Studies on the chemistry of lichens

VII. * Chemical investigations of the lichen species *Lecanora (Aspicilia) Myrinii* (Fr.) Nyl.

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The Norwegian lichen species *Lecanora (Aspicilia) Myrinii* has been chemically investigated with regard to its content of aromatic lichen compounds, hydroxy fatty acids, soluble and bound sugars, and amino acids. Norstictic acid and a tetrahydroxy fatty acid have been isolated. In addition to these two compounds, free galactose, glucose, mannose, sucrose, fructose and 33 ninhydrin-positive compounds were detected in a water extract. The polysaccharides and the protein part of the lichen material were determined after hydrolysis. Great amounts of glucosamine were found in the protein fraction.

The crustaceous lichen species *Lecanora (Aspicilia) Myrinii* is to be found as large, light greyish-white spots with dark coloured apothecia on calciferous rocks in the lofty mountain. I have investigated a material collected from Fjellseter, Nesbyen, Buskerud, during the summer of 1963 and 1966. The lichen grew in great amounts on Hornblende-dioritic gneiss in 1 mm jamdick crust, and the thallus gave a deep red colour with aqueous caustic soda.

Results

The isolation of Norstictic acid and the tetrahydroxy fatty acid

The dried, coarsely powdered lichen (273 g), containing 2.8% water, and 21.4% ash in dry substance, was exhaustively extracted with petroleum ether (b.p. $60-80^\circ$) in order to remove dark coloured products. The residual lichen sample was subsequently extracted with acetone for 60 hours. A crystalline compound was separated out on standing at 2°; yield 4.1g, fraction A. The total acetone filtrate was then concentrated to a small volume, and an almost colourless solid was deposited; yield 3.4 g, fraction B. The acetone mother liquor was at last concentrated to remove all the solvent, and the residue partially purified by washing with acetone, ethanol and ether; yield 1.2 g, fraction C.

Fractions A, B and C from the acetone extraction of the lichen were found to be the same substance, and was recrystallised repeatedly from 80% acetone. A particularly pretty, needle-shaped product, m.p. $270-280^\circ$ (decomp.), was obtained. The crystalline product gave characteristic colour reactions with caustic alkali, ferric chloride, sulphuric acid and with *p*-phenylene-diamine. The substance was very bitter to the taste, did not contain any CH_3O-group, and was identified as Norstictic acid. The acid gave analytical values agreeing with its formula, $C_{18}H_{12}O_9$. (Found: C 58.9%, H 3.80%. Calculated: C 58.1%, H 3.25%.) The identity was further established by preparing its penta-acetate, m.p. $203-204^\circ$. (Found: C 55.6%, H 4.07%. Calculated for $C_{28}H_{24}H_{15}$: C 56.0%, H 4.03%.)

The infra-red absorption spectra of the Norstictic acid from *Lecanora Myrinii* and from *Parmelia acetabulum* showed a surprisingly close similarity. The spectra were compared with the spectra of Salazinic acid. Comparative UV-analyses of the lichen compound from *Lecanora Myrinii* and of Salacinic acid have also been made.

Paper chromatography on buffered paper (Ederol 202, impregnated with 0.1 M Na_2HPO_4, solvent n-butanol + ethanol + water (4 + 1 + 5)), of Norstictic acid from *Lecanora Myrinii* and *Parmelia acetabulum* and of Salazinic acid, further confirmed that the compound from *Lecanora Myrinii* must be Norstictic acid, and additionally that there were no other components in the isolated fractions.

It is of interest to notice that Norstictic acid was discovered from *Lecanora radiosa var. subcircinata* in 1963 by HUNECK[1]. This lichen is a calcicole

* Part VI: Z. Naturforschg. **22 b**, 777 [1967].

[1] S. HUNECK, Naturwissenschaften **50**, 646 [1963].

crustaceous lichen species in conformity with *Lecanora Myrinii*.

A thin layer chromatographic investigation of the aromatic components from some other lichen species in the *Aspicilia*-group was reported in a publication in 1968 by FOLLMANN and HUNECK [2]. Only by *Lecanora esculenta var. jussufii* was a small amount of Stictic acid detected.

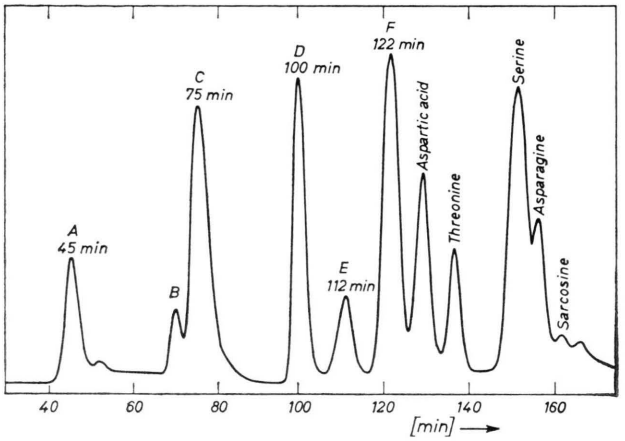

Fig. 1. Chromatographic separation of the water-soluble, ninhydrin-positive compounds of *Lecanora Myrinii*. The elution curve is the first part of the acid fraction obtained with an amino acid analyser (AAA) (Beckman/Spinco 120 B) at 33.5 °C. Column size 53 × 0.9 cm, Resin type PA-28, Column flow rate 30 ml/hr, Buffer pH 3.28.

The lichen residue left after the extraction with acetone was extracted with ethanol for 72 hours. The alcohol extract was evaporated, and the dark coloured residue worked up as described for the investigations of *Parmelia alpicola* in my publication in 1967 [3]. A quite colourless compound was obtained, and identified as a Tetrahydroxy Fatty acid; yield 1.3 g, m.p. 182 – 184°; found C 66.1%, H 11.32 per cent. Work is in progress with a view to establishing the constitution definitely of the tetrahydroxy fatty acid. The results of these investigations will be published in the near future in connection with investigations of similar substances isolated from the lichen species *Parmelia alpicola* [3], *Ochrolechia tartarea* and *Pertusaria* spec.

[2] G. FOLLMANN and S. HUNECK, Z. Naturforschg. **23 b**, 571 [1968].
[3] Y. J. SOLBERG, Z. Naturforschg. **22 b**, 777 [1967].

Soluble carbohydrates and amino acids

Water has successfully been used as an extracting solvent for the determination of individual free sugars and amino acids in dry lichen material. However, boiling ethanol (96%) rapidly inhibits enzymatic action and thus may be essential for the quantitative extraction and determination of these compounds. Another portion of the lichen material (610 g), containing 3.2% water and 30.1% ash was at first treated with boiling ethanol. After the greater part of the alcohol had been distilled off, the material was extracted for 24 hours in a rotary apparatus with cold water. The extract was worked up as described in the experimental part.

Chromatographic evidence for the presence of the free sugars in the extract was obtained with paper chromatography, and the following low molecular carbohydrates were detected:

Sucrose and fructose: Trace or small amounts,
Galactose and mannose: Small amounts,
Glucose: Relatively greater amounts.

Other sugars were not detected, and the results are in harmony with the work of PUAYO [4]. Sucrose, fructose, galactose and glucose were detected by him in the lichen species *Parmelia saxatilis*, *Parmelia caperata* and *Xanthoria parietina*.

The water extract of *Lecanora Myrinii* contained a number of ninhydrin-positive substances. The total composition of the free amino acids in the water extract are given in Table 1. The components have been grouped in order of their contents. Tryptophan was not detected. The extract was examined both by TLC and with column chromatography.

As far as the author is aware, there are comparatively few systematic investigations of free amino acids in the lichen literature published. However, mention must be made here of the publication by RAMAKRISHNAN and SUBRAMANIAN [5], which give an account of the amino acids in the species *Lobaria subisidiosa*, *Umbilicaria pustulata*, *Parmelia nepalensis* and *Ramalina sinensis*. The common amino acids as Ala, Arg, Asp, Glu, Gly, His, Ileu, Leu, Lys, Met, Phe, Pro, Ser, Thr, Try, Tyr and Val were present in an amount of between 30 and 60 mg per 1000 g dry matter.

[4] G. PUAYO, Ann. Biol. **T. 36**, 129 [1960].
[5] S. RAMAKRISHNAN and S. SANKARA SUBRAMANIAN, Curr. Sci. **35**, 124 [1966].

LINKO et al.[6] and HALE, JR.[7] described, in addition to the free amino acids already mentioned above, the detection of $GluNH_2$, $AspNH_2$, Sarc, α-NH_2-But, γ-NH_2-But and Ethanolamine in extracts from the lichen species *Cladonia sylvatica* and *Peltigera canina*. It appears from their investigations that Glutamic acid is predominating, in agreement with the results from *Lecanora Myrinii*.

The free amino acids found in the non-protein-nitrogen fraction by the AAA *, agree well with those detected by TLC. A remarkable feature of the thin layer chromatographic analysis was the probable content of $GluNH_2$ and $AspNH_2$. In the column chromatographic amino acid determination with the acid of AAA, the two acid amides give a single symmetrical peak.

As it appears from Table 1 27 ninhydrin-positive acids and related compounds have been determined in the water extract from *Lecanora Myrinii*. One noticed that Cystathionine, β-Ala, β-NH_2-isobut and Orn have been determined in small amounts.

Asparagine, glutamine, sarcosine, cystine, cystathionine, tyrosine, phenylalanine, ornithine, histidine, ethanolamine, α-NH_2-*n*-butyric acid, β-NH_2-isobutyric acid, β-alanine,	}	<10
Threonine, glycine, valine, methionine, isoleucine, leucine, lysine,	}	$>10, \leqq 50$
Aspartic acid	90	
γ-NH_2-*n*-butyric acid	150	
Serine	180	
Alanine	180	
Arginine	280	
Proline	310	
Glutamic acid	1050	

Table 1. Free amino acids of *Lecanora Myrinii*. The values are calculated as *mg amino acid per 1000 g* ashless tissue.

On the chart from AAA, six ninhydrin-positive peaks which are different from any common amino acid, were observed and termed, as seen in Fig. 1. The figure shows the first part of the elution pattern obtained by applying an aliquot of the desalted water extract to the long coloumn of the AAA. Other unknown peaks did not appear in the rest of the elution pattern and is therefore not reproduced here. All the six unknown peaks are 570 nm peaks before Aspartic acid. The unknown eluting before Aspartic acid, compound F, eluted with Aspartic acid if the temperature was changed to 50°. By the 33.5° run the eluting compounds C and Urea will give a single symmetrical peak. Nevertheless the compound C is very likely not identical with Urea. The same relation exists between the compound A and Phosphoserine. One has not found any unambiguous proof that the compound B is identical with the amino acid Taurine, in spite of the fact that B completely takes the place of Taurine in the elution pattern. It is further clarified that the compound A is not identical with Cysteic acid. By adding Cysteic acid to the sample an unsymmetrical peak occurs. The compound E is not identical with Methionine sulphone.

On the basis of the work published by CONKERTON et al.[8] in 1968, the ratio of the areas under the 440 nm and 570 nm absorption peak tracing for Phosphoserine, Urea, Asp, Thr, Ser and the compounds A, C, D and F, has been calculated. The average of 0.13 for the ratio of the areas of the five standard amino acids and the values of the unknown peaks are compiled in Table 2. The results confirm that the compounds A and C are not identical with Phosphoserine or Urea.

Standard amino acids		Unknown ninhydrin-positive compounds	
o-Phosphoserine	0.13	Compound A	0.25
Urea	0.11	Compound C	0.21
Aspartic acid	0.14	Compound D	0.17
Threonine	0.13	Compound F	0.12
Serine	0.12		

Table 2. The ratio of the areas under the 440 nm and 570 nm absorption peak tracings for standard amino acids and for the unknown compounds A, C, D and F of *Lecanora Myrinii*.

All the unknown compounds in *Lecanora Myrinii*, with the exception of compound B, hydrolyse or decompose by boiling with hydrochloric acid. It is therefore more than likely that more of these ninhydrin-positive compounds may be peptides.

The unidentified compounds could not be characterised completely or identified because of insufficient material. It would appear important to complete the identification of the unknown constituents and to determine their chemical and biological significance. Work is in progress on the preparation of larger amounts of material for that purpose.

[6] P. LINKO, M. ALFTHAN, J. K. MIETTINEN, and A. I. VIRTANEN, Acta chem. scand. **7**, 1310 [1953].

[7] E. HALE, JR., The Biology of Lichens, London 1967.

[8] E. J. CONKERTON, E. E. COLL, and R. L. ORY, Analyt. Letters **1**, 303 [1968].

* AAA = Amino Acid Analysen.

Analysis of the hydrolysates for contents of amino acids and monosaccharides

The dried lichen residue left after the extraction of the soluble sugars and amino acids, was then analysed as to its content of polysaccharides and protein. The material was hydrolysed as described in the experimental part.

The results as regards the individual amino acids in the protein fraction are given in Table 3. It is of interest to make a note of the high Glutamic acid content in the protein part, too, with 0.52 g Glu per

Amino acids		amino acid [%]	N [%]
Aspartic acid		0.32	0.034
Threonine		0.32	0.038
Serine		0.33	0.044
Glutamic acid		0.52	0.050
Proline		0.20	0.024
Glycine		0.21	0.039
Alanine		0.27	0.042
Valine		0.24	0.029
Isoleucine		0.19	0.020
Leucine		0.30	0.032
Tyrosine		0.09	0.007
Phenylalanine		0.17	0.014
Glucosamine		0.23	0.018
Lysine		0.22	0.042
Histidine		0.07	0.019
Arginine		0.22	0.071
Methionine sulphone		0.12	0.009
Tryptophan		0.01	0.001
	Total	4.03	0.533
Unhydrolysed material			0.070
	Total N		0.603

Table 3. Amino acid content of acid hydrolysates of *Lecanora Myrinii*. (The values are given as means of six hydrolysates, calculated on material of 2.6% moisture, 31.3% ash, 0.75% Kjeldahl-nitrogen and 0.03% sulphur.)

100 g material, whereas Tryptophan only is present at a very small amount of about 0.015 g Try per 100 g. Cysteic acid is detected as traces in the hydrolysates. Of great interest, but not unexpected, is the high content of *Glucosamine*. This compound was not detected in the water extract, even not as traces. The sum of the amino acid nitrogen with the addition of the insoluble nitrogen formed during the hydrolysis (estimated by micro-Kjeldahl analysis), accounts for more than *80% of the total nitrogen value*. No attempt was made to determine correction factors for the destroyed fractions of the amino acids under the conditions of hydrolysis in these investigations.

In the elution patterns of the hydrolysates small peaks were detected due to *allo*-Isoleucine, α-NH_2-adipic acid, α-NH_2-*n*-butyric acid, γ-NH_2-*n*-butyric acid and Ornithine. No attempt was made to estimate these small amounts.

The polysaccharide content was determined by means of paper chromatographic analysis of the individual monosaccharides after hydrolysis either with formic acid or with sulphuric acid. The analytical data of the polysaccharide fraction are given in Table 4. As it appears from the table the sum of

	15 N HCOOH 24 hours Weight 3 g	15 N HCOOH 48 hours Weight 3 g	2 N H_2SO_4 24 hours Weight 5 g
Glucose	21.6	18.8	16.2
Galactose + Mannose	15.9	15.7	13.1
Total	37.5	34.5	29.3

Table 4. The constituent sugars in the polysaccharides of *Lecanora Myrinii*. (All values are given as *g per 100 g* material containing 2.6% moisture.)

Galactose, Glucose and Mannose is surprisingly low, and lowest by the hydrolysis with sulphuric acid. The highest value of the sum is to be found by hydrolysis with formic acid for 24 hours with an *average of 37.5 g hexoses per 100 g lichen*. By hydrolysis of a greater amount of *Lecanora Myrinii* (10 g), traces of the pentoses Arabinose and Xylose were detected. It has never been possible to detect the presence of Fructose in the hydrolysates.

A certain amount of interest has been taken in the publication of RAMAKRISHNAN and SUBRAMANIAN [9] in 1965, who called attention to the low polysaccharide values obtained by hydrolysis of the lichen species *Cladonia rangiferina*, *Cladonia gracilis* and *Lobaria isidiosa*, respectively 35%, 30% and 15%, calculated as Glucose.

Experimental procedures

Melting points were determined on a Kofler micro block and are corrected. Evaporations were carried out under reduced pressure at 30 – 35° in a rotary evaporator. The amino acid determinations were carried out with a Beckman/Spinco Model 120 B, automatic amino acid analyser. The resins used in the analysator were the Custom Research Resin, Type PA-28 (long column) and PA-35 (short column) from

[9] S. RAMAKRISHNAN and S. SANKARA SUBRAMANIAN, Current Sci. **11**, 345 [1965].

Beckman. The column flow rate for the long column was 30 ml/h, and for the short column 40 ml/h, and the temperature was $33.5-50^{\circ}$.

Analytical methods. The moisture, ash, total nitrogen and sulphur contents were determined by the same methods as those described in an earlier publication [10].

Thin layer chromatography of the amino acids was carried out on Merck DC-Fertigplatten Cellulose F, by the method of JONES and HEATHCOTE [11]. The sugars in the water extract and in the hydrolysates were determined by the methods of COLOMBO [12] and WILSON [13]. The chromatographic paper used was Whatman No. 4 grade. A descending technique of chromatography was employed, with the solvent *ethylacetate-pyridine-water* $(80+20+10)$. Fructose was detected by the naphthoresorcinol spray reagent, and sucrose with aniline-diphenylamine-phosphoric acid by the method of BAILEY [14].

Extraction procedure. Extraction of the usual lichen substances is partly described above. Otherwise the procedure is in accordance with earlier methods used in this laboratory [3, 15]. The water extract, containing a small amount of xylene as a preservative, was passed through a column containing Dowex 50W, X8, $50-100$ mesh, in the hydrogen form. The amino acids were retained on the resin and the column was treated with distilled water until the effluent showed a negative reaction for carbohydrates. The amino acids were then displaced from the resin with 2 N ammonium hydroxide. The total water and ammonium hydroxide solutions were evaporated separately. The carbohydrate residue was redissolved in a small volume of water with one drop xylene as a preservative. The amino acid residue was redissolved in 0.05 N hydrochloric acid and isopropanol in the mixture $9+1$.

Hydrolysis. The polysaccharides of the lichen material were hydrolysed either with 15 N formic acid or with 2 N sulphuric acid after first having swollen in 72% sulphuric acid at room temperature for 4 hours according to standard methods. The protein part of the material was hydrolysed for 24 hours under dilute conditions with constant boiling hydrochloric acid. The acid was thereupon removed by evaporation, and the residue taken up in a mixture of 0.05 N hydrochloric acid and isopropanol $(9+1)$, and filtered. An aliquot of the filtrate was purified by passing Dowex 50W, x8, $100-200$ mesh, in the acid form, and then Dowex 2, x8, $100-200$ mesh, in the chloride form. The last step was performed in order to remove humin constituents which are produced during the hydrolysis [16].

Cystine, Cysteine and Methionine were at first oxidised to Cysteic acid and Methionine sulphone with performic acid reagent at about 5° overnight. After excess reagent had been removed under vacuum, the residue was hydrolysed in the manner described above.

Tryptophan was determined on hydrolysis with barium hydroxide, and the Tryptophan values estimated photometrically after addition of *p*-dimethylaminobenzaldehyde to an aliquot of the hydrolysate.

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[10] Y. J. SOLBERG, Ann. Bot. Fen. **4**, 29 [1967].
[11] K. JONES and J. G. HEATHCOTE, J. Chromatogr. [Amsterdam] **24**, 106 [1966].
[12] P. COLOMBO, D. CORBETTA, A. PIROTTA, G. RUFFINI, and A. SARTORI, J. Chromatogr. [Amsterdam] **3**, 343 [1960].
[13] C. M. WILSON, Analytic. Chem. **31**, 1199 [1959].
[14] R. W. BAILEY and E. J. BOURNE, J. Chromatogr. [Amsterdam] **4**, 206 [1960].
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[16] I. LARSEN and J. V. MORTENSEN, Analyt. Biochem. [New York] **21**, 466 [1967].