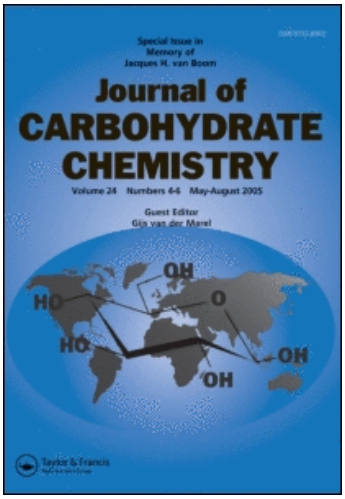

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AN EXTRACELLULAR GALACTOGLUCOXYLOMANNAN PROTEIN FROM THE YEAST *CRYPTOCOCCUS LAURENTII* VAR. *LAURENTII*

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ABSTRACT

An extracellular galactoglucoxylomannan protein composed of D-galactose, D-glucose, D-xylose and D-mannose in a 2.9:1.0:1.1:10.2 mole proportion has been isolated from culture medium of *Cryptococcus laurentii* var. *laurentii*. The polymer of number average molecular mass 19,000 contained 86% carbohydrates, 6.5% protein and 0.7% phosphorus. Results of structural analyses suggested a highly branched comb-like structure of the polysaccharide with a backbone composed of 6-linked α-D-Man residues. Mannose units of the backbone are highly branched at O-2, O-3, and O-4 by side chains composed mainly of 2-linked α-D-Man mostly in the form of dimers and trimers, and to a lesser amount as tetra- and pentamers. Galactosyl units were found to be mostly 6-linked with a very low degree of substitution. Mannose side chains are further branched with D-Xyl, D-Glc, and D-Gal residues preferably in β their forms. The protein part of the glycoprotein was *O*-glycosylated by mannose, mannobiose, and mannotetrose.

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INTRODUCTION

Fungi of the genus *Cryptococcus* are encapsulated nonfermenting yeast-like organisms and some of them can excrete extracellular polysaccharides or glycoproteins into growth medium. It has been found that the main representative of this genus *C. neoformans* is a human and animal pathogen[1] while *C. laurentii* is a zoopathogenic organism only. The extracellular polysaccharides produced by *C. laurentii* showed a close compositional relationship with the capsular polymers[2,3] of *C. neoformans*. Previous studies have been directed toward production and characterisation of the extracellular polysaccharides[4–6] produced by *C. laurentii*. The acidic polysaccharide isolated from an extracellular mixture of *C. laurentii* serotype NRRL-Y-1401 has a linear 3-linked mannopyranose backbone.[7] In our recent work we described a separation of three distinct polymers, i.e., one acidic and two neutral, from a mixture of extracellular polysaccharides of *C. laurentii* var. *laurentii*.[8] One of the neutral polysaccharides was identified as a glucomannan protein[9] with a highly branched 6-linked mannopyranose backbone. In the present paper we report on the structural characterisation of the second neutral polysaccharide isolated from the mixture of extracellular polysaccharides of *C. laurentii* var. *laurentii*, a galactoglucoxylomannan protein.

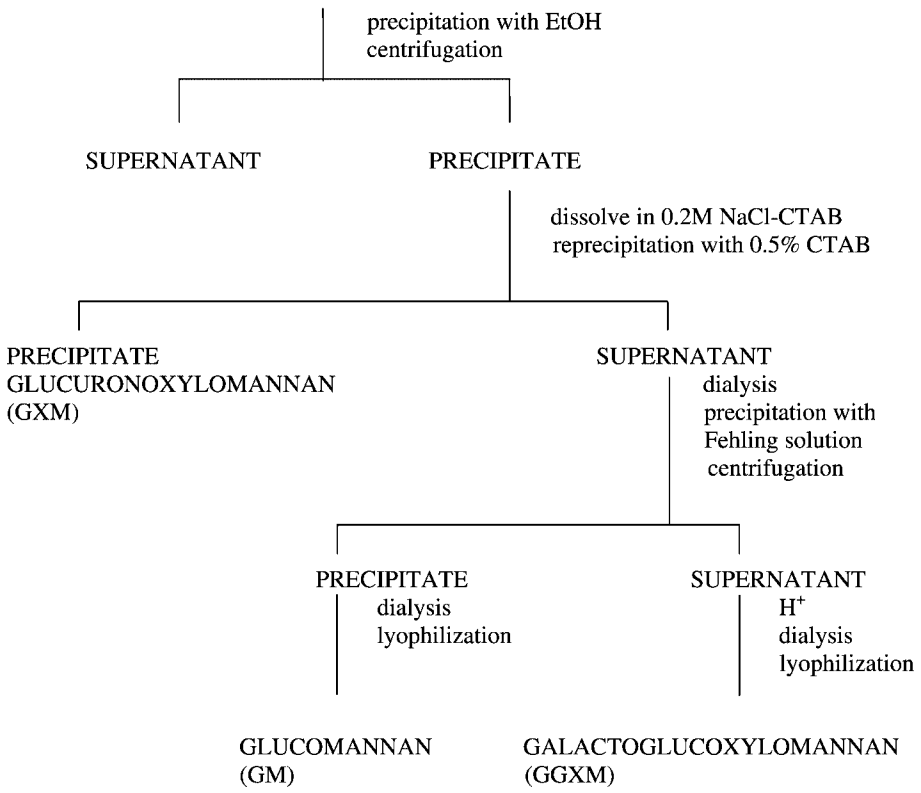
RESULTS AND DISCUSSION

General Properties

The extracellular glycoproteins of *C. laurentii var. laurentii* were recovered by ethanol precipitation of the growth medium. The mixture of glycoproteins was successively fractionated (Scheme 1) by CTABr and Fehlings solution into three different heteropolymers:[8] a glucuronoxylomannan (GXM), a glucomannan protein (GM) already described[9] and a galactoglucoxylomannan protein (GalGXM). Size-exclusion chromatography of the crude GalGXM on Sepharose 2B (Figure 1) afforded the contaminating high molecular-mass (1.9×10^6) GXM ($\sim$10% yield) and the GalGXM ($\sim$90% yield) which was shown to be homogeneous on free-boundary electrophoresis. It contained 6.5% protein, 0.7% phosphorus and had $M_n = 19{,}000$. The polymer was composed of D-galactose, D-glucose, D-xylose and D-mannose in a 2.9:1.0:1.1:10.2 mole proportion.

β-Elimination Reaction

To test the presence of *O*-glycosidic linkages in GalGXM, the polysaccharide was treated with 0.1 M NaOH in the presence of sodium borohydride. Under β-elimination reaction conditions, *O*-glycosidically substituted serine and threonine residues are converted to alanine and α-aminobutyric acid, respectively. Quantitative analysis of the loss of serine and threonine, the increase in alanine and α-aminobutyric acid showed that 44% serine and 64% threonine residues of GalGXM were glycosylated (Table 1). Saccharides released after β-elimination reaction of GalGXM



Scheme 1.

afforded, on hydrolysis, mannose residues only. On TLC in the solvent system S1 three components were observed: mannose, mannobiose, and mannotetrose (used as reference standard) isolated from the GM[9] of *C. laurentii*. MALDI analysis of saccharides released after β-elimination reaction of GalGXM confirmed the presence of hexose (not shown), hexobiose, and hexotetrose (Figure 2). Results of β-elimination showed that the protein part of this glycoprotein is *O*-glycosylated by mannose, mannobiose, and mannotetrose.

Acetolysis of the GalGXM

Partial hydrolysis of the polysaccharide afforded a hydrolyzate mixture which was separated on a Bio-Gel P-2 column into seven distinct fractions, i.e., monosaccharides, oligosaccharides of dp 2–**6** and the fraction eluted in the void volume. The elution profile of GalGXM-derived oligomers (Figure 3) showed that the fractions

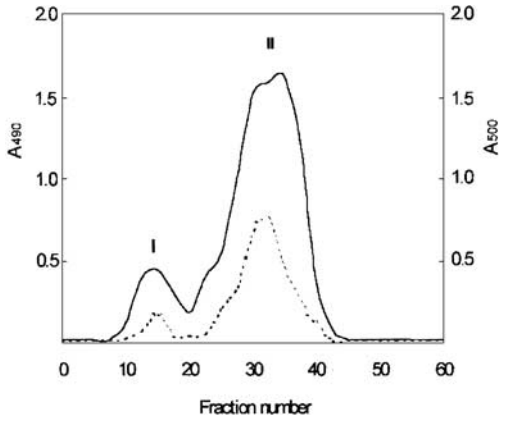


Figure 1. Gel-filtration pattern (Sepharose 2B) of the GGXM. Carbohydrate (–) and protein (–) assay.

with dp **2**, **3** and **4** were major components of the mixture while oligomers of dp **5** and **6** were present only in low amounts. Their degree of polymerization was estimated by comparison with standard mannooligosaccharides. The compositional analysis (Table 2) of individual fractions showed D-Gal, D-Glc, and D-Man in different molar ratios, with D-Man as the dominant sugar residue. D-Xyl was present only in trace amount in all fractions.

Table 1. Changes in the Concentration of Amino Acids Present in GalGXM After β-Elimination Reaction

Amino Acid μmol g^{-1}	Before β-Elimination μmol g^{-1}	After β-Elimination
Asp	15.01	15.22
Ser	**41.46**	**23.32**
Thr	**28.47**	**10.28**
α-aminobutyric acid	–	**7.82**
Glu	18.36	18.68
Pro	traces	traces
Gly	14.92	15.69
Ala	**22.78**	**59.24**
Val	23.11	25.72
Met	7.55	6.82
Ile	7.07	7.34
Leu	6.61	6.08
Lys	7.33	6.98
Tyr	8.57	7.13
Phe	6.92	7.25
Arg	9.45	8.12
His	traces	traces

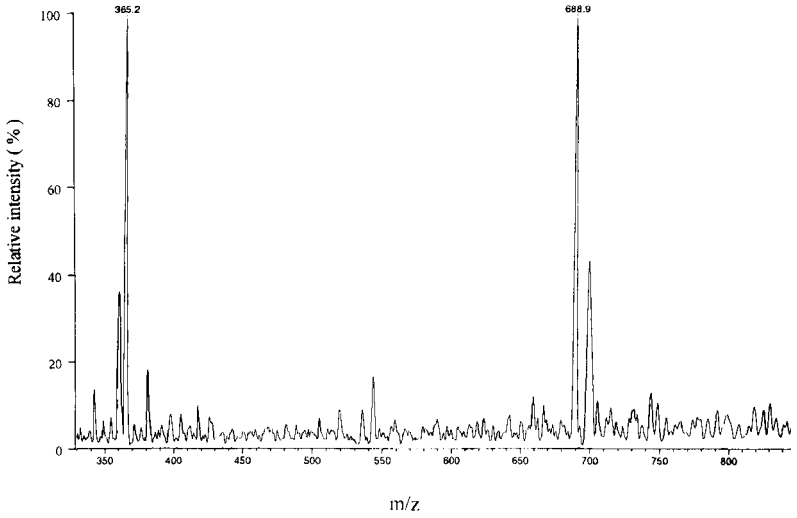


Figure 2. MALDI spectrum of the oligosaccharides released by the alkali-induced β-elimination.

Methylation Analysis of the GalGXM

The backbone of the recently studied extracellular GM,[9] isolated from the same source, was composed of 6-linked Man residues branched at O-2 by side oligosaccharide chains containing 2- and 3-linked Man units. The results of methylation analysis (Table 3) suggest that the backbone of GalGXM is much more branched with

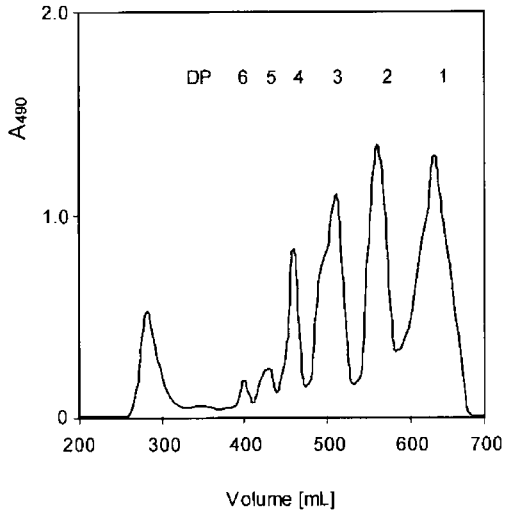


Figure 3. Bio-Gel P-2 pattern of the GGXM-derived oligosaccharides.

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Table 2. Degree of Polymerization (dp) and Sugar Composition of GalGXM-Derived Oligosaccharides

	Monosaccharide Composition (mol%)			
Oligosaccharide (dp)	D-Gal	D-Glc	D-Man	D-Xyl
2	3.4	10.4	86.2	a
3	4.7	7.6	87.7	a
4	5.1	5.4	89.5	a
5	1.9	22.7	75.4	a

a. Traces.

Table 3. Methylation Analysis Data of GalGXM and GalGXM-Derived Oligosaccharides of dp 2, 3, 4, and 5

	Mole %					
Derivative	GGXM	2	3	4	5	Mode of Linkage
2,3,4,6-Me_4-Gal[a]	0.2	b	0.6	–	0.6	Gal-(1 →
2,4,6-Me_3-Gal	1.4	b	–	–	–	→ 3)-Gal-(1 →
3,4,6-Me_3-Gal	–	–	0.6	–	0.7	→ 2)-Gal-(1 →
2,3,6-Me_3-Gal	–	3.1	3.2	4.9	–	→ 4)-Gal-(1 →
2,3,4-Me_3-Gal	10.9	–	–	–	–	→ 6)-Gal-(1 →
4,6-Me_2-Gal	0.3	–	–	–	–	→ 2,3)-Gal-(1 →
2,4-Me_2-Gal	3.4	–	–	–	–	→ 3,6)-Gal-(1 →
3-Me-Gal	1.3	–	–	–	–	→ 2,4,6)-Gal-(1 →
2,3,4,6-Me_4-Glc	2.1	1.1	2.6	–	18.2	Glc-(1 →
2,4,6-Me_3-Glc	1.5	4.7	–	–	–	→ 3)-Glc-(1 →
3,4,6-Me_3-Glc	–	2.6	3.3	–	1.7	→ 2)-Glc-(1 →
2,3,4,-Me_3-Glc	1.1	–	–	–	–	→ 6)-Glc-(1 →
2,3,6-Me_3-Glc	–	–	1.2	5.2	3.2	→ 4)-Glc-(1 →
2,4-Me_2-Glc	0.7	–	–	–	–	→ 3,6)-Glc-(1 →
2-Me-Glc	0.7	–	–	–	–	→ 3,4,6)-Glc-(1 →
2,3,4-Me_3-Xyl	5.1	1.0	0.5	0.4	–	Xyl-(1 →
2,3 + 3,4-Me_3-Xyl	b	b	0.5	–	–	→ 2)-Xyl-(1 → or → 4)-Xyl-(1 →
2,3,4,6-Me_4-Man	27.9	45.6	33.2	31.0	30.9	Man-(1 →
3,4,6-Me_3-Man	5.5	37.7	38.0	39.4	30.5	→ 2)-Man-(1 →
2,3,6-Me_3-Man	–	–	–	–	–	→ 4)-Man-(1 →
2,4,6-Me_3-Man	4.3	4.2	16.3	18.2	4.2	→3)-Man-(1 →
2,3,4,-Me_3-Man	6.3	–	–	–	–	→6)-Man-(1 →
2,6-Me_2-Man	5.6	–	–	–	–	→3,4)-Man-(1 →
4,6-Me_2-Man	1.9	–	–	0.9	10.0	→2,3)-Man-(1 →
2,4-Me_2-Man	16.0	–	–	–	–	→ 3,6)-Man-(1 →
3-Me-Man	3.8	–	–	–	–	→2,4,6)-Man-(1 →

a. 2,3,4,6-Me_4-Gal = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, etc., b. Traces.

a greater variety of linkage types and saccharides as well. Mannose, as the main sugar constituent (~70%) of the polysaccharide, was found to be involved in eight types of linkages with that of 3,6-linked as a dominant one. From the total amount of Man linkages, 37% of Man residues were found to be 6-linked (3,6-, 2,4,6- and 6-linked), 24% involved in 2-, 3-, 2,3- and 3,4-linkages, and 39% as terminal residues. Galactose, the second most abundant sugar (~18%) of the polymer, showed about 60% of the residues as 6-linked non-substituted and 30% with branched 3,6- and 2,4,6-linkages. Glc was present mainly at the non-reducing terminal, 3- and 6-linked positions while xylose was present only at the non-reducing end.

Methylation Analysis of the GalGXM-Derived Oligosaccharides

Similar to GM-derived oligomers,[9] the high content of terminal (~46%) and 2-linked Man residues (~38%) found in the dp **2** fraction indicated that Man-(1→2)-Man is a principal dimer in the mixture. The presence of Man-(1→3)-Man could be deduced as well on the basis of a low amount of 3-linked Man (~4%). 2- and 3-linked Glc derivatives suggest the presence of Glc at the reducing end. Minor portions of terminal glucose (~1%) and xylose (~1%) point to the presence of dimers with non-reducing terminal positions of these sugars. The presence of disaccharides with 4-linked Gal at the reducing end could be deduced on the basis of a 4-linked Gal derivative. Linkage analysis of dp **3** and **4** indicated the prevalence of terminal, 2- and 3-linked Man units (Table 3), thus suggesting similar structures of dominant oligomeric components to GM. In both fractions the occurrence of low amounts of 4-linked Gal, 3- and 2-linked Glc residues also indicated the presence of oligosaccharides with internal or reducing end positions of these sugars in the mixture. The results of the methylation analysis of the pentamer fraction (Table 3) suggest that besides linear pentasaccharide chains, a branched one is present in the mixture with the identical structure of pentasaccharide already identified in GM hydrolyzate.[9]

NMR Spectroscopy of GalGXM and GalGXM-Derived Oligomers

NMR spectra of GalGXM-derived oligosaccharides, namely dp **2** and **3** fraction, reflected also the great variability of linkage types already identified by methylation analysis (Figure 4). Similar to GM-derived oligomers,[9] the prevalent oligosaccharides present in all fractions of GalGXM were composed of 2-linked α-D-Man units, some of which contained terminal 3-linked α-D-Man. Their chemical shifts (Table 4) were in full agreement with already published data.[9] The anomeric region of the dp **2** mixture HSQC spectra is shown in Figure 5A. Besides the signals due to the dominating α-D-Man-(1→2)-D-Man $\mathbf{2_1}$ (Scheme 2) disaccharide, other signals indicated the presence of at least five further species in low amounts. The H1 signal at δ 4.742 as well as those at δ ≈4.62 showed coupling constants $^3J_{1,2} \approx 7.8$ Hz indicating their β anomeric form. C1 chemical shifts δ 97.3–96.6 of these signals confirmed that these signals are due to reducing end residues. On the basis of an easy polarization transfer in the 2D relayed COSY and 2D TOCSY spectra up to H6, H6′, characteristic of the *gluco* configuration,

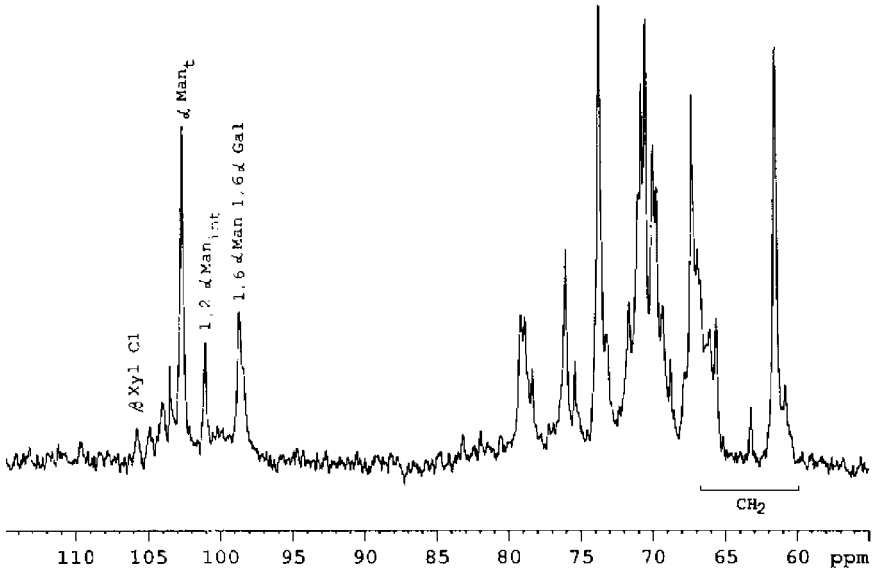

Figure 4. ^{13}C NMR spectrum of GGXM.

the signal at δ 4.742 was attributed to a βGlc residue. For the signal at δ 4.629 the polarization transfer was stopped at H4 δ ≈4.02 showing doublets with $^{3}J_{3,4} \approx 3.6$ and $^{3}J_{4,5} < 1$ Hz, characteristic of Gal residues. Cross peaks for two overlapped signals at δ ≈4.66 did not allow making an unambiguous decision about their sugar origin.

In accord with literature data[10] the cross peak with H1 signal at δ 4.885 was attributed to a β-Man residue and its C1 chemical shift δ 102.44 confirmed its position at the non-reducing end. For inter-glycosidic connection between sugar residues, 1D transient NOESY experiments with selective irradiation of individual anomeric signals in the ^{1}H NMR spectrum (Table 5) were used. After a selective irradiation of this H1 in a 1D transient NOESY spectrum, NOE responses of α- and β-Gal H4 signals at δ 4.112 and 4.058, respectively, were obtained indicating thus the presence of β-D-Man-(1 → 4)-D-Gal (**2$_3$**) (Table 5). Similarly, the NOE response of H2 β-Glc signal at δ 3.408, after the selective irradiation of H1 α-Man signal at δ 5.276, revealed the presence of α-D-Man-(1 → 2)-D-Glc (**2$_4$**) in the mixture. Structures of additional disaccharides present in a mixture were not possible to estimate unambiguously because the signal resolution in the ^{1}H NMR spectrum was not sufficient for the selective irradiation.

In the dp **3** fraction mainly α-D-Man-(1 → 2)-α-D-Man-(1 → 2)-D-Man (**3$_1$**) and α-D-Man-(1 → 3)-α-D-Man-(1 → 2)-D-Man (**3$_2$**) trisaccharides were found. These types of trisaccharides were identified also after the GM acetolysis.[9] Moreover, the presence of a low amount of α-D-Man-(1 → 3)-β-D-Man-(1 → 4)-D-Gal (**3$_3$**) was confirmed, the structure of which was estimated by a 1D transient NOE experiment. The 4-linkage between the β-Man (δ 4.876) and Gal reducing end residue was revealed on the basis of the NOE response of H4 of α,β-Gal after H1β-Man signal irradiation. The H1 signal at δ ∼5.15 (characteristic of terminal 3-linked α-Man) showed the NOE to H3 β-Man

Table 4. Chemical Shifts of Oligosaccharides Found in Hydrolyzate of GalGXM (D_2O at 45°C)

		Chemical Shift δ			
Oligosaccharide	Unit	H1/C1	H2/C2	H3	H4
αMan(1 → 2)	2_{1B}	5.060/102.69	4.089/70.85	3.889	3.670
Manα	$2_{1A}\alpha$	5.386/93.33	3.973/79.80	3.710	3.804
Manβ	$2_{1A}\beta$	4.920/94.19	4.089/79.24	x	x
αMan(1 → 3)	2_{2B}	5.157[a]/102.99	4.130/70.88	x	x
Manα	$2_{2A}\alpha$	5.170[a]/94.80	4.092/78.65	3.970	x
Manβ	$2_{2A}\beta$	5.160[a]	4.090	x	x
βMan(1 → 4)	2_{3B}	4.885/102.44	4.030/70.98	3.992	3.834
Galα	$2_{3B}\alpha$	5.286/93.06	3.856	3.725	4.112
Galβ	$2_{3B}\beta$	4.629/97.33	3.495	3.715	4.058
αMan(1 → 2)	2_{4B}	5.276/100.54	4.022	3.837	x
Glcα	$2_{4B}\alpha$	5.240/92.70	3.582	3.570	3.462
Glcβ	$2_{4B}\beta$	4.742/96.89	3.408/79.99	3.563	3.456
αMan(1 → 2)	3_{1C}	5.061/102.96	4.082/70.89	3.834	3.665
αMan(1 → 2)	3_{1B}	5.299/101.35	4.117/79.27	3.968	3.679
Manα	$3_{1A}\alpha$	5.376/93.35	3.947/79.98	3.710	3.804
Manβ	$3_{1A}\beta$	4.920/94.19	4.069	x	x
αMan(1 → 3)	3_{2C}	5.149/102.81	4.084/70.89	3.892/71.52	x
αMan(1 → 2)	3_{2B}	5.050/102.83	4.224/70.52	3.962/78.89	x
Manα	$3_{1A}\alpha$	5.371/93.35	3.944/78.89	x	x
Manβ	$3_{1A}\beta$	x	x	x	x
αMan(1 → 3)	3_{3C}	5.162/102.98	4.078/70.89	x	x
βMan(1 → 4)	3_{3B}	4.876/102.45	4.169/70.62	3.960/≈78.9	x
Galα	$3_{3A}\alpha$	5.291/93.12	3.856	3.932	4.062/79.44
Galβ	$3_{3A}\beta$	4.623/97.38	3.517	3.715	4.030/78.78
αMan(1 → 3)	4_{1D}	5.162/102.98	4.076/70.96	3.896/70.89	3.664
αMan(1 → 2)	4_{1C}	5.056/102.93	4.223/70.49	3.960/80.04	3.792
αMan(1 → 2)	4_{1B}	5.296/101.37	4.120/79.27	3.963/70.89	3.709
Manα	$4_{1A}\alpha$	5.369/93.40	3.945/80.04	3.759	x
Manβ	$4_{1A}\beta$	4.920/94.22	4.065/79.37	x	x
αMan(1 → 3)	5_{1D}	5.174/103.01	4.093/70.81	3.847	x
αMan(1 → 2)	5_{1C}	5.298/101.39	4.219/78.72	4.103/77.83	3.909
αMan(1 → 2)	5_{1B}	5.282/101.39	4.115/79.06	3.973	x
Manα	$5_{1A}\alpha$	5.372/93.32	3.952/79.97	3.800	x
Manβ	$5_{1A}\beta$	4.919/94.25	x	x	x
αGlc(1 → 2)	5_{1E}	5.208/101.21	3.550/72.48	3.755/74.05	3.418/70.47

x—not assigned, a—assignment may be interchanged, 21_B—compound 2_1, unit B.

(δ 3.960) and together with its downfield shifted C3 signal (δ 78.89) indicated the 3-linkage of terminal α-Man to internal β-Man residue in $\mathbf{3_3}$. Other oligosaccharides present in the mixture were not identified.

In the fraction dp **4** (Figure 5B, Table 4) α-D-Man-(1 → 3)-[α-D-Man-(1 → 2)]$_2$-D-Man ($\mathbf{4_1}$) was found as the main component. COSY and HSQC spectra suggest also the presence of a 2-linked α-D-Man tetramer in low amount ($\mathbf{4_2}$) (Scheme 2). The

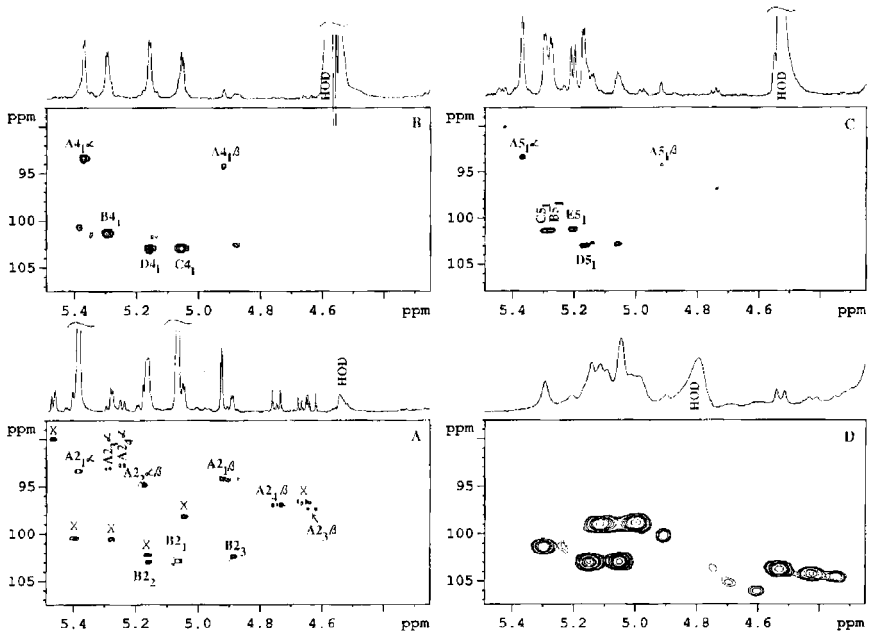

Figure 5. Anomeric region of HSQC spectra of native GalGXM (D) and oligosaccharide fractions isolated after polysaccharide hydrolysis: A-fraction **2**, X-not attributed, B-fraction **4**, C-fraction **5**, D-native GalGXM. The signal assignment corresponds to Table 4, $A2_1\alpha,\beta$-compound $\mathbf{2_1}$, sugar unit A (reducing ends α,β). Spectra A, B, C were recorded at 45°C, spectrum D at 25°C.

pentasaccharide $\mathbf{5_1}$ (Table 4) was found to be the main component in the dp **5** fraction, the presence of which was identified also in the GM polysaccharide.[9]

D C B A

α-D-Man-(1→3)-α-D-Man-(1→2)-α-D-Man-(1→2)-D-Man

2
↑
1
αGlc

E

$\mathbf{5_1}$

In the ^{1}H NMR as well as HSQC spectra of dp **5** fraction (Figure 5C) signals due to the branched pentasaccharide $\mathbf{5_1}$ were dominant. In comparison with the C unit of the linear tetrasaccharide $\mathbf{4_1}$ (Figure 5B), its 2,3-linked αMan H1 signal was downfield shifted to δ 5.298 due to the substitution with αGlc residue (Table 4). The type of linkages between individual sugar residues in $\mathbf{5_1}$ were estimated by NOE responses in 1D transient NOESY spectra (Table 5). Moreover, the low intensity cross peaks at δ 5.16/102.85 and 5.06/102.85 in the HSQC spectrum as well as cross peaks in the COSY spectrum suggest the presence of further oligosaccharides in the mixture with 3-linked and 2-linked αMan residues. In the COSY spectrum the H1 signal at δ 5.06 showed two H1/H2 cross peaks at δ 4.05 and 4.21. The former one

dp **2**$_1$ **αMan(1→2)Man**

2$_2$ αMan(1→3)Man

2$_3$ βMan(1→4)Gal

2$_4$ αMan(1→2)Glc

dp **3**$_1$ **αMan(1→2)αMan(1→2)Man**

3$_2$ **αMan(1→3)αMan(1→2)Man**

3$_3$ αMan(1→3) βMan(1→4)Gal

dp **4**$_1$ **αMan(1→3)αMan(1→2)αMan(1→2)Man**

4$_2$ αMan(1→2)αMan(1→2)αMan(1→2)Man

dp **5**$_1$ **αMan(1→3)αMan(1→2)αMan(1→2)Man**
2
↑
1
αGlc

5$_2$ αMan(1→3)αMan(1→2)αMan(1→2Man(1→2)αMan

5$_3$ αMan(1→2)αMan(1→3)αMan(1→2)αMan(1→2Man

5$_4$ αMan(1→2)αMan(1→2)αMan(1→3)αMan(1→2Man

5$_5$ αMan(1→2)αMan(1→2)αMan(1→2)αMan(1→2Man

Scheme 2.

indicated the presence of the terminal 2-linked α-Man unit, while the latter one could be due to internal 3-linked α-Man,[11] the location of which could not be fixed. Their structures might not be estimated precisely, however, they could be close to **5$_2$–5$_5$** (Scheme 2).

In the anomeric region of the HSQC spectrum of GalGXM (Figure 5D) the cross peaks H1/C1 of internal 2-linked α-D-Man units at δ 5.29/101.20, terminal 3-linked α-D-Man at δ 5.16/102.68, terminal 2-linked α-D-Man at δ 5.07/102.65 could be identified. According to the literature data, the signals at δ 5.11/98.86 correspond to 6-linked α-Man[12] of the backbone, and those at 5.00/98.71 to the 6-linked α-Gal residues.[13] The enzymatic treatment of GalGXM with α-galactosidase showed no effect on the polymer while β-galactosidase yielded a low amount of free galactose. This fact, together with the results of methylation analysis, indicates that besides β-Glc and β-Xyl, some of β-Gal residues also contribute to the branching of mannose side chains. Their H1/C1 cross peaks appeared in the HSQC spectrum at δ 4.75–4.3/103–106 (Figure 5D).

Table 5. NOE Responses in 1D Transient Gradient Enhanced NOE Experiments

Irradiated Signal	NOE Response
2_{1B}-H1	2_{1B}-H2, 2_{1A}-H2α, 2_{1A}-H2β
2_{2B}-H1, 2_{2A}-H1	2_{2B}-H2, 2_{2A}-H2α, 2_{2A}-H2β
2_{3B}-H1	2_{3B}-H2, 2_{3A}-H4β, 2_{3A}-H4α
2_{4B}-H1	2_{4B}-H2, 2_{4A}-H2β
3_{1C}-H1, 3_{2B}-H1	3_{1C}-H2, 3_{1B}-H2, 3_{1B}-H3, 3_{2B}-H2
3_{2C}-H1	3_{2C}-H2, 3_{2B}-H1
3_{3C}-H1, 3_{2A}-H1α	3_{3C}-H2, 3_{3B}-H2, 3_{2A}-H2, 3_{1A}-H2
3_{3B}-H1	3_{3B}-H2, 3_{3A}-H4β, 3_{3A}-H4α
5_{1E}-H1	5_{1C}-H2
5_{1D}-H1	5_{1C}-H3
5_{1B}-H1	5_{1A}-H2α
5_{1C}-H1	5_{1B}-H2

2_{1B}-H1—means compound 2_1, unit B, proton 1.

Compositional analysis of GalGXM showed ~70% of Man and ~18% of Gal residues as the main sugar components. For a capsular GalXM of *C. neoformans*[14] similar values were found (~80% of Man and ~9% of Gal) contrary to an acapsular GalXM produced by the *C. neoformans* mutant[15] (~30% of Man and ~50% of Gal). In addition, both GalXMs contained 10–20% of Xyl residues, no Glc residues, and differed mutually in the structure of the backbone. The backbone of the acapsular GalXM[15] was composed of 6-linked galactan backbone carrying side oligosaccharide chains composed of Man, Gal, and Xyl residues. Its ^{13}C NMR spectrum was very complex showing at least 16 signals in the anomeric region in comparison with that one of the capsular[14] GalXM of *C. neoformans* (only 4 signals). The capsular[14] GalXM ^{13}C NMR spectrum showed close similarity to the spectra of the yeast mannan,[16] glucomannan[9] (having 6-linked mannan backbone branched at C-2 by side oligosaccharide chains with 2- and 3-linked Man residues), and GalGXM. The similarity was found as well in linkage sugar analyses of GM and GalGXM. In GM,[9] 6-linked Man residues represent 37% of the total polysaccharide linkages from which 7% showed no branching and 30% were branched at the position C-2. In GalGXM, from 26% 6-linked Man residues 20% showed branching, however, mainly at position C-3. Data of the acapsular[15] GalXM showed only 2% of 6-linked Man and 29% of 6-linked Gal residues (18% branched at C-3). From this it follows that extracellular GalGXM of *C. laurentii* has a α-(1→6)-mannan backbone in contrast with acapsular[15] GalXM of *C. neoformans* which has a α-(1→6)-galactan backbone.

Acetolysis of GalGXM led to a depolymerization by splitting 6-linkages of the backbone and formation of oligomeric fractions with dp 2–6 due to side chains composed mainly of 2-linked Man. Although 6-linked Gal residues represented 16% of the total linkages in GalGXM, only ~5% of them showed branching. The oligosaccharides β-D-Man-(1→4)-D-Gal (**2_3**) and α-D-Man-(1→3)-β-D-Man-(1→4)-D-Gal (**3_3**) isolated after acetolysis of GalGXM might indicate that some Gal residues could be involved in the (1→6)-linked Man backbone.

The results of β-elimination reaction of GalGXM showed that Man was the only monosaccharide constituent released. This fact led to the conclusion that the protein part of the polymer is *O*-glycosylated mainly with mannose, mannobiose, and mannotetrose. The similar *O*-glycosylation of serine and threonine residues was observed in glucomannan protein[9] isolated from the same source.

In conclusion, the extracellular GalGXM isolated from *C. laurentii* var. *laurentii* showed some similarities with the capsular GalXM isolated from *C. neoformans*, however, both were different form the acapsular GalXM of *C. neoformans*. They differ from each other in the backbone monosaccharide composition, in the molar proportions of constituent monosaccharides, in the side chain lengths, in the degree and positon of monosaccharide residues branching, in protein content, as well as in molecular weigths. Moreover, *C. laurentii* GalGXM was the only glycoprotein which contained Glc residues as well and its protein part was more *O*-glycosylated by mannosyl residues.

EXPERIMENTAL

Culture and Isolation of Extracellular Polysaccharides

Cryptococcus laurentii CCY 17-3-5 from the Culture Collection of Yeast and Yeast-Like Microorganisms, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, was grown at 28°C in a semi-synthetic liquid medium containing per 1 L: 2 g glucose, 0.3 g $(NH_4)_2$ SO_4 0.1 g $MgSO_4$, 0.05 g KH_2PO_4, and 0.3 g yeast autolysate. After one week yeast cells were harvested by centrifugation (8,000 g, 20 min, 4°C). The extracellular polysaccharides were recovered by precipitation of the supernatant with 96% ethanol, dissolved in distilled water, dialyzed and freeze-dried. The mixture of polysaccharides was successively fractionated by CTABr and the resultant supernatant was precipitated with Fehlings solution to give two crude neutral polysaccharides, i.e., a glucomannan protein (GM, precipitate) and a galactoglucoxylomannan protein (GalGXM, supernatant) (Scheme 1). Size-exclusion chromatography of the crude GGXM on a column of Sepharose 2B afforded high (I) and low (II) molecular mass fractions. The major polysaccharide fraction II represented GalGXM.

Analytical Methods

Carbohydrates were detected by phenol-sulfuric acid assay.[17] Protein content was estimated by the method of Lowry using bovine serum albumin as a standard.[18] The average number molecular mass was determined osmometrically at 30°C, using a Knauer Vapour Pressure Osmometer and the relative molecular mass by HPLC chromatography on columns of HEMA BIO 100 and 1000, eluted with 0.1 M Tris-HCl buffer, pH 8.0. Free-boundary electrophoresis of a 1% solution of extracellular polymer was carried out in a Zeiss 35 apparatus, using 0.05 M sodium tetraborate buffer (pH 9.2) at 150 V/cm and 6 mA, for 30 min. The uronic acid content was determined spectrophotometrically with the 3-hydroxybiphenyl reagent.[19] The constituent monosaccharides of the extracelullar glycoproteins were identified after hydrolysis (2 M

trifluoroacetic acid, 2 h, 100°C) and reduction in the form of their trifluoroacetates[20] by gas chromatography on a Hewlett-Packard Model 5890 Series II instrument. It was equipped with a PAS 1701 column (0.32 mm × 25 m) at the temperature program of 110–125°C (2°C /min) – 165°C (20°C/min) and flow rate of nitrogen 20 mL/min. The absolute configuration of monosaccharide residues was determined by GLC of the trimethylsilylated (+)-2-butyl glycosides.[21] The amino acid composition was established with an automatic amino analyzer, type 6020 (Mikrotechna, Prague), after hydrolysis of the glycoprotein (6 M HCl, 20 h, 100°C).

MALDI Analysis of the Oligosaccharides

The MALDI mass spectrometric analysis of the oligosaccharides[22] was carried out on a Kompact MALDI III ToF (Shimadzu Kratos Analytical, Manchester, UK) equipped with a nitrogen laser ($\lambda = 337$ nm, 3 ns pulse width). Positive ions were accelerated from the target in the continuous mode to a final potential of 19 kV. All samples were measured in the reflectron mode. For each spectrum recorded, 50 single laser shots were accumulated. Calibration of the instrument was done externally with the$[M + Na^+]$ ions of standard oligosaccharides. Matrix solution was prepared by dissolving 25 mg of gentisic acid (2,5-dihydroxybenzoic acid) in 1 mL of acetonitrile/water (1/1, v/v). 1 μL mixture of sample : matrix solution (1:3) was placed in the center of one sample well of the stainless-steel 20-positions target. After air-drying of droplet analyte-matrix mixture was transferred into the mass spectrometer.

β-Elimination Reaction

Glycoprotein was treated with 0.1 M NaOH in the presence of 0.8 M $NaBH_4$ at 37°C for 72 h. The sample was neutralized and precipitated with 96% ethanol. Precipitated polymeric residue was removed by centrifugation and analyzed for amino acid composition (Table 1). Non-precipitated, low molecular-weight portion was analyzed for monosaccharide composition. Thin-layer chromatography (TLC) was carried out on Kieselgel 60 in the solvent system S1, 1-butanol-formic acid-water 2:3:1.

Acetolysis of the GalGXM

The native polysaccharide was acetylated by the Kocourek and Ballou[23] method. The mixture of deacetylated products was fractionated on a column (2.5 × 200 cm) of Bio-Gel P-2 by water elution. Fractions of 4 mL were collected and analyzed for the carbohydrate content by phenol-sulfuric acid assay.[17]

Glycosidase Treatment

GalGXM (5 mg) was dissolved in 100 mM citrate-phosphate buffer (2 mL, pH 4.8) and treated with α-galactosidases from *Cryptococcus laurentii* (0.45 U/mL) or

Thermomyces lanuginosus (2 U/mL) at 37°C and 50°C, respectively, for 24 hours. The other portion (5 mg) of GalGXM was dissolved in 100 mM phosphate buffer (2 mL, pH 7.2) or in 100 mM citrate-phosphate buffer (2 mL, pH 4.5) and treated with β-galactosidases from *Sacharomyces fragilis* (Sigma, 4 U/mL) or *Aspergilus oryzae* (Sigma, 4U/mL) at 37°C and 30°C, respectively, for 24 hours. The incubation mixtures were analyzed by TLC on Silica gel 60 (Merck) in the system S2, ethyl acetate-pyridine-water 8:2:1 and the saccharides were detected by spraying the plates with 20% ammonium sulfate and heating.

Methylation Analysis

Dry samples of oligo-and polysaccharides ($\sim$1 mg) were solubilized in dry dimethyl sulfoxide (1 mL) and methylated by the Hakomori method.[24] Methylated products were isolated by partition with dichloromethane, concentrated and hydrolyzed with 2 M trifluoroacetic acid (1 h, 120°C). Partially methylated monosaccharides were reduced with sodium borodeuteride, acetylated and analyzed by gas chromatography-mass spectrometry. GLC-MS of partially methylated alditol acetates[25] was effected on a FINNIGAN MAT SSQ 710 spectrometer equipped with an SP 2330 column (0.25 mm $\times$ 30 m) at 80–240°C (6°C/min), 70 eV, 200 μA, and ion-source temperature 150°C.

NMR Spectroscopy

^{1}H and ^{13}C NMR spectra were acquired at 25°C, 45°C or 60°C in D_2O on 300 MHz Avance DPX Bruker spectrometer, equipped with a selective excitation unit and a kit for gradient enhanced spectroscopy, in 5 mm ^{1}H, ^{13}C, ^{31}P, ^{15}N QNP and 5 mm multinuclear probe with z-gradients and inverse mode of detection. Chemical shifts are referenced to internal acetone with δ 2.225 in ^{1}H and δ 31.07 in ^{13}C NMR spectra. Connectivity from anomeric signals to other protons in each residue were found on the basis of 2D COSY, relayed COSY, TOCSY. Types of glycosidic linkages were found on the basis of 2D NOESY and 1D gradient enhanced transient NOESY[26] experiments using Gaussian pulses for selective excitation. 2D hetero-nuclear ^{1}H-^{13}C HSQC[27] with GARP decoupling was used.

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REFERENCES

1. White, C.W.; Jacobson, E.S. Manosyl transfer in *Cryptococcus neoformans*. Can. J. Microbiol. **1992**, *39*, 129–133.

2. Battacharjee, A.K.; Bennett, J.E.; Glaudemans, C.P.J. Capsular polysaccharides of *Cryptococcus neoformans*. Rev. Infect. Dis. **1984**, *6*, 619–624.
3. Cherniak, R. Soluble Polysaccharides of *Cryptococcus neoformans*. In *Current Topics in Medical Mycology*; McGuinnes, M.R., Ed.; Springer-Verlag: New York, 1988; 40–54.
4. Stoodley, R.J. Thesis; Queens University: Kingston, Ontario, Canada, 1959.
5. Abercrombie, M.J.; Jones, J.K.N.; Lock, M.V.; Perry, M.B.; Stoodley, R.J. The polysaccharides of *Cryptococcus laurentii* (NRRL Y-1401). Can. J. Chem. **1960**, *38*, 1617–1624.
6. Foda, M.S.A.; Badr-Eldijn, S.M.; Phaff, H.J. Biochemical investigation on the capsule-amylose relation in *Cryptococcus laurentii*. Mycologia **1973**, *65*, 365–372.
7. Perry, M.B.; Webb, A.C. Structure of the acidic capsular polysaccharide of *Cryptococcus laurentii* (NRRL Y-1401). Can. J. Biochem. **1982**, *60*, 124–130.
8. Bartek, P.; Kolarova, N.; Capek, P. Isolation and characterization of glycoproteins from the yeast *Cryptococcus laurentii* var. *laurentii*. II. Extracellular glycoproteins. Chem. Pap. **2001**, *55*, 261–268.
9. Kolarova, N.; Matulová, M.; Capek, P. Structure of glucomannan-protein from the yeast *Cryptococcus laurentii* var. *laurentii*. J. Carbohydr. Chem. **1997**, *16*, 609–623.
10. Shibata, N.; Fukasawa, S.; Kobayashi, H.; Tojo, M.; Yonezu, T.; Ambo, A.; Ohkubo, Y.; Suzuki, A. Structural analysis of phospho-D-mannan-protein complexes isolated from yeast and mold cells of *Candida albicans* NIH A-207 serotype A strain. Carbohydr. Res. **1989**, *187*, 239–253.
11. Shibata, N.; Kojima, Ch.; Satoh, Y.; Satoh, R.; Suzuki, A.; Kobayashi, H.; Suzuki, S. Structural study of cell-wall mannan of *Saccharomyces kluyveri* IFO 1685 strain. Eur. J. Biochem. **1993**, *217*, 1–12.
12. Kobayashi, H.; Watanabe, M.; Komido, M.; Matsuda, K.; Ikeda-Hasebe, T.; Suzuki, M.; Shibata, N.; Hisamichi, K.; Suzuki, S. Assignment of ^{1}H and ^{13}C NMR chemical shifts of a D-mannan composed of α-(1 → 2) and α-(1 → 6) linkages obtained from *Candida kefyr* IFO 0586 strain. Carbohydr. Res. **1995**, *267*, 299–306.
13. Bock, K.; Pedersen, Ch.; Pedersen, H. Carbon-13 nuclear magnetic resonance data for oligosaccharides. Adv. Carbohydr. Chem. Biochem. **1984**, *42*, 193–225.
14. Turner, S.H.; Cherniak, R.; Reiss, E. Fractionation and characterization of galactoxylomannan from *Cryptococcus neoformans*. Carbohydr. Res. **1994**, *125*, 343–349.
15. Vaishnav, V.V.; Bacon, B.E.; O'Neill, M.; Cherniak, R. Structural characterization of the galactoxylomannan of *Cryptococcus neoformans* Cap 67. Carbohydr. Res. **1998**, *306*, 315–330.
16. Gorin, P.A.J. Adv. Carbohydr. Chem. Biochem. **1981**, *38*, 13–104.
17. Dubois, M.; Gilles, K.A.; Hamilton, K.J.; Rebers, P.A.; Smith, E. Colorimetric method for determination of sugars and related substances. Anal. Biochem. **1959**, *28*, 350–356.
18. Lowry, O.H.; Rosenbrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the folin phenol reagent. J. Biol. Chem. **1951**, *193*, 265–275.
19. Blumenkrantz, N.; Asboe-Hansen, O. New method for quantitative determination of uronic acid. Anal. Biochem. **1973**, *54*, 484–489.
20. Shapira, J. Identification of sugars as their trifluoroacetyl polyol derivatives. Nature **1969**, *222*, 792–793.

21. Gerwik, G.J.; Kamerling, J.P.; Vliegenthart, J.F.G. Determination of the absolute configuration of monosaccharides in complex carbohydrates by capilary g.l.c.. Carbohydr. Res. **1979**, *77*, 1–8.
22. Harvey, D.J. Matrix-assisted laser desorption/ionization mass spectroscopy of carbohydrates. Mass Spectrosc. Rev. **1999**, *18*, 349–451.
23. Kocourek, J.; Ballou, C.E. Method for finger printing yeast cell wall mannan. J. Bacteriol. **1969**, *100*, 1175–1181.
24. Hakomori, S.J. A rapid permethylation of glycolipid and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. Biochem. (Tokyo) **1964**, *55*, 205–208.
25. Jansson, P.E.; Kenne, L.; Liedgren, H.; Lindberg, B.; Lönngren, J. A practical guide to the methylation analysis of carbohydrates. Chem. Commun. (Univ. Stockholm) **1976**, *8*, 1–75.
26. Kessler, H.; Oschkinat, H.; Griesinger, C. Transformation of homonuclear two-dimensional NMR technique into one-dimensional techniques using Gaussian pulses. J. Magn. Reson. **1986**, *70*, 106–133.
27. Schleucher, J.; Schwendinger, M.; Sattler, M.; Schedletzky, O.; Glaser, S.J.; Sorensen, O.W.; Griesinger, C. A general enhancement scheme in heteronuclear multidimensional NMR employing pulsed field gradients. J. Biomol. NMR **1994**, *4*, 301–306.

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