The Biosynthesis of Flavipin

I. Incorporation of Acetate and Methionine

GÖSTA PETTERSSON

Institute of Biochemistry, University of Lund, Lund, Sweden

Flavipin (I), a metabolic product of *Aspergillus flavipes*, has been biosynthesized in the presence of $1\text{-}^{14}C$-acetate, $2\text{-}^{14}C$-acetate, and $^{14}CH_3\text{-L-}$methionine. The distribution of radioactivity incorporated into flavipin was determined by chemical degradation. The results obtained clearly indicate that the aromatic nucleus as well as the two formyl groups of flavipin are derived from acetate, in consistence with the acetate-polymalonate theory, while the nuclear methyl group of flavipin was found to be an "introduced" C_1-unit.

The culture filtrates of certain strains of *Aspergillus flavipes* grown on Raulin—Thom solution, and of *A. terreus* grown on Czapek—Dox solution, give an intense blue-black ferric reaction and an immediate heavy red precipitate with Brady's reagent (2,4-dinitrophenylhydrazine in aqueous 2 M hydrochloric acid). The metabolic product, called flavipin, responsible for these reactions was isolated by Raistrick and Rudman, who also determined its structure as 1,2-diformyl-4,5,6-trihydroxy-3-methylbenzene (I).[1]

The biosynthesis of flavipin is of interest in its relation to the phenolic mould metabolites gallic acid, pyrogallol, and barnol, in that they each contain three vicinal hydroxyl groups. Barnol (II), which is produced by a certain strain of *Penicillium baarnense*,[2] has recently been shown to be formed by the acetate(-polymalonate) route.[3] On the other hand, Bassett and Tanenbaum have suggested that pyrogallol, isolated from *P. patulum*, is formed by the shikimic acid route,[4] possibly *via* gallic acid which is known to arise from sugar or from tyrosine in *Phycomyces blakesleeanus*.[5] Formally, flavipin may be derived from either of these two pathways toward aromatization.

Flavipin is also a substituted *o*-phthaldialdehyde, a class of compounds to which three other fungal metabolites can be referred; these are cyclopaldic acid (III) from *Penicillium cyclopium*,[6] gladiolic acid (IV) from *P. gladioli*,[7] and quadrilineatin (V) from *A. quadrilineatus*.[8] The acetate origin of cyclopaldic acid has been established by radioactive tracer techniques,[9] and the close structural relationship between cyclopaldic acid and flavipin (see Fig. 1)
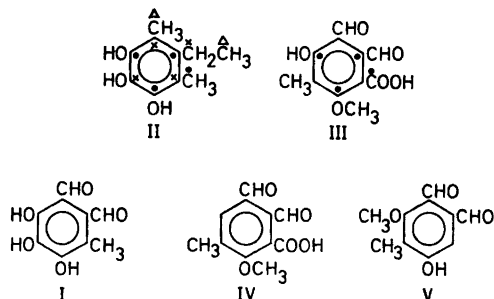
Fig. 1. Experimentally established incorporation of acetate (methyl group, ×; carboxyl group, ●) and methionine (S-methyl group, △) into barnol (II) and cyclopaldic acid (III). The structures of flavipin (I), gladiolic acid (IV), and quadrilineatin (V) are shown for comparison.

makes it probable that the latter compound, also, would be formed by the acetate-polymalonate pathway.

In the present work *A. flavipes* has been grown in the presence of ^{14}C-labelled acetate and methionine. The distribution of the radioactivity incorporated into flavipin was determined by chemical degradation, in order to establish the biogenetic origin of the compound, particularly with reference to the nuclear methyl and formyl groups.

EXPERIMENTAL

Culture conditions. Aspergillus flavipes (Bainier and Sartory) Thom and Church, L.S.H.T.M. No. S.M. 884, obtained from the Commonwealth Mycological Institute, Kew, Surrey, England, was used throughout this work. The mould was grown as submerged cultures in 500 ml Erlenmeyer flasks holding 150 ml portions of a Raulin—Thom medium according to Raistrick and Rudman,[1] on a rotary shaker (300 rpm) at 25°. At 5 days after inoculation from malt agar slope cultures, 1-^{14}C-acetate (0.5 mC), 2-^{14}C-acetate (0.5 mC) and ^{14}CH$_3$-L-methionine (0.1 mC) were added to selected flasks; the radioactive precursors were obtained from the Radiochemical Centre, Amersham, England. After a further three days of growth flavipin was isolated from the culture filtrates as previously described.[1]
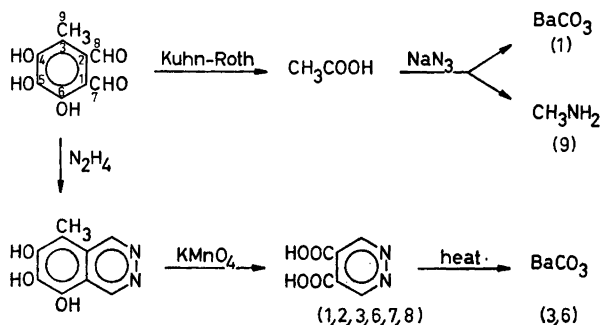

Fig. 2. Chemical degradation of flavipin. Numbers within brackets indicate carbon atoms isolated by the reactions.

· *Degradation reactions.* The radioactive flavipin (5—15 mg) isolated from the culture filtrates was diluted with carrier flavipin (80—120 mg) and the whole was dissolved in acetone (50 ml). The solution was concentrated at 40° to about 15 ml, when, on cooling, pure flavipin crystallized in yellow leaflets, m.p. 233° (decomp.).

The numbering system used to refer to individual carbon atoms of flavipin, and the degradation reactions employed, are shown in Fig. 2. To obtain the total radioactivity of flavipin samples, and of other compounds in these degradations, the wet combustion technique of van Slyke and Folch was used.[18] All determinations of radioactivity were made in a liquid scintillation counter on barium carbonate samples (20 mg) suspended in 10 ml of 0.5 % diphenyloxazole in toluene with the aid of 400 mg Aerosil gel. Kuhn—Roth oxidations on from 30 to 50 mg portions of flavipin were carried out as described by Eisenbraun et al.;[11] a stream of nitrogen gas was passed through the reaction vessel and the evolved carbon dioxide was collected as barium carbonate. Acetic acid was recovered by steam distillation, the fractions being titrated with dilute sodium hydroxide to pH 8.7. The sodium acetate recovered on evaporation was degraded by the Schmidt reaction as described by Phares.[12]

Pyridazine-4,5-dicarboxylic acid was obtained from flavipin as described by Raistrick and Rudman.[1] Treatment of flavipin (80 mg) with hydrazine hydrate (0.1 ml) in 50 % aqueous ethanol yielded the phthalazine derivative (65 mg), which was dissolved in 0.2 M sodium hydroxide (5 ml) and heated on the steam-bath. Potassium permanganate solution (2.5 %) was added drop by drop until its colour persisted (9 ml). A small amount of ethanol was added, the solution filtered, and the filtrate evaporated under reduced pressure. The solid residue was treated with 1 M hydrochloric acid (4 ml), when the pyridazine-dicarboxylic acid separated. After recrystallization from water it had a m.p. of 208°—210° (25—35 mg). This acid (20 mg) was decarboxylated by heating it for 15 min with copper chromite (100 mg) in freshly distilled quinoline (5 ml) under nitrogen, when an almost quantitative amount of carbon dioxide (collected as barium carbonate) was evolved.

RESULTS AND DISCUSSION

The results of the radioactive tracer experiments, which are summarized in Table 1, establish a fundamental role for acetate in flavipin biosynthesis.

The specific activities of the carbon dioxide and acetic acid obtained on Kuhn—Roth oxidation of labelled flavipin, biosynthesised in the presence of 2-^{14}C-acetate, were consistent with the presence of four labelled atoms in the molecule (see Table 2). Location of isotope at C-3 (23.6 % of the total activity) was established by Schmidt degradation of this Kuhn—Roth acetic acid (the nuclear methyl group, C-9, was found to be essentially non-radioactive), and by inference C-1, C-5, and C-8 may also be considered to be labelled by 2-^{14}C-acetate. Confirmatively, one of the labelled atoms was found to be located at C-4 or C-5, since the pyradazinedicarboxylic acid contained

Table 1. ^{14}C-Distribution in flavipin derived from labelled acetate and methionine. The table gives the location of isotope at different carbon atoms (numbered as in Fig. 2) in percentages of the total radioactivity.

Carbon atoms	2-^{14}C-acetate	1-^{14}C-acetate	^{14}CH$_3$-L-methionine
3	24	1	1
9	1	1	93
6	0	22	
4+5	26	27	5
1+2+7+8	52	51	

Table 2. ^{14}C-Distribution in flavipin derived from 2-^{14}C-acetate (0.5 % incorporation).

Material	Carbon atoms isolated	Number of carbon atoms	Activity spec.*	Activity total	Number of labelled found	Number of atoms calc.
Flavipin	all	9	359	3231	4	4
Kuhn—Roth oxidation carbon dioxide	1,2,4,5, 6,7,8	7	350	2450	3.04	3
Kuhn—Roth oxidation methyl group of acetic acid	9	1	21	21	0.03	0
Kuhn—Roth oxidation carboxyl group of acetic acid	3	1	764	764	0.95	1
Pyridazinedicarboxylic acid	1,2,3, 6,7,8	6	397	2382	2.95	3
Carboxyl groups of pyridazinedicarboxylic acid	3,6	2	356	712	0.88	1

* Counts per minute and mg $BaCO_3$.

approximately three fourths of the total activity. Decarboxylation of the latter compound, further, provided evidence that C-6 was non-radioactive.

Similarly, the radioactive assay data were in quantitative agreement with the presence of four labelled atoms, located as shown in Fig. 3, in flavipin samples derived from 1-^{14}C-acetate (see Table 3).

The methyl group of flavipin was found to be derived from the C_1-pool. This is evident from the data given in Table 4; essentially all of the radioactivity incorporated into flavipin from [14]CH_3-L-methionine was located at C-9. It is of interest that neither of the formyl groups was significantly labelled from this precursor, since one of the formyl groups of cyclopaldic acid seems to be derived from the C_1-pool.[9]

Table 3. ^{14}C-Distribution in flavipin derived from 1-^{14}C-acetate (0.2 % incorporation).

Material	Carbon atoms isolated	Number of carbon atoms	Activity spec.*	Activity total	Number of labelled found	Number of atoms calc.
Flavipin	all	9	153	1377	4	4
Kuhn—Roth oxidation carbon dioxide	1,2,4, 5,6,7,8	7	201	1407	4.08	4
Kuhn—Roth oxidation methyl group of acetic acid	9	1	15	15	0.04	0
Kuhn—Roth oxidation carboxyl group of acetic acid	3	1	11	11	0.03	0
Pyridazinedicarboxylic acid	1,2,3, 6,7,8	6	168	1008	2.93	3
Carboxyl groups of pyridazinedicarboxylic acid	3,6	2	156	312	0.91	1

* Counts per minute and mg $BaCO_3$.

Table 4. ^{14}C-Distribution in flavipin derived from [14]CH$_3$-L-methionine (2.7 % incorporation).

Material	Carbon atoms isolated	Number of carbon atoms	Activity spec.	total	Number of labelled atoms found	atoms calc.
Flavipin	all	9	1 650	14 850	1	1
Kuhn—Roth oxidation carbon dioxide	1,2,4, 5,6,7,8	7	108	756	0.05	0
Kuhn—Roth oxidation methyl group of acetic acid	9	1	13 760	13 760	0.93	1
Kuhn—Roth oxidation carboxyl group of acetic acid	3	1	126	126	0.01	0

* Counts per minute and mg BaCO$_3$.

Even though no experiments with 2-^{14}C-acetate were undertaken, Birch *et al.* suggested that the other formyl group (the one adjacent to the carboxyl group) of cyclopaldic acid was derived from the methyl group of acetate.[9] The formyl group of gentisic aldehyde, isolated from *Penicillium patulum*,[13] might also be expected to have this origin;[4] the carboxyl group of gentisic acid (produced by the same mould) has recently been shown to arise by oxidation of an acetate-derived CH$_3$. The present work, however, provides the first experimental demonstration of the oxidation of an acetate-derived CH$_3$ to CHO.

The present work with flavipin, further, adds one more to the increasing number of fungal metabolites that are derived from acetate-polymalonate condensations with reduction of the terminal carboxyl group. The formation of a nuclear methyl group by this process was first observed in barnol;[3] the close biogenetic relationship between this compound (II in Fig. 1) and flavipin (I in Fig. 3) is most striking. In flavipin, however, the terminal carboxyl
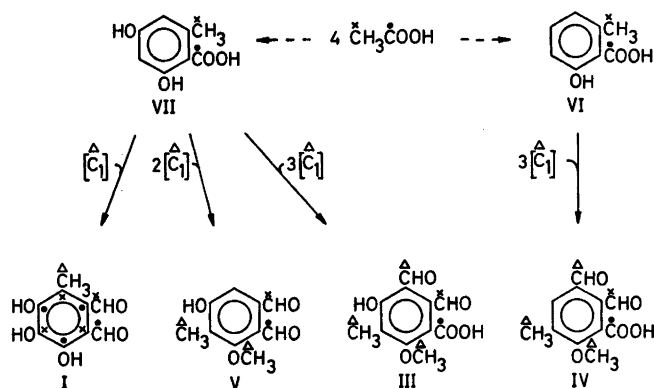

Fig. 3. Proposed biogenetic scheme for the different *o*-phthaldialdehyde derivatives isolated from moulds.

group appears as a formyl group, which has previously been observed in auroglaucin (from *A. novus*), only.[15]

The results with flavipin also indicate that the *o*-phthaldialdehyde derivative quadrilineatin (V) is formed in a similar manner, as shown in Fig. 3. The two formyl groups of the latter compound are thus probably derived from acetate, while the nuclear methyl group would be an "introduced" C_1-unit. Gladiolic acid (IV), on the other hand, seems to be biogenetically related to cyclopaldic acid (III) in the same way as 6-methylsalicylic acid (VI) is related to orsellinic acid (VII); the formyl group adjacent to the carboxyl group would be expected to be derived from the methyl group of acetate, the carbon of the second formyl group being provided from the C_1-pool. Theoretically, it seems likely that 6-methylsalicylic acid is a precursor of gladiolic acid, and orsellinic acid of the other fungal *o*-phthaldialdehydes, as indicated in Fig. 3. Experimental studies on the possible role of orsellinic acid in flavipin biosynthesis are in progress.

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