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Note

Structural investigation of cell-wall polysaccharides from *Neosartorya*: relationships with their putative anamorphs of *Aspergillus*

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Infrageneric taxa in *Aspergillus* have been widely accepted [1–3]. Such different methods as molecular composition and associated immunogenicity [4], the ubiquinone system [5], the yeast killer system [6], and profiles of secondary metabolites [7] have been used to prove the relationship of the genus *Neosartorya* to the subgenus *Fumigati* of *Aspergillus*.

We have proposed that the alkali-extractable water-soluble cell-wall polysaccharides could be used as chemotaxonomic markers at the genus or subgenus level and to compare the teleomorphic and their anamorphic genera [8,9].

In unpublished work, we have studied the structure of the polysaccharide isolated from *Aspergillus fumigatus* which is very similar to that of *Penicillium expansum* [10], and we have also investigated the structure of the cell-wall polysaccharides of four species of *Neosartorya*, in order to ascertain their relationship.

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1. Experimental

Microorganisms and culture media.—The following species of *Neosartorya* were used: *N. stramenia* (CBS 498.65), *N. aureola* (CBS 106.55), *N. quadricincta* (CBS 135.52), and *N. fischeri var. spinosa* (CBS 297.67). The microorganisms were maintained on slants of Bacto potato-dextrose agar supplemented with 1 g L^{-1} of Bacto yeast extract (Difco). The basal medium and growth conditions were as previously described [11].

Wall material preparation and fractionation.—The preparation of wall material [12] and the fractionation procedure [8] were performed as previously described. The fractions obtained from the dry cell-wall material of the different species were as follows: the polysaccharide material extracted with 1 M NaOH at 20°C contained water-soluble polysaccharides (Fraction F1S) and water-insoluble polysaccharides (F1I). Fractions F1S were re-fractionated by treatment with a small portion of water (~ 50 mg mL^{-1}), followed by centrifugation (10,000 *g*, 30 min), giving a solution (F1S-S) and a precipitate (F1S-I).

For preparative chromatography, 100 mg of F1S-S from *N. aureola* and *N. stramenia* were processed according to Leal et al. [8].

Chemical analysis.—Neutral sugars were released by hydrolysis with 2 M H_2SO_4 at 100°C for 5 h and then converted into their corresponding alditol acetates [13]. Identification and quantification were carried out by gas-liquid chromatography (GLC) using 3% SP-2340 on 100–120 Supelcoport [14].

Methylation analyses were performed by the reductive-cleavage method in two steps, as described [15], with trimethylsilyl triflate as catalyst, but the reactions were carried out under N_2 and the time during the reductive-cleavage step was shortened to 5–6 h, to minimise unwanted byproducts.

NMR analysis.—Polysaccharides F1S-S (~ 20 mg) were dissolved in D_2O (0.8 mL) followed by centrifugation (10,000 *g*, 20 min). The supernatant solutions (ca. 0.7 mL) were used for NMR recording. Solutions of samples from Fractions F1I were obtained by dissolving the polysaccharides (~ 15 mg) in 0.3 M NaOD (0.8 mL), followed by centrifugation as above. The sample from *N. stramenia* for 2D experiments was lyophilised, redissolved in D_2O (1 mL), and the process repeated twice for further deuterium exchange. The final sample was dissolved in 0.7 mL of D_2O (99.98% D).

^1H NMR spectra for Fractions F1S-S from all species were recorded at 40°C, and F1S-I at 50°C on a Varian XL-300. 2D ^1H and ^{13}C NMR experiments for *N. stramenia* (F1S-S) were carried out at 40°C on a Varian Unity 500 spectrometer. Proton chemical shifts refer to residual HDO at δ 4.61 and 4.51, respectively, and carbon chemical shifts to internal acetone at δ 31.45.

The 2D NMR experiments (DQF-COSY [16], 2D-TOCSY [17], NOESY and HMQC [18]) were performed as described previously [10].

2. Results and discussion

The proportions of the fractions obtained from the dry cell-wall material of the different species of *Neosartorya* were as follows: Fraction F1S (2 to 4%), Fraction F1I

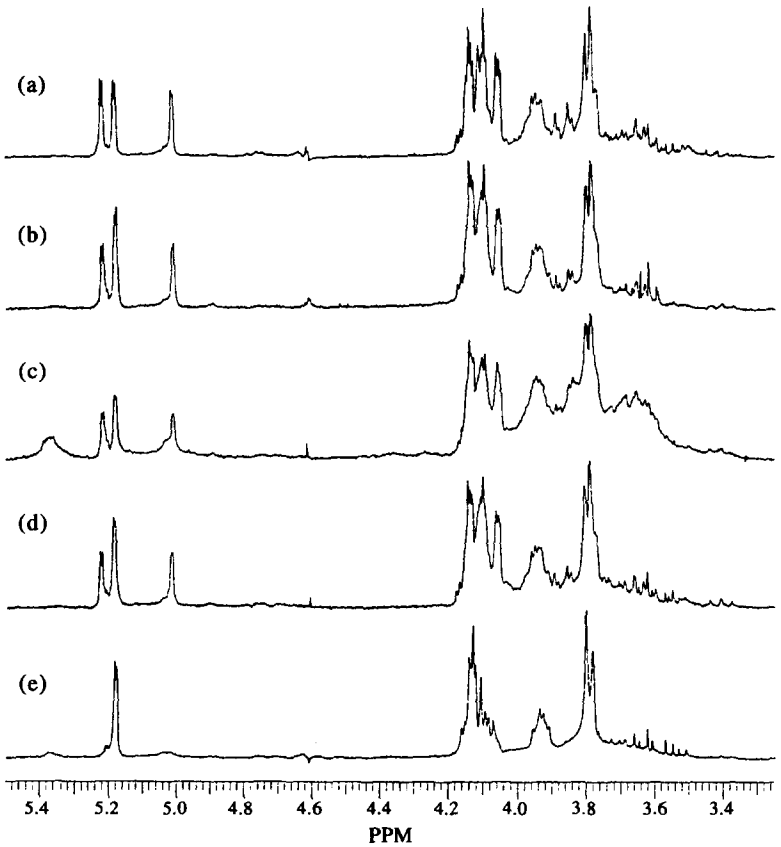

Fig. 1. ^{1}H NMR spectra (300 MHz) of polysaccharides F1S-S from: (a) *N. stramenia*; (b) *N. quadricincta*; (c) *N. aureola*; (d) *A. fumigatus*; (e)*N. fischeri*.

(20 to 36%). Re-fractionation of F1S gave F1S-S (80 to 85%) and F1S-I (5 to 12%). *N. aureola* gave F1S-S (65%) and F1S-I (28%).

Analyses of fractions F1S-S gave D-galactose as practically the only component, with the exception of that from *N. aureola*, which contained important amounts of D-glucose. Fractions F1S-I and F1I gave almost exclusively D-glucose.

Methylation analyses were carried out on the polysaccharides from *N. stramenia* and *N. quadricincta* by the reductive-cleavage procedure [15]. The molar ratios of 5-*O*-acetyl-1,4-anhydro-2,3,6-tri-*O*-methylgalactitol and 6-*O*-acetyl-1,4-anhydro-2,3,5-tri-*O*-methylgalactitol were 2:1 and 2.9:1, respectively, for the two polysaccharides, showing that they contained two and three 5-linked galactofuranosyl residues for each 6-linked galactofuranosyl residue.

The general ^{1}H NMR spectra for the polysaccharides F1S-S of the four species studied are shown in Fig. 1 along with that of *A. fumigatus* [Fig. 1(d)], which has also been included for comparative purposes. It can be seen that the spectra of *N. stramenia* [Fig. 1(a)], *N. quadricincta* [Fig. 1(b)], and *N. aureola* [Fig. 1(c)] are closely related,

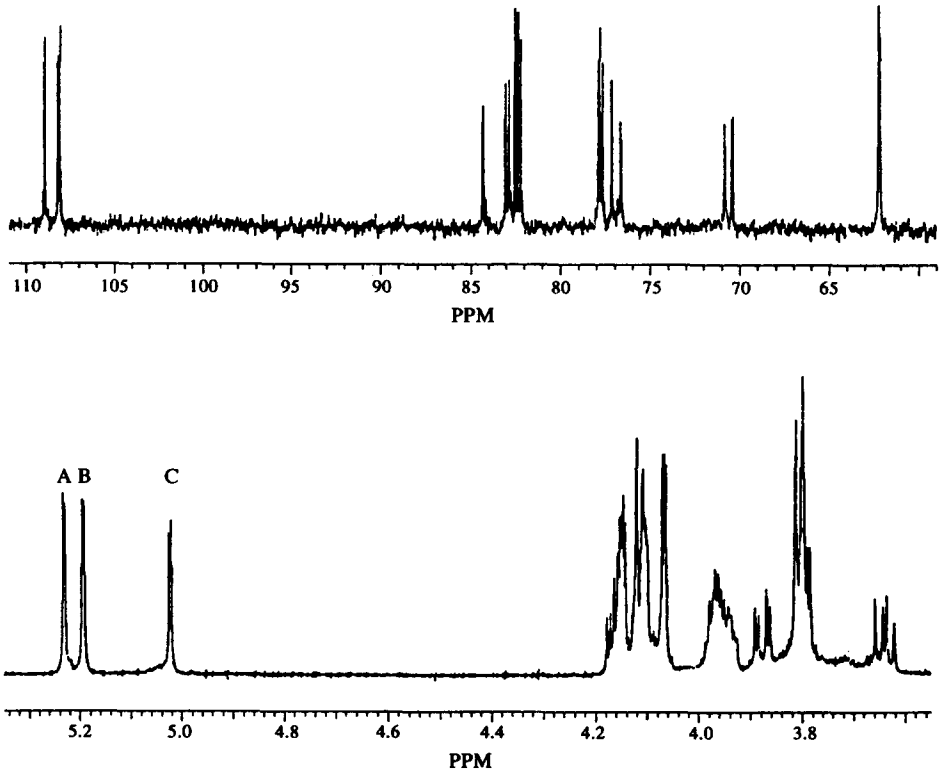

Fig. 2. ^{13}C (125 MHz) and ^{1}H NMR (500 MHz) spectra in D_2O at 40°C for the cell-wall polysaccharide F1S-S from *N. stramenia*. The anomeric protons have been labelled **A–C**.

although the last one shows an additional anomeric signal at δ 5.36, which, in agreement with chemical analysis and ^{1}H NMR data obtained for these and other cases, belongs to the anomeric proton of an α-(1 → 4)-glucan [19]. This glucan is present in the last species in bigger amounts than in the others, and refused to be completely removed from the mixture, even by chromatographic means. On the other hand, the spectrum of *N. fischeri* [Fig. 1(e)] is more similar to that of the polysaccharides isolated from *E. crustaceum* [20] and some *Penicillium* and *Aspergillus* [21]. The spectra of *N. quadricincta* and *A. fumigatus* are very similar to that of the polymer isolated from *P. expansum* [10]. As the proportion of the signals in *N. stramenia* is different to that in *N. quadricincta* and *A. fumigatus*, it seemed advisable to further study its structure.

The high-resolution ^{1}H NMR spectrum and the proton-decoupled ^{13}C NMR spectra of the polysaccharide in D_2O solution showed three anomeric signals of similar intensity (Fig. 2), indicating that the polymer consists of a trisaccharide repeating unit. The three residues were labelled **A**, **B**, and **C**, according to their anomeric protons, in order of increasing field. Assignment of the signals for the different residues was made by performing DQF-COSY and TOCSY experiments. Coherence transfer to all the protons of the three monosaccharides **A–C** was achieved by isotropic mixing, using 2D-TOCSY

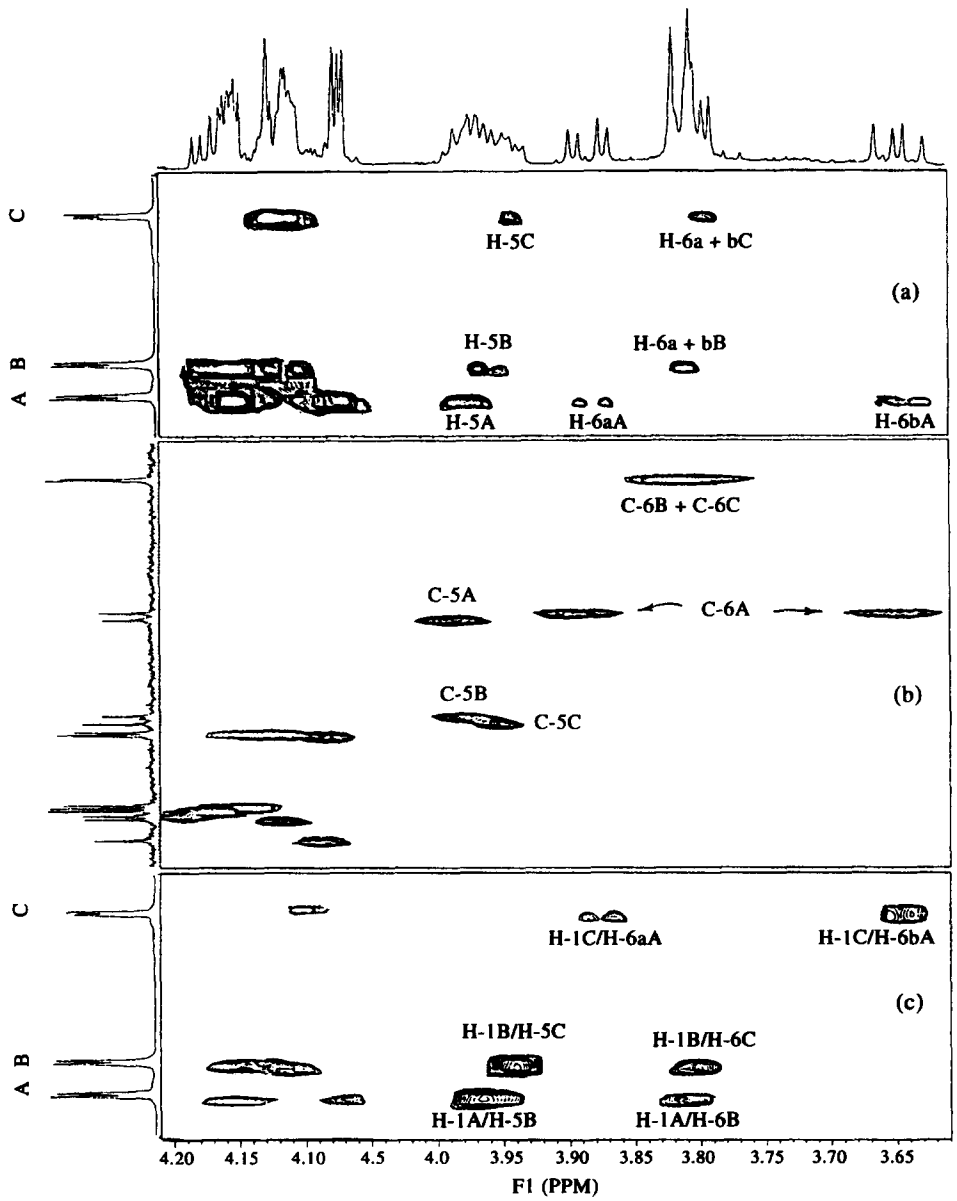

Fig. 3. Selected regions of the 2D-TOCSY (a), HMQC (b), and 2D-NOESY (c) spectra for Fraction F1S-S of *N. stramenia*, showing the connectivities with the rest of the protons. Relevant cross-peaks have been labelled.

with a mixing time of 150 ms. The subspectra through the anomeric signals showed neat connectivities to the rest of the protons [Fig. 3(a)], in spite of the small values of $J_{1,2}$ (2 Hz) for all the three residues. Identification of most cross-peaks was also based on the

Table 1
^1H and ^{13}C NMR chemical shifts (δ) and coupling constants (J, Hz, in square brackets) for the cell-wall polysaccharide F1S-S from *N. stramenia*

Unit	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
F1SS							
A	5.23 [$J_{1,2}$ 2.0]	4.15	4.07	4.08	3.97	3.88 [$J_{5,6a}$ 7.5;$J_{5,6b}$ 3.5;$J_{6a,6b}$ −11.2]	3.64
B	5.20 [$J_{1,2}$ 2.0]	4.16	4.12 [$J_{3,4}$ 6.7]	4.17 [$J_{4,5}$ 3.2]	3.96	3.81	3.81
C	5.03 [$J_{1,2}$ 2.0]	4.12	4.11	4.10	3.94	3.80	3.80
Unit	C-1	C-2	C-3	C-4	C-5	C-6	
F1SS							
A	107.8	82.2	77.6	84.0	70.5	70.1	
B	107.9	82.0	77.5	82.5	76.3	61.9 [a]	
C	108.6	81.9	77.3	82.7	76.8	61.8 [a]	

[a] These values may have to be interchanged.

information obtained from the DQF-COSY. The ^1H NMR chemical shifts are listed in Table 1. The chemical shift values of H-2, H-3, and H-4 were very similar ($\Delta\delta_{max} < 0.2$), and appeared at relatively low field, $\delta > 4$, indicating the presence of galactofuranose residues, as also deduced from the methylation analysis. In addition, according to the $J_{1,2}$ values it can be concluded that all the galactofuranose residues have the β configuration [22].

In order to ascertain the substitution of the residues, we carried out an HMQC experiment [Fig. 3(b)], which maps the connectivities between carbon atoms and their directly bonded protons. The assignment of most of the signals in the ^{13}C NMR spectrum was straightforward, since almost all the ^1H NMR chemical shifts were already known. Carbon chemical shifts are also listed in Table 1. The chemical shifts of the anomeric carbons indicate unequivocally [23] that all the monosaccharides are β-Gal*f* residues. Furthermore, the observed deshielding of C-6 of residue **A** allows its assignment as a 6-*O*-substituted β-Gal*f* unit, while the values for the C-5 chemical shifts show that residues **B** and **C** are 5-*O*-substituted Gal*f* units.

The information concerning the connection of the glycosyl residues **A–C** was obtained from a 2D-NOESY experiment (mixing time = 300 ms). The existence of cross-peaks between signals of different residues indicates their proximity in space and, probably, their connection. Thus, cross-peaks between the anomeric proton of residue **C** and H-6a and H-6b of residue **A** could be observed, along with others connecting H-1 of residue **A** with H-5 and H-6 of unit **B** and H-1 of unit **B** with H-5 and H-6 of residue **C** (Fig. 3c). Although NOE cross-peaks are dependent on the conformation around the glycosidic bonds, and the existence of these cross-peaks does not guarantee the knowledge of the exact position of the linkage, in this case it is obvious that Gal*f* **C** is (1 → 6)-linked to Gal*f* **A**, which is connected via a (1 → 5) linkage to Gal*f* **B**, which is in turn (1 → 5)-bonded to Gal*f* **C**.

The above connectivities and substitutions are in agreement with the analytical results and allow us to propose the structure for the trisaccharide repeating unit of the *N. stramenia* polysaccharide as being:

$$[\rightarrow 6)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow 5)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow 5)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow]_n$$
$$\quad\ \mathbf{A}\qquad\qquad\ \mathbf{B}\qquad\qquad\ \mathbf{C}$$

Concerning the alkali-soluble polysaccharides from Fractions F1I, the ^{1}H NMR spectra showed that all of them consist of a linear α-(1 → 3)-glucan, practically identical to that found in *P. expansum* [10] and also in a wide variety of other fungi.

The slightly soluble polysaccharides from Fractions F1S-I were identified as α-(1 → 4)-glucans, on the basis of their ^{1}H NMR spectra.

The similarity of the water-soluble polysaccharides obtained from the cell wall of *N. quadricincta* and *N. aureola* and those obtained from strains of *A. fumigatus* proves that these microorganisms are related. The polysaccharide of *N. stramenia* contains only three residues of galactofuranose in its repeating unit, although the general structure is very similar. The strain of *N. fischeri* investigated could be misidentified since its polysaccharide consisted of a β-(1 → 5)-galactofuranan, which has been found in species of *Penicillium* and *Aspergillus* [21], and *Eupenicillium* [8,20]. Although a larger number of strains should be investigated the above results support the usefulness as chemotaxonomic markers of the water-soluble cell-wall polysaccharides. On the other hand, all these findings show that the fungal cell wall is a valuable source of new polysaccharides.

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