Available online at www.sciencedirect.com
SCIENCE DIRECT
Carbohydrate Research 341 (2006) 930–934
Carbohydrate RESEARCH

Note

The structure of the O-specific polysaccharide of the lipopolysaccharide from *Burkholderia gladioli* pv. *agaricicola*

Gnuni Karapetyan,[a] Zbigniew Kaczynski,[a] Nicola S. Iacobellis,[b] Antonio Evidente[c] and Otto Holst[a,*]

[a]*Division of Structural Biochemistry, Research Center Borstel, Leibniz-Center for Medicine and Biosciences, D-23845 Borstel, Germany*
[b]*Dipartimento di Biologia, Difesa e Biotecnologie Agro Forestali, Università degli Studi della Basilicata, Viale dell'Ateneo Lucano 10, 85100 Potenza, Italy*
[c]*Dipartimento di Scienze del Suolo, della Pianta e dell' Ambiente, Universitá Degli Studi di Napoli Federico II, I-80055 Portici (NA), Italy*

Received 4 November 2005; received in revised form 16 January 2006; accepted 10 February 2006
Available online 3 March 2006

Abstract—A neutral O-specific polysaccharide containing D-mannose, D-rhamnose and D-galactose was obtained by mild acid hydrolysis of the lipopolysaccharide of the plant pathogenic bacterium *Burkholderia gladioli* pv. *agaricicola*. By means of compositional analyses and NMR spectroscopy, the chemical repeating unit of the polymer was identified as a linear trisaccharide of the structure shown below, in which the mannose residue was quantitatively acetylated at C-2.

Ac
↓
2
→3)-α-D-Man*p*-(1→2)-α-D-Rha*p*-(1→4)-β-D-Gal*p*-(1→

Keywords: Mushroom disease; *Agaricus bitorquis*; *Burkholderia gladioli* pv. *agaricicola*; Lipopolysaccharide; O-Specific polysaccharide; NMR spectroscopy

Many pectolytic bacteria including pseudomonads are found in soil, on root or leaf surfaces, and are known to cause soft rots of vegetables, however none of such bacterial species has so far been implicated in the soft rot of mushrooms.[1] Several potentially devastating cases of wet or soft rot of mushrooms have been observed in the UK. Bacteria pathogenic for cultivated mushrooms are often members of the genus *Pseudomonas*. *Pseudomonas tolaasii* and *P. gingeri* are the cause of brown blotch[2] and ginger blotch,[3] respectively, of *Agaricus bisporus*. *Burkholderia gladioli* pv. *agaricicola* infects sporophores of the edible mushroom *A. bitorquis* causing soft rot disease. The disease is manifested by a rapid development of deep oozing lesions on the pileal which renders the mushroom unmarketable.[4,5] Due to the practical and economical importance of the disease, studies on the isolation and chemical and biological characterization of toxic metabolites are in progress. Preliminary results suggest that these metabolites belong to the class of lipodepsipeptides. Other studies were initiated on the toxicity and structures of the

* Corresponding author. Tel.: +49 4537 188472; fax: +49 4537 188745; e-mail: oholst@fz-borstel.de

0008-6215/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2006.02.010

lipopolysaccharide (LPS) and the exopolysaccharide (EPS) in order to clarify their role in the plant-pathogen interaction and the disease. A putative exopolysaccharide containing D-rhamnose was recently isolated and its repeating unit was determined by chemical and spectroscopic methods.[6]

In this paper, the isolation and the structural determination of the O-specific polysaccharide of the LPS from *B. gladioli* pv. *agaricicola* are reported.

After cultivation and harvest, the bacterial cell mass was lyophilized (yield: 4.57 g bacterial dry mass) and then extracted utilizing the hot phenol/water method.[7] The aqueous phase of the phenol/water treatment was dialyzed, then freeze dried (335 mg, 7.3% of bacterial dry mass), dissolved in water, and the LPS was precipitated by ultracentrifugation and lyophilized (52.2 mg, 18.3% of the dry water phase). The EPS was isolated from the supernatant.[6]

Mild acid hydrolysis of the LPS and centrifugation gave a lipid as sediment (lipid A) and a water soluble carbohydrate fraction. This carbohydrate fraction was purified by gel-permeation chromatography (GPC) on Sephadex G-200, yielding two main fractions, the first of which contained the EPS and the second, the O-specific polysaccharide (OPS) with some EPS. Further GPC separation experiments on Sephadex G-200, Sephadex G-50, Sephacryl S-400, and TSK 55 (S) resins using 0.01 M, or 0.025 M ammonium hydrogencarbonate (pH 8.65) or 0.1 M pyridinium acetate (pH 4.20) did not succeed in a pure OPS preparation. Furthermore, a partial de-O-acetylation was observed during GPC under alkaline conditions, as indicated by an additional anomeric proton signal at δ 5.155 (compare: **a**1 in Table 1). Finally, the second fraction obtained from GPC on Sephadex G-200 was used for further investigations.

Sugar analysis of the OPS fraction identified rhamnose, mannose and galactose in an approximate molar ratio of 5:2:2. Methylation analysis gave mainly 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-rhamnitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-hexitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-hexitol in an approximate molar ratio of 1:1:1, which revealed the OPS to be composed of 2-substituted rhamnopyranose and two 3- and 4-substituted hexopyranose residues. Also, 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-rhamnitol and 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-rhamnitol (approximate molar ratio of 0.75:0.25) were identified which originated from the EPS contamination.[6] The absolute configurations of the sugars were identified as D-mannose, D-galactose and D-rhamnose.[8,9]

The ^{1}H NMR spectrum (Fig. 1, Table 1) of the OPS preparation contained four main signals in the anomeric region, that is, at δ 5.433 (proton **A**2), δ 5.170 (**A**1), δ 4.955 (**B**1) and δ 4.568 (**C**1), a signal characteristic for a methyl group of rhamnose at δ 1.275, and one signal at δ 2.171, characteristic for an *O*-acetyl group. Other resonances in the anomeric region and characteristic

Table 1. ^{1}H and ^{13}C NMR data of the OPS (residues **A**, **B** and **C**) and de-O-acetylated OPS (residues **a**, **b** and **c**) from the LPS of *Burkholderia gladioli* pv. *agaricicola*

Residue	^{1}H, ^{13}C Chemical shifts [δ] ($^3J_{H-1,H-2}/^1J_{C-1,H-1}$ (Hz))								
	1	2	3	4	5	6a	6b	CH_3CO	*CO*
→3)-α-D-Man*p*-	5.170	5.433	4.259	3.910	3.770	3.874	3.839	2.171	—
A	(<1.5)	*70.01*	*76.70*	66.48	74.25	61.79		21.38	174.22
	100.47								
	(175)								
a	5.155	4.289	4.067	3.849	3.731	3.813	3.877		
	(<1.5)	68.64	*79.39*	66.27	74.31	62.04			
	103.03								
	(173)								
→2)-α-D-Rha*p*-	4.955	4.074	3.920	3.491	4.111	1.275	—	—	—
B	(<1.5)	*79.60*	70.40	73.22	70.29	17.63	—	—	—
	100.94								
	(172)								
b	4.975	4.087	3.934	3.494	4.128	1.288	—		
	(<1.5)	*79.36*	71.20	73.30	70.36	17.67	—		
	101.19								
	(172)								
→4)-β-D-Gal*p*-	4.568	3.518	3.733	4.001	3.765	3.761	3.761	—	—
C	(7.6)	72.10	73.15	*78.44*	76.25	61.56		—	—
	101.51								
	(162)								
c	4.613	3.617	3.774	4.024	3.786	3.784	3.784		
	(7.7)	71.92	73.15	*78.53*	76.28	61.67			
	102.06								
	(163)								

Spectra were recorded at 50 °C in ^2H_2O relative to internal acetone (δ_H 2.225; δ_C 31.45). Italicized chemical shifts indicate substituted positions.

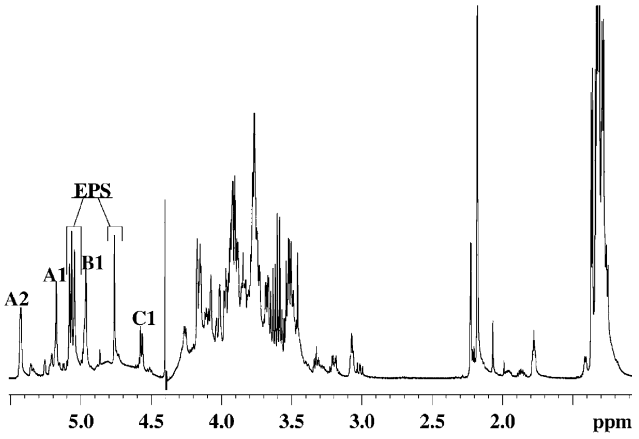


Figure 1. The ^{1}H NMR spectrum of the OPS of the LPS from *Burkholderia gladioli* pv. *agaricicola*. The spectrum was recorded at 600 MHz and 60 °C. The sugar residues were labeled **A**–**C** according to the decreasing chemical shifts of the anomeric protons.

for methyl groups of rhamnose residues originated from the contaminating EPS, which was a rhamnan.[6] The sugar residues of the OPS were labeled **A** to **C** according to decreasing chemical shifts of the anomeric protons. The anomeric signals at δ 5.045–5.080 and at δ 4.760 (labeled EPS) were attributed to the EPS.[6] The couplings of the anomeric signals of residues **A** and **B** ($^{3}J_{\text{H-1,H-2}} < 1.5$ Hz) identified the *manno* and that of residue **C** ($^{3}J_{\text{H-1,H-2}}$ 7.6 Hz) the *galacto* configured residues. A COSY experiment (not shown) assigned the signal at δ 5.433 as H2 of the mannose residue, which was shifted to lower field due to its acetylation. This was confirmed by the down-field shift of C-2 of this residue (at δ 70.01), and by comparison to the respective resonances of the de-O-acetylated LPS (Table 1, H-2 at δ 4.289, C-2 at δ 68.64).

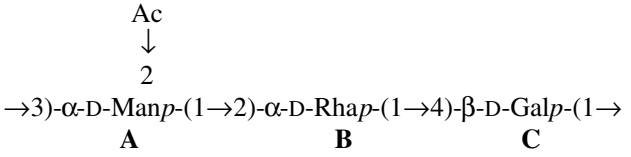
Data from sugar analysis and from NMR spectroscopy indicated that the OPS consisted of a trisaccharidic repeating unit, containing one residue each of D-mannose, D-rhamnose and D-galactose. The ratio of the resonances of protons **A**1:**A**2:**B**1:**C**1:acetyl was about 1:1:1:1:3, proving a quantitative acetylation of O-2 of Man.

The ^{13}C NMR spectrum confirmed the trisaccharidic repeating unit of the OPS (signals for anomeric carbons at δ 100.47, 100.94 and 101.51, Table 1) furnished from two hexose residues (signals for unsubstituted C-6 at δ 61.79 and 61.56), a residue of 6-deoxyhexose (signal for C-6 at δ 17.63) and an *O*-acetyl group (signals for *C*O at δ 174.22 and *C*H$_3$ at δ 21.38).

The complete structural characterization of the polysaccharide was achieved by 1D and 2D ^{1}H and ^{13}C NMR spectroscopy. ^{1}H,^{1}H COSY, double-quantum-filtered COSY (DQFCOSY), ROESY and TOCSY, as well as ^{1}H,^{13}C HMQC and HMBC spectra allowed the complete assignment of all ^{1}H and ^{13}C chemical shifts (Table 1).

Low-field shifted signals of carbon atoms **A**3 (δ 76.70), **B**2 (δ 79.60) and **C**4 (δ 78.44) compared with those of unsubstituted sugars[10] proved glycosylation at C-3 of residue **A**, at C-2 of residue **B**, and at C-4 of **C**. This data confirmed that of methylation analysis. The anomeric configurations of all residues were assigned by the coupling constants $^{1}J_{\text{H-1,C-1}}$ which were identified in another ^{1}H,^{13}C HMQC experiment recorded without decoupling. The $^{1}J_{\text{H-1,C-1}}$ values 175 Hz for residue **A**, 172 Hz for **B** and 162 Hz for residue **C** revealed that the mannosyl and rhamnosyl residues had the α- and the galactosyl residue the β-anomeric configuration.[11,12]

The ROESY and ^{1}H,^{13}C HMBC experiments revealed the sequence of the sugar residues in the repeating unit. *Inter*residual NOE contacts were observed between protons **A**1 and **B**2, **B**1 and **C**4 and **C**6, and **C**1 and **A**3 (Fig. 2A). An additional NOE contact was found between protons **A**1 and **B**1, which is typical for (1→2)-linked residues. Also, *intra*residual NOE contacts were found between **C**1 and **C**3 and **C**1 and **C**5, resulting from the axial orientation of these β-galactopyranosyl ring protons. Thus, the sequence →**A**→**B**→**C**→ was established which was confirmed by the HMBC experiment (not shown) identifying the *inter*residual proton–carbon correlations, that is, between H-1 of **A** and C-2 of **B**, H-1 of **B** and C-4 of **C**, H-1 of **C** and C-3 of **A**. In summary, the data identified the structure of the O-specific polysaccharide from *B. gladioli* pv. *agaricicola* as



The OPS was de-O-acetylated with NaOCH$_3$ and the sample was fractionated on TSK 55 (S) using water as eluent. One main fraction was obtained, sugar analysis of which identified rhamnose, mannose and galactose in an approximate molar ratio of 1:1:1. Methylation analysis gave 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-rhamnitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-hexitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-hexitol in the approximate molar ratio of 1:1:1.

The ^{1}H NMR spectrum (Table 1) showed three anomeric signals at δ 5.155 (**a**, $^{3}J_{\text{H-1,H-2}} < 1.5$ Hz), δ 4.975 (**b**, $^{3}J_{\text{H-1,H-2}} < 1.5$ Hz) and δ 4.613 (**c**, $^{3}J_{\text{H-1,H-2}}$ 7.7 Hz), suggesting a trisaccharidic repeating unit. The sugars giving rise to these ^{1}H resonances were named **a**–**c** according to their decreasing chemical shifts. The ^{13}C NMR spectrum (Table 1) contained anomeric carbon signals at δ 103.03 (**a**, $^{1}J_{\text{C-1,H-1}}$ 173 Hz), δ 101.19 (**b**, $^{1}J_{\text{C-1,H-1}}$ 172 Hz) and δ 102.06 (**c**, $^{1}J_{\text{C-1,H-1}}$ 163 Hz).

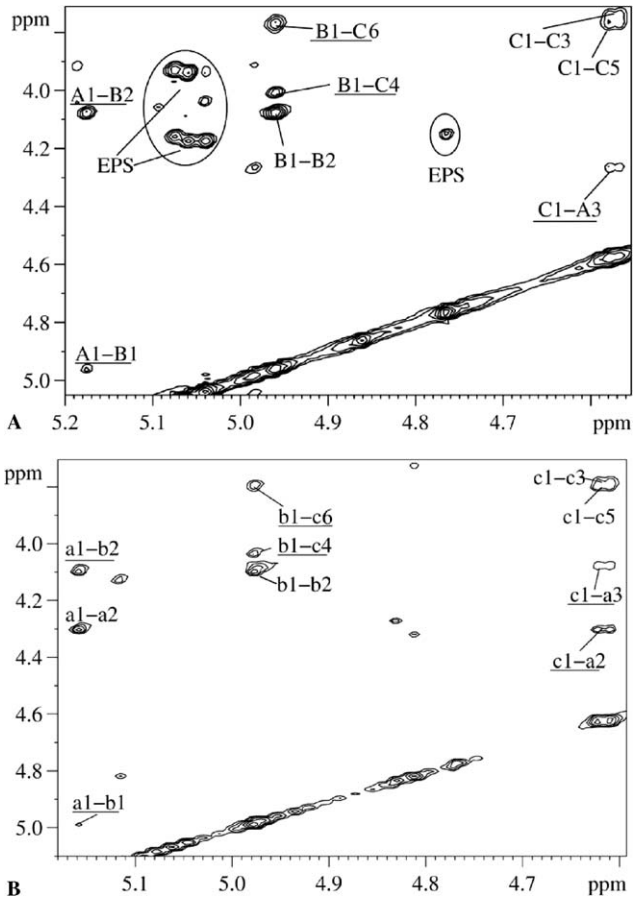


Figure 2. Sections of the ROESY spectra of **A**, the OPS and **B**, the de-O-acetylated OPS, of the LPS from *Burkholderia gladioli* pv. *agaricicola*. The spectra were recorded at 600 MHz and 50 °C. The *letters* refer to the carbohydrate residues as defined in Table 1, and the *arabic numerals* refer to the protons in the respective residues. The *inter*residual NOE contacts are underlined.

Based on their small $^{3}J_{\text{H-1,H-2}}$ spin systems and on COSY data, **a** was assigned as *manno*-pyranosyl and **b** as *rhamno*-pyranosyl residue, thus, **c** was the *galacto*-pyranosyl residue.

Complete assignments were obtained from ^{1}H, ^{1}H correlation (COSY, RELAY COSY, TOCSY) and from ^{1}H,^{13}C correlation (HMQC and HMBC) experiments (Table 1). In 2D ROESY and ^{1}H,^{13}C HMBC experiments, *inter*residual NOE contacts were observed between protons **a**1 and **b**2, **b**1 and **c**4 and **c**1 and **a**3 (Fig. 2B). *Inter*residual NOE contacts were also found between protons **a**1 and **b**1, **b**1 and **c**3, **b**1 and **c**5, and **c**1 and **a**2. and *intra*residual ones between **c**1 and **c**3 and **c**1 and **c**5 (β-*galacto*-pyranosyl residue). Thus, the sequence →**a**→**b**→**c**→ was established which was confirmed by the HMBC experiment (not shown) identifying the *inter*residual proton-carbon correlations, H-1 of **a** and C-2 of **b**, H-1 of **b** and C-4 of **c**, H-1 of **c** and C-3 of **a**.

In conclusion, the structure of the chemical repeating unit of the OPS of the LPS from *B. gladioli* pv. *agaricicola* was established, representing a novel repeating unit not found in other OPS of plant-pathogenic bacteria so far. Rhamnose is a common sugar in such OPS, a number of rhamnan backbones in OPS of LPS from plant-pathogenic *Xanthomonas* and *Pseudomonas* species have been identified.[13–16] The isolation and structural determination of LPS from phytopathogenic bacteria, in particular of the O-specific polysaccharides, is not only of great importance for the identification of the role of this molecule in host–pathogen interactions but also for the taxonomic classification of the microorganisms.[17]

1. Experimental

1.1. Growth of bacteria

Type strain ICMP11096 of *B. gladioli* pv. *agaricicola* was grown at 25 °C under shaking (200 rpm) in 500 mL Erlenmeyer flasks filled with 150 mL of Minimal Medium Broth[18] inoculated with 1.5 mL of a bacterial suspension containing 10^{8} cfu/mL. After 48 h incubation, cultures were centrifuged (20,000*g* for 15 min) and the resulting supernatants were evaluated for the antimicrobial activity against *Bacillus megaterium*,[18] lyophilized and stored at −20 °C. The cells were washed twice with saline and then lyophilized.

1.2. Isolation of LPS

The lyophilized bacteria (4.57 g) were extracted with hot phenol/water,[7] the water phase of which was lyophilized (335 mg, 7.3% of the bacterial dry mass). Of this, 284.6 mg was suspended in 10 mL of ultrapure Mili-Q water and centrifuged (4 °C, 100,000*g*, 5.5 h). The LPS-containing pellet was again suspended in ultrapure Mili-Q water, ultracentrifuged and freeze dried (LPS, 52.2 mg, 18.3%). Both obtained supernatants were combined and lyophilized (181.9 mg, 63.9% of the lyophilized water phase).[6]

1.3. Hydrolysis of LPS

The LPS sample (52.2 mg) was hydrolyzed with aq 1% AcOH at 100 °C for 1.5 h, and the lipid precipitate (lipid A) was removed by ultracentrifugation (100,000*g*, 4 °C, 4 h) and lyophilized (4.6 mg, 8.8%). The supernatant was also lyophilized to yield the carbohydrate portion (42.6 mg, 81.7%), of which 18.7 mg was fractionated by GPC on a column (95 cm × 2.6 cm) of Sephadex G-200 using 0.1 M pyridinium acetate buffer (pH 4.2) as eluent and monitoring with a differential refractometer (Knauer). Two main fractions were obtained and lyophilized [retention volumes of 140 mL (EPS, 4.1 mg, ~22%) and 310 mL (9.2 mg, ~49.5%)].

1.4. De-O-acetylation

Part of the OPS (fraction 2 of GPC 5.4 mg) was treated with 0.05 M $NaOCH_3$ (37 °C, 2 h), then neutralized with 2 M aq HCl, dialyzed against water (molecular mass cut off 3.5 kDa), and freeze dried. Of this material, 2.9 mg was purified by GPC on a column (1.5 cm × 70 cm) of TSK 55 (S) using water as eluent and monitoring by a differential refractometer (Knauer), which yielded a high molecular mass polysaccharide (0.6 mg, 11.1% of the OPS).

1.5. Compositional analyses

Hydrolysis was performed with 2 M CF_3CO_2H (120 °C, 2 h), and the resulting monosaccharides were analyzed by GLC as alditol acetates using a Hewlett–Packard 5880 instrument equipped with a capillary column (30 m × 0.25 mm, 0.25 μm film thickness) of SPB-5 and applying a temperature gradient of 150 °C (3 min) to 260 °C at 3 °C/min. The absolute configurations of the sugars were determined by GLC of their acetylated (*S*)-2-butanol glycosides[8,9] utilizing the same chromatographic conditions as above.

1.6. Methylation analysis

Methylation of the polysaccharide was carried out according to Ciucanu and Kerek.[19] The methylated sample was extracted from DMSO by chloroform, then hydrolyzed (2 M CF_3CO_2H, 120 °C, 2 h), reduced with $NaBH_4$, acetylated and products were analyzed by GLC–MS using a Hewlett–Packard 5989A instrument equipped with a HP-5 MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness) and applying a temperature gradient of 150 °C (3 min) to 320 °C at 5 °C/min.

1.7. NMR spectroscopy

NMR spectra were obtained on solutions of ^2H_2O with a Bruker DRX Avance 600 MHz spectrometer (operating frequencies 600.31 MHz for ^1H NMR and 150.96 MHz for ^{13}C NMR) at 50 °C (1D ^1H at 60 °C). Chemical shifts were reported relative to internal acetone (δ_H 2.225; δ_C 31.45). One-dimensional ^1H and ^{13}C NMR and 2D ^1H,^1H COSY, DQFCOSY, RELAY COSY, TOCSY, ROESY, as well as the ^1H,^{13}C-heteronuclear HMQC and HMBC experiments were recorded applying standard Bruker software.

Acknowledgements

We thank H. Moll, for help with GLC-MS and Dr. A. Cimmino (Università di Napoli Federico II) and P. Lo Cantore (Università degli Studi della Basilicata), for their help in the cultivation of bacteria and the extraction of LPS.

References

1. Lelliot, R. A.; Stead, D. E. *Methods for the Diagnosis of Bacterial Diseases of Plants*; Blackwell Scientific Publications: Oxford, 1987.
2. Paine, S. G. *Ann. Appl. Biol.* **1919**, *5*, 206–219.
3. Wong, W. C.; Fletcher, J. T.; Unsworth, B. A.; Preece, T. F. *J. Appl. Bacteriol.* **1982**, *52*, 43–48.
4. Atkey, P. T.; Fermor, T. R.; Lincoln, S. P. In *Proceedings of the 13th International Congress on The Science and Cultivation of Edible Fungi*; Maher, M. J., Ed.; A. A. Balkema: Rotterdam, 1991; pp 431–435.
5. Lincoln, S. P.; Fermor, T. R.; Stead, D. E.; Sellwood, J. E. *Plant Pathol.* **1991**, *40*, 136–144.
6. Kaczynski, A.; Karapetyan, G.; Evidente, A.; Iacobellis, N. S.; Holst, O. *Carbohydr. Res.* **2006**, *341*, 285–288.
7. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–91.
8. Leontein, K.; Lindberg, B.; Lönngren, J. *Carbohydr. Res.* **1978**, *62*, 359–362.
9. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1979**, *77*, 1–7.
10. Jansson, P. E.; Kenne, L.; Widmalm, G. *Carbohydr. Res.* **1989**, *188*, 169–191.
11. Rundlöf, T.; Weintraub, A.; Widmalm, G. *Eur. J. Biochem.* **1998**, *258*, 139–143.
12. Bock, K.; Lundt, I.; Pedersen, C. *Tetrahedron Lett.* **1973**, *13*, 1037–1040.
13. Knirel, Y. A.; Kochetkov, N. K. *Biochemistry (Moscow)* **1994**, *59*, 1325–1383.
14. Jansson, P. E. In *Endotoxin in Health and Disease*; Brade, H., Opal, S. M., Vogel, S. N., Morrison, D. C., Eds.; Marcel Dekker: New York, 1999; pp 155–178.
15. Corsaro, M. M.; De Castro, C.; Molinaro, A.; Parrilli, M. *Recent. Res. Dev. Phytochem.* **2001**, *5*, 119–138.
16. Evidente, A.; Motta, A. In *Studies in Natural Products Chemistry*; Atta-ur-Rahman, Ed.; Elsevier: Amsterdam, 2002; Vol. 26, pp 581–629.
17. McWain, P.; Gregory, G. F. *Phytochemistry* **1972**, *11*, 2609–2612.
18. Lavermicocca, P.; Iacobellis, N. S.; Simmaco, M.; Graniti, A. *Physiol. Mol. Plant Pathol.* **1997**, *50*, 129–140.
19. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.