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Uronic acid-containing glycopeptides from *Fusarium oxysporum*: Possible significance as chemotypes

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Abstract

Hot aqueous extraction of mycelia of *Fusarium oxysporum*, followed by fractionation on an anionic resin column gave glycopeptides FL-2 and FL-3. Methylation analysis and 1D and 2D NMR data demonstrated β-D-Man*p* units and partial hydrolysis gave α-D-Glc*p*A(1→2)-D-Gal, arising from β-D-Gal*f*-containing groups. Both are chemotaxonomic markers of *Fusarium* spp. FL-3 contained 2,6-di-*O*-substituted Man*p*, as well as 2,6-di-*O*-substituted Gal*f* units, raising the possibility that the former are main-chain constituents, as well as the expected latter structure. The carbohydrate structures of FL-2 and FL-3 differ from those of previously examined polysaccharides of *Fusarium* spp., which are in turn all different from each other, so that they can serve as fingerprints. Possible variations in their main-chain structures can occur as well as those of their side-chains.

Keywords: *Fusarium oxysporum*; Chemotypes; Structure; Glycopeptides; ^{1}H, ^{13}C NMR; Methylation

1. Introduction

Fusarium oxysporum is a phytopathogen capable of infecting economically important crops such as peas and tomatoes (Nelson, Dignani, & Anaissie, 1994). The interaction mechanisms between the fungus and its plant host are complex and little understood. However, it is likely that the molecules expressed on the fungal surface, present in its cell wall, are somehow involved in the phenomena of interaction and invasion.

The chemical composition of the intact cell wall from mycelia of three strains of *F. oxysporum* was studied by Schoffelmeer, Klis, Sietsma, and Cornelissen (1999), who showed the presence of *N*-acetylglucosamine units, presumably from chitin, and those of glucose, galactose, mannose, and uronic acid. The components were similar to that found for the cell wall of *Fusarium graminearum* (Barbosa & Kemmelmeier, 1993).

These neutral monosaccharides and uronic acid were also present in mycelial extracts, obtained from several *Fusarium* spp. under a variety of conditions. Polycarpo et al. (1997) extracted mycelia of *Fusarium solani* with hot aqueous potassium hydroxide to give polysaccharides. Glycoproteins were obtained by Jikibara, Takegawa, and Iwahara (1992), who extracted mycelia of *Fusarium* sp. M7-1 with water at 120 °C, and these were degraded with alkaline borohydride and resulting polysaccharides analyzed in detail (Jikibara, Tada, Takegawa, & Iwahara, 1992), as well as non-reducing oligosaccharides with related structures (Iwahara, Suemori, Ramli, & Takegawa, 1995). Mycelia from seven *Fusarium* spp. were extracted with aqueous alkali at room temperature (Ahrazem et al., 2000) and although the isolated polysaccharides were structurally related, none were identical. However, fractions from *F. javanicum*, *F. graminareum*, and *Fusarium xylarioides* each contained high proportions of 2,6-di-*O*-substituted β-D-Gal*f* units and main-chains of 6-*O*-substituted β-D-Gal*f* units were suggested. Now examined in detail are structurally different glycoprotein fractions obtained from mycelia of *F. oxysporum* on hot aqueous extraction.

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2. Materials and methods

2.1. Extraction of glycopeptides

The mycelia of *F. oxysporum* (500 g dry weight) were extracted with 0.05 M sodium phosphate buffer, pH 7.2, under reflux for 2 h (Haido et al., 1998). After centrifugation at 5000*g* for 15 min, the supernatant was concentrated under reduced pressure to 100 mL, which was added to 3 vols. EtOH and left for 16 h at 4 °C. The resulting precipitate was centrifuged, isolated, dissolved in distilled H_2O, and the solution dialyzed against the same. The retained solution was freeze-dried to give a mixture of glycopeptides (GPB).

2.2. Fractionation of glycopeptides by ion exchange chromatography

GPB (1.0 g) in 0.01 M phosphate buffer, pH 7.0, was applied to a column (11 × 1.6 cm) of SOURCE 30Q anionic resin (Pharmacia AP, Sweden). This was eluted with a discontinuous gradient of 0.2, 0.3, 0.4, and 1 M NaCl in 0.01 M phosphate buffer at pH 7.0 (20, 10, 10, and 100 mL, respectively). The carbohydrate content of the eluates was determined by the method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956), in which 30 μL eluant, 45 μL of 5% phenol, and 150 μL of sulfuric acid were employed: the colorimetric absorbance was measured at 490 nm. Eluant fractions were probed for protein by their absorbance, at 280 nm (Lowry, Rosebrough, & Randall, 1951). Uronic acid contents were determined using the *m*-hydroxybiphenyl colorimetric procedure (Blumenkrantz & Asboe-Hansen, 1973), with absorbance measured at 525 nm, using glucuronic acid as standard.

In the first 30 mL, neutral material was eluted and discarded. The following fractions, eluted with a discontinuous gradient of NaCl, were recovered according to their profile of carbohydrate content. Appropriate eluates were combined, dialyzed, and freeze-dried to give fractions FL-1, FL-2 (0.30 g), FL-3 (0.20 g), and FL-4: FL-1 and FL-4 were not examined due to their low yields.

2.3. HSPEC-MALLS analysis

The molecular weight distribution of FL-2 and FL-3 was determined using Wyatt Technology equipment incorporating columns of 2000, 500, 250, and 120 connected to a differential refractometer (model 2410, Waters). The eluant was aq. 0.1 M $NaNO_2$ + 0.2 g/L NaN_3, at a flow rate of 0.6 mL/min.

FL-2 and FL-3 were each dissolved in aq. $NaNO_2$ (1 mg/mL) and filtered through a cellulose membrane with an average pore diameter of 0.2 μm and a volume of 250 μL was injected into the apparatus. The results were provided directly with the aid of computer software ASTRA 4.70.07.

2.4. Monosaccharide composition of FL-2 and FL-3 glycopeptides

For determination of monosaccharide composition of the glycopeptide fractions, each one (∼5 mg) was hydrolyzed with 2 M TFA at 100 °C for 8 h, the solution evaporated to dryness, and the residue applied to a TLC plate, previously impregnated with 0.3 M Na_2HPO_4 (Ovodov, Evtushenko, Vaskovsky, Ovodova, & Solov'eva, 1967). This was eluted with *n*-BuOH–Me_2CO–H_2O (4:5:1) and developed with 0.05% (w/v) orcinol in 10% (w/v) H_2SO_4 at 100 °C for ∼10 min, using ∼10 μg each of mannose, galactose, mannose, galacturonic acid, and glucuronic acid as standards.

Each glycopeptide (∼1 mg) was hydrolyzed with 2 M TFA for 6 h at 100 °C and the aldoses isolated on evaporation successively reduced with $NaBD_4$ and acetylated with Ac_2O–pyridine at 100 °C for 1 h. The resulting alditol acetates were analyzed by GC–MS using a capillary column of DB-225 (30 m × 0.25 mm i.d.), held at 50 °C during injection, and then programmed at 40 °C/min to 230 °C (constant temperature). The fragments were identified by their typical retention times and electron impact spectra (Jansson, Kenne, Liedgren, Lindberg, & Lönngren, 1976).

2.5. Methylation analysis of oligosaccharide and glycopeptides

Using a modification (Tischer, Gorin, & Iacomini, 2002) of the method of Ciucanu and Kerek (1984), each sample (∼1 mg) was dissolved in a drop of H_2O, to which Me_2SO (2 mL) was added, followed by powdered NaOH. This sequence was used to minimize alkaline degradation of the reducing end-units. After 30 min vigorous shaking, the mixture was left overnight, neutralized with aqueous HOAc, and then extracted with $CHCl_3$, which was washed × 3 with H_2O. On evaporation, the resulting per-*O*-methylated product was converted into partially *O*-methylated alditol acetates by successive treatments with 3% MeOH–HCl for 2 h at 70 °C, 0.5 M H_2SO_4 for 14 h at 100 °C, reduction with $NaBD_4$, and acetylation with Ac_2O–pyridine. The products were analyzed by the GC–MS procedure described above using a capillary column of DB-225 (Jansson et al., 1976), programmed as above, but with maximum temperatures indicated in Tables 1 and 2.

2.6. Partial acid hydrolysis of crude glycopeptide extract (GPB)

GPB (108 mg) was partially hydrolyzed in 0.33 M TFA (2 mL) at 100 °C for 3 h, the solution evaporated to dryness, and the residue fractionated by dialysis. Retained was higher molecular weight material (43 mg), which contained mannose, galactose, and glucose in a 64:26:10 molar ratio, but no glucuronolactone, and which passed through was low molecular weight material (62 mg).

Table 1
Percentage values of *O*-methyl alditol acetates formed on methylation analysis of glycopeptides FL-2 and FL-3

OMe alditol acetate	Retention time (min)	FL-2 (%)	FL-3 (%)	OMe alditol acetate	Retention time (min)	FL-2 (%)	FL-3 (%)
2,3,4,6-Me_4Man	10.02	40	22	2,3,6-Me_3Glc	14.35	1	4
2,3,4,6-Me_4Glc	10.14	1	–	4,6-Me_2Man	16.42	1	1
3,4,6-Me_3Man	12.92	28	29	2,3-Me_2Hex	19.12	1	1
3,5,6-Me_3Gal	13.32	5	7	3,4-Me_2Man	20.44	11	14
2,3,4-Me_3Man	14.14	2	10	3,5-Me_2Gal[a]	22.28	10	12

Column: DB, 225–200 °C.
[a] This derivative gave an e.i. impact profile identical to that of the 2,4-isomer, except that after $NaBD_4$ reduction, ions appeared with *m/z* 117, 130, and 140, instead of 118, 129, and 139, respectively.

The latter was fractionated on Whatman 3 mm filter paper (solvent: *n*-BuOH–pyridine–H_2O, 2:1:1, 42 h) to give an oligosaccharide fraction with R_{Gal} 0.3 (4.9 mg).

2.7. Methylation analysis of oligosaccharides and higher molecular weight material

Methylation analysis of the above oligosaccharide fraction gave rise to alditol acetates of 2,3,4,6-Me_4Glc (9.20, 5%), 3,5,6-Me_3Man (10.52, 3%), 3,4,6-Me_3Man (11.93, 11%), 3,5,6-Me_3Gal (11.08, 25%), and 3,4,6-Me_3Gal (11.50, 56%). Retention times in minutes and percentage contents are in parentheses.

Methylation analysis of $NaBD_4$-reduced oligosaccharide fraction gave rise to alditol acetates of 1,3,4,5,6-Me_5Man (8.00, 17%), 1,3,4,5,6-Me_5Gal (8.28, 79%); 2,3,4,6-Me_4Glc (9.18, 4%).

The oligosaccharide fraction was carboxy-reduced as follows: it was left overnight in 0.5% HCl in MeOH to form its methyl ester, neutralized with Ag_2CO_3 and the product kept overnight at 50 °C in 0.1% NaOMe in MeOH with $NaBD_4$. Methylation analysis furnished alditol acetates of 2,3,4,6-Me_4Man (9.07, 1%), 2,3,4,6-Me_4Glc (9.12, 3%), 3,5,6-Me_3Man (10.50, 11%), 3,4,6-Me_3Man (10.90, 26%), 3,5,6-Me_3Gal (11.05, 21%), 3,4,6-Me_3Gal (11.48, 34%), 2,3,4-Me_3Glc (11.68, 4%): this was the 1,6-d_3 derivative with *m/z* 118, 130, and 191.

2.8. NMR spectroscopy

^1H, ^{13}C NMR, and ^1H (obs.), ^{13}C HMQC, COSY, and TOCSY spectra were obtained using with a 400-MHz Bruker model DRX Avance spectrometer. Samples were dissolved in D_2O and mainly examined at 50 °C. ^1H and HMQC spectra were obtained using DOH presaturation. Chemical shifts are expressed in δ PPM, relative to external Me_4Si ($\delta = 0$). Experiments were carried out as instructed in the Bruker manual.

Aldobiouronic acid containing fraction: ^{13}C NMR (70 °C, 99.97% D_2O), mainly δ 97.9 > 96.4 > 89.7, others at δ 77.3, 76.2 (*O*-subst?), 74.8. ^1H NMR (70 °C, 99.97% D_2O): δ 4.72, $J = 7.55$ Hz (22%); δ 5.07, $J = 0$ (34%); δ 5.27, $J = 0$ (5%); δ 5.33, $J = 3.5$ Hz (22%); δ 5.44, $J = 3.5$ Hz (15%).

3. Results

Mycelia of *F. oxysporum* were extracted with hot phosphate buffer, pH 7.2, which was added to excess ethanol to give a polymeric precipitate (GPB). This was applied to a SOURCE 30-Q anionic resin column, which was eluted with a discontinuous gradient of aqueous NaCl (Fig. 1). Carbohydrate-containing fractions FL-1, FL-2 (30% yield), FL-3 (20% yield), and FL-4 were obtained, but only FL-2 and FL-3 were selected, due to their higher yields. Each was uronic acid-positive and had carbohydrate to protein ratios of 5.8:1 and 10.0:1, respectively.

HPSEC-MALLS analysis showed FL-3 to contain a component with M_w 178 kDa in a much greater quantity than another with M_w 41 kDa, both with d*n*/d*c* 0.11. FL-2 contained two components with M_w 100 and 62 kDa, also both with d*n*/d*c* 0.11 (Fig. 2). Since, it was less heterogeneous, FL-3 was selected for detailed analyses, although some attention was paid to FL-2.

Table 2
Percentage values of *O*-methyl alditol acetates formed on methylation analysis of polysaccharide fraction formed on partial hydrolysis of crude mixture of extract (GPB)

OMe alditol acetate	Retention time (min)	% Content	OMe alditol acetate	Retention time (min)	% Content
2,3,4,6-Me_4Man	8.33	6	2,3,4-Me_3Man	11.13	18
2,3,4,6-Me_4Glc	8.37	1	3,4-Me_2Man	15.05	7
2,3,5,6-Me_4Gal	9.07	4	2,4-Me_2Man	15.17	1
3,4,6-Me_3Man	10.25	48	3,5-Me_2Gal	16.11	4
3,5,6-Me_3Gal	10.39	6	3,4-Me_2Gal	16.35	4

Column: DB, 225–220 °C.

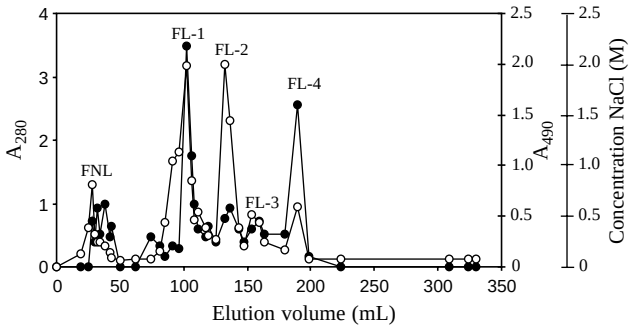

Fig. 1. Elution profile of crude glycopeptide extract (GPB), fractionated on a column of anion-exchange chromatography (SOURCE 30-Q) with a discontinuous gradient of NaCl (discontinuous line). ○, carbohydrate at A_{490}; ●, protein at A_{280}.

Hydrolysis of each fraction followed by TLC examination of the products showed the presence of mannose, galactose, glucose, and a uronic acid, confirmed by GC–MS analysis of derived alditol acetates.

FL-1 contained total carbohydrate (72%), uronic acid (13%), and protein (10%), FL-2 contained total carbohydrate (46%), uronic acid (11%), and protein (8%), suggesting the presence of a salt impurity, and FL-3, carbohydrate (91%) containing uronic acid (22%), and protein (9%).

3.1. Carbohydrate structure of glycopeptide FL-3

Fraction FL-3 had the highest carbohydrate content of the fractions and gave a simpler C-1 region of its ^{13}C NMR spectrum (Fig. 3A) than FL-2 (Fig. 3B), agreeing with its greater homogeneity (Fig. 2). The alditol acetates derived from FL-3 showed Man, Gal, and Glc in a 60:21:15 molar ratio (GC–MS). The use of $NaBD_4$ in the reduction step of the derivatization gave rise to, in addition to the ions at *m/z* 138 and 139 from the hexaacetate of glucitol-1-*d*, a smaller proportion of one at *m/z* 141, from glucitol-1,6-d_3, a reduction product of glucurono-3.6-lactone.

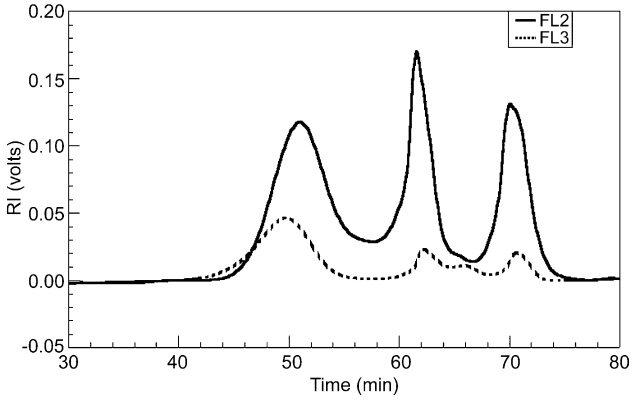

Fig. 2. HSPEC-MALLS of FL-2 and FL-3, obtained using a refractive index detector.

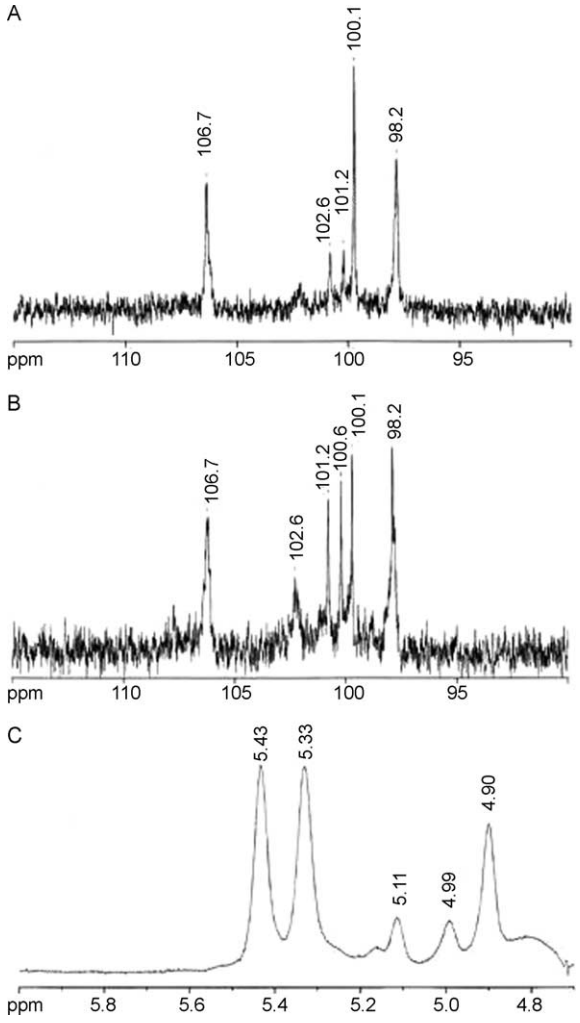

Fig. 3. Anomeric regions of ^{13}C NMR spectra of FL-3 (A) and FL-2 (B) and the ^{1}H NMR spectrum of FL-3 (C).

Methylation–GC–MS analysis of FL-3 gave rise to partially *O*-methylated alditol acetates, which corresponded principally to non-reducing end-units of Man*p* (22%), 2-*O*- (29%), 6-*O*- (10%), and 2,6-di-*O*-subst. Man*p* units (14%), and 2-*O*- (7%) and 2,6-di-*O*-subst. Gal*f* units (12%) (Table 1).

The ratio of non-reducing end- to di-*O*-subst. hexopyranosyl units did not match, due to the presence of 22% of glucuronic acid non-reducing end-units, which would give rise to the alditol acetate of 2,3,4-Me_3-GlcA, retained on GC–MS analysis. The presence of these non-reducing end-units was confirmed, however, when the mixture of *O*-methylaldoses was converted to their methyl glycosides, which was reduced with $NaBD_4$ in MeOH containing NaOMe. The final acetylated product was 7% 2,3,4-Me_3-Glc-1,6-d_3 which had a typical retention time of 14.29 min and gave GC–MS ions at *m/z* 131 and 191 arising from dideuterated C-6 fragments. No other fragments from *O*-substituted Glc*p*A units were detected.

The ^{13}C NMR spectrum of FL-3 at 50 °C showed it to contain carbohydrate and protein, with three main and two

minor C-1 signals, a CO_2-6 signal at δ 175.2, and no signals corresponding to hexosamine and/or its *N*-acetyl derivative. The C-1 region was obtained (Fig. 3A) and a ^{13}C, ^{1}H-coupled spectrum of this region showed the anomeric configuration, which should give $J = \sim 160$ Hz for β-D and ~ 170 Hz for α-D-hexopyranosides (Perlin & Casu, 1969). Values of the order $J = 174$ Hz might be expected for α-D- and β-D-galactofuranosides (Chambat, Joseleau, Lapeyre, & Lefebvre, 1978). These values for FL-3, in the light of the methylation data, showed signals centered at δ 98.2, $J =$ 173 Hz (non-reducing end-units of α-D-Glc*p*A), δ 100.1, $J = 164$ Hz (β-D-Man*p*), and δ 106.7, $J = 174$ Hz (2-*O*-subst. and 2,6-di-*O*-substituted β-D-Gal*f*). Smaller signals from other β-D-Man*p* units were centered at δ 100.6 ($J = 157$ Hz) and 101.2 ($J = 162$ Hz). These three signals of β-D-Man*p* agree with the three Man*p* structures shown by methylation analysis. A small broad signal was present at δ 102.6.

A more accurate quantification of these structures in FL-3 was obtained from the ^{1}H NMR spectrum of FL-3 at 50 °C (Fig. 3C). This contained H-1 signals at δ 4.90 (20%), 4.99 (9%), 5.11 (9%), 5.33 (31%), and 5.43 (31%), none of which exhibited coupling. Its ^{1}H (obs.)^{13}C HMQC spectrum then showed the correlations: δ 4.90 (98.2; 20% of α-D-Glc*p*A non-reducing end-units), 4.99 (101.2; 9% of one β-D-Man*p* component), 5.11 (100.6; 9% of another β-Man*p* component), 5.33 (100.1; 31% of major β-D-Man*p* component), and 5.43 (106.7; 31% of combined 2-*O*-subst. and 2,6-di-*O*-subst. β-D-Gal*f* units) (a broad signal was present at δ 102.6). The three signals were assigned to β-D-Man*p* units since they had the appropriate ^{13}C-1, ^{1}H-1 coupling constants, without observed $H_{1,2}$ coupling, as would occur with both Man*p* anomers (Fig. 3C).

The COSY and TOCSY spectra of FL-3 were of limited value as a basis of further structural elucidation. For example, connectivity of H-1 with H-2 of the β-D-Man*p* units could not be detected. However, limited connectivity of the H-1 signal at δ 5.47 to that of H-2 at δ 4.45 occurred, so that this in turn coupled, in the HMQC spectrum, with a C-2 signal at δ 86.7 whose low field compared with that of the Me β-D-Gal*f* C-2 signal at δ 81.9 (Gorin & Mazurek, 1975; obtained at 33 °C), due to an α-shift, agreed with 2-*O*-substitution of β-D-Gal*f* units (see above methylation data).

3.2. Carbohydrate structure of glycopeptide FL-2

FL-2 contained 46% carbohydrate with 11% uronic acid, and 8% protein, and its HSPEC-MALLS profile contained two principal polymeric peaks (Fig. 2). The C-1 region of its ^{13}C NMR spectrum (Fig. 3B) differed from that of FL-3 (Fig. 3A), as it contained larger C-1 signals of β-D-Man*p* units at δ 100.6 and 101.2 (the spectrum showed that protein and carbohydrate were the only organic components, so that other components were inorganic). This was confirmed by the presence of a low molecular weight component in its HPSEC-MALLS spectrum (Fig. 2). Its neutral monosaccharides were Man, Gal, and Glc in a molar ratio of 56:23:21 (GC–MS), close to 60:21:15 for FL-3, and part of the glucitol acetate also arose from glucuronolactone (GC–MS of *O*-methylated alditol acetates).

Methylation analysis of FL-2 (Table 1) showed, as neutral components, mainly non-reducing end- (40%), 2-*O*- (28%), and 2,6-di-*O*-substituted Man*p* (11%), 4-*O*-substituted Glc*p* (4%), and 2-*O*- (5%) and 2,6-di-*O*-substituted Gal*f* units (10%).

3.3. Characterization of α-D-GlcpA-(1→2)-D-Gal and polysaccharide from partial hydrolysis of GPB

Partial acid hydrolysis of GPB gave a product, which was fractionated by dialysis. The material which passed through the tube was fractionated by paper chromatography to give a product, consisting of an aldobiouronic acid, whose structure was indicated via its ESI-MS spectrum, which contained (−ve mode) a molecular ion with *m/z* 377.1 (GlcANa–Gal$^-$). This was absent in a +ve mode spectrum, which however contained a single molecular ion at *m/z* 365.1 from minor Hex_2-Na^+. The predominance of the aldobiouronic acid was shown by main C-1 signals in a ^{13}C NMR spectrum at 50 °C at δ 97.9 (C-1′) > 96.4 (C_β-1) > 89.7 (C_α-1). An α-configuration for the D-Glc*p*A unit was shown by the high-field C-1′ signal and this was confirmed by a low-field H-1′ signal at δ 5.33 ($J = 3.5$ Hz). Reducing end H-1 signals of D-Gal*p* in the α- and β-form were present at δ 5.44 ($J = 3.5$ Hz) and δ 4.72 ($J = 7.55$ Hz), respectively. These assignments were confirmed by an HMQC spectrum of the isolated fraction at 50 °C using an *hnqcbiphpr* program, which gave the fewest well-defined signals. The anomeric ones were δ 98.0/5.38, 96.6/4.79, 96.3/5.10, and 89.7/5.48. Also present was a signal at δ 75.05/3.68. If this were from C-2 of α-D-Gal*p* reducing-ends, there were COSY correlations of δ 5.38 with 3.865 and δ 5.65 with 3.865 also, but nothing at δ 3.68.

Methylation analysis of the fraction showed non-reducing end-units of Glc*p* (2,3,4,6-Me_4Glc, 5%), and reducing units of 2-*O*-substituted Man (3,5,6-Me_3Man, 3%; 3,4,6-Me_3Man, 11%) and 2-*O*-substituted Gal (3,5,6-Me_3Gal, 25%; 3,4,6-Me_3Gal, 56%).

Methylation analysis of a $NaBD_4$-reduced fraction showed end-units of Glc*p* (2,3,4,6-Me_4-Glc, 4%), 2-*O*-substituted Man-ol (1,3,4,5,6-Me_5Man, 17%), and 2-*O*-substituted Gal-ol (1,3,4,5,6-Me_5Gal, 79%).

The fraction was treated with HCl in methanol to form methyl esters, which were reduced with $NaBD_4$ in MeOH containing NaOMe. Methylation analysis of the product gave rise to a 2,3,4-Me_3Glc-1,6-d_3 derivative, showing the presence of GlcA non-reducing ends.

The higher-molecular weight fraction was subjected to methylation analysis and the main products (Table 2) corresponded to 2-*O*- (48%) and 6-*O*-substituted Man*p* (18%) units, suggesting an unexpected main-chain structure.

4. Discussion

The methylation data now obtained for the FL-3 glycopeptide of *F. oxysporum* (Table 1) shows that its carbohydrate structures differed from those of polysaccharides obtained and examined in detail by two other research groups.

Ahrazem et al. (2000) subjected the mycelia of seven *Fusarium* spp. to hot alkaline extraction followed by Sepharose CL-6B fractionation. The resulting polysaccharides, although related in terms of their monosaccharide components, had various structures, the ^{1}H NMR spectrum of each polysaccharide being typical, so that each might be used as a fingerprint. The structures were determined by methylation analysis and the main ones (>5%) are shown in Table 3, and it can be seen that each carbohydrate structure is typical of the species, and is in turn different from our glycopeptides FL-2 and FL-3 from *F. oxysporum*. In the case of fractions from *F. javanicum*, *F. graminareum*, and *F. xylarioides*, they showed that 3,5-di-*O*-methyl galactose was the only di-*O*-methyl derivative, and suggested a 6-*O*-linked Gal*f* main-chain.

In a sequence of studies initiated by Iwahara, Jikibara, and Takegawa (1990) on a *Fusarium* sp. designated M7-1, its mycelia were extracted with water at 120 °C to give a product with a carbohydrate to protein ratio of 4:1, and which gave on partial hydrolysis, α-D-Glc*p*A-(1→2)-D-Gal characterized as its derived galactitol derivative. Jikibara et al. (1992) treated the preparation with Cetavlon to give water-insoluble GP I (mol. wt 8.8×10^{4}) and water-soluble GP II (mol. wt 3.7×10^{4}). Later, alkaline borohydride treatment provided several oligosaccharides, with mannitol (Jikibara et al., 1992) and mannitol-6-phosphate terminal units (Iwahara et al., 1995). The same alkaline borohydride treatment (Jikibara, Tada, & Iwahara, 1992) gave two high molecular weight fractions, AP I (mol. wt 8.2×10^{4}) and AP II (mol. wt 3.1×10^{4}) from GP I and GP II, respectively, which were analyzed in detail by Jikibara et al. (1992). Each fraction contained principally galactose, with smaller proportions of glucose, glucuronic acid, and mannose. Partial acetolysis provided α-D-Glc*p*-(1→2)-D-Gal, β-D-Man*p*-(1→4)-α-D-Glc*p*A-(1→2)-D-Gal and a trace of β-D-Man*p*-(1→2)-β-D-Man*p*-(1→4)-α-D-Glc*p*A-(1→2)-D-Gal. They proposed a main-chain structure similar to that of Ahrazem et al. (2000) with the refinement of the side-chains defined by their three above partial acetolysis products.

Our methylation studies (Table 1) and NMR data show that the overall structures of glycopeptides FL-2 and FL-3 differ from those present in the polysaccharides present in the seven *Fusarium* spp. and the glycopeptide of *Fusarium* sp. M7-1. FL-2 and FL-3 contain mainly non-reducing end-units of Man*p* and Glc*p*A, 2,6-di-*O*-substituted Gal*f* and Man*p* units (a smaller proportion of 2-*O*-substituted Gal*f* units was also present). Partial acid hydrolysis of the crude *F. oxysporum* glycopeptide extract (GPB) gave rise to an oligosaccharide fraction containing mainly α-D-Glc*p*A-(1→2)-D-Gal, characterized by NMR, methylation analysis, and ESI-MS.

The C-1 region of the ^{13}C NMR spectrum of FL-3 (Fig. 3A) contains three main signals, which was based on the methylation data (Table 1), can be interpreted as arising from non-reducing end-units of α-D-Glc*p*A (δ 98.2), (1→2)-linked to β-D-Gal*p* units, some of which are also substituted at O-6 by β-D-Man*p* units (**1**). β-D-Man*p* is present as non-reducing end- and 2-*O*-(**2**) and 2,6-di-*O*-substituted units. Methylation analysis of partly hydrolyzed GPB gave rise to polysaccharide containing mainly 2-*O*-(48%) and 6-*O*-substituted Man*p* units (Table 2) as its neutral components, showing that mannosyl units are the main core structures.

Table 3
Comparison of principal carbohydrate structures (>5%), represented by the symbol (√), present in glycopeptides of *Fusarium oxysporum* (FL-2, FL-3) and polysaccharides from *Fusarium* spp.

Unit structure	FL-2	FL-3	FN	FCI	FCA	FG	FX	FJ	FA
Glc*p*-(1→	–	–	√	–	–	–	√	√	–
Man*p*-(1→	√	√	–	–	–	√	√	–	–
Gal*f*-(1→	–	–	√	√	√	–	–	–	√
Glc*p*A-(1→	√	√	–	–	–	√	–	–	–
→2)-Man*p*-	√	√	–	–	–	√	–	–	–
→4)-Glc*p*A-	–	–	–	–	√	–	√	–	–
→3)-Glc*p*-	–	–	√	–	–	–	–	–	–
→5)-Gal*f*-	–	–	√	√	√	–	–	–	√
→2)-Gal*f*-	√	√	–	√	–	–	–	–	√
→6)-Man*p*-	–	√	–	–	–	–	√	–	–
→6)-Gal*f*-	–	–	–	–	√	–	√	–	–
→4,6-Man*p*-	–	–	–	–	√	–	–	√	–
→2,6-Man*p*-	√	√	√	√	–	–	–	–	√
→2,6-Gal*f*-	√	√	–	–	–	√	√	√	–

F. (Microdochium) nivale (FN)*, *F. ciliatum* (FCI)*, *F. cavispermum* (FCA)*, *F. graminearum* (FG)*, *F. xylarioides* (FX)+, *F. javanicum* (FJ)*, and *F. aquaeductuum* (FA)*. *, based on data of Ahrazem et al. (2000).

α-D-Glc*p*A (δ 98.2) β-D-Man*p*-(1→2)-β-D-Man*p*-
1 (δ 100.1)
1 ↓ **2**
(minor) 2
[β-D-Man*p* –(1→ 6)]– β-D-Gal*f* (δ 106.7)

This contrasts with the (1→6)-linked β-D-Gal*f* main-chain structure proposed for polysaccharides derived by alkaline extraction of three *Fusarium* spp. (Ahrazem et al., 2000). It follows that a definition of the main-chain structure in the glycopeptides of *F. oxysporum* requires further investigation, especially as these researchers showed that seven of their eight *Fusarium* spp. contained polysaccharides with either Gal*f* or Man*p* units di-*O*-substituted in the 2,6-position, as in FL-2 and FL-3. Such branching could occur either in the main-chain or the side-chains (other structural differences between the polysaccharides of *Fusarium* spp. were also found, although the presence of Glc*p*-(1→2)-Gal*f*- groups and β-D-Man*p* units were constant markers).

It follows that while the methylation analysis and probably the ^{13}C NMR data can serve as fingerprints typical of each *Fusarium* sp., the chemotaxonomic approach based on main-chain and side-chain components, should be used with care.

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