Phytochemistry, 1963, Vol. 2, pp. 335 to 340. Pergamon Press Ltd. Printed in England

THE LICHEN SUBSTANCES OF THE GENUS *EVERNIA*

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(*Received* 28 *March* 1963)

Abstract—The small, morphologically conservative genus *Evernia* shows a simple pattern of chemical variation. Contrary to some recent reports, usnic acid occurs in most specimens of *Evernia prunastri* (L.) Ach. and the microchemical identifications of this constituent were confirmed by the isolation of (+)-usnic acid from a large sample of the lichen. It was possible to identify the lichen substances in most of the other species of *Evernia* by using fragments of herbarium specimens for microanalyses.

INTRODUCTION

LICHENS are noted for their peculiar, crystalline, aromatic constituents[1] that accumulate on the outer surface of the hyphae. Microchemical methods[2-6] for identifying these "lichen substances" have permitted taxonomists to use them, in addition to the traditionally studied morphology, to produce classifications based on a larger number of intrinsic characteristics of these difficult cryptograms. It is well known that large, complex genera such as *Parmelia*,[7-9] *Usnea*,[10] and *Cladonia*[11-13] exhibit highly diverse patterns of chemical compounds even within groups of closely related species. Furthermore, some taxonomists consider the presence or absence of the cortex substances usnic acid and atranorin as a particularly significant, stable taxonomic trait. The present study considers the constituents of *Evernia* and the significance of the distribution of usnic acid in this small, morphologically conservative genus.

A confusion concerning the constituents of *Evernia prunastri* (L.) Ach., a common fruticose species in many north temperate regions and the "mousse de chêne" of the perfume industry, has led to the present re-examination of the chemical variation in the genus. Until recently there was little disagreement that usnic acid occurred, at least as an accessory substance, in *E. prunastri*. Small quantities of this common pigment were extracted from

[1] Y. ASAHINA and S. SHIBATA, *Chemistry of Lichen Substances*, 240 pp. Japan Society for the Promotion of Science, Tokyo (1954).
[2] Y. ASAHINA, *J. Japan. Botany* **12**, 516, 859 (1936); **13**, 529, 855 (1937); **14**, 39, 244, 318, 650, 767 (1938); **15**, 465 (1939); **16**, 185 (1940).
[3] M. MITSUNO, *Pharm. Bull. (Tokyo)* **1**, 170 (1953); **3**, 60 (1955).
[4] C. A. WACHTMEISTER, *Acta Chem. Scand.* **6**, 818 (1952).
[5] C. A. WACHTMEISTER, *Botan. Notiser* **109**, 313 (1956).
[6] C. A. WACHTMEISTER in *Papierchromatographie in der Botanik*, H. F. LINSKENS (editor), p. 135. Springer-Verlag, Berlin (1959).
[7] Y. ASAHINA, *Lichens of Japan*. Vol. II. Genus *Parmelia*, 162 pp. Research Institute for Natural Resources, Tokyo (1952).
[8] H. KROG, *Nytt Mag. for Naturvidenskapene* **88**, 57 (1951).
[9] M. E. HALE, Jr., *Contrib. U.S. Nat. Herb.* **36**, 1 (1960).
[10] Y. ASAHINA, *Lichens of Japan*. Vol. III. Genus *Usnea*, 129 pp. Research Institute for Natural Resources, Tokyo (1956).
[11] Y. ASAHINA, *Lichens of Japan*. Vol. I. Genus *Cladonia*, 255 pp. Hirokawa Publishing Co., Tokyo (1950).
[12] A. W. EVANS, *Rhodora* **52**, 77 (1950).
[13] A. W. EVANS, *Trans. Conn. Acad. Arts Sci.* **38**, 249 (1952).

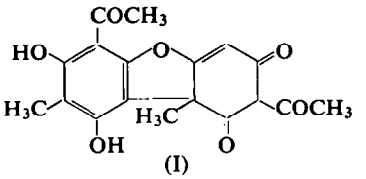
European material of the species by Hesse,[14] Zopf,[15] and St. Pfau.[16] Only Hesse failed to detect it in all the samples that he extracted. Using microcrystallographic methods, Duvigneaud[17] later also concluded that usnic acid was lacking in some specimens, and he established a new species, *E. herinii* Duv., for the chemically aberrant ones. But in 1958 Hess[18] reported the absence of usnic acid in extracts of *E. prunastri* analyzed by paper chromatography. Finally, Ramaut and his co-workers[19] attempted to settle the question by analyzing thirty-one collections of the species (twenty-seven herbarium specimens tested by chromatography and four large samples used for macroextractions). They concluded that usnic acid is lacking in *E. prunastri*.

Since *Evernia prunastri* is a morphologically unique and distinctive lichen, misidentified experimental material can hardly explain the discrepancies in the literature. Either the earlier macroextractions were made on samples contaminated with usnic acid-containing lichens or the chromatographic method used by Hess and by Ramaut and his colleagues is inadequate for determining usnic acid in small quantities.

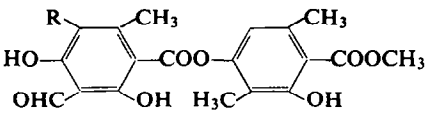
RESULTS AND DISCUSSION

Distribution of Lichen Substances in the Genus Evernia

Table 1 shows the constituents of five species of *Evernia* studied.* The distribution of substances conforms to a pattern found in many genera or species groups of foliose and



(I)



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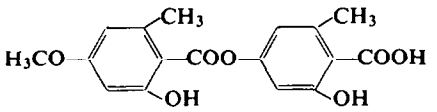

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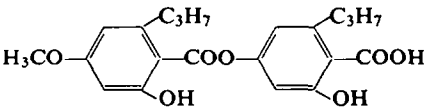

(IV)

fruticose lichens where each species contains one depside or one depsidone in the medulla and either usnic acid (I) or atranorin (II, R = H) in the cortex. In *Evernia*, there are two medullary depsides, evernic acid (III) and its homologue, divaricatic acid (IV). Every species contains usnic acid or atranorin except *E. prunastri* which usually contains both. The commonest pattern of chemical variation among closely related species of lichens is the replacement of the medullary substance by a different depside or depsidone. Changes in the cortex substances, usnic acid and atronorin, seem unrelated to variations in the medullary compounds. Exactly this type of variation is found in *Evernia*, but the entire genus is extremely uniform

[14] O. HESSE, *J. Prakt. Chem.* **65**, 537 (1902); **83**, 22 (1911); **92**, 425 (1915).
[15] ZOPF, *Die Flechtenstoffe*, 450 pp. G. Fischer, Jena (1907).
[16] A. ST. PFAU, *Helv. Chim. Acta* **9**, 650 (1926).
[17] P. DUVIGNEAUD, *Bull. soc. roy. botan. Belg.* **72**, 148 (1940).
[18] D. HESS, *Planta* **52**, 65 (1958).
[19] J. L. RAMAUT, J. LAMBINON and A. TARGÉ, *Lejeunia, Rev. de Bot.*, n. sér. N° 12, 11 pp. (1962).

* Three species attributed to the genus are not included here because they have rarely been collected and because in part there may be taxonomic doubt concerning their generic affinity.

TABLE 1. LICHEN SUBSTANCES IN SPECIES OF THE GENUS *Evernia*

Species	Substances reported	Substances found and analyses used *	Number of specimens tested
Evernia divaricata (L.) Ach.	Divaricatic acid [15]	Divaricatic acid (GE,* chromatography) and usnic acid (GE)	11
		Divaricatic acid alone (GE, chromatography)	1
Evernia illyrica (Zahlbr.) Zahlbr.	Divaricatic acid and atranorin [20]	Divaricatic acid (GE, GAW,*, chromatography) and atranorin (GE, *o*-T*)	3
Evernia mesomorpha Nyl.	Divaricatic acid and usnic acid [21] Evernic acid † [22]	Divaricatic acid (GE, chromatography) and usnic acid (GE)	24
Evernia perfragilis Llano	Divaricatic acid [23]	Divaricatic acid (GE, GAW, chromatography) and usnic acid (GE)	8
Evernia prunastri (L.) Ach.	Evernic acid [15, 19] Atranorin [15, 19] Chloratranorin [1] Usnic acid [1, 15] Thamnolic acid ‡ [17]	Evernic acid (GE, chromatography, extraction), chloratranorin [atranorin] (GE, *o*-T, extraction) and usnic acid (GE, extraction)	28
		Evernic acid (GE, chromatography) and chloratranorin [atranorin] (GE, *o*-T)	4

* The crystallizing reagents [2] used are a solution of glycerol and glacial acetic acid, 1:3 parts by volume (GE); a solution of glycerol, ethanol and water, equal parts by volume (GAW); and a solution of glycerol, ethanol and *o*-toluidine, 2:2:1 parts by volume (*o*-T).

† Probably based on a misidentified specimen of *E. prunastri*.

‡ Crystals of the anil of atranorin or chloratranorin formed in the An solution [2] undoubtedly mistaken for thamnolic acid.

chemically compared to even small species groups in such complex genera as *Parmelia* [24] and *Cladonia*. The conservative morphology of the genus is reflected in the simple pattern of chemical variation.

In the *Everniae* which contain usnic acid, the concentration of this substance is variable. Samples of *Evernia divaricata* and *E. prunastri* which seem to lack usnic acid may contain it in amounts not detectable by our tests. Such variation has not been considered significant taxonomically in other groups of lichens and pale forms of normally yellow, usnic acid-producing species have been thought to result from growth in reduced sunlight. Consequently Duvigneaud's species based on the absence of usnic acid in *E. prunastri* [17] would not seem valid. Furthermore a new species is not justified for the single specimen of *E. divaricata* indicated above to lack usnic acid. The erratic occurrence of usnic acid in *Evernia* suggests that the presence of usnic acid here is not so significant taxonomically as in genera where such variation is less common.

[20] A. ZAHLBRUCKNER, *Ann. K. K. Naturhist. Hofmus.* (*Wein*) **19**, 379 (1904).
[21] Y. ASAHINA, *J. Japan. Botany* **27**, 293 (1952).
[22] M. E. HALE, Jr., *Castanea* **21**, 30 (1956).
[23] M. E. HALE, Jr., *Lecture Notes. Lichenology*, 73 pp. West Virginia University, Morgantown (1957).
[24] W. L. CULBERSON, *Nova Hedwigia* **4**, 563 (1962).

Evernia illyrica (Zahlbr.) Zahlbr., a species found locally in the Mediterranean Region, appears to be a chemical variant if the widely distributed *E. divaricata* (L.) Ach. The three specimens of *E. illyrica* examined contained abundant atranorin but no trace of usnic acid. Although the concentration of usnic acid in *E. divaricata* is variable, no samples of this species contained atranorin. The distinction between *E. illyrica* and *E. divaricata* is one of replacement of one substance by another rather than occasional absence of one compound as in the previously proposed separation of *E. hernii* from *E. prunastri.*

Microchemical Tests and the Determination of Usnic Acid in Evernia prunastri

In the present study, usnic acid was determined by Asahina's[2] microcrystallographic method. Some samples of *Evernia prunastri* give a positive test for usnic acid by microrecrystallization of the residue from the acetone extract in a medium of glycerol and acetic acid (1:3 parts by volume; the GE solution). But usually the relative quantity of evernic acid and of atranorin (or chloratranorin; II, R = Cl) is so large that usnic acid either fails to precipitate or its crystals are lost in the crystalline mass of the abundant substances. But evernic acid is less soluble in benzene than in acetone and if the film of residue on a microslide from the acetone extract is washed with a minimum volume (3–5 drops) of cold benzene, a residue can be obtained from the benzene. If this residue is treated with the GE solution, the characteristic crystals of usnic acid will appear immediately.

By this method usnic acid, atranorin, and evernic acid were demonstrated in fragments of twenty-eight herbarium specimens of *Evernia prunastri.* Four samples gave positive tests for evernic acid and atranorin, but not for usnic acid. While Ramaut and his colleagues[19] failed to identify usnic acid chromatographically in any samples of 100–200 mg of thallus, in the present study this substance was readily demonstrated by the method described above in two samples of a French specimen weighing 5·75 and 5·60 mg. Evernic acid and atranorin are determinable in the same preparations. No attempt was made to devise a method to distinguish atranorin from chloratranorin. The acetone extracts of all samples were chromatographed to confirm the presence of evernic acid. Usnic acid was not detected on these chromatograms.

No known substance gives crystals in the GE solution similar to those produced by usnic acid, but other unidentified yellow pigments in *Evernia prunastri* have been reported.[19] To confirm the microcrystallographic identification of usnic acid, 100 g of *Evernia prunastri* from France was extracted. A yellow pigment was isolated and proved to be (+)-usnic acid by comparison with a sample of (−)-usnic acid isolated from the common arctic-alpine lichen *Cetraria nivalis* and by converting both of these compounds to racemic (±)-usnic acid.

Apparently the high proportion of evernic acid in *E. prunastri* interfered with the previous chromatographic studies. The ratio of usnic acid to evernic acid is so low that the quantity of extract which can be chromatographed without overloading the spot with evernic acid contains too little usnic acid to be detected. On the other hand, the previously reported failures[19] of Ehrlich's reagent to produce the deep blue color characteristic of usnic acid cannot be explained. Most herbarium specimens give a definitely positive test, some are weakly positive, and a few give only a greenish color such as can be obtained with pure atranorin. Prepared mixtures of evernic acid and usnic acid and of atranorin and usnic acid give positive Ehrlich reactions. Although atranorin and usnic acid react in the presence of concentrated sulfuric acid to produce a dark red precipitate, even mixtures containing very small proportions of usnic acid give a positive test and there is no conclusive evidence that this side

reaction might explain the occasional failure of Ehrlich's reagent to give a positive test on crude extracts from specimens of *Evernia prunastri* known to contain usnic acid.

EXPERIMENTAL

Macroextraction of Evernia prunastri (*L.*) *Ach.*

Evernia prunastri collected from the bark of maples near Trelon (Nord), France, in May 1962 (voucher specimen 10, 513 in Duke Herbarium), was carefully sorted to remove contaminating materials. One hundred grams of the coarsely crushed, air-dried lichen was soaked successively in three 2-l. portions of benzene at room temperature for 2 days. Concentration of the combined extracts to about 20 ml gave a tan-coloured solid (A) collected by filtration. The filtrate, evaporated to dryness, provided a second solid fraction (B). Each fraction was digested with chloroform and filtered to remove the slightly soluble evernic acid. The chloroform washing of solid A contained primarily atronorin, but that of solid B also contained a yellow pigment, purified by recrystallizing once from benzene and twice from glacial acetic acid. The small amount (27 mg, p.m. 201–202°) of purified substance was identified as (+)-usnic acid (see below).

The combined acetone extracts from three successive soakings of the benzene-extracted lichen (in 2 l., 2 l., and 1 l. of solvent) were evaporated to dryness and the residue was digested with chloroform. Insoluble crude evernic acid, combined with evernic acid from the benzene extract was recrystallized from dilute acetone yielding 6·3 g of a white solid, m.p. 166–167·5°. A small sample was purified by further recrystallizations, m.p. 169·5–170·5° (reported for evernic acid,[1] m.p. 169–170°). An acetone (30·0 ml) solution of evernic acid (1·04 g) stirred under reflux with methyl iodide (3.0 ml) and potassium carbonate (3.09 g) for 10 hr was cooled and filtered. Evaporation of the filtrate at diminished pressure gave methyl lecanorate trimethyl ether (0.54 g), m.p. 148–149° (reported,[1] m.p. 146–147°).

The chloroform solution from which evernic acid had been separated was combined with the atranorin fraction from the benzene extraction and the residue on evaporation was recrystallized from chloroform and then from acetone. The yield of pale tan solid was 270 mg, m.p. 205–207° (reported for atranorin,[1] m.p. 196°; reported for chloratranorin,[1] m.p. 208–208·5°). This product was not studied further, but the microchemical tests indicated that it was atranorin or chloratranorin. The high melting-point, positive Beilstein test for halogen[25] and depressed melting-point with a sample of atranorin suggest the product is chloratranorin, possibly containing a trace of atranorin.

Identification of (+)-*Usnic Acid from* Evernia prunastri

The yellow pigment isolated from *Evernia prunastri* was compared with a sample of (—)-usnic acid from *Cetraria nivalis*. The two substances had identical color, crystal habit, melting point (201–202°; reported for usnic acid,[1] m.p. 203–204°) and gave identical infrared spectra. But a mixed melting-point was depressed (mixed, m.p. 194·5–195·5°). A small sample of each pigment was racemized in refluxing xylene for 6 hr. A solution of 100 mg (—)-usnic acid from *C. nivalis* in 2·5 ml xylene deposited 74·7 mg racemized product, m.p. 195–196·5° (reported for (±)-usnic acid,[1] m.p. 194°). Similarly, a solution of 22·3 mg of the yellow pigment from *E. prunastri* in 2·0 ml xylene gave 6·6 mg product, m.p. 195·5–196·5°.

[25] F. FEIGL. *Spot Tests*. Volume II. Organic Applications, p. 64. Elsevier Publishing Company, New York (1954).

A mixed melting-point of these compounds was not depressed and their infrared spectra were identical and unchanged.

Microchemical Test for Usnic Acid

The thallus fragment in a vial (1·0 cm in diameter, 3·5 cm in height), is submerged in acetone and warmed for 10–15 min. Three successive acetone extracts evaporated on a microslide leave a film of residue. While the slide is held at one end, 3–5 drops of cold benzene are run over the surface of the residue and collected by touching one corner to a clean slide. This benzene extract, frequently pale yellow, evaporates leaving a residue which is scraped together with a razor blade and covered with a small slip (Corning Glass No. 1, 12 mm) to the lower side of which a drop of the GE solution (glycerol and acetic acid, 1 : 3 parts by volume[2]) has been added. The slide is warmed momentarily over a microflame and if necessary the residue is dispersed by slightly moving the slip. Crystals of atranorin normally appear immediately followed by those of usnic and evernic acids. The order of crystallization depends on many uncontrollable factors. Divaricatic acid is more soluble in benzene than evernic acid, and small quantities of usnic acid mixed with divaricatic acid are better determined by extracting the thallus fragment directly on a slide with 2–4 drops of cold chloroform. The resultant residue is treated with GE solution as described above.

Other Microchemical Tests

In the GE solution, evernic acid forms colorless needles, usually in clusters and more or less curved and branched. The crystals are easily seen with 100 × magnification. The extinction angle is zero. Chromatographed by the ascending method on Whatman No. 1 paper in n-butanol saturated with NH_4OH, evernic acid forms an oval spot at R_f 0·55. When sprayed with *bis* diazotized benzidine,[5] an orange color develops.

Divaricatic acid forms very characteristic, nearly perpendicularly branched, colorless crystals in the GAW solution[2] (glycerol, ethanol and water in equal volumes). The main crystals are large and have an extinction angle of zero; the branch crystals which form upon standing have an oblique extinction (ϕ approximately 42°). Chromatography of divaricatic acid under the conditions described for evernic acid gives an oval spot at R_f 0·70 which develops an orange color with a *bis* diazotized benzidine spray.

Atranorin was not distinguished from chloratranorin. Both substances produce medium to small prisms in the GE solution and yellow needles in *o*-T solution[2] (glycerol, ethanol and *o*-toluidine; 2:2:1 parts by volume). The lower limits of both these tests has been determined to be less than 1 μg and to be dependent at this low level upon the worker's skill in collecting the residue on the slide. The prisms in GE have an extinction angle of zero with respect to the longest axis. In the *o*-T solution, the yellow needles appear blue at the point of extinction ($\phi = 0$). Chromatographed in n-butanol saturated with NH_4OH, the R_f is 0·53. The yellow spot of atranorin or chlorantranorin visible during chromatography in the presence of ammonium hydroxide disappears when the paper is dried. The spot gives a yellow fluorescence in UV light ($\lambda = 360$ mμ) and turns dull orange when sprayed with *bis* diazotized benzidine solution.

Acknowledgements—This investigation was supported in whole by Public Health Service Research Grant GM-08345 from the Division of General Medical Sciences. Dr. W. L. Culberson identified all the lichen specimens.