR, gradient Heteronuclear Multiple Bond Correlation (HMBC)[16] and $^{13}C,^{1}H$ heteronuclear multiple quantum coherence (HMQC)[17] experiments. In addition, the native commercial κ-carrageenan was analyzed by ^{1}H and ^{13}C NMR spectroscopy.

All ^{13}C NMR spectra showed signals consistent with diads G4S-DA and/or G-DA, belonging to κ-[14] and β-[15]carrageenan (Fig. 3).

The $^{13}C,^{1}H$ HETCOR spectrum of product **5** (5% SO_3Na) had only 11 cross-peaks, and overlapping or absence of signals was assumed (spectrum not shown). All the resonances were attributable to β-carrageenan, while the diagnostic regions related to κ-diads—C-4/H-4 of G4S and C-1/H-1 of DA—were free of signals with the selected threshold. When the same sample was subjected to an HMQC experiment, two less-intense, additional cross-peaks were observed (Fig. 4).

The final assignment of the ^{1}H NMR spectrum was made taking into account:

(i) The knowledge of typical coupling constants ^{1}H–^{1}H in β-D-galactopyranosyl and 3,6-anhydro-α-D-galactopyranosyl residues (as reported by Welti,[18] Knutsen and Grasdalen[19] and Falshaw et al.[13]), to predict absence of ^{1}H–^{1}H cross-peaks connecting these protons.
(ii) Distorsionless enhancement by the polarization-transfer (DEPT 135) pulse experiments,[20] to identify both resonances corresponding to methylenes.
(iii) Analogies in chemical shifts, to identify anomeric and methylenic nuclei—^{1}H as well as ^{13}C—derived from β-D-galactopyranosyl and 3,6-anhydro-α-D-galactopyranosyl residues and to provide starting points for the analysis of two-dimensional spectra.

Figure 5 shows the 500-MHz ^{1}H NMR spectra obtained for products **5**, registered at 25 °C (Fig. 5a),

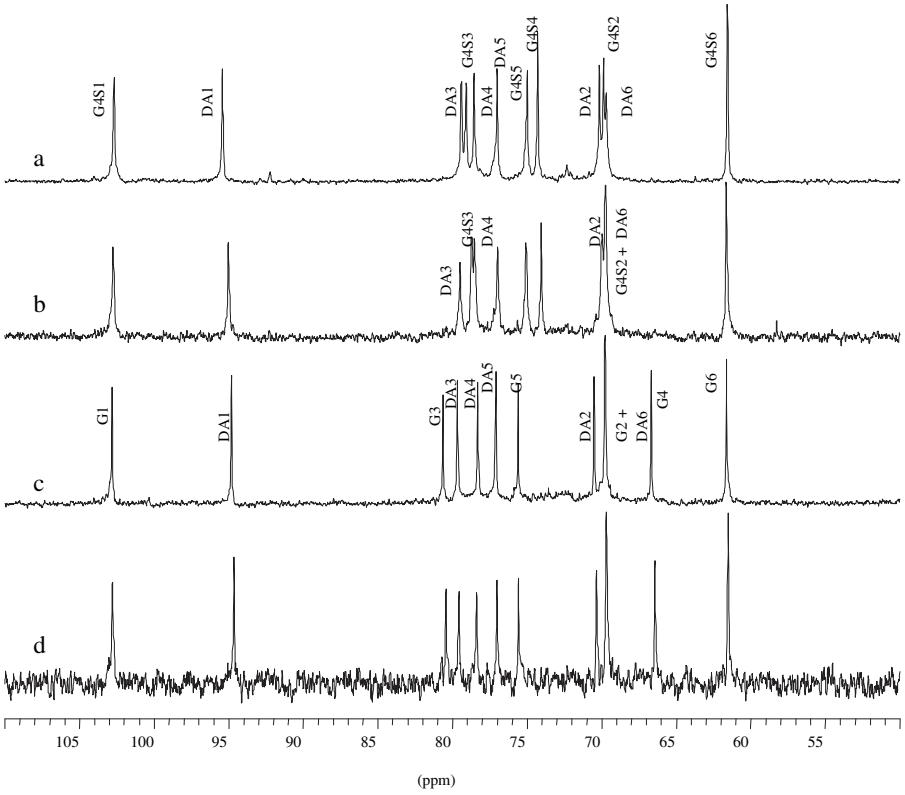


Figure 3. ^{13}C NMR spectra of products **0** (a), **2** (b), **11** (c) and **5** (d) (solvent: D_2O). Spectra (a) and (c) were recorded at 80 °C and 75 MHz; spectra (b) and (d) were recorded at 25 °C and 125 MHz.

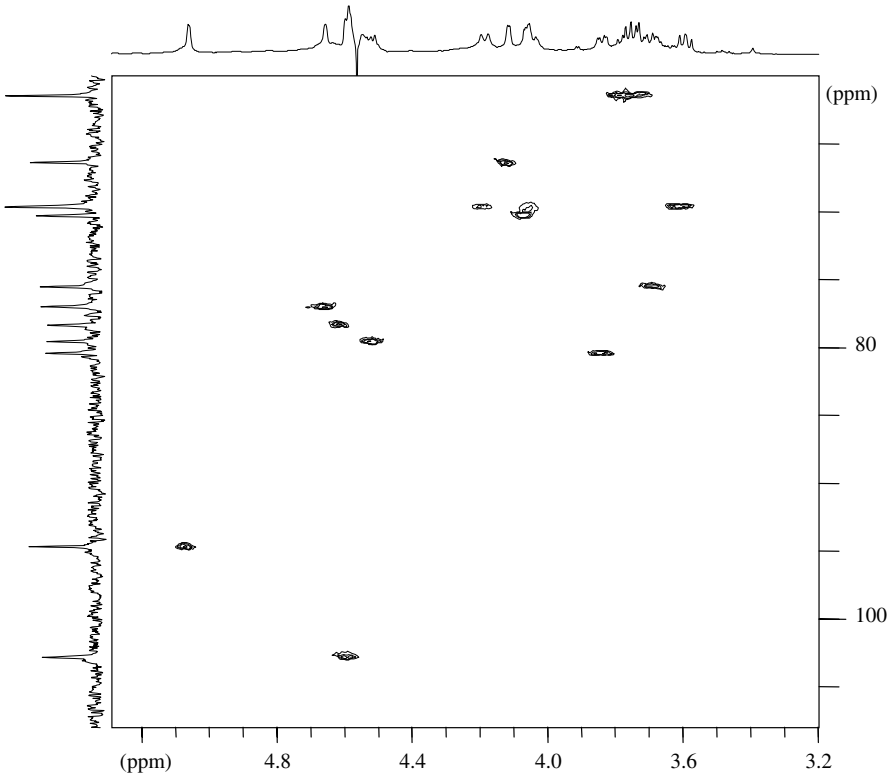


Figure 4. Heteronuclear multiple quantum coherence (HMQC) spectrum of product **5** (solvent D_2O, registered at 45 °C).

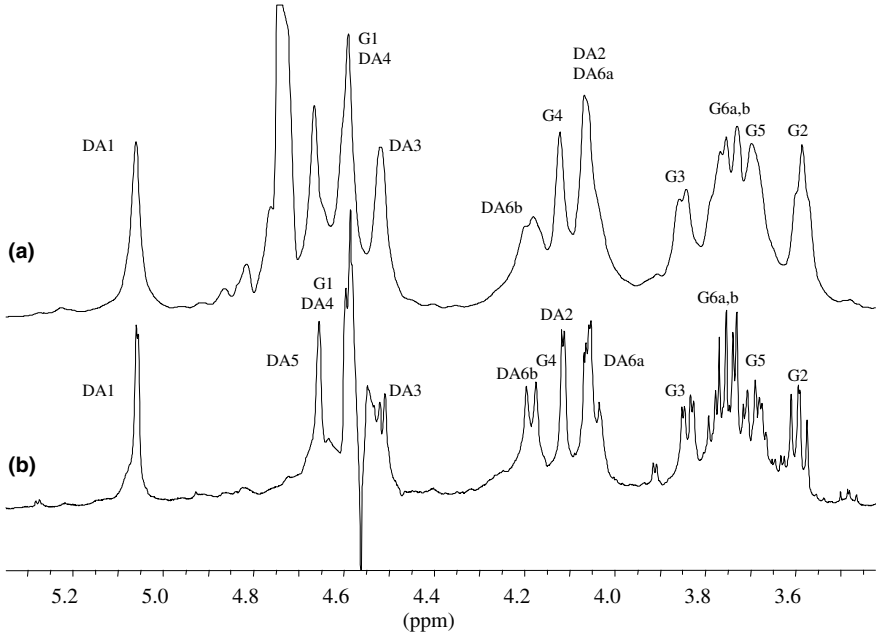


Figure 5. 500-MHz ^1H NMR spectra of: (a) product **5** registered at 25 °C, and (b) product **11**, at 45 °C.

and **11** at 45 °C (Fig. 5b). It can be seen the shift of DOH resonance with temperature, obscuring different signals at both temperatures.[21] More detailed spectral information has been found at 25 °C for the tetrasaccharide DA-G4S-DA-G4S compared to 90 °C;[22] however, for β-carrageenan it was observed that resolution increased at higher temperature.

Table 3 includes the ^1H–^1H connectivities arising from the COSY experiment (Fig. 6). The anomeric chemical shift at 102.9 ppm was considered as the starting point in the HMQC spectrum for the G residue included in β-carrageenan, then the anomeric proton should be at 4.60 ppm. The related homonuclear G1/G2 cross-peak in the COSY spectrum was at 4.61/3.60 ppm,

Table 3. Connectivities from $^{1}H,^{1}H$ COSY spectrum of product **5**

Cross-peak	Coordinates (ppm)
DA1/DA2	5.07/4.08
DA2/DA3	4.07/4.52
DA4/DA5	4.60/4.67
DA5/DA6a	4.67/4.06
DA6a/DA6b	4.06/4.21
G1/G2	4.61/3.60
G2/G3	3.60/3.86
G3/G4	3.86/4.14
G5/G6a,G6b	3.70/3.78
G6a/G6b	3.79/3.74

which was associated to G2/G3 (3.60/3.86 ppm) and G3/G4 (3.86/4.14 ppm) signals. No G4/G5 correlation was observed, as expected for protons with small coupling constants.[13] The other end of the same unit was C-6 (61.4 ppm), linked to H-6a and H-6b (see Scheme 1) at 3.76–3.78 ppm in the HMQC spectrum (3.68–3.73/61.4 ppm in the HETCOR spectrum). Near the diagonal region of the COSY spectrum, two signals at 3.70/3.78 and 3.79/3.74 were attributable to G5/G6a,b and G6a/G6b, respectively.

For the DA unit, the starting point was the anomeric carbon at 94.7 ppm, linked to the proton at 5.07 ppm in the HMQC spectrum (same values for HETCOR spectrum). The homonuclear correlations DA1/DA2 (5.07/4.08 ppm) and DA2/DA3 (4.07/4.52 ppm) were determined, while the DA3/DA4 cross-peak was absent due to the small coupling expected for this protons.[13,19] For the same reason, there was not observed any cross-peak corresponding to DA5/DA6b.

Since it could be possible that C-6 of DA should coincide with another chemical shift, it was necessary to record a DEPT 135 experiment. In this spectrum (not shown), the signal at 69.7 ppm disappeared indicating that the chemical shifts of C-2 of G and C-6 of DA overlapped. In the HETCOR spectrum there was only one cross-peak at 69.7 ppm, related to H-2/C-2 (3.6/69.7 ppm) of the G unit. By comparison with literature data for H-6a and H-6b of the DA residues,[13,18] it was inferred that these protons should be close to 4.10 and 4.20 ppm in β-carrageenan. However, the intensity of any cross-peak related to this methylenic carbon should be low since: (i) one carbon was linked to two protons whose chemical shifts were well resolved (two cross-peaks are to be expected), and (ii) geminal coupling is not lost in an HETCOR experiment; then the total intensity of the signal expected for C-6 should be divided into four subsignals, which could be masked by the spectral noise. These resonances were confirmed by the HMQC spectrum, which had two additional signals at 4.06/69.7 and 4.20/69.7 ppm that corresponded to DA6a and DA6b, respectively.

In the COSY spectrum, two intense cross-peaks at 4.06/4.67 and 4.06/4.21 ppm were consistent with DA6a/

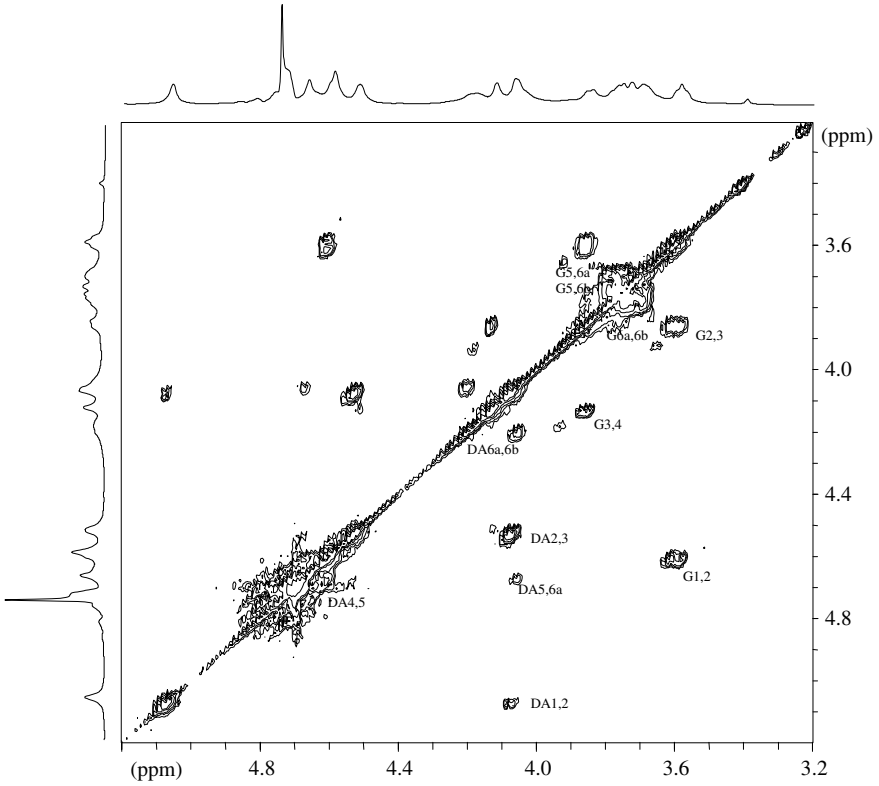


Figure 6. $^{1}H,^{1}H$ COSY spectrum of product **5** (solvent: D_2O, registered at 25 °C, 500-MHz, chemical shifts relative to Me_2SO at 2.71 ppm).

DA5 and DA6a/DA6b signals, respectively. From the HMQC spectrum it was deduced that the proton at 4.67 ppm was linked to the carbon at 77.1 ppm (DA5), while DA4 was assigned at 4.59/78.5 ppm. This implied that the homonuclear signal for DA4/DA5 should be at 4.59/4.66, where a cross-peak was observed but close to the diagonal and water peak. With this assignment, three small signals remained excluded from the β-carrageenan system: 3.65/3.92, 3.93/4.18 (a sequence of three related nuclei) and 4.13/4.51 ppm. There was not any carbon signal in the range 50–115 ppm (region of ^{13}C resonances typical of carbohydrates) that could be linked to the resonance at 3.92–3.93 ppm in the heteronuclear spectra. Therefore, this signal set should derive from some kind of noncarbohydrate compound.

All the signals observed in the HMBC spectrum (not shown) were consistent with the above-mentioned assignments. Some of them—at 4.61/69.6, 4.21/77.3 and 4.06/78.9 ppm, corresponding to DA4/DA6, DA6b/DA5 and DA6a/DA4 (or DA2/DA4), respectively—were useful to confirm the resonances associated to the DA6a and DA6b protons. A cross-peak at 4.52/69.5 ppm was attributed to a DA3/DA6 correlation through the 3,6-anhydro ring. Additionally, pairs of cross-peaks at 5.07/80.6—3.85/94.7 and 4.61/79.0—4.61/102.9 ppm were in agreement with signals related to DA1/G3—G3/DA1 and G1/DA4—DA4/G1 nuclei, according to the linkage positions between the galactose and 3,6-anhydrogalactose residues in carrageenans.

The final assignment of the ^{1}H and ^{13}C NMR spectra of β-carrageenan is included in Tables 4 and 5, respectively. On the one hand, this is the first complete ^{1}H NMR assignment of this carrageenan; on the other hand, it confirms the ^{13}C NMR assignment reported by Usov and Shashkov.[15] Proton chemical shifts corresponding to the G residue are coincident—with a constant displacement of 0.3 ppm—with those reported by Knutsen and Grasdalen[19] for the same unit in the tetrasaccharide DA-G-DA-G4S. This similarity was expected since the residue environment is similar in both cases, and the main difference is the chain length. Less data were available for DA unit. Only three ^{1}H NMR chemical shifts corresponding to β-carrageenan have been previously reported:[13] H-1, 5.04 ppm; H-2, 4.06 ppm and H-3, 4.52 ppm. All of these coincide with those reported herein. Assignments for H-3, H-4, H-5 and H-6a,b nuclei are consistent with the corresponding ones in methyl carrabioside.[23] Besides, H-5, H-6a and H-6b—the protons least influenced by presence or absence of sulfation on the neighbouring units—have chemical shifts similar to the ones reported for DA in a κ-carrageenan hexasaccharide.[19]

In conclusion, desulfation with CTMS in pyridine can be considered as an alternative method—together with acid, solvolytic and pyromellitic acid processes—for structural analysis. It proved to be applicable to sulfate group removal in different positions and polysaccharides—agarans,[12] carrageenans, polysaccharides of the agar-carrageenan hybrid type,[24] galactofucans[25]—some of them including labile units like 3,6-anhydrogalactose. In the case of the isolated fractions of *G. confluens* (Rhodophyta) and *Adenocystis utricularis* (Phaeophyta), it was the only suitable technique that made possible the structural elucidation of their polysaccharides.

Table 4. ^{1}H NMR assignment of β-carrageenan chemical shifts (ppm)[a]

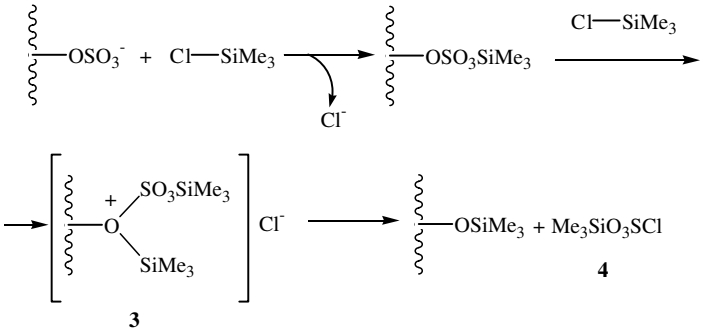
Unit	Chemical shifts (ppm)						
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
G	4.60	3.60	3.86	4.14	3.70[b]	3.79[b]	3.74[b]
DA	5.07	4.08	4.53	4.60	4.67[b]	4.07	4.21

[a] Solvent: D_2O; registered at 25 °C, 500 MHz; chemical shifts relative to acetone at 2.20 ppm.
[b] From two-dimensional spectra.

Table 5. Assignment of chemical shifts (ppm) corresponding to β- and κ-carrageenan diads in the ^{13}C NMR spectra at 25 and 80 °C

Carrageenan	Product	Unit	*T* (°C)	C-1	C-2	C-3	C-4	C-5	C-6
κ	**0**	G4S	80[a]	102.7	69.9	79.1	74.3	75.0	61.5
		DA		95.4	70.1	79.4	78.5	77.0	69.7
κ	**1**	G4S	25[b]	102.7	69.7	78.7	74.0	75.0	61.6
		DA		95.0	70.0	79.5	78.5	76.9	69.7
β	**10**	G	80[a]	102.8	69.8	80.6	66.7	75.6	61.6
		DA		94.8	70.5	79.7	78.3	77.1	69.8
β	**11**	G	25[b]	102.9	69.8	80.4	66.4	75.7	61.6
		DA		94.7	70.4	79.7	78.5	77.1	69.8

[a] Spectra registered at 80 °C; chemical shifts relative to Me_2SO (39.6 ppm).
[b] Spectra registered at 25 °C; chemical shifts relative to Me_2SO (39.5 ppm).



Scheme 2. Proposed mechanism for desulfation of sulfated polysaccharides with CTMS in pyridine.

A complete analysis of the experimental conditions applied to commercial κ-carrageenan and the isolated products was carried out in terms of sulfate content, molecular weight and composition. The results obtained indicated that the best reaction conditions, for highest decrease in sulfate content with lowest degradation and maximum product recovery, were heating of the pyridinium salts at 100 °C during 3 h, and using a CTMS–sulfate molar ratio of 50:1.

When this work started, the mechanism for the selective desulfation of primary positions with BTSA was not completely known; however, a solvolytic process had been discounted.[26] Recently, Horibe and Oshita[27] investigated the desulfation of bis(tri-methylsilyl)ester of 1-*O*-decylglycerol-2,3-sulfate with various silylating agents. It was found that only those reagents containing nitrogen were able to selectively desulfate primary positions, and a mechanism was proposed. In view of the foregoing, the mechanism outlined in Scheme 2 for the desulfation of sulfated polysaccharides with CTMS could be suggested. First, the sulfate silylester would be formed, and this species would give the oxonium salt **3** with chloride as counterion. An electrophylic attack of the trimethylsiloxy sulfonyl cation on chloride would give trimethylsilyl chlorosulfonate **4**. Nevertheless, the present work was not intended to be a mechanistic study, thus the reaction mixtures were not analyzed in order to find evidences of possible intermediates.

Finally, the study by ^{1}H and ^{13}C NMR spectroscopy, using one- and two-dimensional techniques, led to the first complete assignment of the ^{1}H NMR spectrum of β-carrageenan.

3. Experimental

3.1. General methods

κ-Carrageenan (carrageenan type III, from *E. cottonii*, C-1263) was purchased from Sigma Chemical Co. Isolation and fractionation of the sulfated polysaccharides from *G. confluens* has been previously described[12] and led to the crude polysaccharide CP and fraction F2′, among other fractions. Pyridine was dried over 4 Å molecular sieves. Sulfate was measured (on samples previously hydrolyzed in 2 M TFA at 121 °C for 2 h, evaporated to dryness under nitrogen and redissolved in high purity water from a Milli-Q system) with a DIONEX DX-100 ion chromatography system, equipped with an AS4A column (4×250 mm), an AMMS-II micromembrane supressor and a conductivity detector (eluent: 1.8 mM Na_2CO_3/1.7 mM $NaHCO_3$, flow rate: 2 mL min^{-1}). When stated, sulfate content was also measured by the turbidimetric method of Dogdson and Price.[28] Average-number molecular weights were calculated by determination of reducing end-groups using the colorimetric method of Park and Johnson.[29] Unless otherwise stated, dialyses were carried out with tubing with a molecular weight cutoff of 1000.

3.1.1. Pyridinium salts of polysaccharides. Samples were dissolved in water and passed through a cation-exchange column of Amberlite IR 120^{+} (H^{+}), which was eluted with water and the eluate was collected over 20% pyridine. After dialysis (MWCO 6000–8000) against distilled water, pyridinium salts of the polysaccharides were recovered by freeze drying.

3.2. Desulfation of galactans from *G. confluens*

Pyridinium salts of the polysaccharides were suspended in anhydrous pyridine (1% w/v) and then chlorotrimethylsilane (CTMS) was added (according to the CTMS–sulfate molar ratios indicated in Table 1). Reactions were carried out at: (i) 95 °C for 4.5 h; (ii) 100°C for 11 h, then from 100 to 40 °C in 16 h or (iii) 100 °C for 8 h (see Table 1). After dropwise addition of water (to destroy excess of CTMS and remove silylation at hydroxyl groups), the reaction mixture was dialyzed (MWCO 3500) against running tap water, distilled water, 0.1 M NaCl and distilled water, and finally freeze dried to give the desulfated products. Sulfate content was determined by the turbidimetric method above

mentioned. CTMS–sulfate molar ratio of 90:1: CP pyridinium salt, 8.75 mg; desulfated product, 4.4 mg. CTMS–sulfate molar ratio of 470:1: CP pyridinium salt, 10.00 mg; desulfated product, 5.80 mg. CTMS–sulfate molar ratio of 390:1: CP pyridinium salt, 20.86 mg; desulfated product, 14.98 mg. CTMS–sulfate molar ratio of 380:1: CP pyridinium salt, 19.23 mg; desulfated product, 14.88 mg. CTMS–sulfate molar ratio of 470:1: F2′ pyridinium salt, 29.65 mg; desulfated product, 16.34 mg.

3.3. Desulfation of κ-carrageenan

Pyridinium salts of commercial κ-carrageenan were suspended in anhydrous pyridine (1% w/v) and then CTMS was added (according to the CTMS–sulfate molar ratios and reaction conditions indicated in Table 2). After dropwise addition of water, the reaction mixture was dialyzed (MWCO 6000–8000) against running tap water, distilled water, 0.1 M NaCl and distilled water, and finally freeze dried to give products **1**–**11**. Products **1** and **2** were heated in pyridine, without CTMS, and treated in the same way as the other samples. Product **1**: κ-carrageenan pyridinium salt, 29.0 mg; product **1**, 25.85 mg. Product **2**: κ-carrageenan pyridinium salt, 35.0 mg; product **2**, 22.65 mg. Product **3**: κ-carrageenan pyridinium salt, 43.1 mg; product **3**, 22.15 mg. Product **4**: κ-carrageenan pyridinium salt, 47.75 mg; product **4**, 33.6 mg. Product **5**: κ-carrageenan pyridinium salt, 50.1 mg; product **5**, 40.45 mg. Product **6**: κ-carrageenan pyridinium salt, 30.45 mg; product **6**, 24.95 mg. Product **7**: κ-carrageenan pyridinium salt, 43.0 mg; product **7**, 16.6 mg. Product **8**: κ-carrageenan pyridinium salt, 39.0 mg; product **8**, 17.75 mg. Product **9**: κ-carrageenan pyridinium salt, 53.55 mg; product **9**, 34.7 mg. Product **10**: κ-carrageenan pyridinium salt, 54.3 mg; product **10**, 40.1 mg. Product **11**: κ-carrageenan pyridinium salt, 36.75 mg; product **11**, 22.65 mg.

3.4. Methylation analysis

Products **3** (3.10 mg) and **10** (6.50 mg) were methylated according to the method of Ciucanu and Kerek[30] and isolated after dialysis and freeze drying. Yields: product **3**, 1.75 mg; product **10**, 4.20 mg. Linkage analysis was carried out by reductive hydrolysis and acetylation of the sugar mixtures as previously described.[12]

3.5. NMR spectroscopy

3.5.1. ^{13}C NMR spectroscopy. Samples (10–20 mg) were dissolved in 1:1 H_2O/D_2O (0.5 mL), and a 5-mm tube was used. The 75-MHz ^{13}C NMR ^{1}H-decoupled spectra of native κ-carrageenan and product **11** were recorded at 80 °C on a Bruker DPX300 spectrometer, using a spectral width of 9.1 kHz, 90° pulse (5.5 μs), an acquisition time of 0.7 s and a relaxation delay of 4.5 μs for 60,000 and 41,000 scans, respectively. The 125-MHz ^{13}C NMR ^{1}H-decoupled spectra of native κ-carrageenan and products **1**–**11** were recorded at room temperature on a Bruker AM 500 spectrometer, using a spectral width of 29.4 kHz, 51.4° pulse, an acquisition time of 0.56 s and a relaxation delay of 0.6 s, for ca. 3000 scans. Methylene carbons of product **5** were assigned by a DEPT 135 experiment.[18] This spectrum was recorded at 125 MHz and room temperature, on the Bruker AM 500 spectrometer, using a spectral width of 26.3 kHz, an acquisition time of 0.56 s and a relaxation delay of 0.6 s for 5376 scans. In all cases, signals were referenced to internal Me_2SO at 39.6 ppm at 80 °C and at 39.5 ppm at room temperature.

3.5.2. ^{1}H NMR spectroscopy. Samples (7–10 mg) were dissolved in D_2O (0.5 mL), and a 5-mm NMR tube was used. Spectra were recorded on the Bruker AM 500 spectrometer, at room temperature (product **5**) and 45 °C (product **11**), using a spectral width of 5.7 kHz, 90° pulse, an acquisition time of 4.4 s, for 144 scans. Acetone was used as internal standard, at 2.20 ppm at both temperatures.

3.5.3. Two-dimensional NMR spectroscopy. Pulse sequences for ^{1}H,^{1}H COSY and ^{13}C,^{1}H HETCOR techniques were supplied by the spectrometer manufacturers; spectra were recorded on the Bruker AM 500 spectrometer at room temperature.

Gradient HMBC and HMQC 2D spectra were collected on a Bruker Avance DPX 400 spectrometer with an inverse probe at 45 °C. The HMQC spectrum was recorded with a ^{1}H spectral width of 936 Hz, a ^{13}C spectral width of 8050 Hz, a relaxation delay of 1 s, accumulating 64 scans. The HMBC spectrum was recorded with a ^{1}H spectral width of 1055 Hz, a ^{13}C spectral width of 2232 Hz, a relaxation delay of 1 s, accumulating 128 scans.

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