Comparison of cell-wall polysaccharides from *Nectria cinnabarina* with those from the group of *Nectria* with *Sesquicillium* anamorphs

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Alkali-extractable and water-soluble polysaccharides were purified from cell walls of five species of *Sesquicillium* or its teleomorphs, *Nectria lasiacidis* and *Nectria impariphalis*, and from *Nectria cinnabarina*, the type species of *Nectria*, a heterogeneous genus that belongs to the Hypocreales. Methylation and NMR analyses for determination of linkage types and structure were performed and indicated differences between the polysaccharides purified during the present study and those isolated from other nectrioid fungi, namely the presence of 5-O-substituted galactofuranose ($\rightarrow 5$)-Gal-f-(1 $\rightarrow$) in the main chain together with 2,6-di-O-substituted galactofuranose ($\rightarrow 2,6$)-Gal-f-(1 $\rightarrow$) residues in *Sesquicillium buxi* and *Sesquicillium pseudosetosum*. The polysaccharide from *N. impariphalis* was similar to those obtained from the above species, although an additional residue of 6-O-substituted glucopyranose ($\rightarrow 6$)-Glc-p-(1 $\rightarrow$), was detected in some side chains. In *N. lasiacidis* and *Sesquicillium candelabrum* the polysaccharide contained an additional branching point of 5,6-di-O-substituted galactofuranose ($\rightarrow 5,6$)-Gal-f-(1 $\rightarrow$) linked to terminal N-acetylglucosamine GlcNAc-(1 $\rightarrow$). These chains were linked to a small mannan core. All these polysaccharides showed major differences to the polysaccharide of *N. cinnabarina*, which was formed by a main chain of (1 $\rightarrow 6$)- β -linked galactofuranose units almost fully branched at positions 2-O by either single residues of glucopyranose or acidic chains containing glucuronic acid and mannose.

Keywords: complex galactans, Hypocreales, chemotaxonomy

INTRODUCTION

The genus *Sesquicillium* Gams was created by the transfer (Gams, 1968) of *Fusidium buxi* Schmidt in Link and *Verticillium candelabrum* Bonorden. It is characterized by the presence of verticillate conidiophores, whose ultimate branches consist of terminal tapering phialides and subterminal cells with a lateral sporiferous neck. Five new species of *Nectria* with *Sesquicillium* anamorphs were described by Samuels (1989). He found that the anamorphs of *Nectria sesquicilli* and *Nectria lasiacidis* were indistinguishable from *Sesquicillium buxi* and he erected three species of *Sesquicillium* with the anamorphs of the other species of *Nectria*:

Sesquicillium asymmetricum (*Nectria sesquiphialis*), *Sesquicillium impariphiale* (*Nectria impariphalis*) and *Sesquicillium pseudosetosum* (*Nectria parviphalis*). These species of *Nectria* form a homogeneous group characterized by superficial, non-stromatic, smooth globose, non-papillate, orange perithecia. The known species of *Sesquicillium* and their *Nectria* teleomorphs were keyed by Samuels (1989). Recently, Rossman *et al.* (1999) erected a new genus, *Bionectria*, in which the species of *Nectria* with anamorphs in *Sesquicillium* are included.

The type species of the genus *Nectria* is *Nectria cinnabarina* (Tode: Fr.) Fr. The characteristics of species related to *N. cinnabarina*, included in the *N. cinnabarina* group, were discussed by Rossman (1989). In a later work this author expressed the opinion that the genus *Nectria* should be restricted to the *N. cinnabarina* group

Abbreviation: TMS, trimethylsilyl.

Table 1. Sugar composition (%) of fraction F1S-II of *Sesquicillium* species, *N. impariphalis*, *N. cinnabarina* and *N. lasiacidis*

Glc, glucose; Gal, galactose; Man, mannose; GlcA, glucuronic acid; GlcNAc, N-acetylglucosamine.

Micro-organism	Glc	Gal	Man	GlcA	GlcNAc	Recovery (%)
<i>N. cinnabarina</i> CBS 279.48	16.8 ± 1.2	25.9 ± 0.8	7.3 ± 0.6	7.5 ± 0.5	—	57.5
<i>N. cinnabarina</i> CBS 278.48	23.2 ± 1.5	33.7 ± 1.1	12.0 ± 0.8	8.9 ± 1.0	—	77.8
<i>N. cinnabarina</i> CBS 255.47	16.5 ± 2.1	23.6 ± 1.6	11.3 ± 0.6	7.8 ± 0.9	—	59.4
<i>S. buxi</i> CBS 288.62	9.7 ± 1.9	52.2 ± 1.4	7.2 ± 0.9	3.5 ± 0.3	—	72.6
<i>S. pseudosetosum</i> CBS 662.83	10.3 ± 0.7	45.1 ± 1.3	7.7 ± 0.5	2.5 ± 0.4	—	65.6
<i>S. candelabrum</i> CBS 190.38	7.5 ± 1.4	48.5 ± 1.1	6.5 ± 0.4	1.5 ± 0.2	7.0 ± 1.2	71.0
<i>S. candelabrum</i> CBS 205.69	10.5 ± 1.3	45.4 ± 1.7	7.6 ± 0.6	1.0 ± 0.3	7.5 ± 1.1	73.0
<i>S. candelabrum</i> CBS 204.69	12.6 ± 2.9	30.7 ± 1.3	6.5 ± 0.6	1.8 ± 0.3	7.3 ± 1.4	59.6
<i>N. lasiacidis</i> CBS 179.88	16.1 ± 2.5	27.8 ± 0.9	6.1 ± 0.5	3.0 ± 0.8	7.8 ± 1.6	60.8
<i>N. impariphalis</i> CBS 178.88	35.7 ± 1.1	38.4 ± 1.4	7.0 ± 0.6	4.0 ± 0.5	—	85.1

(Rossman, 1993). The large number of species included in the genus *Nectria* made it necessary to divide them into groups according to morphological features (Booth, 1959). Other workers have added new species, elucidated additional characters and substantiated the groups (Samuels & Rossman, 1979; Seifert, 1985; Samuels & Seifert, 1987; Rossman, 1989, 1993).

Various species of *Sesquicillium* have been misidentified because of the morphological similarities with other genera. As an example, some strains of *S. candelabrum* were named *Gliocladium penicilloides* and *Verticillium candelabrum* prior to their final inclusion in *Sesquicillium* (Domsch *et al.*, 1980). Morphological characters are sometimes insufficient to delineate unequivocally fungal genera; therefore, chemical and biochemical characters and, most recently, rDNA sequencing are increasingly used in fungal systematics.

Monosaccharide composition of the cell wall has been used for yeast and fungal taxonomy, systematics and phylogeny (Weijman & Golubev, 1987; Prillinger *et al.*, 1990, 1991, 1993; Messner *et al.*, 1994). Bartnicki-García (1968) proposed the use of dual combinations of major polysaccharides of the cell wall to classify fungal species and divided the whole spectrum of fungi into eight categories. As Pfyffer (1998) stated: 'cell wall polysaccharides appear to be extremely conservative and therefore may be considered reliable markers for fungal taxonomy.' The alkali-extractable and water-soluble polysaccharides from the cell walls have been proposed as taxonomic characters for various fungal genera (Leal & Bernabé, 1998). Among the Hypocreales, some polysaccharides have been proposed as markers for *Calonectria* and its anamorph, *Cylindrocladium* (Ahrazem *et al.*, 1997), and for *Gibberella* and *Fusarium* (Ahrazem *et al.*, 2000). As stated above, the perfect state of *Sesquicillium* species belongs to *Nectria*. This genus is heterogeneous, since it comprises the teleomorphic states of 21 anamorphic genera (Samuels & Seifert, 1987). The concept of *Nectria* has changed according to

the weight given to the morphological and biological characteristics of both the teleomorph and the anamorph by different authors (Rossman, 1993; Rossman *et al.*, 1999).

In this work we characterize the alkali-extractable, water-soluble cell-wall polysaccharides from isolates of *N. cinnabarina*, and of *Sesquicillium* and *Nectria* with *Sesquicillium* anamorphs, in an attempt to find out if the morphological differences which allowed the arrangement of *Nectria* with *Sesquicillium* anamorphs in a group are reflected in the composition of the cell-wall polysaccharides.

METHODS

Micro-organisms and culture media. The strains of *Nectria* and *Sesquicillium* listed in Table 1 were maintained on slants of Bacto potato dextrose agar supplemented with 1 g l⁻¹ yeast extract (Difco). *N. lasiacidis* and *N. impariphalis* are teleomorphs of *S. buxi* (Gams, 1968) and *S. impariphalis* (Samuels, 1989), respectively. The culture medium and growth conditions were as previously described (Gómez-Miranda *et al.*, 1988).

Wall material preparation and fractionation. Wall material was obtained as reported elsewhere (Prieto *et al.*, 1988). Cell-wall material (8 g) was repeatedly extracted with 1 M NaOH (300 ml) at 20 °C. After centrifugation, the supernatants were combined and absolute ethanol (v/v) was added. The precipitate was collected by centrifugation, dialysed against running tap water and then freeze-dried (F1). This polysaccharidic material (500 mg) was suspended in distilled water (10 ml), stirred at room temperature for 2 h and centrifuged. The precipitate was extracted again with distilled water and the supernatant solutions were combined and freeze-dried (F1S).

Isolation of polysaccharides from F1S. A 100 mg aliquot of fraction F1S was dissolved in 1.5 ml distilled water and centrifuged at 13000 g for 15 min to eliminate insolubles. The supernatant was added to a column (90 × 2.6 cm) of Sepharose CL-6B and eluted with distilled water with a flow of 22 ml h⁻¹. Fractions were collected and monitored for carbohydrate by

Fig. 1. Column chromatography of F15 from *Sesquicillium* species, *N. lasiacidis*, *N. cinnabarina* CBS 278.48 and *N. impariphalis* on Sepharose CL-6B. The arrows indicate the elution of the different dextrans (500, 70 and 10 kDa) used as standards for calibration.

the phenol-sulfuric acid method (Dubois *et al.*, 1956). The fractions which gave a positive test for carbohydrates were combined in batches of six successive fractions, concentrated to a small volume and freeze-dried. The column was previously calibrated with a mixture of standards: T500, T70 and T10 dextrans (Pharmacia).

Chemical analysis. Neutral sugars were analysed by methanolysis followed by hydrolysis with 3 M trifluoroacetic acid (1 h at 121 °C). Neutral sugars were converted into their corresponding alditol acetates (Laine *et al.*, 1972) and identified and quantified by GLC as described previously (Gómez-Miranda *et al.*, 1981). Uronic acid content was measured by the carbazole reaction (Bitter & Muir, 1962) and the acidic monosaccharides identified on the hydrolysates as their

trimethylsilyl (TMS)-derivatives by GLC-MS. The carboxylic groups of the uronic acids in the polysaccharide were reduced with sodium borodeuteride (NaBD₄) according to the method of Taylor & Conrad (1972). The complete reduction was checked by IR spectroscopy. IR spectra were obtained by the KBr technique (Price, 1972) on a Perkin-Elmer 1420 infrared spectrophotometer. Aminosugar content was determined after hydrolysis of the polysaccharides (6 M HCl, 4 h, 100 °C) according to the method of Chen & Johnson (1983) and glucosamine was identified by HPLC in an amino acid analyser Biochrom 20 (Pharmacia) using commercial standards.

Absolute configuration of the monosaccharides. The monosaccharides released in the hydrolysates were derivatized according to the method of Gerwig *et al.* (1979) and their

Table 2. Methylation and reductive cleavage analyses (%) from F1S-II of several species of *Sesquicillium*, *N. impariphalis*, *N. cinnabarina* and *N. lasiacidis*

N. cin., *N. cinnabarina*; *N. imp.*, *N. impariphalis*; *N. las.*, *N. lasiacidis*; *S. bux.*, *S. buxi*; *S. can.*, *S. candelabrum*; *S. pse.*, *S. pseudosetosum*.

Linkage type	<i>N. cin</i> *	<i>S. bux</i>	<i>S. pse</i>	<i>S. can</i> †	<i>N. las</i>	<i>N. imp</i>
GlcNAc-(1 → ‡	—	—	—	3.0	2.7	—
Glc p-(1 →	29.1	20.9	29.8	25.4	28.7	13.6
Glc pA-(1 → §	—	3.1	1.8	1.2	2.5	2.5
Man p-(1 →	4.9	—	—	—	—	—
→ 5)-Gal f-(1 →	2.0	33.5	35.1	34.5	24.4	39.5
→ 6)-Man p-(1 →	4.0	2.0	1.5	2.5	2.3	—
→ 6)-Glc p-(1 →	—	—	—	—	—	17.9
→ 4)-Glc pA-(1 →	5.0	—	—	—	—	—
→ 6)-Gal f-(1 →	8.6	2.5	3.0	3.5	5.0	2.3
→ 2,6)-Man p-(1 →	3.0	—	0.6	—	—	—
→ 4,6)-Man p-(1 →	—	1.1	—	2.0	—	—
→ 5,6)-Gal f-(1 →	—	—	—	6.0	7.5	—
→ 2,6)-Gal f-(1 →	33.1	22.5	27.9	20.0	20.5	15.5

* Results for isolate CBS 278.48. Similar residues and proportions were found in the other isolates of *N. cinnabarina*.

† Results for isolate CBS 190.38. Similar residues and proportions were found in the other isolates of *S. candelabrum*.

‡ Detected in the analysis of the native polysaccharide.

§ Detected as 1,5-diacetyl-2,3,4,6-Me₄-Glc with 2 D atoms in C6 (reduced carboxyl).

|| Detected as 1,4,5-diacetyl-2,3,6-Me₃-Glc with 2 D atoms in C6 (reduced carboxyl).

absolute configuration determined by GLC-MS of the tetra-O-TMS-(+)-2-butyglycosides obtained.

Linkage analyses. The reduced polysaccharides (1–5 mg) were methylated according to the method of Ciucanu & Kerek (1984). The polysaccharides that contained aminosugars were also methylated by the same protocol without reducing the carboxylic group. The methylated material was extracted with chloroform/methanol (1:1, v/v), dialysed sequentially against water and 50% ethanol, and evaporated. Methylated fractions, which showed negligible IR absorption for hydroxyl groups, were hydrolysed with 3 M trifluoroacetic acid (121 °C, 1 h), and the products were reduced with NaBD₄, then acetylated and subjected to GLC-MS, using a SPB-1 column (30 m × 0.22 mm i.d. × 0.25 µm film thickness), a temperature programme of 160–200 °C with 1 min initial hold and ramp rate 2 °C min⁻¹, and a mass detector (Q-Mass; Perkin-Elmer). Quantification was performed according to peak area. Analyses by the reductive-cleavage method were performed in two steps (Lee & Gray, 1988), with TMS triflate as catalyst, but the reactions were carried out under Ar and the time during the reductive cleavage step was shortened to 5–6 h, to minimize unwanted by-products. The partially methylated anhydroalditol acetates obtained were analysed by GLC-MS using a fused silica SPB-1 column and a temperature programme of 150–200 °C with 3 min initial hold and ramp rate 3 °C min⁻¹.

NMR analyses. The 1-D ¹H-NMR spectra were recorded for solutions of the polysaccharides previously stirred with Amberlite IR-120 to convert the sodium salt of uronic acids into the free carboxylic acids, deuterated with D₂O and dissolved in 99.9% D₂O. The 1-D ¹H-NMR spectra were determined at 40 °C on a Varian INOVA-300 spectrometer (¹H, 300 MHz). 2-D ¹H- and ¹³C-NMR experiments were

carried out at 40 °C on a Varian Unity 500 spectrometer. Proton chemical shifts refer to residual HDO at δ = 4.61 p.p.m. Carbon chemical shifts refer to internal acetone at δ = 31.07 p.p.m.

RESULTS

Fractionation of polysaccharides from F1S

The alkali-extractable, water-soluble material from all the species was filtered through a column of Sepharose CL-6B (Fig. 1). Two peaks were detected in the three strains of *S. candelabrum* and in *S. pseudosetosum*. The first one (F1S-I) was a minor excluded fraction (≤ 10%) which contained a glucan of high molecular mass, which was eliminated, and the second one (F1S-II) amounted to approximately 90% and eluted in a wide peak. A single peak (F1S-II) was obtained in *S. buxi*, *N. lasiacidis* the three *N. cinnabarina* isolates and *N. impariphalis*. The ¹H-NMR spectra of the polysaccharidic material of different batches from a peak were similar, which indicated that F1S-II was a unique polysaccharide.

Chemical analysis

The polysaccharide of fraction F1S-II from the species analysed gave glucose, mannose, glucuronic acid and higher percentages of galactose (Table 1). Glucuronic acid was detected in all the polysaccharides by GLC-MS analysis of their TMS derivatives. Colorimetric determination of aminosugars gave around 7% in all

Fig. 2. Anomeric region of the ^1H -NMR spectra of fraction F1S-II from *N. cinnabarina*, *N. lasiacidis*, *N. impariphalis* and species of *Sesquicillium*.

strains of *S. candelabrum* and in *N. lasiacidis*, and negative results for the polysaccharides F1S-II from the remaining species. This aminosugar was identified as glucosamine by HPLC in comparable amounts (8 %) to those detected by the colorimetric method. All the monosaccharides had the D configuration.

Linkage analyses

The results of methylation and reductive cleavage analyses are shown in Table 2. The polysaccharides from *Sesquicillium* spp., *N. lasiacidis* and *N. impari-*

phialis contained 5-O-substituted ($\rightarrow 5$)-Galf-(1 $\rightarrow$) and 2,6-di-O-substituted galactofuranose ($\rightarrow 2,6$)-Galf-(1 $\rightarrow$) and terminal residues of glucopyranose (Glc p-(1 $\rightarrow$). All these polysaccharides also contained a small proportion of terminal glucuronic acid. In addition to these residues, the polysaccharide from *N. impariphalis* contained 6-O-substituted glucopyranose ($\rightarrow 6$)-Glc p-(1 $\rightarrow$) and the polysaccharides from *S. candelabrum* and *N. lasiacidis* contained 5,6-di-O-substituted galactofuranose ($\rightarrow 5,6$)-Galf-(1 $\rightarrow$), and terminal *N*-acetylglucosamine (GlcNAc-(1 $\rightarrow$). This last residue was only detected in unreduced samples.

Fig. 3. ^1H -NMR spectrum (D_2O , 40°C , 300 MHz) (a), and ^{13}C -NMR spectrum (D_2O , 40°C , 125 MHz) (b) of the F1S-II polysaccharide from *S. candelabrum* CBS 190.38. The $\text{C}=\text{O}$ and CH_3 singlets are not shown in the carbon spectrum.

The results of the methylation and reductive cleavage (Table 2) indicated that the polysaccharides isolated from strains of *N. cinnabarina* contained 2,6-di-O-substituted galactofuranose ($\rightarrow 2,6$)-Gal f -(1 $\rightarrow$), terminal residues of glucopyranose (Glc p -(1 $\rightarrow$) and mannopyranose (Man p -(1 $\rightarrow$), 6-O-substituted mannopyranose ($\rightarrow 6$)-Man p -(1 $\rightarrow$), and 4-O-substituted glucuronic acid ($\rightarrow 4$)-Glc p A-(1 $\rightarrow$).

^1H -NMR analysis

The ^1H -NMR spectra of F1S-II (Fig. 2) from *S. buxi* and *S. pseudosetosum* revealed a coincidence in the position of the signals, differing in their intensity, and showed five signals at $\delta = 5.03$, 5.09, 5.12, 5.22 and 5.35 p.p.m. The three major peaks ($\delta = 5.03$, 5.09 and 5.35 p.p.m.) appear in the ^1H -NMR spectra of the polysaccharides of all the species analysed, except in that of *N. cinnabarina*.

As the polysaccharide from *S. candelabrum* contained signals observed in most of the spectra of the other fungi, it was chosen for further studies. The ^1H -NMR spectrum of *S. candelabrum* (CBS 190.38) contained six signals in the anomeric region (Fig. 3a). The DQCOSY spectrum displayed H-1/H-2 crosspeaks for six distinct residues, in the proportion 3:1:1:3:3:1, which were labelled A–F, from low to high field, according to their anomeric protons. A 2-D TOCSY (HOHAHA) experiment (mixing time = 85 ms) allowed the establishment of clear connectivities to the rest of the protons for residues A, C, D and F (Fig. 4a). The signals corresponding to unit D readily identified it as α -Glc p , as deduced from the coupling constants ($J_{1,2} = 3.8$, $J_{2,3} = 9.8$, $J_{3,4} = 9.7$ Hz). The low position of proton H-2 (3.94 p.p.m.) and the values of the coupling constants from unit F ($J_{1,2} = 3.6$, $J_{2,3} > 9$, $J_{3,4} = 9.3$ Hz), in addition to the presence of a singlet at 2.05 p.p.m., probably due to an acetyl group, suggested it was a

Glc p Nac moiety. The chemical shifts of protons H-2, H-3 and H-4 corresponding to residues A, B, C and E were very similar ($\Delta\delta < 0.2$ p.p.m.) and appeared at low field, indicating the presence of Gal f units. The ^{13}C -NMR spectrum (Fig. 3b) showed a $\text{C}=\text{O}$ carbon (175.3 p.p.m.), six anomeric singlets, four of them in the range 106–109 p.p.m., and a methyl singlet at 23.0 p.p.m.

To confirm the assignments and to find the glycosylation sites, we used a HMQC experiment (Fig. 4b), which maps the connectivities between carbon atoms and their directly bonded protons. The crosspeaks in the anomeric region corroborated the presence of six residues. For non-anomeric atoms, the chemical shift of C-2 from unit F (54.6 p.p.m.) demonstrated that it was a Glc p Nac unit. The crowding of non-anomeric atoms, for both protons and carbons (Fig. 4b), did not allow unambiguous assignment of all signals; therefore it seemed advisable to run a HMQC-TOCSY experiment which, starting from each HMQC crosspeak of the 2-D spectrum, provides additional signals in the same row, caused by TOCSY transfer, thus allowing the identification of carbon chemical shifts pertaining to each residue (Fig. 5). In this way, most of the remaining carbon and protons were assigned to their corresponding units (Table 3). Comparison of the observed values with those reported in the literature (Bock & Pedersen, 1983; Parra *et al.*, 1994; Leal *et al.*, 1996) led to the identification of residue A as being 2,6-di-O-substituted Gal f , B and E as 5-O-substituted Gal f moieties, and C as 5,6-di-O-substituted Gal f . Concerning the anomeric configuration of these residues, the vicinal $J_{1,2}$ coupling constants of all Gal f moieties ($J < 2$ Hz) suggested that all have β -configuration, which is supported by the chemical shifts of their anomeric carbons ($\delta > 106$ p.p.m.).

A 2-D NOESY spectrum (mixing time = 300 ms, Fig. 4c) allowed the observation of connections H-1A/H-5E,

Fig. 4. 2-D NMR spectra (40 °C, 500 MHz) for selected regions of the F15-II polysaccharide from *S. candelabrum*: (a) TOCSY (HOHAHA, mixing time = 85 ms); (b) HMQC; and (c) NOESY (mixing time = 300 ms) subspectra. The anomeric protons and relevant cross-peaks have been labelled.

Fig. 5. 2-D HMQC-TOCSY spectrum for relevant region of the F15-II polysaccharide from *S. candelabrum*. The crosspeaks corresponding to the different relevant rows are labelled.

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

Residue		1	2	3	4	5	6a	6b
A	H	5.35	4.21	4.24	4.08	4.01	3.88	3.65
	C	106.5	87.9	76.2	83.3†	70.4	70.3	
B	H	5.25	4.13	4.06	4.07	3.96	3.95	3.70
	C	107.7	82.2*	77.9	84.2	76.6‡	62.2	
C	H	5.23	4.14	~4.13	4.07	4.14	3.95	3.70
	C	107.9	82.1*	77.7	84.1	74.5	68.3	
D	H	5.09	3.58	3.72	3.44	3.78	3.88	3.76
	C	99.0	72.1	73.8	70.5	73.5	61.6	
E	H	5.03	4.13	~4.13	~4.12	3.98	3.80	3.80
	C	108.9	82.1*	77.7	83.3†	76.9‡	62.2	
F	H	4.91	3.94	3.72	3.51	3.76	3.88	3.80
	C	98.4	54.6	73.1	71.0	72.0	61.6	

*,†,‡ Values with the same symbol may be interchanged.

Fig. 6. Selected region of the HMBC spectrum for the F15-II polysaccharide from *S. candelabrum*. Relevant crosspeaks are labelled.

H-1D/H-2A, H-1E/H-6a + 6bA and H-1F/H-6a + 6bC, indicating connections $E \rightarrow 6A2 \leftarrow D$, $A \rightarrow 5E$ and $F \rightarrow 6C$.

To find additional connections among residues, we conducted an HMBC experiment, which provides signals corresponding to long-range connections among protons and the carbons placed at two and three bonds from them. In this way, in addition to expected intraring connections, peaks corresponding to H-1A/C-5B, 1A/C-5E, H-1B/C-5C, H-1C/C-5E, H-1D/C-2A, H-1E/C-6A and H-1F/C-6C could be observed (Fig. 6).

Minor signals, corresponding to mannose residues, as deduced from the methylation analyses, are also observed in the proton and carbon 1-D spectra. Due to their low proportion (<6.5%), no additional information on these residues could be obtained, although they are most probably forming part of a small mannan core, which has been found in the polysaccharides isolated from other fungi (Prieto *et al.*, 1997; Ahrazem *et al.*, 1999).

From the combined evidence, it is therefore proposed that the cell-wall polysaccharide isolated from *S. can-*

Fig. 7. Repeating units of polysaccharides from several species of *Sesquicillium*, *N. lasiacidis*, *N. cinnabarina* and *N. impariphalis*, deduced from structural and chemical analyses.

delabrum has an irregular structure (Fig. 7). The derivatives detected in the methylation analyses suggest that the chains are connected to some 4-O positions of a small (1 → 6)-mannan core.

The ^1H -NMR spectra of the other two strains of *S. candelabrum*, and also that of *N. lasiacidis*, are very similar; therefore, the structure of the polysaccharides of these species are closely related, the only expected differences being small variations in the proportions of the different residues. Following analogous methodology, the structure of the polysaccharides of *S. buxi*, *S. pseudosetosum* and *N. impariphialis* were deduced as being those depicted in Fig. 7.

The ¹H-NMR spectra of polysaccharides F1S-II from the three *N. cinnabarina* strains were different from those for the polysaccharides of the *Sesquicillium* species

and the other species of *Nectria*. The signals detected in the anomeric region were at $\delta = 5.03$, 5.10 and 5.16 p.p.m.

DISCUSSION

The polysaccharides from species of *Sesquicillium* and related species of *Nectria* had in common the most abundant residues: terminal glucopyranose (Glc_p-(1 →), 5-O-substituted galactofuranose (→5)-Gal_f-(1 →), and 2,6-di-O-substituted galactofuranose (→2,6)-Gal_f-(1 →), which revealed a fairly similar basic structure and the relatedness of these fungi. The ¹H-NMR spectra of these polysaccharides also revealed a common pattern, with three major signals at δ = 5.03, 5.09 and 5.35 p.p.m. (Fig. 2). Additional residues might permit the identification of certain species. The structures depicted in

Table 4. Assignments of the major signals of the anomeric region of the ^1H -NMR spectra of polysaccharides from *Sesquicillium* spp., *N. cinnabarina*, *N. lasiacidis* and *N. impariphialis*

	H-1 (p.p.m.)	Assignment	References
A	5.32–5.35	$\rightarrow 2,6\text{-}\beta\text{-Gal}f\text{-(1}\rightarrow 5\text{)-}\beta\text{-Gal}f$	Prieto <i>et al.</i> (1995)
B	5.25	$\rightarrow 6\text{-}\beta\text{-Gal}f\text{-(1}\rightarrow 5,6\text{)-}\beta\text{-Gal}f$	Leal <i>et al.</i> (1997)
C	5.22	$\rightarrow 5,6\text{-}\beta\text{-Gal}f\text{-(1}\rightarrow 5\text{)-}\beta\text{-Gal}f$	Leal <i>et al.</i> (1997)
D	5.00–5.06	$\alpha\text{-Glc}p\text{-(1}\rightarrow 2\text{)-}\beta\text{-Gal}f$	Ahrazem <i>et al.</i> (1997)
E	5.03	$\rightarrow 5\text{-}\beta\text{-Gal}f\text{-(1}\rightarrow 6\text{)-}\beta\text{-Gal}f$	Leal <i>et al.</i> (1997)
F	4.91	GlcNAc-(1 $\rightarrow$ 6)- $\beta\text{-Gal}f$	This work
G	5.10–5.12	$\alpha\text{-Glc}pA\text{-(1}\rightarrow 2\text{)-}\alpha\text{-Gal}f$	Ahrazem <i>et al.</i> (1997)
H	4.95	$\alpha\text{-Glc}p\text{-(1}\rightarrow 6\text{)-}\alpha\text{-Glc}p$	This work
I	5.08	$\rightarrow 6\text{-}\alpha\text{-Glc}p\text{-(1}\rightarrow 2\text{)-}\beta\text{-Gal}f$	This work
J	5.16	$\rightarrow 2,6\text{-}\beta\text{-Gal}f\text{-(1}\rightarrow 6\text{)-}\beta\text{-Gal}f$	Ahrazem <i>et al.</i> (1997)
K	5.10–5.12	$\rightarrow 4\text{-}\alpha\text{-Glc}pA\text{-(1}\rightarrow 2\text{)-}\beta\text{-Gal}f$	Ahrazem <i>et al.</i> (1999)

Fig. 7 are proposed from the results of the chemical analyses of the polysaccharides, the full structural analysis of *S. candelabrum* presented here and the coincidence of chemical shifts of the signals of ^1H -NMR spectra with those reported for similar residues (Table 4). The polysaccharides of *S. candelabrum* and *N. lasiacidis* had around 10% of the 5-O-substituted galactofuranose, ($\rightarrow 5\text{-}\beta\text{-Gal}f\text{-(1}\rightarrow$), substituted also at O-6. These species also contain about 10% of N-acetylglucosamine. These results indicated the connection of our isolates of *N. lasiacidis* with *S. candelabrum*. Since it has been reported that the anamorph of *N. lasiacidis* is *S. buxi* (Samuels, 1989), it is possible that this strain of *N. lasiacidis* is misplaced. It is interesting to note that differences in conidium morphology between *S. buxi* and *S. candelabrum* have been described (Bisset, 1983). The polysaccharide from *N. impariphialis* contained 6-O-substituted glucopyranose ($\rightarrow 6\text{-}\text{Glc}p\text{-(1}\rightarrow$), in addition to the three main residues. This species was distinguished from other species of this group by the absence of the ring in the ascus apex (Samuels, 1989).

The polysaccharide obtained from the three isolates of *N. cinnabarina* (structure 1, Fig. 7) was similar to the polysaccharides found in species of *Gibberella* and *Fusarium* (Jikibara *et al.*, 1992; Ahrazem *et al.*, 2000) and in *Penicillium vermoesenii* (= *Gliocladium vermoesenii*) (Ahrazem *et al.*, 1999) since they have similar residues, mainly 2,6-di-O-substituted galactofuranose ($\rightarrow 2,6\text{-}\beta\text{-Gal}f\text{-(1}\rightarrow$), and terminal glucopyranose ($\text{Glc}p\text{-(1}\rightarrow$), and almost identical ^1H -NMR spectra. The differences found in the polysaccharide from *N. cinnabarina* and those species of *Sesquicillium* and *Nectria* with *Sesquicillium* anamorphs were in agreement with the separation of these species according to their morphological characters (Samuels, 1989) and support the creation of the genus *Bionectria* (Rossman *et al.*, 1999).

The results show that changes in morphological features of certain species of a genus are reflected in the structure of the alkali-extractable, water-soluble polysaccharides,

and they confirm that these polysaccharides are reliable characteristics for fungal systematics at genus or sub-genus level and for establishing teleomorph–anamorph and phylogenetic relationships, since: ‘the nature of the polysaccharides in any particular fungus is not capricious, but is related to its taxonomic position and thus reflects its evolutionary history’ (Bartnicki-García, 1987).

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