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Polysaccharides of Lichens and Fungi. VI.[1] Antitumour Active Polysaccharides of Lichens of Stictaceae

Kunio Takahashi, Tadahiro Takeda, Shoji Shibata,[2a]
Motoko Inomata, and Fumiko Fukuoka[2b]

Faculty of Pharmaceutical Sciences, University of Tokyo,[2a] and National Cancer Center Research Institute[2b]

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The water-soluble polysaccharides of several kinds of lichens of Stictaceae, especially *Lobaria orientalis*, have been studied chemically in the relation with their host-mediated antitumour activities against sarcoma 180 in mice. *L. orientalis* contains at least two glycopeptides, LOF-1 and -2, which are the first example of the antitumour active glycopeptides found in lichens. The carbohydrate moiety of LOF-1 consists of 1→6 glucan and 1→3 mannan, which is linked with serine and threonine of the peptide part by O-glycosyl linkages.

In connection with our previous studies[1] on the antitumour activities and the chemical structures of lichen polysaccharides, the present paper describes the structural study of the glycopeptides isolated from some lichens of Stictaceae, *e.g. Lobaria orientalis*, *L. isidiosa*, *L. pseudopulmonaria*, *L. linita*, *L. japonica*, *Sticta gracilis* and *S. wrightii*.

The properties of these samples of lichen polysaccharides closely resemble each other in nitrogen contents and specific rotations as shown in Table I.

Table I. Some Properties of the Polysaccharide Preparations from Lichens

Lichens	*L. orientalis*	*L. isidiosa*	*L. pseudo-pulmonaria*	*L. linita*	*L. japonica*	*Sticta gracilis*	*S. wrightii*
Crude polysaccharide	LO-1	LI-1	LP-1	LL-1	LJ-1	SG-1	SW-1
N (%)	2.13	2.45	2.66	2.82	3.41	2.85	2.77
Yield (%)	5.1	11.5	7.4	8.9	5.7	1.7	2.8
$[\alpha]_D$	+45	+40	+28	+43	+33	+49	+47

Table II. Antitumour Activities of the Water-Soluble Polysaccharide Preparations

Samples	Inhibition ratio (%)	Complete regression
LO-1	89	4/8
LP-1	82	6/10
LI-1	67	6/10

tumour: Sarcoma 180 (Solid)
route: *i.p.*
dose: 150 mg/kg, 10 days
animal: Swiss albino mice

Table III. Dose response of Antitumour activity

Dose (mg/kg × 10 days)	Inhibition ratio	Complete regression
5	56.4	2/10
30	69.7	4/9
50	72.7	7/10
100	87.9	3/10
150	77.1	4/10

1) Part V: T. Takeda, M. Funatsu, S. Shibata, and F. Fukuoka, *Chem. Pharm. Bull.* (Tokyo), **20**, 2445 (1972).

2) Location: *a) Hongo, Bunkyo-ku, Tokyo; Author to whom inquiries should be addressed; b) Tsukiji, Chuo-ku, Tokyo.*

These crude polysaccharides showed characteristic absorptions at 877, 890 cm^{-1} in the infrared (IR) spectrum (in KBr).

As shown in Table II, the present samples of lichen polysaccharides showed remarkable growth-inhibitory effect on the implanted Sarcoma-180 in mice.

The sugar components of these crude polysaccharides are rhamnose, arabinose, xylose, glucose, galactose, mannose and glucosamine. A detailed study has been carried out using the heteropolysaccharides of *Lobaria orientalis*. The crude polysaccharide fraction (LO-1) was obtained by adding ethanol to aqueous extracts. As shown in Table III, LO-1 showed a maximum activity against transplanted sarcoma-180 in a dose of 100 mg/kg, and higher or lower doses gave less effects.

The crude heteropolysaccharide (LO-1) was chromatographed on a column of Sephadex G-200 to give an elution pattern consisting of three fractions (LOF-1, LOF-2, LOF-3) as shown in Fig. 1. The fractions of LOF-1, LOF-2 and LOF-3 were collected separately and lyophilized. Each fraction yielded a single peak on ultracentrifugation and zone electrophoresis.

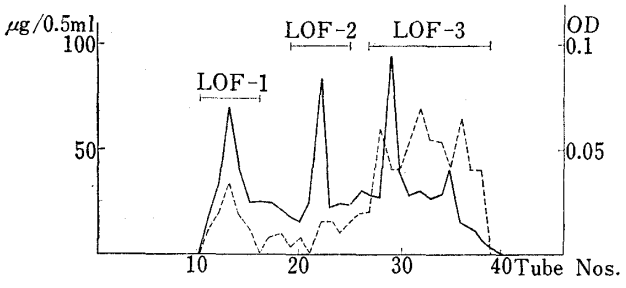

Fig. 1. Separation of LO-1 by Sephadex G-200

—: carbohydrate ——: peptide
column 2.5×45cm flow rate 30 ml/hr flow solvent H_2O
sample LO-1 10.0 mg

TABLE IV. Amino Acid Composition (mole %)

	LOF-1	LOF-2	LOF-3
Lys	4.2	5.0	5.3
Hys			0.6
Arg	2.4		1.1
Asp	10.8	11.8	17.9
Thr	12.4	17.1	12.8
Ser	11.5	13.2	10.5
Glu	10.1	7.9	14.5
Gly	11.9	13.0	6.5
Ala	11.9	9.6	5.7
Val	7.8	8.9	10.7
Ile	6.6	5.0	4.8
Leu	6.1	4.8	4.7
Tyr			1.3
Phe	4.3	3.7	3.6

As shown in Fig. 1, the carbohydrate and peptide peaks coincided each other to reveal that LOF-1 and LOF-2 are glycopeptides, whereas LOF-3 showed a diminishing pattern to the peptide peak on treatment with ion exchange resin. The amino acid compositions given by the hydrochloric acid hydrolysis of LOF-1, 2 and 3 are shown in Table IV. Therefore this hetero-polysaccharide includes a glycopeptide. The specific rotation values and IR spectra (in KBr) given in Table V indicate that β-D-configuration is predominant in the molecules. The sugar compositions of these fractions by a sugar analyzer are given in Table VI.

TABLE V. Properties of LOF-1,2, and 3

	LOF-1	LOF-2	LOF-3
$[\alpha]_D$	+50.0	+31.5	+28.1
IR	875	875	895
N(%)	1.01	1.04	2.58

TABLE VI. Sugar composition of LOF-1, 2, and 3 (μg/ml)

	Rham	Man	Ara	Gal	Xyl	Glc	Glc-NH_2 (%)
LOF-1	22.26	43.06	32.79	85.51	29.81	83.75	3.91
LOF-2	8.68	141.07	13.17	129.64	12.80	41.95	2.92
LOF-3		69.41	2.85	61.06	3.31	23.48	3.50

As shown in Table VI, the major carbohydrate constituents of LOF-1 are glucose (28.2%) and galactose (28.8%), and the minor components are mannose, arabinose, xylose and rhamnose, while LOF-2 consists mainly of mannose and galactose, and LOF-3 is similar to LOF-2.

The quantity of amino sugar was determined by the modified Dische–Borenfreund method[3] and amino acid analyzer to show that both LOF-1 and LOF-2 contain glucosamine.

The antitumour activities of fractions LOF-1, 2, and 3 were shown in Table VII. The antitumour activity of LOF-1 is remarkable in inhibition ratio, and LOF-2 is less active than LOF-1, whereas LOF-3 is inactive in several doses.

Table VII. Antitumour Activities of LOF-1, 2, and 3

Sample	Dose (mg/kg/10 days)	Inhibition ratio	Complete regression
LOF-1	30	48.8	0/8
	10	81.6	4/8
LOF-2	10	76.1	1/7
LOF-3	150	1.2	0/8
	50	−17.8	0/8
	10	7.1	0/8

Thus the active principle, LOF-1 has been studied extensively. After treatment of LOF-1 with alkali, the reaction mixture was reduced with sodium borohydride[4] and the amino acids released were detected with an amino acid analyzer.

On the other hand, the amino acid components in the acid hydrolysate of LOF-1 were determined. An increase of alanine and α-aminobutyric acid shown in the former treatment corresponded approximately to the loss of serine and threonine, respectively determined in the latter measurement. It has, therefore, been revealed that the carbohydrate portion of LOF-1 is linked with the peptide part by O-glycosyl linkages with serine and threonine.

The alkaline degradation products were chromatographed on Sephadex G-200 as shown in Fig. 2 in which a carbohydrate peak (LOF-A6) with a remarkably decreased N value (N: 0.57), and a well-separated peptide peak were manifested.

The N-terminal residues of LOF-1 were determined with 1-fluoro-2,4-dinitrobenzene,[5] and the methyl esters of DNP derivatives were identified by comparison with the authentic

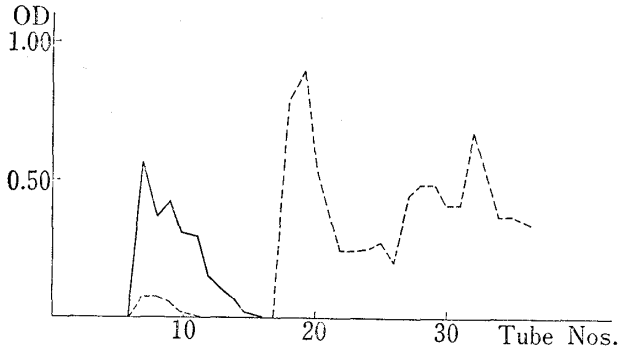

Fig. 2. Elution diagram on Sephadex G-200 of Alkaline degradation Products of LOF-1

——: carbohydrate ——: peptide
column 2.5×45 cm flow rate 10 ml/hr flow solvent H_2O
Sample LOF-A1

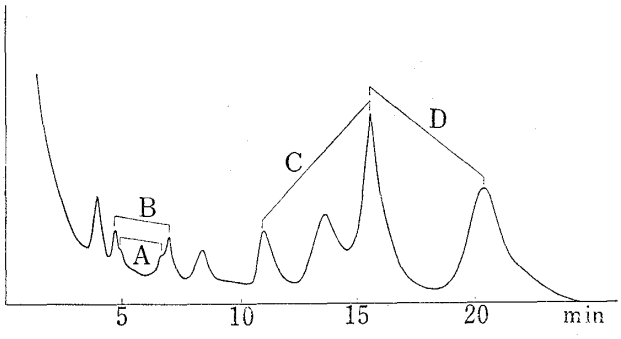

Fig. 3. GLC pattern of Methanolysed Products of LOF-1 Methyl Ether

A: methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside
B: methyl 2,3,4,6-tetra-O-methyl-D-mannopyranoside
C: methyl 2,3,4-tri-O-methyl-D-glucopyranoside
D: methyl 2,4,6-tri-O-methyl-D-mannopyranoside
column: 2% XE-60, column temperature: 145°
carrier gas: N_2 1.0 kg/cm³ H_2, Air: 0.8 kg/cm³

3) T. Kinoshita, *J. Japan. Chem.*, **25**, 1058 (1971).
4) K. Tanaka, M. Bertolini, and W. Pigman, *Biochem. Biophys. Res. Commun.*, **16**, 404 (1964).
5) F. Sanger, Advances in Protein Chemistry Vol. VII, 1952.

samples of DNP-threonine-, DNP-serine methyl esters by mass spectra and thin-layer chromatography (TLC), while DNP- asparagine was not detected.

The methyl ether of LOF-1 prepared by the combined Hakomori and Kuhn methods was methanolysed, and the products were analysed with gas-liquid chromatography (GLC) to reveal the formation of methyl 2,3,4-tri-O-methylglucopyranoside and methyl 2,4,6-tri-O-methyl-mannopyranoside together with a small amount of methyl 2,3,4,6-tetra-O-methyl-glucopyranoside and methyl 2,3,4,6-tetra-O-methyl-mannopyranoside, and some unidentified peaks including those in the higher retention time region, which might be branched chain sugars. These results revealed that of 1→6 glucan and 1→3 mannan are the main components of the carbohydrate moiety of LOF-1.

It has now been shown that the polysaccharide of *L. orientalis* contained at least two antitumour active glycopeptides, LOF-1 and -2, and this is the first example of the antitumour active glycopeptides found in lichens. The water-soluble heteropolysaccharides containing glycopeptide are occurring in several kinds of Stictaceae lichens, which would be noted from the chemotaxonomical viewpoint. We have also found such glycopeptide in lichens of Cladoniaceae, *e.g. Cl. bellidiflora*, and *Cl. graciliformis* as well as in *Acroscyphus sphaerophoroides*.[6] Although several plant glycopeptides have been reported in literatures, the occurrence of glycopeptide in lichens has never been proved except a hemaglutinin obtained from *Parmelia michauxiana*.[7]

Experimental

Sugar analyses were performed with a Japan Electron Optics Lab. liquid chromatographic autoanalyzer Model JLC-6AH; amino acid analyses were made with a JEOL amino acid analyzer Model JLC-5AH. The IR spectra were measured with a Japan Spectroscopic Co. Model DS 402-G Spectrophotometer, the specific rotation with a Yanagimoto Model OR-50 Polarimeter, and optical rotatory dispersion (ORD) curves with a Japan Spectroscopic Co., Model ORD/UV-5 Spectrometer.

A Spinco Model E analitical ultracentrifuge with a Schlieren optical system was used for measurement of sedimentation. Gas-liquid Chromatographic analyses were carried out with a Shimazu Model GC-4APF Gas Chromatograph attached with a hydrogen flame detector.

Assay Method of Antitumour Activities——The test was made by observing the effect on the growth of subcutaneously implanted Sarcoma-180 (solid form) for 5 weeks. Samples suspended in dist. water were injected in mice (Swiss-Albino or ICR mice) intraperitoneally. The details of the assay methods were described in our earlier paper.[8] The results are shown in Table II, III, and VII.

Isolation and Purification——Finely powdered *Lobaria orientalis* (540 g) was extracted with ether and 80% ethanol subsequently in order to remove soluble components, and the residual thalli were extracted further with distilled water on a boiling water-bath. To the hot filtered extracts were added EtOH to form precipitates, which were collected by centrifugation, washed with EtOH and ether, and dried to obtain a pale brownish water-soluble powder (Fraction LO-1). The polysaccharides of other lichens were prepared by the same way. LO-1 was chromatographed on a column of Sephadex G-200, whose elution diagram giving three fractions (LOF-1, LOF-2, LOF-3) was shown in Fig. 1.

Sugar Analysis——Neutral sugar components of LOF-1, LOF-2 and LOF-3 were determined with a sugar analyzer as follows: Each fractions, LOF-1, 2 and 3, was hydrolyzed with 1N H_2SO_4 for 8 hr at 90°. The hydrolysates were dissolved in 0.13 M borate buffer at pH 7.5 and applied to the column of JEOL Resin LC-R-3 transformed into borate form. The determination of sugar components were made with the orcinol-H_2SO_4 method while the absorbances at 510 m and 425 mm were automatically recorded. The sugar contents were indicated in Table VI. Amino sugar was determined by the Modified-Dische-Borenfreund method.

Amino Acid Analysis——LOF-1, 2, and 3 were hydrolyzed with redistilled 6 N HCl in a sealed tube for 16 hr at 110°. The amino acid and amino sugar contents of the hydrolyzate were determined by a JEOL amino acid analyzer.

Alkaline Degradation——LOF-1 (100 mg) was reacted at 5° with 15 ml of 0.3 M $NaBH_4$ dissolved in 0.1 N NaOH, and the reaction mixture was neutralized to pH 5.3 with 1 N AcOH to remove the excess of borohydride. The solution was dialyzed and lyophylized.

6) T. Takeda, S. Shibata, M. Inomata, and F. Fukuoka, *Seikagakv*, **44**, 655 (1972).
7) M.L. Howe and J.T. Barrett, *Biochim. Biophys. Acta*, **215**, 97 (1970).
8) F. Fukuoka, M. Nakanishi, S. Shibata, Y. Nishikawa, T. Takeda, and M. Tanaka, *Gann*, **59**, 421 (1968).

Methylation of LOF-1——LOF-1 (500 mg) was methylated in the usual way, first by the Hakomori's method (2 times) and then by the Kuhn procedure to yield fully methylated LOF-1-Me (200 mg) which gave no OH absorption in the IR spectrum.

Methanolysis of Fully Methylated Polysaccharide——A mixture of methylated LOF-1 (150 mg) and 5% MeOH–HCl (6 ml) was heated at 100° for 8 hr in a sealed tube. After treatment with Amberlite IR-4B, the reaction mixture was evaporated *in vacuo* to yield a syrup. The MeOH solution of the syrup was examined with GLC using 2% XE-60 on Anakrom 50 (2 m) at 145°, under a flow of N_2. The major and minor products were identified, respectively as being methyl 2,3,4-tri-O-methyl-D-glucopyranoside and methyl 2,4,6-tri-O-methyl-D-mannopyranoside and methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside and methyl 2,3,4,6-tetra-O-methyl-D-mannopyranoside by comparison with the authentic samples.

Retention times: Methyl 2,3,4-tri-O-methyl glucoside (α-anomer): 15.5 min, (β-anomer): 10.9 min, methyl 2,4,6-tri-O-methyl mannoside (α-anomer): 20.5 min, (β-anomer): 15.5 min, methyl 2,3,4,6-tetra-O-methyl-glucoside (α-anomer): 7.0 min, (β-anomer): 4.9 min, methyl 2,3,4,6-tetra-O-methyl mannoside (α-anomer): 7.1 min, (β-anomer): 4.8 min.

N-Terminal Determination of LOF-1 by Dinitrophenylation——LOF-1 (30 mg) and 30 mg of $NaHCO_3$ are dissolved together in 2 ml of water. To the solution were added 4 ml of 5% ethanolic solution of dinitrofluorobenzene. The mixture was stirred for 2 hr at room temperature. Ethanol was evaporated, and the residue was washed subsequently with water, EtOH and ether.

DNP-LOF-1 was suspended in 6 ml of 6 N HCl and the mixture was heated for 12 hr in a sealed tube. After cooling, the mixture was extracted 3 times with ether. The ethereal extract was washed with water, and evaporated to dryness. The residue was taken up in ether (30 ml), and the solution was methylated with CH_2N_2. The DNP-methyl esters formed were subjected to TLC and mass spectral (Mass) analysis.

TLC of DNP-methyl Ester——TLC analyses were carried out on a plate of silica gel GF-254, using a solvent system of benzene–acetone (4: 1). DNP-products were identified with the authentic samples of DNP-threonine methyl ester and DNP-serine methyl ester.

Rf value	DNP-threonine methyl ester	0.43
	DNP-serine methyl ester	0.33

mass spectrum	DNP-threonine methyl ester	DNP-serine methyl ester
M^+	299	285
	255 $\left(M-CH_2=C\langle^{H}_{OH}\right)$	
$M-COOCH_3$	240	226

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