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STRUCTURAL STUDIES OF WATER-SOLUBLE GLYCOPROTEINS FROM *Cannabis sativa* L.*

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ABSTRACT

Two carbohydrate–protein fractions, isolated from *Cannabis sativa* L. by extraction with water and chromatography on DEAE-cellulose, contained arabinose, galactose, glucose, mannose, galacturonic acid, 2-acetamido-2-deoxyglucose, and 2-acetamido-2-deoxygalactose. The structure of the carbohydrate moieties was investigated by methylation analysis and Smith degradation. A high percentage of end-groups indicates a large degree of branching, glucose and galactose being the main branch-points, linked at C-3 and C-6. The hexoses are also present as unbranched residues in the chain, largely as (1→3)- and (1→4)-linked units and as end-groups. Arabinofuranosyl units constitute the main part of the non-reducing end-groups, and are also present as part of the chain. The polysaccharide chains are probably linked to protein through the hydroxyl group of hydroxyproline.

INTRODUCTION

The leaves of *Cannabis sativa* L. contain a significant amount of water-soluble, non-dialysable, carbohydrate–protein material. Previous investigations of the carbohydrates in Cannabis have been focused[1,2] largely on the compounds of low molecular weight. We now describe the purification, characterization, and some structural studies of two carbohydrate–protein fractions present in Cannabis leaves, as a part of the comprehensive study of the constituents of *Cannabis sativa* L. initiated by the Division of Narcotic Drugs, United Nations, Geneva.

RESULTS AND DISCUSSION

The water-soluble, non-dialysable extract obtained in ~1% yield from leaves of *Cannabis sativa* contained carbohydrate and protein. By chromatography on DEAE-cellulose, the material was separated into two fractions (*A*, $[\alpha]_D^{20}$ −33°; and *B*, $[\alpha]_D^{20}$ +56°). Fraction *A* passed unretarded through the column in 0.05M Tris-

*Dedicated to the memory of Sir Edmund Hirst, C.B.E., F.R.S.

chloride buffer, whereas fraction *B* was eluted with the same buffer containing 0.25M sodium chloride (Fig. 1). By continued elution of the column with buffer containing higher concentrations of salt (0.5M and M NaCl), a very small proportion of strongly coloured material was obtained; this was not further investigated.

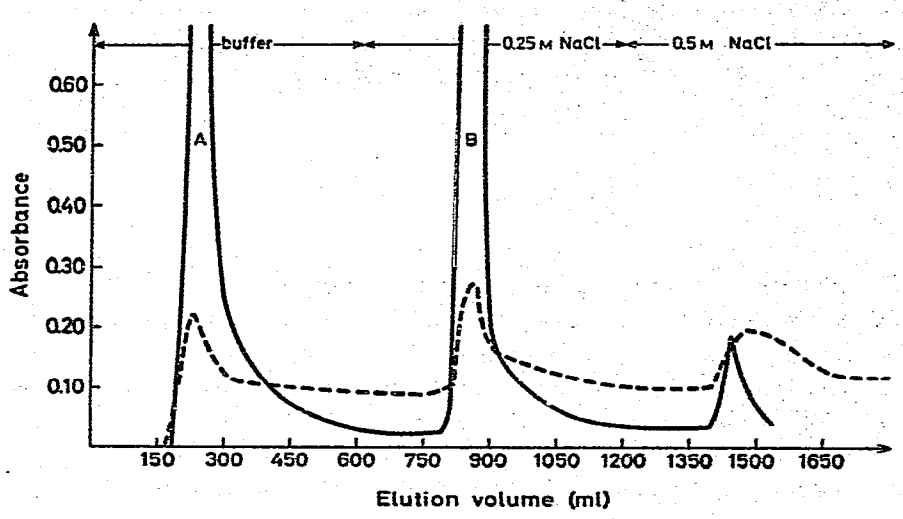

Fig. 1. Chromatography of Cannabis raw-extract on DEAE-cellulose (chloride form). The column (70 × 3.0 cm) was eluted with 0.05M Tris-chloride buffer (pH 6.7) and the same buffer containing 0.25M NaCl and 0.5M NaCl, as indicated by the arrows: ——, carbohydrate; ——, protein.

Fractions *A* and *B* both contained protein (Fig. 1), and when the two fractions were subjected to gel filtration on Sepharose 4B, the protein remained mainly associated with the carbohydrate-containing material (Figs. 2 and 3), suggesting the possibility that both were glycoproteins. Fraction *A* gave an almost symmetric elution pattern in contrast to the more heterogeneous fraction *B* (Fig. 3).

Table I shows the chemical composition of *A* and *B*, and also the carbohydrate composition of Smith-degraded *A*. Fraction *B*, originally retained on the DEAE-cellulose column, contained uronic acid and a higher proportion of protein than *A*. The identity of the uronic acid was established by g.l.c. to be galacturonic acid, which is a characteristic component of the pectic polysaccharides constituting part of the primary cell-wall in plants. The careful studies of Albersheim and co-workers[3,4] on primary plant cell-walls strongly suggest the presence of a covalent linkage between protein and pectic polysaccharides.

Hexosamines normally do not occur in polysaccharides of higher plants. However, 2-acetamido-2-deoxyglucose has been found as a constituent of various plant glycoproteins[5]. In the carbohydrate polymer of Cannabis, the presence of both 2-acetamido-2-deoxyglucose and 2-acetamido-2-deoxygalactose was demonstrated by chromatography (p.c., t.l.c., and g.l.c.) and on the amino acid analyzer[6]. The lower

value for hexosamines found by the amino acid analysis is probably due to partial degradation of the amino sugars, known to occur under strong hydrolytic conditions[7].

The molecular weights of *A* and *B* were estimated approximately by gel filtration on a calibrated column of Sepharose 4B. Calculations based on the elution volume corresponding to the peak of the curve [153 ml for *A* (Fig. 2) and 134 ml for *B* (Fig. 3)] gave values of ~12,000 for *A* and ~34,000 for *B*.

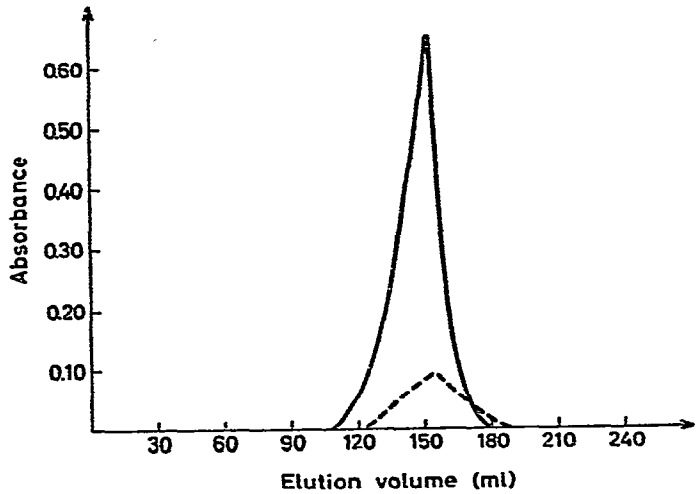

Fig. 2. Gel filtration of fraction *A* on a column (40×2.5 cm) of Sepharose 4B with 0.025M Tris-chloride buffer (pH 7.2): ——, carbohydrate; – – – –, protein.

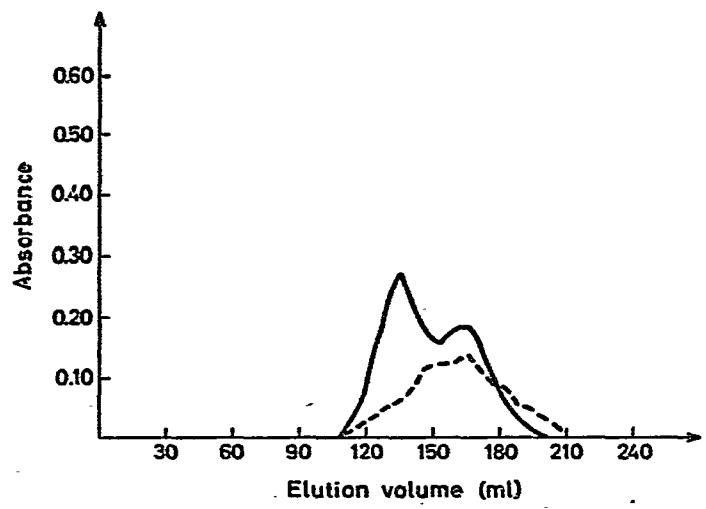

Fig. 3. Gel filtration of fraction *B* on a column (40×2.5 cm) of Sepharose 4B with 0.025M Tris-chloride buffer (pH 7.2): ——, carbohydrate,; – – – –, protein.

TABLE I

CHEMICAL COMPOSITION OF FRACTIONS *A* AND *B*

Component	A	B	A_1[a]	A_2[a]	*Component*	A	B
Total carbohydrate[1,4,b,c]	63	63			Asp[b]	10.8	13.7
Protein	16	27			Thr	7.5	11.4
N-Acetylhexosamine	7.6	3.4			Ser	9.5	16.5
Uronic acid	traces	9.2			Glu	5.7	13.1
Ara	19.4	22.7	2.5	traces	Pro	traces	6.9
Man	traces	7.3	—	—	Gly	3.3	7.9
Gal	10.8	24.6	15.0	3.5	Ala	3.7	12.5
Glc	9.5	6.7	3.6	4.7	0.5 Cys	0.4	—
GalA	—	n.d.	—	—	Val	0.7	3.9
GalNAc	3.1	1.6	6.9	8.3	Leu	traces	—
GlcNAc	6.7	3.7	16.7	9.5	Ile	traces	—
					Lys	—	1.0
					Hyp	7.0	17.0
					GalNAc	18.6	2.5
					GlcNAc	53.4	9.2

[a]Products of first (A_1) and second (A_2) Smith degradations of *A*. [b]The results for carbohydrate and protein are given in %, and for amino acids in μg/mg; n.d. = not determined. [c]Does not include hexosamine.

Mild treatment of *A* and *B* with acid (5mM H_2SO_4 at 100° for 1 h) liberated arabinose, together with a minute proportion of galactose, suggesting that arabinofuranosyl units occupy terminal positions. Incubation of *A* or *B* with β-D-galactosidase released galactose from both fractions, thus indicating the presence also of terminal β-D-galactosyl units.

Fraction *A* consumed 0.62 mol of periodate/mol of "anhydrohexose" unit under conditions selected to avoid over-oxidation. Two consecutive Smith degradations of *A* led to an extensively degraded material, and the composition of periodate-resistant carbohydrate after the first and second degradation is given in Table I. It appears that most of the neutral sugar residues are destroyed by periodate, whereas the hexosamines survive to a larger extent. The amino sugars may be localized in the inner core-region of the polymer, perhaps near a possible link to the protein moiety. The alcohol fractions of low molecular weight obtained after the two Smith degradations of *A* were analysed by g.l.c. Both fractions contained glycerol and ethylene glycol, but only traces of other compounds. Judging from the peak areas, the ratios of glycerol to ethylene glycol were 5:1 and 0.9:1 after the first and second Smith degradations, respectively. The glycerol arises mainly from arabinofuranose and also from galacto- and gluco-pyranose end-groups, and ethylene glycol from arabinopyranose end-groups.

Methylation of *A* and *B*, followed by acid hydrolysis, borodeuteride reduction, and combined g.l.c.–m.s. of the derived alditol acetates, revealed the presence of the compounds listed in Table II. As response factors for the various methylated alditol acetates were not available, the g.l.c. peak area was used as an indication of their

relative proportions. The amount of 2,3,5-tri-*O*-methylarabinitol shows that arabinofuranose is the main terminal sugar in *A* and *B*. In addition, the formation of tetra-*O*-methyl-galactitol and -glucitol indicates galacto- and gluco-pyranose end-groups, respectively. Mannose end-groups may exist in *B*, as tetra-*O*-methyl-glucitol and -mannitol have identical retention times.

TABLE II

ALDITOL ACETATES OBTAINED FROM METHYLATED FRACTIONS *A* AND *B*

T[a]	*Alditol acetate*	*Primary fragments* (m/e)	*Rel. amount*[b]	
			A	B
0.49	2,3,5-Tri-*O*-methylarabinitol	45, 118, 161	6.0	7.6
0.61	2,3,4-Tri-*O*-methylarabinitol	117, 118, 161, 162	0.8	0.4
0.84	3,5-Di-*O*-methylarabinitol	45, 161, 190	0.4	0.9
0.92	2,5-Di-*O*-methylarabinitol	45, 118, 233	1.2	1.3
1.00	2,3,4,6-Tetra-*O*-methylglucitol/mannitol	45, 118, 161, 162, 205	1.0	0.6
1.09	2,3-Di-*O*-methylarabinitol	118, 189	2.5	1.9
1.12	2,3,4,6-Tetra-*O*-methylgalactitol	45, 118, 161, 162, 205	1.0	1.0
1.63	2,4,6-Tri-*O*-methylglucitol	45, 118, 161, 234	0.6	—
1.73	2,4,6-Tri-*O*-methylmannitol	45, 118, 161, 234	—	2.2
1.84	2,4,6-Tri-*O*-methylgalactitol	45, 118, 161, 234	0.5	2.0
1.92	2,3,6-Tri-*O*-methylgalactitol	45, 118, 162, 233	—	traces
2.06	2,3,6-Tri-*O*-methylglucitol	45, 118, 162, 233	1.8	—
4.14	2,4-Di-*O*-methylglucitol	118, 189	1.2	1.8
4.34	2,4-Di-*O*-methylgalactitol	118, 189	0.6	—

[a]Retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol. [b]Area of the peak by g.l.c. relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol.

Thus, the results of methylation analysis are in agreement with the observed release of arabinose on mild hydrolysis with acid, and of galactose on incubation with β-D-galactosidase, and also with the formation of glycerol as the major alcohol in the first Smith degradation. The smaller proportion of ethylene glycol is consistent with terminal arabinopyranosyl units, as indicated by the presence of 2,3,4-tri-*O*-methyl-arabinitol. The formation of 2,3-di-*O*-methylarabinitol reveals (1→4)-linked arabinopyranose and/or (1→5)-linked arabinofuranose residues. Both would also give rise to glycerol on Smith degradation, provided that the sugar linked to the 4-(or 5-)position of the arabinose residue is also oxidised by periodate. As sodium borodeuteride was used for the reduction, it was possible to differentiate between 2,3-di-*O*-methyl- and 3,4-di-*O*-methyl-arabinitol by g.l.c.–m.s. Apart from being end groups, arabinofuranose is also linked in the chain, as shown by the formation of 2,5-di-*O*-methyl- and 3,5-di-*O*-methyl-arabinitol.

The high proportion of end groups indicates a large degree of branching; the formation of 2,4-di-*O*-methyl-glucitol and -galactitol shows the presence of glucose and galactose at branch points linked at C-3 and C-6. The formation of 2,4,6-tri-*O*-methylhexitols indicates (1→3)-linked hexose residues. This is consistent with the

increase in galactose content of the product obtained after the first Smith degradation, and the increase in glucose content after the second Smith degradation of fraction *A*. Table II demonstrates the similarity between *A* and *B*; they give mainly the same methylated sugars, although in somewhat different proportions. The hexosamines were not detected as methylated derivatives. However, the OV-225 column is not entirely satisfactory for chromatography of partially methylated 2-amino-2-deoxy-hexitol acetates, as they tend to be retained on the column[8].

The galacturonosyl residues in the methylated fraction *B* could not be analysed by the g.l.c. procedure employed, as they do not form sufficiently volatile, partially methylated galactonic acid acetate derivatives. The possibility of degradation should not be overlooked during the application of the present methylation method to a glycoprotein. The gas chromatogram of the partially methylated alditol acetates contained certain unidentified peaks, some of which might be ascribed to degradation products.

The close association between carbohydrate and protein in *A* and *B* suggests the presence of a glycoprotein. A number of plant glycoproteins have been isolated recently[9–13]. However, their isolation was usually achieved by the use of alkali or proteolytic enzymes, in contrast to the water-soluble material investigated in this report. In his studies of plant cell-wall glycoproteins, Lamport[9,10] demonstrated the presence of an *O*-glycosyl linkage between arabinose and hydroxyproline in the polypeptide chain. Hydroxyproline-rich glycoproteins seem to play a fundamental

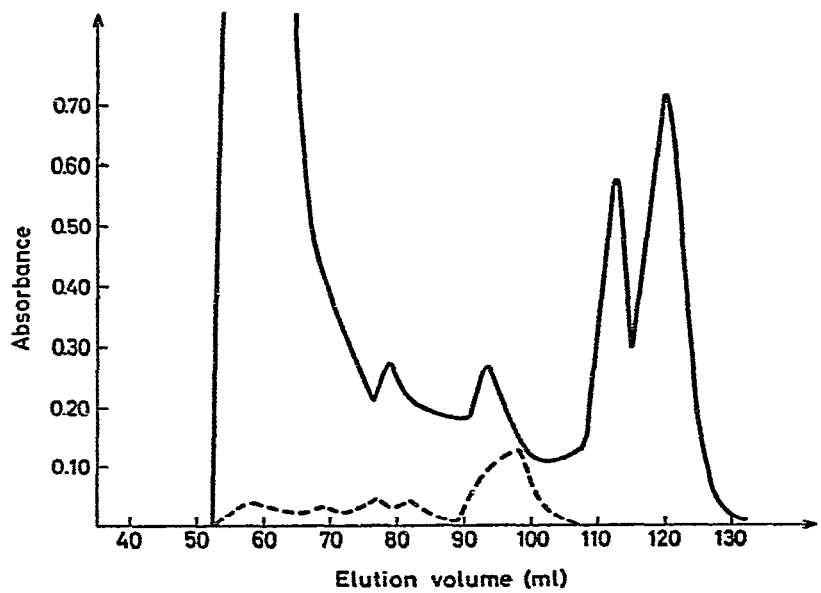

Fig. 4. Gel filtration of Cannabis raw-extract, after alkaline hydrolysis and neutralisation, on a column of Bio-Gel P-2 (34×2.5 cm) by elution with 0.1M acetic acid: ——, carbohydrate; – – –, hydroxyproline. Fractions I, 50–75 ml; II, 75–90 ml; III, 90–108 ml.

role in plant cell-wall structure. Recent work has shown the occurrence of hydroxyproline arabinosides in cell walls of many plants, ranging from flowering plants to the green alga *Chorella*[9]. Table I shows that hydroxyproline occurs in both *A* and *B*, and alkaline hydrolysis of the non-purified extract was performed to study whether this linkage occurs in the polymer. The neutralised hydrolysate was subjected to gel filtration, and the eluate analysed for carbohydrate and non-peptide-bound hydroxyproline (Fig. 4). The hydroxyproline-containing fractions (I, II, and III) were further fractionated by paper electrophoresis. Response to the isatin spray showed the presence, in each fraction, of a hydroxyproline-containing component. No aniline oxalate-positive spots were revealed, while the periodate–benzidine reagent gave a spot coinciding with the isatin-positive spot. These preliminary experiments only indicate the presence of an *O*-glycosyl bond through hydroxyproline to the polypeptide chain. The nature of the carbohydrate–protein linkage is being further investigated.

EXPERIMENTAL

Materials. — *Cannabis sativa* L. was grown from seeds (UNC 335 South Africa) in the Botanical Garden at the University of Oslo during the summer of 1972. The dried leaves were milled, and subjected to Soxhlet extraction with light petroleum (b.p. 60–80°) and chloroform, successively. The residual plant material was stored in a dry place until required.

β-D-Galactosidase (Grade IV, purified from *Escherichia coli*) was obtained from Sigma Chemical Co., and DEAE-Cellulose (Whatman D-11) from Koch–Light Labs. Ltd. Dextrans T40 and T250 and Sepharose 4B were products of Pharmacia Fine Chemicals, and Bio-Gel P-2 (200–400 mesh) was obtained from Bio-Rad Labs.

General methods. — Optical rotations were measured for aqueous solutions at 20° with a Perkin–Elmer Model 141 polarimeter. Concentrations were carried out under diminished pressure below 50° on a rotary evaporator. Dialysis was performed with magnetic stirring against distilled water; toluene was added to prevent microbial growth.

Amino acids were analysed on a BioCal BC 200 automatic amino acid analyzer after hydrolysis of the sample (10 mg) with 6M hydrochloric acid (2 ml) for 20 h at 110° under nitrogen.

Carbohydrate was determined by the phenol–sulfuric acid method[14], with a mixture (1:1.5:2) of D-glucose, D-galactose, and L-arabinose as standard. Hexosamine was estimated by the Elson–Morgan reaction, as modified by Gatt and Berman[15], with 2-amino-2-deoxy-D-glucose as standard. Uronic acid[16] was determined with D-glucurono-6,3-lactone as standard.

Protein was determined by the method of Lowry *et al.*[17], with bovine serum albumin as standard. Free and *O*-substituted hydroxyproline were assayed by the method of Kivirikko[18], after alkaline hydrolysis.

High-voltage electrophoresis was performed in an AB Analysteknik apparatus

on Whatman No. 1 paper in 0.1M hydrochloric acid–citrate buffer (pH 2) at a potential of 60 V/cm for 2 h.

Chromatography. — Paper chromatography was performed on Whatman No. 1 paper with (*A*) ethyl acetate–pyridine–acetic acid–water (5:5:1:3) and (*B*) ethyl acetate–pyridine–water (8:2:1). As spray reagents, aniline oxalate and ninhydrin were used for the detection of reducing sugars and amino acids, respectively. Carbohydrate was detected by periodate–benzidine[19], and hydroxyproline by isatin[20].

G.l.c. was performed on a Varian 1400 gas chromatograph equipped with a flame-ionisation detector; nitrogen was the carrier gas. The following columns were used.

(*a*) 3% of SE-52 on Varaport 30 (200 × 0.2 cm). The temperature programme for the trimethylsilylated methyl glycosides was as reported previously[6]. Trimethylsilylated alcohols from the Smith degradations were chromatographed with a temperature programme starting at 70° and increasing at 2°/min. Samples incubated with β-D-galactosidase were trimethylsilylated, and chromatographed at 180°.

(*b*) 3% of OV-225 on Varaport 30 (400 × 0.25 cm). Partially methylated alditol acetates were chromatographed under isothermal conditions (210°). For g.l.c.–m.s., the column was coupled with a Varian CH-7 mass spectrometer.

Methanolysis. — The dried sample (2–3 mg) was heated with M hydrochloric acid in anhydrous methanol in a sealed tube for 20 h at 80°; mannitol was used as internal standard. Trimethylsilylation of the resulting methyl glycosides was performed with pyridine, hexamethyldisilazane, and chlorotrimethylsilane (5:2:1, v/v) according to Sweeley *et al.*[21].

Hydrolysis of the polysaccharide moiety. — The sample (10 mg) was hydrolysed in M sulfuric acid (2 ml) in a sealed tube for 5 h at 100°. The hydrolysate was neutralised with barium carbonate, and filtered, and the filtrate treated with Dowex 50 (H^+) resin prior to evaporation to dryness.

Gel filtration. — The sample (25 mg) in 25mM Tris-hydrochloric acid buffer (pH 7.2) was applied to a column (40 × 2.5 cm) of Sepharose 4B and eluted with the same buffer; 3-ml fractions were collected. The carbohydrate and protein contents were detected as earlier described[14,17]. For approximate estimation of molecular weight, the column was calibrated with Dextrans T40 (mol. wt. 21,000) and T250 (mol. wt. 90,000). The elution volume corresponding to the peak of the elution curve was plotted against the logarithm of the molecular weight.

Incubation with β-D-galactosidase. — The sample (5 mg) in 0.1M phosphate buffer (pH 7.0, 3 ml) was incubated for 15 h at 37° under toluene with 0.1 ml of β-D-galactosidase suspension (in 2.2M ammonium sulfate). The sample was then deionised with Zerolit DM-F (CO_3^{2-}) resin, and evaporated to dryness. The product was analysed by p.c. and g.l.c.

Periodate oxidation. — The sample (16 mg) was oxidised with 25mM sodium metaperiodate in 0.5M acetate buffer (pH 4.0, 10 ml) at 5° in the dark. The consumption of periodate was followed by the method of Avigad[22], and the reaction stopped after 48 h by addition of ethylene glycol.

Smith degradation. — Oxidation of the sample (150 mg) was achieved in 25mM sodium metaperiodate (100 ml) at 5° in the dark. After 48 h, the oxidation was stopped by addition of ethylene glycol, and the solution dialysed overnight. Sodium borohydride (50 mg) was added, and the mixture kept for 5 h at room temperature prior to acidification with acetic acid and subsequent dialysis. The solution was evaporated to dryness, and hydrolysed with 25mM sulfuric acid for 70 min at 80°. After neutralisation (0.1M NaOH) and dialysis, the retentate was freeze-dried (yield: 20 mg). The dialysable fraction was concentrated, deionised with Zerolit DM-F (CO_3^{2-}) resin, and evaporated to dryness (yield: 70 mg).

For the second Smith degradation (15 mg), the three steps (oxidation–reduction–mild hydrolysis) were repeated (yield: retentate, 4 mg; dialysate, 9 mg).

The products resistant to oxidation were analysed by g.l.c. after methanolysis and trimethylsilylation. The alcohols (dialysable fraction) obtained from the two degradations were silylated directly prior to g.l.c.

Methylation analysis[23]. — The sample (15 mg) was dissolved in dry methyl sulfoxide (1.5 ml) in a 5-ml serum bottle. The bottle was flushed with nitrogen, and 2M methylsulfinyl anion[24] in methyl sulfoxide (1.0 ml) was added. After continuous stirring for 4 h, methyl iodide (0.5 ml) was added dropwise from a syringe, and the stirring was continued for 1 h. The resulting solution was diluted with water, dialysed, and evaporated to dryness.

The methylated polymer was hydrolysed in 2 ml of 90% formic acid (for 4 h at 100°, followed by 1 h at 100° after the addition of 2 ml of water). After removal of formic acid by evaporation to dryness, the residue was dissolved in water (2 ml), treated with sodium borodeuteride (10 mg) overnight in a refrigerator, and then neutralised with acetic acid. The resulting, partially methylated alditols were treated with acetic anhydride (1 ml) for 1 h at 100° in a sealed tube. Acetic anhydride and acetic acid were removed by repeated evaporations with toluene. The acetylated alditols were extracted with chloroform, and the extract was filtered and evaporated to dryness prior to analysis by g.l.c.–m.s.[25,26].

Attempted alkaline degradation. — Cannabis raw extract (130 mg) was treated with refluxing, saturated, aqueous barium hydroxide (10 ml) for 8 h[9]. After neutralisation with 0.5M sulfuric acid and filtration, the solution was evaporated to dryness, and the residue was dissolved in 2 ml of 0.1M acetic acid. This was applied to a column (34 × 2.5 cm) of Bio-Gel P-2 (200–400 mesh), and eluted with 0.1M acetic acid. The fractions (2.4 ml) were assayed for non-peptide-bound hydroxyproline[18] and carbohydrate[14]. Appropriate fractions were combined, and subjected to high-voltage electrophoresis.

Isolation and fractionation of the raw extract. — The pretreated plant material (100 g) was suspended in water (2 l), and stirred for 2 h at ~50° and then at room temperature overnight. The extract was filtered, concentrated, and dialysed, and the retentate fraction was isolated by freeze-drying (yield: 1.0 g).

The lyophilized polymer (0.5 g) was dissolved in 0.05M Tris-hydrochloric acid buffer (pH 6.7, 25 ml) and applied to a column (70 × 3.0 cm) of DEAE-cellulose

(chloride form). The column was eluted with the same buffer, fractions of 15 ml being collected. When the eluate contained no more carbohydrate[14], the column was eluted with the Tris-chloride buffer containing 0.25M sodium chloride. Carbohydrate-positive fractions were combined and concentrated. After dialysis, the two fractions (*A*, 85 mg; *B*, 95 mg) were isolated by freeze-drying.

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