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STRUCTURAL STUDIES ON A GLYCOPEPTIDE FROM THE TREE FUNGUS *Cyttaria harioti* FISCHER*

ELISA M. OLIVA, ALICIA FERNANDEZ CIRELLI†, AND ROSA M DE LEDERKREMER†
Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón II, 1428 Buenos Aires (Argentina)
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ABSTRACT

A glycopeptide (In_1) was isolated by phenol–water extraction from *Cyttaria harioti* Fischer, parasite of *Nothofagus* sps. Neutral sugars account for 89% of In_1 and were characterized as glucose, mannose, and galactose. Glucosamine, identified by g.l.c., was colorimetrically estimated (5.8%). The molar ratio of Glc:Man:Gal:GlcNAc was 17:11:3:2. The linkages between the various monosaccharide residues were established through methylation analysis and periodate oxidation studies. The anomeric configurations of the various glycosyl groups were determined by chromium trioxide oxidation of the acetylated polysaccharide. The results were confirmed by ^{13}C-n.m.r. spectroscopy. The sugar chain is *N*-glycosyl-linked to the peptide. Structural features of the carbohydrate moiety of glycopeptide In_1 are described.

INTRODUCTION

In previous papers[1–4], we reported the isolation of three polysaccharides from *Cyttaria harioti* Fischer, an Ascomycete parasite of *Nothofagus* sps., on which the fungi produce characteristic tumors that infect the tree and finally cause its death. Water extraction and fractional precipitation with ethanol gave[1,2] a heteropolysaccharide composed of D-glucose, D-*arabino*-hexulosonic acid, and D-fructose in the molar ratio of 98:6:1, and an α-D-glucan structurally related to pullulan[4]. The major component was a water-insoluble (1→3)-β-D-glucan, substituted at O-6 of every second or third residue of the backbone[3]. We report herein structural studies on the carbohydrate moiety of a glycopeptide isolated by phenol–water extraction of dried stroma.

RESULTS AND DISCUSSION

Isolation and purification of the glycopeptide (In_1) from *Cyttaria harioti*

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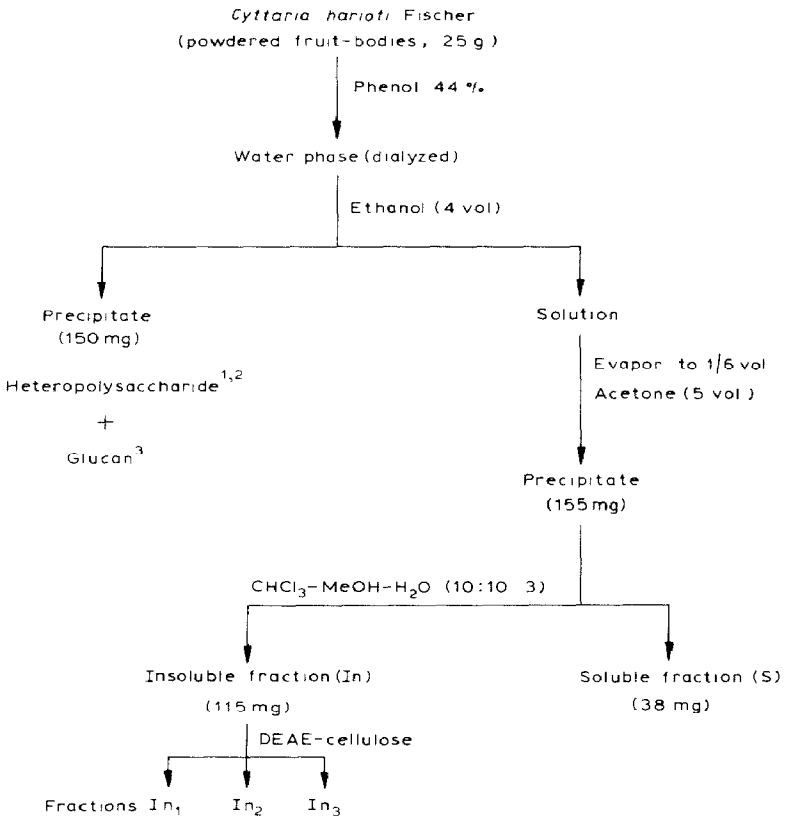


Scheme 1. Isolation and purification of glycopeptide In_1 from *Cyttaria hariotii* Fischer.

Fischer is shown in Scheme 1. DEAE-cellulose column chromatography (Fig. 1) afforded two carbohydrate-containing fractions (In_1 and In_2) and a protein (In_3).

The main fraction (In_1) representing ~90% of the carbohydrate originally present in In, proved to be homogeneous by gel filtration on Bio-Gel P-100 and Sepharose-4 B, respectively, had a mol. wt. estimated at ~6000 by comparison with standard dextrans (Fig. 2); $[\alpha]_D^{20}$ +82.0° (*c* 0.5, water). Neutral sugars accounted for 89% of the material (phenol–sulfuric acid method[5]), and hexosamines for 5.8% (Elson–Morgan[6]). The absorbance at 280 nm suggested the presence of amino acids, which were determined by the method of Lowry *et al.*[7] (4.3%). Neither phosphorus (Bartlett method[8]) nor lipid was present in contrast to the minor Fraction In_2 of lower carbohydrate content[9] (25%, phenol–sulfuric acid method[5]).

In Fraction In_1, neutral sugars were characterized by g.l.c. of the corresponding alditol acetates as glucose, mannose, and galactose. D-Glucose accounted for 52.5% as determined by the D-glucose oxidase method[10]. The hexosamine, liberated by hydrochloric acid hydrolysis, was identified as 2-amino-2-deoxyglucose by g.l.c. of its acetylated anhydrohexitol[11] and its trimethylsilyl derivative[12], respec-

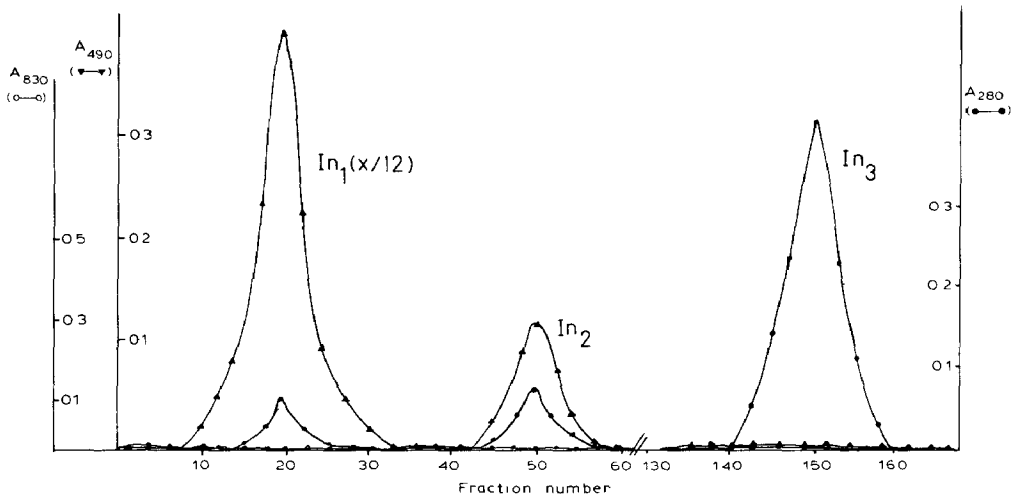


Fig. 1. DEAE-cellulose column chromatography of Fraction In.

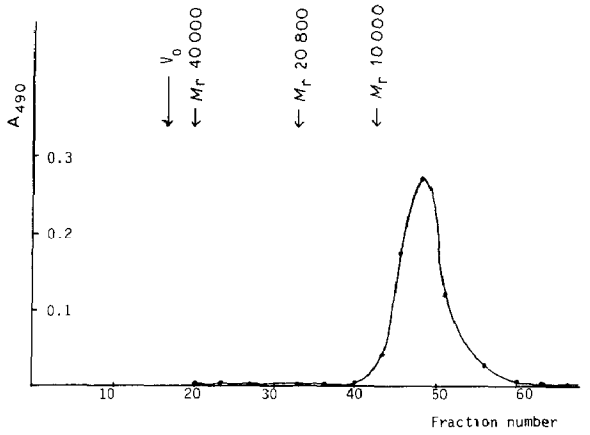


Fig. 2. Determination of the molecular weight of glycopeptide In_1 on a column of Bio-Gel P-100.

tively. The molar ratio of Glc:Man:Gal:GlcNAc was estimated as 17:11:3:2. The amino acid composition is shown in Table I.

On periodate oxidation, the glycopeptide consumed 1.13 mol of periodate/hexose residue with production of 0.15 mol of formic acid. Borohydride reduction of the polyaldehyde, followed by total acid hydrolysis afforded glycerol, erythritol, threitol, mannose, and glucose (molar proportions 86:74:18:9:13) characterized by g.l.c.–m.s. of the corresponding alditol acetates. The amount of threitol (9/100 mol) indicated that all of the galactosyl residues are linked through O-4. The formic acid produced by periodate oxidation and the percentage of glycerol liberated by hydrolysis of the polyalcohol suggested the presence of (1→2)-linked hexose residues.

TABLE I

AMINO ACID COMPOSITION OF GLYCOPEPTIDE In_1

Amino acid	%
Alanine	14.96
Aspartic acid	7.26
Glutamic acid	12.77
Glycine	19 51
Leucine	8.25
Serine	19.25
Threonine	8.57
Tyrosine	9.38

TABLE II

METHYLATION ANALYSIS OF GLYCOPEPTIDE In_1

O-Methyl sugars[a]	*Ret time*[b]		*Molar proport.*[c]	*Primary mass fragments* (m/z)	*Mode of linkage*
	t_1	t_2			
2,3,4,6-Me-Glc	1.00	1.00	12.2	45,117,161,205	Glc*p*-(1→
2,3,4,6-Me-Man	1.00	1.00	1.5	45,117,161,205	Man*p*-(1→
3,4,6-Me-Man	1.94	1.60	28.4	45,161,189	→2)-Man*p*-(1→
2,3,4-Me-Glc	2.67	2.00	6.4	117,161,189,233	→6)-Glc*p*-(1→
2,3,6-Me-Gal	2.67	2.00	10.0	45,117,233	→4)-Gal*p*-(1→
2,3,6-Me-Glc	2 67	1.89	27 8	45,117,233	→4)-Glc*p*-(1→
2,4-Me-Glc	5.42	3.56	8.0	117,189,233	→3,6)-Glc*p*-(1→
2,4-Me-Man	5.42	3 56	5.6	117,189,233	→3,6)-Man*p*-(1→

[a]Determined as alditol acetates. [b]Relative retention-time with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol; t_1, column I; t_2, column II. [c]Relative to 100 mol.

The glycopeptide was methylated by the Hakomori method[13] and hydrolyzed with sulfuric acid. The substitution pattern was determined by g.l.c.–m.s. of the corresponding alditol acetates[14]. Further characterization of the methylated hexoses was achieved by comparison with authentic samples and demethylation studies. The results are shown in Table II. 2,3,4,6-Tetra-*O*-methylglucose and 2,3,4,6-tetra-*O*-methylmannose could not be resolved by g.l.c. of the corresponding alditol acetates under the conditions used. The tetramethylhexose fraction was separated by t.l.c. and, on demethylation, afforded glucose and mannose in the molar proportion of 8:1, as shown by g.l.c. 2,3,6-Tri-*O*-methylglucose and 2,3,4-tri-*O*-methylglucose were resolved by t.l.c. and identified by their characteristic prominent ions at *m/z* 233 and 189, respectively, indicating (1→4) and (1→6) linkages for this sugar. The mannosyl residues were shown to be linked at O-2 by the identification of 3,4,6-tri-*O*-methylmannose. As 2,3,6-tri-*O*-methylgalactose could not be resolved from 2,3,4-tri-*O*-methylglucose by chromatography in two different

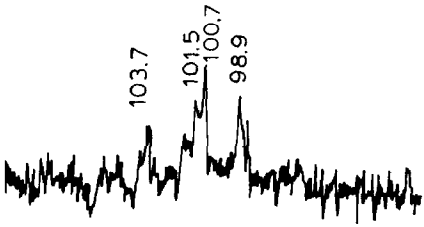


Fig. 3. ^{13}C-N.m.r. spectrum (anomeric region) of glycopeptide In_1.

columns, the molar proportion of the former compound was calculated considering that all the galactose residues were (1→4)-linked, as suggested by the amount of threitol determined after periodate oxidation. The di-*O*-methylhexoses gave a broad peak on g.l.c. but, on demethylation, afforded glucose and mannose in the molar ratio of 3:2. The characterization of 2,4-di-*O*-methylmannose and 2,4-di-*O*-methylglucose indicated the presence of both sugars as branch points and is consistent with the presence of unoxidized mannose and glucose after periodate oxidation.

In order to determine the anomeric configuration of the glycosyl residues, acetylated glycopeptide In_1 was oxidized with chromium trioxide[15,16] in the presence of *myo*-inositol as the internal standard. After 2 h, half of the glucosyl and mannosyl units had been oxidized, while galactose remained unaltered, which suggested both α-D and β-D linkages for the first two sugars, and only α-D linkages for the last one. Resistance to digestion of Fraction In_1 with almond emulsin suggested the α configuration for the D-glucosyl residues in terminal position.

The ^{13}C-n.m.r. spectrum of glycopeptide In_1 (Fig. 3) showed several signals in the anomeric region indicating a complex structure. A main absorption at δ 100.7 would correspond to α-(1→4)-linked D-glucose units[17]. The C-1 signal for 4-*O*-linked α-D-galactopyranosyl residues should appear[18] at δ 100.35 and was not resolved. At δ 101.5, the resonance of 2-*O*-linked α-D-mannopyranose units was observed[19]. At lower field, a signal at δ 103.7 may be due to 3,6-di-*O*-substituted β-D-glucosyl residues[20]. At higher fields in the anomeric region, the signal at δ 98.9 was assigned to 6-*O*-linked α-D-glucopyranosyl residues[21].

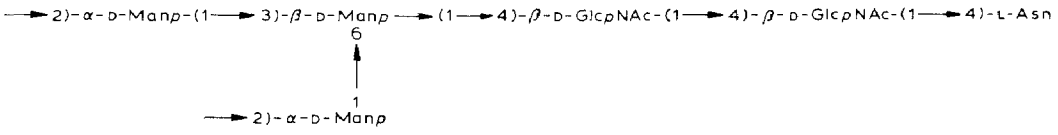
Partial acetolysis of fraction In_1, followed by deacetylation gave a mixture of oligosaccharides that was separated by gel filtration into an excluded and several included fractions. The fraction of lowest mol.-wt. range consisted of oligosaccharides up to d.p. 6, as shown by l.c., and was separated by preparative t.l.c. The fractions corresponding to d.p. 3, 4, 5, and 6 were hydrolyzed and analyzed by g.l.c. of the corresponding alditol acetates. The following molar glucose-to-mannose ratios were found: d.p. 3 (2:1), 4 (1:1), 5 (1:0), and 6 (5:1). The observed ratios for the higher mol.-wt. fractions suggest that the D-glucose units are linked to each other. Maltose was characterized as the main disaccharide by paper chromatography and g.l.c. of the trimethylsilyl derivative, whereas cellobiose could

not be detected, which confirmed that the (1→4)-D-glucosyl linkages are mainly in the α configuration.

After treatment of glycopeptide In_1 under the conditions of β elimination, gel filtration showed no change of the mol. wt. by comparison with the untreated glycopeptide. This observation and the unaltered amino acid composition, suggested the absence of *O*-glycosyl linkages to the peptide residue.

2-Acetamido-1-*N*-L-(aspart-4-*O*-yl)-2-deoxy-β-D-glucopyranosylamine was characterized, by its chromatographic properties[22], among the products of hydrolysis of the Pronase-digested glycopeptide. Isolation of this compound by paper electrophoresis, followed by acid hydrolysis, and further reduction with sodium borotritide gave 2-amino-2-deoxy-D-[1-^{3}H]glucitol. This result indicated an *N*-glycosyl linkage between the carbohydrate and peptide residues.

The proportion of 2-acetamido-2-deoxyglucose agrees with the presence of two units of this amino sugar per mole of glycopeptide, taking into account the mol. wt. determined by gel filtration. The identification of 3,6-disubstituted and (1→2)-linked mannosyl residues suggests the existence of the core **1** that constitutes the invariant fraction of *N*-glycosylpeptides[23]. Other mannosyl residues are linked to this core and glucosyl and galactosyl units may reside in the outer region of the carbohydrate chains. It is of interest that the D-glucose residues are mainly present



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in the glycopeptide chain in α-D-(1→4) linkage, as in the α-D-glucan isolated from the same fungus. D-Glucose is normally not a component of glycoproteins of animal origin or higher plants. Mannose, glucose, galactose, and 2-amino-2-deoxy-D-glucose have been determined[24] as constituents of glycoproteins, isolated from *Aspergillus* and *Saccharomyces* sps., having variable carbohydrate contents (10–60%). Few structural studies have been reported on fungal glycopeptides to be compared with our results.

EXPERIMENTAL

General. — The ^{13}C-n.m.r. spectrum was recorded at 25.2 MHz with a Varian-XL-100-15 spectrometer operating in the F.t. mode for a solution in 3:1 H_2O–D_2O (40 mg/4 mL) in a 12-mm tube, at room temperature. The spectral width was 5120 Hz, the pulse angle 45°, the acquisition time 0.8 s, the pulse width 40 μs, the number of data points 8192, and the number of transients 270 000. 1,4-Dioxane was used as external standard (67.4 p.p.m. downfield from the signal of Me_4Si).

Paper chromatography was conducted by the descending method on What-

man No. 1 paper, with the following solvent systems: (*A*) 6:4:3 1-butanol–pyridine–water; (*B*) 4:3:4 1-butanol–pyridine–water; and (*C*) 4:1 phenol–water. Detection was effected with (*a*) silver nitrate–sodium hydroxide[25]; (*b*) *p*-anisidine hydrochloride[26]; and (*c*) 1% ninhydrin in acetone containing 2% pyridine[22]. T.l.c. was carried out on BC-Alufolien Kieselgel 60 (Merck) plates in (*D*) 7:1:2 1-propanol–ethanol–water; (*E*) 600:150:9:4.5 acetone–benzene–water–NH_3; and (*F*) 70:15:15:2 butanone–pyridine–water–acetic acid; spots were detected with reagent (*c*) or 5% ethanolic H_2SO_4.

G.l.c. was performed with a Hewlett–Packard 5830 gas chromatograph equipped with a flame-ionization detector, on glass columns packed with: (I) 3% ECNSS-M on Gas Chrom Q (0.2 × 180 cm); (II) 10% NPGS on Chromosorb W AW-DMCS (60–80) (0.2 × 180 cm); and (III) 2% OV-101 on Chromosorb W AW DMCS (60–80) (0.2 × 120 cm). G.l.c.–m.s. was performed with a 3% ECNSS-M column in a Varian 1440 chromatograph coupled to a Varian-MAT CH-7 A mass spectrometer controlled by a Varian MAT 166 data system. L.c. was performed on a Hewlett–Packard 1084 B liquid chromatograph equipped with a differential refractometer. A Beckman 119 CL apparatus was used for amino acid automatic analysis.

All evaporations were performed <40° under diminished pressure. Small volumes of aqueous solutions were lyophilized.

Material. — Fruit bodies of *Cyttaria harioti* Fischer were collected in San Martin de los Andes (Neuquén, Argentina) in the spring.

Isolation and purification of the glycopeptide. — Powdered, dried fruit bodies of *Cyttaria harioti* Fischer (25 g) were extracted with 44% phenol at room temperature. The aqueous phase was dialyzed and ethanol (4 vol.) was added. After centrifugation, the solution was concentrated to 100 mL. Upon addition of acetone (500 mL), the yellowish precipitate (0.16 g) was centrifuged and partitioned with 10:10:3 chloroform–methanol–water into a soluble and an insoluble fraction In (0.12 g; 0.5%). Fraction In (46 mg) was chromatographed on a DEAE-cellulose column (32 × 2 cm) eluted with Tris·HCl buffer (pH 8.6; 60 mL) and increasing concentrations of NaCl in the same buffer (0.1, 0.2, 0.3, and 0.5M; 50 mL each), and finally with 0.1M NaOH. Fractions (2 mL) were collected and absorbance at 280 nm was determined. The fractions were analyzed for phosphorus[8] and carbohydrates[5] (see Fig. 1); those corresponding to the major peak (In_1) were desalted and lyophilized.

Fraction In_1 was subjected to gel permeation on columns of: (*a*) Sepharose 4B (1.2 × 100 cm) in phosphate buffer, pH 7.2, 0.1% $NaDodSO_4$, and (*b*) Bio-Gel P-100 (2 × 90 cm) in 0.05M Tris·HCl buffer (pH 8.6). Column (*b*) was previously calibrated with standard dextrans (Fig. 2).

Acid hydrolysis. — (*a*) *Neutral sugars.* A solution of Fraction In_1 (5 mg) in 0.5M H_2SO_4 (1 mL) was heated in a sealed tube for 16 h at 100°. The hydrolyzate was made neutral with $BaCO_3$, filtered, and decationized by stirring with Dowex 50 W (H^+). Paper chromatography (solvent *A*; reagents *a*, *b*) showed the presence of

glucose, mannose, and galactose, which were characterized and estimated by g.l.c. of the corresponding alditol acetates[27] (column *I*, T_i 200°, T_d 210°, T_c 190°; flow 24 mL of N_2/min); the molar ratio of glucose:mannose:galactose was 11:7:2. D-Glucose was also estimated by the D-glucose oxidase method[10].

(*b*) *Amino sugars.* They were determined by a modification of the Elson–Morgan method[6] after acid hydrolysis of Fraction In_1 (5 mg; 4M HCl, 6 h, 100°) and removal of interfering impurities according to the procedure of Boas[28]. The identification of the amino sugars was performed in two ways: (a) g.l.c. of the anhydrohexoses obtained by deamination, after reduction and acetylation[11] (column *I*, T_i 200°, T_d 210°, T_c 185°; flow 27 mL of N_2/min); (b) g.l.c. of the trimethylsilyl ethers[12] (column *III*, T_i 200°, T_d 200°, T_c 150°; flow 28 mL of N_2/min).

(*c*) *Amino acids.* A sample of the glycopeptide was hydrolyzed as recommended[29] to avoid interference by neutral sugars. Analysis was conducted after an hydrolysis of 24 h with 6M HCl at 100–105° under nitrogen.

Periodate oxidation. — Fraction In_1 (9.8 mg) was dissolved in 50mM $NaIO_4$ (10 mL). Oxidation was allowed to proceed at room temperature, in the dark, with occasional shaking. At intervals, the periodate uptake was determined by the arsenite method[30] and, when the oxidation was complete, the released formic acid was estimated by titration with 10mM NaOH. The excess of oxidant was decomposed with 1,2-ethanediol. After extraction with 1-butanol, the aqueous phase was reduced with $NaBH_4$. The solution was passed through a column of Amberlite MB-3 resin and freeze-dried. The polyalcohol was hydrolyzed with 2M HCl for 3 h at 100° under nitrogen, and the products were analyzed by g.l.c.–m.s. of the alditol acetates (column I, T_i 210°, T_d 210°, T_c programmed from 120 to 175°, 3°/min, with t_i 5 min; flow 24.5 mL of N_2/min).

Methylation analysis. — The glycopeptide In_1 (8 mg) was methylated by the Hakomori method[13]. The fully methylated product was hydrolyzed with 72% H_2SO_4 (0.5 mL, 2 h, 25°) and then (4 h) at reflux with 12% H_2SO_4. An aliquot of the partially methylated sugars was converted into the corresponding alditol acetates[27], and these were analyzed by g.l.c. (column I, T_i 210°, T_d 220°, T_c 155°; flow 22 mL of N_2/min; column II, T_i 280°, T_d 225°, T_c 205°; flow 29 mL of N_2/min) and g.l.c.–m.s. (column I, T_i 220°, T_c programmed from 100 to 210°, 6°/min; flow 25 mL of He/min). The results are shown in Table II. The remaining, partially methylated aldoses were separated by t.l.c. (solvent *E*). Individual fractions were demethylated[31] with BBr_3 and analyzed as alditol acetates by g.l.c.

Chromium trioxide oxidation. — A mixture of Fraction In_1 (3.2 mg) and *myo*-inositol (0.7 mg) as the internal standard was dissolved in formamide (0.5 mL) and acetic anhydride (1 mL) and pyridine (1.5 mL) were added. After being stirred for 15 h at room temperature, the solution was evaporated and the residue partitioned between water and chloroform. The organic layer was concentrated and reacetylated in the same way. The acetylation product was dissolved in glacial acetic acid (4.5 mL) and the solution kept at 50°. Chromium trioxide (300 mg) was added and aliquots were removed at intervals, immediately diluted with water (25 mL) to stop

the oxidation, and extracted with chloroform. The dried extracts were deacetylated with sodium methoxide, hydrolyzed, and analyzed as alditol acetates by g.l.c. (column I, T_i 210°, T_d 210°, T_c 185°; flow 25 mL of N_2/min). After 2 h, galactose was not oxidized whereas mannose and glucose were recovered in 50 and 48% yield, respectively.

Enzymic hydrolysis. — The glycopeptide (2 μmol) was incubated with almond emulsin (Worthington Biochemicals Co.) in 0.1M sodium acetate (pH 4.6) for 3 h at 37°. Release of free D-glucose was estimated every hour by the D-glucose oxidase–peroxidase method[10]

Partial acetolysis. — Fraction In_1 (7 mg) was acetolyzed[32] with 10:10:1 acetic acid–acetic anhydride–H_2SO_4 for 65 h. The resulting mixture was deacetylated with sodium methoxide. The oligosaccharides, up to d.p. 6 (Fraction A), were separated from those having a higher mol. wt. on a Bio-Gel P-2 column (1.5 × 100 cm) which had previously been calibrated with maltooligosaccharides. Fraction A was analyzed by l.c. on a LiChrosorb NH_2 column (10 nm; Merck) with 1:1 acetonitrile–water (1.0 mL/s) as the elution solvent, and the retention times were compared with those of known maltooligosaccharides. Preparative t.l.c. of Fraction A (solvent *D*) gave six fractions. Those having d.p. 3, 4, 5, and 6, respectively, were hydrolyzed with 0.5M H_2SO_4 (16 h, 100°) and analyzed by g.l.c. of the corresponding alditol acetates. The disaccharide fraction was reduced, pertrimethylsilylated[33], and analyzed by g.l.c. (column *III*, T_i 300°, T_d 300°, T_c programmed from 200 to 280°, 4°/min, flow 25 mL of N_2/min; t_r 5.83 min).

Carbohydrate–peptide linkages. — (*a*) *O-Glycosyl linkages.* The glycopeptide In_1 (5.4 mg) was treated with 0.1M NaOH–0.2M $NaBH_4$ (2 mL) for 16 h at 35°. The solution was made neutral with acetic acid and passed through a column (1.8 × 70 cm) of Sephacryl S-200 in 0.3M pyridinium acetate buffer (pH 6.1). The eluted material was hydrolyzed for the determination of amino acids, and these were identified and estimated according to the method already described. A sample of untreated glycopeptide was subjected to gel permeation under the same conditions.

(*b*) *N-Glycosyl linkages.* The glycopeptide In_1 (10 mg) was added to a solution of Pronase (*S. griseus;* 100 μg, Sigma Chemical Co.) in 15mM $CaCl_2$ (0.4 mL; pH 8.5) and the mixture incubated for 24 h at 37°, under toluene. The aqueous phase was made 0.1M in acetic acid and subjected to gel filtration on a column (70 × 2 cm) of Sephadex G-25 with the same solvent. Fractions corresponding to the digested glycopeptide (assayed by the phenol–sulfuric acid procedure) were excluded. After freeze-drying, the residue was hydrolyzed[22] with 2M HCl (2 mL) for 20 min at 100°. 2-Acetamido-1-*N*-(L-aspart-4-oyl)-2-deoxy-D-glucopyranosylamine was characterized among the products by p.c. (solvent *C*, reagent *c*). The compound was isolated by preparative paper electrophoresis at pH 3.7 in 1:10:289 (v/v) pyridine–acetic acid–water and 18 V/cm during 1.5 h. The purified material was treated with 2M HCl for 3 h at 100°, evaporated, and reduced with NaB^3H_4 in 0.2M NH_4OH, overnight at room temperature. After neutralization with acetic acid and removal of boric acid as methyl borate, the radioactive mixture was analyzed

by paper electrophoresis under the described conditions together with an authentic sample of 2-amino-2-deoxy-D-glucitol. The electrophoretogram was radioscanned with a Hewlett–Packard 7201 scanner.

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