Biosynthesis of Flaviolin and 5,8-Dihydroxy-2,7-dimethoxy-1,4-naphthoquinone†

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ABSTRACT: Tracer experiments indicate a polyketide origin for the production of flaviolin (2,5,7-trihydroxy-1,4-naphthoquinone) by *Aspergillus niger* and 2,7-dimethoxynaphthazarin (5,8-dihydroxy-2,7-dimethoxy-1,4-naphthoquinone) by *Streptomyces* no. 12396. With the *Streptomycete*, a "solid state fermentation" technology was used for the incorporation studies. Radioactivity from shikimic acid was effectively incorporated into flaviolin; this conversion, however, proceeded by way of acetic acid. The latter stages of biosynthesis of 2,7-dimethoxynaphthazarin by the *Streptomycete* were shown to be as follows: flaviolin → mompain → 2,7-dimethoxynaphthazarin.

In 1953, a naphthoquinone pigment was isolated from the culture medium of an organism which had previously been studied because of its ability to produce citric acid from glucose (Astill and Roberts, 1953). About 2 g of the crystalline pigment was obtained from some 800 l. of culture medium. The organism at that time was named *Aspergillus citricus*, but it has subsequently been reclassified as *Aspergillus niger*. Because of the remarkable color properties in aqueous solution, the pigment was named flaviolin; it was quickly identified as a trihydroxy-1,4-naphthoquinone. Since oxidation of the triacetyl derivative yielded 3,5-dihydroxyphthalic acid, flaviolin was either 2(or 3),5,7-trihydroxy-1,4-naphthoquinone.

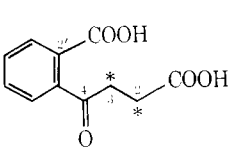
The distribution of the oxygen atoms in flaviolin was consistent with a biosynthetic origin by way of the "polyacetate" pathway (Birch and Donovan, 1954). If this were correct, it followed that the unplaced oxygen in flaviolin was at the 2 position. Birch and Donovan carried out a synthesis of the trimethyl ether of 2,5,7-trihydroxy-1,4-naphthoquinone and found this compound was identical with the trimethyl ether derived from flaviolin itself. They concluded "the acetate hypothesis can probably be used like the isoprene rule in favorable cases to lessen the labor of structure determination by indicating the more probable of alternate structures". Almost simultaneously, Davies et al. also synthesized the trimethyl derivative of flaviolin and, as well, the 3,5,7-trimethyl compound (Davies et al., 1954, 1955); flaviolin, itself, has also been synthesized (Bycroft and Roberts, 1962).

At a later date, two related naphthoquinone pigments were isolated from a nonsporulating *Streptomycete* no. 12396 (Gerber and Wieclawek, 1966). One of these was the 2,7-dimethyl ether of flaviolin (2,7-dimethoxy-5-hydroxy-1,4-naphthoquinone); the other was a naphthazarin (5,8-dihydroxy-2,7-dimethoxy-1,4-naphthoquinone). In 1967 the nonmethylated parent of the latter naphthazarin (2,5,7,8-tetrahydroxy-1,4-naphthoquinone), was isolated from the fungus *Helicobasidium mompa*, and was named mompain (Natori et al., 1967). The Japanese authors assumed that mompain was derived by an acetate pathway (now recognized as an acetate–polymalonate pathway) but attempts to verify this by incorporation experiments using [2-^{14}C]acetate and malonate were inconclusive.

Apparently impressed by the success of the acetate theory in predicting the correct structure for flaviolin, several authors of review articles and books have assigned an acetate–malonate origin to flaviolin although there has never been any experimental verification for this. Furthermore, the usual polyacetate diagnostic features (methyl and carboxyl groups) are completely missing in flaviolin and the dimethoxynaphthazarin from the *Streptomycete*. With the elucidation of the shikimate–ketoglutarate pathway to menaquinones and other plant naphthoquinones, and with other pathways also available, there did not seem any a priori reason to regard flaviolin as an acetate derived metabolite. We have, therefore, undertaken a study of the biosynthesis of flaviolin in *Aspergillus niger* and the dimethoxynaphthazarin in *Streptomycete* no. 12396.

Experimental Section

Labeled Substrates. [2,3-$^{14}C_2$]-4-(2′-Carboxyphenyl)-4-oxobutyrate (**1**) was prepared by the following modification of



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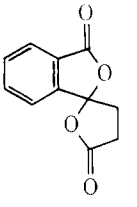

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the method of Roser (1884). In an 18 × 150 mm test tube, [2,3-$^{14}C_2$]succinic acid (sp act. 41 mCi/mmol, 1.44 mg, total activity, 500 μCi) was diluted with unlabeled succinic acid (57.2 mg). Phthalic anhydride (134.2 mg) was added and the mixture heated in an oil bath at 198–200° until a clear liquid had formed. At this time the test tube was swirled to ensure mixing and 15 mg of anhydrous K_2CO_3 was added in one portion. After 20 min the tube was removed from the oil bath and cooled, and the contents extracted with 4 × 4 ml portions of boiling water. The combined water extracts were taken to dryness under reduced pressure and the residue extracted into 5 × 4 ml portions of boiling chloroform. The chloroform extracts were dried over

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$MgSO_4$, reduced in volume, and carefully applied to one 20 × 20 cm preparative silica gel G plate. Following development with chloroform, the area corresponding to the spirolactone (**2**) was collected and extracted with boiling acetone. The white residue obtained on evaporation of the acetone extract was crystallized from 10 ml of boiling water to give colorless needles: yield, 30%; mp 117–117.5° (lit. mp 120°; sp act. 0.79 μCi/μmol). Purity was checked by thin-layer chromatography (TLC)–strip scanning in chloroform and benzene–ethyl acetate–acetic acid (150:75:2).

Prior to tracer experiments, the spirolactone was converted to the disodium *o*-succinylbenzoate by solution in 12% NaOH. All other tracers were dissolved in <5-ml volumes of sterile water before addition to the cultures.

Culture of Organisms. Aspergillus niger, obtained from the Commonwealth Mycological Institute (CMI no. 15954), was maintained on Czapek-Dox agar slants (Difco) with growth at 28°. For pigment production, surface cultures were incubated at 28° on a modified Czapek-Dox medium of the following composition: 2.25 g of NH_4NO_3, 0.30 g of KH_2PO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 2.00 g of yeast extract (Difco), and 60.00 g of D-glucose (anhydrous) per liter of deionized water.

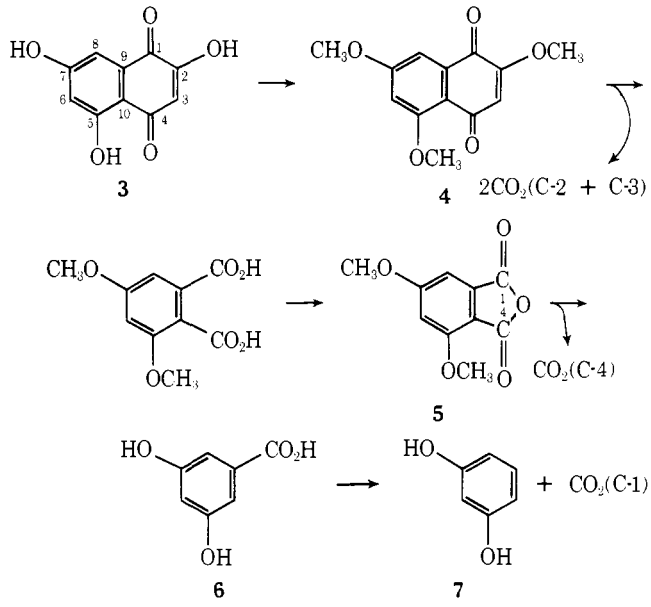
The *Streptomycete* no. 12396, which was kindly provided by Dr. N. N. Gerber, was maintained on slants of the following composition: 50.0 g of D-glucose (anhydrous), 10.0 g of yeast extract (Difco), and 20.0 g of Bacto agar (Difco) per liter of deionized water. For pigment production, the organism was grown in 250-ml erlenmeyer flasks (containing 50 ml of the above medium with the deletion of the agar) for 2 days at 28° as a shake culture. The "seed" flask was then used to inoculate a mixture of 60 ml of sterile H_2O and 250 g of "White River" rice (Fluffy White River Enriched Rice, Riviana Food, Inc.) suspended in 2800-ml Fernbach flasks. The inoculated flasks were shaken on a New Brunswick Scientific Co. Model G-10 reciprocating shaker operating at 275 rpm for the duration of the experiment. After 22 hr the rice grains had assumed a pinkish coloration, and by 72 hr the color was blue-purple. By 120 hr the grains had the color characteristic of new blue jeans. Apart from the color change, there was no obvious alteration of the structure of the rice grains, and no obvious growth of the microorganism externally. Apparentally the mycelial threads penetrate into the rice grains. We have not been able to devise a method to determine the weight of microorganism produced.

Isolation of Metabolites. Flaviolin (**3**) was obtained from the filtered acidified culture fluid of *A. niger* by continuous ether extraction of the medium for 48 hr. Concentration of the ether extract yielded a dark red-brown gum which, after chromatography on Unisil (200–325 mesh) in benzene–ethyl acetate–acetic acid (150:75:2), gave pure flaviolin: yield, 15–25 mg/Fernbach flask; mp 260° with decomposition; infrared (ir) (KBr) 3200, 1675, 1650, 1630, 1590, 1400, 1240, 1180 cm^{-1}; ultraviolet (uv) (C_2H_5OH) 215, 264, 309, 385, and 451 nm (log ϵ 4.39, 4.11, 3.88, 3.32, and 3.35); *m/e* 206 (M^+ and base) > 137 > 178 > 69 > 150 > 180.

2,7-Dimethoxynaphthazarin (**8a** or **8b**) was obtained by extraction of the acidified rice kernels with chloroform. Evaporation of the chloroform and washing of the residue with hexane gave a dark red brown partly crystalline residue which after crystallization from boiling ethanol gave pure 2,7-dimethoxynaphthazarin: yield, 300–350 mg per flask; mp 274–275°; ir (KBr) 3075, 2960, 2850, 1610, 1575, 1480, 1435, 1280, 1230, 1095 cm^{-}; uv (C_2H_5OH) 228, 283, 308, 476, 509, and 545 nm (log ϵ 4.39, 3.71, 3.75, 3.71, 3.75, and 3.55); *m/e* 250 (M^+ and base) > 220 > 232 > 69 > 207 > 151 > 179.

Degradation Experiments. To determine the distribution of radioactivity at the various carbon atoms of flaviolin, the naphthoquinone was first converted to its trimethyl ether (**4**). The reaction sequence outlined in Scheme I allows the isolation of the carbon atoms corresponding to C-2 and C-3 collectively, and C-1 and C-4 individually, of the parent flaviolin molecule. These centers are converted to CO_2 during the degradation reactions and trapped and counted as $BaCO_3$.

Scheme I: Chemical Degradation of Flaviolin (Numbers Refer to the Atoms in Flaviolin).



(A) CONVERSION OF FLAVIOLIN TO TRI-*O*-METHYLFLAVIOLIN (**4**). Flaviolin, 18.6 mg, was dissolved in 5 ml of anhydrous acetone. After addition of 1 g of anhydrous potassium carbonate and 0.8 ml of dimethyl sulfate, the mixture was refluxed with stirring for 2.5–3 hr. The solution was cooled and 4 ml of water was added. Stirring was then continued for an additional 1–1.5 hr to hydrolyze any remaining dimethyl sulfate. An additional 20 ml of water was added and the solution extracted with 3 × 10 ml portions of chloroform. The combined extracts were dried over anhydrous magnesium sulfate, filtered, and taken to dryness on a rotary evaporator. The crude trimethoxyquinone was chromatographed on a 1.0 × 30 cm column of Unisil (200–325 mesh, 20 g) in benzene–ethyl acetate–acetic acid (150:75:2, v/v). Fifty-milliliter fractions were collected. The first four fractions were discarded, and fractions 5–7, containing nearly all the 2,5,7-trimethoxy-1,4-naphthoquinone, were combined and evaporated. The product crystallized from benzene–petroleum ether as yellow needles: mp 199–200°; yield, 10.7 mg (48%).

(B) OXIDATIVE FISSION OF TRIMETHYLFLAVIOLIN TO 3,5-DIMETHOXYPHTHALIC ANHYDRIDE (**5**) AND CO_2. To trimethylflaviolin (45.9 mg) in a 50-ml three-necked flask was added 6 ml of a solution containing 2 ml of 30% unstabilized H_2O_2, 3 ml of 12% NaOH, and 1 ml of deionized H_2O. The flask was immediately immersed in a water bath at 75–80° and stirred until the solution became colorless (2–5 min). At this time the solution was cooled in

an ice bath and the flask purged with N_2. Three milliliters of 5 *N* H_2SO_4 was added and the evolved CO_2 carried through $Ba(OH)_2$ traps by the N_2 stream. The precipitated $BaCO_3$ was collected by centrifugation, washed successively with 2 × 10 ml vol of boiled deionized water, acetone, and ether, and then dried at 100°: yield, 54.8 mg (70%).

The acidified aqueous solution remaining in the reaction vessel was extracted with 2 × 30 ml of ethyl acetate. The combined extracts were dried over anhydrous magnesium sulfate and taken to dryness under reduced pressure to give impure 3,5-dimethoxyphthalic acid. Sublimation of this product at 130° (0.1 mm Hg) and chromatography of the sublimate on a 1 × 25 cm Unisil column (200–325 mesh) in benzene–ethyl acetate–acetic acid (150:75:2) gave a white solid which crystallized from benzene–petroleum ether to give pure 3,5-dimethoxyphthalic anhydride as white needles: yield, 24.3 mg (63%).

(C) DEMETHYLATION AND DECARBOXYLATION OF 3,5-DIMETHOXYPHTHALIC ANHYDRIDE TO 3,5-DIHYDROXYBENZOIC ACID (**6**) AND CO_2. 3,5-Dimethoxyphthalic anhydride (24.3 mg) was refluxed with 7 ml of 57% HI and 0.1 g of red phosphorus for 1 hr and the liberated CO_2 was trapped as $BaCO_3$ (21.5 mg; 90%). The acidic aqueous solution containing the 3,5-dihydroxybenzoic acid was filtered while hot and extracted with 2 × 15 ml vol of ethyl acetate. The combined ethyl acetate extracts were washed successively with 2 × 15 ml vol of distilled water and saturated $Na_2S_2O_3$, then dried over anhydrous magnesium sulfate, and taken to dryness on a rotary evaporator. The 3,5-dihydroxybenzoic acid was crystallized from benzene–ethanol (95%) (4:1, v/v): yield, 10.4 mg (49%).

(D) THE DECARBOXYLATION OF 3,5-DIHYDROXYBENZOIC ACID TO CO_2. 3,5-Dihydroxybenzoic acid (10.4 mg) was dissolved in 2 ml of freshly distilled quinoline, 0.1 g of $CuCr_2O_4$ was added, and the temperature of the mixture was raised to 240°. The CO_2 evolved was collected as $BaCO_3$ (6.2 mg, 54%). No attempt was made to isolate the other reaction product, resorcinol (**7**).

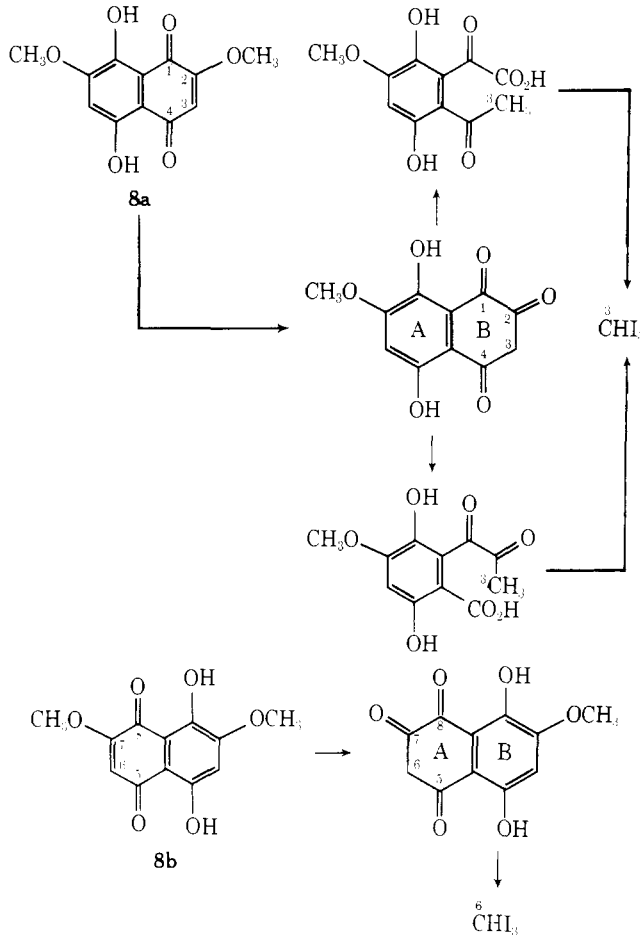
The 2,7-dimethoxynaphthazarin (**8a** or **8b**) was degraded to give C-3,6 as CHI_3 as shown in Scheme II. 2,7-Dimethoxynaphthazarin (50 mg in 5 ml of 12% NaOH) was treated with a solution containing 20 g of KI and 10.0 g of I_2 per 100 ml of H_2O until a light yellow-brown color persisted. A slight excess of 12% NaOH was then added and the solution extracted with 2 × 40 ml of $CHCl_3$. The combined extracts were washed once with 10 ml of 12% NaOH and twice with 10-ml portions of deionized water and then dried over anhydrous magnesium sulfate. The chloroform was removed on a rotary evaporator and the residue of iodoform was crystallized from aqueous methanol: yield, 28 mg (36%).

Radioactivity was determined with a Packard Tri-Carb Model 3310 scintillation counter. Organic ^{14}C-labeled compounds were counted in Bray's solution (Bray, 1960). The activity of barium carbonate samples was obtained by suspension in a thixotropic gel prepared by adding Cab-O-Sil (700 mg) to 20 ml of Bray's solution. All colored samples were corrected for quenching. Incorporation (*I*%) and dilution values (*D*) are defined as follows (Campbell, 1974).

$$\text{incorporation } (I\%) = \frac{\text{total dpm in product pool isolated}}{\text{total dpm added initially to precursor pool}} \times 100$$

$$\text{dilution } (D) = \frac{\text{sp act. of precursor added (dpm/}\mu\text{mol)}}{\text{sp act. of product isolated (dpm/}\mu\text{mol)}}$$

Scheme II: 2,7-Dimethoxynaphthazarin → Iodoform Conversion (Numbers Refer to the Atoms of the Naphthazarin).



Results and Discussion

The *Streptomycete* had been grown originally by Gerber and Wieclawek (1966) on a slurry containing "Pablum" as the main nutrient. This material was no longer available in the 1970's and no pigments were produced by growth of the organism on Czapek-Dox and similar media. In view of a report (Hesseltine, 1972) that increased yields of some secondary metabolites (e.g. aflatoxins) could be obtained by shake culture on natural cereal grains ("solid state fermentation") growth of the *Streptomycete* under these conditions was investigated. Rice grains proved to be very suitable and excellent yields of naphthoquinone pigments were obtained (typically 300–350 mg with 250 g of rice in a 2800-ml Fernbach flask). There was little or no difference between various commercial brands of rice and both "instant" and "slow-cooking" types were suitable. Barley grains, however, gave poor yields. This method is not generally applicable since organisms having an active amylase will quickly hydrolyze the starches in the grains to yield a glutinous mass in the flasks—this was the case with *A. niger*, for example.

No cultural problems were encountered with *A. niger*, although pigments were not produced when shake cultures were used. The results of experiments in which surface cultures of *A. niger* were allowed to metabolize ^{14}C-labeled acetate, malonate, and shikimate are shown in Table I. In these experiments the tracer was added after 12 to 14 days of growth. Experience showed that flaviolin production was just beginning at this time. Metabolism was then allowed to continue for a further 5 to 6 days. The incorporation values

Table I: Precursor Utilization for Flaviolin Production.

Precursor	Amt Added (μCi)	Time of Precursor Addition (Days)	Time of Harvest (Days)	Flaviolin		
				Sp Act. (dpm/μmol)	I(%)	D
[1-^{14}C] Acetic acid	200	12	28	11,062	0.81	4538
[2-^{14}C] Acetic acid	420	14	20	53,800	0.52	2500
[2-^{14}C] Malonic acid	250	12	17	4,376	0.065	4797
[U-^{14}C] Shikimic acid	50	12	16	4,959	0.063	3548

Table II: Comparison of Specific Activities of Flaviolin, Tyrosine, and Fatty Acids.

Biosynthetic Precursor and Amt Added	Time Precursor Present	I^a (%)	D	Sp Act. (dpm/μmol)		
				Flaviolin	Tyrosine	Fatty Acid Methyl Esters
[U-^{14}C] Shikimic acid, 50 μCi	50 min	0.0025	275,510	65	1211	30
[1-^{14}C] Acetic acid, 500 μCi	50 min	0.021	23,457	5400	556	6000
[2,3-$^{14}C_2$] Succinylbenzoic acid, 21 μCi	12 days	0.12	12,700	139		

[a] The I and D values refer to flaviolin.

Table III: Chemical Degradation of Labeled Flaviolin Samples.

Biosynthetic Precursor	Sp Act., dpm/μmol (% Total Act.), in Degradation Fragments[a]							
	4	C-2 + C-3[b]	(C-2 + C-3)[c]	5	C-4[d]	C-4[e]	6	C-1
[1-^{14}C] Acetic acid	570	93		437	127		343	36
		(32.6)	(22.3)	(76.7)	(22.3)	(16.4)	(60.2)	(6.3)
[2-^{14}C] Acetic acid	6722	453.8		5364	336		5156	612
		(13.5)	(20.2)	(79.8)	(5.0)	(3.1)	(76.7)	(9.1)
[2-^{14}C] Malonic acid	693	35.5		554	29.8		536.4	87.3
		(10.2)	(20.1)	(79.9)	(4.3)	(2.5)	(77.4)	(12.6)
[U-^{14}C] Shikimic acid	198	16.9		158	23		142	12
		(17.1)	(20.2)	(79.8)	(11.6)	(8.1)	(71.7)	(6.0)

[a] For reactions and structures, see Scheme I. The specific activity in trimethylflaviolin (4) is taken as 100%. Figures in parentheses are percent total activity relative to 4. [b] Direct value from specific activity of CO_2 released in conversion of 4 → 5. [c] Indirect value from specific activity of trimethylflaviolin (**4**) − specific activity of 3,5-dimethoxyphthalic anhydride (**5**). [d] Direct value from specific activity of CO_2 released in conversion of 5 → 6. [e] Indirect value from specific activity of 3,5-dimethoxyphthalic anhydride (5) − specific activity of 3,5-dihydroxybenzoic acid (**6**).

for the two labeled acetates were in the range 0.5–0.8%; those for malonate were considerably lower. For the incorporation of [U-^{14}C]shikimate the I value was essentially the same as for malonate and the dilution values for all of these precursors were similar (range from 2500 to 5000). In view of these values, shikimate could not be ruled out immediately as a precursor to flaviolin. In short term (50 min) feeding experiments with 14-day-old cultures there was again an approximately tenfold difference in the I value for acetate and shikimate but in this case a much higher dilution for shikimate (D = 275,510) than acetate (D = 23,457) was observed (see Table II).

More information on the possible roles of acetate and shikimate was obtained by degradation of the labeled samples. With an acetate–polymalonate pathway and assuming complete conversion of acetate to malonate, alternate carbon atoms of the chain should be labeled by acetate to the extent of 20% per active center [10 carbon atoms total; 5 centers; 100%]. Therefore, if flaviolin is acetate derived, C-2 and C-3 together should contain maximally 20% of the total activity. Table III shows the $BaCO_3$ corresponding to C-2 and C-3 from a [1-^{14}C]acetate incubation contains 32.6% while that from [2-^{14}C]acetate and [2-^{14}C]malonate contains only 13.5 and 10.2%, respectively. Although the distributions from the methyl- or methylene-labeled samples are lower than expected and that from carboxyl-labeled acetate is higher, a similar situation has been encountered with the plant naphthoquinone, lawsone (2-hydroxy-1,4-naphthoquinone) (Grotzinger and Campbell, 1972). It appears that the carbon carrying the hydroxyl group (C-2) is more readily converted to CO_2 than is the adjacent position (C-3) during oxidation with peroxide. If a similar condition holds for the peroxide oxidation of flaviolin and if C-2 were labeled and C-3 were not, instead of a theoretical value of 20% for C-2 + C-3, a value greater than 20% would be measured experimentally; conversely, if C-3 were labeled while C-2 was not, a value of less than 20% would be obtained. With this understanding it is apparent that C-1 of acetic acid provides C-2 of flaviolin while C-2 of acetic acid and C-2 of malonic acid provide C-3 of flaviolin. To support this argument, an "indirect" value for C-2 + C-3 may be calculated by subtracting the specific activity of the dimethoxyphthalic anhydride (**5**) from that of the trimethylflaviolin (**4**). For all of the precursors shown in Table III, this indirect value was

Table IV: Precursor Utilization and Chemical Degradations of Labeled Dimethoxynaphthazarin Samples.

Biosynthetic Precursors	Amt of Precursor Added (μCi)	Time Precursor Present (Days)	2,7-Dimethoxynaphthazarin			Iodoform (C-3 and C-6)	
			I (%)	D	Sp Act. of Material Degraded[a] (dpm/μmol)	Sp Act. (dpm/μmol)	% Total Act.
[1-^{14}C]Acetic acid	250	5	2.50	11,191	12,303	97	0.79
[2-^{14}C]Acetic acid	250	5	3.66	5,895	21,134	3,403	16.0
[2-^{14}C]Malonic acid	250	5	18.50	362	104,080	19,725	19.0
L-[U-^{14}C]Glutamic acid	50	2	0.165	328,000	1,930	164	8.5
[U-^{14}C]Shikimic acid	25	5	0.86	33,800	273	27	9.9
L-[*methyl*-^{14}C]Methionine[b]	50	5	36.6	4,430	26,950		

[a] In all cases, the 2,7-dimethoxynaphthazarin was used without dilution; hence, these figures represent specific acitivity of the biosynthesized material. [b] In this experiment, mompain was obtained by demethylation of the dimethoxynaphthazarin with HI, and had 0.78% of the total activity of the dimethoxynaphthazarin used in the reaction.

Table V: Utilization of Benzenoid and Naphthalenoid Precursors for Biosynthesis of 2,7-Dimethoxynaphthazarin.

Precursor	2,7-Dimethoxynaphthazarin		
	Sp Act. (dpm/μmol)	I (%)	D
[2,3-$^{14}C_2$]Succinylbenzoate[a]	7	0.0016	247,000
[^{14}C]Flaviolin[b]	473	10.3	17.1
[^{14}C]Mompain[c]	8863	39.1	11.4

[a] Specific activity = 1,756,020 dpm/μmol. [b] Flaviolin was obtained from *A. niger* by growth in the presence of [2-^{14}C] malonic acid; specific activity = 7992 dpm/μmol. [c] Mompain was obtained by demethylation of 2,7-dimethoxynaphthazarin isolated from a culture of the *Streptomycete* no. 12396 when grown in the presence of [2-^{14}C] malonic acid; specific activity = 104,080 dpm/μmol.

always close to 20%. It is also apparent there is a rapid interconversion of acetate and malonate. [1-^{14}C]Acetate also provides activity for C-4 of flaviolin and not C-1, while for [2-^{14}C]acetate and [2-^{14}C]malonate the converse is demonstrated. These data strongly indicate a polyacetate origin for flaviolin.

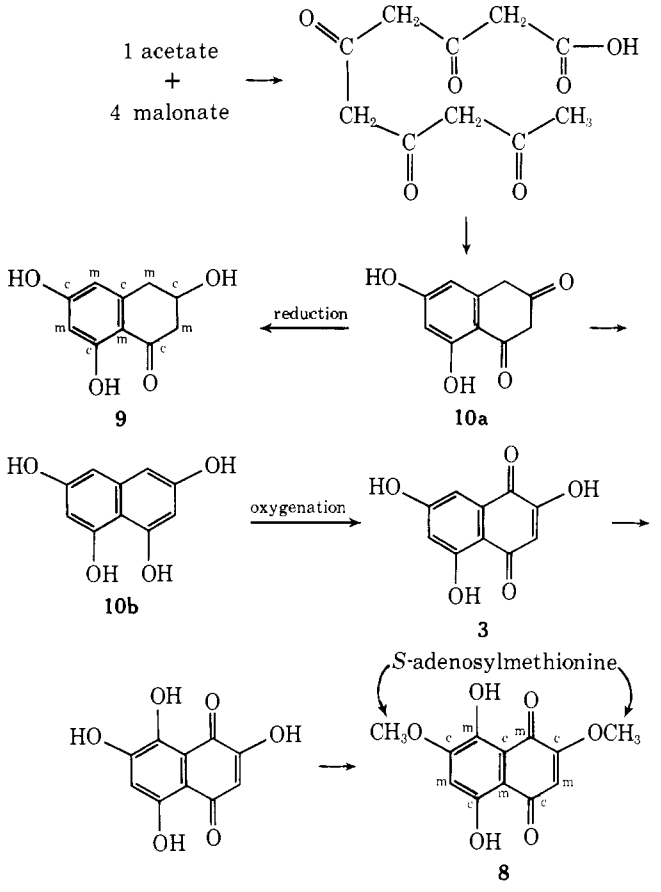
The labeling pattern in flaviolin biosynthesized in the presence of [U-^{14}C]shikimate strongly indicated approximately 10% label at *all* positions (see Table III). This pattern was *not* consistent with that expected for the shikimate–ketoglutarate pathway used for production of menaquinones and some plant naphthoquinones—theory requires 0% activity at C-2 and C-3 (for a review, see Bentley, 1975). The pattern, however, is consistent with conversion of [U-^{14}C]shikimate to [U-^{14}C]acetate. This apparently does occur, and rapidly as well, as indicated by the radioactivity in the fatty acid methyl esters isolated after growth of *A. niger* for 50 min in the presence of [U-^{14}C]shikimate (Table II). The low incorporation of *o*-succinylbenzoate into flaviolin (Table II) (again probably via acetate) is further evidence for the acetate origin, since shikimate derived naphthoquinones generally incorporate this precursor very well (Dansette and Azerad, 1970; Campbell et al., 1971).

The minimum requirement to assemble the flaviolin skeleton is a polyketide chain of ten atoms; the terminal carboxyl must, in this event, eliminate water with the methyl group at the other terminal. The present experiments do not precisely define the position of the "starter acetyl unit"—apparently added malonate was decarboxylated to acetate, as has been observed in several other cases, and location of the acetate unit was not possible. The utilization of a chain of 12 carbon atoms, or an even larger number, is also not eliminated by these results. If a hexaketide (or larger) chain is involved the extra groups must be removed, presumably by oxidation and decarboxylation. Any postulated mechanism must locate an acetate methyl at C-1 and an acetate carboxyl at C-4.

Although distribution problems were anticipated on addition of tracer solutions to the "solid state fermentation" of *Streptomycete* no. 12396, excellent incorporations were obtained (Table IV). The tracers were dissolved in 5 ml of sterile water and an attempt was made to distribute drops of the solution throughout the culture during the addition. These additions were made at 24 hr after inoculation; shaking was then continued for a further 96 hr. Due to the symmetry elements present in 2,7-dimethoxynaphthazarin, either C-3 or C-6 can be eliminated as CHI_3 in the degradation used (see Scheme II); however, due to the alternating structure of the polyketomethylene chain, if C-3 is labeled by [1-^{14}C]acetate, C-6 will be also and conversely. As was the case with flaviolin, if 2,7-dimethoxynaphthazarin is acetate derived each labeled center should represent 20% of the total label in the molecule. Table IV shows C-3 + C-6 is not derived from C-1 of acetate (0.79% activity) but is derived from C-2 (16.0%) of acetate or C-2 (19%) of malonate. This is exactly as for flaviolin. The low values for [2-^{14}C]acetate are assumed to be due to label randomization via the tricarboxylic acid cycle. The values of nearly 10% obtained for both L-[U-^{14}C]glutamic acid and [U-^{14}C]shikimic acid are attributed to prior conversion to doubly labeled acetate before incorporation into the dimethoxynaphthazarin. The incorporation of activity from [2,3-$^{14}C_2$]succinylbenzoate (see Table V) was the lowest obtained with any of the precursors tested. All of these results are consistent only with an acetate–polymalonate origin for the dimethoxynaphthazarin. That the 2- and 7-methoxyl methyl groups are derived from the methyl group of methionine was attested to by the high incorporation (I = 36.6%) value obtained with L-[*methyl*-^{14}C]methionine (see Table V) and by the fact that on demethylation less than 1% of the radioactivity remained in the mompain.

In further experiments, labeled flaviolin and mompain samples were examined as possible precursors to the dimethoxynaphthazarin (see Table V). In both cases, high incorporation values were observed along with low dilution values; these results strongly suggest both of these compounds as intermediates along the pathway to 2,7-dimethoxynaphthazarin. This was further substantiated by an

Scheme III: Biosynthesis of Oxygenated Naphthalenes (m = Methyl and c = Carboxyl of Acetate).



experiment to "trap" any flaviolin intermediate by the addition of unlabeled flaviolin (5.3 mg) to a growing culture containing [2-^{14}C]acetate (100 μCi). On working up this culture it was possible to reisolate sufficient flaviolin for purification and characterization (ca. 0.2 mg). This material was radioactive with a specific activity of 250 dpm/μmol. Hence a small amount of labeled flaviolin was actually present in the culture metabolizing radioactive acetate. These results suggest the pathway shown in Scheme III for the formation of 2,7-dimethoxynaphthazarin. The intermediacy of the tetrahydroxynaphthalene (**10b**) is likely since it is known to be easily oxidized, in vitro, to flaviolin (Baker and Bycroft, 1968). Using the solid state fermentation conditions, no 2,7-dimethylflaviolin could be detected, as had been reported in previous studies with this organism (Gerber and Wieclawek, 1966). Attempts to duplicate the previous results using a slurry of Gerber Baby Food as a nutrient yielded a complex mixture of colored compounds present only in microgram amounts, none of which had ultraviolet and visible spectral properties similar to those reported for the 2,7-dimethylflaviolin. The only major component was again 2,7-dimethoxynaphthazarin. The tracer experiments, and the inability to isolate any 2,7-dimethylflaviolin, suggest that hydroxylation of flaviolin occurs either exclusively or very rapidly, and before the introduction of any methyl groups.

After the completion of this work, we learned that a ^{13}C nuclear magnetic resonance study of the biosynthesis of scytalone had been undertaken (Turner, 1974; Aldridge et al., 1974). Scytalone (**9**) is a hydroxytetralone produced by *Phialophora lagerbergii* and a *Scytalidium* sp. (Findlay and Kwan, 1973). In the former organism, flaviolin is a cometabolite of scytalone. It seems that the initial cyclization product (**10a**) is reduced in scytalone formation and that its tautomeric structure (**10b**) is oxygenated to form flaviolin (**3**) (see Scheme III).

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