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Cell Wall Components of *Leptosphaeria maculans* Enhance Resistance of *Brassica napus*

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Supporting Information

ABSTRACT: Preparations with elicitation activity were obtained from the mycelium of *Leptosphaeria maculans*, a fungal pathogen of oilseed rape (*Brassica napus*). Crude delipidated and deproteinized extract from fungal cell walls induced expression of pathogenesis related gene 1 (*PR1*), hydrogen peroxide accumulation, and enhanced resistance of *B. napus* plants toward infection by *L. maculans*. Elicitation activity significantly decreased after treatment of a crude extract with α- or β-glucanase. Monosaccharide composition analysis of a crude extract purified by ion-exchange chromatography revealed glucose (~58 mol %), mannose (~22 mol %), and galactose (~18 mol %) as the major sugars. FT-IR and NMR spectra confirmed the presence of both carbohydrate and polypeptide components in the purified product. Correlation NMR experiments defined trisaccharide bound to O-3 of serine residue α-D-Glc*p*-(1→2)-β-D-Gal*f*-(1→6)-α-D-Man*p*-(1→3)-L-Ser. Terminal α-D-Glc*p* and (1→6)-β-D-glucan were also detected. The obtained results strongly support the conclusion that these carbohydrates induce defense response in *B. napus* plants.

KEYWORDS: *elicitor, oligosaccharide, Leptosphaeria maculans, Brassica napus, resistance*

INTRODUCTION

During the past two decades, the idea of sustainable agriculture inspired researchers to focus on the feasibility of restricting crop plant chemical treatment to diminish the input of xenobiotics to the food chain. One of the alternatives studied intensively was to take advantage of the plant's own defense potential. Induction of natural disease resistance in crops by biological or chemical elicitors has recently received increasing attention and is now considered the promising strategy for disease management.[1,2]

During their phylogenetic development, plants have evolved numerous defense mechanisms to cope with pathogens and pests. Following pathogen recognition by a plant, a number of defense responses are activated (e.g., callose deposition, phytoalexin synthesis, PR-protein accumulation), which results in increased resistance to subsequent infection. This status is called induced resistance. The advantage of this polygenically based resistance is that it is not easily overcome by new races of pathogen as frequently happens when the resistance is based on the specific interaction of the products of *R* and *Avr* genes. Another advantage is its low specificity. Activated plants could be more resistant to a broader spectrum of pathogens.

Induced resistance could be activated by the application of elicitors, the molecules secreted by pathogens or derived from their cell walls. Elicitors do not have a direct impact on pathogens, which clearly distinguishes them from pesticides.[3] Plants can recognize general chemical structures associated with microorganisms, so-called pathogen-associated molecular patterns (PAMPs) or elicitors. A number of elicitors were described in bacterial pathogens as well as in oomycetes and ascomycetes; for example, bacterial elicitor harpin[4] and flagellin[5] are well characterized including their downstream signaling. Fungal elicitors were also frequently reported, being of proteinaceous or glycoprotein character.[6] Fungal poly- and oligosaccharides have been recognized as potent elicitors as well. Chitin is a component of fungal cell walls, and its fragments act as elicitors in many plants.[7] Oligochitosan, a partially deacetylated form of chitin, induced production of nitric oxide (NO) and hydrogen peroxide in epidermal cells of *Brassica napus*.[8] Surprisingly even neutral saccharides, despite their structural properties (lack of functional side groups) are capable of enhancing plant resistance. Most of the studies in this field are based on β-glucans derived from cellulose or laminarin, that is, of plant or algal origin.[9,10] Studies on neutral poly- or oligosaccharides derived from the cell walls of the pathogens are much less common even if they can be expected to be more effective. Branched hexa(β-D-glucopyranosyl)-D-glucitols prepared by partial acid hydrolysis of *Phytophtora megasperma* mycelial walls induced accumulation of phytoalexins in soybean.[11,12] Oligoglucans (β-1,3 and β-1,6) with a degree of polymerization between 8 and 17 isolated from fungal plant pathogen *Alternaria alternata* 102 induced chitinase activity and ROS accumulation in tobacco BY-2 suspension cells.[13] Disaccharides containing mannose and glucose isolated from the cell walls of *Fusarium oxysporum* rapidly induced the

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phenolic pathway in *Rubus* cells.[14] It is evident that saccharide components of fungal cell walls are capable of triggering defense responses in plants, enhancing resistance toward infection, and even supporting plant growth.[15] Responses differ according to the origin of poly- or oligosaccharide, number of monomer units, and structure.

Our study is focused on the elicitors derived from the mycelium of *Leptosphaeria maculans*, a fungal pathogen of oilseed rape (*B. napus*) producing serious harm to this crop worldwide. Nowadays protection against this disease relies mainly on the use of efficient fungicides, for example, tebuconazole. However, deleterious effects of pesticides on human health and the environment increasingly attract public attention, which necessitates the introduction of alternative methods to protect important crops. Searching for easily biologically degradable resistance inducers of natural origin fits well into this concept. Several preparations of this kind already exist, for example, Messenger (Eden Bioscience), based on bacterial harpin protein.

Here we present the preparation and characterization of the elicitor from the *L. maculans* mycelium, displaying the ability to induce resistance against this pathogen in oilseed rape.

MATERIALS AND METHODS

Cultivation of Plants and Pathogen. *B. napus* seedlings were grown for 10 days in perlite supplied with Steiner's nutrient solution[16] in a cycle of a 14 h day (150 μE m^{-2} s^{-1}, 24 °C) and a 10 h night (19 °C) at 65% relative humidity.

L. maculans, isolate JN2,[17] was cultivated in the medium Gamborg B5 (1000 mL contains 3.16 g of Gamborg B5 with vitamins (Duchefa) in 10 mM MES buffer, pH 6.8, 30 g of saccharose) for 10 days at 26 °C under continuous shaking (130 rpm). Four hundred milliliters of the medium was inoculated with 400 μL of the spore suspension (10^8 spores/mL).

Plant Treatment. Cotyledons of 10-day-old seedlings were sprayed with 4 mL of the sample (6 plants for each experiment). The control plants were treated with water as a negative control and with 32 μM benzothiadiazole (1,2,3-benzothiadiazole-7-carbothioic acid *S*-methyl-ester, commercial product BION 50 WG, Syngenta, (BTH)) as a positive control.

Preparation of Elicitor Fractions from Mycelia of *L. maculans*. Active components from the cell walls of the fungus *L. maculans* were prepared according to the modified procedure of Wolski et al.[18] The mycelium of *L. maculans* was filtered and washed thoroughly with distilled water, and 150 g (fw) was homogenized in 400 mL of distilled water for 5 min in the mixer and filtered again. The mycelium was then washed with a chloroform/methanol mixture (1:1, v/v) to remove lipids, washed with acetone, and dried in air. Cell wall proteins were digested by Proteinase K from *Tritirachium album* (>500 U/mL, Sigma-Aldrich, Prague, Czech Republic) added (10 U/mL) in 15 mL of 20 mM Tris-HCl buffer, pH 7.5, and the suspension was incubated at 37 °C for 2 h in the dark. Enzyme solution was removed by filtration and the mycelium was washed three times with distilled water. Finally, 100 mL of distilled water was added, and the mycelium was extracted by autoclaving for 3 h at 120 °C and filtered, and the final volume was adjusted to 100 mL. The filtrate thus obtained (fraction F) was further purified by ion exchange chromatography on the HiTrap Sepharose FF Q column (AP Czech, Prague, Czech Republic). The anex column was equilibrated with 20 mM Tris-HCl buffer, pH 8.0. Twenty milliliters of filtrate F was applied on the column, and unbound components were washed out with equilibration buffer (fraction F0). Charged compounds were eluted using a linear gradient of NaCl in the elution buffer (0−1.5 M) and collected as fraction F1. The two fractions F0 and F1 were dialyzed against distilled water in the tubing with a membrane cutoff of 6−8 kDa.

Monosaccharide Composition Analysis. Ten milligrams of the lyophilized elicitor fraction F0 was dissolved in 5 mL of deionized water and 1 mL of 3 M trifluoracetic acid just prior to analysis. Samples were hydrolyzed 100 °C for 1 h and evaporated three times with deionized water. Samples were dissolved in 50 mL of deionized water and filtered through a 0.45 μm disc into Dionex vials. Standard mixture solutions of glucose, galactose, glucosamine, xylose, and mannose were prepared containing 0.05, 0.1, and 0.5 μg mL^{-1}, respectively. In addition, xylose and mannose standard solutions were prepared separately in a concentration of 0.1 μg mL^{-1}. Samples were fractionated using a high-performance anion exchange chromatography system with pulsed amperometric detection (HPAEC-PAD), Dionex BIO LC (Dionex, Sunnyvale, CA, USA) with a CarboPac PA1 analytical column and a CarboPac PA1 guard column. Ten microliters of each sample was injected, and fractions were eluted by 16 mM NaOH with isocratic elution at a flow rate of 0.25 mL min^{-1}.

Spectroscopic Measurements. Absorption FT-IR spectrum (400−4000 cm^{-1}) of the elicitor fraction F0 was recorded in KBr pellets by a Nicolet 6700 FT-IR spectrometer (Thermo Fischer Scientific, Waltham, MA, USA); 64 scans were accumulated with a spectral resolution of 2.0 cm^{-1}. The spectrum was smoothed and the baseline corrected using Omnic 8.0 (Thermo Fischer Scientific) software. The ^{1}H and ^{13}C NMR spectra of the elicitor fraction F0 were recorded on a Bruker Avance III TM 600 MHz (Bruker, Billerica, MA, USA) in D_2O solutions. Correlation ^{1}H, ^{1}H PFG-COSY, ^{1}H, ^{1}H TOCSY, ^{1}H, ^{1}H NOESY, and ^{1}H, ^{13}C HMQC, and ^{1}H, ^{13}C HMBC experiments were applied for the resonance signal assignment.

Enzyme Treatment. Fraction F was treated with β-glucanase (β-glucanase from *Aspergillus niger*, 1.2 U/mg, Sigma-Aldrich). Enzyme was added in the concentration 0.1 mg/mL of 100 mM citrate buffer, pH 5.0, and incubated for 120 min at 55 °C with constant shaking in the dark. Fraction F was treated with α-amylase (α-amylase from *Bacillus subtilis*, ∼380 units/mg, Sigma-Aldrich). Enzyme was added in the concentration 0.1 mg/mL of 100 mM citrate buffer, pH 6.9, and incubated for 120 min at 28 °C with constant shaking in the dark. All samples were dialyzed in membrane tubing with a cutoff of 6−8 kDa against distilled water.

Gene Expression. Frozen cotyledons from six 10-day-old seedlings were ground in liquid nitrogen using a mortar and pestle. RNA isolation was performed using a Spectrum plant total RNA kit (Sigma-Aldrich) according to the manufacturer's manual. The concentration of total RNA was measured using spectrophotometer NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and ND-1000 software V3.3.0 for wavelengths 260 and 280 nm. Isolated RNA (2.5 μg) was treated with deoxyribonuclease I (DNA-free Kit, Ambion, Austin, TX, USA) to eliminate possible contamination with genomic DNA. Treated RNA successively served as a substrate for reverse transcription to cDNA. M-MLV reverse transcriptase (Promega, Madison, WI, USA) and oligo dT20 (Metabion, Martinsried, Germany) were used in this step.

cDNA was quantified using DyNAmo Capillary SYBR Green qPCR Kit (Finnzymes, Vantaa, Finland) in the LightCycler 2.0 (Roche, Indianapolis, IN, USA). The reaction mixture preparation for one sample: 5 μL of PCR Master Mix, 1 μL of each primer (500 nM final concentration), 0.5 μL of distilled water, and 2.5 μL of cDNA (equivalent of 6.25 ng of RNA). The conditions of PCR and primers used were described earlier.[19]

Biological Assay. Cotyledons of 10-day-old *B. napus* seedlings were sprayed with elicitor fractions, water, and BTH and inoculated for 4 days after treatment by infiltration of spore suspension (10^5 spores/mL) using a 1 mL plastic syringe without needle. Twelve to 14 days after infiltration, leaves were scanned and lesion areas were evaluated by DPlan 0.5.1 software.[20]

Hydrogen Peroxide Detection. Hydrogen peroxide was detected in situ consistent with the procedure described by Thordal-Christensen et al.[21] The staining solution was prepared by dissolving 20 mg of diaminobenzidine (Sigma-Aldrich) in 200 μL of dimethylformamide and mixing it with 20 mL of 10 mM Tris buffer, pH 7.8. Detached cotyledons were immersed in the staining solution and infiltrated under vacuum. Cotyledons were then kept in a closed Petri dish in darkness at room temperature until reddish-brown staining appeared. The chlorophyll was then removed with 96% ethanol; cotyledons were rehydrated and scanned in a reflective mode.

RESULTS AND DISCUSSION

Production of Biomass for Elicitor Isolation. Starting with production of *L. maculans* mycelia for the preparation of elicitors, we intended to find media yielding a large amount of biomass. Fries medium [22] was not applicable for the experiments in which the elicitation effect has to be studied as it contains yeast hydrolysate. Yeast cells contain up to 1% of ergosterol in dry matter, and Lochman and Mikeš[23] showed that ergosterol treatment leads to the expression of defense genes in tobacco cells. For this reason, it was desirable to find other types of media to eliminate false-positive results. From the media recommended for fungi cultivation,[24] those not containing yeast lysate were chosen (a list of media and their composition can be found in the Supporting Information, Table 1). The highest and comparable amount of biomass was obtained when *L. maculans* was cultivated in a potato medium and in medium Gamborg B5 and modified Gamborg B5[25] with 1 or 50 mM KNO_3 and 1 mM glutamate (Figure 1). The amount of the mycelium obtained by cultivation

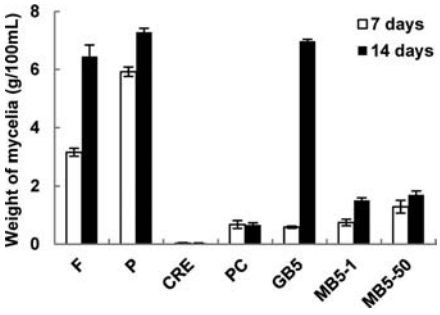

Figure 1. *L. maculans* biomass production in different types of media inoculated with 10^5 spores/mL. Fresh weight of mycelia was measured after 7 and 14 days of cultivation in medium: Fries (F), potato medium (P), creatine medium (CRE), potato−carrot medium (PC), medium Gamborg B5 (GB5), and modified GB5 containing 1 mM KNO_3 and 1 mM glutamate, (MB5-1), or 50 mM KNO_3 and 1 mM glutamate (MB5-50). Error bars indicate ± SE from three replicates.

of *L. maculans* in a potato medium and Gamborg B5 was comparable to the amount produced in Fries medium, but the mycelium grown in a potato medium contained a large amount of starch granules. For this reason, medium Gamborg B5 was finally chosen for preparation of the source material for further experiments.

***L. maculans* Cell Wall Components Trigger Defense Response in *B. napus* Plants.** The mycelium of *L. maculans* was extracted and fractionated as described under Materials and Methods. Obtained fractions (extract F, fractions F0 unbound and F1 bound to annex) were tested for the ability to trigger the response in *B. napus* plants leading to enhanced resistance. First, this was achieved by following the expression of *pathogenesis related gene 1* (*PR1*), which is a marker gene of the salicylic acid mediated plant immune response.[26] Fractions were sprayed on the cotyledons of *B. napus* seedlings, and *PR1* expression was measured by qPCR. Expression of the *PR1* gene was significantly induced by fractions F and F0 (Figure 2A), and the level of the expression was compared with the effect of BTH (BION, Syngenta, SA analogue), plant defense activator (Figure 2A). Second, the capability of the extract of inducing resistance in *B. napus* plants toward *L. maculans* was investigated and, as shown in Figure 2B,C, the area of lesions caused by the infection was significantly reduced and, importantly, the efficiency was the same in fraction F0 unbound to the annex column, whereas

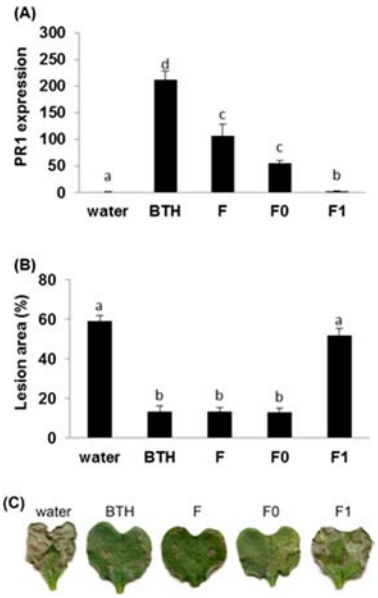

Figure 2. Induction of defense responses in *B. napus* plants treated with *L. maculans* mycelial fractions. Cotyledons of 10-day-old plants were sprayed with fractions F, F0, and F1, water as a negative control, and 32 μM benzothiadiazole (BTH) as a positive control. (A) Expression of *PR1* marker gene estimated 24 h after chemical induction by qPCR. Bars indicate ± SE from at least three separate experiments. (B) Area of lesions on *B. napus* leaves inoculated by *L. maculans* spore suspension (10^5/mL). Bars indicate ± SE from 20 cotyledons. Means with the same letter are not significantly different from each other (ANOVA; $P < 0.05$). (C) Typical symptoms on cotyledons caused by *L. maculans* infection.

fraction F1 was ineffective. The effect of fractions F and F0 was comparable to that of BION.

Changes in cellular redox status are an apparent feature of plant immune responses toward pathogen attack. The production of reactive oxygen species (ROS) in the site of infection is one of the early events of this complex process mediated by NO.[27,28] Production of both ROS and NO, which is considered a hallmark of innate immunity, had been also detected invariably after treatment with numerous elicitors.[27,29] Among others, redox changes mediate the salicylic acid pathway leading to the expression of PR defense genes.

Recently we have demonstrated that hydrogen peroxide accumulates in cotyledons infected with a virulent isolate of *L. maculans* JN2, and the same effect was observed when plants were treated with the elicitor prepared from the cultivation medium of *L. maculans*.[30] Here we show that a similar effect of H_2O_2 accumulation is caused by the extract of the *L. maculans* mycelium very early after the treatment (Figure 3), which indicates rapid activation of defense mechanisms in *B. napus* cotyledons.

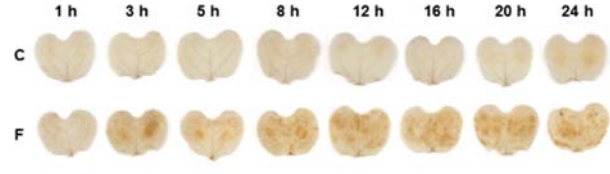

Figure 3. Accumulation of hydrogen peroxide in *B. napus* cotyledons after treatment with fraction F. Hydrogen peroxide was detected by vacuum infiltration of 1 mg mL^{-1} DAB solution (3,3′-diaminobenzidine). C, control plants; F, plants treated with mycelial extract.

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Active Component of the Elicitor Is a Feature of Saccharide. To reveal more clearly the elicitor active components, a mycelial extract (F) was hydrolyzed with enzymes specific for α- and β-glycosidic bonds. Fraction F was treated with microbial α-amylase and β-glucanase, respectively, and plants were sprayed with the digests. The expression of the *PR1* gene was evidently decreased in both cases, and the lesion area significantly increased (Figure 4 A,B), which implicates the involvement of poly- or oligosaccharides in the defense response elicitation effect of the extract. This finding corresponds with data published for elicitors obtained from other fungal plant pathogens. A more complex study was made for β-1,3- and 1,6-oligoglucans isolated from the mycelium of *Alternaria alternata*, which increased the expression of another SA responsive gene *PR3* in tobacco leaves, induced chitinase activity, and caused a weak oxidative burst.[13] Elicitor efficiency is well described for laminarin, a linear β-1,3-oligoglucan from the brown alga *Laminaria digitata*, which triggers accumulation of PR proteins and stimulates resistance of tobacco leaves against pathogenic bacterium *E. carotovora*.[10] Similarly, treatment of the grape vine suspension cells with β-1,3- or β-1,4-oligoglucan led to the transient generation of hydrogen peroxide followed by the differential expression of genes encoding the enzymes of the phenylpropanoid pathway and PR proteins.[9] Evidence for elicitor activity of α-glucans is very sparse. Wolski et al.[31] described that α-1,3-glucan from nonpathogenic *Rhizoctonia* sp. isolate induces expression of PR2, PR3, and PR5 in potato sprouts and enhances protection of potato sprouts and tubers against *Rhizoctonia* canker and dry rot. Our results show that both types of polysaccharides, that is, α- and β-glucans from *L. maculans*, are at least part of the active components of elicitor fraction.

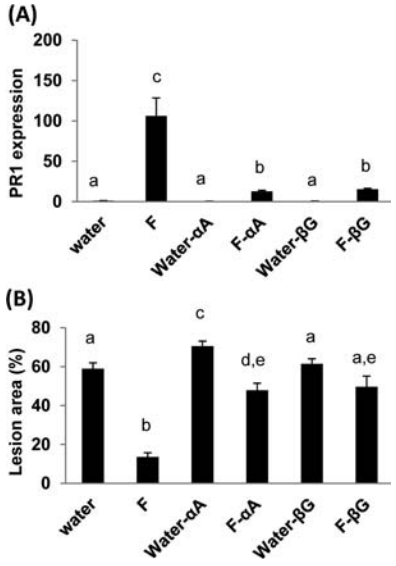

Figure 4. Effect of polysaccharide cleavage on the efficiency of the elicitor fraction F. Plants (10 days old) were sprayed with water as the control, mycelia extract (F), mycelial extract after treatment with α-amylase (F-αA), β-glucanase (F-βG), and respective controls (water-αA, water-βG). (A) Expression of *PR1* marker gene in *B. napus* measured 24 h after chemical induction by qPCR. Bars indicate ± SE from at least three separate experiments. (B) Area of lesions on *B. napus* leaves inoculated by *L. maculans* spore suspension (10^5/mL). Bars indicate ± SE from 20 cotyledons. Means with the same letter are not significantly different from each other (ANOVA; $P < 0.05$).

Structural Analysis of the Active Fraction. The carbohydrate composition of the elicitor fraction F0 is summarized in Table 1. D-Glucose is the main saccharide (~58 mol %), whereas significant amounts of D-mannose (~22 mol %) and D-galactose (~18 mol %) were also found. D-Glucosamine was observed only in a small amount (~2 mol %). The ratio between these sugar units, that is, Glc:Man:Gal:GlcN, is roughly 3:1:1:0.1. The FT-IR spectrum of the elicitor fraction F0 (Supporting Information, Figure 1S) confirmed the presence of both carbohydrate and polypeptide components. The broad intense band centered at 3376 cm^{-1} with two shoulders at lower wavenumbers arose mainly from OH stretching vibrations in sugar hydroxyls with a smaller contribution of NH stretching vibrations in polypeptide. The region of CH stretching vibrations (3000−2800 cm^{-1}) has two narrow bands at 2925 and 2855 cm^{-1} indicating methylene groups; two less pronounced shoulders near 2960 and 2874 cm^{-1} were assigned to methyl groups. The band at 1452 cm^{-1} arose from the CH_2 scissoring vibrations. These features arose from aliphatic amino acids and partially from carbohydrates. The spectrum has intense highly overlapping bands in the regions of both C═O/C═C (1800−1500 cm^{-1}) and CO/CC (1200−950 cm^{-1}) stretching vibrations; the last region is typical for carbohydrates.[32] Intense bands at 1650 cm^{-1} (amide I), 1542 cm^{-1} (amide II), and 1244 cm^{-1} (amide III) indicated the presence of peptide bonds;[33] bands at 1594 and 1404 cm^{-1} were assigned to COO^- stretching vibrations. The shoulder at 1153 cm^{-1} was assigned to COC stretching of glycosidic bonds. The weak bands at 890 and 814 cm^{-1} are indicative of β-glucose and α-mannose residues, respectively.[34,32] The ^1H and ^{13}C NMR spectra of the elicitor fraction F0 are very complex and contain signals of both carbohydrate and protein components (Supporting Information, Figure 2S). Homonuclear (COSY, TOCSY, and NOESY) (Supporting Information, Figure 3S) and heteronuclear (HMQC and HMBC) (Supporting Information, Figure 4S) correlation NMR experiments were used for the assignment of the resonance signals. As a result, several aliphatic and aromatic amino acid residues were identified by the characteristic proton and carbon signals summarized in Table 2. The signals of sugar residues were assigned to specific sugar units (Glc*p*, Gal*f*, and Man*p*) on the basis of the results of COSY, TOCSY, NOESY, and HMQC NMR experiments (Figure 5) and HBMC (not shown); resonance signal assignments are summarized in Table 3. The anomeric proton region (δ 4.4−5.5) contained several resonances of both α- and β-anomers overlapped by a very intense signal of HOD. The major sugar residues corresponding to pronounced H1 signals were assigned as A−H according to decreasing chemical shifts; the minor sugar residues corresponding to much weaker H1 signals were assigned as A′−H′. The anomeric proton signals (doublets) of residues B, B′, D, G, and H were found, respectively, at δ 5.18 ($J_{H1, H2}$ = 3.8 Hz), 5.15 ($J_{H1, H2}$ = 3.6 Hz), 5.02 ($J_{H1, H2}$ = 3.6 Hz), 4.60 ($J_{H1, H2}$ = 8.0 Hz), and 4.49 ($J_{H1, H2}$ = 8.0 Hz). According to the ^1H, ^{13}C HMQC spectrum (Figure 5), the corresponding ^{13}C signals were found at δ 92.1 (B), 98.1 (D), 95.9 (G), and 103.0 (H). Three unresolved

Table 1. Carbohydrate Composition (Mole Percent) of Fraction F0 from *Leptosphaeria maculans*[a]

GlcN	Gal	Glc	Man
2.19 ± 0.09	17.83 ± 0.18	58.05 ± 0.40	21.93 ± 0.56

[a]Values represent the mean ± standard error (n = 4).

Table 2. Chemical Shifts for the Main Amino Acid Units of Fraction F0 from *Leptosphaeria maculans*

	^{1}H (^{13}C) chemical shifts (δ)						
amino acid	C═O	α	β	γ	δ	ε	ξ
Ala	176.2	3.73	1.43 (16.1)				
Asp	173.0	3.84	2.62, 2.74 (39.3)				
Arg	176.0	3.71	1.86 (27.5)	1.63 (24.6)	3.20 (40.5)	(157.1)	
Ser	174.0	3.94 (56.5)	3.74, 3.89 (69.2)				
Thr	173.0	3.54	4.06 (68.6)	1.27 (19.9)			
Val	174.5	3.56	2.23 (29.3)	0.99, 0.94 (17.9, 16.6)			
Leu		2.97 (39.2)	1.67 (39.7)	1.65 (26.3)	0.90 (20.9)		
Ile	174.6	3.61 (58.0)	1.92 (39.7)	1.20, 0.96 (20.9, 14.67)	0.89 (11.2)		
Met	173.0	3.81	2.07, 2.14 (33.4)	2.59 (28.9)		2.08 (13.8)	
		4.12	1.98 (24.6)	2.35 (29.7)		2.00 (22.1)	
Phe	173.7	3.89	3.00, 3.15 (38.8)		7.38 (129.4)	7.32 (129.1)	7.28 (127.7)
Tyr	174.2	3.95 (56.7)	2.80, 2.90 (39.2)	(122.6)	7.15 (130.8)	6.85 (115.9)	
His	173.8	3.94 (56.1)	3.07, 3.24 (36.2)	(130.1)	7.10	8.15	

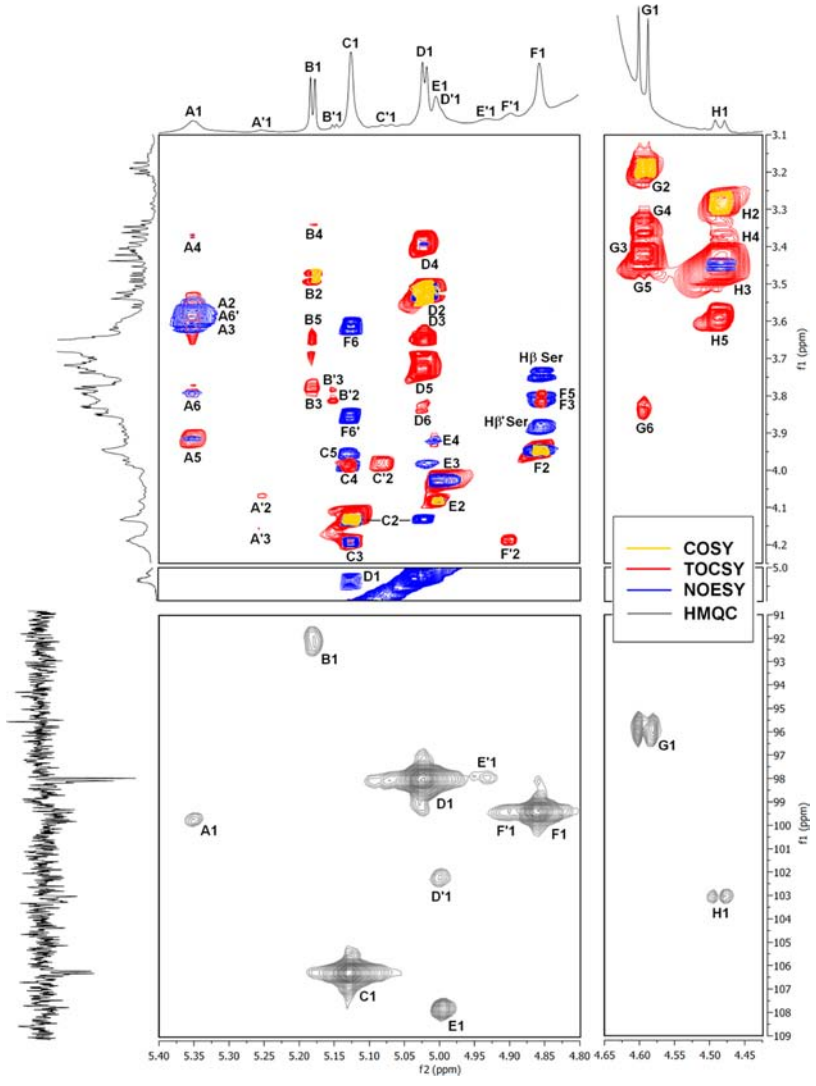

Figure 5. Anomeric region of ^{1}H, ^{1}H COSY (yellow), TOCSY (red), NOESY (blue), and ^{1}H, ^{13}C HMQC (gray) spectra of fraction F0 from *L. maculans*.

proton signals of residues A, C, E, and F were found at δ 5.35, 5.13, 5.00, and 4.86; corresponding carbon resonances were situated at δ 99.8, 106.3, 107.8, and 99.3. The peak positions and coupling constants confirmed that residues B, B′, and D are in α-anomeric configuration, whereas G and H are β-anomers.[35] The positions of proton and carbon signals of residues B and G are typical for nonbound α- and β-glucopyranoses, respectively. In contrast to other sugar resonances, the signals of these two residues demonstrated highly variable intensities after repeated measurements. Such variability could be explained by the

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Table 3. Chemical Shifts for the Saccharide Units of Fraction F0 from *Leptosphaeria maculans*

	^{1}H (^{13}C) chemical shifts (δ)						
unit	1	2	3	4	5	6	assignment
A	5.35 (99.9)	3.58 (72.9)	3.78	3.37	3.92 (73.4)	3.76, 3.83 (61.3)	T-α-Glc*p*
A′	5.25 (102.4)	4.07	4,17				
B	5.18 (92.0)	3.48 (71.1)	3.67 (73.6)	3.34 (70.2)	3.78 (70.6)	3.73, 3.89 (65.7)	α-Glc*p*
B′	5.15	3.81	3.78				α-1,2-Glc*p*
C	5.13 (106.2)	4.13 (86.5)	4.19 (75.5)	3.98 (82.5)	3.95 (70.2)	3.78, 3.80 (62.8)	β-1,2-Gal*f*
C′	5.08	3.98					
D	5.02 (98.1)	3.53 (71.2)	3.67 (72.7)	3.39 (69.5)	3.72 (72.3)	3.83, 3.78 (61.3)	T-α-Glc*p*
D′	5.00 (102.2)	4.03					
E	5.01 (107.8)	4.09 (80.9)	4.02 (77.0)	3.93 (82.5)			β-Gal*f*
E′	4.93 (98.0)	3.51					
F	4.86 (99.2)	3.95 (70.1)	3.80 (70.7)	3.62 (69.2)	3.78 (72.2)	3.84, 3.88 (69.3)	α-1,6-Man*p*
F′	4.90 (99.4)	4.19 (68.7)					T-α-Man*p*
G	4.60 (95.9)	3.19 (74.0)	3.43 (76.2)	3.36 (69.5)	3.45 (76.4)	3.65, 3.84 (68.7)	β-Glc*p*
H	4.49 (103.0)	3.30 (73.0)	3.45 (75.5)	3.42 (69.5)	3.59 (75.1)	3.81, 4.17 (68.9)	β-1,6-Glc*p*

admixture of free glucose in the equilibrium. The residues A, D, and H were also identified as glucopyranoses: the former two are terminal units in α-anomeric configuration, and the latter is a 1,6-linked β-anomeric unit. The C1 signals of C and E in the region of δ 106−108 are typical for the furanose ring and thus indicate β-galactofuranose.[36−38] By contrast, residue F was identified as α-mannopyranose.[39] NMR data for some minor units are insufficient to make exact assignments. The linkages between sugar residues were estimated by NOESY experiments. The interunit NOE signals are summarized in Table 4. These signals

Table 4. Interunit NOE Signals (δ) for Fraction F0 from *Leptosphaeria maculans*

H-1			NOE contact to H-1	
	unit	δ		proton assignment
C1	→2)-β-D-Gal*f*-(1→	5.13	3.62	F4
			3.85	F6
			3.87	F6′
D1	α-D-Glc*p*-(1→	5.02	4.13	C2
			5.12	C1
F1	→6)-α-D-Man*p*-(1→	4.86	3.74	Hβ-Ser
			3.89	Hβ′-Ser

confirm the connection between units C, D, and F. In addition, unit F is probably bound to O-3 of the amino acid serine,[39] which could be a part of a specific peptide. Therefore, the structure of trisaccharide is established as follows:

$$\overset{D}{\alpha\text{-D-Glc}p}\text{-(L} \rightarrow 2)\text{-}\overset{C}{\beta\text{-D-Gal}f}\text{-(L} \rightarrow 6)\text{-}\overset{F}{\alpha\text{-D-Man}p}\text{-(L} \rightarrow 3)\text{-L-Ser}$$

The same trisaccharide motif has been reported for O-linked oligosaccharides obtained from the cell wall of the pathogenic fungus *Fonsecaea pedrosoi*.[40] To our knowledge, this trisaccharide motif has not yet been found in fungal pathogens of plants. Possibly this pattern is recognized by *B. napus* as a PAMP of *L. maculans*. D-Galactose in its furanoid form was found in many oligosaccharides, polysaccharides, and glycoconjugates isolated from microorganisms, including bacteria, fungi, microalgae, lichens, and protozoa, but was not found in higher plants. The Gal*f* residues are able to form various linkages mutually or with other sugars. Being unusual for plants or animals, these units are essential for the survival or virulence of pathogenic mycobacteria.[41] In saprophytic and pathogenic fungi, Gal*f* residues have been found in the species of genera *Aspergillus*,[42−44] *Cladosporium* (*Hormoconis*),[45] *Discula*,[37] *Fonsecaea*,[40] *Guignardia*,[46] *Histoplasma*,[47] *Malassezia*,[48] *Paracoccidioides*,[49,50] and *Trichophyton*.[51]

The rest of glucose is present in the elicitor fraction F0 as free form (B, G), as (1→6)-β-D-glucan fragments (H), or as terminal α-anomeric units of minor oligosaccharides or glycosides (A). These carbohydrate components of *L. maculans* extract, together or individually, act as inducers of defense response in *B. napus* plants, which is in agreement with the decrease of elicitation activity caused by treatment with α- and β-glucanases.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplemental table and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ REFERENCES

(1) Terry, L. A.; Joyce, D. C. Elicitors of induced resistance in postharvest horticultural crops: a brief review. *Postharvest Biol. Technol.* **2010**, *32*, 1−13.

(2) Walters, D.; Newton, A.; Lyon, G. Induced resistance: helping plants to help themselves. *Biologist* **2005**, *52*, 28−33.

(3) Tamm, L.; Thürig, B.; Fleissbach, A.; Goltlieb, A. E.; Karavani, S.; Cohen, Y. Elicitors and soil management to induce resistance against fungal plant diseases. *NJAS−Wageningen J. Life Sci.* **2011**, *58*, 131−137.

(4) Wang, J.; Bi, Y.; Zhang, Z.; Zhang, H.; Ge, Y. Reduction of latent infection and enhancement of disease resistance in muskmelon by preharvest application of harpin. *J. Agric. Food. Chem.* **2011**, *59*, 12527−12533.

(5) Felix, G.; Duran, J. D.; Volko, S.; Boller, T. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* **1999**, *18*, 265−276.
(6) Mao, J.; Liu, Q.; Yang, X.; Long, C.; Zhao, M.; Zeng, H.; Liu, H.; Yuan, J.; Qiu, D. Purification and expression of a protein elicitor from *Alternaria tenuissima* and elicitor-mediated defence responses in tobacco. *Ann. Appl. Biol.* **2010**, *156*, 411−420.
(7) Kombrink, A.; Sanchez-Vallet, A.; Thomma, B. P. H. J. The role of chitin detection in plant-pathogen interactions. *Microbes Infect.* **2011**, *13*, 1168−1176.
(8) Li, Y.; Heng, Y.; Zhao, X.; Du, Y.; Li, F. Oligochitosan induced *Brassica napus* L. production of NO and H_2O_2 and their physiological function. *Carbohydr. Polym.* **2009**, *75*, 612−617.
(9) Aziz, A.; Gauthier, A.; Bezler, A.; Poinssot, B.; Joubert, J. M.; Pugin, A.; Heyraud, A.; Baillieul, F. Elicitor and resistance-inducing activities of β-1,4 cellodextrins in grapevine, comparison with β-1,3 glucans and α-1,4 oligogalacturonides. *J. Exp.Bot.* **2007**, *58*, 1463−1472.
(10) Klarzynski, O.; Plesse, B.; Joubert, J. M.; Yvin, J. C.; Kopp, M.; Kloareg, B.; Fritig, B. Linear β-1,3 glucans are elicitors of defense response in tobacco. *Plant Physiol.* **2000**, *124*, 1027−1037.
(11) Sharp, J. K.; McNeil, M.; Albersheim, P. Purification and partial characterization of a β-glucan fragment that elicits phytoalexin accumulation in soybean. *J. Biol. Chem.* **1984**, *259*, 11312−11320.
(12) Sharp, J. K.; McNeil, M.; Albersheim, P. The primary structures of one elicitor-active and seven elicitorinactive hexa(β-D-glucopyranosyl)-D-glucitols isolated from the mycelial walls of *Phytophthora megasperma* f.sp. *glycinea*. *J. Biol. Chem.* **1984**, *259*, 11321−11336.
(13) Shinya, T.; Ménard, R.; Kozone, I.; Matsuoka, H.; Shibuya, N.; Kauffmann, S.; Matsuoka, K.; Saito, M. Novel β-1,3, β-1,6-oligoglucan elicitor from *Alternaria alternata* 102 for defense response in Tobago. *FEBS J.* **2006**, *273*, 2421−2431.
(14) Nita-Lazar, M.; Heyraud, A.; Gey, C.; Braccini, I.; Lienart, Y. Novel oligosaccharide from *Fusarium oxysporum* L., rapidly induces PAL activity in *Rubus* cells. *Acta Biochim. Pol.* **2004**, *51*, 625−634.
(15) Liu, H.; Cheng, S.; Liu, J.; Du, Y.; Bai, Z.; Du, Y. Synthesis of pentasaccharise and heptasaccharide derivatives and their effects on plant growth. *J. Agric. Food Chem.* **2008**, *56*, 5634−5638.
(16) Steiner, A. A. *The Universal Nutrient Solution in Proceedings of the Sixth International Congress on "Soilless Culture"*; Pudoc: Wageningen, The Netherlandds, 1984; pp 633−650.
(17) Balesdent, M. H.; Attard, A.; Kuhn, A. L.; Rouxel, T. New avirulence genes in the phytopathogenic fungus *Leptosphaeria maculans*. *Phytopathology* **2002**, *92*, 1122−1133.
(18) Wolski, E. A.; Lima, C.; Agusti, R.; Daleo, G. R.; Andreu, A. B.; Lederkremer, R. M. An α-glucan elicitor from the cell wall of a biocontrol binoculate *Rhizoctionia* isolate. *Carbohydr. Res.* **2005**, *340*, 619−627.
(19) Šašek, V.; Nováková, M.; Jindřichová, B.; Boka, K.; Valentová, O.; Burketová, L. Recognition of avirulence gene AvrLm1 from hemi-biotrophic ascomycete *Leptosphaeria maculans* triggers salicylic acid and ethylene signaling in *Brassica napus*. *Mol. Plant−Microbe Interact.* **2012**, *25*, 1238−1250.
(20) Dplan4Lab; Digitální planimetr, http://www.4lab.smyslzivota.cz.
(21) Thordal-Christensen, H.; Zhang, Z. G.; Wei, Y. D.; Collinge, D. B. Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J.* **1997**, *11*, 1187−1194.
(22) Férézou, J. P.; Riche, C.; Quesneau-Thierry, A.; Pascard-Billy, C.; Barbier, M.; Bousquet, J. F.; Boudart, G. Structures de deux toxines isolées des cultures du champignon *Phoma lingam* Tode: La sirodesmine PL et la *desacetylsiro desmin* PL. *J. Chim.* **1977**, *1*, 327−334.
(23) Lochman, J.; Mikeš, V. Ergosterol treatment leads to the expression of a specificset of defence-related genes in tobacco. *Plant Mol. Biol.* **2006**, *62*, 43−51.
(24) Samson, R. A.; Hoekstra, E. S.; Frisvad, J. C. Mycological media for food and indoor fungi. In *Introduction to Food and Airborne Fungi*; Centraalbureau voor Schimmelcultures: Utrecht, Netherlands, 2004; pp 378−382.
(25) Van den Ackerveken, G. F. J. M.; Dunn, R. M.; Cozijnsen, T. J.; Vossen, J. P. M. J.; Van den Broek, H. W. J.; De Wit, P. J. G. M. Nitrogen limitation induces expression of the avirulence gene *Avr9* in the tomato pathogen *Cladosporium fulvum*. *Mol. Gen. Genet.* **1994**, *243*, 277−285.
(26) An, C.; Mou, Z. Salicylic acid and its function in plant immunity. *J. Integr. Plant Biol.* **2011**, *53*, 412−428.
(27) Torres, M. A. ROS in biotic interactions. *Physiol. Plant.* **2010**, *138*, 414−429.
(28) Sun, A.; Shengjuv, N.; Xing, D. Nitric oxide-mediated maintenance of redox homeostasis contributes to NPR1-dependent plant innate immunity triggered by lipopolysaccharides. *Plant Physiol.* **2012**, *160*, 1081−1096.
(29) Delledone, M. NO new is good news for plants. *Curr. Opin. Plant Biol.* **2005**, *8*, 390−396.
(30) Jindřichová, B.; Fodor, J.; Šindelářová, M.; Burketová, L.; Valentová, O. Role of hydrogen peroxide and antioxidant enzymes in the interaction between a hemibiotrophic fungal pathogen, *Leptosphaeria maculans*, and oilseed rape. *Environ. Exp. Bot.* **2011**, *72*, 149−156.
(31) Wolski, E. A.; Maldonado, S.; Daleo, R. G.; Andreu, A. B. A novel α-glucan elicits plant defense responses in potato and induces protection against *Rhizoctonia solani* AG-3 and *Fusarium solani* f.sp. *eumartii*. *Physiol. Mol. Plant Pathol.* **2006**, *69*, 93−103.
(32) Mathlouthi, M.; Koenig, J. L. Vibrational spectra of carbohydrates. *Adv. Carbohydr. Chem. Biochem.* **1986**, *44*, 7−89.
(33) Kong, J.; Yu, S. Fourier transform infrared spectroscopic analysis of protein secondary structures. *Acta Biochim. Biophys. Sin.* **2007**, *39*, 549−559.
(34) Galichet, A.; Sockalingum, G. D.; Belarbi, A.; Manfait, M. FTIR spectroscopic analysis of *Saccharomyces cerevisiae* cell walls: study of an anomalous strain exhibiting a pink-colored cell phenotype. *FEMS Microbiol. Lett.* **2001**, *197*, 179−186.
(35) Bubb, W. A. NMR spectroscopy in the study of carbohydrates: characterizing the structural complexity. *Concept. Magn. Reson. Part A* **2003**, *19A*, 1−19.
(36) Cordeiro, L. M. C.; Beilke, F.; Bettim, F. L.; de Fátima Reinhardt, V.; Rattmann, Y. D.; Iacomini, M. (1→2) and (1→6)-linked β-D-galactofuranan of microalga *Myrmecia biatorellae*, symbiotic partner of *Lobaria linita*. *Carbohydr. Polym.* **2012**, *90*, 1779−1785.
(37) Ahrazem, O.; Prieto, A.; Leal, J. A.; Giménez-Abián, M. I.; Jiménez-Barbero, J.; Bernabe, M. Fungal cell wall polysaccharides isolated from *Discula destructiva* spp. *Carbohydr. Res.* **2007**, *342*, 1138−1143.
(38) Suárez, E. R.; Syvitski, R.; Kralovec, J. A.; Noseda, M. D.; Barrow, C. J.; Ewart, H. S.; Lumsden, M. D.; Grindley, T. B. Immunostimulatory polysaccharides from *Chlorella pyrenoidosa*. A new galactofuranan. Measurement of molecular weight and molecular weight dispersion by DOSY NMR. *Biomacromolecules* **2006**, *7*, 2368−2376.
(39) Helander, A.; Kenne, L.; Oscarson, S.; Peters, T.; Brisson, J.-R. Synthesis and conformational and NMR studies of α-D-mannopyranosyl and α-D-mannopyranosyl-(1→2)-α-D-mannopyranosyl linked to L-serine and L-threonine. *Carbohydr. Res.* **1992**, *230*, 299−318.
(40) Shibata, N.; Okawa, Y. Chemical structure of β-galactofuranose-containing polysaccharide and *O*-linked oligosaccharides obtained from the cell wall of pathogenic dematiaceous fungus *Fonsecaea pedrosoi*. *Glycobiology* **2011**, *21*, 69−81.
(41) Pan, F.; Jackson, M.; Ma, Y.; McNeil, M. Cell wall core galactofuran synthesis is essential for growth of mycobacteria. *J. Bacteriol.* **2001**, *183*, 3991−3998.
(42) Leitao, E. A.; Bittencourt, V. C.; Haido, R. M.; Valente, A. P.; Peter-Katalinic, J.; Letzel, M.; de Souza, L. M.; Barreto-Bergter, E. β-Galactofuranose-containing *O*-linked oligosaccharides present in the cell wall peptidogalactomannan of *Aspergillus fumigatus* contain immunodominant epitopes. *Glycobiology* **2003**, *13*, 681−692.
(43) Tischer, C. A.; Gorin, P. A. J.; de Souza, M. B.; Barreto-Bergter, E. Structures of phosphonogalactomannans isolated from mycelia of *Aspergillus versicolor*. *Carbohydr. Polym.* **2002**, *49*, 225−230.
(44) Schmalhorst, P. S.; Krappmann, S.; Vervecken, W.; Rohde, M.; Müller, M.; Braus, G. H.; Contreras, R.; Braun, A.; Bakker, H.; Routier, F. H. Contribution of galactofuranose to the virulence of the

opportunistic pathogen *Aspergillus fumigatus*. *Eukaryot. Cell* **2008**, *7*, 1268−1277.
(45) Calixto, R.; Mattos, B.; Bittencourt, V.; Lopes, L.; Souza, L.; Sassaki, G.; Cipriani, T.; Silva, M.; Barreto-Bergter, E. β-Galactofuranose-containing structures present in the cell wall of the saprophytic fungus *Cladosporium* (*Hormoconis*) *resinae*. *Res. Microbiol.* **2010**, *161*, 720−728.
(46) Sassaki, G. L.; Ferreira, J. C.; Glienke-Blanco, C.; Torri, G.; De Toni, F.; Gorin, P. A. J.; Iacomini, M. Pustulan and branched β-galactofuranan from the phytopathogenic fungus *Guignardia citricarpa*, excreted from media containing glucose and sucrose. *Carbohydr. Polym.* **2002**, *48*, 385−389.
(47) Barr, K.; Laine, R. A.; Lester, R. L. Carbohydrate structures of three novel phosphoinositol-containing sphingolipids from the yeast *Histoplasma capsulatum*. *Biochem.* **1984**, *23*, 5589−5596.
(48) Shibata, N.; Saitoh, T.; Tadokoro, Y.; Okawa, Y. The cell wall galactomannan antigen from *Malassezia furfur* and *Malassezia pachydermatis* contains β-1,6-linked linear galactofuranosyl residues and its detection has diagnostic potential. *Microbiol.* **2009**, *155*, 3420−3429.
(49) Almeida, I. C.; Neville, D. C.; Mehlert, A.; Treumann, A.; Ferguson, M. A.; Previato, J. O.; Travassos, L. R. Structure of the N-linked oligosaccharide of the main diagnostic antigen of the pathogenic fungus *Paracoccidioides brasiliensis*. *Glycobiology* **1996**, *6*, 507−515.
(50) Levery, S. B.; Toledo, M. S.; Suzuki, E.; Salyan, M. E.; Hakomori, S.; Straus, A. H.; Takahashi, H. K. Structural characterization of a new galactofuranose containing glycolipid antigen of *Paracoccidioides brasiliensis*. *Biochem. Biophys. Res. Commun.* **1996**, *222*, 639−645.
(51) Ikuta, K.; Shibata, N.; Blake, J. S.; Dahl, M. V.; Nelson, R. D.; Hisamichi, K.; Kobayashi, H.; Suzuki, S.; Okawa, Y. NMR study of the galactomannans of *Trichophyton mentagrophytes* and *Trichophyton rubrum*. *Biochem. J.* **1997**, *323*, 297−305.