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Polysaccharides of Lichens and Fungi. IV.[1,2] Antitumour Active O-Acetylated Pustulan-Type Glucans from the Lichens of Umbilicaria Species

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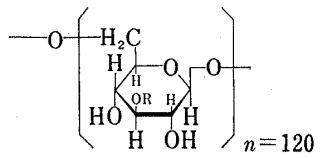
Three species of lichens belonging to *Umbilicaria*, *i.e.*, *U. angulata*, *U. caroliniana*, and *U. polyphylla*, have been examined for their water-soluble polysaccharide consitituents by chemical and physico-chemical methods. The results showed that they contained a partially O-acetylated pustulan as a sole water-soluble polysaccharide. All the polysaccharide specimens obtained here showed a remarkable antitumour effect against the implanted Sarcoma-180 in mice.

In our previous papers[1,4–8] we have reported studies on structure and host mediated antitumour action against the implanted Sarcoma-180 of the water-soluble polysaccharide (designated GE-3) isolated from the lichen *Gyrophora esculenta* Miyoshi ("Iwatake" in Japanese). So far our structural studies have revealed that GE-3 (I) is essentially a linear (1→6)-linked β-glucan (DP *ca.* 120) in which approximately 10% of the glucose units carry O-acetyl groups in the 3-position. A glucan (designated LP-2) closely similar, probably identical to GE-3, has also been obtained as the active principle of another lichen *Lasallia papulosa* (Ach.) Llano.[9] Although the occurrence of a β-(1→6)-glucan, named pustulan, had been known in two species of lichens, *Umbilicaria pustulata* (L.) Hoffm. and *U. hirsuta* (Sw. ex Wester) Ach.,[10–12] GE-3 and LP-2 were the first examples isolated in the partially O-acetylated form.



R=H : R=$COCH_3$ ≒ 10 : 1

I

Taking account of the taxonomically close correlation of the three genera, *Gyrophora*, *Lasallia*, and *Umbilicaria*, all belonging to the family Gyrophoraceae, we assumed from the above finding that the partially acylated pustulan, namely GE-3 type glucan, might commonly distribute as an almost sole water-soluble polysaccharide constituent among the lichens of

1) Part III: Y. Nishikawa, T. Takeda, S. Shibata, and F. Fukuoka, *Chem. Pharm. Bull.* (Tokyo), **17**, 1910 (1969).
2) An outline of this work was presented at the 89th Annual Meeting of the Pharmaceutical Society of Japan, Nagoya, April 1969. Abstracts: *a*) Y. Nishikawa, M. Tanaka, S. Kobayashi, and S. Shibata, p. 616; *b*) T. Takeda, Y. Nishikawa, S. Shibata, F. Fukuoka, and M. Nakanishi, p. 616.
3) Location: *a*) *Hongo, Bunkyo-ku, Tokyo*; *b*) *Tsukiji, Chuo-ku, Tokyo.*
4) F. Fukuoka, M. Nakanishi, S. Shibata, Y. Nishikawa, T. Takeda, and M. Tanaka, *Gann*, **59**, 421 (1968).
5) S. Shibata, Y. Nishikawa, T. Takeda, and M. Tanaka, *Chem. Pharm. Bull.* (Tokyo), **16**, 2362 (1968).
6) Y. Nishikawa, *Japan. J. Clinic. Med.*, **27**, 1744 (1969).
7) Y. Nishikawa, Abstract of the 13th Annual Meeting of the Eastern Branch of the Pharmaceutical Society of Japan, 1969, p. 1.
8) Y. Nishikawa, *Farumashia*, **5**, 543 (1969).
9) According to the private communication from Assoc. Prof. J.M. Aronson, Arizona State University, he has recently isolated the pustulan-type glucan from the North Americal specimen of this lichen.
10) B. Drake, *Biochem. Z.*, **313**, 388 (1943).
11) B. Lindberg and J. MacPherson, *Acta Chem. Scand.*, **8**, 985 (1954).
12) C.G. Hellerqvist, B. Lindberg, and K. Samuelsson, *Acta Chem. Scand.*, **22**, 2736 (1968).

this family. In order to secure it, we have examined here the water-soluble polysaccharide fractions obtained from the additional three species of lichens of *Umbilicaria*, *e.g.*, *U. angulata* Tuck., *U. caroliniana* Tuck., and *U. polyphylla* (L.) Baumg. The fractions, named UA-1, UC-1, and UPo-1, respectively, were readily prepared in good yields by precipitation with ethanol. By analysis of the acid hydrolyzate and determination of the total carbohydrate content it has been shown that all these fractions almost solely consisted of D-glucose units. Comparison of their specific rotations, optical rotatory dispersion curves (ORD), infrared spectra (IR), nuclear magnetic resonance spectra (NMR), and sedimentation patterns revealed that their basic physical properties are closely similar each other and also to those of GE-3. The negative low values of their specific rotations and their negative plain ORD curves suggested that β-D-configuration is predominant in these glucans. It was further confirmed by the features of their IR spectra, which showed the absorption at 910 cm^{-1} and no bands based on the α-glucosidic linkage. The partial acid hydrolysates of these glucans gave the identical paper chromatograms to that of GE-3, showing the liberation of a homologous series of gentio-oligosaccharides. The presence of O-acetyl groups in their molecules was indicated by the absorption band at 1735 cm^{-1} in their IR spectra and by the small signal around δ 2.1 ppm (in D_2O, DSS as an internal reference) in their NMR spectra. The acyl group was conclusively decided to be O-acetyl by identification of the acid liberated on saponification. The present glucans seemed to contain the similar amounts of O-acetyl groups to that of GE-3, since the signal sizes of the methyl protons of acetyl in their NMR spectra were observed to be approximately equal to that of the latter. Their molecular weights were considered to be not so much different from that of GE-3 by comparison of sedimentation behaviour among them. The identity of UA-1, UC-1, and UPo-1 with GE-3 and LP-2 has been supported further from the results of their biological activities. As indicated in Table I, the present samples showed

Table I. Antitumour Effect[a] of Water-Soluble Polysaccharide Preparations Obtained from *U. angulata*, *U. caroliniana*, and *U. polyphylla*

Samples	Inhibition ratio(%)	Complete regression	Mortality(died/total)		Average body wt. change(g)		Liver changes[b]
			Control	Treated	Control	Treated	
UA-1	91.8	3/9	2/10	0/9	+4.9	+5.5	+
UC-1	94.8	2/9	1/9	0/9	+3.5	+4.3	+
UPo-1	97.0	6/10	0/10	0/10	+5.2	+5.9	+
	96.4	6/10	2/10	0/10	+4.9	+5.1	+
GE-3[c]	97.3	7/10	0/10	0/10	—	—	+
LP-1[d,a]	98.4	9/10	0/9	0/10	—	—	+

a) Tumour, sarcoma-180(solid); animal, Swiss albino mouse; dose, 150 (mg/kg)×10 (days); route, *i.p.*; vehicle, aq. dest
b) So far unknown pathologic changes, details of which will be reported elsewhere.[13]
c) For comparison, data were cited from our previous paper[3]
d) LP-1 is almost pure LP-2. In this case, dose administered was 200 mg/kg×10 days.

the remarkable growth-inhibitory effect like GE-3 on the implanted sarcoma-180 in mice. It has incidentally been observed during our experiments on the antitumour activity that the liver of the mice receiving injections of GE-3 underwent hitherto unknown pathologic changes. The liver changes were also encountered, without exception, in the case of the present samples. Details of the study will be reported soon separately.[13]

From the results of the present study, together with our previous findings, it seemed to be probable that there might be some correlation between the taxonomical position of a lichen and its water-soluble polysaccharide constituent(s).

13) R. Tokuzen, W. Nakahara, F. Fukuoka, S. Shibata, and Y. Nishikawa, *Toxicl. Appl. Pharmacol.*, in press.

As water-soluble polysaccharides of lichens, lichenin and isolichenin, are well known to occur in *Cetraria islandica* (L.) ACH. and some other species, co-existingly or individually.[7,14] Another glucan, named everniin, has also been reported from *Evernia prunastri* (L.) ACH.[10,15] So far, however, little has been done on the distribution of these lichen polysaccharides. Therefore, further systematic survey work on the polysaccharide constituents contained in the aqueous extracts of some lichens of other families is now under progress, which may help, in future, to predict which lichens are promissing as the source of the polysaccharides with this type of antitumour effect.

Experimental

The IR spectra were measured with a Japan Spectroscopic Co. Model DS 402G Spectrophotometer, the NMR spectra with a Japan Electron Optics Lab. JMN-3H-60 Spectrometer, the specific rotations with a Yanagimoto Model OR-50 Polarimeter, and the ORD curves with a Japan Spectroscopic Co. Model ORD/UV-5 Spectrometer. A Spinco Model E analytical ultracentrifuge with a schlieren optical system was used for measurements of sedimentation patterns. Gas liquid chromatographic analyses were carried out with a Shimazu Model GC-1B Gas Chromatograph attached with a hydrogen flame detector. Paper chromatograms were run by the descending method with *n*-butanol:pyridine:water in the proportions 6:4:3 (V/V), and sugars were detected on the papers (Toyo Roshi No. 51A) by the ammonia silver nitrate or anilinehydrogenphthalate spraying reagents. Thin-layer chromatography was performed on silica gel (Camag D-5) with $CHCl_3$, and spots were detected by heating the plates after spraying with dil. H_2SO_4.

Preparation of the Polysaccharide Fractions——*U. caroliniana* TUCK. was a generous gift from Dr. S. Kurokawa (a specimen with code number 65172 in his collection), who collected it in Mt. Kimpu, Province Shinano, Japan in July, 1965. *U. angulata* TUCK. and *U. polyphylla* (L.) BAUMG. were collected in the North Peak of Squamish Chief, B.C., Canada, in May, 1968. The respective lichen thalli were crushed into small pieces and extracted three times with distilled water on a boiling water-bath for 5—7 hr. Each extract was filtered while hot and the filtrate was added excessively with EtOH to give a slightly grayish white precipitate, which was collected by centrifugation, washed with acetone and ether, and then dried. The yields and names of the polysaccharide preparations were as follows;

Lichens	*U. angulata* (157 g)	*U. caroliniana* (38 g)	*U. Polyphylla* (6.5 g)
Names of prepn.	UA-1	UC-1	UPo-1
Yields (g) from			
1st. extr.	6.6	5.1	1.0
2nd. extr.	2.7	0.8	0.7
3rd. extr.	2.0	0.3	0.1
Total yields (%)	7.2	16.0	28.1

Further purification is readily attainable by repetition of the freezing-thawing method.

Characterization of the Polysaccharides UA-1, UC-1, and UPo-1——i) Elemental analyses showed that all of these contained negligible amount of nitrogen. ii) The values of their specific rotations $[\alpha]_D^{19}$, (c=0.5, 1N-NaOH)):UA-1, −30°; UC-1, −36°; UPo-1, −32°. iii) They gave similar negative plain ORD curves, which were identical with that of GE-3.[5] iv) Total anhydroglucose content of each preparation was determined to be over 95% by the anthrone-H_2SO_4 method. v) Their IR spectra were identical each other and also with that of GE-3. IR ν_{max}^{KBr} cm^{-1}: 910 (β-glycosidic linkage), 1735, 1250 (acetyl C=O). vi) The NMR spectra were measured at the concentration of 4% in D_2O, using DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) as an internal reference. The sizes of the signals around δ 2.1 ppm (CH_3CO–) were equal mutually and also to that of GE-3, whose acetyl content had been determined to be approximately 2%.[1] vii) Ultracentrifugation of the respective mixture of each polysaccharide (10 mg) and GE-3 (10 mg) in

14) C.F. Culberson, "Chemical and Biochemical Guide to Lichen Products," The University of North Carolina Press, U.S.A., 1969.

15) *a*) F. Stüde, *Ann.*, **131**, 241 (1864); *b*) K. Müller, *Z. Physiol. Chem.*, **45**, 265 (1905); *c*) A. Ullander and B. Tollens, *Ber.*, **39**, 401 (1906); *d*) V. Stefanovic and Ph.D. Thesis, "Fa of cultySciences, University of Belgrade", 1960; *e*) V.M. Mićović, M. Hranisavljević-Jakovljević, and J. Miljković-Stojanović ,*Carbohydr. Res.*, **10**, 525 (1969).

distilled water (4 ml) was carried out in a standard double sector cell at 59,700 rpm, at 14°, and a phase angle of 65°. All of them gave a single symmetric peak in the sedimentation diagrams.

Complete and Partial Acid Hydrolysis——A mixture of 50 mg of each polysaccharide and 5 ml of 1N H_2SO_4 was heated on a boiling water-bath. Heating was continued for 9 hr in the case of complete hydrolysis and for 4 hr in the case of partial hydrolysis. After neutralization by treatment with Amberlite IR-4B (OH- form) followed by concentration into a syrup under a reduced pressure, the resulting hydrolysates in each case were analyzed by paper chromatography. D-Glucose and a negligible amount of galactose were detected as the products of complete acid hydrolysis. The chromatograms of their partial acid hydrolysates were all identical with that of GE-3, showing the liberation of a series of gentio-oligosaccharides.[5]

Identification of Acetic Acid Liberated by Saponification——Each polysaccharide (100 mg) was suspended in 0.2% NaOH solution (4 ml) and the mixture was stirred at room temp. for 30 min. After removal of the precipitated material by filtration, the filtrate was passed through the Amberlite IR-120B (H^+ form) resin. A small portion of the eluate was analyzed by gas liquid chromatography, using Porapak Q column (Waters Associates Inc.): column temp., 223°; detector temp., 210°; carrier gas, N_2. Only one peak was observed in every case, and the retention time (2.4 min) was exactly equal to that of acetic acid. The rest of the acidic solution was neutralized with dil. NaOH and condensed into a syrup *in vacuo*. After acidification with dil. HCl, *p*-bromophenacyl bromide in EtOH was added excessively to the solution and then the whole mixture was refluxed for 1 hr. The resulting ester was identified as *p*-bromophenacyl acetate by thin-layer chromatography.

Assay Method of Antitumour Effect——The test was made by observing the effect on the growth of subcutaneously implanted Sarcoma-180 (solid form) for 5 weeks. Samples were injected intraperitoneally as suspension in distilled water. Details of the method were described in our previous paper.[4] The results are shown in Table I.

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