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Chemical composition of the *Ramalina fraxinea* (L.) Ach. thallus

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Abstract

The folowing lichen acids were isolated from *Ramalina fraxinea* (L.) Ach. thallus: usnic, obtusatic and sekkikaic, as well as d-arabitol and three mucilage and amine fractions. In the mucilage hydrolysates glucose, galactose, glucosamine, arabinose, xylose, rhamnose and glucuronic acid were found. The following amines were discovered chromatographically: choline, betaine, histamine, acetyl-choline and β-phenylethylamine; amino acids: aspartic, glutamic, γ-aminobutyric, serine, threonine, alanine, proline, moreover d-mannitol and free arabinose and glucose.

INTRODUCTION

Lichens as symbiotic organisms contain chemical products of metabolism of fungi, of algae and also components characteristic only for themselves. Among these are lichen acids which do not constitute a homogeneous chemical group. Here belong protolichesteric acid derivatives, those of fulvic acid, depsides of the α- and β-orcin groups, depsidones of α- and β-orcin groups, quinones and combinations of dibenzofurane structure. In some species of the *Ramalina* Ach. genus acids were found from the group of orcin depsides such as evernic, obtusic, sekkikaic, boninic and ramalinic acids; depsidones of the β-orcin group — salacinic, stictic, norstictic and protoceraric acids as wel as d-usnic acid with dibenzofurane structure (K a r r e r 1958).

Sugar alcohols specific for fungi frequently occur in lichens (H e g n a u e r, 1962), particularly erythrytol, arabitol, mannitol and volemitol. Among reserves carbohydrates characteristic in lichens are the β-glucanes, lichenin and pustulane and the α-glucane, isolichenin. From among other carbohydrates trehalose, saccharose and galactosides were revealed.

Lichenin and isolichenin occur in general in the genera *Cetraria*, *Rocella* and *Usnea* (Hegnauer, 1962).

Ramalina fraxinea (L.) Ach. is a common lichen in this country (Motyka, 1962), but its chemical composition is but little known. The occurrence of d-usnic acid and d-arabitol in the thallus of this lichen has been reported from Australia (Karrer et al., 1977).

The aim of the present study was to gain a better knowledge of the chemical composition of this lichen.

MATERIAL AND METHODS

The material consisted of thalli of the lichen *Ramalina fraxinea* (L.) Ach. collected from the trunks of *Acer platanoides* L. in Jantar (Gdańsk District) in August 1975. For chemical analysis 260 g of dried and comminuted material was used (sieve no. 0.5 FP IV).

The crude material was successively extracted with petroleum ether and acetone 60 h with each in a Soxhlet apparatus. Then after drying the material was infused with 10-fold volume of distilled water and kept on a boiling water bath for 30 min. Subsequently it was filtered hot yielding extract W.

The course of purification of lichen acids was checked by thin-layer chromatography on plates coated with a 0.25-mm layer of silica gel H (Merck) under standard conditions (Culberson, Kristinsson, 1970).

Ascending chromatography of saccharides was run on Whatman no. 1 paper in the system n-butanol-pyridin-water (6:4:3) (Kholkin, 1968). The air-dried chromatograms were developed with acidic aniline phthalate, naphtha-resorcin with trichloroacetic acid, p-anisidine with phosphoric acid (Stange, 1959), barbituric acid (Michalska, Jakimowicz, 1969) and ninhydrin (Borkowski, 1973). The chromatograms, after spraying with a developing reagent were heated for 15 min at 105°C.

The sugar alcohols were also chromatographed on Whatman no. 1 paper in the following developing system: (1) methylethyl ketone-acetic acid-saturated aqueous solution of boric acid (9:1:1) (Harborne, 1973), (2) n-butanol-pyridin-water (10:3:3). The chromatograms were developed with $AgNO_3$ solution or by successive spraying with meta-periodate and benzidine (Jerzmanowska, 1970).

Amine chromatography was run on Schleicher Schüll paper no. 2043b Mgl. in the developing systems: n-butanol-ethanol-water (4:1:5) and thin layer chromatography was performed on silica gel G (Merck). The plates were coated with a 0.25-mm gel layer, activated for 1 h at 130°C and developed in the system water-phenol (3:8).

The chromatograms were sprayed with alcoholic iodine solution, acidic butanolic ninhydrin solution and with the modified Dragendorff reagent (Jerzmanowska, 1970).

Two-dimensional amino acid chromatography was run on Whatman no. 3 29×29 cm sheets in the following developing systems (Harborne, 1973): direction I — n-propanol-water (7:3), direction II — isopropanol-water (4:1). The chromatograms after spraying with acidic ninhydrin solution (Borkowski, 1973) were first heated in air over a hot plate, and the sequence of appearance of weak different-coloured spots was followed. Then the paper was transferred to a drier for 10 min at 110°C.

The organic solvents used for extraction of the crude material and for chromatography were anhydrous, of pure for analysis grade. For column chromatography aluminium oxide acid for chromatographic analysis (Schuchardt, München) II° activity was used.

Chromatography was run in the presence of the following standards: (a) saccharides — d-mannitol (Koch-Light, England), glucuronic acid (BDH, England), d-glucose, d-galactose, d-glucosamine pure for analysis (POCH, Poland), d,l-arabinose pure (Schuchardt, West Germany), d(+)-xylose (Fluka AG, Buchs SG Switzerland), l(+)rhamnose (Toseat, England); (b) amines—betaine, choline, acetylcholine and histamine hydrochlorides (POCH, Poland), β-phenylethylamine (Light, England); (c) amino acids — d,l-serine pure, threonin pure (Chemopol, Chechoslovakia), d,l-glutamic acid pure for analysis γ-aminobutyric acid (Reanal, Hungary), l(+)aspartic acid standard (Merck, West Germany) and d,l-alanin (POCH, Poland).

Portions of 0.5 g of mucilage sediment were hydrolysed in 10 cm^3 of 1 N sulphuric acid in sealed ampoules (Öisteth, 1954, Dombrowicz, Broda, 1973) for 8 h at boiling water bath temperature. The hydrolysate was neutralised by a small excess of barium carbonate and filtered. The filtrate was evaporated to dryness in a vacuum evaporator. The residue was washed four times with boiling 90°C ethanol (10 cm^3).

The melting temperatures were determined on a Boetius microscope table.

RESULTS AND DISCUSSION

Ether extract

The petroleum ether extract was concentrated to a 250 cm^3 volume and left to stand in the cold. After 24 h a crystalline sediment precipitated which was filtered off washed with cold petroleum ether, re-

crystallised from acetone and further repeatedly from chloroform with methanol added. The product in the form of yellow needles proved to be chromatographically pure and gave colour reactions characteristic for usnic acid (Asahina, Schibata, 1954; Moiseeva, 1961; Culberson, Kristinsson, 1970) and melted at a temperature (205°C) corresponding to the melting point of usnic acid (Karrer, 1958). The filtrate and the postcrystalline liquids lye were combined and evaporated to dryness. The residue was dissolved in ethyl ether and aqueous 5 per cent KOH solution was added. The ether layer was discarded, the aqueous layer was acidified with diluted HCl and washed with ethyl ether. The ether layer dried (anhydrous Na_2SO_4) and the ether was evaporated. The residues was dissolved hot in anhydrous acetone from which after cooling a crystalline sediment precipitated with usnic acid properties. A total of 1.16 g of usnic acid was obtained, constituting 0.46 per cent of the lichen thallus dry weight.

The acetone filtrate was evaporated to dryness. The residue was dissolved in petroleum ether and subjected to column chromatography on acid Al_2O_3 previously moistened with petroleum ether.

The column was successively developed with petroleum ether the same with 50 per cent cyclohexane added, cyclohexane, cyclohexane with 50 per cent carbon tetrachloride, carbon tetrachloride, the same with 50 per cent benzene added, benzene, benzene with 50 per cent chloroform added, chloroform, the same with 50 per cent ethyl ether added and ethyl ether. From the benzene fraction with 50 per cent chloroform added a crystalline product was obtained which was crystallised from benzene and recrystallized to a constant melting point from ethanol. A chromatographically homogeneous product was obtained in the amount of 0.02 g with m.p. 142°C corresponding to the melting point of sekkikaic acid (Karrer, 1958), which also gives colour reactions characteristic for this acid (Asahina, Schibata, 1954; Culberson, Kristinsson, 1970).

A crystalline product was also obtained from the chloroform fraction. It was slightly contaminated with sekkikaic acid and was not further purified in view of its very small quantity. The products shows on chromatograms (Table 1) an R_f similar to that of obtusatic acid (Culberson, Kristinsson, 1970) and gives reactions characteristic for this acid (Asahina, Schibata, 1954; Culberson, Kristinsson, 1970).

Acetone extract

The acetone extract was concentrated to a 10 cm^3 volume, and an equal volume of ethanol was added. The mixture was left to stand in the cold. After 3 days a coarse-grained crystalline sediment precipitated.

It was filtered off and repeatedly recrystallised from an ethanol-acetone mixture (1:1) and then from anhydrous ethanol until a constant melting point was reached (101—102°C) corresponding to that of d-arabitol (Karrer, 1958). This compound can be visualised on the chromatograms by means of reagents used for sugar alcohols (Jerzmanowska, 1970). Crude d-arabitol was obtained in the amount of 1.61 g, constituting 0.62 per cent of the lichen dry weight.

In the postcrystalline liquids lyes the presence of d-manmitol could be chromatographically demonstrated.

Water extract

The water extract was refrigerated for 12 h, and filtered off the precipitate L. The latter was repeatedly dissolved in hot water and precipitated at +4°C. The sediment thus obtained was washed several times with methanol and ethyl ether, then dried over anhydrous calcium chloride. This yielded 1.12 g of amorphous grey-white sediment which constituted 0.43 per cent of the lichen thallus dry weight.

This sediment does not reduce Fehling's solution, it does not stain with ninhydrin or Lugol's solution. In its solubility in hot water it resembles lichenin (Bystrek, 1972).

Table 1

Results of chromatographic analysis of lichenic acids

Acids	R_f in			spot colour	
	1	2	3	UV	H_2SO_4*
Usnic	0.54	0.49	0.42	absorption	brown
Sekkikaic	0.43	0.48	0.37	light blue	brown-purple
Obtusatic	0.43	0.29	0.25	—	brown-pink

Solvent system: 1 — toluene-dioxane-acetic acid (180:45:5), 2 — n-hexane-ether-formic acid (120:90:20), 3 — toluene-acetic acid (200:30).

* After spraying warmed for 20-30 min at 110°C.

The L sediment hydrolysate was subjected to paper chromatography which revealed glucose (R_f 0.45), galactose (R_f 0.39) and trace amounts of glucosamine (R_f 0.38).

The filtrate from sediment L was treated with an excess of methanol, this yielding sediment L_1. The latter was repeatedly dissolved in a small amount of water and precipitated with methanol. After final precipitation sediment L_1 was washed with methanol, ethyl ether and dried. It weighed 11.01 g (4.25% of lichen thallus dry weight). The purified L_1 sediment does not reduce Fehling's solution, it does not stain with

ninhydrin, but stains blue with Lugol's solution, showing isolichenin properties (Bystrek, 1972).

Sediment L_1 was hydrolysed and the hydrolysate was subjected to paper chromatography. The following saccharides were detected: arabinose (R_f 0.49), galactose (R_f 0.39) and glucosamine (R_f 0.38).

Table 2

Results of chromatographic analysis of sugar alcohols

Sugar alcohols	R_f in	
	1	2
Arabitol	0.30	0.31
Mannitol	0.17	0.16

The lye after sediment L_1 was acidified with HCl to pH 3. The precipitated L_2 sediment was washed with methanol up to disappearance of the acidic reaction, then with ethyl ether and it was dried. The obtained sediment weighed 0.81 g (0.3% of lichen dry weight). The sediment reacted with Fehling's and Lugol's solutions and ninhydrin as did sediment L_1.

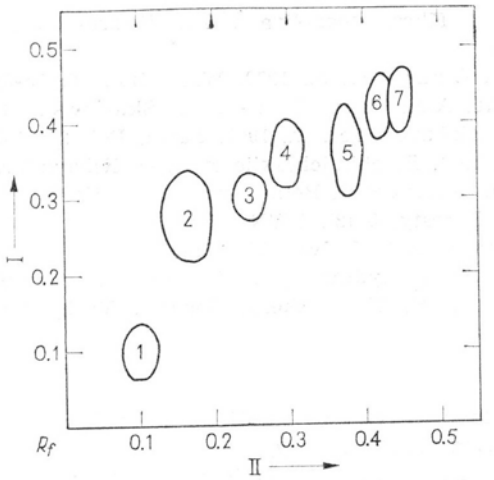
Table 3

Results of chromatographic analysis of amine fraction

Amine	R_f in	
	1	2
Choline	0.03	0.08
Betaine	0.07	0.73
Acetylcholine	0.11	0.24
Histamine	0.16	0.16
Amine x	0.25	0.19
β-phenylethylamine	0.63	0.83

The acidic hydrolysate of this sediment contained: rhamnose (R_f 0.65), xylose (R_f 0.57), arabinose (R_f 0.49), glucose (R_f 0.45), *galactose* (R_f 0.39), glucosamine (R_f 0.38) and glucuronic acid (R_f 0.16).

The filtrate from sediment L_2 was neutralised with lead carbonate, it was filtered off the sediment and methanol was removed in a vacuum evaporator. Amines were precipitated with phospho-tungstic acid (Jerzmanowska, 1970). The sediment was decomposed by means of a saturated barium hydroxide solution and then the excess of Ba^{++} ions was removed with CO_2. The filtrate was acidified with HCl, concentrated in an evaporator and chromatographed. Choline, acetylcholine, betaine, histamine, β-phenylamine and amin X were revealed. Amines have so far not been found in lichens, but many of them were detected in fungi (Karrer, 1958, Kohlmünzner, Grzybek, 1972).



Two dimensional paper chromatogram of free amino acids from *Ramalina fraxinea* (L.) Ach. thallus: 1 — aspartic, 2 — glutamic acid, 3 — serine, 4 — threonine, 5 — alanine, 6 — proline, 7 — γ-aminobutyric acid

The filtrate after amine precipitation was neutralised with barium carbonate, separated from the sediment and evaporated to dryness. The dry residue was repeatedly digested with boiling ethanol. The obtained solution was chromatographed for the presence of sugars and amino acids. Free galactose and arabinose were found. The presence of free amino acids: aspartic, glutamic, γ-aminobutyric, serine, threonine, alanine and proline was also demonstrated (Fig. 1). The detected amino acids with the exception of γ-aminobutyric acid occur as a rule in proteins.

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Składniki chemiczne plechy Ramalina fraxinea (L.) Ach.

Streszczenie

Przeprowadzono systematyczne badania składu chemicznego plechy *Ramalina fraxinea* (L.) Ach. W tym celu wykonano kolejno ekstrakcję rozdrobnionej plechy eterem naftowym, acetonem i wodą. Z ekstraktu eteru naftowego wyizolowano kwasy porostowe: usninowy, obtuzatowy i sekkikowy. Z ekstraktu acetonowego otrzymano arabitol, a w ługach pokrystalizacyjnych wykazano mannitol.

Z ekstraktu wodnego wydzielono:

A) Trzy frakcje śluzu (L, L_1, L_2), przy czym frakcja L pod względem rozpuszczalności i reakcji barwnych odpowiada licheninie, ale różni się od niej składem chemicznym, ponieważ zawiera glukozę, galaktozę i glukozaminę. Frakcja L_1 rozpuszczalnością i reakcją barwną z płynem Lugola przypomina izolicheninę, ale też różni się składem chemicznym, ponieważ zawiera arabinozę, galaktozę i glukozaminę. Frakcja L_2 składem przypomina kwaśne śluzy roślin naczyniowych, ponieważ zawiera galaktozę, arabinozę, ksylozę, ramnozę i kwas glukuronowy.

B) Frakcję amin, w której chromatograficznie wykazano obecność choliny, acetylocholiny, betainy, histaminy, β-fenyloetyloaminy i aminy X.

C) Frakcję wolnych aminokwasów, w której wykryto: kwasy — asparaginowy, glutaminowy i γ-aminomasłowy, oraz serynę, treoninę, alaninę i prolinę.