Naphthaquinone Biosynthesis in Moulds: the Mechanism for Formation of Javanicin

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1. The following compounds, added to the growth medium of *Fusarium javanicum*, were converted into labelled javanicin with the percentage incorporations noted in parentheses: [*Me*-¹⁴C]methionine (0.83); [1-¹⁴C]acetate (0.70); [2-¹⁴C]malonate (0.07). 2. Labelled samples of javanicin were degraded by Zeisel reaction, Kuhn-Roth oxidation and reaction with sodium hypoiodite; acetic acid obtained from the Kuhn-Roth reaction was further degraded by the Schmidt reaction. Labelled methionine was used only for the formation of the methoxyl group, and the remaining carbon atoms were derived by the acetate-plus-polymalonate pathway. The methyl group attached directly to the naphthaquinone ring is derived by the reduction of a carboxyl group. 3. The demonstration of this biosynthetic pathway supports the assignment of the methoxyl group at position 7.

During a search for antibiotic materials found in the culture fluids of moulds, Arnstein & Cook (1947) isolated a red pigment, C₁₅H₁₄O₆, named javanicin from *Fusarium javanicum*. The structure of javanicin was shown to be (I) (see Scheme 1) with the position of the methoxyl group in doubt. From the same culture fluids, a second compound, oxyjavanicin, C₁₅H₁₄O₇, was also isolated. Ruelius & Gauhe (1950, 1951) isolated a material named fusarubin from strains of the same organism, and showed that this material had structure (II) and was identical with oxyjavanicin. The material solanione, which was isolated from *Fusarium solani* by Weiss & Nord (1949), is presumed to be identical with javanicin. In contrast with the relatively large number of benzoquinones and anthraquinones isolated from fungal sources, only a few simple naphthaquinones (flaviolin, 6-methyl-1,4-naphthaquinone, mollisin and bostrycoidin) have so far been identified in moulds. It was therefore decided to investigate the mechanisms used for the biosynthesis of a representative naphthaquinone, and for this study javanicin was chosen. The original of the methyl group was of especial interest; although methyl groups of fungal components are frequently derived from C₁ compounds such as formate and the methyl group of methionine, it was also possible that in javanicin the methyl group was actually derived from the carboxyl group of acetate or malonate (Ehrensward & Gatenbeck, 1960; Thomas, 1961). Further determination of the biosynthetic pathway might

shed light on the position of the methoxyl group, since, if javanicin were found to be derived by the acetate-plus-polymalonate pathway, the methoxyl group would most likely be found on a carbon atom originally carrying a carbonyl function.

EXPERIMENTAL

Isolation of javanicin. For the production of javanicin, *Fusarium javanicum*, IMI 29817, obtained from the Commonwealth Mycological Institute, Kew, Surrey, proved to be the best of a number of organisms that were tested. Cultures were grown as surface pads at 29° on a medium of the following composition: NaNO₃, 2.0 g.; KH₂PO₄, 1.0 g.; KCl, 0.5 g.; MgSO₄·7H₂O, 0.5 g.; FeSO₄·7H₂O, 0.01 g.; Bactotryptone (Difco), 5.0 g.; glucose, 40.0 g.; agar-agar (Difco), 1.0 g.; water to 1 l. The addition of agar was helpful in preventing the mycelium from becoming waterlogged. For large-scale production of 'carrier' material, 500 ml. portions of medium were used in 2 l. Fernbach flasks. In experiments with isotopically labelled substrates, 150 ml. portions of the medium were used in 300 ml. Fernbach flasks. In these cases a mycelial pad was pregrown on the standard medium for 8 or 10 days. The precursor was then added below the pad, after solution in a minimum volume of sterile water. The utilization of the added precursors is shown in Table 1.

After growth, usually for 13 or 14 days, javanicin was isolated as follows. After filtration of the culture medium, the mycelial pads were washed with a little water; the combined filtrate and washings were made alkaline with *n*-NaOH until a deep-purple colour persisted. A vigorous stream of air was then bubbled through the alkaline solution

The numbering system used to refer to individual carbon

Table 3. *Distribution of radioactivity in labelled samples of javanicin*

Experimental details are given in the text.

Carbon atom number	Distribution of radioactivity (% of total)	
	Javanicin from [1- ¹⁴ C]acetate	Javanicin from [2- ¹⁴ C]malonate
3	2.5	11.5
11	11.1	
13	13.9	
14		14.2
Theoretical value per carbon atom if seven atoms in javanicin are labelled equally	14.3	14.3
Observed value per carbon atom if five atoms in Kuhn-Roth CO ₂ are labelled equally	14.9	15.4

atoms of javanicin, and the degradation reactions employed, are shown in Scheme 1. To obtain the total radioactivity of javanicin samples and of other compounds in these degradations, the wet-combustion technique of Van Slyke & Folch (1940) was employed. All determinations of radioactivity were made on BaCO₃ samples in a liquid-scintillation counter, by using 10–15 mg. accurately weighed out and suspended in 10 ml. of 0.5% diphenyloxazole in toluene with the aid of 400 mg. of Aerosil gel. Degradations by the Zeisel method were carried out as described by Bentley & Lavate (1964), and the evolved methyl iodide was trapped as tetramethylammonium iodide. Kuhn–Roth oxidations on 15–45 mg. portions of javanicin were carried out with 7–15 ml. of the oxidizing mixture of Eisenbraun, McElvain & Aycock (1954); a stream of N₂ was passed through the reaction vessel and the evolved CO₂ was collected as BaCO₃. Acetic acid was recovered by steam-distillation, the fractions being titrated with 0.01 N-NaOH to pH 8.7. The sodium acetate recovered on evaporation was degraded by the Schmidt reaction, as described by Phares (1951). Iodoform was obtained by reaction with sodium hypoiodite, essentially as described by Arnstein & Cook (1947).

The specific activities of the javanicin samples actually used for degradation, and of the various degradation products, are shown in Table 2. In Table 3 are shown the percentages of total activity present in those carbon atoms actually isolated by the degradations.

RESULTS AND DISCUSSION

The first experiment, carried out with labelled methionine, immediately indicated that the C-methyl group (C-11) was unlikely to be derived from a C₁ unit. As shown in Table 2, essentially all of the activity (97%) of the javanicin obtained after the addition of [Me-¹⁴C]methionine to *F. javanicum* was present in the methoxyl group (C-15). The acetic acid obtained on Kuhn–Roth oxidation of this javanicin contained only 1.09% of the activity.

The specific activities of the carbon dioxide and acetic acid obtained on Kuhn–Roth oxidation of labelled javanicin biosynthesized in the presence of [1-¹⁴C]acetate were consistent with the presence of seven labelled atoms in the molecule (see Table 3). Schmidt degradation of this Kuhn–Roth acetic acid indicated that the activity was divided roughly equally between the carboxyl (56.8%) and methyl (43.2%) groups. Hence radioactivity was present in C-11 or C-14 or both, as well as in C-3 or C-13 or both. The iodoform reaction on this javanicin sample showed that only a very small proportion of the total activity (2.5%) was present in C-14. It was therefore reasonable to conclude that the Kuhn–Roth acetic acid activity originated almost exclusively from C-11 and C-13. The small amount of activity apparently present in C-14, as indicated by the iodoform reaction, is most probably due to the formation of small amounts of iodoform from C-11. The validity of this interpretation was strengthened by the finding that a compound such as 5,6-dimethoxy-2-methylbenzoquinone, containing a similar methyl group attached to a quinonoid system (but no acetyl group), also gave small amounts of iodoform on treatment with sodium hypoiodite. These results clearly establish the 'polyacetate' origin of the naphthaquinone ring of javanicin and provide proof that the C-methyl group (C-11) was derived from the carboxyl group of acetate.

Many of the compounds derived by the polyacetate pathway have now been shown to be formed by condensation of one molecule of acetyl-CoA with *n* molecules of malonyl-CoA ('acetate-plus-polymalonate pathway'); the early work has been reviewed by Bentley (1962). When an experiment with labelled malonate was carried out in *F. javanicum*, the observed incorporation (see Table 1) was not as marked as might have been expected. Further, on degradation of the labelled javanicin from [2-¹⁴C]malonate, it was found that activity was present in both C-3 and C-14, and in almost equal amounts. If javanicin is formed by the acetate-plus-polymalonate pathway, the 'starter' unit of acetyl-CoA must give rise to C-14 and C-13, and malonate should not contribute significantly to the labelling of these positions. Since considerable activity was actually present in C-14, it seems likely that an extensive conversion of malonate into acetate occurred in these experiments. The labelled malonate had been left in contact with the culture for a total of 5 days, because the rate of javanicin production was low, and since, in many other cases, the expected lack of incorporation of malonate activity into the 'starter' acetate unit has been observed in experiments of comparable duration. Short-term experiments with highly active samples of malonic acid might provide the expected result.