C(6)-Oxidation and C(5)-Epimerization of Locust Bean Galactomannan Studied by High Field NMR and Circular Dichroism

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Galactose depleted locust bean gum was selectively oxidized in C(6) position and epimerized with mannuronan C(5)-epimerases to obtain the corresponding artificial uronanes. These new pseudo-alginates were characterized by NMR spectroscopy and circular dichroism (CD). Specifically, 1D and 2D NMR techniques allowed the degree of epimerization, the distribution of mannuronic and guluronic acid residues in the polysaccharidic chain, and the average G block length to be determined. In addition, NMR diffusion experiments showed that the epimerization reaction did not significantly degrade the polysaccharidic chains. Circular dichroism was used to investigate the kinetics of the epimerization reaction and to evidence the specific interaction between the epimerized locust bean samples with Ca(II) ions in dilute solution. All of the samples considered in this study form wall to wall gels in concentrated polymer solutions.

1. Introduction

Alginates are linear, ionic polysaccharides consisting of statistical sequences of 1→4-linked β-D-mannopyranuronate (M) and α-L-gulopyranuronate (G) residues. Alginates are isolated from seaweeds (*Phaeophyaceae*) or from bacteria (*Pseudomonas aeruginosa* and *Azotobacter vinelandii*). The main step in the biosynthetic pathway of alginates is the epimerization of M residues to G residues by a family of seven mannuronan C(5)-epimerases,[1−7] namely AlgE1−AlgE7. The M/G ratio found in natural alginates depends on the source. The specific properties of different types of alginates are tightly related to the amount and the distribution of M and G residues in the polysaccharidic chain. Because of the gel forming, water-binding, and immunogenic properties of natural alginates, we investigated the possibility of using mannuronan C(5)-epimerases on low cost polysaccharides with a high mannose content for obtaining the corresponding artificial uronanes.

This paper is part of a more extended research program in which konjac glucomamannan[8] and guar gum[9] were examined as potential substrates on which to test the effectiveness of the galactose depletion, the C(6)-oxidation, and the C(5)-epimerization reactions for obtaining artificial uronanes with properties tuned for the desired application. In this paper, we report on the epimerization of locust bean (LB**)** gum.[10] LB is obtained from seeds of the carob tree (*Ceratonia siliqua L*), which can be found throughout the Mediterranean region and in North America. LB is commonly used as a thickening agent, stabilizer, or fat replacement in various foods such as diary products, salad dressings, sauces, and soups.

LB consists of 1→4-linked β-D-mannopyranose residues (m), some of which are substituted at O(6) with an α-D-galactopyranosyl (gal) residue. In LB, the ratio of gal residues to m residues was found to be about 1:4 (see section 3.1), whereas in guar gum, according to literature data,[9,11] this ratio is 1:2. Another difference between guar and LB galactomannans lies in the distribution of gal residues along the polysaccharidic chain; in guar gum, the gal residues are arranged along the chains in an approximately alternating manner.[12] Studies on LB indicated that, although more than one-half of gal residues occupied noncontiguous positions in the chains, the remainder of gal residues was present in a blocklike pattern.

In this paper, a high field NMR characterization of native LB and of the galactose depleted, oxidized and epimerized derivatives is reported. The results obtained using depleted, C(6)-oxidized LB as a substrate are compared with those obtained using mannuronan, which is the most suitable substrate for the action of mannuronan C(5)-epimerases.

The kinetics of the epimerization reactions performed in the presence of different C-5 epimerases and their blends were monitored by circular dichroism (CD). The ability of a few ionic polysaccharides to form gels in the presence of divalent cations such as calcium is the key to biological functions and technological applications. This is particularly true for alginates where the occurrence of G residues generates templates allowing polymer chain associations that are involved in the formation of the physical gel. Therefore, it is of interest to assess, through a preliminary study, the extent of the interaction between the derivatives of LB with Ca(II) ions in dilute solution.[13]

2. Experimental Section

2.1. α-D-Galactosidase Treatment of LB. The galactose-depleted LB was prepared according to the following procedure: 1 g of LB

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(Sigma−Aldrich) dissolved in 500 mL of 0.1 M acetate buffer (pH = 4.6) was treated at 37 °C for 48 h with α-D-galactosidase (37.5 units, where one unit is defined as the amount of enzyme required to release at pH 6.5 and 25 °C 1 μmol of *p*-nitrophenol per min). During the reaction, because of the decreased solubility of the galactose-depleted LB, a precipitate was observed. To inactivate the enzyme, the suspension was brought to 90 °C for about 6 min and then cooled at room temperature.

2.2. Galactose-Depleted LB C(6)−Oxidation. The depleted LB (1 g) was suspended in 500 mL of acetate buffer, and TEMPO (2,2,6,6-tetramethylpiperidin-1-oxy; Sigma; 0.06 g) and NaBr (0.3 g) were added to the reaction mixture; the suspension was kept under stirring. The mixture obtained was cooled overnight at 4 °C. Afterward, sodium hypochlorite (NaOCl) previously adjusted to pH 10, was added. Without further cooling, the pH of the reaction medium was monitored, and if necessary, 0.5 M NaOH was added to maintain the pH at 9.4. After 1 h, the pH tended to decrease slowly, and then EtOH was added to the reaction mixture to deplete NaOCl left. Afterward, $NaBH_4$ was added gradually until the reaction mixture became clear. The solution was kept under stirring for 24 h at room temperature, and then the pH was adjusted to 7.5 by adding CH_3COOH. The resulting solution was extensively dialyzed against distilled water and then freeze-dried.

2.3. Galactose-Depleted, C(6)-Oxidized LB Epimerization and Mannuronan Epimerization. Samples of galactose-depleted oxidized LB and samples of mannuronans were dissolved (C_p = 0.1% w/v) in 50 mM TRIS/HCl buffer (pH 6.9), in the presence of $NaClO_4$ (50 mM) and $CaCl_2$ (2 mM). These samples were epimerized by using three different mannuronan C(5) epimerases: AlgE4 (C = 0.005% w/v), AlgE1 (C = 0.004% w/v), and AlgE6 (C = 0.01% w/v) and their mixtures such as AlgE1+AlgE4+AlgE6, AlgE1+AlgE6, and AlgE4+AlgE6 (each enzyme being used at the previously given concentration). All samples were incubated with the enzyme at 40 °C for 48 h under stirring. The reaction was stopped by chelation of Ca^{2+} ions with the addition of EDTA until a concentration of 13 mM was reached. The pH was adjusted to 7.0 by adding NaOH 0.1 M. To inactivate the enzyme, the solution was heated at 90 °C for about 6 min; afterward the enzyme was removed by filtering the solution using 0.22 μm pore size filters selective for the enzyme but not for the polysaccharides. All solutions were extensively dialyzed against distilled water and freeze-dried.

2.4. Preparation of Ca(II)-Induced Gels from Epimerized LB Samples. Freeze-dried epimerized LB samples were solubilized in water. The Ca(II)−EDTA complex was then added to the solution to obtain a ratio R = 1.1, where R is defined as the ratio of equivalents of Ca(II) ions to equivalents of carboxylic groups. After solubilizing the complex, a number of equivalents of D-glucono-δ-lactone equal to twice that of Ca(II) ions was added, and the system was kept under stirring until D-glucono-δ-lactone was fully solubilized. Afterward, the system was left at rest.

2.5. NMR Measurements. About 3 mg of samples were dissolved in 0.7 mL of a phosphate buffered (pD = 7) D_2O solution containing 0.1 M NaCl. ^1H and ^{13}C NMR spectra were recorded at 343 K on a Bruker AVANCE 600 spectrometer operating at 600.13 and 150.92 MHz, respectively. All one- (1D) and two-dimensional (2D) spectra were recorded using a soft presaturation of the HOD residual signal.[14] ^1H and ^{13}C chemical shifts were reported with respect to a trace of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) used as an internal standard.

^1H and ^{13}C assignments were obtained using $^1H-^1H$ COSY, $^1H-^1H$ TOCSY, and $^1H-^{13}C$ HSQC (heteronuclear single quantum coherence) experiments.[15] All 2D experiments were carried out using 512 and 1024 data points in the F1 and F2 dimension, respectively, and a recycle delay of 2 s. The $^1H-^1H$ TOCSY experiments were acquired with a spin-lock time of 80 ms. The HSQC experiments were performed using a $^1J_{C-H}$ coupling constant of 150 Hz. The number of scans was chosen to achieve a good signal/noise ratio. All 2D experiments were processed with a matrix of 512 × 1024 data points. $^1H-^1H$ TOCSY and HSQC experiments were processed in the phase-sensitive mode, whereas $^1H-^1H$ COSY experiments were processed in the magnitude mode.

Pulsed gradient spin−echo (PGSE) experiments[16] were performed with a pulsed field gradient unit capable of producing a magnetic field gradient in the z direction with a strength of 55.4 G cm^{-1}. The stimulated echo pulse sequence using bipolar gradients with a longitudinal eddy current delay was used.[17] The strength of the sine gradient pulses, of 2.1 ms duration, was logarithmically incremented in 32 steps, from 2 to 95% of the maximum gradient strength, with a diffusion time of 800 ms and a longitudinal eddy current delay of 25 ms. To avoid any overheating of the probehead and any possible convection effect, the diffusion experiments were recorded at 300 K. At this temperature, even though some ^1H resonances of the polysaccharidic samples overlapped, accurate diffusion coefficients could be measured.

After Fourier transformation and baseline correction, the diffusion dimension was processed by means of the DOSY subroutine of the Bruker Xwinnmr software package (version 3.5). For the samples under study, the decay curves could not be described by a single-exponential function. In addition, when trying to fit the data with two exponential functions, multiple solutions were found depending both on the initial guesses chosen and on the constraints imposed. Therefore, to limit at most the user input in fitting the data, we decided to process the diffusion dimension of the DOSY maps with an approximate inverse Laplace transform (ILT) using the ILT module implemented in the Bruker Xwinnmr software package, which is based on a modified version of the numerical procedure CONTIN.[18,19]

The deconvolution of the ^1H NMR spectra was performed using the SHAPE2000 (version 2.1) software package.[20] Applying the deconvolution program, the areas I_i of the peaks resonating in the 4.4−5.2 ppm spectral range were obtained along with the errors ΔI_i, which were always within 4 to 5% of the nominal I_i values. Since it was not possible to obtain errors on the calculated molar fractions, on the doublet and triplet frequencies, and on the average block lengths directly from the deconvolution program, the propagation error theory was applied.

According to our notation: gal = galactose unit; Gal = galacturonic acid unit; m = mannose unit; m* = branched mannose unit; M = mannuronic acid unit; G = guluronic acid unit.

2.6. Circular Dichroism Measurements. Circular dichroism (CD) spectra were recorded at 25 °C with a Jasco J715-A dichograph in the 190−300 nm wavelength range with the following setup: bandwidth, 1 nm; time constant, 2 s; scan rate, 50 nm/min^{-1}; sensitivity, 5 mdeg.; number of scans, 4. All spectra were corrected for the background. The correction was performed subtracting from the current spectrum, the spectrum of a sample containing the TRIS/HCl 50 mM pH 6.9 buffer and the enzyme at the same concentration as the one used for the epimerization of the LB samples.

In our notation, DOLBs indicate galactose depleted, C(6)-oxidized and epimerized Locust Bean samples and, Mans indicates epimerized Mannuronan samples.

3. NMR Results

3.1. Locust Bean Gum. LB consists of 1→4-linked β-D-mannopyranose residues (m) some of which are substituted at O(6) with an α-D-galactopyranosyl (gal) residue, see Figure 1A. The ^1H NMR spectrum of a sample of native LB solubilized in a phosphate buffered D_2O solution at pD = 7 is shown in Figure 2a. The resonances due to the mannose residues appear rather broad, whereas those due to the gal residues are sharp and rather well resolved. The gal/m ratio was obtained by integrating the resonance of the anomeric proton of the gal residues at 5.02 ppm with respect to the resonance of the anomeric proton of the m residues at 4.73 ppm; this gave a gal/m ratio of about 1:4, in agreement with results reported in the literature.[10,11]

The ^1H and ^{13}C (signal) assignments obtained by COSY and TOCSY (data not shown) and HSQC (Figure 3a) experiments

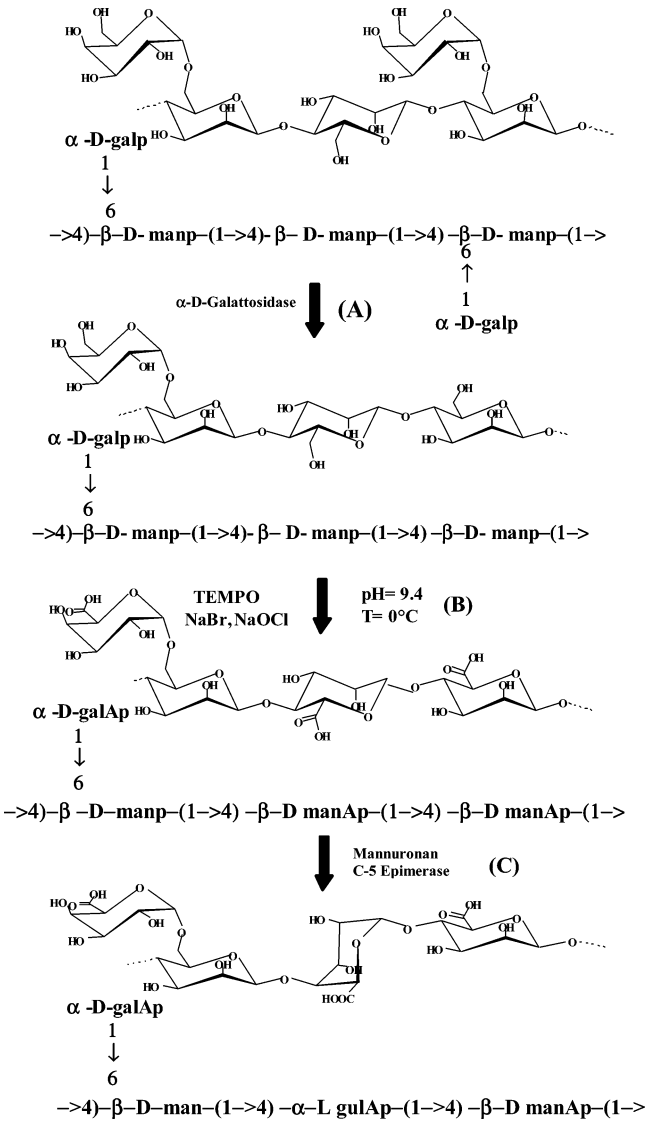


Figure 1. Reactions to which locust bean gum was subjected: (A) partial depletion of the side galactose moieties, (B) TEMPO-mediated oxidation, (C) C(5)-epimerization.

are reported in Table 1. It is worth noting that some chemical shifts of mannose residues m* substituted at O(6) with an α-D-galactopyranosyl (gal) residue were well distinguishable from the chemical shifts of unsubstituted mannose residues (Table 1). In particular, the largest difference in the ^{13}C chemical shift was observed between the cross-peak of the methylene of m residues at 62.6 ppm and the cross-peak of the methylene of m* residues at 68.7 ppm.

3.2. Depletion and C(6)-Oxidation of LB. To further increase the number of the mannose residues available to the selective C(6)-oxidation as well as to the C(5)-epimerization, the side galactose groups were removed by treatment with α-galactosidase. This treatment allowed the gal/m ratio to be decreased from 1:4 down to 1:12.5; hence, after depleting, the fraction of mannose residues carrying galactose side chains was about 8%.

The depleted LB was oxidized in the C(6) position by performing a selective TEMPO-mediated oxidation which transformed m and gal residues into the corresponding acids, namely mannuronic (M) and galacturonic (Gal) acid, see Figure 1B**.** The spectrum of a depleted and C(6)-oxidized sample is shown in Figure 2b; the resonances due to the M and G residues and those due to the residual m* residues were assigned (Table 2). The HSQC spectrum is shown in Figure 3b. Note that, because of the C(6) oxidation, the cross-peaks due to the methylenes of m and gal residues, clearly discernible in the HSQC spectrum of a sample of native LB (Figure 3a), disappeared in the spectrum of the depleted and oxidized LB sample (Figure 3b). Cross-peaks of the methylene of m* residues

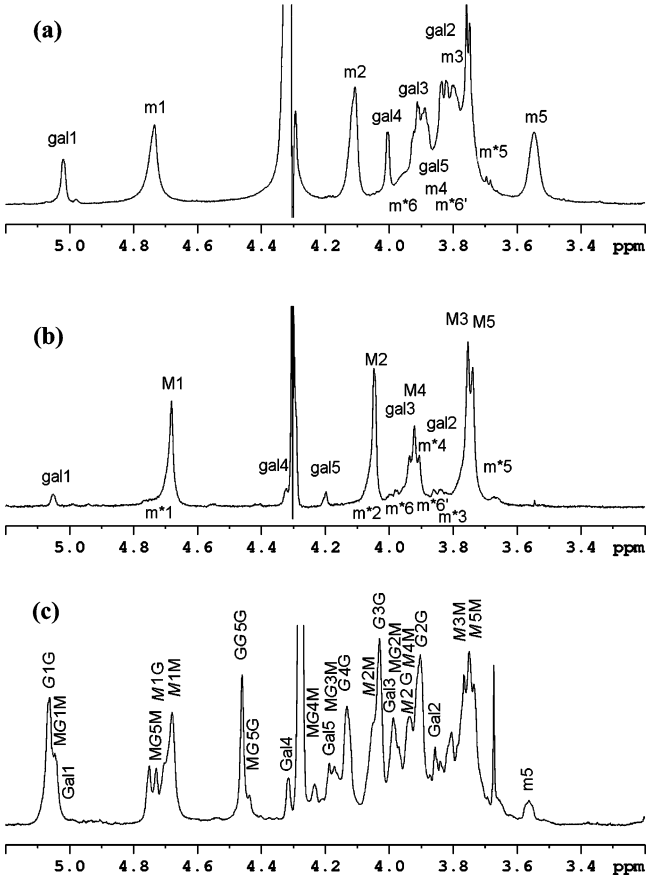


Figure 2. ^1H NMR spectra (600.13 MHz) in a phosphate buffered solution at 343 K of (a) locust bean gum; (b) depleted and C(6)-oxidized locust bean gum; (c) depleted, C(6)-oxidized and epimerized with the AlgE1+AlgE4+AlgE6 mixture locust bean gum.

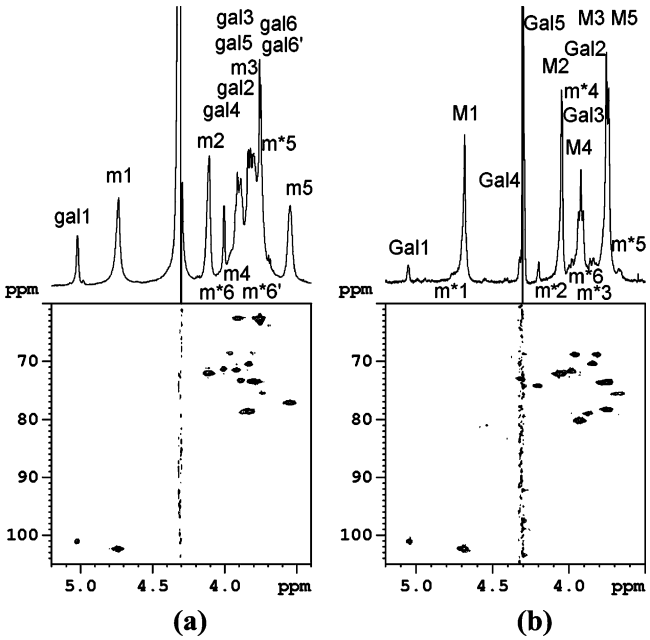


Figure 3. HSQC experiments at $T = 343$ K on (a) locust bean gum, (b) depleted and C(6)-oxidized locust bean gum.

Table 1. ^{1}H and ^{13}C NMR Assignments of Locust Bean Gum in a Buffered Solution (pD = 7) at T = 343 K and m^* = Branched Mannose Residue[a]

mannose residue			galactose residue		
	^{1}H	^{13}C		^{1}H	^{13}C
m1	4.73	102.2	gal1	5.02	100.8
m2	4.11	72.2	gal2	3.83	70.6
m3	3.80	73.6	gal3	3.92	71.6
m4	3.82	78.6	gal4	4.00	71.5
m*4	3.88	78.9			
m5	3.55	77.2	gal5	3.89	73.6
m*5	3.75	75.6			
m6, m6′	3.91, 3.76	62.6	gal6, gal6	3.76	63.3
m*6, m*6′	3.96, 3.80	68.7			

[a] Chemical shifts are reported in ppm with respect to a trace of DSS used as internal standard.

Table 2. ^{1}H and ^{13}C NMR Assignments of Depleted and C(6)-Oxidized Locust Bean Gum in a Buffered Solution (pD = 7) at T = 343 K and m^* = Branched Mannose Unit[a]

mannuronic acid residue			galacturonic acid residue			branched mannose residue		
	^{1}H	^{13}C		^{1}H	^{13}C		1H	^{13}C
M1	4.68	102.3	Gal1	5.06	101.0	m*1	4.700	
M2	4.08	72.2	Gal2	3.86	70.3	m *2	4.075	71.84
M	3.77	73.6	Gal	4.00	71.8	m*3	3.819	73.62
M	3.95	80.0	Gal	4.33	73.0	m *4	3.890	78.92
M	3.77	78.2	Gal	4.21	74.2	m *5	3.677	75.57
						m*6, m*6′	3.980, 3.833	68.82

[a] Chemical shifts are reported in ppm with respect to a trace of DSS used as internal standard.

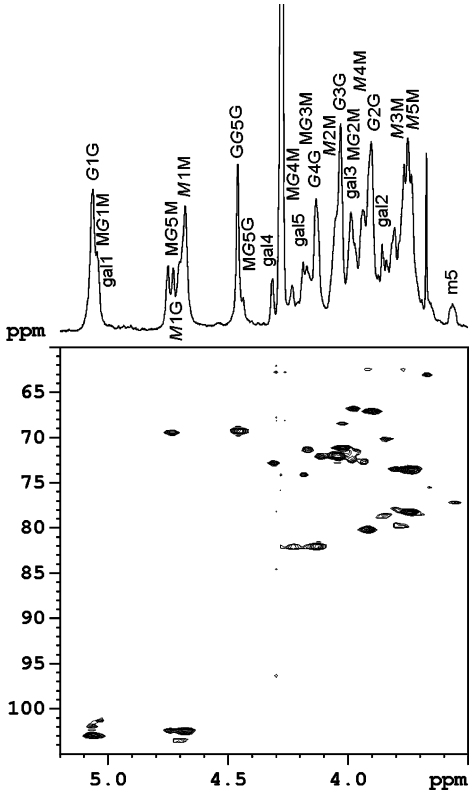


Figure 4. HSQC experiment at T = 343 K on a sample of depleted and C(6)-oxidized locust bean gum epimerized with the AlgE1+AlgE4+AlgE6 mixture.

were still discernible at 3.98 and 3.83 ppm in the ^{1}H dimension and at 68.8 ppm in the ^{13}C dimension.

The percentage of oxidation can be obtained by integrating all resonances due to the anomeric protons with respect to all other resonances due to nonanomeric protons. Because the resonances due to Gal4 and HOD overlapped at 343 K, the integration was performed on a spectrum recorded at 333 K; at this temperature, the HOD residual signal shifts downfield, allowing the Gal4 resonance to be observed. The detailed procedure of integration was previously described.[8,9] According to this procedure, the percentage of C(6)-oxidation was found to be about 76%.

3.3. C(5)-Epimerized, C(6)-Oxidized Locust Bean Gum. NMR spectroscopy has proven to be useful in monitoring the enzymatic epimerization process.[8,9,21] In particular, using ^{1}H NMR spectroscopy, the amount of M residues epimerized to G residues as well as the statistical distribution of M and G residues in terms of diads and triads and the average block length N_G can be quantitatively determined.

The mannuronan C(5)-epimerases introduce G residues in the polysaccharidic chain by C(5)-epimerization of some of the M residues (Figure 1C). Depending on the specific epimerase, the ensuing products may differ in the content as well as in the distribution of M and G residues. These differences in the M and G content and distribution affect the properties of the epimerized compounds. Usually, AlgE4 forms alginates with MG blocks whereas AlgE6 preferentially introduces stretches of G blocks and AlgE1 gives rise to stretches of MG and G blocks.

Galactose depleted and C(6)-oxidized LB samples were used as substrates for mannuronan C(5)-epimerases, AlgE1, Alg4, AlgE6, and their mixtures to obtain the corresponding uronanes, namely DOLBE1, DOLBE4, DOLBE6 and DOLBE1E6, DOLBE4E6, DOLBE1E4E6. All samples were epimerized for 48 h. In Figure 2c, the ^{1}H NMR spectrum of the DOLBE1E4E6 sample is shown. The C(5)-epimerization induced a ring inversion: the axial proton of C(1) of some M residues became equatorial, the equatorial proton of C(2) became axial, and so on. Therefore, new resonances appeared in the spectrum (Figure 2c). ^{1}H and ^{13}C assignments along with the diads and triads assignments were obtained by COSY, TOCSY (data not shown), and ^{1}H−^{13}C HSQC (Figure 4) experiments and were in agreement with the results obtained on alginates and on other artificial uronanes (Table 3).[22,23] Note that the spin system of M*G*M triads as well as the spin system of *G*G diads were well distinguishable.

3.4. Evaluation of Degradation in Epimerized LB Samples Using NMR Diffusion Measurements. A reaction of epimerization may degrade the polysaccharide and hence lower its molecular weight.

As previously reported for guar gum, the occurrence of such degradation may be checked by NMR diffusion measurements.

Molecular self-diffusion can be encoded into NMR data sets by means of pulsed magnetic field gradients.[24] As first proposed by Morris and Johnson,[25] the diffusion information may be conveniently represented in the form of a two-dimensional map showing the chemical shifts and the diffusion coefficients along the horizontal and vertical axis, respectively, according to the so-called diffusion ordered NMR spectroscopy (DOSY) scheme. Basically, DOSY allows compounds to be distinguished according to their size; this technique has been used in many applications.[26−31]

The DOSY maps of the experiments recorded on the C(6)-oxidized LB sample before (a) and after (b) epimerization with AlgE1+Alg4+AlgE6 are shown in Figure 5. These maps clearly show that the self-diffusion coefficient of the polysaccharide before and after epimerization remains the same within experimental errors. Note that, due to the presence of charges on the

Table 3. ^{1}H and ^{13}C NMR Assignments of Depleted, C(6)-Oxidized and C(5)-Epimerized Locust Bean Gum (DOLBE1+E4+E6) in a Buffered Solution (pD = 7) at T = 343 K and m^* = Branched Mannose Unit[a]

mannuronic acid residue			guluronic acid residue			galacturonic acid residue			branched mannose residue		
	^{1}H	^{13}C		^{1}H	^{13}C		^{1}H	^{13}C		^{1}H	^{13}C
*M*1M	4.68	102.2	M*G*1M	5.05	102.8	Gal1	5.05	101.1	m*1	4.76	102.2
*M*1G	4.71	103.4	*G*1G	5.06	102.8						
*M*2 M	4.05	72.1	M*G*2M	3.99	67.0	Gal2	3.85	70.5	m*2	4.13	72.1
*M*2G	3.95	72.1	*G*2G	3.91	67.3						
*M*3M	3.77	73.6	M*G*3M	4.17	71.6	Gal3	3.99	71.9	m*3		
			*G*3G	4.03	71.3						
*M*4M	3.93	80.3	M*G*4M	4.23	82.2	Gal4	4.32	73.0	m*4	3.84	
			*G*4G	4.13	82.1						
*M*5M	3.75	78.3	M*G*5M	4.73	69.7	Gal5	4.20	74.2	m*5	3.57	77.3
			G*G*5G	4.46	69.4						
			M*G*5G	4.44	69.4						
			G*G*5M	4.76	69.4						
									m*6, m*6′	3.83, 3.97	68.9

[a] Chemical shifts are reported in ppm with respect to a trace of DSS used as internal standard.

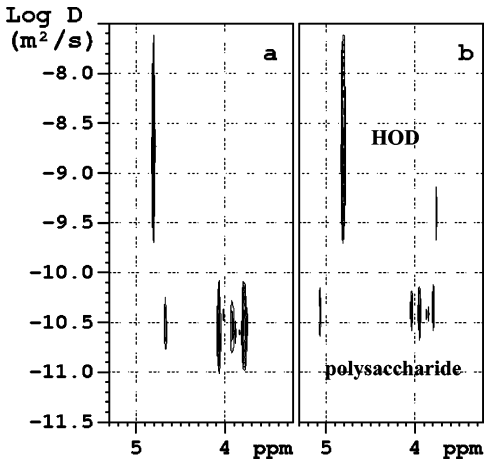


Figure 5. 2D DOSY maps recorded at 300 K on a phosphate buffered D_2O solution (pD = 7) containing 0.01 M NaCl of a depleted, C(6)-oxidized locust bean gum sample before (a) and after (b) epimerization with a mixture of AlgE1, AlgE4, and AlgE6.

polysaccharidic chains, at pD = 7, water is a good solvent both for the oxidized and the epimerized LB samples. Moreover, assuming that the viscosity of both solutions is approximately the same, the self-diffusion coefficients measured in the oxidized LB and in the epimerized derivative solutions are tightly related to the molecular size of the samples; thus any variation in the diffusion coefficient measured before and after the epimerization is related to a change in the molecular size of the polysaccharides which, in turn, implies a variation in their molecular weights. In our case, no significant difference is observed in the self-diffusion coefficient of the polysaccharide in both solutions; see Figure 5. Therefore, NMR diffusion experiments seem to indicate that no significant degradation, i.e., lowering of the molecular weight, occurred during the epimerization reaction. This experimental finding is in agreement with the substantial retention of the molecular weights, as evidenced by SEC-MALLS measurements, of the epimerization products of mannuronan by AlgE4 and AlgE1[32] and of oxided konjac by AlgE4, AlgE1, and AlgE6 (unpublished data) with respect to the starting biopolymers.

The diffusion dimension was processed according to an approximate inverse Laplace transform (ILT). The ILT processing of diffusion data has been extensively investigated by Morris and Johnson.[33] Many applications of the ILT to determine molecular size and molecular weight distributions have been reported in the literature.[34−36] These studies have shown that great care must be used when applying this evaluation scheme because, with default parameters, wide and physically artificial diffusion coefficient distributions can be obtained. This point was further emphasized in a recent study by Håkansson and co-workers.[37] As such, in our case, the width of the correlation spots that appear in the DOSY maps reported in Figure 5 are not to be correlated with the molecular weight distribution of the polysaccharides considered in this study. However, if certain conditions are respected,[33] the center of these spots can be regarded as a reliable measurement of the average self-diffusion coefficient of the polysaccharides. The DOSY maps reported in Figure 5 indicate that the molecular weight of the polysaccharide before and after epimerization remains of the same order of magnitude, although a slight lowering can be observed. Similar findings were obtained for the LB sample epimerized with AlgE1 only (data not shown).

3.5. Quantitative Evaluation of the Enzymatic Activity Using Depleted, C(6)-Oxidized LB as a Substrate. The presence of resonances assigned to G*G*5M, M*G*5M, G*G*5G, and M*G*5G triads as well as the presence of resonances assigned to *G*5G, *M*1M, and *M*1G diads allowed us to quantitatively evaluate the enzymatic activity and to obtain the statistical distribution of G blocks along the polysaccharidic chain. However, due to the overlapping of proton resonances, for evaluating the signal areas, a spectral deconvolution was required. Therefore, a full deconvolution of the 4.4−5.2 ppm region of the ^{1}H spectrum of all epimerized LB derivatives was performed. From the deconvolution, the areas of all signals resonating in this spectral range were obtained, along with the experimental uncertainty.

In Figure 6, the 4.4−5.2 ppm region of the ^{1}H spectra of DOLBE1 (a), DOLBE4 (b), DOLBE6 (c), DOLBE1E6 (d), DOLBE4E6 (e), and DOLBE1E4E6 (f) are shown; the deconvoluted spectra are superimposed on the experimental ones.

The resonance at 4.73 ppm, see Figure 6b, is due to the M*G*5M triads in accordance with the alternating MG sequence introduced by AlgE4. On the other hand, the resonances at 4.46, 4.44, and 4.76 ppm, see Figure 6, are due to the G*G*5G, M*G*5G, and G*G*5M triads, respectively. Indeed, the effect of AlgE1, AlgE6, and mixtures of AlgE1, AlgE4, and AlgE6 is to introduce also G blocks.

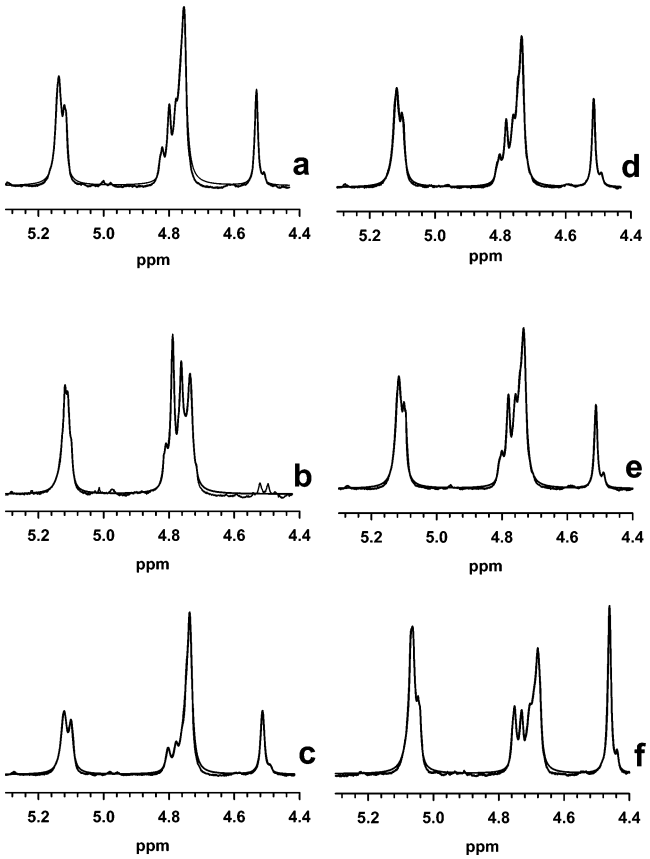


Figure 6. Expansion of the 4.4−5.2 ppm region of the ^{1}H NMR spectra of depleted, C(6)-oxidized locust bean gum samples epimerized with various enzymes: DOLBE1 (a); DOLBE4 (b); DOLBE6 (c); DOLBE1E4 (d); DOLBE4E6 (e); and DOLBE1E4E6 (f). The deconvoluted spectra (smooth lines) have been superimposed on the experimental ones.

The areas obtained from the best fit procedure allowed us to calculate the molar fraction of guluronic acid residues, F_G, the molar fraction of mannuronic acid residues, F_M, as well as the three doublet frequencies F_{G5G}, F_{M1G}, F_{M1M} and the four triplet frequencies F_{GG5M}, F_{MG5M}, F_{GG5G}, and F_{MG5G}. The average block length $N_G = (F_G - F_{MG5M})/F_{GG5M}$ was also obtained (Table 4). Details of the calculation have been previously reported.[8,9]

The highest molar fraction of guluronic acid residues, $F_G = 0.51$, was obtained for the sample epimerized with the mixture of all three enzymes whereas all other samples showed lower degrees of epimerization. The longest average block length, $N_G = 4.8$, was obtained using AlgE1.

3.6. Quantitative Evaluation of the Enzymatic Activity Using Mannuronan as a Substrate. Mannuronan samples were used as substrates for mannuronan C(5)-epimerases, AlgE1, Alg4, AlgE6, and their mixtures, to obtain the corresponding uronanes, namely ManE1, ManE4, ManE6, ManE1E6, ManE4E6, and ManE1E4E6.

For the sake of clarity, the ^{1}H and ^{13}C assignments of mannuronan and epimerized mannuronan samples are reported in Tables 5 and 6, respectively. The ^{1}H NMR spectra of ManE4 and ManE6 are shown in panels a and b of Figure 7, respectively.

Table 5. ^{1}H and ^{13}C NMR Assignments[a] of Mannuronan in a Buffered Solution (pD = 7) at $T = 343$ K

	mannuronic acid residue	
	^{1}H	^{13}C
M1	4.68	102.3
M2	4.05	72.1
M3	3.75	73.7
M4	3.92	80.4
M5	3.76	78.4

[a] Chemical shifts are reported in ppm with respect to a trace of DSS used as internal standard.

Table 6. ^{1}H and ^{13}C NMR Assignments[a] of Mannuronan Epimerized with AlgE6 in a Buffered Solution (pD = 7) at $T = 343$ K

mannuronic acid residue			guluronic acid residue		
	^{1}H	^{13}C		^{1}H	^{13}C
*M*1M	4.66	102.2	M*G*1M	5.04	101.7
*M*1G	4.68	103.3	*G*1G	5.05	102.8
*M*2M	4.02	72.2	M*G*2M	3.97	67.1
*M*2G	3.93	72.9	*G*2G	3.89	67.2
M3	3.72	73.6	M*G*3M	4.16	71.4
			*G*3G	4.02	71.3
*M*4M	3.90	80.1	M*G*4M	4.21	82.1
*M*4G	3.76	79.7	*G*4G	4.12	82.1
*M*5M	3.73	78.3	M*G*5M	4.71	69.8
			*G*5G	4.44	69.4
			G*G*5M	4.74	68.5

[a] Chemical shifts are reported in ppm with respect to a trace of DSS used as internal standard.

A full deconvolution of the 4.4−5.2 ppm region of the ^{1}H spectrum of the epimerized samples was performed. In Figure 8, the deconvoluted spectra are superimposed on the experimental ones. The molar fractions of M and G residues, F_M and F_G; the doublet frequencies, F_{GG}, F_{MM}, and F_{MG}; and the triplet frequencies, F_{MGM} and F_{GGM}, are reported in Table 7. In the case of ManE4, a molar fraction F_G of 0.47 was found, meaning that 47% of M residues were epimerized to G residues. Note that, because AlgE4 introduces MG blocks in the polysaccharidic chain, the degree of epimerization cannot be, in this case, higher than 50%. By contrast, in all other samples, F_G ranged from 0.70 up to 0.86, with the highest F_G value obtained by performing the epimerization in the presence of AlgE1. The shortest block length, $N_G = 9$, was obtained in the case of ManE4E6, whereas the longest ones, $N_G = 48$ and $N_G = 45$, were obtained for ManE1E4E6 and ManE1E6, respectively.

4. Circular Dichroism and Gel Formation Results

4.1. Kinetics of LB Epimerization Studied by Circular Dichroism. The CD technique is fast, requires a very small

Table 4. C(6)-Oxidized, C(5)-Epimerized LB Samples: Mannuronic and Guluronic Acid Molar Fractions (F_M, F_G), Doublet Frequencies (F_{MM}, F_{MG}, F_{GG}), Triplet Frequencies (F_{MGM}, F_{GGM}, F_{GGG}, F_{MGG}), and Average G-Blocks Length N_G

sample	F_M	F_G	F_{MM}	F_{MG}	F_{GG}	F_{MGM}	F_{GGG}	F_{GGM}	F_{MGG}	N_G
DOlBE1	0.66±0.04	0.34±0.02	0.57±0.03	0.087±0.005	0.18±0.01	0.120±0.007	0.17±0.01	0.046±0.0003	0.007±0.001	4.8±0.2
DOLBE4	0.67±0.04	0.33±0.02	0.39±0.02	0.28±0.02		0.33±0.02				
DOLBE6	0.68±0.04	0.32±0.02	0.60±0.03	0.080±0.004	0.19±0.01	0.061±0.004	0.18±0.01	0.067±0.004	0.009±0.001	3.9±0.2
DOLBE1E6	0.63±0.03	0.37±0.02	0.48±0.03	0.15±0.01	0.19±0.01	0.118±0.007	0.17±0.01	0.060±0.003	0.019±0.001	4.2±0.2
DOLBE4E6	0.64±0.03	0.36±0.02	0.49±0.02	0.15±0.01	0.161±0.008	0.151±0.007	0.140±0.007	0.046±0.002	0.020±0.001	4.5±0.2
DOLBE1E4E6	0.49±0.03	0.51±0.03	0.36±0.02	0.126±0.007	0.31±0.02	0.091±0.005	0.29±0.02	0.113±0.007	0.019±0.001	3.7±0.2

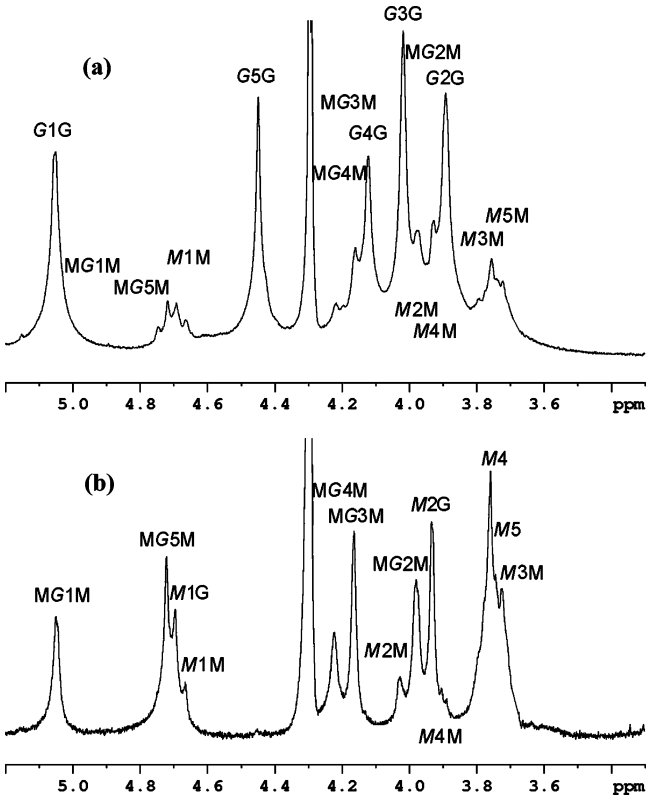


Figure 7. 600.13 MHz ^{1}H NMR spectra in a phosphate buffered solution at 343 K of (a) mannuronan epimerized with AlgE4; (b) mannuronan epimerized with AlgE6.

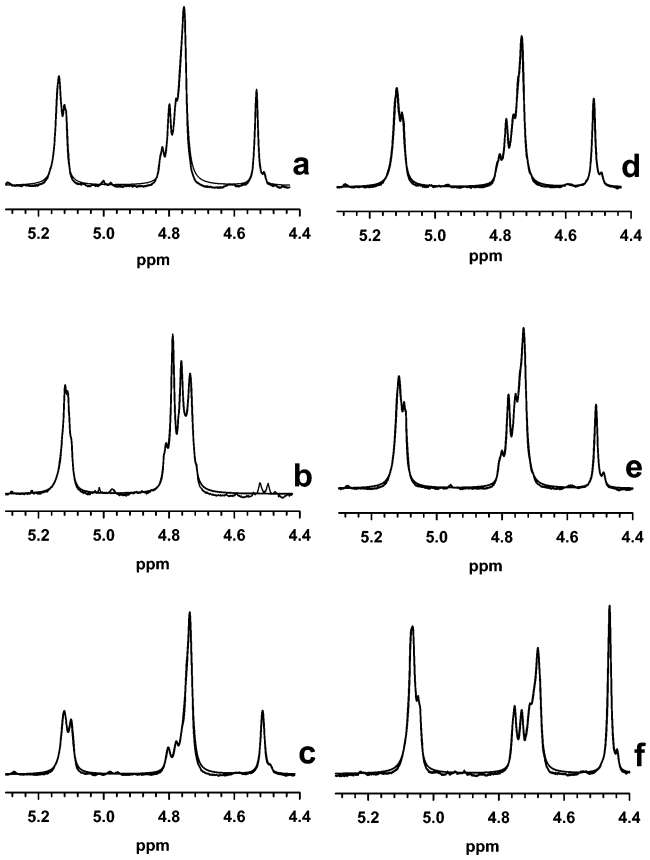


Figure 8. Expansion of the 4.4−5.2 ppm region of the ^{1}H NMR spectra of mannuronan samples with various enzymes: ManE1 (a); ManE4 (b); ManE6 (c); ManE1E4 (d); ManE4E6 (e); and ManE1E4E6 (f). The deconvoluted spectra (smooth lines) have been superimposed on the experimental ones.

amount of sample, and is particularly sensitive to variations in the ring geometry around the carboxyl chromophores; however, no quantitative information about the amount of epimerized residues can be obtained.

A CD spectrum of an alginate[38] results from the superposition of the CD spectra of M and G residues; the M residues show a negative band centered at about 215 nm and a positive band centered at about 200 nm, whereas G residues only show a negative band centered at 205 nm. The evolution of the CD spectra with time qualitatively reflects the transformation of D-mannuronic acid residues into L-guluronic acid residues along the chain.

During the epimerization reaction, the intensity of the positive band observed at 200 nm progressively decreased (Figure 9, panels a and b), indicating a decrease in the mannuronic acid content.

The CD spectra of ManE1 and DOLBE1 recorded at distinct times after the addition of AlgE1 indicate that the rates of epimerization of these two substrates are very different (Figure 9); this can clearly be ascribed to the residual galactose side moieties (8%) and mannose units (66%).

As previously observed in the case of guar gum,[39] the fastest initial epimerization was observed for the sample treated with AlgE4 (data not shown): after 4 h of incubation the spectrum did not change further. The same observation holds for all other samples after 24 h of incubation**.** This observation was fully confirmed by NMR: the ^{1}H spectra of the samples treated for 24 and 48 h were always identical (data not shown). As a consequence the epimerization reaction may be stopped after 24 h.

4.2. Influence of Added Ca(II) Ions on the CD Spectra of Epimerized LB Samples. This work aims at obtaining pseudoalginates that exhibit properties similar to those of naturally occurring alginates. In particular, one of the most interesting properties from an application point of view is their ability to form gels in the presence of divalent cations (e.g., Ca^{2+}). A way for evaluating whether pseudoalginates may undergo gelation in appropriate conditions is to study their behavior in dilute solution in the presence of Ca^{2+} ions. Provided that the proper polymer and Ca^{2+} concentration are chosen, a cooperative conformational transition is a clear indication that a gel can be formed.

Table 7. Epimerized Mannuronan Samples: Mannuronic and Guluronic Acid Molar Fractions (F_M, F_G), Doublet Frequencies (F_{MM}, F_{MG}, F_{GG}), Triplet Frequencies (F_{MGM}, F_{GGM}), and Average G-Blocks Length N_G

sample	F_M	F_G	F_{MM}	F_{MG}	F_{GG}	F_{MGM}	F_{GGM}	N_G
ManE1	0.14±0.01	0.86±0.04	0.053±0.003	0.086±0.004	0.77±0.04	0.061±0.003	0.026±0.006	31±7
ManE4	0.53±0.03	0.47±0.02	0.51±0.03	0.024±0.001		0.47±0.02		
ManE6	0.22±0.01	0.78±0.05	0.157±0.009	0.066±0.004	0.70±0.02	0.054±0.003	0.025±0.001	30±5
ManE4E6	0.30±0.03	0.70±0.06	0.089±0.006	0.21±0.01	0.44±0.03	0.20±0.01	0.06±0.01	9±2
ManE1E6	0.18±0.01	0.82±0.06	0.070±0.005	0.111±0.008	0.70±0.05	0.101±0.008	0.016±0.001	45±4
ManE1E4E6	0.14±0.01	0.86±.04	0.042±0.02	0.101±0.005	0.76±0.03	0.085±0.004	0.016±0.005	48±7

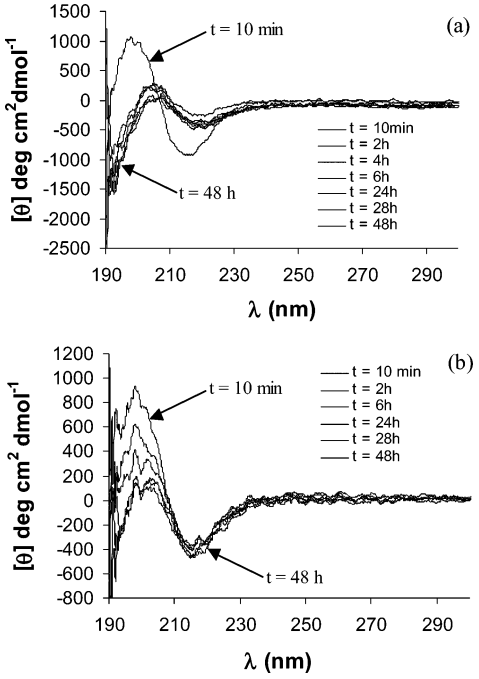


Figure 9. Time course of the partial epimerization of mannuronan (a) and of depleted and C(6)-oxidized locust bean gum (b) in dilute aqueous solution as monitored by circular dichroism at 40 °C (50 mM Tris/HCl buffer; pH = 6.9; 50 mM $NaClO_4$; 2 mM $CaCl_2$; polymer concentration, 0.1% w/v). Enzyme employed: AlgE1, 0.0045% w/v.

For each sample, a series of spectra were run as a function of the *R* ratio (see the Experimental Section). In all samples, as *R* increased, a marked variation of the spectral trend was observed, demonstrating the existence of a well-defined interaction between Ca(II) ions and epimerized LB samples in dilute solutions. In Figure 10, the CD spectra as well as the difference spectra constructed by subtracting the spectrum at $R = 0$ from the spectra at all other values of *R* of MANE1E4E6 and DOLBE1E4E6 as a function of *R* are reported. It is evident that the extent of the variation of the ellipticity is more pronounced in the case of MANE1E4E6: this finding is in agreement with the presence of more extended G blocks in MANE1E4E6 than in DOLBE1E4E6 (Tables 4 and 7). Because this study is primarily aimed at obtaining Ca(II)-induced gels from DOLBs samples, the possibility for epimerized LB samples to specifically interact with Ca(II) ions was assessed.[40] Therefore, CD measurements were made on dilute solutions of these polysaccharides (C_p = 0.02% w/v). By plotting the specific ellipticity $[\theta]_{215}$ as a function of *R*, sigmoid traces were obtained for both MANs and DOLBs samples (Figure 11). This is a clear evidence of the existence of a cooperative interaction between the epimerized polysaccharides (i.e., epimerized mannuronans and locust bean samples) and Ca^{2+} ions even though the extent of the transition is much less marked in the latter case. Again, this is a consequence of the presence of less extended stretches of GG, MG, and MM blocks characterizing the primary structure of epimerized locust bean samples with respect to those of the epimerized mannuronans.

Due to the presence of a specific interaction between the epimerized locust bean samples and the Ca(II) ions, these samples are expected to form aqueous gels in more concentrated solutions.

4.3. Gel Formation in Concentrated Solutions of Epimerized LB Samples. The qualitative behavior evidenced by CD experiments is supported by the experimental conditions required to form the gels. As reported in section 2.4, homogeneous gels from epimerized DOLB samples were obtained following the controlled release of Ca(II) ions from the Ca(II)−EDTA complex. The released Ca(II) ions favor the establishment of lateral association among different chains leading to gel formation.

As previously reported,[41,42] epimerised mannuronans give rise to the formation of strong gels at a polymer concentration ranging between 0.1 and 0.7% w/v. Whereas, the polymer concentration in DOLBs had to be raised to at least 1% w/v. Furthermore, a first set of samples, namely DOLBE1, DOLBE1E6, and DOLBE4E6, gelled at a relatively lower concentration (1% w/v), whereas a second set of samples,

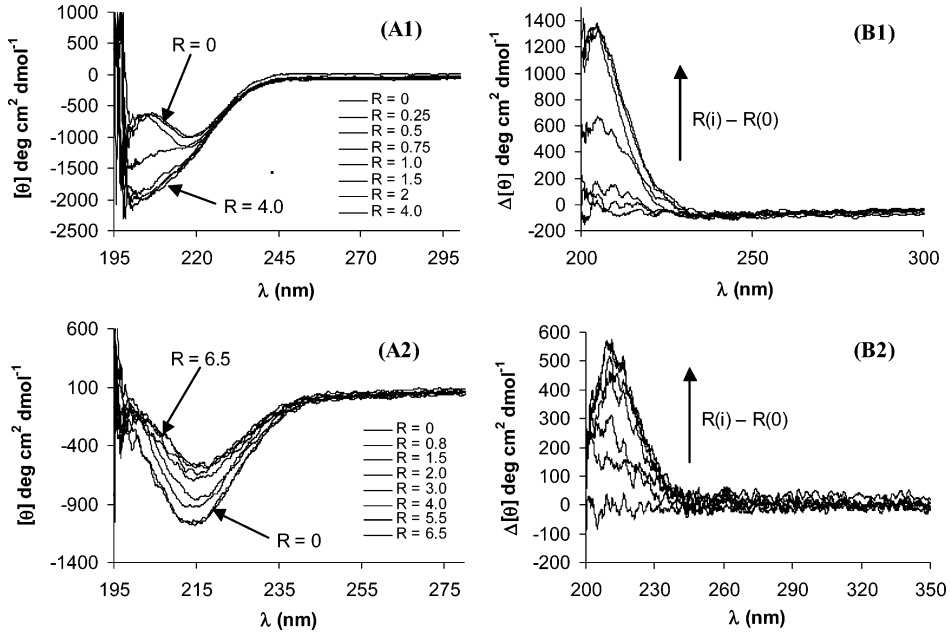


Figure 10. CD spectra dependence on Ca(II) ion concentration of (A) mannuronan and (B) depleted and C(6)-oxidized locust bean gum both partially epimerized with Alg1+4+6 mixed enzymes. In column 1 the CD spectra are reported, in column 2 the differences spectra constructed by subtracting the spectrum at $R = 0$ from the spectra at all other values of *R* ($R(i) - R(0)$) are shown. The polymer concentration is 0.02% w/v (50 mM Tris/HCl buffer; pH = 6.9; 50 mM $NaClO_4$). *R* is the concentration ratio (Ca(II) ions/polymer) in equivalents.

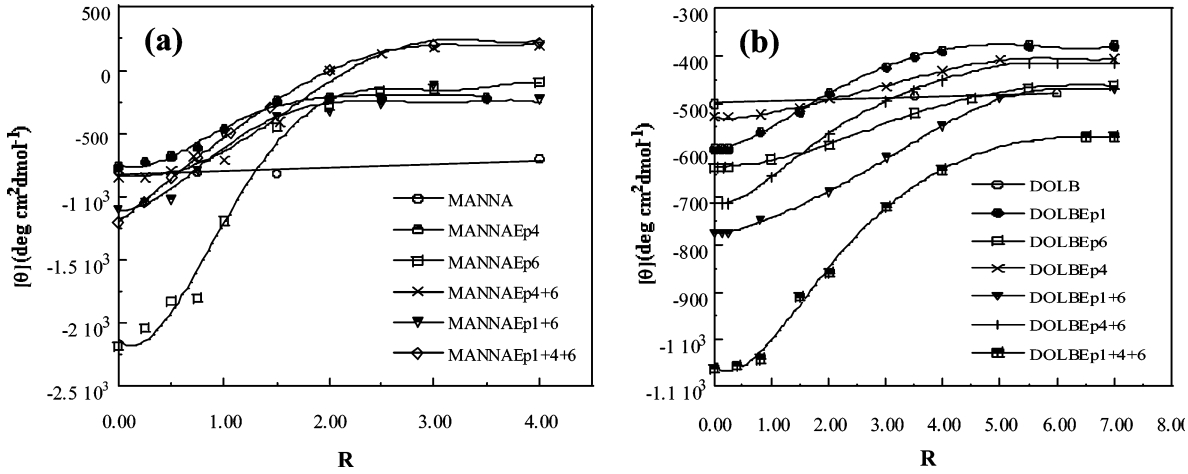
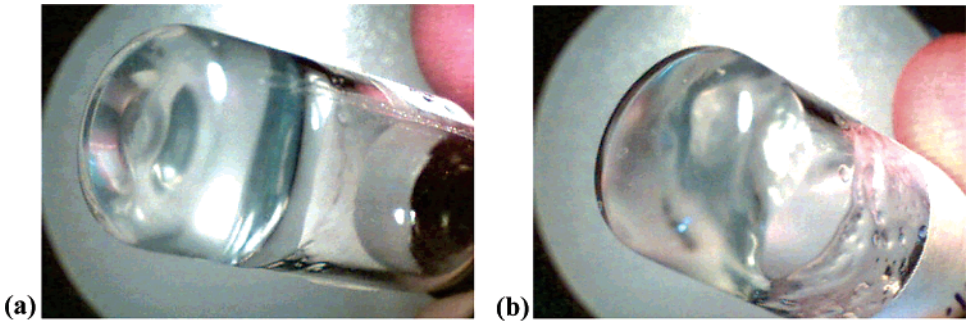


Figure 11. Molar ellepticity [θ] (at 215 nm) dependence on the concentration ratio *R* (Ca(II) ions/polymer) in equivalents of (a) epimerised mannuronans, (b) depleted, C(6)-oxidized and epimerised locust bean samples.



	R (equivalents Ca(II)/equivalents COONa)	C_p (%,w/v)
DOLBE1	1.1	1
DOLBE6	1.1	2
DOLBEp4E6	1.1	1

	R (equivalents Ca(II)/equivalents COONa)	C_p (%,w/v)
DOLBE4	1.1	2
DOLBGE6	1.1	1.2

Figure 12. Examples of Ca(II)-gels obtained from depleted, C(6)-oxidated locust bean gum epimerized with different C(5) epimerases at polymers concentrations and *R* (see the Experimental Section) values indicated in the table underneath. In particular, gels obtained from DOLBE1 and DOLBE4 are shown in (a) and (b), respectively.

DOLBE6 and DOLBE4, gelled at a higher concentration (2% w/v). Moreover, according to the qualitative tilting test, the first set of gels was stable under gravity stress, whereas the second set of gels flowed slightly, indicating that these gels were relatively weaker than those belonging to the first set. Figure 12 summarizes the experimental conditions and illustrates the two different kinds of behavior observed. It is worth noting that this study has confirmed that pseudoalginates that do not have any G blocks, such as those exhibiting an exclusively MG primary structure, may also form gels with calcium ions.[41,42]

5. Conclusions

A new family of artificial uronanes was obtained. Galactose-depleted, C(6)-oxidized and C(5)-epimerized locust bean samples were able to act as a substrate for AlgE1, AlgE4, AlgE6, and their mixtures. The kinetics of epimerization showed that the reaction was complete after 24 h. Although the degree of epimerization in DOLBs samples was lower than that observed in Mans, the nonrandom epimerization pattern of C(5)-epimerases was basically the same: AlgE4 only introduced single G residues, whereas AlgE1 and AlgE6 also formed G blocks with average G block length, N_G, ranging between 3.7 and 4.8. However, even if the average G block length in DOLBs was much shorter than in Mans samples, all epimerized DOLBs samples in dilute solutions interacted with Ca(II) ions and formed wall to wall gels in concentrated solutions.

Furthermore, the gel formation was also observed in pseudo-alginates with exclusively MG blocks.

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