ACTA CHEMICA SCANDINAVICA 21 (1967) 2331–2334

On the Biosynthesis of Lichen Substances

Part 4. The Formation of Pulvic Acid Derivatives by Isolated Lichen Fungi

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The lichen fungus of the lichen *Candellariella vitellina*, grown on solid malt extract medium, was shown to produce four pulvic acid derivatives proving that in the biosynthesis of this group of "lichen substances" no direct participation of the algal partner is required. The four pigments were identified as pulvic acid, pulvic dilactone, calycin and vulpinic acid. The occurrence of vulpinic acid in the fungal culture was surprising since it is not found as product in the corresponding lichen association. In experiments using DL-3-phenyl(alanine-1-^{14}C) as possible precursor all four compounds were labelled.

In studies on the carbon flow from alga to fungus in the composite plant, the lichen *Candellariella vitellina* was used and shown to produce labelled pigments from administered $^{14}CO_2$.

Lichens are known to produce a variety of aromatic compounds such as depsides, depsidones, depsones, dibenzofurans and pulvic acid derivatives, the occurrence of which is, apart from few exceptions, restricted to these symbiotic organisms. It has therefore been suggested that both fungal and algal partner participate in a collaborative effort in the formation of such lichen metabolites. Hess reported the production by cultered lichen fungi of the phenolic carboxylic acids orsellinic and haematommic acid which in the lichen association occur esterified as depsides possibly by the esterase activity of the algal partner.[1] In line with this observation is the finding that in the enzymic breakdown of a depside to its corresponding phenols both symbionts may participate: hydrolysis of the depside linkage through an esterase (phycobiont) followed by decarboxylation of the formed phenolic carboxylic acids through the activity of orsellinic acid decarboxylase (mycobiont).[2]

On the other hand reports have appeared in the literature on the synthesis of "lichen substances" by the isolated mycobiont. Thus Thomas isolated a yellow pigment from the mycobiont of the lichen *Candellariella vitellina*.[3] This compound was identified as "stictaurin" based on a melting point determination. This latter substance, however, as was shown later with pigment

extracts from intact lichens, is in fact a mixture of both calycin and pulvic dilactone. Castle and Kubsch reported on the formation of the dibenzofurans usnic and didymic acid by the mycobiont of the lichen *Cladonia cristatella*.[4] Culberson, however, failed to observe such lichen substances or any other phenolic products in a variety of mycobionts isolated by Ahmadjian from the same lichen.[5] Neither was Fox able to find usnic acid in fungal cultures obtained from the lichen *Ramalina ecklonii* where this compound is normally found.[6]

The picture emerging as to the role of fungal and algal partner in the formation of these compounds is thus confusing. Therefore the present investigation was initiated to establish whether isolated fungal cultures are capable of forming lichen substances in particular those belonging to the pulvic acid group.

EXPERIMENTAL

Culture conditions. A 2 l Fernbach flask containing 500 ml of solid malt extract agar medium (Difco malt extract = 10 g, agar = 15 g, glucose = 5 g, dist. water to make 1000 ml) was inoculated with the lichen fungus isolated from the lichen *Candellariella vitellina*. The inoculum was prepared by macerating fungal mycelium obtained from growth in a test tube with a tissue homogenizer in sterile water. The suspension obtained was then transferred to the flask. After two months of incubation in day light at room temperature, the mycelium started turning yellow. 200 μC of DL-3-phenyl(alanine-1-^{14}C) with a specific activity of 44.2 mC/mmole, dissolved in 1 ml of sterile water, was added to the culture. After additional incubation for two months the pigments formed were isolated.

Isolation. The pigments were extracted using cold chloroform which was added directly on the mycelial growth in the flask. The extract obtained was then evaporated to dryness *in vacuo*.

Identification of vulpinic acid. One half of the extract was separated by preparative thin-layer chromatography in the solvent system butanol:(28 %) NH_4OH, 4:1 (v/v). Silica gel G (Merck) was used as adsorbant and the extract applied in streaks. After developing, four major pigment zones were obtained. Only from the band with R_F = 0.65 the pigment could easily be eluted using organic solvents such as chloroform. This chloroform extract was evaporated to dryness *in vacuo* and recrystallized from ethanol. A crystal was obtained the melting point of which corresponded to that of vulpinic acid (m.p. 148°). To the mother liquor carrier vulpinic acid was added and the mixture recrystallized four times from ethanol to constant radioactivity.

Identification of calycin, pulvic acid, and pulvic dilactone. The other half of the chloroform extract was separated by thin-layer and paper chromatography in a variety of solvent systems. For thin-layer chromatography the above solvent system, acetone:chloroform, 1:1 (v/v), and acetone:chloroform:(99.5 %) ethanol, 4:4:1 (v/v/v) were used [7] and for paper chromatography the systems butanol:(28 %) NH_4OH, 4:1(v/v), and butanol: ethylacetate:(28 %) NH_4OH, 1:8:1 (v/v/v).[8] In all systems four different pigment zones were obtained; the R_F-values, color in visible and UV (365 mμ) light corresponded to that of pulvic acid, calycin, pulvic dilactone, and vulpinic acid, respectively. (The R_F-values of the pigments in the order given were 0.23, 0.30, 0.50, and 0.65 in the system butanol: (28 %) NH_4OH, 4:1 (v/v) using TLC). In addition scanning of the paper chromatograms for radioactivity in a strip counter revealed four major radioactive peaks coinciding with the pigment zones obtained.

Incorporation of $^{14}CO_2$ into pulvic dilactone. 50 mg of fresh thallus of the lichen *Candellariella vitellina* was incubated with 200 μC of $^{14}CO_2$ obtained from a solution of sodium bicarbonate-^{14}C (26.7 mC/mmole) in a Petri dish following in detail a procedure given elsewhere.[9] After different periods of exposure to light the lichen material was rapidly transferred to hot chloroform. The chloroform extract was subsequently applied as a 1 cm wide streak near the edge of a thin-layer plate impregnated with silica gel G and separated by two-dimensionell TLC in the systems: acetone:chloroform:(99.5 %) ethanol, 4:4:1 (v/v/v) and butanol:(28 %) NH_4OH, 4:1 (v/v). The yellow band of pulvic dilactone devel-

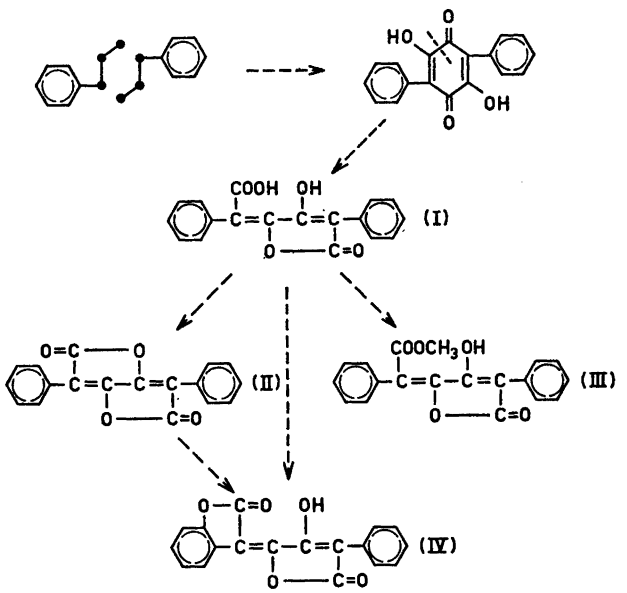

Fig. 1. Probable biosynthetic relationships among the pulvic acid derivatives found in the lichen fungus of *Cand. vit.* (I) pulvic acid, (II) pulvic dilactone, (III) vulpinic acid, (IV) calycin. At what stage in the biosynthetic sequence the additional hydroxyl group in calycin is introduced is yet uncertain.

oped, was scraped off the plate and together with the adsorbant material submitted to total combustion by the van Slyke-Folch method and its radioactivity determined. In the same manner zones of corresponding size above and below the pigment band were taken to detect any radioactivity present. A dark control was run with the petri dish wrapped in aluminium foil.

Determination of radioactivity of the pigment was carried out in the way described since scanning of TLC-plates was found to be unsuitable because of the relatively low activity present.

RESULTS AND DISCUSSION

The isolated lichen fungus of *Candellariella vitellina* was shown to produce the following four pulvic acid derivatives: pulvic acid, pulvic dilactone, calycin and vulpinic acid. Obviously in the biosynthesis of this group of "lichen substances" all the enzymes participating are provided by the fungal partner and the role of the algal symbiont in the natural lichen association is confined to the supply of necessary organic starting material through its photosynthetic activity.

As can be seen from Fig. 1 an intimate biosynthetic relationship exists between the four pigments identified. The formation of their common diphenylbutenolide skeleton has earlier been shown to proceed by condensation of two phenylpropanoid compounds to a symmetrical terphenyl type of structure

such as polyporic acid which is followed by ring fission.[10–12] The first product formed according to this scheme would be pulvic acid. Surprisingly vulpinic acid, which is not found in the corresponding composite plant, was formed by the fungal culture. Why the fungus introduces the additional methylation step leading to vulpinic acid is not understood. It may be that the algal symbiont suppresses the formation of the methylating enzyme of the fungal partner or competes with the fungus for activated methyl groups. It is also conceivable that the observed effect is simply the result of the different nutritional status of the malt extract medium. As a compliment to a previous study [9] on the rate of carbon flow from alga to fungus in the gyrophoric acid producing lichen *Umbilicaria pustulata* using $^{14}CO_2$, a similar experiment was devised with the lichen *Candellariella vitellina*. In these experiments of yet preliminary nature, varying amounts of ^{14}C-labelling appeared in the pigments such as pulvic dilactone from added $^{14}CO_2$ with no activity found in the dark controls. Since we now know that pulvic dilactone is entirely the product of the enzymic activity of the fungus the first appearance of radioactivity in this compound will serve as a measure of the rate of carbon flow. In a series of experiments planned the minimum time necessary to yield labelled pulvic acid derivatives will be determined.

Acknowledgements. This investigation was supported by a grant from the *Swedish Natural Science Research Council*. The author is indebted to prof. V. Ahmadjian, Clark University, Mass., for a culture of the isolated lichen fungus. Thanks are due to Dr. C. Fox from the same university for stimulating discussions and valuable help given. The skilful technical assistance of G. Andersson is acknowledged. Some of the reference material has kindly been provided by Drs. J. Santesson, University of Uppsala, and C. A. Wachtmeister, University of Stockholm.

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Received June 6, 1967.