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THE TERPHENYL QUINONE POLYPORIC ACID: PRODUCTION, ISOLATION AND CHARACTERIZATION

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The naturally-occurring terphenylquinones and biogenetically related pulvic acid derivatives are found mainly in lichens and in the fruiting bodies of fungi living in their natural environment. Of the former, only volucrisporin (I)[3] and phelbiarubrone (II)[4] have been produced in culture flasks under artificial conditions suitable for biosynthetic studies. In particular, polyporic acid (III) "is not produced by the fungal mycelium in artificial culture"[5]. Investigations of the biosynthesis of both classes of compounds have been hampered somewhat by the difficulty of inducing their production under conditions conducive to radioactive tracer studies in whole-cell systems; furthermore no viable cell-free preparations have been reported to our knowledge. However, by use of methods developed for the study of biosynthesis in slow-growing lichens, the "phenylpropanoid" origin of vulpinic acid (IV), pulvic acid dilactone (V) and calycin (VI) has been established.[6,7] Further, polyporic acid administered to lichens is incorporated directly into the latter two compounds,[7] confirming the metabolic relationship suggested by the co-existence of III and some pulvic acid derivatives as found in Sticta species.[8] Polyporic acid can be regarded biogenetically as the "simplest" terphenylquinone in that it requires only formal condensation of two phenylpyruvates for its formation. Subsequent fission of its quinone ring will lead to the various pulvinate derivatives as indicated in the scheme. Pursuing our interest in "fundamental" secondary metabolites (e.g. orsellinic acid, biosynthetically the simplest phenolic polyketide[9]) and those arising from oxidation of aromatic rings (e.g. penicillic acid[10] and stipitatonic acid[11]), we report on the first production of polyporic acid in artificial culture, a prerequisite for further biosynthetic studies.

A strain of Polyporus nidulans Fr. (CBS 422.48, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands) on malt agar was sub-cultured onto slants prepared as follows: standard Czapek Dox medium, 100 ml, was supplemented with glucose (40 g), malt extract (25 g), ferrous sulphate (0.01 g), zinc sulphate (0.01 g), copper sulphate (0.005 g) and agar (20 g), and diluted to one litre with water. A 5 ml portion was used for each slope. After 30 days those with the most prolific growth were homogenized and added (2-3 to each vessel) to 500 ml Erlenmeyer flasks containing a culture medium (130 ml) differing in composition from the slopes in containing only 2 g of agar per litre. After 30 days shaking at 27°C this medium was used as a secondary inoculum which was dispensed (10 ml per flask) into a second batch of identical culture fluid.

Black pigmentation was observed after three weeks shaking at 27°C in the dark. After six weeks mycelial production reached a maximum of 7 g dry weight per litre of medium. The mycelium was collected by centrifugation, air-dried and soxhlet-extracted with ethyl acetate for two days. Preparative thin layer chromatography of the concentrated extract afforded polyporic acid in a yield of 4.5-5.5 mg per gram of dry mycelium. The quinone could not be detected in the liquid medium.

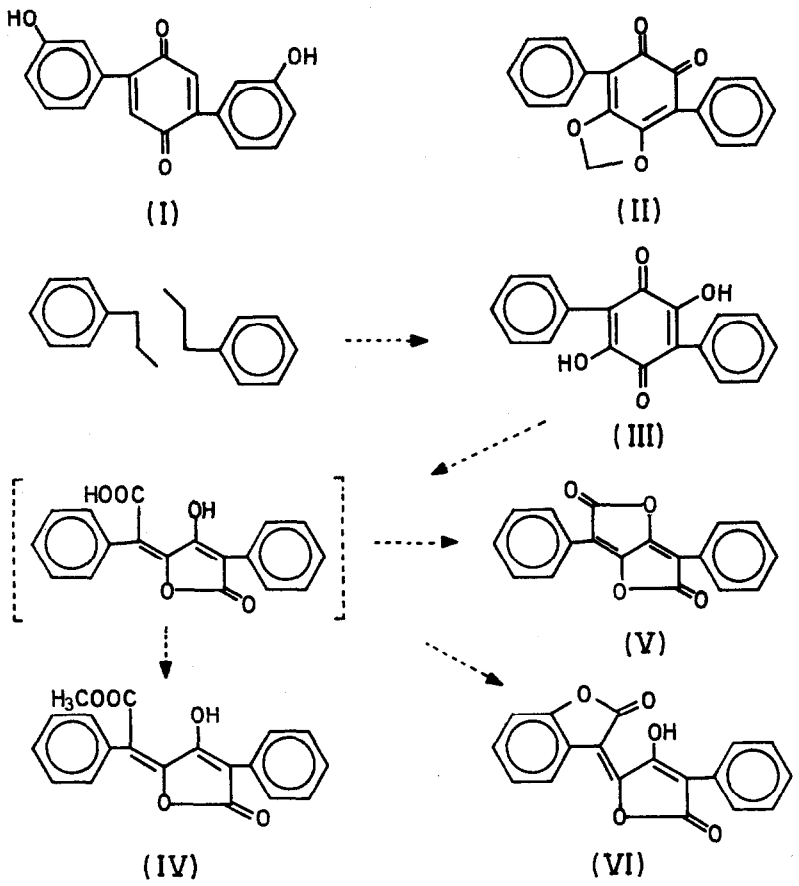
The identity of the purple metabolite (mixed m.p. 305°C, λ_{max} (methanol) 275, 320 (sh) and 530 nm, $E_{275}/E_{530} \sim 20$) was established by comparison with synthetic polyporic acid[12] and with authentic material produced by treatment of phlebiarubrone (II) with alkali.[4] For routine identification, comparative thin layer chromatography and ultraviolet and mass spectrometry were used.

Thin layer chromatograms were run on precoated silica gel plates (Merck), which had been sprayed with 0.2 M EDTA, in benzene - ethyl formate - formic acid 13:3:1 (by volume). Spots were visualized in short-wave ultraviolet light. This system separates polyporic acid (R_f 0.60, black) from phenylalanine (R_f 0.00), phenylpyruvic acid (R_f 0.39), phenyllactic acid (R_f 0.18) and phlebiarubrone (R_f 0.44, red).

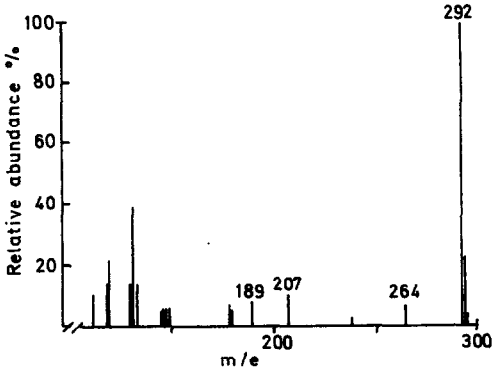
Mass spectra were obtained by using an LKB 9000 instrument. Samples were injected through the direct inlet. Polyporic acid was detected at a source temperature of $\sim$ 110°C. As is apparent from the figure, the spectrum of synthetic reference material closely resembles that obtained by injecting a fragment of dried, pigmented mycelium from a typical culture of P. nidulans.

The conditions described for polyporic acid production seem to be rather specific. Other media e.g. 4% maltose (resting or shake culture medium) or glucose-peptone, supported growth but no black pigmentation could be detected. Growth-rate is markedly reduced in standing culture or at reduced temperatures. Polyporus rutilans (Pers) Fries, known to produce this terphenylquinone as free-living fungus, did not form it in artificial culture. During an earlier study on tyrosine production in micro-organisms the difficulty of growing P. nidulans was also recognized;[13] no mention was made of possible formation of polyporic acid. By contrast, we have found phelbiarubrone (II) to be produced readily by the basidiomycete Phlebia strigozonata (Schw.) Lloyd under a variety of conditions. We therefore tried to induce the formation of polyporic acid, which is not normally a metabolite, by adding ethionine[14] to the media in the hope that C_1-metabolism would be inhibited so that the methylenedioxy ring could not be formed. However, under all conditions tried, no polyporic acid could be detected.

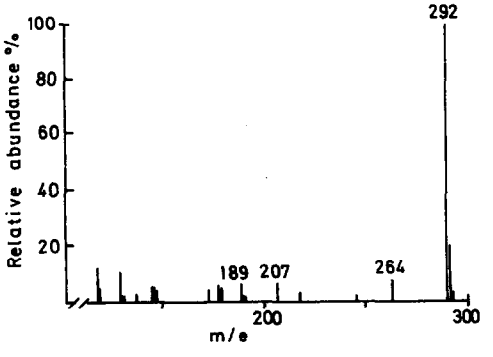
The use of "direct" mass spectrometry for detection of natural products in native state has been used previously in lichen studies[15] in which thallus fragments were introduced directly into the source. This technique has now been extended to fungi by injecting pieces of either whole fruiting body or mycelium. During this study it has been useful for preliminary screening of mycelium from cultures of Polyporus and Phlebia species for the presence of polyporic acid and phlebiarubrone, both of which give relatively abundant molecular ion peaks. In all cases evidence for these quinones could be confirmed by e.g. chromatography of an extract. This technique is very rapid and convenient and may well be of wide application.



Biosynthetic scheme relating polyporic acid to pulvic acid and related metabolites.



Main features of the mass spectrum of synthetic polyporic acid



Mass spectrum of a fragment of dry mycelium from a culture of Polyporus nidulans.

Much speculation has been made as to the biogenesis of polyporic acid and its involvement on the pathways to more complex molecules. Direct evidence should come to light now that the problem of its production in artificial culture has been overcome, although its high degree of symmetry poses severe restrictions on unambiguous interpretation of biosynthetic data. The recent discovery of pulvinamide in the lichen Pseudocyphellaria crocata[16] led to the suggestion of the involvement of phenylalanyl-pyridoxal phosphate Schiff's bases and phenylpyruvyl CoA in the biosynthesis of the quinone and the pulvinates. Some of these postulates should be testable by double-labelling experiments.

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