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Chemical Investigation of the Lichen Species *Anaptychia fusca, Peltigera canina,* and *Omphalodiscus spodochrous*

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Lichens, Allantoin, Taurine, Sterol, Wax Esters

The three lichen species *Anaptychia fusca, Peltigera canina,* and *Omphalodiscus spodochrous* have been studied with regard to their chemical content. Atranorin, gyrophoric acid, tenuiorin, allantoin, taurine, linoleic acid, a new sterol-$C_{31}H_{52}O_2$, and waxes have been determined.

The identity of the compounds was established by consideration of infrared, mass and nuclear magnetic resonance spectra. Besides, thin layer and column chromatographic techniques were used in this work.

Introduction

During our researches of various constituents in lichens, samples of *Anaptychia fusca, Peltigera canina,* and *Omphalodiscus spodochrous* were investigated. *Anaptycha fusca* is known to contain traces of atranorin, arabitol and mannitol. Of interest in the present study of this lichen, is the isolation of *allantoin,* which previously has been found only in the species *Xanthoria parietina* [1]. In addition, the aminoethanesulfonic acid *taurine* was detected in water extract of the same species. So far this compound has been detected in *Xanthoria parietina* [1] and five *Alectoria* species [2].

Peltigera canina has been investigated several times, and tenuiorin, ergosterol, mannitol, and unidentified fatty acids were reported to be the most frequently occurring chemical constituents. Linoleic acid and a sterol, of the composition $C_{31}H_{52}O_2$, were isolated as new compounds of this lichen. Ergosterol, which was found to exist in this lichen by Zellner [3], has not been detected in the sample collected here.

Omphalodiscus spodochrous has been examined earlier by a number of workers and found to contain gyrophoric acid, arabitol, and mannitol. In view of the present work the sample was reexamined and found to contain these substances. Lecanoric and orsellic acids were isolated too, but these compounds must be considered as decomposition products of gyrophoric acid.

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A high-molecular, amorphous mass, was isolated both from *Peltigera canina* and *Omphalodiscus spodochrous*. By means of mass spectrometry, infrared and elemental analyses the identity of the isolated fractions was established as a mixture of waxes.

No attention has been given to the occurrence of sugar alcohols in the species studied. The known depsides of the lichens are only shortly mentioned in the text.

The excellent publications of Culberson [4, 5] have been of great value to the present work.

Experimental

Methods

Elemental analyses were performed by Mikroanalytisches Laboratorium, Elbach-BRD. The melting points are uncorrected. Infrared spectra (IR) were all taken in KBr pellets. The spectra were analyzed on the basis of known structural correlations with absorption frequency.

The mass spectra recorded in this communication were obtained by direct sample insertion into the ion source of the mass spectrometer. Ionising energies of 70 eV were used, and the temperature of the solid heater and in the chamber was lying between 250 and 280 °C. The relative intensities of the fragment-ions are in the text given in parenthesis.

The 60 MHz NMR spectrum of the wax-mixture was run in $CDCl_3$ (100 mg/0.7 ml). Chemical shifts were recorded in δ (ppm down-field from internal standard tetramethylsilane).

Thin layer chromatography (TLC) of the depside and sterol components were carried out on precoated plates with silica gel as absorbant. The solvent C used was the same as reported by Culberson and Kristinsson [6]. The chromatographic technique by TLC and column analyses of taurine is described in a previous communication from this laboratory [1].

Materials and extraction

Dried lichen samples were ground in a Wiley Mill to pass a 1 mm screen. The coarse powder was extracted in a Soxhlet apparatus. The general procedure for extraction of the material and working up of the extracts is described in previous papers in this series [1, 7, 8]. All evaporation of extracts and solutions was performed with a rotating evaporator under reduced pressure.

Isolation and identification of the substances

Anaptychia fusca (Huds.) Vain. The lichen (2.17 kg) was collected on Runde, Møre and Romsdal, and thoroughly extracted with acetone for 70 h.

Allantoin ($C_4H_6O_3N_4$). After crystallization from small quantity of boiling water (20 ml), it melted at 227 °C (d). M^+ 158.

Calcd: C 30.38 H 3.82 O 30.35 N 35.43,
Found: C 30.50 H 3.72 O 30.23 N 35.55.

Peltigera canina (L.) Willd. The sample (1.15 kg) was collected at Harstad, Troms, in 1969, and successively extracted with light petroleum and acetone.

Sativic acid (9,10,12,13-tetrahydroxystearic acid), $C_{18}H_{36}O_6$, was crystallized twice from ethanol, yield 0.29 g; m.p. 156.4 °C.

Calcd: C 62.04 H 10.41 O 27.55,
Found: C 62.23 H 10.00 O 27.54.

Omphalodiscus spodochrous (Hoffm.) Schol. This lichen was collected at Hauge in Dalane, Rogaland, in 1973. The material (3.1 kg) was extracted with acetone for 24 h. The acetone-soluble part of the residue was found to consist of mannitol, gyrophoric-, lecanoric-, and orsellic acid.

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In am deeply indebted to my wife for all her help in the collection of the material.

Results and Discussion

Anaptychia fusca was extracted with acetone for several hours. Without reducing of the extract, a mixture of sugar alcohols was precipitated. Concentrating of the filtrate, followed by purification of the residue readily led to the isolation of the following two fractions.

Fraction I consisted of traces of characteristic, faint yellow crystals identified by colour reactions and TLC as *atranorin*.

Fraction II was a dark coloured crystalline material which was partly purified by washing with warm ethanol. Recrystallizations from small quantities of boiling water gave a colourless product, yield 0.40 g. The elemental analysis conforms to the molecular formula $C_4H_6O_3N_4$. The crystalline compound gave only one peak on the Amino Acid Analyzer with retension value identical to that of authentic *allantoin* [1]. IR and NMR spectra of the isolated compound were in agreement with an authentic sample of allantoin.

The remaining lichen material was then extracted with water in a shaking apparatus for 24 hours, yielding a very dark-coloured and viscous residue. By deionising the residue on a cation exchanger, the evidence of the presence of the aminoethanesulfonic acid, *taurine*, was obtained by means of TLC and column chromatography. The amount of taurine in this lichen was calculated to be about 200 mg per 1000 g material, the same amount as detected in *Xanthoria parietina* [1].

Peltigera canina was extracted with light petroleum for 20 h. Upon concentration of the extract a nearly colourless product (0.24 g) was obtained, provisionally named as *P.c.-X*. By recrystallizing from hot acetic acid a lipid-like substance melting at 77.7 °C was obtained. (Found: C 78.86, H 12.48). Methoxyl groups were not present. IR and MS revealed identity with a fraction isolated from the investigated species, *Omphalodiscus spodochrous*. A description of this compound will therefore be given in the last section of this paper.

The filtrate from P.c.-X was evaporated and the residue afforded colourless needles, which after crystallization from acetic acid yielded 26 mg. TLC with solvent C and visualized with sulfuric acid, demonstrated the presence of two compounds. The uppermost spot was identified as the depside *tenuiorin*.

The depside component was closely followed by a sterol substance, $R_F = 0.43$. It gave a violet colour with sulfuric acid and a strong blue colour with phosphomolybdic acid on TLC plates. This compound was purified by preparative thick layer chromatography with solvent C.

In the lower region of the mass spectrum the sterol stood out prominently with the usual and characteristic peaks [9]. The molecular ion peak was obtained at *m/e* 456 (2%) corresponding to the formula $C_{31}H_{52}O_2$. Important peaks were present at M-CH_3 (1%), M-OH (7%), M-CH_3O (2%), M-H_2O (12%), M-CH_3OH (3%), M-H_2O-CH_3 (5%), M-H_2O-43 (12%), and M-(side chain + 2H) (9%) at *m/e* 285.. Other significant peaks which were observed at *m/e* 369 (14%), 341 (17%), 249 (25%), 248 (100%), 207 (25%), 203 (50%), 190 (14%), and 189 (19%), are due to the ions and fragmentations shown in Fig. 1 a and Fig. 1 b. A metastable peak supports the fragmentation of ion *m/e* 248 to the ion *m/e* 203 by splitting of ring A and loss of CH_3OCH_2. The double bond in position 22 – 23 which was confirmed by the peak *m/e* 341 was ascribed to an elimination of $C_7H_{14}OH$ from the molecular-ion. The existence of a hydroxyl group at C-30 is probable on the basis of the peak at *m/e* 369.

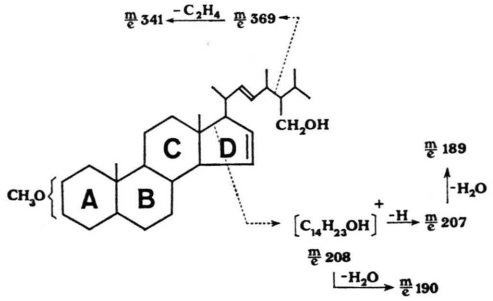

Fig. 1 a. Mass fragmentation pattern of the sterol isolated from *Peltigera canina*.

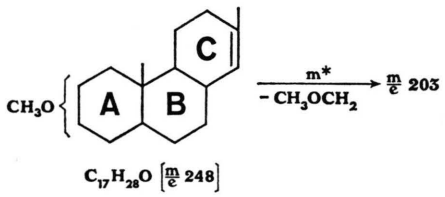

Fig. 1 b. Dominant fragment-ions of the sterol from *Peltigera canina*.

However, owing to the lack of sufficient material, an absolute structural determination of the new sterol could not be accomplished.

The remaining lichen sample was then extracted with acetone. After the volume of the acetone had been diminished, the solution was treated with activated carbon and filtered. Dried in vacuum the acetone-residue yielded 2.33 g of a clear, faintly yellow, viscous liquid. This was found by TLC (Adsorbosil, solv. C) to be an unsaturated fatty acid containing traces of several related substances. The liquid was oxidized by the method of Lapworth [10], and the oxidized product recrystallized from ethanol. By elemental analysis, IR and HrMS, it was found to be a tetrahydroxy-C_{18} fatty acid.

The mass spectrum failed to show the molecular-ion. The base peak was observed at measured mass 155.1058 ($C_9H_{15}O_2$) corresponding to the fragment

$$\overset{+}{HO}=CH-(CH_2)_6-CH=C=O\,.$$

Other prominent fragments in the HrMS were as follows:

	Measured mass	*R.I.*
$\overset{+}{HO}=CH-CH_2-\underset{\diagdown\,O\,\diagup}{CH-CH}-(CH_2)_7-COOH$	229.1438	(18%)
$\overset{+}{HO}=CH-(CH_2)_7-COOH$	173.1184	(40%)
$CH_3-(CH_2)_4-\underset{\diagdown\,O\,\diagup}{CH-CH}-CH_2-CH=\overset{+}{O}H$	157.1211	(51%)
$CH_3-(CH_2)_4-\underset{\diagdown\,\overset{+}{O}\,\diagup}{CH-CH}$	113.0953	(38%)

Metastable ion indicates loss of one molecule of water from the ion at *m/e* 229.

Spectral comparison with authentic 9,10,12,13-tetrahydroxy-stearic acid, m.p. 160.0 °C, demonstrated a complete agreement. Mixed melting point was detected and found to be at 158.8 °C. An eutectic mixture of α and β 9,10,12,13-tetrahydroxy-stearic has its melting point in the region 155 – 159 °C.

The original fatty acid in this lichen species was therefore identical with *linoleic acid*, $C_{18}H_{32}O_2$. This fatty acid has been reported to be found in *Alectoria ochroleuca* (Culberson 1969). Unsaturated C_{18}-acids have also been detected in the species *Hypogymnia physodes, Usnea barbata* and *Cetraria islandica* [11].

It should be emphasized that the epoxi-structures depicted above are only formal representations.

Omphalodiscus spodochrous gave by extraction with acetone a residue from which two fractions were obtained according to solubility. The soluble part of the residue in cold acetone was found to contain the components already known in this lichen.

A small amount of the acetone-residue was insoluble in water and heavily soluble in cold acetone. Crystallization from hot acetic acid yielded 41 mg of a faintly coloured product, *O.sp.-X*, m.p. 82.1 °C. (Found C 78.40, H 12.05, O 9.59). Methoxy groups were not present in the substance, and catalytic hydrogenation did not lead to uptake of hydrogen. A saturated systeme was also supported by IR analysis (Fig. 2).

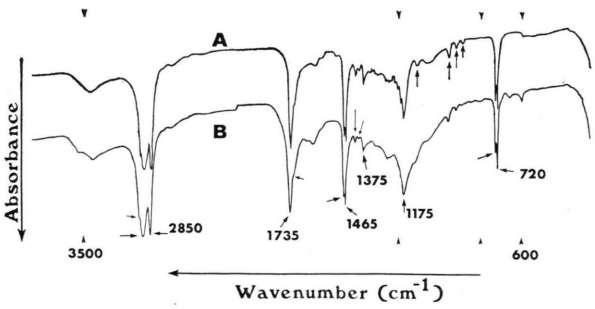

Fig. 2. IR spectra of the waxes isolated from *Peltigera canina* (A) and *Omphalodiscus spodochrous* (B).

UV in chloroform (70 μg/ml) gave maximum absorption at λ 243 nm ($E = 0.380$) and λ 287 nm ($E = 0.295$).

The striking similarity of O.sp.-X and P.c.-X (from *Peltigera canina*) in elemental composition and melting points coupled with the IR absorption spectra evidences, offer convincing arguments for the presence of identical products in the two species.

The asymmetric carbon-hydrogen stretching band at 2920 cm^{-1} shows a constant intensity, compared with the strong ester carbonyl stretching band at 1735 cm^{-1}. Other frequencies common to all esters of *long chain fatty acids* were observed at 1472 – 1465 cm^{-1} (doublet) CH scissoring band, 1375 cm^{-1} (symmetrical bending vibration of CH_3), and at 1170 cm^{-1} (C – O stretching). Characteristic in the spectra was the doublet at 730 – 720 cm^{-1}. The splitting of this CH_2-wag absorption band and the small shoulder at 2960 cm^{-1} correspond to long chain compound with more than sixteen carbon atoms [12]. The band at 1413 cm^{-1} corresponds to the CH_2-group in *alpha*-position to the carbonyl group. None of the spectra exhibit bands in the region 3500 – 3400 cm^{-1} due to free hydroxyl groups. The IR spectra given in Fig. 2 are nearly identical with those spectra demonstrated for wax esters by Hannah *et al.* [13]. Further, they were in good accordance with spectra of octadecylstearate and beeswax run at our laboratory.

The NMR spectra of the isolated products demonstrated the usual picture of alkyl esters consisting of long chain primary alcohols and fatty acids. The protons in the NMR spectra were assigned as follows:

$$\underset{(a)}{CH_3} - \underset{(b)}{(CH_2)_x} - \underset{(c)}{CH_2} - CO - O - \underset{(d)}{CH_2} - \underset{(b)}{(CH_2)_y} - \underset{(a)}{CH_3}$$

(a) δ 0.88, overlapping distorted triplets; (b) δ 1.28, broad, intensive signal; (c) δ 2.25, broad, overlapping triplets; (d) δ 4.07, broad, overlapping triplets.

The close similarity of P.c.-X and O.sp.-X was further established by comparing their respective mass spectra. The upper part of the low resolution mass spectra of the two products with the major fragment-ions are shown in Fig. 3. The parent ions at m/e 285, 313, 341, 369, 397, 453, and 481 could all be assigned to the characteristic ester fragments represented by the formula $C_nH_{2n+1}CO_2H_2^+$, where n has the values of 17, 19, 21, 23, 25, 29, and 31 respectively. Fragments corresponding to the acyl ions were also observed.

The peak at m/e 928 corresponds to the molecular-ion of the compound with the composition $C_{64}H_{128}O_2$. The ions at m/e 648 up to and including m/e 900 may be arised by stepwise loss of

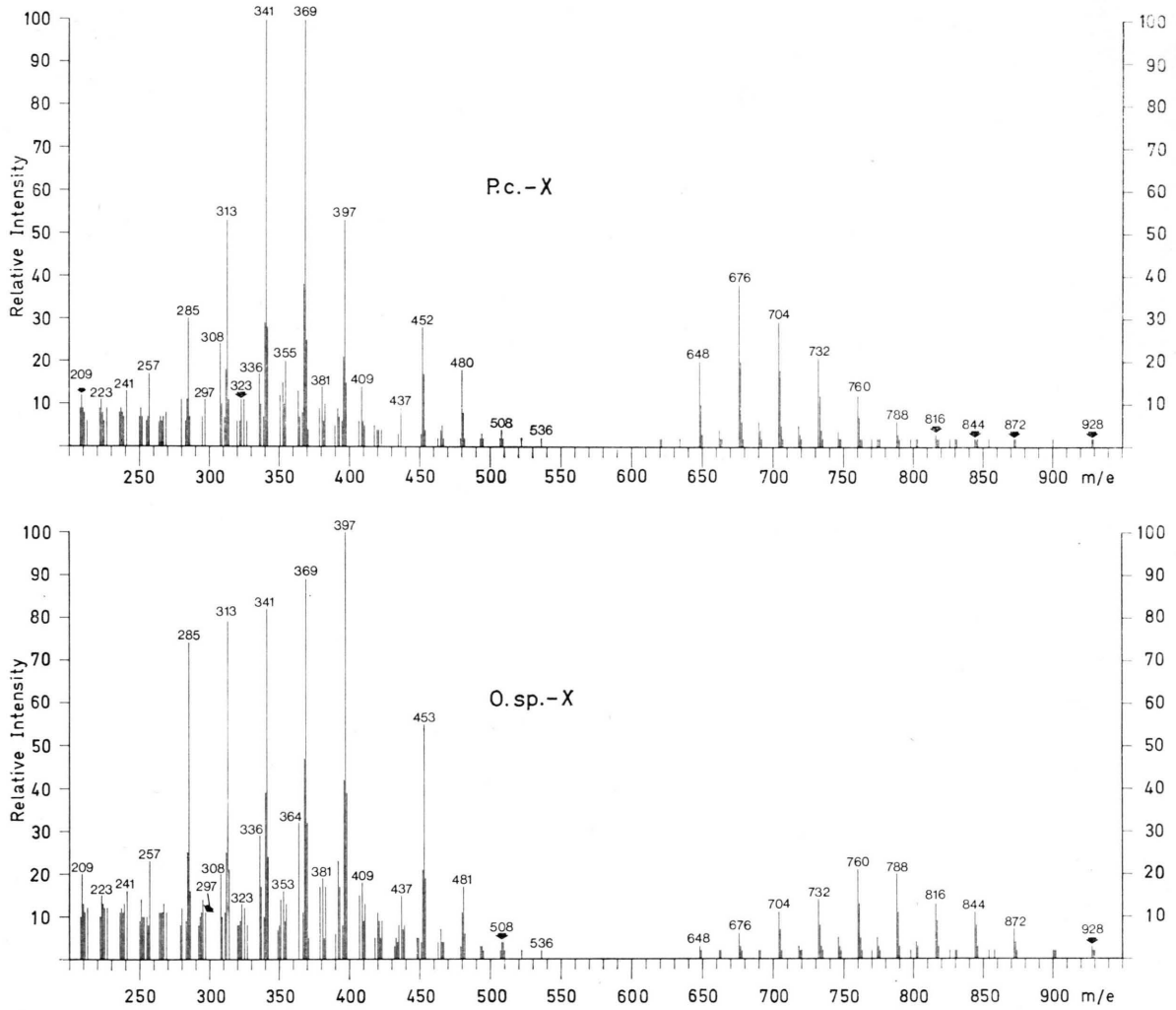

Fig. 3. Low resolution mass spectra of the waxes isolated from *Peltegera canina* (P.c.-X) and *Omphalodiscus spodochrous* (O.sp.-X).

C_2H_4 from the ion at *m/e* 928. More likely they represent the molecular-ions of homologues. The conclusions drawn above are supported by high resolution mass spectrometry. The few effaced bands found in the region $1200-1350\,cm^{-1}$ and the well-defined band at $1375\,cm^{-1}$ in the IR spectra may support the assumption of the presence of homologues. Primary alcohol-fragments represented by the alkenes $C_{28}H_{56}$, $C_{26}H_{52}$, $C_{24}H_{48}$ were recognized as small peaks in the mass spectra.

It is noteworthy that the same product has been isolated at our laboratory from the sporemass of the ascomycete *Elaphomyces granulatus*. The mixture isolated from the fungus has an almost identical composition compared with P.c.-X and O.sp.-X. The elemental composition and the melting points of the products isolated from these species are summarized below.

Product isolated from	Found [%] C	H	O	m.p. [°C]
Peltigera canina	78.86	12.48		77.7
Omphalodiscus spodochrous	78.40	12.05	9.59	82.1
Elaphomyces granulatus	78.12	12.36	9.52	78.6

The high content of oxygen found by elemental analyses is very likely due to the fact that the products crystallized with water of crystallization.

From the present results, there is no doubt that the isolated products, P.c.-X and O.sp.-X, are ester-waxes made up of long-chain acids and the primary alcohols C_{24}, C_{26}, and C_{28}. Small amounts of the paraffin homologues $C_{15}H_{32}$ and $C_{16}H_{34}$ could be recognized in the mass spectra. Free fatty acids could not be detected in the isolated waxes from the lichens.

As far as one can remember, wax substances have previously not been isolated from lichen species. Aliphatic hydrocarbons and wax esters are detected in almost all organisms. In lichens too, these substances must be important constituents as protective coatings on the lichen cortex.

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