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Polysaccharides of Lichens and Fungi. V.[1] Antitumour Active Polysaccharides of Lichens of *Evernia*, *Acroscyphus* and *Alectoria* spp.

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The polysaccharides of some lichens were studied chemically in relation to their host-mediated antitumour activities against sarcoma 180 in mice. A water-insoluble polysaccharide fraction, EP-3, $[\alpha]_D^{17}$ +200°, from *Evernia prunastri* (L.)ACH. was shown to be an α-glucan containing α(1→3)(1→4) linkages in the ratio of 4:1.

The cold water-soluble fraction, EP-4, was separated into EP-7, $[\alpha]_D^{17}$ +12°, and EP-6, $[\alpha]_D^{17}$ +164°. The former was shown to be an antitumour active glucan having β (1→3)(1→4) linkages in the ratio of 3:1, while the latter was revealed to be an α(1→3) (1→4) glucan (approx. ratio 3:2).

The properties of these polysaccharides were discussed in comparing with the data of everniin reported by Mićović, *et al.* A polysaccharide AS-3-IS, $[\alpha]_D^{14}$ +176°, isolated from *Acroscyphus sphaerophoroides* LEV. giving an intensive blue iodine-colour reaction was shown to be a new type of α-glucan consisting of α(1→3), (1→4) and (1→6) linkages. A cold water-soluble polysaccharide, AS-3-S, was revealed to be a new glycopeptide.

The polysacchrides, ASu-2 and ASa-2 isolated from *Alectoria sulcata* (LEV.)NYL. and *A. sarmentosa* (ACH.)ACH. were proved to be lichenin, and ASu-3 and ASa-3 were shown to be isolichenin.

The host-mediated antitumour activities of the polysaccharide fractions isolated from the above lichen species were tested.

In connection with our previous studies[1] on the antitumour activities and the chemical structures of lichen polysaccharides, the present paper concerns chiefly the structural study of the polysaccharides isolated from the lichens of *Evernia prunastri* (L.) ACH., *Acroscyphus sphaerophoroides* LEV., *Alectoria sulcata* (LEV.) NYL., and *Alectoria sarmentosa* (ACH.) ACH.

TABLE I. Antitumour Activities of the Water-Soluble Polysaccharide Preparations obtained from *Evernia prunastri*, *Acroscyphus sphaerophoroides*, *Alectoria sulcata* and *Alectoria sarmentosa*

Samples	Inhibition ratio (%)[a]	Complete regression[a]	Animal[a]
EP-1	84	1/ 9	S
EP-7	100	10/10	I
AS-1	93	5/10	S
ASu-1	85	5/10	I
ASu-2	98	7/10	I
ASa-1	87	0/ 9	I
ASa-2	99	4/ 9	I

Tumour: Sarcoma 180 (solid), route: *i.p.*, dose: 150 mg/kg×10 days, animal: S=Swiss albino mice, I=ICR mice

a) biological assay methods: See ref. (9).

1) Part IV: Y. Nishikawa, M. Tanaka, S. Shibata, and F. Fukuoka, *Chem. Pharm. Bull.* (Tokyo), **18**, 1431 (1970).

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

As shown in Table I the present samples of lichen polysaccharides showed remarkable growth-inhibitory effects on the implanted sarcoma 180 in mice.

The Polysaccharides of *Evernia prunastri* (L.) Ach.

The water extract of *Evernia prunastri* contains three kinds of polysaccharides. The crude polysaccharides were separated by the repeated freezing and thawing procedure into a water-insoluble polysaccharide fraction named EP-3 ($[\alpha]_D^{17}$ +200°), and a cold water-soluble fraction designated EP-4.

EP-4 was fractionated further by precipitation with 0.2M cetyltrimethylammonium hydroxide. The precipitated fraction ($[\alpha]_D^{17}$ +12° (water)) was named EP-7 and the supernatant fraction was named EP-6 ($[\alpha]_D^{17}$ +164°). EP-3 and EP-6 showed characteristic absorption at 845 cm^{-1} in the infrared (IR) spectrum (KBr), while EP-7 showed an absorption maximum at 890^{-1}. The methyl ethers of EP-3, EP-6, and EP-7 prepared by the combined Hakomori and Kuhn methods were methanolysed, and the products were analysed with gas-liquid chromatography (GLC) to reveal the formation of methyl 2,3,6-tri-O-methyl and methyl 2,4,6-tri-O-methyl-D-glucopyranosides together with a small amount of methyl 2,3,4, 6-tetra-O-methyl-D-glucopyranoside. On periodate oxidation EP-3, EP-6, and EP-7 consumed respectively 0.17, 0.35, and 0.26 moles of the reagent per one anhydroglucose unit.

Isolichenin, everniin[3] and PC-3[4] are known as the α-glucan so far isolated from lichens. EP-3 and EP-6 have now been found to be new α-glucans containing α (1→3) (1→4) linkages in the ratio of 4: 1 for EP-3 and approximately 3: 2 for EP-6. The antitumour active principle of *Evernia prunastri*, EP-7, has been shown to be a β-glucan having (1→3) (1→4) linkages in the ratio of 3: 1. The average degree of polymerization (DP) of EP-3, EP-6, and EP-7 measured by the end group assay and the methylation study were 70, 160, and 60 glucose units, respectively.

A cold water-insoluble polysaccharide, everniin, $[\alpha]_D^{19}$ +138°,[5] was isolated from the same lichen and reported to be an α-polyglucan containing (1→3) and (1→4) linkages in the ratio of 4: 1 with a molecular weight of $1.8—3.4\times10^4$.

EP-3 is similar to everniin except the specific rotaion value ($[\alpha]_D^{17}$ +200°). This difference must be due to their purities, since the earlier workers isolated only everniin from the lichen, whereas the present authors revealed that the water-soluble fraction of the same lichen consists of at least three kinds of polysaccharides.

The Polysaccharides of *Acroscyphus sphaerophoroides* Lev.

Acroscyphus sphaerophoroides is a very unique lichen occurring as one species in one genus in some limited higher altitude area in Circum-Pacific region.[6] The crude polysaccharide fraction (AS-1) was isolated by adding ethanol to the aqueous extracts. By the repeated freezing and thawing procedure, the fraction AS-1 was separeted readily into a major, cold water-soluble fraction, AS-3S (Yield: 2.8%), and a minor, cold water-insoluble fraction, AS-3-IS (Yield: 0.16%). Both the fractions gave one spot on a high-voltage paper electrophoresis using a borate buffer and a single sedimentation pattern on ultracentrifugation, respectively. The former seems to be a glycopeptide which will be discussed elsewhere, and the present communication deals with the structure of AS-3-IS. It was shown to be a homoglucan which gives a positive plain optical rotatory dispersion (ORD) curve ($[\alpha]_D^{14}$ +176°) and a characteristic absorption at 845 cm^{-1} in its IR spectrum (KBr) indicating that α-D-configuration is predominant in the molecule.

3) V.M. Mićović, M. Hranisavljević, and J. Miljković-Stojanović, *Carbohyd. Res.*, **10**, 525 (1969).
4) T. Takeda, Y. Nishikawa, and S. Shibata, *Chem. Pharm. Bull.* (Tokyo), **18**, 1074 (1970).
5) V. Stefanovich, *Life Science*, **8**, 122 (1969).
6) S. Shibata, O. Tanaka, U. Sankawa, Y. Ogihara, R. Takahashi, S. Seo, D. Yang, and Y. Iida, *J. Jap. Botany*, **43**, 10—11 (1968).

This glucan showed a distinct blue colouration with iodine, which is very characteristic and remarkable for identification. The GLC of the methanolysis products of AS-3-IS methyl ether prepared by using the Hakomori method and the Kuhn procedure revealed the liberation of methyl 2,3,4-tri-O-methyl-, methyl 2,4,6-tri-O-methyl-, and methyl 2,3,6-tri-O-methyl-D-glucopyranoside, together with a small amount of methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside. (Fig. 1), which were identified with the authentic samples.

Above results have indicated that AS-3-IS is a new type of linear glucan containig $\alpha(1\rightarrow3)$, $\alpha(1\rightarrow4)$, and $\alpha(1\rightarrow6)$ linkages in a molceule. Further studies on the sequence of linkages and colour reaction with iodine are now in progress.

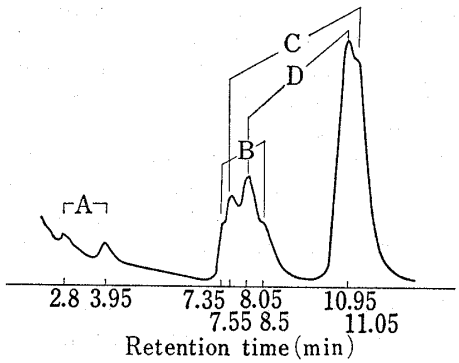

Fig. 1. Methylation Analysis of AS-3-IS

A: methyl 2,3,4,6-tetra-O-methyl glucoside
B: methyl 2,3,4-tri-O-methyl glucoside
C: methyl 2,4,6-tri-O-methyl glucoside
D: methyl 2,3,6-tri-O-methyl glucoside

The Polysaccharides of *Alectoria sulcata* (Lev.) Nyl. *Alectoria sarmentosa* (Ach.) Ach.

It is a well-known fact that the lichen, Iceland moss (*Cetraria islandica* (L.) Ach.) contains two kinds of glucans, lichenin and isolichenin, whose properties and chemical structures have already been investigated extensively by earlier workers.[7]

Isolichenin is soluble even in cold water, while lichenin is soluble only in hot water. In lichenin, $[\alpha]_D$ +18.4°, glucose residues are united through β-1→3 and β-1→4 linkages in the ratio of 3: 7, while in isolichenin, $[\alpha]_D$ +272°, through α-1→3 and α-1→4 linkages in the ratio of 55: 45. The lichens, *Alectoria sulcata* and *A. sarmentosa*, were extracted with hot water and the extracts were added with ethanol to form precipitates (Fractions ASu-1 and ASa-1, respectively), which were separated by the freezing and thawing procedure.

The water-insoluble polysaccharide fractions were named ASu-2, and ASa-2, respectively. The IR spectra of ASu-2 and ASa-2 were identical respectively with the IR spectrum of the authentic samples of lichenin of Iceland moss which was kindly supplied by Dr. S. Peat. The specific rotation of the samples, ASu-2, $[\alpha]_D^{14}$ +17.5°, and ASa-2, $[\alpha]_D^{14}$ +19°, were in good agreement with that of lichenin.[8]

On the other hand, the samples, ASu-3 and ASa-3, were soluble in cold water. Their IR spectra and specific rotations were identical respectively with that of authentic sample of isolichenin (ASu-3, $[\alpha]_D^{14}$ +265°; ASa-3, $[\alpha]_D^{14}$ +275°). The identity of ASu-2 and ASa-2 with lichenin has also been supported from the results of their biological activities.

As indicated in Table I, the present samples showed the remarkable growth-inhibitory effect like lichenin of *Cetraria richardsonii* on the implanted Sarcoma 180 in mice.

Experimental

The infrared (IR) spectra were measured with a Japan Spectroscopic Co. Model DS 402G Spectrophotometer, 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. Gas–liquid chromatographic analyses were carried out with a Shimazu Model GC-4APF Gas Chromatograph attached with a hydrogen flame detector.

Assay Methode of Antitumour Activities——The test was made by observing the effect on the growth of subcutaneously implanted Sarcoma-180 (Solid from) for 5 weeks. Samples suspended in distilled water

7) M. Fleming and D.J. Manners, *Biochem. J.*, **100**, P 24 (1966).
8) M. Fleming and D.J. Manners, *Biochem. J.*, **100**, P 4 (1966).

were injected in mice (Swiss Albino or ICR mice) intraperitoneally. The details of the assay method were described in our earlier paper.[9] The results are shown in Table I.

Isolation and Purification——Finely powdered *Evernia prunastri* (60 g) was extracted with ether and with 80% ethanol in order to remove soluble components. The air-dried residue was further extracted with distilled water on a water-bath. 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 EP-1). Yield, 3%. The fraction EP-1 was warmed in water and a small amount of insoluble substances were removed by filtration. The filtrate was frozen overnight and allowed to thaw at room temperature. By the repeated freezing and thawing procedure, the fraction EP-1 could readily be separated into a cold water-insoluble polysaccharide fraction, named EP-3, and a cold water soluble-fraction, designated EP-4 (the respective yields, 0.75% and 2%). The fraction EP-4 was dissolved in water and 0.2M cetyltrimethylammonium hydroxide was added to the mixture under stirring until precipitation was completed. The precipitate was named EP-7 (yield 0.63%) and the supernatant fraction was added with EtOH to form precipitate, which was collected by a centrifuge, and named EP-6 (yield 0.43%).

The Properties of Polysaccharides EP-3, EP-6, and EP-7——i) The values of their specific rotations. $[\alpha]_D^{17}$ ($c=0.5$, H_2O) EP-3, +200°; EP-6, +164°; EP-7, +12°

ii) EP-3 and EP-6 showed IR ν_{max}^{KBr} 840 cm^{-1}, EP-7 showed ν_{max}^{KBr} 890 cm^{-1}

iii) A sample of these polysaccharides was hydrolyzed with 2N H_2SO_4 for 6 hr at 95°. GLC of the hydrolyzate revealed the presence of glucose only.

Methylation of EP-3, EP-6, and EP-7——EP-3 (50 mg) was methylated in the usual way, first by the Hakomori method (2 times) and then by the Kuhn procedure to yield fully methylated EP-3 (40 mg), which gave no OH absorption in the IR spectrum. EP-6 and EP-7 were treated in the same way.

Methalolysis of Fully Methylated Polysaccharide——A mixture of methylated EP-3 (20 mg) and 5% MeOH–HCl (6 ml) was heated at 100° for 8 hr in a sealed tube. After treatment with Amberlite IR4B, 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 164°, under a flow of N_2. The major and minor products were identified respectively to be methyl 2,4,6-tri-O-methyl, methyl 2,3,6-tri-O-methyl-D-glucopyranoside and methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside by comparison with the authentic samples. Retention times: Methyl 2,4,6-tri-O-methylglucoside (α-anomer): 4.3 min, (β-anomer): 2.8 min, methyl 2,3,6-tri-O-methylglucoside, (α-anomer): 4.0 min, (β-anomer): 3.1 min, methyl 2,3,4,6-tetra-O-methylglucoside, (α-anomer): 2.2 min, (β-anomer): 1.8 min.

Molecular Weights——The number-average degree of polymerzation (DP) was based on the estimation of the reducing end-group by Park-Johnson method and the gravimetric method of the methanolysis products.

Oxidation with Periodate——EP-3 (20 mg) dissolved in water (5 ml) was added with 0.01M $NaIO_4$ (10 ml), and the mixture was allowed to stand at 20° in the dark. A blank experiment was made under the same conditions. Aliquots of the sample and the blank were removed at some intervals, and the absorbances of the resulting solutions were measured in the spectrophotometer at 223 nm in comparing with those of the original solution of periodate and of an equimolar iodate solution. The periodate uptake of EP-6 or EP-7 was also estimated under the same conditions. The number of moles of $NaIO_4$ consumed per anhydroglucose unit of the polysaccharide were as follows (moles/mole): EP-3: 0.1645. EP-6: 0.3505, and EP-7: 0.2559.

Preparation of the Polysaccharide Fractions of *Acroscyphus sphaerophoroides*, *Alectoria sulcata* and *Alectoria sarmentosa*——The lichen thalli were crashed into small pieces and extracted three times with distilled water on a boiling water-bath for 6 hr. Each extract was concentrated and added excessively with EtOH to give slightly grayish white precipitates (AS-1, ASu-1, Asa-1). The precipitates were warmed on suspending in water, and a small amount of insoluble substances remained were removed by filtration. The filtrate was frozen overnight and allowed to thaw at room temperature. The crude fraction could readily be separated into a cold water-insoluble polysaccharide fraction named AS-3-IS, ASu-2 and ASa-2, and a cold water-soluble fraction designated as AS-3S, ASu-3, and ASa-3. The yields of polysaccharide preparations separated as above were shown in Table II.

Methylation of AS-3-IS——AS-3-IS (50 mg) was methylated in the usual way, first by the Hakomori method (2 times) and then by the Kuhn procedure to yield fully methylated AS-3-IS (45 mg) which gave no OH absorption in the IR spectrum.

Methanolysis of the Fully Methylated Polysaccharide——The fully methylated AS-3-IS (30 mg) was heated with 5% MeOH–HCl (6 ml) in a sealed tube on a boiling water-bath for 8 hr. 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 5% neopentylglycolsuccinate on Gaschrom C.L.H. (2 m) at

9) F. Fukuoka, M. Nakanishi, S. Shibata, Y. Nishikawa, T. Takeda, and M. Tanaka, *Gann*, **59**, 421 (1968).

Table II. The Yields of the Polysaccharide Preparations from Lichens

Lichens					
Acroscyphus sphaerophoroides		*Alectoria sulcata*		*Alectoria sarmentosa*	
AS-1	3.5%	ASu-1	26.7%	ASa-1	18.8%
AS-3-IS	0.2	ASu-2	15.0	ASa-2	15.0
AS-3-S	2.8	ASu-3	9.2	ASa-3	1.7

155° under a flow of N_2. The major and minor products were identified, respectively to be methyl 2,3,4-tri-O-methyl-, methyl 2,4,6-tri-O-methyl-, methyl 2,3,6-tri-O-methyl-D-glucopyranoside and methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside by the comparisons with the authentic samples. Retention times: Methyl 2,3,4-tri-O-methylglucoside (α-anomer): 8.5 min, (β-anomer): 7.35 min, methyl 2,4,6-tri-O-methylglucoside (α-anomer): 11.05 min, (β-anomer): 7.55 min, methyl 2,3,6-tri-O-methylglucoside (α-anomer): 10.95 min, (β-anomer): 8.05 min; methyl 2,3,4,6-tetra-O-methylglucoside (α-anomer): 3.95 min, (β-anomer): 2.8 min.

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