Carbohydrate Research, 238 (1993) 241–248
Elsevier Science Publishers B.V., Amsterdam

Determination of the structures of cystocarpic carrageenans from *Gigartina skottsbergii* by methylation analysis and NMR spectroscopy

Marina Ciancia [a], Maria C. Matulewicz [a,1], Paul Finch [b] and Alberto S. Cerezo [a,1]

[a] *Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón 2, Ciudad Universitaria, 1428 Buenos Aires (Argentina)*
[b] *Department of Chemistry, The Bourne Laboratory, Royal Holloway and Bedford New College, Egham, Surrey TW20 0EX (United Kingdom)*

(Received January 13th, 1992; accepted April 24th, 1992)

ABSTRACT

The combined use of methylation analysis and high-field ^1H and ^{13}C NMR spectroscopy allows the determination of the fine structure of the carrageenans produced by the cystocarpic stage of *Gigartina skottsbergii*.

INTRODUCTION

Cystocarpic plants of *Gigartina skottsbergii* synthesize a system of carrageenans, designated $1C_1$, $1C_2$, and $1C_3$, formed by similar amounts of gelling ($1C_1$, 42.6%; $1C_2$, 3.5%) and non-gelling ($1C_3$, 53.9%) carrageenans. Analysis and alkaline treatment of these products as well as their IR spectra suggested κ/ι-structures for the first two and a partially cyclized μ/ν-structure for the latter[1]. Methylation analysis of carrageenan $1C_3$ confirmed this last result[2].

We report now the fine structure of these three carrageenans by the combined use of methylation analysis and high-field ^{13}C and ^1H NMR spectroscopy. Comparison of these structures clearly shows new aspects of their biogenetic relationship.

Correspondence to: Dr. A.S. Cerezo, Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón 2, Ciudad Universitaria, 1428 Buenos Aires, Argentina.

[1] Research Member of the National Research Council of Argentina (CONICET).

EXPERIMENTAL

Cystocarpic *Gigartina skottsbergii* was collected in Bahía Camarones (Provincia de Chubut, Argentina) and sorted in the Instituto Nacional Patagónico (Puerto Madryn, Chubut).

The 3,6-anhydrogalactose content was analysed by the resorcinol method[3]. Molecular weights were determined by the method of Park and Johnson[4]. Sulfate linked to primary hydroxyl groups was determined before and after periodate oxidation according to Rees[5].

Methylation analysis was carried out as described elsewhere[2]. The samples, designated as $1C_1$, $1C_2$, and $1C_3$, were passed down a column of Amberlite IR-120 (Na^+) to remove K^+ ions before the NMR determinations.

The 100-MHz ^{13}C NMR spectra were recorded at 50–65°C, in D_2O solutions, with an external reference of trimethylsilane for $1C_3$ and an external reference of sodium 3-trimethylsilylpropionate-d_4 for $1C_1$ and $1C_2$. The parameters were as follows: pulse angle, 90°; acquisition time, 0.328–0.655 s; relaxation delay, 1.0–0.4 s; spectral width, 25 kHz, and scans 44 142–168 489. A constant offset (−1.5 ppm for $1C_1$, −1.7 ppm for $1C_2$, and −2.2 ppm for $1C_3$) was used to allow for different chemical shift referencing[6].

The ^1H NMR spectra were recorded at 400 MHz, at 50–65°C, in D_2O solutions, with external reference to sodium 3-trimethylsilylpropionate-d_4. The parameters were as follows: pulse angle, 45°; acquisition time, 3.277–3.408 s; relaxation delay, 1.6 s; spectral width, 5 kHz, and scans 270–17 851.

RESULTS

The composition of permethylated carrageenans $1C_1$, $1C_2$, and $1C_3$ are shown in Table I; Table VI indicates the corresponding composition in structural units. The ^{13}C NMR spectra of $1C_1$, $1C_2$, and $1C_3$ are shown in Fig. 1, while their assignments indicating the diads present and the areas of the anomeric signals are given in Tables II, III, and IV. Table V gives the assignments and areas of the

TABLE I

Composition of sugars produced by methylation and hydrolysis of carrageenans $1C_1$, $1C_2$, and $1C_3$ [a]

Carrageenan	Mol% of galactose having methyl groups at the positions indicated								
	2,3,4,6	2,4,6	2,3,6	2,6	4,6	2,4	6	2	3
$1C_1$	tr.[b]	3.5	tr.	92.5			3.1	1.0	tr
$1C_2$		3.8	tr.	91.0			1.0	tr.	4.1
$1C_3$ [c]	1.2	5.7	2.6	75.5	6.9	1.7	1.2	1.9	3.3

[a] The hydrolysates were derivatized to the alditol acetates and to the aldononitrile acetates; 2,6-di-*O*-methyl-, 4,6-di-*O*-methyl-galactose, and the triplet composed of 3-*O*-methyl- and 4-*O*-methyl-galactose, and galactose were readily differentiated using aldononitrile acetates. [b] Percentages lower than 1% are given as trace (tr.). [c] Added for comparison.

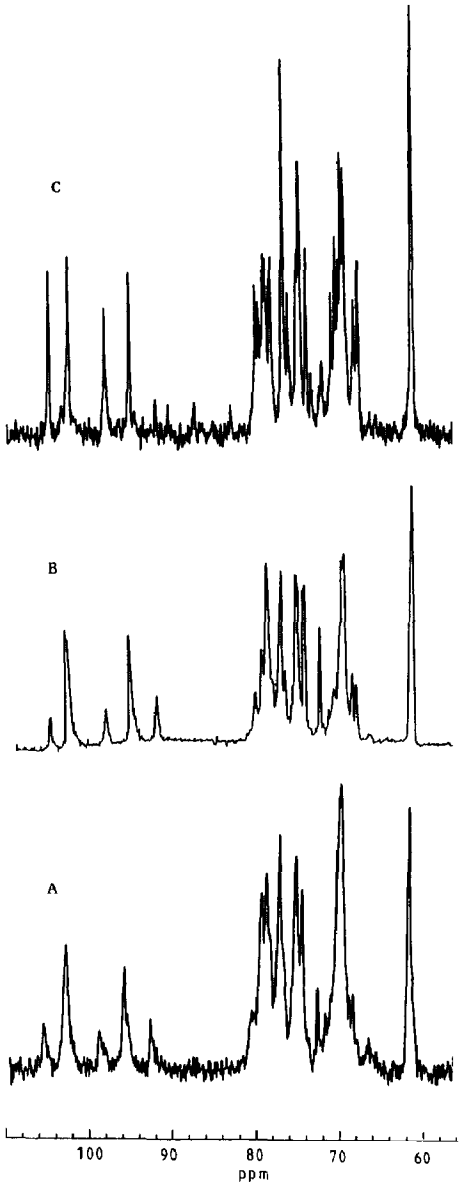

Fig. 1. ^{13}C NMR spectra of carrageenans: (A) $1C_1$, (B) $1C_2$, and (C) $1C_3$.

anomeric protons of the α-units linked to β-D-galactose 4-sulfate[7,8], obtained from the corresponding ^1H NMR spectra of $1C_1$, $1C_2$ and $1C_3$. The spectra of the three fractions showed broad signals and an overlapping for the H-4 of β-D-galactose 4-sulfate units either linked to 3,6-anhydrogalactose, 3,6-anhydrogalactose 2-sulfate or galactose 6-sulfate, and the H-2 of a 3,6-anhydrogalactose 2-sulfate unit linked to β-D-galactose 4-sulfate. Table VI gives the corresponding composition in

TABLE II

^{13}C NMR spectral assignments of carrageenan $1C_1$ [a]

Diad	C-1	C-2	C-3	C-4	C-5	C-6
3-linked β-D-Gal 4-sulfate	105.3 (9)[b]	70.9	80.4	73.4	75.1	61.8
4-linked α-D-Gal 2,6-disulfate	98.7 (7)	76.7	68.8	79.3	68.8	68.3
3-linked β-D-Gal 6-sulfate[c]	105.3 (9)	70.9	78.8	73.4	75.1	61.8
4-linked α-D-Gal 6-sulfate	98.2 (2)	69.0	70.9	79.3	68.8	68.3
3-linked β-D-Gal 2,4-disulfate[c]	103.5 (2)	79.3	79.6	74.5	75.1	61.8
4-linked α-D-Gal 2,6-disulfate	98.7 (7)	76.3	68.8	79.3	68.8	68.3
3-linked β-D-Gal 4-sulfate	102.8 (40)	69.9	79.3	74.5	75.1	61.8
4-linked α-D-3,6-AnGal	95.7 (29)	70.4	79.6	78.8	77.3	69.9
3-linked β-D-Gal 4-sulfate	102.8 (40)	69.9	77.3	72.5	75.1	61.8
4-linked α-D-3,6-AnGal 2-sulfate	92.5 (10)	75.1	78.3	78.8	77.3	70.4
3-linked β-D-Gal	102.8 (40)	69.9	79.3	66.5	75.9	61.8
4-linked α-D-3,6-AnGal 2-sulfate	91.8 (2)	75.9	79.3	78.8	77.3	70.4
3-linked β-D-Gal 2,4-disulfate[c]	101.7 (1)	78.3	78.3	74.5	75.1	61.8
4-linked α-D-3,6-AnGal	95.7 (29)	70.4	79.6	78.8	77.3	69.9

[a] The spectrum was recorded at 100 MHz, at 65°C, in D_2O solution, with proton decoupling. The number of scans was 44 142. [b] Area% of the anomeric signal. [c] Tentative.

structural units from which the contribution of the diads to the whole structure was calculated (Table VII).

$1C_1$ (mol wt 75 000) comprises 76% of diads Gal4SO_3–3,6-AnGal and Gal4SO_3–3,6-AnGal2SO_3 (ratio 3 : 1) together with 15% of classical kink-containing diads. Smaller amounts of nonclassical kink-containing (3%) and unusual (6%) diads were also detected (Table VII).

TABLE III

^{13}C NMR spectral assignment of carrageenan $1C_2$ [a]

Diad	C-1	C-2	C-3	C-4	C-5	C-6
3-linked β-D-Gal 4-sulfate	105.3 (9)[b]	70.8	80.4	74.4	75.3	61.8
4-linked α-D-Gal 2,6-disulfate	98.7 (8)	76.8	68.7	79.7	68.7	68.3
3-linked β-D-Gal 4-sulfate[c]	105.3 (9)	70.8	78.9	74.4	75.3	61.8
4-linked α-D-Gal 6-sulfate	98.4 (1)	69.1	70.9	79.7	68.7	68.3
3-linked β-D-Gal 4-sulfate	103.1 (40)	70.2	79.1	74.4	75.3	61.8
4-linked α-D-3,6-AnGal	95.5 (26)	70.2	79.7	78.9	77.3	70.0
3-linked β-D-Gal 4-sulfate	103.1 (40)	69.9	77.3	72.5	75.3	61.8
4-linked α-D-3,6-AnGal 2-sulfate	92.4 (10)	75.3	78.7	78.9	77.5	70.3
3-linked β-D-Gal[c]	102.5 (3)	70.1	78.9	66.7	76.2	61.8
4-linked α-D-3,6-AnGal 2-sulfate	92.2 (2)	76.2	78.9	78.5	77.6	70.3

[a] The spectrum was recorded at 100 MHz, at 50°C, in D_2O solution, with proton decoupling. The number of scans was 168 489. [b] Area% of the anomeric signals. [c] Tentative.

TABLE IV

^{13}C NMR spectral assignments of carrageenan $1C_3$ [a]

Diad	C-1	C-2	C-3	C-4	C-5	C-6
3-linked β-D-Gal 4-sulfate	105.3 (24)[b]	70.7	80.4	73.8	75.4	61.9
4-linked α-D-Gal 2,6-disulfate	98.8 (15)	76.8	68.9	79.5	68.9	68.4
3-linked β-D-Gal 4-sulfate	105.3 (24)	70.7	78.8	73.8	75.4	61.9
4-linked α-D-Gal 6-sulfate	98.2 (4)	68.9	70.7	79.5	68.9	68.4
3-linked β-D-Gal 4-sulfate	105.3 (24)	70.7	78.8	73.8	75.4	61.9
4-linked α-D-Gal[c]	98.2 (4)	69.9	71.1	79.5	71.1	62.0
3-linked β-D-Gal	105.3 (24)	71.1	79.5	66.2	75.7	61.9
4-linked α-D-Gal 2,6-disulfate[c]	94.8 (3)	76.8	68.9	78.8	68.9	68.4
3-linked β-D-Gal 2-sulfate	103.8 (4)	79.5	78.6	66.2	75.7	61.9
4-linked α-D-Gal 6-sulfate[c]	97.0 (2)	69.6	71.1	78.8	69.9	68.4
3-linked β-D-Gal 2-sulfate	103.8 (4)	79.5	78.6	66.2	75.7	61.9
4-linked α-D-Gal 2,6-disulfate[c]	94.8 (3)	76.8	68.9	78.8	68.9	68.4
3-linked β-D-Gal 4-sulfate	103.0 (25)	70.1	79.5	74.7	75.4	62.0
4-linked α-D-3,6-AnGal	95.8 (23)	70.4	79.7	78.8	77.4	70.1

[a] The spectrum was recorded at 100 MHz, at 65°C, in D_2O solution, with proton decoupling. The number of scans was 53 565. [b] Area% of the anomeric signals. [c] Tentative.

The major diads which contribute to the structure of $1C_2$ (mol wt 124 000) are similar to those of $1C_1$; they comprise 78% of Gal4SO_3–3,6-AnGal and Gal4SO_3–3,6-AnGal2SO_3 (ratio 4:1) together with 19% of μ/ν-structure. Only 4% of unusual diads are present but no nonclassical kink-containing diads were detected.

TABLE V

^1H NMR spectral data of carrageenans $1C_1$, $1C_2$, and $1C_3$. Anomeric assignments of α-D-galactose units linked to β-D-galactose 4-sulfate

	δ (ppm)	α-unit	Area%[a]
$1C_1$	5.52	Gal 2,6-disulfate	10
	5.32	3,6-AnGal 2-sulfate	11
	5.26	Gal 6-sulfate	3
	5.11	3,6-AnGal	28
$1C_2$	5.52	Gal 2,6-disulfate	7
	5.32	3,6-AnGal 2-sulfate	12
	5.11	3,6-AnGal	28
$1C_3$	5.52	Gal 2,6-disulfate	15
	5.32	3,6-AnGal 2-sulfate	5
	5.26	Gal 6-sulfate	9
	5.11	3,6-AnGal	22

[a] The total area% of α-units was estimated as an average of data determined by analytical methods and ^{13}C NMR spectroscopy.

TABLE VI

The composition of carrageenans $1C_1$, $1C_2$, and $1C_3$

Carrageenan	Percentage of the sugar having sulfate at the position indicated										
	3-Linked galactose						4-Linked galactose			3,6-Anhydrogalactose	
	None	4	2	6	2,4	4,6	None	6	2,6	None	2
$1C_1$ [a,b]	2.0	52.7			2.0	tr.[c]	tr.	[d]	4.3[d]	39.0[e]	
$1C_1$ [f]	3	38			2	-	tr.	2	4	43	10
$1C_1$ [g]								2	5	36	8
$1C_2$ [a,b]	1.0	51.0			tr.	1.0	tr.	3.1[d]	5.6[d]	38.3[e]	
$1C_2$ [f]	4	41						1	5	40	9
$1C_2$ [g]									4	35	9
$1C_3$ [a,b]	2.8	36.4	3.4	tr.	tr.	tr.	1.2	7.3[d]	21.2[d]	27.0[e]	
$1C_3$ [f]	44		4					3	11	38	
$1C_3$ [g]								7	9	32	4

[a] From methylation analysis. [b] Corrected for cyclization produced during methylation. [c] Percentages lower than 1% are given as trace (tr.). [d] Contents of 4-linked galactose 6-sulfate and 2,6-disulfate are determined according to Rees. [e] Determined by the method of Yaphe. [f] From the anomeric signals of the ^{13}C NMR spectra. [g] From the anomeric signals of the α-units in the ^1H NMR spectra.

$1C_3$ (mol wt 198 000) is a partially cyclized μ/ν-carrageenan comprising 49% of κ-structure and 37% of classical kink-containing diads. Small proportions of λ-structure (9%), nonclassical kink-containing (4%) and unusual (2%) diads were

TABLE VII

Contribution of the diads to the whole structure

	Diad	(%)
$1C_1$	Gal 4-sulfate–3,6-AnGal	56
	Gal 4-sulfate–3,6-AnGal 2-sulfate	20
	Gal 4-sulfate–Gal 2,6-disulfate	11
	Gal 4-sulfate–Gal 6-sulfate	4
	Gal–3,6-AnGal 2-sulfate[a]	4
	Gal 2,4-disulfate–Gal 2,6-disulfate	3
	Gal 2,4-disulfate–3,6-AnGal	2
$1C_2$	Gal 4-sulfate–3,6-AnGal	63
	Gal 4-sulfate–3,6-AnGal 2-sulfate	15
	Gal 4-sulfate–Gal 2,6-disulfate	16
	Gal 4-sulfate–Gal 6-sulfate	3
	Gal–3,6-AnGal 2-sulfate[a]	4
$1C_3$	Gal 4-sulfate–3,6-AnGal	49
	Gal 4-sulfate–Gal 2,6-disulfate	29
	Gal 4-sulfate–Gal 6-sulfate	8
	Gal 2-sulfate–Gal 2,6-disulfate	5
	Gal 2-sulfate–Gal 6-sulfate	4
	Gal–Gal 2,6-disulfate	4[b]
	Gal 4-sulfate–Gal	2[b]

[a] The presence of nonsulfated galactose was confirmed by the C-4 peak at 66.5 ppm. [b] Determined by methylation analysis.

also detected (Table VII). This carrageenan is soluble in 2 M KCl; this solubility behaviour of "real" (partially cyclized) μ/ν-carragenans has been also found in the μ/ν-carrageenan of *Iridaea undulosa*[9].

DISCUSSION

Methylation analysis has been used for the determination of fine structure of carrageenans. When carried out under carefully controlled conditions, it allows identification of minor structural units[9] which may be the determinants for the rheological behaviour of these polymers. On the other hand, ^{13}C NMR spectroscopy is frequently employed for the identification of different types of gelling carrageenans[10]. Nevertheless, there is no reason for not taking advantage of the full power provided by high-field ^{13}C NMR spectroscopy for determining definite sequences (diads) of structural units in the galactan backbone[6]. Thus, the combined use of both techniques is a very useful tool for structural analysis of carrageenans. ^{1}H NMR spectroscopy has contributed significantly to extend the knowledge on the structure of carbohydrates related to glycoproteins[11]. Here we explore its application to carrageenans. In addition, the signal at 5.52 ppm (Table V) was assigned for the first time to H-1 in α-D-galactose 2,6-disulfate linked to β-D-galactose 4-sulfate taking into account the results obtained from the analytical assay[5] and the corresponding ^{13}C NMR spectra. However, it is also important to consider that, although the use of ^{1}H NMR spectroscopy is more sensitive than the ^{13}C NMR variant in detecting and quantitating minor contents of structural units, for carrageenans the badly defined and overlapping resonances, partly due to the broadness of the signals, do not allow assignments to be made for the diads present in these polysaccharides.

Noteworthy is the analogy in the data obtained by methylation analysis, ^{13}C and ^{1}H NMR spectroscopy (Table VI). All the results indicate that $1C_1$ and $1C_2$ are κ/ι-hybrids. Fraction $1C_2$ (mol wt 124 000) has a similar composition to that of $1C_1$ (mol wt 75 000), but it has a higher solubility in potassium chloride solutions despite its higher molecular weight. This behaviour could be due to a different distribution of kinks in the molecule.

The biosynthetic relationship between gelling and non-gelling carrageenans is evident from Tables VI and VII. The 3,6-anhydrogalactose 2-sulfate units present in $1C_1$ and $1C_2$ are quantitatively formed from the corresponding kinking units of $1C_3$. However, the 3,6-anhydrogalactose units, already found in $1C_3$ and selectively formed from a μ/ν-precursor, indicate that the transformation from a μ/ν- to a κ/ι-carrageenan involves not only a simple cyclization reaction, but also a sequence of steps directed by, at the moment, unknown specificities. Besides, the results show that the galactose 2,6-disulfate units do not give rise to 3,6-anhydrogalactose residues through a previous 2-*O*-desulfation[12].

The μ/ν-carrageenan $1C_3$ is contaminated with a carrageenan containing a partial λ-like structure. This result is in agreement with the fact that after alkaline

treatment and fractionation with potassium chloride, a small amount (6.3%) of a soluble product was obtained. Its methylation analysis indicated the presence of significant quantities of 2,6-di-*O*-methyl- (34.2%) and 4,6-di-*O*-methyl-D-galactoses (22.2%) suggesting a mixed (κ/ι-λ)-structure[2]. Alkaline treatment of $1C_1$ also showed a minor amount (2.9%) of a 2 M KCl-soluble carrageenan[1].

It has been previously reported that the sulfohydrolase which converts 4-linked galactose 6-sulfate and 2,6-disulfate units to the 3,6-anhydrogalactose residues is inhibited by λ-carrageenans, suggesting that sulfation on C-2 of the 3-linked units is deleterious to enzymic activity[13]. The presence of small quantities of diad Gal2, $4SO_3$–3,6-AnGal (2%) in $1C_1$ (Table VII), could indicate that sulfation on C-2 of this 3-linked unit does not inhibit the enzyme but only slows down the rate of the cyclization reaction.

ACKNOWLEDGMENTS

The authors are indebted to Lic. María Luz Piriz (Centro Nacional Patagónico, CONICET) for collecting and sorting the algal material and to UMYMFOR (FCEyN-CONICET) for technical assistance. This work was supported by grants from CONICET, The International Foundation for Science (Sweden), The Third World Academy of Sciences (Italy), and Sigma Xi (The Scientific Research Society). Thanks are due to the University of London Inter-Collegiate Research Services for providing NMR facilities.

REFERENCES

1 M.C. Matulewicz, M. Ciancia, M.D. Noseda, and A.S. Cerezo, *Phytochemistry*, 28 (1989) 2937–2941.
2 M.C. Matulewicz, M. Ciancia, M.D. Noseda, and A.S. Cerezo, *Phytochemistry*, 29 (1990) 3407–3410.
3 W. Yaphe and G.P. Arsenault, *Anal. Biochem.*, 13 (1965) 143–148.
4 J.T. Park and M.J. Johnson, *J. Biol. Chem.*, 181 (1949) 149–151.
5 D.A. Rees, *J. Chem. Soc.*, (1961) 5168–5171.
6 C.A. Stortz and A.S. Cerezo, *Carbohydr. Polym.*, 18 (1992) 237–242.
7 D. Welti, *J. Chem. Res. (M)*, (1977) 3566–3587.
8 S.H. Knutsen, D.E. Myslabodski, and H. Grasdalen, *Carbohydr. Res.*, 206 (1990) 367–372.
9 C.A. Stortz and A.S. Cerezo, *Carbohydr. Res.*, 145 (1986) 219–235.
10 A.I. Usov, *Bot. Mar.*, XXVII (1984) 189–202.
11 J.F.G. Vliegenthart, L. Dorland, and H. van Halbeek, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 209–374.
12 D.W. James, Jr., J. Preiss, and A.D. Elbein, in G.O. Aspinall (Ed.), *The Polysaccharides*, Vol. 3, Academic Press, Orlando, 1985, pp 107–207.
13 K.F. Wong and J.S. Craigie, *Plant Physiol.*, 61 (1978) 663–666.