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Note

Structural characterization of homogalacturonan by NMR spectroscopy—assignment of reference compounds

Bent O. Petersen [a], Sebastian Meier [a], Jens Ø. Duus [a], Mads H. Clausen [b,*]

[a] Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Valby, Denmark
[b] Department of Chemistry, Technical University of Denmark, Kemitorvet, Building 201, DK-2800 Kgs. Lyngby, Denmark

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ABSTRACT

Complete assignment of ^{1}H and ^{13}C NMR of six hexagalactopyranuronic acids with varying degree and pattern of methyl esterification is reported. The NMR experiments were run at room temperature using approximately 2 mg of sample making this method convenient for studying the structure of homogalacturonan oligosaccharides.

Pectin is an important component of plant cell walls and has found widespread application in the food industry as a stabilizer and gelling agent.[1] Due to their key role in plant cell development, the identification of the molecular structure of pectic polysaccharides in different plant tissues is of fundamental importance to the understanding of plant cell physiology. However, pectic polysaccharides are very heterogeneous in their composition, which renders the structural study of these glycans extremely challenging.[2]

Homogalacturonan (HG), an α-(1→4)-linked polymer of galacturonic acid, is the major component of pectic polysaccharides. HG can be methyl esterified and acetylated depending on the plant source. It is believed that HG is synthesized as a highly methyl esterified form in the Golgi apparatus and then de-esterified enzymatically in the plant cell wall.[2,3] Traditionally, the extent and pattern of methyl esterification of oligogalacturonans has been investigated using mass spectrometry.[4] While the degree of esterification of HG has been studied using NMR spectroscopy,[5] the heterogeneity of HG and the lack of well-defined model oligomers have precluded detailed NMR studies of the esterification pattern of HG. Such studies are valuable for the identification of HG oligomers derived from natural sources, the detection of non-random methylation patterns in natural polysaccharides, and eventually in the study of polymer composition and structure.

NMR studies of unmethylated homogalacturonanes with a degree of polymerization ranging from three to sixteen have been reported previously.[6] The ^{13}C signals originating from methylated and unmethylated galacturonic acid residues in native pectin samples have been reported,[7] and the chemical shifts of the C6 carbonyl carbons of the esters were shown to depend on the methylation state of neighboring residues, which has allowed the identification of methylation as primarily blockwise or random.[8]

A recent effort to prepare oligogalacturonides with a well-defined pattern of methyl esterification by de novo chemical synthesis[9] culminated in the preparation of the five hexagalactopyranuronic acids **1–5** shown in Figure 1.[10] The extent and pattern of methyl esterification follows from the synthetic route and was verified by MS–MS studies. So far, these oligomers have served as probes in enzymatic studies[11] and as haptens in the elucidation of the epitopes of several anti-HG monoclonal antibodies.[12]

With their relatively small size and well-defined patterns of methyl esterifications, these molecules are ideal as reference compounds for NMR studies. More knowledge of how the esterification pattern influences the spectral properties of HG will lead to systematic applications of NMR spectroscopy in the study of pectic polysaccharides, in particular HG. To this end, we have obtained complete NMR assignments of all five reference compounds and unmethylated hexagalacturonan (**6**).[13]

Representative examples of a gHSQC and a gHMBC spectrum obtained in this study are shown in Figure 2, and the full assignments of all ^{1}H and ^{13}C resonances for compounds **1–6** are given in Table 1.

As can be seen from the data, it was possible to distinguish and assign the resonances originating from the α- and β-configured reducing end residues. For the remaining sugar residues, the effect from the anomers was not detectable under the conditions used in

* Corresponding author. Tel.: +45 45252131; fax: +45 45933968.
E-mail address: mhc@kemi.dtu.dk (M. H. Clausen).

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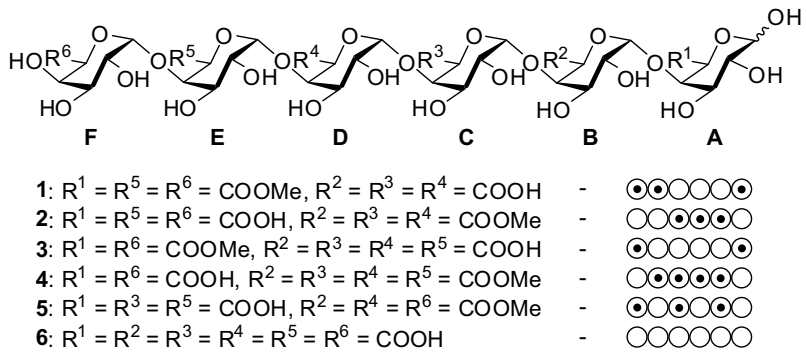

Figure 1. The structures of the six hexagalacturonanes showing methylation pattern and the designation (A–F) of individual residues.

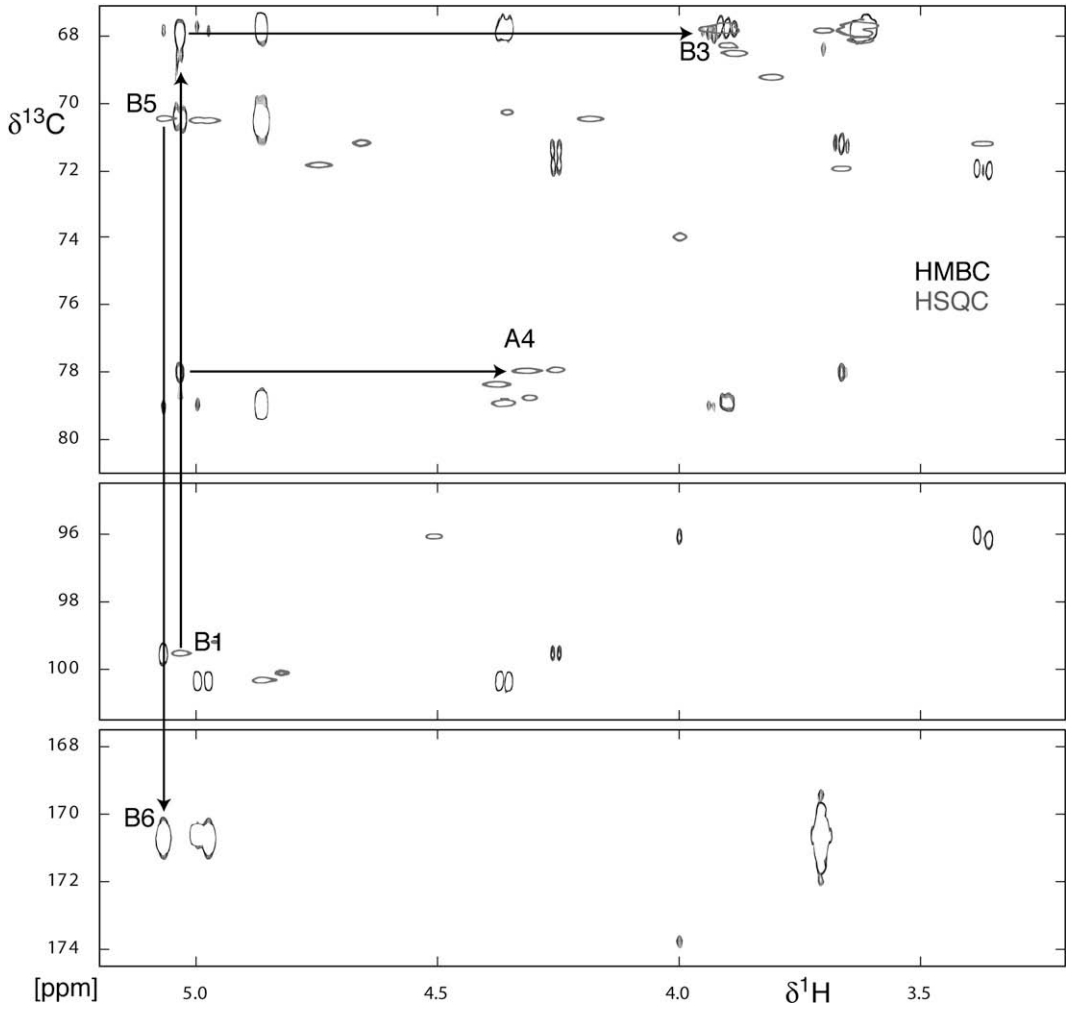

Figure 2. Overlay of HSQC (grey) and HMBC (black) spectra of oligosaccharide **2**. Correlations in the HMBC spectrum from the B5 ^{1}H resonance to B1 and B6 ^{13}C resonances are labeled, as well as the correlations from the B1 ^{1}H to both B3, B5 ^{13}C resonances and to A4 across the glycosidic bond.

this study, although a distinction has previously been reported also for the second pyranose residue from the reducing end of unmethylated oligogalacturonanes.[6]

HMBC correlations from H1′ to C4 through residue 2–6 confirmed the (1→4) glycosidic linkages (Fig. 2). The pyranose rings at the non-reducing end can be distinguished from the other residues by their ^{13}C4 shift of 70–71 ppm, whereas the substituted residues have ^{13}C4 shifts in the range 77–79 ppm, thus further supporting the (1→4) glycosidic linkages.

The presence or absence of a methyl ester in the 6-positions was evident from correlations between H5 and methyl ester protons via scalar couplings and nuclear Overhauser enhancements in COSY and NOESY spectra, respectively. As can be seen from Table 1, the ^{13}C carbonyl shifts also serve as distinctive reporters

Table 1
^{1}H and ^{13}C data for hexagalacturonates **1–6**

	1	2	3	4	5	6	Me
1							
A-α	5.365	3.833	4.044	4.512	4.845		3.827
	93.0	68.4	68.5	79.3	70.5	171.4	53.6
A-β	4.682	3.514	3.815	4.459	4.510		3.827
	97.0	71.8	72.2	78.4	73.9	169.5	53.6
B	4.955	3.785	4.010	4.450	4.725		
	101.0	68.9	69.4	79.6	72.1	176.0	
C	4.945	3.775	4.025	4.450	4.745		
	101.0	68.6	69.3	79.6	72.1	176.0	
D	5.092	3.760	4.028	4.420	4.775		
	99.6	68.9	69.5	79.6	72.1	176.0	
E	5.121	3.744	4.025	4.449	5.177		3.823
	100.3	68.7	68.6	79.6	71.2	171.4	53.5
F	4.925	3.715	3.923	4.339	5.090		3.807
	101.1	68.5	69.3	70.7	72.0	172.0	53.4
	1	**2**	**3**	**4**	**5**	**6**	**Me**
2							
A-α	5.314	3.814	4.008	4.417	4.471		
	92.9	68.6	68.6	79.6	71.1	175.8	
A-β	4.614	3.476	3.772	4.362	4.107		
	96.9	71.9	72.7	78.8	74.8	175.8	
B	5.142	3.729	4.048	4.480	5.180		3.821
	100.4	69.0	68.6	79.8	71.3	171.5	53.6
C	4.973	3.716	4.008	4.466	5.107		3.813
	101.2	68.5	68.5	79.2	71.3	171.3	53.6
D	4.973	3.736	3.999	4.490	5.085		3.813
	101.2	68.6	69.1	79.2	71.3	171.5	53.6
E	4.933	3.755	3.991	4.428	4.772		
	100.9	68.6	69.3	78.8	72.0	175.8	
F	5.075	3.730	3.916	4.293	4.860		
	100.0	68.6	70.0	71.3	72.7	175.8	
	1	**2**	**3**	**4**	**5**	**6**	**Me**
3							
A-α	5.365	3.824	4.035	4.477	4.837		3.822
	93.0	68.3	68.3	79.3	70.2	171.3	53.5
A-β	4.672	3.507	3.800	4.419	4.508		3.822
	97.0	71.6	72.0	78.5	73.8	170.4	53.5
B	4.949	3.759	4.029	4.459	4.339		
	101.0	68.6	69.0	79.3	70.6	174.7	
C	4.949	3.759	4.029	4.459	4.339		
	101.0	68.6	69.0	79.3	70.6	174.7	
D	4.949	3.759	4.029	4.459	4.339		
	101.0	68.6	69.0	79.3	70.6	174.7	
E	4.949	3.759	4.029	4.459	4.339		
	101.0	68.6	69.0	79.3	70.6	174.7	
F	5.093	3.736	3.944	4.336	5.142		3.809
	100.3	68.5	69.2	70.6	71.8	171.9	53.4
	1	**2**	**3**	**4**	**5**	**6**	**Me**
4							
A-α	5.322	3.812	4.021	4.435	4.523		
	93.0	68.7	69.1	79.6	71.0	175.1	
A-β	4.623	3.484	3.792	4.379	4.165		
	96.9	71.9	72.7	78.8	74.6	174.9	
B	5.141	3.735	4.051	4.482	5.179		3.822
	100.4	68.8	68.6	79.8	71.3	171.6	53.3
C	4.978	3.714	4.020	4.477	5.110		3.815
	101.2	68.5	68.7	79.8	71.3	171.6	53.3
D	4.978	3.727	4.009	4.472	5.100		3.815
	101.2	68.5	68.7	79.8	71.3	171.6	53.3
E	4.978	3.741	4.015	4.466	5.090		3.815
	101.2	68.5	68.7	79.4	71.4	171.6	53.3
F	4.931	3.727	3.902	4.307	4.876		
	101.1	68.6	69.8	71.1	72.4	175.1	
	1	**2**	**3**	**4**	**5**	**6**	**Me**
5							
A-α	5.322	3.822	4.030	4.440	4.545		
	93.0	68.5	68.6	79.4	70.8	176.5	
A-β	4.637	3.493	3.786	4.390	4.187		
	96.9	71.8	72.7	78.6	74.5	174.7	
B	5.142	3.759	4.057	4.494	5.172		3.825
	100.4	68.7	68.6	79.1	71.2	171.4	53.5
C	4.943	3.730	4.013	4.436	4.887		
	100.8	68.6	68.9	79.1	71.6	174.7	
D	5.112	3.757	4.032	4.486	5.158		3.825
	100.4	68.7	68.6	79.4	71.2	171.4	53.5
E	4.943	3.730	4.013	4.436	4.887		
	100.8	68.6	68.9	79.1	71.6	174.7	
F	5.078	3.723	3.935	4.335	5.136		3.802
	100.3	68.6	69.3	70.7	71.9	172.1	53.4
	1	**2**	**3**	**4**	**5**	**6**	
6							
A-α	5.303	3.843	3.973	4.429	4.413		
	92.8	68.6	69.6	78.8	71.2	176.0	
A-β	4.593	3.503	3.753	4.387	4.073		
	96.9	72.1	73.0	77.8	74.9	175.2	
B	5.103	3.773	4.018	4.433	4.762		
	99.7	68.9	69.5	78.4	72.0	176.2	
C	5.073	3.758	3.993	4.403	4.756		
	99.6	68.9	69.5	78.6	72.0	176.2	
D	5.073	3.758	3.993	4.403	4.756		
	99.6	68.9	69.5	78.6	72.0	176.2	
E	5.076	3.761	4.003	4.420	4.756		
	99.6	68.9	69.6	78.6	72.0	176.2	
F	5.043	3.703	3.903	4.253	4.713		
	99.7	69.1	70.3	71.6	72.9	176.9	

of the methylation state of C6, as the methylated carbonyl ^{13}C resonances were found in the 174–176 ppm range, while all carboxylic acid signals lie in the well-separated 170–171 ppm range. Also the $^{1}H5$ resonances showed a clear dependence on the methylation state, with methyl ester substituted residues 2–6 resonating from 4.99 to 5.12 ppm and acids from 4.27 to 4.81, while the H5 protons of reducing end residues generally exhibited lower chemical shifts, with the β-configured sugars having $^{1}H5$ resonances with the lowest frequencies. Accordingly, chemical shift values of $^{13}C5$ resonances adjacent to methyl-esters are reduced by 0.5–1 ppm relative to $^{13}C5$ resonances in free galacturonate moieties. As a result, the C5–H5 region of ^{1}H–^{13}C HSQC spectra is a very well-suited indicator of methylation in partially methylated oligogalacturonates, where resonances of galacturonate esters are well separated from resonances of galacturonate.

In conclusion, we have obtained full ^{1}H and ^{13}C chemical shifts assignments of the six reference compounds **1–6** (Fig. 1 and Table 1). The data were obtained using only a few milligram of each oligomer, and NMR data were obtained from millimolar samples at 298 K. This is a clear advantage compared to earlier studies, where larger sample amounts and/or higher temperatures were used for the acquisition. The method described herein and the reference values reported from the NMR spectra of partially methylated oligogalacturonates will aid future applications of NMR in the study of complex HG oligomers from plant sources. Although the samples studied here were more than 95% pure, the method should work equally well for samples of biological origin where purity is often in the 60–80% range. Specifically, the patterns of methyl esterification found in hexagalacturonate **1–5** should be easily identified from NMR data of larger oligomers of plant origin. Similarly, the small amount of material needed for data acquisition should aid in studying HG polysaccharides.

1. Experimental

Unmethylated hexagalacturonan and hexagalactopyranuronic acids were synthesized and purified as previously described.[10,13] All NMR spectra were recorded on a Bruker Avance 800 NMR spectrometer equipped with a TCI cryoprobe at 298 K. Carrier frequencies were 799.96 MHz (^{1}H) and 201.12 MHz (^{13}C). Samples were prepared by dissolving the hexasaccharide (approximately 2 mg)

in 0.6 mL D_2O and transferring the solutions to 5 mm tubes. Acetone was used as an internal standard with ^1H at 2.22 ppm and ^{13}C at 30.89 ppm. Assignments were derived using Bruker standard homo- and hetero-nuclear pulse programs for DQF-COSY, NOESY (800 ms mixing time), TOCSY (100 ms spinlock time), ^1H–^{13}C HSQC, and HMBC. Homonuclear spectra were recorded as data sets with 4096 (F2) × 1024 (F1) complex data points with acquisition times of 328 (F2) and 82 (F1) ms. ^1H–^{13}C correlated gHSQC spectra were recorded as data sets of 2048 (^1H) × 1024 (^{13}C) complex data points with acquisition times of 163 (^1H) and 32 (^{13}C) ms, while multiple bond correlated HMBC spectra with suppression of one-bond correlations were obtained as data sets of 2048 (^1H) × 512 (^{13}C) complex data points (acquisition times 192 (^1H) and 6 (^{13}C) ms). All spectra were processed with zero filling in both dimensions in TOPSPIN 2.0. Spectra were evaluated with the programs TOPSPIN 2.0 and PRONTO.

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