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On the Biosynthesis of Lichen Substances

Part 1. The Depside Gyrophoric Acid

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A method for the *in vivo* study of the biosynthesis of lichen metabolites has been developed. By the administration of diethyl malonate-1-^{14}C, radioactive gyrophoric acid has been obtained with an incorporation of 2 % of the added radioactivity. Results from the chemical degradation of this lichen metabolite clearly indicate that the biosynthesis follows the pattern of "acetate-polymalonate" derived compounds known from other microorganisms.

About 15 000 diverse lichen species occur in nature, many of them forming unique and interesting chemical compounds. Of the lichen substances hitherto investigated, the structure of more than 80 different compounds of low molecular weight has been established. They can be classified into 13 groups on the basis of their chemical structure:[1] (a) *aliphatic* and *alicyclic* compounds: (1) carboxylic acids, (2) polyhydric alkohols, (3) triterpenoid, (4) sulfur containing compounds (b) *aromatic compounds:* (5) pulvic acid derivatives, (6) depsides, (7) depsidones, (8) depsones, (9) quinones, (10) xanthone, (11) chromanone, (12) dibenzofurans (*c*) *heterocyclic* compounds: (13) diketopiperazine.

Of the 13 groups listed, depsides, depsidones, depsones, dibenzofurans, chromanones and the pulvic acid series appear only in lichens. (Exceptions: the nidulins found in the mould *Aspergillus* and *m*-digallic acid found in higher plants). It is known that lichens are symbiotic partnerships of fungi and algae; successful attempts have been made to isolate the fungal part from the lichen. In a few cases these isolated lichen fungi formed lichen metabolites without being in association with their algal partners. Thus, the dibenzofurans usnic acid and didymic acid as well as pulvic anhydride have been identified.[2–4] However, there is evidence that depsides and depsidones cannot be produced by a pure culture of the fungal component, but are the unique products of a collaborative effort.[5]

There exists a bulk of literature in which the possible biogenetic mechanisms of lichen substance formation are discussed; however, no experimental evidence has so far been presented.[6-8] The reason for this might be the fact, that lichens are notoriously slow growers as are their isolated lichenized fungi. This paper deals with attempts made to achieve incorporation of radioactive precursors under various conditions. In an initial test the lichen was lightly packed in a glass cylinder and a water solution of the possible radioactive precursor was added dropwise from a separatory funnel; in such experiments, however, only a slight incorporation was observed. In an another experiment the lichens were added to Erlenmeyer flasks containing autoclaved Czapek-Dox or water medium, respectively, together with the radioactive precursor. They were then placed on the shaker normally used for the submerged cultivation of microorganisms. In this way a surprisingly high degree of incorporation of radioactive material with both media was obtained. Throughout the experiments *Umbilicaria pustulata (= U. papulosa)* was used as test organism and the radioactivity of the isolated gyrophoric acid measured. It was of general interest to show whether the biosynthetic pathway of lichen substances follows the established route of formation known from "acetate-polyacetate" derived compounds produced by other microorganisms. It has previously been shown that orsellinic acid [9] as well as penicillic acid [10] isolated from moulds are formed by condensation of one acetyl-CoA unit and three malonyl-CoA units. Gyrophoric acid represents a tridepside, which is composed of three molecules of orsellinic acid. This made it possible to compare the biosynthetic pathway leading to the formation of orsellinic acid units of different origin.

Umbilicaria pustulata was supplied with diethyl malonate-1-^{14}C by the method just described and gyrophoric acid was isolated from the dried lichen thallus. A part of the depside was hydrolysed with glacial acetic acid and the resulting orsellinic acid degraded. In the course of degradation the total radioactivity was determined. Decarboxylation yielded the radioactivity of the carboxyl group.

Kuhn-Ruth oxidation followed by Schmidt degradation of the acetic acid gave the radioactivity of the terminal methyl group and its adjacent carbon atom. In order to establish the specific radioactivity of each of the three orsellinic acid units making up the gyrophoric acid molecule, the remaining depside was methylated, yielding methyl gyrophorate tetramethyl ether. Hydrolysis gave three different derivatives of orsellinic acid: 6-methyl ether and 4,6-dimethyl ether of orsellinic acid and the methyl ester of 6-methyl ether of orsellinic acid.

EXPERIMENTAL

Culture conditions. 2 g of freshly collected young species of *Umbilicaria pustulata* were transferred to a 100 ml Erlenmeyer flask containing 40 ml of sterilized Czapek-Dox medium. After addition of 0.150 mC of $CH_2(^{14}COOC_2H_5)_2$ the lichen was shaken at 27° for 60 h on a shaker (250 rpm, 1 inch stroke).

Isolation. The lichen was removed by filtration, washed repeatedly with distilled water and air-dried. After excessive extraction with boiling acetone the extract was evaporated to dryness on a steam-bath. The weight of the residue was 30 mg. Chromatography on Whatman 1 paper of a representative sample of the isolated material was carried out in the following system:[11] ethyl methyl ketone:H_2O:diethylamine, 921:77:2. Scanning of the

paper in a strip-counter showed two major radioactive peaks: R_F 0.67 = gyrophoric acid and R_F 0.95 = unknown, probably lipid material. No peak appeared in the place of orsellinic acid with R_F = 0.35. The radioactive gyrophoric acid was then washed out with 650 mg of inactive gyrophoric acid and recrystallized from acetone to constant radioactivity. Yield: 400 mg, m.p. 220°.

Methyl gyrophorate tetramethyl ether. 230 mg of gyrophoric acid were methylated with excess of diazomethane prepared from *p*-tolylsulfonylmethylnitrosamide by dispersing the depside in an etheral solution of diazomethane and keeping it in a closed vessel for two days. The formed flocculent, white precipitate was filtered off and recrystallized from a mixture of acetone-methanol yielding 210 mg of methyl gyrophorate tetramethyl ether, m.p. 196–197°.[12]

4-Hydroxy-6-methoxy-2-methyl-benzoic acid methyl ester. 200 mg of the methyl gyrophorate methyl ester were treated with cold conc. H_2SO_4 for 5 min.[13] After dilution with distilled water, the solution was extracted with ether. The ether phase was shaken with a 0.5 M solution of $NaHCO_3$. The 4-hydroxy-6-methoxy-2-methyl-benzoic acid methyl ester remaining in the ether solution was obtained after evaporation of the ether. Recrystallisation from ligroin yielded 52 mg, m.p. 114°.[14]

4-Hydroxy-6-methoxy-2-methyl-benzoic acid. The $NaHCO_3$ solution from the above experiment was acidified with conc. HCl and extracted with ether. After evaporation of the ether on a steam-bath a residue was obtained containing both the 6-methyl and 4,6-dimethyl ethers of orsellinic acid. Treatment with 6 ml of boiling ether extracted the more soluble dimethyl ether of orsellinic acid from the mixture. The residue was dissolved in hot ethylacetate and precipitated with ligroin. Yield: 40 mg of 4-hydroxy-6-methoxy-2-methyl-benzoic acid, m.p. 175° (decomp.).

4,6-Dimethoxy-2-methyl-benzoic acid. The ether solution from the above experiment was evaporated to dryness on a steam bath. Recrystallisation of the residue from 20 % acetic acid yielded 36 mg of 4,6-dimethoxy-2-methyl-benzoic acid, m.p. 140°.

Orsellinic acid. 130 mg of gyrophoric acid were refluxed in 50 ml of glacial acetic acid for 30 min. After evaporation of the acetic acid *in vacuo* the residue was dissolved in ether and treated with $NaHCO_3$. The bicarbonate phase was separated, acidified with conc. HCl, and reextracted with ether. Recrystallisation of the ether extract from 30 % acetic acid yielded 95 mg of orsellinic acid, m.p. 176°.

Degradation. 5 mg each of radioactive gyrophoric acid, methyl gyrophorate tetramethyl ether, 4,6-dimethoxy-2-methyl-benzoic acid, 4-hydroxy-6-methoxy-2-methyl-benzoic acid, 4-hydroxy-6-methoxy-2-methylbenzoic acid methyl ester, and orsellinic acid were submitted to wetcombustion by the van Slyke-Folch method and the resulting carbon dioxide trapped as barium carbonate. 40 mg of orsellinic acid were decarboxylated to orcinol by heating in glycerol at 180°. 50 mg of the same orsellinic acid were oxidized as described by Kuhn-Roth and the evolved carbon dioxide collected for radioactive analysis. The acetic acid, isolated as sodium acetate after steam distillation, was degraded by a Schmidt reaction and the carboxyl group isolated as barium carbonate. The methylamine produced by the Schmidt reaction was submitted to wet combustion and the carbon dioxide trapped as barium carbonate.

Table 1. Malonate-1-^{14}C derived gyrophoric acid.

compound	cpm/mmole × 10^{-4}
Gyrophoric acid	331
Methyl gyrophorate tetramethyl ether	328
4,6-Dimethoxy-2-methyl-benzoic acid (S)	111
4-Hydroxy-6-methoxy-2-methyl-benzoic acid (Z)	110
4-Hydroxy-6-methoxy-2-methyl-benzoic acid methyl ester (A)	108
Orsellinic acid	110
Carboxyl group (o.a.)	34
Methyl group (o.a)	0
Carbon atom 2 adjacent to methyl group (o.a.)	6
Kuhn-Roth carbon dioxide (o.a.)	101

Radioactive analysis. In the degradation series measurements were performed in a liquid scintillation counter with the samples as barium carbonate suspended in a gel of Aerosil in a toluene solution of 2,5-diphenyloxazol.

RESULTS AND DISCUSSION

The values in Table 1 show that the biosynthetic pathway leading to the formation of the tridepside gyrophoric acid follows the general pattern of biosynthesis of "acetate-polymalonate" derived products known from other microorganisms. The orsellinic acid required for the tridepside is thus formed by condensation of one acetyl-CoA unit with three malonyl-CoA units as indicated in Fig. 2. The carbon atom derived from the carbonyl group of the acetyl-CoA shows only 18 % of radioactivity compared with that of a corresponding malonate derived position. The specific radioactivity of each of the three orsellinic acid units making up the tridepside is about equal. In view of this equal radioactivity in the three orsellinic acid units it is most likely that the same atoms are labelled to the same extent in each unit. Differences in labelling pattern of any individual unit would have been expected to lead to same differences in the observed specific activities.

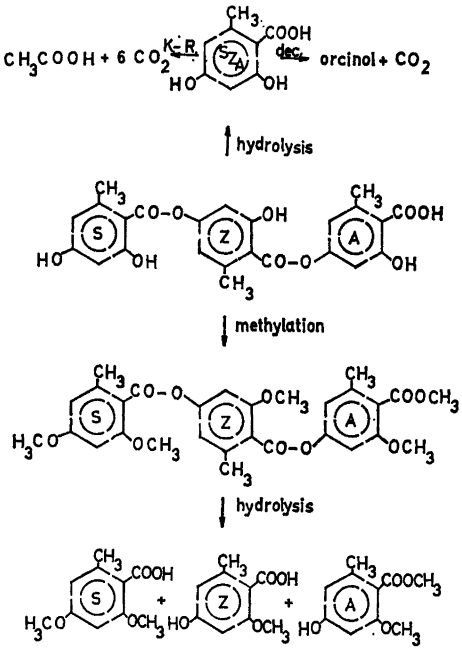

Fig. 1. Scheme of degradation of gyrophoric acid S = Säureglied (acid-part), Z = Zwischenglied (mid-part), A = Alkoholglied (alcohol-part).

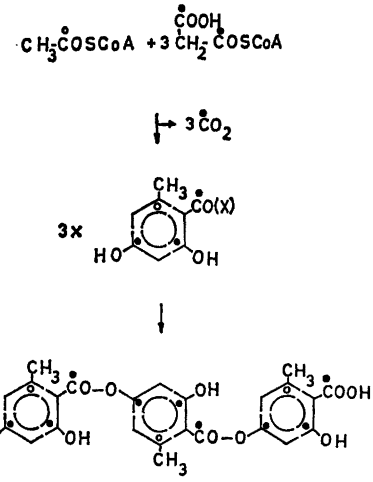

Fig. 2. Pathway postulated for the biosynthesis of gyrophoric acid based on the observed ^{14}C-distribution.

The depside as such can arise in two different ways: (*a*) the ester-linkage is formed between orsellinic acid molecules which are adjacent to each other and are located on the active site of the enzyme complex responsible for the de novo synthesis; (*b*) the orsellinic acid molecules are esterified at a site remote from their place of assembly, possibly by the algal partner.

A successful preparation of a cellfree extract capable of forming gyrophoric acid from added orsellinic acid but without the capacity to form orsellinic acid itself, would favor alternative *b*. As to the question whether the depside-linkage is formed from a free carboxyl group or an activated group of a CO-SCoA type, an experiment with $CH_3C^{18}OOH$ could give the answer. In the latter case the ^{18}O content in the carboxyl group would be of the same magnitude as in the hydroxyl groups.[15]

A variety of questions concerning the mechanism of biosynthesis of lichen substances awaits further investigations. To name one, the widespread phenomenon of phenol coupling among natural products offers in the antibiotic usnic acid a case of special interest with a probable combination of two types of bond formation: intermolecular carbon-carbon coupling followed by an intramolecular oxygen-carbon coupling.[16,17]

Although no attempt was made to sterilize the lichen in these experiments, nevertheless it is extremely unlikely that any adventitious contaminent would be responsible for the observed activity in gyrophoric acid, since this depside is known to be produced only by lichens. The observed incorporation of 2 % of the radioactive precursor into the lichen product gyrophoric acid is comparable to the incorporation obtained with moulds. In fact the incorporation amounts to 4 % if one considers that for each malonic acid molecule incorporated one molecule of carbon dioxide is liberated.

A successful incorporation of radioactive precursors into the depsidone physodic acid produced by the lichen *Parmelia physodes* has also been achieved and the usnic acid producing lichen species *Cladonia sylvatica* as well as the pulvic acid producing *Evernia vulpina* are under investigation. In view of this, the above described simple procedure for the *in vivo* study of the biosynthesis of lichen metabolites should provide a general tool for further investigations in this area.

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