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Determination of the configuration of 3,6-anhydrogalactose and cyclizable α-galactose 6-sulfate units in red seaweed galactans

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Abstract

A combination of two reported procedures was used in order to determine the configuration of the 3,6-anhydrogalactose present in red seaweed polysaccharides. A mild hydrolysis (to cleave only 3,6-anhydrogalactosyl linkages) was followed by a reductive amination with a chiral amine. Then, the total hydrolysis proceeded, followed by a new step of reductive amination. In this way, using (*S*)-α-methylbenzylamine as the chiral amine, it was possible to separate and quantitate both enantiomers of 3,6-AnGal and its 2-*O*-methyl ether as their diastereomeric acetylated aminoalditols. On the other hand, using (*S*)-1-amino-2-propanol, even though the derivatives of both enantiomers of 3,6-AnGal are not separated, the mixture can be safely quantitated with respect to galactose. Furthermore, a one-pot technique was developed to carry out an alkaline treatment of the polysaccharides, followed by the double hydrolysis–reductive amination procedure, which is useful to determine the proportions of both enantiomers of 6-sulfated 4-linked galactose units in the native polysaccharides. The unexpected presence of small amounts of units of this type belonging to the D-series in a porphyran sample is revealed by this novel procedure.

Keywords: Carrageenans; Agarans; 3,6-Anhydrogalactose; Seaweed galactans; Enantiomers

1. Introduction

Red seaweed galactans have structures that are generally based on linear chains of alternating 3-linked β-galactopyranosyl and 4-linked α-galactopyranosyl residues.[1,2] While the former residues always belong to the D-series, the latter may include residues of the D-configuration (in carrageenans) or the L-configuration (in agars), many times occurring totally or partially as 3,6-anhydrogalactopyranosyl moieties.[1,2] In addition, several different substituents may occur (methoxyl, sulfate, pyruvate), and natural galactans are usually molecular hybrids containing differently substituted repeating units,[1] or even α-galactose units with the D- and L-configuration interspersed in the same molecules (D/L-hybrids).[2]

Compositional analysis of red seaweed galactans is complicated by the degradation of 3,6-anhydrogalactose occurring under the strong acid conditions needed for the total hydrolysis of the polymers.[1] This problem has been circumvented by the use of a double-hydrolysis procedure, in which a mild hydrolysis, used to cleave only the 3,6-anhydrogalactosyl linkages is followed by a reduction with sodium borohydride in order to produce stable terminal alditol residues, which are afterwards hydrolyzed under the usual conditions.[3] In another approach ('reductive hydrolysis'), an acid-stable reductant (4-methylmorpholine borane) is used while the 3,6-anhydrogalactosyl linkages are being cleaved.[3,4] Further steps are then used to hydrolyze and derivatize the remaining components. This technique has suffered several modifications in order to improve the yield of 3,6-anhydrogalactose when it is 2-sulfated or the neighboring unit is pyruvylated.[5–7] The derivatives produced

Abbreviations: MMB, 4-methylmorpholine borane; AP, 1-amino-2-propanol; MBA, α-methylbenzylamine.

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can then be separated and quantitated by GLC[3] or HPLC.[7]

The assignment of the D or L configuration to the sugars that form a red seaweed galactan becomes crucial to determine its main characteristics. This was usually carried out by the methods of Gerwig and co-workers[8] and Leontein and co-workers[9] where GLC is applied to derivatives of their glycosides with chiral alcohols. Cases and co-workers used reductive amination with chiral amines to produce separable derivatives of the galactose and its mono-*O*-methyl ethers usually present in red seaweed galactans.[10] These methods were further extended to the hydrolyzates of methylated polysaccharides.[11,12] For the determination of the configuration of 3,6-anhydrogalactose, two different approaches were used: partial reductive hydrolysis[4,13] or methanolysis[14,15] to obtain the derivatives of representative disaccharides (carrabiose and/or agarobiose) amenable to GLC analysis,[13–15] or mild oxidative hydrolysis to generate 3,6-anhydrogalactonic acids, which are then derivatized with chiral alcohols to esters.[16]

Herein we present the determination of the configuration of 3,6-anhydrogalactose (and its 2-*O*-methyl ether) present in red seaweed polysaccharides by a double hydrolysis procedure involving a reductive amination of the anhydro sugar. The procedure is simpler and has a wider scope than previous techniques.[13–16] Furthermore, as 3,6-anhydrogalactose can be generated quantitatively from galactose 6-sulfate by an alkaline treatment,[2,17] a one-pot procedure involving alkaline treatment, mild hydrolysis, reductive amination, total hydrolysis, and final derivatization was developed in order to quantitate both enantiomers of cyclizable 6-sulfate in agars, carrageenans and D/L-hybrids.

2. Results and discussion

The importance of the determination of the configuration of the 3,6-anhydrogalactose present in polysaccharides was recognized in the last decade.[1,13,16] By partial reductive hydrolysis,[4,13] cleavage of the 3,6-anhydrogalactosidic linkages is produced, concomitant with the reduction of those reducing terminals. If consecutive carrabiose or agarobiose units are present in the polysaccharide, disaccharide alditols ('biitols') with characteristic configuration (thus, separable by GLC) are obtained. However, the method fails when no such consecutive units are present, as higher oligosaccharides are produced. Thus, no assessment of the configuration of the total 3,6-anhydrogalactose is obtained, especially for polysaccharides with low 3,6-AnGal content. Another procedure developed for such purpose involves oxidative hydrolysis and derivatization of the 3,6-anhydrogalactonic acid terminals to esters with chiral alcohols.[16] The technique is very laborious, as it requires many steps. Therefore, its sensitivity, at least in our hands, is rather low.

We decided to attempt a quantitation of both 3,6-AnGal enantiomers by a combination of two well-known techniques: a double-hydrolysis procedure,[3] where instead of using sodium borohydride as the reducing agent, a reductive amination with a chiral amine is applied, under the conditions already established.[10] The reaction requires five main steps (mild hydrolysis, reductive amination, strong hydrolysis, reductive amination, and acetylation). When it was carried out using a commercial κ-carrageenan and racemic 1-amino-2-propanol (AP), three main peaks were obtained. One corresponded to the reaction with the 3,6-AnGal, while the other two corresponded to both diastereomers produced by reaction of the D-Gal with the racemic amine. Trace amounts of galactitol and 3,6-anhydrogalactitol peracetates were also encountered. The 3,6-AnGal/Gal ratio deduced from the chromatogram is 1:1.03, i.e., close to that expected from a κ-carrageenan.[1,2] However, as occurred with 2-*O*-methylgalactose[10] no resolution of the enantiomeric pair of 3,6-AnGal (as their diastereomeric aminoalditol derivatives) is obtained. Fortunately, a different picture is obtained by using α-methylbenzylamine (MBA) as the amine. As shown in Table 1, κ-carrageenan gives, by reaction of the racemic amine, two well-resolved peaks of equal area corresponding to the reaction with 3,6-AnGal, together with two peaks of uneven area corresponding to the derivatives of the galactose present (Fig. 1). It was previously established[10] that Gal enantiomers could not be separated efficiently by reaction with MBA, as a marked enantioselectivity precluded its quantitation. The ^{13}C NMR spectrum of a 3,6-AnGal derivative prepared on larger scale (Section 3.6) also indicates similar amounts of both diastereomers. Table 1 also shows other applications of this technique. The use of chiral MBA (Table 1) gives the expected results: 3,6-AnGal of the D-series for κ-carrageenan, L-series for agarose. Only traces of the direct reduction products (alditol acetates) appear if the experimental details are followed strictly, especially those concerning a rigorous evaporation of the trifluoroacetic acid after the first hydrolysis step. The analysis of a methylated κ-carrageenan indicates that the derivatives of both enantiomers of 3,6-anhydro-2-*O*-methylgalactose can be separated even better by the same technique using MBA (but not with AP), with no enantioselectivity. Table 2 shows the chromatographic data for the derivatives of 3,6-AnGal and its 2-*O*-methyl ether. The data obtained for the derivatives of other sugars present in red seaweed galactans is also included in Table 2, considering that the capillary column width is slightly different from that previously reported.[10] In a separate experiment, it was found that no difference appears when the time of the strong hydrolysis step of κ-carrageenan was varied between 1 and 2 h. However,

Table 1
Results of the double hydrolysis–reductive amination method applied to different red seaweed polysaccharides using α-methylbenzylamine (MBA)

Polymer	Amine	Yield [a]	Deduced molar ratio [b]			
			3,6-AnGal		Gal	
			D/(S) = L/(R) [c]	L/(S) = D/(R)	D/(S) = L/(R)	L/(S) = D/(R)
κ-Carrageenan	Racemic	80	30	29	25	16
Agarose	Racemic	94	28	29	26	17
ι-Carrageenan	Racemic	88	28	28	25	19
κ-Carrageenan	(S)	74	52		44	4
Agarose	(S)	95		51	49	

[a] Response of aminoalditols relative to *myo*-inositol = 100.
[b] Traces (< 2%) of 3,6-anhydrogalactitol and/or galactitol peracetates were also encountered.
[c] D/(S) = L/(R) implies condensation product of the D-sugar and the (S)-amine, equivalent to its enantiomer, product of the reaction of the L-sugar and the (R)-amine.

for products carrying methylated galactoses, their recovery after a 1 h hydrolysis is low. Therefore, a 1.5-h hydrolysis is recommended in the standard procedure. GLC–MS analysis of the derivatives generated with MBA shows a characteristic loss of acetyl radical from the molecular ion (see Section 3.5).

Table 1 shows that the recovery of 3,6-AnGal MBA-derivatives is similar for κ-carrageenan and for ι-carrageenan, even though the latter contains 2-sulfated 3,6-AnGal units, which are slower to hydrolyze than the non-sulfated 3,6-AnGal units in κ-carrageenan.[3] Similar results were found for alkali-treated λ-carrageenan (see below), indicating that the method can safely quantitate both 2-sulfated or non-sulfated 3,6-AnGal residues present in the same samples. On the other hand, when the partial reductive hydrolysis method (with MMB)[3]

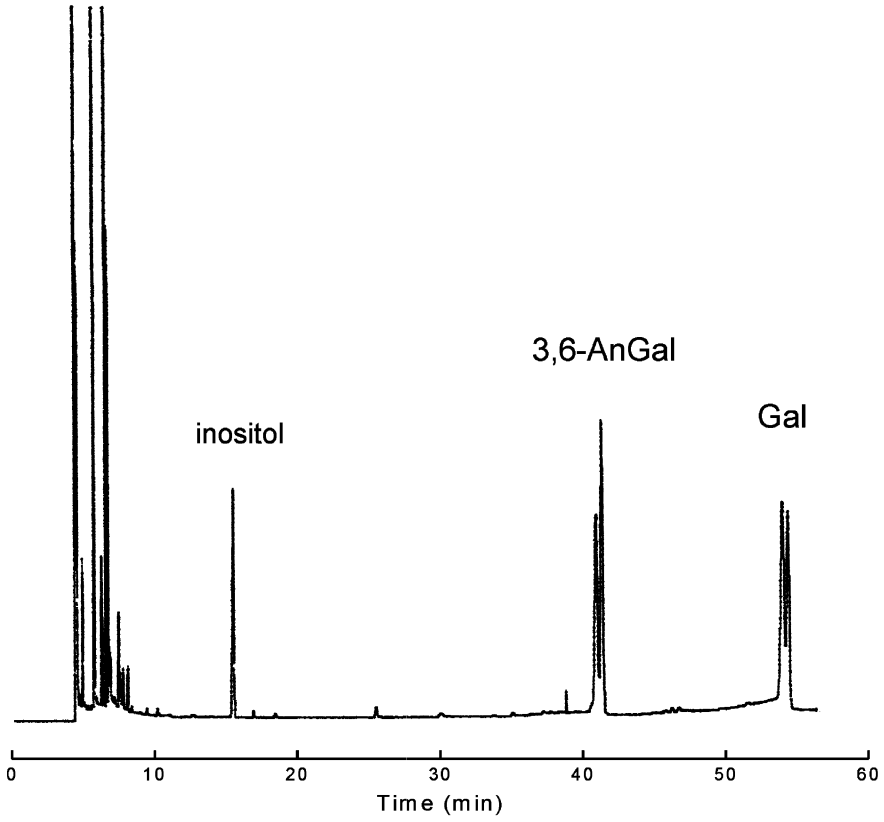

Fig. 1. Gas chromatogram (Program B) of the reaction product of the double hydrolysis method applied to κ-carrageenan, followed by reaction with racemic MBA. In each doublet, the first peak corresponds to the reaction of the D-sugar with the (S)-amine, whereas the second is equivalent to that of the L-sugar and the (S)-amine.

Table 2
Retention times [a] and separation factors (r) of acetylated aminoalditols originated from enantiomeric sugars using an Ultra-2 column

	(*S*)-α-methylbenzylamine (MBA) (Program B)			(*S*)-1-amino-2-propanol (AP) (Program A)		
	D	L	R	D	L	r
3,6-An-2-*O*-Me-Gal	2.232	2.278	1.020	1.601		1
3,6-AnGal	2.708	2.732	1.009	1.840		1
2-*O*-Me-Gal	2.986	3.029	1.014	2.230		1
Gal	3.439	3.464	1.007 [b]	2.505	2.520	1.006

[a] Relative to peracetylated *myo*-inositol = 1 (15.51 min in both programs).
[b] Enantioselectivity was observed.

was applied to ι-carrageenan under the original conditions, the 3,6-AnGal recovery was low (Gal–3,6-AnGal ratio = 1.5:1, determined as their alditol acetates). However, when the modifications to this technique suggested by Jol and co-workers[7] were applied (prolonged time for the first hydrolysis, and an extra evaporation step), a 1:1 ratio of both sugars was achieved.

When the reaction was carried out with no addition of amine in the second step, in order to save expensive chiral reagent, the D/L ratio and recovery of the 3,6-AnGal was similar to that produced using the full reaction. However, the reaction produces galactitol partly at the expense of the aminogalactitol, probably due to the direct reducing action of the sodium cyanoborohydride in the second step, when the amine present was less abundant. Attempts to use two different amines, one for each step of the hydrolysis resulted in complicated mixtures, as expected considering that these amines are added in excess with respect to the carbohydrates.[10] The current procedure requires some steps, but is simpler, faster and more sensitive than other methods reported elsewhere for the same purpose.[16]

It is well known that the 4-linked, 6-sulfated α-galactose units present in red seaweed galactans undergo a cyclization to 3,6-anhydrogalactose when submitted to an alkaline treatment.[17] This fact was the basis for the use of a 3,6-AnGal determination (usually colorimetric[18]) before and after alkaline treatment to quantify the galactose 6-sulfate present in a polysaccharide preparation.[17,19] Therefore, the currently proposed method could be useful to determine not only the proportion of galactose 6-sulfate, but also its configuration. Porphyran was the polysaccharide chosen for this study, as it carries high proportions of 'precursor' 6-sulfate.[17] The crude polysaccharide from *Porphyra columbina*[20–22] was obtained in 16% yield. Its compositional analysis (Table 3) agrees with previous reports.[20–22] Slightly more D-units than L-units were obtained (54:46), suggesting the possible presence of small amounts of D/L-hybrids. Although at first such hybrids were mostly reported as present in carrageenophytes, which contained small amounts of L-galactose,[2] recent studies have shown the occurrence of D/L-hybrids in agarophytes also.[14,15] It might be argued that a D:L ratio higher than unity arises from analytical errors. Thus, an alkaline treatment, followed by a determination of the configuration of the 3,6-AnGal, would be helpful to ascertain such structural details. As this study shows, in order to carry out such procedure, there is no need to perform a stepwise method with intermediate isolation, as all the reactions can be carried out in a one-pot fashion, following a simple technique (Section 3.3). The compositional analysis of the isolated product and that obtained by the one-pot analysis are very similar (Table 3). Furthermore, although most of the 3,6-AnGal that was produced by the alkaline treatment (approx 35% of the total sugars) belongs to the L-series, about 3–4% of the 3,6-AnGal corresponds to the D-series, though there was no 3,6-An-D-Gal in the original polysaccharide. This data shows clearly that the native polysaccharide of *P. columbina* is a D/L-hybrid (or a mixture of two polysaccharides), as it contains about 3% of 4-linked D-galactose 6-sulfate units. As none of the D-units was present as the 3,6-anhydro ring originally, it may be inferred that the enzyme (sulfohydrolase) that converts those units to 3,6-An-D-Gal moieties is not present in *P. columbina*. The deduced proportion of 3- and 4-linked units is 50:50, as expected for a red algal galactan with a disaccharidic repeating structure.[2]

The alkaline treatment of porphyran,[22] as well as those of carrageenans of the κ-family[23] proceeds quantitatively in about 3 h. However, carrageenans of the λ-family require harsher conditions.[19,23] The half-life determined for the alkaline treatment of a λ-carrageenan from *Gigartina skottsbergii* is 170 min.[23] Thus, a reaction of at least 16 h would be required to achieve constancy in the 3,6-AnGal content. For a λ-carrageenan from *Iridaea undulosa*[19,24] we have found that after 10 h of alkaline treatment the production of 3,6-AnGal reaches a constant value of 48% (by reductive hydrolysis), which remains unaltered for at least 16 h. A previous report indicated the presence of 45% of cyclizable 6-sulfate for the same sample.[19]

Table 3
Compositional analysis performed by different methods to the crude polysaccharides from *P. columbina*[20–22] and cystocarpic *I. undulosa*[19]

Polysaccharide/method	Molar ratio					
	3,6-AnGal		Gal		6-O-Me-	D-Gal
	D	L	D	L		
P. columbina, native						
Reductive hydrolysis, MMB		11		54		35
Total hydrolysis, reductive amination, AP		n.d.	23	41		36
Double hydrolysis, reductive amination, AP		10	19	37		33
Double hydrolysis, reductive amination, MBA		8	25	30		37
Deduced analysis [a]		10	19	36		35
P. columbina, alkali treated						
PS isolated by dialysis, reductive hydrolysis, MMB		46		17		37
One-pot, reductive hydrolysis, MMB		48		21		31
One-pot. total hydrolysis, reductive amination, AP		n.d.	44	2		54
One-pot, double hydrolysis, reductive amination, MBA	4	48	15	2		31
Deduced analysis [a]	3 [c]	45 [b]	19	2		31
Cystocarpic *I. undulosa*, native [c]						
Reductive hydrolysis, MMB		37		63		
Double hydrolysis, reductive amination, MBA	35		64	1		
Deduced analysis [a]	37		62	1		
Cystocarpic *I. undulosa*, alkali treated [c]						
Reductive hydrolysis, MMB		52		48		
Double hydrolysis, reductive amination, MBA	49		50	1		
Deduced analysis [a]	52[d]		47	1		

[a] The proportion of each sugar (either enantiomer) was determined from the partial reductive hydrolysis, whereas the proportion of each enantiomer was determined by the use of the corresponding chiral amine (AP for Gal and 6-*O*-Me-Gal, MBA for 3,6-AnGal).
[b] Indicates that about 3% of the D-Gal and 35% of the L-Gal of the native polysaccharide were 6-sulfated.
[c] Traces of glucose ($< 2\%$) were also detected.
[d] Indicates that about 15% of the D-Gal of the native polysaccharide was 6-sulfated.

The procedure was also applied to the crude cystocarpic carrageenan from *I. undulosa*[19] (Table 3). The two main fractions of this product were analyzed by NMR spectroscopy, which shows they are mixtures (or hybrids) of carrageenans of the κ-family.[24] An approximate ratio of κ/ι/μ/ν-diads of 55:20:5:20 was inferred for the crude carrageenan under analysis.[24] However, minor fractions containing L-galactose and other sugars appear after fractionation of an alkali-treated sample.[25] The present study (Table 3) indicates 74% of κ/ι-diads in the product, whereas the remainder corresponds to μ/ν-diads, i.e., results very close to those obtained by NMR spectroscopy.[24] The L-galactose present in low proportions in the native polysaccharide[25] is not precursor of 3,6-AnGal, as the alkaline treatment did not generate any 3,6-An-L-Gal.

Table 4 summarizes the main techniques available for the compositional analysis of red seaweed galactans by gas–liquid chromatography. None of those techniques allow a complete quantitation (including configurations) of all the sugars present in red seaweed galactans, though many of them complement each other. In the present paper we have introduced the double hydrolysis–reductive amination procedure. The use of AP as the chiral amine gives the possibility of quantitating the enantiomers of galactose and some of its mono-*O*-methyl ethers together with the whole 3,6-anhydrogalactose, whereas the use of MBA generates separable derivatives of both enantiomers of 3,6-AnGal and its 2-*O*-methyl ether, together with those of 2-*O*-methylgalactose, although the quantitation of galactose and the other methylated derivatives is not possible. The extension of these techniques to the determination of the configuration of 6-sulfate in a simple one-pot determination expands even further the possibilities of the current method.

3. Experimental

3.1. Materials

Samples of κ-carrageenan and ι-carrageenan were purchased from Sigma (St. Louis, MO, USA), while

Table 4
Summary of the methods used for the compositional analysis of red seaweed galactans by application of gas chromatographic (GLC) techniques

	Reactions involved [a]	Derivative	Reference	Quantitation of acid-stable sugars	Quantitation of 3,6-AnGal	Determination of configurations	Comments
1	1. Hydrolysis, 2. $NaBH_4$	Alditols	[26]	X			3-*O*-Me-Gal and 4-*O*-Me-Gal not differentiated
2	1. Hydrolysis, 2. $HONH_2$	Aldononitriles	[27]	X			
3	1. Hydrolysis, 2. Chiral ROH/H^+	Glycosides	[8,9]			X	Many peaks (anomeric forms) per sugar. Severe overlapping if methylated Gals are present.
4	1. Hydrolysis, 2. $NaCNBH_3$/(*S*)-MBA	Aminoalditols	[10]			X	Only for 2-*O*-Me-Gal. Enantioselective for other Gal units
5	1. Hydr., 2. $NaCNBH_3$/(*S*)-AP	Aminoalditols	[10]	X		X	2-*O*-Me-Gal enantiomers not separated
6	Hydrolysis/MMB (mild, then strong)	Alditols	[3,4]	X	X		Also for 3,6-An-2-*O*-Me-Gal, see comment on **1**
7	As **1**, twice (mild, then strong hydrolysis)	Alditols	[3]	X	X		Also for 3,6-An-2-*O*-Me-Gal, see comment on **1**
8	1. Mild hydrolysis, 2. MMB	Disacch. alditols	[4,13]			X	Only for 3,6-AnGal in consecutive carrabiose or agarobiose units
9	1. Mild hydrolysis, 2. Br_2, 3. H^+, 4. $NaBH_4$, 5. $SOCl_2$, 6. *s*-BuOH	Esters	[16]			X	Only for 3,6-AnGal
10	As **5**, twice (mild, then strong hydrolysis)	Aminoalditols	[b]	X	X	X	3,6-AnGal and 2-*O*-Me-Gal enantiomers not separated
11	As **4**, twice (mild, then strong hydrolysis)	Aminoalditols	[b]			X	Only for 3,6-An-2-*O*-Me-Gal, 3,6-AnGal and 2-*O*-MeGal

[a] A further step to make volatile derivatives (acetylation or trimethylsilylation) is necessary in all cases.
[b] Current work.

agarose is from K & K Lab (Plainview, NY, USA). Gametophytic samples of *P. columbina* were collected near Comodoro Rivadavia (Chubut Province, same batch as previously reported).[22] A voucher specimen of *P. columbina* has been deposited at the HRP (Regional Herbarium of the Patagonia, FCN,UNPSJB) and bears the number 5795. The crude polysaccharide was isolated after extraction of the milled seaweed with water at room temp for 24 h with stirring. The slurry was centrifuged, and the polysaccharide was isolated from the supernatant after precipitation with 3 vols of 2-PrOH. The carrageenans from *I. undulosa* were obtained and fractionated as reported elsewhere.[19] The fractions used in the current paper are those labelled as C (cystocarpic carrageenan) and T_1 (λ-carrageenan). Other standards were obtained as reported earlier.[10]

3.2. Recommended procedure for the double hydrolysis–reductive amination method

One milligram of polysaccharide was hydrolyzed with 0.1 M CF_3COOH (0.25 mL, 3 h, 80 °C), as reported previously.[3] The solvent was evaporated, and additions of water (3 × 0.5 mL) were made followed by evaporations to remove traces of CF_3COOH. The residue was submitted to reductive amination with chiral or racemic MBA or AP as described by Cases and co-workers[10] Briefly, the following reagents were added: a 1:8 solution of the amine in MeOH (20 μL for AP, 32 μL for MBA), 17 μL of a 20% solution of glacial AcOH in MeOH, and 13 μL of a 3% solution of $NaBH_3CN$ in MeOH. The vial was capped, and the mixture was allowed to react (1–2 h, 65 °C). Finally, the excess of $NaBH_3CN$ was destroyed with 3 M aq CF_3COOH (dropwise, in the fume hood). The mixture was evaporated and coevaporated with water (3 × 0.5 mL) and MeOH (5 × 0.5 mL). The residue was treated with 2 M CF_3COOH for 90 min at 120 °C. The solvent was perevaporated, and the residue was allowed to react with the same amine as described previously. After drying overnight in the desiccator, the residue was treated with 1:1 pyridine–Ac_2O (45 min, 100 °C). After cooling, the derivatives were extracted with $CHCl_3$ and washed with water (3 × 0.5 mL) and satd $NaHCO_3$ (3 × 0.5 mL). The organic phase was dried with anhyd Na_2SO_4 and injected onto the GLC column.

GLC was carried out with a Hewlett–Packard 5890A apparatus equipped with a flame-ionization detector. A Hewlett–Packard Ultra 2 column (50 m × 0.32 mm; thickness of liquid phase, 0.17 μm) was used, with N_2 as the gas carrier (approx 1 mL/min), with a head pressure of 11.5 psi, and a split ratio of 70:1. Program A (for hydroxypropylaminoalditols) started at 180 °C, 4 °C/min to 220 °C (held for 2 min) and then 1 °C/min to 250 °C (held for 10 min). Program B for methylbenzylaminoalditols) started similarly, but the final ramp was taken up to 270 °C and then held for 5 min. The injector and detector temp were set at 270 °C.

3.3. Recommended procedure for the one-pot alkaline treatment–double hydrolysis procedure

One milligram of polysaccharide was dissolved in 0.2 mL of water, and 1 mg of $NaBH_4$ was added. After 1 h, 0.1 mL of 3 M NaOH were added, and the solution was allowed to react at 80 °C. For polysaccharides not belonging to the λ-family, 3 h of reaction are sufficient, whereas for λ-carrageenans a treatment of 16 h is suggested. In the present work, for carrageenans of the κ-family the reaction was extended to 5 h, for comparison with previous work.[25] The solution was neutralized with 0.12 mL of 3 M CF_3COOH, and Amberlite 120 (H^+) resin was added. After filtration, the solvent was evaporated-off (with an intermediate water addition), and the residue was derivatized to the acetylated aminoalditols as stated in Section 3.2 or the acetylated alditols as stated in Section 3.4.

3.4. Reductive hydrolysis procedure

The procedure of Stevenson and Furneaux was used,[3] with the modifications proposed by Jol and co-workers[7] (prehydrolysis of 30 min and an extra evaporation step), especially when the presence of 2-sulfated 3,6-AnGal units was suspected. The final hydrolysis was carried out for 2 h, and the acetylation step with 1:1 CF_3COOH–Ac_2O (10 min, 50 °C) The alditols were determined by GLC in the above-mentioned apparatus, using an SP-2330 column (30 m × 0.25 mm, thickness 0.20 μm) using a program which started at 200 °C, then at 2 °C/min up to 230 °C, and finally held at 230 °C, while the injector and detector were set at 240 °C.

3.5. GLC–MS

The reaction products of various samples with α-methylbenzylamine were analyzed on a Shimadzu QP 5050 A GC–MS apparatus working at 70 eV using conditions similar to those described above with an Ultra 2 column, but using He as the gas carrier at a flow rate of 7 mL/min and a split ratio of 11:1. Besides the base peak at *m*/*z* 105 ($PhCH^+CH_3$) observed in the mass spectra of all the derivatives, a characteristic peak at high *m*/*z* ratios appears in relatively high proportion (approx 20% of the base peak), originated in the loss of acetyl radical from the molecular ion. For the derivative of Gal it appears at *m*/*z* 494, for 3,6-AnGal at *m*/*z* 392, for 2-*O*-Me-Gal at *m*/*z* 466 and for 3,6-An-2-*O*-Me-Gal at *m*/*z* 364. For the derivatives produced by the reaction with 1-amino-2-propanol, the mass spectra are those expected from the predicted fragmentations of the galactose derivative.[10]

3.6. Preparation of 3,6-anhydro-1-deoxy-1-(1′-phenylethylamino)-D-galactitol for NMR studies

A sample of κ-carrageenan (64 mg) was submitted to the double hydrolysis–reductive amination procedure with racemic MBA (Section 3.2), up to the strong hydrolysis step. After complete evaporation of the solvent, the residue was dissolved in water and treated with Amberlite 120 (H^+) resin. The resin was washed with water to remove the galactose and other neutral materials, and the diastereomeric mixture of the title compound was isolated after elution with 2 M NH_3 and evaporation of the solvent. The residue was dissolved in 1:1 H_2O–D_2O, and subjected to ^{13}C NMR spectroscopy, using a Bruker AM 500 spectrometer equipped with a 5-mm probe operating at 125 MHz. Chemical shifts were measured relative to acetone as internal standard, and referred to Me_4Si by calibrating the acetone methyl group to 31.1 ppm. Putative assignments[28,29] for the mixture (in most cases two peaks are indicated for each carbon, due to the splitting produced by the presence of diastereomers) are as follows: δ 145.3 (C-1″), 129.7 (C-3″), 128.2 (C-4″), 127.8 and 127.7 (C-2″), 88.2 and 88.1 (C-3), 80.5 (C-4), 77.4 and 77.3 (C-5), 74.9 and 74.8 (C-6), 70.9 and 70.4 (C-2), 58.5 and 57.7 (C-1), 51.1 and 50.5 (C-1′) and 23.0 (C-2′).

3.7. Other procedures

κ-Carrageenan was methylated as described previously.[19] The alkaline treatment with isolation of the intermediate product was carried out as reported.[25] The molar ratios deduced from the chromatograms were calculated considering that the FID-integrated areas were proportional to the molecular weight of the derivatives. The yield of the reactions, relative to *myo*-inositol was calculated in the same manner, taking into account the proportions of carbohydrates present in each polysaccharide, which were determined by the phenol–sulfuric acid method.[30]

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