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Agars from nine species of red seaweed in the genus *Curdiea* (Gracilariaceae, Rhodophyta)

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Abstract

Agars have been isolated from the nine currently recognised species of red seaweed in the genus *Curdiea*. Characterisation of their structures by chemical and spectroscopic methods showed all had a basic repeating structure of alternating 3-linked β-D-galactopyranosyl and 4-linked 3,6-anhydro-α-L-galactopyranosyl units but substituted with high levels of methyl ether groups. The native agars, isolated with hot aqueous buffer solution, had only weak gelling abilities owing to some of the 4-linked units being present as precursor α-L-galactopyranosyl-6-sulfate units. Conversion of these precursor units to the corresponding 3,6-anhydrides by treatment with hot alkali generally led to increased gel strength. Agars from *Curdiea angustata*, *C. codioides*, *C. crassa* and *C. flabellata* were predominantly methylated on position 6 of the 3-linked β-D-galactopyranosyl units, while agars from *C. irwinii*, *C.* sp. nov. (Three Kings), and *C. racovitzae* were almost completely methylated on position 2 of the 4-linked 3,6-anhydro-α-L-galactopyranosyl units. The agars from *C. coriacea* and *C. obesa*, however, were nearly completely methylated at both these positions. The alkali-modified agars from these latter two algae had gel-melting temperatures significantly above the boiling point of water. Small amounts of unusual 4-*O*-methylxylopyranosyl branching units were detected in the agars from *C. irwinii* and *C. obesa*.

Keywords: Agar; Methylation; *Curdiea*; Gel properties

1. Introduction

Agars, the gel-forming polysaccharides isolated from certain red seaweeds, are linear polymers based on a disaccharide repeat structure of alternating 3-linked β-D-galactopyranosyl and 4-linked 3,6-anhydro-α-L-galactopyranosyl units. Using the nomenclature of Knutsen et al. [1], these are referred to as G and LA units, respectively (Fig. 1A). Each idealised disaccharide repeat structure has three hydroxyl groups on the 3-linked β-D-galactopyranosyl unit, at positions identified herein as G-2, G-4 and G-6, and one on the 4-linked 3,6-anhydro-α-L-galactopyranosyl unit, at LA-2. Certain of these can be substituted by various *O*-linked groups, particularly methyl ether, sulfate ester, pyruvate acetal or glycosyl units such as xylopyranosyl (X). The common positions of methyl ether substitution are shown in Fig. 1. Some of the 4-linked units in native agars (i.e., as they exist in seaweeds) can occur in the form of 6-sulfated α-L-galactopyr-

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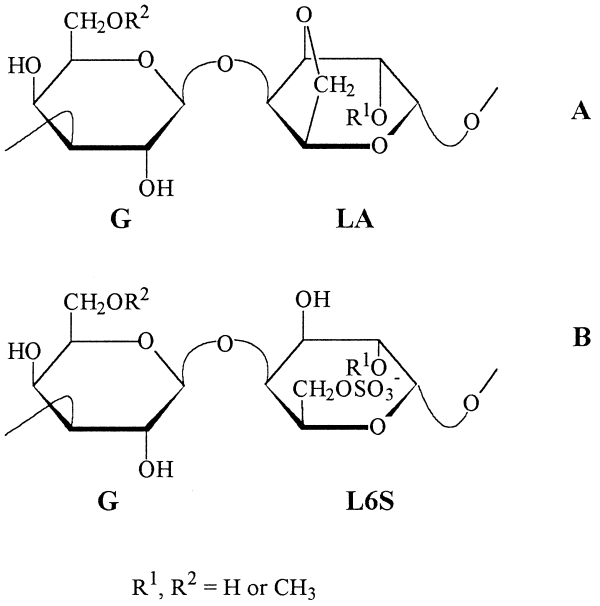

Fig. 1. Structures of idealised agar (A) and agar precursor (B) with common positions of methyl ether substitution.

anosyl (L6S) units (L2M,6S when 2-*O*-methylated) instead of the corresponding 3,6-anhydride (Fig. 1B). These 6-sulfated units are often referred to as "precursors" because they are considered the biosynthetic precursors of the LA units and because the same conversion, involving intramolecular displacement of the 6-sulfate group by O-3, can also be effected by treatment with hot aqueous alkali [2]. The presence of too many (methylated) precursor units in agar molecules hinders the molecular association responsible for gelation, so where required an alkaline treatment can be used to improve gel strength.

Species from the genus *Curdiea* (which is in the family Gracilariaceae) have been reported only from the temperate or subantarctic waters of Australasia and Antarctica. Previous structural characterisation of agars prepared from two New Zealand *Curdiea* species (*C. coriacea* and *C. flabellata*) revealed high levels of methyl ether substituents [3]. The native agars also contained high levels of precursor units in common with many *Gracilaria* species. In the case of *C. coriacea*, alkaline treatment yielded a gel-forming agar in which both positions G-6 and LA-2 were essentially completely methylated. Agars from *Gracilaria* species frequently have methylation at G-6, at levels up to around 50%, and occasionally have significant levels at LA-2 [4] or both G-6 and LA-2 [5]. Agars from *Pterocladia* and *Gelidium* species, by comparison, have relatively little methylation [6].

Recently, a highly methylated agar very similar to that of *C. coriacea* has been reported as one component of the polysaccharide from *Gracilaria eucheumoides* [7]. Both Takano et al. [7] and Minghou et al. [4] independently examined agars extracted from seaweed samples identified as *G. eucheumoides* collected in Japan and China, respectively, but reported markedly different chemical structures. The latter workers reported a high level of methylation on position LA-2 but not on position G-6 in the agar from their sample (results which we have independently confirmed [8]). Neither publication, however, permits unambiguous determination of the species.

Methyl ether substituents are known to influence the properties of agar gels. Natural methylation can raise both the gel-setting and gel-melting temperatures, whilst synthetic introduction of methyl groups has been shown to lower the former [9,10]. *C. coriacea* agar gives a particularly high-melting gel, a 1% w/v gel having a melting point above 100 °C compared to 90–95 °C for an unsubstituted agar. An equivalent observation was made for a similarly highly methylated agar from Japanese *G. eucheumoides* [7].

The unusual thermostability of the *C. coriacea* agar gel led us to investigate other *Curdiea* species to determine whether they contain similar materials. Another New Zealand *Curdiea* species, the rather rare, turf-like *C. codioides* was examined in 1994. It too contained a highly methylated agar yet methylation was exclusively on position G-6 [10]. We now report a comparative study of the isolation, chemical structure, and gelling properties of all nine *Curdiea* species that can be identified with certainty at this time.

2. Experimental

Materials.—Algal samples were air-dried after collection. Voucher specimens of the following algal materials used in this study have been deposited in the herbarium of the Museum of New Zealand (WELT): *C. obesa*, North side of Rottnest Island, Western Australia, November 1992, WELT A21180; *C. codioides*, Lottin Point, East Cape, New Zealand, June 1997, WELT A21958; *C. flabellata*, Patti Point, Timaru, New Zealand, October 1993, WELT A21183; *C. crassa*, Bongin

Bongin Bay, North Sydney, NSW, Australia, March 1994, WELT A21184; *C. angustata*, Port MacDonnell, South Australia, WELT A21179; *C. racovitzae*, King George Island, Antarctica, January 1986, WELT A21185; *C.* sp. nov. (Three Kings) Great Island, Three Kings Island, New Zealand, March 1997, WELT A21970 (for which the name *C. balthazor* is to be proposed); and *C. coriacea*, Northland, New Zealand, 1994, WELT A21186. A voucher specimen of the following algal materials has been deposited in the herbarium of the University of Melbourne (MELU): *C. irwinii*, Avalon Beach, Miami, Western Australia, March 1978, MELU K6561. A sample of agarose prepared from *Pterocladia lucida* was a gift from Dr I.J. Miller, Carina Chemical Laboratories Ltd, Lower Hutt, New Zealand.

General method for isolation of polysaccharide directly from seaweed.—A sample of air-dried weed (5 g) was cut into pieces and soaked in sodium phosphate buffer (25 mM, pH 6.8, 175 mL) overnight. The liquid was discarded and replaced with fresh buffer, then the mixture was transferred to a pressure bomb with a glass liner. An insert tube in the lid extended below the surface of the liquid allowing direct measurement, under pressure, of the solution temperature with a calibrated thermocouple. The bomb was sealed, placed in a contact-thermometer controlled oil bath heated to 140 °C and allowed to warm up to 110–120 °C. Timing was started, and the extraction was allowed to proceed for 90 min. During this time, the temperature was allowed to rise to at least 137 °C. After cooling to 90–95 °C, the bomb was opened and the liner removed. The extract was filtered hot through a Whatman GF/C (1.2 mm) filter under pressure. The weed debris and filter were removed, re-extracted for 30 min in fresh buffer (80 mL) and re-filtered. The combined extracts were allowed to cool. If they gave a reasonably firm gel, the extracts were freeze-thawed. The matrix recovered from the freeze-thawed gel was allowed to stand in excess distilled water, freeze-thawed again and dried in the oven at 60 °C. In the case of *C. flabellata*, the combined extracts did not gel, so they were dialysed against distilled water (10 vol.) in tubing with a nominal 10–12 kDa molecular weight cut-off, then lyophilised.

General method for alkali pre-treatment of seaweed.—A sample of air-dried weed (5 g) was cut into pieces and soaked in $NaOH/NaBH_4$ (4%/0.4% w/v, 40 mL) at room temperature for 2 h. The solution was decanted off, and the container of weed were immersed in a water bath at 80 °C for 3 h. The weed was then washed with flowing tap water, added to aqueous NaH_2PO_4 (7.5% w/v, 40 mL), and left to stand for 20 min. The weed was washed again with tap water for 30 min and immersed in sodium phosphate buffer (25 mM, pH 6.8, 175 mL). The pH was checked after 1 h and adjusted back to 6.8 with dilute HCl. The weed was then soaked overnight and extracted as described above for native weed.

Alkali treatment of native agar from C. racovitzae.—Sodium hydroxide (4 g) and sodium borohydride (0.4 g) were dissolved in water (50 mL). A sample of native agar from *C. racovitzae* (0.32 g) was dissolved in water (50 mL), and the two solutions were mixed and heated in an oil bath for 3 h at 80 °C. After cooling, the mixture was neutralised with glacial acetic acid, dialysed and lyophilised to give 0.24 g (75% recovery by weight) of alkali-treated agar.

Gel strength measurements.—Dried agar (1 g) was placed in a 250-mL beaker and dissolved in distilled water (100 mL) by pressure cooking for 15 min. The *C. obesa* agar did not dissolve under these conditions, so it was heated in the bomb at 135 °C for 15 min. The weight of the solution was then adjusted to 100 g with additional water. After stirring the solution to ensure that it was homogeneous, it was left at room temperature overnight to gel. The beaker was then placed on a top-pan balance, a circular stainless steel rod (1 cm^2 cross-sectional area) was pressed by hand into the gel until it collapsed, and the maximum balance reading was noted. The calculated gel strength was an average of four determinations on the same sample.

Gel-melting and -setting temperature measurements.—Agar (30 mg), distilled water (3 mL) and a small piece of lead as a weight were placed in a screw-capped culture tube (13×100 mm) and pressure-cooked for 20 min (twice if not properly dissolved). After shaking, the solution was allowed to set overnight in a vertical position. A 5-mm glass bead was then added, and the tube was suspended in a glycol bath heated on a hotplate, with the temperature being increased at approximately 2 °C/min. The gel-melting range was determined as the temperatures of the glycol bath between which the bead started to sink and reached the bottom of the tube. The heating was then stopped, and the tube was allowed to cool. Every minute the tube was removed briefly from the bath and turned horizontally. The gel-setting range was determined as

the temperatures between which the solution showed signs of gelling and at which it would no longer flow. All determinations were performed in duplicate.

Constituent sugar analysis of polysaccharides.—The reductive hydrolysis method of Stevenson and Furneaux [8] was used to convert the constituent sugars in the agar samples into alditol acetate derivatives. This method utilises in situ reduction with *N*-methylmorpholine·borane during hydrolysis to prevent degradation of 3,6-anhydrogalactose units. These derivatives were then analysed by GLC according to the methods of Falshaw and Furneaux [11], in which quantitation is based on experimentally determined response factors. The location of the methyl group on xylopyranosyl units in *C. irwinii* and *C. obesa* agars was determined by hydrolysis (2 M TFA, 1 h, 120 °C), reduction ($NaBD_4$) and acetylation (TFA/Ac_2O, 1:1 v/v, 10 min, 50 °C) of samples [8], and analysis of the resulting C-1-deuterated alditol acetate derivatives by GLC–EIMS according to the method of Falshaw and Furneaux [11]. The position of the methylated xylopyranosyl units on the galactan backbone of *C. irwinii* agar was determined by trideuteromethylation of the polysaccharide [8] prior to reductive hydrolysis. The location of the methyl group on the xylopyranosyl units was also confirmed by this technique.

^{13}C NMR spectroscopy.—Spectra were recorded on 3% w/v solutions in 1:1 D_2O–H_2O at 90 °C on a Varian Unity-500 spectrometer at a carbon frequency of 125 MHz, using a 10 mm broad-band probe, acquisition time 1.17 s, delay 0.8 s, and an 80° pulse. For each spectrum, 3600 transients were collected and a 5 Hz exponential line broadening was applied. Chemical shifts are quoted relative to internal Me_2SO at 39.47 ppm.

3. Results and discussion

Production of agar samples.—The structures and certain properties of the polysaccharides from *C. coriacea*, *C. flabellata*, and *C. codioides* have been reported previously [3,10]. The conditions used for extraction and/or analysis of these samples were slightly different, however, so for consistency, they were re-extracted and analysed under the same conditions as the other six *Curdiea* species examined here for the first time.

A sample of each species was extracted in buffered phosphate solution, and the native polysaccharide (N) was recovered by freeze-thawing if possible, otherwise by dialysis and lyophilisation. The yields obtained are shown in Table 1. Whilst most species gave a native extract that gelled, the gel strengths were too weak to warrant measurement.

For eight species, enough seaweed was available for samples of alkali-modified agar (A) to be prepared by pre-treatment of the seaweed with alkali prior to extraction. In the case of *C. racovitzae*, a sample of alkali-modified agar (AP) was prepared by treatment of a portion of the native polysaccharide with alkali.

Methyl ether substitution of Curdiea *agars*.—Constituent sugar analyses for the agar polysaccharides from all the Curdiea species examined are shown in Table 1. Of most interest is the fact that all the agars were highly substituted with methyl ether groups. Three general substitution patterns were observed, reflecting the sites of methylation in the various species. The agars from *C. angustata*, *C. codioides*, *C. crassa*, and *C. flabellata* were all highly methylated on G-6 (> 60%), while complete methylation on LA-2 was observed for *C.* sp. nov. (Three Kings) and to a slightly lesser extent for *C. irwinii* and *C. racovitzae*. *C. coriacea* and *C. obesa* agars, however, exhibited almost complete methylation on *both* G-6 and LA-2 positions. High levels of methylation have also been observed in gel-forming agars from a few red algal species, for example a highly 6-*O*-methylated agar from *Euptilota formosissima* (in the Order Ceramiales) [12], a 2-*O*-methylated agar from a Chinese sample of *G. eucheumoides* [4,8], and an agar methylated on both G-6 and LA-2 positions from a Japanese sample of *G. eucheumoides* [7]. In general, however, levels of methylation in agars are much lower. Agars from *Gelidium* and *Pterocladia* species (as well as a few *Gracilaria* species) have less than 10% methylation on any position. Typical *Gracilaria* agars have between 10 and 50% overall methylation, predominantly on G-6, and little on LA-2. High levels of methylation are not unique to *Curdiea* species, but the presence of substantial methylation (at whatever position) can be used as a taxonomic marker for *Curdiea* species in combination with other characteristic features such as a lack of sterile, thick-walled gonimoblast tissue and the presence of tetrasporangial and spermatangial nemathecia [13]. This has already led to the reassessment of the identity of one mislabelled sample.

Table 1
Yields and constituent sugar analyses of agars isolated from *Curdiea* species

Species	Sample[a]	%Yield	Constituent sugar[b] (normalised mol%)								Estimated % precursors	
			2-Me-AnGal	AnGal	6-Me-Gal	2-Me-Gal	Gal	4-Me-Xyl	Xyl	Glc	ΔGal[c]	ΔAnGal[d]
C. angustata	N	47	Tr	39	48	Tr	11	0	0	2	6	6
	A	36	2	41	49	0	5	0	1	2		
C. codioides	N	20	0	37	40	0	16	0	4	3	5	5
	A	45	Tr	44	41	Tr	10	0	2	3		
C. crassa	N	32	0	36	46	0	12	0	0	6	6	8
	A	25	1	44	47	0	3	0	0	5		
C. flabellata	N	18	0	19	23	Tr	13	0	0	45	3	11
	A	11	3	26	23	0	14	0	3	31		
	E	54	4	37	32	0	19	0	4	4		
C. irwinii	N	16	44	0	0	5	40	2	1	8	5	6
	A	19	49	1	0	1	39	4	1	5		
C. sp. nov. (Three Kings)	N	17	43	—	—	9	42	—	—	6	9	9
	A	19	46	—	—	1	39	—	—	14		
C. racovitzae	N	16	42	1	3	11	40	0	1	2	8	7
	AP	75	50	1	4	1	42	0	1	1		
C. coriacea	N	17	47	1	45	5	1	0	0	1	0	1
	A	29	48	1	43	2	2	0	0	4		
C. obesa	N	30	48	Tr	39	4	4	1	2	2	3	0
	A	31	47	Tr	42	1	4	1	2	3		

[a]N = native agar, isolated directly from seaweed; A = alkali-modified agar, isolated from alkali-pretreated seaweed; E = agar recovered from alkali-modified agar after treatment with amyloglucosidase; AP = agar recovered after treatment of the native agar with alkali.
[b]AnGal determined as 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol, Gal as galactitol hexaacetate, etc.
[c]{Gal(N) + 2-Me-Gal(N)} − {Gal(A) + 2-Me-Gal(A)} on data normalised without (substituted) xylose and glucose.
[d]{AnGal(A) + 2-Me-AnGal(A)} − {AnGal(N) + 2-Me-AnGal(N)} on data normalised without (substituted) xylose and glucose.

The propensity for methylation in *Curdiea* agars extends to the xylopyranosyl units found in *C. irwinii* agar and, to a lesser extent, *C. obesa* agar. The site of methylation was determined to be the 4-position by GLC–EIMS of C-1 deuterium-labelled alditol acetates. A sample of *C. irwinii* agar was also trideuteromethylated prior to the preparation of alditol acetates. GC–EIMS analysis revealed the presence of terminal xylopyranosyl units (as the chromatographically indistinguishable 1,5-di-*O*-acetyl-2,3,4-tri-*O*-trideuteromethylxylitol and 1,5-di-*O*-acetyl-4-*O*-methyl-2,3-di-*O*-trideuteromethylxylitol) and 3,6-Gal (as 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-trideuteromethylgalactitol) in similar molar proportions. The relative proportions of 4-*O*-methyl xylopyranosyl and xylopyranosyl units were calculated from the ratio of the intensities of the ion pairs *m*/*z* 104:107, *m*/*z* 117:120, and *m*/*z* 164:167, and was consistent with that determined by constituent sugar analysis. The presence of 3,6-Gal indicated that these terminal (4-*O*-methyl)xylopyranosyl units occur as single branches off the main agar chain at the G-6 position.

Starch.—All the polysaccharide extracts studied contained some glucose. Neither the native nor alkali-modified *C. flabellata* extracts formed firm gels, and so were recovered by dialysis and lyophilisation. They had particularly high levels of glucose, representing nearly half of the total sugar content of the native extract. A significant level of floridean starch had been identified previously in the extract from another sample of *C. flabellata* from the South Island of New Zealand, and this had been effectively removed by treatment with amyloglucosidase followed by dialysis [3]. Amyloglucosidase treatment of the current extract from an alkali-pretreated *C. flabellata* sample similarly reduced the level of glucose in the recovered agar, from 31% to 4%, confirming that the majority of the glucose residues are from starch. The recovered *C. flabellata* agar (labelled E in Table 1) gelled only very weakly. It is possible that the agar polymers in

this alga are of relatively low molecular weight as the agar from alkali-modified *C.* sp. nov. (Three Kings) gelled well in the presence of a significant amount of floridean starch (Tables 1 and 3).

Precursor residues.—In an idealised agar structure, the total number of 4-linked 3,6-anhydrogalactopyranosyl units (determined by constituent sugar analysis as the alditol acetate species AnGal + 2-Me-AnGal) should equal the total number of 3-linked galactopyranosyl units (determined as Gal + 6-Me-Gal). A lower level of total 3,6-anhydrogalactopyranosyl units may indicate the presence of precursor (L6S/L2M,6S) units. Constituent sugar analysis would generate the alditol acetate species Gal and 2-Me-Gal from L6S and L2M,6S units, respectively. The level of these precursor units in the native agars can be estimated from either the decrease in the level of the alditol acetate species Gal and/or 2-Me-Gal (ΔGal) or the increase in AnGal and/or 2-Me-AnGal (ΔAnGal) values in the constituent sugar analyses in going from native to alkali-modified agars once normalised to exclude the variations in level of other sugars [14]. From these values it can be judged that levels of precursor (L6S/L2M,6S) units ranged up to 11 mol% (Table 1).

^{13}C NMR spectroscopy.—^{13}C NMR spectroscopy of hot aqueous solutions of alkali-treated agar samples enabled the major structures identified from constituent sugar analysis to be confirmed by

Table 2
^{13}C NMR spectral assignments (δ, ppm, referenced to internal Me_2SO at 39.47 ppm)

Species	Unit[a]	Carbon shifts						
		1	2	3	4	5	6	O–CH_3
C. angustata	**G6M**	102.3	70.1	82.1	69.0	73.5	71.7	59.0
	G						61.5	
	LA	98.2	69.8	80.1	77.3	75.5	69.3	
	LA2M	98.7	78.8	78.5				
C. codioides	**G6M**	102.3	70.1	82.1	68.9	73.5	71.7	59.0
	G						61.5	
	LA	98.2	69.9	80.1	77.3	75.6	69.3	
C. crassa	**G6M**	102.3	70.1	82.1	69.0	73.5	71.7	59.0
	LA	98.2	69.9	80.1	77.3	75.6	69.3	
	LA2M	98.7	78.8	78.5				
	FS	100.1	72.2	73.8			61.2	
C. flabellata	**G6M**	102.3	70.1	82.1	68.9	73.5	71.7	59.0
	G			82.5[b]			61.6	
	LA	98.2	69.8	80.1	77.3	75.5	69.3	
	LA2M	98.6	78.8	78.5	77.6			
	X	103.8		76.3		65.6		
C. irwinii	**G(LA2M)**	102.6	70.1	82.7	68.7	75.2	61.3	
	LA2M	98.7	78.8	78.5	77.5	75.7	69.4	59.1
	FS	100.1	72.2	73.8		71.8		
	X	103.8		76.3	69.7	65.6		
	X4M	103.6	73.5	75.0	79.2	63.1		58.3
C. sp. nov. (Three Kings)	**G(LA2M)**	102.6	70.2	82.7	68.8	75.2	61.3	
	LA2M	98.6	78.8	78.5	77.5	75.7	69.4	59.1
	FS	100.0	72.1	73.8		71.8		
C. racovitzae	**G(LA2M)**	102.6	70.1	82.6	68.7	75.2	61.3	
	G6M					73.5	71.7	
	LA2M	98.6	78.8	78.4	77.5	75.7	69.4	59.1
C. coriacea	**G6M(LA2M)**	102.6	70.1	82.6	69.1	73.5	71.8	58.9
	LA2M	98.6	78.8	78.5	77.6	75.7	69.4	59.0
C. obesa	**G6M(LA2M)**	102.6	70.1	82.6	69.1	73.5	71.8	58.9
	LA2M	98.6	78.8	78.5	77.6	75.7	69.4	59.0
	X	103.8		76.3	69.7	65.6		
	X4M	103.6		75.0	79.2	63.1		

[a]Units for which major signals were observed are shown in bold.
[b]Shifts for C-3 of a 3-linked unit (G or G6M) adjacent to an LA2M unit.

Table 3
Comparison of gelling characteristics of alkali-modified agars from *Curdiea* species with their methylation patterns

Species	Sample[a]	Gel strength (g/cm^2 at 1% w/v)	Gel-melting range (°C)	Gel-setting range (°C)	% Methylation[b] on G-6	LA-2
C. angustata	A	370	93.5–94.5	46–42	91	5
C. codioides	A	315	92–92.5	48–46	80	0
C. crassa	A	1560	101–102.5	49–46	94	2
C. flabellata	E	Non-gelling	—	—	62	10
C. sp. nov. (Three Kings)	A	920	97.5–98.5	35–34	0	100
C. irwinii	A	340	90–91	37–34	0	98
C. racovitzae	AP	70	88–90	35–33	9	98
C. coriacea	A	560	112–113	44.5–42	96	98
C. obesa	A	1100	120–121	48–46	91	100

[a]For codes A, N, AP, and E see footnotes to Table 1.
[b]% G-6 methylation determined as 6-Me-Gal/{6-Me-Gal + Gal}; % LA-2 methylation determined as 2-Me-AnGal/{2-Me-AnGal + AnGal}, as in Table 1.

comparison of the chemical shifts observed (Table 2) with literature values for agars methylated at G-6, LA-2 [4], and both G-6 and LA-2 [3]. The nomenclature for ^{13}C NMR assignments used here is an abbreviated version of that proposed by Knutsen et al. [1], i.e., C-3 in a 6-*O*-methyl-β-D-galactopyranosyl unit is abbreviated to G6M-3. When in a single spectrum there are two chemical shift values for a given carbon atom, owing to the differing nature of the adjacent units, the adjacent units are indicated, e.g., G6M(LA) or G6M(LA2M) (Table 2). Strictly, a triad should be shown, e.g., (LA)G6M(LA), but for simplicity, and because it is unclear whether it is the unit at the reducing end or the non-reducing end of the chain that controls the chemical shift, just diads are shown.

The spectra produced were well-resolved, and signals corresponding to minor components were observed in certain cases (Table 2). For, example, signals corresponding to floridean starch (FS) were observed in the spectra from *C. crassa*, *C. irwinii*, and *C.* sp. nov. (Three Kings) agars, and for 6-*O*-methylation in *C. racovitzae* agar. The ^{13}C NMR spectra of *C. flabellata*, *C. irwinii*, and *C. obesa* agars all contained signals at 103.8, 76.3, and 65.6 ppm corresponding to X-1, X-3, and X-5 of single xylopyranosyl branch units [3], and an unassigned signal at 74.0 ppm. This is approximately 0.8 ppm higher than that expected for X-2 [15,16]. The *C. irwinii* and *C. obesa* agar spectra also contained a minor signal at 69.7 ppm corresponding to X-4. The presence of this signal would be obscured in the *C. flabellata* agar spectrum by larger resonances. Further signals were observed in the spectra of both *C. irwinii* and *C. obesa* agars at 103.6, 79.2, 75.0, and 63.1 ppm. These were all more intense in the *C. irwinii* spectrum, suggesting that they result from 4-*O*-methylated xylopyranosyl (X4M) units. In the xylan from *Leptosarca simplex* [17], glycosylation of xylopyranose at *O*-4 strongly deshields C-4, but somewhat shields C-3 and C-5 relative to the equivalent carbons in a single branch unit while having little effect on C-1 and C-2. 4-*O*-Methylation would be expected to have a similar effect to glycosylation, and so the signals are, therefore, assigned to X4M-1, X4M-4, X4M-3 and X4M-5, respectively, as shown in Table 2. The signal at 73.5 ppm in the spectrum of *C. irwinii* agar is assigned to X4M-2 (cf. 73.3 ppm for C-2 of 4-linked units in the xylan from *Leptosarca simplex*). The presence of this signal in the *C. obesa* agar spectrum would be obscured by the G6M(LA2M)-5 resonance. The *C. irwinii* agar spectrum also contained a signal at 58.3 ppm probably corresponding to the O-CH_3 of 4-*O*-methylated xylopyranosyl units. Further signals at 102.4 and 69.0 ppm may be due to carbons-1 and -6 of a G unit substituted with a xylopyranosyl unit (i.e., G6X), by analogy with the shifts observed for C-1 and C-6 of a β-D-galactoside on 6-*O*-β-D-galactosylation [18].

The ^{13}C NMR spectra of all the 6-*O*-methylated agars contained a small unidentified signal at 76.4 ppm. In addition, other small signals were observed in various spectra: 103.1 ppm (*C. codioides*, *C. irwinii* and *C. flabellata* agars), 109.1 and 80.4 ppm (*C. flabellata* and *C. coriacea* agars), 103.9 and 101.4 ppm (*C. angustata* agar), 74.1 ppm (*C. codioides* agar), 70.4 and 73.9 ppm (*C. flabellata* agar), 100.3 ppm [*C.* sp. nov. (Three Kings) agar], 68.2 ppm [*C. irwinii*, *C.* sp. nov. (Three Kings), and *C. coriacea* agars], and 67.4 ppm [*C. angustata*, *C.*

irwinii, *C.* sp. nov. (Three Kings), and *C. coriacea* agars]; the latter two having been observed previously [3].

Gel strengths.—Treatment of the seaweeds with hot alkali prior to extraction had a considerable positive effect on the gel strengths of the agars produced. Gels made from 1% w/v concentrations of the alkali-modified *Curdiea* agars were, with the exception of *C. flabellata*, all of sufficient strength for gel-strength measurements to be made, and values ranged from a rather modest 70 g/cm^2 for *C. racovitzae* to a very strong 1560 g/cm^2 for *C. crassa* (Table 3). The presence of precursor units in the corresponding native agars (see above) significantly weakened their ability to form gels, a common observation for agars and carrageenans generally.

Gel-melting temperatures.—Given the known effects of methylation on gel-melting temperature (see Introduction) some correlations may be expected between the gel-melting temperature and the extent and type of methylation for the alkali-modified agars studied here. The alkali-modified agars prepared from *C. irwinii* and *C. racovitzae* melt in the range 88–91 °C (Table 3), while an essentially unmethylated agar from *Pterocladia lucida* tested for comparison has a gel-melting temperature of 92–93 °C. This might suggest that methylation on position LA-2 alone has a slightly negative, if any, impact on gel-melting temperature. However, both these agars came from specimens over 10 years old, and the 2-*O*-methylated agar from a recently collected specimen of *C.* sp. nov. (Three Kings), does have a melting point higher than that of *Pterocladia lucida* agar, which suggests, not surprisingly, that other factors such as molecular weight can also influence gelling properties. Of the four agars methylated predominantly on G-6, gel-melting temperatures were obtained for only three samples due to the non-gelling nature of *C. flabellata* agar. The melting temperature of the agar from *C. crassa* was higher (101–102 °C) than that of *P. lucida*, but that from *C. angustata* was only slightly higher (about 94 °C) and that for *C. codioides* was not significantly different. A high level of methylation on position G-6 alone or on LA-2 alone thus appears to raise the gel-melting temperature to near the boiling point of water only if the agar has a high gel strength.

It is the gels from the agars of *C. coriacea* and *C. obesa* that are methylated on both G-6 and LA-2 positions, however, that have dramatically higher melting temperatures (about 121 and 113 °C, respectively). A high melting point (121 °C) was also observed by Takano et al. for the gel made from an agar fraction of similar structure obtained from a Japanese sample of *G. eucheumoides* [7]. Thus, the presence of high levels of methyl ether substituents on both G-6 and LA-2 appears to be required to obtain gels that are stable significantly above the boiling point of water.

It is generally accepted that agar gelation involves two molecules pairing to form double helices, then segments of a number of these double helices clustering in junction zones. The junction zones will be held together by a mixture of hydrogen bonding and hydrophobic interactions. Modelling of the double helix [19] revealed that the G-2 hydroxy groups are on the inside of the double helix and involved in a hydrogen-bonding network with encased water molecules. The other hydroxy groups, at positions G-4, G-6, and LA-2, are on the outside of the double helix. In the agars from *C. coriacea* and *C. obesa*, those at G-6 and LA-2 are replaced with lipophilic methoxy groups, leaving only one exterior hydroxy group per disaccharide repeat unit. This dramatically increases the hydrophobic character of the outside surfaces of the double helices, which must interact to form junction zones. It seems probable then that gels of agars with two methyl groups per disaccharide repeat unit have higher melting temperatures than those of less substituted agars because of increased hydrophobic interactions in the junction zones, with a resulting overall increase in thermodynamic stability.

Gel-setting temperatures.—Evidence in the literature for the influence of naturally occurring methyl ether substituents on gel-setting temperatures is less clear. In a detailed study, Guiseley found a correlation between increasing natural methoxy content in alkali-modified *Gracilaria* agars (which can be presumed to be due largely to methylation on G-6) and increasing gel setting temperature [9]. He observed some anomalies which may have been due to agars methylated on other positions. At odds with this correlation, however, is the observation that the gel-setting temperature of alkali-modified porphyran, an agarose in which G-6 is about 50% methylated, was reported not to differ from that of unsubstituted agarose [20]. Chemical methylation, by contrast, lowers the gel-setting temperature, and we have correlated this with the introduction of G-2 methoxy groups (not found naturally in agars),

which are less easily accommodated inside the double helix and hence destabilise it [10]. In this study, we find that agars highly methylated at LA-2 have gel-setting temperatures (33–37 °C) similar to that of unsubstituted *P. lucida* agar (34–38 °C), but that agars highly methylated at G-6 have much higher gel-setting temperatures (by about 10 °C). Surprisingly perhaps, the introduction of a second methyl ether group at LA-2 has little impact, as agars highly methylated at both LA-2 and G-6 melt in the same range (42–49 °C) as those which are only G-6 methylated (Table 3).

4. Conclusions

The nine currently recognised species of *Curdiea* red seaweeds all contain significantly methylated agars. Methylation can occur extensively at position G-6 alone, at position LA-2 alone, or at both positions, and examples of agars of each type having strong gel-forming ability have been encountered. Unusual 4-*O*-methylxylopyranosyl units were detected in agars from two of the species examined. The extent and position of methylation can affect the gelling/melting temperature of the agar gels. Most notably, it is confirmed that the gel-melting point is very significantly raised only by the "double methylation" pattern. Thermostable agar gels provide an interesting test case in the examination of gelation mechanisms, and an opportunity for the discovery of new biotechnological and food products.

While potential uses for a thermostable agar/agarose can be envisaged, the lack of sufficient seaweed suitable for industrial processing remains an unresolved problem. A survey of *C. coriacea* in the far North of New Zealand has identified only a modest standing stock and it does not appear likely that the situation will be much different for *C. obesa* in Western Australia. Attempts to cultivate *C. coriacea* have been unsuccessful so far, but this appears to be the best way forward.

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References

[1] S.H. Knutsen, D.E. Myslabodski, B. Larsen, and A.I. Usov, *Bot. Marina*, 37 (1994) 163–169.
[2] J.A. Hemmingson, R.H. Furneaux, and H. Wong, *Carbohydr. Res.*, 296 (1996) 285–292.
[3] R.H. Furneaux, I.J. Miller, and T.T. Stevenson, *Hydrobiologia*, 204/205 (1990) 645–654.
[4] J. Minghou, M. Lahaye, and W. Yaphe, *Bot. Marina*, 28 (1985) 521–528.
[5] R. Falshaw, R.H. Furneaux, T.D. Pickering, and D.E. Stevenson, *Bot. Marina*, (1998) submitted for publication.
[6] W.A. Nelson, G.A. Knight, R. Falshaw, R.H. Furneaux, A. Falshaw, and S.M. Lynds, *J. Appl. Phycology*, 6 (1994) 497–507.
[7] R. Takano, K. Hayashi, and S. Hara, *Phytochemistry*, 40 (1995) 487–490.
[8] T.T. Stevenson and R.H. Furneaux, *Carbohydr. Res.*, 210 (1992) 277–298.
[9] K.B. Guiseley, *Carbohydr. Res.*, 13 (1970) 247–256.
[10] I.J. Miller, R. Falshaw, and R.H. Furneaux, *Carbohydr. Res.*, 262 (1994) 127–135.
[11] R. Falshaw and R.H. Furneaux, *Carbohydr. Res.*, 252 (1994) 171–182.
[12] I.J. Miller and R.H. Furneaux, *Bot. Marina*, 40 (1997) 333–339.
[13] W.A. Nelson and G.A. Knight, *New Zealand J. Bot.*, 35 (1997) 195–202.
[14] J.A. Hemmingson, R.H. Furneaux, and V.H. Murray-Brown, *Carbohydr. Res.*, 287 (1996) 101–115.
[15] M.I. Errea and M.C. Matulewicz, *Phytochemistry*, 42 (1996) 1071–1073.
[16] K. Bock, C. Pedersen, and H. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 42 (1984) 193–225.
[17] N.M. Adams, R.H. Furneaux, I.J. Miller, and L.A. Whitehouse, *Bot. Marina*, 31 (1988) 9–14.
[18] D.E. Stevenson, A.D. Woolhouse, R.H. Furneaux, D. Batcheler, and C.T. Eason, *Carbohydr. Res.*, 256 (1994) 185–188.
[19] S. Arnott, H. Fulmer, W.E. Scott, I.C.M. Dea, R. Moorhouse, and D.A. Rees, *J. Mol. Biol.*, 90 (1974) 269–284.
[20] W. Yaphe and M. Duckworth, *Proc. Int. Seaweed Symp.*, 7 (1971) 15–22.