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WATER-SOLUBLE GLYCOPROTEINS FROM *CANNABIS SATIVA* (SOUTH AFRICA)

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Key Word Index—*Cannabis sativa;* Cannabinaceae; marijuana; glycoprotein; cell wall protein; serine-*O*-galactoside; hydroxyproline.

Abstract—The water-soluble glycoproteins obtained from *Cannabis* leaves of plants grown from South African seeds have been further studied. Treatment of the glycoprotein fractions with NaOH in the presence of $NaBH_4$ resulted in a significant decrease in the serine content and a corresponding increase in alanine. The carbohydrate side chains released contained the sugar alcohol, galactitol. By treatment of the glycoprotein fractions with NaOH in the presence of Na_2SO_3 and subsequent acid hydrolysis, cysteic acid was formed. These data indicate that carbohydrate and protein are connected via serine-*O*-galactoside linkages. Further investigation of the structure of the carbohydrate part of the glycoproteins was carried out by methylation analysis, Smith-degradation and enzyme incubation. The present glycoprotein material of plants grown from South African seeds is similar to the material previously investigated, but in contrast to the latter, it is devoid of hexosamine.

INTRODUCTION

The leaves of *Cannabis sativa* contain *ca* 1% of water-soluble glycoprotein which has been shown to consist essentially of two fractions by ion exchange and gel chromatography [1]. Both fractions contain a significant amount of hydroxyproline. Electrophoretic and chromatographic examination of an alkaline hydrolysate indicated that the protein chain is linked to carbohydrate through this amino acid [1].

Recently Lamport *et al.* [2] reported the existence of a serine-*O*-galactoside linkage in a glycopeptide isolated from tomato cell wall. It has been postulated that this bond may be involved in cross-linking other cell wall macromolecules. The cell wall polysaccharides may be attached to the hydroxyproline-rich protein, extensin, via the serine hydroxyl group. The hydroxyproline arabinosides are thought to stabilize the extensin polypeptide backbone, conferring on it a rigid, rod-like structure [3].

Apart from further structural examination of the carbohydrate moiety of the glycoprotein, the aims of the present study were to establish whether the serine-*O*-galactoside linkage is also present in the *Cannabis* leaf glycoprotein. Previously, only cell walls from cultured cells in well defined systems have been investigated [2,4,5] in contrast to the material reported here, which is an extract from leaves of a full-grown plant with cells in various stages of development.

RESULTS AND DISCUSSION

In contrast to most other plant glycoproteins investigated, the present material was soluble in water, avoiding any pretreatment with enzymes or alkaline agents. Fraction A ($[\alpha]_D^{20}$ + 21°), passed unretarded through the DEAE-cellulose column, and was obtained in 12.5% yield, whereas fraction B ($[\alpha]_D^{20}$ 0°) was eluted with Tris buffer containing 0.25 M NaCl and was obtained in 24.5% yield (Fig. 1).

IR spectra of A and B showed no absorption bands at 1735 and at 1250 cm^{-1} suggesting the absence of ester linkages [6]. This is in agreement with the results obtained by the GLC method used for estimation of *O*-acetyl groups [6]. The only peak present corresponded with acetic acid in a quantity of less than 1% (Table 1).

Fraction A was examined for the presence of reducing carbohydrate end groups by cautious treatment with $NaBH_4$ [7]. The presumed alditol fraction was acetylated and GLC of the product gave 5 peaks, one of which had the R_t of mannitol hexaacetate. GC–MS analysis, however,

Table 1. Optical rotation and % composition of fractions A and B

	A	A_1	A_2	B	B_1	B_2
$[\alpha]_D^{20}$	+ 21°			0°		
O-Acetyl	0.22			0.70		
Total carbohydrate	88.3	69.0	55.0	72.3	53.1	40.6
Protein	16.8	23.0	26.3	25.2	35.0	37.8
Uronic acid	—	—	—	15.8	8.1	3.8
Arabinose	35.7	13.5	5.2	21.1	6.6	4.4
Rhamnose	—	—	—	6.7	4.1	1.4
Xylose	—	—	—	2.7	tr	tr
Mannose	3.0	1.1	tr	3.0	tr	tr
Galactose	23.8	35.6	35.0	22.0	28.9	24.8
Glucose	21.5	4.0	2.1	7.5	tr	tr
Galacturonic acid	—	—	—	9.3	5.6	2.8

A_1 and B_1: Residual glycoprotein after the first Smith-degradation of A and B.

A_2 and B_2: Residual glycoprotein after the second Smith-degradation of A and B.

* This investigation was carried out in collaboration with the United Nations Narcotics Laboratory in Geneva, as part of the U.N. *Cannabis* research programme established by resolution 8 (XIV) of the Commission on Narcotic Drugs.

For Part I in this series see ref. [1].

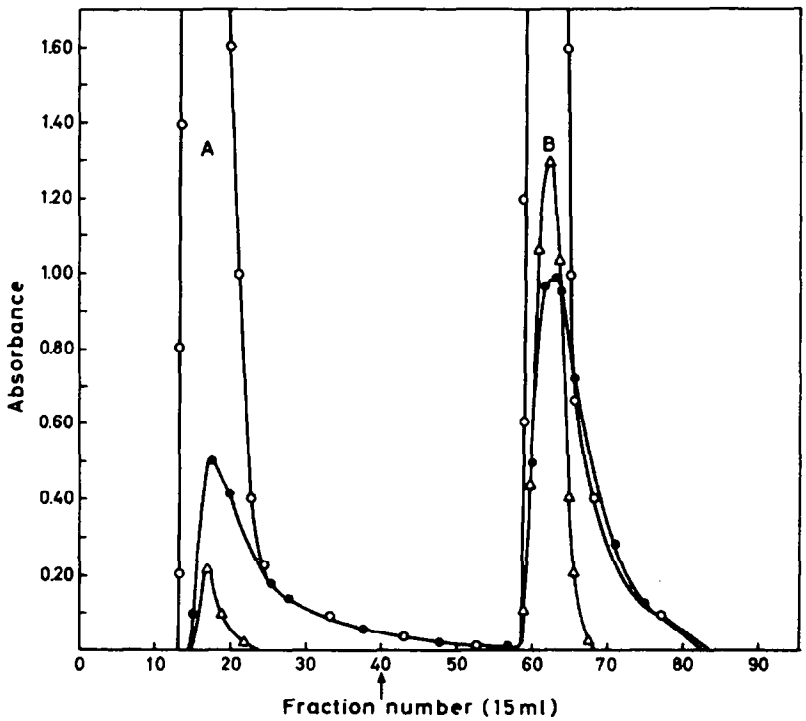

Fig. 1. Elution of *Cannabis* (South Africa) raw extract on DEAE-cellulose in the chloride form. The column (45 × 3 cm) was eluted with 0.05 M Tris–HCl buffer pH 6.8. At fraction no. 40 the eluting agent was changed to buffer containing 0.25 M NaCl, as indicated by the arrow. —○—○— carbohydrate; —●—●— protein; —△—△— uronic acid.

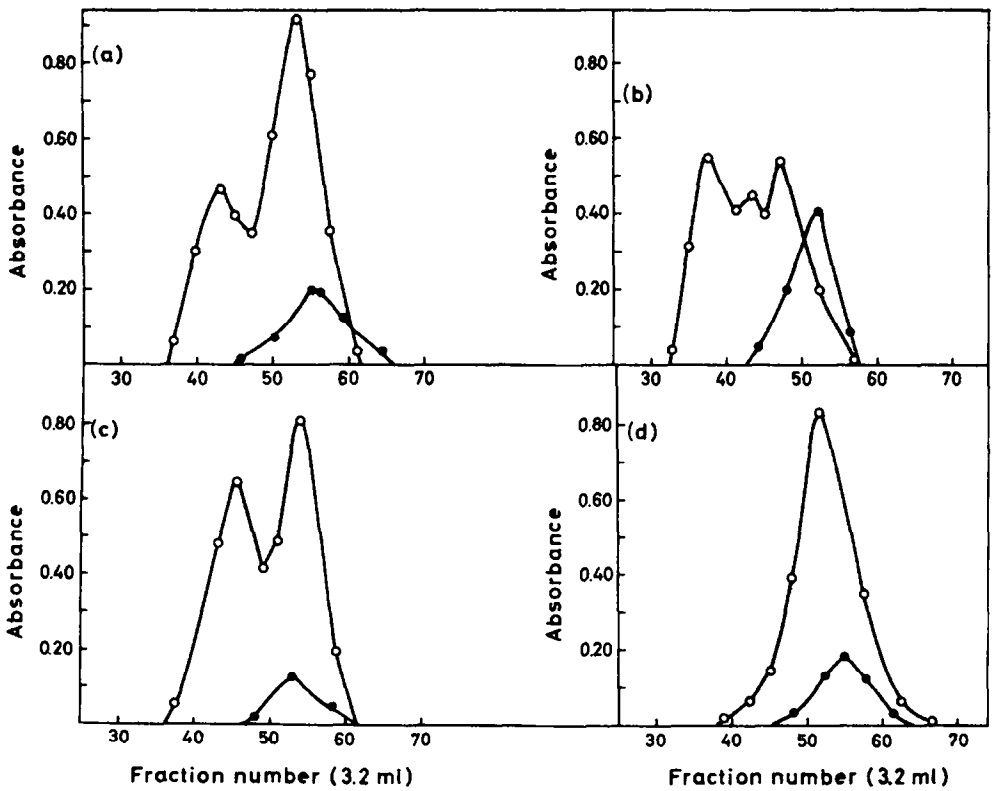

Fig. 2. Gel filtration on Sepharose 4B (36 × 2.5 cm) with 0.025 M Tris–HCl buffer pH 7.2. (a) Fraction A; (b) Fraction B; (c) Fraction A_1 (residual glycoprotein after 1. Smith-degradation of A). (d) Fraction A_2 (residual glycoprotein after 2. Smith-degradation of A). —○—○— carbohydrate; —●—●— protein.

revealed primary fragments different from those produced from a peracetylated hexitol. Evidently this and the other peaks obtained represent degradation products. The absence of any alditol is in full agreement with the carbohydrate–protein complex of *Cannabis* leaves being a glycoprotein.

MW was estimated by gel filtration on a calibrated Sepharose 4B column. The elution profile for both A and B (Figs 2a and b) consists of two peaks, which were combined for further studies. Calculations based on the curve, gave a MW for the two peaks in A of *ca* 83000 and 16500, and for the two peaks in B of *ca* 125000 and 42000, respectively. These MW values are considerably higher than those found previously [1].

Gel filtration of the residual glycoproteins A_1 and B_1, obtained after the first Smith-degradation of A and B, gave almost the same elution pattern on Sepharose as A and B, in contrast to the curves given by A_2 and B_2, resulting from the second Smith-degradation of A and B. A_2 and B_2 both showed a symmetrical elution profile. The elution profiles on Sepharose of products A_1 and A_2 are shown in Figs 2c and d, respectively.

Fractions A, A_1, B and B_1 were subjected to incubation with pronase. It has been reported [8] that cell wall protein is exceptionally resistant to proteolytic degradation—probably a result of its unusually high imino acid content and its close association with carbohydrate. The samples incubated with pronase were applied on a Sepharose 4B column. Fraction A was apparently unaffected by the incubation—the elution pattern coinciding with the pattern for untreated A. Fraction A_1 and to a greater extent B and B_1 had their elution pattern altered to a somewhat lower MW relative to untreated glycoprotein fractions.

Smith-degradation analysis was largely carried out as before [1]. The chemical composition of the resistant products is included in Table 1, and the consumption of periodate and the yields obtained after the two degradations are summarized in Table 2. It appears that galactose is most resistant to periodate, indicating that this sugar is (1 → 3)-linked or constitutes branch points. Rhamnose and galacturonic acid, only occurring in B, seem to be destroyed to the same extent; presumably these sugars constitute a rhamnogalacturonan, reported to be present in the cell wall of higher plants [4, 9].

The low MW alcohol fractions obtained after the two Smith-degradations were analysed by GLC as peracetylated derivatives. All of the 4 alcohol fractions contained a major peak corresponding to glycerol triacetate. Glycerol would arise mainly from arabinofuranose and manno-, galacto-, and glucopyranose end groups. Alcohols obtained after the first Smith-degradation of A contained additional peaks with R_ts corresponding with erythritol- and threitol tetraacetate; the 3 other alcohol fractions contained only traces of tetritols. Erythritol would arise from (1 → 4)-linked glucose and/or mannose and threitol from (1 → 4)-linked galactose.

Table 2. Consumption of periodate and yields (mg) obtained after the first and the second Smith-degradation of fractions A and B

	A	B
Consumption of IO_4^- in first Smith-degradation	1.00	1.04*
Consumption of IO_4^- in second Smith-degradation	0.98	0.76
Material before first Smith-degradation	400	400
Residual glycoprotein after first Smith-degradation	127.6	188.0
Material before second Smith-degradation	100	100
Residual glycoprotein after second Smith-degradation	32.0	42.3
Dialysable fraction after first Smith-degradation	141.7	50.9
Dialysable fraction after second Smith-degradation	24.1	20.8

* Mole IO_4^-/anhydrosugar unit.

Table 3. Methylation analysis of fraction A, B and B after carboxyl-reduction of the methylated polymer (B')

RTMG	Alditol acetate	Primary fragments MS m/e				Area in % of total peak area A	B	B'
0.51	2,3,5-Tri-*O*-methylarabinitol	45	118	161		18.2	19.7	
0.63	2,3,4-Tri-*O*-methylarabinitol	117	118	161	162	1.9	1.5	
0.84	3,5-Di-*O*-methylarabinitol	45	161	190		1.4	1.6	
0.90	2,5-Di-*O*-methylarabinitol	45	118	233		3.2 }	3.9	
0.90	3,4-Di-*O*-methylrhamnitol	131	190			— }		
1.00	2,3,4,6-Tetra-*O*-methylglucitol/mannitol	45	118	161	162 205	4.7	1.7	
1.07	2,3-Di-*O*-methylarabinitol	118	189			23.4	12.2	
1.13	2,3,4,6-Tetra-*O*-methylgalacitol	45	118	161	162 205	3.9	3.0	
1.38	2-Mono-*O*-methylrhamnitol	118				—	0.9	
1.55	3-Mono-*O*-methylrhamnitol	190	203			—	1.0	
1.63	2,4,6-Tri-*O*-methylglucitol	45	118	161	234	7.3	7.4	
1.78	2,4,6-Tri-*O*-methylgalactitol	45	118	161	234	12.4	5.7	
1.89	2,3,6-Tri-*O*-methylgalactitol	45	118	162	233	10.2	3.4	
1.96	2,3,6-Tri-*O*-methylglucitol	45	118	162	233	3.5	1.5	
2.30	Degr. product of galacturonic acid					—	18.8	5.2
2.36	2,3,4-Tri-*O*-methylgalacitol	118	162	189	233	1.1	4.7	4.0
2.64	2,6-Di-*O*-methylgalacitol	45	118			} 0.5	—	—
2.64	4,6-Di-*O*-methylgalactitol	45	161			}	—	—
2.96	3,6-Di-*O*-methylglucitol	45	190	233		0.4	—	—
3.45	2,3-Di-*O*-methylglucitol	118	261			0.4	0.8	0.8
3.63	2,3-Di-*O*-methylgalacitol	118	261			0.4	1.1	1.1
3.87	2,4-Di-*O*-methylgalactitol	118	189			5.7	7.3	6.6
4.72	2-Mono-*O*-methylgalactitol	118				1.5	3.8	4.3

RTMG—R_t relative to 1,5,Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol.

Methylation analysis of uronic acid-containing polysaccharides may lead to a significant degradation. For this reason, and also because the MS fragmentation pattern of partly methylated hexuronic acid acetates has not been fully elucidated, the uronic acid units in the permethylated glycoprotein were reduced to the corresponding neutral sugar residues. Half of fraction B was carboxyl-reduced before hydrolysis to compare the non-reduced methylated with the reduced methylated B. Fraction A was not reduced as it is almost devoid of uronic acid.

Incorporation of deuterium at C-6 of the uronic acid by reduction of its Me ester with $NaBD_4$ made it possible to distinguish between methylated hexitol acetates originating from galactose and from the carboxyl-reduced galacturonic acid.

The results (Table 3) are in good agreement with those obtained previously [1]. Both fractions contain mainly arabinofuranose end groups but also gluco- and/or manno- and galactopyranose end groups. The 2,3-di-*O*-methylarabinitol formed shows that the chains consist of (1 → 4)-linked arabinopyranose and/or (1 → 5)-linked arabinofuranose. This is consistent with glycerol being formed by Smith-degradation. Rhamnose, only present in B, appears in the methylation analysis as 3,4-di-*O*-methylrhamnitol and as 2-mono- and 3-mono-*O*-methylrhamnitol.

The amount of 2,4,6-tri-*O*-methylgalactitol, 2,4-, 2,6 and 4,6-di-*O*-methylgalactitol formed is in accordance with the resistance to periodate oxidation displayed by galactose, as demonstrated by the Smith-degradation. A large peak (T_{TMG} 2.3) arising from fraction B (Table 3) may most likely be ascribed to partly methylated galacturonic

Table 4. Destruction of serine and threonine and formation of alanine after $NaOH/NaBH_4$ treatment

	A	A'	%Δ
Serine	9.84	8.84	−10.16
Threonine	7.21	6.84	−5.13
Alanine	11.02	12.16	+10.34
Glycine	11.36	11.32	−0.35

A': Fraction A after treatment with $NaOH/NaBH_4$. The results are given in mol% of total amino acids.

acid or to a uronic acid degradation product, since this peak is absent in fraction A and decreases by more than 70% after carboxyl-reduction of fraction B. However, its MS fragmentation pattern has so far not been interpreted. By carboxyl-reduction the galacturonic acid would be reduced to galactose, thus giving an increase in this sugar. However, Table 3 does not demonstrate this clearly. Only the content of 2-mono-*O*-methylgalactitol showed any increase, revealing (1→3)(1→4)-linked galacturonic acid residues.

In the structural examination of a glycoprotein it is of primary importance to determine the mode of linkage between carbohydrate and protein. Recently Lamport *et al.* [2] reported the existence of a serine-*O*-galactoside bond in a glycopeptide isolated from tomato cell wall; also other workers have established the presence of this linkage in plant glycoproteins [4, 5].

An *O*-glycosidic linkage between a sugar and the hydroxyl group of peptide-linked serine or threonine will be labile to alkali, resulting in β-elimination of the carbohydrate moiety. By performing the alkali treatment in the presence of excess borohydride [2] the carbohydrate residues liberated would be expected mainly to terminate in the respective sugar alcohol. Of the unsaturated amino acid units formed in the β-elimination reaction the α-aminopropenoic acid residues (from serine) will be hydrogenated quantitatively to give alanine, whereas the α-aminobutenoic acid residues (from threonine) will remain largely unchanged unless a catalyst (e.g. $PdCl_2$) is added [10].

Treatment of the *Cannabis* glycoproteins with $NaOH–NaBH_4$ resulted in a *ca* 10% increase in alanine, a corresponding decrease in serine, and a *ca* 5% decrease in the threonine content (Table 4). The content of the other amino acids changed insignificantly, exemplified by glycine.

Only one sugar alcohol, galactitol, was formed during the reductive base-catalyzed hydrolysis of the glycoprotein. As the TMSi-ethers of glucitol and galactitol have identical GLC R_ts, the identity of galactitol was established by GLC of its peracetylated derivative. This suggests that galactose was the only sugar involved in linking carbohydrate to serine/threonine in the polypeptide chain. This was found for both fraction A and B.

Further evidence for the nature of the glycopeptide linkage was obtained by treatment of A and B with dilute alkali in the presence of Na_2SO_3. By this procedure glycosylated serine and threonine residues are converted to cysteic acid and to 2-amino-3-sulphonylbutyric acid, respectively [11]. As these sulphonic acids are not separated on the amino acid analyzer [12] the reaction products were silylated and analysed by GLC. Although the TMSi-sulphonic acids had a low response factor on GLC, the $NaOH–Na_2SO_3$-treated samples gave a peak with the same R_t as TMSi-cysteic acid but no peak corresponding with TMSi-2-amino-3-sulphonylbutyric acid was observed. Thus clear evidence is presented that serine is involved in glycopeptide bonding whereas a corresponding function of threonine is still questionable.

Planter and Carlson [13] have described an alternative method for determination of *O*-glycosidic linkages to serine and threonine by alkaline degradation. Here the rate of β-elimination is followed by measuring the increase in *A* at 240 nm. Subsequent acid hydrolysis yields pyruvate and α-ketobutyrate from glycosylated serine and threonine, respectively, which can then be measured spectrophotometrically in the presence of lactic acid dehydrogenase and NADH. However, this method proved unsatisfactory in our hands.

In addition to serine-*O*-galactoside linkages evidence has been obtained previously [1] for the presence in the *Cannabis* glycoprotein also of a glycopeptide bond between hydroxyproline and a neutral sugar, possibly arabinose, although this still awaits experimental proof. The present work has further confirmed the complexity of the carbohydrate portion of the *Cannabis* leaf glycoprotein. Although the detailed molecular architecture is too intricate for suggesting a definite structure, the Smith-degradation and methylation analysis clearly show that arabinose is located mainly in the outer part of the molecule, constituting arabinofuranose end groups and (1→4)-linked arabinopyranose and/or (1→5)-linked arabinofuranose units. In contrast the other major component sugar, galactose, makes up most of the interior part of the molecule, being (1→3)-linked, and serving as branch points.

In our previous work on a different batch of South African *Cannabis* leaf glycoprotein [1] the fractions corresponding to A and B had a lower MW and also contained hexosamine. The reason for the discrepancy between the previous and the present material is not clear, but may possibly be related to the stage of leaf development.

EXPERIMENTAL

Materials. Cannabis sativa L. (South Africa) was grown in the Botanical Garden at the University of Oslo. Identification of the plants was carried out by Prof. A. Nordal, Dept. of Pharmacognosy, Institute of Pharmacy, University of Oslo. The leaves were harvested and pretreated with organic solvents as previously described [1].

Extraction and purification. The pretreated material (200 g) was suspended in H_2O (2 l.) and stirred for 3 hr at 50° and 18 hr at room temp. The extract was filtered by suction, concd, dialysed and the retentate fraction isolated by freeze-drying (yield: 4 g). The lyophilized polymer (1 g) was applied to a DEAE-cellulose column and eluted with Tris–HCl buffers as before [1]. Yield of A: 125 mg, B: 245 mg.

General methods. IR spectra were recorded for samples (1–2 mg) in KBr discs. Estimation of carbohydrate and protein, and measurement of optical rotation, were carried out as described in [1], but uronic acid was estimated by the method of ref. [14]. MWs were estimated on a column of Sepharose 4B (36 × 2.5 cm). The column was calibrated with dextrans of the T Dextran series and eluted with 0.025 M Tris–HCl buffer pH 7.2; 3.2 ml fractions being collected. All GLC analyses were performed on a FID instrument using N_2 as the carrier gas. For GC–MS the column was coupled with a Varian CH-7 mass spectrometer.

O-Acetyl groups were estimated by the method of ref. [6]. The products (20 mg) were analysed by GLC on a column

(400 × 0.2 cm) of 10% SP-1200/1% H_3PO_4 on Chromosorb at 90° with propionic acid as int. stand.

Reducing end groups [7]. The sample (20 mg) in H_2O (2 ml) was incubated with $NaBH_4$ (40 mg) at 40° for 18 hr. The soln was neutralized with 2 M HOAc (1.5 ml) and subjected to repeated evaporations with MeOH for the removal of boric acid. The product was hydrolysed with 2 N H_2SO_4 (1 ml) for 5 hr at 100°, followed by neutralization of the soln with $BaCO_3$, filtration, and treatment of the filtrate with Dowex 50 × 8 (H^+). The residue was treated with Dowex 1 × 8 (OH^-), evapd to dryness, and acetylated with Ac_2O–Py (1:1) for 1 hr at 100°. The acetylated product was analysed by GLC isothermally at 205° on a glass column (200 × 0.25 cm) packed with 3% OV-225 on Varaport.

Incubation with pronase. The sample (20 mg) was incubated with pronase (Pronase B Grade 45.000 P.U.K./g. *ex. Streptomyces griseus* (Calbiochem)) (5 mg) in 0.05 M Tris–HCl buffer containing 0.01 M $CaCl_2$, pH 7.85 (5 ml) for 24 hr at 37°. Toluene was added to prevent microbial growth. The digest was filtered, concd to dryness and applied on the Sepharose column for gel filtration.

Smith-degradation. The sample (400 mg) was oxidized with 50 mM $NaIO_4$ (100 ml) at 5° in the dark for 26 hr. The consumption of periodate was followed spectrophotometrically at 223 nm [15]. The reaction was stopped by addition of ethylene glycol and the soln dialysed for 6 hr. $NaBH_4$ (120 mg) was added and the mixture left at room temp. for 18 hr, prior to acidification (HOAc), dialysis 5–6 hr and lyophilization (yield A: 322 mg, B: 305 mg). Hydrolysis was achieved in 0.05 N H_2SO_4 (15 ml) at 80° for 70 min. The hydrolysate was neutralized (0.1 M NaOH) and dialysed. The retentate fractions and the dialysable fractions (deionized with Zerolit DM-F, CO_3^{2-}) were finally freeze-dried. For the second Smith-degradation (100 mg) the 3 steps (oxidation–reduction–mild hydrolysis) were repeated. The products resistant to periodate were analysed by GLC after methanolysis [1] and TMSi-derivativatization on a 3% SE-52 column (390 × 0.2 cm) on Varaport. The temp. programme used started at 145° with an increase of 1°/min for 10 min, followed by an increase of 2°/min to 175°, and finally 4°/min to 225°. The low MW alcohol fractions (dialysable fractions) were analysed as acetylated derivatives on the 3% OV-225 column (200 × 0.25 cm) at 80° for 10 min, followed by an increase of 4°/min to 210°.

Methylation analysis. The procedure of Hakomori was performed as already described [1], except that a modification was introduced for the acidic fraction B. After methylation with MeI half of the material B was carboxyl-reduced with $NaBD_4$ (10 mg) for 18 hr. It was neutralized (HOAc) and evapd to dryness for the removal of boric acid. The methylated carboxyl-reduced B and the methylated non-reduced B were both hydrolysed with HCO_2H, reduced with $NaBD_4$ and acetylated. The partially methylated alditol acetate fractions were analysed by GC–MS on an OV-225 column (400 × 0.25 cm) at 210°.

Alkaline degradation in the presence of $NaBH_4$. The samples (A and B, 200 mg) dissolved in 2 M $NaBH_4$ (20 ml) and 0.25 M NaOH (20 ml) was kept for 6 hr at 50°. Excess $NaBH_4$ was decomposed with 3 M HCl. Boric acid was removed as Me borate by additions of MeOH and subsequent evaporations. The material was then desalted by chromatography on a Bio-Gel P-2 column by elution with 10% EtOH in H_2O. The low MW fraction was concd, lyophilized and analysed by GLC on the SE52 column after methanolysis and silylation [1] exactly as for the analysis of the products resistant to periodate. Part of the sample was hydrolysed, acetylated and chromatographed at 205° on the OV-225 (200 × 0.25 cm) column. Another part of the low MW fraction of fraction A from the Bio-Gel column was hydrolysed with 6 M HCl for 20 hr at 110° under N_2 and analysed on a BioCal BC 200 automatic amino acid analyzer.

Alkaline degradation in the presence of Na_2SO_3. The sample (15–20 mg) was dissolved in a mixture of MNa_2SO_3 (1.5 ml) and 0.1 M NaOH (1.5 ml) and kept for 40 hr at room temp. The NaOH–Na_2SO_3-treated samples were neutralized with 2 M HCl and dialysed for 2 hr. The residue was hydrolysed with 6 M HCl, filtered and concd. The hydrolysate was applied on a Dowex 50 × 8 (200–400 mesh) H^+ column and eluted with 0.2 M Py acetate buffer pH 3. The eluate was subjected to several evaporations to dryness. Trimethylsilylation was performed with BSTFA (*N,O-bis*(trimethylsilyl) trifluoro-acetamide) according to ref. [12], although anhydrous Py was used as solvent instead of MeCN. The TMSi samples were analysed by GLC isothermally at 145° on a 3% SE-52 column (200 × 0.2 cm) on Varaport.

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