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Studies on the Water Soluble Constituents of Lichens. II.[1] Antitumor Polysaccharides of *Lasallia*, *Usnea*, and *Cladonia* Species[2]

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From six species of lichens, a variety of polysaccharide fractions were prepared and their antitumor effects were tested against the sarcoma 180 implanted in mice. These fractions were characterized by chemical and physicochemical methods. The active principle of *Lasallia pensylvanica* has been determined as GE-3 type glucan (a partially O-acetylated β-(1→6)-glucan). Another highly effective β-glucan, containing (1→3)- and (1→4)-linkages in the ratio 3 : 7, was isolated from *Usnea rubescens*, and it has been identified with lichenin. In addition, presence of so far unknown heteropolysaccharides with moderate antitumor effect has been suggested in the minor fraction of the lichen. Four *Cladonia* lichens, *i.e. C. crispata*, *C. mitis*, *C. rangiferina* subsp. *grisea*, and *C. squamosa*, were also examined, and it has been revealed that, in all of them, complex heteroglycans consisting chiefly of mannose, galactose, and glucose occur commonly as predominant, cold-water soluble polysaccharides, together with a small amount of α-glucan presumed to be PC-3 type (an α-glucan possessing (1→3)- and (1→4)-linkages alternately). Both the heteroglycan fraction and the glucan fraction of *C. squamosa* were shown to exert moderate antitumor effect.

Since 1968 the antitumor polysaccharides of various lichens have been studied extensively, and they were proved to exert a remarkable host-mediated inhibitory effect on the growth of sarcoma 180 implanted in mice.[5] The active principles of some lichens have been isolated in pure state and structurally determined. Among them two kinds of linear β-D-glucan, *i.e.* GE-3 (or pustulan) type and lichenin type, were revealed to be especially effective. Occurrence of complete regression of the tumor could be observed in almost all the mice treated

1) Part I: Y. Nishikawa, K. Michishita, and G. Kurono, *Chem. Pharm. Bull.* (Tokyo), **21**, 1014 (1973).
2) This paper includes the combined results presented at the 91st, 92nd, and 93rd Annual Meetings of the Pharmaceutical Society of Japan: Fukuoka, April, 1971; Osaka, April, 1972; and Tokyo, April, 1973, respectively. The earlier papers related to this work have been reported by two (Y.N. and F.F.) of the present authors in the series entitled "Polysaccharides of Lichens and Fungi" (Part IV: Y. Nishikawa, M. Tanaka, S. Shibata, and F. Fukuoka, *Chem. Pharm. Bull.* (Tokyo), **18**, 1431 (1970)).
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with both types of polysaccharide, without accompanying significant toxicity. On the other hand, α-D-glucans so far obtained, such as isolichenin and PC-3 (nigeran-like polysaccharide) types, were all demonstrated to be only moderately effective. These results have been briefly reviewed.[5f,g,l,n]

The present paper deals with the antitumor polysaccharide fractions prepared from the six species of lichens, *i.e. Lasallia pensylvanica* (HOFFM.) LLANO (Gyrophoraceae), *Usnea rubescens* STIRT. (Usneaceae), *Cladonia crispata* (ACH.) FLOT., *C. mitis* SANDST., *C. rangiferina* (L.) WEB. subsp. *grisea* AHTI. and *C. squamosa* (SCOP.) HOFFM. (Cladoniaceae). These lichens, with one exception of *C. mitis* which had been preliminarily examined,[5d] have never been reported on their antitumor polysaccharide constituents.

From the lichens mentioned above, the crude polysaccharide fractions, designated LPe-1, UR-1, CC-1, CM-1, CRa-1, and CS-1, respectively, were prepared by adding ethanol to the aqueous extracts, and then further separated by repetition of the freezing and thawing procedure. The antitumor effect of some of the fractions thus resulted was assayed under the similar conditions as employed in the previous works.[5d] The results are shown in Table I. In order to clarify the active polysaccharides contained in these lichens, characterization of the respective fractions has now been attempted.

TABLE I. Antitumor Effect of the Polysaccharide Fractions[a]

Lichen	Sample	Inhibition ratio (%)	Complete regression	Mouse[b]
Lasallia pensylvanica	LPe-1-1[c]	99	6/ 8	I
Usnea rubescens	UR-1	99	9/10	I
	UR-1-1[d]	100	9/ 9	I
	UR-1-2	70	4/ 9	I
Cladonia crispata	CC-1	48	1/ 8	I
Cladonia mitis	CM-1[e]	99	8/ 9	S
Cladonia rangiferina subsp. *grisea*	CRa-1	78	3/10	I
Cladonia squamosa	CS-1	55	2/10	I
	CS-1-1[f]	67	5/ 9	I
	CS-1-2	72	1/ 7	I
	CS-1-2	69	5/10	I
	CS-1-2	62	1/10	I
cf. Authentic samples[g]				
Gyrophora esculenta	GE-3	97	7/10	S
Cetraria islandica var. *orientalis*	Lichenin	100	8/ 8	S
Parmelia caperate	PC-3	67	0/ 9	S

a) conditions used for assay: tumor, sarcoma 180 (solid); vehicle, aq. dest.; route, *i.p.*; dose, 150 mg/kg×10 days except for CM-1 (200 mg/kg×10 days)
b) I, ICR; S, Swiss albino
c) identified with GE-3 type
d) identified with lichenin
e) Data were cited from ref. 5*d*).
f) identified with PC-3 type
g) Data were cited from ref. 5*l*).

Lasallia pensylvanica

Presence of the GE-3 type of antitumor polysaccharide could be readily anticipated in the present lichen *L. pensylvanica*, since it has already been demonstrated that this type of glucan was found almost solely in all of the species belonging to Gyrophoraceae previously examined (*Gyrophora esculenta* MIYOSHI, *Umbilicaria angulata* TUCK., *U. caroliniana* TUCK., *U. polyphylla* (L.) BAUMG., and *Lasallia papulosa* (ACH.) LLANO).[5a–g,i,j,l,n] In fact, from the crude polysaccharide fraction, LPe-1, of the lichen, a homogeneous glucan, LPe-1-1,

$[\alpha]_D^{19}$ −42.2° (*cf.* GE-3, $[\alpha]_D^{19}$ −37.5°) was isolated by the freezing and thawing procedure, and, as described in the experimental part, the basic properties including the marked antitumor activity (Table I) were proved to be similar to those of the authentic specimen of GE-3 obtained from *G. esculenta*. The infrared (IR) and nuclear magnetic resonance (NMR) spectra, the optical rotatory dispersion (ORD) curve, and the ultracentrifugal and electrophoretic behaviors of the glucan were also identical with those of the latter. It consumed two moles of periodate per anhydroglucose unit and gave a series of gentiooligosaccharides on partial acid hydrolysis. From the results of methylation study, it was concluded that the basic skeleton of the polysaccharide is linear and built up entirely with (1→6)-linked glucose residues. The glucan was fully methylated by the method of Hakomori[6] and the methanolysates were analysed by gas–liquid chromatography (GLC). As shown in Fig. 1, the chromatogram indicated the liberation of methyl 2,3,4-tri-O-methyl-D-glucoside as a sole product other than permethylated glucose. The content of acetyl groups, whose presence in the molecule was suggested from the IR- and NMR-spectra, was determined to be 2.7% (*cf.* GE-3, *ca.* 2%) by GLC using Polapak Q column.

On the basis of the above findings, the antitumor polysaccharide contained in *L. pensylvanica* has now been identified with GE-3 type. No attempt was made in the present study to locate the substituent, since it has already been revealed that the O-acetyl groups are attached to the 3-position of the glucose units in the molecule of GE-3.[5e] Considering from their undistinguishable sedimentation behaviors, the molecular weight of the present sample appeared to be similar to that of the authentic specimen of GE-3, to which the value 20000 had been given by the method of equilibrium ultracentrifugation.[5e]

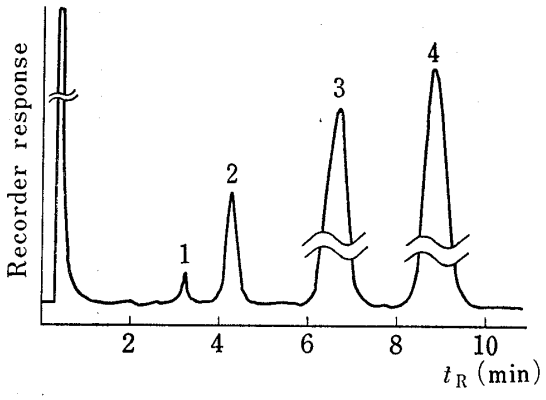

Fig. 1. Gas Chromatogram of the Methanolysate of the Methylated LPe-1-1

1 and 2: methyl 2,3,4,6-tetra-O-methyl-D-glucoside
3 and 4: methyl 2,3,4-tri-O-methyl-D-glucoside
conditions: 5% NPGS (neopentylglycol succinate) on Shimalite W (60—80 mesh); 2 m×4 mm i.d.; 180°; N_2 (40 ml/min)

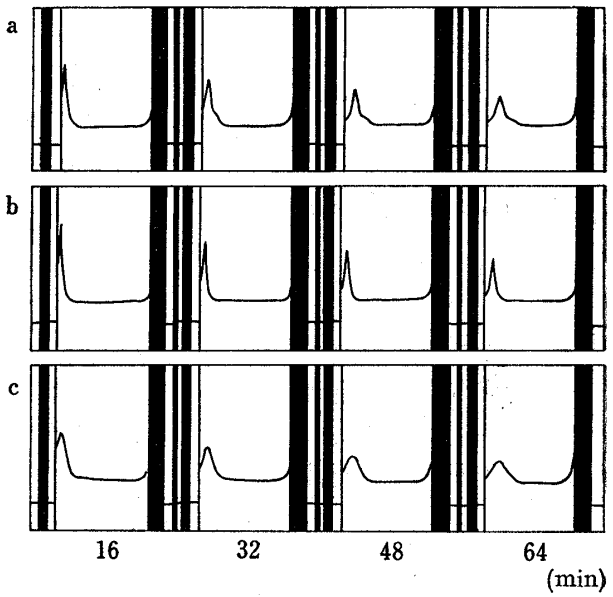

Fig. 2. Sedimentation Patterns (1% Aq. solution, 59780 rpm, at 20°)

a) fraction UR-1; b) fraction UR-1-1; c) fraction UR-1-2

Usnea rubescens

The marked antitumor effect of lichenin was first demonstrated with *Cetraria islandica* (L.) Ach. var. *orientalis* Asahina.[5d] Additional confirmatory evidences were obtained in the subsequent studies on various other lichens, including *Usnea bayleyi* (Stirt.) Zahlbr. and *U. pseudo-montis Fuji* Asahina.[5i–n] As expected, the predominant existence of this type

6) S. Hakomori, *J. Biochem.*, **55**, 205 (1964).

of glucan in the present lichen *U. rubescens* was suggested by the facts that the crude polysaccharide fraction, UR-1, was highly effective (Table I) and showed almost identical IR-spectrum with that of the authentic specimen of lichenin isolated from *C. islandica* var. *orientalis*. The fraction UR-1 was, however, not homogeneous ultracentrifugally (Fig. 2), and on complete acid hydrolysis it yielded not only glucose but also various other sugars, *e.g.* galactose, mannose, xylose, *etc.*

By repetition of the freezing and thawing procedure, the fraction UR-1 was separated into a major, ultracentrifugally (Fig. 2) and electrophoretically homogeneous glucan (fraction UR-1-1), $[\alpha]_D^{18}$ +21.3° (*cf.* lichenin, $[\alpha]_D^{19}$ +18.4°), and a minor, cold-water soluble fraction (UR-1-2), $[\alpha]_D^{18}$ +75.8°. The former exerted a remarkable antitumor effect, while the latter possessed only a moderate activity (Table I). It is also important to note that liver of the mice receiving injections of the sample UR-1-1 (but not of the sample UR-1-2) underwent the same pathologic changes as encountered previously in the cases of lichenin and GE-3 type of glucan.[5j] The glucan UR-1-1 was assumed to be identical with the authentic specimen of lichenin by comparison of their basic properties, IR-spectra, ORD curves, and the sedimentation behaviors. In order to secure its identity with lichenin, methylation study was carried out. As shown in Fig. 3, the gas chromatogram of the methanolysates derived from the methylated glucan consisted of the peaks corresponding to methyl 2,3,6- and methyl 2,4,6-tri-O-methyl- and methyl 2,3,4,6-tetra-O-methyl-D-glucosides. In addition, the periodate consumption of the glucan was determined to be 0.66 moles per anhydroglucose unit. From these results it has been confirmed that the glucan UR-1-1 is, like lichenin, essentially linear and composed of (1→3)- and (1→4)-glucosidic linkages in the ratio 3: 7.

It is well known that a small amount of isolichenin co-exists with lichenin in many lichens. In the present lichen, however, this type of α-glucan seemed to be absent or, if present, in negligible amount, since the minor fraction UR-1-2 gave negative coloration with iodine. This was further supported by the following nature of the fraction: i) The value of specific rotation was significantly lower than that ($[\alpha]_D$ +272°) of isolichenin. ii) The IR-spectrum (900 (broad) and 804 cm^{-1}) was devoid of the characteristic absorption bands due to isolichenin (925, 845, and 800 cm^{-1}) (Fig. 4). iii) On acid hydrolysis it liberated considerable amounts of

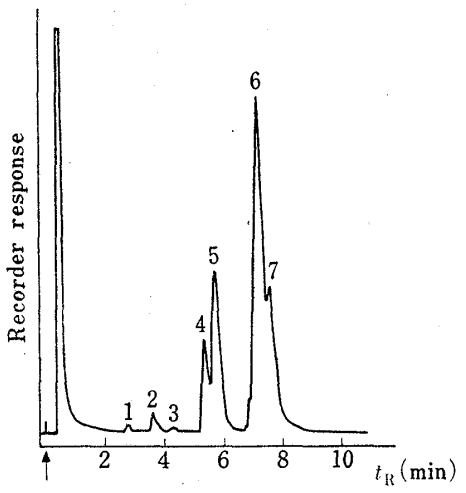

Fig. 3. Gas Chromatogram of the Methanolysate of the Methylated UR-1-1

1,2 and 3: methyl 2,3,4,6-tetra-O-methyl-D-glucoside
4 and 7: methyl 2,4,6-tri-O-methyl-D-glucoside
5 and 6: methyl 2,3,6-tri-O-methyl-D-glucoside
conditions: 2% XE-60 on Chromosorb W (AW-DMCS) (60—80 mesh); 2 m×4 mm i.d.; 164°; N_2 (40 ml/min)

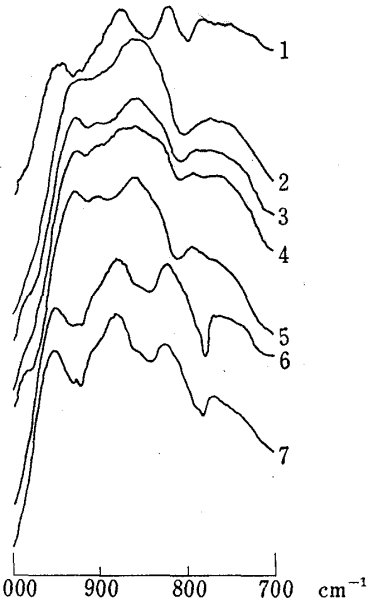

Fig. 4. IR-Spectra (in KBr)

1, isolichenin (authentic specimen); 2, fraction UR-1-2; 3, fraction CS-1; 4, fraction CC-1; 5, fraction CS-1-2; 6, PC-3 type α-glucan (authentic specimen); 7, fraction CS-1-1

galactose and mannose, in addition to glucose, together with trace amounts of arabinose, xylose, and rhamnose (anhydroglucose content determined by the anthrone method, 50.2%). The fraction appeared to be still heterogeneous, as suggested by the sedimentation pattern in which a single but broad peak could be observed (Fig. 2). These facts are indicative of the presence of complex heteropolysaccharides in the fraction. Our preliminary examination has suggested that the fraction UR-1-2 is separable into, at least, two fractions by DEAE-cellulose column chromatography. Details of the results will be reported soon.

***Cladonia* Lichens (*C. crispata*, *C. mitis*, *C. rangiferina* subsp. *grisea* and *C. squamosa*)**

Previously, the crude polysaccharide fraction (CM-1) of *C. mitis* has been preliminarily examined and shown to have a marked antitumor effect (Table I).[5d] In this connection, we now studied on the antitumor polysaccharides of three other *Cladonia* species so far unexamined, giving some additional informations on *C. mitis* for comparison. From the present lichens, three fractions (CC-1, CRa-1, and CS-1) corresponding respectively to CM-1 were prepared, but they were all found to be less effective than the latter (Table I). These four fractions were similar, each other, in many respects: i) They are readily soluble even in cold water, leaving only small amounts of undissolved solid materials. ii) On acid hydrolysis, they liberate predominantly glucose, galactose, and mannose, together with arabinose, xylose, and rhamnose in trace quantities. iii) Their IR-spectra are mutually identical, showing the absorption pattern previously never encountered (Fig. 4). iv) They have $[\alpha]_D$ values around 50°, and give the positive plain ORD curves. v) They contain nitrogen, albeit in less than 1%, probably due to the presence of amino acid components. Thus, it has been strongly suggested that so far unknown, closely related heteroglycans (or heteroglycan-peptide complexes) occur commonly as major polysaccharides in *Cladonia* lichens.

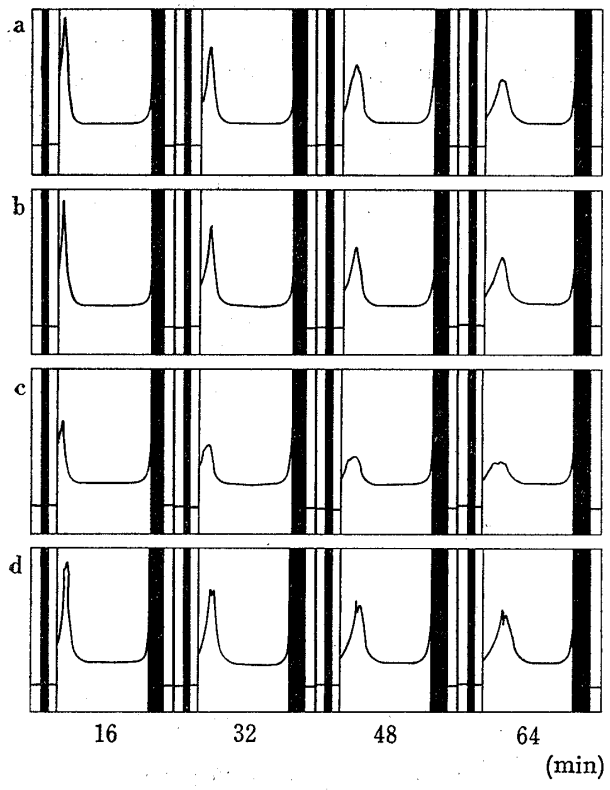

Fig. 5. Sedimentation Patterns (1% Aq. solution, 59780 rpm, at 20°)

a) fraction CC-1-2; b) fraction CRa-1-2; c) fraction CS-1-2; d) fraction CM-1-2

However, some significant differences in the basic properties could also be observed among these fractions: i) Ultracentrifugally, the fractions CC-1 and CRa-1 appear to be homogeneous, while the fractions CM-1, in particular, and CS-1 are clearly heterogeneous (Fig. 5), and ii) by treatment with Fehling solution, the latter fractions yielded considerable amounts of precipitates, but the formers failed virtually to form insoluble copper-complexes. Variation of the antitumor effect among these fractions might be correlated with such differences. In addition, it should not be overlooked that the conditions used for antitumor assay of the fraction CM-1 were slightly different from those employed for other samples (see Table I).

By repetition of the freezing and thawing procedure, the respective crude polysaccharide fractions were separated into the major heteroglycan fractions (CC-, CM-, CRa-, and CS-1-2) and the minor, cold-water insoluble fractions (CC-, CM-, CRa-, and CS-1-1). The basic properties (Table II), including the IR-spectra

TABLE II. Properties of the Heteroglycan Fractions of *Cladonia* Lichens

Lichen	*C. crispata*	*C. rangiferina* subsp. *grisea*	*C. squamosa*	*C. mitis*
fraction	CC-1-2	CRa-1-2	CS-1-2	CM-1-2
Solubility	readily soluble in cold water			
$[\alpha]_D$ (in H_2O)	+29.7° (c=0.40; at 15°)	+22.2° (c=0.41; at 15°)	+39.5° (c=0.51; at 18°)	+19.9° (c=0.30; at 25°)
ORD (in H_2O)	positive plain curve			
N%	0.14	0.23	0.53	1.04
Glucose content (%)[a]	31.3	49.4	39.7	41.5
Sugar composition[b]	mannose (###); galactose, and glucose (##); rhamnose (+); arabinose, and xylose (trace)			
Coloration with I_2	—	—	—	—
Precipitation with				
Fehling solution	trace	trace	##	##
$Ba(OH)_2$	###	###	###	###
IR spectra (KBr)[c]	mutually similar; 1725, 1250, 915, and 810 cm^{-1}			
Sedimentation pattern	apparently homogeneous		clearly heterogeneous	

a) determined by the anthrone method
b) Analysed by TLC and GLC. Representative gas chromatogram is shown in Fig. 6.
c) Representative absorption patterns are shown in Fig. 4.
d) see Fig. 5

(Fig. 4) and the sedimentation behaviors (Fig. 5), of the resulting major fractions were found to be almost identical with those of the corresponding parent fractions, respectively. Sugar compositions of these fractions were analysed semiquantitatively by thin-layer chromatography (TLC) and by GLC. GLC was performed according to the method of Tamura,[7] and the typical chromatogram obtained is shown in Fig. 6. From the results, it has been

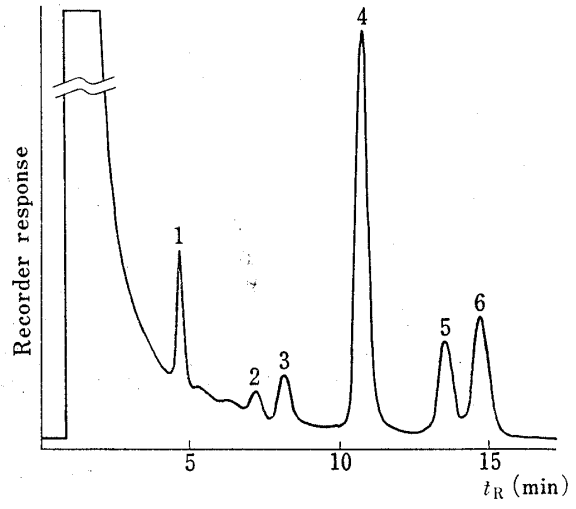

Fig. 6a. Analysis of the Sugar Composition of Fraction CS-1-2 by GLC as TFA Derivatives of Alditols

1, rhamnose; 2, arabinose; 3, xylose; 4, mannose; 5, glucose; 6, galactose

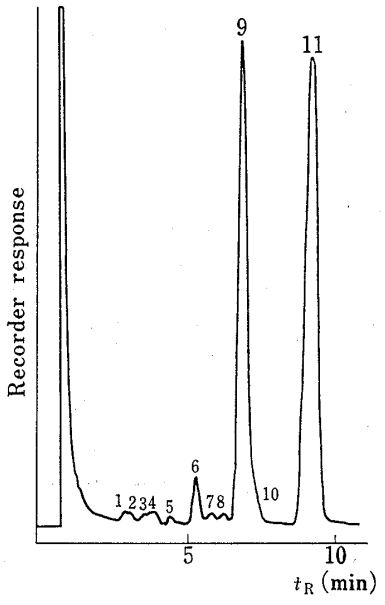

Fig. 6b. Analysis of the Sugar Composition of Fraction CS-1-1 by GLC as TMS Derivatives

1 and 7, arabinose; 2 and 3, rhamnose; 4 and 5, xylose; 6 and 10, mannose; 8 and 10, galactose; 9 and 11, glucose

GLC conditions and the relative t_R are shown in Table III.

7) H. Nakamura and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **18**, 2314 (1970).

revealed that these heteroglycan fractions contain commonly a variety of sugars in the following order, mannose≫galactose>glucose>rhamnose >arabinose and xylose; though the relative content of each sugar varies slightly from sample to sample. The fraction CS-1-2 was subjected to the antitumor test and the inhibition ratio was determined to be 60—70% without significant fluctuation (Table I). In the present study, other fractions were not tested, due to the shortage of material. Further purification and structure elucidation of this type of antitumor heteropolysaccharides are now under progress.

On the other hand, the minor fractions (CC-1-1, $[\alpha]_D^{22}$ +232°; CM-1-1, $[\alpha]_D^{26}$ +242°; CRa-1-1, $[\alpha]_D^{20}$ +215°; and CS-1-1, $[\alpha]_D^{19}$ +245°) were composed almost entirely of glucose, accompanying with some other sugars in negligible quantities (Fig. 6). Unlike isolichenin, they were insoluble in cold water, and gave negative coloration with iodine. Occurrence of another α-glucan, called PC-3 type ($[\alpha]_D$ +201°), has been found recently in *Parmelia caperata* as a main polysaccharide.[5h] This is a nigeran-like, linear glucan containing (1→3)- and (1→4)-linkages alternately. The PC-3 type of glucan seems to be distributed widely in *Parmelia* lichens,[8] but its presence in other genera has never been reported as yet. It shows a characteristic IR-spectrum (925, 845, and 780 cm^{-1}), which is readily distinguishable from that of isolichenin (Fig. 4). The close relationship between the minor polysaccharides and the PC-3 type of glucan has been suggested by comparison of their IR-spectra, as well as their ultracentrifugal and electrophoretic behaviors with those of the authentic specimen of the latter. Using the fraction CS-1-1, methylation study, periodate oxidation, and antitumor assay (Table I) were carried out. These results supported its identity with PC-3 type of glucan.

As a conclusion, common occurrence of the so far unknown heteroglycans and a small amount of the PC-3 type glucan has been revealed in all of the *Cladonia* lichens examined.

In 1906 Ulander and Tollens mentioned the presence of lichenin in *C. rangiferina*,[9] but our careful examination failed to detect the glucan in *C. rangiferina* subsp. *grisea* used in the present study.

Experimental

The IR-spectra were measured with a JASCO (Japan Spectroscopic Co.) Model DS-402G Spectrophotometer, the specific rotations with a JASCO Model DIP-SL Automatic Polarimeter, and the ORD curves with a JASCO Model ORD/UV-5 Spectrometer. The NMR-spectra (in D_2O; internal reference, sodium 2,2-dimethylsilapentane-5-sulfonate (DSS)) were measured with a Japan Electron Optics Lab. Model JNM-C60-H Spectrometer. The carolimetric determinations were performed with a Hitachi Model 101 Spectrophotometer. A Beckmann Spinco Model E Analytical Ultracentrifuge with a schlieren optical system was used for measurements of the sedimentation patterns (1% aqueous solution, at 59780 rpm, at 20°). GLC was carried out with a Shimadzu Gas Chromatograph Model GC-4BPF attached with a hydrogen flame ionization detector, using glass columns.

Assay Method of Antitumor Effect——Antitumor effect was determined under the similar conditions as described previously.[5d] But it is notewothy to mention that, in the present study, ICR mice were employed instead of Swiss albino mice. Seven-day-old sarcoma 180 ascites, 0.05 ml (*ca.* 8×10^6 cells), were transplanted subcutaneously into the right groins of mice. The test samples, dissolved or suspended in distilled water, were injected intraperitoneally with 150 mg/kg/day doses for 10 days, starting 24 hr after tumor implantation. After observing the tumor growth for 5 weeks, the tumor weights of treated mice were compared with those of untreated mice. The inhibition ratios were calculated by the following formula: Inhibition ratio (%)$=[(C-T)/C]\times100$; where C is the average tumor weight of the control group, and T is that of the treated group. Complete regression of the tumors was also recorded. The results obtained by the present samples are listed in Table I.

Analyses of Sugar Composition——Complete acid hydrolysis was carried out by heating a sample in 1N H_2SO_4, at the concentrations less than 1%, on a boiling water-bath for 9 hr. After neutralization with Amberlite IR-4B (OH^-), the reaction mixture was concentrated under a reduced pressure to give a syrup, which was analysed by TLC: adsorbent, Avicel SF (microcrystalline cellulose powder); solvent systems, A) AcOEt: pyridine: AcOH: H_2O (5: 5: 1: 3) and B) phenol: 1% NH_4OH (2: 1); spray reagents, anilinehydro-

8) unpublished data.
9) A. Ulander and B. Tollens, *Chem. Ber.*, 39, 401 (1906).

genphthalate or diphenylamine-aniline. Sugars liberated were identified by comparison of their *Rf* values with those of the authentic specimens (Table III). For GLC analyses, sugars were converted into the trimethylsilyl (TMS) derivatives, or into the trifluoroacetyl (TFA) derivatives of the corresponding alditols. TMS derivatives were prepared by Sweeley's method,[10] using the well-dried residue obtained after evaporation of the syrup to dryness. The standard procedure[7] adopted for preparation of TFA derivatives was as follows: To the syrup, 1% $NaBH_4$ in water was added excessively. The solution was allowed to stand at room temperature for several hours and the excess borohydride was destroyed by addition of AcOH. The mixture was evaporated to dryness, and the residue was co-distillated with MeOH (five times). The residue was azeotroped, repeatedly, with benzene, and dried over P_2O_5. The well-dried sample was treated with N,N-dimethylformamide and trifluoroacetic anhydride (1: 1) at room temperature for 10 min, and an aliquot of the reaction mixture was injected directly into the gas chromatograph. Peaks detected were identified by comparison with those of the corresponding derivatives prepared from the authentic specimens. The relative retention times (relative t_R) and the GLC conditions are described in Table III.

Table III. TLC *Rf* Values and GLC Relative t_R of Sugars

Sugar	TLC[a] *Rf* values		GLC relative t_R	
	Solv. A[b]	Solv. B[c]	TMS[d]	TFA[e]
Rhamnose	0.91	0.56	0.63 0.69	0.43
Xylose	0.74	0.44	0.74 0.83	0.75
Arabinose	0.67	0.50	0.56 1.09	0.66
Mannose	0.66	0.42	1.00[f] 1.39	1.00[g]
Glucose	0.60	0.36	1.31 1.76	1.25
Galactose	0.53	0.40	1.19 1.39	1.36

a) adsorbent, Avicel SF; double developments
b) AcOEt: pyridine: AcOH: H_2O (5:5:1:3)
c) phenol: 1% NH_4OH (2:1)
d) column packing, 1.5% SE-30 on Chromosorb W (AW-DMCS) (60—80 mesh); column size, 3.5 m×4 mm i.d.; column temp., 200°; carrier gas, N_2 (40 ml/min)
e) TFA means the TFA derivative of the alditol resulted from the corresponding aldose. column packing, 2% XF-1105 on Chromosorb W (AW-DMCS) (60—80 mesh); column size, 3.5m×4 mm i.d.; column temp., 155°, carrier gas, N_2 (50 ml/min)
f) 5.4 min
g) 11.0 min

Determinations of Periodate Consumption and Anhydroglucose Content——Periodate uptake was determined spectrophotometrically according to the method of Aspinall and Ferrier.[11] Anhydroglucose content was measured by the anthrone method in the usual manner.

Methylation Study——By employment of Hakomori's method,[6] methylation was performed repeatedly, usually three times, until no absorption bands of OH groups could be observed in the IR-spectrum of the product. Methanolysis was carried out by heating the fully methylated derivative at 100° for 8 hr with 5% MeOH–HCl in a sealed tube. After neutralization with Amberlite IR-4B (OH^-), the reaction mixture was evaporated *in vacuo*, and then the $CHCl_3$ extract of the residue was analysed by GLC.

Preparation of the Crude Polysaccharide Fractions (LPe-1, UR-1, CC-1, CRa-1, and CS-1)——The dry lichens listed in Table IV were broken into small pieces and pre-extracted with MeOH for removal of the low molecular weight constituents. Then the residual lichen thalli were extracted thrice with distilled water in a boiling water-bath (one extraction for 9—10 hr). The filtrates (if necessary, after concentration *in vacuo*) were poured into three volumes of EtOH to form precipitates, which were collected by centrifugation, washed well with acetone and $(C_2H_5)_2O$, and then dried. The supernatants were concentrated *in vacuo* and worked up as above to give some additional materials. Thus, from the respective lichens, the crude polysaccharide fractions, LPe-, UR-, CC-, CRa-, and CS-1, were obtained as slightly brownish flakes. The fractions isolated from the 1st, 2nd, and 3rd extracts were kept separately (lot Nos. 1, 2, and 3, respectively). Unless otherwise stated, lot No. 1 of each fraction was used throughout the present work. The yields are shown in Table IV. Fraction CM-1 employed in the present study was the same batch that prepared previously from *Cladonia mitis*.[5d]

Preparation of the Fraction LPe-1-1——The fraction LPe-1 was warmed in water and small amounts of undissolved materials were filtered off. The filtrate was frozen solid overnight and allowed to thaw at

10) C.C. Sweeley, R. Bentley, M. Makita, and W.W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).
11) G.O. Aspinall and R.J. Ferrier, *Chem. Ind.* (London), **1957**, 1216.

Table IV. Lichens Used and Yields of the Crude Polysaccharide Fractions[a)]

Lichen			Crude polysaccharide fraction				
				Yield[b)]			
Species	Collected in	Dry wt. (g)	Name	Lot 1 (g)	Lot 2 (g)	Lot 3 (g)	Total (%)
Lasallia pensylvanica	Mt. Kimpu	292	LPe-1	34.8	25.4	6.5	22.8
Usnea rubescence	Subarbs of Sendai	119	UR-1	12.4	8.5	5.6	22.3
Cladonia crispata	Kumogahata, Kyoto	90	CC-1	1.6	1.0	0.8	3.8
Cladonia rangiferina subsp. *grisea*	Kumogahata, Kyoto	90	CRa-1	1.9	1.1	1.1	4.6
Cladonia squamosa	Kumogahata, Kyoto	1292	CS-1	35.4	20.0	16.1	5.5

a) As for the crude polysaccharide fraction, CM-1, of *Cladonia mitis*, the same batch that reported previously[5d)] was employed in the present study.
b) Lots 1, 2, and 3 correspond, respectively, to the fractions obtained from the 1st, 2nd, and 3rd extracts.

room temperature. The precipitate was collected by centrifugation, washed with acetone and $(C_2H_5)_2O$, and then dried. After several repetition of the procedure, slightly greyish white buff flakes were afforded (fraction LPe-1-1). Yield, 19.5% from the dry weight of the lichen.

Characterization of the Fraction LPe-1-1 (GE-3 Type Glucan)——By direct comparison, it was confirmed that all the properties mentioned below were identical with those of the authentic specimen of GE-3 type glucan obtained previously from *Gyrophora esculenta*.[5c)] $[\alpha]_D^{19}$ −42.2° (c=0.33, H_2O) (*cf.* GE-3, $[\alpha]_D^{19}$ −37.5° (c=0.5, 1N NaOH)). ORD, a negative plain curve. IR ν_{max}^{KBr} cm^{-1}: 1735 (acetyl) and 910 (β-glucosidic linkage). NMR (D_2O) ppm: 2.1 ($C\underline{H}_3CO$–). N%, negligible. Solubility; soluble readily in hot water, but sparingly in cold water. Coloration with I_2-KI, negative. Coloration with $FeCl_3$, negative. Treatment with Fehling solution, able to form the insoluble Cu-complex. Treatment with $Ba(OH)_2$ solution, no precipitate. Sedimentation pattern; a single, symmetric peak (A mixed sample of LPe-1-1 and the authentic specimen (1: 1) also gave a single peak.). Electrophoresis (buffer, 0.05M borate buffer (pH 9.3); glass paper, Toyo GB 60 (10×30 cm); development, 1 hr at 500 V; spray reagent, p-anisidine): one spot; mobility, 9.0 cm. Anhydroglucose content, 98.5%. Periodate consumption, 2.04 moles of $NaIO_4$/anhydroglucose unit. Sugar composition, glucose only (TLC and GLC). Paper chromatogram (solvent, same as solvent A used in TLC; spray reagent, $AgNO_3$) of the partial acid hydrolysates (1N H_2SO_4, 2.5 hr): *Rf* values (double developments) of glucose, gentiobiose, -triose, and -tetraose; 0.77, 0.47, 0.38, and 0.26, respectively. Methylation study: t_R (min); 3.2 and 4.2 (methyl 2,3,4,6-tetra-O-methyl-β- and -α-D-glucoside), 6.6 and 8.9 (methyl 2,3,4-tri-O-methyl-β- and -α-D-glucoside) (the representative chromatogram and the GLC conditions, see Fig. 1).

Determination of Acetyl Group: A suspension of LPe-1-1 (1 g) in 0.2% NaOH (30 ml) was stirred at room temperature for 30 min. After centrifugation, the supernatant was passed through the column of Dowex 50 W-X8 (H^+) and the eluate was analysed by GLC (column packing, Porapac Q (Water Associate Inc.); column temperature, 200°; column size, 2 m×4 mm i.d.; carrier gas, N_2 (40 ml/min)). Only one peak was detected; t_R (min), 3.0 (*cf.* t_R (min): AcOH, 3.0; C_2H_5COOH, 5.6). A calibration curve for AcOH was prepared with good lineality using C_2H_5COOH as an internal standard (I.S.): relative response factor (peak height ratio (AcOH/I.S.)/weight ratio (AcOH/I.S.))=1.1. By employment of the calibration curve, the acetyl content was determined to be 2.7% (*cf.* acetyl content of GE-3: estimated value, 2.5%; reported value, *ca.* 2%[5e)]). Antitumor effect, see Table I.

Properties of the Fraction UR-1——IR ν_{max}^{KBr} cm^{-1}: 890 (β-glucosidic linkage); The whole pattern was similar to that of lichenin. N%, negligible. Coloration with I_2–KI, negative. Sedimentation pattern; In addition to a major peak, presence of a small, faster moving peak could be observed (Fig. 2). Sugar composition: glucose, +++; galactose, and mannose, +; arabinose, xylose, and rhamnose, trace. (by TLC). Antitumor effect, see Table I. Pathologic liver change,[5f)] positive.

Preparation of the Fractions UR-1-1 and UR-1-2——Using the fraction UR-1, the freezing and thawing procedure was repeated several times. After the final thawing, the precipitate was collected centrifugally, washed with acetone and $(C_2H_5)_2O$, and then dried (Fraction UR-1-1). The supernatants were combined together, and concentrated *in vacuo*. To the solution, EtOH was added excessively fo form a precipitate, which was worked up as above (Fraction UR-1-2). Both fractions were obtained as slightly greyish white buff flakes. The ratios of UR-1-1 to UR-1-2 were significantly different among the three lots (lot Nos. 1, 2, and 3) of UR-1, *i.e.* 63: 37, 46: 54, and 32: 68, respectively.

Characterization of the Fraction UR-1-1 (Lichenin Type Glucan)——By direct comparison, all the properties mentioned below were found to be identical with those of the authentic specimen of lichenin isolated

from *Cetraria islandica* var. *orientalis*.[5d] $[\alpha]_D^{18}$ +21.3° (*c*=0.61, 1N NaOH) (*cf.* lichenin, $[\alpha]_D^{19}$ +18.4° (*c*=0.4, 2N NaOH)). ORD, a negative plain curve. IR ν_{max}^{KBr} cm^{-1}: 890 (β-glucosidic linkage). N%, negligible. Solubility; soluble readily in hot water, but sparingly in cold water. Coloration with I_2–KI, negative. Sedimentation pattern: a single, symmetric peak (Fig. 2); A mixed sample of UR-1-1 and the authentic lichenin (1: 1) also gave a single peak. Electrophoresis (Conditions used were same as employed in the case of LPe-1-1, except for voltage (200 V)): one spot; mobility, 4.3 cm. Anhydroglucose content, 95.7%. Periodate consumption, 0.66 moles of $NaIO_4$/anhydroglucose unit. Sugar composition, solely glucose (TLC and GLC). Paper chromatogram (solvent system, BuOH: pyridine: H_2O (6: 4: 3); double developments) of the partial acid hydrolysates (0.33N H_2SO_4, 7 hr), identical with the corresponding chromatogram derived from chenin.

Methylation Study: The fraction UR-1-1 and the authentic specimen of lichenin were respectively subjected to methylation followed by methanolysis under the similar conditions. The gas chromatograms of these methanolysates were found to be identical, both qualitatively and quantitatively. The chromatogram and the GLC conditions are shown in Fig. 3. t_R (min): 5.8 and 7.2 (methyl 2,3,6-tri-O-methyl-D-glucoside); 5.4 and 7.7 (methyl 2,4,6-tri-O-methyl-D-glucoside); 2.8, 3.6, and 4.3 (methyl 2,3,4,6-tetra-O-methyl-D-glucoside). Antitumor effect, see Table I. Pathologic liver change,[5j] positive.

Properties of the Fraction UR-1-2——$[\alpha]_D^{18}$ +75.8° (*c*=0.52, H_2O). ORD, a positive plain curve. IR ν_{max}^{KBr} cm^{-1}: 900 (broad), and 804; Characteristic absorption bands of isolichenin (925, 845, and 800 cm^{-1}) were undetectable (Fig. 4). N%, negligible. Solubility, soluble in cold water. Coloration with I_2–KI, negative. Fehling solution, no precipitate. Sedimentation pattern; a single, but broad peak (Fig. 2). Anhydroglucose content, 50.2%. Sugar composition: glucose, galactose, and mannose, +++; arabinose, xylose, and rhamnose, trace (TLC and GLC). Antitumor effect, see Table I. Pathologic liver change,[5j] negative.

Properties of the Fractions CC-1, CRa-1, CS-1, and CM-1——Basic properties of these fractions are summarized in Table V. Antitumor effect, see Table I.

Table V. Properties of the Crude Polysaccharide Fractions of *Cladonia* Lichens

Lichen	*C. crispata*	*C. rangiferina* subsp. *grisea*	*C. squamosa*	*C. mitis*
fraction	CC-1	CRa-1	CS-1	CM-1
Solubility	most parts readily soluble in cold water			
$[\alpha]_D^{28}$ (1N NaOH)	+48.5° (*c*=0.26)	+42.5° (*c*=0.19)	+60.7° (*c*=0.25)	+36.0°[a] (*c*=0.50)
ORD (1N NaOH, 28°)	positive plain curve			
N%	0.59	0.86	0.46	0.91
Glucose content (%)[b]	52.3	48.6	49.9	43.6
Sugar composition[c]	mannose, glucose, and galactose (###) rhamnose, arabinose, and xylose (trace)			
Coloration with I_2	—	—	—	—
Precipitation with				
Fehling solution	trace	trace	###	##
$Ba(OH)_2$	###	###	###	###
IR spectra (KBr)[d]	mutually similar; 915 and 812 cm^{-1}			
Sedimentation pattern[e]	apparently homogeneous		clearly heterogeneous	

a) The data were cited from ref. 5d).
b) determined by the anthrone method
c) analysed by TLC
d) Representative absorption patterns are shown in Fig. 4.
e) The patterns of CC-, CRa-, CS-, and CM-1 were almost identical with those of CC-, CRa-, CS-, and CM-1-2, respectively (see Fig. 5).

Preparation of the Fractions CC-1-1, CRa-1-1, CS-1-1, and CM-1-1 and the Fractions CC-1-2, CRa-1-2, CS-1-2, and CM-1-2——In the similar way as described in the case of *U. rubescens*, the crude polysaccharide fractions CC-, CRa-, CS-, and CM-1 were separated into the major, cold-water soluble fractions CC-, CRa-, CS-, and CM-1-2 and the minor, insoluble fractions CC-, CRa-, CS-, and CM-1-1, correspondingly, in the following ratios; 85: 15, 90: 10, 95: 5, and 89: 11. All of these fractions were obtained as slightly greyish white buff flakes.

Properties of the Fractions CC-1-2, CRa-1-2, CS-1-2, and CM-1-2——The basic properties of these fractions are summarized in Table II. Antitumor effect of the fraction CS-1-2, see Table I.

Characterization of the Fractions CC-1-1, CRa-1-1, CS-1-1, and CM-1-1 (PC-3 Type Glucan)——These fractions showed commonly the following properties, which were also identical with those of the authentic specimen of PC-3 isolated previously from *Parmelia caperata*.[5h] Specific rotations of CC-, CRa-, CS-, and

CM-1-1: $[\alpha]_D^{22}$ +232° (c=0.15, 1N NaOH), $[\alpha]_D^{20}$ +215° (c=0.15, 1N NaOH), $[\alpha]_D^{19}$ +245° (c=0.15, 1N NaOH), and $[\alpha]_D^{26}$ +242° (c=0.11, 1N NaOH), respectively (PC-3, $[\alpha]_D^{20}$ +231° (c=0.15, 1N NaOH). *cf.* reported value,[5h] +201° (2N NaOH)). ORD, similar positive plain curves. IR ν_{max}^{KBr} cm^{-1}: 925, 845, and 780; The spectra were mutually identical, showing the absorption pattern which agreed with that of PC-3, but did not with that of isolichenin (Fig. 4). Solubility, soluble in hot water, but almost insoluble in cold water. N%, negligible. Coloration with I_2–KI, negative. Sedimentation pattern, identical each other, giving a single peak whose behavior was undistinguishable from that of PC-3. Electrophoresis (conditions used were same as described in the case of UR-1-1): one spot; mobility, 3.3 cm. Anhydroglucose content, from 95 to 98%. Periodate consumption, 0.5±0.02 moles of $NaIO_4$/anhydroglucose unit. Sugar composition: glucose as a overwhelmingly predominant sugar, together with mannose, galactose, arabinose, xylose, and rhamnose in negligible quantities. (TLC and GLC (Fig. 6)).

Methylation Study: This was carried out using the fraction CS-1-1. The resulting methanolysates were analysed by GLC under the same conditions as adopted in the methylation study of UR-1-1. The chromatogram was similar, qualitatively, though not quantitatively, to the corresponding chromatogram resulted from UR-1-1 (Fig. 3). Thus, the equimolar liberation of methyl 2,3,6- and methyl 2,4,6-tri-O-methyl-D-glucosides, accompanying with a small amount of permethylated glucose, was confirmed. Other peaks detected were all negligibly small in size, so that they were not identified. Antitumor effect of the fraction CS-1-1, see Table I.

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