Carbohydrate Research, 86 (1980) 69–76
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

STRUCTURAL INVESTIGATIONS OF TWO CAPSULAR POLYSACCHARIDES FROM *Cryptococcus neoformans*

EDWIN H. MERRIFIELD AND ALISTAIR M. STEPHEN

C.S.I.R. Carbohydrate Research Unit, Department of Organic Chemistry, University of Cape Town (South Africa)

(Received December 3rd, 1979; accepted for publication, February 26th, 1980)

ABSTRACT

Two capsular polysaccharides from *Cryptococcus neoformans* serotype A have been shown to be chemically equivalent. One of these polysaccharides was further investigated and shown to consist of a chain of (1→3)-linked D-mannosyl residues, each of which is substituted at O-2 by a D-glucosyluronic acid or D-xylosyl group.

INTRODUCTION

Fungal strains of the genus *Cryptococcus* all produce capsules, some of which are much thicker than the enclosed cell. Approximately twelve species have been identified on the basis of antigenic and morphological characteristics[1], but only one species, *C. neoformans*, is pathogenic in man. *C. neoformans* possesses an unusual affinity for the central nervous system, presumably due to the presence of vitamins and metabolites in this system that satisfy the nutritional requirements of the organism[2]. The capsule of *C. neoformans*, its most distinctive feature, is composed of a polysaccharide containing mainly glucuronic acid, mannose, and xylose residues[1].

In the present study, two strains (R and H) of *C. neoformans* were isolated at Groote Schuur Hospital, Cape Town, from the cerebrospinal fluid of patients suffering from culture-proven cases of meningitis. They were identified by the nature of the typical capsules produced, as seen in Indian ink preparations on microscopy, their demonstration of urease activity, and their pathogenicity, which was confirmed by intracerebral inoculation of mice.

RESULTS AND DISCUSSION

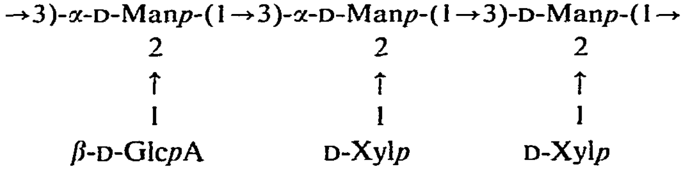
The two strains were grown on Littman's[2] capsule medium, and the acidic polysaccharides produced were purified by precipitation with Cetavlon bromide according to the method of Nimmich[3]. The physical and chemical characteristics of the purified R and H polysaccharides are shown in Table I.

Polysaccharide R, as isolated initially, contained appreciable quantities of bound galactose, and was found to consist of two molecular species [mol. wts.

was hydrolysed and the resulting sugars were analysed by p.c. and by g.l.c. as the alditol acetates; 2,3,4-tri-*O*-methyl-D-xylose (2.0), 2,4,6-tri-*O*-methyl-D-mannose (0.8), and 4,6-di-*O*-methyl-D-mannose (2.2) were found in the molar proportions shown. These results indicate that most of the glucuronic acid residues in the polysaccharide were degraded by base and confirm that they are linked to O-2 of D-mannose.

Polysaccharide H consumed ~ 1 mol of periodate per sugar residue. Reduction of the oxidised polysaccharide followed by mild hydrolysis with acid yielded a water-insoluble product that gave only D-mannose on more vigorous hydrolysis with acid. When the fully methylated derivative of the Smith-degraded product was hydrolysed, only 2,4,6-tri-*O*-methyl-D-mannose was detected in the hydrolysate. These results indicate that all the D-glucuronic acid and D-xylose residues are present as end groups and that the polysaccharide chain is composed of (1→3)-linked residues of D-mannose; there was no indication of branching.

The foregoing data are consistent with a polysaccharide structure (**5**) in which each of the (1→3)-linked residues of D-mannose in the chain is substituted at O-2 by either D-glucuronic acid or D-xylose. A consideration of the acid content of the polysaccharide H (~15%), together with the proportions of the neutral sugar constituents, indicates that two terminal D-xylosyl groups are present for every terminal D-glucosyluronic acid group.



$$\begin{array}{ccc} \rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1 & \rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1 & \rightarrow 3)\text{-D-Man}p\text{-}(1\rightarrow \\ 2 & 2 & 2 \\ \uparrow & \uparrow & \uparrow \\ 1 & 1 & 1 \\ \beta\text{-D-Glc}p\text{A} & \text{D-Xyl}p & \text{D-Xyl}p \end{array}$$

5

The modes of linkage of the D-glucuronic acid and D-mannose residues in polysaccharide H were determined from the $[\alpha]_D$ values of the oligosaccharides **1**–**4**. The β-D linkage indicated by the isolation of mannobiose **1** could be accommodated in the structure **5** at the mannosyl residue for which the anomeric configuration is not specified, but only with some reservation, as it is not easy to understand why a mannobiose unit so positioned should be preferentially released during partial, acid hydrolysis of polysaccharide H. The terminal D-xylosyl groups in the repeating sequence do not appear in any of the oligosaccharides. However, the values calculated for the optical rotation of polysaccharide H by Hudson's rules of isorotation[7], on the assumption that the D-xylosyl groups are all α-linked or all β-linked, are $[\alpha]_{589}$ +57° and $[\alpha]_{589}$ −12°, respectively. The actual value, +5°, is much closer to the latter, which suggests that these groups are β-D-linked.

Comparison with other polysaccharides from Cryptococcus neoformans. — Little has been published concerning the polysaccharides from *C. neoformans*. Detailed structures have been reported by Miyazaki[8] for an untyped *Cryptococcus* polysaccharide and by Bhattacharjee *et al.* for serotypes C[9] and D[10]. The polysaccharide from serotype D closely resembles that of polysaccharide H (type A) in that both D-glucosyluronic acid and D-xylosyl groups are present as non-reducing end-groups attached to O-2 of α-D-mannosyl residues which are (1→3)-linked in the backbone. However, evidence was found to suggest a limited degree of double-branching in the polysaccharide from type D, which was not detected in polysaccharide H. The polysaccharide investigated by Miyazaki differs from polysaccharide H in that it consists of chain residues of D-mannose linked through O-2, each of which is alternately substituted at O-4 by D-xylose or the aldobiouronic acid 4-*O*-(glucopyranosyluronic acid)-D-mannose. Blandamer and Danishefsky[11] reported that the polysaccharide from type B consists of a mannan backbone with branches of xylose and glucuronic acid, but the positions of the linkages in the polysaccharide structure have not been determined. The type-specific polysaccharide from *C. neoformans* A contains xylose, galactose, mannose, and glucuronic acid, and has been shown by Rebers *et al.*[1] to consist of a mixture of at least two acidic polysaccharides. Since polysaccharide H examined in the present study has been serotyped as A, it is possible that the mixture examined by Rebers *et al.* included polysaccharide H and one or more polysaccharides containing residues of galactose.

EXPERIMENTAL

General methods. — The techniques used have been fully described in a recent paper[12]. Solvent systems *a*,*b*,*d* and *f* were employed in paper chromatography.

Mild, acid hydrolysis of polysaccharides R and H. — Samples (40 mg) of each polysaccharide were hydrolysed with 0.1M trifluoroacetic acid (20 mL) at 100° for 24 h. Samples (2 mL) of the hydrolysates were withdrawn at intervals and the acid was removed by distillation with methanol. Each residue was dissolved in water and analysed by p.c. in solvents *a* and *d*.

Hydrolysis of polysaccharides R and H. — Each of the polysaccharides (50 mg) was hydrolysed with 2M trifluoroacetic acid at 100° for 18 h. P.c. of the hydrolysates (solvents *a* and *d*) showed the presence of D-xylose, D-mannose, D-glucuronic acid, and an aldobiouronic acid giving the same colour (when sprayed with *p*-anisidine) and having the same mobility as 2-*O*-(β-D-glucopyranosyluronic acid)-D-mannose. The ratios of neutral sugars (see Table I) in each hydrolysate were determined by g.l.c. of the derived alditol acetates. Preparative g.l.c. of the acetates in each case permitted the recovery of D-mannitol hexa-acetate, the circular dichroism spectrum of which was identical with that of a standard sample, and xylitol penta-acetate which was found to be achiral by this method. Isolation of samples of xylose from the hydrolysates of the polysaccharides R and H by preparative p.c. (solvent *a*) and measurement of their o.r.d. curves indicated that these residues also have the D configuration.

Methylation analyses. — Samples (200 mg) of polysaccharides R and H in the acid form were methylated once by Hakomori's method[13] and three times by the method of Purdie and Irvine[14] to give, in each case, a colourless, amorphous product which showed no hydroxyl absorption in the i.r. spectrum. An estimate of the uronic acid content of each polysaccharide was obtained by measuring the relative intensities of the absorptions due to the methyl ester carbonyl (1740 cm^{-1}) and methoxyl (1100 cm^{-1}) groups in the methylated derivatives, and making comparisons with methylated polysaccharides of known uronic acid content.

The fully methylated polysaccharides (20 mg) were hydrolysed with 2M trifluoroacetic acid at 100° for 18 h. The methyl sugars in the hydrolysate were identified by p.c. (solvents *b* and *f*) and by g.l.c. of their trimethylsilylated, derived alditols. After methanolysis, g.l.c. of the resulting mixtures of methylated glycosides revealed the glycosides of methyl 2,3,4-tri-*O*-methyl-D-glucuronate (removed by saponification), 2,3,4-tri-*O*-methyl-D-xylose, and 4,6-di-*O*-methyl-D-mannose. The proportions of each sugar are shown in Table I.

Partial hydrolysis of polysaccharide H; isolation of oligosaccharides. — Polysaccharide H (100 mg) was hydrolysed with 2M trifluoroacetic acid at 100° for 3 h, and four products (**1–4**) were isolated by preparative p.c. (solvent *a*). Each product was homogeneous in paper chromatography (solvents *a*, *b*, and *d*). The characteristics of each component are shown in Table II. Oligosaccharides **1–4** were characterised as follows.

Methylated, neutral disaccharide **1** was hydrolysed with 2M trifluoroacetic acid at 100° for 4 h. P.c. of the hydrolysate (solvents *b* and *f*) showed the presence of 2,3,4,6-tetra-*O*-methyl-D-mannose and 2,4,6-tri-*O*-methyl-D-mannose. The identity of these sugars was confirmed by g.l.c. of the trimethylsilyl derivatives of the anomers of the methylated sugars.

Methanolysis of fully methylated aldobiouronic acid **2** gave (g.l.c.) methyl glycosides of methyl 2,3,4-tri-*O*-methyl-D-glucuronate (removed by saponification) and 3,4,6-tri-*O*-methyl-D-mannose in the same proportions as were given upon methanolysis of methylated 2-*O*-(β-D-glucopyranosyluronic acid)-D-mannose. The identity of 3,4,6-tri-*O*-methyl-D-mannose was confirmed by hydrolysis of the methylated aldobiouronic acid followed by g.l.c. of the trimethylsilyl derivatives of the anomers of the tri-*O*-methyl sugar, and also by p.c. in solvents *b* and *f*.

Hydrolysis of the aldotriouronic acid **3** with 2M trifluoroacetic acid at 100° for 8 h, followed by p.c. of the hydrolysate (solvents *a* and *d*), gave D-mannose, the aldobiouronic acid **2**, and a trace of D-glucuronic acid. Fully methylated **3** was hydrolysed with 2M trifluoroacetic acid at 100° for 18 h. P.c. of the hydrolysate (solvents *b* and *f*) showed the presence of 2,4,6-tri-*O*-methyl-D-mannose and 3,4,6-tri-*O*-methyl-D-mannose, the identities of which were confirmed by g.l.c. of their trimethylsilyl derivatives. Reduction of the hydrolysate followed by trimethylsilylation and g.l.c. analysis gave the proportions of sugars shown in Table II.

Results obtained from hydrolysis, p.c., and methylation analysis of the aldotetraouronic acid **4** were identical with those for **3**, except for an increase in the proportion of 2,4,6-tri-*O*-methyl-D-mannose. Methanolysis and g.l.c. of the methylated derivatives of both **3** and **4** gave glycosides of methyl 2,3,4-tri-*O*-methyl-D-glucuronate in addition to the methyl glycosides of the neutral sugars, as shown in Table II.

Reduction of methylated polysaccharide H. — Fully methylated polysaccharide H (110 mg) was reduced with lithium aluminium hydride (700 mg) in tetrahydrofuran (10 mL) for 18 h. After the addition of aqueous ethanol, the solution was evaporated to dryness and the residue extracted with chloroform (3 × 30 mL). The extracts were combined and the chloroform was removed, to give a product (60 mg) which showed no i.r. absorption at 1740 cm^{-1} (methyl ester CO). This product (30 mg) was hydrolysed with 2M trifluoroacetic acid at 100° for 8 h. P.c. of the hydrolysate (solvents *b* and *f*) showed the presence of a component giving the same colour with *p*-anisidine and having the same mobility as 2,3,4-tri-*O*-methyl-D-glucose. A portion of the hydrolysate was reduced, acetylated, and analysed by g.l.c. with column (*a*). Preparative g.l.c. allowed the recovery of the individual, methylated alditol acetates, which were further characterised by mass spectrometry. The 2,3,4-tri-*O*-methyl-D-glucitol triacetate was demethylated[15] with BCl_3 and re-acetylated to give D-glucitol hexa-acetate, which was dissolved in acetonitrile and the circular dichroism spectrum recorded. Comparison of the c.d. spectrum with that of a standard sample confirmed the D configuration of the glucose.

Alkaline degradation of methylated polysaccharide H. — The fully methylated polysaccharide (22 mg) was degraded with base and then re-methylated[6]. The resulting solution was diluted with water and dialysed against running tap-water for 48 h. The modified polysaccharide (15 mg) was isolated by extraction of the aqueous solution with chloroform and hydrolysed with 2M trifluoroacetic acid for 8 h at 100°, and the sugars thereby released were analysed by p.c. (solvent *b*) and by g.l.c. of the derived alditol acetates (column *b*).

Smith degradation of polysaccharide H. — A solution of polysaccharide H (300 mg) in aqueous sodium metaperiodate (50 mL, 0.113M) was kept in the dark for 3 days, the consumption of periodate (1.12 mol per hexose residue) being monitored by the arsenite method[16]. The reaction was terminated by addition of ethylene glycol (1 mL), and the solution was stirred for 3 h, dialysed against running tap-water for 2 days, concentrated, and then treated with sodium borohydride (1 g) for 4 days. After acidification with Amberlite IR-120(H^+) resin, the solution was co-distilled with methanol but was not taken to dryness. The final traces of borate were removed by dialysing the solution against running tap-water for 3 days before freeze-drying, which yielded the polyalcohol (200 mg). A solution of this product in M trifluoroacetic acid was stirred at room temperature for 6 days. The trifluoroacetic acid was removed by freeze-drying, the residue was extracted with methanol (3 × 20 ml), and the methanol-soluble material was examined by p.c. (solvent *b*). When sprayed with silver nitrate, the paper chromatogram showed the presence of glycolaldehyde and ethylene glycol.

The methanol-insoluble material, which was also almost totally insoluble in water, was freeze-dried to yield the Smith-degraded product (71 mg). After hydrolysis of this product with 2M trifluoroacetic acid at 100° for 8 h, p.c. (solvents *a* and *d*) showed only D-mannose. A portion of the hydrolysate was reduced, acetylated, and analysed by g.l.c. (column *b*); only D-mannitol hexa-acetate was detected.

Because of the insolubility of the Smith-degraded product, the Hakomori methylation procedure was modified as follows. A suspension of Smith-degraded product (35 mg) in dry dimethyl sulphoxide was heated in an oil bath at 60° for 90 min. After being cooled to room temperature, the solution was treated with methylsulphinyl anion (3 mL, 1.6M), stirred for 48 h, and then cooled in ice–water whilst methyl iodide (5 mL) was slowly added. The solution was diluted with water (10 mL) and dialysed against running tap-water for 2 days before being extracted with chloroform (3 × 20 mL). The extracts were combined and the solvent was removed, to give a product (24 mg), $[\alpha]_D^{20}$ +29° (*c* 0.2, chloroform), which showed no hydroxyl absorption in the i.r. After hydrolysis of a sample (10 mg) of the methylated product, p.c. (solvents *b* and *f*) and g.l.c. (of the trimethylsilyl derivatives) revealed only 2,4,6-tri-*O*-methyl-D-mannose. Reduction and acetylation of the hydrolysate gave 2,4,6-tri-*O*-methyl-D-mannitol triacetate, which was isolated by preparative g.l.c. and further characterised by mass spectrometry.

ACKNOWLEDGMENTS

The authors thank Dr. J. van den Ende (Department of Bacteriology, University of Cape Town) for the isolation and identification of the strains R and H, Dr. S. C. Churms (C.S.I.R. Carbohydrate Research Unit, University of Cape Town) for gel-permeation experiments, and the South African Council for Scientific and Industrial Research and the University of Cape Town for financial assistance.

REFERENCES

1 P. A. Rebers, S. A. Barker, M. Heidelberger, Z. Dische, and E. E. Evans, *J. Am. Chem. Soc.*, 80 (1958) 1135–1137, and references cited therein.
2 M. L. Littman, *Trans. N.Y. Acad. Sci., Ser. 2*, 20 (1958) 623–648.
3 W. Nimmich, *Z. Med. Mikrobiol. Immunol.*, 154 (1968) 117–131.
4 R. W. Bailey, *Int. Ser. Monogr. Pure Appl. Biol., Vol. 4, Oligosaccharides*, Pergamon Press, Oxford, 1965, p. 50.
5 B. Lindberg, J. Lönngren, and J. L. Thompson, *Carbohydr. Res.*, 28 (1973) 351–357.
6 G. O. Aspinall and K.-G. Rosell, *Carbohydr. Res.*, 57 (1977) c23–c26.
7 C. S. Hudson, *J. Am. Chem. Soc.*, 31 (1909) 66–86.
8 T. Miyazaki, *Chem. Pharm. Bull.*, 9 (1961) 829–833.
9 A. K. Bhattacharjee, K. J. Kwon-Chung, and C. P. J. Glaudemans, *Immunochemistry*, 15 (1978) 673–679.
10 A. K. Bhattacharjee, K. J. Kwon-Chung, and C. P. J. Glaudemans, *Carbohydr. Res.*, 73 (1979) 183–192.
11 A. Blandamer and I. Danishefsky, *Biochim. Biophys. Acta*, 117 (1966) 305–313.
12 E. H Merrifield and A. M. Stephen, *Carbohydr. Res.*, 74 (1979) 241–257.
13 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1966) 205–208.
14 T. Purdie and J. C. Irvine, *J. Chem. Soc.*, 83 (1903) 1021–1037.
15 T. G. Bonner, E. J. Bourne, and S. McNally, *J. Chem. Soc.*, (1960) 2929–2934.
16 P. Fleury and J. Lange, *J. Pharm. Chim.*, 17 (1933) 107–113.