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Polysaccharides in Lichens and Fungi. I. Antitumour Active Polysaccharides of *Gyrophora esculenta* Miyoshi and *Lasallia papulosa* (Ach.) Llano[1]

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A marked inhibiting effect against implanted sarcoma 180 was shown by the so far unknown glucan of a lichen, *Gyrophora esculenta* Miyoshi. The glucan was isolated in a pure state and proved to be a partially acylated β-1,6-glucan. The antitumour active component of another lichen, *Lasallia papulosa* (Ach.) Llano, has been almost established to be identical with that of *G. esculenta*.

Our previous short communication[3a] described the remarkable antitumour activities of water extracts of some lichens against the implanted sarcoma 180 in mice. As for the antitumour activities more details with additional data will be reported soon.[3b]

The purpose of this investigation was to isolate the active components and to elucidate their structures.

Lichens used in this work were *Gyrophora esculenta* Miyoshi (an edible lichen with popular name in Japanese, "Iwatake") and *Lasallia papulosa* (Ach.) Llano. From both the specimens active components have been isolated in pure state, and they were found to be similar glucans with so far unknown structures. Certain polysaccharides from higher plants, algae, and higher fungi have been shown to inhibit growth of transplanted tumours, but few were well characterized. In lichens, occurrence of three glucans, lichenin,[4,5] isolichenin,[4,5d,6] and pustulan,[7,8] has been reported, and the active glucans obtained in this work were proved to be closely related to the latter in the molecular structure. In 1943, pustulan was isolated by Drake[7] from *Umbilicaria pustulata* (L.) Hoffm. and *U. hirsuta* (Sw.) Ach. He proposed the structure as β-1,6-glucan, and later in 1954, the structure was confirmed by Lindberg and MacPherson.[8]

Both active components have been revealed to be β-1,6-glucans bearing small amounts of ester residues in the molecules. Glucans with ester moiety have never been reported

1) Presented at the 88th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, April 1968.
2) Location: *Hongo, Tokyo.*
3) *a*) S. Shibata, Y. Nishikawa, M. Tanaka, F. Fukuoka, and M. Nakanishi, *Zeitschrift für Krebsforschung*, 71, 102 (1968); *b*) F. Fukuoka, M. Nakanishi, S. Shibata, Y. Nishikawa, T. Takeda, and M. Tanaka, *Gann*, 59, 421 (1958).
4) O.P. Mittal and T.R. Seshadri, *J. Sci. Ind. Res.* (India), **13A**, 174 (1954).
5) *a*) W.L. Cunningham and D.J. Manners, *Biochem. J.*, **90**, 596 (1964); *b*) A.S. Perlin and S. Suzuki, *Can. J. Chem.*, **40**, 50 (1962); *c*) S. Peat, W.J. Whelan, and J.G. Roberts, *J. Chem. Soc.*, **1957**, 3916; *d*) N.B. Chanda, E.L. Hirst, and D.J. Manners, *ibid.*, **1957**, 1951; *e*) K.H. Meyer and P. Gürtler, *Helv. Chim. Acta*, **30**, 751 (1947); *f*) R.A. Boissonas, *ibid.*, **30**, 1703 (1947).
6) *a*) S. Peat, W.J. Whelan, J.R. Turvey, and K. Morgan, *J. Chem. Soc.*, **1961**, 623; *b*) K.H. Meyer and P. Gürtler, *Helv. Chim. Acta*, **30**, 761 (1947).
7) B. Drake, *Biochem. Z.*, **313**, 388 (1943).
8) B. Lindberg and J. MacPherson, *Acta Chem. Scand.*, **8**, 985 (1954).

from lichens. Two acidic polysaccharides, luteic acid[9] and islandic acid,[10] produced by *Penicillium* SPP. ARE KNOWN to be malonyl hemi-esters of β-1,6-glucan, but it is evident that the active glucans, being neutral, are not identical with them.

***Gyrophora esculenta* MIYOSHI**

The lichen thallus (collected specimen) was extracted twice with hot water. The first extract was directly evaporated to dryness to give a fraction called GE-1. The second extract was added with ethanol to form a precipitate (fraction GE-2), which was further purified by freezing and thawing method to yield slightly greyish white fibrous flakes (fraction GE-3) (see Fig. 1). The respective yields of fractions GE-1, GE-2, and GE-3 were about 5%, 5.8%, and 5.2%.

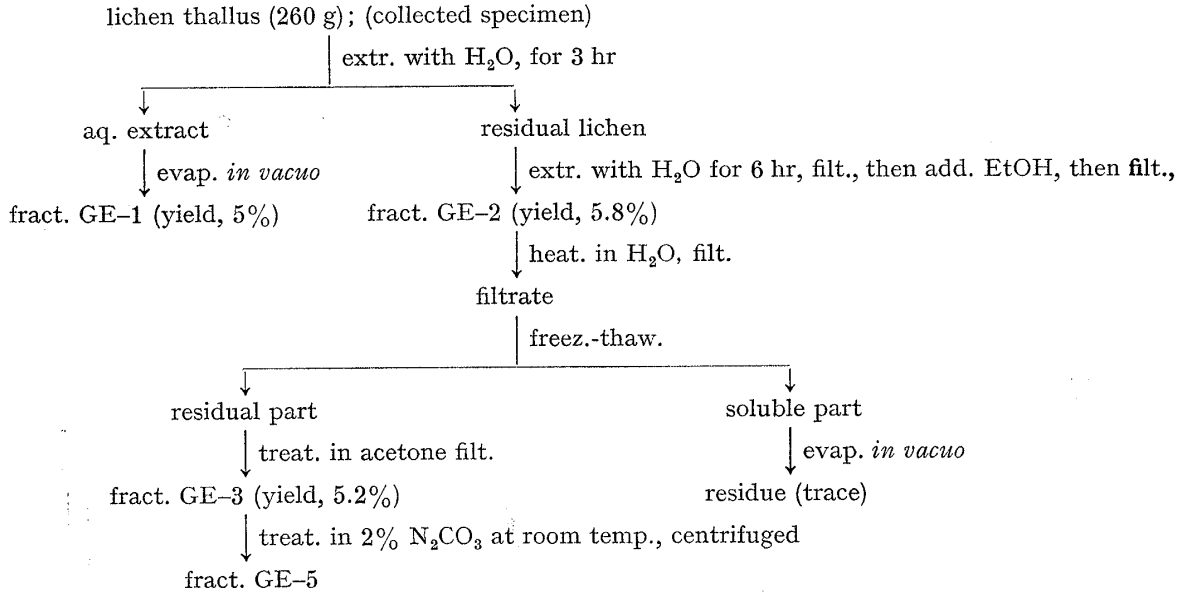

Fig. 1. Fractionation of Aqueous Extract of *Gyrophora esculenta* MIYOSHI

In order to examine the antitumour activity of different lot of the same species, fractions CGE-2 and CGE-3, which correspond respectively to fractions GE-2 and GE-3, were prepared from the commercial specimen available in Chichibu district in Japan. As shown in Table I, some data on antitumour activity cited from our forthcoming paper,[3b] dealing with the details of that field, show their marked effectiveness against implanted sarcoma 180.

These results prompted us to elucidate the molecular structure of the active component.

By the ferric chloride tests and the elemental analyses, it was found that all of these fractions contained neither phenolic components, such as tannin or depside, nor nitrogen containing substances, like protein or peptide. The major constituent of these fractions was suggested to be polysaccharide by the features of their infrared patterns.[11] Since they gave no colouration with iodine, the presence of isolichenin and starch was excluded. On complete acid hydrolysis, each fraction gave D-glucose as a sole product. The total glucose contents were determined by anthrone method to give the following results: GE-1, 57.4%; GE-2, 86.2%; and GE-3, 98.4%. From the above results it was found that the increase

9) *a*) H. Raistrick and M.L. Rintoul, *Trans Royal. Soc.* (London), **B220**, 255 (1931); *b*) C.G. Anderson, W.N. Haworth, H. Raistrick, and M. Stacey, *Biochem. J.*, 33, 272 (1939); *c*) G.G. Freeman and C.S. McPherson, *ibid.*, **45**, 179 (1949); *d*) P.F. Lloyd, G. Pon, and M. Stacey, *Chem. Ind.* (London), **1956**, 172.

10) *a*) E. Ebert and M.H. Zenk, *Archiv. für Microbiologie*, **54**, 276 (1966); *b*) B.H. Howard and H. Raistrick, *Biochem. J.*, **44**, 227 (1949); *c*) J. Baddiley, J.G. Buchanan, and E.M. Thain, *J. Chem. Soc.*, **1953**, 1944.

11) S.A. Barker, E.J. Bourne, and D.H. Whiffen, "Methods in Biochem. Anal.," ed. by D. Glick, Vol. 3, Interscience Publishers, N.Y., 1960, p. 213.

Table I. Basic Properties of Fractions prepared in This Study

Source	Fr. No.	$[\alpha]_D$	IR (KBr), cm^{-1} ester	IR (KBr), cm^{-1} β-linkage	Antitumour activity[b] (inhibit. ratio (%))
Gyrophora esculenta Miyoshi	GE-1				65.9
	GE-2	−38.3° (NaOH)	1735 1250	910	90.0, 93.7 (GE-2) 95.9 (CGE-2)
	GE-3	−37.5° (NaOH) (acetate, +10.3° ($CHCl_3$))	1735 1250	910	99.1, 96.1 (GE-3) 97.9 (CGE-3)
	GE-5	−37.4° (NaOH)	none	910	85.9
Lasallia papulosa (Ach.) Llano	LP-1	−36.7° (NaOH)	1735 1250	910	98.4
	LP-2	−38.8° (NaOH) (acetate, +9.7° ($CHCl_3$))	1735 1250	910	
	LP-4	−40.0° (NaOH)	none	910	
Umbilicaria pustutata (L.) Hoffm.	Pustulan	−37.3° (NaOH)[a] (acetate[8], +9.1° ($CHCl_3$))	none	910[a]	

a) The value was obtained by our measurements using the authentic specimen kindly given by Prof. B. Lindberg.
b) These data are cited from our forthcoming paper, in which the antitumour activities of these fractions will be described in details.

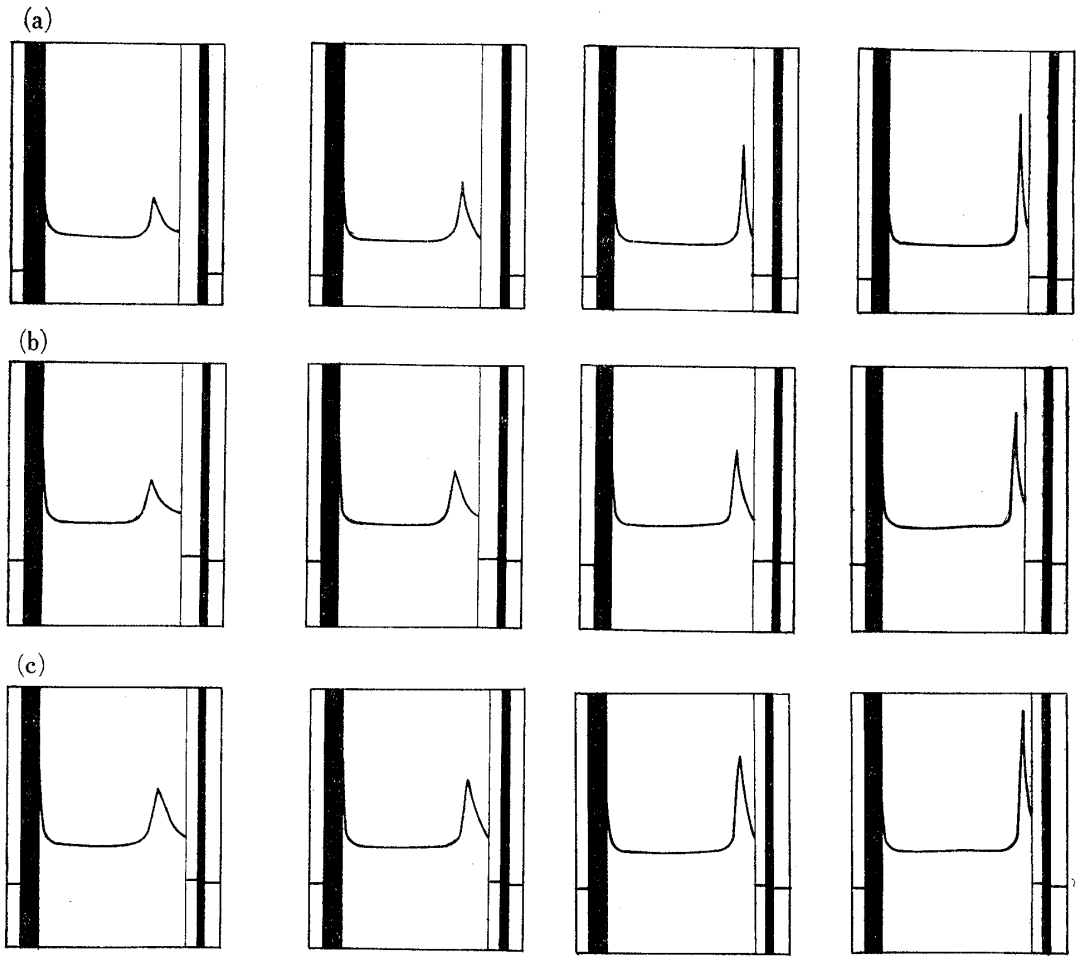

Fig. 2. Sedimentation Patterns of (a) GE-3, (b) GE-3: Pustulan (1:1) and (c) LP-1: Pustulan (1:1) in Borate Buffer

Exposures were made 16, 32, 48 and 64 min after 59780 rpm had been reached.
In these cases sedimentation is from right to left. temperature 7°

of the glucose contents was in accordance with the increase of effectiveness and the most active fraction GE-3 almost entirely consisted of glucose units. Moreover, fraction GE-3 was suggested to be homogeneous, since it gave only one peak in the sedimentation diagram (see Fig. 2).

These findings clearly indicated that a certain glucan would have the antitumour activity, and in analogy to this it has been strongly suggested that the polysaccharide components would be responsible for the antitumour activities of aqueous extracts of other lichens.

The biologically active glucan (GE-3) showed a negative low specific rotation ($[\alpha]_D^{19}$ $-37.5°$ in NaOH), suggesting β-configuration. The configuration was further confirmed by the absorption at 910 cm^{-1} in the infrared spectrum[11]; no absorptions based on α-linkage could be observed.

The products liberated by partial acid hydrolysis of the glucan (GE-3) were analysed by paper chromatography. According to the method proposed by French,[12] log α'-values ($\alpha'=Rf/1-Rf$) of the detected spots were plotted against n, the number of glucose units in the oligosaccharide. A straight line obtained, as shown in Fig. 3, strongly indicated that all the oligosaccharides belong to the same series. Partial acid hydrolysis of the authentic sample of pustulan was carried out under the same conditions, and the chromatogram was found to be identical with that of the hydrolysates of the fraction GE-3.

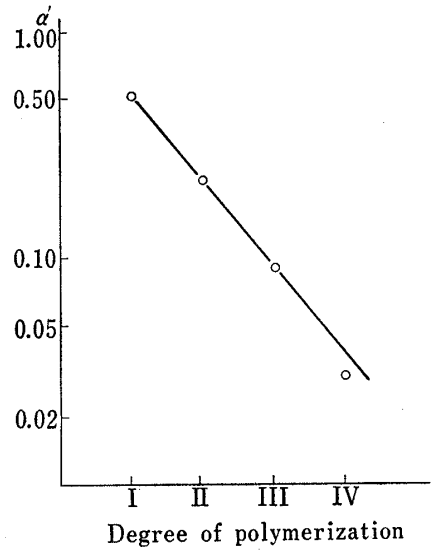

Fig. 3. Relation between log α'-values and Chain Length

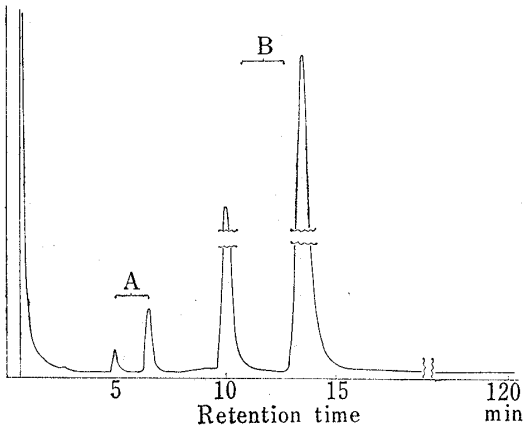

Fig. 4. Gas Chromatogram of the Methanolysis Products of the Methyl Ether of GE–3

A: α- and β-methyl 2,3,4,6-tetra-O-methylglucosides
B: α- and β-methyl 2,3,4-tri-O-methylglucosides

The results showed that the products of GE-3 consisted of glucose, gentiobiose, gentiotriose and gentiotetraose; even traces of other di-, tri-, and tetra–saccharides could not be detected. The same series of oligosaccharides were produced by enzymolysis with a β-1,6-glucanohydrolase, which was prepared from the culture filtrate of *Gibberella* spp. following the procedure reported by A. Shibata.[13] Pustulan also gave the same chromatogram after digestion by the enzyme. Rapid and complete methylation of the fraction GE-3 was achieved according to the Hakomori's method[14] with sodium hydride and methyl iodide in dimethyl sulphoxide. A treatment gave an almost permethylated product, but it still showed a week absorption band of hydroxyl groups in the infrared spectrum. Therefore, the methylation was repeated to yield a product without absorptions of free hydroxyl groups, which showed

12) D. French and G.M. Wild, *J. Am. Chem. Soc.*, **75**, 2612 (1953).
13) A. Shibata, *J. Japan Biochem. Soc.*, **39**, 558 (1967).
14) S. Hakomori, *J. Biochem.*, **55**, 205 (1964).

$[\alpha]_D^{13}$ −15.4° (in $CHCl_3$). Methanolysis of the methylated product gave methyl 2,3,4-tri-O-methyl-D-glucoside as a major product together with a trace amount of methyl 2,3,4,6-tetra-O-methyl-D-glucoside. The methylated glucoses were identified by gas chromatographic analysis and by comparison with authentic specimens. No other peaks could be observed during 2 hours' scanning (Fig. 4).

All the results mentioned above suggested that the fraction GE-3 was built up with a homogeneous linear glucan closely related to pustulan (β-1,6-glucan). The similarity of physical properties of the fraction GE-3 and pustulan has been demonstrated as follows:

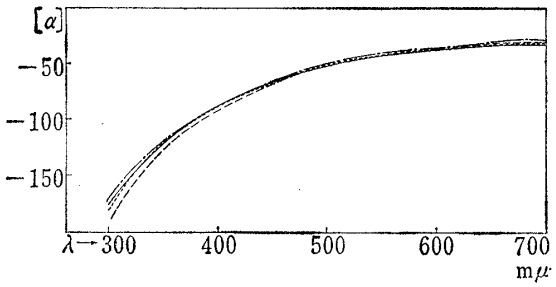

Fig. 5. ORD Curves of Pustulan (————), GE-5 (– – – – –), GE-3 (- - - - - - - - - - -), and LP-1 (·····) (in NaOH)

i) The specific rotation of pustulan was in good agreement with that of the fraction GE-3 (pustulan, $[\alpha]_D^{19}$ −37.3°[15] (c=0.5, 1 N NaOH); GE-3, $[\alpha]_D^{19}$ −37.5° (c=0.5, 1 N NaOH)).

ii) Both compounds gave the similar optical rotatory dispersion curves, as shown in Fig. 5.

iii) The acetate of GE-3 prepared following the method of Carson and Maclay[16] had a low positive rotation ($[\alpha]_D^{18}$ +10.3° in $CHCl_3$), and the value was found to be very close with that of pustulan triacetate reported by Lindberg[8] ($[\alpha]_D$ +9.1° in $CHCl_3$).

iv) Ultracentrifugation of a mixed sample of GE-3 and pustulan (1:1) in borate buffer showed a single peak in the sedimentation diagram (see Fig. 2).

However, a comparison of their infrared spectra indicated that there was a significant difference between the chemical structures of these two polysaccharides. The spectrum of GE-3 had absorption bands at 1735 cm^{-1} and 1250 cm^{-1}, indicating the presence of ester portion in the molecule, while the spectrum of pustulan did not have these absorptions. It is not probable that these absorptions depended upon some low molecular impurities contaminated in this fraction, since successive extractions with organic solvents, such as ethyl ether, acetone, and ethanol, resulted no change in the infrared patterns. Treatment of the fraction GE-3 with 2% sodium carbonate solution at room temperature yielded a product (fraction GE-5,) whose infrared spectrum was quite identical with that of pustulan, and the specific rotation ($[\alpha]_D^{16}$ −37.4° (c=0.5, 1 N NaOH)) was also very close to that of pustulan.

It was concluded from these results that fraction GE-5 entirely consisted of a pustulan-type polysaccharide, and the highly active fraction GE-3 was proved as a new β-1,6-glucan bearing ester residues in a small portion in the molecule. Further investigations are now under progress in order to determine the locations and the total amount of the ester portions and the nature of the acyl grouping.

Lasallia papulosa (Ach.) Llano

As will be reported in another paper,[3b] the precipitated material (fraction LP-1), $[\alpha]_D^{21}$ −36.7° (c=0.5, 2 N NaOH), obtained by adding ethanol to the aqueous extract of this lichen shows a remarkable inhibiting effect on sarcoma 180.

The present study has revealed that the active component of this lichen was closely related chemically to that of *Gyrophora esculenta* Miyoshi. The fraction LP-1 gave the same results as in GE-3, in the experiments of complete and partial acid hydrolysis and also enzymolysis with β-1,6-glucanohydrolase.[13] The infrared spectrum of fraction LP-1 was identical with that of fraction GE-3, including the absorptions of ester at 1735 cm^{-1} and

15) This value was obtained by our own measurement of the authentic sample of pustulan kindly given by Prof. B. Lindberg.

16) J.F. Carson and W.D. Maclay, *J. Am. Chem. Soc.*, **68**, 1015 (1946).

1250 cm^{-1}. In addition, the infrared spectra of acetyl derivatives of both materials were superimposable. Their optical rotatory dispersion curves also supported their similarity (Fig. 5). Moreover, a mixture of LP-1 and GE-3 (1:1) gave only one peak in the sedimentation pattern (Fig. 2). After further purification by freezing and thawing method, the resulting material (fraction LP-2) was treated with 2% sodium carbonate solution. The infrared spectrum of this product (fraction LP-4) was identical with that of the authentic sample of pustulan, having no absorption bands of ester. Both substances had approximately the same values of the optical rotation (LP-4, $[\alpha]_D^{13}$ −40.0° (c=0.45, 2 N NaOH)).

It is evident on the basis of above results that the fraction LP-4 consisted of a β-1,6-glucan, like pustulan, while the active fraction LP-1 contained the gulcan bearing ester grouping. Although more precise comparisons are necessary, it is suggested strongly from the present data that this partially acylated glucan is almost identical with that (fraction GE-3) obtained from *G. esculenta*.

As both the species of lichens used in this study are closely related to *Umbilicaria pustulata* from the taxonomical point of view, it might be probable that pustulan also existed in the latter lichen as a partially acylated form, and the acyl grouping might be separated during the process of isolation taken by Lindberg and MacPherson,[8] since they treated the lichen with 2% sodium carbonate solution for fourteen days, prior to hot water extraction.

Experimental

Paper partition chromatography was carried out on Toyo-Roshi No. 51 with the following solvent systems: *a*) pyridine–AcOEt–AcOH–H_2O (5:5:1:3), and *b*) *n*-BuOH–pyridine–H_2O (6:4:3). Spraying reagents used for detection were $AgNO_3$ and aniline hydrogen phthalate. Antitumour activity shown in this paper is cited from our forthcoming paper,[3b] which will deal wih the details in that part. The assay was made on implanted sarcoma 180 in mice.

Gyrophora esculenta Miyoshi

Isolation and Purification——The lichen "Iwatake," (*Gyrophora esculenta* Miyoshi), collected at Mt. Ryogami, (dry weight, 260 g) was fragmented and extracted with water in a water-bath for three hours. The aqueous extract was filtered and evaporated to dryness under a reduced pressure to give a dark brown powder (fraction GE-1). Yield, 5%. The residual lichen thallus was again extrated with hot water for 6 hr. The hot extract was filtered and ethanol was added to form precipitates which were collected by centrifugation, washed thoroughly with EtOH and ether, and dried. A greyish white powder was obtained. (fraction GE-2). Yield, 5.8%. The fraction GE-2 was warmed in water and small amounts of insoluble substances were removed by filtration. The filtrate was frozen solid overnight and allowed to thaw at room temperature. The remaining solid part was centrifuged and the sedimented portion was stirred in acetone. The fibrous powder was readily collected by filtration, washed with ether and dried. Slightly greyish white buff flakes were yielded (fraction GE-3). Yield, 5.2% (calculated from the weight of starting material) (see Fig. 1). This lichen is commercially available in Chichibu district in Japan, as food-stuff. The commercial specimen (73 g) was also extracted with hot water (1.5 liter) for 5 hr. The aqueous extract was separated according to the same method described as in the case of the second extract of the specimen collected in the mountain, and the fractions CGE-2 and CGE-3, which correspond to GE-2 and GE-3 respectively, were prepared. Yields, CGE-2, 26%; CGE-3, 18%. Some basic properties described below were examined, and it was proved that the fraction GE-2 was undistinguishable from CGE-2, and GE-3 from CGE-3.

Properties of the Fraction GE-2 (or CGE-2)——It gave a negative colouration with iodine–reagent, $FeCl_3$–reagent, and Elson–Morgan reagent. It was soluble in hot water, but only slightly in cold water. $[\alpha]_D^{19}$ −38.3° (c=0.5, 1 N NaOH). IR ν_{max}^{KBr} cm^{-1}: 1735, 1250 (ester), 910 (β-linkage). Antitumour activity—— Inhibition ratio: 93.7% (GE-2), 95.9% (CGE-2); Complete regression: 4/10 (GE-2), 1/6 (CEG-2).

Properties of the Fraction EG-3 (or CGE-3)——It gave no colouration with iodine reagent, $FeCl_3$–reagent, Elson–Morgan reagent, and naphtharesorcinol reagent. It was soluble in hot water, but slightly in cold water. $[\alpha]_D^{19}$ −37.5° (c=0.5, 1 N NaOH) (*cf.* pustulan,[15] $[\alpha]_D^{19}$ −37.3° (c=0.5, 1 N NaOH). As shown in Fig. 5, the ORD curve was superimposable with that of pustulan.[15] Nitrogen content found by elemental analysis was negligible. IR ν_{max}^{KBr} cm^{-1}: 1735, 1250 (ester), 910 (β-linkage). The aqueous solution was shown to be neutral by measurement of pH-value. Sedimentation pattern——As shown in Fig. 2, only one peak was observed. Mixed sample of pustulan and this material (1:1) gave a single peak. Antitumour activity——Inhibition ratio: 99.1% (GE-3), 97.9% (CGE-3); Complete regression: 8/10 (GE-3), 5/8 (CGE-3).

Total Carbohydrate Contents of Fractions GE-1, GE-2, and GE-3——These were determined by anthrone method using glucose as a standard. The results were as follows: GE-1, 57.4%; GE-2, 86.2%; GE-3, 98.4%; and GE-5, 103.7%.

Complete Acid Hydrolysis of Fractions GE-2 and GE-3——Each sample (20 mg) was heated in 1 N H_2SO_4 (2.5 ml) in a boiling water bath for 6 hr. The reaction mixture was neutralized with aq. $Ba(OH)_2$ solution, and $BaSO_4$ was filtered off. The filtrate was treated with Dowex 1-X8 (OH^- form) and Dowex 50W-X8 (H^+ form) for complete removal of salt and concentrated under a reduced pressure to give a syrup, which was analysed by paper chromatography. Only glucose was detected and no other spots could be observed. *Rf*, 0.86 (Solvent system a)); 0.41 (Solvent system b)).

Partial Acid Hydrolysis of Fraction GE-3——Fraction GE-3 (67 mg) was warmed in 1 N H_2SO_4 (4.5 ml) for 2.5 hr in a water bath (bath temp. 90°). After neutralization with aq. $Ba(OH)_2$ solution followed by filtration, the filtrate was treated with Dowex 50W-X8 (H^+ form) and Dowex 1-X8 (OH^- form) to remove salt, then concentrated *in vacuo*. The authentic sample of pustulan was also hydrolyzed under the same conditions. Both products gave the similar paper chromatograms. *Rf* values: 0.34 (glucose); 0.18 (gentiobiose); 0.08 (gentiotriose); and 0.03 (gentiotetraose) (Solvent system b) (see Fig. 3).

Methylation of the Fraction GE-3 by Hakomori's Method——A mixture of NaH (200 mg) and dimethyl sulphoxide (5 ml) was refluxed at 65—70° for 45 min, and then added to the solution (20 ml) of GE-3 (100 mg) in dimethyl sulphoxide. The whole mixture was stirred for 6 hr at room temp. Methyl iodide (1.2 g) was added carefully with further stirring for 16 hr at room temp. in a glass-stoppered flask. After dilution with water, the resulting precipitate was collected by filtration, washed well with water and dried. Eighty mg of pale yellow powder was obtained. The supernatant was extracted with $CHCl_3$ and removal of the solvent yielded 20 mg of residues. These products still had absorptions of OH-groups in their infrared spectra, so that both the fractions were combined together. The repeated methylation yielded 80 mg of the product which gave no absorption near 3400 cm^{-1} in infrared the spectrum (absorption band based on β-linkage, at 900 cm^{-1}). $[\alpha]_D^{13}$ −15.4° (c=0.26, in $CHCl_3$).

Methanolysis of the Fraction GE-3 Methylate——A mixture of GE-3 (24.4 mg) and 5% methanolic HCl (6 ml) was heated at 100° in a sealed tube for 8 hr. After treatment with Amberlite IR4B (OH^- form), the reaction mixture was evaporated *in vacuo* to yield a syrup. The chloroform solution of the syrup was applied to gas liquid chromatography using a Hitachi model F-6 with 5%-Neopentyl glycol succinate on Gaschrom C.L.H. (2 m) at 180°, under a flow of nitrogen. The major and minor products were identified respectively to be methyl 2,3,4-tri-O-methyl-D-glucoside and methyl 2,3,4,6-tetra-O-methyl-D-glucoside by comparison with the authentic samples. Retention times: Major methyl glucoside, α-anomer, 13.6 min, β-anomer, 10.1 min; Minor methyl glucoside, α-anomer, 6.5 min, β-anomer, 5.0 min (see Fig. 4).

Acetylation of the Fraction GE-3——The fraction GE-3 (1 g) was suspended in formamide (10 g), and the mixture was vigorously stirred at 50°. Pyridine (16 g) and Ac_2O (6 g) were added dropwise in this order, and the mixture was stirred for 5 hr at 40°. After being allowed to stand at room temperature for 2 days, the reaction mixture was poured into 2% HCl solution (125 ml) with ice(12.5 g), and the precipitate formed was collected by centrifugation. The product was washed with 0.5% HCl solution and water, and then dried. The infrared spectrum still had an absorption of OH-groups near 3600 cm^{-1}. Therefore, the material was reacetylated with a mixture of pyridine (20 ml) and Ac_2O (6 ml) by the usual manner. The product gave a strong absorption band of acetyl group at 1760 cm^{-1}, but no absorption of OH-groups in its infrared spectrum. Yield, 680 mg. $[\alpha]_D^{13}$ +10.3° (c=0.126, in $CHCl_3$) (*cf.* pustulan triacetate,[8] $[\alpha]_D$ +9.1° (c=0.3, in $CHCl_3$)).

Enzymatic Hydorlysis of the Fraction GE-3 with β-1,6-Glucanohydrolase——β-1,6-Glucanohydrolase was prepared from the culture filtrate of *Gibberella* spp. following A. Shibata's method.[13] To a suspended solution of GE-3 (20 mg) in 0.005 M KOAc buffer (pH 5.0) (2 ml), the enzyme in phosate buffer buffer (pH(6.8) (0.5 ml) was added, and the mixture was incubated at 37° for 2 hr. A part of the reaction mixture was taken out and heated at 100° for 30 min to inactivate the enzyme. The authentic sample of pustulan was also digested by the enzyme under the same conditions. Both the products gave the identical chromatogram on paper, showing the liberation of glucose, gentiobiose, gentiotriose, and gentiotetraose. The incubation was continued for further 24 hr and samples were taken at intervals for determination of the copper-reducing power by Somogyi-Nelson method. Reducing power was increasing gradually during this perjod.

Extraction of the Fraction GE-3 with Organic Solvents——The finely powdered fraction GE-3 (700 mg) was successively extracted with EtOH, acetone, and ether, using 500 ml of solvent in each extraction under refluxing for several hours. The infrared spectra of each residual part were all identical with that of fraction GE-3, giving the absorption bands of ester at 1735 and 1250 cm^{-1}. Each extracted portion gave only negliegible amounts of residues on evaporation.

Cleavage of Ester Bond of the Fraction GE-3 with Alkali——The fraction GE-3 (30 mg) was suspended in 0.1 N $NaHCO_3$ solution (5 ml) and the mixture was stirred for 30 min at room temp. The insoluble part was collected by centrifugation, washed with water, EtOH, and ether, and then dried. Its infrared spectrum gave still the absorption of ester. Further treatment with 1 N $NaHCO_3$ solution did not cause any changes in the infrared spectrum. GE-3 (100 mg) was stirred in 1% Na_2CO_3 solution (5 ml) for 15 min at room temp. The absorption bands at 1735 and 1250 cm^{-1} remained sitll, but became very weak. Further

treatment of the material with 2% Na_2CO_3 solution for 20 min yielded a product without these absorptions (fraction GE-5). The whole spectrum was superimposable with that of the authentic sample of pustulan. It had $[\alpha]_D^{16}$ −37.4° (c=0.5, 1 N NaOH) (*cf.* pustulan,[15] $[\alpha]_D^{19}$ −37.3° (c=0.5, 1 N NaOH)) The ORD curves of both samles were also similar as shown in Fig. 5. Antitumour activity——Inhibition ratio: 85.5%, complete regression: 1/8.

***Lasallia papulosa* (ACH.) LLANO**

Isolation and Purification——The lichen thallus (collected in Virginia, U.S.A.) (10 g) was fragmented into small pieces and extracted with hot water for 8 hr. After filtration and concentration, EtOH was added to the extract. The resulting precipitate was collected by centrifugation. (fraction LP-1). Yield, 3.9 g (39%). Three gram of the fraction LP-1 was heated with water and small amounts of the insoluble parts were removed by filtration. The filtrate was frozen overnight and allowed to thaw at room temperature, giving 2.5 g of slightly greyish white powder (fraction LP-2). Yield, 83%, based on the fraction LP-1, and 32%, based on the starting material.

Properties of the Fractions LP-1 and LP-2——Basic properties were examined at the stage of the fraction LP-1. It gave no colouration with iodine, $FeCl_3$–reagent, and Elson–Morgan reagent. Elemental analysis showed that the sample contained negligible amounts of nitrogen. The sedimentation pattern of mixed sample of this material and the authentic sample of pustulan (1:1) gave only one peak, as shown in Fig. 2. Both the fractions LP-1 and LP-2 gave similar infrared spectra, which were also identical with that of the fraction GE-3, a fraction (partially acylated β-1,6-glucan) isolated from *G. esculenta* MIYOSHI, including the absorptions at 1735 and 1250 cm^{-1} (ester) and 910 cm (β-linkage). Specific optical rotation: the fraction LP-1, $[\alpha]_D^{21}$ −36.7° (c=0.305, 2 N NaOH); the fraction LP-2, $[\alpha]_D^{21}$ −38.8° (c=0.32, 2 N NaOH). The ORD curve of the fraction LP-1 was identical with that of pustulan (Fig. 5). Antitumour activity of the fraction LP-1——Inhibition ratio: 98.4%; complete regression: 9/10.

Complete and Partial Acid Hydrolysis and Enzymolysis of the Fraction LP-1——These experiments were carried out under the same conditions used in the case of the fraction GE-3, and the same results were obtained.

Treatment of the Fraction LP-2 with Alkali——The fraction LP-2 was stirred in 2% Na_2CO_3 solution at room temperature for 30 min to give a product (fraction LP-4), $[\alpha]_D^{13}$ −40.0° (c=0.45, 2 N NaOH), giving no absorption bands of ester in its infrared spectrum. The spectrum was superimposable with that of the authentic sample of pustulan.

Acetylation of the Fraction LP-2——The fraction LP-2 was acetylated in the same way as described in the case of the fraction GE-3. The infrared spectrum of the product was superimposable with that of the fraction GE-3 acetate. Both acetates showed similar $[\alpha]_D$ values: LP-1 acetate, $[\alpha]_D^{19}$ +9.7° (c=0.20, in $CHCl_3$); *cf.* GE-3 acetate, $[\alpha]_D^{13}$ +10.3° (c=0.12, in $CHCl_3$).

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