Carbohydrate Research, 229 (1992) 233–244
Elsevier Science Publishers B.V., Amsterdam

The use of neocarrabiose oligosaccharides with different length and sulphate substitution as model compounds for ^{1}H-NMR spectroscopy

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(Received August 5th, 1991; accepted November 21st, 1991)

ABSTRACT

The high-field ^{1}H-NMR spectra of various carrageenan oligosaccharides at room temperature are given. The assignments were facilitated by the use of proton double-quantum coherence (DQCOSY) and ^{1}H–^{13}C chemical shift correlation 2D NMR spectroscopy, and by comparing high-field ^{1}H-NMR spectra of various 4-sulphated oligosaccharides of the neocarrabiose type. The effects of anomeric configuration on the ^{1}H resonances on the same or neighbouring units are discussed. The ^{13}C-NMR shift data are given for the tetrasaccharide of kappa-carrageenan.

INTRODUCTION

Carrageenan is a family of water-soluble galactans extracted from marine red algae consisting of alternating 3-linked β-D-galactopyranose (G units) and 4-linked α-D-galactopyranose residues (D units). Each unit may be sulphated, and the 4-linked units can be in the 3,6-anhydro form (A units), as found in the carrageenans that can form a gel. The carrageenan molecules are hybrid in nature, and the sulphate substitution and the presence or absence of A units form the basis for naming carrageenan fractions from different algae[1]. Mercaptolysis and methanolysis of these galactans give products with A units at the reducing end[2,3], and hydrolysis with specific carrageenases produces a series of oligosaccharides of the neocarrabiose type[4–].

NMR spectroscopy has been applied to such algal polysaccharides and intact algal tissue[7]. In order to determine the fine structure, the galactans have to be depolymerised and the fragments isolated. Major progress in the elucidation of the structure of red algal galactans followed the use of ^{13}C-NMR spectroscopy on the

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polysaccharides[8,9] or on oligosaccharides and non-degradable galactan fractions produced enzymically from agar and carrageenan-type polysaccharides[10–14]. Although the sensitivity of ^{1}H-NMR spectroscopy is much higher, this method has been utilised much less and mainly restricted to monosaccharide derivatives[15,16], the disaccharide neocarrabiose[17], and various substituted neoagarobiose oligosaccharides[18]. The analysis of the ^{1}H-NMR spectra of polysaccharides has been based on the results of Welti[19]. In agars, pyruvyl, methoxyl, 6-sulphate[8], and 4-sulphate[18] groups have been detected, as have methoxyl[7], pyruvyl[20], or sulphate groups in different positions in carrageenans[7,19,21].

In order to elucidate structure–function relationships in the carrageenan series, especially when using model compounds in conformational studies, an exact knowledge of the sulphate-distribution pattern is required. The higher sensitivity of ^{1}H-NMR spectroscopy should allow the detection of 5% of contamination or irregularities.

We now report the assignments of the ^{1}H resonances of several neocarrabiose oligosaccharides.

EXPERIMENTAL

The 500-MHz ^{1}H-NMR spectra were recorded at 25° with a Bruker AM-500 spectrometer, using 18K data points and a pulse recycling time of 2.7 s. Chemical shifts (δ) are given in ppm relative to that of sodium 4,4-dimethyl-4-silapentane-1-sulphonate (DSS) but measured indirectly via acetone in D_2O (δ 2.225)[22]. ^{1}H-NMR spectra of mixtures of oligosaccharides and the 2D NMR spectra of the kappa-tetrasaccharide neocarratetraose 4^1,4^3-disulphate were recorded at ambient temperature with a Jeol EX400 spectrometer with a 5-mm tunable probe. The homonuclear double quantum COSY spectrum was acquired using a 1K × 1K matrix, 16 scans per block, and 2.41 s between aquisitions. The ^{1}H–^{13}C chemical-shift-correlated spectrum was acquired using standard parameters in the automode menu in Jeol EX400 (2K × 2K matrix, 256 scans per block, and a total repeat time of 1.2 s). The interpretations of the 500-MHz spectra are based on COSY (Fig. 1) and the 2D ^{1}H–^{13}C chemical-shift-correlated spectrum (Fig. 2) of the tetrasaccharide with ^{13}C-NMR shift assignments (Table I) based on Rochas et al.[23].

In order to confirm the differences in chemical shifts, some spectra were obtained for mixtures of the different oligosaccharides (see Fig. 5). The oligosaccharides were purchased from Sigma.

RESULTS AND DISCUSSION

The nomenclature used (see **1**) to designate the positions of the different residues and the positions of the sulpate groups in the oligosaccharides is a modification of that of Rochas et al.[13]. The disaccharide at the non-reducing end comprises 3,6-anhydro-α-D-galactopyranose and β-D-galactopyranose (or β-D-

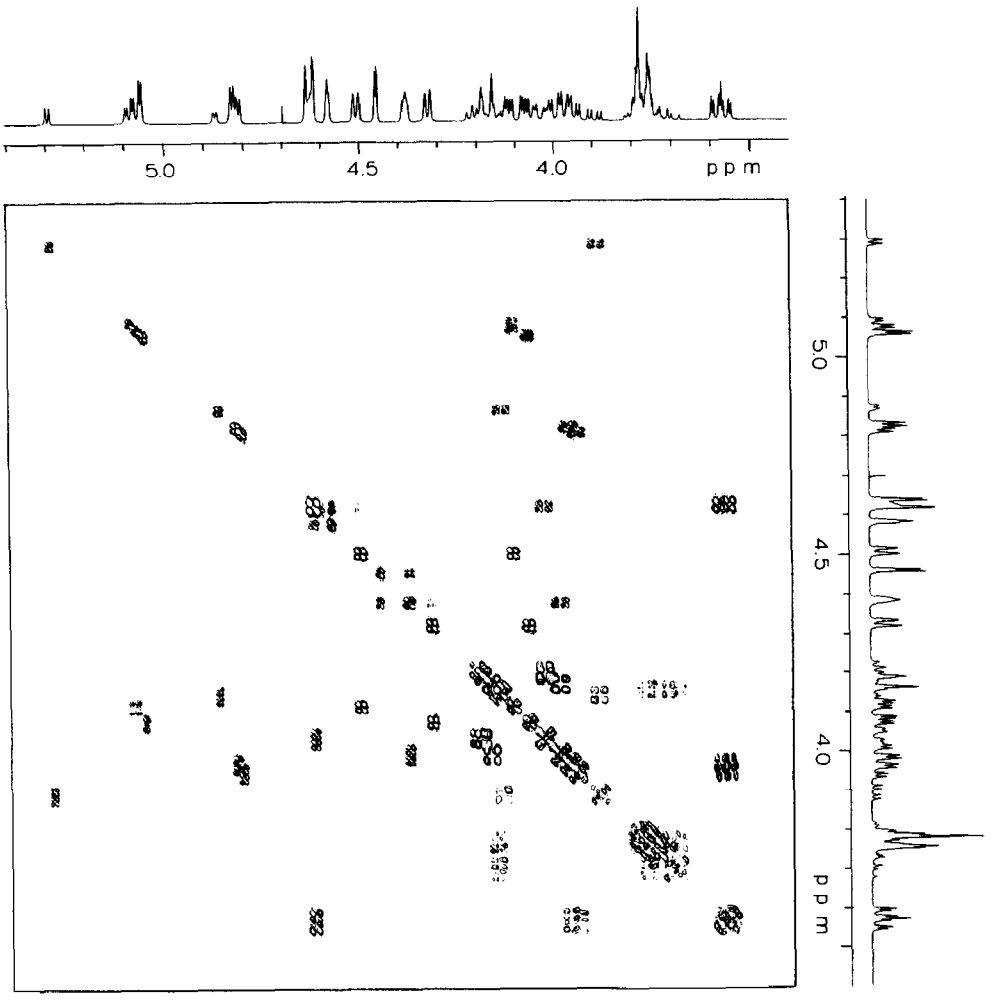


Fig. 1. 400-MHz ^{1}H DQCOSY spectrum of neocarratetraose 4^1,4^3-disulphate (A–G4S–A–G4S) at 25°.

galactopyranose 4-sulphate) which are designated as Anr and Gnr (or G4Snr), respectively. Internal 3,6-anhydro-α-D-galactopyranose and β-D-galactopyranose (or β-D-galactopyranose 4-sulphate) residues are designated as A and G (or G4S), respectively, and 3,6-anhydro-α-D-galactopyranose and α- and β-D-galactopyranose (or D-galactopyranose-4-sulphate) residues at the reducing end are designed as Ar and Gα,β (or G4Sα,β), respectively. Since ^{1}H resonances of the A residues are affected by the α,β equilibrium on the neighbouring G4Sα,β reducing end, these effects, when observed, are designated by Arα or Arβ. When the identity of the neighbour(s) in the chain does not result in an observable shift for a certain ^{1}H resonance at the field strength used, the nomenclature used is R′ towards the reducing end and R towards the non-reducing end. For disaccharides,

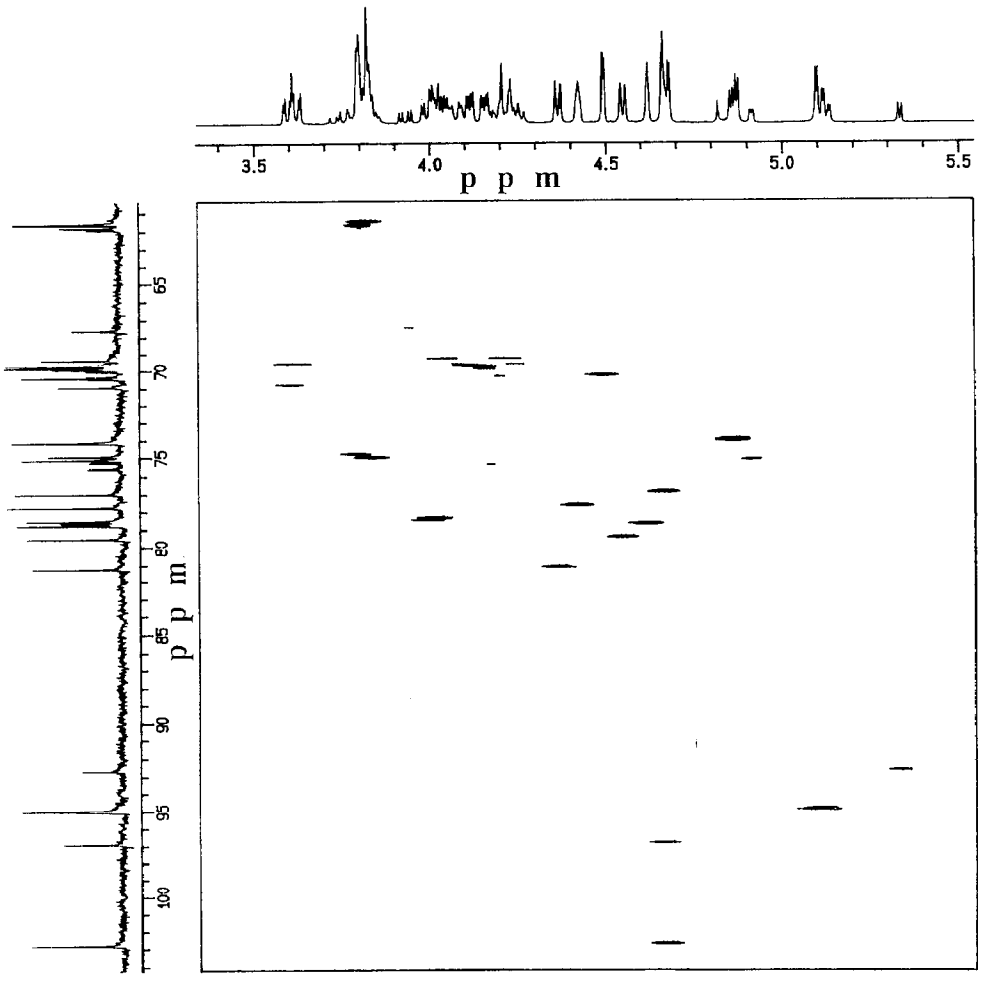


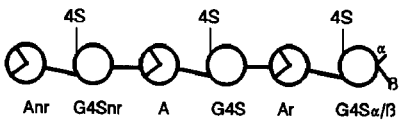
Fig. 2. Heteronuclear $^{1}H-^{13}C$ chemical-shift-correlated spectrum of A–G4S–A–G4S at 20°.

the notation has priority in the reducing end, regardless of the fact that the A residue is more affected by occupying the non-reducing end. Using this system, the β anomer of neocarrabiose 4^{1}-sulphate is designated Ar-G4Srβ with, where

TABLE I

^{13}C-NMR chemical shift data (δ in ppm) for neocarratetraose $4^{1},4^{3}$-disulphate (A–G4S–A–G4S) at 20°

Unit	C-1	C-2	C-3	C-4	C-5	C-6
G4Sα	92.77	67.60	75.58	75.28	70.49	61.95
G4Sβ	96.94	70.97	78.65	74.19	74.97	61.84
G4snr	102.84	69.78	78.52	74.15	75.15	61.64
Anr	95.00	69.89	81.28	70.44	77.79	69.45
Ar	95.00 (β)	69.96 (β)	79.56	78.83	77.02	69.78
	94.94 (α)	70.10 (α)				



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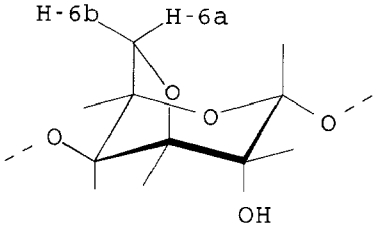

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appropriate, a subscript indicating the particular proton. In the A units, H-6a and H-6b are as shown in **2**.

The ^{1}H-NMR spectra of three oligosaccharides of neocarrabiose 4-sulphate are shown in Fig. 3, the chemical shift data are given in Tables II and III, and the coupling constants in Table IV. The most downfield resonance (5.32 ppm, $J_{1,2}$ 3.9 Hz) in the spectra arises from the reducing end, i.e., R-G4S$\alpha_{H\text{-}1}$. R-G4S$\beta_{H\text{-}1}$ and G4S$_{H\text{-}1}$ resonate at ~4.65 ppm ($J_{1,2}$ 7.9 Hz).

Based on the intensities of the H-1 signals, the α/β-ratio of 3-linked D-galactose 4-sulphate in D_2O at room temperature is estimated as 0.31–0.36 : 0.61–0.64. The values are close to those found for D-galactose[24] and D-galactose 6-sulphate[25]. Deviations in ratios for the various samples were probably due to integration errors, differences in the concentrations of salt and oligosaccharides, and the existence of a small proportion of furanose forms. Low-intensity signals, especially those occurring in the spectrum of the tetrasaccharide, could be due to furanose forms or to unknown impurities. Similar characteristic low-intensity signals were also found in the ^{1}H-NMR spectrum of neoagarobiose (data not shown).

The H-1 signals were used as entry points for the analysis of the COSY spectrum. The connectivities to the respective H-2 signals are observed readily (Fig. 1). The most upfield resonances (~3.6 ppm) are for H-2 of the (1 → 3)-linked β-D-galactose residues and the β form of the reducing-ends. The H-1 signal of the corresponding α anomers is at ~3.92 ppm. Most of the resonances in the 2D COSY spectrum are resolved sufficiently to confirm the assignments. The signals (dd) at 4.00, 3.98, and 4.17 ppm are identified as G4Snr$_{H\text{-}3}$, G4S$\beta_{H\text{-}3}$, and G4S$\alpha_{H\text{-}3}$, respectively. Due to the α,β equilibrium, the H-4 protons of the reducing ends of the kappa-oligosaccharides (R-G4S$\alpha_{H\text{-}4}$ and R-G4S$\beta_{H\text{-}4}$) resonate at ~4.90 and ~4.84 ppm, respectively. G4Snr$_{H\text{-}4}$ and G4S$_{H\text{-}4}$ for the hexasaccharides, which are partly masked by the large solvent peak, resonate at

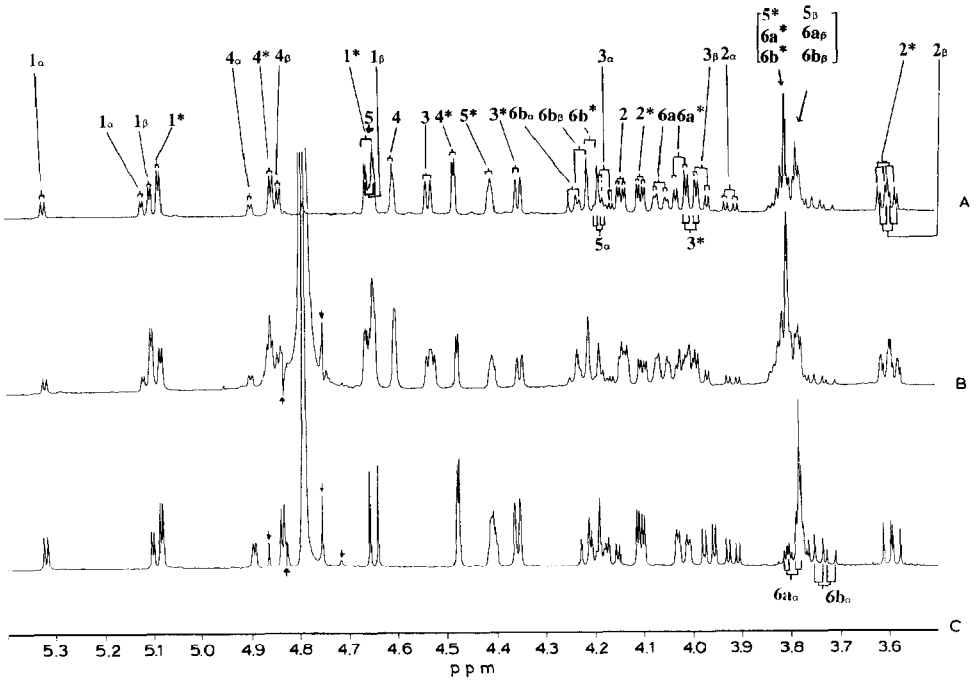

Fig. 3. 500-MHz ^{1}H-NMR spectra of 4-sulphated neocarrabiose oligosaccharides at 25°: A, $(A–G4S)_2$; B, $(A–G4S)_3$; and C, A–G4S; protons of the G units are designated on the upper line (and, due to lack of space, below the spectrum) and those of the A units on the lower line, * denotes a non-reducing end or a galactose 4-sulphate unit next to a non-reducing end, α and β refer to the anomers of D-galactose 4-sulphate or the effect of the α,β-equilibrium on the neighbouring 3,6-anhydro unit, and the small arrows identify spinning sidebands from residual water.

4.86 ppm. For the 3-linked D-galactose residues, the connectivity between H-4α and H-5α is missing due to small spin–spin coupling. Furthermore, because the resonances of H-5 and H-6a,6b of 3-linked D-galactose overlap, the COSY spectrum does not allow their assignments. However, after comparing the spectra of oligosaccharides of the kappa-type (Fig. 3), it is reasonable to assume that H-5 and H-6a,b of the reducing-end unit resonate at slightly higher field compared to the corresponding protons of the non-reducing end and internal G residues. In this region, the resonances of R-G4S$\alpha_{\text{H-6b}}$ ($\sim$ 3.71 ppm) are completely separated from those of the remaining H-6 and H-5 of the 3-linked units, whereas that of R-G4S$\alpha_{\text{H-6a}}$ ($\sim$ 3.79 ppm) is only partly separated from these resonances. The assignments of the remaining H-6 and H-5 resonances were made by the heteronuclear shift-correlated spectrum (Fig. 2).

For the A units, all connectivities, except those between H-3 and H-4, and H-5 and H-6b, are clearly displayed in the COSY spectrum. However, since each of these protons is coupled to two protons, their assignments can be made unambiguously.

TABLE II

^{1}H-NMR shift data (δ in ppm) of the G units in neocarrabiose oligosaccharides at 25°

Proton	$(A-G4S)_3$	$(A-G4S)_2$	A–G4S	A–G–A–G4S	A–G
GH-1α	5.320	5.320	5.320	5.320	5.299
GH-1β	4.654	4.653	4.650	4.650	4.615
GnrH-1	4.658	4.656		4.618	
GH-1	4.660				
GH-2α	3.917	3.919	3.919	3.921	3.909
GH-2β	3.593	3.595	3.594	3.597	3.587
GnrH-2	3.596	3.601		3.603	
GH-2	3.599				
GH-3α	4.165	4.174	4.165	4.171	4.055
GH-3β	3.983	3.979	3.967	3.974	3.837
GnrH-3	4.003	4.000		3.866	
GH-3	4.013				
GH-4α	4.901	4.899	4.895	4.897	4.185
GH-4β	4.835	4.840	4.835	4.837	4.128
GnrH-4	4.855	4.855		4.139	
GH-4	4.855				
GH-5α	4.186	4.186	4.190	4.185	4.066
GH-5β	3.774	3.774	3.774	3.77	3.670
GnrH-5	3.817	3.817		3.72	
GH-5	3.817				
GH-6aα	3.79	3.79	3.793	3.79	3.726
GH-6aβ	3.783	3.785	3.78	3.78	3.772
GnrH-6a	3.800	3.800		3.70–3.72	
GH-6a	3.800				
GH-6bα	3.731	3.731	3.731	3.73	3.723
GH-6bβ	3.783	3.783	3.783	3.78	3.733
GnrH-6b	3.800	3.800		3.70–3.72	
GH-6b	3.800				

The α,β equilibrium influences the resonances of some of the protons on the neighbouring A unit. Thus, the resonances of R-$A_{H\text{-}1}$–G4S-R′ and R-$Ar_{H\text{-}1}$–G4Sβ are at ~5.103 ppm and those of R-$Ar_{H\text{-}1}$–G4Sα of both the tetrasaccharide and the hexasaccharide are shifted downfield to ~5.12, whereas those of $Anr_{H\text{-}1}$–G4S-R′ of both residues are shifted upfield to ~5.085 ppm. For the disaccharide, the resonances for Ar$\alpha_{H\text{-}1}$ and Ar$\beta_{H\text{-}1}$ of ArG4Sr are at ~5.102 and ~5.084 ppm, respectively. These resonances merge with those of R-$Ar_{H\text{-}1}$–G4Sβ ($dp \geq 4$) or R-$A_{H\text{-}1}$–G4S-R′ ($dp \geq 6$) and $Anr_{H\text{-}1}$–G4S-R′ ($dp \geq 4$), respectively. The Ar of the disaccharide differs from that of the higher oligosaccharides in that it has the characteristics of both a unit next to a reducing end (Arα,β) and a unit occupying the non-reducing end (Anr).

Another effect due to the α,β equilibrium is found for the H-6b of the Ar units. The resonances centred at 4.234 and 4.224 ppm are due to R-$Ar_{H\text{-}6b}$–G4Sα and R-$Ar_{H\text{-}6b}$–G4Sβ, respectively. The latter resonance occupies the same position as that of the H-6b of internal A units in the hexasaccharide which are next to G4S. The splitting is commonly observed for the $Ar_{H\text{-}6b}$ resonance regardless of 4-

TABLE III

^{1}H-NMR chemical shift data (δ in ppm) of the A units in neocarrabiose oligosaccharides at 25°

Proton	$(A-G4S)_3$	$(A-G4S)_2$	A-G4S	A-G-A-G4S	A-G
ArH-1α	5.120	5.121	5.102	5.119	5.080
ArH-1β	5.103	5.104	5.084	5.101	5.062
AnrH-1	5.084	5.085		5.065	
AH-1	5.103				
ArH-2α	4.142	4.144	4.106	4.143	4.055
ArH-2β	4.142	4.144	4.106	4.143	4.055
AnrH-2	4.102	4.103		4.048	
AH-2	4.138				
ArH-3α	4.535	4.535	4.357	4.529	4.372
ArH-3β	4.535	4.535	4.357	4.529	4.372
AnrH-3	4.352	4.352		4.367	
AH-3	4.529				
ArH-4α	4.607	4.606	4.478	4.592	4.500
ArH-4β	4.607	4.606	4.478	4.592	4.500
AnrH-4	4.479	4.483		4.502	
AH-4	4.607				
ArH-5α	4.650	4.648	4.408	4.667	4.423
ArH-5β	4.650	4.648	4.408	4.667	4.423
AnrH-5	4.409	4.409		4.423	
AH-5	4.650				
ArH-6aα	4.061	4.062	4.020	4.053	4.032
ArH-6aβ	4.061	4.062	4.020	4.053	4.032
AnrH-6a	4.020	4.021		4.032	
AH-6a	4.061				
ArH-6bα	4.234	4.234	4.218	4.236	4.214
ArH-6bβ	4.224	4.224	4.202	4.219	4.195
AnrH-6b	4.203	4.203		4.196	
AH-6b	4.224				

TABLE IV

Coupling constants (J in Hz) for oligosaccharides of the 4-sulphated neocarrabiose type

D-Gal		3,6-AG	
$J_{1,2\alpha}$	3.9	$J_{1,2}$	2.4
β/nr/int [a]	7.9		
$J_{2,3\alpha}$	10.3	$J_{2,3}$	5.4
β/nr/int	9.9		
$J_{3,4\alpha}$	3.1 (2.5) [c]	$J_{3,4}$	TS [b]
β/nr/int	3.3		
$J_{4,5\alpha}$	TS	$J_{4,5}$	1.9
β/nr/int	0.95		
$J_{5,6a\alpha}$	4.4 (6.2)	$J_{5,6a}$	3.1
β/nr/int	8.2 [d] (7.7)		
$J_{5,6b\alpha}$	8.1 (6.2)	$J_{5,6b}$	TS
β/nr/int	4.3 [d] (4.4)		
$J_{6a,6a\alpha}$	−11.9 (NM) [e]	$J_{6a,6b}$	−10.6
β/nr/int	−12.0 [d] (11.6)		

[a] Internal G4S units. [b] Too small to be measured. [c] Deviating coupling constants measured from neocarrabiose (A–B) are given in parenthesis. [d] Data from ref. 19. [e] Not measured due to similar chemical shifts.

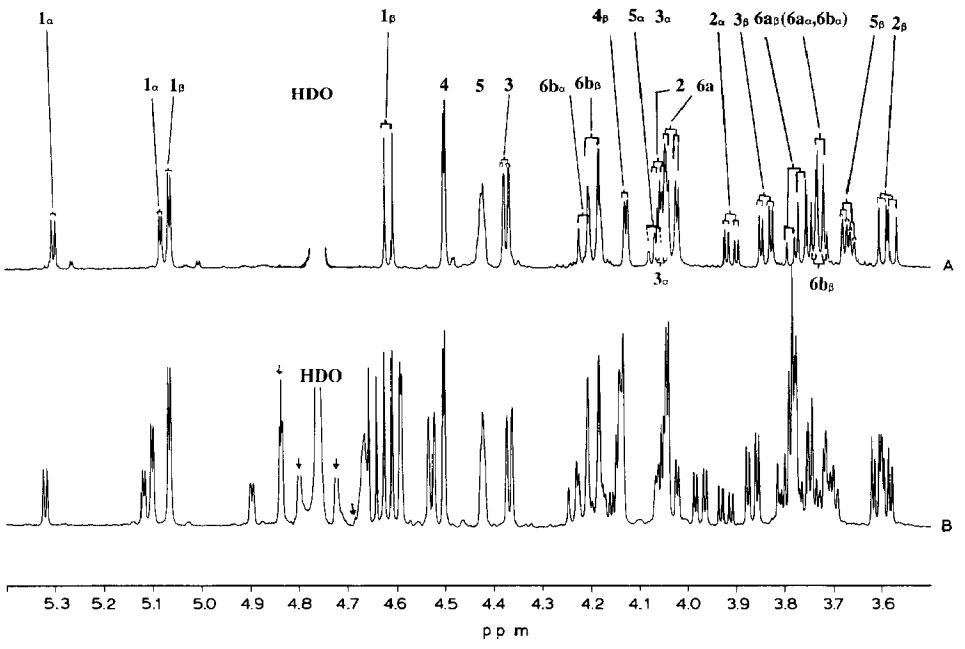

Fig. 4. 500-MHz ^{1}H-NMR spectra of derivatives of neocarrabiose at 25°: A, A–G; B, A–G–A–G4S (see legend to Fig. 3).

sulphation of the neighbouring unit(s). This effect is not observed for $Ar_{H\text{-}6a}$. For the Ar residue, H-6a of both the tetra- and the hexa-saccharide resonates at ~ 4.020 ppm, which is the same position as that of $Ar_{H\text{-}6a}$ of the disaccharide. In general, increasing the number of neocarrabiose units does not affect the chemical shifts of the ^{1}H resonances of the A unit occupying the non-reducing end. The coupling constants for A are $J_{6a,6b} - 10.6$ and $J_{5,6a}$ 3.1 Hz, whereas $J_{5,6b}$ is too small to be measured. Values close to these have been reported for derivatives of 3,6-anhydrogalactose[15,17].

The NMR spectra of the partly desulphated tetrasaccharide and neocarrabiose are given in Fig. 4. Removing the 4-sulphate group of the galactose unit, giving A–G–A–G4S or A–G, introduces an upfield shift of 0.02 ppm for the H-1 resonances of Anr and Ar. A similar difference in chemical shift has been found in the ^{1}H-NMR spectra of carrageenan extracted from *Furcellaria lumbricalis*[26,27], *Eucheuma gelatinae,* and *Endocladia muricata* (data not published), which are undersulphated kappa-carrageenans. The largest difference in chemical shift caused by desulphation of the D-galactose residue is found for the H-2 resonance of the neighbouring A unit towards the non-reducing end. Replacement of a 4-sulphated unit, characteristic for kappa-carrageenan or A–G4S disaccharide sequences, with a desulphated or A–G sequence, results in an upfield shift of ~ 0.05 ppm for the H-2 resonances. Similar, but smaller, downfield displacements of the resonances of H-3,4,5 of anhydrogalactose in sequences of Anr–G4S-R′ relative to those of

Anr–G-R′ are found in the region 4.35 to 4.5 ppm. The data for the two sets of tetra- and di-saccharides show that H-6a and H-6b, which are in fixed positions due to the anhydro ring, are affected differently by the presence of a 4-sulphate group. For H-6a and H-6b of both Anr–G-R′ and Ar–Gα/β, the substitution with G4S causes a small downfield shift for the H-6b resonance of ~ 0.007 ppm and a somewhat larger upfield shift (~ 0.012 ppm) for that of H-6a.

For the Anr–Gnr–Ar–G4Sα/β oligosaccharide, the resonances of $Ar_{H\text{-}1}$ and $Ar_{H\text{-}2}$ occupy the same positions as for corresponding Ar protons from the tetrasaccharide and hexasaccharide of the pure kappa type. This finding shows that desulpation of the galactose residue does not have any significant effect on the position of the resonances of the most distant protons on the nearest A unit towards the reducing end. However, effects are found for H-3,4,5, due to a closer proximity to O-4 of the neighbouring residue.

The chemical shifts of the resonances of corresponding reducing-end protons (R-G4S$_{H\text{-}n}$) of the different oligosaccharides are similar and affected only slightly by the dp. For oligosaccharides, the resonances of the β anomers in general have the same, or similar, δ values to those of the G-4Snr units or internal G4S residues in oligosaccharides or polysaccharides. For the internal or non-reducing end G residues, no "long-distance effect" is found from the α,β equilibrium. The resonances from α anomers, with the exception of that of G4S$\alpha_{H\text{-}6b}$, are shifted downfield compared to those of the corresponding β anomers.

By removing the 4-sulphate group, the ring protons are affected to a different degree (see Table II) compared with the sulphated analogues, with the largest effect (~ 0.7 ppm) for the H-4 resonance. At the same time, the $J_{3,4}$ value for the α anomer is reduced from 3.1 (G4S) to 2.5 Hz (G). The introduction of a 4-sulphate group also shifts the resonances of R-A–G4S$\alpha_{H\text{-}1}$ and R-A–G4S$\beta_{H\text{-}1}$ downfield (0.02 and 0.035 ppm, respectively), compared to that of H-1 of a neocarrabiose (R-A–Gα/β) end (see Fig. 5 for the mixture of A–G and A–G4S oligosaccharides).

The H-1 resonance of α-D-galactose occurs at ~ 5.3 ppm, and is slightly affected when there is a 4-sulphate group. Thus, two resonances in this region, with $J_{1,2}$ values of 3.9 Hz, indicate that the sample contains a mixture of oligosaccharides with different reducing ends. Further, the H-1 of (1 → 4)-linked 3,6-anhydro-α-D-galactose 2-sulphate, the iota-carrageenan structural character, which resonates only ~ 0.02 ppm upfield of H-1 of R-G4Sα, can be distinguished[19] by the $J_{1,2}$ value of 2.3 Hz. This characteristic signal occurs in oligosaccharides enzymically prepared from commercial samples of kappa-carrageenan[27] and can be used to obtain a more accurate measurement of the iota-content than an estimation based on ^{13}C-NMR spectroscopy.

Comparing oligosaccharides with different distributions of sulphate groups reveals some relatively large effects, e.g., $Anr_{H\text{-}2}$–G4S-R′ and $Anr_{H\text{-}2}$–G-R′ at ~ 4.1 and ~ 4.05 ppm, respectively, but gives little information due to the overlapping with signals from other protons. However, the small differences, found

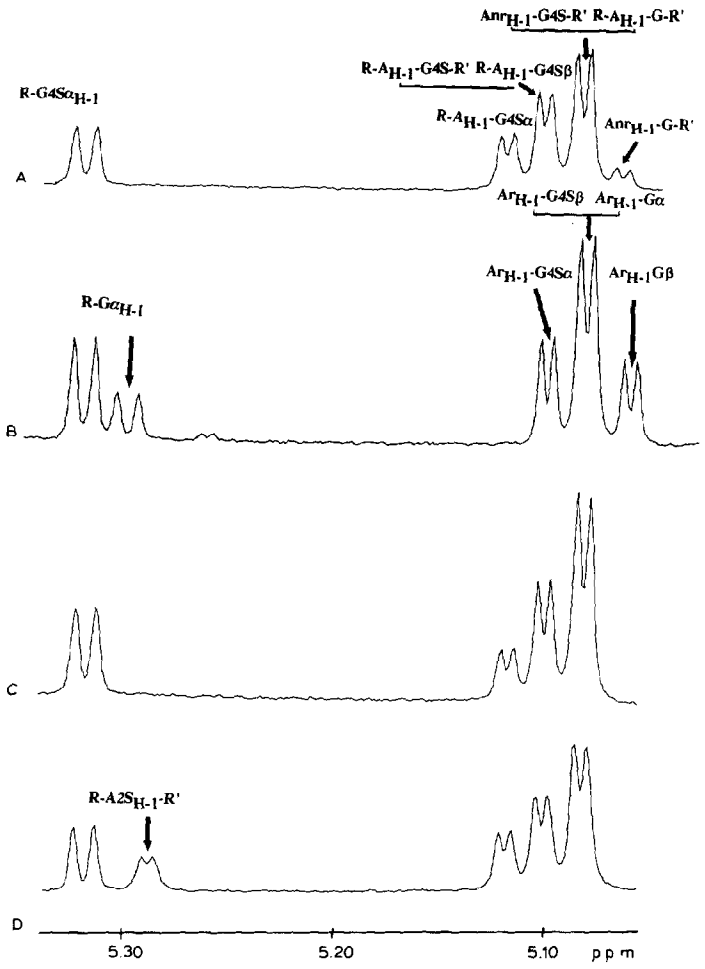

Fig. 5. Region for the H-1 resonances in the 400-MHz ^{1}H-NMR spectra at 20° of mixtures of some neocarrabiose derivatives: A, A–G4S–A–G4S and A–G–A–G4S (5:1); B, A–G4S and A–G (2:1); C, A–G4S–A–G4S and A–G4S (3:2); and D, A–G4S–A–G4S and A–G4S–A2S–G4S–A–G4S (2:1).

in the region 4.35–4.50 ppm, from the well-resolved resonances of H-3,4,5 of Anr residues, give information about the sulphation of the nearest neighbour. The occurrence of sequences of Anr–G4S-R′ or Anr–G-R′, as can be found in oligosaccharide mixtures enzymically produced from a carrageenan of the *Furcellaria* type, can be distinguished [27].

A knowledge of the identity and proportions of the products obtained upon treatment of different kappa-type or kappa-like carrageenans is needed in order to determine the mode of action of the kappa-carrageenase and related enzymes. For kappa-carrageenase, the information available on the substrate specificity is limited with respect to the pattern of distribution of 4-sulphate groups, and the

minimum chain length required is not known. Basic information about chain length and sequences can be obtained with ^{1}H- and ^{13}C-NMR spectroscopy. To achieve this aim, a variety of model compounds of neocarrabiose oligosaccharides with different lengths and patterns of sulphate groups, enzymically produced from desulphated carrageenans of the *Furcellaria* type, are needed.

ACKNOWLEDGMENTS

The Bioenergy Program of the Norwegian Agricultural Research Council is thanked for support (to S.H.K.). The 500-MHz NMR spectra were recorded at SINTEF's MR-Centre im Trondheim by Eng. A. Østensen, and the DQCOSY spectrum was recorded by Jeol (Tokyo). We thank Professor B. Larsen for helpful discussions.

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