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THE FORMATION OF ROCCELLIC ACID, EUGENITOL, EUGENETIN, AND RUPICOLON BY THE MYCOBIONT *LECANORA RUPICOLA*

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Abstract—Ether extracts of cultures of the lichen fungal symbiont *Lecanora rupicola* contain roccellic acid, eugenitol, eugenetin, rupicolon, and a fourth as yet unidentified product. This is the first report of aliphatic carboxylic acids, chromones, or chlorinated compounds formed by cultivated lichen fungi, and seems to be one of the few instances of the formation in culture of substances found in the composite lichen.

INTRODUCTION

ONE CHARACTERISTIC of most lichen symbioses is the formation of secondary natural products. The metabolic and biogenetic pathways leading to the formation of these substances have recently been reviewed.[1,2]

An intriguing question concerning the formation of lichen substances is the degree to which each member of the symbiosis participates in the biosynthesis of these substances. In a few cases, the origin of lichen compounds has definitely been ascribed to the fungal partner, i.e. the pulvic acid derivatives vulpinic acid, pulvinic acid, pulvinic dilactone and calycin.[3,4] The formation of the hydroxyanthraquinone parietin has also been reported in cultures of lichen fungi[5–7] but the structural identity of these compounds have not been established by conventional chemical methods. A report of finding usnic, rhodocladonic and didymic acids in cultures of the mycobiont *Cladonia cristatella*[8] was based on very questionable chemical methods, and attempts to confirm this work have been completely unsuccessful.[9]

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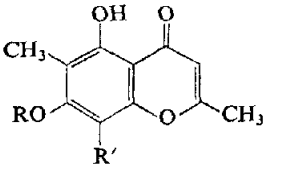
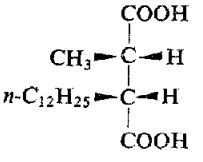
In this report, we wish to describe several new compounds isolated from cultures of a lichen mycobiont and to comment on the biosynthesis of lichen substances by lichen symbionts growing in pure culture.

RESULTS AND DISCUSSION

An ether extract of agar cultures of *Lecanora rupicola* (L.) Zahlbr. was evaporated to dryness. The resulting residue was extracted with benzene and the soluble portion was chromatographed on silica gel with benzene followed by benzene–ethyl ether mixtures. Four compounds were obtained from the eluates. The first of these, eugenetin (I) is unknown in lichen thalli. The second compound (II) rupicolon is a chlorinated chromone previously isolated and identified from the composite lichen in nature.[10] The third compound was found to be identical with eugenitol (III) also previously unknown as a lichen product. The fourth substance was found by high resolution mass spectrometry to be $C_{23}H_{20}O_8$ (measured mass 424·1158, calculated mass 424·1157). The u.v. spectrum with maxima at 230, 258, 294, and 330 nm is typical of a chromone. Possibly this compound is a dimeric chromone but further studies will be required for positive identification. The residue of the ether extract that was insoluble in benzene contained (+)-roccellic acid (IV).



	R	R′
(I) Eugenetin	CH_3	H
(II) Rupicolon	H	Cl
(III) Eugenitol	H	H



(IV) Roccellic acid

The substances described above represent two new classes of compounds produced by lichen fungi: chromones and aliphatic dicarboxylic acids. While both types are known from cultured free-living fungi, this is to our knowledge the first report of the occurrence of such compounds chemically identified in isolated lichen fungi. In addition, the formation of eugenetin, eugenitol, and rupicolon probably represents a product-precursor relationship in which eugenitol accumulates and is subsequently chlorinated. The reverse possibility, that rupicolon is converted to eugenitol is also worthy of consideration, but seems less likely.

Roccellic acid is probably derived from an acetate–malonate derived fatty acid which has been condensed with a three-carbon carboxylic acid. Several similar compounds have been studied in free-living fungi.[11]

These results are made more interesting by the fact that "typical" lichen acids are unknown in isolated lichen fungi. In a study of 100 lichen fungi in culture, Fox[2] was unable to detect a single authentic lichen depside, depsidone, dibenzofuran, depsone, anthraquinone or precursor of these substances. These results, coupled with the occurrence of the metabolites reported here, indicate that the metabolic pathways that result in the formation of "typical" lichen acids are not operative in lichen fungi in culture, and that the depside, depsidone, depsone or dibenzofuran type compounds are unique to the lichen association. It appears,

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therefore, that the formation of the above-named lichen compounds is the result of the provision of a particular substance to the fungus by the alga in the lichen association that either serves as a precursor of acetate-malonate phenolics or serves to induce the formation of these substances by the lichen fungi.

In confirmation of this, although *L. rupicola* is known also to produce atranorin and chloratranorin in the lichenized state, no atranorin, chloratranorin or phenolic precursors of either compound has been found in our cultures.

One interesting facet of these findings is that it has been the practice of some taxonomic lichenologists to use the occurrence of lichen acids as the single determinant of a lichen species. If the lichen species name actually is the name for the fungal symbiont, the use of a particular lichen acid to decide a species would seem to depend on the consistent production of that acid whether in culture or in the lichen symbiosis. Since the lichen acids used for these species determinations are evidently the result of the symbiosis rather than a consistent expression of the fungal metabolism, such a practice of determining a new species of fungus seems irrational unless the understanding exists that the species in question produces a particular substance as a result of its particular environment.

The extremely slow growth of the fungus used in these studies has so far proscribed isotopic investigations of the pathways involved in the formation of the products reported above, but further studies with larger cultures are in progress.

EXPERIMENTAL

Cultivation of the Fungus

The fungus used in this work was the mycobiont *Lecanora rupicola* (L.) Zahlbr. It was isolated in pure culture from a specimen collected at Karkevagge, Sweden. Collection, identification, and isolation were performed by Professor V. Ahmadjian of the University of Massachusetts, U.S.A. Since isolation, the fungus has been maintained at 10° on a malt–yeast extract agar. Subcultures have been made at yearly intervals.

In this study, cultures were grown for 18 months on malt–yeast extract medium after which they were removed from the surface of the medium and transferred to a blender containing distilled water. The mycelium was thoroughly homogenized and the resulting suspension used to inoculate 6 1-l. flasks each containing 250 ml of sterile malt–yeast extract agar. The cultures were then incubated for 1 yr at 20°.

When harvested the colonies were densely compacted, being about 1–1·5 cm in dia., and did not extend into the agar to any appreciable extent. Small wart-like clusters of microcrystalline substances were found around the colonies.

Extraction and Purification

The cultures were harvested by cutting the bottoms from the flasks and removing the agar whole. The agar disks were sliced into quarters and soxhlet extracted for 2 days with peroxide-free Et_2O. A control flask containing uninoculated medium was also examined; with negative results.

The Et_2O extract was evaporated in N_2 to yield a slightly gummy yellow solid, which was dissolved in C_6H_6 and chromatographed on SiO_2 gel. The column was eluted sequentially with the following solvents: C_6H_6, C_6H_6–Et_2O (9:1) and (1:1). The fractions obtained from the column were recrystallized from C_6H_6.

Identification of Products

When the crude extract (500 mg) was chromatographed on Kieselgel G TLC in C_6H_6–dioxane–HOAc (90:25:4), four distinct spots were found to give a blue or brown colour with 2% alcoholic $FeCl_3$. 20% H_2SO_4, followed by charring in an oven at 120°, revealed a fifth spot. Column chromatography of the benzene-soluble material (100 mg) yielded four fractions. The first was eluted with C_6H_6, and on recrystallization from benzene was found to have a melting point of 159–160°. The second fraction (5 mg), also eluted with C_6H_6, had a melting point of 263–264°. A third fraction (20 mg) was obtained from elution with C_6H_6–Et_2O (9:1) and melted at 288–289°. The fourth substance (15 mg) melted at 280–281° and was obtained from elution with C_6H_6–Et_2O (1:1). The first compound was found by high resolution mass spectrometry to have the formula $C_{12}H_{12}O_4$ (measured mass 220·0733, calculated mass 220·0735) and showed the following NMR signals: 2·07 (3H), 2·34 (3H), 3·89 (3H), 4·66 (1H), 6·02 (1H), and 6·34 (1H) ppm when measured at 60 MHz in $CDCl_3$. Spectra and melting points were identical to eugenetin.

After recrystallization, the material from the second fraction was found to be identical with rupicolon prepared by synthesis. The third fraction was found to be identical with eugenitol, also by comparison with a known synthetic sample. The fourth phenolic substance obtained from the column has not as yet been completely identified.

The residue from the original Et_2O extract that was not soluble in benzene was recrystallized from acetone (yield 200 mg) and was found to be identical to (+)-roccellic acid ($[\alpha]_D + 18°$) on the basis of melting points, mixed melting points, and spectral data.

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