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Note

Fungal cell wall polysaccharides isolated from *Discula destructiva* spp.

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Abstract—The alkali-extractable water-soluble polysaccharides F1SS isolated from the cell wall of four species of *Discula destructiva* have been studied by methylation analysis and NMR spectroscopy, and their idealized structures established as

$$\{[\rightarrow 6)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow 5)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow]_n\rightarrow 6)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow 5)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow\}_m\rightarrow \text{Mannan core}$$
$$\begin{array}{c} 2 \\ \uparrow \\ 1 \\ \alpha\text{-L-Rha}p \end{array}$$

where $n \approx 2$ for strains CBS 109771 and CBS 133.91, $n \approx 1$ for CBS 132.91, and it has an intermediate value in strain CBS 130.91. The mannan core was obtained by mild hydrolysis of the F1SS polysaccharide and its structure consisted of a skeleton of α-(1→6)-mannopyranan, with around one out of eleven residues substituted at C-2 by short chains (one to six units) of 2-substituted mannopyranoses.

Keywords: Rhamnose; Galactofuranose; NMR spectroscopy; Chemotaxonomy

The fungus *Discula destructiva* is the causal agent of a severe disease in species of *Cornus* (dogwood anthracnose). The devastation of these trees in North America is often dramatic since their fruits and twigs serve as food for wildlife and there is a million dollars nursery industry. The colorful floral bracts make these trees and its cultivars highly prized landscape ornamentals. For a description and historical review of the fungus and the disease see Redling.[1]

The fungal alkali-extractable water-soluble cell wall polysaccharides (F1SS), which form part of peptido-polysaccharides, are minor components of the fungal cell wall (around 2–8%). Polysaccharide moieties similar to the polysaccharides F1SS have been shown to occur in glycoproteins.[2,3] The complex carbohydrates of these molecules are antigenically relevant[4–10] and serve for different biological functions, being one of the most important its participation in cell–cell and/or cell–host recognition phenomena.[11] We herein report on a novel structure isolated from *D. destructiva* which extends previous reports on fungal polysaccharides F1SS. The main aim of these studies is the utilization of these polysaccharides in the establishment of taxonomic and evolutionary relationships in Ascomycetes.

Polysaccharides F1SS amounted around 3% of the cell wall in all isolates. Acid hydrolysis revealed the presence of galactose, rhamnose, and mannose in a molar ratio close to 6.7:2.5:1, respectively, and less than 1.5% of glucose (see Table 1), which is due to the presence of a small amount of glycogen, occurring as impurity

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Table 1. Neutral sugars (mol %) released from polysaccharides F1SS from four isolates of *Discula destructiva*

Strain	Species name	Rha	Man	Gal	Glc
CBS 130.91	*Discula destructiva*	23.9	7.9	67.0	1.2
CBS 132.91	*Discula destructiva*	15.8	12.7	70.2	1.3
CBS 133.91	*Discula destructiva*	24.0	9.7	65.1	1.2
CBS 109771	*Discula destructiva*	24.1	9.6	65.2	1.1

in many other cell wall polysaccharides.[12,13] The absolute configuration analysis showed D-configuration for galactose and mannose and L-configuration for rhamnose. Methylation analysis gave partially methylated alditol acetates corresponding to terminal Rha*p*, 5-O-substituted, 6-O-substituted, and 2,6-di-O-substituted Gal*f*, in variable proportions, and, in some cases, small amounts of terminal Gal*f* and 5,6-di-O-substituted Gal*f* (Table 2).

^{1}H NMR spectra of the polysaccharides F1SS of all four isolates studied are shown in Figure 1. The general pattern indicated that all of them consist of very similar structures varying only in the relative intensities of the signals from the different residues. Thus, that polysaccharide with the maximum content in rhamnose (CBS 109771) was selected for further studies. The high resolution ^{1}H NMR spectrum contained, *inter allia*, a doublet at ca. 1.32 ppm, suggesting the presence of 6-deoxyhexopyranose, and four unresolved doublets due to anomeric signals, with integrated areas 2.3:1:3.3:2.3, which were labeled **K**–**N** in order of increasing field (Fig. 1a).

The ^{13}C NMR spectrum (Fig. 2a) showed also four singlets in the anomeric region, and one at 17.6 ppm, derived from the C-6 of the deoxyhexopyranose residue. A series of ^{1}H–^{1}H (DQFCOSY, TOCSY), and ^{1}H–^{13}C

Table 2. Percentages of the linkage types deduced from methylation analysis of the polysaccharides F1SS isolated from four strains of *Discula destructiva*

RT[a] (min)	Linkage type	Characteristic fragments (*m*/*z*)	Relative abundance (%)			
			CBS 130.91	CBS 132.91	CBS 133.91	CBS 109771
5.76	Rha*p*-(1→	89, 102, 115, 118, 131, 162, 175	18.2	8.8	9.8	19.4
8.59	Gal*f*-(1→	89, 102, 118, 162, 205	3.2	5.5	9.6	2.5
10.87	→5)-Gal*f*-(1→	87, 102, 113, 118, 131, 162, 173, 233	49.9	47.9	42.6	53.3
12.36	→6)-Gal*f*-(1→	102, 117, 118, 130, 162, 173, 233	7.2	21.7	18.5	5.4
14.70	→5,6)-Gal*f*-(1→	118, 201, 261	0.0	4.0	0.0	0.0
15.34	→2,6)-Gal*f*-(1→	88, 101, 117, 130, 173, 190, 233	21.5	12.1	19.6	19.4

[a] Retention time.

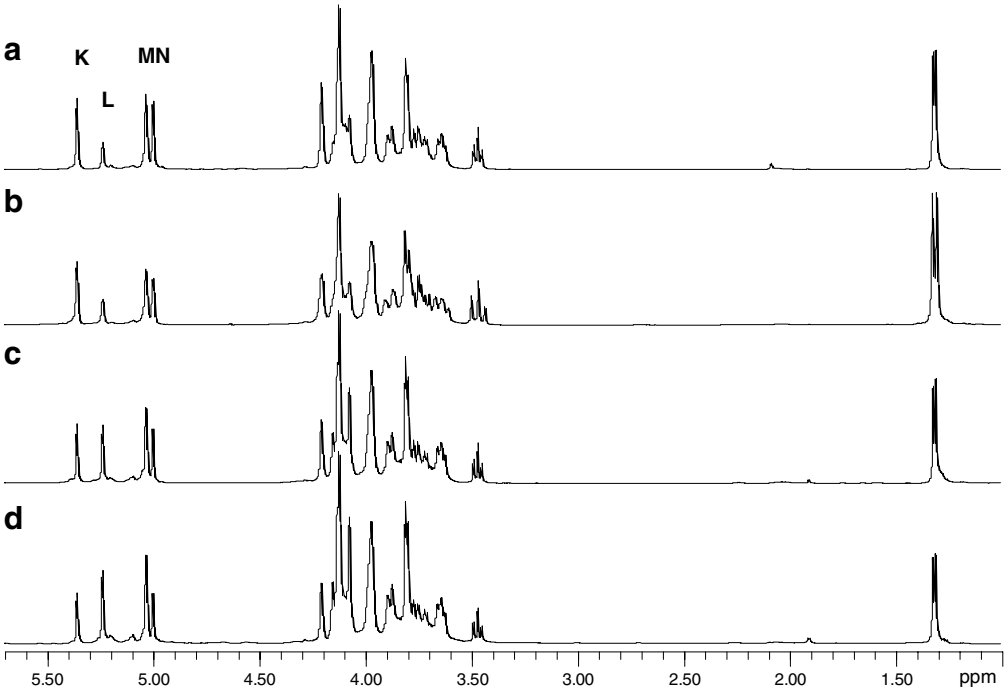


Figure 1. ^{1}H NMR spectra of the F1SS polysaccharides isolated from the cell wall of *Discula destructiva*, strains CBS 109771 (a), CBS 133.91 (b), CBS 130.91 (c), and CBS 132.91 (d). The anomeric protons have been labelled **K**–**N**.

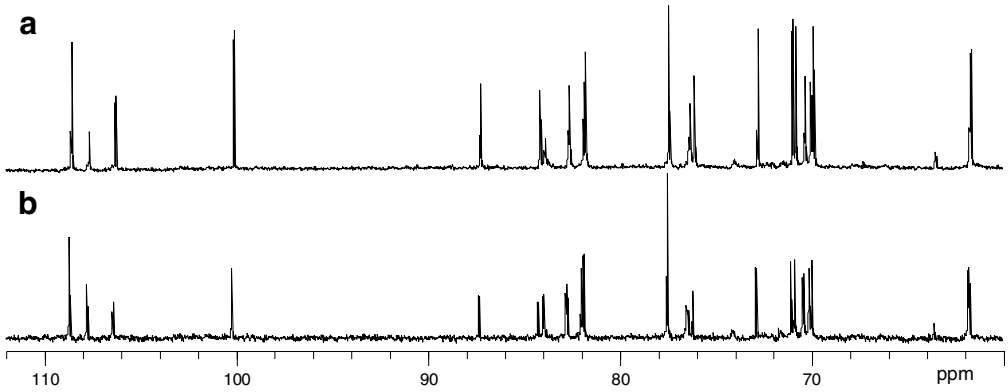
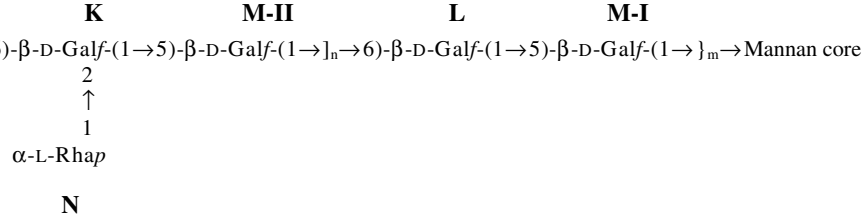


Figure 2. ^{13}C NMR spectra of the F1SS polysaccharides isolated from the cell wall of *Discula destructiva*, strains CBS 109771 (a) and 132.91 (b).

(HMQC, HSQC-TOCSY, and HMBC) 2D-correlation experiments allowed the assignment of most of the main signals of all the residues. The proton and carbon chemical shifts values are shown in Table 3. Comparison of these values with those of reference analogous compounds[14,15] revealed that **K** was a 2,6-di-O-substituted Gal*f*, **L**, a 6-O-substituted Gal*f*, **M**, a 5-O-substituted Gal*f*, and **N**, a terminal Rha*p* moiety. The **M** signal showed a shoulder at ca. 5.035 ppm, which suggested the presence of two kinds of slightly different 5-O-substituted Gal*f* units, approximately in analogous proportion than **K**/**L**. In addition to the slight difference in the chemical shifts of the anomeric protons, slight splitting were also observed for C-4, C-5, and C-6 carbons of residues **M**, which were labeled as **M-I** and **M-II**. In order to confirm these differences, we also studied the proton and carbon NMR spectra of the polysaccharide with **N**→2**K**→5**M-I**→6**K**′ (or 6**L**)→5**M-II**, where **K**′ represent a second unit of **K**. The slight differences in **M-I** and **M-II** are probably due to the proximity of the substitution at position 2 of residue **K**, as compared with residue **L**. Concerning the anomeric configuration of the different units, the anomeric coupling constants of the galactofuranose residues were around 1.8 Hz, which corresponds to β anomeric configurations.[16] The C-1 chemical shifts were also in complete agreement with the proton deductions, displaying values from 106 to 109 ppm, according with β-configurations. A carbon coupled HMQC experiment gave $^{1}J_{\text{C-1,H-1}} = 176$ Hz for the rhamnose unit, which demonstrated α-configuration for residue **N**.[17]

From all the combined data, the idealized structure of the polysaccharides F1SS from *D. destructiva* were deduced to be



the lower rhamnose content (strain CBS 132.91). The analysis of the ^{1}H NMR spectrum shows that the shoulder of **M-I** signal has now about the same proportion than **M-II**, and also than the clear, although very slight splitting (differences ⩽0.1 ppm) of C-4, C-5, and C-6 carbons in the ^{13}C NMR spectrum (Fig. 2a and b).

Regarding the geometry and arrangement of the different residues, they were unequivocally deduced from an HMBC experiment (Fig. 3), which showed H-1**K**/C-4**K**, H-1**K**/C-5**M**, H-1**L**/C-4**L**, H-1**L**/C-5**M**, H-1**M**/C-4**M**, H-1**M**/C-6**K** (or **L**), H-1**N**/C-5**N**, and H-1**N**/C-2**K** crosspeaks, which revealed both the furanoid character of **K**, **L**, and **M**, the pyranoid structure of **N**, and also the connections of the residues in the sequence:

From integration of the anomeric protons it was deduced that $n \approx 2$ for the polysaccharide of strains CBS 109771 and CBS 133.91, $n \approx 1$ for that of CBS 132.91, and it has an intermediate value in strain CBS 130.91. The small amounts (from traces to 1%) of terminal and 5,6-di-O-substituted Gal*f* are most probable due to scarce residues of **L** and/or **M** with units of Gal*f* attached at positions 5 or 6, respectively, as has been shown to occur in similar structures.[18,19] The average molecular mass of the polydisperse polysaccharides are in the range of 70–80 kDa, as calculated by gel permeation chromatography on a Sepharose CL-6B column, previously calibrated with different dextrans.

Table 3. ^{1}H and ^{13}C NMR chemical shifts (δ) for the alkali-extractable water-soluble cell wall polysaccharide (F1SS) isolated from *Discula destructiva*

Residue		1	2	3	4	5	6a	6b
K	H	5.36	4.20	4.19	4.09	3.97	3.88	3.64
	C	106.4	**87.4**	76.2	84.2	70.4	**70.1**	
L	H	5.24	4.14	4.07	4.08		3.88	3.64
	C	107.8	82.0	77.5	84.0	ca. 70.5	**70.1**	
M-I	H	5.04	4.13	4.12	4.12	3.96	3.80	3.80
	C	108.7	81.9	77.5	82.7	**76.4**	61.7	
M-II	H	5.035	4.13	4.12	4.12	3.96	3.80	3.80
	C	108.7	81.9	77.5	82.8	**76.5**	61.8	
N	H	5.00	3.96	3.76	3.47	3.73	1.32	
	C	100.3	71.1	70.9	72.9	70.0	17.6	

Bold numbers represent glycosylation sites.

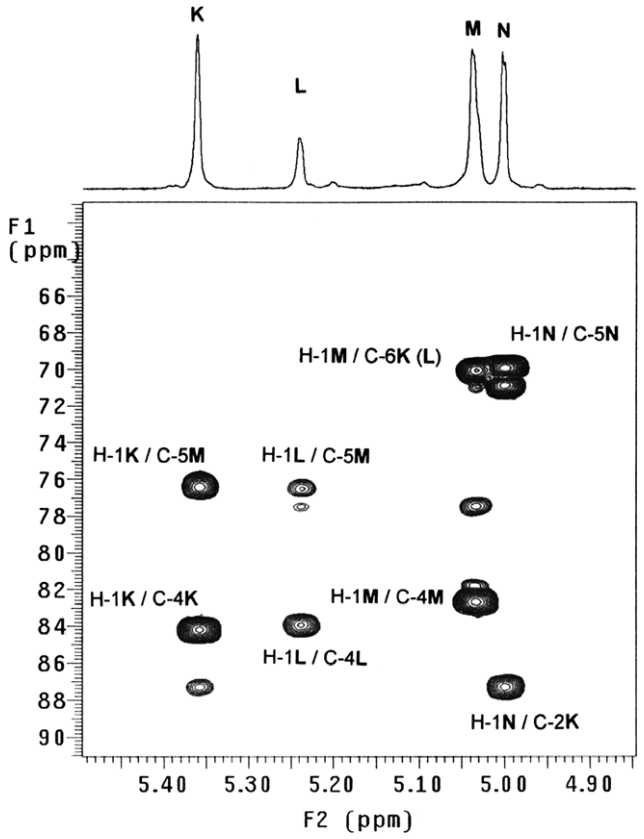


Figure 3. Anomeric region of the 2D HMBC spectrum of the F1SS polysaccharide isolated from the cell wall of *Discula destructiva*, strain CBS 109771. The crosspeaks showing relevant connections have been labelled.

The polysaccharide F1SS from strain CBS 109771 was treated with diluted acid, which selectively hydrolyzed the furanosidic chains, giving a degraded polysaccharide (the core). The material recovered represents around 3.8% of the native polysaccharide F1SS, and was exclusively composed of mannose. The discrepancy between the amount of mannose estimated from the analysis of the native polysaccharide (9.6%, Table 1) and the amount of mannan core recovered may be due to losses of this material during its preparation process (hydrolysis, dialysis, and lyophilization). On the other hand, due to the lability of the galactofuranoses under the hydrolysis conditions (3 M TFA), it is possible that they were partially destroyed (in our experience, particularly those substituted at C-6) and consequently the much more stable mannopyranoses and glucopyranoses were overestimated. Methylation analysis demonstrated the presence of only terminal Man*p*, 2-O-substituted, 6-O-substituted, and 2,6-di-O-substituted Man*p* (Table 4). There is not good agreement between the proportions of branch points and of terminal residues detected by this technique. These proportions are better estimated by integration of the anomeric proton signals of its NMR spectrum. The ^{1}H NMR spectrum of this material (Fig. 4) contained five major anomeric signals and two minor signals between 5.4 and 4.8 ppm, labelled **A**–**G**, from low to high field. Indeed, this spectrum was very similar to those obtained for the mannan cores after partial hydrolysis of several fungal polysaccharides F1SS.[6,20–23] These mannan cores are constituted by a main chain of α-(1→6) mannopyranose, substituted at C-2 by variable amounts of either a single unit of Man*p* or small chains (two to seven units) of α-(1→2) Man*p* residues. We have reported on similar structures for the cores of *Chaetosartorya chrysella* and *Aspergillus* spp.[22] According to the values of the chemical shifts found in the ^{1}H NMR spectrum of the core of *D. destructiva* and, after comparison with the mannan core of *C. chrysella*, it seems obvious that **A**–**D** are 2-O-substituted Man*p* units, located at different places along the side chains of the core. Thus, **C** is the 2-Man*p* moiety linked to the C-2 of the main α-(1→6) mannopyranose backbone, **D** is the terminal residue of the side chains, and **A** and **B** are 2-Man*p* residues located in between **C** and **D**. **E** is a 2,6-di-O-substituted Man*p*; **F**, a terminal Man*p*, linked either to C-2 of the backbone residues or to the last unit in the side chains (**D**), and **G**, 6-O-substituted Man*p* residues of the backbone. Integration of the

Table 4. Percentages of the linkage types deduced from methylation analysis of the mannan core from the polysaccharide F1SS isolated from *Discula destructiva* CBS 109771

Retention time (min)	Linkage type	Characteristic fragments (*m*/*z*)	Relative abundance (%)
8.44	Man*p*-(1→	102, 118, 129, 145, 161, 162, 205	18.5
10.75	→2)-Man*p*-(1→	87, 88, 101, 129, 130, 161, 190	56.5
11.71	→6)-Man*p*-(1→	87, 88, 99, 102, 118, 129, 162, 189	10.2
14.73	→2,6)-Man*p*-(1→	117, 118, 129, 130, 189, 190	14.8

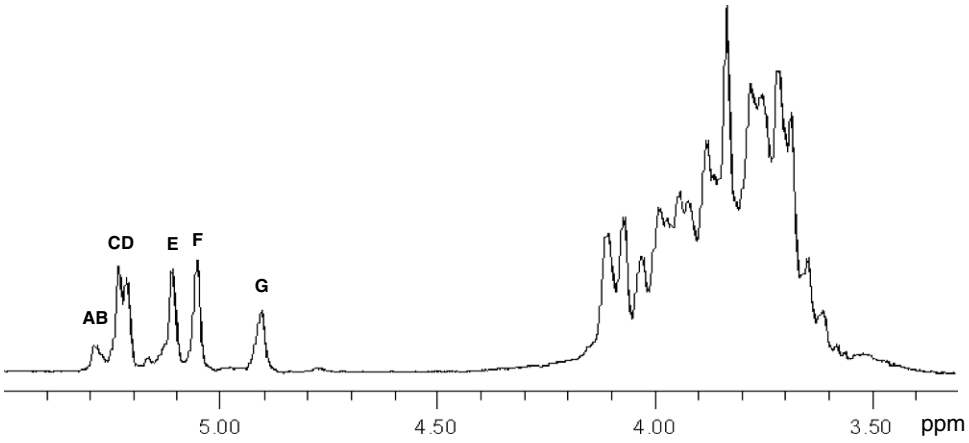


Figure 4. ^{1}H NMR spectrum of the polysaccharide obtained after mild hydrolysis of the F1SS polysaccharide isolated from the cell wall of *Discula destructiva*, strain CBS 109771. The anomeric peaks have been labelled **A**–**G**.

different anomeric protons allowed estimating the relative proportion of the residues. Thus, 2-*O*-Man*p*, 2,6-di-*O*-Man*p*, *t*-Man*p*, and 6-*O*-Man*p* amount near 45%, 21%, 21%, and 13%, respectively.

Polysaccharides containing galactofuranose chains with different linkage types attached to an α-(1→6) mannan core have been reported for several groups of fungi. The characteristic polysaccharide of fungi related to *Discula* (the Diaporthales) consists of chains with β-(1→6) and β-(1→5)-galactofuranose residues linked to a mannan core.[24] Since the polysaccharide F1SS from the strains of *D. destructiva* investigated herein also presents those chains, it may indicate that it belongs to the diaporthalean lineage.[25] Nevertheless, the presence of rhamnose distinguish these isolates from the other members of the group. Single rhamnose residues attached to a mannan backbone have also been reported in several species of fungi[20,26–28] but, to the best of our knowledge, it is the first time that they have been described linked to a galactofuranose chain.

1. Experimental

1.1. Microorganisms and growth conditions

The isolates of *D. destructiva* (CBS 130.91, 132.91, 133.91, and 109771) were maintained in slants of Bacto potato dextrose agar supplemented with Bacto yeast extract (Difco) 1 g L^{-1}. The culture medium and growth conditions were as previously described.[29]

1.2. Wall material preparation and fractionation

Wall material was obtained as reported elsewhere.[30] Polysaccharide F1SS was obtained according to Ahrazem et al.[31]

1.3. Chemical analysis

For analysis of neutral sugars the polysaccharides were hydrolyzed with 3 M TFA (1 h at 121 °C). The resulting monosaccharides were converted into their corresponding alditol acetates[32] and identified and quantified by gas–liquid chromatography (GLC) using a SP-2380 fused silica column (30 m × 0.25 mm ID × 0.2 μm film thickness) with a temperature program (210–240 °C, initial time 3 min, ramp rate 15 °C min^{-1}, final time 7 min) and a flame ionization detector. The monosaccharides released after hydrolysis were derivatized according to Gerwig et al.[33] and their absolute configuration determined by GC–MS of the tetra-*O*-TMSi-(+)-2-butyl-glycosides obtained.

1.4. Methylation analyses

The polysaccharide (1–5 mg) was methylated according to the method of Ciucanu and Kerek.[34] The methylated

material was treated and processed according to Ahrazem et al.,[31] with the exception that the partially methylated F1SS polysaccharide was hydrolyzed with TFA 1.5 M, 121 °C, 60 min.

1.5. Partial hydrolysis of the polysaccharide F1SS

A 50 mg sample of the polysaccharide was hydrolyzed with 5 mL of 0.15 M TFA for 5 h at 100 °C. The degraded polysaccharide was then recovered by dialysis (molecular weight cutoff ca. 3 kDa) and lyophilization.

1.6. NMR analysis

1D- and 2D- ^{1}H and ^{13}C NMR experiments were carried out at 40 °C on a Varian Unity 500 spectrometer with a reverse probe and a gradient unit. Proton chemical shifts refer to residual HDO at δ 4.61 ppm. Carbon chemical shifts refer to internal acetone at δ 31.07 ppm. The polysaccharide F1SS (ca. 20 mg) was dissolved in D_2O (1 mL) followed by centrifugation (10,000*g*, 20 min) and lyophilization. The process was repeated twice and the final sample was dissolved in D_2O (0.7 mL, 99.98% D). 2D-NMR experiments (DGF-COSY, TOCSY, NOESY, HMQC, HSQC-TOCSY, and HMBC) were performed by using the standard Varian software.

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