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Structural investigation of the polysaccharide fraction from the mucilage of *Dicerocaryum zanguebaricum* Merr.

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Abstract

The polysaccharide fraction from the mucilage of *Dicerocaryum zanguebaricum* (Pedaliaceae) appears to be mainly constituted of a chemically homogeneous polysaccharide. By NMR and chemical degradative methods its structure appeared to consist of alternate → 4)-β-D-Glc*p*A-(1 → and → 2)-α-D-Man*p*-(1 → units. Single branch units of β-D-Xyl*p* and α-D-Gal*p* are linked to the O-3 positions of Man*p* and a significant number of Glc*p*A residues.

Keywords: Dicerocaryum zanguebaricum; Pedaliaceae; Mucilage; Glucuronomannoglycan

1. Introduction

Dicerocaryum zanguebaricum is a herb common along the coast in South and South-East Africa. Its leaves have specialised mucilaginous hairs, like those of the other members of Pedaliaceae [1,2]. When boiled or simply steeped in cold water, they give a mucilaginous preparation that is used as a folk remedy to aid the expulsion of retained placenta in women, for treating gonorrhoea and hydrocile, as well as a shampoo for the hair [3].

We are unaware of any investigation on the structure of the mucilage of this plant.

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2. Experimental

Origin and purification of the polysaccharide.—Leaves of *Dicerocaryum zanguebaricum* were collected by one of the authors in the coast area of Maputo (Mozambique) near the Agricultural Faculty during November–December 1991, dried at room temperature under ventilation, and stored at room temperature. The dry leaves (10 g) of *Dicerocaryum zanguebaricum* were stirred in water (500 mL) at 80 °C for 1 h. The leaves were removed by filtration and the solution was freeze-dried to give a residue (3.9 g). To an aqueous solution (700 mL) of the latter an equal volume of a 1:1 mixture of Tris-saturated phenol and $CHCl_3$ was added to eliminate the protein fraction. After centrifugation, the supernatant solution was washed with $CHCl_3$ (three times) and then dialysed (Spectrapor 4 cellulose tube, cut-off 12,000–14,000 Da). The solid (2.3 g) was dissolved in H_2O (100 mL) at 50 °C and then further purified by precipitation with EtOH (250 mL) overnight at −24 °C, washed with EtOH, and dried in a vacuum desiccator. The carbohydrate content of the residue (1.2 g) was tested by the phenol–H_2SO_4 [4] assay. Since it was shown to contain uronic acid by the *m*-hydroxybiphenyl test [5], it was chromatographed on Q-Sepharose eluting first at pH 7.5 with 20 mM Tris-HCl (320 mL) and then with a linear gradient up to 800 mM (640 mL). Based on colorimetric tests for total sugars, the fractions between 1500 and 1800 mL eluted with 800 mM buffer were pooled to give a fraction (900 mg) which showed a negligible absorption at 280 nm. Chemical homogeneity of the polysaccharide fraction was checked by finding a homogeneous peak in all of the following chromatographic conditions: Bio-Gel A 5m (50 mM NaOAc, pH 5.2), Bio-Gel A 1.5m (50 mM NaOAc, pH 5.2), and Sephacryl S-400 HR (100 mM Tris, pH 7.0). Since methanolysis of fractions taken across the peak of the latter chromatography gave similar molar ratios for monosaccharides, the structural investigation was performed on the whole polysaccharide fraction, $[\alpha]_D$ +40.3° (*c* 3.60, H_2O), without further purification. An apparent molecular mass of 590 kDa was found by calibration of the Bio-Gel A 1.5-m column with dextran standards.

Another batch of dry leaves (1 g) was stirred in water (80 mL) for 1 h at room temperature. After workup as above, a polysaccharide fraction (30 mg) identical (^1H, ^{13}C NMR, chromatographic behaviour) to that extracted at 80 °C was obtained.

General.—The ^1H and ^{13}C NMR spectra were obtained in D_2O at 400 and 100 MHz, respectively, with a Bruker AM 400 spectrometer equipped with a dual probe, in the FT mode at 85 °C. The ^{13}C and ^1H chemical shifts were measured using 1,4-dioxane (67.4 ppm) and sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate, respectively, as internal standards. The DEPT experiment was performed using a polarisation transfer pulse of 135° and a delay adjusted to an average C,H coupling of 160 Hz. The standard Bruker software was used for the heteronuclear C,H COSY experiment (XHCORR) under the following conditions: the time domain in f_2 was 1K, 64 spectra were collected with 1280 scans, the spectral width was 8000 Hz in f_2 and 1200 Hz in f_1, and delays were optimised for $^1J_{C,H}$ 160 Hz. Fourier transformation was performed with a shifted sine-bell function in both dimensions. Optical rotations were determined on a Perkin–Elmer 141 polarimeter. IR spectra were recorded for KBr pellets on a Perkin–Elmer 1760 Fourier-transform spectrometer. HPLC was performed with a Varian 5020 instru-

Table 1
Molar ratio of the monosaccharides obtained by hydrolysis and methanolysis of native polysaccharide, and methanolysis of carboxyl-reduced polysaccharide, fraction **a**, fraction **b**, and Smith-degraded polysaccharide

Monosaccharide	Native			Carboxyl-reduced	Fr. **a**	Fr. **b**	Smith-degraded
	Hydrolysis	Methanolysis					
D-Man	1.0	1.5 [a]	1.4 [b]	1.7 [a]	1 [a]	1 [a]	1 [a]
D-GlcA (Glc)		1.0	(1.0)	(1.5)	1	1	1
D-Xyl	1.7	1.9	1.6	1.0			
D-Gal	1.4	2.0	1.6	1.5			

[a] Determinated as Me_3Si ethers.
[b] Determinated as alditol acetates.

ment, using a Waters R 401 differential refractometer as the detector. TLC was carried out on Silica Gel F_{254} (Merck). GLC was performed with a Carlo Erba instrument equipped with a flame-ionisation detector, and GLC–MS with a Hewlett–Packard 5890 instrument. Mass spectra were recorded with a VG ZAB HF instrument equipped with an FAB source, using a glycerol–thioglycerol (1:1) matrix.

Acid hydrolysis.—Polysaccharide samples were hydrolysed with 2 M CF_3CO_2H [6] according to the following procedure: each sample was kept at 120 °C up to 5 h, analysing every hour the content of neutral sugars; the molar ratios of the sugars were evaluated, using *myo*-inositol as internal standard, by GLC on an SPB-1 capillary column (SUPELCO, 30 m × 0.25 mm i.d., flow rate 1 mL/min, N_2 as carrier gas) at 235 °C. The maximum values for each monosaccharide, taken at the appropriate time, are reported in Table 1.

Methanolysis.—Methanolysis was performed with M HCl in MeOH for 16 h at 80 °C. Part of the methanolysate was analysed as Me_3Si ethers as follows: the resulting mixture of methyl glycosides was dried over P_2O_5, treated with Trisil (Pierce) for 20 min at 80 °C, and subjected to GLC on an SPB-1 capillary column (SUPELCO, 30 m × 0.25 mm i.d., flow rate 1 mL/min, N_2 as carrier gas), with the temperature programme: 160 °C for 3 min, 160 → 200 °C at 2 °C/min, 200 → 260 °C at 10 °C/min, 260 °C for 10 min. The other part was treated with M $LiEt_3BD$ in THF for 12 h at room temperature. After destruction of excess of hydride by adding a few drops of glacial AcOH, the sample was deionised on Dowex 50W-X8 (H^+ form) resin, eluting by 1:1 H_2O–EtOH. The sample was then acid-hydrolysed and analysed as alditol acetates.

Carboxyl reduction of polysaccharide.—This was carried out with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate at pH 4.75 with 2-(4-morpholino)ethanesulfonic acid as buffer agent [7], followed by reduction with 2 M $NaBH_4$ at pH 7.0. The reaction mixture was dialysed against water, freeze-dried, and subjected four times to reduction under the same conditions. In this way 89% of the acid was reduced, as deduced from the *m*-hydroxybiphenyl test.

Methylation.—Polysaccharide samples were methylated as described [8]. The crude product was filtered on a C_{18} Sep-Pak cartridge (Waters), previously washed with EtOH (20 mL), MeCN (2 mL), and water (10 mL). The fractions were eluted with water (50 mL), 4:1 water–MeCN (8 mL), MeCN (2 mL), and EtOH (6 mL). The last two fractions

Table 2
Molar ratios of methylated monosaccharides from methylated native and autohydrolysed polysaccharide, fraction **a**, fraction **b**, 'core', and Smith-degraded polysaccharide

Monosaccharide	Native	Autohydrolysate at 6 h	Fraction **a**	Fraction **b**	'Core'	Smith-degraded
2,3,4-Tri-OMe-Xyl*p*	2.5	1.0				
2,3,4,6-Tetra-OMe-Gal*p*	2.7	3.1				
4,6-Di-OMe-Man*p*	2.7	2.2				
2,3-Di-OMe-Glc*p*A	1.0	2.5	1		1	1
2-OMe-Glc*p*A	1.9	1.3				
3,4,6-Tri-OMe-Man*p*		1.3	1		1	1
2,3,4-Tri-OMe-Glc*p*A			1	1		
1,3,4,6-Tetra-OMe-Man(ol)			1	1		

were pooled and evaporated to give the methylated polysaccharide which was carboxyl-reduced with M $LiEt_3BD$ in THF and, after desalting on Dowex 50W-X8 (H^+), hydrolysed with 2 M CF_3CO_2H. The partially methylated products in the hydrolysates were reduced with $NaBD_4$, acetylated, and analysed by GLC–MS on an SP-2330 capillary column (SUPELCO, 30 m × 0.25 mm i.d., flow rate 0.8 mL/min, He as carrier gas), with the temperature programme: 170 °C for 3 min, 170 → 240 °C at 4 °C/min, 240 °C for 10 min. GLC of the methylated alditol acetates was carried out on a column identical with that used for GLC–MS (flow rate 1 mL/min, N_2 as carrier gas), with the same temperature programme, using effective response factors [9], and normalising the peak areas with respect to that of *myo*-inositol hexa-acetate, used as the internal standard.

Autohydrolysis of the native polysaccharide.—A solution of the polysaccharide (20 mg) in water (1.5 mL) was kept at 95 °C for 6 h at pH 3.5, then dialysed (Spectrapor 4 cellulose tube, cut-off 12,000–14,000 Da). Both the inside and the outside contents were freeze-dried. The residue from the inside content (17 mg) was submitted to ^{13}C NMR analysis (see text) and methylation reaction (Table 2). The residue from the outside content (2 mg) was submitted to monosaccharide analysis. Only xylose was detected. Another sample (80 mg) of the native polysaccharide was submitted to autohydrolysis at pH 3.5 for 25 h at 95 °C. Usual workup gave residues of 62 mg (^{13}C NMR analysis, see text) and 13 mg from the inside and outside solutions, respectively. The residue from the outside content was submitted to TLC (4:1 2-propanol–water) monosaccharide analysis. Xylose and traces of galactose were detected.

Isolation of the 'core' by acid hydrolysis of the autohydrolysate at 25 h.—The above inner residue (40 mg) was acid-hydrolysed with 0.1 M CF_3CO_2H at 115 °C for 4 h. After dialysis and lyophilisation, the inner residue (12 mg) was chromatographed on Bio-Gel P10 and eluted with water, to give one peak (8 mg). For ^1H and ^{13}C NMR data, see text.

Isolation of oligosaccharides.—The outside solution of the above hydrolysis was lyophilised (28 mg) and chromatographed on Bio-Gel P2 by elution by water. Three fractions were collected: **a** (8 mg), **b** (6 mg), and **c** (13 mg). For the composition of fractions **a** and **b**, see text.

TLC analysis (4:1 2-propanol–water) of fraction **c** showed it to be composed of a mixture of galactose and xylose, which was resolved by HPLC (Hypersil-NH_2; eluant 8:2 MeCN–H_2O, flow 1 mL/min), to yield galactose (4 mg) and xylose (3 mg).

Smith degradation of the native polysaccharide.—A sample of the native polysaccharide (80 mg) was subjected to Smith degradation [10] as follows: 80 mg were dissolved in water (3 mL), treated with 0.1 M $NaIO_4$ (25 mL) at 4 °C, and kept in the dark for 3 days. After addition of a few drops of ethylene glycol to reduce the excess of periodate, the sample was extensively dialysed against tap water, using a Spectrapor 6 cellulose tube with a cut-off of 1000 Da. The inside content was treated with $NaBH_4$ (120 mg) for 1 h at room temperature. After the usual workup, the sample was submitted to mild acid hydrolysis with 0.5 M CF_3CO_2H (9 mL) for 16 h and dialysed as above. The inside content was freeze-dried (11 mg), chromatographed on Bio-Gel P 10, and eluted in the void volume by water; $[\alpha]_D$ +6° (*c* 1.95, H_2O).

Enzymatic hydrolysis of polysaccharide.—A sample of native polysaccharide (6 mg) was submitted to enzymatic hydrolysis with α-galactosidase from *Aspergillus niger* [11], at 37 °C in 0.1 M sodium citrate buffer for 30 min. The enzymatic reaction was stopped by immersing the tube in a boiling water bath. The galactose present in the digest was identified as alditol acetates by GC.

Determination of the absolute configuration of the monosaccharides.—The configurations of galactose and xylose were established by polarimetry. The configurations of mannose and glucuronic acid were established by GC analysis of their trimethylsilylated (−)-2-butyl glycosides [12,13].

3. Results and discussion

The extraction of the polysaccharide fraction was tested at two temperatures, but no difference was found for the polysaccharide structure. The extraction at higher temperature was chosen because of the larger amount of material obtained. The qualitative and quantitative monosaccharide composition (Table 1) of the polysaccharide contained in the *Dicerocaryum zanguebaricum* mucilage was obtained both by methanolysis and acid hydrolysis. The methanolysis crude reaction was, in part, analysed as trimethylsilyl ethers and, in part, as alditol acetates, after reduction of carboxymethyl groups, acid hydrolysis, and reduction of hemiacetal functions. In addition, the monosaccharide composition was confirmed by methanolysis of carboxyl-reduced polysaccharide.

The methylation data (Table 2) of the native polysaccharide indicated a highly branched structure and a quite similar ratio among the four monosaccharides. This finding disagreed with the low ratio found for the GlcA from the methanolysis data (Table 1). However, the acid content of polysaccharide was evaluated by spectroscopic analysis (calibration of the absorption of the *m*-hydroxybiphenyl test) and a value of 27% in weight for the acid content was found. The mannose ratio (Table 1) was also lower than those of Xyl and Gal but this might be explained both by the reduced exposure of the mannose unit to the hydrolytic medium, owing to its branching nature and/or a linkage with the less-hydrolysable uronic acid units.

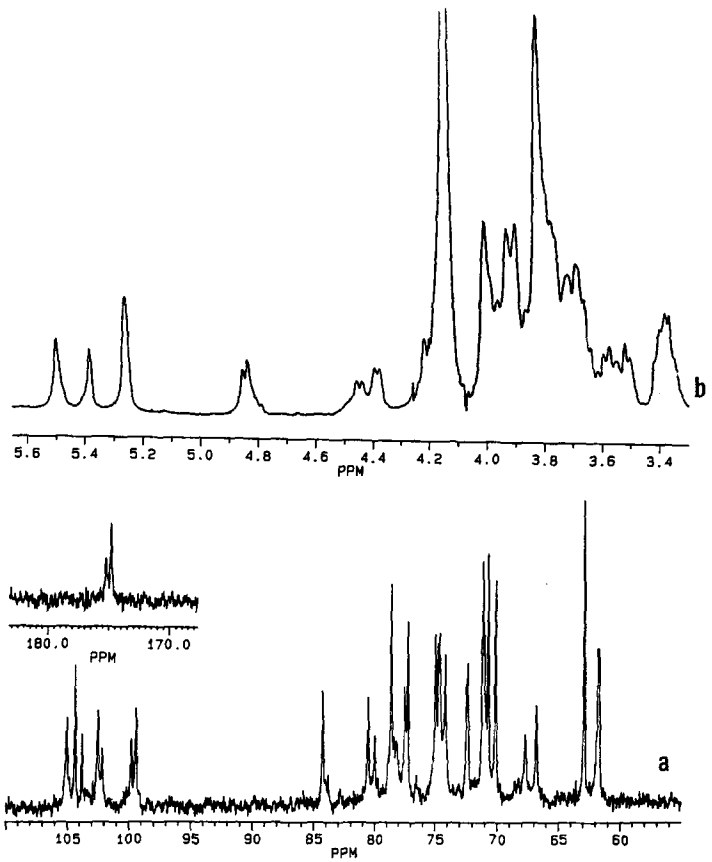

Fig. 1. ^{13}C (100 MHz) (a) and ^{1}H NMR (400 MHz) (b) spectra of the native polysaccharide of *Dicerocaryum zanguebaricum* in D_2O (pH 7) at 85 °C.

The D configuration for all monosaccharides was established by polarimetry and GC analysis.

Further information was drawn from the ^{1}H and ^{13}C NMR spectra (Fig. 1) of the native polysaccharide. Correlations among anomeric atoms, via a 2D NMR heterocorrelated experiment, are reported in Table 3 together with $^{3}J_{H,H}$ and $^{1}J_{C,H}$ values.

Table 3
Correlation of anomeric signals (δ) for the native polysaccharide from 2D NMR, one-bond C,H COSY data [a]

Proton	Carbon	Assignment
5.54 bs	99.4 (177)	α-Man*p*
5.47 bs	99.8 (171)	α-Man*p*
5.39 bs	102.5 (177) and 102.2 (170)	α-Gal*p*
4.93 d (7.3)	105.0 (166)	β-Xyl*p*
4.62 d (7.3) and 4.48 d (6.7)	104.3 (165) and 103.8 (160)	β-GlcA*p*

[a] In parentheses, $^{3}J_{H,H}$ and $^{1}J_{C,H}$ values (Hz).

The assignments were based on the ^{1}H and ^{13}C chemical shifts, and the anomeric configurations were deduced from the $^{3}J_{H,H}$ and $^{1}J_{C,H}$ values. In particular, the upfield chemical shifts of α-Man C-1 carbons were in accord with the O-2 glycosylation [14]. The other monosaccharide in the α configuration was believed to be α-Gal, since the latter is more common in nature than α-Xyl and α-GlcA. Moreover, the presence of α-Gal, suggested by the band at 817 cm^{-1} [15] in the IR spectrum of the native polysaccharide, was confirmed by the enzymatic hydrolysis. Finally, distinction between xylose signals and those of uronic units was suggested by the lower field for the H-1 chemical shift of xylose (δ 4.93) [16]

Other valuable ^{13}C signals were that at 84.2 ppm, attributed to the glycosylated C-3 position of a glucopyranosyluronic unit, and that for the oxymethylene carbon at 66.8 ppm, assignable to C-5 of xylopyranose. This chemical shift value supported the β configuration [17] for this monosaccharide. Further identifiable signals were those of carboxyl carbons at 175.2 and 174.8 ppm, and of the oxymethylene carbons at 62.9 and 61.8 ppm.

Comparison of the ^{13}C NMR spectra for the autohydrolysate at 6 h and the native polysaccharide showed a decreased signal at 66.8 ppm for xylose, which was the only monosaccharide freed during the autohydrolysis. Chemical analysis of the methylated autohydrolysate revealed (Table 2) the presence of 2-substituted mannose and an increase of the 4-substituted Glc*p*A/3,4-substituted Glc*p*A ratio with respect to that of the native polysaccharide. These results indicated that both the mannose and the glucopyranosyluronic acid were glycosylated by xylose and by galactose.

The acid hydrolysis with 0.1 M CF_3CO_2H at 115 °C for 4 h of the autohydrolysate at 25 h yielded a polysaccharide whose ^{1}H and ^{13}C NMR spectra both showed two anomeric signals in ca. 1:1 ratio at δ 5.39 (bs) and 4.44 (d, *J* 7.3 Hz) and at δ 101.7 and 99.9, respectively. In addition, a carboxyl signal at δ 175.6 was also present in the ^{13}C NMR spectrum These data suggested a polysaccharide constituted of mannose and glucuronic acid in 1:1 ratio, as was confirmed by chemical analysis. The methylation, followed by acid hydrolysis and alditol acetate analysis, revealed only two types of linkages, namely 4-substituted glucopyranosyluronic acid and 2-substituted mannopyranose in the 1:1 ratio (Table 2). These structural features suggested for this polysaccharide, that represents the 'core', a linear structure made up of →4)-β-D-Glc*p*A and →2)-α-D-Man*p* units.

As well as the 'core', two oligosaccharides were also obtained from the acid hydrolysis. The first, indicated as **a**, contained Man and GlcA in 1:1 ratio (Table 1) by methanolysis and Me_3Si GC analysis. Methylation analysis of the reduced oligosaccharide showed 1:1:1:1 ratios (Table 2) for terminal Glc*p*A, 4-substituted Glc*p*A, 2-substituted Man*p*, and 2-substituted mannitol, indicating a tetrasaccharide structure.The ^{1}H NMR spectrum of fraction **a** showed three broad singlets at δ 5.42, 5.30, and 4.96, which were assigned [18] to the anomeric protons of the inner and the reducing mannose residue, respectively, and three doublets at δ 4.58, 4.51, and 4.49, $^{3}J_{H,H}$ 7.8 Hz, assignable to H-1 of the inner and terminal glucuronic residues. The signals of the reducing unit were identified by their disappearance after $NaBH_4$ reduction. The ^{13}C NMR spectrum displayed two carboxyl signals at δ 176.3 and 175.5, four anomeric signals at δ 102.0 and 101.8, both with $^{1}J_{C,H}$ 160 Hz, attributed to β-glucopyranosyl-

uronic units, and at δ 98.9 ($^1J_{C,H}$ 180 Hz) and 92.5 ($^1J_{C,H}$ 177 Hz), assignable to the inner and the reducing mannose residue, respectively. The FAB MS spectrum in positive mode of the methylated tetrasaccharide showed pseudomolecular ions at m/z 913 and 908 (Na^+ and NH_4^+ forms), but no fragmentations appeared. In order to gain insight into the sequence, the tetrasaccharide was per-*O*-deuterioacetylated and analysed by FAB MS. The spectrum showed fragments at m/z 1109, due to $[MH - 2\ CD_3COOH]^+$, at m/z 609, attributable to $[Man\text{-}GlcA]^+$ or $[GlcA\text{-}Man]^+$, and at m/z 906, assignable to $[Man\text{-}GlcA\text{-}Man]^+$. These latter peaks indicated an alternating structure for the tetrasaccharide whose structure can be therefore depicted as: β-D-Glc*p*A-(1 → 2)-α-D-Man*p*-(1 → 4)-β-D-Glc*p*A-(1 → 2)-D-Man.

The other oligosaccharide fraction, indicated as **b**, was shown to be a disaccharide composed of reducing mannose 2-*O*-glycosylated by a β-D-glucopyranosyluronic unit, using methanolysis data (Table 1), which indicated a 1:1 ratio between GlcPA and Man, and methylation analysis of the reduced derivative. The latter data (Table 2) indicated a 1:1 ratio between terminal GlcPA and 2-substituted mannitol. The ^1H NMR spectrum showed at δ 5.31 (d, 1 H, J 2.0 Hz, H-1 α-anomer) and 4.97 (d, 1 H, J 1.0 Hz, H-1 β-anomer) two signals of reducing mannose, and at δ 4.62 and 4.52 two doublets (J 7.8 Hz) assignable to the glucuronic residue [18]. The ^{13}C NMR spectrum displayed a carboxyl signal at δ 175.1, and anomeric signals at δ 102.5, attributed to the β-glucopyranosyluronic unit, and at δ 94.5 and 92.9 assigned to the reducing mannose residue.

The results above suggested the alternating structure → 2)-α-D-Man*p*-(1 → 4)-β-D-Glc*p*A-(1 → for the backbone of the polysaccharide of *Dicerocaryum zanguebaricum.*

The ^1H and ^{13}C NMR spectra of the Smith-degraded polysaccharide were identical to those of the 'core'. Integration of the two ^1H NMR anomeric signals at δ 5.42 (bs) and 4.52 (d, J 7.8 Hz) revealed a 1:1 ratio between Man and GlcA (Table 1). This was confirmed by methylation analysis, which gave a 1:1 ratio of 2-substituted mannose and 4-substituted glucuronic acid (Table 2). Therefore, the structure of the Smith-degraded polysaccharide was a linear chain built up by 2-substituted mannose and 4-substituted uronic acid in a 1:1 ratio. The finding of a linear polysaccharide from the Smith reaction suggested that neither mannose nor uronic units were in the side chains of the backbone.The possibility that the 4-substituted uronic units were in the side chain was ruled out by finding the same mannose/uronic acid ratio for the Smith-degraded polysaccharide and for the native polysaccharide.

From the foregoing data, it is possible to suggest the following average fragment, irrespective of the sequence of branches along the backbone, for the polysaccharide of *Dicerocaryum zanguebaricum.*

→ 4)-β-D-Glc*p*A-(1 → 2)-α-D-Man*p*-(1 → 4)-β-D-Glc*p*A-(1 → 2)-α-D-Man*p*-(1 → 4)-β-D-Glc*p*A-(1

3 3 3 3
↑ ↑ ↑ ↑
1 1 1 1
R R R R

R = β-D-Xyl*p* or α-D-Gal*p*

The repeating fragment → 4)-β-D-Glc*p*A-(1 → 2)-α-D-Man*p*-(1 → of the backbone of this structure appears to be widespread in nature [16,19–22]. However, the side

chains are usually branched, whereas in *Dicerocaryum zanguebaricum* the polysaccharide has a 'comb' structure with arms consisting of a single monosaccharide unit.

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