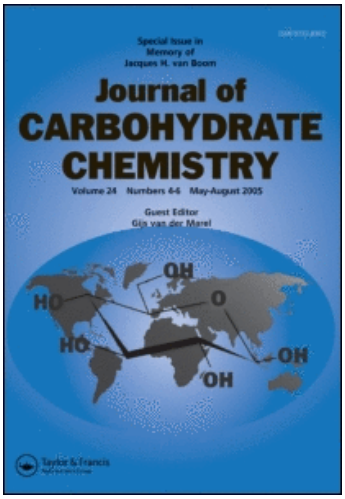


Journal of Carbohydrate Chemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

IDENTIFICATION OF THE EXOPOLYSACCHARIDE AMYLOVORAN BY NMR[1]

Roger Busson[a]; André De Bruyn[b]; Piet Herdewijn[a]; Martine Maes[c]; Eric Messens[d]
[a] Laboratory of Medicinal Chemistry, Rega Institute, Katholieke Universiteit Leuven, Leuven, Belgium [b] Department of Organic Chemistry, Krijgslaan, Belgium [c] Department of Crop Protection, Agricultural Research Centre, Merelbeke, Belgium [d] Department of Molecular Genetics, Ghent University, Gent, Belgium

Online publication date: 31 March 2001

To cite this Article Busson, Roger , De Bruyn, André , Herdewijn, Piet , Maes, Martine and Messens, Eric(2001) 'IDENTIFICATION OF THE EXOPOLYSACCHARIDE AMYLOVORAN BY NMR[1]', Journal of Carbohydrate Chemistry, 20: 2, 109 — 120
To link to this Article: DOI: 10.1081/CAR-100103952
URL: http://dx.doi.org/10.1081/CAR-100103952

J. CARBOHYDRATE CHEMISTRY, 20(2), 109–120 (2001)

IDENTIFICATION OF THE EXOPOLYSACCHARIDE AMYLOVORAN BY NMR[1]

Roger Busson,[1] André De Bruyn,[2,*] Piet Herdewijn,[1] Martine Maes,[3] and Eric Messens[4]

[1] Laboratory of Medicinal Chemistry, Rega Institute, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium
[2] Department of Organic Chemistry, Krijgslaan 281 S4, Belgium
[3] Department of Crop Protection, Agricultural Research Centre, B-9820 Merelbeke, Belgium
[4] Department of Molecular Genetics, Ghent University, K. L. Ledeganckstraat 35, B-9000 Gent, Belgium

Dedicated to Professor Joachim Thiem on the occasion of his 60th birthday.

ABSTRACT

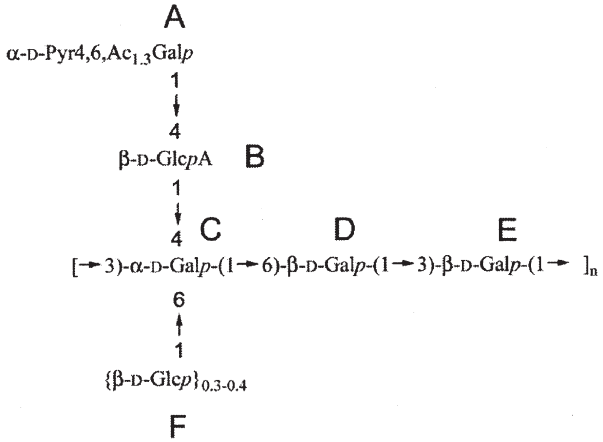
Structural analysis of the exopolysaccharide (EPS) amylovoran produced by different natural *Erwinia amylovora* isolates revealed repeating pentasaccharide substructures substituted 30–40% with a sixth monosaccharide when isolated from host plants of the *Malaceae* species. Only a pentasaccharide substructure was found for pathogens isolated from *Rubus* plants. The differences between both substructures, obtained after treatment of the amylovorans with a depolymerase, were shown with NMR. The host range of fire blight bacteria could be partially found in this difference.

*Corresponding author.

INTRODUCTION

Fire blight is an important quarantine disease on pear and apple trees. Besides fruit trees, other host plants have been described for the fire blight pathogen *Erwinia amylovora.* They all belong to the Rosales order. Among these are the Malaceae hosts, and the *Rubus* spp. (e.g., raspberries) that belong to the family of the Rosaceae. *E. amylovora* isolates from *Rubus* are not infective on Malaceae[2–5] and strains infective on Malaceae hosts show highly reduced pathogenicity towards *Rubus* spp.[3] To control further spread of the disease, more understanding is needed on the pathogenicity of *E. amylovora* and its occurrence and compatibility with the different hosts. In the process of infection, the bacterial coat is an essential virulence factor because it participates in the first intimate contact with the host plant system. The exopolysaccharide amylovoran is the main compound of the *E. amylovora* coat. Structural analysis of the exopolysaccharide (EPS) amylovoran produced by different natural *E. amylovora* isolates is now accessible. A phage depolymerase enzymatic cleavage of the polymer led to the isolation of oligosaccharide repeating substructures such as penta- or hexasaccharides.[6, 7] When produced by virulent strains infective on host plants of the *Malaceae* species, the penta-saccharide repeating substructure is substituted about 30–40% with a sixth monosaccharide (glucose). With fire blight isolates from the *Rubus* sp., the EPS showed not a mixture of hexa- and pentasaccharide substructures, but only a pentasaccharide repeating unit.

Nimtz *et al.*[7] elucidated the structure of the pentasaccharide-repeating unit from mass spectral data and a combination of ^{1}H and ^{13}C 1D and 2D NMR techniques. They also proposed a structure for the hexasaccharide, based mainly on mass spectral data and on partial NMR data. The oligosaccharide-repeating unit of the *E. amylovora* strain Ea1/79 according to Nimtz *et al.*[7] is given in Scheme 1 with structure and letter code of the monosaccharide residues. F is the additional (1→6)-β-D-glucopyranosyl residue, shown to be present at only 10%, and not well characterized. Only four chemical shifts for residue F were given in a Table footnote. In the strains that we have studied, we followed the EPS isolation procedure of



Scheme 1.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Nimtz *et al.*[7] to exclude levan or other polysaccharide contamination in the samples. We find that the proportion of hexasaccharide *vs.* pentasaccharide is 30–40%. The higher hexasaccharide content allows a more thorough NMR study and the extraction of a complete set of parameters for the F-residue. Our results substantiate the structural proposals.[7]

RESULTS AND DISCUSSION

Rearrangements

In the native form of the *E. amylovora* strain Ea1/79 according to Nimtz *et al.*[7] residue A is a non-*O*-acetylated (10%), 2-*O*-acetylated (26%), 3-*O*-acetylated (24%), or 2,3-di-*O*-acetylated (40%) (1→4)-α-D-galactopyranosyl residue and bears a 4,6-bound pyruvate group in the R-configuration. Two months after first recording the ^{1}H NMR spectrum of the compounds, we noticed important changes in the spectrum. The disappearance of the resonances in the region δ 5.40–5.55, accompanied with an increase of the δ 5.35 signal, indicated that the compounds, and especially the diacetate, slowly deacetylated under the measuring conditions (pH=3 and 27°C). This observation was confirmed by the changes in the acetyl region at δ 2.04–2.10 where all peaks, but especially the two peaks at higher field, became much weaker while the methyl proton peak of the free acetic acid at δ 1.994 showed an increase in intensity. The appearance of new small peaks in this region may possibly be explained by some transesterification instead of hydrolysis. The new acetylation sites, however, could not be identified. This result adds some uncertainty about the real acetylation assignments for native amylovoran.

^{1}H and ^{13}C NMR Data

The ^{1}H NMR spectra for the investigated samples are given in Figure 1A (malaceae-type compound) and Figure 1B (rubus-type compound), and their corresponding ^{13}C NMR spectra (only the glycosidic region, δ 90 − δ 106) in Figures 2A and 2B. More detailed ^{1}H NMR data for the malaceae-type hexasaccharide compound are presented in Table 1.

For the analysis of the ^{1}H NMR spectra, published data[7] were taken as a starting point and were used in a comparative way. The ^{1}H NMR resonances were unequivocally assigned using selective excited TOCSY experiments. Otherwise, correlations with directly bonded carbons as observed in GHSQC spectra were of great help for the assignments (Table 2). Only the ^{13}C shifts of glycosidic and hydroxymethylene carbons could be assigned with certainty.

Two groups of relevant ^{1}H resonances (data not in the tables) are the methyl peaks found in the region δ 2.0–2.1 and δ 1.4–1.5. In the former region, the methyl resonances for the acetyl groups were found as was mentioned above. Two intense peaks at high field side (δ 2.04 and δ 2.06) could be assigned to the acetyl methyls of the diacetylated compound, and the two peaks δ 2.08 and δ 2.10 to the acetyl

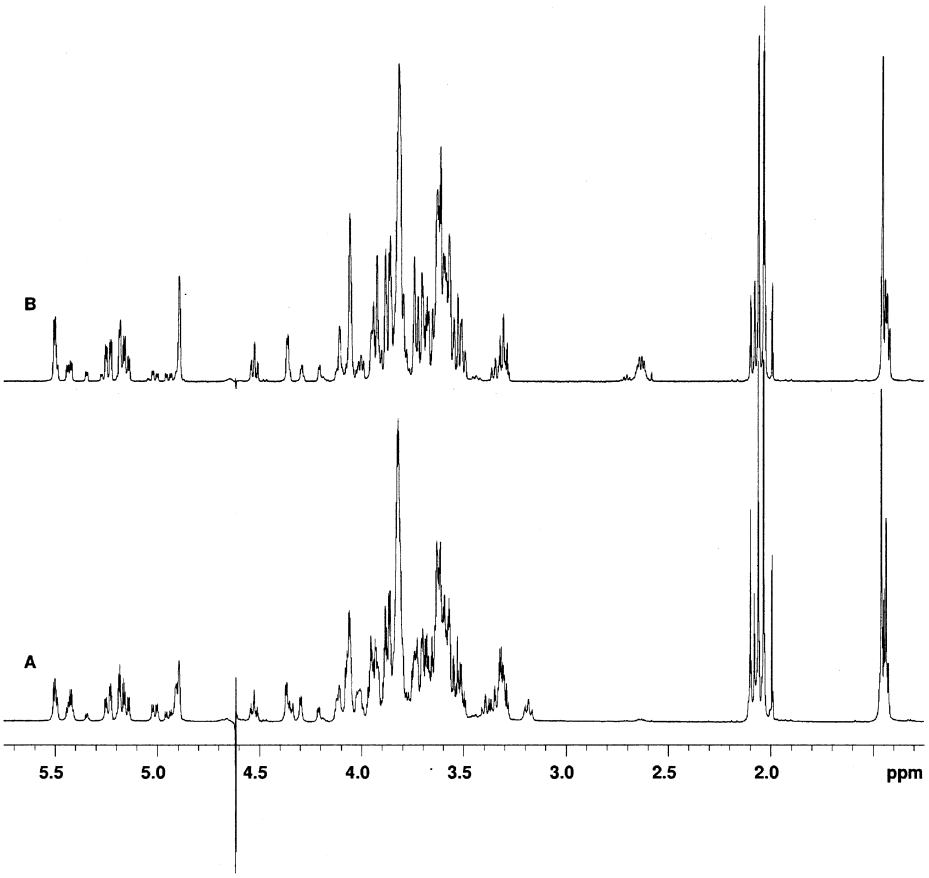


Figure 1. A. ^{1}H NMR spectrum of the malaceae-type amylovoran penta-(and hexa)-oligomers. B. Idem of the rubus-type amylovoran penta-oligomer. See experimental part for the recording conditions.

methyls of the two monoacetylated compounds. There is also a peak at δ 1.994 that is most likely due to some free acetic acid present in the sample. At δ 1.46, 1.44/1.43 and 1.42 we found the resonances for the pyruvate methyl group of the diacetylated, the monoacetylated or non-acetylated A residue.

Much more information, however, is derived from study of the resonances of the glycosidic protons (δ 4.3–5.6) and carbons (δ 90–106). The latter can be typically divided into four groups:[8, 9] β anomeric carbons of non-reducing sugars normally absorb at the low field end around δ 104 and α-anomers at about δ 100, while reducing sugars usually show absorption peaks at around δ 96 or 92 for β- or α-anomers, respectively. Since in the investigated oligomers only two ^{13}C signals are visible in the region δ 92–96, it is immediately apparent that in the samples not more than one reducing residue is present. Thus the doublet at δ 5.19 (correlating with δ 92.9) and part of the multiplet at δ 4.53 (correlating with δ 96.8) could be readily assigned to the α- and β-anomer, respectively, of reducing residue E.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

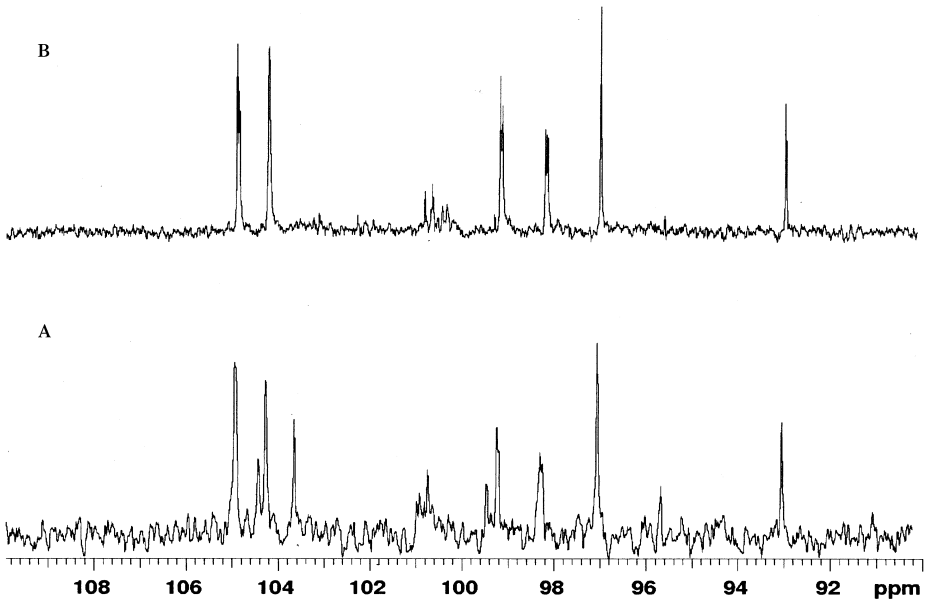


Figure 2. A. The region of the ^{13}C NMR resonances of the glycosidic carbons of the malaceae-type penta-(and hexa)-saccharides. B. Idem for the rubus-type pentasaccharide.

In accordance with previous data,[7] the four different acetylation patterns for residue A are reflected by the peaks in the region δ 5.30–5.55. Correlation of these signals with ^{13}C peaks at δ 100.5/100.7 and at δ 98.0/98.1 (typically upfield shifted 2-*O*-acetylated isomers) confirmed the α- configuration of the A residue. The other α-anomer (of residue C) is visible as a broadened singlet at δ 4.91 corresponding to a single line at δ 99.1 (rubus-compound) or to a split peak at δ 99.1/99.2

Table 1. ^{1}H NMR Data of the Malaceae-type Hexasaccharide Compound

RESIDUE	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
$A_{\text{-diOAc}}$	5.55	5.25	5.15	—	—	—	
$A_{\text{-2-OAc}}$	5.45	4.95	4.06	4.22	3.68	3.92	3.73
$A_{\text{-3-OAc}}$	5.42	4.06	5.02	4.32	3.70	3.90	3.80
A	5.35	3.80	3.84	4.13	3.63	3.92	3.77
Eα	5.19	3.89	3.89	4.12	—	—	—
C	4.91	3.82	3.82	4.07	—	3.88	3.72
B	4.61	3.35	3.72	3.92	—	—	—
D	4.52	3.56	3.56	4.08	—	3.77	3.55
Eβ	4.54	3.53	3.75	3.87	—	3.60	3.55
F	4.38	3.18	3.40	3.33	3.33	3.85	3.68
(a)	$^{3}J[1,2]$	$^{3}J[2,3]$	$^{3}J[3,4]$	$^{3}J[4,5]$	$^{3}J[5,6a]$	$^{3}J[5,6b]$	$^{2}J[6a,b]$
	8.9	9.0	8.6	(nd)	1.8	4.5	−12.3

a: J values for the F residue.

Table 2. Glycosidic ^{1}H- and ^{13}C Chemical Shifts of the Malaceae-type Hexasaccharide

Residue	^{1}H Resonance	^{13}C Resonance
A-2,3-di-OAc (α)	5.55	98.0
A-2-OAc (α)	5.45	98.1
A-3-OAc (α)	5.42	100.5
A (α)	5.35	100.7
Eα	5.19	92.9
C (α)	4.91	99.1 and 99.2
Eβ and D (β)	4.53 (quasi triplet)	96.8 and 104.8
B (β)	4.61 (under HOD)	104.1 and 104.2
F (β)	4.38 (multiplet)	103.7

(malaceae-compound). The broadening or splitting is very likely due to the non-coincidence of the different sub-species (Eα or β, presence or absence of F) present in the samples. In the ^{13}C spectra of both the malaceae- and rubus-type compound, several other overlapping peaks are seen in this region at δ 100. They are, however, not glycosidic carbon resonances but signals due to the quaternary ketal carbons of the pyruvate moiety of the A units. Their presence was easily confirmed by their disappearance in a DEPT spectrum or by their long-range correlation with the methyl protons at δ 1.4 as shown in a GHMBC spectrum. Likewise, in the ^{1}H spectra from δ 4.85–5.25, two kinds of resonances were found: besides the doublets for the glycosidic protons we also observed double doublets at δ 5.25, 5.15, 5.02 and 4.95, all showing a typical downfield acetylation shift, and thus pointing to H-2 and H-3 of the acetylated modifications of residue A.

From δ 4.52–4.61, we found the anomeric protons of the non-reducing residues that occur in the β-configuration, namely residue B and residue D. For B the signal was obscured under the saturated water peak at δ 4.61 and showed a correlation with a peak at δ 104.1 in the rubus sample and at δ 104.1/104.2 in the malaceae sample. We suggest that the two closely spaced C-1 peaks of uronic acid residue B in the malaceae sample are caused most likely by the presence or absence of residue F at the nearby 6-position of unit C. For D the anomeric proton which correlated in both samples with a peak at δ 104.8, was found as part of a multiplet at δ 4.54–4.52 together with H-1 of the reducing sugar Eβ. In the malaceae sample there was also a second extra ^{13}C resonance in this region at δ 103.7 which correlated with a proton obscured in a multiplet at δ 4.38, a chemical shift diagnostic for β-anomeric protons.[9] As will be shown further from TOCSY experiments, this additional β-linked residue F in the malaceae compound is a β-D-glucopyranosyl moiety coupled via a β(1→6) link to unit C.

Assignment of the other ring protons was based on 1D-TOCSY experiments with selective excitation of the respective glycosidic protons using EBURP-1 excitation pulses[10] and an array of three or more different mixing times.

In general, our experimental data compared very well with those of Nimtz *et al.*,[7] except for an almost constant but small difference of about δ 0.10. This is most probably due to a difference in referencing or to changes in experimental condi-

tions such as pH or concentration. Complete data for the diacetylated derivative were missing, because as mentioned above, substantial hydrolysis of the acetyl groups had occurred at the time of the TOCSY experiments.

It should be noted that in this communication we provide complete ^{1}H chemical shifts and coupling constants for the F residue. Also, the data for the rubus-type compound are not explicitly given because they are very similar to those of the malaceae-type hexasaccharide (except for residue F). The only differences are the chemical shifts of the H-6 protons of residue C, which are found at δ 3.62 and δ 3.72 in the rubus-type compound as seen in figure 4.

Structural Assignment: (i) Relative Presence of Pentasaccharide and Hexasaccharide in Malaceae-type Amylovoran

Compared to the malaceae-type compound, one residue was missing in the rubus-type compound, as evidenced by the absence of the diagnostic δ 103.7 resonance. The malaceae-type EPS has been suggested earlier to consist of a pentasaccharide-repeating unit substituted with a sixth residue F (glucose) forming a hexasaccharide unit for about 10%.[7] Because in the ^{1}H spectrum no isolated hexasaccharide resonances were found, it was not possible to estimate correctly the proportion between pentasaccharide and hexasaccharide in the strains that we have studied. Nevertheless, by considering the ^{13}C NMR pattern at δ 103–104, we estimated that F must be present about 30–40% in the malaceae-type oligosaccharide mixture. It cannot be known by this study whether the F-substituted pentasaccharide in the native malaceae-type amylovoran, occurs clustered or randomly distributed over the EPS biopolymer.

(ii) Identification of Residue F and Structure Confirmation of the Other Residues in the Malaceae-type Compounds

For the identification of the residues in the proposed structures, we performed selective 1D-TOCSY experiments for each of the glycosidic protons at three or more different isotropic mixing times (40, 80, and 120 ms).[10]

The selective TOCSY on the pattern at δ 4.38 (Figure 3) clearly revealed the typical resonances of a β-D-glucopyranosyl residue. Three triplet-shaped signals for H-2, H-3, and H-4 with a characteristic[11, 12] J value of about 9 Hz for each, as well as the typical pattern for the signals of H-6a and H-6b, were found. This additional glucoside was assigned to be residue F, and thus constitutes the sixth residue of the hexasaccharide.

(iii) Identification of the Link Between Residue F and the Rest of the Repeating Hexasaccharide Unit

After the identification of the residues, the linkages between these residues had to be identified. For simple oligosaccharides, the links can be derived by com-

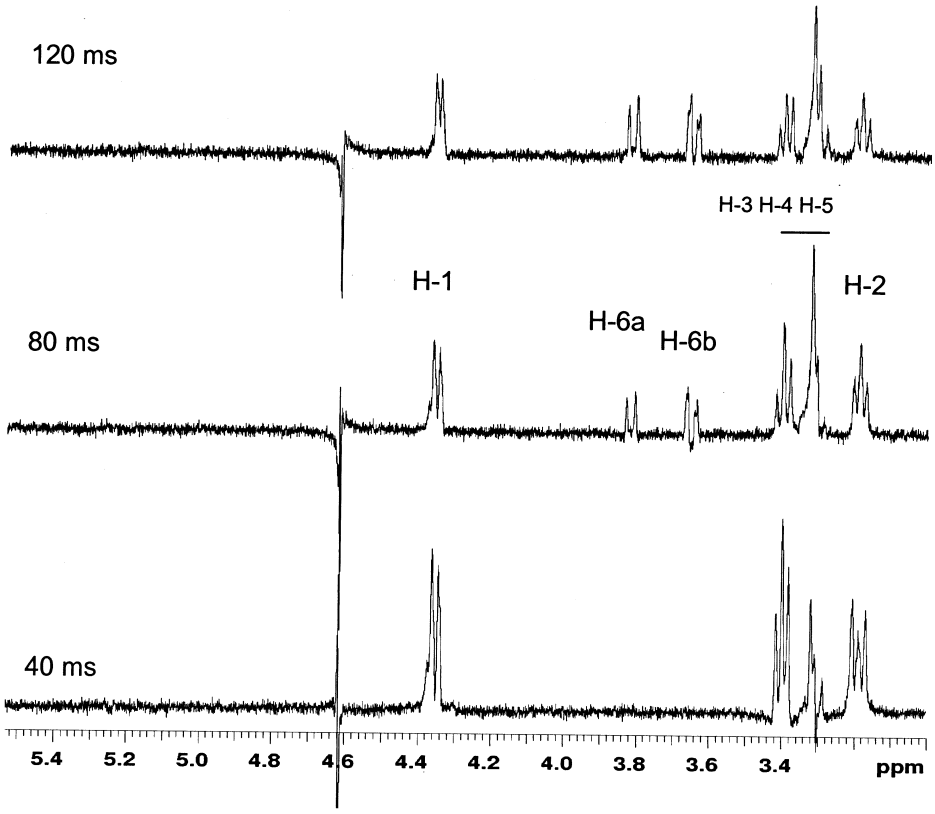


Figure 3. Selective TOCSY experiment on H-1 of residue F at δ 4.38 for malaceae-type compound.

paring the chemical shifts of the ring protons of each residue with those of the monosaccharide.[11, 12] Here, however, because of the presence of multi-substituted residues and because of a uronic acid instead of a glycoside, predictions based on chemical shifts are rather risky. Therefore, we used edited GHSQC experiments, for identification of exocyclic hydroxymethylene groups. Normally, C-6 in aldohexopyranose[13] resonates at δ 61. However, when the hydroxymethylene group is involved in a glycosidic bond or is otherwise substituted, the carbon resonance shifts downfield to δ 67–72.[10, 14, 15] When this experiment was performed on the rubus-type compound (Figure 4B), only two carbon resonances, each showing two proton connectivities, were observed at frequencies higher than δ 65. Two residues are present with a 1→6 linkage or with a substituted 6-OH group, namely residue D which is bound to residue C, and residue A which is pyruvated at C_4–C_6. In the experiment for the malaceae-type compounds (Figure 4A), three CH_2 carbon resonances each with their corresponding two-proton connectivities were observed at a lower field than δ 65 (see also Table 3). This result clearly indicated that in this compound, in addition to the expected two linkages, a third 1→6 link at δ 70.2 is present. The new 1→6 link can only occur between residues C and F, because the

two correlating H-6 protons at δ 3.88/3.72 belong to the C unit, as determined from a TOCSY with long mixing time on H-1 at δ 4.91.

Another interesting observation is the low intensity of the GHSQC cross peaks at δ 70.2, which is in accordance with the proposed 30–40 % proportion of the F unit in the oligosaccharide mixture.

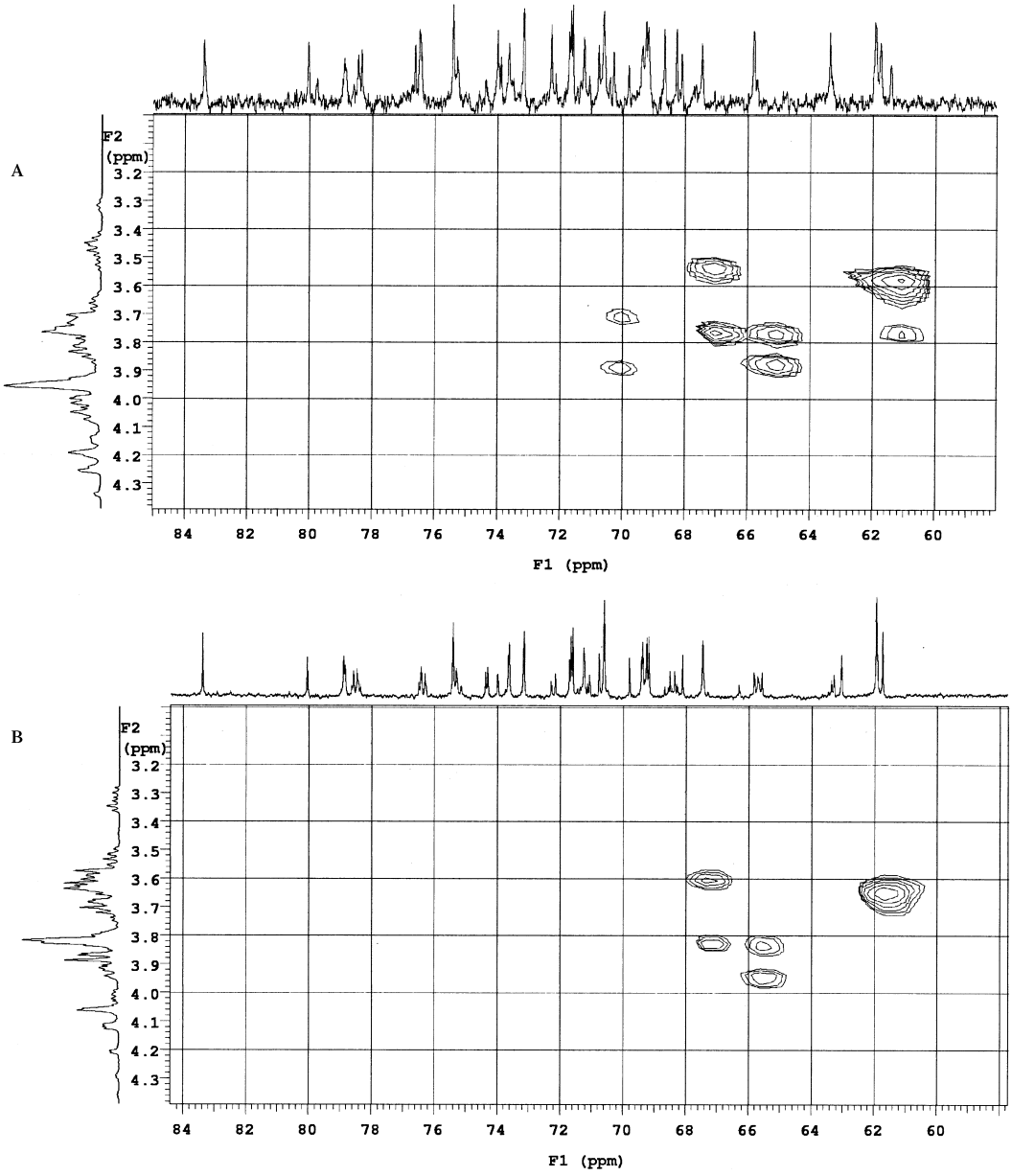


Figure 4. The edited HSQC (only methylene groups are shown) experiments. A. The malaceae-type compound. B. The rubus-type compound.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Table 3. Results from the Edited (CH_2) GHSQC Experiment for the Malaceae-type Compound

C-6	H-6a	H-6b	Residue
65.6	3.92	3.77	A (non-acetylated)
66.7	3.77	3.55	D
70.2	3.88	3.72	C

The peak at δ 65.8 could be easily assigned to carbon 6 of the pyruvated A unit because of its correlation with the two H-6 protons of the nonacetylated A residue at δ 3.92 and δ 3.77. The latter were assigned by a selective TOCSY experiment on the δ 5.35 resonance and using a long mixing time of 400 ms. The second downfield shifted CH_2 signal at δ 67.4 correlates with proton resonances at δ 3.77 and δ 3.55 that belong to H-6a/b of residue D (Table 1). The latter assignment however, was not straightforward because the glycosidic protons of D and Eβ collapsed at δ 4.53, so that a selective TOCSY at that multiplet did not give unequivocal results. However, careful analysis of these TOCSYs in function of different mixing times and measurement of additional selective TOCSYs on the two equatorially oriented H-4 protons at δ 3.87 and at δ 4.08 allowed an unequivocal assignment of the different hydroxymethylene protons involved.

Final confirmation of the deduced structures is obtained from mass spectral data: 1074.3 Da corresponding to $C_{39}H_{62}O_{34}$ for the non-*O*-acetylated hexasaccharide unit from malaceae-type strain, and 912.2 Da corresponding to $C_{33}H_{52}O_{29}$ for the non-*O*-acetylated pentasaccharide unit from rubus-type strain.

CONCLUSIONS

Using sophisticated NMR techniques, including edited GHSQC and selective excited TOCSY experiments with different mixing times, the detailed structure of amylovoran from different *Erwinia amylovora* strains could be elucidated. Our work not only confirmed the structure of the pentasaccharide repeating unit as proposed by Nimtz *et al.*,[7] but also allowed a more detailed description of the additional F residue that is present at a much higher (30–40%) degree in the amylovoran of malaceae-type exopolysaccharides than previously suggested.[7] Direct evidence is given that residue F is a β-D-glucopyranosyl residue that is connected via a β 1→6 link to residue C, thereby accounting for the 30–40% hexasaccharide-repeating units in the oligosaccharide mixture. It is probable that the structure difference for both types of compounds is at least partially responsible for the host range, as will be discussed in more detail elsewhere.

EXPERIMENTAL

Samples (about 0.5 mg) were dissolved in 0.7 mL deuterium oxide (Aldrich, D Atom 99.98%) with no internal reference added. All the NMR spectra were mea-

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

sured on a Varian UNITY-500 spectrometer operating at 499.475 MHz for ^{1}H NMR and at 125.599 MHz for ^{13}C NMR. All the spectra were measured at 27°C. ^{1}H spectra were referenced to the HOD resonance. Standard Varian software Vnmr version 5.3B was used throughout. All experiments were performed in a broadband 5mm inverse-detection PFG probe (typical values for 90° proton pulses are 5.8 μs and for ^{13}C 90° pulses 18 μs). Gradient pulses were produced by Performa II PFG source (Varian). The Pandora box program calculated the shaped pulses for selective excitation.

The NMR techniques used included 1D-TOCSY,[13, 16] double-quantum filtered COSY,[17] DEPT,[18] GHSQC [19, 20] (edited) or gradient heteronuclear single-quantum coherence experiments, GHMBC[19, 20] or gradient heteronuclear multiple bond correlated experiments optimized for 2- or 3-bond C, H correlation.

The purification, enzymatic cleavage of amylovoran by a phage ϕ-EaIh depolymerase enzyme and the isolation of the oligosaccharides by gel permeation was completely analogous to that described by Nimtz *et al.*[7]

ACKNOWLEDGMENTS

We thank J. S. Hartung[6] for providing ϕ-EaIh depolymerase clone, and S. K. Mohan for strain deliverance. The Belgian Ministry of Small Enterprises and Agriculture (to M.M.) and the Fund for Scientific Research (Flanders) ("Krediet aan Navorsers" nr 1.5.515.98N to E.M.) supported this work.

REFERENCES

1. Presented at the *20th International Carbohydrate Symposium,* Hamburg, Germany, August 27–September 1, 2000.
2. Evans, I. R. Fire blight in raspberries in Alberta. Acta Horticulturae **1996**, *411*, 69–72.
3. Garbeva, P.; Crepel, C.; Maes, M. Phytopathological differentiation of *Erwinia amylovora* strains. Parasitica **1996**, *52*, 17–22.
4. McManus P. S.; Jones, A. L. Genetic fingerprinting of *Erwinia amylovora* strains isolated from tree-fruit crops and *Rubus* spp. Phytopathology **1995**, *85*, 1547–1553.
5. Norelli, J. L.; Aldwinckle, H. S.; Beer, S. V. Differential host and pathogen interactions among cultivars of apple and strains of *Erwinia amylovora*. Phytopathology **1984**, *74*, 136–139.
6. Hartung, J. S.; Fulbright, D. W.; Klos, E. J. Cloning of a bacteriophage polysaccharide depolymerase gene and its expression in *Erwinia amylovora*. Mol. Plant-Microbe Interact. **1988**, *1*, 87–93.
7. Nimtz, M.; Mort, A.; Domke, T.; Wray, V.; Zhang, Y.; Qiu, F.; Coplin, D.; Geider, K. Structure of amylovoran, the capsular exopolysaccharide from the fire blight pathogen *Erwinia amylovora*. Carbohydr. Res. **1996**, *287*, 59–76.
8. Bock, K.; Pedersen, C. Carbon-13 nuclear magnetic resonance spectroscopy of monosaccharides. Adv. Carbohydr. Chem. Biochem. **1983**, *41*, 27–66.
9. Bock, K.; Pedersen, C. A study of ^{13}C–^{1}H coupling constants in hexopyranoses. J. Chem. Soc., Perkin Trans. 2 **1974**, 293–297.

10. Maras, M.; De Bruyn, A.; Schraml, J.; Herdewijn, P.; Claeyssens, M.; Fiers, W.; Contreras, R. Structural characterization of *N*-linked oligosaccharides from cellobiohydrolase I secreted by the filamentous fungus *Trichoderma reesei* RUTC 30. Eur. J. Biochem. **1997**, *245*, 617–625.
11. De Bruyn, A.; Anteunis, M.; Verhegge, G. ^{1}H NMR study of the di-glucopyranoses in D_2O. Bull. Soc. Chim. Belges **1975**, *84*, 721–734.
12. De Bruyn, A.; Anteunis, M.; Van Beeumen, J. Chemical shifts of aldohexopyranoses revisited and application to gulosylglucose. Bull. Soc. Chim. Belges **1977**, *86*, 259–265.
13. Bax, A.; Davis, D. G. MLEV-17 based two-dimensional homonuclear magnetization transfer spectroscopy. J. Magn. Reson. **1985**, *65*, 355–360.
14. Bock, K.; Thøgersen, H. Nuclear magnetic resonance spectroscopy in the study of mono- and oligosaccharides. Annu. Rev. NMR Spectrosc. **1982**, *13*, 1–57.
15. Bock, K.; Pedersen, C.; Pedersen, H. Carbon-13 nuclear magnetic resonance data for oligosaccharides. Adv. Carbohydr. Chem. Biochem. **1984**, *42*, 193–225.
16. Bax, A.; Davis, D. G. Practical aspects of two-dimensional transverse NOE spectroscopy. J. Magn. Reson. **1985**, *63*, 207–213.
17. Piantini, U.; Sørensen, O. W.; Ernst, R. R. Multiple quantum filters for elucidation NMR coupling networks. J. Am. Chem. Soc. **1982**, *104*, 6800–6801.
18. Doddrell, D.; Pegg, D.; Bendall, M. Distortionless enhancement of NMR signals by polarization transfer. J. Magn. Reson. **1982**, *48*, 323–327.
19. Wagner, G. Two-dimensional relayed coherence transfer spectroscopy of a protein. J. Magn. Reson. **1983**, *55*, 151–156.
20. Wilker, W.; Leibfritz, D.; Kerssebaum, R.; Bermel, W. Gradient selection in inverse heteronuclear correlation spectroscopy. Magn. Reson. Chem. **1993**, *31*, 287–292.

Received August 21, 2000
Accepted March 1, 2001

Request Permission or Order Reprints Instantly!