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Note

Structural determination of the phytotoxic mannan exopolysaccharide from *Pseudomonas syringae* pv. *ciccaronei*

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Abstract

The structural determination was performed of a mannan exopolysaccharide from the Gram negative bacterium *Pseudomonas syringae* pv. *ciccaronei*, which is the pathogenic agent responsible for the leaf spots of carob plants. The structure, obtained by chemical, enzymatic and spectroscopic methods, consisted of a backbone of α-(1→6)-linked mannopyranose units with 80% substituted at C-2 by mono-, di- and trisaccharide side chains. In addition, terminal glucose units and phosphate groups were found to be present. This is, to the best of our knowledge, the first report of a mannan exopolysaccharide structure from a phytopathogenic bacterium. The pure polysaccharide showed phytotoxic effects, i.e., chlorosis and necrosis on tobacco leaves.

Keywords: Mannan; Exopolysaccharide; *Pseudomonas*; Carob; Phytotoxicity

The role of extracellular polysaccharides (EPS) from phytopathogenic pseudomonads was until now not completely understood. Rudolph et al.[1] included EPS into a group of bacterial virulence factors together with enzymes, toxins and membrane active substances. In a recent review on EPS from bacterial plant pathogens,[2] Jahr et al. confirmed the role of EPS in infection and colonization of host tissue and in the survival of the pathogen. The structural determination of the exopolysaccharides from phytopathogenic bacteria is the first step in understanding their function in plant pathogenesis.

In this study, the EPS fraction of *Pseudomonas syringae* pv. *ciccaronei*, the causal agent of leaves spots of carob tree[3] was purified, characterized and the phytotoxic effect on tobacco plants reported.

The EPS fraction, which was obtained by precipitation from ethanol of the aqueous phase of the cultural filtrate, contained, on the basis of Lowry assay,[4] approx. 30% protein.

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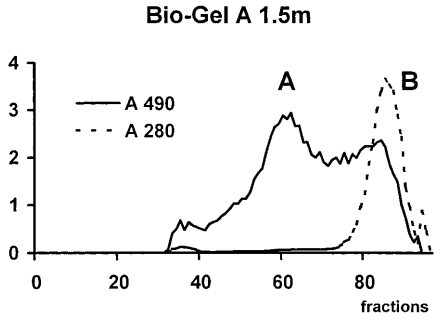


Fig. 1. Bio-Gel A 1.5 m gel-filtration chromatography of EPS fraction from *Pseudomonas syringae* pv. *ciccaronei*.

Table 1
Methylation data of EP fraction from *Pseudomonas syringae* pv. *ciccaronei*

Residue	Molar ratio
2,6-Substituted Man*p*	3.0
t-Man*p*	5.1
2-Substituted Man*p*	2.7
3-Substituted Man*p*	0.9
6-Substituted Man*p*	0.9
t-Glc*p*	1.2

This fraction was then subjected to a purification on Bio-Gel A 1.5 m obtaining two peaks, namely fraction A (protein content 1%) and fraction B (protein content 60%) (Fig. 1). Since the phytotoxicity bioassay was negative for fraction B and positive for fraction A, the structural analysis was performed only on this latter. In order to verify the homogeneity of fraction A, a Sephacryl S-300 gel-filtration chromatography was performed, obtaining a single peak (EP fraction) with an average molecular weight of 56 kDa, $[\alpha]_D$ +11.2°.

The glycosyl composition, obtained by analysis of alditol acetates, indicated a monosaccharide composition consisting of mannose with traces of glucose. The absolute configuration of these sugars was determined to be D on the basis of the GLC analysis of their 2-octyl glycoside acetates.[5] The results of the methylation analysis (Table 1) showed the presence of differently linked mannose units suggesting a mannan structure.

The ^{13}C and ^{1}H NMR spectra (Fig. 2) were in good agreement with the previous observations, because they exhibited seven broad anomeric ^{1}H singlets, at δ 5.284, 5.153, 5.130, 5.105, 5.082, 5.037, and 4.889, and four anomeric ^{13}C signals at δ 103.5, 101.6, 100.6, and 99.5, all attributable to mannose units. Besides these signals we also found in the ^{1}H spectrum three peculiar signals at δ 5.427 (t, 8.6 Hz) and δ 4.517 (d, 8.1 Hz) and 4.548 (d, 8.1 Hz). The use of HMQC, COSY, TOCSY, NOESY and HMBC experiments allowed the identification of most of the ^{1}H and ^{13}C signals of the sugar residues (Table 2). The anomeric proton chemical shifts together with $^{1}J_{C,H}$ and $^{3}J_{H,H}$ values, indicated the α and the β configuration for mannose and glucose units respectively. From an HMQC experiment, the proton anomeric signal at δ 5.427 was found to be correlated to an anomeric carbon signal at δ 97.1 and both values were in agreement with a phosphorylated mannose unit.[6] The presence of a signal at δ −1.608 in a ^{31}P NMR spectrum confirmed this and suggested a phosphodiester linkage. In order to define the phosphate content, both chemical analysis and proton anomeric signal integration between the signal at δ 5.427 and all of the other anomeric signals were performed, giving a 3% value.

The chemical data and the close similarity of chemical shifts with those reported in the literature,[6,7] supported a sugar backbone consisting of α-(1→6)-linked mannopyranose units branched at C-2. The integration of proton and carbon anomeric signals of 6-linked and 2,6-linked mannopyranose units suggested a highly branched structure (80%). This was confirmed by molar ratios of permethylation data (Table 1) between terminal and 6-linked units, considering that the low recovery of 2,6-OMe-Man with respect to the terminal units was in agreement with a peculiar acid lability of 3,4-di-OMe-mannose residues.[8] The arms are made up of 2- and/or 3-linked mannose units ending with mannose or glucose as deduced by methylation analysis and NMR data.

An exomannosidase enzymatic hydrolysis performed on EP fraction gave, after Sephadex G-15 gel-filtration chromatography, two products. The more retained fraction showed to be only mannose on the basis of chemical and spectroscopic data. The polysaccharide fraction, eluted in the void volume, was analysed by NMR experiments (Table 3).

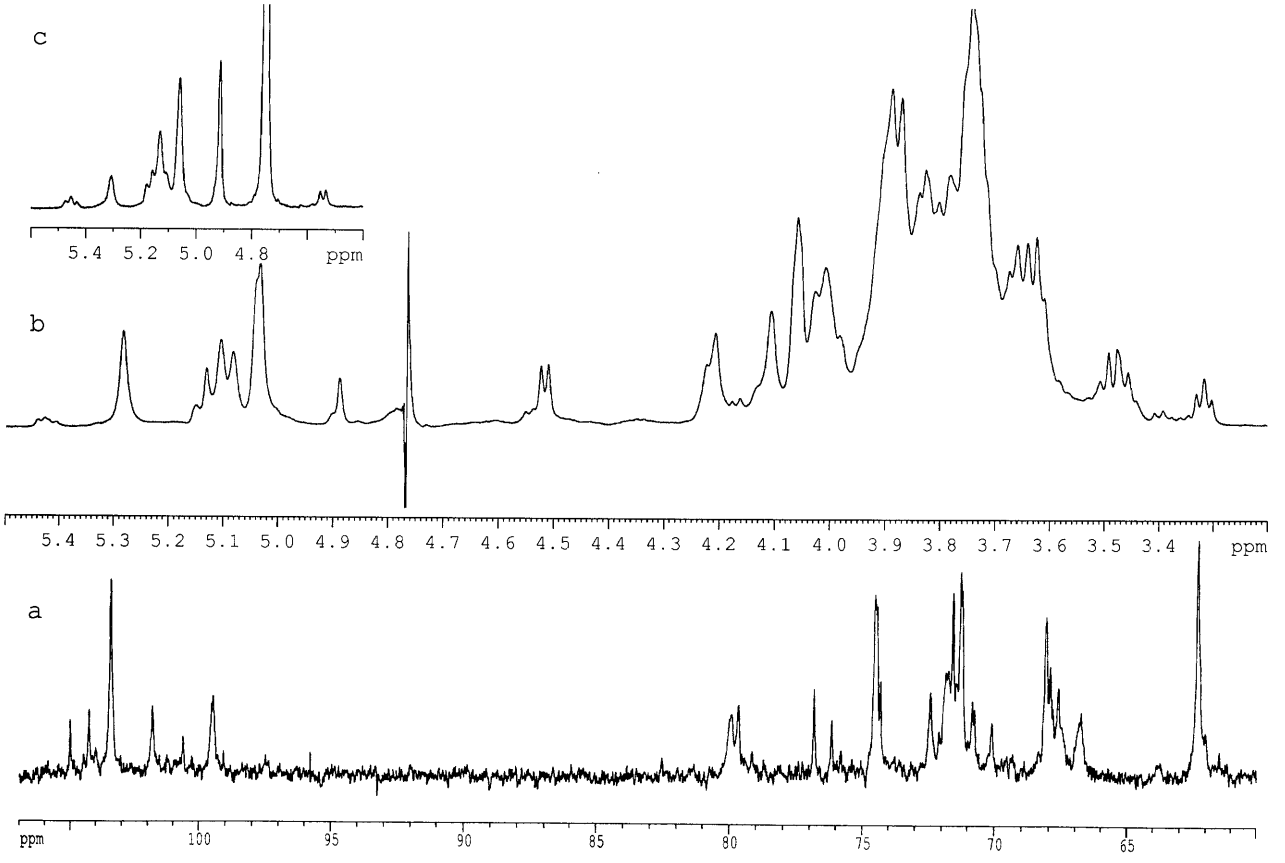


Fig. 2. ^{13}C (a), ^{1}H-NMR (b) spectra of the EP fraction from *Pseudomonas syringae* pv. *ciccaronei* and anomeric region of ^{1}H-NMR spectrum (c) of the EP fraction after enzymatic hydrolysis (600 MHz, D_2O, 30 °C).

Table 2
^{1}H and ^{13} C data of EP fraction from *Pseudomonas syringae* pv. *ciccaronei*

Residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
1-P Man*p*	5.427	4.000				
	97.1	71.3				
2-Man*p*	5.284	4.109	4.001	3.669	3.759	3.886, 3.736
	101.6	79.5	71.0	67.4	74.6	61.9
3-Man*p*	5.153	4.213	3.931			3.886, 3.736
	103.5	71.1	79.0			62.1
3-Man*p*	5.130	4.210	3.942		3.752	3.886, 3.736
	103.5	71.1	79.1		74.6	62.1
2,6-Man*p*	5.105	4.022	3.756		3.794	4.000, 3.699
	99.5	79.8	71.5		72.3	67.8
2,6-Man*p*	5.082	4.012	3.904		3.790	4.000, 3.699
	99.5	79.8	71.5		72.4	67.8
t-Man*p*	5.037	4.074	3.827			3.893, 3.711
	103.5	70.0	71.8			62.1
t-Man*p*	5.037	4.056	3.828			3.893, 3.711
	103.5	71.1	71.8			62.1
6-Man*p*	4.889	3.981	3.815	3.818		3.937, 3.756
	100.6	71.0	71.7	68.5		66.8
t-Glc*p*	4.517	3.316	3.483	3.457	3.625	3.843, 4.220
	104.4	74.1	76.9	70.5	75.6	62.1

Table 3
^{1}H and ^{13}C data of EP fraction from *Pseudomonas syringae* pv. *ciccaronei* after enzymatic hydrolysis

Residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
1-P Man*p*	5.435	4.001	3.913	3.802	3.700	
	96.8	70.5	70.8	67.1		
2-Man*p*	5.280	4.105	3.906	3.718	3.714	3.870, 3.721
	101.2	79.3	71.0	67.4	74.4	61.8
3-Man*p*	5.159	4.209	3.881		3.761	3.886, 3.736
	103.2	71.1	79.1		74.3	62.0
3-Man*p*	5.137	4.226	3.876		3.756	3.886, 3.736
	103.2	71.1	79.1		74.6	62.0
2,6-Man*p*	5.105	4.027	3.932		3.800	4.005, 3.701
	99.2	79.6	71.5		72.3	67.6
t-Man*p*	5.037	4.059	3.860	3.663	3.754	3.893, 3.711
	103.2	71.0	71.2	67.5	74.4	62.1
t-Man*p*	5.037	4.204	3.932		3.864	3.893, 3.711
	103.2	70.1	70.7			62.1
6-Man*p*	4.889	3.981	3.818	3.708		3.913, 3.729
	100.6	70.6	71.0	67.3		66.5
t-Glc*p*	4.517	3.318	3.480	3.617	3.843	3.846, 4.214
	104.2	74.1	76.3	70.5	75.6	62.2

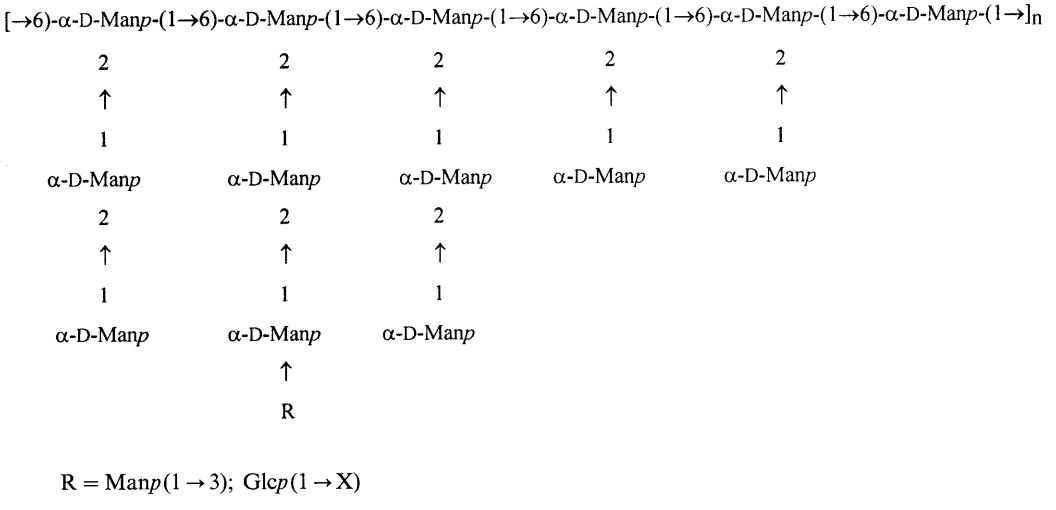
The comparison of proton anomeric signal intensities between the EP fraction and the mannosidase degraded product (Fig. 2) showed a remarkable increase in the signal at δ 4.889 (6-Man) with respect to signals at δ 5.284 (2-Man), and δ 5.153 and 5.130 (3-Man). The methylation data were in good agreement with this observation, actually the GC analysis showed a significant higher content of 6-Man. Since the mannosidase hydrolyses the EP from the terminal mannose units, this result confirmed the presence of 6-Man in the backbone and of 2- and 3-Man in the arms. The presence of *t*-Man residue in the mannosidase product (Table 3), which is due to an incomplete enzymatic hydrolysis, could be explained by a high number of branching points which makes the hindered arms not easily approachable by the enzyme.

In order to establish the length of the branches, an acetolysis reaction, whereby 6-linked sugars are preferentially cleaved,[9] was performed. The crude reaction mixture was separated on Bio-Gel P-2 to give, besides fraction A, eluted in the void volume, fractions B–E. Fraction E consisted of only reducing mannose. Fractions D, C and B were found to be oligosaccharides and consisted of α-D-Man*p*-(1→2)-D-Man, α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-D-Man and α-D-Man*p*-(1→3)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-D-Man, respectively, on the basis of their NMR data identity with those reported in literature,[10] and confirmed by methylation analysis and by FABMS data of their acetate derivatives. A molar ratio of about 1:2:2:1 for Man:Man_2:Man_3:Man_4 was found from the acetolysis reaction. Only for fraction B, ^{1}H and ^{13}C NMR spectra showed, besides to the more abundant mannose anomeric signals, a doublet at δ 4.550 (7.8 Hz) and δ 103.57, respectively, both attributed to *t*-Glc units. Thus, it is possible to suggest that some of the trisaccharide branches end with glucose units.

Fraction A was a mixture of oligosaccharides which were phosphorylated as deduced by the signal at δ 4.394 in the ^{31}P NMR spectrum. In particular, this value indicated monophosphorylated units. Since all chromatographic attempts to separate this mixture were unsuccessful, the crude oligosaccharides were analysed by FABMS as permethylated alditol derivatives. This resulting spectrum showed two *pseudo* molecular ion peaks at m/z 974 $(M+H)^+$ and 770 $(M+H)^+$ corresponding to phosphorylated tetra- and trisaccharide structures, respectively. Besides these ions there were three peaks at m/z 721, 517,

299, attributable to fragments, P–Hex–Hex–Hex$^+$, P–Hex–Hex$^+$ and P–Hex$^+$, respectively, indicating the phosphorylated mannose, unit as a terminal non-reducing one. After hydrolysis, reduction and acetylation of this fraction, GLC–MS analysis showed the presence of 2-Man, 3-Man, 2-linked mannose as terminal reducing units and the unexpected 6-Man instead of the terminal non-reducing mannose. This means that in some branches the terminal non-reducing residue is phosphorylated at C-6 and that this phosphate is linked to an oligosaccharide whose length can not be longer than four residues.

This result together with molar ratio data from methylation analysis (Table 1) allowed us to suggest an average repeating unit built up of mannose α-(1→6) backbone highly branched at C-2 with mono, di- and trisaccharide side chains as here reported and only few tri- and disaccharides chains are phosphorylated at the last mannose residue:



Among EPSs of plant pathogen bacteria, pathovars of *P. syringae* were found to produce alginate and levans.[11] The EPS structure from *P. ciccaronei* shows the typical arrangement common to mannans already isolated from fungi, with some peculiar features, i.e., the presence of a *t*-β-glucopyranose unit and phosphate which are not commonly reported.[6] Furthermore, to the best of our knowledge, this is the first report of mannan exopolysaccharide from phytopathogenic bacteria.

The EP fraction was assayed for its phytotoxic effect on tobacco leaves (Table 4). Starting with the concentration of 0.01 mg/mL, it determined chlorotic reactions after 24–48 h in tissue of tobacco leaves, chlorotic areas evolved during the following days, and became necrotic 4 days after injection. Preliminary results obtained with tobacco cells indicated that EPS affected the metabolism of ascorbate, an enzymatic system involved in the induction of plant defence response to phytopathogens.[12]

Table 4
Effect on tobacco leaves of EP fraction from *Pseudomonas syringae* pv. *ciccaronei*

Concentration (mg/mL)	Symptoms observed on the infiltrated area after			
	2 days		4 days	
	Chlorosis	Necrosis	Chlorosis [a]	Necrosis
2.5	+	−	++	+++
1.0	+	−	++	+++
0.5	+	−	++	+++
0.25	+	−	+	+
0.05	+	−	+	+
0.01	+	−	+	+

[a] Chlorosis around necrotic area.

1. Experimental

General methods.—The ^{1}H and ^{13}C NMR spectra were recorded in D_2O at 600 and 150.9

MHz, respectively, with a Bruker DRX 600 spectrometer equipped with a reverse probe, in the FT mode at 30 °C. ^{13}C and ^{1}H chemical shifts are expressed in δ relative to internal 1,4-dioxane (67.4 ppm) and TSP (sodium 3-trimethylsilylpropionate-2,2,3,3-d_4), respectively. The intensity ratio of proton signals was estimated from an ^{1}H NMR spectrum performed with an interpulsed delay of 3 s. ^{31}P NMR spectra were measured at 243 MHz in D_2O using a coaxial tube containing 85% H_3PO_4 as the internal standard. Two-dimensional homonuclear and heteronuclear NMR spectra were recorded using standard Bruker software. A mixing time of 200 ms was employed for NOESY spectrum. Mass spectra were recorded in positive mode, with a VG ZAB HF instrument equipped with a FAB source. TLC was carried out on Silica Gel F_{254} (E. Merck). All compounds were revealed by sprying plates with a saturated solution of chromic oxide in concd H_2SO_4, followed by heating at 120 °C for 15 min. Total carbohydrates were determined by the phenol–H_2SO_4 assay. Optical rotations were determined on a Perkin–Elmer 141 polarimeter. UV absorbance was determined on a Perkin–Elmer Lambda 7 instrument. GLC was performed with a Carlo Erba EL 490 instrument equipped with a flame-ionization detector. Protein contents were measured by the Lowry method. Partially methylated alditol acetates were analyzed on a Hewlett–Packard 5890 GLC–MS instrument. Oligosaccharide fractions were acetylated with (2:1) C_5H_5N and Ac_2O overnight at rt. All reagents were of analytical grade. Solvents were purchased from Romil.

Bacterial growth and culture conditions.—*P. syringae* pv. *ciccaronei* NCPPB2355 (National Collection of Plant Pathogenic Bacteria, Harpenden, UK), was maintained on nutrient agar containing glycerine (NGA) at 4 °C and was grown on King's B medium (KB).[13]. In order to produce EPS, the bacterial suspension (200 mL; A_{600} nm, 0.3) was added to 250 mL Erlenmeyer flasks containing 100 mL of Luria–Bertani (LB) medium. Bacteria were grown at 26 °C, and after 4 days the cultures were centrifuged (9000*g*, 10 min, 4 °C) and filter sterilized (0.45 mm cellulose acetate, Millipore Corp., Bedford, MA, USA). The culture filtrate was lyophilized.

Purification of bacterial extracellular polysaccharides.—The lyophilised culture filtrate (22 g/1.6 L) was suspended in ultrapure Milli-Q water and processed as previously reported[12] to give the 'crude' EPS (450 mg). This fraction was applied to a column of Bio-Gel A 1.5 m (Biorad, 1.5 × 96 cm), eluted with 50 mM NH_4HCO_3 at a flow rate of 30 mL/h at rt; 1.5 mL fractions were collected and assayed for protein content (280 nm) and the phenol test (490 nm). On the basis of the chromatogram reported in Fig. 1, fractions A and B were pooled and freeze dried (A, 205 mg and B, 214 mg). The proteins content was found to be 1% for A and 60% for B. The homogeneity of fraction A was then tested on Sephacryl S-300 column (Pharmacia, 1.5 × 90 cm, flow rate 50 mL/h, fraction volume 4 mL) eluting with 50 mM NH_4HCO_3, obtaining a single peak by the phenol test. Fractions were pooled and freeze-dried (200 mg).

Acid hydrolysis.—Polysaccharide samples were hydrolysed with 2 M CF_3CO_2H at 120 °C for 1 h.[10] The molar ratios of alditol acetates were evaluated using inositol as the internal standard by GLC on a SP2330 capillary column (Supelco, 30 m × 0.25 mm i.d.; flow rate 1 mL/min, N_2 as carrier gas), at 235 °C.

Methylation analysis.—Polysaccharide samples were methylated as reported.[14] The partially methylated products were reduced with $NaBD_4$, acetylated and analysed by GLC–MS on a SPB-1 capillary column (Supelco, 30 m × 0.25 mm i.d., flow rate 0.8 mL/min, He as carrier gas) with a gradient temperature as follows: 80 °C for 2 min, up to 240 °C at 8 °C/min, 240 °C for 15 min. Molar ratios were obtained using effective response factors,[15] and normalising the peak areas with respect to that of inositol hexa-acetate used as the internal standard.

Acetolysis of mannan.—Acetolysis of 100 mg mannan was performed as reported.[10] The deacetylated products were applied to a column (1.5 × 96 cm) of Bio-Gel P-2, and eluted with distilled water at a flow rate of 14 mL/h at rt; 2.5 mL fractions were collected. The fractionation yielded five fractions, namely, the exclusion volume fraction A (20 mg), the oligosaccharides B–D (46, 10, 5 mg), and mannose (15 mg). A sample (1 mg) of each was reduced with $NaBD_4$ for 1 h at rt, before

methylation. The latter was performed according to Ciucanu procedure[16] and the products were analysed by FABMS in the positive-ion mode. The oligosaccharides glycosyl linkage analysis was obtained as above reported for the polysaccharide sample.

Enzymatic hydrolysis.—The sample (30 mg) was dissolved in 50 mM $Na^+CH_3COO^-$ (2 mL) and treated with α-mannosidase (200 μL, Sigma) at 30 °C for 17 days. After lyophilisation the sample was chromatographed on a Sephadex G-15 (Pharmacia, 1.5 × 89 cm, 10 mM NH_4HCO_3, flow rate 45 mL/h, fraction volume 2.3 mL) obtaining two fractions, one in the void volume (10 mg) and the other more retained (16 mg).

Determination of absolute configuration.—A sample of mannan (1 mg) was hydrolysed with a mixture of 2 M CF_3COOH for 1 h at 120 °C. After neutralisation the crude reaction was treated with (+)-2-octanol (0.5 mL) and a drop of TFA overnight at 130 °C while stirring [5]. After usual work-up, the crude reaction was acetylated with Ac_2O and C_5H_5N. The GLC–MS analysis was performed on a ZB-5 capillary column (Phenomenex, 30 m × 0.25 mm i.d.), with this temperature programme: 150 °C for 5 min, up to 260 °C at 6°/min, 260 °C for 15 min.

Tobacco leaf assay.—Tobacco leaves of 40-day-old plants cv. Samsun, grown in the greenhouse (25 °C, 15 h daylight, 70% RH) were injected in their abaxial surface with EP solutions at concentrations of 2.5, 1.0, 0.5, 0.25, 0.05 and 0.01 mg/mL. Distilled water was injected as control. Plants were maintained at 23 °C, and after 1 and 4 days appearance of necrosis and/or chlorosis was registered.

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References

1. Rudolph, K. W. E.; Gross, M.; Ebrahim-Nesbat, F.; Nollemburg, M.; Zomoriadan, A.; Widra, K.; Neugebauer, M.; Hettwer, U.; El-Shouny, W.; Sonnemberger, B.; Klement, Z. In *Molecular Mechanism of Bacterial Virulence*; Kado, C. I.; Crosa, J. H., Eds.; Kluwer: The Netherlands, 1994; pp. 357–378.
2. Jahr, H.; Bahro, R.; Eichenlaub, R. *Prog. Botany* **1999**, *60*, 119–138.
3. Ercolani, G. L.; Caldarola, M. *Phytopathol. Mediterr.* **1972**, *11*, 71–73.
4. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
5. Leontein, K.; Lindberg, B.; Lönngren, J. *Carbohydr. Res.* **1978**, *62*, 359–362.
6. Vinogradov, E.; Petersen, B.; Bock, K. *Carbohydr. Res.* **1998**, *307*, 177–183.
7. Kogan, G.; Pavliak, V.; Masler, L. *Carbohydr. Res.* **1988**, *172*, 243–253.
8. Barreto-Bergter, E. M.; Travassos, L. R. *Carbohydr. Res.* **1980**, *86*, 273–285.
9. Lee, Y. C.; Ballou, C. E. *Biochemistry* **1965**, *4*, 287–298.
10. Corsaro, M. M.; De Castro, C.; Evidente, A.; Lanzetta, R.; Molinaro, A.; Mugnai, L.; Parrilli, M.; Surico, G. *Carbohydr. Res.* **1998**, *308*, 349–357.
11. Leigh, J. A.; Coplin, D. L. *Ann. Rev. Microbiol.* **1992**, *46*, 307–346.
12. De Gara, L.; Lavermicocca, P.; Pinto, M. C.; Bartoli, B.; Lonigro, S. L.; Evidente, A. *Petria* **1998**, *8*, 236–237.
13. King, E. O.; Ward, M. K; Raney, D. E *J. Lab. Clin. Med.* **1954**, *44*, 301–307.
14. Hakomori, S. *J. Biochem.* (*Tokyo*) **1964**, *55*, 205–208.
15. Sweet, D. P.; Shapiro, R. H; Albersheim, P. *Carbohydr. Res.* **1975**, *40*, 217–225.
16. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.